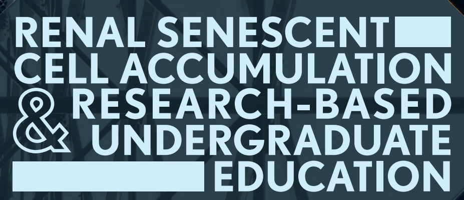

RENAL SENESCENT CELL ACCUMULATION & RESEARCH-BASED UNDERGRADUATE EDUCATION

FLORIS VALENTIJN

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Senescent cell accumulation
& Research-based undergraduate education

Senescente niercel accumulatie
& Onderzoek gebaseerd bachelor onderwijs
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
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CHAPTER 1
General introduction

Chronic kidney disease is a major health burden lacking effective treatment

Chronic kidney disease (CKD) is a common, progressive and irreversible disorder. In 2016, the global prevalence of CKD was estimated to be 13.4%. (Hill et al. 2016) In the Netherlands, the prevalence of CKD is estimated to be around 10%. (Nierstichting-a) People with CKD have an increased risk of end-stage kidney disease (ESKD) and cardiovascular disease. Lifestyle and drug interventions (e.g. ACE inhibition or angiotensin II receptor type 2 inhibition) are currently the cornerstone for treatment for CKD, but do not sufficiently prevent CKD progression. A large number of CKD patients (approximately 2000 patients per year in the Netherlands (Nierstichting-b)) develop ESKD and will need to switch to renal replacement therapy such as dialysis or transplantation. During dialysis, quality of life is poor and morbidity and mortality are high. ESRD patients who start dialysis between the ages of 45 and 65 have a 5-year survival rate of under 50% in the United States and Europe. (Robinson et al. 2016) Donor kidneys are scarce and long-term renal allograft survival is poor. The half-life of kidney transplants is approximately 9 years for deceased donor grafts and 12 years for living donor grafts. (Lamb et al. 2011) Thus, the demand for better treatment options to stop CKD progression and improve survival of a graft kidney is high.

Current therapies for CKD patients (e.g. angiotensin-converting enzyme inhibitors and angiotensin receptor blockers) show minimal efficacy on reversing disease progression. Treatment of Acute Kidney Injury (AKI) with better immunosuppressive therapies may delay the development of CKD, but do not sufficiently halt CKD progression to ESRD. New kidney function replacement therapies (e.g. bioartificial kidneys) have the potential of reducing ESRD complications and thus improve the survival of kidney patients, but cannot reverse the damage.

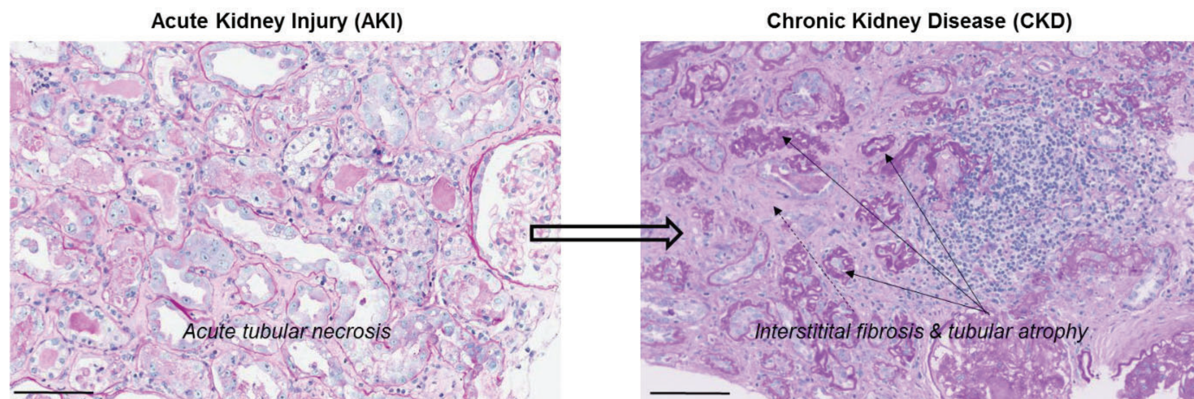

Figure 1. Representative micrographs of renal cortex tissue stained with periodic acid-Schiff (PAS) derived from kidney allograft biopsies in the acute phase (left) and chronic phase (right) after transplantation. Right picture: dotted arrow indicates area of interstitial fibrosis; continuous arrows indicate atrophic tubules. Bar = 200 µm.

AKI to CKD transition

The renal cortex consist of glomeruli and tubules, that in healthy conditions are positioned back to back. This back-to-back morphology is lost in CKD: tubules become atrophic and there is deposition of extracellular matrix, which together is called tubulointerstitial fibrosis (Figure 1). Acute kidney injury (AKI) is characterized by acute tubular damage or necrosis and an acute but transient decline in kidney function. Ischemia reperfusion injury (IRI), in which lack of blood flow is followed by re-oxygenation injury, is a major cause of AKI and subsequent CKD. Unfortunately, kidney transplantation, currently the best therapeutic option for ESRD patients, causes IRI, which contributes to CKD and poor graft survival. (Zhao et al. 2018) CKD is characterized by tubulointerstitial fibrosis with progressive loss of renal function. Tubulointerstitial fibrosis is the main predictor of loss of renal function and the final endpoint of CKD, regardless of etiology. (Liu 2011) The mechanisms underlying renal fibrosis may serve as potential targets to prevent and/or inhibit CKD progression. Improving renal

function under the given circumstances has the potential to counteract CKD complications and prevent patients from requiring kidney function replacement therapy.

Targeting cellular senescence in CKD

Recent preclinical research shows that cellular senescence (hereinafter "senescence") plays an important role in the development of chronic damage and fibrosis in the kidney. (Sturmlechner et al. 2017) Senescence is induced by various triggers, in particular DNA damage. Upon DNA damage to the tubular epithelium, part of the damaged tubular epithelial cells fail to undergo efficient DNA repair or apoptosis and become senescent (Figure 2). A senescent cell is stuck in a unique state (usually due to DNA damage) where it 1) has lost its original function, 2) ceases to divide due to a prolonged cell cycle arrest, 3) secretes a variety of pro-fibrotic and pro-inflammatory cytokines (so-called senescence associated secretory phenotype or “SASP”) and 4) does not go into apoptosis due to strong overexpression of anti-apoptotic factors. Studies in genetically modified mice show that the controlled elimination of senescent cells (i.e. “senolysis”) extends healthy lifespan and is associated with preserved kidney function. (Baker et al. 2016) The extent to which targeting senescence in CKD or transplantation leads to less fibrosis, and improvement of kidney function and graft survival is unknown.

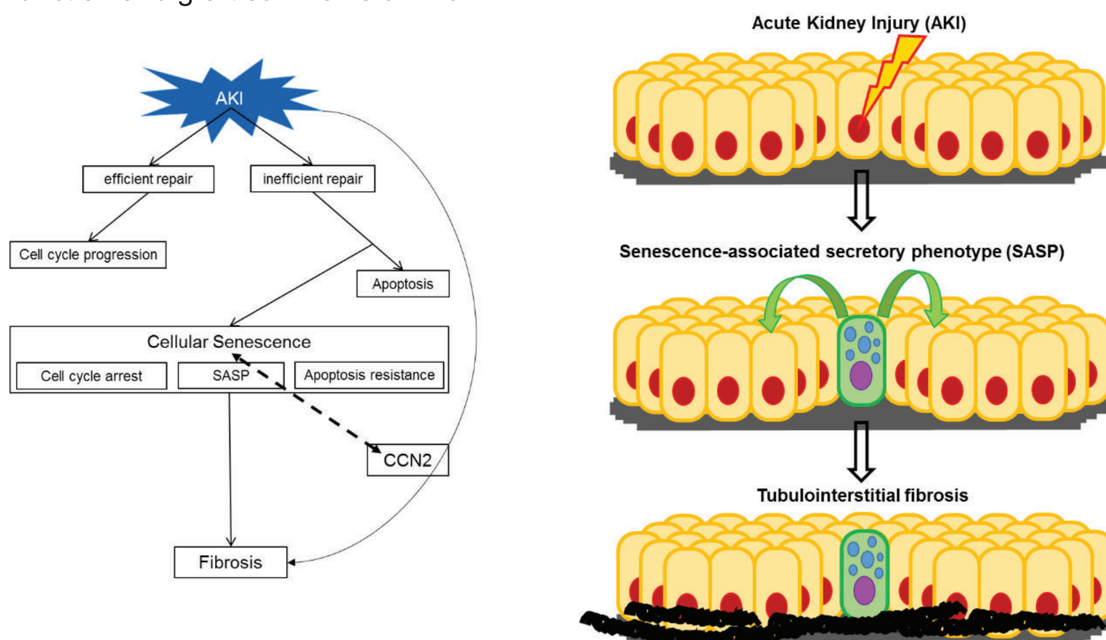

Figure 2. AKI induces renal senescence and subsequent tubulointerstitial fibrosis in case of inefficient repair and evasion of apoptosis. Dotted arrow indicated a possible link between Cellular Communication Nertwork factor 2 (CCN2) and senescence in AKI to fibrosis progression.

Promising results from senolysis experiments have sparked the interest in clinically applicable therapeutic compounds that target senescence-associated pathways. Senolysis is one of the various potential interventional approaches to target the adverse effects of cellular senescence. Other approaches include prevention of senescence, modulation of SASP (termed “senomorphics”), and stimulation of immune system-mediated clearance of senescent cells. Opposed to senolytics, therapeutic agents that modulate SASP and prevent senescence do not induce apoptosis in senescent cells, but interfere with senescent-associated intracellular pathways.

Cellular senescence and Cellular Communication Network factor 2 in CKD

The matricellular protein cellular communication network factor 2 (CCN2), previously known as connective tissue growth factor (CTGF) is implicated in AKI and contributes to CKD. CCN2 is involved in various biological processes, including cell proliferation, differentiation, adhesion and angiogenesis, and contributes to loss of kidney function by promoting inflammation and fibrosis. (Falke et al. 2014; Ramazani et al. 2018; Sánchez-López et al.

2009) CCN2 is also implicated in senescence associated pathways. It is not only a prominent SASP factor secreted in large amounts by senescent cells (Yang et al. 2010), but can also function by itself as a cellular survival factor and as inducer of cellular senescence in vitro. (Jun & Lau 2017; Wahab et al. 2007) However, the interplay between CCN2 and renal senescence remains to be elucidated (Figure 2).

Translational medicine in research based biomedicine education

Translational medicine (TM) is the (bio)medical science that focuses on the translation of (fundamental) biomedical research to the patient, and vice versa. (S and B 2009) The aim of translational medicine is to improve healthcare, for instance by bringing new scientific insights from the lab (bench) to the patient (bedside) more efficiently, and by enabling scientific research on rare diseases. (Cohrs et al. 2015) Translational medicine requires optimal cooperation and communication between different disciplines (interdisciplinary) and an efficient interaction between the main domains of an academic center: scientific research, education and clinic. TM programs have thus far mainly be focused on teaching graduate students, scientists and clinicians. Insufficient light is shed on educating the next generation of translational (physician-)scientists by focusing on undergraduate students.

Students develop academic skills and employ deep learning in a didactic framework of research-based education. (Binkley et al. 2012; Elken and Wollscheid 2016; Healey and Jenkins 2009; Kahn and O'rourke 2005) This is a research-minded, student-centered approach, based on learning by addressing relevant questions and complex authentic research tasks. Students learn academic skills, including core TM skills, by elaborating on authentic research in collaboration with experienced researchers. Active participation of undergraduate students in biomedical research, characteristic for research-based education, is underrepresented in undergraduate science programs.

Outline of thesis

Part 1:
The first part of this thesis is about the biomedical research conducted during the PhD track. It focuses on the role of cellular senescence and CCN2 in response to renal injury. **Chapter 2** describes the concept of cellular senescence in general and provides an overview of the evidence for senescence occurrence in the kidney in mainly proximal tubular epithelial cells (PTECs), and causality with organ injury, followed by a section providing a general overview of different therapeutic targeting strategies. In **Chapter 3**, the role of CCN2 in DNA damage response and subsequent senescence and fibrosis in delayed graft function and chronic allograft dysfunction following kidney transplantation, and in experimental renal IRI is elucidated. **Chapter 4** describes how CCN2 is also implicated in oxidative stress induced DNA damage response in the immediate early response of experimental IRI. **Chapter 5** shows the effect of aging on senescence and other proinflammatory processes, following toxic acute kidney injury. **Chapter 6** show the overtime activation of senescence-associated mechanisms due to aging. **Chapter 7** describes how a senescent cell state can be induced in cultured PTECs and how such an *in vitro* model may be used to screen for potential senotherapeutic drugs. In **Chapter 8**, the identification of senescent cells is discussed, followed by a detailed description of the pharmacological options for targeting senescent cells in general and ways to specifically target PTECs. **Chapter 9** describes the identification of a prominent tubular senescence phenotype in childhood cancer survivors that developed karyomegalic interstitial nephritis (KIN) after ifosfimide therapy, linking KIN to senescent cell accumulation and unexplained loss of kidney function associated with KIN.

Bridging research (part 1) and education (part 2):
In **Chapter 10,** the design for a research-based bachelor course design to provide novel insights into underlying mechanisms of the rare renal disease KIN is proposed.

Part 2:
The second part of this thesis describes educational research performed in order to evaluate the effect of this research-based course concept on student learning. **Chapter 11** describes the course design and evaluation of two intertwined interdisciplinary courses encompassing the full research cycle from formulating hypotheses to hands-on labwork. In **Chapter 12**, the course design and evaluation of a laboratory course focused on executing research in the lab is described. **Chapter 13** describes how and why providing education and performing research can be combined in a hybrid PhD track.

Finally, **Chapter 14** is an overarching discussion of the novel insights gained in this thesis, including implications and opportunities for follow-up of the biomedical research insights, and implementation and follow-up of the educational research findings.

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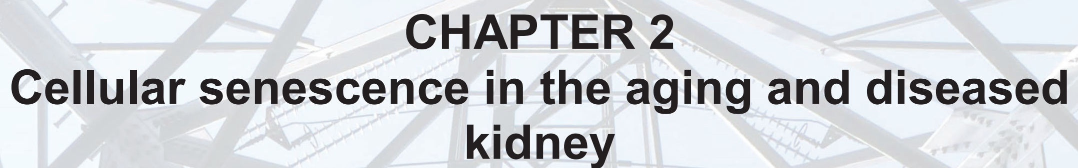

CHAPTER 2
Cellular senescence in the aging and diseased kidney

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Chapter 2. Cellular senescence in the aging and diseased kidney

Abstract

The program of cellular senescence is involved in both the G1 and G2 phase of the cell cycle, limiting G1/S and G2/M progression respectively, and resulting in prolonged cell cycle arrest. Cellular senescence is involved in normal wound healing. However, multiple organs display increased senescent cell numbers both during natural aging and after injury, suggesting that senescent cells can have beneficial as well as detrimental effects in organismal aging and disease. In the kidney, senescent cells accumulate in various compartments with advancing age and renal disease. In experimental studies, forced apoptosis induction through the clearance of senescent cells leads to better preservation of kidney function during aging. Recent groundbreaking studies demonstrate that senescent cell depletion through INK-ATTAC transgene-mediated or cell-penetrating FOXO4-DRI peptide induced forced apoptosis, reduce age-associated damage and dysfunction in multiple organs, in particular the kidney, and increased performance and lifespan. Senescence is also involved in oncology and therapeutic depletion of senescent cells by senolytic drugs has been studied in experimental and human cancers. Although studies with senolytic drugs in models of kidney injury are lacking, their dose limiting side effects on other organs suggest that targeted delivery might be needed for successful application of senolytic drugs for treatment of kidney disease. In this review, we discuss (i) current understanding of the mechanisms and associated pathways of senescence, (ii) evidence of senescence occurrence and causality with organ injury, and (iii) therapeutic strategies for senescence depletion (senotherapy) including targeting, all in the context of renal aging and disease.

Chronic kidney disease is a major health burden

The pathophysiological substrate of chronic kidney disease (CKD) is kidney injury leading to fibrosis and reduced kidney function reflected in a lower glomerular filtration rate (GFR). This might be due to the normal wear and tear associated with aging, and/or to renal disease. Chronic kidney disease (CKD) is a common, progressive and irreversible disorder. In 2016, the global prevalence of CKD was estimated to be 13.4%. (Hill et al. 2016) People with CKD have an increased risk of end-stage renal failure (ESRD) and cardiovascular disease. Lifestyle and drug interventions (e.g. ACE inhibition or angiotensin II receptor type 2 inhibition) are currently the cornerstone for treatment for CKD, but do not sufficiently prevent CKD progression. A large number of CKD patients develop ESRD and will need to switch to renal replacement therapy such as dialysis or transplantation. During dialysis, quality of life is poor and morbidity and mortality are high. ESRD patients that start dialysis between the ages of 45 and 65 have a 5-year survival rate of under 50% in the United States and Europe. (Robinson et al. 2016) Donor kidneys are scarce and long-term renal allograft survival is poor. The half-life of kidney transplants is approximately 9 years for deceased donor grafts and 12 years for living donor grafts. (Lamb et al. 2011) Thus, the demand for better treatment options to stop CKD progression and improve survival of a graft kidney is high.

The mechanisms leading to fibrosis in both renal aging and renal damage are complex and involve multiple pathological phenomena and signaling pathways, such as proinflammatory/fibrotic signaling, loss of renoprotective factors (e.g. Klotho and bone morphogenetic proteins), vascular rarefaction and oxidative stress (O'Sullivan et al. 2017). Tubulointerstitial fibrosis is the main predictor of loss of renal function and the final endpoint of CKD, regardless of etiology. (Liu et al. 2011) The mechanisms underlying renal fibrosis may serve as potential targets to prevent and/or inhibit CKD progression. Improving renal function under the given circumstances has the potential to counteract CKD complications and prevent patients from requiring renal function replacement therapy. A relatively new theory suggests involvement of cellular senescence as a central process in both early and late phases of renal aging and injury, connecting existing mechanisms of fibrosis.

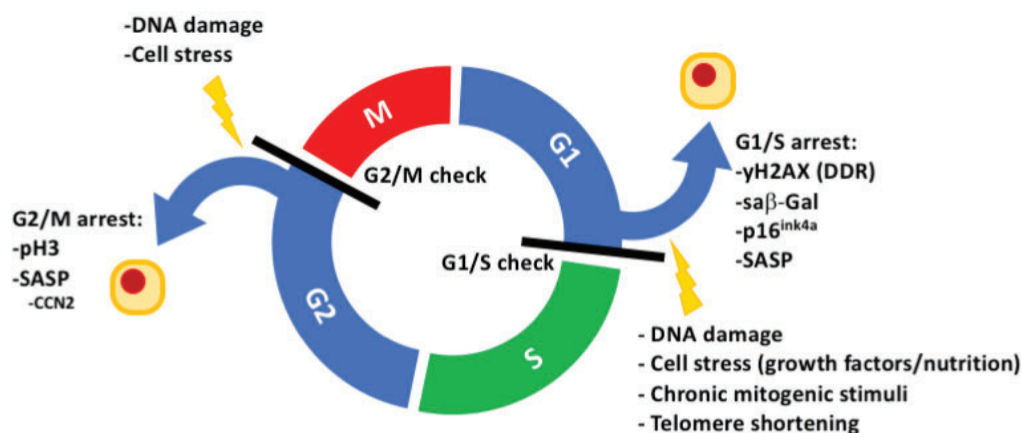

Figure 1. The cell cycle, relevant cell cycle arrest points, and their inducers and associated markers

Cellular senescence and associated pathways

Cellular senescence traditionally refers to a permanent cell cycle arrest (CCA) that can be initiated by various cellular stresses despite the presence of growth-inducing stimuli. This is opposed to quiescence, which is a temporary state of CCA due to a lack of growth stimulation (Blagosklonny 2011). Senescence has typically been linked to the G1-phase of the cell cycle, but it does occur also in G2 (see below) (Stein and Dulić 1995; Smith and Pereira-Smith 1996). Major triggers of senescence include repeated cell division and telomere shortening (also referred to as replicative senescence), and factors such as oxidative stress or genotoxic injury (stress–induced premature senescence) (Figure 1) (Campisi 1997; Toussaint et al. 2000). Additionally, a number of cytokines/growth factors

have been implicated to induce senescense, including transforming growth factor-β (TGF-β) and the matricellular protein CCN1 (Datto et al. 1995; Jun and Lau 2010; Kim et al. 2013). Mechanistically, senescent cells can act cell-autonomously by induction of CCA and non-cell-autonomously by influencing neighboring cells through proteins that are part of the senescence-associated secretory phenotype (SASP) (Xue et al. 2007).

Senescence-associated secretory phenotype
Senescent cells have a distinct secretome termed the senescence-associated secretory phenotype (SASP) (Krtolica et al. 2001; Coppé et al. 2008). Via this SASP, senescent cells affect neighboring cells by producing profibrotic and proinflammatory factors, including interleukin 6, plasminogen activator inhibitor-1 (PAI-1), TGF-β and connective tissue growth factor (CCN2/CTGF), several of which are considered markers of senescence (Table 1) (Matjusaitis et al. 2016a). Normally, this immunogenic phenotype is part of a cancer defence mechanism that enables senescent cells to be eliminated (senescent-cell clearance) by the immune system through a process known as immune surveillance (Kang et al. 2011). However, an impaired immune system due to aging, disease or immunosuppressive therapy may cause senescent cells to evade elimination and maintain their metabolic activity. Although the exact composition of the SASP secrotome is unknown, it is considered to be a driving force behind senescence-induced fibrosis of the kidney (Sturmlechner et al. 2017).

Senescent cells express a broad spectrum of features, rendering a distinct morphology *in vivo* (reviewed by ref. (Sharpless and Sherr 2015)). Typical phenotypic traits are permanent CCA, persistent DDR, SASP, epigenetic changes like senescence-associated heterochromatic foci (SAHFs), altered metabolism including increased lysosomal and proteasomal activity, and telomere shortening and dysfunction (Sharpless and Sherr 2015; Matjusaitis et al. 2016b). The most commonly used biomarkers of senescence are senescence-associated β-galactosidase (SA-β-Gal) and p16. (Dimri et al. 1995; Krishnamurthy et al. 2004; Burd et al. 2013)). Despite the availability of several markers and detection techniques (e.g. immunohistochemistry), accurate detection of senescent cells is complicated by (i) heterogeneity of senescent cells, (ii) organismal and possibly even individual variation of senescent markers and (iii) low sensitivity and specificity of senescent markers. (Gil and Peters 2006; Aan et al. 2013). Therefore, it is important to use combinations of different markers to reliably identify senescent cells.

Table 1. Features of senescent cells

Feature	Senescence marker	Method of detection
Senescence-associated secretory phenotype	Cytokines (IL-6, IL-8, GROα, GROβ, IL- α, PAI-1, CCL2/MCP-1) Growth factors (GM-CSF, G-CSF, HGF/SF, IGF, TGF-β, CCN2/CTGF) Proteases (MMP-1, -2, and -3) Non-soluble extracellular matrix proteins (collagens, fibronectin, laminin)	ELISA, FACS
DNA-associated	DNA damage markers (γH2AX, ATM, ATR, TP53, Rad17, MDC1, TIF)	IHC
	DNA synthesis (Ki67, EdU, BrdU)	Ki67 IHC; EdU or BrdU incorporation
	Telomere length/dysfunction	qPCR, FISH
	Epigenitic changes (senescence-associated heterochomatin foci)	CiA, DAPI staining, IHC
DNA-damage response	Proteasome activity	Fluorogenic peptide substrate assay
	Lysosomal activity (β-galactosidase, α-Fucosidase)	IHC, qPCR, EM, WB
	ROS	DCFDH-DA fluorometry, chemiluminescent oxygen detection reagents, FACS
Cell cycle arrest	Cyclin-dependent kinase inhibitors (p21CIP1, p16INKa, p19ARF, p14ARF, p27KIP1, p15Ink4b)	IHC, qPCR

Abbreviations: IHC = immunohistochemistry; qPCR = quantitative PCR; EM = electron microscopy; WB = Western blot

Prolonged cell cycle arrest

Prolonged CCA is a key feature of senescence and is mediated via induction of the DDR. Following DNA damage, the DDR arrests cell cycle progression at specific checkpoints, particularly the G1/S checkpoint, thereby allowing time for DNA repair to prevent that errors are replicated or passed on to daughter cells in mitosis (Jackson and Bartek 2009). Cells with repairable DNA lesions go into transient CCA (quiescence), eventually re-entering the cell cycle in case of adequate DNA damage response by the DDR machinery. In contrast, severe or irreparable DNA lesions trigger prolonged DDR signaling, resulting in apoptosis or permanent growth arrest (senescence) (Campisi and d'Adda di Fagagna 2007).

Senescence is classically linked to the G1-phase of the cell cycle (Stein and Dulić 1995; Smith and Pereira-Smith 1996). However, accumulating evidence indicates that senescence also occurs in the G2 phase, generally referred to as G2-arrest. (reviewed in ref. (Gire and Dulic 2015)). It is widely accepted that senescence associated prolonged G1- and G2-arrest occurs via late anti-proliferative DDR signaling in response to persistent DNA damage (Malaquin et al. 2015). Cell cycle progression requires activation of cyclin dependent kinases (CDKs). DDR induced prolonged CCA in senescence is characterized by accumulation of cyclin dependent kinase inhibitors (CKIs) like tumor protein p53 (TP53 or p53), p21CIP1 (p21) and p16INK4a (p16) (el-Deiry et al. 1993; Harper et al. 1993). These CKIs inactivate CDKs and block CDK-mediated phosphorylation of the retinoblastoma tumor suppressor (Rb). This causes Rb to remain attached to and thereby inhibit the transcriptionally active E2F protein complex, thus preventing G1/S transition and DNA replication, or G2/M progression and mitosis, ultimately limiting cellular proliferation (Zhang et al. 1993; Serrano et al. 1993; Jullien et al. 2013) (Figure 2).

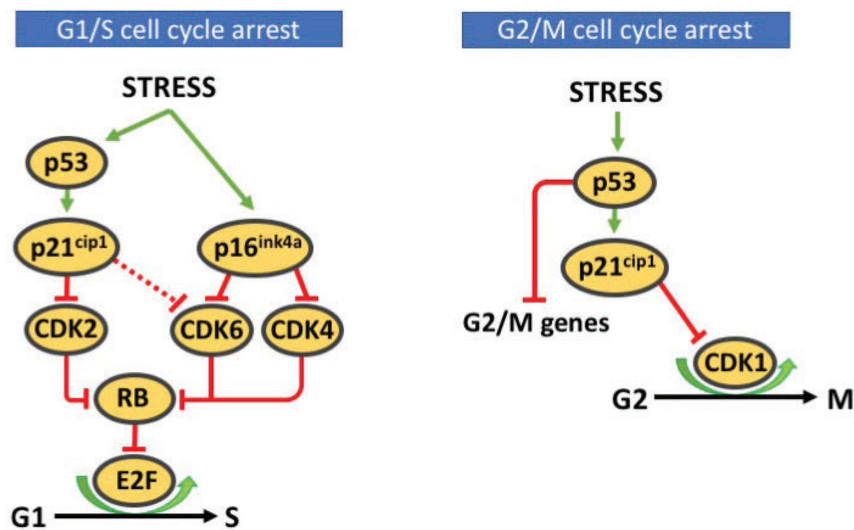

Figure 2. Cell cycle arrest signaling. Left panel: major signaling pathway associated with G1S arrest. Right panel: major signaling pathway associated with G2 M arrest

Several relevant differences between G1- and G2-arrest are postulated. Firstly, replicative senescence applies mainly to G2 arrest as telomere attrition preferentially triggers DDR at the G2/M checkpoint (d'Adda di Fagagna 2008; Jullien et al. 2013; Mao et al. 2014). Secondly, p53 mediates senescence independent of p21 in the G2 phase (Johmura et al. 2014). Thirdly, the G2/M checkpoint is not as efficient in inducing CCA as the G1-S checkpoint, which relies on strong p21 induction (Löbrich and Jeggo 2007; Cesare et al. 2013). Prolonged (i.e. senescent) G2-arrested cells express enhanced levels of profibrotic growth factors like TGF-β1 and CCN2 (Yang et al. 2010). Hence, the term "maladaptive regeneration/repair" has been postulated (Bonventre 2014a). Yet, to our knowledge the only specific marker for G2-arrest to date is histone H3 phosphorylation at Ser10 (p-H3), as cells in G2 phase show a distinct phosphorylation and IHC staining pattern of p-H3. Other markers to make a clear distinction between G1 and G2-arrested cells are lacking, and also the

possible variability of the exact composition of the SASP, is still poorly defined, thus it seems appropriate to refer to cellular senescence-like features, rather than suggesting that these identify a homogeneous phenotype (Crosio et al. 2002; Yang et al. 2010).

The apoptotic balance

The p53 protein plays an important role in controlling cell fate by mediating temporary CCA, senescence, or apoptosis in case of irreversible DNA damage. The absolute levels of p53 expression seem to be decisive in determining cell fate (Khoo et al. 2014). High levels of p53 lead to apoptosis and lower levels result in temporary CCA (Chen et al. 1996; Kracikova et al. 2013). However, the effect of p53 on senescence remains unclear. Thus far, low levels of p53 have only been described to induce temporary CCA. In case of irreversible DNA damage, p53 plays a prominent role in DDR-mediated apoptosis via induction and inhibition of pro-apoptotic and anti-apoptotic proteins, respectively (reviewed by ref. (Khoo et al. 2014)). Pro-apoptotic proteins like phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1; also known as NOXA) and p53-upregulated modulator of apoptosis (PUMA) bind and inhibit multiple mitochondrial anti-apoptotic BCL family members (Chen et al. 2005). Pro-apoptotic proteins that are directly upregulated by p53 also include BAX and p53-inducible protein 3 (PIG3) (Samuels-Lev et al. 2001).

The exact mechanisms of p53 and its impact on the pro/anti-apoptotic balance in senescence remain unclear. Strikingly, senescent IMR90 cells show upregulation of pro-apoptotic PUMA and BIM and reduced anti-apoptotic BCL-2, but this does not induce these cells to actually undergo apoptosis (Baar et al. 2017). This evasion of apoptosis could be explained by secondary factors that influence the function of p53, thereby favoring senescence over apoptosis. Such factors include the Forkhead box protein O4 (FOXO4) that binds and retains p53 to persistent nuclear foci containing DDR proteins termed ‘DNA segments with chromatin alterations reinforcing senescence’ (DNA-SCARS) that associate with PML (promyelocytic leukemia protein) nuclear bodies (Rodier et al. 2011; Baar et al. 2017). The association between DNA-SCARS and PML nuclear bodies, where many repair and chromatin-modifying proteins localize, promotes senescence-associated gene expression (Rodier et al. 2011). An anti-apoptotic role has also been assigned to CCN2 in various organs and tissues, including mesangial cells of human kidneys (Wahab et al. 2007).

Evidence for senescence in the kidney from observational studies

It is a well-established fact that senescent cell numbers are increased during physiological renal aging as well as in response to renal injury. Tables 2 and 3 list available reports to date, regarding quantification of senescent cell numbers in the human kidney during renal physiological aging and disease, and their relation with glomerulosclerosis and/or interstitial injury. To what extent senescence might be an epiphenomenon, or causally linked to clinically relevant parameters such as renal interstitial and/or glomerular disease and renal functionality, remains controversial and is further discussed below.

Senescence in renal aging

To date no published data are available that specifically address G2 arrest in aging kidneys. However, varying combinations of senescence markers, such as p16 and SA-β-Gal, have consistently been found to be increased in aged kidneys (Table 2). Thus, increased numbers of senescent cells were found in association with glomerulosclerosis and tubulointerstitial changes (Ding et al. 2001; Chkhotua et al. 2003; Melk et al. 2003; Melk et al. 2004). Additionally, in a murine model of allograft nephropathy, old donor kidneys displayed increased p16 levels, a reduced proliferation of tubular epithelial cells after transplantation, and increased susceptibility to transplantation related stress compared to kidneys from young donors (Melk et al. 2009). Also, old mice displayed a relatively higher increase of SA-β-Gal, p53 and p21, compared to young mice upon IRI (Clements et al. 2013). Together, these observations suggest an age-dependent increase in the susceptibility of the kidney to induction of senescence and concomitantly reduced regenerative capacity.

Table 2. Observations regarding quantification of senescent cell numbers in renal aging

Study	Species (comparison or range in age)	Senescence marker	Sublocalization	Association with aging
Aging				
(Krishnamurthy et al. 2004)	Mouse and rat (3 vs 28 months)	SA-β-Gal, p16, p19	Cortical tubules	Nephritis
(Chkhotua et al. 2003)	Human (21–80 years)	p16, p27	Cortical tubules and interstitium	Nephron atrophy
(Melk et al. 2004)	Human (8 weeks-88 years)	p16, p53, TGFβ1, p14	Glomeruli, tubules, arteries	Glomerulosclerosis, interstitial fibrosis and tubular atrophy
(Ding et al. 2001)	Rat (3 vs12 vs 24 months)	SA-β-gal, TGF-β1, p21	Tubulointerstitium	Interstitial fibrosis and tubular trophy
(Melk et al. 2003)	Rat (9–24 months)	p16, SA-β-gal	Glomeruli, tubulointerstitium	Interstitial fibrosis
(Sis et al. 2007)	Human (mean age 36.4 years)	p16	Glomeruli, tubulointerstitium, arteries	None
Aging in (a model of) renal transplantation				
(Melk et al. 2009)	Mouse (3 vs 18 months)	p16, Ki-67	Glomeruli, tubulointerstitium	Tubular atrophy, reduced tubular proliferation
(Clements et al. 2013)	Mouse 8–10 vs 46–49 weeks)	SA-β-Gal, p53, p21	Tubules	Mortality and kidney function, interstitial fibrosis, inflammation
(Chkhotua et al. 2003)	Human (19–60 years)	p16, p27	Glomeruli, tubulointerstitium	None

Senescence in renal disease

In response to injury, renal senescent cell numbers, indicated by varying combinations of markers mainly consisting of SA-β-Gal, p16 and p21 expression, are increased in various experimental animal models and human renal diseases (Table 3). For example, an increase of senescence markers was observed upon renal injury in rodent models including DOCA-salt-induced hypertension, streptozotocin (STZ)-induced diabetic nephropathy (DN), and cisplatin-induced nephrotoxicity (Zhou et al. 2004; Westhoff et al. 2008; Kitada et al. 2014). Increased expression of senescence markers has also been found in diseased human and mouse kidneys in hypertension, DN, CKD, delayed graft function (DGF) after kidney transplantation, and in various glomerular diseases, including membranous nephropathy, minimal change disease, IgA nephropathy (IgAN) and focal segmental glomerulosclerosis (FSGS) (Melk et al. 2005; Sis et al. 2007; Westhoff et al. 2008; Verzola et al. 2008; Liu et al. 2012a; Quimby et al. 2013). Importantly, in hypertension, DN, MG, IgAN, FSGS and also in DGF after renal transplantation, senescent cell accumulation correlated with renal histopathological changes (glomerulosclerosis, interstitial fibrosis and tubular atrophy), with renal function, and/or with proteinuria, while in IgAN, tubular expression of p16, p21 and SA-β-Gal also correlated with blood pressure (Liu et al. 2012b). In DN, tubular SA-β-Gal expression correlated with body mass index (BMI) and blood glucose, and tubular p16 was associated with BMI, LDL cholesterol and HbA1c (Verzola et al. 2008). Furthermore, the increment of renal senescent cells was associated with disease progression in IgAN (Liu et al. 2012b).

In line with these observations, therapeutic intervention in rodents reduced the accumulation of senescent cells induced by DOCA-salt, STZ and cisplatin respectively (Zhou et al. 2004; Westhoff et al. 2008; Kitada et al. 2014). Remarkably, only one study failed to show a relation between senescent cell accumulation and disease progression, where in kidney biopsies from diabetic patients with proteinuria, both p16 and SA-β-Gal were strongly

upregulated in an early phase, but did not further increase during disease progression (Verzola et al. 2008).

Taken together, these data indicate that cellular senescence is associated with detrimental effects contributing to histopathological and functional deterioration and that it can be caused by various distinct disease-associated triggers. Moreover, it appears that in various renal diseases, senescence might be prevented or overcome by the application of appropriate therapeutic strategies. Thus, disease- and therapy-induced damage might induce renal senescence and contribute to histopathological and functional changes in the kidney and even play a deleterious role in disease progression. These detrimental effects of senescence could be explained by (i) reduced organ function via SASP effects, (ii) persistence of functionally incompetent cells, and (iii) impairment of the proliferation required to replace damaged cells.

Table 3. Observations regarding quantification of senescent cell numbers in renal disease

Study	Disease or context	Species	Senescence marker	Sublocalization	Association
Renal disease					
(Westhoff et al. 2008)	Hypertension	Human	p16	Glomeruli, tubulointerstitium, arteries	Tubular atrophy, interstitial fibrosis, glomerulosclerosis and vascular damage
(Liu et al. 2012b)	IgA nephropathy	Human	SA-β-Gal, p16, p21	Tubules	Blood pressure, disease progression, glomerulosclerosis, tubular atrophy, interstitial fibrosis, inflammatory cell infiltration, matrix accumulation,
(Verzola et al. 2008)	Diabetic nephropathy	Human	SA-β-Gal, p16	Glomeruli, tubules	BMI, blood glucose, proteinuria, LDL cholesterol, HbA1c, glomerular ischemic lesions, tubular atrophy
(Kitada et al. 2014)	Diabetic nephropathy (STZ)	Mouse	SA-β-Gal, p21	Glomeruli, tubules	None
(Sis et al. 2007)	Glomerular disease (MN, FSGS and MCD)	Human	p16	Glomeruli, tubulointerstitium	Proteinuria, age, tubular atrophy and interstitial fibrosis, interstitial inflammation
(Park et al. 2007)	ADPKD	Human and rat	p21 (decreased)	Not assessed	None
(Lu et al. 2016)	Nephronophtisis	Mouse	SA-β-Gal, p16	Tubules	None
(Quimby et al. 2013)	Chronic kidney disease	Cat	SA-β-Gal	Tubules	Telomere shortening
Therapy induced					
(Zhou et al. 2004)	Cisplatin	Rat	p21, p27	Tubules	DNA repair
(Melk et al. 2005)	Delayed graft function and diseased native kidneys	Human	p16	Glomeruli, tubulointerstitium, arteries	Tubular atrophy and interstitial fibrosis (delayed graft function)
(Chkhotua et al. 2003)	CAN	Human	p16 and p27	Glomeruli, tubulointerstitium	Severity of CAN
G2-arrest					
(Bonventre 2014b)	Multiple tubular injury	Mouse	TGF-β1	Not assessed	Increased creatinine, glomerulosclerosis, tubular atrophy, interstitial fibrosis, myofibroblast proliferation, vascular rarefaction

Abbreviations: STZ = streptozotocin; MN = membranous nephropathy, FSGS = focal segmental glomerulosclerosis, MCD = minimal change disease; ADPKD = autosomal dominant polycystic kidney disease; CAN = chronic allograft nephropathy

Type and localization of renal senescent cells

In the aging or injured kidney, senescent cells have been found in both the medulla and cortex, and include tubular, glomerular, interstitial and vascular cells. Senescent cells are predominantly found in the cortex and represent mainly proximal tubular cells (PTECs). This holds true especially for G2-arrested senescent cells (Yang et al. 2010; Bonventre 2014a). Markers of senescence are also found in glomeruli of diseased kidneys, including DN, membranous nephropathy, FSGS, minimal change disease and glomerulonephritis (Melk et al. 2005; Sis et al. 2007; Verzola et al. 2008). The affected cell types in these conditions are podocytes, mesangial and/or endothelial cells, and parietal epithelial cells. Interstitial and vascular cells are among the cell types undergoing senescence in hypertension and glomerular disease (Sis et al. 2007; Westhoff et al. 2008). Thus, the type and localization of senescent cells seems to be dependent on the specific stressors involved and on the exact location of injury. In the context of aging, senescence might be induced mainly in tubular cells due to increased oxidative and cellular stress (Melk et al. 2004). Although tubular epithelium also seems to account for the majority of senescent cells in renal disease, other cell types may be affected as well, corresponding with the location of injury. For instance, kidneys with glomerular disease typically display increased expression of p16 in glomerular cell nuclei (Sis et al. 2007).

Table 4. Outcome of intervention of senescence in renal aging and after kidney injury in mice

Study	Model of kidney injury	Method of senescence intervention	Acute and long-term outcome (days after kidney injury)	Effect of senescence
(Baker et al. 2016)	Natural aging (1-year old)	p16-KO	Attenuated glomerulosclerosis	Detrimental: contributing to renal aging
(Wolstein et al. 2010)	UUO	p16-KO	Acute (10d): increased renal fibrosis	Beneficial: part of anti-fibrotic mechanism
(Megyesi et al. 2001)	IRI	p21-KO	Acute (<7d): impaired renal recovery, higher renal damage, higher mortality	Beneficial: responsible for recovery after acute ischemic renal failure
(Baisantry et al. 2016)	IRI	ATG5-KO	Acute (3d): increased renal damage, increased cell death	Beneficial: responsible for recovery after acute ischemic renal failure
			Long-term (30d): attenuated interstitial fibrosis, better kidney function	Detrimental: promoting renal fibrosis
(Lee et al. 2012)	IRI	INK4a-KO	Long term: improved kidney regeneration (14d), decreased capillary rarefaction (1-28d)	Detrimental: promoting renal fibrosis
(Braun et al. 2012)	Kidney transplantation	p16-KO	Long term (21d): reduced interstitial fibrosis, reduced nephron atrophy	Detrimental: contributing to adverse long-term allograft outcomes
(Hochegger et al. 2007)	IRI	p53 inhibition via pifithrin-α	Acute (<48h of reperfusion): reduced serum creatinine, reduced tubular necrosis score	Detrimental: contributing to acute renal failure after ischemia
(Yang et al. 2010)	IRI, AAN, UUO	p53 inhibition via pifithrin-α	Reduced fibrosis	Detrimental: promoting renal fibrosis

Abbreviations: IRI=ischemia reperfusion injury; UUO=unilateral ureteral obstruction; AAN=acute aristolochic acid toxic nephropathy; KO = genetic knock-out

Eliminating senescent cells in renal injury

Possible benefits of intervention in the process of senescence have been explored through transgenic depletion and pharmaceutical inhibition or elimination of senescent cells (i.e. senotherapy). Table 4 summarizes the data published to date regarding the depletion or inhibition of formation of senescent kidney cells in experimental kidney injury. Many mouse studies have shown already that depletion/inhibition of senescent cells via genetic

modification or pharmaceutical inhibition reduces renal injury, while senotherapeutics have been studied less extensively in the kidney and are discussed in the next section. In the context of aging, senescent cell depletion through the INK-ATTAC transgene that removes p16(Ink4a)-positive senescent cells upon drug treatment, led to attenuated glomerulosclerosis and lower blood urea nitrogen levels later in life (Baker et al. 2016). In contrast, knockdown of p16 increased renal fibrosis following unilateral ureteral obstruction (UUO), indicating a beneficial effect of senescence on tissue remodeling upon acute kidney injury (Wolstein et al. 2010). Similarly, deletion of p21 and autophagy protein 5 (ATG5, which is critically involved in tubular epithelial senescence) aggravated ischemia-reperfusion injury (IRI), leading to increased renal damage and cell death, impaired renal recovery and higher mortality (Megyesi et al. 2001; Baisantry et al. 2016). On the other hand, inactivation of p16 or ATG5 resulted in reduction of interstitial fibrosis and nephron atrophy later after IRI, indicating a protective longterm effect of inhibition of senescence on the development of fibrosis (Lee et al. 2012; Braun et al. 2012; Baisantry et al. 2016). Furthermore, pharmaceutical p53 and JNK inhibition led to reduced numbers of G2-arrested cells together with less fibrosis in a model of severe bilateral IRI (Yang et al. 2010).

In summary, the fact that senescent-cell depletion induces a maladaptive, fibrotic repair response in UUO-related obstructive injury and in in the acute phase after IRI, while it leads to less apoptosis and enhanced regenerative proliferation in the chronic phase after IRI (O'Sullivan et al. 2017) points to a beneficial effect of cellular senescence in the early phase of acute kidney injury, where it might support regeneration while, in contrast, prolonged senescence during later stages appears to have detrimental effects in more chronic renal injury, a feature demonstrated in Figure 3. Of note, similar paradoxical effects have been attributed to cellular senescence also in other conditions, including tumor biology and liver regeneration, where SASP can mediate paracrine effects of senescent cells, inducing either stemness or senescence in neighboring cells, depending on short or long duration, respectively (Ritschka et al. 2017). Obviously, this time-dependent effect on outcome will be of key importance for translational opportunities. For example, in kidney transplantation, acute as well as chronic factors may drive accumulation of senescent cells. These include tacrolimus nephrotoxicity which, in rodents, involves the production of ROS and subsequent DDR, and possibly also reduction of physiological clearance of senescent cells as a result of immunosuppressive therapies (Khanna and Pieper 2007).

The interpretation and applicability of experimental studies with reference to human kidney aging and disease is complex. Renal disease models are mostly performed in young rodents and of relatively short duration. They thereby reflect acute, rather than chronic kidney injury and may be most relevant to studying the role of senescence in acute, regenerative responses to injury (Le Clef et al. 2016). Furthermore, it should be kept in mind that most rodent experimental models of renal disease are performed in relatively young animals, potentially affecting their relevance to the aging kidney. As for understanding the role of cellular senescence in progression from acute to chronic kidney injury, and in CKD itself. The chronic IRI and multiple hit models with their cumulative, diverse stresses, and also the observations on senescent cell depletion during normal and accelerated aging will be most relevant (Bonventre 2014a; Le Clef et al. 2016; Sturmlechner et al. 2017).

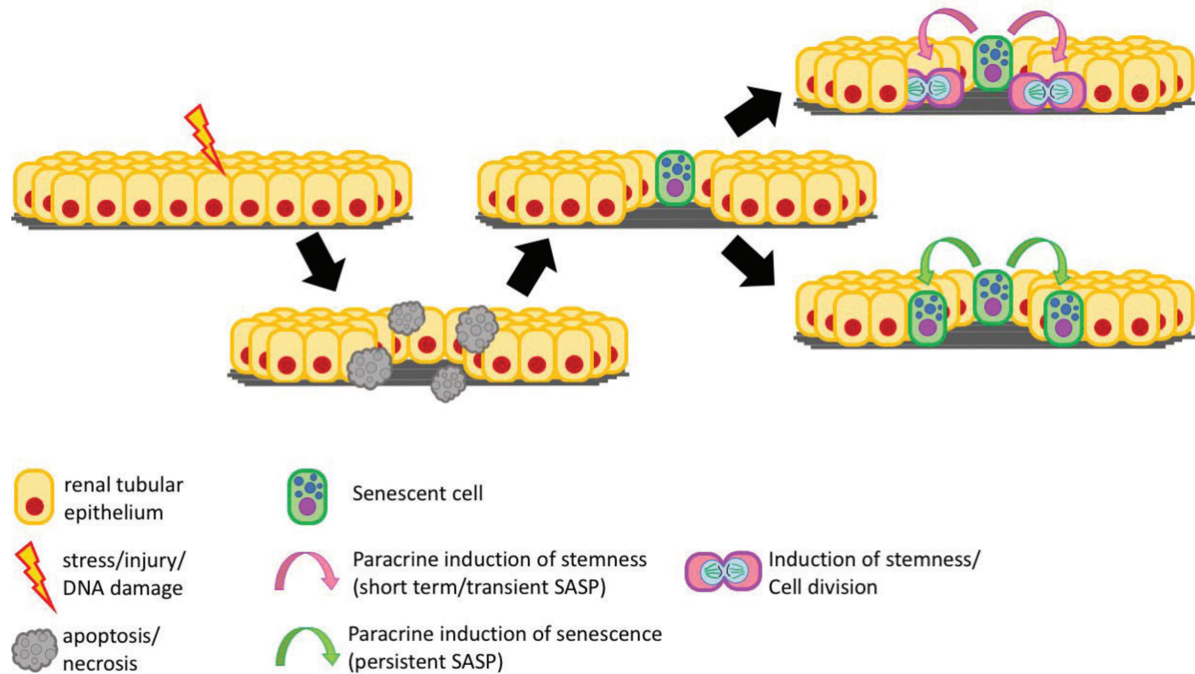

Figure 3. Paracrine effects of senescent cells in early and late phases of tissue injury.

Pharmaceutical senotherapies

Clearance of senescent cells through transgenic depletion has revealed promising beneficial effects on kidney homeostasis during aging and upon damage. For translational purposes, development of non-transgenic intervention is now of major interest and treatment with several different compounds targeting a variety of senescence-associated pathways are under study. These so-called ‘senotherapies’ can be broadly divided according to three different approaches (Childs et al. 2015):

Prevention of senescence

The first approach is to prevent senescence by limiting triggers inducing senescence through lifestyle and anti-aging drugs (e.g. caloric restriction, antioxidant agents, etc.), and by inhibiting proinflammatory pathways. The role of cellular senescence in the renal effects of successful lifestyle interventions linked with extended (healthy) lifespan, such as a healthy diet, exercise and avoidance of smoking, is not clear. However, such a role would be compatible with the known efficacy of caloric restriction in rats, where it extended healthy lifespan and also reduced oxidative DNA damage, proinflammatory factors, senescence and fibrosis in the kidney (Heydari et al. 2007; Ning et al. 2013; Xu et al. 2015). The life extending effect of caloric restriction in rodents is mediated through the MAPK and mTOR growth promoting pathways, which are linked to the SASP (Inoki et al. 2012). Antioxidants might be beneficial by reducing ROS-mediated DNA damage and thereby preventing DDR-induced senescence (Holmström and Finkel 2014). Mitochondria-targeted therapy with SS-31 (also known as Elamipretide) is thought to stabilize cardiolipin on the inner mitochondrial membrane, thereby limiting mitochondrial dysfunction (Kloner et al. 2015). Administration of this peptide reduces senescence of renal parietal epithelial cells (PECs) in aged mice, accompanied by attenuated glomerulosclerosis in treated mice compared to controls (Sweetwyne et al. 2017). The authors linked these results to the detrimental effect of mitochondrial dysfunction, indicated by the observations that PECs of treated mice displayed reduced mitochondrial damage (also observed in podocytes), reduced upregulation of the ROS-generating enzyme Nox4, and reduced senescence compared to controls. Moreover, 8 weeks of treatment with Elamipretide resulted in increased PEC density and attenuated PEC activation, but also led to reduced podocyte injury, and increased glomerular endothelial capillary integrity. Metformin also reduces the production of ROS (Algire et al. 2012).

Elimination of senescent cells

The second approach is to aim at the removal of senescent cells. So-called ‘senolytics’ interfere with anti-apoptotic and pro-survival signaling, thereby eliminating senescent cells (Zhu et al. 2015). Senescent cells have much in common with cancer cells, including similarities in metabolic activity, the DDR and activation of pro-survival pathways or inhibition of pro-apoptotic pathways (Ghosal and Chen 2013; Dörr et al. 2013). Therefore, similar strategies used for mediating apoptosis in cancer cells, are explored for removal of senescent cells. In other words, antitumor drugs are investigated for their potential as senolytic agents. ABT-263 (also known as navitoclax) is one of the most widely studied senolytic agents. It is regarded as a pan-BCL inhibitor, as it is known to cause apoptosis in various cell types by inhibiting the anti-apoptotic BCL family members, including BCL-2, BCL-xL and BCL-W (Chang et al. 2016; Yosef et al. 2016). However, navitoclax has not yet been tested for its impact on viability, phenotype, and function of kidney cells *in vitro* or *in vivo*. Other agents studied for targeting senescent cells (although also not in the kidney) include the cancer drugs quercetin and dasatinib (Zhu et al. 2015). These anti-tumor agents are known for inhibiting a broad spectrum of protein kinases and tyrosine kinases (O'Hare et al. 2005; Russo et al. 2012). *In vitro*, quercetin and dasatinib reduce expression of the anti-apoptotic regulator PAI-2 and induce apoptosis in senescent primary human pre-adipocytes and HUVECs, respectively (Zhu et al. 2015). *In vivo*, combined administration of quercetin and dasatinib leads to reduced markers of senescence (SA-β-Gal and p16) in fat and liver tissue from old mice, and to functional improvement. However, the effect of these drugs has been reported to be non-specific as it remains unclear in how far they are due to senescent cell depletion, or secondary to intervention in a multitude of unrelated pathways (Chang et al. 2016). More recently, the selective BCL-xL inhibitors A1331852 and A1155463, were identified as potential senolytics, inducing senescence-specific apoptosis in human umbilical vein endothelial cells (HUVECs) and human lung fibroblast (IMR90) (Zhu et al. 2017). As selective BCL-xL inhibitors, they may have less hematological toxicity than navitoclax.

Elimination of senescent cells may also be induced by Fisetin, a naturally occurring flavone that causes senescence-specific apoptosis in HUVECs, and by the natural product piperlongumine which caused apoptosis of senescent human WI-38 fibroblasts, no matter whether senescence was induced by either ionizing radiation, or replicative exhaustion, or ectopic expression of the Ras oncogene (Zhu et al. 2016; Wang et al. 2016). The precise mechanism of action by which fisetin and piperlongumine induce apoptosis in senescent cells still remains unclear.

Another possible target for interfering in the apoptotic balance is CCN2 which is part of the SASP. In human mesangial cells, CCN2 stabilizes the anti-apoptotic protein Bcl-2, by activation of MAPK phosphatase-1 (MKP-1), resulting in reduced apoptosis and cell survival (Wahab et al. 2007). Furthermore, in human BJ fibroblasts, CCN2 induces senescence (Jun et al. 2017). In addition, CCN2 plays a key role in the pathogenesis of kidney disease and can activate profibrotic pathways. (Falke et al. 2014; Ramazani et al. 2018) Theoretically, targeting senescence-induced CCN2 might thus have beneficial effects at multiple levels, including triggering apoptosis of senescent cells. Indeed, genetic silencing of MKP-1 using siRNA or antisense oligonucleotides was able to induce cell apoptosis in mesangial cells treated with CCN2 (Wahab et al. 2007). However, direct evidence for CCN2-induced upregulation of anti-apoptotic proteins in the context of senescence is lacking. Additionally, the tumor suppressor p53, a known target for cancer treatment, can also be targeted for halting senescence. The FOXO4-D-Retro-Inverso(DRI) peptide (also known as Proxofim) selectively induces targeted apoptosis of senescent cells (TASC) by competing with normal anti-apoptotic FOXO4-p53 binding (Baar et al. 2017). Similar to trans-genetic elimination of senescent cells discussed above, administration of FOXO4DRI cell-penetrating peptide reduced tubular senescent cell numbers, preserving and even restoring renal function in both rapidly aging trichothiodystrophy (TTD) mice and naturally aging wild type mice. As discussed above, these promising data should be seen in the context that several possible adverse effects of eliminating senescent cells have been pointed out, including impaired

cutaneous wound healing and increased fibrosis upon liver damage, underscoring the critical positive contribution of naturally occurring cellular senescence to (acute) regenerative processes (Krizhanovsky et al. 2008; Demaria et al. 2014). Another, maybe largely theoretical, consideration might be that application of senolytics in advanced disease states with high numbers of accumulated senescent cells, might lead to a cell lysis syndrome due to sudden elimination of massive numbers of senescent cells. In most known conditions, however, this seems unlikely as senescent cells only seem to make up for a small percentage of total cells.

Modulation of the SASP

SASP modulating drugs target proinflammatory signaling pathways such as NF-κB, JNK or p38 MAPK. SP600125 is an inhibitor of c-Jun N-terminal kinase (JNK), a member of the growth promoting pathway MAPK playing an essential role in inflammatory responses, including the SASP (Bennett et al. 2001). In the kidney, G2-arrested PTECs activate JNK-signaling, thereby upregulating profibrotic cytokines like CCN2 and TGF-β. As discussed above, IRI after treatment with a SP600125 (a pan-JNK inhibitor) leads to lower numbers of senescent cells in G2-phase and reduces fibrosis (Yang et al. 2010). The proinflammatory pathways of the SASP can also be targeted by the mTOR inhibitor rapamycin or the AMPK activator metformin, leading to prevention of senescence (Iglesias-Bartolome et al. 2012; Noren Hooten et al. 2016). Although SASP modulation is expected to limit detrimental paracrine effects of prolonged presence of senescent cells, SASP modulation could also lead to harmful side-effects. First, SASP factors are not senescence-specific but are upregulated in a broad spectrum of different pathways, and intervention could thus interfere with vital processes. Secondly, SASP modulation could impede immune surveillance, and hamper elimination of senescent cells.

Stimulating senescence

Strikingly, certain triggers inducing senescence ultimately limit, rather than stimulate fibrosis in various organs and conditions. For instance, the matricellular protein CCN1 (also known as CYR61) induces senescence of fibroblasts in cutaneous wound healing and of liver myofibroblasts in hepatic injury, and its expression is linked to reduced fibrosis (Jun and Lau 2010; Kim et al. 2013). Therefore, CCN1-induced senescence might be used as therapy to limit fibrosis. Although its role in the kidney remains unclear, CCN1 expression is also elevated in several senescence-associated human pathologies beyond the liver, including atherosclerosis, which suggests a possible role in age-related diseases (Littlewood and Bennett 2007). On the other hand, the expression of CCN2, another, closely related, member of the CCN protein family, is associated with fibrosis of different organs, including the liver (Krizhanovsky et al. 2008). This indicates that the dual role of senescence on fibrosis is dependent on varying triggers.

Targeting strategies

Currently available senolytic drugs have several limitations, the major challenge being to target the right cells at the right time. The clinical applicability of systemically administered senolytic drugs is impeded by dose limiting side-effects on other organs. For instance, navitoclax caused dose-dependent thrombocytopenia, due to inhibition of BCL-xL in platelets resulting in platelet apoptosis (Mason et al. 2007; Zhang et al. 2007; Wilson et al. 2010). Theoretically, such limitations of senolytic drugs may be overcome by targeted delivery to specific organs and cell types or by specific targeting of senescent cells.

Targeted accumulation of senolytic agents in the kidney might be achieved using nanomedicines (i.e. nanoparticulate carriers) like conjugates and liposomes. Delivery of therapeutic of such functionalized compounds should enable high enough drug concentrations where needed, while sharply limiting systemic drug exposure and thereby side effects. The delivery of an effective senolytic drug dose inside targeted cells would be accomplished by internalization of the functionalized compound triggered by binding to the cell surface receptors specifically expressed by certain cell types in the kidney (Falke et al.

2015). There is a variety of compounds that can carry therapeutic agents, including glucosamine conjugates, poly vinylpyrrolidone (PVP)-derivatives, lysozyme conjugates and other low molecular weight protein (LMWP)carriers, and also the targeting peptide (KKEEE)3 K (Franssen et al. 1993; Kamada et al. 2003; Lin et al. 2013; Falke et al. 2015; Wischnjow et al. 2016). The kidney is eminently qualified for such targeting strategies, as kidney cells possess several relatively specific cell surface receptors, including LDL receptor-related protein 2 (commonly known as megalin/ cubulin), integrin α8, E-selectin, podocyte-specific antigen and folate receptor 1α (Falke et al. 2015).

Targeting of PTECs, which display features of senescence with aging and pathology, may be the most promising approach, as these cells are highly active in accumulating compounds from the filtered urine via their receptors in the luminal brush-border (Christensen et al. 2012). Among these, megalin and folate receptor 1α have been successfully employed for targeting the proximal tubular epithelium (Prakash et al. 2008; Shillingford et al. 2012). Another promising approach to target PTECs is the therapeutic use of small interfering RNA (siRNA). In mouse models of ischemic and cisplatin-induced acute kidney injury, intravenous administration of siRNA against p53 reduces cellular p53 and attenuates p53-mediated apoptosis (Molitoris et al. 2009). The validity of this approach is currently being addressed in a phase 3 clinical trial testing p53 siRNA for preservation of kidney function after major cardiothoracic surgery.

Targeting other renal cell types (i.e. mesangial cells, endothelial cells and podocytes) is theoretically possible through targeting of different surface receptors, using nanoparticles, or with liposomes (Tuffin et al. 2005; Choi et al. 2011; Kamaly et al. 2016). For instance, mesangial cells, that predominantly become senescent as a consequence of hypertension and diabetic nephropathy, have been targeted through integrin α8 and Thy 1.1 using liposomes in rodents (Scindia et al. 2008; Suana et al. 2011).

Summary and future

In the kidney, both G1- and G2-arrested senescent cells accumulate with advancing age and renal disease in various areas, particularly in cortical proximal tubular cells. In aging mice, genetic clearance of senescent cells leads to better preservation of kidney function and morphology. In diseased kidneys, there is a time-dependent effect of senescence on the development of fibrosis, with early beneficial effects and detrimental long-term consequences. Studies examining therapeutic options for depletion of senescent cells in humans are complicated because of dose limiting side effects on other organs. Therefore, specific targeting of senescent cells in the kidney might be essential.

Further research is also needed to understand in how far accumulation of renal senescent cells in renal aging and disease (i) is due to increased production or reduced clearance via immune surveillance, (ii) is a direct cause or a consequence of progressive organ injury and organismal aging and (iii) if elimination of these cells (at the right time) improves kidney function and histopathological changes. Novel therapeutic approaches for elimination of senescent cells *in vivo*, including targeting strategies to overcome dose limiting side effects on other organs, will be important to find answers to these questions.

Chapter 2. Cellular senescence in the aging and diseased kidney

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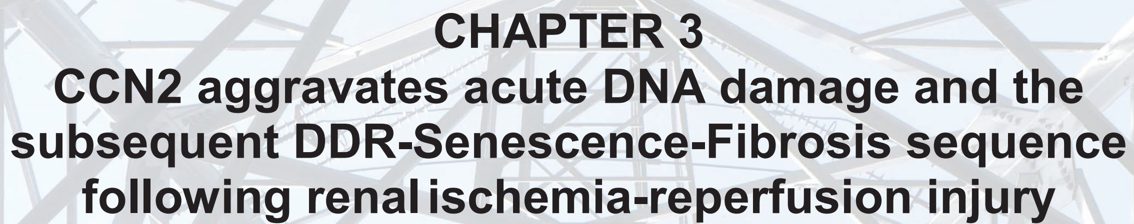

CHAPTER 3
CCN2 aggravates acute DNA damage and the subsequent DDR-Senescence-Fibrosis sequence following renal ischemia-reperfusion injury

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Chapter 3. CCN2 aggravates the DDR-Senescence-Fibrosis sequence following renal IRI

ABSTRACT

Chronic allograft dysfunction with progressive fibrosis of unknown cause remains a major issue after kidney transplantation, characterized by ischemia-reperfusion injury (IRI). One hypothesis is that "spontaneous" progressive tubulointerstitial fibrosis following IRI is driven by cellular senescence evolving from a prolonged, unresolved DNA damage response (DDR). Cellular communication network factor 2 (CCN2, formerly called CTGF), an established mediator of kidney fibrosis, is also involved in senescence associated pathways. We therefore investigated the relation between CCN2 and cellular senescence. Tubular CCN2 overexpression was associated with DDR, and with loss of kidney function and tubulointerstitial fibrosis in early and late phase human kidney allograft biopsies. Consistently, CCN2 deficient mice developed reduced senescence and tubulointerstitial fibrosis in the late phase, i.e. 6 weeks after IRI. Moreover, tubular DDR markers and plasma urea were less elevated in knockout than in wild type mice. Finally, CCN2 administration or overexpression in epithelial cells induced upregulation of tubular senescence-associated genes including p21, while silencing of CCN2 alleviated DDR induced by anoxia-reoxygenation injury in cultured PTECs. Together, our observations indicate that inhibition of CCN2 can mitigate IRI-induced AKI, DNA damage, and the subsequent DDR-Senescence-Fibrosis sequence. Thus, targeting CCN2 might help to protect the kidney from transplantation associated post-IRI chronic kidney dysfunction.

INTRODUCTION

Chronic Allograft dysfunction (CAD) due to tubulointerstitial fibrosis is the leading cause of renal allograft loss and may develop without identifiable cause, despite adequate immunosuppression. Ischemia-reperfusion injury (IRI), in which ischemia is followed by re-oxygenation injury, is the main cause of acute kidney injury (AKI) associated with transplantation surgery and considered a major cause of CAD. CAD is defined by progressive tubulointerstitial fibrosis, functional decline and eventual loss of the kidney graft. (Li & Yang., 2009; Situmorang & NS., 2019) Short-term allograft survival has improved substantially over the past decades, but long-term outcomes remain poor. (Lamb et al., 2011; Wekerle et al., 2017) Given the high demand for suitable donor kidneys and their limited availability, prolonging survival of allografts is of utmost importance.

Cell cycle arrest (CCA) is a physiological process and essential for repair of DNA damage immediately after injury. (Branzei & Foiani, 2008) Unsuccessful DNA repair either results in apoptosis or alternatively, in cellular senescence with persistence of a DNA damage response (DDR). Cellular senescence has been defined as a state of persistent, irreversible CCA that may lead to detrimental adverse tissue remodelling via dedifferentiation, and by the associated phenomenon dubbed the Senescence-Associated Secretory Phenotype (SASP) characterized by the secretion of pro-inflammatory and pro-fibrotic stimuli. (de Keizer, 2017; Gire & Dulic, 2015; Gorgoulis et al., 2019; Wang et al., 2017) In the kidney, cellular senescence is associated with the development of tubulointerstitial fibrosis following transplantation surgery-induced IRI. (Ferlicot et al., 2003; Günther et al., 2017; McGlynn et al., 2009; Melk et al., 2004) Interestingly, in experimental ageing, elimination of senescent cells preserved kidney function. (Baar et al., 2017; Baker et al., 2016) In unilateral IRI, treatment with the “senolytic” agents dasatinib and quercetin reduced tubulointerstitial fibrosis. (Li et al., 2021)

Cellular communication network factor 2 (CCN2), previously known as connective tissue growth factor (CTGF), is a matricellular protein involved in IRI and fibrosis. It contributes to tubulointerstitial fibrosis in CAD and has successfully been targeted to limit fibrosis. (Falke et al., 2014) CCN2 is involved in various processes, including cell proliferation, differentiation, adhesion and angiogenesis, and promotes inflammation and fibrosis. (Falke et al., 2014; Ramazani et al., 2018; Sánchez-López et al., 2009) CCN2 is also implicated in cellular senescence. It is not only a prominent SASP factor, but can also function by itself as a cellular survival factor and as inducer of cellular senescence *in vitro*. (Jun & LF., 2017; Wahab et al., 2007; Yang et al., 2010)

Observing co-localization of CCN2 with DDR and senescence markers in an early and late biopsy of the same human kidney allograft led us to hypothesize that CCN2 could contribute to senescent cell accumulation and tubulointerstitial fibrosis during CAD development following IRI. To elucidate this hypothesis, we investigated the relation between CCN2 and cellular senescence in early and late phase human kidney allograft biopsies, in a bilateral IRI model in CCN2 knockout mice, in CCN2-treated mice and cultured cells and finally, in an anoxia-reoxygenation model in CCN2 silenced cultured cells.

MATERIALS & METHODS

Human kidney specimens

Tissue sections were derived from routine clinical kidney allograft biopsies at the UMC Utrecht. Follow-up biopsies were taken 7 days and 8 months after transplantation from a 55-year old male, with a creatinine at biopsy of 1498 µmol/L and 779 µmol/L, respectively. Indication biopsies for delayed graft function (DGF) 6-8 days after transplantation and protocol biopsies 5-29 years after transplantation served as early and late phase specimens. All transplants were derived from non-living donors. All patient samples were leftover body material from clinical biopsies and were collected according to the institutional ethical guidelines. Samples were anonymized, which allowed us to use this redundant tissue for research purposes, without requirement of informed patient consent. (van Diest PJ., 2002)

Animals

All animal procedures were performed according to the ARRIVE guidelines and with consent of the local Experimental Animal Ethics Committees. (Kilkenny et al., 2012) Generation of tamoxifen inducible CCN2 full knockout mice is described elsewhere. (Fontes et al., 2015) In brief, CCN2$^{Flox/Flox}$ mice were crossbred with ROSA26CreERT2 mice (Gt(ROSA)26Sor$^{tm(cre/ERT2)Tyj}$/J, The Jackson Laboratory, Maine, USA), both on a C57Bl6/J background. For intraperitoneal injection, tamoxifen citrate was dissolved in Corn oil (Sigma Aldrich, Missouri, USA) in a 10mg/ml concentration. In order to induce recombination, 12-14 week old male mice received four intraperitoneal injections on alternate days over a course of 7 days with 100µl tamoxifen-corn oil solution. Littermates injected with vehicle Corn oil using the same regimen were used as control mice (referred to as WT). Treatments were performed in a blinded fashion. After the last injection, a 14-day washout period was followed by the IRI operation.

Ischemia-reperfusion injury model

IRI was executed as previously described. (Kinashi et al., 2017) Renal pedicles were located through an abdominal midline incision and bilaterally clamped for 25 minutes with neurovascular clamps. Clamping and subsequent reperfusion associated colour changes were visually confirmed. IRI mice without colour changes were excluded. Sham operated mice underwent the same procedure without the pedicle clamping. Mice were anesthetized with 2% isoflurane and body temperature was maintained at 37°C. The operator was blinded for treatment group. After 3 days or 6 weeks, mice were euthanized by lethal dose Ketamine-Xylazine-Acepromazine injection and plasma and organs were collected and stored at -80°C.

Cell cultures

Mouse primary PTECs (RPTECs, C57-6015, Cell Biologics, Chicago, IL, USA) were cultured as described previously using 100ng/mL recombinant CCN2 (Peprotech, 120-19, Cranbury, NJ, USA) and a GasPak EZ Anaerobe Container System with Indicator (cat. no. 26001, BD Biosciences, S. Plainfield, NJ, USA). (Eleftheriadis et al., 2021) Etoposide 1.5µg/mL (E1383-25MG, Sigma-Aldrich) was used as positive control for senescence. Gene silencing was performed using predesigned siRNA corresponding to CCN2 (siRNA ID MSS274358, Thermo-fisher, Massachusetts, USA) according to the manufacturer's protocol. In short, sub-confluent cells were transfected in Opti-MEM reduced serum medium (Invitrogen) for 24 hours with 5 ng/ml siRNA using 50 nM Lipofectamine RNAi-MAX (Invitrogen), followed by 24h incubation in 20% FBS medium and 24h incubation in serum-free medium. Controls were non-transfected cells treated with lipofectamine vehicle. Cells were subjected to 24 hours of anoxia and 2 hours of reoxygenation.

HK-2 human tubular epithelial cell line was cultured in DMEM media supplemented with 5% FBS. Semiconfluent HK-2 cells were infected with lentiviruses bearing a CMV promoter driven human CCN2 cDNA construct (LPP-L5140-Lv105) or control vector (GeneCopoeia; Rockville, MD, USA) using 5µg/ml Polybrene in DMEM/5%FBS for 24 hrs. Following a 24-hour recovery period, stable cultures were selected in 5µg/ml puromycin containing media. To maintain selection pressure, media were changed every 3 days.

Plasma urea and creatinine

Plasma urea (DiaSys, Holzheim, Germany) and plasma creatinine (Arbor Assays, Ann Arbor, USA) were measured using colorimetric assay conform manufacturer's protocol.

Histology and immunohistochemistry

Tissue was fixed in a buffered 4% formalin solution for 24 hours and embedded in paraffin. 3µm sections were mounted on adhesive slides (Leica Xtra), rehydrated through xylene and alcohol washes, and rinsed in distilled water. For Periodic Acid Schiff (PAS) and Masson's trichrome (MTC) staining, standard procedures were used (Dako, Glostrup, Denmark).

Immunohistochemistry (IHC) for gamma H2AX (γH2AX) and CCN2 was performed as described previously and IHC for p21^{CIP1} (p21) was set up based on the manufacturer's

protocol. (Falke et al., 2012; Lachaud et al., 2016) First, endogenous peroxidase was blocked using H_2O_2, followed by antigen retrieval by boiling in pH6 citrate buffer and primary antibody incubation (*For human and mouse:* anti-γH2AX [pSer139], Novus Biologicals NB100-2280, 1:250; anti-CCN2, Santa Cruz sc-14939, 1:200; *For human*: anti-p21, Cell Signaling Technology #2947, 1:100; *For mouse:* anti-p21, Abcam ab188224, 1:4000; anti-Ki-67, Thermofisher RM9106S, 1:100; anti-cleaved caspase 3, BD Pharmingen 559565, 1:500) diluted in 1% BSA blocking solution. For p21, CCN2 and Ki-67, secondary HRP conjugated antibodies were applied and visualised using Nova Red substrate (Vector Laboratories, Burlingame, CA, USA). For γH2AX and cleaved caspase 3, alkaline phosphatase conjugated antibody and liquid permanent red substrate (Dako) were used. Slides were counterstained with Mayer's hematoxylin.

Images were acquired using a Nikon Eclipse E800 microscope or scanned (Hamamatsu NanoZoomer) and digitally photographed in ImageScope to allow manual aligning of serial slides. For assessment of histopathological damage, a kidney pathologist (TQN) blinded for experimental conditions, graded acute tubular injury (ATI) on PAS slides. ATI was graded on a scale from 0 to 3 as a percentage of the total cortical area of the tissue section (0 = 0%; 1 = <25%; 2 = <50%; 3 = >50%). ATI was defined as tubular dilatation, epithelial necrosis, cast formation and loss of brush border. (Pieters et al., 2019; Stokman et al., 2005) For MTC, positive area percentages were calculated based on 10 random microscopy images with 200x magnification using Photoshop CS6 (Adobe, San Jose, CA, USA) and ImageJ1 (NIH, USA). For nuclear stains (γH2AX and p21), whole slides were scanned and the number of positive cells and total cells was calculated in QuPath. (Bankhead et al., 2017) For all scores, the score is displayed as the mean of the left and right kidney. In human biopsies, cortical tubules containing one or more positive cell(s) were annotated and manually counted using QuPath. For assessing the localization of positive staining, tubular structures were identified by kidney pathologists (TQN and RG). TECs were defined as cross sections through tubular structures with basal membranes.

RNA in-situ hybridisation

To visualize RNA, the viewRNA system (ThermoFisher) was used with a human CCN2 probe set (VA1-12672-VT) following the manufacturer's protocol. In brief, freshly cut 3 µm sections were mounted on adhesive slides and dried for 1 hour at 60°C. After deparaffinization and rehydration, slides were heat pretreated for 10 minutes at 95°C followed by protease (1:250) treatment for 10 minutes at 40°C. Subsequent hybridisation steps with the probe set, pre-amplifier, amplifier and label probe were all carried out at 40°C. Fast Red substrate was applied for 1 hour at room temperature and the slides were slightly counterstained with Mayer's hematoxylin.

Quantitative real-time PCR

DNA and full RNA were extracted from kidney cortical poles using Trizol (Thermo-Fisher). Purity and quantity were determined using Nanodrop 2000 (Thermo-Fisher). For RNA analysis, a cDNA library was synthesized using 3µg RNA per kidney with SuperScript III reverse transcriptase (Thermo-Fisher). qPCRs were run on a ViiA 7 real-time PCR system (Applied Biosystems, California, USA). The SYBR green primer sequences and Taqman probes qPCR are shown in Table S1. To assess CCN2 DNA expression, we used intron/exon spanning SYBR green primers complementary to CCN2 intron2-exon3 and CCN2 exon4-intron4. For mRNA analysis, TATA-box binding protein (TBP) was used as internal reference. Samples were run in duplicate. Samples free of mRNA and reverse transcriptase were used to control for potential contamination. ΔΔCT method was used to calculate relative expression levels.

Western blot

RPTECs were lysed and Western blot and ELISA analyses were performed as described previously. (Eleftheriadis et al., 2021) Experiments were repeated three times. Membranes were incubated with antibodies for the following proteins: CCN2 (E2W5M; 1:1000; Cell

Signaling Technology #10095), γH2AX (1:1000; Novus Biologicals NB110-2280), p21Waf1/Cip1 (1:1000, Cell Signaling Technology #37543), Ki-67 (1:1000, NBP2-22112, Novus Biologicals, Abingdon, Oxon, UK), GLB1 (1:1000, ab55176, Abcam), andβ-actin (1:2500; Cell Signaling Technology #4967) for murine cultures and CCN2 (L-20 goat polyclonal; 1:1000; Santa Cruz-14939), p21 (1:1000; Cell Signaling-2947), PAI-1 (1:1000; #9163) and β-tubulin (1:1000; Abcam-ab6046) for human cultures.Furthermore, a mouse IL-6 ELISA Kit (Elabscience E-EL-M0044) was used.

SA-β-Galactosidase activity

SA-*β*-galactosidase (SA-*β*-Gal) activity was performed following the manufacturer's protocol (Senescence Cells Histochemical staining Kit, CS0030-1KT, 069M4101V, Sigma) after 72h of CCN2 stimulation.

Statistical analysis

Two-way ANOVA with post-hoc Tukey correction was used to compare the means of continuous variables in the 4 IRI groups. Discrete dependent variables in the IRI experiment and CCN2 administration experiments were tested non-parametrically with Mann-Whitney and Kruskal-Wallis with post-hoc Dunn's test. Correlation of two independent variables was assessed using Pearson for continuous variables and Spearman for discrete variables. Values exceeding >1.5 interquartile ranges from the mean of a group were labelled as outliers and excluded. Data that showed abnormal distribution (i.e. right skewness) was log-transformed. Homogeneity of variances was tested with Levene's test because of unequal sample sizes. All statistical analyses were executed using statistical program SPSS (IBM SPSS Statistics 25). Error bars represent SEM. P-values < 0.05 were considered statistically significant.

RESULTS

Tubular CCN2 and DDR co-localize in acute IRI and subsequent tubulointerstitial fibrosis of the same kidney allograft

Phosphorylation of H2AX (γH2AX) marks DDR and induces CCA via the p53/p21 pathway. (Fragkos et al., 2009) P21 activation halts the cell cycle, usually transient in case of efficient DNA repair, but permanent in senescence. (Romanov et al., 2012) **Index case:** Histological and immunohistochemical examination of a kidney allograft biopsy 8 months after transplantation revealed co-localization of tubular γH2AX, p21, and CCN2 in fibrotic areas (Figure 1A). Evaluation of a previous biopsy from that same allograft obtained for DGF 7 days post transplantation, revealed co-localization of tubular γH2AX, p21, and CCN2 in areas with acute tubular injury (i.e. tubular dilatation, epithelial necrosis, cast formation and loss of brush borders; Figure 1B).

Tubular CCN2 and DDR are associated with loss of kidney function in CAD biopsies

Tubular expression of γH2AX, p21 and CCN2 were assessed in kidney allograft biopsies taken for CAD (5-29 years post transplantation) or for DGF (6-8 days post transplantation) with no apparent cause other than tubulointerstitial fibrosis or IRI, respectively. Patient characteristics are shown in Table S2. In CAD biopsies, estimated glomerular filtration rate (eGFR) correlated with γH2AX ($r=-0.73$; $p=0.02$) and p21 ($r=-0.68$; $p=0.04$) expression (Figure 2A,B). Additionally, tendencies were observed between CCN2 and DDR in late biopsies (Figure S1A,B), and in DGF biopsies between eGFR, DDR and CCN2 (Figure S1C-E) and CCN2 and DDR (Figure S1F,G). Finally, CCN2 mRNA co-localized with CCN2 protein, as well as γH2AX and p21 in CAD and DGF biopsies, indicating a tubular source of CCN2 transcription (Figure S2).

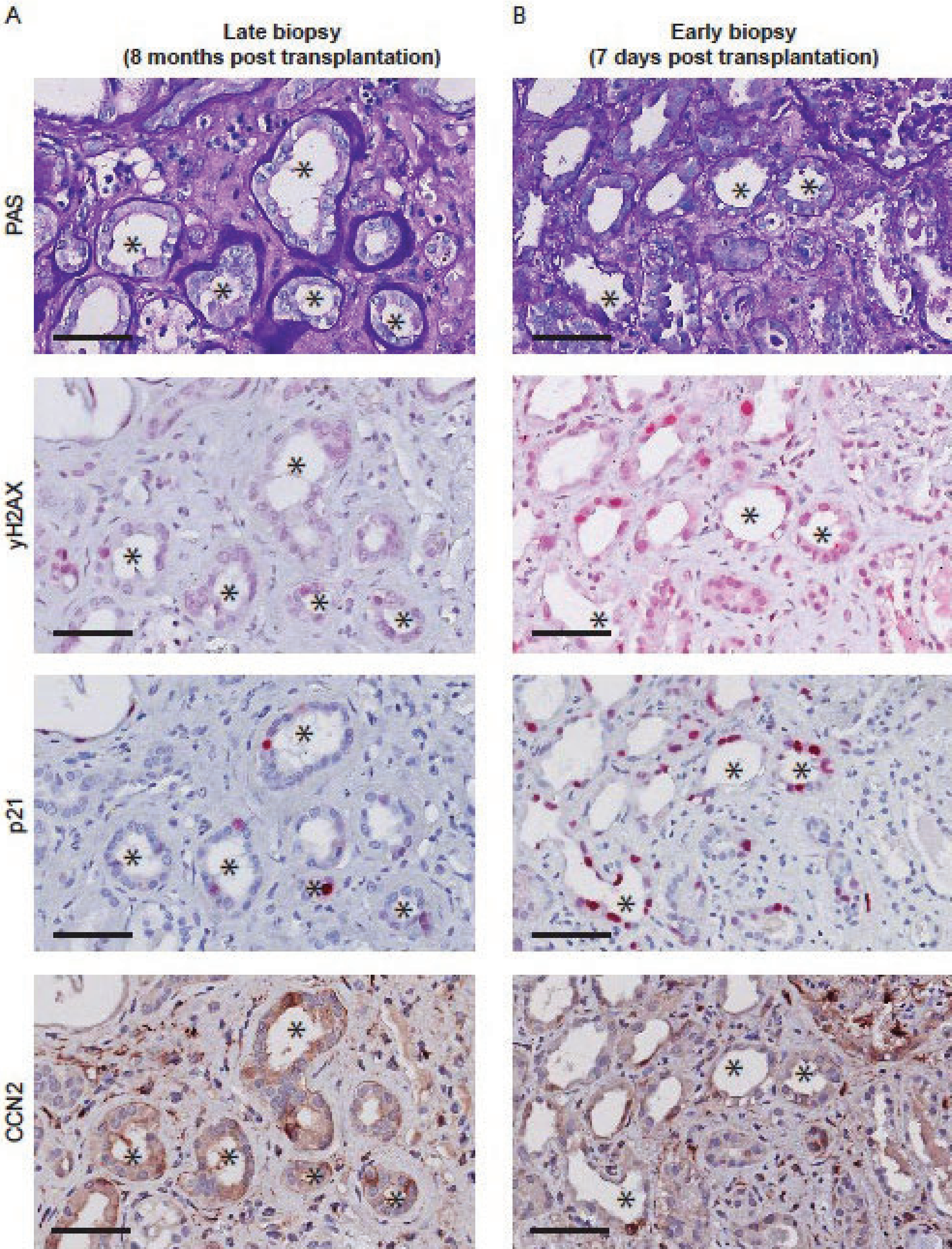

Figure 1. Tubular expression of cellular communication network factor 2 (CCN2) co-localizes with DNA damage response (DDR) in an early and late biopsy of the same kidney allograft
(A, B) Representative micrographs of renal cortex tissue derived from the same kidney allograft biopsied 8 months (A) and 7 days (B) after transplantation. Images depict consecutive sections stained with periodic acid-Schiff (PAS), gamma H2AX (γH2AX), p21CIP1 (p21) and CCN2. Asterisks indicate the same renal tubules expressing γH2AX, p21CIP1 (p21) and CCN2. Bar = 100 µm.

