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Unilateral ischemia-reperfusion as a model for acute-to-chronic kidney disease

DEVELOPMENT AND APPLICATION

Unilaterale ischemie-reperfusie als model voor acuut-naar-chronisch nierfalen

ONTWIKKELING EN TOEPASSINGEN

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Unilateral ischemia-reperfusion as a model for acute-to-chronic kidney disease: development and application / N. Le Clef

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List of Abbreviations

α-SMA	α smooth muscle
AdC	decitabine
AKI	acute kidney injury
BIRI	bilateral ischemia-reperfusion injury
BMP	bone morphogenetic protein
BUN	blood urea nitrogen
CCN1, 2, 3	acronym, Cyr61, CTGF, and NOV
CKD	chronic kidney disease
CpG	cytosine nucleotide followed by a guanine nucleotide
CTGF	connective tissue growth factor (CCN2)
Cyr61	cysteine-rich protein 61
DC	dendritic cell
DGF	delayed graft function
DNMT	DNA-methyltransferase
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
EndMT	endothelial-mesenchymal transition
ESRD	end-stage renal disease
GFR	glomerular filtration rate
HAVCR1	hepatitis A virus receptor 1 (kidney injury molecule-1; KIM-1)
IL	interleukin
IRI	ischemia-reperfusion injury
JNK	c-Jun N-terminal kinases
KDIGO	Kidney Disease: Improving Global Outcomes
LCN2	lipocalin 2 (neutrophil gelatinase-associated lipocalin; NGAL)
MCP-1	monocyte chemoattractant protein
MSC	mesenchymal stem cell
NBF	neutral buffered formalin
NFkB	nuclear factor NF-kB
NO	nitric oxide
NOV	nephroblastoma overexpressed
NSAID	non-steroidal anti-inflammatory drugs
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
qPCR	quantitative polymerase chain reaction
rhCCN3	recombinant human CCN3
ROS	reactive oxygen species
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
UIRI	unilateral ischemia-reperfusion injury
UPLC	ultra-pressure liquid chromatography
UUO	unilateral ureteral obstruction
WT	wild-type

Chapter 1

Introduction

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Chapter 1: Contents

Abstract

Despite decades of scientific research, the incidence and prevalence of acute kidney injury (AKI) is still increasing worldwide. In addition, it is becoming increasingly clear that patients with a history of AKI often do not completely recover renal function, and thus contribute to the growing patient population with chronic kidney disease (CKD). Ischemia is an important aetiology of AKI, as it is inherent to renal transplantation, and a complication of major bleeding, shock and surgery, such as coronary artery bypass graft, aortic aneurysm surgery or thoraco-abdominal procedures. The pathophysiology underlying acute ischemic kidney injury is multifactorial and involves many different pathways (such as apoptosis, vascular dysfunction and inflammation) and cell types (such as fibroblasts, tubular, endothelial and inflammatory cells) which are discussed in this chapter. Furthermore, mechanisms possibly underlying progression from acute to chronic renal injury, such as prolonged production of pro-fibrotic cytokines and chemokines, inflammation and hypoxia, are reviewed in this chapter.

1.1 Incidence and clinical features of acute renal injury

Acute kidney injury (AKI) refers to a clinical syndrome characterized by a rapid decrease in renal function, occurring over hours to days, with the accumulation of waste products such as creatinine and urea, and dysregulation of fluid, electrolyte, and acid-base homeostasis[1,2]. According to the KDIGO (Kidney Disease: Improving Global Outcomes) guidelines, AKI is defined as one of the following: an increase in serum creatinine of more than 0.3 mg/dl within 48 hours; or an increase in serum creatinine to 1.5 times baseline within the prior 7 days; or a urine output of less than 0.5 ml/kg/h for 6 hours[3]. AKI comprises a variety of aetiologies, including intrinsic renal pathology (acute interstitial nephritis, acute glomerular and vascular renal diseases); non-specific renal aetiologies (ischemia-reperfusion, toxic injury); as well as extra-renal pathology (pre-renal azotaemia and acute post-renal obstructive nephropathy)[3]. Hypoperfusion, a complication of major surgery and sepsis, can induce ischemia-reperfusion injury to the kidney and, together with nephrotoxins, is the main cause of AKI[4,5]. Also, renal ischemia is an inevitable phenomenon in kidney transplantation[6] and is one of the factors contributing to delayed graft function (DGF)[7]. The manifestations and clinical consequences of AKI are similar regardless of aetiology and comprise both direct injury to the kidney as well as acute impairment of renal function[3].

The incidence of AKI is difficult to estimate. The epidemiology of AKI is not well known because of the lack of a broadly accepted general clinical definition which resulted in more than 30 operational definitions of AKI used in published studies[2]. As a consequence, reported incidence vary according to the definitions used and the populations under study[8]. Also, diagnosis and severity of AKI is based on a rise in serum creatinine levels, which is considered a suboptimal marker of glomerular filtration rate (GFR) and an insensitive late marker of AKI, as it may rise 2-3 days after the insult[5,9]. Nonetheless, an increased incidence of AKI was noted between 1988 and 2003[8]. Furthermore, each year the incidence for non-dialysis-requiring AKI increases with more than 5000 cases per million people (0.5%) and 295 cases per million people requiring dialysis (0.03%). Each year, 19% of hospitalized patients are affected, especially critically ill patients, with a prevalence greater than 40% at admission to the intensive-care unit if sepsis is present, more than 36% on the day after admission to an intensive-care unit, and more than 60% during intensive-care-unit admission[1]. Thus, AKI is a common complication of critical illness, which is associated with high mortality.

Renal ischemia is inevitable in kidney transplantation. During the transplant procedure the kidney first suffers from a period of warm ischemia, i.e. the period between clamping of blood flow and removal of the kidney, followed by a period of cold ischemia, i.e. the storage of the organ prior to transplantation, and a second period of warm ischemia during the transplant procedure itself.

Ischemia-reperfusion injury is an important risk factor for delayed graft function and adverse outcomes[10-12]. In addition, ischemic AKI is a relatively frequent complication of major surgery, especially in the elderly or in patients with underlying diseases[13,14]. Especially, surgical procedures such as coronary artery bypass graft, aortic aneurysm surgery or thoraco-abdominal procedures are associated with a high rate of AKI-related complications[15,16]. In a minority of cases ischemic AKI is caused by stenosis, i.e. occlusion of the renal vessels. Thrombosis of the renal vein is an infrequent complication in renal transplantation[17].

Currently, there are no efficient treatments to improve outcomes in established AKI; despite intensive research within the field and in our understanding of the pathogenesis. Therefore, disease management has remained largely supportive in nature[18,19]. Patients with severe AKI in need of renal replacement treatment receive dialysis or, on the long-term, transplantation, both of which are limited in duration with a high rate of complications and premature death: the 5-year survival of dialysis patients is 40.6% in Europe[20] and 40.2% in the United States[21] whilst the average 10-year survival after transplantation is 56% in Europe and 43% in the United States[22].

1.2 Pathophysiology of ischemic acute kidney injury

The pathophysiology of ischemic AKI is complex, with various anatomical, hemodynamic and cellular factors contributing to injury. Two components play a major role in the pathophysiology of ischemic AKI: a vascular component, including intrarenal vasoconstriction with a fall in glomerular filtration pressure, vascular congestion in the outer medulla, and activation of tubulo-glomerular feedback; and a tubular component, with tubular obstruction, transtubular back-leak of the filtrate, and tubulo-interstitial inflammation[4].

This section aims to briefly summarize our current understanding of the most important pathophysiological mechanisms in the development of ischemic acute renal failure.

1.2.1 Vulnerability of the renal outer medulla

The kidneys receive 25% of the cardiac output, but renal blood flow is not uniformly distributed within the kidney. Most of the blood supply (20 % of the cardiac output) is directed to the renal cortex, with a tissue pO_2 of 50-60 mmHg. By contrast, the blood supply to the medulla is limited to 5-10 % of the total renal blood flow with a tissue pO_2 of 10-20 mmHg[23,24] (Figure 1.1 C).

The hypoxic state of the medulla is due to counter current diffusion of oxygen from the descending to the ascending vasa recta (Figure 1.1 B), which is necessary to build the medullary solute gradient that ensures the urinary concentration and

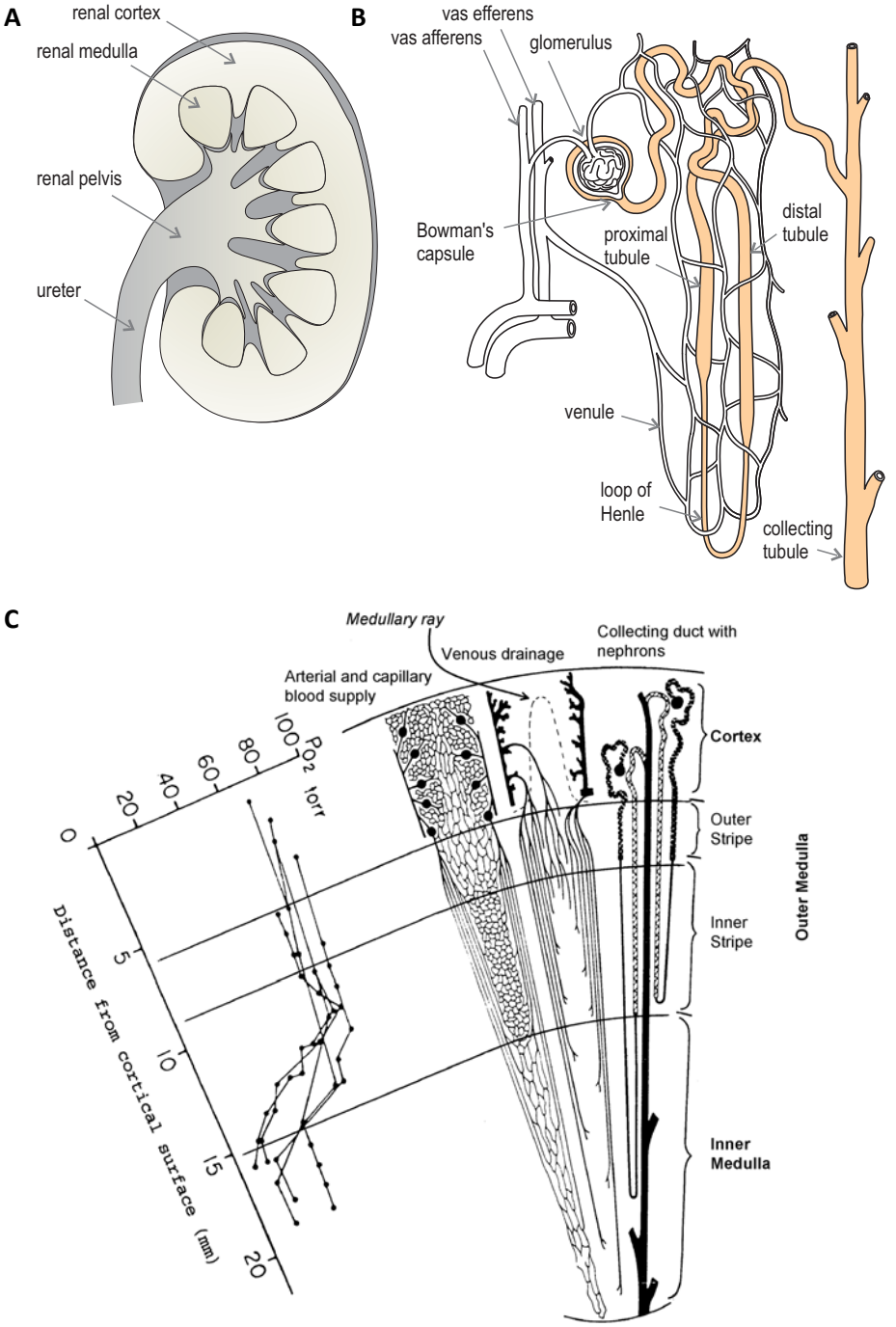

Figure 1.1: A: Structure of the kidney. **B**: Detailed structure of the nephron.
C: Intrarenal oxygen tension (left) and renal vascularization (right).
Adapted from Brezis et al. (1991)[25].

dilution capacity of the kidney and prevent washout of the solute gradient[24,26]. At the same time, metabolically highly active cells, responsible for reabsorption of solutes by active tubular transport, are present in this low oxygen tension zone, rendering the juxtamedullary cortical region and outer medulla most sensitive to hypoxia[27,28]. In the outer stripe of the outer medulla, it is the S3 segment of the proximal tubule in particular that is most susceptible to ischemic injury because these tubular cells have a limited capacity to undergo anaerobic metabolism (i.e., glycolysis), whereas the cells of the thick ascending tubules in this region contain higher amounts of glycolytic enzymes and can reduce their oxygen demand upon reduction in workload[29]. Hence, even modest reductions in renal blood flow could lead to hypoxia in the S3 proximal tubule region[30]. Depending on the degree of resulting hypoxia, this entails tubulo-interstitial injury, cell death and tissue remodelling[31].

1.2.2 Tubular epithelial injury

The most prominent effects of renal ischemia are seen in the tubules. Injured tubules become diversely dilated, and lined by a flat undifferentiated epithelium or poorly differentiated proximal tubule cells with breakdown of the brush border and 'blebbing' of the apical membrane[32,33]. Injured tubular cells release tubular debris into the tubular lumen, contributing to cast formation, obstruction, increased tubular pressure and a marked reduction in single-nephron GFR[34]. In addition, the interstitium widens with infiltration of immune cells and (myo)fibroblasts around atrophic and dilated tubules displaying abnormally thick basement membranes (Figure 1.2 A). Furthermore, there is increased deposition of type I collagen and reduction of capillary density in the interstitium[32]. In patients with ischemic nephropathy, athero-embolic intrarenal disease or severe small vessel disease are frequently observed[35].

The cellular and molecular responses of the kidney to an ischemic insult can be subdivided in 2 phases. In the early phase after an ischemic insult the pathophysiological processes leading to necrosis and apoptosis predominate. The second phase, which overlaps with the first, involves a repair response during which the kidney utilizes its inherent capacity to restore its structure and function[33,36].

1.2.2.1 Alterations in tubular cell metabolism

The profound reduction in cellular energy content accompanying the oxygen deficiency (cellular hypoxia) during ischemia influences many cellular systems and induces a variety of metabolic processes in tubular cells[34,37] (Figure 1.2 B).

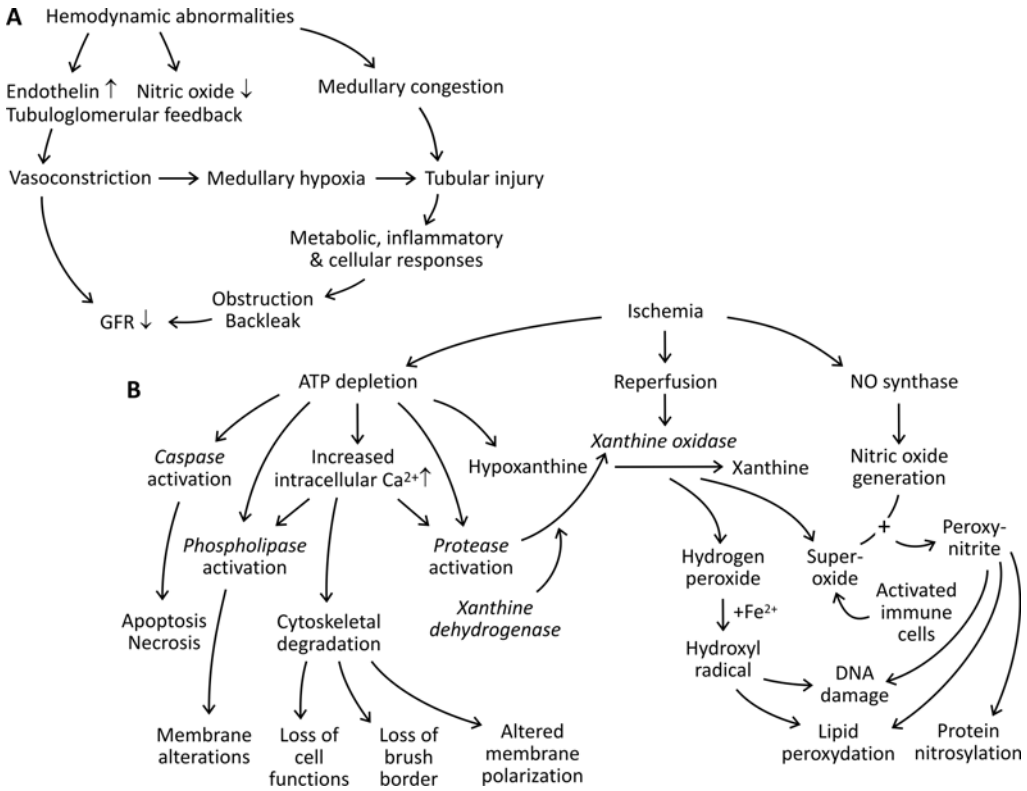

Figure 1.2: Pathophysiological mechanisms leading to a decrease of the GFR (**A**), and the consequences of ischemia reperfusion on cellular integrity (**B**).

Alterations in adenine nucleotide metabolism

Upon ischemia, cellular ATP levels fall rapidly: oxygen deprivation leads to a rapid degradation of ATP to ADP and AMP[34]. As mentioned before, proximal tubules have little capacity for glycolysis, i.e. anaerobic metabolism for ATP production[27]. With prolonged ischemia, AMP is metabolized to adenine nucleotides and hypoxanthine. Hypoxanthine accumulation contributes to generation of injurious reactive oxygen molecules. Adenine nucleotides can diffuse out of cells, and their depletion prohibits replenishment of ATP upon reperfusion[37], thereby aggravating cellular injury.

Alterations in intracellular calcium

Damaged cells and tissues accumulate calcium as its homeostasis is impaired by ATP depletion. This increase of intracellular free calcium leads to activation of proteases, phospholipases, cytoskeletal degradation[33,37] and further impairment of the energy metabolism by interfering with the production of ATP[33]. This rise in intracellular calcium is a well-documented event after AKI[37].

Generation of reactive oxygen molecules

Reactive oxygen species (ROS) are derived from several sources in post-ischemic tissue: (1) In mitochondria, intermediates of the electron transport chain become more reduced, resulting in generation of superoxide upon restoration of oxygenation[34]. (2) During reperfusion, the conversion of accumulated hypoxanthine to xanthine generates hydrogen peroxide and superoxide. Hydrogen peroxide reacts with iron to form the highly reactive hydroxyl radicals[37]. (3) Activated neutrophils also produce superoxide[34]. At the same time, ischemia induces nitric oxide (NO) synthase in tubule cells. The generated NO interacts with superoxide resulting in oxidation of proteins, peroxidation of lipids, and DNA damage, contributing to development of necrosis, dysfunction of sublethally injured cells and generation of the signals for activation of apoptotic pathways[37,38].

1.2.2.2 Alterations in tubular cell structure

Sublethal injury to proximal tubule cells after an ischemic episode results in alterations of tubular cell structure, which consists in dysfunction of the actin cytoskeleton and leads to loss of brush border and cellular polarity, redistribution of transcellular transporters, and loss of cell junctions with detachment of viable cells[33,36,37,39]. The remaining viable cells “flatten out” along the basement membrane and repopulate the denuded basement membrane area[34]. Regenerated proximal tubular cells redifferentiate and restore their proximal tubular morphology[40] (Figure 1.3). The mechanisms underlying these morphological changes are discussed briefly below.

Alterations in the apical cytoskeleton

Ischemia provokes structural alterations in surface membrane polarity and junctional complexes mediated by the apical cortical cytoskeleton, which reflects in intracellular actin delocalization from the apical to the lateral cell membrane[33,37]. The subsequent alterations in microvilli structures cause breakdown of the brush border of the proximal epithelial cells. This goes along with the release of membrane-bound, free-floating extracellular vesicles termed “blebs” which either undergo internalization or are released into the tubular lumen and thus contribute to cast formation and obstruction. These casts and vesicles which contain actin and actin depolymerizing factor have been detected in the urine of animals and humans with AKI[37].

The ATP depletion also results in disruption of cell-cell junctions, i.e. tight junctions and adherens junctions[33,37,41]. Dissociation of cellular junctional complexes allows membrane lipids and integral and peripheral membrane proteins to diffuse laterally into the alternate surface membrane domain[41]. Changes in the expression, distribution and aggregation of proteins associated with the junctional com-

plex have been documented after ischemic injury in animals and humans with AKI[39]. As a result, both paracellular permeability and cell polarity are altered, which can magnify the transtubular back-leak of glomerular filtrate to the peritubular capillaries[33,34].

Alterations in the basolateral cytoskeleton

Ischemia causes the disruption of the polarized proteins Na^+-K^+-ATPase and integrins[39]. After ischemic AKI, the adherent epithelium continues to express apical and basolateral membrane proteins, however, their distribution is no longer polarized[36]. As a consequence, the efficacy of transcellular sodium uptake by the proximal tubular cells decreases, resulting in an increased intraluminal sodium chloride delivery to the macula densa, which activates tubulo-glomerular feedback, with a subsequent decrease in GFR[29,42]. The decreased sodium reabsorption due to the disturbed localization of Na^+-K^+-ATPase thus leads to an increase in fractional excretion of sodium, which is a diagnostic signature of ARF[29,37].

Upon ischemic injury, β1-integrins are redistributed to the apical membrane. Integrins mediate cell-substratum adhesions in the basolateral membrane[37]. In the adult kidney, members of the β1-integrin subfamily are the most common and localize to basal surfaces of tubular epithelia where they interact with extracellular matrix (ECM) components of the basement membrane[43]. Redistribution of β1 integrin to the apical membrane causes detachment of viable cells from the basement membrane[37], leaving these denuded areas as the only barrier between the filtrate and the peritubular interstitium. This allows for back-leak of the filtrate, especially under circumstances where the pressure in the tubule is increased due to intratubular obstruction by exfoliated cells and cellular debris[33]. In experimental ischemic AKI, it was shown that the state of β1-integrin activation is also critical for maintenance of tubule epithelial integrity[43]: in response to ischemia-reperfusion injury, the β1-subunit reorganizes predominantly in the S3 segment epithelia that remain attached to basement membranes[36].

Alterations in cell viability

After ischemic AKI, most tubular cells are sublethally injured and undergo repair after adequate reperfusion[4]. Sublethal injury is manifested by a loss of the brush border because of interiorization of brush border microvilli on the one hand and shedding of brush border microvilli into the tubular lumen on the other hand. Restoration of brush border includes repositioning of previously internalized microvilli membranes[44].

A subset of tubular cells displays patchy cell death resulting from necrosis and apoptosis[37]. Cell necrosis is induced by rapid and overwhelming depletion of cellular energy stores and by irreversible injury to the lipid bilayer of the plasma membrane and subcellular organelles, causing substantial cell swelling and subse-

quent disruption of the integrity of plasma membrane and cell organelles with injury to and inflammation of surrounding tissues[26]. Apoptosis, however, is a highly regulated, active and energy-dependent form of 'programmed cell death', which is induced by orchestrated changes in gene expression. Morphologically, apoptosis is characterized by cell shrinking and formation of apoptotic bodies, consisting of condensed chromatin and cytoplasmic elements, which are rapidly ingested by neighbouring cells and resident macrophages, without causing inflammation[45].

In the post-ischemic kidney, necrotic cell death of proximal tubules is initially more prominent than apoptotic cell death because the apoptotic pathway requires ATP for full execution, and pro-apoptotic proteins of the intrinsic apoptosis pathway are downregulated before injury[38]. In addition, necrosis usually occurs after more severe injury and in the more susceptible nephron segments, whereas apoptosis predominates after less severe injury and in the ischemia-resistant distal nephron segments. Recent research *in vivo* indicates that apoptosis occurs in two waves: the first wave is detectable 6 to 12 hours after the insult, and eliminates previously healthy tubule cells. The second wave occurs approximately one week after injury and removes inflammatory, hyperplastic and redundant cells and therefore may play a role in the remodelling of injured tubules[37].

1.2.2.3 Mechanisms of repair

Surviving tubule cells have a remarkable regeneration potential[37]. Under normal circumstances, proximal tubule cells divide at a low rate. The classical view of tubular regeneration states that compensatory hyperplasia, i.e. mitosis of cells during the differentiation state, provides effective replenishment of randomly eliminated tubular cells by self-renewal of adjacent cells under physiological conditions[46,47]. This view is supported by findings from fate-tracing studies, which have confirmed that restoration of the tubule epithelium after ischemic AKI predominantly occurs via proliferation of mature tubular cells that undergo dedifferentiation[37,46,48] (Figure 1.3).

The *first* phase of this regeneration process consists of the death and exfoliation of the proximal tubular cells and is characterized by expression of stress response genes and the accumulation of mononuclear cells. The *second* phase is a dedifferentiation stage. Local epithelial cells undergo dedifferentiation[4,33]. These cells possess a morphologically flattened appearance with a poorly differentiated brush border[38]. The dedifferentiation of proximal tubule cells is considered a recapitulation of embryogenesis and characterized by expression of a number of proteins that are expressed at high levels in the developing metanephric mesenchyme, such as vimentin, but not expressed to the same degree in the adult kidney epithelial cell[38,49]. This protein expression pattern is also likely to be important for the spreading of the viable epithelial cells over the basement membrane

during the repair process[33]. In the *third* phase, there is a pronounced increase in proliferation of the surviving proximal tubule cells, and growth factors could have an important role in this response[4]. The poorly differentiated regenerative cells spread and migrate over the exposed areas of the basement membrane[33]. In the *last* phase, the regenerative tubular cells regain their differentiated character and

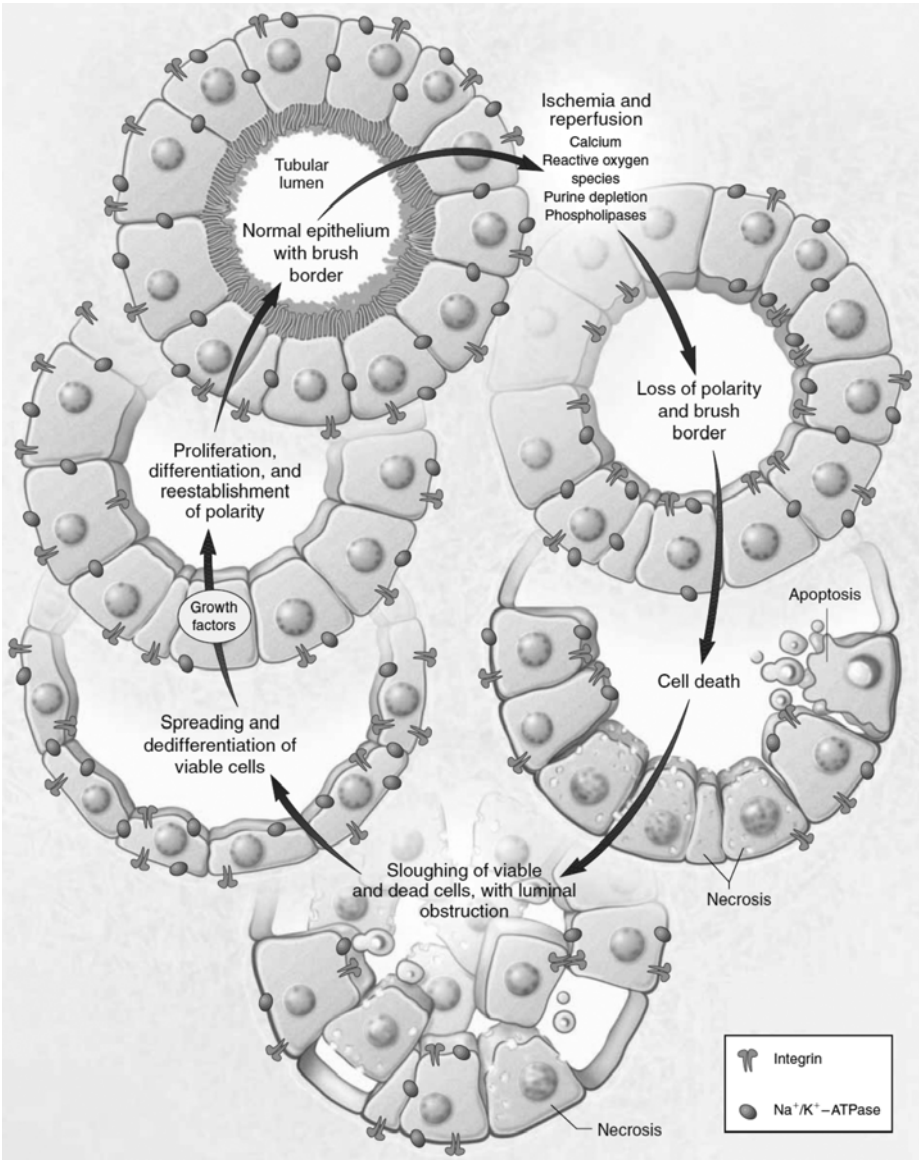

Figure 1.3: Mechanism of tubular repair. Adapted from Thadani et al. (1996)[40].

produce a normal proximal-tubule epithelium, which results in restoration of the functional integrity of the nephron[4,33]. This dedifferentiation process must be a beneficial mode of regeneration for rapid reparation of focal areas following injury to the tubule, such as after ischemia reperfusion[46].

Recently, stem and progenitor cell populations have been implicated in tubular regeneration. However, fate-tracing experiments have shown that the contribution of these cells is only indirect by providing soluble factors that support regeneration[47,50].

1.2.3 Vascular dysfunction

After reperfusion, total renal blood flow increases again, however, it reaches only 40 to 50% of pre-ischemic values in both human and experimental ischemic AKI[37,51]. In many cases in animals and humans, the decrease in total renal blood flow alone cannot entirely explain the reduction in GFR that is observed during an episode of AKI[52]. Remarkable regional disturbances in blood flow persist beyond the ischemic episode: oxygenation of the cortico-medullary junction and the outer medulla in particular remain poor, with blood flow after reperfusion in this region being only approximately 10% of normal[53,54]. In addition, intravital microscopy has revealed that sporadic cessation and reversal of blood flow occurs in peritubular capillaries during reperfusion[37]. These regional alterations in oxygen delivery prolong the ischemic insult in the most vulnerable region of the kidney and explain why single nephron GFR is relatively well maintained in superficial cortical nephrons, but severely reduced in the deeper juxtamedullary nephrons[53].

Endothelial cells are important determinants of vascular tone, leukocyte function and smooth muscle responsiveness[52]. Disruption of the actin cytoskeleton, as described in tubular cells, has also been documented in endothelial cells in experimental AKI[37] and results in alterations in endothelial cell-cell contacts and breakdown of the perivascular matrix, which ultimately leads to increased microvascular permeability and loss of fluid into the interstitium[52]. In addition, ischemic AKI also leads to increased endothelial expression of a variety of adhesion molecules that promote endothelial-leukocyte interactions[37]. Moreover, changes occur in the production of vasoactive agents such as a decreased in NO and prostacyclin, and an increase of platelet activating factor (PAF), and endothelin[42,55]. Increased expression of adhesion molecules together with increased expression of counter receptors on leukocytes[52] leads to enhanced leukocyte-endothelial interactions. As a result, leukocytes are activated, promoting injury and swelling of the endothelial cell, physically obstruct blood flow, further activate leukocytes. In addition, activated leukocytes contribute to the production of cytokines promoting vasoconstriction and add to the effects of vasoconstriction on local blood flow and tubule cell metabolism[38,52,55], especially in the outer medulla of the kidney[52].

The resulting thrombosis and congestion may prolong and exacerbate local ischemia, i.e. “no reflow” phenomenon, causing further tubular necrosis and renal failure[42]. Microthrombus formation has been described in experimental ischemia as well as in transplant biopsies[56]. Also, ROS production by platelets and in the hypoxic tubular cells contributes to vascular dysfunction after ischemic AKI is, as ROS may influence the effects of vasoconstrictors and vasodilators leading to an increase in renal vascular resistance[56]. Hence, platelets have the potential to aggravate endothelial cell damage and contribute to leukocyte activation and recruitment at the site of injury[57].

Although the renal tubular system has an impressive capacity for regeneration, the renal vascular system lacks comparable regenerative potential[58]. After experimental ischemia-reperfusion injury, the number of microvessels in the inner stripe of the outer medulla declines, which is associated with chronic hypoxia and prolongation of the ischemic insult in the most vulnerable region of the kidney, which in turn leads to increased tubular injury and tubulo-interstitial fibrosis[52].

1.2.4 Immune response on I/R

Post-ischemic tissue infiltration by neutrophils, macrophages, and different subtypes of T cells is a hallmark of renal ischemic injury. Inflammation has a major role in the pathophysiology of ischemic AKI. The inflammatory process is initiated by both the endothelial and tubular cell dysfunction[59]. Injury to the endothelium results in an increase in vascular permeability, as explained in the previous paragraph, enabling extravasation of leukocytes into the kidney[60]. Injured tubular cells secrete an array of cytokines (TNF-α, IL-6, IL-1b, and TGFβ) and chemokines (monocyte chemoattractant protein (MCP)-1, IL-8 and RANTES) and growth factors into the renal tissue, hereby contributing to the inflammatory cascade[4,59,61]. The secretion of these cytokines and chemokines can attract and activate leukocytes, reducing their deformability and enhancing their tendency to be sequestrated[29,38,62]. They also activate the endothelium, resulting in upregulation of adhesion molecules. Sequestered leukocytes can then potentiate injury via a positive feedback pathway by generating more ROS and eicosanoids, enhancing inflammation and vascular tone[29,38]. Adherence of neutrophils to the vascular endothelium is an early crucial process in the initiation of damage to ischemic tissues.

Several leukocyte subtypes have been shown to aggregate in peritubular capillaries, interstitial space, and even within tubules after ischemic AKI[37].

Neutrophils. Neutrophils are the first leukocyte subtype to accumulate in the post-ischemic kidney, particularly in the peritubular capillary network of the outer medulla[52]. Neutrophils produce proteases, myeloperoxidase, ROS and cytokines, resulting in increased vascular permeability and reduced tubular epithelial and

endothelial cell integrity[52]. However, in experimental AKI, neutrophil depletion or blockade of neutrophil function provides only partial protection of renal function in some (not all) models, suggesting little clinical significance of neutrophil infiltration[37].

Macrophages. There are two different sources of macrophages in the ischemic kidney: 1) tissue-resident macrophages, which are seeded during development and are maintained through local proliferation, and 2) monocyte-derived macrophages, which are recruited into inflamed tissues[63]. Macrophages infiltrate the post-ischemic kidney shortly after neutrophils[53,64]. Activation of macrophages can be classified into two major categories: classically activated M1 macrophages, and alternatively activated M2 macrophages[65]. M1 macrophages are pro-inflammatory, producing large amounts of pro-inflammatory cytokines, which can stimulate the activity of other leukocytes, ROS and nitrogen intermediates[52,60]. Although they may cause tissue damage, they can play an important role in clearing apoptotic cells and debris after sterile injury, thereby initiating the repair response, as seen after muscle injury[65]. M2 macrophages are diverse, and can be subcategorized into wound-healing and immune-regulatory macrophages, although there is likely to be considerable overlap[52,65]. Activation of the wound-healing M2 macrophages leads to upregulation of growth factors and stimulates co-factors important for cell proliferation and ECM production. This increase in the production of growth factors is important in normal wound healing, but can promote fibrosis if sustained or abnormally regulated. Immune-regulatory M2 macrophages produce high levels of IL-10, an immunosuppressive cytokine, thus limiting inflammation through dampening of the immune response. In addition, these macrophages produce TGFβ which can inhibit the production of pro-inflammatory cytokines, but is also considered to have a pro-fibrotic potential[65]. Selective macrophage depletion ameliorates ischemic AKI, but the induction of tissue injury by macrophages seems to require the coordinated action of T cells and neutrophils[66].

T cells. Infiltration of T cells occurs both in the early and late phases of AKI, and like macrophages, T cells can facilitate injury (Th1 phenotype) but also promote repair (Th2 phenotype) after ischemia-reperfusion[52]. It can be assumed that individual biological properties of the different subsets of T-cells are fundamentally important in the process of kidney repair after ischemia[59]. Consistent with a causal role for T lymphocytes, CD4+/CD8+ knockout mice display improved recovery from ischemic AKI, with decreased tubular necrosis and a reduced amount of infiltrating neutrophils[67]. Blocking the T-cell costimulatory pathway is protective in experimental ischemic AKI by preventing vascular congestion of the vasa recta[68]. However, RAG-1– deficient mice, which have no T or B cells and do not produce immunoglobulins or T cell receptor proteins, were not protected[69].

Alongside of leukocyte infiltration, activation of the complement system in is-

chemic AKI amplifies the inflammatory response in the kidney[37]. After ischemia-reperfusion, the compliment system is activated, predominantly via the alternative pathway by macrophage-derived C3[52]. Selective inhibition of the alternative compliment activation pathway has been proven to protect the kidney from ischemic AKI in animal models[70,71]. Also, factor C5a of the classical complement activation pathway, has been shown to play an important role in ischemia-reperfusion-induced kidney inflammation[52,59]. The C5a receptor is expressed by tubular epithelial cells, and its expression increases upon ischemic injury, and by certain interstitial macrophages. C5a also acts as a potent chemoattractant which results in the recruitment of neutrophils, monocytes and T cells[59]. Selective inhibition of the alternative complement pathway protects the kidney in experimental ischemic injury[52].

As local inflammation worsens, in part driven by inflammatory mediators produced by tubule cells, well-reperfused areas can become under-perfused again[38].

1.3 The AKI to chronic kidney disease (CKD) connection

Chronic kidney disease (CKD) is defined as histopathological kidney damage and/or decreased GFR (<60 ml/min/1,73m²) for at least 3 months, irrespective of the cause[72]. In Western countries, age, diabetes, hypertension, obesity and cardiovascular disease are the most important factors associated with increased risk of developing CKD[72]. Also, glomerular and tubule-interstitial diseases, as a result of infections, and exposure to drugs and toxins, contribute to the growing population of patients suffering from CDK[73]. Furthermore, in spite of the kidney's large regenerative capacity, a significant proportion of AKI patients do not recover complete renal function[37,74].

After an episode of AKI, complete resolution of normal structure and function would be the optimal outcome[75]. However, long after ischemia of the solitary kidney, histological indications of developing CKD are present, such as dilated and shrunken tubules, markedly thickened tubule basement membranes, interstitial fibrosis with increased numbers of fibroblasts, and diminished capillary density[32]. Long-term follow-up studies report that between 35 and 71% of patients surviving an episode of AKI had incomplete renal function as assessed by creatinine clearance or serum creatinine measurements[76] (Figure 1.4). Moreover, these patients are more likely to develop end-stage renal disease (ESRD) as compared to patients without a history of either AKI or CKD[2,8,52,77,78]. Although incomplete recovery from severe AKI is a well-recognized pathway to persistent and progressive CKD, recent studies have suggested that even complete recovery from AKI is associated with a subsequent risk for CKD development[8,79]. The severity and outcome of an episode of acute kidney injury is predicted by its duration, and even transient disease is associated with increased mortality[8].

Many injury factors are thought to contribute to post-ischemic fibrosis: (1) the production of pro-fibrotic cytokines, chemokines and growth factors[78], (2) a persistent inflammatory response with chronic activation of macrophages[52,79,80], (3) long-term hypoxia from sustained loss of peritubular microvessels[24,52,80], and (4) arrest of tubular cells in the G2/M phase of the cell cycle[81]. It was hypothesized that once renal damage reaches a certain threshold, the progression of renal disease is consistent, irreversible, and largely independent of the initial insult[23].

1.3.1 Fibroblasts and extracellular matrix production (fibrosis)

Extracellular matrix (ECM) is a dynamic superstructure of self-aggregating macromolecules, including fibronectin, collagens and proteoglycans, to which cells attach by integrins[82]. The matrix surrounding cells is continuously degraded by proteases[82]. ECM turnover is regulated primarily by the matrix metalloproteinases (MMPs) and their endogenous inhibitors, i.e. the tissue inhibitor of metalloproteinase (TIMP) and the plasmin – plasminogen activator – plasminogen activator inhibitor (PAI) cascade[82,83]. In tissue fibrosis, matrix accumulates as a consequence of both increased production and decreased turnover of ECM proteins. In addition, the fibrotic matrix also contains residual fragments of collagen IV, which normally are found exclusively in basement membranes, as well as several fibronectin splice variants that modulate the fibrogenic potential[84]. In kidney fibrosis, the volume of the injured kidney decreases as residual renal parenchyma collapses and the vacated space of the

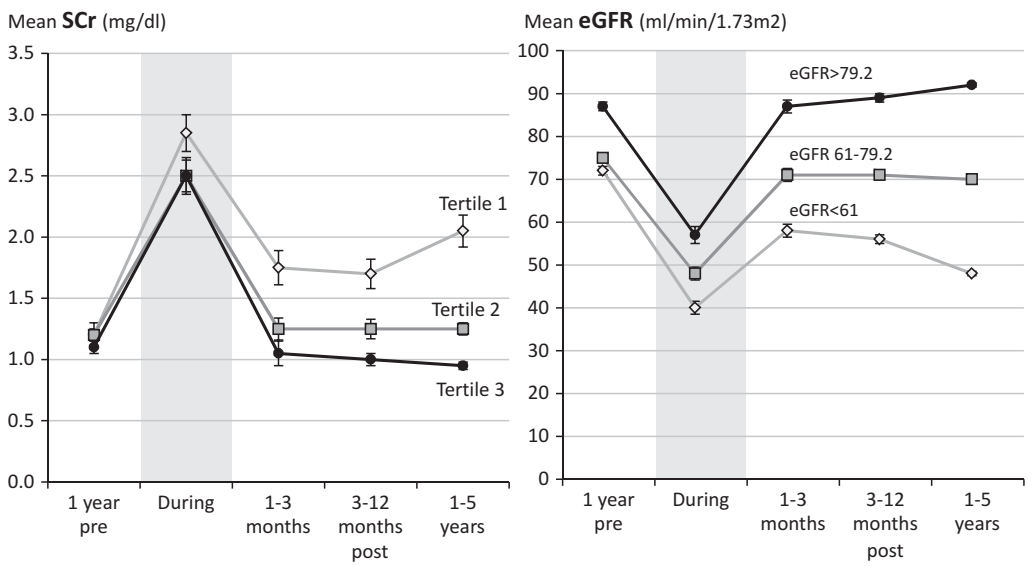

Figure 1.4: Incomplete recovery of AKI is associated with a risk for CKD development.
Adapted from Chawla et al. (2012)[78].

degenerated nephrons is contracted and filled by fibrous tissue, predominantly consisting of collagens type I and III and fibronectin[84-86].

