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Citation

Bijkerk, R., Florijn, B. W., Khairoun, M., Duijs, J. M. G. J., Ocak, G., Vries, A. P. J. de, ... Reinders, M. E. J. (2017). Acute Rejection After Kidney Transplantation Associates With Circulating MicroRNAs and Vascular Injury. *Transplantation Direct*, 3(7).
doi:10.1097/TXD.0000000000000699

Version: Not Applicable (or Unknown)
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Downloaded from: <https://hdl.handle.net/1887/94758>

Note: To cite this publication please use the final published version (if applicable).

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Acute Rejection After Kidney Transplantation Associates With Circulating MicroRNAs and Vascular Injury

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Background. Acute rejection (AR) of kidney transplants is associated with the loss of endothelial integrity, microvascular rarefaction and, ultimately, graft dysfunction. Circulating angiogenic microRNAs (miRNAs) may serve as markers for microvascular injury. Here, we investigated the short- and long-term effects of AR after kidney transplantation on systemic vascular injury and the associated circulating miRNA profile. **Methods.** Systemic vascular injury was determined by measuring capillary tortuosity and density within the oral mucosa as well as by assessing circulating levels of angiopoietin-2/angiopoietin-1 ratio, vascular endothelial growth factor and soluble thrombomodulin. After a pilot study, we selected 48 miRNAs to assess the AR- and microvascular injury associated circulating miRNAs. **Results.** In stable transplant recipients (n = 25) and patients with AR (n = 13), which were also studied longitudinally (1, 6, and 12 months post-AR), we found an AR-associated increase in markers of systemic vascular injury, of which vascular endothelial growth factor and soluble thrombomodulin normalized within 1 year after AR. Of the 48 selected miRNAs, 8 were either decreased (miR-135a, miR-199a-3p, and miR-15a) or increased (miR-17, miR-140-3p, miR-130b, miR-122 and miR-192) in AR. Of these, miR-130b, miR-199a, and miR-192 associated with markers of vascular injury, whereas miR-140-3p, miR-130b, miR-122, and miR-192 normalized within 1 year after AR. **Conclusions.** AR after kidney transplantation is characterized by systemic microvascular injury and associates with specific circulating miRNA levels.

(*Transplantation Direct* 2017;3:e174; doi: 10.1097/TXD.0000000000000699. Published online 19 June, 2017.)

In the context of transplantation, microvascular endothelial cells (ECs) are very susceptible to injury that can result from episodes of acute rejection (AR). After the alloimmune response, cytokines and growth factors are produced that can lead to EC activation and microvascular destabilization.¹⁻⁶ These rejection-associated events can result in perpetual EC damage and

promotion of (aberrant) angiogenesis within the allograft.^{1,3,5} Together, these insults lead to the loss of the microvasculature, chronic ischemia, and cell death^{7,8} and, ultimately, to the development of interstitial fibrosis/tubular atrophy and graft dysfunction.^{1,2,5} The rate-limiting role of microvascular

Received 10 April 2017. Revision requested 1 May 2017.

Accepted 15 May 2017.

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This work was supported by a Veni grant from ZonMW (01086089) to M.E.J. Reinders and grants from the Dutch Heart Foundation (Queen of Hearts: Improving diagnosis of CVD in women; 2013T084), the Dutch kidney foundation (NSN 140113) and the European Foundation for the study of Diabetes to A.J. van Zonneveld and R. Bijkerk.

The authors declare no conflicts of interest.

A.J.vZ. and M.E.J.R. contributed equally.

R.B., B.W.F., and M.K. participated in the design, coordination of the study and carried out the experiments and wrote the article. G.O. and J.M.G.J.D. carried out the experiments. A.P.J.d.V., A.F.S., and J.W.d.F. contributed to the discussion. M.M. contributed to the statistical analysis. T.J.R., A.J.vZ. and M.E.J.R. contributed to the design and supervision of the study and helped to draft the article. All authors read and approved the final article.

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Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantationdirect.com).

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ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000000699

integrity in AR associated graft loss was supported in a study demonstrating that when EC injury was inhibited (by limiting ongoing inflammation) and the microvasculature was maintained intact, physiological remodelling occurred and allograft tissue returned to normal.^{5,8,9}

We previously demonstrated that microRNAs (miRNAs) play a major role in maintaining microvascular integrity.¹⁰ Additionally, miRNA dysregulation is involved in the pathophysiology of diseases associated with microvascular injury.^{11,12} Recently, we also demonstrated that circulating miRNAs could serve as markers of such microvascular injury after simultaneous pancreas kidney (SPK) transplantation.¹³ Furthermore, these circulating miRNAs might provide insight in and contribute to the underlying pathology.^{14,15} Several microRNAs, both in the allograft tissue as well as circulating, have been described to associate with AR.¹⁶⁻²⁴ However, their relation to vascular injury during and after the rejection episode is largely unexplored. Here, we investigated whether circulating miRNAs could serve as markers of microvascular injury in the context of AR. To that end, we first explored the relation of AR with systemic microvascular injury. Then, in a cross-sectional study of patients with AR, we measured circulating miRNA levels and associated them with markers of systemic vascular injury, including vascular endothelial growth factor (VEGF), soluble thrombomodulin (sTM) and angiopoietin-2 (Ang-2), which all play a central role in the angiogenic and inflammatory responses.^{1-3,6,25-27} Additionally, we used sidestream darkfield (SDF) imaging, a noninvasive tool to visualize the human microcirculation²⁸ that we previously used to investigate the labial mucosal capillary tortuosity as marker for systemic microvascular disease in diabetes mellitus type I patients before and after SPK.²⁹ Finally, in a longitudinal study, patients were followed up for 1 year after the AR episode to evaluate longer term effects on vascular injury and circulating miRNAs. Taken together, we

demonstrate that circulating miRNAs associate with microvascular injury in AR and could potentially be used to monitor microvascular integrity in disease progression.

