The role of miR-486-5p in renal vascular endothelium in ischemic acute kidney injury

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Abstract

Acute kidney injury (AKI) refers to a rapid decline in kidney function and increases the risk for chronic kidney disease (CKD). Yet, no effective treatments exist. Ischemia-reperfusion (I/R) injury is a major cause of AKI in clinical settings such as cardiac surgery and kidney transplant. In I/R AKI, endothelial injury contributes to capillary rarefaction, tubulointerstitial fibrosis, and the transition to CKD. microRNAs are short non-coding RNAs that potently regulate gene expression. We previously showed that miR-486-5p protects against kidney I/R injury in mice. However, the effect of miR-486-5p on long-term vascular outcomes after kidney I/R injury were unexplored, and its targets in endothelial cells undefined. The aims of this project were to (1) evaluate the effect of miR-486-5p on kidney I/R injury in rats, focusing on long-term vascular function and the AKI to CKD transition, (2) determine the effect of miR-486-5p in cultured endothelial cells, and (3) conduct a systematic review of dysregulated miRNAs in human AKI.

We demonstrated that only early delivery of miR-486-5p to rats with kidney I/R prevents kidney injury and protects against CKD development and endothelial dysfunction. Unexpectedly, early and delayed delivery of miR-486-5p attenuated I/R-induced kidney endothelial nitric oxide synthase (eNOS) expression. In cultured endothelial cells, miR-486-5p decreases eNOS protein levels without targeting the eNOS 3' untranslated region. miR-486-5p also inhibits angiogenesis *in vitro* in an eNOS-dependent manner. Biotinylated miR-486-5p pull-down identified multiple predicted and novel transcripts, including MAML3 – a transcriptional coactivator of Notch signaling – as targeted by miR-486-5p. Selective MAML3 knockdown inhibits eNOS protein levels, suggesting that miR-486-5p inhibits angiogenesis via targeting of MAML3 and resultant eNOS downregulation. For the third aim, our systematic review of miRNAs in human AKI

identified potential AKI biomarkers and highlighted important clinical miRNA study methodological limitations.

This thesis advances our earlier work on the therapeutic potential of miR-486-5p in pre-clinical AKI. The prevention of capillary rarefaction and systemic vascular dysfunction only by early miR-486-5p administration suggest that early gene responses to I/R injury are critical for the transition from acute to chronic kidney disease. Although no adverse vascular effects were observed *in vivo,* miR-486-5p conferred an eNOS-dependent anti-angiogenic effect in cultured endothelial cells, possibly by targeting MAML3. These findings support a novel pathway regulating endothelial function.

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List of Publications (2021-2025)

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List of Abbreviations

ABMR	Antibody-mediated rejection
Ach	Acetylcholine
AGO	Argonaute
AKI	Acute kidney injury
AR	Acute rejection
BP	Blood pressure
BrdU	5-bromo-2'-deoxyuridine
BUN	Blood urea nitrogen
CCR2	C-C chemokine receptor 2
CKD	Chronic kidney disease
Cr	Creatinine
CS-AKI	Cardiac surgery-associated AKI
CX3CL1	C-X3-C motif ligand 1
CX3CR1	C-X3-C chemokine receptor 1
DCT	Distal convoluted tubule
DGF	Delayed graft function
ECFC	Endothelial colony forming cell
eNOS	Endothelial nitric oxide synthase
EMT	Endothelial-to-mesenchymal transition
FOXO1	Forkhead Box O1
GFR	Glomerular filtration rate
HITS-CLIP	High-throughput sequencing of RNAs by crosslinking immunoprecipitation

H/R	Hypoxia-reoxygenation
HPMEC	Human pulmonary microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin-like growth factor-1
IGFBP7	Insulin-like growth factor-binding protein 7
I/R	Ischemia-reperfusion
KIM-1	Kidney injury molecule-1
MAML3	Mastermind-like transcriptional coactivator 3
miRNA	Micro-RNA
Mmp-19	Matrix metalloproteinase-19
MPO	Myeloperoxidase
MRE	miRNA response element
mRNA	messenger RNA
MSC	Mesenchymal stem cell
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
PAR-CLIP	photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PCT	Proximal convoluted tubule
PI3K	phosphatidyl-inositol-3-kinase
PDGFRβ	platelet-derived growth factor receptor
PTEN	Phosphatase and tensin homolog
PST	Proximal straight tubule

RISC	RNA-induced silencing complex
Scb	scramble
siRNA	small interfering RNA
SMA	α-smooth muscle actin
SNP	Sodium nitroprusside
TCMR	T-cell mediated rejection
TGF-β	Transforming growth factor-β
TIMP-2	Tissue inhibitor of matrix metalloproteinase 2
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UTR	Untranslated region
VEGFA	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor 2

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Chapter 1: Introduction

1-1 Overview of microRNA biogenesis

microRNAs (miRNAs) are short non-coding RNAs of approximately 22-25 nucleotides in length that were originally discovered in *Caenorhabditis elegans* in 1993[1]. Since then, over 2,000 miRNA species have been identified in humans, although the functional significance of many is uncertain[2]. miRNAs are highly conserved across species and potent regulators of gene expression[3]. In this regard, it is estimated that over 60% of human protein-coding genes are miRNA targets[3].

Most miRNAs are produced via canonical miRNA biogenesis whereby RNA polymerase II generates the pri-miRNA transcript[4]. The pri-miRNA is cleaved in the nucleus by the microprocessor complex comprised of Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8) to produce the precursor-miRNA (pre-miRNA)[5]. The pre-miRNA is exported to the cytoplasm and processed by the RNase III endonuclease Dicer to generate the mature miRNA duplex via removal of the terminal loop[4]. The final mature miRNA form depends on directionality as either the 5p or 3p strand of the mature miRNA duplex can be the guide strand that is loaded onto an Argonaute (AGO) protein to form the miRNA-induced silencing complex (RISC)[4]. For any particular miRNA, the proportion of AGO-loaded 5' or 3' strands is influenced by the identities of the 5' nucleotides[6] and the thermodynamic stability at the 5' ends of the miRNA duplex[7]. However, tissue-specific differences in 5p/3p ratios support a role for other regulatory mechanisms determined by the cellular environment[8].

1-2 miRNA mechanisms of action: regulation of target gene expression

The miRNA-RISC complex primarily mediates post-transcriptional gene silencing by binding to the 3' untranslated region (UTR) of its target mRNAs, resulting in either inhibition of translation or mRNA de-adenylation and degradation[9]. However, miRNA functions and cellular localization are more complex[4]. For instance, miRNA binding sites for target gene repression have been detected in other regions of mRNAs including the 5' UTR and within coding sequences[4]. Although miRNAs are traditionally known to exert their effects in the cytoplasm, there is evidence that miRNA-RISC complex shuttles to the nucleus. Human AGO interacts with TNRC6A that functions as a nuclear-cytoplasmic shuttling protein to bring AGO into the nucleus[10]. In addition, the karyopherin importin 8 regulates the movement of RISC into the nucleus by binding the AGO-miRNA complex[11]. Studies have identified mature miRNAs within nuclei using RNA sequencing or microarray methods, and emerging nuclear miRNA functions including gene silencing at the transcriptional and post-transcriptional levels as well as activation of mRNA transcription[12]. Moreover, nuclear miRNAs have been associated with euchromatin at sites of active gene transcription, although the role of the miRNA-RISC complex in the regulation of chromatin structure and gene transcription is not clearly defined[13].

Canonical binding of a miRNA to its target mRNA 3' UTR involves the miRNA seed sequence comprising nucleotides 2-7 at the 5' end[14]. Genome analysis of 3' UTR sequences across different species suggests that over 30% of human protein coding genes demonstrate evolutionary conservation for miRNA targeting[14]. Yet, there is increased complexity behind miRNA-mRNA interactions, including in the 3' UTR where non-canonical interactions have been reported[15]. In this regard, other miRNA responsive elements (MREs) for pairing of a miRNA to its mRNA target include miRNA centered sites[16] and extended base pairing within the

3’ end of the miRNA, referred to as 3’ supplemental or compensatory binding[17]. Although the predominant miRNA-mRNA interactions involve binding of the miRNA seed sequence to its 3’ UTR target, individual miRNAs can exhibit distinct binding patterns with additional target sites within coding exons, introns, 5’ UTRs, and non-mRNA targets including circular RNAs, ribosomal RNA, and long non-coding RNAs[18]. Not surprisingly, an individual miRNA can have hundreds to thousands of targets[19].

1-3 miR-486-5p biogenesis and tissue distribution

miR-486-5p is a muscle-enriched miRNA that is conserved among mammals[20]. miR-486-5p was initially discovered as a skeletal muscle-enriched miRNA that is downregulated in muscle biopsies from patients with Duchenne muscular dystrophy[21]. miR-486-5p is highly expressed in skeletal and cardiac muscle, with moderate expression in lung, liver, bladder, and brain[22, 23], and is also highly expressed in erythroid cells with increasing levels throughout erythropoiesis[24].

miR-486-5p is transcribed from an intron within the *ANK1* locus on chromosome 8p11.21, which encodes both the Ankyrin 1 gene expressed primarily in erythroid cells and the muscle-specific isoform sAnk1. Human miR-486-1 and mouse miR-486a-5p are transcribed in the same direction as Ank1 to generate either miR-486-5p or -3p from opposite ends of the pre-miRNA hairpin. Unlike typical pre-miRNA duplex strand dissociation, miR-486-5p biogenesis in erythroid cells specifically exhibits Argonaute 2 (AGO2) – Slicer dependence to remove the star (3p) strand, generating mature miR-486-5p[25]. Further, catalytically inactive AGO2 promotes miR-486 duplex arrest and decreased miR-486-5p activity in erythroid cells[25]. Since the only other tissue where AGO2 is dominantly expressed is skeletal muscle[25], AGO2 enzymatic activity appears to be linked to endogenous miR-486-5p activity.

1-4 miR-486-5p targets and functions in pre-clinical disease models

1-4.1 Overview of most prominent miR-486-5p targets

miR-486-5p has been implicated in a wide variety of diseases. Based on 3' UTR sequence homology (https://miRDB.org), over 300 predicted miR-486-5p targets have been identified, although not all have been validated as direct targets, a process typically done via luciferase reporter assay. The most prominent miR-486-5p targets are discussed here. For an in-depth exploration of miR-486-5p targets in non-malignant diseases, we refer to our narrative review on this topic[20], included in Appendix A.

1-4.2 Skeletal muscle: myogenic differentiation and skeletal muscle catabolism

Phosphatase and tensin homolog (PTEN) and *Forkhead Box Protein 01* (FOXO1) are the earliest validated miR-486-5p targets and the most prominent in skeletal muscle disorders[22]. PTEN is a lipid phosphatase that negatively regulates the phosphatidyl-inositol-3-kinase (PI3K)/Akt (protein kinase B) pathway by dephosphorylation of the intracellular messenger phosphatidyl-inositol-triphosphate (PIP3) to PIP2, resulting in inhibition of cellular growth, proliferation, and survival[26]. The FOXO1 transcription factor is involved in insulin and insulin-like growth factor-1 (IGF-1) signaling. More specifically, FOXO1 is a negative regulator of Akt activation but is also inhibited by phosphorylated Akt[27].

miR-486-5p is a regulator of myogenesis as it is required for myoblast function and viability[28]. Transient upregulation of miR-486-5p after muscle injury has been associated with normal muscle regeneration[28], and miR-486-5p expression is downregulated in muscle biopsies from patients with Duchenne muscular dystrophy[21], thus implicating miR-486-5p in primary skeletal muscle disorders. In addition to downregulated skeletal muscle expression in patients

with Duchenne muscular dystrophy[21], miR-486-5p levels are reduced in dystrophin-deficient mice[23]. Muscle-specific miR-486-5p over-expression in dystrophin-deficient mice targeted 'dedicator of cytokinesis 3' (DOCK3) and PTEN in dystrophin-deficient skeletal muscle, resulting in increased Akt phosphorylation and improved muscle structure and function[23].

Further, miR-486-5p protects against muscle catabolism in pre-clinical models of chronic kidney disease (CKD) and glucocorticoid-induced muscle wasting through targets PTEN and FOXO1[29, 30]. In CKD, the uremic environment increases glucocorticoid production and suppresses Akt signaling, ultimately resulting in FOXO1 dephosphorylation and translocation to the nucleus, where it activates the transcription of E3 ubiquitin ligases that promote muscle proteolysis[31, 32]. In mice with CKD-induced muscle wasting, intra-muscular administration of miR-486-5p targets FOXO1 and PTEN, resulting in decreased E3 ubiquitin ligase levels and improved muscle mass[30]. In a mouse model of glucocorticoid-induced muscle wasting, exosomal miR-486-5p also suppressed the nuclear translocation of FOXO1, associated with downregulation of muscle atrophy markers[29]. These data suggest that miR-486-5p is a potential therapeutic target for disorders of muscle catabolism and that its protective effect is associated with targeting of PTEN and FOXO1.

1-4.3 Cardiac developmental and myocardial ischemia

In post-natal cardiac growth, miR-486-5p expression is driven by a member of the myocardin family of transcription factors (MRTF-A). In this regard, Small *et al* demonstrated an inverse relationship between miR-486-5p and PTEN protein levels during post-natal cardiac growth with miR-486-5p levels rising over 4 weeks after birth while PTEN protein levels decline[22]. Moreover, miR-486-5p overexpression in cardiomyocytes decreases PTEN and FOXO1 protein

levels and increases Akt activation, thereby implicating miR-486-5p as a regulator of post-natal cardiac growth associated with Akt signaling activation[22].

miR-486-5p has also been studied as a potential therapeutic intervention in animal models of cardiac ischemia-reperfusion (I/R) injury and myocardial infarction. In mice with cardiac I/R injury, ventricular miR-486-5p overexpression[33], exosomal miR-486-5p[34], or administration of either systemic or cardiomyocyte-specific adeno-associated virus-mediated miR-486-5p[35] reduced infarct size and inhibited cardiomyocyte apoptosis, associated with downregulated PTEN expression and Akt activation. Further, adeno-associated virus-expressing miR-486-5p preserved long-term cardiac function and prevented cardiac fibrosis with maintained suppression of PTEN and FOXO1 protein levels[35].

Moreover, in mice and non-human primates subjected to myocardial infarction via left anterior descending coronary artery ligation, intra-myocardial administration of exosomal miR-486-5p reduced infarct size, improved heart function and increased long-term cardiac vascular density[36]. RNA sequencing of mouse hearts revealed downregulation of miR-486-5p target mRNA for matrix metalloproteinase-19 (Mmp-19), which encodes a protein highly expressed in cardiac fibroblasts that modulates vascular endothelial growth factor A (VEGFA) secretion[36]. Thus, miR-486-5p confers a pro-angiogenic effect in experimental myocardial infarction, possibly via a paracrine mechanism involving fibroblast Mmp-19 and cleavage of extracellular VEGFA.

1-4.4 Ischemic stroke

The therapeutic potential of exosomal miR-486-5p was recently evaluated in a rat model of ischemic stroke via middle cerebral artery occlusion[37]. The authors demonstrated that exosomal miR-486-5p delivery after middle cerebral artery occlusion improved neurological function and

increased brain microvascular density in association with decreased PTEN expression and increased Akt phosphorylation[37]. These data suggest that miR-486-5p targeting of PTEN to activate Akt signaling contributes to the promotion of angiogenesis and neurological recovery after cerebral ischemic injury[37].

1-4.5 Pulmonary and cardiac fibrosis

Transforming growth factor-beta (TGF-β) signaling drives the nuclear translocation of Smad proteins, thereby activating the transcription of pro-fibrotic genes such as alpha-smooth muscle actin, collagens, and fibronectin, ultimately leading to organ fibrosis[38]. miR-486-5p targeting of the Smad family of transcription factors protects against organ fibrosis in pre-clinical models. In mouse fibroblasts, miR-486-5p targets and decreases Smad2 levels, thus inhibiting pro-fibrotic gene expression and proliferation[39]. Further, administration of miR-486-5p to mice exposed to silica or bleomycin attenuates pulmonary fibrosis[39]. In experimental models of heart failure, IgE signaling has been shown to promote pathological cardiac remodeling in association with upregulated Smad1 levels and TGFβ signaling[40]. Treatment of IgE-stimulated cardiac fibroblasts with miR-486-5p decreased Smad1 and collagen levels[41]. Further, lentivirus-mediated miR-486-5p delivery by tail vein injection in mice attenuated myocardial fibrosis[41]. Thus, miR-486-5p confers anti-fibrotic effects in select pre-clinical models of organ fibrosis via targeting of Smad family members, resulting in inhibition of TGF-β signaling and decreased expression of pro-fibrotic genes.

1-5 Acute kidney injury and the transition to chronic kidney disease

1-5.1: Overview of kidney structure, function, and microvasculature

The kidneys regulate fundamental processes including the elimination of toxins and waste as well as fluid, electrolyte, and acid-base homeostasis. The basic kidney anatomy from the outermost layers moving inward includes the kidney cortex, medulla, and papilla which protrudes into the renal pelvis (Figure 1A)[42]. The medulla is further sub-divided into the outer and inner medulla, and the outer medulla consists of the outer stripe (corticomedullary junction) and inner stripe. The basic kidney structure consists broadly of nephrons, collecting ducts, and the microvasculature. A human kidney contains approximately 1 million nephrons – although this number varies between individuals – and importantly, the number of nephrons is set at birth and lost nephrons cannot be replaced[42]. Structurally, a nephron consists of a glomerulus, the kidney's filtering unit, connected to a tubule that ultimately drains into a collecting duct. The nephron tubule is further subdivided into distinct functional components including: proximal convoluted tubule (PCT), proximal straight tubule (PST), the thin descending and ascending limbs and thick ascending limb of the loop of Henle, distal convoluted tubule (DCT), connecting tubule, cortical connecting duct, and outer medullary and inner medullary collecting ducts.

Glomeruli form the selectively permeable filtration barrier that allows for the passage of water and small solutes but excludes blood cells and macromolecules based on size and charge[42]. Plasma is filtered from the glomerulus to Bowman's space through the fenestrated capillary endothelium, the glomerular basement membrane, and the epithelial cell layer comprised of podocytes[42]. Filtrate enters the proximal tubule where most of the filtered water and solutes are reabsorbed. Proximal tubular cells have a prominent brush border and large mitochondria for active and transcellular secondary transport processes. Of note, the S3 segment of the proximal tubule is involved in drug secretion via organic anion transporters[42] and is also considered to be the most susceptible to ischemic injury[43 44], presumably because this segment has high energy

consumption required for transport function and faces an inability to sustain ATP levels in ischemic injury[45, 46]. Filtrate progresses through the tubular segments (loop of Henle, distal tubule), where luminal water and electrolyte composition is modified by reabsorption or secretion until the filtrate is delivered to the collecting ducts for the formation and elimination of urine.

The kidney has a complex microvasculature equipped to withstand extreme differences in osmolality and oxygenation between the outer cortex and inner medullary regions[47]. In humans, overall renal blood flow averages 4mL/gram tissue/min, although the outer cortex receives approximately 5mL/g/min with consecutively deeper regions receiving less flow per unit weight, down to 0.5mL/g/min at the inner medulla[47]. The renal artery delivering blood flow enters the renal pelvis where it branches into the interlobar arteries. The interlobar arteries extend through the renal column and give rise to the arcuate arteries that run along the corticomedullary junction without entering the medulla. The arcuate arteries form the cortical radial arteries (i.e. interlobular arteries) that ascend radially to deliver blood to the outer cortex. The interlobular arteries give rise to the glomerular afferent arterioles, which are also the major resistance vessels controlling the glomerular filtration rate (GFR). Afferent arterioles terminate to form the glomerular capillary network (where the primary filtrate is formed) which gives rise to the efferent arteriole. Blood leaves glomeruli via the efferent arterioles, and perfusion of cortical peritubular capillaries and the renal medulla is post-glomerular. Cortical efferent arterioles give rise to the oxygen-rich cortical peritubular capillaries that course along proximal and distal tubules. Efferent arterioles of juxtamedullary glomeruli give rise to the descending arteriolar vasa recta which form the capillary networks that supply the renal medulla. The venous ascending vasa recta drain the renal medulla to return to the cortex[42, 47] (Figure 1B).

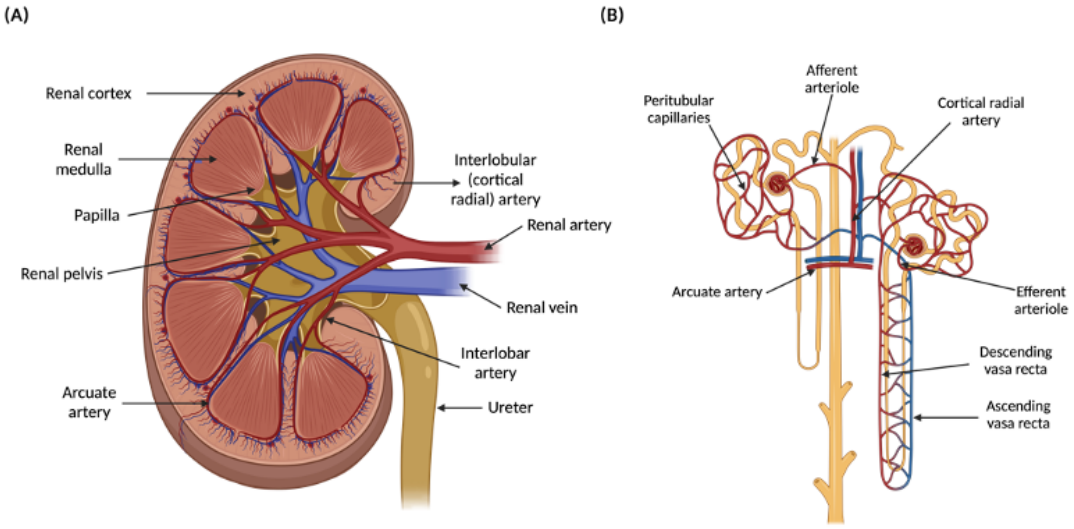

Figure 1. Schematic representation of (A) kidney structural organization and (B) the kidney microvasculature. Figure created with Biorender.

1-5.2 Acute kidney injury: Clinical definition, prevalence, and associated complications

Acute kidney injury (AKI) refers to a rapid decline in kidney function. The causes of AKI in clinical settings are diverse, including I/R injury (for example in the context of cardiovascular surgery, shock, or delayed graft function in kidney transplantation), sepsis, nephrotoxins, immune-mediated injury, or obstruction. Clinically, the diagnosis of AKI is made based on serum creatinine (SCr) concentration and/or urine output[48], although these measures have limitations including a lack sensitivity for early AKI diagnosis and inability to distinguish AKI etiology[49]. AKI affects up to 20% of hospitalized patients[50] with a higher prevalence in intensive care unit settings and confers a high in-hospital mortality rate that rises up to 50% for severe AKI[51]. Renal replacement therapy with dialysis is a supportive strategy for the management of refractory AKI-related complications related to electrolytes, acid-base status, uremia, pericarditis, or fluid management[48].

Patients who recover from AKI remain at risk of long-term adverse consequences. A meta-analysis of 82 studies comprising 2 million hospitalized adults followed for at least 1 year demonstrated that those who experienced an episode of AKI had an approximate 2-fold increased risk of death, 2-fold increased risk of new or progressive CKD, and 4-fold increased risk of kidney failure compared to patients without AKI[52]. Although pre-hospitalization variables including kidney function, rate of kidney function decline and proteinuria may have some confounding effect on cardiovascular complications, AKI remains an independent risk factor for long-term cardiovascular outcomes and mortality[53]. A retrospective cohort study of hospitalized pediatric patients with AKI not requiring dialysis demonstrated a higher risk of developing CKD and hypertension over a median 9.7 year follow up[54]. Consequently, considering the prevalence of AKI, its associated in-hospital complications and long-term outcomes, its incremental cost in Canada is substantial, estimated at $200 million annually[55]. Yet, despite the health and economic burden of AKI, no effective treatments exist and preventative measures are limited[48].

1-5.3 Pathophysiology of I/R AKI: tubular and endothelial cell injury

The pathophysiology of I/R AKI has been studied using rodent models whereby renal artery clamping for a set amount of time temporarily inhibits oxygen and nutrient delivery to the kidneys with subsequent reperfusion, initiating a cascade of pathological cellular responses[56]. Kidney I/R injury is characterized by acute tubular injury and necrosis, and renal vascular endothelial injury and dysfunction. Kidney ischemia leads to decreased renal blood flow and cellular ATP depletion, initiating renal tubular epithelial cell and microvascular injury. Histological findings of acute tubular injury include tubular cell necrosis and apoptosis, loss of the brush border in proximal tubular cells, tubular epithelial cell detachment from the basement membrane and sloughing into the tubular lumen with the formation of intra-tubular casts[43]. In

addition to tubular injury, kidneys with acute tubular necrosis develop interstitial edema due to microvascular permeability and back-leak of tubular filtrate from intra-tubular obstruction[43]. Along with histological changes, I/R injury results in decreased GFR, and thus decreased kidney function. In addition to decreased renal blood flow, the reduction in GFR during the initiation phase may be mediated in part via activation of tubuloglomerular feedback[43]. In this regard, acute tubular injury impairs the proximal tubule's ability to reabsorb sodium, resulting in increased sodium delivery to the macula densa, leading to afferent arteriolar vasoconstriction.

The extension phase of ischemic kidney injury – a result of prolonged decreased renal blood flow with hypoxia and inflammation – is most pronounced at the corticomedullary junction. In contrast, the return of kidney cortical blood flow during this phase promotes the recovery of kidney tubular epithelial cells of the outer cortex[43]. Early endothelial cell injury alters vascular reactivity, compromises capillary barrier function, and increases vascular permeability, all of which contribute to the extension of tubular injury and declining kidney function[57]. Earlier studies in rodents demonstrated impaired renal hemodynamics after kidney I/R injury. For instance, post-ischemic rat kidneys exhibited impaired renal blood flow auto-regulation extending up to 1 week after AKI recovery, despite restoration of total renal blood flow[58]. Moreover, altered vascular reactivity is linked to loss of endothelial nitric oxide synthase (eNOS) function[59], a key enzyme that produces nitric oxide (NO). eNOS is expressed at high levels in the medulla and is a known regulator of medullary blood flow[60]. In kidney I/R injury, impaired perfusion of peritubular capillaries occurs early upon reperfusion and can be ameliorated by restoring eNOS function[61]. Consequently, eNOS is also an important regulator of microvascular blood flow in ischemic AKI.

Increased vascular permeability is a consequence of the activation of injured endothelium, resulting in leukocyte adhesion and infiltration, thereby exacerbating kidney injury. Endothelial activation is characterized by increased endothelial cell surface expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), leukocyte adhesion and infiltration within hours of I/R injury[62, 63]. In addition, injured endothelial cells express chemokine (C-X3-C motif) ligand 1 (CX3CL1), a chemoattractant for chemokine receptors C-X3-C chemokine receptor 1 (CX3CR1) and C-C chemokine receptor 2 (CCR2) on the surface of monocytes and macrophages, thus promoting macrophage infiltration early in kidney I/R injury, with exacerbation of kidney injury from the production of pro-inflammatory cytokines[64].

1.5-4 Maladaptive tubular repair after I/R AKI leads to kidney fibrosis

Although tubular epithelial cells undergo apoptosis, migration and proliferation to repair and restore tubular integrity and kidney function, some populations of tubular epithelial cells exhibit abnormal repair with adverse long-term consequences[65]. Functionally, maladaptive repair has been linked to abnormal cell cycle kinetics, and pro-fibrotic and pro-inflammatory signaling between tubular epithelial cells and other cell types. Canaud *et al* reported that a sub-population of injured proximal tubular cells undergo cell cycle arrest and assume a senescent pro-fibrotic phenotype[66]. Further, Kirita *et al* performed single nucleus sequencing of mouse kidneys up to 6 weeks after bilateral I/R injury and identified sub-clusters of proximal tubular cells that persisted and exhibited down-regulated proximal tubular cell terminal differentiation markers along with up-regulated genes involved in pro-inflammatory and pro-fibrotic signaling[67]. More specifically, ligand-receptor interactions between these “failed repair proximal tubular cells” and other cell types revealed vasoconstrictor and pro-fibrotic signaling to endothelium, pro-fibrotic signaling to

fibroblasts, and pro-inflammatory and pro-fibrotic cytokines to leukocytes[67]. Moreover, in a mouse model of unilateral kidney I/R (with intact contralateral kidney), Xu *et al* demonstrated a role for immune-mediated tubular atrophy driven by macrophage persistence in the injured kidney, which promoted a T-cell and neutrophil-mediated pro-inflammatory environment[68]. In kidney biopsies from patients with acute tubular necrosis, the authors showed that T cell and neutrophil numbers negatively correlated with recovery of kidney function[68]. Thus, maladaptive repair of proximal tubular cells after injury is associated with persistent inflammation and pro-fibrotic signaling, leading to interstitial fibrosis and tubular atrophy, thus contributing to the transition from AKI to CKD. However, the underlying molecular mechanisms remain incompletely understood.

1-5.5 Impaired vascular recovery after AKI contributes to the development of CKD

Unlike the injured tubular epithelium, the renal microvasculature displays reduced regenerative capacity after ischemic AKI, which might predispose to CKD development due to persistent intra-renal hypoxia and impaired medullary function[57]. In rats with bilateral kidney I/R injury, Basile *et al* demonstrated that although kidney function normalized by 1 week after I/R with normal histological tubular morphology at 8 weeks, post-ischemic kidneys had a permanent 30-50% reduction in peritubular capillary density localized to the inner stripe of the outer medulla by 4 weeks and tubulointerstitial fibrosis by 40 weeks[69]. Functionally, the rats also developed urinary concentrating defects suggesting impaired medullary function[69]. In addition, rats post-AKI exhibited a blunted pressure-natriuresis response along with impaired medullary blood flow when subjected to elevations in renal perfusion pressure, and developed hypertension in response to increased dietary salt intake[70]. While ischemic AKI had no impact on baseline

blood pressure (BP) long-term, post-AKI rats fed a high salt diet following renal recovery developed sodium-dependent hypertension, albuminuria, and tubulointerstitial fibrosis[71]. These pre-clinical studies suggest that permanent reduction in microvascular capillary density impairs medullary function, thus contributing to sodium-sensitive hypertension and interstitial fibrosis after AKI.

Although many studies have demonstrated a direct relationship between peritubular capillary loss and kidney fibrosis in kidney I/R injury models[72], kidney fibrosis is not necessarily progressive[65] and the relationship between these entities is likely more complex. A comparison of different pre-clinical AKI models in mice including cisplatin, glycerol-induced rhabdomyolysis, and unilateral kidney I/R injury with delayed contralateral nephrectomy found that the level of peritubular capillary rarefaction was more closely associated with CKD progression than the level of kidney fibrosis[73]. This suggests a possible direct causal relationship between peritubular capillary rarefaction (and not fibrosis *per se*) and decline in long-term kidney function.

Since reversible peritubular capillary loss has also been observed in kidney I/R injury[73], it is not clear if the consequences of early endothelial injury confer permanent microvascular loss. Potential mechanisms contributing to permanent peritubular capillary rarefaction include endothelial cell apoptosis, endothelial-to-mesenchymal transition (EMT), and pericyte activation and loss[43]. The contribution of endothelial cell apoptosis to capillary rarefaction following I/R AKI has been inconsistent in the pre-clinical literature, although the kidney transplant literature suggests that endothelial cell apoptosis as a cause of microvascular loss impacts long-term allograft survival[74]. Burger *et al* demonstrated kidney endothelial cell apoptosis after kidney I/R injury in mice, which was attenuated by systemic administration of human cord blood-derived

endothelial colony-forming cell (ECFC)-derived exosomes[75]. Yet, Horbelt *et al* could not identify apoptotic endothelial cells after kidney I/R injury using Tie-GFP mice to label kidney endothelial cells[76]. Thus, endothelial cell apoptosis after kidney I/R injury has been difficult to capture, possibly because of the transient nature of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive structures in immunohistochemical analyses or perhaps due to other pathways driving endothelial cell fate. In this regard, Basile *et al* employed a transgenic mouse model to trace renal endothelial cell fate following I/R AKI[77]. The authors demonstrated interstitial expansion of endothelial cells in areas lacking blood flow that co-localized with myofibroblast markers, suggesting that reduced vascular density post-I/R AKI is mediated, in part, from an endothelial phenotypic change termed EMT[77].

Lastly, the loss of interactions between endothelial cells and neighbouring cells may contribute to the permanent reduction in microvascular density. Pericytes are smooth muscle cells directly connected to endothelial cells that support vascular stability[78]. Renal pericytes are most concentrated within the outer medulla where they detect and respond to vasoactive signals from endothelial cells by regulating medullary blood flow via the vasa recta[79]. In kidney I/R injury, pericytes become activated, detach from the peritubular capillaries and migrate to the interstitium where they differentiate into myofibroblasts[78]. Inhibition of platelet-derived growth factor receptor (PDGFR)α/β activation after AKI decreases pericyte activation, downregulates PDGF and TGFβ expression, and decreases macrophage infiltration and fibrosis[80]. Consequently, pericyte activation and detachment can de-stabilize the endothelium, increase vascular permeability, and contribute to capillary rarefaction and fibrosis[78].

1-6 miRNAs for acute kidney injury

miRNAs can be detected in biological fluids including plasma/serum, and urine[4]. Indeed, circulating miRNAs are highly stable and resistant to nuclease digestion[81]. Circulating miRNAs exist in non-membrane bound form associated with the AGO2-ribonucleoprotein complex – the predominant form identified in human plasma – and within extracellular vesicles[82, 83]. Given their stability in biological fluids, miRNAs have been evaluated as potential diagnostic biomarkers for a variety of human diseases including but not limited to cancers[84], cardiovascular diseases[85], neurodegenerative diseases[86] and CKD[87]. Dysregulated miRNAs have also been studied as potential biomarkers in specific populations with AKI. For example, urinary and plasma miR-21 has been reported dysregulated in cardiac surgery-associated AKI[88, 89]. In kidney transplantation, there has been interest in both circulating miRNAs and miRNA profiles from graft biopsies as diagnostic signatures for acute rejection. For instance, miR-210 was downregulated in urine samples from patients with acute T-cell mediated rejection (TCMR) and its expression correlated with severity of TCMR and immunosuppression therapy[90], thus suggesting clinical relevance.

miRNAs regulate important biological processes related to AKI pathophysiology and the development and progression of CKD, a topic extensively reviewed by Mahtal *et al*[91]. The earliest evidence supporting an important role for miRNAs in kidney function derived from animal models of CKD. More specifically, inhibition of miRNA biogenesis in mouse podocytes via conditional Dicer knockout resulted in loss of podocyte miR-30a expression, with functional consequences including glomerular injury and progression to kidney failure[92]. Mouse models of diabetic nephropathy demonstrated that miR-192 is upregulated in glomeruli of diabetic mice, associated with increased TGFβ and Col1A2 levels[93], and that miR-377 overexpression was

associated with increased fibronectin production[94], thus suggesting a pathogenic role for these miRNAs in kidney fibrosis.

In AKI, proximal tubular and endothelial cell injury involve pathways regulating apoptosis, inflammation, metabolism, angiogenesis, and fibrosis[43]. Wei *et al* demonstrated that mice with targeted deletion of Dicer from proximal tubular cells exhibited global downregulation of microRNAs within the kidney cortex and were more resistant to kidney I/R injury, suggesting a pathogenic role for microRNAs in ischemic AKI for the first time[95]. However, there is increasing evidence for the protective effect of miRNAs in pre-clinical AKI models. A systematic review and meta-analysis of miRNAs as therapeutics for pre-clinical AKI included 70 studies with 42 miRNAs as potential therapeutic targets[96]. Of these, miR-21 (14 studies), miR-146 (4 studies), and miR-155 (3 studies) were the most frequently investigated miRNAs. Most interventional miR-21 studies involved the administration of miR-21 antagomirs. Overall, miR-21 antagonism in kidney I/R injury was associated with increased apoptosis, inflammation, and exacerbation of kidney injury[96].

1-6.1 miR-486-5p in ischemic acute kidney injury and the renal vasculature

Our laboratory's work in kidney I/R injury began with endothelial colony forming cells (ECFCs) and ECFC-derived small extracellular vesicles (exosomes)[75] which ultimately identified miR-486-5p as a potential therapy for ischemic AKI[97]. miR-486-5p is highly enriched within extracellular vesicles including human adipose and bone marrow mesenchymal stem cell-derived exosomes[98]. Our lab previously showed that intravenous (i.v.) administration of human cord blood ECFC-derived exosomes to mice with kidney I/R injury protected kidney function and attenuated histological injury and apoptosis[97]. MicroRNA profiling demonstrated that these

exosomes were most highly enriched in miR-486-5p[97]. To determine whether the kidney-protective effect of exosomes could be attributed to miR-486-5p, ECFCs were transfected with miR-486-5p antagomir, resulting in the production and isolation of miR-486-5p-deficient exosomes. Administration of miR-486-5p-deficient exosomes to mice with kidney I/R-injury had no kidney-protective effects[97]. These data strongly suggested that ECFC-derived exosomes protected against ischemic kidney injury via exosomal transfer of miR-486-5p. A subsequent study from our laboratory demonstrated that direct i.v. administration of lipid-packaged miR-486-5p to mice with kidney I/R injury at the time of reperfusion also conferred the same renal protective effect[99]. Further, at 24 hr after kidney I/R, miR-486-5p decreased protein levels of its target PTEN, and downregulated the expression of genes involved in apoptosis and tumor necrosis factor-α (TNF-α) signaling in proximal tubular cells[99].

Although miR-486-5p targets PTEN and decreases apoptosis, associated with the prevention of kidney[97, 99], cardiac[33-35], and cerebral[37] ischemic injury in pre-clinical models, its effect on endothelial injury, the vasculature, and the AKI to CKD transition remains unknown. In human umbilical vein endothelial cells (HUVECs) subjected to hypoxia-reoxygenation (H/R), ECFC-derived exosomes decreased PTEN expression and apoptosis, while exosomes from ECFCs transfected with miR-486-5p antagomir did not[97]. These data suggest that exosomal transfer of miR-486-5p could plausibly target PTEN and prevent endothelial cell apoptosis in kidney ischemic injury. In this regard, our laboratory infused ECFC-derived exosomes to mice with kidney I/R injury and showed decreased renal endothelial cell apoptosis and attenuated endothelial cell proliferation induced by I/R[75]. Yet, RNA sequencing of kidney $CD31^+$ endothelial cells isolated from mice with kidney I/R injury and treated with miR-486-5p did not identify differentially expressed genes related to apoptosis. RNA sequencing did reveal

decreased expression of pro-inflammatory genes with miR-486-5p treatment – including members of the chemokine ligand (CXCL) subfamily and ICAM-1[99].

Although these data suggest that miR-486-5p might protect against renal microvascular injury via decreasing endothelial cell apoptosis, endothelial activation and inflammation, the pre-clinical literature contains conflicting evidence regarding the role of miR-486-5p on the vasculature. Exosomal transfer of miR-486-5p promoted angiogenesis in animal models of cutaneous wound healing[100], myocardial infarction[36], and ischemic stroke[37] associated with targeting Sp5, Mmp-19, and PTEN, respectively. In contrast, administration of miR-486-5p mimic to HUVECs targeted CADM1, resulting in increased cell permeability and invasion without impacting angiogenesis[101]. Yet, when administered to HUVEC spheroids, miR-486-5p mimic inhibited sprouting angiogenesis[102]. Thus, effects of miR-486-5p on the vasculature remain unclear and could have long-term implications on microvascular recovery following AKI.

Moreover, there is controversy surrounding the biological effect of potential PTEN suppression by miR-486-5p. In the context of the AKI-CKD transition, adverse effects of PTEN inhibition have been reported[103]. In this regard, pharmacological inhibition of PTEN worsened ischemic[104] and cisplatin-induced AKI[105] in mice. Further, kidneys from mice treated with PTEN inhibitor and subjected to kidney I/R injury had increased levels of phosphorylated Akt (S473) and demonstrated increased interstitial fibroblast proliferation, inflammatory cytokine expression, and tubulointerstitial fibrosis[106]. In addition, after AKI, regenerating proximal tubular cells with persistent PTEN loss failed to differentiate and displayed a pro-fibrotic phenotype[107], suggesting that suppressed PTEN levels are associated with maladaptive tubular epithelial cell repair and contribute to kidney fibrosis[107] and the transition to CKD.

1-7 Rationale and hypothesis

AKI is a common complication among hospitalized patients with a high in-hospital mortality rate. Patients who recover from AKI are at increased risk of adverse long-term consequences including death, progressive CKD, and kidney failure. Although maladaptive tubular epithelial cell repair and permanent renal microvascular loss following AKI contribute to the development of tubulointerstitial fibrosis, the molecular mechanisms driving these processes and the AKI-CKD transition are not well-defined. Consequently, no effective therapies exist, and preventative measures for AKI are limited. miRNAs are potent regulators of gene expression that regulate signaling pathways involved in fundamental biological processes relevant to AKI pathophysiology. Despite over a decade of research involving miRNA therapeutics for pre-clinical AKI, less is known about miRNAs in human AKI. While miR-486-5p attenuates ischemic AKI in mice, its effect on the vasculature and the AKI to CKD transition is unclear and its targets in endothelium remain undefined.

The three major aims of this thesis were (1) to evaluate the effect of miR-486-5p in ischemic AKI in rat with a focus on the vasculature and the AKI to CKD transition; (2) to determine the effect of miR-486-5p in endothelium; (3) to conduct a systematic review of miRNAs in human AKI to identify potential miRNAs that hold promise as clinical bionmarkers or therapeutic candidates. In the first aim, the rat model was selected specifically for ease of conducting vascular studies but also to determine if miR-486-5p effectively protects against AKI in a rodent model other than mouse. Our overall hypothesis was that (1) early administration of miR-486-5p to rats with kidney I/R injury would protect against ischemic AKI and prevent renal microvascular injury and (2) delayed miR-486-5p administration

might confer adverse long-term renal and vascular outcomes after AKI, based on possible inhibitory effects on PTEN and conflicting data on angiogenic responses.

The main objectives of Manuscript 1 were to: (1) determine if early administration of miR-486-5p in kidney I/R protects against ischemic AKI in rats, (2) determine whether early administration of miR-486-5p impacts the development of peritubular capillary rarefaction, kidney fibrosis, and global vascular function, and (3) determine whether the timing of miR-486-5p administration impacts long-term kidney and vascular outcomes. These objectives were accomplished using a rat model of kidney I/R injury with administration of miR-486-5p mimic (or scramble [scb] miRNA negative control) at the start of reperfusion (early) or after 4 and 21 days of reperfusion (delayed), with assessment of kidney and vascular outcomes after 10 weeks.

The main objectives of Manuscript 2 were to: (1) determine the effect of miR-486-5p on endothelial cell functions *in vitro*, and (2) identify the mRNA targets for miR-486-5p in cultured endothelial cells. These objectives were accomplished using HUVECs treated with miR-486-5p mimic (or scb miRNA) and subjected to normoxia or H/R, followed by assessments of gene expression and cellular functions. miR-486-5p targets were identified using a biotinylated miRNA pulldown approach.

The main objective of Manuscript 3 was to conduct a systematic review of dysregulated miRNAs in human AKI to identify potential clinical miRNA biomarker and therapeutic candidates. This objective was accomplished using a systematic search strategy, and data were reported descriptively as a qualitative synthesis.

Preface to Manuscripts

All manuscripts included in this thesis were written during my PhD studies. The references for each manuscript are included at the end of each chapter, while references for the Introduction and Discussion chapters are found in the Reference section at the end of this thesis. The supplemental information for main manuscripts is included in the thesis Appendix section, and I provide links to the online information for published manuscripts. In addition, two manuscripts related to this thesis are included in the Appendix section. Thesis chapters are prefaced with a brief summary (including author contributions) connecting the manuscript to the thesis.

List of manuscripts in the main thesis

Manuscript I is published in *Clinical Science* (2024): I am first author

Manuscript II is accepted for publication at the *Journal of Cellular and Molecular Medicine* (2025): I am first author

Manuscript III is published in *Renal Failure* (2024): I am first author

List of manuscripts in the Appendix

Manuscript IV (narrative review) is published in *Cellular and Molecular Life Sciences* (2022): I am first author

Manuscript V (systematic review protocol) is published in the *Canadian Journal of Kidney Health and Disease* (2021): I am first author

Chapter 2: Manuscript I

Connection to thesis: In this manuscript, we show that miR-486-5p effectively mitigates ischemic kidney injury in rats and provide evidence that it prevents I/R-induced renal endothelial injury. The manuscript demonstrates that early administration of miR-486-5p prevents long-term peritubular capillary rarefaction, kidney fibrosis, and systemic endothelial dysfunction. We also evaluated the effect of delayed miR-486-5p administration, revealing no protective or additional adverse impact on long-term kidney or systemic vascular outcomes. Lastly, this study showed that miR-486-5p inhibits I/R-induced upregulation of kidney eNOS protein expression, a finding that is further explored in Manuscript II.

Manuscript status: published in *Clinical Science* (PMID:38739452)

Author contributions: The study was conceived and designed by Kevin D. Burns, Adrianna Douvris, and José L. Viñas. Adrianna Douvris and José L. Viñas conducted experiments and performed data analyses. Joseph Zimpelmann assisted with animal surgeries, tissue extraction, and myography. Alexey Gutsol conducted histopathologic analyses. Dylan Burger provided assistance with vascular function studies. Adrianna Douvris wrote first drafts of the manuscript, which were edited by Kevin D. Burns. All authors reviewed and approved the final manuscript.

miR-486-5p protects against rat ischemic kidney injury and prevents the transition to chronic kidney disease and vascular dysfunction

Running title: miR-486-5p prevents AKI-CKD transition in rat

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Abstract

Aim

Acute kidney injury (AKI) increases the risk for progressive chronic kidney disease (CKD). MicroRNA (miR)-486-5p protects against kidney ischemia reperfusion (IR) injury in mice, although its longterm effects on the vasculature and development of CKD are unknown. We studied whether miR-486-5p would prevent the AKI to CKD transition in rat, and affect vascular function.

Methods

Adult male rats were subjected to bilateral kidney IR followed by i.v. injection of liposomal-packaged miR-486-5p (0.5 mg/kg). Kidney function and histologic injury were assessed after 24 h and 10 weeks. Kidney endothelial protein levels were measured by immunoblot and immunofluorescence, and mesenteric artery reactivity was determined by wire myography.

Results

In rats with IR, miR-486-5p blocked kidney endothelial cell increases in intercellular adhesion molecule-1 (ICAM-1), reduced neutrophil infiltration and histologic injury, and normalized plasma creatinine ($p<0.001$). However, miR-486-5p attenuated IR-induced kidney endothelial nitric oxide synthase (eNOS) expression ($p<0.05$). At 10 weeks, kidneys from rats with IR alone had decreased peritubular capillary density and increased interstitial collagen deposition ($p<0.0001$), and mesenteric arteries showed impaired endothelium-dependent vasorelaxation ($p<0.001$). These changes were inhibited by miR-486-5p. Delayed miR-486-5p administration (96 h, 3 weeks after IR) had no impact on kidney fibrosis, capillary density, or endothelial function.

Conclusion

In rats, administration of miR-486-5p early after kidney IR prevents injury, and protects against CKD development and systemic endothelial dysfunction. These protective effects are associated with inhibition of endothelial ICAM-1 and occur despite reduction in eNOS. miR-486-5p holds promise for the prevention of ischemic AKI and its complications.

Keywords:
acute kidney injury, ischemia, microRNA, endothelium, vasculature, myography

Clinical Perspectives

- Ischemia-reperfusion (IR) acute kidney injury is associated with permanent reduction of peritubular capillary density, tubulointerstitial fibrosis, and transition to chronic kidney disease (CKD). miR-486-5p protects against kidney IR injury in mouse, but its long-term effect on the vasculature and CKD development is unknown.
- In rats with kidney ischemia, miR-486-5p administered at the start of reperfusion inhibited ischemia-induced endothelial intercellular adhesion molecule-1 and preserved kidney function, despite inhibiting upregulation of kidney endothelial nitric oxide synthase. After 10 weeks, rats with early administration of miR-486-5p showed no evidence of CKD, or systemic endothelial dysfunction.
- This study highlights the potent protective effects of early intervention with miR-486-5p on rat kidney ischemic injury and its longterm complications. The prevention of late capillary loss and vascular dysfunction by miR-486-5p support the critical importance of early gene responses in programing the transition from acute to chronic kidney disease.

Introduction

Acute kidney injury (AKI) affects up to 20% of hospitalized patients and confers a high mortality risk (1). Patients who recover are at increased risk of progressive chronic kidney disease (CKD) (2). An important cause of AKI is ischemia-reperfusion (IR) injury, characterized by acute tubular injury, necrosis/apoptosis, and endothelial cell injury (3). Recovery involves repair and regeneration of tubular epithelial cells, although this process can be incomplete, resulting in interstitial fibrosis and tubular atrophy (4). Further, in kidney IR injury, endothelial damage alters vascular reactivity (5, 6) and increases the surface expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (7), thereby compromising capillary barrier function (8), and driving inflammatory cell infiltration (7, 9, 10). Endothelial injury results in capillary loss, exacerbating chronic hypoxia and propagating tubular injury. These changes contribute to the transition to CKD (11-13), marked by tubulointerstitial fibrosis.

Treatment options for AKI remain limited, and microRNAs (miRNAs) have emerged as potential novel therapeutics. miRNAs are short non-coding RNAs that potently regulate gene expression by binding the 3‘untranslated region (UTR) of target messenger RNAs (mRNAs) to induce degradation or inhibit translation (14). A systematic review identified 42 miRNA species as therapeutic targets in rodent models of AKI (15). We have shown that one of these miRNAs, miR-486-5p, protects against kidney IR injury in mice, associated with inhibition of apoptosis and decreased kidney expression of the miR-486-5p target gene *phosphatase and tensin homolog* (PTEN) (16, 17). miR-486-5p also significantly downregulated proximal tubular activation of genes involved in apoptosis and tumor necrosis factor (TNF)-α signaling (17).

Despite these findings, the effect of miR-486-5p on the transition to CKD remains unknown. In this regard, persistent PTEN loss in proximal tubules following IR injury may cause

dysfunctional tubule regeneration and fibrosis (18), conferring a negative impact on recovery from AKI. Long-term effects of miR-486-5p on the vasculature are also unclear. In a pre-clinical study of myocardial infarction, exosomal transfer of miR-486-5p increased vascular endothelial growth factor (VEGFA) signaling and enhanced cardiac angiogenesis (19). Exosomal miR-486-5p promoted microvascular cell proliferation, migration and angiogenesis after cutaneous wound healing, by targeting the transcriptional repressor *Sp5* (20). In contrast, in cultured endothelial cells miR-486-5p was found to inhibit sprouting angiogenesis (21).

The uncertainty regarding potential adverse effects of miR-486-5p on long-term kidney and vascular function prompted the current studies, in which we first determined if miR-486-5p administered at the start of reperfusion would protect against ischemic AKI in rat, and then studied the impact on late development of peritubular capillary rarefaction and kidney fibrosis. We also tested if delayed administration of miR-486-5p after IR would impact peritubular capillary density, development of kidney fibrosis, and systemic endothelium-dependent vascular function.

Materials and Methods

Kidney IR Injury

Male Sprague Dawley rats aged 6-8 weeks, weighing 250-300 g, were purchased from Charles River Laboratories (Senneville, QC, Canada) and housed at the University of Ottawa Animal Care Facility. All animal experiments were conducted at the University of Ottawa. Animal protocols were approved by the Animal Care Ethics Committee at the University of Ottawa and performed according to the recommendations of the Canadian Council for Animal Care. All rats were anesthetized with inhaled isoflurane. Briefly, the vaporizer was set to 4% then 2% isoflurane at 1.5 litres per minute for induction and maintenance of anesthesia, respectively.

There was no significant difference in the duration of anesthesia amongst all groups. Bilateral flank incisions were performed, followed by bilateral renal artery clamping for 45 min. Lipid-encapsulated miR-486-5p mimic (Invivofectamine 3.0, Thermo Fisher Scientific, Waltham, MA, USA) or scramble (scb) miRNA (Thermo Fisher Scientific) was administered as a single dose of 0.5 mg/kg by tail vein injection at the start of reperfusion (17), or as delayed administration of two doses 96 h and 3 weeks after reperfusion. Sham rats underwent surgery without renal artery clamping. The number of rats ranged from 6 to 14 per experimental group. 24 h and 10 weeks after reperfusion, rats were anesthetized with inhaled isoflurane for kidney blood flow measurements followed by euthanasia by decapitation (while anesthetized with isoflurane) for blood and tissue collection. Figure 1 provides a timeline schematic of the experimental protocol including number of rats used and mortality rate (3/114 rats, 2.6%).

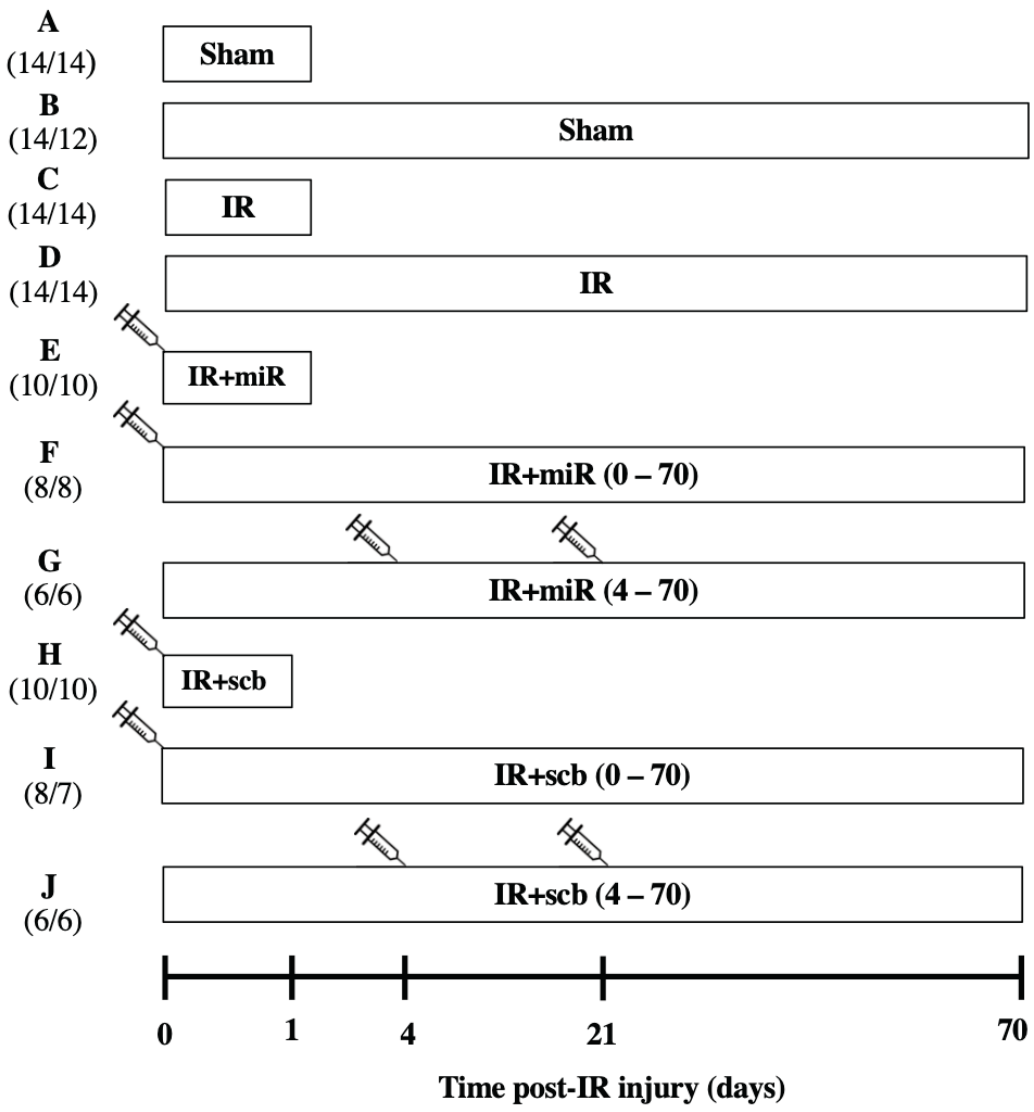

Figure 1. Experimental timeline illustrating study groups

Rats were subjected to sham surgery or bilateral kidney ischemia-reperfusion (IR) for 45 min (time 0). End-points were set at 24 h (1 day) or 10 weeks (70 days) after reperfusion. Groups E, F, H and I received a single dose of miR-486-5p (0.5 mg/kg, IR+miR-486-5p) or scramble miRNA (0.5 mg/kg, IR+scb) by tail vein injection at the start of reperfusion. Groups G and J received two doses of miR-486-5p or scramble miRNA: the first dose on day 4 and second dose on day 21 of reperfusion. To the left of the figure, the number of animals in each group is shown in parentheses. The first number refers to the starting number of rats in each group while the second number indicates the rats that completed the study. Not shown in this timeline: a separate group of 10 rats received delayed miR-486-5p injection at 4 days after IR and were sacrificed at 5 days post-IR (group numbers included n=3 (sham), n=4 (IR), n=3 (IR+miR-486-5p)).

Regional kidney blood flow

At 24 h or 10 weeks after surgery, rats were anesthetized with inhaled isoflurane and placed in the right lateral decubitus position. A left flank incision exposed the left kidney, which was decapsulated, and regional blood flow was measured by laser doppler flowmetry (ABLPHN20 20G probe, Transonic Scisense Inc, London, ON, Canada) with the laser doppler probe mounted onto a micromanipulator attached to a stereotaxic frame. Baseline cortical blood flow was measured by resting the probe on the kidney surface and baseline medullary blood flow was measured by inserting the probe to a depth of 3.0-4.5 mm (measured with the frame) into the kidney parenchyma (22). Probe placement within the medulla was verified at the end by dissection. Results are reported as absolute values of tissue perfusion (arbitrary units).

Biochemistry

Blood samples were collected in heparinized tubes for plasma separation. Plasma creatinine (Cr) and blood urea nitrogen (BUN) were measured by IDEXX Laboratories (Toronto, ON, Canada) (17). Urine albumin and creatinine were measured by rat albumin ELISA (Nephrat II) and Creatinine Companion assay (both from Ethos Biosciences, Logan Township, NJ, USA).

Real-time PCR

Total RNA was isolated from whole kidney, liver, heart, and spleen using the miRNeasy micro kit (Qiagen Inc., Toronto, ON, Canada). Reverse transcription and real-time PCR for miR-486-5p was performed via TaqMan^{TM} MicroRNA Assay (Life Technologies Inc, Toronto, ON, Canada) with the Applied Biosystems 7300 real-time PCR system (Foster City, CA, USA). Endogenous

U6 snRNA was used for normalization, and the relative levels of miR-486-5p were calculated using the $2^{-\Delta\Delta Ct}$ method (23).

Histology

Kidneys were fixed in 3% paraformaldehyde and embedded in paraffin. All histological analyses were performed in a blinded manner by a pathologist (co-author A.G.). Tubular injury was detected by periodic acid Schiff stain, and semi-quantified using a scoring system (0-4) as described (24). Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (ApopTag Plus Kit, MilliporeSigma Canada Ltd, Etobicoke, ON, Canada), as described (17), with counting of stained nuclei. Collagen was detected by picrosirius red staining as the percentage of collagen relative to the total area.

ICAM-1 (1:100, mouse monoclonal, Abcam Inc, Toronto, ON, Canada), endothelial nitric oxide synthase (eNOS, 1:100, rabbit monoclonal, Abcam Inc), CD31 (1:250 goat anti-CD31, R&D Systems Inc., Burlington, ON, Canada), and α-smooth muscle actin (1:500 mouse α-SMA, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were detected by immunofluorescence on frozen cryostat sections (OCT embedded, 10 μm thickness). For quantification of eNOS signal, only arterial segments ≥ 200 μm in length were counted to eliminate the effect of varying angles on fluorescence intensity (25).

Levels of α-smooth muscle actin (1:1000 mouse α-SMA, Santa Cruz Biotechnology, Inc.), neutrophil myeloperoxidase (1:1000 rabbit anti-MPO, Abcam Inc), macrophage-specific glycoprotein F4/80 (1:250 rabbit anti-F4/80, Abcam Inc), vascular endothelial growth factor receptor-2 (1:1000 VEGFR2, rabbit, Cell Signaling, Whitby, ON, Canada), and CD31 (1:500 goat anti-CD31, R&D Systems Inc.) were measured in kidney corticomedullary sections by

immunohistochemistry. Interstitial peritubular capillary density was estimated with CD31 staining as the percent positive area per field of view. All primary antibodies were visualized with ImmPRESS Polymer Detection Kits (Vector Laboratories, Inc., Newark, CA, USA). All images were acquired with the Zeiss Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) equipped with the Olympus camera DP73 (Olympus Canada, Richmond Hill, ON, Canada). Images were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

In-situ hybridization (ISH)

miR-486-5p, random sequence (negative control) miRNA, and positive control probes were prepared by Advanced Cell Diagnostics (Newark, CA, USA). ISH was performed on paraffin-embedded kidney sections using the miRNAscope^{TM} HD (RED) Assay according to the manufacturer's protocol (Advanced Cell Diagnostics).

Immunoblots

Lysates from whole kidney or human umbilical vein endothelial cells (HUVECs) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked in 5% milk for 1 h, and incubated for 16 h at 4˚C with primary antibodies against PTEN, eNOS, VEGFR2 (all 1:1000, Cell Signaling), VEGFA (1:1000, Santa Cruz Biotechnology, Inc.), and ICAM-1 (1:1000, R&D Systems (rat), 1:1000, Cell Signaling; human). Loading controls β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (both 1:4000, Cell Signaling) were incubated for 1 h at room temperature. Washed membranes were incubated with horseradish peroxidase-conjugated secondary antibodies anti-rabbit (1:5000, Abcam) or anti-goat (1:5000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, and visualized by

chemiluminescence. Densitometry was performed using ImageJ software (NIH) Bethesda, MD, USA.

Cell culture and hypoxia/reoxygenation (H/R)

HUVECs were obtained from American Type Culture Collection (ATCC, via Cedarlane Corp., Burlington, ON, Canada) and cultured at 37ºC in 5% CO_2 in EBM2 medium supplemented with microvascular growth factors and 2% fetal bovine serum (Lonza, Basel, Switzerland, catalog # CC-3156). Cells were transfected with 1 nM miR-486-5p mimic or scramble miRNA in RNA-iMax (all from Thermo Fisher Scientific) and subjected to H/R as described (24).

Blood pressure

Systolic blood pressure (SBP) was measured via tail-cuff plethysmography (CODA®, Kent Scientific Corp, Torrington, CT, USA). 8 weeks after surgery, average SBP was calculated from sessions comprised of 15 cycles each to achieve at least 10 accepted readings. Rats underwent 3 to 4 days of training prior to measurements.

Wire myography

Rat mesenteries were dissected and placed in ice-cold physiological saline solution (PSS). Segments (1.6-2.0 mm) of second-order branches (vessel diameter 300-550 μm) were mounted onto a Multi-Wire Myograph system (DMT, Ann Arbor, MI, USA) and equilibrated in PSS with 95% O_2 and 5% CO_2 at 37˚C. Initial vessel viability was confirmed by contractile response to 60 mM potassium chloride (KCl), and vascular function assessment was performed by addition of acetylcholine (Ach, 10 μM) to vessels pre-constricted with phenylephrine (5 μM). Only vessels

with viable endothelium (minimum 45% vasodilatory response of phenylephrine pre-constricted vessels to Ach) were included in analyses. Endothelium-dependent and -independent vasorelaxation was evaluated in response to Ach or sodium nitroprusside (SNP), respectively (1 nM to 10 μM). Data are presented as percentage of maximal constriction.

Statistical Analyses

Results are expressed as mean ± SEM, and statistical comparisons were conducted using 1- or 2-way analysis of variance (ANOVA) with Tukey post-test as appropriate. Statistical analyses were performed with GraphPad Prism 9.4 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $p<0.05$.

Results

miR-486-5p localizes to the kidney and protects against ischemic injury

Preliminary experiments with the bilateral IR model in rat demonstrated peak elevation of plasma Cr after 24 h, with partial recovery by 72 h and return to baseline Cr by 4 weeks. A dose of 0.5 mg/kg of miR-486-5p was selected from preliminary experiments (n=3) showing efficacy.

At 24 h after kidney IR injury, miR-486-5p levels were increased in the kidneys of rats administered miR-486-5p by real-time PCR (Figure S1). miR-486-5p levels were also significantly increased in the liver and spleen of miR-486-5p-treated rats, but not in the heart. By *in situ* hybridization, rats treated with miR-486-5p demonstrated its localization within proximal tubular cells and interstitial capillary endothelial cells (Figure 2). Probe signal intensity was highest in rats that received miR-486-5p (Figure 2), and was absent in the negative control using a scramble probe. *In situ* hybridization did not detect miR-486-5p within macrophages from kidneys of rats with IR.

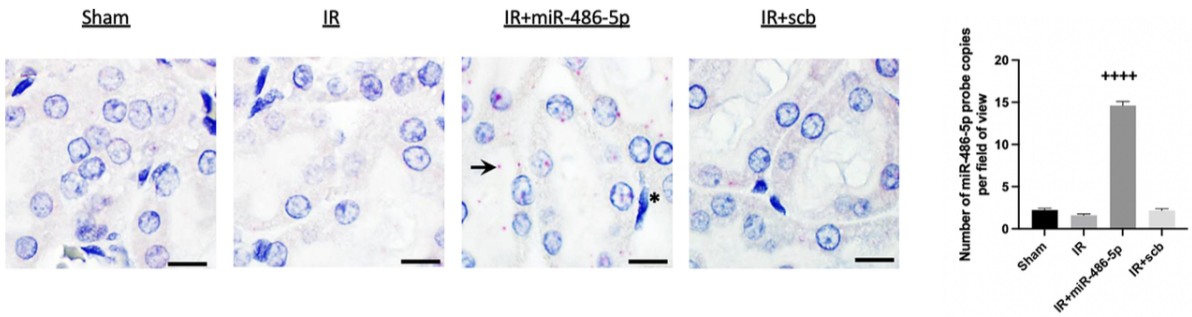

Figure 2. *In situ* hybridization for miR-486-5-p in rat kidney cortex

Localization of miR-486-5p in the rat kidney cortex 24 h post-IR, with injection of miR-486-5p (0.5 mg/kg, IR+miR-486-5p) or scramble miRNA (0.5 mg/kg, IR+scb) at the time of reperfusion. At 24 h, enhanced miR-486-5p signal (pink-red areas) was observed in rats injected with miR-486-5p, localized to proximal tubular cells (arrow) and endothelial cells (asterisk). Background nuclear staining with hematoxylin. Scale bar = 10 μm, magnification ×1000. The graph shows the quantification of miR-486-5p probe copies/field of view (++++P<0.0001 vs all groups)

miR-486-5p potently protected against kidney ischemic injury, attenuating the ischemic injury-induced rise in plasma Cr and BUN levels (Figure 3).

Laser doppler flowmetry demonstrated that miR-486-5p preserved cortical blood flow and enhanced medullary blood flow (Figure S2). At 24 h, kidneys from miR-486-5p-treated rats showed decreased tubular injury, decreased infiltration of neutrophils and macrophages, and diminished apoptosis (Figure 4).

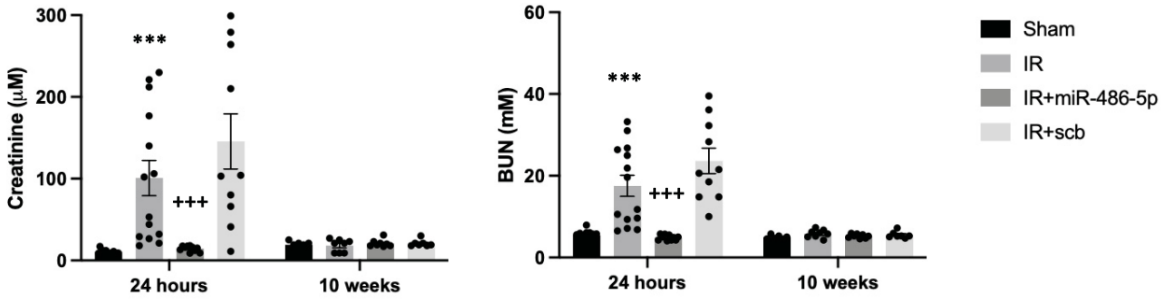

Figure 3. miR-486-5p protects against ischemic kidney injury in rat

Graphs depict plasma creatinine (Cr, left) and blood urea nitrogen (BUN, right) at 24 h and 10 weeks after kidney IR injury, with administration of miR-486-5p or scramble (scb) miRNA by tail vein injection at the start of reperfusion. Plasma Cr and BUN are reported in standard SI units. To convert plasma Cr from μM to mg/dL, multiply by 0.0113, and to convert mM BUN to mg/dL, multiply by 2.8. ***P<0.001 vs sham, IR+miR-486-5p; +++P<0.001 vs IR+scb. Number of rats: n = 10–14 rats per group (24 h) and n = 6–8 rats per group (10 weeks).

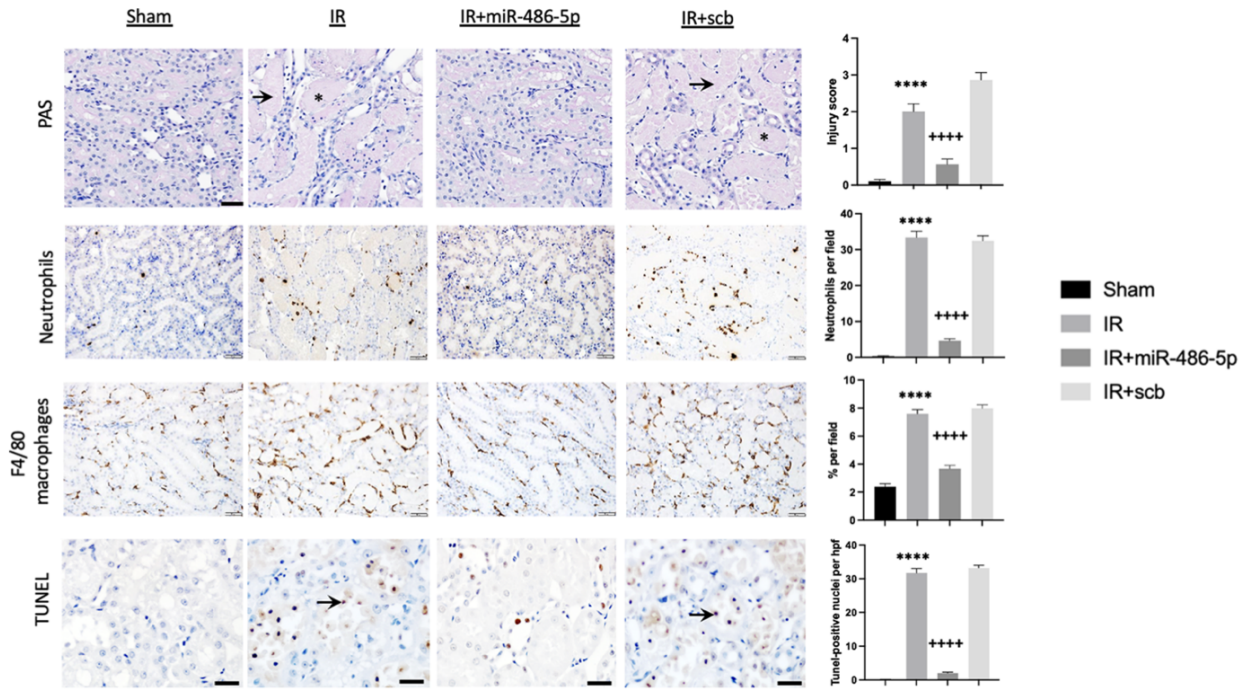

Figure 4. Effect of miR-486-5p on kidney injury scores, inflammatory cell infiltration and apoptosis 24 h after IR injury
Groups include sham rats, rats with kidney IR injury alone (IR), or rats with kidney IR injury and treated with either miR-486-5p mimic (IR+miR-486-5p) or scramble miRNA (IR+scb) at the start of reperfusion. Representative images from sections of outer medulla. Periodic acid schiff (PAS) staining depicts acute tubular injury in IR and IR+scb rat groups (asterix: tubular cast; arrow: tubular dilatation, nuclear loss) (scale bar = 50 μm, magnification ×200). Representative images of kidney neutrophil and macrophage infiltration (scale bar = 50 μm, magnification ×200). Representative images of terminal deoxynucleotidyl transferase-mediated dUTP nick-end label (TUNEL) staining (scale bar = 20 μm, magnification ×400), with arrows indicating stained nuclei. Graphs on the right depict semiquantitative analyses. ****$P<0.0001$ vs sham, IR+miR-486-5p; ++++$P<0.0001$ vs IR+scb; $n = 7$ rats per group.

Effect of miR-486-5p on endothelial protein levels

Since miR-486-5p was detected within endothelial cells 24 h after injection and preserved regional kidney blood flow and kidney function, we performed immunoblots and histological analyses for proteins involves in vascular function and endothelial activation (3, 24, 26). ICAM-1 was detected in capillary endothelium, with significantly increased levels after kidney IR injury by both immunoblot and immunofluorescence (Figure 5). This effect was blocked by miR-486-5p (Figure 5). We also transfected HUVECs with miR-486-5p and subjected them to H/R; this revealed significant attenuation of the H/R-induced upregulation of ICAM-1 (Figure S3).

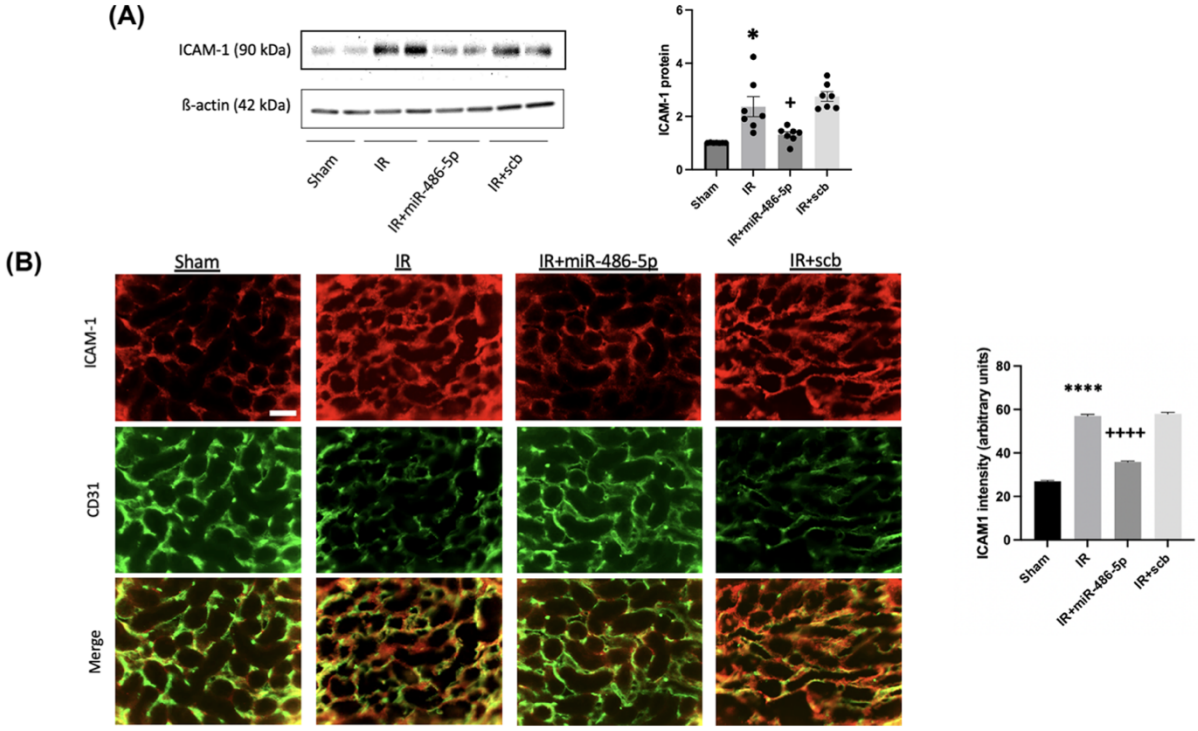

Figure 5. Effect of miR-486-5p on kidney protein levels of ICAM-1 24 h after IR injury

(**A**) Graph shows kidney protein expression of ICAM-1 with representative immunoblot. Protein expression was normalized to β-actin. (**B**) Representative immunofluorescence images of ICAM-1 (red), CD31 (green), and co-localization (scale bar = 50 μm, magnification ×400) with semiquantative analysis for ICAM-1 signal ****$P<0.0001$, *$P<0.05$ vs sham, IR+miR-486-5p; ++++$P<0.0001$, +$P<0.05$ vs IR+scb miRNA. $n = 7$ rats per group.

Kidney IR injury upregulated eNOS protein levels, observed within endothelial cells of terminal arterioles, and this was inhibited by miR-486-5p on immunoblot and immunofluorescence (Figure 6). IR injury also increased whole kidney VEGFA by immunoblot analysis, and this was inhibited by miR-486-5p (Figure 7), although immunohistochemistry did not permit precise localization on either paraffin-embedded or frozen sections (not shown). To determine if the effects of miR-486-5p on eNOS and VEGFA protein levels were specific to kidney, we conducted immunoblot analysis on liver tissue isolated from the rat groups. The results show that kidney IR had no significant effect on liver expression of eNOS or VEGFA, and miR-486-5p injection did not alter levels (Figure S4).

