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microRNAs are dysregulated in the cerebral microvasculature of CKD mice

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TABLE OF CONTENTS

1. ABSTRACT

Vascular calcification arises during chronic kidney disease (CKD), and increases the risk of cardiovascular mortality. In CKD, alterations of cerebral circulation were linked with an increase in ischemic strokes and behavioral troubles. Studying pathophysiological mechanisms of calcifications and detecting new biomarkers in the cerebral circulation is thus an important issue. microRNAs are small non-coding, single-stranded RNAs that regulate messenger RNAs at the post-transcriptional level. They are involved in numerous pathologies and represent new opportunities to develop disease predictors. We used RT-qPCR to quantify endothelial-specific microRNAs in cerebral arterioles from WT mice and from pathological models of CKD. We used four mice groups: WT SHAM, WT CKD, Apolipoprotein E Knock-Out (ApoE-KO) SHAM, ApoE-KO CKD. Brains were removed after two and ten weeks of uremia and RNA from cerebral arterioles was extracted. miR-17 and miR-126 were the most dysregulated in the pathological conditions, at both the second week and tenth week of uremia. Our results suggest that miR-17 and miR-126 are potential new biomarkers of cerebral troubles of CKD patients and new therapeutic targets for innovative treatments.

2. INTRODUCTION

Vascular calcifications develop during the later stages of chronic kidney disease (CKD) and contribute to an increased risk of cardiovascular mortality and morbidity (1) (2). Profound alterations of cerebral circulation have been reported in CKD patients and are responsible for an increase in ischemic strokes, which represent the third most common cause of death in these patients, and behavioral troubles (3). It has also been demonstrated that patients with end-stage renal disease have greater risks of developing cognitive impairment and dementia (4, 5). It is thus important to detect new predictors of the cerebral microvasculature damage, for diagnosis purposes and also to identify targets for innovative treatments. Various blood-based biomarkers of the brain microvasculature have been described in the literature (6). For example, Advanced Glycation End (AGE) products such as Carboxymethyl-Lysine have been described as markers for microvascular complications in type 2 diabetic patients (7). Knowledge about their usefulness in CKD-induced cerebral disorders is very limited. We recently reported that CKD alters endothelial function in the brain of uremic mice, notably by modulating the levels of peroxisome proliferator-activated receptor-gamma (PPARγ) and of the inactive form of

eNOS (3). Here, we studied the time-course of expression levels of endothelial-specific microRNAs (miRNAs) in cerebral arterioles of Wild type C57/BL6 (WT) mice and of pathological models of CKD, in order to detect new biomarkers in the brain endothelial cells. Both WT and Apolipoprotein-E Knock-Out (Apo-E KO, which share the same C57/BL6 genetic background) mice were overlaid with a partial nephrectomy, thereby inducing CKD. Apo-E KO mice were used in order to recognize supplementary clues in our study, as a murine model of CKD with atherosclerosis, increased aortic stiffness and vascular calcification (Apo-E KO CKD mice). Apo-E KO mice display large atheromatous plaques in aortas and thus enable one to understand the role of atherosclerosis in vascular calcifications. Additionally, ApoE-KO mice where CKD is surgically induced develop vascular calcifications more rapidly than WT CKD mice.

miRNAs are small non-coding, single-stranded RNAs, that regulate target messenger RNAs at the post-transcriptional level (8). They are involved in a vast number of pathologies and represent new opportunities to develop biomarkers (9). One study has shown that miRNAs are differentially expressed in the endothelium of brain tumor (10). However, no study to date has looked at the expression of endothelial cell-specific miRNAs in the context of CKD and its related cerebral complications. The pro-angiogenic miR-126 is highly expressed and specific of endothelial cells (11). miR-126 plays an instrumental role in vascular dysfunction (12) and inhibits the expression of its target Vascular Cell Adhesion Molecule-1 (V-CAM 1) (13). The miR-17-92 cluster is a polycistronic gene which encodes six mature miRNAs (miR-17, miR-18a, miR-19a, miR19-b, miR-20a and miR-92a). All are highly expressed in endothelial cells and regulate vascular integrity and angiogenesis(14). miR-17 has an anti-angiogenic activity and miR-92a has been reported to promote cell proliferation and to induce tumoral angiogenesis (15). The pro-angiogenic miR-296 favors endothelial cell migration and plays a critical role in angiogenesis, by enhancing the expression of growth factor receptors (16). The anti-angiogenic homolog miRNAs miR-221 and miR-222 both target c-Kit (a Tyrosine Kinase receptor) and e-NO Synthase (e-Nos) (17, 18). These small RNAs are highly expressed by the vascular endothelial cell, where they have been shown to regulate survival, proliferation and differentiation. Lastly, miR-223 has been implicated as an inflammatory miRNA in various normal and tumorous cell types (19) (20), and has also been described as a neuroprotective miRNA (21). Here, we studied the expression of endothelial-specific miRNAs in cerebral arterioles of CKD and non-CKD WT mice and of CKD and non-CKD Apo-E KO mice.