MATERIALS AND METHODS

Transplantation

All procedures were approved by the Leiden University Medical Center's Medical Ethical Committee; all methods were performed in accordance with the relevant guidelines and regulations and all patients in the studies were enrolled after given informed consent on all aspects of the study. All patients underwent solitary kidney transplantation at the Leiden University Medical Center between 2003 and 2012. Kidney transplantation was performed as described previously (24). Average treatment regimen involved prednisone (tapered to a dose of 10 mg by 6 weeks and a dose of 7.5 mg by 3 months), cyclosporine (targeted to an area under the curve (AUC) 5400 ng/h per mL first 6 weeks then 3250 ng/h per mL) or tacrolimus (AUC, 210 ng/h per mL first 6 weeks, then 125 ng/h per mL) and mycophenolate mofetil (MMF) (AUC, 30-60 ng/h per mL). In case of side effects patients were converted to everolimus (AUC, 120-150 ng/h per mL). All patients received induction treatment with basiliximab (40 mg at days 0 and 4) or daclizumab (100 mg/day on the day of transplantation and 10 days after transplantation).

Cross Sectional and Longitudinal AR Study Population

In the cross-sectional study, a total of 38 patients (24 men and 14 women) were enrolled. This cohort (marked as "cohort A" for clarity purposes) is used in the studies found in Figures 1 to 5. All measurements were performed before patients received treatment for AR. None of the 38 patients had signs of infection at the time of the measurements. From

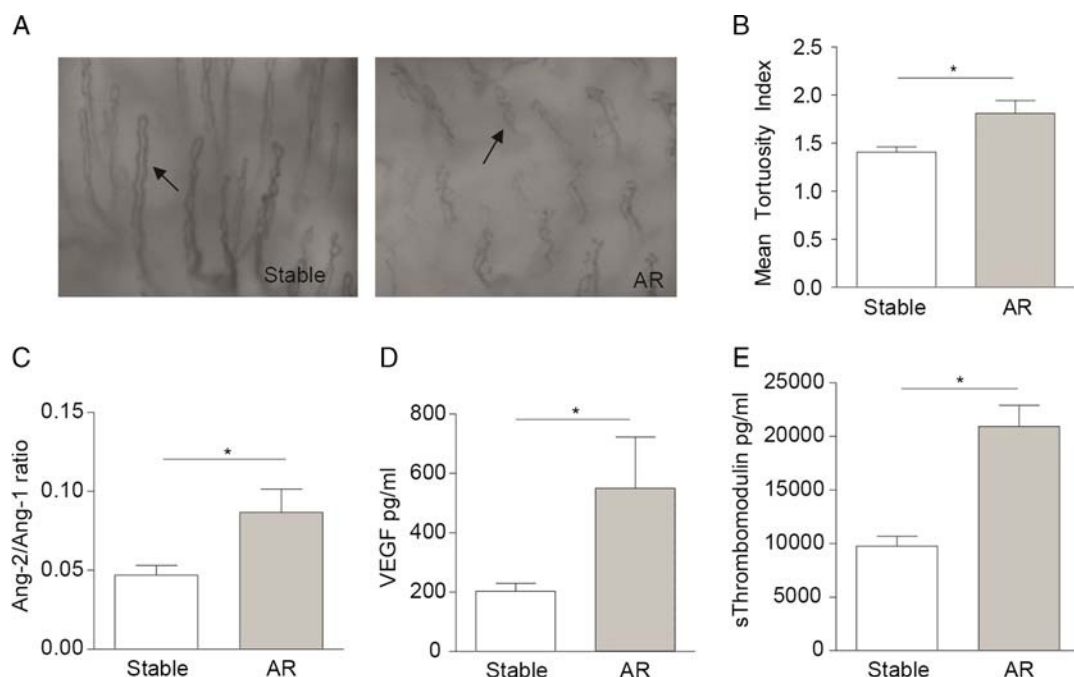


FIGURE 1. Increase in markers of vascular injury in AR. A, SDF images of the oral mucosa visualizing the microvascular capillaries of a representative patient in stable and AR group. Black arrow: capillary loops. B, Mean tortuosity index of microvascular capillaries in the stable ($n = 25$) and AR ($n = 13$) group. C-E, Ang-2/Ang-1 ratio (C), VEGF-A (D) and sTM (E) serum levels (pg/mL) in stable and AR group. Data shown are mean $\pm$ SEM. * $P < 0.05$ compared to stable group.