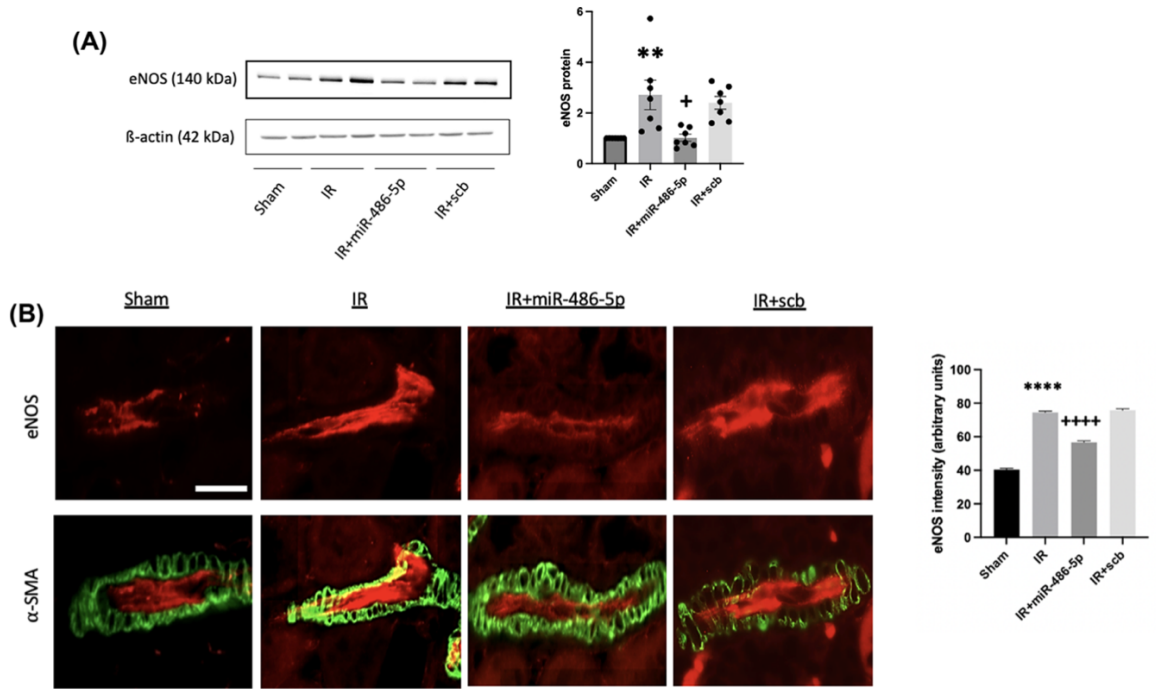

Figure 6. Effect of miR-486-5p on kidney protein levels of eNOS 24 h after IR injury

(**A**) Graph shows kidney protein expression of eNOS with representative immunoblot. Protein expression was normalized to β-actin. (**B**) Representative immunofluorescence images of eNOS (red) and α-smooth muscle actin (α-SMA; green) in renal terminal arterioles (scale bar = 30 μm, magnification ×400) with semiquantitative analysis for eNOS signal. ****$P<0.0001$, **$P<0.01$ vs sham, IR+miR-486-5p; ++++$P<0.0001$, +$P<0.05$ vs IR+scb miRNA. $n = 7$ rats per group.

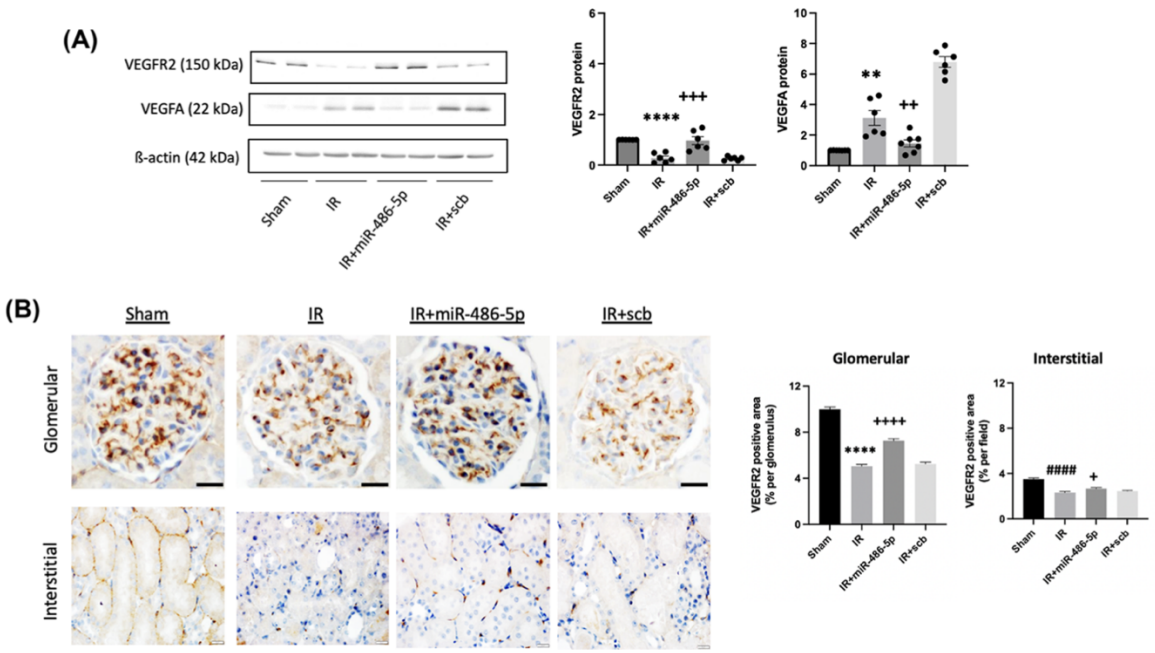

Figure 7. Effect of miR-486-5p on kidney protein levels of vascular endothelial growth factor receptor-2 (VEGFR2) and vascular endothelial growth factor-A (VEGFA) 24 h after IR injury

(**A**) Graphs show kidney protein expression of VEGFR2 and VEGFA, with representative immunoblots. Protein expression was normalized to β-actin. (**B**) Representative images of VEGFR2 staining within glomeruli (top, scale bar = 20 μm, magnification ×400) and interstitium (bottom, scale bar = 20 μm, magnification ×400) by immunohistochemistry with semiquantitative analysis. ****P<0.0001, **P<0.01 vs sham, IR+miR-486-5p; ++++P<0.0001, +++P<0.001, ++P<0.01 vs IR+scb miRNA; +P<0.05 vs IR; ####P<0.0001 vs sham. n = 7 rats per group.

Decreased VEGFR2 levels were observed after kidney IR injury by immunoblot. Immunohistochemistry demonstrated staining localized within glomeruli and interstitial endothelial cells and showed that miR-486-5p attenuated this IR-induced decrease (Figure 7).

In our previous studies involving mice with kidney IR injury, administration of miR-486-5p was associated with decreased PTEN protein levels (16, 17). In rat, however, miR-486-5p had no effect on kidney PTEN protein levels at 24 h (Figure 8).

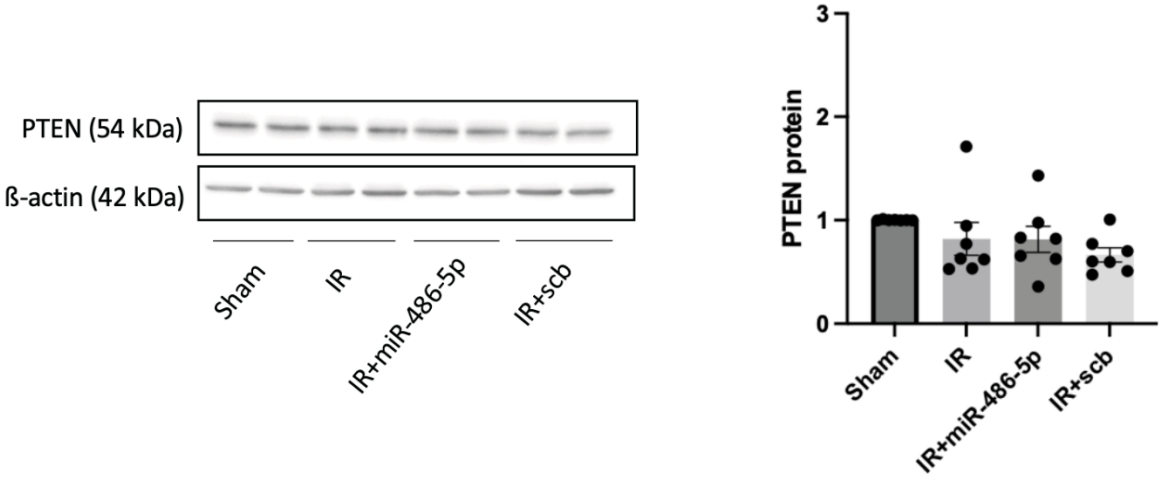

Figure 8. Effect of miR-486-5p on kidney protein levels of phosphatase and tensin homolog (PTEN) 24 h after IR injury in rats

Graph shows kidney protein expression of PTEN with representative immunoblots. Protein expression was normalized to β-actin. $n = 7$ rats per group.

Effect of early administration of miR-486-5p on peritubular capillary density and endothelial function at 10 weeks

Since miR-486-5p inhibited ischemia-stimulated kidney eNOS and VEGF protein at 24 h, rats were followed for 10 weeks to determine the impact on development of capillary rarefaction and interstitial fibrosis (13, 27). At 10 weeks, rats with IR injury alone recovered kidney function with normalization of plasma Cr and BUN (Figure 2) and without significant increase in albuminuria (Figure S5). Kidneys from these rats had increased collagen fiber content by picrosirius red staining and decreased $CD31^+$ peritubular capillary density (Figure 9). Post-ischemic kidneys showed increased staining for the pro-fibrotic myofibroblast marker α-SMA, and $F4/80^+$ macrophages (Figure 9). By contrast, kidneys from rats treated with miR-486-5p had preserved peritubular capillary density and decreased staining for interstitial collagen fibers, α-SMA and F4/80 macrophages.

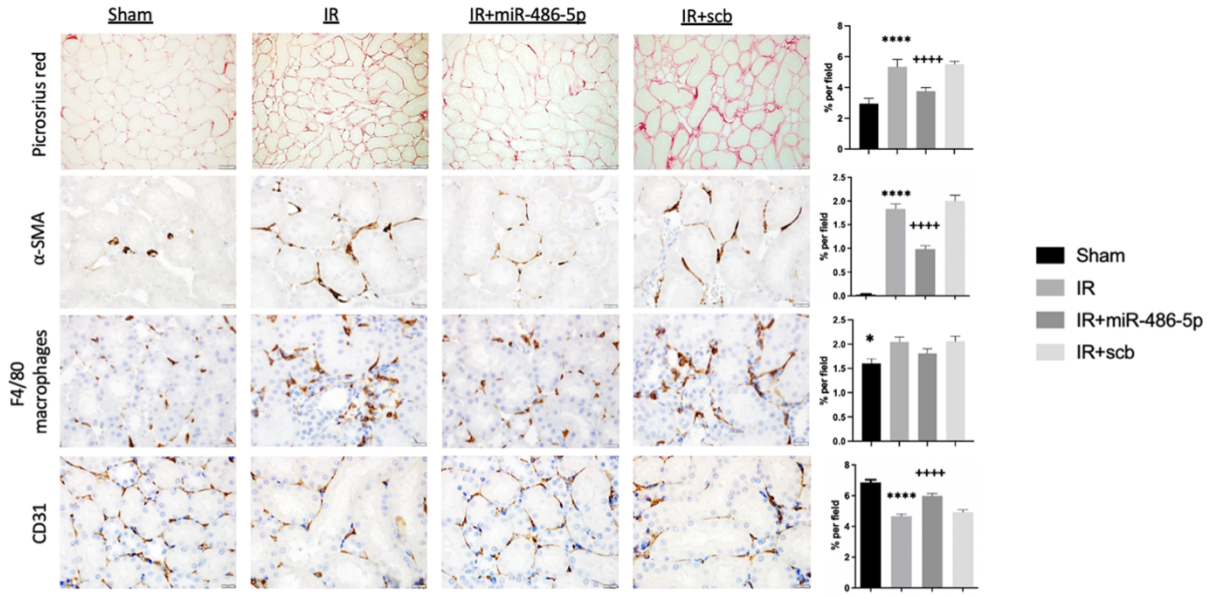

Figure 9. Effect of early miR-486-5p administration on kidney fibrosis and peritubular capillary density 10 weeks after kidney IR injury

Semiquantitative analysis and representative images of picrosirius red staining for interstitial collagen deposition (scale bar = 50 μm, magnification ×200), smooth muscle -actin (-SMA, scale bar = 20 μm, magnification ×400), kidney macrophage infiltration (F4/80, scale bar = 20 μm, magnification ×400), and CD31$^+$ peritubular capillary density (scale bar = 20 μm, magnification ×400) from kidneys of sham rats, rats with kidney IR injury (IR) alone, or rats with kidney IR injury administered either miR-486-5p (IR+miR-486-5p) or scramble miRNA (IR+scb) at the start of reperfusion. ****P<0.0001 vs sham, IR+miR-486-5p, *P<0.05 vs IR; ++++P<0.0001 vs IR+scb. N = 6–8 rats per group.

Recovery from AKI is associated with impaired kidney hemodynamics (28), hypertension (28, 29), and peripheral vascular dysfunction (29). At 10 weeks, all groups of rats had similar kidney cortical and medullary blood flows, and systolic blood pressure (SBP) (Figure S6). However, mesenteric arteries from rats with IR alone (or treated with scb miRNA) showed impaired endothelium-dependent vasorelaxation, which was prevented by early administration of miR-486-5p (Figure 10). There were no significant differences in the endothelium-independent vasodilatory responses to SNP amongst the groups.

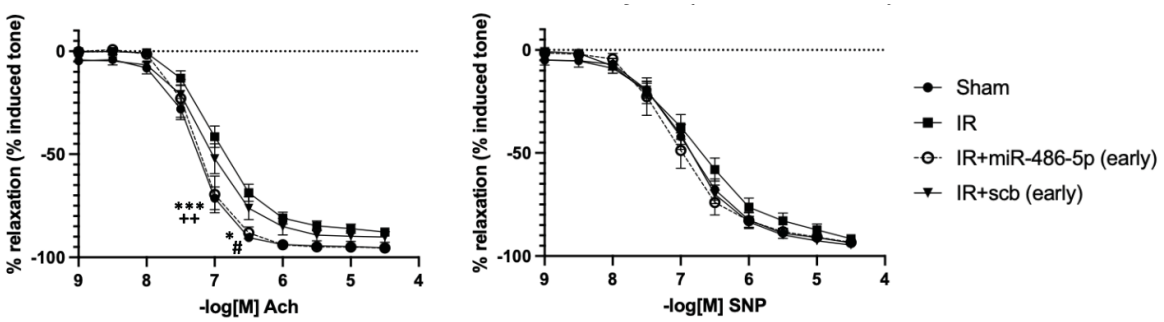

Figure 10. Effect of early administration of miR-486-5p on mesenteric artery reactivity 10 weeks after kidney IR injury
Left: Acetylcholine (Ach), Right: Sodium nitroprusside (SNP); ***$P<0.001$, *$P<0.05$, sham vs IR, IR+scramble (scb); ++$P<0.01$, IR+miR-486-5p vs IR, IR+scb, #$P<0.05$, IR+miR-486-5p vs IR; $n = 9–14$ rats per group for sham and IR groups, and $n = 6–7$ rats per group for IR+miR-486-5p and IR+scb groups.

Late administration of miR-486-5p does not affect AKI to CKD transition

We next determined if late administration of miR-486-5p might affect vascular function or transition to CKD. Rats were administered a first dose of miR-486-5p (0.5 mg/kg i.v.) at 96 h after IR, and a second dose at 3 weeks. At 24 h after the first dose, RT-qPCR revealed increased kidney levels of miR-486-5p (Figure S7). At that time point (120 h after kidney IR), kidney eNOS protein expression remained significantly increased in rats with IR alone, but not in rats with IR injury treated with miR-486-5p (Figure S7).

In all rats subjected to kidney IR injury, plasma Cr and BUN were significantly increased 24 h after IR, with return towards baseline by 96 h, and complete recovery by 3 weeks, sustained to 10 weeks (Figure S8). Late and repeated miR-486-5p dosing did not prevent development of kidney fibrosis or preserve capillary density at 10 weeks (Figure 11). Similarly, unlike the protective effect of early miR-486-5p treatment, delayed administration of miR-486-5p did not preserve Ach-mediated vasodilation in mesenteric arteries at 10 weeks (Figure 12).

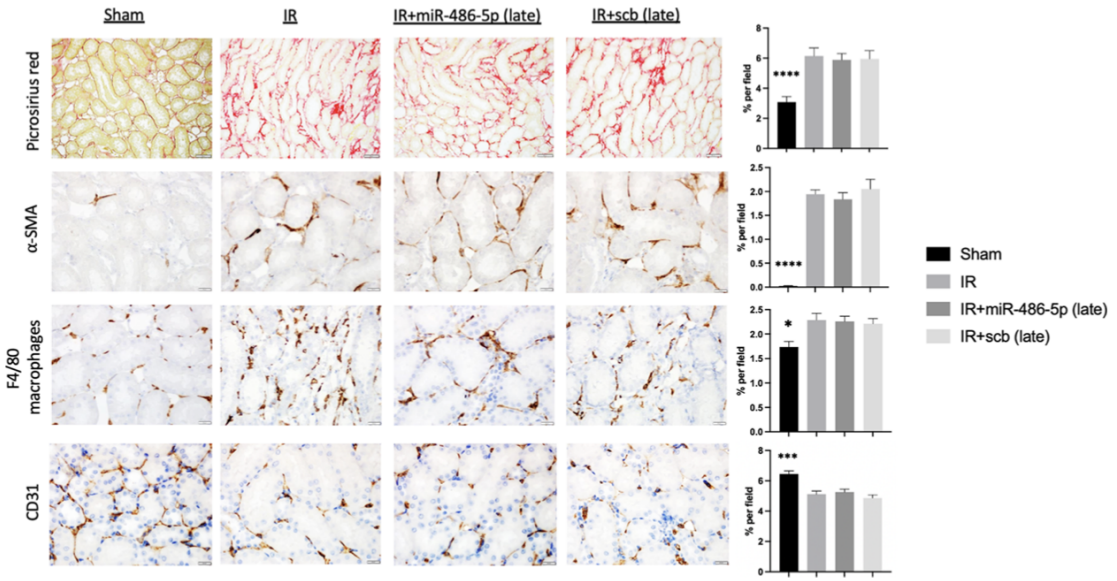

Figure 11. Effect of delayed miR-486-5p administration on kidney fibrosis and peritubular capillary density 10 weeks after IR injury

Semiquantitative analysis and representative images of picrosirius red staining for interstitial collagen deposition (scale bar = 50 μm, magnification ×200), smooth muscle -actin (-SMA, scale bar = 20 μm, magnification ×400), kidney macrophage infiltration (F4/80, scale bar = 20 μm, magnification ×400), and CD31$^+$ peritubular capillary density (CD31$^+$, scale bar = 20 μm, magnification ×400) from kidneys of sham rats, rats with kidney IR injury alone (IR), or rats with kidney IR injury administered either miR-486-5p (IR+miR-486-5p) or scramble miRNA (IR+scb) at 96 h and 3 weeks after kidney IR injury. ****P<0.0001, ***P<0.001, *P<0.05, sham vs all groups, n = 6 rats per group.

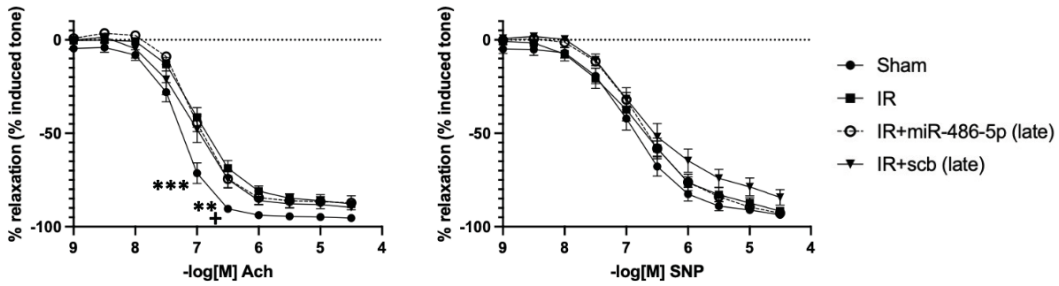

Figure 12. Effect of late administration of miR-486-5p on mesenteric artery reactivity 10 weeks after kidney IR injury

Left: Acetylcholine (Ach), Right: Sodium nitroprusside (SNP); ***P<0.001, sham vs all groups, **P<0.01, sham vs IR, IR+miR-486-5p, +P<0.05, sham vs IR+ scramble miRNA (scb); n = 9–14 rats per group for sham and IR groups and n = 6 rats per group for IR+miR-486-5p and IR+scb groups.

Discussion

Treatment options for AKI are limited (30). We previously showed that direct administration of miR-486-5p protects against kidney IR injury in mice, associated with downregulation of PTEN

expression (17). Although pre-clinical studies support the pro-angiogenic potential of miR-486-5p (19, 20), an anti-angiogenic effect has been reported in cultured endothelial cells (21). Furthermore, persistent PTEN loss following kidney injury may promote dysfunctional tubule regeneration and fibrosis (18). We hypothesized that early administration of miR-486-5p would prevent ischemic AKI and the transition to CKD, with potential for delayed miR-486-5p administration to exert a negative impact on the vasculature. Our results indicate that miR-486-5p protects against ischemic AKI in rat, associated with inhibition of ICAM-1 upregulation and decreased inflammatory cell infiltration. Early administration of miR-486-5p prevented peritubular capillary loss, interstitial fibrosis, and systemic endothelial dysfunction after 10 weeks, whereas delayed administration of miR-486-5p after peak kidney injury had no impact on long term kidney or systemic vascular outcomes.

The mechanism by which miR-486-5p prevents ischemic AKI remains unclear but may involve multiple gene targets. In the present studies, *in situ* hybridization localized miR-486-5p within rat proximal tubular cells and interstitial capillary endothelial cells, suggesting a direct effect of miR-486-5p on these cells. Although miR-486 levels are increased within mouse kidney macrophages after unilateral ureteral obstruction (31), we were unable to detect miR-486-5p in kidney macrophages in rats with ischemic injury alone or rats treated with scramble miR, suggesting that endogenous levels are below detectability by this method. Relatively few macrophages were present within kidneys from mice treated with miR-486-5p, precluding quantification by *in situ* hybridization. Taken together, our data suggest that macrophage miR-486-5p is unlikely to contribute to the protective effect.

We showed that miR-486-5p inhibited IR-induced upregulation of ICAM-1, eNOS, and VEGFA while preserving VEGFR2 expression. Since these are not predicted targets of miR-486-

5p, the regulatory mechanism remains unknown. We previously identified differentially expressed genes involved in apoptosis and TNF-α signaling in proximal tubular cells from mice with kidney IR injury treated with miR-486-5p (17). In pre-clinical models of kidney (17) and cardiac (32, 33) IR injury, miR-486-5p decreases PTEN protein expression and inhibits apoptosis. miR-486-5p also transiently downregulated kidney PTEN mRNA at 4 h after kidney IR injury in mice (17), yet the present study found no effect of miR-486-5p on PTEN protein expression at 24 h after kidney IR injury in rat. It is plausible that early, transient downregulation of miR-486-5p target gene expression contributes to its protective effect while minimizing potential long-term negative consequences. In this regard, sustained PTEN knockdown in kidney IR injury confers a pro-fibrotic phenotype (18), while pharmacological inhibition of PTEN has been shown by Zhou et al to exacerbate ischemic kidney injury in mice (34).

In kidney IR injury, outer medullary hypoperfusion exacerbates tubular epithelial cell injury and contributes to permanent peritubular capillary loss (35). Collett *et al* demonstrated that infusion of conditioned medium from endothelial-colony forming cells (ECFCs) protected against rat kidney IR injury and early loss of medullary blood flow, and inhibited IR-induced ICAM-1 expression (10). Interestingly, we previously showed that cord blood ECFC-derived conditioned medium contains small extracellular vesicles (exosomes) that inhibit ICAM-1 upregulation in HUVECs subjected to H/R injury (24) and confer a protective effect against kidney IR injury in mice via exosomal transfer of miR-486-5p (16). In the present studies, miR-486-5p preserved renal blood flow and attenuated endothelial injury as evidenced by inhibition of IR-induced ICAM-1 upregulation along with decreased inflammatory cell infiltration. Although ICAM-1 is not a predicted direct miR-486-5p target, miR-486-5p inhibited its upregulation *in vivo* and *in vitro* in transiently transfected HUVECs subjected to H/R injury.

These results strongly suggest that miR-486-5p preserves endothelial function by targeting pathways involved in endothelial cell activation and leukocyte adhesion/migration. Indeed, targeting of this pathway by miR-486-5p may represent an important mechanism for its protective effect in ischemic AKI.

Although eNOS is also not a predicted direct target of miR-486-5p, its administration early or late after IR injury decreased total kidney eNOS expression compared to rats with IR alone. Other studies have reported IR-induced upregulated eNOS protein levels (36, 37), presumably as a protective mechanism to preserve kidney perfusion. Nitric oxide (NO) is an important regulator of kidney microvascular blood flow (38) with evidence for beneficial effects (5, 39-41) but also potential for worsening (42) in kidney IR injury. In this regard, both non-selective NOS or eNOS inhibition decrease apoptosis after kidney IR injury, and eNOS activity has been linked to inducible NOS (iNOS) overexpression and increased apoptosis (43, 44). IR-induced eNOS upregulation may also contribute to eNOS uncoupling, a phenomenon that results in superoxide and free radical production and scavenging of NO to form peroxynitrite (45, 46). Further, in mice, eNOS overexpression increased liver IR injury, inducing protein nitration, transaminitis, and apoptosis (47). Thus, it is plausible that inhibition of IR-induced eNOS expression by miR-486-5p might prevent injury related to reactive nitrogen species. On the other hand, our data indicate that miR-486-5p preserves endothelial function, and enhanced NO production as a response to IR may not be necessary. Further studies are required to determine role of miR-486-5p-induced inhibition of eNOS expression in the protective response to kidney IR injury.

AKI is associated with long-term increased risk of systemic vascular disease and hypertension (48). Post-ischemic rats developed mesenteric artery dysfunction at 10 weeks,

while early administration of miR-486-5p preserved endothelium-dependent vasorelaxation. In mice, systemic vascular dysfunction with impaired endothelium-dependent vasorelaxation has been reported 4 weeks after kidney IR injury, with a role for activation of the endothelin system as mediator (29). While the role of miR-486-5p in modulating the endothelin system after kidney IR injury is unknown, in human pulmonary artery smooth muscle cells miR-486-5p increased the expression and secretion of endothelin-1 (49). However, the effect of miR-486-5p on biological pathways via its target genes depends on cell type or experimental model (50).

In this study, we showed that delayed administration of miR-486-5p at 96 h and 3 weeks after kidney IR injury did not prevent peritubular capillary rarefaction, development of tubulointerstitial fibrosis, or systemic endothelial dysfunction. In rats with kidney IR injury, Leonard *et al*. showed that VEGF administration preserved microvascular density when administered early post-IR but lacked effect on vessel recovery when administered late (3 weeks) after IR (36). miR-486-5p has an anti-fibrotic effect in pre-clinical models of pulmonary and cardiac fibrosis via targeting of Smad1/2 and inhibition of transforming growth factor (TGF)-β signaling (51, 52). The pathogenesis of tubulointerstitial fibrosis after kidney IR injury is likely complex beyond TGF-β signaling, involving permanent microvascular damage (13, 27) and release of inflammatory mediators such as high mobility group box-1 (*HMGB1*) from injured or necrotic tubular cells (53). In mice, early delivery of miR-486-5p targets genes involved in injury pathways in kidney IR, including TNF-α signaling and apoptosis (17). In rat, we show that miR-486-5p inhibits endothelial activation and preserves endothelial function. Early administration of miR-486-5p after kidney IR injury is therefore critical to prevent permanent peritubular capillary loss and the transition to CKD, and indeed our data suggest this process is programmed to occur soon after reperfusion.

Our study has certain limitations. First, experiments were performed exclusively on male rats. In this regard however, in previous studies we showed that female mice are significantly less susceptible to kidney IR injury (54). Second, considerable variability in extent of injury 24 h after reperfusion was observed in the rat model of bilateral IR, a feature that is widely recognized (55). Third, we used a volatile anesthetic (isoflurane) in our rat kidney IR model, as such anesthetics are routinely administered in clinical practice, including cardiovascular surgeries that represent an important cause of kidney IR injury in humans. We recognize that volatile anesthetics have renoprotective effects (56), although we carefully controlled for this issue by providing all rats with the same anesthetic treatment. Fourth, SBP evaluation by non-invasive tail cuff plethysmography may not have been sensitive enough to detect mild but significant changes in blood pressure. Furthermore, laser doppler flowmetry is ideally suited for measuring relative blood flow changes, and not absolute flow.

In summary, miR-486-5p administered as a single dose at the start of reperfusion protects against ischemic AKI in rat, preserves early endothelial function, prevents peritubular capillary loss and tubulointerstitial fibrosis and preserves endothelium-dependent vasorelaxation. Further, despite inhibition of eNOS protein expression, delayed administration of miR-486-5p after peak injury has no long-term adverse or protective effects. These results suggest that miR-486-5p is a promising treatment for the prevention of ischemic AKI and associated long-term complications.

Data availability statement: All data are available by contacting the corresponding author.

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Conflict of interest statement: The authors have no conflicts of interest to declare.

Additional information

Supplementary data is available for this paper online at https://doi.org/10.1042/CS20231752. We also provide the supplemental data figures in Appendix A.

References

1. Wang HE, Muntner P, Chertow GM, Warnock DG (2012) Acute Kidney Injury and Mortality in Hospitalized Patients. Am J Nephrol. **35**:349-55 https://doi.org/10.1159/000337487
2. See EJ, Jayasinghe K, Glassford N, Bailey M, Johnson DW, Polkinghorne KR, et al. (2019) Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. Kidney International. **95**:160-72 https://doi.org/10.1016/j.kint.2018.08.036
3. Basile DP, Anderson MD, Sutton TA (2012) Pathophysiology of acute kidney injury. Compr Physiol. **2**(2):1303-53 https://doi.org/10.1002/cphy.c110041
4. Basile DP, Bonventre JV, Mehta R, Nangaku M, Unwin R, Rosner MH, et al. (2016) Progression after AKI: Understanding Maladaptive Repair Processes to Predict and Identify Therapeutic Treatments. J Am Soc Nephrol. **27**:687-97 https://doi.org/10.1681/ASN.2015030309
5. Brodsky SV, Yamamoto T, Tada T, Kim B, Chen J, Kajiya F, et al. (2002) Endothelial dysfunction in ischemic acute renal failure: rescue by transplanted endothelial cells. Am J Physiol Renal Physiol. **282**:F1140-F9 https://doi.org/10.1152/ajprenal.00329.2001

6. Conger JD, Robinette JB, Schrier RW (1988) Smooth Muscle Calcium and Endothelium-derived Relaxing Factor in the Abnormal Vascular Responses of Acute Renal Failure. J Clin Invest. **92**:532-7 https://doi.org/10.1172/JCI113628
7. Kelly KJ, Williams WW, Colvin RB, Bonventre JV (1994) Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. Proc Natl Acad Sci U S A. **91**(2):812-6 https://doi.org/10.1073/pnas.91.2.812
8. Sutton TA, Mang HE, Campos SB, Sandoval RM, Yoder MC, Molitoris BA (2003) Injury of the renal microvascular endothelium alters barrier function after ischemia. Am J Physiol Renal Physiol. **285**:F191-8 https://doi.org/10.1152/ajprenal.00042.2003
9. Kelly KJ, Sutton TA, Weathered N, Ray N, Caldwell EJ, Plotkin Z, et al. (2004) Minocycline inhibits apoptosis and inflammation in a rat model of ischemic renal injury. Am J Physiol Renal Physiol. **287**:F760-F6 https://doi.org/10.1152/ajprenal.00050.2004
10. Collett JA, Mehrotra P, Crone A, Shelley WC, Yoder MC, Basile DP (2017) Endothelial colony-forming cells ameliorate endothelial dysfunction via secreted factors following ischemia-reperfusion injury. Am J Physiol Renal Physiol. **312**:F897-F907 https://doi:10.1152/ajprenal.00643.2016
11. Basile DP (2007) The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function. Kidney International. **72**(2):151-6 https://doi.org/10.1038/sj.ki.5002312
12. Bonventre JV, Yang L (2011) Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest. **121**(11):4210-21 https://doi.org/10.1172/JCI45161
13. Menshikh A, Scarfe L, Delgado R, Finney C, Zhu Y, Yang XH, et al. (2019) Capillary rarefaction is more closely associated with CKD progression after cisplatin, rhabdomyolysis, and ischemia-reperfusion-induced AKI than renal fibrosis. Am J Physiol Renal Physiol. **317**:F1383-F97 https://doi.org/10.1152/ajprenal.00366.2019
14. Jonas S, Izaurralde E (2015) Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet. **16**(7):421-33 https://doi.org/10.1038/nrg3965
15. Zankar S, Trentin-Sonoda M, Viñas JL, Rodriguez RA, Bailey A, Allan D, et al. (2021) Therapeutic effects of micro-RNAs in preclinical studies of acute kidney injury: a systematic review and meta-analysis. Scientific Reports. **11**:9100 https://doi.org/10.1038/s41598-021-88746-y
16. Viñas JL, Burger D, Zimpelmann J, Haneef R, Knoll W, Campbell P, et al. (2016) Transfer of microRNA-486-5p from human endothelial colony forming cell–derived exosomes reduces ischemic kidney injury. Kidney International. **90**:1238-50 http://dx.doi.org/10.1016/j.kint.2016.07.015
17. Viñas JL, Spence M, Porter CJ, Douvris A, Gutsol A, Zimpelmann JA, et al. (2021) micro-RNA-486-5p protects against kidney ischemic injury and modifies the apoptotic transcriptome in proximal tubules. Kidney International. **100**:597-612 https://doi.org/10.1016/j.kint.2021.05.034
18. Lan R, Geng H, Polichnowski AJ, Singha PK, Saikumar P, McEwen DG, et al. (2012) PTEN loss defines a TGF-ß-induced tubule phenotype of failed differentiation and JNK signaling during renal fibrosis. Am J Physiol Renal Physiol. **302**:F1210-F23 https://doi.org/10.1152/ajprenal.00660.2011

19. Li Q, Xu Y, Lv K, Wang Y, Zhong Z, Xiao C, et al. (2021) Small extracellular vesicles containing miR-486-5p promote angiogenesis after myocardial infarction in mice and nonhuman primates. Sci Transl Med. **13**:eabb0202 https://doi.org/10.1126/scitranslmed.abb0202
20. Lu Y, Wen H, Huang J, Liao P, Liao H, Tu J, et al. (2020) Extracellular vesicle-enclosed miR-486-5p mediates wound healing with adipose derived stem cells by promoting angiogenesis. J Cell Mol Med. **00**:1-15 https://doi.org/10.1111/jcmm.15387
21. Rosano S, Parab S, Noghero A, Corà D, Bussolino F (2022) Long Non-Coding RNA LINC02802 Regulates In Vitro Sprouting Angiogenesis by Sponging microRNA-486-5p. Int J Mol Sci. **23**:1653 https://doi.org/10.3390/ijms23031653
22. Guan Z, Miller SB, Greenwald JE (1995) Zaprinast accelerates recovery from established acute renal failure in the rat. Kidney International. **47**:1569-75 https://doi.org/10.1038/ki.1995.220
23. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research. **29**(9):e45 https://doi.org/10.1093/nar/29.9.e45
24. Burger D, Viñas JL, Akbari S, Dehak H, Knoll W, Gutsol A, et al. (2015) Human Endothelial Colony-Forming Cells Protect against Acute Kidney Injury: Role of Exosomes. Am J Pathol. **185**:2309-23 https://doi.org/10.1016/j.ajpath.2015.04.010
25. Shihan MH, Novo SG, Marchand SJL, Wang Y, Duncan MK (2021) A simple method for quantitating confocal fluorescent images. Biochem Biophys Rep. **1**:100916 https://doi.org/10.1016/j.bbrep.2021.100916
26. Hörbelt M, Lee S-Y, Mang HE, Knipe NL, Sado Y, Kribben A, et al. (2007) Acute and chronic microvascular alterations in a mouse model of ischemic acute kidney injury. Am J Physiol Renal Physiol. **293**:F688-F95 https://doi.org/10.1152/ajprenal.00452.2006
27. Basile DP, Donohue D, Roethe K, Osborn JL (2001) Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. Am J Physiol Renal Physiol. **281**:F887-F99 https://doi.org/10.1152/ajprenal.00050.2001
28. Pechman KR, Miguel CD, Lund H, Leonard EC, Basile DP, Mattson DL (2009) Recovery from renal ischemia-reperfusion injury is associated with altered renal hemodynamics, blunted pressure natriuresis, and sodium-sensitive hypertension. Am J Physiol Renal Physiol. **297**:R1358-R63 https://doi.org/10.1152/ajpregu.91022.2008
29. Czopek A, Moorhouse R, Gallacher PJ, Pugh D, Ivy JR, Farrah TE, et al. (2022) Endothelin blockade prevents the long-term cardiovascular and renal sequelae of acute kidney injury in mice. Sci Transl Med. **14**:eabf5074 https://doi.org/10.1126/scitranslmed.abf5074
30. James M, Bouchard J, Ho J, Klarenbach S, LaFrance J-P, Rigatto C, et al. (2013) Canadian Society of Nephrology Commentary on the 2012 KDIGO Clinical Practice Guideline for Acute Kidney Injury. Am J Kidney Dis. **61**(5):673-85 http://dx.doi.org/10.1053/j.ajkd.2013.02.350
31. Connor KL, Teenan O, Cairns C, Banwell V, Thomas RAB, Rodor J, et al. (2020) Identifying cell-enriched miRNAs in kidney injury and repair. JCI Insight. **5**(24):e140399. https://doi.org/10.1172/jci.insight.140399.
32. Zhu H-h, Wang X-t, Sun Y-h, He W-k, Liang J-b, Mo B-h, et al. (2019) MicroRNA-486-5p targeting PTEN Protects Against Coronary Microembolization-Induced Cardiomyocyte

Apoptosis in Rats by activating the PI3K/AKT pathway. Eur J Pharmacol. **855**:244-51 https://doi.org/10.1016/j.ejphar.2019.03.045
33. Bei Y, Lu D, Bär C, Chatterjee S, Costa A, Riedel I, et al. (2022) miR-486 attenuates cardiac ischemia/reperfusion injury and mediates the beneficial effect of exercise for myocardial protection. Molecular Therapy. **30**(4):1675-91 https://doi.org/10.1016/j.ymthe.2022.01.031
34. Zhou J, Jia L, Hu Z, Wang Y (2017) Pharmacological Inhibition of PTEN Aggravates Acute Kidney Injury. Scientific Reports. **7**:9503 https://doi.org/10.1038/s41598-017-10336-8
35. Regner KR, Roman RJ (2012) Role of Medullary Blood Flow in the Pathogenesis of Renal Ischemia-Reperfusion Injury. Curr Opin Nephrol Hypertens. **21**(1):33-8 https://doi.org/10.1097/MNH.0b013e32834d085a
36. Leonard EC, Friedrich JL, Basile DP (2008) VEGF-121 preserves renal microvessel structure and ameliorates secondary renal disease following acute kidney injury. Am J Physiol Renal Physiol **295**:F1648 –F57 https://doi.org/10.1152/ajprenal.00099.2008
37. Ge Y-Z, Wu R, Xin H, Liu H, Lu T-Z, Zhao Y-C, et al. (2015) Effects of ischemic preconditioning on the systemic and renal hemodynamic changes in renal ischemia reperfusion injury. Int J Clin Exp Pathol. **8**(2):1128-40
38. Allen W. Cowley J, Mori T, Mattson D, Zou A-P (2003) Role of renal NO production in the regulation of medullary blood flow. Am J Physiol Regul Integr Comp Physiol. **284**:R1355-R69 https://doi.org/10.1152/ajpregu.00701.2002
39. Sucher R, Gehwolf P, Oberhuber R, Hermann M, Margreiter C, Werner ER, et al. (2010) Tetrahydrobiopterin protects the kidney from ischemia-reperfusion injury. Kidney International. **77**(8):681-9 https://doi.org/10.1038/ki.2010.7
40. Schneider R, Raff U, Vornberger N, Schmidt M, Freund R, Reber M, et al. (2003) L-Arginine counteracts nitric oxide deficiency and improves the recovery phase of ischemic acute renal failure in rats. Kidney International. **64**(1):216-25 https://doi.org/10.1046/j.1523-1755.2003.00063.x
41. Garcia-Criado FJ, Eleno N, Santos-Benito F, Valdunciel JJ, Reverte M, Lozano-Sánchez FS, et al. (1998) Protective effect of exogenous nitric oxide on the renal function and inflammatory response in a model of ischemia-reperfusion. Transplantation. **66**(8):982-90 https://doi.org/10.1097/00007890-199810270-00003
42. Devarajan P (2006) Update on Mechanisms of Ischemic Acute Kidney Injury. J Am Soc Nephrol. **17**:1503-20 https://doi.org/10.1681/ASN.2006010017
43. Viñas JL, Sola A, Genescà M, Alfaro V, Pí F, Hotter G (2006) NO and NOS isoforms in the development of apoptosis in renal ischemia/reperfusion. Free Radical Biology & Medicine. **40**:992-1003 https://doi.org/10.1016/j.freeradbiomed.2005.10.046
44. Viñas JL, Sola A, Hotter G (2006) Mitochondrial NOS upregulation during renal I/R causes apoptosis in a peroxynitrite-dependent manner. Kidney International. **69**:1403-9 https://doi.org/10.1038/sj.ki.5000361
45. Janaszak-Jasiecka A, Płoska A, Wierońska JM, Dobrucki LW, Kalinowski L (2023) Endothelial dysfunction due to eNOS uncoupling: molecular mechanisms as potential therapeutic targets. Cell Mol Biol Lett. **28**(1):21 https://doi.org/10.1186/s11658-023-00423-2

46. Granger DN, Kvietys PR (2015) Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biology. **6**:524-51 http://dx.doi.org/10.1016/j.redox.2015.08.020
47. Palanisamy AP, Cheng G, Sutter AG, Liu J, Lewin DN, Chao J, et al. (2014) Adenovirus-Mediated eNOS Expression Augments Liver Injury after Ischemia/Reperfusion in Mice. PLoS ONE. **9**(3):e93304 https://doi.org/10.1371/journal.pone.0093304
48. Noble RA, Lucas BJ, Selby NM (2020) Long-Term Outcomes in Patients with Acute Kidney Injury. CJASN. **15**:423-9 https://doi.org/10.2215/CJN.10410919
49. Yen T-A, Huang H-C, Wu E-T, Chou H-W, Chou H-C, Chen C-Y, et al. (2022) Microrna-486-5P Regulates Human Pulmonary Artery Smooth Muscle Cell Migration via Endothelin-1. Int J Mol Sci. **23**:10400 https://doi.org/10.3390/ijms231810400
50. Douvris A, Viñas J, Burns KD (2022) miRNA-486-5p: signaling targets and role in non-malignant disease. Cellular and Molecular Life Sciences. **79**:376 https://doi.org/10.1007/s00018-022-04406-y
51. Ji X, Wu B, Fan J, Han R, Luo C, Wang T, et al. (2015) The Anti-fibrotic Effects and Mechanisms of MicroRNA-486-5p in Pulmonary Fibrosis. Scientific Reports. **5**:14131 https://doi.org/10.1038/srep14131
52. Zhao H, Yang H, Geng C, Chen Y, Tang Y, Li Z, et al. (2021) Elevated IgE promotes cardiac fibrosis by suppressing miR-486a-5p. Theranostics. **11**(15):7600-15 https://doi.org/10.7150/thno.47845
53. Zhao ZB, Marschner JA, Iwakura T, Li C, Motrapu M, Kuang M, et al. (2023) Tubular Epithelial Cell HMGB1 Promotes AKI-CKD Transition by Sensitizing Cycling Tubular Cells to Oxidative Stress: A Rationale for Targeting HMGB1 during AKI Recovery. J Am Soc Nephrol. **34**:394-411 https://doi.org/10.1681/ASN.0000000000000024
54. Viñas JL, Porter CJ, Douvris A, Spence M, Gutsol A, Zimpelmann JA, et al. (2020) Sex diversity in proximal tubule and endothelial gene expression in mice with ischemic acute kidney injury. Clinical Science. **134**:1887-909 https://doi.org/10.1042/CS20200168
55. Fu Y, Tang C, Cai J, Chen G, Zhang D, Dong Z (2018) Rodent models of AKI-CKD transition. Am J Physiol Renal Physiol. **315**:F1098-F106 https://doi.org/10.1152/ajprenal.00199.2018
56. Fukazawa K, Lee HT (2014) Volatile Anesthetics and AKI: Risks, Mechanisms, and a Potential Therapeutic Window. J Am Soc Nephrol. **25**:884-92 https://doi.org/10.1681/ASN.2013111215

Chapter 3: Manuscript II

Connection to thesis: This manuscript evaluates the effect of miR-486-5p on eNOS expression in cultured endothelial cells and determines the impact of miR-486-5p-induced eNOS downregulation on endothelial cell function *in vitro*. The manuscript provides novel information regarding direct miR-486-5p targets in endothelial cells and introduces a pathway regulating eNOS expression and angiogenesis via its target MAML3. These data suggest there could be potential for miR-486-5p to have negative effects on endothelial function, although this has not yet been shown *in vivo*.

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miR-486-5p inhibits eNOS and angiogenesis in cultured endothelial cells by targeting MAML3

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Abstract

Background: Kidney ischemia-reperfusion (I/R) is associated with endothelial injury. Administration of miRNA (miR)-486-5p protects against rat kidney I/R injury, with localization to capillary endothelial cells, although it inhibits I/R-induced endothelial nitric oxide synthase (eNOS) protein expression. Here, we studied the effect of miR-486-5p on eNOS and endothelial cell function and determined its mRNA targets.

Methods: Human umbilical vein endothelial cells (HUVECs) were transfected with miR-486-5p mimic and assayed for proliferation, migration, and network formation. Biotinylated miR-486-5p was transfected for pulldown of bound mRNA, followed by RNA sequencing.

Results: miR-486-5p markedly decreased eNOS mRNA and protein in HUVECs ($p<0.0001$) and decreased eNOS protein in human pulmonary microvascular endothelial cells ($p<0.05$), although eNOS was not a direct target of miR-486-5p. miR-486-5p inhibited angiogenesis, which was rescued with eNOS plasmid transfection. RNA sequencing of biotinylated miR-486-5p pulldown RNA revealed highly significant enrichment in predicted targets FOXO1, FOXP1, TNFSF4, MAML3, and CELSR3, and in the non-predicted target SPCS2. RT-qPCR validated these transcripts as inhibited by miR-486-5p. While silencing of FOXO1 had no impact on eNOS protein, MAML3 silencing inhibited eNOS levels.

Conclusion: miR-486-5p inhibits angiogenesis in endothelial cells via eNOS down-regulation, which involves selective targeting of MAML3. These data support a novel pathway regulating endothelial cell function.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs, averaging 22 nucleotides in length, and are evolutionarily conserved post-transcriptional regulators of genes involved in key biological

processes including cell differentiation, growth, and proliferation (1). More than 60% of human protein-coding genes are estimated to be miRNA targets (2). miRNAs primarily regulate protein expression by binding the 3' untranslated region (UTR) of target mRNA to induce post-transcriptional gene silencing via mRNA degradation or inhibition of translation (1). However, other binding sites have been identified within 5' UTRs, introns, and exons of mRNAs as well as non-mRNA targets including long non-coding and circular RNAs (3). In addition, individual miRNA exhibit distinct target binding patterns (3). Not surprisingly, studies aimed at identifying miRNA-mRNA interactions have identified hundreds to thousands of targets per miRNA (4-6).

miR-486-5p is a muscle-enriched miRNA that regulates signaling pathways involved in apoptosis, cell proliferation, migration, and angiogenesis (7). Pre-clinical studies have demonstrated that miR-486-5p improves muscle function in muscular dystrophy (8), protects against skeletal muscle wasting (9, 10) and cardiac ischemia-reperfusion (I/R) injury (11), and attenuates pulmonary (12) and cardiac fibrosis (13). We previously showed that both direct miR-486-5p administration and exosomal transfer of miR-486-5p protected against kidney I/R injury in mice associated with down-regulation of its target gene phosphatase and tensin homolog (PTEN) and genes involved in apoptosis and inflammation (14, 15). miR-486-5p administered early to rats with kidney I/R localized to peritubular capillary endothelial cells and prevented development of late kidney fibrosis and systemic endothelial dysfunction after ischemic kidney injury (16).

Despite its protective short and long-term effects in kidney I/R injury, in rats miR-486-5p unexpectedly inhibited I/R-induced upregulation of kidney endothelial nitric oxide synthase (eNOS) (16), suggesting potential adverse effects on vascular function. In this regard, whether miR-486-5p directly affects angiogenesis is unclear. Administration of miR-486-5p to cultured

human umbilical vein endothelial cells (HUVECs) inhibited its target CADM1, resulting in increased cell permeability and invasion, without affecting angiogenesis (17), while in HUVEC spheroids, miR-486-5p inhibited sprouting angiogenesis (18). In contrast, exosomal miR-486-5p promoted angiogenesis in animal models of myocardial infarction (19), cutaneous wound healing (20), and ischemic stroke (21). Consequently, the effects of miR-486-5p on endothelial cell function are unclear and its mRNA targets remain undefined. Here, we studied the effect of miR-486-5p on eNOS and endothelial cell function *in vitro*. Experiments were conducted in normoxic endothelial cells and with exposure to hypoxia-reoxygenation (H/R) to mimic I/R injury from our *in vivo* studies (14, 16). We also conducted a biotinylated miRNA pull-down assay to identify mRNA targets for miR-486-5p in cultured endothelial cells.

Methods

Cell culture

HUVECs were purchased from American Type Culture Collection (PCS-100-013, ATCC, via Cedarlane Corp., Burlington, ON, Canada), and cultured at 37ºC in 5% CO_2 in EBM2 medium supplemented with microvascular growth factors and 2% FBS (Lonza, Basel, Switzerland, catalog # CC-3156). HUVECs between passages 4-7 were used for all experiments. Human pulmonary microvascular endothelial cells (HPMECs) were purchased from ScienCell™ (Catalog # 3000, Carlsbad, CA, USA) and cultured at 37ºC in 5% CO_2 in Endothelial Cell Medium (ScienCell™ catalog # 1001), supplemented with 5% FBS, growth supplement, and penicillin/streptomycin. Only HPMECs between passages 4-6 were used. For all functional assays and target validation experiments, HUVECs were transfected with 1 nM miRVana™ miR-486-5p mimic or scramble (scb) miRNA in lipofectamine RNAiMax (Thermo Fisher Scientific, Waltham, MA, USA). For miR-486-5p inhibition, HUVECs were transfected with 10 nM

miRVana^{TM} miR-486-5p inhibitor (Thermo Fisher Scientific). For gene silencing, HUVECs were transfected with 5 nM eNOS siRNA, 5 nM FOXO1 siRNA, or 10 nM MAML3 siRNA (SilencerSelect, Thermo Fisher).

For H/R experiments, HUVECs were transfected with miR-486-5p or scb miRNA and after 24 hr were subjected to hypoxia (1% O_2, 5% CO_2, 94% N_2) for 24 hr, followed by re-oxygenation in standard culture conditions for up to 24 hr.

Immunoblot and phospho-kinase array

At 48 hr post-transfection, HUVECs were lysed in radioimmunoprecipitation assay (RIPA) buffer or nuclear and cytoplasmic extraction (NETN) buffer (latter specifically for MAML3 immunoblot). Lysates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked in 5% milk for 1 hr, and incubated for 16 hr at 4ºC with primary antibody against eNOS (#32027), phospho-eNOS (#9571), FOXO1 (#2880), PTEN (#9552), (all 1:1000, Cell Signaling, Whitby, ON, Canada) or MAML3 (NB100-2129; 1:1000; Novus Biologicals, Toronto, ON, Canada). Membranes were incubated with primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH,1:5000, Cell Signaling, #2118) for 1 hr at room temperature. Washed membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000, Abcam, Toronto, ON, Canada) for 1 hr at room temperature, and visualized by chemiluminescence. Densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA).

Screening for relative levels of phosphorylated protein kinases in HUVEC lysates from miR-486-5p or scb miRNA-transfected cells was performed using the Proteome Profiler Phospho-Kinase array kit as per manufacturer's recommendations (Catalog # ARY003C, R&D

Systems Inc, Burlington, ON, Canada). Images were obtained using Image Lab Software (Bio-Rad, Mississauga, ON, Canada).

Luciferase reporter assay

Reporter vectors containing Firefly and Renilla Luciferases with wild type or mutant eNOS 3'UTR were obtained from Genecopeia (Rockville, MD, USA). The pEZX-MT06-eNOS 3' UTR-f/rLuc or mutant eNOS 3' UTR-f/rLuc vectors (50 ng) were transfected into HUVECs in a 96-well plate with Lipofectamine 3000 alone, or with 10 nM miR-486-5p mirVana mimic (Thermo Fisher). Luciferase activity was determined after 24 hr using a Dual Luminescence assay kit (Genecopeia) by an Orion II microplate luminometer (Berthold Detection Systems, Pforzheim, Germany).

5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay

Cell proliferation was evaluated by measuring BrdU incorporation during DNA synthesis (Cell Proliferation ELISA, BrdU (colorimetric), Roche, Laval, QC, Canada) as per the manufacturer's protocol. HUVECs were seeded at a density of $7.5x10^3$ cells per well (normoxia) or $5x10^3$ cells per well (H/R) in a 96-well plate and transfected with 1 nM miR-486-5p, 1 nM scb miRNA, or 5 nM eNOS siRNA in lipofectamine RNAiMax, or with lipofectamine alone. After 24 hr, the media was replaced. Normoxic cells were incubated for another 24 hr, and then labeled with 10μM BrdU. For H/R, the cells were then placed in 1% O_2 for 24 hr, followed by reoxygenation for 24 hr, and then labeled with 10μM BrdU. Normoxic and H/R HUVECs were incubated for 2 hr at 37ºC for labeling. The labeling medium was removed, cells were dried at 60ºC for 1 hr, fixed, incubated with anti-BrdU for 90 min, washed, and incubated with substrate solution. After

15 min, absorbances were read at 370 nm with reference wavelength 492 nm. The data are presented as absorbance 370 nm minus absorbance 492 nm, normalized relative to untreated controls.

Scratch wound assay

HUVECs were seeded at a density of $1x10^4$ cells per well onto a 96-well ImageLock plate (Sartorius, Ann Arbor, MI, USA) coated with an attachment factor (Thermo Fisher Scientific). Cells were transfected with 1 nM miR-486-5p, 1 nM scb miRNA, or 5 nM eNOS siRNA in Lipofectamine RNAiMax or with Lipofectamine alone for 24 hr. After another 24 hr of normoxia or 24 hr of hypoxia (1% O_2), a WoundMaker (Sartorius) was used to create a uniform scratch in each well as per the manufacturer's protocol. The plate was placed in the Incucyte S3 Live-Cell Analysis System (Sartorius), and images for cell migration were acquired every 2 hr for 24 hr. Wound area was determined using Image J (22).

Matrigel network formation assay

HUVECs were seeded into 6 well plates and transfected with 1 nM miR-486-5p, 1 nM scb miRNA, or 5 nM eNOS siRNA in Lipofectamine RNAiMax. To determine whether eNOS rescues *in vitro* network formation, HUVECs were reverse-transfected with miR-486-5p and eNOS plasmid together or with miR-486-5p and mutated eNOS S1179A plasmid. pcDNA3-eNOS-GFP (23) and pcDNA-eNOS S1179A (24) were purchased from Addgene (# 22444 and #22485). *In vitro* network formation assays were performed 48 hr post-transfection. Briefly, each well of a 96 well plate was coated with 50µL of Matrigel (Corning, Bedford, MA, USA) and incubated at 37ºC for 30 min for polymerization. HUVECs were collected by trypsinization,

counted using Trypan Blue with a hemocytometer, and seeded at a density of $1.5x10^4$ cells per well. At 4 hr, 8 hr, and 24 hr post-seeding, Tag Image File Format (TIFF) images of capillary-like networks were captured using a Zeiss Axio Image M2 microscope equipped with digital camera. For hypoxia, cells plated onto Matrigel were incubated in 1% O_2 for 8 hours post-seeding, followed by imaging. Images were not quantified at 24 hr post-seeding (16 hr of reoxygenation) due to significant cell loss in Matrigel coated plates. Images were processed using the Angiogenesis function of ImageJ (25).

Biotinylated miRNA pulldown

3' biotinylated miR-486-5p and 3' biotinylated cel-miR-67 negative control were purchased from Horizon Discovery Biosciences (Waterbeach, Cambridge, UK). HUVECs were seeded into 10 cm^2 plates. The pulldown protocol was adapted from Wani *et al* (26) and Martin *et al* (6). 3'Bi-miR-486-5p or 3'-Bi-cel-miR were transfected in Lipofectamine RNAiMax at a final concentration of 10 nM. After 24 hr, cells were lysed in ice-cold hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol (DTT), 0.5% Igepal CA-630, 60 U/mL Superase-In RNase inhibitor (Thermo Fisher Scientific), and 1x protease inhibitor (MilliporeSigma, Oakville, ON, Canada)). Lysates were placed on dry ice for 5 min and allowed to thaw. Lysates were then centrifuged at 12,000 x g for 2 min at 4ºC to clear debris. The supernatants were transferred to clean 1.5 mL microfuge tubes, and NaCl was added to a final concentration of 1 M.

Streptavidin magnetic beads (Dynabeads MyOne Streptavidin C1, Thermo Fisher Scientific) were prepared on the day of transfection. 125 µL of MyOne C1 beads (per 600 pmol biotinylated RNA) were washed with 1x bead binding and wash buffer (5 mM Tris-HCl (pH

7.5), 0.5 mM EDTA, 1 M NaCl), and made RNase-free by incubating in Solution A (0.1 M NaOH, 0.05 M NaCl), then in Solution B (0.1 M NaCl) (26). Beads were blocked overnight at 4ºC with 1 mg/mL Ultrapure bovine serum albumin and 1 mg/mL yeast tRNA (Thermo Fisher Scientific). The pre-blocked beads were incubated with lysate supernatant for 30 min at room temperature and then washed in hypotonic lysis buffer plus 1 M NaCl. After the last wash, the beads were resuspended in 100 µL nuclease-free water for RNA isolation.

RNA isolation and RNA sequencing

RNA was isolated with the miRNeasy micro kit (Qiagen Inc., Toronto, ON, Canada) with modifications to purify RNAs > 200 nucleotides. Briefly, 700 µL of Qiazol was added to the beads, followed by 140 µL of chloroform. After incubation at room temperature for 3 min, the samples were centrifuged at 12,000 x g for 15 min at 4ºC. For each sample, the aqueous phase was transferred to a new 1.5 mL microfuge tube containing 1x volume of 70% ethanol. The mixture was then transferred to a RNeasy micro-spin column and purified according to the manufacturer's protocol. The RNA was eluted in 25 µL of nuclease-free water. RNA sample quality assessment was performed with the Fragment Analyzer (Agilent) and concentration measured with the Qubit 3.0 (Thermo Fisher Scientific). Next-generation sequencing libraries were prepared with the Ultra II directional RNA kit (New England Biolabs Ltd., Ipswich, MA, USA) using 15 ng of RNA input. Sequencing (RNA-Seq) was performed with the Nextseq 2000 P2 100 cycle flow cell (Illumina, San Diego, CA, USA).

Bioinformatics

After sequencing of pulldown RNA, the libraries were quantified using salmon v1.10.1 (27) in version 3.14.0 of the nf-core RNAseq pipeline (28). The gene/sample count matrix generated from salmon results was loaded into the R statistical analysis package and filtered to remove genes with no detected reads in any sample. Differential expression between biotinylated miR-486-5p and biotinylated cel-miR-67 pulldown RNA was evaluated using DESeq2 (29). Principal component analysis (PCA) was performed using the DESeq2 plotPCA function. Given the expected lower level of biological variability between replicates, the alpha (FDR/q-value) cutoff parameter was set to 0.01. Raw fold changes were moderated using the 'apeglm' method for lfc shrinkage (30) in DESeq2; the presented log2FoldChange values represent these shrunken log fold change estimates. Significantly differentially expressed genes were identified using an adjusted p-value (padj; Benjamin-Hochberg corrected p-value) cut-off of 0.01. Predicted targets of miR-486-5p were obtained from the miRDB target prediction database (31, 32) (downloaded 2024-06-27).

Real-time qPCR

Total RNA was isolated from HUVECs 24 hr post-transfection using the miRNeasy micro kit (Qiagen). Reverse transcription and real-time qPCR for eNOS, FOXO1, FOXP1, TNFSF4, CELSR3, MAML3, SPCS2, and PTEN were performed via TaqMan^{TM} Gene Expression Assays (Life Technologies, Inc., Toronto, ON, Canada) using the Applied Biosystems 7300 real-time PCR system (Foster City, CA, USA). Endogenous GAPDH was used for normalization of mRNA levels. The relative levels of genes of interest were calculated using the $2^{-\Delta\Delta Ct}$ method (33).

Statistical Analyses

All experiments were performed in duplicate or triplicate technical replicates, with at least 3 biological replicates. Results are expressed as mean ± SEM, and statistical comparisons were conducted using one- or two-way analysis of variance (ANOVA) with Tukey's post-test as appropriate. Statistical analyses were performed with GraphPad Prism 10 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $p<0.05$. Statistical analysis of RNA-seq results is described above in 'Bioinformatics'.

Results

miR-486-5p reduces eNOS mRNA and protein levels in cultured endothelial cells

A phosphokinase array was first performed to screen for the impact of miR-486-5p mimic on protein phosphorylation in HUVECs. miR-486-5p decreased the levels of phosphorylated eNOS at serine 1177, but also had an inhibitory effect on the levels of several other phosphorylated kinases (Supplemental Figure 1). We then tested the effect of miR-486-5p transfection on eNOS mRNA and protein in HUVECs. At 1 nM or 10 nM, miR-486-5p markedly decreased eNOS mRNA and protein, as well as phospho-eNOS (S1177) protein levels (Figure 1a,b). We attempted miR-486-5p inhibition in HUVECs with miR-486-5p antagomir, although the Ct values for endogenous miR-486-5p indicated low levels, and a reduction in miR-486-5p with miR-486-5p antagomir was not demonstrated (data not shown). Furthermore, there was no effect of miR-486-5p antagomir on eNOS protein levels, while eNOS silencing confirmed antibody specificity (Figure 1c). Accordingly, further experiments focused on a gain-of-function approach in endothelial cell functional assays. To determine if the inhibition of eNOS was specific to HUVECs, we studied the effect of miR-486-5p in a different endothelial cell line. In cultured

human pulmonary microvascular endothelial cells, miR-486-5p also significantly inhibited eNOS protein levels (Figure 1d). From the miRNA target prediction database (miRDB; https://mirdb.org) eNOS has not been identified as a predicted miR-486-5p target. We therefore conducted a luciferase reporter assay, which demonstrated that miR-486-5p does not directly target the eNOS 3' UTR (Figure 1e).

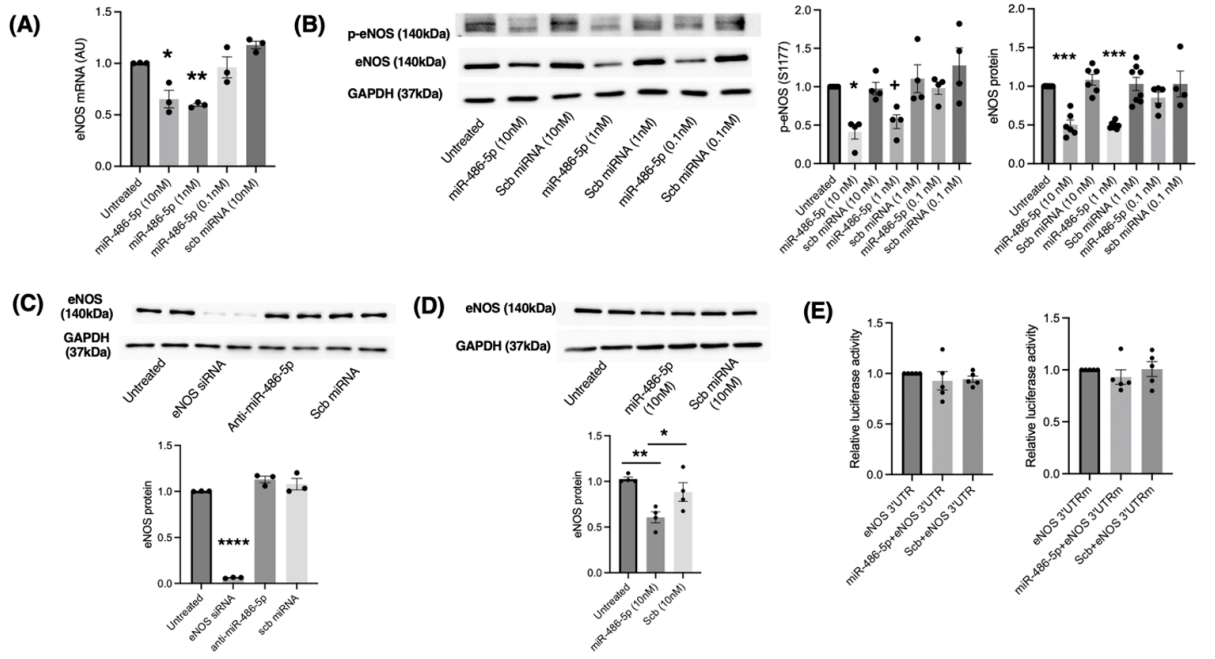

Figure 1. miR-486-5p inhibits eNOS mRNA and protein levels but does not target the eNOS 3' UTR. (A) RT-qPCR for eNOS mRNA in HUVECs, normalized to GAPDH. **p<0.01, *p<0.05 miR-486-5p 10 nM and 1 nM vs untreated, scb miRNA; n=3 **(B)** eNOS and phospho-eNOS (S1177) protein levels in HUVECs. Representative immunoblots and densitometry. Protein levels were normalized to GAPDH. ***p<0.001, miR-486-5p 10 nM, 1 nM vs untreated, scb miRNA (10 nM, 1 nM, 0.1 nM); *p<0.05, miR-486-5p (10 nM) vs untreated, scb (1nM, 0.1 nM); +p<0.05 miR-486-5p (1 nM) vs scb (0.1 nM); n=4-8 experiments. (C) Immunoblot of eNOS protein levels from HUVECs transfected with 5 nM eNOS siRNA, 10 nM miR-486-5p antagomir, or 10 nM scb miRNA. eNOS protein levels were normalized to GAPDH. ****p<0.0001; n=3 experiments. (D) Immunoblot of eNOS protein levels from human pulmonary microvascular endothelial cells (HPMECs) transfected with 10 nM miR-486-5p mimic or scb miRNA. eNOS protein levels were normalized to GAPDH. **p<0.01, *p<0.05; n=4 experiments. (E) Luciferase reporter assay to assess for miR-486-5p binding to the eNOS 3' UTR (left graph) and a mutated 3' UTR construct (right graph); n=5 experiments.

Effect of miR-486-5p on HUVEC proliferation and migration

We evaluated the functional effects of miR-486-5p in HUVECs under normoxic conditions and upon exposure to H/R. In either condition, miR-486-5p had no impact on cell proliferation, although selective knockdown with eNOS siRNA significantly inhibited this process (Figure 2a). HUVECs subjected to H/R exhibited decreased migration during re-oxygenation compared to normoxic cells (Figure 2b). Selective eNOS knockdown significantly inhibited cell migration under both conditions (Figure 2b). In contrast, miR-486-5p had no impact on cell migration in normoxia but tended to decrease migration by 24 hr in H/R (Figure 2b).

Effect of miR-486-5p on network formation (angiogenesis)

miR-486-5p transfection inhibited angiogenesis in normoxic HUVECs, with decreased number of network nodes at all time points post-seeding up to 24 hr (Figure 3a), and decreased network nodes after 8 hr of H/R (Figure 3b). eNOS siRNA transfection had a similar inhibitory effect on angiogenesis in normoxia and with H/R (Figure 3a,b). To determine the impact of miR-486-5p-induced eNOS downregulation on HUVEC angiogenesis, we first treated cells with a nitric oxide (NO) donor to attempt to rescue the inhibitory effect of miR-486-5p. DETA-NONOate releases NO at a slow rate and has been shown to induce a pro-angiogenic response at 10 μM (34). At a concentration of 10 μM, DETA-NONOate did not rescue the inhibitory effects of miR-486-5p or eNOS siRNA, while at a higher dose (100 μM), it inhibited network formation (data not shown). Consequently, HUVECs were co-transfected with miR-486-5p and eNOS plasmid (to restore eNOS expression) for attempted rescue of impaired angiogenesis. eNOS plasmid transfection increased levels of eNOS protein in these cells (Supplemental Figure 2). Co-transfection of eNOS plasmid with miR-486-5p restored angiogenesis, with increased network nodes at 8 hr and

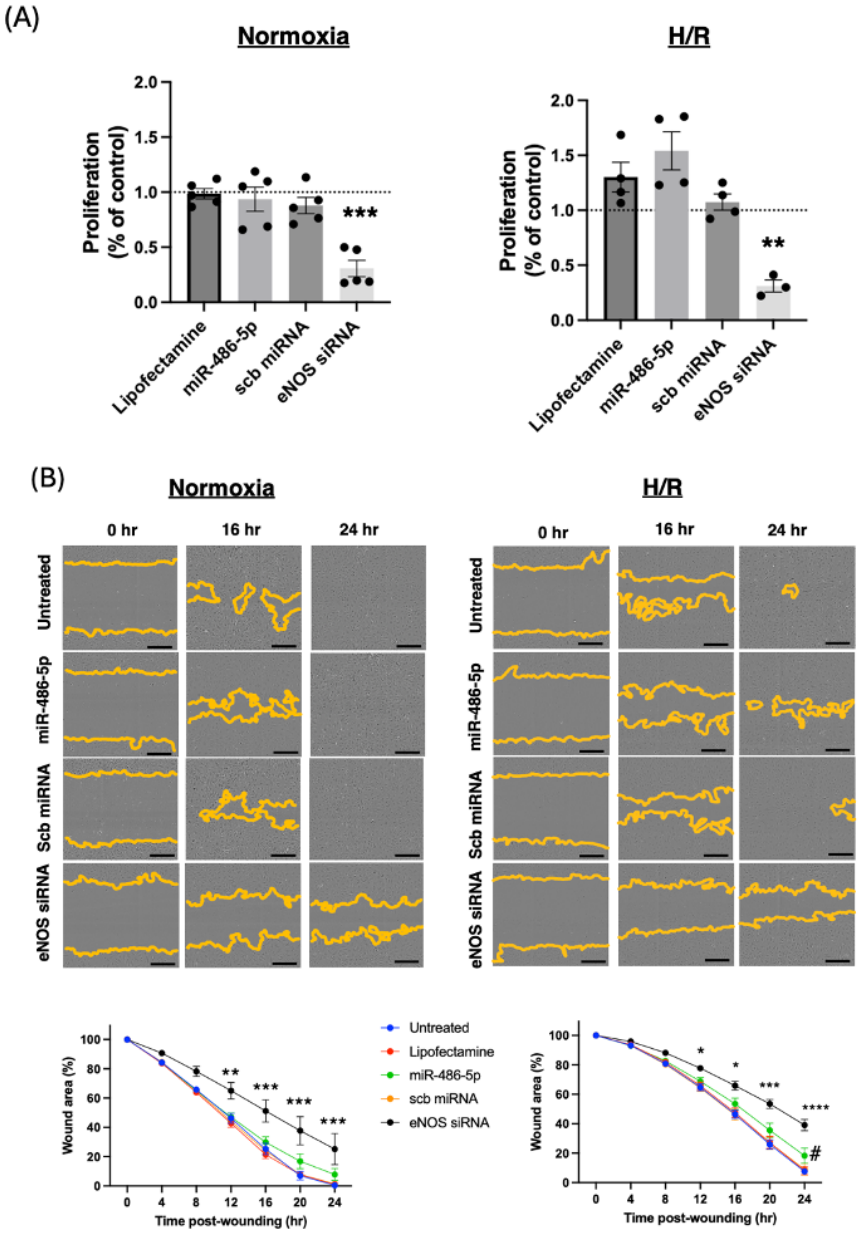

Figure 2. Effect of miR-486-5p on cell proliferation and migration in HUVECs under normoxic conditions or subjected to H/R. (A) Cell proliferation was measured by 5-bromo-2'-deoxyuridine (BrdU) assay. ***p<0.001, **p<0.01 eNOS siRNA vs all groups; n=4-5 experiments. (B) Cell migration was evaluated using a scratch wound-healing assay. Representative images at 0 hr, 16 hr, and 24 hr after wounding are shown, with orange lines indicating the cellular migration front (top). Quantification of wound area closure showed no effect of miR-486-5p in normoxia, but a trend towards inhibition of migration by 24 hr of re-oxygenation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 eNOS siRNA vs all groups;
# p<0.05, miR-486-5p vs untreated, lipofectamine; n=3-5 experiments. Scale bar = 300 μm.

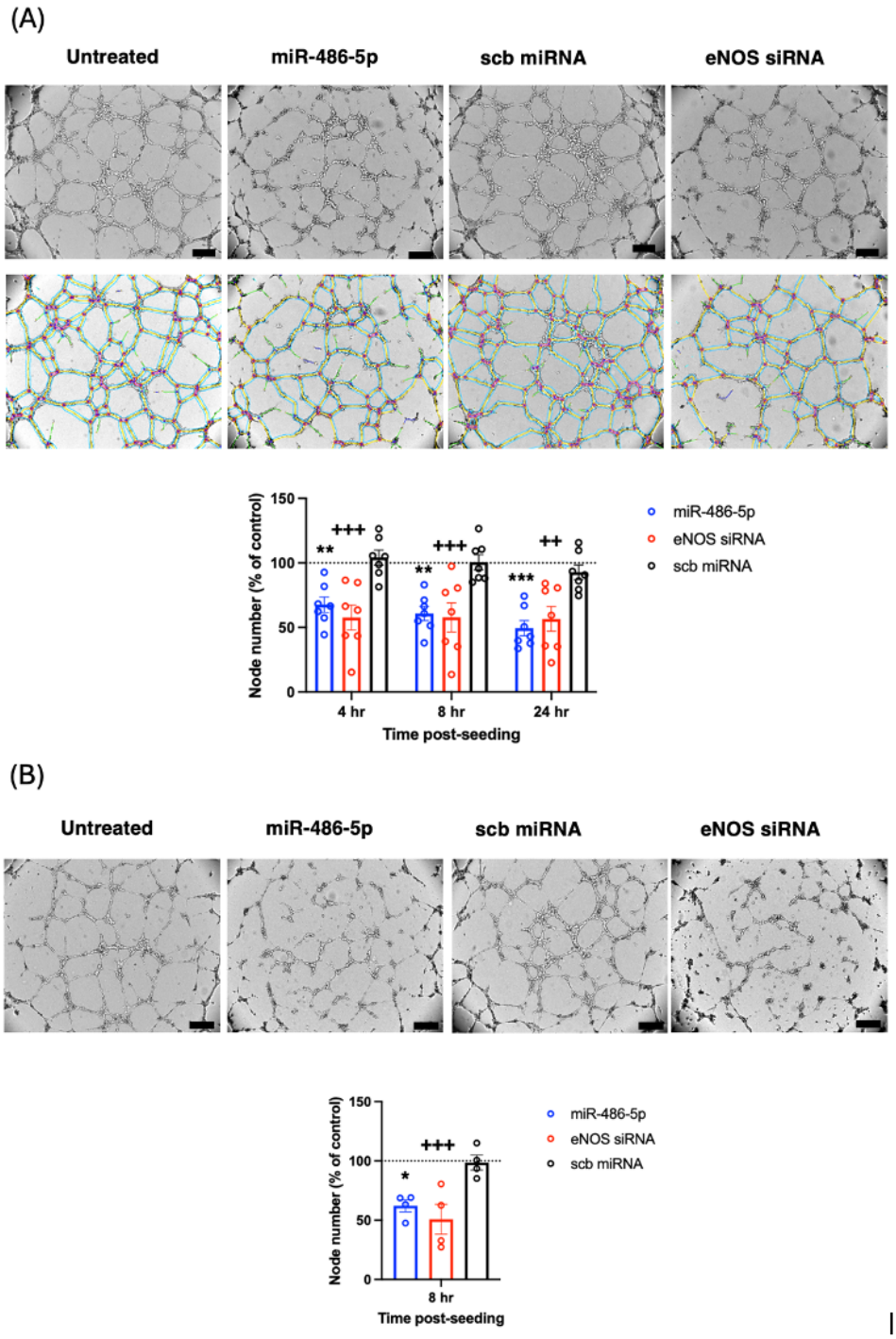

Figure 3. miR-486-5p inhibits network formation in HUVECs in (A) normoxia or (B) subjected to H/R. Representative images 8 hr after seeding HUVECs in Matrigel, with quantification of network nodes. Representative network traces obtained from Image J are shown (A, bottom). Scale bar = 200 μm. *p<0.05, **p<0.01, ***p<0.001, miR-486-5p vs scb; ++p<0.01, +++p<0.001, eNOS siRNA vs scb; n=4-7 experiments (2-way ANOVA and Tukey's *post-hoc* test).

24 hr post-seeding (Figure 4a). In contrast, co-transfection with mutated eNOS at S1179A, lacking the phosphorylation site for activation, did not rescue the anti-angiogenic effect of miR-486-5p (Figure 4b).

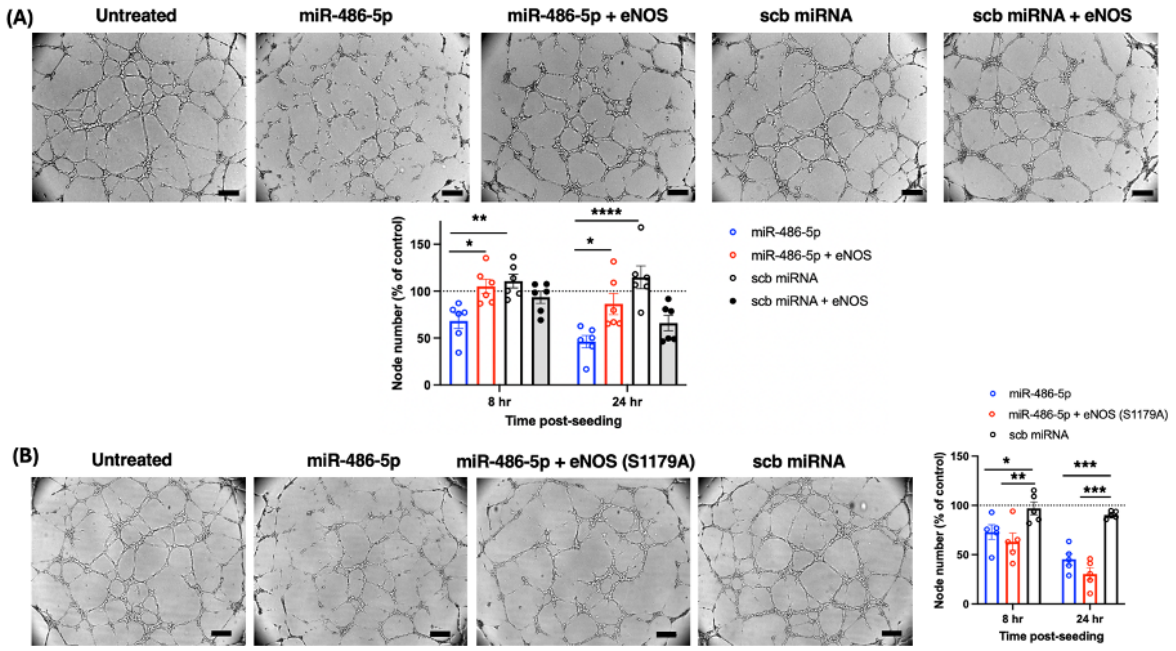

Figure 4. Co-transfection of HUVECs with miR-486-5p and eNOS plasmid restores network formation. (A) Effect of co-transfection with wild-type eNOS (n=6) and (B) effect of co-transfection with mutant inactive eNOS S1179A (n=5). Representative images 8 hr after seeding HUVECs in Matrigel, with quantification of network nodes at 8 hr and 24 hr post-seeding. Scale bar = 200 μm; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (2-way ANOVA and Tukey's *post-hoc* test).

