MicroRNAs 223-3p and 93-5p in Patients with Chronic Kidney Disease before and after Renal Transplantation

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Abstract

Chronic kidney disease (CKD) is associated with a multifactorial dysregulation of bone and vascular calcification and closely linked to increased cardiovascular mortality and concomitant bone disease. We aimed to investigate specific microRNA (miRNA) signatures in CKD patients to find indicators for vascular calcification and/or bone mineralization changes during CKD and after kidney transplantation (KT).

A miRNA array was used to investigate serum miRNA profiles in CKD patients, then selected miRNAs were quantified in a validation cohort comprising 73 patients in CKD stages 3 to 5, 67 CKD patients after KT, and 36 healthy controls. A spectrum of biochemical parameters including markers for kidney function, inflammation, glucose, and mineral metabolism was determined.

The relative expression of miR-223-3p and miR-93-5p was down-regulated in patients with CKD stage 4 and 5 compared to healthy controls. This down-regulation disappeared after kidney transplantation even when lower glomerular filtration rates (eGFR) persisted. MiR-223-3p and miR-93-5p were associated with interleukin-6 (IL-6) and eGFR levels, and by trend with interleukin-8 (IL-8), C-peptide, hematocrit, and parathyroid hormone (PTH).

This study contributes new knowledge of serum miRNA expression profiles in CKD, potentially reflecting pathophysiological changes of bone and calcification pathways associated with inflammation, vascular calcification, mineral and glucose metabolism. Identified miRNA signatures can contribute to future risk markers or future therapeutic targets in bone and kidney disease.
1 Introduction

Chronic kidney disease (CKD), in particular end-stage renal disease (ESRD), is frequently associated with disorders of mineral and bone metabolism (CKD-MBD) [1,2], and an increased cardiovascular risk [3] with progressive vascular calcification leading to a high cardiovascular mortality [4,5]. Therefore, the identification of biomarkers to predict the risk of vascular calcification and bone diseases is of utmost relevance for CKD patients.

CKD-MBD is a systemic disorder of mineral and bone metabolism due to CKD, manifesting as either one or a combination of abnormalities in the metabolism of calcium, phosphorus, parathyroid hormone (PTH) and vitamin D, as well as abnormalities in bone-turnover, bone mineralization, bone volume, and extra-skeletal calcification relevant to the cardiovascular system [6]. Vascular calcification is highly connected to vascular smooth muscle cells (VSMCs) of the media or intimal vessel layer [7]. Media calcification has been found in the majority of ESRD patients at the time of kidney transplantation (KT) accompanied by an expression of osteogenic factors, which might contribute to a phenotypic switch of VSMCs to osteoblast-like cells, resulting in vessel calcification [8,9]. Also, disturbances of mineral metabolism including hyperphosphatemia, metabolic acidosis, and inflammatory processes are common during CKD, involving parameters such as PTH [10], C-reactive protein (CRP) [11], interleukin 6 (IL-6) [12], and interleukin 8 (IL-8) [13].

MiRNAs have been proposed to be involved in different aspects of CKD development and deterioration of the disease, but also in systemic effects such as CKD-MBD and vascular calcification [14]. An association of the decrease of miR-125b, -145 and -155 with vascular calcification has been shown in ex-vivo and in-vitro experiments [15]. The total amount of miRNAs is generally decreased during CKD, but the renal excretion system is not involved in the excretion of circulating miRNAs [16]. Reduced miRNA levels might potentially occur due to secondary effects of the decreased kidney function, such as accumulation of RNases [16]. On the other hand, miRNA levels are very stable in serum and were shown to be protected from endogenous RNase activity, most likely as a result of a dense packaging into exosomes, an association to protein complexes and other molecules, or potential molecular modifications [17].

Distinctive patterns of circulating miRNA levels have been described as biomarkers for disease, such as coronary artery disease (CAD) and diabetes mellitus (DM) [18]. In type 2 diabetes mellitus (T2DM), a strong confounding element during CKD, miR-126 and other miRNAs were suggested as novel biomarkers for DM risk estimation [19]. The importance of miRNAs in CKD-MBD has been indicated by their role as important regulators of function and differentiation of osteoblasts and osteoclasts during bone development and homeostasis [20]. First data of miRNAs as biomarkers in bone disease have been published [21]. MiR-550a-5p and miR-382-3p have been suggested as promising circulating biomarkers.

Keywords
microRNA; chronic kidney disease; bone metabolism; kidney transplantation; biomarker; CKD-MBD
biomarkers for diabetic bone disease in postmenopausal osteoporotic women [22]. Another study in patients with recent osteoporotic fractures has indicated different serum levels for several miRNAs compared to healthy controls [23].

