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Chapter 3

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

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Abstract

**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 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 (Ang-2)/angiopoietin-1 (Ang-1)-ratio, vascular endothelial growth factor (VEGF) and soluble thrombomodulin (sTM). Following 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), that 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 VEGF and sTM normalized within one year after AR. Out of 48 selected miRNAs, eight 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 while 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.

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Introduction

In the context of transplantation, microvascular endothelial cells (ECs) are very susceptible to injury that can result from episodes of acute rejection (AR). Following 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 (IFTA) and graft dysfunction (1, 2, 5). The rate-limiting role of microvascular 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 transplantation (SPK) (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 employed sidestream darkfield (SDF) imaging, a non-invasive tool to visualize the human microcirculation (28) that we previously used to investigate the labial mucosal capillary tortuosity as marker for systemic microvascular disease in diabetes mellitus type I (DM) patients before and after SPK (29). Finally, in a longitudinal study, patients were followed...
up for one 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.

**Subjects 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 (LUMC) 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/ml first 6 weeks then 3250 ng.h/ml) or tacrolimus (AUC 210 ng.h/ml first 6 weeks, then 125 ng.h/ml) and mycophenolate mofetil (MMF) (AUC 30-60 ng.h/ml). In case of side effects patients were converted to everolimus (AUC 120-150 ng.h/ml). All patients received induction treatment with basiliximab (40 mg at day 0 and 4) or daclizumab (100 mg/day on the day of transplantation and 10 days after transplantation).

**Cross sectional and longitudinal acute rejection (AR) study population**

In the cross sectional study, a total of 38 patients (24 males and 14 females) 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 those 38 patients, the control group (stable group) consisted of 25 kidney transplant recipients with of eGFR>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 acute rejection. Biopsies were evaluated using the Banff criteria by two independent pathologists. Pathological characteristics including Banff grade are summarized in Table 1 and Supplementary Table 1 (http://links.lww.com/TXD/A45). All transplant recipients were negative for pre-transplant donor specific antibodies (DSA), as well as de
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novo DSA after transplantation. Also, viral (BK, CMV) or urinary tract infections were absent. Patients with SPK, active infection, liver failure, active auto-immune 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, two 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 Supplementary Figures 1 and 2 (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 (CKD) patients were included because they received a kidney transplant. The patients in the CKD 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 Supplementary Table 2 (http://links.lww.com/TXD/A45).

Microcirculatory imaging
The SDF microscan (MicroVision Medical Inc., Wallingford, PA, U.S.A) 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 two 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/mm$^2$) 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-hours urine. The eGFR
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was calculated with plasma creatinine concentration using the Modification of Diet in Renal Disease (MDRD) formula. For miRNA analysis, plasma was harvested by centrifugation of EDTA-anti-coagulated blood for 10 min at 3000 rpm and subsequently stored at -80˚C. At the same time, blood was collected 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, USA) according to the manufacturer supplied protocol. Likewise, VEGF and sTM (Gen-Probe Diaclone Research, Besançon, France) levels were assessed. Since 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 TRlzol µL reagent (Invitrogen, Breda, The Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) with an adapted protocol. In short, 160 µL of chloroform was added to the plasma/TRlzol sample and centrifuged for 15 min at 12000 rpm. The aqueous phase was combined with 1.5 volumes of 100% ethanol and transferred to a MinElute Spin column and centrifuged for 15 sec at 13000 rpm. Subsequently the column was washed with 700 µL RWT buffer, twice with 500 µL RPE buffer and centrifuged for 15 sec at 13000 rpm after the first two washing steps and 2 min at 13000 rpm after the last washing step. Finally, RNA was eluted with 15 µL 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, USA). cDNA was preamplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) according to 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 (i.e. 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 ± SEM, unless stated otherwise. Differences between two groups in the cross-sectional study were analysed 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. For categorical variables cross-tables were used and analysed with the chi-square 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 ANOVA analysis. Correlations between interval variables were calculated using the Spearman rank correlation coefficient. 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**