Biotinylated miR-486-5p pulldown identifies miR-486-5p targets in HUVECs

To identify potential targets of miR-486-5p that might mediate eNOS inhibition, HUVECs were transfected with biotinylated miR-486-5p or biotinylated cel-miR-67 negative control for 24 hr, followed by biotinylated RNA pulldown with streptavidin beads and RNA sequencing. Initial transcript mapping with filtering to remove genes that were not detected yielded a total 24,605 genes. Principal component analysis (PCA) revealed a clear separation between the miR-486-5p and control RNA pulldowns (Figure 5). After DESEq2 normalization with a cut-off adjusted p-value of 0.01, filtering out genes with low expression levels (7,631 genes, 31%) and genes with

extreme outlier replicates (126 genes, 0.5%), we retained 2,325 significantly differentially expressed genes. Of these, 1,729 had higher abundance and 596 lower abundance in the miR-486-5p pulldown (Supplemental File 1).

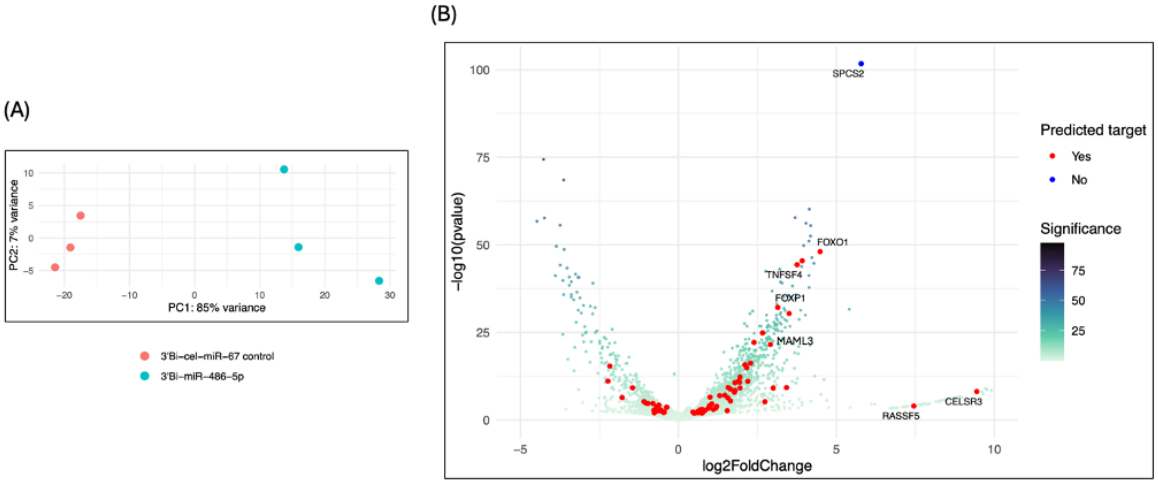

Figure 5. RNA-sequencing of biotinylated miR-486-5p pulldown RNA. (A) Principal component analysis of biotinylated miRNA pulldown RNA-seq data. The data is segregated between miR-486-5p and cel-miR-67 negative control pulldown RNA; n=3 samples per condition. **(B) Volcano plot of differentially expressed genes in miR-486-5p pulldown RNA relative to cel-miR-67 control.** Differential expression is represented as log2 fold change (relative to cel-miR-67). The red dots indicate predicted miR-486-5p targets from miRDB miRNA target prediction database (https://mirdb.org). All smaller points are significantly differentially expressed genes that are not miRDB predicted miR-486-5p targets, coloured according to level of significance. Significance was calculated as –log10(padj).

Comparing RNA-seq results to predicted miR-486-5p targets from miRDB (331 predicted targets), 40 transcripts (12% of predicted targets) were identified as having a fold change significantly different from zero and enriched in the miR-486-5p pulldown compared to control. Thus, the majority of genes (1,689/1,729, 97.7%) identified as enriched in the miR-486-5p pulldown are not found in the miRDB predicted target database. From predicted miR-486-5p targets, the ones most significantly increased in the miR-486-5p pulldown included FOXO1, ZNF37A, TNFSF4, FOXP1, RASSF3, GLIPR2, and GOLGA3, while CELSR3 had the highest fold change (Table 1, Figure 5). Notably, the most statistically significantly enriched transcript

from the miR-486-5p pulldown RNA was SPCS2, which is not a predicted target of miR-486-5p (Figure 5). As expected, eNOS mRNA was not identified in the pulldown.

Table 1: Most significantly enriched transcripts in miR-486-5p pull-down RNA from HUVECs

Predicted miR-486-5p targets			Enriched, not predicted miR-486-5p targets		
Gene	**Log2FC**	**p-value (adj)**	**Gene**	**Log2FC**	**p-value (adj)**
FOXO1	4.48	9.65×10^{-46}	SPCS2	5.78	2.98×10^{-98}
ZNF37A	3.92	3.41×10^{-43}	STK39	4.14	2.83×10^{-57}
TNFSF4	3.76	4.92×10^{-42}	LSM4	3.69	5.86×10^{-55}
FOXP1	3.14	7.31×10^{-30}	MTRES1	4.18	4.94×10^{-50}
RASSF3	3.50	1.03×10^{-28}	SP2	4.13	1.28×10^{-48}
GLIPR2	2.66	2.04×10^{-23}	H3-3B	3.96	2.07×10^{-47}
GOLGA3	2.39	8.47×10^{-21}	NDEL1	4.22	4.50×10^{-44}
MAML3	2.91	3.04×10^{-20}	ETV1	4.29	1.71×10^{-42}
UBASH3B	2.28	3.87×10^{-15}	SRC	3.90	1.24×10^{-41}
FGD6	2.16	5.15×10^{-14}	FMC1	2.76	2.48×10^{-40}
NCOA6	2.16	6.19×10^{-14}	MTF2	4.13	3.61×10^{-39}
MAVS	1.93	5.31×10^{-10}	NABP1	3.23	2.07×10^{-37}
RELT	1.91	2.64×10^{-10}	RHOBTB2	3.61	2.61×10^{-36}
ZNF740	3.42	1.52×10^{-8}	EIF4EBP1	2.96	6.71×10^{-35}
CELSR3	9.44	1.67×10^{-7}	ZFP91	3.48	9.60×10^{-34}

We selected transcripts for validation by qPCR based on their adjusted p-values and log fold changes. HUVECs were transfected with 1 nM miR-486-5p mimic or scb miRNA, and total RNA was isolated after 24 hr. FOXO1, FOXP1, TNFS4, MAML3, CELSR3 and SPCS2 mRNAs were significantly decreased by miR-486-5p (Figure 6). PTEN mRNA, a validated miR-486-5p target reported in several studies including kidney (15) and cardiac I/R injury (11, 35), skeletal muscle disorders (36), and ischemic stroke (21), was not identified in our miR-486-5p pulldown. Although miR-486-5p mimic transfection in HUVECs did not decrease PTEN mRNA across a range of concentrations, a small decrease in PTEN protein levels was observed (Supplemental Figure 3).

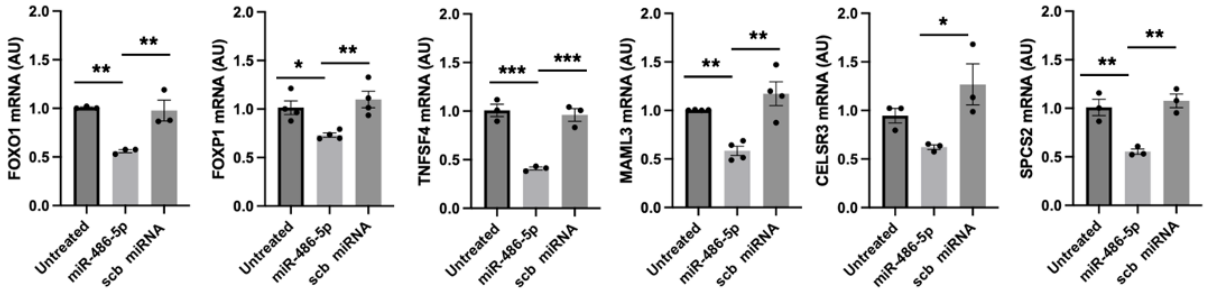

Figure 6. miR-486-5p target validation by RT-qPCR. The levels of 6 enriched transcripts in the pulldown RNA were measured in total RNA from HUVECs transfected with miR-486-5p mimic (1 nM) or scb miRNA (1 nM). All 6 transcripts were decreased by miR-486-5p. *p<0.05, **p<0.01, ***p<0.001; n=3-4 experiments.

Selective knockdown of MAML3 inhibits eNOS protein expression

We used our miR-486-5p pulldown data to probe the link between miR-486-5p and eNOS expression. FOXO1 regulates eNOS transcription (37), and FOXO1 protein levels are significantly inhibited by miR-486-5p (Figure 7a). siRNA-mediated FOXO1 knockdown had no statistically significant impact on eNOS protein levels, although there was a trend towards increased eNOS protein (Figure 7b). MAML3 knockdown inhibits angiogenesis linked to miR-486-5p (18), and MAML3 is a positive regulator of eNOS activation (38). We show that miR-486-5p significantly decreased MAML3 mRNA (Figure 6) and protein levels (Figure 7c). In contrast to FOXO1, siRNA-mediated MAML3 knockdown in HUVECs significantly inhibited eNOS protein expression (Figure 7d).

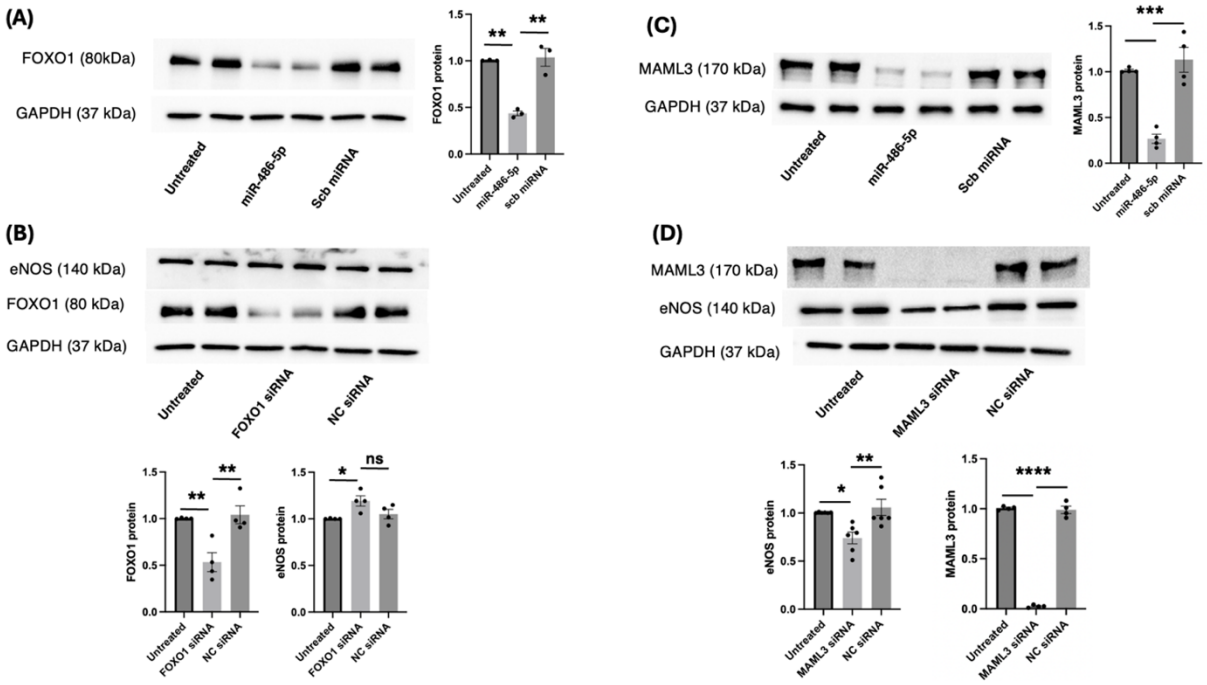

Figure 7. **MAML3 silencing decreases eNOS protein levels in HUVECs.** (A) Immunoblot demonstrating decreased FOXO1 protein levels by miR-486-5p. **p<0.01; n=3 experiments (B) Selective FOXO1 silencing and eNOS protein levels. *p<0.05, **p<0.01, ns=not significant; n=4 experiments (C) Immunoblot demonstrating decreased MAML3 protein levels by miR-486-5p. ***p<0.001; n=4 experiments (D) Immunoblot of eNOS protein from HUVECs treated with MAML3 siRNA demonstrates decreased eNOS protein levels; *p<0.05, **p<0.01, ****p<0.0001; n=4-6 experiments.

Discussion

Angiogenesis is the biological process of new blood vessel formation from existing ones (39). Tight regulation of angiogenesis is critical because while this process is physiological during embryogenesis, wound healing, and I/R injury, it can be pathological in other contexts such as diabetic retinopathy, tumour growth and metastases (39). In this regard, regulatory miRNA networks play an important role in the temporal coordination of angiogenic processes in endothelial cells including proliferation and migration (40). miR-486-5p has been reported to have variable effects on angiogenesis, depending on cell type, target organ or direct delivery versus exosomal transfer (18-21). On the other hand, eNOS, which generates nitric oxide (NO),

is a major regulator of endothelial function (41) and stimulates angiogenesis (42). Here, we demonstrate that administration of miR-486-5p mimic to cultured endothelial cells reduces eNOS protein expression and inhibits angiogenesis. Inhibition of eNOS expression was observed in two cell lines, suggesting conservation of effect across human endothelial cell types. The inhibitory effect of miR-486-5p on angiogenesis was rescued by co-transfecting eNOS plasmid. Although our data cannot completely exclude an effect of miR-486-5p on kinases targeting eNOS phosphorylation (S1177), our phosphokinase array revealed no significant differences in the levels of phosphorylated Akt (S473, T308) or in checkpoint kinase (Chk-2) by miR-486-5p, and kinases targeting eNOS phosphorylation (S1177) were not enriched in the miR-486-5p pull-down RNA. Taken together, these data suggest that the anti-angiogenic effect of miR-486-5p is mediated through inhibition of eNOS expression. Finally, since eNOS mRNA is not a direct target of miR-486-5p, biotinylated miRNA pull-down of transcripts was conducted to identify a potential pathway regulating eNOS expression. We identified MAML3 as a highly enriched target of miR-486-5p, and its silencing significantly inhibited eNOS protein expression. These data suggest a pathway whereby miR-486-5p targets MAML3 mRNA, leading to inhibition of eNOS expression and reduction in angiogenesis.

MAML3 is a nuclear protein that functions as a transcriptional co-activator for Notch signaling, a highly conserved pathway that influences cell fate decisions (43), organogenesis, and tumour angiogenesis (38). Notch signaling activates eNOS during embryonic cardiac development (44). In a mouse model, Patenaude *et al* demonstrated that endothelial Notch was required for tumour growth and perfusion in response to vascular endothelial growth factor (VEGF) by regulating eNOS activation (38). Rosano *et al* showed that long non-coding RNA LINC02802 promotes sprouting angiogenesis in HUVECs by acting as a competing endogenous

RNA for miR-486-5p (18). Furthermore, overexpression of MAML3 counter-acted miR-486-5p's inhibitory effect, supporting a role for Notch signaling in angiogenesis (18). Our data in HUVECs are in concordance with Rosano *et al* (18) although the mechanism by which miR-486-5p targeting of endothelial MAML3 regulates eNOS expression and NOTCH signaling requires further study.

Unexpectedly, we found that miR-486-5p had no significant effect on endothelial cell migration or proliferation while selective eNOS knockdown inhibited both processes. Although we focused on MAML3, individual miRNAs have numerous gene targets. For example, we validated that miR-486-5p decreased FOXO1 mRNA and protein levels, but also showed that silencing of FOXO1 had no statistically significant impact on eNOS protein levels, although there was a trend towards increased eNOS protein levels. FOXO1 is a transcription factor involved in the regulation of cell proliferation, apoptosis, and metabolism (45). Endothelial-specific deletion of FOXO1 in mice increased endothelial cell proliferation whereas overexpression restricted vascular expansion, thus implicating FOXO1 as a regulator of vascular growth (46). In addition, a study of postnatal neovascularization demonstrated that FOXO1 bound the eNOS promoter, inhibited eNOS protein expression, and inhibited angiogenesis in cultured endothelial cells (47). Thus, targeting of FOXO1 by miR-486-5p could plausibly compensate for the functional consequences of miR-486-5p-mediated eNOS inhibition, although the effect of FOXO1 on NO production remains unknown. Consequently, the absence of effect of miR-486-5p on endothelial cell proliferation or migration could be due to targeting of FOXO1 (or other targets) involved in these cellular processes.

Several techniques are available to probe for miRNA-mRNA interactions. High-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) (48)

and photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) (4) identify mRNA fragments bound to Argonaute proteins, but, infer interactions between protein bound mRNAs and miRNAs bioinformatically (6). In contrast, the method used here, involving transfection of biotinylated miRNA followed by streptavidin purification and RNA sequencing of bound mRNA identifies transcripts with a high degree of specificity (49, 50). The number of transcripts enriched in our pulldown RNA is consistent with other studies that used either biotinylated miRNA pulldown (6) or other target identification approaches (4). For instance, Martin *et al* used the biotin pulldown technique on ten miRNAs, identifying an average of 1,500 genes per miRNA that were significantly enriched in the pulldown RNA, with the majority as non-predicted targets (6).

False positive targets could arise from non-specific interactions, but the complexity of miRNA-mRNA interactions also provides a plausible explanation for the large number of transcripts enriched in the miR-486-5p pull-down RNA, exceeding the predicted targets from miRDB (331 transcripts, 40 of which were identified in our pull-down). Similarly, a study by Tan *et al* used biotinylated miR-522 pulldown and identified 547 enriched transcripts, of which only 53 were predicted by TargetScan (49). Although canonical binding involves the miRNA seed in its 5' region (nucleotides 2-7), the presence of imperfect seed matches has been reported (51). Other miRNA responsive elements (MREs) for miRNA/mRNA pairing include miRNA centered sites (52), and extended base pairing within the 3' region of miRNA, known as 3' supplemental binding (53) or 3' compensatory binding (2). MREs have also been identified within 5' UTRs (3) and within coding regions (3, 54). In this regard, Martin *et al* showed that 82.7% of their significantly enriched transcripts contained a seed or centered miRNA binding site, suggesting that this approach identifies miRNA targets with high sensitivity while minimizing false positive

target identification (6). In the current studies, we identified SPCS2 as a highly enriched target, although it is absent from miR-486-5p target prediction databases. However, manual analysis of the SPCS2 3' UTR sequence (UCSC Genome Browser, https://genome.ucsc.edu) reveals that it contains a binding site to the miR-486-5p seed region (position 2-7 from the 5' end of miR-486-5p). Consequently, our miR-486-5p pulldown RNA-seq data are likely to include novel miR-486-5p targets in cultured endothelial cells.

We acknowledge certain limitations of our study due to cell model and other methodologies. HUVECs are macrovascular endothelial cells and are therefore not the ideal model to recapitulate microcirculatory pathophysiology (55) and our study provides limited data on pulmonary microvascular endothelial cells. Biotinylated miRNA pulldown can detect targets that are translationally repressed but is less likely to detect targets that are degraded (6). In this regard, inhibition of PTEN protein by miR-486-5p has been reported in cardiomyocytes (11), skeletal muscle (36), brain microvascular endothelial cells (21), and in HUVECs subjected to H/R and treated with exosomes enriched in miR-486-5p (15). However, PTEN was not enriched in our pulldown RNA. Given there was a trend for decreased PTEN protein levels by miR-486-5p, transcript degradation remains a possibility. Other considerations include the effects of cell type (7, 56) and cellular environment (57) on miRNA-target interactions and cellular functions. In addition, changes in mRNA or protein levels also depend on rates of transcription (58) and protein stability or half-life (59). Finally, administration of exogenous miRNA mimic *in vitro* could account for reported differences in miR-486-5p targets and functions. For example, only exosomal miR-486-5p confers pro-angiogenic effects across diverse experimental models (19-21), in contrast to the inhibitory effects of miR-486-5p mimic in the present studies, suggesting other cargo within exosomes such as the RNA-induced silencing complex (RISC) along with the

transferred miRNA. Transfection of exogenous miRNA mimic *in vitro* delivers supraphysiological levels that compete for the RISC, reducing the availability of miRNA binding proteins for endogenous miRNAs (57). Consequently, administration of exogenous miRNAs may reduce the effectiveness of endogenous miRNA target gene repression (60).

In summary, we show that miR-486-5p inhibits angiogenesis in HUVECs, mediated by down-regulation of eNOS expression. Biotinylated miR-486-5p pulldown identified an enrichment of multiple transcripts in HUVECs, reflecting both predicted and novel targets. Targeting of MAML3 by miR-486-5p contributes to inhibition of eNOS expression and thereby angiogenesis, implicating the Notch pathway as a regulator of eNOS expression and endothelial cell function.

Data availability statement

The data set from the biotinylated miR-486-5p pull-down RNA sequencing is available from the GEO repository (GSE281065). All data are available by contacting the corresponding author.

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Additional information supplemental data figures are provided in Appendix B.

References:

1. Jonas S and Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nature Reviews Genetics* 2015; 16: 421-433. DOI: https://doi.org/10.1038/nrg3965.
2. Friedman RC, Farh KK-H, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research* 2009; 19: 92-105. DOI: https://doi.org/10.1101/gr.082701.108.
3. Broughton JP, Lovci MT, Huang JL, et al. Pairing beyond the Seed Supports MicroRNA Targeting Specificity. *Molecular Cell* 2016; 64: 320-333. DOI: http://dx.doi.org/10.1016/j.molcel.2016.09.004.
4. Hafner M, Landthaler M, Burger L, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 2010; 141: 129-141. DOI: https://doi.org/10.1016/j.cell.2010.03.009.

5. Lal A, Thomas MP, Altschuler G, et al. Capture of MicroRNA–Bound mRNAs Identifies the Tumor Suppressor miR-34a as a Regulator of Growth Factor Signaling. *PLoS Genet* 2011; 7: e1002363. DOI: https://doi.org/10.1371/journal.pgen.1002363.
6. Martin HC, Wani S, Steptoe AL, et al. Imperfect centered miRNA binding sites are common and can mediate repression of target mRNAs. *Genome Biology* 2014; 15: R51. DOI: https://doi.org/10.1186/gb-2014-15-3-r51.
7. Douvris A, Viñas JL and Burns KD. miRNA-486-5p: signaling targets and role in non-malignant disease. *Cellular and Molecular Life Sciences* 2022; 79. DOI: https://doi.org/10.1007/s00018-022-04406-y.
8. Alexander MS, Casar JC, Motohashi N, et al. MicroRNA-486–dependent modulation of DOCK3/PTEN/AKT signaling pathways
improves muscular dystrophy–associated symptoms. *J Clin Invest* 2014; 124: 2651-2667. DOI: https://doi.org/10.1172/JCI73579.
9. Li Z, Liu C, Li S, et al. BMSC-Derived Exosomes Inhibit Dexamethasone- Induced Muscle Atrophy via the miR-486-5p/FoxO1 Axis. *Front Endocrinol* 2021; 12: 681267. DOI: https://doi.org/10.3389/fendo.2021.681267.
10. Xu J, Li R, Workeneh B, et al. Transcription factor FoxO1, the dominant mediator of muscle wasting in chronic kidney disease, is inhibited by microRNA-486. *Kidney International* 2012; 82: 401-411. DOI: https://doi.org/10.1038/ki.2012.84.
11. Bei Y, Lu D, Bär C, et al. miR-486 attenuates cardiac ischemia/reperfusion injury and mediates the beneficial effect of exercise for myocardial protection. *Molecular Therapy* 2022; 30: 1675-1691. DOI: https://doi.org/10.1016/j.ymthe.2022.01.031.
12. Ji X, Wu B, Fan J, et al. The Anti-fibrotic Effects and Mechanisms of MicroRNA-486-5p in Pulmonary Fibrosis. *Scientific Reports* 2015; 5: 14131. DOI: https://doi.org/10.1038/srep14131.
13. Zhao H, Yang H, Geng C, et al. Elevated IgE promotes cardiac fibrosis by suppressing miR-486a-5p. *Theranostics* 2021; 11: 7600-7615. DOI: https://doi.org/10.7150/thno.47845.
14. Viñas JL, Spence M, Porter CJ, et al. micro-RNA-486-5p protects against kidney ischemic injury and modifies the apoptotic transcriptome in proximal tubules. *Kidney International* 2021; 100: 597-612. DOI: 10.1016/j.kint.2021.05.034.
15. Viñas JL, Burger D, Zimpelmann J, et al. Transfer of microRNA-486-5p from human endothelial colony forming cell–derived exosomes reduces ischemic kidney injury. *Kidney International* 2016; 90: 1238-1250. DOI: 10.1016/j.kint.2016.07.015.
16. Douvris A, Viñas JL, Gutsol A, et al. miR-486-5p protects against rat ischemic kidney injury and prevents the transition to chronic kidney disease and vascular dysfunction. *Clinical Science* 2024; 138: 599-614. DOI: https://doi.org/10.1042/CS20231752.
17. Sun B, Han Y and Shi M. Stromal-derived miR-486-5p promotes metastasis of non-small-cell lung cancer cells by targeting the CADM1/tight junctions axis in vascular endothelial cells. *Cell Biol Int* 2021; 45: 849-857. DOI: https://doi.org/10.1002/cbin.11531.
18. Rosano S, Parab S, Noghero A, et al. Long Non-Coding RNA LINC02802 Regulates In Vitro Sprouting Angiogenesis by Sponging microRNA-486-5p. *Int J Mol Sci* 2022; 23: 1653. DOI: 10.3390/ijms23031653.

19. Li Q, Xu Y, Lv K, et al. Small extracellular vesicles containing miR-486-5p promote angiogenesis after myocardial infarction in mice and nonhuman primates. *Sci Transl Med* 2021; 13: eabb0202. DOI: 10.1126/scitranslmed.abb0202.
20. Lu Y, Wen H, Huang J, et al. Extracellular vesicle-enclosed miR-486-5p mediates wound healing with adipose derived stem cells by promoting angiogenesis. *J Cell Mol Med* 2020; 00: 1-15. DOI: 10.1111/jcmm.15387.
21. Bao H, Mao S, Hu X, et al. Exosomal miR-486 derived from bone marrow mesenchymal stem cells promotes angiogenesis following cerebral ischemic injury by regulating the PTEN/Akt pathway. *Scientific Reports* 2024; 14: 18086. DOI: https://doi.org/10.1038/s41598-024-69172-2.
22. Suarez-Arnedo A, Figueroa FT, Clavijo C, et al. An image J plugin for the high throughput image analysis of in vitro scratch wound healing assays. *PLoS ONE* 2020; 15: e0232565. DOI: https://doi.org/10.1371/journal.pone.0232565.
23. Sowa G, Liu J, Papapetropoulos A, et al. Trafficking of Endothelial Nitric-oxide Synthase in Living Cells. *J Biol Chem* 1999; 274: 22524-22531. DOI: https://doi.org/10.1074/jbc.274.32.22524.
24. Fulton D, Gratton J-P, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; 399: 597-601. DOI: https://doi.org/10.1038/21218.
25. Carpentier G. Contribution: angiogenesis analyzer. *ImageJ News* 2012; 5.
26. Wani S and Cloonan N. Profiling direct mRNA-microRNA interactions using synthetic biotinylated microRNA-duplexes. *bioRxiv* 2014. DOI: https://doi.org/10.1101/005439.
27. Patro R, Duggal G, Love MI, et al. Salmon: fast and bias-aware quantification of transcript expression using dual-phase inference. *Nat Methods* 2017; 14: 417-419. DOI: https://doi.org/10.1038/nmeth.4197.
28. Ewels PA, Peltzer A, Fillinger S, et al. The nf-core framework for community-curated bioinformatics pipelines. *Nat Biotechnol* 2020; 38: 276-278. DOI: https://doi.org/10.1038/s41587-020-0439-x.
29. Love MI, Huber W and Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 2014; 15: 550. DOI: https://doi.org/10.1186/s13059-014-0550-8.
30. Zhu A, Ibrahim JG and Love MI. Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics* 2018. DOI: https://doi.org/10.1093/bioinformatics/bty895.
31. Liu W and Wang X. Prediction of functional microRNA targets by integrative modeling of microRNA binding and target expression data. *Genome Biology* 2019; 20: 18.
32. Chen Y and Wang X. miRDB: an online database for prediction of functional microRNA target. *Nucleic Acids Research* 2020; 48: D127-D131.
33. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 2001; 29: e45. DOI: https://doi.org/10.1093/nar/29.9.e45.
34. Ridnour LA, Isenberg JS, Espey MG, et al. Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. *PNAS* 2005; 102: 13147-13152. DOI: https://doi.org/10.1073/pnas.0502979102.

35. Zhu H-h, Wang X-t, Sun Y-h, et al. MicroRNA-486-5p targeting PTEN Protects Against Coronary Microembolization-Induced Cardiomyocyte Apoptosis in Rats by activating the PI3K/AKT pathway. *European Journal of Pharmacology* 2019; 855: 244-251. DOI: https://doi.org/10.1016/j.ejphar.2019.03.045.
36. Alexander MS, Casar JC, Motohashi N, et al. Regulation of DMD pathology by an ankyrin-encoded miRNA. *Skeletal Muscle* 2011; 1: 27. DOI: https://doi.org/10.1186/2044-5040-1-27.
37. Xia N, Strand S, Schlufter F, et al. Role of SIRT1 and FOXO factors in eNOS transcriptional activation by resveratrol. *Nitric Oxide* 2013; 32: 29-35. DOI: http://dx.doi.org/10.1016/j.niox.2013.04.001.
38. Patenaude A, Fuller M, Chang L, et al. Endothelial-Specific Notch Blockade Inhibits Vascular Function and Tumor Growth through an eNOS-Dependent Mechanism. *Cancer Res* 2014; 74: 2402-2411. DOI: https://doi.org/10.1158/0008-5472.CAN-12-4038.
39. Walsh DA. Pathophysiological Mechanisms of Angiogenesis. *Advances in Clinical Chemistry* 2007; 44: 187-212. DOI: https://doi.org/10.1016/S0065-2423(07)44006-9.
40. Rosano S, Corà D, Parab S, et al. A regulatory microRNA network controls endothelial cell phenotypic switch during sprouting angiogenesis. *eLife* 2020; 9: e48095. DOI: https://doi.org/10.7554/eLife.48095.
41. Fulton DJR. Transcriptional and Posttranslational Regulation of eNOS in the Endothelium. *Advances in Pharmacology* 2016; 77: 29-51. DOI: http://dx.doi.org/10.1016/bs.apha.2016.04.001.
42. Smith TL, Oubaha M, Cagnone G, et al. eNOS controls angiogenic sprouting and retinal neovascularization through the regulation of endothelial cell polarity. *Cellular and Molecular Life Sciences* 2022; 79: 37. DOI: https://doi.org/10.1007/s00018-021-04042-y.
43. Wu L, Sun T, Kobayashi K, et al. Identification of a Family of Mastermind-Like Transcriptional Coactivators for Mammalian Notch Receptors. *Molecular and Cellular Biology* 2002; 22: 7688-7700. DOI: https://doi.org/10.1128/MCB.22.21.7688–7700.2002.
44. Chang ACY, Fu Y, Garside VC, et al. Notch Initiates the Endothelial-to-Mesenchymal Transition in the Atrioventricular Canal through Autocrine Activation of Soluble Guanylyl Cyclase. *Developmental Cell* 2011; 21: 288-300. DOI: https://doi.org/10.1016/j.devcel.2011.06.022.
45. Tzivion G, Dobson M and Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim Biophys Acta* 2011; 1813: 1938-1945. DOI: https://doi.org/10.1016/j.bbamcr.2011.06.002.
46. Wilhelm K, Happel K, Eelen G, et al. FOXO1 couples metabolic activity and growth state in the vascular endothelium. *Nature* 2016; 529: 216-220. DOI: **https://doi.org/10.1038/nature16498**.
47. Potente M, Urbich C, Sasaki K-i, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest* 2005; 115: 2382-2392. DOI: https://doi.org/10.1172/JCI23126.
48. Chi SW, Zang JB, Mele A, et al. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature* 2009; 460: 479-486. DOI: https://doi.org/10.1038/nature08170.

49. Tan SM, Kirchner R, Jin J, et al. Sequencing of Captive Target Transcripts Identifies the Network of Regulated Genes and Functions of Primate-Specific miR-522. *Cell Reports* 2014; 8: 1225-1239. DOI: http://dx.doi.org/10.1016/j.celrep.2014.07.023.
50. Tan SM and Lieberman J. Capture and Identification of miRNA Targets by Biotin Pulldown and RNA-seq. *Methods Mol Biol* 2016; 1358: 211-228. DOI: https://doi.org/10.1007/978-1-4939-3067-8_13.
51. Moore MJ, Scheel TKH, Luna JM, et al. miRNA–target chimeras reveal miRNA 3'-end pairing as a major determinant of Argonaute target specificity. *Nature Communications* 2015; 6: 8864. DOI: https://doi.org/10.1038/ncomms9864.
52. Shin C, Nam J-W, Farh KK-H, et al. Expanding the MicroRNA Targeting Code: Functional Sites with Centered Pairing. *Molecular Cell* 2010; 38: 789-802. DOI: https://doi.org/10.1016/j.molcel.2010.06.005.
53. Grimson A, Farh KK-H, Johnston WK, et al. MicroRNA Targeting Specificity in Mammals: Determinants Beyond Seed Pairing. *Mol Cell* 2007; 27: 91-105. DOI: https://doi.org/10.1016/j.molcel.2007.06.017.
54. Zhang K, Zhang X, Cai Z, et al. A Novel Class of MicroRNA Recognition Elements That Function Only in Open Reading Frames. *Nat Struct Mol Biol* 2018; 25: 1019-1027. DOI: https://doi.org/10.1038/s41594-018-0136-3.
55. Chi J-T, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA* 2005; 100: 10623-10628. DOI: https://doi.org/10.1073pnas.1434429100.
56. Kohram F, Fallah P, Shamsara M, et al. Cell type-dependent functions of microRNA-92a. *J Cell Biochem* 2018; 119: 5798-5804. DOI: https://doi.org/10.1002/jcb.26765.
57. Diener C, Keller A and Meese E. The miRNA-target interactions: An underestimated intricacy. *Nucleic Acids Research* 2024; 52: 1544-1557. DOI: https://doi.org/10.1093/nar/gkad1142.
58. Cloonan N, Brown MK, Steptoe AL, et al. The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. *Genome Biology* 2008; 9: R127. DOI: https://doi.org/10.1186/gb-2008-9-8-r127.
59. Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. *Nature* 2011; 473: 337-342. DOI: https://doi.org/10.1038/nature10098.
60. Khan AA, Betel D, Miller ML, et al. Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. *Nat Biotechnol* 2009; 27: 549-555. DOI: https://doi.org/10.1038/nbt.1543.

Chapter 4: Manuscript III

Connection to thesis: This manuscript is a systematic review of dysregulated miRNAs in human AKI. The manuscript identified the most frequently reported dysregulated miRNAs within and across categories of AKI that have potential as AKI biomarkers and as candidates for further mechanistic studies. The manuscript also reports on important limitations that impact the interpretation of results due to study design, methodological, and data normalization factors. Thus, these findings can help inform study design for higher quality clinical studies in this area.

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Author Contributions: Kevin D. Burns conceptualized the study. Risa Shorr, Adrianna Douvris, and Kevin D. Burns designed and conducted the literature search strategy. Adrianna Douvris, José L. Viñas, Shareef Akbari, Karishma Tailor, Dylan Burger and Kevin D. Burns conducted study screening, data extraction and quality assessment in pairs. Adrianna Douvris conducted qualitative data analyses and drafted the initial manuscript. Manoj M. Lalu provided important input on study design and data analysis. Adrianna Douvris, Dylan Burger, Manoj M. Lalu, and Kevin D. Burns contributed to manuscript review and editing. All authors approved the final version. Kevin D. Burns is the guarantor of the manuscript.

Systematic Review of MicroRNAs in Human Acute Kidney Injury

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Abstract

Introduction: Early diagnosis of acute kidney injury (AKI) is limited with current tools. MicroRNAs (miRNAs) are implicated in AKI pathogenesis in preclinical models, but less is known about their role in humans. We conducted a systematic review to identify dysregulated miRNAs in humans with AKI.

Methods: We searched Ovid MEDLINE, Embase, Web of Science, and CENTRAL (August 21, 2023) for studies of human subjects with AKI. We excluded reviews and pre-clinical studies without human data. The primary outcome was dysregulated miRNAs in AKI. Two reviewers screened abstracts, reviewed full texts, performed data extraction and quality assessment (Newcastle Ottawa Scale).

Results: We screened 2,456 reports and included 92 for synthesis without meta-analysis. All studies except one were observational. Studies were grouped by etiology of AKI: cardiac surgery-associated (CS-AKI, n=13 studies), sepsis (n=25), nephrotoxic (n=9), kidney transplant (n=26), and other causes (n=19). In total, 128 miRNAs were identified to be dysregulated across AKI studies (45 miRNAs upregulated, 55 downregulated, 28 both). miR-21 was the most frequently reported (n=17 studies) and it was increased in all etiologies except CS-AKI where it was decreased (n=3 studies). Study limitations included bias due to targeted approaches, absence of clinical data/controls, and miRNA normalization methods. Overall study quality was fair (median 5/9, range 2-8 points).

Conclusion: Dysregulated miRNAs, particularly miR-21, have potential as AKI biomarkers. These results should be interpreted cautiously due to methodological limitations. Standardized methods and unbiased approaches are needed to validate candidate miRNA biomarkers.

Registration: International Prospective Register of Systematic Reviews (PROSPERO CRD42020201253)

Introduction

Acute kidney injury (AKI) is a common complication among hospitalized patients (1) with the highest prevalence in intensive care units (ICU) (2). AKI confers an increased risk of progressive chronic kidney disease (CKD) (3) and mortality (1, 2). Prompt diagnosis may have specific treatment implications since underlying causes of AKI are diverse, including ischemia-reperfusion injury, sepsis, nephrotoxins, and immune-mediated injury.

Currently, the standard for AKI diagnosis is based on serum creatinine concentration (SCr) and/or urine output (4), but important limitations include the lack of sensitivity for early AKI identification, and the inability to distinguish etiology (5). Other AKI biomarkers have been explored to address this gap, including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), insulin-like growth factor-binding protein 7 (IGFBP7), and tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) (5). NGAL, produced primarily by the thick ascending limb of the loop of Henle and collecting duct intercalated cells (5), has been shown to predict AKI in cardiac surgery (6) and in critically ill patients (7). It can also aid in distinguishing pre-renal from intrinsic injury (8). Proximal tubular KIM-1 is increased in response to ischemic and nephrotoxic injury (9). In addition to its biomarker role, KIM-1 has protective effects in ischemic AKI (10). In clinical studies, the product of IGFBP7 and TIMP-2 (termed “Nephrocheck”) was superior to other biomarkers in predicting severe AKI (11, 12). While of potential utility, these biomarkers do not differentiate between causes of intrinsic kidney injury. In this regard, urinary chemokine C-X-C motif ligand 9 (CXCL9) has recently

been validated as a diagnostic biomarker for acute interstitial nephritis (13), a potentially treatable cause of AKI.

MicroRNAs (miRNAs) are non-coding RNAs consisting of 18-22 nucleotides that are evolutionarily conserved across species and that regulate gene expression by post-transcriptional gene silencing (14). miRNAs are secreted in biological fluids including human plasma or serum, and urine (15), and are enriched within circulating extracellular vesicles (16). Accordingly, miRNAs have been studied in humans as potential diagnostic biomarkers for cancers (17, 18), cardiovascular diseases (19, 20), abdominal aortic aneurysm (21), neurodegenerative diseases (22), and non-alcoholic fatty liver disease (23).

miRNAs regulate diverse biological processes related to AKI pathophysiology including apoptosis, cell proliferation, angiogenesis, inflammation, and cell differentiation (14). A systematic review of miRNAs in pre-clinical studies of lab animal models of AKI identified 42 miRNAs with therapeutic potential (24). Of these, miR-21 was the most frequently studied, with gene deletion studies demonstrating exacerbation of kidney injury with increased apoptosis and inflammation (24). While miRNAs have been measured in humans with varying causes of AKI (14, 25), their role as biomarkers or prognostic indicators remains unclear. We therefore conducted a systematic review to identify and characterize the most promising miRNA candidates as biomarkers and potential therapeutic targets in humans with AKI.

Methods

This systematic review was conducted according to our protocol that was published (26) and registered in the International Prospective Register of Systematic Reviews (PROSPERO

CRD42020201253). This report follows the Preferred Reporting items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (see PRISMA checklist, Supplementary file S1).

Eligibility Criteria

We included original research studies (observational and interventional) of human participants evaluating miRNAs in AKI, and excluded reviews, editorials, conference abstracts, and pre-clinical (i.e. lab cell culture and animal based) studies of AKI without human data. Studies of both pediatric and adult populations were considered. The exposure (AKI) was defined by AKIN or RIFLE criteria according to the KDIGO clinical practice guidelines for AKI (4), and comparator groups were human subjects without AKI. We included all etiologies of AKI and excluded patients with end-stage kidney disease receiving renal replacement therapy. The primary outcome included miRNA levels relative to study controls from all biological samples (26).

Search Strategy

The systematic search was performed in Ovid MEDLINE, Embase, and Web of Science. The search strategy included terms related to AKI and miRNAs in human subjects and was constructed in consultation with an information specialist (Risa Shorr, MLS, The Ottawa Hospital Learning Services; full search strategy can be found in Supplementary file S2). The search was originally run July 15, 2020 and last updated August 21, 2023. The search was also supplemented by manually screening the reference list of all included studies, which did not yield additional material.

Study Selection

Search results were uploaded to Covidence (Veritas Health Innovation, Melbourne, Australia). Study screening by titles and abstracts, and full text review was performed by pairs of independent reviewers. Disagreements were resolved by consensus after discussion. Attempts to contact study authors occurred in some instances for clarification.

Data Extraction

Data extraction and quality assessment were performed independently by pairs of reviewers using Research Electronic Data Capture (REDCap) (27). Information included study characteristics, cause of AKI, kidney function details, methods of miRNA measurements (sample type, unbiased or targeted approach, normalization method), and dysregulated miRNAs. Disagreements were resolved by consensus after discussion.

Quality Assessment and Data Synthesis

We used the Newcastle Ottawa Scale (NOS) for the quality assessment of observational studies (28). A meta-analysis of the primary outcome was not performed due to the degree of clinical and methodological heterogeneity between studies (26). Data are instead reported descriptively as a qualitative synthesis, using the SWiM (29) reporting guidelines as a framework, focusing on the methodologies used for miRNA measurements and the identification of dysregulated miRNAs. Studies were categorized according to etiology of AKI. We reported the overall sources of miRNA measurements across all studies and organized sources of miRNAs with respect to AKI etiology. Only validated dysregulated miRNAs were reported for studies that included a validation cohort after initial high-throughput screen. Dysregulated miRNAs within and across AKI etiologies were identified as reported in (i) ≥ 3 studies, (ii) 2 studies, or in single

studies.

Deviations from protocol

As described in the protocol, all observational and interventional published studies evaluating miRNAs in human AKI that met our inclusion criteria were included, but conference abstracts were ultimately excluded. Secondary outcomes associated with miRNA levels (ie kidney function, AKI severity, recovery of kidney function, and in-hospital mortality) were recorded in Supplementary table 1 when available, but not analyzed further due to overall paucity of data. Based on available study data, exploratory outcomes were not evaluated. We were ultimately unable to account for confounders (ie age, medical comorbidities, medications). Finally, meta-analysis was not performed due to clinical heterogeneity (miRNA variant, sample source, AKI etiology).

Results

Search results and overview

Study Design

We identified 2,456 studies (PRISMA diagram, Figure 1). Following screening, 92 studies met eligibility criteria. Detailed information on each study including design, study populations, miRNA measurement methods, miRNA findings, and references is provided in Supplementary Table 1. All studies (with the exception of one randomized controlled trial of remote ischemic preconditioning for cardiac surgery) were observational, including case-control, prospective cohort, and retrospective cohort studies. There were no interventional studies of miRNAs in humans with AKI. The majority of studies (60, 65%) focused on miRNAs as

diagnostic or prognostic biomarkers, while 32 (35%) were mechanistic pre-clinical lab cell culture and animal studies that also contained limited human data. Measurements of kidney function were not reported in 22%, while 32% lacked information on the timing of miRNA measurements.

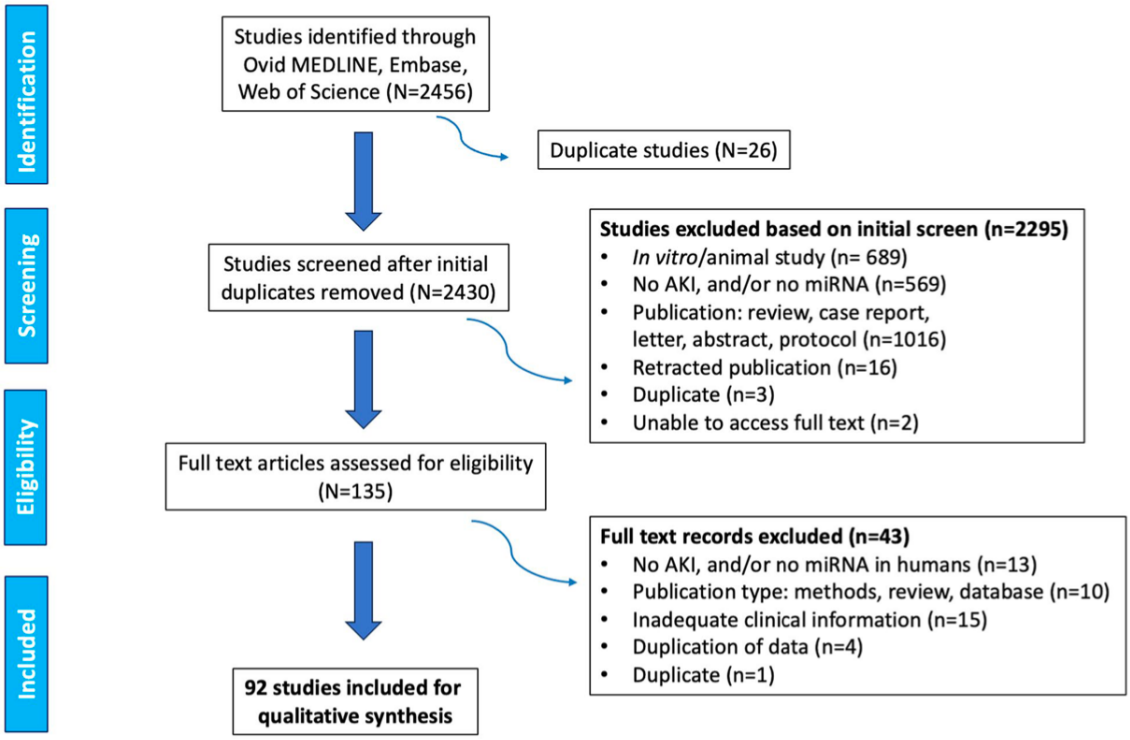

Figure 1. PRISMA flow diagram detailing the number of abstracts and full texts screened and reasons for study exclusion. 92 studies of microRNAs in human AKI were included for qualitative synthesis

The 92 studies were categorized as AKI associated with cardiac surgery (CS-AKI, n=13), sepsis (n=25), nephrotoxins (n=9), kidney transplant recipients (n=26), or other causes (n=19) (Figure 2 and Supplementary Table 1). Within the category of other causes, 12 studies included mixed etiologies of AKI (acute tubular necrosis (ATN), sepsis, nephrotoxic, acute glomerulonephritis (GN), obstruction, pre-renal). The others included acute GN (n=1), acute

obstruction (n=1), hepatorenal syndrome (n=1), cardiorenal syndrome (n=3), and one study did not report on cause of AKI. Most studies were restricted to adult subjects and only 3 included pediatric subjects. Although most studies used appropriate disease controls, within the sepsis-associated and other causes of AKI categories, 40% and 37%, respectively, used control groups comprised exclusively of healthy individuals.

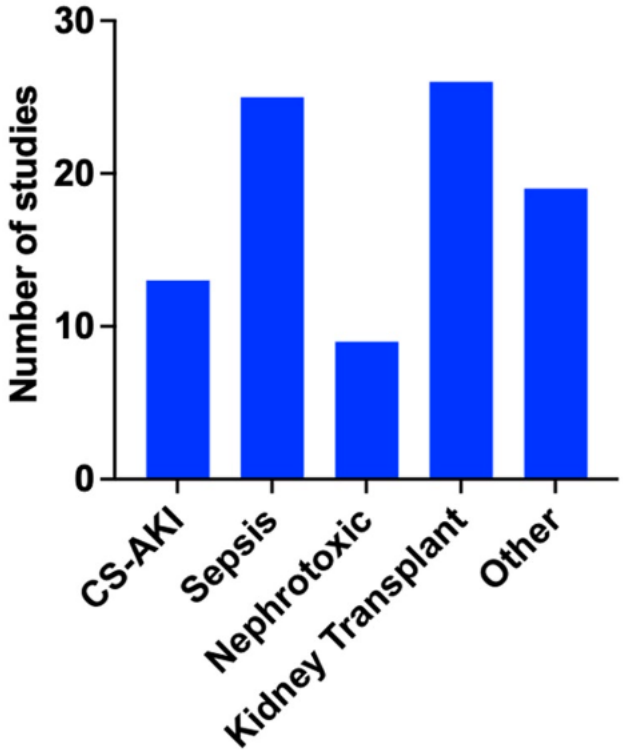

Figure 2. Overview of study groupings ($n = 92$ included studies) within AKI categories. Studies were grouped into distinct AKI categories included AKI associated with cardiac surgery (CS-AKI, $n = 13$), sepsis ($n = 25$), nephrotoxins ($n = 9$), kidney transplantation ($n = 26$), and other causes ($n = 19$). The group of studies under "other" causes of AKI includes studies of mixed causes of AKI, acute glomerulonephritis, obstructive AKI, hepatorenal syndrome, acute cardiorenal syndrome, or unspecified AKI.

miRNA sample sources, measurement methods, and data normalization

Among all studies, the most common sources for miRNA measurements were serum or plasma (n=42), urine (n=27), and kidney tissue (n=16) (Figure 3a). Plasma or serum miRNAs were most commonly reported in CS-AKI, sepsis, nephrotoxic, and other causes of AKI, whereas studies of kidney transplant-associated AKI reported mostly miRNAs from kidney tissue, urine, and peripheral blood mononuclear cells (PBMCs) (Figure 3b).

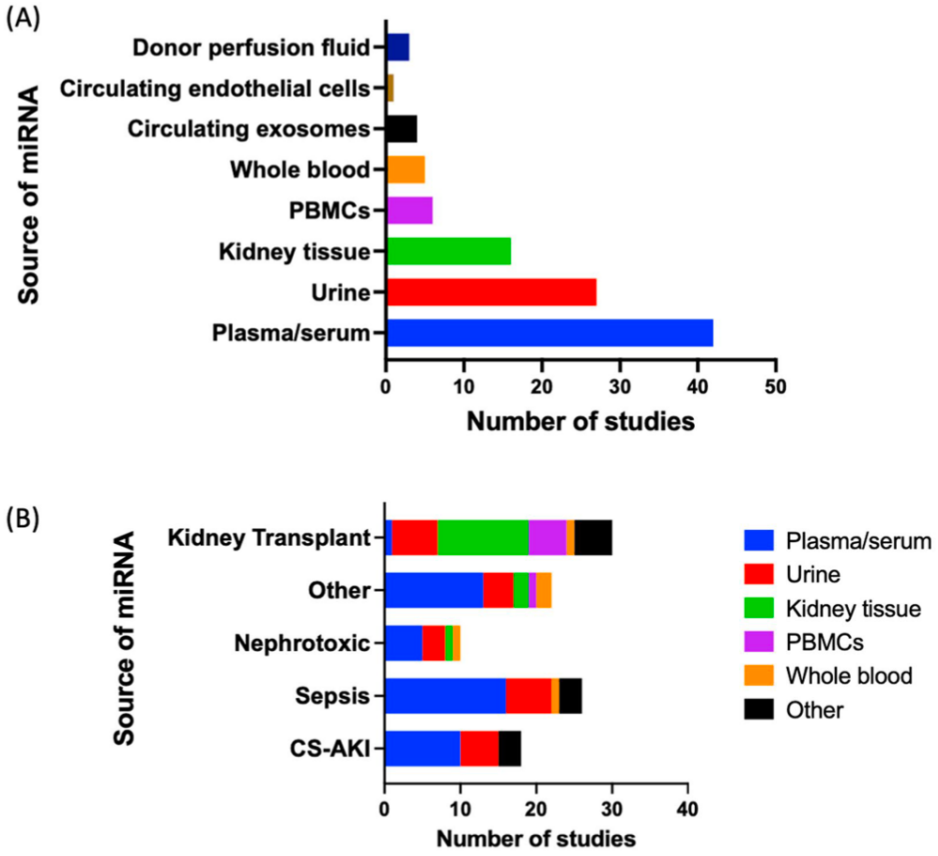

Figure 3. Overview of biological fluids and tissues for miRNA measurements in human AKI. (A) Sample source of miRNAs from all included studies. Of the 92 studies, 13 reported on miRNA levels from more than one source. The majority of urinary miRNAs measurements was from urine supernatant ($n=21$), but studies also used urine sediment ($n=3$) or urine exosomes ($n=3$). PBMCs: peripheral blood mononuclear cells; ECs: endothelial cells (B) Distribution of miRNA sample sources within each kidney injury category. "other" includes circulating exosomes ($n=4$), urinary exosomes ($n=3$), circulating endothelial cells ($n=1$), and donor perfusion fluid ($n=3$). The biological samples used in the individual studies are listed in Supplementary Table 1.

miRNA levels were measured using high-throughput (36%) or targeted (64%) approaches. Of the studies with high-throughput screens, 85% included a validation step with RT-qPCR. The sepsis-associated AKI category included the highest number of targeted studies

(20/25, 80%). The most frequent normalization method for circulating miRNAs was with endogenous U6 (26/60, 43%), followed by exogenous 'spike-in' controls (21/60, 35%), or other endogenous controls (10/60, 17%). For urinary miRNAs, normalization occurred using exogenous (13/27, 48%) or endogenous controls (13/27, 48%), while 3 studies did not report a normalization method.

Most common dysregulated miRNAs in human AKI

We identified 30 miRNAs reported in 3 or more studies (Figure 4a), and 14 miRNAs reported in 2 studies (Figure 4b) across all categories of AKI. miR-21 was the most commonly reported dysregulated miRNA, with up-regulation in AKI due to nephrotoxins (30), kidney transplants (31-35), and other causes (36-38). In contrast, miR-21 was mostly down-regulated in CS-AKI (39-41). In studies of sepsis-associated AKI, miR-21 was either increased (42, 43) or decreased (44, 45). Of note, studies in CS-AKI demonstrated transient miR-21 dysregulation. For instance, baseline (pre-surgery) miR-21 was decreased in patients who developed CS-AKI (39, 40). Post-operatively, serum and urine miR-21 levels were lowest after 6 hours, and gradually rose over 24 hr, suggesting transient down-regulation. Kang *et al* performed remote ischemic preconditioning in pediatric cardiac surgery and demonstrated protection against CS-AKI, associated with transient up-regulation of miR-21 over 24 hr post-operatively, followed by a return to baseline by 5 days (41).

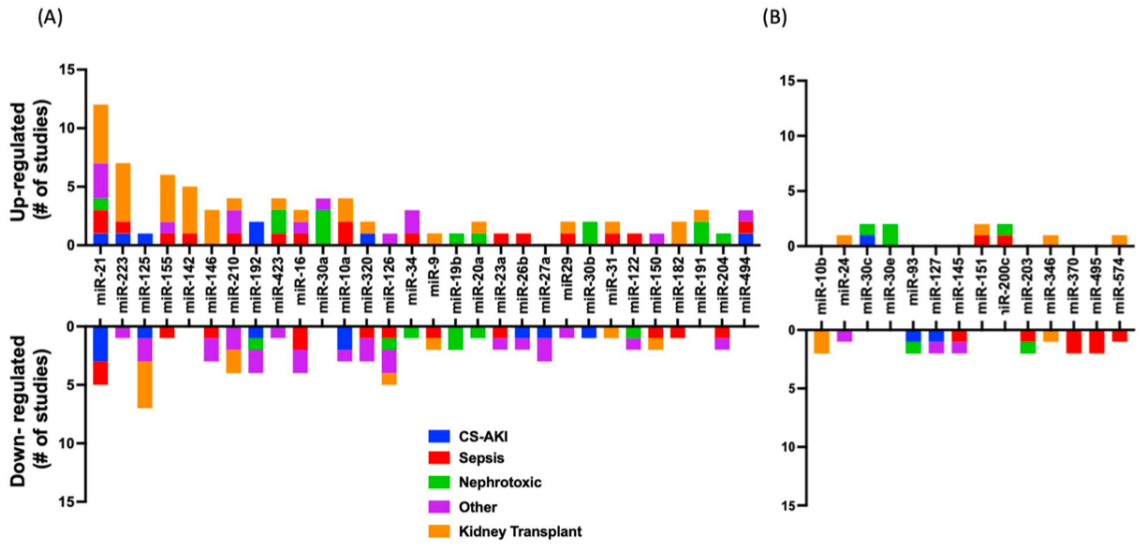

Figure 4. Dysregulated miRNAs in humans with AKI. (A) miRNAs reported in at least 3 studies. (B) miRNAs reported in two studies. Top: miRNAs up-regulated in human AKI. Bottom: miRNAs down-regulated in human AKI. The number of studies is represented by the Y-axis, while the X-axis represents the miRNA of interest. AKI: acute kidney injury. CS-AKI: cardiac surgery-associated AKI

miR-423 was up-regulated in AKI due to nephrotoxins (30, 46), sepsis (43), and kidney transplants (47), and down-regulated in cardiorenal syndrome (48). miR-223 was up-regulated in CS-AKI (49), kidney transplants (34, 35, 47, 50, 51), and sepsis (44), while miR-30a was increased in nephrotoxic AKI (46, 52, 53) or AKI due to other causes (36). miR-155, implicated in immune system function (54), was increased in acute transplant rejection (31, 34, 51, 55) and in patients with acute GN (56), and either increased or decreased in sepsis-associated AKI (42, 57). Similarly, miR-142 was increased in acute rejection (51, 55, 58), but also in kidney transplant recipients with delayed graft function (58) and unspecified graft dysfunction (50), and in sepsis-associated AKI (59). miR-16 was both increased and decreased in AKI associated with sepsis (44, 59, 60) and other causes (36, 61, 62), and increased in delayed graft function in kidney transplantation (63). Changes in miR-16 levels in AKI were dependent on the biological fluid sampled for miRNA measurement, with increased levels in urine, and decreased levels in plasma/serum. miR-192 was both increased and decreased in CS-AKI (49, 64, 65), and decreased in AKI associated with nephrotoxins (66) and other causes (36).

The most frequently reported down-regulated miRNAs were miR-126 and miR-125. miR-126 was down-regulated across several categories including AKI due to sepsis (67), nephrotoxins (68), kidney transplants (35), or other causes (69, 70). miR-125 was down-regulated in transplant-associated AKI (31, 35, 71, 72), CS-AKI (73), and AKI due to other causes (74, 75). Lastly, of the miRNAs identified from at least 2 studies, 70% were reported as up- or down-regulated in AKI. In nephrotoxic AKI, two studies showed that the source of biological fluid impacted the outcomes as levels of miR-19b and miR-20a changed in opposing directions between serum and urine (68, 76).

Table 1 summarizes the most prominent dysregulated miRNAs in AKI, reported in 5 or more studies, highlighting the sample sources, biological functions, and potential clinical applications.

Table 1: Most prominent differentially expressed miRNAs in human AKI

miRNA	AKI category	miRNA source	Biological role	Clinical relevance
miR-10a	Transplant[33, 77] Sepsis[44,78], CS-AKI[49, 73], Other[70]	Urine, Plasma/ serum	Aggravates kidney IR injury in mice, increases apoptosis[79]	Potential biomarker
miR-16	Sepsis[44,59,60], Other[36,61,62], Transplant[63]	Urine, Plasma/ serum	Increases tubular cell apoptosis and kidney injury[80]	Potential urinary biomarker
miR-21	CS-AKI[39-41] Transplant[31-35] Other[36-38]	Serum, Urine, Kidney	Increased by ischemic preconditioning[41], Inhibits apoptosis and inflammation in AKI[81,72]	Potential biomarker: peri-operative levels for CS-AKI, DGF
miR-125	Transplant[31, 35, 7`, 72], Other[74, 75]	Kidney, PBMCs, Serum	Cell adhesion, proliferation, immune responses[83]	Potential biomarker for DGF and AR
miR-126	Transplant[35] Nephrotoxic[68] Sepsis[67] Other[33,69,70]	Kidney, Plasma/ serum, Urine	Attenuates kidney IR injury and microvascular damage[84]	Potential marker of vascular injury and therapeutic target
miR-142	Transplant[50, 51, 55, 58] Sepsis[59]	Kidney, PBMCs, Urine	Deficiency may promote allograft tolerance; regulator of T_{reg} cell homeostasis[85]	Potential biomarker for acute graft dysfunction and therapeutic target

miR-146	Transplant[31, 86, 87] Other[37, 70] Sepsis[44]	Kidney, Urine, Plasma/ serum	Decreases inflammation via inhibition of IRAK1 and CXCL8[86]	Potential therapeutic target for kidney IR injury
miR-155	Transplant[31, 34, 51, 55] Acute GN[56]	Kidney, Urine	Adaptive immunity, T cell response[56]	Potential biomarker for TCMR and therapeutic target
miR-192	CS-AKI[49, 64, 65] Other[36, 38] Nephrotoxic[66]	Urine, Plasma/ serum	Promotes kidney injury[88], mediates tubular cell growth arrest[89]	Potential early biomarker for CS-AKI
miR-210	Transplant[34, 55, 77], Other[36, 37, 61, 70], Sepsis[44]	Urine, Plasma/ serum	Stimulates angiogenesis, inhibits inflammation[90]	Potential biomarker for AR, predictor of long-term graft function
miR-223	Transplant[34, 35, 47, 50, 51], Sepsis[44], CS-AKI[49]	Kidney, PBMCs	Decreases inflammation and pyroptosis in septic AKI[91]	Potential biomarker for AR
miR-320	Sepsis[44], Other[36, 61], Transplant[72], CS-AKI[49]	Kidney, Plasma/ serum	Kidney levels increased in IR injury[92]	Potential biomarker for AKI
miR-423	Nephrotoxic[30, 46],Transplant[47] Sepsis[43] Other[48]	PBMCs, Urine	Pro-apoptosis, endoplasmic reticulum and oxidative stress[93]	Potential biomarker for nephrotoxic AKI

Abbreviations: AKI: acute kidney injury; AR: acute rejection; CS-AKI: cardiac surgery associated AKI, CXCL8: C-X-C chemokine motif ligand 8; DGF: delayed graft function; GN: glomerulonephritis; IR: ischemia reperfusion; IRAK1: interleukin 1 receptor associated kinase 1; PBMCs: peripheral blood mononuclear cells, TCMR: T-cell mediated rejection

Dysregulated miRNAs in human AKI reported only once

The majority of dysregulated miRNAs in human AKI (84/128, 66%) were identified from single studies both within and across kidney injury categories. Of these 84 miRNAs, 17 (20%) were reported from targeted studies, with the majority identified from high-throughput screening. The miRNAs reported only once are listed in Table 2, separated into kidney injury category.

Table 2: **Differentially expressed miRNAs in human AKI reported only once**

Kidney Injury Category	Up-regulated	Down-regulated
CS-AKI	miR-487a, -490, -688, -301, -106b	miR-152, let-7g

Sepsis	miR-4321, -4270, -107, -143, -214, -497	miR-22, -205, -290, -590, -580, -671, -886, -299, -557, -376b, -335, -4299, -136, -342
Nephrotoxic	miR-188, -512, -3168, -6125, -4718, -660	miR-19a, -3187, -17, -30d, -92a, -106a, -195, -451, -484
Other	miR-489, -216, -709, -141, -556	miR-329, -200a, -129, -5100, -2861, -15a, 652, -199a, -101, let-7i
Kidney Transplant	miR-381, -629, -658, -100, -99a, -650, -221, -429, -503, -33a, -98, -373, -18a, -181d, -218	miR-663, -326, -324, -217, -187, -148, -23b, -147, -383, -181b, -4488, -4532

Dysregulated miRNAs in kidney transplant-associated AKI

Kidney transplant-associated AKI is a unique category that includes distinct etiologies, reflecting ischemic, immunologic, nephrotoxic, vascular, and/or infectious insults (31-33, 94) as well as acute rejection(31, 34, 35). MiRNAs in transplant studies were sampled from several sources, including kidney tissue, urine supernatant, or urinary exosomes. In acute rejection, urinary levels of miR-155 (31, 34, 55) and miR-223 (34, 35, 47, 51, 55) were up-regulated, while miR-210 was downregulated (55). Lorenzen *et al* found that urinary miR-210 levels were inversely correlated with severity of acute rejection, and normalized following successful treatment, while lower miR-210 levels associated with long-term decline in graft function (77). Wilflingseder *et al* compared graft biopsies from delayed graft function (DGF), T cell-mediated rejection (TCMR), and antibody-mediated rejection and found that miR-155 up-regulation was unique to TCMR (31). miR-125 was down-regulated in both DGF (71) and acute rejection (31, 35, 72) while all other down-regulated miRNAs in transplant AKI were only reported with acute rejection (Figure 5).

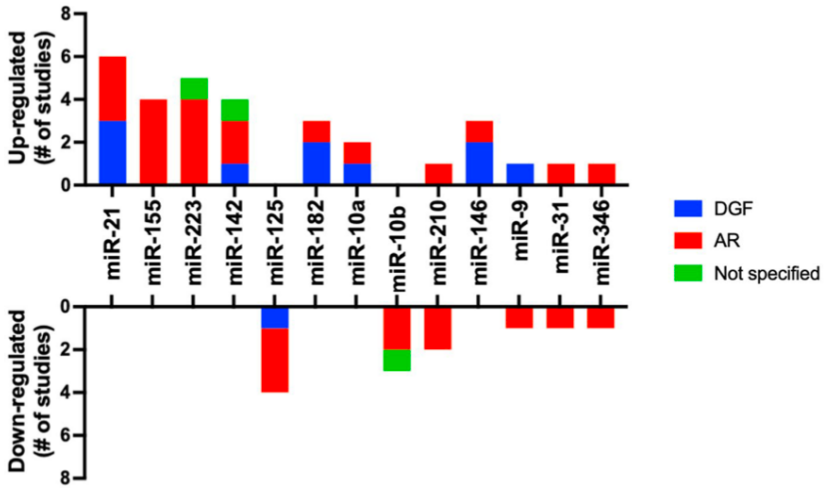

Figure 5. Dysregulated miRNAs in kidney transplant recipients with acute graft dysfunction. Graph depicts miRNAs reported in at least 3 studies. Top: miRNAs up-regulated in acute graft dysfunction. Bottom: miRNAs down-regulated in acute graft dysfunction. AR: acute rejection; DGF: delayed graft function

Quality Assessment and Risk of Bias

Quality assessment was performed using the Newcastle Ottawa Scale (28), which evaluates study population selection, study group comparability, outcome assessment and follow-up using a 9 point scale. Figure 6 depicts the overall quality assessment scoring (scoring details can be found in Supplementary Table 1). Studies that scored ≥7 points were considered 'good quality', while ≤3 points were ranked as 'poor quality'. The overall study quality was fair and varied between AKI categories, with a median score of 5/9 (range 2-8 points). CS-AKI, nephrotoxic AKI, and kidney transplant categories had the highest quality scores, with a median score of 6 for each. No studies scored 3 points or less within the CS-AKI and nephrotoxic AKI categories. Sepsis-associated AKI and AKI due to other causes each included a higher proportion of lower quality studies, with median scores of 5 and 4, respectively.

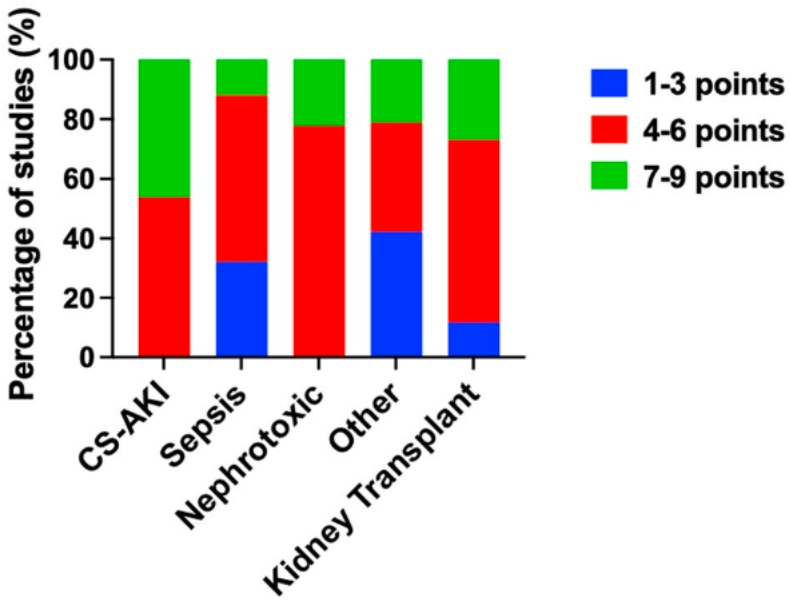

Figure 6. Quality assessment scoring using the Newcastle Ottawa Scale. Graphical representation of the percentage of studies within each AKI category (Y-axis) scoring 1-3 points (considered poor quality), 4-6 points, and 7-9 points (considered good quality). AKI: acute kidney injury. CS-AKI: cardiac surgery-associated AKI

Discussion

This systematic review provides an overview of dysregulated miRNAs in humans with AKI. We performed qualitative synthesis of 92 studies that met inclusion criteria and categorized them as AKI due to cardiac surgery, sepsis, nephrotoxins, kidney transplant, or other causes. Our results reveal distinct dysregulated miRNAs across all AKI categories, with the most frequently reported being miR-21, although the quality of studies was modest and methodological weaknesses limit the strength of current evidence for clinical application. Consequently, these dysregulated miRNAs may represent candidates for validation as prognostic biomarkers.

A recent systematic review and meta-analysis of miRNAs in human AKI by Brown *et al* (95) highlighted 3 dysregulated miRNAs (miR-21, -370, -495), which were also identified in our review (Figure 4). Similar to our systematic review, Brown *et al*. found that the majority of

differentially expressed miRNAs were reported in no more than one study and noted that methodological differences could contribute to the lack of reproducibility. However, our systematic review differs in certain respects, including more expansive inclusion criteria and our method of data analysis. Notably, we also reported on dysregulated miRNAs in kidney transplant recipients with acute graft dysfunction, an area of active biomarker research. Further, the data in our review are presented as a detailed narrative synthesis, since the substantial clinical and methodological heterogeneity across studies did not justify a formal meta-analysis.

miR-21 was the most frequently dysregulated miRNA in AKI studies across etiologies: transiently decreased in CS-AKI but increased in sepsis and kidney transplant AKI. In children undergoing cardiac surgery, Kang *et al* showed that remote ischemic preconditioning protected against CS-AKI and was associated with increased urinary and plasma miR-21 levels (41). In kidney transplantation, perfusion fluid miR-21 levels correlated with kidney function at 6 and 12 months post-transplant (96). A systematic review of the therapeutic effects of miRNAs in animal models of AKI identified miR-21 as the most promising therapeutic target, impacting apoptosis and inflammation (24). In mice, Xu *et al* demonstrated that kidney ischemia induced miR-21 upregulation, which inhibited programmed cell death protein 4 (PDCD4) (a pro-apoptotic miR-21 target gene) (97) contributing to the renoprotective effect of delayed ischemic preconditioning (81). Exosomal miR-21 also protects against septic AKI in pre-clinical studies, associated with inhibition of inflammation and apoptosis (82). While there are no interventional studies of miR-21 therapy in humans with AKI, it is noteworthy that the effects of an anti-miR-21 oligonucleotide (lademirsen) were recently reported in a Phase 2 clinical trial for adults with Alport syndrome (98). Although the study did not demonstrate therapeutic benefit, serious adverse events did not occur, supporting the feasibility of translating miRNA therapy to clinical

trials. In summary, our review reveals prominent dysregulation of miR-21 in human AKI, in agreement with pre-clinical studies (24). These data support the evolutionary conservation of miRNAs and their biological targets across species. Consequently, enhancing kidney miR-21 may be a therapeutic strategy for human AKI, and miR-21 could be a biomarker in CS-AKI.

miR-423 and miR-30a were most frequently up-regulated in nephrotoxic AKI. miR-423 was also up-regulated in sepsis-associated and kidney transplant AKI but decreased in acute cardiorenal syndrome. In cultured proximal tubular cells exposed to hypoxia-reoxygenation, miR-423 has been shown to increase apoptosis (99), while administration of miR-423 to rats with kidney ischemia increases endoplasmic reticulum stress and oxidative stress (93). Increased urinary miR-30a levels correlate with gentamicin-induced AKI in canines (100), and, sponging of miR-30a by long non-coding RNA protects against ischemic AKI in mice (101). The results of our review suggest that miR-423 and miR-30a have potential as biomarkers for nephrotoxic AKI and may be mediators of kidney injury.

miR-223 was up-regulated in CS-AKI, and in AKI due to sepsis or kidney transplantation. *In vitro* and pre-clinical studies have suggested protective effects of miR-223 in septic AKI (102, 103), although the role of dysregulation in kidney transplant dysfunction is unknown. On the other hand, our review uncovered a group of 3 miRNAs (miR-155, miR-142, miR-146) that were up-regulated in kidney transplant recipients with acute graft dysfunction. Of these, miR-155 was up-regulated in TCMR and increased in sepsis-AKI and acute GN due to anti-neutrophil cytoplasmic antibody (ANCA) vasculitis. Krebs *et al* demonstrated increased miR-155 expression in the kidneys of patients with ANCA vasculitis, with localization within infiltrating inflammatory cells, and also showed decreased pathogenic T_{h17} response and attenuated nephritis in miR-155-deficient mice (56). Serum levels of miR-155 increase in

humans with AKI due to sepsis, with a sensitivity of 91.1% and specificity of 84.5% for diagnosis, and levels associate with AKI severity and prognosis (104). Thus, miR-155 may be a potential biomarker for acute TCMR and sepsis-associated AKI, and its inhibition may represent a potential therapeutic target.

miR-142 and miR-146 are implicated in immune system regulation. In experimental heterotopic cardiac transplantation, T-cell-specific miR-142 deficiency promoted cardiac allograft survival in the absence of immunosuppression medications, associated with absent donor-specific antibodies and an increase in the population of regulatory T cells (85). In contrast, miR-146 is induced by IR injury and attenuates the inflammatory response via targeting interleukin-1 receptor associated kinase 1 (IRAK1), inhibiting the C-X-C motif ligand 8 (CXCL8) pathway (86). A possible common pathway for miR-155, miR-142, and miR-146 involves interleukin-6 (IL-6) signaling, which also increases pathogenic Th_{17} cells, inhibits the development of regulatory T cells, and is associated with TCMR and ABMR in kidney transplantation (105). Experimental and clinical studies of anti-IL-6 therapies for ABMR have demonstrated inhibition of donor-specific antibody development with induction of regulatory T cell responses (106, 107). Consequently, miR-155, miR-142 and miR-146 may be mediators of immune system regulation in acute rejection, possibly linked to IL-6 signaling.

Lastly, miR-126 and miR-125 were down-regulated across multiple AKI categories. miR-125 was most frequently reported in transplant-associated AKI, whereas miR-126 was studied in transplant, sepsis, nephrotoxic, and other causes of AKI. miR-126 is enriched in endothelial cells, and, circulating miR-126 levels are decreased in ANCA vasculitis and in patients with end-stage kidney disease on dialysis, suggesting it may be a marker of vascular injury (108).

Administration of miR-126 attenuates ischemic kidney injury in animal models, associated with microvascular protection (84, 109). Thus, miR-126 holds potential as a biomarker of AKI and vascular injury in humans, and as a therapeutic target.

There are several strengths to our study. We conducted a comprehensive search strategy of all AKI etiologies, and performed rigorous data extraction, with a requirement for two independent reviewers at each step. The profiles of dysregulated miRNAs can be used to identify commonalities and differences amongst AKI categories, which may represent candidate miRNAs for validation as prognostic or diagnostic biomarkers in AKI. This study also identified frequently reported and under-studied miRNAs that may have pathogenic or protective mechanisms of action in AKI, and therefore could represent therapeutic candidates.

We acknowledge certain weaknesses that warrant caution when interpreting the findings. Formal meta-analysis for select differentially expressed miRNAs could not be performed, and data on Receiver Operating Characteristic (ROC) curves was not independently analyzed. Methodological weaknesses in many of the studies must be acknowledged. For example, the reporting of dysregulated miRNAs was biased by the large number of studies that targeted specific miRNAs. Specifically, miR-21 was frequently selected for analyses across targeted studies. Due to the large number of studies that performed an initial screen followed by validation of candidate miRNAs, we only reported validation data in our study. The majority of identified miRNAs were also derived from single studies, although most of those studies used an unbiased approach. Further, we identified many pre-clinical studies with limited human data. Several studies (notably in septic AKI) used healthy volunteers as controls rather than more appropriate disease controls. Further, the timing of AKI diagnosis and miRNA measurements was not clearly specified in nearly one third of studies, thereby limiting the utility of miRNAs as

AKI diagnostic biomarkers. Finally, while most studies controlled for age and sex, the absence of other clinical information increased the potential for residual confounding.