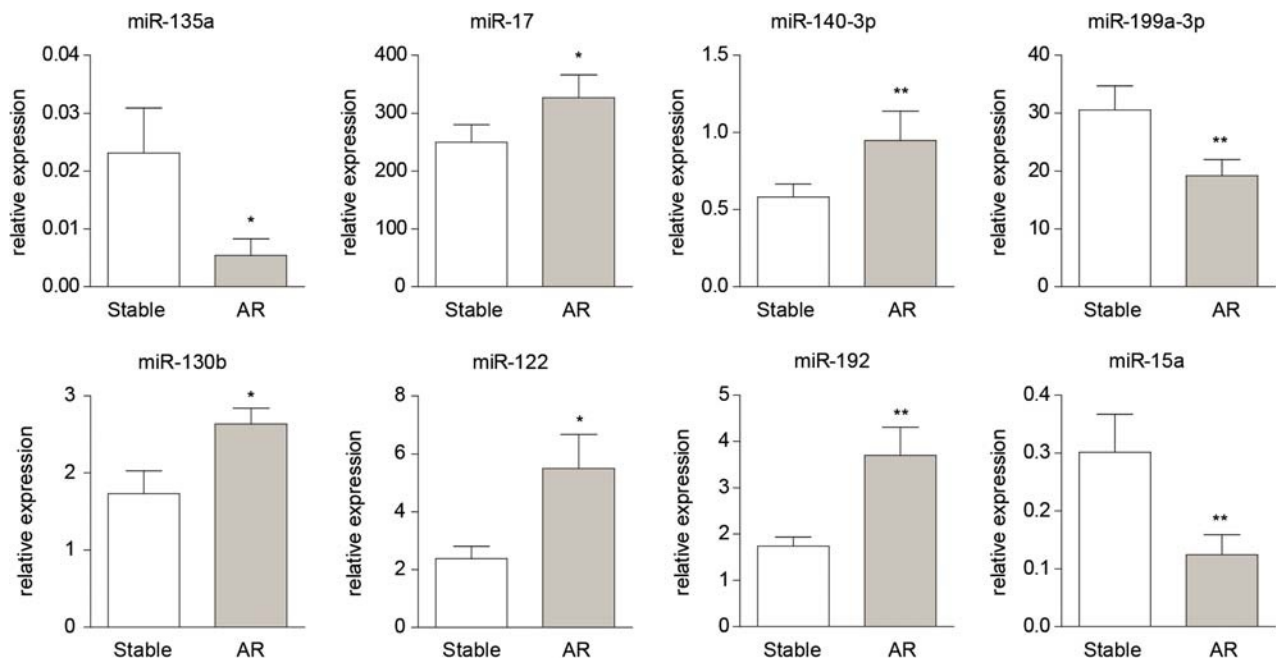


FIGURE 2. Differential miRNA levels in AR. MiRNAs that show statistically significant differences between stable and AR group (* $P < 0.05$, ** $P < 0.001$). Means are relative expression levels, and can also be compared among miRNAs. Stable group, $n = 13$; AR, $n = 13$. Data shown are mean $\pm$ SEM, and adjusted for sex and age as possible confounders.

those 38 patients, the control group (stable group) consisted of 25 kidney transplant recipients with of estimated glomerular filtration rate (eGFR) greater than 30 mL/min and stable renal function after transplantation. Thirteen patients were included in the AR group because of a decrease in renal function and biopsy proven AR. Biopsies were evaluated using

the Banff criteria by 2 independent pathologists. Pathological characteristics including Banff grade are summarized in Table 1 and Table S1, SDC (<http://links.lww.com/TXD/A45>). All transplant recipients were negative for pretransplant donor-specific antibodies, as well as de novo donor-specific antibodies after transplantation. Also, viral (BK, cytomegalovirus)