3. MATERIAL AND METHODS

3.1. Animals: Diet, surgical and histological procedures

All experiments were performed in female mice purchased from Charles Rivers (Lyon, France). The animals were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a 12:12-hour light-dark cycle and were given standard chow (Harlan Teklad Global Diet 2016, Harlan, Oxon, UK) and tap water *ad libitum*. The components of the diet (as listed by the manufacturer) were 4.2% (wt/wt) fat, 16.7% protein, 60.89% carbohydrates, 0.98% calcium, 0.25% sodium, and 0.65% phosphorus. The study was performed in WT and ApoE-KO mice. All of the animal studies conform to the principles of the Directive 2010/63/EU of the European Parliament and all protocols were approved by our Institution's Animal Care and Use Committee (Comite Regional d'Ethique en Matière d'Experimentation Animale de Picardie, CREMEAP). Mice were anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, respectively), and all efforts were made to minimize suffering.

At 8 weeks of age, mice were assigned to the following four groups: WT mice that underwent sham operations (WT SHAM); WT mice with CKD (WT CKD); ApoE-KO mice which underwent sham operations (ApoE-KO SHAM), and ApoE-KO mice with CKD (ApoE-KO CKD), as described in Figure 1. To induce CKD, we applied cortical electrocautery to the right kidney and then performed left kidney total nephrectomy 2 weeks after the first operation. Control animals underwent sham operations, including decapsulation of both kidneys. Special care was taken to avoid damage to the adrenal glands. Female mice were used since ApoE KO CKD female mice have a faster progression of vascular calcification than male counterparts (22). Blood samples were taken at indicated time points and serum urea, total cholesterol, triglycerides, inorganic phosphorus, and calcium levels were measured as previously described (23).

3.2. Cerebral arterioles preparation

Cerebral arterioles were prepared as previously described (24). Briefly, animals were weighed, anesthetized with ketamine (80 mg/kg) plus xylazine (8 mg/kg) and perfused with 20 ml of saline through a cardiac puncture in order to wash the vessels from blood. The brain was removed and immediately frozen in liquid nitrogen and stored at -80°C until use. Isolated brains were washed in ice-cold sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4) and homogenized with a Dounce homogenizer in 5 ml of sucrose buffer. The homogenate was centrifuged at 1000g for 10 min. The supernatant containing neuronal cells was discarded and the white layer of myelin in the upper part of the pellet was removed. The pellet was suspended in 5 ml of the same buffer and centrifuged at 1000g for 10 min for three subsequent washes. The pellet was resuspended in the sucrose buffer and centrifuged at 300g to eliminate detached cells. This step was repeated three times and the resulting pellet was resuspended in 1 ml of sucrose buffer and centrifuged at 13000 g for 5 min. The resulting supernatant was used as the cerebral microvessels preparation.

3.3. RNA isolation and real-time PCR

RNA isolation was performed using the *mir*Vana™ miRNA Isolation Kit (Applied Biosystem) as per manufacturer's instructions (25). For miRNAs, Taqman assays available from Applied Biosystems were used for