Concerns regarding miRNA isolation and measurement methods also deserve mention. The wide variety of biological sources for miRNA measurements, the effect of timing of sample collection, and lack of standardization of collection and storage practices can all impact data reproducibility (110). Some miRNAs are enriched within erythrocytes and hemolysis may impact the circulating miRNA profile (111), and most studies did not report on the possible effects of hemolysis. Data normalization, a process used in RT-qPCR to minimize technical variability between samples, was not standardized across studies and may affect miRNA reporting (110). Stably expressed internal control genes such as U6 are commonly used in RT-qPCR for cell or tissue samples, but standardization is lacking for the normalization measurements of circulating or urinary miRNAs (112). Concerns have been raised regarding the suitability of endogenous U6 for the normalization of circulating miRNAs due to its fluctuating expression and stability (113). Further, regarding normalization of urinary miRNAs, only one study measuring urinary miRNAs corrected data for urinary Cr concentration (30), and one study of urinary exosome miRNAs reported urinary particle concentration-adjusted miRNA expression data (73).

In summary, our review indicates that dysregulated miRNAs occur across several human AKI categories. Certain miRNAs are primarily up-regulated in AKI (miR-21, miR-223, miR-155, miR-142, miR-146, miR-423) while others are down-regulated (miR-126, miR-125). AKI etiology, tissue source, or timing of measurement could influence the direction of dysregulation. For example, miR-21 was upregulated in most AKI etiologies but down-regulated early and transiently post-operatively in CS-AKI. Most reported miRNAs have been studied in pre-clinical

AKI models (24), although 3 miRNAs (miR-125, miR-142, miR-30a) have not been directly studied *in vivo* or *in vitro*. Future research should focus on improving study design in human AKI and optimizing methods for miRNA detection and quantitation. Overall, miRNAs have potential as diagnostic and prognostic biomarkers for human AKI, and as therapeutic targets for injury and AKI recovery.

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References

1. Wang HE, Muntner P, Chertow GM, et al. Acute Kidney Injury and Mortality in Hospitalized Patients. *Am J Nephrol* 2012; 35: 349-355. DOI: https://doi.org/10.1159/000337487.
2. Uchino S, Bellomo R, Goldsmith D, et al. An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. *Crit Care Med* 2006; 34: 1913-1917. DOI: https://doi.org/10.1097/01.CCM.0000224227.70642.4F.
3. See EJ, Jayasinghe K, Glassford N, et al. Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. *Kidney International* 2019; 95: 160-172. DOI: https://doi.org/10.1016/j.kint.2018.08.036.
4. KDIGO clinical practice guidelines for acute kidney injury. *Kidney International Suppl* 2012; 2: 19-36.
5. Schrezenmeier EV, Barasch J, Budde K, et al. Biomarkers in acute kidney injury – pathophysiological basis and clinical performance. *Acta Physiol* 2017; 219: 556-574. DOI: https://doi.org/10.1111/apha.12764.
6. Ho J, Tangri N, Komenda P, et al. Urinary, Plasma, and Serum Biomarkers' Utility for Predicting Acute Kidney Injury Associated With Cardiac Surgery in Adults: A Meta-analysis. *Am J Kidney Dis* 2015; 66: 993-1005. DOI: https://doi.org/10.1053/j.ajkd.2015.06.018.
7. de Geus HRH, Bakker J, Lesaffre EMEH, et al. Neutrophil gelatinase-associated lipocalin at ICU admission predicts for acute kidney injury in adult patients. *Am J Respir Crit Care Med* 2011; 183: 907-914. DOI: https://doi.org/10.1164/rccm.200908-1214OC.
8. Singer E, Elger A, Elitok S, et al. Urinary neutrophil gelatinase-associated lipocalin distinguishes pre-renal from intrinsic renal failure and predicts outcomes. *Kidney International* 2011; 80: 405-414. DOI: https://doi.org/10.1038/ki.2011.41.
9. Ichimura T, Hung CC, Yang SA, et al. Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. *Am J Physiol Renal Physiol* 2004; 286: F552-F563. DOI: https://doi.org/10.1152/ajprenal.00285.2002.
10. Ismail OZ, Zhang X, Wei J, et al. Kidney injury molecule-1 protects against Gα12 activation and tissue damage in renal ischemia-reperfusion injury. *Am J Pathol* 2015; 185: 1207-1215. DOI: https://doi.org/10.1016/j.ajpath.2015.02.003.
11. Kashani K, Al-Khafaji A, Ardiles T, et al. Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury. *Crit Care* 2013; 17: R25. DOI: https://doi.org/10.1186/cc12503.
12. Bihorac A, Chawla LS, Shaw AD, et al. Validation of cell-cycle arrest biomarkers for acute kidney injury using clinical adjudication. *Am J Respir Crit Care Med* 2014; 189: 932-939. DOI: https://doi.org/10.1164/rccm.201401-0077OC.
13. Moledina DG, Obeid W, Smith RN, et al. Identification and validation of urinary CXCL9 as a biomarker for diagnosis of acute interstitial nephritis. *J Clin Invest* 2023; 133: e168950. DOI: https://doi.org/10.1172/JCI168950.
14. Mahtal N, Lenoir O, Tinel C, et al. MicroRNAs in kidney injury and disease. *Nature Reviews Nephrology* 2022; 18: 643-662. DOI: https://doi.org/10.1038/s41581-022-00608-6.
15. O'Brien J, Hayder H, Zayed Y, et al. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol* 2018; 9: 402. DOI: https://doi.org/10.3389/fendo.2018.00402.

16. Baglio SR, Rooijers K, Koppers-Lalic D, et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes
enriched in distinctive miRNA and tRNA species. *Stem Cell Research & Therapy* 2015; 6: 127. DOI: https://doi.org/10.1186/s13287-015-0116-z.
17. Carter JV, Galbraith NJ, Yang D, et al. Blood-based microRNAs as biomarkers for the diagnosis of colorectal cancer: a systematic review and meta-analysis. *British Journal of Cancer* 2017; 116: 762–774. DOI: https://doi.org/10.1038/bjc.2017.12.
18. Nguyen THN, Nguyen TTN, Nguyen TTM, et al. Panels of circulating microRNAs as potential diagnostic biomarkers for breast cancer: a systematic review and meta-analysis. *Breast Cancer Research and Treatment* 2022; 196: 1-15. DOI: https://doi.org/10.1007/s10549-022-06728-8.
19. Kaur A, Mackin ST, Schlosser K, et al. Systematic review of microRNA biomarkers in acute coronary syndrome and stable coronary artery disease. *Cardiovascular Research* 2020; 116: 1113-1124. DOI: https://doi.org/10.1093/cvr/cvz302.
20. Templeton EM, Cameron VA, Pickering JW, et al. Emerging microRNA biomarkers for acute kidney injury in acute decompensated heart failure. *Heart Failure Reviews* 2021; 26: 1203-1217. DOI: https://doi.org/10.1007/s10741-020-09928-w.
21. Iyer V, Rowbotham S, Biros E, et al. A systematic review investigating the association of microRNAs with human abdominal aortic aneurysms. *Atherosclerosis* 2017; 261: 78-89. DOI: http://dx.doi.org/10.1016/j.atherosclerosis.2017.03.010.
22. Juźwik CA, Drake SS, Zhang Y, et al. microRNA dysregulation in neurodegenerative diseases: A systematic review. *Progress in Neurobiology* 2019; 182: 101664. DOI: https://doi.org/10.1016/j.pneurobio.2019.101664.
23. Liu C-H, Ampuero J, Gil-Gómez A, et al. miRNAs in patients with non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Journal of Hepatology* 2018; 69: 1335-1348. DOI: http://dx.doi.org/10.1016/j.jhep.2018.08.008.
24. Zankar S, Trentin-Sonoda M, Viñas JL, et al. Therapeutic effects of micro-RNAs in preclinical studies of acute kidney injury: a systematic review and meta-analysis. *Scientific Reports* 2021; 11: 9100. DOI: https://doi.org/10.1038/s41598-021-88746-y.
25. Brandenburger T and Lorenzen JM. Diagnostic and Therapeutic Potential of microRNAs in Acute Kidney Injury. *Front Pharmacol* 2020; 11: 657. DOI: https://doi.org/10.3389/fphar.2020.00657.
26. Douvris A, Burger D, Rodriguez RA, et al. MicroRNA in Human Acute Kidney Injury: A Systematic Review Protocol. *Canadian Journal of Kidney Health and Disease* 2021; 8: 1-8.
27. Harris PA, Taylor R, Thielke R, et al. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009; 42: 377-381. DOI: https://doi.org/10.1016/j.jbi.2008.08.010.
28. Wells GA, Shea B, O'Connor D, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses.
29. Campbell M, McKenzie JE, Sowden A, et al. Synthesis without meta-analysis (SWiM) in systematic reviews: reporting guideline. *BMJ* 2020; 368: 16890. DOI: https://doi.org/10.1136/bmj.l6890.

30. Pavkovic M, Robinson-Cohen C, Chua AS, et al. Detection of Drug-Induced Acute Kidney Injury in Humans Using Urinary KIM-1, miR-21, -200c, and -423. *Toxicol Sci* 2016; 152: 205-213. DOI: https://doi.org/10.1093/toxsci/kfw077.
31. Wilflingseder J, Regele H, Perco P, et al. miRNA Profiling Discriminates Types of Rejection and Injury in Human Renal Allografts *Transplantation* 2013; 95: 835-841. DOI: https://doi.org/10.1097/TP.0b013e318280b385.
32. Wilflingseder J, Sunzenauer J, Toronyi E, et al. Molecular Pathogenesis of Post-Transplant Acute Kidney Injury: Assessment of Whole-Genome mRNA and MiRNA Profiles. *PLoS ONE* 2014; 9: e104164. DOI: https://doi.org/10.1371/journal.pone.0104164.
33. Khalid U, Newbury LJ, Simpson K, et al. A urinary microRNA panel that is an early predictive biomarker of delayed graft function following kidney transplantation. *Scientific Reports* 2019; 9: 3584. DOI: https://doi.org/10.1038/s41598-019-38642-3.
34. Seo J-W, Lee YH, Tae DH, et al. Development and validation of urinary exosomal microRNA biomarkers for the diagnosis of acute rejection in kidney transplant recipients. *Front Immunol* 2023; 14: 1190576. DOI: https://doi.org/10.3389/fimmu.2023.1190576.
35. Gallon L, Mathew JM, Bontha SV, et al. Intragraft Molecular Pathways Associated with Tolerance Induction in Renal Transplantation. *J Am Soc Nephrol* 2018; 29: 423-433. DOI: https://doi.org/10.1681/ASN.2017030348.
36. Ma Y, Fu J, Qian L, et al. Serum miRNA expression and correlation with clinical characteristics in acute kidney injury. *Int J Clin Exp Pathol* 2017; 10: 8721-8726.
37. Watany MM, Hagag RY and Okda HI. Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study. *Clin Chem Lab Med* 2018; 56: 739-747. DOI: https://doi.org/10.1515/cclm-2017-0483.
38. Newbury LJ, Simpson K, Khalid U, et al. miR-141 mediates recovery from acute kidney injury. *Scientific Reports* 2021; 11: 16499. DOI: https://doi.org/10.1038/s41598-021-94984-x.
39. Gaede L, Liebetrau C, Blumenstein J, et al. Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery. *Nephrol Dial Transplant* 2016; 31: 760-766. DOI: https://doi.org/10.1093/ndt/gfw007.
40. Arvin P, Samimagham HR, Montazerghaem H, et al. Early detection of cardiac surgery-associated acute kidney injury by microRNA-21. *Bratisl Med J* 2017; 118: 626-631. DOI: https://doi.org/10.4149/BLL_2017_120.
41. Kang Z, Li Z, Huang P, et al. Remote ischemic preconditioning upregulates microRNA-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass. *Pediatric Nephrology* 2018; 33: 911-919. DOI: https://doi.org/10.1007/s00467-017-3851-9.
42. Saikumar J, Hoffmann D, Kim T-M, et al. Expression, Circulation, and Excretion Profile of MicroRNA-21, -155, and -18a Following Acute Kidney Injury. *Toxicological Sciences* 2012; 129: 256-267. DOI: https://doi.org/10.1093/toxsci/kfs210.
43. Ramachandran K, Saikumar J, Bijol V, et al. Human miRNome Profiling Identifies MicroRNAs Differentially Present in the Urine after Kidney Injury. *Clinical Chemistry* 2013; 59: 1742-1752. DOI: https://doi.org/10.1373/clinchem.2013.210245.

44. Lin Y, Ding Y, Song S, et al. Expression patterns and prognostic value of miR-210, miR-494, and miR-205 in middle-aged and old patients with sepsis-induced acute kidney injury. *Bosn J Basic Med Sci* 2019; 19: 249-256. DOI: http://dx.doi.org/10.17305/bjbms.2019.4131.
45. Zhang F, Luo X, Wang Y, et al. LncRNA PMS2L2 Is Downregulated in Sepsis-Induced Acute Kidney Injury and Inhibits LPS-Induced Apoptosis of Podocytes. *Kidney and Blood Press Res* 2023; 48: 515-521. DOI: https://doi.org/10.1159/000528053.
46. Shihana F, Wong WKM, Joglekar MV, et al. Urinary microRNAs as non-invasive biomarkers for toxic acute kidney injury in humans. *Scientific Reports* 2021; 11: 9165. DOI: https://doi.org/10.1038/s41598-021-87918-0.
47. Alfaro R, Legaz I, Jimenez-Coll V, et al. MicroRNA Expression Changes in Kidney Transplant: Diagnostic Efficacy of miR-150-5p as Potential Rejection Biomarker, Pilot Study. *J Clin Med* 2021; 10: 2748. DOI: https://doi.org/10.3390/jcm10132748.
48. Bruno N, Maaten JMt, Ovchinnikova ES, et al. MicroRNAs relate to early worsening of renal function in patients with acute heart failure. *International Journal of Cardiology* 2016; 203: 564-569. DOI: http://dx.doi.org/10.1016/j.ijcard.2015.10.217.
49. Sullo N, Mariani S, JnTala M, et al. An Observational Cohort Feasibility Study to Identify Microvesicle and Micro-RNA Biomarkers of Acute Kidney Injury Following Pediatric Cardiac Surgery. *Pediatr Crit Care Med* 2018; 19: 816-830. DOI: https://doi.org/10.1097/PCC.0000000000001604.
50. Li F, Qian W, Quan X, et al. Differential MicroRNA Expressions in Human Peripheral Blood Mononuclear Cells Are Predictive of Renal Allograft Function. *Transplantation Proceedings* 2019; 51: 715-721. DOI: https://doi.org/10.1016/j.transproceed.2019.01.051.
51. Soltaninejad E, Nicknam MH, Nafar M, et al. Differential expression of microRNAs in renal transplant patients with acute T-cell mediated rejection. *Transplant Immunology* 2015; 33: 1-6. DOI: http://dx.doi.org/10.1016/j.trim.2015.05.002.
52. Gutiérrez-Escolano A, Santacruz-Vázquez E and Gómez-Pérez F. Dysregulated microRNAs involved in contrast-induced acute kidney injury in rat and human. *Renal Failure* 2015; 37: 1498-1506. DOI: https://doi.org/10.3109/0886022X.2015.1077322.
53. Sun S-q, Zhang T, Ding D, et al. Circulating MicroRNA-188, -30a, and -30e as Early Biomarkers for Contrast-Induced Acute Kidney Injury. *J Am Heart Assoc* 2016; 5: e004138. DOI: https://doi.org/10.1161/JAHA.116.004138.
54. Vigorito E, Kohlhaas S, Lu D, et al. miR-155: an ancient regulator of the immune system. *Immunol Rev* 2013; 253: 146-157. DOI: https://doi.org/10.1111/imr.12057.
55. Millán O, Budde K, Sommerer C, et al. Urinary miR-155-5p and CXCL10 as prognostic and predictive biomarkers of rejection, graft outcome and treatment response in kidney transplantation. *Br J Clin Pharmacol* 2017; 83: 2636-2650. DOI: https://doi.org/10.1111/bcp.13399.
56. Krebs CF, Kapffer S, Paust H-J, et al. MicroRNA-155 Drives TH17 Immune Response and Tissue Injury in Experimental Crescentic GN. *J Am Soc Nephrol* 2013; 24: 1955-1965. DOI: https://doi.org/10.1681/ASN.2013020130.
57. You T and Kuang F. Circ_0008882 Stimulates PDE7A To Suppress Septic Acute Kidney Injury Progression by Sponging miR-155-5p. *Shock* 2023; 59: 657-665. DOI: https://doi.org/10.1097/SHK.0000000000002093.

58. Domenico TD, Joelsons G, Montenegro RM, et al. Upregulation of microRNA 142-3p in the peripheral blood and urinary cells of kidney transplant recipients with post-transplant graft dysfunction. *Brazilian Journal of Medical and Biological Research* 2017; 50: e5533. DOI: http://dx.doi.org/10.1590/1414-431X20175533.
59. Han R, Li W, Tian H, et al. Urinary microRNAs in sepsis function as a novel prognostic marker. *Experimental and Therapeutic Medicine* 2023; 26: 346. DOI: https://doi.org/10.3892/etm.2023.12045.
60. Pan W, Zhang J, Hu L, et al. Evaluation Value of Serum miR-4299 and miR-16-5p in Risk Stratification of Sepsis-Induced Acute Kidney Injury. *BioMed Research International* 2022; 2022: 8. DOI: https://doi.org/10.1155/2022/5165892.
61. Lorenzen JM, Kielstein JT, Hafer C, et al. Circulating miR-210 Predicts Survival in Critically Ill Patients with Acute Kidney Injury. *Clin J Am Soc Nephrol* 2011; 6: 1540-1546. DOI: https://doi.org/10.2215/CJN.00430111.
62. Chen H-H, Lan Y-F, Li H-F, et al. Urinary miR-16 transactivated by C/EBPβ reduces kidney function after ischemia/reperfusion–induced injury. *Scientific Reports* 2016; 6: 27945. DOI: https://doi.org/10.1038/srep27945.
63. Connor KL, Teenan O, Cairns C, et al. Identifying cell-enriched miRNAs in kidney injury and repair. *JCI Insight* 2020; 5: e140399. DOI: https://doi.org/10.1172/jci.insight.140399.
64. Zhang L, Xu Y, Xue S, et al. Implications of dynamic changes in miR-192 expression in ischemic acute kidney injury. *Int Urol Nephrol* 2017; 49: 541-550. DOI: https://doi.org/10.1007/s11255-016-1485-7.
65. Zou Y-F, Wen D, Zhao Q, et al. Urinary MicroRNA-30c-5p and MicroRNA-192-5p as potential biomarkers of ischemia–reperfusion-induced kidney injury. *Experimental Biology and Medicine* 2017; 242: 657-667. DOI: https://doi.org/10.1177/1535370216685005.
66. Vliegenthart ADB, Shaffer JM, Clarke JI, et al. Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury. *Scientific Reports* 2015; 5: 15501. DOI: https://doi.org/10.1038/srep15501.
67. Li P, Wu Y, Goodwin AJ, et al. Circulating extracellular vesicles are associated with the clinical outcomes of sepsis. *Front Immunol* 2023; 14: 1150564. DOI: https://doi.org/10.3389/fimmu.2023.1150564.
68. Shihana F, Joglekar MV, Raubenheimer J, et al. Circulating human microRNA biomarkers of oxalic acid-induced acute kidney injury. *Archives of Toxicology* 2020; 94: 1725-1737. DOI: https://doi.org/10.1007/s00204-020-02679-5.
69. Kölling M, Seeger H, Haddad G, et al. The Circular RNA ciRs-126 Predicts Survival in Critically Ill Patients With Acute Kidney Injury. *Kidney Int Rep* 2018; 3: 1144-1152. DOI: https://doi.org/10.1016/j.ekir.2018.05.012.
70. Aguado-Fraile E, Ramos E, Conde E, et al. A Pilot Study Identifying a Set of microRNAs As Precise Diagnostic Biomarkers of Acute Kidney Injury. *PLoS ONE* 2015; 10: e0127175. DOI: https://doi.org/10.1371/journal.pone.0127175.
71. McGuinness D, Leierer J, Shapter O, et al. Identification of Molecular Markers of Delayed Graft Function Based on the Regulation of Biological Ageing. *PLoS ONE* 2016; 11: e0146378. DOI: https://doi.org/10.1371/journal.pone.0146378.

72. Sui W, Lin H, Peng W, et al. Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA. *Genomics* 2013; 102: 310-322. DOI: http://dx.doi.org/10.1016/j.ygeno.2013.05.002.
73. Miller D, Eagle-Hemming B, Sheikh S, et al. Urinary extracellular vesicles and micro-RNA as markers of acute kidney injury after cardiac surgery. *Scientific Reports* 2022; 12: 10402. DOI: https://doi.org/10.1038/s41598-022-13849-z.
74. Wang S, Wu L, Du L, et al. Reduction in miRNA-125b-5p levels is associated with obstructive renal injury. *Biomedical Reports* 2017; 6: 449-454. DOI: https://doi.org/10.3892/br.2017.875.
75. Xue Q, Yang L, Wang J, et al. lncRNA ROR and miR-125b Predict the Prognosis in Heart Failure Combined Acute Renal Failure. *Disease Markers* 2022; 2022: 1-6. DOI: https://doi.org/10.1155/2022/6853939.
76. Shihana F, Mohamed F, Joglekar MV, et al. Urinary versus serum microRNAs in human oxalic acid poisoning: Contrasting signals and performance. *Toxicology Letters* 2020; 334: 21-26. DOI: https://doi.org/10.1016/j.toxlet.2020.09.003.
77. Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a Mediator of Acute T-Cell Mediated Rejection in Renal Allograft Recipients. *American Journal of Transplantation* 2011; 11: 2221-2227. DOI: https://doi.org/10.1111/j.1600-6143.2011.03679.x.
78. Xun L, Li Z, Wang H, et al. The Value of Combining miR-10a-5p Levels and PLR to Evaluate the Prognosis of Sepsis Patients with Acute Kidney Injury. *Acta Medica Mediterranea* 2022; 38: 3303.
79. Xu D, Li W, Zhang T, et al. miR-10a overexpression aggravates renal ischemia–reperfusion injury associated with decreased PIK3CA expression. *BMC Nephrology* 2020; 21: 248. DOI: https://doi.org/10.1186/s12882-020-01898-3.
80. Li H, Duan J, Zhang T, et al. miR-16-5p aggravates sepsis-associated acute kidney injury by inducing apoptosis. *Renal Failure* 2024; 46: 2322688. DOI: https://doi.org/10.1080/0886022X.2024.2322688.
81. Xu X, Kriegel AJ, Liu Y, et al. Delayed ischemic preconditioning contributes to renal protection by upregulation of miR-21. *Kidney International* 2012; 82: 1167-1175. DOI: https://doi.org/10.1038/ki.2012.241.
82. Pan T, Jia P, Chen N, et al. Delayed Remote Ischemic Preconditioning Confers Renoprotection against Septic Acute Kidney Injury via Exosomal miR-21. *Theranostics* 2021; 9: 405-423. DOI: https://doi.org/10.7150/thno.29832.
83. Sun Y-M, Lin K-Y and Chen Y-Q. Diverse functions of miR-125 family in different cell contexts. *Journal of Hematology & Oncology* 2013; 6: 6. DOI: https://doi.org/10.1186/1756-8722-6-6.
84. Bijkerk R, Solingen Cv, Boer HCd, et al. Hematopoietic MicroRNA-126 Protects against Renal Ischemia/Reperfusion Injury by Promoting Vascular Integrity. *J Am Soc Nephrol* 2014; 25: 1710-1722. DOI: https://doi.org/10.1681/ASN.2013060640.
85. Anandagoda N, Roberts LB, Willis JCD, et al. Dominant regulation of long-term allograft survival is mediated by microRNA-142. *Am J Transplant* 2020; 20: 2715-2727. DOI: https://doi.org/10.1111/ajt.15907.

86. Amrouche L, Desbuissons G, Rabant M, et al. MicroRNA-146a in Human and Experimental Ischemic AKI: CXCL8-Dependent Mechanism of Action. *J Am Soc Nephrol* 2017; 28: 479-493. DOI: https://doi.org/10.1681/ASN.2016010045.
87. Milhoransa P, Montanari CC, Montenegro R, et al. Micro RNA 146a-5p expression in delayed graft function. *Braz J Nephrol* 2019; 41: 242-251. DOI: https://doi.org/10.1590/2175-8239-JBN-2018-0098.
88. Zhang C, Guan G, Wang J, et al. MicroRNA-192-5p downregulates Fat Mass and Obesity-associated Protein to aggravate renal ischemia/reperfusion injury. *Renal Failure* 2023; 45: 2285869. DOI: https://doi.org/10.1080/0886022X.2023.2285869.
89. Jenkins RH, Davies LC, Taylor PR, et al. miR-192 Induces G2/M Growth Arrest in Aristolochic Acid Nephropathy. *American Journal of Pathology* 2014; 184: 996-1009. DOI: http://dx.doi.org/10.1016/j.ajpath.2013.12.028.
90. Zaccagnini G, Greco S, Longo M, et al. Hypoxia-induced miR-210 modulates the inflammatory response and fibrosis upon
acute ischemia. *Cell Death and Disease* 2021; 12: 435. DOI: https://doi.org/10.1038/s41419-021-03713-9.
91. Xie Z, Tang J, Chen Z, et al. Human bone marrow mesenchymal stem cell-derived extracellular vesicles reduce inflammation and pyroptosis in acute kidney injury via miR-223-3p/HDAC2/SNRK. *Inflammation Research* 2023; 72: 553-576. DOI: https://doi.org/10.1007/s00011-022-01653-4.
92. Güçlü A, Koçak C, Koçak FE, et al. Micro RNA-320 as a novel potential biomarker in renal ischemia reperfusion. *Renal Failure* 2016; 38: 1468-1475. DOI: https://doi.org/10.1080/0886022X.2016.1227915.
93. Yuan X-P, Liu L-S, Chen C-B, et al. MicroRNA-423-5p facilitates hypoxia/reoxygenation-induced apoptosis in renal proximal tubular epithelial cells by targeting GSTM1 via endoplasmic reticulum stress. *Oncotarget* 2017; 8: 82064-82077.
94. Cooper JE and Wiseman AC. Acute kidney injury in kidney transplantation. *Curr Opin Nephrol Hypertens* 2013; 22: 698-703. DOI: https://doi.org/10.1097/MNH.0b013e328365b388.
95. Brown N, Roman M, Miller D, et al. A Systematic Review and Meta-Analysis of MicroRNA as Predictive Biomarkers of Acute Kidney Injury. 2024; 12: 1695. DOI: https://doi.org/10.3390/biomedicines12081695.
96. Khalid U, Ablorsu E, Szabo L, et al. MicroRNA-21 (miR-21) expression in hypothermic machine perfusate may be predictive of early outcomes in kidney transplantation. *Clin Transplant* 2016; 30: 99-104. DOI: https://doi.org/10.1111/ctr.12679.
97. Hu H, Jiang W, Xi X, et al. MicroRNA-21 Attenuates Renal Ischemia Reperfusion Injury via Targeting Caspase Signaling in Mice. *Am J Nephrol* 2014; 40: 215-223. DOI: https://doi.org/10.1159/000368202.
98. Gale DP, Gross O, Wang F, et al. A Randomized Controlled Clinical Trial Testing Effects of Lademirsen on Kidney Function Decline in Adults with Alport Syndrome. *Clin J Am Soc Nephrol* 2024; Online ahead of print. DOI: https://doi.org/10.2215/CJN.0000000000000458.
99. Chen J, Zheng Y and Li L. LncRNA RPSAP52 regulates miR-423-5p/GSTM1 axis to suppress hypoxia-induced renal proximal tubular epithelial cell apoptosis. *Archives of*

Physiology and Biochemistry 2020; 128: 1066-1070. DOI: https://doi.org/10.1080/13813455.2020.1750657.
100. Sun B, Qu Z, Cheng G, et al. Urinary microRNAs miR-15b and miR-30a as novel noninvasive biomarkers for gentamicin-induced acute kidney injury. *Toxicology Letters* 2021; 338: 105-113. DOI: http://dx.doi.org/10.1016/j.toxlet.2020.12.006.
101. Haddad G, Kölling M, Wegmann UA, et al. Renal AAV2-Mediated Overexpression of Long Non-Coding RNA H19 Attenuates Ischemic Acute Kidney Injury Through Sponging of microRNA-30a-5p. *J Am Soc Nephrol* 2021; 32: 323-341. DOI: https://doi.org/10.1681/ASN.2020060775.
102. Tan J, Fan J, He J, et al. Knockdown of LncRNA DLX6-AS1 inhibits HK-2 cell pyroptosis via regulating miR-223-3p/NLRP3 pathway in lipopolysaccharide-induced acute kidney injury. *Journal of Bioenergetics and Biomembranes* 2020; 52: 367-376. DOI: https://doi.org/10.1007/s10863-020-09845-5.
103. Gao M, Li H, Liu Q, et al. KLF6 promotes pyroptosis of renal tubular epithelial cells in septic acute kidney injury. *Shock* 2022; 57: 417-426. DOI: https://doi.org/10.1097/SHK.0000000000001881.
104. Fan H, Sun M and Zhu Jh. Clinical role of serum microRNA-155 in early diagnosis and prognosis of septic patients with acute kidney injury. *International Urology and Nephrology* 2024; 56: 1687-1694. DOI: https://doi.org/10.1007/s11255-023-03855-z.
105. Jordan SC, Ammerman N, Huang E, et al. Importance of IL-6 inhibition in prevention and treatment of antibody-mediated rejection in kidney allografts. *Am J Transplant* 2022; 22: 28-37. DOI: https://doi.org/10.1111/ajt.17207.
106. Louis K, Fadakar P, Macedo C, et al. Concomitant loss of regulatory T and B cells is a distinguishing immune feature of antibody-mediated rejection in kidney transplantation. *Kidney International* 2022; 101: 1003-1016. DOI: https://doi.org/10.1016/j.kint.2021.12.027.
107. Jordan SC, Ammerman N, Choi J, et al. Evaluation of Clazakizumab (Anti-Interleukin-6) in Patients With Treatment-Resistant Chronic Active Antibody-Mediated Rejection of Kidney Allografts. *Kidney Int Rep* 2022; 7: 720-731. DOI: https://doi.org/10.1016/j.ekir.2022.01.1074.
108. Scullion KM, Vliegenthart ADB, Rivoli L, et al. Circulating argonaute-bound microRNA-126 reports vascular dysfunction and treatment response in acute and chronic kidney disease. *iScience* 2020; 24: 101937. DOI: https://doi.org/10.1016/j.isci.2020.101937.
109. Cantaluppi V, Gatti S, Medica D, et al. Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells. *Kidney International* 2012; 82: 412-427. DOI: https://doi.org/10.1038/ki.2012.105.
110. Chorley BN, Atabakhsh E, Doran G, et al. Methodological considerations for measuring biofluid-based microRNA biomarkers. *Critical Reviews in Toxicology* 2021; 51: 264-282. DOI: https://doi.org/10.1080/10408444.2021.1907530.
111. Shkurnikov MY, Knyazev EN, Fomicheva KA, et al. Analysis of Plasma microRNA Associated with Hemolysis. *Bulletin of Experimental Biology and Medicine* 2016; 160: 748-750. DOI: https://doi.org/10.1007/s10517-016-3300-y.

112. Faraldi M, Gomarasca M, Banfi G, et al. Free Circulating miRNAs Measurement in Clinical Settings: The Still Unsolved Issue of the Normalization. *Adv Clin Chem* 2018; 87: 113-139. DOI: https://doi.org/10.1016/bs.acc.2018.07.003.
113. Xiang M, Zeng Y, Yang R, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. *Biochem Biophys Res Commun* 2014; 454: 210-214. DOI: https://doi.org/10.1016/j.bbrc.2014.10.064.

Chapter 5: General Discussion

5-1 Summary of results

AKI is a serious complication among hospitalized patients with limited treatment options. Patients who recover from an episode of AKI are also at risk of long-term progressive CKD[52]. In kidney I/R injury, endothelial cell injury and dysfunction lead to peritubular capillary rarefaction which is associated with tubulointerstitial fibrosis and the transition to CKD[43]. We previously showed that miR-486-5p protects against kidney I/R injury in mice, associated with PTEN targeting and inhibition of apoptosis[97, 99]. However, the effect of miR-486-5p on endothelial function and long-term kidney and vascular outcomes post-AKI is unknown.

This thesis evaluated the role of miR-486-5p on endothelial function in ischemic AKI. Manuscript I demonstrated that only early administration of miR-486-5p to rats with kidney I/R injury prevented long-term renal microvascular loss and systemic endothelial dysfunction[108], supporting the importance of early gene responses for the AKI to CKD transition. This manuscript also highlighted an unexpected finding – inhibition of eNOS protein expression by miR-486-5p. Manuscript II provided *in vitro* evidence that miR-486-5p inhibits eNOS expression in cultured endothelial cells, but not by 3' UTR targeting. It also demonstrated the anti-angiogenic potential of miR-486-5p via eNOS downregulation. Multiple miR-486-5p targets were identified by pull-down assay in endothelial cells and suggested that eNOS downregulation is mediated by targeting of MAML3 by miR-486-5p, thus implicating the Notch pathway as a regulator of eNOS expression and endothelial function. In contrast to miR-486-5p's protective effect in animal models of I/R injury, our *in vitro* data suggests that miR-486-5p has the potential for adverse vascular effects mediated via inhibition of eNOS expression. Lastly, manuscript III, a systematic review of miRNAs in human AKI, identified several dysregulated miRNAs – the

most frequently reported being miR-21 – that may have potential as AKI diagnostic and prognostic biomarkers[109]. This study also acknowledged important methodological limitations confounding the outcomes.

5-2 Early administration of miR-486-5p *in vivo* protects against kidney I/R injury and prevents late peritubular capillary loss and systemic vascular dysfunction

These studies revealed that early administration of miR-486-5p to rats with kidney I/R injury was associated with its localization to tubular cells and interstitial capillary endothelial cells. miR-486-5p protected against kidney injury and preserved peritubular capillary density. In addition to decreased acute tubular injury, miR-486-5p preserved regional kidney blood flow, decreased inflammatory cell infiltration, and attenuated ICAM-1 protein expression, suggesting that miR-486-5p inhibits endothelial injury. In contrast, delayed administration of miR-486-5p, with a first dose at 96 hr and second dose at 3 weeks after kidney I/R, did not prevent loss of peritubular capillary density or kidney fibrosis[108].

These data highlight three key findings. First, our data supports the notion that there is a critical therapeutic window in AKI to prevent the permanent loss of renal microvasculature. Despite pro-angiogenic[36, 37] and anti-fibrotic[39, 41] effects in other pre-clinical disease models, delayed and repeated dosing of miR-486-5p failed to preserve peritubular capillary density and prevent kidney fibrosis. Second, rats subjected to kidney I/R injury demonstrated long-term systemic vascular dysfunction, which was also prevented by early administration of miR-486-5p. Lastly, miR-486-5p inhibited I/R-induced eNOS protein expression, yet no vascular adverse effects were seen regardless of dosing strategy.

Although the localization of miR-486-5p to kidney tubular and interstitial capillary endothelial cells suggests a direct effect on these cell types, its mechanism of action and gene targets in kidney I/R injury are unknown. We previously showed that the protective effect of miR-486-5p in mice with kidney I/R injury was associated with decreased PTEN protein expression and inhibition of apoptosis after 24 hr[97] but we also showed transient PTEN mRNA downregulation 4 hr after I/R[99]. Thus, the lack of effect on PTEN protein expression in rats does not exclude the possibility that PTEN was transiently downregulated in the present study, which could confer an early protective effect while minimizing potential adverse effects on tubular cell repair[106] and recovery.

miR-486-5p may have multiple gene targets in proximal tubular cells. Our transcriptomic analysis of differentially expressed genes in mouse proximal tubular cells at 24 hr after kidney I/R injury primarily revealed downregulation of genes involved in apoptosis and inflammation pathways such as tumor necrosis factor-α signaling[99]. Interestingly, proximal tubular cell expression of the transcription factor Sox9 was increased by I/R and decreased by miR-486-5p[99]. Sox9 is required for kidney organogenesis[110] and has been linked to the kidney epithelial injury-induced repair response[111]. A recent study demonstrated that induced expression of Sox9 in tubular epithelial cells after AKI led to two possible long-term cell fates. Tubular epithelial cells either underwent successful repair and turned off Sox9 expression, or had unrestored apicobasal polarity and continued to express Sox9. Cells that maintained Sox9 expression accumulated activated myofibroblasts via secretion of Wnt ligands, suggesting that sustained epithelial Sox9 expression signals a fibroproliferative response that contributes to the development of kidney fibrosis[111]. Thus, proximal tubular downregulation of Sox9 by miR-486-5p might protect against the development of kidney fibrosis after AKI, although this pathway was not evaluated in the

present study. Further, Sox9 is not a predicted miR-486-5p target from online target prediction databases (miRDB). However, Manuscript II revealed that miR-486-5p also targets MAML3 in endothelium, a transcriptional co-activator of Notch signaling. Given that Sox9 is also a Notch target gene[112], this represents one plausible mechanism for Sox9 downregulation by miR-486-5p for future study.

The present study demonstrated a protective effect of miR-486-5p on the renal vascular endothelium in rats with kidney I/R injury, although the miR-486-5p targets in renal endothelial cells remain unknown. By inhibiting endothelial injury, miR-486-5p effectively attenuates the extension phase of kidney I/R injury. Regional kidney blood flow measurements also indicated that miR-486-5p preserved cortical flow and enhanced medullary blood flow at 24 hr after I/R. Indeed, outer kidney medullary hypoperfusion exacerbates kidney injury and contributes to peritubular capillary loss[113]. In this regard, a study by Collett *et al* demonstrated that infusion of human cord blood-derived ECFCs – which we have previously shown as enriched in miR-486-5p[97] – to rats subjected to kidney I/R prevented the early reduction in medullary blood flow, inhibited I/R-induced ICAM-1 expression, and protected against kidney injury[114]. Similarly, our present study demonstrated that miR-486-5p inhibited I/R-induced kidney ICAM-1 expression and inflammatory cell infiltration[114]. Further, we showed that miR-486-5p inhibited ICAM-1 expression in HUVECs subjected to H/R injury, although ICAM-1 is not a predicted miR-486-5p target. Early studies of rats with bilateral kidney I/R injury or uninephrectomized rats subjected to unilateral renal pedicle occlusion and administered either ICAM-1 antisense oligonucleotides or anti-ICAM-1 antibodies also demonstrated that early ICAM-1 inhibition protects against inflammatory cell infiltration and the onset of kidney injury[115, 116]. Taken together, our data suggest that miR-486-5p protects against endothelial injury by targeting pathways involved in

endothelial activation and leukocyte adhesion, thus preserving the peritubular capillaries after kidney ischemic injury[108].

The lack of effect with delayed miR-486-5p administration at 96 hr and at 3 weeks after I/R emphasizes a narrow window of opportunity for intervention to prevent permanent renal microvascular loss and systemic endothelial dysfunction. Consistent with a narrow therapeutic window, an early study of rats subjected to kidney I/R injury demonstrated that administration of anti-ICAM-1 antibodies prevented kidney functional and histological injury when administered at or within 0.5 and 2 hr of reperfusion, but not after 8 hr[117]. Another factor associated with negative microvascular effects is the loss of endogenous VEGFA expression by 1 week after kidney I/R injury[118]. In this regard, Leonard *et al* evaluated the role of exogenous VEGF-121 (a non-heparin binding form of VEGF) on kidney microvascular structure after ischemic AKI in rats that were challenged with a high salt diet[119]. Their study demonstrated that administration of VEGF-121 from day 0 through 35 or day 3 through 35 had no effect on the initial loss of kidney function but preserved long-term microvascular density and prevented the development of salt sensitive CKD[119]. In contrast, delayed exogenous VEGF-121 administration after 3 weeks failed to preserve peritubular capillary density and did not protect against the transition to CKD[119]. Although the exact mechanism of action of miR-486-5p is unknown, our data suggests that when administered late after kidney I/R injury, it has no impact on the pathogenesis of peritubular capillary loss[108]. Lastly, we acknowledge that both early and delayed miR-486-5p administration inhibited I/R-induced eNOS protein expression[108], although our data reveal no adverse effects on the kidney microvasculature or systemic endothelial dysfunction compared to untreated post-ischemic rats.

5-3 miR-486-5p targets MAML3, downregulates eNOS protein levels and inhibits *in vitro* network formation in cultured endothelial cells

In Manuscript II, we evaluated the functional effect of miR-486-5p and characterized its mRNA targets in cultured endothelial cells. Our data revealed that miR-486-5p markedly decreased eNOS protein and mRNA levels in HUVECs, and also inhibited eNOS protein expression in human pulmonary microvascular endothelial cells. Functionally, miR-486-5p inhibited *in vitro* network formation in isolation in an eNOS-dependent manner, with no significant effect on HUVEC proliferation or migration in normoxic standard culture conditions. To determine the mechanism by which miR-486-5p inhibits eNOS expression and angiogenesis, we conducted a biotinylated miR-486-5p pull-down followed by RNA sequencing of the pull-down RNA. This approach identified MAML3, a transcriptional co-activator of Notch signaling, as highly enriched in the pull-down RNA, and its silencing significantly inhibited eNOS protein expression. Given that miR-486-5p-mediated targeting of MAML3 conferred an anti-angiogenic effect in HUVECs[102], our data suggests that miR-486-5p-mediated inhibition of eNOS expression and inhibition of angiogenesis may occur via miR-486-5p targeting of MAML3, although the mechanism by which inhibition of MAML3 regulates Notch signaling and eNOS expression requires further study.

Of the available techniques to probe for miRNA-mRNA interactions, we selected the biotinylated miRNA pull-down approach because sequencing of the pull-down RNA allows for the identification of direct miRNA targets. Our approach revealed 1,729 significantly enriched transcripts in the miR-486-5p pull-down RNA, similar to reported yields in the literature[19, 120]. Interestingly, while miRDB lists 331 predicted miR-486-5p targets, only 40 transcripts (12% of predicted targets) were identified as having a significantly enriched fold-change in the miR-486-

5p pull-down RNA compared to control, Thus the vast majority of enriched transcripts were not bioinformatically predicted miR-486-5p targets. A study by Martin *et al* evaluated the specificity of mRNA interactions with several biotinylated miRNAs, demonstrating an average of 1,500 genes per miRNA significantly enriched in the pull-down RNA, the majority of which were not found in target prediction databases[19]. This study showed that predicted miRNA targets were enriched in the pull-down fraction compared to the cell lysate, but also found differences in the proportion of previously validated targets in their pull-down RNA depending on cell type. The authors subsequently conducted luciferase reporter assays on untested miRNA targets, resulting in an experimental validation rate of 87.5%, which exceeds the validation rate of target prediction databases alone[19]. Overall, the high number of enriched transcripts in the biotinylated miRNA pull-down RNA could reflect the presence of non-canonical binding including miRNA centered sites, 3' supplemental or compensatory binding sites, and binding to miRNA responsive elements (MREs) within 5' UTRs and coding regions. Martin *et al* also demonstrated that 82.7% of significantly enriched transcripts contained either a seed or centered miRNA binding site[19], indicating an element of non-canonical binding. These data provide compelling evidence that the biotinylated miRNA pull-down approach enriches for true direct miRNA targets.

One important limitation to this approach is the potential for false negatives for miRNA targets that are degraded rather than translationally repressed. This remains a plausible explanation for why PTEN was not detected in the pull-down RNA, particularly as targeting of PTEN by miR-486-5p has been reported in cardiomyocytes[22, 33-35], skeletal muscle[23], brain microvascular endothelial cells[37], and HUVECs subjected to H/R injury[97]. The present study did demonstrate a trend for decreased PTEN protein levels in HUVECs transfected with miR-486-5p.

Our study also highlights discordant functional data pertaining to miR-486-5p in endothelial cells. Consistent with the study by Rosano *et al*[102], we demonstrate that miR-486-5p inhibits angiogenesis in cultured endothelial cells. In addition, we show that miR-486-5p's anti-angiogenic effect is conferred via inhibition of eNOS expression. In contrast, miR-486-5p confers pro-angiogenic effects in other pre-clinical models. In this regard, intra-myocardial administration of miR-486-5p-enriched mesenchymal stem cell (MSC)-derived exosomes to mice and non-human primates with experimental myocardial infarction reduced infarct size, improved heart function, increased cardiac vascular density after 4 weeks for mice and 17 months for non-human primates, and increased vessel sprouting from isolated aortic rings *ex-vivo*[36]. The authors showed that the miR-486-5p target, matrix metalloproteinase 19 (Mmp-19), was downregulated in exosome-treated hearts, associated with upregulation of VEGFA signaling. Further, *in vitro* network formation assays in HUVECs cultured with conditioned medium from fibroblasts transfected with miR-486-5p revealed increased tube length, suggesting a pro-angiogenic paracrine mechanism from cardiac fibroblasts[36]. Exosomal transfer of miR-486-5p promoted microvascular endothelial cell proliferation, migration, and angiogenesis in a cutaneous wound study by targeting and downregulating the transcriptional repressor Sp5[100]. Further, in a rat model of ischemic stroke, delivery of bone marrow MSC-derived exosomes, enriched in miR-486-5p, improved neurological function, and increased microvascular density[37]. To selectively determine the impact of miR-486-5p on microvascular function, rat brain microvascular endothelial cells were treated with exosomes derived from bone marrow MSCs transfected with either miR-486-5p mimic or miR-486-5p antagomir. These studies demonstrated that exosomal transfer of miR-486-5p promoted *in vitro* angiogenesis in rat brain microvascular endothelial cells subjected to oxygen-glucose deprivation/re-oxygenation injury[37].

Mechanistically, the promotion of angiogenesis and functional recovery after cerebral ischemic injury was associated with targeting of PTEN and enhanced Akt signaling[37]. Our lab has also demonstrated that exosomal transfer of miR-486-5p in HUVECs subjected to H/R injury was associated with targeting of PTEN and inhibition of apoptosis[97].

There are several possible contributors to discordant miR-486-5p target identification and functional effects between studies. For instance, we used HUVECs for our *in vitro* functional assays, which are macrovascular endothelial cells[121] and thus do not necessarily recapitulate microcirculatory pathophysiology. However, as discussed above, other pre-clinical studies also used HUVECs and demonstrated PTEN targeting[97] and pro-angiogenic effects with exosomal delivery of miR-486-5p[36]. Consideration must also be made to rates of transcription and protein stability as factors that impact target gene mRNA[122] and protein levels[123]. Finally, an important methodological difference involves the delivery of exosomal miR-486-5p versus direct miR-486-5p administration by transient transfection.

Exosomes are generated by invagination of the endosomal membrane forming intraluminal vesicles within multivesicular bodies (MVBs)[124]. Exosomes mediate intercellular communication and are enriched in a variety of RNA species including miRNAs. Multiple mechanisms have been studied for packing RNA species into exosomes, a process that remains incompletely understood[124]. Studies have identified an association between the RISC and exosomal miRNA sorting, suggesting the presence of miRNA binding proteins within exosomes[124]. AGO2 and GW182, the main components of RISC, were shown to co-localize with MVBs[125], and inhibiting MVB formation resulted in miRISC loss[126]. In addition, in cancer cells AGO2-associated miRNAs have been shown to sort into exosomes[127]. Thus, it is plausible that exosomal transfer of miRNA also involves RISC components, reducing the chance of miRNA

machinery saturation upon miRNA delivery. In contrast, an *in vitro* study with transfected miRNA mimics demonstrated upregulation of endogenous miRNA targets, suggesting impaired effectiveness of endogenous miRNAs due to competition for RISC from the supraphysiological levels of exogenous miRNA[128]. In HeLa cells, transient transfection of miRNA led to the accumulation of high molecular weight RNA species and non-specific changes in gene expression while lentiviral infection or plasmid transfection caused smaller increases in mature miRNA levels without these adverse effects[129]. Although our functional assays in HUVECs were conducted with a transfection concentration of only 1 nM, it remains plausible that exosomal miRNA delivery leads to smaller increases in mature miRNA levels, which might contribute to the functional differences observed between studies.

5-4 Dysregulated miRNAs may have diagnostic and prognostic potential for kidney injury in humans

Over the past decade, there has been an expanding effort to uncover the roles of miRNAs in AKI from human and pre-clinical studies[130]. Manuscript III, a systematic review of 92 studies of dysregulated miRNAs in human AKI, aimed to identify potential miRNA biomarker and therapeutic candidates in humans[109]. Our systematic review identified a total 128 miRNAs as dysregulated across AKI etiologies, with the majority (84/128, 66%) identified once from single studies only, which raises concerns regarding the reproducibility of results[109]. Interestingly, we did not identify miR-486-5p among dysregulated miRNAs, and therefore it may not have biomarker potential despite the therapeutic effects in rodent models of AKI. In this regard, a previous study of miR-486-5p in mice with kidney I/R injury demonstrated that I/R led to non-significant increases in endogenous miR-486-5p levels in whole kidney and in isolated proximal

tubules[131], and we did not demonstrate any significant difference in endogenous miR-486-5p levels in rats with kidney I/R injury[108]. Further, miR-486-5p is abundant in human plasma and its levels are impacted by hemolysis as miR-486-5p is also an erythroid-enriched miRNA[20], thereby potentially impacting the interpretation of circulating miR-486-5p in biomarker studies.

miR-21 was the most frequently reported dysregulated miRNA across human AKI etiologies, transiently downregulated in cardiac surgery-associated AKI (CS-AKI) but increased in sepsis and kidney transplant-associated AKI[109]. miR-21 was also identified as the most frequently studied and most promising therapeutic target in rodent AKI models, demonstrating that miR-21 antagonism in the setting of ischemic pre-conditioning aggravated AKI[96]. The therapeutic effect of miR-21 appears limited to the AKI initiation stage, and there are no studies that evaluating the effect of delayed administration[96]. Interestingly, in Alport syndrome, a genetic cause of progressive CKD attributed to mutations in type IV collagen, miR-21 levels were increased in human kidney samples and correlated with disease severity, while administration of anti-miR-21 in a mouse model of Alport syndrome attenuated kidney function decline[132]. Pre-clinical data on miR-21 antagonism in Alport syndrome led to the use of the miR-21 antagonist Levasimendan in the first phase II randomized control trial of miRNA therapeutics for kidney disease[133]. Although this trial demonstrated no benefit of miR-21 antagonism on CKD progression in Alport syndrome, no serious adverse effects were demonstrated[133]. This supports the feasibility of translating miRNA therapies with robust pre-clinical data to clinical trials for other kidney diseases.

Finally, although this systematic review identified dysregulated miRNAs that have potential pathogenic or protective mechanisms relevant to AKI, we also reported on important methodological limitations that introduce bias, impacting data reproducibility and interpretation.

These limitations relate to factors in study design, miRNA isolation and measurement strategies, and data normalization. Specifically, many studies conducted only targeted approaches, including most miR-21 studies. Interestingly, most dysregulated miRNAs reported from single studies used an unbiased approach. Other serious study design concerns included limited human data, use of healthy volunteers as controls rather than appropriate disease controls, and lack of information on timing of AKI diagnosis and miRNA measurements.

We also identified methodological heterogeneity in miRNA isolation and measurement methods across the studies. Major areas of concern include the different biological sources of miRNAs, lack of information regarding sample hemolysis (which impacts the circulating miRNA profile[134]), lack of anticoagulant standardization for blood collection (affects miRNA profiles[135]), and absence of data normalization, particularly for circulating or urinary miRNAs. Thus, study design improvements in these areas are needed to improve the reliability and impact of this research.

5-5 Conclusions and future directions

AKI is a common complication in hospitalized patients with a high in-hospital mortality, and patients with recovered AKI remain at risk of long-term adverse vascular and kidney outcomes. There is a critical need for effective treatments for this condition. This thesis focused on the therapeutic potential of miR-486-5p in rat kidney I/R injury and associated long-term systemic and renal microvascular complications. Our data revealed that early administration of miR-486-5p to rats with kidney I/R injury prevented endothelial injury and highlighted the importance of early treatment for the prevention of peritubular capillary rarefaction and systemic endothelial dysfunction after ischemic AKI. Importantly, despite inhibition of I/R-induced kidney

eNOS expression by miR-486-5p, we found no evidence of long-term adverse vascular outcomes in our rat model. Yet, this thesis also revealed adverse effects on endothelial function via eNOS downregulation in cultured endothelial cells, likely mediated by targeting of MAML3. To further explore these differences, future research comparing alternative miR-486-5p delivery methods on target gene repression and cell functions is needed, particularly since the use of miR-486-5p antagomiRs in cultured endothelial cells is ineffective due to baseline low endogenous miR-486-5p levels. Overall, the findings in this thesis contribute an important step towards the development of a novel therapy for patients with ischemic AKI.

References

1. Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; 75: 843-854. DOI: https://doi.org/10.1016/0092-8674(93)90529-Y.
2. Plotnikova O, Baranova A and Skoblov M. Comprehensive Analysis of Human microRNA-mRNA Interactome. *Front Genet* 2019; 10: 933. DOI: https://doi.org/10.3389/fgene.2019.00933.
3. Friedman RC, Farh KK-H, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19: 92-105. DOI: https://doi.org/10.1101/gr.082701.108.
4. O'Brien J, Hayder H, Zayed Y, et al. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol* 2018; 9: 402. DOI: https://doi.org/10.3389/fendo.2018.00402.
5. Denli AM, Tops BBJ, Plasterk RHA, et al. Processing of primary microRNAs by the Microprocessor complex. *Nature* 2004; 432: 231-235. DOI: https://doi-org.proxy.bib.uottawa.ca/10.1038/nature03049.
6. Frank F, Sonenberg N and Nagar B. Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* 2010; 465: 818-822. DOI: https://doi.org/10.1038/nature09039.
7. Khvorova A, Reynolds A and Jayasena SD. Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* 2003; 115: 209-216. DOI: https://doi.org/10.1016/S0092-8674(03)00801-8.
8. Medley JC, Panzade G and Zinovyeva AY. microRNA strand selection: unwinding the rules. *Wiley Interdiscip Rev RNA* 2021; 12: e1627. DOI: https://doi.org/10.1002/wrna.1627.
9. Jonas S and Izaurralde E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet* 2015; 16: 421-433. DOI: https://doi.org/10.1038/nrg3965.
10. Nishi K, Ai Nishi TN and Ui-Tei K. Human TNRC6A is an Argonaute-navigator protein for microRNA-mediated gene silencing in the nucleus. *RNA* 2013; 19: 17-35. DOI: https://doi.org/10.1261/rna.034769.112.
11. Wei Y, Li L, Wang D, et al. Importin 8 Regulates the Transport of Mature MicroRNAs into the Cell Nucleus. *J Biol Chem* 2014; 289: 10270-10275. DOI: https://doi.org/10.1074/jbc.C113.541417.
12. Catalanotto C, Cogoni C and Zardo G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci* 2016; 17: 1712. DOI: https://doi.org/10.3390/ijms17101712.
13. Cernilogar FM, Onorati MC, Greg O. Kothe4 AMB, et al. Chromatin-associated RNAi components contribute to transcriptional regulation in Drosophila. *Nature* 2011; 480: 391-395. DOI: https://doi.org/10.1038/nature10492.
14. Lewis BP, Burge CB and Bartel DP. Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. *Cell* 2005; 120: 15-20. DOI: https://doi.org/10.1016/j.cell.2004.12.035.

15. Moore MJ, Scheel TKH, Luna JM, et al. miRNA–target chimeras reveal miRNA 30-end pairing as a major determinant of Argonaute target specificity. *Nat Commun* 2015; 6: 8864. DOI: https://doi.org/10.1038/ncomms9864.
16. Shin C, Nam J-W, Farh KK-H, et al. Expanding the MicroRNA Targeting Code: Functional Sites with Centered Pairing. *Mol Cell* 2010; 38: 789-802. DOI: https://doi.org/10.1016/j.molcel.2010.06.005.
17. Grimson A, Farh KK-H, Johnston WK, et al. MicroRNA Targeting Specificity in Mammals: Determinants Beyond Seed Pairing. *Molecular Cell* 2007; 27: 91-105. DOI: https://doi.org/10.1016/j.molcel.2007.06.017.
18. Broughton JP, Lovci MT, Huang JL, et al. Pairing beyond the Seed Supports MicroRNA Targeting Specificity. *Molecular Cell* 2016; 64: 320-333. DOI: http://dx.doi.org/10.1016/j.molcel.2016.09.004.
19. Martin HC, Wani S, Steptoe AL, et al. Imperfect centered miRNA binding sites are common and can mediate repression of target mRNAs. *Genome Biology* 2014; 15: R51. DOI: https://doi.org/10.1186/gb-2014-15-3-r51.
20. Douvris A, Viñas J and Burns KD. miRNA-486-5p: signaling targets and role in non-malignant disease. *Cell Mol Life Sci* 2022; 79: 376. DOI: https://doi.org/10.1007/s00018-022-04406-y.
21. Eisenberg I, Eran A, Nishino I, et al. Distinctive patterns of microRNA expression in primary muscular disorders. *Proc Natl Acad Sci U S A* 2007; 104: 17016-17021. DOI: https://doi.org/10.1073/pnas.0708115104.
22. Small EM, O'Rourke JR, Moresi V, et al. Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. *Proc Natl Acad Sci U S A* 2010; 107: 4218-4223. DOI: https://doi.org/10.1073/pnas.1000300107.
23. Alexander MS, Casar JC, Motohashi N, et al. MicroRNA-486–dependent modulation of DOCK3/PTEN/AKT signaling pathways
improves muscular dystrophy–associated symptoms. *J Clin Invest* 2014; 124: 2651-2667. DOI: https://doi.org/10.1172/JCI73579.
24. Lulli V, Romania P, Morsilli O, et al. MicroRNA-486-3p Regulates gamma-Globin Expression in Human Erythroid Cells by Directly Modulating BCL11A. *PLoS ONE* 2013; 8: e60436. DOI: https://doi.org/10.1371/journal.pone.0060436.
25. Jee D, Yang J-S, Park S-M, et al. Dual Strategies for Argonaute2-Mediated Biogenesis of Erythroid miRNAs Underlie
Conserved Requirements for Slicing in Mammals. *Molecular Cell* 2018; 69: 265-278. DOI: https://doi.org/10.1016/j.molcel.2017.12.027.
26. Ghafouri-Fard S, Abak A, Shoorei H, et al. Regulatory role of microRNAs on PTEN signaling. *Biomed Pharmacother* 2021; 133: 110986. DOI: https://doi.org/10.1016/j.biopha.2020.110986.
27. Tzivion G, Dobson M and Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochim Biophys Acta* 2011; 1813: 1938-1945. DOI: https://doi.org/10.1016/j.bbamcr.2011.06.002.
28. Alexander MS, Casar JC, Motohashi N, et al. Regulation of DMD pathology by an ankyrin-encoded miRNA. *Skeletal Muscle* 2011; 1: 27. DOI: https://doi.org/10.1186/2044-5040-1-27.

29. Li Z, Liu C, Li3 S, et al. BMSC-Derived Exosomes Inhibit Dexamethasone-Induced Muscle Atrophy via the miR-486-5p/FoxO1 Axis. *Front Endocrinol* 2021; 12: 681267. DOI: https://doi.org/10.3389/fendo.2021.681267.
30. Xu J, Li R, Workeneh B, et al. Transcription factor FoxO1, the dominant mediator of muscle wasting in chronic kidney disease, is inhibited by microRNA-486. *Kidney International* 2012; 82: 401-411. DOI: https://doi.org/10.1038/ki.2012.84.
31. Stitt TN, Drujan D, Clarke BA, et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* 2004; 14: 395-403. DOI: https://doi.org/10.1016/s1097-2765(04)00211-4.
32. Sandri M, Sandri C, Gilbert A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 2004; 117: 399-412. DOI: https://doi.org/10.1016/s0092-8674(04)00400-3.
33. Zhu H-H, Wang X-T, Sun Y-H, et al. MicroRNA-486-5p targeting PTEN Protects Against Coronary Microembolization-Induced Cardiomyocyte Apoptosis in Rats by activating the PI3K/AKT pathway. *Eur J Pharmacol* 2019; 855: 244-251. DOI: https://doi.org/10.1016/j.ejphar.2019.03.045.
34. Sun X-H, Wang X, Zhang Y, et al. Exosomes of bone-marrow stromal cells inhibit cardiomyocyte apoptosis under ischemic and hypoxic conditions via miR-486-5p targeting the PTEN/PI3K/AKT signaling pathway. *Thromb Res* 2019; 177: 23-32. DOI: https://doi.org/10.1016/j.thromres.2019.02.002.
35. Bei Y, Lu D, Bär C, et al. miR-486 attenuates cardiac ischemia/reperfusion injury and mediates the beneficial effect of exercise for myocardial protection. *Molecular Therapy* 2022; 30: 1675-1691. DOI: https://doi.org/10.1016/j.ymthe.2022.01.031.
36. Li Q, Xu Y, Lv K, et al. Small extracellular vesicles containing miR-486-5p promote angiogenesis after myocardial infarction in mice and nonhuman primates. *Sci Transl Med* 2021; 13: eabb0202. DOI: https://doi.org/10.1126/scitranslmed.abb0202.
37. Bao H, Mao S, Hu X, et al. Exosomal miR-486 derived from bone marrow mesenchymal stem cells promotes angiogenesis following cerebral ischemic injury by regulating the PTEN/Akt pathway. *Scientific Reports* 2024; 14: 18086. DOI: https://doi.org/10.1038/s41598-024-69172-2.
38. Meng X-M, Nikolic-Paterson DJ and Lan HY. TGF-β: the master regulator of fibrosis. *Nat Rev Nephrol* 2016; 12: 325-338. DOI: https://doi.org/10.1038/nrneph.2016.48.
39. Ji X, Wu B, Fan J, et al. The Anti-fibrotic Effects and Mechanisms of MicroRNA-486-5p in Pulmonary Fibrosis. *Scientific Reports* 2015; 5: 14131. DOI: https://doi.org/10.1038/srep14131.
40. Zhao H, Yang H, Geng C, et al. Role of IgE-FcεR1 in Pathological Cardiac Remodeling and Dysfunction. *Circulation* 2021; 143: 1014-1030. DOI: https://doi.org/10.1161/CIRCULATIONAHA.120.047852.
41. Zhao H, Yang H, Geng C, et al. Elevated IgE promotes cardiac fibrosis by suppressing miR-486a-5p. *Theranostics* 2021; 11: 7600-7615. DOI: https://doi.org/10.7150/thno.47845.
42. Feehally J, Floege J, Tonelli M, et al. Section 1: Essential Renal Anatomy and Physiology. *Comprehensive Clinical Nephrology (Sixth Edition)* 2019: 1-29.
43. Basile DP, Anderson MD and Sutton TA. Pathophysiology of Acute Kidney Injury. *Compr Physiol* 2012; 2: 1303-1353. DOI: https://doi.org/10.1002/cphy.c110041.

44. Venkatachalam MA, Bernard DB, Donohoe JF, et al. Ischemic damage and repair in the rat proximal tubule: Differences among the S1, S2, and S3 segment. *Kidney International* 1978; 14: 31-49. DOI: https://doi.org/10.1038/ki.1978.87.
45. Bastin J, Cambon N, Thompson M, et al. Change in energy reserves in diferent segments of the nephron during brief ischemia. *Kidney International* 1987; 31: 1239-1247. DOI: https://doi.org/10.1038/ki.1987.137.
46. Uchida S and Endou H. Substrate specificity to maintain cellular ATP along the mouse nephron. *Am J Physiol* 1988; 255: F977-983. DOI: https://doi.org/10.1152/ajprenal.1988.255.5.F977.
47. Kumar S and Molitoris BA. Renal Endothelial Injury and Microvascular Dysfunction in Acute Kidney Injury. *Semin Nephrol* 2015; 35: 96-107. DOI: https://doi.org/10.1016/j.semnephrol.2015.01.010.
48. KDIGO clinical practice guidelines for acute kidney injury. *Kidney International Suppl* 2012; 2: 19-36.
49. Schrezenmeier EV, Barasch J, Budde K, et al. Biomarkers in acute kidney injury – pathophysiological basis and clinical performance. *Acta Physiol* 2017; 219: 556-574. DOI: https://doi.org/10.1111/apha.12764.
50. Wang HE, Muntner P, Chertow GM, et al. Acute Kidney Injury and Mortality in Hospitalized Patients. *Am J Nephrol* 2012; 35: 349-355. DOI: https://doi.org/10.1159/000337487.
51. Uchino S, Bellomo R, Goldsmith D, et al. An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. *Crit Care Med* 2006; 34: 1913-1917. DOI: https://doi.org/10.1097/01.CCM.0000224227.70642.4F.
52. See EJ, Jayasinghe K, Glassford N, et al. Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. *Kidney International* 2019; 95: 160-172. DOI: https://doi.org/10.1016/j.kint.2018.08.036.
53. McCoy IE, Hsu JY, Zhang X, et al. Probing the Association between Acute Kidney Injury and Cardiovascular Outcomes. *Clin J Am Soc Nephrol* 2023; 18: 850-857. DOI: https://doi.org/10.2215/CJN.0000000000000163.
54. Robinson CH, Jeyakumar N, Luo B, et al. Long-Term Kidney Outcomes after Pediatric Acute Kidney Injury. *J Am Soc Nephrol* 2024; 35: 1520-1532. DOI: https://doi.org/10.1681/ASN.0000000000000445.
55. Collister D, Pannu N, Ye F, et al. Health Care Costs Associated with AKI. *Clin J Am Soc Nephrol* 2017; 12: 1733-1743. DOI: https://doi.org/10.2215/CJN.00950117.
56. Fu Y, Tang C, Cai J, et al. Rodent models of AKI-CKD transition. *Am J Physiol Renal Physiol* 2018; 315: F1098-F1106. DOI: https://doi.org/10.1152/ajprenal.00199.2018.
57. Basile DP. The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function. *Kidney International* 2007; 72: 151-156. DOI: https://doi.org/10.1038/sj.ki.5002312.
58. Conger JD, Robinette JB and Hammond WS. Differences in vascular reactivity in models of ischemic acute renal failure. *Kidney International* 1991; 39: 1087-1097. DOI: https://doi.org/10.1038/ki.1991.138.

59. Conger JD, Robinette JB and Schrier RW. Smooth Muscle Calcium and Endothelium-derived Relaxing Factor in the Abnormal Vascular Responses of Acute Renal Failure. *J Clin Invest* 1988; 82: 532-537. DOI: https://doi.org/10.1172/JCI113628.
60. Mattson DL, Lu S and CowleyJr AW. Role of Nitric Oxide in the Control of the Renal Medullary Circulation. *Clin Exp Pharmacol Physiol* 1997; 24: 587-590. DOI: https://doi.org/10.1111/j.1440-1681.1997.tb02096.x.
61. Brodsky SV, Yamamoto T, Tada T, et al. Endothelial dysfunction in ischemic acute renal failure: rescue by transplanted endothelial cells. *Am J Physiol Renal Physiol* 2002; 282: F1140-F1149. DOI: https://doi.org/10.1152/ajprenal.00329.200.
62. DeGreef KE, Ysebaert DK, Persy V, et al. ICAM-1 expression and leukocyte accumulation in inner stripe of outer medulla in early phase of ischemic compared to HgCl2-induced ARF. *Kidney International* 2003; 63: 1697-1707. DOI: https://doi/org/10.1046/j.1523-1755.2003.00909.x.
63. Kelly KJ, Sutton TA, Weathered N, et al. Minocycline inhibits apoptosis and inflammation in a rat model of ischemic renal injury. *Am J Physiol Renal Physiol* 2004; 287: F760-F766. DOI: https://doi.org/10.1152/ajprenal.00050.2004.
64. Li L, Huang L, Sung S-SJ, et al. The chemokine receptors CCR2 and CX3CR1 mediate monocyte/macrophage trafficking in kidney ischemia-reperfusion injury. *Kidney International* 2008; 74: 1526-1537. DOI: https://doi.org/10.1038/ki.2008.500.
65. Venkatachalam MA, Weinberg JM, Kriz W, et al. Failed Tubule Recovery, AKI-CKD Transition, and Kidney Disease Progression. *J Am Soc Nephrol* 2015; 26: 1765-1776. DOI: https://doi.org/10.1681/ASN.2015010006.
66. Canaud G and Bonventre JV. Cell cycle arrest and the evolution of chronic kidney disease from acute kidney injury. *Nephrol Dial Transplant* 2015; 30: 575-583. DOI: https://doi.org/10.1093/ndt/gfu230.
67. Kirita Y, Wu H, Uchimura K, et al. Cell profiling of mouse acute kidney injury reveals conserved cellular responses to injury. *Proc Natl Acad Sci U S A* 2020; 117: 15874-15883. DOI: https://doi.org/10.1073/pnas.2005477117.
68. Xu L, Guo J, Moledina DG, et al. Immune-mediated tubule atrophy promotes acute kidney injury to chronic kidney disease transition. *Nature Communications* 2022; 13: 4892. DOI: https://doi.org/10.1038/s41467-022-32634-0.
69. Basile DP, Donohoe D, Roethe K, et al. Renal ischemic injury results in permanent damage to peritubular capillaries and influences long-term function. *Am J Renal Physiol* 2001; 281: F887-F899. DOI: https://doi.org/10.1152/ajprenal.00050.2001.
70. Pechman KR, Miguel CD, Lund H, et al. Recovery from renal ischemia-reperfusion injury is associated with altered renal hemodynamics, blunted pressure natriuresis, and sodium-sensitive hypertension. *Am J Physiol Regul Integr Comp Physiol* 2009; 297: R1358-R1363. DOI: https://doi.org/10.1152/ajpregu.91022.2008.
71. Spurgeon-Pechman KR, Donohoe DL, Mattson DL, et al. Recovery from acute renal failure predisposes hypertension and secondary renal disease in response to elevated sodium. *Am J Physiol Renal Physiol* 2007; 293: F269-F278. DOI: https://doi.org/10.1152/ajprenal.00279.2006.

72. Bábícková J, Klinkhammer BM, Buhl EM, et al. Regardless of etiology, progressive renal disease causes ultrastructural and functional alterations of peritubular capillaries. *Kidney International* 2017; 91: 70-85. DOI: https://doi.org/10.1016/j.kint.2016.07.038.
73. Menshikh A, Scarfe L, Delgado R, et al. Capillary rarefaction is more closely associated with CKD progression after cisplatin, rhabdomyolysis, and ischemia-reperfusion-induced AKI than renal fibrosis. *Am J Physiol Renal Physiol* 2019; 317: F1383-F1397. DOI: https://doi.org/10.1152/ajprenal.00366.2019.
74. Hall AV and Jevnikar AM. Significance of Endothelial Cell Survival Programs for Renal Transplantation. *Am J Kidney Dis* 2003; 41: 1140-1154. DOI: https://doi.org/10.1016/S0272-6386(03)00345-7.
75. Burger D, Viñas JL, Akbari S, et al. Human endothelial colony-forming cells protect against acute kidney injury: role of exosomes. *Am J Pathol* 2015; 185: 2309-2323. DOI: https://doi.org/10.1016/j.ajpath.2015.04.010.
76. Hörbelt M, Lee S-Y, Mang HE, et al. Acute and chronic microvascular alterations in a mouse model of ischemic acute kidney injury. *Am J Physiol Renal Physiol* 2007; 293: F688-F695. DOI: https://dio.org/10.1152/ajprenal.00452.2006.
77. Basile DP, Friedrich JL, Spahic J, et al. Impaired endothelial proliferation and mesenchymal transition contribute to vascular rarefaction following acute kidney injur. *Am J Physiol Renal Physiol* 2011; 300: F721-F733. DOI: https://doi.org/10.1152/ajprenal.00546.2010.
78. Verma SK and Molitoris BA. Renal Endothelial Injury and Microvascular Dysfunction in Acute Kidney Injury. *Semin Nephrol* 2015; 35: 96-107.
79. Crawford C, Kennedy-Lydon TM, Callaghan H, et al. Extracellular nucleotides affect pericyte-mediated regulation of rat in situ vasa recta diameter. *Acta Physiol* 2011; 202: 241-251. DOI: https://doi.org/10.1111/j.1748-1716.2011.02310.x.
80. Chen Y-T, Chang F-C, Wu C-F, et al. Platelet-derived growth factor receptor signaling activates pericyte-myofibroblast transition in obstructive and post-ischemic kidney fibrosis. *Kidney International* 2011; 80: 1170-1181. DOI: https://doi.org/10.1038/ki.2011.208.
81. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008; 105: 10513-10518. DOI: https://doi.org/10.1073/pnas.0804549105.
82. Turchinovich A, Weiz L, Langheinz A, et al. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 2011; 39: 7223-7233. DOI: https://doi.org/10.1093/nar/gkr254.
83. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A* 2011; 108: 5003-5008. DOI: https://doi.org/10.1073/pnas.1019055108.
84. Sehovic E, Urru S, Chiorino G, et al. Meta-analysis of diagnostic cell-free circulating microRNAs for breast cancer detection. *BMC Cancer* 2022; 22: 634. DOI: https://doi.org/10.1186/s12885-022-09698-8.
85. Kaur A, Mackin ST, Schlosser K, et al. Systematic review of microRNA biomarkers in acute coronary syndrome and stable coronary artery disease. *Cardiovasc Res* 2020; 116: 1113-1124. DOI: https://doi.org/10.1093/cvr/cvz302.

86. Juźwik CA, Drake SS, Zhang Y, et al. microRNA dysregulation in neurodegenerative diseases: a systematic review. *Prog Neurobiol* 2019; 182: 101664. DOI: https://doi.org/10.1016/j.pneurobio.2019.101664.
87. Garmaa G, Nagy R, Kói T, et al. Panel miRNAs are potential diagnostic markers for chronic kidney diseases: a systematic review and meta-analysis. *BMC Nephrol* 2024; 25: 261. DOI: https://doi.org/10.1186/s12882-024-03702-y.
88. Gaede L, Liebetrau C, Blumenstein J, et al. Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery. *Nephrol Dial Transplant* 2016; 31: 760-766. DOI: https://doi.org/10.1093/ndt/gfw007.
89. Kang Z, Li Z, Huang P, et al. Remote ischemic preconditioning upregulates microRNA-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass. *Pediatr Nephrol* 2018; 33: 911-919. DOI: https://doi.org/10.1007/s00467-017-3851-9.
90. Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. *Am J Transplant* 2011; 11: 2221-2227. DOI: https://doi.org/10.1111/j.1600-6143.2011.03679.x.
91. Mahtal N, Lenoir O, Tinel C, et al. MicroRNAs in kidney injury and disease. *Nat Rev Nephrol* 2022; 18: 643-662. DOI: https://doi.org/10.1038/s41581-022-00608-6.
92. Harvey SJ, Jarad G, Cunningham J, et al. Podocyte-specific deletion of dicer alters cytoskeletal dynamics and causes glomerular disease. *J Am Soc Nephrol* 2008; 19: 2150-2158. DOI: https://doi.org/10.1681/ASN.2008020233.
93. Kato M, Zhang J, Wang M, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. *Proc Natl Acad Sci U S A* 2008; 104: 3432-3437. DOI: https://doi.org/10.1073/pnas.0611192104.
94. Wang Q, Wang Y, Minto AW, et al. MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *FASEB J* 2008; 22: 4126-4135. DOI: https://doi.org/10.1096/fj.08-112326.
95. Wei Q, Bhatt K, He H-Z, et al. Targeted deletion of Dicer from proximal tubules protects against renal ischemia-reperfusion injury. *J Am Soc Nephrol* 2010; 21: 756-761. DOI: https://doi.org/10.1681/ASN.2009070718.
96. Zankar S, Trentin-Sonoda M, Viñas JL, et al. Therapeutic effects of micro-RNAs in preclinical studies of acute kidney injury: a systematic review and meta-analysis. *Scientific Reports* 2021; 11: 9100. DOI: https://doi.org/10.1038/s41598-021-88746-y.
97. Viñas JL, Burger D, Zimpelmann J, et al. Transfer of microRNA-486-5p from human endothelial colony forming cell–derived exosomes reduces ischemic kidney injury. *Kidney International* 2016; 90: 1238-1250. DOI: http://dx.doi.org/10.1016/j.kint.2016.07.015.
98. Baglio SR, Rooijers K, Koppers-Lalic D, et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes
enriched in distinctive miRNA and tRNA species. *Stem Cell Research & Therapy* 2015; 6: 127. DOI: https://doi.org/10.1186/s13287-015-0116-z.
99. Viñas JL, Spence M, Porter CJ, et al. micro-RNA-486-5p protects against kidney ischemic injury and modifies the apoptotic transcriptome in proximal tubules. *Kidney International* 2021; 100: 597-612. DOI: https://doi.org/10.1016/j.kint.2021.05.034.

100. Lu Y, Wen H, Huang J, et al. Extracellular vesicle-enclosed miR-486-5p mediates wound healing with adipose-derived stem cells by promoting angiogenesis. *J Cell Mol Med* 2020; 24: 9590-9604. DOI: https://doi.org/10.1111/jcmm.15387.
101. Sun B, Han Y and Shi M. Stromal-derived miR-486-5p promotes metastasis of non-small-cell lung cancer cells by targeting the CADM1/tight junctions axis in vascular endothelial cells. *Cell Biol Int* 2021; 45: 849-857. DOI: https://doi.org/10.1002/cbin.11531.
102. Rosano S, Parab S, Noghero A, et al. Long Non-Coding RNA LINC02802 Regulates In Vitro Sprouting Angiogenesis by Sponging microRNA-486-5p. *Int J Mol Sci* 2022; 23: 1653. DOI: https://doi.org/10.3390/ijms23031653.
103. Cao F, Li Y, Peng T, et al. PTEN in kidney diseases: a potential therapeutic target in preventing AKI-to-CKD transition. *Front Med* 2024; 11: 1428995. DOI: https://doi.org/10.3389/fmed.2024.1428995.
104. Zhou J, Jia L, Hu Z, et al. Pharmacological Inhibition of PTEN Aggravates Acute Kidney Injury. *Scientific Reports* 2017; 7: 9503. DOI: https://doi.org/10.1038/s41598-017-10336-8.
105. Zhou J, Fan Y, Tang S, et al. Inhibition of PTEN activity aggravates cisplatin-induced acute kidney injury. *Oncotarget* 2017; 8: 103154-103166. DOI: https://doi.org/10.18632/oncotarget.20790.
106. Zhou J, Zhong J, Lin S, et al. Inhibition of PTEN Activity Aggravates Post Renal Fibrosis in Mice with Ischemia Reperfusion-Induced Acute Kidney Injury. *Cell Physiol Biochem* 2017; 43: 1841-1854. DOI: https://doi.org/10.1159/000484070.
107. Lan R, Geng H, Polichnowski AJ, et al. PTEN loss defines a TGF-beta-induced tubule phenotype of failed differentiation and JNK signaling during renal fibrosis. *Am J Physiol Renal Physiol* 2012; 302: F1210-F1223. DOI: https://doi.org/10.1152/ajprenal.00660.2011.
108. Douvris A, Viñas JL, Gutsol A, et al. miR-486-5p protects against rat ischemic kidney injury and prevents the transition to chronic kidney disease and vascular dysfunction. *Clinical Science* 2024; 138: 599-614. DOI: https://doi.org/10.1042/CS20231752.
109. Douvris A, Viñas JL, Akbari S, et al. Systematic review of microRNAs in human acute kidney injury. *Renal Failure* 2024; 46: 2419960. DOI: https://doi.org/10.1080/0886022X.2024.2419960.
110. Reginensi A, Clarkson M, Neirijnck Y, et al. SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Geneti* 2011; 20: 1143-1153. DOI: https://doi.org/10.1093/hmg/ddq558.
111. Aggarwal S, Wang Z, Pacheco DRF, et al. SOX9 switch links regeneration to fibrosis at the single-cell level in mammalian kidneys. *Science* 2024; 383: eadd6371. DOI: https://doi.org/10.1126/science.add6371.
112. Kramer J, Schwanbeck R, Pagel H, et al. Inhibition of Notch Signaling Ameliorates Acute Kidney Failure and Downregulates Platelet-Derived Growth Factor Receptor β in the Mouse Model. *Cells Tissues Organs* 2016; 201: 109-117. DOI: https://doi.org/10.1159/000442463.
113. Regner KR and Roman RJ. Role of medullary blood flow in the pathogenesis of renal ischemia-reperfusion injury. *Curr Opin Nephrol Hypertens* 2012; 21: 33-38. DOI: https://doi.org/10.1097/MNH.0b013e32834d085a.