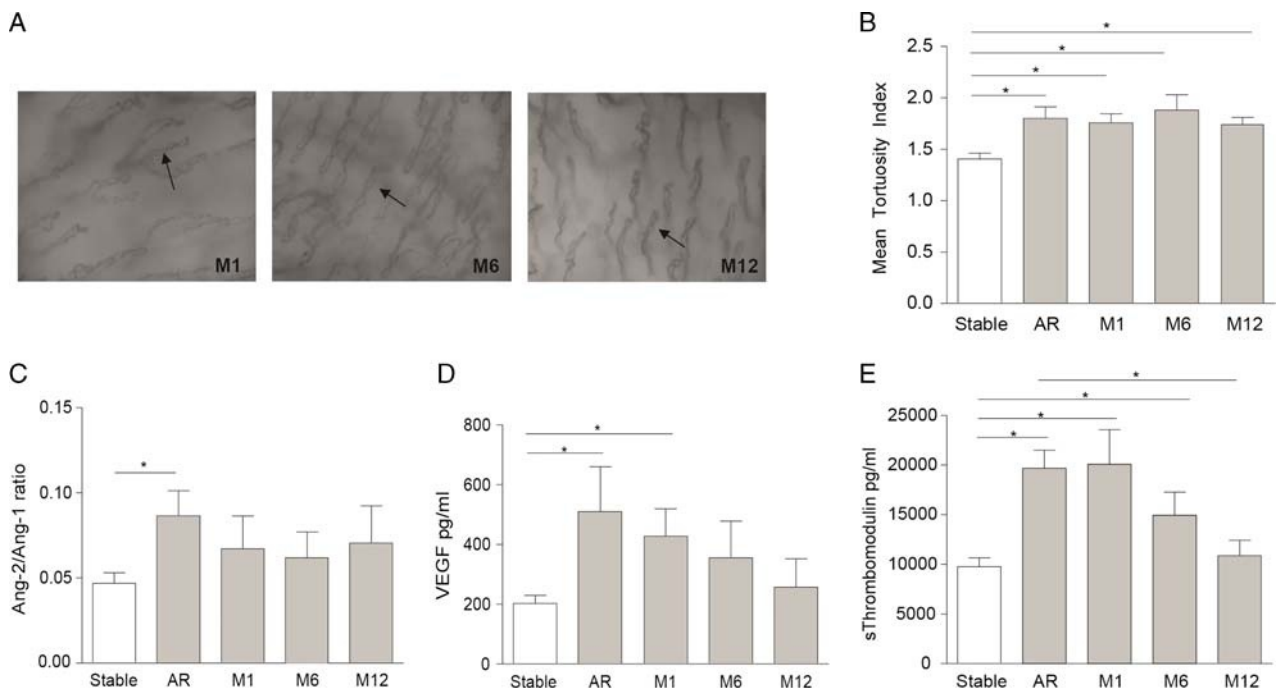


FIGURE 3. Normalization of VEGF and sTM, but not of Ang-2/Ang-1 ratio and microvascular tortuosity, within 1 year after AR. A, Sidestream dark field images of the oral mucosa visualizing the microvascular capillaries of a representative stable kidney transplant patient, 1 (M1), 6 (M6), and 12 (M12) months after rejection in the longitudinal rejection study. Black arrows: capillary loops. B, Longitudinal course of the mean tortuosity index of microvascular capillaries in the stable group ($n = 25$), AR group ($n = 13$) and at 1 (M1, $n = 13$), 6 (M6, $n = 13$) and 12 (M12, $n = 11$) months after rejection. C-E, Longitudinal course of serum Ang-2/Ang-1 ratio (C), VEGF-A (D) and sTM (E) levels at 1, 6 and 12 months after rejection. Data are shown as mean $\pm$ SEM. * $P < 0.05$.

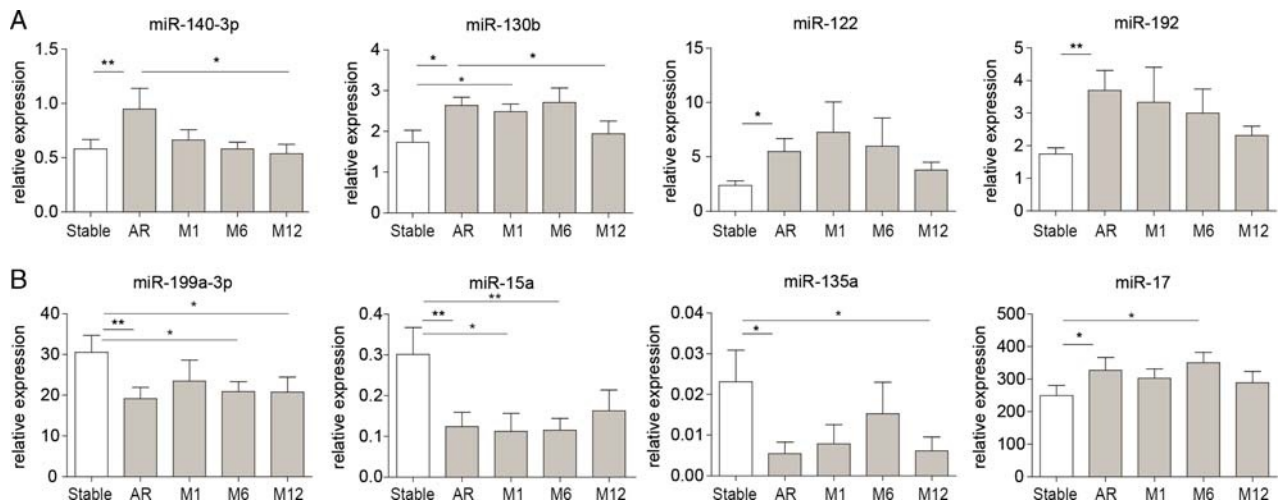


FIGURE 4. Circulating miRNA levels in patients with AR, followed up for 1 year. A-B, Longitudinal course of the circulating miRNA levels in the stable group, AR group and at 1 (M1), 6 (M6), and 12 (M12) months after rejection. A, miRNAs that show (a trend towards) normalization within 12 months after AR. B, miRNAs that do not normalize within 12 months after AR. * $P < 0.05$, statistically significant. Means are relative expression levels, and can also be compared among miRNAs. Stable group, $n = 13$; AR D0, $n = 13$; AR M1, $n = 8$; AR M6, $n = 9$; AR M12, $n = 11$. Data shown are mean $\pm$ SEM.

or urinary tract infections were absent. Patients with SPK, active infection, liver failure, active autoimmune disease, epilepsy, or malignancy in the last 5 years (except patients treated for basal cell carcinoma who were in full remission) were excluded from the study. The patients in the AR group were also studied prospectively in a longitudinally study at 1 (M1, $n = 13$), 6 (M6, $n = 13$), and 12 months after the AR episode (M12, $n = 11$, 2 patients discontinued their participation after a second rejection episode).

Stable Transplantation Study Population

In this control cohort (marked as “cohort B” for clarity purposes), a total of 48 patients were enrolled. This cohort is used in the studies found in Figures S1 and S2, SDC (<http://links.lww.com/TXD/A45>) and served to assess the effect of transplantation itself on the levels of markers of vascular injury and circulating miRNAs. The control group of this cohort (controls) consisted of 20 healthy controls. Twenty-eight chronic kidney disease patients were included because they received a kidney transplant. The patients in the chronic kidney disease group were studied in a longitudinally study at 1 (M1, $n = 28$), 6 (M6, $n = 28$) and 12 months after transplantation (M12, $n = 28$). Patient characteristics are summarized in Table S2, SDC (<http://links.lww.com/TXD/A45>).

