Identification and analysis of urine-derived exosomal miRNAs and BDNF

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II Summary

Morphological changes of the complex 3-D architecture of podocytes as well as the loss of these post-mitotic cells often result in severe kidney disease. Since currently, there are no curative drugs, we focused on the identification of non-invasive biomarkers, allowing an early detection of the onset of such diseases. Therefore, we analyzed the cellular- and the cell-free fractions of urine samples from patients suffering from chronic kidney disease (CKD), especially for injury markers as well as for exosome-derived miRNAs.

We identified the mRNA of the neuronal protein brain-derived neurotrophic factor (BDNF) in the cellular fraction of 120 CKD patients and found that the expression was highly correlated with the mRNA expression of the kidney injury marker molecule 1 (KIM-1). Furthermore, we found that both were correlated with the mRNA expression of the podocyte-specific gene Nephrin (NPHS1), suggesting that podocytes are very likely the cellular source.

Beside this, we observed that BDNF is upregulated in biopsies of diabetic patients and seems to be involved in the differentiation of podocytes. Immunofluorescence staining clearly showed that BDNF is localized in the cell body and major processes of podocytes within the glomerulus. Knockdown experiments in zebrafish larvae, a well-established animal model to study kidney function, showed the importance of BDNF on kidney function, morphology and filtration *in vivo*.

Additionally, we analyzed circulating exosomal microRNAs (miRs) isolated from the cell-free urine fraction. After the optimization of a column-based isolation protocol for exosomes, we identified miR-16 from a pre-selected set of candidates as a suitable endogenous reference gene for data normalization. Subsequently, we analyzed the exosomal levels of miR-21, miR-30a-5p and miR-92a in urine samples of 41 CKD patients and 5 healthy controls. We found significantly enhanced levels of miR-21 in CKD patients that were also negatively correlated with the eGFR, suggesting a negative influence on kidney function. MiR-21 was also highly upregulated in de-differentiated glomeruli and in kidneys of nephrotoxic serum- (NTS-) treated mice as an *in vivo* kidney injury model.

To summarize, we identified two promising new and non-invasive biomarkers for CKD in the urine of patients which may also have a functional relevance on kidney function.

1 Introduction

1.1 Background

Podocytes are highly specialized epithelial cells, which form the outer aspect of the glomerular filtration barrier and are therefore responsible for a proper kidney filtration function. It became clear that injury of podocytes contributes to the development of glomerulopathies as the main cause for chronic kidney disease (CKD) [1, 2].

With 10% of the population affected worldwide, CKD is a major public health burden [3]. As presented by the GANI_MED study, especially North-Eastern Germany displays an area with increasing incidence [4]. Beside genetic defects, medication, infections and autoimmune diseases, the main causes for CKD are diabetes mellitus type II and arterial hypertension in the western world. CKD is characterized by a decrease and subsequently by a loss of kidney function [2].

Podocytes are highly differentiated, post-mitotic cells that form the glomerular filtration barrier (GFB), together with glomerular endothelial cells and the glomerular basement membrane (GBM). They attach to the GBM, where they form the outer layer of the GFB, by their complex morphology composed of foot processes interdigitating with those of neighboring podocytes in a zipper-like mode. Proteins like Nephrin, Neph1, FAT1/2 and P-cadherin link the adjacent foot processes and are thereby forming the slit diaphragm which is essential for the proper filtration function of the kidney [5]. Changes of this complex morphology lead to effacement, a flattening and loss of foot processes and subsequently to a loss of podocytes. Podocyte effacement affects the permselectivity of the GFB, leading to the development of proteinuria, which is a main symptom of the

nephrotic syndrome. Podocyte effacement and loss are associated with the irreversible loss of nephron function, which can lead to the development of CKD, if appearing globally. CKD, if left untreated, progresses to end-stage renal disease (ESRD), which makes the requirement of renal replacement therapy inevitable [2].

Since CKD is a terminal disease without any causal therapy, it is essential to detect this disease early to decelerate its progression. Therefore, the development of predictive biomarkers is of vital relevance. Currently, CKD is diagnosed at a relatively late time point, due to its unobtrusive symptoms and a lack of appropriate non- or minimal-invasive diagnostic instruments. This led to an increasing research effort on the development of non-invasive biomarkers.

In the context of kidney research especially urine plays a prominent role as a potential biomarker source. Urine displays a sample type that can be separated into two main components: the cellular fraction and the cell-free fraction (Figure 1). Both fractions have a promising potential as source for biomarkers.

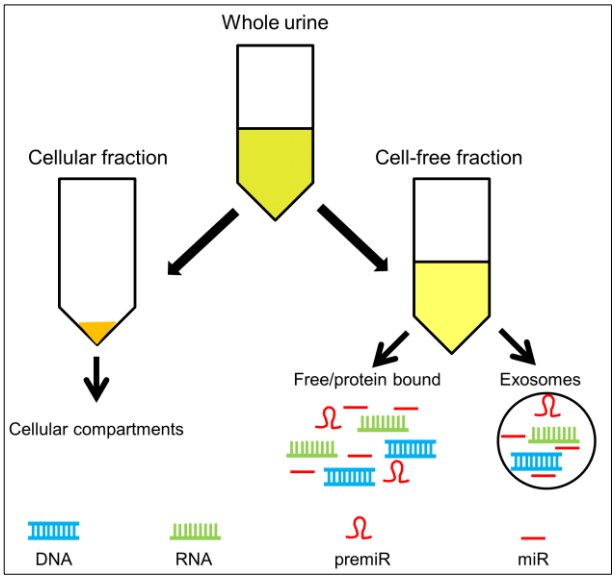

Figure 1: Urine fractions and their nucleic acid composition. Two main fractions can be distinguished: the cellular fraction containing all intracellular nucleic acids and the cell-free fraction with free- and vesicular nucleic acids.

It could be shown that differentially expressed mRNAs from the urine sediment, representing the cellular fraction, are able to indicate CKD [6–9]. For example, Fukuda et al. demonstrated that the urinary ratio of the podocyte genes Podocin (NPHS2) to NPHS1 serves as a podocyte stress marker [10]. Despite these and other findings, further research is needed to discover additional target molecules beside podocyte genes as potential biomarkers for CKD.

It became clear that podocytes and neurons share certain features in terms of morphological and molecular biological characteristics, like the expression of the proteins Synaptopodin [11] and Nephrin [12].

As one of those proteins, the BDNF recently appeared. Originally known as a protein responsible for neuronal growth, survival and branching that is essential for neuronal development and survival *in vivo,* it could be identified in podocytes together with its receptor Tropomyosin receptor kinase B (TrkB). It was found out that BDNF is influencing the actin polymerization, which is essential for a proper podocyte morphology, adhesion and filtration function. This is mediated through the regulation of the two miRs miR-132 and miR-133, resulting in cell survival and repair after induced podocyte damage [13]. Therefore, BDNF displays a promising potential as novel podocyte protein and potential biomarker.

Another potential biomarker is the KIM-1. This is a well-known marker for acute kidney injury (AKI) that is not expressed in the healthy kidney [14]. It is highly upregulated in tubular cells upon injury [15] and could recently be identified in podocytes and as glomerular damage marker [16].

However, only little is known about the mRNA expression of both genes in the urinary cell pellet and their exact glomerular localization in physiological as well as in pathological conditions.

The second urine fraction is generally referred to as cell-free urine (CFU). Amongst other molecules, CFU contains all sorts of RNAs that are mostly present bond to proteins, to other nucleic acids or are packed in vesicles. Especially miRs became a research object of increasing interest since their discovery 1993 and their designation as such in 2001. MiRs are short (~25 nt) non-coding RNAs that block protein expression by either translation repression or degradation of their target mRNAs [17–19]. Most miRs are detectable in physiological as well as pathological conditions in all types of tissues, cells and body fluids, including urine [20, 21]. Non-cellular associated miRs are referred to as circulating miRs (cmiRs) and are present in the urine mainly bond to carrier proteins like Ago2 or High-density lipoprotein and empacked in vesicles like exosomes [22–25] (Figure 1). Due to their resistance to degradation by nucleases, exosomal miRs display a special sample type in terms of sampling, handling and storage issues, lowering the risk of bias compared to free or protein bound miRs [26, 27].

Unfortunately, little is known about the usability of urinary exosomal miRs as potential biomarkers for CKD. Most studies are focusing on single diseases or perform whole miRnome analysis of a relatively small sample set. It could be shown that urinary exosomes of patients suffering from focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) display a unique miR pattern differing from healthy controls [28]. In Lupus nephritis miR-29c serves as a renal fibrosis marker [29]. For diabetic nephropathy (DN) diverse urinary exosomal miRs could be identified as

potential predictive biomarkers [30, 31]. But further investigation is of essential need, to discover new, non-invasive, predictive biomarkers.

Exosome isolation and data normalization are currently the pitfalls in the context of urinary exosomal miR research. There are different approaches addressing these problems. Ultracentrifugation and column based methods are the commonly used methods for exosome isolation at the moment [24, 32–35]. Ultracentrifugation is relatively cost and time intensive and requires equipment not available at every lab. This makes this method unsuitable for high sample counts. Column based methods represent a practical alternative to ultracentrifugation, even though most kits lack some critical steps, like removal of free RNAs. Currently, the gold standard for miR quantification is the RT-qPCR. Although there is no consent about data normalization, usage of endogenous reference controls is the most commonly applied method [36]. Endogenous references require a stable and abundant expression over all samples, which needs to be evaluated for each sample set, especially in cmiR research.

1.2 Aim of this work

The aim of the present work was to detect potential, non-invasive biomarkers for CKD in the cellular and the cell-free fractions of urine samples and to investigate their functional roles.

To this end, we measured the mRNA expression levels of different podocyte genes and three potential candidate genes including BDNF and KIM-1 in the urine sediment of CKD patients. We then investigated the localization as well as the functional role of BDNF within the glomerulus *in vitro* and *in vivo*.

Additionally, we wanted to establish an optimized column based urine exosome isolation procedure and an endogenous normalization strategy for urinary exosomal miR expression data in CKD by the use of preselected normalization candidates. After that, we measured the levels of miR-21, miR-30a-5p and miR-92a in urinary exosomes isolated from the urine supernatant of CKD patients and compared them with a healthy control group, to test, whether they could serve as potential, new biomarkers. MiR-21 was further investigated concerning its role in different glomerular injury models.

2 Results

2.1 Biomarkers and their function in urine sediment of CKD patients

2.1.1 BDNF mRNA in urine sediment of CKD patients

Previous studies showed that mRNAs present in the urine sediment of CKD patients could serve as potential biomarkers making them a promising non-invasive diagnostic tool. As described by Endlich et al. [37], we measured the mRNA expression levels of BDNF, KIM-1 and Cathepsin-L (CTSL1) in the urine sediment of 120 CKD patients by RT-qPCR, to proof their capability as potential biomarkers for CKD. Additionally, we investigated the mRNA expression levels of the three podocyte genes NPHS1, NPHS2 and Podocalyxin (PODXL), to see if podocytes contribute to the urine sediment and if they show a deregulation of these genes in CKD.

The results revealed measurable mRNA amounts of NPHS1, NPHS2 and PODXL and the potential biomarkers BDNF, KIM-1 and CTSL1 in the urine sediment of all 120 CKD patient samples. We correlated the expression data and found a very strong positive correlation between BDNF and KIM-1 (R=0.87). There was also a positive correlation between BDNF and NPHS1 (R=0.27) as well as between KIM-1 and NPHS1(R=0.37). Correlation analysis between the measured mRNA levels and the clinical data hemoglobin A1c (HbA1C), estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR) revealed a significant negative correlation between BDNF expression and UACR but not between the other parameters [37].

Diabetes mellitus, as the main cause of CKD, contributes to a superior proportion of all nephropathies. Since our cohort displays a patient set with a typical distribution of nephropathies, diabetics are the biggest subgroup within our cohort. Therefore, as shown in the supplementary part of Endlich et al. [37], we compared the expression levels of BDNF and KIM-1 of CKD patients with and without diabetes. Diabetes was defined as an HbA1C value ≥6.5. Interestingly, we could observe significantly higher levels of BDNF and KIM-1 mRNA in diabetics compared to non-diabetic patients. We could also find sex dependent differences concerning the expression of BDNF. The BDNF levels of diabetic females and males differed significantly, while other constellations concerning the sex showed no significant differences (Figure 2).

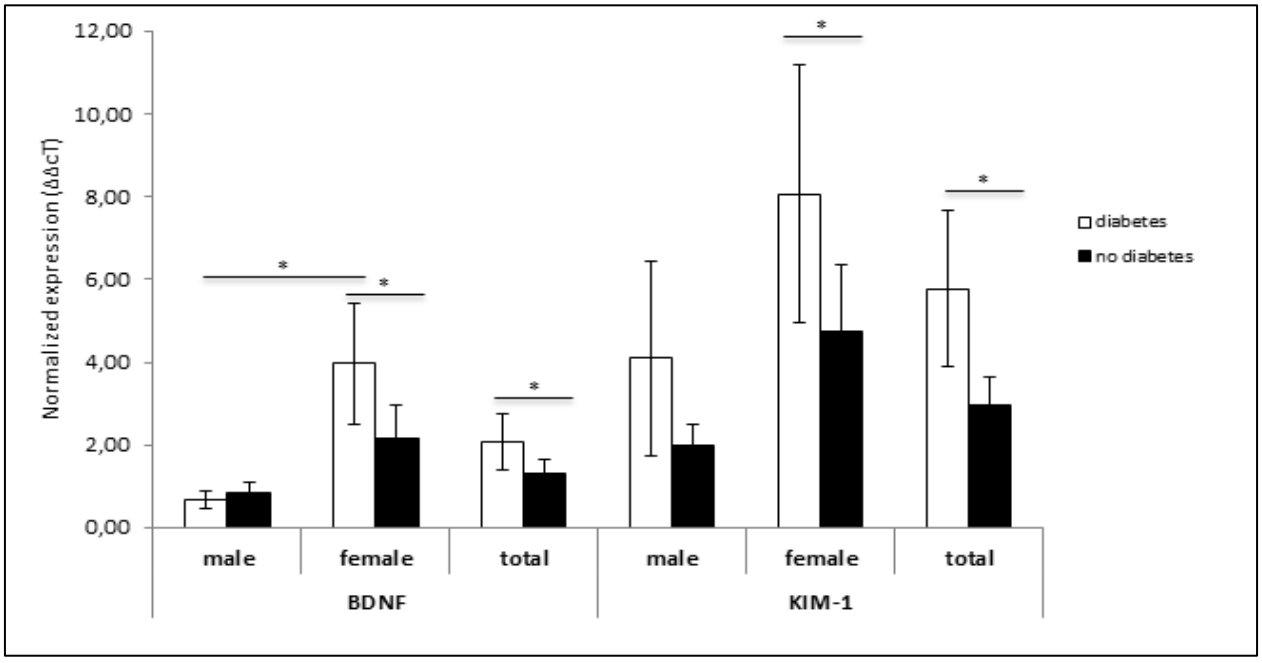

Figure 2: Sex dependent BDNF and KIM-1 mRNA expression in urine sediment of CKD patients with and without diabetes mellitus. BDNF and KIM-1 expression is enhanced in CKD patients with diabetes mellitus. BDNF expression is also sexdependent. Expression is shown as normalized against an inter-run calibrator and against GAPDH. Error bars=SD. (From the supplemental part of [37])

2.1.2 Glomerular localization and expression of BDNF and KIM-1 in kidney sections of healthy individuals and in diabetic nephropathy

Because we found the mRNA levels of BDNF and KIM-1 being upregulated in the urine sediment of diabetic patients, we wanted to investigate their expression on the protein level in kidney sections of patients suffering from DN in comparison to healthy individuals. As revealed by co-immunostaining for Synaptopodin, we found BDNF being expressed in the cell body and main processes of podocytes but not in foot processes as no co-staining with Synaptopodin could be observed. We confirmed these results by 3D-structured illumination microscopy (3D-SIM) of kidney sections co-stained for Nephrin (Figure 3).

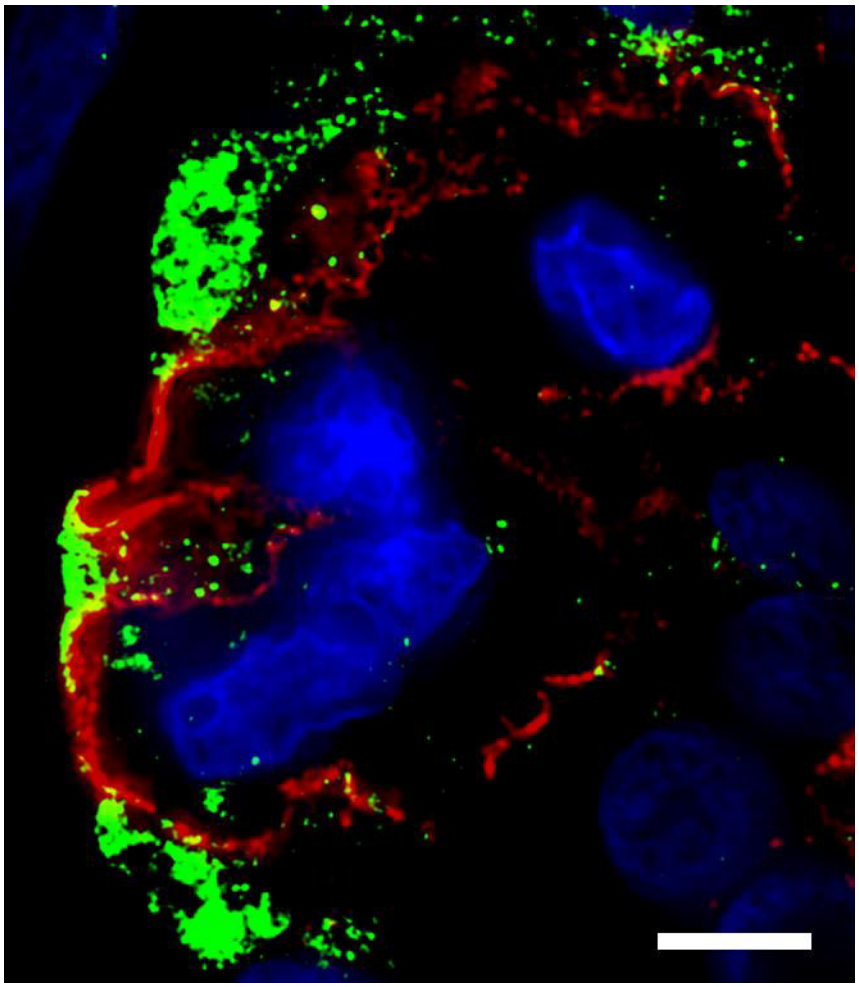

Figure 3: 3D-structured illumination micrograph (3D-SIM) of BDNF expression in human kidney sections. BDNF (green) is expressed in the cell body of podocytes and not in foot processes, as no co-localization with the slit diaphragm protein Nephrin (red) can be detected. nuclei=blue. Scale bar=5 µm. (Adapted from the supplemental part of [37].

The expression of BDNF was enhanced in kidney sections of DN patients. These results could also be confirmed by microarray analysis of human glomeruli. KIM-1 is a well-known marker for acute kidney injury, mainly observed in damaged tubular cells. Here we could show a massive upregulation of KIM-1 not only in tubular cells but also in podocytes of DN patients, whereas almost no KIM-1 signal could be observed in healthy kidney sections. RNA-Seq results of 3 day (d) cultured glomeruli compared to freshly isolated glomeruli confirmed that observation [37].