Fibroblasts are considered to be the main effector cells that produce ECM, cytokines and growth factors which sustain and further promote the fibrotic process and attract inflammatory cells during fibrosis[87,88]. Fibroblasts are stellate shaped cells with a stellate nucleus and abundant rough endoplasmatic reticulum, collagen secreting granules, actin filaments under the plasma membrane and multiple cell processes, which connects them to tubular and capillary basement membranes and dendritic cells (DCs)[80]. In normal physiological wound repair, fibroblast activation is transient, and fibrosis is initiated to maintain the structural integrity of tissue, to contain the deleterious consequences of inflammatory reactions, and to recruit and stimulate cells necessary for the repair of damaged tissue and subsequent restoration of function[85,89]. When tissue repair is accomplished, the fibrotic tissue is resolved and fibroblasts return to their resting state[89,90]. In chronic fibrosis, fibroblasts are unable to return to their resting state and, as a consequence, continue to proliferate and produce excess ECM[88-93]. The resultant disruption and destruction of tissue architecture, and the subsequent loss of function can lead to glomerulosclerosis, tubular atrophy and dilatation, tubulo-interstitial fibrosis and rarefaction of the glomerular, as well as peritubular capillaries[31,80,85]. The origin of fibroblasts in organ fibrosis is still controversial. Possible sources are resident fibroblasts[83], bone marrow-derived progenitor cells[83], epithelial-mesenchymal transition (EMT), endothelial-mesenchymal transition (EndMT), mesenchymal stem cells (MSC), pericytes[94], … At the moment there is general consensus that fibroblasts are a heterogeneous population, with cells from multiple lineages contributing to the final mix of fibroblast population in tissue[94,95].

For years it has been assumed that myofibroblasts are the principal mediators of tubulo-interstitial fibrosis, mainly based on the observation that fibrosis associates with *de novo* accumulation of α-smooth muscle actin (SMA) positive cells. However, this assumption has been mitigated by a number of recent observations: (1) α-SMA positive fibroblasts express stress fibres, making them unable to actively migrate[96]; (2) α-SMA deficient animals display increased procollagen I gene expression as compared to wild-type animals[95,97]; (3) the number of α-SMA positive fibroblasts decreases, despite the progression of renal fibrosis[98]; and (4) mice with an α-SMA deficiency develop more fibrosis and their fibroblasts produce more collagen I, which indicates that expression of α-SMA affects suppression of proliferation, procollagen synthesis, and migration of myofibroblasts[97].

1.3.2 Fibrosis-related proteins

Injured and regenerating tubule cells, endothelial and interstitial cells, and inflammatory cells can contribute to the progression of fibrosis by providing pro-inflammatory chemokines, cytokines, and adhesion molecules that are involved in repair, regeneration and remodelling[29,37,38,60,99-101], but can also be pro-fibrotic[84,101,102]. Epithelial signalling, activated acutely in response to injury, may persist late during kidney recovery (at a time when it should become suppressed)[32,103] thereby mediating primary interstitial injury[84].

1.3.2.1 Transforming growth factor (TGF) β

The transforming growth factor (TGF) β superfamily consists of TGFβ1, -β2 and -β3; activins; and bone morphogenic proteins (BMP). These proteins are expressed in virtually all mammalian cell types, as are their downstream signalling mediators, i.e. the Smad proteins. However, TGFβ may also act through pathways that are not directly linked to Smad activation[104] (Figure 1.5).

TGFβ is considered an important regulator of cell proliferation, differentiation, apoptosis, immune response, and ECM remodelling, depending on the physiological context[104,105].

Expression. Interstitial and tubule cells can produce TGFβ1[91,107]. Platelets contain high concentrations of TGFβ1 and platelet-derived growth factor (PDGF) that are released into the tissue at the site of injury. Inactive (latent) TGFβ1, bound locally to the ECM, can also be activated after injury. TGFβ1 is also strongly chemotactic for neutrophils, T cells, monocytes and fibroblasts[82]. TGFβ production may be a self-sustaining, autocrine event in injured renal cells[104] as TGFβ1 can induce both infiltrating cells and resident cells to produce more of itself. This auto-induction amplifies the biological effects of TGFβ1 at the injury site and may have a central role in fibrosis[82,108].

Regulation. The effects of TGFβ on ECM synthesis and deposition are mediated by the type I receptor. The effects of TGFβ on cell growth and proliferation are mediated by the type II receptor[82]. Since TGFβ mediates different events, multiple mechanisms are involved determining the outcome of TGFβ signalling. Regulation involves availability and activation of TGFβ, receptor expression and localization, and control of the TGFβ family-specific Smad signalling proteins and their interaction with signalling pathways[104]. Phosphorylated Smad-2 (pSmad-2) and Smad-3 associate to form a heteromultimer with Smad-4 (Co-Smad). This complex is then translocated to the nucleus, where it can regulate target gene expression[109]. Inhibitory Smads, such as Smad 7, negatively regulate TGFβ1 signalling by competition for association with the type I receptor, thus preventing the recruitment and

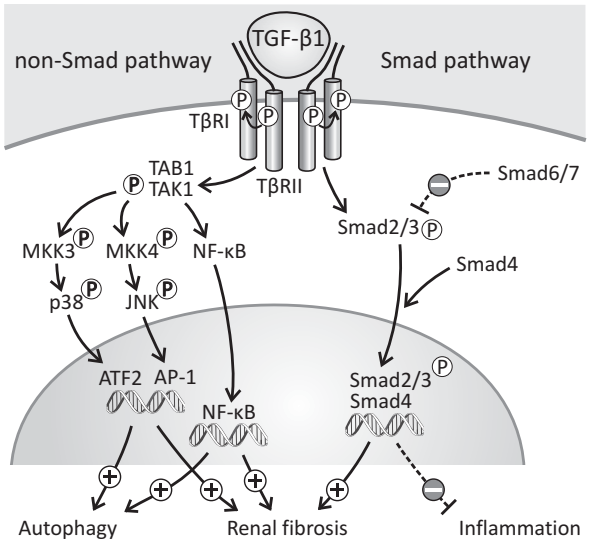

Figure 1.5: TGFβ signalling through Smad-dependent and –independent pathways. Adapted from Lee et al. (2015)[106].

phosphorylation of Smad 2 and 3. In addition to the canonical pathway (Smad-dependent), TGFβ1 also activates Smad-independent pathways to mediate its diverse actions[106].

Fibrogenic effects. Excessive or sustained production of TGFβ1 is a key molecular mediator of tissue fibrosis[82]. Elevated expression of all three TGFβ isoforms has been demonstrated in the glomeruli and tubulo-interstitium of patients with renal diseases characterized by excessive ECM accumulation. Elevated TGFβ has also been observed in various animal models of kidney fibrosis[110]. Neutralizing antibodies against TGFβ have shown to attenuate renal pathologies in animal models of obstructive nephropathy[111,112], diabetic nephropathy[113-116], glomerulonephritis[117], and hypertensive nephropathy[118,119]. Fibrogenic responses of TGFβ include ECM deposition by simultaneously stimulating cells leading to a several fold increase in the synthesis of most matrix proteins, decreased production of matrix-degrading proteases, increased production of inhibitors of these proteases, and modulated expression of integrins in a manner that increases cellular adhesion to the matrix[80,82,91,108]. Interaction of the cell with its environment can also influence TGFβ signalling. As with fibrosis, a cell makes more inappropriate or abnormal ECM. The changes in matrix-cell interaction going along herewith will further stimulate irregularities in cell function, and more abnormal ECM, resulting in a vicious cycle.

In addition, TGFβ exerts direct effects on fibroblasts, can induce expression of other cytokines, such as connective tissue growth factor (CTGF)[104], and trigger focal inflammatory processes[86].

1.3.2.2 Tumour necrosis factor (TNF) α

The tumour necrosis factor (TNF) superfamily includes at least 19 cytokines that play critical roles in regulating the development and function of the immune system. TNFα is a potent pro-inflammatory cytokine and an important mediator of inflammatory tissue damage, as it can upregulate its own expression[120], induce the production of other pro-inflammatory cytokines (IL-1, IL-6 and macrophage migration inhibitory factor (MIF)), chemokines (MCP-1) and growth factors (TGFβ)[106,121-123] and stimulate a variety of immune cells[120]. TNFα has been implicated in the pathogenesis of many inflammatory diseases of the kidney including autoimmune lupus nephritis, glomerulonephritis, septic acute renal failure, and renal ischemia-reperfusion injury[124]. TNFα exists in both a transmembrane form and a soluble form, to which most of the biological effects are attributable[121,123].

Expression. TNFα is produced primarily by macrophages in response to various inflammatory stimuli[121]. After ischemic injury, TNFα is also produced by intrinsic renal cells, such as mesangial and tubular epithelial cells[106,125,126]. Upon injury, at physiological concentrations, TGFβ1 regulates TNFα in monocytes by stimulating or inhibiting their production or modulating their actions to synchronize and control the repair process[82]. TNFα, synthesized by mesangial cells and podocytes, acts to enhance inflammatory cell recruitment, stimulate mesangial cell production of TGFβ, and promote glomerular fibrin deposition[123].

Regulation. The biological effects of TNFα are mediated by two distinct cell surface receptors: TNFR1, which predominates and TNFR2[125]. Both receptors act synergistically for cell proliferation and maturation, cytotoxicity and antiviral activity. Binding of TNFα on its receptor results either in activating caspase 8 or c-Jun N-terminal kinases (JNK), promoting apoptosis or triggering a cascade of events activating nuclear factor NF-kB (NFkB) signalling, which suppresses apoptosis but initiates gene transcription of factors, such as TGFβ and MCP-1, involved in acute and chronic inflammatory responses[123]. Since TNFα can activate both survival and apoptotic signalling, its cytotoxicity is highly contextual and dependent on the presence of sensitizing contributors[127]. In normal conditions, TNFR1 is not expressed in renal tubular cells. After ischemia-reperfusion TNFR1 is highly expressed in tubular cells, in a pattern that is similar to TNFα, suggesting the regulation of TNFR1 by its own ligand[125]. The induction of the TNF gene transcription following ischemia-reperfusion injury is due to the direct activation of p38 mitogen-activated protein kinases (p38 MAP) and NFkB by locally formed ROS[124].

CCN1 and CCN2, either in soluble form or as adhesion substrates, enable TNFα to induce apoptosis: TNFα alone induces a transient JNK activation and a low level of ROS that is quickly dampened by NFkB-induced anti-oxidant proteins. Despite NFkB actions, the combination of CCN1 and TNFα induces a sufficient amount of ROS to trigger a robust and biphasic activation of JNK[127].

Fibrogenic effects. TNFα plays a role in glomerular inflammation and scarring[123], however it may have anti-fibrotic effects as demonstrated *in vitro* and *in vivo*, by suppression of matrix gene expression[128,129]. Blockade of TNFα with a monoclonal antibody suppresses inflammation and renal fibrosis in experimental crescentic glomerulonephritis[123], diminishes apoptosis[126], and results in better recovery of tubular structures and suppression of interstitial fibrosis after renal ischemia-reperfusion injury[125]. In addition, TNFα neutralization after obstructive renal injury results in a significant reduction in TGFβ1 mRNA expression and protein content, approaching sham levels[120].

1.3.2.3 CCN2 (CTGF)

Connective tissue growth factor (CTGF or CCN2) is a member of the CCN family of secreted matricellular proteins, all possessing similarities in multi-modular structure, but differences in function[130]. Each CCN protein contains 4 domains, each domain being capable of binding multiple ligands. Because of this large amount of interactions, CCN proteins can affect many different biological functions[88]. CCN2 is involved in endothelial cell migration and proliferation, angiogenesis and vascular smooth muscle cell apoptosis[131]. In addition, CCN2 is a cytokine that promotes ECM synthesis and turnover and is a chemo-attractant and mitogen for fibroblasts[88,132].

Expression. CCN2 is produced by kidney mesangial cells[130]. CCN2 is induced by TGFβ1 in wound healing and may be involved in tissue repair[88,133]. CCN2 binds to TGFβ, promoting interaction with its receptor and increasing downstream signalling[88,109]. TGFβ1 increases CCN2 expression, and the increase in CCN2 induced by TGFβ1 may provide an autocrine mechanism to enhance the bioactivity of TGFβ1[109]. In addition, a number of factors, including mechanical strain, appear to stimulate CCN2 expression without the upregulation of TGFβ, supporting the idea that CCN2 may provide a more downstream and essential target for regulation of matrix metabolism in fibrosis[130]. CCN2 can also bind to several other growth factors to modify their function, cell surface proteins by which intracellular signalling may be initiated, and ECM proteins that act as a sink for CCN proteins and modify their turnover[106]. In this manner, CCN2 upregulates the expression of integrins on the cell surface, facilitating the deposition and assembly of ECM proteins[134].

Regulation. CCN2 is primarily regulated at the level of transcription[134] and its regula-

tion is dependent on the cellular context and the cell type in which it is produced or acting[135]. Synthesis of CCN2 protein and mRNA is stimulated by specific growth factors, such as endothelin1 and TGFβ, in addition to environmental changes (e.g. hypoxia) and biomechanical stimuli (e.g. stretch) in a cell type-specific manner[134].

Fibrogenic effects. CCN2 mRNA and protein are overexpressed in renal fibrotic lesions[135]. CCN2 has emerged as an essential downstream mediator of many of the effects of TGFβ1, e.g. CCN2 induces the synthesis and secretion of ECM proteins, in particular fibrillary collagens[128,136]. Furthermore, CCN2 is thought to play an important role in the recruitment of inflammatory cells[88], at least *in vitro*, where CCN2 was chemotactic for monocytes[137]. *In vivo* and *in vitro* studies have shown that antagonism of CCN2 activity can block the TGFβ-mediated collagen synthesis[128,138-141]. However, blocking CCN2 only partially blocks TGFβ-stimulated ECM production by proximal tubule cells[104] as the activation of CCN2 expression can proceed independently of TGFβ[135]. Also, treatment with antisense CCN2 ameliorated unilateral ureteral obstruction (UUO)-induced fibrosis despite continued expression of TGFβ[142].

1.3.2.4 CCN3 (NOV)

CCN3 is another member of the CCN family of secreted matricellular proteins.

Expression. CCN3 expression is relatively high in the developing kidney[143]. CCN3 is an inhibitor of cell proliferation in a variety of both normal and tumour cells[135,144] and high CCN3 concentrations are found in quiescent cells. From these observations, it was concluded that CCN3 is a proto-oncogene with anti-proliferative activity. In addition, because of the observation that truncated and full length CCN3 proteins exist and in view of the fact that the amount of nuclear CCN3 varies greatly among different cell lines, biological properties of the CCN3 proteins are likely to depend on their structure and subcellular localization[135].

Regulation. The existence of a truncated form with a similar N-terminal sequence indicates that specific proteolysis of CCN3 may be a key element in the regulation of its biological activity. In addition, *in vitro* experiments suggest that protein kinase C might play a role in the downregulation of CCN3 gene expression[135].

Fibrogenic effects. CCN3 has anti-proliferative effects in several cell systems[146,147] and is an inhibitor of mesangial cell proliferation *in vitro*[130,135,148]. Evidence from *in vitro* experiments indicates that CCN3 could be an endogenous negative regulator of CCN2 and in this way of ECM production and fibrosis[130,145,149,150] (Figure 1.6). Cell types expressing high levels of CCN2 generally express low levels of CCN3 whilst both CCN3 gene and protein expression is downregulated upon exposure to TGFβ[130]. Addition of CCN3 or induction of CCN3 overexpression in cultured

mesangial cells caused down-regulation of CCN2 gene expression and collagen I secretion[130]. In addition, in a mouse model of diabetic nephropathy (BTBR ob/ob mice) administration of recombinant human CCN3 reduced the expression of fibrosis-related genes CCN2, collagen Iα2, TGFβ1 and PAI-1[151]. Furthermore, histological changes characteristic of human diabetic nephropathy were reversed by treatment[151]. The mechanism underlying CCN2 expression inhibition is unknown, but is not due to downregulation of Smad3 signalling[88].

1.3.2.5 PAI-1 (Serpin 1)

Plasminogen Activator Inhibitor (PAI)-1 is a member of the serine protease inhibitor (serpin) family[152] and a potent inhibitor of plasminogen activators that convert plasminogen to plasmin[153], thereby inhibiting matrix degradation[108]. In addition, PAI-1 has been attributed a mediating role in fibrosis, rheumatoid arthritis, atherosclerosis, tumour angiogenesis and bacterial infections. It also modulates cellular adhesion or migration, wound healing, angiogenesis, and tumour cell metastasis[154].

Regulation. PAI-1 gene expression is tightly regulated by a wide variety of cytokines and growth factors, coagulation factors and hormones, including TGFβ, IL-1β, endothelial growth factors (EGF), insulin, lipopolysaccharide and lipoproteins[152,155]. TGFβ has been shown to potently induce PAI-1 production[108,152].

Fibrogenic effects. Excessive or sustained production of TGFβ1 is a key molecular mediator of tissue fibrosis[82], which can induce sustained activity of PAI-1, contributing to excessive collagen accumulation, and in turn assisting in tissue fibrosis[152]. In a renal transplantation rat model, PAI-1 was upregulated and persistently expressed during the progressive phase of chronic rejection, synchronously with fibrin deposition in the grafts, suggesting that induction of PAI-1 may be responsible for the fibrin deposition, leading to irreversible damage and CKD[154]. Indeed, in CKD patients, circulating concentrations of PAI-1 are increased[156], resulting in increased synthesis of matrix components and inhibition of matrix degradation, which are mechanisms underlying fibrogenesis in human disease as well as in ex-

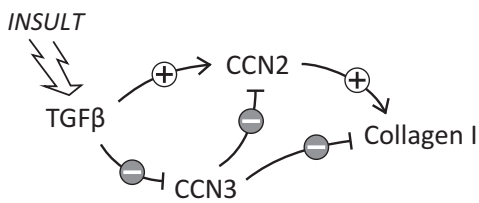

Figure 1.6: A hypothesis for the anti-fibrotic effect of CCN3 in tissue fibrosis.
Adapted from Riser et al. (2010)[145].

perimental models[108,157]. Also, in transplant patients, significantly higher levels of PAI-1 were present upon acute and chronic rejection[158,159]. Moreover, a correlation was found between PAI-1 serum levels and the rate of decline in renal function in transplant patients[160].

1.3.3 Interstitial inflammation

Distinction between inflammatory processes underlying the acute or chronic kidney injury phase is not easily made. Nevertheless, it is well-known that inflammation is an important mediator underlying the progression from acute-to-chronic kidney disease[101]. Foci of interstitial fibrosis display ongoing local inflammatory processes associated with injured nephrons, which will accelerate the degeneration of those nephrons and encroach to neighbouring healthy tubules, thereby becoming an active contributor to progression[86]. Endothelial activation and injury during reperfusion can potentiate interactions with leukocytes[31,38,101]. Sustained leukocyte accumulation and activation in the kidney can promote extended periods of ischemia because of vascular congestion[161]. In turn, hypoxia can provide a homing signal for inflammatory cells[83]. Macrophages accumulate at sites of vascular disruption and respond to the hypoxic environment by altering a wide array of genes involved in survival, tissue revascularization and recruitment and activation of more inflammatory cells[83]. Indeed, there is a strong correlation between macrophage infiltration and the extent of fibrosis[84]. Macrophage depletion experiments in rats and mice have shown that macrophages can promote fibrosis after ischemia-reperfusion injury (M1 phenotype)[66,107]. However, macrophages are also critical for normal repair and inhibition of fibrosis and CKD (M2 phenotype)[162-164] (see also above, paragraph 'Macrophages'). In addition, it was demonstrated that M1 macrophages that traffic to the post-ischemic kidney can change their phenotype *in situ* to the anti-inflammatory M2 phenotype[162]. Furthermore, infiltration of T-lymphocytes is characteristic for both the early and later phases of AKI which, like macrophages and DCs, can facilitate injury but also promote repair after ischemia-reperfusion injury[52,84,165]. Lastly, renal epithelial cells contribute to inflammation by secreting pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and TGFβ, and chemokines such as MCP-1, IL-8 and RANTES[37,38]. Anti-inflammatory agents may act directly on the epithelial cell in protecting against ischemia-reperfusion associated inflammation[166-168].

1.3.4 Hypoxia

Histologic studies of human kidney biopsies and experimental AKI models have shown that extensive tubulo-interstitial injury is associated with damage to renal arterioles and arteries as well as with damage to and loss of peritubular capillaries[23]. Furthermore, vascular restorative capacity after AKI is more blunted than

the robust tubular repair[78]. It has been demonstrated in experimental AKI models that the reduction in peritubular capillary density following AKI persists on the long-term despite an overall recovery response[24,52,76,169], thus causing chronic hypoxia and prolonging the ischemic insult in the most vulnerable region of the kidney[52,76]. Injured tissue that has insufficient vascular supply becomes hypoxic[78], which can be expected to lead to increased tubular injury and tubulo-interstitial fibrosis[52,170], potentially setting into motion a self-propagating injury cascade[78]. Rarefactions of the peritubular vasculature are also seen in human kidneys following recovery from delayed graft function[24,84,101,171,172]. The chronic hypoxia can be reinforcing and progressive -since increased fibrosis will further compromise the microvasculature and further decrease the availability of oxygen and nutrients to the tubules-, enhance tubular stress and epithelial cell injury, possibly interfere with normal regenerative processes and thus lead to more severe fibrosis[23,52,101,170]. Even when the peritubular capillaries are essentially intact, tubular oxygen supply is still impaired by interstitial fibrosis in the setting of CKD. The extended distance between the capillaries and tubular cells, caused by the fibrotic plaques, reduces the efficiency of oxygen diffusion. This is also a self-sustaining process as hypoxia can also activate fibroblasts and change ECM metabolism of resident renal cells. Their fibrogenic response in turn leads to the obliteration of peritubular capillaries[23,24,83].

In addition, significant evidence exists that altered renal endothelial function contributes to a reduction in renal blood flow following ischemia-reperfusion injury. Renal auto-regulatory responses are impaired for at least 7 days post-ischemia, suggesting prolonged endothelial dysfunction despite an overall recovery response, which has been suggested to prolong injury and predispose the kidney to further damage, as evidenced by biopsies from AKI patients[56].

Also, collective results from human and animal studies led to the reasonable hypothesis that the essential pathophysiology that connects CKD, AKI and ESRD is the reduction of renal mass that occurs during CKD. After an episode of AKI, tubule cells proliferate vigorously. However, depending on the severity of damage, recovery of normal structure is frequently incomplete. Studies in animal models have shown that a subpopulation of regenerating tubules fails to re-differentiate and becomes defective, which is associated with fibrosis[173]. Tubule stress imposed by reduced renal mass may impair the regenerative process by compromising epithelial re-differentiation[101]. The volume of nephron loss will be contracted and filled by fibrous tissue, i.e. fibrosis, a process that occurs in conjunction with tubule degeneration[86].

1.3.5 Recent mechanistic discoveries

1.3.5.1 DNA-methylation

DNA-methylation is an epigenetic modification, whereby a methyl group is added to the DNA, on the 5-carbon of cytosines in CpG sequences[174]. Most frequently, epigenetics is defined as changes in gene expression that occur without changes in the DNA-sequence[175]. Overall, most CpGs in the genome are methylated, with the exception of CpG islands, which are stretches of more densely CpG sites, predominantly found in the promoter regions of genes. DNA-methylation in promoter regions is most often associated with gene silencing[176]. It is becoming clear that DNA-methylation, in addition to cancer pathologies, also plays an important role in renal pathologies, as independent investigators found that aberrant DNA-methylation is associated with inflammation[176,177] and the development of renal fibrosis[89,178-180] and occurs during cold ischemia in transplantation[181,182].

It was hypothesized by Bechtel et al. (2010) that fibroblast activation in fibrotic kidney disease is the result of epigenetic alterations, more precisely DNA-methylation, which renders them terminally activated[89]. A genome-wide methylation screen in primary human fibrotic fibroblasts identified 12 genes that were selectively hypermethylated. Among the identified genes, RASAL1, an inhibitor of Ras was of particular interest. Silencing of RASAL1, by means of DNA-hypermethylation induced fibroblast activation and fibrogenesis[89]. Additional studies are required to gain further insights into the role of DNA-methylation in fibrotic kidney disease and its potential therapeutic application.

1.3.5.2 Micro-RNA

Another type of epigenetic modification is RNA-interference by means of microRNAs (miRNA). MiRNAs are endogenous short (20-22 nucleotides), non-coding, single stranded RNA molecules, which regulate gene expression by cleaving target transcripts or arresting translation[174,183]. In the kidney, miR-192, -194, -204, -215 and -216 are more abundant compared to other organs[184]. Also, a number of miRNAs are not expressed in the kidney, such as miR-1a, -1d, -122a, -124a, -133a, -133b and -296[185]. MicroRNAs are crucial for renal development, homeostasis and cellular senescence[174,183,185]. Disruption of microRNA biogenesis may result in rapid progression of ESRD[183]. Differential expression of miRNAs has been described in polycystic[186,187], diabetic[188,189] and fibrotic[183] kidney disease, IgA nephropathy[190], lupus nephritis[191] and after transplantation[192]. However, since every miRNA can potentially regulate the translation of a large number of different mRNA and each mRNA can possess multiple binding sites for a single or many different miRNA, neutralization of a miRNA can result in off-target effects and potential adverse effects[193].

1.3.5.3 Cell cycle and maladaptive repair

Under normal physiologic conditions, cell proliferation in kidneys is limited to a small percentage of cells (<1%), and most cells are quiescent (G0 phase of the cell cycle) at any given point in time[194,195]. After acute injury, kidney epithelial cells rapidly re-enter the cell cycle[81,195] and this high proliferative capacity has been interpreted to reflect an intrinsic ability of surviving epithelial cells to adapt to the loss of neighbouring cells by proliferating, ultimately replacing the cells that died as a result of the insult[50,196]. However, the stress of the acute injury that causes the kidney cells to enter the cell cycle also induces the production of p21, a cell cycle inhibitor[195]. Indeed, it was shown by Yang et al. that after severe or sustained kidney injury, proliferation of a subset of proximal tubular cells becomes arrested at the G_2/M phase, activating JNK signalling, stimulating the production of pro-fibrotic cytokines such as TGFβ and CCN2[81], inducing sustained fibroblast activation and proliferation, ultimately resulting in excessive ECM production and CKD[161]. To illustrate the importance of this cell cycle arrest, fibrosis can be reduced by treatment with a JNK inhibitor, or by administration of p53, thus bypassing the G_2/M phase[81]. The protective effect of bypassing the cell cycle seems counter-intuitive, but it could be due to a combination of increased proliferation and a reduced inflammatory response to tissue damage with less production of pro-fibrotic cytokines[195].

1.4 Animal models of acute and chronic kidney disease

Animal models are indispensable for research on complex diseases such as renal pathologies. The pathogenesis of AKI and CKD and the progression from acute-to-chronic renal injury involves complex multi-cellular interplays within the heterogeneous renal tissue, which cannot be adequately captured in simplified cell culture or *in silico* settings[197]. An ideal animal model of a specific renal pathology would reproduce most or all of the lesions of the human disease[197]. Rodent (mouse and rat) disease models are favourable, for several reasons: 1) widely available, 2) relative low cost as compared to higher order mammals, 3) the possibility of inducing genetic modifications, which allows both testing the role of specific proteins as well as tracking the fate of cells in disease[197,198]. There is a large spectrum of animal models to mimic both acute and chronic kidney disease (Table 1.1)[199-204]. However, few experiments study the long-term consequences in AKI models and vice versa.

Table 1.1: *Overview of the most frequently used animal models of AKI and CKD*[199-204].

Surgical	Toxic	Genetic	Type
ACUTE KIDNEY DISEASE MODELS			
Ischemia-reperfusion injury (IRI)			Tubular
	Cisplatin		Tubular
	Aristolochic acid		Tubular
	Folic acid		Tubular
	Mercuric chloride ($HgCl_2$)		Tubular
	Glycerol		Tubular
	Gentamicin		Tubular
	NSAID-induced AKI		Tubular
	(Acetaminophen/Diclofenac sodium)		
	Ifosfamide		Tubular
	Uranium		Tubular
	Ferric-nitrilotriacetate		Tubular
	S-(1,2-dichlorovinyl)-L-cysteine (DCVC)		Tubular
	[a] Cecal ligation and puncture (CLP)		Tubular
	[a] Lipopolysaccharide		Tubular
CHRONIC KIDNEY DISEASE MODELS			
Unilateral ureteral obstruction (UUO)			Tubular
Subtotal nephrectomy			Tubular
	Radiation nephropathy		Glomerular
(+ unilateral nephrectomy)	Puromycin aminonucleoside nephrosis (PAN)		Glomerular
	Adriamycin		Glomerular
	Anti-Thy 1.1 glomerulonephritis		Glomerular
	Cyclosporine A (CsA)		Tubular
	Adenine		Tubular
		FGS/Nga mice	Glomerular
		ZSF1 rats	Glomerular
	[b] Ethylene glycol		Tubular
[b] 2-kidney 2-clip			Glomerular Vascular
(+ unilateral nephrectomy)	[c] Deoxycorticosterone acetate (DOCA)(+ high salt diet and/or angiotensin II)		Glomerular Vascular
	[c] Angiotensine II infusion		Glomerular Vascular
	[c] nitro-L-arginine methyl ester (L-NAME)		Glomerular Vascular
		[c] Munich-Wistar-Frömter (MWF) rats	Glomerular Vascular
		[c] Spontaneous hypertensive rats (SHR)	Glomerular Vascular
		[c] RenTgMK mice	Glomerular Vascular
(+ uninephrectomy)	[d] Streptozotocin (STZ)		Glomerular
		[d] Ins2 Akita mice (type I)	Glomerular
(+ uninephrectomy)		[d] db/db mice (type II)	Glomerular

(continued on next page)

a: Sepsis; b: renal stenosis; c: hypertension; d: diabetic nephropathy; e: IgA nephropathy; f: crescentic glomerulonephritis; g: membranous glomerulonephropathy; h: lupus nephritis; i: Alport nephropathy; j: polycystic kidney disease.

Table 1.1 Continued (CHRONIC KIDNEY DISEASE MODELS)

Surgical	Toxic	Genetic	Type
		[d] BTBR ob/ob mice (type II)	Glomerular
		[d] ZDF rats (type II)	Glomerular
		[d] Agouti (Ay) mice (type II)	Glomerular
		[d] OLETF rats (type II)	Glomerular
		[d] eNOS-/-/db/db (type II)	Glomerular
		[e] ddY mice	Glomerular
	[f] Anti-GBM glomerulonephritis		Glomerular
	[g] Bovine serum albumin overload proteinuria		Glomerular
		[g] Heymann nephritis rats	Glomerular
		[h] NZB x NZW	Glomerular
		[h] NZB/W	Glomerular
		[h] MRL lpr/lpr	Glomerular
		[h] BXSB	Glomerular
		[i] Alport mice	Glomerular
		[j] cpk mice/rats	Glomerular

a: Sepsis; b: renal stenosis; c: hypertension; d: diabetic nephropathy; e: IgA nephropathy; f: crescentic glomerulonephritis; g: membranous glomerulonephropathy; h: lupus nephritis; i: Alport nephropathy; j: polycystic kidney disease.

1.5 Conclusion

The pathophysiology underlying acute ischemic kidney injury is multifactorial and involves many different pathways (such as apoptosis, vascular dysfunction and inflammation) and cell types (such as fibroblasts, tubular, endothelial and inflammatory cells). The research field on the mechanisms underlying progression from acute-to-chronic renal injury is a dynamic domain, where the recent discoveries such as the role of DNA-methylation, cell cycle arrest and micro-RNAs may pave the way to new therapeutic strategies. Animal models are essential both in fundamental research and in the search for novel or alternative therapeutic strategies. Animal models grant the opportunity to investigate molecular pathways and disease-specific mechanisms, as well as to evaluate potential novel therapies.

1.6 Aims of the thesis

Animal models are indispensable to unravel the mechanisms underlying the progression from acute-to-chronic kidney disease, and to evaluate potential or novel therapeutic strategies. Several animal models of AKI are available, however, they are often of little clinical relevance, or unsuited to study the progression from acute-to-chronic kidney disease. In view of this, the first aim of this thesis was to develop and characterize the physiological and clinically relevant mouse model of

ischemia-reperfusion as a model of acute-to-chronic kidney injury. In addition, it is known from transplant biology that both warm ischemia time, i.e. the duration between clamping of blood flow and removal of the kidney (which corresponds with body temperature during ischemia in the mouse model) and cold ischemia time, i.e. the duration of extra-corporal ischemia time (which corresponds with duration of ischemia in the mouse model) are risk factors for adverse outcomes and delayed graft function. Hereto, we additionally investigated the influence of these parameters on the long-term fibrotic outcome.

Fibroblasts are considered to be the main effector cells that produce ECM, in concert with cytokines and growth factors which sustain and further promote the fibrotic process and attract inflammatory cells. In fibrosis, fibroblasts are terminally activated, rendering them unable to return to their resting state once the pathological insult has been resolved. It has been shown that aberrant DNA-methylation, i.e. hyper-methylation of RASAL1, is one of the mechanisms underlying this hyper-activation. Therefore, the second aim of this thesis was to investigate whether progression of renal fibrosis can be halted by prevention of the copying of the pathological DNA-methylation pattern of terminally activated fibroblasts to their daughter cells.

Research in both animal models and patients indicates that both persistence of inflammation as well as prolonged activity of the TGFβ-pathway are possible mechanisms underlying progression from acute-to-chronic kidney injury. Hereto, the third aim of this thesis was to investigate whether attenuation of inflammation or antagonism of TGFβ is able to prevent the progression from acute-to-chronic renal injury.

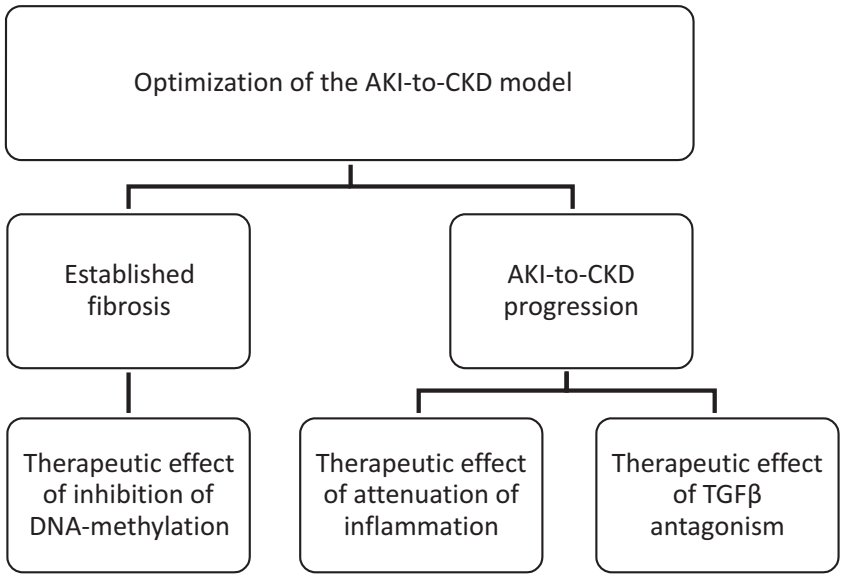

Figure 1.7: Aims of the thesis.

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Chapter 2

Methods

2

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Chapter 2: Contents

Despite the intuitive simplicity of the renal ischemia-reperfusion (IRI) model, i.e. obstructed renal blood flow for a given period of time, researchers are often confronted with problems concerning reproducibility of the model and within-group variation. Body temperature during ischemia and ischemia time are the most important and the most acknowledged factors influencing long-term outcome after UIRI, as described in detail in chapter 3. However, other minor technical factors, such as anaesthesia, mouse sex and strain, also influence the renal pathological outcome, making this model in its execution more complicated and less reproducible than generally anticipated. Furthermore, in the literature regarding nephrology, there is no consensus in methodology to quantify chronic renal damage and fibrosis. In order to cover the whole practical finesse of ischemia-reperfusion, a detailed description of the procedure is provided here, complemented with a referenced overview on crucial technical issues with the specific aim of putting starters in the right direction of implementing IRI in their research. Furthermore, we describe a methodology for quantifying immunostainings of fibrosis-related proteins using image analysis software and Western blotting.

2.1 Mouse model of renal ischemia-reperfusion injury

2.1.1 Pre-operative preparation

2.1.1.1 Anaesthesia and analgesia

Anaesthesia

General anaesthesia in laboratory animals involves loss of consciousness, loss of sensation (analgesia) and muscle relaxation[1]. An ideal anaesthetic agent is easy to administer, produces a fast and adequate immobilization, has limited side effects, and is reversible and safe for animals and operators. Unfortunately such an anaesthetic is not available, and the best drug selection is highly variable according to different experimental circumstances[2] e.g. interference with pathology. Inhalation anaesthesia is usually preferred to injection anaesthetics. Induction and recovery of inhalation anaesthesia are rapid, safer (as it causes less cardiovascular depression) and allows accurate control over the depth of anaesthesia[3]. However, compared to injection anaesthesia, inhalation anaesthetics are counter-indicated for use during IRI as it was shown that some volatile anaesthetics confer profound protection against renal IRI by attenuating inflammation[4]. Injection anaesthetics most commonly used for laboratory mice are barbiturates, dissociative anaesthetics such as ketamine, and α2 agonists. Barbiturates such as sodium pentobarbital (Nembutal, CEVA Sante Animale) are counter-indicated for IRI, as they reduce blood flow to the kidney, secondary to lowered blood pres-

sure, with reduced glomerular filtration rate (GFR) and urine output[1]. In addition, barbiturates have a narrow margin of safety. Dissociative anaesthetics, e.g. ketamine (Ketalar, Pfizer) and tiletamine (Zoletil, Virbac), have a wide margin of safety, analgesic potential and prevent spinal sensitization (wind-up). Ketamine is often combined with other anaesthetic agents such as α2 agonists to improve quality of anaesthesia while reducing its side effects. Ketamine combined with xylazine (Rompun, Bayer) is the most used ketamine combination in mice, producing short surgical anaesthesia (30-45 minutes) with good immobilization and some analgesia[2]. Ketamine is metabolized in the liver, producing inactive metabolites that are excreted by the kidney[1], and as such is safe to use in mice with compromised renal function as is the case with IRI. Xylazine is also metabolized in the liver, producing inactive metabolites, however it is recommended to lower the dose in case of renal failure[1]. When used during the induction of IRI, and certainly when a healthy kidney is left in place, remnant renal function is sufficient to allow its safe use.

Analgesia

The use of analgesia should be based on the species, the type of procedure performed, the pharmacokinetics of available agents, and any known adverse effects of the specific drugs[5]. Also, it is currently believed that analgesia administered pre-operatively (pre-emptive analgesia) provides a more efficient and readily pain control[3]. Analgesics most commonly used for laboratory mice are opioids and non-steroidal anti-inflammatory drugs (NSAIDs)[6]. Opioids are part of the most potent analgesic agents. Fentanyl (Durogesic, Janssen-Cilag), oxymorphone (Opana, Endo Pharmaceuticals), buprenorphine (Temgesic, Reckitt Benckiser) and butorphanol (Stadol, Hospira) are the most commonly used opioids in laboratory animal care. Fentanyl is the most powerful, but is also the shortest acting. In addition, it is given transdermally by skin patch, making it less favourable for being administered routinely[2]. Buprenorphine seems to be the most appropriate analgesic for use in mice undergoing IRI because of its long lasting (12 hours) effect, high therapeutic index and its potential for being used in animals with compromised renal function since it is metabolized in the liver[7]. However, caution has to be taken with buprenorphine as it can suppress respiration, cause sleepiness or slow down the recovery of anaesthesia[6]. NSAID's such as carprofen (Rimadyl, Pfizer), ketoprofen (Rofenid, Sanofi-Aventis), ketorolac (Taradyl, Roche), and meloxicam (Mobic, Boehringer Ingelheim) are also useful in laboratory animals, all the more since they exhibit some pleiotropic effects, such as reduction of fever and inflammation. However, as NSAID's also have renal side-effects, they are contra-indicated for being used as analgesic during induction of IRI[1]. Shortly after induction of anaesthesia, buprenorphine, diluted in sterile saline, is provided via intraperitoneal injection. In general, it is not necessary to provide additional analgesia during the post-operative care since the behaviour of mice

does not show significant signs of distress after the initial dose of buprenorphine[8].