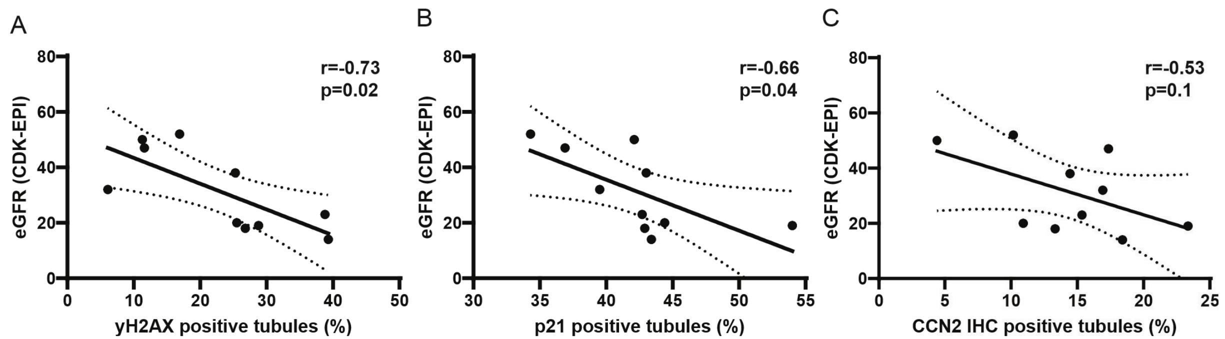

Figure 2. Tubular expression of cellular communication network factor 2 (CCN2) and DNA damage response (DDR) are associated with kidney function in chronic allograft dysfunction (CAD) biopsies
(A-C) Estimated Glomerular Filtration Rate (eGFR) correlated with cortical tubular γH2AX (A) and p21 (B) and trended to correlate with CCN2 (C) expression in CAD biopsies.

CCN2 deletion reduces IRI-induced late tubulointerstitial fibrosis, functional decline and persistent tubular injury
To study the association between CCN2 and kidney function, damage, fibrosis and senescence, a 6-week IRI model was conducted using ROSA26Cre tamoxifen inducible CCN2 full KO mice. Tamoxifen induced recombination resulted in 99% reduction in CCN2 mRNA in both sham and IRI kidneys ($p<0.005$; Figure 3A), as well as reduced CCN2 protein expression ($p=0.03$ and $p=0.04$, resp.; Figure 3B and Figure S3). Plasma urea increase upon IRI was reduced by CCN2 KO ($p=0.01$; Figure 3C). Similar trends for plasma creatinine were observed (Figure S4A). Furthermore, tubulointerstitial fibrosis in MTC staining was impeded by CCN2 KO ($p<0.005$; Figure 3D). Concordantly, IRI-induced upregulation of gene expression levels of tubular injury markers KIM-1 and NGAL, and of regeneration factor SOX9 was reduced in CCN2 KO IRI mice ($p=0.02$, $p=0.009$ and $p<0.005$; Figure 3E-G). Thus, CCN2 deficient mice had reduced tubular damage and fibrosis 6 weeks after IRI.

CCN2 deletion reduces IRI-induced late cellular senescence
To elucidate if senescence might be implicated in the fibrosis mitigating effect of CCN2 reduction, markers related to DDR, proliferation, p53 signaling and SASP were analyzed by IHC and qPCR. Reduced numbers of γH2AX-positive cells in CCN2 KO IRI mice indicated reduced DDR ($p<0.005$; Figure 4A). The great majority of these cells were not cycling as evidenced by absent co-staining with Ki-67 (not shown). Additionally, p21 mRNA expression and the numbers of p21-positive cells in IRI kidneys were reduced by CCN2 KO ($p=0.02$ and $p=0.04$, resp.; Figure 4B). In CCN2 KO IRI kidneys, mRNA levels of SASP factors IL-1β, CCL2 and PAI-1 were all reduced compared to WT IRI kidneys ($p<0.005$, $p<0.005$ and $p<0.005$; Figure 4C-E). Of note, in WT IRI kidneys, γH2AX- and p21-positive cells were mainly PTEC in fibrotic areas of the outer cortex (Figure 4A, B). Overall, this indicated that CCN2 deficient mice had a reduced senescence phenotype 6 weeks after IRI.

CCN2 deletion preserves kidney function despite similar acute tubular injury marker expression at 3 days after IRI
To evaluate possible CCN2 involvement in early phases of the transition from acute IRI to the long term pro-fibrotic senescent state, a 3-day IRI model was performed. Tamoxifen administration resulted in a near total reduction of CCN2 mRNA in sham and IRI kidneys ($p<0.005$; Figure 5A). The IRI-induced increases of plasma urea and creatinine were reduced in CCN2 KO mice ($p<0.005$ and $p<0.005$; Figure 5B and Figure S4B, respectively). However, histological examination of the cortices revealed similar degrees of acute tubular damage in WT and CCN2 KO IRI mice (Figure 5C). Concordantly, gene upregulation of tubular injury markers KIM-1 and NGAL and regeneration factor SOX9 were equally upregulated in CCN2 KO as in WT kidneys (Figure 5D-F). In addition, IRI-induced proliferation and apoptosis of PTECs were similar in WT and CCN2 KO mice (Figure S5).

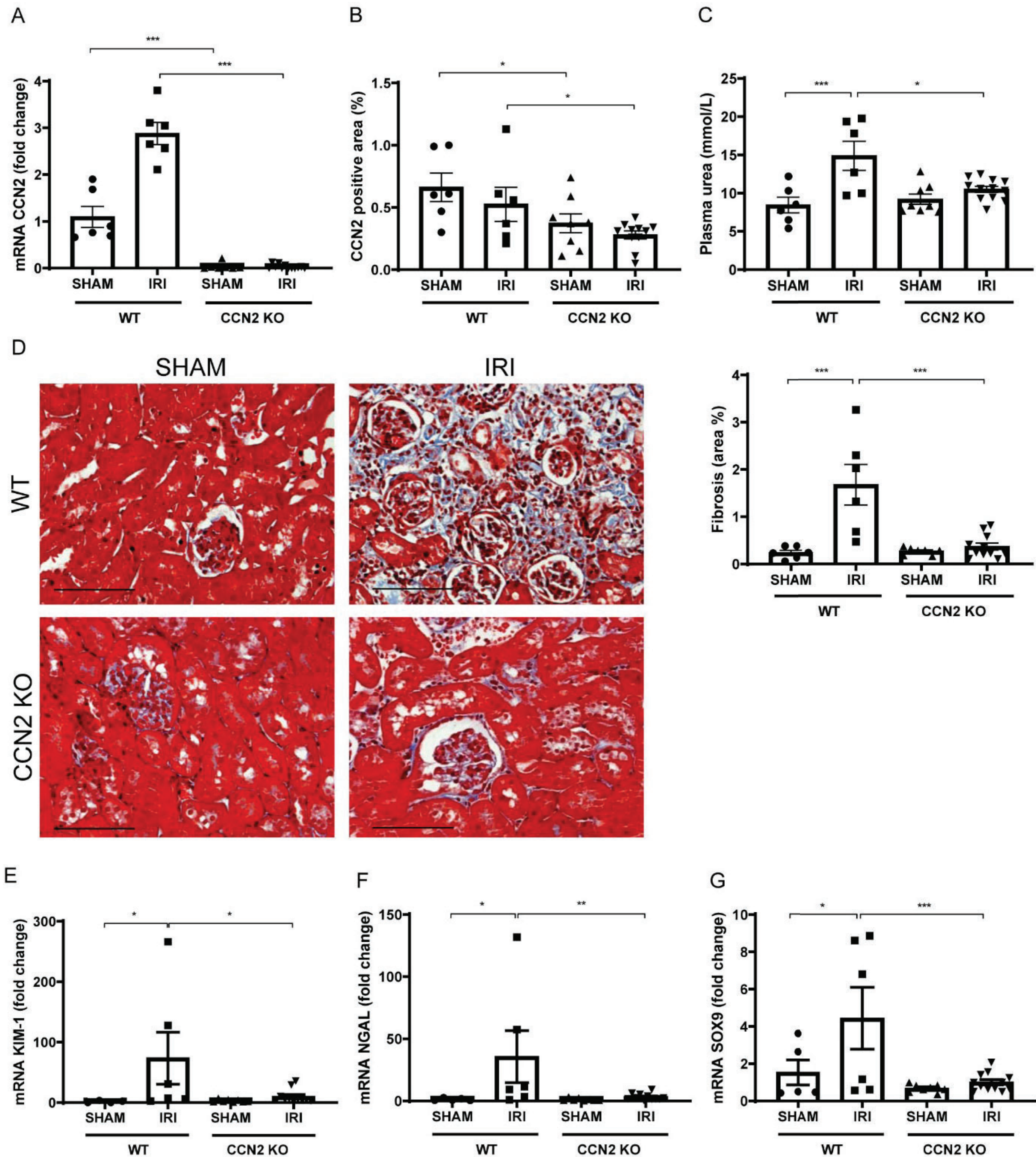

Figure 3. Near total deletion of cellular communication network factor 2 (CCN2) expression reduces late tubulointerstitial fibrosis, functional decline and persistent tubular injury 6 weeks after ischemia-reperfusion injury (IRI)

(A, B) Quantitative real-time polymerase chain reaction (qPCR; A) and immunohistochemistry (B) analysis showed that CCN2 mRNA and protein expression were decreased after sham and IRI surgery in CCN2 KO mice compared with wild type (WT) mice. **(C)** Measurement of plasma urea levels showed that the decline in kidney function was reduced in CCN2 KO mice compared with WT mice. **(D)** Representative micrographs and quantification of mouse renal cortex stained with Masson's trichrome showed that increased tubulointerstitial fibrosis in IRI kidneys was decreased in CCN2 KO mice compared with WT mice. **(E-G)** qPCR analysis showed that increased mRNA expression of KIM-1 (C), NGAL (D) and SOX9 (E) in IRI kidneys was reduced in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=4-6 for WT sham; N=5-6 for WT IRI; N=7-8 for KO sham; N=11-13 for KO IRI). TATA-box binding protein (TBP) was used as an internal control. *P<0.05, **P < 0.01, and ***P < 0.005. Bar = 100 μm.

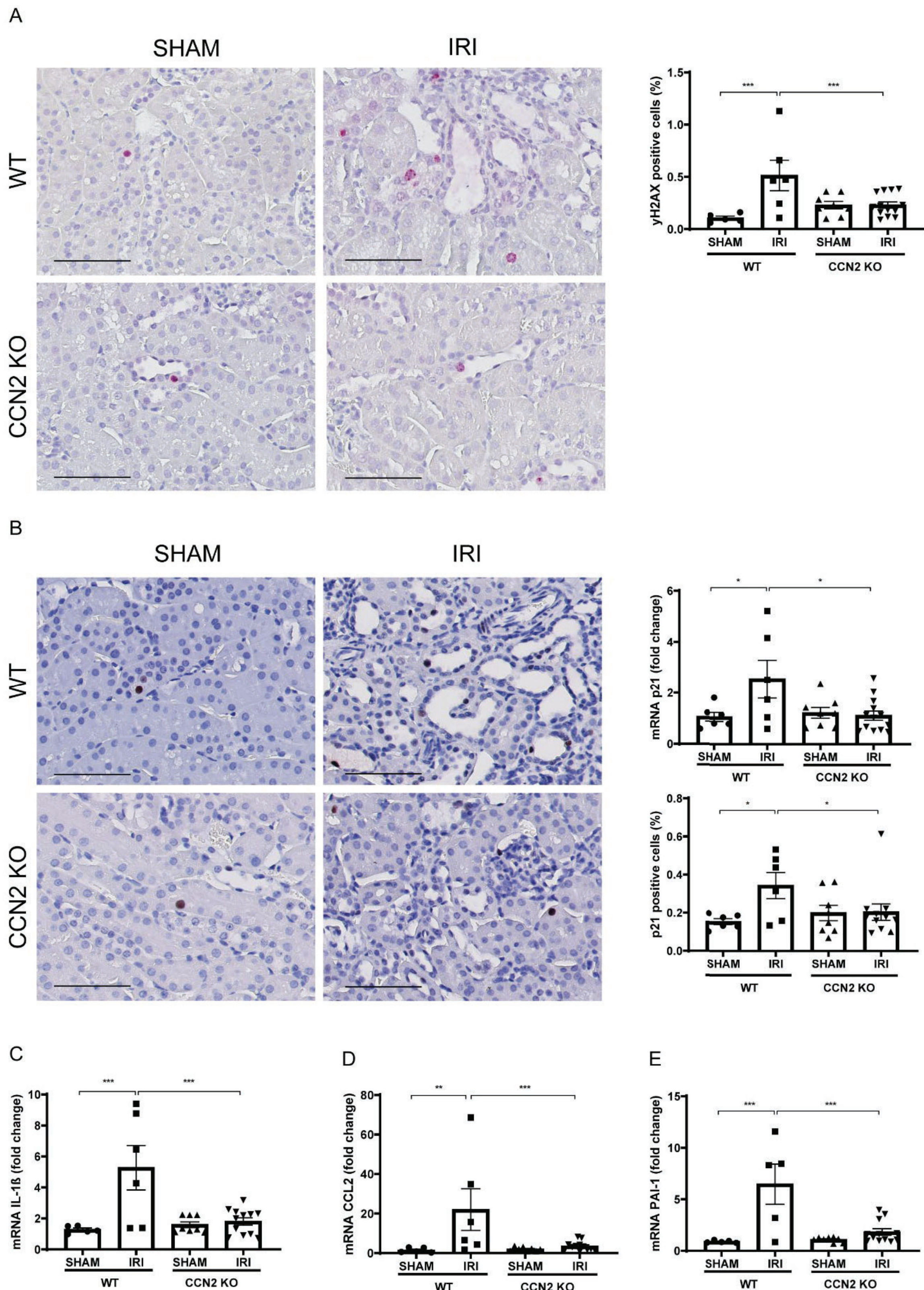

Figure 4. Near total deletion of cellular communication network factor 2 (CCN2) expression reduces cellular senescence 6 weeks after ischemia-reperfusion injury (IRI)
(A, B) Representative micrographs and quantification of mouse renal cortex stained with gamma H2AX (γH2AX; A) and p21CIP1 (p21; B) showed that increased DDR in IRI kidneys was decreased in CCN2 KO mice compared with wild type (WT) mice. Additionally, quantitative real-time polymerase chain reaction (qPCR) analysis showed that increased mRNA expression of p21 in IRI kidneys was reduced in CCN2 KO mice compared with WT mice. **(C-E)** Quantitative real-time polymerase chain reaction (qPCR) analysis showed that increased mRNA expression of IL-1β (E), CCL2 (F) and PAI-1 (G) in IRI kidneys were reduced in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=5-6 for WT sham; N=5-6 for WT IRI; N=8 for KO sham; N=12-13 for KO IRI). TATA-box binding protein (TBP) was used as an internal control. *$P<0.05$, **$P < 0.01$ and ***$P < 0.005$. Bar = 50 μm.

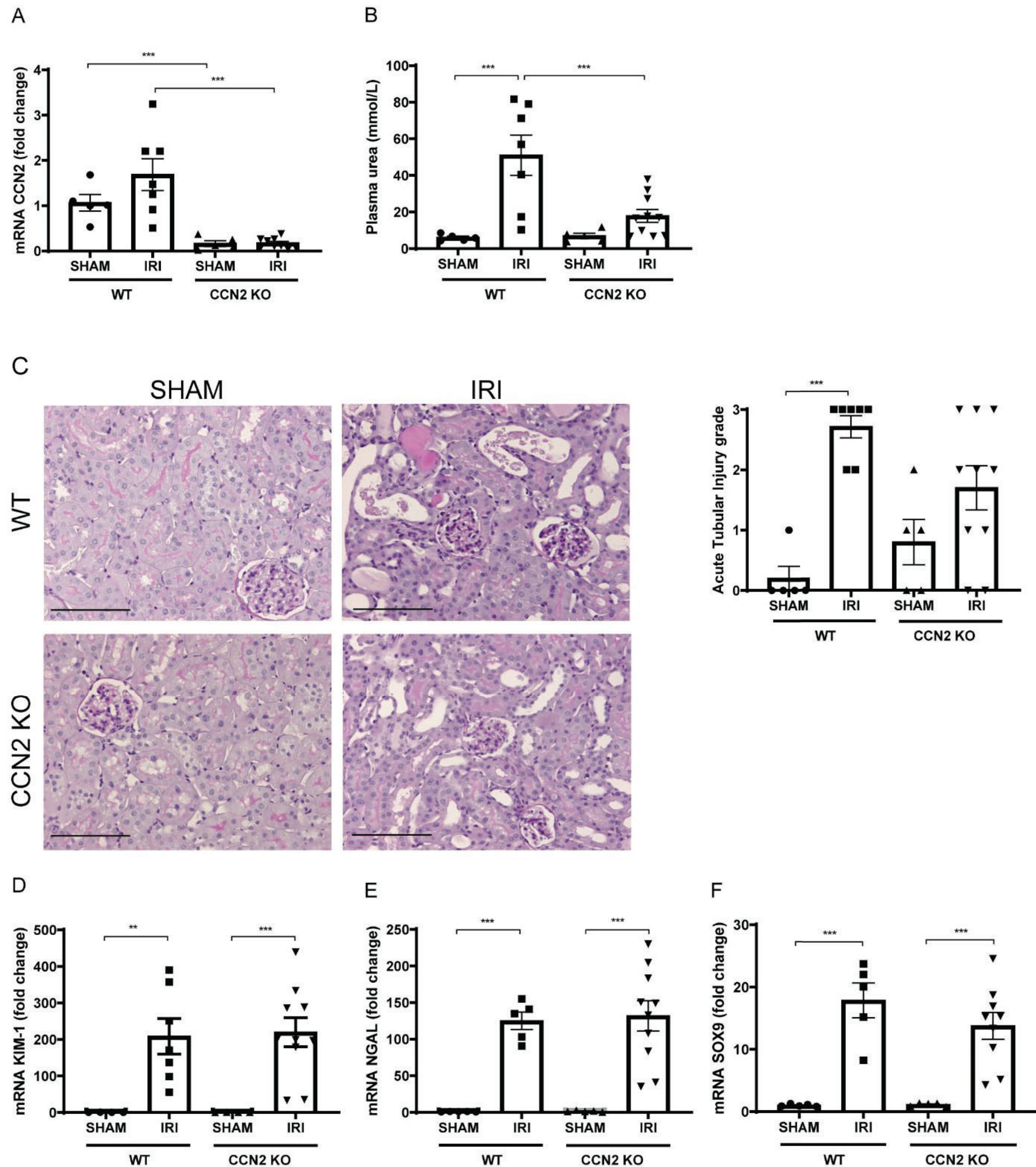

Figure 5. Near total deletion of cellular communication network factor 2 (CCN2) expression preserves kidney function despite similar acute tubular injury 3 days after ischemia-reperfusion injury (IRI)
(A) Quantitative real-time polymerase chain reaction (qPCR) analysis showed that CCN2 mRNA expression was decreased after sham and IRI surgery in CCN2 KO mice compared with wild type (WT) mice. **(B)** Measurement of plasma urea levels showed that the decline in kidney function was reduced in CCN2 KO mice compared with WT mice. **(C)** Representative micrographs and quantification of acute tubular injury histology of mouse renal cortex stained with periodic acid-Schiff (PAS) showed that increased acute tubular injury in IRI kidneys was not decreased in CCN2 KO mice compared with WT mice. **(D-F)** qPCR analysis showed increased mRNA expression of KIM-1 (D), NGAL (E) and SOX9 (F) in IRI kidneys in WT and CCN2 KO mice. Data are expressed as mean ± SEM (N=4-5 for WT sham; N=7 for WT IRI; N=5 for KO sham; N=10 for KO IRI). TATA-box binding protein (TBP) was used as an internal control. $^{**}P < 0.01$ and $^{***}P < 0.005$. Bar = 100 μm.

CCN2 deletion reduces IRI-induced early DNA damage and DDR at 3 days after IRI

CCN2 KO IRI kidneys had lower numbers of γH2AX-positive cells (p=0.005; Figure 6A) and lower p21 mRNA and protein expression (p=0.02 and p<0.005 resp.; Figure 6B) indicating reduced DNA damage and DDR. In WT IRI kidneys, γH2AX- and p21-positive cells were mainly PTECs localized in the inner cortex (Figure 6A, B). IRI-induced mRNA levels of SASP

factors IL-1β, CCL2 and PAI-1 were similar in KO and WT mice (Figure 6C-E). Furthermore, in CCN2 KO IRI mice, knockout efficiency marked by CCN2 exon 3 DNA levels containing the floxed genomic site, correlated with plasma urea, ATI grade, NGAL mRNA, p21 mRNA, number of p21-positive cells and CCL2 mRNA, and a trend was observed for CCN2 mRNA, KIM-1 mRNA, Sox9 mRNA and PAI-1 mRNA (Figure S6). Overall, this indicated that CCN2 deficient kidneys had reduced DNA damage and DDR 3 days after IRI.

CCN2 induces DNA damage and DDR in PTECs

CCN2 induced DDR was evaluated in RPTECs and HK-2 cells. Stimulation of RPTECs with 100ng/mL CCN2 increased the expression of γH2AX and p21 protein, and had an anti-proliferative effect marked by reduced Ki-67 expression (($p=0.04$, $p=0.02$ and $p=0.08$, respectively; Figure 7A-D). Moreover, the mRNA expression of p21 and SASP factors PAI-1 and CCL2 ($p<0.005$; Figure 7E-G), and SA-β-Gal activity ($p<0.05$; Figure 7H) were increased by CCN2. Additionally, in HK-2 cells, stable CCN2 overexpression resulted in increased expression of γH2AX, p21 and PAI-1 ($p=0.001$, $p<0.001$ and $p=0.01$, respectively; Figure 7I-M), and an increased proportion of cells in G2/M phase (Figure 7N).

CCN2 silencing reduces DNA damage and DDR induced by anoxia-reoxygenation injury in PTECs

The direct impact of CCN2 on DDR in tubular cells was evaluated by silencing CCN2 in RPTECs subjected to anoxia-reoxygenation (AR) injury, as an *in vitro* model for IRI. (Eleftheriadis et al., 2018) AR injury induced upregulation of CCN2, γH2AX and p21 was alleviated when CCN2 was silenced, comparable to the expression levels of control cells that were cultured under normoxic conditions ($p=0.007$; $p=0.005$ and $p<0.005$, respectively; Figure 8A-D). Conversely, increased Ki-67 expression suggested alleviation of CCA in CCN2 silenced AR-injured cells ($p<0.005$; Figure 8A,E). Finally, the expression of GLB1, marking senescence-associated β-gal (SA-β-gal) activity, was suppressed in CCN2 silenced cells ($p=0.006$; Figure 8A,F).

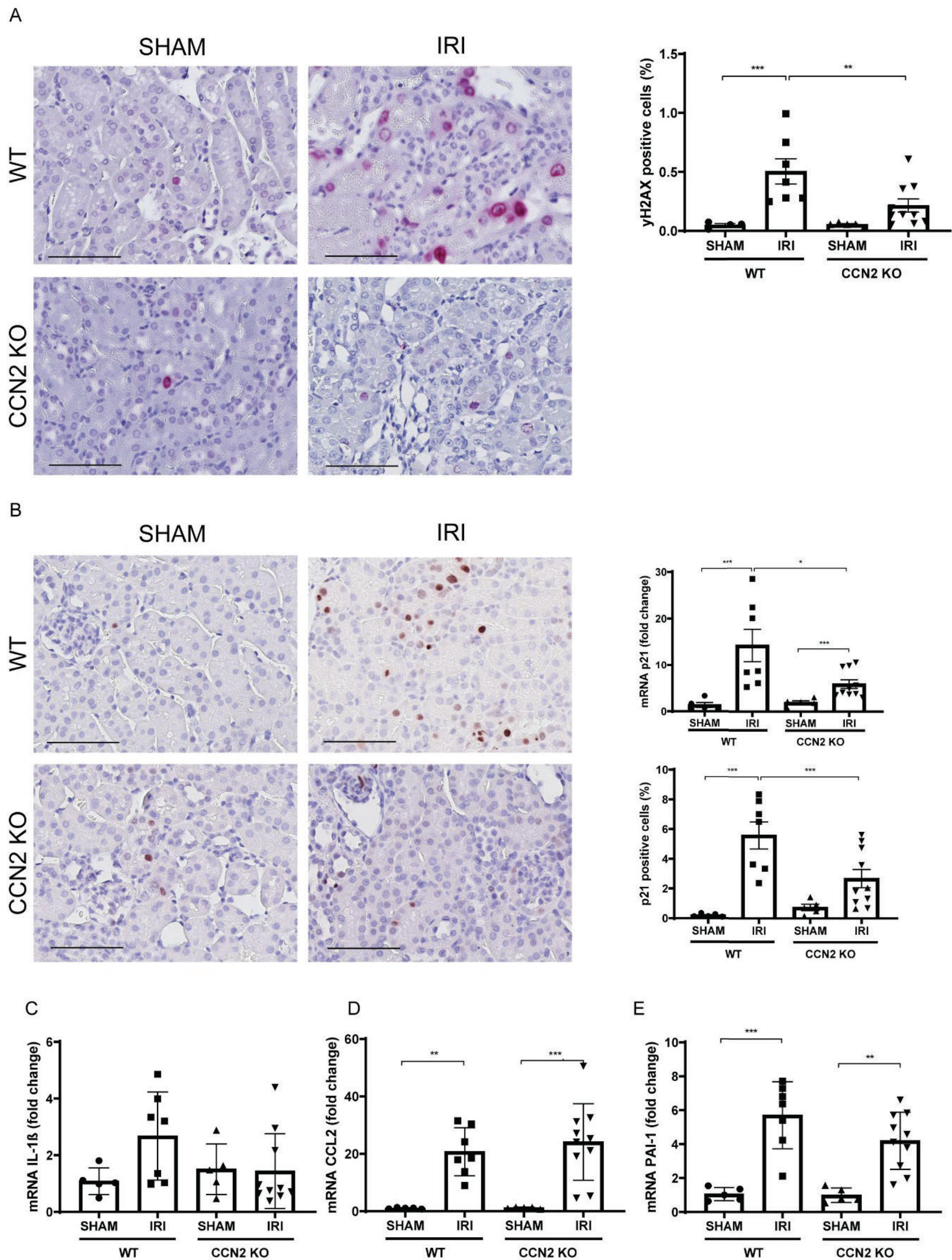

Figure 6. Near total deletion of cellular communication network factor 2 (CCN2) expression reduces DNA damage and DNA damage response 3 days after ischemia-reperfusion injury (IRI)
(A, B) Representative micrographs and quantification of mouse renal cortex stained with gamma H2AX (γH2AX; A) and p21CIP1 (p21; B) showed that increased DDR in IRI kidneys was decreased in CCN2 KO mice compared with wild type (WT) mice. Additionally, quantitative real-time polymerase chain reaction (qPCR) analysis showed that increased mRNA expression of p21 in IRI kidneys was reduced in CCN2 KO mice compared with WT mice. **(C-E)** Quantitative real-time polymerase chain reaction (qPCR) analysis showed that increased mRNA expression of IL-1β (E), CCL2 (F) and PAI-1 (G) in IRI kidneys were similar in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=4-5 for WT sham; N=6-7 for WT IRI; N=4-5 for KO sham; N=9-10 for KO IRI). TATA-box binding protein (TBP) was used as an internal control. *P<0.05, *P<0.01 and ***P < 0.005. Bar = 50 μm.

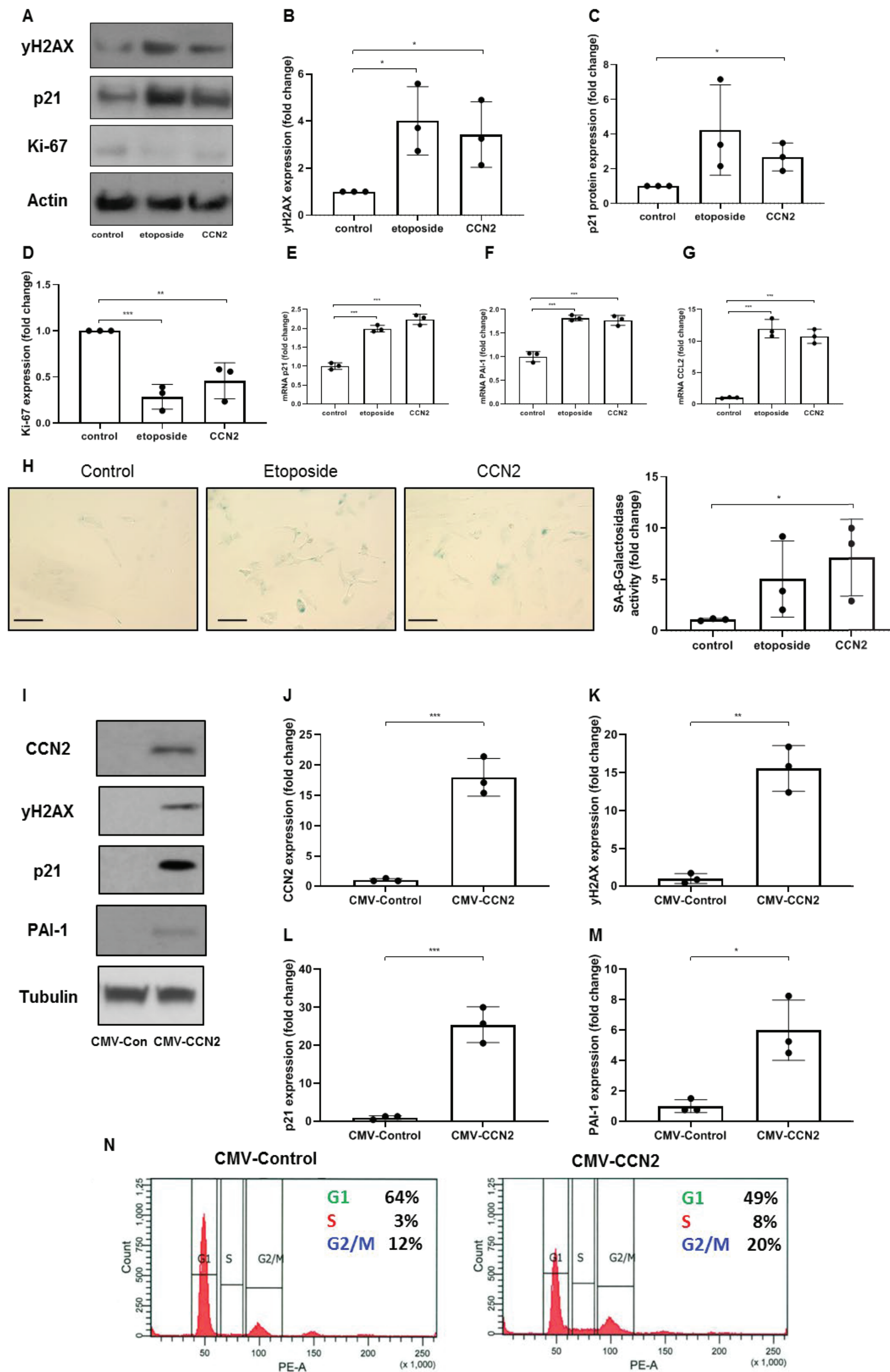

Figure 7. Cellular communication network factor 2 (CCN2) induces DNA damage and DNA damage response (DDR) in cultured renal proximal tubular epithelial cells (PTECs)
(A) Representative western blotting of protein extracts from cultured primary murine RPTECs stimulated with 100ng of CCN2 for gamma H2AX (γH2AX), p21CIP1 (p21) and Ki-67 showed that CCN2 increased DDR and reduced proliferation. **(B-D)** Corresponding protein expression levels of γH2AX (B), p21 (C) and Ki-67 (D). Data are expressed as mean ± SEM relative to basal of 3 independent experiments. **(E-G)** Quantitative real-time polymerase chain reaction (qPCR) analysis showed that CCN2 increased the mRNA expression of p21 (E), PAI-1 (F) and CCL2 (G). Data are expressed as mean ± SEM. TATA-box binding protein (TBP) was used as an internal control. **(H)** Representative micrographs and quantification of RPTECs stained with SA-β-galactosidase stimulated with 100ng of CCN2 showed that CCN2 induced senescence. **(I)** Representative western blotting of cell extracts isolated from HK-2 cells stably expressing empty vector (CMV-Control) and CCN2 construct (CMV-CCN2) at a similar confluence for CCN2, γH2AX, p21 and PAI-1 showed that CCN2 increased DDR and the expression of SASP factor PAI-1.**(J-M)** Corresponding protein expression levels of CCN2 (I), γH2AX (J), p21 (K) and PAI-1 (L). Data are expressed as mean ± SEM relative to basal of 3 independent experiments. **(N)** Flow cytometry analysis of propidium iodide-stained CMV-Control and CMV-CCN2 expressing cells determined the cell cycle distributions in G1, S, G2/M phases as indicated. *P<0.05, *P<0.01 and ***P < 0.005. Bar = 50 μm.

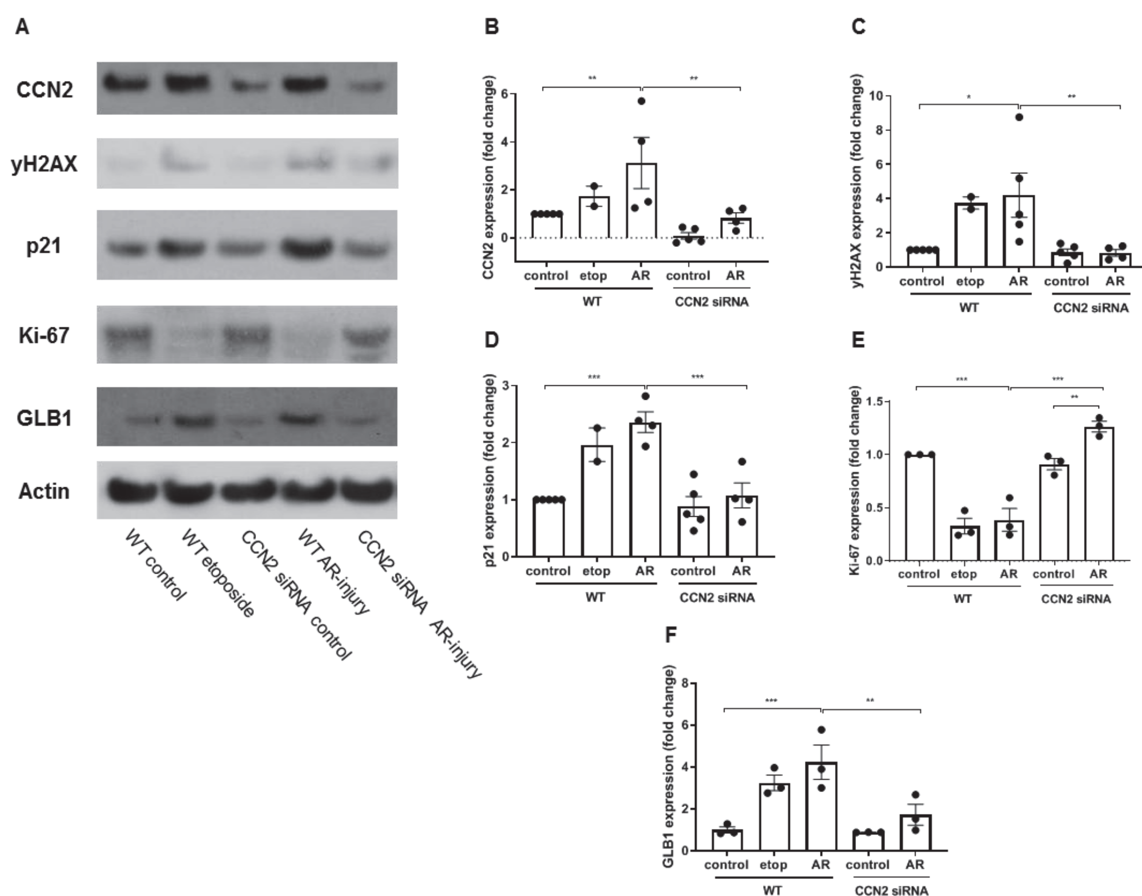

Figure 8. Silencing of cellular communication network factor 2 (CCN2) alleviates DNA damage and DNA damage response induced by anoxia-reoxygenation (AR) injury in cultured renal proximal tubular epithelial cells (PTECs)
(A) Representative western blotting of protein extracts from cultured cells for CCN2, gamma H2AX (γH2AX) and p21CIP1 (p21) showed that increased DDR in AR-injured PTECs was alleviated by silencing of CCN2. Data represent three independent experiments. **(B-E)** Corresponding protein expression levels of CCN2 (B) γH2AX (C), p21 (D) and Ki-67 (E). Data are expressed as mean ± SEM relative to basal of 3 independent experiments.

DISCUSSION

Our observations in human kidney allograft biopsies reveal an association of eGFR with tubular CCN2 expression, DDR and cellular senescence, reflecting a sequence probably initiated by transplantation surgery-induced IRI. This is supported by our experimental data showing that CCN2 deletion preserves renal function and reduces tubulointerstitial fibrosis and tubular senescence 6 weeks after IRI. Remarkably, this is preceded by preserved renal function and reduced tubular DDR, despite similar acute tubular damage 3 days after IRI. Direct impact of CCN2 on proximal tubular DDR is further supported by *in vitro* data showing that CCN2 silencing in PTEC also reduces DDR and conversely, CCN2 administration induces p21 expression *in vivo* and *in vitro*. CCN2 has previously been shown to contribute to fibrosis development in CAD. (Vanhove et al., 2017; Vitalone et al., 2010) These novel findings suggest that CCN2 negatively contributes also to the acute response to IRI, in particular DNA damage and the DDR, thereby contributing to cellular senescence and fibrosis at later stages (Figure 9). This implicates that anti-CCN2 therapy may help to prevent post-IRI chronic kidney dysfunction by limiting IRI-induced acute DNA damage, senescent cell accumulation and subsequent fibrosis.

These observations provide novel insights into the link between CCN2, senescence and fibrosis upon IRI. Several *in vitro* studies have suggested that CCN2 is a crucial factor in cellular senescence. For instance, CCN2 induces CCA in mesangial cells and bronchial epithelial cells, and a senescence phenotype in fibroblasts. (Abdel-Wahab et al., 2002; Capparelli et al., 2012; Jang et al., 2017; Jun & LF., 2017) *In vivo*, CCN2 inhibition prevents

fibrosis induced CAD and PTEC-specific conditional CCN2 KO reduces IRI-induced fibrosis in combination with reduced G2/M arrest. (Inoue et al., 2019; Luo et al., 2008) Accordingly, renal fibrosis elicited by folic acid was prevented in CCN2 knockout mice. (Rayego-Mateos et al., 2018) Moreover, CCN2 induced G2/M arrest of TECs associated with induction of fibrotic-related responses. (Rayego-Mateos et al., 2018) In human, anti-CCN2 therapy reduces albuminuria, an important predictor of CKD, in diabetic patients with mild albuminuria. (Adler et al., 2010) However, the effects of CCN2 inhibition on senescence have not been investigated to date.

Beneficial effects of CCN2 expression upon organ injury have also been observed. For instance, cardiac-specific CCN2 overexpression and post-ischemic administration of recombinant CCN2 protect the myocardium from acute IRI. (Ahmed et al., 2011) Also, CCN2 induces a SASP including also anti-fibrotic matrix metalloproteinases in cultured fibroblasts and in specific stages of cutaneous wound healing, and reduces collagen formation in the latter. (Jun & LF., 2017) This could be explained by a context-dependent beneficial anti-fibrotic role of cellular senescence in specific conditions, including cutaneous wound healing and upon liver injury. (Krizhanovsky et al., 2008; Demaria et al., 2014) Negative effects of CCN2 inhibition have not been demonstrated in the setting of renal injury.

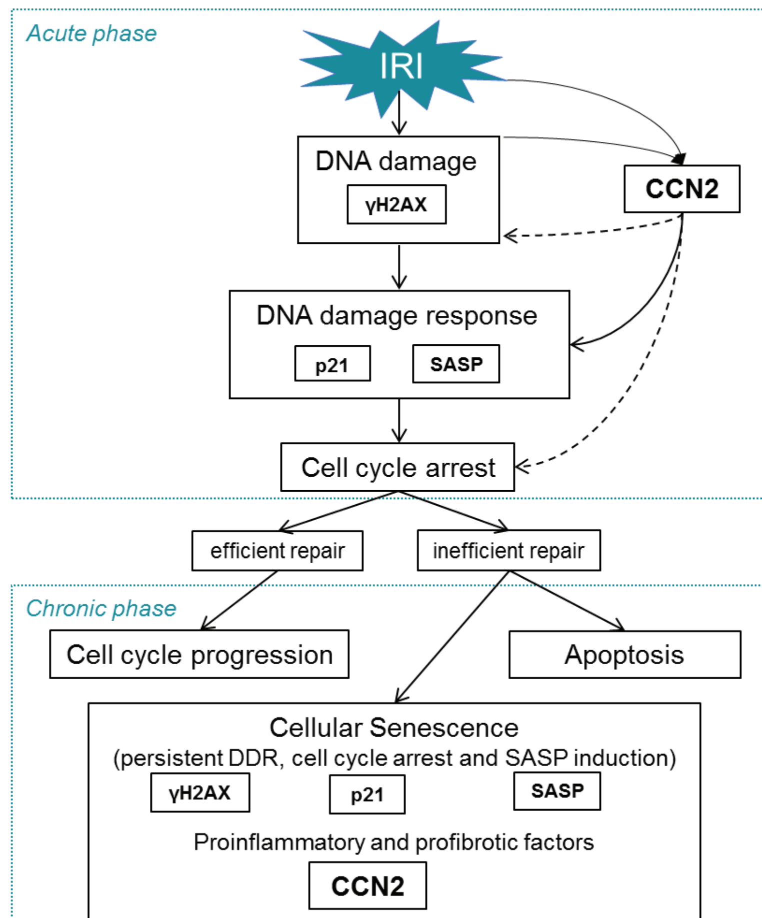

Figure 9. Mechanisms involved in ischemia-reperfusion injury (IRI) linked with cellular communication network factor 2 (CCN2) in CCN2 KO IRI mice.
Near absence of CCN2 reduced the expression of gamma H2AX (γH2AX)- and p21CIP1 (p21) positive cells, marking reduced DNA damage response and cell cycle arrest early after IRI. In the late phase upon IRI, this was followed by sustained reduction in γH2AX- and p21-positive cells, along with reduced SASP, indicating reduced senescence phenotype. This coincided with reduced fibrosis and preserved kidney function. Dotted arrows are used to acknowledge that the exact mechanisms by which CCN2 contributes to senescent cell accumulation remain to be established.

Main mechanisms of how CCN2 contributes to IRI-induced senescence might involve DDR and the SASP. IRI induces DNA damage in TECs and subsequent DDR to facilitate regeneration of the injured tubules. (Zhou et al., 2012) Unresolved DNA damage leads to

prolonged DDR, evolving in cellular senescence of the tubular epithelium within 3 days of injury that increases gradually over time. (Jin et al., 2019) CCN2-induced senescence in cultured cells is mediated through accumulation of cell cycle regulators like p53 and p21. (Abdel-Wahab et al., 2002; Jang et al., 2017; Jun & LF., 2017) Concordant with a role for CCN2 in the development of a senescence phenotype, we observed that DDR was attenuated in CCN2 KO mice in the early phase of IRI and in CCN2 inhibited PTEC subjected to AR injury, and conversely, that CCN2 induced p21 expression. This suggests that CCN2 affects development of a senescence phenotype by altering DDR and subsequent CCA in the early phase upon renal injury.

The senescence-associated secretory phenotype (SASP) promotes fibrosis and inflammation, reinforces the CCA and contributes to paracrine senescence induction. (Chien et al., 2011; Myrianthopoulos et al., 2019; Xu et al., 2018) Development of the SASP relies on activation of several transcription factors including NF-κB. (Chien et al., 2011) CCN2 activates NF-κB in murine kidneys, induces expression of SASP factors including IL-1β and MMP-1 in cultured fibroblasts and is itself also a constituent of SASP in senescent renal TEC. (Chien et al., 2011; Jun & LF., 2017; Liu et al., 2019; Sánchez-López et al., 2009; Yang et al., 2010) Moreover, sustained tubular expression of the SASP factor PAI-1 promotes p21 expression, G2/M arrest, and fibrotic tubular maladaptive repair (including CCN2 upregulation). (Gifford et al., 2021) In the late phase upon IRI, we observed that CCN2 KO mouse kidneys had reduced upregulation of SASP markers, suggesting that reduced autocrine and paracrine senescence induction might explain at least in part the association of reduced fibrosis with reduced accumulation of senescent cells.

In conclusion, our data add to the well-established role of CCN2 in kidney fibrosis providing evidence that it plays a role also early in the development of tubular damage and subsequent tubulointerstitial fibrosis following IRI, in particular in the DNA damage-DDR-Cellular Senescence-Fibrosis sequence. This implies that CCN2-targeted therapy might benefit graft function and survival outcomes of transplanted kidney patients and warrants further evaluation of pharmacological interventions in the early and later stages of transplant-surgery related IRI.

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SUPPLEMENTARY MATERIAL

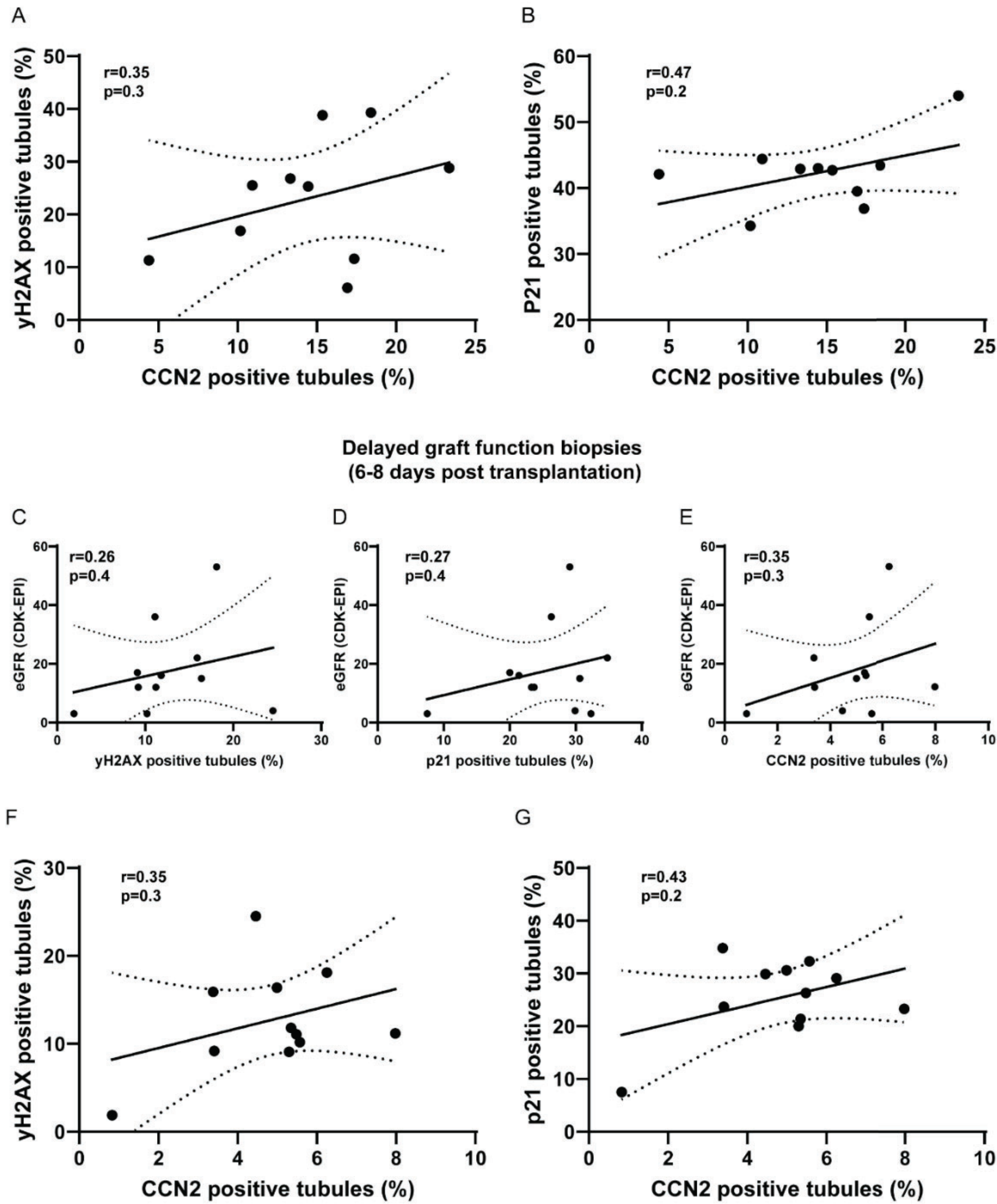


Figure S1. Tubular expression of cellular communication network factor 2 (CCN2) was not significantly associated with DNA damage response (DDR) in late kidney allograft biopsies, nor with kidney function and DDR in early kidney allograft biopsies
(A, B) Tubular CCN2 expression showed a tendency to correlate with gamma H2AX (γH2AX; A) and p21CIP1 (p21; B) in chronic allograft dysfunction biopsies. **(C-E)** Estimated Glomerular Filtration Rate (eGFR) did not correlate with cortical tubular γH2AX (C), p21 (D) and CCN2 (E) expression in delayed graft function biopsies. **(F, G)** Tubular CCN2 expression showed a tendency to correlate with γH2AX (F) and p21 (G) in delayed graft function biopsies.

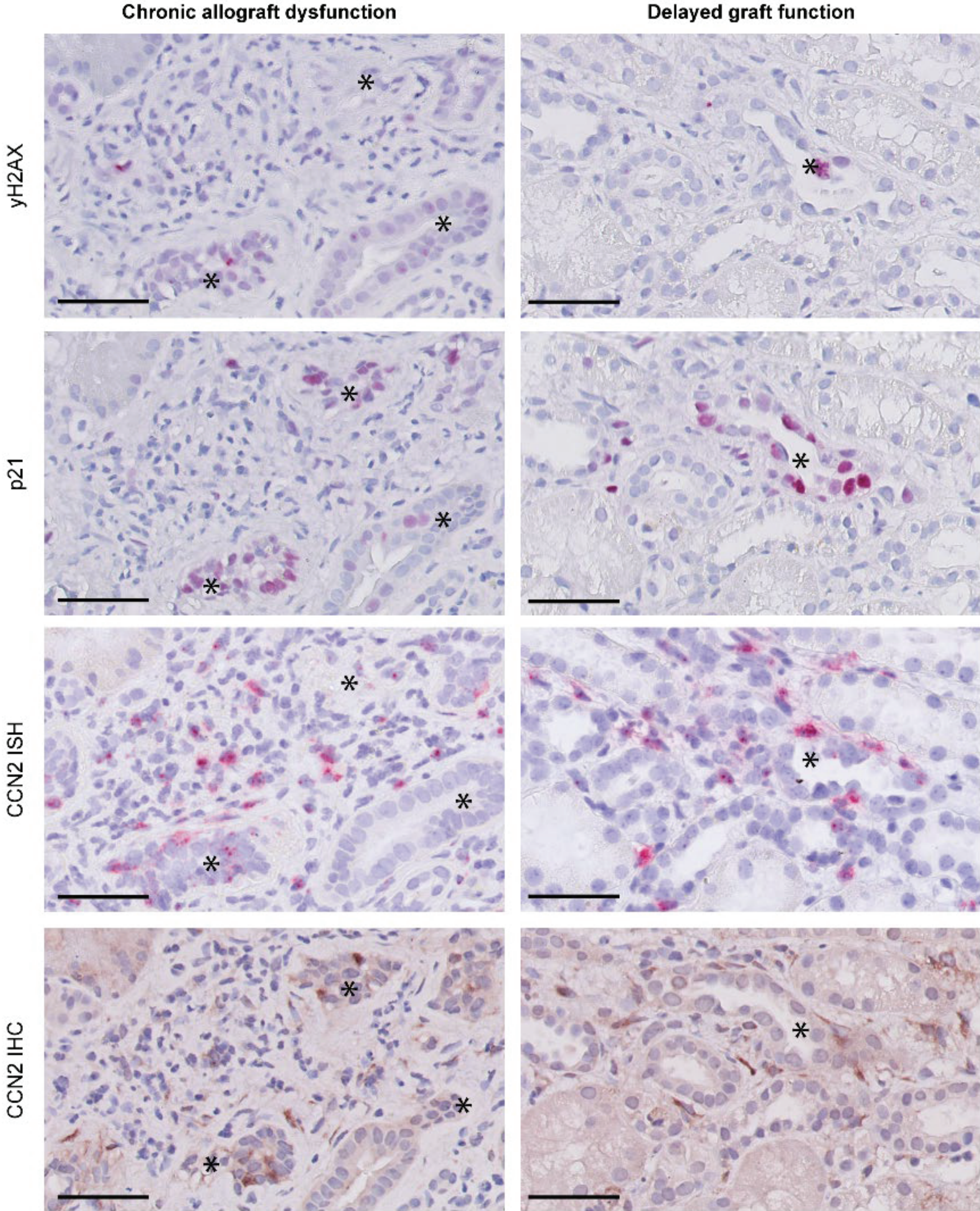


Figure S2. Tubular expression of cellular communication network factor 2 (CCN2) co-localized with DNA damage response (DDR) in late and early kidney allograft biopsies
Representative micrographs of renal cortex tissue derived from kidney allograft biopsies taken for chronic allograft dysfunction or for delayed graft function with no apparent cause other than tubulointerstitial fibrosis or IRI, respectively. Images depict consecutive sections stained with gamma H2AX (γH2AX), p21CIP1 (p21), CCN2 mRNA and CCN2 protein. Asterisks indicate the same renal tubules expressing γH2AX, p21CIP1 (p21), CCN2 mRNA and CCN2 protein. Bar = 50 μm. ISH = in situ hybridization; IHC = immunohistochemistry

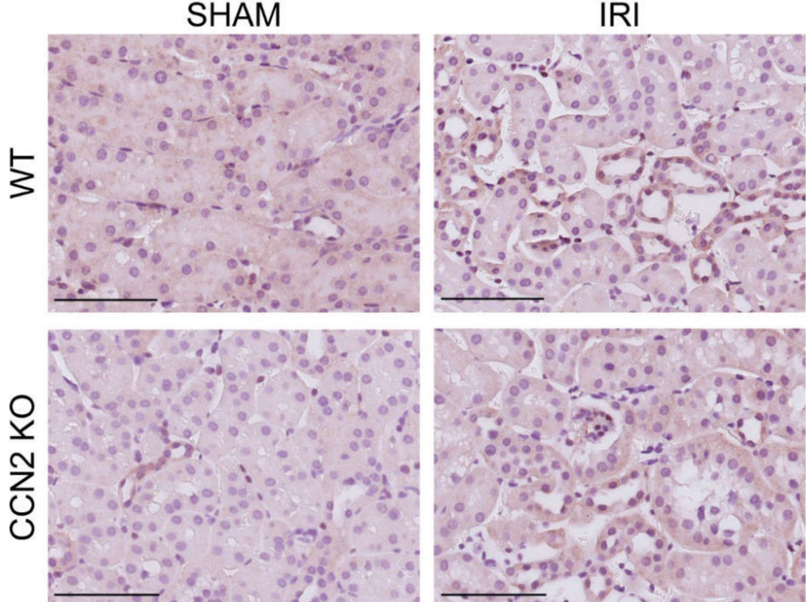


Figure S3. Near total deletion of cellular communication network factor 2 (CCN2) expression reduced CCN2 protein expression 6 weeks after ischemia-reperfusion injury (IRI)
(A, B) Representative micrographs of mouse renal cortex stained with CCN2 showed that CCN2 protein expression was decreased after sham and IRI surgery in CCN2 KO mice compared with wild type (WT) mice. Bar = 100 μm.

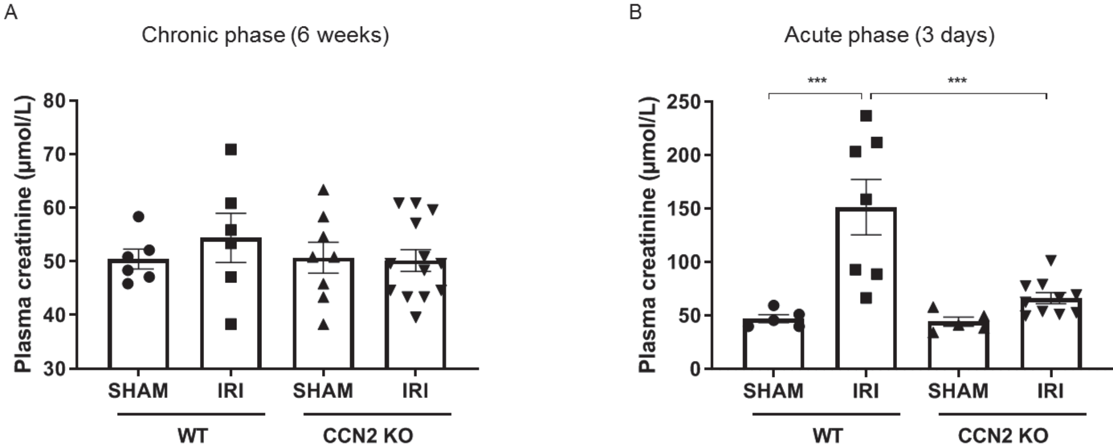


Figure S4. Near total deletion of cellular communication network factor 2 (CCN2) expression results in reduced plasma creatinine 3 days after ischemia-reperfusion injury (IRI) and in similar plasma creatinine 6 weeks after IRI
(A, B) Measurement of plasma creatinine levels showed that the decline in kidney function was reduced in CCN2 KO mice compared with WT mice 3 days after IRI (A) and trended to be lower in CCN2 KO mice compared with WT mice 6 weeks after IRI. Data are expressed as mean ± SEM (N=5/6 for WT sham; N=6/7 for WT IRI; N=5/8 for KO sham; N=10/13 for KO IRI in the 3day IRI and 6week IRI model respectively.). ***P < 0.005.

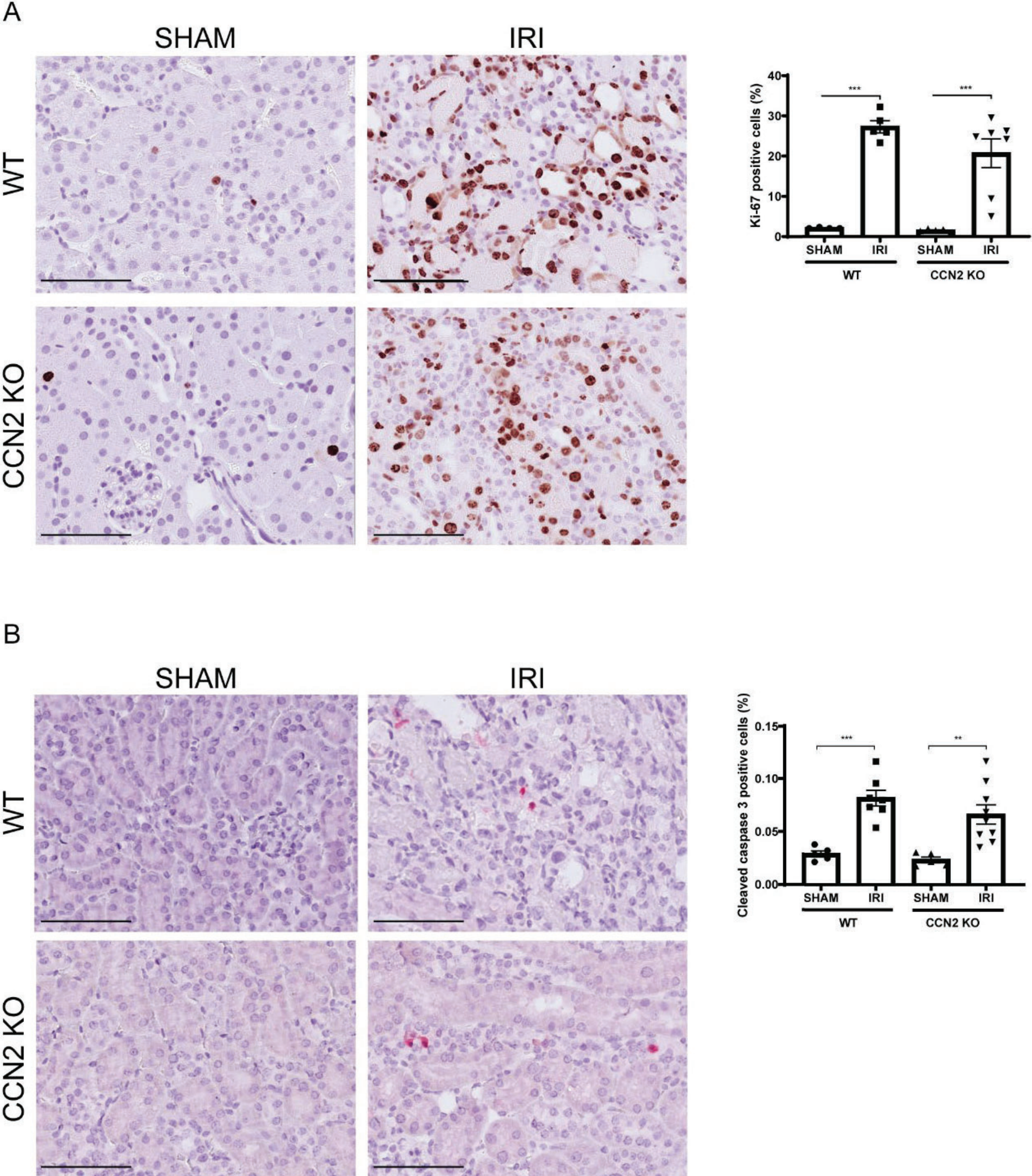


Figure S5. Near total deletion of cellular communication network factor 2 (CCN2) expression results in similar levels of proliferation and apoptosis 3 days after ischemia-reperfusion injury (IRI)
(A, B) Representative micrographs and quantification of mouse renal cortex stained with proliferation marker Ki-67 (A) and apoptosis marker Cleaved caspase 3 (B) showed that increased proliferation and apoptosis in IRI kidneys was similar in CCN2 KO mice compared with wild type (WT) mice. Data are expressed as mean ± SEM (N=5 for WT sham; N=6-7 for WT IRI; N=5 for KO sham; N=9-10 for KO IRI). **P < 0.01, and ***P < 0.005. Bar = 100 μm.

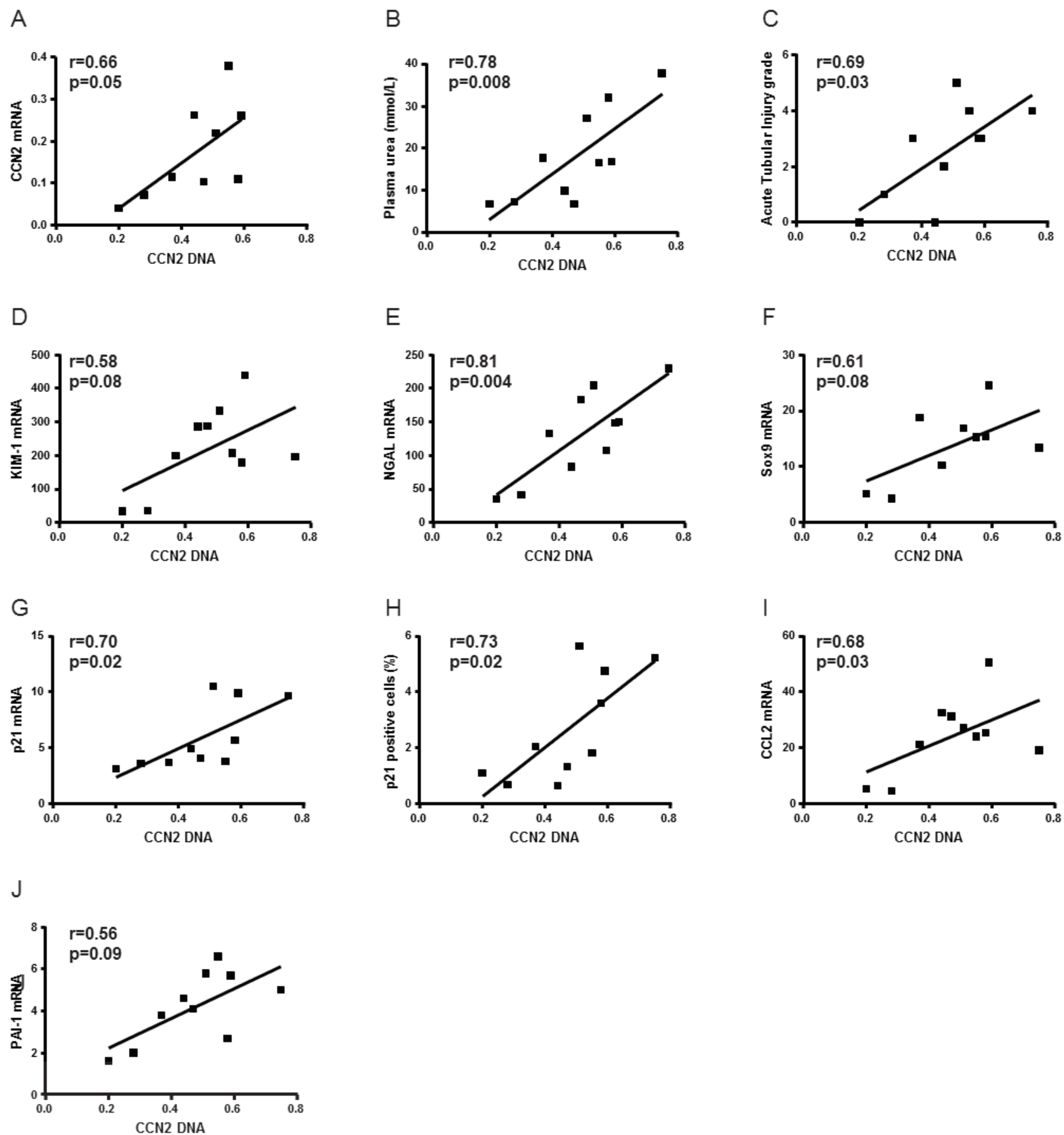


Figure S6. Cellular communication network factor 2 (CCN2) genetic knockout efficiency correlated with CCN2 mRNA, plasma urea, acute tubular injury, NGAL mRNA, p21 mRNA and PAI-1 mRNA in CCN2 KO mice 3 days after ischemia-reperfusion injury (IRI)
(A-F) Correlation of CCN2 exon 3 DNA with CCN2 mRNA (A), plasma urea (B), acute tubular injury (C) KIM-1 mRNA (D), NGAL mRNA (E), Sox9 mRNA (F) p21 mRNA (G) number of p21-positive cells (H), CCL2 mRNA (I) and PAI-1 mRNA (J) in CCN2 KO IRI mice.

Table S1. Primers used for real-time polymerase chain reaction

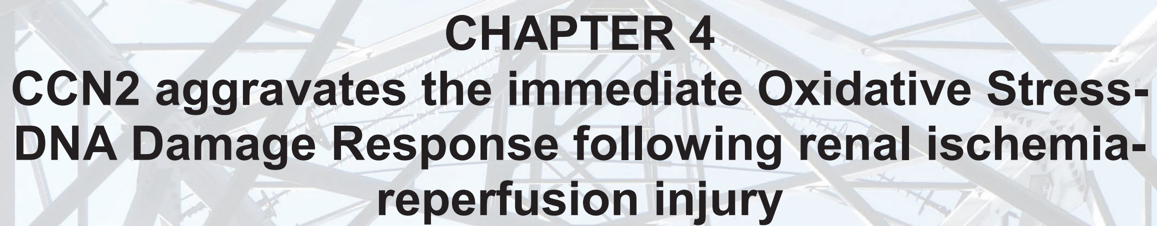
Gene	SYBR Green primer sequence or Taqman Gene Expression Assay
CCN2 intron2-exon3	Fwd: AGCTGCGATCACAGACTGAC
	Rev: CACGCTCCGTACACAGTTCT
CCN2 exon4-intron4	Fwd: GCATCTCCACCCGAGTTACC
	Rev: AGGGGCAGAGGATGTACCTT
TBP	Mm00446971_m1
CCN2	Mm01192932_g1
KIM1	Mm00506686_m1
NGAL	Mm01324470_m1
SOX9	Mm00448840_m1
CDKN1A	Mm00432448_m1
IL1B	Mm00434228_m1
CCL2	Mm00441242_m1
SERPINE1	Mm00435860_m1

Fwd = forward primer; Rev = reverse primer.

Table S2. Kidney transplant patient characteristics

	DGF (N=11)	CAD (N=10)	Total (N=21)
Sex			
Female	5 (45.5%)	5 (50.0%)	10 (47.6%)
Male	6 (54.5%)	5 (50.0%)	11 (52.4%)
Age (years)			
Mean (SD)	50.5 (17.6)	50.1 (21.5)	50.3 (19.1)
Median [Min, Max]	55.0 [15.0, 69.0]	48.0 [14.0, 79.0]	50.0 [14.0, 79.0]
Graft age			
Mean (SD)	51.3 (15.7)	62.8 (16.5)	56.2 (16.5)
Median [Min, Max]	54.0 [24.0, 75.0]	62.0 [40.0, 83.0]	55.0 [24.0, 83.0]
Missing	3 (27.3%)	4 (40.0%)	7 (33.3%)
Days after transplant			
Mean (SD)	11.6 (8.18)	5860 (2790)	2800 (3530)
Median [Min, Max]	7.00 [6.00, 32.0]	5090 [2230, 10300]	32.0 [6.00, 10300]
Donor status			
Postmortem	11 (100%)	10 (100%)	21 (100%)
Donor status (circulation)			
Non heart-beating	9 (81.8%)	7 (70.0%)	16 (76.2%)
Heart-beating	2 (18.2%)	3 (30.0%)	5 (23.8%)
eGFR at biopsy (ml/min/1,73m2)			
Mean (SD)	17.5 (15.1)	31.3 (14.5)	24.1 (16.1)
Median [Min, Max]	15.0 [3.00, 53.0]	27.5 [14.0, 52.0]	19.0 [3.00, 53.0]
Creatinine at biopsy (µmol/L)			
Mean (SD)	531 (456)	223 (95.6)	385 (364)
Median [Min, Max]	333 [164, 1500]	228 [112, 408]	269 [112, 1500]

DGF = delayed graft function; CAD = chronic allograft dysfunction; eGFR = estimated glomerular filtration rate



CHAPTER 4
CCN2 aggravates the immediate Oxidative Stress-DNA Damage Response following renal ischemia-reperfusion injury

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Chapter 4. CCN2 aggravates immediate Oxidative Stress and DDR following renal IRI

ABSTRACT

AKI due to altered oxygen supply after kidney transplantation is characterized by renal ischemia-reperfusion injury (IRI). Recent data suggest that AKI to CKD progression may be driven by cellular senescence evolving from prolonged DNA damage response (DDR) following oxidative stress. Cellular communication factor 2 (CCN2, formerly called CTGF) is a major contributor to CKD development and was found to aggravate DNA damage and the subsequent DDR-Cellular Senescence-Fibrosis sequence following renal IRI. We therefore investigated the impact of CCN2 inhibition on oxidative stress and DDR in vivo and in vitro. Four hours after reperfusion, full transcriptome RNA sequencing of mouse IRI kidneys revealed CCN2-dependent enrichment of several signaling pathways, reflecting a different immediate stress response to IRI. Furthermore, decreased staining for γH2AX and p-p53 indicated reduced DNA damage and DDR in tubular epithelial cells of CCN2 knockout (KO) mice. Three days after IRI, DNA damage and DDR were still reduced in CCN2 KO and this was associated with reduced oxidative stress, marked by lower lipid peroxidation, protein nitrosylation and kidney expression levels of Nrf2 target genes (HMOX1 and NQO1). Finally, silencing of CCN2 alleviated lipid peroxidation induced by anoxia-reoxygenation injury in cultured PTECs. Together, our observations suggest that CCN2 inhibition might mitigate AKI by reducing oxidative stress induced DNA damage and the sub-sequent DDR. Thus, targeting CCN2 might help to limit post-IRI AKI.

INTRODUCTION

Acute kidney injury (AKI) that requires renal replacement therapy is associated with over 50% mortality, and the severity of AKI in hospitalized patients correlates to mortality, length of hospital stay and healthcare costs (Chertow et al., 2005; Faubel & Shah, 2016). Also, AKI predisposes to chronic kidney disease (CKD) development later in life, in part due to excessive fibrosis associated with tubular injury (Parr & Siew, 2016). Ischemia-reperfusion injury (IRI) is a leading cause of AKI, that occurs in 30-50% of patients receiving deceased donor kidneys, and in this context, often leads to delayed graft function (Siedlecki et al., 2011; Ympa et al., 2005). Understanding the immediate pathophysiological changes that underlie IRI in the acute phase is paramount for identifying early intervention options to limit subsequent AKI to CKD progression.

During renal IRI, excessive amounts of reactive oxygen species (ROS) are produced (Esteve et al., 1999; Jassem & Heaton 2004). ROS induce DNA damage and consequently lead to a DNA damage response (DDR) to facilitate cell repair and regeneration of the injured tubules through DNA repair or apoptosis (Ratliff et al., 2016; Srinivas et al., 2019). However, a subset of cells fails to go into apoptosis, despite unresolved DNA damage, resulting in persistent DDR and senescence (Hernandez-segura et al., 2018). Senescent cells alter their microenvironment through a proinflammatory and profibrotic secretome dubbed the Senescence-Associated Secretory Phenotype (SASP), thereby affecting organ morphology and function (Coppé et al., 2010). Preventing cellular senescence by interfering with senescent-associated intracellular pathways has been proposed as novel strategy to prevent progression to CKD (Knoppert et al., 2019).

Cellular communication network factor 2 (CCN2), previously known as connective tissue growth factor (CTGF), is a well-known modulator in CKD development (Kok et al., 2014; Metalidis et al., 2013). CCN2 is a matricellular protein involved in various processes, including matrix biology, angiogenesis, inflammation, cell proliferation, hypoxia sensing, senescence and apoptosis (Capparelli et al., 2012; Chien et al., 2006; Higgins et al., 2004; Inoki et al., 2002; Kothapalli et al., 1998). Furthermore, CCN2 is associated with DDR and subsequent cellular senescence in delayed graft function and chronic allograft dysfunction following kidney transplantation, and in experimental IRI (Valentijn F.A., 2021), and is also a potent inducer of vascular oxidative stress (Rodrigues-Diez et al., 2015).

Differential regulation of gene expression in multiple signaling pathways associated with CCN2 in the immediate response, 4 hours after experimental IRI led us to hypothesize that CCN2 can contribute to oxidative stress induced DDR in the immediate phase during IRI. To elucidate this hypothesis, we assessed kidney function, damage, DDR and oxidative stress markers in wild type (WT) and CCN2 knockout (KO) mice 4 hours and 3 days after IRI. In addition, we also evaluated the effect of CCN2 treatment in mice, and of CCN2 silencing in an IRI-like model in cultured renal epithelial cells.

MATERIALS AND METHODS

Animals

Experiments were performed according to the European Community guidelines for animal experiments and the ARRIVE guidelines, and with consent of the Experimental Animal Ethics Committee of the University of Utrecht, The Netherlands (Kilkenny et al., 2012). Generation of tamoxifen inducible CCN2 full knockout mice is extensively described elsewhere (Fontes et al., 2015). In brief, CCN2$^{Flox/Flox}$ mice were crossbred with ROSA26CreERT2 mice (Gt(ROSA)26Sor$^{tm(cre/ERT2)Tyj}$/J, The Jackson Laboratory, Maine, USA), both on a C57Bl6/J background. Male 12-14 week old mice received four intraperitoneal injections on alternate days over a course of 7 days with either 100µl Corn oil (Sigma-Aldrich, St. Louis, Missouri, US) or 100µl of 10mg/ml tamoxifen-corn oil solution (Sigma Aldrich, St. Louis, Missouri, US). After the last injection, a 14-day washout period was followed by the IRI operation. Sample sizes were based on published studies and pilot experiments. (Kinashi et al., 2017)

Ischemia-reperfusion injury model

The surgical procedures were executed as previously described (Kinashi et al., 2017). In short, renal pedicles were located through an abdominal midline incision and bilaterally

clamped for 25 minutes with atraumatic neurovascular clamps. Clamping and subsequent reperfusion associated color changes were visually confirmed. IRI mice without color changes were excluded from further analyses. Sham operated animals underwent the same procedure with the exception of the pedicle clamping. The operations were executed under 2% isoflurane anaesthesia and body temperature was maintained at 37°C. After 4 hours, mice were euthanized by lethal dose Ketamine-Xylazine-Acepromazine injection, and plasma and organs were collected and stored at −80°C.

Mouse recombinant CCN2 model
CCN2 administration studies were performed in 3 month old male C57Bl/6 mice as previously described (Sánchez-López et al., 2009). Mice received 2.5 ng/g body weight recombinant human CCN2 (corresponding to the C-terminal module IV of CCN2; MBL International Corporation, Woburn, MA, USA) or saline by intraperitoneal administration.

Cell culture
Mouse primary renal PTECs (RPTECs, cat. no. C57-6015, Cell Biologics, Chicago, IL, USA) were cultured as described previously using a GasPak EZ Anaerobe Container System with Indicator (cat. no. 26001, BD Biosciences, S. Plainfield, NJ, USA) (Eleftheriadis et al., 2021). Gene silencing was performed using a predesigned siRNA corresponding to CCN2 (siRNA ID MSS274358, Thermo-fisher, Massachusetts, USA) according to the manufacturers protocol. In short, sub-confluent cells were transfected in Opti-MEM reduced serum medium (Invitrogen) for 24 hours with 5 ng/ml siRNA using 50 nM Lipofectamine RNAi-MAX (Invitrogen), followed by a 24h incubation in 20% FBS medium and a 24h incubation in serum-free medium. Controls were non-transfected cells treated with lipofectamine vehicle. Cells were subjected to 24 hours of anoxia and 2 hours of reoxygenation.

Plasma urea
Plasma urea was measured using colorimetric assay conform manufacturers protocol (DiaSys, Holzheim, Germany).

Histology and immunohistochemistry
Renal tissue was fixed in a buffered 4% formalin solution for 24 hours and subsequently embedded in paraffin blocks. Sections were mounted on adhesive slides (Leica Xtra) and rehydrated through a series of xylene and alcohol washes after which slides were rinsed in distilled water.

For Periodic Acid Schiff (PAS) staining, standard procedures were used (Dako, Glostrup, Denmark). Immunohistochemistry (IHC) for gamma H2AX (γH2AX), 4-Hydroxynonenal (4-HNE) and Bcl-xL were performed as described elsewhere (Marquez-Exposito et al., 2021; Lachaud et al., 2016; Schreiber et al., 2019). IHC for serine 15 phosphorylated p53 (p-p53) and nitrotyrosine was set up based on the manufacturer's protocol. First endogenous peroxidase was blocked using H_2O_2, followed by antigen retrieval by boiling in pH6 citrate buffer and primary antibody incubation for two hours at room temperature (anti-γH2AX [p Ser139], Novus Biologicals NB100-2280, 1:500; anti-p-p53 [p Ser15], R&D Systems AF1043, 1:800; anti-Bcl-xL, Abcam ab178844, 1:16.000; anti-nitrotyrosine, Bioss bs-8551R, 1:500) or overnight at 4°C (anti-4-HNE, Abcam ab46545, 1:150), diluted in 1% BSA blocking solution. For 4-HNE IHC, endogenous peroxidase was blocked after the primary antibody to prevent extra lipid oxidation. For p-p53, Bcl-xL, 4-HNE and nitrotyrosine, secondary HRP conjugated antibodies were applied and visualised using Nova Red substrate (Vector Laboratories, Burlingame, CA, USA). For γH2AX, alkaline phosphatase conjugated antibody and liquid permanent red substrate (Dako) were used. Slides were counterstained with Mayer's hematoxylin.

Slides were scanned (NanoZoomer, Hamamatsu, Japan) and images were acquired by taking photographs in ImageScope. For assessment of ATN severity, a blinded pathologist (TQN) graded cortical ATN severity using an arbitrary score ranging from 0 (none) to 3 (severe) on PAS stained slides. ATN was scored based on loss of TECs, luminal debris and mitotic activity. The score is displayed as the mean of the left and right kidney. Nuclear expression of γH2AX and p-p53 was quantitated in QuPath (QuPath 0.2.3, Edinburgh, Scotland) by counting the number of IHC-positive cells, relative to the number of

hematoxylin stained nuceli in whole slides (Bankhead et al., 2017). Total expression of p-p53, 4-HNE, nitrotyrosine and Bcl-xL was quantitated in QuPath by setting a pixel classifier based on selected annotations corresponding to positive and negative areas, resulting in the percentage of positive area relative to total tissue area in whole slides.

RNA-seq

Full transcriptome RNA-sequencing was performed on a cDNA library constructed from RNA extracted from 18 selected samples of kidney cortex (4 WT sham; 4 WT IRI; 4 KO sham; 6 KO IRI). mRNA was isolated using NEXTflex Poly(A) Beads (Bioo scientific, Austin, TX). Libraries were prepared using the Rapid Directional RNA-Seq Kit (NEXTflex), looped in equimolarly and sequenced on Illumina NextSeq500 to produce single-end 75 base long reads, and read-count analysis was performed by the Utrecht DNA Sequencing Facility (Utrecht, the Netherlands). RNA-seq reads were aligned to the reference genome GRCm38 using STAR version2.4.2a (Dobin et al., 2013). Read groups were added to the BAM files with Picard's AddOrReplaceReadGroups (v1.98). The BAM files are sorted with Sambamba v0.4.5 and transcript abundances are quantified with HTSeq-count version 0.6.1p1 using the union mode (Anders et al., 2015). Subsequently, reads per kilobase of transcript per million mapped reads (RPKM's) were calculated with edgeR's RPKM function (Robinson et al., 2009). The full dataset with RPKM values has been deposited in the GEO database (accession number GSE186316).

Differentially expressed genes were identified using the DESeq2 package with standard settings (Anders & Huber, 2010). False discovery rate (FDR) adjusted p-values were used to determine significance (Klipper-Aurbach et al., 1995). Pathway enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA; MSigDB, UC San Diego, US) (Subramanian et al., 2005). GSEA analysis was performed on the expression dataset file containing quantile normalized log2 transformed RPKM values, the Hallmarks and KEGG gene sets databases and the 'Mouse_Symbol_with_Remapping_to_Human_Orthologs' chip platform. Gene sets with a FDR<25% were identified as significantly enriched and ranked based on the normalized enrichment score (NES) A full list of differentially expressed genes (DEGs) and enriched pathways between all groups is included in Table S2.

Quantitative real-time PCR

Full RNA was extracted from kidney cortical poles using Trizol reagent (Thermo-Fisher, Waltham, Massachusetts, US). RNA purity and quantity was determined using Nanodrop 2000 (Thermo-Fisher). Using 3µg RNA per kidney, a cDNA library was synthesized with SuperScript III reverse transcriptase (Thermo-Fisher). Relative RNA expression levels were determined on a ViiA 7 real-time PCR system (Applied Biosystems, Pleasanton, CA, USA). The SYBR green primer sequences and Taqman probes used for quantitative real-time polymerase chain reaction (qPCR) are shown in Table S1. TATA-box binding protein (TBP) was used as internal reference gene. Samples were run in duplicate. Samples free of mRNA and reverse transcriptase were used to control for potential contamination of the reaction. ΔΔCT method was used to calculate relative expression levels.

Western blot

Cultured RPTECs were lysed and Western blot analysis was routinely performed as described previously (Eleftheriadis et al., 2021). Experiments were repeated three times. Membranes were incu-bated with antibodies specific for the following proteins: 4-HNE (1:500; Abcam ab46545), nitrotyrosin (1:500; Bioss bs-8551R; Bcl-xL (1:500; Abcam ab178844), HMGB1 (1:500; Pro-teintech 10829-AP-1), p-p53 (1:1000, Cell Signaling Technology #9284) and β-actin (1:2500; Cell Signaling Technology #4967).