2.1.3 BDNF in podocyte (de-)differentiation

Podocyte dedifferentiation is a major mechanism in the pathogenesis of DN. To investigate whether BDNF is involved in this process, we applied our podocyte dedifferentiation assay (Glom-Assay). In this procedure, we use magnetic beads isolated glomeruli of a mouse strain that expresses enhanced cyan fluorescent protein (eCFP) under the control of the Nephrin promotor. Nephrin is a podocyte specific protein within the kidney, which expression decreases as one of the first podocyte proteins during de-differentiation. So the decreasing eCFP fluorescence can be used as a surrogate marker for dedifferentiation [38]. Glomeruli were either control treated or treated with the TrkB inhibitor ANA-12 in serial concentrations for 6 d. We found a significantly reduced eCFP signal in the ANA-12 treated glomeruli in a concentration dependent manner, indirectly showing a positive effect of BDNF on podocyte differentiation (Figure 4) [37].

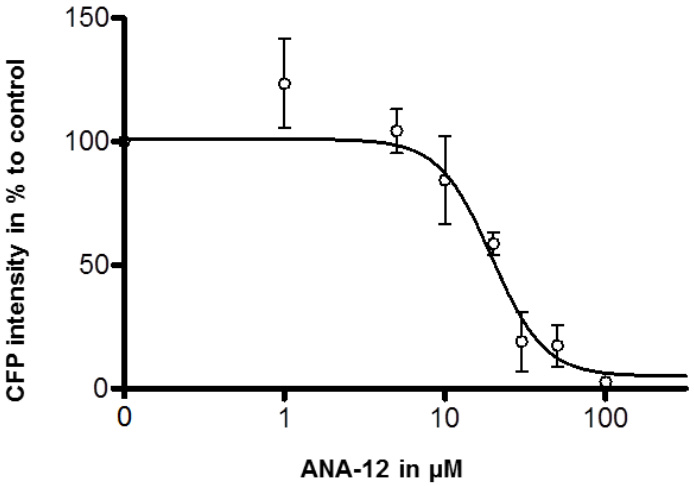

Figure 4: Inhibition of the TrkB receptor triggers podocyte dedifferentiation. ANA-12 decreases the eCFP intensity of glomeruli compared to control treated glomeruli after 6 d cultivation concentration dependently. Error bars=SD. (From the supplemental part of [37]).

2.1.4 Bdnf knockdown in zebrafish

To investigate the influence of BDNF on kidney function *in vivo*, we used the zebrafish *Danio rerio* as model organism. We knocked down (KD) bdnf protein expression by the injection of bdnf morpholinos (bdnfMOs) and control morpholinos (CtrlMOs) into larvae

of different transgenic zebrafish strains. We first used the transparent ET strain that expresses enhanced green fluorescent protein (eGFP) under the promoter control of the podocyte specific gene wt1a (Tg(wt1a:eGFP); $^{mitfaw2/w2}$; roy$^{a9/a9}$), resulting in green fluorescent glomerular cells, including podocytes. The KD was verified by RT-PCR and RT-qPCR. Zebrafish larvae treated with bdnfMOs showed a slightly lowered viability compared to CtrlMOs treated larvae. Interestingly 83% of bdnfMOs injected larvae developed pericardial edema, which is a hallmark of an impaired kidney function. By two photon microscopy (2-PM) of ET larvae 6 days past fertilization (dpf), we could observe two main phenotypes. One phenotype showed a failed fusion of the pronephric glomeruli in the midline. The second phenotype was characterized by a reduced podocyte number, no visible major processes and a dilated Bowman`s space. This was also confirmed by fluorescence staining of cryosections of ET larvae 3 dpf for F-actin. In contrast to this, the CtrlMOs treated larvae presented a normal glomerular morphology with typically shaped podocyte major foot-processes. BdnfMOs injected larvae also showed a downregulation of the slit diaphragm protein nephrin as revealed by immunofluorescence staining and of podocin as revealed by RT-PCR and RT-qPCR. Due to these observations, we wanted to see if the downregulation of the podocyte genes, the loss of podocytes and the altered glomerular morphology after KD of bdnf have an effect on the glomerular filtration function. To address this, we used another zebrafish strain, called CADE, which expresses a eGFP-tagged form of the vitamin D-binding protein (Tg(fabp10a:gc-eGFP); mitfa$^{w2/w2}$; roy$^{a9/a9}$). This 78 kDa protein cannot pass the intact GFB resulting in green fluorescent blood plasma. In case of an impaired filtration, the vitamin D-binding protein passes the GFB resulting in a loss of green blood

fluorescence. In larvae treated with bdnfMOs we observed a massive loss of blood fluorescence 3 dpf as well as 6 dpf compared to CtrlMOs treated larvae [37].

2.2 MiRs in urinary exosomes of CKD patients

2.2.1 Exosome isolation and characterization

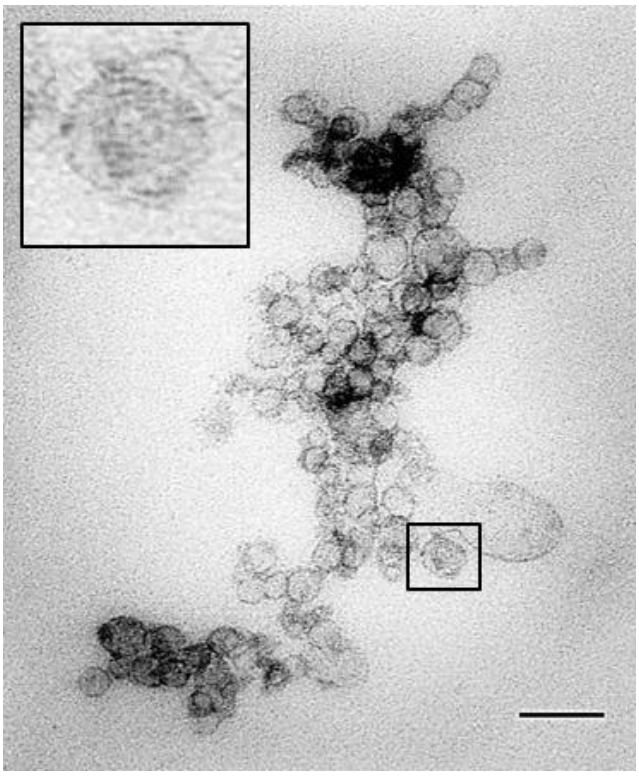

Figure 5: Transmission electron micrograph of urinary exosomes. The isolated vesicles show a typical exosome cup-shape (magnification) and size of 20-80 nm. Scale bar=100 nm.

As described in the introduction, there is no consent about exosome isolation from urine samples. Here we wanted to establish an optimized column based method using the commercially available Urine Exosome Purification and RNA Isolation Midi Kit® (Norgen, Thorold, ON, Canada). Therefore, we increased the centrifugation time to 15 min in the second step as well as the centrifugation speed to 2000 x g in the first step and to 3000 x g in the second step, to ensure a cell- and debris-free urine supernatant. Exosomes from the urine supernatant were prepared for transmission electron microscopy (TEM) for characterization. TEM revealed vesicles with a cup-shape, which is typical for exosomes and a size of 20-80 nm (Figure 5). The size was also confirmed by particle tracking analysis.

We also added an RNase digestion step with the Riboshredder® for 30 min at 37°C after elution of intact exosomes, in order to remove remaining non-exosomal freeRNAs from

the exosome preparation. This was ensured by a positive control digestion of RNA with a predefined concentration in every single preparation. Samples were only used for downstream analysis, if the control RNA was fully digested. Further characterization was performed exemplarily with three representative samples on three different steps of the preparation procedure. Using Western Blot (WB), we could confirm the presence of exosome marker proteins CD63, CD9 and TSG101 in the slurry pellet, the exosome filtrate and after RNase treatment with decreasing band intensities from step to step. The whole preparation protocol is shown in Figure 6 [39].

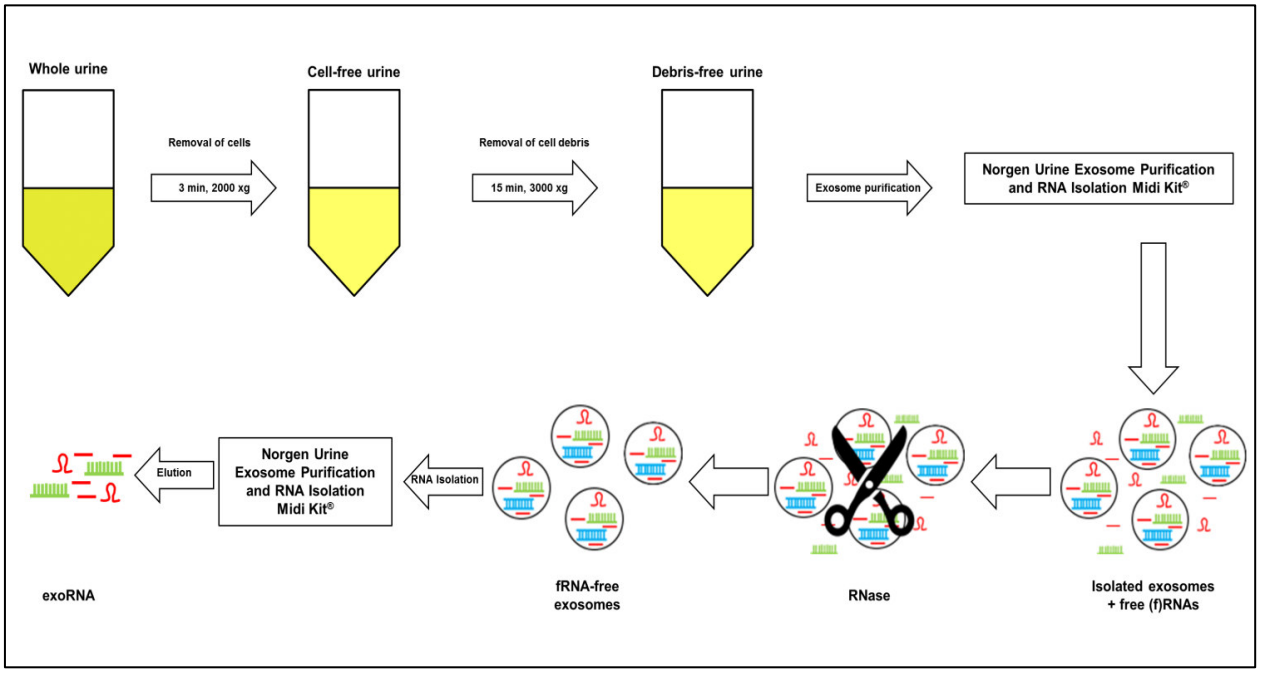

Figure 6: Urinary exosomal RNA isolation protocol. The protocol is based on the Urine Exosome Purification and RNA Isolation Kit® from Norgen. The centrifugation steps were adjusted and RNase treatment was included.

2.2.2 Identification of suitable endogenous reference genes for urinary exosomal miR expression data

Since data normalization is one of the biggest issues in circulating miR research, the search for a suitable normalization strategy is very important. For every study, it is essential to elaborate a normalization strategy that fits to the specific context. Here we decided to use an endogenous reference gene as normalizer. Endogenous reference genes require an abundant as well as a stable expression throughout a specific sample set. We used preselected endogenous normalizers that are evidentially expressed in urinary exosomes and/or have been used as endogenous normalizers in circulating miR studies before; namely miR-16, miR-21, miR-92a and miR-124a. Additionally, we used RNU6B as one of the most used reference genes throughout diverse miR studies. We measured the levels of these normalization candidates by RT-qPCR in exosomes from urine samples of 33 randomly picked CKD patients and 5 healthy controls. The results revealed an abundant expression of miR-16, miR-21, miR-92a and miR-124a in samples from CKD patients and from healthy controls but not of RNU6B.

The expression data was analyzed with the four different data normalization tools normFinder, BestKeeper, Genorm and DeltaCt. All softwares ranked miR-92a as the most stable normalization candidate. MiR-92a was followed by miR-16 as second stable normalizer that was ranked second by all softwares except for BestKeeper. Both were followed by miR-21 and miR-124a that were ranked third and fourth, respectively (Figure 7).

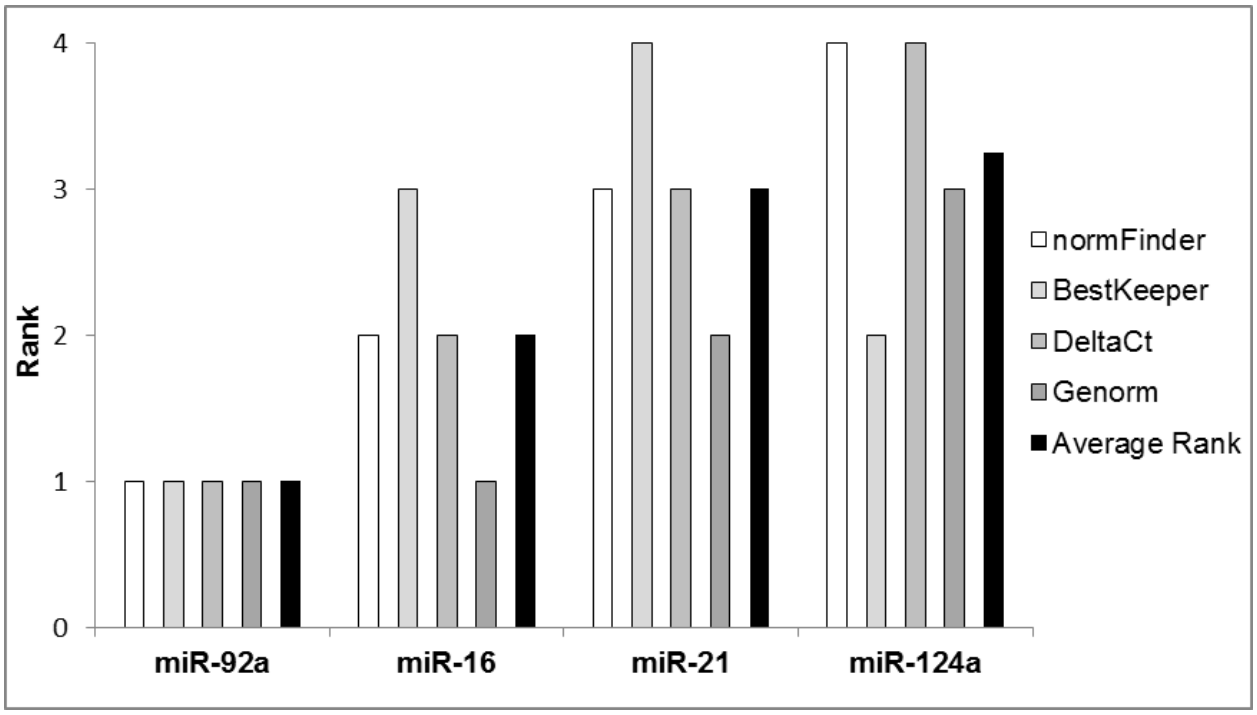

Figure 7: Ranking of normalization analysis. The expression data was adjusted to RNA input and inter-run calibrator corrected before analysis with four different normalization tools.

After that we tested the expression data for differences between healthy controls and CKD patients, because a suitable normalizer should show a stable expression over the whole data set without significantly differential expression in certain patient groups. We could only find a significant difference between CKD patients and healthy controls in the expression of miR-92a. Thereby we had to reject miR-92a as potential endogenous reference gene and picked miR-16 [39].

2.2.3 MiR-21 in urinary exosomes of CKD patients

After evaluation of an exosome isolation and data normalization strategy, we analyzed a new patient set consisted of 41 CKD patients and 5 healthy controls. This patient set also differs from the first one in a lower average age of 47 years compared to 62 years and in a higher proportion of diabetics with 73% compared to 12% in the first set. Herein we measured the expression levels of miR-21, miR-30a and miR-92a by RT-qPCR.

Interestingly, we could find significantly enhanced levels of miR-21 in urinary exosomes of patients suffering from CKD compared to healthy control individuals. The miR-21 levels were more than three times elevated (3.3 ±1.9). We could not find any differences between both groups regarding the levels of miR-30a and miR-92a. There were also no significant differences between miR levels of CKD patients with and without diabetes mellitus. Even though miR-21 was not correlated with UACR and HbA1C, it was negatively correlated with the eGFR (R=-0.434). This was the only significant correlation between miR levels and the described clinical parameters [40].

2.2.4 MiR-21 expression in (de-)differentiated murine glomeruli

Podocyte dedifferentiation is a major event in the pathogenesis of diverse nephropathies including DN. Since our patient cohort consisted of diabetics to a considerable amount, we wanted to investigate the role of miR-21 in that process. Therefore, we used our well established podocyte dedifferentiation model (GlomAssay). We isolated the RNA from freshly isolated (0 d) and from 9 d cultured glomeruli and measured the expression of miR-21. In 9 d cultured glomeruli we found massively enhanced miR-21 levels (108.8 ±78.0-fold) as compared to controls [40].

2.2.5 MiR-21 expression in nephrotoxic serum-induced glomerulonephritis

Furthermore, we investigated miR-21 expression levels in an *in vivo* injury model. In this model, mice were injected with nephrotoxic serum (NTS), leading to the development of glomerulonephritis or with PBS as control. We could observe two main phenotypes. The more abundant, mild phenotype displayed glomerular basement membrane accumulation and mesangial matrix expansion, whereas the severe phenotype displayed crescent formation and podocyte loss. In contrast to that, control mice

exhibited a normal glomerular morphology. We isolated RNA from formalin-fixed paraffin-embedded (FFPE) blocks of these kidneys and measured the miR-21 levels by RT-qPCR. We observed that miR-21 was significantly upregulated in NTS-treated mice compared to control mice. Additionally, miR-21 was correlated with the UACR of the treated mice [40].

3 Discussion

The early detection of CKD is an essential step in the treatment of the disease. Routinely used diagnostic markers like urinary albumin [41], creatinine [42] and cystatin C [43–45] are either incapable of detecting early disease stages or cannot differentiate subclinical forms of CKD. Precise diagnosis mostly requires invasive methodology like biopsying or labor intense downstream procedures. Therefore, there is a great need for non-invasive biomarkers with the ability of early detection and differentiation of disease stages as well as disease groups. Urine displays a powerful source for the discovery of such biomarkers. In the cell pellet of urine samples from patients suffering from CKD, we have found that mRNA levels of BDNF, a neuronal growth factor which has recently been discovered as podocyte protein [13], were highly correlated with the mRNA of KIM-1, a well-established urinary marker for AKI [14], tubular damage and also for glomerular injury. This suggests that BDNF could serve as a new biomarker for glomerular damage.

Within our cohort, we observed that BDNF as well as KIM-1 are significantly upregulated in patients suffering from diabetic mellitus. This is in agreement with our results received from biopsies of DN patients as well as with the data from other groups [46, 47]. We have not detected relevant KIM-1 positive signals in control stainings, but found a strong upregulation in tubuli of DN patients. This was also detected in podocytes of biopsies from DN patients. Additionally, we could confirm these results by RNASeq analysis of dedifferentiated glomeruli in our GlomAssay [38]. Furthermore, we determined the cellular localization of BDNF in human podocytes of FFPE kidney sections by confocal laser scanning microscopy (LSM) and 3D-SIM. By immunofluorescence, we have found

that BDNF was mainly localized in the cell body and major processes, but not in the podocyte foot processes. Moreover, in patients suffering from DN, BDNF expression was up-regulated, which was also confirmed by microarray analysis of human glomeruli.