The aim of this study was to use human CKD serum samples to identify a pattern of systemic deregulated miRNAs which have a putative involvement in mineral metabolism and calcification and which are changed during CKD progression and potentially after kidney transplantation. These miRNAs are thought to be involved in key pathways of disease-associated complications such as CKD-MBD and may serve as biomarkers and targets for subsequent diagnostic and putative therapeutic studies in CKD patients.

2 Materials and Methods

2.1 Patient cohorts

Seventy-one patients with CKD stages 3 to 5 (without renal replacement therapy (RRT)), sixty-six CKD patients after KT (mean time since transplantation: 7 years, range 1 - 28), and 36 healthy controls were included in this cross-sectional study. CKD patients were separated into subgroups according to their estimated glomerular filtration rate (eGFR) (calculated via CKD-EPI creatinine equation [24]). A total of five groups was compared, including CKD stages 3-5 without RRT (eGFR: 30-44, 15-29 and below 15 [ml/min/1.73m$^3$]) and patients after KT (eGFR: >15 [ml/min/1.73m$^3$]). Blood samples were drawn in the morning after a 12-hour overnight fast and serum as well as plasma samples were centrifuged according to a standardized protocol, aliquoted, and stored at -80 °C until analysis.

2.2 Ethical approval

This study was approved by the Research Ethics Committee of the Medical University of Graz, Austria, (23-056 ex 10/11) and is registered at ClinicalTrials.gov (NCT01362569). Written informed consent was obtained from all patients and controls and the study was performed according to the principles laid out in the Declaration of Helsinki.

2.3 MiRNA techniques

MiRNAs were isolated from serum using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). 1200 μl Qiazol lysis reagent were added to 200 μl of serum and incubated at room temperature for 5 minutes. 2 fmol of non-human miRNA (ath-miR-159a) were spiked in as an internal control. After mixing, 200 μl of chloroform were added, followed by 15 seconds of vortexing. After incubation for 3 minutes at room temperature, the samples were centrifuged for 15 minutes at 12000 g and 4 °C. The upper phase (approximately 800 μl) was transferred to a new tube and 1200 μl 100 % ethanol and 20 μl glycerol (Ambion, ThermoFischer, Waltham, USA) were added. The subsequent isolation was done using miRNeasy Kit column tubes and solutions following the manufacturer’s instructions. RNA was eluted from the columns in 40 μl RNase-free water and stored at −80 °C.

The digital multiplexed nanoString nCounter human miRNA expression assay (nanoString Technologies, Seattle, USA) was performed with 10–30 ng total RNA isolated from a net volume of 250 μl serum as input material in a separate isolation also using the miRNeasy
Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Small RNA samples were prepared by ligating a specific DNA tag (miR-tag) onto the end of each mature miRNA. Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. Data was processed according to the instructions by nanoString using the nSolver software. In the pilot step, changes in miRNAs in 10 CKD stage 5 patients compared to healthy controls were assessed. Based on the human miRNA expression assay results, covering over 800 human miRNAs, 12 miRNAs were selected for further analysis based on expression differences seen in the array and their putative involvement in pathways relevant to CKD, vascular calcification, bone mineralization, and VSMC biology according to relevant literature and bioinformatics tools (mirPath, TargetScan, miRDB).

The 12 selected miRNAs were analyzed using quantitative real-time PCR (qPCR, polymerase chain reaction) in a discovery group of random samples from all 5 available groups (CKD 3 – 5, KT, controls). Based on reproducible results for 4 miRNAs (miR-223-3p, miR-93-5p, miR-142-3p, and miR146a-5p), a subsequent qPCR analysis of these miRNAs was performed in all samples. The qPCR was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA). 3 μl isolated RNA was used for reverse transcription to synthesize complementary DNA using TaqMan miRNA-specific primers and the TaqMan reverse transcription kit (Applied Biosystems). After reverse transcription, an amplification step using the TaqMan PreAmp Master Mix was conducted. Real-time PCR amplifications were performed using TaqMan miRNA assays (TaqMan MGP probes, FAM dye-labeled) on the LightCycler® 480 (Roche, Basel, Switzerland). For all qPCRs, a maximum of 40 cycles was performed and the cycle number at which the amplification plot crossed the threshold was calculated (CT)\[25.\] Relative expression levels were calculated using miRNA levels after normalization to non-human spiked miRNA ath-miR-159a. Expression differences were calculated using the mean value of the controls as the factor for normalization. Calculations were done according to the ΔΔct-method \[25.\]

