INTERACTIONS between the **kidney** and its **environment**

chronic intervention studies

Colofon

The research described in this thesis was supported by a grant from the Netherlands Organisation for Scientific Research (NWO. VIDI grant 016.096.359).

Cover illustration & book design: Ilse Schrauwers - isontwerp.nl
Printed by: Gildepint.nl
ISBN: 978-94-92303-00-4

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Interactions between the kidney and its environment

chronic intervention studies

Interacties tussen nier en omgeving:
chronische interventie studies
(met een samenvatting in het Nederlands)

Взаимодействие между бъбреците и системната среда:
хронични интервенции
(с резюме на български)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen

op vrijdag 16 oktober 2015 des ochtends te 10.30 uur

door

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geboren op 1 december 1984
te Gotse Delchev, Bulgarije

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Copromotor:
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Contents

List of abbreviations

ACE1	angiotensin converting enzyme 1
ADP	adenosine diphosphate
AdrB2	beta-2 adrenergic receptor
ANP	atrial natriuretic peptide
AT1	angiotensin II receptor type 1
ATP	adenosine triphosphate
BA	bilateral ablation
BM	bone marrow
BMC	bone marrow cells
BNP	brain natriuretic peptide
BOLD-MRI	blood oxygen level-dependent magnetic resonance imaging
BP	blood pressure
BSA	bovine serum albumin
BW	body weight
CI	confidence interval
CKD	chronic kidney disease
Cx43	connexin43
CTGF	connective tissue growth factor
CV	conduction velocity
CVD	cardiovascular disease
DGF	delayed graft function
DMEM	Dulbecco's modified Eagle medium
DN	diabetic nephropathy
DOCA	deoxycorticosterone acetate
ECD	expanded criteria donors
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
ESKD	end-stage kidney disease
FE	fractional excretion
GDF	growth differentiation factor
GDP	guanosine diphosphate
GFP	green fluorescent protein
GFR	glomerular filtration rate
GS	glomerulosclerosis
HMGCR 3	hydroxy-3-methyl- glutaryl Coenzyme A reductase
HW	heart weight
I/R	ischemia/reperfusion
IF/TA	interstitial fibrosis/tubular atrophy
IRI	ischemia-reperfusion injury
KW	kidney weight
L-NNA	(omega)-nitro-L-arginine
LK	left kidney
LV	left ventricle
LVH	left ventricular hypertrophy
LVMI	left ventricle mass index

MAP	mean arterial pressure
MCP-1	monocyte chemotactic protein 1
MDCT	multi-detector computed tomography
MSC	mesenchymal stem cells
NO	nitric oxide
NOS	nitric oxide synthase
NOx	stable nitric oxide metabolites
PAH	para-aminohippuric acid
PAS	periodic acid Schiff
PEG	polyethylene glycol
PFA	paraformaldehyde
PKB	protein kinase B
RAS	renin-angiotensin system
RBC	red blood cells
RBF	renal blood flow
RECA	rat endothelial cell antigen
REML	restricted maximum likelihood estimation
RK	right kidney
ROS	reactive oxygen species
RPF	renal plasma flow
RV	right ventricle
RVR	renal vascular resistance
SBP	systolic blood pressure
SCD	standard criteria donors
SD	standard deviation
SDF1α	stromal derived factor 1α
SE	standard error
SHR	spontaneously hypertensive rats
sICAM	soluble intercellular adhesion molecule
SMD	standardized mean difference
SNx	subtotal nephrectomy (5/6 nephrectomy)
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TMRM	tetramethylrhodamine methyl ester
TH	tyrosine hydroxylase
TI	tubulo-interstitial injury
TNFα	tumor necrosis factor α
TPE	total protein excretion
TX	transplantation
UCP	uncoupling protein
VEGF	vascular endothelial growth factor
VT	ventricular tachyarrhythmia

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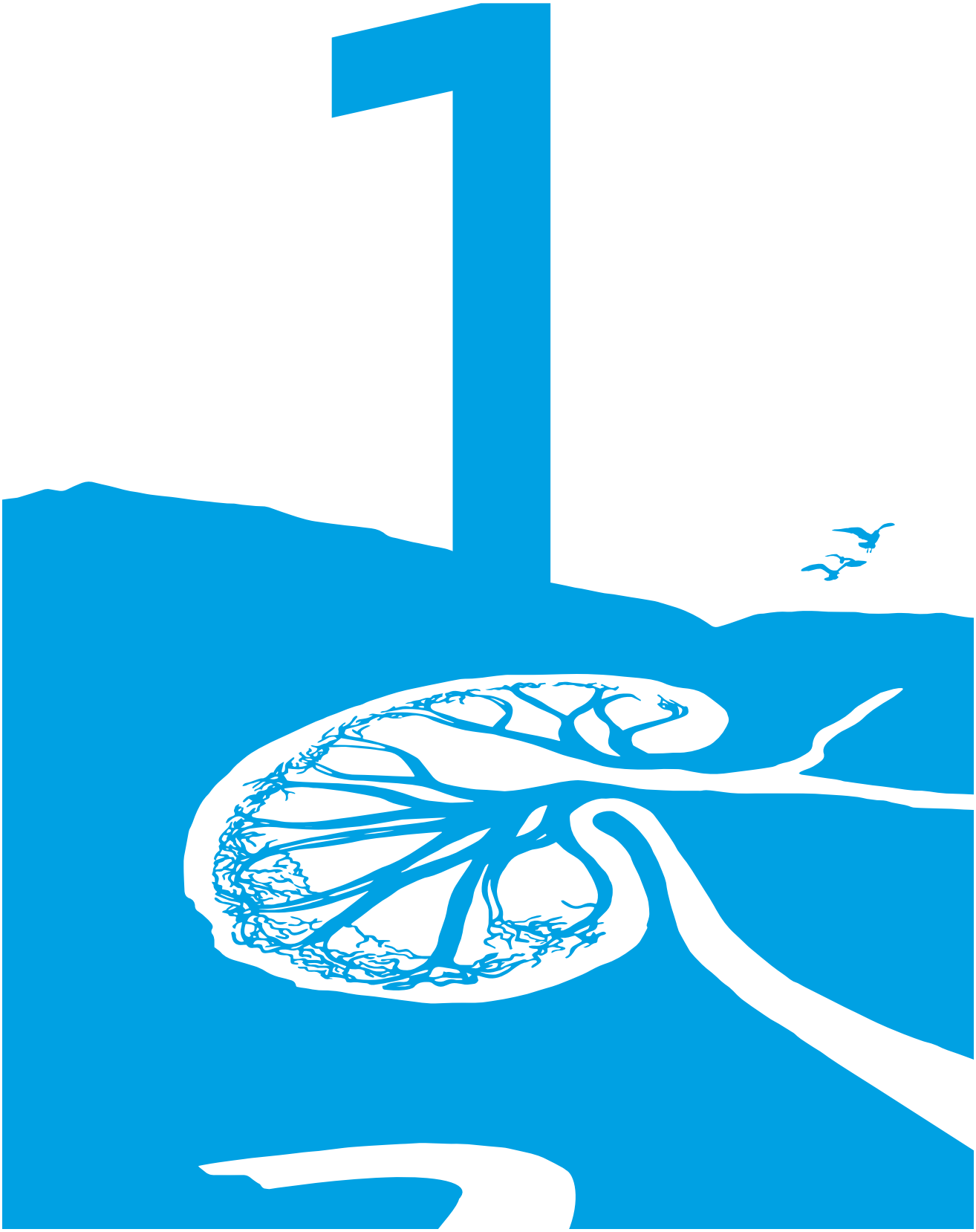

Introduction and outline of this thesis

Introduction

Chronic kidney disease (CKD) is a progressive condition causing significant morbidity and mortality. Worldwide the number of patients with CKD is growing, mainly due to a dramatic increase in atherosclerosis and diabetes[1] with a consequent rise in the number of patients with end stage kidney disease (ESKD) requiring renal replacement therapies.

Progression of CKD: influence of inflammation and oxidative stress

The increasing incidence of CKD cannot be fully explained by traditional risk factors such as hypertension and diabetes (Figure 1). Studies have demonstrated that relative to healthy counterparts oxidative stress and inflammation are significantly elevated in CKD patients, and that these contribute to CKD progression. Oxidative stress develops from an imbalance between free radical production, often increased through dysfunctional mitochondria[2], and reduced anti-oxidant defences. The primary pathological mechanism that links oxidative stress, inflammation, and CKD progression is characterized by injury in the kidney due to the activities of intra- and extracellular oxygen-

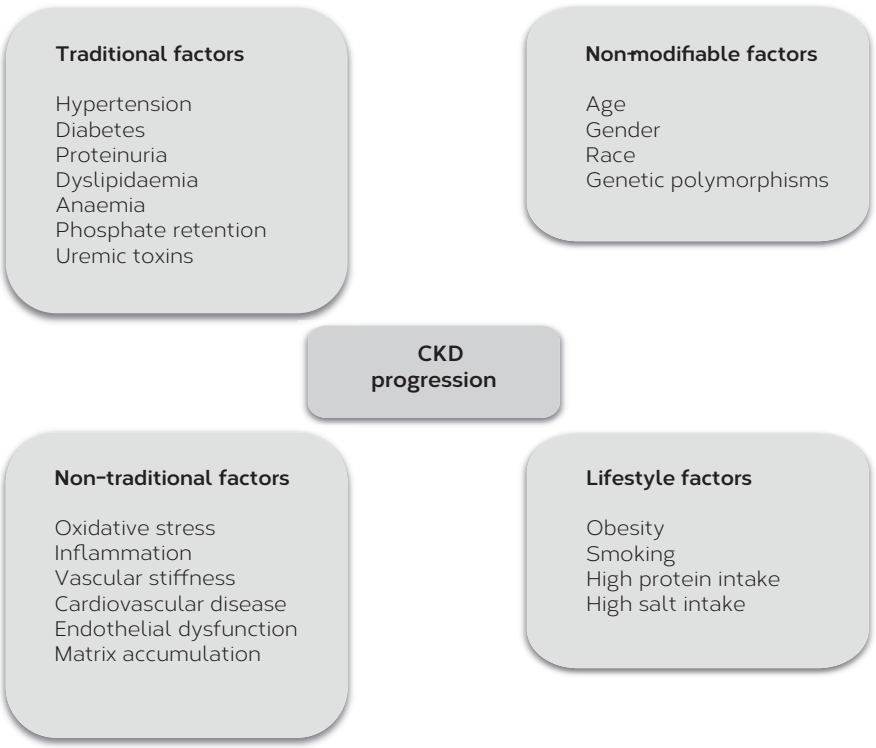

Figure 1. *Factors that influence CKD progression.*

derived radicals and the resultant inflammatory response. In addition, increased oxidative stress deteriorates endothelial function[3]. Endothelial dysfunction contributes to the development of hypertension and progression of CKD[4]. Thus, reducing oxidative stress and inflammation and improving endothelial cell health in patients with CKD are promising therapeutic strategies[5].
Morbidity and mortality in the CKD and ESKD population is increased mainly due to cardiovascular (CV) diseases, with sudden cardiac death due to arrhythmia being one of the most common reasons [6]. Sudden cardiac death is defined as an unexpected death from a cardiovascular cause with or without structural heart disease, arising from arrhythmia such as fibrillation. Mechanisms that lead to sudden cardiac death in CKD are still largely undefined.