Due to the fact that podocyte dedifferentiation is a key mechanism in the pathogenesis of DN [48, 49] and that our patient group consisted of a high amount of diabetics, we wanted to find out whether BDNF might play a role in the diabetes-associated dedifferentiation process of podocytes. Therefore, we used the GlomAssay [38] and specifically inhibited the BDNF receptor TrkB expressed in podocytes [13]. We observed that the fluorescence intensity of eCFP, the *Read out* of the dedifferentation of podocytes in the GlomAssay, was significantly decreased after TrkB inhibition, underlining the importance of BDNF on podocyte differentiation. Similar findings were observed by Li et al. [13], who found an upregulation of TrkB and Nephrin after treating podocytes with exogenous BDNF *in vitro*.

Interestingly, we found evidence for a sex-dependent BDNF mRNA expression in diabetic CKD patients, which currently is a relatively under attended phenomenon [50, 51] that needs further research. However, a bigger cohort is needed to confirm these results.

Besides these results, we have found that BDNF as well as KIM-1 were positively correlated with NPHS1. This led to the conclusion that the urinary cell pellet associated BDNF and KIM-1 expression is, at least to a certain amount, podocytes contributed. BDNF was also positively correlated with the UACR, indicating a direct connection between BDNF expression and kidney function, which has been shown for plasma derived BDNF before [52]. For KIM-1 we could not find such a correlation.

To further examine the role of BDNF on kidney function, we used the larval zebrafish as *in vivo* model organism. Zebrafish larvae are easy to breed and have a high reproduction rate within short time periods. Other advantages are their suitability for *in vivo* imaging techniques like 2-PM [53–55] and the relatively easy to perform KD with morpholinos [56]. Zebrafish larvae have a pronephric kidney consisting of a single glomerulus attached to two tubules, resembling those of humans [57]. They express a bdnf orthologue that matches human BDNF to 91% [58]. The morpholino-mediated KD of bdnf led to pericardial edema formation, which is a hallmark for impaired kidney function, confirming the importance of bdnf for kidney homeostasis in zebrafish larvae. This could be further corroborated by the use of a zebrafish strain (CADE) that expresses the vitamin-D binding protein fused to eGFP, which cannot pass the normal GFB, resulting in green fluorescent blood. Here we found a loss of intravascular eGFP fluorescence in bdnfKD CADE larvae indicating a leaky GFB. Further analysis revealed that the knockdown of bdnf in 3 dpf larvae lead to the downregulation of the podocyte genes nephrin and podocin, indirectly showing a negative influence on the podocyte integrity possibly leading to podocyte loss. Furthermore, this could be confirmed by the use of the ET strain that exhibits green fluorescent podocytes. After bndfKD these larvae showed less eGFP positive cells in the glomerulus. Similar results have been reported by the group of Li et al. [13]. In order to analyze structural glomerular changes, we stained cryosections of zebrafish larvae for the cytoskeletal protein F-actin. This revealed an impaired glomerular morphology, which could also be confirmed by 2-PM, that was caused by podocyte loss as well as a developmental delay in bdnfKD larvae. These findings are in agreement with findings of Li and coworkers, who have shown the importance of bdnf for proper pronephros function in zebrafish larvae [13]. They have

also demonstrated that the application of bdnf restored podocyte integrity and kidney function in an adriamycin-induced kidney injury model.

The cell-free urine fraction is a relatively new sample type in biomarker research. Especially miRs from urine-derived exosomes have a great potential as a source for non-invasive biomarkers, since they are protected from degradation [59, 60]. Unfortunately, they also expose some methodical issues [33]. As already described, there is no consent about exosome purification from the urine supernatant. Here we used a column-based method which has advantages over ultracentrifugation in terms of time consumption, costs, material availability and sample throughput but also regarding exosome recovery and RNA yield [33, 61].

Our isolated exosomes showed a typical shape and size in TEM and in dynamic light scattering analysis [25]. The slight size difference between both methods might be due to shrinking processes while sample fixation for TEM [62, 63] and to clumping of exosomes in dynamic light scattering which we could also observe in TEM. The presence of exosomes was also confirmed by WB signals for exosomal marker proteins [25] throughout the whole preparation procedure. To avoid contamination with non-exosomal RNAs, we digested non-exosomal RNAs with an RNase cocktail during the preparation procedure [26, 27].

Another inconsistency in cmiR research is data normalization. There are different approaches to address this problem [64, 65]. One method is the use of spike-in controls which are synthetic miRs from unrelated species, mostly from *Caenorhabditis elegans*, that are added to the exosome-containing solution to function as artificial endogenous controls. However, this normalization method indeed considers the procedural

influences on the samples but neglects the endogenous miR expression status in each sample [66–68]. Another approach is the normalization against the global mean miR expression. Even this might be the most accurate normalization technique, it is not applicable in every laboratory, since it requires decent amounts of different miRs to be measured, making it very time-consuming and cost-intensive [69]. This qualifies the method rather for array- and sequencing-based techniques than for RT-qPCR. In the present work, we used the gold standard for RT-qPCR normalization which currently is the use of an endogenous reference gene that is stably expressed throughout the whole sample set [36, 70].

We tested miR-16 miR-92a, miR124a and miR-21 for their suitability as endogenous reference genes within a representative CKD patient set. Additionally, we tested the widely used endogenous normalizer RNU6B that is not only taken for cellular but also for cmiR data normalization in the literature [64, 65, 71]. We could not detect any expression of RNU6B in our samples which was expected since it is a nuclear RNA that should not appear in the exosome preparation.The results are in agreement with other groups [72, 73] that used RNU6B expression as quality control.

The four other candidates were abundantly expressed and got further analyzed by four different softwares for their stability within our tested sample set. Every software ranked miR-92a as most stable followed by miR-16, miR-21 and miR-124a. MiR-92a and miR-16 have been identified as suitable normalizers before [73, 74]. However, we found significantly differing miR-92a levels between healthy subjects and CKD patients, so that it had to be rejected. Resulting from that, miR-16 was designated as endogenous

reference gene that is frequently used in other studies with different scientific backgrounds [73, 75–78].

Then we tested three different miRs, namely miR-21, miR-30a-5p and miR-92a, if they could serve as potential, new and non-invasive biomarkers in a new set of patients suffering from CKD. This patient set showed a lower average age as well as a higher amount of diabetics compared to the first one [39]. After exosome preparation, RNA isolation and RT-qPCR analysis with the previously described method [39], we observed a significant upregulation of miR-21 in CKD patients but not for miR-92a and miR-30a. This upregulation was independent of the diabetes state. The results indicate that urinary exosomal miR-21 could serve as a non-invasive biomarker for CKD. It might also be predictable for the kidney disease state, since it was also negatively correlated with the eGFR.

MiR-21 has been identified as biomarker for kidney injury before. Other studies showed a deregulation of miR-21 expression in biopsies [79, 80] and body fluids [81] including urine [82–84] where it is mainly considered as fibrotic marker [85, 86]. But here we could show for the first time that miR-21 is upregulated in urinary exosomes of CKD patients as a new and promising sample type.

The exact role of miR-21 is controversial. Whereas Lai and coworkers postulate that miR-21 knockout leads to a disease aggravation in DN mice [87], other groups showed contrary results for DN [88, 89], IgAN [90] and Alport`s Syndrome [88, 91]. Thus, this discrepancy needs further clarification. To address this, we applied different injury models and measured the miR-21 levels.

As previously described, our cohort consisted of diabetics to a decent amount. Since podocyte dedifferentiation is a main driver of DN pathogenesis, we applied our GlomAssay [38] to see if miR-21 is involved in this process. The results show a massive upregulation of miR-21 in dedifferentiated glomeruli, indicating, at least an indirect, influence of miR-21 on podocyte dedifferentiation.

To clarify the role of miR-21 expression in other subtypes of CKD, we used NTS-treated mice as *in vivo* injury model for glomerulonephritis (GN) [92]. Here again, we found an upregulation of miR-21 in kidneys of NTS-treated mice. Together with the positive correlation of miR-21 expression and proteinuria in these mice, this corroborates the important role of miR-21 in glomerular pathogenesis.

In summary, in the cellular urine fraction, we could identify BDNF mRNA as potential, new biomarker for CKD and we could determine its exact localization within podocytes. Additionally, we highlighted its important role in podocyte homeostasis *in vivo* and *in vitro*. In the cell-free urine fraction we could establish a protocol for the purification of exosomes and the normalization of RT-qPCR data of patients suffering from CKD. We could identify urinary exosomal miR-21 as potential, new, non-invasive biomarker for CKD and could show its role in the pathogenesis of glomerulopathies in different kidney injury models.

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5 Publications

5.1 Identification of miR-16 as an endogenous reference gene for the normalization of urinary exosomal miRNA expression data from CKD patients

Tim Lange, Sylvia Stracke, Rainer Rettig, Uwe Lendeckel, Jana Kuhn, Rabea Schlüter, Volkhard Rippe, Karlhans Endlich, Nicole Endlich
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Own work

- Project planning and administration; manuscript writing; figures; literature research; method establishment; urine processing and organization; exosome isolation and characterization; microRNA isolation; RT-qPCR analysis; data-acquisition; -analysis and –curation; statistics

Sylvia Stracke

- Urine processing; urine organization; patient recruitment

Rainer Rettig

- Project administration; manuscript corrections

Uwe Lendeckel

- Project administration; manuscript corrections

Jana Kuhn

- Urine processing

Rabea Schlüter

- Transmission electron microscopy; manuscript correction

Volkhard Rippe

- Manuscript correction; Sample supply

Karlhans Endlich

- Project administration; data analysis; manuscript correction

Nicole Endlich

- Project administration; data analysis; manuscript writing

PLOS ONE

RESEARCH ARTICLE

Identification of miR-16 as an endogenous reference gene for the normalization of urinary exosomal miRNA expression data from CKD patients

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Abstract

Chronic kidney disease (CKD) is a severe disorder with an increasing incidence worldwide. An early detection may help to prevent its progression and to minimize the risk of cardiovascular diseases as one of the major comorbidities. Recently, extracellular miRNAs like urinary exosomal miRNAs became of great interest as non-invasive biomarkers which can be determined by RT-qPCR. But until now, there is no consensus regarding the normalization of miRNAs isolated from body fluids. The present study analyzed the miRNAs miR-16, miR-92a, miR-21, miR-124a and the small nuclear RNA RNU6B for their applicability as an endogenous reference gene in expression studies of exosomal miRNAs isolated from CKD patients. For this purpose, miRNA expression levels were determined by RT-qPCR after the isolation of urinary exosomes from 33 CKD patients and from 5 healthy controls. Expression data was analyzed with the normalization determination software NormFinder, BestKeeper, GeNorm and DeltaCt. Our results revealed an abundant expression of the four candidate miRNAs in urinary exosomes and no detectable expression of RNU6B. We identified miR-16 as the most stable endogenous reference gene in our data set, making it a suitable endogenous reference gene for miRNA studies of urinary exosomes derived from CKD patients.

Introduction

With 10% of the population affected in Europe [1], chronic kidney disease (CKD) represents a major public health burden. Unfortunately, there is no causal therapy, terminally leading to renal replacement therapy like hemodialysis or transplantation. Besides inflammatory and

autoimmune diseases, CKD mostly arises from diabetes mellitus type II and or arterial hypertension, with glomerulopathies representing the main pathophysiological correlate leading to end-stage renal disease (ESRD) [2]. Effacement or loss of podocytes, a terminally postmitotic cell type and essential part of the glomerular filtration barrier, have been identified as key players in the pathogenesis of glomerulopathies [3]. Since early detection could help to decelerate the progress of CKD and could lower the risk for cardiovascular diseases as secondary diseases, identification of suitable biomarkers plays an essential role in CKD research. As already demonstrated, urinary expression of essential podocyte genes could have been shown to be a more sensitive stress biomarker than standard clinical parameters [4]. miRNAs, small (22 nt) non-coding RNAs regulating the gene expression by their binding to a target mRNA, have recently been discovered as useful clinical markers. They are expressed intracellularly and in all body fluids in a tissue- and developmental stage-specific manner [5]. It was recently shown that miRNAs can be secreted in microvesicles as well as in exosomes [6]. As part of the RNA-induced-silencing-complex (RISC), miRNAs block the translation or induce the degradation of the specific target mRNA [7–9].

Urine plays a prominent role as a non-invasive source of exosomal miRNAs that have previously been identified as biomarkers for several diseases. Currently, little is known about exosomes derived from injured podocytes. Lv and coworkers have identified miR-29c as being significantly down-regulated in CKD patients compared to healthy people. Furthermore, miR-29c expression correlated with estimated glomerular filtration rate (eGFR) and the degree of fibrosis [10]. Moreover, the expression of miR-145 and miR-130 was significantly up-regulated in microalbuminuric patients, whereas the expression of miR-155 and miR-424 was down-regulated. These results were confirmed in animal experiments [11].

To guarantee a high specificity and reproducibility, independently of the applied RT-qPCR (SYBR or Taqman™ probes), a reliable normalization strategy is essential [12]. Currently, the most common method to standardize the assay is to use an endogenous control for relative expression analyses [13,14]. A suitable reference gene needs to be stably and abundantly expressed, which is often not fulfilled.

In the present study, we investigated the suitability of the four different miRNAs miR-16, miR-21, miR-92a and miR-124a as reference genes for the analysis of urinary exosomes miRNAs isolated from CKD patients.

Material and methods

Samples

The samples were obtained from urine of 33 CKD patients within the scope of the Greifswald Approach to Individualized Medicine (GANI_MED) [15]. All individuals signed a written informed consent to participate in the study. The study follows the ethical rules of the declaration of Helsinki. Urine provided by five healthy employees of the Department of Anatomy and Cell Biology of the University Medicine Greifswald.

Urine processing

Morning urine of the patients was processed within 4 hours after voiding, since no degradation was observed within this time span in pre-trials. Approximately 50–100 ml urine were transferred to Falcon tubes and centrifuged for 3 min at 2000 x g to remove any cells or cell debris. The supernatant was transferred to fresh Falcon tubes and stored at -80˚C. For further procession, samples were thawed overnight at 4˚C. Then the samples were centrifuged again at 2500 x g for 15 min to remove any residual cell debris. 10 ml of the supernatant were transferred to 15 ml tubes for exosome preparation.

Electron microscopy

For transmission electron microscopy (TEM), 10 mL of cell- and debris-free urine were treated with ExoQuick-TC (System Biosciences, Mountain View, CA, USA) for exosome isolation purposes according to the manufacturer's instructions with minor modifications. To 10 ml urine, 3.3 mL ExoQuick-TC were added and stored overnight at 4˚C. Then, samples were centrifuged at 10,000 x g for 60 min and the supernatant was discarded. The pellet was prepared for TEM according to Théry and coworkers [16] with minor modifications in step 6: 1% uranyl acetate was used to contrast the samples instead of uranyl-oxalate.

Exosome preparation and miRNA isolation

Exosome preparation was performed with the Urine Exosome Purification and RNA Isolation Midi Kit (Norgen, Thorold, ON, Canada) according to manufacturer's instructions with minor modifications. The centrifugation steps were performed as described in the urine processing part, to ensure cell- and debris-free urine. After elution of the exosome fraction, the exosome solution was treated with 0.0125 U/μl of the RNase cocktail RiboShredder (Epicentre, Madison, WI, USA) for 30 min at 37˚C, to eliminate cell-free non-exosomal RNA. After RNase treatment, the exosome solution was stored on ice and Lysis Buffer A plus Lysis Additive B were added immediately. RNA was eluted in 75 μl Elution buffer. The miRNA concentrations were determined with the Qubit miRNA-Assay and the Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions using 15 μl sample volume.

Exosome characterization

Three different exosome fractions from two samples obtained with the Urine Exosome Purification and RNA Isolation Midi Kit (Norgen, Canada) were taken for exosome characterization by Western blot. The first fraction was obtained from 400 μl supernatant of the resuspended and centrifuged Slurry Buffer pellet. The second fraction was the first filtrate containing the cleaned up exosomes. The third fraction was the RiboShredder treated exosome suspension. To each 400 μl fraction, 100 μl of ExoQuick-TC were added, mixed by inversion and incubated at 4˚C for 4 h, to precipitate exosomes. Then the samples were centrifuged at 14,000 x g for 30 min and the supernatants were discarded. Exosomes from cell culture supernatants of human podocytes (kindly provided by Dr. Pavenstädt, Münster, Germany) and HEK293 cells served as positive controls for Western blot. Exosomes were isolated with ExoQuick-TC following the manufacturer's instructions. The pellets from urine samples and cell culture samples were resuspended in Pierce IP Lysis Buffer (500 mM, Thermo Fisher Scientific) with 1 x Halt Proteinase Inhibitor Cocktail (Thermo Fisher Scientific) and 1 x EDTA (0.1%, Thermo Fisher Scientific). Then, the suspension was shaken for 20 min at 1,400 rpm at 4˚C in a Thermo Mixer Comfort (Eppendorf, Hamburg, Germany). Afterwards, the samples were centrifuged for 20 min at 14,000 x g at 4˚C and the supernatants were transferred to a fresh tube and stored at -20˚C.

Western blot analyses

The protein samples were measured with the Piece BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Then, the samples were adjusted to 20 μg/lane (TSG101) and to 10 μg/lane (CD9 and CD63), mixed with 6 x sample buffer (0.35 M Tris pH 6.8, 0.35 M SDS, 30% v/v Glycerol, 0.175 mM bromophenol blue) and boiled at 95˚C for 5 min. The protein samples were separated on a 4–20% gradient Mini-Protean TGX

Gel Stain-free (Bio-Rad, Hercules, CA, USA). The separated proteins were blotted on nitrocellulose membranes using the Trans-Blot Turbo RTA Transfer Kit (Bio-Rad) and the Trans-Blot Turbo Transfer System (Bio-Rad) at 2.5 A/25 V for 5 min. Membranes were washed in 1x TBS+T wash buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$ supplemented with Tween-20 0.1%; AppliChem) and blocked in wash buffer supplemented with 5% milk powder (blocking solution) for 1 h at room temperature. Primary antibodies were diluted in blocking solution and incubated with the membranes overnight. After washing 3 x 5 min with wash buffer, the membranes were incubated with secondary antibodies for 45 min, washed again 4 x 5 min, developed with the ECL Prime Western Blotting Detection Reagent (Amersham) and visualized on X-ray films (Amersham, Hyperfilm ECL) by using Carestream Kodak autoradiography GBX developer/fixer solutions. For normalization and usage of alternative antibodies on the same blot, blots were stripped. Antibodies were used at the following final concentrations: anti-TSG101 (Sigma-Aldrich, 1:1000), anti-CD9 (Invitrogen, 1:2000) and anti-CD63 (Invitrogen; 1:8000).

Dynamic light scattering analysis

For dynamic light scattering analysis, 10 ml of three different cell- and debris-free, representative urine samples were treated with ExoQuick-TC as already described. To 10 ml urine, 3.3 ml ExoQuick-TC was added and stored overnight at 4˚C. Samples were centrifuged at 10,000 x g for 60 min and the supernatant was discarded. The pellets were resuspended in 1.5 ml ice cold PBS. Then samples were vacuum-degassed for 20 min at RT in a ThermoVac (GE, Little Chalfont, UK). Afterwards 200 μl of each sample were transferred to a 1 cm path lenght cuvette (Brandt, Wertheim, Germany). Each sample was measured 5 times with a Zetasizer Nano SZ (Malvern, Herrenberg, Germany) at 25˚C with a refraction index of 1.36 and an absorption of 0.001 with standard diluent parameters as referred to H_2O. Each measurement consisted of 20 runs ā 10 sec. Data was analyzed with the Zetasizer Software 7.11 and displayed as size/intensity plots.