2.4 Biomarkers

Biomarkers were measured in serum and/or plasma according to the different manufacturers’ instructions. Levels of IL-6, TNFα, IL-8, and IL-10 were measured using FlowCytomix™ Multiplex Technology (eBioscience, San Diego, CA, USA). IL-6R (human IL-6R Platinum ELISA, eBioscience, San Diego, CA, USA) and MPO (Myeloperoxidase ELISA K6631B, Immundiagnostik, Bensheim, Germany) were measured with standard ELISA technology. bALP, CRP, iron, ferritin, gamma-glutamyl transferase (GGT), glucose, calcium, magnesium, sodium, phosphate, and potassium were measured on an automated system via the Cobas® 8000/6000 modular analyzer series, PTH, OC, and CTX on the Cobas® 4000 analyzer series (Roche Diagnostics, Rotkreuz, Switzerland). C-peptide was determined by the ADVIA Centaur XP Immunoassay System (Siemens Healthcare, Erlangen, Germany). Hematocrit was measured by ABL800 FLEX blood gas analyzer (Radiometer GmbH, Willich, Germany). 25(OH)vitamin D (25(OH)D) levels were assessed via an automated chemiluminescence immunoassay on the iSYS automated system ( Immunodiagnostic Systems, Boldon NE35 9PD, UK).
2.5 Target predictions

To predict possible miR-223-3p and miR-93-5p targets which might play a role during CKD-MBD and vascular calcification, the online resources miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/path-self.html) and TargetScan 7.0 (http://www.targetscan.org) were used. Predicted target genes and their conserved miRNA binding sites were annotated. To check for validated targets miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) was used. Gene functions and disease relations were checked via the Genecards (http://www.genecards.org), UniProt (http://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov/gene) webpages.

2.6 Statistical analysis

Patient characteristics and biomarker levels are reported as mean ± standard error of the mean (SEM) unless otherwise stated.

Quantitative qPCR data are reported as mean ± standard deviation (SD). The expression levels of miRNAs were analyzed using the 2^(-ΔΔCT) method [25]. The non-human spiked miRNA ath-miR-159a was used as a normalization control in the calculations. MiRNA changes in CKD groups are expressed as the relative fold change compared to miRNA levels of healthy controls as reference. Quantitative data were compared using the Kruskal-Wallis test for non-parametric samples with post-hoc Bonferroni correction for multiple comparisons and adjustments for multivariate analysis.

To evaluate the associations between specific miRNA concentrations and biological markers, univariate linear regression analyses were used. Standardized coefficients B and their standard errors (SEs) were obtained.

In all cases, a p-value of <0.05 was considered statistically significant.

3 Results

3.1 Patient characteristics for CKD, KT and control groups

To discover changes in miRNA expression 171 recruited patients were divided in 5 groups including CKD stages 3 – 5, KT patients, and controls (Table 1). eGFR levels were lower in later CKD stages. The amounts of patients with T2DM are indicated (Table 1).

Levels of surrogate parameters for inflammatory processes (IL-6, IL-8, IL-10, TNFα), glucose metabolism (glucose, C-peptide), electrolyte homeostasis (calcium, magnesium, sodium, potassium, phosphate), hematocrit, PTH, 25(OH)D and further parameters are shown in Table 2. OC, CTX, bALP and TRAP were measured in a subgroup of CKD patients.

IL-6 was significantly elevated in CKD stages compared to healthy controls (p<0.01) and this effect became more pronounced in late CKD stages and persisted in KT recipients. Furthermore, inflammatory (IL-8, IL-6R) as well as anti-inflammatory (IL-10) interleukins were significantly increased in CKD and KT patients compared to the controls.
TNFα and CRP showed no significant differences between CKD, KT or control groups. Hematocrit decreased significantly with the reduction in eGFR. Phosphate as well as urea, creatinine, magnesium, and PTH showed a significant increase parallel to the decrease in kidney function.

The bone turnover markers OC and CTX were increased in late-stage CKD compared to early CKD stages. Additionally, OC was also increased compared to the KT group (Table 2).

### 3.2 Primary discovery sample analysis

In a primary discovery step, 12 miRNAs (Suppl. Table 1) were selected for further analysis based on expression differences seen in a miRNA array and their putative involvement in bone and calcification during CKD according to existing knowledge and databases. These 12 miRNAs were analyzed via qPCR in a small discovery group of random samples selected from all available groups (CKD3-5, KT and controls). Based on reproducible results of 4 miRNAs (miR-223-3p, miR-93-5p, miR-142-3p and miR146a-5p) a subsequent qPCR analysis of these 4 selected miRNAs was performed in all samples.

### 3.3 MiR-223-3p and miR-93-5p are down-regulated over the course of chronic kidney disease and normalize after kidney transplantation

Systemic expression levels of miR-223-3p and miR-93-5p were significantly reduced in CKD patients in stages 4 and 5 compared to CKD stage 3, healthy controls, and KT recipients (Figure 1A, B).

