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MicroRNAs and Drug-induced Kidney Injury

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Abstract

Drug-induced kidney injury (DIKI) is a severe complication in hospitalized patients associated with higher probabilities of developing progressive chronic kidney disease or end-stage renal diseases. Furthermore, DIKI is a problem during preclinical and clinical phases of drug development leading to high rates of project terminations. Understanding the molecular perturbations caused by DIKI would pave the way for a new class of therapeutics to mitigate the damage. Yet, another approach to ameliorate DIKI is identifying sensitive and specific translational biomarkers that outperform the current diagnostic analytes like serum creatinine and facilitate early diagnosis. MicroRNAs (miRNAs), a class of non-coding RNAs, are increasingly being recognized to have a two-pronged approach towards DIKI management: 1) miRNAs have a regulatory role in gene expression and signaling pathways thereby making them novel interventional targets and 2) miRNAs enable diagnosis and prognosis of DIKI because of their stable presence in biofluids. In this review, apart from summarizing the literature on miRNAs in DIKI, we report small RNA sequencing results showing miRNA expression profiles at baseline in normal kidney samples from mice and humans. Additionally, we also compared the miRNA expression in biopsies of normal human kidneys to patients with acute tubular necrosis, and found 76 miRNAs significantly downregulated and 47 miRNAs upregulated (FDR adjusted $p<0.05$, +/−2-fold change).

In summary, we highlight the transformative potential of miRNAs in therapeutics and translational medicine with a focus on drug-induced kidney damage.

Keywords

microRNA; Kidney; Kidney toxicity; Acute kidney injury; Biomarker; Therapeutic targets

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Conflict of Interest

The authors declare that there are no conflicts of interest.

1. Introduction

Drug-induced kidney injury

The high susceptibility of the kidney to toxicity is mainly due to its function of eliminating endogenous waste products as well as xenobiotics. These substances can induce toxic responses due to a high local concentration and/or transformation into reactive metabolites (Kahl, Schmuck, & Vohr, 2010). Commonly prescribed drugs (Table 1) are known to cause acute kidney injury (AKI) that is a severe condition associated with high probabilities of developing progressive chronic kidney disease or end-stage renal diseases, thus leading to high mortality rates (Chawla, Eggers, Star, & Kimmel, 2014). In fact, the incidence of dialysis-requiring AKI increased from 222 to 533 cases per million person-years from 2000 to 2009 in the US (Hsu, McCulloch, Dudley, Lo, & Hsu, 2013). Epidemiological studies show that drugs are the cause of 18–27% of hospitalizations and 19% of intensive care unit patients within the group of AKI patients (Taber & Pasko, 2008; Uchino, et al., 2005). Taking into account that treating patients with end stage renal diseases accounted for over $40 billion in public and private US funds in 2009 (niddk.nih.gov, 2016), drug-induced AKI (DIKI) is a major public health concern. Additionally, DIKI accounts for approximately 10% of the failures in the preclinical and clinical stages (Cook, Hansen, Siu, & Abdul Razak, 2015) thus having a high relevance and a significant economic impact in drug development.

Kidney injury in humans is measured using functional biomarkers like blood urea nitrogen and/or serum creatinine. Although theses biomarkers are considered to be the standard diagnostic analytes in routine care, they are known to be modified by nutrition, muscle mass, age, sex, muscle injury, and aggressive fluid resuscitation (Waikar, Betensky, Emerson, & Bonventre, 2012). Furthermore, they increase only when glomerular filtration rate decreases by more than 50% and they do not reflect dynamic changes in filtration rates (Uchino, 2010). Novel sensitive and specific biomarkers are urgently needed to provide for cost-effective and non-invasive methods of detecting and treating early stage kidney injury. Early diagnostic and predictive biomarkers would also allow for stratification of patients that may be susceptible to develop AKI thereby facilitating clinical trials. Currently, in the absence of any therapeutics for AKI, renal replacement therapy remains the only option (Bellomo, 2015) for severe AKI, leading to an indispensable need for improved kidney injury management, i.e. detection as well as improved therapy.

In the last two decades, due to significant advances in understanding the molecular pathogenesis of AKI using state-of-the-art genome sequencing technologies, microRNAs (miRNAS) have emerged as novel therapeutic targets as well as biomarker candidates for AKI.

microRNA Biogenesis, Function and Extracellular Features

MiRNAs are approximately 20–25 nucleotides long, non-coding and evolutionary conserved small RNAs. MiRNAs were first discovered in *C. elegans* (R. C. Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993) followed by the recognition of their conservation in

a wide range of species (Pasquinelli, et al., 2000), leading to the current status of 788 known miRNAs in rats, 1899 in mice and 2585 in humans (miRBase, 2014).

In the cell, miRNAs regulate gene expression at the post-transcriptional level. As part of a ribonucleoprotein complex called miRISC (miRNA-induced silencing complex) they bind to complementary sequences in the 3′-untranslated regions of target mRNAs thus inhibiting mRNA translation. The process of miRNA maturation, miRISC incorporation and subsequent mRNA binding is relatively well explored and reviewed in detail in several review articles (Desvignes, et al., 2015; Garcia-Lopez, Brieno-Enriquez, & Del Mazo, 2013; Krol, Loedige, & Filipowicz, 2010). The complementarity between miRNA and mRNA does not have to be perfect for translational inhibition, therefore one miRNA regulates several hundred mRNAs and likewise, one mRNA is regulated by several miRNAs (Filipowicz, Bhattacharyya, & Sonenberg, 2008). In fact, it is estimated that over 50% of all protein-coding genes are regulated by miRNAs in mammals (Krol, et al., 2010) revealing their overall involvement in diverse physiological as well as pathological processes (Ceman & Saugstad, 2011; T. Li, et al., 2011; Szabo & Bala, 2013; Visone & Croce, 2009; Y. Wang & Lee, 2009; Wiemer, 2007). Many miRNAs are found to be highly enriched in particular organs or at a particular stage of development or disease progression in human body (Kriegel, et al., 2013; Landgraf, et al., 2007) – for instance the liver specific miR-122 (Lagos-Quintana, et al., 2002), kidney cortex enriched miR-192 (Tian, Greene, Pietrusz, Matus, & Liang, 2008), skeletal muscle enriched miR-133a and -b (Sempere, et al., 2004), or the cardiomyocyte specific miR-208a (van Rooij, et al., 2007). The expression of the miR-17~92 cluster, consisting of miR-17, -18a, 19a, -20a, -19b1 and -92a1, seems to be essential for normal nephrogenesis since ablation of the cluster in a mouse model resulted in reduced numbers of nephrons (Marrone, et al., 2014). Furthermore, miR-21 and miR-150 were found highly enriched in kidney cysts of patients with polycystic kidney disease and kidney biopsies from patients with lupus nephritis, respectively (Lakhia, et al., 2015; H. Zhou, et al., 2013).