Selection of the normalization candidates

Candidates were selected by evidential expression in urinary exosomes and/or evidential use as normalizers in previous circulating miRNA studies. RNU6B was also chosen since it is broadly used as normalizer for intracellular miRNA studies as well as for circulating miRNA studies (Table 1).

Reverse transcription

cDNA was synthesized starting from 1 ng miRNA using Taqman™ miRNA Assays and the Taqman™ miRNA Reverse Transcription Kit (Thermo Fisher Scientific). The following Taqman™ miRNA Assays for candidate normalizers were used in this study: Hsa-miR-16-5p: ID #000391; Hsa-miR-21-5p: ID #000397; Hsa-miR-92a-3p: ID #000431; Hsa-miR-124a-3p: ID #001182.

Table 1. Endogenous normalization candidates.

Candidate	Type	Accession# MiRBase / NCBI	Evidence
hsa-miR-16-5p	miRNA	MIMAT0000069	[17,18]
hsa-miR-21-5p	miRNA	MIMAT0000076	[17,18]
hsa-miR-92a-3p	miRNA	MIMAT0000092	[17–19]
hsa-miR-124a-3p	miRNA	MIMAT0000422	[18]
RNU6B	snRNA	NR_002752	

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We also tested a broadly used Taqman™ miRNA Assay for miRNA normalization: RNU6B: ID #001219. In case of less than 1 ng RNA, 5 μL of RNA solution were used. The setup for RT-reactions was performed after the manufacturer's instructions. One ng placenta RNA was used as positive control and inter-run-calibrator and was co-synthesized with every cDNA synthesis run. Negative controls included no template and no reverse transcriptase controls.

RT-qPCR

The qPCR was performed with the Taqman™ miRNA Assays described above and the Taqman™ Universal Master Mix II, no UNG (Thermo Fisher Scientific) following the manufacturer's instructions. The reaction volumes contained 1.33 μl undiluted cDNA solution and 18.67 μl Master Mix. The qPCR was performed on the Light Cycler® Nano (Roche Applied Biosystem, Mannheim, Germany) with the following cycler scheme: 10 min at 95˚C followed by 45 cycles of 15 sec at 95˚C and 60 sec at 60˚C. All samples were run in triplicate and placenta cDNA was used as inter-run-calibrator. Negative controls included the ones from cDNA synthesis and an extra no template control for the qPCR reaction. Standard curves with standard cDNA samples were used for efficiency determination of every single Taqman™ miRNA Assay. C_t-values were calculated by the Light Cycler® Nano SW 1.1 software (Roche Applied Biosystem) with automatically set thresholds and baselines. Raw C_t-values $\geq$38 were excluded from analysis. All C_t-values were normalized against starting RNA amount and were inter-run calibrator corrected.

Normalization analysis

Normalization analysis was performed using different online available normalization tools. The first one is NormFinder v0.953 (http://moma.dk/NormFinder-software), which works with an algorithm for linear data to determine the most stable normalization candidate gene [20]. The tool calculates a stability value (SV) for every single candidate. The lower the stability value, the more stable is the expression of the corresponding candidate gene. The data output comes with the stability values for all candidates together with standard deviations (SD) and announces the most stable candidate gene. Splitting data into study groups, results in additional intra- and intergroup variation and determination of the best endogenous normalizer combination. The second tool used in this study is the online-based tool RefFinder [21] (http://fulxie.0fees.us/). It comprises four different commonly used normalization tools, namely BestKeeper [22], comparative DeltaCt [23,24], NormFinder [20] and GeNorm [25], working with different algorithms to evaluate the most stably expressed gene or gene pair of a specific sample set.

Statistical analyses

All statistical analyses were performed with IBM SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, USA). Data was tested for normality by Kolmogorov-Smirnov test. Differences between two groups were tested with Mann-Whitney-*U*-test. The two-tailed tests with p-values ≤ 0.05 were considered statistically significant.

Results

Characterization of urinary exosomes

For characterization of exosomes in the urine supernatants, exosomes were isolated from 10 ml urine of 3 CKD patients, using ExoQuick-TC and were prepared for electron microscopy. Fig 1A shows the results obtained from TEM, revealing vesicles with a size of 20–80 nm and a cup-like shape as typical characteristics of exosomes. Furthermore, a dynamic light scattering analysis

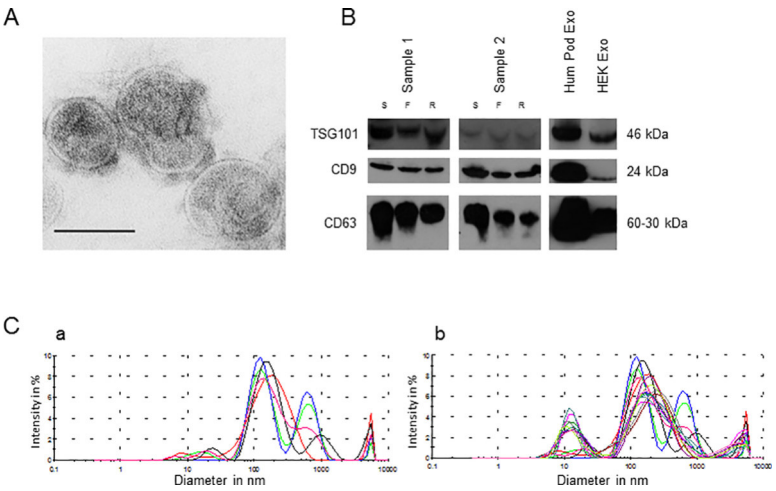

Fig 1. Characterization of urinary exosomes. Urinary extracellular vesicles were isolated with ExoQuick-TC and prepared for TEM. Vesicles showed an exosome-like shape and size. Scale bar = 100 nm (A). Exosomes of two samples from Norgen urine exosome preparation kit were precipited with ExoQuick-TC and analyzed by Western blot for exosomal markers TSG101, CD9 and CD63. Protein extracts display the presence of exosomal marker proteins over the whole preparation procedure with a slight decrease of band intensities. S = slurry pellet, F = filtrated exosome suspension and R = filtrated exosome suspension after RiboShredder treatment (B). Zetasizer analysis of urinary exosome samples (C). The results of the 5 measurements of the urine sample displayed in TEM (A) are shown (C, a). Beside this the results of the 5 measurements per sample of three urinary exosome samples are shown (C, b). The figures show vesicles with typical exosomal size and some bigger structures that might be due to clumping artefacts.

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revealed two main peaks for the sample displaying the sample shown in TEM (Fig 1C a). The biggest peak shows vesicles with a size ranging from approximately 45 nm to 300 nm with its maximum at approximately 130 nm. The second smaller peak has its maximum at 600 nm which is caused by vesicle clumping. The analysis of three different representative urinary exosomal samples showed a relatively homogeneous size distribution throughout the different samples with a similar peak situation as described for the first sample alone (Fig 1C b). Additionally, we performed our exosome preparation protocol as described above on urine samples from two individuals. To confirm that the vesicles obtained were in fact exosomes, protein extracts were analyzed by Western blot for the presence of exosome markers TSG101, CD9 and CD63 (Fig 1B). The Western blot showed specific signals for all three exosome markers in all three preparation fractions with decreasing band intensity of CD63 and CD9 from Slurry Buffer pellet, over exosome filtrate to the RiboShredder-treated fraction. Lysates of human podocytes and HEK293 exosomes served as positive controls.

Patient characteristics

The patient characteristics are shown in Table 2. In total, 38 individuals were included in this study with a mean age of 62 (±19) years. Eighteen participants were female. The study group included four diabetic patients that were all in the CKD group. The CKD group had a mean age of 66 (±16) years. Fifteen members of this group were female. The healthy control group had a mean age of 40 (±15) years and included three females.

Expression of mature miR-16, miR-21, miR-92a and miR-124a

Normalized expression of all four candidates (miR-16, miR-21, miR-92a and miR-124a) showed a relatively high homogeneity over all 38 samples (S1 Table), since the slopes of the

Table 2. Patient characteristics. Patient characteristics are displayed as total patients, CKD-patients and the normal group with parameters: mean age in years, SD = Standard deviations; sex in amount patients, percentage and diabetes in amount patients, percentage.

Characteristics	Total	CKD patients	Healthy controls
Age; mean (SD)	62 (±19)	66 (±16)	40 (±15)
Sex; n female/male (%)	18 (47.4)/20 (52.6)	15 (45.5)/18 (54.5)	3 (60.0)/2 (40.0)
Diabetes; n (%)	4 (10.5)	4 (12.1)	0 (0.0)

Patient characteristics are displayed as total patients, CKD-patients and the normal group with parameters: mean age in years, SD = Standard deviations; sex in amount patients, percentage and diabetes in amount patients, percentage

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trend lines were zero or, in the case of miR-21, close to zero (Fig 2A). These results also showed an abundant expression of the four mature miRNAs in urinary exosomes with median normalized C_t-values of 17.6, 17.1, 15.3 and 15.1 for miR-16, miR-21, miR-92a and miR-124a, respectively. For RNU6B we could not detect any expression, indicated by raw C_t-values ≥ 38 (data not shown).

Stability analysis

NormFinder. For stability analysis the whole data set of input RNA and inter-run calibrator-normalized C_t-values was analyzed with the NormFinder software ignoring classification. As shown in Fig 2B, miR-92a showed the highest stability (SV = 0.38±0.14) followed by miR-16 (SV = 0.51±0.13), miR-21 (SV = 1.07±0.14) and miR-124a (SV = 1.13±0.15). From these results the NormFinder software identified miR-92a as the best normalizer. In a second analysis, data was split into groups comprised of CKD patients and healthy individuals. The results showed that miR-16 appears to be the most stable candidate (SV = 0.18) followed by miR-92a (SV = 0.27), miR-21 (SV = 0.35) and miR-124a (SV = 0.66) (Fig 2B). The best combination of two genes was the combination of miR-16 and miR-92a with a SV of 0.25.

RefFinder. Additionally, the data set was analyzed with the online tool RefFinder. This tool combines the normalization determination algorithms GeNorm, BestKeeper, DeltaCt and

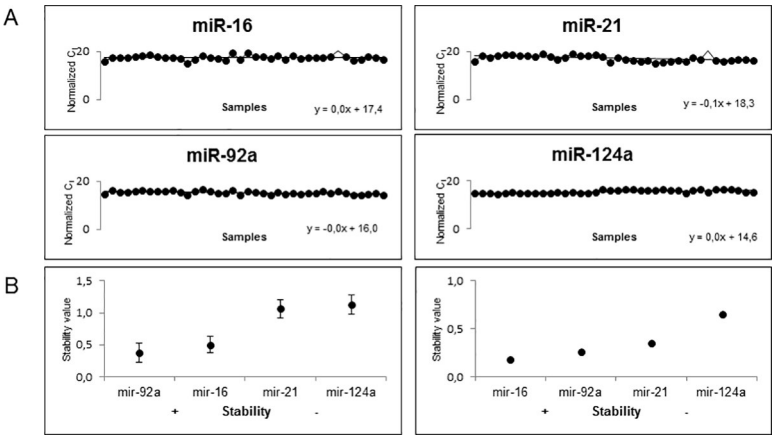

Fig 2. Expression distribution and stability of normalization candidate mature miRNAs. All four candidate miRNAs are abundantly expressed in urinary exosomes of CKD patients and the normal group. Exosomal RNA was isolated with the Norgen urinary exosome preparation and RNA isolation kit. Expression data was generated by qRT-PCR using Taqman® miRNA Assays. The raw Ct-values were normalized against RNA input and an inter-run calibrator (A). MiR-92a is the most stable candidate normalizer within the data set, as revealed by NormFinder software. The best combination is miR-92a/16 with miR-16 as the most stable one, when input data is split into CKD patients and normal group. Error bars = SD (B).

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NormFinder. Stability analysis with BestKeeper identified miR-92a as the most stable normalization candidate in the presented data set. miR-92a was followed by miR-124a as the second stable candidate, which in turn was followed by miR-21 and miR-16 (Fig 3). Looking at significant correlations of the potential normalizers with the BestKeeper index, the only candidate that showed a non-significant correlation was miR-124a (data not shown). The comparative DeltaCt method showed the same results as the NormFinder software or the GeNorm analysis that stated miR-16/92a as the most stable gene-pair (Fig 3). The NormFinder results obtained from the software were also confirmed by the online version included in RefFinder (Fig 3).

Expression differences between CKD patients and healthy controls

Since it is essential for an endogenous reference gene to be stably expressed in both, diseased and healthy states, we analyzed our data set for differences in mean C_t-values between both groups. Fig 4 shows no significant differences between healthy controls and CKD patients in the mean expression of miR-16, miR-124a and miR-21. The only significant difference between these two groups was observed with respect to the expression of miR-92a (Fig 4).

Discussion

In this study we analyzed miR-16, miR-21, mir-92a and miR-124a as potential reference miRNA genes in urinary exosomes of 33 CKD patients and five healthy controls. Since ultracentrifugation is not very applicable when it comes to high sample throughput due to its low availability and the high time consumption, we used a low-speed centrifugation kit approach which is established for the isolation of exosomes. Furthermore, it has been shown that low-speed centrifugation approaches are superior over ultracentrifugation in terms of total exosome isolation and especially in RNA recovery [26,27]. The presence and characterization of exosomes were confirmed by TEM and Western blot. Transmission electron micrographs

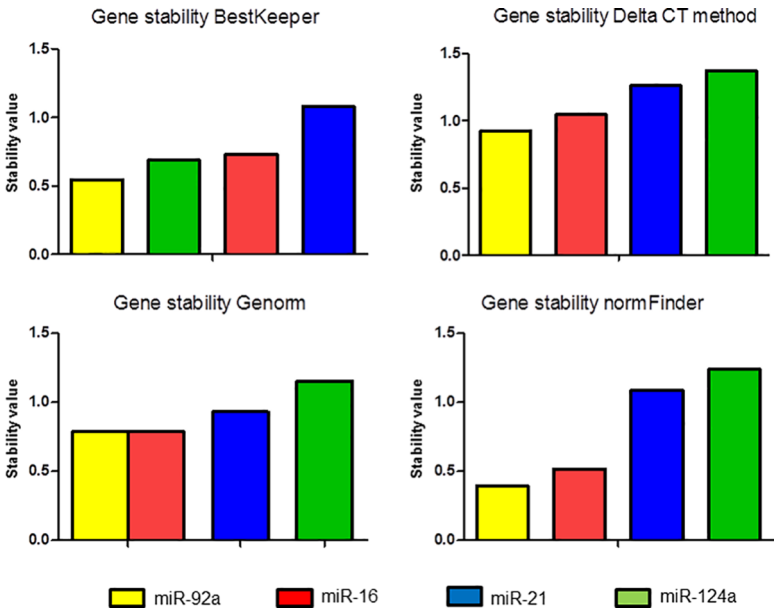

Fig 3. Stability of normalization candidate mature miRNAs determined with different algorithms. Stability values were calculated by the online available tools BestKeeper, DeltaCt, Genorm and NormFinder. The lower the stability value, the higher the stability. Each tool reveals miR-92a as the most stable one or the combination of miR-92a/miR-16. miR-92a = yellow, miR-16 = blue, miR-21 = purple and miR-124a = green.

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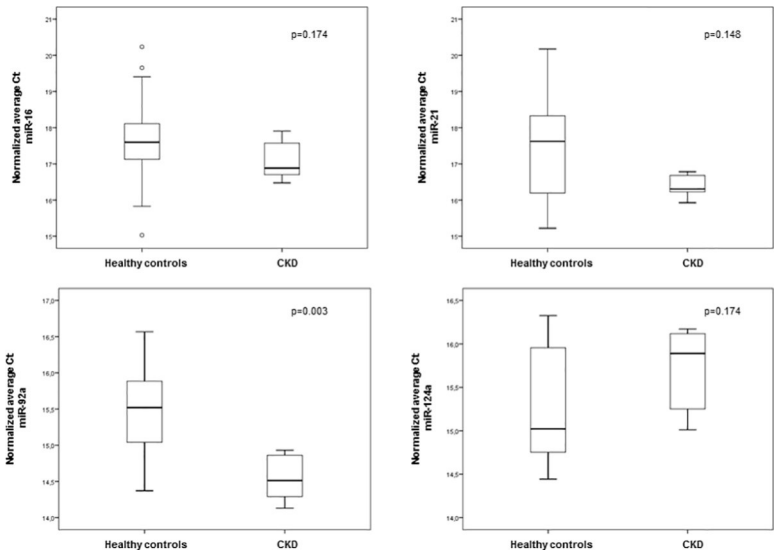

Fig 4. Expression differences of the normalization candidates between healthy controls and chronic kidney disease (CKD) patients. Box-Whisker Plot of the RNA input- and inter-run calibrator-normalized expression values determined by Taqman® qRT-PCR. The midline represents the median and the box borders represent the inter-quantile range. Percentiles are displayed by whiskers.

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revealed extracellular vesicles with typical exosomal characteristics in shape and size [28]. The relatively small vesicle size in urine samples has been described previously [29–31]. This might be due to shrinking processes during TEM preparation procedure, as reported previously [32,33]. Beside TEM we analyzed the size of the exosomes with dynamic light scattering. By using this method we found that the isolated vesicles showed the size typical for exosomes [34] beside some bigger structures that might be due to clumping, which we have also identified by TEM. Since extracellular vesicle cargo is protected against RNase treatment [6,35–37], we included an additional RNase treatment step in our isolation procedure, to eliminate contamination with non-exosomal free-miRNAs. Furthermore, we were able to detect strong signals for the typical exosome markers CD9, CD63 and TSG101 [34] throughout the whole preparation procedure, by Western blots. The TSG101 intensity varied between both samples. It is known that the proportions of exosomal marker proteins can vary between different samples. Royo and co-workers could also observe different TSG101 levels in different samples from prostate adenoma patients with relatively equal CD63 protein levels, considering different exosomal protein loading as a possible reason [38]. The stability of the purified exosomes was also confirmed by the consistent measurement of the described miRNAs.

To limit variations due to different urine concentrations, we used timed urine samples [39,40]. Although three different normalization strategies for miRNA RT-qPCR experiments are available, there is no consensus over universally applicable endogenous controls. One strategy is the normalization by global mean miRNA expression [41]. However, this method requires a remarkable amount of miRNAs per sample and is expensive and time consuming. Another method normalizes the amount of miRNA against a spike-in control like a synthesized miRNA from *Caenorhabditis elegans* such as ce-miR-37. This normalization may consider the experimental influences on the samples, but it does not consider the endogenous state of the overall miRNA expression [42–44]. The most utilized method is the endogenous control method that determines the relative expression of the target gene using abundant and stably expressed endogenous miRNAs [13,14,23]. One of the most frequently used endogenous

reference genes for circulating and exosomal miRNA normalization is RNU6B. RNU6B is a small noncoding RNA, but not a miRNA, which is exclusively expressed in nuclei and should not be detectable in isolated exosomes or in fractions of circulating miRNA. Nevertheless, serals studies [10,45–47] recommend RNU6B as a reference gene for quantification of miRNA in body fluids. In the present study, RNU6B was not detectable suggesting that our exosome fraction was pure and did not show any cellular contamination. These results are in agreement with those obtained by Solayman et al. and Sole et al. [17,46], who proposed to use RNU6B as a quality indicator for cellular contaminations and cellular degradation processes. It was reported that miR-92a or the combination of miR-92a with miR-16 are the most reliable candidates for normalization, followed by miR-16 alone [17,48]. In agreement with these reports, the stability analysis of our data with the NormFinder software identified miR-92a as the most stable miRNA followed by miR-16. Using the BestKeeper software, we also found miRNA-92 as the most reliable candidate. The second candidate of the BestKeeper analysis was miR-124a, directly followed by miRNA-16. However, the BestKeeper software did not consider the non-significant correlation of miRNA-124a with the BestKeeper value. Therefore, our analysis revealed that miR-92a and miR-16 are the most stably expressed normalization candidates, which is in agreement with several miRNA expression studies [17,48]. The disadvantage of miR-92a as a normalizer is that it cannot be used as an endogenous reference gene since we found a significant difference in the expression of miR-92a in the control group compared to the CKD group.