MiR-223-3p was down-regulated in CKD stage 4 (p<0.0001 compared to controls; p<0.01 compared to CKD3; p<0.0001 compared to KT) and in CKD stage 5 (p=0.003 compared to controls; p=0.06 compared to CKD 3; p=0.025 compared to KT). After KT, these expression levels normalized to the levels found in serum samples of healthy controls (Figure 1A).

MiR-93-5p showed a stable down-regulation in all three CKD stages with a significant decrease in CKD 4 (p=0.0093) and CKD 5 (p=0.048) compared to healthy controls. After KT, the expression was normalized, showing no difference between KT and control samples, but a significant increase compared to both CKD 4 (p<0.0001) and CKD 5 (p<0.0001) (Figure 1B).

The expression differences in the miRNA array experiments initially seen for miR-142-3p and miR-146a-5p were mitigated in qPCR experiments in all CKD and KT groups. Nevertheless, miR-142-3p showed a small, but significant down-regulation in CKD 4, compared to healthy controls (p=0.025) and KT recipients (p<0.001) (Figure 1C). For miR-146a-5p, there was no statistically significant difference between any of the groups (Figure 1D).

To exclude a confounding effect of T2DM on miRNA expression, differences in expression for all 4 miRNAs were analyzed according to manifestation of T2DM (Suppl. Figure 1 and 2). However, there was no overall change of the results except for decrease in statistical power due to decrease in sample size.
3.4 Associations of miR-223 and miR-93 with relevant clinical parameters

Univariate linear regression analyses for miR-223-3p and miR-93-5p showed significant associations of these miRNAs with kidney function, hematocrit, inflammation, PTH, 25(OH)D, and glucose metabolism (Table 3).

MiR-223-3p was significantly associated with eGFR in CKD stage 5 with a trend towards an association in CKD stage 4. In patients with CKD stage 4, miR-223-3p was significantly associated with levels of IL-6 and CTX. Furthermore, in this stage, miR-223-3p showed a trend towards an association with IL-8 and 25(OH)D (Table 3). In CKD stage 5, miR-223-3p showed a significant association with OC levels.

MiR-93-5p was significantly associated with eGFR in CKD stage 5 and with levels of IL-6 and hematocrit, both in CKD stages 4 and 5. In CKD stage 5, miR-93-5p showed an association with C-peptide and a trend towards an association with PTH. A trend for an association with PTH was also seen in CKD stage 4. OC was significantly associated with miR-93-5p in CKD stage 4 and showed a trend towards an association in stage 5 (Table 3).

3.5 MiRNA target prediction indicates binding of miR-223-3p and miR-93-5p to genes regulating bone metabolism during CKD

Based on miRWalk 2.0 predictions, miR-223-3p and miR-93-5p were found to interact with IL-6, TGFβ, Wnt signaling, and osteoblast differentiation. Genes which showed a connection to these miRNAs were further investigated using TargetScan 7.0.

MiR-223-3p was predicted to regulate the following genes, CHUK (conserved helix-loop-helix ubiquituous kinase; IKKα), RPS6KB1 (ribosomal protein S6 kinase B1) and IL6ST (interleukin 6 signal transducer) (detailed results in Suppl. Table 2).

MiR-93-5p was predicted to regulate STAT3 (Signal transducer and activator of transcription 3), TNFSF11 (tumor necrosis factor superfamily member 11, RANKL), SMAD5 (SMAD family member 5), SMAD6 (SMAD family member 6), BMR2 (bone morphogenetic protein receptor type 2), PPARD (peroxisome proliferator activated receptor delta), and NFAT5 (nuclear factor of activated T-cells 5, tonicity-responsive) (detailed results in Suppl. Table 3).

Using miRTarBase we evaluated targets outside of the CKD-MBD spectrum, which have already been confirmed in studies via molecular methods (Suppl. Table 4 and 5).

3.6 MiRNA expression differences between CKD and KT patients with comparable eGFR

When comparing selected CKD 4 patients with an eGFR of 15 – 29 to KT patients with a similar eGFR level (eGFR 15 – 29), the expression of miR-223-3p (p<0.01), miR-93-5p (p<0.01), and miR-142-3p (p<0.01) was significantly lower in CKD compared to KT patients with matched eGFR (Figure 2).

4 Discussion

In this study, we report a significantly lower systemic expression of two specific miRNAs, miR-223-3p and miR-93-5p, during later stages of CKD and a reversal of this effect after
kidney transplantation. Expression levels tended to normalize after kidney transplantation for both miRNAs, even exceeding expression levels found in healthy controls. MiR-223-3p and miR-93-5p levels were significantly associated with CKD stages, parameters of inflammation and kidney function, and indices of glucose metabolism. Target predictions indicated miRNA involvement in bone metabolism via different pathways which makes them interesting as putative biomarkers for CKD-MBD, vascular calcification, and other osteogenic diseases connected to CKD.