Outside the cell, miRNAs were discovered for the first time in serum/plasma from cancer patients (X. Chen, et al., 2008; Mitchell, et al., 2008) and afterwards in other body fluids like urine, breast milk, saliva and cerebral fluid (Weber, et al., 2010). Extracellular miRNAs are very stable and resistant to degradation even with long-time storage at room temperature, pH variability and multiple freeze-thaw cycles (Y. Li, et al., 2011; J. S. McDonald, Milosevic, Reddi, Grebe, & Algeciras-Schimnich, 2011; Mitchell, et al., 2008; Mraz, Malinova, Mayer, & Pospisilova, 2009). Their stability is probably due to an association with RNA-binding proteins or being packed into vesicles (Arroyo, et al., 2011; Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2011; Vickers & Remaley, 2012; K. Wang, Zhang, Weber, Baxter, & Galas, 2010; Xu, Yang, & Ai, 2013). The function of extracellular miRNAs is not yet understood and some studies suggest that they might be active signaling molecules (Melo, et al., 2014; Y. Zhang, et al., 2010). However, unique features of extracellular miRNAs have made them promising biomarker candidates. Consequently, due to their intracellular function and their stable presence in biofluids, miRNAs could significantly contribute to improved kidney injury management as both potential biomarkers and interventional targets (Figure 1).

2. Mechanistic Role of microRNAs in Drug-induced Kidney Injury

MicroRNA Expression in the Kidney

By functioning as regulators of gene expression miRNAs play a crucial role in a variety of molecular processes in multiple organs including the kidney. To gain insight into kidney miRNA expression at baseline, we conducted small RNA sequencing in normal human and mouse kidney samples. After ranking the normalized read counts, the top expressed miRNAs were compared between human and mouse kidneys (Figure 2A). Sixty percent of the top 20 miRNAs were overlapping in human and mouse kidney, of which for example miR-10b was in the top five of both. This confirms the high conservation between species and leads to the assumption that these kidney-enriched miRNAs are involved in normal kidney homeostasis. Less is known about miR-10b in kidney physiology, but deregulation was reported in the context of clear cell renal cell carcinoma and acute allograft rejection (Fritz, Lindgren, Ljungberg, Axelson, & Dahlback, 2014; X. Liu, et al., 2015). However, the role of other kidney-enriched miRNAs like miR-192 or the miR-30 family are better elucidated. MiR-192 targets the β1 subunit of the Na^+/K^+-ATPase and by inhibiting the expression of this subunit it negatively affects the enzyme activity thereby contributing to renal handling of fluid balance, whereas the miR-30 family is involved in the nephron development and glomerular integrity (Agrawal, Tran, & Wessely, 2009; Mladinov, Liu, Mattson, & Liang, 2013; Wu, et al., 2014). Overall, several studies have demonstrated miRNA involvement in kidney physiology and pathophysiology (Bhatt, Kato, & Natarajan, 2016). When we conducted small RNA sequencing comparing the miRNA expression in biopsies of normal human kidneys to patients with acute tubular necrosis (ATN), we found 76 miRNAs significantly downregulated and 47 miRNAs upregulated (FDR adjusted $p<0.05$, +/-2-fold change; Figure 2B and Table 2). Using target prediction software, the miRNA expression profile revealed affected pathways in the ATN as well as associated kidney disease states that highlight the importance of miRNA analysis for understanding the pathogenesis of DIKI.

In line with these findings as well as based on results from other disciplines, miRNAs have emerged as promising therapeutic targets either by restoring or more commonly by inhibiting their function with synthetic miRNA mimics and anti-miRs, respectively.

Wei and colleagues (2010) showed for the first time the essential involvement of miRNAs in AKI by using a proximal tubule specific Dicer knockout (a miRNA processing enzyme). They reported a differential miRNA expression profile at baseline (80% and 16% of miRNAs were decreased and increased, respectively) due to the knockout which resulted in a significant protection against ischemic AKI (Wei, et al., 2010). The majority of published studies have focused on understanding the regulation of miRNAs during ischemia/reperfusion (I/R)-induced AKI. For example, miR-24 was shown to be increased in mouse kidneys after I/R injury in human allograft biopsies as well as in primary human proximal tubule cells after anoxia/hypoxia (Lorenzen, et al., 2014). Further, heme oxygenase 1 and H2A histone family, member X, were verified as miR-24 targets and silencing miR-24 resulted in decreased injury in cells and in mouse kidneys probably via apoptosis inhibition. Similarly, miR-687 was found to be up-regulated in mouse kidneys after ischemic injury and

again anti-miR-687 treatment led to significantly less injury (Bhatt, et al., 2015). PTEN was identified as a direct target and thus blocking of miR-687 preserved PTEN expression and attenuated cell cycle activation and apoptosis.

The potential use of miRNAs for treating kidney injury is an extremely exciting area of ongoing investigation and since there are distinct as well as common mechanisms between different kinds of AKI, results for I/R injury also provide new insights and innovative targets for DIKI.

