Received: 7 January 2020 | Revised: 12 April 2020 | Accepted: 13 April 2020
DOI: 10.1111/ajt.15961

ORIGINAL ARTICLE

AJT

Diabetic nephropathy alters circulating long noncoding RNA levels that normalize following simultaneous pancreas–kidney transplantation

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Funding information
Dutch Kidney Foundation, Grant/Award Number: 16OKG16; European Foundation for the Study of Diabetes

Simultaneous pancreas–kidney transplantation (SPKT) replaces kidney function and restores endogenous insulin secretion in patients with diabetic nephropathy (DN). Here, we aimed to identify circulating long noncoding RNAs (lncRNAs) that are associated with DN and vascular injury in the context of SPKT. Based on a pilot study and a literature-based selection of vascular injury–related lncRNAs, we assessed 9 candidate lncRNAs in plasma samples of patients with diabetes mellitus with a kidney function >35 mL/min/1.73 m^2 (DM; n = 12), DN (n = 14), SPKT (n = 35), healthy controls (n = 15), and renal transplant recipients (KTx; n = 13). DN patients were also studied longitudinally before and 1, 6, and 12 months after SPKT. Of 9 selected lncRNAs, we found *MALAT1*, *LIPCAR*, and *LNC-EPHA6* to be higher in DN compared with healthy controls. SPKT caused *MALAT1*, *LIPCAR*, and *LNC-EPHA6* to normalize to levels of healthy controls, which was confirmed in the longitudinal study. In addition, we observed a strong association between *MALAT1*, *LNC-EPHA6*, and *LIPCAR* and vascular injury marker soluble thrombomodulin and a subset of angiogenic microRNAs (*miR-27a*, *miR-130b*, *miR-152*, and *miR-340*). We conclude that specific circulating lncRNAs associate with DN-related vascular injury and normalize after SPKT, suggesting that lncRNAs may provide a promising novel monitoring strategy for vascular integrity in the context of SPKT.

KEYWORDS
clinical research/practice, translational research/science, pancreas/simultaneous pancreas-kidney transplantation, molecular biology, diabetes: secondary complications, molecular biology

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Abbreviations: Ang-2, angiopoietin-2; D0, before transplantation; DM, diabetes mellitus; DN, diabetic nephropathy; EV, extracellular vesicle; KTx, kidney transplantation; lncRNA, long noncoding RNA; LUMC, Leiden University Medical Center; M1, 1 month after transplantation; M12, 12 months after transplantation; M6, 6 months after transplantation; miRNA, microRNA; SPKT, simultaneous pancreas–kidney transplantation; sTM, soluble thrombomodulin.

1 | INTRODUCTION

Diabetes mellitus (DM) is a major cause of end-stage renal disease and leads to microvascular complications such as retinopathy and neuropathy.[1-3] Because diabetic nephropathy (DN) is characterized by albuminuria and elevated blood pressure, the main early goals in preservation of kidney function, in addition to preventing hyperglycemia, are reducing microalbuminuria and hypertension.[4] Ultimately, when end-stage renal disease develops, simultaneous pancreas–kidney transplantation (SPKT) is a preferred treatment option that replaces kidney function and restores endogenous insulin secretion in patients with DN.

However, integrity of the vasculature is a rate-limiting factor in the long-term outcome of organ transplants.[5] Although the endothelial dysfunction associated with DN is partly restored after transplantation,[6-8] the endothelium in SPKT is further challenged by transplant-specific adverse effects such as ischemia–reperfusion injury and following the use of immunosuppressive drugs, such as steroids and calcineurin inhibitors, that exhibit unfavorable effects on the vasculature. In addition, viral infections or acute rejection are known to affect microvascular integrity.[9-11] Taken together, due to these risk factors, the vasculature is continually challenged. Thus, to preserve graft function, monitoring of microvascular integrity may be of high clinical value as patients could receive targeted treatment.

We previously demonstrated that SPKT reversed microvascular damage in DN[8] and found that specific microRNAs (miRNAs) are associated with DN and microvascular impairment and vascular injury markers, such as angiopoietin-2 (Ang-2) and soluble thrombomodulin (sTM).[7,12] Recently, long noncoding RNAs (lncRNAs) have been recognized as important regulators of gene expression and may be promising candidate biomarkers for early recognition of disease progression.[13] LncRNAs are defined as noncoding transcripts longer than 200 nucleotides, interfere with a variety of cellular processes, and are involved in the pathophysiology of a broad range of diseases including kidney and vascular diseases.[14,15] For example, increased levels of the lncRNA *MALAT1* have been described to associate with DM and the development of organ dysfunction, such as retinopathy and nephropathy, by contributing to inflammation and the impaired response of endothelial cells to glucose.[16] Also, the long noncoding megacluster (lnc-MGC), hosting a cluster of nearly 40 miRNAs has been described to be involved in the development of diabetic kidney disease, most likely via endothelium reticulum stress–dependent mechanisms.[17] However, although an initial study demonstrated differences in circulating lncRNA levels in DN patients compared with healthy controls,[18] little is known about the relation of circulating lncRNAs with DN and vascular injury, in particular in the unique context of SPKT.

The aim of this study was to identify lncRNAs that are associated with SPKT and (micro)vascular injury. This could provide more insight in the development of vascular complications and may identify specific lncRNAs to be of benefit for predicting or combating vascular injury progression.

2 | MATERIALS AND METHODS

2.1 | Study cohort

Study design and all study procedures were approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC), and written informed consent was obtained from all participants.