New therapies for CKD

Halting the progression of CKD is a major health care priority. The current treatment for CKD is mainly symptomatic and there are no specific strategies to halt its progression. Cell therapies represent an attractive therapeutic strategy and several promising results from preclinical and phase 1 studies have been published in the last years. However, the design of preclinical studies is very diverse, varying in terms of models of CKD, timing of interventions, cell type or cell product, number of cells, administration route and read-out of kidney function and morphology. Furthermore, there is growing evidence that the systemic environment affects cells and can impair the therapeutic efficacy of (autologous) cell therapy. We and others have shown that the therapeutic efficacy of cell therapies is impaired by age and disease state [7,8], pointing out the importance to investigate ways to treat/restore cell dysfunction.

Kidney transplantation outcome: importance of donor, recipient, and ischemia/reperfusion injury

Kidney transplantation is the best treatment for ESKD patients. Thanks to the fast improvement in the field in the last few decades, including improved surgical techniques, immunosuppression and screening, it is possible nowadays to perform incompatible kidney allo-transplantations and transplant successfully kidneys from living-related and unrelated donors, and donors after brain or cardiac death. However, two main problems for CKD patients remain: shortage of donors and limited survival of the renal grafts. Although the short-term results of kidney transplantation have improved over the past 20 years, the long- term results have improved either minimally or not at all[9]. The donor and recipient quality and transplantation associated events such as ischemia/reperfusion (I/R) are possible explanations for this observation.

Donor factors

There is significant variability in the quality of donor kidneys that are used for transplantation. The quality of the donor kidney has a direct effect on clinical outcomes such as acute rejection, delayed graft function, and patient and allograft survival. Kidney grafts can be derived from deceased donors or from living donors. Deceased donors are further divided into standard criteria donors (SCD) and expanded criteria donors (ECD). ECDs refer to older kidney donors (≥60 yr) or donors who are aged 50 to 59 yr and have two of the following three features: hypertension, terminal serum creatinine >130 μmol/l, or death from cerebrovascular accident. Increasing numbers of patients on the waiting list is the reason to widen the pool of donors and ECD. Transplantation from ECDs has a poorer short-term outcome than transplantation from SCDs[10]. Nevertheless, despite reports demonstrating that ECD have a higher rate of delayed graft function and a greater susceptibility to preservation injury as well as drug toxicity, the long-term outcome is quite satisfactory[11,12].

The non-immunological reasons for superiority of living donor kidneys include the precise evaluation of kidney donor function prior transplantation and short ischemic damage. The living donors can be further divided into (un) related, (un)directed anonymous, and (multiple) paired exchange living donor. The disparity between donor kidney availability and demand has increased utilization of kidneys from marginal living donors [13]. The term 'marginal living donors', also called 'complex living donors' [14], is used to name the ECD but in living situation. These are living donors with hypertension, proteinuria, haematuria, elevated body mass index, dyslipidaemia, advanced age, nephrolithiasis. Data on long-term outcome when using marginal living donors are scarce and careful evaluation of long-term outcomes is needed.

Recipient factors

The key recipient factors that influence outcome after kidney transplantation include their age, recurrence of native disease, dialysis, and cardiovascular comorbidities. Premature CV death with a functioning graft is one of the leading factors in reducing long-term graft survival overall. The annual risk for CV death in transplant recipients remains 50-fold higher than in the general population[15]. The impact of kidney transplantation on recipient aortic stiffness remains poorly defined. Short-term improvement after living-donor kidney transplantation[16] and no change at 1 year after cadaveric-kidney transplantation[17] have been described.

Ischemia/reperfusion

Ischemia–reperfusion is a complex pathophysiological process involving hypoxia and/or re-oxygenation, ionic imbalance-induced edema and acidosis, oxidative stress, mitochondrial uncoupling and endothelium activation[18]. The total ischemia time of a graft can be divided in the following consecutive periods of time: short warm ischemia during organ retrieval, cold ischemia associated with preservation and storage, warm ischemia time during transplantation. Ischemia of the kidneys (like that of other tissues) deprives cells of adenosine triphosphate (ATP), as a result of which the cells are then unable to maintain essential homeostatic processes. This ultimately leads to cell death by apoptosis or necrosis if timely reperfusion does not occur. Reperfusion injury is multifactorial and is partly attributable to rapid re-oxygenation of hypoxic tissues, resulting in oxidative damage, and calcium overload because of loss of ion pump homeostasis. The main consequences of renal ischemia–reperfusion are kidney graft primary non-function and delayed graft function (DGF) or chronic graft dysfunction, all of which involve a mandatory return of the patient to dialysis. Mitochondria play a pivotal role in the IRI process, by participating in energy production and generation of ROS (figure 2). Targeting

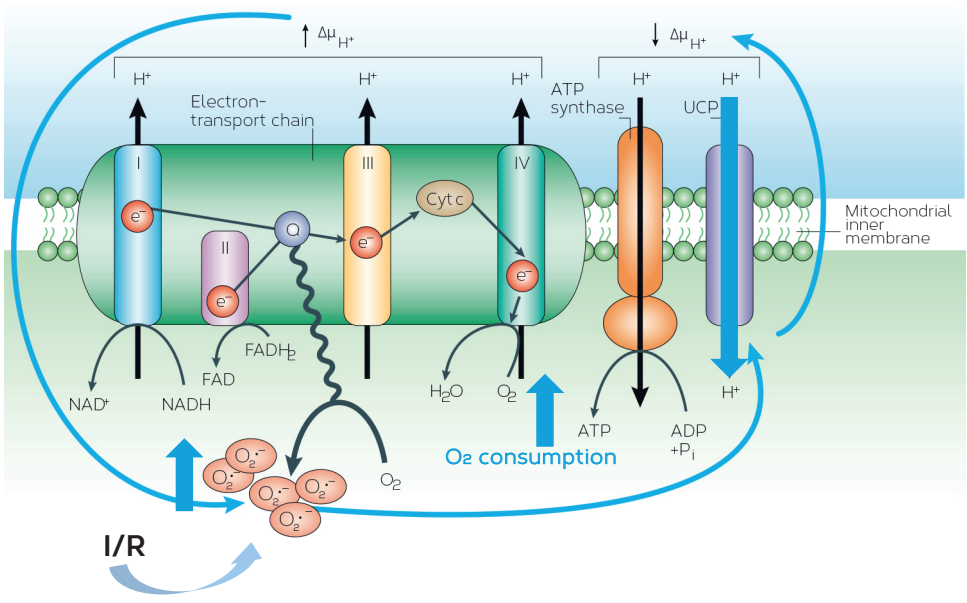

Figure 2. *Schematic view of mitochondrial inner membrane and electron transport chain. Superoxide activation of the UCP-2 after ischemia/reperfusion (I/R).*

Adapted from Krauss et al. Nature Reviews Molecular Cell Biology 2005.

mitochondria's overproduction of ROS during I/R could be a promising preventive strategy to avoid chronic graft dysfunction.

Conclusion

Most of the understanding in the pathophysiology in kidney transplantation comes from animal models using healthy donors and recipients that fail to reproduce the complexity of factors involved in the human situation, notably, the influence of the CKD environment, inflammation and oxidative stress on the graft. Experiments in parabiotic pairings have stressed the importance of the systemic environment for muscle and liver regeneration[19], as well as reversal of cardiac hypertrophy[20]. Furthermore, a clinical report described reversal of diabetic nephropathy in human cadaver kidneys after transplantation into non-diabetic recipients[21]. If true for CKD, the environment in CKD patients (characterized by inflammation, oxidative stress and vascular stiffness) receiving a kidney allograft might influence the transplantation outcome and improving this environment could have beneficial effects on the donor kidneys.

Aim and outline of this thesis

The aim of this thesis was to investigate the influence of systemic environment on the kidney, and vice versa, in the setting of chronic kidney disease and in the setting of kidney transplantation.

Kidney transplantation is known to be the best treatment option for patients with ESKD. The growing difference between decreasing supply of and increasing need for donors led to introducing the marginal living donors as a strategy providing more kidneys for transplants. However, no experimental kidney transplantation model has been described so far where a kidney from marginal living donors (with hypertension background, glomerular damage, etc) is used. Organ shortage demands fundamental research on marginal living donors including accurate predictors of function and injury prior to experimental transplantation. Traditional models of CKD always include uninephrectomy [22]. Avoiding uninephrectomy in ablation models would allow prediction of function and injury at the time-point of experimental transplantation. To this end we developed a novel bilateral renal ablation model that was staged by the level of proteinuria. Chapter 2 gives a detailed description of this model.

CKD is characterized by hypertension and concomitant oxidative and inflammatory systemic injury[23]. Oxidative damage as such is responsible for hypertension. While the presence of oxidative stress as a feature of CKD is well

established[24], its relation to hypertension and related hemodynamics in established experimental CKD has not been systematically addressed. In Chapter 3 we studied the interaction between oxidative damage and renal hemodynamics in long-term, established experimental CKD.

Cardiovascular disease (CVD) is the most common cause of mortality in patients with CKD[25], having important economical and public health implications. The most common causes of CV death are sudden cardiac death and heart failure[26] which differs from the general population. In Chapter 4 we investigated the underlying mechanisms for enhanced arrhythmogenicity in CKD in two mouse models.