MDA assay

Lipid peroxidation was assessed in RPTECs cultured in 6-well-plates. At the end of the reoxygenation period, malondialdehyde (MDA), the end product of lipid peroxidation, was measured fluorometrically in cell extracts with the Lipid Peroxidation (MDA) Assay Kit (cat. no. ab118970, Abcam, Cambridge, UK). Before MDA measurement, Bradford assay was

performed to adjust lysate volumes to equal protein concentration. These experiments were performed six times.

Statistical analysis
Two-way ANOVA with post-hoc Tukey correction was used to compare means of the various parameters in all 4 groups at both time points. Discrete dependent variables were tested non-parametrically with the Kruskal-Wallis test (ATN grade). Correlation of two independent variables was assessed using Pearson. Values exceeding >1,5 interquartile ranges from the mean were labelled as outliers and excluded. Data that showed abnormal distribution (i.e. right skewness) was log-transformed. Otherwise, normal distribution was assumed. Homogeneity of variances was tested with Levene's test because of unequal sample sizes. All statistical analyses were executed using statistical program SPSS (IBM SPSS Statistics 25). Error bars represent SEM and p-values smaller than 0.05 were considered statistically significant.

RESULTS

CCN2 deletion does not reduce IRI-induced functional decline and acute tubular necrosis 4 hours after IRI
To evaluate CCN2 mediated changes in immediate IRI, a 4 hour IRI model was conducted using tamoxifen inducible CCN2 full KO mice (Figure 1A). Tamoxifen induced recombination resulted i n 77% reduction in CCN2 mRNA in both sham and IRI kidneys (p=0.005 and p=0.008, resp.; Figure S1A). Plasma urea increase upon IRI was similar in WT and in CCN2 KO mice (Figure S1B). Histological examination revealed IRI-induced acute tubular necrosis, but no significant difference between WT- and CCN2 KO IRI mice (Figure S1C). Concordantly, tubular injury markers KIM-1, NGAL and SOX9 were equally upregulated in CCN2 KO IRI and WT IRI kidneys (Figure S1D-F).

CCN2 regulates gene transcription of several signaling pathways 4 hours after IRI
To find CCN2-related changes in primary major regulatory events associated with the immediate response to IRI in the renal cortex, full transcriptome RNA-sequencing was performed. According to literature, distinct differential gene expression patterns can be recognized in the kidney, as early as three hours after IRI, but the increase of a pro inflammatory transcriptome in those reports was limited (Supavekin et al., 2003; Stroo et al., 2010). In order to allow sufficient time for alteration of transcriptome profile with limited risk of analyzing secondary regulatory mechanisms, we harvested kidneys 4 hours after reperfusion.

Principal component analysis (PCA), as a method to identify similarities and differences in overall gene expression, showed that the data clearly segregated WT IRI, CCN2 KO IRI and SHAM groups, but no evident sub-clusters formed when comparing WT SHAM with CCN2 KO SHAM mice (Figure 1B). Pairwise comparison of transcriptome reads between WT IRI and CCN2 KO IRI mice yielded a total of 4167 differentially expressed genes (DEGs) (Table S2.3), of which 2169 genes were significantly downregulated and 1998 upregulated in CCN2 KO IRI mice (Figure 1C). DEGs with the largest fold down- or upregulation are presented in Table S3. In sham mice, a total of 21 DEGs were identified, of which 15 downregulated and 6 upregulated in CCN2 KO SHAM compared to WT SHAM (Figure 1C).

Gene set enrichment analysis (GSEA) on DEGs in CCN2 KO IRI compared to WT IRI mice using the Hallmarks and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases identified significant enrichment of 16 pathways with the WT IRI phenotype (Figure S2A) and 18 pathways with the KO IRI phenotype (Figure S2B) (Kanehisa & Goto, 2000). WT IRI phenotype enriched pathways included cytokine- (TNFα and IL-6/JAK/STAT3), growth factor- (TGFβ) and downstream transcription factor signaling (p53, PI3K/AKT/mTOR and mTORC1) as well as inflammatory response, hypoxia and oxidative phosphorylation, suggesting overall lower activation of these pathways in CCN2 KO compared to WT IRI-mice.

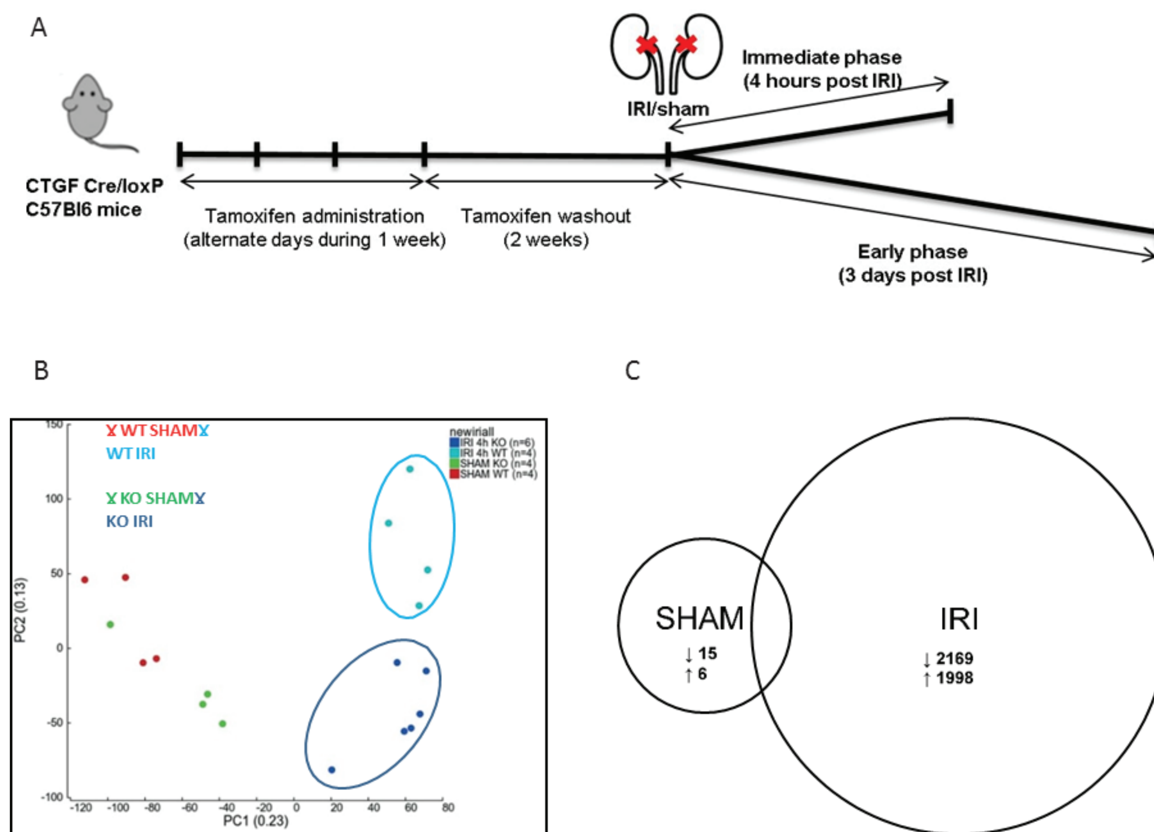

Figure 1. CCN2 regulates gene transcription 4 hours after renal IRI
(A) Time course of the IRI mouse model. **(B)** Principal component analysis plot of sham and IRI RNA sequence data in both WT and CCN2 KO genotypes showed clustering of WT IRI, CCN2 KO IRI and sham groups, but no clustering within the sham group. **(C)** Pairwise comparisons of RNA sequence data of sham and IRI data in both WT and CCN2 KO genotypes showing the amount of differentially expressed genes.

CCN2 deletion reduces DNA damage and p53 activation 4 hours after IRI

Oxidative stress in IRI involves excessive ROS production, driving DNA damage and a DDR involving p53 pathway activation (Lakin & Jackson, 1999; Terryn & Devuyst, 2011; Srinivas et al., 2019). Markers related to DNA damage, p53 signaling and oxidative stress were analyzed by immunohistochemistry (IHC) and qPCR. Lower cell numbers positive for the double strand break marker phosphorylated H2AX (γH2AX) were observed in CCN2 KO IRI kidneys compared to WT IRI kidneys, indicating reduced DNA damage ($p<0.005$; Figure 2A). Additionally, p-p53 expression, indicative for DNA damage-induced p53 activation (Shieh et al., 1997), was reduced by CCN2 KO ($p=0.01$; Figure 2B). Furthermore, in CCN2 KO IRI kidneys, CCN2 mRNA correlated with p-p53 ($r=0.77$; $p=0.04$; Figure 2B). Of note, γH2AX and p-p53 were mainly expressed in tubular epithelial cells (TECs). However, although we observed upregulation of p53 transcriptional target genes GADD45A, an established immediate IRI response gene (Cippà et al., 2018; Liu et al., 2017) and p21Cip1 (p21), CCN2 KO did not reduce the expression level of these genes on this time point (Figure 2C,D).

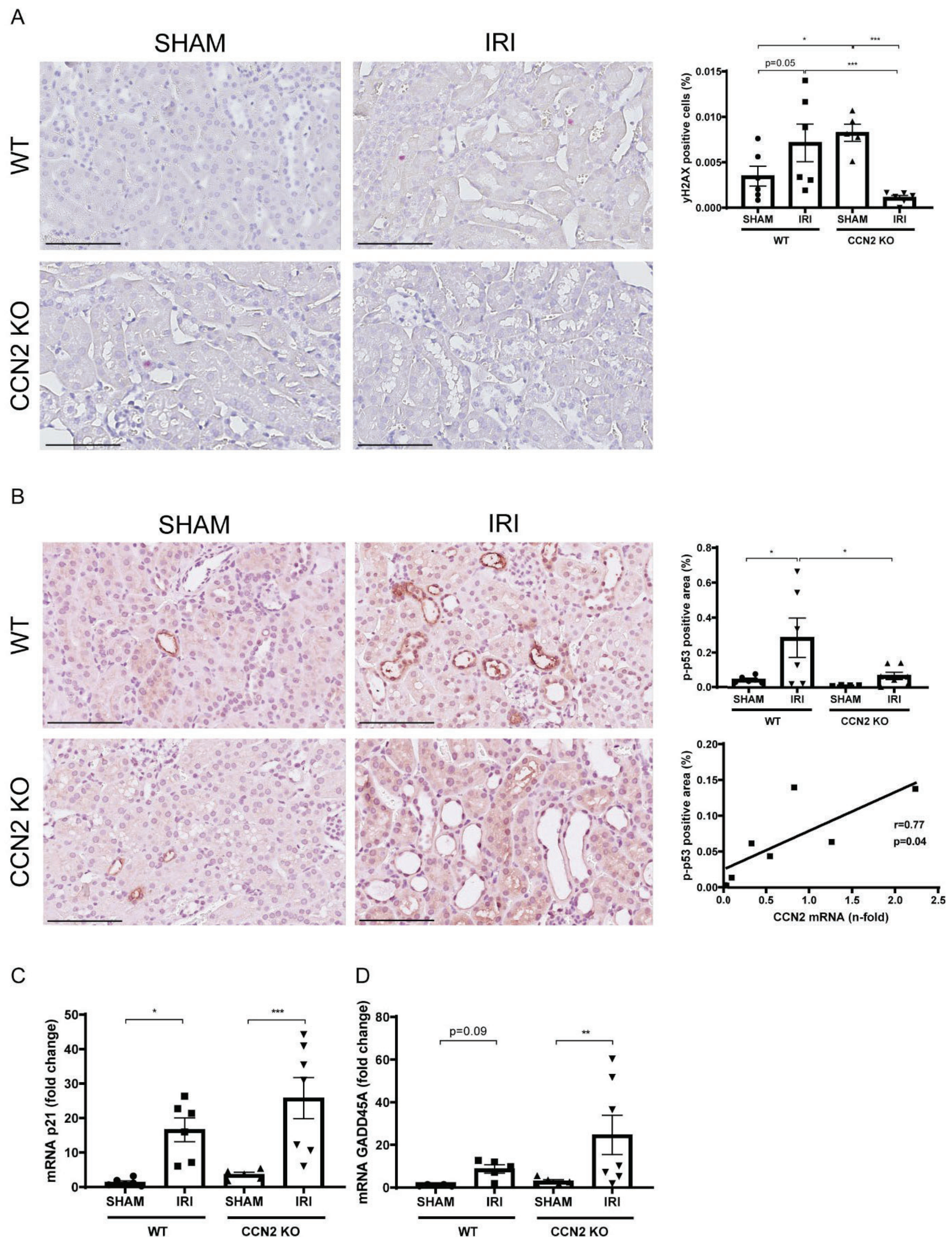

Figure 2. Near total deletion of CCN2 resulted in reduced DNA damage and p53 activation 4 hours after IRI
(A, B) Representative micrographs of mouse renal cortex stained with gamma H2AX (γH2AX; A) and phosphorylated p53 (p-p53; B). Quantification of γH2AX (A) and p-p53 (B) staining showed that increased DNA damage and p53 activation in IRI kidneys, was decreased in CCN2 KO mice compared with WT mice. Additionally, p-p53 expression correlated with CCN2 mRNA in CCN2 KO kidneys (B). **(C, D)** Quantitative real-time polymerase chain reaction (qPCR) analysis showed that mRNA expression levels of p21CIP1 (p21) and GADD45A were increased by IRI in WT and in CCN2 KO kidneys. Data are expressed as mean ± SEM (N=6 for WT sham; N=6 for WT IRI; N=4-5 for KO sham; N=6-7 for KO IRI). TBP was used as an internal control. *P<0.05, **P < 0.01, and ***P < 0.005. Bar = 100 μm.

CCN2 deletion reduces DDR and apoptosis resistance 3 days after IRI

To further evaluate protective effects of CCN2 deletion, we performed a 3 day IRI model. In this model, we previously observed less DNA damage and p21 upregulation in CCN2 KO kidneys compared to WT kidneys 3 days after IRI (Valentijn F.A., 2021). Furthermore, CCN2 KO IRI kidneys had lower numbers of p-p53 positive cells (p=0.02; Figure 3A) and reduced expression of GADD45A (p<0.005; Figure 3B) 3 days after IRI. Expression of p-p53 was predominantly nuclear and mainly observed in TECs (Figure 3A,B). Additionally, gene and protein expression levels of anti-apoptotic mediator Bcl-xL (p=0.04 and p=0.02; resp.; Figure 4A,B and gene expression level of HMGB1 (p<0.005; Figure 4C) were reduced in CCN2 KO injured mice.

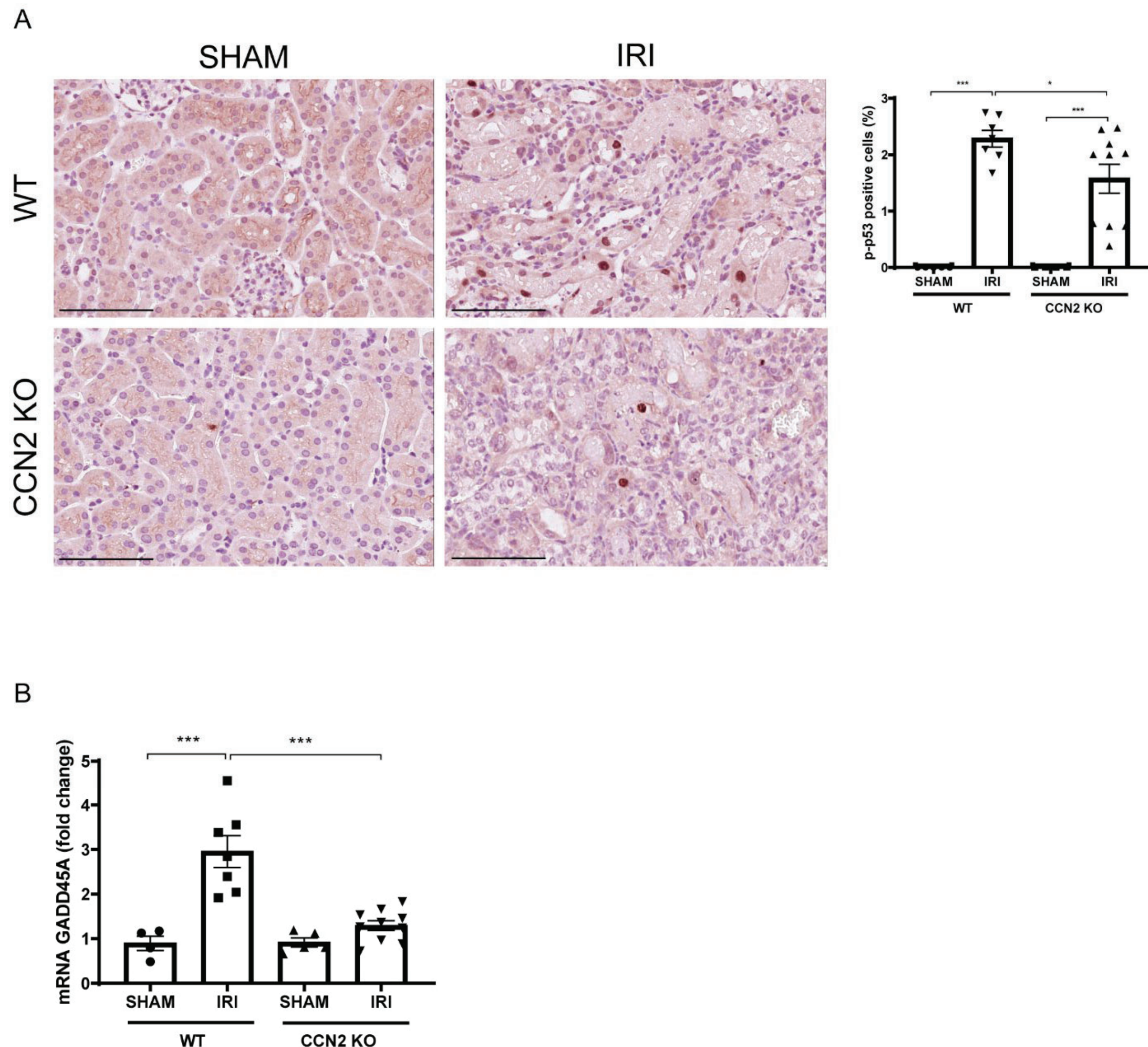

Figure 3. Near total deletion of CCN2 resulted in reduced p53 activation 3 days after IRI.
(A) Representative micrographs of mouse renal cortex stained with phosphorylated p53 (p-p53). Quantification of p-p53 staining showed that increased p53 activation in IRI kidneys, was decreased in CCN2 KO mice compared with WT mice. **(B)** qPCR analysis showed that increased mRNA expression of GADD45A in IRI kidneys was reduced in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=4-5 for WT sham; N=7 for WT IRI; N=4-5 for KO sham; N=10 for KO IRI). TBP was used as an internal control. *P<0.05 and ***P < 0.001. Bar = 100 μm.

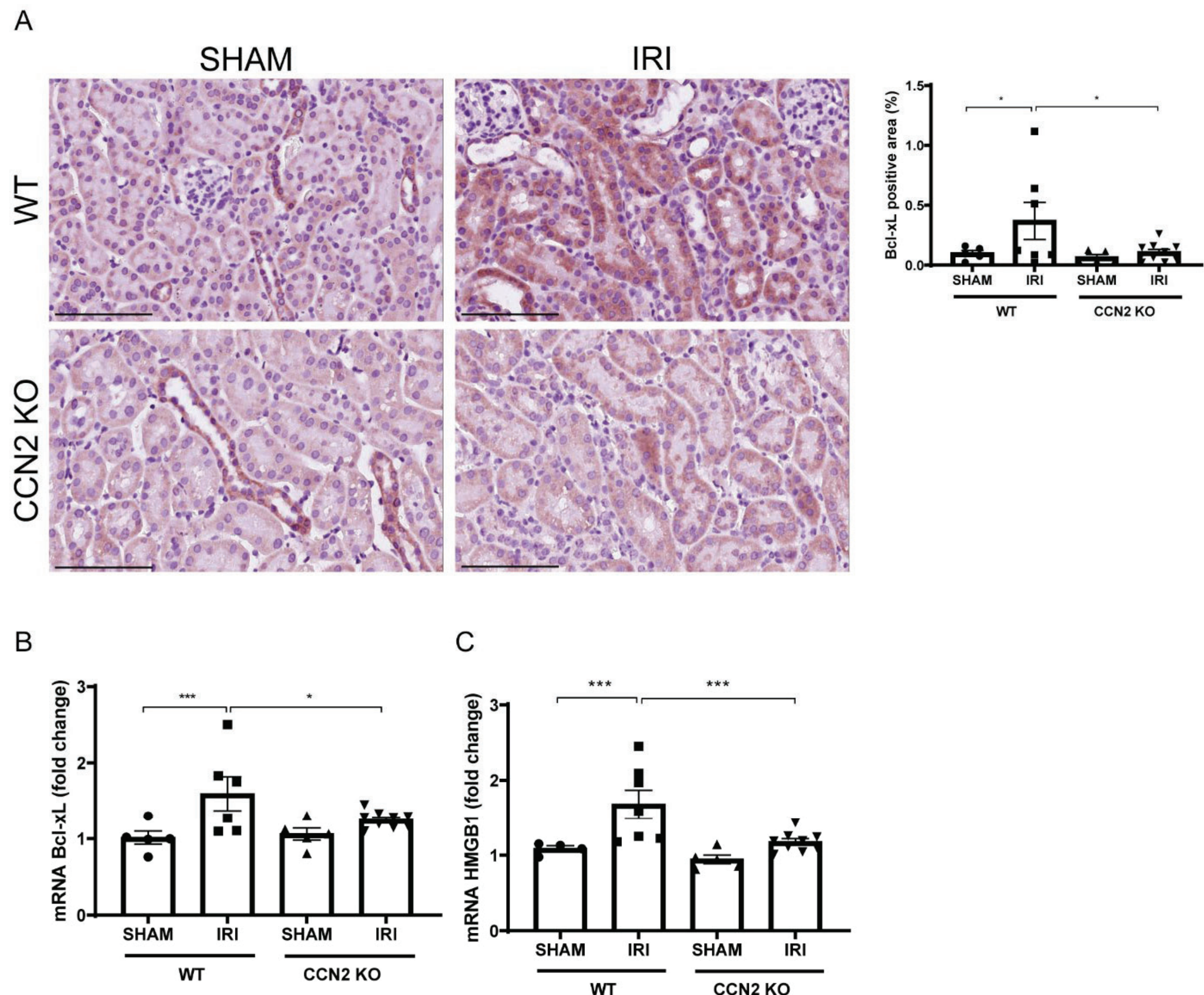

Figure 4. Near total deletion of CCN2 expression resulted in reduced Bcl-xL and HMGB1 anti-apoptosis expression 3 days after IRI
(A) Representative micrographs of mouse renal cortex stained with BclCL-xL suggested showed that increased Bcl-xL expression in IRI kidneys, was decreased in CCN2 KO mice compared with WT mice. **(B,C)** qPCR analysis showed that increased mRNA expression of Bcl-xL (B) and HMGB1 (C) in IRI kidneys wereas reduced in CCN2 KO mice compared with WT mice. (B) qPCR analysis showed that increased mRNA expression of HMGB1 in IRI kidneys was reduced in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=4-5 for WT sham; N=7 for WT IRI; N=5 for KO sham; N=9-10 for KO IRI). TBP was used as an internal control. *P<0.05 and ***P < 0.001. Bar = 100 µm.

CCN2 deletion reduces oxidative stress response 3 days after IRI

The role of CCN2 in oxidative stress response was evaluated by assaying ROS production and redox signaling. The lipid peroxidation product 4-Hydroxynonenal (4-HNE) and protein nitrosylation product nitrotyrosine, that mark ROS tissue levels, were not expressed 4 hours after IRI (data not shown). IRI induced upregulation of ROS scavenging erythroid 2-related factor 2 (Nrf2 target) genes Heme Oxygenase 1 (HMOX1) and Glutamate-Cysteine Ligase Catalytic Subunit (GCLC) (p=0.009 and p<0.005, resp.; Figure S3A,C) and tendencies for increased expression after IRI were observed for (NAD(P)H Quinone Dehydrogenase 1 (NQO1) and Glutamate-Cysteine Ligase Modifier Subunit (GCLM), but no differences in expression levels were observed between CCN2 KO and WT IRI kidneys (Figure S3A-D).

However, in the later model, 3 days after IRI, CCN2 KO kidneys showed lower 4-HNE expression (Figure 5A; p<0.005) and lower nitrotyrosine expression (Figure 5B; p<0.005) indicating that CCN2 KO reduced excessive ROS production upon IRI. Expression of these oxidative stress markers was mainly observed in proximal tubules of the inner cortex. Furthermore, reduced mRNA levels of Nrf2 target genes HMOX1 and NQO1 were observed in CCN2 KO compared to WT IRI kidneys (p<0,001 and p=0,001; Figure 5C, D).

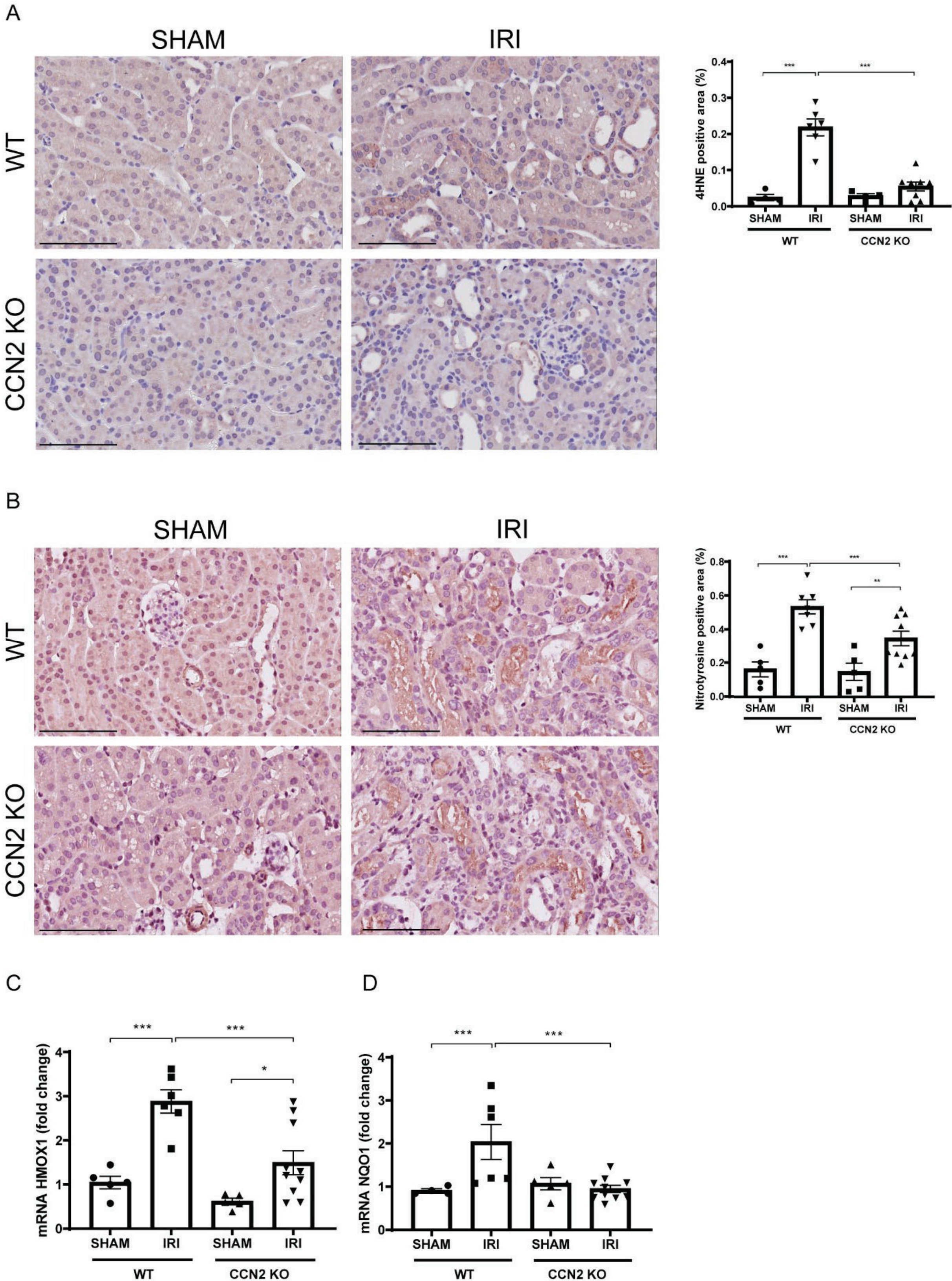

Figure 5. Near total deletion of CCN2 expression resulted in reduced oxidative stress response 3 days after IRI
(A, B) Representative micrographs of mouse renal cortex stained with 4-Hydroxynonenal (4-HNE; A) and nitrotyrosine (B). Quantification of 4-HNE (A) and nitrotyrosine (B) staining showed that IRI-induced increase of lipid peroxidation and protein nitrosylation respectively, were decreased in CCN2 KO mice compared with WT mice. **(C, D)** qPCR analysis showed that increased mRNA expression of HMOX-1 (C) and NQO1 (D) in IRI kidneys was significantly reduced in CCN2 KO mice compared with WT mice. Data are expressed as mean ± SEM (N=4-5 for WT sham; N=6-7 for WT IRI; N=4-5 for KO sham; N=8-10 for KO IRI). TBP was used as an internal control. *P<0.05, *P<0,01 and ***P < 0.001. Bar = 100μm.

CCN2 induces oxidative stress in mouse kidneys

To evaluate if CCN2 itself can induce an oxidative stress response, mice were injected with recombinant CCN2 and kidneys were harvested 24 hours later. Kidneys from CCN2 injected mice showed an increase in 4HNE expression compared to control mice. ($p<0.05$; Figure 6). Expression of 4-HNE was mainly observed in tubules (Figure 6).

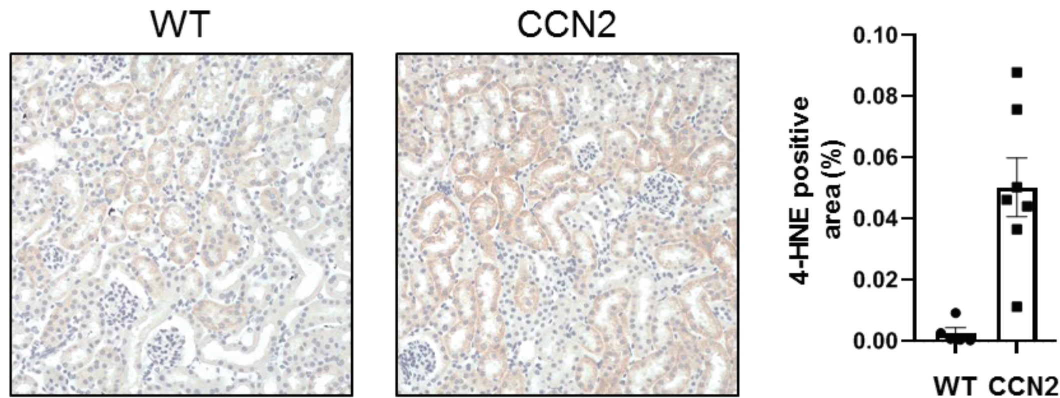

Figure 6. Administration of CCN2 induces oxidative stress in murine kidneys. Mice received intraperitoneal injections with 2,5ng/g of CCN2 and were sacrificed 24 hours later. Representative micrographs and quantification of mouse renal cortex stained with 4-Hydroxynonenal (4-HNE), showing 4-HNE positive tubules. Data are expressed as mean ± SEM of 4-HNE positive area per field (N=5 for control and N=7 for CCN2).

CCN2 silencing reduces oxidative stress induced by anoxia-reoxygenation injury in PTECs

The direct impact of CCN2 on oxidative stress in renal tubular cells was evaluated by silencing CCN2 in primary murine RPTECs subjected to anoxia-reoxygenation (AR) injury, mimicking *in vivo* IRI (Eleftheriadis et al., 2018), and showing a similar immediate upregulation of CCN2. (Valentijn F.A., 2021) AR-injured RPTEC showed more expression of p-p53, 4-HNE and nitrotyrosine compared to control cells that were cultured under normoxic conditions, and p-p53 and 4-HNE expression was alleviated when CCN2 was silenced (Figure 7A). No marked differences in Bcl-xL or HMGB1 expression were observed (data not shown). Finally, AR-injured RPTEC showed increased levels of lipid peroxidation marker MDA compared to controls cells that were cultured under normoxic conditions, and this was alleviated when CCN2 was silenced ($p<0.005$; Figure 7B). Taken together, silencing of CCN2 allevi-ated DDR and lipid peroxidation induced by anoxia-reoxygenation in-jury in cultured PTECs.

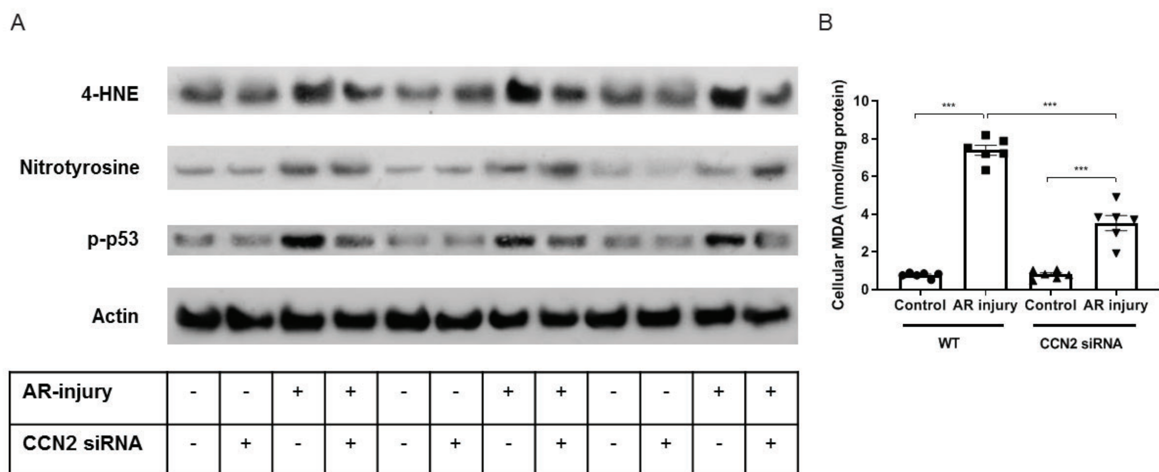

Figure 7. Silencing of CCN2 alleviates oxidative stress response induced by AR-injury in cultured PTECs
(A) Representative western blotting of protein extracts from cultured cells for 4-HNE, nitrotyrosine and p-p53 showed that increased oxidative stress response and DDR in AR-injured PTECs was alleviated by silencing of CCN2. Data represent three independent experiments. **(B)** Malondialdehyde (MDA) levels indicated that increased oxidative stress response in AR-injured PTECs was alleviated by silencing of CCN2. Data represent six independent experiments. Data are expressed as mean ± SEM. ***$P < 0.001$.

DISCUSSION

Our observations in the immediate early response phase following experimental IRI identify CCN2-dependent enrichment of several signaling pathways involved in the stress response to IRI. This was evidenced by differential gene expression at 4 hours after reperfusion, and follow-up analyses revealed that CCN2 deletion reduces DNA damage and DDR already at that time point, despite similar decline in kidney function and acute tubular damage. Subsequently, reduced DDR, anti-apoptosis and oxidative stress response were observed 3 days after IRI. Moreover, CCN2 silencing also reduced DDR and oxidative stress in cultured PTECs and intraperitoneally injected recombinant CCN2 induced oxidative stress in murine kidneys. CCN2 has previously been shown to contribute to vascular ROS generation (Rodrigues-Diez et al., 2015), and to DDR and subsequent cellular senescence in delayed graft function and chronic allograft dysfunction following kidney transplantation, and in experimental IRI (Valentijn F.A., 2021). These novel findings suggest that CCN2 not only acts as a profibrotic factor in later stages of adverse tissue remodeling, but that it also negatively contributes to the immediate early response to IRI, in particular to oxidative stress induced DDR (Figure 8). This could explain our previous findings that CCN2 deletion preserves kidney function 3 days after IRI, and contribute to reduced cellular senescence and fibrosis at later stages (Valentijn F.A., 2021). This implicates that anti-CCN2 therapy, currently in phase 2 and phase 3 trials for other indications, may be developed to reduce post-IRI acute and chronic kidney dysfunction by limiting IRI-induced oxidative stress induced DNA damage, senescent cell accumulation and subsequent fibrosis.

This study provides novel insights into the effect of CCN2 on the oxidative stress response following renal IRI. Few studies have previously observed that CCN2 is a potent inducer of oxidative stress in other organs and cell types. CCN2 induces oxidative stress in murine aorta and in cultured vascular smooth muscle cells and endothelial cells (Rodrigues-Diez et al., 2015). Furthermore, CCN2 induces accumulation of ROS in cultured fibroblasts (Juric et al., 2009). However, to our knowledge, this is the first report regarding CCN2-induced oxidative stress in the kidney, and the first demonstration of a protective effect of CCN2 inhibition on oxidative stress induced by other factors. Remarkably, CCN2 overexpression has also been described to limit oxidative stress. For instance, CCN2 protected cultured cardiac myocytes from doxorubicin-induced oxidative stress and cell death (Moe et al., 2013). Possibly, the complex and diverse mechanisms of action of CCN2, might lead to different outcomes depending on the cell type and context, including matrix, cytokine environment, and genotype (Lipson et al., 2012).

Main mechanisms by which CCN2 might contribute to oxidative stress following renal IRI include increased mitochondrial oxidative phosphorylation and ROS generation, and decreased ROS scavenging. First, CCN2 may interfere with mitochondrial oxidative phosphorylation, which is implicated in renal IRI (Huang et al., 2018). GSEA revealed enrichment of the oxidative phosphorylation pathway 4 hours after IRI in the direction of lower activation in CCN2 KO IRI- compared to WT IRI-mice. Consistently, in murine chondrocytes, CCN2 deletion impaired metabolism with reduced intracellular ATP levels (Maeda-Uematsu A et al., 2014). Upon reperfusion of the kidney, restored levels of oxygen stimulate mitochondrial oxidative phosphorylation to produce ATP, the main source of cellular energy, with the concurrence of harmful ROS (Devarajan 2006; Jassem & Heaton, 2004). Thus, altered ATP production depending on CCN2 expression level could affect the level of ROS and subsequent DNA damage upon renal IRI. However, a contrary effect of CCN2 on ROS generation has also been described in Oral Squamous Cell Carcinoma cells, where CCN2 overexpression impaired mitochondrial oxidative phosphorylation (Lai et al., 2018). A possible effect of CCN2 inhibition on oxidative phosphorylation and underlying mechanisms in the setting of renal IRI remains to be elucidated.

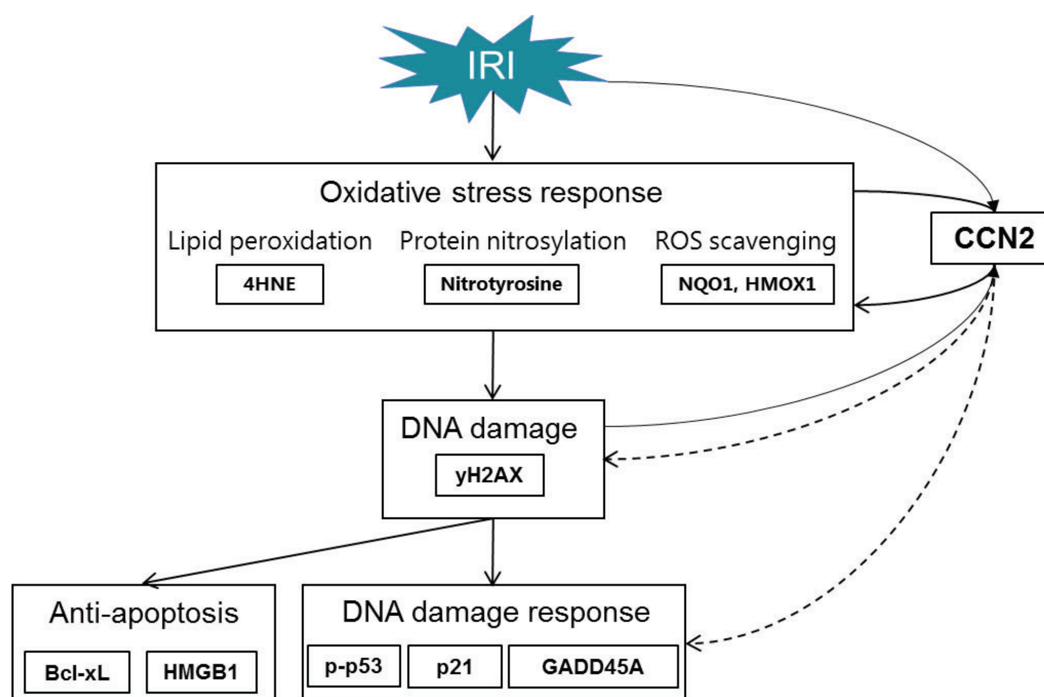

Figure 8. Mechanisms involved in IRI linked with CCN2. Inhibition of CCN2 reduced the expression of γH2AX and p-p53 marking reduced DNA damage and DDR in the immediate early response following IRI. In the early phase upon IRI, this was followed by sustained reduction in DNA damage and DDR, along with reduced anti-apoptosis. This was associated with reduced oxidative stress response. Dotted arrows are used to acknowledge that the exact mechanisms by which CCN2 contributes to DNA damage and DDR remain to be established.

Underlying pathways linking CCN2 and the oxidative stress response may include epidermal growth factor receptor (EGFR) activation and NAD(P)H oxidases (Nox)1 activity, which have previously been linked to CCN2 induced ROS generation (Rodrigues-Diez et al., 2015). Crosstalk between CCN2 and ROS producing metabolic pathways may also involve integrin interactions. CCN1 induces ROS through integrin signaling (Chen et al., 2007), and engegament of integrin α6β1 and cell surface heparan sulfate proteoglycans (HSPGs) (Juric et al., 2009). The mechanisms of action of CCN2 and CCN1 may well be similar in several respects, since they are highly homologous and bind the same receptors (Chen et al., 2001, 2007). Alternatively, reduced CCN2 might improve the handling of ROS by antioxidant mechanisms. However, regarding Nrf2 target genes, no differences in gene expression levels of GCLC and GCLM were observed, and reduced rather than increased expression levels of HMOX1 and NQO1 were found in the CCN2-depleted mice.

In aggregate, our observations indicate that DNA damage and DDR in the kidney following IRI, are mitigated by CCN2 inhibition through reduction of the oxidative stress response in tubular epithelial cells. Anti-CCN2 therapy might therefore be explored for its potential to help limiting post-IRI AKI, and AKI to CKD progression.

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Chapter 4. CCN2 aggravates immediate Oxidative Stress and DDR following renal IRI

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SUPPLEMENTARY MATERIAL

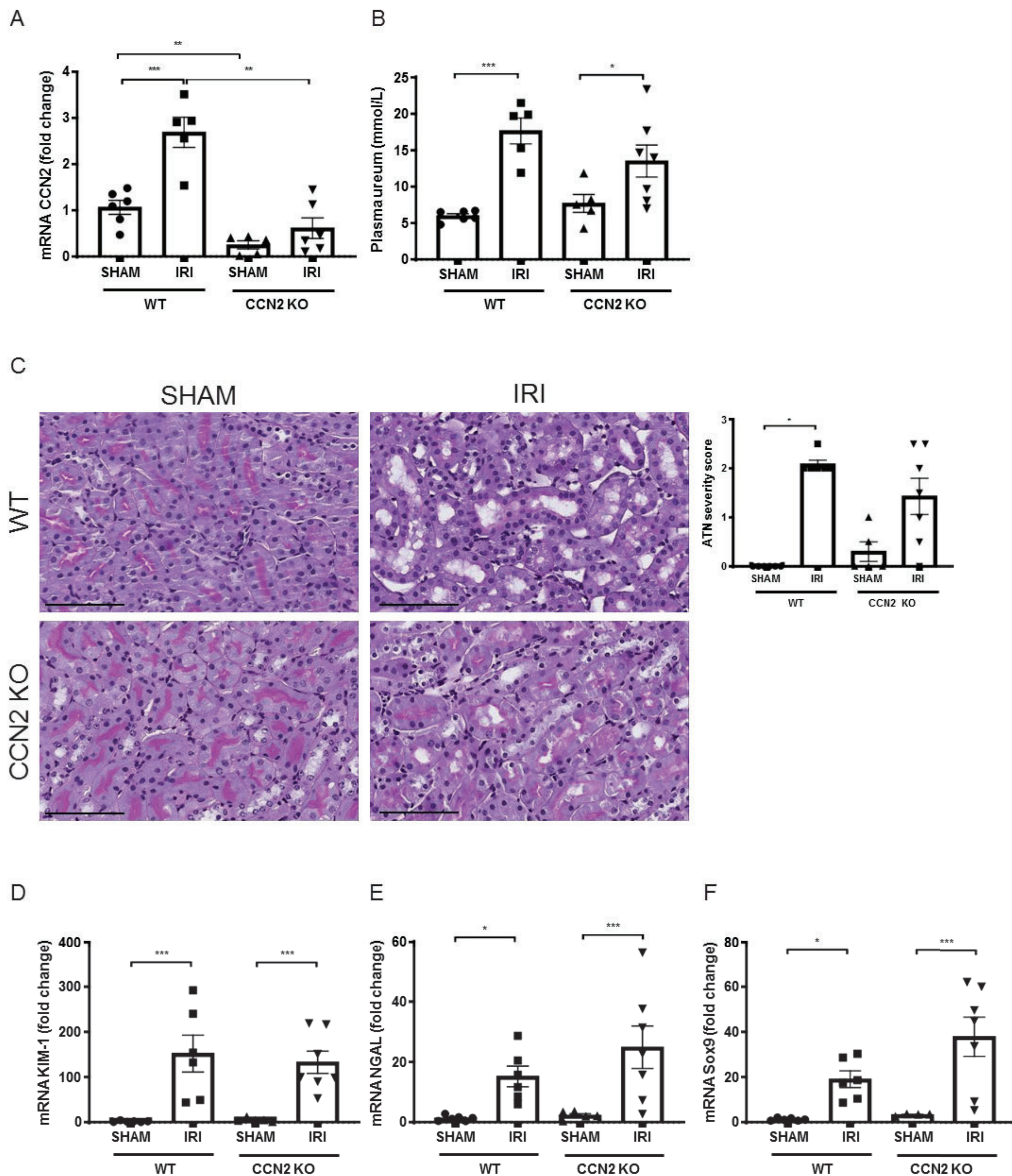
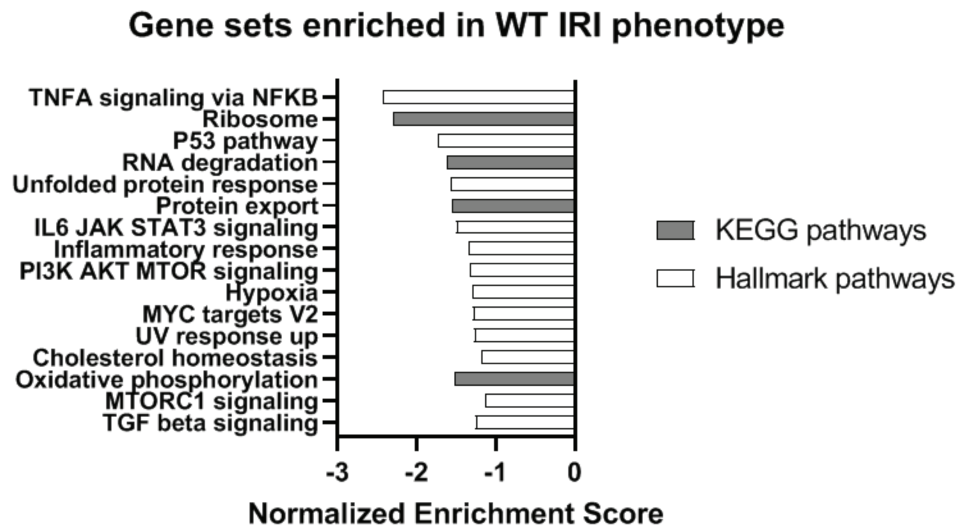


Figure S1: Near total deletion of CCN2 does not reduce functional decline and acute tubular necrosis 4 hours after IRI **(A)** qPCR analysis showed that CCN2 mRNA expression was decreased after sham and IRI surgery in CCN2 KO mice compared with wild type (WT) mice. **(B)** Measurement of plasma urea levels showed that IRI-induced decline in kidney function was similar in CCN2 KO mice and WT mice. **(C)** Representative micrographs and quantification of acute tubular necrosis (ATN) histology of mouse renal cortex stained with periodic acid-Schiff (PAS) showed that increased acute tubular injury in IRI kidneys was not decreased in CCN2 KO mice compared with WT mice. **(D-F)** qPCR analysis showed that mRNA expression levels of KIM-1 (C), NGAL (D) and SOX9 (E) were increased by IRI in WT and in CCN2 KO kidneys. Data are expressed as mean ± SEM (N=6 for WT sham; N=5-6 for WT IRI; N=4-5 for KO sham; N=6-7 for KO IRI). TBP was used as an internal control. *P<0.05, **P < 0.01, and ***P < 0.005. Bar = 100 μm.

A



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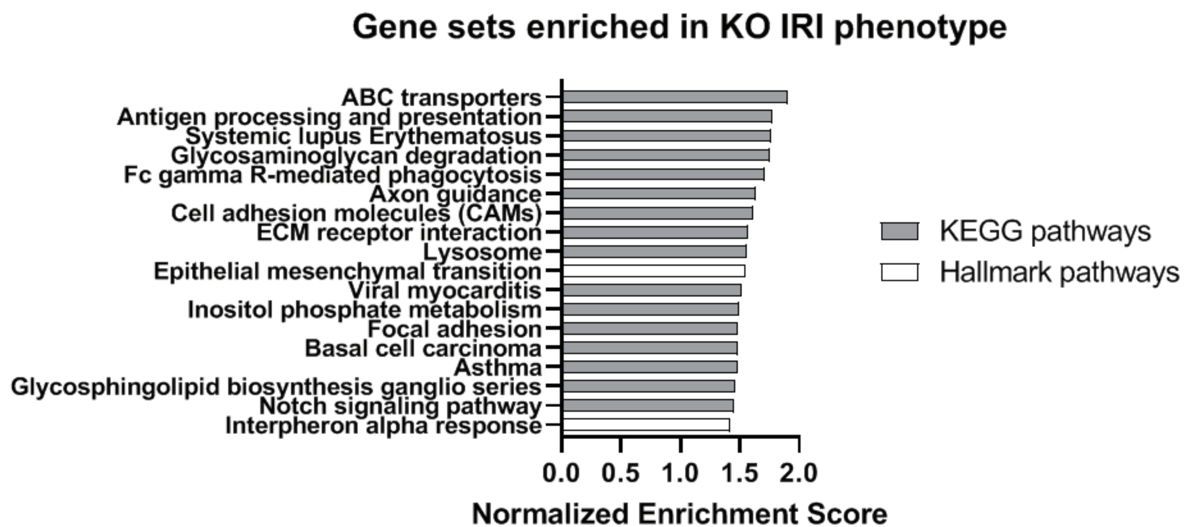

Figure S2: Near total deletion of CCN2 expression resulted in differential regulation of several pathways 4 hours after renal IRI
Gene set enrichment analysis (GSEA) on the whole gene set of kidneys from CCN2 KO IRI mice compared to WT IRI mice revealed enrichment of several KEGG pathways and Hallmark pathways in the WT IRI phenotype (A) and in the CCN2 KO IRI phenotype (B). Pathways are ranked based on the normalized enrichment score.

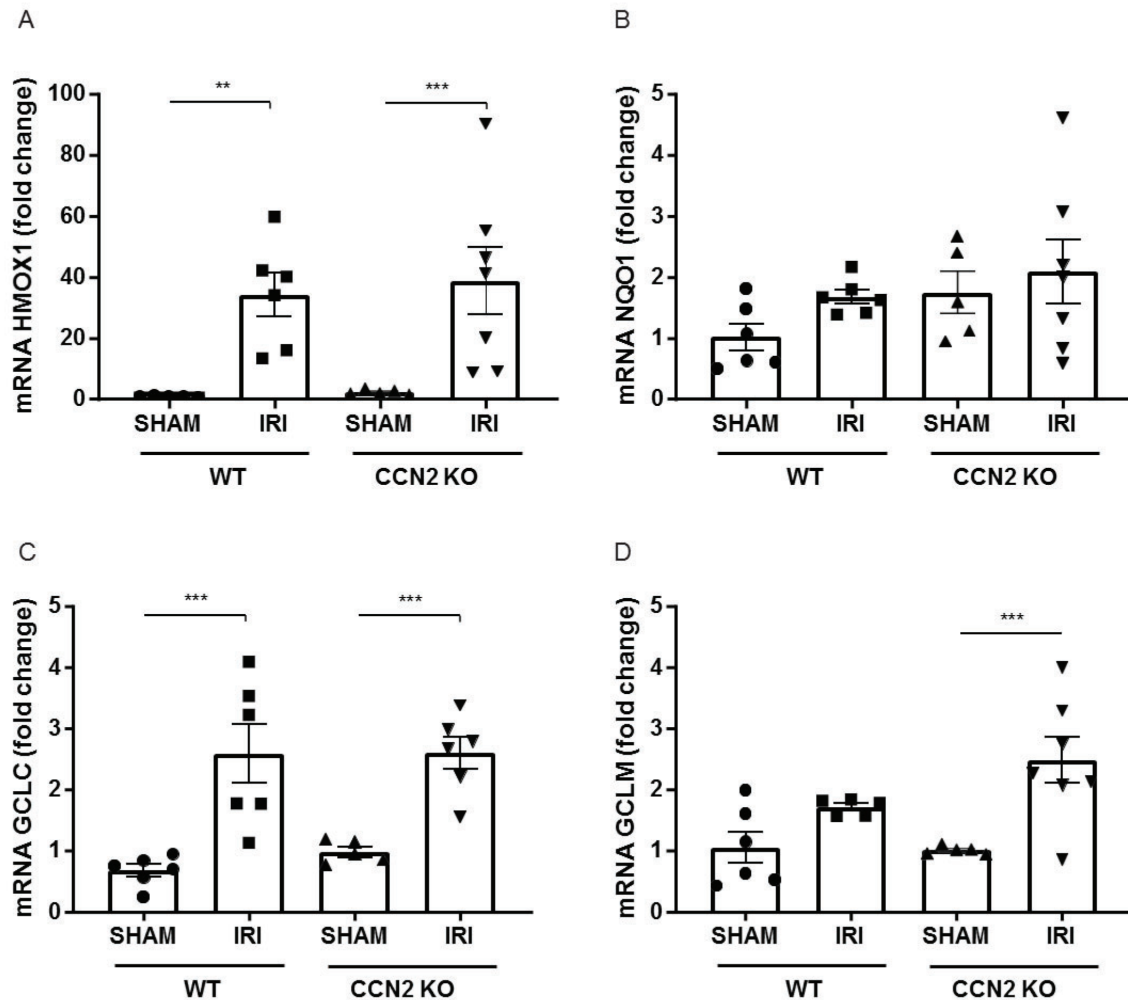

Figure S3: Near total deletion of CCN2 expression resulted in similar Nrf2 target gene expression 4 hours after IRI
qPCR analysis of HMOX1 (A), NQO1 (B), GCLC (C) and GCLM (D). Data are expressed as mean ± SEM (N=5-6 for WT sham; N=5-6 for WT IRI; N=5 for KO sham; N=6-7 for KO IRI). TATA-box binding protein (TBP) was used as an internal control. *P<0.05 and ***P < 0.001.

Table S1: Primers used for real-time polymerase chain reaction

Gene	SYBR Green primer sequence or Taqman Gene Expression Assay	accession	amplicon size
HMOX1	Fwd: AGGGTCAGGTGTCCAGAGAA Rev: CTTCCAGGGCCGTGTAGATA	NM_010442.2	72
NQO1	Fwd: AGCGTTCGGTATTACGATCC Rev: AGTACAATCAGGGCTCTTCTCG	NM_008706.5	68
GCLC	Fwd: AGATGATAGAACACGGGAGGAG Rev: TGATCCTAAAGCGATTGTTCTTC	NM_010295.2	62
GCLM	Fwd: TGACTCACAATGACCCGAAA Rev: TCAATGTCAGGGATGCTTTCT	NM_008129.4	79
HMGB1A	Fwd: TGGGCGACTCTGTGCCTC Rev: GCCTCTCGGCTTTTTAGGATC	NM_010439.4	72
TBP	Mm00446971_m1	NM_013684.3	93
CCN2	Mm01192932_g1	NM_010217.2	61
KIM1	Mm00506686_m1	NM_134248.2	94
NGAL	Mm01324470_m1	NM_008491.1	84
SOX9	Mm00448840_m1	NM_011448.4	101
CDKN1A	Mm00432448_m1	NM_007669.4	96
GADD45A	Mm00432802_m1	NM_007836.1	59

Fwd = forward primer; Rev = reverse primer.

Table S2: Differentially expressed genes and enriched pathways in pairwise comparisons between all groups
Table S2 is available on: https://www.mdpi.com/2076-3921/10/12/2020

Table S3: Differentially expressed genes with the largest fold down- or upregulation in the pairwise comparison between CCN2 KO IRI mice and WT IRI mice

Gene	Full gene name	log2 FC §	lfcSE	p value &
Rap2b*	RAP2B (member of RAS oncogene family)	2,106	0,238	0,000
Frat2*	FRAT regulator of WNT signaling pathway 2	1,902	0,297	0,000
Ier2*	immediate early response 2	1,780	0,209	0,000
Cxcl1	C-X-C motif chemokine ligand 1	1,718	0,306	0,000
Cebpb	CCAAT enhancer binding protein beta	1,686	0,215	0,000
Cxcl10	C-X-C motif chemokine ligand 10	1,667	0,200	0,000
Kif23	kinesin family member 23	1,660	0,237	0,000
Csrnp1	cysteine and serine rich nuclear protein 1	1,505	0,227	0,000
Vgf	VGF nerve growth factor inducible	1,486	0,313	0,000
Maff	MAF bZIP transcription factor F	1,484	0,307	0,000
Junb	jun B proto-oncogene	1,482	0,186	0,000
Btg1	BTG anti-proliferation factor 1	1,478	0,190	0,000
Pde10a	phosphodiesterase 10A	1,449	0,268	0,000
Ppp1r15a	protein phosphatase 1 regulatory subunit 15A	1,427	0,279	0,000
Btg2	BTG anti-proliferation factor 2	1,376	0,210	0,000
Klhdc7a	kelch domain containing 7A	-1,681	0,212	0,000
Flrt1	fibronectin leucine rich transmembrane protein 1	-1,665	0,231	0,000
Fads6	fatty acid desaturase 6	-1,644	0,227	0,000
Il22ra1	interleukin 22 receptor subunit alpha 1	-1,612	0,313	0,000
Ccnd1	cyclin D1	-1,526	0,182	0,000
Prox1	prospero homeobox 1	-1,500	0,303	0,000
Gpam	glycerol-3-phosphate acyltransferase (mitochondrial)	-1,485	0,159	0,000
Tmem88b	transmembrane protein 88B	-1,482	0,246	0,000
Adamts15	ADAM metallopeptidase with thrombospondin type 1 motif 15	-1,468	0,248	0,000
Syt17	synaptotagmin 17	-1,462	0,251	0,000
Cyp2u1	cytochrome P450 family 2 subfamily U member 1	-1,455	0,279	0,000
Bsnd	barttin CLCNK type accessory beta subunit	-1,449	0,186	0,000
Fam78a	family with sequence similarity 78 member A	-1,447	0,272	0,000
Cyp26b1	cytochrome P450 family 26 subfamily B member 1	-1,408	0,299	0,000
Tnrc18	trinucleotide repeat containing 18	-1,399	0,210	0,000

§ Positive Log2 fold change values represent lower expression in CCN2 KO IRI vs WT IRI; Negative Log2 fold change values represent higher expression in CCN2 KO IRI vs WT IRI. & False discovery rate adjusted p-values. Abbreviations: CCN2 = cellular communication network factor 2; KO = knockout; IRI = ischemia-reperfusion injury; WT = wild type; FC = fold change; SE = standard error

CHAPTER 5
Acute kidney injury is aggravated in aged mice by the exacerbation of proinflammatory processes

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Chapter 5. AKI is aggravated by exacerbated proinflammatory processes in elderly

ABSTRACT

Acute kidney injury (AKI) is more frequent in elderly patients. Mechanisms contributing to AKI (tubular cell death, inflammatory cell infiltration, impaired mitochondrial function, and prolonged cell cycle arrest) have been linked to cellular senescence, a process implicated in regeneration failure and progression to fibrosis. However, the molecular and pathological basis of the age-related increase in AKI incidence is not completely understood. To explore these mechanisms, experimental AKI was induced by folic acid (FA) administration in young (3-months-old) and old (1-year-old) mice, and kidneys were evaluated in the early phase of AKI, at 48 h. Tubular damage score, KIM-1 expression, the recruitment of infiltrating immune cells (mainly neutrophils and macrophages) and proinflammatory gene expression were higher in AKI kidneys of old than of young mice. Tubular cell death in FA-AKI involves several pathways, such as regulated necrosis and apoptosis. Ferroptosis and necroptosis cell-death pathways were upregulated in old AKI kidneys. In contrast, caspase-3 activation was only found in young but not in old mice. Moreover, the anti-apoptotic factor BCL-xL was significantly overexpressed in old, injured kidneys, suggesting an age-related apoptosis suppression. AKI kidneys displayed evidence of cellular senescence, such as increased levels of cyclin dependent kinase inhibitors p16ink4a and p21cip1, and of the DNA damage response marker γH2AX. Furthermore, p21cip1 mRNA expression and nuclear staining for p21cip1 and γH2AX were higher in old than in young FA-AKI mice, as well as the expression of senescence-associated secretory phenotype (SASP) components (*Il-6, Tgfb1, Ctgf,* and *Serpine1*). Interestingly, some infiltrating immune cells were p21 or γH2AX positive, suggesting that molecular senescence in the immune cells (“immunosenescence”) are involved in the increased severity of AKI in old mice. In contrast, expression of renal protective factors was dramatically downregulated in old AKI mice, including the antiaging factor Klotho and the mitochondrial biogenesis driver PGC-1α. In conclusion, aging resulted in more severe AKI after the exposure to toxic compounds. This increased toxicity may be related to magnification of proinflammatory-related pathways in older mice, including a switch to a proinflammatory cell death (necroptosis) instead of apoptosis, and overactivation of cellular senescence of resident renal cells and infiltrating inflammatory cells.

Chapter 5. AKI is aggravated by exacerbated proinflammatory processes in elderly

INTRODUCTION

Acute kidney injury (AKI) is a common and devastating pathologic condition in part due its higher incidence in the elderly and its association with an increased short- and longterm mortality (Levey and James, 2017; Hounkpatin et al., 2019; Martin-Cleary et al., 2019; Logan et al., 2020). Moreover, AKI is closely related to chronic kidney disease (CKD) as AKI may accelerate CKD progression to end-stage renal disease (ESRD) and CKD predisposes to AKI (Venkatachalam et al., 2015; Siew et al., 2016; Ruiz-Ortega et al., 2020). All these facts underscore the importance of the research in this area. Furthermore, the cellular and molecular mechanisms of the increased sensitivity to AKI in elderly patients are incompletely understood (Mehran et al., 2019; Infante et al., 2020; Aleckovic-Halilovic et al., 2021), hampering the design of any preventive or therapeutic approaches.

Kidney tubular cells comprise the bulk of the kidney cell mass and may be injured by hypoxia, toxic compounds, metabolic disorders and proteinuria, among other factors. In response to an insult, tubular epithelial cells undergo phenotype changes associated with tubular function impairment and activation of inflammatory, fibrotic and cell death pathways, which may reflect a state of cellular senescence (Linkermann et al., 2014; RuizOrtega et al., 2020). The initial phase of AKI is followed by a recovery phase characterized by activation of protective and regenerative mechanisms that restore epithelial properties and functions in surviving cells (Yang et al., 2010). Tubular cell death in AKI can involve several cell death pathways, such as apoptosis and regulated necrosis (Linkermann and Green, 2014). Cells dying by regulated necrosis release intracellular molecules, called damage-associated molecular patterns (DAMPs), which amplify the inflammatory response by the activation of neutrophils and other immune cells in a process termed necroinflammation. There are several forms of regulated necrosis, including necroptosis, ferroptosis, and pyroptosis (Newton and Manning, 2016). Necroptosis, the best characterized form of regulated apoptosis, is elicited by the binding of the receptor-interacting protein 1 (RIPK1) to RIPK3, leading to its oligomerization and autophosphorylation. Then, the active RIPK1-RIPK3 complex (also called necrosome) activates the pseudokinase mixed lineage kinase domain-like protein (MLKL), which translocates to the cellular membrane, causing cell membrane permeabilization, rupture, and subsequent cell death (Newton and Manning, 2016). Necroptosis plays an important role in experimental AKI, as described in renal ischemia/reperfusion injury (IRI), folic acid (FA)-AKI and cisplatin nephropathy (Linkermann et al., 2013; Linkermann and Green, 2014; Xu et al., 2015; Martin-Sanchez et al., 2017; Martin-Sanchez et al.,2018a). Ferroptosis, a caspase-independent cell death pathway, is characterized by reduced glutathione activity or content, reduced glutathione peroxidase 4 (GPX4) protein levels, massive lipid peroxidation and cell loss (Martin-Sanchez et al., 2020). Targeting ferroptosis by chemical inhibition or gene expression modulation reduced tubular injury and improved renal function in different experimental models, including IRI and FA-AKI (Martin-Sanchez et al., 2017; Martin-Sanchez et al., 2020).

Cellular senescence represents a maladaptive response to AKI, characterized by prolonged cell cycle arrest (Melk et al., 2004; Bonventre, 2014; Gorgoulis et al., 2019). Following an initial insult, DNA damage activates a protective mechanism consisting in the arrest of the cell cycle and the activation of the DNA damage response (DDR) to facilitate DNA repair. After successful DNA repair, cells re-enter the cell cycle (Branzei and Foiani, 2008). Nevertheless, persistent activation of this protective mechanism can contribute to damage, as observed in disease conditions associated with cellular senescence (Gire and Dulic, 2015). Regarding the kidney, accumulation of senescent (in particular tubular epithelial) cells has been implicated in regeneration failure and AKI-to-CKD transition (Schmitt and Cantley, 2008; O'Sullivan et al., 2017; Kim et al., 2019). In this sense, prolonged tubular epithelial cell cycle arrest, sustained inflammation, and impaired mitochondrial function can contribute to CKD progression (Levey and James, 2017; Andrade et al., 2018; Sato and Yanagita, 2018; Jiang et al., 2020). Cellular senescence or premature aging in the kidney is characterized by increased expression of some cell cycle-related molecules such as the cyclin kinase inhibitors p16ink4a, p21cip, and p53 (Melk et al., 2004; Andrade et al., 2018; Knoppert et al., 2019; Koyano et al., 2019). Senescent cells are also

characterized by a detrimental secretome known as senescence-associated secretory phenotype (SASP) (Melk et al., 2004; Bonventre, 2014). This secretome is enriched with proinflammatory cytokines, growth factors and profibrotic proteins such as IL-6, TGF-β, CTGF/CCN2 and PAI-1 (Acosta et al., 2013; Zhou et al., 2020) and is able to spread the senescence phenotype to neighboring cells (paracrine senescence) (Acosta et al., 2013), and to promote kidney fibrosis (Ferlicot et al., 2003; Melk et al., 2004; McGlynn et al., 2009; Yang et al., 2010; Günther et al., 2017; Valentijn et al., 2018). While inflammation is one of the first steps in tissue repair, persistent inflammation contributes to CKD progression (Cao et al., 2015; Rabb et al., 2016; Sato and Yanagita, 2018). Cytokines and interleukins within the SASP contribute to enduring inflammation and to further tubular cell injury and dysfunction (Kirkland and Tchkonia, 2017). Moreover, a low-grade inflammatory milieu is known to be present in the aged tissues, a condition named “inflammaging” (Greene and Loeser, 2015; Rea et al., 2018). “Immunosenescence” is a related concept, in which the dysfunctional immune response in the elderly presents characteristics related to cellular senescence and promotes inflammation, thus playing a crucial role in inflammaging (Sato and Yanagita, 2019; Schroth et al., 2020).

In the present study, we sought to elucidate the potential cellular and molecular mechanisms contributing to the increased severity of AKI in old age. To this aim, we have investigated whether aging-related processes, such as induction of cellular senescence, inflammaging and loss of renal protective factors, can modulate tubular damage, including cell death pathways activation and phenotype changes induced by AKI. Previous experimental studies have reported age-related exacerbation of renal injury in different AKI models. Now, we have investigated the FA-AKI model that presents a different mechanism of kidney injury (crystalluria with intratubular obstruction) than those in prior studies (cytokine storm) or on exogenous (cisplatin) or endogenous (heme) molecules that are directly toxic to tubular cells (Maddens et al., 2012; Nath et al., 2013; Wen et al., 2015). Aging is a process that has no fixed start date and does not occur suddenly. Rather, human glomerular filtration rate starts decreasing progressively from age 18–24 years (Wetzels et al., 2007). Most previous experimental studies on AKI and aging used mice from 15 to 18 months old (Maddens et al., 2012; Nath et al., 2013; Wen et al., 2015). However, in a sepsis AKI model an increase in mortality was already observed at 12 months (Maddens et al., 2012). Therefore, this time point was chosen to investigate early age-associated changes that could be responsible for increased AKI susceptibility.

MATERIALS AND METHODS

Experimental Model of Acute Kidney Injury Induced by Toxins

C57BL/6 mice were originally obtained from JAX™ Mice (Charles River Europe laboratory) and then the mouse colony breeding was maintained in the Fundación Jimenez Diaz Animal facilities, following JAX™ recommendations (Jackson Laboratory, 2007). Animals were fed with a standard diet provided by the animal facilities. Young (3-month-old) and old (1-year-old) C57BL/6 male mice were injected intraperitoneally with 125 mg/kg folic acid (FA) dissolved in sodium bicarbonate. Body weight was similar in young and old mice (26.1 g in young vs. 29.8 g in older mice). Previous studies have demonstrated that the lethal dose for FA (lethality dependent on AKI) varies by more than 3-fold in different mouse strains (Parchure et al., 1985). In addition, in a sepsis AKI model has reported lethality at 12 months (Maddens et al., 2012). Therefore, to decrease the high risk of death in old mice, we used a lower FA dose than in prior studies (125 mg/kg instead of 250 mg/kg) (Linkermann et al., 2013; Martin-Sanchez et al., 2018a), and mice of 12 months. As observed in results, this dose induced a significant tubular damage with no death associated at this time point. Five to ten mice per group were studied in the early phase of AKI, after 48 h of FA injection. Untreated mice of the same age were used as their corresponding controls.

Animals were euthanatized by CO2 inhalation. The kidneys were perfused in situ with saline before removal, and half of each kidney (2/4) was fixed, embedded in paraffin, and used for immunohistochemistry, while the rest was snap-frozen in liquid nitrogen for renal

cortex RNA and protein studies. Kidneys from all groups were compared to control kidneys from young mice, expressing results as fold-change over control values of 1.

Protein Studies
Total proteins were isolated from frozen kidney tissue using an appropriate lysis buffer as previously described (Rodrigues-Diez et al., 2013) and quantified using a BCA protein assay kit (ThermoScientific). Proteins (50 µg) were separated on 8–15% acrylamide gels using the SDS-PAGE, as described (Rodrigues-Diez et al., 2013). Briefly, after electrophoresis, samples were transferred on to polyvinylidenedifluoride membranes (Millipore) blocked in TBS containing 0.1% Tween 20 (TBST) and 5% dry non-fat milk for 1 h at room temperature and incubated in the same buffer with different primary antibodies overnight at 4°C. After washing with TBST, membranes were incubated with the appropriate HRP (horseradish peroxidase)conjugated secondary antibody (Invitrogen) 1 h at room temperature and developed using an ECL kit (Amersham Biosciences). Results were analyzed by LAS 4,000 and Amersham Imager 600 (GEHealthcare) and densitometered by Quantity One software (Biorad). The following primary antibodies were employed [dilution]: MLKL ([1:1,000], ab172868, abcam), α-tubulin ([1:5,000], T5168, Sigma-Aldrich) and α-Cleaved Caspase 3 ([1:1,000, #9661S, Cell Signaling). The evaluation of IL-6 in kidney tissue was done by ELISA (BD Biosciences, Cat. No. 555240) following the instructions provided by the manufacturer.

Histology and Immunohistochemistry
Paraffin-embedded kidney sections were stained using standard histology procedures, as described elsewhere (Rodrigues-Diez et al., 2013). Tubular damage and inflammatory infiltrate were scored as arbitrary units on periodic acid-Schiff (PAS, SigmaAldrich) stained slides as previously described (Zoja et al., 2002). Immunostaining was carried out in 3 µm thick tissue sections. Antigens were retrieved using the PTlink system (DAKO) with sodium citrate buffer (10 mM) adjusted to pH 6–9, depending on the immunohistochemical marker. Endogenous peroxidase was blocked. Tissue sections were incubated for 1 h at room temperature with 1X Casein Solution (Vector Laboratories) to eliminate non-specific protein binding sites. Primary antibodies were incubated overnight at 4°C and diluted in antibody solution (DAKO). Specific HRP-conjugated (DAKO) or biotinylated secondary antibodies (Amersham Biosciences) were used for 1 h followed by Avidin-Biotin Complex incubation (Vector Laboratories). Signal was developed with substrate solution and 3,3-diaminobenzidine as a chromogen (Abcam). Then sections were counterstained with Carazzi's haematoxylin (Richard Allan Scientific). The primary antibodies used were [dilution]: KIM-1 ([1:500]; AF 1817, R&D), P21 ([1:2,000, Ab188224, Abcam), γH2AX [1:1,000], NB1002280 Novus Biological), BCL-xL ([1:4,000], ab178844, Abcam), F4/80 ([1: 50]; MCA497, Bio-Rad), CD3 ([1:100], A0452, DAKO), Myeloperoxidase ([1X], IS511, DAKO) and 4-Hydroxynonenal ([1:1,000], Ab46545, Abcam). Specificity was checked by omission of primary antibodies (not shown). Quantification was made by using the Image-Pro Plus software (Maryland, United States) determining the positive staining area relative to the total area or counting positive staining manually (in the case of inflammatory cells), in 5–10 randomly chosen fields (× 200 magnification).

Gene Expression Studies
RNA from renal cortex was isolated with TriPure reagent (Roche). cDNA was synthesized by a High Capacity cDNA Archive kit (Applied Biosystems) using 2 µg of total RNA and following the manufacturer's instructions. Quantitative gene expression analysis was performed on an AB7500 fast realtime PCR system (Applied Biosystems) using fluorogenic TaqMan MGB probes and primers designed by Assay-onDemand™ gene expression products. Mouse assays IDs were: *p21cip1*: Mm00432448_m, *p16ink4a*: Mm00494449_m1, *Klotho*: Mm00502002_m1, *Bcl2l1*: Mm004337783_m1, *Il6:* Mm00446190_m1, *Lcn2*: Mm01324470_m1, *Havcr1*/*Kim1*: Mm00506686_m1, *Ctgf/Ccn2*: Mm01192933_g1, *Ccl-2*:

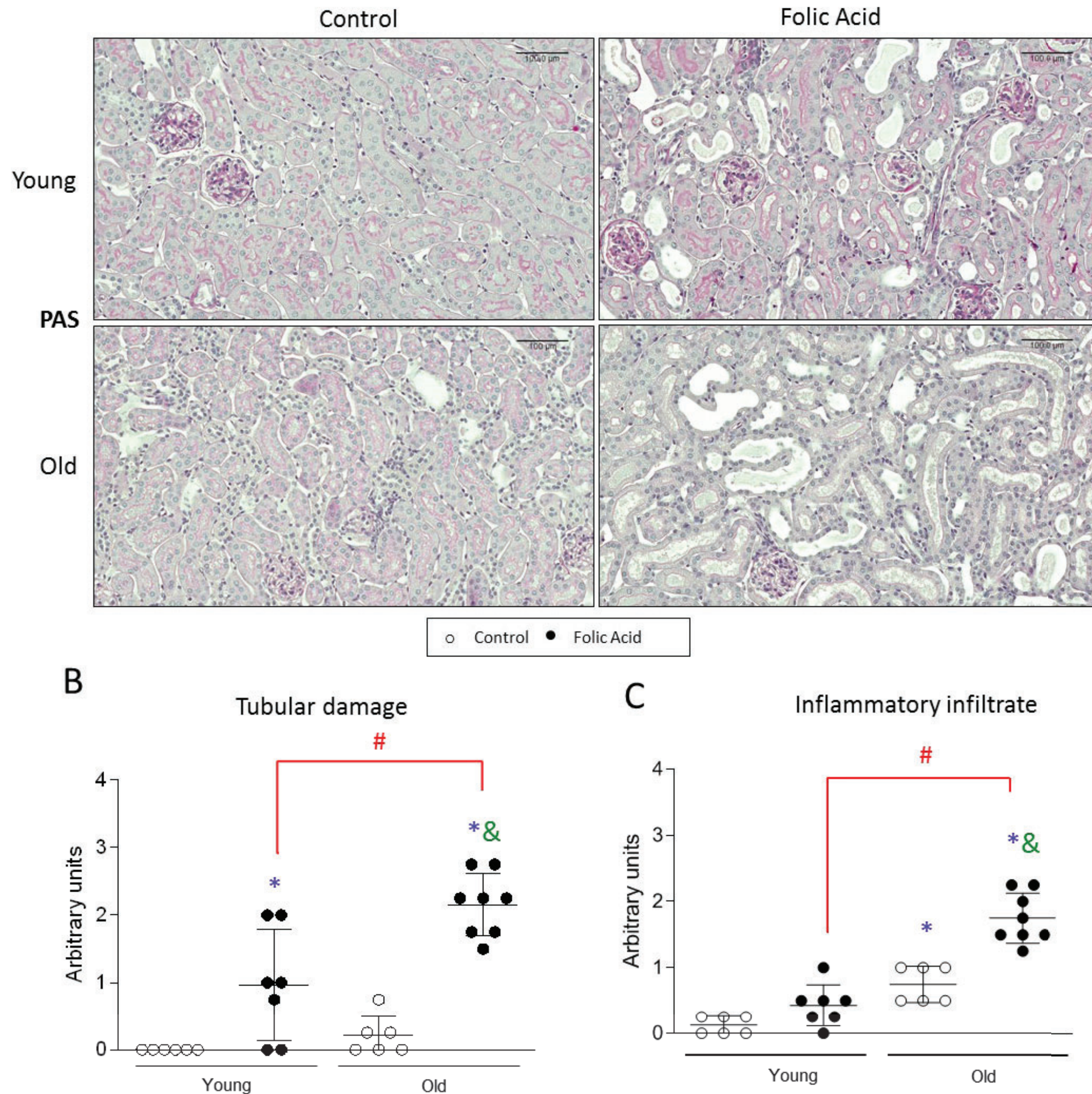

FIGURE 1 | Histological characterization of renal lesions in the acute phase of the folic acid nephropathy in young and old mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 mice and kidneys were studied after 48 h. The morphological lesions were evaluated by Periodic Acid- Schiff stained kidney sections. (A) Figure shows representative micrographs from each group and the quantification, from 0 to 4, of (B) tubular damage and (C) inflammatory infiltrate. Scale bars = 100 μm. Data are shown as arbitrary units and expressed as mean ± SD of *n* = 6–8 animals per group. $^{*}p < 0.05$ vs. control young mice, $^{\#}p < 0.05$ vs. FA-injected young mice, $^{\&}p < 0.05$ vs. control old mice. The non-parametric Kruskal-Wallis statistical test was performed.

Mm00441242_m1, *Ppargc1a*: Mm01208835_m1, *Tgfβ1*: Mm01178820, *Mlkl*: Mm01244219_m1, *Ripk3*: Mm00444947_m1, *Serpine1*: Mm00435858_m1, *Ccl5*: Mm01302428_m1, *Cxcl1*: Mm04207460_m1, *Cxcl2*: Mm00436450_m1, *Cxcl5*: Mm00436451_m1, *Cxcl10*: Mm00445235_m1, and *Gpx4*: Mm00515041_m1. Data were normalized to *Gapdh*: Mm99999915_g1. The mRNA copy numbers were calculated for each sample by the instrument software using Ct value (“arithmetic fit point analysis for the lightcycler”). Results were expressed in n-fold, calculated relative to young mice control group after normalization against *Gapdh*.

Statistical Analysis

Results are expressed as n-fold increase with respect to the average of young control mice as mean ± standard deviation of the mean (±SD). The Shapiro-Wilk test was used to evaluate sample Normality distribution. If the samples followed the Gaussian distribution, a one-way ANOVA followed by the corresponding post-hoc analyses, were used. To compare

nonparametric samples, a Kruskal-Wallis and a subsequent posthoc analysis was performed. Statistical analysis was conducted using GraphPad Prism 8.0 (GrahPad Software, San Diego California United States). Values of $p < 0.05$ were considered statistically significant.

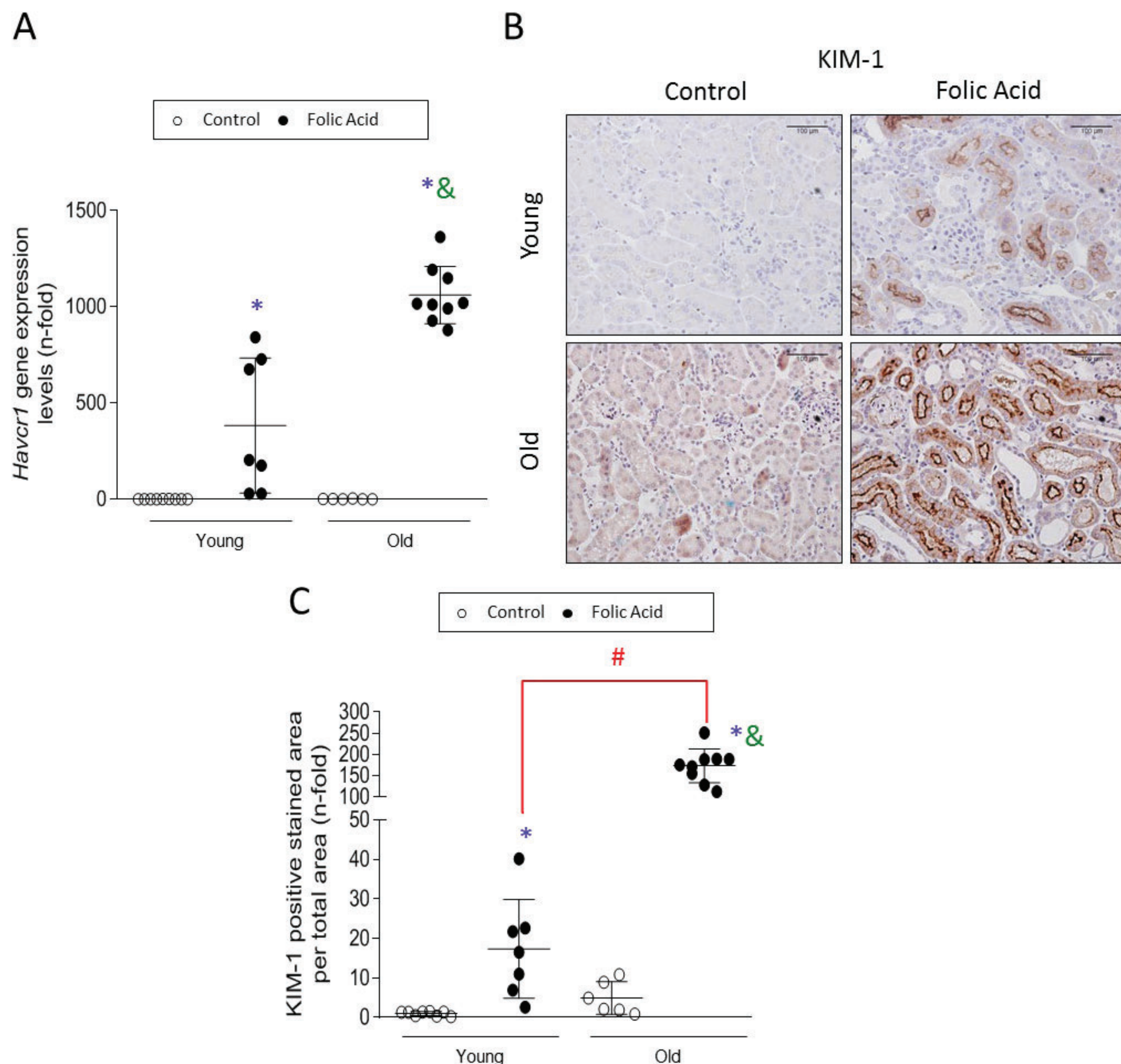

FIGURE 2 | The damage biomarker KIM-1 is overexpressed in injured tubules of old mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 mice and kidneys were studied after 48 h. (A) Total mRNA was isolated from frozen sections of whole kidneys and qRT-PCR was performed to determine gene expression levels of Havcr-1. (B) Representative microphotographs of KIM-1 expression levels evaluated by immunohistochemistry and (C) its quantification of stained area per total area. Scale bars _ 100 μm. Data are shown as n-fold and expressed as mean ± SD of n _ 6–9 animals per group. *$p < 0.05$ vs. control young mice, #$p < 0.05$ vs. FA-injected young mice, &$p < 0.05$ vs. control old mice. The non-parametric Kruskal-Wallis statistical test was performed.