2.1.1.2 Optimized pre-operative preparation of the animal for the induction of IRI

The mouse is anaesthetized with a mixture of ketamine (Ketalar, Pfizer, Elsene, Belgium; 80 mg/kg) and xylazine (Rompun, Bayer, Wuppertal, Germany; 16 mg/kg), diluted in sterile saline to a final volume of 2.4 ml/100 g body weight. The mixture is administered intraperitoneally whereupon the animal is immediately transferred to an incubator set at a fixed temperature until loss of righting reflex has occurred. It is of paramount importance that body temperature is monitored throughout the procedure since it may decrease by several degrees following administration of ketamine and xylazine[5] and hypothermia is one of the most common causes of anaesthetic death. Following induction of anaesthesia, which usually takes 3-5 minutes, the left side of the abdomen is depilated and disinfected with 70% ethanol. Depilation of the abdomen is done with Veet cream (Reckitt Benckiser, Brussel, Belgium), followed by sufficient cleaning with moist sterile tissues to remove any cream remnants. Preferably, depilation is conducted one day in advance, for several reasons: 1) it improves working efficacy on the day of surgery, 2) it allows a more efficient time use during which the animal is sedated (45-60 minutes) 3) it avoids the need to administer additional anaesthesia, 4) it induces less skin irritation and, 5) depilation of the skin just before surgery substantially contributes to the decrease of body temperature that occurs after anaesthesia even when placed on a heating pad. Overall, depilation of the skin 24 hours in advance improves the reproducibility of the surgery. Next, eye ointment (Duratears, Alcon-Couvreur, Puurs, Belgium) is applied to make sure the cornea is protected from drying and trauma, and buprenorphine (Temgesic, Reckitt Benckiser, Brussel, Belgium; 0.05 mg/kg), diluted in sterile saline, is provided via intraperitoneal injection. The animal is placed with its back on the heating pad (Physitemp, Clifton, New Jersey) in a position with its head and neck extended to ensure that its airway remains unobstructed[3]. The body temperature is monitored trough a rectal probe, with a feedback system to the heating pad (Physitemp, Clifton, New Jersey). Before initiating surgery, anaesthetic depth is determined by touching the medial corner of the eye, which should not result in a response and by testing the withdrawal response by applying pressure with a fingernail to the back foot of the animal, who should not withdraw[1]. Respiration should be monitored to ensure that it is of adequate depth and normal frequency[2].

2.1.1.3 Considerations on the ischemia-reperfusion procedure

Ischemia time

Renal ischemia time is an important determinant of AKI severity and subsequent

renal pathology[9, 10]. The most commonly used ischemia times for bilateral (BIRI) and unilateral (UIRI) ischemia-reperfusion injury (IRI) with contralateral nephrectomy are 30 minutes in mice and 45 minutes in rats. For UIRI without nephrectomy 30, 45 and 60 minutes of ischemia are most frequently used in mice and 45 minutes in rats. Ischemia induces inhibition of active ion transmembrane transport because of depletion of intracellular energy stores, resulting in increased ion and water influx, causing cell swelling and oedema. The influx of water and ions results in local hemoconcentration because of the transmigration of water into the cells, which causes increased blood viscosity. Stiffening of leukocytes and the increased leukocyte-endothelial cell and neutrophil-neutrophil interactions further impairs blood flow properties, which hinder the restoration of microvascular blood flow upon reperfusion, also known as the "no reflow" phenomenon. Severity of the no reflow phenomenon and the cellular oedema are dependent on the time of ischemia[10-12]. The duration of ischemia necessary to induce a progressive and persistent renal injury depends on the properties of the vascular clip, mouse strain[13], gender[14], mouse weight (as fat tissue can insulate) and thus needs to be optimized and standardized empirically.

Body temperature during ischemia-reperfusion

Another important determinant of renal outcome after renal ischemia-reperfusion is body temperature during ischemia. The effect of body temperature on the severity of acute IRI is connected to the body metabolism, and relates to three different processes: 1) higher body temperature during ischemia results in a more severe decrease of intracellular energy stores, 2) the concentration of degradation products inosine and hypoxanthine increases with increasing body temperature during ischemia, which results in increased production of free radicals upon reperfusion, and 3) increasing the body temperature during ischemia produces an increased damage of cell membranes[9, 15, 16]. It is known that hypothermia during experimental IRI provides renal protection, as it delays degradation processes and extends cell tolerance to ischemic stress[16]. In addition, hypothermia reduces inflammatory processes and limits the increase in vascular permeability[17]. Thus, temperature control during ischemia is one of the most important aspects of IRI models, and is necessary for reproducibility, yet far more difficult to standardize than ischemia time. Appropriate equipment for temperature control is required since lack of active temperature control exposes the animals to daily and seasonal variations in room temperature and air drafts, even when placed on a heating pad[8]. The latter is nicely illustrated by experimental work of Delbridge et al. (2007) demonstrating the difference in AKI severity, as measured by serum creatinine, when rats underwent BIRI on either a heating pad without temperature control, heating pad with rectal temperature-control or without heating pad[15]. These and our own observations illustrate (chapter 3) that monitoring body temperature

during the procedure is of particular importance, as the body temperature of the animal needs to remain stable during ischemia, preferably up to the moment the animal regains consciousness. With regard to adequate temperature control, it is important to keep in mind that the body temperature of animals, sedated at room temperature can decrease several degrees following administration of ketamine and xylazine[5]. Also, heating the animal with a heat lamp and heating pad needs to be closely monitored as body temperature can spike at 38°C or higher, increasing the variability within the group. As an alternative, a neonatal incubator provides a more stable temperature controlled environment which avoids body temperature spikes and prevents the anaesthesia-associated temperature drop when animals are put inside immediately after sedation (own observations). Overall, it should be noted that temperature settings depend on the lab environment, the mouse strain and weight (as fat tissue can insulate) and therefore require an empiric optimization and standardization.

2.1.2 Surgery

Surgery should not be started before the core body temperature of the mouse, as measured with a rectal probe, is stabilized at the set point and the mouse is in deep anaesthesia. Stabilization of the core body temperature can take up to 15 minutes, may require a heat lamp (in addition to the heating pad) and a draft-free location to facilitate this process. The abdomen is opened with a midline incision; approximately 1-1.5 cm. Using a wound spreader the intestines are carefully pushed aside and the left kidney is exposed, however not exteriorized to avoid rigorous cooling of the kidney and ischemic preconditioning during manipulation of the kidney. The renal pedicle is carefully dissected with fine-point tweezers to remove the perihilar adipose tissue, exposing the blood vessels for renal pedicle clamping. It is important that the blood supply to the adrenal gland remains unaffected. The renal pedicle is clamped with an atraumatic vascular clip (Scanlan, Saint Paul, Minnesota) using holding forceps, ensuring minimal vascular damage, and as little as possible perihilar fat in the clamp. Successful ischemia is characterized by a gradual colour change of the kidney from red to dark purple within 1-2 minutes. The right kidney is left untouched. The abdomen is temporarily closed with a suture and the animal is transferred to an infant incubator, kept at the temperature that allows the body temperature of the animal to remain stable at the set-point temperature for the duration of ischemia. Temperature of the animal is monitored continuously with a rectal thermometer (Bioseb, Vitrolles, France). Fluctuations in body temperature during ischemia should be kept less than 0.5°C, as higher variations will increase variability in the degree of renal injury. The vascular clip is released at the desired time to start reperfusion. After verification of kidney colour to change back to red (roughly within 10 seconds), a Vicryl 4-0

suture (Ethicon, Norderstedt, Germany) is used to first close the muscle layer, followed by closing of the skin. Sham-operated animals are subjected to the exact same surgical procedure, aside from clamp placement.

2.1.3 Post-operative care

Immediately after surgery, 1 ml saline or Plasma-Lyte (Baxter, Lessen, Belgium; i.e. buffered low chloride saline solution) is given intraperitoneally to compensate for the fluid loss during surgery. Even though the superiority of Plasma-Lyte over saline is not proven, clinical reports allow us to assume that high chloride solutions are associated with worse AKI as compared to low-chloride solutions[18]. The animals are kept on a warm water mat (water temperature at 37°C) until awakening after which they are placed in an open grid recovery cage under a heating lamp until full consciousness is regained. Heating lamp and cage are placed in such a way that one end of the cage maintains room temperature whilst the distance between lamp and animals is held large enough to avoid overheating. After 24 hours the animals are transferred to their housing cage. During the first 3 days after the surgery, the animals, in addition to the standard chow, are supplemented with DietGel Recovery Purified Soft Diet for Rodents (Clear H_2O, Portland, Maine), rich in water and sugars, to reduce the post-operative weight loss and allow faster recovery after surgery. Weekly follow up of the body weight and behaviour is performed as measures of good health.

2.2 Quantification of (immuno)histological stainings

Renal morphology is evaluated on left kidney tissue fixed in NBF, stained with Masson's trichrome after fixation in Bouin's fixative.

Immunostainings. Briefly, paraffin embedded 4 µm thick sections of ischemic kidney tissue were blocked with normal serum of the animal in which the secondary antibody was generated, and incubated overnight with the primary antibody. After washing, sections were incubated with a biotinylated secondary antibody and subsequently incubated with avidin and biotinylated horseradish peroxidase (AB-complex, Vector Laboratories, Burlingame, California). A dark brown colour was developed with diaminobenzidine in the presence of 3% H_2O_2. Sections were counterstained with methyl green to visualize nuclei.

A computerized image analysis system (Axiovision) running a custom made program is used for the quantification of Masson's stain and the various immunostainings. A digital camera captures images directly from the slide on the microscope and these images are transferred to the computer and used for the histomorphometric analysis (Figure 2.1 A). In a first step a relevant set of RBG-pixels is chosen which detects the colour of interest in the stain, i.e. blueish green for Mas-

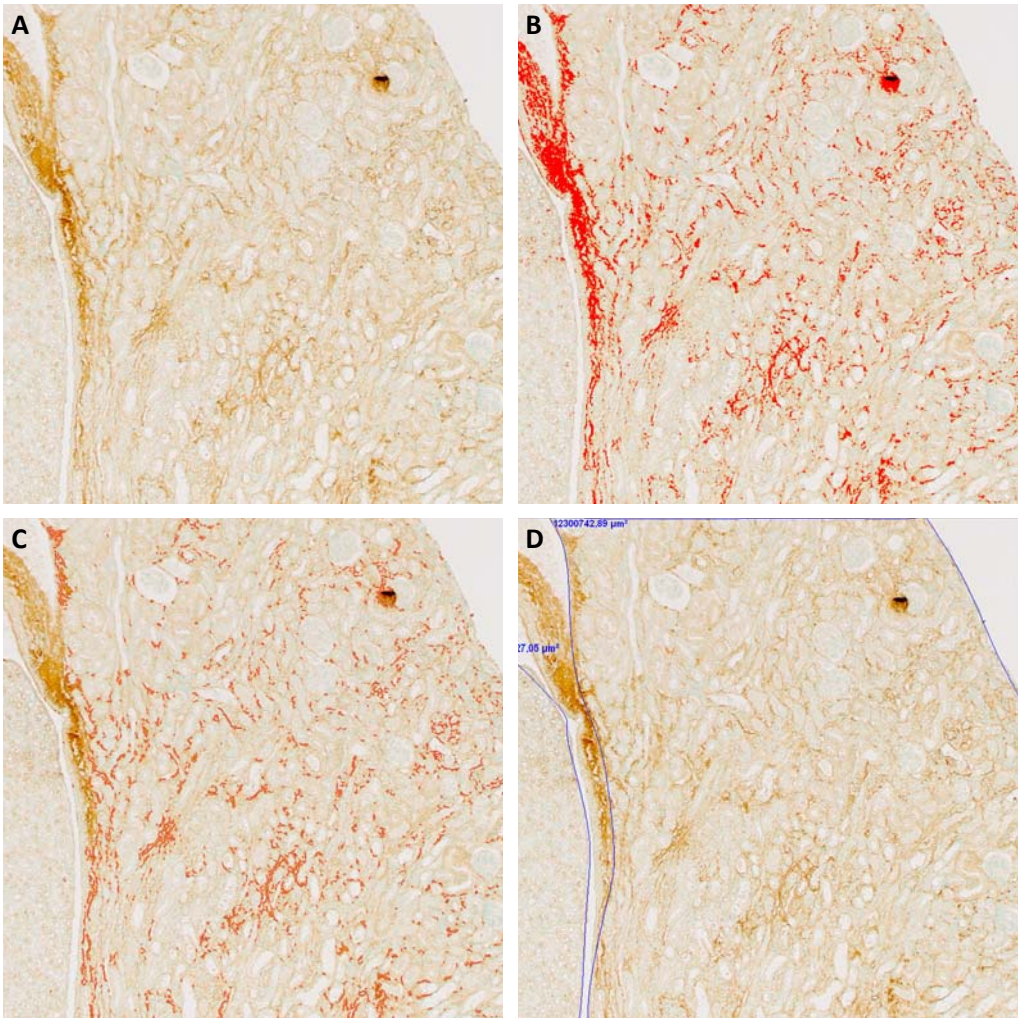

Figure 2.1: Overview of the image quantification.
A: The image from the slide on the microscope is transferred to the computer. **B**: A relevant set of RBG-pixels is chosen which detects the colour of interest in the stain. **C**: A merged image is then created, with pixels that fall within the defined range. **D**: Tissue outline is manually drawn per image.

son's stain, brown for the immunostaining (Figure 2.1 B). The same set of pixels is used to quantify all slides of the experiment. A merged image is then created, with pixels that fall within the defined range encircled in red (Figure 2.1 C). Now there is the possibility of manually changing the pixel selection (box), allowing correction of wrongly selected pixels or addition of non-selected pixels. After this step, the number of selected pixels is calculated in the entire image. Tissue outline is then manually drawn per image (Figure 2.1 D), allowing calculation of the area % stain per slide, by calculating the ratio of the summed absolute areas of staining versus the total tissue surface.

This quantification is performed on at least 5 digital images per tissue slide, randomly chosen from renal cortex and outer medulla.

2.3 Western blot

Total protein is isolated from a pole section of the ischemic kidney using RIPA buffer, after which the protein concentration is determined by a colorimetric method, Pierce™ BCA protein assay kit (Fisher Scientific, Landsmeer, The Netherlands). The sample is diluted to 3mg/ml, and Laemli buffer (161-0737, Bio-Rad, Temse, Belgium) is added. Proteins are separated through SDS-PAGE gel electrophoresis (35 min at 165 V) on 4-20% precast polyacrylamide gels (456-1096, Bio-Rad). After electrophoresis, proteins are transferred onto a PVDF blotting membrane with 0.45 µm pore size (GE Healthcare Life Sciences; Diegem, Belgium) for 60 min at 100V. The membrane is then incubated in 5% non-fat milk (blotting grade blocker; Biorad) for 2 hours and subsequently overnight with the primary antibody, diluted in 1% non-fat milk. Following several wash steps, the membrane is incubated with a peroxidase conjugated secondary antibody. After several wash steps, the immune complexes are detected using a chemiluminiscence kit (Super-Signal West; Fisher Scientific).

2.4 Analysis of genomic DNA-methylation

DNA is extracted from a pole section of the ischemic kidney (Allprep Mini Kit; Qiagen, Antwerpen, Belgium). Isolated genomic DNA samples are hydrolysed to individual deoxyribonucleosides in a simplified one-step procedure. A digest mix is prepared by adding 300 mU Phosphodiesterase I (P3134-100MG, Sigma Aldrich, Diegem, Belgium), 200 U alkaline phosphatase, (P7923-2KU Sigma Aldrich) and 250 U Benzonase® Nuclease, (E1014-5KU Sigma Aldrich) to 5 ml Tris–HCl buffer (pH 7.9, 20 mM) containing 100 mM NaCl and 20 mM $MgCl_2$. Extracted DNA (1 µg diluted in 50 µl HPLC-grade water) is hydrolysed by adding 50 µl digest mix and incubating at 37°C for at least 12 hours. After hydrolysis, water (HPLC-grade) is added to the samples up to a total volume of 1 ml. Reference standards for 5'-methyl-2'-deoxycytidine (5-mdC) and 2'-deoxycytidine (dC) are purchased from Jena Bioscience (N-1044, Jena Bioscience, Germany) and Sigma (D3897-1G) respectively. Stock solutions of 5-mdC and dC are prepared by dissolving the purchased solid reference standards in pure water (HPLC-grade). Using these stock solutions, a series of calibration solutions are prepared for 5-mdC and dC in a range of 0.1-10 ng/mL and 1–100 ng/mL respectively. The same calibration standards are used in all of the experiments. Global DNA methylation is obtained by quantifying 5-mdC and dC using UPLC, in combination with tandem mass spectrometry (MS-MS). LC/MS-MS analysis of the samples is conducted on a Waters® Acquity UPLC™, coupled to a Waters® Micromass Quattro Premier™ Mass Spectrometer. With this technique injections are performed on a Waters® UPLC column (BEH C18, 50 mm

x 2.1 mm, 1.7 µm) which is held at a temperature of 40°C during analysis. The mobile phase consists of a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The volume of injection is 10 µl at a flow rate of 0,35 ml/min. The analyses are performed using electro spray ionization (ESI) in the positive mode and a multiple reaction monitoring (MRM) method is used with argon as collision gas. Mass-to-charge (m/z) transitions monitored were 242.2 →125.95 for 5-mdC and 228.1 → 111.9 for dC.

The global DNA methylation is expressed as a percentage of 5-mdC versus the sum of 5-mdC and dC [5-mdC/(5-mdC + dC)] %. We measured samples in duplicate to account for technical variation that resulted in a R2 of 0.8 (ICC = 0.90). The average methylation value of both measurements was used in the statistical analysis.

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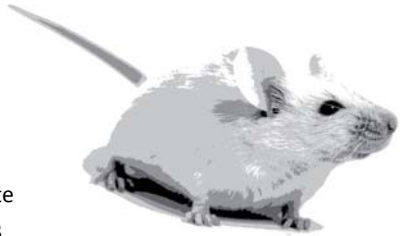
Chapter 3

The animal model of unilateral ischemia-reperfusion: a model of acute-to-chronic kidney disease or renal repair

3



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Chapter 3: Contents

Abstract

Background: Acute kidney injury (AKI) is an underestimated, yet important risk factor for development of chronic kidney disease (CKD). Even after initial total recovery of renal function, some patients develop progressive and persistent deterioration of renal function and these patients are more likely to progress to end-stage renal disease (ESRD). Animal models are indispensable for unravelling the mechanisms underlying this progression towards CKD and ESRD and for the development of new therapeutic strategies in its prevention or treatment. Ischemia (i.e. hypoperfusion after surgery, bleeding, dehydration, shock, or sepsis) is a major aetiology in human AKI, yet unilateral ischemia-reperfusion is a rarely used animal model for research on CKD and fibrosis.

Methods: To evaluate the model of unilateral ischemia-reperfusion (UIRI) without contralateral nephrectomy as a model for acute-to-chronic kidney injury, and to evaluate the effect of body temperature during ischemia and ischemia time, UIRI was performed at different body temperatures and for different durations. In addition, the UIRI model with contralateral nephrectomy was evaluated as a model for renal repair.

Results: Here, we demonstrate in C57Bl/6J mice, by both histology and gene expression, that UIRI without contralateral nephrectomy is a very robust model to study the progression from acute renal injury to long-term tubulo-interstitial fibrosis, i.e. the histopathological hallmark of CKD. Furthermore, we report that the extent of renal fibrosis, in terms of *collagen I*, *TGFβ*, *CCN2* and *CCN3* expression and collagen I immunostaining, increases with increasing body temperature during ischemia and ischemia-time. In addition, we report that UIRI with contralateral nephrectomy is a model of renal repair.

3.1 Introduction

Despite decades of scientific research, chronic kidney disease (CKD) still has an increasing incidence and prevalence[1]. In addition, it is becoming increasingly clear that acute kidney injury (AKI) is an underestimated, yet important risk factor for the development of CKD[2]. Long-term follow-up studies (4 months to 6 years) report that between 35 and 71% of patients surviving an episode of AKI had incomplete recovery of renal function as assessed by creatinine clearance or serum creatinine measurements[3]. Even after initial total recovery of renal function, some patients develop progressive and persistent deterioration of renal function[4]. Moreover, these patients are more likely to progress to end-stage renal disease (ESRD) compared to patients without a history of AKI[2]. Getting insight in the mechanisms underlying the progression from acute to chronic renal injury is a major focus of recent research in the field[5]. Since the pathogenesis of acute-to-chronic renal injury involves a complex multi-cellular interplay within the heterogeneous renal tissue, animal models play a crucial role in unravelling these complexities towards development of new and efficient therapeutic modalities[6].

Rodent (mouse and rat) disease models are favourable, for several reasons: 1) widely available, 2) relative low cost as compared to higher order mammals, 3) the possibility of inducing genetic modifications, which allows both testing the role of specific proteins as well as tracking the fate of cells in disease[7, 8]. Renal ischemia reperfusion injury (IRI) is one of the most used animal models for both fundamental and therapeutic intervention studies in AKI. Yet, despite the nephropathological relevance of ischemia, only a few studies applied IRI to study long-term sequelae of an acute ischemic insult[9-13]. The potential of this model as an initiator of CKD has not been systematically investigated. The IRI model comes in different flavours, each with their own natural course of renal dysfunction and histopathology. Importantly, not all IRI variants are suited to study the progression from AKI to CKD and fibrosis. A distinction has to be made between cold and warm renal ischemia reperfusion (Figure 3.1). Cold ischemia, where ischemia is either performed at 32°C body temperature[14, 15] or by cooling the kidney to 4°C[16-18], is a rarely used variant of the IRI model. Most often cold ischemia actually refers to cold storage of the kidney before transplantation, either with[19-21] or without[22-24] warm ischemia prior to the cold storage period. Warm ischemia, on the other hand, is most frequently used and can be subdivided into bilateral ischemia-reperfusion (BIR) and unilateral ischemia-reperfusion (UIR). Depending on the presence of the contralateral kidney, UIR can be further subdivided into UIR without contralateral nephrectomy and UIR with contralateral nephrectomy. An additional variation on the latter model consists in the timing at which the uninjured contralateral kidney is removed (cfr. Skrypnyk et al. (2013)[25]).

As a model of AKI, bilateral ischemia-reperfusion injury (BIRI) affects total renal

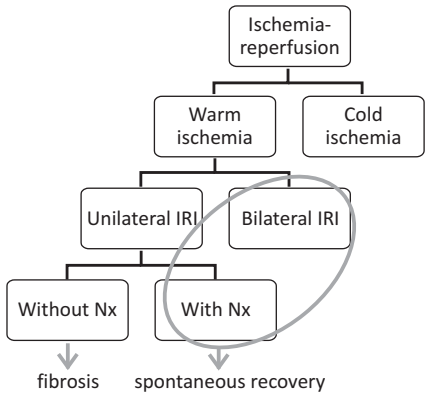

Figure 3.1: Overview of the different variations of the ischemia-reperfusion model.

mass and induces a measurable increase in serum creatinine and blood urea nitrogen (BUN), both functional hallmarks of AKI in patients[26]. However, with respect to its application for studying chronic renal fibrosis, a strict control of the severity of the induced ischemic renal injury is critical: when renal injury is too mild, near complete recovery of the kidneys ensues without progression towards chronic renal injury and fibrosis[27, 28]. On the other hand, when the induced ischemic insult is too severe, animals are very likely to die of acute renal failure within 48 hours[25]. Although long-term studies with BIRI have been performed[3, 29, 30], most studies indicate that kidney morphology returns to almost normal 2 weeks after the bilateral ischemic insult. A few studies reported a limited number of tubules with signs of damage and some lymphocyte infiltration in the interstitium[29, 30]. Microvascular rarefication was noted to be present 4 weeks after BIRI[31] and some glomerular atrophy and hypertrophy and interstitial scarring was observed 40 weeks after BIRI[3]. Serum creatinine returned to sham-levels 16 days after BIRI and remained stable up to 40 weeks after BIRI[3, 31], indicating no long-term functional decay.

The pathological course of unilateral ischemia-reperfusion injury (UIRI) with immediate contralateral nephrectomy (i.e. during the same surgery) is expected to be quite similar to BIRI in the sense that in both models, the animals leave the surgical procedure with injured renal tissue only. Comparing the restoration capacities of the affected kidneys in the nephrectomy, unilateral and bilateral IRI models respectively, higher repair capacity was observed in the nephrectomy model followed by bilateral then the unilateral model[32]. Due to the presence of the healthy kidney, which can compensate for the loss of function of the ischemic kidney, the risk of mortality caused by acute renal failure is highly reduced[27] and the consequences of AKI can be investigated well beyond the first days of acute injury[33]. Furthermore, UIRI without nephrectomy allows to conduct longer ischemia times[29] (up to 60 minutes in mice[9] and 190 minutes in rats[34]), thus al-

lowing studies in a larger range of severity of kidney injury. This potential of the UIRI model without nephrectomy in inducing a range of histopathological changes more closely resembles the nephropathological spectrum seen in patients[35]. Moreover, creating an uremic milieu, as occurs with BIRI, and which is part of the renal pathology in patients, is avoided[26]. This allows the assessment of the natural course of post-ischemic renal damage without possible protective effects inherent to uraemia, i.e. cytoprotective[36] and anti-inflammatory effects[37]. It should be noted, however, that due to the presence of the non-injured contralateral kidney, the functional course after UIRI cannot be assessed by simply taking a blood sample and collect 24-hours urine to calculate creatinine clearance[25]. One way is to remove the healthy contralateral kidney and measure glomerular filtration rate (GFR) hours after removal[13], which can be an end-point analysis in itself. Alternatively, split renal function measurement by use of ureter catheterization has been performed in dogs[38, 39], pigs[40] and rats[41, 42]. In rats, this is done shortly before euthanasia (end-point analysis), a technique which is unlikely to be applied in mice.

Generally, consequences of AKI induction through ischemia-reperfusion are studied 24-48 hours up to 2 weeks after the insult[13, 28, 43]. We hypothesize that the model of UIRI without nephrectomy is valuable to study the evolution of the histopathology of acute ischemic kidney injury progressing to CKD with long-term development of fibrosis, and that the model of UIRI with nephrectomy is a model of AKI progressing to renal repair. In the present study we therefore investigated the long-term renohistopathological outcome of UIRI with and without nephrectomy with emphasis on evaluating the development of fibrosis. In addition, in the UIRI model without contralateral nephrectomy, we aimed at investigating the effect of the two most important determinants of ischemic injury on long-term fibrotic outcome, i.e. core body temperature during and duration of ischemia. Since we studied AKI-induced CKD and renal repair, we also included analysis of long-term expression of tubular injury markers and inflammatory cytokines.

3.2 Materials and methods

3.2.1 Study set-up

All surgical procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics Committee (approval number 2011-51).

3.2.1.1 Study no. 1: Unilateral renal ischemia-reperfusion without contralateral nephrectomy as a robust model for acute to chronic kidney injury in mice

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously[44] (see chapter 2). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent warm UIRI, with continuously monitoring of body temperature. The right kidney was left untouched. Sham-operated animals received the same surgical procedure except placement of the clamp.

Study set-up: Male C57Bl/6 mice subjected to an acute ischemic kidney injury by unilateral ischemia-reperfusion (UIR) without contralateral nephrectomy consistently develop post injury renal fibrosis. As already mentioned above, the extent of acute renal injury depends on both body temperature and ischemia time[45]. To examine (1) the effect of body temperature during ischemia on fibrotic outcome after UIRI, the left kidney was clamped for 30 minutes at 37°C (n=5), 36°C (n=4), 35°C (n=10) or 34°C (n=5) and animals were euthanized 12 weeks after UIRI; (2) the effect of ischemia time on fibrotic outcome after UIRI, the left kidney was clamped for 30, 21 or 18 minutes at 36°C and animals were euthanized 6 weeks (resp. n=5, n=12, n=6) and 12 weeks (resp. n=4, n=5, n=10) after UIRI.

Table 3.1: *Overview of the different ischemia conditions (body temperature during ischemia and ischemia time) that were used in study no. 1.*

	37°C	36°C	35°C	34°C
18 min		●		
21 min		●		
30 min	●	●	●	●

Sample size (n=6) was determined by power analysis with respect to the 3R principle of animal ethics. Some UIRI conditions were repeated to verify reproducibility, i.e. UIRI at 35°C for 30 minutes and at 36°C for 21 and 18 minutes. On aver-

age, we encountered 6% mortality, mainly due to post-anaesthetic complications. In addition, upon statistical analysis, on average 8% of the animals were excluded from analysis, because their values were considered outliers for the different parameters under study. Prior to surgery, animals were randomly allocated into the different groups. Animals had free access to standard chow and tap water.

3.2.1.2 Study no. 2: Unilateral renal ischemia-reperfusion with contralateral nephrectomy as a model of renal repair in mice

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously[44] (see chapter 2). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent warm (36°C body temperature) UIRI, with continuously monitoring of body temperature. Three days after ischemia-reperfusion of the left kidney, nephrectomy of the right kidney or sham operation was performed. Sham-operated animals received the same surgical procedure except placement of the clamp and nephrectomy of the right kidney.

Study set-up: Male C57Bl/6 mice subjected to an acute ischemic kidney injury by unilateral ischemia-reperfusion (UIR) without contralateral nephrectomy consistently develop post injury renal fibrosis. UIRI with contralateral nephrectomy is the most frequently used model of experimental AKI, however, on the long-term it is considered a model of renal repair[32]. To examine the effect of contralateral nephrectomy after UIRI on fibrotic outcome, the left kidney was clamped for 21 minutes at 36°C, and after 3 days, right nephrectomy (n=6) or sham operation (n=6) was performed. Animals were euthanized 6 weeks after UIRI.

3.2.2 Real-time PCR

Total mRNA is extracted from a pole section of the ischemic kidney (PureLink RNA Mini Kit; Life Technologies, Gent, Belgium) and converted to cDNA (High Capacity cDNA archive kit; Life Technologies, Gent, Belgium). To quantify gene expression, qPCR, based on the Taqman fluorescence method (ABI Prism 7000 sequence detection system; Life Technologies), was used. Taqman probes and primers for *GAPDH* (Mm99999915_g1), *collagen I α1* (Mm00801666_g1), *TGFβ1* (Mm01178820_m1), *CCN2* (Mm01192931_g1), *CCN3* (Mm00456855_m1), *Havcr1* (Mm00506686_m1), *Lcn2* (Mm01324470_m1), *TNFα* (Mm00443258_m1) and *IL-6* (Mm00446190_m1) were purchased from Life Technologies (Gent, Belgium). Each gene was analysed in triplicate and the expression was normalized to the reference gene *GAPDH*. Calculations were made conform the comparative Cq-method.

3.2.3 Histology

Renal morphology was evaluated on ischemic kidney tissue fixated in NBF (Neutral Buffered Formalin), stained with Masson's trichrome after post-fixation in Bouin's fixative. Masson's trichrome stain is the standard for visualizing fibrosis in tissue as it provides a useful sense of tissue morphology and allows evaluation of localization and severity of extracellular matrix deposition.

For collagen I immunostaining, paraffin embedded 4 μm thick sections of ischemic kidney tissue were blocked with normal goat serum and incubated overnight with the primary antibody, polyclonal rabbit anti-mouse collagen I antibody (dilution 1/3500, Catalogue number T40777R, Lot number 20I25000, Biodesign International, Saco, Maine). After washing, sections were incubated with a biotinylated secondary antibody, goat anti-rabbit IgG antibody (dilution 1/200, PK-4001, Vector Laboratories, Burlingame, California) and subsequently incubated with avidin and biotinylated horseradish peroxidase (AB-complex, Vector Laboratories, Burlingame, California). A dark brown colour was developed with diaminobenzidine in the presence of 3% H_2O_2. Sections were counterstained with methyl green to visualize nuclei. Collagen I immunostaining was quantified using the Axiovision image analysis software (Carl Zeiss, Jena, Germany) and quantification was performed blinded. The area % stain represents the ratio of the summed absolute areas of staining versus the total tissue.

3.2.4 Statistics

All statistical analysis was performed with SPSS Statistics 22 (IBM, Brussel, Belgium). Data are presented as mean ± standard deviation, or as individual values. Comparisons between groups are assessed using a Kruskal-Wallis test, followed by a two-tailed Mann-Whitney U test. Values of $p<0.05$ are considered significant.

3.3 Results

3.3.1 Study no. 1: Unilateral renal ischemia-reperfusion without contralateral nephrectomy as a robust model for acute to chronic kidney injury in mice

3.3.1.1 Effect of body temperature during ischemia on fibrotic outcome

Unilateral renal ischemia-reperfusion injury (UIRI) results in a significant reduction of renal mass ($p<0.05$) at all temperature conditions tested. As depicted in Figure 3.2 A, UIRI at 37°C caused a ±75% reduction ($p<0.05$) in renal mass, whereas the mildest temperature condition tested, i.e. 34°C, also caused a less pronounced ($p<0.05$) but still severe reduction in renal mass (±70%). Masson's stain showed prominent renal damage and severe loss of structure, atrophic renal cortex with disruption of tubular architecture, marked tubule necrosis and intratubular casts, and extensive interstitial inflammatory infiltration (Figure 3.3 A).

Quantification of fibrosis by collagen I immunostaining demonstrated an increased deposition of collagen I for all body temperatures under study as com-

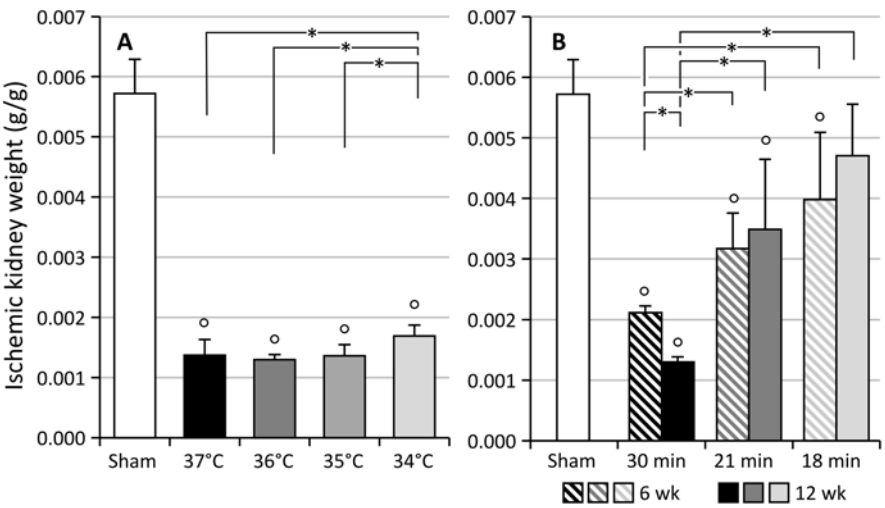

Figure 3.2: Ischemic kidney weight at euthanasia. Kidney weights are corrected for body weight. **A**: UIRI was performed for 30 minutes at 37°C (n=5), 36°C (n=4), 35°C (n=10) or 34°C (n=5) and animals were euthanized 12 weeks after UIRI. UIRI results in a significant reduction of renal mass ($p<0.05$) at all temperature conditions tested. **B**: UIRI was performed for 30, 21 or 18 minutes at 36°C and animals were euthanized 6 weeks (resp. n=5, n=12, n=6) and 12 weeks (resp. n=4, n=5, n=10) after UIRI. UIRI causes an ischemia time-dependent reduction in renal mass, with a significantly more severe reduction in renal mass with longer ischemia times. *: $p<0.05$, °: $p<0.05$ vs. Sham.

pared to sham ($p<0.05$), with a more pronounced increase in collagen I staining for UIRI at 37°C as compared to the lower body temperatures (35°C and 34°C) ($p<0.05$) (Figure 3.4 C). As shown in Figure 3.5, 12 weeks after UIRI a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed in renal cortex tissue in all core body temperature conditions tested as compared to sham ($p<0.05$). The long-term UIRI-induced expression of these genes is also temperature-dependent: higher expression with higher temperature during ischemia (37°C and 36°C vs. 35°C and 34°C; $p<0.05$) (Figure 3.5).

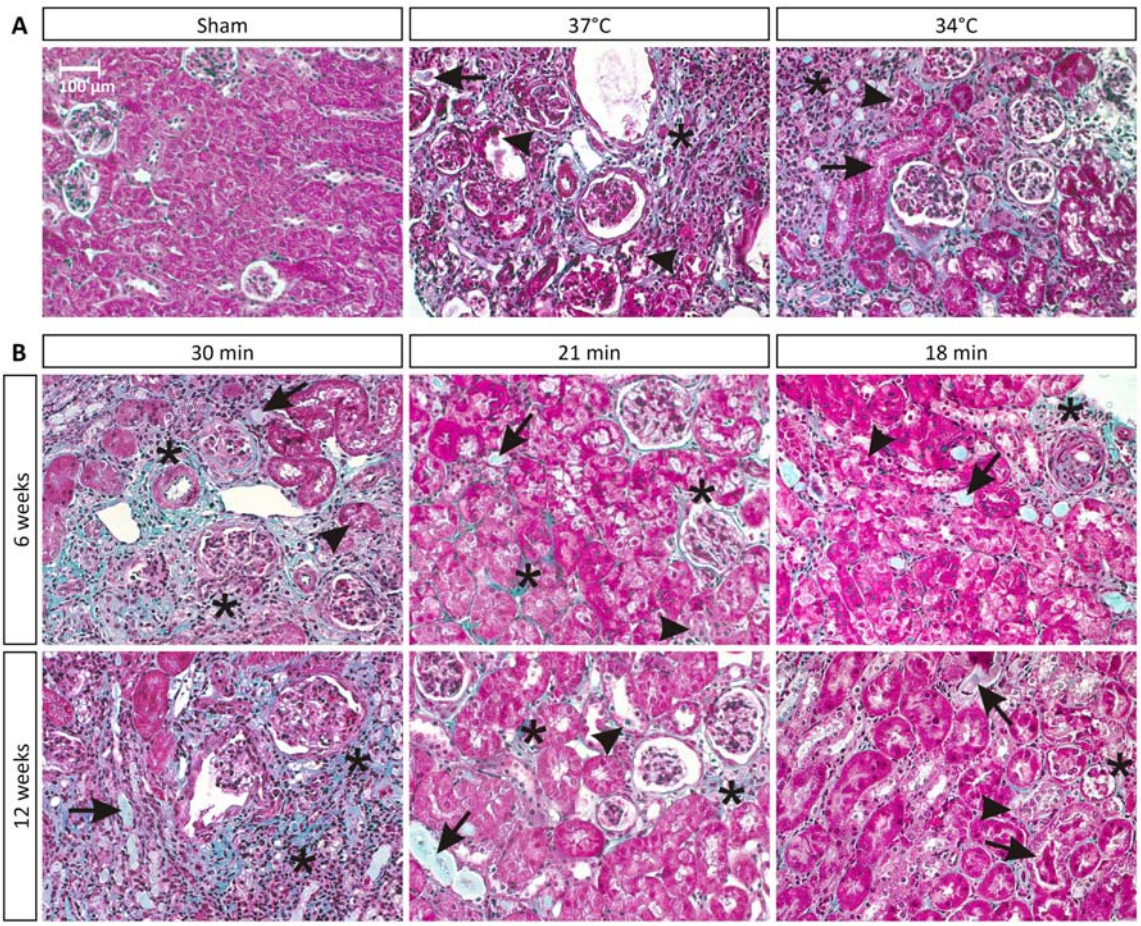

Figure 3.3: **Masson's stained sections of ischemic kidney tissue**. The images shown are representative of the group. Masson's stain showed prominent renal damage and severe loss of structure, with necrotic cells (arrowhead), casts or intraluminal debris (arrow), inflammatory infiltration and fibrosis (*). Blue stain represents extracellular matrix deposition (i.e. fibrosis). Magnification: 200x.
A: Effect of body temperature on long-term fibrotic outcome 12 weeks after UIRI.
B: Effect of ischemia time on long-term fibrotic outcome 6 and 12 weeks after UIRI.