The current treatment for CKD is mostly supportive and new therapies are needed. It has been demonstrated that anti-oxidative treatment has little effect on blood pressure and renal hemodynamics in established CKD. Cell-based therapy has proven to be a promising clinical approach for several pathological conditions and might represent a novel strategy to treat kidney disease[27]. These preclinical observations have already translated into pioneering clinical trials[28]. In Chapter 5 we performed a systematic review and meta-analysis to evaluate the efficacy of cell-based therapy in animal studies of CKD, and to determine whether local or systemic factors affect cell-based therapy efficacy.

Most cell-based therapy studies used cells or cell products derived from healthy animals. In the clinical situation, however, the use of autologous cells, exposed to the CKD/uremic environment, would be preferred. While administration of healthy bone marrow cells in a rat model of established CKD significantly reduced CKD progression, administration of cells obtained from a rat CKD bone marrow donor had a markedly attenuated effect suggesting a pivotal influence of the diseased environment on the efficiency of the bone marrow cells[8]. Pretreatment of the cells in order to improve their therapeutic efficacy might be useful for developing strategies for cell-based therapies for CKD. In Chapter 6 we aimed to improve the therapeutic efficacy of cells acquired from CKD donors.

Ischemia-reperfusion (I/R) is accompanied by an increased mitochondrial production of reactive oxygen species (ROS)[29] and is an inevitable event accompanying kidney transplantation. It is considered a common cause for delayed graft function (DGF) and acute renal failure, ultimately resulting in interstitial fibrosis/tubular atrophy (IF/TA, previously reported as chronic

allograft nephropathy)[30]. Mechanisms leading to DGF and IF/TA after renal transplantation are poorly understood, and, at present, we lack therapies to prevent I/R injury. In **Chapter 7** we studied the early events that accompany kidney transplantation, more specifically the hypoxia associated with IRI and that is preceded damage. Furthermore, we explored whether pre-treatment of the donor (environment) could preserve graft function. This study focuses on the 'ideal' kidney transplantation: healthy donor and recipient from the same strain with minimal ischemia time. That is why in **Chapter 8** we further explored the kidney graft and environment interaction in the presence of oxidative damage, inflammation, uremia and high blood pressure. We used the bilateral model described in chapter 2 and performed cross transplantation between healthy and CKD donors/recipients. Moreover, we investigated whether the healthy systemic environment could halt the progression of CKD.

Cardiac fibrosis with accompanying left ventricular hypertrophy (LVH), can mechanically impede electrical propagation which induces electrical instability leading to arrhythmias and sudden cardiac death[31]. Normalization of hypertension and correction of the uremic state in CKD patients receiving a healthy kidney allograft are known to reverse LVH[32]. However, effects on cardiac fibrosis, a characteristic of CKD, are unknown, as are effects of marginal donor kidneys on recipient LVH and cardiac fibrosis. In **Chapter 9** we studied the effect of the healthy or diseased graft (hypertension and uremic state) on left ventricular hypertrophy and cardiac fibrosis in kidney transplantation.

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Systemic Predictors of Single Kidney Function and Injury in a Bilateral Ablation Model of CKD in the Rat

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Abstract

Organ shortage demands fundamental research on marginal donors including accurate predictors of graft function and injury prior to experimental transplantation (TX). Our aim was to develop a novel marginal donor model involving bilateral renal injury and to identify systemic predictors of kidney function and injury at TX.

Male Lewis rats (8 weeks of age) underwent 2/3 ablation of each kidney in a one-step procedure (BA, n=20), followed by 6% salt and NOS-inhibitor (L-NNA) supplementation. L-NNA was withdrawn when proteinuria exceeded 200 mg/24h. Controls underwent bilateral sham surgery (CON, n=16). Urea and proteinuria were measured regularly. A terminal measurement, performed when proteinuria exceeded 100mg/24h in BA rats, included mean arterial pressure (MAP) measurement and split-urine collection for single kidney function (GFR: inulin and RPF: PAH) in left and right kidney (LK and RK). Glomerulosclerosis (GS) and tubulo-interstitial injury (TI) were scored. CON rats were age-matched.

Proteinuria, in comparison to MAP and urea, was the best predictor for GFR, RPF, TI and GS (r=-0.72; r=-0.63, r=0.81 and r=0.82 respectively, all P<0.001). Symmetry: LK-GS and LK-TI correlated strongly with RK-GS and RK-TI (r=0.79, P<0.001, r=0.89, P<0.001) respectively. These findings firmly position this new bilateral ablation model in the field of TX of marginal donors.

Introduction

Despite significant improvement in patient management, chronic kidney disease (CKD) is still a devastating illness with high rate of morbidity and mortality worldwide. Ultimately, CKD progresses to end-stage renal disease and requires renal replacement therapies. Understanding and dissecting the pathogenic mechanisms of kidney dysfunction in the setting of CKD are essential for finding new and more effective treatments or optimizing current renal replacement therapies.

Both CKD and kidney transplantation (TX) are characterized by complex derangements of homeostasis and therefore animal models are necessary to study their development and progression. Animal models of CKD provide opportunity to investigate disease-specific mechanisms, to investigate molecular pathogenesis, and assess potential novel therapies. The most used and well-know model of CKD is 5/6th (subtotal) nephrectomy (SNX) model that mimics the course of progressive renal failure irrespective of etiology in humans [1]. SNX rats develop systemic and glomerular hypertension, proteinuria, uremia and progressive sclerotic glomerular and tubular injury of the initially normal nephrons in the remnant. The natural history of this model depends on the methods used [2]. Approaches with infarction typically are associated with more severe and rapid development of proteinuria and hypertension than those with excision of renal mass. The more severe hypertension with ligation is probably due to marked upregulation of the renin-angiotensin system in the peri-infarct zone.

Organ shortage demands fundamental research on marginal donors including accurate predictors of function and injury prior to experimental TX. However, traditional models of CKD always include uninephrectomy. Symmetrical ablation, thus avoiding uninephrectomy, in ablation models would allow prediction of function and injury in the graft at the time-point of experimental TX, based on pre-TX function and contralateral injury, respectively.

The aim of this study was to develop a novel, bilateral ablation model of CKD for further transplantation studies. Furthermore, we investigated symmetry of injury and identified systemic predictors of single kidney function and injury.

Methods

Animals

Male inbred Lewis rats (Charles River Germany) were housed under standard conditions in a light-, temperature- and humidity-controlled environment. The protocol was approved by the Utrecht University Committee of Animal Experiments (DEC number 2010.II.05.097 and DEC number 2012.II.03.053).

CKD development

Rats at 8 weeks of age underwent 2/3 ablation of each kidney in a one-step procedure (bilateral ablation: BA, n=21). First, median laparotomy was performed and the branches of left and right renal artery were coagulated until 2/3 of each kidney turned ischemic. The intestines of the rat were gently moved aside on moist sterile gauze and were kept warm at 37°C. After coagulation, the abdomen was checked for bleeding and then was sutured. Controls underwent bilateral sham surgery (CON, n=16) by manipulating the branches of both renal arteries. The surgery was performed using isoflurane anaesthesia (5% induction, 1.5–2% maintenance). All rats received an intramuscular injection of analgesia directly and one day after surgery (Buprenorphine, 0.05 mg/kg). Development of CKD was accelerated with N(omega)-nitro-L-arginine (L-NNA), a NO-synthase inhibitor (50 mg/L) in drinking water[3,4] and by was supplementing standard powdered chow (CRM-FG; Special Diet Services Ltd., Witham, Essex, UK) with 6% NaCl until proteinuria exceeded 200 mg/day after a median of 8 weeks (range: 6–9 weeks). Subsequently L-NNA and supplemental NaCl were withdrawn causing proteinuria to initially fall and subsequently increase slowly [5].

Measurements

Urea and proteinuria were measured regularly. 24-h urine samples were collected weekly for determination of total protein excretion (TPE), with the rats in individual metabolic cages while fasting. Blood samples were collected from the tail vein. Plasma urea was determined by DiaSys Urea CT FS (DiaSys-Diagnostic Systems, Holzheim, Germany).

Terminal experiments were planned within a week when proteinuria exceeded 100 mg/day. This time point was reached after a median of 35 weeks (range: 22–56 weeks). This approach ensured that staging of CKD was similar in all rats. Timing of terminal experiments in sham-operated controls was determined by their age-matched CKD littermates. The terminal measurement included split-urine collection for single kidney function (glomerular filtration rate, GFR: inulin clearance and effective renal plasma flow, ERPF: PAH clearance) in left and right kidney (LK and RK). On the day of the experiment the trachea was intubated with a 16-G catheter (Venisystems, Abbocath-T, Abbott, Ireland) under isoflurane anesthesia (5% induction, 1.5–2% maintenance). The femoral artery was cannulated in order to obtain direct measurement of mean arterial pressure (MAP) and a Transonic flow probe was placed on the left renal artery to measure renal blood flow (RBF) [6]. Urine was collected allowing measurement of kidney function. During surgery, animals received

an intravenous infusion of a 150 mmol/L NaCl solution containing 6% bovine serum albumin (BSA) at a rate of 100 µl/kg/min. Following surgery, the infusion was switched to a 150 mmol/L NaCl solution with 1% BSA, containing inulin for measurement of GFR, which was maintained at the same infusion rate throughout the experiment. Following a 60 min equilibration period, after which both MAP and RBF signals were stable, data were collected for 30 min.

Renal morphology

Directly after performing the terminal experiment protocol, rats were sacrificed and tissues were collected and fixed in 4% paraformaldehyde for embedding in paraffin or were snap frozen. Glomerulosclerosis (GS) and tubulo-interstitial injury (TI) were scored on periodic acid-Schiff (PAS)-stained paraffin-embedded slides.