Table 1. Mice biological samples two weeks post-surgery

	WT Sham	WT CKD	Apo-E KO Sham	Apo-E KO CKD	Effect ApoE KO	Effect CKD
Urea (mM)	10.55 ± 1.18	79.16 ± 37.78	6.53 ± 0.41	30.07 ± 2.19	0.0156	0.0001
Cholesterol (mM)	1.57 ± 0.32	3.59 ± 0.82	7.09 ± 0.87	13.56 ± 1.22	< 0.0001	< 0.0001
Triglycerides (mM)	1.07 ± 0.22	1.26 ± 0.085	1.35 ± 0.51	1.89 ± 0.66	NS	NS
Calcium (mM)	2.08 ± 0.045	2.83 ± 0.30	2.09 ± 0.05	2.52 ± 0.16	0.0313	< 0.0001
Phosphorus (mM)	3.07 ± 0.43	3.72 ± 0.69	2.5 ± 0.5	2.28 ± 0.60	0.0002	NS

Table 2. Mice biological samples ten weeks post-surgery

	WT Sham	WT CKD	Apo-E KO Sham	Apo-E KO CKD	Effect ApoE KO	Effect CKD
Urea (mM)	10.3 ± 1.04	31.70 ± 7.64	8.87 ± 0.87	34.02±10.73	NS	0 .0001
Cholesterol (mM)	2.21 ± 0.42	3.01 ± 0.38	7.75 ± 0.63	11.39 ± 0.62	<0.0001	0.0020
Triglycerides (mM)	0.75 ± 0.10	1.04 ± 0.21	1.08 ± 0.13	1.93 ± 0.72	0.0057	0.0090
Calcium (mM)	2.34 ± 0.04	2.93 ± 0.38	2.38 ± 0.22	2.66 ± 0.23	Not significant	Not significant
Phosphorus (mM)	2.18 ± 0.21	3.56 ± 0.39	2.38 ± 0.39	3.99 ± 0.77	Not significant	<0.0001

both cDNA synthesis and real-time PCR. The Ct value obtained on each miRNA was normalized using the U6 spliceosomal RNA as endogenous control, and the expression relative to the median value was determined by $2^{-\Delta\Delta Ct}$.

3.4. Statistical Analysis

Data are shown as mean ± standard error of mean (SEM). Statistical significance was determined by two tailed student's *t*-test except for mice data which was examined in a 2-way ANOVA that took into account the status of the animal (presence or absence of ApoE-KO presence or absence of CKD, and the interaction between ApoE-KO and CKD). Results were considered statistically significant at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.005$ (***).

RESULTS

4.1. Serum biochemistry

Plasmatic concentrations of urea were measured to determine the renal function in our models. After two weeks of CKD (T2), urea levels were significantly increased in WT CKD and ApoE-KO CKD mice compared with their respective SHAM controls, confirming that these mice were in severe uremia throughout the experiment, and had thus an impaired renal function (Table 1). Triglycerides levels were not significantly different in all mice groups. The cholesterol serum levels were increased in ApoE-KO SHAM and ApoE-KO CKD mice compared with WT SHAM and WT CKD respectively. The cholesterol levels were also significantly higher in WT CKD and Apo-E KO CKD mice compared to SHAM counterparts. Phosphorus levels were significantly lower in ApoE-KO mice than in WT. At T2, calcium levels were significantly increased in WT CKD and ApoE-KO CKD mice by comparison with the respective SHAM mice.

After ten weeks of CKD (T10), serum urea levels were also significantly increased in WT CKD and Apo-E KO CKD mice in comparison with their respective SHAM controls (Table 2). Calcium levels in sera were not significantly altered in any mice groups. At this time point, the cholesterol levels were close to those observed after 2 weeks. We observed an important effect of ApoE-KO and a smaller effect of CKD resulting in elevated cholesterol serum levels. Triglycerides levels showed significant differences at T10 with a significant effect of ApoE-KO and CKD. Phosphorus levels were significantly higher in CKD mice when compared with counterparts. This indicates an effect of CKD on phosphorus serum at T10 which was not detected at T2.

4.2. MicroRNA expression levels in the various murine groups

4.2.1. miR-17 and miR-92 expression

MiR-17 is a small RNA which is part of the 17-92a cluster and is implicated in the tumoral and angiogenic processes. At T2, there was no significant difference in miR-17 expression between the two groups of ApoE-KO mice. In contrast, the expression of this miRNA was significantly lower in ApoE-KO mice compared with WT counterparts ($p<0.01$). In WT mice, miR-17 expression level was significantly reduced by 30% in CKD mice compared with SHAM controls (Figure 2A). After 10 weeks of CKD, miR-17 levels were still significantly lower in ApoE-KO mice compared to WT, in both SHAM and CKD conditions. miR-17 was however increased by 50% in WT CKD mice versus SHAM (Figure 2B).