Since this is not the case for the second stable candidate of our data analyses, the miR-16, which is already used as an endogenous reference gene in several cancer studies [49–52] and also in studies with hypertensive patients [17], we conclude that miR-16 is the best normalizer for miRNAs of urinary exosomes isolated from CKD patients within the tested candidates.

Supporting information

S1 Table. S1 Table shows the raw miRNA expression data normalized against RNA input and the inter-run calibrator of CKD patients (numbers) and healthy control individuals (N-number).
(PDF)

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5.2 BDNF: mRNA expression in urine cells of patients with chronic kidney disease and its role in kidney function

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Own work

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ORIGINAL ARTICLE

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BDNF: mRNA expression in urine cells of patients with chronic kidney disease and its role in kidney function

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Abstract

Podocyte loss and changes to the complex morphology are major causes of chronic kidney disease (CKD). As the incidence is continuously increasing over the last decades without sufficient treatment, it is important to find predicting biomarkers. Therefore, we measured urinary mRNA levels of podocyte genes *NPHS1*, *NPHS2*, *PODXL* and *BDNF*, *KIM-1*, *CTSL* by qRT-PCR of 120 CKD patients. We showed a strong correlation between BDNF and the kidney injury marker *KIM-1*, which were also correlated with *NPHS1*, suggesting podocytes as a contributing source. In human biopsies, *BDNF* was localized in the cell body and major processes of podocytes. In glomeruli of diabetic nephropathy patients, we found a strong *BDNF* signal in the remaining podocytes. An inhibition of the *BDNF* receptor TrkB resulted in enhanced podocyte dedifferentiation. The knockdown of the orthologue resulted in pericardial oedema formation and lowered viability of zebrafish larvae. We found an enlarged Bowman's space, dilated glomerular capillaries, podocyte loss and an impaired glomerular filtration. We demonstrated that *BDNF* is essential for glomerular development, morphology and function and the expression of *BDNF* and *KIM-1* is highly correlated in urine cells of CKD patients. Therefore, *BDNF* mRNA in urine cells could serve as a potential CKD biomarker.

KEYWORDS
BDNF, biomarker, CKD, diabetes, podocyte

1 | INTRODUCTION

The "Greifswald Approach to Individualized Medicine (GANI_MED)" aims at the development of individualized diagnosis, prevention and therapy strategies for common diseases.[1,2] Therefore, patient cohorts were recruited and investigated under standardized and routine conditions. The cohort investigated in this report consisted of patients diagnosed with chronic kidney disease (CKD).[3]

CKD mostly affects risk groups like patients suffering from diabetes mellitus or hypertension.[4-6] The disease is characterized by a decrease and subsequently by a loss of kidney function named end-stage renal disease. Loss of renal function can only be compensated by renal replacement therapies like haemodialysis or transplantation. Until today, CKD is not reversible and it is therefore important to identify predictive biomarkers and possible target molecules allowing early detection and prevention.

As it became obvious that podocytes are associated with the development of CKD, this specific cell type was brought into the focus of research.[7,8] Podocytes, a post-mitotic cell type, maintain the glomerular filtration barrier by their unique cellular structure, which includes major processes and foot processes covering the glomerular basement membrane (GBM) in a zipper-like fashion.[9,10] Changes in these structures lead to an impairment of glomerular function and are related to several kidney diseases like focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD) and diabetic nephropathy (DN).

As podocytes share certain structural and molecular biological characteristics with neurons, proteins involved in neuronal structural and physiological maintenance[11,12] are of great interest for podocyte research and might play a potential role as biomarkers. One of those neuron-specific proteins is *brain-derived neurotrophic factor* (*BDNF*), a neurotrophic factor which is involved in neurogenesis, neuronal survival,[13,14] branching[15,16] and synaptic growth.[17] Thus, Ernfors et al[18] have already shown that *BDNF* plays a key role in neuronal development, because heterozygous knockout (KO) mice showed decreased neuronal development and homozygous KO mice often die directly after birth. It has already been shown that *BDNF* binds to 2 different receptors—TrkB and p75, that are involved in cell survival and differentiation processes.[19,20] Recently, it has been reported that *BDNF* and TrkB are expressed in podocytes *in vivo*, being essential for actin polymerization and cell survival.[21] As the actin cytoskeleton plays an important role for podocyte morphology and adhesion *in vivo*, and podocyte detachment is a major event in glomerulopathies, we investigated the expression of *BDNF* in cells appearing in the urine of patients suffering from CKD in an attempt to find out whether *BDNF* could be a suitable marker for the detection of DN.

As a second potential biomarker for glomerulosclerosis, we chose Hepatitis A virus cellular receptor 1 (*HAVCR1*) or kidney injury molecule-1 (*KIM-1*), a transmembrane protein that is not or at very low levels expressed in healthy kidneys.[22-25] Interestingly, Zhao et al[26] found *KIM-1* being up-regulated in parietal epithelial cells and dedifferentiated podocytes of diabetic rats. Furthermore, recent findings show that the expression of *KIM-1* reduces the negative effects of acute kidney injury by inducing phagocytosis.[27] Therefore, we selected *KIM-1* to proof whether it could be used as a potential glomerular biomarker.

To study the influence of *BDNF* on podocyte development and glomerular morphology *in vivo*, we took larval zebrafish as a well-established model organism. The zebrafish larva is ideal for podocyte research[28-30] as it develops a functioning glomerulus during 48-56 hours post-fertilization (hpf),[31,32] which can be studied directly in living larvae by 2-photon microscopy (2-PM).[28,33,34] Moreover, by the use of the morpholino technology, specific proteins can easily be knocked down.

Zebrafish express a *bdnf* orthologue, whose amino acid sequence is 91% identical to human *BDNF*.[35] Although the *BDNF* sequence is rather conserved among these species, little is known about the function of *bdnf* in the zebrafish pronephros. A recent study has shown that there is a beneficial, microRNA-mediated effect on actin polymerization in adriamycin-induced podocyte damage emphasizing the important role for *BDNF* in kidney homoeostasis.[21]

Our study shows that the mRNA expressions of *BDNF*, a newly identified podocyte gene, and of *KIM-1*, an injury-induced protein, are highly correlated in urine cells of CKD patients and secondly that the expression is associated with DN. Moreover, we show the importance of *BDNF* for glomerular function in zebrafish larvae and in isolated murine glomeruli.

2 | METHODS

2.1 | Study participants

Participants were recruited in the GANI_MED nephrology cohort.[3] All participants signed informed written consent forms. The study is consistent with the principles of the declaration of Helsinki, as reflected by an a priori approval of the Ethics Committee of the University of Greifswald.

2.2 | Clinical sample collection

A total of 120 urine samples were collected from participants who had known CKD with or without hypertension and/or diabetes. We used 50-100 mL morning urine. Only in 5% of cases, the urine volume was <50 mL. Unfortunately, the HbA1c value of 1 patient was not available. The time period from urine voiding until processing never exceeded 4 hours, as we found out that in this time viable cells could still be cultivated.

2.3 | Urine processing

Urine was centrifuged in a 50 mL centrifuge tube at room temperature (RT) for 3 minutes at 2100 *g*. The urine pellet was resuspended in 1 mL phosphate-buffered saline (PBS), transferred to a 1.5 mL centrifuge tube and then centrifuged at 12 000 *g* for 1 minute at RT. The supernatant was discarded. The washed urine pellet was resuspended in 900 μL Phenol/Guanidine-based Qiazol lysis

reagent (Qiagen, Hilden, Germany) and then short-term stored at −20°C until use.

2.4 | Kidney specimens

Kidney tissue for immunofluorescence was obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation. Biopsies from 2 subjects with diagnosed DN were investigated. The histopathological diagnosis included the following: diabetic glomerulosclerosis (patient 1) and FSGS with tubular changes (patient 2). Control kidney tissue was taken from normal kidney parts of a renal tumour surgery patient. The clinical-functional diagnosis included the following: slight restriction in GFR and arterial hypertrophy. An informed consent was obtained from the donor.

2.5 | Podocyte de-/differentiation assay

All animal experiments were performed in accordance with national animal protection guidelines that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local governmental authorities. The podocyte dedifferentiation assay was performed as described by Kindt et al.[36] Glomeruli were treated with ANA-12 (1-100 μmol/L, Sigma-Aldrich). After 6 days, the cyan fluorescent protein (CFP) intensity was quantified. Therefore, *z*-stacks of 50 to 80 glomeruli were recorded with the aforementioned confocal laser scanning microscope. Mean fluorescence intensity per glomerulus was calculated after background correction. Half maximal inhibitory concentrations (IC_{50}) were calculated by fitting the data to a sigmoidal dose-response regression curve using Prism 5.01 (GraphPad Software, San Diego, CA, USA). RNA sequencing was performed as previously described.[37]

2.6 | Zebrafish strains

The following zebrafish strains were used: ET (Tg(*wt1a*:eGFP); mitfa$^{w2/w2}$; roy$^{a9/a9}$), CADE (Tg(*fabp10a*:DBP-eGFP); mitfa$^{w2/w2}$; roy$^{a9/a9}$).[38] All zebrafish strains were raised, mated and maintained in E3 medium at 28.5°C, as previously described.[30,39]

2.7 | Morpholinos injection

Translation-blocking bdnf morpholinos (bdnfMO) were manufactured by Gene Tools LCC (Philomath, OR, USA). As negative control, we used standard control morpholinos (CtrlMO) offered by Gene Tools. The morpholinos were diluted to 1 mmol/L. A volume of approximately 3 nL per zebrafish was injected into 2 to 4-cell stage fertilized eggs using a microinjector (Transjector 5246, Eppendorf, Hamburg, Germany).

2.8 | Immunohistology

Immunohistology for cryosections was performed as described previously.[39,40]

2.9 | Zebrafish in vivo microscopy

In vivo imaging was performed as previously described.[28,34,40]

2.10 | Statistical analysis

Urine expression data were log-transformed for all correlation analyses. Associations between potential biomarkers were assessed using Pearson correlation followed by the Benjamini-Hochberg procedure. Comparisons between groups were performed as indicated. All comparisons between 2 groups concerning zebrafish experiments were done with the Mann-Whitney *U* test. All statistical analyses were performed using LABMAT version 2013 and SPSS V. 21.

3 | RESULTS

3.1 | Baseline characteristics of patients

To identify individual prognostic biomarkers for CKD, we analysed a panel of potential urinary biomarkers in 120 GANI_MED renal study participants. The baseline characteristics are shown in Table 1. The investigated patient group consisted of 45 women (37.5%) and 75 men (62.5%) with a mean age of 64.3 years. All patients were afflicted with CKD. The group included 33 diabetes patients and 75 dialysis patients. Mean estimated glomerular filtration rate (eGFR) was 23.2 mL/min/1.73 m^2, and 110 patients had an eGFR less than 60 mL/min/1.73 m^2. The mean urinary albumin-to-creatinine ratio (uACR) was 1245 mg/g. The diabetic group consisted of 19 male and 14 female patients, whereas the non-diabetics included 55 men and 31 women. For one study participant, there was no information about the diabetic status. The mean age was 65.9 years in diabetics and 63.6 years in non-diabetics. The diabetic patients had a mean eGFR of 23.9 mL/min/1.73 m^2 compared to 22.7 mL/min/1.73 m^2 in non-diabetic patients. This difference was not statistically significant. There was also no statistically significant difference concerning

TABLE 1 Patient characteristics

Variables	Total	Diabetics	Non-diabetics
n	120	33	86
Sex (m/f)	75/45	19/14	55/31
Mean age (y)	64.3 ± 15.7	65.9 ± 13.3	63.6 ± 16.5
Mean eGFR mL/min/1.73 m^2	23.2 ± 20.9	23.9 ± 16.1	22.7 ± 22.4
eGFR<60 mL/min/1.73 m^2	110/120	32/33	77/86
Mean UACR (mg/g)	1245 ± 2199	1531 ± 2654	1149 ± 1994
Dialysis	75/120	16/33	59/86

m = male; f = female; eGFR = estimated glomerular filtration rate, UACR = urinary creatinine-albumin ratio.

the uACR, which was 1531 mg/g in diabetics and 1149 mg/g in non-diabetics. The group of diabetics included 32 patients with an eGFR lower than 60 mL/min/1.73 m^2 and 16 dialysis patients. The group of non-diabetics included 77 patients with an eGFR lower than 60 mL/ min/ 1.73 m^2 and 59 dialysis patients. Again, these differences were not statistically significant.

3.2 | Correlations between urine mRNA levels

To investigate whether the mRNA levels were interrelated, we performed a correlation analysis (Figure 1A) followed by the Benjamini-Hochberg procedure to determine statistical significance (Figure 1B). We found a strong positive correlation between the expressions of *BDNF* and *KIM-1* ($R = 0.87$, $P = 2.3 \times 10^{-38}$, Figure 1C). Interestingly, we also observed significant correlations between the expressions of *BDNF* and the podocyte marker *NPHS1* ($R = 0.27$, $P = .0025$, Figure 1D) as well as between the expressions of *KIM-1* and *NPHS1* ($R = 0.37$, $P = 3.7 \times 10^{-5}$). These findings suggest that podocytes might contribute to the population of *BDNF*- and *KIM-1*-expressing cells that are detectable in the urine. Additionally, there were statistically significant correlations of the podocyte marker *NPHS2* with *NPHS1* ($R = 0.23$, $P = .011$) as well as with *CTSL-1* ($R = 0.27$, $P = .0026$). Other significant correlations were not detected.

3.3 | Correlation analysis between urine mRNA levels and clinical parameters revealed a negative correlation between *BDNF* and uACR

To identify possible correlations between the investigated expressions of urinary cell pellet mRNA species and clinical parameters, we applied the Pearson correlation analysis corrected by the Benjamini-Hochberg procedure for the expression of the 3 podocyte markers *NPHS1*, *NPHS2*, *PODXL*, the 3 biomarker candidates *BDNF*, *KIM-1* and *CTSL-1* and the clinical parameters eGFR, HbA1c and uACR. These calculations revealed a significant negative correlation between *BDNF* and uACR ($P = .0046$; Figure 1E). *BDNF* and eGFR were not correlated with each other. Additionally, there was no significant difference in BDNF expression between patients of different CKD stages (Figure 1F). Furthermore, we did not find any significant correlation between any of the other parameters in the observed patient group (data not shown).

3.4 | Sex-specific expression of *BDNF* and *KIM-1* in diabetic and non-diabetic patients

As our cohort shows a typical frequency distribution of nephropathies with the largest proportion suffering from diabetes, which is

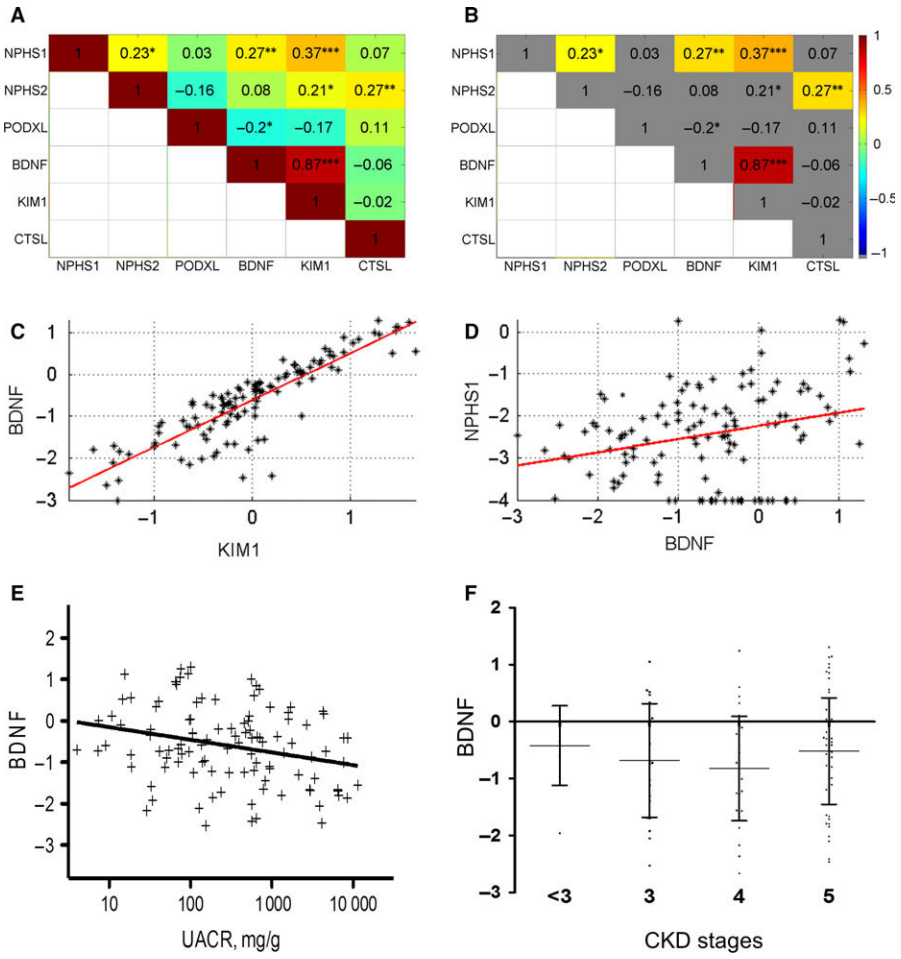


FIGURE 1 Pearson correlation of urine sediment mRNA expression. mRNA expression was determined by qRT-PCR and normalized to *GAPDH* and the interrun calibrator. Pearson correlations are shown in the coloured boxes (A). Pearson correlations after Benjamini-Hochberg procedure are shown in B, where grey boxes indicate non-significant correlations and coloured boxes indicate significant values. *BDNF* mRNA was significantly correlated with mRNA of *KIM-1* and *NPHS1* as also shown in C and D. *KIM-1* mRNA is significantly correlated with *NPHS1* as well. *BDNF* mRNA is correlated with uACR (E). There is no significant difference in BDNF mRNA expression between different CKD stages [stage <3: n = 9, stage 3: n = 21, stage 4: n = 31, stage 5: n = 58]; (F). [*$P < .05$; **$P < .01$; ***$P < .001$]