In accordance to the expression differences of miR-223-3p in our study, miR-223 deregulation was recently associated with vascular calcification processes, osteoclast differentiation, VSMC phenotype but also in inflammation via various targets [26]. In ApoE mice, a murine model of CKD, miR-223 was found to be up-regulated in atherosclerotic aortas and low systemic miR-223 levels were associated with increased calcification and CKD stages [27]. Supporting this finding, an in-vitro study of miR-223 in VSMCs suggested an association with inorganic phosphate metabolism, with an increased expression of miR-223 in primary VSMCs after phosphate treatment, which led to increased calcification of VSMCs [28]. Accordingly, in-vivo studies have suggested that decreased systemic levels of miR-223 indicate negative metabolic effects.

We detected a down-regulation of miR-93-5p during deterioration of CKD. MiR-93 has previously been suggested as a potential regulator of vascular endothelial growth factor (VEGF), a key regulator for angiogenesis and vascular homeostasis. A down-regulation of miR-93, similar to our findings, has been associated with higher levels of VEGF and IL-6 in T1DM [29]. Furthermore, down-regulation of miR-93 was associated with upregulation of VEGF levels with subsequent increase of collagen and fibronectin in renal glomeruli, causing diabetic nephropathy [30].

The down-regulation of miR-93-5p in our study might also indicate an enhanced calcification process, as miR-93 has been shown to be inversely correlated to Sp7, an important transcription factor for osteoblast mineralization, where higher Sp7 levels are maintained by down-regulated miR-93 [31]. Sp7 has been indicated to play a role in the calcification process of VSMCs and their differentiation to osteoblastic cells, regulated via miR-125b [32]. Therefore, the link between miR-93 and SP7 might be an important factor during CKD-MBD.

In osteoporotic patients with normal kidney function, miR-93 and other miRNAs were differentially expressed in serum and bone tissue samples [33]. MiR-93-5p was also shown to be downregulated in serum of osteoporotic patients with low traumatic fractures and has also been associated with bone mineral density [34].

In renal biopsies of progressive CKD patients, miR-142-5p, miR-223 as well as miR-146b were upregulated compared to stable CKD patients, indicating a role during deterioration of the disease [35]. In ADPKD (autosomal dominant polycystic kidney disease) patients, miR-223 was upregulated in urine compared to patients with CKD diseases of other etiology such as diabetes or glomerulonephritis [36]. While the loss of specific miRNAs might be a
key mechanism for decreased miRNA patterns in serum in our study we could not analyze urinary samples due to strongly decreased kidney function in many patients.

We found no differences in miR-223-3p and miR-93-5p expression between CKD patients with and without T2DM, although an earlier study found decreased miR-223 plasma levels in T2DM patients compared to healthy controls, with a possible effect of miR-223 already early in T2DM disease manifestation [37]. We hypothesize that inflammatory processes rather than T2DM caused the downregulation in our study, but the lack of significant differences could have also been caused by the small subgroup sample.

We see IL-6, an inflammatory marker, to be associated with decreased miR-223-3p and miR-93-5p expression. There is already some evidence for regulatory effects of miR-223 on IL-6. The production of IL-6 promoted by STAT3 was shown to be regulated via miR-223 but vice-versa, IL-6 was found to inhibit miR-223 via a negative feedback loop [38]. Similar to miR-223, miR-93 has been suggested to interact with STAT3 [39], which indicates that, comparable to miR-223, it might also be involved in the regulation of IL-6 [38]. A significant association of miR-93-5p with IL-6 in CKD 4 stages and, to a lesser degree, CKD stage 5 in our study, supports previous reports indicating effects of miR-93 during inflammation. For instance, an anti-inflammatory effect of miR-93 has been reported, when miR-93 was shown to directly target interleukin-1 receptor-associated kinase 4 (IRAK4) mRNA, and thereby inhibiting the pro-inflammatory effect of IRAK4 on NF-κB signaling [40]. Therefore, down-regulation of miR-93 might increase the pro-inflammatory effect of IRAK4, which has been shown to be an important regulator for cytokines such as IL-6 and a driving force of renal failure during CKD [41]. Considering that miR-93 is down-regulated during inflammation, miR-93 might be up-regulated during anti-inflammatory conditions. In our study miR-93-5p was up-regulated in the KT group compared to CKD patients and healthy controls which could be due to the immunosuppressive medication KT patients usually receive.