MiR-92a expression level was significantly decreased in ApoE-KO mice compared with WT mice after 10 weeks of CKD (Figure 2D, $p<0.05$). At T2 there was a clear, although not significant, tendency towards decrease in ApoE-KO mice when compared to WT counterparts (Figure 2C) In contrast, there was no effect of CKD.

4.2.2. miR-221 and miR-222 expression

miR-221 and miR-222 are both anti-angiogenic miRNAs, sharing the same cluster. After 2 and 10 weeks of CKD, they did not show any effect of CKD, but displayed a tendency towards decrease in ApoE-KO mice compared with WT mice (Figure 3). This decrease in Apo-E KO mice (compared with WT counterparts, operated or not) became significant for miR-222 after 10 weeks of CKD (Figure 3, C and D) $p<0.01$)

4.2.3. miR-126, miR-223 and miR-296 expression

miR-126 is predominant in endothelial cells. VCAM-1 (Vascular cell adhesion molecule-1), a marker of vascular inflammation, is one of its main targets. At T2, mir-126 expression in mice arterioles was increased by 60% (Figure 4A, $p<0.05$) in WT CKD mice compared with WT SHAM. A decrease of 20 % was found in ApoE-KO

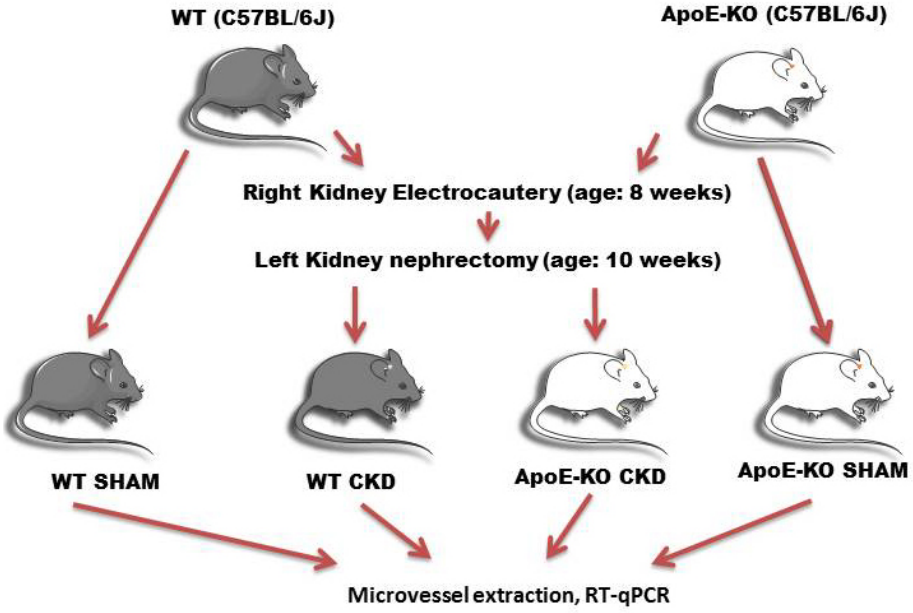

Figure 1. Microvessels were isolated from the brain of animal models of CKD and/or atherosclerosis for this study. Wild type C57/BL6 (WT SHAM), WT CKD (mice were overlaid with a partial nephrectomy, inducing CKD), ApoE-KO SHAM (from the same C57/BL6 background), and ApoE-KO CKD. Apo-E KO mice display large atheromatous plaques and enable us to understand the role of atherosclerosis in CKD. ApoE-KO CKD mice were used since they develop calcifications more rapidly than WT CKD mice and/or ApoE-KO mice alone.