RESULTS

Experimental AKI Induced by Folic Acid Administration is Characterized by More Severe Tubular Injury and Inflammatory Cell Infiltration in Old Mice.

Kidney injury was studied 48 h after the injection of a low dose (125 mg/kg) of FA to young (3-months-old) and old (1-year-old) mice. The morphological changes showed that FA administration induced tubular injury was more severe in old than in young mice (Figures 1A,B). Moreover, in FA-treated mice the recruitment of inflammatory cells in the kidney was higher in old than in young mice (Figures 1A,C).

Tubular damage was further evaluated at molecular levels by studying the gene expression levels of the tubular injury biomarker *Havcr-1*, that encodes the KIM-1 protein

(Beker et al., 2018; Griffin et al., 2019; Gohda et al., 2020). Havcr-1/ KIM-1 is an early marker of kidney injury in rodent AKI induced by IRI or nephrotoxic drugs (Bignon et al., 1976; Amin et al., 2004; Prozialeck et al., 2007). Kidney *Havcr-1* gene expression was increased in FA-induced AKI in both young and old mice (Figure 2A). In control young kidneys, KIM-1 protein expression was minimal (Figures 2B,C). However, in FA-injured kidneys apical KIM-1 staining was observed (Figure 2B), in accordance with previous studies (Han et al., 2002). Importantly, the quantification of KIM-1 staining showed dramatically higher tubular KIM-1 protein expression levels in old than in young injured kidneys (Figure 2C).

To further characterize the kidney infiltrating cells, immunohistochemistry was done using specific markers of neutrophils (Mieloperoxidase), macrophages (F4/80 + cells) and T-lymphocytes (CD3+ cells) (Figure 3A). Infiltration by neutrophils, monocytes/macrophages and CD3+ T cells was significantly higher in old than in young mice with FA-induced AKI (Figures 3B–D). Next, changes in gene expression of key inflammatory markers were evaluated by qRT-PCR in mouse kidneys. There were no differences in gene expression levels of proinflammatory factors between young and old control mice. In contrast, all of them were increased in response to FA administration both in young and old mice compared to untreated mice (Figure 4). The proinflammatory marker *Lcn2*, which encodes the kidney damage biomarker N-GAL (Wang et al., 2007), and the chemokine *Cxcl1*, which plays a key role in neutrophil recruitment (Chung and Lan, 2011), were significantly upregulated in FA kidneys from old compared to AKI young ones (Figure 4). In addition, other cytokines and chemokines, such as *Ccl2* and *Cxcl2* were also higher in old vs. young FA kidneys, but no differences were found in the case of *Ccl5* and *Cxcl10* (Figure 4).

Ferroptosis is Increased in Old Folic Acid-Induced Acute Kidney Injury When Compared to Young Folic Acid Kidneys

Ferroptosis is a regulated death pathway involved in the first wave of death in FA-AKI (Martin-Sanchez et al., 2017). To determine if ferroptosis was overactivated in response to FA administration in old mice, lipid peroxidation, a final ferroptosis target, was evaluated by HNE immunohistochemistry. An increase in HNE staining was found in FA-injected kidneys from old mice compared to young ones (Figure 5A). GPX4 reduction was previously described in FA-AKI (Martin-Sanchez et al., 2017). However, gene expression levels of *Gpx4* were not diminished in young FA-kidneys at the low dose used in the present study, whereas in old mice there was a slight, but not significant, diminution of *Gpx4* mRNA levels (Figure 5B).

Necroptosis Components are Overexpressed in Response to Folic Acid Administration in Old Mice.

Necroptosis is a cell death pathway associated with inflammation (Newton and Manning, 2016). The renal expression of the main components of the necroptosis pathway was evaluated in the AKI model. Renal *Ripk3* and *Mlkl* gene expression levels were increased in FA-injected mice compared to controls, as previously described (Figures 6A,B) (Martin-Sanchez et al., 2017; Martin-Sanchez et al., 2018a). These increases were markedly higher in old mice showing a significantly higher gene expression of both markers (Figure 6A), as well as MLKL total protein expression (Figures 6C,D).

Apoptosis is not Involved in the Folic Acid-Acute Kidney Injury severity Observed in Old Mice Compared to Young Mice.

Apoptosis is also involved in FA-AKI (Justo et al., 2006). As previously described, activation of caspase 3 was found during AKI in young mice, as evidenced by increased levels of mature caspase 3 (Justo et al., 2006). However, this was not the case for old mice with AKI, in whom active caspase 3 was not increased (Figures 6E,F). These data would suggest a possible switch of AKI-induced cell death pathway from a non-inflammatory apoptotic cell death to a proinflammatory necroptosis cell death in old age.

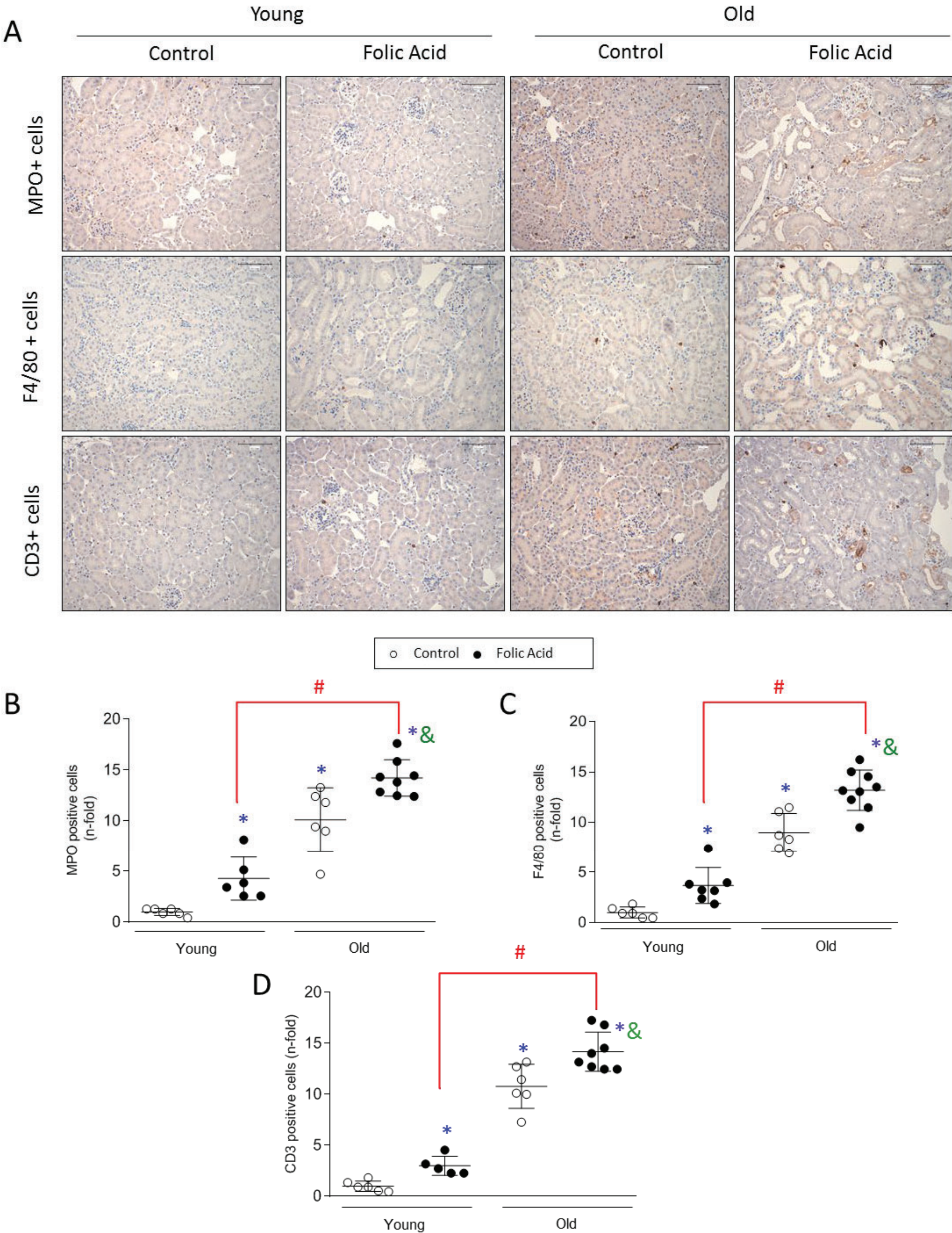

FIGURE 3 | Characterization of inflammatory infiltrate in the acute phase of folic acid (FA) nephropathy in young and old mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 mice and kidneys were studied after 48 h. Inflammatory cell infiltration was evaluated using antibodies against myeloperoxidase (neutrophils), F4/80 (monocytes/macrophages/dendritic cells) and CD3 (T lymphocytes). (A) Representative micrographs from each group. Scale bars 100 µm. (B–D) Quantification of MPO (B), F4/80 (C), and CD3 (D) positive cells. Data are shown as n-fold and expressed as mean ± SD of n 6–9 animals per group. *p < 0.05 vs. control young mice, #p < 0.05 vs. FA-injected young mice, &p < 0.05 vs. control oldmice. The parametric one-way ANOVA statistical test was performed.

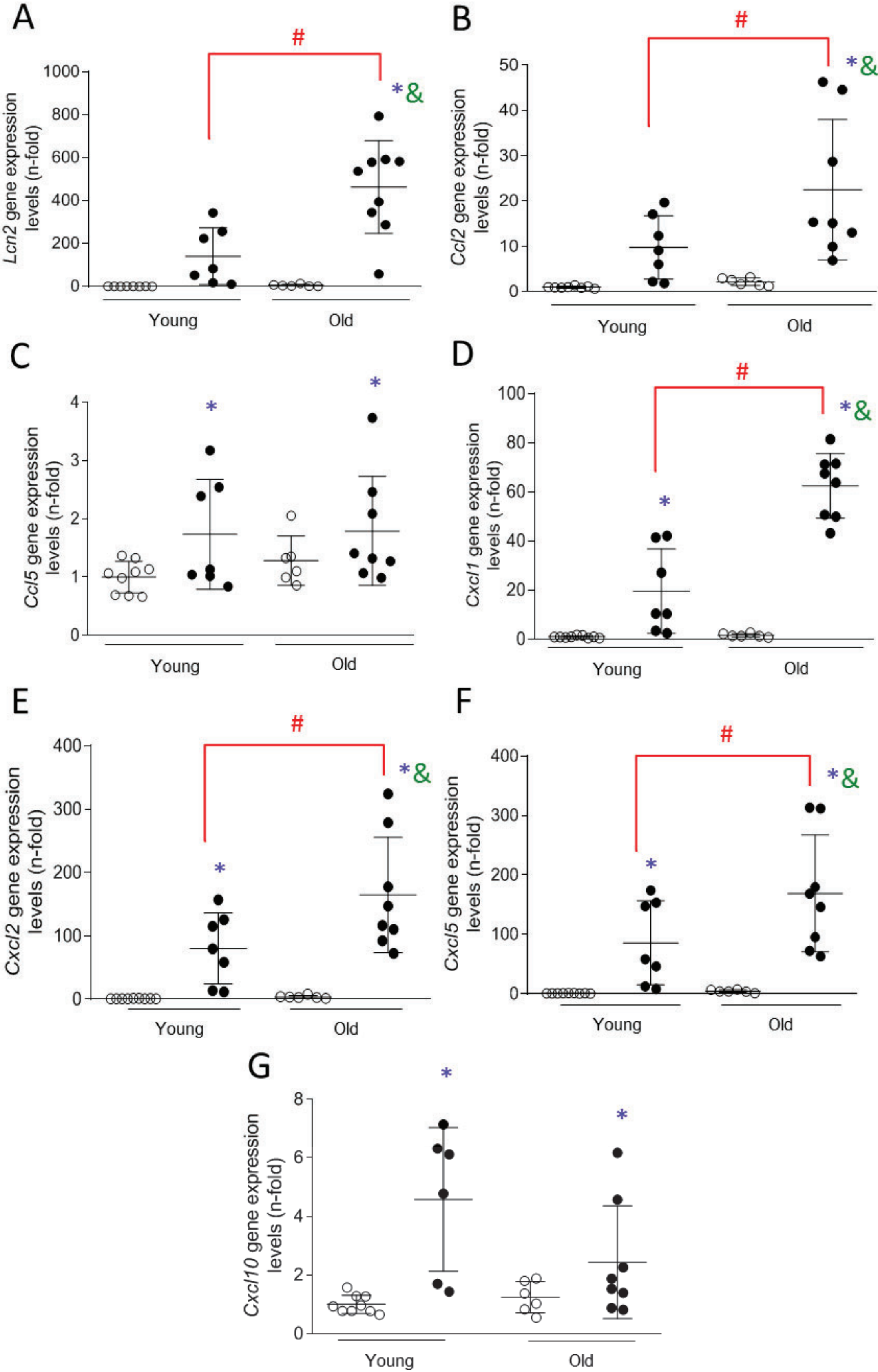

FIGURE 4 | Kidney expression of proinflammatory genes in the acute phase of folic acid (FA) nephropathy in young and old mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 mice. Kidneys were studied after 48 h and qRT-PCR was performed to assess Lcn2 (A), Ccl2 (B), Ccl5 (C), Cxcl1 (D), Cxcl2 (E), Cxcl5 (F), and Cxcl10 (G) gene expression levels. Data are shown as n-fold and expressed as mean ± SD of n 6–9 animals per group. *$p < 0.05$ vs. control young mice, #$p < 0.05$ vs. FA-injected young mice, &$p < 0.05$ vs. control old mice. The parametric one-way ANOVA statistical test was performed.

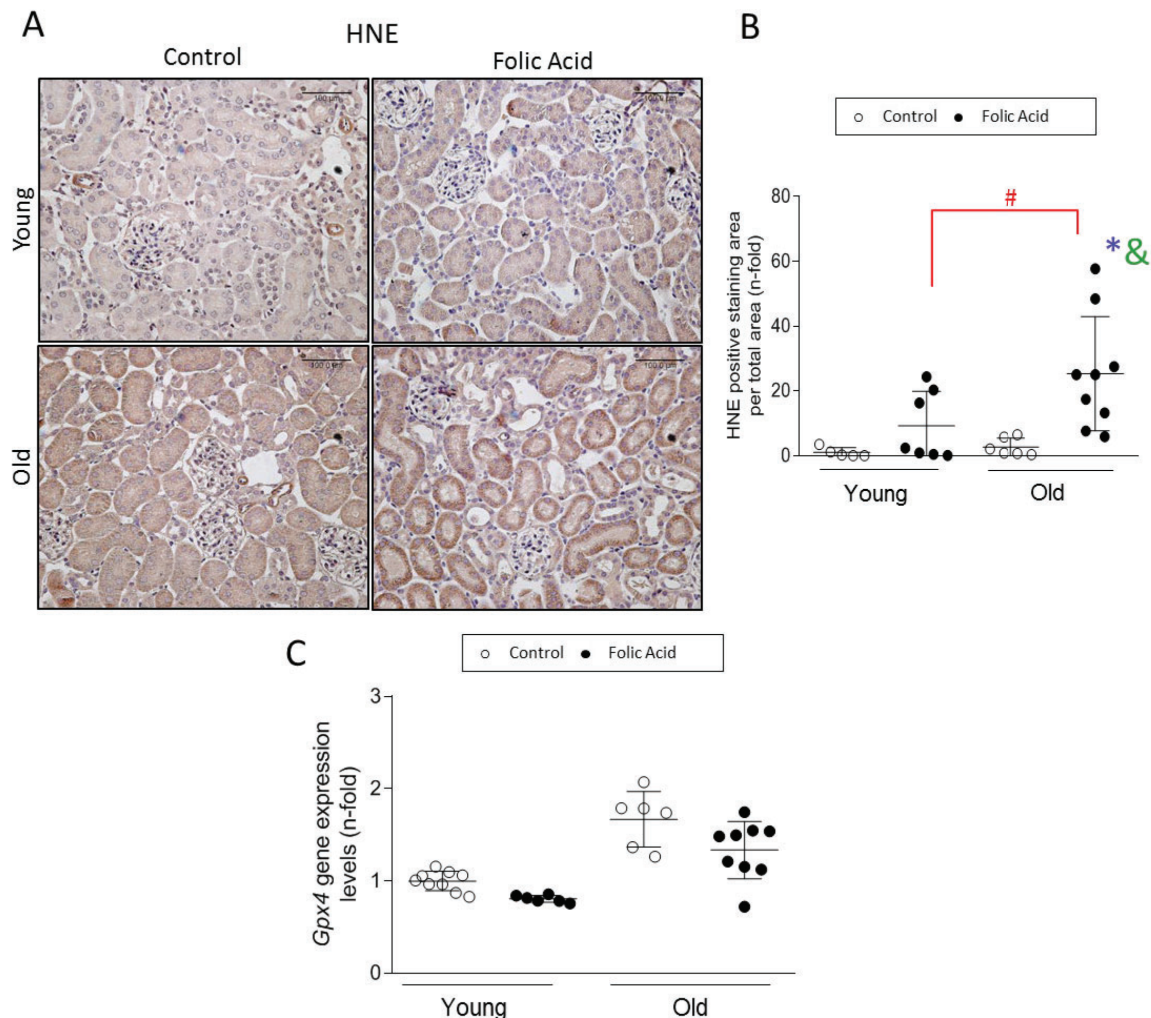

FIGURE 5 | Ferroptosis death pathway is significantly increased in FA-induced AKI in old vs. young mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 and kidneys were studied after 48 h (A, B) 4-Hidroxynonenal (HNE) immunohistochemistry was performed. (A) Representative pictures from each group and (B) the quantification of stained area per total area was performed. (C) Renal gene expression levels of Gpx4 were studied by qRT-PCR. Scale bars 100 µm. Data are expressed as mean ± SD of n 6–9 animals per group. *p < 0,05 vs. Young mice control. The non-parametric Kruskal-Wallis statistical test was performed.

Molecular Senescence is Activated in Acute Kidney Injury and Exacerbated in Old Mice

Cellular senescence was induced in the early phase of FA-AKI, as observed by increased gene expression levels of the cyclin dependent kinase inhibitors *p16ink4a* and *p21cip1* at 48 h (Figures 7A,B). Importantly, *p16ink4a* and *p21cip1* upregulation was exacerbated by aging, evidenced by higher expression in old than in young FA-injured kidneys (Figures 7A,B). Moreover, nuclear p21cip1 immunostaining was observed in FA-injected mice (Figures 7C,D) and the number of p21cip1 positive nuclei was significantly higher in old than in young FA-injured kidneys (Figure 7E). To further analyze senescent-related mechanisms, the DNA damage response marker γH2AX was evaluated. In response to FA administration, nuclear γH2AX expression was also increased in both young and old mice and showed a higher upregulation in old mice (Figures 7F–H). Interestingly, some infiltrating immune cells were also p21cip1 or γH2AX positive (Figures 7C,D,F,G), suggesting that immunosenescence and inflammaging are involved in the aggravated AKI response to FA in old mice.

Another feature of senescent cells is the increased production of SASP. The analysis of the gene expression levels of SASP components *Tgfβ1, Il6, Ctgf/Ccn2* and *Serpine1* (which encodes PAI-1) and the IL-6 protein levels assayed by ELISA, in the early phase of

AKI, showed that all of the evaluated SASP components were higher in old AKI mice compared to the young ones (Figures 8A–E).

Senescent cells are protected from apoptosis (Knoppert et al., 2019). Here, the anti-apoptotic factor BCL-xL, an important B-cell lymphoma 2 (BCL-2) family member central to senescent cell apoptosis resistance (Chang et al., 2016; Yosef et al., 2016) was evaluated at gene (*Bcl2l1*) and protein (BCL-xL) levels. Both *Bcl2l1* gene and BCL-xL protein expression levels were upregulated in injured kidneys of old mice compared to the young ones (Figures 8F–H). Interestingly, overexpression of BCL-xL was observed in old control mice compared to young mice (Figures 8G,H).

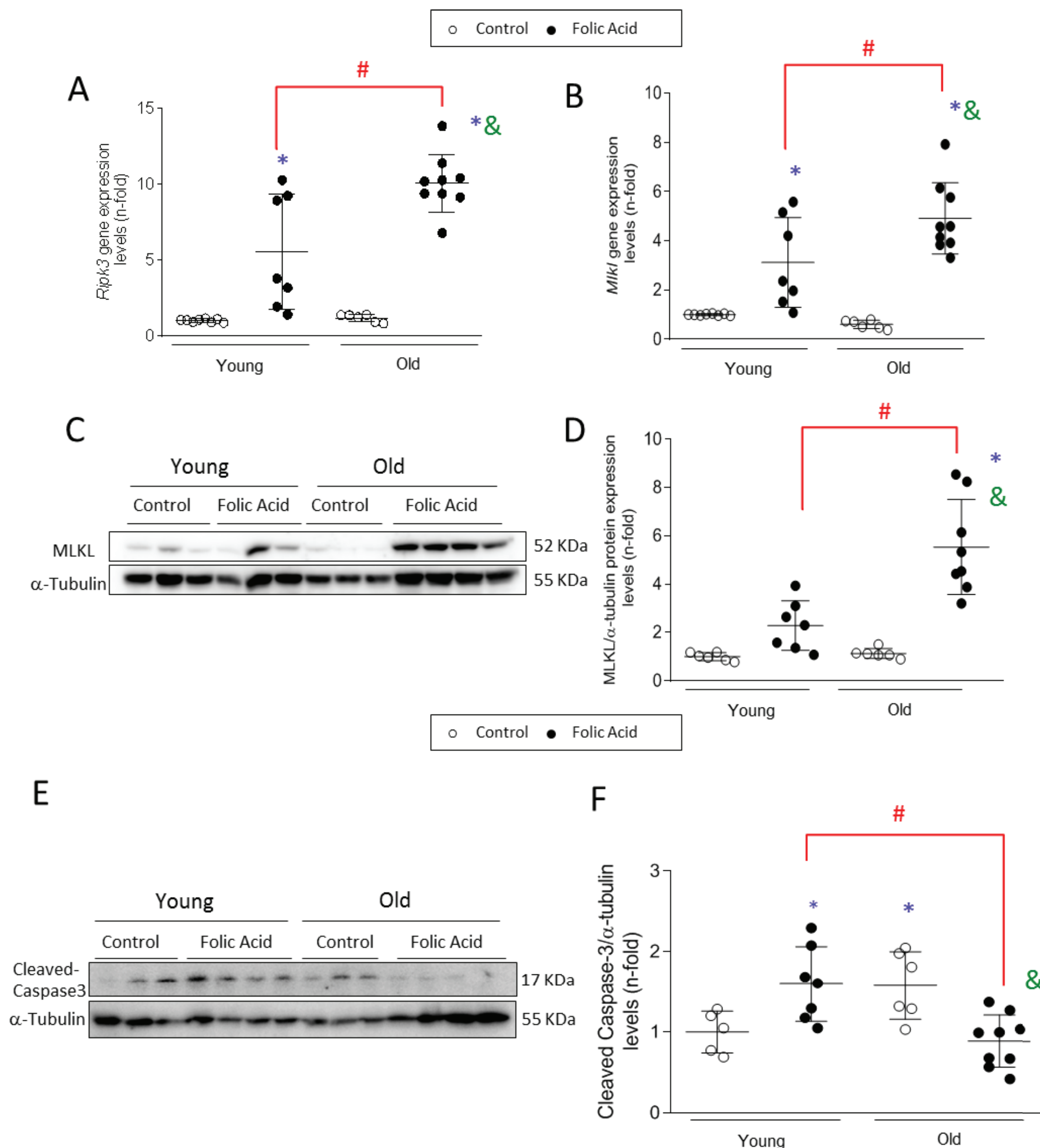

FIGURE 6 | Upregulation of the necroptosis pathway in the acute phase of folic acid (FA) nephropathy in old and young mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6mice and kidneys were studied after 48 h. The renal gene expression levels of Ripk3 (A) and mlkl (B) were evaluated by qRT-PCR. (C–F) MLKL protein and active caspase-3 (represented by cleaved caspase-3) were determined by Western blot. α-tubulin was used as loading control. (C, E) Representative blots and (D, F) their quantification. Data are shown as n-fold and expressed as mean ± SD of n 5–9 animals per group. *p < 0.05 vs. control young mice, #p < 0.05 vs. FA-injected young mice, &p < 0.05 vs. control old mice. The parametric one-way ANOVA statistical test was performed.

Age-Related Loss of Protective Factors

Klotho is an anti-aging protein of kidney origin that is lost very early in the course of AKI or CKD (Moreno et al., 2011; Fernandez-Fernandez et al., 2018; Sanchez-Niño et al., 2019;

Fernández-Fernández et al., 2020). As expected, K*lotho* gene downregulation was found in FA-induced AKI of young mice and in control and FA-AKI old kidneys (Figure 9A). Interestingly, kidney *Klotho* mRNA levels were far lower in old FA-injected mice that in young FA-injected mice (Figure 9A), suggesting that *Klotho* is a key target gene in AKI in the elderly. PGC-1α is a master regulator of mitochondrial biogenesis with anti-inflammatory and protective functions (Fontecha-Barriuso et al., 2019; Fontecha-barriuso et al., 2020). There was no change in kidney PGC-1α expression at gene level (named *Ppargc1a*) in young FA-injected mice at the lower than usual FA dose used, whereas decreased levels were found in control old kidney and a further downregulation in old FA kidneys (Figure 9B).

DISCUSSION

The studies done in the murine model of AKI by exposure to the toxic compound FA revealed increased acute tubular damage in aging mice. Similarly, more severe drug-related AKI effects in elderly subjects have been described in humans (Metz-Kurschel et al., 1990; Khan et al., 2017), supporting the relevance of this experimental model to explore kidney disease (Miyauchi, 1991; Fan et al., 2017; Montgomery et al., 2017). The characterization of acute tubular damage at molecular level reveals an exacerbation of the tubular injury marker KIM-1 in old mice. The observations regarding the mechanisms triggered by AKI point to an age-related magnification of several proinflammatory-related processes, including gene overexpression of some proinflammatory factors (*Lcn-2*, *Cxcl1*, and *Il-6*), overactivation of inflammatory-cell-death pathways such as necroptosis, and amplification of cellular senescence including immunosenescence (Figure 10). Moreover, our experimental data, showing an exacerbation of renal damage in 12-monthsold mice associated with the loss of renal protective factors, support the idea that age-associated susceptibility to AKI may start earlier than previously thought.

After an ischemic or nephrotoxic AKI insult, a wide range of pathophysiological events occur, including changes in tubular cell phenotypes, such as loss of physical cell–cell interactions and partial epithelial-to-mesenchymal transition (EMT) (Ruiz-Ortega et al., 2020), or even tubular cell death mediated by apoptosis and prominent programmed and unprogrammed necrosis (Martin-Sanchez et al., 2018b; Martin-Sanchez et al., 2018a). The proximal tubular cell is an important target of AKI (Chevalier, 2016), as we have confirmed in the FA model by de novo expression of the tubular damage biomarker KIM-1 in these cells. Some reports have found a lack of difference in initial severity of IRI, as described by no changes in tubular injury score, between aged and young mice after 1 day post IRI (Sato et al., 2016; Kim et al., 2019), whereas in our model of low-dose of FA-induced AKI, we found an exacerbated increase in KIM-1 positive tubular cells in old mice. Accordingly, in other murine AKI models induced by kidney exposure to toxic compounds, such as heme proteins (Nath et al., 2013), cisplatin (Wen et al., 2015) or bacteria inoculation (Maddens et al., 2012), a significant tubular damage in the acute phase was also described in old mice, showing age-related predisposition of tubular injury in response to toxic-induced damage.

Injured tubular cells are an important source of proinflammatory cytokines and chemokines, which contribute to the amplification of the inflammatory response (Liu et al., 2018). In this sense, FA-injected old murine kidneys presented a synergistic upregulation of proinflammatory genes, such as *Lcn-2, Cxcl1*, and *Il-6*, that codify cytokines and chemokines involved in the recruitment of infiltrating immune cells in the kidney. Previous experimental studies have also investigated the inflammatory response in the initial phase of AKI in old mice. In the IRI model, the number of macrophages significantly increased after 1 day in both young and aged mice (Kim et al., 2019), as found in other models of toxic exposure (Nath et al., 2013; Wen et al., 2015). These data clearly indicate that the combination of advanced age and exposure to toxics or ischemia induces an exacerbated innate inflammatory response in the injured kidney at this acute time point and suggest an increased susceptibility of the elderly to AKI. In addition, in the IRI model, exacerbation of immune response and changes in macrophage phenotypes is involved in the AKI-to-CKD transition (Kim et al., 2019).

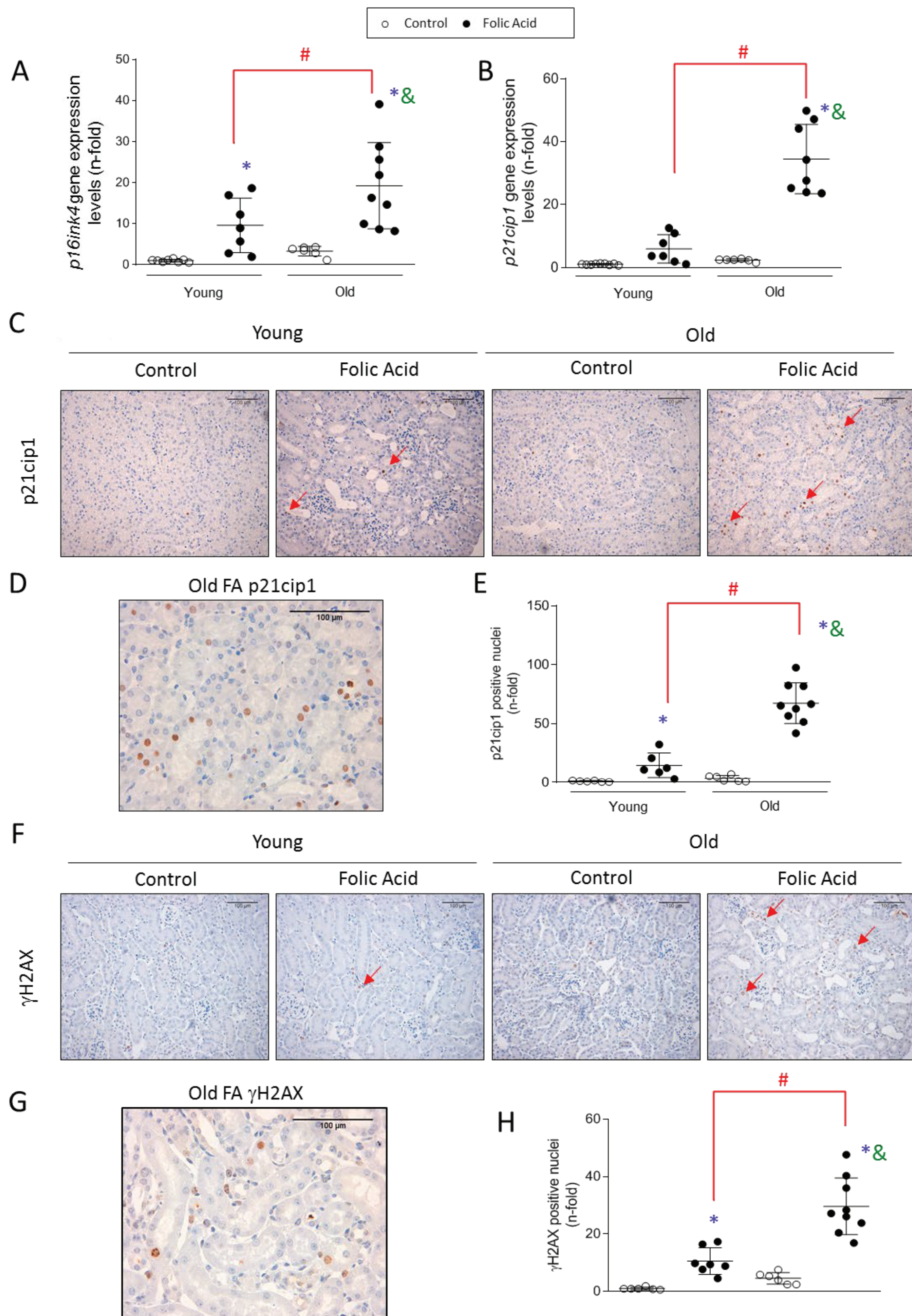

FIGURE 7 | Kidney expression of cell-cycle arrest and DDR markers pathway in the acute phase of folic acid (FA) nephropathy in old and young mice. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6mice and kidneys were studied after 48 h (A, B) Kidney p16ink4a (A) and p21cip1 (B) gene expression levels. (C–E) p21cip1 immunohistochemistry was conducted. (C) Representative microphotographs of p21cip1 showing nuclear staining and (D) detail of a FA-injured old mouse kidney showing positive nuclear p21cip1 staining. (E) Nuclear p21cip1 quantification. (F–H) γH2AX immunohistochemistry was performed. (F) Representative micrographs of γH2AX and (G) detail of a FA-injured old mouse kidney showing positive nuclear γH2AX staining. (H) Nuclear γH2AX quantification. Red arrows indicate positive nuclear staining. Scale Bars 100 μm.Data are expressed asmean ± SD of n 6–9 animalsper group. *p < 0.05 vs. control youngmice, #p < 0.05 vs. FA-injected young mice, &p < 0.05 vs. control oldmice. The parametric one-way ANOVA statistical test was performed, except for γH2AX quantification, in which a non-parametric Kruskal-Wallis statistical test was conducted.

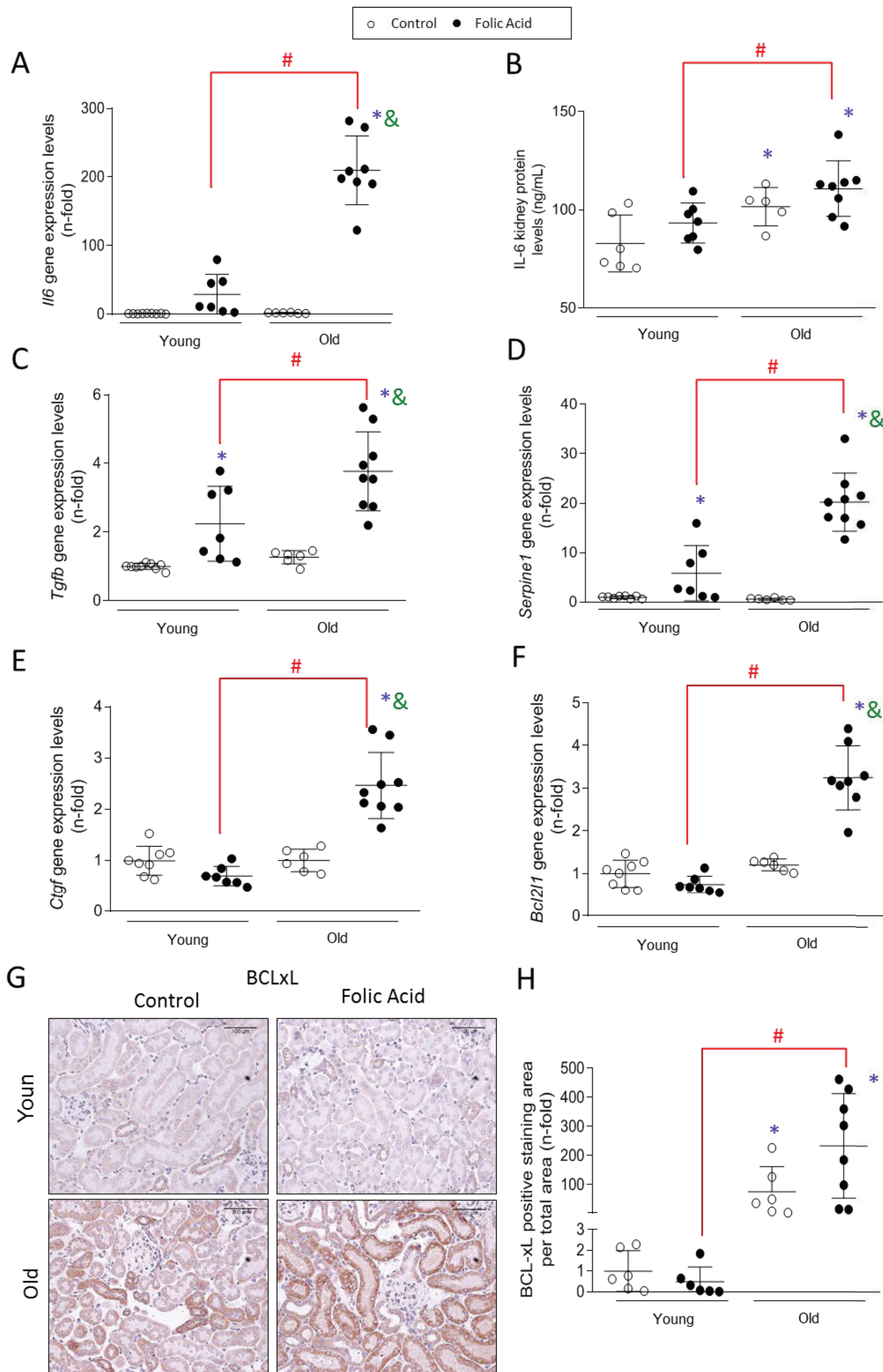

FIGURE 8 | Increased expression of senescence-associated secretory phenotype components (SASP) and anti-apoptotic proteins in old injured kidneys. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6 mice and kidneys were studied after 48 h. Gene expression levels of Il6 (A), Tgfβ (C), Serpine1 (D), and Ctgf (E) were determined by qRT-PCR. (B) Total protein of renal extracts of IL-6 were evaluated by ELISA. (F) Gene expression levels of Bcl2l1 were evaluated by qRT-PCR. (G, H) BCL-xL protein was evalated by immunohistochemistry. (G) Representative microphotographs of BCL-xL and (H) its quantification of stained area per total area. Data are shown as n-fold and expressed as mean ± SD of n 6–9 animals per group. *p < 0.05 vs. control young mice, #p < 0.05 vs. FA-injected young mice, &p < 0.05 vs. control old mice. The parametric one-way ANOVA statistical test was performed, except for the BCL-xL quantification, in which a non-parametric Kruskal-Wallis statistical test was conducted.

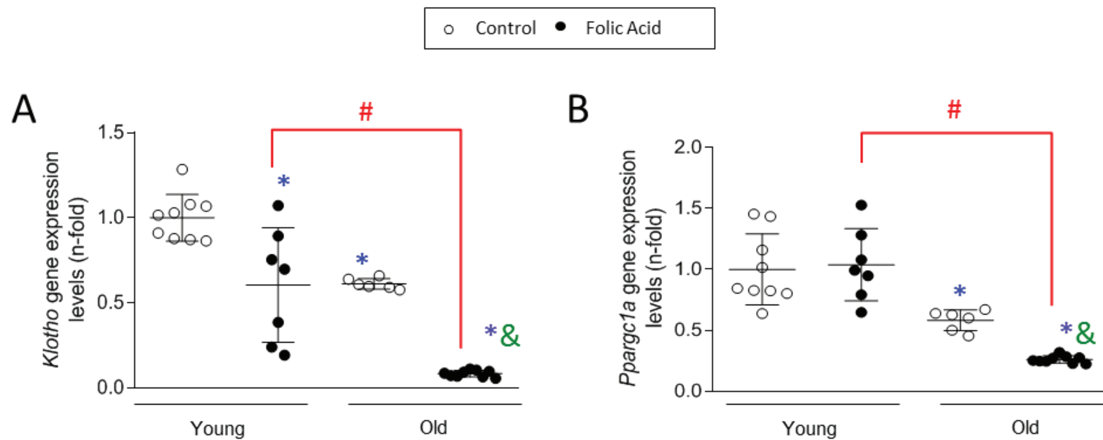

FIGURE 9 | Loss of protective factors in old kidneys is exacerbated in response to folic-acid (FA) administration. Folic Acid (FA; 125 mg/kg) was injected in 3-months-old (Young) and 1-year-old (Old) C57BL/6mice and kidneys were studied after 48 h. Kidney Klotho (A) and Ppargc1a (B) gene expression levels were assessed by qRT-PCR. Data are shown as n-fold and expressed as mean ± SD of n 6-9 animals per group. *p < 0.05 vs. control young mice, #p < 0.05 vs. FA-injected young mice, &p < 0.05 vs. control old mice. The parametric one-way ANOVA statistical test was performed.

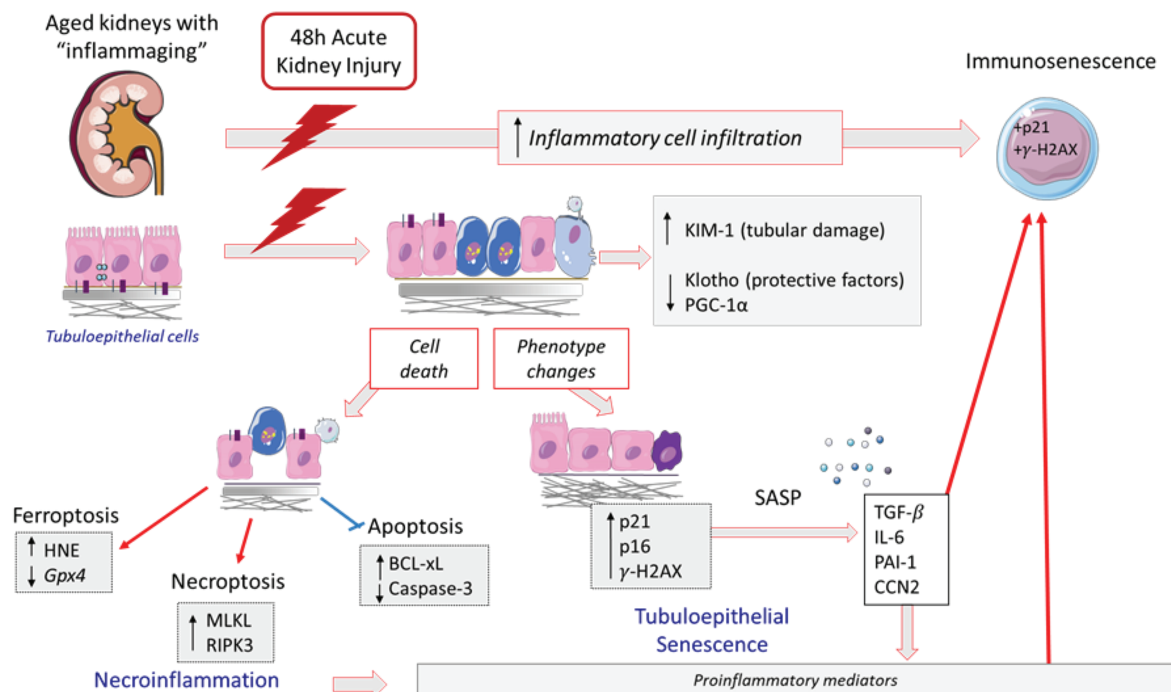

FIGURE 10 | Proposed mechanisms involved in aging-related FA-AKI increased susceptibility. In response to FA injury, aging kidneys present an increase of KIM-1 expression, indicator of tubular damage, lower levels of nephroprotective factors and immunosenescent infiltrating cells. The tubular cell damage can be lethal; in FA-aging kidneys there is an activation of inflammatory forms of cell death, such as necroptosis and ferroptosis, as well as an inhibition of apoptosis. In aging kidneys, injured tubular cells change their phenotype to a proinflammatory and senescent one, being IL-6 one of the most upregulated cytokines. These cellular and molecular changes may partially underlie the age-related increased susceptibility to developing more severe AKI in response to FA.

Many evidences in humans indicate that the elderly exhibit low-grade systemic chronic inflammation even in healthy conditions (Goronzy and Weyand, 2013; Franceschi and Campisi, 2014). Moreover, aging-related dysregulation of several innate and acquired immune responses have been described (Goronzy and Weyand, 2013; Montecino-Rodriguez et al., 2013; Franceschi and Campisi, 2014), and in human kidney transplant patients, aged donor kidneys were observed to attract more infiltrating inflammatory cells than young ones (Øien et al., 2007). Inflammaging of the kidney has also been demonstrated by a microarray analysis of human samples (Rodwell et al., 2004). However, no significant renal changes were found at gene level for proinflammatory factors in healthy old (12 month) mice.

Outstandingly, a magnification of the FA-AKI-induced proinflammatory response was observed in aging mice, which could be either a cause or a consequence of increased tubular damage. Among the proinflammatory mediators potentially involved in AKI exacerbation, IL-6 has special relevance. We have found that *Il-6* gene expression was synergistically upregulated in FA-AKI in old mice, as previously described in a model of hemoglobin-induced AKI (Nath et al., 2013). Since IL6 is a proinflammatory cytokine and a SASP component, targeting IL-6 or its downstream signaling could be an interesting therapeutic option in AKI in the elderly.

Tubular cell death is a feature of AKI and both apoptosis and regulated necrosis pathways are activated during FA-AKI (Sanz et al., 2008; Martin-Sanchez et al., 2017; Martin-Sanchez et al.,2018a). Caspase 3 activation is a central event in apoptosis (Justo et al., 2006; Linkermann et al., 2013; Martin-Sanchez et al., 2018a). In the present study, FA-AKI was associated with caspase 3 activation in young but not in old mice. In cultured tubular epithelial cells, inhibition of caspases is known to switch the mode of cell death induced by inflammatory cytokines from apoptosis to necrosis pathways (Justo et al., 2006; Martin-Sanchez et al., 2018a). In accordance with the latter, lack of caspase 3 activation in old FA-AKI mice was associated with evidence of involvement of the necroptosis pathway, i.e., RIPK3 and MLKL upregulation. This is a key difference to point out between young and old FA-AKI mice in our study, since apoptosis is a non-inflammatory form of cell death while necroptosis promotes inflammation (Martin-Sanchez et al., 2018b). In addition, ferroptosis is also overactivated in old FA-AKI, as shown by increased lipid peroxidation. Treatment with Ferrostatin-1, a ferroptosis inhibitor, prevented the inflammatory response and the expression of necroptotic proteins in FA-injected mice (Martin-Sanchez et al., 2017; Martin-Sanchez et al., 2018a), showing that this form of cell death is also related to inflammation. The observed downmodulation of apoptosis in old FA mice is also in line with the induction of a senescent phenotype of tubular cells in injured kidneys, since senescent cells are characteristically protected from apoptosis (Knoppert et al., 2019). These results are supported by the increased baseline and post-FA-induced AKI expression of the anti-apoptotic protein BCL-xL showed in old, but not in young mice. In summary, our findings indicate an aging-related change in cell death mechanisms linked to increased tubular injury, characterized by an activation of proinflammatory cell death pathways (necroptosis and ferroptosis) and suppression of non-inflammatory cell death pathways (apoptosis) (Figure 10).

As mentioned above, regulated necrosis can also contribute to age-related amplification of AKI-induced renal inflammatory response. Thereby, DAMPs released by necrotic cells can produce innate immunity cell-derived cytokines by the activation of identical pattern recognition receptors, such as Toll-like receptors expressed on tissue-resident or infiltrating immune cells (Ujiie, 1989; Wu et al., 2007; Kurts et al., 2013). DAMP-associated inflammation is one of the earliest processes following AKI and contributes to an amplification of the loop of cell death/inflammation (Mulay et al., 2016b). Among the immune cells, some studies have demonstrated that macrophages actively participate in necroptosis (Linkermann et al., 2014; Mulay et al., 2016a). In this sense, in the model of IRI, the gene deletion of RIPK3 or MLKL reduced macrophage infiltration and NLRP3 inflammasome activation (Chen et al., 2018). Our results demonstrated that the number of infiltrating macrophages was significantly higher in old FA-injected kidneys associated with an overexpression of the necroptosis components RIPK3 or MLKL, supporting an exacerbation of necroptosis-macrophage inflammatory pathway in aging AKI mice.

Apart from kidney pathologies, necroptosis-mediated inflammation plays an important role in a variety of age-related diseases such as Alzheimer's disease, Parkinson's disease, and atherosclerosis (Royce et al., 2019). Some studies have found an association of age-related increase in DAMPs circulating levels, such as mitochondrial DNA or high mobility group protein B1 (Davalos et al., 2013; Pinti et al., 2014), with circulating proinflammatory cytokines (TNF-α, IL-6) in humans, suggesting that DAMPs might play a role in low-grade systemic chronic inflammation described in the elderly (Goronzy and Weyand, 2013; Franceschi and Campisi, 2014). In the same way, some experimental data support a relation

between necroptosis and inflammaging. For example, accelerated aging Cu/Zn superoxide dismutase (Sod1) deficient mice that exhibit increased levels of circulating proinflammatory cytokines (Zhang et al., 2013; Deepa et al., 2019) had elevated MLKL protein and gene expression in adipose tissue at 9 months compared with age-matched wild type mice (Royce et al., 2019). Although we have found increased inflammatory cell infiltration in the old mice kidneys, the evaluation of key components of the necroptosis pathway, such as RIPK3 or MLKL, in 1-year old C57BL/6 mice showed no changes at gene and protein levels in healthy kidneys compared to young ones, suggesting that there is no age-related activation of necroptosis in our experimental conditions.

Cellular senescence may occur as a result of cell cycle arrest due to increased expression of cyclin kinase inhibitors (Knoppert et al., 2019). Previous studies in different AKI models have described a rapid upregulation of p21cip1 expression in the early phase of AKI (Megyesi et al., 1998; Yu et al., 2005; Hodeify et al., 2011). Accordingly, we found increased expression of p21cip1 and p16ink4a in FA-AKI mice. Some studies have proposed that p21cip1 prevents DNA-damaged cells from entering the cell cycle by directly inhibiting CDK2 activity (Yu et al., 2005), thus avoiding cell death by necrosis or apoptosis (Megyesi et al., 1998). Indeed, p21cip1 knockout mice showed increased susceptibility to AKI mediated by ischemia or nephrotoxins (Megyesi et al., 1998; Megyesi et al., 2001; Nishioka et al., 2014). In contrast, the model of renal ablation in p21cip1 knockout mice presented diminished cell cycle arrest, amelioration of renal dysfunction and lower interstitial fibrosis (Megyesi et al., 1999). On the other hand, renal p21cip1 is essential for the beneficial effects of renal ischemic preconditioning (Nishioka et al., 2014). Moreover, distinct types and severity of kidney injury can behave differently regarding cell cycle arrest (Yang et al., 2010). Therefore, the functional consequences of p21cip1 expression are cell and disease context specific. In the present study, renal p21cip1 mRNA expression and tubular p21cip1 nuclear staining were significantly higher in old FA-induced AKI than in young mice. Furthermore, the DNA damage response marker γH2AX was also significantly activated in old AKI mice, showing mainly nuclear positive staining in tubular cells. Similarly, activation of prolonged cell cycle arrest have also been reported in other experimental AKI models, but in this case, linked to fibrosis (Yang et al., 2010). In IRI-AKI mice, treatment with a p53 inhibitor has demonstrated the importance of G1 cell cycle arrest in the progression of fibrosis (Lim et al., 2018). Another mechanism involved in senescence-mediated renal damage is related to the induction of SASP in injured tubular cells (Acosta et al., 2013). Here, we observed that in FA-induced AKI there was a significant increase in SASP gene expression (including *Tgfβ1, Ctgf/Ccn2*, *Il6*, and *Serpine1*) in old mouse kidneys. Taken together, this data suggests that there is a magnification of the senescence phenotype in aged AKI mice (Figure 10). Interestingly, our results showed that in old murine injured kidneys, also some infiltrating immune cells were p21cip1 or γH2AX positive, suggesting molecular senescence in the immune cells in the aging kidney may be involved in the aggravated AKI response to FA in old mice (Figure 10). Although the exact cause of inflammaging is not known, cellular senescence (Campisi and D'Adda Di Fagagna, 2007) and immune senescence (Franceschi et al., 2000; McElhaney and Effros, 2009) have been proposed to play a key role in this process.

Finally, yet another remarkable finding was the reduced expression of nephroprotective factors Klotho and PGC-1α and their dramatic further downregulation induced by AKI in old mice. Klotho is normally expressed and secreted by tubular cells and has anti-aging, anti-inflammatory and anti-fibrotic properties (Kuro-o et al., 1997; Kurosu et al., 2005; Sanchez-Niño et al., 2013). Klotho downregulation can be both a consequence and driver of inflammaging in kidney disease (Moreno et al., 2011; Izquierdo et al., 2012; Fernandez-Fernandez et al., 2018; Sanchez-Niño et al., 2019; Fernández-Fernández et al., 2020). For example, Klotho protects endothelial cells from senescence (Carracedo et al., 2012). PGC-1α is the master regulator of mitochondrial biogenesis and PGC-1α deficiency is known to promote spontaneous kidney inflammation and to increase the severity of AKI (Fontecha-Barriuso et al., 2019; Fontecha-barriuso et al., 2020). Thus, the loss of the nephroprotective factors Klotho and PCG-1α due to aging could contribute to an increased inflammatory and fibrotic response to FA-AKI.

In conclusion, our data indicate that aging kidneys lose local nephroprotective factors and reveal a switch to a proinflammatory cell death (necroptosis and ferroptosis) instead of apoptosis (Figure 10), associated to a synergistic upregulation of several proinflammatory (Lcn-2 and Cxcl1) and SASP mediators, such as IL-6. Moreover, these changes may partially underlie the age-related increased susceptibility to developing more severe AKI in response to toxic compounds, as clearly showed by a dramatic increase of KIM-1 expressing tubular cells (Figure 10). Another characteristic of severe AKI in aging kidneys includes the induction of cellular senescence in intrinsic renal cells and inflammatory cells. These features could interfere with the resolution of acute injury and favor the AKI-to-CKD transition. All these data point out the relevance of investigating the effects of senolytic drugs on cell-death pathways involved in AKI. Better understanding of inflammaging and immunosenescence could contribute to identifying prevention and/or intervention points to mitigate the structural and functional impairment of the kidneys in elderly people. Given the increasing frequency of AKI in the elderly, this information may help to come up with age-specific interventions to prevent or treat kidney injury in this age group.

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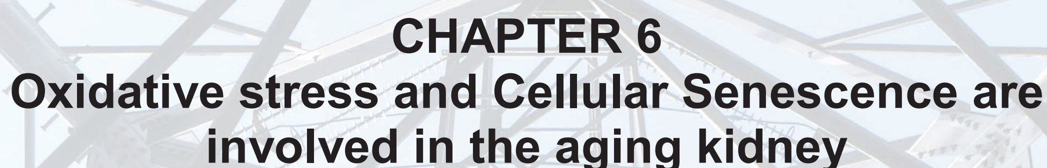

CHAPTER 6
Oxidative stress and Cellular Senescence are involved in the aging kidney

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Chapter 6. Oxidative stress and Cellular Senescence are involved in the aging kidney

ABSTRACT

Chronic kidney disease (CKD) can be considered as a clinical model for premature aging. However, non-invasive biomarkers to detect early kidney damage and the onset of a senescent phenotype are lacking. Most of the preclinical senescence studies in aging have been done in very old mice. Furthermore, the precise characterization and overtime development of age-related senescence in the kidney remain unclear. To address these limitations, the age-related activation of cellular senescence-associated mechanisms and their correlation with early structural changes in the kidney were investigated in 3- to 18-month-old C57BL6 mice. Inflammatory cell infiltration was observed by 12 months, whereas tubular damage and collagen accumulation occurred later. Early activation of cellular senescence-associated mechanisms was found in 12-month-old mice, characterized by activation of the DNA-Damage-Response (DDR) in tubular cells and infiltrating immune cells, activation of the antioxidant NRF2 pathway and Klotho downregulation. However, induction of tubular cell cycle-arrest (CCA) and renal Senescent-Associated Secretory Phenotype (SASP) was only found in 18-month-old mice. In aging mice, both inflammation and oxidative stress (marked by elevated lipid peroxidation and NRF2 inactivation) remained increased. These findings support the hypothesis that prolonged DDR and CCA, loss of nephroprotective factors (Klotho), dysfunctional redox regulatory mechanisms (NRF2/antioxidant defense) and infiltrating immunosenescent cells can be early drivers of age-related kidney damage progression.

INTRODUCTION

Chronic kidney disease (CKD) is emerging as an important health problem due to the absence of early diagnostic biomarkers and effective treatments. Although recent clinical trials have reported promising results with SGLT2 inhibition, the majority of CKD patients still progresses towards end-stage renal diseases (ESRD), needing renal replacement therapies, such as dialysis or transplantation. (Ortiz 2021) The aged population is constantly increasing, and kidney aging is a risk factor for both Acute Kidney Injury (AKI) and CKD. (Infante et al., 2020) In this regard, CKD is predicted to become the 5th global cause of death by 2040. Parmar & Bashir, 2021) Recent studies show that many features of aging characterize CKD, suggesting that this disease can be considered as a clinical presentation of premature aging (Stenvinkel & Larsson, 2013; Sturmlechner et al., 2017). Therefore, there is an urgent unmet medical need to understand the mechanisms of age-related kidney damage, as well as early biomarkers, towards a better management of CKD.

Aging is related to telomere shortening due to replicative stress, and this can lead to cellular senescence (Banerjee et al., 2021). This process is defined as an irreversible cell cycle arrest (CCA), characterized by alterations in chromatin organization and gene expression, which induces profound phenotypic changes. Senescent cells possess a specific secretome known as senescence-associated secretory phenotype (SASP) (Bonventre, 2014; Melk et al., 2004), which is enriched with pro-inflammatory cytokines, growth factors and profibrotic proteins (Acosta et al., 2013; Zhou et al., 2020). Cellular senescence can be deleterious or beneficial depending on the biological context and timing. In this sense, the SASP can evoke a local inflammatory response with intricate and divergent effects, including the removal of senescent cells by phagocytosis, thus contributing to tissue remodeling and damage resolution (Freund et al., 2010; Hoenicke & Zender, 2012; Krizhanovsky et al., 2008), whereas persistent senescence-mediated inflammation leads to aberrant tis-sue remodeling and fibrosis (Valentijn et al., 2018). In addition, the SASP, acting in an autocrine or paracrine manner in neighboring cells, can reinforce and propagate cellular senescence, by a process named secondary senescence (Acosta et al., 2013).

Cellular senescence is induced in response to multiple types of damage, such as in-tense oncogenic signaling, DNA damage, telomere shortening, inflammation, oxidative stress and toxins (Zhou et al., 2020). In the kidney, senescence has been involved in AKI, regeneration, AKI-to-CKD transition, CKD progression, transplant rejection and aging (Valentijn et al., 2018). The senescence mechanisms in the kidney include activation of the DNA Damage Response (DDR), which can be prolonged in time and activate the p53/p21cip1 axis and p16ink4a. Both p21cip1 (from now on p21) and p16ink4a (from now on p16) play a key role as inhibitors of cyclin-dependent kinases (CDK), blocking retinoblastoma tumor suppressor (Rb) phosphorylation mediated by CDKs, thus limiting cell proliferation and provoking CCA (Valentijn et al., 2018). This triggers a cascade of phenotypic changes in tubular epithelial cells which release the aberrant SASP secretome, enriched in proinflammatory and profibrotic factors, aggravating kidney dysfunction and contributing to renal damage progression (Knoppert et al., 2019a; Muñoz-Espín & Serrano, 2014; Valentijn et al., 2018). Among the SASP, proinflammatory components, several cytokines and chemokines, such as Interleukin-6 (IL-6) and CCL-2 (MCP-1), contribute to persistent renal inflammation and to further tubular cell injury and dysfunction (Kirkland et al., 2017; Kirkland & Tchkonia, 2017) whereas SASP profibrotic factors, such as trans-forming growth factor-β (TGF-β) and cellular communication network factor 2 (CCN2/CTGF), induce profibrotic responses, leading to kidney fibrosis (K.-H. Kim et al., 2004; Ungvari et al., 2017).

Oxidative stress plays a key role in the premature aging associated to CKD (Stenvinkel et al., 2021). Reactive oxygen Species (ROS) accumulation and redox imbalance can lead to tissue damage due to increased breakage of the DNA and subsequent DDR, resulting in CDK inhibitors activation (Banerjee et al., 2021; Ungvari et al., 2017). This state of cells involves the activation of one of the molecular pathways to remove ROS, the Nuclear Factor (Erythroid-Derived 2)-Related Factor 2/Hemooxigenase-1 (NRF2/HO-1) axis (Zhou et al., 2020). In physiological conditions, NRF2 is sequestered by Keap1, but upon a redox

imbalance, NRF2 is released from Keap-1 and then translocated to the nucleus to bind to ARE sequences in DNA, initiating the transcription of genes related to antioxidant defense, such as HO-1 and NAD(P)H dehydrogenase (quinone 1) (NQO-1), among others (Ucar et al., 2021). Moreover, ROS and the NRF2/HO-1 pathway are involved in the increase and decrease, respectively, of inflammatory mediators (Stenvinkel et al., 2021). In aging tissues, the NRF2/HO-1 pathway has been described to be dysfunctional, being unable to reduce neither ROS production nor inflammaging, the term to describe a low-grade inflammatory state of aged tissues (González-Bosch et al., 2021). Indeed, inflammaging and NRF2 impairment are relevant characteristics of CKD (Stenvinkel et al., 2021).

One important question in kidney research is the lack of non-invasive biomarkers to detect early kidney damage, including the onset of a senescence phenotype. Although p16 expression has been used as a classical senescence biomarker, there is not a specific biomarker to define the senescent cell state (Hernandez-segura et al., 2018; Sharpless & Sherr, 2015; Valentijn et al., 2018). One recent study tried to identify robust senescence biomarkers *in vivo*, evaluating the mRNA expression profiles of a panel of known molecular hallmarks of senescence in multiple tissues in very aged (30 months) hybrid CB6F1 female mice, showing that p16 expression was upregulated in all tissues analyzed, including the kidney, whereas SASP components vary among tissues (Hudgins et al., 2018). Despite this, the precise extent of senescent cell accumulation in aged animals, and a deeper characterization of age-related senescence in the kidney and its functional outcome remain unclear. Most of senescence-related experimental studies have been done in very old mice (Kadota et al., 2021; Kim et al., 2021). However, in a recent preclinical study we demonstrated an age-related increased susceptibility to develop more severe AKI in mice by the age of 12 months through exacerbation of senescence-related mechanisms (Marquez-Exposito et al., 2021). Therefore, in this paper our aim was to investigate the age-related activation of cellular senescence, the redox-related mechanisms, and their correlation with early structural changes in the kidney, evaluating 3-, 12- and 18-month-old C57BL6 mice.

MATERIALS AND METHODS

Animals

Experiments were performed according to the European Community guidelines for animal experiments and the ARRIVE guidelines, and with consent of the Experimental Animal Ethics Committee of the Health Research of the IIS-Fundación Jiménez Díaz and Proex065/18 of the Comunidad de Madrid. Six to nine C57BL/6 male mice per age group (3-, 12- and 18-month-old) were studied.

All animals were sacrificed with an overdose of CO_2 in a special chamber. Blood and urine were collected, and kidneys were perfused *in situ* with saline before removal. Half of each kidney (2/4) was fixed, embedded in paraffin, and used for immunohistochemistry, and the rest was snap-frozen in liquid nitrogen for renal cortex RNA and protein studies.

Protein studies

Total proteins were isolated from frozen kidney tissue in lysis buffer as previously described (Marquez-Exposito et al., 2021) and quantified using a BCA protein assay kit (ThermoScientific). Proteins (50 µg) were separated on 8-15% acrylamide gels using the SDS-PAGE, as described (Marquez-Exposito et al., 2021). After electrophoresis, samples were transferred on to polyvinylidenedifluoride membranes (Millipore) blocked in TBS containing 0.1% Tween 20 and 5% dry non-fat milk for 1 h at room temperature and incubated in the same buffer with different primary antibodies over-night at 4ºC. After washing, membranes were incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (Invitrogen) 1 h at room temperature and developed using an ECL kit (Amersham Biosciences). Results were analysed by LAS 4000 and Amersham Imager 600 (GEHealthcare) and densitometered by Quantity One software (Biorad). The following primary antibodies were employed [dilution]: NRF2 ([1:500]; sc-365949, Sta. Cruz Biotechnology) and ERK1/2 ([1:500]; sc-514302, Sta. Cruz Biotechnology).

Histology and immunohistochemistry

Paraffin-embedded kidney sections were stained using standard histology procedures, as described elsewhere (Marquez-Exposito et al., 2021). Periodic acid-Schiff (PAS, Sigma-Aldrich) stained slides were quantified, assessing tubular damage as tubular dilation and interstitial inflammatory infiltrate as arbitrary units as previously described (Zoja et al., 2002). Picrosirius red staining was performed using a mixing of 1% Direct Red 80 (Sigma) and Picric Acid Solution (Sigma), and slides were quantified using Image Pro-plus Software (Maryland, USA) determining the positive red staining area relative to the total area.

Immunohistochemistry (IH) was carried out in 3 µm thick tissue sections. The PTlink system (DAKO) was used for antigen retrieving using sodium citrate buffer (10 mM) adjusted to pH 6–9, depending on the immunohistochemical marker. Endogenous peroxidase was blocked. Sections were incubated for 1 h at room temperature with 1X Casein Solution (Vector Laboratories) to remove non-specific protein binding sites. Then, primary antibodies were incubated overnight at 4ºC. Specific HRP-conjugated (DAKO) or biotinylated secondary antibodies (Amersham Biosciences) were used. The latter were followed by Avidin-Biotin Complex incubation (Vector Laboratories). Signal was developed with Substrate solution and 3,3-diaminobenzidine as a chromogen (Abcam). Finally, slides were counterstained with Carazzi's haematoxylin (Richard Allan Scientific). The primary antibodies used were: p21 ([1:2000, Ab188224, Abcam), γH2Ax [1:500], NB1002280 Novus Biological), F4/80 ([1:50]; MCA497, Bio-Rad), Myeloperoxidase ([1X], IS511, DAKO), 4-Hydroxynonenal (HNE) ([1:1,000], Ab46545, Abcam) and phosphorilated-NRF2 serine 40 ([1:2000]; Ab76026, Abcam). Specificity was checked by omission of primary antibodies (not shown). Quantification was made by using the Image-Pro Plus software (Maryland, USA) determining the positive staining area relative to the total area or counting positive staining manually (in the case of P21, γH2AX, myeloperoxidase, and F4/80 staining), in 5-10 randomly chosen fields (×200 magnification).

Gene expression studies

RNA from renal cortex was isolated with TriPure reagent (Roche). cDNA was synthesized by a High Capacity cDNA Archive kit (Applied Biosystems) using 2µg total RNA primed with random hexamer primers following the manufacturer's instructions. Quantitative gene expression analysis was performed on an AB7500 fast real-time PCR system (Applied Biosystems) using fluorogenic TaqMan MGB probes and primers de-signed by Assay-on-DemandTM gene expression products. Mouse assays IDs were: *p21*: Mm00432448_m, *p16* (Cdkn2a): Mm00494449_m1, *Klotho* (*Kl*): Mm00502002_m1, Il6: Mm00446190_m1, *Lcn2*: Mm01324470_m1, *Havcr1* (*Kim-1*): Mm00506686_m1, *Ctgf/Ccn2*: Mm01192933_g1, *Ccl-2*: Mm00441242_m1, *Tgfb1*: Mm01178820_m1,*Hmox*: Mm00516005_m1, *Nfe2l2* (*Nrf2*): Mm00477784_m1, *Serpine1*: Mm00435858_m1, *Serpinb2*: Mm00440905_m1, *Catalase:* Mm00437992_m1 and *Sod1*: Mm01344233_g1. Data were normalized to *Gapdh*: Mm99999915_g1 (Vic). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to young mice control group after normalization against *Gapdh*.

Statistical analysis

Results are expressed as n-fold increase with respect to the average of 3-month-old mice as mean ± standard error of the mean (±SEM), except for the PAS, p21, gH2AX, MPO and F4/80 quantification which are expressed in arbitrary units. The Shapiro-Wilk test was used to evaluate sample Normality distribution. If the samples followed the Gaussian distribution, a one-way ANOVA followed by the corresponding post-hoc analyses were used. To compare non-parametric samples, a Kruskal-Wallis and a subsequent post-hoc analysis was performed. Graphics and statistical analysis were conducted using GraphPad Prism 8.0 (GrahPad Software, San Diego California USA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Inflammation precedes tubular and glomerular lesions in kidneys of aged mice

Kidney morphology was evaluated by PAS staining. Interstitial and perivascular inflammatory cell infiltration was observed in the kidneys of 12- and 18-month-old mice (Figure 1A). Moreover, the inflammatory infiltration grade showed a significant increase in 12-month-old mice compared to 3-month-old mice and was even higher at 18 months (Figure 1B). In addition, glomerular damage was observed in 18-month-old mice (Figure 1A). Despite the presence of inflammatory infiltrate in kidneys from 12-month-old mice, significant tubular dilation was not found until the age of 18 months, showing only a tendency to increase at the age of 12 months (Figure 1A and B). To confirm these changes, we also evaluated mRNA levels of the kidney injury biomarkers Lcn2 (which encodes for NGAL protein) and Havcr-1 (which encodes for KIM-1 protein) (Amin et al., 2004; Beker et al., 2018; Gohda et al., 2020; Griffin et al., 2019; Prozialeck et al., 2007). The renal gene expression levels of both genes were upregulated only at 18 months (Figure 1C).

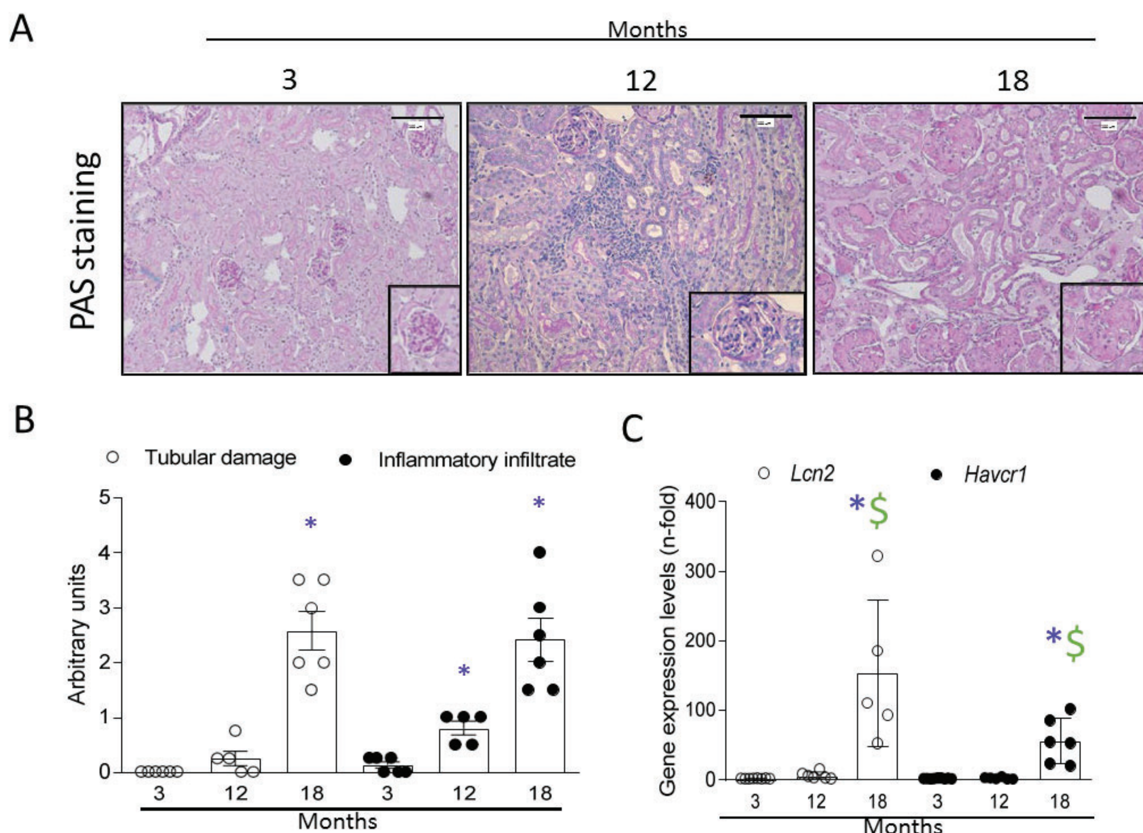

Figure 1. Incipient kidney damage was observed in 12-month-old mice and was increased in 18-month-old mice
Kidneys from 3-, 12- and 18-month-old C57BL/6 mice were studied. **(A)** Representative PAS staining microphotographs per group at 200X magnification, and glomeruli detail. Scale Bar: 100μm. **(B)** PAS staining score (From 0 to 4) was categorized as: tubular damage (defined as tubular dilatation and tubular atrophy), and inflammatory infiltrate (interstitial and perivascular cells). Data are presented as arbitrary units. **(C)** qRT-PCR from kidney extracts analyzing *Lcn2* (which encodes NGAL) and *Havcr1* (which encodes KIM-1). Data are represented as n-fold and expressed as mean ± SEM of 6-9 animals per group. *p <0.05 vs. 3-month-old mice and $p <0.05 vs. 12-month-old mice. The non-parametric Kruskal-Wallis statistical test was performed.

Kidney infiltrating cells were further characterized by immunohistochemistry using specific markers for neutrophils (Myeloperoxidase, MPO) and macrophages (F4/80+ cells). Infiltration by neutrophils and monocytes/macrophages was observed in the kidney cortex of 12- and 18-month-old mice, not finding substantial differences between aged mice in MPO but higher F4/80+ infiltrating cells in 18-month-old mice (Figure 2). Taken together, the spontaneous development of tubular and glomerular lesions in naturally aging mice was

preceded by kidney inflammatory infiltration.

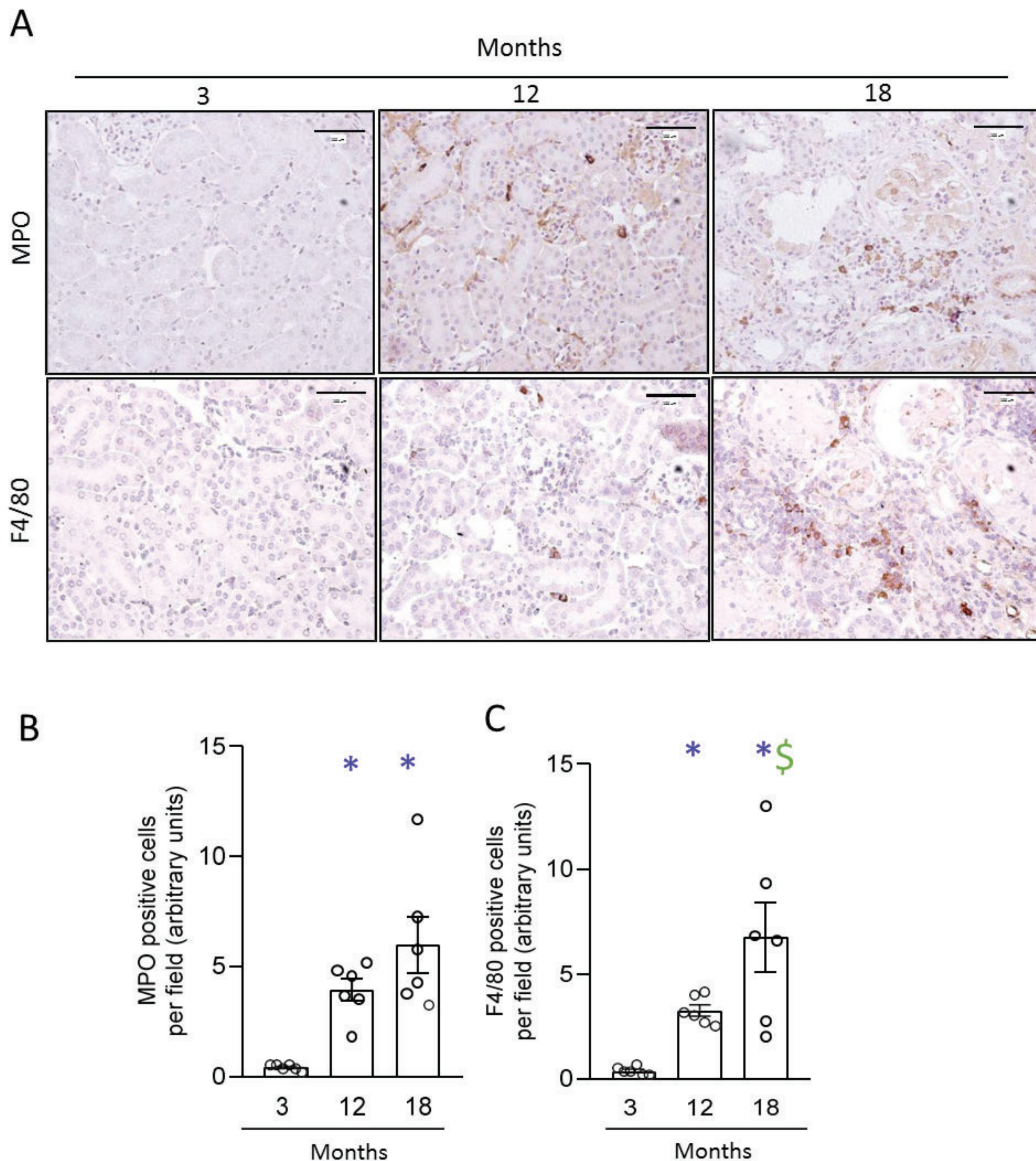

Figure 2. Interstitial inflammatory infiltration was already observed in 12-month-old mice and maintained at 18-months Kidneys from 3-, 12- and 18-month-old C57BL/6 mice were stained for myeloperoxidase (MPO) and F4/80 by immunohistochemistry. **(A)** Representative microphotographs of MPO (neutrophil marker) and F4/80 (macrophage and dendritic cell marker) at 200X magnification. Scale Bar: 100μm. **(B)** MPO quantification and **(C)** F4/80 quantification of the average of positive cells per field, presented as arbitrary units and expressed as mean ± SEM of 6 animals per group. *p <0.05 vs. 3-month-old mice and $$p$ <0.05 vs. 12-month-old mice. The One-way ANOVA statistical test was performed.

Aged kidneys display increased collagen accumulation

To determine whether aged kidneys presented aberrant ECM accumulation a picrosirius red staining was performed and collagen content was evaluated. Kidney cortex showed a slight increase on collagen accumulation by 12 months, but only in 18-month-old mice collagen deposition was significantly higher than in 3-month-old mice (Figure 3). Thus, 18-month-old

mice developed fibrosis.

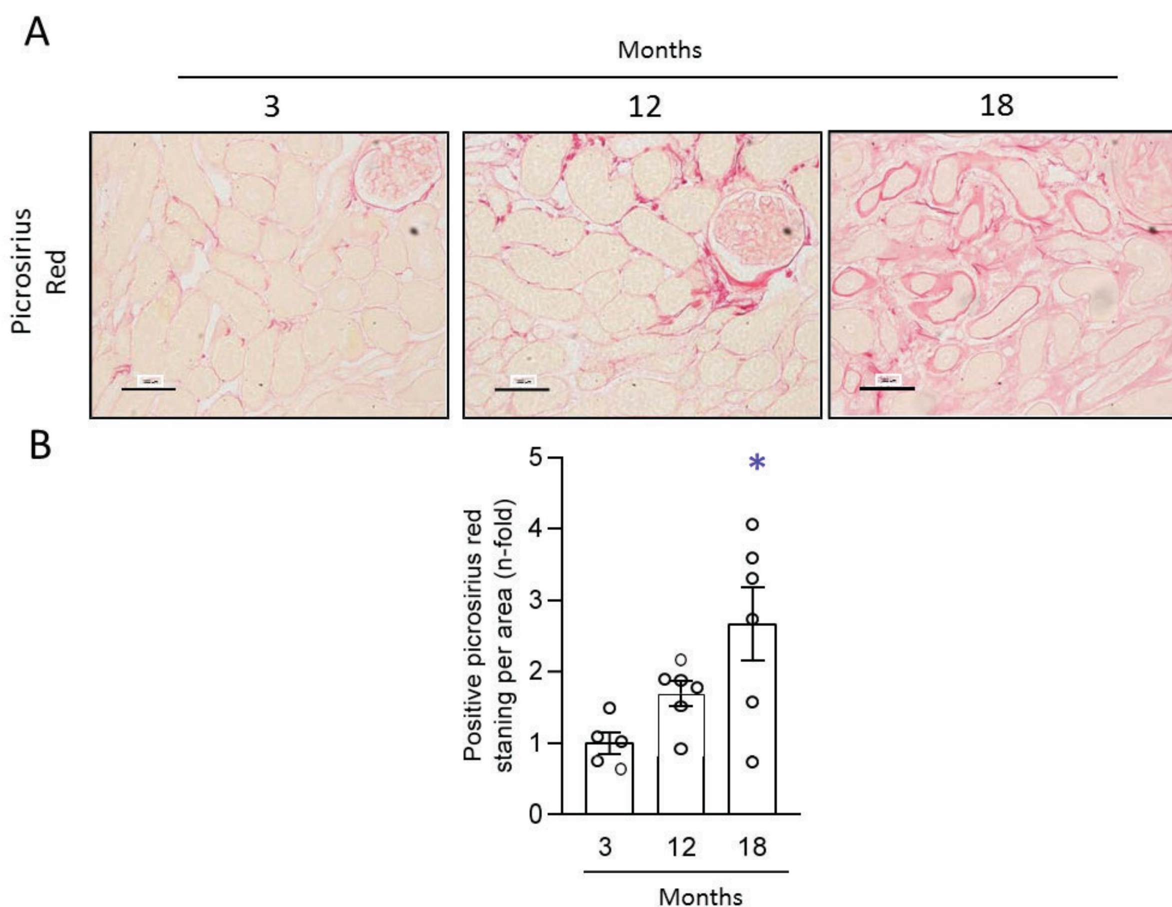

Figure 3. Collagen accumulation was found in the kidneys of 18-month-old mice
Paraffin-embedded kidneys from 3-, 12- and 18-month-old C57BL/6 mice were stained with picrosirius red. **(A)** Representative microphotographs of picrosirius red staining at 200X magnification. Scale Bar: 100 μm. **(B)** Positive picrosirius red staining quantification per total area, presented as n-fold and expressed as mean ± SEM of 6 animals per group. *p <0.05 vs. 3-month-old mice and \$$p$ <0.05 vs. 12-month-old mice. The One-way ANOVA statistical test was performed.

Activation of senescence mechanisms: DNA-Damage-Response precedes cell cycle arrest and SASP

To evaluate the development of a senescence phenotype in the aging kidney, several markers of senescence-associated mechanism were assayed. First, activation of DNA-Damage-Response (DDR) was evaluated using the DDR marker γH2AX by IH. In kidneys of 12-month-old mice, γH2AX positive nuclei were found, mainly in tubular cells and in some infiltrating immune cells, whereas almost no γH2AX positive nuclei were observed in 3-month-old mice. Similar levels of γH2AX positive nuclei were observed in 18-month-old mice (Figure 4A,B).