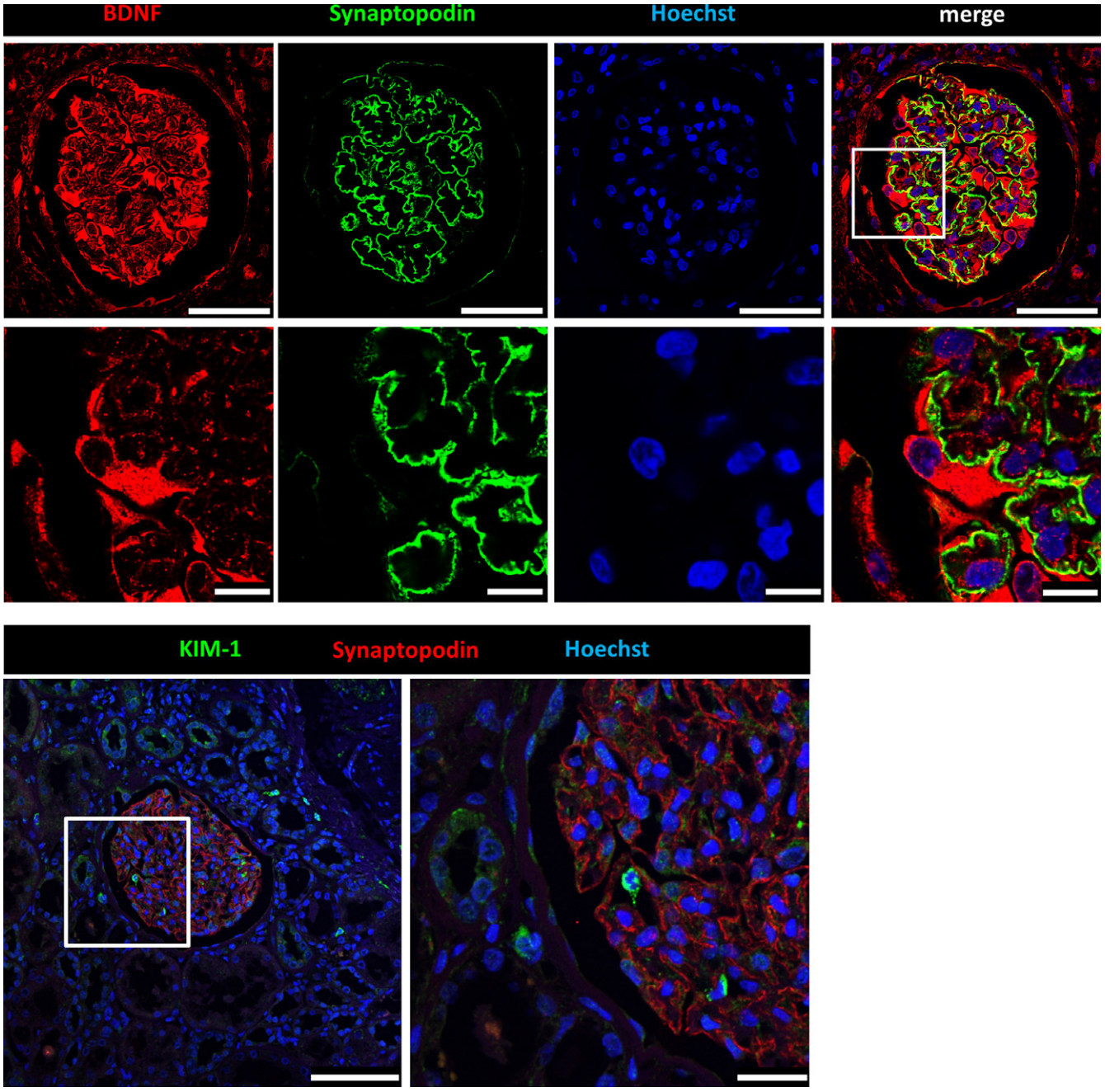


FIGURE 2 Immunofluorescence staining of healthy human kidney sections for *BDNF* and *KIM-1*. Normal formalin-fixed paraffin embedded kidney sections were stained for *BDNF* (red), the podocyte marker protein synaptopodin (green) and nuclei (blue) by Hoechst. *BDNF* was mainly expressed in the cell body and major processes of podocytes. There was moderate expression of synaptopodin in parietal epithelial cells. [Scale bars upper panel = 50 μm. Scale bars lower panel = 10 μm] Kidney sections were also stained for *KIM-1* (green), synaptopodin (red) and nuclei (blue) by Hoechst. Very few *KIM-1* positive cells were visible within the glomerulus. Only a few non-glomerular cells were stained. [Scale bar left picture = 100 μm. Scale bar right picture = 25 μm]

one of the major causes of CKD, we investigated the expression of *BDNF* and *KIM-1* in diabetic and non-diabetic patients (Figure S1). We observed that the expressions of *BDNF* and *KIM-1* were significantly higher in diabetic than in non-diabetic patients ($P = .04$ and $P = .02$, respectively). The mean *BDNF* expression in diabetics reached a value of 2.07, whereas non-diabetics showed a lower expression value of 1.30. Similar results were found for *KIM-1*. The expression of *KIM-1* was almost twice as high in diabetic patients than in non-diabetic patients (5.79 vs 2.97).

Interestingly, we found that the expressions of *BDNF* and *KIM-1* were almost twice as high in females than in males (Figure S1), indicating a sex-dependent expression of *BDNF* and *KIM-1*. Thus, in females suffering from DN, the expressions of *BDNF* and *KIM-1* were 3.97 and 8.07, respectively, compared to 2.15 and 4.75,

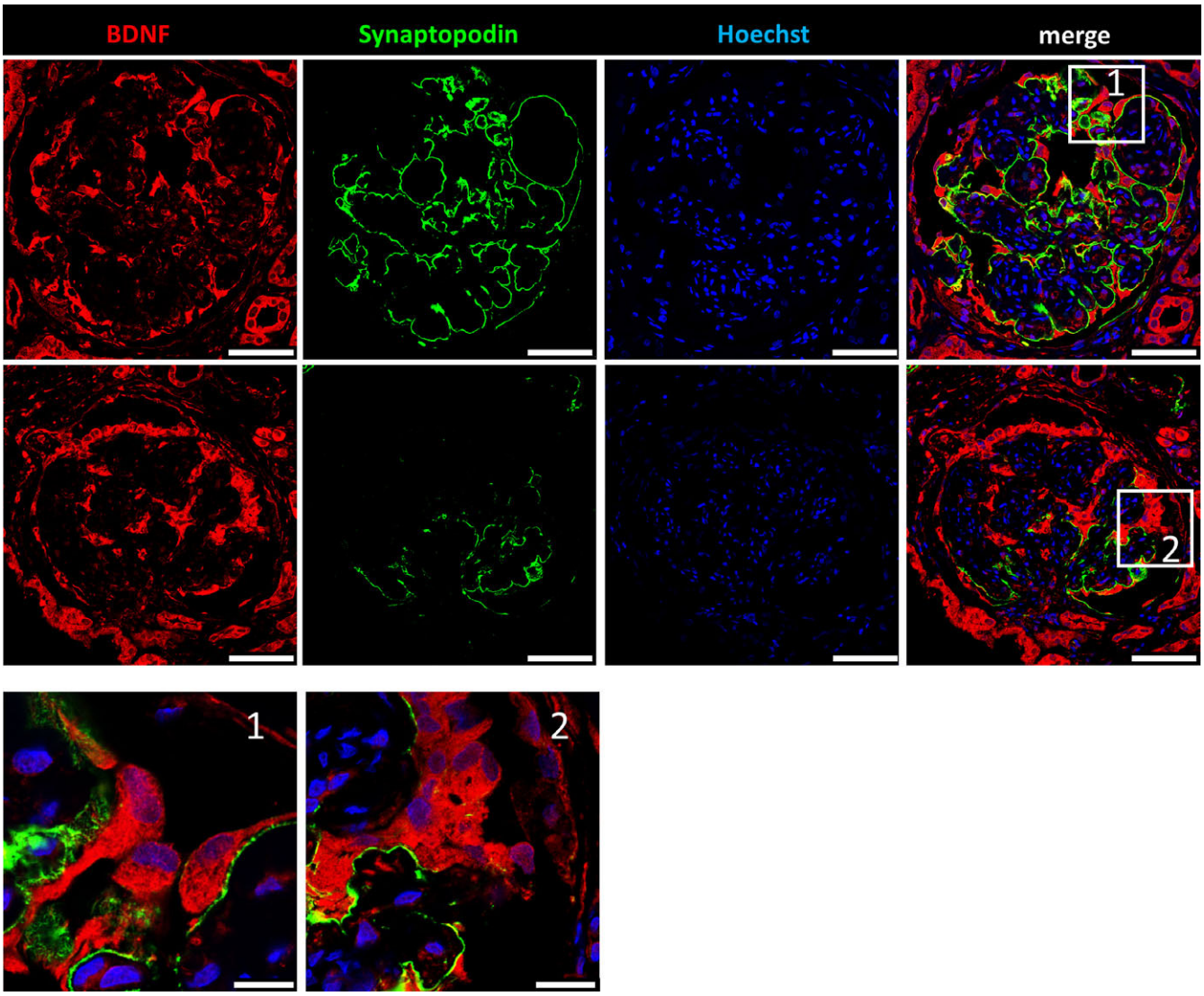


FIGURE 3 Immunofluorescence staining of human diabetic nephropathy (DN) kidney sections for *BDNF*. Formalin-fixed paraffin embedded DN kidney sections were stained for *BDNF* (red), synaptopodin (green) and nuclei (blue) by Hoechst. Fewer *BDNF*-expressing cells were found in glomeruli of DN kidney sections than in healthy kidneys from Figure 2. Cells still expressing the podocyte marker protein synaptopodin show an enhanced *BDNF* intensity (magnification 1 and 2). [Scale bars upper panels = 50 μm. Scale bars 1/2 = 10 μm]

respectively, in non-diabetic females. The associations of *BNDF* and *KIM-1* with DN were not detected in males (Figure S1). There was a statistically significant difference in *BDNF* mRNA expression between female and male diabetics but not between female and male non-diabetics. In contrast, *KIM-1* mRNA expression did not significantly differ between female and male diabetics or non-diabetics.

3.5 | *BDNF* and *KIM-1* expressions are up-regulated in podocytes of patients with DN

As described previously, *BDNF* is expressed in a range of different tissues and cell types. On the basis of our finding that the urine mRNA levels of *BDNF* and the podocyte-specific marker *NPHS1* were statistically associated with each other, we investigated the expression of *BDNF* in human glomeruli by immunofluorescence staining. As shown in Figure 2, *BDNF* was expressed in glomeruli of normal human kidney sections, especially in podocytes and to some extent also in parietal epithelial cells. As the localization of synaptopodin was mainly restricted to podocyte foot processes and no co-localization was found between synaptopodin and *BDNF* in podocytes, we conclude that *BDNF* is mainly localized in the major processes and in the cell body of podocytes. This was confirmed by co-staining with the slit diaphragm protein nephrin (Figure S3). In renal biopsies from patients suffering from DN, we found an up-regulation of the fluorescence intensity of the remaining podocytes as identified by co-staining with synaptopodin (Figure 3). The subpodocyte space was lost in these biopsies. Confirming these findings, microarray analysis of glomeruli from patients suffering from DN showed a significant 2.0-fold enhanced expression of *BDNF* and a 1.3-fold enhanced expression of its receptor TrkB compared to

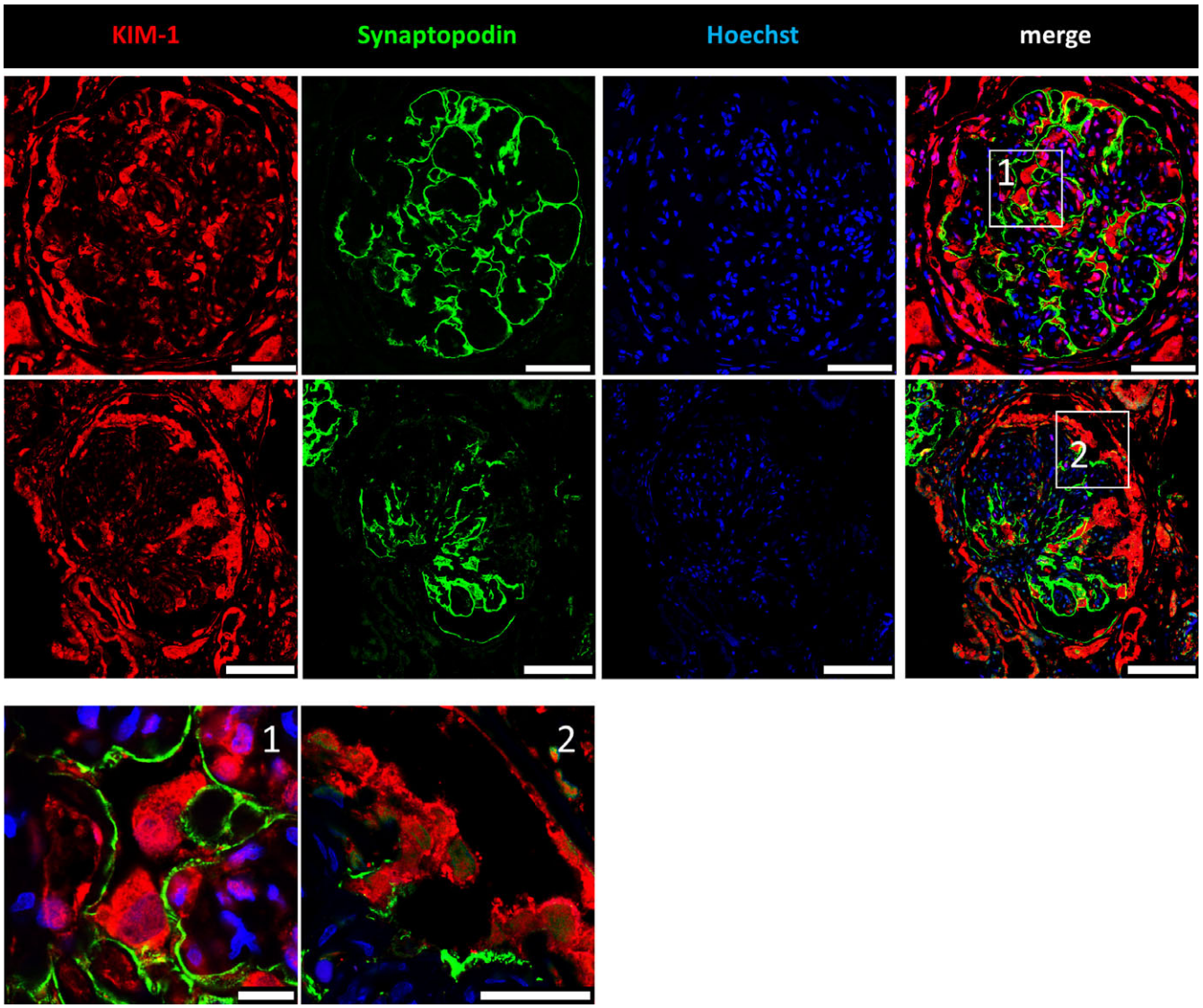


FIGURE 4 Immunofluorescence staining of human diabetic nephropathy (DN) kidney sections for *KIM-1*. Formalin-fixed paraffin embedded DN kidney sections were stained for *KIM-1* (red), synaptopodin (green) and nuclei (blue) by Hoechst. A significant increase in the *KIM-1* expression was found in podocytes as well as in other epithelial cells. Higher magnifications are shown in 1 and 2. [Scale bars upper panel = 50 μm. Scale bars lower panel = 75 μm. Scale bars 1/2 = 10 μm]

glomeruli from control individuals. Moreover, we further confirmed the increase of BDNF expression (1.2-fold) in protein-overload experiments in cultured murine podocytes (Figure S4). In contrast to *BDNF*, *KIM-1* was never observed in podocytes and rarely found in other cells of normal glomeruli (Figure 2). However, in patients with DN, a strong up-regulation of *KIM-1* in podocytes was detected (Figure 4). Additionally, sequencing data of glomeruli from our dedifferentiation assay[36] revealed a 139-fold increased Kim-1 expression in 3-day cultivated glomeruli compared to freshly isolated glomeruli.

3.6 | *BDNF* stabilizes podocyte differentiation in cultured glomeruli

We recently established a novel assay to study podocyte de-/differentiation in isolated glomeruli of mice expressing CFP under control of the *Nphs1* promoter.[36] As podocytes of isolated glomeruli spontaneously dedifferentiate in cell culture accompanied by a decrease in *Nphs1* expression, we used the *Nphs1*-dependent CFP expression to quantify podocyte dedifferentiation. Isolated glomeruli were incubated with various concentrations (1-100 μmol/L) of the selective TrkB inhibitor ANA-12. After 6 days, CFP intensity was decreased concentration-dependently by ANA-12 with an IC_{50} value of 19.6 μmol/L (Figure S2), indicating an involvement of *BDNF* in podocyte differentiation.

3.7 | The knockdown of *BDNF* induces proteinuria in zebrafish larvae

To study the function of *bdnf in vivo*, we performed a *bdnf* knockdown (KD) with specific morpholinos. To this end, we generated a