In a single-center, cross-sectional, observational study, 78 individuals aged 18 years or older were enrolled. Four groups of patients with DM type 1, all treated in the outpatient clinic of the LUMC, were included: a group of DM patients with signs of early DN (eGFR > 35 mL/min/1.73 m^2) (DM; n = 12), a group of DM patients with DN on the waiting list for SPKT (DN; n = 14), a group of DM patients with functioning pancreas and kidney grafts (SPKT; n = 35), and a group of DM patients with a functioning kidney graft (KTx; n = 13) consisting of 10 patients with a solitary kidney transplant and 3 patients who initially received an SPKT but lost their pancreatic graft within 4 days after transplantation due to vascular thrombosis. A control group consisted of 15 healthy, age-matched volunteers. Only patients with a sufficient amount of plasma for all required assays were included in this study. Exclusion criteria were active infection or autoimmune disease, liver failure, epilepsy, and malignancy in the past 5 years (excepted full remission after treatment for basal cell carcinoma).

This cohort was previously described and was studied for a selection of circulating miRNAs for microvascular endothelial injury, sTM, and angiopoietin-2 (Ang-2) in plasma samples of all participants.[8]

Sixteen DN patients who received an SPKT were followed longitudinally during the first year after transplantation. Plasma samples of these patients were obtained before and 1, 6, and 12 months after transplantation, but plasma samples for all 4 timepoints were not available for all 16 patients. The available group size for each timepoint is shown in Table 2.

2.2 | Identification of candidate lncRNAs

To identify candidate lncRNAs, we performed a pilot study and a literature-based selection. For the pilot study, we selected candidate lncRNAs by assessing plasma profiles of 40 173 lncRNAs in 6 randomly selected healthy controls and 6 DN patients. lncRNAs were selected based on differential expression ($P < .001$ or a fold change > 50 combined with a value of $P < .05$). Second, we performed a literature search to select a set of candidate lncRNAs that have been described to associate with vascular injury (described in detail in Results). Together, this resulted in the selection of 22 lncRNAs. Using RT-qPCR validation, only 9 of these 22 lncRNAs were detectable and assessed in the whole patient cohort. To ensure robust expression, only lncRNAs with >95% of the samples showing detectable expression were selected for further analysis, yielding 4 lncRNAs: *LNC-EPHA6*, *MALAT1*, *LIPCAR*, and *LNC-RPS24*.

2.3 | Transplantations and follow-up

All vital parameters and blood and urine samples were measured and collected at the outpatient clinic of the LUMC. Both KTx and SPKT were performed in the LUMC, and these procedures were described previously.[8,19] Frequent follow-up of transplanted patients took place at the transplantation outpatient clinic in the LUMC.

All SPKT patients and 86% of KTx patients were treated with calcineurin inhibitors (65% tacrolimus, 35% cyclosporine). Prednisone use in SPKT and KTx was 70% and 60%, respectively. Most SPKT and KTx patients were treated with triple therapy including mycophenolate mofetil (73% and 93%, respectively).

2.4 | RNA isolation

By using the RNeasy Micro Kit (Qiagen) with an adapted protocol, total RNA was isolated from 200 μL plasma using 800 μL TRIzol reagent (Invitrogen). In summary, the plasma/TRIzol sample was centrifuged for 15 minutes (15 000*g*) after the addition of 160 μL chloroform. After the aqueous phase was combined with 100% ethanol (1.5 volume), it was transferred to a MinElute Spin column (Qiagen) and centrifuged for 15 seconds (18 000*g*). The column was then washed with 700 μL RWT buffer and twice with 500 μL RPE buffer. This was centrifuged for 15 seconds (18 000*g*) after the first 2 washing steps and 2 minutes (18 000*g*) after the third washing step. Then, 15 μL RNase-free water was added to elute the RNA.

2.5 | Profiling lncRNAs

The lncRNA profiling was performed by Arraystar Inc. In brief, for the microarray analysis, the Agilent Array platform was used. Sample preparation and microarray hybridization were performed according to the manufacturer's protocols with some minor modifications. Samples were amplified and transcribed into fluorescent cRNA along the entire length of the transcripts with no 3′ bias using a random priming method (Arraystar Flash RNA Labeling Kit; Arraystar). The labeled cRNA was hybridized onto the Human lncRNA Array v4.0 (8 × 60K; Arraystar), containing 40 173 lncRNAs. After washing of the slides, the arrays were scanned using the Agilent Scanner G2505C.

Agilent Feature Extraction software (version 11.0.1.1) was used for analysis of the acquired array images. The GeneSpring GX v12.1 software package (Agilent Technologies) was used for quantile normalization and subsequent data processing. After this quantile normalization of the raw data, lncRNAs that have flags in present or marginal (“all targets value”; in at least 6 of 12 samples) were selected for further data analysis. Volcano Plot filtering was used to identify statistically significant differentially expressed lncRNAs between the 2 groups. Finally, hierarchical clustering was performed to show distinguishable lncRNAs expression pattern among the groups.

2.6 | RT-qPCR

For validation of identified lncRNAs, we performed RT-qPCR. To quantify lncRNA levels, isolated RNA was reverse transcribed using Iscript (Bio-Rad) according to the manufacturer’s protocol. Quantitative PCR of target genes was done using SYBR Green Master Mix (Applied Biosystems). Used primer sequences of target lncRNAs are given in Table S1.

2.7 | Statistical analyses

All parametric data are described as mean ± SD, and nonparametric data are presented as median and IQR. Categorical variables are given as numbers and percentages. Testing for differences in Tables 1 and 2 was performed by using 1-way ANOVA for parametric data, Kruskall-Wallis test for nonparametric data, and Fisher exact test for categorical data.