Next, CCA changes were investigated. First, p21 was determined by IH and RT-PCR. Although a slight increase on p21 protein and mRNA levels was observed in the kidneys of 12-month-old mice, only at 18 months both gene and protein levels were significantly upregulated compared to young mice (Figure 4A, C and D). Remarkably, some infiltrating immune cells were also p21 positive (Figure 4A), suggesting that immunosenescence and inflammaging are already present in the aged kidney. The CKD inhibitor p16 was also explored. Although a tendency for higher p16 mRNA levels was observed in 12-month-old mice, a significant upregulation of p16 mRNA levels was only observed in 18-month-old mice (Figure 4E).

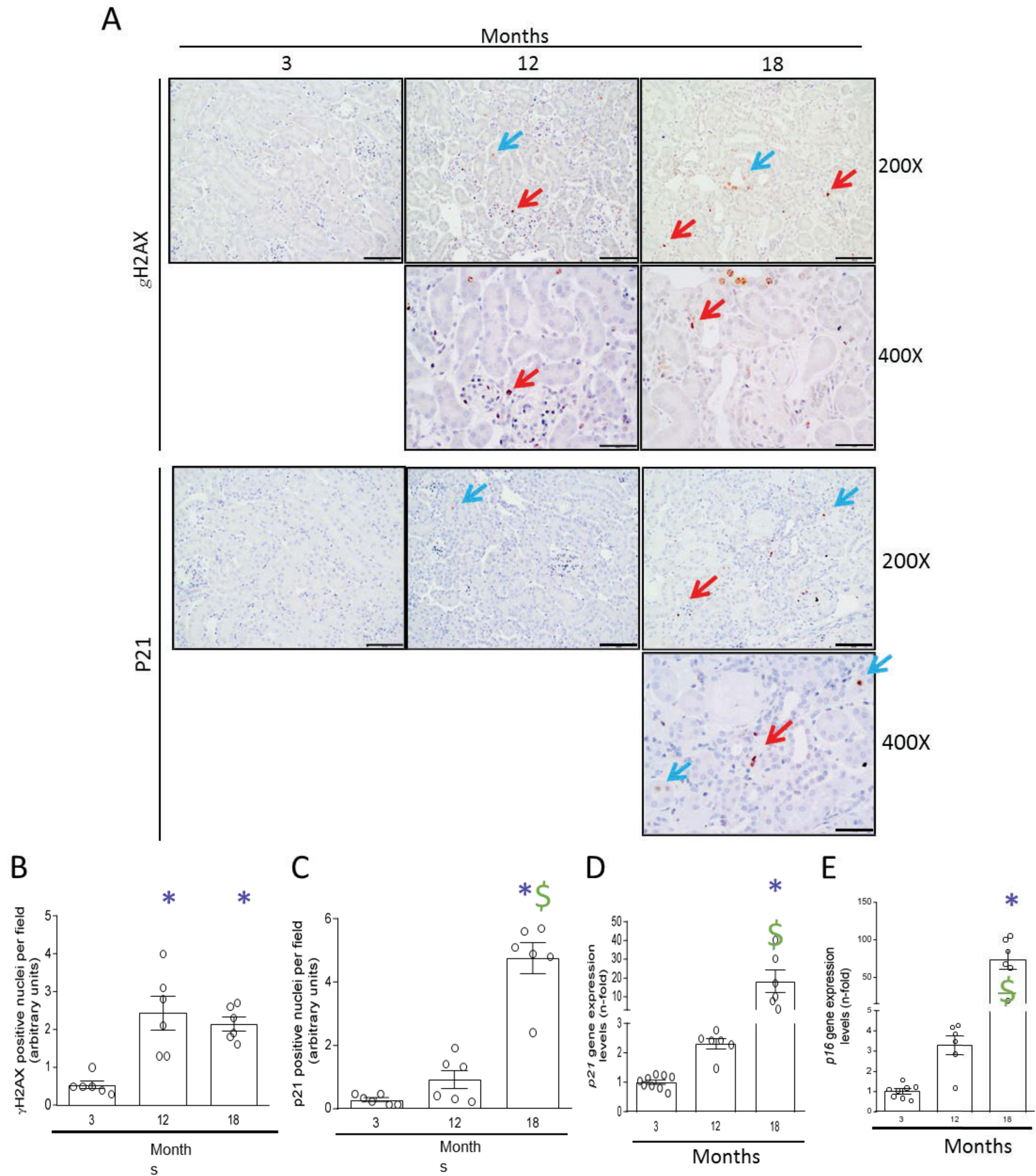

Figure 4. Early activation of DNA Damage Response (DDR) in 12-month-old mice kidneys was followed by induction Cell Cycle-Arrest (CCA) in 18-month-old kidneys
Kidneys from 3-, 12- and 18-month-old C57BL/6 mice were studied. **(A)** Representative immunohistochemistry microphotographs for γH2AX, a DDR marker, and p21, a cyclin-dependent kinase inhibitor which is a CCA marker, at 200X (Scale Bar: 100 μm) and 400X (Scale Bar: 50 μm) magnification. Red arrows mark positive interstitial nuclei and blue arrows mark positive tubular epithelial cell nuclei. **(B,C)** Quantification of γH2AX (B) and p21 (C) positive nuclei per field, respectively. Data are presented as arbitrary units. **(D,E)** qRT-PCR from kidney extracts for *p16* (D) and *p21* (E), markers of CCA and cellular senescence. Data are presented as n-fold and expressed as mean ± SEM of 6-9 animals per group. **p* <0.05 vs. 3-month-old mice and $*p* <0.05 vs. 12-month-old mice. The non-parametric Kruskal-Wallis statistical test was performed.

Another feature of senescent cells is a SASP, including the increased production of proinflammatory and profibrotic factors (Hernandez-segura et al., 2018; Knoppert et al., 2019b). The analysis of the gene expression levels of the proinflammatory SASP components *Ccl2*, *Il6* and *Il1b* and the profibrotic factors *Tgfb1, Ccn2/Ctgf* and *Serpine1* (which encodes PAI-1) showed that all of them were only upregulated in 18-month-old mice (Figure 5), but not in 12-month-old mice. Taken together, this data indicates that aging-associated senescent cell

accumulation involves initial DDR as first mechanism of damage, subsequently followed by CCA and SASP.

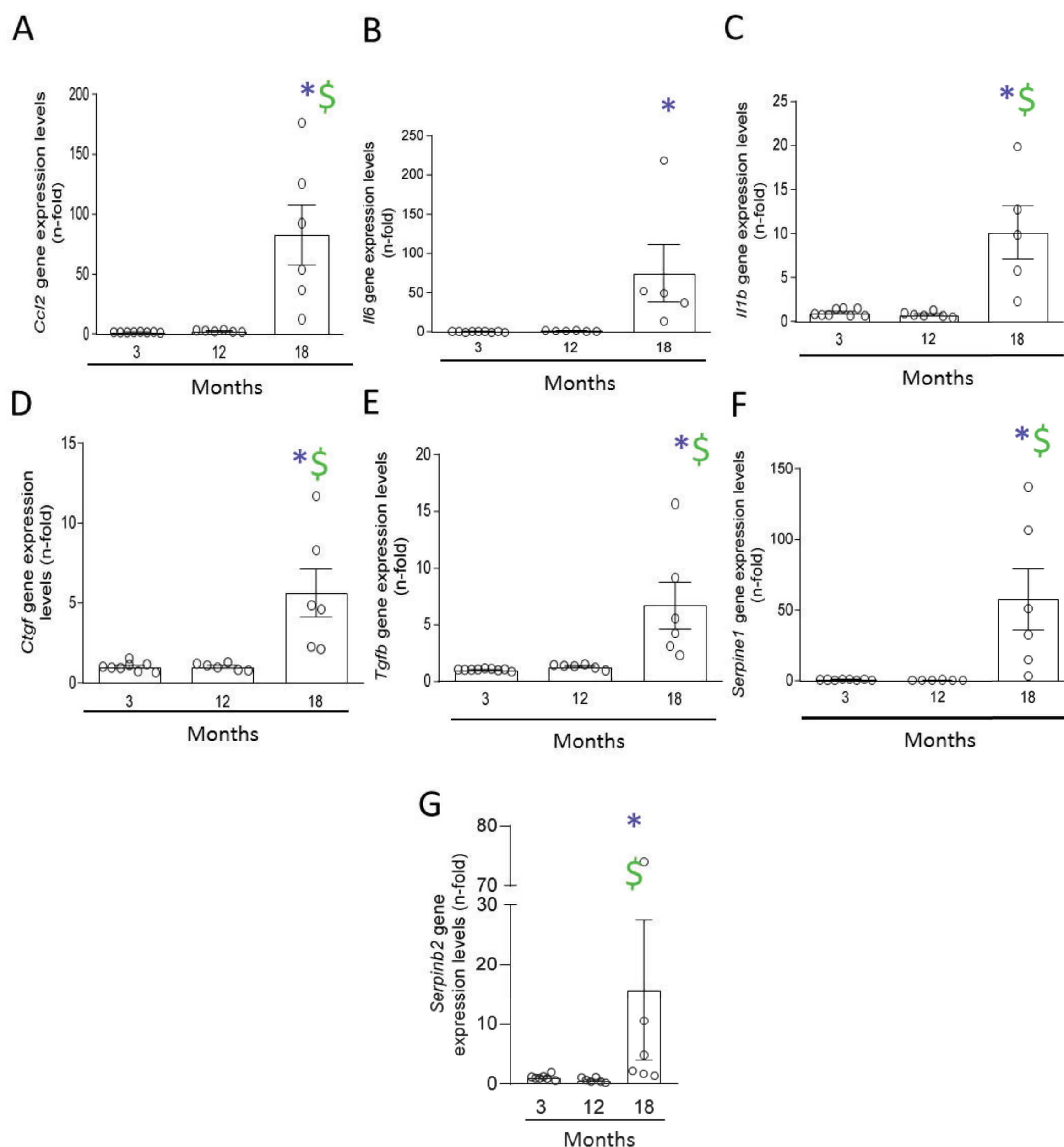

Figure 5. Senescence-associated secretory phenotype (SASP) was found in 18-month-old mice kidneys
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. **(A**) *Ccl2,* **(B)** *Il6,* **(C)** *Il1b,* **(D)** *Ctgf,* **(E)** *Tgfb1,* **(F)** *Serpine1* and **(G)** *Serpinb2* were analysed by qRT-PCR from kidney extracts. Data are represented as n-fold and expressed as mean ± SEM of 6-9 animals per group. *$p < 0.05$ vs. 3-month-old mice and \$$p < 0.05$ vs. 12-month-old mice. The One-way ANOVA statistical test was performed.

The anti-aging factor Klotho is lost early during renal aging

Klotho is rapidly downregulated when kidneys are injured, making this factor a marker of early kidney damage, that has been associated with the aging and cellular senescence in the kidney (Fernandez-Fernandez et al., 2018; Fernández-Fernández et al., 2020; Marquez-Exposito et al., 2021; Moreno et al., 2011; Maria Dolores Sanchez-Niño et al., 2020). As expected, Klotho mRNA levels were downregulated in 12-month-old mice, and a significant loss of Klotho was found in 18-month-old mice when compared to 12-month-old mice (Figure 6), suggesting an overtime progressive aging-associated loss of Klotho.

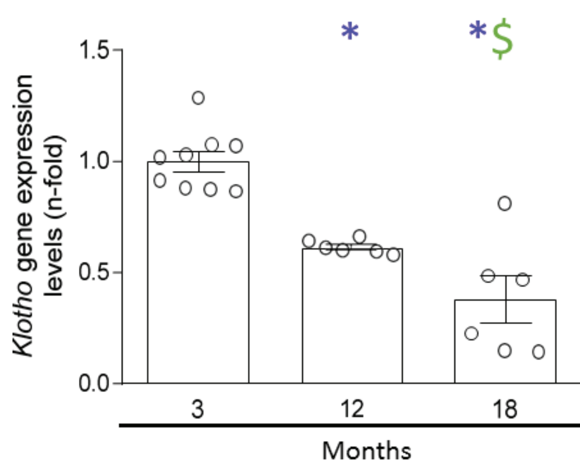

Figure 6. Klotho gene expression levels showed a marked decline in 12-month-old mice which was more downregulated in 18-month-old mice
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. Klotho qRT-PCR from kidney extracts were done. Data are represented as n-fold, and expressed as mean ± SEM of 6-9 animals per group. *p < 0.05 vs. 3-month-old mice and $$p$ < 0.05 vs. 12-month-old mice. The One-way ANOVA statistical test was performed.

NRF2 pathway is deregulated in the aging kidney

The timeline of NRF2 pathway was evaluated over time at kidney gene, protein, and activation levels. Both Nfe2l2 (the gene that encodes for the NRF2 protein) gene expression and NRF2 total protein expression levels were elevated in the kidneys from 18-month-old mice, but not from 12 months, compared to 3-month-old mice (Figure 7A and B). Finally, the activation of NRF2 pathway was explored by evaluating NRF2 phosphorylation by IH. Interestingly, IH quantification showed that NRF2 overactivation peaked at 12 months as assessed by increased phosphorylated NRF2 in cell nuclei. NRF2 activation diminished thereafter, and phosphorylated-NRF2 levels were similar in 18- and 3-month-old mice (Figure 7). These data show an early activation of the NRF2 pathway, observed at 12 months, followed by an upregulation of NRF2 protein levels associated to increase de novo gene expression, but not associated to sustained activation of the pathway.

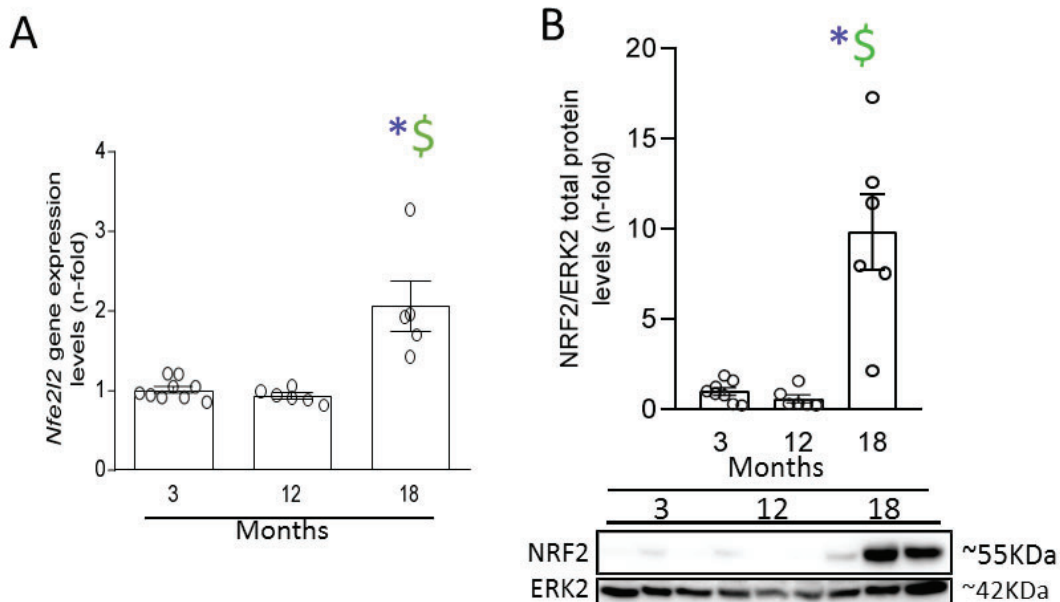

Figure 7. Early activation of antioxidant NRF2 pathway in 12-month-old mice was followed by a deregulation in 18-month-old mice kidneys
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. **(A)** qRT-PCR from kidney extracts of *Nfe2l2* gene expression levels. **(B)** Quantification of NRF2 total protein levels by Western blot in the upper panel and the representative blots in the lower panel, using ERK2 protein levels as loading control.

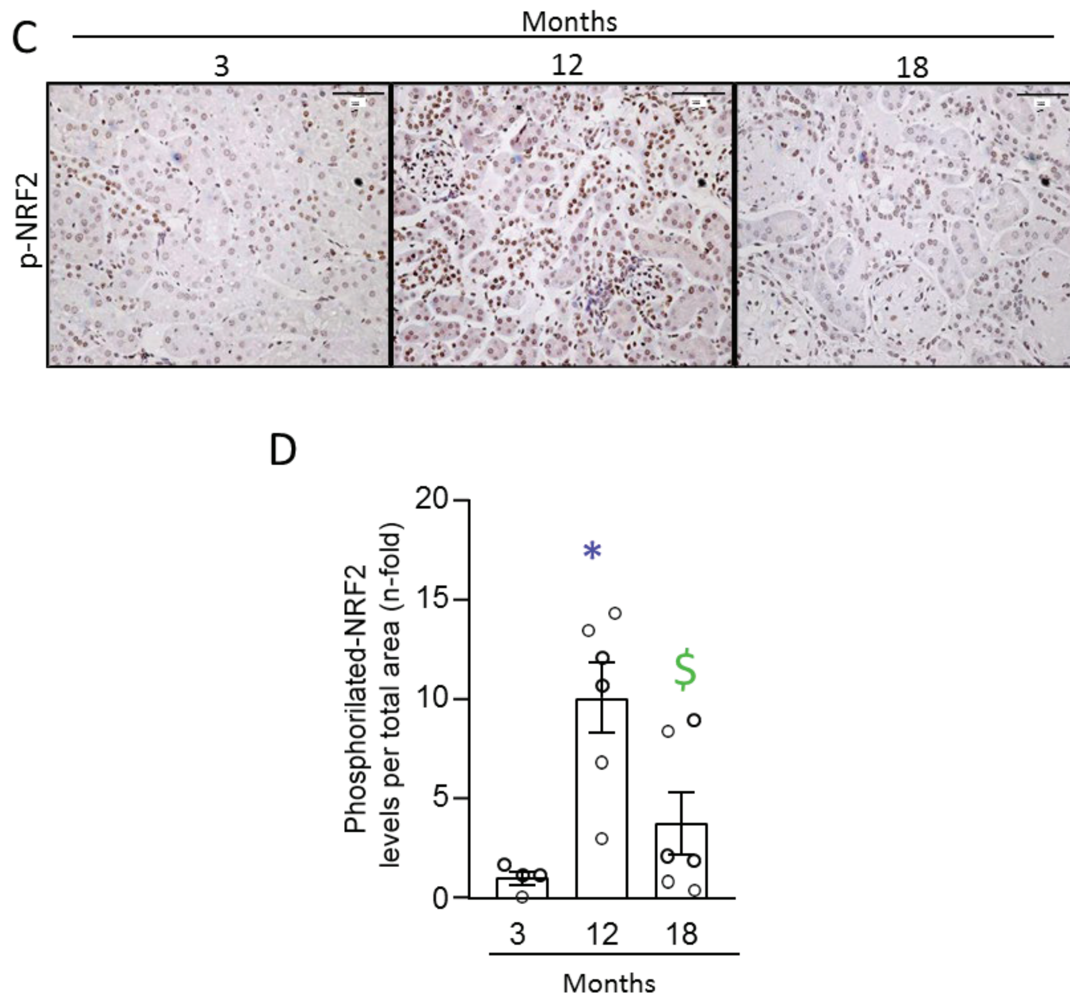

Figure 7 (continued)
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. **(C)** Representative microphotographs of phosphorilated-NRF2 staining at 200X magnification. Scale Bar: 100 μm. **(D)** Quantification of positive phosphorilated-NRF2 nuclei per total area. Data are represented as n-fold and expressed as mean ± SEM of 4-9 animals per group. *$p < 0.05$ vs. 3-month-old mice and \$$p < 0.05$ vs. 12-month-old mice. The non-parametric Kruskal-Wallis statistical test was performed.

A redox imbalance is progressive in the aging kidney

Oxidative stress is another mechanism associated with senescence and kidney damage (Banerjee et al., 2021; Stenvinkel et al., 2021; Ungvari et al., 2017). Gene expression of redox response-related factors, including Catalase, Sod1 and Hmox-1 (which encodes for HO-1 protein) were analyzed. Renal gene expression of Catalase was downregulated at 12 months compared to 3 months, and further decreased at 18 months (Figure 8), whereas Sod-1 gene expression was only diminished at 18 months. In contrast, Hmox-1 mRNA levels were elevated in the kidneys from 18-month-old mice, but not at earlier time points (Figure 8).

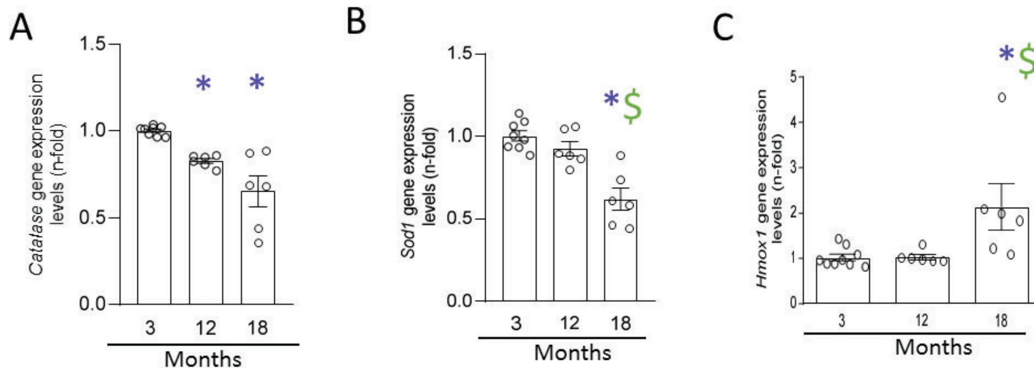

Figure 8. The antioxidative NRF2 target genes are modified during murine kidney aging
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. **(A)** *Hmox1* (which encodes for hemooxygenase-1 protein), **(B)** *Catalase* and **(C)** *Sod1* (which encodes for Superoxide dismutase 1 protein) were analysed by qRT-PCR from kidney extracts. Data are represented as n-fold and expressed as mean ± SEM of 4-9 animals per group. $*p < 0.05$ vs. 3-month-old mice and $\$p < 0.05$ vs. 12-month-old mice. The non-parametric Kruskal-Wallis statistical test was performed.

Finally, lipid peroxidation, one of the final responses of oxidation in CKD (Guo et al., 2021; Soulage et al., 2020), was studied by staining for 4-HNE. A significant increase of lipid peroxidation was only observed in the kidneys of 18-month-old mice, but not at 12-month-old (Figure 9).

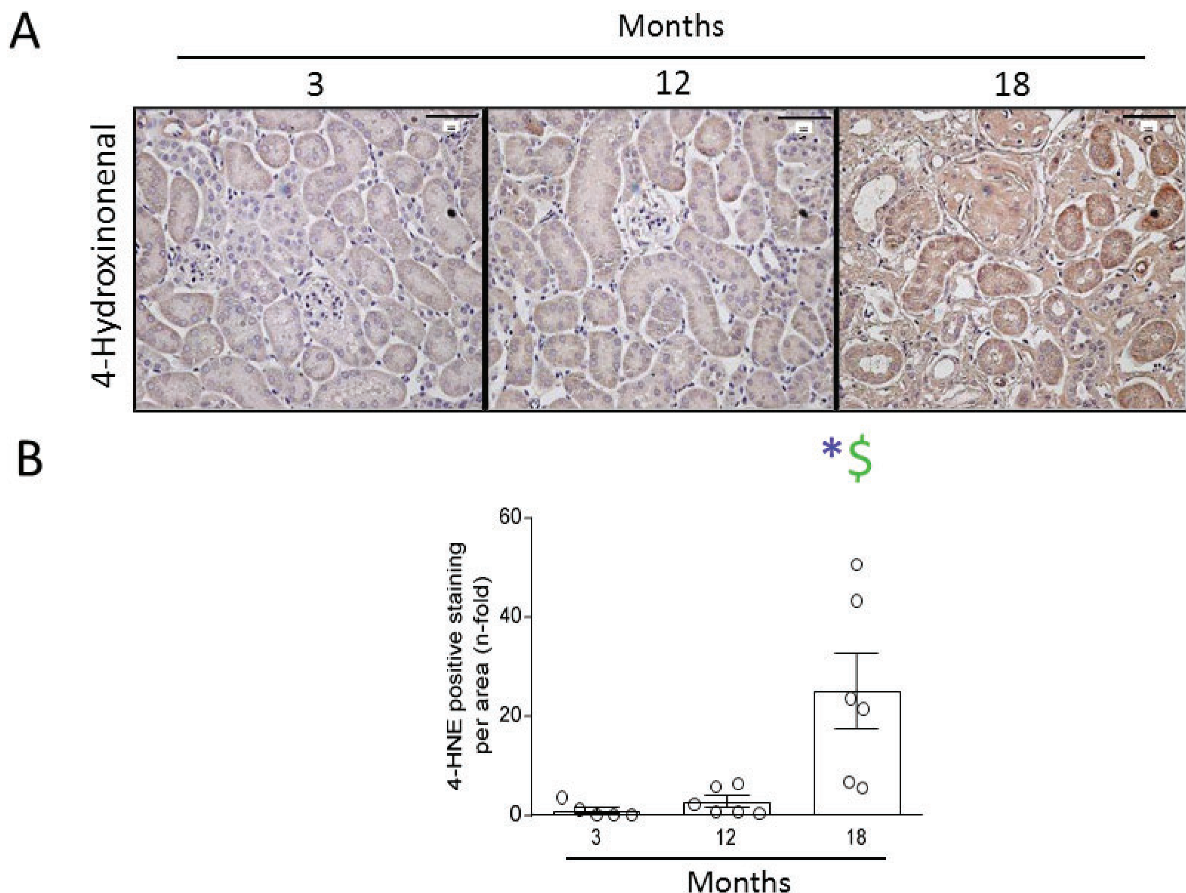

Figure 9. The lipid peroxidation marker 4-Hydroxinonenal is increased in 18-month-old mice kidneys
Kidneys from 3, 12 and 18-month-old C57BL/6 mice were extracted and studied. **(A)** Representative microphotographs of the 4-Hydroxinonenal (4-HNE) immunohistochemistry at 200X magnification. Scale Bar: 100μm. **(B)** Quantification of 4-HNE protein levels per total area. Data are represented as n-fold and expressed as mean ± SEM of 5-9 animals per group. $*p < 0.05$ vs. 3-month-old mice and $\$p < 0.05$ vs. 12-month-old mice. The non-parametric Kruskal-Wallis statistical test was performed.

DISCUSSION

Our findings support the hypothesis that loss of protective factors (Klotho), dysfunctional redox regulatory mechanisms and senescent cells (both tubular cells and immune cells) can be early drivers of age-related kidney damage progression (Figure 10).

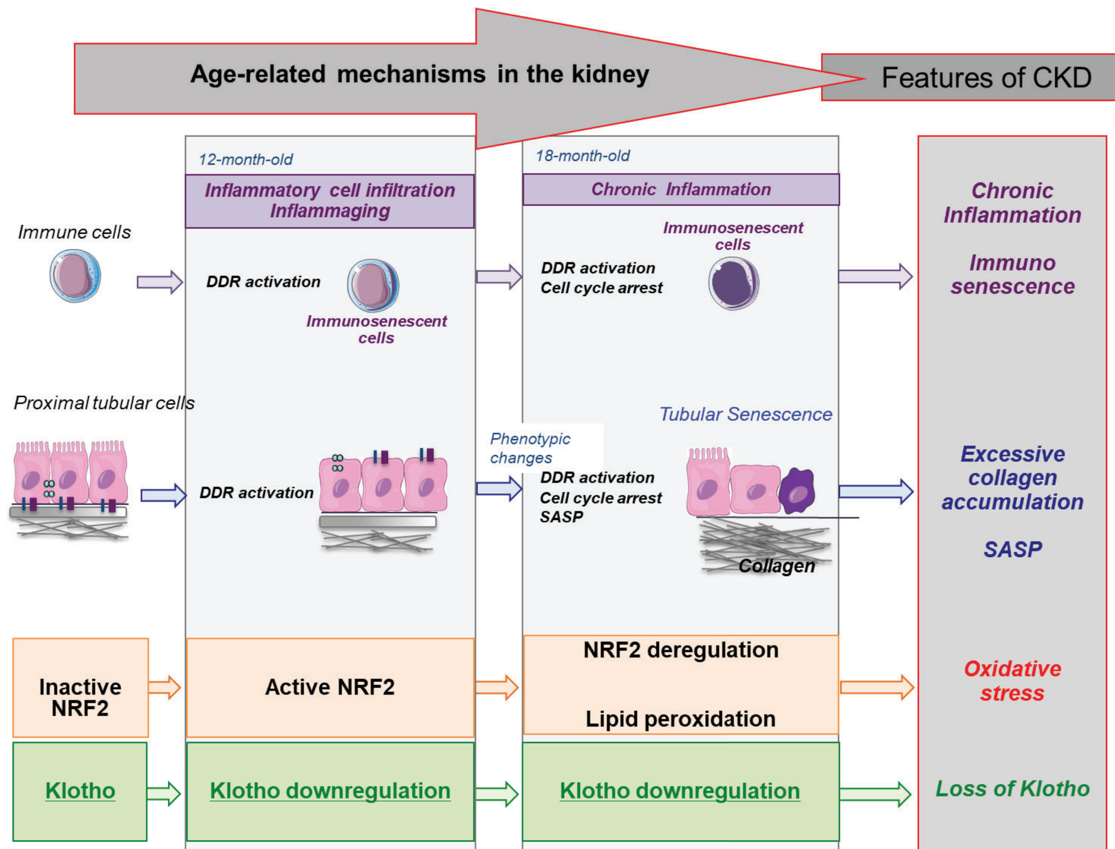

Figure 10. Conceptual representation of age-associated mechanisms in murine kidneys
The earliest changes in aging kidneys include the activation of pro-senescent responses, such as DDR in both proximal-tubular and immune cells, the activation of NRF2 antioxidant defense and the loss of the nephroprotective factor klotho before morphological changes occur. In the long term, DDR activation is maintained and is a driver of CCA-induced cellular senescence, which is observed in tubular and immune cells. Moreover, there is an induction of SASP, accumulation of collagen and loss of the nephroprotective responses NRF2 and Klotho, leading to increase oxidative stress. All of these are hallmarks of CKD.

In humans, glomerular filtration rate (GFR) decreases progressively, starting from age 18-24 years (Wetzels et al., 2007). At the age of 50–60 years, even healthy human kidneys from human suffer macrostructural changes, such as a decrease in the cortex and increase in the medullary volume, increasing the surface roughness and the number of cysts. Moreover, nephron loss is directly related to GFR decline, and nephrons are lost with aging (Hommos et al., 2017), but there is no clear relation between activation of senescence mechanisms and loss of kidney function in healthy humans. Although many experimental studies have investigated the activation of senescence mechanisms in response to kidney injury, little attention has been given to the characterization of the activation of senescence in healthy mice, and most of the studies have been done in very old mice (Maddens et al., 2012; Nath et al., 2013; Wen et al., 2015).

Our studies demonstrate that 12-month-old C57Bl6 mice present an early activation of pro-senescence mechanisms in tubular cells, characterized by high nuclear γH2AX in some tubular epithelial cells, suggesting DNA damage, and subsequent DDR activation. DDR markers can activate p21/p53 downstream pathways to induce cellular senescence (Hernandez-segura et al., 2018; Valentijn et al., 2018). However, in 12-month-old mice, the presence of p21 positive cells in the kidney was scarce and renal mRNA levels were slightly, but not significantly upregulated. Interestingly, in 18-month-old mice, an increase in p21 positive tubular cells, increased p21 and p16 gene expression levels, and overexpression of

SASP genes were found. Furthermore, this was associated with tubular damage and fibrosis, exclusively in 18-month-old mice. These data show that at the age of 12 months, the mouse kidney exerts DDR activation, but at this age is not associated with tubular damage and fibrosis. However, the deleterious age-related changes continue along the time. At 18 months, tubular cells presented positive senescence markers (nuclear γH2AX and p21 expression), associated with tubular damage and collagen accumulation. Accordingly, in a study done in CB6F1 hybrid female mice, a strain reported to have an average lifespan of 30 months (Miller et al., 2005), which is considerably longer than the average lifespan of 25 months reported for C57BL/6mice (Turturro et al., 1999), p21 gene expression was increased at 12 months, whereas p16 only increased after 24 months (Miao et al., 2019). In previous studies in aged kidneys from mice and humans with allograft nephropathy, p16 and p21 expression were highly correlated with structural and functional histological changes (Melk, 2003; Yang & Fogo, 2010). These studies support our data, since we observed tubular damage and peak expression of p16 and p21 only in 18-month-old mice, indicating that these cellular senescence markers could represent a late stage of dysfunction when the kidney damage is already established. Morphological changes and loss of mitochondria function would lead to ROS production, triggering oxidative stress and therefore accelerating the progression of renal fibrosis (Miao et al., 2019). In a previous study in C57Bl6 mice, mitochondrial dysfunction was only found at 24 months, but not earlier (Miao et al., 2019). Our findings showing a tendency for increased collagen accumulation already at the earliest time points suggest that other mechanisms, besides mitochondrial dysfunction, could be involved in age-related kidney fibrosis.

SASP-mediated secondary senescence can be another mechanism of age-related kidney damage, and could contribute to kidney damage progression, causing sustained inflammation and fibrosis. In this sense, 18-month-old murine kidneys presented a significant upregulation of SASP components, including profibrotic factors, such as TGF-β and CCN2, and maintained the γH2AX and p21/p16 activation in tubular-epithelial cells, contributing to amplify tubular damage by secondary senescence and fibrosis. The release of SASP components by senescent cells can affect the growth, migration and differentiation of neighboring cells, mainly impacting overall tissue architecture, and promoting chronic inflammation (Ovadya & Krizhanovsky, 2014; Van Deursen, 2014). Changes in SASP components have been previous investigated in very old (30 months) female mice showing an upregulation of proinflammatory factors *Il1b, ccl8, Cxcl1* and *Cxcl2* in kidneys (Hudgins et al., 2018). Moreover, SASP factors can contribute to NF-κB pathway activation, which is known to have a relevant role in establishing and maintaining SASP components production and DDR activation (Franzin et al., 2021). Taken together, both the DDR and SASP are involved in releasing more pro-senescence secretome factors, thus amplifying the inflammatory and fibrotic response which is one of the mechanisms involved in the AKI-to-CKD transition and in CKD progression (Franzin et al., 2021).

Aging has been related to low-grade chronic inflammation, in a process termed “inflammaging”, in which both the innate and acquired immune responses are dysregulated (Franceschi & Campisi, 2014; Goronzy & Weyand, 2013; Montecino-Rodriguez et al., 2013). Human kidney transcriptomics disclosed evidence of inflammaging in aging kidneys (Rodwell et al., 2004). Moreover, healthy and transplanted aged human kidneys have higher inflammatory infiltration than young ones (Øien et al., 2007). Consistently, we have found the presence of inflammatory cell infiltration, including macrophages and neutrophils, in the kidneys from 12-month-old mice, but at this point there were no significant changes in kidney gene expression levels for several proinflammatory factors, including SASP components or chemokines, that could be responsible for immune recruitment into the kidney. Importantly, “immunosenescence” has been described as the decline of immune cell efficiency due to aging (Aiello et al., 2019). Immunosenescent cells differ from healthy immune cells, as they express more proinflammatory factors, display different the CD membrane expression markers (for T cells, overexpression of CD57 and loss of CD28), express CCA proteins such as γH2AX, p21 and p16 (Rodriguez et al., 2020), and an altered secretome (Aiello et al., 2019). Immunosenescent cells accumulate in different tissues during natural aging (Aiello et al., 2019). In our study, γH2AX-positive cells infiltrated the kidneys of 12-month-old mice,

indicating incipient immunosenescence. Moreover, accumulation of γH2AX and p21 positive infiltrating cells was clearly found in 18-month-old kidneys, supporting the involvement of immunosenescent cells in kidney damage progression. Experimental studies have demonstrated that the selective, senolytic-mediated elimination of senescent cells or the disruptions of the SASP program could be used as potential therapeutic strategies against aging (Baar et al., 2017). All these data remark the complexity of senescence regulation and functional consequences, indicating the necessity of further research in this area.

Oxidative stress is one of the possible inducers of senescence, since ROS production can damage the DNA and activate the DDR (Muñoz-Espín & Serrano, 2014). Moreover, prolonged ROS production activates the NRF2/Are pathway, implicated in detoxification, through NRF2 phosphorylation and translocation to the nucleus, leading to the regulation of transcription of NRF2 target genes. Several of these genes are involved in the antioxidant response, such as *Catalase*, *Sod-1*, *Hmox-1* and others (Khan et al., 2021; Ungvari et al., 2019; Yu et al., 2021). Some evidences show that cellular senescence is directly associated with NRF2 pathway impairment, since increased NRF2 pathway decreases senescence marker expression such as p21 (Yu et al., 2021) and its inhibition produces cellular senescence (Khan et al., 2021). Different preclinical studies have demonstrated the role of the NRF2 in kidney damage. In murine subtotal nephrectomy, NRF2 nuclear levels and the expression of the target genes *Hmox-1*, *Catalase* and *Gpx4*, was decreased 12 weeks postrenal ablation, indicating an impairment of the NRF2 antioxidant pathway in CKD (H. J. Kim & Vaziri, 2010). In another study, NRF2 was overactivated initially following unilateral ureteral obstruction in mice but downregulated in the chronic phase, indicating that NRF2 pathway inactivation is associated to sustained inflammation and damage progression (Stenvinkel et al., 2021). Our data support these findings, since NRF2 is overactivated in 12-month-old mice, indicating a redox imbalance and highlighting that mild damage is already occurring in the kidney. However, al later time points, NRF2 pathway is impaired, as evidenced by the downregulation of active NRF2 and *Catalase* and *Sod-1* gene expression levels in 18-month-old mice. The NRF2 total mRNA and protein upregulation observed at this time-point could represent a compensatory mechanism against tissue damage but this pathway is already dysfunctional. On the other hand, NRF2 activation goes beyond cytoprotective properties, as it is implicated in lipid metabolism (Stenvinkel et al., 2020). In CKD, aberrant quantities of plasma lipids are a target of ROS, creating subproducts such as 4-HNE and accompanied by decreased antioxidant enzymes such as SOD-1 and Catalase, among others (Guo et al., 2021). In our study, we only observed 4-HNE production at 18 months, suggesting excess ROS production and lipid deposition, not compensated by efficient antioxidant responses, as evidenced by low *Catalase* and *Sod-1* gene expression. These data indicate that 18-month-old mice already have evidence of CKD that is not observed in 12-month-old mice. These findings suggest that targeting the NRF2 pathway should be explored as a potential therapy for kidney aging.

Another remarkable factor involved with ROS production and cellular senescence is the nephroprotective hormone Klotho, which was downregulated in kidneys from 12-month-old mice (Marquez-Exposito et al., 2021; Miao et al., 2019). Klotho is normally expressed and secreted by tubular cells and has anti-aging, anti-inflammatory and anti-fibrotic properties (Kuro-o et al., 1997; Kurosu et al., 2005; Maria D. Sanchez-Niño et al., 2013). For example, Klotho protects endothelial cells from senescence (Carracedo et al., 2012), and is a marker of aging and cellular senescence (de Oliveira, 2006; Kanbay et al., 2021). Klotho downregulation can be both a consequence and driver of inflammaging and increased ROS production in kidney disease (Fernández-Fernández et al., 2020; Izquierdo et al., 2012; Moreno et al., 2011; Maria Dolores Sanchez-Niño et al., 2020; Yang & Fogo, 2010). The increase in ROS production due to a Klotho downregulation could explain the overactivation of NRF2 in 12-month-old mice, as in these mice we already found low levels of Klotho. Despite NRF2 activation, in 18-month-old mice Klotho is further downregulated, NRF2 activation impaired and pro-fibrotic gene expression and collagen deposition highly increased, in line with the role of Klotho in diminishing cellular senescence and renal fibrosis, the hallmark of CKD (Maique et al., 2020).

In conclusion, our preclinical studies in C57Bl6 mice describe early age-related changes in 12-month-old mice kidneys characterized by the loss of the nephroprotective factor Klotho, the activation of several protective responses, such as DDR and NRF2/antioxidant defense, and the presence of infiltrating immunosenescent cells. At this time point the investigation of biomarkers of early damage could be very interesting. However, the deleterious age-related changes progress over time, since in 18 month-old mice, tubular damage and kidney fibrosis were already associated with tubular senescence phenotype changes, including cell growth arrest and SAPS overexpression, supporting the hypothesis of senescent cells as drivers of age-related kidney damage progression (Figure 10). These processes present many similarities with mechanisms involved in CKD progression and support further research in this area to prevent kidney damage even in healthy population.

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CHAPTER 7

A human conditionally immortalized proximal tubule epithelial cell line as a novel model for studying senescence and response to senolytics

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ABSTRACT

Accumulating evidence suggests that senescence of renal tubular epithelial cells leads to fibrosis. These cells show resistance to apoptosis and secrete senescence-associated secretory phenotype (SASP) factors that are involved in diverse signaling pathways, influencing kidney fibrosis. Here, we investigated whether our previously established conditionally immortalized proximal tubule epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1) can be used as a valid *in vitro* model to study renal senescence and senolytics response. CiPTEC-OAT1 proliferate rapidly at 33℃ and exhibit a "senescence-like" arrest at 37℃, most likely due to suppression of SV40T expression and subsequent reactivation of the p53 and Rb pathways. To understand how permissive (33 ℃) and non-permissive (37 ℃) temperatures of the cell culture affect the senescence phenotype, we cultured ciPTEC-OAT1 for up to 12 days and evaluated SASP and apoptosis markers. Day 0 in both groups is considered as the non-senescence group (control). Further, the potential of established senolytics navitoclax, dasatinib, quercetin and the combination of the latter two to clear senescent cells, was evaluated. Maturation of ciPTEC-OAT1 at non-permissive temperature affected mRNA and protein levels of senescence markers. Decreased expression of lamin b1 and upregulation of p21 gene expression were found in the non-permissive temperature group. SASP factors, including CCN2, PAI-1A, IL-1β, and IL-6 were also upregulated, but no difference in Bcl-2 and Bcl-xL mRNA levels were found in the non-permissive temperature group. After culturing ciPTEC-OAT1 up to 12 days, cells in the non-permissive temperature group showed an upregulation in the apoptosis-associated proteins Bcl-2, BID and Bax and a downregulation in Mcl-1, Bad, Bak and Bim at various time points. Further, Bcl-xL, Puma, Caspase 3, Caspase 7 and Caspase 9 showed initial upregulations followed by downregulations at later time points. The loss of lamin B1, upregulation of p21 levels and downregulation of p53, along with the upregulation of SASP factors were indicative for a senescence phenotype promoted by maturation at 37 ℃. Finally, the senolytics response was evaluated by testing cell viability following exposure to senolytics, to which cells appeared dose-dependently sensitive. Navitoclax was most effective in eliminating senescent cells. In conclusion, culturing ciPTEC-OAT1 at 37 ℃ induces a senescence phenotype characterized by increased expression of cell cycle arrest and anti-apoptosis markers, SASP factors and responsiveness to senolytics treatment.

INTRODUCTION

Renal fibrosis is the common end point for progressive kidney diseases, including kidney failure and excessive accumulation of extracellular matrix (Boor et al., 2010). Accumulating evidence suggest that senescence of renal tubular epithelial cells (TECs) influences kidney fibrosis (Schafer et al., 2018). Senescence is a state of prolonged cell cycle arrest, which limits cellular proliferative life span. Opposed to acute (short-term) senescent cells that can be cleared by immune cells, chronic (long-term) senescent cells keep accumulating and contribute to tissue pathology (Munoz-Espin and Serrano, 2014;Kobbe, 2019). Different hallmarks of senescence have been recognized which are involved in diverse signaling pathways, including cell cycle arrest, a senescence-associated secretory phenotype (SASP), and apoptosis resistance (Hernandez-Segura et al., 2018).

Cell cycle arrest is a typical characteristic of senescent cells, which is largely mediated through activation of either one or both p53/p21$^{CIP1/WAF1}$ (p21) and p16^{Ink4a} (p16)/pRb pathways (Kumari and Jat, 2021). p53/p21 is activated during DNA damage response, resulting in a p21-dependent G0/G1 cell cycle arrest (Ceccaldi et al., 2012;Ou and Schumacher, 2018). On the other hand, p16 inactivates Retinoblastoma 1 (pRb) thereby inhibiting the action of the cyclin dependent kinases, leading to G1 cell cycle arrest (Rayess et al., 2012). Acute DNA damage cause a cell cycle arrest via the p53/p21 pathway, while chronic DNA damage followed by the induction of the p16/pRB pathway maintains cell cycle arrest and senescence (Sperka et al., 2012). As a key mediator of cell cycle arrest, p21 also shows a p53-independent upregulation according to some research (Zhang et al., 2011;Ruan et al., 2020). Furthermore, p53 is also involved in apoptosis (Rufini et al., 2013;Ou and Schumacher, 2018). SASP factors are related to a DNA damage response and are generally proinflammatory and/or profibrotic compounds including numerous cytokines (e.g., IL-6 and IL-8), growth factors (e.g., TGF-β and CCN2), chemokines (e.g., CCL2), and matrix-metalloproteinases (e.g., MMP-1 and MMP-3) (Hernandez-Segura et al., 2018;Birch and Gil, 2020). These proteins induce or maintain senescence through different pathways, contributing to kidney fibrosis (Docherty et al., 2019). Senescent cells also show resistance to apoptosis (Childs et al., 2014) and accumulate dysfunctional mitochondria (Korolchuk et al., 2017). Mitochondrial outer membrane permeabilization (MOMP) is responsible for apoptosis in numerous cell death pathways (Chipuk et al., 2006). In the intrinsic apoptosis pathway, Bcl-2 and caspase family proteins play important roles (Van Opdenbosch and Lamkanfi, 2019;Ngoi et al., 2020). The Bcl-2 family is divided into three main groups: anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1), pro-apoptotic (Bax and Bak), and pro-apoptotic BH3-only (Bim, Bid, Bad and Puma) (Anantram and Degani, 2019). Senescent cells are known to be in a primed apoptotic state, triggered by the abnormal upregulation of anti-apoptotic and pro-apoptotic proteins (Fan et al., 2020). Caspase family proteins are downstream players of MOMP in the intrinsic apoptosis pathway (Shalini et al., 2015), and after the activation of the Bax-Bak-dependent MOMP, cytochrome C is released from the mitochondria stimulating caspase-9 activation and its downstream executioners, caspases-3 and -7, to initiate apoptosis (Van Opdenbosch and Lamkanfi, 2019).

Current efforts are focused on clearing senescent cells as treatment option for prevention of kidney fibrosis development and progression. Senolytics represent a good option as they can selectively eliminate senescent cells participating in senescence associated pathways by interfering with anti- and pro-survival signaling (Zhu et al., 2015). However, suitable cell models are required to evaluate senescence development in TECs and their response to senolytics.

We previously developed a conditionally immortalized proximal tubule epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1) and applied it successfully for pharmacological and toxicological investigations, including drug disposition and interaction studies (Wilmer et al., 2010;Nieskens et al., 2016). OAT1 is a first step in the elimination of organic anions in humans and is responsible for the uptake of many anionic (waste) products in renal proximal tubule (Pou Casellas et al., 2021). Since the expression of OAT1 is rapidly lost when culturing (primary) PTEC *in vitro*, OAT1 was stably expressed in ciPTEC by lentiviral transduction. This cell line now allows prediction of drug-induced

nephrotoxicity and drug-drug interactions of organic anions *in vitro* (Nieskens et al., 2016). OAT1 is also involved in the uptake of uremic toxins, known to participate in the uremic syndrome typical of chronic kidney disease (Nigam and Bush, 2019). Since senescence is an important factor contributing to chronic kidney disease, uremic toxins might play a role in this process as well.

CiPTEC were created by means of a temperature sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) and the essential catalytic subunit of human telomerase (hTERT), to keep the characteristics of primary cells (Wilmer et al., 2010). Temperature-sensitive SV40T allows cells to proliferate at the permissive low temperature of 33 °C but induces a proliferation block that resembles senescence at non-permissive temperature of 37 °C (Larsson et al., 2004;Wilmer et al., 2010). The hTERT maintains telomere length, preventing replicative senescence induced by telomere shortening (Bodnar et al., 1998). Some studies already showed a relation between senescence and SV40T conditional models, because both pRb and p53 are activated by SV40T at the non-permissive temperature leading to a senescence-like arrest in the cells (Larsson et al., 2004;Brookes et al., 2015). Therefore, we hypothesized that ciPTEC-OAT1 exhibit a senescence phenotype when cultured at non-permissive temperature that can be used to study senescence development in TECs and their response to senolytics. In the present study, we evaluated common senescence markers and apoptosis markers in ciPTEC-OAT1 cultured at permissive and non-permissive temperatures at different time points, to investigate whether these cells can be implemented as a valid *in vitro* model to study renal senescence. Day 0 in both groups is considered as the non-senescence group (control). Finally, the senolytics response was detected by means of cell viability assessment and senescence-associated β-galactosidase (SA-β-gal) activity.

MATERIALS AND METHODS

Quantitative real-time PCR

CiPTEC-OAT1 cells were seeded into 6-well format plates and grown at 33 ℃; then half of the plates were transferred to 37 ℃ and cultured for up to 7 days. Afterwards, cells were lysed in Trizol (Thermo-Fisher, Massachusetts, USA) followed by 5 min centrifugation at 4 °C. After RNA isolation, RNA quantity was determined using Nanodrop 2000 (Thermo-Fisher, Massachusetts, USA). For RNA analysis, a cDNA library was synthesized using 3μg RNA per sample with SuperScript III reverse transcriptase (Thermo-Fisher, Massachusetts, USA). Samples were mixed with TaqMan Gene Expression Assays (Table S1) and run on a ViiA 7 real-time PCR system (Applied Biosystems, California, USA). TATA-box binding protein (TBP) was used as internal reference gene. Samples were run in duplicate and H_2O samples were used to control for potential contamination of reaction. ΔΔCT method was used to calculate relative expression levels.

ciPTEC-OAT1 maturation process

CiPTEC-OAT1 were grown and expanded at 33 ℃. Following the seeding, cells were either kept at permissive temperature of 33 ℃ or incubated for desired time up to 12 days at non-permissive temperature of 37 ℃. The culture medium and cell lysate were collected on day 0, 3, 6, 9 and 12, and used for the assessment of senescence markers and phenotype.

The ciPTEC-OAT1 cell line was cultured as reported previously (Mihajlovic et al., 2017). Briefly, cells were cultured in phenol-red free DMEM-HAM's F12 medium (Gibco, Life Technologies, Paisly, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Greiner Bio-One, Alphenaan den Rijn, the Netherlands), 5 μg/mL insulin, 5 μg/mL transferrin, 5 μg/mL selenium, 35 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor (EGF) and 40 pg/mL tri-iodothyronine to form complete culture medium, up to a maximum of 60 passages. Cells were cultured at 33 °C and 5% (v/v) CO2 to allow proliferation. Cells were grown up to 90% confluence at 33 °C, then transferred for 7 days or 9 days at 37 °C, 5% (v/v) CO2 for maturation, refreshing the medium every other day.

Cell viability assay

Cell viability was measured using PrestoBlue® cell viability reagent (Thermo Scientific, Vienna, Austria). CiPTEC-OAT1 cells were seeded into 96-well format plates at a density of 63000 per well, cultured for 24 h at 33 ℃ and matured for 9 days at 37 ℃. Matured cells (37 ℃) were exposed to 100 µL medium with different concentrations of navitoclax, dasatinib, quercetin or dasatnib and quercetin combinations. Senolytics were obtained from MedchemExpress, the Netherlands. All experiments were performed in a 96-well plate setup in triplicates with a minimum of three independent experiments.

SA-β-gal staining assay

CiPTEC-OAT1 cells were seeded into 12-well format plates, grown at 33 ℃, then transferred to 37 ℃ for maturation and culturing for 9 days. The cells matured at 37 ℃ for 0 day and 9 days were exposed to 1 mL medium with different concentrations of navitoclax, dasatinib, or dasatnib and quercetin combinations. The SA-β-gal-positive cells were detected using Senescence Detection Kit (ab65351, Abcam, UK), and evaluated for blue colorization using an optical microscope (200x magnification).

Western Blot

CiPTEC-OAT1 cells were lysed in ice-cold RIPA Lysis Buffer (Thermo Scientific, Vienna, Austria) for 30 min followed by 20 min centrifugation at 4 °C and obtain protein samples were quantified by BCA Protein Assay Kit (Thermo Scientific, Vantaa, Finland). Proteins were loaded and separated on 14–20% acrylamide gradient SDS gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) in appropriate transferring conditions (25V, 7min). The membranes were blocked in 5% skim milk-TBST for 2 h and incubated with the primary antibody overnight at 4 °C and anti-rabbit (1:3000, Dako, P0448, USA) or anti-mouse (1:3000, Dako, P0260, USA) secondary antibodies for 1h at room temperature. The membrane was exposed to Clarity Western ECL Blotting Substrate followed manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA) then imaged using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) to detect the protein bands, which were quantified using ImageJ software (version 1.53c, National Institutes of Health, USA).

The following proteins were detected by Western blotting: Bcl-2, Bcl-xL, Mcl-1, Bad, Bak, Bim, BID, Bax, Puma, Caspase-3, Caspase-7, Caspase-9, p53, p21 and β-gal, for which primary antibodies were purchased from Cell Signaling Technology (UK). Lamin B1 primary antibody was purchased from Abcam (UK). The dilution of all primary antibodies was 1:1000.

ELISA

Cell culture supernatants were centrifuged for 10 min, 240 x g, 4 °C, and stored at −20 °C. To determine the concentration of SASP factors in the culture supernatants, the ELISA Kits of IL-6 (88-7066-88, Invitrogen, Carlsbad, CA, USA), IL-8 (88-8086-88, Invitrogen, Carlsbad, CA, USA), CCN2 (DY9190-05, R&D System, UK), TNF-α (88-7346-88, Invitrogen, Carlsbad, CA, USA) and TGF-β1(88-8350-88, Invitrogen, Carlsbad, CA, USA) were used according to the manufacturer's instructions.

Statistics

All data analysis and statistics were performed using the GraphPad Prism (version 8.3.0; GraphPad software, La Jolla, CA, USA), and expressed as mean ± SEM. For comparison of two groups at different temperature and different time points, multiple t-test was used followed by Holm-Sidak multiple comparison test. To compare multiple groups in the same condition, the one-way ANOVA was used followed by Dunnett's multiple comparison test. $P<0.05$ was considered significant. Cell viability was expressed as inhibitory constants at 50% of control viability levels (IC_{50} values), which were calculated by plotting log senolytics concentration versus-viability following background subtraction. Nonlinear regression with a variable slope constraining the bottom to 0 was used to fit the normalized data.

RESULTS

Growing ciPTEC-OAT1 at non-permissive temperature increases SA-β-gal activity and expression of senescence-associated genes

To understand if non-permissive temperature leads to cellular senescence, we compared senescence-associated beta-galactosidase (SA-β-gal) activity and the expression of several senescence markers on mRNA level after culturing ciPTEC-OAT1 for 7 days at permissive (33 ℃) and non-permissive temperature (37 ℃). Increased SA-β-gal activity was observed in cells cultured at non-permissive temperature compared to permissive temperature (Figure 1A). Furthermore, lamin B1 mRNA levels were decreased (p=0.008; Figure 1B) and p21 mRNA levels were increased (p<0.001; Figure 1C) in the non-permissive temperature group. SASP factors including, CCN2, PAI-1, IL-1β and IL-6 were all upregulated in the non-permissive temperature group (p<0.001, p=0.02, p=0.02, and p=0.03, resp.; Figure 1D-G). Similar mRNA levels of Bcl-2 and Bcl-xL were observed across both conditions (Figure 1,I).

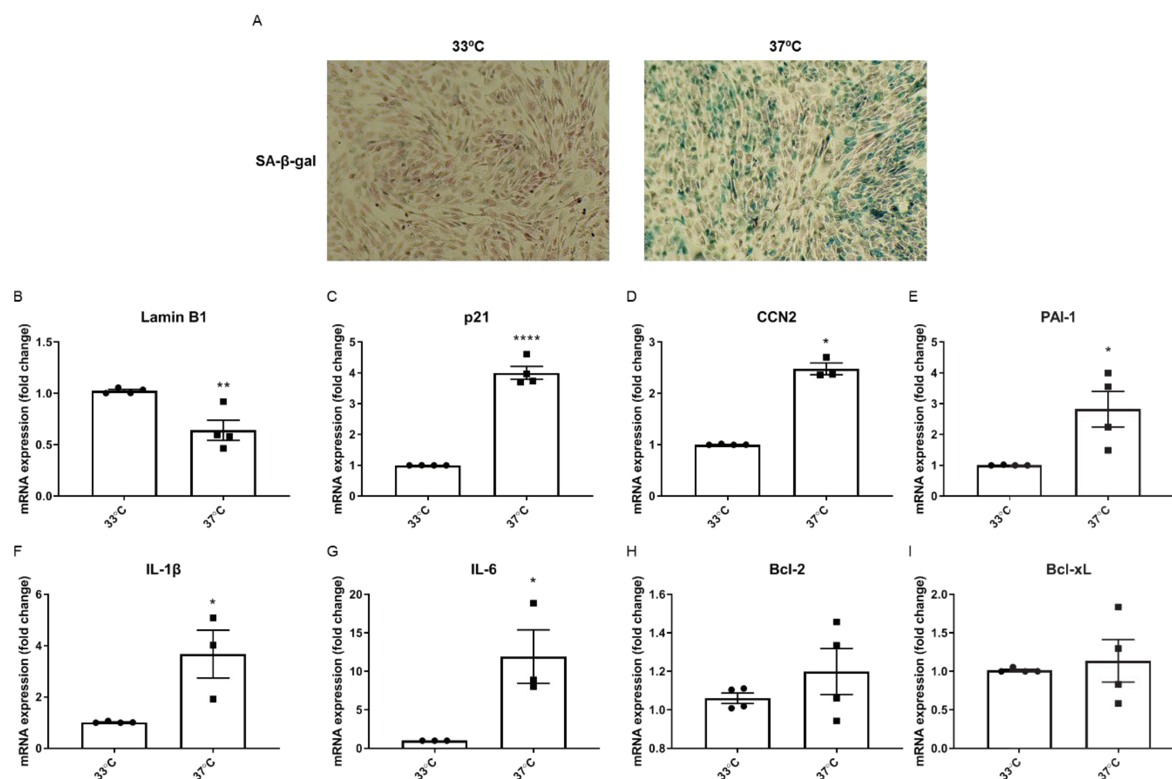

Figure 1. Growing ciPTEC at non-permissive temperature (37 ℃) increases the expression of senescence-associated genes and SA-β-gal activity
(A) Representative images of SA-β-gal staining counterstained with Nova-red. **(B-I)** Gene expression levels of lamin B1 (B), p21 (C), CCN2 (D), PAI-1 (E), IL-1β (F), IL-6 (G) Bcl-2 (H) and Bcl-xL (I) in ciPTEC-OAT1 cultured for 7 days at 37 ℃. Data are expressed as mean ± SEM. Four independent experiments in triplicates were performed. TBP was used as an internal control. *P<0.05, **P<0.01, and ***P<0.001 (unpaired t-test).

Maturation of ciPTEC-OAT1 at non permissive temperature affects expression levels of common senescence makers

To further characterize the development of a senescence phenotype in ciPTEC-OAT1, the expression levels of senescence markers (p53, lamin B1, p21 and β-gal) were evaluated over time at permissive and non-permissive temperatures of 33 ℃ and 37 ℃, respectively (Hernandez-Segura et al., 2018). Total-p53 (T-p53) expression showed a time-dependent upregulation in the proliferation group at 33 ℃ that was not observed in the maturation group at 37 ℃, and T-p53 expression levels were significantly lower at 37 ℃ compared to 33 ℃ (Figure 2A,C). The expression of lamin B1 varied, but showed a trend for decreased levels after longer culture (days 9 and 12) at both temperatures, and a trend for lower expression at 37 ℃ compared to 33 ℃ (Figure 2B,D). Furthermore, p21 expression showed an overtime increase at both 33 ℃ and 37 ℃ with upregulation on day 12 at 33 ℃ and on days 3 through

12 at 37 ℃ (Figure 2E,F). In addition, in non-permissive conditions there was a significant increase of p21 expression compared to permissive temperature at days 3, 6 and 12. Increased SA-β-gal activity in senescent cells is in part due to increased expression of the lysosomal β-galactosidase protein (Lee et al., 2006). β-gal showed a tendency for increased overtime expression at both temperatures, and a tendency for higher expression at 37 ℃ on day 3 and 6 (Figure 2E,G).

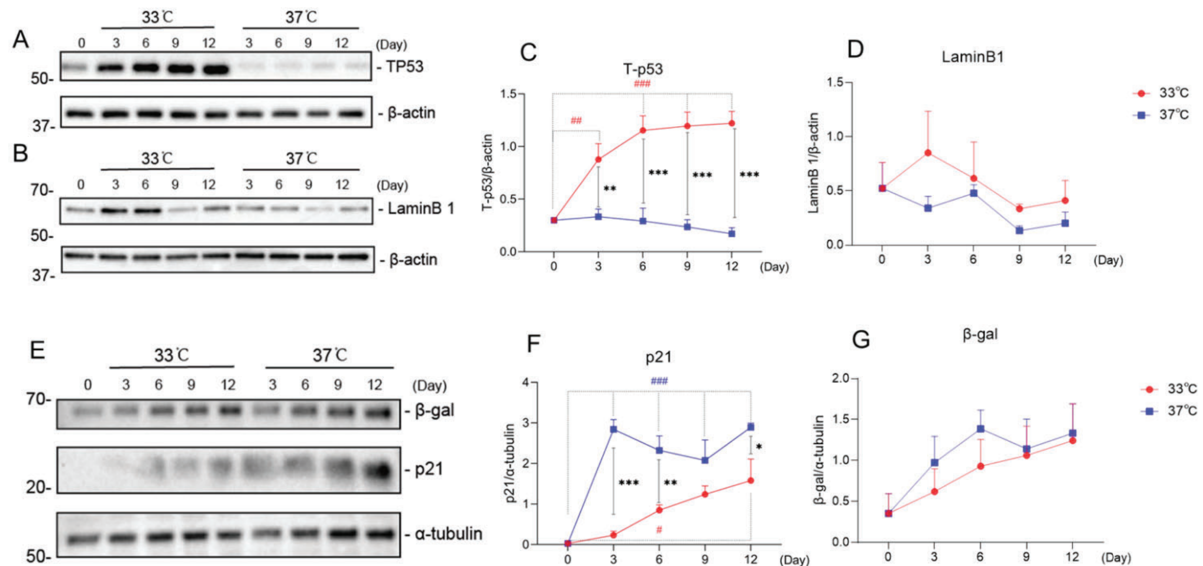

Figure 2. Maturation at non permissive temperature of 37℃ affects common senescence markers expression in ciPTEC-OAT1
(A,B,E) Representative western blots showing expression of Total-p53 (Tp53; A), lamin B1 (B), p21 (E) and β-gal (E). **(C,D,F,G)** Relative expression levels of Tp53 (C), lamin B1 (D), p21 (F) and β-gal (G) over time (day 0 to day 12), at permissive (33 ℃) and non-permissive (37 ℃) temperatures. Protein expression levels were normalized against α-tubulin or β-actin and expressed as mean ± SEM. Three independent experiments in triplicates were performed. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (expression levels at 37 ℃ compared to 33 ℃ at the same time point; Multiple t-test, Holm-Sidak multiple comparison test). # $P<0.05$, ## $P<0.01$, ### $P<0.001$ (expression levels at day 3, 6, 9 or 12 compared to day 0, at 33℃ or 37℃; One-way ANOVA, Dunnett's multiple comparison test). For clarity, only one-sided error bars are shown.

Maturation of ciPTEC-OAT1 at non-permissive temperature increases common SASP factors secretion

The overtime secretion profile of some typical SASP factors (IL-6, TGF-β1, TNF-α, IL-8 and CCN2) were evaluated by ELISA on supernatants from both culture conditions. IL-6 was increasingly secreted over time, both at 33℃ and 37 ℃ culture conditions, and levels were significantly higher at the non-permissive temperature compared to the permissive temperature at days 3, 6 and 12 (Figure 3A). The secreted levels of TGF-β1 (Figure 3B) and TNF-α (Figure 3C) were not markedly different over time and between the culture conditions. The secretion profile of IL-8 showed a time-dependent increase at both temperature conditions, with a tendency for higher expression levels at 37 ℃ compared to 33 ℃ (Figure 3D). Finally, CCN2 showed an initial increasing trend in secretion, followed by a trend for reduction and return to basal levels starting from day 3, at both culture conditions (Figure 3E). Compared to the permissive culture conditions, the concentration of CCN2 at non-permissive temperature was slightly higher on all time points.

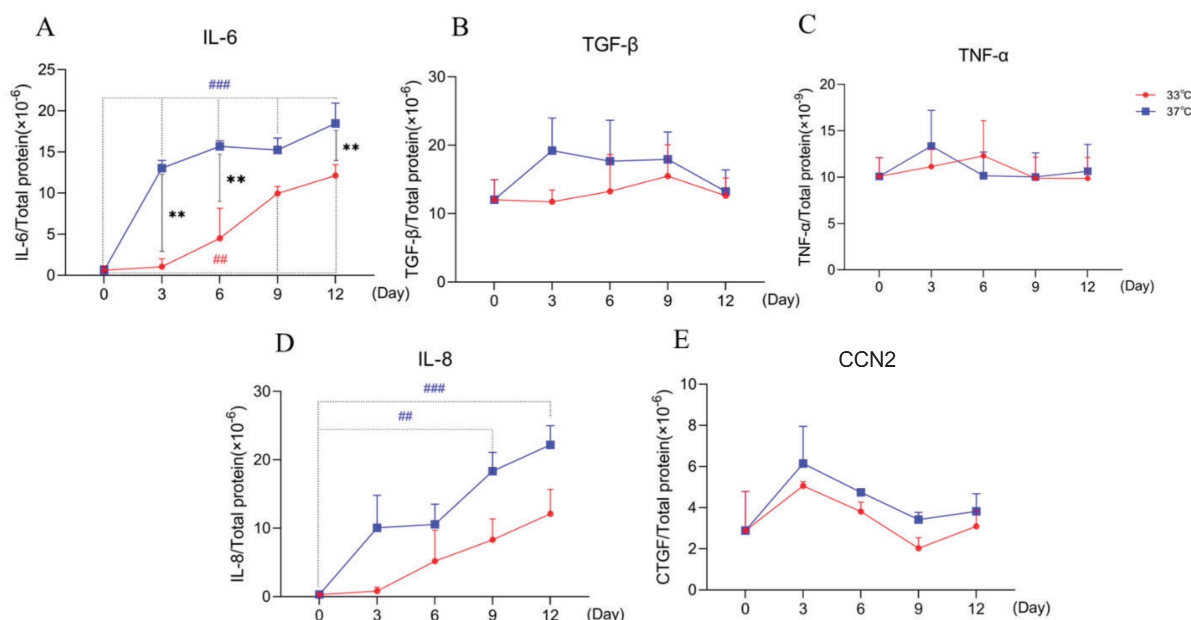

Figure 3. Maturation at non-permissive temperature of 37℃ affects common SASP factors secretion by ciPTEC-OAT1 (A-E) Release of IL-6 (A), TGF-β1 (B), TNF-α (C), IL-8 (D) and CCN2 (E) by ciPTEC-OAT1 over time at permissive (33 ℃) and non-permissive (37 ℃) temperatures. Concentration is expressed as pg/ml and normalized for total protein (ug/ml). Three independent experiments were performed in triplicates. *P<0.05, **P<0.01, ***P<0.001 (secreted levels at 37 ℃ compared to 33 ℃ at the same time point; Multiple t-test, Holm-Sidak multiple comparison test). # P<0.05, ## P<0.01, ### P<0.001 (secreted levels at day 3, 6, 9 or 12 compared to day 0, at 33 ℃ or 37 ℃; One-way ANOVA, Dunnett's multiple comparison test). For clarity, only one-sided error bars are shown.

Maturation at non-permissive temperature affects protein levels of apoptosis-associated markers in ciPTEC-OAT1

To test whether maturation at the non-permissive temperature of 37 ℃ induced an anti-apoptotic profile characteristic for senescent cells, both anti- (Figure 4A-D) and pro-apoptotic (Figure 4E-P) protein markers were evaluated. Bcl-2 expression increased over time regardless of temperature, but there was a trend of higher expression levels at non-permissive temperature (Figure 4B). The protein levels of Mcl-1 decreased with time at both conditions, and trended to be lower at permissive temperature (Figure 4C). Bcl-xL (Figure 4D) levels showed an upregulation at both 33 ℃ and 37 ℃ at almost all time points, with levels significantly higher at 37 ℃ compared to 33 ℃ at day 9. Taken together, pro-apoptotic Bcl-2 and Bcl-xL showed overall higher expression levels at non-permissive temperature (37℃) compared to permissive temperature (33℃), whereas Mcl-1 levels tended to be lower at non-permissive temperature.

With respect to the pro-apoptotic proteins, over time increased expression levels were observed only at non-permissive temperature for Bim (Figure 4G), and at both permissive and non-permissive temperature for all the other Bcl-2 family members tested, including Puma (Figure 4F), Bax (Figure 4H), BID (Figure 4I) and Bad (Figure 4L). Significant lower expression levels at non-permissive-temperature compared to permissive temperature were observed for Bim and Bak on day 9 and 12. Conversely, Bax (day 3-12) and BID (day 3) showed significant higher expression levels at non-permissive-temperature.

For the pro-apoptotic caspases, procaspase-3 (Figure 4N), procaspase-7 (Figure 4O) and procaspase-9 (Figure 4P) we found a trend for over time upregulation at permissive temperature, while at non-permissive temperature, after an initial upregulation, there was a trend of downregulation at later time points, starting at day 3 for procaspases-3 and 9, and at day 6 for procaspase-7.

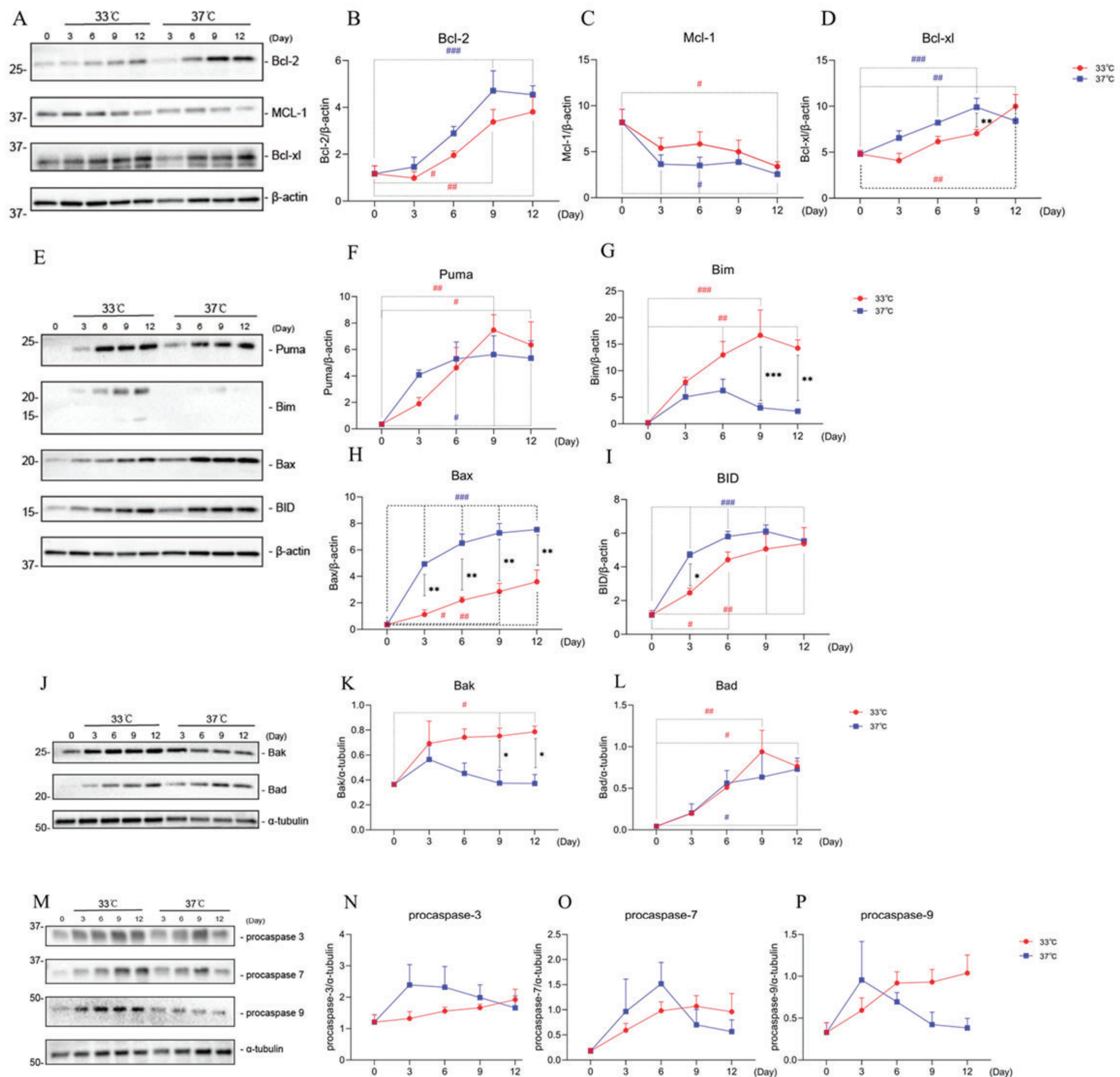

Figure 4. Maturation at non-permissive temperature of 37℃ affects protein levels of apoptosis-associated markers in ciPTEC-OAT1
(A) Representative Western blots showing expression of anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 belonging to the Bcl-2 family. **(B-D)** Relative expression of Bcl-2 (B), Mcl-1 (C) and Bcl-xL (D) over time (day 0 to day 12) at permissive (33 ℃) and non-permissive (37 ℃) temperatures. **(E,J)** Representative Western blots of the pro-apoptotic proteins (Puma, Bim, Bax and BID shown in E, Bak and Bad shown in J) of Bcl-2 families. **(F-L)** Relative expression levels of Puma (F), Bim (G), Bax (H), BID (I), Bak (K) and Bad (L) over time at 33 ℃ and 37 ℃. **(M)** Representative Western blots showing expression of procaspase-3, procaspase-7 and procaspase-9. **(N-P)** Relative expression of procaspase-3 (N), procaspase-7 (O) and procaspase-9 (P) at different time points, at 33 ℃ and 37 ℃. Protein expression levels were normalized against α-tubulin or β-actin and expressed as mean ± SEM. Three independent experiments in triplicates were performed. *P<0.05, **P<0.01, ***P<0.001 (expression levels at 37 ℃ compared to 33 ℃ at the same time point; Multiple t-test, Holm-Sidak multiple comparison test). # P<0.05, ## P<0.01, ### P<0.001 (expression levels at day 3, 6, 9 or 12 compared to day 0, at 33 ℃ or 37 ℃; One-way ANOVA, Dunnett's multiple comparison test). For clarity, only one-sided error bars are shown.

ciPTEC-OAT1 exhibiting a senescence-like phenotype are susceptible to common senolytics

The senescence-like phenotype in ciPTEC-OAT1 cultured in non-permissive conditions was evident especially after 9 days of culturing (Figure S1). Therefore, this time point was selected to test the effects of senolytics, a class of small molecules that can selectively eliminate senescent cells by interfering with anti- and pro-survival signaling pathways (Zhu et al., 2015) For this, senescent cells (day 9 of culture) and non-senescent cells (day 0) were exposed to navitoclax, dasatinib and quercetin to assess the cell viability. Navitoclax only reduced the cell viability of senescent cells, with significantly lower levels in senescent cells from 3nM-10μM (Figure 5A). For dasatinib, a dose-dependent reduction in cell viability was

observed in both culture conditions, but cell viability was significantly lower in senescent cells compared to non-senescent cells from 3nM-3µM (Figure 5B). Quercetin reduced cell viability in both culture conditions in a similar dose-dependent manner (Figure 5C). IC50 values of these senolytics were lower at day 9 of cell maturation at non-permissive temperature compared to 0 days (Table 1). Thus, navitoclax and dasatinib showed efficacy in selectively reducing senescent cell viability. The combination of dasatinib and quercetin (D+Q) has been shown to more effectively eliminate senescent cells than either agent alone (Zhu et al., 2015). The combination of D+Q was tested with quercetin concentrations outside the toxic range (regardless of culture condition) presented in Figure 5C and increasing concentrations of dasatinib. D+Q reduced cell viability in both culture conditions in a similar dose-dependent manner (Figure 5D,E). However, dasatinib combined with the highest quercetin dose (2µM) significantly reduced the cell viability in the senescent cell culture, but not in the control cells. This suggests that the co-treatment of dasatinib and quercetin can selectively target senescent ciPTEC-OAT1.

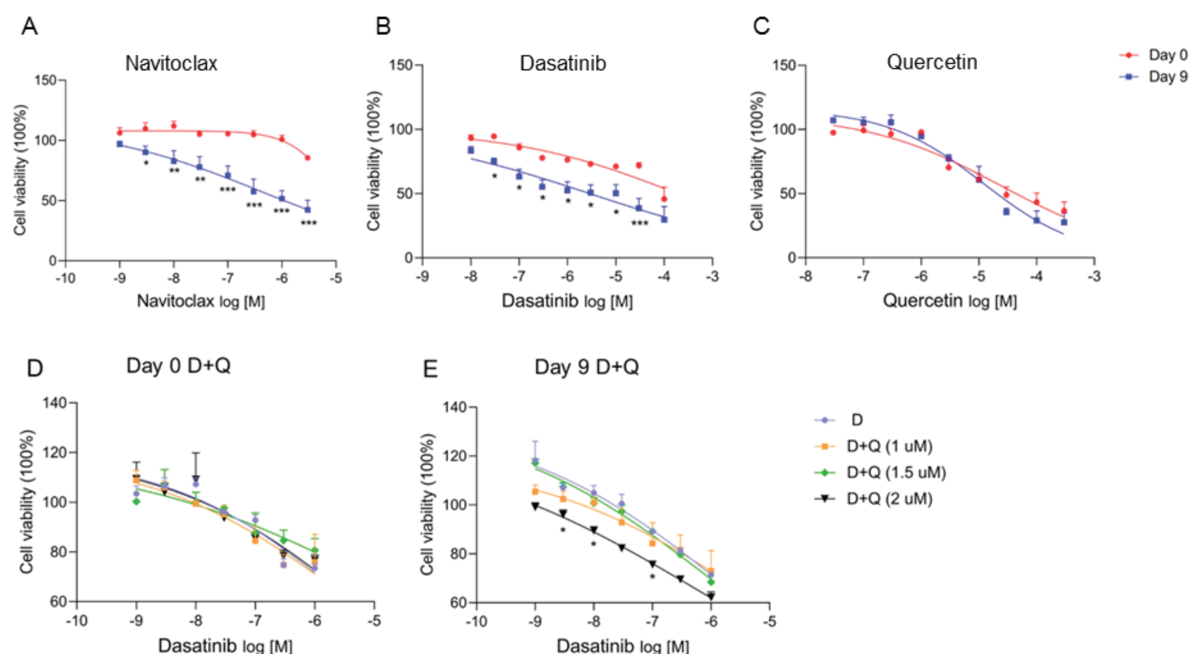

Figure 5. ciPTEC-OAT1 cultured at non-permissive temperature and exhibiting a senescence-like phenotype are susceptible to common senolytics
(A-C) Cell viability of ciPTEC-OAT1 cultured for 0 or 9 days at non-permissive temperature of 37 ℃ and exposed to 100 µL medium with increasing concentrations of navitoclax (A) dasatinib (B), quercetin (C) for 24 h. **(D,E)** Cell viability of ciPTEC-OAT1 cultured for 0 (D) and 9 days (E) at non-permissive temperature end exposed to 100 µL medium with increasing concentrations of dasatinib combined with quercetin (1 µM, 1.5 µM or 2 µM) for 24h. Four independent experiments were performed in triple. Data are presented as mean ± SEM, for which results were normalized to unexposed cells. *P<0.05, **P<0.01, ***P<0.001 (A to C, cell viability at Day 9 compared to Day 0 at the same concentration; Multiple t-test, Holm-Sidak multiple comparison test. D and E, cell viability of increasing concentrations of dasatinib combined with quercetin (1 µM, 1.5 µM or 2 µM) compared to dasatinib alone; Two-way ANOVA, Dunnett's multiple comparison test). For clarity, only one-sided error bars are shown.

Table 1. IC_{50} of Figure 5A-E.

				D+Q			
IC50	**Q**	**D**	**N**	**D(Q=0µM)**	**D(Q=1µM)**	**D (Q=1.5µM)**	**D(Q=2µM)**
Day 0	>10 µM	>3µM	>3µM	>3µM	>4µM	>3µM	>4µM
Day 9	>3 µM	>1µM	>0.4µM	>1µM	>6µM	>1µM	>0.7µM

Senolytics clear senescent ciPTEC-OAT1 as evaluated by functional SA-β-gal expression

Senolytic treatment response was further evaluated by assaying SA-β-gal activity and β-gal expression levels in ciPTEC-OAT1 cultured for 9 days at 37 ℃ compared to day 0. Following exposure to navitoclax, lower SA-β-gal positive cell numbers were observed in a dose-dependent fashion (Figure 6A), and β-gal expression showed a downregulation trend in navitoclax treated senescent cells, with concentration of 100 nM being the most effective

(Figure 6B). D+Q treatment also resulted in a dose-dependent reduction in SA-β-gal positive cell numbers (Figure 6C), and a tendency for lower β-gal expression levels (Figure 6D).

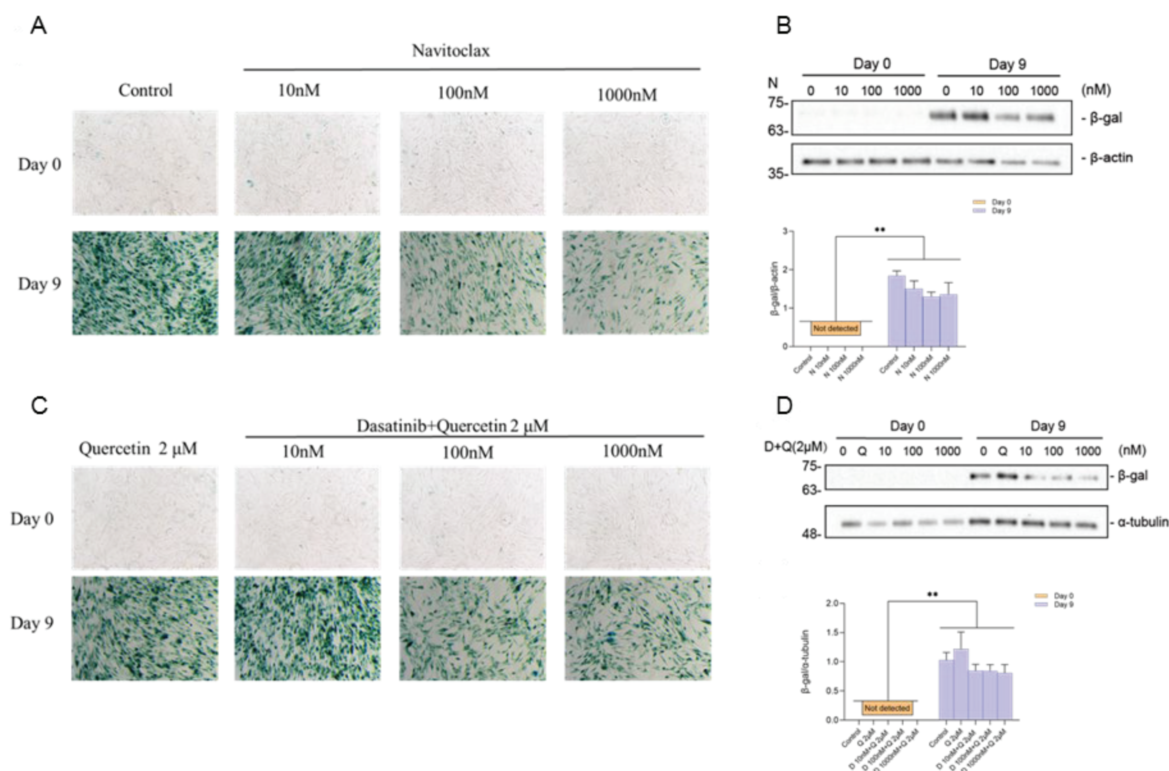

Figure 6. Effects of senolytics on SA-β-gal activity and protein levels in ciPTEC-OAT1 cultured for 9 days at non-permissive temperature
(A,C) Representative images of SA-β-gal staining in ciPTEC-OAT1 cultured for 0 and 9 days at 37 ℃ and after 24 h exposure to 1 mL medium with different concentrations (10 nM, 100 nM, or 1000 nM) of navitoclax (A) and of dasatinib combined with quercetin 2 μM (C). **(B,D)** Representative western blots and relative expression levels showing expression of total β-gal after 24 h exposure to different concentrations of navitoclax (B) and dasatinib combined with quercetin 2 (D) in ciPTEC-OAT1 cultured for 0 or 9 days at non-permissive temperature. (D) Relative expression of total β-gal after 24 h exposure to different concentrations of navitoclax. Protein expression levels were normalized to α-tubulin or β-actin, and expressed as mean ± SEM. Three independent experiments were performed in triple. *P<0.05, **P<0.01, (expression levels at 37 ℃ compared to 33 ℃ at the same time point; Multiple t-test, Holm-Sidak multiple comparison test). For clarity, only one-sided error bars are shown.