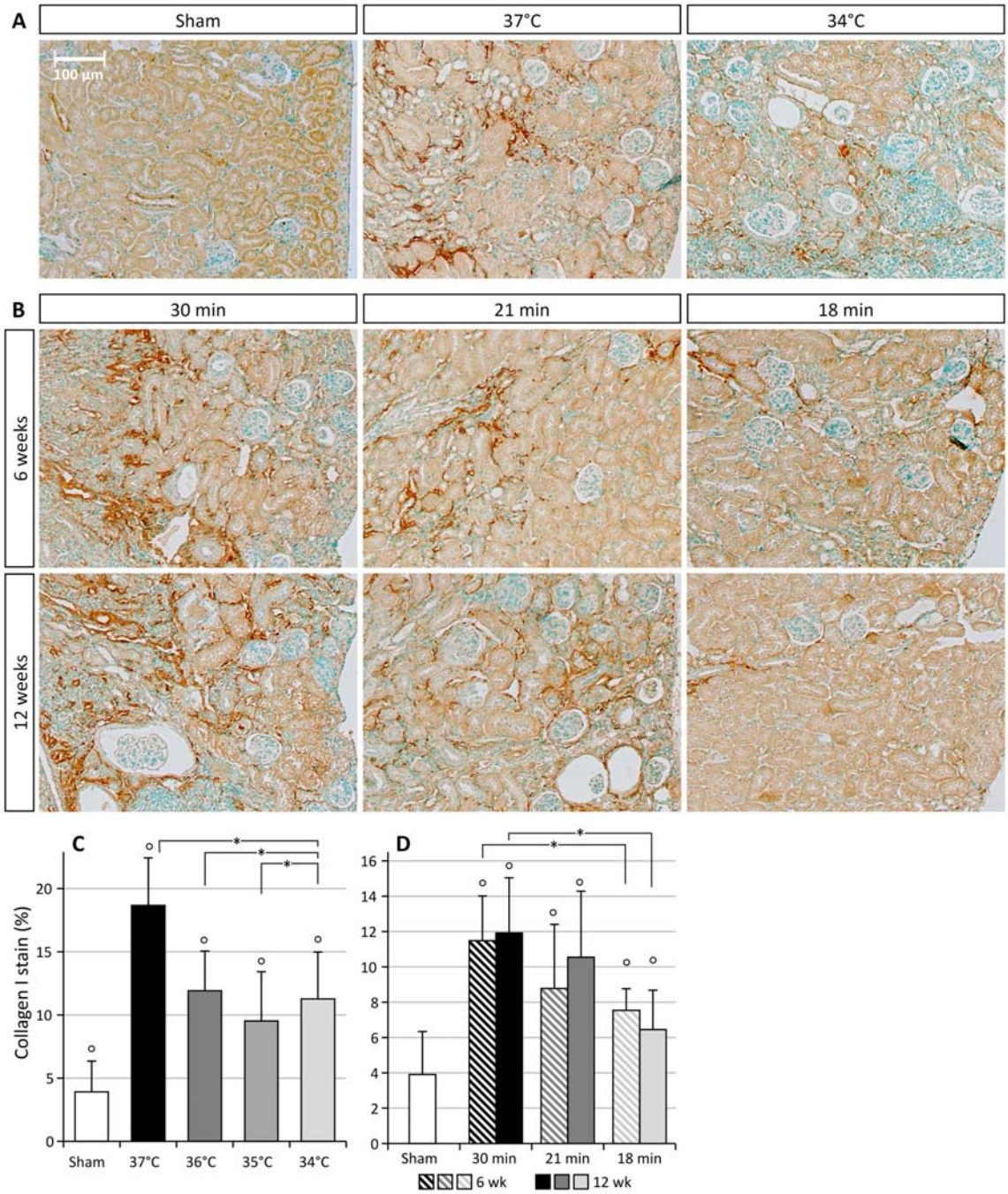

Figure 3.4: Collagen I immunostaining in the ischemic kidneys.
A: Effect of body temperature on long-term collagen I deposition in the ischemic kidney, 12 weeks after UIRI (magnification: 100x). **B**: Effect of ischemia time on long-term collagen I deposition in the ischemic kidney, 6 and 12 weeks after UIRI (magnification: 100x). **C**: UIRI was performed for 30 minutes at 37°C (n=5), 36°C (n=4), 35°C (n=10) or 34°C (n=5) and animals were euthanized 12 weeks after UIRI. Collagen I deposition seems to be dependent on body temperature during ischemia: more collagen I deposition after UIRI at higher body temperatures. **D**: UIRI was performed for 30, 21 or 18 minutes at 36°C and animals were euthanized 6 weeks (resp. n=5, n=12, n=6) and 12 weeks (resp. n=4, n=5, n=10) after UIRI. Collagen I deposition seems to be ischemia time-dependent: more collagen I deposition after longer ischemia times. *: $p<0.05$, °: $p<0.05$ vs. Sham.

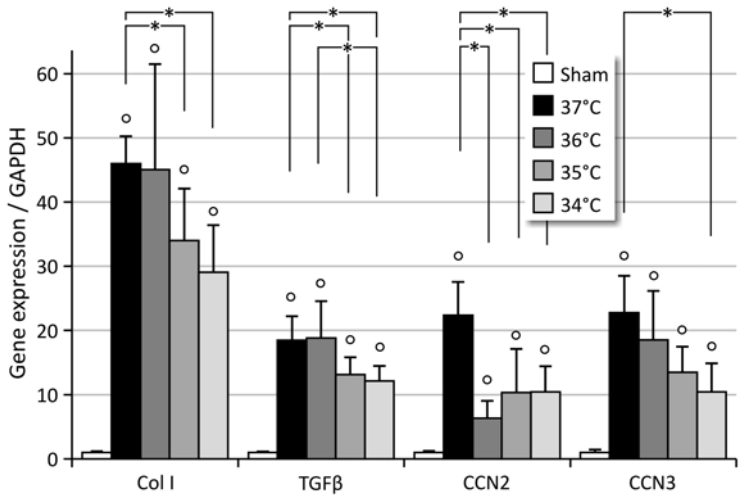

Figure 3.5: Relative quantification of long-term IRI-induced expression of fibrosis-related genes. Core body temperature during ischemia determines degree of long-term fibrotic outcome.
UIRI was performed for 30 minutes at 37°C (n=5), 36°C (n=4), 35°C (n=10) or 34°C (n=5) and animals were euthanized 12 weeks after UIRI. Twelve weeks after UIRI, a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed in renal cortex tissue in all core body temperature conditions tested. The expression of these genes is also temperature-dependent: higher expression with higher temperature during ischemia. *: $p<0.05$, °: $p<0.05$ vs. Sham.

3.3.1.2 Effect of body temperature during ischemia on long-term expression of inflammatory and tubular injury markers

Analysis of gene expression of hepatitis A virus receptor 1 *(Havcr1*; T cell immunoglobulin mucin protein 1 (TIM-1)-producing gene in mice; KIM-1, human homolog) and lipocalin 2 *(Lcn2*; neutrophil gelatinase-associated lipocalin; NGAL) as markers for sustained tubular injury showed a significant upregulation of both markers ($p<0.05$) at all temperature conditions tested (Figure 3.6 A). In addition, expression of the inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-6 were significantly higher ($p<0.05$) at all temperature conditions tested (Figure 3.6 B). However, no temperature-dependence was observed for the gene expression of the tubular injury markers and inflammatory cytokines.

3.3.1.3 Effect of ischemia time on fibrotic outcome

As depicted in Figure 3.2 B, UIRI caused a significant reduction in renal mass at all ischemia time-conditions tested as compared to sham. In addition, longer

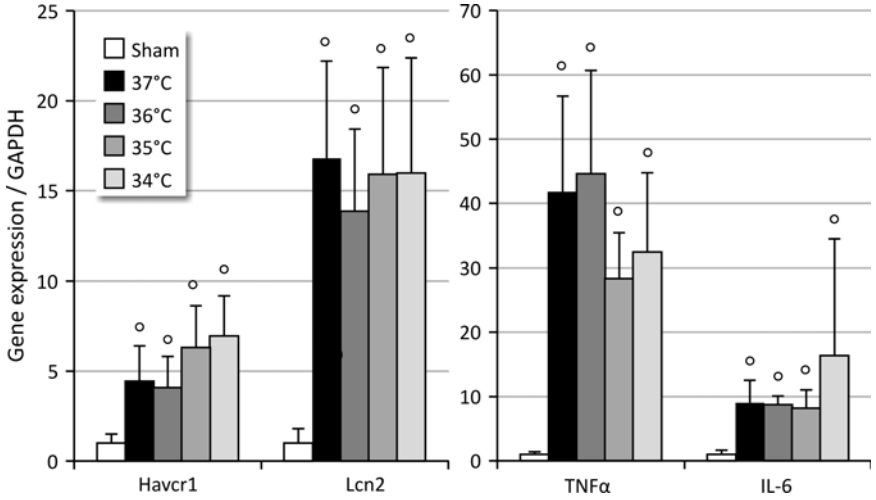

Figure 3.6: Relative quantification of long-term IRI-induced expression of tubular injury and inflammatory markers.
UIRI was performed for 30 minutes at 37°C (n=5), 36°C (n=4), 35°C (n=10) or 34°C (n=5) and animals were euthanized 12 weeks after UIRI. **A**: Twelve weeks after UIRI, a significant increase in gene expression of tubular injury markers *Havcr1* (KIM-1) and *Lcn2* (NGAL) was observed in renal cortex tissue in all core body temperature conditions tested. **B**: Twelve weeks after UIRI, a significant increase in gene expression of inflammatory cytokines *TNFα* and *IL-6* was observed in renal cortex tissue in all core body temperature conditions tested. °: p<0.05 vs. Sham.

ischemia times induce a more severe reduction in renal mass: 30 minutes UIRI caused a ±75% reduction in renal mass (p<0.05), whereas the mildest ischemia time condition tested, i.e. 18 minutes UIRI, caused a ±20% reduction in renal mass (p<0.05). The severity of histologic renal damage is dependent on ischemia time: 30 minutes of UIRI caused prominent renal damage and severe loss of structure (Figure 3.3 B), as was also seen in the previous experiment on the effect of body temperature during ischemia. On the other hand, 6 and 12 weeks after 18 minutes of UIRI, renal tissue had a more or less normal appearance with some intratubular casts and necrotic tubuli (Figure 3.3 B). Quantification of fibrosis by collagen I immunostaining shows an ischemia time-dependent effect, with significantly less collagen I staining after 18 minutes UIRI as compared to 30 minutes, both at week 6 and 12 (p<0.05; Figure 3.4 D). In addition, a tendency towards progression of renal fibrosis from week 6 to week 12 is seen with 30 and 21 minutes of UIRI. However, the mildest ischemia time-condition, i.e. 18 minutes UIRI, shows tendency towards reduction in collagen I deposition from week 6 to week 12 (Figure 3.4 D). As shown in Figure 3.7, 12 weeks after 30, 21 and 18 minutes UIRI, a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed as compared to sham (p<0.05). At week 12, the in-

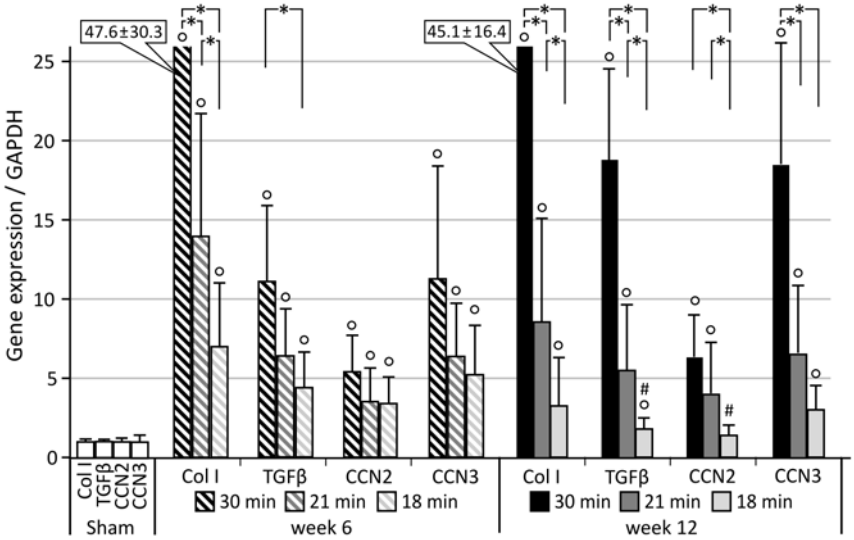

Figure 3.7: Relative quantification of long-term IRI-induced expression of fibrosis-related genes. Duration of ischemia determines degree of long-term fibrotic outcome.
UIRI was performed for 30, 21 or 18 minutes at 36°C and animals were euthanized 6 weeks (resp. n=5, n=12, n=6) and 12 weeks (resp. n=4, n=5, n=10) after UIRI. Six weeks after 30, 21 and 18 minutes of UIRI, a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed. 12 weeks after 30 and 21 minutes of UIRI, although not statistically significant, a further increase in gene expression of these genes is observed. However, 12 weeks after 18 minutes of UIRI, a trend to decreased gene expression of *collagen I* and *CCN3* and a significant decrease in of *TGFβ* and *CCN2* is observed.
*: p<0.05, °: p<0.05 vs. Sham, #: p<0.05 vs. week 6.

crease in gene expression of these fibrosis-related genes is less pronounced with shorter ischemia times, i.e. 21 and 18 minutes UIRI, as compared to 30 minutes UIRI (p<0.05). Also, 12 weeks after 18 minutes UIRI, expression of the pro-fibrotic genes *collagen I*, *TGFβ* and *CCN2* is even lower as compared to 21 minutes UIRI (p<0.05). There is a tendency towards higher gene expression of the fibrosis-related genes 12 weeks after 30 and 21 minutes UIRI as compared to 6 weeks (p>0.05). However, 12 weeks after 18 minutes of UIRI, gene expression of *TGFβ* and *CCN2* is significantly lower as compared to week 6 (p<0.05).

3.3.1.4 Effect of ischemia time on long-term expression of inflammatory and tubular injury markers

As shown in Figure 3.8 A, 6 weeks after 30, 21 and 18 minutes UIRI, a significant increase in gene expression of the tubular injury marker *Havcr1* (KIM-1) was observed as compared to sham (p<0.05). At week 12, expression of *Havcr1* is reduced after 30 minutes of UIRI as compared to week 6. Also, at week 12,

the mildest ischemia-time condition (18 minutes of UIRI) induced a significant lower upregulation of *Havcr1* expression as compared to the most severe condition (30 minutes of UIRI) ($p<0.05$). Upregulation of the gene expression of the tubular injury marker *Lcn2* (NGAL) shows an ischemia time-dependent effect, with significantly reduced upregulation after 18 and 21 minutes of UIRI as compared to 30 minutes at week 6 ($p<0.05$; Figure 3.8 A). Also, as for *Havcr1*, the expression of *Lcn2* is reduced 12 weeks after 30 minutes of UIRI as compared to week 6 ($p<0.05$). In addition, at week 12, expression of *Lcn2* is significantly lower after 21 minutes of UIRI as compared to 30 minutes. Likewise for 18 minutes of UIRI as compared to 21 and 30 minutes. As shown in Figure 3.8 B, 6 weeks after 30, 21 and 18 minutes of UIRI, a significant increase in gene expression of the inflammatory cytokines *TNFα* and *IL-6* was observed as compared to sham ($p<0.05$). Shorter ischemia times, i.e. 21 and 18 minutes, induced significantly lower upregulation of *TNFα* and *IL-6* ($p<0.05$) (Figure 3.8 B). At week 12, upregulation of *TNFα* shows an ischemia-time dependent effect, with significantly reduced upregulation after 21 minutes of UIRI as compared to 30 minutes, and likewise for 18 minutes of UIRI as compared to 21 and 30 minutes. Gene expression of *IL-6* is significant higher at week 12 as compared to week 6 after 30 minutes of UIRI. In addition, 12 weeks after 18 minutes of UIRI, i.e. the mildest condition, gene expression of *IL-6* is significantly lower as compared to both 30 and 21 minutes of UIRI (Figure 3.8 B).

3.3.2 Study no. 2: Unilateral renal ischemia-reperfusion with contralateral nephrectomy as a model of renal repair in mice

3.3.2.1 Effect on fibrotic outcome

As mentioned previously, renal ischemia-reperfusion (21 minutes at 36°C) induces a reduction in renal mass of approximately 52%, 6 weeks after UIRI. Nephrectomy of the healthy contralateral kidney rescues this atrophy of the ischemic kidney, and renal mass of the ischemic kidney is even increased with 16% as compared to sham (Figure 3.9).

As shown in Figure 3.10, 6 weeks after UIRI without contralateral nephrectomy a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed in renal cortex tissue. However, 6 weeks after UIRI with contralateral nephrectomy, expression of all fibrosis-related genes returned to values seen in sham-operated animals, with exception of *collagen I (*Figure 3.10). Even though *collagen I* gene expression in the ischemic kidney was still significantly higher in UIRI with nephrectomy animals as compared to sham animals ($p<0.05$), its expression was significantly lower as compared to UIRI without nephrectomy animals (3.23±1.74 vs. 17.88±5.83-fold) ($p<0.05$).

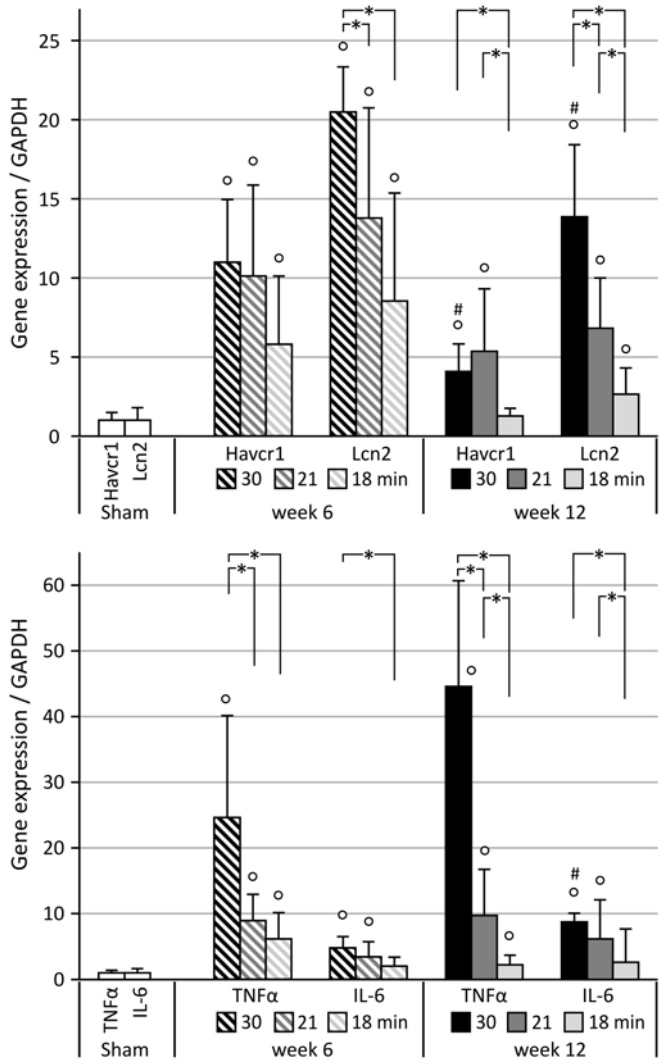

Figure 3.8: Relative quantification of long-term IRI-induced expression of tubular injury and inflammatory markers.
UIRI was performed for 30, 21 or 18 minutes at 36°C and animals were euthanized 6 weeks (resp. n=5, n=12, n=6) and 12 weeks (resp. n=4, n=5, n=10) after UIRI. **A:** Six weeks after 30, 21 and 18 minutes of UIRI, a significant increase in gene expression of tubular injury markers *Havcr1* (KIM-1) and *Lcn2* (NGAL) was observed. At 12 weeks after UIRI, upregulation of these markers is ischemia-time dependent, with higher upregulation with longer ischemia times. **B:** Six weeks after 30, 21 and 18 minutes of UIRI, a significant increase in gene expression of inflammatory cytokines *TNFα* and *IL-6* was observed. In addition, short ischemia times, i.e. 18 minutes of UIRI, induced significantly lower gene expression of these markers. At 12 weeks after UIRI, upregulation of these inflammatory cytokines shows an ischemia-time dependent effect, with shorter ischemia times inducing less upregulation of gene expression of these inflammatory markers.
*: $p<0.05$, °: $p<0.05$ vs. Sham, #: $p<0.05$ vs. week 6.

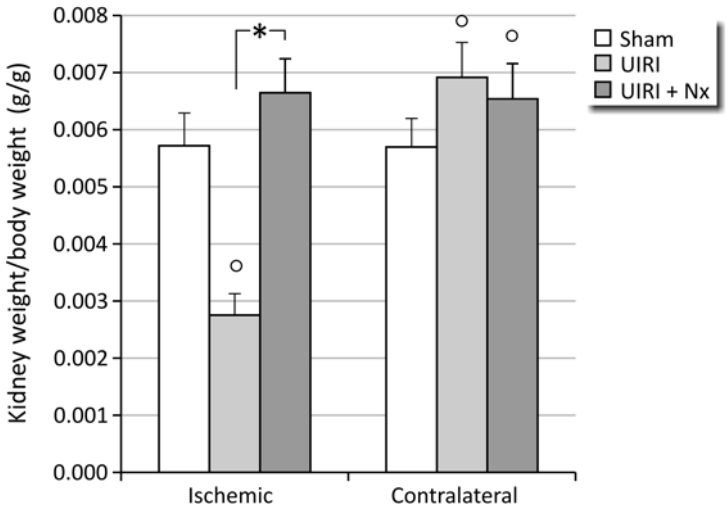

Figure 3.9: Weight of ischemic kidneys, with and without controlateral nephrectomy, corrected for body weight (B).
UIRI was performed for 21 minutes at 36°C, 3 days later contralateral nephrectomy (n=6) or sham operation (n=6) was performed, and animals were euthanized 6 weeks after UIRI. UIRI results in a significant reduction of renal mass ($p<0.05$) when the contralateral kidney is present. After UIRI with nephrectomy, renal atrophy is prevented, moreover, renal hypertrophy occurs. *: $p<0.05$, °: $p<0.05$ vs. Sham.

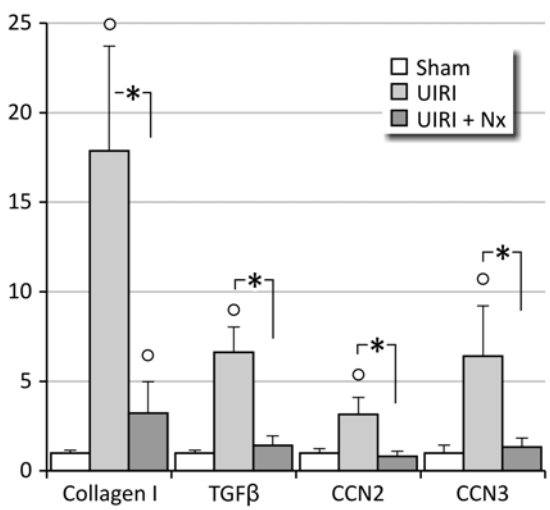

Figure 3.10: Relative quantification of long-term IRI-induced expression of fibrosis-related genes.
UIRI was performed for 21 minutes at 36°C, 3 days later contralateral nephrectomy (n=6) or sham operation (n=6) was performed, and animals were euthanized 6 weeks after UIRI. Six weeks after UIRI without nephrectomy, a significant increase in gene expression of fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* and *CCN3* was observed in renal cortex tissue. Contralateral nephrectomy after UIRI rescues the kidney from degeneration as evidenced by normalization of expression of fibrosis-related genes. *: $p<0.05$, °: $p<0.05$ vs. Sham.

3.3.2.2 Effect on long-term expression of tubular injury and inflammatory markers

Six weeks after UIRI without contralateral nephrectomy, tubular injury markers *Lcn2 (*NGAL) and *Havcr1* (KIM-1) are still distinctly elevated (15.51±4.24 and 11.48±5.09-fold resp.) (Figure 3.11). However, 6 weeks after UIRI with contralateral nephrectomy, expression of both tubular injury markers returned to values seen in sham-operated animals. The same phenomenon was observed for the inflammatory markers. Marked upregulation of *TNFα* and *IL-6* gene expression was present 6 weeks after UIRI without nephrectomy (9.17±3.01 and 2.59±0.80-fold resp.) (Figure 3.11). Six weeks after UIRI with nephrectomy, expression of TNFα and IL-6 in the ischemic kidney was not different from the expression pattern seen in sham animals.

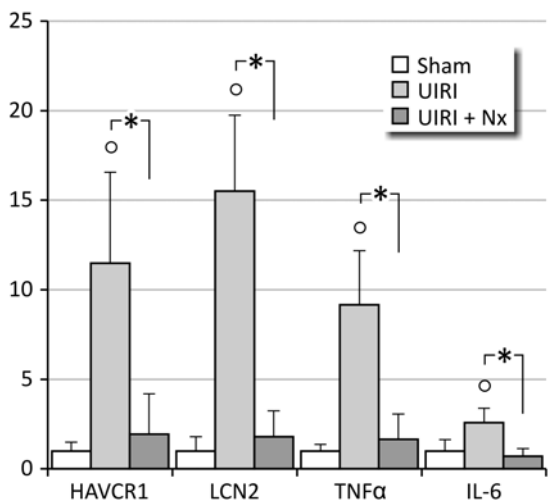

Figure 3.11: Relative quantification of long-term IRI-induced expression of tubular injury and inflammatory markers.
UIRI was performed for 21 minutes at 36°C, 3 days later contralateral nephrectomy (n=6) or sham operation (n=6) was performed, and animals were euthanized 6 weeks after UIRI. Six weeks after UIRI without nephrectomy, a significant increase in gene expression of tubular injury markers *Havcr1* (KIM-1) and *Lcn2* (NGAL) and inflammatory markers *TNFα* and *IL-6* was observed. Contralateral nephrectomy after UIRI induces repair as evidenced by normalization of expression. *: $p<0.05$, °: $p<0.05$ vs. Sham.

3.4 Discussion

Amongst the realm of models to study or intervene with the development of chronic kidney disease (CKD), the IRI model is rarely used[25, 46, 47]. In acute kidney injury (AKI) however, renal UIRI with contralateral nephrectomy is one of the most used animal models for both fundamental and therapeutic intervention studies. Yet, despite the long-term functional and pathological consequences of ischemia, only a few studies applied IRI to study chronic effects ensuing from an acute ischemic insult[48-50]. Yet, together with nephrotoxic injury from drugs (poly pharmacy, radiocontrast drugs, poison, or metals), ischemia (hypoperfusion after surgery, bleeding, dehydration, shock, or sepsis) is a major aetiology in human AKI[51, 52], and recent clinical studies clearly demonstrate a pathological link between AKI and CKD. The hazard ratio for developing end-stage renal disease (ESRD) in patients with AKI without previous CKD is 13.0[53]. Delayed graft function following renal transplantation, dialysis-requiring acute renal failure, old age and incomplete recovery from AKI are associated with an increased risk for renal nephropathy and progression to CKD[54-57]. Experimental work on mechanisms underlying progression from AKI to CKD in ischemic and renal ablation models indicates that a persistent inflammatory response[27, 58], alterations in renal microvasculature[31, 59, 60] and derangements of the endocrine response and abnormalities in circulating mediators[2] may contribute to progressive injury and lack of recovery. Hence, IRI is a clinically relevant model to study the AKI to CKD connection. However, most of the experimental research on CKD and fibrosis is performed in the unilateral ureteral obstruction (UUO) model. Although undoubtedly valuable, this model is a correlate for a rather rare cause of human renal disease[46, 47, 51]. Here, we present evidence for UIRI to be a suitable animal model to study the progression from acute to chronic kidney injury, with minimal mortality and very consistent development of fibrosis in the injured kidney. In this model, we evaluated the impact of the two main determinants of acute ischemic injury, i.e. core body temperature during and duration of ischemia, on long-term fibrotic outcome and concomitant expression of tubular injury and inflammatory markers. In the second study, we demonstrated that UIRI with contralateral nephrectomy is a model of renal repair.

A macroscopic parameter indicative of progressive fibrotic renal lesions is a reduction in renal mass[29, 32]. In accordance with this, in the first study (UIRI without nephrectomy), the data show a significant reduction in renal mass at all conditions tested (Figure 3.2). Histological analysis, by means of Masson's trichrome stain and collagen I immunostaining, reflect the expected clinically relevant histopathology of CKD[29, 61, 62], characterized by the presence of tubular casts and debris, atrophic tubuli, ongoing inflammation, and tubulo-interstitial fibrosis (Figure 3.3 and 3.4). Complementary to the histological analysis, the expression of a panel of fibrosis-related genes was determined by qPCR, i.e. *collagen I*, an extracellular

matrix component; *TGFβ*, an important pro-inflammatory and cell proliferative cytokine; and *CCN2* and *CCN3*, growth factors. In all currently investigated conditions of ischemia, a significant increase in expression was observed for all fibrosis genes under study (Figure 3.5 and 3.7). However, when the contralateral (healthy) kidney is removed after UIRI, atrophy of the ischemic kidney did not occur (Figure 3.9). Moreover, ischemic kidney weights tended to be higher in this group as compared to sham, as was previously reported[32, 49]. Analysis of the expression of the fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* (CTGF) and *CCN3* showed normalization of gene expression to levels similar to shams, except for *collagen I* (Figure 3.10).

Since our model consists in the induction of an acute ischemic injury, we also investigated whether the expression of early tubular injury markers KIM-1 and NGAL was still elevated on the long-term. Indeed, we confirmed that renal expression of these markers remained increased in ischemia-induced progressive renal disease (UIRI without nephrectomy) (Figure 3.6 A and 3.8 A), as was previously reported in UUO and cisplatin-induced fibrosis[63-65]. In addition, we observed an ischemia-time dependent effect, in particular for NGAL and less pronounced for KIM-1, such that long-term expression of the tubular injury markers increased with longer ischemia times (Figure 3.8 A). These findings are in accordance to the findings of van Timmeren et al. (2007) who found an association between tubular KIM-1 expression and interstitial fibrosis in renal biopsies from patients with a variety of renal pathologies[66]. In our study, although upregulation of NGAL and KIM-1 persisted up to week 12, expression at week 6 was higher (Figure 3.8 A). Interestingly, a similar decreased expression from week 6 to week 12 after UIRI without contralateral nephrectomy has been reported previously[67]. The fact that it has been demonstrated in renal biopsies that completely atrophic (as well as normal) tubules do not express KIM-1, might explain the decreased expression at week 12 as compared to week 6 (Figure 3.8 A)[66, 68]. When nephrectomy is performed after UIRI, no upregulation of these tubular injury markers was observed in the ischemic kidney, indicating limited tubular injury at 6 weeks after UIRI (Figure 3.11). These findings are in accordance to findings of Jain et al. (2000), who reported no change in protein/creatinine ratio up to 8 weeks in this model[50].

Since it is known that the model of ischemia-reperfusion features a pronounced inflammatory response, we examined the gene expression of 2 inflammatory cytokines, i.e. TNFα and IL-6, that have already been shown to be upregulated after an acute injury and as well as during the chronic renal injury phase[29, 69, 70]. We confirmed that renal expression of these inflammatory cytokines remained increased in ischemia-induced progressive renal disease (Figure 3.6 B and 3.8 B). As in the case of the tubular injury markers, we observed an ischemia-time dependent effect, in particular for TNFα and less pronounced for IL-6, with higher expression of the inflammatory cytokines with longer ischemia times (Figure 3.8

B). Thus, our results show that an acute unilateral ischemic insult results in long-term, active and progressive fibrotic lesions, rendering UIRI without contralateral nephrectomy a suitable model to study the histopathological progression from acute to chronic kidney injury. Interestingly, UIRI with nephrectomy induced no upregulation of these cytokines nor of the tubular injury markers (Figure 3.11). Since inflammation after UIRI is associated with renal injury[71] and is an important mechanism underlying the progression from acute to chronic kidney injury[72], lack of inflammatory cytokine upregulation further supports renal repair in this model.

Next, we set out to investigate to what extent alterations in body temperature during ischemia and ischemia time influence long-term fibrotic outcome in the UIRI model. Hereto we performed UIRI in a range of conditions commonly used in short-term UIRI experiments, i.e. variations in body temperature from 34°C-37°C and variations in ischemia time from 18-30 minutes. In transplant biology, it is known that both warm ischemia time, i.e. duration between clamping of blood flow and prelevation of the kidney, and cold ischemia time, i.e. duration of extra-corporal ischemia time, are risk factors for delayed graft function and adverse outcomes[35, 73, 74]. The results of the experiments described in this chapter demonstrate that both determinants influence the severity and natural course of the subsequent renal pathology that develops after ischemic AKI. However, taking into account the expression of the tubular injury markers, inflammatory cytokines and fibrosis-related genes, body temperature during ischemia should be particularly thought of as an important factor of variance within the model, and should not be taken lightly in view of standardization of the ischemia-reperfusion model. Ischemia time, on the other hand, is the main factor that determines the severity of the long-term fibrotic outcome. This is a finding that is also true for other variants of the IRI model (bilateral IRI and unilateral IRI with contralateral nephrectomy)[11, 45, 75-77]. Contrary to these other variants of the IRI model, where spontaneous recovery of the ischemic kidneys is seen despite similar ischemia-conditions[25, 78], it should be noted that all ischemia conditions tested in our study, both severe and mild, induced renal fibrosis consistently. Only 18 minutes of ischemia, which generally is a rather mild ischemia condition, did not appear to result in progressive fibrosis. Nevertheless, as higher core body temperatures during ischemia and/or longer ischemia times both cause a more severe reduction in renal mass (Figure 3.2), the model of UIRI can be considered a tuneable model for either acute to chronic kidney injury or reversibility of the acute injury. Indeed, we also showed that depending on the severity of the ischemic insult, i.e. high (37°C) vs. lower body temperature (34°C) and 30 minutes vs. 18 minutes of UIRI, either progression or reversal of the renal pathology can be achieved. The latter is in accordance with findings from others who also showed that short ischemia times (<18 minutes of warm ischemia) induced reversible renal injury without long-term effects[79, 80].

In view of the above, it is not surprising that the increase in expression of fibro-

sis-related genes also depends on the duration of ischemia and core body temperature during ischemia, with higher body temperatures (37°C and 36°C) having a more pronounced effect than lower temperatures (35°C and 34°C) as reflected by the higher increase in gene expression of *collagen I*, *TGFβ*, *CCN2*, *CCN3* at higher body temperature during ischemia and longer ischemia times (Figure 3.5 and 3.7). Besides body temperature (to a certain extent) and duration of ischemia as determinants of renal pathology, a number of factors must also be taken into account as possible sources of variation, such as strain[81], gender[82], age, anaesthesia[83] and pre-operative preparation of the animal. However, in a consistent experimental setup, these factors of variation are expected to be standardized such that fine-tuning of the ischemia conditions only relies on duration of ischemia and body temperature.

In conclusion, in the first study we demonstrated that UIRI without nephrectomy is a very robust model for induction of long-term tubulo-interstitial fibrosis. In addition, we demonstrate that varying the two main determinants of IRI induced AKI, i.e. body temperature during and duration of ischemia, in the unilateral IRI without nephrectomy model allows tuning of these long-term effects. In the second study we demonstrated that UIRI with contralateral nephrectomy is a model of renal repair. Indeed it was hypothesized by others that the presence of the normal kidney may retard the restoration of damaged kidney in the UIRI model[32]. However, despite an apparently complete resolution of the initial ischemic insult, as evidenced by normalization of tubular injury, inflammatory and fibrosis markers, research by others in this UIRI with nephrectomy model, indicates that between weeks 16 and 52, extensive glomerular and interstitial injury evolves[48-50]. In order to explain this phenomenon it has been stated that ischemia-reperfusion injury reduces the number of nephrons in the kidney, thus exacerbating hypertrophy and hyperfunction in surviving nephrons, ultimately resulting in renal injury and deterioration of renal function[49]. Nevertheless, further investigation of the differences between both UIRI models will contribute to a better knowledge of mechanisms underlying either renal repair or development of chronic injury.

3.5 References

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Chapter 4

No therapeutic efficacy of untargeted DNA-demethylation therapy initiated during established, ischemia-reperfusion-induced renal fibrosis

4

Submitted to Nephron

Chapter 4: Contents

Abstract

Background: Current treatment options for chronic kidney disease (CKD) are limited and focussed on slowing progression by tight control of co-morbidities. Fibrosis, the common underlying process in CKD, is a major research focus for development of alternative therapeutic approaches. In CKD, fibroblasts are terminally activated because of alterations in their DNA-methylation pattern, particularly hypermethylation. Therefore, prevention of the copying of the pathological DNA-methylation pattern in proliferating fibroblasts could be a new effective therapeutic strategy for treatment of CKD.

Methods: To evaluate the therapeutic effect of short-term treatment (10 days) with the DNA-methyltransferase (DNMT)-inhibitor decitabine on early and late established renal fibrosis, male C57Bl/6 mice (10-12 weeks of age) underwent severe unilateral ischemia-reperfusion injury (30 minutes at 37°C body temperature). Respectively 3 and 6 weeks after surgery, decitabine treatment (0.25 mg/kg) was initiated for 10 days. Efficacy of therapy on fibrosis was evaluated by *collagen I* and *TGFβ* gene expression and histological quantification of collagen I staining. In addition, the effect of decitabine treatment on tubular injury and inflammation was determined.

Results: We report a significant increase in gene expression of the various *Dnmts* both in the early and chronic fibrosis phase. Upon decitabine treatment, only *Dnmt3b* gene expression displayed a persistent reduction. Yet, even though decitabine treatment induced a significant reduction in genomic DNA-methylation, progression of fibrosis was not attenuated or prevented. Furthermore, neither tubular injury nor inflammation in the more chronic phase of the ischemic injury was influenced by decitabine treatment.

4.1 Introduction

Existing strategies for treatment of chronic kidney disease (CKD) in essence are systemic in nature and focus on slowing progression in order to improve survival and quality of life[1]. Patients with end-stage renal disease (ESRD) have to rely on dialysis treatment or transplantation, both of which still are temporary solutions with a high rate of complications and premature death: the 5-year survival of dialysis patients is 40.6% in Europe[2] and 40.2% in the United States[3] whilst the average 10-year survival after transplantation is 56% in Europe and 43% in the United States[4]. As in CKD, surviving transplanted kidneys cannot escape development of fibrosis, a detrimental accumulation of extracellular matrix (ECM) and the histopathological hallmark of progressive renal decay[5]. Currently, therapeutic approaches directly targeting cell biological mechanisms underlying fibrosis, are intensively investigated[6]. It is believed that effective therapeutic prevention, attenuation or reversal of the fibrotic process will significantly slow down the progressive decline of both chronically injured and transplanted kidney function[7].

Fibroblasts, independent of their origin[8], are considered to be the main effector cells that produce ECM, in concert with cytokines and growth factors which sustain and further promote the fibrotic process and attract inflammatory cells[9]. In normal physiological wound repair, fibroblast activation is transient, and fibroblasts return to their resting state after resolution of the primary insult. In chronic fibrosis, fibroblasts are unable to return to their resting state and, as a consequence, continue to proliferate and produce excess ECM[10]. It was hypothesized by Bechtel et al. (2010) that fibroblast activation in fibrotic kidney disease is the result of epigenetic alterations, more precisely DNA-methylation, which renders them terminally activated[11]. It is becoming clear that DNA-methylation, in addition to cancer pathologies, also plays an important role in renal pathologies, as independent investigators found that aberrant DNA-methylation is associated with inflammation[12, 13] and the development of renal fibrosis[11, 14-16] and occurs during cold ischemia in transplantation[17, 18]. Importantly, aberrant DNA-methylation is thought to influence allograft survival since advanced donor age, differentiation and polarization of immune cells as well as ischemia are associated with alterations in the DNA-methylation pattern[5, 19, 20]. Targeting DNA-methylation has thus become a new hopeful avenue in development of CKD therapeutics.

Since DNA-methylation patterns are propagated through cell division by the "maintenance" DNA-methyltransferase (DNMT1)[21, 22], and findings of Bechtel et al. (2010) demonstrated that DNMT1 suppression (*Dnmt1* expression is decreased by 70% in DNMT1$^{-/+}$ heterozygous mice), ameliorates folic acid induced fibrosis[11], prevention of the copying of the pathological DNA-methylation pattern in proliferating fibroblasts, by inhibition of DNMT1, could be a new valuable therapeutic

strategy for treatment of CKD. Therefore, we hypothesized that by interfering with the DNMTs, copying of the DNA-methylation pattern during proliferation of terminally activated fibroblasts could be prevented, thereby breaking the vicious circle of terminal fibroblast activation, and thus resulting in the attenuation of progressive fibrosis. Hereto, we evaluated the therapeutic effect of short-term treatment (10 days) with the DNMT-inhibitor decitabine, on established renal fibrosis in the early and late chronic phase following severe unilateral ischemia-reperfusion injury in the mouse, a renal transplant correlate model.

4.2 Methods

4.2.1 Animals and experimental design

All animal procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics Committee (approval number 2011-51). Animals had free access to standard chow and tap water.

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously[23] (see chapter 2). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent 30 minutes of warm (37°C body temperature) UIRI, with continuously monitoring of body temperature. The right kidney was left undisturbed. Sham-operated animals received the same surgical procedure except placement of the clamp.

Dose-finding experiment: Male C57Bl/6 mice (10-12 weeks of age) underwent UIRI as described above. Afterwards, the mice (n=4/treatment group) were injected with 0.10, 0.25 or 0.50 mg/kg decitabine (A3656-10MG, Sigma-Aldrich, Missouri, USA) for 2 weeks after which genomic DNA-methylation in kidney tissue was determined by Ultra-Performance Liquid Chromatography (UPLC; Waters® Acquity UPLC™)[24].