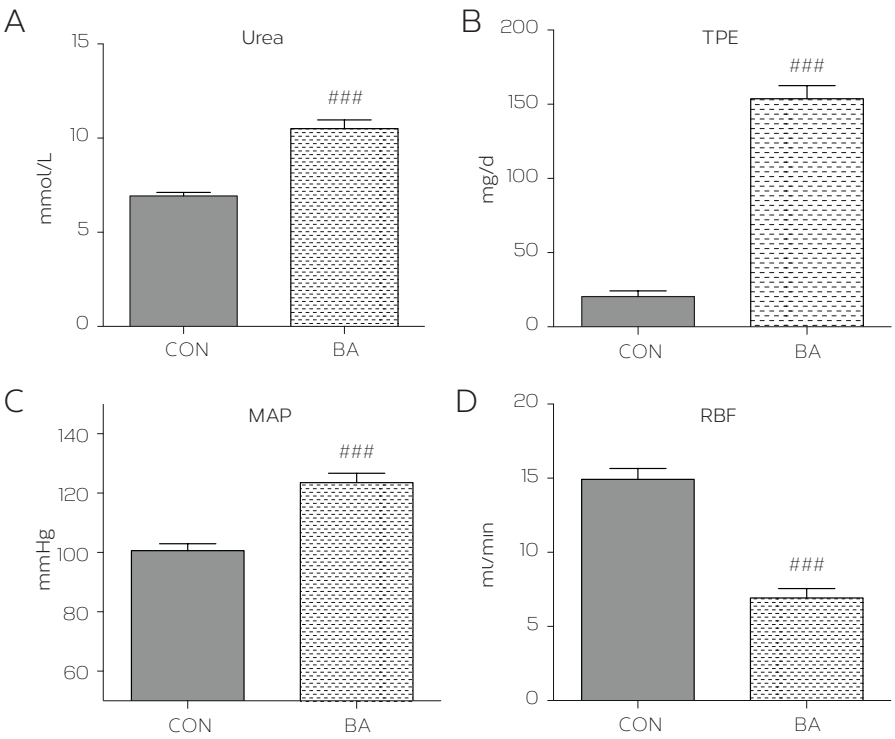

Figure 1. *Bilateral ablation leads to established chronic kidney disease (panel A: urea; panel B: TPE; panel C: MAP; panel D: RBF).*

CON: age-matched-control rats; BA: bilateral ablation rats; TPE: total protein excretion; MAP: mean arterial pressure RBF: renal blood flow. Means ± SEM. Unpaired T-test, ###P<0.0001

Statistics: Data are means ± SEM. T-test and linear regression were used when appropriate.

Representative results

BA leads to established CKD

In the CKD group, 3 of the initial 23 animals died during follow-up which resulted in n = 18 of CKD animals, an 87% survival rate. Mortality was either spontaneous (1 rat) or caused by intestinal ischemia in the first week after bilateral ablation possibly due to manipulation of the intestines during surgery (2 rats). Survival rate of all sham-operated CON rats was 100%.
In comparison to the CON rats, the BA rats had higher urea levels, TPE, MAP and RBF (all P<0.001, Figure 1).

Symmetry of injury

LK-GS and LK-TI correlated strongly with RK-GS and RK-TI (r=0.79, P<0.001, r=0.89, P<0.001) respectively (Figure 2A, B). We plotted these data using Bland Altman method in order to show agreement between LK and RK morphology (figure 2C, D).

Systemic predictors for function and injury

Proteinuria, in comparison to MAP and urea, was the best predictor for GFR, RPF, TI and GS (r=-0.72; r=-0.64, r=0.81 and r=0.82 respectively, all P<0.001, Figure 3). MAP (Figure 4) and urea (Figure 5) correlated with both function (GFR and RPF) and injury parameters (TI and GS): r=-0.51; r=-0.48; r=0.68; r=0.57 and r=-0.64; r=-0.60; r=0.70; r=0.57, respectively, all P<0.001.

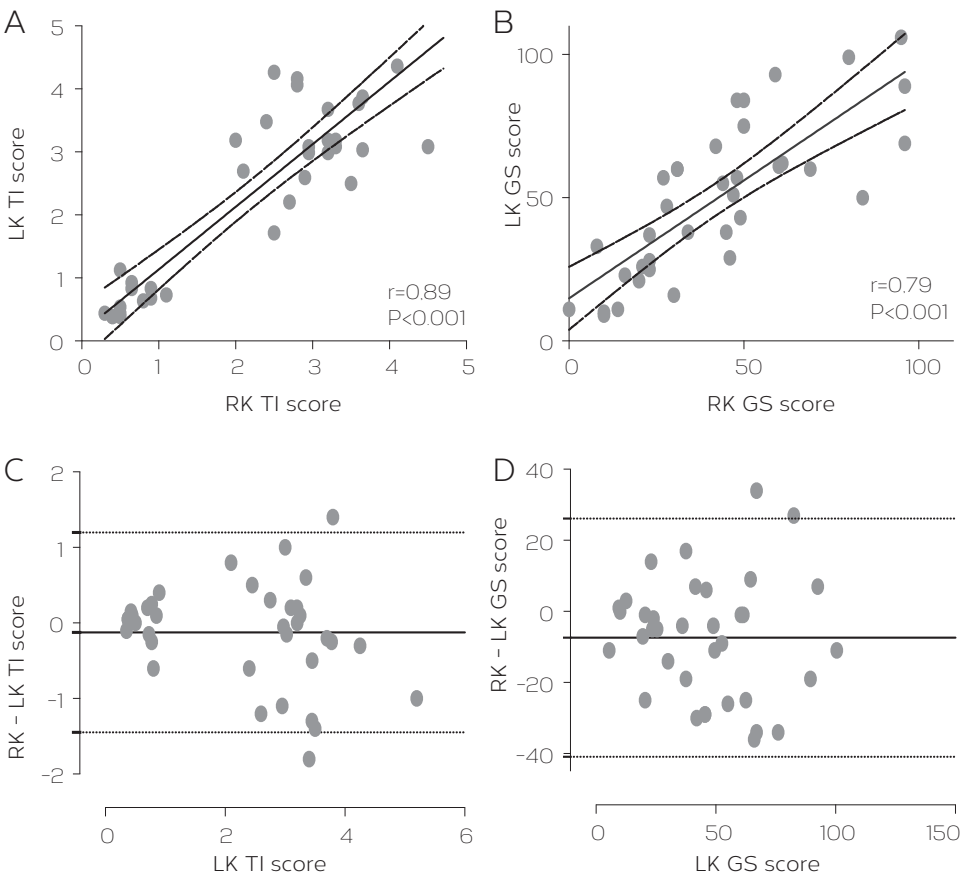

Figure 2. *Symmetry of injury. Correlation between LK and RK TI score (panel A) and LK and RK GS score (panel B). Bland-Altman plots for TI and GS (panel C and D).*

LK: left kidney; RK: right kidney; TI: tubulo-interstitial injury; GS: glomerulosclerosis.

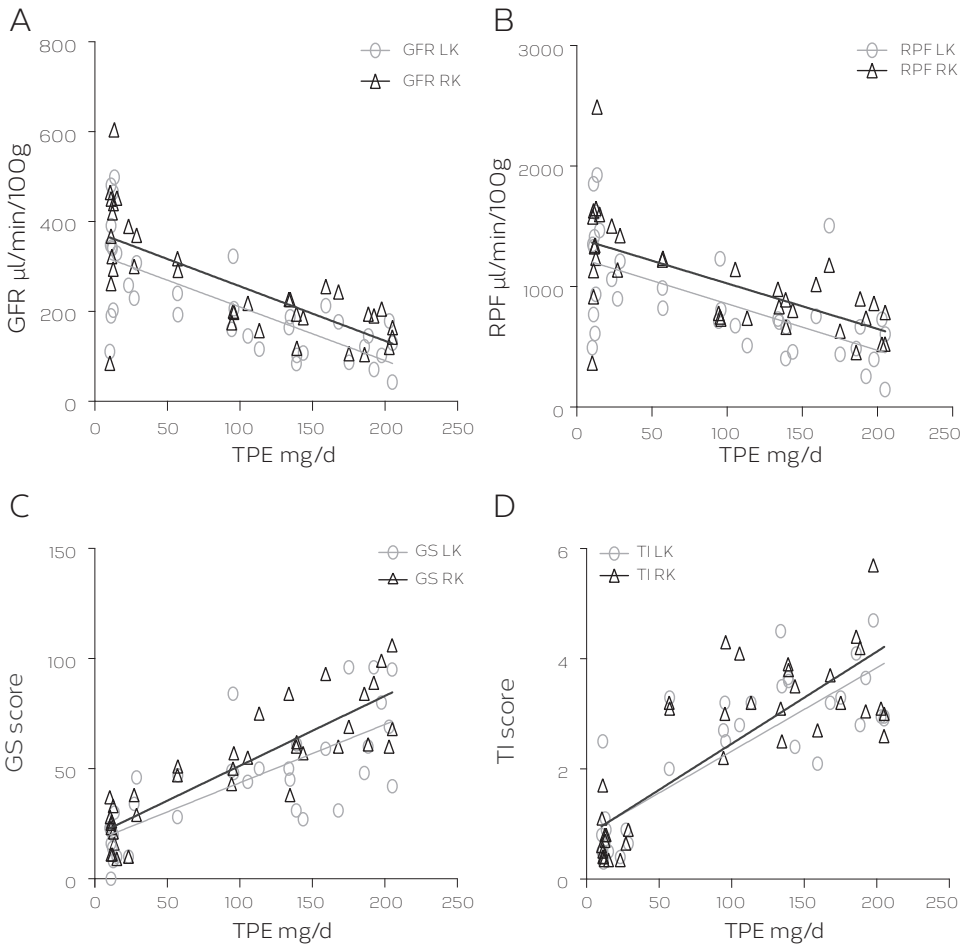

Figure 3. *Proteinuria is the best systemic predictor for function (panels A and B) and injury (panels C and D).*

LK: left kidney; RK: right kidney; TI: tubulo-interstitial injury; GS: glomerulosclerosis; TPE: total protein excretion.