DISCUSSION

Cellular senescence is an irreversible condition with cell cycle arrest, SASP and apoptosis resistance, which contributing to fibrosis and chronic kidney disease (Stenvinkel and Larsson, 2013;Hernandez-Segura et al., 2018). We previously developed ciPTEC-OAT1 to be used in drug screening and nephrotoxicity studies (Nieskens et al., 2016). In the present study, we demonstrate that the cell model also provides opportunities for studying tubular senescence in the kidney. ciPTEC-OAT1 cultured at non-permissive temperature for nine days obtain a senescence-like phenotype, including differential expression of markers for cell cycle arrest and SASP and apoptosis-associated markers, and are sensitive to senolytic drugs.

In the present study, we demonstrated that the ciPTEC-OAT1 cultured at non-permissive temperature expressed common senescence markers. In particular, the decrease in lamin B1 (Shimi et al., 2011) and upregulation in p21 (Calcinotto et al., 2019) have been described as characteristic features, that are involved in maintaining senescence phenotype by regulating JNK and caspase signaling (Yosef et al., 2017). Further, increased SASP factors have been reported as proinflammatory and matrix-degrading molecules (Childs et al., 2015), including PAI-1 (Sun et al., 2019), IL-1β (Shi et al., 2019), CCN2 (Jun and Lau, 2017) and IL-6 (Mosteiro et al., 2018).

The observed loss of lamin B1 and increase in SA-β-gal activity, well-known indicators

of senescence (Hernandez-Segura et al., 2018), in ciPTEC-OAT1 cultured at non-permissive temperature further confirm the phenotypical changes. Silencing lamin B1 immediately leads to inhibition of proliferation and the induction of senescence (Shimi et al., 2011). Our previous research regarding cell cycle analysis of ciPTEC-OAT1 at permissive and non-permissive temperature (Mihajlovic et al., 2019), has shown that ciPTEC-OAT1 when cultured at 37℃ for 1 or 7 days exhibit significantly reduced proliferation (less cells in S phase) and an increased number of cells in G0/G1 phase of the cell cycle, indicating halted proliferation at non-permissive temperature. This is in line with our current results. The activity of the lysosomal β-gal reflects increased metabolic activity and enhanced lysosomal content typical of senescent cells (Hernandez-Segura et al., 2018). Transfection with the temperature sensitive SV40T gene allows the cells to become conditionally immortalized (Wilmer et al., 2010). Although downregulation of SV40T at 37 ℃ allows activation of p53 and pRb, factors involved in both p53/p21 and p16/pRb pathways, there was no significant difference in their mRNA expression (Figure S2) and p16 protein expression appeared undetectable. This suggests that senescence of ciPTEC-OAT1 may not be induced by the p16/pRb pathway. On the other hand, p53 is pivotal in determining the fate of the cells, implying that the p53/p21 pathway is key in the initiation of senescence (Mijit et al., 2020). Previously published works described a decline of total-p53 levels in stress-induced senescence in mice (Feng et al., 2007) and p21 role in maintaining senescence in mice (Yosef et al., 2017), which is also in accordance with our *in vitro* data showing a decline in p53 levels and an increase in p21 levels at 37 ℃. Therefore, our results suggest that ciPTEC-OAT1 cultured at 37℃ promote senescence through p53/p21 pathway.

In addition to previously tested markers, SASP factors are also important players in senescence. IL-6 maintains senescence through the p53/p21 pathway (Effenberger et al., 2014;Li et al., 2020b), shared by IL-8, which is expressed as a function of IL-6 (Kuilman et al., 2008). TGF-β1 and CCN2 are other SASP factors reported to mediate senescence (Jun and Lau, 2017;You et al., 2019). CCN2 is a downstream mediator of TGF-β1 and is regulated by TGF-β1 (Ou et al., 2020). TGF-β1 induces kidney fibrosis by accumulation of extracellular matrix and CCN2 expression by activation of Smad3 and p53 (Li et al., 2020a). Meanwhile, both CCN2 and TGF-β1 induce senescence and are accompanied with the upregulation of IL-6 and IL-8 (Jun and Lau, 2017;Fan et al., 2019). Although not significant, our results show an increasing trend of both TGF-β1 and CCN2 secretion in 37℃ group compared to 33℃ group. PAI-1 is a major TGF-β1/p53 target gene in kidney fibrosis and is known to be elevated in senescent cells, correlating with increased tissue TGF-β1 levels (Samarakoon et al., 2019;Rana et al., 2020). In our study, PAI-1 and CCN2 increased remarkably on mRNA level, which may in part be due to increased SASP. TNF-α is another SASP factor and inducer of senescence (Guo et al., 2019), but our results showed no important differences over time and between the culture conditions, indicating that senescence of ciPTEC-OAT1 is not induced or maintained by TNF-α. There are some discrepancies between mRNA and protein levels of the obtained results. Despite being difficult to explain, these discrepancies might be due to differences in the regulation of transcription and protein translation processes, as well as protein turnover rate.

Another senescence feature entails apoptosis, responsible for cell turnover and maintaining extracellular environment. For instance, the Bcl-2 family members modulate the delicate balance between pro and anti-apoptosis (Ngoi et al., 2020). The trends for upregulation of Bcl-2 and Bcl-xL we observed, suggest that ciPTEC-OAT1 became anti-apoptotic upon maturation at a non-permissive temperature. Senescent cells show downregulation of Mcl-1 on protein levels (Lee et al., 2018), which is consistent with our findings. Furthermore, the effectors Bax and Bak shuttle between cytosol and mitochondrial outer membrane with different rates (Pena-Blanco and Garcia-Saez, 2018), which might explain why we observed a differential expression in the proteins. BH3-only proteins bind to the BH3 domain of the anti-apoptotic Bcl-2 proteins via hydrophobic interactions, thereby promoting cellular apoptosis (Anantram and Degani, 2019). Of BH3-only proteins, Bid showed higher protein levels at different time points in maturation group. The increasing trend in all other proteins point towards a priming of cells to undergo apoptosis, but that the

execution of the death program is restrained. These findings are in line with previous reports showing that following senescence induction by ionizing radiation, senescent cells upregulate pro-apoptosis markers (Chang et al., 2016;Baar et al., 2017). Therefore, cellular senescence in our model with an upregulation of pro-apoptotic markers despite having an anti-apoptotic phenotype, argue for cells searching for a new balance to maintain homeostasis. Caspases are another group of proteins involved in cell death mediated by apoptosis and important senescent markers (Shalini et al., 2015). After MOMP, caspase activation takes place often within minutes, leading to cell death (Pena-Blanco and Garcia-Saez, 2018). Inhibition of caspases therefore blocks apoptosis. Here we detected that the activator (procaspase-9) and executioner (procaspase-3 and procaspase-7) were upregulated at permissive temperature, but showed a differential pattern when cells were cultured at non-permissive temperature. When mitochondrial-mediated apoptosis is induced and caspase-9 and caspase-3 are activated, the expression of Bax and Bcl-2 has been reported to show different levels to maintain their balance (Zhang et al., 2021). Irradiation-induced senescence is accompanied by an upregulation of procaspase-3, -7 and -9 (Chang et al., 2016;Baar et al., 2017) but a downregulation of activated caspase-3 (Chang et al., 2016), in line with our results. The final downregulation of procaspases observed in our study might be explained by the cleavage of procaspase-9 finally to active caspase-3 and caspase-7. But because of senescence induction and an adapted balance in Bcl-2 family proteins procaspase-9 is inhibited, finally leading to apoptosis-resistance (Figure 7).

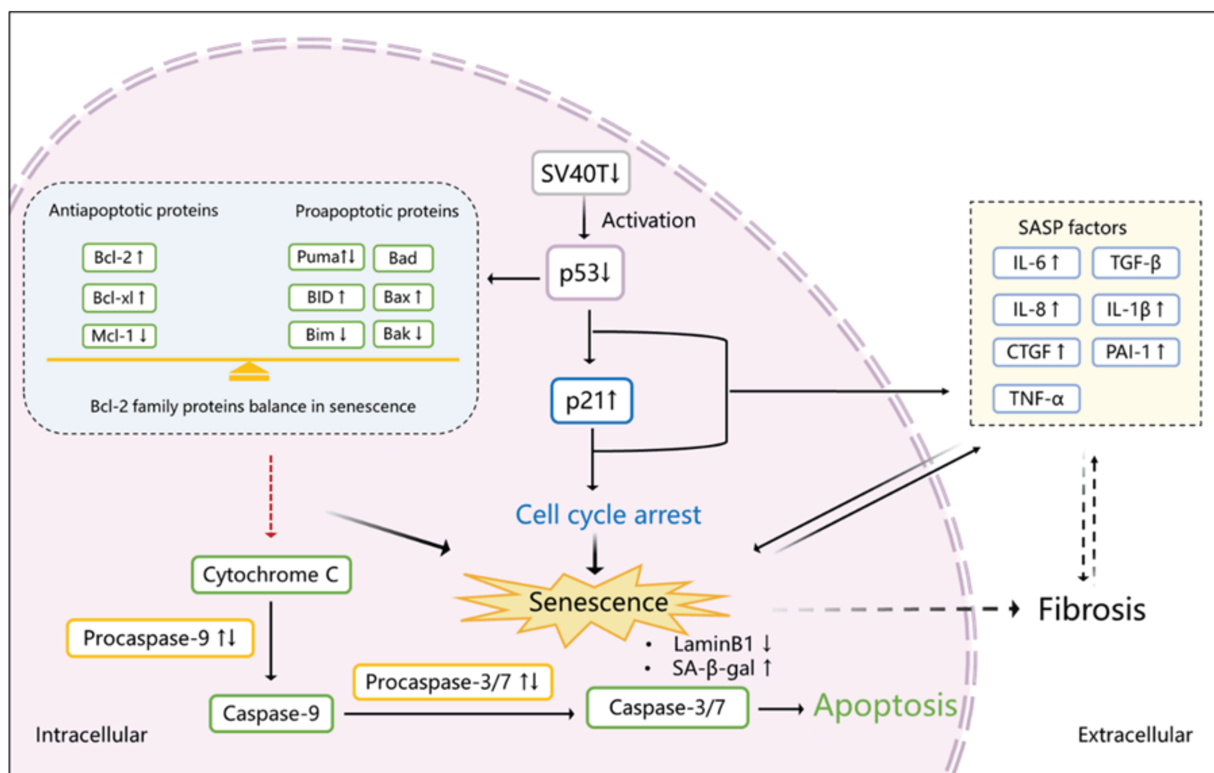

Figure 7. Proposed scheme of senescence induction in ciPTEC-OAT1 after maturation at non-permissive temperature. After transfer of cells to 37 ℃ and subsequent downregulation of SV40T, p53 is activated which transcriptionally upregulates p21, inducing cell cycle arrest and eventually leading to senescence. SASP factors are released during this process as well. Activated p53 also influences the expression of Bcl-2 family proteins that become abnormally upregulated. Procaspase 9 and its downstream proteins procaspases 3/7 are activated at the beginning of this process, and with time going by, the Bcl-2 family reach a balance between anti-apoptotic and pro-apoptotic proteins expression, halting the activation of procaspases and inhibiting apoptosis. In addition, senescent cells show a downregulation of lamin B1 and an upregulation of SA-β-gal and some SASP factors, including IL-6, IL-8, CCN2, IL-1β and PAI-1, which may further contribute to kidney fibrosis. ↑, upregulation; ↓, downregulation; ↑↓, initial upregulation following with downregulation (expression levels at 37 ℃ compared to 33 ℃).

Finally, the senolytics navitoclax, dasatinib and quercetin were evaluated. Our results suggest that ciPTEC-OAT1 cultured at non-permissive temperature were sensitive to senolytics. The Bcl-2 family inhibitor navitoclax (Tse et al., 2008;Chang et al., 2016)

appeared most effective in selectively reducing viability of senescent cells. A clinical trial with the combination of dasatinib and quercetin demonstrated a decrease in p21 and p16 positive human adipose tissue cells and plasma SASP factors of diabetic kidney disease participants (Hickson et al., 2019). Senolytic treatment of ciPTEC-OAT1 led to a dose-dependent reduction of SA-β-gal positive cells, in line with previous results (Zhu et al., 2015). It has been suggested that SA-β-gal activity may be an outcome rather than a cause of senescence (Lee et al., 2006;Piechota et al., 2016), and our findings argue for a clearance of senescent cells leading to a reduction in total cell number. Our follow-up research will focus on investigating the underlying mechanisms of senolytics used in this study.

In conclusion, our results suggest ciPTEC-OAT1 can be used as a valid proximal tubule cell model both for mechanistic studies inherent to renal senescence and fibrosis and for senolytic effects of newly developed drugs and their combinations.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used for real-time polymerase chain reaction

Gene	Taqman Gene Expression Assay
TBP	Hs00427620_m1
Lamin B1	Hs01059210_m1
p21 (CDKN1A)	Hs00355782_m1
CCN2	Hs00170014_m1
PAI-1(SERPINE1)	Hs00167155_m1
IL-1β	Hs01555410_m1
IL-6	Hs00174131_m1
BCL-2	Hs00608023_m1
Bcl-xL (BCL2L1)	Hs00236329_m1

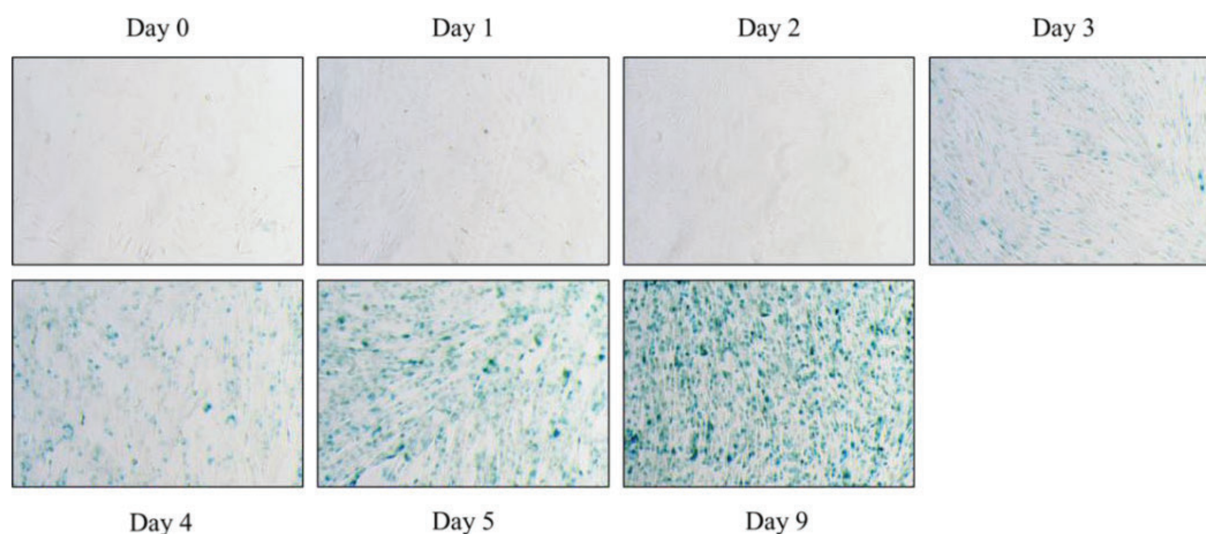


Figure S1. SA-β-gal activity in ciPTEC-OAT1 during culture at non-permissive temperature.
Representative images of SA-β-gal staining in ciPTEC-OAT1 cultured for 0 through 9 days at non-permissive temperature (37 ℃). Three independent experiments were performed.

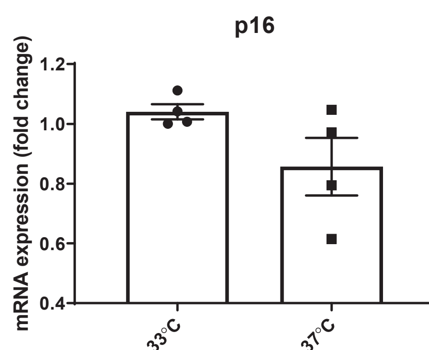
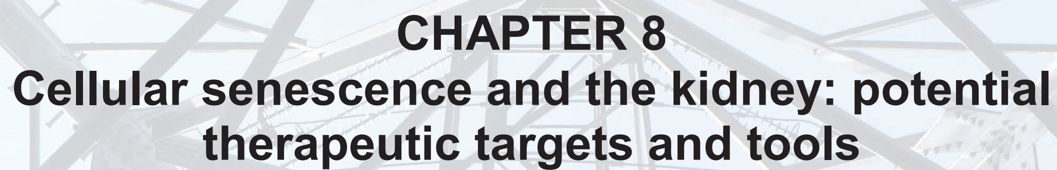


Figure S2. Gene expression levels of p16 at permissive and non- permissive temperatures
Gene expression levels p16 in ciPTEC-OAT1 cultured for 7 days at permissive (33 ℃) and non-permissive temperature (37 ℃). Data are expressed as mean ± SEM. Four independent experiments in triplicates were performed. TBP was used as an internal control.



CHAPTER 8
Cellular senescence and the kidney: potential therapeutic targets and tools

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Chapter 8. Potential therapeutic targets for cellular senescence in the kidney

Abstract

Chronic kidney disease (CKD) is an increasing health burden (affecting approximately 13.4% of the population). Currently, no curative treatment options are available and treatment is focused on limiting the disease progression. The accumulation of senescent cells has been implicated in the development of kidney fibrosis by limiting tissue rejuvenation and through the secretion of profibrotic and proinflammatory mediators termed as the senescence-associated secretory phenotype. The clearance of senescent cells in aging models results in improved kidney function, which shows promise for the options of targeting senescent cells in CKD. There are several approaches for the development of "senotherapies", the most rigorous of which is the elimination of senescent cells by the so-called senolytic drugs either newly developed or repurposed for off-target effects in terms of selectively inducing apoptosis in senescent cells. Several chemotherapeutics and checkpoint inhibitors currently used in daily oncological practice show senolytic properties. However, the applicability of such senolytic compounds for the treatment of renal diseases has hardly been investigated. A serious concern is that systemic side effects will limit the use of senolytics for kidney fibrosis. Specifically targeting senescent cells and/ or targeted drug delivery to the kidney might circumvent these side effects. In this review, we discuss the connection between CKD and senescence, the pharmacological options for targeting senescent cells, and the means to specifically target the kidney.

Chapter 8. Potential therapeutic targets for cellular senescence in the kidney

Introduction of renal disease and senescence

Renal disease as a major individual and global burden

Chronic kidney disease (CKD) is defined by the persistent loss of kidney function and currently affects approximately 13.4% of the global population (Hill et al., 2016; Jager and Fraser, 2017). The progressive nature of CKD often leads to end-stage renal disease (ESRD), requiring renal replacement therapy. To date, there are no curative therapeutic options for CKD/ESRD. Survival on dialysis remains poor (Collins et al., 2010), and there is a global shortage of kidney donors, because (among other reasons) the kidney transplant 10-year graft survival rate is only 60% at best due to rejection and the progressive loss of graft function (Matas et al., 2015). Furthermore, for transplant recipients, the lifelong use of immunosuppressive therapy is mandatory. Therefore, any progress in the development of therapies that could prevent CKD progression (and ultimately ESRD altogether) could have a major societal impact.

Current therapy consists of treating CKD complications and slowing CKD progression by targeting known risk factors for disease progression, such as salt and protein intake, hypertension, and glomerular hyperfiltration.

An as yet untreatable final common pathway irrespective of the etiology in CKD is kidney fibrosis, characterized histologically by glomerulosclerosis, tubular atrophy, and interstitial fibrosis (Liu, 2011). Numerous compounds directly targeting factors involved in fibrosis driving pathways are currently being studied with varying results [e.g., transforming growth factor-β (TGF-β) signaling pathway inhibitors (pirfenidone and fresolimumab; Meng et al., 2016), anti-CCN2/connective tissue growth factor (CTGF; pamrevlumab; Kok et al., 2014), and tyrosine kinase inhibitors (e.g., nintedanib, gefitinib, and imatinib)]. This approach shows some promise, but clinical trial results vary (Klinkhammer et al., 2017). Apart from the use of the renin-angiotensin-aldosteron pathway interfering agents such as ACE inhibitors or angiotensin receptor blockers to reduce the progressive remodeling of renal parenchyma, no therapeutics addressing pathophysiological mechanisms underlying CKD are used clinically. However, increasing effort is currently put into investigating the efficacy of targeting senescent cells during renal disease

Cellular senescence: general aspects

During life, cells are unavoidably exposed to various stresses that potentially cause DNA damage [e.g., reactive oxygen species (ROS), ionizing/ultraviolet (UV) radiation, sheer stress, chemical injury, or replicative stress]. To ensure integrity, DNA is checked during cell cycle progression and, when needed and possible, repaired. Major DNA integrity checks occur during the G1/S or G2/M transition phases of the cell cycle (Moonen et al., 2018). When DNA injury is irreparable, either apoptosis or a permanent inhibition of cell cycle progression occurs despite growth factor stimulation. The latter situation/condition is also known as cellular senescence (Campisi and d'Adda di Fagagna, 2007). Replicative senescence is triggered by telomere attrition resulting from repeated cell division, whereas stress-induced premature senescence is due to oxidative and genotoxic stresses (Campisi and d'Adda di Fagagna, 2007; Toussaint et al., 2000). The exact mechanism of DNA damage detection, cell cycle checkpoint control, and the mechanism of renal senescence are expertly reviewed elsewhere (Campisi and d'Adda di Fagagna, 2007; Ashwell and Zabludoff, 2008; Sturmlechner et al., 2017; Singh and Wu, 2019).

As depicted in Figure 1, p53 and p16 are key regulators of cell fate in the setting of DNA damage. Particularly, the level of p53 expression determines whether an arrested cell i) continues replication upon DNA damage resolution, ii) becomes senescent, or iii) goes into apoptosis (Purvis et al., 2012). The expression of either pro-cell cycle arrest or pro-apoptotic molecules shows a linear increase with p53. p53-mediated apoptosis induction is threshold dependent and low levels of p53 can be sufficient to induce cell cycle arrest (Kracikova et al., 2013).

Senescence is an important driver of fibrosis. Senescent cells acquire a senescence-associated secretory phenotype (SASP) characterized by the expression and secretion of

profibrotic and proinflammatory factors. These SASP factors act upon neighboring healthy cells in a paracrine fashion, thereby driving the progression of fibrosis in CKD (Coppe et al., 2010; de Keizer, 2017). Senescent cells are mostly cleared by the immune system but accumulate during the aging process (Hoenicke and Zender, 2012).

Recent evidence suggests that senescence may play a key role in CKD progression (Valentijn et al., 2018). As many factors associated with SASP are known to induce fibrosis in the kidney [e.g., TGF-β, CCN2 (also known as CTGF), interleukin (IL)-1, and IL-6] (Wang et al., 2017), targeting senescence might prove an effective alternative strategy for CKD treatment. This review aims to 1) provide a concise description of the pathophysiology of cellular senescence in the kidney and 2) discuss the various potential intervention points within the senescence network.

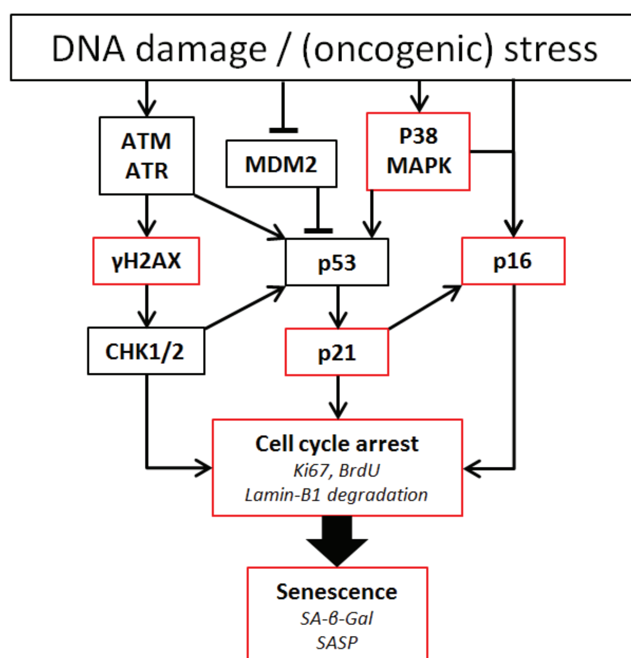

Figure 1. Pathways involved in cell cycle arrest Means to identify senescent cells are indicated in red. Ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases play a central role in DNA damage detection and response. Both proteins rapidly phosphorylase histone 2AX. Furthermore, ATM and ATR can both lead to phosphorylation of the tumor suppressor p53(pSer15) upon DNA damage. Furthermore, ATM phosphorylates the checkpoint kinases Chk1 and Chk2. These in turn target the effector molecule Cdc25C. Chk2 directly phosphorylates p53 at pSer20, leading to expression of cyclin dependent kinase inhibitor p21. Both phosphorylation of Cdc25C and p21 inhibit cyclin dependent kinase, leading to hypophosphorylation of retinoblastoma tumor suppressor (Rb). This enables Rb to bind to E2F, inhibiting cell cycle progression. Likewise p53 activity can beincreased by p38 MAPK activity, induced by reactive oxygen species (ROS), or by binding of p14ARF to murine double minute 2 (MDM2) preventing degradation of p53 (Elledge & Zhou, 2000; Fischer, Quaas, Steiner, & Engeland, 2016; Hirao et al., 2002) . In addition to p53, accumulation of the tumor suppressor $p16^{Ink4a}$ also leads to cell cycle arrest via the inhibition of cyclin dependent kinase 4/6 and subsequent hypo phosphorylation of Rb (Moonen et al., 2018).

Pro-survival pathways in senescence

Apoptosis resistance is an important characteristic of senescent cells and the most widely and intensely explored target for therapeutic intervention. Telomere attrition, DNA damage, and other stressors typically induce cells to upregulate proapoptotic factors, the effect of which is counteracted by the simultaneous increase of anti-apoptotic factors to prevent their premature cell loss. Thus, shifting the balance toward the dominance of antiapoptotic factors constitutes the “Achilles’ heel” of senescent cells that circumvent apoptosis (Wang, 1995; Sasaki et al., 2001; Sagiv et al., 2013; Zhu et al., 2015).

As a result, senescent cells become even more resistant to intrinsic and extrinsic pro-apoptotic stimuli than non-senescent cells, as exemplified by higher survival and less apoptosis after tumor necrosis factor-α (TNF-α) treatment and UV irradiation (Yosef et al., 2016). An expert review on the apoptotic balance in cellular senescence is provided by Childs et al. (2014).

The activation of mitochondrial anti-apoptotic B-cell lymphoma 2 (BCL-2) family members (BCL-2, BCL-W, BCL-xL, MCL-1, and A1) has been identified as the central molecular mechanism by which senescent cells resist apoptosis. Knockout (KO) of a combination of BCL-W, BCL-xL, and BCL-2 leads to the reduced viability of senescent cells,

showing that these cells depend on the (over)expression of anti-apoptotic factors to prevent "spontaneous" apoptosis (Chang et al., 2016; Yosef et al., 2016).

Tied to their dependence on BCL-2 protein family members, senescent cells are reliant on pro-survival pathways involving the p53-p21-serpine and phosphoinositide 3-kinase (PI3K)/ AKT pathways. As such, the ephrin-dependent receptor ligands ephrin B1 and B3 and plasminogen activator inhibitor-1 (PAI-1) are also implicated in pro-survival signaling in senescent cells (Zhu et al., 2015).

The exact interplay of pro- and anti-apoptotic proteins is complex (Fuchs and Steller, 2015). In case of irreversible DNA damage, the DNA damage response (DDR) mediates apoptosis via the activation of pro-apoptotic proteins such as phorbol-12myristate-13-acetate-induced protein 1 (also known as NOXA) and the BCL-2 homology domain 3 (BH3)-only protein Bim and the activation of p53 upregulated modulator of apoptosis (PUMA) that binds and inhibits multiple anti-apoptotic BCL family members (O'Connor et al., 1998; Chen et al., 2005).

Interestingly, Baar et al. (2017) found that the pro-apoptotic PUMA and BIM were upregulated in radiation-induced senescent IMR90 fibroblasts, whereas the anti-apoptotic BCL-2 was reduced in senescent cells, making these cells, in theory, more susceptible to apoptosis. However, the reduction of BCL-2 in irradiated fibroblasts is not consistently seen and the expression of pro- and anti-apoptotic proteins may vary depending on the cell type (Zhu et al., 2016), which could explain the observed differences in response to therapies targeting specific apoptotic pathways.

Detecting cellular senescence

To date, a definitive sensitive and specific marker for cellular senescence has not been identified. Hallmarks of senescent cells are their resistance to apoptosis and phenotypic changes, including altered morphology with large flattened cell bodies. Another interesting characteristic of senescent cells is the increased lysosomal content resulting in lysosomal β-galactosidase (β-gal) activity (also known as senescence-associated β-Gal or SA-βGal) being readily detectible at the enzymatically suboptimal and relatively high pH of 6.0 (Lee et al., 2006). However, SA-β-Gal is typically also increased in non-senescent, high-density, and confluent cell cultures, which limits its applicability as a standalone marker for the detection of senescent cells. Furthermore, senescent cells can be detected based on the activity in the pathways leading to cell cycle arrest (Figure 1), e.g., increased phosphorylation of histone H2AX (γ-H2AX) and accumulation of cyclin-dependent kinase (CDK) inhibitors (CKIs) such as tumor protein p53 (TP53 or p53), p21Cip1 (p21), and p16Ink4a (p16), and by the increase of senescence-associated heterochromatin foci. The importance of p16 is illustrated by the beneficial effect the elimination of p16-expressing cells has on age-related deterioration (Baker et al., 2011). In contrast, senescent cells stop expressing proliferation markers (e.g., Ki-67) and produce a senescence-associated secretome (SASP; see SASP in CKD).

Another particularly interesting feature of senescent cells is the loss of the structural nuclear lamina component lamin B1. The importance of reduced nuclear lamina integrity is underlined by the progeria phenotype of the Hutchinson-Gilford syndrome caused by the loss-of-function mutations in the lamin A gene (De Sandre-Giovannoli et al., 2003). In apoptotic cells, lamin B1 is degraded by caspases, whereas, in senescent cells, lamin B1 decrease results from the reduced lamin B1 mRNA stability. Of note, no decrease in lamin B1 is seen in quiescent cells (Freund et al., 2012).

Combinations of several of the markers mentioned above have been used to identify senescent cells *in vitro* and *in vivo* (Myrianthopoulos, 2018). Table 1 shows the most frequently used markers of senescence in mammal studies.

Table 1. Markers used to identify senescent cells

Marker	Reference
Degradation of Lamin B1	(Freund et al., 2012; Hernandez-Segura et al., 2017; Sadaie et al., 2013; Shimi et al., 2011)
Senescence associated β-galactosidase at pH6.0	(Dimri et al., 1995; Lee et al., 2006)
Lipofuscin accumulation	(Rizou et al., 2019; von Zglinicki, Nilsson, Docke, & Brunk, 1995)
HMGB1 relocalization	(Davalos et al., 2013)
Senescence associated heterochromatin foci	(Masashi Narita et al., 2003)
Increased SIRT2 (NAD+ dependent histone deacetylase III class enzyme) expression	(Anwar, Khosla, & Ramakrishna, 2016)
(phosphorylated) p38 MAPK	(W. Wang et al., 2002)
$p16^{Ink4a}$ expression	(Alcorta et al., 1996; Stein, Drullinger, Soulard, & Dulic, 1999)
$p21^{WAF/CIP1}$ expression	(Alcorta et al., 1996; Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013; Stein et al., 1999)
Flattened and enlarged phenotype	(Serrano, Lin, McCurrach, Beach, & Lowe, 1997)
Absence of proliferation markers (Ki-67, BrdU, EdU)	(Hernandez-Segura, Nehme, & Demaria, 2018; Lawless et al., 2010)
yH2AX foci	(Lawless et al., 2010)
Plasminogen activator inhibitor type-1 (PAI-1) overexpression	(Goldstein, Moerman, Fujii, & Sobel, 1994)
Expression of SASP factors (e.g. . IL-1α, IL-1β, IL-6, IL-8, MCP-1, CCN2)	(Freund et al., 2010; K.-H. Kim et al., 2004)
Expression of Dec1 (class E basic helix-loop-helix protein 40 (BHLHE40))	(Collado et al., 2005)
Expression of DcR2 (TNF receptor superfamily member 10D (TNFRSF10D))	(Collado et al., 2005)

Cellular senescence in the aging and injured kidney

Aging

Aging is associated with the decline of kidney function. During aging, increased renal p16 expression is most notably seen in tubular epithelium and to a lesser extent in glomerular (podocytes and parietal epithelium) and interstitial cells. Changes in p16 were more pronounced in the cortex compared to the medulla (Melk et al., 2004; Sis et al., 2007). In rodents, the amount of senescent proximal tubular cells increases with age, whereas no increase of senescent cells is seen in the glomeruli. Renal tubular cell senescence correlates with tubular atrophy, interstitial fibrosis, and glomerulosclerosis (Verzola et al., 2008; Liu et al., 2012). Furthermore, the removal of senescent tubular cells leads to decreased glomerulosclerosis (Baker et al., 2016). Along with increased tubular senescence, an increase in p21 and TGF-β1 expression is measured in the tubulo-interstitium (Ding et al., 2001). In aging mice kidney, an age-dependent increase is seen in cortical p21 mRNA expression and an increase in p21 plasma concentration, but no increase in urinary p21 excretion was observed (Johnson and Zager, 2018).

Injury

Acute damage of tubular epithelial cells [e.g., transplantation associated ischemia-reperfusion injury (IRI) or acute kidney injury (AKI)] leads to DNA damage and induces an intrinsic DDR. Proximal tubular epithelial cells are especially susceptible to injury (ischemia or toxic injury) (Bonventre et al., 2011). Upon injury, a substantial number of tubular cells undergo apoptosis or are shed via the urine. Due to the regenerative capacity of tubular cells, lost cells are largely replaced via the proliferation of neighboring tubular epithelium (Duffield et al., 2005; Humphreys et al., 2008). However, prolonged or repeated renal injury leads to maladaptive repair (i.e., inflammation, myofibroblast accumulation, fibrosis, and vascular rarefaction) leading to CKD. AKI predisposes kidneys to CKD development with age or after a second hit. Alternatively, CKD patients are more prone to AKI development upon injury (Chawla and Kimmel, 2012). A possible explanation lies in the accumulation of senescent cells during aging and post-injury, given that after AKI the senescent cell burden slowly accumulates over time (Jin et al., 2019).

The severity of allograft nephropathy, diabetic nephropathy, and IgA nephropathy is associated with the level of senescence (Joosten et al., 2003; Verzola et al., 2008; Liu et al., 2012). Additionally, the level of senescence before kidney transplantation could predict the outcome in terms of graft function (McGlynn et al., 2009), suggesting that targeting senescent cells could be an effective therapeutic intervention in kidney disease.

As proof of principle for the therapeutic potential of targeting senescence, several studies were conducted showing the attenuation of functional decline and fibrosis. Small interfering RNA-based p53 inhibition after IRI reduces cellular senescence and is associated with the attenuation of kidney fibrosis in rats (Molitoris et al., 2009). This phenomenon is thought to be in part related to the effects of G2/M cell cycle-arrested tubular cells, which have been shown to produce excessive amounts of TGF-β and CCN2 as part of SASP (Yang et al., 2010; Bonventre, 2014). These factors are also associated with kidney fibrosis (Phanish et al., 2010).

Renal diseases such as IgA nephropathy and lupus nephritis associate with increased senescent cell burden (Liu et al., 2012; Yang et al., 2018), but diabetes mellitus is the most studied disease regarding cellular senescence in the kidney.

Exposure of the kidney to high blood glucose levels in patients with type 2 diabetes significantly increases their renal senescent cell burden. This induction of senescence is specifically seen in tubular epithelium cells and podocytes (Verzola et al., 2008). Only 7 days of (streptozotocin-induced) hyperglycemia already increases senescent cell burden in the mouse kidney and contributes to the acquisition of SASP (Prattichizzo et al., 2018). The accumulation of senescent cells is also seen in hyperglycemic rats, where the most pronounced effect is seen in the cortical tubules of the kidney at 10 days (Satriano et al., 2010). Interestingly, the clearance of senescent cells improves glucose homeostasis and insulin sensitivity in a mouse model of obesityinduced metabolic dysfunction. The clearance of senescent cells in this model also resulted in improved renal podocyte function and reduced microalbuminuria (Palmer et al., 2019).

Benefits of Senescence During Kidney Injury

Contrary to the general opinion backed by evidence, some contradictory evidence exists regarding the beneficial role of senescence during renal injury. One study shows in a shortterm model of unilateral ureter obstruction (UUO), a robust model for kidney atrophy and fibrosis, that cell cycle arrest might be favorable in the acute phase of injury. p16-KO mice show increased kidney damage and fibrosis compared to wildtype littermates (Wolstein et al., 2010). Similar results were seen in a renal ischemia-reperfusion mice model, where senescence induction with a CDK4 and CDK6 inhibitor improved serum creatinine and blood urea nitrogen (DiRocco et al., 2014). This is consistent with previous evidence in other tissues that the effects of SASP secretion (e.g., stemness induction) might have benefits in the handling of acute injuries, whereas prolonged SASP exposure results in impaired tissue repair (de Keizer, 2017).

SASP in CKD

In the long term, senescent cells may impair tissue function and the rejuvenation of their environment. Underlying the detrimental effects of prolonged cell cycle arrest is SASP. The precise SASP composition varies between cell types and mode of senescence induction and depends on time after senescence induction (Hernandez-Segura et al., 2017). Furthermore, all known SASP factors are also implicated in other non-senescent cell conditions (Coppe et al., 2010). In addition to the previously mentioned profibrotic factors, SASP consists of an array of inflammatory chemokines and cytokines, allowing cross-talk between senescent cells and neighboring cells and facilitating the detection and elimination of senescent cells by the immune system (i.e., immune surveillance) (Kang et al., 2011). See Table 2. For a more extensive list, see Freund et al. (2010) and Wang et al. (2017). Although SASP has detrimental effects in the long term, the short-term effects of SASP can be beneficial (e.g., during embryogenesis and wound healing) (Storer et al., 2013; Demaria et al., 2014). Moreover, the secretion of SASP components such as vascular endothelial growth factor

(VEGF) and fibroblast growth factor 2 promote tissue repair during kidney injury (Bonventre, 2014; Yan et al., 2017).

SASP factors can influence both the innate and adaptive immune responses in either promoting clearance by the immune system or causing immunosuppression, thereby promoting the elimination or persistence of senescent cells, respectively (Burton and Stolzing, 2018). An impaired immune system (due to aging, disease, or immunosuppressive therapy) may cause some senescent cells to evade elimination and maintain an SASP secretome (Lujambio, 2016). Exposure to dedifferentiating SASP factors (primarily IL-1β and IL-6) induces a state of pluripotency in neighboring cells, leaving these cells unable to differentiate ("stem lock") (Brady et al., 2013; Pietras et al., 2016; de Keizer, 2017). Thus, through SASP, senescent cells can harm healthy neighboring cells and also block the renewal of lost or damaged cells. This paracrine effect of senescent cells is exemplified by Xu et al. (2018), showing that intraperitoneal injection of senescent preadipocytes in mice resulted in increased physical dysfunction and increased cellular senescence in the recipient tissues along with increased SASP factors. Furthermore, da Silva et al. (2019) showed that xenotransplantation of senescent human fibroblasts to immunodeficient mice led to an increased expression of senescence markers in the surrounding tissues.

Table 2. SASP factors associated with inflammation or fibrosis.

Proinflammatory	Profibrotic
IL-1α	TGF-β
IL-1β	CNN2 (CTGF)
IL-6	VEGF
IL-7	PDGF
IL-8	
MCP-1	
MCP-2	
MIP-1α	
IFN-γ	
TNF-α	

The ability of the kidney to acquire a SASP has been illustrated by Prattichizzo et al. (2018) and Zhang et al. (2017). Both in mice with a hyperglycemic milieu and in mice with an accelerated aging phenotype, the kidneys showed increased transcription of key SASP mRNAs (most notably IL-1β and IL-6) and increased amounts of SASP proteins (namely IL-1β) (Zhang et al., 2017; Prattichizzo et al., 2018). Interestingly, Prattichizzo et al. (2018) showed that endothelial cells and macrophages are an important source of SASP factors in the kidney in a hyperglycemic milieu. Recently, Yao et al. (2019) showed that knockdown of fibroblastspecific PAI-1 (a well-established SASP factor) reduced renal fibrosis after kidney injury. The secretion of SASP factors contribute to the formation of kidney fibrosis after kidney injury. Thus, multiple cell types in the kidney are implicated in the formation of kidney fibrosis.

Although all known constituents of SASP can also be induced independent of cellular senescence, the overlap between SASP and the factors increased in CKD models and patients is quite remarkable (Wang et al., 2017). For example, in a mouse model of kidney fibrosis after IRI, increased expression of MCP-1 (also known as CCL2) and TNF-α is observed (Clements et al., 2013). Increased levels of MCP-1, epidermal growth factor, VEGF, IL-6, and IL-1α are also detected early in the peripheral blood of patients with CKD progression (Perlman et al., 2015). In addition, spot-urine analysis in CKD patients showed the presence of MCP-1, IL-8, and TGF-β1, whereas only MCP-1 could be detected in healthy control urine. However, increased plasma and urinary concentration of MCP-1, IL-8, and TGFβ1 did not correlate with increase of CKD stage (Vianna et al., 2013).

SASP is regulated by several transcription factors, the main regulators being nuclear factor-κB (NF-κB) and C/EBPβ (Orjalo et al., 2009). Aside from SASP, NF-κB signaling mediates the balance between apoptosis and autophagy (Salminen et al., 2012). Autophagy is seen as both pro-senescent (mainly in oncogene induced) and anti-senescent (less defective mitochondria and ROS) (Kwon et al., 2017). Another regulatory protein involved in SASP secretion are sirtuins. Sirtuin activity regulates DNA repair, apoptosis, inflammation

control, and antioxidative defense. Sirtuins (mainly SIRT1) inhibits NF-κB, decreasing SASP secretion (Salminen et al., 2012; Grabowska et al., 2017). Mammalian target of rapamycin (mTOR) serves as an important mediator of SASP secretion. During senescence, autolysosomes and mTOR accumulate in a specific compartment called the TOR autophagy spatial coupling compartment (TASCC). Recently, it was discovered that the TASCC is associated with the secretion of TGF-β and CCN2 in tubular epithelium cells and is associated with increased fibrosis after kidney injury (Narita et al., 2011; Canaud et al., 2019). Remarkably, CCN2 can by itself increase AKT kinase activity and promote senescence in epithelial cells and thus play a role in the paracrine spread (furtherance) of senescence driving CKD (Jang et al., 2017).

Kidney Fibrosis

When epithelial cells become senescent, this leads to maladaptive repair and contributes to the progression of kidney fibrosis (Ferenbach and Bonventre, 2015). Furthermore, senescent cells contribute to a profibrotic milieu through their SASP (Tchkonia et al., 2013), and senescence markers correlate with the amount of kidney fibrosis in mice (Clements et al., 2013). Additionally, the importance of senescence in the process of kidney fibrosis is illustrated by the decrease of fibrosis after IRI in mice lacking p16 expression either due to the loss of the INK4a locus or the short hairpin RNA-mediated silencing of p16 (Braun et al., 2012; Luo et al., 2018). Although these studies mostly pertain animals, evidence suggests that senescence is also implicated in human kidney fibrosis. In human transplant biopsies, the senescent cell burden correlates with the amount of fibrosis and tubular atrophy, and the senescent cell burden can predict transplant kidney function (Ferlicot et al., 2003; Melk et al., 2004; McGlynn et al., 2009; Gunther et al., 2017).

Malignancy

The role of cellular senescence in tumor suppression is well established (Sharpless et al., 2004). Mitosis of cells containing DNA damage or the activation of oncogenes can be inhibited by senescence mechanisms (Bielak-Zmijewska et al., 2018). Thus, inactivating senescent pathways makes these cells more susceptible to malignant transformation. For example, p21deficient mice with reduced senescence show an increased susceptibility for tumor development (Martin-Caballero et al., 2001). Furthermore, signs of loss of this tumor-suppressive mechanism in renal cell carcinoma are associated with a more proliferative phenotype and a higher tumor grade (Macher-Goeppinger et al., 2013).

Aside from preventing tumorigenesis, cellular senescence can also reduce the proliferative ability of malignancies (Zeng et al., 2018). This is used in clinical practice by senescence induction by (a combination of) chemotherapy, radiation therapy, or CDK4 and CDK6 inhibitors (Shah et al., 2018; Zeng et al., 2018). However, systemic chemotherapy also induces senescence in otherwise healthy tissues, leading to local and systemic inflammation and detrimental short- and long-term effects. These effects have been implicated in increased cancer recurrence and cancer metastasis (Demaria et al., 2017). Furthermore, SASP factors such as CCN2 promote chemotherapy resistance of cancer cells (Sun et al., 2012; Yang et al., 2016; Zeng et al., 2017).

Immunosurveillance is an important mechanism of senescent cell clearance (Sagiv and Krizhanovsky, 2013), and in line with this, chronic immunosuppression might contribute to the accumulation of senescent cells after kidney transplantation. Clinically, this is supported by the observation that calcineurin inhibition leads to an altered natural killer cell phenotype (a major contributor to immunosurveillance) and thus reduced immunosurveillance and persistence of senescent cells (Hoffmann et al., 2015). In addition to the loss of kidney function resulting from the deleterious effect of SASP on the microenvironment, evidence suggests that failure of senescent cell elimination might also be oncogenic (Coppe et al., 2010).

Thus, cellular senescence can be both beneficial and detrimental in the prevention and treatment of malignancies (Zhang et al., 2019).

Senotherapy

Eliminating senescent cells through transgenic depletion and pharmaceutical inhibition reduces kidney dysfunction and longterm kidney injury in experimental models of kidney damage, obesity-induced metabolic dysfunction, and during aging (Valentijn et al., 2018). These promising results have spurred interest in the development of clinically applicable therapeutic compounds that target senescence-associated pathways.

Eliminating senescent cells (dubbed as senolysis) is just one of the various potential interventional approaches to target the adverse effects of cellular senescence (so-called “senotherapy”), including the prevention of senescence, modulation of SASP (termed senomorphics), and stimulation of immune system-mediated clearance of senescent cells (reviewed by Kim and Kim, 2019).

Senomorphics and Prevention of Senescence

Several therapeutic options have been shown to modulate SASP and prevent senescence by interfering with senescent-associated intracellular pathways. These therapeutic agents have not been shown to specifically induce apoptosis in senescent cells, as opposed to senolytics. For instance, glucocorticoids suppress SASP and reduce IL-6 in senescent HCA2 medium (Laberge et al., 2012). The inhibition of the JAK/STAT pathway by JAK inhibitor 1 reduces the transcription of senescent preadipocyte SASP factors such as IL-6, IL-8, IP-10, CXCL-1, MCP-1, and MCP-3 (also known as CCL7) (Xu et al., 2015). The p38 inhibitors UR-13756 and BIRB 796 both decreased IL-6 suppression (Alimbetov et al., 2016). Several other well-established senomorphic and senescence preventing agents are discussed below.

Metformin

Metformin, a biguanide used in the treatment of type 2 diabetes, shows promise as an anti-aging agent as reviewed by Kanigur Sultuybek et al. (2019). The decrease in blood glucose by metformin is mainly achieved by the reduction of gluconeogenesis and increased glucagon signaling. However, metformin has several other effects, most notably the activation of AMP-activated protein kinase (AMPK) and the downregulation of the mTOR pathway (Pernicova and Korbonits, 2014). In 2016, the Food and Drug Administration (FDA) revised the guidelines for metformin treatment in patients with CKD, now allowing patients with CKD to be treated with metformin. This is a very promising step given the renal protective abilities of metformin in patients with diabetic nephropathy and even nondiabetic kidney disease, as reviewed by Ravindran et al. (2017). Although the precise mechanism by which metformin attenuates age-related disease is unknown, it is suggested that metformin inhibits NF-κB signaling, thus inhibiting SASP (Moiseeva et al., 2013; Noren Hooten et al., 2016; Kanigur Sultuybek et al., 2019). An *in vitro* study has shown that metformin treatment delayed senescence in human diploid fibroblasts and human mesenchymal stem cells (MSCs), probably through increasing GPx7 and Nrf2 (Fang et al., 2018). Furthermore, metformin reduces ROS formation, γ-H2AX foci, and ATM, in turn reducing the formation of senescent cells (Halicka et al., 2011; Park and Shin, 2017). Besides age-related disease, metformin has a beneficial effect in the treatment of cancer by inhibiting metabolism, reducing protumorigenic signaling through NF-κB inhibition, and reducing stemness in cancer cells that escape oncogene-induced senescence (Deschenes-Simard et al., 2019).

Rapamycin

Rapamycin, an mTOR inhibitor, can potently reduce oxidative injury by inhibiting protein synthesis and stimulating intracellular repair and autophagy. Rapamycin (and its derivatives) are currently used as a potent immunosuppressive drug after solid organ transplantation (Nguyen et al., 2019) and are used (in higher concentrations) for their oncolytic properties in advanced renal cell carcinoma (Boni et al., 2009).

In UUO mice, treatment with rapamycin reduces the fibrotic response in the kidney (Falke et al., 2015) and rapamycin treatment increases the lifespan of middle-aged mice (Bitto et al., 2016). *In vitro* experiments with rapamycin resulted in an increased proliferation of senescent cells (Demidenko et al., 2009). As such, this drug is suggested to alleviate the

senescent burden and could explain the positive effects seen in the mice models. However, to achieve this effect, even higher concentrations are needed than the concentrations used for the oncolytic properties and this may limit the clinical translation (Kaeberlein, 2014).

Senescent cells, like cancer cells, are highly metabolically active and thus are susceptible to the inhibition of protein synthesis and cell growth by rapamycin via the inhibition of both mTOR complexes 1 and 2 (Li et al., 2014). Thus, mTOR inhibition can both induce apoptosis in malignant cells and reduce senescent cell burden.

Although it is not entirely clear whether especially senescent tumor cells are sensitive to the oncolytic activity of rapamycin, generally speaking, senescent as well as non-senescent cells with high metabolic activity might share increased dependence on the mTOR pathway for their survival. mTOR inhibition has shown beneficial effects in the setting of organ transplantation, cancer, and senescence, but the inhibition of mTOR can also lead to serious side effects (Nguyen et al., 2019). These side effects include insulin resistance, glomerular dysfunction, dyslipidemia, hematologic side effects (anemia, leucopenia, and thrombocytopenia), mucositis, pneumonitis, lymphedema, angioedema, and osteonecrosis. This wide variety of side effects likely reflects the broad involvement of the mTOR pathway in cell and tissue homeostasis.

Niacin and Resveratrol

Another option to reduce the development of cellular senescence is via sirtuin activation. As mentioned above, SIRT1 inhibits NF-κB signaling, which is a key transcriptional regulator of SASP, but sirtuins also alleviate cell cycle arrest by regulation of p53, NF-κB, STAT, FOXO1, and FOXO3 (Wakino et al., 2015). A decrease in sirtuin activation is implicated in the reduction of kidney function with age (Ugur et al., 2015) and increased activation of SIRT1 attenuates high glucose-induced kidney mesangial hypertrophy (Zhuo et al., 2011). Increased sirtuin activation can be achieved via dietary supplementation of NAD+ with niacin, such as nicotinamide riboside (Rajman et al., 2018). SIRT activation can also be achieved by resveratrol, although the beneficial effect of resveratrol on senescence markers is seen in analogy to resveratrol without SIRT1 activation (Latorre et al., 2017). Although nicotinamide riboside and resveratrol have a positive effect on the health and lifespan of animals (Gambini et al., 2015; Rajman et al., 2018), they do not induce a senolytic effect (Latorre et al., 2017; Grezella et al., 2018).

Senolytics

The removal of senescent cells with so-called “senolytics” may be the most feasible and most attractive approach for clinical application, as the prevention of senescence and modulation of SASP would require chronic treatment with prolonged exposure to therapeutics.

Senolytic agents are a class of small molecules that can selectively kill senescent cells that participate in senescenceassociated pathways by interfering with anti- and pro-survival signaling (Zhu et al., 2015).

Because of the same reliance on anti-apoptotic signaling of tumor cells as seen in senescence, similar therapeutic strategies are being explored in oncology as can be used for effective/ specific senescence depletion, and the kidney community may well benefit from the yield of such studies.

Several senolytic drugs are already in clinical use or in advanced phases of anti-tumor drug development, providing relevant information concerning efficacy and safety that may be extrapolated at least in part to non-oncological kidney diseases.

Therefore, the use of existing antitumor drugs as senolytics has gained interest. Although very few of these drugs have been tested on renal tubular epithelium, many have already been tested on a number of different cell types and tissues both *in vivo* (Table 3) and *in vitro* (Table 4), which may render a first impression of their potential for application in kidney diseases. Below, we therefore review the effects of candidate senolytic drugs on different cell types exposed to various senescenceinducing triggers. For their targets, see Figure 2.

Table 3. *in vivo* effect of potential senolytic therapy

Group	Drug	Target	Model	Result	Reference
BH3 mimetics	Navitoclax (ABT-263)	BCL-2, BCL-xL, BCL-W inhibitor	-Body radiation	-Reduced lung fibrosis -improved lung tissue elasticity	(Chang et al., 2016)
			-Normal aging	-Improved hematopoietic parameters -decreased SASP factors	
			-Induced emphysema (porcine pancreatic elastase (PEE))	-Improved lung tissue elasticity	(Mikawa et al., 2018)
	ABT-737	BCL-2, BCL-xL, BCL-W inhibitor	-Body radiation -$p14^{ARF}$ induced senescence	-Decreased cellular senescence in lungs and skin	(Yosef et al., 2016)
FOXO4 inhibitor	FOXO4-DRI	Foxo4-p53 interaction inhibition	-Doxorubicin	-Decreased doxorubicin-induced body weight and liver toxicity.	(Baar et al., 2017a)
			-Normal aging -Premature aging model	-improved fur density and responsiveness -improved kidney function	
UBX0101	UBX0101	MDM2-p53 pathway	-post traumatic osteoarthritis (ACLT transection)	-reduced pain -decreased cartilage erosion/thinning -decreased SASP factors	(Jeon et al., 2017)
Flavenoids-TKI combination	Quercetin and dasantinib	BCL-2 pathway inhibitor (proteasome activator)	-natural aging	-decreased osteoclast numbers -improved left ventricular ejection fraction	(Zhu et al., 2015; Roos et al., 2016; Farr et al., 2017; Ogrodnik et al., 2017; Schafer et al., 2017; Xu et al., 2018)
		PI3K and serpines inhibitor	-single leg radiation	-increased exercise time, distance and total work to exhaustion.	
			-premature aging model	-decreased age related symptoms	
		Tyrosine kinase inhibitor (EFNB dependent suppression of apoptosis)	-atherosclerosis model	-reduced intimal plaque calcification -improved vascular relaxation	
			-liver steatosis model	-reduced hepatic fat deposition	
			-transplant of senescent adipocytes	-attenuated decrease of physical function -attenuated weight reduction	
			-pulmonary fibrosis (bleomycin induced)	-attenuated lung compliance -increased distance until exhaustion	
Flavenoids	Fisetin	(hydrophobic groove of) BCL-2	-natural aging	-extension of median and maximal lifespan	(Yousefzadeh et al., 2018)
			-premature aging model	-reduced senescent mesenchymal stem/progenitor cells -reduced SASP factors	
HSP90 inhibitors	17-DMAG	Inhibits the molecular chaperone HSP90, leading to AKT and ERK destabilization.	-premature aging model	-reduced age related symptoms and increased body condition -reduced kidney $p16^{Ink4a}$ expression	(Fuhrmann-Stroissnigg et al., 2017)

BH3 Mimetics

BH3 protein (e.g., BIM, BID, and PUMA) is upregulated by stress signals and binds with pro-survival BCL-2 protein family. This inhibits the interaction of BCL-2 family protein with BAX and BAK. Released BAX and BAK form oligomers and initiate the caspase cascade by perforating the outer mitochondrial membrane leading to apoptosis, a process circumvented by senescence via an increase in BCL-2 family member expression (Adams and Cory, 2018).

BH3 mimetics inhibit the binding of BCL-2 family to BAX and BAK, enabling BAX and BAK to form oligomers and induce apoptosis. There are several BH3 mimetics targeting a different (combination of) BCL-2 family protein(s). One of the more successful being

venetoclax (ABT-199; BCL-2 inhibitor), which has been FDA approved for the treatment of refractory chronic lymphocytic leukemia (Adams and Cory, 2018). Although successful in oncology, the *in vitro* senolytic effect of venetoclax in human fibroblasts is variable and dependent on the manner of senescence induction (Chang et al., 2016; Yosef et al., 2016).

A promising and more extensively tested BH3 mimetic is navitoclax (ABT-263; BCL-2, BCL-xL, and BCL-w inhibitor). In several studies, navitoclax induced a senolytic effect in different human and mouse cells, including renal epithelial cells (Chang et al., 2016; Zhu et al., 2016; Fuhrmann-Stroissnigg et al., 2017; Kim et al., 2017; Pan et al., 2017; Grezella et al., 2018). However, in human MSCs, navitoclax showed only low selectivity for senescent cells compared to non-senescent cells and no senolytic effect was observed in human preadipocytes (Zhu et al., 2016; Grezella et al., 2018). Navitoclax clears senescent cells and reduces SASP in old and irradiated mice. Furthermore, treatment of mice with navitoclax results in improved myeloid function and reversed pulmonary fibrosis (Chang et al., 2016; Pan et al., 2017).

The BH3 mimetic ABT-737 (BCL-2 and BCL-xL inhibitor) causes a selective reduction in senescent human and mouse fibroblasts in several models of senescence (etoposide, H-ras, replicative), but a lesser reduction is seen in radiation-induced senescent fibroblasts (Yosef et al., 2016; Baar et al., 2017). The *in vivo* application of ABT-737 results in a significant reduction of senescent cells, i.e., in the basal layer of skin epidermis after radiation and p14ARF-induced senescence (Yosef et al., 2016).

Several other BH3 mimetics have been studied in the context of senescence. The BCL-xL inhibitors A1331852 and A1155463 both selectively reduce the viability of radiation-induced senescent HUVECs and IMR90s compared to non-senescent cells. Like navitoclax, no selective reduction of senescent cell viability is seen in preadipocytes (Zhu et al., 2017). TW-37 (BCL-2 and MCL-1 inhibitor) is less senolytic compared to navitoclax in radiation-induced senescent endothelial cells and fibroblasts (Zhu et al., 2016). No senolytic effect is observed in preadipocytes. WEHI-539 (BCL-xL inhibitor) shows no senolytic effect in radiation-induced senescent WI-38 cells (Chang et al., 2016). Obatoclax (BCL-2, BCL-xL, and BCL-W inhibitor) induces a significant reduction in both control cell and senescent human fibroblasts (Yosef et al., 2016).

Flavenoids

Quercetin

Quercetin is a flavonol. It belongs to the polyphenols and is subclassified as a flavonoid. It is found in fruits and vegetables and the average daily consumption amounts to approximately 10 mg/day. Quercetin has an anti-oxidative effect, targeting ROS and reactive nitrogen species (ROS). Apart from this direct antioxidative effect, quercetin has an indirect anti-oxidative effect via activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and paraoxonase 2 (PON2). Both pathways have an antioxidant effect (Costa et al., 2016). Interestingly, quercetin activates sirtuin 1 (Sirt1), a nicotinamide dinucleotide-dependent deacetylase that has a renoprotective effect, mediated by the deacetylation of p53, improving mitochondrial function and decreasing NF-κB, resulting in decreased fibrogenesis (Wakino et al., 2015).

Quercetin has most often been studied in combination with the tyrosine kinase inhibitor dasatinib. *In vitro*, this combination selectively reduces senescent human endothelial cells and mouse fibroblasts while being less effective in pre-adipocytes and human lung fibroblasts (Zhu et al., 2015; Baar et al., 2017; FuhrmannStroissnigg et al., 2017; Schafer et al., 2017; Grezella et al., 2018). In replicative senescent human MSCs, no clear senolytic effect was observed, whereas in Werner syndrome and in a HutchinsonGilford progeria model quercetin (without dasatinib) caused a decrease in senescence markers in human MSCs (Zhu et al., 2015; Geng et al., 2019). Treatment with quercetin and dasatinib of primary human adipose tissue from obese patients resulted in the decreased expression of senescence markers and reduced SASP production (Xu et al., 2018).

Table 4. *in vitro* senolytic effect of tested drugs on different cell lines and different manners of senescence induction.

Group	Drug	Target	Cell line	Induction	Senolytic	Reference
BH3 mimetics	Navitoclax (ABT-263)	BCL-2, BCL-xL, BCL-W inhibitor	-WI-38	-radiation -replicative exhaustion -oncogenic RAS expression	-yes -yes -yes	(Chang et al., 2016) (Schafer et al., 2017) (Zhu et al., 2016) (Fuhrmann-Stroissnigg et al., 2017) (Grezella et al., 2018) (Pan et al., 2017)
			-IMR90	-radiation -etoposide	-yes -no	
			-RECs	-radiation	-yes	
			-MEFs	-rediation -premature aging	-yes -yes	
			-HUVECs	-radiation	-yes	
			-human preadipocytes	-radiation	-no	
			-human mesenchymal stromal cells (MSCs)	-replicative exhaustion	-yes	
			AECIIs primary pneumocytes (C57BL/6J mice)	-radiation	-yes	
	Venetoclax (ABT-199)	BCL-2 inhibitor	-WI-38	-radiation	-yes, in combination with WEHI-539	(Chang et al., 2016)
			-IMR90	-etoposide -oncogenic H-Ras expression	-no -yes	(Yosef et al., 2016)
	ABT-737	BCL-2, BCL-xL, BCL-W inhibitor	-IMR90	-radiation -etoposide -Oncogenic H-Ras expression -replicative exhaustion	-yes -yes -yes -yes	(Baar et al., 2017b) (Yosef et al., 2016)
	WEHI-539	BCL-xL inhibitor	-WI-38	-radiation	-yes in combination with Venetoclax	(Chang et al., 2016)
	TW-37	BCL-2, MCL-1 inhibitor	-IMR90	-radiation	-yes	(Zhu et al., 2016)
			-HUVECs	-radiation	-yes	
			-human readipocytes	-radation	-yes	
			-MEFs	-oxidative stress -replicative exhaustion	-no -no	
	A1331852	BCL-xL inhibitor	-IMR90	-radiation	-yes	(Zhu et al., 2017)
			-HUVECs	-radiation	-yes	
			-human preadipocytes	-radiation	-no	
	A1155463	BCL-xL inhibitor	-IMR90	-radiation	-yes	
			-HUVECs	-radiation	-yes	
			-human preadipocytes	-radiation	-no	
	Obatoclax (GX15-070)	BCL-2, BCL-xL, BCL-W inhibitor	-IMR90	-etoposide -oncogenic H-Ras expression	-no -no	(Yosef et al., 2016)
FOXO4 inhibitor	FOXO4-DRI	Foxo4-p53 interaction inhibition	-WI-38	-radiation	-yes	(Baar et al., 2017b)
			-IMR90	-radiation -doxorubicin	-yes -yes	
			-BJ	-radiation	-yes	
UBX0101	UBX0101	MDM2/P53	-primary human chondrocytes	-osteoarthritis patient	-yes	(Jeon et al., 2017)

Flavenoids and TKI	Quercetin and Dasatinib	BCL-2 pathway inhibitor (proteasome activator) PI3K and serpines inhibitor Tyrosine kinase inhibitor (EFNB dependent suppression of apoptosis)	-IMR90	-radiation -etoposide	-no (yes*) -yes	(Zhu et al., 2015; Baar et al., 2017; Fuhrmann-Stroissnigg et al., 2017; Lehmann et al., 2017; Schafer et al., 2017; Geng et al., 2019; Grezella et al., 2018; Xu et al., 2018)
			-pulmonary mouse ATII cells	-bleomycin	-yes	
			-HUVECs	-radiation -replicative exhaustion	-yes -yes	
			-human preadipocytes	-radiation -replicative exhaustion	-yes/no -yes	
			-MEFs	-radiation -premature aging	-yes -yes	
			-MSCs	-replicative exhaustion -premature aging	-no -no	
	Fisetin	BCL-2 pathway inhibitor (hydrophobic groove of BCL-2)	-IMR90	-etoposide	-no (decreased senescence, no apoptosis)	(Yousefzadeh et al., 2018)
			-HUVECs	-etoposide	-yes	
			-MEFs	-etoposide -premature aging	-no -no	
			-MSCs	-replicative exhaustion	-no	(Zhu et al., 2017)
HSP90 inhibitors	17-DMAG	Inhibits the molecular chaperone HSP90, leading to AKT and ERK destabilization.	-WI-38	-replicative exhaustion	-yes	(Fuhrmann-Stroissnigg et al., 2017)
			-IMR90	-etoposide	-yes	
			-MEFs	-premature aging	-yes	
			-MSC	-oxidative stress	-yes	
	Geldanamycin		-MEFs	-premature aging	-yes	
Niacine	Nicotinamide riboside	Nicotinamide adenine dinucleotide (NAD^+) increase	-MSCs	-replicative exhaustion	-no	(Grezella et al., 2018)
Resveralogues	Resveratrol	Activation of SIRT1 (NAD-dependent protein deacetylase)	-NHDF (human fibroblasts)	-replicative exhaustion	-no	(Latorre et al., 2017)
			-HF043 (human dermal fibroblasts)	-replicative exhaustion	-no	
			-MRC5 (human lung fibroblasts)	-replicative exhaustion	-no	

Senolytic effect is defined as a selective induction of apoptosis in senescent cells. Cell lines: WI-38, human lung fibroblast; IMR90, human lung fibroblast; REC, human renal epithelial cells; MEF, mouse embryonic fibroblasts; HUVEC, human umbilical vein endothelial cell; BJ, human foreskin fibroblasts; MSC, mesenchymal stromal cells (femoral bone marrow); NHDF, normal human dermal fibroblasts.

Quercetin and the quercetin-dasatinib combination have been applied in several mouse models, including naturally and accelerated aging mice, obesity-induced metabolic dysfunction, single-leg radiation, atherosclerosis model, liver steatosis, bleomycin- induced pulmonary injury, and transplantation of senescent cells. Combined treatment with quercetin and dasatinib of mice with metabolic syndrome not only resulted in improved glucose metabolism but also resulted in a decreased albuminuria (Palmer et al., 2019). In naturally aged mice, combined treatment with quercetin and dasatinib resulted in decreased cell senescence in fat and liver, improved left ventricular ejection fraction, decreased senescence in endothelial and smooth muscle layers, reduced physical dysfunction, reduced SASP expression, and improved bone microarchitecture (Zhu et al., 2015; Roos et al., 2016; Farr et al., 2017; Xu et al., 2018). Treatment of mice results in a remarkable improvement of several

aging-related parameters, such as condition, muscle function, and coordination (Zhu et al., 2015).

Fisetin
Fisetin is another member of the flavonoid family and is also found in fruits and vegetables. Fisetin targets a plethora of signaling pathways including PI3K/AKT, NF-κB, p38 mitogenactivated protein kinase (MAPK), and BCL-2/BCL-xL (Sundarraj et al., 2018). Interestingly, fisetin has been shown to be beneficial in cisplatin-induced AKI (Sahu et al., 2014).

In vitro, fisetin reduces (etoposide-induced) senescent cells in human and mouse cells but not in replicative senescent human fibroblasts. Interestingly, in mouse fibroblasts, fisetin does not induce apoptosis, suggesting that the effect is not senolytic in these cells but that fisetin alleviates the cell cycle arrest (Yousefzadeh et al., 2018).

In naturally aged mice (22–24 months) and a XFE progeria model (p16+/Luc; Ercc1-/Δ), fisetin treatment results in an extension of median and maximal lifespan and a decrease in cellular senescence in liver, kidney, fat, spleen, and peripheral CD3+ T cells and a decrease in circulating SASP factors (Yousefzadeh et al., 2018).

p53-Mediated Senolytics
FOXO4
The FOXO4 D-retro inverso (DRI) peptide targets the FOXO4-p53 axis, leading to an increase in active p53 in senescent cells (release from DNA-SCARS). FOXO4 DRI increases the mouse quality of life (fur and activity) in naturally old and genetically aged models of senescence in mice. In a mouse model of folic acid-induced kidney damage, a significant reduction in senescent tubular cells was seen, although no reduction in renal fibrosis, cellular infiltrate, and tubular damage was seen (Jin et al., 2019). Moreover, in the XdpTTD/TTD p16:3MR mouse model of accelerated aging, FOXO4 DRI treatment was associated with preserved kidney function (Baar et al., 2017). FOXO4 DRI treatment of senescent human fibroblasts resulted in a selective reduction of senescent cells compared to non-senescent cells (Baar et al., 2017).

UBX0101
UBX0101 is a small-molecule inhibitor targeting MDM2/p53 interaction. Treatment of primary human chondrocytes isolated from osteoarthritis patients with UBX0101 showed a senolytic effect of UBX0101 and reduced SASP expression. In a posttraumatic osteoarthritis mouse model (ACTL transection), treated mice showed reduced pain and reduced articular cartilage erosion and less senescent markers in the cartilage. The improvement in physical function lasted 84 days (Jeon et al., 2017). A phase 1 clinical trial regarding osteoarthritis is ongoing (ClinicalTrials.gov Identifier: NCT03513016).

Heat Shock Protein 90 Inhibitors
Heat shock protein 90 (HSP90 is a molecular chaperone that is especially transcribed during cellular stress, aiding protein stabilization and preventing protein misfolding and aggregation. The so-called client proteins of HSP90 include transcription factors (e.g., p53), kinases (e.g., CDK4), eNOS, TERT, and mitochondrial proteins. In tumor cells, HSP90 has an anti-apoptotic effect mediated by mTOR, NF-κB, and FOXO3A; furthermore, it leads to AKT stabilization mitigating apoptosis (Taipale et al., 2010; Fuhrmann-Stroissnigg et al., 2017).

The senolytic potential of HSP90 inhibitors was discovered in a screening assay for senotherapeutics using Ercc1-/- mouse embryonic fibroblasts (MEFs). Several possible senolytic agents involved in regulating autophagy were analyzed and treatment with the HSP90 inhibitors 17-DMAG and geldanamycin resulted in the strongest senolytic effect. The senolytic effects of 17-DMAG are also seen in senescent human MSCs and fibroblasts (Fuhrmann-Stroissnigg et al., 2017). Furthermore, progeroid mice (Ercc1-/Δ) treated with 17-DMAG showed reduced age-related symptoms and increased overall condition (Fuhrmann-Stroissnigg et al., 2017).

Panobinostat

Panobinostat (LBH-589) is a histone deacetylase (HDAC) inhibitor and is FDA and European Medicines Agency approved for the treatment of refractory multiple myeloma (Tzogani et al., 2018). *In vivo* studies with HDAC inhibitors show attenuation of renal inflammation and fibrosis in several animal models for kidney disease (Brilli et al., 2013; Van Beneden, et al., 2013). HDAC causes the deacetylation of lysine residues of histone tails, promoting the interaction of histones and DNA, inhibiting mRNA transcription. Other targets of HDAC besides the histones are E2F, p53, and NF-κB. Inhibitors of HDAC increase p21 transcription levels and result in the acetylation of p53, thereby promoting cell cycle arrest (Yoon and Eom, 2016). However, HDAC inhibitors also have a marked effect on the apoptotic balance in the cell, increasing transcription of proapoptotic proteins Bax, Bak, Bim, Bad, Noxa, Puma, Bid, and Apaf1 and decreasing expression of BCL-2, BCL-xL, Mcl-1, and survivin (Marchion and Münster, 2007). This pro-apoptotic effect could explain the senolytic effect seen in senescent non-small cell lung cancer and head and neck squamous cancer after chemotherapy (Samaraweera et al., 2017).

2-Deoxy-D-glucose (2DG)

2DG is a glucose analog that competitively inhibits the uptake of glucose by GLUTs. Once inside the cell, 2DG inhibits ATP production, in turn activating AMPK and furthermore leading to cell cycle arrest, decreased cell growth, increased autophagy, and cell death (Zhang et al., 2014). 2DG shows a senolytic effect on senescent vascular smooth muscle cells. The potential senolytic effect of 2DG relies on the increased metabolic activity (glucose consumption) of senescent cells (Gardner et al., 2015).

To summarize, the identified senolytics, senomorphics, and SASP modulators show varying results on *in vivo* and *in vitro* models for aging and disease. This variance is highly dependent on the cell type and manner of senescence induction. It is therefore important to investigate the senotherapeutic drugs in the cell type of interest using the appropriate induction of senescence. The senotherapies tested in *in vivo* kidney disease models show promising signs of kidney function preservation and reduced kidney fibrosis.

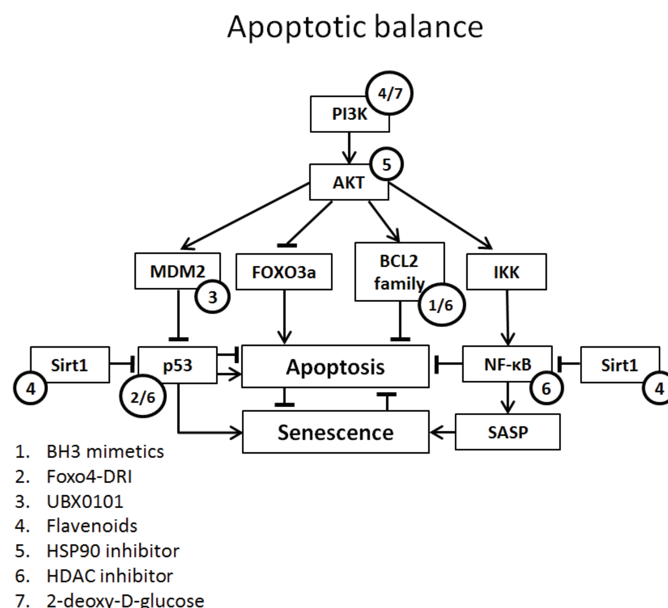

Figure 2. The apoptotic balance of senescent cells with potential targets of senolytics
The apoptotic balance is influenced by a plethora of mediators. Abbreviations: PI3K, phosphoinositide 3-kinases; MDM2, murine double minute 2; FOXO3, forkhead box O3; BCL2, B-cell lymphoma 2; IKK, IkB kinase complex; Sirt1, sirtuin 1; SASP, senescence-associated secretory phenotype.

Targeted therapy

Although senolytic therapy is potentially beneficial in reversing age-related diseases, off-target effects might occur. For instance, loss of the beneficial role of cellular senescence in cutaneous wound healing and in the prevention of fibrosis upon liver injury (Krizhanovsky et al., 2008; Demaria et al., 2014). Furthermore, several potent senolytic agents such as navitoclax and ABT-737 show major systemic side effects such as thrombocytopenia and neutropenia when administered systemically (Wilson et al., 2010; Kipps et al., 2015).

Other hurdles in senolytic therapy for kidney disease are the first-pass hepatic clearing of molecules reducing bioavailability and thus kidney exposure and the rapid passaging of molecules through the kidney, leaving little time for the molecules to have effect.

These hurdles can in part be overcome by the intermittent administration of the senolytic drugs while still achieving the desired effect. Moreover, a targeted delivery of senolytic compounds to senescent renal cells would allow for a decrease in systemic exposure and toxicity.

Targeted accumulation of senolytic agents in the kidney might be achieved using nanomedicines (i.e., nanoparticulate carriers) such as conjugates and liposomes. The delivery of therapeutic of such functionalized compounds should enable high enough drug concentrations where needed.

Major methods of targeting proximal renal tubular cells are the use of protein- or peptide-based carriers and the use of nanoparticles (Liu et al., 2019). Protein- or peptide-based carriers consist of a low molecular weight protein (LMWP; e.g., lysozyme and immunoglobulins) to which the drug is linked (Zhou et al., 2014). In case of proximal tubular epithelium, the LMWP has a high affinity for tubular cell membrane receptors such as low-density lipoprotein receptor-related protein 2 (megalin and cubilin) (Christensen et al., 2012). Upon binding to the membrane receptor, the drug and LMWP are internalized via endocytosis. After degradation of the bond between drug and LMWP in the lysosome, the drug enters the cytoplasm (Nasiri et al., 2018).

Nanoparticles are engineered organic or inorganic carriers typically smaller than 150 nm, designed to deliver drugs to specific organs or cell types, depending on the nanoparticle's characteristics. Depending on the desired target, nanoparticles can be designed varying in size, shape, charge, and composition. Furthermore, the surface of the nanoparticle can be coated with ligands (e.g., immunoglobulins) targeting specific cell types (Kamaly et al., 2016). To pass the glomerular filtration barrier and reach luminal surface of the tubular epithelium, nanoparticles need to be smaller than 5 to 7 nm. A positive charge facilitates, whereas a negative charge hampers passage (Kamaly et al., 2016). Interestingly, however, much larger mesoscale nanoparticles of 400 nm were found to selectively target proximal epithelial cells possibly by transcytosis across capillaries and endocytosis into the epithelium (Williams et al., 2015).

Examples of drug targeting to the proximal tubular epithelium include the conjugation of imatinib — platelet-derived growth factor receptor kinase inhibitor—to lysozyme via a platinum (II)-based Universal Linkage System (ULS). This resulted in a bioavailability of 100% when to imatinib-ULS-lysozyme was administered either intravenously or in the intraperitoneal cavity and a decreased exposure of other organs to imatinib in mice (Dolman et al., 2012).

Challenges and opportunities

The timing of the senolytic treatment of CKD is of great importance. Due to the limited regenerative potential of the kidney in CKD, treatment with senolytics would seem most beneficial in the early stages of CKD when the number of senescent cells is still limited and little progression of fibrosis has occurred. The identification of biomarkers to identify this "window of opportunity" will be of great help to make the best use of senolytics.

Because the actual disease burden of early CKD is relatively limited, (potential) side effects of senolytics will constitute major hurdles to the application of such drugs at this stage of disease progression. Therefore, it will be essential to keep side effects to a minimum.

For this, it is important to realize that senescent cells can express specific surface markers such as NKG2D ligands (MIC and RAET1/ULBP related), identifying them for elimination by surveilling immune cells. The fact that perforin KO in mice leads to the accumulation of senescent cells suggests that targeted intracellular delivery of granzymes might constitute an effective approach for novel senolytic therapies (Ovadya et al., 2018). Senescence-induced surface markers might also be explored for the targeted delivery of senolytic drugs in general.

Research regarding senescence in the kidney has pointed to the proximal tubular epithelium as the culprit, and the removal of senescent tubular epithelial cells is therefore a promising approach to the attenuation of fibrosis in CKD, an otherwise untreatable and progressive disease (Baar et al., 2017). Due to the specific nature of proximal tubular epithelium, several specific targeting options are available, by which therapeutic drug efficacy can be potentiated and side effects can be reduced. Repurposing senolytic drugs that have been tested in clinical trials for other, mostly oncological, indications by functionalization for targeted delivery is a promising method to make a fast translation to clinical nephrology practice.

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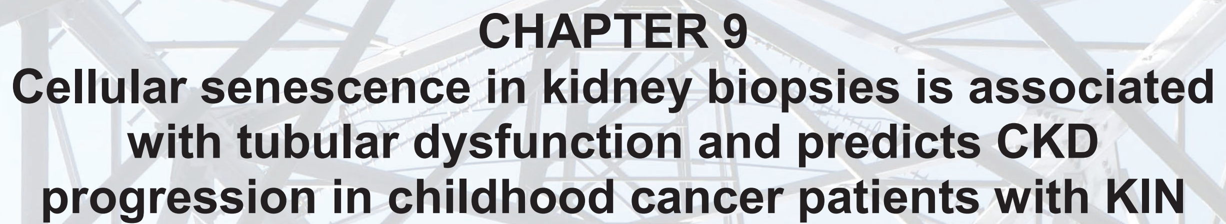

CHAPTER 9
Cellular senescence in kidney biopsies is associated with tubular dysfunction and predicts CKD progression in childhood cancer patients with KIN

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ABSTRACT

Karyomegalic interstitial nephropathy (KIN) has been reported as an incidental finding in patients with childhood cancer treated with ifosfamide. It is defined by presence of tubular epithelial cells (TECs) with enlarged, irregular and hyperchromatic nuclei. Cellular senescence has been proposed to be involved in kidney fibrosis in hereditary KIN patients. We report that KIN could be diagnosed 7-32 months after childhood cancer diagnosis in 6/6 consecutive patients biopsied for progressive chronic kidney disease (CKD) of unknown cause between 2018 and 2021. Morphometry of nuclear size distribution, and markers for DNA damage (γH2AX), cell-cycle arrest (p21+, Ki67-), and nuclear lamina decay (loss of lamin B1), identified karyomegaly and senescence features in TECs of all 6 patients. The number of p21-positive TECs by far exceeded the typically small numbers of truly karyomegalic cells and p21-positive TECs contained less lysozyme, testifying to defective resorption which explains the consistently observed low molecular weight (LMW) proteinuria. Moreover, in the 5 patients with the largest nuclei, the percentage of p21-positive TECs tightly correlated with eGFR-loss between biopsy and last follow-up ($R^2=0.93$, $p<0.01$). Cellular senescence is associated with tubular dysfunction and predicts CKD progression in childhood cancer patients with KIN, and appears to be a prevalent cause of otherwise unexplained CKD and LMW proteinuria in children treated with ifosfamide.

INTRODUCTION

Chronic kidney disease (CKD) in childhood cancer patients typically shows a tubular injury phenotype with high urine output and low molecular weight (LMW) proteinuria. Karyomegalic interstitial nephritis/nephropathy (KIN) has been described as a rare cause for CKD in ifosfamide treated patients. (McCulloch et al., 2011) KIN is defined by tubular cell degeneration featuring abnormally large irregular and hyperchromatic nuclei. (Burry, 1974; Mihatsch et al., 1979) It has been proposed that cellular senescence due to compromised DNA damage repair might contribute to kidney fibrosis in hereditary KIN patients with mutations in the FANCD2/FANCI-Associated Nuclease 1 (FAN1) gene, and in humans and experimental animals exposed to ochratoxin A, busulfan or pyrrolizidine alkaloids. (Burry, 1974; Godin et al., 1996; Zhou et al., 2012)

To explore the prevalence of ifosfamide induced early KIN and the role of cellular senescence, we analyzed the biopsies and clinical histories of six consecutive patients from the pediatric nephology department of the Wilhelmina Children's Hospital, UMC Utrecht between 2018 and 2021 with unexplained and progressive loss of kidney function after ifosfamide treatment for pediatric cancers.