All lncRNA results were normalized by the $CT^{\Delta\Delta}$ method to β-actin, as previously described.[20-23] After logarithmic transformation (with base 10), all lncRNAs showed a normal distribution and were then further analyzed. In the cross-sectional study, differences in logarithmic mean lncRNA levels were analyzed using a univariate general linear model including adjustment for sex and age. For analysis of data in the longitudinal study, a linear-mixed model analysis was used (with inclusion of repeated-measures analysis and adjustment for multiple testing). Categorical data were analyzed for differences using Friedman 2-way ANOVA by ranks. Correlations between vascular markers and lncRNAs were analyzed using the Spearman rank correlation.

A value of $P < .05$ was considered to be statistically significant. All data analysis was performed using SPSS version 23.0 (SPSS Inc.), and graphs were created using GraphPad Prism version 8.0 (GraphPad Prism Software Inc.).

3 | RESULTS

3.1 | Identification of candidate lncRNAs

To identify candidate lncRNAs that are associated with DN, we assessed plasma levels of 40 173 lncRNAs in a pilot study in 6 healthy individuals and 6 DN patients. In addition, we selected a subset of lncRNAs from the literature that were previously described to associate with vascular injury (Figure 1A describes our identification strategy). Figure 1B,C illustrates a clear differential lncRNA profile in our pilot study in plasma of DN patients compared with healthy controls (full profiling data of this pilot study can be found in Table S2). Of 40 173 lncRNAs, 11 517 (29%) were detectable in the microarray analysis: 185 were significantly upregulated and 103 were significantly downregulated ($P < .05$). We subsequently selected 13 candidate lncRNAs that were differentially expressed between DN patients and healthy controls, based

TABLE 1 Cross-sectional study patient characteristics

	HC (n = 15)	DM (n = 12)	DN (n = 14)	SPKT (n = 35)	KTx (n = 13)
Sex, male, n (%)	8 (53%)	6 (50%)	9 (64%)	23 (66%)	4 (31%)
Age (y)	44 ± 10	54 ± 14	44 ± 6[b]	48 ± 8	48 ± 10
BMI (kg/m^2)	24.8 ± 3.6	23.7 ± 2.6	24.3 ± 2.8	24.1 ± 4.2	24.6 ± 4.8
Systolic blood pressure (mm Hg)	133 ± 13	130 ± 14	141 ± 21	140 ± 23	133 ± 25
Diastolic blood pressure (mm Hg)	83 ± 7	70 ± 9[a]	84 ± 10[b]	84 ± 13[b]	78 ± 12
Haemoglobin (mmol/L)	8.8 ± 0.7	8.4 ± 1.3	7.4 ± 0.7[a,b]	8.2 ± 1.1	7.9 ± 1.0
Haematocrit (L/L)	0.42 ± 0.03	0.41 ± 0.05	0.36 ± 0.04[a,b]	0.41 ± 0.05[c]	0.40 ± 0.04
HbA_{1c} (mmol/mol)	—	53.7 ± 8.9	63.6 ± 15.6	37.7 ± 8.7[b]	67.5 ± 8.7[b]
Glucose (mmol/L)	5.2 ± 1.1	12.7 ± 5.1[a]	12.5 ± 5.7[a]	5.9 ± 2.9[b,c]	11.2 ± 5.3[a,d]
eGFR (mL/min/1.73 m^2)	93 ± 17	71 ± 24	26 ± 17[a]	52 ± 19[a,b,c]	60 ± 24[a,c]
Proteinuria (g/24 h) median (IQR)	—	0.29 (0.13-0.53)	0.68 (0.31-1.16)	0.27 (0.18-0.81)[c]	0.21 (0.21-0.36)[c]
Smoking, n (%)	0 (0%)	2 (17%)	0 (0%)	3 (9%)	1 (8%)
Antihypertensive drugs, n (%)					
ACE inhibitor	—	6 (50%)	7 (54%)	13 (37%)	6 (46%)
Angiotensin II antagonist	—	3 (25%)	5 (39%)	8 (23%)	0 (0%)[c,d]
Calcium antagonist	—	1 (8%)	6 (46%)[b]	20 (57%)[b]	5 (38%)
Diuretic	—	5 (42%)	6 (46%)	7 (20%)	4 (31%)
Statin, n (%)	—	6 (50%)	8 (62%)	24 (69%)	5 (38%)
Duration of diabetes (y)	—	33 ± 9	31 ± 9	28 ± 9	36 ± 9[d]
Time since Tx (mo) median (IQR)	—	—	—	45 (19-102)	21 (9-74)
Diabetes after SPKT, n (%)	—	—	—	3 (9%)	—

Abbreviations: BMI, body mass index; DM, diabetes mellitus (eGFR > 35 mL/min/1.73 m^2); DN, diabetic nephropathy; HC, healthy controls; KTx, kidney transplantation; SPK, simultaneous pancreas–kidney transplantation; Tx, transplantation (SPKT or KTx).

[a] $P < .05$ vs HC.

[b] $P < .05$ vs DM.

[c] $P < .05$ vs DN.

[d] $P < .05$ vs SPKT.

TABLE 2 Longitudinal study patient characteristics (n = 16)

	D0 (n = 12)	M1 (n = 12)	M6 (n = 15)	M12 (n = 14)
Sex, male, n (%)	9 (75%)	9 (75%)	12 (80%)	12 (86%)
Age (y)	44 ± 6	44 ± 6	45 ± 6	45 ± 6
BMI (kg/m^2)	24.9 ± 3.3	23.9 ± 2.7	24.1 ± 2.0	23.8 ± 2.3
Systolic blood pressure (mm Hg)	148 ± 19	127 ± 25	135 ± 23	129 ± 15
Diastolic blood pressure (mm Hg)	86 ± 12	76 ± 14	79 ± 13	78 ± 6
Haemoglobin (mmol/L)	7.5 ± 0.7	6.7 ± 1.0	7.3 ± 0.8	7.9 ± 0.9
Haematocrit (L/L)	0.36 ± 0.04	0.33 ± 0.05	0.37 ± 0.04	0.39 ± 0.05
Glucose (mmol/L)	14.5 ± 6.6	6.4 ± 1.1[a]	5.3 ± 1.4[a]	5.7 ± 1.5[a]
Proteinuria (g/24 h) median (IQR)	0.68 (0.36-0.76)	0.74 (0.39-1.40)	0.52 (0.18-0.98)	0.53 (0.14-1.08)

Abbreviations: BMI, body mass index; D0, before transplantation; M1, 1 months after transplantation; M12, 12 months after transplantation; M6, 6 months after transplantation.