Treatment regimen: After UIRI, animals were randomly allocated to 6 treatment groups (Figure 4.1). 1) Sham (n=6); 2) UIRI + untreated (n=6); 3) UIRI + decitabine starting 3 weeks after UIRI (n=16), 4) UIRI + vehicle starting 3 weeks after UIRI (n=12); 5) UIRI + decitabine starting 6 weeks after UIRI (n=16) and 6) UIRI + vehicle starting 6 weeks after UIRI (n=12). Decitabine was diluted in phosphate buffered saline (vehicle) in a concentration of 0.25 mg/kg and administered for 10 days through subcutaneous injection, as this route of administration has been proven superior[25, 26]. Optimal decitabine dosage (0.25mg/kg/day) was determined in a dose-finding experiment where different dosages of decitabine (0.10, 0.25 and 0.50 mg/kg/day) were evaluated on their capacity to induce a pronounced reduc-

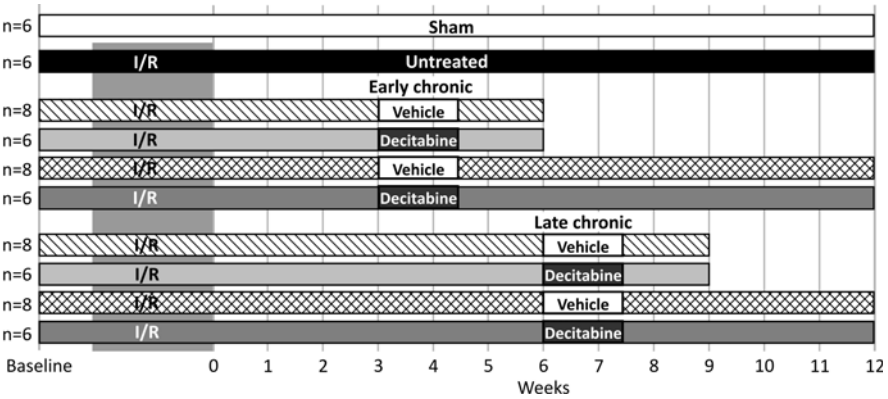


Figure 4.1: Study set-up. After UIRI, animals were randomly allocated to 6 treatment groups. 1) Sham; 2) UIRI + untreated; 3) UIRI + decitabine starting 3 weeks after UIRI, 4) UIRI + vehicle starting 3 weeks after UIRI; 5) UIRI + decitabine starting 6 weeks after UIRI and 6) UIRI + vehicle starting 6 weeks after UIRI. Half of the animals of the groups receiving decitabine (3 and 5) and vehicle treatment groups (4 and 6) were euthanized 12 days after treatment whilst the remaining animals were euthanized 12 weeks after UIRI.

tion of genomic DNA-methylation in both healthy and ischemic kidneys.

Euthanasia: Half of the animals of the groups receiving decitabine (3 and 5) and vehicle treatment groups (4 and 6) were euthanized 12 days after treatment whilst the remaining animals were euthanized 12 weeks after UIRI (Figure 4.1). Animals were euthanized through exsanguination via the abdominal vena cava under ketamine-xylazine anesthesia. Kidneys were excised, renal pole fractions were snap frozen in liquid nitrogen and transversal slices of renal tissue were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and 10% NBF for 4 hours and 24 hours, resp., rinsed with 70% isopropanol and embedded in paraffin for histology.

4.2.2 Analysis of genomic DNA-methylation in ischemic kidney tissue

The efficacy of a DNA-methyltransferase inhibitor is generally determined by its ability to induce demethylation and reactivation of one or a few marker genes[27]. In the current study, the reactivation of marker genes was not evaluated. Instead, the amount of genomic DNA-methylation in the ischemic kidney was determined by Ultra-Performance Liquid Chromatography (UPLC), as this is one of the most significant and experimentally straightforward parameters for determining the efficacy of a DNA-methyltransferase inhibitor[27]. Here, DNA is extracted from a pole section of the ischemic kidney (Allprep Mini Kit; Qiagen, Belgium) and global DNA-methylation is determined through UPLC (see chapter 2) as reported previously[24]. Briefly, isolated genomic DNA samples were enzymati-

cally hydrolysed to individual deoxyribonucleosides and dissolved in water. Using reference standards for 5'-methyl-2'-deoxycytidine (5-mdC; N-1044, Jena Bioscience, Germany) and 2'-deoxycytidine (dC; D3897-1G, Sigma-Aldrich), a series of calibration solutions was prepared in a range of 0.1-10 ng/mL and 1–100 ng/mL respectively. The same calibration standards were used in all of the experiments. Global DNA-methylation was obtained by quantifying 5-mdC and dC using UPLC, in combination with tandem mass spectrometry (MS-MS; Waters® Micromass Quattro Premier™ Mass Spectrometer) with injections performed on a Waters® UPLC column (BEH C18, 50 mm x 2.1 mm, 1.7 μm) at 40°C.

The global DNA methylation is expressed as a percentage of 5-mdC versus the sum of 5-mdC and dC [5-mdC/(5-mdC + dC)] %. Samples were measured in duplicate. The average methylation value of both measurements was calculated and used for further statistical analysis.

4.2.3 Real-time PCR

Total mRNA is extracted from a pole section of the ischemic kidney (PureLink RNA Mini Kit; Life Technologies, Merelbeke, Belgium) and converted to cDNA (High Capacity cDNA archive kit; Life Technologies). To quantify gene expression, qPCR, based on the Taqman fluorescence method (ABI Prism 7000 sequence detection system; Life Technologies), was used. Taqman probes and primers for *GAPDH* (Mm99999915_g1), *collagen I α1* (Mm00801666_g1), *TGFβ1* (Mm01178820_m1), *Dnmt1* (Mm00599763_m1), *Dnmt3a* (Mm00432881_m1) and *Dnmt3b* (Mm01240113_m1), *Havcr1* (Mm00506686_m1), *Lcn2* (Mm01324470_m1), *TNFα* (Mm00443258_m1) and *IL-6* (Mm00446190_m1) were purchased from Life Technologies. Each gene was analyzed in triplicate and the expression was normalized to the reference gene *GAPDH*. Calculations were made conform the comparative Cq-method.

4.2.4 Histology

For collagen I immunostaining, paraffin embedded 4 μm thick sections of ischemic kidney tissue were blocked with normal goat serum and incubated overnight with the primary polyclonal rabbit-anti-mouse collagen I antibody (Biodesign International, Maine). After washing, sections were incubated with a biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, California) and subsequently incubated with avidin and biotinylated horseradish peroxidase (AB-complex, Vector Laboratories). A dark brown color was developed with diaminobenzidine in the presence of 0.03% H_2O_2. Sections were counterstained with methyl green to visualize nuclei. Collagen I immunostaining was quantified using the Axiovision image analysis software (Carl Zeiss, Jena, Germany; see chapter 2). Data are pre-

sented as area % positive stain, calculated as the ratio of the absolute area of positive staining versus the total surface of the tissue section.

4.2.5 Statistics

For statistical analysis, IBM SPSS Statistics 22 software was used. Data are presented as mean ± standard deviation (SD), or as individual values. Comparisons between groups were made using a Kruskal-Wallis test, followed by a Mann-Whitney U test when significant. Values of $p<0.05$ are considered significant.

4.3 Results

4.3.1 Dose-finding experiment for decitabine

Of the 3 doses dectitabine (0.10, 0.25 and 0.50 mg/kg/day) that were tested in mice with healthy and ischemic kidneys, the dose of 0.5 mg/kg caused severe weight loss and mortality (Figure 4.2). Hence, these animals had to be euthanized prematurely, which led to the conclusion that this dose was unsuitable for further use. The dose of 0.25 mg/kg induced on average an 11% reduction in genomic DNA-methylation (Figure 4.3), with some weight loss (Figure 4.2), without mortality. The dose of 0.10 mg/kg induced on average a 5% reduction in genomic DNA-methylation without weight loss or mortality. As the dose of 0.25 mg/kg decitabine induced the most reduction in DNA-methylation without mortality, and showed a promising decrease on *collagen I* mRNA expression (data not shown) in the ischemic injured kidneys, this dose was chosen for the actual experiment. Given the noted weight loss after 14 days of treatment, the actual treatment period for the main study was set at 10 days for ethical reasons.

4.3.2 Confirmation that unilateral ischemia-reperfusion induces atrophy and fibrosis

After vehicle treatment in the early chronic fibrosis phase (week 6 after UIRI), a severe reduction in renal mass is observed as compared to sham (0.050±0.008 g vs. 0.164±0.014 g) ($p<0.05$) (Figure 4.4 A). The severity of the renal atrophy remains similar after vehicle treatment in the late chronic fibrosis phase (week 9 after UIRI), and after the additional follow-up period until the endpoint of the experiment (week 12 after UIRI) (Figure 4.4 B). The weights of the contralateral uninjured kidneys show a tendency to increase in untreated and vehicle-treated animals as compared to sham, which is in accordance with the physiological fact that the contralateral kidney becomes hypertrophic to compensate for the loss of function of the ischemic kidney (Figure 4.4).

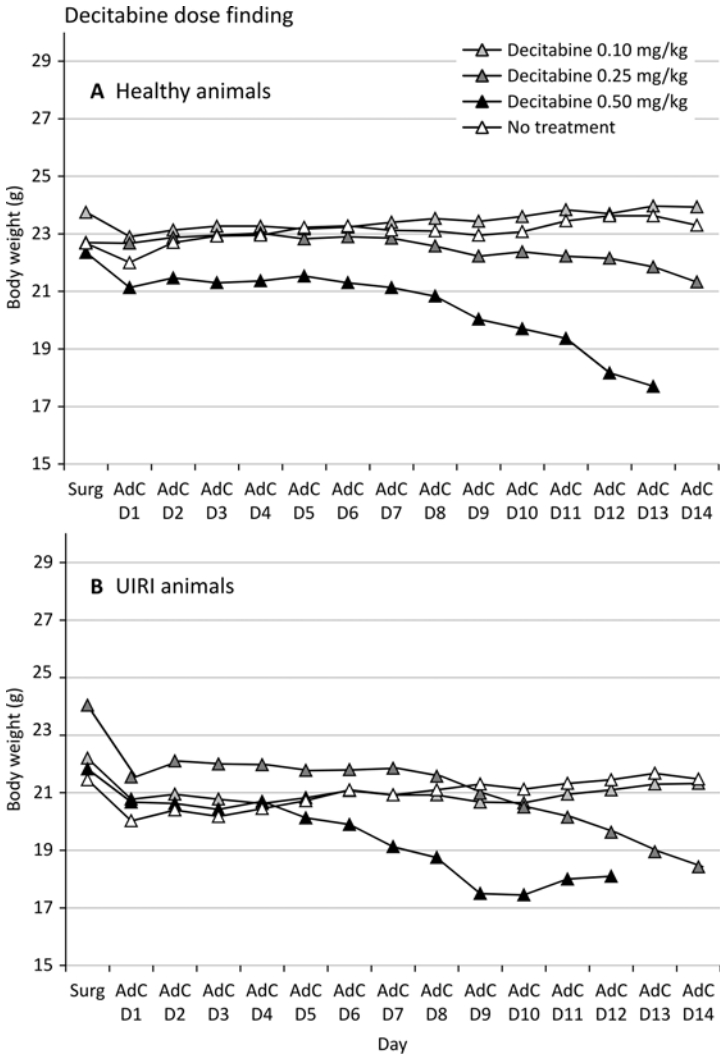


Figure 4.2: Follow-up of body weight of mice, during treatment with different dosages of decitabine. Weight loss was observed as a side effect of decitabine treatment. The highest dose, 0.5 mg/kg/day induced severe weight loss, and animals had to be euthanized earlier than anticipated. AdC: decitabine.

After vehicle treatment in the early chronic fibrosis phase (week 6), significant upregulation of the fibrosis-related genes *collagen I* and *TGFβ* (33.17±10.54 and 9.81±4.25-fold resp.) was observed in the ischemic kidneys (Figure 4.5 B and C). Additionally, renal fibrosis was quantified on collagen I immunostaining (Figure 4.5 A). Significantly more positivity was present in the ischemic kidneys of vehicle-treated animals (25.70±9.31%) as compared to sham (9.84±5.15 %) ($p<0.05$) at this time point (Figure 4.5 D).

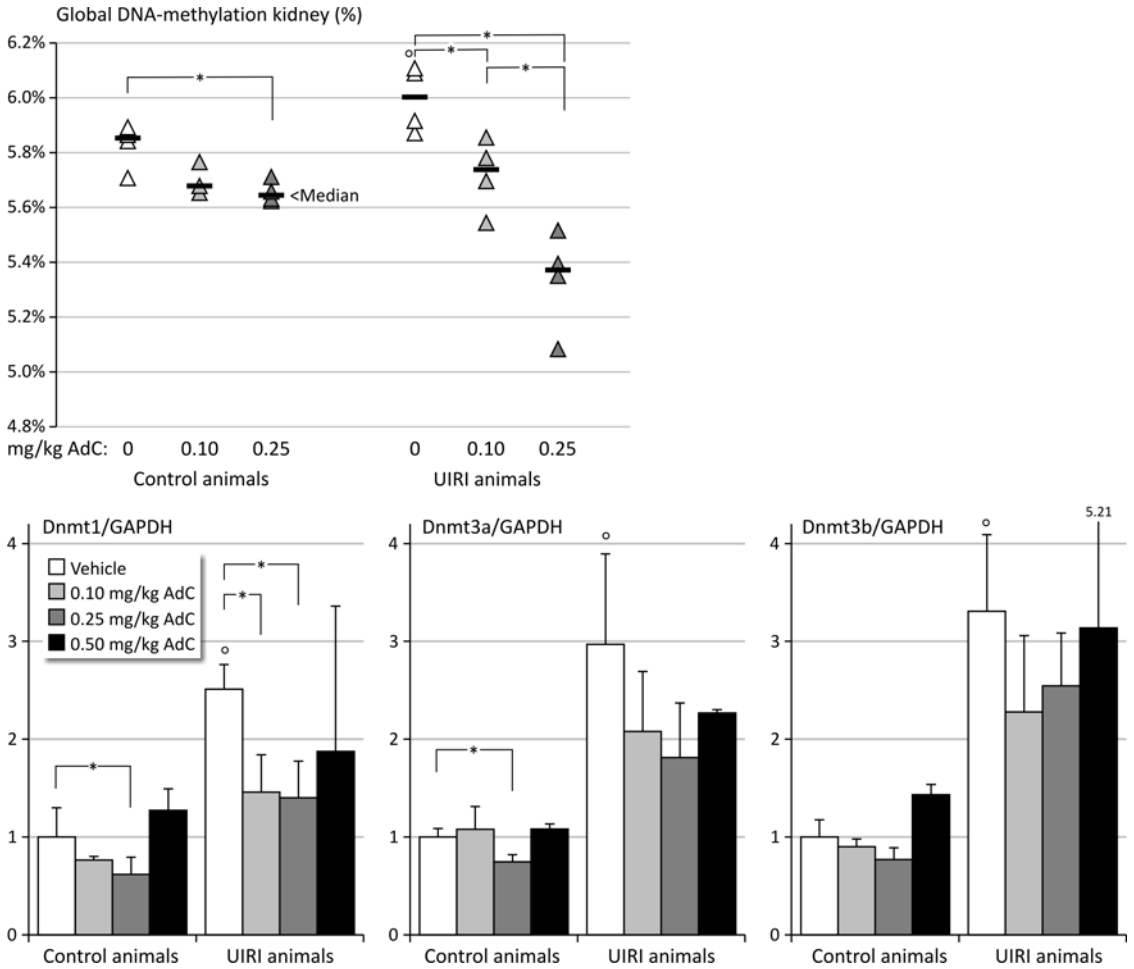


Figure 4.3: Efficacy of inhibition of DNA-methylation in the dose-finding experiment.
A: Percentage of genomic DNA-methylation in the ischemic kidney after a treatment period with different dosages of decitabine. A significant increase in genomic DNA-methylation was observed after UIRI. Decitabine induced a dose-dependent reduction in genomic DNA-methylation in UIRI-animals. **B**: Gene expression analysis of *Dnmt1*, *Dnmt3a*, *Dnmt3b.* UIRI resulted in higher gene expression of all genes under investigation. As expected from literature, treatment with decitabine induced a significant reduction in *Dnmt1* gene expression. °: p<0.05 vs. Sham, *: p<0.05. AdC: decitabine.

These observations were confirmed by quantitative analysis of Masson stained sections (data not shown). The expression of the fibrosis-related genes *collagen I* and *TGFβ* remained stably upregulated in vehicle-treated animals after treatment in the late chronic fibrosis phase (week 9) and after the additional follow-up period (week 12) as compared to shams (Figure 4.6 A and B). Similarly, the amount of collagen I positivity was still significantly higher in the ischemic kidneys of vehicle-treated animals as compared to both untreated and sham animals at week 9 and 12 after UIRI (Figure 4.6 C).

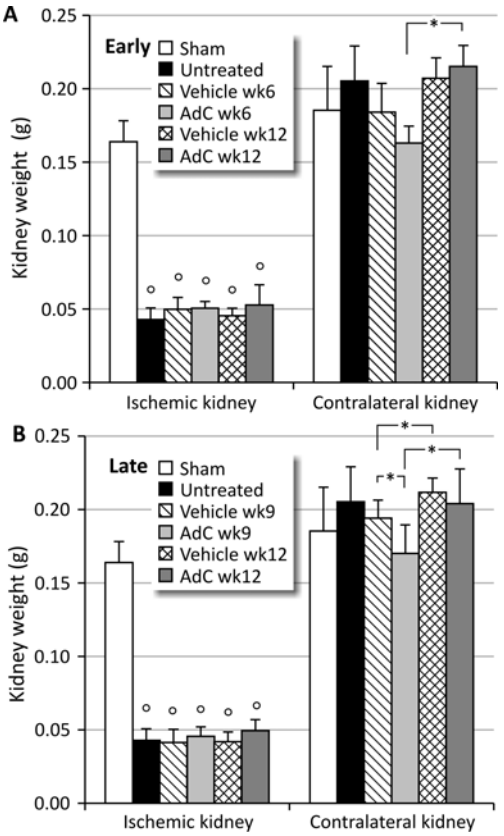


Figure 4.4: Mass of the kidneys at euthanasia. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of early treatment (wk6 after UIRI) and 12 weeks after UIRI (endpoint of experiment), 12 days after end of late treatment (wk9 after UIRI) and 12 weeks after UIRI (endpoint of experiment).
A: Mass of the ischemic and contralateral kidneys, not corrected for body weight, after treatment in the early chronic fibrosis phase. **B**: Mass of the ischemic and contralateral kidneys, not corrected for body weight, after treatment in the late chronic fibrosis phase.
°: $p<0.05$ vs. Sham, *: $p<0.05$. AdC: decitabine.

4.3.3 Early nor late treatment with decitabine could attenuate renal fibrosis

In this study, 16% mortality was observed upon decitabine treatment, whilst no mortality was seen in vehicle-treated animals. A short-term treatment with decitabine in the early or late chronic fibrosis phase had no effect on the loss of renal mass (Figure 4.4). However, the contralateral, uninjured kidney of decitabine-treated animals in the early chronic fibrosis phase shows a tendency towards decreased renal mass as compared to vehicle-treated animals (Figure 4.4 A), which

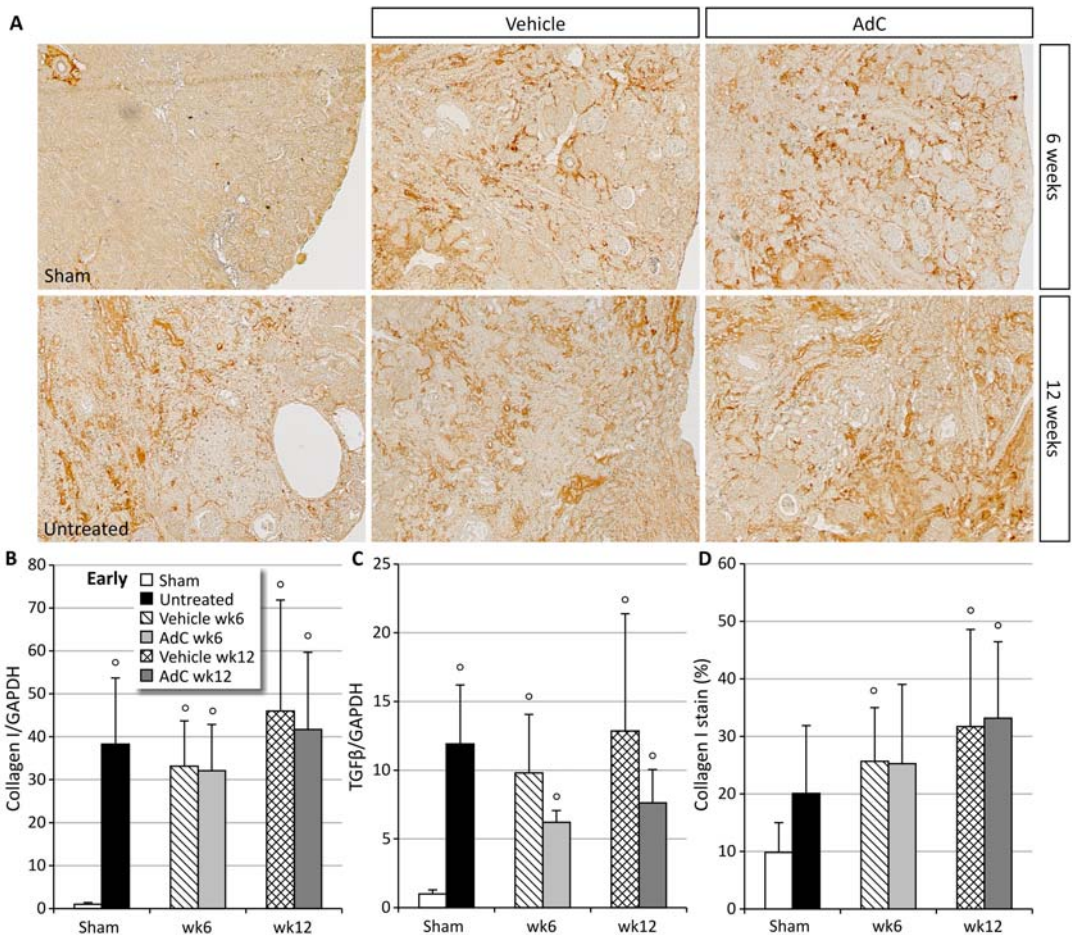


Figure 4.5: Evaluation of long-term fibrosis development in the ischemic kidney after treatment in the early chronic fibrosis phase. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of treatment (wk6 after UIRI) and 12 weeks after UIRI (endpoint of experiment). **A**: Representative images of collagen I immunostained ischemic kidney tissue, 12 days after the end of treatment (6 and 9 weeks after UIRI resp.) (magnification: 100x). **B**: Quantification of *collagen I* gene expression (qPCR). **C**: Quantification of *TGFβ* gene-expression (qPCR). **D**: Histological quantification of collagen I positivity of ischemic kidneys (x50 magnification). °: p<0.05 vs. Sham, *: p<0.05. AdC: decitabine.

became statistically significant when treatment was initiated in the late chronic fibrosis phase (0.170±0.019 g vs. 0.194±0.012 g) (Figure 4.4 B). However, after additional follow-up (week 12 after UIRI), this effect was no longer seen (Figure 4.4).

A short-term treatment with decitabine in the early chronic fibrosis phase did not significantly influence the expression of the fibrosis-related genes *collagen I* and *TGFβ* as compared to vehicle treatment (Figure 4.5 B and C), however, a tendency towards decreased expression of *TGFβ* was observed (p=0.29). Quantification of renal fibrosis on collagen I immunostaining showed no effect of

decitabine treatment on the amount of staining in the ischemic kidneys (Figure 4.5 D). These observations were confirmed by quantification of Masson's stain (data not shown). When decitabine treatment was initiated in the late chronic phase, no significant changes in *collagen I* and *TGFβ* gene expression could be observed either (Figure 4.6 A and B). Also, quantification of collagen I staining showed no effect of decitabine treatment on the degree of positivity in the ischemic kidney (Figure 4.6 C).

4.3.4 Ischemia-reperfusion induces severe long-term tubular injury and inflammation

Expression of tubular injury markers *Havcr1* (KIM-1) and *Lcn2* (NGAL) was highly upregulated (19.27±11.33 and 37.42±13.91-fold resp.) ($p<0.05$) after vehicle treatment in the early chronic fibrosis phase (6 weeks after UIRI) (Figure 4.7 A and B). Expression of these markers was similar with vehicle treatment in the late chronic fibrosis phase (week 9 after UIRI) (Figure 4.8 A and B). A tendency towards decreased expression of both markers was observed after the follow-up period with vehicle treatment both in the early and late chronic fibrosis phase.

Expression of the inflammatory cytokines TNFα and IL-6 was significantly upregulated after vehicle treatment in the early chronic fibrosis phase (week 6) as compared to sham (10.48±6.15 and 6.42±3.93-fold resp.) ($p<0.05$) (Figure 4.7 C and D). Expression of these cytokines was similar with vehicle treatment in the late chronic fibrosis phase (week 9) and after the additional follow-up (week 12) (Figure 4.8 C and D).

4.3.5 Early nor late treatment with decitabine could attenuate tubular injury or inflammation

A short-term treatment with decitabine in the early chronic fibrosis phase (week 6) did not influence the gene expression of the tubular injury markers *Havcr1* (KIM-1) or *Lcn2* (NGAL) as compared to vehicle treatment (Figure 4.7 A and B). Decitabine-treatment in the late chronic fibrosis phase (week 9) did not influence the expression of *Havcr1*, however, induced significant higher expression of *Lcn2* as compared to vehicle-treatment (63.86±34.34 vs. 33.25±10.95-fold) ($p<0.05$) (Figure 4.8 A and B). After the additional follow-up period, this difference in expression was no longer seen.

Treatment with decitabine did not have an effect on the expression of the inflammatory cytokines *TNFα* and *IL-6* both in the early chronic (week 6) or late chronic fibrosis phase (week 9) or after the additional follow-up period (week 12) (Figures 4.7 C-D and 4.8 C-D).

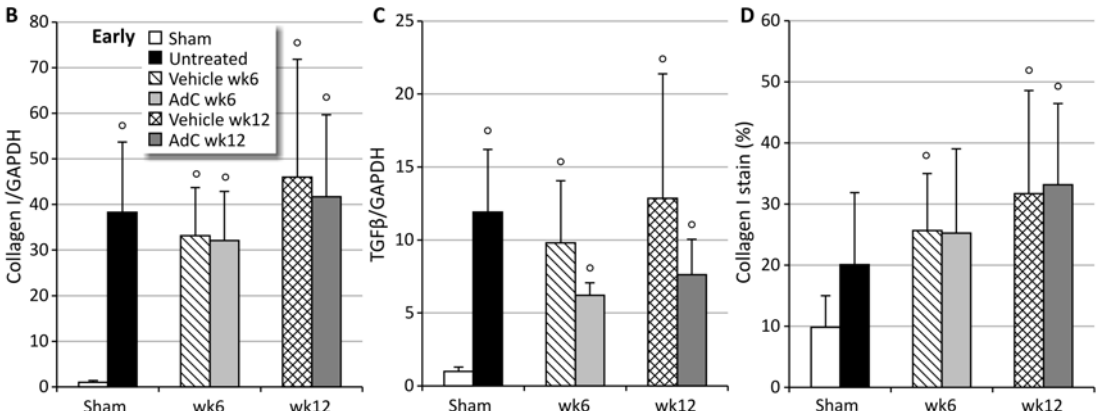
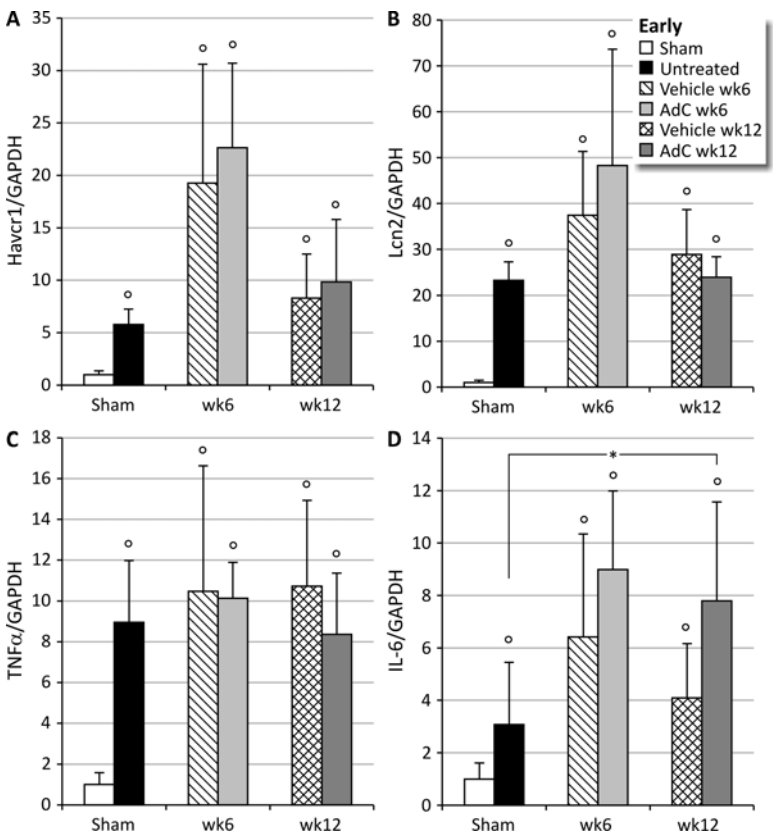


Figure 4.6: Evaluation of long-term fibrosis development in the ischemic kidney after treatment in the late chronic fibrosis phase. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of treatment (wk9 after UIRI) and 12 weeks after UIRI (endpoint of experiment). **A**: Quantification of *collagen I* gene expression (qPCR). **B**: Quantification of *TGFβ* gene expression (qPCR). C: Histological quantification of collagen I positivity of ischemic kidneys (x50 magnification).
°: p<0.05 vs. Sham, *: p<0.05. AdC: decitabine.



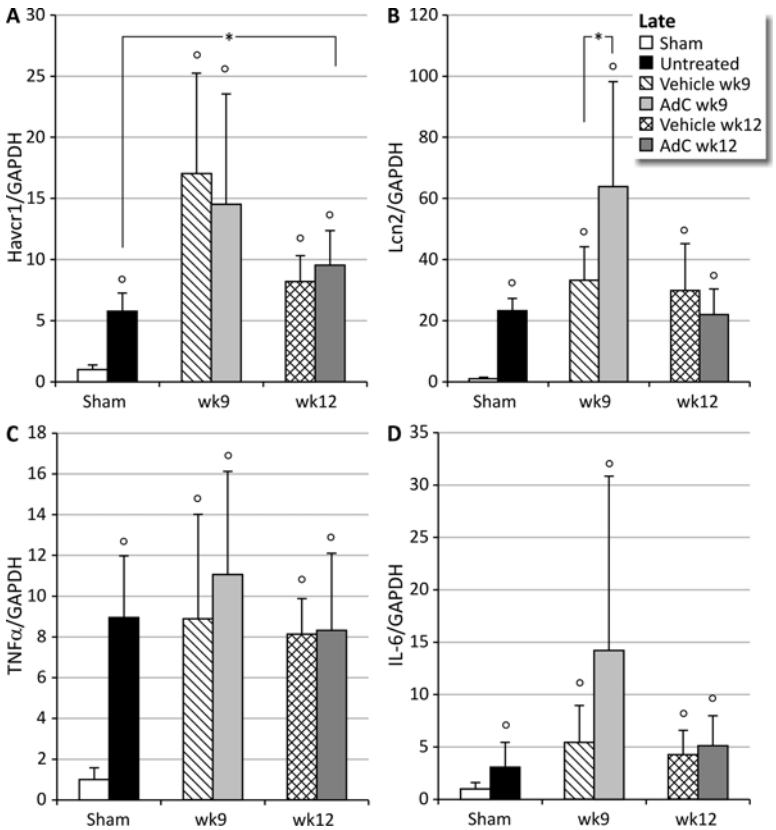


Figure 4.7 (left page): Analysis of expression of tubular injury markers and inflammatory cytokines after treatment in the early chronic fibrosis phase. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of treatment (wk6 after UIRI) and 12 weeks after UIRI (endpoint of experiment). **A:** Quantification of *Havcr1* (KIM-1) gene expression (qPCR). **B:** Quantification of *Lcn2* (NGAL) gene-expression (qPCR). **C:** Quantification of *TNFα* gene expression (qPCR). **D:** Quantification of *IL-6* gene expression (qPCR). °: p<0.05 vs. sham, *: p<0.05. AdC: decitabine.

Figure 4.8 (above): Analysis of expression of tubular injury markers and inflammatory cytokines after treatment in the late chronic fibrosis phase. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of treatment (wk9 after UIRI) and 12 weeks after UIRI (endpoint of experiment). **A**: Quantification of *Havcr1* (KIM-1) gene expression (qPCR). **B**: Quantification of *Lcn2* (NGAL) gene-expression (qPCR). **C**: Quantification of *TNFα* gene expression (qPCR). **D**: Quantification of *IL-6* gene expression (qPCR). °: p<0.05 vs. sham, *: p<0.05. AdC: decitabine.

4.3.6 Ischemia-reperfusion induces upregulation of Dnmts

After vehicle treatment in the early chronic fibrosis phase, expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* was significantly upregulated in the ischemic kidneys as compared to sham (4.97±1.01, 5.27±1.75 and 6.94±1.23-fold resp.) ($p<0.05$) (Figure 4.9 A). Genomic DNA-methylation in the ischemic kidney at this time point, as measured by UPLC, showed no difference between vehicle-treated and sham animals (Figure 4.9 B). The gene expression of *Dnmt1*, *3a* and *3b* remained stably upregulated in vehicle-treated animals euthanized at week 9 and week 12 after UIRI as compared to shams ($p<0.05$) (Figure 4.9 C). Genomic DNA-methylation in the ischemic kidney showed a tendency to increase from week 6 up to week 12 after UIRI in vehicle-treated animals (Figure 4.9 B and D).

4.3.7 Early but not late treatment with decitabine affects Dnmt gene expression

A short-term treatment with decitabine in the early chronic fibrosis phase (week 6) did not influence the gene expression of *Dnmt1* and *Dnmt3a* as compared to vehicle treatment at this time point; however, a consistent trend towards decreased expression was observed ($p=0.11$) (Figure 4.9 A). The gene expression of *Dnmt3b* was significantly lower after decitabine treatment as compared to the vehicle group (4.51±1.45 vs. 6.94±1.23) ($p<0.05$). Genomic DNA-methylation in the ischemic kidney, as measured by UPLC, showed no difference between decitabine-treated and vehicle-treated animals at this time point (Figure 4.9 B). Whilst decitabine treatment, initiated in the early chronic fibrosis phase, resulted in a consistent tendency towards decreased expression of *Dnmt1* and *Dnmt3a*, this effect was not observed after decitabine treatment in the late chronic fibrosis phase (week 9 after UIRI) (Figure 4.9 C). Also, the significant reduction in *Dnmt3b* gene expression upon decitabine treatment in the early chronic phase was not present after decitabine treatment in the late chronic phase (Figure 4.9 C). At the endpoint of the experiment (week 12 after UIRI), the gene expression of *Dnmt1* and *Dnmt3a* remained stably upregulated, both after treatment in the early and late chronic fibrosis phase (Figure 4.9 A and C). Gene expression of *Dnmt3b* remained significantly lower after decitabine treatment in the early but not the late chronic fibrosis phase as compared to the untreated group (4.41±1.03 vs. 7.13±2.32) at the endpoint of the experiment ($p<0.05$). Genomic DNA-methylation in the ischemic kidney at this time point was significantly increased in decitabine-treated animals (5.77±0.28%), treated in the early not late fibrosis phase as compared to untreated animals (5.39±0.15%) ($p<0.05$) (Figure 4.9 B and D).

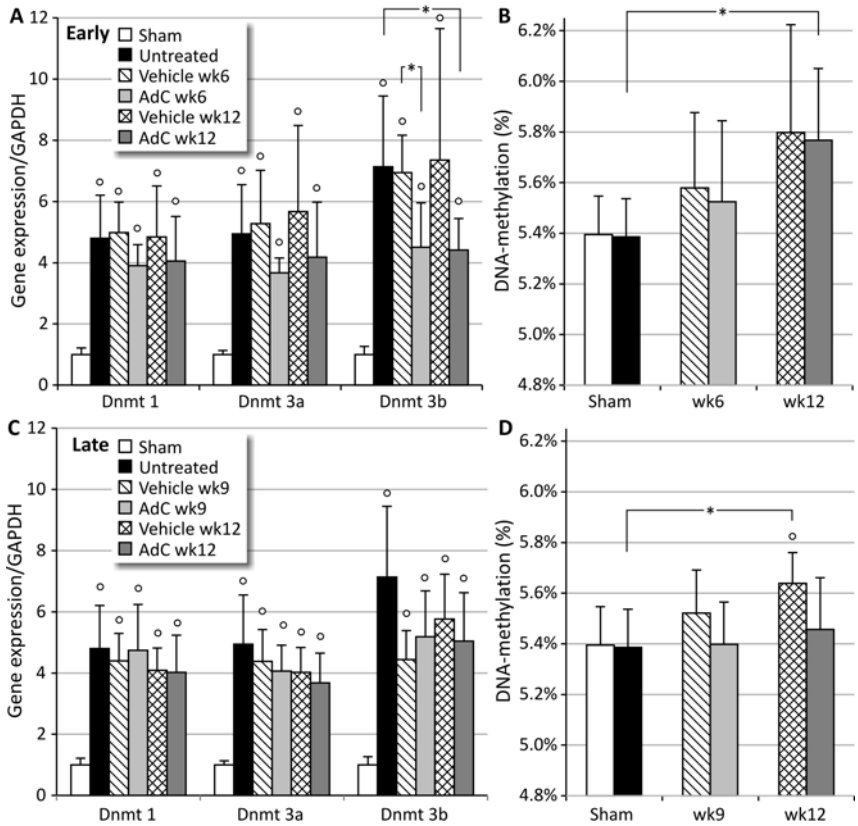


Figure 4.9: Parameters of DNA-methylation in the ischemic kidney. UIRI was performed for 30 minutes at 37°C, n=6 in sham, untreated and vehicle groups, n=8 in decitabine treatment groups. Animals were euthanized 12 days after end of early treatment (wk6 after UIRI) and 12 weeks after UIRI (endpoint of experiment), 12 days after end of late treatment (wk9 after UIRI) and 12 weeks after UIRI (endpoint of experiment).
A: Quantification of gene expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* (qPCR) after treatment in the early chronic fibrosis phase. **B**: Percentage of genomic DNA-methylation in the ischemic kidney (UPLC) after treatment in the early chronic fibrosis phase. **C**: Quantification of gene expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* (qPCR) after treatment in the late chronic fibrosis phase. **D**: Percentage of genomic DNA-methylation in the ischemic kidney (UPLC) after treatment in the late chronic fibrosis phase. °: $p<0.05$ vs. Sham, *: $p<0.05$. AdC: decitabine.

4.4 Discussion

The (myo)fibroblast, independent of its origin, is the principal effector cell for the development of fibrosis in organs. Bechtel et al. (2010) have demonstrated that in folic acid renal fibrosis, fibroblasts become terminally activated because of hypermethylation of the RASAL1 gene, rendering fibroblasts unable to return to their resting state after the resolution of the primary insult[11]. This permanent activation is particularly characterized by proliferation and excess production of extracellular matrix. Importantly, during proliferation, established DNA-methylation patterns of CpG island promoters are copied to the daughter cells[28], providing them with a terminally activated phenotype as well. Since the maintenance DNA-methyltransferase 1 (DNMT1) is responsible for this, one may hypothesize that by inhibiting DNMT1 activity, copying of the DNA-methylation pattern of proliferating, terminally activated fibroblasts to daughter cells might be prevented. As in clinical practice most patients already present with chronic fibrosis at the moment of CKD diagnosis, we investigated whether treatment with the DNMT inhibitor decitabine is able to attenuate progression of established renal fibrosis. Decitabine is a 5-aza substituted nucleoside analogue[29, 30] that is incorporated into the DNA, serves as a substrate for DNMTs[30] and induces depletion of genomic DNMT stores[27, 29]. Since decitabine is neither cell type- nor DNA-sequence specific, we opted for a short-term treatment (10 days) to minimize adverse effects, whilst ensuring to cover multiple fibroblast cell divisions.

This study was conducted in a mouse model of severe unilateral renal ischemia-reperfusion injury (UIRI) without contralateral nephrectomy, which we recently demonstrated to result in development of long-term renal fibrosis[23]. Ischemia-reperfusion is a major obligate injurious factor in renal transplant pathology and a common clinical cause of AKI for which a role for pathologic DNA-methylation has been suggested[17, 31]. To the best of our knowledge in the present study we report for the first time persistent long-term upregulation of *Dnmt1*, *3a* and *3b* following UIRI (Figures 4.9 A and C), thereby complementing reports from experimental studies using models of folic acid-induced renal fibrosis[11] and hyperhomocysteinemia nephropathy[32]. Furthermore, we found that genomic DNA-methylation was significantly increased 14 days after UIRI (Figure 4.3) and demonstrated a tendency towards persistent elevation of DNA-methylation at 6, 9 and 12 weeks after UIRI (Figures 4.9 B and D). These data, in combination with reports of altered DNA-methylation of specific genes in CKD patients[11, 14, 16, 18, 33], further corroborate that aberrant DNA-methylation is a pathological entity also in ischemia-reperfusion induced AKI/CKD, supporting the use of this model in a tentative therapeutic intervention study based on DNA-demethylation.

To assess the efficacy of DNA-methyltransferase inhibition by decitabine, we investigated the DNA-methylation dynamics. In the current study, a 14-day treat-

ment regimen with 0.25 mg/kg/day decitabine induced a significant reduction in DNA-methylation of approximately 3% in healthy animals and 11% in UIRI animals (Figure 4.3). Thus, upon decitabine treatment, a small yet significant reduction in genomic DNA-methylation was observed. This result is not surprising as merely approximately 3% of CpG island promoters are methylated in normal cells[28]. However, 12 days after the end of decitabine treatment, both in the early or late fibrosis phase, there was no longer a significant difference in genomic DNA-methylation in decitabine treated animals as compared to those receiving vehicle (Figures 4.9 B and D), suggesting recovery of genomic DNA-methylation. This is in line with the finding of Kagey et al. (2010) noting that re-silencing after decitabine removal is possible due to the preservation of some repressive histone modifications upon DNA-demethylation[34], thereby rendering the effects of epigenetic drugs transient. The fact that demethylated genes can be remethylated within a few days after the end of treatment[27, 28, 35] may explain the observed normalisation of DNA-methylation by day 12 after treatment (Figure 4.9 B and D), as was also seen in human blood samples[36].