MicroRNAs in Kidney Toxicity

The involvement of specific miRNAs in DIKI is overall less well explored. The currently known miRNAs with the respective DIKI models are summarized in Table 3. One of the earliest reports showed miR-34a, a p53 target, to be increased in mouse proximal tubular epithelial cells after cisplatin treatment (Bhatt, et al., 2010). Silencing of miR-34a increased cisplatin toxicity leading to the conclusion that miR-34a has a protective role during kidney injury. Although miR-34a was also increased in an I/R mouse model, its inhibition was associated with decreased autophagy and thus aggravated injury (X. J. Liu, et al., 2015). However, miR-155 was shown to be significantly upregulated in rats with kidney injury either induced by gentamicin administration or following I/R and, subsequently, miR-155 KO-mice exhibited significantly enhanced kidney toxicity in response to cisplatin administration (Pellegrini, et al., 2014; Saikumar, et al., 2012). This finding suggests a protective role of miR-155 during DIKI but also an involvement in a common mechanism between I/R and drug-induced AKI. A comparable protective effect was also seen for miR-125b, which was increased in *in vivo* and *in vitro* models of cisplatin-induced kidney injury and was suggested to be part of the Nrf2 pathway (Joo, Lee, Koo, & Kim, 2013). There are some published reports that show the contribution of miRNA deregulation in the onset and progression of injury. Treating the immortalized human proximal tubule epithelial cell line HK-2 with cisplatin increased the expression of miR-181a which subsequently inhibited its known target BCL-2 resulting in apoptosis (G. Chen, et al., 2010; Zhu, et al., 2012) and correspondingly less apoptosis was detected when cells were treated with anti-miR-181a. In mouse kidneys and in a rat kidney cell line, miR-122 was decreased after cisplatin, gentamicin and doxorubicin treatment (C. G. Lee, et al., 2014). Since FOXO3 is a verified target of miR-122, FOXO3 stimulated downstream activation of p53 in the absence of miR-122 and resulted in progression of apoptosis and kidney injury. Similar findings were observed in a mouse toxicity model with doxorubicin where miR-133a was increased which in turn was found in other models to directly inhibit the multidrug resistance-associated protein 2 (MRP2; (Loeser, et al., 2015)). MRP2 is one of the numerous transporters in proximal tubule epithelial cells located on the apical membrane and is known to be decreased in injured proximal tubules and thereby further augmenting kidney toxicity (Wen, et al., 2014).

Besides the kidney toxicity of cytostatic drugs including cisplatin or doxorubicin and aminoglycoside antibiotics like gentamicin, miRNAs have been studied in cyclosporine A toxicity. Cyclosporine A is an immunosuppressive agent commonly given to transplant or autoimmune disease patients but it is known for its long-term kidney toxicity characterized

by severe renal tubulointerstitial fibrosis. Treating human proximal tubular epithelial cells *in vitro* with cyclosporine A deregulated 46 miRNAs (J. Chen, Zmijewska, Zhi, & Mannon, 2015). One of the few increased miRNAs was miR-21 (~5.5-fold), which is widely explored in the context of kidney disease and injury (Li, et al., 2013; T. B. Zhou & Jiang, 2014). In the cyclosporine A model, miR-21 up-regulation was associated with AKT activation, PTEN decrease and the increase of several markers of epithelial-mesenchymal transition (EMT) including vimentin and αsmooth muscle actin. Epithelial to mesenchymal transition has been shown to play an important role in kidney fibrosis (Lovisa, et al., 2015) and also in the context of cyclosporine A toxicity (Slattery, Campbell, McMorrow, & Ryan, 2005). These results correspond with a therapeutic study on kidney fibrosis, demonstrating that inhibition of miR-21 was protective against TGF-β-induced fibrogenesis in a mouse model of Alport nephropathy (Gomez, et al., 2015). Further, Yuan et al. (2015) found another miRNA, miR-494, to be involved in cyclosporine A induced EMT (Yuan, Benway, Bagley, & Iacomini, 2015). MiR-494 was increased approximately 2-fold in mouse kidneys as well as in HK-2 cells after cyclosporine A treatment. Again a PTEN decrease was observed (~4-fold), which was identified as a direct target of miR-494. Counteracting a PTEN inhibition by using anti-miR-494 prevented cyclosporine A induced EMT.

In summary, the further exploration of the miRNA role in the pathogenesis of DIKI could lead to the development of miRNA-based therapeutics and this seems promising based on the current findings and results seen in other related disciplines.

3. MicroRNAs as Biomarkers for Drug-induced Kidney Injury

Biomarker Candidates

With the 21st century advances in omics technologies, biomarker science has emerged as a very exciting multidisciplinary approach to understand and classify disease pathogenesis. However, the biomarker science pipeline that involves carrying the biomarker from discovery to confirmation, evaluation, qualification and validation steps requires a significant commitment of resources and time (Figure 3). Based on the platform of choice for a biomarker discovery effort, starting with >10,000 candidates, one could identify a handful of successfully qualified and validated biomarkers that enables improved patient care as well as a more efficient drug development. Over a decade ago, this path was formed and successfully taken, culminating in 2008 with a qualification of seven urinary protein biomarkers by the US Food and Drug Administration (FDA) and European Medicines Agency for the assessment of DIKI in preclinical studies. In spite of this breakthrough work, implementation of these pre-clinically qualified biomarkers into clinical practice is still awaited (Dieterle, et al., 2010; EMA, 2009; Murray, et al., 2014). Protein-based biomarker evaluation is often challenging because of the high diversity of proteins and their post-translational modifications and the resulting issues in assay development.

On the contrary, referring to the FDA's description of an ideal biomarker (Wallace, et al., 2001), extracellular miRNAs fit all the criteria: *1*) easy accessibility in diverse body fluids, *2*) stability, 3) conservation across species, 4) association with particular tissues or pathological states and 5) a sensitive measurement method. Due to these unique features miRNAs have shown great promise as non-invasive biomarkers. They were detected in

almost all body fluids including the clinically most relevant blood and urine (Weber, et al., 2010). Although high concentrations of RNA degrading enzymes are present in the extracellular space, miRNAs are found to be remarkably stable (McDonald, Milosevic, Reddi, Grebe, & Algeciras-Schimnich, 2011), which is probably due to their packing into microvesicles and exosomes or their association with proteins and high-density lipoproteins (Arroyo, et al., 2011; Hoy & Buck, 2012; Turchinovich, Weiz, Langheinz, & Burwinkel, 2011). Furthermore, miRNAs not only show organ specificity many times but are also highly conserved in sequence across species (Landgraf, et al. 2007; Sun, et al. 2004). Commonly, they are measured by real-time PCR which is a sensitive and well-established method for nucleic acids. To date, several hundred studies have evaluated the potential of miRNAs as biomarkers for various pathological conditions including cancers, cardiovascular and neurodegenerative diseases. For circulating miRNAs i.e. miRNAs from blood, only, 35 different clinical studies are currently registered assessing their performance as biomarkers for human diseases (clinicaltrials.gov, 2015); several of these are also associated with kidney diseases like autosomal dominant polycystic kidney disease, renal cell carcinoma and AKI after cardiac surgery.