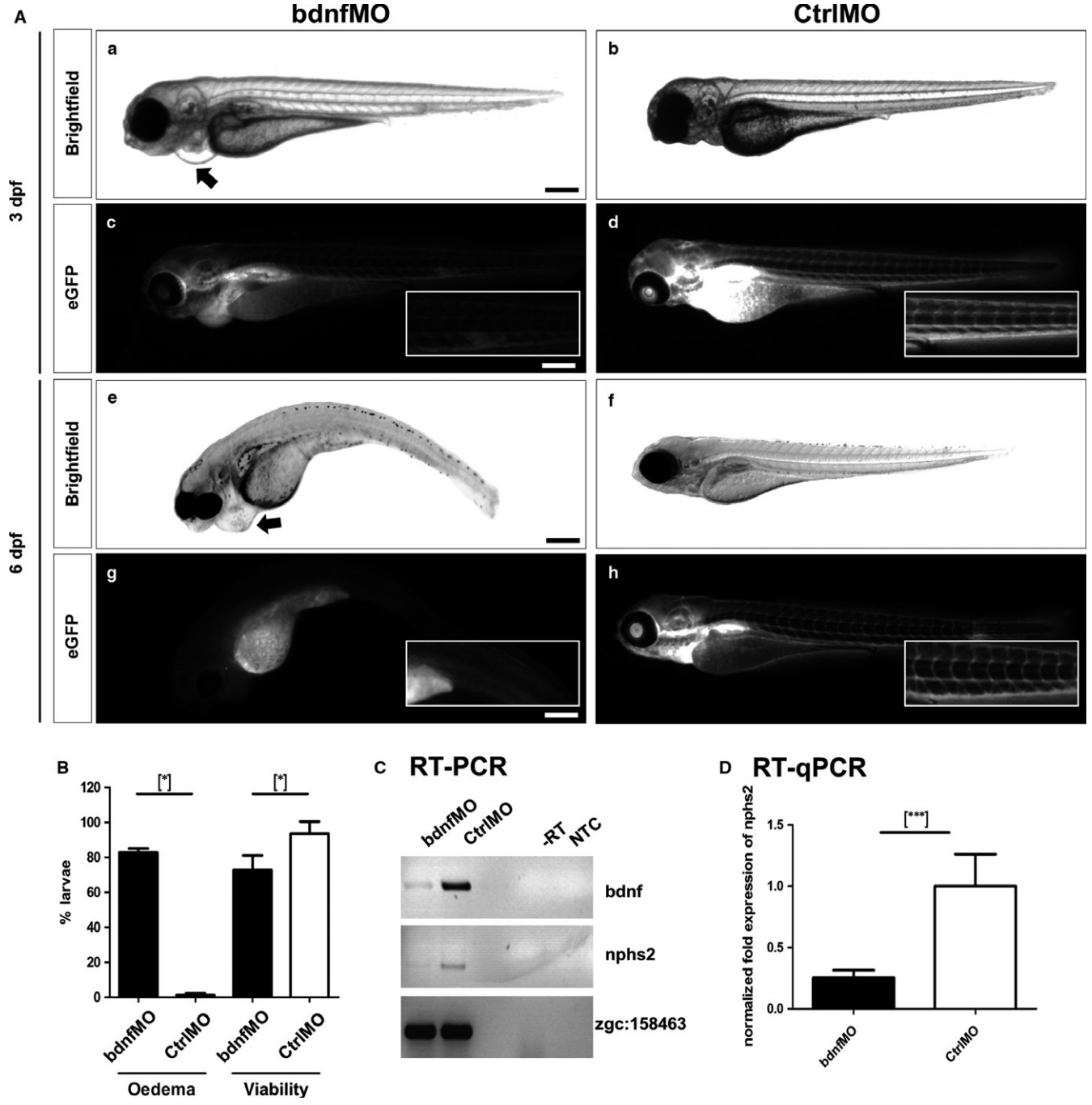


FIGURE 5 *Bdnf* knockdown in zebrafish larvae leads to pericardial oedema formation, impaired glomerular filtration and down-regulation of podocyte-specific genes. *Bdnf* morpholinos (bdnfMO) and control morpholinos (CtrlMO) were injected in *ET* zebrafish eggs. Brightfield pictures were taken 3 dpf (A; a and b) and 6 dpf (A; e and f). bdnfMO-injected larvae exhibit a higher rate of pericardial oedema development and a lower viability than normal controls (B). Morpholino injection in *CADE* larvae reveals an impairment of glomerular filtration barrier function in bdnfMO-injected larvae 3 dpf (A; c) and 6 dpf (A; g) compared to CtrlMO-injected larvae (A; d and f). The knockdown of *bdnf* was verified by RT-PCR (C). The down-regulation of the podocyte marker *nphs2* was verified on the mRNA level by RT-PCR (C) and qRT-PCR (D). Expression levels were normalized to *zgc:158463* in RT-PCR and to *zgc:158463* and *eef1a1/1* in qRT-PCR by the ΔΔCt method. [Scale bars = 500 μm] [$^{*}P < .05$, $^{***}P < .001$]

translation-blocking morpholino (bdnfMO). Three days after injection of the bdnfMO, 82.7 ± 2.0% of the zebrafish larvae had developed severe pericardial oedema (arrow in Figure 5A), a hallmark of impaired kidney function. In contrast, only 1.3 ± 0.8% of CtrlMO-injected larvae developed pericardial oedema (Figure 5A,B). In addition, bdnfMO-injected larvae were less viable (72.9 ± 7.1%) compared with larvae injected with CtrlMO (93.6 ± 6.0%) (Figure 5B). To investigate whether the pronephros of the zebrafish larvae was affected by the KD of *bdnf*, we used the transgenic zebrafish strain CADE expressing an eGFP-tagged vitamin D-binding protein in

the blood which cannot pass the intact filtration barrier. In contrast to CtrlMO-treated larvae, we observed a nearly complete loss of eGFP fluorescence in the blood of *bdnf* KD larvae at 3 and 6 dpf (Figure 5A), indicating leakage of the filtration barrier due to *bdnf* KD.

3.8 | *BDNF* is important for the proper morphology of zebrafish glomeruli as well as for the expression of nephrin and podocin

The KD of *bdnf* was verified by RT-PCR showing reduced intensities of the specific amplicon in bdnfMO-injected larvae compared with CtrlMO-injected larvae (Figure 5C). Furthermore, *bdnf* KD larvae showed reduced expression of *nphs2* (podocin) in RT-PCR analysis (Figure 5C), which was confirmed by qRT-PCR (Figure 5D). To study the glomerular morphology, we stained cryosections of zebrafish larvae, utilizing the ET strain that expresses eGFP specifically in podocytes.[28,30] After staining the F-actin cytoskeleton with Alexa-546 phalloidin, we observed significant changes in the morphology of the glomeruli in response to the KD of *bdnf*. In addition to an enlargement of the glomerular tuft and Bowman's space, we observed a reduced number of podocytes in *bdnf* KD larvae (3 dpf) in contrast to CtrlMO-treated zebrafish larvae (Figure 6A). Moreover, immunohistological staining for nephrin revealed a significant reduction of the slit membrane protein due to the KD of *bdnf* in the zebrafish larvae (Figure 6A).

3.9 | *In vivo* microscopy of *bdnf* knockdown larvae

Using *in vivo* 2-photon microscopy (2-PM) of *bdnf*MO-injected *ET* larvae, we found 2 different phenotypes. Both phenotypes showed fewer podocytes and a dilated bowman's space (Figure 6Bc, asterisks) compared with controls (Figure 6Be,f). No major processes could be distinguished in z-stacks of glomeruli of KD larvae (Movie S1), while control larvae showed a normal glomerular morphology and well-shaped major processes (Movies S3 and S4). The second phenotype showed hindered fusion of the 2 glomeruli in the midline (Figure 6Ba, white line, Movie S2), indicating disturbance of the migration of the renal primordia (Figure 6A).

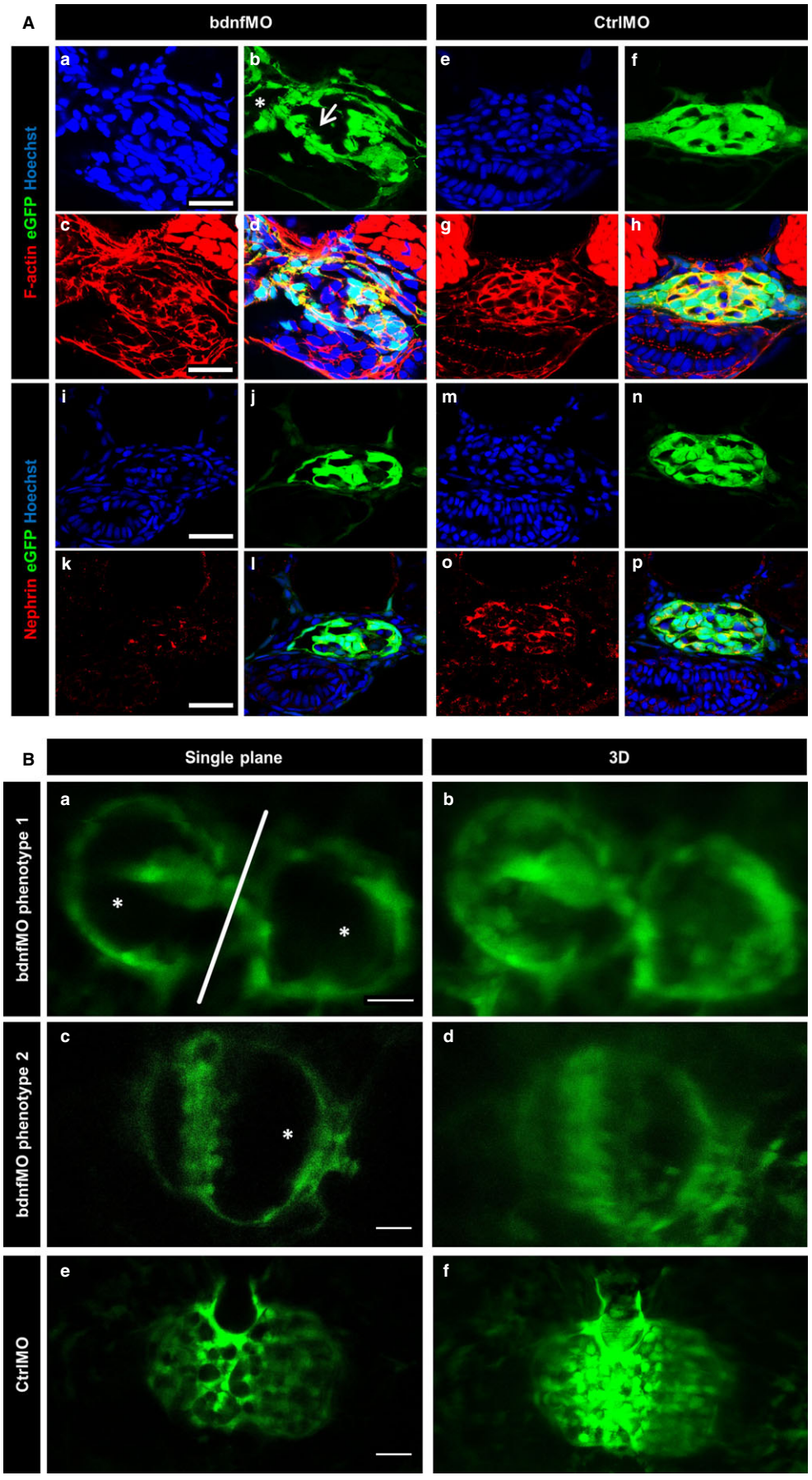
4 | DISCUSSION

Biomarkers from non-invasive sources like urine are of growing interest in CKD research, as they seem to be a promising diagnostic tool for early detection of the disease. Conventional parameters like proteinuria, cystatin C and creatinine are established markers of kidney function, which are positively correlated with kidney dysfunction.[41-45] Because they are only measurable at a relatively progressed disease state, the establishment of markers for an earlier disease state is needed. In this study, we investigated the mRNA expression levels of potential predictive biomarkers for CKD in the urine sediment. Previous studies identified a variety of predictive biomarkers from urine sediment mRNA for non-malignant kidney diseases.[46-49] In the present study, we found a strong and highly significant positive correlation between *BDNF* mRNA levels and *KIM-1* mRNA levels. *KIM-1*, which is endogenously expressed at a very low levels, is a well-established urinary biomarker for acute kidney injury and is also known to be positively correlated with tubular and tubulointerstitial injury as well as with glomerular damage.[26,50-52] This is in a very good agreement with our findings, as we could also detect glomerular expression of *KIM-1* by immunofluorescence staining and in murine glomeruli of our podocyte dedifferentiation assay. Therefore, *BDNF* mRNA levels might serve as a new potential biomarker for glomerular kidney injury. Interestingly, we observed a statistically significant up-regulation of both mRNA species in diabetic patients compared with non-diabetic patients and for *BDNF* in a sex-specific manner. Sex specificity in kidney injury has been described earlier,[53,54] but seems to be relatively underestimated. *BDNF* mRNA levels were also negatively correlated with uACR, indicating a possible influence on glomerular filtration barrier function. The non-correlation of urinary *BDNF* levels and eGFR may suggest that podocytes with high levels of BDNF possibly detach at a lower rate. Podocyte loss to a certain extent is a main event in glomerulopathies.[55,56] Accordingly, we also found a positive correlation of *KIM-1* and *BDNF* with the podocyte marker *NPHS1*, suggesting that their expression might be, at least partially, podocyte-derived. Currently, there is no known cellular mechanism directly linking *KIM-1* and *BDNF*. Interestingly, recent studies have shown that both proteins are involved in autophagy, an important process for cell survival.[57-59]

Due to the findings described above, we further investigated the role of *BDNF in vitro* and *in vivo*. To date, only one study[21] has addressed the role of *BDNF* in kidney function with a special focus on podocytes. Li and coworkers revealed a critical role for *BDNF* in podocyte cytoskeletal maintenance. They showed that *BDNF* TrkB-dependently up-regulates actin polymerization in podocytes through the regulation of the microRNA-132/134 *in vitro*. Exogenous application of *BDNF* led to more developed stress fibres and ramifications of podocytes and could ameliorate podocyte damage by puromycin aminonucleoside, adriamycin and protamine sulphate. They showed that *BDNF*-mediated TrkB activation has a stabilizing effect on podocyte homoeostasis as well as a rescuing effect in different podocyte injury models. These results suggest that the detection of *BDNF* in the urine of patients might be an indication of stressed podocytes that started to activate their "survival factor" *BDNF*.

As podocytes and neurons share some common features, it was predictable that *BDNF* may also be expressed in both cell types, as is the case for other proteins.[11,12] In keeping with this notion, we found that *BDNF* was expressed in the cell body and in major processes of podocytes in human kidney biopsies. Surprisingly, we did not see any co-localization with synaptopodin, a podocyte-specific protein which is exclusively expressed in podocyte foot processes, indicating that the localization of *BDNF* is restricted to the cell body and the major processes.

Looking at sectioned kidney biopsies of patients, which were diagnosed with DN, we observed less *BDNF*-expressing podocytes, but podocytes that still expressed *BDNF* showed an increased


A
bdnfMO
CtrlMO
F-actin eGFP Hoechst
Nephrin eGFP Hoechst
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l
m
n
o
p
B
Single plane
3D
bdnfMO phenotype 1
bdnfMO phenotype 2
CtrlMO

FIGURE 6 *Bdnf* knockdown in zebrafish larvae leads to morphological changes in the glomerulus and the down-regulation of podocyte marker proteins. BdnfMO and CtrlMO were injected in ET eggs expressing eGFP specifically in podocytes. Cryosections were counterstained for F-actin by phalloidine (red) and nuclei by Hoechst (blue) (A; a-h). bdnfMO-treated larvae show an enlarged glomerular tuft (A; b arrow) and Bowman's space (A; b asterisk) compared with the normal morphology of the CtrlMO-treated larvae (A; e-h). Counterstaining of the slit diaphragm protein nephrin (red) and nuclei by Hoechst (blue, A; i-p) reveals a down-regulation of nephrin due to bdnfMO treatment (A; k) compared to CtrlMO-treated larvae (A; o). [Scale bars = 20 μm] *In vivo* microscopy reveals 2 different phenotypes of bdnfMO-treated larvae. Phenotype 1 is characterized by unfused glomeruli (B; a white line and b), a reduced number of podocytes and a dilatation of Bowman's space and the glomerular tuft (B; a asterisks). The second phenotype is characterized by the absence of podocyte major processes, a reduced podocyte number and a dilatation of Bowman's space and the glomerular tuft (B; c asterisk and d). CtrlMO-injected larvae show a normal glomerular morphology with well-shaped major processes (B; e and f). [Scale bars = 20 μm]

expression, which could serve as a potential biomarker in the diagnostic evaluation of renal biopsies. These results were confirmed by microarray analyses of renal tissue from DN patients, where we have also detected an up-regulation of the BDNF receptor TrkB, underlining the importance of *BDNF* for podocyte homoeostasis.

As podocyte dedifferentiation is a critical step in the progression of DN, we applied our well-established dedifferentiation assay to study the influence of *BDNF* on podocyte differentiation.[36] We could show that the inhibition of the TrkB receptor, which mediates for *BDNF* signalling, led to decreased nephrin promotor activity and therefore to increased podocyte dedifferentiation in murine glomeruli.

Only little is known about the effect of a *BDNF* KO on kidney homoeostasis *in vivo*. As *BDNF* KO mice die directly after birth and no kidney-specific phenotypical impacts have been published,[18] we selected the zebrafish larva as model organism. Zebrafish larvae are relatively easy to breed and show a glomerular morphology similar to that of mammals with one glomerulus connected to two tubules in their first functional state, the pronephros.[31,32] Another advantage is their applicability for *in vivo* microscopic techniques like 2-PM which can track changes in morphology and function.[28,34] *bdnf* KD larvae developed pericardial oedema as an indicator for an impairment of the glomerular filtration barrier. This finding could be confirmed by a decrease in the intravascular eGFP intensity in bdnfMO-treated *CADE* larvae, also indicating a leaky filtration barrier.[28,30] We also found a reduced expression of the podocyte markers podocin and nephrin, and a disrupted F-actin structure in bdnfMO-treated larvae, which are suggestive of podocyte loss.

A positive influence of *BDNF* on the expression of podocyte markers like nephrin has been shown before.[21] We were able to confirm this effect using 2-PM microscopy, where two phenotypes were visible: The first phenotype supports the hypothesis of podocyte loss, with less podocytes visible on the glomerular tuft. The second phenotype showed an unfused glomerulus, which might be a hint for developmental delay. It has been demonstrated previously that *bdnf* has beneficial effects on zebrafish kidney function and podocyte homoeostasis in a model of kidney injury. In this model, exogenous *bdnf* treatment rescued the expression of nephrin and almost completely restored podocyte morphology.[21]

Here, we show that *BDNF* mRNA may potentially serve as a new prognostic urinary biomarker for CKD. We also show that the expression of *BDNF* in human podocytes is locally restricted to the cell body and major processes. Furthermore, we give first insights into the involvement of *BDNF* in podocyte dedifferentiation and into its deregulation in DN. The KD of *bdnf* leads to impaired glomerular filtration as well as to podocyte loss and/or hindered migration of glomerular progenitors in an *in vivo* zebrafish model.

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CONFLICT OF INTEREST

The authors disclose no conflict of interests.

AUTHOR CONTRIBUTIONS

N.E. and K.E. designed the study. S.S. and J.K. contributed to urine processing. J.K. performed urine mRNA PCR experiments. T.L. and P.K. contributed to statistical data analysis; N.E., U.Z., J.K., M.T.L., C.D.C. handled and analysed the biopsies. F.K. performed the (de-)differentiation assay experiments. T.L., A.M.K. and F.S. performed zebrafish experiments. N.E., T.L. and K.E. analysed experimental data. T.L. did the figure design and literature search. N.E., T.L. and K.E. wrote the main manuscript text. All authors had approval of the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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5.3 MiR-21 is upregulated in urinary exosomes of CKD patients and after glomerular injury

Tim Lange, Nadine Artelt, Frances Kindt, Sylvia Stracke, Rainer Rettig, Uwe Lendeckel, Christos E. Chadjichristos, Panagiotis Kavvadas, Christos Chatziantoniou, Karlhans Endlich, Nicole Endlich
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Own work

- Project planning and administration; manuscript writing; figures; literature research; method establishment; urine processing and organization; exosome isolation; microRNA isolation; RT-qPCR analysis urine samples; RNA isolation and RT-qPCR analysis of murine glomeruli samples; RNA isolation and RT-qPCR analysis of murine kidney sections, data-acquisition; -analysis and -curation; statistics

Nadine Artelt

- NTS-treatment of mice; FFPE of mice kidneys

Frances Kindt

- Glom-Assay

Sylvia Stracke

- Urine processing; urine organization; patient recruitment

Rainer Rettig

- Project administration; manuscript corrections

Uwe Lendeckel

- Project administration; manuscript corrections

Christos E. Chadjichristos

- NTS-treatment of mice

Panagiotis Kavvadas

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Christos Chatziantoniou

- NTS-treatment of mice

Karlhans Endlich

- Project administration; data analysis; manuscript correction

Nicole Endlich

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SHORT COMMUNICATION

WILEY

MiR-21 is up-regulated in urinary exosomes of chronic kidney disease patients and after glomerular injury

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KEYWORDS: biomarker, CKD, miR-21, miRNA, urinary exosomes

1 | INTRODUCTION

With 500 million people affected worldwide, chronic kidney disease (CKD) constitutes a major public health problem.[1] Mostly arising from arterial hypertension and diabetes mellitus, it is a terminal disease without any causal therapy, leading to dialysis and/or kidney transplantation. Glomerulopathies are the main cause of end stage renal disease (ESRD), which are mainly caused by podocyte effacement or loss.[2]

A relevance of microRNAs (miRs) on podocyte function and the pathogenesis of glomerulopathies[3] could have been clarified. Both, their molecular influence on translation and their potential role as biomarkers for kidney diseases, including CKD, became of major research interest. Especially miRs derived from urinary exosomes appear to have a promising potential as biomarkers, since they can be accessed non-invasively and they are protected against degradation,[4] making sample preparation more unsusceptible against environmental influences. Unfortunately, only little is known about exosomal miR expression in urine of CKD patients.