Even though the focus of this study was on the therapeutic potential of inhibition of DNA-methylation, we also investigated its effects on the expression of Dnmts. In our experiment, gene expression of all *Dnmts* was clearly upregulated following UIRI (Figure 4.9 A and C). When decitabine was administered in the early (but not late) chronic phase, upregulation tended to be attenuated for all *Dnmts* (Figure 4.9 A). Interestingly, although we observed a significant decrease only for *Dnmt1* gene expression immediately after a 14-day treatment period with decitabine (Figure 4.3), only *Dnmt3b* suppression reached long-lasting significance (Figure 4.9 A). This indicates that the effect of decitabine treatment on *Dnmt1* expression is rather transient, whereas that for *Dnmt3b* appears to be delayed. This is interesting since overexpression experiments have revealed that the *de novo* DNA-methylation activity of DNMT3a and 3b has a broad range of targets, whereas that of DNMT1 is limited to selected genes[37, 38]. Although DNMT1 is the most abundant methyltransferase in mammalian cells[39] and *in vitro* experiments suggest that it is a prominent hypermethylator in somatic cells[11, 40], it remains unclear how DNMT1 would facilitate aberrant *de novo* DNA-methylation of selected genes in fibrosis[28], since the substrate of DNMT1 is hemimethylated DNA[41]. In addition, DNMT1 knockout cells display almost normal genomic DNA-methylation levels, while cells lacking both DNMT1 and DNMT3B undergo a substantial loss of DNA-methylation in the promoter region of tumor suppressor genes[42]. These observations resulted in the hypothesis that DNMT3b is thought to be the additional activity that cooperates with DNMT1 to maintain cellular DNA-methylation patterns[30]. Although it remains a fact that DNMT1 depletion ($DNMT^{-/+}$ heterozygous mice) attenuates folic acid-induced fibrosis[11], the precise role of DNMT3b in

fibrosis certainly warrants further investigation.

Regardless of its transient effects on DNA-methylation, inhibition of DNMTs by short-term decitabine treatment did not prevent the progression of fibrosis in this study. In particular, decitabine treatment, neither initiated in the early nor late chronic phase of fibrosis development, was unable to attenuate the increased gene expression of *collagen I* or *TGFβ* (Figures 4.5 B-C and 4.6 A-B). Quantitative histological analysis by collagen I immunostaining confirmed these observations (Figures 4.5 D and 4.6 C). In addition, analysis of gene expression of KIM-1 (*Havcr1*), NGAL (*Lcn2*), *TNFα* and *IL-6* indicated that decitabine was unable to attenuate tubular injury (Figures 4.7 A-B and 4.8 A-B) as well as inflammation (Figure 4.7 C-D and 4.8 C-D). However, one remark to the above should be made. Although not significant, when decitabine treatment was initiated at the early chronic fibrosis phase, a clear tendency towards decreased *TGFβ* could be observed as compared to vehicle and untreated animals (Figure 4.5 C). One could speculate that in conditions of mild renal injury, as could reasonably be achieved with milder conditions of ischemia-reperfusion (i.e. lower body temperature, shorter ischemia times[23], or with prolonged treatment (at lower dose), decitabine might be able to attenuate renal fibrosis. Indeed, gene expression of the tubular markers KIM-1 and NGAL indicates that the ischemic insult in this study induced severe renal injury. However, this hypothesis is subject to further investigation.

Overall, we conclude that even though decitabine treatment induced a significant reduction in genomic DNA-methylation, progression of fibrosis, as evaluated by *collagen I* and *TGFβ* gene expression and histological quantification of Masson stain and collagen I immunostaining, was not attenuated or prevented in this model. We would like to note that the effect of treatment with nucleoside analogues was shown valuable against fibrosis mainly in *in vitro* experiments[43-45]. However, the only evidence of prevention of fibrosis by inhibition of DNMT's *in vivo* was provided by Bechtel et al. (2010)[11] and Pushpakumar et al. (2015)[32] in the kidney and by Kim et al. (2014)[46] in cardiac fibrosis. Taken into account (i) the limited positive results of decitabine treatment on renal fibrosis in the current experiment, (ii) the overall limited amount of positive publications on nucleoside analogues as a treatment strategy for fibrosis and (iii) the severity of adverse effects (e.g. weight loss) of this compound (even for relative short-term treatments), administration of nucleoside analogues as such seems unsuited as a first-line treatment in CKD. Evaluating the efficacy of precisely targeted DNA-demethylation strategies would therefore be invaluable to fully reveal the putative therapeutic potential of DNA-demethylation in targeting development or progression of renal fibrosis[47].

4.5 References

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Chapter 5

Short-term dexamethasone treatment transiently attenuates fibrosis in acute-to-chronic kidney injury

5

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Chapter 5: Contents

Abstract

Background: Acute kidney injury (AKI) is an underestimated, yet important, risk factor for the development of chronic kidney disease (CKD). Currently, there are no therapeutics targeting the progression towards chronic injury after an AKI episode. Persistence of inflammation after a renal ischemic injury has been observed, both in experimental models and patients, and may be one of the most important mechanisms underlying progression of acute-to-chronic renal injury. Attenuation of inflammation after AKI might therefore be a good first-line therapeutic strategy.

Methods: Male C57Bl/6 mice (10-12 weeks of age) underwent warm (36°C body temperature) unilateral ischemia-reperfusion of the kidney for 21 minutes. One day after surgery, treatment with the corticosteroid dexamethasone was initiated for 3 weeks. Fibrosis was quantified by collagen I gene expression and immunostaining, as well as gene expression analysis of fibrosis-related genes *TGFβ*, *CCN2* (CTGF), *PAI-1* and *CCN3*. Furthermore, inflammation was evaluated by *TNFα* gene expression and protein expression of the F4/80 macrophage marker and the α-SMA fibroblast marker.

Results: Treatment with dexamethasone attenuated development of fibrosis, as evidenced by reduced collagen I gene expression and immunostaining, in combination with reduced gene expression of the pro-fibrotic *CCN2* and increased expression of the anti-fibrotic *CCN3*. The effects of dexamethasone on renal fibrosis persisted up to 3 weeks after the end of treatment, as evidenced by stagnation of collagen I deposition in the ischemic kidney, in contrast to vehicle-treatment, where further progression of fibrosis was observed. However, following the treatment period expression levels of the pro-fibrotic genes re-approached those of vehicle-treated injured kidneys suggesting that the effects of dexamethasone on fibrosis beyond the treatment period are temporary. Thus, immune suppressive treatment strategies can attenuate the development of renal fibrosis after an acute ischemic event, and can provide a basis for a multi-factorial treatment strategy. However, persistent treatment until near complete resolution of inflammation may be required to maintain long-term effects.

5.1 Introduction

Acute kidney injury (AKI) is an underestimated, yet important, risk factor for the development of chronic kidney disease (CKD)[1]. Long-term follow-up studies (4 months to 6 years) report that between 35 and 71% of patients surviving an episode of AKI had incomplete recovery of renal function as assessed by creatinine clearance or serum creatinine measurements[2]. These patients are more likely to progress to end-stage renal disease (ESRD) as compared to patients without a history of either AKI or CKD[1,3] and contribute to the growing population of CKD patients. Currently, there are no therapeutic interventions targeting disease progression after AKI[4] highlighting the urgent need for novel therapeutic approaches that aim at preventing and/or reversing the pathophysiologic sequelae of AKI[5].

Renal ischemia-reperfusion injury, due to hypoperfusion after surgery, bleeding or dehydration, is a major aetiology in human AKI[6,7]. Renal IRI is of particular importance in the setting of kidney transplantation[8,9]. We previously optimized a mouse model of AKI to CKD by unilateral ischemia-reperfusion (UIRI) without contralateral nephrectomy, with development of moderate renal fibrosis and significant long-term inflammation[10] (Chapter 3).

Inflammation plays a major role in the pathophysiology of ischemic AKI[11]. Post-ischemic tissue infiltration by neutrophils, macrophages, and different subtypes of T-cells is a hallmark of acute renal ischemic injury, both in patients and experimental models[6,9,11]. Persistence of inflammation may contribute to maladaptive cellular repair responses after acute injury and may be an intrinsic component of progression of renal injury[4,12]. In view of the above, attenuation of inflammation after acute ischemic kidney injury may be a suitable therapeutic strategy in the prevention of progression of renal injury. Dexamethasone is a glucocorticoid, widely used in renal diseases as an anti-inflammatory and immunosuppressive agent[13]. Corticosteroids inhibit the synthesis of chemokines and cytokines resulting in protection against inflammation, and at high doses inhibit the immune response[14]. It was already shown in experimental models that dexamethasone has a protective effect against ischemic damage in liver and hart[15,16]. In the kidney, pre-treatment with dexamethasone has been demonstrated to ameliorate the severity of an acute ischemic insult[13,17-19] (Table 5.1). These studies, however, particularly covered the acute injury phase up to 24 hours after the ischemic insult. In the light of the recently appreciated link between AKI and CKD, we here evaluated for the first time the long-term (up to 6 weeks) impact of temporary (3 weeks) inflammatory suppression by dexamethasone on pathology progression and development of fibrosis in ischemia-reperfusion injured kidneys.

Table 5.1: *Therapeutic use of dexamethasone in experimental AKI.*

Dexamethasone	Model	Species	Dosage	Administration	Treatment	Effect
Suzuki et al.	HgCl2	Rats	1 mg/kg	s.c.	Daily	Delay of early tubular lesions, inhibition of lymphocyte infiltration and unaltered development of fibrosis.
Chen et al.	LPS endotoxin	Mice	2.5 mg/kg	i.p.	1 dose	Anti-oxidant stress properties, improved capillary blood flow and renal function.
Choi et al.	CLP-induced sepsis	Mice	2.5 mg/kg	i.v. or i.p.?	1 dose	Improved renal function, reduced inflammatory cytokine expression and apoptosis.
Yuan et al.	NZB/NZW systemic lupus erythematosus	Mice	1 mg/kg	i.p.	Daily	Reduced albuminuria and renal immune complexes.
Yuan et al.	NZB/NZW systemic lupus erythematosus	Mice	1 mg/kg	i.p.	Daily	Attenuation of progression of renal dysfunction, glomerulonephritis, tubulointerstitial disease and immune cell infiltration.
Zhang et al.	Unilateral IRI + Nx	Mice	4 mg/kg	i.p.	1 dose	Improved renal function, attenuated histological changes and neutrophil infiltration.
Zhang et al.	Unilateral IRI + Nx	Mice	4 mg/kg	i.p.	1 dose	Improved renal function and attenuated post-ischemic histological changes.
Speir et al.	Unilateral IRI + Nx	Rats	3 mg/kg	i.p.	1 dose	Histologic ischemic changes and tubular necrosis unaltered, higher tubular regeneration.
Takahira et al.	Bilateral IRI	Rats	1 mg/kg	i.v.	1 dose	Attenuated neutrophil infiltration, no effect on renal function or tubular necrosis.
Kumar et al.	Bilateral IRI	Rats	3 mg/kg	i.p.	1 dose	Improved renal function and attenuated histological damage.
Kumar et al.	Bilateral IRI	Mice	8 mg/kg	i.p.	1 dose	Improved renal function and reduced tubular cell apoptosis.
Rusai et al.	Unilateral IRI (?)	Rats	2 mg/kg	i.p.	?	
Zager et al.	Unilateral IRI	Mice	250 µg/ injection	i.p.	Daily for 3 days then alternate days	Blunted inflammatory cytokine upregulation, attenuation of renal atrophy.

5.2 Methods

5.2.1 Animals and experimental design

All animal procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics Committee (approval number 2015-37). On average we encountered 5% mortality, mainly due to post-anaesthetic complications. In addition, on average 10% of the animals were excluded from analysis, because their values were marked as outliers for different parameters upon statistical analysis. Prior to surgery, animals were randomly allocated to the different treatment groups. Animals had free access to standard chow and tap water.

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously[10] (see chapter 2). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent 21 minutes of warm (36°C body temperature) UIRI, with continuously monitoring of body temperature. The right kidney was left undisturbed. Sham-operated animals received the same surgical procedure except placement of the clamp.

Treatment regimen: Animals were divided over 4 groups (Figure 5.1): 1) UIRI + no treatment (n=8), 2) UIRI + dexamethasone (n=20, 10 mg/kg, daily for 3 days, then every other day as reported previously[20]), 3) UIRI + vehicle (n=20, PBS, same volume as dexamethasone, daily for 3 days, then every other day), and 4) Sham + no treatment (n=10). Treatment was initiated approximately 2 hours after UIRI, for 3 weeks and administrated via intraperitoneal injection. Animals of groups 1 and 4, and half of the animals of group 2 and 3 were euthanized at the end of the 3-week treatment regimen. The other half of group 2 and 3 were euthanized at week 6, after an additional 3 weeks of follow-up without treatment.

Euthanasia: Animals were euthanized by exsanguination via the abdominal vena cava under ketamine-xylazine anaesthesia. Kidneys were excised, renal pole fractions were snap frozen in liquid nitrogen and transversal slices of renal tissue were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and NBF (10% neutral buffered formalin) for 4 hours and 24 hours, resp., rinsed with 70% isopropanol and embedded in paraffin for histology.

5.2.2 Real-time PCR

Total mRNA was extracted from a pole section of the ischemic kidney (PureLink RNA Mini Kit; Life Technologies, Gent, Belgium) and converted to cDNA (High Capacity cDNA archive kit; Life Technologies). To quantify gene expression, qPCR, based on the Taqman fluorescence method (ABI Prism 7000 sequence detection system; Life Technologies), was used. Taqman probes and primers for *GAPDH*

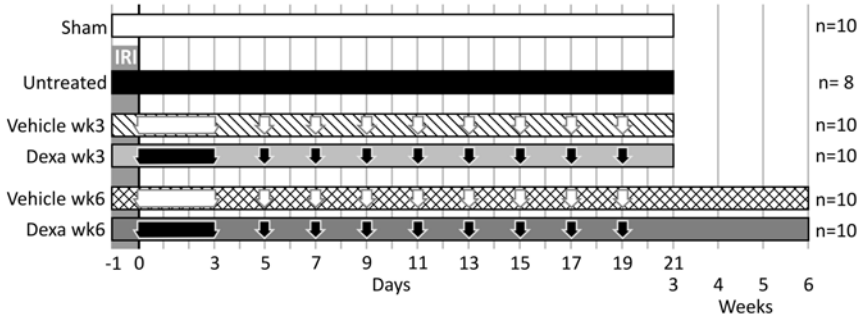

Figure 5.1: Study set-up.

(Mm99999915_g1), *collagen I α1* (Mm00801666_g1), *TGFβ1* (Mm01178820_m1), *CCN2* (Mm01192931_g1), *CCN3* (Mm00456855_m1), *PAI-1* (Mm00435860_m1) and *TNFα* (Mm00443258_m1) were purchased from Life Technologies. Each gene was analysed in triplicate and the expression was normalized to the housekeeping gene *GAPDH*. Calculations were made conform the comparative Cq-method.

5.2.3 Histology

For the immunostainings, paraffin embedded 4 µm thick sections of ischemic kidney tissue were blocked with goat normal serum and incubated overnight with the primary antibody, resp. polyclonal rabbit anti-mouse collagen I antibody (T40777R, Biodesign International, Saco, Maine) for evaluation of fibrosis, and polyclonal rabbit anti-mouse Ki67 (Novus, Abingdon, UK) for evaluation of cell proliferation. After washing, sections were incubated with a biotinylated goat anti-rabbit IgG antibody (PK-4001, Vector Laboratories, Burlingame, California) and subsequently incubated with avidin and biotinylated horseradish peroxidase (ABcomplex, Vector Laboratories). A dark brown colour was developed with diaminobenzidine in the presence of 3% H_2O_2. Sections were counterstained with methyl green to visualize nuclei. The various immunostainings were quantified using the Axiovision image analysis software (Carl Zeiss, Jena, Germany). For the quantification (see chapter 2) of collagen I immunostaining, digital photographs were taken (complete slide, original magnification x100). The area % stain represents the ratio of the summed absolute areas of staining versus the total tissue. Quantification of the Ki67 immunostaining was performed on 5 random cortical and outer medulla fields per kidney (x500 original magnification). Data are presented as the mean number of Ki67 positive tubular nuclei per 5 fields.

5.2.4 Western blotting

Total protein was isolated, separated through SDS-PAGE gel electrophoresis and transferred onto a PVDF blotting membrane (see chapter 2). The membrane

was then incubated with the primary antibody, resp. polyclonal rabbit-anti-mouse β-actin (4970 S, Cell Signalling, Leiden, Netherlands), polyclonal rabbit-anti-mouse F4/80 (sc-25830, Santa Cruz, Heidelberg, Germany) and rabbit-anti-mouse α-SMA (ab5694, Abcam, Cambridge, UK). Following several wash steps, the membrane was incubated with a HRP conjugated goat-anti-rabbit IgG (sc-2004, Santa Cruz). Immune complexes are detected using a chemiluminiscence kit (SuperSignal West; Fisher Scientific).

5.2.5 Statistics

All statistical analysis was performed with SPSS Statistics 22 (IBM, Brussel, Belgium). Data are presented as mean ± standard deviation, or as individual values with median. Comparisons between groups are assessed using a Kruskal-Wallis test, followed by a Mann-Whitney U test. Values of $p<0.05$ are considered significant.

5.3 Results

5.3.1 Progression from AKI to CKD in untreated and vehicle-treated animals

As depicted in Figure 5.2, renal unilateral ischemia-reperfusion injury (UIRI) without contralateral nephrectomy induced a significant reduction of renal mass as compared to sham-operated animals (3.29±0.29 mg/g vs. 6.04±0.58 mg/g)

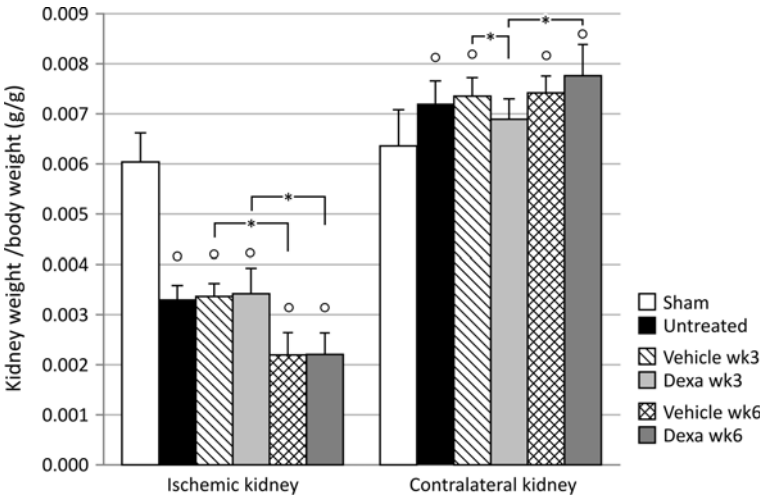

Figure 5.2: Mass of the kidneys at euthanasia, corrected for body weight. UIRI was performed for 21 minutes at 36°C, n=8 in the untreated group, n=10 in sham, dexamethasone and vehicle treatment groups. Animals were euthanized after treatment (3 weeks after UIRI) and 3 weeks after treatment (6 weeks after UIRI). °: $p<0.05$ vs. Sham, *: $p<0.05$.

(p<0.05) 3 weeks after UIRI. The severity of the renal atrophy is equal in ischemic kidneys of vehicle-treated animals and untreated animals (Figure 5.2). The weights of the contralateral uninjured kidney increased after UIRI in untreated animals as compared to sham (7.21±0.45 mg/g vs. 6.36±0.73 mg/g) (p<0.05), which is in accordance with the physiological fact that the contralateral kidney becomes hypertrophic to compensate for the loss of function of the ischemic kidney (Figure 5.2). The extent of hypertrophy is similar in vehicle-treated animals and untreated animals.

Significant upregulation of expression of fibrosis-related genes *collagen I* (16.7±3.0 fold), *TGFβ* (13.4±1.1 fold), *CCN2* (CTGF) (3.6±1.1 fold), *CCN3* (10.1±4.6 fold), *PAI-1* (20.5±4.1 fold) and *TNFα* (11.3±4.8 fold) was observed in the ischemic kidneys of untreated animals as compared to shams (p<0.05) 3 weeks after UIRI (Figure 5.3 C and 5.4 A-D). Vehicle-treatment did not significantly influence this

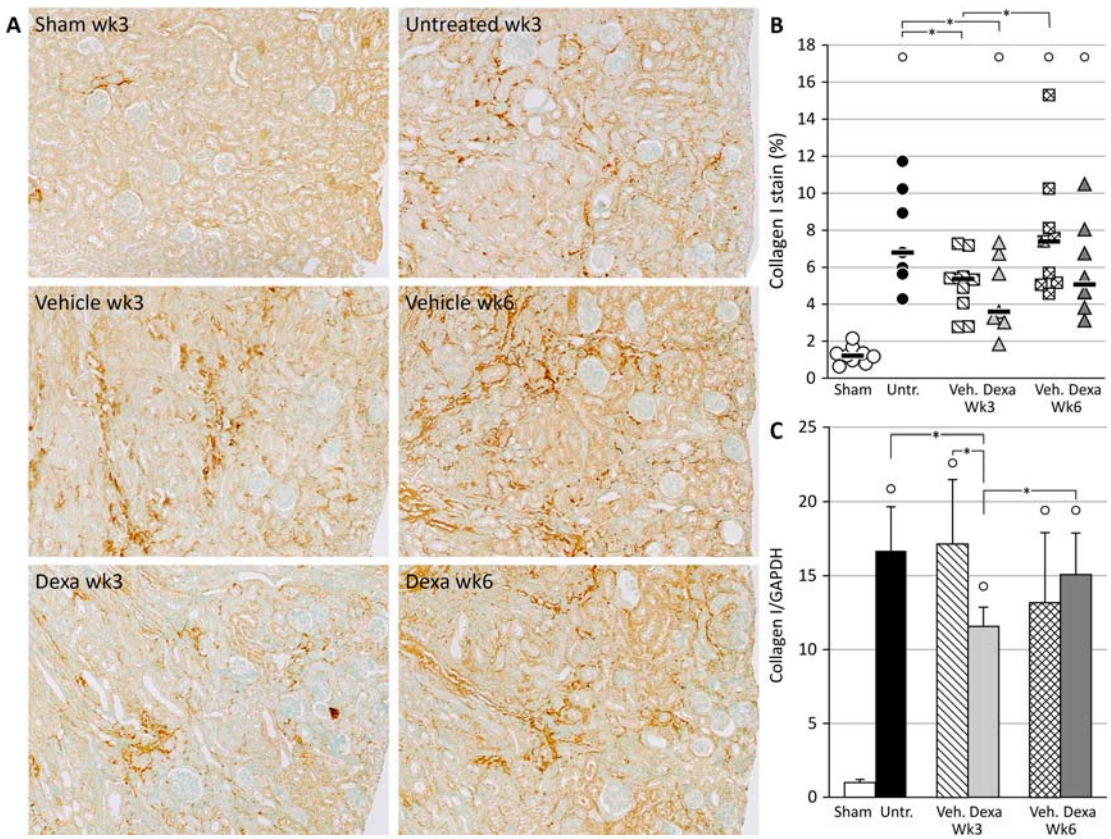

Figure 5.3: Evaluation of long-term fibrosis development in the ischemic kidneys. UIRI was performed for 21 minutes at 36°C, n=8 in the untreated group, n=10 in sham, dexamethasone and vehicle treatment groups. Animals were euthanized after treatment (3 weeks after UIRI) and 3 weeks after treatment (6 weeks after UIRI). **A**: Representative images of collagen I immunostained ischemic kidney tissue (magnification: 100x). **B**: Histological quantification of collagen I positivity of ischemic kidneys (x50 magnification). **C**: Quantification of *collagen I* gene expression (qPCR). °: p<0.05 vs. Sham, *: p<0.05.

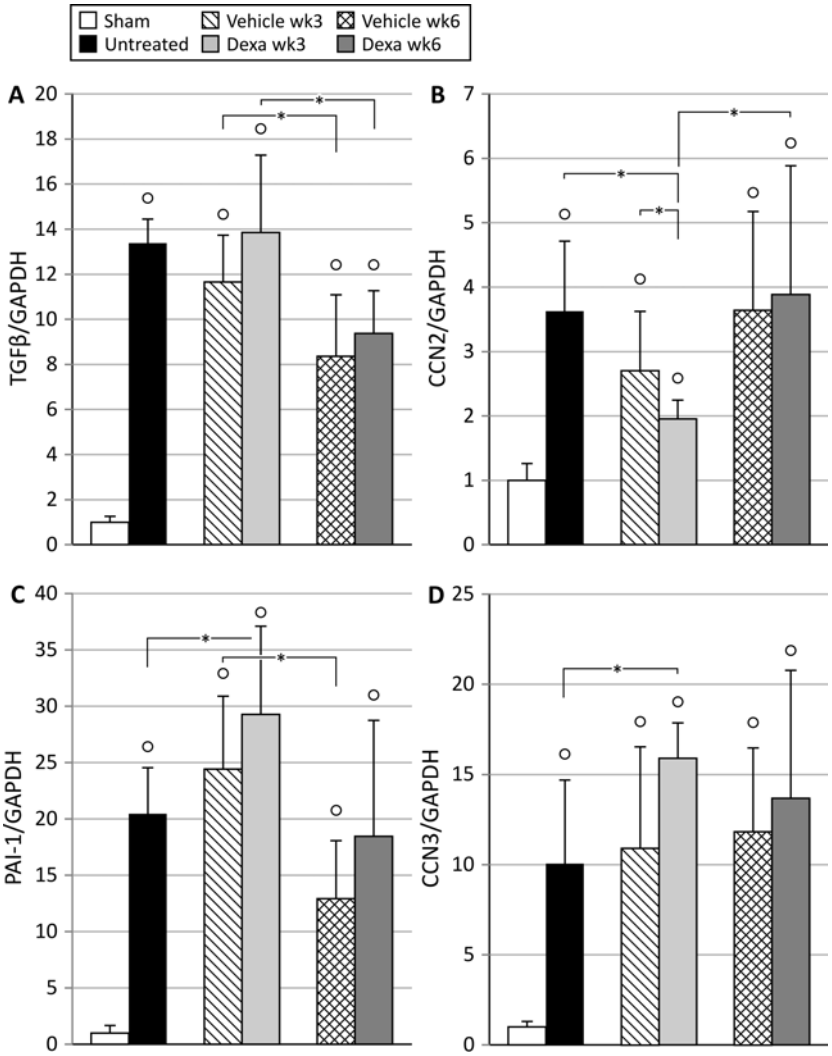

Figure 5.4: **Analysis of expression of fibrosis-related genes in the ischemic kidney**. UIRI was performed for 21 minutes at 36°C, n=8 in the untreated group, n=10 in sham, dexamethasone and vehicle treatment groups. Animals were euthanized after treatment (3 weeks after UIRI) and 3 weeks after treatment (6 weeks after UIRI). **A**: Quantification of *TGFβ* gene expression (qPCR). **B**: Quantification of *CCN2* (CTGF) gene-expression (qPCR). **C**: Quantification of *PAI-1* gene expression (qPCR). **D**: Quantification of *CCN3* gene expression (qPCR). °: p<0.05 vs. Sham, *: p<0.05.

expression pattern. In addition, collagen I immunostaining demonstrated significantly more collagen I deposition (i.e. fibrosis) in the kidneys of untreated UIRI animals as compared to shams (7.6±2.7% vs. 1.3±0.5%) ($p<0.05$) (Figure 5.3). After vehicle treatment, significant less collagen I staining was present in the ischemic kidneys as compared to the untreated group (5.1±1.5% vs. 7.6±2.7%) ($p<0.05$).

Using Ki67 immunostaining, we evaluated the proliferative response in the ischemic kidneys (Figure 5.4). Untreated ischemic kidneys showed significant increased proliferation as compared to sham (115±20 vs. 25±12 Ki67 positive cells/field) ($p<0.05$) 3 weeks after UIRI. A similar amount of Ki67-positive cells were observed in the kidneys of vehicle-treated animals as compared to untreated animals.

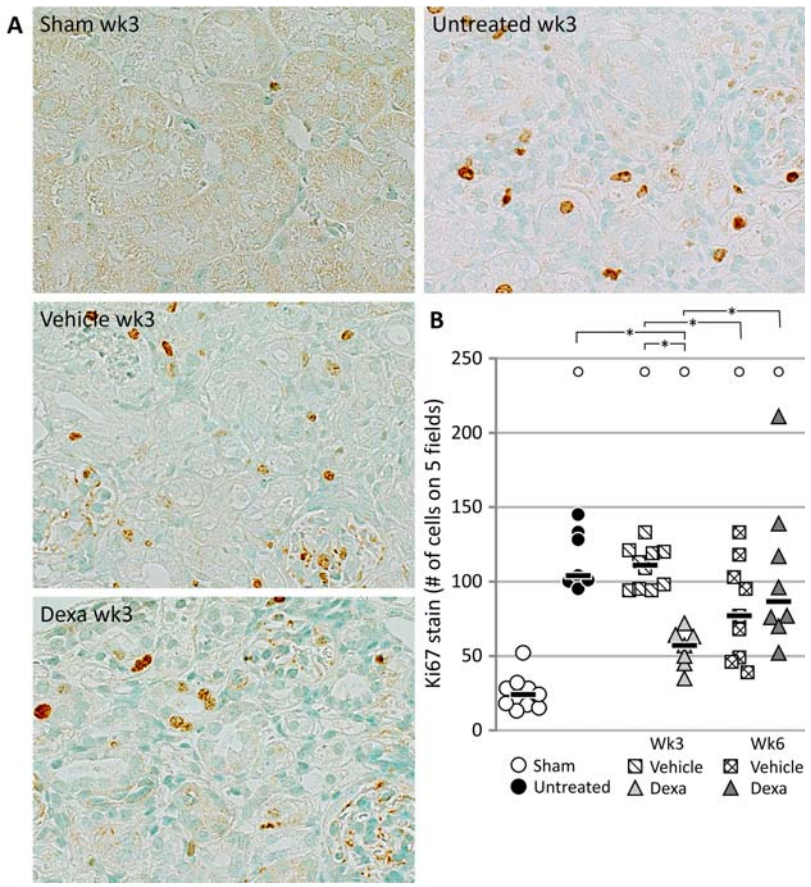

Figure 5.5: **Evaluation of cellular proliferation (Ki67 immunostaining) in the ischemic kidneys.** UIRI was performed for 21 minutes at 36°C, n=8 in the untreated group, n=10 in sham, dexamethasone and vehicle treatment groups. Animals were euthanized after treatment (3 weeks after UIRI) and 3 weeks after treatment (6 weeks after UIRI). **A**: Representative images of Ki67 immunostained ischemic kidney tissue (magnification: 500x). **B**: Histological quantification of Ki67 positivity in ischemic kidneys (x500 magnification). °: $p<0.05$ vs. Sham, *: $p<0.05$.

As it is known that both macrophages and myofibroblasts have an important role in the pathology of renal IRI and its repair, we quantified the amount of the F4/80 glycoprotein, which is expressed by murine monocytes/macrophages, and α-SMA (myofibroblasts) protein. Similar amounts of F4/80 protein were detected in healthy and ischemic kidneys of untreated or vehicle treated animals, 3 weeks after UIRI (Figure 5.6 B). As shown in Figure 5.6 C, there is a significant increased α-SMA protein expression in the ischemic kidney of untreated animals as compared to sham (1.213±0.379 ng vs. 0.214±0.144 ng). Vehicle-treated animals showed decreased α-SMA protein expression as compared to untreated animals 3 weeks after UIRI (0.757±0.386 ng vs. 1.213±0.379 ng). In addition, expression of α-SMA showed a tendency to increase from week 3 to week 6 after UIRI (Figure 5.6 C).

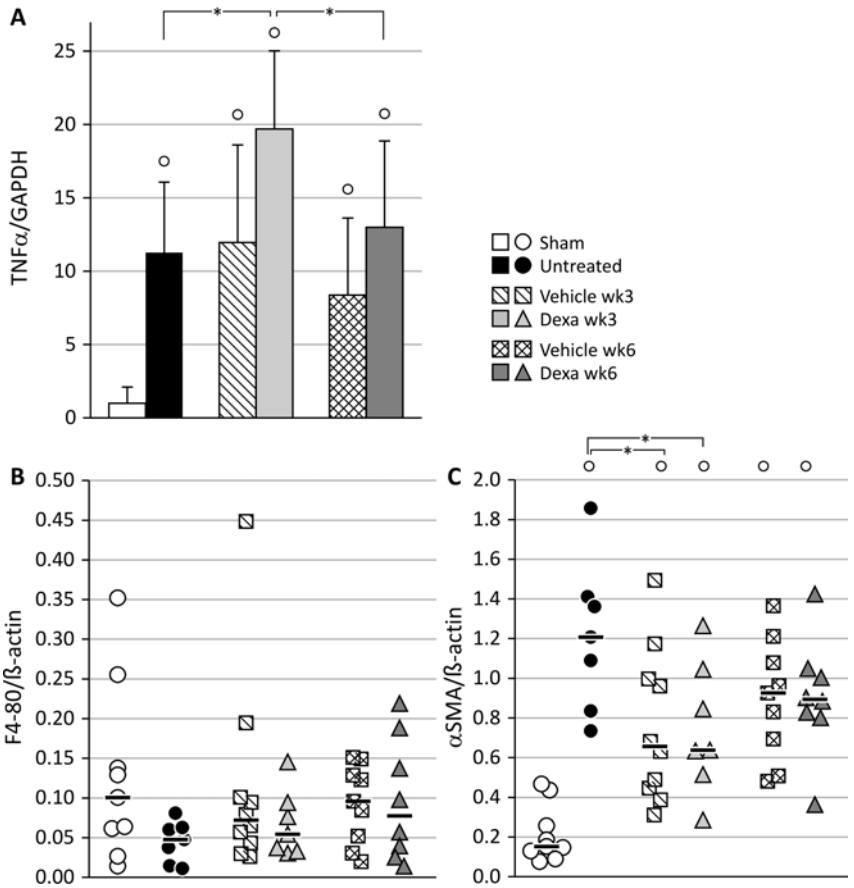

Figure 5.6: **Evaluation of inflammatory and fibroblast markers in the ischemic kidneys**. UIRI was performed for 21 minutes at 36°C, n=8 in the untreated group, n=10 in sham, dexamethasone and vehicle treatment groups. Animals were euthanized after treatment (3 weeks after UIRI) and 3 weeks after treatment (6 weeks after UIRI). **A**: Quantification of *TNFα* gene expression (qPCR). **B**: Quantification of F4/80 macrophage/monocyte protein expression (Western blot). **C**: Quantification of α-SMA fibroblast protein expression (Western blot). °: $p<0.05$ vs. Sham, *: $p<0.05$.

5.3.2 Temporary dexamethasone treatment attenuates development of fibrosis after ischemic AKI

Animals received dexamethasone treatment during 3 weeks at a dose of 10 mg/kg/day, after which they were euthanized. No noticeable adverse health effects were observed upon dexamethasone treatment. The renal atrophy inherent to the UIRI model was not prevented or attenuated by dexamethasone treatment (Figure 5.2). The mass of the contralateral kidneys, on the other hand, was significantly less hypertrophied as compared to the vehicle group (6.89±0.41 mg/g vs. 7.36±0.37 mg/g) ($p<0.05$) and did not significantly differ from the sham group (Figure 5.2).

Expression of the pro-fibrotic genes *collagen I* and *CCN2* was significantly lower after dexamethasone-treatment as compared to vehicle treatment (11.6±1.3 vs. 17.1±4.4 fold and 2.0±0.3 vs. 2.7±0.9 fold resp.) ($p<0.05$) (Figure 5.3 C and 5.4 B). The decrease in *collagen I* gene expression upon dexamethasone treatment was confirmed by collagen I immunostaining (Figure 5.3 B-C), where significantly less deposition was present in the dexamethasone treated ischemic kidney as compared to untreated animals (4.5±1.9 vs. 7.6±2.7%) ($p<0.05$) but not vehicle-treated animals. Also, after 3 weeks of dexamethasone treatment, gene expression of *PAI-1* and *CCN3* increased further as compared to the untreated group (15.9±2.0 vs. 10.1±4.6-fold and 29.3±7.8 vs. 20.5±4.1-fold resp.) ($p<0.05$), but not as compared to the vehicle treated group (Figure 5.4 C-D). Of these, only *CCN3* gene expression tended towards higher expression upon dexamethasone treatment as compared to vehicle treatment ($p=0.097$) (Figure 5.4 D). There was no difference in the expression of *TGFβ* after dexamethasone treatment as compared to vehicle treatment (Figure 5.4 A).

The expression of the pro-inflammatory cytokine *TNFα* was significantly increased after dexamethasone treatment as compared to untreated animals (19.7±5.3 vs. 11.3±4.8-fold) ($p<0.05$), but not vehicle treated animals (Figure 5.6 A). Dexamethasone treatment did not have an effect on the amount of F4/80 protein in the ischemic kidney (Figure 5.6 B), however it induced a significant decrease in α-SMA protein expression in the ischemic kidney as compared to the untreated group (0.746±0.331 ng vs. 1.213±0.379 ng) ($p<0.05$). No difference was seen when compared to vehicle treatment (Figure 5.6 C). Upon dexamethasone treatment, significantly less cell proliferation can be observed in the ischemic kidney as compared to vehicle treatment (56±16 vs. 110±14 Ki67 positive cells/field) and no treatment (56±16 vs. 4.53±1.92 Ki67 positive cells/field) ($p<0.05$) (Figure 5.5 B).

5.3.3 Temporary dexamethasone treatment does not appear to permanently attenuate renal decay after ischemic AKI

Since 3 weeks of dexamethasone treatment exerted beneficial effects on the ischemic kidneys, an additional 3-week follow-up period (without treatment) was implemented, to evaluate whether these effects persist during the further course of the fibrotic process. As depicted in Figure 5.2, renal atrophy further aggravated both in vehicle-treated as well as dexamethasone-treated animals from week 3 to week 6 after UIRI, with a nearly identical reduction in renal mass of 45% to approximately 65% of control kidney weight. The contralateral kidney of dexamethasone-treated animals demonstrated increased hypertrophy at 6 weeks after UIRI as compared to 3 weeks, to similar levels as vehicle-treated animals (Figure 5.2).

The expression of the fibrosis-related genes *collagen I* and *CCN2* remained stably upregulated in vehicle-treated animals after the follow-up period (Figure 5.3 C and 5.4 B). In dexamethasone-treated animals, *collagen I* and *CCN2* gene expression was significantly increased (15.1±2.8 vs. 11.6±1.3 and 3.9±2.0 vs. 2.0±0.3-fold resp.) ($p<0.05$) to match the expression of vehicle-treated animals (Figure 5.3 C and 5.4 B). Quantification of collagen I immunostaining showed increased collagen I deposition in the ischemic kidneys of vehicle-treated animals (7.7±3.4% vs. 5.1±1.5%) ($p<0.05$), whereas collagen I deposition in ischemic kidneys of dexamethasone-treated animals was not significantly increased as compared to immediately after treatment (Figure 5.3 B). After the follow-up period, gene expression of *PAI-1* and *TGFβ* was decreased in both in vehicle and dexamethasone treated groups as compared to immediately after treatment (Figure 5.4 A and C). Gene expression of *CCN3* remained stably upregulated in both vehicle-treated and dexamethasone-treated animals.

The expression of the pro-inflammatory *TNFα* remained stably upregulated in vehicle treated animals after the follow-up period. However, its gene expression was decreased in dexamethasone-treated animals at this time-point as compared to immediately after treatment (13.0±5.9 vs. 19.7±5.3-fold) ($p<0.05$) to similar levels as vehicle-treated animals (Figure 5.6 A). Protein expression of F4/80 and α-SMA remained stably upregulated in both vehicle- and dexamethasone-treated animals (Figure 5.6 B-C). The amount of proliferating cells in the ischemic kidneys of vehicle-treated animals was decreased after the additional follow-up period as compared to immediately after treatment (81±33 vs. 110±14 Ki67 positive cells/field) ($p<0.05$). However, the amount of proliferating cells in the ischemic kidney of dexamethasone-treated animals was increased at this time-point as compared to immediately after treatment (105±51 vs. 55±13 Ki67 positive cells/field) ($p<0.05$) to similar amounts as present in vehicle-treated animals (Figure 5.5 B).