114. Collett JA, Mehrotra P, Crone A, et al. Endothelial colony-forming cells ameliorate endothelial dysfunction via
secreted factors following ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2017; 312: F897-F907. DOI: https://doi.org/10.1152/ajprenal.00643.2016.
115. Rabb H, Mendiola CC, Saba SR, et al. Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury. *Biochem Biophys Res Commun* 1995; 211: 67-73. DOI: https://doi.org/10.1006/bbrc.1995.1779.
116. Haller H, Dragun D, Miethke A, et al. Antisense oligonucleotides for ICAM-1 attenuate reperfusion injury and renal failure in the rat. *Kidney International* 1996; 50: 473-480. DOI: https://doi.org/10.1038/ki.1996.338.
117. Kelly KJ, WilliamsJr WW, Colvin RB, et al. Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. *Proc Natl Acad Sci U S A* 1994; 91: 812-816. DOI: https://doi.org/10.1073/pnas.91.2.812.
118. Zhong X, Tang T-T, Shen A-R, et al. Tubular epithelial cells-derived small extracellular vesicle-VEGF-A promotes peritubular capillary repair in ischemic kidney injury. *NPJ Regen Med* 2022; 7: 73. DOI: https://doi.org/10.1038/s41536-022-00268-x.
119. Leonard EC, Friedrich JL and Basile DP. VEGF-121 preserves renal microvessel structure and ameliorates secondary renal disease following acute kidney injury. *Am J Physiol Renal Physiol* 2008; 295: F1648-F1657. DOI: https://doi.org/10.1152/ajprenal.00099.2008.
120. Lal A, Thomas MP, Altschuler G, et al. Capture of MicroRNA–Bound mRNAs Identifies the Tumor Suppressor miR-34a as a Regulator of Growth Factor Signaling. *PLoS Genetics* 2011; 7: e1002363. DOI: https://dopi.org/10.1371/journal.pgen.1002363.
121. Chi J-T, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 2003; 100: 10623-10628. DOI: https://doi.org/10.1073/pnas.1434429100.
122. Cloonan N, Brown MK, Steptoe AL, et al. The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. *Genome Biology* 2008; 9: R127. DOI: https://doi.org/10.1186/gb-2008-9-8-r127.
123. Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression. *Nature* 2011; 473: 337-342. DOI: https://doi.org/10.1038/nature10098.
124. O'Brien K, Breyne K, Ughetto S, et al. RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nature Reviews Molecular Cell Biology* 2020; 21: 585-606. DOI: https://doi.org/10.1038/s41580-020-0251-y.
125. Gibbings DJ, Ciaudo C, Erhardt M, et al. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* 2009; 11: 1143-1149. DOI: https://doi.org/10.1038/ncb1929.
126. Lee YS, Pressman S, Andress AP, et al. Silencing by small RNAs is linked to endosome trafficking. *Nat Cell Biol* 2009; 11: 1150-1156. DOI: https://doi.org/10.1038/ncb1930.
127. McKenzie AJ, Hoshino D, Hong NH, et al. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. *Cell Rep* 2016; 15: 978-987. DOI: https://doi.org/10.1016/j.celrep.2016.03.085.

128. Khan AA, Betel D, Miller ML, et al. Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs. *Nat Biotechnol* 2009; 27: 549-555. DOI: https://doi.org/10.1038/nbt.1543.
129. Jin HY, Gonzalez-Martin A, Miletic AV, et al. Transfection of microRNA Mimics Should be Used with Caution. *Front Genet* 2015; 6: 340. DOI: https://doi.org/10.3389/fgene.2015.00340.
130. Douvris A, Burger D, Rodriguez RA, et al. MicroRNA in Human Acute Kidney Injury: A Systematic Review Protocol. *Can J Kidney Health Dis* 2021; 8: 1-8. DOI: https://doi.org/10.1177/20543581211009999.
131. Viñas JL, Spence M, Gutsol A, et al. Receptor-Ligand Interaction Mediates Targeting of Endothelial Colony Forming Cell-derived Exosomes to the Kidney after Ischemic Injury. *Scientific Reports* 2018; 8: 16320. DOI: https://doi.org/10.1038/s41598-018-34557-7.
132. Guo J, Song W, Boulanger J, et al. Dysregulated Expression of microRNA-21 and Disease-Related Genes in Human Patients and in a Mouse Model of Alport Syndrome. *Hum Gen Ther* 2019; 30: 865-881. DOI: https://doi.org/10.1089/hum.2018.205.
133. Gale DP, Gross O, Wang F, et al. A Randomized Controlled Clinical Trial Testing Effects of Lademirsen on Kidney Function Decline in Adults with Alport Syndrome. *Clin J Am Soc Nephrol* 2024; 19: 995-1004. DOI: https://doi.org/10.2215/CJN.0000000000000458.
134. Shkurnikov MY, Knyazev EN, Fomicheva KA, et al. Analysis of Plasma microRNA Associated with Hemolysis. *Bull Exp Biol Med* 2016; 160: 748-750. DOI: https://doi.org/10.1007/s10517-016-3300-y.
135. Zhelankin AV, Iulmetova LN and Sharova EI. The Impact of the Anticoagulant Type in Blood Collection Tubes on Circulating Extracellular Plasma MicroRNA Profiles Revealed by Small RNA Sequencing. *Int J Mol Sci* 2022; 23: 10340. DOI: https://doi.org/10.3390/ijms231810340.

Appendices

Appendix A

Supplemental data for Manuscript I

miR-486-5p protects against rat ischemic kidney injury and prevents the transition to chronic kidney disease and vascular dysfunction

Running title: miR-486-5p prevents AKI-CKD transition in rat

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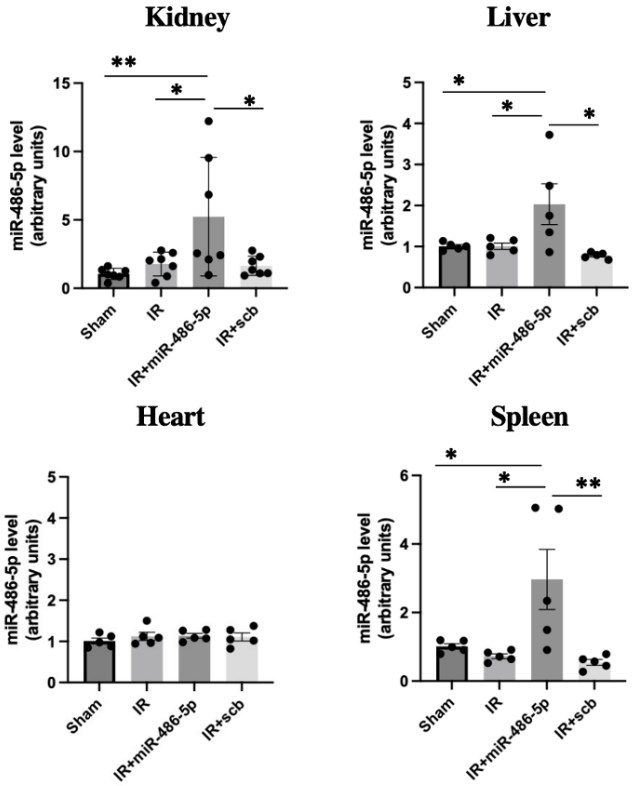

Figure S1. miR-486-5p levels in rat kidney, liver, heart and spleen at 24 h after kidney ischemia-reperfusion (IR) injury. Groups include sham-operated, kidney ischemia-reperfusion injury alone (IR), or kidney IR injury with administration of 0.5 mg/kg miR-486-5p (IR+miR-486-5p) or scramble miRNA (IR+scb) by tail vein injection at the start of reperfusion. miR-486-5p levels were normalized to endogenous U6 snRNA. **Kidney**: **p<0.01 for sham vs IR+miR-486-5p; *p<0.05 for IR and IR+scb vs IR+miR-486-5p; **Liver:** *p<0.05 for sham and IR+scb vs IR+miR-486-5p. **Spleen**: *p<0.05 for sham and IR vs IR+miR-486-5p, **p<0.01 for IR+scb vs IR+miR-486-5p. N=7 rats per group for kidney, and N=5 rats per group for liver, heart, and spleen.

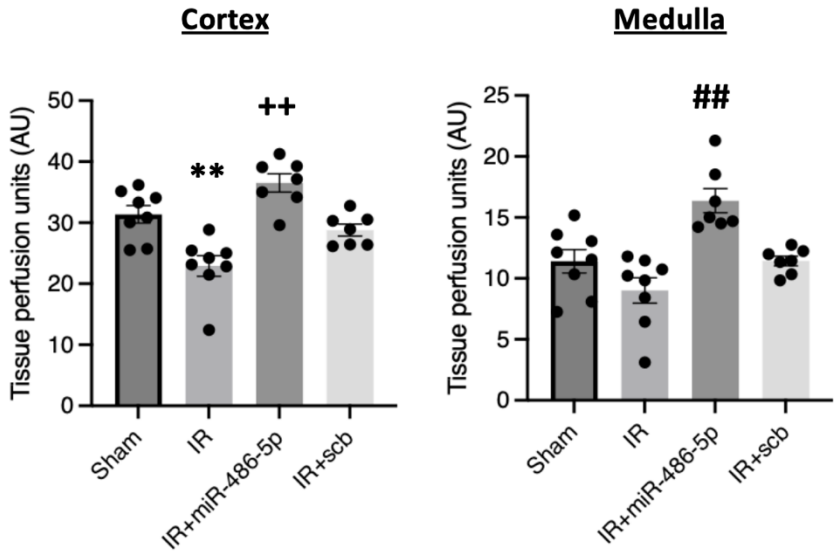

Figure S2. Effect of miR-486-5p on regional kidney blood flow 24 h after ischemia-reperfusion (IR) injury in rat. Cortical (left) and medullary (right) blood flows by laser doppler flowmetry 24 h after kidney IR injury. **p<0.01 vs sham, IR+miR-486-5p; ++p<0.01 vs IR+scb; ##p<0.01 IR+miR-486-5p vs all groups; n=7 rats per group.

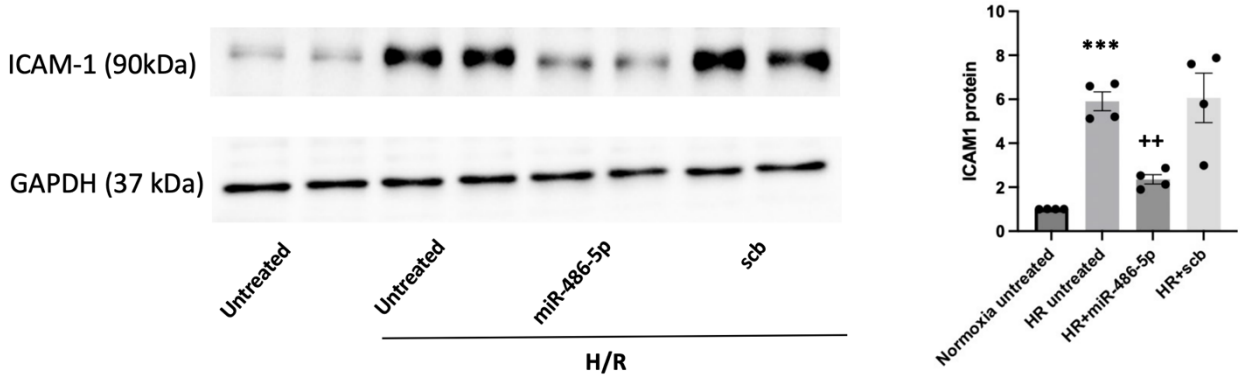

Figure S3. Effect of miR-486-5p on intercellular adhesion molecule-1 (ICAM-1) protein levels in HUVECs subjected to hypoxia/reoxygenation (H/R). ICAM-1 protein expression was evaluated by immunoblot and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Representative immunoblot is shown. Groups include untreated normoxic control HUVECs, and untreated, miR-486-5p- or scramble miRNA-transfected HUVECs exposed to H/R (H/R untreated, H/R+miR-486-5p, H/R+scb). ***p<0.001 H/R untreated vs normoxia untreated and H/R+miR-486-5p; ++p<0.01 H/R+miR-486-5p vs H/R+scb (n=4).

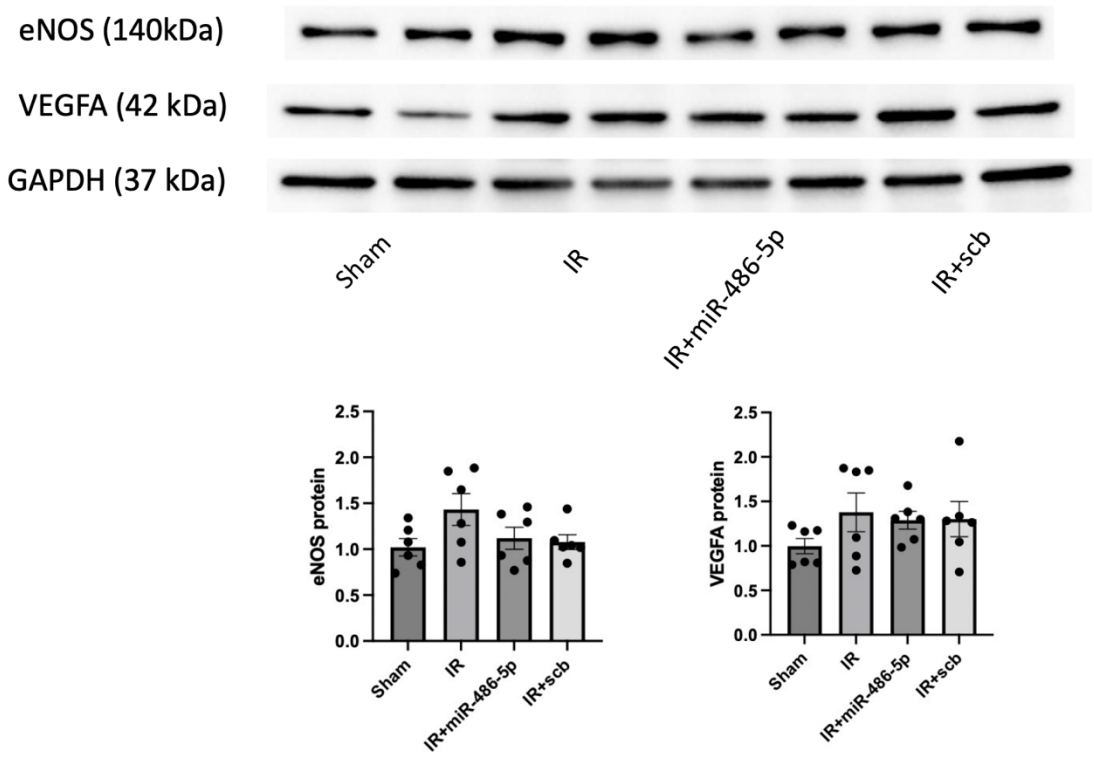

Figure S4: Effect of miR-486-5p on liver protein levels of endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor-A (VEGFA) 24 h after ischemia-reperfusion (IR) injury in rats. Graphs show liver protein expression of eNOS and VEGFA (dimer only) with representative immunoblots. Protein expression was normalized to GAPDH (n=6 rats per group)

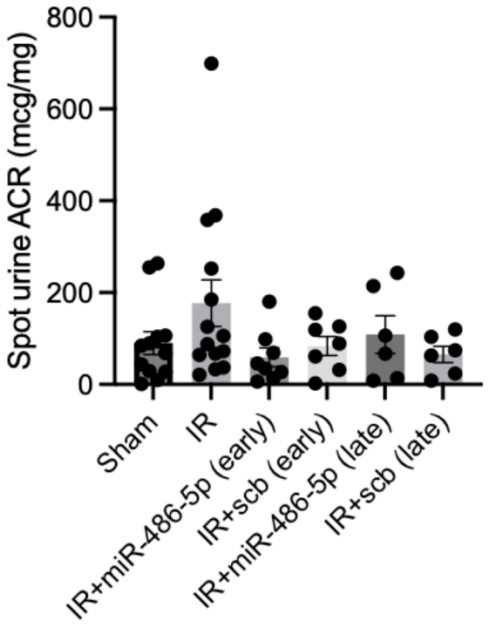

Figure S5. Urine albumin to creatinine ratio (ACR) in rats 10 weeks after bilateral kidney ischemia-reperfusion (IR) injury, with early or late administration of miR-486-5p. Groups include sham-operated, kidney IR injury alone (IR), kidney IR injury with early administration of miR-486-5p or scramble miRNA (scb) at the start of reperfusion, and kidney IR injury with late administration of miR-486-5p (or scb miRNA) at 96 h (dose #1) and 3 weeks (dose #2) after reperfusion. n=12-14 rats per group for sham and IR groups, and n=6-8 rats per group for IR+miR-486-5p and IR +scb groups

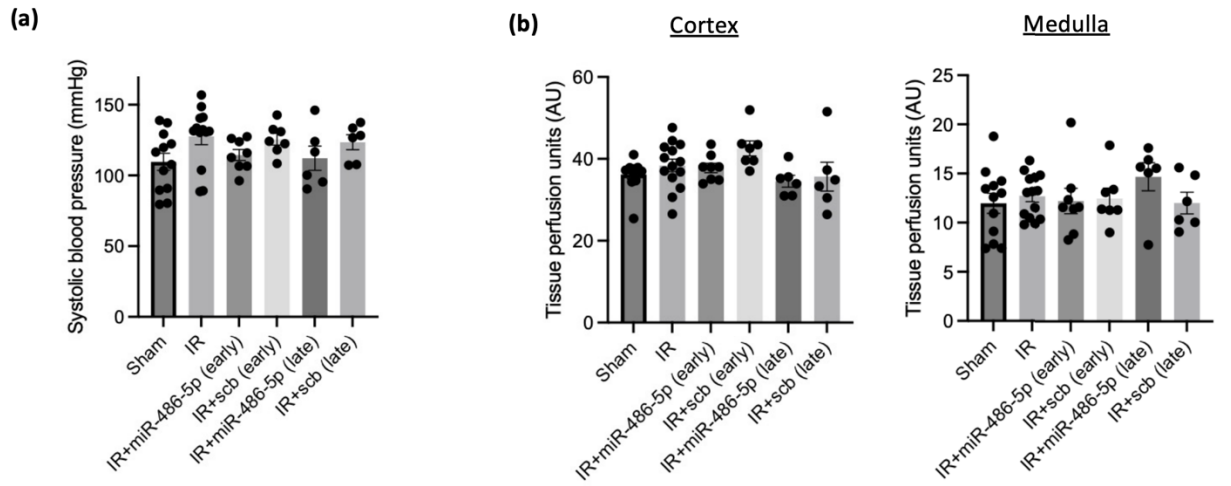

Figure S6. Blood pressure and regional kidney blood flow 8-10 weeks after kidney ischemia-reperfusion (IR) injury. (A) Systolic blood pressure 8 weeks after kidney IR injury. (B) Kidney cortical and medullary blood flows 10 weeks after kidney IR injury; n=12-14 rats per group for sham and IR groups, and n=6-8 rats per group for IR+miR-486-5p and IR+scramble (scb) groups.

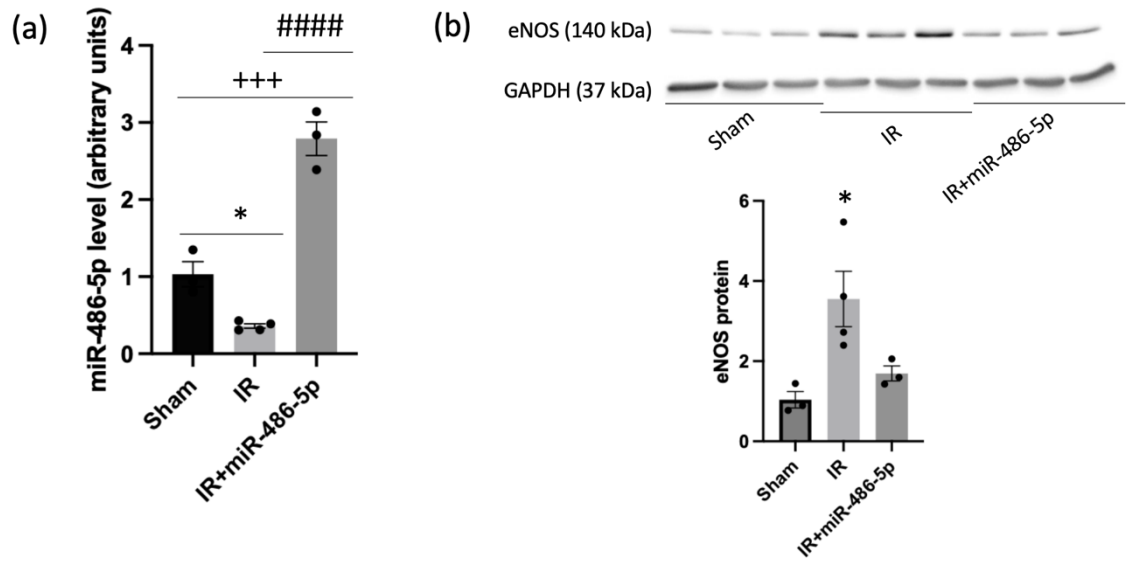

Figure S7. Kidney miR-486-5p levels (a) and endothelial nitric oxide synthase (eNOS) protein expression (b) 24 h after late administration of miR-486-5p. Rats were subjected to kidney ischemia-reperfusion (IR) injury, followed by administration of miR-486-5p 96 h after reperfusion and sacrifice 24 h later. Rat groups include sham-operated, kidney ischemia-reperfusion (IR) injury alone (IR), or kidney IR injury with administration of 0.5 mg/kg miR-486-5p (IR+miR-486-5p) (a) miR-486-5p levels were normalized to endogenous U6 snRNA. *p<0.05 (sham vs IR), +++p<0.001 (sham vs IR+miR-486-5p), ####p<0.0001 (IR vs IR+miR-486-5p) (b) Graph shows kidney protein expression of eNOS, with representative immunoblot. Protein expression was normalized to GAPDH. *p<0.05 sham vs IR; n=3 rats per group for sham and IR+miR-486-5p groups, and n=4 for IR group

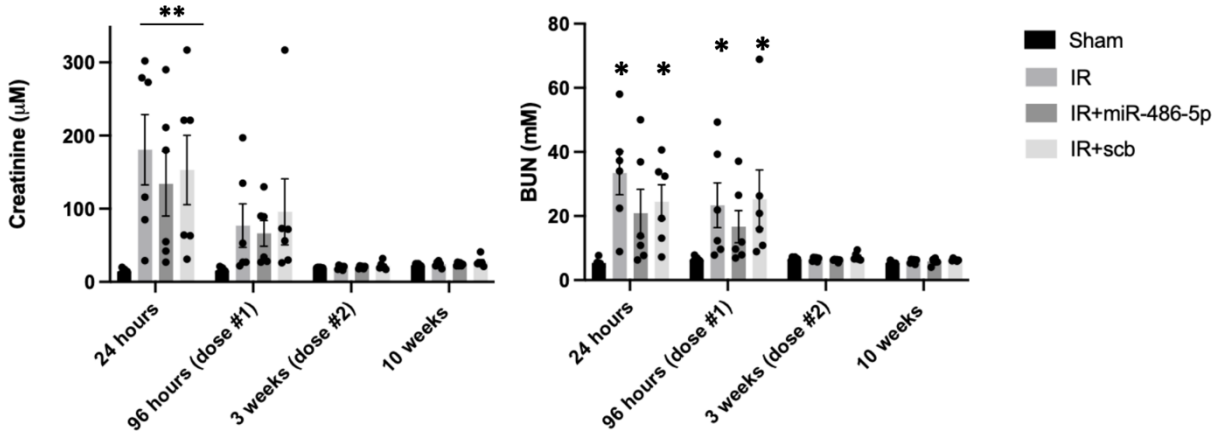

Figure S8. Effect of delayed miR-486-5p administration on kidney function after ischemia-reperfusion (IR) injury. Kidney function by plasma creatinine (Cr) and BUN levels with late administration of miR-486-5p or scramble (scb) miRNA at 96 h and 3 weeks after reperfusion. Plasma Cr and BUN are reported in standard SI units. To convert plasma Cr from μM to mg/dL, multiply by 0.0113, and to convert mM BUN to mg/dL, multiply by 2.8. **p<0.01, *p<0.05 vs sham; n=6 rats per group.

Appendix B

Supplemental data for Manuscript II

miR-486-5p inhibits eNOS and angiogenesis in cultured endothelial cells by targeting MAML3

Adrianna Douvris[1,2], Ali Maadelat[1], Christopher J. Porter[3], Dylan Burger[1,2], Kevin D. Burns[1,2]

Affiliations

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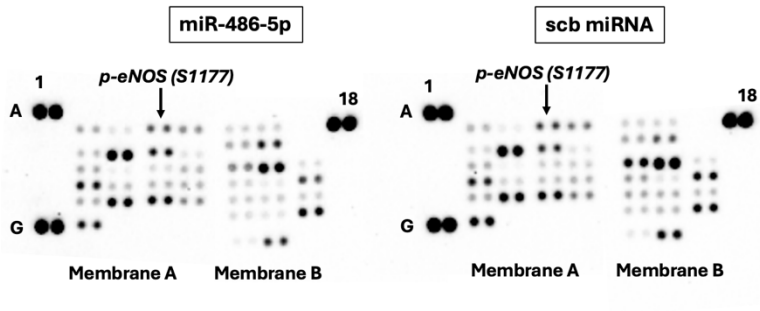
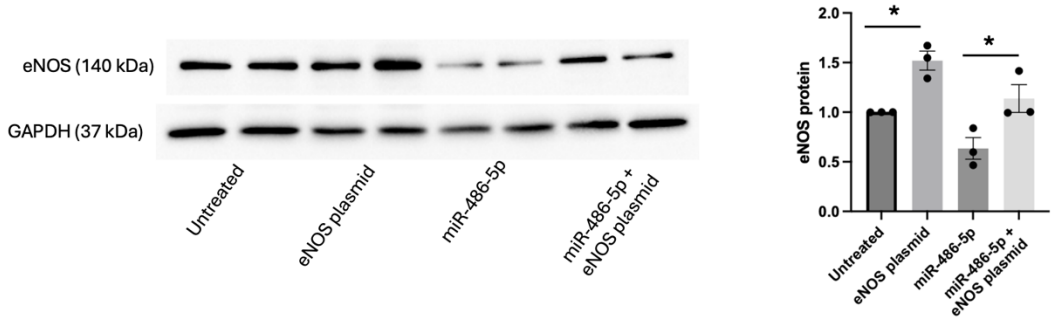


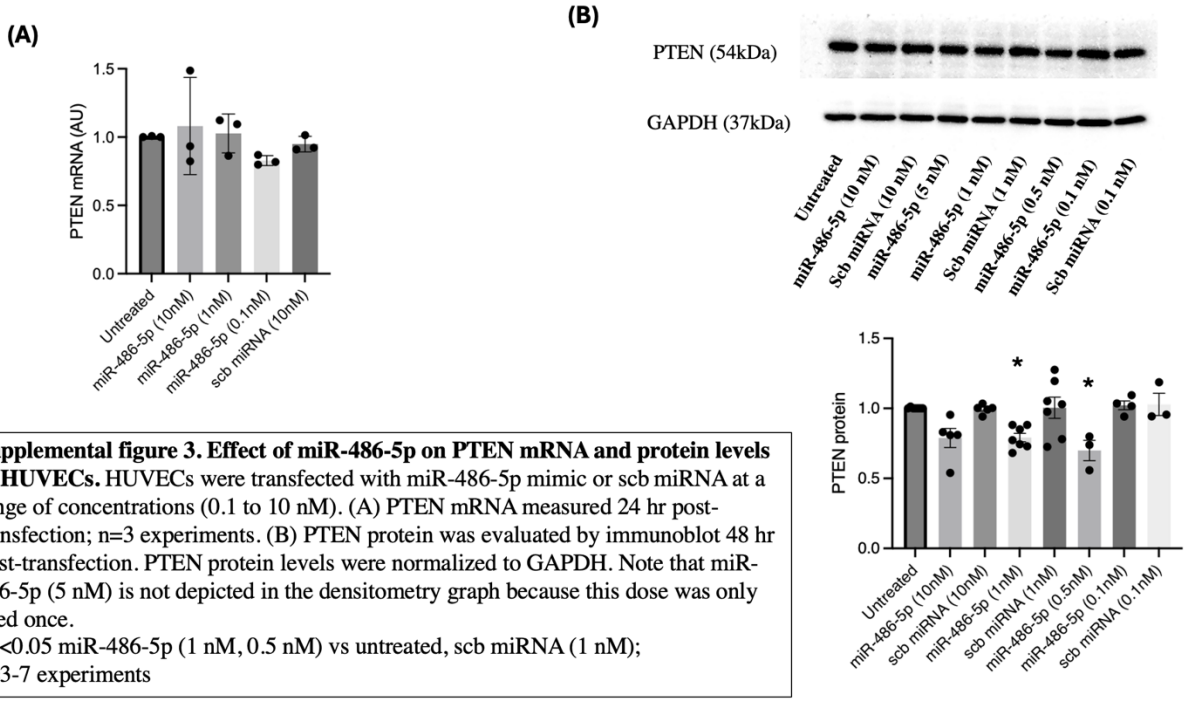
Phosphokinase Array Legend

Spot	Protein	Phospho-site
A-A1,2	Reference spot	---
B-A11,12	Akt 1/2/3	T308
B-A13,14	Akt 1/2/3	S473
B-A17,18	Reference spot	---
A-B3,4	CREB	S133
A-B5,6	EGFR	Y1086
A-B7,8	eNOS	S1177
A-B9,10	ERK1/2	T202/Y204 T185/Y187
B-B11,12	Chk-2	T68
B-B13,14	c-Jun	S63
A-C3,4	Fgr	Y412
A-C5,6	GSK-3α/8	S21/S9
A-C7,8	GSK-3ß	S9
A-C9,10	HSP27	S78/S82
B-C11,12	p53	S15
B-C13,14	p53	S46
B-C15,16	p53	S392
A-D3,4	JNK1/2/3	T183/Y185/ T221/Y223
A-D5,6	Lck	Y394
A-D7,8	Lyn	Y397
A-D9,10	MSK1/2	S376/S360
B-D11,12	P70 S6 kinase	T389
B-D13,14	P70 S6 kinase	T421/S424
B-D15,16	PRAS40	T246
A-E3,4	p38α	T180/Y182
A-E5,6	PDGFRß	Y751
A-E7,8	PLC-ð1	Y783
A-E9,10	Src	Y419
B-E11,12	PYK2	Y402
B-E13,14	RSK1/2	S221/S227
B-E15,16	RSK1/2/3	S380/S386/ S377
A-F3,4	STAT2	Y689
A-F5,6	STAT5a/b	Y694/Y699
A-F7,8	WNK1	T60
A-F9,10	Yes	Y426
B-F11,12	STAT1	Y701
B-F13,14	STAT3	Y705
B-F15,16	STAT3	S727
A-G1,2	Reference spot	---
A-G3,4	ß-catenin	---
A-G9,10 B-G17,18	PBS (negative control)	---
B-G11,12	STAT6	Y641
B-G13,14	HSP60	---

Supplemental figure 1. Phosphokinase antibody array from HUVECs transfected with miR-486-5p mimic or scb miRNA.
Left: images of membranes (phospho-proteins are in duplicate).
Right: Array legend for coordinates, protein target and phosphorylation site



Supplemental figure 2. eNOS plasmid transfection in HUVECs. HUVECs were reverse-transfected with eNOS plasmid, miR-486-5p mimic alone, or eNOS plasmid with miR-486-5p mimic. HUVECs were lysed after 48 hr for immunoblot. eNOS protein levels were normalized to GAPDH. *p<0.05 (n=3 experiments)



Supplemental figure 3. Effect of miR-486-5p on PTEN mRNA and protein levels in HUVECs. HUVECs were transfected with miR-486-5p mimic or scb miRNA at a range of concentrations (0.1 to 10 nM). (A) PTEN mRNA measured 24 hr post-transfection; n=3 experiments. (B) PTEN protein was evaluated by immunoblot 48 hr post-transfection. PTEN protein levels were normalized to GAPDH. Note that miR-486-5p (5 nM) is not depicted in the densitometry graph because this dose was only used once.
*p<0.05 miR-486-5p (1 nM, 0.5 nM) vs untreated, scb miRNA (1 nM);
n=3-7 experiments

Appendix C

Supplemental data for Manuscript III

Systematic Review of MicroRNAs in Human Acute Kidney Injury

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PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	File S2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6-7
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6-7
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	n/a
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	7

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	7
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	n/a
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	8 (and fig 1)
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8-11, Supplementary table 1
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	11, Supplementary Table 1
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	11 (and fig 4)
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	n/a
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	11
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	n/a
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12-14, 16
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	14-15
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	16
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	17

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

Supplementary file s2

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Ovid MEDLINE(R) ALL <1946 to August 21, 2023>
EBM Reviews - Cochrane Central Register of Controlled Trials <July 2023>

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6 or/1-5 632639
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68 55 and 67 3476
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Supplementary Table 1

Author	Study Title	Country	Study Design	Study population
Kidney Transplant				
Lorenzen J.M. et al (2011) [1]	Urinary miR-210 as a Mediator of Acute T-Cell Mediated Rejection in Renal Allograft Recipients	Germany	Case-control	Kidney transplant recipients
Wilflingseder J. et al (2013) [2]	miRNA Profiling Discriminates Types of Rejection and Injury in Human Renal Allografts	Austria	Case-control	Graft biopsies
Sui W. et al (2013) [3]	Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA	China	Case-control	Kidney transplant recipients
Lorenzen J.M. et al (2014) [4]	MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury	Germany	Case-control	Kidney transplant recipients
Wilflingseder J. et al (2014) [5]	Molecular Pathogenesis of Post-Transplant Acute Kidney Injury: Assessment of Whole-Genome mRNA and MiRNA Profiles	Hungary	Case-control	Kidney transplant recipients
Liu X. et al (2015) [6]	MicroRNA-10b downregulation mediates acute rejection of renal allografts by derepressing BCL2L11	China	Case-control	Kidney transplant recipients
Soltaninejad E. et al (2015)[7]	Differential expression of microRNAs in renal transplant patients with acute T-cell mediated rejection	Iran	Case-control	Kidney transplant recipients
Tao J. et al (2015)[8]	Serum MicroRNA-99a Helps Detect Acute Rejection in Renal Transplantation	China	Retrospective cohort	Kidney transplant recipients
McGuinness D. et al (2016)[9]	Identification of Molecular Markers of Delayed Graft Function Based on the Regulation of Biological Ageing	Scotland	Retrospective cohort	Pre-implantation graft biopsies
Amrouche L. et al (2017) [10]	MicroRNA-146a in Human and Experimental Ischemic AKI: CXCL8-Dependent Mechanism of Action	France	Case-control	Kidney transplant recipients
Domenico T.D. et al (2017)[11]	Upregulation of microRNA 142-3p in the peripheral blood and urinary cells of kidney transplant recipients with post-transplant graft dysfunction	Brazil	Case-control	Kidney transplant recipients
Millán O. et al (2017)[12]	Urinary miR-155-5p and CXCL10 as prognostic and predictive biomarkers of rejection, graft outcome and treatment response in kidney transplantation	Spain, Germany	Prospective cohort	1st time adult kidney transplant recipients
Jin P. et al (2017)[13]	Essential role of microRNA-650 in the regulation of B-cell CLL/lymphoma 11B gene expression following transplantation: A novel mechanism behind the acute rejection of renal allografts	China	Prospective cohort	Kidney transplant recipients
Gallon L. et al (2018)[14]	Intragraft Molecular Pathways Associated with Tolerance Induction in Renal Transplantation	USA	Retrospective cohort	Kidney transplant recipients
Cheng K. et al (2018)[15]	Role of peripheral blood microRNA-181b in acute vascular rejection after renal transplantation	China	Prospective cohort	1st time adult kidney transplant recipients
Gremmels H. et al (2019)[16]	The Small RNA Repertoire of Small Extracellular Vesicles Isolated From Donor Kidney Preservation Fluid Provides a Source for Biomarker Discovery for Organ Quality and Posttransplantation Graft Function	Netherlands	Case-control	Donor organ preservation fluid from DCD kidneys
Khalid U. et al (2019)[17]	A urinary microRNA panel that is an early predictive biomarker of delayed graft function following kidney transplantation	Wales	Prospective cohort	New Kidney transplant recipients
Li F. et al (2019)[18]	Differential MicroRNA Expressions in Human Peripheral Blood Mononuclear Cells Are Predictive of Renal Allograft Function	China	Retrospective cohort	Kidney transplant recipients
Milhoransa P. et al (2019)[19]	microRNA 146a-5p expression in Kidney transplant recipients with delayed graft function	Brazil	Prospective cohort	Kidney transplant recipients
Roest H.P. et al (2019)[20]	Cell-free MicroRNA miR-505-3p in Graft Preservation Fluid Is an Independent Predictor of Delayed Graft Function After Kidney Transplantation	Netherlands	Case-control	Preservation fluid from deceased donor kidneys
Wang J. et al (2019)[21]	Expression Profiling of Exosomal miRNAs Derived from the Peripheral Blood of Kidney Recipients with DGF Using High-Throughput Sequencing	China	Case series	Kidney transplant recipients who received a DCD kidney
Seo J-W. et al (2023)[22]	Development and validation of urinary exosomal microRNA biomarkers for the diagnosis of acute rejection in kidney transplant recipients	Korea	Prospective cohort	Kidney transplant recipients
Connor K.L. et al (2020)[23]	Identifying cell-enriched miRNAs in kidney injury and repair	United Kingdom	Retrospective cohort	Kidney transplant recipients
Alfaro R. et al (2021)[24]	MicroRNA Expression Changes in Kidney Transplant: Diagnostic Efficacy of miR-150-5p as Potential Rejection Biomarker, Pilot Study	Spain	Retrospective cohort	Kidney transplant recipients
Rutman A.K. et al (2022)[25]	Extracellular Vesicles From Kidney Allografts Express miR-218-5p and Alter Th17/Treg Ratios	Canada	Retrospective cohort	Deceased donor kidney perfusion fluid
Shi L. et al (2023[26])	MiR-20a-5p alleviates kidney ischemia/reperfusion injury by targeting ACSL4-dependent ferroptosis	China	Case-control	Kidney transplant recipients
Cardiac				
Du J. et al (2013)[27]	MicroRNA-21 and Risk of Severe Acute Kidney Injury and Poor Outcomes after Adult Cardiac Surgery	China	Prospective cohort	Cardiac surgery patients
Aguado-Fraile E. et al (2015)[28]	A Pilot Study Identifying a Set of microRNAs As Precise Diagnostic Biomarkers of Acute Kidney Injury	Spain	Prospective cohort	(1) AKI (ATN) vs healthy controls (2) ICU patients with multi-organ failure vs healthy controls (3) Cardiac surgery
Gaede L. et al (2016)[29]	Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery	Germany	Prospective cohort	Cardiac surgery patients

Arvin P. et al (2017)[30]	Early detection of cardiac surgery-associated acute kidney injury by microRNA-21	Iran	Case-control	Elective cardiac surgery
Zhang L. et al (2017)[31]	Implications of dynamic changes in miR-192 expression in ischemic acute kidney injury	China	Case-control	Cardiac surgery
Zou Y-F. et al (2017)[32]	Urinary MicroRNA-30c-5p and MicroRNA-192-5p as potential biomarkers of ischemia-reperfusion-induced kidney injury	China	Prospective cohort	Cardiac surgery
Kang Z. et al (2018)[33]	Remote ischemic preconditioning upregulates microRNA-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass	China	RCT	Pediatric congenital heart disease, cardiac surgery
Sullo N. et al (2018)[34]	An Observational Cohort Feasibility Study to Identify Microvesicle and Micro-RNA Biomarkers of Acute Kidney Injury Following Pediatric Cardiac Surgery	United Kingdom	Prospective cohort	Children undergoing cardiac surgery
Wei Q. et al (2018)[35]	MicroRNA-668 represses MTP18 to preserve mitochondrial dynamics in ischemic acute kidney injury	China	Case-control	Cardiac surgery cohort and medical patients with AKI
Gong Q. et al (2021)[36]	Hsa-miR-494-3p attenuates gene HtrA3 transcription to increase inflammatory response in hypoxia/ reoxygenation HK2 Cells	China	Case-control	Cardiac surgery
Miller D. et al (2022)[37]	Urinary extracellular vesicles and micro-RNA as markers of acute kidney injury after cardiac surgery	United Kingdom	Case-control (RCT subset)	Cardiac surgery
Chen Y. et al (2022)[38]	Exosomal transfer of microRNA-590-3p between renal tubular epithelial cells after renal ischemia-reperfusion injury regulates autophagy by targeting TRAF6	China	Retrospective cohort	Cardiac surgery
Li X. et al (2023)[39]	Inhibition of MiR-106b-5p mediated by exosomes mitigates acute kidney injury by modulating transmissible endoplasmic reticulum stress and M1 macrophage polarization	China	Case-control	Cardiac surgery
Sepsis				
Saikumar J. et al (2012)[40]	Expression, Circulation, and Excretion Profile of MicroRNA-21, -155, and -18a Following Acute Kidney Injury	USA	Case-control	Intensive care unit
Ramachandran K. et al (2013)[41]	Human miRNome Profiling Identifies MicroRNAs Differentially Present in the Urine after Kidney Injury	USA	Case-control	Critically ill patients with sepsis
Ge Q-M. et al (2017)[42]	Differentially expressed miRNAs in sepsis- induced acute kidney injury target oxidative stress and mitochondrial dysfunction pathways	China	Prospective cohort	Critically ill patients with sepsis from bacteremia
Wang S. et al (2017)[43]	MiR-107 induces TNF-a secretion in endothelial cells causing tubular cell injury in patients with septic acute kidney injury	China	Case-control	Pediatric patients with sepsis
Zhang J. et al (2018)[44]	Urinary miR-26b as a potential biomarker for patients with sepsis-associated acute kidney injury: a Chinese population-based study	China	Prospective cohort	Hospitalized patients (ICU) with sepsis from bacteremia
Bukauskas T. et al (2019)[45]	Values of circulating molecular biomarkers (microRNAs) for the evaluation of renal failure during urgent abdominal sepsis anaesthesia	Lithuania	Prospective cohort	Patients undergoing surgery for intra-abdominal sepsis
Lin Y. et al (2019)[46]	Expression patterns and prognostic value of miR-210, miR-494, and miR-205 in middle-aged and old patients with sepsis-induced acute kidney injury	China	Case-control	Hospitalized patients (ICU) with sepsis
Sun J. et al (2020)[47]	Correlation Between Single Nucleotide Polymorphisms at the 3'-UTR of the NFKB1 Gene and Acute Kidney Injury in Sepsis	China	Case-control	Hospitalized patients (ICU) with sepsis
Liu S. et al (2021)[48]	Downregulation of miR-574-5p inhibits HK-2 cell viability and predicts the onset of acute kidney injury in sepsis patients	China	Prospective cohort	Hospitalized patients with sepsis
Shi L. et al (2021)[49]	MiR-150-5p protects against septic acute kidney injury via repressing the MEKK3/JNK pathway	China	Case-control	Hospitalized patients (ICU) with sepsis
Zhang H. et al (2021)[50]	Deregulated microRNA-22-3p in patients with sepsis-induced acute kidney injury serves as a new biomarker to predict disease occurrence and 28-day survival outcomes	China	Prospective cohort	Hospitalized patients with sepsis
Zheng C. et al (2021)[51]	miR-34b-5p promotes renal cell inflammation and apoptosis by inhibiting aquaporin-2 in sepsis-induced acute kidney injury	China	Case-control	Hospitalized patients with sepsis
Gong J. et al (2022)[52]	Downregulation of circ-ZNF644 alleviates LPS-induced HK2 cell injury via miR-335-5p/HIPK1 axis	China	Case-control	Hospitalized patients with sepsis
Ma W. et al (2022)[53]	The Potential of miR-370-3p and miR-495-3p Serving as Biomarkers for Sepsis-Associated Acute Kidney Injury	China	Retrospective cohort	Hospitalized patients with sepsis
Pan W. et al (2022)[54]	Evaluation Value of Serum miR-4299 and miR-16-5p in Risk Stratification of Sepsis-Induced Acute Kidney Injury	China	Prospective cohort	Hospitalized patients with sepsis
Xun L. et al (2022)[55]	The value of combining miR-10a-5p levels and PLR to evaluate the prognosis of sepsis patients with acute kidney injury	China	Prospective cohort	Hospitalized patients (ICU) with sepsis
Xu L. et al (2022)[56]	Circ_0114427 promotes LPS-induced septic acute kidney injury by modulating miR-495-3p/TRAF6 through the NF-kB pathway	China	Case-control	Hospitalized patients with sepsis
Ye J. et al (2022)[57]	miR-23a-3p inhibits sepsis-induced kidney epithelial cell injury by suppressing Wnt/b-catenin signaling by targeting wnt5a	China	Case-control	Hospitalized patients (ICU) with sepsis
Zhang F. et al (2023)[58]	LncRNA PMS2L2 Is Downregulated in Sepsis-Induced Acute Kidney Injury and Inhibits LPS-Induced Apoptosis of Podocytes	China	Retrospective cohort	Hospitalized patients with sepsis

Kuang F. et al (2023)[59]	CIRC_0001818 targets miR-136-5p to increase lipopolysaccharide-induced HK2 cell injuries by activating TXNIP/NLRP3 inflammasome pathway	China	Case-control	Hospitalized patients with sepsis
Zhang B. et al (2023)[60]	CIRC_0114428 influences the progression of septic acute kidney injury via regulating miR-370-3p/TIMP2 axis	China	Case-control	Hospitalized patients with sepsis due to bacteremia
You T. et al (2023)[61]	CIRC_0008882 stimulates PDE7A to suppress septic acute kidney injury progression by sponging miR-155-5p	China	Case-control	Hospitalized patients with sepsis
Han R. et al (2023)[62]	Urinary microRNAs in sepsis function as a novel prognostic marker	China	Retrospective cohort	Elderly patients with bacteremia
Liu W. et al (2023)[63]	Exosomal microRNA-342-5p secreted from adipose-derived mesenchymal stem cells mitigates acute kidney injury in sepsis mice by inhibiting TLR9	China	Case-control	Hospitalized patients with sepsis
Li P. et al (2023)[64]	Circulating extracellular vesicles are associated with the clinical outcomes of sepsis	USA	Prospective cohort	Hospitalized patients (ICU) with sepsis
Nephrotoxic				
Vliegenthart A.D.B. et al (2015)[65]	Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury	United Kingdom	Prospective cohort	Patients hospitalized for acetaminophen overdose
Gutiérrez-Escolano A. et al (2015)[66]	Dysregulated microRNAs involved in contrast-induced acute kidney injury in rat and human	Mexico	Case-control	Patients undergoing percutaneous coronary intervention
Pavkovic M. et al (2016)[67]	Detection of Drug-Induced Acute Kidney Injury in Humans Using Urinary KIM-1, miR-21, -200c, and -423	USA, United Kingdom	1. Case-control 2. Prospective cohort	1. Patients with acetaminophen overdose 2. Patients with malignant mesothelioma
Sun S. et al (2016)[68]	Circulating MicroRNA-188, -30a, and -30e as Early Biomarkers for Contrast-Induced Acute Kidney Injury	China	Case-control	Patients undergoing coronary angiography or percutaneous coronary intervention
Shihana F. et al (2020)[69]	Circulating human microRNA biomarkers of oxalic acid-induced acute kidney injury	Sri Lanka	Case-control	Patients hospitalized for oxalic acid ingestion
Shihana F. et al (2020)[70]	Urinary versus serum microRNAs in human oxalic acid poisoning: Contrasting signals and performance	Sri Lanka	Case-control	Patients hospitalized for oxalic acid ingestion
Zhang L. et al (2020)[71]	Dysregulation of HULC promotes contrast-induced nephropathy (CIN) via regulating signaling pathway of miRNA-512 and prostaglandin E1 (PGE1)	China	Retrospective cohort	Hospitalized patients with CAD treated by percutaneous coronary intervention
Quintanilha J.C.F. et al (2021)[72]	MiR-3168, miR-6125, and miR-4718 as potential predictors of cisplatin-induced nephrotoxicity in patients with head and neck cancer	Brazil	Case-control	Patients with head and neck cancer treated with cisplatin
Shihana F. et al (2021)[73]	Urinary microRNAs as non-invasive biomarkers for toxic acute kidney injury in humans	Sri Lanka	Retrospective cohort	Patients hospitalized for toxin/ ingestion (Russell viper venom, oxalic acid, paraquat, glyphosate)
Other				
Lorenzen J.M. et al (2011)[74]	Circulating miR-210 Predicts Survival in Critically Ill Patients with Acute Kidney Injury	Germany	Post-hoc measure of prospective cohort samples	Hospitalized patients (ICU) with AKI requiring RRT
Lan Y-F. et al (2012)[75]	MicroRNA-494 Reduces ATF3 Expression and Promotes AKI	China	Case-control	ICU
Krebs C.F. et al (2013)[76]	MicroRNA-155 Drives TH17 Immune Response and Tissue Injury in Experimental Crescentic GN	Germany	Case-control	Kidney biopsies with ANCA-associated GN
Bruno N. et al (2016)[77]	MicroRNAs relate to early worsening of renal function in patients with acute heart failure	Netherlands	Case-control	Acute heart failure
Chen H-H. et al (2016)[78]	Urinary miR-16 transactivated by C/EBPβ reduces kidney function after ischemia/reperfusion-induced injury	Taiwan	Case-control	ICU
Ma Y. et al (2017)[79]	Serum miRNA expression and correlation with clinical characteristics in acute kidney injury	China	Case-control	Patients with AKI
Wang S. et al (2017)[80]	Reduction in miRNA-125b-5p levels is associated with obstructive renal injury	China	Case-control	Patients with ureteral obstruction
Guo Y. et al (2018)[81]	MicroRNA-709 Mediates Acute Tubular Injury through Effects on Mitochondrial Function	China	Case-control	Kidney biopsies from patients with various AKI causes
Watany M.W. et al (2018)[82]	Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study	Egypt	Case-control	Patients with liver cirrhosis and AKI
Kölling M. et al (2018)[83]	The Circular RNA ciRs-126 Predicts Survival in Critically Ill Patients With Acute Kidney Injury	Germany	Post-hoc measure of prospective cohort samples	ICU
Fan P-C. et al (2019)[84]	A circulating miRNA signature for early diagnosis of acute kidney injury following acute myocardial infarction	Taiwan	Prospective cohort	CCU
Newbury L.J. et al (2021) [85]	miR-141 mediates recovery from acute kidney injury	United Kingdom	Retrospective cohort	Hospitalized patients with stage 3 AKI
Mao H. et al (2021)[86]	MEG3 aggravates hypoxia/reoxygenation induced apoptosis of renal tubular epithelial cells via the miR-129-5p/ HMGB1 axis	China	Case-control	Hospitalized patients with AKI
Aomatsu A. et al (2021)[87]	MicroRNA expression profiling in acute kidney injury	Japan	Case-control	ICU
Phulkerd T. et al (2022)[88]	Circulating and urinary microRNAs profile for predicting renal recovery from severe acute kidney injury	Thailand	Prospective cohort	Hospitalized patients with stage 3 AKI
Liu J. et al (2022)[89]	Correlation between the expression of serum miR-2861 and miR-34 and the prognosis of CVVH in patients with acute renal failure	China	Prospective cohort	Hospitalized patients with AKI receiving CVVH

Petejova N. et al (2022)[90]	Expression and 7-day time course of circulating microRNAs in septic patients treated with nephrotoxic antibiotic agents	Czech Republic	Prospective cohort	Hospitalized patients (ICU) with sepsis	
Xue Q. et al (2022)[91]	lncRNA ROR and miR-125b Predict the Prognosis in Heart Failure Combined Acute Renal Failure	China	Case-control	Heart failure and AKI	
Xie Z. et al (2023)[92]	Human bone marrow mesenchymal stem cell-derived extracellular vesicles reduce inflammation and pyroptosis in acute kidney injury via miR-223-3p/HDAC2/SNRK	China	Case-control	Patients with AKI	

Supplementary table continued

Author	AKI cause	Population		Sample size (N = # of patients)		Age (years)		Sex (% male)	
		Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Kidney Transplant									
Lorenzen J.M. et al (2011)	AR	Biopsy-proven acute cellular rejection	-stable graft function	N=62	N=19	51 (21-72)	51 (18-67)	58.1	52.6
Wilflingseder J. et al (2013)	DGF, AR	DGF, TCMR, ABMR	-protocol biopsies without histological injury	N=14 (DGF) N=41 (AR)	N=10	**Donor** ***DGF***: 46-64 ***AR:*** 44-64 **Recipient** ***DGF:*** 47-65 ***AR:*** 36-65	**Donor:** 54.0-67.0 **Recipient:** 41-59	**Donor** ***DGF:*** 61.5 ***AR:*** 60.0 **Recipient** ***DGF:*** 84.6 ***AR:*** 70.0	**Donor:** 40.0 **Recipient:** 70.0
Sui W. et al (2013)	AR	Biopsy-proven AR	Kidney cortex from nephrectomy patients with kidney tumor	N=3	N=3	NR	NR	NR	NR
Lorenzen J.M. et al (2014)	Ischemia-reperfusion injury	Long cold ischemia time	Short cold ischemia time	N=5	N=5	47.0±4.7	46.2±3.9	40	20
Wilflingseder J. et al (2014)	DGF	Biopsy-proven ATN without rejection	Protocol biopsies	N=8	N=10	**Donor:** 53 (46.8; 56.5) **Recipient**: 55.3 (50.1-58.7)	**Donor**: 42.5 (40.3; 50) **Recipient:** 57.6 (47.9-62.9)	**Donor:** 62.5 **Recipient:** 75	**Donor:** 40 **Recipient:** 80s
Liu X. et al (2015)	AR	Biopsy-proven AR	Normal graft biopsy	N=15	N=15	NR	NR	NR	NR
Soltaninejad E. et al (2015)	AR (TCMR)	Graft dysfunction, biopsy	Normal graft function and histology	N=17	N=18	47 (21-61)	55 (34-67)	58.8	61.1
Tao J. et al (2015)	AR, DGF	Biopsy-proven AR, DGF	Stable graft function	N=12 (AR) N=15 (DGF)	N=11	AR: 35.1±8.4 DGF: 40.1±13.0	38.8±9.7	AR: 75.0 DGF: 60.0	63.6
McGuinness D. et al (2016)	DGF	DGF	No DGF	N=27	N=67	Cohort (n=94) Donor: 46.6 (11-78) Recipient: 50.7 (20-75)		Cohort (n=94) Donor: 55.3 Recipient: 62.8	
Amrouche L. et al (2017)	DGF	(1) Biopsy-proven ATN; (2) DCD	(1) Normal graft biopsy (2) LD	(1) N=19 (2) N=35	(1) N=15 (2) N=16	(1) 56±16 (2) 53±15	(1) 56±15 (2) 42±15	(1) 42 (2) 60	(1) 67 (2) 69
Domenico T.D. et al (2017)	Graft dysfunction	ATN, AR	Stable graft function, normal biopsy	N=41	N=8	41±16	54±11	43.9	37.5
Millán O. et al (2017)	AR	Biopsy-proven AR	No AR	n=8	N=72	Donor: 57.5±16.1 Recipient: 48.0±12.7	Donor: 53.0±13.0 Recipient: 49.0±12.4	Recipient: 50.0	Recipient: 47.2
Jin P. et al (2017)	Graft dysfunction	Biopsy-proven AR	Stable graft function	N=19	N=10	38.7 (22-54)	41.5 (25-65)	80	63
Gallon L. et al (2018)	AR	Biopsy-proven AR	No AR	N=10	N=17	43.7±13.2	48.7±14.5	70.0	47.0
Cheng K. et al (2018)	Acute vascular rejection	Recipients with vascular rejection	Recipients without vascular rejection	N=14	N=20	37.5±4.1	36.1±3.8	42.8	50
Gremmels H. et al (2019)	Graft dysfunction	DGF	No DGF	N=8	N=8	**Donor**: 61.5 (54.5-63.3) **Recipient:** 65.5 (57.0-68.3)	**Donor:** 54.0 (49.5-65.3) **Recipient:** 55.5 (50.3-63.3)	**Recipient:** 62.5	**Recipient:** 75
Khalid U. et al (2019)	Graft dysfunction	DGF	No DGF	N=13	N=10	**Donor:** 65 (12-76) **Recipient:** 56 (39-76)	**Donor:** 54 (20-75) **Recipient:** 43 (19-74)	**Donor:** 76.9 **Recipient:** 76.9	**Donor:** 40 **Recipient:** 70
Li F. et al (2019)	Graft dysfunction	Graft dysfunction	Normal graft function	N=45	N=59	39.5±10.3	37.9±10.2	73.3	39.0
Milhoransa P. et al (2019)	Graft dysfunction	DGF, AR	Stable graft function	N=33 (DGF) N=9 (AR)	N=13	DGF: 46.8±14.5 AR: 42.8±11.6	48.3±12.2	DGF: 45 AR: 44	69

Roest H.P. et al (2019)	Graft dysfunction	DGF	No DGF	N=20	N=20	Donor: 63±11 Recipient: 65 (29-77)	Donor: 56±13 Recipient: 62 (24-76)	Donor: 45 Recipient: 60	Donor: 30 Recipient: 80
Wang J. et al (2019)	Graft dysfunction	DGF	No DGF	N=4	N=5	43.7±13.3	35.8±5.0	100	60
Seo J-W. et al (2023)	Graft dysfunction	AR Other graft injury	Stable graft function	Discovery: N=60 (AR) Validation: N=180 (AR)	Discovery: N=48 Validation: N=100	**Discovery:** 47.6±11.1 **Validation:** 47.4±11.4 (AR), 47.4±13.1 (other)	**Discovery:** 53.4±10.7 **Validation:** 47.6±12.1	**Discovery:** 65 **Validation:** 69 (AR) 75 (other)	**Discovery**: 39.6 **Validation:** 52.5
Connor K.L. et al (2020)	Graft dysfunction	DGF	No DGF	N=9	N=6	Donor: 44.9 (17.4) Recipient: 54.2 (17.7)	Donor: 48.2 (9.8) Recipient: 50.2 (14.3)	Donor: 67 Recipient: 44	Donor: 83 Recipient: 33
Alfaro R. et al (2021)	Acute rejection	Biopsy-proven AR	Non-AR	N=5	N=10	61.2±6.7	53.8±11.6	60	60
Rutman A.K. et al (2022)	Graft dysfunction	DGF	No DGF	N=11 (screen) N=9 (validation)	N=8 (screen) N=9 (validation)	Donor: 54.6 Recipient: 55.6	Donor: 47.2 Recipient: 53.3	Donor: 82 Recipient: 73	Donor: 62.5 Recipient: 63
Shi L. et al (2019)	Graft dysfunction	DGF	Non-transplant patients with GN	N=5	N=8	39.6±9.4	27.6±9.9	80	62.5
Cardiac surgery									
Du J. et al (2013)	CS-AKI	CS-AKI	CS, no AKI	N=80	N=40	59-61 (±14)	60±10	68	75
Aguado-Fraile E. et al (2015)	ATN CS-AKI	(1)ATN (2) ICU MOF (3)CS-AKI	(1,2) healthy controls (3)CS, no AKI (includes pediatrics)	1,2: N=39 3. NR	1,2. N=30 3. NR	1,2. 65±16 3. NR	1,2. 38±11 3. NR	1,2. 50-80 3. NR	1,2. 50-60 3. NR
Gaede L. et al (2016)	CS-AKI	CS-AKI	CS, no AKI	N=42	N=73	74 (73-79)	71 (64-76)	50	67
Arvin P. et al (2017)	CS-AKI	CS-AKI	CS, no AKI	N=14	N=14	58.6±12.0	58.9±10.3	50	50
Zhang L. et al (2017)	CS-AKI	CS-AKI	CS, no AKI	N=35	N=35	66.0±9.0	62.7± 7.1	63	60
Zou Y-F. et al (2017)	CS-AKI	CS-AKI	CS, no AKI	N=27	N=44	57.6±2.5	59.2±2.2	77.8	59.1
Kang Z. et al (2018)	CS-AKI	CS with RIPC	CS without RIPC	N=200	N=249	Mths 41.5±33.0	Mths 31.5±22.2	57.0	51.8
Sullo N. et al (2018)	CS-AKI	CS-AKI	CS, no AKI	N=14	N=10	Mths 7.9 (0.4-30.4)	Mths 46.4 (36.4-90.0)	50	40
Wei Q. et al (2018)	1. CS-AKI 2. Mixed AKI	1. CS-AKI 2. Medical patients with AKI	1. CS, no AKI 2. GN without AKI	1. N=32 2. N=8	1. N=30 2. N=7	1. 61.5±9.5 2. 46.8±13.5	1. 59.3±10.1 2. 38.6±15.5	1. 68.6 2. 50.0	1. 66.7 2. 85.7
Gong Q. et al (2019)	CS-AKI	CS-AKI	CS, no AKI	N=10	N=8	61.9±10.3	64.6±8.9	60.0	62.5
Miller D. et al (2022)	CS-AKI	CS-AKI	CS, no AKI	N=45	N=49	71 (66-75)	74 (70-78)	86.7	77.6
Chen Y. et al (2022)	CS-AKI	Older with AKI	Younger with AKI	N=13	N=12	>60	18-44	NR	NR
Li X. et al (2023)	CS-AKI	CS-AKI	CS, no AKI	N=36	N=30	51.8±7.7	49.3±6.5	58.3	63.3
Sepsis									
Saikumar J. et al (2012)	Septic, ATN	ICU patients with AKI	Healthy volunteers	N=22	N=25	53.5±16.7	35.6±10.7	59	32
Ramachandran K. et al (2013)	Septic, ATN	1. ICU patients AKI 2. Transplant with ATN	1. Healthy volunteers 2. ICU patients no AKI	1. N=71 2. N=27	1. N=74 2. N=23	1. 61.1±13.1 2. 54.6±12.9	1. 36.3±10.7 2. 55.2±16.8	1. 62 2. 63	1. 53 2. 48
Ge Q-M. et al (2017)	Septic	Sepsis AKI	Sepsis no AKI Healthy controls	N=35	N=30 N=20 HCs	67.0±10.1	65.3±19.1 55.8±13.2	34.3	43.3 50.0
Wang S. et al (2017)	Septic	Sepsis AKI Non-sepsis AKI	Sepsis no AKI Healthy controls	N=30	N=30	(mths) 64-67±22	(mths) 71-74±11	54-60	53-60
Zhang J. et al (2018)	Septic	Sepsis (with and without AKI)	SIRS, without organ failure	N=155 (68 AKI 87 no AKI)	N=56	56.1±14.2	54.5±12.9	60.6	57.1
Bukauskas T. et al (2019)	Septic	Sepsis AKI Sepsis CKD	Sepsis normal kidney function	N=8, N=8	N=11	62.81 (23-90)		44.4	
Lin Y. et al (2019)	Septic	Sepsis AKI	Healthy controls	N=110	N=110	65.7±8.3	66.3±8.1	51.8	52.7
Sun J. et al (2020)	Septic	Sepsis AKI	Sepsis no AKI	N=235	N=235	61.6±11.5	61.5±13.2	61.7	63.8

Liu S. et al (2021)	Septic	Sepsis AKI	Sepsis no AKI	N=58	N=78	54.0±15.9	49.7±15.2	77.5	73.0
Shi L. et al (2021)	Septic	Sepsis AKI	Healthy controls	N=30	N=15	54.2±15.1	49.2±14.2	73.3	53.3
Zhang H. et al (2021)	Septic	Sepsis AKI	Sepsis no AKI	N=69	N=89	49.1±14.9	50.2±16.1	79.7	75.3
Zheng C. et al (2021)	Septic	Sepsis AKI	Healthy controls	N=30	N=30	39.3±7.8	37.8±8.1	63.3	56.7
Gong J. et al (2022)	Septic	Sepsis AKI	Healthy controls	N=38	N=41	54.1±8.1	53.6±7.8	52.6	53.7
Ma W. et al (2022)	Septic	Sepsis AKI	Sepsis no AKI	N=96	N=88	49.8±10.0	51.2±9.8	58.3	54.5
Pan W. et al (2022)	Septic	Sepsis AKI	Sepsis no AKI Healthy controls	N=64	N=51 (sepsis no AKI)	72.9±8.1	71.6±9.9	54.7	52.9
Xun L. et al (2022)	Septic	Sepsis AKI	Sepsis no AKI	N=62	N=80	45.3±18.6	45.2±18.6	51.6	55.0
Xu L. et al (2022)	Septic	Sepsis AKI	Sepsis no AKI Healthy controls	N=26	N=16 N=24 HCs	63.8±13.6	65.3±15.2 60.1±16.1 (HC)	42.3	56.2 45.8 (HC)
Ye J. et al (2022)	Septic	Sepsis AKI	Healthy controls	N=25	n=20	45.8±3.2	44.4±2.5	60.0	60.0
Zhang F. et al (2023)	Septic	Sepsis AKI	Sepsis no AKI Healthy controls	N=50	N=50 N=50 HCs	51.6±5.8	51.9±5.9	80.0	80.0
Kuang F. et al (2023)	Septic	Sepsis AKI	Healthy controls	N=25	N=20	65.2±7.1	64.4±6.1	56.0	55.0
Zhang B. et al (2023)	Septic	Sepsis AKI	Healthy controls	N=45	N=45	67.2±12.9	65.2±15.1	64.4	51.1
You T. et al (2023)	Septic	Sepsis AKI	Healthy controls	N=36	N=36	54.3±6.7	NR	61.1	NR
Han R. et al (2023)	Septic	Sepsis AKI	Sepsis no AKI Healthy controls	N=29	N=28 N=17 HCs	All sepsis: Mean age 81 (65-97) HC: 75.3±5.5		48.3	53.6 41.1 (HC)
Liu W. et al (2023)	Septic	Sepsis AKI	Healthy control	N=30	N=30	39.3±7.8	37.4±8.2	63.3	53.3
Li P. et al (2023)	Septic	Sepsis with ARDS	Sepsis no ARDS, non-septic ICU	N=75	N=33	58.5±1.9	52.3-58.7±6.3	53.3	38.1-58.3
Nephrotoxic									
Vliegenthart A.D.B. et al (2015)	Nephrotoxic (APAP)	1. APAP-ALI 2. APAP-ALI with AKI	1. APAP no tox 2. APAP-ALI no AKI	1. N=68 2. N=30	1. N=67 2. N=38	1. 39 (26-54) 2. 41 (31-49	1. 38 (25-55) 2. 40 (27-58	1. 33.8 2. 46.7	1. 38.8 2. 36.8
Gutiérrez-Escolano A. et al (2015)	Nephrotoxic (CI-AKI)	CI-AKI	Contrast no AKI	N=92	N=92	67.6±11.1	65.8±10.6	69.6	70.7
Pavkovic M. et al (2016)	Nephrotoxic (APAP, cisplatin)	1. APAP AKI 2. CP-AKI	1. APAP no AKI 2. CP no AKI	1. n=43 2. n=45	1. n=27 1. n=61	1. 39.8±13.4 2. 67.5±10.6	1. 39.3±15.9 2. 62.5±10.5	1. 44.2 2. 84.4	1. 37.1 2. 70.5
Sun S. et al (2016)	Nephrotoxic (CI-AKI)	CI-AKI	Contrast no AKI	N=71	N=71	60.8±1.3	61.3±1.3	63.4	52.1
Shihana F. et al (2020)	Nephrotoxic (OA)	OA AKI	1. OA no AKI 2. HC	N=41	1. n=15 2. n=23	25 (18-32)	1. 20 (20-27) 2. 26 (21-28)	43.9	1. 46.7 2. 65.2
Shihana F. et al (2020)	Nephrotoxic (OA)	OA AKI	1. OA no AKI 2. HC	N=26 (AKIN2,3)	1. n=14 2. n=27	25 (19-28)	1. 20 (19-23) 2. 26 (24-27)	53.8	1. 36.0 2. 65.0
Zhang L. et al (2020)	Nephrotoxic (CI-AKI)	CI-AKI	Contrast no AKI	N=78	N=242	66.1±7.9	65.6±8.4	29.5	30.2
Quintanilha J.C.F. et al (2021)	Nephrotoxic (cisplatin)	CP-AKI	CP no AKI	N=6 (seq) Validation numbers NR	N=6 (seq) Validation numbers NR	57.5±7.4	60.3±4.9	66.7	83.3
Shihana F. et al (2021)	Nephrotoxin (mixed)	Toxic AKI	1. Toxic no AKI 2. HC	N=143	1. N=55 2. N=27	36.5 (means range 23-56)	1. 23.5 (means range 18-32) 2. 26 (24-27)	72.0	1. 67.3 2. 65.0
Other									
Lorenzen J.M. et al (2011)	ATN Septic	AKI	1. HC 2. AMI	N=77	1. N=30 2. N=18	49 (39-60)	1. age-matched 2. NR	52.0	NR
Lan Y-F. et al (2012)	Septic, ATN, nephrotoxic	ICU patients with AKI	ICU patients without AKI	16 patients	10 patients	67.25 (43-87)	70.00 (42-89)	69	60
Krebs C.F. et al (2013)	GN	ANCA GN	Kidney donor biopsies	N=10	N=13	NR	NR	NR	NR
Bruno N. et al (2016)	Cardio-renal	Acute HF with AKI	Acute HF no AKI	17 patients	81 patients	69.5±12.7	68.8±11.3	23.5	55.6
Chen H-H. et al (2016)	Mixed	ICU AKI	ICU no AKI	N=7	N=11	20-89	30-87	86.0	75.0
Ma Y. et al (2017)	Mixed	AKI	Healthy controls	N=28	N=58	53.2±21.6	52.5±23.6	67.9	67.2
Wang S. et al (2017)	Obstruction	Ureteral obstruction	Healthy controls	N=91	N=76	53.8±12.4	42.2±12.8	58.2	53.9
Guo Y. et al (2018)	Mixed	AKI	Paracarcinoma kidney tissue	N=21	N=7	49.0±12.7	NR	52.0	NR

Watany M.W. et al (2018)	ATN HRS	AKI	Healthy controls	N=50	N=30	48-53±6-7	45±8	76.0	60.0
Kölling M. et al (2018)	Mixed	ICU AKI	1. ICU no AKI 2. ESKD HD 3. HC	N=109	1. N=25 2. N=20 3. N=30	52 (40-63)	1. 52 (42-71) 2. 49 (39-62) 3. 54 (46-66)	59.0	1. 48.0 2. 55.0 3. 53.3
Fan P-C. et al (2019)	Cardiorenal	1. AMI with AKI 2. No AMI with AKI	1. AMI without AKI 2. No AMI, without AKI	1. 23 patients 2. 29 patients	1. 26 patients 2. 30 patients	1. 73±2 2. 71±2	1. 59±2 2. 61±3	1. 82.6 2. 65.5	1. 84.6 2. 63.3
Newbury L.J. et al (2021)	Pre-renal (47%), ATN (34%), GN, obstruction	Stage 3 AKI non-recovered and recovered	Archived controls	N=29	N=20	62.4±19.9	NR	56.7	NR
Mao H. et al (2021)	Mixed	AKI	Healthy controls	N=25	N=25	≤60: 60% >60: 40%	NR	64.0%	NR
Aomatsu A. et al (2021)	ATN, septic, other	AKI	Healthy controls	N=30	N=30	63.6±11.1	42.5±13.2	70.0	70.0
Phulkerd T. et al (2022)	ATN, septic, nephrotoxic	AKI, recovery	AKI, no recovery	N=64	N=46	58±17	65±16	68.8	56.6
Liu J. et al (2022)	Mixed	AKI 'good prognosis'	AKI 'poor prognosis'	N=84	N=28	53.0±5.2	52.8±5.4	59.5	60.7
Petejova N. et al (2022)	Septic Nephrotoxic	Sepsis with nephrotoxic antibiotics	Sepsis, other antibiotics	1. Vanco: 7 patients 2. Gent: 20 patients	Other antibiotics: 19 patients	1. 65 (47-76) 2. 59 (47-72)	65 (63-68)	1. 57.0 2. 65.0	68.0
Xue Q. et al (2022)	HF (presumed cardiorenal)	HF with AKI	Healthy controls	90 patients	90 patients	60.9±5.8	62.1±6.7	50.0	50.0
Xie Z. et al (2023)	NR	AKI	Healthy controls	N=20	N=20	52-66	51-65	75.0	60.0

Supplementary Table continued

Author	AKI definition	Timing of AKI diagnosis	Kidney function	
			Cases	Controls
Kidney Transplant				
Lorenzen J.M. et al (2011)	Subclinical: Cr<20% increase Clinical: Cr > 20% increase Banff Classification	Protocol biopsies 6 wks, 3 mths, 6 mths post-transplant	Cr<20%: 81% Cr>20%: 19%	NR
Wilflingseder J. et al (2013)	DGF: ATN without rejection; ≥1 HD within 1st wk post-transplant CMR: Banff criteria ABMR: rising Cr, C4d staining and humoral tissue injury	Time of biopsy (days post-transplant): DGF: 7 (2; 8) CMR:16.5 (7; 44.5) ABMR: 14 (12; 55.5) Control: 104.5 (102; 109.5)	NR	NR
Sui W. et al (2013)	Banff criteria '97	NR	NR	NR
Lorenzen J.M. et al (2014)	Long cold ischemia time	NR	NR	NR
Wilflingseder J. et al (2014)	≥1 HD sessions within 1st wk after transplant and Cr > 4 mg/dL after 1 wk	Within 12 d after transplant	Cr at biopsy (mg/dL): 9.81 (5.74; 10.14)	Cr at biopsy (mg/dL): 1.54 (1.32; 1.73)
Liu X. et al (2015)	Graft dysfunction, Banff classification	NR	NR	NR
Soltaninejad E. et al (2015)	Graft dysfunction, Banff classification	Biopsies within 9-18 mths post-transplant	Cr at biopsy: 5.18±1.49 mg/dL	Cr at biopsy: 1.24±0.21 mg/dL
Tao J. et al (2015)	Graft dysfunction, Banff classification	NR	NR	NR
McGuinness D. et al (2016)	DGF: need for dialysis within 1-wk post-transplant	NR	Donor Cr at retrieval (μmol/L): 92.2 (23-786)	
Amrouche L. et al (2017)	AKI: DGF (need for HD within 1st wk post-transplant) with biopsy-proven ATN	DGF: Within first wk post-transplant	mGFR 3-mth (ml/min) (1)Biopsy: 32±17 (2)Urine: 53±16	mGFR 3-mth (ml/min) (1)Biopsy: 54±13 (2)Urine: 67±22
Domenico T.D. et al (2017)	Biopsy-proven ATN or AR (Banff 20078)	Indication biopsies within 1-month post-transplant (protocol biopsies at 3 mths)	Cr at biopsy (mg/dL): 4.0±1.7 (AR) 5.4±2.7 (ATN)	Cr at biopsy (mg/dL): 1.0±0.4
Millán O. et al (2017)	Biopsy-proven acute cellular rejection	8 patients, 12 AR episodes total within 1st 6 mths	1st week: 567±108 μmol/L 6 months: 221±65 μmol/L	1st week: 195±99 μmol/L 6 months: 117± 34 μmol/L
Jin P. et al (2017)	Biopsy-proven AR (Banff 2007)	At hospital admission	Cr (μmol/L): 568±20	Cr (μmol/L): 223±16
Gallon L. et al (2018)	Biopsy-proven acute cellular rejection	0.2-11 mths post-transplant	At biopsy (mg/dL): 1.8±1.9	At biopsy (mg/dL): 1.2±0.3
Cheng K. et al (2018)	Biopsy-proven vascular rejection	Within 6 mths post-transplant	Pre-transplant: 372±86 μmol/L	Pre-transplant: 364±79 μmol/L
Gremmels H. et al (2019)	DGF: need for HD within first wk post-transplant	Up to 7 d post-transplant	NR	NR
Khalid U. et al (2019)	DGF: need for HD within first wk post-transplant	7 d post-transplant	5 d: 606±218μmol/L	5 d: 184±114μmol/L
Li F. et al (2019)	NR	At initial sample collection, and after 3-4 wks	NR	NR