Therefore, the present study aims at the investigation of the expression levels of the well-investigated miRs miR-21, miR-30a-5p and miR-92a in urinary exosomes of CKD patients, representing a new promising, non-invasive sample type and their functional roles in different injury models.

2 | MATERIAL AND METHODS

2.1 | Patient urine samples

Patient urine samples were obtained within the scope of the Greifswald Approach to Individualized Medicine (GANI_MED).[5] A written informed consent was signed by all participants. The study followed the ethical rules of the declaration of Helsinki. For a precise description see Data S1.

2.2 | Urinary exosomal miRNA isolation, reverse transcription and RT-qPCR

Urine processing, exosome preparation, miR isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed as described previously.[6] Taqman™ miRNA Assays: See Data S1. For a precise description see Data S1.

2.3 | Glomeruli dedifferentiation assay, RNA isolation and RT-qPCR

The isolation, cultivation and RNA isolation of murine glomeruli was performed as described by Kindt et al.[7] Taqman™ miRNA Assays: See Data S1. For a precise description see Data S1.

2.4 | Nephrotoxic serum treatment, RNA isolation and RT-qPCR

Mice handling and nephrotoxic serum (NTS) treatment were performed as previously described.[8] All mouse experiments were performed in accordance with the national animal protection guidelines that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local governmental authorities. For a precise description see Data S1.

2.5 | Statistical analysis

See Data S1.

3 | RESULTS

3.1 | Patient characteristics

The patient set consisted of 41 patients suffering from CKD and five healthy controls. CKD patients had a mean age of 47 (±10) years and healthy controls had a mean age of 40 (±15) years. There were 17 females in the CKD group and three in the healthy control group. In the CKD group 30 individuals exhibited diabetes mellitus, which was defined as HbA1C values of ≥6.5%. The mean urinary albumin to creatinine ratio (UACR) was 1598.7 (±2297.5) mg/g in CKD patients. The mean estimated glomerular filtration rate (eGFR) was 22.5 (±18.7) mL/min/1.73 m^2 in the CKD group. For the healthy control group we did not collect data regarding UACR and eGFR (Table S1).

3.2 | Levels of miR-21 are enhanced in urinary exosomes of CKD patients

We found a significantly ($P < 0.001$) higher average level of miR-21 in CKD patients (3.3 ± 1.9) compared to healthy controls (0.9 ± 0.5) (Figure 1A). Only three CKD patients had a lower miR-21 level than the average level of healthy controls (Figure S1). We also found significantly ($P = 0.001$, $P < 0.001$, respectively) higher average levels of miR-21 in CKD patients with (3.5 ± 1.9) and without diabetic nephropathy (DN) (3.2 ± 2.0) compared to healthy controls (0.9 ± 0.5) (Figure 1B). Receiver operating characteristic (ROC) analysis (ROC) revealed an area under the curve of 0.92 (Figure 1 E). We did not observe significant differences between CKD patients and healthy controls with regard to miR-30a-5p and miR-92a, respectively (Figure 1A,B).

3.3 | MiR-21 is negatively correlated with eGFR

We found a statistically significant positive correlation between miR-30a-5p and miR-92a of 0.660 ($P < 0.001$). The only statistically significant correlation ($P = 0.005$) between clinical parameters HbA1c, eGFR and UACR and miR expression levels we detected, was a negative correlation between miR-21 and eGFR of −0.434 (Figure 1C,D).

3.4 | MiR-21 expression is up-regulated in (de-) differentiated glomeruli

Freshly isolated glomeruli showed a strong cyan fluorescent protein (CFP) signal in all glomeruli driven by a nephrin promoter fragment. In glomeruli cultured for 9 days the CFP signal almost totally disappeared (Figure 2A). Glomeruli cultured for 9 days showed a statistically significant enhanced relative miR-21 expression of 108.8 (±78.0) compared to 1.0 (±0.5) in freshly isolated glomeruli ($P = 0.037$) (Figure 2B). Interestingly, dedifferentiated glomeruli also showed an up-regulation of genes involved in MAP-ERK signalling (Data S1).

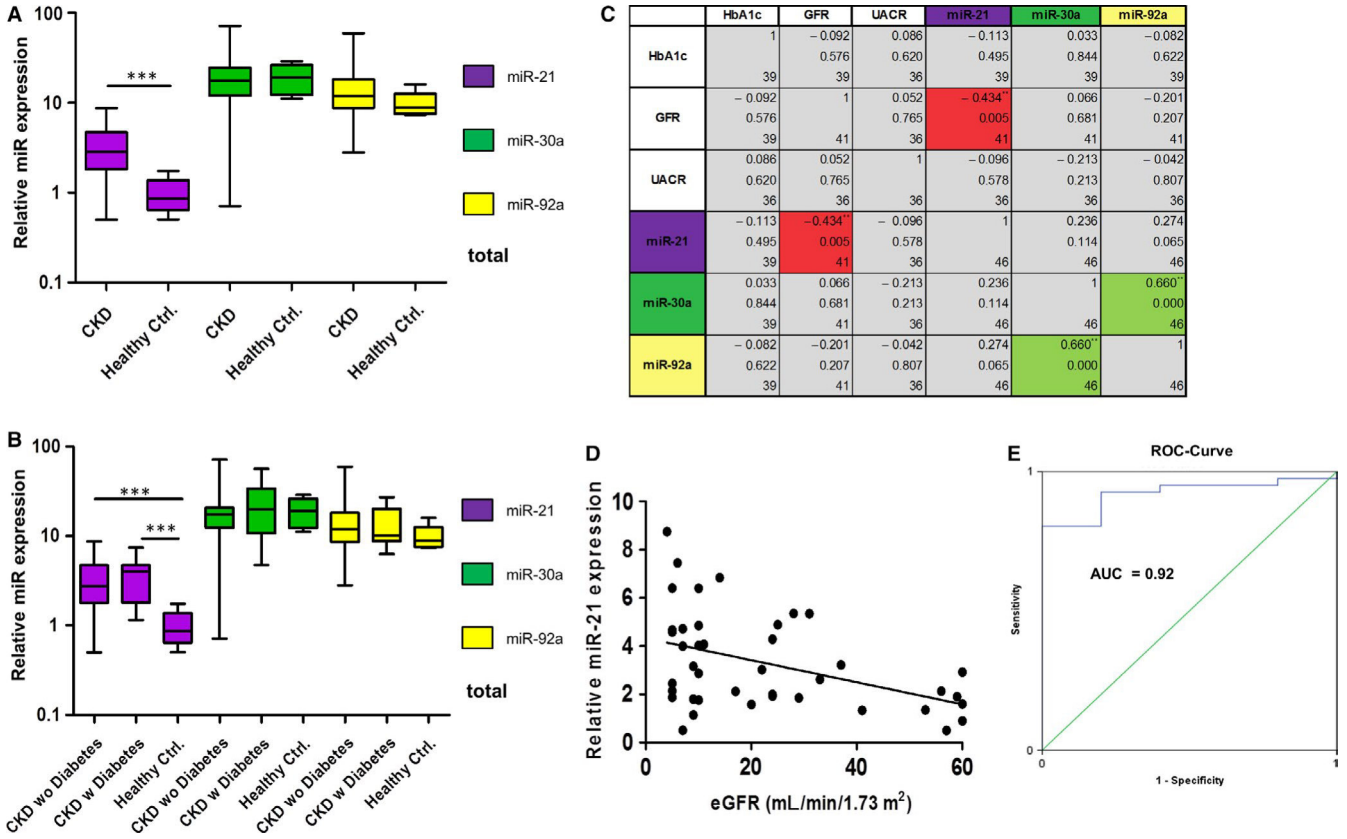
3.5 | MiR-21 is overexpressed in NTS-treated mice

Nephrotoxic serum-treated mice showed development of glomerulonephritis as shown by Hematoxylin and eosin (H&E) staining and Periodic acid–Schiff (PAS) staining (Figure 2C). We could observe glomeruli with two main phenotypes. The more abundant mild phenotype displayed glomerular basement membrane accumulation and mesangial matrix expansion, whereas the severe phenotype displayed crescent formation and podocyte loss. The PBS-injected control mice showed a normal histological morphology. Interestingly, we could find a significant ($P = 0.035$) average 8.9-fold up-regulation of miR-21 in mice injected with NTS in relation to PBS-injected control mice (Figure 2D). The miR-21 expression was correlated with UACR ($R = 0.632$; $P = 0.037$) as shown in Figure 2E.

4 | DISCUSSION

Besides conventional biomarkers, urinary miRs display a promising non-invasive tool as potential biomarkers for CKD. In contrast with total urine, urine sediment or cell-free urine samples, miRs derived from urinary exosomes are protected against degradation by nucleases and are protected against other environmental factors potentially compromising RNA integrity.[4] This makes them a new sample type with a promising potential in simplifying the handling and storage of patient urine samples.

Most of the current studies performed total miR profiling on relatively small patient sets or concentrated on a single miR in a specific disease. In the present study, we used a randomly picked, widespread set of patients suffering from CKD.



	HbA1c	GFR	UACR	miR-21	miR-30a	miR-92a
HbA1c	1	−0.092	0.086	−0.113	0.033	−0.082
		0.576	0.620	0.495	0.844	0.622
	39	39	36	39	39	39
GFR	−0.092	1	0.052	−0.434**	0.066	−0.201
	0.576		0.765	0.005	0.681	0.207
	39	41	36	41	41	41
UACR	0.086	0.052	1	−0.096	−0.213	−0.042
	0.620	0.765		0.578	0.213	0.807
	36	36	36	36	36	36
miR-21	−0.113	−0.434**	−0.096	1	0.236	0.274
	0.495	0.005	0.578		0.114	0.065
	39	41	36	46	46	46
miR-30a	0.033	0.066	−0.213	0.236	1	0.660**
	0.844	0.681	0.213	0.114		0.000
	39	41	36	46	46	46
miR-92a	−0.082	−0.201	−0.042	0.274	0.660**	1
	0.622	0.207	0.807	0.065	0.000	
	39	41	36	46	46	46

FIGURE 1 Levels of urinary exosomal miR-21, miR-30a-5p and miR-92a in chronic kidney disease (CKD) patients and healthy controls. MiR levels were measured by RT-qPCR normalized against inter-run calibrator and miR-16. MiR-21 is significantly up-regulated in CKD patients. *y*-axis = Log_{10}, error bars = SD (A). There is no difference between CKD patients with or without diabetes *y*-axis = Log10, error bars = SD (B). MiR-21 is negatively correlated with eGFR (red boxes). MiR-30a-5p is positively correlated with miR-92a (green boxes) (C,D). Boxes top down: *R* (correlation coefficient), *P*-value, n. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$ (C), *y*-axis = Log_2 (D). ROC-curve analysis of miR-21. Green line = diagonal; blue line = ROC-curve; AUC, area under the curve (E)

We could not find significant differences in expression levels between CKD patients and healthy controls with regard to miR-30a-5p. Interestingly, miR-30a-5p was recently proposed as a potential biomarker for FSGS in human urine samples.[9] Since our cohort represents a randomly picked patient set, it is likely that it is composed of FSGS patients to a statistically insufficient number. This might also be the case for miR-92a, which is known to be up-regulated in crescentic rapid progressive glomerulonephritis.[10]

In the present study, we found miR-21 being significantly up-regulated in urinary exosomes of patients suffering from CKD. However, miR-21 expression cannot discriminate between CKD patients with or without diabetes. Even though miR-21 was not correlated with UACR, which can be explained by high within-person variability,[11] it was found to be negatively correlated with eGFR indicating a possible opposing effect on kidney function.

Our results are in agreement with those of other groups describing that miR-21 is up-regulated in biopsies and different body fluids of patients suffering from diverse kidney diseases [12,13] including CKD.

Since podocyte dedifferentiation is a main event in the pathogenesis of glomerulopathies,[14] we studied the expression of miR-21 in our glomeruli dedifferentiation assay.[7] Here, we found a strong up-regulation of miR-21 during the dedifferentiation of mouse podocytes during 9 days and an up-regulation of genes involved in MAP-ERK signalling as described before.[15]

Moreover, we applied NTS to mice that induces glomerulonephritis as an CKD injury model.[16] We found a strong up-regulation of miR-21, underlining the important role of miR-21 in glomerular pathogenesis.

Summarizing, we found an up-regulation of miR-21 urinary exosomes of CKD patients and in different podocyte injury models, indicating that urinary exosomal miR-21 could be used as a potential new non-invasive, prognostic biomarker for CKD.

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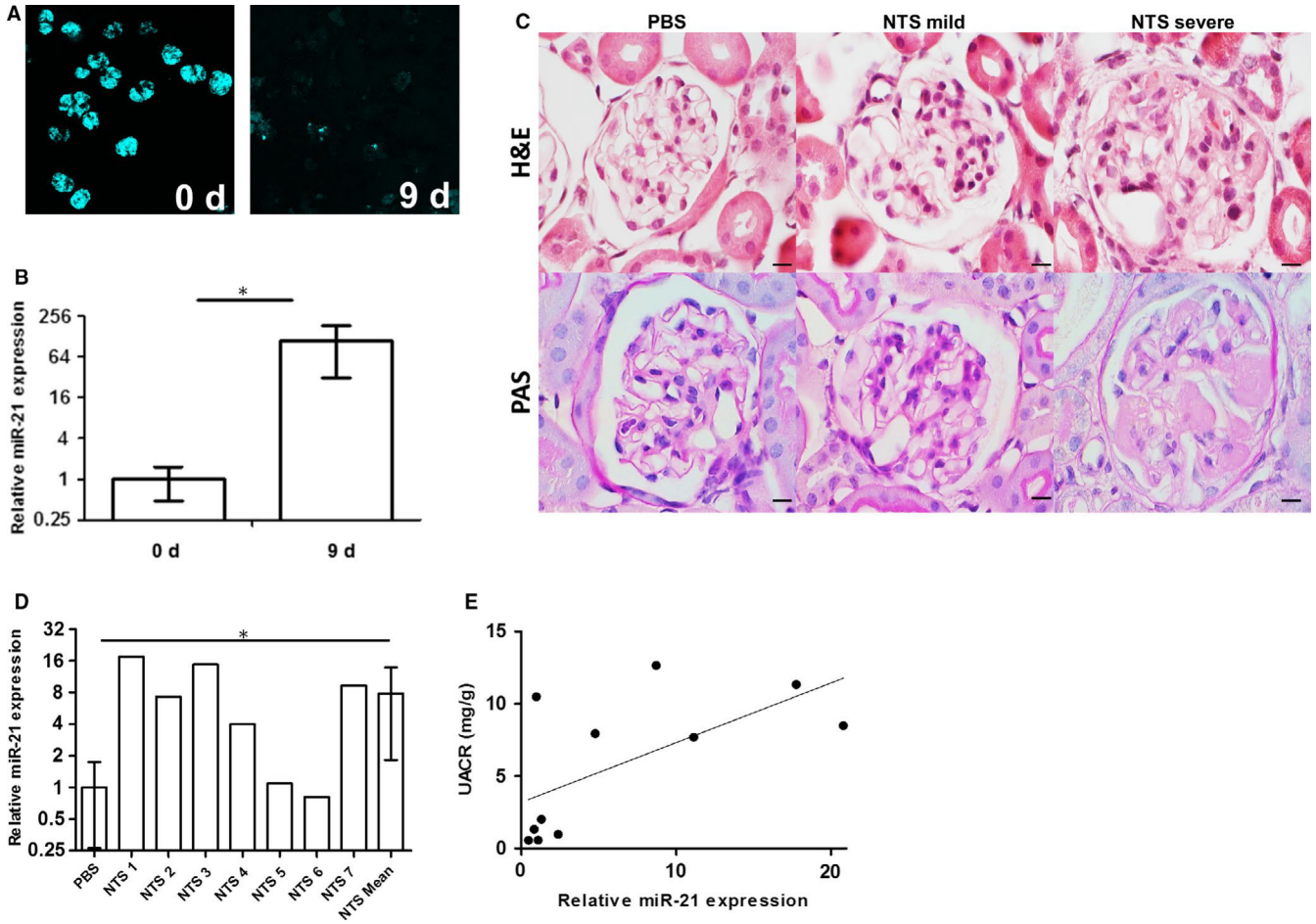

FIGURE 2 MiR-21 levels in dedifferentiated podocytes and in nephrotoxic serum (NTS)-treated mouse kidneys. Dedifferentiated glomeruli show almost no cyan fluorescent protein fluorescence (A). MiR-21 is 108.8 times up-regulated in dedifferentiated glomeruli. *y*-axis = Log_2; error bars = SD; n = 3. (B) The glomeruli of NTS treated mice show two main phenotypes in H&E and PAS staining. Scale bars = 10 µm. (C). MiR-21 is 8.9 times up-regulated in NTS-treated formalin-fixed paraffin-embedded (FFPE) mice kidneys. *y*-axis = Log_2; error bars = SD; PBS n = 4; NTS n = 7. (D). Pearson correlation of relative miR-21 expression and UACR. *x*-axis = Log_{10} (E)

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CONFLICT OF INTEREST

There are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

TL and NE did the study design. SS and TL contributed to urine processing. TL performed the urinary exosomal miRNA experiments including statistical analysis. FK and TL performed the experiments regarding the (de-)differentiation assay. TL, NE and KE analysed the experimental data. NA, TL, PK, CEC, CC did the NTS treatment experiments. TL did the figure design and literature search. TL, NE and KE wrote the main manuscript text. RR and UL contributed to manuscript revisal and to the GANI_MED study. All authors had approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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6 Scientific achievements

Publications:

- Lange T, Stracke S, Rettig R, Lendeckel U, Kuhn J, et al. Identification of miR-16 as an endogenous reference gene for the normalization of urinary exosomal miRNA expression data from CKD patients. PloS one. 2017; 12: e0183435.
- Endlich N, Lange T, Kuhn J, Klemm P, Kotb AM, et al. BDNF: mRNA expression in urine cells of patients with chronic kidney disease and its role in kidney function. Journal of cellular and molecular medicine. 2018 Nov;22(11):5265-5277
- Lange T, Artelt N, Kindt F, Stracke S, Rettig R, et al. MiR-21 is upregulated in urinary exosomes of CKD patients and after glomerular injury. Journal of cellular and molecular medicine. 2019 Jul;23(7):4839-4843.
- Iervolino A, Lange T, Hansen K, Siegerist F, Hammer E, et al. Proteome analysis of glomeruli isolated from zebrafish larvae. Nephrology Dialysis Transplantation, Volume 34, Issue Supplement_1, June 2019, gfz106.FP015

Contributions to scientific conferences

- *"BDNF and KIM-1 mRNA expression in urine cells of patients with chronic kidney disease."* 27th Meeting of the European Renal Cell Study Group, Louth, Ireland, 2015 (selected for oral presentation)
- *"BDNF: mRNA expression in urine cells of patients with chronic kidney disease and its role in kidney function."* 111th Meeting of the Anatomische Gesellschaft, Göttingen, Germany, 2016 (selected for oral presentation)

- *„Urinary exosomal miRNA expression in CKD: miR-21 as a potential biomarker. “* 29th Meeting of the European Renal Cell Study Group, Göttingen, Germany, 2017 (selected for oral presentation)
- *“Urinary exosomal miRNA expression in CKD: miR-21 as a potential biomarker.”* 113th Meeting of the Anatomische Gesellschaft, Rostock, Germany, 2018 (selected for oral presentation)

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