MiR-223 has been described as a regulator in the NF-κB pathway and the toll-like receptor 4 (TLR4)-pathway, both important for the development of CKD [42,43]. MiR-223 has been shown to target the inhibitor of NF-κB kinase α (IKKα) in macrophages. An increase of miR-223 was proposed to increase NF-κB, resulting in a controlled pro-inflammatory state, leading to an immune response in macrophages without systemic autoimmune effects [44]. We did not detect any association of miR-93-5p with IL-8 even though down-regulation of miR-93 has been proposed to be associated with an up-regulation of IL-8 in infected IB3-1 cells, mimicking the inflammatory state in fibrotic lungs. IL-8 was hypothesized as a direct target of miR-93 due to potential target sites in the 3’-UTR of IL-8 mRNA [45]. In our study, the missing association could have been caused by sample size in the subgroups or other confounding elements.

OC is a well-known indicator of bone turnover and bone formation and was shown to be elevated during renal failure as an indication for high bone turnover [46]. Associations of miR-223-3p with OC are difficult to interpret in our study and in general during CKD progression, due to the potential OC accumulation. For both miR-223-3p and miR-93-5p we found no association with the bone associated turnover enzymes TRAP5b and BALP, but
this might also be an effect of the low number of samples that were available for the analysis.

The association of miR-93-5p with hematocrit seen in CKD stages 4 and 5 are in line with a previously published hypothesis that circulating miRNAs are potentially excreted from red blood cells [47]. However, results differ in our KT patients with low hematocrit alongside increased miR-93-5p expression.

When looking at putative targets for miR-223-3p using TargetScan we identified IKKα, RPS6KB1 and IL6ST, the latter two active in the IL-6 signaling pathway. RPS6KB1, an effector of the PI3K (phosphatidylinositol 3-kinase) pathway important for ROS downstream signaling, was increased in a CKD rat model on a high calcium-phosphate diet, developing vascular calcification and VSMC differentiation to osteoblast-like cells in combination with IL-6 and TNF [48]. IL6ST is as an important factor during osteoblast differentiation in response to PTH in mice [49].

We also looked at several predict targets for miR-93-5p. Predicted genes of interest were STAT3, TNFSF11 (RANKL), SMAD5, SMAD6, BMR2, PPARD, and NFAT5, well-known regulators of mineral and vascular changes in CKD.

Influences of RANKL on osteoclastogenesis and calcification of VSMCs link bone and vasculature [50,51]. SMAD5, SMAD6, and BMPR2 are involved in BMP signaling. Studies in the pluripotent cell line C2C12 as well as rat aortic smooth muscle cells have shown that activated SMAD5 induces osteoblast differentiation via RUNX2 [52,53]. SMAD6 plays a role as an inhibitor of BMP signaling [54] and as an important factor for the normal development and tissue specific modulation of vessels [55]. BMPR2, activates SMAD proteins and downstream signaling and leads to a switch of VSMCs to the osteogenic phenotype [56]. In mice, loss of BMPR2 resulted in increased bone mass due to osteoblasts activation [57]. In VSMCs, PPARD was overexpressed during vascular lesion formation, suggesting a role during VSMC modulation in atherosclerosis [58]. The transcription factor NFAT5 up-regulates expression of RUNX2 via the transcription factor SOX9 [59], playing a role in the osteogenic transformation of VSMCs, which might be enhanced by simultaneous inflammation [60].

To minimize the effect of kidney impairment we analyzed CKD and KT patients with a similar eGFR and found significant differences for miR-223-3p and miR-93-5p expression, with higher levels in the KT group. This indicates a regulation of the circulating amount of both miRNAs, which appears to be independent of kidney function.

Other miRNAs analyzed in our study, such as miR-142-3p, showed a similar trend, which was not statistically significant, probably due to pronounced scattering and the restricted sample size of the subgroups. MiR-142-3p was indicated as an important factor during osteoblast differentiation via the Wnt signaling pathway [61], which is regulated by several miRNAs [62].

In our study, miR-146a-5p and other miRNAs previously reported to be involved in calcification and mineralization processes during normal kidney function [63], had a stable
miRNA expression profile in CKD and KT patients and did not differ from healthy controls. Differences of miR-146a-5p in serum samples were detected during our primary array experiment, but subsequent qPCR results were no longer significant. This is in line with a study that only found downregulation of miR-146a plasma levels during CKD when patients underwent acute exercise, not during normal physical activity [64]. Interestingly, also higher miR-146a plasma levels were also associated with impaired renal function and increased arterial stiffness [65].

Despite increased inorganic phosphate levels in CKD patients there was no association of miR-223-3p or miR-93-5p with systemic phosphate levels probably due to a contribution of tissue or intracellular phosphate. The importance of high phosphate levels was described for bone mineralization in CKD [66], and for increased VSMC migration and calcification, additionally modified by miR-223 [28].

Our study focused on serum to detect overall miRNA changes because it is a common material used in many studies, it is easy to handle and minimally invasive. The stability of miRNAs in serum is probably achieved due to their transport in exosomes and binding to several proteins [67]. Further analyses of the miRNA origin of specific tissues and cells will be necessary to investigate underlying mechanisms.