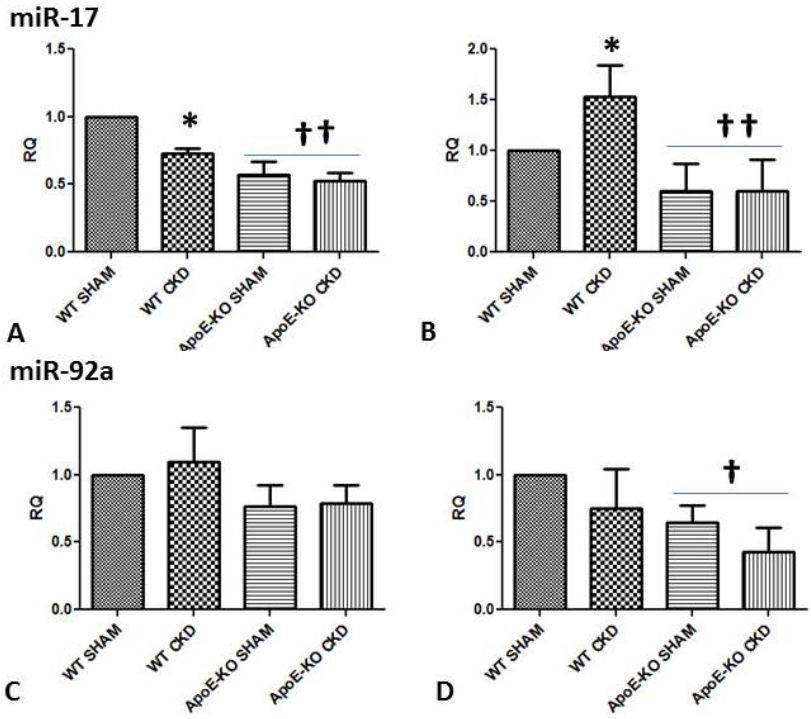

Figure 2. Expression of miRNAs from the miR17-92a cluster in CKD and atherosclerotic mice. Mouse microvessels were isolated from WT and ApoE-KO mice for miRNA studies at indicated times. miR-17 and miR-92a expression expressed as RQ normalized to U6, two weeks after nephrectomy (A) and ten weeks after nephrectomy (B). Statistical significance was determined by a two-way ANOVA (n =4 ± S.E.M, *P< 0.05 CKD mice *vs* Sham mice, † P< 0.05 Apo-E KO mice *vs* WT mice).

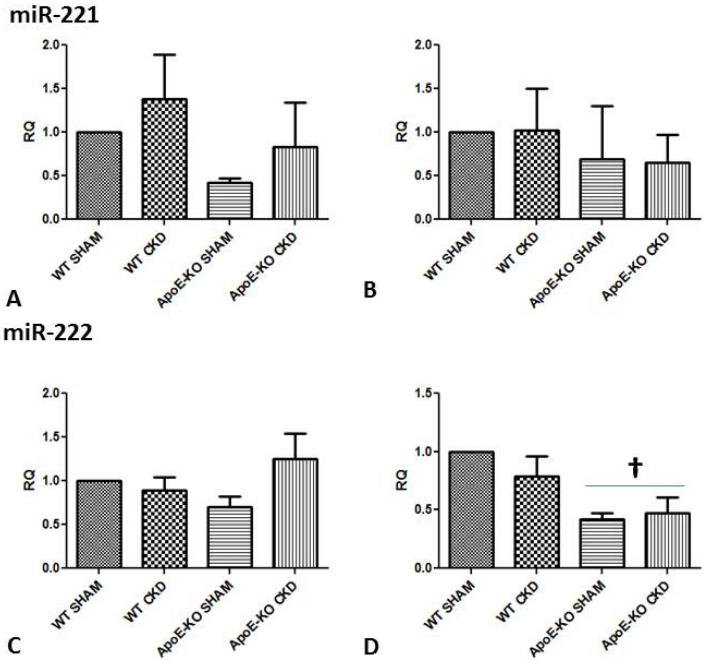

Figure 3. Expression of miR-221 and miR-222 in CKD and atherosclerotic mice. Mouse microvessels were isolated from WT and ApoE-KO mice for miRNA studies at indicated times. miR-221 and miR-222 expression was expressed as RQ normalized to U6, two weeks after nephrectomy (A) and ten weeks after nephrectomy (B). Statistical significance was determined by a two-way ANOVA (n =4 ± S.E.M, *P< 0.05 CKD mice *vs* Sham mice, † P< 0.05 Apo-E KO mice *vs* WT mice).

($p<0.05$). In a pooled analysis, the ApoE-KO animals exhibited a significantly lower expression of miR-126 (relative to the pooled WT animals).