**Characteristics of cross sectional and longitudinal acute rejection (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 Supplementary Table 1 (http://links.lww.com/TXD/A45) (Banff Scores and pathological characteristics). All rejections in this cohort involved T-cell-mediated rejections (TCMR). Renal function of the patients with stable allograft function (47.2±13 ml/min/1.73 mm²) was better compared to AR patients (38.1±13 ml/min/1.73 mm², p<0.05). In the longitudinal study (Table 1) patients showed significant decreased renal function during all time points after AR compared to stable patients.
**Table 1:** Patients characteristics of acute rejection patients during (D0) and one (M1), six (M6) and twelve (M12) months after AR compared with stable renal transplant recipients.

| Patients characteristics                  | Stable renal transplant (N=25) | D0 (N=13) | M1 (N=13) | M6 (N=13) | M12 (N=11) |
|-------------------------------------------|---------------------------------|-----------|-----------|-----------|------------|
| **Age (years)**                           | 53.2 ± 13                       | 51.7 ± 10 | 51.8 ± 10 | 52.4 ± 10 | 52.7 ± 10  |
| **Sex, male N (%)**                       | 15 (60%)                        | 9 (69%)   | 1 (8%)    | 1 (8%)    | 1 (8%)     |
| **Smoking, N (%)**                        | 3 (12%)                         | 1 (8%)    | 1 (8%)    | 1 (8%)    | 0          |
| **Median time since transplantation (months) (IQR)** | 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/m²)**                           | 25.8 ± 4                        | 24.3 ± 3  | 24.3 ± 3  | 23.8 ± 4  | 25.2 ± 4   |
| **Dialysis, N (%)**                       | 4 (16%)                         | 0         | 0         | 0         | 0          |
| **Systolic BP (mmHg)**                    | 137 ± 20                        | 142 ± 19  | 132 ± 10  | 132 ± 16  | 127 ± 19   |
| **Diastolic BP (mmHg)**                   | 82 ± 9                          | 83 ± 13   | 77 ± 9    | 79 ± 12   | 72 ± 10    |
| **eGFR (ml/min/1.73 m²)**                 | 47.2 ± 13                       | 38.1 ± 13*| 35.4 ± 15*| 40.2 ± 16*| 36.0 ± 18* |
| **Median proteinuria (g/24hr) (IQR)**     | 0.2 (0.2-0.4)                   | 0.4 (0.3-1.0)* | 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*  | 6.3 ± 3   | 5.9 ± 1*   |
| **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|
### Table 1: Continued.

| Patients characteristics       | Stable renal transplant (N=25) | D0 (N=13) | M1 (N=13) | M6 (N=13) | M12 (N=11) |
|-------------------------------|-------------------------------|-----------|-----------|-----------|------------|
| **Anti-hypertensives, 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%)    |
| Angiote nsin-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%) | 11 (100%) | (82%)      |
| Everolimus                    | 2 (8%)                        | 1 (8%)    | 0         | 0         | 1 (9%)     |
| Mycophenolate Mofetil         | 21 (84%)                      | 9 (69%)   | 9 (69%)   | 10 (77%)  | 7 (64%)    |
| **Anti-rejection, N (%)**     |                               |           |           |           |            |
| Methylprednisolone            |                               | 11        |           |           |            |
| ATG                           | 1 (8%)                        |           |           |           |            |
| Alemtuzumab                   | 1 (8%)                        |           |           |           |            |
| **Donor characteristics**     |                               |           |           |           |            |
| Age (years)                   | 52.3 ± 9                      |           |           |           |            |
| Sex, male N (%)               | 8 (32%)                       |           |           |           |            |
| Donortype, deceased donors, N (%)| 5 (20%)       | 5 (39%)   |           |           |            |

All data are mean ±SD, unless otherwise specified. *p<0.05 compared to stable patients. †P<0.05 compared to AR. BMI, body mass index; BP, blood pressure; ACE, angiotensin converting enzyme; eGFR, estimated glomerular filtration rate; IQR, interquartile range.

**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) (Fig 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=NS, data not shown).
Figure 1. Increase in markers of vascular injury in acute rejection.
(A) Sidestream darkfield 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 soluble thrombomodulin (E) serum levels (pg/ml) in stable and AR group. Data shown are mean ± SEM. *P<0.05 compared to stable group.