[a] $P < .05$ vs D0.

on either a fold change above 50 combined with $P < .05$ or with $P < .001$. Of these lncRNAs, *DUSP4*, *G010782*, *G012233*, *G050505*, *G090324*, *GPC6-AS2*, *HOTAIR*, and *uc.48* were upregulated in DN and *LNC-EPHA6*, *G014780*, *MIR31HG*, *LNC-RPS24*, and *ZNF131* were downregulated in DN. In addition, our literature-based selection yielded 9 additional, vascular injury–related lncRNAs:

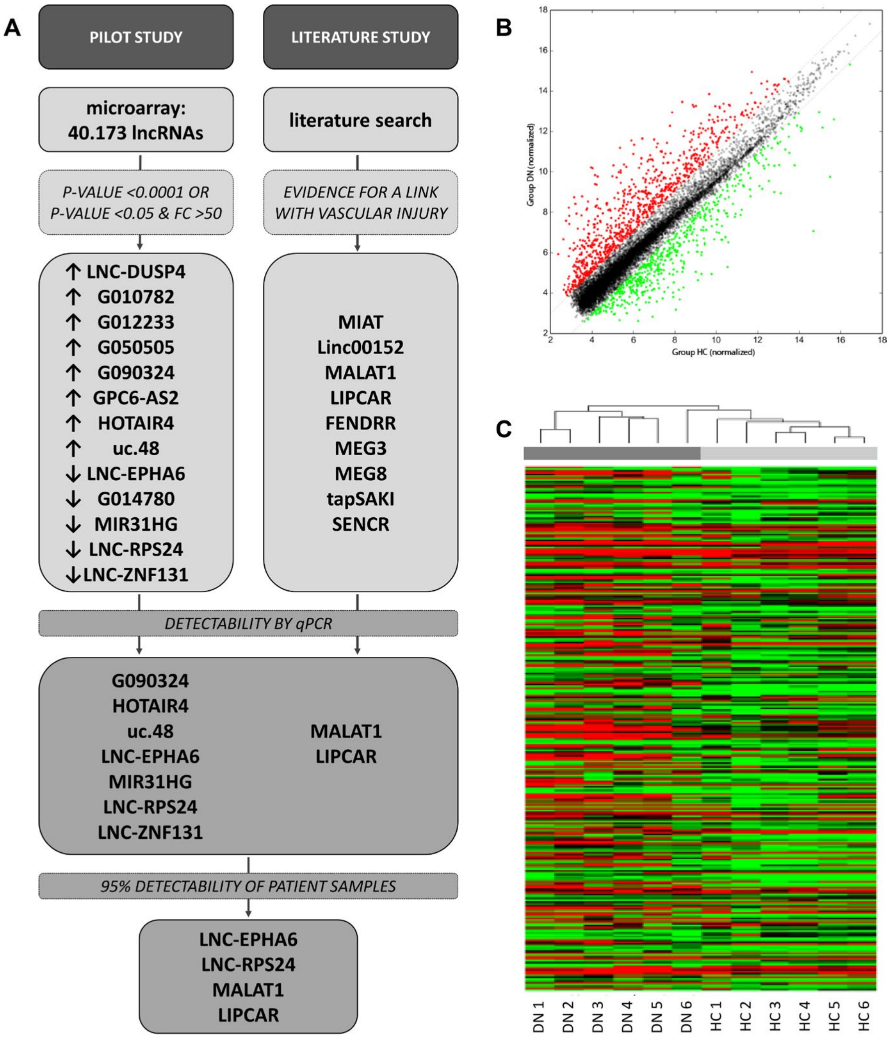

FIGURE 1 Identification of candidate lncRNAs that associate with diabetic nephropathy. A, Schematic overview of identification strategy of candidate lncRNAs, based on a pilot profiling study in plasma of 6 healthy controls (HC) and 6 diabetic nephropathy (DN) patients, as well as a literature-based selection of lncRNAs that have been described to associate with vascular injury. B, Scatterplot visualizing differential lncRNA expression between indicated conditions. The red and the green points in the plot represents the statistically significant up and downregulated lncRNAs, respectively, in DN compared with HC. C, Hierarchical clustering shows a distinguishable lncRNA expression profiling among patient plasma samples, visualized in a heatmap. Red depicts high expression; green, low expression [Color figure can be viewed at wileyonlinelibrary.com]

MIAT,[24,25] *Linc00152*,[26,27] *MALAT1*,[28,29] *LIPCAR*,[30,31] *FENDRR*,[32,33] *MEG3*,[34,35] *MEG8*,[24,36] *tapSAKI*,[37] and *SENCR*.[38,39] Next, we determined whether these lncRNAs could be validated and detected using RT-qPCR. Nine of the 22 selected lncRNAs (*MALAT1*, *LNC-EPHA6*, *LIPCAR*, *LNC-RPS24*, *G090324*, *HOTAIR*, *MIR31HG*, *uc.48*, and *ZNF131*) were detectable using RT-qPCR in the same plasma samples and were selected for analysis in the main cohort.