At T10, miR-126 expression tended to decrease with kidney disease in both WT and ApoE-KO mice, although significance could not be reached (Figure 4B).

miR-223 is involved in inflammation (20) and alteration of its expression was already described in various diseased tissues (25). An increase of this miRNA was reported in damaged muscle areas and this miRNA was also linked with osteogenesis, and thus to calcium-phosphate (Ca*Pi) deposits detected in vascular calcifications (25). In our hands however, no significant difference of this miRNA was found between the different groups after 2 or 10 weeks of CKD (Figure 4, C and D).

miR-296 is involved in angiogenesis *via* targeting of HGF (26). In our hands, miR-296 levels in arterioles decreased in ApoE-KO mice, from both groups, when compared with WT counterparts. This was true at both time points, *ie* after 2 (Fig 4.E, $p<0.05$) and 10 weeks of CKD (Fig 4.F, $p<0.01$).

5. DISCUSSION

In the present study, we analyzed changes over time in endothelial miRNAs expression in microvessels extracted from brains of murine models of CKD. To that end, four different groups were used: WT SHAM, WT CKD (overlaid with a partial nephrectomy which induced CKD), ApoE-KO SHAM (from the same genetic background, which enabled us to distinguish the roles of atherosclerosis and CKD), as well as ApoE-KO CKD, which combine CKD and atherosclerosis and develop calcifications more rapidly than WT CKD mice and/or ApoE-KO mice alone.

Brains were removed after two and ten weeks of uremia and total RNA from cerebral arterioles were extracted. The biochemical analysis was as expected considering our mice model: Apo-E KO mice developed early cholesterolemia and later triglycemia. We expectedly show that uremia is increased from the early stages of CKD throughout the experiment and that phosphorus levels were higher in the later stage as expected (22, 23, 27). Indeed phosphoremia is directly related with progression of CKD, as patients gradually lose the ability to excrete phosphorus (22).

We quantitated the expression of miRNAs of interest in endothelial cells of cerebral arterioles during the process of vascular calcification and atherosclerosis. We demonstrate here that miR-17, miR-92a, miR-126, miR-221, miR-222, miR-223 and miR-296 were significantly detected in a preparation of endothelial cells from cerebral mice arterioles. To the best of our knowledge, this is the first time that microRNA expression levels are detected in endothelial cells from murine cerebral arterioles. Podolska *et al* (28) already studied miRNA expression in the porcine brain and showed the presence of miR-17, miR-221 and miR-222, but they did not look more precisely at their tissue distribution. In addition, we were unable to find in