In terms of kidney toxicity biomarkers, work with miRNAs has thus far been focused on urine as it is non-invasive, directly derived from kidneys and has been shown to contain miRNAs. Our laboratory was amongst the first few laboratories to demonstrate the isolation of miRNAs from urinary supernatants and showed the differential expression of miR-21 and miR-155 in the urines of rats with AKI or gentamicin-induced AKI (Saikumar, et al., 2012). These results were confirmed by a precompetitive consortium of pharmaceutical industries, ILSI Health and Environmental Sciences Institute. In spite of using a different cisplatin doses, fasting/ feeding conditions and different rat strains, approximately 20 miRNA candidates were found to be the same in both studies (Kanki, et al., 2014; Pavkovic, Riefke, & Ellinger-Ziegelbauer, 2014). Increases of specific miRNAs were also measured in urine from rats treated with gentamicin or doxorubicin (Church, et al., 2014; Nassirpour, et al., 2014). All miRNA levels correlated with histopathological changes as well as the qualified protein biomarker Kim-1 and were increased before serum creatinine and blood urea nitrogen. Almost all these studies were conducted in rats, (Table 4), but for example, miR-21 was also found in urine from human AKI patients from the intensive care unit (Ramachandran, et al., 2013) as well as in patients with acetaminophen or cisplatin induced AKI (Pavkovic, Robinson-Cohen, et al., 2015) thereby strengthening the translational potential of miRNAs in DIKI settings. A recent study evaluated miRNAs in plasma as biomarkers for contrast-induced kidney injury (Gutierrez-Escolano, Santacruz-Vazquez, & Gomez-Perez, 2015). First, the miR-30 family (miR-30a, -c, and -e) was found to be increased in rat plasma after the administration of contrast agent and these results were validated in a patient cohort where especially miR-30a performed very well in differentiating patients with contrast-induced nephropathy from those without. Even though the number and diversity of miRNA biomarker studies specifically for DIKI are small, the data is promising and results from other kidney diseases including immunoglobulin A nephropathy, ischemic AKI, glomerulonephritis or focal segmental glomerulosclerosis (Ichii, et al., 2014; Pavkovic, Riefke, Frisk, Groticke, & Ellinger-Ziegelbauer, 2015; G.

Wang, et al., 2011; J. F. Wang, et al., 2014; W. Zhang, et al., 2014) support the value of further exploration.

In addition, extracellular miRNA biomarkers could have more advantages in drug development. Since they are conserved and ubiquitously expressed, they could not only be implemented in preclinical and clinical studies but also *in vitro* studies for screening of compounds with potential toxic effects. The presence of miRNAs in cell culture medium was mostly demonstrated while exploring their potential role in inter-cellular communication (M. K. McDonald, Capasso, & Ajit, 2013; Rani, 2014; Y. Zhou, et al., 2014). However, assuming that miRNAs can be actively as well as passively released from injured kidney cells, cell culture media of treated and untreated cells could have different miRNA profiles. We had previously found miR-21, -200c and -423 to be increased in patients with AKI and here we show that when primary human proximal tubular epithelial cells are treated with the contrast agent sodium diatrizoate all three miRNAs, miR-21, -200c and -423 significantly increased in medium of cells (Figure 5). Furthermore, due to their intracellular function, extracellular miRNAs could mirror the events taking place in the injured kidney, thus being not only indicative of the injury itself but also of the affected pathways. For instance, cisplatin induces miR-34a expression in rat kidneys which itself is involved in the p53-mediated apoptosis pathway and increased miR-34a levels were also measured in urine of the same cisplatin treated rats (Pavkovic, et al., 2014).

All findings and hypotheses around miRNA biomarkers for kidney toxicity seem promising and valuable; nonetheless more replication and validation of the biomarkers are needed in large multi-centered cohorts to confirm the reproducibility and performance.

Challenges of microRNAs as Biomarkers

Despite all the promising results with miRNA biomarkers it is worth mentioning that the field is facing several basic challenges that hinder a smooth process of biomarker qualification and validation.

One of the biggest challenges in clinical miRNA biomarker studies is the comparison with serum creatinine (SCr) as a gold standard definition for acute kidney injury. Although in preclinical studies renal histopathological examination is the gold standard for DIKI diagnosis, in clinical assessments SCr remains widely used. In fact, moderate performances of new AKI biomarker candidates are frequently seen in clinical studies, where AKI is mostly defined based on increased SCr levels (Pavkovic, Robinson-Cohen, et al., 2015). A potential solution for DIKI studies could be to use the treatment with the nephrotoxic drug per se for comparison.

Yet, another challenge is the lack of standardization in not only miRNA isolation and measurement approaches but also sample collection, handling and storage conditions. The most common methods of miRNA isolation are phenol/chloroform-based techniques including silica column purification, but these have also been shown to vary greatly depending on the vendor (El-Khoury, Pierson, Kaoma, Bernardin, & Berchem, 2016; Martinez-Fernandez, Paramio, & Duenas, 2016). In terms of miRNA measurement, qRT-PCR is widely used especially from biospecimens having overall low RNA yields like serum

or urine. The issue here is the absence of generally agreed endogenous control miRNAs. The reported normalization strategies differ greatly, including normalization with 1.) synthetic miRNAs that were spiked in during RNA isolation (Argyropoulos, et al., 2013; J. F. Wang, et al., 2010), 2.) small RNAs like RNU48 or U6 (G. Wang, et al., 2012; N. Wang, et al., 2012) or 3.) invariant miRNAs identified within the specific study (Hanke, et al., 2010; Yang, et al., 2012). All three approaches have disadvantages: normalization to spike-ins does not account for a true biological variability but rather accounts for a technical variation; normalizing to small RNAs could bias the results because the small RNA itself could have been differentially expressed during injury or the isolation and transcription efficiency is different for small RNAs vs. miRNAs (e.g. U6 ~106 nucleotides vs. miRNAs 20–25 nucleotides); finally the use of invariant miRNAs for normalization, although popular, seems not to be universal and only limited to specific studies or even specific datasets. Furthermore, urine as a biospecimen has its own challenges regarding normalization, which is necessary to account for variations in urine flow rate/concentrations due to hydration or diuresis. Yet, urine is very interesting since it is directly associated with the kidneys and thereby serves as a relevant and most-proximal non-invasive matrix to perform miRNA biomarker discovery for AKI. Normalizing biomarker levels with urinary creatinine as is usually done for protein biomarkers was suggested in a recent rat study (Pavkovic, et al., 2014). This approach, however, has several limitations due to potential changes of urinary creatinine induced by factors like variations in diurnal production, physical activity, diet, muscle mass (Greenblatt, et al., 1976; Heymsfield, Arteaga, McManus, Smith, & Moffitt, 1983; Waikar, Sabbisetti, & Bonventre, 2010). Furthermore, simulations on creatinine kinetics revealed that protein biomarker performance is actually affected by this way of normalization (Waikar, et al., 2010) which could also be the case for miRNAs.