MATERIALS AND METHODS

Patients and biopsies

All children undergoing a kidney biopsy at the Wilhelmina Children's Hospital, UMC Utrecht between 2018 and 2021 for progressive decline of kidney function after ifosfamide treatment were included. The biopsies were taken 7-32 months (median 20 months) after initiation of ifosfamide treatment for various childhood cancers at the Princes Máxima Center for Pediatric Oncology. Biopsies were reviewed by two expert renal pathologists (TN and RG) by standard light microscopy, immunofluorescence, and electron microscopy, unless otherwise specified. Additional stainings and morphometry were performed (see below). For comparison, six protocol kidney allograft biopsies without any pathological findings taken 3 months after kidney transplantation were included. Clinical data were retrieved from the electronic patient files.

Additional stainings

Double-stranded DNA-breaks were detected by staining for yH2AX (NB100-2280, Novus biologicals). For nuclear lamina staining Lamin B1 antibody ab16048 (Abcam) was used. Cell cycle inhibitor p21 was detected using ventana RTU 760-4453 (Cellmarque), or, for double staining with lysozyme, antibody 2947S from Cell signaling. Tubular reabsorption of lysozyme was assessed using antibody A099 (Dako), and Ki67 expression was detected using ventana RTU M7240 (DAKO). Chromogenic color development was by Horse Radish Peroxidase (HRP) linked BrightVision (ImmunoLogic, Duiven, NL) species-specific secondary antibody, followed by 3,3'-diaminobenzidine (Sigma Aldrich, St. Louis, MO). For fluorescent visualization anti-rabbit IgG/alexa 594 715-585-150 (Jackson) and 4',6-diamidino-2-phenylindole were used.

Morphometry and image analysis

Whole slides were obtained with NanoZoomer scanners (Hamamatsu, Japan) and selected images were acquired by taking snapshots in ImageScope. Nuclear size was measured using QuPath 0.2.3 (University of Edinburgh, Edinburgh, Scotland), automatically detecting nuclear outlines and calculating nuclear surface of total available cortical area in three H&E sections per biopsy. (Bankhead et al., 2017) The number of p21 positive cells was automatically counted with QuPath (positive cell detection) in one entire cross-section of the available cortex in the biopsy. The cortical surface area staining for lysozyme was quantified using the QuPath pixel classifier.

Statistical analysis

Cell distribution analysis, percentile calculations, and statistics (Mann-Whitney test, simple linear regression and Wilson/Brown for ROC curves) were performed on exported measurements in R and GraphPad Prism (8.3.0).

RESULTS

Case reports

Clinical information is summarized in Table 1 and Figure 1A and a detailed individual patient history is described in appendix A. One patient had neuroblastoma, three had Ewing sarcoma, one embryonal rhabdomyosarcoma, and one Wilms tumor. Age at diagnosis ranged from 3.2 to 16.2 years. All patients had a normal kidney function at diagnosis of malignancy. Cumulative ifosfamide doses were between 12-100 g/m2. In addition, one patient received cisplatin, and three patients cyclofosfamide. Four patients (2 Ewing sarcoma, one neuroblastoma and one Wilms tumor) had received radiotherapy including the kidney region, with a mean kidney dose range of 0.2-26 Gray. At latest follow-up (22-69 months after diagnosis of malignancy) kidney function declined in all patients, reaching CKD stage 2 (n=3), stage 3 (n=2) or stage 4 (n=1). All had LMW proteinuria. Blood pressure was normal in all patients. The clinical picture was consistently that of tubulointerstitial nephropathy. Two patients received steroids, for inflammation observed in the biopsy. Kidney function initially improved in case 1, but progressed to CKD stage 4 despite steroid treatment. In case 3 kidney function stabilized at CKD stage 2. Of the four patients that did not receive steroid treatment, cases 2 and 4 stabilized (at CKD stage 2 and 3a), while cases 5 and 6 progressed to CKD stage 3b and 2 at latest follow-up.

Detection of KIN and senescence markers

Identification of KIN by automated assessment of nuclear size and correlation with kidney function

Tubular epithelial cells (TECs) with enlarged nuclei are the hallmark of KIN. (Bhandari et al., 2002) The initial diagnosis of KIN in five of our six patients was evident at standard microscopic assessment, but the biopsy of one patient (case 6) initially appeared normal in routine evaluation (Figure 1B). To validate these findings, nuclear sizes were measured using QuPath. Although the mean nuclear size in KIN and protocol biopsies was comparable (18.83 µm2 and 19.87 µm2 respectively), there was a clear difference between groups regarding the size of the largest nuclei (Figure 1C-D), with a small subpopulation in all six biopsies showing abnormal enlargement. ROC analysis revealed that all six biopsies could be distinguished from protocol biopsies by comparing the 999th promille of largest nuclei (Supplemental figure 1), while case 3 was still misclassified when comparing the 99th percentile, and discriminative value was completely lost when comparing the 10% largest nuclei. The 999th percentile of nuclear size trended to associate with urinary β2-microglobulin/creatinine ratio (Figure 1G), but did not correlate with decline in eGFR or increase in serum creatinine (Figure 1E-F).

Thus, morphometry of nuclear size distribution was a valuable tool in identifying KIN(-like pathology), but the relevance of such small proportions of karyomegalic cells might be questioned. However, also decline in eGFR was neither correlated with glomerulosclerosis (R^2=0.35, p=0.2120, data not shown) or tubulointerstitial fibrosis (R^2=0.32, p=0.2407, data not shown).

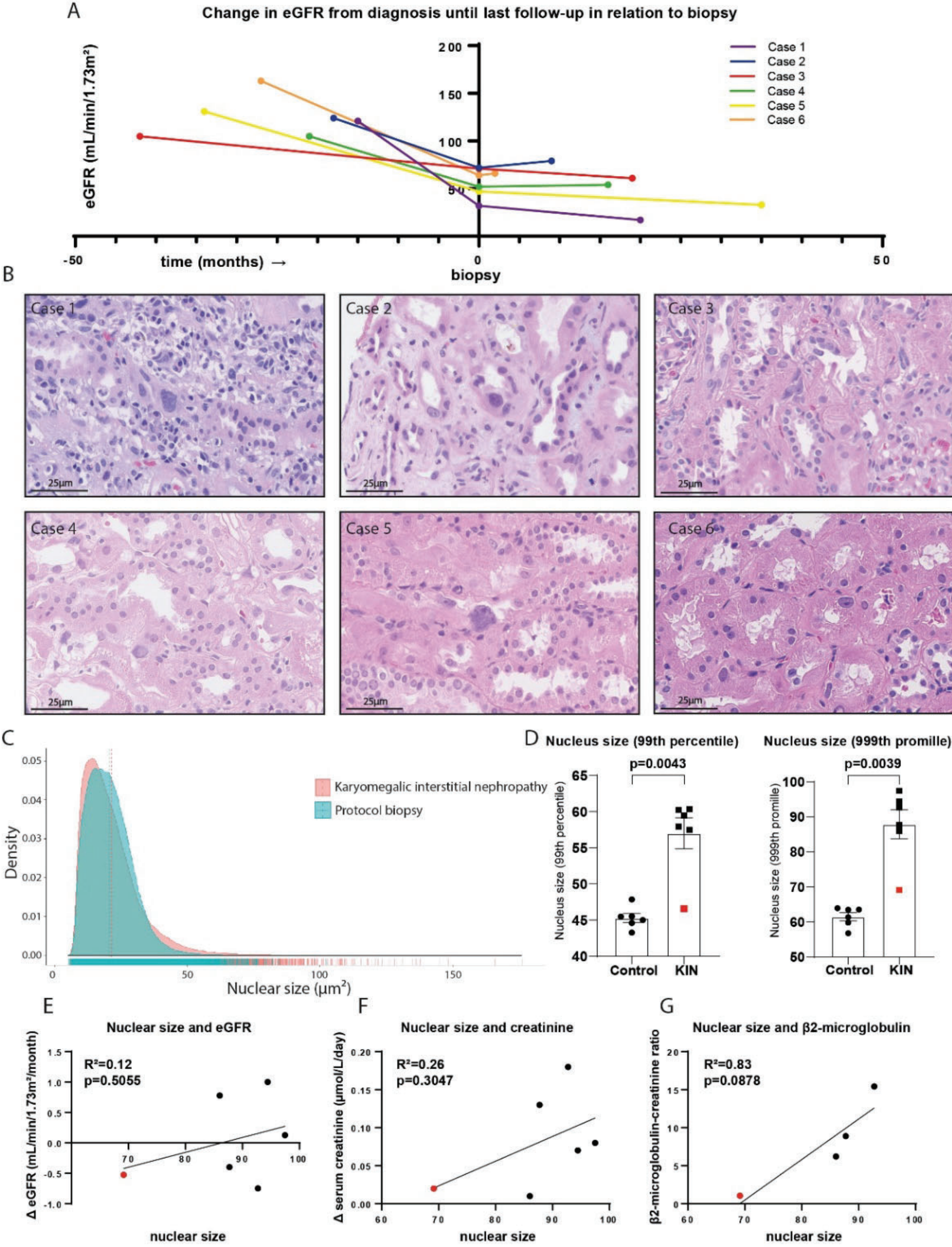

Figure 1. Kidney function decline and nuclear size
(A) Change in eGFR from diagnosis of the malignancy (first point in time) to biopsy (time=0), to last moment of follow-up (last point in time). All six patients had a decline in eGFR from moment of the diagnosis of malignancy until the last moment of follow-up (last point). After biopsy three patients showed progressive decline of eGFR, while the other three patients showed improvement of eGFR during follow-up. **(B)** Representative pictures of the H&E stain for all six biopsies. The six biopsies showed a variation in inflammation and nuclear size. On visual observation the nuclei ranged from bizarre, irregularly enlarged nuclei to almost inconspicuously enlarged nuclei. **(C)** Comparison of the nuclear size distribution of the KIN biopsies with protocol kidney graft biopsies (aggregate of 6 biopsies per group). The dotted vertical line indicates the mean, which is comparable for both groups. The small lines on the X-axis represent single nuclei. **(D)** Comparison of the 99th percentile and 999th promille of nuclear size in individual KIN biopsies and protocol kidney graft biopsies. Although a significant difference is seen when comparing the 99th percentile of nuclear size between the two groups, one of the biopsies in the KIN groups (case 3, indicated with the red dot) shows remarkable overlap with the control group. When focusing on an even smaller subpopulation of nuclei (999th promille) the overlap is lost. **(E, F, G)** Scatter diagram with the best-fit line of nuclear size (999th promille) in relation to kidney function and urinary β2-microglobulin/creatinine ratio. No correlation or trend is seen between the size of the 999th percentile of nuclei and change in eGFR or serum creatinine (E, R^2=0.12, p=0.5055; F, R^2=0.26, p=0.3047). A non-significant trend is seen between nuclear size (999th promille) and urinary β2-microglobulin/creatinine ratio (for two patients no urinary β2-microglobulin/creatinine ratio was available at time of biopsy)(G, R^2=0.83, p=0.0878). In all graphs the red dot represents the KIN biopsy with the smallest average nuclear size in the 99th and 999th promille.

Features of cellular senescence in and beyond karyomegalic cells

Cellular senescence has been implicated in KIN caused by impaired DNA damage response due to a FAN1 mutation. (Zhou et al., 2012) We therefore looked for cellular senescence features in the patient kidney biopsies. We found that most of the enlarged nuclei in the six biopsies were Ki67 negative and p21 positive, consistent with cell cycle arrest, the key feature of cellular senescence (Figure 2A). (Knoppert et al., 2019) In addition, several enlarged nuclei were yH2AX positive, indicating unresolved double-strand DNA breaks (Figure 2E), and showed decreased expression of the nuclear envelope protein Lamin B1 (Figure 2F), both indicative of cellular senescence. (Knoppert et al., 2019; Kuo & Yang, 2008) Of note, KIN biopsies contained more p21 positive cortical cells compared to protocol biopsies (p=0.0022; Figure 2B-D), showing that many morphologically normal appearing TECs were senescent, or at least cell cycle arrested (Figure 2B), possibly contributing to function loss.

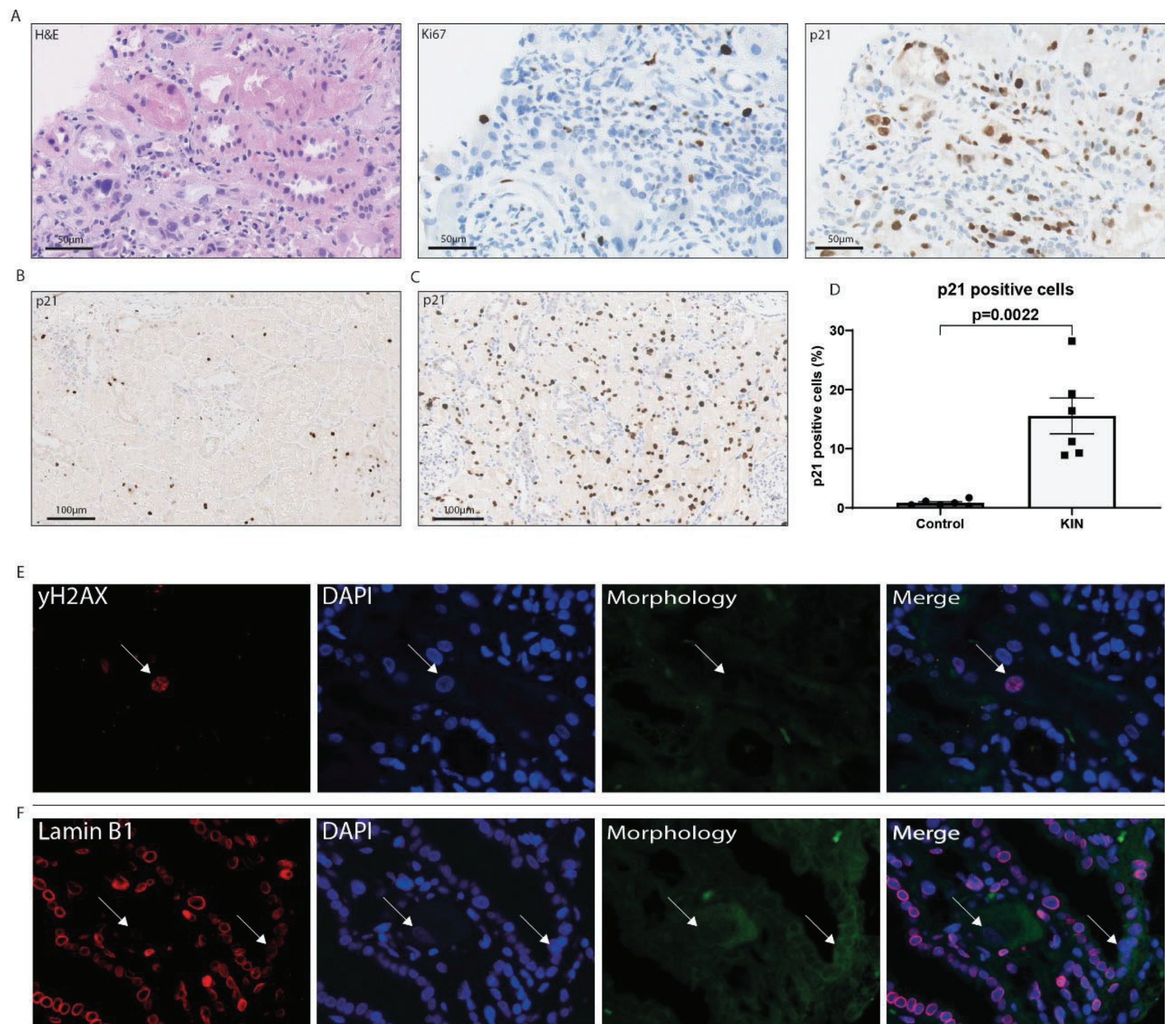

Figure 2. Senescence phenotype in karyomegalic interstitial nephropathy
(A) Representative pictures of H&E, Ki67 and p21 stains in a KIN biopsy. The enlarged nuclei visible in the H&E stain are negative for Ki67 and positive for p21. **(B, C, D)** Representative pictures and quantification of p21 positive cells in (B) protocol kidney graft biopsies and (C) KIN biopsies. KIN biopsies showed significantly more p21 positive cells compared to protocol kidney graft biopsies (D, p=0.0022). **(E)** Representative image of yH2AX positive cells in KIN biopsies. Some, but not all of the enlarged nuclei in the KIN biopsies showed yH2AX foci, indicating unresolved double stranded DNA breaks. **(F)** Representative image of nuclear Lamin B1 stain in KIN biopsies. Lamin B1 loss was present in both enlarged and regular sized nuclei.

Correlation of P21 positivity with LMW proteinuria and kidney function

The percentage of p21 positive cortical cells correlated with the increase of serum creatinine (R^2=0.83, p=0.0119), but not with eGFR decline (R^2=0.43, p=0.1578) (Figure 3A-B). Remarkably, however, p21 strongly correlated with eGFR decline when case 3 (with the least enlarged nuclei) was excluded (R^2=0.93, p=0.0079), and the percentage of p21 positive cortical cells also correlated with the urine β2-microglobulin/creatinine ratio available for four of the six patients (Figure 3C, R^2=0.93, p=0.0377).Staining for lysozyme as a marker for tubular reabsorption was reduced in p21 positive cells (Figure 3D), and there was a trend towards inverse association between percentage of cortical p21 positive cells and lysozyme staining (R^2=0.63, p=0.0586) (Figure 3E). Lysozyme staining also showed a trend towards negative correlation with the urinary β2-microglobulin/creatinine ratio (Figure 3F, R^2=0.88, p=0.0596).

Together, these findings suggest that dysfunctional TECs with senescence(-like) features may be responsible for both LMW proteinuria, and, via tubular-glomerular feedback, also loss of GFR. Urinary β2-microglobulin/creatinine ratios were not related to interstitial fibrosis (R^2=0.01, p=0.9115, data not shown).

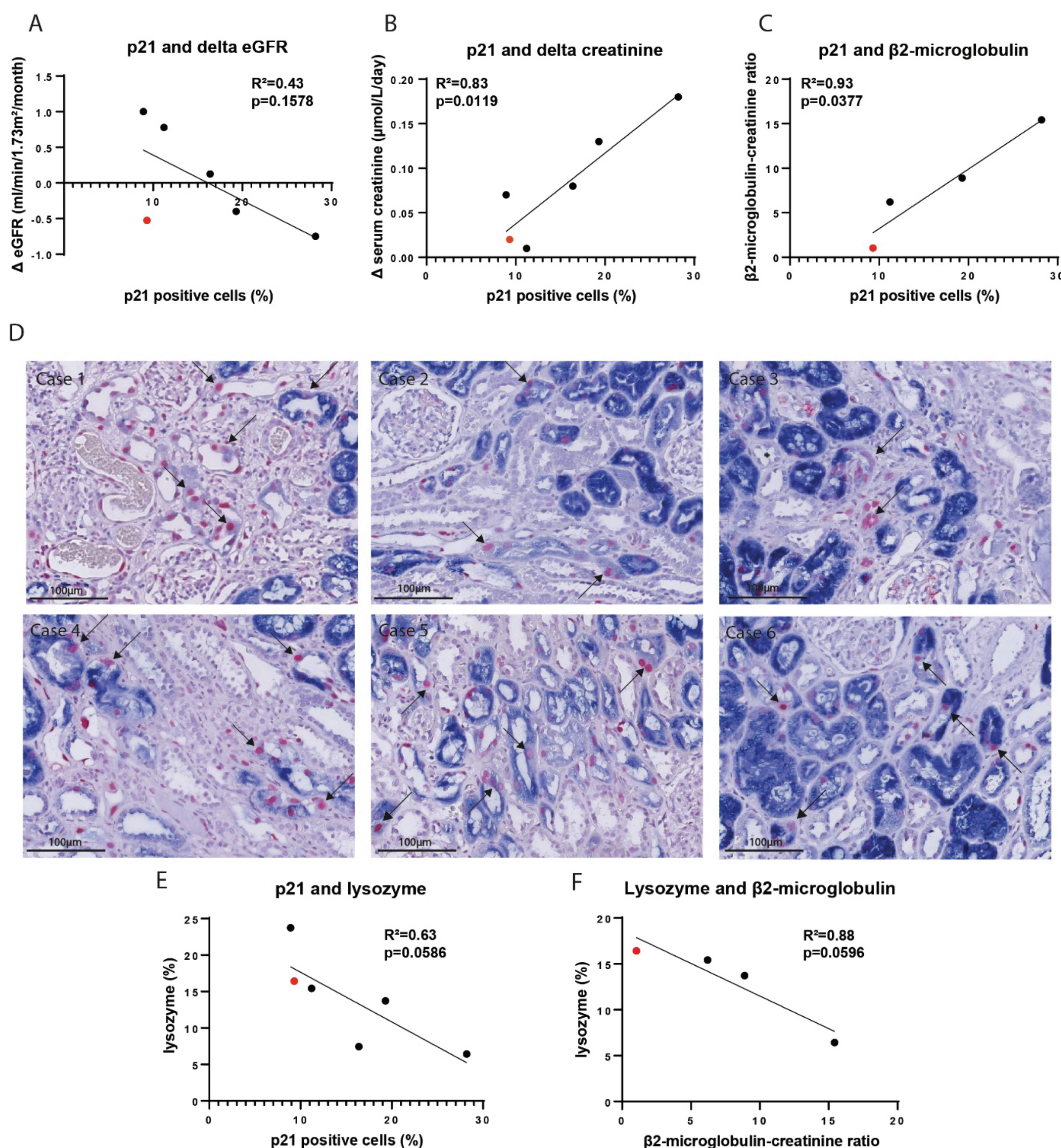

Figure 3. p21 expression in relation to kidney function and tubular reabsorption.
(A, B, C) Scatter diagram with the best-fit line of p21 positive cell percentage in relation to kidney function and urinary β2-microglobulin/creatinine ratio. The red dot indicates the biopsy with the lowest 999th promille in nuclear size. **(A)** p21 and change in eGFR show a non-significant negative trend (A, R^2=0.43, p=0.1578) but when the biopsy with the smallest nuclei is excluded a clear correlation is present between p21 expression and a decrease in kidney function (R^2=0.93, p=0.0079). **(B)** p21 and change in serum creatinine show a positive correlation (R^2=0.83, p=0.0119) in the KIN biopsies. This correlation is maintained when to biopsy with the smallest 999th promille of nuclear size is excluded (R^2=0.79, p=0.0429). **(C)** p21 and β2-microglobulin/creatinine ratio show a positive correlation (R^2=0.93, p=0.0377), although this correlation is lost when the biopsy with the smallest 999th promille of nuclear size is excluded (R^2=0.96, p=0.1332). For two patients no urinary β2-microglobulin/creatinine ratio was available at time of biopsy. **(D)** Double stain for p21 and lysozyme. Tubules and single cells expressing p21 showed reduced staining for lysozyme. **(E)** Scatter diagram with the best-fit line of the percentage of p21 positive cells in relation to percentage of lysozyme positive surface area in the biopsy cortex. Lysozyme and p21 show a non-significant negative trend (R^2=0.63, p=0.0586), the p-value increases when the biopsy with the smallest 999th promille of nuclear size is excluded (R^2=0.64, p=0.1047). **(F)** Scatter diagram with the best-fit line of the relation between β2-microglobulin/creatinine ratio and lysozyme positive surface area (R^2=0.88, p=0.0596), the p-value and R^2 increases when the biopsy with the smallest 999th promille of nuclear size is excluded (R^2=0.99, p=0.0685). For two patients no urinary β2-microglobulin/creatinine ratio was available at time of biopsy.

DISCUSSION

The major finding of this study is that tubular dysfunction associated with features of cellular senescence in TECs appears to be an important aspect of early CKD development in childhood cancer patients. This phenotype has been described as KIN. (Burry, 1974; Godin et al., 1996; Mihatsch et al., 1979) With morphometry and p21 staining, we could readily diagnose this condition in kidney biopsies from all six consecutive pediatric oncology patients treated with ifosfamide and presenting with polyuria, LMW proteinuria, and with loss of GFR.

KIN has been linked to FAN1 mutations, suggesting a role for defective DNA damage repair and ensuing cellular senescence, but this hypothesis has not been substantiated to date.(Zhou et al., 2012) Several studies have reported KIN in patients treated for childhood cancer as a rare event related to ifosfamide treatment, a nephrotoxic drug that is known to cause tubulopathy, reduced glomerular filtration and renal Fanconi syndrome, but details on clinical features and pathology are lacking. (Loebstein et al., 1999; Matsuura et al., 2014; McCulloch et al., 2011; Jaysurya et al., 2016; R. Skinner et al., 2000; Skinner et al., 2010; Stöhr et al., 2007) Despite the small sample size, our observations confirm that tubular injury after ifosfamide therapy is responsible for the clinical picture and suggest that KIN and cellular senescence should be considered as underlying cause of early CKD in childhood cancer patients.

Because all patients received additional nephrotoxic therapies, including other chemotherapeutic agents and radiotherapy to the kidney region (limited to four patients), kidney damage may not be attributable to ifosfamide alone. Nevertheless, tubular karyomegaly, the most specific feature of KIN, has thus far only been reported after ifosfamide and cyclophosphamide therapy, and not for other chemotherapeutic agents or radiotherapy. (Matsuura et al., 2014; McCulloch et al., 2011; Jaysurya et al., 2016; Radha et al., 2014)

All six biopsies in the current study contained TECs with large, irregular and hyperchromatic nuclei. Tubulointerstitial fibrosis was observed in four patients, of whom three had little (<5%) fibrosis and only one patient had prominent (20%) fibrosis. Only two biopsies (including the one with prominent fibrosis) also showed mild to moderate interstitial inflammatory infiltrate. Previous reports of ifosfamide induced KIN cases reported moderate to severe fibrosis with a mild to severe lymphocytic infiltrate. (Matsuura et al., 2014; McCulloch et al., 2011; Jaysurya et al., 2016) Although KIN is classically referred to as interstitial nephritis (Mihatsch et al., 1979), the (near) absence of inflammation in our cases suggests that the abbreviation “KIN” might better be used to refer to nephropathy, rather than nephritis.

The karyomegalic TECs in all six biopsies had senescence features, including unresolved DNA damage, cell cycle arrest and nuclear lamina alterations. Strikingly, these features were also observed in far larger numbers of morphologically normal cells. Senescence is a prolonged anti-proliferative cell state that is typically induced when DNA repair and apoptosis fail, and is associated with maladaptive repair and progressive fibrosis in CKD. (Docherty et al., 2019; Hernandez-segura et al., 2018; Sharpless & Sherr, 2015) Defective DNA damage repair was previously linked to genetic KIN caused by FAN1 mutations. (Zhou et al., 2012) Furthermore, high rates of DNA damage has been observed in pediatric oncology patients treated with ifosfamide, and low rates of cellular proliferation (Willits et al., 2005), indicative of cell cycle arrest are observed in ifosfamide induced KIN. (Matsuura et al., 2014; McCulloch et al., 2011) Our data underscore that senescence is a central mechanism in KIN. Furthermore, these senescent cells can contribute to progressive loss of kidney function through senescence-associated secretory phenotype (SASP) factors that drive progressive tubulointerstitial fibrosis. (Wang et al., 2017)

Although KIN is considered a rare condition, it may be a more prevalent cause of reduced kidney function among childhood cancer patients than previously suggested. Of note, our study only refers to children in which a biopsy was performed because of kidney function decline of unknown cause was detected. However, development of some degree of (even subclinical) kidney dysfunction is not a rare event in childhood cancer patients, but can easily be overlooked. (Calderon-Margalit et al., 2021; Green et al., 2021; Kooijmans et al.,

2019) Subtle changes in proteinuria are frequently not noticed in standard urinalysis second to diluted urine, a frequent phenomenon in tubulopathy. Hence, KIN/cellular senescence might be even more prevalent than thus far realized. Therefore, we would suggest that standard urinalysis, protein to creatinine ratio, and LMW protein detection, urinary glucose and phosphate should be pursued as a standard of care in high risk patients to optimize surveillance of kidney toxicity. (Levin et al., 2013)

The importance of recognition of KIN as a separate entity in children suspected of CKD, early after childhood cancer treatment, is the feasibility of early intervention to avoid later sequelae. The use of steroids may have prevented further kidney function decline in one of our patients, but steroids were only administered in two patients with interstitial inflammation. Although alternative therapies are currently not available, our data may provide perspectives for treatment of KIN with novel, so called “senomorphic” and “senolytic” therapies. (Knoppert et al., 2019)

In conclusion, cellular senescence due to unresolved DNA damage might be the pathogenesis of KIN. KIN can develop during or after childhood cancer treatment. This urges the implementation of surveillance of kidney function, as standard of care, as early recognition of this condition may benefit from the development of novel interventions targeting senescent cells.

Table 1: baseline and follow up characteristics cases

Case #	1	2	3	4	5	6
Age diagnosis (yr)	3.4	3.2	12.6	16.2	11.3	4.2
Gender	Male	Male	Male	Male	Male	Female
Diagnosis	NBL Adrenal gland Stage IV	eRMS Maxilla Localized disease	Ewing Fibula Localized disease	Ewing 2nd rib + Metastatic disease	Ewing 12th Rib Localized disease	Wilms Left kidney Stage IV
eGFR (ml/min/1.73m2) at diagnosis	121	124	105	105	131	163
Referral nephrology (months after diagnosis)	7	12	28	19	32	21
eGFR (ml/min/1.73m2) at referral	59	89	64	54	48	82
Steroid treatment	yes	no	yes	no	no	no
FU time (months after diagnosis)	35	27	61	37	69	22
eGFR (ml/min/1.73m2) at last FU	17	79	61	54	33	66
Urine PCR FU (mg/mg)	2.63	0.80	0.57	1.70	1.19	1.98
B2MG/cr ratio FU (mg/mg)	0.093	0.003	0.030	0.099	0.047	0.162
Cumulative dose Ifosfamide (g/m^2)	22.5	54.0	100.0	54.0	80.0	12.0
Cumulative dose cisplatin (g/m^2)	0.5	0	0	0	0	0
Cumulative dose cyclophosphamide (g/m^2)	0	4.1	0	10.7	0	4.4
Radiotherapy	Abdominal	Maxilla	None	2nd rib + Proximal humerus vertebra T11 vertebra S1 costa 10	12th rib	Initial therapy Flank Left, including inferior vena cava Re-irradiation Whole abdomen
Mean kidney dose (Gray)	LK 13 Gy RK 3 Gy	0	0	LK 0.2 Gy RK 0.2 Gy	LK 7 Gy RK 26 Gy	LK n.a. RK 1 Gy + 9 Gy at re-irradiation
Histological findings						
Glomerulosclerosis	15%	3%	7%	None	None	None
IF/TA	20%	<5%	<5%	<5%	None	None
Inflammation	Moderate	None	Mild	None	None	None

NBL: neuroblastoma; eRMS: embryonal rhabdomyosarcoma; Ewing: Ewing sarcoma; Wilms: Wilms tumor; eGFR: estimated GFR by bedside Schwartz equation in ml/min/1.73m^2; PCR: protein to creatinine ratio (mg/mg); RK right kidney LK left kidney FU: follow-up visit; B2MG/cr: beta-2-microglobulin to creatinine ratio (normal range < 0.00035 mg/mg) IF/TA: interstitial fibrosis and tubular atrophy.

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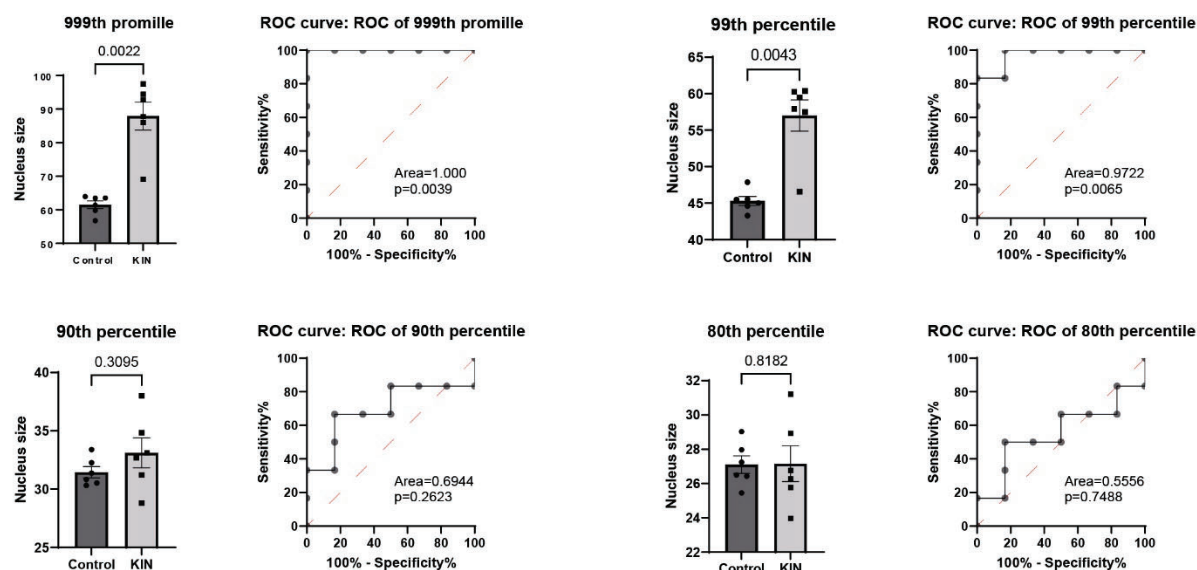
SUPPLEMENTARY MATERIAL



Supplemental figure 1. Different percentiles of nuclear size in protocol transplant biopsies compared with KIN biopsies with corresponding ROC-curves
Differences in nuclear size become apparent when the largest 0,1% - 1% of nuclear size are compared. When looking at a larger percentile the difference is lost.

Supplementary Results. More detailed case description

Case 1

Case 1 was diagnosed at the age of 3 years with a neuroblastoma (N-myc positive) originating from the left adrenal gland with metastatic lesions of the bone (stage IV). Kidney function at the time of diagnosis was normal. He was treated according to the NBL-2009 High Risk protocol and received chemotherapy including cycles of cisplatinum, etoposide, vindesin and dacarbazine, doxorubicin, ifosfamide, vincristine. After tumor resection, he received high-dose chemotherapy (Busalphan/Melphalan) with autologous stem cell reinfusion followed by loco-regional radiotherapy. During oncologic treatment several episodes of acute kidney injury were observed, second to chemotherapeutic treatment or sepsis. Kidney function gradually declined to 59 ml/min/1.73m2 during oncologic treatment, and was accompanied by hypokalemia, nephrotic range proteinuria, both glomerular and LMW proteinuria.

Kidney biopsy (at age 5) showed moderate chronic injury with approximately 15% of glomeruli showing global glomerulosclerosis and 20% interstitial fibrosis and tubular atrophy. The biopsy was remarkable for the presence of many tubular epithelial cells with enlarged, hyperchromatic nuclei with irregular outlines (figure 1B). There was slight interstitial edema with a dispersed moderate lymphocytic infiltrate. IF for immunoglobulin heavy and light chains and for complement factors C1q and C3c were negative. Also EM showed no deposits or other diagnostic abnormalities. The biopsy was signed out as "consistent with KIN".

As the biopsy also showed inflammation, steroid treatment was initiated by which, however, kidney function did not improve. Proteinuria did not respond to ACEi treatment, consistent with tubular dysfunction as a cause, as reflected also by a large proportion of urinary LMW proteins, and was discontinued. Over the 28 months after referral to the nephrology clinic, CKD progressed to stage 4 (eGFR 17 ml/min/1.73m2) and work up for kidney replacement therapy was initiated.

Case 2

Case 2 was a patient diagnosed with an embryonal rhabdomyosarcoma of the left maxillary sinus at the age of 3 years. Kidney function at time of diagnosis was normal (eGFR: 121 ml/min/1.73m2). He was treated according to the EPSSG RMS 2005 protocol, including IVA (ifosfamide, vincristine, actinomycine D), and VC (vinorelbine, cyclophosphamide), and brachytherapy after tumor resection. He was referred to the nephrology outpatient clinic because of kidney function decline (eGFR 89 ml/min/1.73m2) with proteinuria during his oncologic treatment, i.e. 12 months after primary diagnosis.

Kidney biopsy showed minimal chronic injury with approximately 3% of glomeruli showing global glomerulosclerosis. The remaining glomeruli appeared normal by light microscopy and IF. EM was not performed. There was less than 5% of interstitial fibrosis and tubular atrophy. A small minority of the tubular epithelial cells showed slightly enlarged hyperchromatic nuclei with irregularity of the nuclear outlines (figure 1B). There were no signs of inflammation The conclusion of the pathology report of the biopsy was “consistent with KIN”.

No treatment was installed. Two years after diagnosis of KIN, eGFR has stabilized at 79 ml/min/1.73m2 while LMW proteinuria persisted.

Case 3

Case 3 was diagnosed with Ewing sarcoma of the left fibula at the age of 12 years. Kidney function at time of diagnosis was normal (eGFR:105 ml/min/1.73m2). Treatment according to the EWING-2008 protocol was started including VIDE (vincristine, ifosfamide, doxorubicin, etoposide) and VAI (vincristine actinomycine D and ifosfamide) schedules. Surgery without radiotherapy was applied to obtain local disease control. At the age of 15, he was referred to the outpatient clinic of pediatric nephrology with LMW proteinuria and a declining kidney function (eGFR 64 ml/min/1.73m2).

Kidney biopsy revealed mild chronic injury with approximately 7% globally sclerosed glomeruli and less than 5% interstitial fibrosis. There were no signs of glomerular abnormalities, and immunofluorescence and electron microscopy were not performed. However, scattered tubular epithelial cells with enlarged hyperchromatic nuclei and irregular nuclear outlines were noted (Figure 1B). There was a mild diffuse lymfocytic infiltrate in the interstitium. The pathological result of the biopsy concluded that this was “consistent with KIN”.

Because of lymphocyte infiltration (with limited interstitial fibrosis and tubular atrophy), oral steroid treatment was initiated which resulted in stabilization of kidney function over time (eGFR of 61 ml/min/1.73m2 33 months after referral to nephrology clinic). LMW proteinuria however remained unchanged/stable.

Case 4

Case 4 was diagnosed at age 16 years with a Ewing sarcoma of the 2nd rib and metastatic lesions to the bone/bone marrow (proximal humerus, vertebrae T11, S1 and costa 10) . Kidney function at the time of diagnosis was normal (105 ml/min/1.73m2). He started the EWING-2008 protocol with VIDE, VAC and VC treatments, and radiotherapy to the primary tumor and metastatic sites at presentation). His kidney function declined towards 54 ml/min/1.73m2 with LMW proteinuria, 6 months after high-dose Threosulphan/Melphalan with autologous stem cell reinfusion.

Kidney biopsy revealed minimal chronic injury with less than 5% interstitial fibrosis and no globally sclerosed glomeruli. There were no signs of glomerular abnormalities. Immunofluorescence and electron microscopy were not performed. However, throughout the biopsy, multiple tubular epithelial cells were identified, with enlarged, hyperchromatic nuclei and irregular nuclear outlines (figure 1B). There were no signs of acute tubular injury or interstitial infiltration. The biopsy result was “consistent with KIN”.

No additional treatment was initiated and kidney function stabilized up to end of follow-up 37 months after initial diagnosis. LMW proteinuria did not improve over time.

Case 5

Case 5 was diagnosed at age 11 years with a Ewing sarcoma arising from costa 12 with extension in the abdomen and neuro-foramen. Kidney function at the time of diagnosis was normal(eGFR:131 ml/min/1.73m2). He was treated according to the EWING-2008 protocol, including VIDE and VAI cycles. As the tumor was unresectable primary radiotherapy was applied. Kidney function declined during chemotherapy towards an eGFR of 48 ml/min/1.73m2 accompanied by LMW proteinuria.

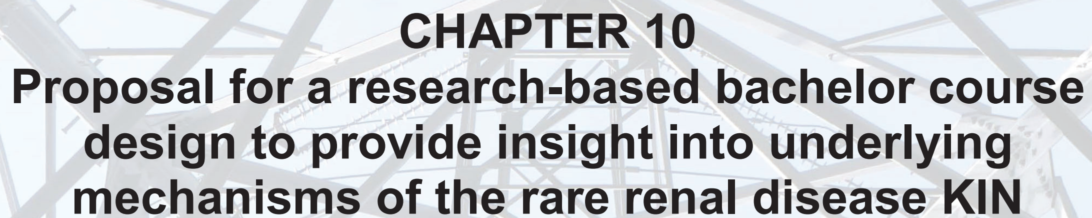
Kidney biopsy showed no chronic injury (no glomerulosclerosis, interstitial fibrosis or tubular atrophy), but a small minority of the tubular epithelial cells had enlarged hyperchromatic nuclei with slightly irregular nuclear outlines (figure 1B). There were no signs of acute tubular injury or interstitial infiltration. Immunofluorescence was negative. The biopsy report was “consistent with KIN”.

Despite renoprotective supportive care with ACEi, kidney function continued to decrease (eGFR 33 ml/min/1.73m2 at latest visit, 3 years after initial malignancy diagnosis) with persistent LMW proteinuria and normal blood pressure. No steroids were prescribed.

Case 6

Case 6 was a 4 year old girl with an intermediate risk Wilms tumor of the left kidney and lung metastases (stage IV; loco-regional stage III) who was treated preoperative with three vincristine/actinomycin courses, and three vincristin/actinomycin/doxorubicin courses. Nephrectomy followed by radiotherapy to the flank and inferior renal vein, and post-operative nine vincristin/actinomycin courses were given. After 10 months an abdominal recurrence was observed, and treated with two ICE (ifosfamide/carboplatin/etoposide) and two CCE (cyclophosphamide/carboplatin/etoposide) courses followed by high dose Melphalan and autologous stem cell reinfusion, and whole abdominal radiotherapy. One year later she presented at the pediatric nephrology outpatient clinic with compromised kidney function (eGFR 82 ml/min/1.73m2) and LMW proteinuria.

Kidney biopsy was postponed because of a single functioning kidney, but with further progression of compromised kidney function and reluctance to start steroids as a proof of principle we decided to biopsy. The histological evaluation showed no chronic injury (no glomerulosclerosis, interstitial fibrosis or tubular atrophy), and on routine examination initially no apparent abnormalities were noted, except only some very subtle enlargement of tubular nuclei, and was considered non-conclusive. She did not receive steroid treatment. Follow-up time was too short to describe in this article.



CHAPTER 10
Proposal for a research-based bachelor course design to provide insight into underlying mechanisms of the rare renal disease KIN

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Karyomegalic interstitial nephropathy

Karyomegalic interstitial nephropathy (KIN) is a rare but severe fibrotic kidney disease with renal tubular dysfunction of unknown origin. Histology shows atypical tubular epithelial cells (TECs) with large abnormal hyperchromatic nuclei with irregular outlines. KIN affects <1 per million people worldwide. (*Orphanet*) It is an orphan disease with lack of support and resources for discovering treatments for it. Therefore, understanding of the pathophysiology is limited and causative treatment options are currently lacking.

Clinical presentation

KIN usually presents as a slowly progressive chronic kidney disease (CKD), eventually leading to end-stage renal disease before the age of 50 years. (Bhandari et al., 2002) KIN patients usually present in the second or third decade of life with asymptomatic and progressive signs of CKD, including mild to moderate renal dysfunction, mild proteinuria and glucosuria, and less than one-third present with hematuria. (Monga et al., 2006) Extrarenal features are absent or mild. Almost half of the patients display a past medical history of recurrent upper respiratory tract infections and abnormal liver function tests. (Monga et al., 2006; Bhandari et al., 2002) Paramount to the diagnosis of KIN is a renal biopsy showing karyomegalic tubular epithelial cells.

Pathology

Histologically, KIN is characterized by severe chronic interstitial fibrosis and tubular changes, associated with glomerulosclerosis and vascular lesions. The presence of karyomegalic tubular epithelial cells, lining the proximal and distal tubules, and characterized by markedly enlarged and hyperchromatic nuclei, represents the disease hallmark that makes it distinguishable from other common causes of chronic tubulointerstitial nephritis. (Bhandari et al., 2002) Consistently, karyomegaly has been described in the liver and lung, but also in other organs, including the brain, skin, and digestive tissues. (Monga et al., 2006; Mihatsch et al., 1979) Nevertheless, and in sharp contrast to the kidney, extrarenal karyomegaly is usually associated with only subtle clinical and biological changes. Karyomegalic cells can also be detected in urine samples. (Monga et al., 2006)

Etiology

KIN was described for the first time over 40 years ago as a hereditary kidney disease. (Burry 1974; Mihatsch et al., 1979) Ever since, KIN was thought to be a hereditary disorder because almost half of the patients had a familial history of nephropathy. Recently, KIN has been linked to mutations in the FAN1 (FANCD2/FANCI-Associated Nuclease 1) gene, a gene involved in the DNA damage response (DDR) pathway, devoted to repair of DNA interstrand crosslink damage. (Knipscheer et al., 2009; Zhou et al., 2012) To date, 12 families with an autosomal recessive inheritance have been reported with FAN1 mutations. (Palmer et al., 2007; Baba et al., 2006)

The past decades, similar KIN lesions were described in long-term survivors of malignancies treated with the alkylating agent ifosfamide (IFO). (Jayasurya et al., 2016; Matsuura et al., 2014; McCulloch et al., 2011) IFO is an oxazaphosphorine chemotherapeutic agent that is widely used in the treatment of solid tumours. It is a recognised nephrotoxic drug, causing tubulopathy, reduced glomerular filtration and renal Fanconi syndrome, but rarely causes end-stage renal failure. (Loebstein et al., 1999; Skinner et al., 2000, 2010; Stöhr et al., 2007) Chloroacetaldehyde, a metabolite of IFO, acts by depleting glutathione and thus making cells vulnerable to oxidative stress. The karyomegaly may be secondary to IFO-induced tubular damage.

Furthermore, renal karyomegaly may also be related to alternative causes, including viral infections, immunosuppressive therapy such as alkylating agents, and exposure to heavy metals and mycotoxins, particularly ochratoxin A. (McCulloch et al., 2011; Hassen et al., 2004) It is postulated that KIN results from defective cell division due to multiple events including exposure to toxins, viruses, or genetic predisposition.

New insights in KIN pathophysiology

Clinic

Response to corticosteroid treatment with improvement in kidney function has been noted in some, but not all of the published KIN case studies. (Jayasurya et al., 2016; Matsuura et al., 2014) Why some KIN patients do respond to corticosteroid treatment in spite of signs of irreversible chronic histological damage, and why functional decline is progressive in other patients remains unclear.
At the pediatric Nephrology department of our center, different responses are seen after treatment of IFO-induced KIN patients with corticosteroids, ranging from initial stabilization of kidney function to progressive decline of kidney function and persistent proteinuria. (Knoppert et al., 2021)

Patho(physio)logy

Previously, trends for reduced cellular proliferation of TECs (marked by lower Ki-67-positive TEC numbers), an essential process for tubular regeneration upon AKI, were observed in KIN biopsies of IFO-treated patients compared to AKI biopsies. (Jayasurya et al., 2016; McCulloch et al., 2011) Furthermore, experimental studies revealed a role for DNA damage response signaling in KIN. (Zhou et al., 2012)
Recently, at the UMCU, immunohistochemical staining of kidney biopsies from childhood cancer survivors with proven KIN after receiving IFO treatment, revealed a pronounced cellular senescence phenotype, including unresolved DNA damage (yH2AX positive foci), cell cycle arrest (marked by limited Ki67 expression and positive p21 expression) and decreased expression of the nuclear envelope marker Lamin B1 of mainly proximal tubular epithelial cells. (Knoppert SN, 2021) Furthermore, senescent cell marker expression was not only observed in enlarged nuclei that are typical for KIN, but also in normal appearing tubular epithelial cells. In line with these observations, it can be postulated that IFO-induced tubular DNA damage prevents the normal cellular regenerative process resulting in senescent TEC with karyomegaly and a senescent microenvironment driving renal fibrosis and progressive functional decline.

Current challenges / important research questions

With increased survival from paediatric cancer, KIN due to ifosfamide treatment may become an increasing prevalent complication. Understanding KIN pathophysiology is therefore of utmost importance to limit renal complications of childhood cancer treatment.

Important questions that need to be addressed include:
- Why do KIN patients develop CKD at a young age even though the initial damage trigger (ifosfamide therapy) is gone?
- How can KIN patients be treated to limit/prevent CKD progression?
- Why do some KIN patients respond to corticosteroid treatment while others do not?
- Is there a genetic predisposition for IFO-induced KIN patients to develop the KIN phenotype?

We here propose to bring in these burning research questions into an innovative translational medicine- and research-based education concepts to study KIN.

Innovative Translational medicine- and research-based education concepts to study KIN

Orphan diseases like KIN represent a pressing medical need, but face financial problems because they are often not prioritized by medical centers nor industry for gaining support and resources for discovering treatments. Translational medicine (TM) an interdisciplinary branch of biomedicine that seeks to bridge the gap between (fundamental) biomedical research and patients by improving the process of bringing lab discoveries to the benefit of patients, plays an important role in rare disease innovation, discovery and drug development. (Cohrs et al., 2015; Albani & Prakken, 2009) A novel approach to stimulate optimal interplay between

different disciplines, resources, expertise and techniques in biomedicine, and thereby foster TM, are interdisciplinary laboratory courses. (Drost et al., 2019) Herein, students collaborate with scientists and doctors hands-on in a laboratory on a real patient case that is introduced by the faculty and includes patient participation. Financial hurdles are overcome, because expanses are covered by the University and patient societies contribute to the bench-fee.

For this, the 'Bachelor Research Hub' and a novel education concept embedded into the key principles of research-based learning and TM, were created (www.bachelorresearchhub.com). The Bachelor Research Hub is a well-equipped physical laboratory, centrally positioned within the UMC Utrecht, where students perform research together with researchers, doctors, physician-researchers and in which patients (associations) are also involved. Students work on cases of patients with unknown conditions or on a disease where good diagnostics or treatment are not available. In organized courses (of 15 ECs), students work together in subgroups on the same problem approaching it from different disciplinary perspectives, resulting in a joint end product. Students come together from the Biomedical Sciences and Medicine programmes. The courses include the Bachelor Research Lab (BRL) course and the dual course continuum Pathology and Experimental Translational Medicine (ETM).

BRL course

The Bachelor Resrach Lab (BRL) course is a full-time 15 ECTS elective course positioned in the second or third period of the third year of the undergraduate program Biomedical Sciences. Four groups of students work together in a dedicated laboratory on an actual ongoing research problem of a faculty member. Faculty will introduce the subject and their main hypothesis through a plenary interdisciplinary session with patients, patient societies, treating medical doctors, researchers and industry representatives. All groups work on their own subhypotheses, which will be executed from different methodological angles. Students will propose the research, execute the experiments, and collectively report in a single research article. The students are supervised by experienced researchers that are facilitated by the Bachelor Research Hub.

Pathology and ETM course

This course design covers the entire research cycle, from designing unique research proposals in the Pathology course to executing the best selected proposal in the subsequent ETM course. (Drost et al., 2019)

In the Pathology course, a real-world clinical problem is introduced to the students in a plenary session with a patient suffering from unknown disease or disease with inadequate diagnosis/treatment options, treating medical doctors from distinct disciplines, researchers and the funding patient society. All these players (including patients) present their stories (personal experiences, patient file, state-of-the art research) in 15 min each, ending with posing the challenge to the students. Next, divided into groups of 4-6, students start to critically think about and to write an empiric research proposal to potentially uncover the cause of the patient's disease, or to improve diagnostics or therapies. Each proposal consists of feasible experiments that are doable within a well-equipped research laboratory in a timespan of several weeks. All proposals are peer-reviewed and ranked by both fellow students and faculty based on consensus. Students present their work via oral or poster presentations in a real-world symposium setting in the presence of all stakeholders.

In the ETM course, the best and most achievable proposal is selected and submitted to the patient society that might be willing to contribute to research costs. The selected proposal will be executed hands-on by 4 groups of 4 students in a well-equipped research lab during the ETM course. The students form interdisciplinary groups, consisting of 2 biomedical sciences students and 2 medical students. The students are supervised in the lab by experienced researchers and are in close connection to ongoing research, clinicians, and researchers. Students report progress on their research project in lab journals and report preliminary results to supervising researchers, clinicians and patients. Students report their

work in the form of a mini-scientific report and they present their work via oral presentations in a real-world symposium setting in the presence of all stakeholders.

Methods and stakeholders

Bachelor (bio)medical students, doctors and scientists from the pediatric nephrology, genetics and pathology departments and supervisors from the Bachelor Research Hub, will join forces to study KIN, with the help of KIN patients and representatives from patient societies. Educational evaluations will be conducted by educational scientists from the Bachelor Research Hub. The participating parties are displayed in table 1.

Table 1. Participating parties

Stakeholders	
Bachelor students	Biomedical sciences students
	Medical students
KIN experts	Pediatric Nephrologist
	Pathologist
	Clinical geneticist
	PhD candidate
Bachelor Research Hub supervisors	Researchers/research technicians
Patient societies	Kidney patients foundation
	Cancer patients foundation
Educational experts	Educational scientists

Research tools UMCU

In the fully equipped Bachelor Research Hub students can work with:
-Patient material: frozen and paraffin embedded kidney biopsies of several acute and chronic kidney disease, including IFO-induced KIN patients
-Renal cell cultures, including genetic transformation tools and/or therapeutic intervention testing
-Mouse material: frozen and paraffin embedded kidney tissue of several acute and chronic kidney injury models
-Basic lab techniques including genomics, transcriptomics and proteomic technologies

Outcomes and course evaluation

Research

Ifosfamide therapy and FAN1 mutations cause KIN, a rare but severe fibrotic kidney disease, characterized by progressive CKD, leading to end-stage renal disease before the age of 50 years. Kidney biopsies from KIN patients, regardless of etiology, reveal karyomegalic tubular epithelial cells on renal biopsy. Recently, genetic KIN has been linked to defective DNA damage response, a hallmark of cellular senescence, and senescent cell were observed in biopsies from IOF-induced KIN patients. These studies suggest a role of senescent cells in sustained DNA damage response and progressive decline of kidney function in KIN patients. However, the link between karyomegalic TECs, senescence and eventually fibrosis in the kidneys of KIN patients remains unknown.

Therefore, in this project, we aim to elucidate the molecular mechanisms related to cellular senescence in the karyomegalic interstitial nephropathy phenotype. Cellular senescence and the availability of therapeutic agents that eliminate (i.e. senolytics) or prevent accumulation of senescent cells, provide novel therapeutic strategies to limit chronic kidney disease progression in KIN patients.

Education

These novel courses address several significant teaching issues. First, ‘research-tutored’ and ‘research-based’ elements of the Healey’s framework of research-based education are often poorly developed and underrepresented in undergraduate science programs. (Healey 2005)
Therefore, our courses put a strong emphasis on inquiry-based learning and student-centeredness. For instance, students perform hands-on research and experience the full

research cycle in a safe learning environment facilitated by a specific well-equipped and dedicated laboratory that is assigned to the students. Second, academies are dominated by a lack of connection between education, research, clinic and society, and also lack interdisciplinary teamwork in education. Our previous studies have established that students benefit from the interplay with researchers, clinicians and patients, and from interdisciplinary collaboration. (Drost et al., 2019; Valentijn et al., 2021; Schot et al., 2021) Furthermore, this lack of interdisciplinary connection contributes to delay of patient benefits from research discoveries. (Albani & Prakken, 2009) Therefore, we connected these disciplines to create synergy between education, research, clinic and society.

The educational aim is to stimulate students' development of academic skills, knowledge and attitudes towards translational medicine, teachers' development of didactic skills, patient involvement in research and synergy between education, research, clinic and society. Reported effects of students, supervisors and patients participating in the course will be evaluated and analysed for scientific publication.

Dissemination

The results of the lab work will be presented at scientific conferences and through publications in peer-reviewed journals, and be used by the PhD candidate and supervisors for scientific publications. Student evaluation of the course(s) will be presented on scientific conferences and through publication of articles in peer-reviewed journals.

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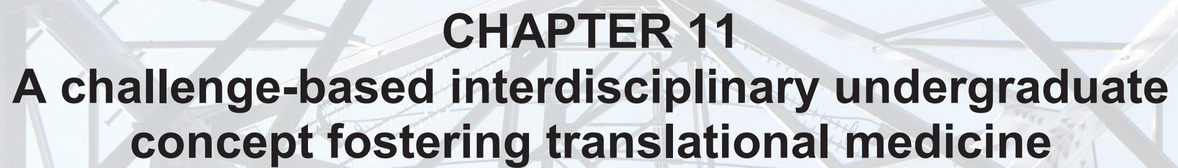

CHAPTER 11
A challenge-based interdisciplinary undergraduate concept fostering translational medicine

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ABSTRACT

Translational medicine (TM) is an interdisciplinary branch of biomedicine that bridges the gap between (fundamental) biomedical research and patients from bench-to-bedside. The goal of TM is to improve global health by combining disciplines, resources, expertise, and techniques in biomedicine. Fundamental TM skills include interdisciplinary collaboration, communication, critical thinking, and creative problem-solving (so-called 4C's). TM is currently limited in undergraduate biomedical education programs -which are mainly designed towards educating future professionals- with limited patient contact and opportunities for collaboration between different disciplines. In this study, we aimed to develop a novel challenge-based educational concept, grounded in the theoretical framework of research-based education, to implement TM in undergraduate biomedical education. Students were introduced to an authentic clinical problem through an interdisciplinary session with patients, medical doctors, and scientists. Next, students collaborated in groups to design unique laboratory-based research proposals addressing this problem. Finally, the best proposal was executed hands-on by student teams in a consecutive interdisciplinary laboratory course. Written questionnaires and focus groups were used to evaluate the efficacy of the educational concept on student learning, especially regarding the 4C's and student motivation. Evaluation results revealed that students developed 4C skills and acquired a 4C mindset. Working on an authentic patient case positively contributed to communication, critical thinking and creative problem-solving skills. Working in an interdisciplinary setting helped students to develop collaboration and communication skills. Furthermore, students were motivated by (i) the relevance of their work that made them feel taken seriously and competent, (ii) the patient involvement that highlighted the societal relevance of their work, and (iii) the acquisition of a realistic view of science. In conclusion, we have showcased a widely applicable challenge-based undergraduate concept fostering TM.

INTRODUCTION

In academia, communication gaps between basic scientists and physicians and the lack of interdisciplinary collaboration between research area's contribute to fragmentation of the bench-to-bedside translation. (Albani & Prakken, 2009) Translational medicine (TM) focuses on optimizing this route of translating scientific knowledge into real-world health impact by promoting enhancements in clinical application, combining disciplines, resources, expertise and techniques in biomedicine. (Abernethy & Wheeler, 2011; Cohrs et al., 2015; Weber, 2013; Morris et al., 2011) Thus far, the educational programs that have been developed to train translational (physician-)scientists are mostly aimed at teaching graduate students, scientist and clinicians. (Rubio et al., 2010; Kurpinski et al., 2014; Foty et al., 2018)

Core TM skills include interdisciplinary collaboration, communication, critical thinking and creative problem-solving, so-called "4C's". (Albani & Prakken, 2009) Previously, these 4C's have been successfully adopted in graduate-level TM training programs (Foty et al., 2018) and are widely accepted 21st century academic skills.(Binkley et al., 2012) Students develop academic skills and employ deep learning in a didactic framework of constructivism (Olusegun, n.d.) and inquiry-based learning.(Binkley et al., 2012; Elken & Wollscheid, 2016; Healey & Jenkins, 2009; Kahn & O'rourke, 2005) Furthermore, students are motivated by authentic learning (Herrington et al., 2003; Lombardi, 2007) and patient participation (Sacristán et al., 2016; Sharma et al., 2018), enabling them to acquire these academic skills. Herein, students acquire a research mindset by learning academic skills and to think divergently (in finding multiple solutions) rather than convergently (only one right answer). (Chamorro-Premuzic & Reichenbacher, 2008) Students elaborate on doing authentic (i.e. actual and relevant) research with uncertainty of outcomes and clear links to urgent healthcare and research problems, patients and society, representative for real-world TM research. Also, improving TM skills and mindset prepares students for subsequent master programs and their future biomedical carriers. (Rubio et al., 2010) Therefore, there is a pressing and more sustainable need to teach competent bridgers between lab and clinic already at the early undergraduate phase.

TM is currently limited in undergraduate biomedical education programs -which are mainly designed towards educating future professionals- with limited patient contact and opportunities for collaboration between students from different disciplines. Recently, we started the "Bachelor Research Hub", a well-equipped and dedicated laboratory at the University Medical Center Utrecht (faculty of Medicine, Utrecht University), in which students can execute their research. (Drost et al., 2019) Herein, integration of education, research and clinic created synergy in learning and cooperation between these three main pillars. (Drost et al., 2019) In this study, we aimed to develop a novel challenge-based educational concept to implement TM in undergraduate biomedical education, grounded in the theoretical framework of research-based education. First, in the Pathology course, students were introduced to an authentic clinical problem through an interdisciplinary session with patients, medical doctors, and scientists. All students worked in groups on their own unique research proposals. Second, in the Experimental Translation Medicine (ETM) course, the best proposal was executed hands-on by interdisciplinary student teams. We hypothesized that the authentic patient case and working in an interdisciplinary team would positively influence student learning. We evaluated these courses at the level of effects on (1) self-perceived student learning of the following academic skills and mindset: interdisciplinary collaboration, communication, critical thinking, and creative problem-solving, and (2) motivation.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Faculty of Social and Behavioral Sciences of Utrecht University (nr. 20-506).

Written questionnaire

All participating students completed a written questionnaire at the end of the course. Items were either scored on a 5-point Likert scale (--, -, ±, +, ++), or on a 10-point scale (10 being the highest). Additionally, students could make remarks to substantiate their experience.

Focus group

Focus groups were conducted throughout the academic year 2020-2021 with both students and supervisors of the courses. In total, 5 focus groups with 5-6 students and 2 focus groups with 3-5 supervisors were conducted. These focus groups were aimed at gaining further insight how the course contributed to student learning, focused on 4C's skills and mindset. Focus groups were led by authors WDS, MAH and FAV, who were not involved in teaching the courses. All students and supervisors gave informed consent prior to participation to the focus groups.

Data analysis

Focus groups were transcribed based on video recordings. The written records were coded for the course elements ('authentic patient case' and 'interdisciplinary teamwork') and learning outcomes ('collaboration', 'communication', 'creative problem-solving', 'critical thinking' 'other skills and mindset' and 'motivation') and creating matrix coding queries connecting course elements with learning outcomes using Nvivo 12 software (QSR International Pty Ltd. 2020). Subsequently, a second round of Nvivo analysis was performed, in which the learning outcomes were coded as 'positive effect on learning', 'no effect on learning' or 'unclear effect on learning', and on 'biomedical student' or 'medical student'.

RESULTS

COURSE DESIGN

Educational environment

In the undergraduate program Biomedical Sciences at the faculty of Medicine, Utrecht University (Utrecht, The Netherlands), each academic year is divided into 4 equal periods of 10 weeks that each harbor 1-2 courses. The pathology course is a ten-week halftime 7.5 ECTS (European Credits Transfer System) course, positioned in the first period of the second year, attended by 96 students. The ETM course is a ten-week full-time 15 ECTS elective course positioned in the third period of the second year, with a maximum of 16 Biomedical Sciences and Medicine students.

This study describes one full research cycle from introduction of a patient case offered in the Pathology course to execution of a research proposal in the ETM course. Main aims of the courses are clustered in knowledge, skills, and attitude, and include training of scientific and academic skills focused on the 4C's, completing the research cycle including hands-on research and dealing with the uncertainties of experimental results. The specific learning goals are listed in Table 1.

Content and design

Our educational concept consists of two phases, coinciding with two courses: (1) writing research proposals in the Pathology course and (2) executing the best ranked research proposal in the ETM course (Figure 1).

Table 1. Learning goals of the courses Pathology and ETM

Domain	After these courses students are able to:	
	Pathology	**ETM**
Knowledge and insights	Make the link between disease processes and scientific research	Explain the most important concepts and theories of the subject of study. Integrate and discuss these concepts and theories: predict experimental results based on theory, develop theory based on experimental results to contribute to new scientific insights.
Skills	Describe and apply commonly used methods and techniques in experimental pathology Prepare a scientific essay to further describe and study a pathobiological problem Make a well-founded poster/presentation regarding a pathobiological problem	Find and critically evaluate scientific literature. Formulate (sub-)hypotheses based on scientific literature and ongoing (unpublished) research in the faculty. Determine methods to approach the research question (from various angles). Use lab techniques to obtain experimental data to answer the research question. Draw conclusions based on the data and scientific literature. Analyze, combine, and integrate the data to apply it to a scientific discussion. Present the study in a scientific article. Present the study in an oral presentation. Formulate the (societal) relevance of the study.
Attitude	Working together in such a way that the best achievable group result is achieved	Take responsibility for their research. Cooperate to obtain the best possible group outcome. Be critical towards themselves and other students. Keep to the rules of the laboratory. Process the results with scientific integrity.

Pathology course aimed to design research proposal (phase 1)

The Pathology course was performed as previously described. (Drost et al., 2019) Briefly, students were introduced to the problem of phospholamban (PLN) hereditary heart disease, in a plenary session with PLN patients, treating medical doctors from distinct disciplines, researchers and a member from the funding PLN patient society. All involved parties presented their stories and described their involvement in the TM project (e.g. personal experiences of patients, patient files by clinicians, state-of-the-art research by scientists), ultimately posing the challenge to students. This was the starting point for students to work in 16 teams of 4 students on their own, unique, empiric research proposals aimed at better understanding of PLN and finding therapeutic targets. During the assignment, students were supervised by experienced researchers. Moreover, all treating physicians were available for questions to support the students. All research proposals were peer-reviewed by both fellow students and faculty and subsequently ranked by faculty based on consensus. On the final course day, all students presented their work via an oral or poster presentation in a symposium setting in the presence of all stakeholders. Based on proposals and presentations, the best and most feasible study was selected and submitted to the PLN patient foundation (Figure 1) for their approval.

ETM course aimed to execute research proposal (phase 2)

In this second course, grounded in research-based learning in line with the previous Pathology course, the best proposal was executed hands-on by 4 groups of 4 students in

well-equipped biomedical research laboratories. The students formed interdisciplinary groups, consisting of 2-3 biomedical sciences students and 1-2 medical students. The students were supervised in the lab by 8 experienced researchers. During the hands-on practical part of the course, students reported progress on their research project in lab journals and reported preliminary results to supervising researchers, clinicians, and patients. In the last week of the course, students reported their work in the format of a scientific manuscript and presented their work via oral presentations in a symposium-like setting in the presence of all stakeholders (Figure 1).

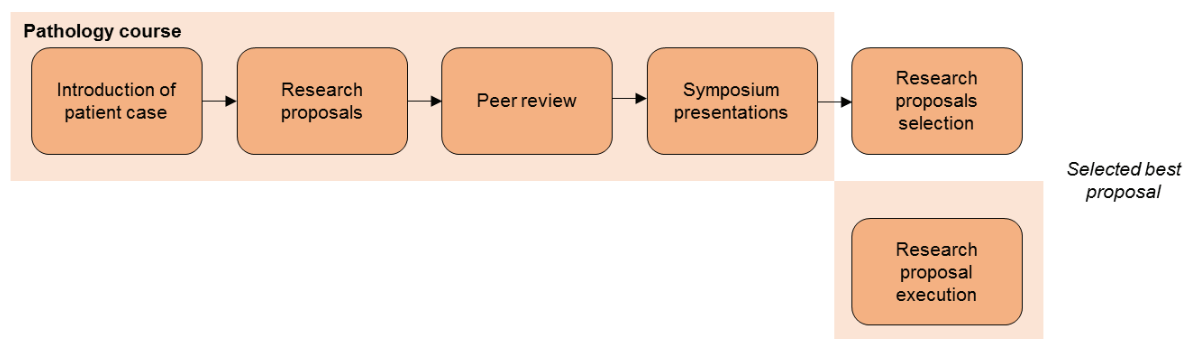

Figure 1. Schematic overview of the Pathology-ETM course continuum curriculum design

Assessment
To pass the Pathology course, students needed to obtain a mean passing grade (5.5 on a 10-point scale) on the following assessment criteria: Performance during group work (25%), Final oral presentation (10%), General effort (10%), Mid-course tests (25%) and Final test (30%). To pass the ETM course, students needed to obtain a passing grade (5.5 on a 10-point scale) on all the following assessment criteria: Performance during lab work (25%), Final written scientific report (10%), Final oral presentation (20%), Journal club (10%), Lab journal (5%), and General effort (30%).

Supervisor training
All faculty and supervisors followed an educational training program from an experienced educational specialist (author MAH, who was not involved in teaching the courses). Prior to both courses, a 4-hour workshop was provided in which supervisors were taught how to guide undergraduate students in a lab, with a particular focus om giving feedback and how to motivate students. During the courses, four peer consultation sessions were planned to discuss problems and challenges in a peer consultation setting.

COURSE EVALUATION
Written questionnaire
Authentic learning in an interdisciplinary setting stimulated student development and their motivation to learn
The written questionnaires revealed that students appreciated the Pathology (Table 2) and ETM (Table 3) course, as is evident from the high evaluation of the courses in general (8.4 ± 0.7 and 8.5 ± 0.8, resp.; 1–10-point scale) and the high scores on the questionnaire items (ranging from 4.0-4.6 and 3.6-4.9, resp.). Students found working on an authentic clinical research problem inspiring (4.4 ± 0.6 and 4.9 ± 0.4, resp.): "*the research-based learning principle gave me a better picture of reality*". In addition, it stimulated development of academic skills (4.2 ± 0.6 and 4.6 ± 0.5, resp.) and to process theory into practice (4.0 ± 0.8 and 4.6 ± 0.5, resp.): "*pressure cooker course to gain lab skills and academic skills*". Furthermore, students found patient participation and their personal interaction with patients and doctors inspiring (4.6 ± 0.5 and 4.2 ± 0.7, resp.): "*I found it really inspiring that patients and treating doctors were available during the plenary session*". Specific to the ETM course, teamwork between biomedical and medical students was motivating (4.0 ± 0.6) and

stimulated development of academic skills (3.6 ± 0.9). Finally, ETM students found teamwork with experienced laboratory researchers motivating (4.8 ± 0.4) and beneficial for their development of academic skills (4.8 ± 0.4). These data indicate that students appreciate authentic learning in an interdisciplinary setting including patients.

Table 2. Pathology post-course student evaluation

Survey Item	*n*	Mean	SD
Course design			
Working on an actual and relevant clinical (research) problem was motivating	88	4.4	0.6
Working on an actual and relevant clinical (research) problem stimulated the development of my academic skills	86	4.2	0.6
Working on actual and relevant clinical (research) problems stimulated me to process the theory	87	4.0	0.8
The presence of the PLN patient and doctors specialized in PTN was motivating	89	4.6	0.5
The possibility to do the proposed experiment in a real lab was stimulating	88	4.4	0.6
Course content			
Writing a research proposal was informative	88	4.2	0.5
Giving and receiving peer-feedback on the report was informative	88	3.5	0.7
Making and presenting a poster was informative	77	3.4	0.8
Making and presenting a powerpoint-presentation was informative	73	3.4	0.7
I give this course the following grade (10 point scale)	91	8.4	0.7

Academic year 2019–2020 (n = 88 students). Likert scale rating from 1 (‘I highly disagree’) to 5 (‘I highly agree’).

Table 3. ETM post-course student evaluation

Survey item	*n*	Mean	SD
Course design			
Working on an actual and relevant clinical (research) problem was motivating	14	4.9	0.4
Working on an actual and relevant clinical (research) problem stimulated the development of my academic skills	14	4.6	0.5
Working on actual and relevant clinical (research) problems stimulated me to process the theory	14	4.6	0.5
Working in an interdisciplinary team with biomedical and medical students was motivating	14	4.0	0.6
Working in an interdisciplinary team with biomedical and medical students stimulated the development of my academic skills	14	3.6	0.9
Working in a team with experienced laboratory researchers as supervisors was motivating	14	4.8	0.4
Working in a team with experienced laboratory researchers as supervisors stimulated the development of my academic skills	14	4.8	0.4
The presence of the PLN patient was motivating	14	4.3	0.6
The presence of doctors specialized in PLN was motivating	14	4.0	0.8
Course content			
Formulating a hypothesis and a research proposal with description of the experimental approach (first 2 weeks) was instructive to me	14	4.1	0.5
Carrying out a research proposal in the laboratory was instructive to me	14	4.8	0.4
Presenting research data during work meetings was instructive to me	14	3.9	0.9
Reading and presenting an article in a powerpoint presentation (Article presentations week 1) was instructive to me	14	3.9	0.8
Reading and presenting an article in a powerpoint presentation during the Journal club (week 9) was instructive to me	14	3.9	0.8
Documenting experimental data in a lab journal was instructive to me	14	3.9	0.6
Writing a scientific article was instructive to me	14	4.9	0.3
Making and presenting a final powerpoint presentation (week 10) was instructive to me	14	4.5	0.7
I give this course the following grade (10 point scale)	10	8.5	0.8

Academic year 2020–2021 (n = 14 students). Likert scale rating from 1 (‘I highly disagree’) to 5 (‘I highly agree’).

Focus group

Authentic patient case

Students develop 4C skills through working on an authentic patient case

Focus groups were conducted to evaluate the effect of the authentic patient case and interdisciplinary working on the development of 4C’s skills and mindset. When asked about the 4C skills the students gained through working on an authentic patient case, they mentioned positive effects on their development of all 4C’s. Pathology students reported improvements in collaboration (especially regarding consultation of other students for peer-feedback), communication (especially regarding communication to laymen patients), creative problem-solving and critical thinking skills. Development of critical thinking skills included critical appraisal of different ideas and to think a few steps further about the feasibility of experiments. ETM students mentioned that they gained critical thinking and creative

problem-solving skills although working on an authentic patient case did not enhance their collaboration and communication skills. Critical thinking included how to plan and perform experiments, how to interpret own or others' results, critical reading of articles and discussions during work meetings. Creative problem-solving included thinking out of the box and looking at problems with a broader view and from different angles.

Supervisors also noted positive effects on 4C skills. They observed that Pathology students developed critical thinking skills regarding searching and reviewing the literature, assessing feasibility of experiments, and balancing this with novelty, critical appraisal of different techniques and their time courses, and formulating the research problem and question. Supervisors also noted creative problem-solving skills and were impressed by the students' out-of-the-box ideas: "*I am not sure if the proposal ideas proposed by the students would have been proposed by PhD candidates*". According to the supervisors, ETM students developed collaboration skills (both within and between groups, and regarding division of tasks, communication skills (especially regarding presenting and group discussions), and critical thinking and creative problem-solving skills (related to reviewing literature, handling difficult situations independently and connecting different ideas).

Students acquire a 4C mindset through working on an authentic patient case

In the focus groups, students mentioned that working on an authentic patient case changed their mindset regarding the 4C's. Pathology students reported development of a mindset including collaboration, communication, and critical thinking. Regarding collaboration, students became aware of the importance of peer-feedback and each other's perspective: "*I became more inclined to seek peer feedback and became aware of the importance of others' different perspectives. And also that you can be useful to other groups.*" Regarding communication, students became aware of their importance in informing patients: "*because of the societal relevance, you take extra steps to communicate your research seriously to the outside*". Finally, students acquired a critical mindset evidenced by the following reflections: "*The authentic case stimulated to think a few steps further whether the experiment can really be executed in the lab*" and this "*stimulated long discussions and consideration of ideas, and to ask the question what is really the best idea and is this socially relevant?*".

ETM students reported development of a mindset including all 4C's. The authentic patient case made them aware of the importance of collaboration and communication: "*The format of the course makes you more inclined to collaborate, because you are thrown into the deep. You cannot say in your group 'I'll do it myself'. It encourages more – and better – collaboration and communication.*" It also contributed to their mindset regarding critical thinking and creative problem-solving: "*because not much was known about it yet, you start to think critically*" and "*creative thinking is sparked by being thrown into the deep in the first week*".

Students are motivated by working on an authentic patient case

Students from both courses indicated that working on an authentic patient case was motivating because their work is being used: "*something is really done with our research*", it mattered to PLN patients, and it made them feel taken seriously. Students appreciated the patient involvement because it highlighted the societal relevance of their research: "*seeing a patient makes research more concrete. It became visible what illness does in a person's life*" and the patient case made them feel emotionally connected to the patient: "*The patient case increased my motivation to make something out of the teamwork. It made me look for extra information by reading a lot. To improve and get the best out of myself*". One student mentioned: "*The course has brought research to life, has made it possible to think about research in a different way, an eye-opener that research can be so relevant and close by.*" ETM students appreciated that their experiments were never done by someone else and the uncertainty in outcomes motivated them.

Supervisors also noted that the students were very motivated. One supervisor observed that: "*interaction was much bigger, students asked more questions and worked*

harder than in other courses” and “*students felt important. They had a sense of contributing to a real problem.*”.

Interdisciplinary setting

Students develop collaboration and communication skills through working in an interdisciplinary setting

When asked about the 4C skills the students gained through working in an interdisciplinary setting, their perceived effects were dependent on the course. Pathology students noted no positive effects of working in an interdisciplinary setting on their development of 4C skills. However, ETM students noted positive effects on their development of collaboration and communication skills. Especially biomedical students gained these skills because “*it was necessary to guide the medical students because they had less experience in the lab and with reading scientific papers*”. Biomedical students learned to communicate on a different level: “*you learn how to transfer knowledge appropriately to the audience*” and to improve the teamwork: “*you learn to communicate better so that someone who is a little less far than you, can come along too*”.

Pathology supervisors noted a positive effect of the interdisciplinary introduction session on creative problem-solving, as this contributed to the originality of the students’ ideas. ETM supervisors also noted positive effects on collaboration and communication skills. Biomedical students learned to “*step down to explain technical aspects in such a way that medical students understood*” and became competent in helping medical students: “*sometimes they took over the role of the supervisor*”.

Students acquire a 4C mindset through working in an interdisciplinary setting

The interdisciplinary introduction in the Pathology course contributed to an open and creative mindset, as evidenced by the following reflections: “*The presence of the PLN experts from different disciplines made me aware of the different routes you can look at, outside of the usual roads*” and “*This enlarged my world: different ways of looking at a problem*”. It made students more aware of the importance of interdisciplinary teamwork in research: “*The course made me realize that interdisciplinarity is important in research*”.

ETM students reported development of a collaborative and communicative mindset. For biomedical students, working in a team with medical students stimulated them to explain the content of scientific papers and lab techniques to medical students, consult routinely with them so they could keep up, divide tasks and letting go of doing everything yourself. Medical students benefited from the biomedical students’ research skills and knowledge by asking them for help. Biomedical students mentioned that they realized the importance of communication at the same level, for good teamwork with medical students.

Students are motivated by working in an interdisciplinary setting

Students from both courses indicated that working in an interdisciplinary setting was motivating because of several reasons. Pathology students indicated that the presence of PLN experts was motivating because it made them relate to the problem of PLN and see the relevance of it: “*The fact that the problem is looked at from different angles, makes it much more real and closer to you. More relevant to make an effort*”. Furthermore, it contributed to a realistic view of science: *“puts your research in a broader context, providing a more realistic picture for later research, who and what is involved? That motivates.”* That students were consulted to help with a real and unsolved problem made them feel competent and this motivated them: “*It helped to hear from all of the PLN experts that we don't have the answer to the problem yet and that you as a student, can potentially make a difference.”* and “*That was the first time in my bachelor that I had the idea that you can make a difference.”* ETM students indicated that working in interdisciplinary teams was motivating because of the personal aspect: teachers and students knew each other and teachers “*want the best for you*”. Medical students were also motivated because they felt that biomedical students knew more about fundamental aspects of PLN and about labwork. Therefore, they wanted to

understand it too and showed more commitment. Biomedical students noted that working with medical students had no effect on their motivation.

Pathology supervisors also noted that students were very motivated and remarked that the presence of PLN experts played an important role in motivating the students. One supervisor stated: "*Students became highly motivated by making links between different disciplines*". ETM supervisors noted that "*Biomedical students were less demotivated if an experiment fails than medical students and could motivate medical students to carry on the experiments*".

Taken together, these data indicate that students develop 4C skills and acquire a mindset including these 4C's through authentic learning in an interdisciplinary setting including patients.

DISCUSSION

In this study, we describe the course design of two consecutive undergraduate courses that foster Translational Medicine (TM) education, thereby enhancing the bench to bedside to society axis. This novel challenge-based educational concept is embedded in the theoretical framework of research-based learning, and includes a patient-centered and interdisciplinary approach. (Griffiths, 2007; Healey & Jenkins, 2009) Herein, students learn by addressing relevant questions and complex, authentic research tasks with uncertainty of outcomes and clear links to urgent healthcare and research problems, patients and society. (Binkley et al., 2012; Elken & Wollscheid, 2016; Healey & Jenkins, 2009; Kahn & O'rourke, 2005) We have previously shown that working on authentic patient cases enhances student motivation and development of academic skills. (Schot et al., 2021) Implementing TM in undergraduate education, in which students from the start of their studies get acquainted with TM and develop their skills and mindset is a novel approach to stimulate implementation of TM.

Written questionnaire and focus group data revealed that students developed 4C skills and acquired a mindset including these 4C's. The authentic patient case mainly contributed to development of communication, critical thinking and creative problem-solving skills, and helped students to acquire a translational mindset (i.e. including all 4C's). The interdisciplinary setting mainly contributed to collaboration and communication skills, and stimulated a collaborative, communicative and creative mindset. Our findings are in accordance with other course based undergraduate research experiences (CUREs) that report student development of 4C skills. (Hunter et al., 2007; Indorf et al., 2019; Harrison et al., 2011; Delventhal & Steinhauer, 2020; Seymour et al., 2004) Moreover, CUREs stimulate positive student attitudes towards science and research. (Ballen et al., 2017; Lopatto 2007; Olimpo et al., 2016; Harrison et al., 2011) In addition to such general attitudinal changes, we observed that students gained positive attitudes towards the 4C's. Students became aware of the importance of good collaboration and communication, and a critical and creative mindset to perform research. In CUREs, students learn that good communication skills are necessary to professional practice. (Hunter et al., 2007) Traditional CURE hallmarks include scientific practices, development of new knowledge, relevant or meaningful work, collaboration and iteration. (Krim et al., 2019; Olimpo et al., 2016; Auchincloss et al., 2014; Harvey et al., 2014) Our course concept also includes an interdisciplinary setting, including collaboration between students of different disciplines, and between students, faculty, and patients. Regarding interdisciplinary teamwork, our findings are compatible with other undergraduate research experiences involving interdisciplinary student teamwork and collaboration with faculty, that report perceived student gains in collaboration and communication skills, (Casson et al., 2018) and positive attitudes about teamwork. (Sturner et al., 2016) Interdisciplinary collaboration in our courses can be further elaborated towards a transdisciplinary approach by involving students and scientists from other disciplines from both in- and outside the biomedical field (e.g. pharmacology, beta sciences, bioengineering, social sciences, economics, and humanities) and from different (inter)national institutions. (Bovenschen N et al., n.d.) This transdisciplinary network model may further enhance

student learning and motivation, and may also overcome evaluation limitations on sample size and provide insights of applicability to other fields of study.

Patient participation is associated with increased understanding of the importance of communication (Towle et al., 2014; Collins et al, 2011), and development of communication skills. (G. A et al., 2005; Rees et al., 2007) In the focus groups, students indicated that the patient involvement did not affect their learning because actual collaboration with them was lacking. In the future, further patient (society) involvement could be an important addition to this course concept. This includes informing patients about research progression (e.g. through student presentation to patient audiences and laymen summaries) and inclusion of patients in different steps of the research cycle (e.g. formulating/prioritizing important research questions).