Strengths of our study are the overall large sample size and the presence of all late CKD stages without the confounding influence of RRT and the inclusion of a large control group with a variety of parameters measured during the development of the disease and after KT. A number of important serum parameters including interleukins and markers of glucose and bone metabolism have been investigated in this study. Furthermore, miRNA array screening results have been confirmed by individual qPCR validation. However, smaller group sizes in several CKD subgroups might have reduced the detection of underlying regulatory associations and the possibility of multivariate analyses, but subgrouping via eGFR was necessary due to the distinct characteristics of each CKD stage.

Based on our findings, miR-223-3p and miR-93-5p should be further investigated as systemic markers for CKD, their role in bone metabolism, vascular calcification and potential VSMC differentiation. Due to various effects on different pathways miR-223-3p and miR-93-5p are of great interest for CKD disease development, both because of inflammatory effects and effects on mineral metabolism. Various potential downstream targets of miR-223-3p and miR-93-5p do point to an involvement in osteoblast and VSMC differentiation during CKD-MBD with possible diagnostic and therapeutic potential.

5 Conclusion

In conclusion, this human CKD and KT study shows significant changes of systemic miR-223-3p and miR-93-5p levels, associated with CKD stage as well as inflammation and bone parameters. Further in-vivo and in-vitro experiments to validate these pilot results are necessary to provide more detailed insights into the regulatory network of CKD.

MiRNA signatures might help to assess pathophysiological changes during CKD and potential bone specific and vascular risks as well as therapy aspects in CKD patients.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Fold changes of circulating miRNAs (miR-223-3p, miR-93-5p, miR-142-3p and miR-146a-5p) in CKD patients at different CKD stages and in patients after renal transplantation (KT) compared to healthy controls. CKD stages 3-5 (CKD 3…eGFR 30-44; CKD 4…eGFR 15-29; CKD 5…eGFR <15).
Data are depicted as mean ± SD. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Figure 2.
Comparison of miRNA expression between the CKD4 and KT patients with a similar eGFR ("KT (CKD4 equivalent)", eGFR between 15 and 29 [ml/min/1.73m²]).
Data are depicted as mean ± SD.
Table 1

General characteristics of CKD patients, KT (kidney transplant) recipients and healthy controls.

| Characteristic [Units] | Chronic Kidney Disease | KT (n=66) | Controls (n=34) |
|------------------------|------------------------|-----------|-----------------|
|                        | CKD 3 (n=26)           | CKD 4 (n=34) | CKD 5 (n=11)    |
| Age [years]            | 51±14                  | 64±12      | 54±14*          | 45±15          |
| Sex [male]             | 69%                    | 60%        | 73%             | 64%            |
| BMI [kg/m²]            | 27.2±5.2               | 30.5±7.3  | 29±6.1          | 26±4.4         | 24.7±2.7       |
| eGFR [ml/min/1.73m³]   | 56.9±23.8*             | 21.5±4.2  | 11.9±2.3 < f    | 45.3±17.7c     | 85±19.1        |
| Diabetes [Yes]         | 31%                    | 44%*       | 27%             | 26%            | 0% **          |
| Time after Transplantation [months] | n.a.                   | n.a.      | n.a.            | 86±86          | n.a.           |

CKD patients were divided into 3 groups according to their eGFR (CKD 3 – 5) and none of the CKD patients received hemodialysis (renal replacement therapy, RRT). Data are depicted as mean ± SD.

\( a \) \( p<0.05 \)

\( b \) \( p<0.01 \)

\( c \) \( p<0.001 \), compared to controls

\( d \) \( p<0.05 \)

\( e \) \( p<0.01 \)

\( f \) \( p<0.001 \), compared to KT. Kruskal-Wallis tests for independent samples and Bonferroni post hoc testing for multiple comparison correction were used.

Diabetes incidence was compared using Chi-square test with post-hoc Bonferroni correction. *\( p=0.04 \); **\( p=0.0016 \)

n.a. not applicable.

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Table 2
Serum parameters in CKD patients, KT recipients and healthy controls.