In general the harmonization of miRNA biomarker evaluation is important to enable the establishment of reference ranges accounting for potential differences in strain, species, fasting/feeding conditions in preclinical studies and age, sex, ethnicity, comorbid conditions in clinical studies. Expression analyses in kidneys during the life span of F344 rats have shown that miRNA levels were sex and age dependent (Kwekel, Vijay, Desai, Moland, & Fuscoe, 2015), therefore also miRNA levels in biofluids could be affected by these factors. Thus, a systematic effort towards standardization could accelerate qualification of these miRNAs and improve the interpretation of miRNA biomarker candidates from different studies.

4. Conclusion

DIKI is still a large problem in clinical as well as non-clinical settings. The detection of DIKI is hindered by poorly performing standard diagnostic analytes and once AKI is diagnosed therapeutic approaches are nearly nonexistent. On the one hand, miRNAs regulate over 50% of all protein-coding genes at the post-transcriptional level. Thus miRNAs are involved in almost all physiological as well as pathological processes, which make them interesting therapeutic targets. On the other hand, extracellular miRNAs are remarkably stable and found to be valuable biomarker candidates in diverse disease settings. Therefore, due to the intracellular function of miRNAs and their presence in biofluids, miRNAs could significantly contribute to improved management of DIKI as interventional targets but also

as potential biomarkers. Positive results from other disciplines and the limited yet promising data in the field of kidney medicine hold great promise for a successful application of miRNA-based interventions in the context of DIKI.

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Abbreviations

AKI	acute kidney injury
ATN	acute tubular necrosis
DIKI	drug-induced kidney injury
EMT	epithelial to mesenchymal transition
FDR	false discovery rate
I/R	ischemia/reperfusion
miRNA	microRNA
qRT-PCR	quantitative real time PCR

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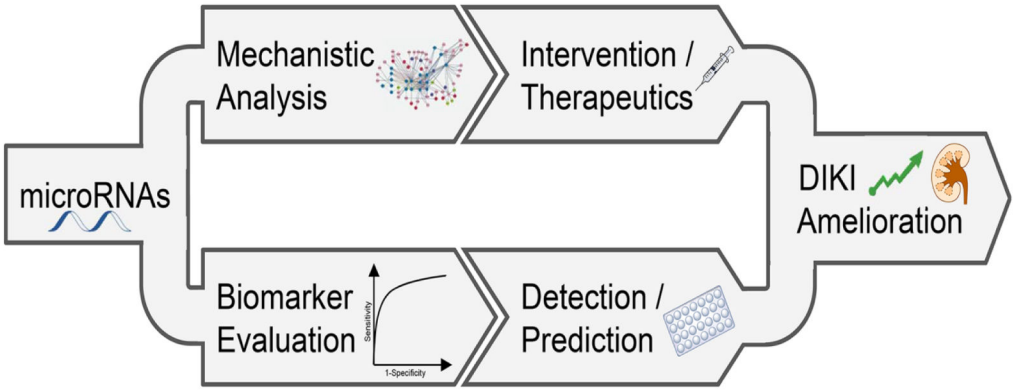

Figure 1. MicroRNAs ameliorate DIKI via a two-pronged approach: identifying interventional strategies and facilitating early diagnosis

Due to the intracellular function of miRNAs and their stable presence in biofluids, miRNAs could significantly contribute to ameliorate kidney injury outcomes as interventional targets and potential biomarkers.