5.4 Discussion

It is becoming increasingly clear that incomplete recovery from severe AKI is an important pathway to persistent and progressive CKD with underlying fibrosis. Recent studies have suggested that even complete recovery from AKI is associated with a strongly increased risk for CKD development[3,4,21]. A recent meta-analysis reported that patients surviving an episode of AKI have an 8.8-fold increased risk for CKD and a 3.3-fold increased risk for ESRD[4]. Understanding the mechanisms underlying the progression from acute-to-chronic renal injury is the focus of recent research in the field[22]. Since renal fibrosis is nearly always preceded by and closely associated with inflammation, both in patients[12] and experimental models of fibrosis[23,24], it is thought to be one of the major processes that contributes to progression of renal disease[24,25]. Also, experimental studies demonstrated that even when renal function recovers after AKI, pro-inflammatory and pro-fibrotic pathways remain active[26]. In accordance with this, we recently confirmed progression from AKI to CKD in a murine model of renal ischemia-reperfusion[10] (cfr. transplantation, major surgery, hypoperfusion, stenosis[27-33]). In particular, we observed features of CKD as seen in patients[34,35], such as development of tubulo-interstitial fibrosis, renal atrophy and inflammation, within 3 weeks after the ischemic insult, and verified the persistent nature of these CKD features up to 12 weeks post-ischemia[10]. With respect to inflammation, several laboratories demonstrated that suppression of the inflammatory response can reduce post-ischemic injury 24-48 hours after the ischemic insult[36-38] (Table 5.1). Yet, few studies investigated the long-term effects of inflammatory modulation on development of CKD, which, in the context of the recently appreciated AKI-to-CKD link, is of major therapeutic interest. Therefore, we here evaluated whether temporary treatment (3 weeks) with immune-suppressive dexamethasone is able to attenuate the development of post-ischemic renal fibrosis and avert the progression from acute to chronic renal injury.

In the current study, renal atrophy is pronounced and progressive, with loss of renal mass up to 44% and 64% within 3 and 6 weeks after the ischemic insult, respectively (Figure 5.2). Treatment with dexamethasone was unable to attenuate or prevent this loss of renal mass; not immediately after the 3-week treatment period, nor in the subsequent follow-up period. However, adaptive growth of the contralateral kidney to compensate for the loss of functional renal tissue[39] of the ischemic kidney did not occur immediately after dexamethasone treatment. This lack of compensatory hypertrophy could indicate that dexamethasone was able to rescue a certain degree of renal function (however not renal mass) of the ischemic kidney. Although the treatment regimen and dosing was the same as in the experiment of Zager et al. (2011), who observed 50% loss of renal mass in dexamethasone treated animals as compared to 66% in untreated animals[20] we

can only speculate as to why dexamethasone did not influence renal atrophy in our study. Most likely this is due to differences in the severity of the ischemic insult, which is very subjective to interlaboratory variation as well as mouse strain dependent susceptibility to ischemic AKI[40]. The presumption that the loss of function of the ischemic kidney was attenuated by dexamethasone treatment, is further supported by the mitigated development of post-ischemic fibrosis, as evidenced by a reduction in collagen I gene- and protein expression, and *CCN2* gene expression (Figure 5.3 B-C and 5.4 B). Moreover, gene expression of *CCN3*, that has been shown to have anti-fibrotic properties[41], was significantly elevated upon dexamethasone treatment (Figure 5.4 D). Furthermore, although gene expression of the pro-fibrotic *PAI-1*, an inhibitor of matrix degradation, appeared to be significantly elevated after dexamethasone treatment in comparison to the untreated condition, it did not differ from the vehicle-treated group. Similar results were observed in mercury chloride-induced nephropathy[42]. To further examine the effect of dexamethasone treatment on the fibrotic response after an acute ischemic insult, protein expression of α-SMA, a marker for activated fibroblasts, was determined. In normal kidney tissue, α-SMA staining is only found in smooth muscle cells, mostly in blood vessels[43]. In fibrotic diseases, α-SMA expression of myofibroblasts is recognized as a hallmark of their emergence and an indicator of disease severity[44]. In our study, dexamethasone did not have an effect on the amount of activated, α-SMA expressing fibroblasts as compared to vehicle-treatment. However, evidence suggests that α-SMA in myofibroblasts appears to have a suppressing role in tissue fibrosis progression, and forced expression in α-SMA$^{-/-}$ animals ameliorates fibrosis in the model of ureter obstruction and mesangioproliferative glomerulonephritis[44].

In addition to fibrosis, the post-ischemic period is characterized by an active inflammatory response, resulting from both activation of resident inflammatory cells and recruitment of circulating inflammatory cells[12]. Baeck et al. (2015) have shown, by means of a fluorescent double stain (Ki67 and F4/80), that significant proliferation of monocyte-derived macrophages occurs in the ischemic kidney, both in the acute (day 3, day 5) and early chronic phase (day 20)[45], thereby amplifying and prolonging the local inflammatory response[46]. In accordance with this, we observed significant infiltration of inflammatory cells in the ischemic kidney, quantified by protein expression of the F4/80 glycoprotein, which is expressed by murine monocytes/macrophages. Also, significantly elevated gene expression of the inflammatory cytokines *TNFα* and *TGFβ* supports ongoing post-ischemic inflammation. Increased gene expression of the inflammatory *TNFα* was observed in the ischemic kidney after dexamethasone treatment as compared to untreated animals. Although unexpected at first sight, it has been shown that TNFα can modulate the expression of the glucocorticoid receptor isoforms in such a manner that glucocorticoid resistance may occur[47]. Consistent with this is the

fact that, steroid insensitivity has been described in renal epithelial cells[48] and macrophages in the lung[49]. Thus, it might not be surprising that dexamethasone treatment did not have an effect on the amount of F4/80 macrophage protein in the ischemic kidney. However, it was shown by Castano et al. (2009) that it is possible to achieve attenuation of fibrosis without affecting the number of interstitial macrophages, quantified by F4/80 protein expression, which is also in line with our observations[50].

In normal physiological conditions, renal tubular cells have a low proliferation rate. The current consensus on renal tubular regeneration states that restoration of the tubular epithelium after an acute injury occurs predominately via proliferation of surviving epithelial cells that undergo dedifferentiation, primarily within the first 2 weeks[51-53]. In our study, a significant increase in cell proliferation in the injured kidney is seen up to 6 weeks after UIRI. Since successful proliferation of proximal tubule cells, i.e. no G2/M cell cycle arrest, is associated with attenuation of fibrosis[54], it was quite unexpected that temporary attenuation of renal decay immediately after dexamethasone treatment went along with an overall decrease in cell proliferation in the ischemic kidney. It should be noted, however, that quantification of proliferation in our study made no distinction between the renal cell types (epithelial cells, fibroblasts, inflammatory cells), nor cell cycle phase or their location (i.e. tubules vs. interstitium) due to severe distortion of the physiological tubulo-interstitial structure. However, independent studies reported decreased infiltration of lymphocytes[42], T-cells[55] and dendritic cells[55] upon dexamethasone treatment. The question which proliferative cell type and cell cycle phase was primarily affected by dexamethasone treatment in the current setting lies outside the scope of the current report and can only be solved by evaluating Ki67/ phosphorylated histon 3 expression in mice with distinctly genetically labelled cell types.

As a three-week dexamethasone treatment regimen attenuated the development of renal fibrosis after UIRI, we included an additional three weeks of follow-up without treatment, to evaluate whether the beneficial effects of dexamethasone treatment persist during the further course of the ischemic renal pathology. Dexamethasone treatment was unable to prevent long-term loss of renal mass. Moreover, as compared to immediately after end of treatment, a further loss of renal mass was observed, indicating that the progressive nature of the pathological course could not be attenuated by dexamethasone. The fact that compensatory hypertrophy of the contralateral kidney emerged after the three-week follow-up period indicates that either the positive effect of dexamethasone is temporary, or that longer or continuous treatment is necessary to reach persistent benefit from treatment. After the follow-up period, expression of the pro-fibrotic genes *collagen I* and *CCN2* was significantly increased as compared to the end-of-treatment time point (Figures 5.3 C and 5.4 B), indicating that the beneficial effect of

dexamethasone-treatment on pro-fibrotic gene expression is transient. However, analysis of collagen I deposition by immunostaining showed no further progression of fibrosis during the follow-up period in the dexamethasone group, whereas vehicle treated animals displayed progressive renal fibrosis and increasing collagen I staining in the ischemic kidney (Figure 5.3 B).

In conclusion, immune suppressive treatment strategies can attenuate the development of renal fibrosis after an acute ischemic event, evidenced by decreased upregulation of *collagen I* and *CCN2* gene expression and decreased collagen I immunostaining immediately after a 3-week treatment period. Dexamethasone did not exert an effect on macrophage F4/80 expression, and tended to increase *TNFa* gene expression. Additional follow-up of the dexamethasone-treated animals indicated that temporary dexamethasone treatment does not appear to permanently attenuate upregulation of fibrosis-related genes, whereas collagen I protein deposition tends to be affected longer. Overall immune suppressive treatment strategies can attenuate the development of CKD/fibrosis after an acute ischemic event, and can provide a basis for a multi-factorial treatment strategy. However, persistent treatment until near complete resolution of inflammation might be required.

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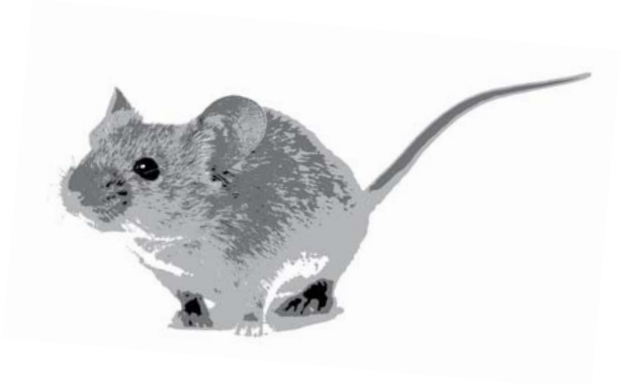
Chapter 6

Interference with the TGFβ-pathway in a mouse model of acute-to-chronic renal injury

6



Chapter 6: Contents

Abstract

Background: Acute kidney injury (AKI) still has a worldwide increasing incidence and prevalence. It is becoming increasingly clear that these patients often progress towards chronic kidney disease (CKD). Currently there are no therapeutics that can halt this progression towards chronic injury and development of fibrosis. Since TGFβ is involved in both AKI and fibrosis, antagonism of TGFβ is thought to be a promising therapeutic strategy.

Methods: To evaluate the therapeutic efficacy of anti-TGFβ antibody treatment and supplementation of CCN3, the endogenous counterpart of the downstream-to-TGFβ pro-fibrotic CCN2 (CTGF), on the progression of acute to chronic kidney injury, C57Bl/6 mice underwent unilateral ischemia-reperfusion (UIRI; 21 minutes at 36°C). Treatment with antibody to TGFβ was initiated both 24 hours before and 2 hours after UIRI and administered every other day for 3 weeks. Supplementation with recombinant human CCN3 was initiated 2 hours after UIRI and administered daily for 3 weeks.

Results: We demonstrate, both by histology and gene expression, that in the model used and under the conditions tested, CCN3 treatment was unable to alter the development of renal fibrosis. Also, TGFβ antagonism had a very limited effect on the deposition of collagen I, hence fibrosis, in the ischemic kidney.

Conclusion: Antagonism of TGFβ may not be suited as a first-line therapeutic strategy after acute severe ischemic renal injury.

6.1 Introduction

Chronic kidney disease (CKD) is a worldwide public health problem with an increasing incidence and prevalence[1]. In Western countries, age, diabetes, hypertension, obesity and cardiovascular disease are the most common risk factors for CKD. However, it is increasingly appreciated that after initial recovery from an episode of acute kidney injury (AKI), a significant proportion of patients exhibit persistent or progressive deterioration in renal function[2]. Long-term follow-up studies (4 months to 6 years) report that between 35 and 71% of patients surviving an episode of AKI had incomplete renal function as assessed by creatinine clearance or serum creatinine measurements[3]. Moreover, these patients are more likely to develop end-stage renal disease (ESRD) compared with patients without a history of AKI[2]. Thus far, there is no therapy available to halt the progression from acute to chronic renal injury.

The mechanisms underlying the progression of AKI to CKD remain to be elucidated[4]. TGFβ1 is a key molecular mediator of tissue fibrosis[5]. Elevated expression of TGFβ isoforms has been demonstrated in the glomeruli and tubulo-interstitium of patients with renal diseases and in various animal models of kidney fibrosis[6]. However, overexpression of TGFβ is also observed in AKI[7-9]. Since TGFβ is chemotactic for inflammatory cells, can influence cell growth and proliferation, and has pro-fibrotic properties by stimulation of extracellular matrix (ECM) production and auto-inducing its secretion[5], TGFβ may be an important player in the progression from acute to chronic renal injury (Table 6.1). However, given the prominent anti-inflammatory and anti-proliferative roles of TGFβ[10], therapies solely aimed at inhibiting action of TGFβ1 may be inappropriate[11]. Inhibition of proteins further downstream in the TGFβ-signalling pathway might therefore be a more subtle and better controllable therapeutic approach to attenuate progression of renal fibrosis. Several major pathways modulate or are modulated by TGFβ1 activity, such as CCN2[11]. CCN2 (previously named CTGF) is a direct downstream early response gene of TGFβ[11] and has emerged as an essential downstream mediator of many of the effects of TGFβ1, particularly those associated with fibrosis[10]. CCN3, another family member, has recently been shown to work in a yin-yang fashion with CCN2 to regulate fibrosis development in a cell culture model of diabetic nephropathy[12] (Table 6.2). In addition, *in vitro* experiments revealed that TGFβ1 treatment increased CCN2 levels while simultaneously decreased CCN3 expression, followed by an increase in collagen I transcription and translation[13]. Further, the addition of CCN3 or the overexpression of CCN3 mRNA by viral transfection blocked this pathway, preventing the pathological actions of CCN2[14]. Most recently this effect of CCN3 was substantiated in a “best” model of type 3 diabetes associated renal fibrosis[15]. These findings lead us to hypothesize that CCN3 might be a promising treatment strategy to prevent or attenuate the progression from acute-to-chronic renal injury.

Table 6.1: *Therapeutic use of anti-TGFβ antibody treatment in experimental renal pathologies.*

Anti-TGFβ	Model	Species	Dosage	Administration	Treatment	Effect
Sharma et al.	STZ-induced diabetes	Mice	300 µg/injection	i.p.	Alternate days	
Benigni et al.	Unilateral Nx + STZ	Rats	0.5 mg/kg	i.p.	3x/week	Limited progression of proteinuria and partial renoprotection.
Ziyadeh et al.	db/db	Mice	300 µg/injection	i.p.	3x/week	Reduced collagen IV and fibronectin expression, minimal mesangial matrix expansion.
Chen et al.	db/db	Mice	?	i.p.	3x/week	Reversal of GBM thickening and reduction of mesangial expansion.
Liang et al.	NEP25 podocyte ablation	Mice	3 mg/kg	i.p.	Alternate days	Decreased glomerular fibrosis, no effect on proteinuria.
Dahly et al.	Dahl S	Rats	0.5 and 5 mg/kg	i.p.	Alternate days	Reduced blood pressure, proteinuria and albinuria; reduced matrix deposition and tubular necrosis.
Murphy et al.	Dahl S	Rats	0.5 mg/kg	i.p.	Alternate days	Reduced proteinuria, glomerular injury, tubular necrosis and protein casts.
Yu et al.	Anti-Thy1 glomerulonephritis	Rats	0.5 and 5 mg/kg	i.p.	Alternate days	Dose-dependent reduction of glomerular fibrotic disease.
Mesnard et al.	Anti-GBM glomerulonephritis	Mice	150 µg/injection	i.v.	1 dose	Exacerbated renal disease.
Lavoie et al.	5/6e Nx	Rats	0.5 mg/kg	i.p.	3x/week	Anti-hypertensive effect and attenuation of vascular, glomerular and tubular damage.
Ma et al.	Unilateral Nx + PAN	Rats	0.5 and 5 mg/kg	i.p.	3x/week	Improved renal function, no effect on proteinuria, ameliorated renal sclerosis and fibrosis.
Islam et al.	CsA nephrotoxicity	Rats	3 mg/injection	i.p.	Alternate days	Improved histological damage and renal function, normalization of collagen I expression.
Ling et al.	CsA nephrotoxicity	Mice	2.5 mg/kg	i.p.	3x/week	Reduced tubular damage, attenuated inflammatory response, and a decreased tubulo-interstitial expansion and fibrosis.
Egger et al.	Adriamycin	Rats	2 mg/kg	i.p.	1x/week	No influence on collagen deposition.
Liang et al.	Adriamycin	Mice	1, 5 and 10 mg/kg	i.p.	Alternate days	Decreased expression of collagen I and fibronectin, no effect on proteinuria.
Fukasawa et al.	ATS nephritis	Rats	2 mg	i.p.	2x/week	Ameliorated proteinuria, renal dysfunction, and glomerular and tubulointerstitial ECM deposition.
El Chaar et al.	UUO	Rats	0.5 mg/kg	i.p.	Daily	Decreased apoptosis, macrophage and fibroblast infiltration and collagen in the kidney.
Wu et al.	UUO	Mice	5 mg/kg	i.p.	Alternate days	Attenuation of epithelial G2/M arrest, fibrosis and α-SMA expression.
Miyajima et al.	UUO	Rats	0.5, 2 and 4 mg/injection	i.p.	Alternate days	Decreased tubular apoptosis and fibrosis.
Guan et al.	Transplantation	Rats	5 mg/kg	i.p.	3x/week	Improved renal function, reduced proteinuria, cellular infiltration, tubular injury and fibrosis.
Basile et al.	Bilateral IRI	Rats	2.5 and 10 mg/kg	i.v.	Alternate days	Reduced expression of ECM-associated genes.
Spurgeon et al.	Bilateral IRI	Rats	0.5 mg/kg	i.v.	Alternate days	No effects on renal function, marked reduction expansion of intertitial compartiment with a reduction in interstitial cells and fibroblasts.

Table 6.2: *Therapeutic use of CCN3* ***in vitro*** *and* ***in vivo****.*

CCN3	Model	Species	Dosage	Administration	Treatment	Effect
In vitro						
Abd El Kader et al.	NIH3T3 fibroblast cells	Mouse	CCN3 overexpression			Repression of TGFβ-induced fibrogenic phenotype.
Ren et al.	Palatal fibroblasts	Rat	CCN3 overexpression			Inhibition of proliferation, promotion of fibroblast apoptosis and reduced collagen I, III and α-SMA expression.
Riser et al.	16KC2 mesangial cells	Rat	CCN3 overexpression			Reduced collagen I and CCN2 production, no effect on proliferation.
Riser et al.	16KC2 mesangial cells	Rat	CCN3 overexpression			Reduced collagen I and CCN2 production, no effect on proliferation.
Riser et al.	Dermal fibroblasts	Human	?			Inhibition of PDGF-induced responses (proliferation and MMP-1 production).
van Roeyen et al.	Mesangial cells	Human	10, 50, 100 and 150 nM			Endogenous growth inhibitor, CCN3 is regulated by PDGF-BB and –DD.
In vivo						
Marchal et al.	UUO	Mice	CCN3-/- mice			Reduction in inflammatory markers, reduced monocyte infiltration and fibrosis.
Riser et al.	ob/ob	Mice	0.604 and 6.04 µg/kg	i.p.	3x/week	Dose-dependent reduction in renal fibrosis gene activation.
van Roeyen et al.	Anti-Thy 1.1 glomerulonephritis	Rats	CCN3 overexpression			Increased glomerular expression of pro-angiogenic factors and reduced mesangial cell proliferation and matrix protein accumulation.

Renal ischemia-reperfusion injury (IRI), due to hypoperfusion after surgery, bleeding or dehydration, is a major aetiology in human AKI[4, 15] and of particular importance in the setting of kidney transplantation[16, 17]. Although relevant in the context of the AKI-CKD connection, ischemic models are rarely used to study or intervene with long-term renal decay. To this end, we previously optimized an ischemic mouse model of AKI to CKD, i.e. unilateral ischemia-reperfusion (UIRI) without contralateral nephrectomy, and confirmed development of moderate renal fibrosis, atrophy and long-term inflammation up to 12 weeks after the ischemic insult[18]. Although TGFβ-inhibition therapy proved beneficial in bilateral ischemia-reperfusion[19, 20] it was never tested in the setting of confirmed progression to long-term CKD as observed in the unilateral IRI mouse model[18]. Therefore, we here evaluated the therapeutic potential of interference with the TGFβ-pathway by means of an antibody to TGFβ on the one hand and supplementation of endogenous CCN3 on the other.

6.2 Methods

6.2.1 Animals and experimental design

All animal procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Antwerp Ethics Committee (approval number 2015-37). On average we encountered 15% mortality, mainly due to post-anaesthetic complications. Prior to surgery, animals were randomly allocated into the different treatment groups. Animals had free access to standard chow and tap water.

Surgery: Renal unilateral ischemia-reperfusion injury (UIRI) was performed as described previously[18] (see chapter 2). Briefly, male C57Bl/6J mice (10-12 weeks of age; Charles River, Saint-Germain-Nuelles, France) underwent 21 minutes of warm (36°C body temperature) UIRI, with continuously monitoring of body temperature. The right kidney was left undisturbed. Sham-operated animals received the same surgical procedure except placement of the clamp.

Treatment regimen: Animals were randomly divided over 7 groups (Figure 6.1): 1) Sham (n=10), 2) UIRI + no treatment (n=8), 3) UIRI + rhCCN3 (n=8, recombinant human CCN3, Peprotech, 5 µg/kg, daily), 4) UIRI + vehicle (n=5, PBS, daily), 5) UIRI + antibody to TGFβ (n= 10, BioXcell, 0.5 mg/kg, every other day, starting day -1[21, 22]), 6) UIRI + antibody to TGFβ (n= 8, 0.5 mg/kg, every other day, starting day 0), 7) UIRI + vehicle (n=5, PBS, every other day, starting day 0). Treatment was administered via intraperitoneal injection and initiated approximately 2 hours after UIRI, except for group 4, according to the treatment regimen described above, for 3 weeks.

Euthanasia: Animals were euthanized by exsanguination via the abdominal vena cava under ketamine-xylazine anaesthesia. Kidneys were excised, renal pole fractions were snap frozen in liquid nitrogen and transversal slices of renal tissue were

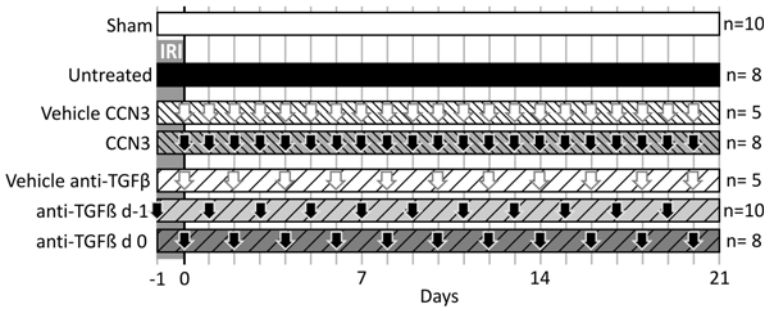


Figure 6.1: Study set-up.

fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid) and NBF (10% neutral buffered formalin) for 4 hours and 24 hours, resp., rinsed with 70% isopropanol and embedded in paraffin for histology.

6.2.2 Real-time PCR

Total mRNA is extracted from a pole section of the ischemic kidney (PureLink RNA Mini Kit; Life Technologies, Gent, Belgium) and converted to cDNA (High Capacity cDNA archive kit; Life Technologies). To quantify gene expression, qPCR, based on the Taqman fluorescence method (ABI Prism 7000 sequence detection system; Life Technologies), was used. Taqman probes and primers for *GAPDH* (Mm99999915_g1), *collagen I α1* (Mm00801666_g1), *TGFβ1* (Mm01178820_m1), *CCN2* (Mm01192931_g1), *CCN3* (Mm00456855_m1), *PAI-1* (Mm00435860_m1) and *TNFα* (Mm00443258_m1) were purchased from Life Technologies. Each gene was analysed in triplicate and the expression was normalized to the housekeeping gene *GAPDH*. Calculations were made conform the comparative Cq-method.

6.2.3 Histology

For the immunostainings, paraffin embedded 4 µm thick sections of ischemic kidney tissue were blocked with goat normal serum and incubated overnight with the primary antibody, resp. polyclonal rabbit anti- mouse collagen I antibody (T40777R, Biodesign International, Saco, Maine) and polyclonal rabbit anti-mouse Ki67 (cell cycle, Novus, Abingdon, UK). After washing, sections were incubated with a biotinylated goat anti-rabbit IgG antibody (PK-4001, Vector Laboratories, Burlingame, California) and subsequently incubated with avidin and biotinylated horseradish peroxidase (AB-complex, Vector Laboratories). A dark brown colour was developed with diaminobenzidine in the presence of 3% H_2O_2. Sections were counterstained with methyl green to visualize nuclei. The various immunostainings were quantified (see chapter 2) using the Axiovision image analysis software (Carl Zeiss, Jena, Germany). For the quantification of collagen I immunostaining, digital photographs were taken (complete slide, original magnification x100). The area % stain represents the ratio of the summed absolute areas of staining versus the total tissue. Quantification of the Ki67 immunostaining was performed on 5 random cortical and outer medulla fields per kidney (x500 original magnification). Indicated is the mean number of Ki67 positive tubular nuclei per 5 fields.

6.2.4 Western blotting

Total protein was isolated, separated through SDS-PAGE gel electrophoresis and transferred onto a PVDF blotting membrane (see chapter 2). The membrane was then incubated with the primary antibody, resp. polyclonal rabbit-anti-mouse

β-actin (4970 S, Cell Signalling, Leiden, Netherlands), polyclonal rabbit-anti-mouse F4/80 (sc-25830, Santa Cruz, Heidelberg, Germany) and rabbit-anti-mouse α-SMA (ab5694, Abcam, Cambridge, UK). Following several wash steps, the membrane was incubated with a HRP conjugated goat-anti-rabbit IgG (sc-2004, Santa Cruz). Immune complexes are detected using a chemiluminiscence kit (SuperSignal West; Fisher Scientific).

6.2.5 Statistics

All statistical analysis was performed with SPSS Statistics 22 (IBM, Brussel, Belgium). Data are presented as mean ± standard deviation, or as individual values with median. Comparisons between groups are assessed using a Kruskal-Wallis test, followed by a Mann-Whitney U test. Values of $p<0.05$ are considered significant.

6.3 Results

6.3.1 Progression from AKI to CKD in untreated and vehicle-treated animals

As depicted in Figure 6.2, renal unilateral ischemia-reperfusion (UIRI) without contralateral nephrectomy induced a significant reduction of renal mass as compared to sham-operated animals (3.29±0.29 mg/g vs. 6.04±0.58 mg/g) ($p<0.05$) 3 weeks after UIRI (Figure 6.2). The severity of the renal atrophy is equal in ischemic kidneys whether vehicle-treated or untreated. The weights of the contralateral uninjured kidney increased slightly, but significantly after UIRI in untreated animals as compared to sham (7.21±0.45 mg/g vs. 6.36±0.73 mg/g) ($p<0.05$), which

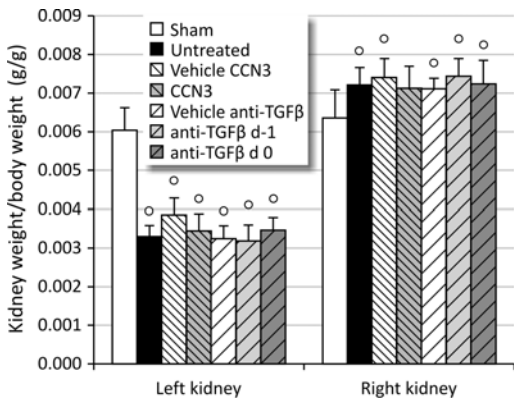


Figure 6.2: Mass of the kidneys at euthanasia, corrected for body weight. UIRI was performed for 21 minutes at 36°C, n=8 in the untreated, rhCCN3 and anti-TGFβ groups, n=4 in vehicle groups, n=10 in sham group. Animals were euthanized after treatment (3 weeks after UIRI). °: $p<0.05$ vs. Sham, *: $p<0.05$.

is in accordance with the physiological fact that the contralateral kidney becomes hypertrophic to compensate for the loss of function of the ischemic kidney (Figure 6.2). The extent of hypertrophy is similar in vehicle-treated animals and untreated animals.

Significantly elevated expression of the pro-fibrotic genes *TGFβ* (13.38±1.06 fold), *CCN2* (CTGF) (3.63±1.09 fold), *PAI-1* (20.47±4.09 fold) and *collagen I* (16.68±2.98 fold) was observed in the ischemic kidney of untreated animals as compared to the healthy kidney of sham animals (Figures 6.3 and 6.4 C). In addition, significantly elevated *CCN3* gene expression (10.07±4.62 fold) was observed in untreated animals. Vehicle treatment did not induce significant changes in the expression pattern of the fibrosis-related genes under study as compared to untreated animals. These results are supported by collagen I immunostaining (Figure 6.4 B). Significantly more collagen I was present in the kidneys of untreated UIRI animals as compared to shams (7.64±2.70% vs. 1.25±0.46%) ($p<0.05$). The amount of collagen I in the ischemic kidney was similar in untreated and vehicle-

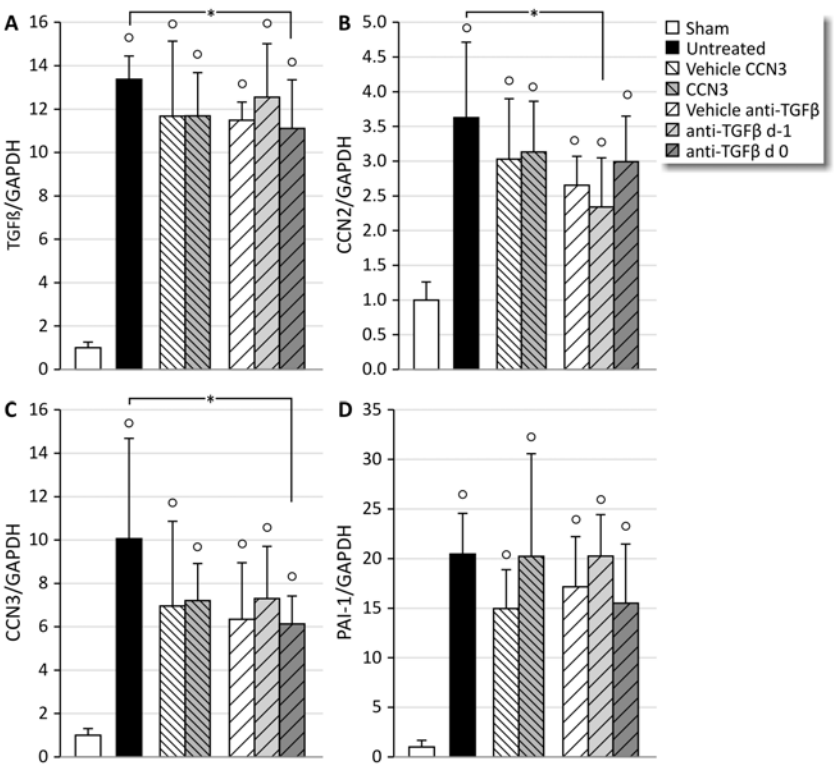


Figure 6.3: Analysis of expression of fibrosis-related genes in the ischemic kidney.
UIRI was performed for 21 minutes at 36°C, n=8 in the untreated, rhCCN3 and anti-TGFβ groups, n=4 in vehicle groups, n=10 in sham group. Animals were euthanized after treatment (3 weeks after UIRI). **A**: Quantification of *TGFβ* gene expression (qPCR). **B**: Quantification of *CCN2* (CTGF) gene-expression (qPCR). **C**: Quantification of *CCN3* gene expression (qPCR). **D**: Quantification of *PAI-1* gene expression (qPCR). °: $p<0.05$ vs. Sham, *: $p<0.05$.

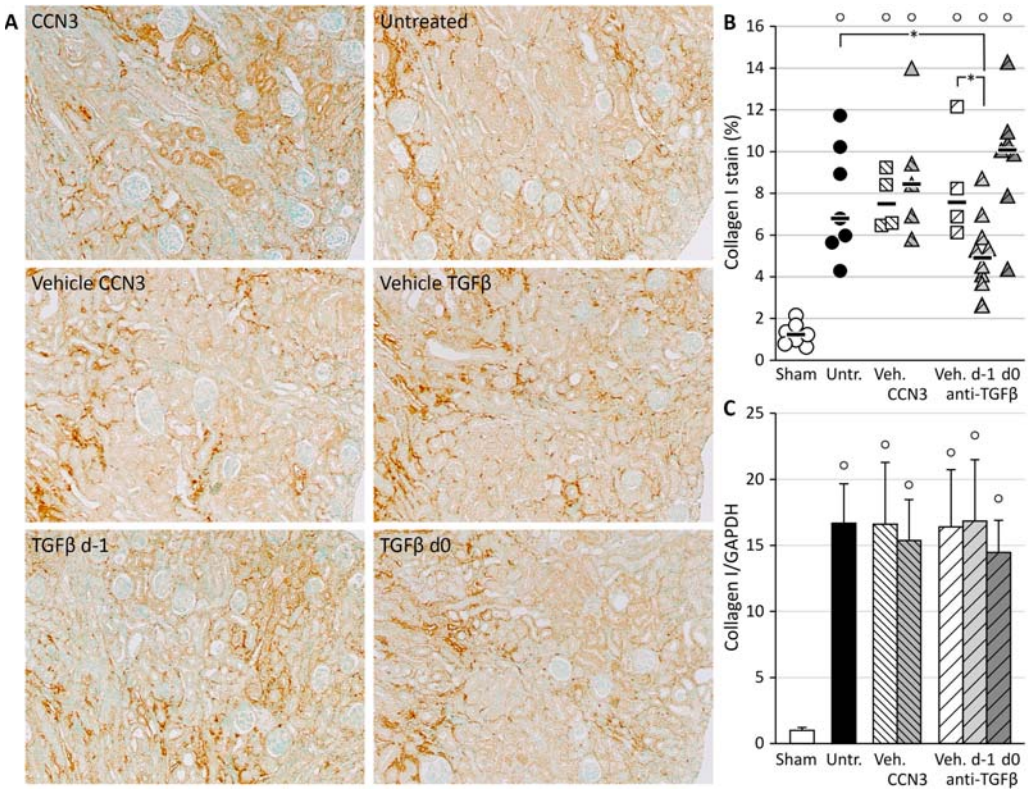


Figure 6.4: Evaluation of long-term fibrosis development in the ischemic kidneys.
UIRI was performed for 21 minutes at 36°C, n=8 in the untreated, rhCCN3 and anti-TGFβ groups, n=4 in vehicle groups, n=10 in sham group. Animals were euthanized after treatment (3 weeks after UIRI).
A: Representative images of collagen I immunostained ischemic kidney tissue (magnification: 100x).
B: Histological quantification of collagen I positivity of ischemic kidneys (x50 magnification).
C: Quantification of *collagen I* gene expression (qPCR). °: $p<0.05$ vs. Sham, *: $p<0.05$.

treated animals.

Also, significantly more activated fibroblasts (myofibroblasts), i.e. increased α-SMA protein expression, were present in the ischemic kidney of untreated animals as compared to sham (Figure 6.5 B). There was no difference in amount of activated fibroblasts in vehicle treated animals as compared to untreated animals. Furthermore, significantly elevated gene expression of the inflammatory cytokine TNFα (11.27±4.81 fold) was observed in the ischemic kidney of untreated animals as compared to sham ($p<0.05$) (Figure 6.5 A). Using Ki67 immunostaining, we evaluated the proliferative response in the ischemic kidneys. Untreated ischemic kidneys showed increased proliferation as compared to sham (115±20 vs. 25±12 Ki67 positive cells/field) ($p<0.05$; Figure 6.6). Vehicle treated groups (either rhCCN3 or anti-TGFβ treatment regimen) did not differ from untreated animals.

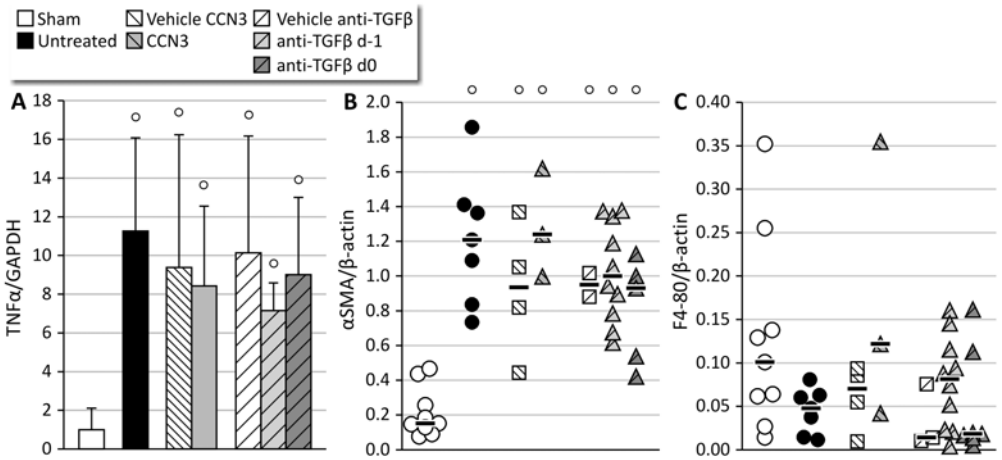


Figure 6.5: Evaluation of inflammatory and fibroblast markers in the ischemic kidneys.
UIRI was performed for 21 minutes at 36°C, n=8 in the untreated, rhCCN3 and anti-TGFβ groups, n=4 in vehicle groups, n=10 in sham group. Animals were euthanized after treatment (3 weeks after UIRI). **A**: Quantification of *TNFα* gene expression (qPCR). **B**: Quantification of F4/80 macrophage/monocyte protein expression (Western blot). **C**: Quantification of α-SMA fibroblast protein expression (Western blot). °: p<0.05 vs. Sham, *: p<0.05.

6.3.2 Administration of rhCCN3 or anti-TGFβ antibody has no distinct effect on development of post-ischemic fibrosis.

Neither recombinant human CCN3 (rhCCN3) nor antibody to TGFβ (anti-TGFβ) treatment could prevent or attenuate the atrophy of the ischemic kidney (Figure 6.2). However, rhCCN3, but not anti-TGFβ treatment prevented contralateral hypertrophy (Figure 6.2 A).

Treatment with rhCCN3 did not have a noticeable effect on the expression of the fibrosis-related genes *TGFβ*, *CCN2*, *PAI-1* or *collagen I*, nor on the endogenous *CCN3* expression (Figure 6.3 and 6.4 C). Also, no effect of rhCCN3 treatment could be observed on the amount of collagen I deposition in the ischemic kidney as compared to its vehicle group (Figure 6.4 B). Anti-TGFβ treatment, initiated 24 hours before UIRI, induced a significantly lower gene expression of *CCN2* as compared to the untreated group (2.34±0.71 vs. 3.63±1.09; p<0.05), however this was not the case when compared to its vehicle group (Figure 6.3 B). When anti-TGFβ treatment was initiated hours after UIRI, a slightly lower gene expression of *TGFβ* (11.11±2.24 vs. 13.38±1.06) and *CCN3* (6.14±1.28 vs. 10.07±4.62) was observed as compared to the untreated group (p<0.05). However, again this was not the case when compared to its vehicle group (Figure 6.3 A and C). Furthermore, anti-TGFβ treatment, initiated 24 hours before UIRI, demonstrated no effect on *collagen I* gene expression, yet, there was less deposition of collagen I in the ischemic

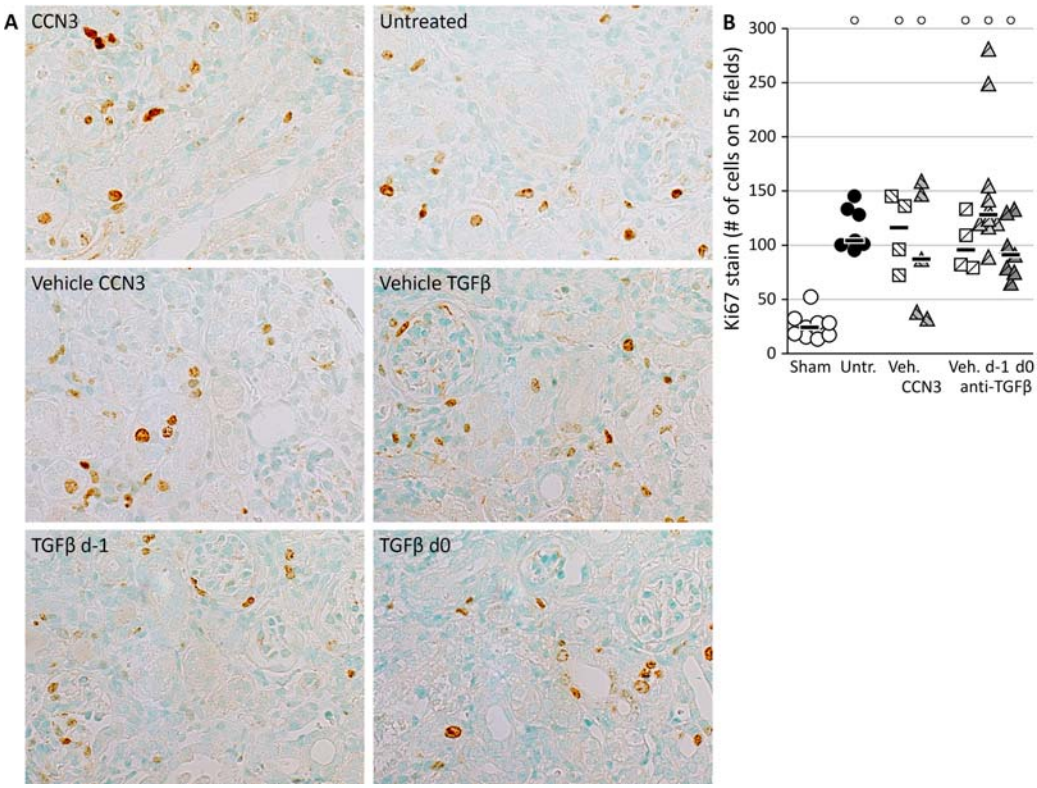


Figure 6.6: Evaluation of cellular proliferation (Ki67 immunostaining) in the ischemic kidneys.
UIRI was performed for 21 minutes at 36°C, n=8 in the untreated, rhCCN3 and anti-TGFβ groups, n=4 in vehicle groups, n=10 in sham group. Animals were euthanized after treatment (3 weeks after UIRI).
A: Representative images of Ki67 immunostained ischemic kidney tissue (magnification: 500x).
B: Histological quantification of Ki67 positivity in ischemic kidneys (x500 magnification).
°: $p<0.05$ vs. Sham, *: $p<0.05$.

kidney as compared to its vehicle group (4.98±1.90% vs. 8.34±2.68%) ($p<0.05$) (Figure 6.4 B). Treatment with anti-TGFβ, initiated hours after UIRI, did not have an effect on the collagen I gene expression nor deposition in the ischemic kidney, as compared to its vehicle treated group. The amount of activated fibroblasts, as quantified by α-SMA protein expression, was not influenced by rhCCN3 nor anti-TGFβ treatment (Figure 6.5 B).