Milhoransa P. et al (2019)	Diagnostic classification of AKI based on clinical assessment and biopsy (Banff 2013)	Simultaneous biopsy and blood sampling Range: 12 – 331 d post-transplant	Cr at biopsy (mg/dL) DGF: 5.7±4.0 AR: 3.8±3.3	Cr at biopsy (mg/dL) 2.5±1.9
Roest H.P. et al (2019)	DGF: poor kidney function (low urine output, high serum Cr) and need for HD within the 1st wk post-transplant	Within 1-wk post-transplant	NR	NR
Wang J. et al (2019)	DGF: ≥ 1 HD session within the 1st wk post-transplant	1-wk post-transplant	565±224 µmol/L	74±15 µmol/L
Seo J-W. et al (2023)	Biopsy-proven AR (TCMR, ABMR, chronic active ABMR) based on Banff 2017. Other graft injury based on biopsy: ATN, IFTA, CNi toxicity, BK virus nephropathy	1.5±2.6 – 4.4±4.4 yr after transplant (AR) 5.8±7.4 – 9.9±8.9 yr after transplant (controls)	eGFR ml/min/1.73m^2 Discovery: 38±22 Validation:33±15(AR) 39±17 (other)	eGFR ml/min/1.73m^2 Discovery: 70±13 Validation: 77±20
Connor K.L. et al (2020)	DGF: 'increased or stable serum Cr, or decrease < 10% per day for 3 d in the 1st week post-transplant'	First-passed urine post-transplant	eGFR ml/min/1.73m^2 30 d: 39 (20) 90 d: 44 (15)	eGFR ml/min/1.73m^2 30 d: 52 (17) 90 d: 54 (13)
Alfaro R. et al (2021)	AR (TCMR): Cr ≥ 20% above baseline and biopsy (Banff 2017). ABMR: histopathology, +C4d staining, +DSA	Within 1-yr post-transplant	Cr (mg/dL): 2.5±1.7	Cr (mg/dL): 1.9±2.4
Rutman A.K. et al (2022)	DGF: temporary need for dialysis post-transplant	Early post-transplant	NR	NR
Shi L. et al (2019)	DGF	Early post-transplant	Cr: 4.57±0.98 mg/dL	Cr: 0.69±0.07 mg/dL
Cardiac surgery				
Du J. et al (2013)	AKIN: post-operative increase in plasma Cr ≥ 50% baseline or ≥0.3mg/dL.	Within 20 ±10 hr post-surgery	Cr: 1.6±0.4 mg/dL	Cr: 1.1±0.3 mg/dL
Aguado-Fraile E. et al (2015)	AKIN and RIFLE serum Cr criteria	(1) NR (2) during admission (3) within 7 d post-cardiac surgery	(1) 1.5-13.0 mg/dL (2) 2.7±4.4 mg/dL (3) NR	(1) 0.8±0.12 (2) 0.8±0.2 (3) NR
Gaede L. et al (2016)	AKI ≥ Stage 2 within 7 d post-cardiac surgery	Up to 7 d post-cardiac surgery	Peak Cr (2 d post-op) 2.4 (1.3-2.9) mg/dL	Cr 2 d post-op: 1.0 (0.8-1.3) mg/dL
Arvin P. et al (2017)	Increased serum Cr ≥ 0.3 mg/dL within 24 hr after cardiac surgery	24 hr after cardiac surgery	Cr 24 hr after surgery: 1.44±0.16 mg/dL	Cr 24 hr after surgery: 0.80±0.08 mg/dL
Zhang L. et al (2017)	Post-surgery rise in plasma Cr ≥ 50% from baseline or ≥ 26.5 µmol/L	Peak plasma Cr 24 hr after surgery	Cr 24 hr after surgery: 141±56 µmol/L	Cr 24 hr after surgery: 83±18 µmol/L
Zou Y-F. et al (2017)	KDIGO definition	Up to 72 hr post-cardiac surgery	Cr 24 hr after surgery: 1.69±0.12 mg/dL	Cr 24 hr after surgery: 0.84±0.03 mg/dL
Kang Z. et al (2018)	KDIGO definition	Within 48 hr after surgery	Cr 48 hr after surgery 32.1±12.7 µmol/L	Cr 48 hr after surgery: 38.7±11.7 µmol/L
Sullo N. et al (2018)	KDIGO definition with modification for infants (pRIFLE)	Within 24 to 48 hr after surgery	Cr 24 hr after surgery 52.9±20.1 µmol/L	Cr 24 hr after surgery 28.7±8.6 µmol/L
Wei Q. et al (2018)	NR	1. Within 24 hr after surgery 2. NR	1. Cr (24 hr): 1.4±0.4 2. 6.1±1.8 (mg/dL)	1. Cr (24 hr): 0.8±0.2 2. 0.8±0.2 (mg/dL)
Gong Q. et al (2021)	KDIGO definition: increased Cr > 26.5µmol/L by 48 hr or > 1.5x baseline by 7 d after cardiac surgery	Within 24 hr after surgery	Cr (24 hr), µmol/L 112.8±29.6	Cr (24 hr), µmol/L 76.7±8.3
Miller D. et al (2022)	KDIGO definition	Peak Cr 48 hr after surgery	AKI stages 1-3: 1: n=36; 2/3: n=9	No AKI
Chen Y. et al (2022)	NR	Timing after surgery NR	AKI stages: 1: n=8; 2/3: n=4	NR
Li X. et al (2023)	KDIGO definition	Within 48 hr after surgery	Peak Cr (µmol/L): 174.6±89.3	Peak Cr (µmol/L) 66.9±13.1
Sepsis				
Saikumar J. et al (2012)	Increase Cr ≥ 100% from baseline	NR	Cr 3.7±2.8 mg/dL	NR
Ramachandran K. et al (2013)	Increase Cr ≥ 50% over baseline	NR	Peak Cr (mg/dL) 2.6-3.6±(1.7-1.9)	Peak Cr (mg/dL) 1.3±1.4
Ge Q-M. et al (2017)	KDIGO definition	NR	Cr (mg/dL): 3.24±2.92	Cr (mg/dL): 0.82±0.2
Wang S. et al (2017)	KDIGO criteria	At the emergency department	Cr (mg/dL) 1.9-2.0±0.8	Cr (mg/dL) 0.44-0.48±0.1
Zhang J. et al (2018)	KDIGO and AKIN criteria	Within 7 d	AKI stages: 1: 55.8%, 2: 16.1%, 3: 27.9%	NR
Bukauskas T. et al (2019)	Defined as eGFR < 60mL/min/1.73m^2	NR	(AKI+CKD) Cr in µmol/L 195.7±152.7	Cr in µmol/L 86.2±36.0
Lin Y. et al (2019)	AKIN criteria	NR	Cr: 252.3±56.4 µmol/L	Cr: 43.3±7.2 µmol/L
Sun J. et al (2020)	AKIN criteria	Within 1st 72 hr of ICU admission	Approx. Cr range: 141 – 840 µmol/L	Approx. Cr range: 44 – 120 µmol/L
Liu S. et al (2021)	KDIGO/AKIN criteria	Within 24 hr of admission	Cr (µmol/L): 156.2±26.1	Cr (µmol/L): 91.7±33.2
Shi L. et al (2021)	KDIGO criteria	Before ICU admission	NR	NR
Zhang H. et al (2021)	KDIGO/AKIN criteria	At admission, up to 7 d (based on AKI definition)	Admission Cr (µmol/L) 155.1±25.3	Admission Cr (µmol/L) 97.2±32.9
Zheng C. et al (2021)	KDIGO criteria: absolute increase Cr by 26.5µmol/L within 48 hr, increase Cr by ≥50% within 7 d, or urine output ≤ 0.5mL/kg/hr for 6 hr.	Up to 7 d (based on definition)	Cr at AKI diagnosis (µmol/L): 159.4±26.7	Cr 64.2±17.1 µmol/L

Gong J. et al (2022)	NR	NR	Cr (μmol/L) 301.5±35.9	Cr (μmol/L) 62.9±4.8
Ma W. et al (2022)	KDIGO/AKIN criteria	NR	Cr (μmol/L) 151.8±28.1	Cr (μmol/L) 94.4±27.0
Pan W. et al (2022)	Amsterdam Cooperative Research Association criteria	NR	Cr (μmol/L) 425.3±63.6	Cr (μmol/L) 84.4±27.8
Xun L. et al (2022)	NR	NR	Cr (μmol/L) 433.2±80.2	Cr (μmol/L) 83.2±10.3
Xu L. et al (2022)	NR	NR	NR	NR
Ye J. et al (2022)	AKIN criteria	NR	Cr (μmol/L): 260.2±20.6	Cr (μmol/L) 46.8±17.5
Zhang F. et al (2023)	Increased serum Cr ≥ 0.3 mg/dL or 1.5x from baseline; urine output < 0.5 mL/kg/hr for > 6 hr	NR	Urea (μmol/L) 19.1 (12.6-29.3) No Cr data	Urea (μmol/L) 7.2 (5.9-9.9) No Cr data
Kuang F. et al (2023)	NR	NR	Cr (μmol/L): 253.5±52.4	Cr (μmol/L): 62.5±13.4
Zhang B. et al (2023)	NR	NR	NR	NR
You T. et al (2023)	NR	NR	Cr: 334.5±27.4 μmol/L	NR
Han R. et al (2023)	KDIGO criteria	NR	NR	NR
Liu W. et al (2023)	KDIGO criteria	At time of diagnosis of sepsis AKI	179.5±24.6	70.5±24.5
Li P. et al (2023)	KDIGO criteria	1 d or 3 d post-admission	NR	NR
Nephrotoxic				
Vliegenthart A.D.B. et al (2015)	NR	Up to 100 hr post-ingestion	Cr (μmol/L): 209 (120-286)	Cr (μmol/L): 61 (56-73)
Gutiérrez-Escolano A. et al (2015)	>25% increase Cr or absolute increase Cr by ≥ 0.5mg/dL at 24-48 hr after CM exposure	Pre-PCI, and 3-48 hr post-PCI	Peak Cr after 48 h (mg/dL) 1.19±0.4	Peak Cr after 48 h (mg/dL) 0.78±0.21
Pavkovic M. et al (2016)	1. Serum Cr > 1mg/dL 2. AKIN criteria	1. NR 2. Any time up to 144 hr post-chemotherapy	1. Cr 2.44mg/dL (1.3-3.0) 2. Stage 1: 66.7%, Stages 2&3: 33.3%	1. Cr 0.62mg/dL (0.54-0.7) 2. Cr NR
Sun S. et al (2016)	Absolute increase in Cr ≥ 0.3mg/dL or relative increase in Cr ≥25% over baseline.	Admission and 24-48 hr after procedure	NR	NR
Shihana F. et al (2020)	AKIN criteria	Admission samples within 8 hr of ingestion. Serial samples up to 24 h of admission.	Peak Cr (24 hr), mg/dL AKIN1 (n=4): 0.97 (0.8-1.1) AKIN2 (n=14): 1.3 (1.1-2.0) AKIN3 (n=19): 2.8 (2.1-4.4)	Peak Cr (24 hr), mg/dL 1. 0.87 (0.8-0.9) 2. 0.84 (0.6-1.1)
Shihana F. et al (2020)	AKIN criteria	Admission samples within 8 hr of ingestion. Serial samples up to 24 h of admission.	Peak Cr (24 hr), mg/dL AKIN2: 0.88 (0.8-1.0) AKIN3: 1.61 (1.3-1.8)	Peak Cr (24 hr), mg/dL 1. 0.72 (0.7-0.8) 2. 0.80 (0.7-0.9)
Zhang L. et al (2020)	Rise in Cr > 25% from baseline, or absolute increase from baseline of > 0.5mg/dL	NR	NR	NR
Quintanilha J.C.F. et al (2021)	Nephrotoxicity: grade ≥ 2 increased Cr (≥ 2x baseline Cr) according to Common Toxicity Criteria for Adverse Events (CTCAE).	5 d post-cisplatin	Cr (mg/dL): 0.94±0.21 (seq group)	Cr (mg/dL): 3.39±2.13 (seq group)
Shihana F. et al (2021)	AKIN criteria	24 hr after admission	AKIN stages: 1: 31%, 2: 26%, 3: 43%	n/a
Other				
Lorenzen J.M. et al (2011)	AKI by RIFLE criteria. RRT indicated by: >30% decrease eGFR and oliguria (<30ml/hr) or hyperkalemia (K>6.5mmol/L) or metabolic acidosis (pH < 7.15, bicarbonate<12).	Within 48 hr before inclusion	100% receiving RRT	NR
Lan Y-F. et al (2012)	Increase Cr ≥ 1.5x (RIFLE and AKIN criteria)	NR	Peak Cr (mg/dL): 1.8-8.3 (3.99). AKIN stages: 1 – 44%, 2 – 31%, 3 – 25%	Peak Cr (mg/dL): 0.8-2.5 (1.52)
Krebs C.F. et al (2013)	ANCA GN confirmed on kidney biopsy	NR	NR	NR
Bruno N. et al (2016)	Increase in serum Cr of ≥ 0.3 mg/dL from admission to 3 d	Up to 3 d post-admission	Baseline Cr (mg/dL) 1.5 (1.3-1.9)	Baseline Cr (mg/dL) 1.4 (1.2-1.9)
Chen H-H. et al (2016)	RIFLE and AKIN criteria	Established AKI	Cr (mg/dL): 0.8-5.9	Cr (mg/dL): 1.5-21.3
Ma Y. et al (2017)	AKIN criteria	Established AKI	NR	NR
Wang S. et al (2017)	Obstruction confirmed by ultrasound.	NR	Cr (μmol/L): 84.3±34.9	Cr (μmol/L): 64.8±12.6
Guo Y. et al (2018)	NR	Established AKI	Cr at biopsy (μmol/L): 188.0 (104-393)	NR
Watany M.W. et al (2018)	HRS: Cr > 132.6μmol/L +advanced liver failure with portal hypertension. ATN: increased Cr > 50% above baseline or > 26.5μmol/L within 48 hr with identified ATN cause.	Established AKI	Cr (μmol/L): HRS: 277.6±100.8 ATN: 409.3±142.3	NR

Kölling M. et al (2018)	AKI by RIFLE criteria. RRT indicated by: >30% decrease eGFR and oliguria (<30ml/hr) or hyperkalemia (K>6.5mmol/L) or metabolic acidosis (pH < 7.15, bicarbonate<12).	At RRT initiation	100% receiving RRT	NR
Fan P-C. et al (2019)	Change in serum Cr from KDIGO criteria	Within 24 hr of admission	1. 3.4±0.5 mg/dL 2. 3.0±0.3 mg/dL	1. 0.9±0.1 mg/dL 2. 0.8±0.1 mg/dL
Newbury L.J. et al (2021)	Stage 3 AKI: Increase Cr ≥ 3x baseline, or increase Cr ≥ 353.6µmol/L, or RRT initiation. 90 d recovery: resolution to baseline Cr, or to stage 1 (Cr ≤ 1.9x baseline).	Stage 3 AKI at 0 d (study entry)	Cr (µmol/L) at: 0 d: 493.6±283.4 90 d: 168.4±106.4	NR
Mao H. et al (2021)	NR	NR	Approx mean Cr (µmol/L) 226-343±(120-155)	NR
Aomatsu A. et al (2021)	KDIGO criteria	Within 24 hr of AKI onset	KDIGO AKI grade: 1: 63%; 2: 20%; 3: 17%	NR
Phulkerd T. et al (2022)	Stage 3 AKI (KDIGO): Cr >3x baseline or >4mg/dL, or RRT initiation. Recovery at 28 d: dialysis-independent; Cr levels returned to within 1.5x baseline.	Cr 28 d after AKI diagnosis.	Admission Cr (mg/dL) 3.85 (2.52-4.87)	Admission Cr (mg/dL) 2.94 (2.29-4.13)
Liu J. et al (2022)	AKI definition and RRT indications NR. Poor prognosis: deterioration of kidney function and death within 30 d.	Within 24 hr of admission	Cr (µmol/L): 206±38	Cr (µmol/L): 227±52
Petejova N. et al (2022)	KDIGO criteria	Up to 7 d of antibiotics	**AKI stage at start:** **1. Vanco:** No AKI: 57%, stage 1: 14%, stage 3: 29%. **2. Gent:** No AKI: 40%, Stage 1: 25%, stage 2: 25%, stage 3: 10%	**AKI stage at start:** Other antibiotics: No AKI: 15% Stage 1: 21% Stage 2: 32% Stage 3: 15%
Xue Q. et al (2022)	NR	NR	NR	NR
Xie Z. et al (2023)	NR	NR	NR	NR

Supplementary Table continued

Author	miRNA source	Timing of sample collection	RNA isolation method	Unbiased or targeted	miRNA measurement method	Normalization
Kidney Transplant						
Lorenzen J.M. et al (2011)	Urine	6 wks, 3 and 6 mths after transplant	MasterPureTM (Epicentre Biotechnologies)	Both	miRNA array RT-qPCR	Cel-miR-39 spike-in
Wilflingseder J. et al (2013)	Kidney	See 'Timing of AKI diagnosis'	miRNeasy FFPE kit (Qiagen)	Both	miRNA array RT-qPCR	Endogenous U6
Sui W. et al (2013)	Kidney	NR	mirVana miRNA isolation kit (Ambion)	Unbiased	miRNA array	per-gene on median normalization
Lorenzen J.M. et al (2014)	Kidney	NR	Trizol (Invitrogen)	Targeted	RT-qPCR	Endogenous RNU-48
Wilflingseder J. et al (2014)	Kidney	0 hr, within 12 d post-transplant	Trizol (Invitrogen)	Both	miRNA array RT-qPCR	Endogenous U6
Liu X. et al (2015)	Kidney	NR	miRNeasy (Qiagen)	Both	NGS RT-qPCR	NGS analysis NR Endogenous U6.
Soltaninejad E. et al (2015)	Kidney PBMC	9-18 mths post-transplant	mirVana miRNA isolation kit (Ambion)	Targeted	RT-qPCR	Endogenous RNU44
Tao J. et al (2015)	Serum	NR	miRNeasy serum/plasma kit (Qiagen)	Both	miRNA array RT-qPCR	Endogenous RNU6-2
McGuinness D. et al (2016)	Kidney	Pre-transplant	TRI Reagent (Invitrogen)	Both	miRNA array RT-qPCR	Endogenous RNU44, RNU48, MammU6, or U6snoRNA
Amrouche L. et al (2017)	Kidney Urine pellet	Early post-transplant	miRNeasy (Qiagen)	Targeted	RT-qPCR	Endogenous RNU48
Domenico T.D. et al (2017)	PBMCs Urine sediment	Within 1 mth post-transplant	mirVana PARIS (Ambion)	Targeted	RT-qPCR	Exogenous cel-miR-39
Millán O. et al (2017)	Urinary cell pellet post-centrifugation	1 wk, 1, 2, 3, 6 mths post-transplant, preceding AR episodes.	TRIzol (Life Technologies)	Targeted	RT-qPCR	Endogenous miR-103.
Jin P. et al (2017)	Kidney	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Gallon L. et al (2018)	Kidney	Up to 25 mths post-transplant	High Pure RNA Paraffin kit (Roche)	Targeted	RT-qPCR	NR
Cheng K. et al (2018)	Whole blood	Pre-transplant; 1, 2, 3, 4 wks post-transplant	mirVana miRNA isolation kit (Ambion)	Targeted	RT-qPCR	Endogenous U6
Gremmels H. et al (2019)	Perfusion fluid exosomes	Pre-transplant	Total exosome RNA isolation kit (Invitrogen)	Unbiased	miRNA panel	On-chip SNORD49A

Khalid U. et al (2019)	1st urine post-transplant	Post-transplant 0 to 5 d	miRNeasy mini kit (Qiagen)	Both	miRNA array RT-qPCR	Exogenous cel-miR-39
LI F. et al (2019)	PBMC	NR	Tripure Isolation Reagent (Roche)	Targeted	RT-qPCR	Endogenous RNU44
Milhoransa P. et al (2019)	Kidney PBMC	Post-transplant (d): Controls: 99 (86-116); DGF: 14 (12-26);AR: 58 (14-331)	mirVana PARIS (Ambion)	Targeted	RT-qPCR	PBMC: spike-in cel-miR-39 Kidney: endogenous RNU48
Roest H.P. et al (2019)	Cell-free donor perfusion fluid	Pre-transplant	-Anti-miRNA magnetic beads -miRNeasy mini kit (Qiagen)	Both	miRNA array RT-qPCR	-Exogenous cel-miR-39
Wang J. et al (2019)	Blood exosomes	1 wk post-transplant	mirVana miRNA isolation kit (Ambion)	Unbiased	High throughput sequencing	NR
Seo J-W. et al (2023)	Urinary exosomes	NR	exoRNeasy serum/plasma kit (Qiagen)	Both	nCounter miRNA assay RT-qPCR	internal positive control Endogenous miR-16-5p
Connor K.L. et al (2020)	Urine	0 and 1-5 d post-transplant	miRNeasy kit (Qiagen)	Targeted	RT-qPCR	Exogenous cel-miR-39
Alfaro R. et al (2021)	PBMCs	At time of AR	Maxwell 16 miRNA kit (Promega)	Targeted	miScriptmiRNA PCR Array T-Cell, B-cell panel (RT-qPCR)	SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6-2, 28S rRNA
Rutman A.K. et al (2022)	Perfusion fluid exosomes	Pre-transplant	miRNeasy kit (Qiagen)	Both	RNA sequencing RT-qPCR	Exogenous UniSp6
Shi L. et al (2019)	Kidney	10 d post-transplant (range 6-14 d)	n/a	Targeted	Fluorescence in-situ hybridization for miR-10a-5p	NR
Cardiac surgery						
Du J. et al (2013)	Plasma, Urine	Within 24 hr post-operatively	mirVana PARIS	Targeted	RT-qPCR	Exogenous cel-miR-39
Aguado-Fraile E. et al (2015)	Serum	(1) Biobank (AKI) (2) AKI diagnosis (3) Before AKI	miRNeasy (Qiagen)	Both	miRNA array RT-qPCR	Spike-in (exogenous) synthetic oligo
Gaede L. et al (2016)	Serum	Pre-surgery, 4 hr post-surgery	miRNeasy (Qiagen)	Targeted	RT-qPCR	Exogenous cel-miR-39
Arvin P. et al (2017)	Serum Urine	Prior to, and 6-72 hr post-surgery	miRNeasy (Qiagen)	Targeted	RT-qPCR	Exogenous cel-miR-39
Zhang L. et al (2017)	Plasma	Prior to, and 2-72 hr post-surgery	mirVana PARIS	Targeted	RT-qPCR	Exogenous cel-miR-39
Zou Y-F. et al (2017)	Urine	Prior to and up to 72 hr post-surgery	miRNeasy serum/plasma kit	Targeted	RT-qPCR	Exogenous cel-miR-39
Kang Z. et al (2018)	Plasma Urine	Prior to, up to 5 days post-surgery	mirVana PARIS	Targeted	RT-qPCR	Exogenous cel-miR-39
Sullo N. et al (2018)	Serum	Pre-op, and 6, 12, 24 hr post-op	miRNeasy	Unbiased	miRNA array	Endogenous U6
Wei Q. et al (2018)	Serum (1,2) Urine (1,2) Kidney (2)	1. Pre-op, 0, 3, 9, 24 hr 2. NR	mirVana PARIS	Targeted	RT-qPCR	**Urine, serum:** Exogenous cel-miR-39. **Kidney**: endogenous snoRNA-202
Gong Q. et al (2021)	Serum	Pre-op, up to 24 hr post-op	Trizol	Targeted	RT-qPCR	Endogenous U6
Miller D. et al (2022)	Urinary EV	Before and 24 hr after surgery	exoRNeasy	Both	RNA sequencing RT-qPCR	Endogenous let-7a-5p
Chen Y. et al (2022)	Plasma exosomes	Pre and post-surgery	Trizol	Both	RNA sequencing RT-qPCR	Endogenous U6
Li X. et al (2023)	Urinary exosomes	Post-surgery	Trizol	Targeted	RT-qPCR	Endogenous U6
Sepsis						
Saikumar J. et al (2012)	Urine	NR	Trizol	Targeted	RT-qPCR	Endogenous U87
Ramachandran K. et al (2013)	Urine	NR	miRNeasy serum/plasma kit	Both	miRNA microarray RT-qPCR	Exogenous cel-miR-39, miRTC
Ge Q-M. et al (2017)	Serum	At time of AKI diagnosis	miRNeasy	Both	miRNA microarray RT-qPCR	Endogenous miR-423-5p
Wang S. et al (2017)	Circulating endothelial cells	At time of AKI diagnosis	miRNeasy TaqMan micRNA Cells-to-CT kit	Both	miRNA microarray RT-qPCR	Endogenous U6
Zhang J. et al (2018)	Urine supernatant	Daily for 1st wk of ICU admission. Before AKI.	mirVana miRNA isolation	Targeted	RT-qPCR	Exogenous cel-miR-39
Bukauskas T. et al (2019)	Whole blood	NR	NR	Targeted	RT-qPCR	Exogenous cel-miR-39
Lin Y. et al (2019)	Plasma	NR but after AKI	NR	Unbiased	miRNA microarray	Exogenous cel-miR-39
Sun J. et al (2020)	Plasma	NR but after AKI	Trizol	Targeted	RT-qPCR	Endogenous GAPDH
Liu S. et al (2021)	Serum	Within 24 hr of admission	Trizol	Targeted	RT-qPCR	Endogenous U6
Shi L. et al (2021)	Serum	NR, but before ICU admission	Trizol	Targeted	RT-qPCR	Endogenous U6

Zhang H. et al (2021)	Serum, urine	At admission (before AKI)	Trizol	Targeted	RT-qPCR	Endogenous U6
Zheng C. et al (2021)	Serum	At AKI diagnosis	Trizol	Targeted	RT-qPCR	Endogenous U6
Gong J. et al (2022)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Ma W. et al (2022)	Urine	NR	Trizol	Targeted	RT-qPCR	NR
Pan W. et al (2022)	Serum	NR	Serum/plasma mIRNA extraction klt	Targeted	RT-qPCR	Endogenous U6
Xun L. et al (2022)	Serum	NR	NR	Targeted	RT-qPCR	NR
Xu L. et al (2022)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Ye J. et al (2022)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Zhang F. et al (2023)	Plasma	NR	RNAzol	Targeted	RT-qPCR	Endogenous U6
Kuang F. et al (2023)	Plasma exosomes	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Zhang B. et al (2023)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
You T. et al (2023)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Han R. et al (2023)	Urine	Within 24 hr of sepsis diagnosis	mirVana miRNA isolation kit	Both	RNA sequencing RT-qPCR	Endogenous U6
Liu W. et al (2023)	Serum	At diagnosis	Trizol	Targeted	RT-qPCR	Endogenous U6
Li P. et al (2023)	Circulating EC-derived and total exosomes	Post-admission day 1	miRNeasy serum/plasma kit	Targeted	RT-qPCR	Exogenous cel-miR-39
Nephrotoxic						
Vliegenthart A.D.B. et al (2015)	Plasma	Up to 100 hr post-ingestion	miRNeasy serum/plasma	Both	miRNA PCR array RT-qPCR	Exogenous cel-miR-39
Gutiérrez-Escolano A. et al (2015)	Plasma	Pre-PCI, 3-48 hr post-PCI	Trizol	Targeted	RT-qPCR	Proprietary synthetic miRNA oligonucleotides
Pavkovic M. et al (2016)	Urine Kidney (ATN)	1. After AKI 2. Pre , and up to 144 hr post-treatment	miRNeasy serum/plasma kit	Targeted	RT-qPCR In-situ hybridization	qPCR positive control (not specified), and urine Cr
Sun S. et al (2016)	Plasma	Pre-contrast, 4-6 hr post-contrast	mirVana	Targeted	RT-qPCR	Exogenous cel-miR-39
Shihana F. et al (2020)	Serum	4 – 24 hr post-ingestion	RNeasy-HT	Both	miRNA panel Custom array (validation)	Exogenous ath-miR-159a, ath-miR-172a
Shihana F. et al (2020)	Urine	4 – 24 hr post-ingestion	RNeasy-HT	Both	miRNA panel custom array (validation)	Exogenous ath-miR-172a
Zhang L. et al (2020)	Whole blood	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Quintanilha J.C.F. et al (2021)	Plasma	Baseline and 5 d post-cisplatin	miRNeasy serum/plasma	Both	RNA seq RT-qPCR	Exogenous cel-miR-39
Shihana F. et al (2021)	Urine	Within 8 hr of ingestion	RNeasy-HT	Both	miRNA microarray custom array (validation)	Exogenous ath-miR-172a
Other						
Lorenzen J.M. et al (2011)	Plasma	Before RRT initiation	MasterPure RNA kit	Both	miRNA array RT-qPCR	Exogenous cel-miR-54
Lan Y-F. et al (2012)	Serum Urine	NR	QiAmp circulating nucleic acid kit	Targeted	RT-qPCR	Endogenous U6
Krebs C.F. et al (2013)	Kidney	NR	High Pure FFPE RNA kit (Roche)	Both	miRNA array RT-qPCR. ISH	18S rRNA or small RNAs
Bruno N. et al (2016)	Plasma	Post-admit 1 d	miRCURY RNA isolation for bio-fluids (Exiqon)	Targeted	RT-qPCR	Geometrical mean of endogenous miR-30a-5p, miR-194-5p
Chen H-H. et al (2016)	Serum, urine	After AKI	QiAMP	Both	miRNA array RT-qPCR	Endogenous RNU6B
Ma Y. et al (2017)	Serum	After AKI	Trizol	Both	miRNA array RT-qPCR	Endogenous U6
Wang S. et al (2017)	PBMCs	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Guo Y. et al (2018)	Kidney	After AKI	n/a	Targeted	ISH	n/a
Watany M.W. et al (2018)	Serum	After AKI	miRNeasy	Targeted	RT-qPCR	Endogenous U6
Kölling M. et al (2018)	Whole blood	Before RRT initiation	Trizol	Targeted	RT-qPCR	Exogenous cel-miR-39
Fan P-C. et al (2019)	Serum	24 hr, 48 hr, and 1 wk of admission	miRNeasy	Targeted	RT-qPCR	Exogenous cel-miR-39

Newbury L.J. et al (2021)	Urine	After AKI	NR	Both	miRNA array RT-qPCR	Endogenous miR-191-5p
Mao H. et al (2021)	Serum	After AKI	NR	Targeted	RT-qPCR	Endogenous U6
Aomatsu A. et al (2021)	Serum	Within 24 hr of AKI onset	NucleoSpin miRNA plasma column	Targeted	RT-qPCR	Endogenous RNU-6
Phulkerd T. et al (2022)	Serum, urine	1st day of AKI diagnosis	NR	Both	nCounter miRNA array RT-qPCR	Endogenous miR-16-5p
Liu J. et al (2022)	Serum	Within 24 hr of admission	RNA extraction kit	Targeted	RT-qPCR	NR
Petejova N. et al (2022)	Whole blood	Up to 7 d of antibiotics	Qiazol	Targeted	RT-qPCR	Exogenous cel-miR-39-3p Endogenous miR-486-5p
Xue Q. et al (2022)	Serum	NR	Trizol	Targeted	RT-qPCR	Endogenous U6
Xie Z. et al (2023)	Plasma	NR	Trizol	Targeted	RT-qPCR	Endogenous U6

Supplementary Table continued

Author	Measured miRNAs	Dysregulated miRNAs and conclusions
Kidney Transplant		
Lorenzen J.M. et al (2011)	384 miRNAs screened	**miRNA array**: 21 miRs **Validation:** Urine miR-10b, -210 decreased, and miR-10a increased in AR. Lower miR-210 associated with decline in GFR 1-yr post-transplant
Wilflingseder J. et al (2013)	904 miRNAs screened	**Validation:** **DGF vs controls**. ***Increased:*** miR-182-5p, -21-3p ; **TCMR vs controls**. ***Increased***: miR-155-5p; ***Decreased:*** miR-125-5p **ABMR vs controls. *Increased:*** miR-182-5p, -21-3p, -146b-5p **miR-182, miR-21 were increased in DGF and ABMR,** suggesting overlap in signaling pathways. **miR-146-5p, miR-1228, let-7i levels separated ABMR from TCMR and DGF**, and **differential expression of miR-155-5p, miR-125-5p were unique to TCMR.** miRNA signatures have the potential to differentiate between DGF, TCMR, and ABMR.
Sui W. et al (2013)	Microarray: 71 miRNAs were detected	20 dysregulated miRNAs (12 down, 8 up) in AR **Decreased miRNAs listed:** miR-663, -326, -346, -125b, -324-3p; **Increased miRNAs listed**: miR-658, -320, -381, -629 5 transcription factors correlated with miRNA data: AP-1, AP-4, STATx, c-Myc, p53
Lorenzen J.M. et al (2014)	miR-24	miR-24 increased in transplant kidneys with long cold ischemia times, therefore upregulated in kidney IR injury post-transplant.
Wilflingseder J. et al (2014)	49 miRNAs identified	**29 miRNAs upregulated in DGF:** miR-21-3p, -212-3p, -132-3p, -4505, -3679-5p, -4530, -4299, -4433-3p, -4507, -3648, -182-5p, -1587, -4667-5p, -1224-5p, -4685-5p, -4430, -4749-5p, -2392, -4690-5p, -4463, -762, -4508, -149-3p, -3196, -4516, -1268a/b, -3687, -4532. **Validation**: miR-182-5p, -21-3p increased in AKI miR-182-5p, -21-3p identified and validated as AKI-specific miRNA signature miR-182-5p expression also correlated with transcripts associated with post-transplant AKI (LCN2, S100A8, MMP7)
Liu X. et al (2015)	75 dysregulated miRNAs	**Top 10 increased**: miR-145-5p, -146a, -155, -650, -223, -125a, -629, -602, -21, -628 **Top 10 decreased**: miR-10b, -524-3p, -611, -30c, -17-3p, -330, -30a-3p, -483, -663, -32 **Validation**: miR-10b down-regulated in AR
Soltaninejad E. et al (2015)	miR-142-5p, -142-3p, -155, -223	**Biopsies:** miR-142-5p, -142-3p, -155, -223 increased in TCMR vs controls **PBMCs:** miR-223, -142-3p increased in TCMR vs controls. In TCMR, miR-223, -142-3p are up-regulated in both graft biopsies and in PBMCs
Tao J. et al (2015)	miRNA array: 384 miRs Validation: 6 miRs	**Increased in AR vs controls (array):** let-7a-5p, let-7c-5p, let-7f-5p, miR-151a-5p, -99a-5p, -100-5p. **Increased in AR vs controls (validation):** miR-100, -99a. Increased levels of miR-99a, -100-5p in serum of patients with AR vs stable function. By ROC analysis, miR-99a discriminated AR from DGF.
McGuinness D. et al (2016)	miRNA array: 754 miRs Validation: miR-217, -125b	**Array (good vs poor graft function within 2 years of transplant):** miR-505, -34a, -1275, -125a-5p, -449a, -155, -101, -375, -96, -217, -125b. **Targeted:** miR-217, -125b down-regulated in grafts that develop DGF Pre-transplant miR-125b, -217 levels were associated with DGF. Recovery from DGF correlated with miR-125b levels
Amrouche L. et al (2017)	miR-146a	Kidney miR-146a levels increased in ATN. Urine miR-146a increased in DCD kidney transplant recipients. Higher urinary miR-146a levels associated with more severe kidney ischemic injury
Domenico T.D. et al (2017)	miR-142-3p	**PBMCs:** miR-142-3p increased in ATN (vs AR and controls). **Urine sediment**: miR-142-3p increased in ATN and AR vs controls. miR-142-3p increased in PBMCs of transplant recipients with ATN (vs AR); candidate biomarker for post-transplant ATN
Millán O. et al (2017)	miR-142-3p, -210-3p, -155-5p	miR-155-5p, -142-3p increased in AR; mmiR-210-3p **decreased** in AR Urinary pellet miR-155-5p has potential as diagnostic and prognostic biomarker for AR. miR-155-5p correlated with anti-rejection medication trough levels.
Jin P. et al (2017)	miR-650	miR-650 is **increased** in graft biopsies from kidneys with AR compared to control grafts
Gallon L. et al (2018)	84 miRNAs	**Upregulated in controls vs AR:** miR-31-5p, -9-5p, -125b-5p, -187-3p, -148-3p, -126-3p, 23b-3p, 147a, -383-5p) **Downregulated in controls vs AR:** miR-21-5p, -223-5p The up-regulated miRNAs in grafts of recipients with immune tolerance vs AR have target genes involved in inflammatory pathways.
Cheng K. et al (2018)	miR-181b	miR-181b is down-regulated in vascular rejection at 1-4 weeks post-transplant. No difference in pre-transplant miR-181b levels. Blood miR-181b is (1) decreased in vascular rejection, and (2) increased in the treatment-responsive subgroup
Gremmels H. et al (2019)	223 miRNAs detected	10 up-regulated miRNAs in DGF in ***unadjusted*** analysis but n**one significant after adjustment.** Donor kidney perfusion exosomal miRNAs were not statistically significantly associated with DGF after adjustments
Khalid U. et al (2019)	Array: 377 miRNAs Validation: 8 miRNAs	**Array (8 miRNAs increased):** miR-9, -10a, -21, -29a, -221, -429, -506, -574-3p **Targeted (increased in DGF):** miR-9, -10a, -21, -29a, -221, -429 Urinary miR-21 increased over 5 days in DGF. Urinary miRNA profile can be predictive of DGF.

Li F. et al (2019)	miR-142-3p, -142-5p, -10b, -30a-3p, -223, -486-5p, -155, -211, let-7c	PBMC miR-142-3p, -142-5p are increased in patients with graft dysfunction After 3-4 weeks: miR-142-3p/5p, miR-223 increased and miR-10b decreased in PBMCs from recipients with abnormal Cr miR-142-3p/5p, miR-10b, and miR-223 in PBMCs of kidney recipients have potential to predict graft dysfunction.
Milhoransa P. et al (2019)	miR-146a-5p	**PBMC:** miR-146a not statistically significantly increased in DGF (vs stable function and AR) **Kidney:** miR-146a increased in DGF vs stable function, no significant difference between AR vs stable graft function miR-146a levels were increased in grafts with DGF vs stable function. In PBMCs, there is a trend towards increased miR-146a in DGF vs stable controls and AR.
Roest H.P. et al (2019)	223 miRNAs	32 miRNAs were differentially expressed in perfusion fluid from grafts with DGF vs without DGF, but only 1 (miR-505-3p) statistically significant. miR-505-3p increased in perfusion fluid of DCD kidneys with DGF; potential predictive marker for DGF.
Wang J. et al (2019)	-87 identified miRNAs	Increased in DGF vs controls: miR-33a-5p, -98-5p, -151a-5p. Peripheral blood exosomal miR-33a-5p, -98-5p, -151a-5p increased in DGF; potential as biomarkers for DGF
Seo J-W. et al (2023)	-798 miRNAs measured	-14 urinary exosomal miRs **(screen). Increased in AR**: miR-197-5p; **Decreased in AR**: miR-575, -489-3p, -4532, -4516, -4488, -320e, -3195, -3185, -3158-3p, -1915-3p, -187-3p, -1305, -1268a. **(Targeted)** miR-4488, -4532 decreased in AR. miR-21-5p, -155-5p, 210-3p, 223-3p, -31-5p, 373-3p increased in AR. Urinary exosomal miRNAs are differentially expressed in AR (vs controls) but may not be specific to rejection-associated graft injury.
Connor K.L. et al (2020)	miR-16-5p, -18a-5p, -194-5p	DGF: Increased urinary miR-16-5p, -18a-5p Macrophage-enriched miRNAs (miR-16-5p, -18a-5p) are potential predictors of DGF, whereas proximal tubular cell-enriched miR-194-5p was not.
Alfaro R. et al (2021)	84 miRNAs related to B, T-cell activation	**PBMC miRNAs increased in AR**: miR-574-3p, -181d, -191-5p, -423-5p, -346, -223-3p. **Decreased in AR**: miR-150-5p Differentially expressed miRNAs related to B and T cell activation were identified in PBMCs (AR vs non-AR)
Rutman A.K. et al (2022)	RNA-seq Targeted: miR-218-5p	Perfusion fluid miRNAs Increased in DGF: miR-218-5p, -151-b, -675-3p RT-qPCR validated increased miR-218-5p in perfusion fluid from grafts with DGF miR-218-5p levels inversely correlated with recipient eGFR at post-transplant days 7 through 180 miR-218-5p may contribute to pro-inflammatory environment in DGF via Th17/Treg ratio
Shi L. et al (2019)	miR-20a-5p	miR-20a-5p signal increased in DGF kidneys vs non-transplant controls miR-20a-5p signal was detected in kidney tubular epithelial cells
Cardiac surgery		
Du J. et al (2013)	miR-21	miR-21 increased in urine and plasma post-cardiac surgery and positively correlated with AKI progression. Urinary, plasma miR-21: associated with severe AKI, other poor outcomes post-cardiac surgery.
Aguado-Fraile E. et al (2015)	Array: 786 miRNAs 10 miRs selected for validation	**Decreased in ICU AKI vs healthy controls:** miR-101-3p, -210-3p, -126-3p, -26b-5p, -29a-3p, -146a-5p, -27a-3p, -93-3p, -10a-5p, -127-3p. **Decreased in cardiac surgery AKI:** pre-and post-surgery miR-26b-5p, -27a-3p, -93-3p, -127-3p miR-210-3p, -126-3p, -29a-3p, -146a-5p decreased in ICU patients with AKI, correlated with AKI severity. miR-26b-5p, -27a-3p, -93-3p, -127-3p decreased in CS-AKI; potential cardiac surgery AKI biomarkers
Gaede L. et al (2016)	miR-21	Pre-surgery miR-21 decreased in patients who developed post-cardiac surgery AKI miR-21 may be a predictive biomarker for CS-AKI. miR-21 levels did not correlate with long-term outcomes (mortality, death)
Arvin P. et al (2017)	miR-21	Baseline serum and urinary miR-21 decreased in CS-AKI. Serum and urine miR-21 most decreased after 6 hr in CS-AKI. No significant difference in pre-post-surgery miR-21 in control group. Serum and urinary miR-21 levels are decreased early (6 hr) after cardiac surgery in CS-AKI. Baseline levels are lower despite matching groups.
Zhang L. et al (2017)	miR-192	Plasma miR-192 increased immediately post-surgery in both groups but remained significantly higher 2 hr post-surgery in AKI. miR-192 decreased 24 hr after surgery in both groups. Plasma miR-192 levels were altered by cardiac surgery, but transiently higher at 2 hr post-surgery in patients with AKI.
Zou Y-F. et al (2017)	miR-30c-5p, miR-192-5p, miR-378a-3p	In patients who develop CS-AKI, urinary miR-30c-5p and miR-192-5p significantly increased at 2 hr post-surgery and return to baseline by 6 hr. Early transient rise in urine miR-30c-5p, -192-5p in AKI; potential as early diagnostic biomarkers
Kang Z. et al (2018)	miR-21	Plasma , urine miR-21 peaked 24 hr post-surgery in RIPC group, returned to baseline by day 5. Increased urine miR-21 sustained for up to 72 hr. RIPC protected against CS-AKI, associated with increased urinary and plasma miR-21, decreased TNFa.
Sullo N. et al (2018)	NR	**Children**: **Pre-op** let-7g-5p, miR-152-3p, -30b-5p decreased, miR-320a increased in AKI. **Post-op**: miR-125a-3p/5p, -301-3p, -223-3p increased in AKI. **All ages (including infants)**: **pre-op** miR-192-5p decreased, miR-487a-3p, -490-3p increased in AKI. **Post-op**: miR-10a-5p decreased in AKI. Circulating miRNAs are differentially expressed pre- and post-surgery in children with CS-AKI and may have biomarker potential, although the miRNA profile in children and infants differs.
Wei Q. et al (2018)	miR-688	1. Serum: increased miR-688 post-surgery in both groups. **Urine:** increased miR-688 post-surgery in AKI 2. miR-688 increased in kidney tissue from AKI patients, but not differentially expressed in urine and serum from AKI patients. Kidney miR-688 is increased in ischemic AKI.
Gong Q. et al (2021)	miR-494-3p	miR-494 was increased by 1 hr post-surgery in AKI with return to baseline by 12 hr post-surgery Serum miR-494-3p transiently increased post-op in CS-AKI; potential early biomarker for CS-AKI
Miller D. et al (2022)	8/13 miRNAs: miR-10a, -125a-5p, -196a-5p, -320a-3p, -93a-5p, -99a-3p/5p,-21-5p	(Urinary particle-adjusted) miR-10a-5p decreased post-surgery in AKI, associated with AKI severity; pre-surgery miR-125a-5p decreased in AKI Urinary EV miR-125a-5p might be predictive of CS-AKI; miR-10a-5p may have prognostic value in CS-AKI
Chen Y. et al (2022)	56 miRNAs identified	Post-op miR-590-3p (1) increased in younger and (2) decreased in older CS-AKI patients. Exosomal miR-590-3p decreased post-op in older patients with CS-AKI
Li X. et al (2023)	miR-106-5b	Urine exosomal miR-106-5b increased in CS-AKI, and correlated with AKI severity (stages 1-3) Urine exosomal miR-106-5b may have biomarker potential for CS-AKI.
Sepsis		
Saikumar J. et al (2012)	miR-21, -155, -18a	Urine miR-21 increased and miR-155 decreased in AKI Higher urinary miR-21 and lower miR-155 in AKI: potential urinary AKI biomarkers.
Ramachandran K. et al (2013)	1809 miRNAs Validation: 7 miRNAs	Urinary miR-21, -200c, -423 increased in ICU patients with AKI. Relative levels of these miRNAs were significantly higher than in transplant AKI. Urinary miR-21, -200c, -423 increased in AKI and differentiated between ICU patients with and without AKI. Possible AKI biomarkers in ICU.

Ge Q-M. et al (2017)	40 miRNAs Validation: miR-4270, -4321, -3165, -23a-3p, -4456, -142-5p, -miR-22-3p, -191-5p	**Array**: miRNAs **increased** septic AKI (serum): miR-4270, -4321, -3165. miRNAs **decreased** in septic AKI (serum): miR-142-5p, -22-3p, -191-5p, -23a-3p, -4456 **Validation:** miR-4321 and miR-4270 increased in septic AKI (vs septic and healthy controls). Serum miR-4321 and -4270 are upregulated in septic AKI vs septic non-AKI controls. Potential biomarkers for septic AKI.
Wang S. et al (2017)	38 miRNAs	miR-107 is increased in circulating endothelial cells (ECs) of pediatric patients with septic AKI. miR-107 is increased in circulating ECs of pediatric patients with established septic AKI; may be a mediator of septic AKI.
Zhang J. et al (2018)	miR-26b	Increased urinary miR-26b levels in septic patients who developed AKI. Urinary miR-26b levels were higher in septic AKI group 24 h prior to AKI development, associated with AKI severity. Higher levels were associated with in-hospital mortality.
Bukauskas T. et al (2019)	miR-30d-5p, -23a-3p, -146a-5p	None significantly different between any groups Blood levels of miR-30d-5p, -23a-3p, -146a-5p were not altered between patients with peritonitis and normal kidney function, AKI, or CKD.
Lin Y. et al (2019)	30 miRNAs	11 miRNAs increased in sepsis AKI: miR-210, -494, -23a, -26a, -29a, -10a-5p, -122, -143, -214, -223, -497 11 miRNAas decreased in sepsis AKI: miR-205, -146a, -182-5p, -16, -21, -145, -203, -204, -290, -320, -590 miR-210, and miR-494 had the largest increase while miR-205 had the largest decrease in sepsis AKI. These miRNAs were associated with in-hospital survival.
Sun J. et al (2020)	miR-580, -671-3p, -886-5p, -299-5p, -557, -9	Plasma miR-580, -671-3p, -886-5p, -299-5p, -557, -9 levels were all decreased in AKI NF-κB locus SNPs were associated with AKI risk in sepsis and associated with levels of plasma miR-299-5p, -557, -9, linking these miRNAs in the pathophysiology of sepsis-associated AKI.
Liu S. et al (2021)	miR-574-5p	Serum miR-574-5p levels are significantly decreased in sepsis AKI (vs sepsis no AKI), with progressive decrease with AKI stage. Serum miR-574-5p levels correlated with AKI severity.
Shi L. et al (2021)	miR-150-5p	Serum miR-150-5p is decreased in sepsis AKI vs healthy controls
Zhang H. et al (2021)	miR-22-3p	Serum and urine miR-22-3p decreased in septic patients who develop AKI vs septic controls. miR-22-3p was also associated with poor 28-d survival in sepsis AKI. Urine and serum miR-22-3p have potential as diagnostic and prognostic biomarkers for sepsis-associated AKI.
Zheng C. et al (2021)	miR-34b-5p	Serum miR-34b-5p levels are increased in patients with septic AKI vs healthy controls.
Gong J. et al (2022)	miR-335-5p	Serum miR-335-5p decreased in sepsis AKI vs healthy controls
Ma W. et al (2022)	miR-370-3p miR-495-3p	Urine miR-370-3p, miR-495-3p are decreased in septic patients with AKI. Urinary miR-370-3p, -495-3p are lower in septic patients with AKI compared to septic controls and associated with lower 28-d survival. Potential as prognostic biomarkers.
Pan W. et al (2022)	miR-4299 miR-16-5p	Serum miR-4299, miR-16-5p are decreased in septic AKI; potential as diagnostic biomarkers for sepsis-associated AKI
Xun L. et al (2022)	miR-10a-5p	Serum miR-10a-5p increased in sepsis AKI and associated with increased mortality. Serum miR-10a-5p levels significantly higher in septic patients with AKI; may have prognostic value in sepsis AKI.
Xu L. et al (2022)	miR-495-3p	Serum miR-495-3p was decreased in sepsis vs healthy controls, and also significantly decreased in patients with septic AKI vs septic patients without AKI
Ye J. et al (2022)	miR-23a-3p	Serum miR-23a-3p decreased in septic AKI vs healthy control
Zhang F. et al (2023)	miR-21	Serum miR-21 decreased in septic AKI vs septic patients without AKI Lower serum miR-21 levels were found in septic patients with AKI vs without AKI. Endogenous miR-21 is associated with renoprotection.
Kuang F. et al (2023)	miR-136-5p	Plasma exosomal miR-136-5p was decreased in sepsis AKI vs healthy controls
Zhang B. et al (2023)	miR-370-3p	Serum miR-370-3p is decreased in sepsis AKI vs healthy controls.
You T. et al (2023)	miR-155-5p	Serum miR-155-5p is increased in sepsis AKI vs healthy controls.
Han R. et al (2023)	miR-31-5p, -151a-3p, -142-5p, -16-5p	Screen: 6 miRNAs increased in sepsis AKI, 27 miRNAs increased in sepsis no AKI (vs healthy controls) Targeted: Urine miR-31-5p, -151a-3p, -142-5p, -16-5p are increased in sepsis (AKI and non-AKI) vs healthy controls Urinary miR-142-5p, miR-16-5p had good predictive value for septic AKI and were associated with 28-d mortality.
Liu W. et al (2023)	miR-342-5p	Serum miR-342-5p is decreased in sepsis AKI vs healthy controls
Li P. et al (2023)	miR-126-3p/-5p	Circulating total exosomal miR-126-5p decreased over 3 d between sepsis and non-sepsis ICU controls. Decreased circulating exosomal miR-126-5p was associated with AKI, ARDS, and increased mortality.
Nephrotoxic		
Vliegenthart A.D.B. et al (2015)	93 miRNAs (screen); 16 miRNAs (targeted)	5 miRNAs significantly decreased in APAP-ALI with AKI (vs no AKI): miR-19a-3p, -19b-3p, -192-5p, -34a-5p, -3187-3p Plasma miR-19a-3p, -19b-3p, -192-5p, -34a-5p, -3187-3p are potential novel AKI biomarkers in acetaminophen overdose with liver toxicity.
Gutiérrez-Escolano A. et al (2015)	miR-30a, -30c, -30e, -320	Plasma miR-30a, -30c, and -30e increased within 6 hr after PCI in CI-AKI vs no AKI. Plasma miR-30a, -30c, -30e levels increase early after contrast exposure prior to AKI development. Potential biomarkers for CI-AKI risk post-PCI.
Pavkovic M. et al (2016)	miR-21, -200c, and -423	1. miR-21, -200c, -423 increased in APAP with AKI 2. No significant difference in miRNA levels in mesothelioma cohort with AKI 3. miR-21 signal by ISH in ATN kidneys (but not normal tissue), with localization to injured tubules. Urinary miR-21, -200c, -423 increased in patients with acetaminophen overdose with AKI, and associated with AKI risk. No association between these miRNAs and AKI development in mesothelioma patients undergoing cisplatin treatment.
Sun S. et al (2016)	miR-188-5p, -30a-5p, -30e-5p	Plasma miR-188, -30a, -30e levels increased in CI-AKI compared to baseline (pre-contrast) Plasma miR-188, -30a, -30e levels are increased hours after CM exposure; potential diagnostic biomarkers for CI-AKI.
Shihana F. et al (2020)	Screen: 212 dysregulated miRNAs	13 miRNAs were significantly **decreased** between patients with OA ingestion with and without AKI: miR-17, -19b, **-20a**, -30d, **-92a, -93**, -106a, -122, -126**, -195**, -203**, -451**, -484. Bolded: most associated with AKI.

	Validation: 53 selected miRNAs	5 miRNAs detected 4-8 hr post-ingestion (miR-20a, -92a, -93, -195, -451) significantly decreased in patients with OA ingestion and AKI; potential AKI biomarkers.
Shihana F. et al (2020)	Total 754 miRNAs 30 differentially expressed miRNAs selected.	25/30 urinary miRNAs **increased** in OA AKI. Urinary miRNAs rise before AKI development, with decline over 24 hr. Top 4 increased urinary miRNAs: miR-191, -19b, -20a, -30b Urinary miRNA profile correlated more with AKI in OA poisoning than did plasma miRNAs. Urinary miR-191, -19b, -20a, -30b are potential biomarkers for OA AKI.
Zhang L. et al (2020)	miR-512	Blood miR-512 levels increased in CI-AKI after PCI
Quintanilha J.C.F. et al (2021)	RNA-seq: 33 dysregulated miRNAs	Increased in AKI by RNA-seq: miR-3168, -6125, -4718. **Validation**: baseline plasma miR-3168, -6125, -4718 trended higher in AKI group, but no difference at 5 d. Plasma miR-3168, -6125, -4718 are potential predictors of cisplatin-induced AKI.
Shihana F. et al (2021)	Screen: 112 miRNAs Validation: 53 miRNAs	7 common urinary miRNAs increased in all groups of nephrotoxic AKI (vs no AKI): miR-30b, -30a-3p, -30a-5p, -204, -191, -660, -423-5p Potential early biomarkers for detection of nephrotoxic AKI.
Other		
Lorenzen J.M. et al (2011)	Screen: 13 miRNAs. Validation: miR-16, -320, -210	Plasma miR-16, -320 decreased, miR-210 increased in ICU patients with RRT-requiring AKI vs disease controls (acute MI). miR-210 levels increased in non-survivors with AKI (at 28 d).
Lan Y-F. et al (2012)	miR-494	Urinary miR-494 increased with AKI. No difference in serum miR-494 between groups. Compared with ICU patients without AKI, those with AKI have higher urinary miR-494 levels.
Krebs C.F. et al (2013)	12 miRNAs	Kidney miR-155 increased in ANCA GN, inversely correlated to kidney function. ANCA GN kidneys had glomerular staining for miR-155 within infiltrating inflammatory cells. Data suggests infiltrating cells as a source of miR-155.
Bruno N. et al (2016)	12 miRNAs measured	**Cases:** Decreased levels of miR-199-3p, -423-3p, -let-7i-5p. **Patients with highest increase in serum Cr:** decreased miR-199a-3p, miR-27a-3p, miR-652-3p, miR-423-5p, miR-let-7i-5p. Worsening kidney function in acute HF was associated with lower baseline levels of several circulating miRNAs. MiR199a-3p was the strongest predictor of AKI.
Chen H-H. et al (2016)	Screen: 30 miRNAs Validation: miR-16	Urine let-7d, -26-3p, miR-16, miR-451, miR-486-5p, miR-518, miR-720 up-regulated, 21miRNAs down-regulated in AKI. **Validation:** Urinary miR-16 increased in ICU patients with AKI vs ICU patients without AKI. No change in serum miR-16.
Ma Y. et al (2017)	Screen: 24 dysregulated miRNAs	**(Validation)** Serum miR-210, -21, -30a, -489, -216, -34, -150 increased in AKI. Serum miR-320, -122, -16, -192, -329, -200a decreased in AKI (vs healthy controls)
Wang S. et al (2017)	miR-125b-5p, -324-5p, -326	PBMC miR-125b-5p levels decreased in obstructive kidney injury vs healthy controls, correlated with kidney function decline.
Guo Y. et al (2018)	miR-709 ISH	miR-709 was increased in kidneys of AKI patients compared to controls, co-localized with AQP1 to proximal tubules, and levels correlated with higher tubular injury scores and Cr.
Watany M.W. et al (2018)	miR-21, -210, -146	Serum miR-210 is decreased in AKI vs healthy controls, and in ATN vs HRS. miR-21 increased in AKI vs healthy controls, and in ATN vs HRS; miR-146a decreased in AKI vs healthy controls, and in ATN vs HRS miR-210, miR-146a are decreased while miR-21 is increased in patients with cirrhosis with ATN vs HRS. These miRNAs have potential to differentiate between HRS and ATN in cirrhosis patients with AKI.
Kölling M. et al (2018)	miR-126-5p	Blood miR-126-5p levels are decreased in severe AKI vs healthy controls, whereas circ-126 is increased in AKI vs (1) ICU no AKI, (2) ESKD HD, and (3) healthy controls. miR-126-5p levels are decreased in AKI, likely due to sponging from increased circ-miR-126 (which was associated with mortality in AKI).
Fan P-C. et al (2019)	36 miRNAs	Circulating miR-24, -23a, -145 decreased in post-MI AKI.
Newbury L.J. et al (2021)	Screen: 377 miRNAs Validation: 5 miRNAs	Urinary miR-21-5p, -126-3p, -141-3p increased in severe AKI vs controls. miR-192-5p, -204-5p decreased in severe AKI vs controls. Increased urinary miR-141-3p and decreased miR-192-5p were associated with AKI non-recovery at 90 d: potential prognostic markers for AKI recovery.
Mao H. et al (2021)	miR-129-5p	Serum miR-129-5p decreased in patients with AKI vs healthy controls. Lower miR-129-5p levels were associated with higher serum Cr.
Aomatsu A. et al (2021)	miR-5100	Serum miR-5100 is decreased in early AKI vs healthy controls.
Phulkerd T. et al (2022)	Screen: 18 (urine) and 11 (serum) miRNAs.	**Validation:** Only urinary miR-556-3p levels significantly increased in the renal recovery group (vs non-recovery): potential predictor of renal recovery. Serum miRNAs were not significantly different.
Liu J. et al (2022)	miR-2861, -34a	Serum miR-2861 was decreased and miR-34a was increased in the group of AKI patients with poor prognosis (vs good prognosis). Decreased serum miR-2861 and increased miR-34a were associated with poor prognosis in AKI.
Petejova N. et al (2022)	miR-15a-5p, miR-155-5p, miR-192-5p, miR-423-5p	4 miRNAs showed a similar pattern of levels over 7 d regardless of antimicrobial therapy miR-15a-5p was decreased by day 7 in subset of patients with AKI receiving Gentamicin: potential gentamicin nephrotoxicity biomarker
Xue Q. et al (2022)	miR-125b	Serum miR-125b decreased in heart failure with AKI (vs healthy controls) and associated with long-term mortality, hospital re-admission.
Xie Z. et al (2023)	miR-223-3p	Plasma miR-223-3p is decreased in AKI patients vs healthy controls

Supplementary Table continued: Quality Assessment and Risk of Bias (Newcastle Ottawa Scale)

Author	Study Title	Selection				Comparability		Outcome			Total
		S1	S2	S3	S4	C1	C2	O1	O2	O3	
Kidney Transplant											
Lorenzen J.M. et al (2011)	Urinary miR-210 as a Mediator of Acute T-Cell Mediated Rejection in Renal Allograft Recipients	1	1	1	1	1	1	1	1	0	8
Wilflingseder J. et al (2013)	miRNA Profiling Discriminates Types of Rejection and Injury in Human Renal Allografts	1	0	1	1	0	1	1	0	0	5

Sui W. et al (2013)	Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA	0	0	1	0	0	0	1	0	0	2
Lorenzen J.M. et al (2014)	MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury	1	1	1	1	0	1	1	0	0	5
Wilflingseder J. et al (2014)	Molecular Pathogenesis of Post-Transplant Acute Kidney Injury: Assessment of Whole-Genome mRNA and MiRNA Profiles	1	1	1	1	0	1	1	0	0	6
Liu X. et al (2015)	MicroRNA-10b downregulation mediates acute rejection of renal allografts by derepressing BCL2L11	0	0	0	1	0	0	1	0	0	2
Soltaninejad E. et al (2015)	Differential expression of microRNAs in renal transplant patients with acute T-cell mediated rejection	1	1	1	1	0	1	1	0	0	6
Tao J. et al (2015)	Serum MicroRNA-99a Helps Detect Acute Rejection in Renal Transplantation	1	1	1	1	0	1	1	0	0	6
McGuinness D. et al (2016)	Identification of Molecular Markers of Delayed Graft Function Based on the Regulation of Biological Ageing	1	1	1	1	0	1	1	1	0	7
Amrouche L. et al (2017)	MicroRNA-146a in Human and Experimental Ischemic AKI: CXCL8-Dependent Mechanism of Action	1	1	1	0	0	1	1	1	0	6
Domenico T.D. et al (2017)	Upregulation of microRNA 142-3p in the peripheral blood and urinary cells of kidney transplant recipients with post-transplant graft dysfunction	1	1	1	0	0	1	1	0	0	5
Millán O. et al (2017)	Urinary miR-155-5p and CXCL10 as prognostic and predictive biomarkers of rejection, graft outcome and treatment response in kidney transplantation	1	1	1	1	0	1	1	1	1	8
Jin P. et al (2017)	Essential role of microRNA-650 in the regulation of B-cell CLL/lymphoma 11B gene expression following transplantation: A novel mechanism behind the acute rejection of renal allografts	0	1	1	1	0	0	1	0	0	4
Gallon L. et al (2018)	Intragraft Molecular Pathways Associated with Tolerance Induction in Renal Transplantation	0	1	1	1	0	0	1	0	0	4
Cheng K. et al (2018)	Role of peripheral blood microRNA-181b in acute vascular rejection after renal transplantation	1	1	1	1	0	1	1	0	0	5
Gremmels H. et al (2019)	The Small RNA Repertoire of Small Extracellular Vesicles Isolated From Donor Kidney Preservation Fluid Provides a Source for Biomarker Discovery for Organ Quality and Posttransplantation Graft Function	1	1	1	1	0	1	0	1	0	6
Khalid U. et al (2019)	A urinary microRNA panel that is an early predictive biomarker of delayed graft function following kidney transplantation	1	1	1	1	0	1	1	1	0	7
Li F. et al (2019)	Differential MicroRNA Expressions in Human Peripheral Blood Mononuclear Cells Are Predictive of Renal Allograft Function	1	1	0	1	0	1	0	1	0	5
Milhoransa P. et al (2019)	microRNA 146a-5p expression in Kidney transplant recipients with delayed graft function	1	1	1	0	0	1	1	0	0	5
Roest H.P. et al (2019)	Cell-free MicroRNA miR-505-3p in Graft Preservation Fluid Is an Independent Predictor of Delayed Graft Function After Kidney Transplantation	1	1	0	1	0	1	1	1	1	7
Wang J. et al (2019)	Expression Profiling of Exosomal miRNAs Derived from the Peripheral Blood of Kidney Recipients with DGF Using High-Throughput Sequencing	0	1	1	0	0	0	1	1	0	4
Seo J-W. et al (2023)	Development and validation of urinary exosomal microRNA biomarkers for the diagnosis of acute rejection in kidney transplant recipients	1	1	1	1	0	1	1	0	0	6
Connor K.L. et al (2020)	Identifying cell-enriched miRNAs in kidney injury and repair	1	1	1	1	0	1	1	1	0	7
Alfaro R. et al (2021)	MicroRNA Expression Changes in Kidney Transplant: Diagnostic Efficacy of miR-150-5p as Potential Rejection Biomarker, Pilot Study	1	1	1	1	0	1	1	0	0	6
Rutman A.K. et al (2022)	Extracellular Vesicles From Kidney Allografts Express miR-218-5p and Alter Th17/Treg Ratios	1	1	1	1	0	1	1	1	0	7
Shi L. et al (2023)	MiR-20a-5p alleviates kidney ischemia/reperfusion injury by targeting ACSL4-dependent ferroptosis	1	0	1	0	0	0	1	0	0	3
Cardiac surgery											
Du J. et al (2019)	MicroRNA-21 and Risk of Severe Acute Kidney Injury and Poor Outcomes after Adult Cardiac Surgery	1	1	1	1	0	1	1	1	0	7
Aguado-Fraile E. et al (2015)	A Pilot Study Identifying a Set of microRNAs As Precise Diagnostic Biomarkers of Acute Kidney Injury	1	0	1	1	0	0	1	1	0	5
Gaede L. et al (2016)	Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery	1	1	1	1	1	1	1	1	0	8
Arvin P. et al (2017)	Early detection of cardiac surgery-associated acute kidney injury by microRNA-21	1	1	1	0	0	1	1	1	0	6
Zhang L. et al (2017)	Implications of dynamic changes in miR-192 expression in ischemic acute kidney injury	1	1	1	0	0	1	1	1	0	6

Zou Y-F. et al (2017)	Urinary MicroRNA-30c-5p and MicroRNA-192-5p as potential biomarkers of ischemia-reperfusion-induced kidney injury	1	1	1	1	0	1	1	1	1	8
Kang Z. et al (2018)	Remote ischemic preconditioning upregulates microRNA-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass	0	1	1	1	1	0	1	1	0	6
Sullo N. et al (2018)	An Observational Cohort Feasibility Study to Identify Microvesicle and Micro-RNA Biomarkers of Acute Kidney Injury Following Pediatric Cardiac Surgery	0	1	1	1	0	1	1	1	0	6
Wei Q. et al (2018)	MicroRNA-668 represses MTP18 to preserve mitochondrial dynamics in ischemic acute kidney injury	1 / 0	1 / 0	1 / 1	1 / 0	1 / 0	1 / 0	1 / 1	1 / 0	0 / 0	8 / 2
Gong Q. et al (2021)	Hsa-miR-494-3p attenuates gene HtrA3 transcription to increase inflammatory response in hypoxia/ reoxygenation HK2 Cells	0	1	1	1	0	1	1	1	0	6
Miller D. et al (2022)	Urinary extracellular vesicles and micro-RNA as markers of acute kidney injury after cardiac surgery	1	1	1	1	0	1	1	1	0	7
Chen Y. et al (2022)	Exosomal transfer of microRNA-590-3p between renal tubular epithelial cells after renal ischemia-reperfusion injury regulates autophagy by targeting TRAF6	1	1	1	0	0	0	1	0	0	4
Li X. et al (2023)	Inhibition of MiR-106b-5p mediated by exosomes mitigates acute kidney injury by modulating transmissible endoplasmic reticulum stress and M1 macrophage polarization	1	1	1	1	0	1	1	1	0	7
Sepsis											
Saikumar J. et al (2012)	Expression, Circulation, and Excretion Profile of MicroRNA-21, -155, and -18a Following Acute Kidney Injury	1	0	1	0	0	0	1	0	0	3
Ramachandran K. et al (2013)	Human miRNome Profiling Identifies MicroRNAs Differentially Present in the Urine after Kidney Injury	1	1	1	0	0	1	1	0	0	5
Ge Q-M. et al (2017)	Differentially expressed miRNAs in sepsis- induced acute kidney injury target oxidative stress and mitochondrial dysfunction pathways	0	1	1	0	0	1	1	1	0	5
Wang S. et al (2017)	MiR-107 induces TNF-a secretion in endothelial cells causing tubular cell injury in patients with septic acute kidney injury	0	1	1	0	0	1	1	0	0	4
Zhang J. et al (2018)	Urinary miR-26b as a potential biomarker for patients with sepsis-associated acute kidney injury: a Chinese population-based study	1	1	1	1	0	0	1	1	0	6
Bukauskas T. et al (2019)	Values of circulating molecular biomarkers (microRNAs) for the evaluation of renal failure during urgent abdominal sepsis anaesthesia	0	1	1	0	0	0	1	1	0	4
Lin Y. et al (2019)	Expression patterns and prognostic value of miR-210, miR-494, and miR-205 in middle-aged and old patients with sepsis-induced acute kidney injury	0	0	1	0	0	1	1	1	1	5
Sun J. et al (2020)	Correlation Between Single Nucleotide Polymorphisms at the 3'-UTR of the NFKB1 Gene and Acute Kidney Injury in Sepsis	1	1	1	1	0	0	1	0	0	5
Liu S. et al (2021)	Downregulation of miR-574-5p inhibits HK-2 cell viability and predicts the onset of acute kidney injury in sepsis patients	1	1	1	1	1	0	1	0	0	6
Shi L. et al (2021)	MiR-150-5p protects against septic acute kidney injury via repressing the MEKK3/JNK pathway	1	0	1	0	0	0	1	0	0	3
Zhang H. et al (2021)	Deregulated microRNA-22-3p in patients with sepsis-induced acute kidney injury serves as a new biomarker to predict disease occurrence and 28-day survival outcomes	1	1	1	1	0	1	1	1	1	8
Zheng C. et al (2021)	miR-34b-5p promotes renal cell inflammation and apoptosis by inhibiting aquaporin-2 in sepsis-induced acute kidney injury	1	0	1	0	0	1	1	0	0	4
Gong J. et al (2022)	Downregulation of circ-ZNF644 alleviates LPS-induced HK2 cell injury via miR-335-5p/HIPK1 axis	0	0	1	0	0	1	1	0	0	3
Ma W. et al (2022)	The Potential of miR-370-3p and miR-495-3p Serving as Biomarkers for Sepsis-Associated Acute Kidney Injury	0	1	1	0	0	1	1	1	0	5
Pan W. et al (2022)	Evaluation Value of Serum miR-4299 and miR-16-5p in Risk Stratification of Sepsis-Induced Acute Kidney Injury	1	1	1	0	0	1	1	1	0	6
Xun L. et al (2022)	The value of combining miR-10a-5p levels and PLR to evaluate the prognosis of sepsis patients with acute kidney injury	1	1	1	1	0	1	1	0	1	7
Xu L. et al (2022)	Circ_0114427 promotes LPS-induced septic acute kidney injury by modulating miR-495-3p/TRAF6 through the NF-kB pathway	1	1	1	1	0	0	1	0	0	5
Ye J. et al (2022)	miR-23a-3p inhibits sepsis-induced kidney epithelial cell injury by suppressing Wnt/b-catenin signaling by targeting wnt5a	0	0	1	0	0	1	1	0	0	3

Zhang F. et al (2023)	LncRNA PMS2L2 Is Downregulated in Sepsis-Induced Acute Kidney Injury and Inhibits LPS-Induced Apoptosis of Podocytes	0	1	1	1	0	1	1	0	0	5
Kuang F. et al (2023)	CIRC_0001818 TARGETS MIR-136-5P TO INCREASE LIPOPOLYSACCHARIDE-INDUCED HK2 CELL INJURIES BY ACTIVATING TXNIP/NLRP3 INFLAMMASOME PATHWAY	0	0	1	0	0	1	1	0	0	3
Zhang B. et al (2023)	CIRC_0114428 influences the progression of septic acute kidney injury via regulating miR-370-3p/TIMP2 axis	0	0	1	0	0	1	1	0	0	3
You T. et al (2023)	CIRC_0008882 stimulates PDE7A to suppress septic acute kidney injury progression by sponging miR-155-5p	0	0	1	0	0	0	1	0	0	2
Han R. et al (2023)	Urinary microRNAs in sepsis function as a novel prognostic marker	0	1	1	1	0	1	1	1	1	7
Liu W, et al (2023)	Exosomal microRNA-342-5p secreted from adipose-derived mesenchymal stem cells mitigates acute kidney injury in sepsis mice by inhibiting TLR9	0	0	1	0	0	1	1	0	0	3
Li P. et al (2023)	Circulating extracellular vesicles are associated with the clinical outcomes of sepsis	0	1	1	0	0	1	1	1	0	5
Nephrotoxic											
Vliegenthart A.D.B. et al (2015)	Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury	0	1	1	0	0	0	1	1	0	4
Gutiérrez-Escolano A. et al (2015)	Dysregulated microRNAs involved in contrast-induced acute kidney injury in rat and human	0	1	1	1	1	1	1	1	0	7
Pavkovic M. et al (2016)	Detection of Drug-Induced Acute Kidney Injury in Humans Using Urinary KIM-1, miR-21, -200c, and -423	0/0	1/1	1/1	0/0	0/0	1/1	1/1	0/1	0/1	4/6
Sun S. et al (2016)	Circulating MicroRNA-188, -30a, and -30e as Early Biomarkers for Contrast-Induced Acute Kidney Injury	0	1	1	1	0	1	1	1	0	6
Shihana F. et al (2020)	Circulating human microRNA biomarkers of oxalic acid-induced acute kidney injury	0	1	1	1	0	1	1	1	0	6
Shihana F. et al (2020)	Urinary versus serum microRNAs in human oxalic acid poisoning: Contrasting signals and performance	0	1	1	1	0	1	1	1	0	6
Zhang L. et al (2020)	Dysregulation of HULC promotes contrast-induced nephropathy (CIN) via regulating signaling pathway of miRNA-512 and prostaglandin E1 (PGE1)	0	1	1	0	0	1	1	0	0	4
Quintanilha J.C.F. et al (2021)	MiR-3168, miR-6125, and miR-4718 as potential predictors of cisplatin-induced nephrotoxicity in patients with head and neck cancer	0	1	1	1	0	1	1	1	1	7
Shihana F. et al (2021)	Urinary microRNAs as non-invasive biomarkers for toxic acute kidney injury in humans	0	1	1	1	0	0	1	0	0	4
Other											
Lorenzen J.M. et al (2011)	Circulating miR-210 Predicts Survival in Critically Ill Patients with Acute Kidney Injury	0	0	1	0	1	1	1	1	1	6
Lan Y-F. et al (2012)	MicroRNA-494 Reduces ATF3 Expression and Promotes AKI	1	1	1	0	0	0	1	0	0	4
Krebs C.F. et al (2013	MicroRNA-155 Drives TH17 Immune Response and Tissue Injury in Experimental Crescentic GN	0	0	1	0	0	0	1	0	0	2
Bruno N. et al (2016)	MicroRNAs relate to early worsening of renal function in patients with acute heart failure	1	1	1	1	1	1	1	1	0	8
Chen H-H. et al (2016)	Urinary miR-16 transactivated by C/EBPβ reduces kidney function after ischemia/reperfusion-induced injury	1	1	1	0	0	0	1	0	0	4
Ma Y. et al (2017)	Serum miRNA expression and correlation with clinical characteristics in acute kidney injury	0	0	1	0	0	1	1	0	0	3
Wang S. et al (2017)	Reduction in miRNA-125b-5p levels is associated with obstructive renal injury	0	0	1	0	0	0	1	0	0	2
Guo Y. et al (2018)	MicroRNA-709 Mediates Acute Tubular Injury through Effects on Mitochondrial Function	0	0	1	0	0	0	1	0	0	2
Watany M.W. et al (2018)	Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study	0	0	1	0	0	1	1	0	0	3
Kölling M. et al (2018)	The Circular RNA ciRs-126 Predicts Survival in Critically Ill Patients With Acute Kidney Injury	0	0	1	0	0	1	1	1	1	5
Fan P-C. et al (2019)	A circulating miRNA signature for early diagnosis of acute kidney injury following acute myocardial infarction	1	1	1	1	0	0	1	1	0	6
Newbury L.J. et al (2021)	miR-141 mediates recovery from acute kidney injury	1	0	1	1	0	0	1	1	1	6
Mao H. et al (2021)	MEG3 aggravates hypoxia/reoxygenation induced apoptosis of renal tubular epithelial cells via the miR-129-5p/ HMGB1 axis	0	0	1	0	0	0	1	0	0	2
Aomatsu A. et al (2021)	MicroRNA expression profiling in acute kidney injury	0	0	1	0	0	0	1	0	0	2

Phulkerd T. et al (2022)	Circulating and urinary microRNAs profile for predicting renal recovery from severe acute kidney injury	0	1	1	1	0	1	1	1	1	7
Liu J. et al (2022)	Correlation between the expression of serum miR-2861 and miR-34 and the prognosis of CVVH in patients with acute renal failure	0	1	1	0	0	1	1	1	1	7
Petejova N. et al (2022)	Expression and 7-day time course of circulating microRNAs in septic patients treated with nephrotoxic antibiotic agents	1	1	1	1	0	1	1	1	1	8
Xue Q. et al (2022)	lncRNA ROR and miR-125b Predict the Prognosis in Heart Failure Combined Acute Renal Failure	1	0	1	0	0	1	1	1	0	5
Xie Z. et al (2023)	Human bone marrow mesenchymal stem cell-derived extracellular vesicles reduce inflammation and pyroptosis in acute kidney injury via miR-223-3p/HDAC2/SNRK	0	0	0	0	0	1	1	0	0	2

List of Abbreviations:
AKI: Acute kidney injury; AKIN: Acute Kidney Injury Network; ANCA GN: Anti-neutrophil cytoplasmic antibody glomerulonephritis; APAP: acetaminophen; ATN: acute tubular necrosis; AR: Acute rejection; ABMR: Acute antibody-mediated rejection; PBMCs: peripheral blood mononuclear cells; Cr: creatinine; CS-AKI: cardiac surgery-associated AKI; CVVH: continuous veno-venous hemofiltration; DGF: delayed graft function; EC: endothelial cell; EV: extracellular vesicles; HC: healthy controls; HRS: hepatorenal syndrome; ICU: intensive care unit; KDIGO: Kidney Diseases Improving Global Outcomes; MOF: multi-organ failure; NR: not reported; OA: oxalic acid; PCI: percutaneous coronary intervention; RIPC: remote ischemic preconditioning; RRT: renal replacement therapy; TCMR: acute T-cell-mediated rejection

References

1. Lorenzen JM, Volkmann I, Fiedler J, et al. Urinary miR-210 as a Mediator of Acute T-Cell Mediated Rejection in Renal Allograft Recipients. *American Journal of Transplantation* 2011; 11: 2221-2227. DOI: https://doi.org/10.1111/j.1600-6143.2011.03679.x.
2. Wilflingseder J, Regele H, Perco P, et al. miRNA Profiling Discriminates Types of Rejection and Injury in Human Renal Allografts *Transplantation* 2013; 95: 835-841. DOI: https://doi.org/10.1097/TP.0b013e318280b385.
3. Sui W, Lin H, Peng W, et al. Molecular dysfunctions in acute rejection after renal transplantation revealed by integrated analysis of transcription factor, microRNA and long noncoding RNA. *Genomics* 2013; 102: 310-322. DOI: http://dx.doi.org/10.1016/j.ygeno.2013.05.002.
4. Lorenzen JM, Kaucsar T, Schauerte C, et al. MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury. *J Am Soc Nephrol* 2014; 25: 2717-2729. DOI: https://doi.org/10.1681/ASN.2013121329.
5. Wilflingseder J, Sunzenauer J, Toronyi E, et al. Molecular Pathogenesis of Post-Transplant Acute Kidney Injury: Assessment of Whole-Genome mRNA and MiRNA Profiles. *PLoS ONE* 2014; 9: e104164. DOI: https://doi.org/10.1371/journal.pone.0104164.
6. Liu X, Dong C, Jiang Z, et al. MicroRNA-10b downregulation mediates acute rejection of renal allografts by derepressing BCL2L11. *Experimental Cell Research* 2015; 333: 155-163. DOI: http://dx.doi.org/10.1016/j.yexcr.2015.01.018.
7. Soltaninejad E, Nicknam MH, Nafar M, et al. Differential expression of microRNAs in renal transplant patients with acute T-cell mediated rejection. *Transplant Immunology* 2015; 33: 1-6. DOI: http://dx.doi.org/10.1016/j.trim.2015.05.002.
8. Tao J, Yang X, Han Z, et al. Serum MicroRNA-99a Helps Detect Acute Rejection in Renal Transplantation. *Transplantion Proceedings* 2015; 47: 1683-1687. DOI: http://dx.doi.org/10.1016/j.transproceed.2015.04.094.
9. McGuinness D, Leierer J, Shapter O, et al. Identification of Molecular Markers of Delayed Graft Function Based on the Regulation of Biological Ageing. *PLoS ONE* 2016; 11: e0146378. DOI: https://doi.org/10.1371/journal.pone.0146378.
10. Amrouche L, Desbuissons G, Rabant M, et al. MicroRNA-146a in Human and Experimental Ischemic AKI: CXCL8-Dependent Mechanism of Action. *J Am Soc Nephrol* 2017; 28: 479-493. DOI: https://doi.org/10.1681/ASN.2016010045.
11. Domenico TD, Joelsons G, Montenegro RM, et al. Upregulation of microRNA 142-3p in the peripheral blood and urinary cells of kidney transplant recipients with post-transplant graft dysfunction. *Brazilian Journal of Medical and Biological Research* 2017; 50: e5533. DOI: http://dx.doi.org/10.1590/1414-431X20175533.
12. Millán O, Budde K, Sommerer C, et al. Urinary miR-155-5p and CXCL10 as prognostic and predictive biomarkers of rejection, graft outcome and treatment response in kidney transplantation. *Br J Clin Pharmacol* 2017; 83: 2636-2650. DOI: https://doi.org/10.1111/bcp.13399.