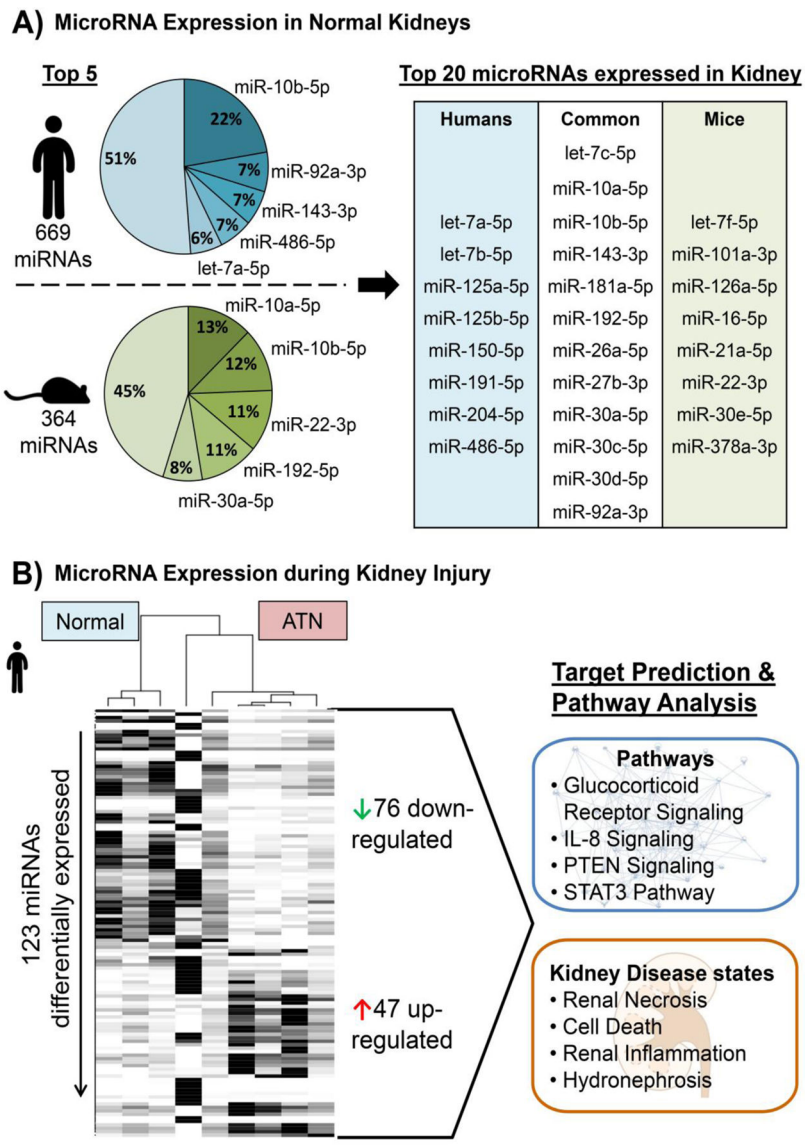

Figure 2. MicroRNA Expression in Healthy and Injured Kidneys

A) To measure the baseline expression levels of microRNAs in the kidney total RNA was extracted from normal human (n=5) and mice (n=3) kidney samples followed by a small RNA sequencing using the Illumina platform. The normalized mean read counts were ranked by defining read counts >10 as being expressed. The proportions of the top expressed microRNAs were compared within each species as well as between human and mouse and revealed a big overlap of highly abundant microRNAs in the kidneys. B) Total RNA was also extracted and small RNAs were sequenced from kidney samples of patients with acute tubular necrosis (ATN; n=5). Differentially expressed microRNAs between normal and ATN kidney were identified using the DEseq2 algorithm including an adjustment for age and gender of the patients. This resulted in 47 up- and 76 down-regulated microRNAs in ATN vs. normal (FDR adjusted $p<0.05$ and fold-change cut-off +/− 2). Subjecting the 123 total deregulated microRNAs to a target prediction and pathway analysis utilizing Ingenuity

Pathway Analysis demonstrated that these miRNAs are associated with specific pathways as well as kidney disease states.

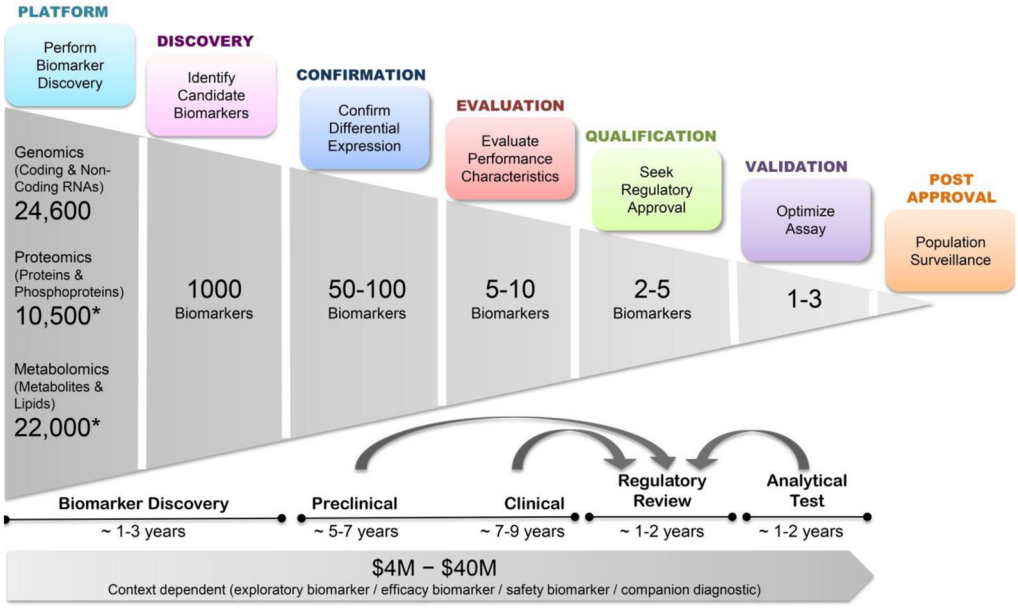

Figure 3. Biomarker Development Pipeline

The overview is based on estimations and the experiences gained during the Critical Path's Predictive Safety Testing Consortium's work on protein biomarkers for kidney injury.

*Dependent on platforms used as well as type of biospecimen.

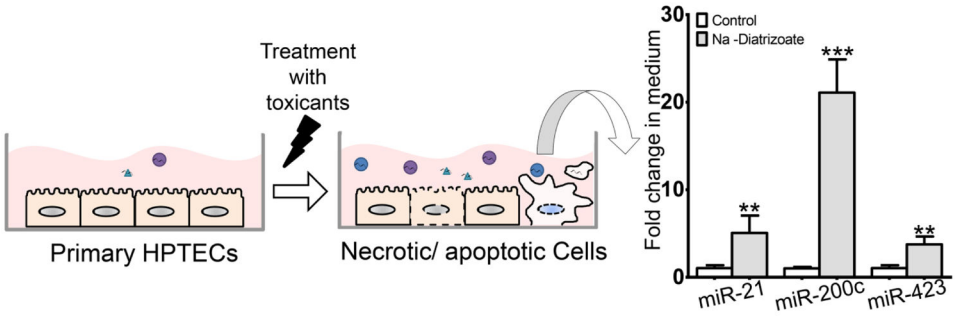

Figure 4. MicroRNAs as Biomarkers for in vitro Toxicity Testing

Normal cells have been shown to release miRNA-containing vesicles. Likewise, injured cells also release miRNAs actively as well as passively (apoptosis/necrosis) which then can be measured in different biofluids. In *in vitro* studies, this would correspond to miRNA profiles in the supernatant of untreated and treated cultured cells. Differential levels could enable an evaluation of these extracellular miRNAs as biomarker candidates for screening of potential kidney toxic agents. To test this hypothesis, primary human proximal tubular epithelial cells (HPTECs) were cultured and treated with the contrast agent sodium diatrizoate (200 mM) for 24h. Total RNA was isolated from 200 µl medium and miR-21, -200c and -423 were measured by qRT-PCR as these miRNAs have been reported to increase following kidney injury in human urines ([Ramachandran, et al., 2013]). All three miRNAs were found to be significantly increased in the medium of treated HPTECs as compared to untreated cells. Data is presented as mean fold changes with standard deviation (n=4) relative to the untreated cells ($2^{-\Delta\Delta Ct}$ with internal reference). 1-way ANOVA with Dunnett's test was used for p-value calculation: ** $p<0.01$ and *** $p<0.001$.