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) (Fig 1C). In line with these observations, both VEGF (567 ±188 pg/ml vs. 202 ±27 pg/ml, p=0.02) and sTM (19667 ±1809 pg/ml vs. 9667±921 pg/ml, p<0.0001) levels were increased in AR patients compared to stable recipients (Fig 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, while the change in capillary tortuosity became borderline significant (p=0.07).

**Acute rejection results in a shift in circulating miRNA levels**

Next, we sought to determine circulating miRNA levels, since 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 acute rejection (Supplementary Table 3A and 3B (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 Supplementary Table 3A (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 to the stable group, after adjustment for sex and age as possible confounders (Figure 2). Complete miRNA data (AR vs. stable) can be found in Supplementary Table 4 (http://links.lww.com/TXD/A45).

Figure 2. Differential miRNA levels in acute rejection.
MiRNAs that show statistically significant differences between stable and acute rejection (AR) group (*P<0.05, **P<0.001). Means are relative expression levels and can also be compared amongst miRNAs. Stable group, n=13; AR, n=13. Data shown are mean ± SEM and adjusted for sex and age as possible confounders.
Selective effects on vascular injury markers and miRNAs in the first year after acute rejection

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 (Fig 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 (Fig 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) (Fig 3D). Mean sTM levels similarly decreased at 6 months (14927 ±2330 pg/ml, p=0.02) and 12 months (10875 ±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.

Figure 3. Normalization of VEGF and sTM, but not of Ang-2/Ang-1 ratio and microvascular tortuosity, within one year after acute rejection.

(A) Sidestream dark field images of the oral mucosa visualizing the microvascular capillaries of a representative stable kidney transplant patient, one (M1), six (M6) and twelve (M12) months after rejection in the longitudinal rejection study. Black arrows: capillary loops. (B) Longitudinal course of the mean tortuosity index of microvascular capillaries. (C) Evolution of the Ang-2/Ang-1 ratio with time. (D) VEGF serum levels with time. (E) Mean sTM levels with time.
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Figure 3. Continued.
capillaries in the stable group (n=25), acute rejection (AR) group (n=13) and at one (M1, n=13), six (M6, n=13) and
twelve (M12, n=11) months after rejection. (C-E) Longitudinal course of serum Ang-2/Ang-1 ratio (C), VEGF-A (D)
and soluble thrombomodulin (E) levels at one, six and twelve months after rejection. Data are shown as mean ±
SEM. *P<0.05.

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 Supplementary Table 5 (http://links.lww.com/TXD/A45).

Figure 4. Circulating miRNA levels in patients with acute rejection, followed up for one year.
(A-B) Longitudinal course of the circulating miRNA levels in the stable group, acute rejection (AR) group and at one
(M1), six (M6) and twelve (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 signifi-
cant. Means are relative expression levels, and can also be compared amongst 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 ± SEM.

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 Supplementary Table 2 (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 (Supplementary Figure 1 (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 Supplementary Figure 2 (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 post-transplantation.

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 Supplementary Table 6, 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, three miRNAs that 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, e.g. miR-29a, miR-93 and miR-361-5p significantly correlated with Ang-2-1 ratio, capillary tortuosity and VEGF levels, respectively (Supplementary Table 6 (http://links.lww.com/TXD/A45)).

![Figure 5](http://links.lww.com/TXD/A45)

**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.
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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 Supplementary Figure 1 (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).

While 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 exosomes (32) or associated with RNA-binding proteins (Argonaute2 [Ago2]) (33) or lipoprotein complexes (high-density lipoprotein [HDL]) (34). In particular 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 response (36) 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), while 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 endothelial cells where it may have pathogenic effects e.g. via targeting PPARγ (39), which subsequently leads to endothelial cell 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, while miR-192 was previously found to be increased in exosomes in the setting of cardiovascular disease (43) and to play an essential role in (tumour) angiogenesis (44). Additionally, it has been demonstrated that exosomal miR-192 can be transferred to endothelial cells 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, since it is increasingly recognized that miRNAs play a major role in maintaining microvascular integrity (10), while 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, as 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 co-stimulation 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.

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Disclosures
The authors declare no conflicts of interest.

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