Microcirculatory Imaging

The SDF microscan (MicroVision Medical Inc., Wallingford, PA) was performed as described previously.^{28,29} Before analysis, the video files were anonymized so that the assessor was blinded to the subject’s identity. Capillary loops were assessed by 2 individual assessors in a randomized, blind fashion. From the forty video files obtained of the microcirculation in each subject, four technically best files were selected from each lip quadrant for analysis. Capillary density (capillaries/mm²) was calculated by counting the number of vessels per screen shot. Subsequently, tortuosity of capillary loops was assessed using a validated scoring system described previously and the average of assessed capillary tortuosity was used to calculate mean tortuosity index per patient.²⁸

Laboratory and Urinary Assessments

All patients underwent routine venous blood sampling that was collected before the morning intake of immunosuppression to assess creatinine, urea, HbA1c, glucose, and hemoglobin. Proteinuria was measured in collected 24-hour urine. The eGFR was calculated with plasma creatinine concentration using the Modification of Diet in Renal Disease formula. For miRNA analysis, plasma was harvested by centrifugation of EDTA-anti-coagulated blood for 10 minutes at 3000 rpm and subsequently stored at -80°C . At the same time, blood was collected

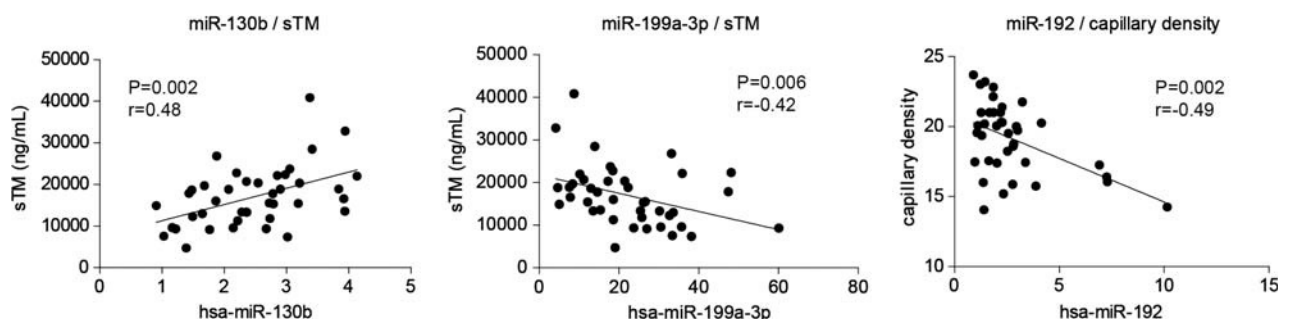


FIGURE 5. Correlation of circulating miRNAs with markers of (micro)vascular injury. MiRNAs that were different between stable and AR group correlate with markers of systemic vascular injury.

TABLE 1.**Patients characteristics of AR patients during D0 and M1, M6, and M12 after AR compared with stable renal transplant recipients**

Patients characteristics	Stable renal transplant (n = 25)		AR							
			D0 (n = 13)		M1 (n = 13)		M6 (n = 13)		M12 (n = 11)	
Age, y	53.2	±13	51.7	±10	51.8	±10	52.4	±10	52.7	±10
Sex: male, n (%)	15	(60%)	9	(69%)						
Smoking, n (%)	3	(12%)	1	(8%)	1	(8%)	1	(8%)	0	
Median time since transplantation (IQR), mo	5	(1-19)	12	(3-22)						
Primary kidney disease, n (%)										
Glomerulonephritis	8	(32%)	0		0		0		0	
Diabetes mellitus	3	(12%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
Pyelonephritis or interstitial nephritis	1	(4%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Focal segmental glomerulosclerosis	3	(12%)	0		0		0		0	
Urologic	1	(4%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
Polycystic kidney disease	4	(16%)	4	(31%)	4	(31%)	4	(31%)	4	(31%)
Hypertension	2	(8%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Unknown	2	(8%)	2	(15%)	2	(15%)	2	(15%)	2	(15%)
Other	1	(4%)	1	(8%)	1	(8%)	1	(8%)	1	(8%)
BMI, kg/m ²	25.8	±4	24.3	±3	24.3	±3	23.8		25.2	
Dialysis, n (%)	4	(16%)	0		0		0		0	
Systolic BP, mm Hg	137	±20	142	±19	132	±10	132	±16	127	±19
Diastolic BP, mm Hg	82	±9	83	±13	77	±9	79	±12	72	±10
eGFR, mL/min per 1.73 m ²	47.2	±13	38.1	±13 ^a	35.4	±15 ^a	40.2	±16 ^a	36.0	±18 ^a
Median proteinuria (IQR), g/24 h	0.2	(0.2-0.4)	0.4	(0.3-1.0) ^a	0.3	(0.2-0.7)	0.2	(0.2-0.3)	0.3	(0.1-0.3)
Glucose, mmol/L	6.0	±2	5.5	±1	5.7	±2 ^b	6.3	±3	5.9	±1 ^b
Hemoglobin, mmol/L	7.9	±1	7.6	±1	7.3	±1	7.7	±1	7.7	±1
Hematocrit, L/L	0.40	±0.06	0.38	±0.04	0.36	±0.05	0.39	±0.05	0.39	±0.05
Antihypertensives, n (%)										
ACE inhibitor	5	(20%)	2	(15%)	3	(23%)	3	(23%)	2	(18%)
Diuretics	6	(24%)	3	(23%)	4	(31%)	2	(15%)	1	(9%)
β blockers	11	(44%)	6	(46%)	7	(54%)	7	(54%)	6	(55%)
Calcium antagonists	14	(56%)	8	(62%)	9	(69%)	6	(46%)	6	(55%)
Angiotensin-II antagonists	1	(4%)	3	(23%)	3	(23%)	2	(15%)	3	(27%)
Statins, n (%)	9	(36%)	5	(39%)	6	(46%)	7	(54%)	7	(64%)
Immunosuppressive, n (%)										
Cyclosporine	3	(12%)	3	(23%)	3	(23%)	2	(15%)	0	
Tacrolimus	19	(76%)	9	(69%)	9	(69%)	10	(77%)	9	(82%)
Prednisone	24	(96%)	13	(100%)	13	(100%)	13	(100%)	11	(100%)
Everolimus	2	(8%)	1	(8%)	0		0		1	(9%)
Mycophenolate mofetil	21	(84%)	9	(69%)	9	(69%)	10	(77%)	7	(64%)
Antirejection, n (%)										
Methylprednisolone			11	(84%)						
ATG			1	(8%)						
Alemtuzumab			1	(8%)						
Donor characteristics										
Age, y	52.3	±9	46.4	±11						
Sex: male, n (%)	8	(32%)	4	(31%)						
Donor type, deceased donors, N (%)	5	(20%)	5	(39%)						

^aP < 0.05 compared to stable patients.^bP < 0.05 compared with AR.