3.2 | Patient characteristics

The baseline characteristics of all individuals of the cross-sectional study (HC, DM, DN, SPKT, and KTx; N = 89) are presented in Table 1. Mean duration of DM in the DM, DN, SPKT, and KTx groups was > 27 years. Diabetic retinopathy was described in all patients with DN and 94% of patients with an SPKT. Due to restoration of endogenous insulin production, glucose and HbA_{1c} levels were lower in patients who received an SPKT compared with DN patients or patients who received a solitary KTx. eGFR was significantly higher in SPKT and KTx compared with DN.

3.3 | Circulating levels of specific lncRNAs associate with DM and DN

To determine the association between DN and circulating lncRNAs, we measured levels of *G090324*, *HOTAIR4*, *uc.48*, *LNC-EPHA6*, *MIR31HG*, *LNC-RPS24*, *ZNF131*, *MALAT1*, and *LIPCAR* using RT-qPCR in plasma samples of all individuals in the cohort. Only 4 of these 9 lncRNAs (*MALAT1*, *LNC-EPHA6*, *LNC-RPS24*, and *LIPCAR*) met our criteria of being detectable in >95% of the samples (Figure 1A) and were selected for further analysis.

Circulating levels of *MALAT1* and *LNC-EPHA6* were strongly increased in patients with DM compared with HC after adjustment for sex and age (P = .005 and P = .001, respectively). We also observed increased levels of *LNC-RPS24* and *LIPCAR*. Circulating levels of *MALAT1* and *LIPCAR* were significantly higher in DN patients compared with HC (P = .008 and P = .047, respectively) and a trend was observed for *LNC-EPHA6* (Figure 2). No lncRNAs showed significantly lower levels in DN patients. We also analyzed whether dialysis treatment before transplantation affected circulating lncRNA levels, but no correlation was found (data not shown).

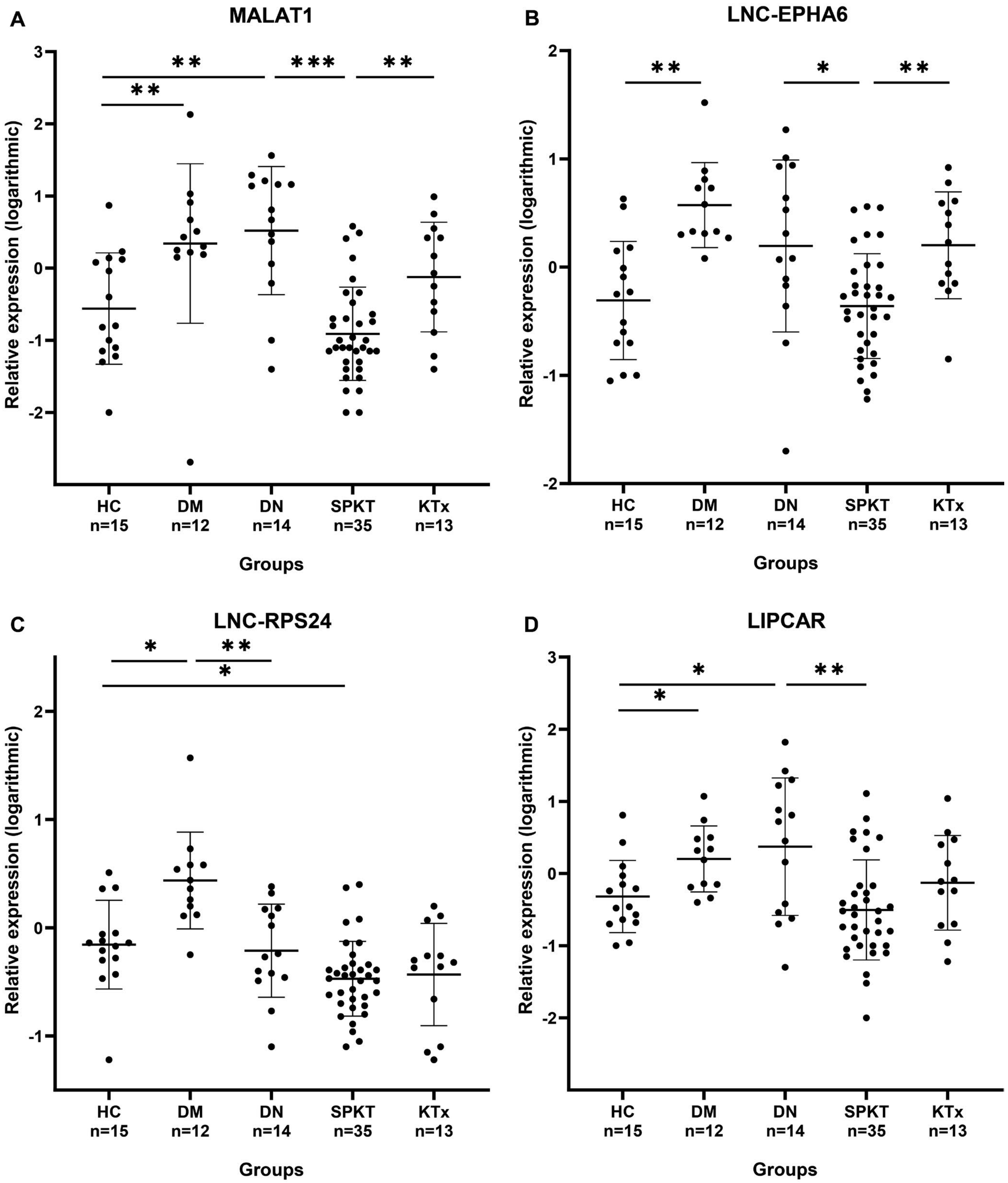

FIGURE 2 Circulating lncRNA levels are affected by diabetic nephropathy and simultaneous pancreas–kidney transplantation. Relative expression of *MALAT1* (A), *LNC-EPHA6* (B), *LNC-RPS24* (C), and *LIPCAR* (D) in the cross-sectional cohort; healthy controls (HC; n = 15), diabetes mellitus with eGFR > 35 mL/min/1.73 m^2 (DM; n = 12), diabetic nephropathy (DN; n = 14), simultaneous pancreas–kidney transplantation (SPKT; n = 35), and kidney transplantation (KTx; n = 13). lncRNA relative expression levels are depicted as logarithmic values. Data are represented as mean ± SD, *P < .05, **P < .01, ***P < .001