| Parameter [Unit] | CKD 3 (n=26) | CKD 4 (n=34) | CKD 5 (n=11) | KT (n=66) | Controls (n=34) |
|------------------|--------------|--------------|--------------|-----------|----------------|
| Creatinine [μmol/l] | 131±42\text{a,b,c,d} | 248±57\text{a,b,e} | 415±90\text{a,b,e} | 157±68\text{a,c,d} | 80±11\text{b,c,d,e} |
| Urea [mmol/l] | 8.6±3.6\text{c,d} | 18.2±6.1\text{a,b,e} | 22.8±3.6\text{a,b,e} | 11.6±6.3\text{a,c,d} | 5.2±1\text{b,c,d,e} |
| IL-6 [pg/ml] | 1.9±3\text{a} | 2.4±1.9\text{a} | 5.5±5.3\text{a} | 3.3±5.1\text{a} | 0.1±0.4\text{b,c,d,e} |
| IL-6R [pg/ml] | 259±67\text{a} | 260±64\text{a} | 234±55 | 224±63\text{a} | 180±47\text{b,d,e} |
| TNFα [pg/ml] | 3.1±5.2\text{a} | 3.5±4.6\text{a} | 2.6±4.7 | 3.2±5.2\text{a} | 0.7±2.5\text{b,d,e} |
| IL-8 [pg/ml] | 20±45\text{a} | 12.8±9.2\text{a} | 73±125\text{a,e} | 24.1±4.7\text{a} | 1.5±1.2\text{b,c,d,e} |
| IL-10 [pg/ml] | 2.1±3.1\text{a} | 3±2.3\text{a} | 4.5±5.6\text{a} | 4.3±7.8\text{a} | 0.1±0.4\text{b,c,d,e} |
| MPO [ng/ml] | 402±153 | 390±181\text{a} | 469±255 | 431±179 | 522±185\text{d} |
| CRP [mg/l] | 4±5.9 | 4.1±3.7 | 7.3±6.3 | 3.6±4 | 2.6±3.7 |
| Iron [μg/dl] | 87±27 | 74±25 | 71±22 | 87±31 | 102±39 |
| Ferritin [μg/ml] | 248±57 | 36.8±3.8\text{a} | 35.1±4.6\text{a} | 37.6±3.8 | 41.5±3.1\text{b,c,d} |

CKD patients were divided into 3 groups according to their eGFR (CKD 3 – 5) and none of the CKD patients received haemodialysis (renal replacement therapy, RRT). Data are depicted as mean ± SD.

Significant differences between the groups: \text{a}...p<0.05; compared to control; \text{b}...p<0.05 compared to KT; \text{c}...p<0.05 compared to CKD 5; \text{d}...p<0.05 compared to CKD 4; \text{e}...p<0.05 compared to CKD 3 using Kruskal-Wallis tests for independent samples with Bonferroni post hoc testing for multiple comparison correction.

\text{f} Parameters were measured in a subgroup of 79% (OC), 80% (CTX), 50% (bALP) and 47% (TRAP) of samples from CKD 3-5 and KT patients.

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n.d. not done
Table 3
Regression analysis of miR-223-3p and miR-93-5p expression with parameters of kidney function, inflammation, and mineral and glucose metabolism.

|                   | Statistical Associations | miR-223-3p |          | miR-93-5p |          |
|-------------------|--------------------------|------------|----------|-----------|----------|
|                   |                          | adjusted $r^2$ | p-value  | adjusted $r^2$ | p-value  |
| CKD 4             |                          |              |          |            |          |
| IL-6              |                          | 0.155       | 0.018    | 0.136      | 0.043    |
| IL-8              |                          | 0.092       | 0.061    | -0.48      | 0.988    |
| eGFR              |                          | 0.09        | 0.059    | -0.01      | 0.388    |
| Hematocrit        |                          | 0.09        | 0.272    | 0.127      | 0.049    |
| C-peptide         |                          | -0.024      | 0.575    | -0.044     | 0.845    |
| OC                |                          | 0.046       | 0.778    | 0.2        | 0.036    |
| PTH               |                          | -0.029      | 0.642    | 0.091      | 0.088    |
| CTX               |                          | 0.226       | 0.015    | -0.062     | 0.932    |
| Inorganic phosphate|                        | 0.026       | 0.391    | -0.023     | 0.49     |
| 25(OH)D           |                          | 0.092       | 0.057    | -0.045     | 0.977    |
| CKD 5             |                          |              |          |            |          |
| IL-6              |                          | -0.091      | 0.582    | -0.123     | 0.915    |
| IL-8              |                          | -0.67       | 0.503    | 0.16       | 0.138    |
| eGFR              |                          | 0.584       | 0.01     | 0.41       | 0.027    |
| Hematocrit        |                          | 0.137       | 0.175    | 0.59       | 0.006    |
| C-peptide         |                          | -0.136      | 0.847    | 0.492      | 0.014    |
| OC                |                          | 0.517       | 0.041    | 0.384      | 0.06     |
| PTH               |                          | -0.058      | 0.461    | 0.339      | 0.058    |
| CTX               |                          | 0.272       | 0.166    | 0.062      | 0.291    |
| Inorganic phosphate|                        | 0.052       | 0.301    | -0.154     | 0.806    |
| 25(OH)D           |                          | 0.034       | 0.306    | -0.143     | 0.969    |

Associations which were statistically significant or showed a trend towards significance are shown in bold print.