Table 1

Commonly used drugs with nephrotoxic side effects

Name	Pharmacological Class	Name	Pharmacological Class
Capreomycin	aminoglycosides antibiotics	**5-Fluoruracil**	antineoplasic
Gentamicin	aminoglycosides antibiotics	**Arsenic Trioxide**	antineoplasic
Kanamycin	aminoglycosides antibiotics	**Camptothecin**	antineoplasic
Neomycin	aminoglycosides antibiotics	**Carmustine**	antineoplastic
Streptomycin	aminoglycosides antibiotics	**Cisplatin**	antineoplasic
Tobramycin	aminoglycosides antibiotics	**Doxorubicin**	antineoplasic
Acetaminophen	analgesic	**Idarubicin**	antineoplasic
Bacitracin	antibiotics	**Mitomycin C**	antineoplasic
Ciprofloxacin	antibiotics	**Paclitaxel**	antineoplasic
Demeclocycline	antibiotics	**Puromycin**	antineoplasic
Imipenem	antibiotics	**Aldesleukin**	antineoplasic, immunomudulating
Methoxyflurane	analgesic	**Ifosfamide**	antineoplasic, immunosuppressive
Polymyxins	antibiotics	**Methotrexate**	antineoplastic, antimetabolite, immunosuppressant
Rifampicin	antibiotics	**Pentamidine**	antiprotozoal
Streptozocin	antibiotics	**Acyclovir**	antiviral
Sulfamethoxazole	antibiotics	**Cidofovir**	antiviral
Tetracyclines	antibiotics	**Foscarnet**	antiviral
Trimethoprim	antibiotics	**Tenofovir**	antiviral
Vancomycin	antibiotics	**Deferoxamine**	chelating agent
Sulfonamides	antibiotics, anti-diabetics, diuretics	**EDTA**	chelating agent
Pentamidine isethionate	antifungal	**Sodium aurothiomalat**	immunosuppressive, anti-rheumatic
Amphotericin B	antifungal, antiprotozoal	**Cyclosporine A**	immunsupressive
Chlorpropamide	anti-hyperglycemic	**Penicillamine**	immunsupressive
Gallium nitrate	anti-hypercalcemia	**Tacrolimus**	immunsupressive
Pamidronate	anti-hypercalcemia	**Lithium**	psychiatric medication
Aspirin	anti-inflammatory, analgesic, antipyretic	**Diatrizoate**	radiocontrast
Ibuprofen	anti-inflammatory, analgesic, antipyretic	**Iodipamide**	radiocontrast

Table 2

MicroRNA expression changes in kidney injury

List of 123 differentially expressed microRNAs between normal human kidneys and kidneys from patients with acute tubular necrosis (ATN). Data corresponds to Figure 2B; FDR adjusted p-value.

			76 downregulated microRNAs			**47 upregulated microRNAs**		
microRNA	**fold change**	**adjusted p-value**	**microRNA**	**fold change**	**adjusted p-value**	**microRNA**	**fold change**	**adjusted p-value**
miR-124-3p	**−13.45**	<0.001	miR-424-5p	**−3.19**	0.004	miR-339-5p	**2.03**	0.0240
miR-206	**−12.90**	<0.001	miR-204-3p	**−3.18**	0.020	miR-130b-5p	**2.05**	0.0159
miR-582-3p	**−12.74**	<0.001	miR-10a-3p	**−3.18**	0.002	miR-15b-5p	**2.10**	0.0251
miR-892a	**−10.28**	<0.001	miR-26a-1-3p	**−3.16**	0.049	miR-574-3p	**2.17**	0.0355
miR-184	**−9.72**	<0.001	miR-500b-5p	**−3.14**	0.003	miR-4742-3p	**2.18**	0.0283
miR-4482-3p	**−9.12**	0.002	miR-10b-3p	**−3.08**	0.004	miR-361-3p	**2.19**	0.0209
miR-135a-5p	**−8.80**	0.001	miR-218-5p	**−3.06**	0.028	miR-3173-5p	**2.26**	0.0388
miR-20b-5p	**−7.58**	<0.001	miR-10a-5p	**−3.01**	0.005	miR-125b-1-3p	**2.27**	0.0390
miR-190a-5p	**−7.25**	<0.001	miR-99a-3p	**−2.97**	0.010	miR-25-5p	**2.34**	0.0190
miR-194-5p	**−7.19**	<0.001	miR-548q	**−2.90**	0.048	miR-5701	**2.36**	0.0082
miR-1251-5p	**−7.10**	0.006	miR-4510	**−2.89**	0.025	miR-4301	**2.36**	0.0040
miR-891a-5p	**−7.09**	0.001	miR-30d-3p	**−2.88**	0.020	miR-423-5p	**2.51**	0.0235
miR-106a-5p	**−7.06**	<0.001	miR-30b-3p	**−2.86**	0.013	miR-6747-3p	**2.54**	0.0090
miR-216b-5p	**−6.79**	0.006	miR-30a-5p	**−2.85**	0.001	miR-342-5p	**2.62**	0.0101
miR-192-5p	**−6.63**	0.001	miR-30a-3p	**−2.82**	0.029	miR-7977	**2.62**	0.0010
miR-3065-3p	**−6.56**	0.001	miR-501-5p	**−2.78**	0.001	miR-663b	**2.64**	0.0290
miR-9-5p	**−6.45**	<0.001	miR-338-3p	**−2.70**	0.039	miR-342-3p	**2.70**	0.0081
miR-6500-3p	**−5.71**	0.012	miR-629-5p	**−2.70**	0.016	miR-941	**2.70**	0.0355
miR-26a-2-3p	**−5.54**	0.003	miR-22-5p	**−2.63**	0.016	miR-330-3p	**2.72**	<0.0019
miR-3065-5p	**−5.40**	0.006	miR-1270	**−2.60**	0.034	miR-4286	**2.94**	0.0034
miR-10b-5p	**−5.38**	<0.001	miR-500a-3p	**−2.55**	0.002	miR-197-5p	**2.96**	0.0284
miR-362-5p	**−5.37**	<0.001	miR-17-5p	**−2.47**	0.011	let-7i-3p	**3.00**	0.0284
miR-187-3p	**−5.14**	0.002	miR-30e-3p	**−2.42**	0.044	miR-629-3p	**3.07**	0.0049
miR-20a-5p	**−5.11**	<0.001	miR-30c-2-3p	**−2.40**	0.033	miR-642a-3p	**3.15**	0.0420