3.4 | Normalization of lncRNAs in SPKT patients

Given the increased lncRNA levels as a result of DN, we next sought to determine if SPKT would normalize lncRNA levels. Compared with DN patients, levels of *MALAT1*, *LIPCAR*, and *LNC-EPHA6* were significantly lower in patients with SPKT after adjustment for sex and age (P < .001, P = .007, and P = .037, respectively). *LNC-RPS24* did not differ significantly. Although *LIPCAR* levels did not significantly differ between SPKT and KTx, *MALAT1* and *LNC-EPHA6* showed higher values in the KTx group compared with the SPKT group, which implies that changes other than kidney function play a role in these altered lncRNA levels.

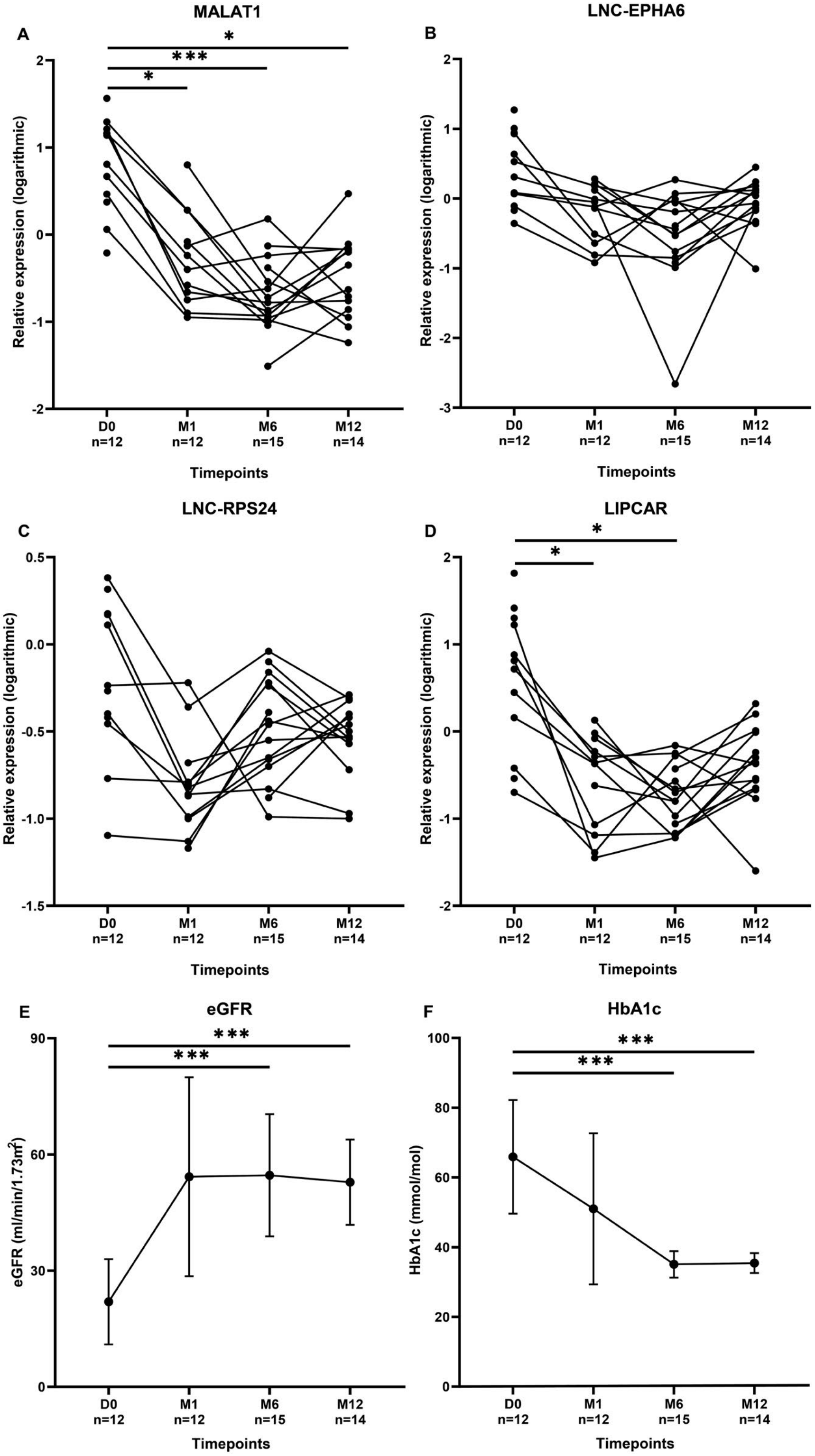

FIGURE 3 Longitudinal study validates differential lncRNA expression and indicates dynamics. Relative expression of *MALAT1* (A), *LNC-EPHA6* (B), *LNC-RPS24* (C), and *LIPCAR* (D) before (D0) and 1, 6, and 12 months (M1, M6, and M12, respectively) after simultaneous pancreas–kidney transplantation. lncRNA relative expression levels are depicted as logarithmic values. eGFR (E) improves after transplantation, and HbA_{1c} (F) declines to steady levels. Data are represented as mean ± SD, *P < .05, ***P < .001

3.5 | Dynamics of lncRNAs after SPKT

To validate the changes of lncRNAs after SPKT, we followed DN patients who received a successful SPKT in time. Plasma samples for detecting lncRNA expression were obtained before (D0) and 1, 6, and 12 months after transplantation (M1, M6, and M12, respectively). The patient characteristics are presented in Table 2. Significant improvement of eGFR and significant decline in HbA_{1c} levels after transplantation were observed and are presented in Figure 3E,F.