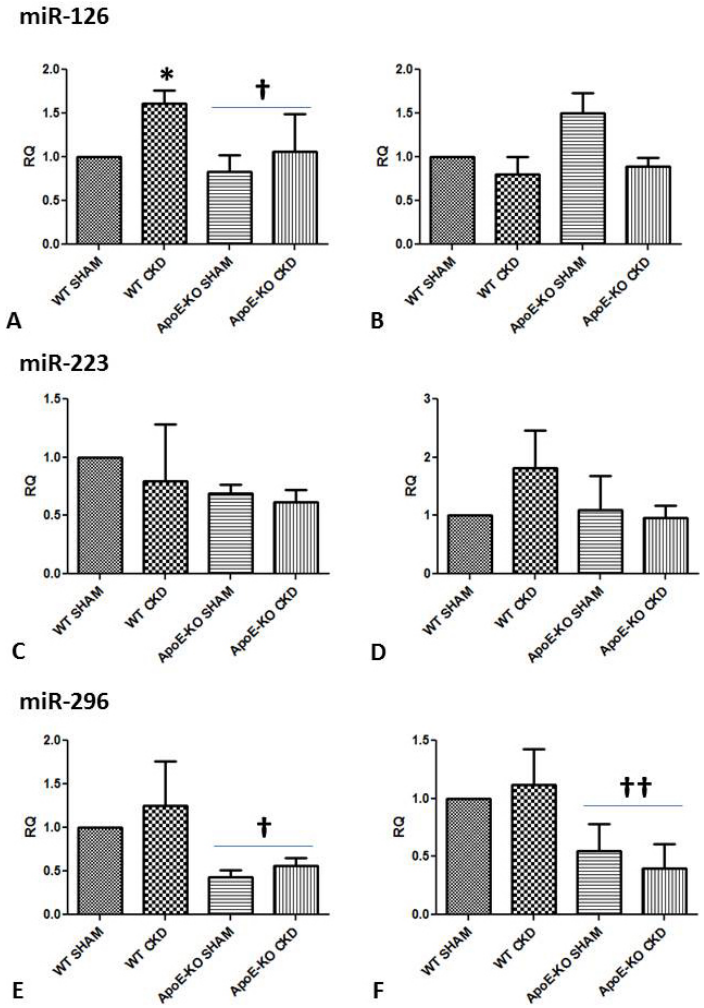

Figure 4. Expression of miR-126, miR-223 and miR-296 in CKD and atherosclerotic mice. Mouse microvessels were isolated from WT and ApoE-KO mice for miRNA studies at indicated times. miR-126, miR-223 and miR-296 expression was expressed as RQ normalized to U6, two weeks after nephrectomy (A) and ten weeks after nephrectomy (B). Statistical significance was determined by a two-way ANOVA (n =4 ± S.E.M, *P< 0.05 CKD mice *vs* Sham mice, † P< 0.05 Apo-E KO mice *vs* WT mice).

the literature any work about mice arterioles during CKD or atherosclerosis. Other markers of cerebral damage during the course of CKD have been described in the literature. Bugnicourt *et al* (3) demonstrated in our laboratory that endothelial function is altered in the cerebral microvasculature of 4 week-old CKD mice. More precisely, they found that endothelium-dependent relaxation was impaired during CKD, but endothelium-independent relaxation was not. In addition CKD had no effect on cerebral arteriolar structure and composition. Quantitative expression of VCAM-1 was similar in CKD and non-CKD mice. On the other hand, quantitative expression of PPARγ, a protector of blood vessels, was significantly lower in CKD mice than in non-CKD mice and was significantly lower in Apo-E KO mice than in WT mice. In this work we describe to the best of our knowledge for the first time a deregulation of two microRNAs, miR-17 and miR-126, in CKD conditions in WT mice.

The apolipoprotein Apo-E has an important role in the brain as a lipid acceptor as part of the transport of cholesterol and other essential lipids to neurons. ApoE-containing lipoprotein particles are essentially produced by astrocytes. Many studies indicate that astrocytes are involved in the control of endothelium blood-brain barrier properties and show that Apo-E deficiency leads to damages in tight junction integrity and to blood-brain barrier leakage (29). Our study demonstrates that Apo-E deficiency induces a significant decrease in the expression of five distinct microRNAs (miR-17, miR-92a, miR-126, miR-222, miR-296) in endothelial cells derived from mice arterioles of ApoE-KO mice compared with WT mice. Interestingly, miR-126, miR-222 and miR-296 were down-modulated in endothelial cells exposed to inflammatory stimuli (30), and we suggest here a link between the damage in the blood brain barrier of Apo-E KO and a deregulation of miRNA expression.

With regard to miR-126, we found that two weeks of CKD were associated with higher levels of this miRNA in WT and Apo-E KO mice than in the corresponding non-CKD mice. Zernecke *et al* (12) demonstrated that a pathological stress induces an increase of miR-126 in endothelial cells from the general circulation vessels. It has been shown that this miR-126 overexpression, due to an increase in endothelial cell apoptotic bodies containing miR-126, can reduce atherosclerosis *via* CXCL12 expression (12), and allows endothelial cells from vessels to maintain their integrity (11). In CKD pathology, kidney peritubular capillary endothelial cells were shown to undergo apoptosis leading to capillary loss, tissue hypoxia, and oxidative stress (31). Interestingly, Lorenzen *et al* (32) found miR-126 to be up-regulated in serum of patients with hemolytic uremic syndrome. There was no association between miR-126 and renal function but levels of miR-126 were associated with neurological symptoms at baseline and during follow-up. Our own results expand on these and suggest that the high levels of miR-126 we found at T2 in both WT and Apo-E KO endothelial cells are part of a protective effect against stress during the installation of CKD.