13. Jin P, Chen H, Xie J, et al. Essential role of microRNA-650 in the regulation of B-cell CLL/lymphoma 11B gene expression following transplantation: A novel mechanism behind the acute rejection of renal allografts. *International Journal of Molecular Medicine* 2017; 40: 1840-1850. DOI: https://doi.org/10.3892/ijmm.2017.3194.
14. Gallon L, Mathew JM, Bontha SV, et al. Intragraft Molecular Pathways Associated with Tolerance Induction in Renal Transplantation. *J Am Soc Nephrol* 2018; 29: 423-433. DOI: https://doi.org/10.1681/ASN.2017030348.
15. Cheng K, Wan J, Luo A, et al. Role of peripheral blood microRNA-181b in acute vascular rejection after renal transplantation. *Int J Clin Exp Med* 2018; 11: 728-734.
16. Gremmels H, Jong OGd, Toorop RJ, et al. The Small RNA Repertoire of Small Extracellular Vesicles Isolated From Donor Kidney Preservation Fluid Provides a Source for Biomarker Discovery for Organ Quality and Posttransplantation Graft Function. *Transplantation Direct* 2019; 5: e484. DOI: https://doi.org/10.1097/TXD.0000000000000929.
17. Khalid U, Newbury LJ, Simpson K, et al. A urinary microRNA panel that is an early predictive biomarker of delayed graft function following kidney transplantation. *Scientific Reports* 2019; 9: 3584. DOI: https://doi.org/10.1038/s41598-019-38642-3.
18. Li F, Qian W, Quan X, et al. Differential MicroRNA Expressions in Human Peripheral Blood Mononuclear Cells Are Predictive of Renal Allograft Function. *Transplantation Proceedings* 2019; 51: 715-721. DOI: https://doi.org/10.1016/j.transproceed.2019.01.051.
19. Milhoransa P, Montanari CC, Montenegro R, et al. Micro RNA 146a-5p expression in delayed graft function. *Braz J Nephrol* 2019; 41: 242-251. DOI: https://doi.org/10.1590/2175-8239-JBN-2018-0098.
20. Roest HP, Ooms LSS, Gillis AJM, et al. Cell-free MicroRNA miR-505-3p in Graft Preservation Fluid Is an Independent Predictor of Delayed Graft Function After Kidney Transplantation. *Transplantation* 2019; 103: 329-335. DOI: https://doi.org/10.1097/TP.0000000000002527.
21. Wang J, Li X, Wu X, et al. Expression Profiling of Exosomal miRNAs Derived from the Peripheral Blood of Kidney Recipients with DGF Using High-Throughput Sequencing. *BioMed Research International* 2019; 2019: 1-14. DOI: https://doi.org/10.1155/2019/1759697.
22. Seo J-W, Lee YH, Tae DH, et al. Development and validation of urinary exosomal microRNA biomarkers for the diagnosis of acute rejection in kidney transplant recipients. *Front Immunol* 2023; 14: 1190576. DOI: https://doi.org/10.3389/fimmu.2023.1190576.
23. Connor KL, Teenan O, Cairns C, et al. Identifying cell-enriched miRNAs in kidney injury and repair. *JCI Insight* 2020; 5: e140399. DOI: https://doi.org/10.1172/jci.insight.140399.
24. Alfaro R, Legaz I, Jimenez-Coll V, et al. MicroRNA Expression Changes in Kidney Transplant: Diagnostic Efficacy of miR-150-5p as Potential Rejection Biomarker, Pilot Study. *J Clin Med* 2021; 10: 2748. DOI: https://doi.org/10.3390/jcm10132748.

25. Rutman AK, Negi S, Saberi N, et al. Extracellular Vesicles From Kidney Allografts Express miR-218-5p and Alter Th17/Treg Ratios. *Front Immunol* 2022; 13: 784374. DOI: https://doi.org/10.3389/fimmu.2022.784374.
26. Shi L, Song Z, Li Y, et al. MiR-20a-5p alleviates kidney ischemia/reperfusion injury by targeting ACSL4-dependent ferroptosis. *American Journal of Transplantation* 2023; 23: 11-25. DOI: https://doi.org/10.1016/j.ajt.2022.09.003.
27. Du J, Cao X, Zou L, et al. MicroRNA-21 and Risk of Severe Acute Kidney Injury and Poor Outcomes after Adult Cardiac Surgery. *PLoS ONE* 2013; 8: e63390. DOI: https://doi.org/10.1371/journal.pone.0063390.
28. Aguado-Fraile E, Ramos E, Conde E, et al. A Pilot Study Identifying a Set of microRNAs As Precise Diagnostic Biomarkers of Acute Kidney Injury. *PLoS ONE* 2015; 10: e0127175. DOI: https://doi.org/10.1371/journal.pone.0127175.
29. Gaede L, Liebetrau C, Blumenstein J, et al. Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery. *Nephrol Dial Transplant* 2016; 31: 760-766. DOI: https://doi.org/10.1093/ndt/gfw007.
30. Arvin P, Samimagham HR, Montazerghaem H, et al. Early detection of cardiac surgery-associated acute kidney injury by microRNA-21. *Bratisl Med J* 2017; 118: 626-631. DOI: https://doi.org/10.4149/BLL_2017_120.
31. Zhang L, Xu Y, Xue S, et al. Implications of dynamic changes in miR-192 expression in ischemic acute kidney injury. *Int Urol Nephrol* 2017; 49: 541-550. DOI: https://doi.org/10.1007/s11255-016-1485-7.
32. Zou Y-F, Wen D, Zhao Q, et al. Urinary MicroRNA-30c-5p and MicroRNA-192-5p as potential biomarkers of ischemia–reperfusion-induced kidney injury. *Experimental Biology and Medicine* 2017; 242: 657-667. DOI: https://doi.org/10.1177/1535370216685005.
33. Kang Z, Li Z, Huang P, et al. Remote ischemic preconditioning upregulates microRNA-21 to protect the kidney in children with congenital heart disease undergoing cardiopulmonary bypass. *Pediatric Nephrology* 2018; 33: 911-919. DOI: https://doi.org/10.1007/s00467-017-3851-9.
34. Sullo N, Mariani S, JnTala M, et al. An Observational Cohort Feasibility Study to Identify Microvesicle and Micro-RNA Biomarkers of Acute Kidney Injury Following Pediatric Cardiac Surgery. *Pediatr Crit Care Med* 2018; 19: 816-830. DOI: https://doi.org/10.1097/PCC.0000000000001604.
35. Wei Q, Sun H, Song S, et al. MicroRNA-668 represses MTP18 to preserve mitochondrial dynamics in ischemic acute kidney injury. *J Clin Invest* 2018; 128: 5448-5464. DOI: https://doi.org/10.1172/JCI121859.
36. Gong Q, Shen Zm, Sheng Z, et al. Hsa-miR-494-3p attenuates gene HtrA3 transcription to increase inflammatory response in hypoxia/reoxygenation HK2 Cells. *Scientific Reports* 2021; 11: 1665. DOI: https://doi.org/10.1038/s41598-021-81113-x.
37. Miller D, Eagle-Hemming B, Sheikh S, et al. Urinary extracellular vesicles and micro-RNA as markers of acute kidney injury after cardiac surgery. *Scientific Reports* 2022; 12: 10402. DOI: https://doi.org/10.1038/s41598-022-13849-z.
38. Chen Y, Zhang C, Du Y, et al. Exosomal transfer of microRNA-590-3p between renal tubular epithelial cells after renal ischemia-reperfusion injury regulates autophagy by targeting TRAF6. *Chinese Medical Journal* 2022; 135: 2467-2477. DOI: https://doi.org/10.1097/CM9.0000000000002377.
39. Li X, Zhong Y, Yue R, et al. Inhibition of MiR-106b-5p mediated by exosomes mitigates acute kidney injury by modulating transmissible endoplasmic reticulum stress and M1 macrophage polarization. *J Cell Mol Med* 2023; 27: 2876-2889. DOI: https://doi.org/10.1111/jcmm.17848.
40. Saikumar J, Hoffmann D, Kim T-M, et al. Expression, Circulation, and Excretion Profile of MicroRNA-21, -155, and -18a Following Acute Kidney Injury. *Toxicological Sciences* 2012; 129: 256-267. DOI: https://doi.org/10.1093/toxsci/kfs210.
41. Ramachandran K, Saikumar J, Bijol V, et al. Human miRNome Profiling Identifies MicroRNAs Differentially Present in the Urine after Kidney Injury. *Clinical Chemistry* 2013; 59: 1742-1752. DOI: https://doi.org/10.1373/clinchem.2013.210245.
42. Ge Q-M, Huang C-M, Zhu X-Y, et al. Differentially expressed miRNAs in sepsis- induced acute kidney injury target oxidative stress and mitochondrial dysfunction pathways. *PLoS ONE* 2017; 12: e0173292. DOI: https://doi.org/10.1371/journal.pone.0173292.
43. Wang S, Zhang Z, Wang J, et al. MiR-107 induces TNF-a secretion in endothelial cells causing tubular cell injury in patients with septic acute kidney injury. *Biochem Biophys Res Commun* 2017; 483: 45-51. DOI: http://dx.doi.org/10.1016/j.bbrc.2017.01.013.
44. Zhang J, Wang C-J, Tang X-M, et al. Urinary miR-26b as a potential biomarker for patients with sepsis-associated acute kidney injury: a Chinese population-based study. *Eur Rev Med Pharmacol Sci* 2018; 22: 4606-4610. DOI: https://doi.org/10.26355/eurrev_201807_15518.
45. Bukauskas T, Kairytė M, Mickus R, et al. Values of circulating molecular biomarkers (microRNAs) for the evaluation of renal failure during urgent abdominal sepsis anaesthesia. *Acta Medica Lituanica* 2019; 26: 17-24. DOI: https://doi.org/10.6001/actamedica.v26i1.3951.
46. Lin Y, Ding Y, Song S, et al. Expression patterns and prognostic value of miR-210, miR-494, and miR-205 in middle-aged and old patients with sepsis-induced acute kidney injury. *Bosn J Basic Med Sci* 2019; 19: 249-256. DOI: http://dx.doi.org/10.17305/bjbms.2019.4131.
47. Sun J, Cai X, Shen J, et al. Correlation Between Single Nucleotide Polymorphisms at the 3'-UTR of the NFKB1 Gene and Acute Kidney Injury in Sepsis. *Genetic Testing and Molecular Markers* 2020; 24: 274-284. DOI: https://doi.org/10.1089/gtmb.2019.0222.
48. Liu S, Zhao L, Zhang L, et al. Downregulation of miR-574-5p inhibits HK-2 cell viability and predicts the onset of acute kidney injury in sepsis patients. *Renal Failure* 2021; 43: 942-948. DOI: https://doi.org/10.1080/0886022X.2021.1939051.

49. Shi L, Zhang Y, Xia Y, et al. MiR-150-5p protects against septic acute kidney injury via repressing the MEKK3/JNK pathway. *Cellular Signalling* 2021; 86: 110101. DOI: https://doi.org/10.1016/j.cellsig.2021.110101.
50. Zhang H, Che L, Wang Y, et al. Deregulated microRNA-22-3p in patients with sepsis-induced acute kidney injury serves as a new biomarker to predict disease occurrence and 28-day survival outcomes. *International Urology and Nephrology* 2021; 53: 2107-2116. DOI: https://doi.org/10.1007/s11255-021-02784-z.
51. Zheng C, Wu D, Shi S, et al. miR-34b-5p promotes renal cell inflammation and apoptosis by inhibiting aquaporin-2 in sepsis-induced acute kidney injury. *Renal Failure* 2021; 43: 291-301. DOI: https://doi.org/10.1080/0886022X.2021.1871922.
52. Gong J, Zhao S, Luo S, et al. Downregulation of circ-ZNF644 alleviates LPS-induced HK2 cell injury via miR-335-5p/HIPK1 axis. *Environmental Toxicology* 2022; 37: 2855-2864. DOI: https://doi.org/10.1002/tox.23642.
53. Ma W, Miao X, Xia F, et al. The Potential of miR-370-3p and miR-495-3p Serving as Biomarkers for Sepsis-Associated Acute Kidney Injury. *Computational and Mathematical Models in Medicine* 2022; 2022: 5. DOI: https://doi.org/10.1155/2022/2439509.
54. Pan W, Zhang J, Hu L, et al. Evaluation Value of Serum miR-4299 and miR-16-5p in Risk Stratification of Sepsis-Induced Acute Kidney Injury. *BioMed Research International* 2022; 2022: 8. DOI: https://doi.org/10.1155/2022/5165892.
55. Xun L, Li Z, Wang H, et al. The Value of Combining miR-10a-5p Levels and PLR to Evaluate the Prognosis of Sepsis Patients with Acute Kidney Injury. *Acta Medica Mediterranea* 2022; 38: 3303.
56. Xu L, Cao H, Xu P, et al. Circ_0114427 promotes LPS-induced septic acute kidney injury by modulating miR-495-3p/TRAF6 through the NF-kB pathway. *Autoimmunity* 2022; 55: 52-64. DOI: https://doi.org/10.1080/08916934.2021.1995861.
57. Ye J, Feng H and Peng Z. miR-23a-3p inhibits sepsis-induced kidney epithelial cell injury by suppressing Wnt/b-catenin signaling by targeting wnt5a. *Brazilian Journal of Medical and Biological Research* 2022; 55: e11571. DOI: https://doi.org/10.1590/1414-431X2021e11571.
58. Zhang F, Luo X, Wang Y, et al. LncRNA PMS2L2 Is Downregulated in Sepsis-Induced Acute Kidney Injury and Inhibits LPS-Induced Apoptosis of Podocytes. *Kidney and Blood Press Res* 2023; 48: 515-521. DOI: https://doi.org/10.1159/000528053.
59. Kuang F, Wang B, You T, et al. CIRC_0001818 Targets miR-136-5p to Increase Lipopolysaccharide-induced HK2 Cell Injuries By Activating TXNIP/NLRP3 Inflammasome Pathway. *Shock* 2023; 6: 110-120. DOI: https://doi.org/10.1097/SHK.0000000000002140.
60. Zhang B, You T, Liu Y, et al. Circ_0114428 Influences the Progression of Septic Acute Kidney Injury Via Regulating miR-370-3p/TIMP2 Axis. *Shock* 2023; 59: 505-513. DOI: https://doi.org/10.1097/SHK.0000000000002077.
61. You T and Kuang F. Circ_0008882 Stimulates PDE7A To Suppress Septic Acute Kidney Injury Progression by Sponging miR-155-5p. *Shock* 2023; 59: 657-665. DOI: https://doi.org/10.1097/SHK.0000000000002093.
62. Han R, Li W, Tian H, et al. Urinary microRNAs in sepsis function as a novel prognostic marker. *Experimental and Therapeutic Medicine* 2023; 26: 346. DOI: https://doi.org/10.3892/etm.2023.12045.
63. Liu W, Hu C, Zhang B, et al. Exosomal microRNA-342-5p secreted from adipose-derived mesenchymal stem cells mitigates acute kidney injury in sepsis mice by inhibiting TLR9. *Biological Procedures Online* 2023; 25: 10. DOI: https://doi.org/10.1186/s12575-023-00198-y.
64. Li P, Wu Y, Goodwin AJ, et al. Circulating extracellular vesicles are associated with the clinical outcomes of sepsis. *Front Immunol* 2023; 14: 1150564. DOI: https://doi.org/10.3389/fimmu.2023.1150564.
65. Vliegenthart ADB, Shaffer JM, Clarke JI, et al. Comprehensive microRNA profiling in acetaminophen toxicity identifies novel circulating biomarkers for human liver and kidney injury. *Scientific Reports* 2015; 5: 15501. DOI: https://doi.org/10.1038/srep15501.
66. Gutiérrez-Escolano A, Santacruz-Vázquez E and Gómez-Pérez F. Dysregulated microRNAs involved in contrast-induced acute kidney injury in rat and human. *Renal Failure* 2015; 37: 1498-1506. DOI: https://doi.org/10.3109/0886022X.2015.1077322.
67. Pavkovic M, Robinson-Cohen C, Chua AS, et al. Detection of Drug-Induced Acute Kidney Injury in Humans Using Urinary KIM-1, miR-21, -200c, and -423. *Toxicol Sci* 2016; 152: 205-213. DOI: https://doi.org/10.1093/toxsci/kfw077.
68. Sun S-q, Zhang T, Ding D, et al. Circulating MicroRNA-188, -30a, and -30e as Early Biomarkers for Contrast-Induced Acute Kidney Injury. *J Am Heart Assoc* 2016; 5: e004138. DOI: https://doi.org/10.1161/JAHA.116.004138.
69. Shihana F, Joglekar MV, Raubenheimer J, et al. Circulating human microRNA biomarkers of oxalic acid-induced acute kidney injury. *Archives of Toxicology* 2020; 94: 1725-1737. DOI: https://doi.org/10.1007/s00204-020-02679-5.
70. Shihana F, Mohamed F, Joglekar MV, et al. Urinary versus serum microRNAs in human oxalic acid poisoning: Contrasting signals and performance. *Toxicology Letters* 2020; 334: 21-26. DOI: https://doi.org/10.1016/j.toxlet.2020.09.003.
71. Zhang L, Li P, Zhang Bl, et al. Dysregulation of HULC promotes contrast-induced nephropathy (CIN) via regulating signaling pathway of miRNA-512 and prostaglandin E1 (PGE1). *Scientific Reports* 2020; 10: 11691. DOI: https://doi.org/10.1038/s41598-020-68634-7.
72. Quintanilha JCF, Cursino MA, Borges JB, et al. MiR-3168, miR-6125, and miR-4718 as potential predictors of cisplatin-induced nephrotoxicity in patients with head and neck cancer. *BMC Cancer* 2021; 21: 575. DOI: https://doi.org/10.1186/s12885-021-08317-2.
73. Shihana F, Wong WKM, Joglekar MV, et al. Urinary microRNAs as non-invasive biomarkers for toxic acute kidney injury in humans. *Scientific Reports* 2021; 11: 9165. DOI: https://doi.org/10.1038/s41598-021-87918-0.
74. Lorenzen JM, Kielstein JT, Hafer C, et al. Circulating miR-210 Predicts Survival in Critically Ill Patients with Acute Kidney Injury. *Clin J Am Soc Nephrol* 2011; 6: 1540-1546. DOI: https://doi.org/10.2215/CJN.00430111.

75. Lan Y-F, Chen H-H, Lai P-F, et al. MicroRNA-494 Reduces ATF3 Expression and Promotes AKI. *J Am Soc Nephrol* 2012; 23: 2012-2023. DOI: https://doi.org/10.1681/ASN.2012050438.
76. Krebs CF, Kapffer S, Paust H-J, et al. MicroRNA-155 Drives TH17 Immune Response and Tissue Injury in Experimental Crescentic GN. *J Am Soc Nephrol* 2013; 24: 1955-1965. DOI: https://doi.org/10.1681/ASN.2013020130.
77. Bruno N, Maaten JMt, Ovchinnikova ES, et al. MicroRNAs relate to early worsening of renal function in patients with acute heart failure. *International Journal of Cardiology* 2016; 203: 564-569. DOI: http://dx.doi.org/10.1016/j.ijcard.2015.10.217.
78. Chen H-H, Lan Y-F, Li H-F, et al. Urinary miR-16 transactivated by C/EBPβ reduces kidney function after ischemia/reperfusion–induced injury. *Scientific Reports* 2016; 6: 27945. DOI: https://doi.org/10.1038/srep27945.
79. Ma Y, Fu J, Qian L, et al. Serum miRNA expression and correlation with clinical characteristics in acute kidney injury. *Int J Clin Exp Pathol* 2017; 10: 8721-8726.
80. Wang S, Wu L, Du L, et al. Reduction in miRNA-125b-5p levels is associated with obstructive renal injury. *Biomedical Reports* 2017; 6: 449-454. DOI: https://doi.org/10.3892/br.2017.875.
81. Guo Y, Ni J, Chen S, et al. MicroRNA-709 Mediates Acute Tubular Injury through Effects on Mitochondrial Function. *J Am Soc Nephrol* 2018; 29: 449-461. DOI: https://doi.org/10.1681/ASN.2017040381.
82. Watany MM, Hagag RY and Okda HI. Circulating miR-21, miR-210 and miR-146a as potential biomarkers to differentiate acute tubular necrosis from hepatorenal syndrome in patients with liver cirrhosis: a pilot study. *Clin Chem Lab Med* 2018; 56: 739-747. DOI: https://doi.org/10.1515/cclm-2017-0483.
83. Kölling M, Seeger H, Haddad G, et al. The Circular RNA ciRs-126 Predicts Survival in Critically Ill Patients With Acute Kidney Injury. *Kidney Int Rep* 2018; 3: 1144-1152. DOI: https://doi.org/10.1016/j.ekir.2018.05.012.
84. Fan PC, Chen CC, Peng CC, et al. A circulating miRNA signature for early diagnosis of acute kidney injury following acute myocardial infarction. *J Transl Med* 2019; 17: 139. DOI: https://doi.org/10.1186/s12967-019-1890-7.
85. Newbury LJ, Simpson K, Khalid U, et al. miR-141 mediates recovery from acute kidney injury. *Scientific Reports* 2021; 11: 16499. DOI: https://doi.org/10.1038/s41598-021-94984-x.
86. Mao H, Huang Q and Liu Y. MEG3 aggravates hypoxia/reoxygenation induced apoptosis of renal tubular epithelial cells via the miR-129-5p/HMGB1 axis. *J Biochem Mol Toxicol* 2021; 35: e22649. DOI: https://doi.org/10.1002/jbt.22649.
87. Aomatsu A, Kaneko S, Yanai K, et al. MicroRNA expression profiling in acute kidney injury. *Transl Res* 2022; 244: 1-31. DOI: https://doi.org/10.1016/j.trsl.2021.11.010.
88. Phulkerd T, Lertussavavivat T, Limothai U, et al. Circulating and urinary microRNAs profile for predicting renal recovery from severe acute kidney injury. *Journal of Intensive Care* 2022; 10: 45. DOI: https://doi.org/10.1186/s40560-022-00637-0.
89. Liu J, Zhang L, Guo H, et al. Correlation Between the Expression of Serum miR-2861 and miR-34a and the Prognosis of CVVH in Patients with Acute Renal Failure. *Acta Medica Mediterranea* 2022; 38: 903. DOI: https://doi.org/10.19193/0393-6384_2022_2_138.
90. Petejova N, Martinek A, Zadrazil J, et al. Expression and 7-day time course of circulating microRNAs in septic patients treated with nephrotoxic antibiotic agents. *BMC Nephrology* 2022; 23: 111. DOI: https://doi.org/10.1186/s12882-022-02726-6.
91. Xue Q, Yang L, Wang J, et al. lncRNA ROR and miR-125b Predict the Prognosis in Heart Failure Combined Acute Renal Failure. *Disease Markers* 2022; 2022: 1-6. DOI: https://doi.org/10.1155/2022/6853939.
92. Xie Z, Tang J, Chen Z, et al. Human bone marrow mesenchymal stem cell-derived extracellular vesicles reduce inflammation and pyroptosis in acute kidney injury via miR-223-3p/HDAC2/SNRK. *Inflammation Research* 2023; 72: 553-576. DOI: https://doi.org/10.1007/s00011-022-01653-4.

Appendix D

Manuscript IV

Connection to thesis: This manuscript is a detailed review of the current literature pertaining to the biogenesis, regulation, and biological functions of miR-486-5p in non-malignant diseases. This narrative review highlights the complexity of miRNA functions by reporting on differences in biological effects between experimental models and validated 3' UTR targets.

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REVIEW

miRNA-486-5p: signaling targets and role in non-malignant disease

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Abstract

MicroRNAs (miRNAs) are short non-coding RNAs, highly conserved between species, that are powerful regulators of gene expression. Aberrant expression of miRNAs alters biological processes and pathways linked to human disease. miR-486-5p is a muscle-enriched miRNA localized to the cytoplasm and nucleus, and is highly abundant in human plasma and enriched in small extracellular vesicles. Studies of malignant and non-malignant diseases, including kidney diseases, have found correlations with circulating miR-486-5p levels, supporting its role as a potential biomarker. Pre-clinical studies of non-malignant diseases have identified miR-486-5p targets that regulate major signaling pathways involved in cellular proliferation, migration, angiogenesis, and apoptosis. Validated miR-486-5p targets include *phosphatase and tensin homolog* (*PTEN*) and *FoXO1*, whose suppression activates phosphatidyl inositol-3-kinase (PI3K)/Akt signaling. Targeting of *Smad1/2/4* and *IGF-1* by miR-486-5p inhibits transforming growth factor (TGF)-β and insulin-like growth factor-1 (IGF-1) signaling, respectively. Other miR-486-5p targets include *matrix metalloproteinase-19* (*MMP-19*), *Sp5, histone acetyltransferase 1 (HAT1),* and *nuclear factor of activated T cells-5 (NFAT5)*. In this review, we examine the biogenesis, regulation, validated gene targets and biological effects of miR-486-5p in non-malignant diseases.

Keywords microRNA · Extracellular vesicles · Apoptosis · Angiogenesis · Fibrosis · Ischemia reperfusion injury

Introduction

MicroRNAs (miRNAs) are short non-coding RNAs, approximately 22–25 nucleotides long that were first identified in *Caenorhabditis elegans* in 1993 [1]. The human genome alone contains over 1500 miRNAs, although the functional significance of many remains uncertain [2]. miRNAs are highly conserved across species and potently regulate gene expression, as over 60% of human protein-coding genes are conserved targets of miRNAs [3]. The biogenesis of miRNAs is a multistep process that can be divided into canonical and non-canonical pathways, which have been reviewed elsewhere [4]. miRNAs function primarily by post-transcriptional gene silencing via the RNA-induced silencing complex (RISC) pathway in the cytoplasm, wherein the miRNA associates with an Argonaute protein in the RISC, and binds to the 3′ untranslated region (UTR) of its target mRNA [5]. Argonaute proteins associate with a GW182 protein that interacts with poly(A)-binding protein and a cytoplasmic deadenylase complex. Consequently, the miRNA–RISC complex mediates mRNA deadenylation and degradation, although inhibition of mRNA translation may also occur via an unclear mechanism [5]. miRNAs may also bind other regions of target genes, including the 5′UTR and coding sequences, and recent studies have identified a nuclear role as activators or silencers of gene transcription [6, 7]. miRNAs have garnered attention for their potential roles in disease diagnosis, pathogenesis, and therapy, as their expression is dysregulated in both malignant and non-malignant diseases [8–10].

miR-486-5p is a muscle-enriched miRNA [11] that circulates at relatively high levels in plasma [12], is enriched within small extracellular vesicles [13], and has been linked

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to signaling pathways involved in several cancers [9] as well as non-malignant conditions, such as skeletal muscle disorders [14, 15], ischemia–reperfusion injury (IRI) [16–18], and organ fibrosis [19–21]. Here, we review miR-486-5p biogenesis and regulation of expression, discuss circulating miR-486-5p as a potential biomarker in non-malignant diseases, and the role of exosomal transfer in experimental disease models. We also report major targets of miR-486-5p and affected biological pathways in non-malignant diseases, and highlight the controversies regarding downstream effects on cell and organ function. For information on the role of miR-486-5p in malignant diseases, the reader is referred to a recent comprehensive review [9].

Biogenesis of miR-486-5p

miR-486-5p is highly conserved in mammals, and was first identified as a muscle-enriched miRNA whose expression is downregulated in Duchenne's muscular dystrophy [22]. miR-486-5p is highly expressed in myoblasts during myogenesis, and in skeletal muscle [14], and is found at high levels in cardiac muscle, with moderate expression in lung, brain, liver, and bladder [11, 23, 24]. miR-486-5p is also a dominant erythroid miRNA, and its expression is upregulated in erythropoiesis [25, 26].

miR-486 is first transcribed from an intron within the *ANK1* locus, located on chromosome 8p11.21 (Fig. 1). The *ANK1* locus encodes the Ankyrin 1 gene, which is primarily expressed in erythroid cells. The mouse genome contains miR-486a-5p and miR-486b-5p at this locus, both of which consist of the same mature sequence but are transcribed in opposite directions. Similarly, the human genome encodes miR-486-1 and miR-486-2, also consisting of the same mature sequence but transcribed in opposite directions. The biogenesis and expression of miR-486-5p discussed here refers to miR-486a-5p/miR-486-1, as these are both transcribed in the same direction as *Ank1*. Two miRNAs, miR-486-5p and miR-486-3p [9], are generated from opposite ends of the pre-miRNA hairpin [4]. miRNA strand selection is determined by intrinsic features of the miRNA duplex including the identity and thermodynamic stability of the 5′ nucleotides of each strand, but tissue-specific differences in strand selection (miRNA 5p/3p ratios) also support other regulatory mechanisms involving miRNA processing, duplex remodeling, and degradation [27].

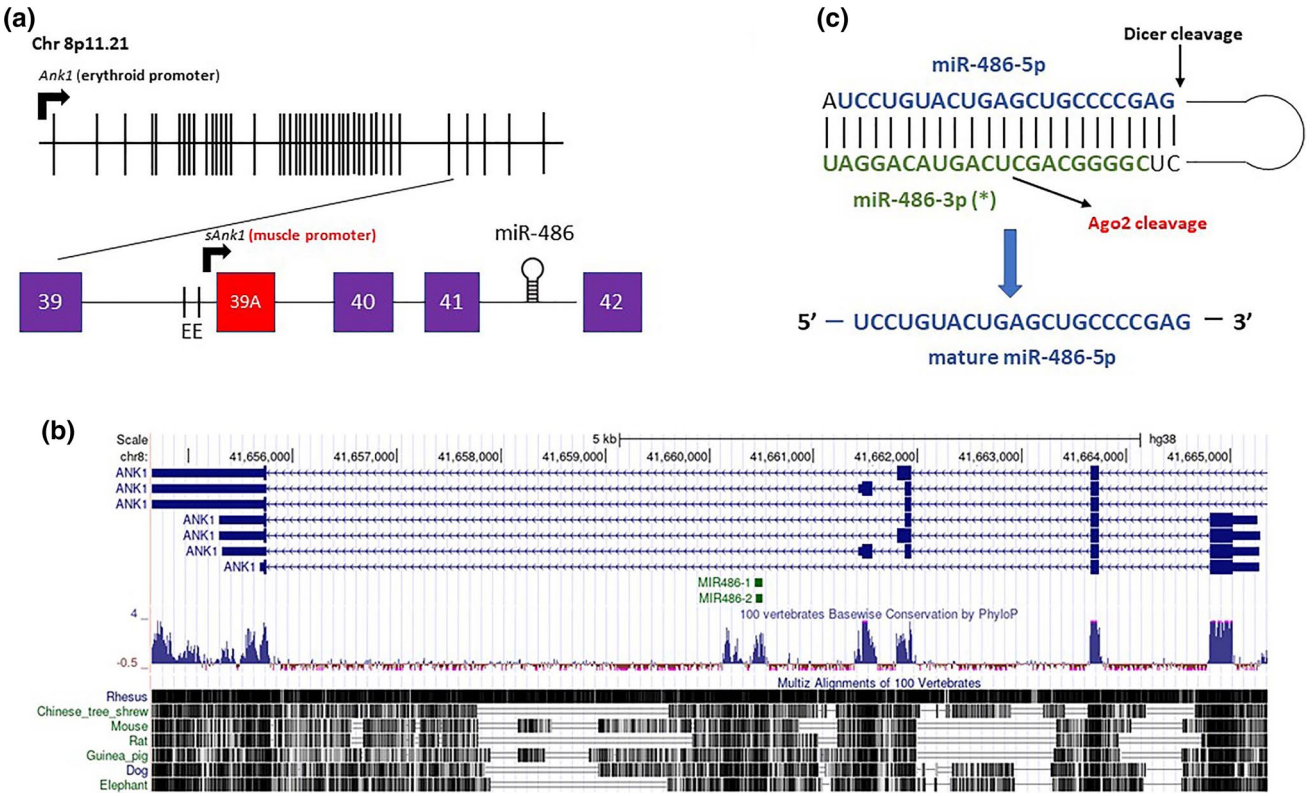

Fig. 1 miR-486-5p biogenesis **a** miR-486-5p is transcribed from the last intron of the *Ank1* gene, located downstream from alternative exon (39A) of the muscle-specific *sAnk1* isoform **b** genomic organization of miR-486-5p and *Ank1* is conserved among mammals **c** pre-miR-486 requires Dicer cleavage, and also requires Argonaute-2 catalytic activity to remove the 3p star (*) strand, generating functional mature miR-486-5p (adapted from Refs. [11, 25])

miR-486 processing exhibits Argonaute-2-Slicer dependence, whereby Argonaute-2 catalytic activity is required to generate functional mature miRNA via slicing to remove the star (3p) strand; catalytically inactive Argonaute-2 results in miR-486-3p accumulation, miR-486 duplex arrest, and blunted miR-486-5p activity [25].

miR-486-3p expression and function

The function and expression of the star strand, miR-486-3p, has been less well studied within different tissues. Within the hematopoietic system, miR-486-5p and miR-486-3p are highly expressed in erythroid cells [26]. However, their expression patterns differ in that miR-486-5p increases throughout the differentiation process, whereas miR-486-3p peaks earlier and then declines slightly [26]. Structurally, the pre-miR-486 miRNA/star duplex is perfectly paired and conserved across mammalian species, possibly reflecting conserved activity of miR-486-3p, although functional sensor assays used to validate miRNA activity showed that unlike mature miRNA-486-5p, miR-486-3p failed to repress its sensor [28]. Furthermore, immunoprecipitation of Argonaute proteins revealed that miR-486-3p associated with Argonautes 1 and 3, but functional sensor assays in wild type, Argonaute-2 knockout and Argonaute-2 catalytically inactive mouse embryonic fibroblasts showed that despite its accumulation, miR-486-3p did not exhibit gene regulatory activity [25].

Nonetheless, miR-486-3p target genes have been identified in malignant and non-malignant diseases [9], suggesting a role in gene regulation. In erythroid cells, miR-486-3p targets and downregulates the zinc finger protein *BCL11A*, associated with increased expression of the γ-globin gene and fetal hemoglobin synthesis [26]. miR-486-3p targets and downregulates the deacetylase *Sirtuin 2 (SIRT2),* resulting in decreased α-synuclein-induced toxicity in vitro, and suggesting that miR-486-3p may protect against Parkinson's disease progression [29]. A recent systematic review of 34 studies found that miR-486-3p is among the most frequently altered miRNAs in patients with autism spectrum disorder (ASD) [30]. miR-486-3p was reported to be upregulated in the serum of patients with ASD, and targets and downregulates *AT-rich interaction domain 1B* (*ARID1B*) [31], a gene mutated in ASD that also confers an ASD-like phenotype in *Arid1b* haploinsufficient mice [32]. Although the processing of pre-miR-486 and distribution of miR-486-5p and miR-486-3p within different tissues is not fully understood, these data support miR-486-3p as a functional miRNA with a potential role in human diseases. The remainder of this review will focus on miR-486-5p. For further information on miR-486-3p in human diseases, the reader is referred to a recent review [9].

Regulation of miR-486-5p expression and cellular localization

Although Ank1 mRNA is primarily expressed in erythroid cells, an isoform (*sAnk1*/*Ank1.5*) containing a muscle-specific first exon and the last three exons of the *Ank1* erythroid gene is produced from an alternate promoter [33] (Fig. 1a, b). The tissue distribution of *sAnk1* mirrors that of miR-486-5p, enriched in heart and skeletal muscle [11]. Furthermore, there is increased expression of both *sAnk1* mRNA and miR-486-5p during myoblast differentiation [24]. In rat neonatal cardiomyocytes, Small et al.showed that myocardin-related transcription factor A (MRTF-A) induces the expression of both miR-486-5p and *sAnk1*. The *sAnk1* promoter also contains two conserved E boxes for MyoD transcriptional activity, and MyoD activity directly regulates *sAnk1* and miR-486-5p expression [24]. These data support the co-regulation of miR-486-5p and *sAnk1* and suggest that miR-486-5p is produced from the processing of *sAnk1* intronic RNA [11].

The regulation of miR-486-5p expression has derived largely from studies pertaining to malignancies, where its dysregulation is paradoxically associated with either tumor suppression or oncogenesis [9]. Epigenetic modifications of the *ANK1* promoter [34] and reduced p53 expression or activity [35] are associated with downregulation of miR-486-5p. The miR-486-5p promoter also contains a binding site for hypoxia-inducible-factor-1α (HIF-1α). HIF-1α overexpression activates the miR-486-5p promoter in HeLa cells, and induces expression of miR-486-5p in prostate cancer cell lines, suggesting that hypoxia stimulates its transcription [36]. The effect of hypoxia on miR-486-5p expression is not limited to malignant cells as hypoxic rat cardiomyocytes also exhibit upregulated miR-486-5p [37]. miR-486-5p expression is also regulated at the post-transcriptional level. For example, long non-coding RNAs (lncRNAs) can directly bind to miR-486-5p, inhibiting its expression and activity in experimental models [21, 38, 39].

Traditionally, miRNA biogenesis is a multi-step process involving nuclear and cytosolic components, with the classical function of mature miRNA being post-transcriptional gene silencing in the cytoplasm via miRISC and the 3′ UTR of the target gene [7]. Studies with high-throughput profiling techniques have identified mature miRNAs that are enriched in cell nuclei [40–42], and RISC components including Argonaute-2 and trinucleotide repeat-containing gene 6A protein (TNRC6) have been identified in the nucleus and thus likely play a role in miRNA nuclear transport and activity [43, 44]. Nuclear miRNAs can (1) mediate post-transcriptional gene silencing to downregulate the expression of long non-coding RNAs, (2) interact

with pri-miRNAs to inhibit their maturation; (3) activate or suppress gene transcription by interacting with gene promoters in association with Argonaute-2; and (4) induce gene expression by enhancer activation, which may also require Argonaute-2 [7].

The cellular localization of mature miR-486-5p is indeed not limited to the cytoplasm. Deep sequencing of the nuclear and cytoplasmic pools of small RNAs from a human nasopharyngeal carcinoma cell line identified 339 nuclear and 324 cytoplasmic miRNAs, the majority of which overlap, including miR-486-5p [42]. The large degree of overlap suggests that miRNAs are imported into the nucleus. Viñas et al. administered intravenous lipid-encapsulated miR-486-5p mimic to mice with bilateral kidney IRI and by *in-situ* hybridization revealed that miR-486-5p localized to both cytoplasm and nuclei of cortical tubular cells [45]. Thus, it is conceivable that miR-486-5p may exert biological effects in the nucleus via one or more of the identified nuclear roles of miRNAs [7], such as post-transcriptional gene silencing of other non-coding RNAs or regulation of protein-coding gene expression through direct interaction with promoter or enhancer sites.

Circulating miR-486-5p and extracellular vesicles

Circulating miRNAs are stable in plasma and resistant to nuclease digestion [46]. An estimated 90% of circulating miRNAs are present in a non-membrane bound form, associated with the Argonaute-2 ribonucleoprotein complex, whereas a smaller proportion are found within small extracellular vesicles, such as exosomes (50–150 nm diameter) [46, 47]. miR-486-5p is abundant in human plasma [12, 48], where it circulates freely or within exosomes [49]. However, as an erythropoietic miRNA, miR-486-5p is also released by red blood cell hemolysis, thereby increasing miR-486-5p levels in hemolyzed samples. This feature can thereby complicate the interpretation of circulating miR-486-5p levels in biomarker studies [50, 51].

miR-486-5p is differentially expressed in human plasma or serum in a wide range of conditions including, but not limited to solid tumor malignancies [9], sepsis [52], primary muscle diseases (e.g., Duchenne muscular dystrophy [22]), cardiorespiratory diseases (e.g., chronic heart failure [19], cystic fibrosis [53]), diabetic kidney disease [54], osteoarthritis [55], neurological conditions (e.g., vascular dementia [56], Huntington's disease [57], ASD [58]), and various endocrine disorders (e.g., metabolic syndrome [59], childhood obesity [60], type 2 diabetes mellitus [61], polycystic ovary syndrome [62], and recurrent miscarriage [63]). Accordingly, circulating levels may have utility for diagnosis or prognosis in a variety of diseases (outlined in Supplementary Table 1). Although these studies provide data on clinical associations, further research is required to determine the diagnostic and prognostic potential of circulating miR-486-5p. Of the studies outlined in Supplementary Table 1, the diagnostic accuracy of circulating miR-486-5p was evaluated only in type 2 diabetes [54], vascular dementia [56], and outcomes of embryo transfer with in vitro fertilization (IVF) [63]. Regmi et al.evaluated serum miRNAs in patients with diabetic kidney disease, revealing that miR-486-5p was downregulated, with a receiver operating characteristic area under the curve (ROC-AUC) of 0.853 [54]. In patients with vascular dementia due to cerebral small vessel disease, plasma miR-486-5p had a sensitivity of 75%, specificity of 83%, and ROC–AUC of 90% as a diagnostic marker [56]. In a study of embryo transfer outcomes in IVF, a plasma miRNA signature that included miR-486-5p had a sensitivity of 100% and specificity of 83% for recurrent miscarriage, but sensitivity of only 68.1% and specificity of 54% for successful outcome [63]. Given the variety of non-malignant diseases with dysregulated circulating miR-486-5p levels, its potential as a non-invasive diagnostic and prognostic biomarker warrants further study.

miR-486-5p is enriched within exosomes, which are important mediators of intercellular communication [64] and have shown protective effects in experimental models of organ injury [16, 17, 65, 66]. Indeed, miR-486-5p is among the most abundant miRNAs in both human adipose and bone marrow mesenchymal stem cell (BMSC)-derived exosomes, and over-represented compared to its expression within the parent cells [13]. Furthermore, human cord blood endothelial colony-forming cell (ECFC)-derived exosomes are enriched in miR-486-5p, with levels that are markedly higher compared to ECFC-derived microparticles (100–1000 nm diameter) [16]. Mechanisms involved in selective cargo loading of miRNA and other RNA species into extracellular vesicles remain unclear. However, factors that influence the miRNA profile within extracellular vesicles include the pathophysiological state of the source cell, RNA properties (such as small size, affinity for membrane lipids, and cytoplasmic localization), RNA sequence motifs, post-transcriptional modifications, and associations with RNA binding proteins [64].

Targets of miR-486-5p

Based on 3′UTR sequence homology (http://mirdb.org/cgi-bin/search.cgi), more than 300 predicted miR-486-5p targets have been identified. However, not all predicted targets have been validated by demonstrating a direct interaction between miR-486-5p and the gene transcript 3′ UTR, typically done

by luciferase reporter assay. Below, we review key validated targets of miR-486-5p in non-malignant diseases.

Skeletal muscle *PTEN* and *FoXO1*

Phosphatase and tensin homolog (PTEN) is a tumor suppressor protein whose expression is tightly regulated at multiple levels [67], and represents the original validated target of miR-486-5p [11]. As a lipid phosphatase, *PTEN* negatively regulates the phosphatidyl-inositol-3-kinase (PI3K)/Protein kinase B (Akt) signaling pathway by de-dephosphorylating the intracellular messenger and PI3K product phosphatidyl-inositol-triphosphate (PIP3) to PIP2 [68]. Consequently, *PTEN* inhibits several cellular functions including proliferation, migration, survival, and angiogenesis [68, 69].

FoxO1 belongs to the family of forkhead transcription factors (FoxOs), which are involved in insulin and insulin-like growth factor-1 (IGF-1) signaling, thus affecting cell growth, proliferation, differentiation, apoptosis, oxidative stress, ageing, and metabolism [70]. *FoxO1* is a negative regulator of Akt signaling, and its activity is also inhibited via Akt-mediated phosphorylation [71].

Small et al. [11] first identified *PTEN* and *FoxO1* as miR-486-5p targets, by luciferase reporter assay. In mice, an inverse correlation was shown between the expression of miR-486-5p and *PTEN* in postnatal cardiac growth. Furthermore, miR-486-5p overexpression in cardiomyocytes downregulated endogenous *PTEN* and *Fox01* protein expression and increased p-Akt (at Ser473). These data implicate miR-486-5p as a promoter of cardiac muscle growth by targeting and downregulating *PTEN* and *Fox01*, thereby activating PI3K/Akt signaling [11].

PTEN and PI3K/Akt signaling has also been implicated in primary muscle disorders, including Duchenne muscular dystrophy (DMD), caused by mutations in the *dystrophin* gene [72]. miR-486-5p expression is downregulated in skeletal muscle of patients with DMD compared to healthy controls [22] and in dystrophin-deficient muscles in mice [23]. Dystrophin-deficient animals have increased *PTEN* expression and decreased Akt phosphorylation within dystrophin-deficient muscle tissue [73], and modulation of Akt signaling improves muscle function in these models [74]. At the cellular level, miR-486-5p inhibition is detrimental to myoblasts, resulting in reduced myotube formation, failed migration, increased caspase-3/7 levels and enhanced apoptosis [14]. In human myotubes that overexpress *DOCK3* (a validated target of miR-486-5p in skeletal muscle), *PTEN* expression is increased, associated with reduced Akt phosphorylation, higher expression of activated caspases-3/7 and increased myotube apoptosis [23]. These results provide strong evidence that miR-486-5p modulates these pathways via targeting *PTEN* and *DOCK3* (Fig. 2).

The dynamic expression of miR-486-5p in normal muscle regeneration is critical for its regenerative effect [14]. A comparison of wild type and *dystrophin*-deficient mice subjected to cardiotoxin-induced tibialis anterior muscle injury revealed that miR-486-5p expression is transiently upregulated after injury, with normal muscle regeneration, while *dystrophin*-deficient mice have reduced miR-486-5p expression following injury and impaired muscle regeneration. The authors also evaluated muscle regeneration in transgenic mice that overexpress miR-486-5p exclusively in heart and skeletal muscle. Transgenic mice with muscle-specific miR-486-5p overexpression are viable, with no significant phenotypic differences from wild-type controls at 6 months of age, other than slight weight gain. However, when subjected to cardiotoxin-induced skeletal muscle injury, the transgenic mice displayed slowed muscle regeneration and altered architecture, with increased multinucleated myofibers and a higher proliferative index [14]. Post-skeletal muscle injury, mice with muscle-specific miR-486-5p overexpression had decreased expression of target genes *PTEN* and *FoxO1* (compared to wild type mice) along with downregulation of cyclin-dependent kinase inhibitors p21and p27 [14], which are *FoxO1* targets [75]. These findings suggest that maintaining high miR-486-5p expression (rather than dynamic miR-486-5p expression) influences muscle satellite cell kinetics and fusion associated with targeting of *PTEN* and *FoxO1*, resulting in delayed and abnormal skeletal muscle regeneration [14].

In addition to myogenic differentiation and DMD, miR-486-5p protects against muscle wasting through targets *PTEN* and *FoxO1* [15, 66]. In experimental chronic kidney disease (CKD), the catabolic environment increases glucocorticoid production and suppresses insulin signaling, resulting in decreased PI3K/Akt activity [76, 77]. Suppression of Akt signaling induces *FoxO1* dephosphorylation, which translocates to the nucleus and activates expression of E3 ubiquitin ligases responsible for muscle proteolysis [77]. In mice with muscle-specific gene deletion of *FoXO1*, Xu et al. reported protection against CKD-induced muscle wasting [15]. In vivo, muscle miR-486-5p levels were decreased in CKD mice, and electroporation of miR-486-5p mimic into muscle improved mass, and decreased expression of ubiquitin E3 ligases, *FoxO1* and *PTEN* [15]. In dexamethasone-treated cultured myotubes, Li et al.demonstrated that BMSC-derived exosomes (enriched in miR-486-5p) improved myotube quantity, reduced expression of muscle atrophy markers, and downregulated the nuclear translocation of *FoxO1*, whereas these effects were blunted using exosomes treated with miR-486-5p inhibitor [66]. Accordingly, these data suggest that miR-486-5p is a potential therapeutic strategy for disorders of muscle catabolism via its targeting of *PTEN* and *FoxO1*.

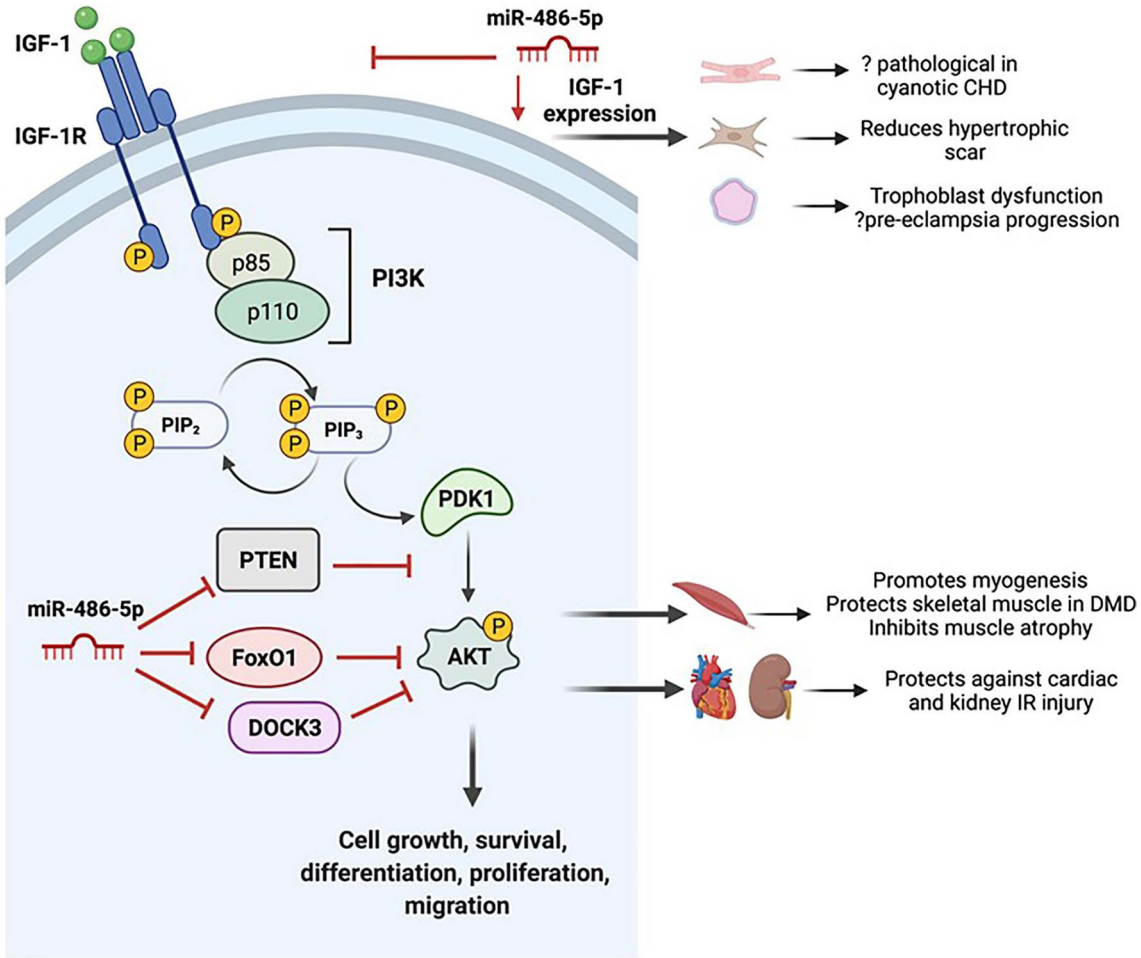

Fig. 2 miR-486-5p can activate or suppress PI3K/Akt signaling via its targets. miR-486-5p targets *PTEN, DOCK3,* and *FoxO1* to activate PI3K/Akt signaling, and confers protective effects in skeletal muscle disorders, and kidney and cardiac IR injury (top). miR-486-5p targets *IGF-1* to inhibit PI3K/Akt signaling, with possible negative consequences in cyanotic congenital heart disease and pre-eclampsia. *CHD* congenital heart disease, *DMD* Duchenne muscular dystrophy, *IGF-1* insulin-like growth factor 1, *IR* ischemia reperfusion, *PI3K* phosphatidylinositol-3-kinase, *PTEN* phosphatase and tensin homolog (created with BioRender.com)

Targeting of *PTEN* and PI3K/Akt signaling in cardiac ischemia

Acute myocardial infarction is associated with IRI [78] and coronary microembolization [79], which lead to myocardial cell apoptosis. In a swine model of coronary microembolization, activation of the *PTEN*/Akt pathway contributes to cardiomyocyte apoptosis [80]. In rats with coronary microembolization, ventricular miR-486-5p expression is downregulated, and its overexpression activates PI3K/Akt signaling by reducing *PTEN* expression, resulting in decreased cardiomyocyte apoptosis, reduced microinfarct area and improved cardiac function [81]. In a preclinical study of myocardial IRI, hypoxia/re-oxygenation triggers cardiomyocyte apoptosis with increased *PTEN* expression and reduced miR-486-5p expression [17]. Exosomes derived from rat BMSCs overexpressing miR-486-5p have a pronounced inhibitory effect on *PTEN* expression, leading to Akt activation, apoptosis reduction and significant reduction in myocardial infarct size compared to unaltered exosomes [17].

Bei et al. demonstrated that endogenous miR-486-5p was downregulated in cardiac tissue from mice subjected to IRI [18]. Injection of adeno-associated virus 9 (AAV9)-expressing miR-486-5p to mice subjected to cardiac IRI significantly reduced infarct size and cardiomyocyte apoptosis at 24 h, preserved cardiac function and reduced cardiac fibrosis at 3 weeks. Long term cardiac remodeling and dysfunction (at 6 weeks) was also prevented [18]. At 3 weeks after IRI, there was decreased expression of *PTEN* and *FoxO1* in cardiac tissue of mice treated with miR-486-5p. Moreover, cardiomyocyte-specific expression of miR-486-5p suppressed apoptosis, attenuated cardiac dysfunction and fibrosis at 3 weeks, and downregulated *PTEN* and *FoxO1* expression.

On the other hand, miR-486-5p inhibition by miR-486-5p sponge AAV9 in cardiac IRI did not further increase infarct size at 24 h or worsen cardiac dysfunction at 3 weeks [18]. Of interest, swimming exercise upregulated miR-486-5p expression in isolated cardiomyocytes from mouse heart tissue, downregulated cardiac *PTEN* and *FoxO1* expression, and protected against cardiac IRI [18]. Furthermore, miR-486-5p knock out mice subjected to exercise prior to cardiac IRI had significantly increased infarct size at 24 h compared to wild type controls [18]. miR-486-5p mimic suppressed apoptosis in cultured rat neonatal and human induced pluripotent stem cell-derived (hiPSC) cardiomyocytes, along with decreased expression of *PTEN* and *FoxO1* and activation of Akt signaling. Finally, while anti-miR-486-5p increased apoptosis in rat cardiomyocytes, silencing either *PTEN* or *FoxO1* in anti-miR-486-5p-transfected cardiomyocytes attenuated the pro-apoptotic effect of miR-486-5p inhibition [18].

These compelling results suggest that exosomal transfer or direct administration of miR-486-5p protects against myocardial IRI by targeting *PTEN* and *FoxO1*, thereby activating Akt signaling and suppressing cardiomyocyte apoptosis (Fig. 2). Furthermore, these data also support an important role of miR-486-5p for the cardioprotective benefit of exercise in cardiac IRI.

Matrix metalloproteinase-19 (MMP-19) and promotion of angiogenesis in myocardial infarction

Besides targeting *PTEN*/PI3K/Akt signaling, miR-486-5p may protect against myocardial ischemic injury via other pathways. De novo angiogenesis may protect injured cardiomyocytes, prevent adverse cardiac remodeling and thereby reduce the risk of heart failure after myocardial infarction [82]. In BM-MSCs under hypoxia, the expression of miR-486-5p and the pro-angiogenic vascular endothelial growth factor (VEGF) is upregulated [83]. miR-486-5p overexpression increases VEGF mRNA, and miR-486-5p inhibition downregulates VEGF mRNA and secretion, suggesting that miR-486-5p confers a proangiogenic effect [83].

Matrix metalloproteinase 19 (*Mmp-19*) is an inhibitor of angiogenesis whose expression is downregulated in invasive carcinomas [84]. *Mmp-19*-deficient mice display earlier onset tumor angiogenesis [85] and *Mmp-19* reduces endothelial cell angiogenesis by proteolytic cleavage of plasminogen, generating angiostatin-like fragments as endogenous angiogenesis inhibitors [86]. Li et al.studied the effect of exosomal miR-486-5p on angiogenesis after myocardial infarction in mice and non-human primates [65]. In mice with myocardial infarction, intra-myocardial administration of hypoxia-preconditioned MSC-derived exosomes, enriched in miR-486-5p, reduced infarct size, improved left ventricular ejection fraction and increased vascular density after 4 weeks. Exosomes also increased vessel sprouting from isolated aortic rings, further supporting their pro-angiogenic effect. Hypoxia-preconditioned exosomes from MSCs treated with anti-miR-486-5p failed to promote myocardial repair and angiogenesis, suggesting that their protective effect is mediated in part by miR-486-5p. RNA sequencing of mouse myocardial tissues identified *Mmp-19* as significantly downregulated in the exosome-treated hearts. *Mmp-19* is highly expressed in cardiac fibroblasts, and is a direct target of miR-486-5p by luciferase reporter assay. Furthermore, pathway analysis revealed that VEGF signaling is upregulated in myocardial tissue from exosome-treated mice, and levels of uncleaved VEGF-A are higher in fibroblasts overexpressing miR-486-5p or with silencing of *Mmp-19* [65], thus linking miR-486-5p to angiogenesis through its target *Mmp-19*.

Further studies in a non-human primate model of myocardial infarction revealed that hypoxia-preconditioned exosomes (enriched in miR-486-5p) promote cardiac angiogenesis, reduce infarct size, and improve cardiac function [65]. After 17 month follow-up, increased vascular and arterial density was found within exosome-treated hearts, indicating long-lasting cardiac angiogenesis. Intramyocardial injection of miR-486-5p-overexpressing exosomes improves cardiac function, reduces infarct size, and increases vascular density [65]. These results suggest that miR-486-5p promotes angiogenesis and functional myocardial recovery in two pre-clinical models of myocardial infarction. The pro-angiogenic effect may occur via a paracrine mechanism from fibroblasts that targets *Mmp-19* expression, resulting in decreased cleavage of extracellular VEGF-A.

Targeting of the PTEN/Akt pathway in kidney ischemic injury

Acute kidney injury (AKI) refers to a rapid decline in kidney function and is a common complication of hospitalization, affecting up to 20% of patients with a higher prevalence in critical care settings [87]. In-hospital mortality rises with increasing severity of AKI with a rate for severe AKI up to 50%[88]. Patients who recover from AKI are at risk of adverse outcomes including new or progressive CKD, and kidney failure [89]. Yet, no effective treatments for AKI exist and preventative measures are limited [90].

The role of miR-486-5p has been evaluated in the context of kidney IRI, an experimental model of human ischemic AKI characterized by tubular cell damage and apoptosis/necrosis, as well as endothelial cell dysfunction and loss [91]. Human cord blood ECFC-derived exosomes, highly enriched in miR-486-5p, suppress apoptosis of cultured

human endothelial cells subjected to hypoxia/reoxygenation by transfer of miR-486-5p, targeting *PTEN* and activating Akt signaling [16, 92]. In mice with kidney IRI, ECFC-derived exosomes decrease kidney *PTEN* expression (and activate Akt), associated with reduced apoptosis, histologic injury and improved kidney function [16]. These data suggest that ECFC-derived exosomes protect against kidney IR injury via transfer of miR-486-5p.

A recent study by Viñas et al. examined the effects of direct intravenous administration of lipid-encapsulated miR-486-5p mimic to mice with ischemic AKI, and evaluated the transcriptome of kidney proximal tubules and endothelial cells [45]. miR-486-5p mimic significantly improved kidney function and decreased histological injury and apoptosis in mice subjected to kidney IRI, and prevented the proximal tubular activation of genes commonly associated with ischemic injury. Kidney *PTEN* protein expression was decreased by miR-486-5p, associated with activation of Akt.

Importantly, the protective effects of miR-486-5p in ischemic AKI may involve other distinct miR-486-5p targets besides *PTEN*. In particular, distinct proximal tubular genes associated with apoptosis and the tumor necrosis family (TNF) pathway are significantly downregulated by miR-486-5p 24 h after IRI [45]. Furthermore, RNA sequencing of kidney proximal tubular cells and endothelial cells revealed only few known miR-486-5p targets downregulated by mimic 24 h after IRI. Notably, *in-situ* hybridization revealed that miR-486-5p localizes to the cytoplasm and nucleus of kidney cortical tubular cells [45], raising the possibility that miR-486-5p may directly regulate gene transcription at the nuclear level, in addition to its effects via 3′UTR targeting.

Targeting of *NFAT5* in chronic kidney disease: diabetic nephropathy

CKD is defined by the presence of abnormalities of kidney structure or function for at least 3 months [93]. Diabetes mellitus is the leading cause of CKD world-wide, and diabetic nephropathy (DN) affects approximately 30% of patients with diabetes [94]. The pathophysiology of DN involves hemodynamic and metabolic factors including increased intraglomerular pressure and hyperfiltration, and the production of advanced glycation end-products. This promotes the generation of growth factors and hormones, such as transforming growth factor (TGF)-β and angiotensin II, reactive oxygen species, and inflammatory mediators, all of which contribute to kidney histological changes including glomerular basement membrane thickening, extracellular matrix (ECM) deposition within the mesangium, proliferative changes, and ultimately, tubulointerstitial fibrosis and glomerulosclerosis [94, 95].

The *nuclear factor of activated T-cells* (*NFAT*), the substrate for calcineurin, represents a family of calcium-dependent transcription factors. *NFAT5* is ubiquitously expressed in all tissue types, and is an important gene regulator in organs with high hyperosmotic pressure risk, such as kidneys, heart, and brain [96]. However, *NFAT5* can have a pathogenic role in disease as it regulates the expression of pro-inflammatory cytokines [96], increases nuclear factor (NF)-κB activity [97], and increases the expression of genes involved in vascular smooth muscle cell and macrophage migration, platelet activation, and angiogenesis [96]. In experimental DN, the *NFAT* family of transcription factors is required for ECM protein accumulation and glomerular hypertrophy [98], and *NFAT* inhibition reduces podocyte injury [99] and renal fibrosis [100].

In this context, Duan et al. demonstrated that *NFAT5* is a validated target of miR-486-5p in DN [21]. In addition, miR-486-5p is a downstream target of lnc-ISG20 RNA: lnc-ISG20 expression increases in kidneys of diabetic mice and in mesangial cells cultured in high glucose, while miR-486-5p decreases; *NFAT5* expression also increases in both models [21]. *NFAT5*-induced Akt phosphorylation promotes fibrosis by increasing the expression of collagen, fibronectin and TGF-β in mesangial cells cultured in high glucose. In mice with DN, lnc-ISG20 overexpression promotes kidney fibrosis via Akt phosphorylation, while *NFAT5* knockdown prevents fibrosis [21]. Thus, lnc-ISG20 downregulates miR-486-5p expression, resulting in increased *NFAT5* expression, Akt activation, and increased expression of pro-fibrotic genes [21]. These data support an anti-fibrotic role of miR-486-5p in diabetic nephropathy.

Of note, the effect of glucose on miR-486-5p expression may be cell-specific. Although high glucose downregulates miR-486-5p expression in mesangial cells [21], exposure of human adipose tissue-derived MSCs to high glucose upregulates miR-486-5p expression, which inhibits its cellular proliferation by targeting and downregulating *SIRT1* [101], a deacetylase that regulates gene expression and is implicated in insulin sensitivity in type 2 diabetes [102]. *SIRT1* expression is downregulated in adipose tissue of patients with diabetes [102], and in endothelial progenitor cells exposed to high glucose [103]. Bouchareychas et al. showed that miR-486-5p is upregulated in monocytes from hyperglycemic mice, in exosomes derived from BM-derived macrophages exposed to hyperglycemia, and in plasma-derived exosomes from patients with diabetes and peripheral arterial disease [104]. Thus, although miR-486-5p may protect against kidney fibrosis by targeting *NFAT5* in DN, its upregulation in other cell types in the context of hyperglycemia may have adverse consequences.

Targeting of *IGF-1*

Insulin-like growth factor-1 (IGF-1) is a polypeptide trophic factor whose biological actions are mediated by the IGF-1 receptor, a tyrosine kinase that phosphorylates intracellular proteins to activate multiple signaling pathways, including PI3K/Akt and mitogen-activated protein kinase (MAPK) [105]. Consequently, *IGF-1* is involved in several cellular functions including survival, growth, and differentiation. Targeting of *IGF-1* by miR-486-5p in malignant diseases has been reported to reduce cancer cell growth and migration, and stimulate apoptosis [35, 106].

Studies of miR-486-5p and *IGF-1* signaling in non-malignant diseases have been limited to in vitro models, and reveal conflicting effects on proliferation, migration, and apoptosis. *IGF-1* is an essential regulator of cardiac structure and homeostasis [107] and reduced serum levels of *IGF-1* are found in patients with cyanotic congenital heart disease, who experience chronic hypoxemia [108]. Erythrocyte levels of miR-486-5p levels are increased in pediatric patients with cyanotic congenital heart disease compared to healthy controls [109]. Fan et al. showed that miR-486-5p expression is upregulated in rat cardiomyocytes exposed to hypoxia, and demonstrated *IGF-1* is a direct target of miR-486-5p by luciferase reporter assay. Downregulation of miR-486-5p increases *IGF-1* expression and cell viability, and suppresses hypoxia-induced cardiomyocyte apoptosis, whereas silencing of *IGF-1* has the opposite effect [37]. The data suggest that miR-486-5p may have a pathological role in cyanotic congenital heart disease by targeting *IGF-1*, thereby promoting cardiomyocyte apoptosis (Fig. 2).

The role of miR-486-5p and *IGF-1* targeting has also been studied in pre-eclampsia, a serious pregnancy-specific complication mediated by placental dysfunction. Human placental microvascular endothelial cell-derived exosomes are enriched in miR-486-5p [110] and levels are further upregulated following exposure to hypoxia/reoxygenation. Administration of hypoxia-exposed exosomes to trophoblast cells results in transfer of miR-486-5p, associated with decreased cell viability, proliferation, migration and invasion, via targeting of *IGF-1* [110]. These data suggest that miR-486-5p may have a pathological role in the development and progression of pre-eclampsia by causing trophoblast dysfunction. Similarly, an in vitro study in hypertrophic scar fibroblasts showed that miR-486-5p overexpression inhibits cell viability, migration and expression of collagens and promotes apoptosis by targeting *IGF-1* [111]. Interestingly, miR-486-5p has opposing biological effects in this model, suppressing PI3K/Akt signaling through *IGF-1*, in contrast to skeletal muscle [15, 23] and models of cardiac IRI [17, 81] Thus, cell-specific factors influence the regulation of miR-486-5p expression and its affected target genes, which ultimately determine biological effects. Indeed, in malignant diseases miR-486-5p has been reported to have both oncogenic and tumor suppressor roles [9].

Targeting *SMAD1/2/4* and inhibition of TGF-β signaling

TGF-β belongs to a superfamily of related growth factors and comprises three isoforms in mammals (TGF-β1, TGF-β2, TGF-β3), all of which bind the TGF-β receptor 2 (TGFR2) to recruit TGFR1 and activate signaling [112]. TGF-β signaling is the primary driver of tissue fibrosis, but also has effects on cell proliferation, differentiation, apoptosis, and immunity [112]. TGF-β signaling activates *Smad*-based pathways by phosphorylation and activation of *Smad2/3* by the TGFR1, resulting in nuclear translocation of the *Smad* complex and transcription of pro-fibrotic genes, such as smooth muscle actin (α-SMA), collagens, and fibronectin [112]. TGF-β also interacts with non-*Smad*-based signaling including the MAPK and PTEN/PI3K/Akt pathways. Ultimately, TGF-β signaling through *Smad* and non-*Smad*-based pathways induces fibrosis via myofibroblast activation, excessive ECM production, and inhibition of ECM degradation [112].

In mice with pulmonary fibrosis, lung miR-486-5p levels diminish, and decreased levels are found in the serum and lung tissue of patients with silicosis and idiopathic pulmonary fibrosis [20]. Administration of miR-486-5p attenuates pulmonary fibrosis in mice exposed to silica or bleomycin [20]. In cultured mouse fibroblasts miR-486-5p directly targets *Smad2*, inhibits TFG-β-induced expression of pro-fibrotic genes and reduces fibroblast proliferation [20]. In human hypertrophic scar fibroblasts, miR-486-5p targets *Smad2* to inhibit proliferation and induce apoptosis [113]. These data support an anti-fibrotic role for miR-486-5p in models of pulmonary fibrosis and hypertrophic scar.

Epithelial–mesenchymal transition (EMT) is another TGF-β-mediated mechanism that contributes to fibrosis [112], and is implicated in development of posterior capsular opacification, (also known as secondary cataract), a complication of cataract surgery. In vitro studies using cultured human lens epithelial cells reported that miR-486-5p expression is downregulated in TGF-β2-induced lens cells, and overexpression of miR-486-5p reduces proliferation, migration, and EMT by targeting *Smad2* and *Smad4*, suggesting that miR-486-5p may prevent the progression of cataracts [114, 115].

Heart failure, a common end-stage manifestation of cardiac diseases, is associated with cardiac remodeling, characterized by cardiomyocyte hypertrophy, apoptosis,

cardiac fibroblast activation and ECM deposition [116]. Serum IgE levels are elevated in patients with heart failure, and the IgE receptor (FCεR1) is upregulated in heart tissue from these patients [117]. Zhao et al. showed that blocking the IgE-FCεR1 pathway alleviates pathological cardiac remodeling in two distinct mouse models of heart failure [117]. Treatment of rat cardiac fibroblasts with IgE results in fibroblast activation, matrix protein production, and upregulation of TGF-β signaling in an FCεR1-dependent manner [117].

Zhao et al. [19] subsequently reported that miR-486-5p is downregulated (and its predicted target *Smad1* is upregulated) in mouse cardiac fibroblasts treated with IgE in an FCεR1-dependent manner, while the expression of collagens and α-SMA is also increased. Luciferase reporter assay revealed that *Smad1* is a direct target of miR-486-5p, while miR-486-5p mimic decreases expression of *Smad1*/p-*Smad1* in primary mouse cardiac fibroblasts. *Smad1* also promotes activation and collagen expression in cardiac fibroblasts treated with IgE. These data support a role for the miR-486-5p/*Smad1* pathway in IgE-induced collagen expression, and suggest that miR-486-5p protects against cardiac fibrosis by targeting and downregulating *Smad1*.

Although targeting of TGF-β/*Smad* signaling by miR-486-5p may protect against select fibrotic diseases, other processes may be negatively impacted. Shi et al. reported that patients with osteoarthritis (the most common form of arthritis caused by degeneration of joint cartilage) exhibit low levels of *Smad2* and elevated miR-486-5p levels in cartilage tissue compared to control patients. In cultured human chondrocytes, miR-486-5p targets *Smad2* and decreases cell proliferation and type II collagen expression. These data suggest that miR-486-5p may promote osteoarthritis progression by targeting *Smad2* [118] (Fig. 3). However, using collagen-induced arthritic mice as a model for the inflammatory autoimmune disease rheumatoid arthritis, Chen et al. demonstrated that exosomes containing miR-486-5p alleviate disease severity by decreasing the expression of *Tob1*, an antiproliferative protein that interacts with *Smad* family proteins [119], inducing osteoblast differentiation [120]. Thus the role of miR-486-5p in arthritis is complex and may differ based on arthritis etiology.

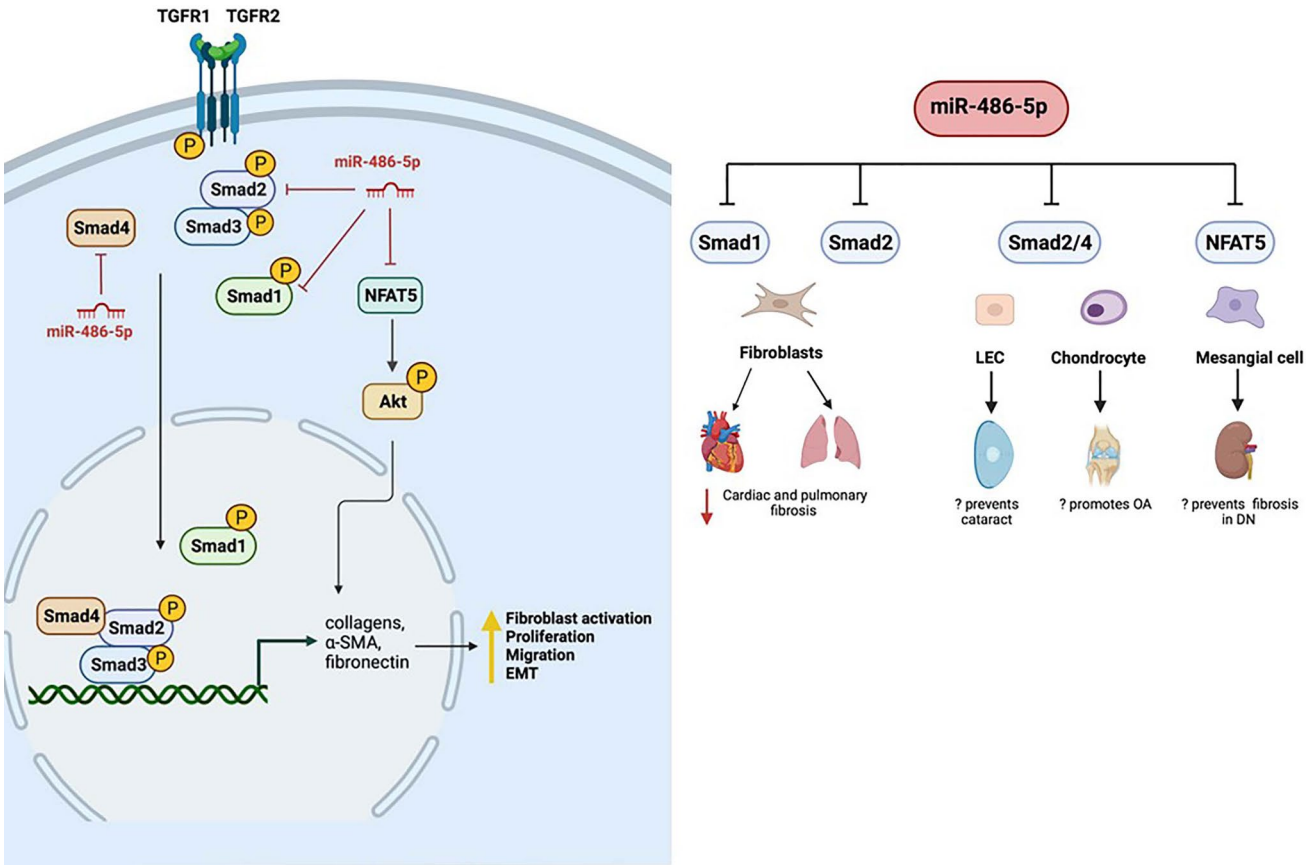

Fig. 3 miR-486-5p targets *Smad*-dependent TGF-β signaling. miR-486-5p confers protective effects in models of cardiac and pulmonary fibrosis, hypertrophic scar, and cataract progression but may have a role in osteoarthritis pathogenesis. *EMT* epithelial-to-mesenchymal transition, *NFAT-5* nuclear factor of activated T cells-5, *OA* osteoarthritis, *α-SMA* α-smooth muscle actin, *TGF-β* transforming growth factor β (created with BioRender.com)

Targeting *Sp5*/Wnt signaling

Successful wound healing requires cellular proliferation, migration, and angiogenesis [121]. The Wnt/β-catenin pathway involves evolutionary conserved signaling that is essential for cell fate and organization during embryogenesis, and plays a role in adult tissue homeostasis and regeneration [122]. The *Sp5* transcription factor is a Wnt target gene that negatively regulates Wnt signaling by transcriptional repression of downstream genes, such as *p21* and cyclin-D2, both of which regulate cellular proliferation, and the latter has also been implicated in endothelial cell repair [123].

Lu et al.investigated the role of adipose-derived stem cell-secreted extracellular vesicles, enriched in miR-486-5p, on cutaneous wound healing and the validated target *Sp5* [121]. In vitro experiments revealed that vesicle transfer of miR-486-5p enhances proliferation and migration of human skin fibroblasts, and stimulates proliferation, migration, and angiogenesis of human microvascular endothelial cells by inhibiting the expression of *Sp5*, thereby increasing cyclin-D2 expression. Thus, the relationship between miR-486-5p targeting of the transcriptional repressor *Sp5* and endothelial repair warrants further exploration in other models of organ injury and recovery that require de novo angiogenesis.

miR-486-5p targets *Histone acetyltransferase 1*

Not all pre-clinical data demonstrate protective effects of miR-486-5p in disease. In this regard, histone acetylation is an epigenetic modification that changes chromatin structure and affects DNA replication, repair, and activation of gene transcription [124]. *Histone acetyltransferase 1 (HAT1)* partially localizes to the cytoplasm and deacetylates newly synthesized histone H4 on lysines 5 and 12 (H4K5, H4K12) [125]. *HAT1* is involved in several biological processes including chromatin assembly, DNA replication, DNA repair, cell proliferation and glucose metabolism [124].

Liu et al. investigated the relationship between miR-486-5p and ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux in macrophage-derived foam cells [126]. ABCA1 expression and atherogenesis are regulated by epigenetic modifications [127], but ABCA1 is not a predicted target of miR-486-5p. However, in this study miR-486-5p directly targeted *HAT1* by luciferase reporter assay. Treatment of macrophage-derived foam cells with miR-486-5p mimic decreases *HAT1* expression, decreases H4K5/H4K12 acetylation, and blocks cholesterol efflux. *HAT1* overexpression increases ABCA1 expression, while miR-486-5p mimic inhibits ABCA1 expression [126]. Further supporting the role of miR-486-5p in atherosclerosis, $Apoe^{-/-}$ mice treated with exosomes from BM-derived macrophages (enriched in miR-486-5p) develop atherosclerotic lesions with macrophage foam cells [104]. Together, these studies suggest that macrophage miR-486-5p may promote atherosclerosis.