MALAT1 and *LIPCAR* levels decreased during the first year in accordance with the differences between DN and SPKT patients as demonstrated in the cross-sectional study cohort (Figure 3). Moreover, they appear to have normalized as early as 1 month after transplantation. *LNC-EPHA6* showed the same trend although it was not statistically significant. No further significant changes were observed following the 1-month timepoint, whereas *LNC-RPS24* levels did not change within the first year after SPKT.

3.6 | lncRNAs associate with soluble thrombomodulin and miRNAs

To assess the relationship between lncRNAs levels and vascular injury, we analyzed their correlation with vascular injury markers sTM and Ang-2. In addition, we assessed the correlation between lncRNAs and previously determined angiogenic miRNA levels in these patients (*miR-25*, *miR-27a*, *miR-126*, *miR-130b*, *miR-132*, *miR-152*, *miR-181a*, *miR-223*, *miR-320*, and *miR-326*), because previous studies showed that these miRNAs may serve as markers for vascular injury. Moreover, we analyzed the correlation between lncRNAs and kidney function (eGFR) and diabetes (HbA_{1c}). Interestingly, we found that *LIPCAR* negatively correlated with eGFR while MALAT1 correlated significantly with HbA_{1c}. When we related lncRNA levels to markers of vascular injury, we found sTM to show a positive correlation with *LNC-EPHA6* and *LIPCAR*. Furthermore, *miR-27a*, *miR-130b*, *miR-152*, and *miR-340* were correlated with MALAT1, LNC-EPHA6, and *LIPCAR*, whereas *LNC-EPHA6* also correlated with *miR-25*, after adjustment for sex, age, and multiple testing (Table 3).

4 | DISCUSSION

This study shows that plasma levels of specific lncRNAs (*MALAT1* and *LIPCAR*) are significantly higher in patients with DN compared with in healthy individuals. Both *MALAT1* and *LIPCAR*, as well as *LNC-EPHA6*, are significantly lower in patients who received an SPKT compared with DN patients. This phenomenon is confirmed in our longitudinal study where these lncRNAs show a significant decrease during the first year after transplantation. In addition, *MALAT1*, *LNC-EPHA6*, and *LIPCAR* strongly associate with sTM and angiogenic miRNAs, suggesting that the identified lncRNAs are associated with vascular injury.

TABLE 3 Correlation of lncRNAs with kidney function (eGFR), diabetes (HbA1c), and vascular injury markers sTM, Ang-2, and angiogenic miRNAs

	MALAT1	*LNC-EPHA6*	*LIPCAR*	*LNC-RPS24*
eGFR (mL/min/1.73 m^2)	−0.198 (ns)	−0.244 (ns)	**−0.412 (*P* = .003)**	−0.132 (ns)
HbA_{1c} (mmol/mol)	**0.357 (*P* = .010)**	0.244 (ns)	0.218 (ns)	0.095 (ns)
Vascular injury markers				
sTM	0.250 (ns)	**0.284 (*P* = .031)**	**0.342 (*P* = .009)**	0.091 (ns)
Ang-2	0.069 (ns)	−0.015 (ns)	0.030 (ns)	−0.200 (ns)
Angiogenic miRNAs				
miR-25	−0.062 (ns)	**−0.409 (*P* = .02)**	0.320 (ns)	−0.301 (ns)
miR-27a	**0.384 (*P* = .05)**	**0.670 (*P* < .001)**	**0.616 (*P* < .001)**	−0.006 (ns)
miR-126	−0.128 (ns)	0.074 (ns)	0.056 (ns)	0.006 (ns)
miR-130b	**0.539 (*P* < .001)**	**0.711 (*P* < .001)**	**0.658 (*P* < .001)**	0.026 (ns)
miR-132	0.243 (ns)	0.361 (ns)	0.285 (ns)	0.221 (ns)
miR-152	**0.447 (*P* = .004)**	**0.557 (*P* < .001)**	**0.503 (*P* < .001)**	−0.024 (ns)
miR-181a	0.074 (ns)	0.247 (ns)	0.216 (ns)	0.052 (ns)
miR-223	−0.297 (ns)	0.004 (ns)	−0.095 (ns)	0.002 (ns)
miR-320	0.252 (ns)	0.232 (ns)	0.269 (ns)	0.118 (ns)
miR-326	0.082 (ns)	0.245 (ns)	0.261 (ns)	0.084 (ns)
miR-340	**0.532 (*P* < .001)**	**0.657 (*P* < .001)**	**0.603 (*P* < .001)**	−0.011 (ns)
miR-574	−0.332 (ns)	−0.240 (ns)	−0.273 (ns)	0.070 (ns)
miR-660	0.319 (ns)	0.109 (ns)	0.119 (ns)	0.169 (ns)

Note: Values represent correlation coefficients and *P*-values. Bold values are significant correlations (*P* < 0.05).

Abbreviations: Ang-2, angiopoietin-2; sTM, soluble thrombomodulin.