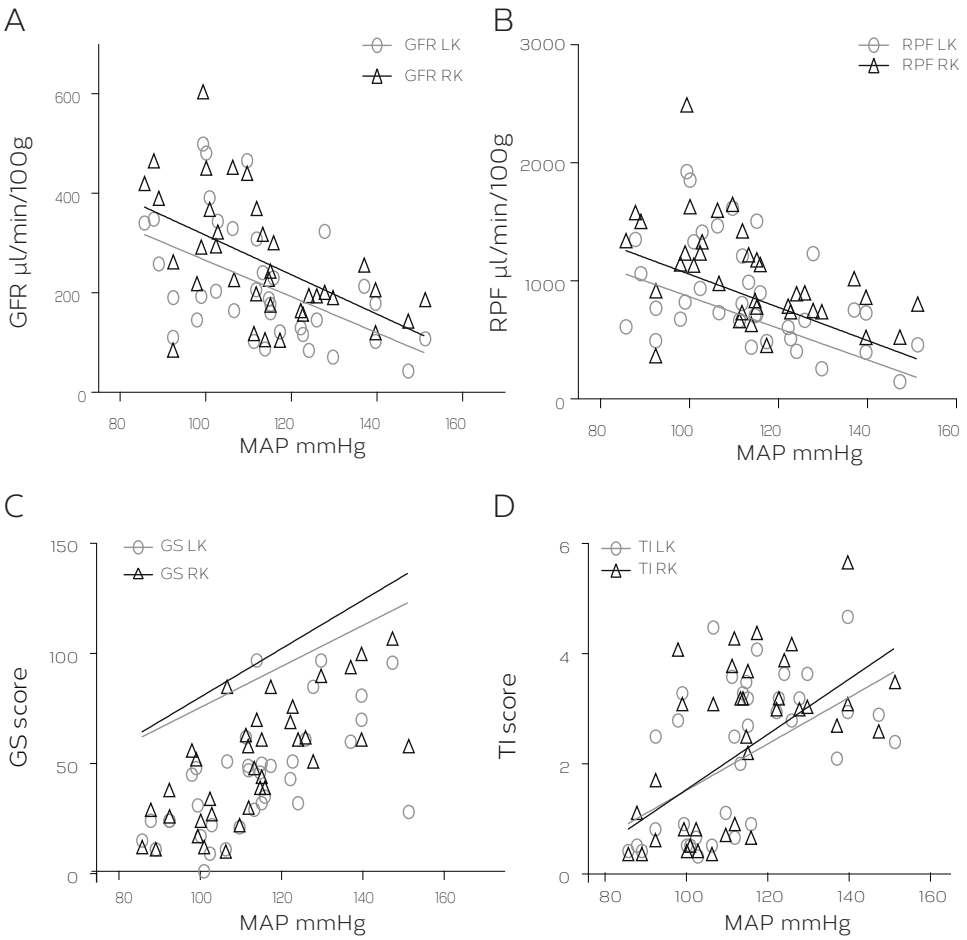

Figure 4. *Correlation between mean arterial pressure (MAP) and for function (panels A and B) and injury (panels C and D) parameters.*

LK: left kidney. RK: right kidney. TI: tubulo-interstitial injury. GS: glomerulosclerosis.

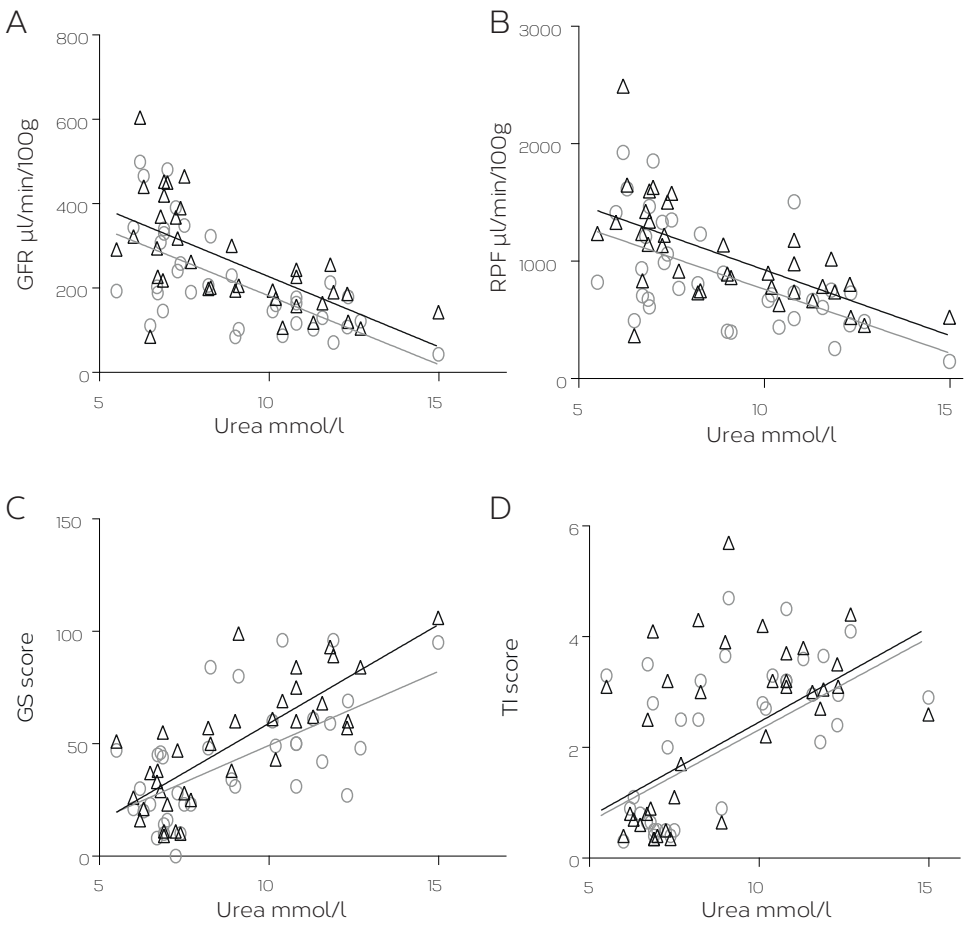

Figure 5. *Correlation between urea and for function (panels A and B) and injury (panels C and D) parameters.*

LK: left kidney; RK: right kidney; TI: tubulo-interstitial injury; GS: glomerulosclerosis.

Discussion

Our study shows that bilateral ablation, together with 6% salt and L-NNA (NOS inhibitor) supplementation, lead to development of established CKD in Lewis rats. The injury, measured by scoring GS and TI, was symmetrical and was best predicted by proteinuria. These findings firmly position this new bilateral ablation model in the field of TX of marginal donors.

The first major decision when contemplating the use of animal models is to choose the species and strain involved. Rodent models remain the most popular species to model human disease. Lewis-to-Lewis TX is a well-estab-

lished model for isogenic TX that allows us to study exclusively the influence of the CKD environment in TX setting [7]. Moreover, GFP+ Lewis rats allow differentiation between donor and recipient cell involvement[8]. However, the Lewis rat is relatively resistant to development of kidney injury[9] and because in our study the volume of remnant kidney tissue is twice as much as in the classical 5/6th nephrectomy model, we expected that development of CKD would be slower. Therefore, to accelerate the course of injury, bilateral ablation was combined with supplementation of 6% salt to the chow and L-NNA in the drinking water as described [4].

Although flank incision is characterized by lower risk of wound infection and herniation, we preferred median laparotomy to approach both kidneys. In this way both kidneys could be ablated in a one-step procedure. Moreover, because of the much shorter renal vessels of the right kidney, exposing the branches of the right renal artery via flank incision was technically challenging and carried more bleeding risk. It has been shown by Griffin et al, that infarction or incision also makes a difference in the mass reduction models[10]. In this case, the infarction approach was preferred because of two reasons: 1. It is characterized with higher renin release, more pronounced rise in blood pressure and more glomerular injury[11]; 2. When using the incision model, extrarenal tissue adheres to the wound and cause adhesions to the kidney surface. As our aim was to use these kidneys in further experiments as marginal donors in TX setting, we chose the infarction model in order to avoid such technical complications.

To our knowledge, this is the first bilateral model of CKD that is suitable for TX studies. It allows us to assess the kidney damage at the time-point of TX by using the contralateral kidney as a "protocol biopsy". Moreover, this model can be used to investigate effects on both kidneys of unilateral interventions (for example, unilateral denervation [12] or administration of progenitor cells via renal artery[13]).

Acknowledgements

We thank Paula Martens for her excellent technical assistance.

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Maintenance of hypertensive hemodynamics does not depend on ROS in established experimental chronic kidney disease

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Published in PLoS One. 2014 Feb 12;9(2):e88596.

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Abstract

While the presence of oxidative stress in chronic kidney disease (CKD) is well established, its relation to hypertensive renal hemodynamics remains unclear. We hypothesized that once CKD is established blood pressure and renal vascular resistance (RVR) no longer depend on reactive oxygen species. CKD was induced by bilateral ablation of 2/3 of each kidney. Compared to age-matched, sham-operated controls all ablated rats showed proteinuria, decreased glomerular filtration rate (GFR), more renal damage, higher mean arterial pressure (MAP), RVR and excretion of oxidative stress markers and hydrogen peroxide, while excretion of stable nitric oxide (NO) metabolites tended to decrease. We compared MAP, RVR, GFR and fractional excretion of sodium under baseline and during acute Tempol, PEG-catalase or vehicle infusion in rats with established CKD vs. controls. Tempol caused marked reduction in MAP in controls (96±5 vs.79±4 mmHg, P<0.05) but not in CKD (130±5 vs. 127±6 mmHg). PEG-catalase reduced MAP in both groups (controls: 102±2 vs. 94±4 mmHg, P<0.05; CKD: 118±4 vs. 110±4 mmHg, P<0.05), but did not normalize MAP in CKD rats. Tempol and PEG-catalase slightly decreased RVR in both groups. Fractional excretion of sodium was increased by both Tempol and PEG-catalase in both groups. PEG-catalase decreased TBARS excretion in both groups. In sum, although oxidative stress markers were increased, MAP and RVR did not depend more on oxidative stress in CKD than in controls. Therefore reactive oxygen species appear not to be important direct determinants of hypertensive renal hemodynamics in this model of established CKD.

Introduction

Chronic kidney disease (CKD) is associated with hypertension. Patients with mild to moderate renal insufficiency have increased levels of oxidative stress [1–5] i.e. unfavorable redox balance in which pro-oxidants gain the upper hand over anti-oxidants. This results in a net increase in reactive oxygen species (ROS), leading to cellular and tissue damage. Experimentally increasing ROS (superoxide anion and hydrogen peroxide) in the renal medulla induces hypertension [6,7].

Several studies support the hypothesis that antioxidants may play an important role in the pathogenesis of chronic renal failure and that antioxidant intervention can slow the progression of renal insufficiency in different experimental models of renal disease [8]. On the other hand, with the notable exception of a single study in hemodialysis patients [9], clinical studies showed no beneficial effects of antioxidants in the CKD population [8,10,11].

Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) is a stable low-molecular-weight (172.25 g/mol) cell-permeable superoxide dismutase (SOD) mimetic that has been used to reduce oxidative injury in cell and animal models. Chronic Tempol administration has been shown to ameliorate oxidative stress and lower arterial pressure in various rat models of hypertension: spontaneously hypertensive rats (SHR) [12], Dahl salt-sensitive rats [13], mineralocorticoid-induced hypertension [14], lead-induced hypertension [15], and erythropoietin-induced hypertension in uremic rats [16]. Acute Tempol administration decreases mean arterial pressure (MAP) and renal vascular resistance (RVR) in SHR [17,18] and in two-kidney one-clip hypertension [19]. Although in the remnant kidney model, chronic Tempol administration decreases oxidative stress, it has only been shown to prevent or reduce increase of blood pressure for 10-14 days after nephrectomy [20,21].