The relationship between miR-486-5p and *HAT1* has also been evaluated in the context of chronic obstructive pulmonary disease (COPD). Zhang et al. reported that miR-486-5p is upregulated in lung tissues of patients with COPD compared to smokers without COPD, and in alveolar macrophages and peripheral monocytes of COPD patients and smokers compared to healthy non-smokers [128]. Rat pulmonary macrophages exposed to cigarette smoking extract upregulate endogenous miR-486-5p and toll-like receptor 4 (TLR4), a known inflammatory trigger, and downregulate *HAT1*, a direct miR-486-5p target. miR-486-5p negatively regulates *HAT1* expression in rat pulmonary macrophages, and *HAT1* suppression increases expression of TLR4 and inflammatory cytokines [128]. miR-486-5p may therefore play a pathological role in COPD by regulating TLR-4 triggered inflammation via its target *HAT1*.

miR-486-5p targets in development

In skeletal muscle development, the transcription factor *Pax7* is expressed in muscle satellite cells, and is downregulated in activated satellite cells for differentiation [129]. In C2C12 myoblasts, Dey et al.showed that miR-486-5p expression is upregulated during myoblast differentiation [24]. miR-486-5p overexpression accelerates myoblast differentiation, while miR-486-5p inhibition delays differentiation with persistent expression of *Pax7* protein, which is a target of miR-486-5p. Thus, miR-486-5p promotes myogenesis by targeting and downregulating *Pax7*, a transcription factor required for satellite cell biogenesis and survival [24].

In hematopoiesis, miR-486-5p expression is upregulated throughout erythroid differentiation [25, 26]. miR-486-5p overexpression in cord blood CD34+ cells (megakaryocyte–erythroid progenitors) enhances cell growth, erythroid differentiation and cell survival, while miR-486-5p inhibition suppresses these processes [130]. miR-486-5p inhibition also upregulates *PTEN* and *FoxO1* protein expression, decreases p-Akt, and promotes apoptosis and cell growth inhibition. *FoxO1* knockdown rescued the effects of miR-486-5p inhibition but did not influence erythroid differentiation. Thus contributing targets of miR-486-5p other than *FoxO1* remain to be uncovered [130]. The role of

miR-486-5p in erythropoiesis has been further characterized in homozygous miR-486-5p knockout mice [25]. The mice are viable, but display defects within the erythroid lineage in bone marrow and spleen, with accumulation of early stage erythroblasts, and a reduced proportion of mature erythrocytes. When subjected to oxidative stress, peripheral blood from miR-486-5p knockout mice shows a greater reduction of red blood cells and increased proportion of reticulocytes [25].

miR-486-5p is also implicated in neurogenesis. Dori et al. isolated discrete populations of neural progenitor cells (proliferative progenitors that expand the stem cell pool and differentiative progenitors that generate neurons) and profiled global miRNA expression during cortical development [131]. miR-486-5p expression is transiently downregulated in differentiative progenitors compared with proliferative progenitors and neurons, termed an 'off-switch' transcript. In utero delivery of a locked nucleic acid miR-486-5p inhibitor to mice at embryonic day 13.5 (the developmental stage, where cortical progenitor cells are mostly proliferative progenitors) increases the proportion of cortical progenitor cells at the expense of neurons without affecting neuron migration or survival [131]. These findings suggest that miR-486-5p regulates cell fate in neurogenesis, but the precise targets of miR-486-5p in cortical development remain unknown. Thus, understanding the mechanisms by which miR-486-5p regulates developmental processes could provide additional insight into its role in disease states and identify new targets and pathways for further study.

Conclusions

miR-486-5p is implicated in several non-malignant diseases as an important regulator of critical signaling pathways including *PTEN*/Akt, *IGF-1, MMP-19*/VEGF, *Smad*-dependent TGF-β, and Sp5/Wnt/β-catenin, affecting biological processes including apoptosis, cellular

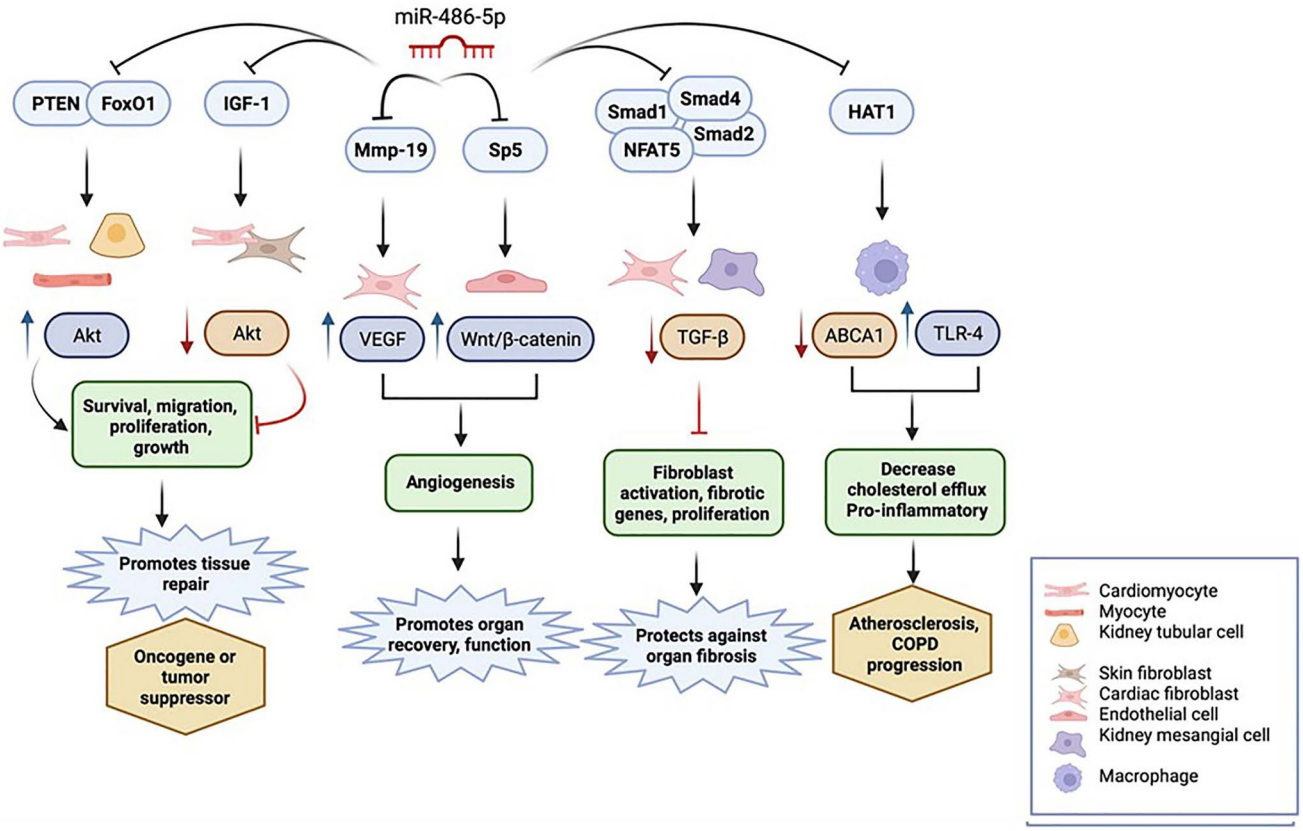

Fig. 4 Overview of miR-486-5p target genes, affected signaling pathways, cellular processes, and possible biological effects. miR-486-5p has therapeutic potential in non-malignant diseases by modulating signaling pathways that control critical cellular processes that are involved in tissue regeneration, recovery of organ function, prevention of adverse long-term consequences after organ injury, and organ fibrosis. *ABCA1* ATP binding cassette subfamily A member 1, *COPD* chronic obstructive pulmonary disease, *HAT-1* histone acetyltransferase 1, *IGF-1* insulin-like growth factor-1, *Mmp-19* matrix metalloproteinase-19, *NFAT5* nuclear factor of activated T cells-5, *OA* osteoarthritis, *PI3K* phosphatidylinositol-3-kinase, *PTEN* phosphatase and tensin homolog, *TGF-ß* transforming growth factor-ß, *TLR-4* toll-like receptor-4, *VEGFA* vascular endothelial growth factor-A (created with BioRender.com)

migration and proliferation, angiogenesis, and fibrosis (summarized in Fig. 4). miR-486-5p also regulates epigenetic modifications by targeting and downregulating *HAT1*. However, varying biological effects of miR-486-5p have been reported depending on the targeted genes, which may also be specific to cell type and injury model. In addition to its validated 3′UTR targets that are downregulated by post-transcriptional gene silencing via the cytoplasmic RISC pathway, miR-486-5p also localizes to the nucleus, and thus may affect gene expression beyond classical post-transcriptional gene regulation.

Pre-clinical studies support miR-486-5p as a promising therapy for several non-malignant diseases, including cardiac and kidney disorders, due to its proliferative, pro-angiogenic, anti-apoptotic, and anti-fibrotic effects. miR-486-5p protects against cardiac ischemic injury and targets *PTEN,* suppressing cardiomyocyte apoptosis in cardiac IRI [17, 81], improves cardiac function and promotes angiogenesis after myocardial infarction associated with targeting *Mmp-19* in cardiac fibroblasts [65], and reduces IgE-mediated cardiac fibrosis by targeting *Smad1* [19]. miR-486-5p also protects against ischemic kidney injury and targets *PTEN* [16, 45], associated with decreased expression of select genes involved in apoptosis and the TNF inflammatory pathway, and may confer a protective anti-fibrotic role in diabetic kidney disease by targeting *NFAT5* [21]. Although there are no human clinical trials involving miR-486-5p therapy to date, clinical trials are underway for other miRNAs [132]. For instance, a phase I trial of intravenous liposomal miR-34a mimic administered to patients with advanced solid tumors was found to target tumors but unfortunately led to serious immune-mediated adverse events, resulting in early trial termination [133]. In Alport nephropathy—a hereditary nephropathy—Phase 1 trials involving use of the miR-21 inhibitor RG-012 have been completed and will move to Phase 2 [132]. Accordingly, miR-486-5p has potential as a bio-therapeutic agent in several non-malignant diseases. Nonetheless, studies addressing the safety profile and longer term effects of miR-486-5p delivery are warranted, since miR-486-5p affects cell cycle kinetics [14], its expression is tightly regulated in growth and development [11], and dysregulated expression has been associated with malignancy potential [9].

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Author contributions KDB and JV conceptualized the study. AD, JV, and KDB drafted the initial manuscript. AD, JV, and KDB all contributed to the literature search, manuscript review and editing, and approved the final version. KDB is the guarantor of the manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval Not applicable.

Consent to publish Not applicable.

References

1. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854. https://doi.org/10.1016/0092-8674(93)90529-y
2. Ameres SL, Zamore PD (2013) Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 14:475–488. https://doi.org/10.1038/nrm3611
3. Friedman RC, Farh KK-H, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19:92–105. https://doi.org/10.1101/gr.082701.108
4. O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol 9:402. https://doi.org/10.3389/fendo.2018.00402
5. Jonas S, Izaurralde E (2015) Towards a molecular understanding of microRNA-mediated gene silencing. Nat Rev Genet 16:421–433. https://doi.org/10.1038/nrg3965
6. Catalanotto C, Cogoni C, Zardo G (2016) MicroRNA in control of gene expression: an overview of nuclear functions. Int J Mol Sci 17:1712. https://doi.org/10.3390/ijms17101712
7. Liu H, Lei C, He Q, Pan Z, Xiao D, Tao Y (2018) Nuclear functions of mammalian MicroRNAs in gene regulation, immunity and cancer. Mol Cancer 17:64. https://doi.org/10.1186/s12943-018-0765-5
8. Brandenburger T, Lorenzen JM (2020) Diagnostic and therapeutic potential of microRNAs in acute kidney injury. Front Pharmacol 11:657. https://doi.org/10.3389/fphar.2020.00657
9. ElKhouly AM, Youness RA, Gad MZ (2020) MicroRNA-486-5p and microRNA-486-3p: multifaceted pleiotropic mediators in

oncological and non-oncological conditions. Non-coding RNA Res 5:11–21. https://doi.org/10.1016/j.ncrna.2020.01.001
10. Gadde S, Rayner KJ (2016) Nanomedicine meets microRNA: Current advances in RNA- based nanotherapies for atherosclerosis. Arterioscler Thromb Vasc Biol 36:e73–e79. https://doi.org/10.1161/ATVBAHA.116.307481
11. Small EM, O'Rourke JR, Moresi V, Sutherland LB, McAnally J, Gerard RD, Richardson JA, Olson EN (2010) Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. PNAS 107:4218–4223. https://doi.org/10.1073/pnas.1000300107
12. Bayés-Genis A, Lanfear DE, Ronde MWJD, Lupón J, Leenders JJ, Liu Z, Zuithoff NPA, Eijkemans MJC, Zamora E, Antonio MD et al (2018) Prognostic value of circulating microRNAs on heart failure-related morbidity and mortality in two large diverse cohorts of general heart failure patients. Eur J Heart Fail 20:67–75. https://doi.org/10.1002/ejhf.984
13. Baglio SR, Rooijers K, Koppers-Lalic D, Verweij FJ, Lanzón MPR, Zini N, Naaijkens B, Perut F, Niessen HWM, Baldini N et al (2015) Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. Stem Cell Res Ther 6:127. https://doi.org/10.1186/s13287-015-0116-z
14. Alexander MS, Casar JC, Motohashi N, Myers JA, Eisenberg I, Gonzalez RT, Estrella EA, Kang PB, Kawahara G, Kunkel LM (2011) Regulation of DMD pathology by an ankyrin-encoded miRNA. Skelet Muscle 1:27. https://doi.org/10.1186/2044-5040-1-27
15. Xu J, Li R, Workeneh B, Dong Y, Wang X, Hu Z (2012) Transcription factor FoxO1, the dominant mediator of muscle wasting in chronic kidney disease, is inhibited by microRNA-486. Kidney Int 82:401–411. https://doi.org/10.1038/ki.2012.84
16. Viñas JL, Burger D, Zimpelmann J, Haneef R, Knoll W, Campbell P, Gutsol A, Carter A, Allan DS, Burns KD (2016) Transfer of microRNA-486-5p from human endothelial colony forming cell-derived exosomes reduces ischemic kidney injury. Kidney Int 90:1238–1250. https://doi.org/10.1016/j.kint.2016.07.015
17. Sun X-H, Wang X, Zhang Y, Hui J (2019) Exosomes of bone-marrow stromal cells inhibit cardiomyocyte apoptosis under ischemic and hypoxic conditions via miR-486-5p targeting the PTEN/ PI3K/AKT signaling pathway. Thromb Res 177:23–32. https://doi.org/10.1016/j.thromres.2019.02.002
18. Bei Y, Lu D, Bär C, Chatterjee S, Costa A, Riedel I, Mooren FC, Zhu Y, Huang Z, Wei M et al (2022) miR-486 attenuates cardiac ischemia/reperfusion injury and mediates the beneficial effect of exercise for myocardial protection. Mol Ther 30:1675–1691. https://doi.org/10.1016/j.ymthe.2022.01.031
19. Zhao H, Yang H, Geng C, Chen Y, Tang Y, Li Z, Pang J, Shu T, Nie Y, Liu Y et al (2021) Elevated IgE promotes cardiac fibrosis by suppressing miR-486a-5p. Theranostics 11:7600–7615. https://doi.org/10.7150/thno.47845
20. Ji X, Wu B, Fan J, Han R, Luo C, Wang T, Yang J, Han L, Zhu B, Wei D et al (2015) The anti-fibrotic effects and mechanisms of MicroRNA-486-5p in pulmonary fibrosis. Sci Rep 5:14131. https://doi.org/10.1038/srep14131
21. Duan Y-R, Chen B-P, Chen F, Yang S-X, Zhu C-Y, Ma Y-L, Li Y, Shi J (2021) LncRNA lnc-ISG20 promotes renal fibrosis in diabetic nephropathy by inducing AKT phosphorylation through miR-486-5p/NFAT5. J Cell Mol Med 25:4922–4937. https://doi.org/10.1111/jcmm.16280
22. Eisenberg I, Eran A, Nishino I, Moggio M, Lamperti C, Amato AA, Lidov HG, Kang PB, North KN, Mitrani-Rosenbaum S et al (2007) Distinctive patterns of microRNA expression in primary muscular disorders. PNAS 104:17016–17021. https://doi.org/10.1073/pnas.0708115104
23. Alexander MS, Casar JC, Motohashi N, Vieira NSM, Eisenberg I, Marshall JL, Gasperini MJ, Lek A, Myers JA, Estrella EA et al (2014) MicroRNA-486-dependent modulation of DOCK3/PTEN/AKT signaling pathways improves muscular dystrophy-associated symptoms. J Clin Investig 124:2651–2667. https://doi.org/10.1172/JCI73579
24. Dey BK, Gagan J, Dutta A (2011) miR-206 and -486 induce myoblast differentiation by downregulating Pax7. Mol Cell Biol 31:203–214. https://doi.org/10.1128/MCB.01009-10
25. Jee D, Yang J-S, Park S-M, Farmer DJT, Wen J, Chou T, Chow A, McManus MT, Kharas MG, Lai EC (2018) Dual strategies for argonaute2-mediated biogenesis of erythroid miRNAs underlie conserved requirements for slicing in mammals. Mol Cell 69:265–278. https://doi.org/10.1016/j.molcel.2017.12.027
26. Lulli V, Romania P, Morsilli O, Cianciulli P, Gabbianelli M, Testa U, Giuliani A, Marziali G (2013) MicroRNA-486-3p regulates γ-globin expression in human erythroid cells by directly modulating BCL11A. PLoS ONE 8:e60436. https://doi.org/10.1371/journal.pone.0060436
27. Medley JC, Panzade G, Zinovyeva AY (2021) microRNA strand selection: unwinding the rules. WIREs RNA 12:e1627. https://doi.org/10.1002/wrna.1627
28. Yang J-S, Phillips MD, Betel D, U P, Ventura A, Siepel AC, Chen KC, Lai EC (2011) Widespread regulatory activity of vertebrate microRNA* species. RNA 17:312–326. https://doi.org/10.1261/rna.2537911
29. Wang Y, Cai Y, Huang H, Chen X, Chen X, Chen X, Mai H, Li X, Zhao J, Yang J et al (2018) miR-486-3p influences the neurotoxicity of a-synuclein by targeting the SIRT2 gene and the polymorphisms at target sites contributing to Parkinson's disease. Cell Physiol Biochem 51:2732–2745. https://doi.org/10.1159/000495963
30. Huang Z-X, Chen Y, Guo H-R, Chen G-F (2021) Systematic review and bioinformatic analysis of microRNA expression in autism spectrum disorder identifies pathways associated with cancer, metabolism, cell signaling, and cell adhesion. Front Psychiatry 12:630876. https://doi.org/10.3389/fpsyt.2021.630876
31. Yu D, Jiao X, Cao T, Huang F (2018) Serum miRNA expression profiling reveals miR-486-3p may play a significant role in the development of autism by targeting ARID1B. NeuroReport 29:1431–1436. https://doi.org/10.1097/WNR.0000000000001107
32. Shibutani M, Horii T, Shoji H, Morita S, Kimura M, Terawaki N, Miyakawa T, Hatada I (2017) *Arid1b* haploinsufficiency causes abnormal brain gene expression and autism-related behaviors in mice. Int J Mol Sci 18:1872. https://doi.org/10.3390/ijms18091872
33. Gallagher PG, Forget BG (1998) An alternate promoter directs expression of a truncated, muscle-specific isoform of the human ankyrin 1 gene*. J Biol Chem 273:1339–1348. https://doi.org/10.1074/jbc.273.3.1339
34. Tessema M, Yingling CM, Picchi MA, Wu G, Ryba T, Lin Y, Bungum AO, Edell ES, Spira A, Belinsky SA (2017) ANK1 methylation regulates expression of microRNA-486-5p and discriminates lung tumors by histology and smoking status. Cancer Lett 410:191–200. https://doi.org/10.1016/j.canlet.2017.09.038
35. Peng Y, Dai Y, Hitchcock C, Yang X, Kassis ES, Liu L, Luo Z, Sun H-L, Cui R, Wei H et al (2013) Insulin growth factor signaling is regulated by microRNA-486, an underexpressed microRNA in lung cancer. PNAS 110:15043–15048. https://doi.org/10.1073/pnas.1307107110
36. Yang Y, Ji C, Guo S, Su X, Zhao X, Zhang S, Liu G, Qiu X, Zhang Q, Guo H et al (2017) The miR-486-5p plays a causative role in prostate cancer through negative regulation of multiple tumor suppressor pathways. Oncotarget 8:72835–72846. https://doi.org/10.18632/oncotarget.20427

37. Fan J, Shi S, Qiu Y, Zheng Z, Yu L (2019) MicroRNA-486-5p down-regulation protects cardiomyocytes against hypoxia-induced cell injury by targeting IGF-1. Int J Clin Exp Pathol 12:2544–2551
38. Xiong F, Wei W-P, Liu Y-B, Wang Y, Zhang H-Y, Liu R (2021) Long noncoding RNA XIST enhances cerebral ischemia-reperfusion injury by regulating miR-486-5p and GAB2. Eur Rev Med Pharmacol Sci 25:2013–2020. https://doi.org/10.26355/eurrev_202102_25103
39. Li D, Wu L, Knox B, Chen S, Tolleson WH, Liu F, Yu D, Guo L, Tong W, Ning B (2020) Long noncoding RNA LINC00844-mediated molecular network regulates expression of drug metabolizing enzymes and nuclear receptors in human liver cells. Arch Toxicol 94:1637–1653. https://doi.org/10.1007/s00204-020-02706-5
40. Park CW, Zeng Y, Zhang X, Subramanian S, Steer CJ (2010) Mature microRNAs identified in highly purified nuclei from HCT116 colon cancer cells. RNA Biol 7:606–614. https://doi.org/10.4161/rna.7.5.13215
41. Chen B, Zhang B, Luo H, Yuan J, Skogerbø G, Chen R (2012) Distinct microRNA subcellular size and expression patterns in human cancer cells. Int J Cell Biol 2012:672462. https://doi.org/10.1155/2012/672462
42. Liao J-Y, Ma L-M, Guo Y-H, Zhang Y-C, Zhou H, Shao P, Chen Y-Q, Qu L-H (2010) Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 39 trailers. PLoS ONE 5:e10563. https://doi.org/10.1371/journal.pone.0010563
43. Nishi K, Nishi A, Nagasawa T, Ui-Tei K (2013) Human TNRC6A is an argonaute-navigator protein for microRNA-mediated gene silencing in the nucleus. RNA 19:17–35. https://doi.org/10.1261/rna.034769.112
44. Kalantari R, Hicks JA, Li L, Gagnon KT, Sridhara V, Lemoff A, Mirzaei H, Corey DR (2016) Stable association of RNAi machinery is conserved between the cytoplasm and nucleus of human cells. RNA 22:1085–1098. https://doi.org/10.1261/rna.056499.116
45. Viñas JL, Spence M, Porter CJ, Douvris A, Guts A, Zimpelmann JA, Campbell PA, Burns KD (2021) micro-RNA-486-5p protects against kidney ischemic injury and modifies the apoptotic transcriptome in proximal tubules. Kidney Int 100:597–612. https://doi.org/10.1016/j.kint.2021.05.034
46. Turchinovich A, Weiz L, Langheinz A, Burwinkel B (2011) Characterization of extracellular circulating microRNA. Nucleic Acids Res 39:7223–7233. https://doi.org/10.1093/nar/gkr254
47. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL et al (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. PNAS 108:5003–5008. https://doi.org/10.1073/pnas.1019055108
48. LaBelle J, Bowser M, Brown A, Farnam L, Kho A, Li J, McGeachie M, Chase R, Piehl S, Allen K et al (2021) Commercially available blocking oligonucleotides effectively suppress unwanted hemolysis-related miRNAs in a large whole-blood RNA cohort. J Mol Diagn 23:671–682. https://doi.org/10.1016/j.jmoldx.2021.03.006
49. Wu Q, Yu L, Lin X, Zheng Q, Zhang S, Chen D, Pan X, Huang Y (2020) Combination of serum miRNAs with serum exosomal miRNAs in early diagnosis for non-small-cell lung cancer. Cancer Manag Res 12:485–495. https://doi.org/10.2147/CMAR.S232383
50. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, Tait JF, Tewari M (2012) Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. Cancer Prev Res (Phila) 5:492–497. https://doi.org/10.1158/1940-6207.CAPR-11-0370
51. Shkurnikov MY, Knyazev EN, Fomicheva KA, Mikhailenko DS, Nyushko KM, Saribekyan EK, Samatov TR, Alekseev BY (2016) Analysis of Plasma microRNA Associated with Hemolysis. Bull Exp Biol Med 160:748–750. https://doi.org/10.1007/s10517-016-3300-y
52. Sun B, Guo S (2021) miR-486-5p serves as a diagnostic biomarker for sepsis and its predictive value for clinical outcomes. J Inflamm Res 14:3687–3695. https://doi.org/10.2147/JIR.S323433
53. Ideozu JE, Zhang X, Rangaraj V, McColley S, Levy H (2019) Microarray profiling identifies extracellular circulating miRNAs dysregulated in cystic fibrosis. Sci Rep 9:15483. https://doi.org/10.1038/s41598-019-51890-7
54. Regmi A, Liu G, Zhong X, Hu S, Ma R, Zafar LGMI, Chen L (2019) Evaluation of serum microRNAs in patients with diabetic kidney disease: a nested case-controlled study and bioinformatic analysis. Med Sci Monit 25:1699–1708. https://doi.org/10.12659/MSM.913265
55. Kong R, Gao J, Si Y, Zhao D (2017) Combination of circulating miR-19b-3p, miR-122-5p and miR-486-5p expressions correlates with risk and disease severity of knee osteoarthritis. Am J Transl Res 9:2852–2864
56. Prabhakar P, Chandra SR, Christopher R (2017) Circulating microRNAs as potential biomarkers for the identification of vascular dementia due to cerebral small vessel disease. Age Ageing 46:861–864. https://doi.org/10.1093/ageing/afx090
57. Hoss AG, Lagomarsino VN, Frank S, Hadzi TC, Myers RH, Latourelle JC (2015) Study of plasma-derived miRNAs mimic differences in Huntington's disease brain. Mov Disord 30:1961–1964. https://doi.org/10.1002/mds.26457
58. Ghahramani-Seno MM, Hu P, Gwadry FG, Pinto D, Marshall CR, Casallo G, Scherer SW (2011) Gene and miRNA expression profiles in autism spectrum disorders. Brain Res 1380:85–97. https://doi.org/10.1016/j.brainres.2010.09.046
59. Zaki MB, Abulsoud AI, Elsisi AM, Doghish AS, Mansour OAE, Amin AI, Elrebehy MA, Mohamed MY, Goda MA (2019) Potential role of circulating microRNAs (486–5p, 497, 509–5p and 605) in metabolic syndrome Egyptian male patients. Diabetes Metab Syndr Obes 12:601–611. https://doi.org/10.2147/DMSO.S187422
60. Prats-Puig A, Ortega FJ, Mercader JM, Moreno-Navarrete JM, Moreno M, Bonet N, Ricart W, López-Bermejo A, Fernández-Real JM (2013) Changes in circulating microRNAs are associated with childhood obesity. J Clin Endocrinol Metab 98:E1655-1660. https://doi.org/10.1210/jc.2013-1496
61. Matsha TE, Kengne AP, Hector S, Mbu DL, Yako YY, Erasmus RT (2018) MicroRNA profiling and their pathways in South African individuals with prediabetes and newly diagnosed type 2 diabetes mellitus. Oncotarget 9:30485–30498. https://doi.org/10.18632/oncotarget.25271
62. Butler AE, Ramachandran V, Sathyapalan T, David R, Gooderham NJ, Benurwar M, Dargham SR, Hayat S, Najafi-Shoushtar SH, Atkin SL (2020) microRNA expression in women with and without polycystic ovarian syndrome matched for body mass index. Front Endocrinol (Lausanne) 11:206. https://doi.org/10.3389/fendo.2020.00206
63. Yang Q, Gu W-W, Gu Y, Yan N-N, Mao Y-Y, Zhen X-X, Wang J-M, Yang J, Shi H-J, Zhang X et al (2018) Association of the peripheral blood levels of circulating microRNAs with both recurrent miscarriage and the outcomes of embryo transfer in an in vitro fertilization process. J Transl Med 16:186. https://doi.org/10.1186/s12967-018-1556-x
64. O'Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO (2020) RNA delivery by extracellular vesicles in mammalian

cells and its applications. Nat Rev Mol Cell Biol 21:585–606. https://doi.org/10.1038/s41580-020-0251-y
65. Li Q, Xu Y, Lv K, Wang Y, Zhong Z, Xiao C, Zhu K, Ni C, Wang K, Kong M et al (2021) Small extracellular vesicles containing miR-486–5p promote angiogenesis after myocardial infarction in mice and nonhuman primates. Sci Transl Med 13:eabb0202. https://doi.org/10.1126/scitranslmed.abb0202
66. Li Z, Liu C, Li S, Li T, Li Y, Wang N, Bao X, Xue P, Liu S (2021) BMSC-derived exosomes inhibit dexamethasone-induced muscle atrophy via the miR-486-5p/FoxO1 axis. Front Endocrinol 12:681267. https://doi.org/10.3389/fendo.2021.681267
67. Brito MB, Goulielmaki E, Papakonstanti EA (2015) Focus on PTEN regulation. Front Oncol 5:166. https://doi.org/10.3389/fonc.2015.00166
68. Ghafouri-Fard S, Abak A, Shoorei H, Mohaqiq M, Majidpoor J, Sayad A, Taheri M (2021) Regulatory role of microRNAs on PTEN signaling. Biomed Pharmacother 113:110986. https://doi.org/10.1016/j.biopha.2020.110986
69. Gomez-Manzano C, Fueyo J, Jiang H, Glass TL, Lee H-Y, Hu M, Liu J-L, Jasti SL, Liu T-J, Conrad CA et al (2003) Mechanisms underlying PTEN regulation of vascular endothelial growth factor and angiogenesis. Ann Neurol 53:109–117. https://doi.org/10.1002/ana.10396
70. Lee S, Dong HH (2017) FoxO integration of insulin signaling with glucose and lipid metabolism. J Endocrinol 233:R67–R79. https://doi.org/10.1530/JOE-17-0002
71. Tzivion G, Dobson M, Ramakrishnan G (2011) FoxO transcription factors; regulation by AKT and 14-3-3 proteins. Biochem Biophys Acta 1813:1938–1945. https://doi.org/10.1016/j.bbamcr.2011.06.002
72. Emery AE (2002) The muscular dystrophies. Lancet 359:687–695. https://doi.org/10.1016/S0140-6736(02)07815-7
73. Feron M, Guevel L, Rouger K, Dubreil L, Arnaud M-C, Ledevin M, Megeney LA, Cherel Y, Sakanyan V (2009) PTEN contributes to profound PI3K/Akt signaling pathway deregulation in dystrophin-deficient dog muscle. Am J Pathol 174:1459–1470. https://doi.org/10.2353/ajpath.2009.080460
74. Kim MH, Kay DI, Rudra RT, Chen BM, Hsu N, Izumiya Y, Martinez L, Spencer MJ, Walsh K, Grinnell AD et al (2011) Myogenic Akt signaling attenuates muscular degeneration, promotes myofiber regeneration and improves muscle function in dystrophin-deficient mdx mice. Hum Mol Genet 20:1324–1338. https://doi.org/10.1093/hmg/ddr015
75. Lees SJ, Childs TE, Booth FW (2008) Age-dependent FOXO regulation of p27Kip1 expression via a conserved binding motif in rat muscle precursor cells. Am J Physiol Cell Physiol 295:C1238-1246. https://doi.org/10.1152/ajpcell.00349.2008
76. Bailey JL, Zheng B, Hu Z, Price SR, Mitch WE (2006) Chronic kidney disease causes defects in signaling through the insulin receptor substrate phosphatidylinositol 3-kinase/Akt pathway: implications for muscle atrophy. J Am Soc Nephrol 17:1388–1394. https://doi.org/10.1681/ASN.2004100842
77. Robinson KA, Baker LA, Graham-Brown MPM, Watson EL (2020) Skeletal muscle wasting in chronic kidney disease: the emerging role of microRNAs. Nephrol Dial Transplant 35:1469–1478. https://doi.org/10.1093/ndt/gfz193
78. Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M, Eckle T (2012) Myocardial ischemia reperfusion injury: from basic science to clinical bedside. Semin Cardiothorac Vasc Anesth 16:123–132. https://doi.org/10.1177/1089253211436350
79. Bekkers SC, Yazdani SK, Virmani R, Waltenberger J (2010) Microvascular obstruction: underlying pathophysiology and clinical diagnosis. J Am Coll Cardiol 55:1649–1660. https://doi.org/10.1016/j.jacc.2009.12.037
80. Wang J, Che H, Su Q, You Zhou M, Liu T, Li L (2016) The PTEN/Akt signaling pathway mediates myocardial apoptosis in swine after coronary microembolization. J Cardiovasc Pharmacol Ther 21:471–477. https://doi.org/10.1177/1074248415624158
81. Zhu H-H, Wang X-T, Sun Y-H, He W-K, Liang J-B, Mo B-H, Li L (2019) MicroRNA-486-5p targeting PTEN protects against coronary microembolization-induced cardiomyocyte apoptosis in rats by activating the PI3K/AKT pathway. Eur J Pharmacol 855:244–251. https://doi.org/10.1016/j.ejphar.2019.03.045
82. Gogiraju R, Bochenek ML, Schäfer K (2019) Angiogenic endothelial cell signaling in cardiac hypertrophy and heart failure. Front Cardiovasc Med 6:20. https://doi.org/10.3389/fcvm.2019.00020
83. Shi X-F, Wang H, Xiao F-J, Yin Y, Xu Q-Q, Ge R-L, Wang L-S (2016) MiRNA-486 regulates angiogenic activity and survival of mesenchymal stem cells under hypoxia through modulating Akt signal. Biochem Biophys Res Commun 470:670–677. https://doi.org/10.1016/j.bbrc.2016.01.084
84. Velinov N, Aebersold D, Haeni N, Hlushchuk R, Weinstein F, Sedlacek R, Djonov V (2007) Matrix metalloproteinase-19 is a predictive marker for tumor invasiveness in patients with oropharyngeal squamous cell carcinoma. Int J Biol Markers 22:265–273. https://doi.org/10.5301/JBM.2008.2632
85. Jost M, Folgueras AR, Frérart F, Pendas AM, Blacher S, Houard X, Berndt S, Munaut C, Cataldo D, Alvarez J et al (2006) Earlier onset of tumoral angiogenesis in matrix metalloproteinase-19-deficient mice. Cancer Res 66:5234–5241. https://doi.org/10.1158/0008-5472.CAN-05-4315
86. Brauer R, Beck IM, Roderfeld M, Roeb E, Sedlacek R (2011) Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen. BMC Biochem 12:38. https://doi.org/10.1186/1471-2091-12-38
87. Wang HE, Muntner P, Chertow GM, Warnock DG (2012) Acute kidney injury and mortality in hospitalized patients. Am J Nephrol 35:349–355. https://doi.org/10.1159/000337487
88. Uchino S, Bellomo R, Goldsmith D, Bates S, Ronco C (2006) An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. Crit Care Med 34:1913–1917. https://doi.org/10.1097/01.CCM.0000224227.70642.4F
89. See EJ, Jayasinghe K, Glassford N, Bailey M, Johnson DW, Polkinghorne KR, Toussaint ND, Bellomo R (2019) Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. Kidney Int 95:160–172. https://doi.org/10.1016/j.kint.2018.08.036
90. Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group (2012) KDIGO clinical practice guideline for acute kidney injury. Kidney Int Suppl 2:19–36. https://doi.org/10.1159/000339789
91. Bonventre JV, Yang L (2011) Cellular pathophysiology of ischemic acute kidney injury. J Clin Investig 121:4210–4221. https://doi.org/10.1172/JCI45161
92. Viñas JL, Spence M, Gutsol A, Knoll W, Burger D, Zimpelmann J, Allan DS, Burns KD (2018) Receptor-ligand interaction mediates targeting of endothelial colony forming cell-derived exosomes to the kidney after ischemic injury. Sci Rep 8:16320. https://doi.org/10.1038/s41598-018-34557-7
93. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group (2013) KDIGO 2012 clinical practice guideline for the evaluation and management of chronic kidney disease. Kidney Int Suppl 3:1–150
94. Umanath K, Lewis JB (2018) Update on diabetic nephropathy: core curriculum 2018. Am J Kidney Dis 71:884–895. https://doi.org/10.1053/j.ajkd.2017.10.026
95. Kato M, Natarajan R (2014) Diabetic nephropathy—emerging epigenetic mechanisms. Nat Rev Nephrol 10:517–530. https://doi.org/10.1038/nrneph.2014.116

96. Cen L, Xing F, Xu L, Cao Y (2020) Potential role of gene regulator NFAT5 in the pathogenesis of diabetes mellitus. J Diabetes Res. https://doi.org/10.1155/2020/6927429
97. Zhai S, Li M, Sun B, Han Y (2019) Amelioration of lipopolysaccharide-induced nephrotic proteinuria by NFAT5 depletion involves suppressed NF-κB activity. Inflammation 42:1326–1335. https://doi.org/10.1007/s10753-019-00993-4
98. Gooch JL, Barnes JL, Garcia S, Abboud HE (2003) Calcineurin is activated in diabetes and is required for glomerular hypertrophy and ECM accumulation. Am J Physiol Renal Physiol 284:F144–F154. https://doi.org/10.1152/ajprenal.00158.2002
99. Zhang L, Li R, Shi W, Liang X, Liu S, Ye Z, Yu C, Chen Y, Zhang B, Wang W et al (2013) NFAT2 inhibitor ameliorates diabetic nephropathy and podocyte injury in db/db mice. Br J Pharmacol 170:426–439. https://doi.org/10.1111/bph.12292
100. Lu A, Pallero MA, Owusu BY, Borovjagin AV, Lei W, Sanders PW, Murphy-Ullrich JE (2020) Calreticulin is important for the development of renal fibrosis and dysfunction in diabetic nephropathy. Matrix Biol Plus 8:100034. https://doi.org/10.1016/j.mbplus.2020.100034
101. Kim YJ, Hwang SH, Lee SY, Shin KK, Cho HH, Bae YC, Jung JS (2012) miR-486-5p induces replicative senescence of human adipose tissue-derived mesenchymal stem cells and its expression is controlled by high glucose. Stem Cells Dev 21:1749–1760. https://doi.org/10.1089/scd.2011.0429
102. Rutanen J, Yaluri N, Modi S, Pihlajamäki J, Vänttinen M, Itkonen P, Kainulainen S, Yamamoto H, Lagouge M, Sinclair DA et al (2010) SIRT1 mRNA expression may be associated with energy expenditure and insulin sensitivity. Diabetes 59:829–835. https://doi.org/10.2337/db09-1191
103. Balestrieri ML, Rienzo M, Felice F, Rossiello R, Grimaldi V, Milone L, Casamassimi A, Servillo L, Farzati B, Giovane A et al (2008) High glucose downregulates endothelial progenitor cell number via SIRT1. Biochim Biophys Acta 1784:936–945. https://doi.org/10.1016/j.bbapap.2008.03.004
104. Bouchareychas L, Duong P, Phu TA, Alsop E, Meechoovet B, Reiman R, Ng M, Yamamoto R, Nakauchi H, Gasper WJ et al (2021) High glucose macrophage exosomes enhance atherosclerosis by driving cellular proliferation & hematopoiesis. Science 24:102847. https://doi.org/10.1016/j.isci.2021.102847
105. Zheng W-H, Quirion R (2006) Insulin-like growth factor-1 (IGF-1) induces the activation/phosphorylation of Akt kinase and cAMP response element-binding protein (CREB) by activating different signaling pathways in PC12 cells. BMC Neurosci 7:51. https://doi.org/10.1186/1471-2202-7-51
106. Youness RA, El-Tayebi HM, Assal RA, Hosny K, Esmat G, Abdelaziz AI (2016) MicroRNA-486-5p enhances hepatocellular carcinoma tumor suppression through repression of IGF-1R and its downstream mTOR, STAT3 and c-Myc. Oncol Lett 12:2567–2573. https://doi.org/10.3892/ol.2016.4914
107. Laustsen PG, Russell SJ, Cui L, Entingh-Pearsall A, Holzenberger M, Liao R, Kahn CR (2007) Essential role of insulin and insulin-like growth factor 1 receptor signaling in cardiac development and function. Mol Cell Biol 27:1649–1664. https://doi.org/10.1128/MCB.01110-06
108. Dündar B, Akçoral A, Saylam G, Unal N, Meşe T, Hüdaoğlu S, Büyükgebiz B, Böber E, Büyükgebiz A (2000) Chronic hypoxemia leads to reduced serum IGF-1 levels in cyanotic congenital heart disease. J Pediatr Endocrinol Metab 13:431–436. https://doi.org/10.1515/jpem.2000.13.4.431
109. Mukai N, Nakayama Y, Murakami S, Tanahashi T, Sessler DI, Ishii S, Ogawa S, Tokuhira N, Mizobe T, Sawa T et al (2018) Potential contribution of erythrocyte microRNA to secondary erythrocytosis and thrombocytopenia in congenital heart disease. Pediatr Res 83:867–873. https://doi.org/10.1038/pr.2017.327
110. Ma R, Liang Z, Shi X, Xu L, Li X, Wu J, Zhao L, Liu G (2021) Exosomal miR-486-5p derived from human placental microvascular endothelial cells regulates proliferation and invasion of trophoblasts via targeting IGF1. Hum Cell 34:1310–1323. https://doi.org/10.1007/s13577-021-00543-x
111. Xiao Y (2020) MiR-486-5p inhibits the hyperproliferation and production of collagen in hypertrophic scar fibroblasts via IGF1/PI3K/AKT pathway. J Dermatol Treat. https://doi.org/10.1080/09546634.2020.1728210
112. Meng X-M, Nikolic-Paterson DJ, Lan HY (2016) TGF-β: the master regulator of fibrosis. Nat Rev Nephrol 12:325–338. https://doi.org/10.1038/nrneph.2016.48
113. Shi Y, Wang L, Yu P, Liu Y, Chen W (2019) MicroRNA-486-5p inhibits the growth of human hypertrophic scar fibroblasts by regulating Smad2 expression. Mol Med Rep 19:5203–5210. https://doi.org/10.3892/mmr.2019.10186
114. Liu B, Sun J, Lei X, Zhu Z, Pei C, Qin L (2017) MicroRNA-486-5p suppresses TGF-ß2-induced proliferation, invasion and epithelial mesenchymal transition of lens epithelial cells by targeting Smad2. J Biosci 42:575–584. https://doi.org/10.1007/s12038-017-9709-2
115. Wang H, Zheng G (2020) LncRNA NEAT1 promotes proliferation, migration, invasion and epithelial-mesenchymal transition process in TGF-β2-stimulated lens epithelial cells through regulating the miR-486-5p SMAD4 axis. Cancer Cell Int 20:529. https://doi.org/10.1186/s12935-020-01619-8
116. Piek A, de-Boer RA, Silljé HHW (2016) The fibrosis-cell death axis in heart failure. Heart Fail Rev 21:199–211. https://doi.org/10.1007/s10741-016-9536-9
117. Zhao H, Yang H, Geng C, Chen Y, Pang J, Shu T, Zhao M, Tang Y, Li Z, Li B et al (2021) Role of IgE-FcεR1 in pathological cardiac remodeling and dysfunction. Circulation 143:1014–1030. https://doi.org/10.1161/CIRCULATIONAHA.120.047852
118. Shi J, Guo K, Su S, Li J, Li C (2018) miR-486-5p is upregulated in osteoarthritis and inhibits chondrocyte proliferation and migration by suppressing SMAD2. Mol Med Rep 18:502–508. https://doi.org/10.3892/mmr.2018.8931
119. Tzachanis D, Freeman GJ, Hirano N, Puijenbroek AAV, Delfs MW, Berezovskaya A, Nadler LM, Boussiotis VA (2001) Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. Nat Immunol 2:1174–1182. https://doi.org/10.1038/ni730
120. Chen J, Liu M, Luo X, Peng L, Zhao Z, Hea C, He Y (2020) Exosomal miRNA-486-5p derived from rheumatoid arthritis fibroblast-like synoviocytes induces osteoblast differentiation through the Tob1/BMP/Smad pathway. Biomater Sci 8:3430. https://doi.org/10.1039/c9bm01761e
121. Lu Y, Wen H, Huang J, Liao P, Liao H, Tu J, Zeng Y (2020) Extracellular vesicle-enclosed miR-486-5p mediates wound healing with adipose-derived stem cells by promoting angiogenesis. J Cell Mol Med 00:1–15. https://doi.org/10.1111/jcmm.15387
122. Schunk SJ, Floege JR, Fliser D, Speer T (2021) WNT-β-catenin signalling—a versatile player in kidney injury and repair. Nat Rev Nephrol 17:172. https://doi.org/10.1038/s41581-020-00343-w
123. Huang R, Hu Z, Cao Y, Li H, Zhang H, Su W, Xu Y, Liang L, Melgiri ND, Jiang L (2019) MiR-652-3p inhibition enhances endothelial repair and reduces atherosclerosis by promoting Cyclin D2 expression. EBioMedicine 40:685–694. https://doi.org/10.1016/j.ebiom.2019.01.032
124. Poziello A, Nebbioso A, Stunnenberg HG, Martens JHA, Carafa V, Altuccia L (2021) Recent insights into histone acetyltransferase-1: biological function and involvement in pathogenesis. Epigenetics 16:838–850. https://doi.org/10.1080/15592294.2020.1827723

125. Parthun MR (2012) Histone acetyltransferase 1: more than just an enzyme? Biochim Biophys Acta 1819:256–263. https://doi.org/10.1016/j.bbagrm.2011.07.006
126. Liu D, Zhang M, Xie W, Lan G, Cheng H-P, Gong D, Huang C, Lv Y-C, Yao F, Tan Y-L et al (2016) MiR-486 regulates cholesterol efflux by targeting HAT1. Biochem Biophys Res Commun 472:418–424. https://doi.org/10.1016/j.bbrc.2015.11.128
127. Cao Q, Rong S, Repa JJ, Clair RS, Parks JS, Mishra N (2014) Histone deacetylase 9 represses cholesterol efflux and alternatively activated macrophages in atherosclerosis development. Arterioscler Thromb Vasc Biol 34:1871–1879. https://doi.org/10.1161/ATVBAHA.114.303393
128. Zhang J, Xu Z, Kong L, Gao H, Zhang Y, Zheng Y, Wan Y (2020) miRNA-486-5p promotes COPD progression by targeting HAT1 to regulate the TLR4-triggered inflammatory response of alveolar macrophages. Int J Chron Obstruct Pulmon Dis 15:2991–3001. https://doi.org/10.2147/COPD.S280614
129. Zammit PS, Relaix F, Nagata Y, Ruiz APR, Collins CA, Partridge TA, Beauchamp JR (2006) Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci 119:1824–1832. https://doi.org/10.1242/jcs.02908
130. Wang L-S, Li L, Li L, Chu S, Shiang K-D, Li M, Sun H-Y, Xu J, Xiao F-J, Sun G et al (2015) MicroRNA-486 regulates normal erythropoiesis and enhances growth and modulates drug response in CML progenitors. Blood 125:1302–1313. https://doi.org/10.1182/blood-2014-06-581926
131. Dori M, Cavalli D, Lesche M, Massalini S, Alieh LHA, Toledo BCD, Khudayberdiev S, Schratt G, Dahl A, Calegari F (2020) MicroRNA profiling of mouse cortical progenitors and neurons reveals miR-486-5p as a regulator of neurogenesis. Development 147:190520. https://doi.org/10.1242/dev.190520
132. Hanna J, Hossain GS, Kocerha J (2019) The Potential for microRNA therapeutics and clinical research. Front Genet 10:478. https://doi.org/10.3389/fgene.2019.00478
133. Hong DS, Kang Y-K, Borad M, Sachdev J, Ejadi S, Lim HY, Brenner AJ, Park K, Lee J-L, Ki T-Y et al (2020) Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. Br J Cancer 122:1630–1637. https://doi.org/10.1038/s41416-020-0802-1

Appendix E

Manuscript V

Connection to thesis: This manuscript is the protocol for the systematic review of miRNAs in human AKI.

Manuscript status: Published in the *Can J Kidney Health Dis* (PMID: 33996109)

Author contributions: Kevin D. Burns conceptualized the study. Adrianna Douvris drafted the initial manuscript. Risa Shorr, Adrianna Douvris, and Kevin D. Burns drafted the proposed search strategy. Rosendo A. Rodriguez, Manoj M. Lalu, and Edward G. Clark provided important input into the study design and data analysis components. Adrianna Douvris, Dylan Burger, Rosendo A. Rodriguez, Edward G. Clark, Manoj M. Lalu, José L. Viñas, and Kevin D. Burns all contributed to manuscript review and editing and approved the final version. Kevin D. Burns is the guarantor of the manuscript.

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MicroRNA in Human Acute Kidney Injury: A Systematic Review Protocol

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Abstract

Background: Acute kidney injury (AKI) is a common complication of hospitalization with high morbidity and mortality for which no effective treatments exist and for which current diagnostic tools have limitations for earlier identification. MicroRNAs (miRNAs) are small non-coding RNAs that have been implicated in the pathogenesis of AKI, and some miRNAs have shown promise as therapeutic tools in animal models of AKI. However, less is known about the role of miRNAs in human AKI.

Objective: To evaluate the role of miRNAs in human subjects with AKI.

Design: Systematic review and meta-analysis

Measurements: Quantification of miRNA levels from human blood, urine, or kidney biopsy samples, and measures of renal function as defined in the study protocol.

Methods: A comprehensive search strategy for Ovid MEDLINE All, Embase, Web of Science, and CENTRAL will be developed to identify investigational studies that evaluated the relationship between miRNA levels and human AKI. Primary outcomes will include measurements of kidney function and miRNA levels. Study screening, review and data extraction will be performed independently by 2 reviewers. Study quality and certainty of evidence will be assessed with validated tools. A narrative synthesis will be included and the possibility for meta-analysis will be assessed according to characteristics of clinical and statistical heterogeneity between studies.

Limitations: These include (1) lack of randomized trials of miRNAs for the prevention or treatment of human AKI, (2) quality of included studies, and (3) sources of clinical and statistical heterogeneity that may affect strength and reproducibility of results.

Conclusion: Previous studies of miRNAs in different animal models of AKI have generated strong interest on their use for the prevention and treatment of human AKI. This systematic review will characterize the most promising miRNAs for human research and will identify methodological constraints from miRNA research in human AKI to help inform the design of future studies.

Systematic review registration: PROSPERO CRD42020201253

Abrégé

Contexte: L'insuffisance rénale aiguë (IRA) est une complication fréquente des hospitalisations avec morbidité et mortalité élevées. Il n'existe aucun traitement efficace contre l'IRA et les outils diagnostiques actuels qui permettent son dépistage précoce comportent des limites. Les microARN (miARN) sont de petits ARN non codants ayant été impliqués dans la pathogenèse de l'IRA; certains d'entre eux se sont révélés prometteurs comme outils thérapeutiques dans les modèles animaux de l'IRA. Le rôle des miARN dans l'IRA chez l'humain est cependant moins connu.

Objectif: Évaluer le rôle des miARN chez les sujets humains atteints d'IRA.

Type d'étude: Examen systématique et méta-analyze

Mesures: La quantification des taux de miARN chez l'humain à partir d'échantillons de sang, d'urine ou de biopsie rénale, et mesure de la fonction rénale telle que définie dans le protocole de l'étude.

Méthodologie: Une stratégie de recherche exhaustive des bases de données Ovid MEDLINE All, Embase, Web of Science et CENTRAL sera élaborée afin de répertorier les études expérimentales ayant évalué la relation entre les taux de miARN et l'IRA chez l'humain. Les principaux critères d'évaluation comprendront la mesure de la fonction rénale et des taux de miARN. Deux examinateurs procéderont de façon indépendante à la sélection des études, à leur examen et à l'extraction des données. La qualité des études et la robustesse des données seront évaluées à l'aide d'outils validés. Une synthèse descriptive

sera incluse et la possibilité d'une méta-analyze sera évaluée en fonction des caractéristiques de l'hétérogénéité clinique et statistique entre les études.
Limites: Les limites de l'étude concernent notamment (i) le manque d'essais randomisés examinant les miARN pour la prévention ou le traitement de l'IRA humaine; (ii) la qualité des études incluses; et (iii) les sources d'hétérogénéité clinique et statistique susceptibles d'affecter la robustesse et la reproductibilité des résultats.
Conclusion: Des études antérieures sur les miARN dans différents modèles animaux de l'IRA ont suscité un vif intérêt pour leur utilization dans la prévention et le traitement de l'IRA chez l'humain. Cet examen systématique caractérisera les miARN les plus prometteurs pour la recherche sur l'IRA humaine et définira les contraintes méthodologiques de telles études, ce qui aidera à orienter la conception des études futures.

Keywords
acute kidney injury, systematic review, human, microRNA, biomarker

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Background

Acute kidney injury (AKI) refers to a rapid decline in kidney function. Acute kidney injury affects up to 20% of hospitalized patients particularly in those admitted to intensive care units (ICU).[1] Patients who develop AKI have an increased risk of death[1] and are more likely to have long term adverse outcomes if they recovered from AKI. In a meta-analysis of 82 studies with 2 million hospitalized adults followed for minimum 1 year, AKI was associated with a nearly 2-fold increased risk of death, a 3-fold increased risk of new or progressive chronic kidney disease (CKD), and a 4-fold increased risk of kidney failure compared to patients without AKI.[2] The incremental cost of AKI in Canada is estimated to exceed CAN$200 million per year, and greater severity AKI is associated with incremental increases in both hospital length of stay and costs.[3] Despite the health and economic burden of AKI, no effective interventions currently exist for treatment,[4] preventative measures are limited,[5] and current diagnostic tools including serum creatinine and urine output have major limitations for earlier identification.[6]

AKI can be caused by different mechanisms, including ischemia-reperfusion injury (IRI), sepsis, and nephrotoxins. At the cellular level, proximal tubular and endothelial cell injury involve pathways in inflammation, apoptosis, angiogenesis, and fibrosis.[7] MicroRNAs (miRNAs) are small noncoding RNAs, well conserved across species, that regulate gene expression at the post-transcriptional level by binding the 3′-untranslated region (UTR) of messenger RNA (mRNA), thereby inhibiting mRNA translation and promoting mRNA degradation.[8] Thus far, more than 1500 miRNAs have been identified in humans and have been found to be involved in many biological processes including cell cycle regulation, apoptosis, hypoxia, metabolism, immunity, and oncogenesis.[7] Over the last 10 years, there has been an expanding effort to evaluate the role of miRNAs in AKI. This is reflected by the increasing annual number of publications on this topic over this time period in PubMed: from 4 publications in 2010, to an average of 50-60 publications annually over the last 5 years.

Several miRNAs have been studied in animal models of AKI—the most frequent of which is miR-21—for their expression patterns, mechanisms of action and therapeutic potential.[9] For instance, Song *et al* evaluated the effect of miR-21 knockdown on AKI using in vitro hypoxia/re-oxygenation and in vivo mouse IRI models.[10] These knockdown studies suggest that miR-21 protects against ischemic AKI. miR-21 knockdown intensified ischemia-induced renal epithelial cell injury by (1) enhanced apoptosis through the phosphatase and tensin homolog (PTEN)/Akt/mammalian target of rapamycin (mTOR)/hypoxia-inducible factor (HIF) pathways and (2) increased inflammation by promoting dendritic cell maturation.[10]

Pre-clinical research is an important step for the translation of basic science to human studies, and a systematic review of miRNAs as therapy for pre-clinical models of AKI

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is underway.[11] Although miRNAs are showing promise as therapies in pre-clinical AKI models, much less is known about their potential as a therapy to prevent or treat AKI in humans.[12] Furthermore, pre-clinical studies have not readily translated to human application and study design flaws are a contributing factor. For example, many studies of IRI-AKI are conducted in male animals.[13] However, animal experimental models have shown that female sex confers protection against IRI-AKI,[14-17] an observation that has also been noted from observational studies and meta-analysis in humans.[18,19]

Currently, no clinical trials on the therapeutic use of miRNA in human AKI have been registered (clinicaltrials.gov, https://www.isrctn.com/), but several miRNAs have been evaluated as biomarkers of AKI and other kidney diseases.[12] In cardiac surgery and critically ill patients, small studies that measured urinary or plasma levels of miRNAs as a prognostic marker of AKI have reported conflictive results and this highlights the complexity of miRNA pathways.[7] One such example is miR-21, whose levels have been shown to be increased in the urine and plasma of critically ill and cardiac surgery patients with AKI,[20-22] yet another study of cardiac surgery patients found that lower pre-operative plasma miR-21 levels conferred an increased risk of developing cardiac surgery-associated AKI.[23] This systematic review will evaluate many aspects of miRNAs related to human AKI, including an improved understanding of the pathophysiology related to different AKI causes, and will identify methodological limitations, sources of heterogeneity and outcome measure differences, which will help inform the planning and design of clinical trials.

Methods

Study Design

This systematic review will be reported in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements, and the Cochrane Handbook for Systematic Reviews of Interventions will be used as best-practice guidance.[24,25] The study protocol was written in accordance with PRISMA for protocols (PRISMA-P) and is registered with the International Prospective Register of Systematic Reviews (PROSPERO CRD42020201253). A populated PRISMA-P checklist is provided in supplementary file 1.

Objectives

The primary aim is to evaluate the role of miRNAs in human AKI. The secondary aims will be to evaluate the association between miRNAs and renal outcomes including AKI severity, the incidence of dialysis-requiring AKI, renal recovery, and in-hospital mortality. The relationship between timing of miRNA measures in AKI and clinical outcomes, and the effect of sex on miRNA expression in AKI will be evaluated if possible.

Types of Studies

Observational and interventional studies (randomized and non-randomized) evaluating miRNAs in human AKI that meet the inclusion criteria below will be included. Narrative reviews, editorials, and case reports will be excluded.

Population. Studies of human subjects (adult and pediatric populations) with both dialysis-requiring and non-dialysis-requiring AKI will be included. AKI will be defined broadly using RIFLE and AKIN definitions outlined in the KDIGO clinical practice guidelines for AKI.[5] All AKI causes will be included (ie, etiology), but if there are sufficient number of studies, subgroup analyses will be conducted to determine any differences in the etiology of AKI. Studies of patients with end-stage kidney disease treated with kidney replacement therapy (maintenance dialysis) will be excluded.

Studies involving non-human animal and cell culture models will be excluded.

Exposure. The exposure of interest is AKI. Studies will characterize the relationship between the presence of AKI, AKI severity and etiology, and miRNA levels.

Comparator(s). Studies will be comparing miRNA levels in patients with AKI to those without AKI. In the event there is no comparator group, studies may compare miRNA levels with differing severity of AKI.

Confounders. These include any variables that can influence miRNA levels. As miRNAs affect a range of biological processes and their expression is dysregulated in many diseases, confounders include AKI cause and CKD,[12] diabetes mellitus,[26] infection/sepsis,[27] cancer,[28,29] and medications (ie anticoagulants[30] and anti-cancer agents).[29] Age will also be explored as a confounding factor.

Outcomes

(a) *Primary outcome*: miRNA levels from blood, urine, or kidney tissue in association with AKI. miRNA quantification methods may include but are not limited to quantitative PCR, microarray, or quantification using fluorescence *in situ* hybridization.

(b) *Secondary outcomes*: (1) Measurement of renal function by serum Cr, BUN or urea, or urine volume; (2) biomarkers of AKI[31] (ie neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1) or others), and kidney structural changes by kidney biopsy for histological analyses if available; (3) measures of association between miRNA levels and other outcomes of interest: (i) severity of AKI, (ii) dialysis-requiring AKI, and (iii) complete or

partial renal recovery (broad criteria due to lack of uniform definition[32] includes serum Cr $< 1.5\times$ baseline, or, no longer requiring renal replacement therapy, or, serum Cr decreases but does not return to $<1.5\times$ baseline), and (iv) in-hospital mortality.

(c) *Exploratory outcomes*: (i) the association between sex and miRNA levels in AKI, (ii) between miRNA level and AKI cause, and (iii) timing of miRNA measurement and clinical outcomes.

Search Strategy

We performed an initial search using PubMed for prior systematic reviews on this topic. We also searched PROSPERO to identify currently registered systematic reviews. We did not identify any prior or ongoing systematic review.

We will implement a comprehensive search strategy in collaboration with a research librarian (provided in supplementary file 2). The search will include terms related to acute kidney injury, miRNA, and extracellular vesicles (which may contain miRNA as cargo).

We will search the following databases from years 1947 to present:

- Ovid MEDLINE All
- Embase
- CENTRAL (Cochrane Central Register of Controlled Trials)
- Web of Science

The search will be supplemented by manually scanning the reference list of all included studies for additional material. Editorials, review articles, case reports, patent applications, and studies involving only animal or in-vitro experiments will be excluded. Abstracts from the last 5 years from the American Society of Nephrology scientific meetings will be reviewed. A search of clinicaltrials.gov for any contemporary or previous protocols will be conducted to verify eligible studies.

Study Selection

Titles and abstracts initially identified from our search will be exported to Covidence for independent screening by at least 2 investigators. Duplicate citations will be removed.

The following requirements must be met for an article to be considered for full text review:

- Original study (observational or interventional) of human AKI with miRNA expression measures. We will exclude non-original research (ie review or editorials), and pre-clinical studies.
- Possible causes of AKI include but are not limited to (1) pre-renal, hepatorenal, or cardio-renal syndrome; (2) acute tubular necrosis; (3) major surgery (ie cardiac, vascular, intra-abdominal); (4) shock; (5) sepsis; (6) nephrotoxins; (7) acute obstruction; (8) acute interstitial nephritis; (9) acute glomerulonephritis; (10) malignancy-associated AKI and (11) AKI in renal transplant recipients including delayed graft function and acute rejection. Studies of patients with end-stage kidney disease treated with kidney replacement therapy (maintenance dialysis) will be excluded.
- Studies in English and French languages will be included.
- Studies that do not specifically address miRNA or kidney function will be excluded.

The 2 reviewers will also document the primary reason for article exclusion after full text review. Any discrepancies between included and excluded studies between the 2 independent reviewers will be discussed for a consensus decision. If a consensus cannot be reached, a third reviewer will provide an independent opinion. We will calculate the kappa statistic for inter-reviewer reliability.

Data Extraction

A data extraction form will be created and piloted prior to duplicate data extraction by 2 independent reviewers. The data extracted will include the following:

- *Study characteristics*: authors, journal information, publication year, geographic location, in-patient setting (ward, ICU, post-cardiac surgery, transplant), study design, type of publication (abstract or full manuscript).
- *Population characteristics*: Age, sex, ethnicity, comorbidities, AKI setting (ie ICU, post-cardiac surgery, etc), admission diagnosis, AKI cause.
- Type of exposure and comparator:
    - Type of kidney insult responsible for AKI if applicable to the study (ie cardiac surgery, sepsis, nephrotoxin) and timing of AKI if available.
    - Control group characteristics
    - Duration of follow-up.
- Outcomes:
    - *Primary outcome*: miRNA characteristics: variant, type of sample (blood, urine, kidney tissue), timing of measurement relative to AKI, method of miRNA measurement including assay and instrument. Normalization methods and measurement units for miRNA expression will be identified.
    - *Secondary and exploratory outcomes*: (i) measures of renal function and markers of AKI as described above; (ii) AKI severity, incidence of dialysis-requiring AKI, in-hospital mortality, renal recovery;

(iii) comparison of miRNA levels with presence of AKI, renal outcomes, and sex; (iv) relationship between miRNA and AKI cause; and (v) timing of miRNA measurement and clinical outcomes

If information is missing from a study, attempts will be made to contact the study authors. The data will be exported from Covidence for data analysis.

Risk of Bias and Quality Assessment

Two independent reviewers will assess the included studies for potential bias and quality. Non-randomized studies will be evaluated for risk of bias using the Newcastle-Ottawa Scale (NOS),[33] which evaluates study group selection, comparability, and outcomes. If randomized studies meeting inclusion criteria are identified, risk of bias will evaluated with the Cochrane Handbook Risk of Bias Assessment Tool.[34] Any disagreements will be resolved by discussion, and, if not possible, then a third reviewer.

Data Synthesis and Analysis

The decision to perform a meta-analysis on the primary outcome will depend on the assessment of statistical and clinical heterogeneity. Assessment of clinical heterogeneity between studies will be based on miRNA variant, sample type (blood, urine, kidney biopsy), miRNA normalization methods, AKI cause, and timing of measurement relative to AKI if available. We will assess statistical heterogeneity using the I^2 statistic.[35] If statistical heterogeneity between studies is high ($I^2 > 50\%$ and deemed to represent considerable heterogeneity), then data will be reported descriptively for the outcomes of interest. In these cases, we will provide a narrative synthesis of included studies using the Synthesis Without Meta-analysis (SWiM) reporting guideline as a framework.[36] If clinical and statistical heterogeneity are acceptable, studies with similar outcomes measures and miRNAs will be pooled to calculate pooled weight effect estimates using the inverse variance method, and data will be modeled according to the DerSimonian-Laird Method (random effects model).[37]

If sufficient studies are eligible, we will report on likely confounders of miRNA expression, including cause of AKI, underlying comorbidities (ie CKD, diabetes mellitus, cancer), medications, and acute medical conditions (ie type of surgery, infection, shock, acute coronary syndrome). We will assess the quality of control for confounders by reporting whether studies: (1) had balanced groups, (2) matched subjects for the confounders, and (3) adjusted for the confounders in their statistical analyses. Where possible, we will perform sub-group analyses listed in the following.

Subgroup Analysis

If data are available from included studies, subgroup analyses will include the following:

(a) Population characteristics:
   a. Pediatric vs adult population
   b. Male vs female sex

(b) Exposures:
   a. AKI cause
   b. Severity of AKI

(c) Outcomes:
   a. Timing of miRNA measurement relative to AKI: categorized as (1) prior to AKI onset, (2) early AKI (within 48 hours of injury), or (3) late (after 48 hours)
   b. Sample type (blood, urine, or tissue)
   c. Requirement for renal replacement therapy (yes/no)
   d. Renal recovery (yes/no)
   e. In-hospital mortality (yes/no)

Assessment of reporting biases will be performed on the primary outcome by constructing a funnel plot if an adequate number of studies is identified.[35]

Data analysis will be performed using RevMan 5.3.

Assessing the Quality of Evidence

The quality of evidence for the primary outcome will be assessed as "very low" to "high" in accordance to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Workgroup.[38] Limitations of included studies will also be identified with suggestions for improvement where possible.

Discussion

AKI occurs frequently in hospitalized patients and is associated with increased morbidity and mortality. There is an important need for tools for early detection of AKI, and the development of novel therapies for its prevention and treatment. Pre-clinical miRNA studies in AKI have shown promise as potential AKI markers and therapies. Those studies have uncovered plausible biological mechanisms implicating several miRNAs in the pathogenesis of AKI. More than 50 different miRNAs are differentially expressed in AKI,[8] but their significance in human AKI is unclear. In addition, translating pre-clinical AKI research to clinical practice has proven to be challenging due to pre-clinical methodological issues that are beyond the scope of this review, and important differences between experimental AKI models and human AKI.[13] For instance, rodents and other mammals lack modeling for common medical comorbidities such as CKD, diabetes mellitus, or hypertension which impact AKI prognosis. There are sex-specific differences in AKI susceptibility, thus most pre-clinical studies are conducted in male animals. Of note, gender may have effects independently of biological sex;[39] however, this is unlikely to be reported. Pre-clinical models are also not truly representative of AKI in humans which is often multifactorial in the setting of systemic

illness. Another consideration is the timing of intervention in human AKI: with the exception of specific circumstances (ie planned cardiac surgery), the onset of AKI is often unknown or identified late.

A systematic review of miRNAs in human AKI will identify the miRNAs selected for study in humans thus far, the reason for their selection (ie biological mechanism, pre-clinical results) and may uncover other miRNAs that have not yet been studied in pre-clinical models, creating new research opportunities for scientists. Our systematic review of miRNAs in experimental AKI models[11] will serve to facilitate the comparison of miRNAs in pre-clinical versus human AKI. Further, a systematic review of miRNAs in human AKI will address the heterogeneity and controversy among studies of miRNA in AKI by identifying the directionality and magnitude of miRNA associations with AKI. This information can be used to determine if pre-clinical studies support the direction of effects seen in human AKI, and, if quantitative analysis of effect size is possible, can be helpful to calculate an estimated sample size in future clinical trials. It will also identify important limitations from existing studies that could be used by researchers to improve future clinical trial design. These include methodological limitations, sources of heterogeneity, and confounders that could impact accuracy of results, and outcome measures. Overall, this information will inform researchers and clinicians on better planning further well-designed studies for both AKI diagnosis and treatment.

There are limitations to this planned systematic review. We do not expect to identify human clinical trials of miRNA delivery systems for the prevention or treatment of AKI. Our data will come from descriptive clinical studies which will not provide mechanistic information on their own without pre-clinical experimental models. The timing of miRNA measures relative to AKI may not be possible to control in some clinical settings which may affect the primary outcome, but timing will be considered in the analysis. Finally, small sample sizes and study design with respect to case and control cohorts may also affect the strength and reproducibility of results.

The field of miRNA therapeutics is beginning to evolve from pre-clinical studies to phase I/II clinical trials in other disciplines. In Oncology, the first such phase I trial used a miR-34 mimic (MRX34) with an intravenous nanoparticle liposomal delivery system for 85 adult patients with refractory advanced solid tumors.[40] Although the study was able to show miR-34 delivery to tumors and dose-dependent target gene modulation, it was stopped early due to serious immune-related adverse effects.[41] In the treatment of hepatitis C infection, a retrospective follow up analysis of a phase 2a multicentre trial found that the locked nucleic acid antisense inhibitor of miR-122 miravirsen had a sustained virological response in 58% (7/12) of trial participants and no significant adverse effects over a 35-month follow-up period.[42] Given miRNA therapeutics are being studied in clinical trial settings for other illnesses, this could be a promising direction for the prevention and treatment of AKI.

Conclusions

A systematic review and meta-analysis of the role of miRNAs in human AKI will be conducted. This study will identify and characterize the most promising miRNAs for further AKI research and methodological limitations from current miRNA research in human AKI that will help improve the design of future clinical and experimental studies in this area.

List of Abbreviations

AKI, acute kidney injury; AKIN, acute kidney injury network; BUN, blood urea nitrogen; CKD, chronic kidney disease; Cr, creatinine; GRADE, grading of recommendations assessment, development and evaluation; ICU, intensive care unit; IRI, Ischemia reperfusion injury; KDIGO, kidney disease improving global outcomes; mRNA, messenger RNA; miRNA, microRNA; NOS, Newcastle Ottawa Scale; RIFLE, risk, injury, failure, loss, end-stage kidney disease; UTR, untranslated region.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

All authors have given their consent for publication.

Availability of Data and Materials

All data are available upon request.

Author Contributions

KDB conceptualized the study. AD drafted the initial manuscript. RS, AD, and KDB drafted the proposed search strategy. RAR, MML, and EC provided important input into the study design and data analysis components. AD, DB, RAR, EC, MML, JV, and KDB all contributed to manuscript review and editing and approved the final version. KDB is the guarantor of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

References

1. Wang HE, Muntner P, Chertow GM, Warnock DG. Acute kidney injury and mortality in hospitalized patients. *Am J Nephrol*. 2012;35:349-355.
2. See EJ, Jayasinghe K, Glassford N, et al. Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. *Kidney Int*. 2019;95(1):160-172.
3. Collister D, Pannu N, Ye F, et al. Health care costs associated with AKI. *Clin J Am Soc Nephrol*. 2017;12:1733-1743.
4. Jo SK, Rosner MH, Okusa MD. Pharmacological treatment of acute kidney injury: why drugs haven't worked and what is on the horizon. *Clin J Am Soc Nephrol*. 2007;2:356-365.
5. KDIGO clinical practice guidelines for acute kidney injury. *Kidney Int Suppl*. 2012;2:19-36.
6. Romagnoli S, Ricci Z, Ronco C. Perioperative acute kidney injury: prevention, early recognition, and supportive measures. *Nephron*. 2018;140(2):105-110.
7. Jones TF, Bekele S, O'Dwyer MJ, Prowle JR. MicroRNAs in acute kidney injury. *Nephron*. 2018;140:124-128.
8. Fan P-C, Chen C-C, Chen Y-C, Chang Y-S, Chu P-H. MicroRNAs in acute kidney injury. *Hum Genom*. 2016;10:29.
9. Brandenburger T, Somoza AS, Devaux Y, Lorenzen JM. Noncoding RNAs in acute kidney injury. *Kidney Int*. 2018; 94:870-881.
10. Song N, Zhang T, Xu X, et al. MiR-21 protects against ischemia/reperfusion-induced acute kidney injury by preventing epithelial cell apoptosis and inhibiting dendritic cell maturation. *Front Physiol*. 2018;9:790.
11. Zankar S, Rodriguez RA, Vinas JL, Burns KD. The therapeutic effects of microRNAs in preclinical studies of acute kidney injury: a systematic review protocol. *Syst Rev*. 2019;8:235.
12. Brandenburger T, Lorenzen JM. Diagnostic and therapeutic potential of microRNas in acute kidney injury. *Front Pharmacol*. 2020;11:657.
13. de Caestecker M, Humphreys BD, Liu KD, et al. Bridging translation by improving preclinical study design in AKI. *J Am Soc Nephrol*. 2015;26(12):2905-2916.
14. Viñas JL, Porter CJ, Douvris A, et al. Sex diversity in proximal tubule and endothelial gene expression in mice with ischemic acute kidney injury. *Clinical Science*. 2020;134:1887-1909.
15. Tanaka R, Tsutsui H, Ohkita M, Takaoka M, Yukimura T, Matsumura Y. Sex differences in ischemia/reperfusion-induced acute kidney injury are dependent on the renal sympathetic nervous system. *Eur J Pharmacol*. 2013;714:397-404.
16. Kher A, Meldrum KK, Wang M, Tsai BM, Pitcher JM, Meldrum DR. Cellular and molecular mechanisms of sex differences in renal ischemia–reperfusion injury. *Cardiovasc Res*. 2005;67:594-603.
17. Kang KP, Lee JE, Lee AS, et al. Effect of gender differences on the regulation of renal ischemia-reperfusion-induced inflammation in mice. *Mol Med Rep*. 2014;9(6):2061-2068.
18. Neugarten J, Golestaneh L, Kolhe NV. Sex differences in acute kidney injury requiring dialysis. *BMC Nephrology*. 2018; 19:131.
19. Neugarten J, Golestaneh L. Female sex reduces the risk of hospital-associated acute kidney injury: a meta- analysis. *BMC Nephrology*. 2018;19:314.
20. Saikumar J, Hoffmann D, Kim T-M, et al. Expression, circulation, and excretion profile of MicroRNA-21, -155, and -18a following acute kidney injury. *Toxicol Sci*. 2012;129(2): 256-267.
21. Ramachandran K, Saikumar J, Bijol V, et al. Human miRNome profiling identifies MicroRNAs differentially present in the urine after kidney injury. *Clin Chem*. 2013;59(12): 1742-1752.
22. Du J, Cao X, Zou L, et al. MicroRNA-21 and risk of severe acute kidney injury and poor outcomes after adult cardiac surgery. *PLoS ONE*. 2013;8:e63390.
23. Gaede L, Liebetrau C, Blumenstein J, et al. Plasma microRNA-21 for the early prediction of acute kidney injury in patients undergoing major cardiac surgery. *Nephrol Dial Transplant*. 2016;31(5):760-766.
24. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Plos Medicine*. 2009;6:e1000097.
25. Higgins J, Thomas J, Chandler J, et al. *Cochrane Handbook for Systematic Reviews of Interventions Verion 6.1*. 2nd ed. Chichester, England: John Wiley & Sons; 2020.
26. Zampetaki A, Kiechl S, Drozdov I, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circ Res*. 2010;107:810-817.
27. Benz F, Roy S, Trautwein C, Roderburg C, Luedde T. Circulating MicroRNAs as Biomarkers for Sepsis. *Int J Mol Sci*. 2016;17:78.
28. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov*. 2017;16(3):203-222.
29. To KK, Tong CW, Wu M, Cho WC. MicroRNAs in the prognosis and therapy of colorectal cancer: from bench to bedside. *World J Gastroenterol*. 2018;24:2949-2973.
30. Barraclough JY, Joan M, Joglekar MV, Hardikar AA, Patel S. MicroRNAs as prognostic markers in acute coronary syndrome patients—A systematic review. *Cells*. 2019;8:1572.
31. Kashani K, Cheungpasitporn W, Ronco C. Biomarkers of acute kidney injury: the pathway from discovery to clinical adoption. *Clin Chem Lab Med*. 2017;55:1074-1089.
32. Kellum JA. How can we define recovery after acute kidney injury? Considerations from epidemiology and clinical trial design. *Nephron Clin Pract*. 2014;127(1-4):81-88.
33. Wells G, Shea B, O'Connell D, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses. http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp. Published 2012. Accessed April 7, 2021.
34. Higgins JPT, Altman DG, Gøtzsche PC, et al. The Cochrane Collaboration's tool for assessing risk of bias in randomized trials. *BMJ*. 2011;343:d5928.

35. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Statistics in Medicine*. 2002;21:1539-1558.
36. Campbell M, McKenzie JE, Sowden A, et al. Synthesis without meta-analysis (SWiM) in systematic reviews: reporting guideline. *BMJ*. 2020;368:16890.
37. Deeks J, Higgins J, Altman D. Chapter 10: analysing data and undertaking meta-analyses. In: Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, Welch VA, eds. *Cochrane Handbook for Systematic Reviews of Interventions*. Version 6.1. Cochrane. www.training.cochrane.org/handbook. Published 2020. Accessed April 7, 2021.
38. Guyatt GH, Oxman AD, Vist GE, et al. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ*. 2008;336:924-926.
39. Pelletier R, Khan NA, Cox J, et al. Sex versus gender-related characteristics: which predicts outcome after acute coronary syndrome in the young? *J Am Coll Cardiol*. 2016;67:127-135.
40. Beg MS, Brenner AJ, Sachdev J, et al. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs*. 2017;35(2):180-188.
41. Hong DS, Kang Y-K, Borad M, et al. Phase I study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours. *Br J Cancer*. 2020;122:1630-1637.
42. van der Ree MH, van der Meer AJ, de Bruijne J, et al. Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. *Antiviral Res*. 2014;111:53-59.