All data are mean ± SD, unless otherwise specified.

BMI, body mass index; BP, blood pressure; ACE, angiotensin converting enzyme; IQR, interquartile range.

for analysis of serum Ang-1, Ang-2, VEGF (VEGF-A) and sTM concentrations. Both Ang-1 and Ang-2 were measured by ELISA (R&D Systems, Minneapolis, MN) per the manufacturer supplied protocol. Likewise, VEGF and sTM (Gen-Probe Diaclone Research, Besançon, France) levels were assessed. Because the Ang-2/Ang-1 rather than the absolute levels of either cytokine has been considered to determine the

functional status of the microvasculature,^{26,27,30} this ratio was calculated for the different groups and time points.

RNA Isolation

Total RNA was isolated from 200 µL plasma using 800 TRIzol µL reagent (Invitrogen, Breda, The Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands)

with an adapted protocol. In short, 160 μ L of chloroform was added to the plasma/TRIzol sample and centrifuged for 15 minutes at 12 000 rpm. The aqueous phase was combined with 1.5 volumes of 100% ethanol and transferred to a MinElute Spin column and centrifuged for 15 seconds at 13 000 rpm. Subsequently the column was washed with 700- μ L RWT buffer, twice with 500- μ L RPE buffer and centrifuged for 15 seconds at 13 000 rpm after the first 2 washing steps and 2 minutes at 13 000 rpm after the last washing step. Finally, RNA was eluted with 15- μ L RNase-free water.

Profiling miRNAs

For miRNA cDNA synthesis, reverse transcription of total RNA was performed using the miRNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was preamplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) per the protocol of the manufacturer. For the pilot, for each cDNA sample, 384 miRNAs including 6 controls (RNU44, RNU48, 4*U6) were profiled using TaqMan Array MicroRNA Human Card A V2.0 (Applied Biosystems). For the main study, custom designed Megaplex cards were generated to determine the expression of selected set of miRNAs. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). miRNAs levels were assessed in patients for which sufficient amounts of plasma were available (ie, stable group, $n = 13$; AR D0, $n = 13$; AR M1, $n = 8$; AR M6, $n = 9$; AR m12, $n = 11$). We used the difference in total signal of all miRNAs as a correction factor for normalization.

Statistical Analyses

Continuous normally distributed data are presented as mean $\pm$ SEM, unless stated otherwise. Differences between 2 groups in the cross-sectional study were analysed using the unpaired 2-sample T test. When criteria for parametric testing were not met, median and interquartile range are presented and tested with the Mann-Whitney test. For categorical variables, cross-tables were used and analyzed with the χ^2 test. In addition, multivariable linear regression was used to adjust for possible confounders. Comparisons of mean differences between the different time points in the longitudinal study were performed using analysis of variance. Correlations between interval variables were calculated using the Spearman rank correlation coefficient. Differences were considered statistically significant with P less than 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

Characteristics of Cross Sectional and Longitudinal AR Study Population

To investigate the relation of circulating miRNAs and markers of systemic vascular injury with AR, we set up a cross sectional study ("cohort A") including AR patients and stable controls (stable allograft function after transplantation), allowing us to collect plasma for the assessment of vascular injury markers and circulating miRNAs. In addition, these AR patients were studied longitudinally at 1, 6, and 12 months after rejection to determine long-term effects. Baseline subject and transplantation characteristics are presented in Table 1 and Table S1, SDC (<http://links.lww.com/TXD/A45>)

(Banff Scores and pathological characteristics). All rejections in this cohort involved T cell-mediated rejections. Renal function of the patients with stable allograft function (47.2 ± 13 mL/min per 1.73 mm^2) was better compared with AR patients (38.1 ± 13 mL/min per 1.73 mm^2 , $P < 0.05$). In the longitudinal study (Table 1) patients showed significant decreased renal function during all time points after AR compared with stable patients.

Patients With AR Have Increased Capillary Tortuosity and Elevated Levels of Ang-2/Ang-1 Ratios, VEGF and sTM

We investigated whether AR resulted in changes in capillary tortuosity, indicating systemic vascular injury. Indeed, patients with AR showed significantly increased capillary tortuosity compared to stable patients (1.70 ± 0.1 vs. 1.41 ± 0.06 , $P > 0.01$) (Figure 1AB), also after adjustment for age, sex, BMI, blood pressure, glucose levels and smoking. Capillary density showed no significant difference between the stable renal function group and patients with AR ($P = \text{NS}$, data not shown).

Next, to evaluate the effects of AR on the expression of angiogenic factors, we calculated the Ang-2/Ang-1 ratio and measured serum levels of VEGF and sTM. The Ang-2/Ang-1 ratio was significantly increased in AR group (0.09 ± 0.02) compared with stable renal transplant recipients (0.05 ± 0.01 , $P = 0.01$) (Figure 1C). In line with these observations, both VEGF (567 ± 188 pg/mL vs 202 ± 27 pg/mL, $P = 0.02$) and sTM ($19\,667 \pm 1809$ pg/mL vs 9667 ± 921 pg/mL, $P < 0.0001$) levels were increased in AR patients compared with stable recipients (Figure 1DE). The differences remained significant after adjustment for age, sex, BMI, blood pressure, glucose levels, and smoking ($P < 0.05$). When adjustment for kidney function was performed, sTM, VEGF, and Ang2/1 ratios remained significantly different between stable and AR, whereas the change in capillary tortuosity became borderline significant ($P = 0.07$).