Catalase, an H_2O_2 detoxifying enzyme, has been shown to prevent hypertension induced by the infusion of H_2O_2 in the renal medulla [7]. Polyethylene glycol (PEG)-catalase was preferred to catalase, since the conjugation of catalase with PEG enhances cell association and increases cellular enzyme activity [22]. PEG-catalase prevents the markedly increased vascular and urinary H_2O_2 levels and rise in blood pressure in hypertension induced by adenosine receptor blockade [23]. In angiotensin-induced hypertension, although blood pressure was markedly decreased during the first days of PEG-catalase administration, this effect waned after only three days [24].

While the presence of oxidative stress as a feature of CKD is well established, its relation to hypertension and related hemodynamics in CKD has not been systematically addressed. In the current study we hypothesized that ROS are not important determinants of hypertensive renal hemodynamics in long-

term, established experimental CKD. To this end we developed a novel bilateral renal ablation model that was staged by the level of proteinuria. In order to differentiate hypertensive effects of superoxide and H_2O_2, we studied acute effects of the SOD mimetic Tempol or PEG-catalase on blood pressure (BP) and renal hemodynamics in rats with established CKD and age-matched sham-operated control rats. Furthermore, we investigated the effect of both these interventions on oxidative stress in CKD and control rats.

Materials and Methods

Ethics statement

The study protocol was approved by the Utrecht University Committee on Animal Experiments, and conformed to Dutch Law on Laboratory Animal Experiments (DEC number 2010.II.05.097 and DEC number 2012.II.03.053).

Animals

Male inbred Lewis rats (Lew/CRl), 180-200g, were purchased from Charles River, Germany and housed in a climate-controlled facility with a 12:12-hour

Table 1. *Characterisation of CKD vs. control (CON) rats: organ weights, clinical signs and renal injury.*

	CON	CKD
N	13	18
Body weight (BW) g	560 ± 14	540 ± 11
Total renal weight (mg/100g BW)	664 ± 13	591 ± 10 ###
Heart weight (mg/100g BW)	244 ± 5	280 ± 14 #
Total wet lung weight (mg/100g BW)	309 ± 6	337 ± 6 ##
Diuresis (ml/24h/100g)	3.15 ± 0.3	5.45 ± 0.68 #
Proteinuria (mg/24h)	16 ± 4	152 ± 9###
Hematocrit (%)	45.2 ± 0.3	42.6 ± 0.5 ###
Plasma urea (mmol/L)	6.76 ± 0.18	10.47 ± 0.38 ###
Plasma creatinine (µmol/L)	34 ± 4	50 ± 6 #
Plasma Na (mmol/L)	146.6 ± 1.5	145.7 ± 1.3
Plasma K (mmol/L)	4.25 ± 0.12	4.22 ± 0.07

Mean ± SEM. t-test: # P<0.05, ##P<0.01, ###P<0.001 vs. CON

light: dark cycle under standard conditions.
In order to develop established CKD in this strain, the rats were subjected to partial ablation of both kidneys. Via laparotomy under isoflurane anaesthesia (5% induction, 1.5-2% maintenance), branches of both renal arteries were coagulated, resulting in loss of approximately 2/3 of total renal mass in a one-step procedure. Age-matched control rats were sham-operated (CON). All rats received an intramuscular injection of analgesia straight after and one day after surgery (Buprenorphine, 0.05 mg/kg). 24-h urine samples were collected weekly for determination of protein excretion, with the rats in individual metabolic cages while fasting, as described [25]. Blood samples were collected from the tail vein for determination of plasma urea and creatinine. CKD was initially accelerated with N(omega)-nitro-L-arginine (L-NNA), a NO-synthase inhibitor (50 mg/L) in drinking water and the standard powdered chow (CRM-FG; Special Diet Services Ltd., Witham, Essex, UK) was supplemented with 6% NaCl until proteinuria exceeded 200 mg/day after a median of 8 weeks (range: 6- 9 weeks). Subsequently L-NNA was withdrawn causing proteinuria to initially fall and subsequently increase slowly as described by Quiroz et al. [26](data not shown). Terminal experiments were planned within a week when proteinuria exceeded 100 mg/day. This time point was reached after a median of 35 weeks (range: 22 – 56 weeks). This approach ensured that staging of CKD was similar in all rats. Previously we have shown that proteinuria predicts target organ injury in hypertensive rats [27]. Timing of terminal experiments in sham-operated controls was determined by their age-matched CKD litter mates.
One week prior to termination 24h urinary excretion of markers of oxidative stress (thiobarbituric acid reactive substances (TBARS), 8-isoprostane (EIA kit, Cayman Chemical, Michigan, USA) and hydrogen peroxide (Amplex

Table 2. *Gene expression of renin, AT1, ACE1 and VEGF-A in CON and CKD rats. Data are presented as log fold change relative to CON.*

	CON	CKD
renin	0.0 ± 0.37	-1.6 ± 0.35 #
AT1	0.0 ± 0.15	-0.7± 0.24
ACE1	0.0 ± 0.17	-0.1 ± 0.59
VEGF-A	0.0 ± 0.10	-1.4 ± 0.46 #

Means ± SEM. Unpaired T-test. #P<0.05 vs. CON.

Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular probes, OR, USA)) were measured. Urinary excretion of stable NO metabolites NO_2 + NO_3 (NOx) were determined by fluorometric quantification of nitrite content [28]. Rats underwent a terminal measurement under anaesthesia as described. L-NNA, Tempol, PEG-catalase, BSA and Buprenorphine were purchased from Sigma-Aldrich. Isoflurane was purchased from Abbott.

Terminal experiment protocol

On the day of the experiment the trachea was intubated with a 16-G catheter (Venisystems, Abbocath-T, Abbott, Ireland) under isoflurane anesthesia (5% induction, 1.5-2% maintenance). The femoral artery was cannulated in order to obtain direct measurement of MAP and a Transonic flow probe was placed on the left renal artery to measure renal blood flow (RBF) [17,29], allowing calculation of renal vascular resistance (RVR: MAP/RBF). Urine was collected allowing measurement of kidney function (glomerular filtration rate, GFR: inulin clearance). During surgery, animals received an intravenous infusion of a 150 mmol/L NaCl solution containing 6% bovine serum albumin (BSA) at a rate of 100 µl/kg/min. Following surgery, the infusion was switched to a 150 mmol/L NaCl solution with 1% BSA, containing inulin for measurement of GFR, which was maintained at the same infusion rate throughout the experiment. Following a 60 min equilibration period, after which both signals were stable, baseline data were collected for 15 min. Thereafter, to investigate renal vascular reactivity we continuously infused the SOD mimetic Tempol (180 µmol/kg/h, CKD n=6, CON n=4), PEG-catalase (2000 units/kg/h, CKD n=8, CON n=5) or vehicle (NaCl, 0.9% 6 ml/kg/h, CKD n=4, CON n=4) after baseline measurements. Following a 45 min equilibration period, after which both signals were stable, intervention data were collected for 15 min. This dose for Tempol was chosen because it has already been shown by others that 72-90 µmol/kg is an effective dose and acute response was very rapid to intravenous Tempol in anaesthetized rat with spontaneous hypertension [30]. A dose of 174 µmol/kg caused a decrease in MAP with more than 30 mmHg and when given in an effective dose (72-90 µmol/kg). Tempol reduced the blood pressure in all hypertensive models with evidence of oxidative stress [31]. Moreover, Tempol administration ameliorated 8-isoprostane excretion in several hypertensive models [32,33]. Fractional excretions of sodium and potassium (FE Na and FE K) were calculated using standard formulae.

Oxidative stress protocol

To investigate the effect of antioxidants on oxidative stress in our CKD model, we administered Tempol (180 µmol/kg), PEG-catalase (2000 IU/kg) or vehicle

(0.9 % NaCl) iv (tail vein) in a separate cohort of CKD rats. Administration of antioxidant or vehicle was time-matched (between 17:30 and 18:30h) and followed by collection of urine in metabolic cages overnight. We compared TBARS excretion between age-matched CKD (n=6) and CON (n=6) rats, treated in a repeated-design experiment with Tempol, PEG-catalase or vehicle in random sequence.

Renal morphology

Directly after performing the terminal experiment protocol, rats were sacrificed and tissues were collected and fixed in 4% paraformaldehyde for embedding in paraffin or were snap frozen. Glomerulosclerosis (GS) and tubulo-interstitial injury (TI) were scored on PAS-stained paraffin-embedded slides [34]. Furthermore, endothelial cells in the glomeruli and tubuli were stained with rat endothelial cell antigen (RECA). $(RECA)^+$ pixels were counted in glomeruli and tubular fields using ImageJ Software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD) [35]. In order to evaluate whether Tempol and PEG-catalase caused changes in the sympathetic nervous system, we performed immunohistochemistry using an antibody against marker for sympathetic nerves: tyrosine hydroxylase (TH) [36]. Snap frozen kidney slices were incubated overnight with anti-TH antibody (P40101-0, Pel-Freez Biologicals, 1:500).

Gene expression

To determine whether Tempol and PEG-catalase affected renin-angiotensin system (RAS), gene expression of angiotensin II receptor type 1 (AT1), angiotensin converting enzyme 1 (ACE1) and renin in renal tissue was assessed by qPCR as described [35]. Using the same method we assessed the renal expression of vascular endothelial growth factor (VEGF-A), which is responsible for angiogenesis and endothelial cell proliferation [37]. The following TaqMan Gene Expression Assays (Applied Biosystems) were used : (AT1: Rn01435427_m1), (ACE1:Rn00561094_m1), (renin: Rn00561847_m1), (VEGF-A: Rn00582935_m1), (beta-actin: Rn00667869_m1) and (beta-2-microglobulin: Rn00560865_m1). Cycle time (Ct) values for all genes were normalized for mean Ct-values of beta-actin and beta-2-microglubulin which we previously determined to be the two most stable housekeeping genes for renal tissue for all groups.

Statistics

Values are expressed as mean ± SEM. Data were compared with unpaired T-test, one way analysis of variance (ANOVA) and two-way ANOVA for repeated measurements when appropriate. Tukey test was used as a post-hoc test ($P<0.05$).