Interestingly, *MALAT1* and *LNC-EPHA6* levels decreased after SPKT but exhibited higher levels in patients who received a KTx only. This suggests that the reduction in plasma levels of these lncRNAs is not related to restoration of renal function but might be associated with glycemic control. In line with this, we did find a significant correlation between MALAT1 and HbA_{1c} levels. The clear difference in *MALAT1* levels between the HC and DM group further supports this finding. In contrast, *LIPCAR* levels did not statistically differ between the SPKT and KTx groups, suggesting that *LIPCAR* levels are more dependent on renal function. This is confirmed by the strong correlation of *LIPCAR* with eGFR, whereas *MALAT1*, *LNC-EPHA6*, and *LNC-RPS24* do not correlate with kidney function. Nonetheless, although we have a well-defined cohort, further studies are necessary to validate these findings, as group sizes in the current study are limited. However, the longitudinally study also serves as an internal validation to confirm the results of the cross-sectional study while it illustrates the natural course of lncRNAs after restoring endogenous insulin secretion and kidney function.

Furthermore, we cannot exclude that altered levels of lncRNAs were caused by the immunosuppressive therapy that patients with SPKT received, although the KTx group serves as a control group with comparable immunosuppressive therapy. Because *MALAT1* and *LNC-EPHA6* levels differ significantly in SPKT compared with KTx, this suggests that these altered lncRNAs levels are not due to the immunosuppressive therapy.

In our study, we selected 22 candidate lncRNAs, of which only 9 were detectable using RT-qPCR. These included *MALAT1*, which was below the detection threshold in our microarray pilot. *LIPCAR* did show increased plasma levels in patients with DN compared with healthy controls in the pilot study, but this was not statistically significant due to a large range in data values (while the group sizes in the pilot were limited to n = 6). Of the 9 lncRNAs that were detectable using RT-qPCR, only 4 of these were detectable in the majority of the samples (>95%) and therefore further analyzed to enable a robust interpretation of these lncRNAs. Nonetheless, probably due to the fact that lncRNAs are often expressed at very low levels,[40] the majority of lncRNAs were either undetectable or sporadically detectable, which is consistent with previous reports,[41] suggesting only highly abundant lncRNAs may prove to be useful as biomarkers.

Given that DN and SPKT strongly associate with vascular injury,[1] we analyzed the relation of lncRNA levels with previously assessed markers of vascular injury (sTM, Ang-2, and angiogenic miRNAs).[7] We observed a correlation of *LIPCAR* and *LNC-EPHA6* with sTM, whereas we found several strong correlations of *LIPCAR*, *MALAT1*, and *LNC-EPHA6* with a specific subset of angiogenic miRNAs (*miR-27a*, *miR-130b*, *miR-152*, and *miR-340*). Although these associations may prove to be not causally related, it is interesting that, for example, *MALAT1* has been described previously in the pathogenesis of several vascular diabetic complications, such as DR and cardiomyopathy.[42-44] The reduced *MALAT1* levels after SPKT suggest an improved state of vascular health that may associate with diminished development of these secondary diabetic complications. Moreover, *LIPCAR* was previously described to be correlated with the presence of heart failure and predicts subsequent patient survival,[30] whereas in DM patients, *LIPCAR* is strongly correlated with left ventricular diastolic dysfunction, waist circumference, and plasma fasting insulin.[45] Although *LNC-EPHA6* has not been described previously, lncRNAs are often coexpressed and coregulated with their neighboring genes.[46] As such, it may be speculated that *LNC-EPHA6* function relates to the biological role of EPHA6, which is part of a family of EPH receptor tyrosine kinases, which interact with ephrins and hereby regulate important processes such as angiogenesis.[47,48] In addition, lncRNAs have often been described to influence miRNA levels by serving as an miRNA sponge,[49] providing a potential link between angiogenic miRNAs and differentially regulated lncRNAs in the context of DN. Furthermore, circulating lncRNAs are carried in extracellular vesicles (EVs) and as such may contribute to vascular injury via distant cell–cell communication. In fact, EV-containing lncRNAs and miRNAs are described as important factors in communication between organs in diabetes.[50] It is important to note that we previously described that the RNA obtained using our isolation methods contains all EV RNA, as evidenced by, among others, CD63 expression, which is a marker of EVs and electron microscopy confirmation of EV content of plasma.[51] Interestingly, we see a strong correlation of lncRNAs *MALAT1*, *LNC-EPHA6*, and *LIPCAR* with *miRNA-27a*, *miRNA-130b*, *miRNA-152*, and *miRNA-340*, which may be explained by the coappearance of these noncoding RNAs in the same EV. Indeed, these miRNAs have been previously demonstrated to be involved in cell–cell communication via EV.[51-54] Taken together, these results emphasize the potential of lncRNAs in the pathogenesis of the disease, whereas both *MALAT1* and *LIPCAR*, as well as *LNC-EPHA6*, may play an important role in angiogenesis and the development of vascular injury, suggesting changed circulating levels of these lncRNAs may reflect vascular injury in the context of DN and SPKT.

In conclusion, we are the first to demonstrate that several lncRNAs are altered in DN patients and normalize after SPKT. Our data suggest that certain lncRNAs reflect (micro)vascular damage and that these lncRNAs might provide better insight in the pathophysiology of DN and SPKT and could potentially serve as a novel tool to monitor vascular integrity.

ACKNOWLEDGMENTS

Dr Bijkerk is supported by a grant from the Dutch Kidney Foundation (16OKG16), and Drs Bijkerk and van Zonneveld are supported by a grant from the European Foundation for the Study of Diabetes (EFSD).

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Groeneweg KE, Au YW, Duijs JMGJ, et al. Diabetic nephropathy alters circulating long noncoding RNA levels that normalize following simultaneous pancreas–kidney transplantation. *Am J Transplant*. 2020;20:3451–3461. https://doi.org/10.1111/ajt.15961