AR Results in a Shift in Circulating miRNA Levels

Next, we sought to determine circulating miRNA levels, because they could serve as markers of microvascular injury as they are central regulators of microvascular pathophysiology. To select candidate miRNAs that are associated with AR we performed a pilot study assessing plasma profiles of 384 miRNAs from 4 stable controls and 6 patients with AR (Table 3A and 3B, SDC, <http://links.lww.com/TXD/A45>). Based on (i) differential expression (based on P value) between the AR and stable group, (ii) high range expression levels and (iii), a reported relation of a miRNA with rejection or processes related to microvascular destabilization, we selected 48 miRNAs (indicated in Table S3A, SDC, <http://links.lww.com/TXD/A45>) that associated with AR, for further evaluation in the main study.

The 48 selected miRNAs were subsequently assessed in the plasma of AR and stable patients. We found 8 of them to be significantly decreased (miR-135a, miR-199a-3p, and miR-15a) or increased (miR-17, miR-140-3p, miR-130b, miR-122, and miR-192) in AR compared with the stable group, after adjustment for sex and age as possible confounders (Figure 2). Complete miRNA data (AR vs stable) can be found in Table S4, SDC (<http://links.lww.com/TXD/A45>).

Selective Effects on Vascular Injury Markers and miRNAs in the First Year After AR

During the follow-up period after AR, capillary tortuosity remained significantly increased at 1 month (1.72 ± 0.1 , $P < 0.01$), 6 (1.71 ± 0.4 , $P < 0.05$) and 12 months (1.74 ± 0.1 , $P < 0.001$) compared to patients with a stable renal function (Figure 3AB). Also, the Ang-2/Ang-1 ratio remained elevated at 1 month (0.07 ± 0.02 , $P = 0.35$), 6 months (0.06 ± 0.02 , $P = 0.44$) and 12 months (0.07 ± 0.02 , $P = 0.36$) after AR compared with stable renal transplant recipients (0.05 ± 0.01), although not statistically significant (Figure 3C). Furthermore, VEGF serum levels remained significantly increased up to 1 month after rejection (465 ± 109 pg/mL) compared with stable renal transplant recipients (202 ± 27 pg/mL, $P = 0.03$) but decreased at 6 (368 ± 150 pg/mL, $P = 0.23$) and 12 months (257 ± 95 pg/mL, $P = 0.42$) (Figure 3D). Mean sTM levels similarly decreased at 6 months ($14\,927 \pm 2330$ pg/mL, $P = 0.02$) and 12 months ($10\,875 \pm 1549$ pg/mL, $P < 0.05$) after rejection compared to stable patients (Figure 3E), while 12 months after rejection sTM was also significantly different from the AR group ($P < 0.05$). The differences remained significant after correction for age, sex, BMI, blood pressure, glucose levels and smoking.

We next assessed whether we could detect differences in the levels of the 48 selected miRNAs in the patients with AR after 1-year follow-up. For the 8 miRNAs that differed statistically significant between stable and AR, we found 4 of them (miR-140-3p, miR-130b, miR-122, and miR-192) to show (a trend towards) normalization of their levels within 1 year after AR (Figure 4A). In contrast, miR-199a-3p, miR-15a, miR-135a, and miR-17 remained either decreased or increased until 1 year after AR (Figure 4B). The longitudinal course of all 48 miRNAs can be found in Table S5, SDC (<http://links.lww.com/TXD/A45>).

Given that (time after) transplantation itself might contribute to changes in these vascular injury markers and miRNA levels, we included an additional control patient cohort ("cohort B") that includes kidney transplantation patients with stable renal function after transplantation, that were followed up to 12 months after transplantation. This allows distinguishing the effects of transplantation itself versus AR for these markers. Patient characteristics of this cohort are included as Table S2, SDC (<http://links.lww.com/TXD/A45>). In this cohort, we determined capillary tortuosity, Ang-2 (as we found Ang-2/Ang-1 ratio to be mainly determined by Ang-2 levels) and sTM. We found capillary tortuosity, Ang-2 and sTM levels to restore within 12 months after transplantation (Figure S1, SDC, <http://links.lww.com/TXD/A45>). Furthermore, we measured the levels of the 8 miRNAs from Figure 2 in this additional control patient cohort (cohort B). These data are illustrated in Figure S2, SDC (<http://links.lww.com/TXD/A45>) demonstrating that the differences induced by transplantation itself are occurring within 1 month after transplantation, suggesting that the differences in the AR cohort cannot be the result of the transplantation or differences in time posttransplantation.

Specific miRNAs Associate With Markers of Vascular Injury

Given the relation between AR and vascular injury, we next sought to determine if we could establish correlations of

vascular injury markers with specific circulating miRNAs (in cohort A). In addition, we determined correlation with renal function (creatinine levels, proteinuria and eGFR). As shown in Table S6, SDC (<http://links.lww.com/TXD/A45>), which contains P values and correlation coefficients for all comparisons, we found multiple significant correlations. Figure 5 illustrates the highlights of the significant correlations that we observed. Specifically, miR-130b, miR-199a-3p, and miR-192, 3 miRNAs, which were significantly different between the stable and AR group, showed significant correlations with sTM (miR-130b and miR-199a-3p) and capillary density (miR-192). In addition, we also found several correlations for miRNA with vascular injury markers, although their levels were not affected by AR, for example, miR-29a, miR-93, and miR-361-5p significantly correlated with Ang-2-1 ratio, capillary tortuosity and VEGF levels, respectively (Table S6, SDC, <http://links.lww.com/TXD/A45>).