Results

Ablation of 2/3 of each kidney leads to established CKD

In the CKD group, 3 of the initial 21 animals died during follow-up which resulted in n=18 of CKD animals, an 85% survival rate. Mortality was either spontaneous (1 rat) or caused by intestinal ischemia in the first week after bilateral ablation possibly due to manipulating the intestines during surgery (2 rats). Survival rate of all sham-operated CON rats was 100%. CKD rats showed slightly lower body weight vs. CON rats (Table 1). All organ weights were corrected for body weight. Renal mass was lower (P<0.01) and heart and wet lungs heavier in CKD rats (P<0.05 and P<0.01 respectively). CKD rats showed increased diuresis (P<0.01) and proteinuria (P<0.001). All CKD rats had mild anemia (P<0.001), higher plasma urea (P<0.001) and creatinine (P<0.05). Markers of oxidative stress were increased in CKD: TBARS and 8-isoprostane excretion were significantly higher (P<0.01 and P<0.001 respectively), whereas H_2O_2 excretion tended to increase (P=0.07) vs. CON rats (Fig. 1). NOx excretion tended to decrease in CKD vs. CON (P=0.06). In CKD rats, the expression of renin and VEGF-A were lower in comparison to CON rats. No differences in expression of AT1 and ACE1 were found (Table 2). CKD rats showed marked glomerulosclerosis and tubulo-interstitial injury (both P<0.001) (Fig. 2). Counts of RECA-positive pixels indi-

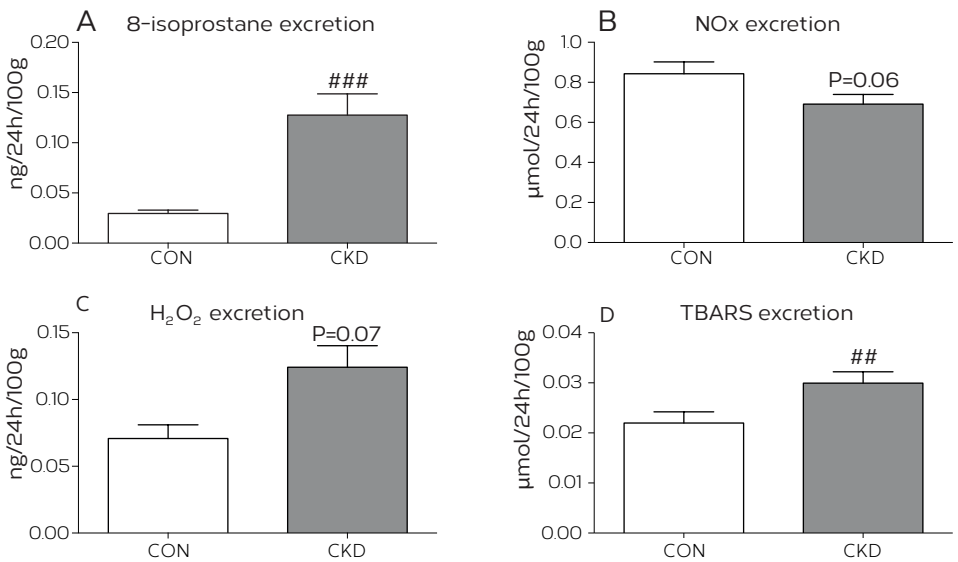


Figure 1. *24h excretions of 8-isoprostanes (panel A), NO metabolites (NOx, panel B), hydrogen peroxide (panel C) and lipid peroxides (TBARS, panel D) in CKD vs. CON rats.*

Mean ± SEM. ###P<0.001, ##P<0.01 vs. CON.

cated lower numbers of endothelial cells in the glomeruli and the tubular fields of CKD rats vs. CON rats (Fig. 3). Visual impression showed no difference in tyrosine hydroxylase expression (Supplemental Figure 1).

Tempol decreased MAP in CON but not in CKD and did not affect RVR

CKD increased MAP (P=0.001) and Tempol decreased MAP (P<0.001, Fig. 4A). However, the effect of Tempol was different in CKD than in CON, resulting in strong interaction (P<0.01), and when individual groups were compared with the post-hoc test, we found that infusion of Tempol significantly decreased MAP in CON (P<0.001) but had no effect on MAP in CKD. All CKD rats had higher RVR vs. CON rats (P<0.01) and Tempol had no significant effect on RVR (Fig. 4B).

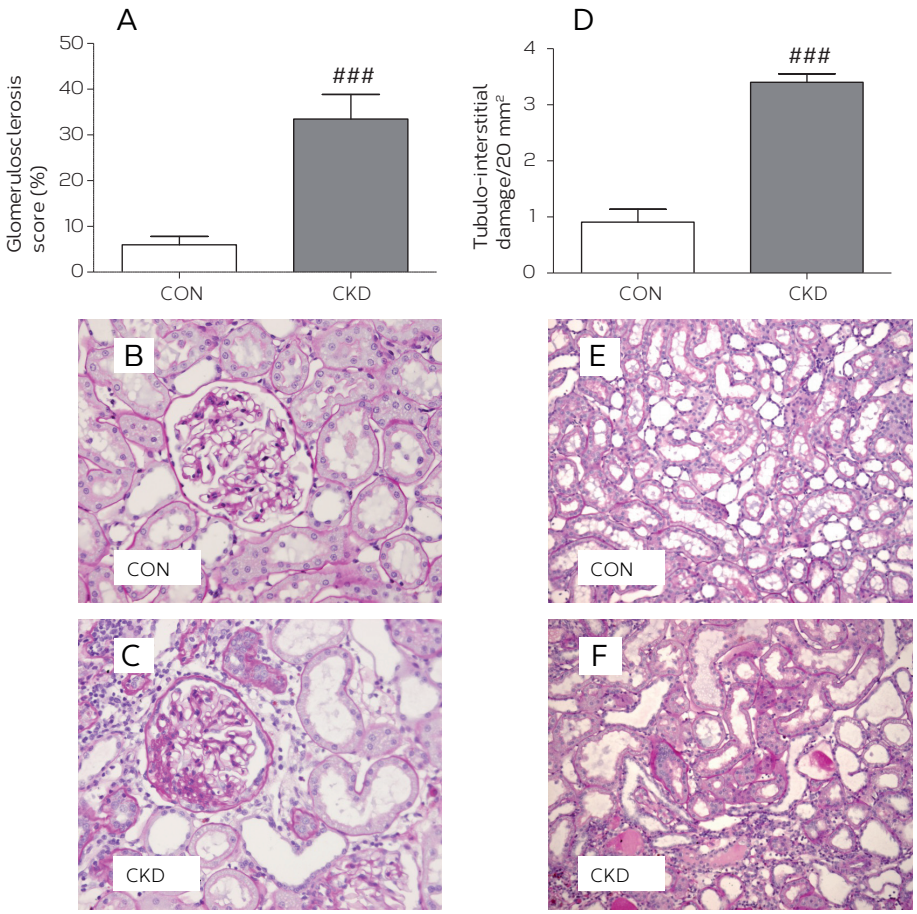


Figure 2. *Bilateral ablation (C and F) induced more glomerulosclerosis (panel A) and tubulo-interstitial damage (panel D) in CKD rats compared to controls (B and E) on PAS-stained sections. Means ± SEM. Unpaired t-test: ###P<0.001 vs. CON.*

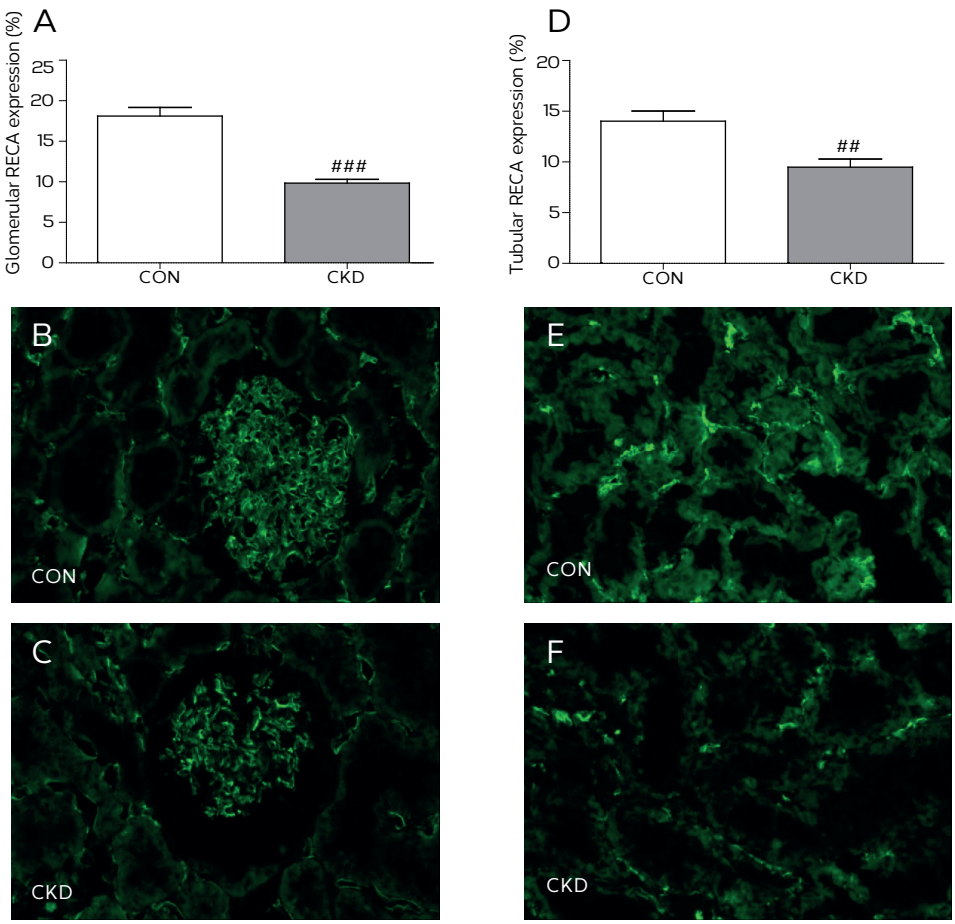


Figure 3. *Less (RECA)+ pixels (green) were found in CKD rats compared to CON rats in both glomeruli (panel A) and tubular fields (panel D). Immunohistochemical labeling is shown in CON rats (panels B and E) and in CKD rats (panels C and F). Means ± SEM. Unpaired t-test: ###P<0.001; ##P<0.01 vs. CON.*

PEG-catalase decreased MAP and RVR in CON and CKD

PEG-catalase significantly reduced MAP (P<0.001) in both CON and CKD, and in the post-hoc analysis, PEG-catalase-induced reductions in MAP were all significant (Fig. 5A). For RVR, the same pattern was observed: PEG-catalase decreased RVR in both CON and CKD (P<0.001), and in the post-hoc analysis all PEG-catalase-induced reductions in RVR were significant (Fig. 5B).