76 downregulated microRNAs					47 upregulated microRNAs		
microRNA	**fold change**	**adjusted p-value**	**microRNA**	**fold change**	**adjusted p-value**	**microRNA**	**fold change**	**adjusted p-value**
miR-577	**−5.07**	0.003	miR-6511b-5p	**−2.38**	0.021	miR-197-3p	**3.17**	0.0012
miR-1269a	**−4.97**	0.008	miR-200c-3p	**−2.31**	0.016	miR-4767	**3.18**	0.0156
miR-138-2-3p	**−4.93**	0.016	miR-664a-5p	**−2.19**	0.044	miR-4516	**3.27**	0.0137
miR-204-5p	**−4.90**	0.001	miR-152-3p	**−2.18**	0.034	miR-6812-3p	**3.27**	0.0165
miR-211-5p	**−4.77**	0.001	miR-151a-3p	**−2.12**	0.003	miR-5010-5p	**3.29**	0.0242
miR-5588-5p	**−4.45**	0.014				miR-766-3p	**3.51**	<0.0016
miR-4461	**−4.33**	0.014				miR-6769b-3p	**3.53**	0.0137
let-7f-5p	**−4.19**	0.005				miR-34b-3p	**3.59**	0.0110
miR-660-5p	**−4.18**	0.001				miR-935	**3.66**	0.0157
miR-338-5p	**−4.12**	0.008				miR-483-3p	**3.69**	0.0290
miR-6723-5p	**−4.05**	0.008				miR-3960	**3.71**	0.0375
miR-194-3p	**−3.94**	0.021				miR-142-3p	**3.96**	0.0269
miR-532-5p	**−3.84**	<0.001				miR-3690	**3.99**	0.0206
miR-502-5p	**−3.71**	0.006				miR-150-3p	**4.10**	0.0027
miR-379-5p	**−3.67**	0.029				miR-4792	**4.26**	0.0075
miR-26b-5p	**−3.67**	0.008				miR-150-5p	**4.36**	0.0034
miR-98-5p	**−3.61**	0.002				miR-3195	**4.54**	0.0128
miR-138-5p	**−3.60**	0.028				miR-642a-5p	**5.39**	<0.0017
miR-374b-5p	**−3.56**	0.002				miR-4448	**6.16**	0.0027
miR-188-5p	**−3.43**	0.008				miR-3168	**6.36**	0.0049
let-7e-5p	**−3.34**	0.001				miR-6731-3p	**6.43**	0.0017
miR-107	**−3.27**	0.003				miR-4492	**6.67**	0.0030
miR-196a-5p	**−3.26**	0.025				miR-4508	**7.20**	<0.0019

Table 3

MicroRNAs involved in drug-induced kidney injury (DIKI)

microRNA	DIKI Models	*in vitro* Models	*in vivo* Models	Expression	Upstream Regulator	Targets[a]	Proposed Effect	Reference
miR-122	Cisplatin, Gentamicin, Doxorubicin		Mouse	Decrease		FOXO3	p53 Activation	(C. G. Lee, et al., 2014)
miR-124	Cyclosporin A	HPTEC	Mouse	Increase				(J. Chen, et al., 2015)
miR-133a	Doxorubicin	HPTEC [c]	Mouse, Human kidney biopsies	Increase	ET-1/ ET-B Receptor	MRP2	Increasing Injury	(Loeser, et al., 2015)
miR-146b	Cisplatin	HPTEC	Mouse[c]	Decrease				(Pellegrini, et al., 2015)
miR-155	Cisplatin, Gentamicin		Mouse, Rat	Increase		c-Fos[b]		(Saikumar, et al., 2012) (Pellegrini, et al., 2014)
miR-181a	Cisplatin	HK-2		Increase		BCL-2	Apoptosis	(Zhu, et al., 2012)
miR-18a	Cisplatin	HPTEC	Mouse[c]	Decrease				(Pellegrini, et al., 2015)
miR-21	Cyclosporin A	HPTEC	Mouse	Increase		AKT/PTEN Pathway	EMT	(J. Chen, et al., 2015)
miR-21	Gentamicin		Mouse	Increase				(Saikumar, et al., 2012)
miR-21	Gentamicin		Rat	Increase				(Jia, et al., 2013)
miR-34a	Cisplatin	BUMPT- 306	Mouse	Increase	p53		Cytoprotection	(Bhatt, et al., 2010)
miR-34a	Cisplatin		Rat	Increase	p53[b]			(Pavkovic, et al., 2014)
miR-34a	Cisplatin, Gentamicin, Doxorubicin		Mouse	Increase		Sirt1[b]	p53 Activation via FOXO3 Acetylation	(C. G. Lee, et al., 2014)
miR-494	Cyclosporin A	HK-2	Mouse	Increase		PTEN	EMT	(Yuan, et al., 2015)

[a] in the context of DIKI;

[b] no direct interaction shown in this study;

[c] kidney injury model but not DIKI; EMT, Epithelial-Mesechymal Transition

Table 4

MicroRNA biomarker candidates for drug-induced kidney injury (DIKI)

microRNARNA	DIKI (Model)	Species	Biofluid	Direction	Reference
let-7g, miR-93, -191, -195	Cisplatin	Rat	Urine	UP	(Kanki, et al., 2014)
miR-15, -16, -20a, -192, -193, -210	Cisplatin	Rat	Urine	UP	(Pavkovic, et al., 2014)
miR-21, -200c, -423	Cisplatin, Acetaminophen	Patients	Urine	UP	(Pavkovic, Robinson- Cohen, et al., 2015)
miR-203a, let-7d	Gentamicin	Rat	Urine	DOWN	(Nassirpour, et al., 2014)
miR-21, miR-155	Gentamicin	Rat	Urine	DOWN	(Saikumar, et al., 2012)
miR-30a,c,e	Contrast agent *	Rat Patients	Plasma	UP	(Gutierrez-Escolano, et al., 2015)
miR-320	Gentamicin	Rat	Urine	UP	(Nassirpour, et al., 2014)
miR-34c	Doxorubicin	Rat	Urine	UP	(Church, et al., 2014)

* iohexhol + furosemide + indomethacin were used for rats