DISCUSSION

In the present study, we found that the systemic microvasculature is markedly disturbed in patients with AR compared with stable renal transplant recipients. In addition, we demonstrated that specific circulating miRNAs associate with AR and vascular injury.

Although it cannot be excluded that transplantation itself contributes to altered levels of vascular injury markers, our data regarding systemic vascular injury markers in the longitudinal AR study show that Ang-2 and capillary tortuosity maintain elevated after AR, but reverse within 1 year after transplantation, as determined in stable transplantation patients that were followed for a similar time (Figure 3 and Figure S1, SDC, <http://links.lww.com/TXD/A45>). These data suggest AR-specific effects on capillary tortuosity and Ang-2. This prolonged Ang-2 induced destabilization promotes the dissociation of pericytes from ECs, resulting in the formation of abnormal capillary networks and abnormal blood flow.²⁶ Hence, early induced endothelial injury appears to be sustained which might impact long-term graft function.³¹

Although initially, it was thought that circulating miRNA populations were merely shed by-products of tissue damage, recent studies suggest that circulating miRNAs can be transferred to vascular target cells thereby playing an active role in vascular (dys)function.¹⁴ MiRNAs in the bloodstream are found packaged in exosomes³² or associated with RNA-binding proteins (Argonaute2 [Ago2])³³ or lipoprotein complexes (high-density lipoprotein).³⁴ Exosomal miRNAs have been described to be transferred to and be functional in target cells in various tissues.³⁵ The source of these miRNA-containing exosomes during AR may be the immune cells that drive the inflammatory response³⁶ or they possibly originate from the donor graft.³⁷ As such, we believe that the circulating miRNAs we identified may play a role in the pathophysiology of AR and associated vascular injury. A promising candidate miRNA potentially relevant to AR pathophysiology that emerged from our study is miR-130b. In our previous study on circulating miRNAs in the setting of diabetic nephropathy,¹³ we found a strong correlation of miR-130b with sTM levels as well, similar to the findings in this study. Furthermore, miR-130b was previously found to be present and increased in

(adipocyte-derived) exosomes in metabolic disease,³⁸ whereas it was recently demonstrated that these adipose-derived exosomal circulating miRNAs are transferred to target cells in various tissues exerting their function,³⁵ providing proof of principle of (distant) cell-cell communication via exosomal miRNAs. As such, in our study, it can be argued that exosomal-miR-130b (regardless of the source) can be transferred to ECs where it may have pathogenic effects, for example, via targeting PPAR γ ,³⁹ which subsequently leads to EC activation (and thus increased sTM levels) and an aberrant angiogenic response.^{40,41} Of note, increased levels of circulating miR-130b were also found to be associated with renal damage in lupus nephritis.⁴²

Similarly, we found high miR-192 levels to correlate with decreased capillary density, whereas miR-192 was previously found to be increased in exosomes in the setting of cardiovascular disease⁴³ and to play an essential role in (tumor) angiogenesis.⁴⁴ Additionally, it has been demonstrated that exosomal miR-192 can be transferred to ECs and subsequently abrogate the normal angiogenic program by targeting IL-8, ICAM, and CXCL1.⁴⁵ Furthermore, miR-192 has been described to be involved in the development of renal fibrosis,⁴⁶ whereas IF/TA is often observed in rejection and causing deterioration of renal allograft function.⁴⁷ These data suggest a pathogenic link of these miRNAs to AR and vascular injury, although additional functional studies are required to further validate these associations and mechanisms. Circulating miRNAs thus represent promising candidate biomarkers for early monitoring of microvascular disease, because it is increasingly recognized that miRNAs play a major role in maintaining microvascular integrity,¹⁰ whereas these circulating miRNAs may also provide insight in the identification of disease specific deregulated molecular mechanisms.^{14,15}

Furthermore, although most patients received a similar drug treatment regimen, we cannot exclude a role for (dose-dependent) immunosuppression-mediated effects on vascular injury and miRNA levels in these patients. Also, because levels of several miRNAs associate with kidney function, it can be argued that in patients with kidney dysfunction, a reduced clearance effect could cause increased levels of these circulating miRNAs. However, a recent study provided evidence against such an effect,⁴⁸ suggesting kidney function independent effects as a cause for these differential miRNA levels. Indeed, statistical adjustment for kidney function (eGFR) showed limited influence on our results (in Figure 1).

Although speculative, selected circulating miRNAs could potentially serve as a biomarker for AR, but additional validation studies may be necessary to evaluate this potential. An additional application for the use of these noninvasive biomarkers might be found in the monitoring of the impact of novel therapeutics on vascular integrity. For instance, belatacept, which is a selective blocker of costimulation in acquired immunity designed to provide effective immunosuppression while avoiding the toxicities associated with calcineurin inhibitors,⁴⁹ may be sparing the vasculature as well.⁵⁰ Furthermore, assessment of vascular integrity might be valuable in currently performed therapies with mesenchymal stromal cells in renal transplant recipients.^{51,52} Circulating miRNAs might serve as such vascular injury markers, and could potentially be ideally suited to monitor these effects.

Taken together, the current study demonstrates that circulating microRNAs associate with AR and (systemic) microvascular injury. Although additional (functional) studies are necessary to gain more insight, our data suggest that specific circulating miRNAs might participate and provide insight in the pathophysiology of AR, and may potentially serve as a readily assessable biomarker for monitoring the injury status of the microvasculature.

ACKNOWLEDGMENTS

The authors want to acknowledge and thank Rients de Boer and Rohit Timal for participating in the data collection and SDF measurements.

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