6.3.3 Administration of rhCCN3 or anti-TGFβ antibody has no effect on the inflammatory response after UIRI

As it is known that macrophages have an important role in the pathology of renal IRI and its repair, we quantified the F4/80 monocytes/macrophages protein expression. As shown in Figure 6.5 C, treatment with rhCCN3 did not have an effect on the amount of F4/80 protein in the ischemic kidney as compared to

its vehicle treated group. Likewise, no effect was noted in animals treated with anti-TGFβ, both initiated 24 hours before and shortly after UIRI (Figure 6.5 C). The macrophage influx was accompanied by significantly elevated gene expression of the inflammatory cytokine *TNFα*. In line with the observations of the macrophage protein expression, treatment with rhCCN3 did not influence the gene expression of *TNFα* (Figure 6.5 A). Also, anti-TGFβ treatment, both initiated 24 hours before or shortly after UIRI, did not have an effect on the gene expression of *TNFα*. Also, treatment with rhCCN3 did not induce changes in the amount of proliferating cells in ischemic kidneys as compared to the untreated and its vehicle treated group (Figure 6.6). The same applies to anti-TGFβ treatment, initiated 24 hours before or hours after UIRI (Figure 6.6).

6.4 Discussion

It is becoming increasingly clear that incomplete recovery from severe AKI is an important pathway to persistent and progressive CKD and recent studies have suggested that even complete recovery from AKI is associated with an increased risk for CKD development[23]. Ishani et al. (2009) performed a large study in CKD patients without a history of AKI or ESRD and found an adjusted hazard ratio of 13.0 for patients with AKI without previous CKD[24]. Understanding the mechanisms underlying the progression from acute to chronic renal injury is the focus of recent research in the field[4], and will undoubtedly contribute to development of much needed effective therapeutic strategies.

In patients, ischemia-reperfusion is a major cause of AKI (transplantation, major surgery, hypoperfusion, stenosis[25-31]). Recently, we optimized a murine ischemic model of AKI that progresses to CKD as evidenced by development of major histopathological hallmarks[32, 33] such as tubulo-interstitial fibrosis, inflammation and renal atrophy within 3 weeks after the ischemic insult[18]. Importantly, we verified the persistent and progressive nature up to 12 weeks post-ischemia, making this a suitable model to test our hypothesis. Excessive or sustained production of TGFβ is a key molecular mediator of tissue fibrosis[5, 7, 34-36] and was therefore proposed as a therapeutic target[7, 31, 37]. In addition, TGFβ levels are also elevated immediately after renal injury because of increased secretion by tubular epithelial cells and infiltrating leucocytes, in particular macrophages[7, 38]. Therefore, TGFβ is thought to be one of the mediators underlying progression from acute to chronic renal injury. Treatment with anti-TGFβ has already proven beneficial in other models of nephropathy such as obstructive nephropathy[21, 39], diabetic nephropathy[40-42], glomerulonephritis[43] and hypertensive nephropathy[44, 45] (Table 6.1). The present study evaluated for the first time the effect of TGFβ antagonism, using an anti-TGFβ-antibody (1D11), on progression from acute-to-chronic kidney injury and the development of fibrosis in a unilateral ischemia-reperfusion model with

confirmed development to CKD. Since CCN3, a downstream and independent mediator of the effects of TGFβ has been proposed as a new therapeutic strategy, and shown to not only block, but also to reverse progression of established CDK associated with diabetes and obesity[15], we also tested the effects of this agent.

In the current study, renal atrophy is pronounced and progressive, with loss of renal mass up to 45% within 3 weeks after the ischemic insult (Figure 6.2). This is additionally accompanied by a persistent fibrotic and inflammatory process as evidenced by significant elevated collagen I gene and protein expression in the ischemic kidney (Figure 6.4), and elevated expression of the pro-fibrotic genes *PAI-1*, i.e. an important regulator of the cellular proteolytic degradation of extracellular matrix proteins and CCN2 (CTGF), i.e. a pro-fibrotic matricellular signalling molecule (Figure 6.3 B). Anti-TGFβ treatment was unable to prevent or attenuate the loss of renal mass. When antibody treatment was initiated hours after UIRI, not 24 hours before UIRI, upregulation of *TGFβ1* gene expression was slightly suppressed as compared to untreated animals, but not when compared to vehicle-treated animals (Figure 6.3 A), in contrast to reports in bilateral ischemia-reperfusion injury (BIRI)[20] and unilateral ureteral obstruction (UUO) experiments[22,47]. Whether the lack of effect of anti-TGFβ treatment on *TGFβ* gene expression is due to the higher dosages used in these experiments, a feedback mechanism to compensate for decreased TGFβ protein expression or represents failure of treatment, remains to be determined. Yet, upon anti-TGFβ pre-treatment, attenuation of fibrosis, i. e. collagen I deposition in the ischemic kidney, was observed (Figure 6.4). In addition, gene expression of the pro-fibrotic CCN2 was significantly decreased in this treatment group (Figure 6.3 B). However, when anti-TGFβ treatment was initiated after UIRI, collagen I gene expression and deposition in the ischemic kidney were unaltered by treatment (Figure 6.4) in contrast to the report of Spurgeon et al. (2004) where normal renal morphology was observed after anti-TGFβ-antibody treatment in the BIRI model[21]. Histological analysis of the ischemic kidneys of untreated animals verified a prominent expansion of the interstitial compartment after UIRI (data not shown) by infiltration of inflammatory cells and fibroblasts. It was demonstrated *in vitro* and *in vivo* that TGFβ induces proliferation of fibroblasts and accelerates the transition into the myofibroblast phenotype[48]. Also, TGFβ exerts both pro-inflammatory and anti-inflammatory properties, depending on the physiological context[49]. Indeed, Spurgeon et al. (2004) reported a marked reduction in interstitial cells in combination with a significant reduction in interstitial fibroblasts (S100A4-positivity) upon anti-TGFβ-antibody treatment initiated in the BIRI model[21]. However, in our experiment, treatment with anti-TGFβ antibody, initiated 24 hours before or 2 hours after UIRI, did not have a significant effect on the amount of cell proliferation (Figure 6.6), infiltrating fibroblast (α-SMA protein expression) (Figure 6.5 B) or macrophages (F4/80 protein expression) in the ischemic kidney (Figure 6.5 C). Even though the administration route was dif-

ferent (intraperitoneal vs. intravenous), it is important to keep in mind that spontaneous repair is inherent to the BIRI model. Long-term follow-up studies report that renal morphology returns to almost normal 2 weeks after the bilateral ischemic insult with minor signs of structural damage, microvascular rarefication and interstitial infiltration[3,50-52]. However, in this model serum creatinine returned to sham-levels 16 days after BIRI and remained stable up to 40 weeks after BIRI[3,53], indicating no long-term functional decay. On the other hand, the model of unilateral IRI is a model of ischemia-induced CKD, as mentioned before[18].

As TGFβ influences multiple systems, complete inhibition might not be eligible[11]. In renal transplantation for instance, TGFβ can exert beneficial immunosuppressive properties and plays an important role in the generation of T-regulatory cells[37]. Therefore, inhibition of proteins further downstream in the TGFβ-signalling pathway might be preferable. TGFβ is a potent regulator of CCN expression: it highly induces CCN2 mRNA expression and represses CCN3 gene expression[53]. CCN2 is described as a downstream mediator of TGFβ[7]. As CCN3 is regulated in an antithetical manner to CCN2, CCN3 is believed to be an endogenous counterpart to CCN2, acting as negative regulator of CCN2's pro-fibrotic actions[13] (Table 6.2).

In this study, we evaluated for the first time the effect of supplementation of recombinant human CCN3 on progression from acute to chronic kidney injury and the development of fibrosis in the unilateral ischemia-reperfusion injury model. Treatment with rhCCN3 did not have an effect on the renal atrophy after UIRI. Supplementation of rhCCN3 after UIRI did not have an effect on *CCN3* gene expression in this study (Figure 6.3 C). In contrast to the results of Riser et al. (2014) in the *ob/ob* diabetic mouse model, where suppression of *collagen I*, *CCN2*, *TGFβ* and *PAI-1* gene expression was observed[54], expression of these pro-fibrotic genes was unaltered in our experiment (Figures 6.3 and 6.4C). However, comparing the expression patterns of these pro-fibrotic genes in untreated animals in both models, renal pathology is far more severe after UIRI as compared to *ob/ob* mice (e.g. upregulation of *collagen I* mRNA expression: 2-fold in *ob/ob* vs. 7-fold after UIRI; for CCN2: 2-fold in *ob/ob* vs. 4-fold after UIRI). Beneficial results of experiments in experimental models of diabetes[12, 54] and glomerulonephritis[55] in combination with the negative results reported in this manuscript and in the UUO model[56] indicate that CCN3 is a potential novel therapeutic strategy in glomerular nephropathies, however, unsuited to treat progressive tubular nephropathies and fibrosis.

Overall, effect of TGFβ antagonism on the ischemic kidney and the development of fibrosis was very limited in this study. Perhaps the inflammatory response inherent to the UIRI model was too prominent, masking or impeding beneficial results of TGFβ antagonism. However, clinical trials on TGFβ inhibitors have been disappointing. In addition, very recently it was reported that fibrosis after renal injury was not reduced when the TGFβ receptor was "knocked out" in matrix-producing cells in experimental models of renal fibrosis (i.e. UUO and aristolochic

acid-induced renal injury)[57]. Thus, blocking TGFβ signalling in interstitial cells is not sufficient to attenuate fibrosis, implying that a better understanding of how TGFβ mediates response to injury is necessary to evaluate its therapeutic potential in renal fibrosis.

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Chapter 7

General Discussion

7

Chapter 7: Contents

General discussion and perspectives

The incidence and prevalence of chronic kidney disease (CKD) is still increasing worldwide[1]. In addition to the well-known risk factors, such as old age, diabetes, hypertension, obesity and cardiovascular disease[2], it is becoming increasingly clear that acute kidney injury (AKI) is an underestimated, yet important risk factor for the development of CKD[3]. It has long been thought that, because of the large regenerative capacity of the kidney, complete resolution of normal structure and function occurs after an AKI episode[4]. However, a significant proportion of AKI patients exhibit persistent or progressive deterioration of renal function[5]. Indeed, clinical evidence indicates that between 35 and 71% of patients surviving an episode of AKI had incomplete recovery of renal function as assessed by creatinine clearance or serum creatinine measurements[6]. These patients importantly contribute to the growing population of chronic kidney disease (CKD) patients. Furthermore, they are more likely to develop end-stage renal disease (ESRD) compared with patients without a history of either AKI or CKD[7-10]. Since the pathogenesis of acute to chronic renal injury involves a complex multicellular interplay within the heterogeneous renal tissue, animal models play a crucial role in unravelling these complexities towards development of new and efficient therapeutic modalities[11]. A multitude of animal models of AKI and CKD are available (Table 1.1, Chapter 1). However, animal models of AKI are used for AKI research only, and the same applies to animal models of CKD. The crosstalk between both pathologies or the progression from AKI to CKD is rarely studied. Renal ischemia reperfusion injury (IRI) is one of the most used animal models for both fundamental and therapeutic intervention studies in AKI. Despite this, few experimental studies applied IRI to study the long-term consequences of an acute ischemic insult[12-16] or attempted to intervene with the progression from AKI-to-CKD. Therefore, in this thesis we optimized a mouse model of ischemia-induced AKI progressing to CKD (Chapter 3) and evaluated the therapeutic potential of targeting pathways that are most likely involved in AKI-to-CKD progression, i.e. DNA-methylation (Chapter 4), inflammation (Chapter 5) and TGFβ (Chapter 6). Finally, from the perspective of a natural phenomenon driving renal recovery, we also evaluated the effect of contralateral nephrectomy on the long-term outcome of the ischemically-injured kidney (Chapter 3).

The mouse model of ischemia-reperfusion injury

The IRI model comes in different flavours, each with their own natural course of renal dysfunction and histopathology. Importantly, not all IRI variants are suited to study the progression from AKI to CKD and fibrosis. Bilateral ischemia-reperfusion injury (BIRI) affects total renal mass and induces a measurable increase

in serum creatinine and blood urea nitrogen (BUN), both functional hallmarks of AKI in patients[17]. However, most long-term studies with BIRI indicate that kidney function returns to almost normal 2 weeks after the bilateral ischemic insult as evidenced by serum creatinine measurements[6,18]. The pathological course of unilateral ischemia-reperfusion injury (UIRI) with immediate nephrectomy of the unharmed contralateral kidney (i.e. during the same surgery) is quite similar to BIRI. Indeed, when early death does not occur due to severe renal injury, several studies indicate recovery of renal function/histology in this model[6,18-20]. Since in both models, the animals leave the surgical procedure with injured renal tissue only, an inherent tendency to recover appears to be associated with these conditions. However, when the contralateral unharmed kidney is left in place, the natural course of the injured kidney turns out to be quite different. Early reports by Finn et al. (1984) already indicated that the response of the kidney to a temporary unilateral ischemic insult is dependent on the presence or absence of the contralateral, unharmed kidney[21]. In particular, non-removal of a healthy kidney appears to favour progressive degeneration of the injured contralateral kidney in rats[21]. Since this thesis is focussed on the AKI-CKD link, we conducted a series of experiments in mice in which the long term outcome of UIRI without contralateral nephrectomy, was evaluated. The underlying aim was to develop/optimize a relevant murine model of ischemic renal injury suitable to study AKI and CKD as well as their interrelation.

In addition, with respect to ischemic conditions in transplant biology, it is known that both warm ischemia time, i.e. duration between clamping of blood flow and prelevation of the kidney, and cold ischemia time, i.e. duration of extracorporal ischemia time, are risk factors for delayed graft function and adverse outcomes[22-24]. Since there is no systematic report on the impact of these two parameters, i.e. body temperature during ischemia and ischemia time, on the long-term outcome after UIRI, we included variations of these parameters in our studies (Chapter 3). Also, to confirm the importance of the contralateral kidney, we performed unilateral ischemia-reperfusion with contralateral nephrectomy, and evaluated whether the absence of the healthy kidney would indeed result in enhanced repair of the injured kidney in our model.

Analysis of the results of the first series of experiments confirmed our hypothesis that unilateral ischemia-reperfusion without contralateral nephrectomy progresses towards chronic injury and fibrosis. Histological analysis, by means of Masson's trichrome stain and collagen I immunostaining, reflected the expected clinically relevant histopathology of CKD[19,25,26], characterized by the presence of tubular casts and debris, atrophic tubuli, ongoing inflammation, and tubulointerstitial fibrosis. In addition, a significant increase in expression was observed for all of the fibrosis-related genes under study, i.e. *collagen I*, an extracellular matrix component; *TGFβ*, an important pro-inflammatory and cell proliferative cy-

tokine; and *CCN2* and *CCN3*, matricellular signalling molecules. Furthermore, we confirmed that renal expression of the tubular injury markers KIM-1 (Havcr1) and NGAL (Lcn2) remained increased in ischemia-induced progressive renal disease (UIRI without nephrectomy), as was previously reported in unilateral ureteral obstruction (UUO) and cisplatin-induced fibrosis[27-29]. Also, since it is known that the model of ischemia-reperfusion features a pronounced inflammatory response, we evaluated and were able to confirm that renal expression of the inflammatory cytokines *TNFα* and *IL-6* remained increased in ischemia-induced progressive renal disease further corroborating the presence of common histopathological features of CKD.

With respect to ischemic conditions, we set out to investigate to what extent alterations in body temperature during ischemia and ischemia time influence long-term fibrotic outcome in the UIRI model. Hereto we performed UIRI in a range of conditions commonly used in short-term UIRI experiments, i.e. variations in body temperature from 34°C-37°C and variations in ischemia time from 18-30 minutes. The results of these experiments indicate that both determinants influence the severity and natural course of the subsequent renal pathology that develops after ischemic AKI. However, taking into account the expression of the tubular injury markers, inflammatory cytokines and fibrosis-related genes, body temperature during ischemia should be particularly thought of as an important factor of variance within the model, and should not be taken lightly in view of the establishment of standardized procedures for use of the ischemia-reperfusion model. Ischemia time, on the other hand, is the main factor that determines the severity of the long-term fibrotic outcome. Importantly, it should be noted that all ischemia conditions tested in our study, both severe and mild, induced renal fibrosis consistently. Only 18 minutes of ischemia, which generally is a rather mild ischemia condition, did not appear to result in progressive fibrosis. Taken together, the model of unilateral ischemia-reperfusion without contralateral nephrectomy is a clinically relevant AKI model which progresses towards chronic injury and development of fibrosis with clinical features of CKD as seen in patients[19,25,26]. As such, this model allows to study the histo-(patho)logical progression of acute-to-chronic kidney injury, a topic gaining much research attention in the field.

In the second study we confirmed our hypothesis that unilateral ischemia-reperfusion with contralateral nephrectomy is a model of renal repair. Renal atrophy - a macroscopic parameter indicative of progressive fibrotic renal lesions is a reduction in renal mass[19,30] - which was observed in the first series of experiments, was not seen here. In addition, analysis of the expression of the fibrosis-related genes *collagen I*, *TGFβ*, *CCN2* (CTGF) and *CCN3* showed normalization of expression to levels similar to shams, except for *collagen I*. Furthermore, 6 weeks after UIRI with contralateral nephrectomy no upregulation of the tubular injury markers was observed in the ischemic kidney, indicating limited to no persisting tubular injury.

Also, no upregulation of the inflammatory cytokines was observed in the ischemic kidney after nephrectomy. Since inflammation after UIRI is associated with renal injury[31] and is an important mechanism underlying the progression from acute to chronic kidney injury[32], lack of cytokine upregulation further supports renal repair in this model. Studying the differences between both UIRI models can contribute to our knowledge of mechanisms underlying either renal repair or chronic injury.

Despite the intuitive simplicity of the IRI model, i.e. obstruct renal blood flow for a given period of time, researchers are often confronted with problems concerning reproducibility of the model and within-group variation. Therefore, we also set out to optimize and convey the surgical procedure of unilateral IRI. Based on an extensive literature study and the existing expertise in our lab with ischemia-reperfusion in the rat[33-35] we identified factors, aside from body temperature during ischemia and ischemia time, which are possible sources of variation of the model, such as anesthetics and analgetics, mouse gender, strain and body weight (Chapter 2).

In the AKI-to-CKD UIRI model, the presence of the healthy contralateral kidney has a dual effect; on the one hand it determines the decay of the ischemic kidney, on the other hand, it poses a shortcoming of the model as it prohibits assessment of renal function[36]. Nonetheless, in order to acquire a sense of the degree of renal injury, gene expression of tubular injury markers KIM-1 (Havcr1) and NGAL (Lcn2) were included in our analysis. Both KIM-1 and NGAL have been found to be present in the urine of men and animals after renal ischemia-reperfusion[37,38]. Furthermore, van Timmeren et al. (2007) have demonstrated that urinary KIM-1 levels in renal disease are associated with tubular KIM-1 expression[39] and Kashiwagi et al. (2014) have demonstrated that the pattern of immunohistochemical expression of NGAL in the affected renal tubules corresponds well with its mRNA expression[27]. Hence, analysing gene expression of these markers is a proper technique to assess renal tubular damage/renal dysfunction after UIRI.

Evaluation of different anti-fibrotic therapeutic strategies

Several processes play a crucial role in the development and progression of fibrosis, such as production of pro-fibrotic cytokines, chemokines and growth factors[8], a persistent inflammatory response with chronic activation of macrophages[9,40,41], long-term hypoxia from sustained loss of peritubular microvessels[9,41,42], and arrest of tubular cells in the G2/M phase of the cell cycle[43]. These pathways are currently the focus of therapeutic-driven research. In this thesis we particularly focussed on DNA-methylation, inflammation and the TGFβ-pathway, as for these there is little to no information on the long-term outcome of the kidney after ischemic AKI.

Fibroblasts, independent of their origin[44], are considered to be the main effec-

tor cells that produce extracellular matrix (ECM), in concert with cytokines and growth factors which sustain and further promote the fibrotic process and attract inflammatory cells[45]. In chronic fibrosis, fibroblasts are unable to return to their resting state and, as a consequence, continue to proliferate and produce excess ECM[46]. It was demonstrated by Bechtel et al. (2010) that this terminal fibroblast activation in fibrotic kidney disease is the result of alterations in the DNA-methylation status of specific genes, amongst which RASAL1[47]. Therefore, we hypothesized that by interfering with the DNA-methyltransferases (DNMTs), copying of the DNA-methylation pattern during proliferation of terminally activated fibroblasts could be prevented, thereby breaking the vicious circle of terminal fibroblast activation, and thus resulting in the attenuation of progressive fibrosis. Hereto, the therapeutic effect of short-term treatment (10 days) with the DNMT-inhibitor decitabine on established renal fibrosis in the early and late chronic fibrotic phase following severe UIRI was evaluated. Even though decitabine treatment induced a significant reduction in genomic DNA-methylation, progression of fibrosis was not attenuated or prevented, as evaluated by *collagen I* and *TGFβ* gene expression and quantification of Masson stain and collagen I immunostaining. Overall, the amount of positive publications on nucleoside analogues as a treatment strategy for fibrosis is limited[47-49] and taken into account the severity of adverse effects (e.g. weight loss) of this compound (even for relative short-term treatments), administration of nucleoside analogues as such seems unsuited as a first-line treatment in CKD. Evaluating the efficacy of precisely targeted DNA-demethylation strategies would therefore be invaluable to fully reveal the putative therapeutic potential of DNA-demethylation in targeting development or progression of renal fibrosis[50].

In chapter 5, the therapeutic potential of dexamethasone in preventing or attenuating progression towards chronic injury and fibrosis after an acute ischemic insult to the kidney was investigated. Inflammation plays a major role in the pathophysiology of ischemic AKI[51]. Persistence of inflammation after an acute ischemic insult may contribute to maladaptive cellular repair responses and may be an intrinsic component of progression of renal injury[4,52]. In view of the above, we hypothesized that attenuation of inflammation after acute ischemic kidney injury may be a suitable therapeutic strategy in the prevention of progression of renal injury. The use of immunosuppressive corticosteroids is somewhat controversial in treating fibrosis, as these drugs are often found ineffective in blocking the progress of fibrosis[53]. Furthermore, prolonged use of anti-inflammatory steroids can delay wound repair and increase local wound complications [54,55]. Therefore, we opted to evaluate a temporary (3 weeks) treatment regimen of dexamethasone, initiated immediately after the induction of the ischemic renal injury. The development of renal fibrosis after UIRI was attenuated by the glucocorticoid dexamethasone, evidenced by decreased upregulation of *collagen I* and *CCN2* gene ex-

pression and decreased collagen I deposition in the ischemic kidney immediately after the 3-week treatment period. Additional follow-up of the dexamethasone-treated animals, however, indicated that the beneficial effect of dexamethasone is most likely transient, as pro-fibrotic gene expression approached the levels seen in vehicle-treated animals. Most likely, treatment until complete resolution of inflammation is necessary to achieve prevention of the development of fibrosis after ischemic AKI.

A remarkable issue we have observed in these experiments was that administration of vehicle solvent alone (i.e. without effective compound) often also resulted in alterations of pro-fibrotic gene expression and collagen I deposition in the kidney (for example in Figure 5.3). This observation stresses the importance to include both an untreated and a vehicle-treated group in intervention studies to be able to correctly interpret study results. The effect of fluid administration has not been systematically investigated in experimental AKI models. However, in critically ill patients and patients at risk of an AKI episode, the importance of fluid therapy is generally recognized. Fluid management is a delicate process; as fluid overload may result in respiratory complications[56]. Moreover, the choice and amount of fluid, and assessment of fluid status are controversial[57]. In conclusion, short-term dexamethasone treatment transiently attenuated the development of fibrosis after ischemic AKI.

In chapter 6, it was investigated whether interference with the TGFβ-pathway is able to prevent the progression from acute to chronic renal injury and development of renal fibrosis after UIRI. TGFβ1 is a key molecular mediator of tissue fibrosis[58]. Elevated expression of TGFβ isoforms has been demonstrated in the glomeruli and tubulo-interstitium of patients with renal diseases and in various animal models of kidney fibrosis[59]. In addition, expression of TGFβ is also observed in AKI[60-62]. Since TGFβ is chemotactic for inflammatory cells, can influence cell growth and proliferation, and has pro-fibrotic properties by stimulation of ECM production and auto-inducing its secretion[58], TGFβ may be an important player in the progression from acute to chronic renal injury. However, given the prominent anti-inflammatory and anti-proliferative roles of TGFβ[63], therapies solely aiming to inhibit the action of TGFβ1 may be too radical[64]. Inhibition of proteins further downstream in the TGFβ-signalling pathway might therefore be a subtler and therapeutically better controllable approach to attenuate progression of renal fibrosis. Therefore, we hypothesized that antagonism of TGFβ by antibody treatment to TGFβ and supplementation of CCN3, the endogenous counter-actor of the pro-fibrotic CCN2 and downstream protein in the TGFβ-pathway, could be a promising treatment strategy to prevent or attenuate the progression from acute to chronic renal injury. Even though antibody treatment to TGFβ attenuated renal pathology in other experimental models[65-71], the effect of TGFβ antagonism on the ischemic kidney and the development of fibrosis in this experiment was very

limited. A decrease in collagen I immunostaining was observed, in combination with decreased *CCN2* gene expression. In addition, effect of TGFβ antagonism was observed only when treatment is initiated before the ischemic insult. Despite the earlier proven effects of supplementation or depletion of CCN3 on the development of fibrosis in different models of renal pathology[72,73], we have to conclude that administration of rhCCN3 showed no effect on the development or progression of renal fibrosis in the UIRI model. The results of this study are somewhat unexpected, however, they do not preclude the relevance of TGFβ as a therapeutic target. The central role of the TGFβ-pathway in the pathogenesis and progression of tissue fibrosis has been abundantly proven[62,74-78]. Therefore, it is possible that the inflammatory response in the UIRI model is overpowering the beneficial effects of interference with the TGFβ-pathway. Combining TGFβ-antagonism with inflammation-suppressing therapeutics might shed light on the matter.

Taken together, we found overall little persistent effect of any therapeutic strategy in the UIRI model. One possible explanation could be that the pathways we interfered with in these studies are not of particular importance in ischemia-induced progressive renal disease. However, genomic DNA-methylation was increased upon UIRI in the decitabine study (Chapter 4), indicating that aberrant DNA-methylation emerged after ischemic AKI as was previously reported in other models of renal fibrosis[47,79,80]. In addition, a reduction in genomic DNA-methylation and *Dnmt1* gene expression were observed immediately after the end of treatment and suppression of *Dnmt3b* gene expression persisted in the long-term, confirming therapeutic efficacy of the decitabine treatment regimen. Thus, even though the results of this experiment were negative, the therapeutic potential of altering pathological DNA-methylation patterns remains a promising new strategy. However, this would probably require gene-specific targeting.

Likewise, with respect to the role of TGFβ in the UIRI model (Chapter 6), we have consistently observed significant and persistent upregulation of TGFβ and the downstream CCN2 (CTGF). Another possible explanation could be a lack of effect of the compounds themselves. However, even though we did not repeat the previously reported in vivo experiments in other models as positive control, the doses and treatment regimens often match those previously published and were aimed to maximize therapeutic effect, whilst taking animal health into account. The transient beneficial effects of attenuation of inflammation suggest that most likely, the inflammatory response after UIRI is overpowering the other pathological pathways investigated here (DNA-methylation and the TGFβ-pathway), and thus masking or prohibiting the beneficial effect of the treatment strategies tested in this thesis. At the moment a breeding program has been initiated to acquire mice with genetically labelled proximal tubule cells. These mice will allow us to i) directly investigate effects of treatment on these cells, such as antagonism of TGFβ, and ii) investigate unexpected observations, such as the decreased pro-

liferation in the ischemic kidney in the dexamethasone experiment. Furthermore, labelled proximal epithelial cells can be undoubtedly identified in histopathological analysis and, after isolation, enable to study the cellular factors/pathways that determine the outcome after UIRI with or without contralateral nephrectomy on different omics-levels (epigenomics, genomics, transcriptomics, proteomics, metabolomics).

Overall, the current work demonstrates that unilateral ischemia-reperfusion without contralateral nephrectomy is a robust model for acute-to-chronic renal injury and is useful to study mechanisms underlying the AKI-CKD progression as well as to evaluate therapeutic strategies. The results of the various therapeutic intervention studies lead us to conclude that a single-target strategy for acute-to-chronic renal injury and fibrosis appears to be highly insufficient in this condition.

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Summary

Acute kidney injury (AKI) is an underestimated, yet important risk factor for development of chronic kidney disease (CKD). Even after initial total recovery of renal function, a significant proportion of patients exhibit progressive and persistent deterioration of renal function, rendering them more likely to progress to end-stage renal disease (ESRD). Animal models are indispensable for unravelling the mechanisms underlying this progression towards CKD and ESRD, and for the development of new strategies for its prevention and treatment. Amongst a variety of AKI causes, ischemia (i.e. hypoperfusion after surgery, bleeding, dehydration, shock, or sepsis) is a major aetiology in human AKI. Yet, although clinically relevant, the long-term therapeutic effects are rarely investigated in the rodent model of ischemic AKI. In particular, the model of unilateral ischemia-reperfusion (UIRI), despite its interesting pathological features, is a seldom used animal model for research on AKI-to-CKD and fibrosis. The aims of this thesis were threefold: (i) to develop and characterize the physiological and clinically relevant mouse model of ischemia-reperfusion as a model of acute-to-chronic kidney injury, (ii) to investigate whether progression of renal fibrosis can be halted by prevention of the copying of the pathological DNA-methylation pattern of terminally activated fibroblasts to their daughter cells, and (iii) to investigate whether attenuation of inflammation or antagonism of TGFβ is able to prevent the progression from acute-to-chronic renal injury.

In a first series of experiments in C57Bl/6J mice, we demonstrated, by both histology and gene expression, that UIRI without contralateral nephrectomy is a very robust model to study the progression from acute renal injury to long-term tubulo-interstitial fibrosis, i.e. the histopathological hallmark of CKD. Furthermore, we report that the extent of renal fibrosis, in terms of collagen I, TGFβ, CCN2 and CCN3 expression and collagen I immunostaining, increases with increasing body temperature during ischemic surgery and with duration of ischemia. In a second series of experiments, we demonstrated that when contralateral nephrectomy is performed after UIRI, renal repair is promoted.

In fibrosis, activated fibroblasts play a major role in progression of renal disease. Whereas in physiological repair their activation is only transient, it has been shown in CKD that they are terminally activated, rendering them unable to return

to their resting state once the pathological insult has been resolved. It has been demonstrated that aberrant DNA-methylation, more precisely hypermethylation of RASAL1, is one of the mechanisms underlying this hyperactivation. In this thesis, it was therefore investigated in the UIRI AKI-to-CKD model whether progression of renal fibrosis can be halted through administration of the DNA-methyltransferase inhibitor decitabine, since this would prevent the copying of the pathological DNA-methylation pattern of terminally activated fibroblasts to their daughter cells. Despite the significant reduction in genomic DNA-methylation and *Dnmt3b* gene expression upon decitabine treatment, progression of fibrosis was not attenuated or prevented. Furthermore, neither tubular injury nor inflammation in the more chronic phase of the ischemic injury was influenced by decitabine treatment. In addition to the terminal activation of fibroblasts, persistence of inflammation after a renal ischemic injury has been observed, both in experimental models and patients. Therefore, it was investigated whether attenuation of inflammation was able to halt or attenuate the progression towards CKD after ischemic AKI. Indeed, treatment with the immunosuppressive corticosteroid dexamethasone attenuated the development of fibrosis, as evidenced by reduced collagen I gene expression and immunostaining, in combination with reduced gene expression of the pro-fibrotic *CCN2* and increased expression of the anti-fibrotic *CCN3*. The effects of dexamethasone on renal fibrosis persisted up to 3 weeks after the end of treatment, as evidenced by stagnation of collagen I deposition in the ischemic kidney, in contrast to vehicle-treatment, where progression of fibrosis was observed. However, following the treatment period expression levels of the pro-fibrotic genes re-approached those of vehicle-treated injured kidneys suggesting that the effects of dexamethasone on fibrosis beyond the treatment period are temporary. Persistent treatment until near complete resolution of inflammation may therefore be required to maintain long-term effects. Since the TGFβ-pathway is involved in both AKI and fibrosis, antagonistic treatment of TGFβ is thought to be a promising strategy. Therefore, the effect of anti-TGFβ antibody treatment or supplementation of the endogenous anti-fibrotic recombinant human CCN3 on the progression of acute-to-chronic kidney injury was evaluated. Supplementation of rhCCN3 was unable to prevent or attenuate the development of renal fibrosis. Also, TGFβ antagonism had a very limited effect on the deposition of collagen I, hence fibrosis, in the ischemic kidney. Therefore, antagonism of TGFβ appears not to be suited as a first-line therapeutic strategy after acute ischemic renal injury.

In conclusion, this thesis demonstrated the development of renal fibrosis after unilateral AKI and evaluated the efficacy of new and existing therapeutic strategies. The work performed in this thesis leads to conclude that UIRI is a very robust model of renal fibrosis and that, as expected for human disease, development of a multi-factorial treatment strategy needs to be pursued.

Samenvatting

Acuut nierfalen (AKI) is een belangrijke risicofactor voor het ontwikkelen van chronisch nierfalen (CKD), waarvan de significantie onderschat wordt. Zelfs na volledig herstel van de nierfunctie, ondervindt een groot deel van de patiënten een continue, progressieve verslechtering van de nierfunctie. Deze patiënten vertonen een verhoogd risico voor het ontwikkelen van eindstadium nierfalen (ESRD). Diermodellen zijn niet alleen noodzakelijk om de mechansimen te onderzoeken die aan de basis liggen van deze progressie naar CKD en ESRD, maar ook voor het ontwikkelen van nieuwe curatieve en profylactische behandelingen. Ischemie is een belangrijke oorzaak van AKI bij de mens. Het is inherent is aan transplantatie en is een vaak voorkomende complicatie is van zware bloedingen, schock en operaties zoals bypass van de hart coronairen, aneurisma van de aorta of thoraco-abdominale procedures. Ondanks de klinische relevantie worden de effecten van therapeutica zelden op lange termijn onderzocht in het ischemie-reperfusie diermodel. Bovendien wordt het diermodel van unilaterale ischemie-reperfusie (UIRI) zelden gebruikt voor onderzoek naar de AKI-CKD connectie en fibrose, ondanks zijn interessante pathologische gelijkenissen met de patient. De doelen van deze thesis zijn daarom als volgt: (i) optimalisatie en karakterisatie van het fysiologisch en klinisch relevante muismodel van ischemie-repefusie als een model voor acuut-naar-chronisch nierfalen, (ii) het onderzoeken of de progressie van renale fibrose kan worden vertraagd door te verhinderen dat het pathologische DNA-methylatie patroon van terminaal geactiveerde fibroblasten naar de dochtercellen kan gekopieerd worden en (iii) het onderzoeken of het onderdrukken van de inflammatoire respons of de TGFβ-pathway de progressie van acuut-naar-chronisch nierfalen kan vertragen.

In een eerste reeks experimenten hebben we aangetoond dat UIRI zonder contralaterale nefrectomie een robuust model is om de progressie van een acute renale beschadiging naar chronische tubulo-interstitiele fibrose, het histopathologisch kenmerk van CKD, te bestuderen. We hebben aangetoond dat zowel de expressie van fibrose-geassocieerde genen (collageen I, TGFβ, CCN2 and CCN3), als de hoeveelheid collageen I (immunokleuring) in de ischemische nier toeneemt met stijgende lichaamstemperatuur en langere ischemietijden. In een tweede

reeks experimenten hebben we aangetoond dat wanneer de onbeschadigde, contralaterale nier wordt weggehaald na UIRI, renaal hesteld wordt bevorderd.

Bij fibrose spelen geactiveerde fibroblasten een belangrijke rol in de progressie van renaal verval. Terwijl in normaal fysiologisch herstel is de activatie van fibroblasten transiënt is, zijn bij CKD de fibroblasten terminaal geactiveerd, waardoor ze niet meer kunnen terugkeren naar hun rusttoestand. Het werd reeds aangetoond dat abberante DNA-methylatie, met name hypermethylatie van RASAL1, een van de mechanismen is die aan de basis ligt van deze terminale activatie. Daarom werd in deze thesis onderzocht of de progressie van de renale fibrose kan vertraagd worden door het toedienen van de DNA-methyltransferase inhibitor decitabine in het UIRI AKI-naar-CKD model. Op deze manier zou namelijk verhinderd kunnen worden dat het pathologische DNA-methylatie patroon van de terminaal geactiveerde fibroblasten wordt gekopieerd naar de dochtercellen. Ondanks de significante reductie in genomische DNA-methylatie en *Dnmt3b* genexpressie heeft de decitabine behandeling de progressie van de fibrose niet kunnen verminderen. Daarnaast werd er ook geen effect geobserveerd op de tubulaire beschadiging of inflammatie in de chronische fase van de ischemische beschadiging. We kunnen dus concluderen dat een behandeling met decitabine een zeer beperkt effect heeft op de progressie van nierfibrose.

Een ander fenomeen dat vaak geobserveerd wordt bij zowel proefdieren als patienten na een ischemische beschadiging van de nier is een aanhoudende inflammatoire respons. Daarom werd in deze thesis onderzocht of het onderdrukken van de inflammatoire respons na een acute ischemische insult de progressie naar chronisch nierfalen kan verminderen. We konden inderdaad vastellen dat de ontwikkeling van fibrose onderdrukt werd met de dexamethasone behandeling, gekenmerkt door een verminderde collageen I afzetting in de ischemische nier in combinatie met een verlaagde expressie van het pro-fibrotische CCN2 en een verhoogde expressie van het anti-fibrotische CCN3 gen. Het fibrose-onderdrukkend effect van de behandeling met dexamethasone bleef aanwezig tot 3 weken na het einde van de behandeling, in tegenstelling tot vehikel-behandelde dieren, waar progressie van fibrose werd waargenomen. Echter, na de opvolgingsperiode benaderden de expressielevels van de pro-fibrotische genen deze van de vehikel-behandelde nieren, wat suggereert dat het effect van een korte dexamethasone behandeling op fibrose slechts van tijdelijke aard is. Een continue behandeling tot wanneer de inflammatie (bijna) volledig verdwenen is waarschijnlijk nodig zijn om een gunstig effect op lange termijn te kunnen bestendigen.

Aangezien de TGFβ-pathway een rol speelt bij zowel acuut nierfalen als fibrose, kan antagonistische behandeling van TGFβ een beloftevolle nieuwe strategie zijn. Daarom werd in deze thesis het therapeutisch effect van een antilichaam tegen TGFβ enerzijds en supplementatie van CCN3, de endogene tegenhanger van het downstream van TGFβ agerende CCN2 (CTGF) anderzijds, op de progressie van

acuut naar chronisch nierfalen onderzocht. Supplementatie van CCN3 was niet in staat de ontwikkeling van fibrose na een episode van ischemisch acuut nierfalen te voorkomen of te verminderen. Ook de direct antagonistische behandeling van TGFβ had een zeer beperkt effect op de afzetting van collageen I en dus op de ontwikkeling van fibrose in de ischemische nier. Concluderend kunnen we stellen dat antagonistische behandeling van TGFβ niet geschikt lijken als eerstelijns therapie na een episode van ischemisch acuut nierfalen.

Samenvattend werd in deze thesis hebben we aangetoond dat fibrose ontstaat na een unilateraal ischemische insult en werd de efficientie van nieuwe en bestaande therapieën getest. Het werk in deze thesis laat toe te concluderen dat UIRI een zeer robuust model is voor renale fibrose en dat de ontwikkeling van een multi-factoriële behandeling voor CKD een na te streven doelstelling is.

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Le Clef N., Godderis L., Poels K., D'Haese P. C., Vervaet B. A. No therapeutic efficacy of untargeted DNA-demethylation therapy initiated during established, ischemia-reperfusion-induced renal fibrosis. Submitted to Nephron.

Le Clef N., Moonen L., Geryl H., D'Haese P. C., Vervaet B. A. Dexamethasone attenuates fibrosis after acute-to-chronic kidney injury. Submitted to Am J Physiol Renal Physiol.

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Nathalie