Students found working on an authentic patient case and interdisciplinary working motivating for various reasons. First, students valued the relevance of their work which made them feel taken seriously and competent. This fits with the self-determination theory of intrinsic motivation, which states that autonomy and a feeling of competence and relatedness, support motivation. (Ryan & Deci, 2000) Second, patient involvement enhanced student motivation by emphasizing the societal relevance of students' work. Although it has been established that involving patients motivates students in medical education, patients are rarely involved in CUREs. (Dammers et al., 2001; Spencer et al., 2000; Vail et al., 1996) Our findings show the motivating effect of patient encounters in a CURE setting. Third, students were motivated by the realistic view of science they gained. Additionally, in the ETM course, medical students were motivated by the technical skills and biomedical knowledge of biomedical students.

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CHAPTER 12
A novel undergraduate biomedical laboratory course concept in synergy with ongoing faculty research

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Chapter 12. A novel undergraduate biomedical laboratory course concept

ABSTRACT

Optimal integration of education and ongoing faculty research in many undergraduate science programs is limited to the capstone project. Here, we aimed to develop a novel course-based undergraduate research experience (CURE) in synergy with ongoing faculty research. This 10-week course called Biomedical Research Lab is embedded in the curriculum of the undergraduate program Biomedical Sciences and grounded in the theoretical framework of research-based learning. Four groups of four students work together in a dedicated laboratory on an actual ongoing research problem of faculty. All groups work on the same research problem, albeit from different (methodological) perspectives, thereby stimulating interdependence between all participants. Students propose new research, execute the experiments, and collectively report in a single research article. According to students, the course enhanced scientific, laboratory, and academic skills. Students appreciated ownership and responsibilities of the research, laboratory teachers as role models, and they were inspired and motivated by doing authentic actual research. The course resulted in a better understanding of what doing research entails. Faculty valued the didactical experience, research output and scouting opportunities. Since topics can change per course edition, we have showcased a widely applicable pedagogy creating synergy between ongoing research and undergraduate education.

INTRODUCTION

To prepare students for the demands from the labor market, universities pay much attention to train students the required academic skills. Among others, these include communication, critical thinking, creative thinking, (interdisciplinary) problem solving, collaboration, project management, and self-organization skills. (Binkley et al., 2012) Academic skills and deep-learning of many students prosper in a didactic framework of constructivism (Bada & Olusegun, 2015.) and research-based education. (Elken & Wollscheid, 2016; Binkley et al., 2012; Healey & Jenkins, 2009; Prince & Felder, 2006; Kahn & O'rourke, 2005) This latter pedagogy is generally considered as a research-minded, student-centered approach, based on learning by addressing relevant questions and complex authentic research tasks. (Elken & Wollscheid, 2016; Binkley et al., 2012; Healey & Jenkins, 2009; Kahn & O'rourke, 2005) Students elaborate on real-world actual research and actively learn the academic skills to facilitate a better transition to master programs and the labor market. (Healey 2005) Working with role models further enhances student motivation and inspiration. (Healey et al., 2010; Healey & Jenkins, 2009)

Research-based education is grounded in a widely accepted framework developed by Griffiths (Griffiths, 2007) and further shaped by Healey. (Healey & Jenkins, 2009) This framework expresses two axes, one with the dimensions "teacher-focused" and "student-focused," and the other one with "research content" and "research processes/problems." The resulting quadrants form four scenarios that describe the relationship between teaching and research: (a) Research-led: where students learn about research findings and information transmission is the main teaching mode, (b) Research-oriented: where students learn about research processes, including state-of-the-art technology, (c) Research-tutored: where students learn to discuss and write research papers, and (d) Research-based: where students learn as researchers with inquiry-based activities and the research-cycle, including doing hands-on research. (Healey & Jenkins, 2009) While all quadrants are important in curriculum design, the latter two student-focused quadrants are often limited and underrepresented in educational undergraduate science programs. (Healey 2005) This is a missed opportunity, since in these quadrants students most actively participate in research.

Next to the positive effects of research-based education on student learning, it has been well established that scientific research can also benefit from teaching. (Healey et al., 2010; Jenkins et al., 1998) Teaching tasks force researchers to hold a broad overview and perspective on their discipline, lead to better reflection on their research, and raise talent scouting opportunities. (Visser-Wijnveen et al., 2010) Students can generate new research questions and hypotheses, give feedback and new ideas, and enhance (societal) relevance by continuing to ask (global) questions. Students generate new data and insights that can be beneficial for researchers. (Visser-Wijnveen et al., 2010) However, apart from the classical bachelor thesis (capstone project), creating synergy between research and teaching to enhance the research-teaching nexus in undergraduate programs often does not occur naturally and this relationship even has the tendency to diverge due to political and institutional policies and cultures. (Elken & Wollscheid, 2016; Jenkins et al., 1998)

In the present study, we aimed to develop a novel course-based undergraduate research experience (CURE) that creates strong synergy with ongoing faculty research, in which scientists and students are seen as partners. All students work in subgroups on the same research problem of a faculty, albeit from different methodological perspectives. Within the boundaries of this research problem, students propose hypotheses and the research, execute the experiments and collectively interpret and report their data. We evaluated this course at the levels of technical (laboratory) skills, academic skills, views and attitudes toward science, the research-teaching synergy, and the effects of following this course on the bachelor thesis (undergraduate capstone project). This widely applicable novel educational CURE concept not only enhances student academic and scientific skills, but also fosters ongoing faculty research.

MATERIALS AND METHODS

The study is approved by the Ethics Committee of the Faculty of Social and Behavioral Sciences of Utrecht University. The approval is based on the documents send by the researchers as requested in the application form of the Ethics committee and filed under number 20-506.

Written questionnaire

All participating students (n = 53) from the four editions of the BRL course completed a written questionnaire at the end of the course. The results are displayed in Table 2. Most items were scored on a 5-point Likert scale (, , ±, +, ++), with the exception of the items “the level of this course was” and “the required time investment was.” These items were rated from “much too low (score 1)” to “much too high (score 5).” Finally, students were asked to rate the course as a whole on a 10-point scale (a 10 being the highest rate) and there were comments fields where students could make open remarks related to learning activities, supervision, workload, and any remaining issues.

Focus group

Next to written evaluations, we conducted two focus groups with the students that followed the first edition of the BRL course in the academic year 2017–2018: one immediately after the BRL course and one after completion of their bachelor thesis (capstone project) in the fourth period of the third year (academic year 2017– 2018). The latter was aimed at gaining further insight into how the course contributed to the students' technical and academic skills, their views and attitudes toward science, and their performance in the capstone project. In addition, we conducted a focus group with the supervisors of BRL course edition 2017–2018 to investigate how they experienced the supervision. The focus groups were led by authors WDS, MAH and IM, who did not teach or supervise the students in the BRL course. All students and supervisors gave informed consent prior to participation to the focus groups.

RESULTS

COURSE DESIGN

Educational environment

In the undergraduate program Biomedical Sciences (approximately 175 students per year) at the faculty of Medicine, Utrecht University (Utrecht, The Netherlands), each academic year is divided into four equal periods of 10 weeks that each harbor 1–2 courses. Our novel Biomedical Research Lab (BRL) course is a full-time 15 ECTS (European Credits Transfer System) elective course positioned in the second and third period of the third year, with a maximum of 16 students per course (Figure 1). In the event that more than 16 students want to enroll in a BRL course, students are selected via allotment. Alternatively, students can choose among several other elective courses in the same period. All students had basic textbook knowledge of molecular biology, cell biology, physiology, and research methods, as well as basic laboratory skills (standard cook-book practicals) during year 1 and 2 courses.

In the BRL course, all students get the opportunity to participate in one authentic ongoing research project from faculty (principal investigator) of the Faculty of Medicine at Utrecht University. The current study describes the first four consecutive editions of the course that were offered in 2017–2020. Each course edition highlighted a different scientific theme, that is, virus immunology (2017–2018, period 2), tumor immunology (2018–2019, period 2 and 2019–2020, period 3), and oncology (2019–2020, period 2). Main aims of the course are clustered in knowledge, skills, and attitudes, including training of scientific and academic skills, completing the research cycle with hands-on research and dealing with the uncertainties of experimental results. The specific learning goals are listed in Table 1.

The laboratory costs (bench fees) and part of the supervisor salaries were financially compensated by the standard educational compensation fee of the program Biomedical Sciences of Utrecht University.

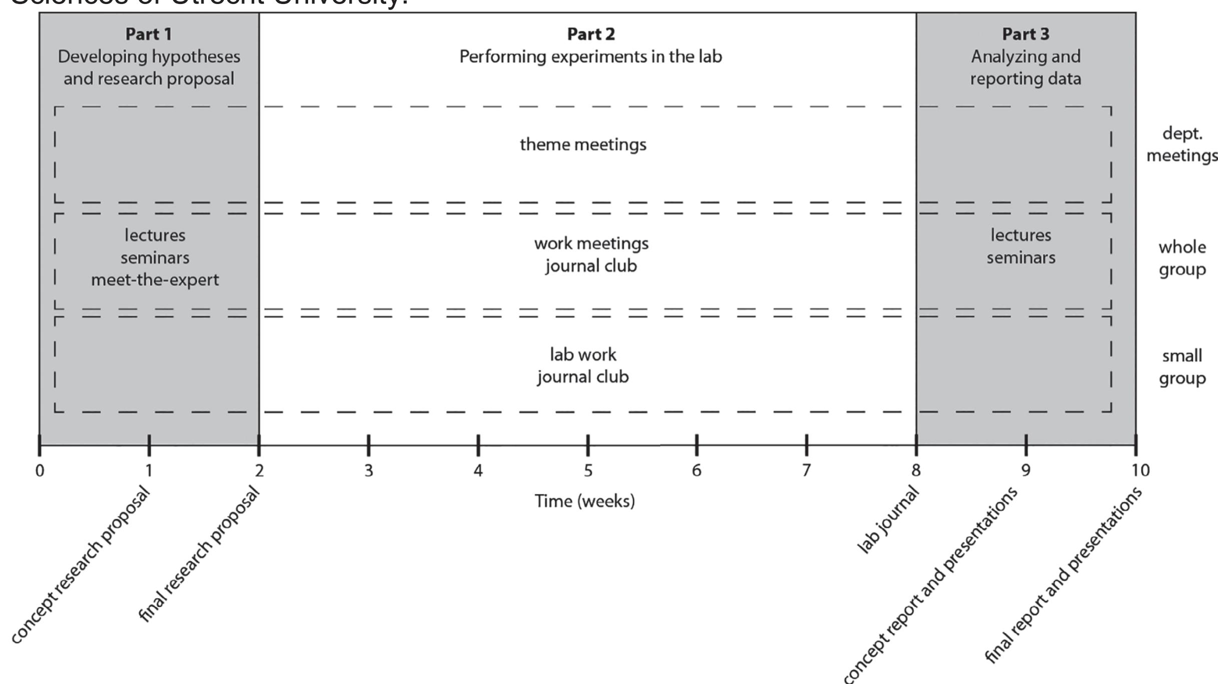

Figure 1. Set-up of the course Biomedical Research Lab

Content and design

The course consisted of three separate parts; (a) Defining hypotheses and writing research proposals (week 1–2), (b) performing experiments in the lab (week 3–8), and (c) analyzing and reporting data (week 9–10).

Part 1: Defining hypotheses and writing research proposals

At the start of the course, a principal investigator introduces the subject and poses the research problem that is cutting-edge and topical in the faculty's lab. This problem is embedded in literature, which was provided to the students through scientific papers and lectures, including unpublished data. During the first 2 weeks of the course, students framed hypotheses and wrote a proposal for the research they would perform in the next part of the course. For this, the 16 students were divided into 4 groups of 4 students each, resulting in 4 hypotheses and covered by 4 research proposals. Importantly, all groups addressed the same overall research problem, albeit from different (methodological) perspectives. Staff members were readily available for moderation and questions during the proposal conceptions and through planned meet-the-expert sessions (1 h every day). Moreover, tutorials were offered on skills, i.e., how to keep a lab journal, basics for writing a research proposal and how to write an introduction of a scientific article. Each group wrote their concept proposal including an introduction section in week 1 (max three pages), gave and received feedback from peers and faculty on the proposals, and delivered a pitch of the final version of their proposal at the end of week 2. The four introduction sections (1 per group) were used later in phase 3, writing a research article. Part 1 of the course ended with a tour in the research lab where they were going to perform their proposed experiments in the next phase. Nearly all contact time in part 1 was provided by a principal investigator supported by his/her research staff, including PhD students, postdocs, technicians and (under)graduate students.

Table 1. Learning goals of the course Biomedical Research Lab

Domain	After this course students are able to:
Knowledge and insights	Explain the most important concepts and theories of the subject of study.
	Integrate and discuss these concepts and theories: predict experimental results based on theory, develop theory based on experimental results to contribute to new scientific insights.
Skills	Find and critically evaluate scientific literature.
	Formulate (sub-)hypotheses based on scientific literature and ongoing (unpublished) research in the faculty.
	Determine methods to approach the research question (from various angles).
	Use lab techniques to obtain experimental data to answer the research question.
	Draw conclusions based on the data and scientific literature.
	Analyze, combine, and integrate the data to apply it to a scientific discussion.
	Present the study in a scientific article.
	Present the study in an oral presentation.
	Formulate the (societal) relevance of the study.
Attitude	Take responsibility for their research.
	Cooperate to obtain the best possible group outcome.
	Be critical toward themselves and other students.
	Keep to the rules of the laboratory.
	Process the results with scientific integrity.

Part 2: Performing experiments in the lab

In the second part of the course (weeks 3–8), students performed their proposed experiments in a well-equipped research laboratory at the University Medical Center Utrecht (UMCU) that was fully assigned to the students. When necessary, more specific techniques were done in the lab of the principal investigator or in core facilities at the UMCU. Each group of four students was guided by a supervisor affiliated with the laboratory of the principal investigator. In the 2017–2020 editions of the course, the lab supervisors were composed of mixtures of a postdoc, a PhD student, a senior lab technician, a graduate student, or undergraduate student. Experiments performed by the students were directly relevant to the research of the postdoc, PhD student, or PI. In addition to lab work, students participated once a week in (a) a journal club, in which students selected a scientific article that they presented and discussed with their peers and supervisors, (b) a general theme meeting, in which PhD students and postdocs of the UMCU presented their work (only for course editions 1, 2, and 4), and (c) a work meeting in which students presented the (raw) data obtained from their experiments to peers, supervisors, and staff member. At the end of part 2, students handed in their lab journals.

Part 3: Analyzing and reporting data

The last 2 weeks of the course, students analyzed and reported their data. There were two tutorials covering writing a scientific article and preparing an oral presentation. Whereas all four groups of students prepared their own final presentation, all 16 students were reshuffled in new groups, each addressing one particular section of a full scientific manuscript. As such, all students, supervisors, and the principal investigator worked together on the same final end product of the course, a scientific article. For this, one student was appointed as coordinator of the writing process. The introduction section of the manuscript was extracted from the four proposals as written in phase 1. After 1 week, the students gave and received peer feedback on the concept version of their scientific article. Also, they practiced their oral presentations together with the faculty member. On the final day of the course, students gave their oral presentations to their peers, lab supervisors, faculty member, the course coordinator, and other interested faculty. In addition, they handed in the final version of their scientific article.

Assessment
To pass the course, students needed to obtain a passing grade (5.5 on a 10-point scale) on all of the following assessment criteria: Performance during lab work (20%), Final written scientific report (20%), Final oral presentation (20%), Lab journal (20%), and General effort (20%).

Supervisor training
All faculty and supervisors received an educational training from experienced educational specialist (author MAH). Prior to the BRL course, a 4-h workshop was provided in which supervisors were taught how to guide undergraduate students in a lab, with a particular focus on giving feedback and how to motivate students. During the BRL course there were three moments of peer consultation. In addition, when master students were involved in the guidance, they were offered the opportunity to reflect on their educational development in a thesis. For this, they were awarded with nine ECTS (educational internship).

COURSE EVALUATION

Written questionnaire

Students appreciated the BRL course as is evident from the high scores on the statements (Table 2), the high grade for the course in general (8.6–9.2; 1–10 point scale), and the answers to the open questions in the written evaluation. Students appreciated going through the research cycle, ranging from framing a research proposal to executing the experiments and interpreting and reporting their data. One student mentioned: “working on ongoing new research and coming up with the hypotheses and experimental design ourselves was fun and I learned a lot from it. I also learned a lot because we were allowed to do almost all the lab work ourselves” (2017), and “I appreciate the free choice in your own research and developing your academic skills” (2019). Students found the BRL course inspiring and motivating, thereby enhancing academic skills: “I found executing current and our own proposed research so much more motivating compared to pre-arranged ‘cookbook’ practicals” (2018), “An opportunity to really deal with research and the feeling that you are taken seriously as an undergraduate student” (2019), and “A course where you perform research within a current ongoing investigation, while working fanatically for 6 weeks, was very motivating for me. I would recommend this course to everyone that wants to experience working in a lab” (2020). Students also indicated that the BRL course gave them a more realistic view of doing research. Students wrote “this course is really close to research. I really know much better what research entails thanks to this course” (2017), and “It really gives a sneak preview and a good preparation for future graduate internships. Also appreciated the supervision, very easy to reach and knowledgeable” (2020). Finally, some students made comments about the time investment of the course, stating that it was high but that they knew this in advance and that it was necessary and worth it.
The most important points of improvement that the students initially mentioned in 2017–2018 were related to (a) the structure of the journal club with statements such as “the journal club was a good initiative and also important but as it was organized now, there was too little time to read and prepare all the articles,” (b) combining all the research done in the course into one paper with statements such as “I think it is better to write four separate papers because writing one paper with sixteen people is too chaotic,” and (c) adjusting the group size or the activities within each group with statements such as “a group of four people was too much in our case. We couldn't really divide the practical work. Therefore, at times two to three people had nothing to do.” These comments prompted us to substantially improve these aspects (a–c) of the course toward 2020. (a) Students prepared and discussed a research paper on a weekly basis during the journal clubs in the 2017–2018 edition. This took too much preparation time while also performing lab experiments. Therefore, in 2019 we implemented one collective journal club in part 3 of the course according to the “jig-saw” method.[12] In this method, students had 6 weeks (part 2) to search, read, and make a presentation of one article per group, after which groups were reshuffled to discuss the four papers with an extra

focus on how to use this article in the discussion section of their final paper. Students appreciated this form of the journal club better, as was supported by the mean score for the statement “I learned from the journal club (part 2)” that gradually increased from 2.8 in the first edition to 4.4 in the latest edition. (b) Students of the 2017–2018 course edition also noted that it was quite a challenge to write one professional scientific article with 16 people in part 3 of the course. This concern was addressed in the next courses by appointing one student as “paper coordinator,” which provided much more structure for the writing process. (c) Students experienced that it can be difficult to divide and plan the work efficiently in a group of four people during part 2 of the course. Therefore, in subsequent BRL courses we stressed in advance that waiting times are inherent in doing research and that time-management is an important academic skill to organize the workload better within their own subgroup. These approaches, in which students were given more autonomy to direct and influence their course activities, led to much more positive feedback on these issues during the years (Table 2).

Table 2. Post-course student evaluation

	2017–2018 (Period 2) n= 16		2018–2019 (Period 2) n= 16		2019–2020 (Period 2) n= 16		2019–2020 (Period 3) n= 5	
Course edition	M	SD	M	SD	M	SD	M	SD
Survey items								
Content and organization								
This course fitted well with my prior knowledge	4.2	0.5	4.3	0.8	4.3	0.4	4.4	0.5
I was informed well about this course	4.3	0.6	4.2	0.5	4.3	0.6	4.6	0.5
The course was well designed	4.4	0.5	4.5	0.5	4.6	0.5	4.6	0.5
I obtained a lot of knowledge during this course	4.7	0.6	4.9	0.4	4.9	0.3	5.0	0.0
I was able to explore doing scientific research during this course	4.8	0.6	5.0	0.0	4.9	0.3	5.0	0.0
My enthusiasm for scientific research increased during this course	4.4	0.7	4.1	0.6	4.4	0.6	5.0	0.0
The course was scheduled well	3.9	0.6	3.9	0.5	4.3	0.7	4.4	0.5
The rooms for this course were adequate	4.1	0.9	4.2	0.4	4.1	0.7	4.6	0.5
The required time investment was[a]	3.5	0.5	3.5	0.5	3.1	0.3	3.0	0.0
The level of this course was[a]	3.2	0.4	3.2	0.4	3.2	0.4	3.4	0.5
Learning activities								
I learned from conceiving the hypotheses (part 1)	4.3	0.5	4.1	0.4	4.2	0.4	4.0	0.0
I learned from writing the research proposal (part 1)	4.3	0.5	4.1	0.3	4.3	0.5	4.6	0.5
I learned from performing the research in the lab (part 2)	4.9	0.3	4.8	0.4	4.9	0.3	5.0	0.0
Working on actual, relevant, ongoing research was motivating and inspiring	4.7	0.6	4.7	0.5	4.8	0.4	4.6	0.5
I learned from keeping a lab journal (part 2)	4.0	0.8	3.5	0.7	4.0	0.7	4.2	0.4
The group size (four students per sub-hypothesis) was adequate	3.9	1.0	4.0	0.5	4.5	0.6	N/A	N/A
I learned from the work meetings (part 2)	4.1	0.6	3.9	0.6	4.1	0.8	4.8	0.4
I learned from the journal club (part 2)	2.9	0.7	3.3	0.7	3.8	0.8	4.4	0.5
I learned from the Immunology theme meeting (part 2)	3.6	0.8	3.4	0.7	N/A	N/A	—	—
I learned from writing the scientific report (part 3)	4.5	0.5	4.3	0.6	4.4	0.6	4.6	0.5
I learned from giving the oral presentation (part 3)	4.1	0.6	4.1	0.6	4.2	0.7	4.0	0.0
During this course I improved my Academic skills	4.5	0.5	4.5	0.5	4.6	0.5	4.4	0.5
Supervision								
The teachers were enthusiastic and involved	4.7	0.5	4.6	0.5	4.9	0.4	5.0	0.0
The teachers were knowledgeable	4.5	0.7	4.2	0.4	4.7	0.5	5.0	0.0
The daily supervision in the lab was adequate	4.5	0.5	4.3	0.6	4.9	0.4	4.8	0.4
My fellow students put in their best effort	4.3	0.5	4.4	0.5	4.7	0.5	4.8	0.4
Overall								
There was a good atmosphere during the course	4.6	0.5	4.6	0.5	4.8	0.4	4.6	0.5
I give this course the following grade (10 point scale)	8.8	0.4	8.6	0.6	8.9	0.4	9.2	0.4

Note: Likert scale rating from 1 (“I highly disagree”) to 5 (“I highly agree”). Abbreviations: M, mean; N/A, not applicable; SD, standard deviation; , response rate insufficient. [a]These items were poled from “much too low (score 1)” to “much too high (score 5).” Bold values represent means of course evaluations.

Focus groups

Technical and academic skills

When asked about the research skills the students gained through the BRL course, they listed a wide range of skills covering the entire empirical cycle, including critical thinking, problem solving, collaboration, writing and pitching their idea, planning the experiment (especially regarding the logistics of ordering materials and incorporating factor time between subsequent steps), gathering and keeping track of the data in lab journals and reporting results ethically and understandable for the readers. Group cohesion was mentioned as a positive side effect of exchanging ideas and problem solving. Because all student groups worked on the same question with different lab techniques, students appreciated that one group could solve the problem of another group from a different perspective. Students also indicated that the BRL course made them more critical on research papers they read due to their experience with the journal clubs. Moreover, they focused more on the details and the required statistics in literature: "before, I would have never noticed that a control was missing from an experiment." Writing the scientific article also made the students more critical about their own data: "what data is still missing?" and it made them aware that researchers need to "sell the story at conferences and to get grant money."

Views and attitudes toward science

Students indicated that the main reasons for signing up for the BRL course were gaining research experience and finding out whether research was something they would like to pursue in their Master and further careers. This means that the students that participated in the course actively sought out the opportunity to investigate their own views and attitudes toward (doing) science. In the focus groups, students mentioned that the BRL gave them a more realistic view of what doing science actually is. They specifically mentioned that doing research takes much more time then they anticipated as illustrated by these statements made by the students: "when I read a textbook, I always thought I can do that too and very quickly. Now I know it's much harder than that," "I know now that a single paragraph in a paper can be weeks of work,""many things can go wrong, an article in a journal only shows the pretty picture," "I understand now why obtaining a PhD takes four years." In addition, students indicated that joining research meetings and journal clubs gave them a good understanding of the collaborative nature of a research group. They did feel, however, that they collaborated more than in an average research group, because they worked in groups of four. In one of the focus groups, the students agreed on the idea that "in real life, our group of four is probably like one researcher but with extra hands so it was easier for us to divide the work."

Performance in the capstone project

The Bachelor Thesis (capstone project) of the Biomedical Sciences program at Utrecht University has a stronger emphasis on literature review than on lab work (8 weeks literature review and writing and 2 weeks lab work). When we asked students about the influence of attending the BRL course on their performance in the capstone project, students initially mentioned the technical lab skills they acquired during the BRL course. Students also mentioned other skills, such as searching for and synthesizing literature, placing your research within the literature and thinking about the implications and follow-up questions of your research. Students indicated that they were more skilled in reading scientific literature and keeping lab journals. In addition, students mentioned that their experiences in the BRL course were very valuable when writing a scientific article during their capstone project. They specifically mentioned "making choices and limiting what you present to your reader" and "being brief and concise when presenting your data" as learning gains from the BRL that they could transfer to their capstone project. Finally, the students mentioned that participating in the BRL course had advantages in other courses because they had a better understanding of lab techniques, including those that lecturers mentioned during talks in the theme meetings about their own research.

Research-teaching synergy

In the BRL course, students contributed to relevant realworld research and performed complex authentic research tasks under supervision of faculty as role models. We hypothesized that this would lead to a synergistic symbiosis between researchers and students. Indeed, we found that both students and faculty mutually benefited from the course.

In the focus groups with the students, they indicated that working on real-life research was motivating because it "really mattered." They compared the BRL course to "cookbook practicals" which are quite common in the biomedicine undergraduate program and found the BRL course more motivating and inspiring because the outcome of the research was unknown in advance. In addition, they stated that they learned more because when things did not go as expected, they had to think of solutions to the problem rather than see what the outcome of their neighbor's experiment was and continue from there. Students stated that the BRL course also had added value compared to coming up with "fictional experiments" because they had to consider constraints such as time, money, and the availability of resources.

In the focus group with the supervisors, they too mentioned that they felt that the real-life experience and the enthusiasm of the students as well as the supervisors made the BRL course an inspiring and motivating learning environment. This made supervising within the BRL a fun experience well worth the time investment. Importantly, in addition to the learning gains they saw for the students (in line with those described by the students themselves), the supervisors also indicated that they themselves benefitted from supervising in the BRL course. For instance, the supervisors got an opportunity to supervise students and strengthen their didactical skills as they needed to explain the theory behind the experiment the students performed. The fact that they could do so adequately boosted their self-confidence. Also, the data the students gathered lead to research output for the faculty members and working with the students gave the opportunity to scout excellent students for future intern positions. PhD Student (supervisor): "The data the students generated are incorporated in a research article of my thesis and will be further worked out as a research paper for publication in a scientific journal. This research otherwise could not have been done." Supervisor/ PI: "Students that followed the BRL course know better what research in my lab entails and are therefore able to make a better informed decision to do a future Master internship in my lab."

Both the students and the supervisors emphasized the importance of the supervision in the success of the BRL course. The students specifically mentioned patience, trust, enthusiasm, optimism and creating a safe learning environment in which students are allowed to make mistakes, as stimulating characteristics of the supervision. They also see their supervisor as a role model of whom you can copy behaviors and skills. The supervisors mention that an important skill for example is to clarify the theory behind the experiments and explaining procedures and techniques to the students.

DISCUSSION

In this study, we developed a novel undergraduate educational concept that integrates ongoing faculty research with teaching. This concept harbors the classical hallmarks of a course based undergraduate research experience (CURE), including (a) scientific practices, such as asking questions, proposing hypotheses, designing studies, collecting and analyzing data, and communicating results, (b) development of new knowledge, (c) relevant or meaningful work, (d) collaboration, and (e) iteration. (Krim et al., 2019; Olimpo et al., 2016; Harvey et al., 2014) Additive and, to our knowledge, new in our concept is that all 16 students work on the same research problem that comes from ongoing research of a faculty, and that students address this problem in subgroups from different scientific perspectives. This strengthens the research-teaching nexus by creating mutual incentives and benefits for students and researchers that are multipronged.

All elements of Healey's framework of research-based education (Healey & Jenkins, 2009) are represented in the BRL course. Students learn about research (research-led) and research processes (research-oriented) in lectures, theme meetings, and journal clubs. Students also learn by doing in that the BRL course puts a strong emphasis on "research-tutored" and "research-based" elements, that is, writing/ peer-review and going through the research cycle, respectively. (Healey & Jenkins, 2009) These

latter two inquiry-based and student-centered elements are often poorly developed and underrepresented in undergraduate science programs. (Healey 2005) To this end, we have created a learning environment that is facilitated by a specific well-equipped and dedicated laboratory that is assigned to the students. This recently founded Bachelor Research Hub is positioned in the middle of the research laboratory center at the University Medical Center Utrecht, with short lines to ongoing research and facilities. (Bovenschen et al., 2020; Drost et al., 2019) Here, undergraduate students can meet other students, researchers, clinician-scientists and medical doctors, and have the opportunity to do research both in course-based research within the curriculum (i.e. BRL course) or extracurricular. (Drost et al., 2019)

We investigated how the BRL course affects students' academic skills and their perception toward science. Student data revealed that students gain a better perception of the research cycle. This ranged from dealing with details in experimental logistics up to placing their work in a broad and societal context. According to the students, this has significantly improved their academic skills. Students valued the autonomy and trust that were given thereby enhancing their motivation, which is compatible with the self-determination theory of motivation. (Ryan & Deci; 2000) This theory states that autonomy, together with a feeling of competence and relatedness, fosters deep learning and academic skills. (Ryan & Deci; 2000) Students recognized the authenticity of their own real research and felt part of a larger research team. (Indorf et al., 2019) Interestingly, students found the group strategy and organization within the BRL course to be stimulating and constructive as many problems could be solved either within the individual student group or the whole groups, while working toward an answer to the common research question. This fits with the concept of interdependence and collaborative learning. (Scager et al., 2016) Our findings are compatible with other more traditional CUREs that report gains in research skills such as critical thinking, problem-solving skills, data analysis, and oral and written communication, as well as personal development skills such as self-confidence and self-efficacy. (Indorf et al., 2019; Harrison et al., 2011; Hunter et al., 2007; Russel et al., 2007; Seymour et al., 2004; Gregerman et al., 1998) CURE student' perceptions of collaboration and making relevant novel discoveries are positively related to their cognitive and emotional ownership, and impact on clarifying students' career intentions. (Cooper et al., 2019; Corwin et al., 2018) Moreover, CUREs stimulate learning a topic in depth, learning to work independently, building tolerance for obstacles faced in the research process, and transforming the student-teacher relationship. (Harrison et al., 2011; Hunter et al., 2007; Russel et al., 2007; Seymour et al., 2004) In the future, further interdisciplinary intra- and inter-group cohesion and interdisciplinary dynamics could be an important addition to the BRL course. Collaboration with other disciplines can be a significant addition to the set of academic skills that are important for students' preparedness for their careers in academia and industry. (Drost et al., 2019; Spelt et al., 2009; Brew 2001)

Our study indicates that the BRL course concept enhances the synergy between research and teaching. On the one hand, the course format is successful in shaping research attitudes and transferring the major academic research skills to undergraduate students that are valuable for a better transition to master programs and labor market. This already becomes apparent in the students' progress during the capstone projects later in the same academic year, in which students realized advantages of skills developed during the BRL course. On the other hand, faculty researchers benefit by acquiring new data output, lots of relevant and critical questions and insights from students, financial support for laboratory costs and guidance, and scouting opportunities of excellent students for future research (internship) positions. Moreover, faculty, PhD students and even Master students benefit from training by educational specialists and the Master students get credited (ECTS) for participating as supervisor. Such a teaching experience for PhD and master students tends to improve their preparedness for a research career. (Shortlidge & Eddy, 2018) Thus, we have showcased a mutually rewarding interplay between ongoing research and undergraduate education that is not only applicable to life sciences, but also constitutes a basic pedagogy applicable to other disciplines.

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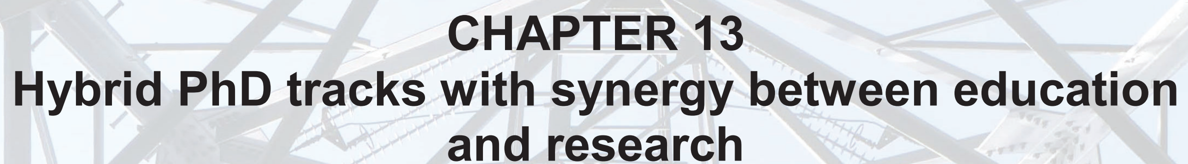

CHAPTER 13
Hybrid PhD tracks with synergy between education and research

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Based on:
Onderwijs moet een essentieel onderdeel zijn van een promotietraject. ScienceGuide 05/05/2021. https://www.scienceguide.nl/2021/05/onderwijs-moet-een-essentieel-onderdeel-zijn-van-een-promotietraject-2/ (Valentijn and Bovenschen, 2021)

Providing education should be an essential part of a PhD trajectory
PhD candidates are currently mainly assessed on their research performances. Hybrid PhD trajectories, based on the creation of synergy between research and education, could break with this uniform way of evaluation. Hybrid PhD trajectories support recognition of both teaching and research performances and can contribute to diversity in academic work. By properly combining teaching practices with conducting research, PhD candidates not only become better teachers, but also better researchers.

Educational competences and achievements have become subordinate to publishing and obtaining research grants. In a joint position paper (NWO, 2019), important Dutch research institutes and funders note this one-sided emphasis on research performance. In addition, they regret the fact that this leads to undervaluation of other academic domains. Several academic centres have therefore already taken steps towards better support and appreciation for teaching activities (VSNU, 2017). However, PhD candidates still receive too little space and recognition for their teaching activities.

Teaching promotes scientific development
A frequently heard comment from supervisors is that providing education comes at the expense of research time and output. This could lead to a delay in the PhD trajectory and therefore reduce PhD candidates' academic employment opportunities. However, this idea is completely at odds with the traditional academic ideal of an interaction between conducting research and providing education.

Several studies indicate that there are positive and synergistic connections between conducting research and providing education (Elken et al., 2016). A national sample of PhD candidates at a medical faculty in the United States shows that training in evidence-based education actually increases their confidence in being well prepared for an academic career and increases their research output (Shortlidge et al., 2018). This suggests that PhD candidates who invest in their educational development benefit not only as teachers, but also as researchers.

Hybrid PhD programme stimulates diverse academic development
The starting point for a hybrid PhD trajectory is creating synergy between conducting research and providing education. A hybrid PhD trajectory differs from a regular PhD trajectory in that PhD candidates are not only assessed on their research performances, but also on their educational performances. By correctly combining teaching with research practice, PhD candidates not only become better teachers, but also better researchers. Hybrid PhD tracks promote the quality of education, the recognition of educational achievements and career diversity by creating space for academic profiles in both research and education.

Supervisors can contribute to the revaluation of providing education by creating and stimulating hybrid PhD trajectories. After all, a PhD track is more than a dissertation consisting of scientific articles (ScienceGuide, 2020). It is an academic training program, and providing education, together with conducting scientific research, is the core task of academics. Teaching activities like educational innovation and evaluation and a "BKO" teaching qualification should therefore all count in the assessment of a PhD trajectory. Moreover, a dissertation can also contain reflections on didactic development (for example a BKO portfolio) and chapters devoted to educational articles (for example educational evaluations).

Providing education in addition to your own research
A new way of combining research and teaching is education evaluation with the goal of obtaining more insight into student learning in one's own educational courses. This

knowledge can be disseminated in the form of (scientific) publications and experience with this form of education can count towards for a BKO teaching qualification.

Supervisors could also involve their PhD students more in the innovation and evaluation of the education they provide. Financial support for educational evaluation by PhD students can be provided through education grants for promoters, for example through support from the university or through the NRO Comenius programme, or through individual grants for PhD candidates, for example through talent programs of academic institutions.

Another new development in line with the revaluation of providing education by PhD candidates are the so-called 'PhD *teaching tracks*'. This combines research with developing and providing education, at the same time as obtaining a BKO teaching qualification. Some universities already support PhD *teaching tracks* by financing contract extensions for PhD candidates. This gives PhD candidates more space for providing education during their PhD trajectory.

Education in synergy with own research

A new educational development with a synergistic interaction between conducting one's own research and providing education can be found in research-based courses. In this, PhD students, in collaboration with other professionals, involve (interdisciplinary) teams of students in their own research.

This creates synergy between research and education because the supervising PhD candidate teaches the students critical thinking and creative problem-solving skills in a real-world scientific environment. This not only contributes to both the learning of the students and the didactic development of supervising PhD candidates, but may also provide the lecturers with new, original ideas and relevant research data (Drost et al, 2019).

Conclusion

Thanks to educational developments, providing education can be easily combined with conducting research in a hybrid PhD trajectory. Educational innovation and evaluation, PhD *teaching tracks* and research-based courses give PhD candidates the opportunity to efficiently integrate teaching into their PhD trajectory. This stimulates broad academic development and the acquisition of a strong educational profile of PhD candidates. In addition, it contributes to improving the quality of education and a structural revaluation of providing of education in the academy. Therefore, promoters should give PhD candidates more space to provide education and give their educational performances the attention it deserves.

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CHAPTER 14
Summarizing discussion and future perspectives

Chapter 14. Summarizing discussion and future perspectives

In this thesis, cellular senescence in Chronic Kidney Disease (CKD), and targets for intervention are explored. Special attention is given to the effect of CCN2 inhibition on renal senescence in the context of renal ischemia-reperfusion injury (IRI). In this chapter, first the concept and detrimental effects of cellular senescence in the kidney are defined. This is followed by a more detailed description of the molecular modifier CCN2 and aging to the renal response to injury. Next, options for interfering with cellular senescence (i.e. senotherapeutics), and an *in vitro* model for screening of senotherapeutics that eliminate senescent cells (i.e. senolysis) are described. The translational implications of these novel insights are highlighted by the identification of a senescence phenotype in the rare kidney disease karyomegalic interstitial nephropathy (KIN). The last section integrates findings from a novel educational concept that fosters translational medicine, providing a perspective to base further translational research on, and perform this research in synergy with bachelor biomedicine education. Finally, a central role for the PhD candidate in coordinating such educational advancements in the form of a hybrid PhD track, is motivated.

Occurrence of cellular senescence in the kidney
As described in the introduction and **Chapter 2**, cellular senescence is defined by DNA damage response (DDR), permanent cell cycle arrest and a senescence-associated secretory phenotype (SASP). Cellular senescence is implicated in renal aging and in renal disease and senescent cells can be targeted to combat CKD. Current understanding of the occurrence and localization of senescent cells in renal aging and disease, causation between senescence and renal aging and injury, and (seno)therapeutic strategies are reviewed in this chapter. From the reviewed literature, we conclude that senescent cells accumulate with advancing age and in various renal diseases and that the affected cells are mainly proximal tubular epithelial cells (PTECs). In the context of renal aging and renal injury, senescence is associated with detrimental effects on renal histopathology and kidney function. Eliminating renal senescent cells in experimental studies can reduce such detrimental effects (Li et al., 2021), providing the first evidence for a causal role of senescence in renal functional and histopathological deterioration, and opportunities for development of clinically applicable therapeutic agents that interfere with senescence-associated pathways (i.e “senotherapy”).

Identifying novel modifiers and contributing factors of renal senescence in response to injury
One senotherapeutic approach is to interfere with modifiers of senescence-associated pathways, including DDR, cell cycle arrest and SASP. As described in the introduction, CCN2 is a well-established mediator of kidney fibrosis after renal transplantation (Vanhove et al., 2017; Vitalone et al., 2010), and has previously been identified as SASP factor (Yang et al., 2010) and inducer of senescence. (Jun & Lau., 2017) In **Chapter 3** we show a spatial relation between CCN2 and DDR markers, and their association with kidney function in human kidney allograft biopsies that are subjected to transplantation surgery-induced IRI. Furthermore, inhibition of CCN2 reduces senescence of mainly PTECs following renal IRI, and this is associated with preserved kidney function and reduced fibrosis. We could trace this protective effect back to the early phase, as CCN2 inhibition reduces tubular DNA damage and the DDR in a 3day IRI mouse model and in a cultured PTEC IRI-like model.

DNA damage and DDR are induced by reactive oxygen species (ROS), that are excessively produced as part of the oxidative stress response in IRI. (Terryn & Devuyst, 2011; Srinivas et al., 2019) In addition to the previous chapter, in **Chapter 4** we show that CCN2 already modulates the immediate stress response to IRI, 4 hours after reperfusion. Furthermore, inhibition of CCN2 also reduces DNA damage and DDR in this immediate phase. Subsequent reduced DDR and anti-apoptosis, 3 days after IRI, is associated with a reduced oxidative stress response in CCN2 knockout mice. Concordantly, *in vitro* analysis revealed that CCN2 inhibition reduces oxidative stress response in cultured PTECs and, *in vivo*, CCN2 induces tubular oxidative stress in mouse kidneys. Together these observations suggest that CCN2 inhibition mitigates the ROS-DDR-Senescence-Fibrosis sequence in the AKI-to-CKD transition upon renal IRI.

Acute kidney injury (AKI) is a pathological condition predisposing to CKD, with various aetiologies (e.g. IRI, toxic injury) and increased incidence is seen in elderly patients. (Levey & James, 2017) In **Chapter 5**, the increased AKI susceptibility associated with aging, is addressed by describing the differential cellular and molecular response to toxic AKI in young versus old mice. We found that old mice have increased acute tubular damage in response to folic acid injury. This is associated with an age-related magnification of senescence, inflammation and proinflammatory cell death, and with loss of the nephroprotective factor Klotho, that might explain age-related increased AKI susceptibility.

In **Chapter 6**, the overtime age-related activation of cellular senescence-associated mechanisms in the kidney is evaluated in 12- and 18-month-old mice. We found that aging induced "spontaneous" early inflammatory infiltration and subsequent later structural changes and fibrosis. This is associated with early DDR activation and loss of the nephroprotective factor Klotho, and subsequent later redox imbalance, cell cycle arrest and SASP, supporting the hypothesis of senescent cells as drivers of age-related kidney damage progression.

Novel tools to study and interfere with cellular senescence in the kidney

Identification of potential candidates that target senescent PTECs requires a senescent cell culture of proximal tubular epithelium for senotherapeutic drug screening. **Chapter 7** describes that conditionally immortalized proximal tubule epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1) may be used for studying renal tubular senescence and for senotherapeutic drug screening by simply adjusting the culture conditions. Culturing ciPTEC-OAT1 at the non-permissive temperature of 37°C, via SV40T downregulation and subsequent pRb and p53 activation (Larsson et al., 2004), induces a senescence-like phenotype, that is more pronounced compared to ciPTEC-OAT1 cultured at the permissive temperature of 33°C. Furthermore, we found that treatment with senolytics may reduce cell viability and SA-B-Gal activity of cells cultured for 9 days at the non-permissive temperature, suggesting responsiveness to senolytics.

As reviewed in **Chapter 8**, there are various potential interventional approaches to target the adverse effects of cellular senescence, including prevention of senescence, SASP modulation (i.e senomorphics) and senescent cell elimination (i.e senolysis). The pharmacological options for targeting senescent cells, associated hurdles, and the means to specifically target the kidney are reviewed in this chapter. From the reviewed literature, we conclude that there are several promising senomorphic-, senescence preventing- and senolytic agents that show potential for targeting senescence in the kidney. However, the application of these senotherapies may be complicated by chronic treatment with prolonged systemic exposure and systemic side effects. These hurdles could be overcome by targeted delivery to the proximal tubular epithelium, for example by using nanomedicines with high affinity for tubular cell membrane receptors. Repurposing senotherapeutic drugs that are already tested in clinical trials for other indications by functionalization for targeted delivery may accelerate translation to clinical application for CKD patients.

Identifying senescence as novel mechanism implicated in a prevalent cause of CKD in childhood cancer survivors

In **Chapter 9** we identify a senescence-like phenotype due to unresolved DDR in ifosfamide induced karyomegalic interstitial nephropathy (KIN), which is a prevalent cause of CKD in childhood cancer survivors. Selected kidney biopsies taken for renal failure after childhood cancer treated with the alkylating agent ifosfamide, all show a state of DNA damage induced senescence. These senescent cells include karyomegalic PTECs that are typical for KIN, but also include morphologically normal appearing PTECs that show strong positive staining for P21. This data suggests that senescence due to unresolved DNA damage, is a prevalent feature in therapy induced KIN, and is more widespread than only karyomegaly observed by routine histological examination. This could provide new opportunities for effective treatment of KIN with senotherapeutics to limit CKD progression.

Bridging research (part 1) and education (part 2)
However, orphan diseases like KIN face financial problems because they are often not prioritized by medical centers nor industry for gaining support and resources for discovering treatments. **Chapter 10** describes a proposal to overcome this problem by studying KIN in an research-based educational setting, in which expanses are covered by the University and patient societies contribute to the bench-fee. Herein, bachelor students collaborate with experienced scientists, doctors from associated disciplines (e.g. Pediatric Nephrology, Genetics and Pathology), patients and patient societies to gain insights into the underlying mechanisms of KIN.

Novel educational concept to stimulate translational medicine
To facilitate and improve synergistic interdisciplinary collaboration between students, scientists, and doctors from different disciplines and patients, a novel educational concept embedded into the key principles of research-based learning and translational medicine (TM), was employed. This educational concept encompasses multiple courses, including the Bachelor Research Lab (BRL) course and the dual course continuum Pathology and Experimental Translational Medicine (ETM).

In **Chapter 11** we describe the Pathology and ETM course design, and show that students and supervisors perceive positive effects on student development of academic skills and motivation. Students and supervisors note that authentic learning and interdisciplinary working have a positive effect on student learning of the so-called 4C skills (communication, collaboration, critical thinking and creative problem-solving). In addition, authentic learning and interdisciplinary working motivate students to make the most out of their teamwork.

In addition to the previous chapter, in **Chapter 12** we describe the BRL course design and show that students and teachers appreciate this course. Our evaluations reveal that students appreciate ownership and responsibilities of the research, laboratory teachers as role models, they are inspired and motivated by doing authentic, actual research and they gain better understanding of what doing research entails. Faculty values the didactical experience, research output and scouting opportunities. Together these observations suggest that this novel undergraduate biomedical educational concept has a positive effect on student learning and creates synergy between education, research and clinic in academia.

Vision on integration of research and education in hybrid PhD tracks
In **Chapter 13**, the synergy between providing education and performing research in hybrid PhD tracks is motivated. Through several novel educational activities, including educational innovation and evaluation, PhD teaching tracks and research-based courses, PhD candidates can efficiently integrate teaching into their PhD trajectory. Providing education combined with conducting research in a hybrid PhD trajectory stimulates broad academic development and the acquisition of a strong educational profile of PhD candidates.

Translation and Future perspectives
To date effective pharmacological treatment options to prevent CKD progression are lacking. The review of literature we performed in Chapter 2 reveals that elimination of senescent cells is associated with beneficial effects on kidney homeostasis during aging and upon renal damage in the setting of experimental CKD. If this can be translated to the clinical setting (i.e if CKD patients benefit from senolytic treatment) is currently addressed in clinical trials. Recently, the first clinical trials have reported effects of senolytics on organ function and efficacy in reducing senescent cell abundance in humans. Intermittent treatment with the combination of dasatinib and quercetin (D&Q) improves physical function in patients with senescence-associated idiopathic pulmonary fibrosis (iPF) (Justice et al., 2019), and a 3-day treatment with D&Q reduces senescent cell burden in adipose tissue and skin of diabetic kidney disease patients. (Hickson et al., 2019) Completion of the latter ongoing clinical trial will also reveal the effect of this DQ regimen on change of kidney function (ClinicalTrials.gov Identifier: NCT02848131).

Our data pointed to PTECs as the culprit of cellular senescence in the kidney. They made out the majority of senescent cells in delayed graft function and chronic allograft dysfunction following kidney transplantation, and in experimental IRI (Chapter 3), as is the case in CKD due to aging and renal injury, and in various other renal diseases (Chapter 2). This could be explained by the susceptibility of PTECs to ischemic or toxic injury. (Bonventre & Yang, 2011) In CKD, PTECs that fail to resolve DNA damage or undergo apoptosis, become senescent, driving CKD progression through the secretion of proinflammatory and profibrotic factors, including CCN2.

CCN2 inhibition might be a novel senotherapeutic approach to combat CKD. The experimental and human studies executed in Chapter 3 imply that inhibition of CCN2 may help to prevent post-IRI CKD by limiting senescent cell accumulation and fibrosis. This suggests that there is senotherapeutic potential in anti-CCN2 therapy for kidney transplant patients and warrants further evaluation of anti-CCN2 therapy in the setting of renal IRI. Several pharmacological inhibitors of CCN2 are in clinical trials for fibrotic diseases, including CCN2-targeted neutralizing antibodies, small interfering RNA's and antisense oligonucleotides (Brigstock 2009; Chen et al., 2020), but none of these address kidney disease.

Monoclonal antibody therapy has become an established strategy for a range of diseases. For example, in the kidney rituximab and eculizimab are successfully used for treatment of immune-mediated glomerular diseases. (Santoro et al., 2015) FG-3019 (pamrevlumab), a human recombinant neutralizing antibody to CCN2 holds promise as potential therapeutic. In animal studies, FG-3019 has shown anti-fibrotic effects in the liver, pancreas, lung, and skeletal muscles. (Chen et al., 2020) In a phase II trial, long-term treatment was well tolerated and reduced iPF disease progression. (Richeldi et al., 2020) Furthermore, in a phase I trial, it was well tolerated and reduced albuminuria, an important predictor of CKD, in patients with diabetic nephropathy. (Adler 2010) Phase 2 and -3 clinical trials with FG-3019 are currently being conducted for several indications, but lack kidney disease. Thus, FG-3019 may be a promising candidate for reproducing the results from our transgenic mouse studies presented in Chapter 3 and 4. Novel CCN2-target strategies that could be explored for senotherapeutic potential include aptamer-based drugs (Chen et al., 2020) and nanoparticle-based siRNA delivery systems for transporting CCN2-targeted siRNA's. (Kang et al., 2020)

The work presented in Chapter 8 reviews the various other potential interventional approaches to target senescent cells in the kidney, and possibilities for targeting PTECs to overcome dose limiting side effects on other organs. Given the similarities of senescent cells and cancer cells in metabolic activity, DDR and activation of pro-survival pathways, repurposing antitumor drugs that have been tested in clinical trials for other indications to target senescent cells may lead to a fast translation to clinical nephrology practice.

For this, senotherapeutic screening tools with predictive value for response to senotherapeutics are needed. In our AKI-to-CKD mouse models, IRI (Chapter 3 and 4) and folic acid injury (Chapter 5) induce a prominent tubular senescence phenotype, including key senescence features of DDR, cell cycle arrest, SASP and anti-apoptosis. This suggests these models may be used to study tubular senescence and hold potential to study the response to senotherapeutics *in vivo*. Indeed, treatment with senolytics reduces senescence and fibrosis in the setting of IRI and toxic AKI. (Li et al., 2021) Furthermore, there is substantial overlap in the early and late response transcriptome of murine IRI kidneys and human kidney transplantation biopsies, suggesting translational value of experimental IRI for studying the AKI to CKD transition following kidney transplantation. (Cippà et al., 2018) Advanced technologies like single-cell RNA sequencing allow for more detailed identification of gene expression profiles and pathway identification at the individual cell level. Comparison of RNA senescence signatures of human CKD and mouse CKD models may help to identify

the most relevant mouse model that reflects the human state based on best matching transcriptomic senescence signatures.

As described in Chapter 7, a senescence-like phenotype can be induced by culturing temperature-sensitive ciPTEC-OAT1 at the non-permissive temperature of 37°C. Moreover, these cells show sensitivity to senolytics agents, suggesting this PTEC culcture model may be used for screening potential candidates that target senescent PTECs described in Chapter 8.

Another major challenge of translating senotherapies for CKD to clinical application will be finding the right timing of treatment. The reviewed literature described in Chapter 2 points to a time-dependent effect of senolysis on outcome in the context of experimental renal injury. Senescence exerts beneficial effects in the early phase of AKI, where it might support regeneration while, in contrast, prolonged senescence exerts detrimental effects in CKD. On the other hand, limited regenerative potential of the kidney warrants treatment with senotherapeutics in the early stages of CKD when senescent cell numbers are still limited and little progression of fibrosis has occurred. In preclinical studies, senolytic treatment initiation early after IRI and toxic AKI has shown efficacy in reducing senescence and fibrosis. (Li et al., 2021)

The work presented in Chapter 5 shows that toxic AKI results in a magnification of the renal injury response, including a more pronounced senescence phenotype. In kidney transplantation, timing may even be more complex, as acute as well as chronic factors drive accumulation of senescent cells. These include IRI around kidney transplantation and tacrolimus nephrotoxicity, which both involve the production of ROS and subsequent DDR (Jassem & Heaton, 2004; Khanna & Pieper, 2007), and may also include reduction of physiological clearance of senescent cells (immune surveillance) as a result of immunosuppressive therapies. (Kang et al., 2011)

The experimental and human studies executed in Chapter 4 suggest that CCN2 not only acts as a profibrotic factor in later stages of adverse tissue remodeling, but that it also negatively contributes to the immediate early response to IRI, in particular to oxidative stress induced DDR. Thus, transplanted kidneys may benefit from early anti-CCN2 therapy around kidney transplantation, in the recipient following transplantation, or even in the donor prior to transplantation. Although causation and underlying mechanisms remain to be elucidated, our observations suggest that CCN2 inhibition reduces IRI-induced senescent cell accumulation by alleviating oxidative stress induced DNA damage. Future studies should address the optimal timing of CCN2 inhibition around IRI and reproducibility of our results presented in Chapter 3 and 4 with a clinical relevant CCN2 inhibitor.

Implementation and Future perspectives

The work presented in Chapter 11 and 12 provides the first indications that integrating undergraduate biomedical education in faculty research is a succesfull strategy to foster TM. Our evaluations focused on perceived student learning outcomes point to beneficial effects on the development of TM skills. Other short-term outcomes included increased satisfaction of both students and supervisors of the learning environment, original research study designs, succesfull funding acquisition based on research grant proposals and execution of proposals in the lab. All of these outcomes are predefined indicators of success of TM training programs. (Rubio et al., 2010) To elaborate in more detail on student learning in our educational concept, research including assessment of assessed learning outcomes on 4C's as constructs should be conducted to confirm our findings. Future studies aimed at evaluating the success of these TM courses, could also elaborate further on gains of other involved parties including clinicians and patients, and on long-term impact of translational research studies and on students' academic trajectories.

This challenge-based educational concept provides opportunities for application in various fields of research. Implementation aimed at involving students and scientists from other disciplines from both in- and outside the biomedical field (e.g. pharmacology, beta sciences, bioengineering, social sciences, economics, and humanities) and from different

(inter)national institutions (transdisciplinary collaboration) may further foster TM by educating a new generation of translational scientists that can more effectively work together at interdisciplinary interfaces to bridge the gap between bench and bedside.

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APPENDICES
Nederlandse samenvatting

Introductie

Nierfalen

Bij nierfalen (ook wel nierschade) is er sprake van een verminderde nierfunctie. Hierdoor lukt het de nieren minder goed om afvalstoffen uit het lichaam te verwijderen. Nierfalen kan plotseling ontstaan (acute nierschade) of langzaam en sluipend (chronische nierschade). Chronische nierschade is een groot en groeiend gezondheidsprobleem. Het komt wereldwijd bij meer dan 13% van de bevolking voor, met name bij ouderen. (1) De zorgkosten bedragen in Nederland alleen al meer dan 800 miljoen euro per jaar. (2) Bij vergevorderde chronische nierschade, vermindert de kwaliteit van leven en is er risico op vroegtijdig overlijden. De behandeling richt zich op het voorkomen van verder verlies van de nierfunctie. Voor de meeste nierziekten bestaan echter geen medicijnen die achteruitgang van de nierfunctie voorkomen. Hierdoor worden nierpatiënten uiteindelijk afhankelijk van nierfunctie-vervangende behandelingen: dialyse (kunstmatig overnemen van de nierfunctie) of transplantatie (vervanging van de nier).

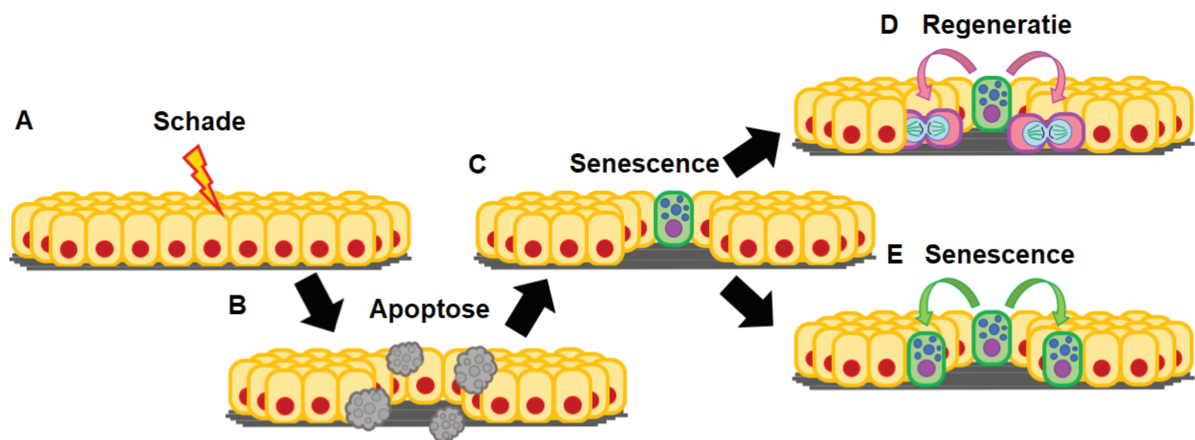
Chronische nierschade treedt op bij nierziekten en/of normale slijtage die gepaard gaat met veroudering. Schade aan de nieren leidt tot afname van functionerend nierweefsel en achteruitgang van de nierfunctie, die beide niet alleen onomkeerbaar maar ook progressief zijn. De afname van functionerend nierweefsel wordt grotendeels veroorzaakt door verlittekening (fibrose) in de nieren. Verlittekening na nierschade is vergelijkbaar met een litteken na een huidwond: een diepe wond leidt tot een litteken waarbij functionerend weefsel verandert in bindweefsel. Er bestaan meerdere theorieën, maar de precieze mechanismen van chronische nierschade zijn nog niet volledig opgehelderd. Nieuwe studies tonen aan dat zogenaamde “ouderdomscellen” (senescente cellen) een belangrijke rol spelen bij chronische nierschade.

Ontstaan van senescente niercellen

Senescente cellen kunnen ontstaan ten gevolge van zowel acute nierschade (bijvoorbeeld door zuurstoftekort bij niertransplantatie) en bij chronische nierschade (bijvoorbeeld door veroudering van niercellen). Bij beide vormen van nierschade raken niercellen beschadigd, meestal in de vorm van DNA schade. De meeste beschadigde niercellen die onherstelbare DNA schade oplopen gaan over tot geprogrammeerde celdood (apoptose). Om deze gestorven niercellen te vervangen gaan omringende, onbeschadigde niercellen zich delen (regeneratie). Echter, in de cellen met DNA schade die wél zijn blijven leven wordt de celdeling permanent stopgezet. Deze cellen stoppen met het doorlopen van de celcyclus (celcyclus arrest) om celdeling en daarmee het doorgeven van DNA schade aan nieuwe cellen te voorkomen. Deze beschadigde cellen zijn gevangen in een permanente toestand waarin ze niet meer kunnen delen en niet meer goed functioneren als normale niercellen. Omdat deze cellen niet opgeruimd worden door apoptose, blijven ze wel in de weg zitten. Ze scheiden bovendien signaalstoffen uit die ontsteking en verlittekening van nog gezond naburig nierweefsel veroorzaken. Dit leidt tot minder functionerend nierweefsel en verdere verslechtering van de nierfunctie.

Normaal gesproken zijn de signaalstoffen die senescente cellen uitscheiden nuttig, omdat ze het afweersysteem aansporen om opgeruimd te worden (immuunrespons). Als een cel door bijvoorbeeld zuurstoftekort onherstelbare DNA schade oploopt, maar niet genoeg om in apoptose te gaan, zal deze beschadigde cel senescent worden en via signaalstoffen afweercellen aantrekken om opgeruimd te worden en zo naburig weefsel toestaan om te herstellen. Een verstoord immuunsysteem als gevolg van veroudering, ziekte of afweer onderdrukkende medicatie kan er echter voor zorgen dat senescente cellen deze eliminatie ontwijken, zich opstapelen in organen en schadelijk signaalstoffen blijven produceren. Doordat veroudering gepaard gaat met DNA foutjes stapelen senescente cellen zich naarmate we ouder worden in diverse organen in kleine getalen op. Daarom zijn we ze senescente cellen – van het Latijn voor verouderen – gaan noemen.

In de nieren hopen senescente cellen zich op door veroudering en bij nierziekten. (3) Recent wetenschappelijk onderzoek naar veroudering in muizen heeft aangetoond dat het regelmatig opruimen van senescente cellen de leeftijd en gezondheid kan verlengen. (4, 5) Bovendien hebben deze muizen een betere nierfunctie en minder verlittekening van de nieren. Hieruit blijkt dat senescence een nadelig effect heeft op de nieren bij veroudering. Het effect van senescence in de nieren is ook getest in experimentele muismodellen van nierschade die representatief zijn voor nierziekten bij mensen. Hieruit blijkt dat senescence cellen gunstige effecten uitoefenen in de periode kort na het ontstaan van nierschade en nadelige effecten op de lange termijn. Dit tijdsafhankelijke effect van senescence is mogelijk te verklaren doordat senescente cellen op de korte termijn regeneratie stimuleren, maar op de lange termijn een omgeving met nog meer senescente cellen en schadelijke signaalstoffen stimuleren (Figuur 1).



Figuur 1. Celschade van niercellen ten gevolge van ziekte en veroudering (A) leidt tot geprogrammeerde celdood (apoptose) (B) en senescence (C). Na schade hebben senescente niercellen via signaalstoffen in de vroege fase een positief effect op hun omgeving door stimulatie van celdeling (regeneratie) (D), maar juist een schadelijk effect in de late fase door nóg meer senescence van naburige niercellen (E).

Elimineren van senescente niercellen

Momenteel worden medicijnen die het aantal senescente cellen verminderen (zogenaamde senolytica) getest op hun bruikbaarheid voor oude of zieke patiënten. Deze medicijnen hebben als doel senescente cellen te elimineren. Het kan echter nog jaren duren voordat senolytica door artsen voorgeschreven kunnen worden. Senescente cellen hebben veel gemeen met kankercellen, omdat ze net als kankercellen beschikken over "overlevingsnetwerken". Deze netwerken zorgen ervoor dat de cellen apoptose kunnen weerstaan. Dit betekent dat medicijnen die apoptose uitlokken in kankercellen, ook gebruikt zouden kunnen worden voor verwijdering van senescente cellen. Oftewel: bestaande kanker medicijnen kunnen dienen als senolytica. Deze medicijnen kunnen momenteel nog niet worden toegepast vanwege bijwerkingen op andere organen, maar deze bijwerkingen kunnen in de toekomst mogelijk voorkomen worden door senolytica alleen op niercellen toe te passen middels gerichte afgifte in de nieren. Dit wordt ook wel targeting genoemd. Het idee van targeting is simpel: de juiste cellen worden op het juiste moment aangepakt. Dit kan bijvoorbeeld door medicijnen in te pakken in speciale omhulsels die door niercellen worden herkend en alleen hieraan binden. Zo kan een senolytisch medicijn gericht én in de juiste dosering in de niercellen terecht komen, en worden bijwerkingen op andere organen voorkomen.

Kortom, senescente niercellen dragen bij aan het ontstaan en de progressie van chronisch nierfalen, zowel bij nierziekten als veroudering, en het elimineren van deze niercellen is een veelbelovende behandeling voor chronische nierpatiënten.

From bench to bedside
Biomedisch onderzoek zoals beschreven in deel 1 van dit proefschrift kan nieuwe interessante inzichten opleveren over ziektes en/of ziekteprocessen. Voordat patiënten hier in de praktijk ook daadwerkelijk baat bij hebben moet er echter nog een vertaalslag (translatie) gemaakt worden. Translationele geneeskunde (translational medicine) is de (bio)medische wetenschap die zich bezighoudt met de translatie van (fundamenteel) biomedisch onderzoek naar de patiënt, en vice versa. (6) Het doel van translationele geneeskunde is om de gezondheidszorg te verbeteren door onder andere nieuwe wetenschappelijke inzichten uit het lab (bench) sneller naar de patiënt (bedside) te brengen en wetenschappelijk onderzoek voor patiënten met zeldzame, onbegrepen ziektes mogelijk te maken. (7) Translationale geneeskunde vereist een optimale samenwerking en communicatie tussen verschillende disciplines (interdisciplinair) en een efficiënte wisselwerking tussen de hoofddomeinen van een academisch centrum: wetenschappelijk onderzoek, onderwijs en kliniek.

Onderzoek naar ernstige aandoeningen die zeldzaam zijn en waarvoor geen behandeling bestaat (weesziekten) wordt bemoeilijk door financiële problemen. Door de lage prioriteit van medische centra en de farmaceutische industrie voor wetenschappelijk onderzoek naar weesziekten is het verkrijgen van steun en middelen voor het ontdekken van behandelingen erg moeilijk. Een voorbeeld is de zeldzame nierziekte KIN (karyomegale interstitiële nefropathie) die optreedt bij kinderkanker patiënten die zijn behandeld met zware chemo. Het koppelen van bachelor biomedisch onderwijs aan translationeel onderzoek in samenwerking met ervaren onderzoekers, artsen en patiënten, maakt onderzoek naar weesziekten mogelijk doordat universiteit en patiëntenverenigingen bijdragen aan de kosten. Daarnaast wordt er synergie gecreëerd tussen onderzoek, onderwijs, kliniek en maatschappij. Studenten leren in een op onderzoek gebaseerde onderwijsomgeving, en vergaren nieuwe inzichten die nuttig zijn voor onderzoekers, artsen en patiënten.

Inhoud proefschrift

Dit proefschrift bestaat uit twee delen. Het eerste deel beschrijft biomedisch onderzoek en het tweede deel onderwijskundig onderzoek.

Deel 1:

In deel 1 van dit proefschrift wordt ingegaan op de rol van senescente niercellen in chronische nierschade. Er wordt ingegaan op factoren die van invloed zijn op het ontstaan van senescente niercellen en op mogelijke behandelingen die aangrijpen op mechanismes die lijden tot senescence en deze celstaat in stand houden.

Hoofdstuk 2 beschrijft wat senescente cellen zijn, waar ze voorkomen in de nier, door welke typen nierschade ze ontstaan en bij welke nierziektes ze voorkomen. Hier wordt toegelicht dat DNA schade, en een gebrekkige reactie van de beschadigde cellen om deze DNA schade te repareren, een belangrijke trigger is voor het ontstaan van senescente cellen.

Hoofdstuk 3 beschrijft dat ischemie-reperfusie (IR) schade, een veelgebruikt schademodel om chronische nierschade mee te bestuderen in proefmuizen, leidt tot senescente cellen. Bovendien wordt hier aangetoond dat remming van de signaalstof Cellular Communication Network factor 2 (CCN2; cellulaire communicatie netwerk factor 2), een molecuul dat betrokken is bij verlittekening van de nier, zorgt voor minder senescente niercellen en minder verlittekening van de nier ten gevolg van IR schade. In **Hoofdstuk 4** wordt hier een mogelijke verklaring voor gegeven door te kijken naar het effect van CCN2 remming in de vroege fases van IR schade. Kort nadat de muizen IR schade ondergaan, komen er schadelijke zuurstofradicalen vrij die zorgen voor DNA schade. Remming van CCN2 zorgt voor minder schadelijke zuurstofradicalen en daaropvolgende DNA schade. Kortom, remming van CCN2 bij IR schade kan bijdragen aan minder senescente cellen en minder verlittekening van de nieren. **Hoofdstuk 5** laat zien dat niet alleen moleculaire factoren zoals CCN2, maar ook leeftijd bijdraagt aan een hevige response op nierschade. Uit de vergelijking van de response op nierschade tussen jonge en oude muizen blijkt dat het nierweefsel van oude muizen heviger is aangedaan, met daarnaast ook o.a. meer DNA schade en senescente cellen. Dit verklaart mogelijk waarom oudere patiënten gevoeliger zijn voor acute nierschade. In **Hoofstuk 6** laten we zien dat verlittekening van de nier ook optreedt ten gevolge van veroudering, en processen die hierbij betrokken zijn op verschillende momenten worden geactiveerd. Eerst ontstaan DNA schade en ontsteking, en pas op nóg latere leeftijd worden cellen volledig senescent met verlittekening van de nier. Dit ondersteunt de gedachte dat senescente cellen CKD door veroudering veroorzaken.

Om medicijnen te ontwikkelen die zich richten op senescente niercellen (senotherapeutische geneesmiddelen) kan een screeningsmodel voor geschikte kandidaten helpen. Voordat deze medicijnen worden getest op muizen kan hun werking eerst getest worden in een kweek met niercellen. In **Hoofdstuk 7** wordt beschreven hoe een kweekmodel met ciPTEC niercellen gebruikt kan worden om senescente niercellen te bestuderen en voor screening van senotherapeutische geneesmiddelen. **Hoofdstuk 8** geeft een uitgebreid overzicht van in de literatuur beschreven geneesmiddelen met senotherapeutische potentie. Er worden daarnaast aanbevelingen gedaan hoe eventuele bijwerkingen voorkomen kunnen worden door senotherapeutische geneesmidellen specifiek te laten aanrijpen op senescente niercellen.

In **Hoofdstuk 9** tonen we aan dat senescente niercellen ook voorkomen in overlevenden van kinderkanker die door hun behandeling de zeldzame nierziekte KIN ontwikkelen. Dit zou nieuwe mogelijkheden voor behandeling kunnen geven, maar medicijn onderzoek/ontwikkeling voor zeldzame ziektes als KIN wordt belemmerd door gebrek aan geld en interesse. **Hoofdstuk 10** beschrijft een voorstel om dit probleem op te lossen door KIN te bestuderen in een op onderzoek gebaseerde cursus, waarin studenten hun eigen onderzoek naar KIN opzetten en uitvoeren, en de universiteit en patiëntenverenigingen voorzien in de kosten. Hierin werken bachelor studenten samen met ervaren wetenschappers, artsen uit aanverwante disciplines (o.a. Pediatrische Nefrologie, Genetica

en Pathologie), patiënten en patiëntenverenigingen om inzicht te krijgen in de onderliggende mechanismen van KIN.

Deel 2:
Deel 2 van dit proefschrift beschrijft onderwijskundig onderzoek naar het effect van op onderzoek gebaseerde cursusconcepten op het leren van studenten. **Hoofdstuk 11** beschrijft het onderzoek gebaseerde cursusontwerp en de evaluatie van twee interdisciplinaire laboratorium cursussen waarin bachelor studenten een volledige onderzoekscyclus doorlopen. **Hoofdstuk 12** beschrijft het cursusontwerp en de evaluatie van een soortgelijke laboratoriumcursus die volledig gericht is op het uitvoeren van laboratorium onderzoek. In **Hoofdstuk 13** wordt beschreven hoe en waarom het geven van onderwijs en het doen van onderzoek gecombineerd kunnen worden in een hybride promotietraject.

Hoofdstuk 14, ten slotte, is een overkoepelende bespreking van de nieuwe inzichten die in dit proefschrift zijn verkregen, inclusief implicaties en mogelijkheden voor follow-up van de biomedische onderzoeksinzichten, en implementatie en follow-up van de onderwijskundige onderzoeksresultaten.

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2. Volksgezondheidenzorg.info. Ranglijst ziekten op basis van zorgkosten. 2018. Geraadpleegd op 7 februari 2018 via: https://www.volksgezondheidenzorg.info/ranglijst/ranglijst-ziekten-op-basis-van-zorgkosten.
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4. Baker DJ, Childs BG, Durik M et al. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. Nature. 2016; 530: 184–189.
5. Baar MP, Brandt RMC, Putavet DA et al. Targeted apoptosis of senescent cells restores tissue homeostasis in response to Chemotoxicity and aging. Cell. 2017; 169:132–147.e16.
6. Albani S, Prakken B. The advancement of translational medicine-from regional challenges to global solutions. Nat Med. 2009;15(9):1006-1009. doi:10.1038/nm0909-1006 [19734876]
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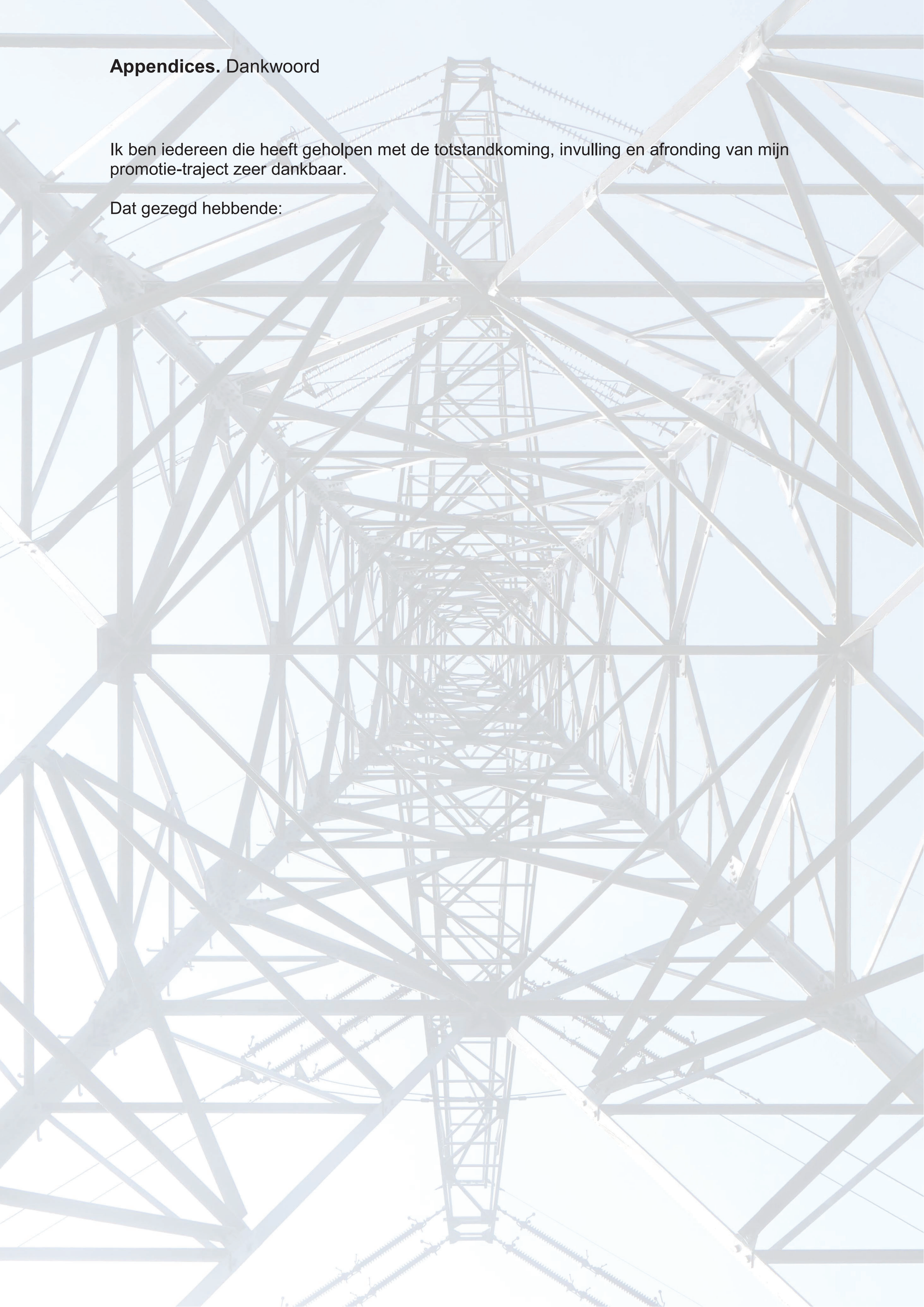

Appendices. Dankwoord

Ik ben iedereen die heeft geholpen met de totstandkoming, invulling en afronding van mijn promotie-traject zeer dankbaar.

Dat gezegd hebbende:

APPENDICES
Curriculum Vitae

RESEARCH PROFESSIONALISATION

Certificates/competences

2019: Art 9. Wet op de dierproeven certificaat, Universiteit Utrecht

2018: ML-II bevoegdheid voor werken met genetisch gemodificeerde micro-organismen, Farmaceutische Wetenschappen, David de Wied, Universiteit Utrecht

Courses

General scientific courses:

2020: PhD Activating Career Event (0.25 EC)

2020: Statistics for in the Lab: in vivo et in vitro (1.5 EC)

2020: This thing called science (2.0 EC)

2019: Writing a scientific paper (1.5 EC)

2019: GSLS PhD Day 2019 (0.3 EC)

2019: The art of presenting science (0.3 EC)

2019: Winter school Nefrologie, Nierstichting (1.45 EC)

2018: Cursus proefdierkunde (3.0 EC)

2018: Summer school Translational Medicine: Doing the Right Research Right (1.8 EC)

Regenerative Medicine PhD programme courses

2019: Introduction to RM (1.5 EC)

2019: Perspectives in RM (0.6 EC)

2018: Scientific publishing course by EMBO (0.25 EC)

2018: Enabling technologies (1.0 EC)

CONFERENCE PRESENTATION

2021 Oral presentation, ASN Kidney Week, San Diego, United States.
CTGF aggravates the Oxidative stress-DNA damage-Cellular senescence sequence following renal IRI

2021 Oral presentation, Nederlandse Nefrologiedagen, virtual conference
CCN2 in Renal Ischemia Reperfusion Injury: Direct effects on Oxidative stress-DNA damage response

2021 Oral presentation, Nederlandse Nefrologiedagen, virtual conference
CCN2 in Renal Ischemia Reperfusion Injury: Role in development of Cellular Senescence and Fibrosis

2021 Short communication, ERCSG 2021, virtual conference
CCN2 aggravates acute DNA damage and the subsequent DDR-Senescence-Fibrosis sequence following renal IRI.

2019 Poster presentation, ASN Kidney Week, Washington DC, Unites States. *CTGF/CCN2 knockdown prevents AKI-induced cellular senescence and subsequent fibrosis*

PUBLICATIONS

Valentijn FA, Knoppert SN, Marquez-Exposito L, Rodrigues-Diez RR, Pissas G, Tang J, Tejedor-Santamaria L, Broekhuizen R, Samarakoon R, Eleftheriadis T, Goldschmeding R, Nguyen TQ, Ruiz-Ortega M, Falke LL. Cellular communication network 2 (connective tissue growth factor) aggravates acute DNA damage and subsequent DNA damage response-senescence-fibrosis following kidney ischemia reperfusion injury. *Kidney Int.* 2022 Jul 31:S0085-2538(22)00553-1. doi: 10.1016/j.kint.2022.06.030.

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Valentijn FA, Falke LL, Nguyen TQ, Goldschmeding R. Cellular senescence in the aging and diseased kidney. *J Cell Commun Signal*. 2018;12(1):69-82. doi:10.1007/s12079-017-0434-2

AWARDS

2021: **Best basic science abstract**, Nederlandse Nefrologiedagen

2018: **Talma Eykman thesis award**, UMC Utrecht

COMMUNITIES

2018 – 2021: Alexandre Suerman PhD/MD-traject, UMC Utrecht
UMC Utrecht talentprogramma gekoppeld aan een beurs voor wetenschappelijk onderzoek en een mentorprogramma met masterclasses gericht op persoonlijke en wetenschappelijke groei en ontwikkeling

2020-2021: Young Science in Transition, UMC Utrecht
Thinktank of young researchers developing ideas and organizing events for open science, team science and recognition and rewards

ORGANIZATION

2020: Mini-symposium *Team science and Rewards*, Young Science in Transition, UMC Utrecht

2020: Masterclass *‘Talent meets talent’ with Michael Dudok de Wit*, Alexandre Suerman PhD/MD-traject, UMC Utrecht

DIDACTIC PROFESSIONALISATION

Certificates

2021: Basiskwalificatie Onderwijs (BKO)

Courses

2021: Providing Effective Supervision in the Lab, Training for lab supervisors, Educational Consultancy & Professional Development, Universiteit Utrecht

2021: Toetsing I; toetsvragen ontwikkelen, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding, UMC Utrecht.

2021: Bias training voor docenten, Training voor dLAB docenten, Platform Diversiteit en Inclusie, UMC Utrecht.

2020: Online onderwijs geven, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding, UMC Utrecht.

2020: Blended learning, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding, UMC Utrecht.

2020: Coachende gespreksvoering, Training voor tutoren en mentoren, Onderwijscentrum UMC Utrecht.

2019: Activerende Werkvormen, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding.

2019: Basistraining Didactiek, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding, UMC Utrecht.

2018: Onderwijs in kleine groepen, Trainingen voor de docent, Expertisecentrum voor Onderwijs en Opleiding.

Teaching activities

2020-2021: Docent onderwijs evaluator (SoTL), BRL cursus, Pathologie cursus en Experimental Translational Medicine cursus, bachelor Biomedische wetenschappen en Geneeskunde, UMC Utrecht
werkzaamheden: opzetten en wetenschappelijke evaluatie van de laboratorium cursussen BRL, Pathologie en ETM volgens de principes van 'Scholarship of Teaching & Learning (SoTL)

2020/10+11: Werkgroep docent, Pathologie cursus, bachelor Biomedische wetenschappen, UMC Utrecht
werkzaamheden: begeleiden van studenten bij het bedenken en uitwerken van een onderzoeksvoorstel o.b.v. een klinisch relevante casus

2016-2021: Practicum docent, practica Nefropathologie, Circulatie III bachelor en SUMMA Geneeskunde, UMC Utrecht
werkzaamheden: doceren van praktische beoordeling van een nierbiopt d.m.v. plenair inleidend college van een casus/nierbiopt – individuele begeleiding van studenten bij beoordeling van nierbiopten – plenair college met nabespreking (ca 50 studenten per groep).

2018-2021: Tutor, studieonderdeel Studiereflectie en Tutoraat, bachelor Geneeskunde, UMC Utrecht

werkzaamheden: voorbereiden en begeleiden van werkgroep- en individuele bijeenkomsten, met focus op professionele ontwikkeling, reflectie en studievoortgang (ca 12 studenten/groep in werkgroep onderwijs en individuele begeleiding van studenten)

2020/09+10: Werkgroep docent, Circulatie III, jaar 2 bachelor Geneeskunde, UMC Utrecht
werkzaamheden: voorbereiden en begeleiden van werkgroep bijeenkomsten, met focus op nier- en longziekten (ca 15 studenten/groep)

2020/01/22: Werkgroep docent, KLO Werkgroep Casus 1.2. Keelpijn, jaar 1 bachelor Geneeskunde, UMC Utrecht
werkzaamheden: voorbereiden en begeleiden van een eenmalige werkgroep bijeenkomst, met focus op klinisch redeneren (ca 12 studenten/groep)

2018: Werkgroep docent, startblok Vorm & Functie, jaar 1 bachelor Geneeskunde, UMC Utrecht
werkzaamheden: voorbereiden en begeleiden werkgroep bijeenkomsten, met focus op de basisbeginselen van Anatomie en Fysiologie en contextueel denken (ca 12 studenten/groep)