Although miR-17 and miR-92a come from the same polycistronic cluster, we show a different expression profile in our CKD model. Induction of CKD at T2 and T10 did not provoke any significant difference when one looks at miR-92a whereas miR-17 level was decreased at 2 weeks and increased at 10 weeks. This cluster is highly expressed in human endothelial cells (33). These two miRNAs have different effects in angiogenesis, as miR-17 has an anti-angiogenic activity (34) whereas miR-92a has been implicated in functional recovery after myocardial infarction and limb ischemia by enhancing blood vessel growth (35). In the healthy kidney, a balance exists between the expression of proangiogenic and antiangiogenic molecules, which is disrupted in CKD, leading to an antiangiogenic environment (31). Plasma levels of endostatin, one of the most potent endothelial inhibitors of angiogenesis, are elevated in CKD (36). Moreover, in response to kidney injury, endothelial apoptosis is induced by deprivation of survival growth factors such as VEGF and increased apoptotic stimuli (31). miR-17 could participate to this late defective angiogenesis phase found in CKD. Indeed, our data showed a significant increase of miR-17 in WT CKD mice vs WT sham after 10 weeks of uremia and Yin *et al* (37) demonstrated recently that miR-17 induced an inhibition of the angiogenic phenotype of EC by down-regulating Flk-1 in the cell signal pathway of VEGF. Also, in our study, both miRNAs were significantly decreased in Apo-E KO mice (Sham and CKD) at 10 weeks of CKD, suggesting a role for these miRNAs in blood vessel pathology during the latter stages of CKD.

Both miR-221 and 222 are highly expressed in endothelial cells and in vascular smooth muscle cells (VSMC) (18). We found lower miR-222 expression in Apo-E KO mice (pooled) versus WT (pooled) after 10 weeks of CKD. miR-221 and miR-222 are anti-angiogenic factors (18). Liu *et al* (18) found that an overexpression of miR-221 and 222 in HUVEC significantly reduced endothelial cell migration. miR-221 and 222 also regulate eNOS in the endothelium. Interestingly, we already found in a previous study that quantitative expression of eNOS phosphorylated on threonine 495 (the inactive form of eNOS) was significantly higher at T4 in CKD mice than in non-CKD mice and significantly higher in Apo-E KO mice when compared with WT mice (3). The plasma concentration of ADMA, a uremic toxin and an endogenous inhibitor of eNOS was elevated and plasma concentration of L-Arginine (eNOS substrate) was low in CKD. L-arginine levels were also significantly lower in Apo-E KO mice than in WT mice (3). Our results thus suggest a role for miR-222 in the regulatory mechanisms of Nitric Oxide (NO) which is essential for angiogenesis, capillary network maturation, and vessel permeability, during the course of CKD.

miR-223 was first described as a key regulator of the homeostasis of the immune system. It was later shown to be involved in cancerogenesis, inflammatory and autoimmune diseases and other pathological processes (25). miR-223 has been shown to be neuroprotective by targeting glutamate receptor subunits GluR2 and NR2B in the brain (21). Surprisingly, in our hands miR-223 which is also involved in inflammation shows no significant difference of expression levels in the four groups of mice. Lastly, miR-296 has been shown to be induced in brain endothelial cells during glioma progression. This miRNA contributes to angiogenesis by directly decreasing the expression of Hepatocyte-growth factor regulated tyrosine kinase substrate (HGS) and indirectly increases the levels of VEGFR2 and PDGFRβ and the response to VEGF (16, 35). On the other hand, increases in VEGF and EGF concentrations have been shown to induce miR-296 expression. miR-296 is also increased in response to exposure to human brain glioma cells in culture (16). In our study, miR-296 expression decreased in Apo-E KO mice versus WT after 2 and 10 weeks of CKD. In accordance with our results, Dentelli *et al* (30) demonstrated that miR-296 is down-modulated in endothelial cells exposed to inflammatory stimuli.

In conclusion, our results showed a significant decrease in the expression of five different microRNAs (miR-17, miR-92a, miR-126, miR-222, miR-296) in ApoE-KO mice compared with WT mice which represent thus potential biomarkers of the brain damage due to atherosclerosis. Interestingly, two of them, miR-17 and miR-126 were also deregulated in cerebral arterioles with CKD, so they can be used as potential new biological markers for human brain disorders and could be developed as novel therapeutic targets.

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