Vehicle

Infusion of vehicle (0.9 % NaCl) did not affect either MAP (Fig. 6A) or RVR (Fig 6B).

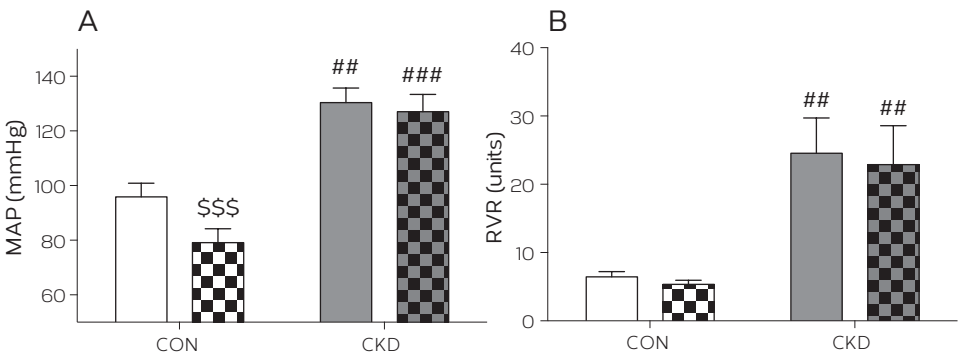


Figure 4. *Mean arterial pressure (MAP) (panel A) and renal vascular resistance (RVR) (panel B) prior to baseline (plain bars) and during Tempol (bars with squares) in CON (n=4, white bars) and CKD (n=6, grey bars) rats. Mean ± SEM. Two-way RM ANOVA (P CKD vs. CON =0.001, P Tempol vs. baseline <0.001, P Interaction =0.002 for panel A; P CKD vs. CON = 0.030, P Tempol vs. baseline = NS, P Interaction = NS for panel B). ## P<0.01, ### P<0.001 vs. CON. $$$ P<0.001 vs. baseline (paired observations).*

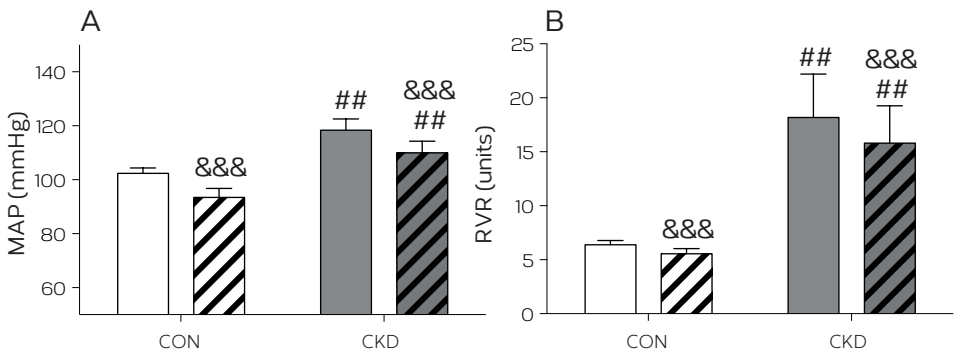


Figure 5. *Mean arterial pressure (MAP) (panel A) and renal vascular resistance (RVR) (panel B) prior to baseline (plain bars) and during PEG-catalase (striped bars) in CON (n=5, white bars) and CKD (n=8, grey bars) rats. Mean ± SEM. Two-way RM ANOVA (P CKD vs. CON = 0.017, P PEG-catalase vs. baseline <0.001, P Interaction = NS for panel A; P CKD vs. CON = 0.001, P PEG-catalase vs. baseline <0.001, P Interaction = NS for panel B). ## P<0.01, ### P<0.001 vs. CON. && P<0.01, &&& P<0.001 vs. baseline (paired observations).*

Tempol and PEG-catalase reduced GFR in CON and increased FE Na in CKD

CKD rats had lower GFR vs. CON rats (P<0.001, Table 3). Tempol had different effects on GFR in CKD and CON, resulting in interaction (P<0.05), and when groups were compared with the post-hoc test, Tempol markedly reduced GFR in CON (P<0.001), but not in CKD. Similarly, PEG-catalase had different effects on GFR in CKD and CON (P<0.05), and reduced GFR in CON (P<0.001), but not

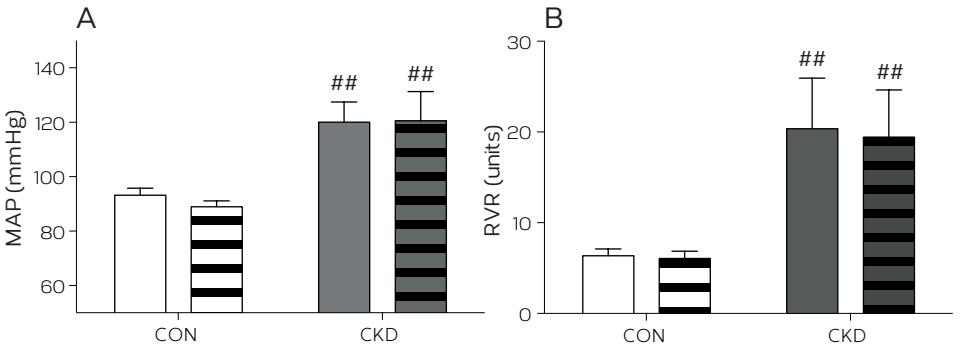


Figure 6. *Mean arterial pressure (MAP) (panel A) and renal vascular resistance (RVR) (panel B) prior to baseline (plain bars) and during infusion of vehicle (bars with horizontal lines) in CON (n=4, white bars) and CKD (n=4, grey bars) rats. Mean ± SEM. Two-way RM ANOVA (P CKD vs. CON = 0.020, P vehicle vs. baseline = NS, P Interaction = NS for panel A; P CKD vs. CON = 0.040, P vehicle vs. baseline = NS, P Interaction =NS for panel B). ## P<0.01 vs. CON.*

Table 3. *Glomerular filtration rate (GFR) and fractional electrolyte excretion prior to (baseline) and after intervention (Tempol, PEG-catalase, vehicle).*

	CON	
	baseline	Tempol
N	4	
GFR (µl/min/100g)	672 ± 65	485 ± 60 $$
FE Na (%)	0.28 ± 0.12	0.23 ± 0.13
FE K (%)	26.09 ± 1.94	25.11 ± 0.73
	baseline	PEG-catalase
N	5	
GFR (µl/min/100g)	784 ± 105	510 ± 112 &&&
FE Na (%)	0.15 ± 0.04	0.12 ± 0.04
FE K (%)	30.47 ± 3.53	28.67 ± 3.56
	baseline	vehicle
N	4	
GFR (µl/min/100g)	741 ± 102.13	523 ± 107 §
FE Na (%)	0.17 ± 0.04	0.16 ± 0.05
FE K (%)	30.67 ± 0.79	26.77 ± 1.94

Mean ± SEM. ANOVA RM. Tukey post-hoc test for comparison between groups ### P<0.001, ## P<0.01, #P<0.05 vs. CON; $$P<0.01 Tempol vs. baseline; &&& P<0.001 PEG-catalase vs. baseline; § P<0.05 vehicle vs. baseline.

in CKD. This pattern for GFR was also observed during vehicle infusion.
CKD rats had higher FE Na (P<0.01) and FE K (P<0.001) than CON rats (Table 3). During Tempol infusion FE Na tended to decrease in CON (NS), but was markedly increased in CKD (P<0.01), both compared to their own baseline. This pattern resulted in a significant interaction for FE Na (P<0.05). During PEG-catalase effects on FE Na were similar to those observed for Tempol: no change in CON but a marked increase in CKD (P<0.001), resulting in significant interaction (P<0.01). FE K was not affected by either Tempol or PEG-catalase, and neither FE Na nor FE K were affected by vehicle infusion.

Comparison of changes induced by Tempol, PEG-catalase and vehicle

Figure 7 depicts the changes in MAP and RVR after acute administration of Tempol, PEG-catalase or vehicle in CON and CKD rats. For change in MAP an overall effect of all three interventions was observed (P<0.001) as well as interaction (P<0.01, Fig. 7A). Tempol infusion decreased MAP by nearly 15 mmHg in CON rats (P<0.001) but MAP remained unchanged in CKD rats when compared with the change caused by vehicle infusion in the same condition. However, when comparing change in MAP caused by PEG-catalase adminis-

CKD		P-value		
baseline	Tempol	CKD-cat	Tempol	Interaction
6				
302 ± 18 ###	262 ± 21 ##	<0.001	=0.003	=0.029
1.02 ± 0.26	1.91 ± 0.54 # $$	=0.042	=0.066	=0.043
64.22 ± 4.61 ###	62.43 ± 3.90 ###	<0.001	=0.665	=0.898
baseline	PEG-catalase	CKD	PEG-catalase	Interaction
8				
354 ± 40 ###	273 ± 24 ##	<0.001	<0.001	=0.021
0.47 ± 0.08	0.79 ± 0.14 ### &&&	=0.005	=0.026	=0.009
63.42 ± 5.41 ###	60.96 ± 4.98 ###	<0.001	=0.501	=0.915
baseline	vehicle	CKD	vehicle	Interaction
4				
303 ± 32 ##	240 ± 28 #	=0.013	=0.007	=0.066
1.27 ± 0.60	1.60 ± 0.92	=0.145	=0.364	=0.342
73.50 ±8.58 ##	73.45 ± 7.64 ##	=0.001	=0.273	=0.284

tration, the opposite was observed: MAP decreased slightly in CON rats but was significantly lower in CKD rats (P<0.05) when compared to change caused by vehicle infusion in the same condition. Changes in RVR were not significantly different (Fig. 7B). One-hour acute infusion of Tempol or PEG-catalase in terminal setting did not cause any changes in the renal expression of RAS and VEGF-A genes or in tyrosine hydroxylase staining in comparison to vehicle infusion in both CON and CKD (Supplemental Table 1 and Supplemental Figure 1).

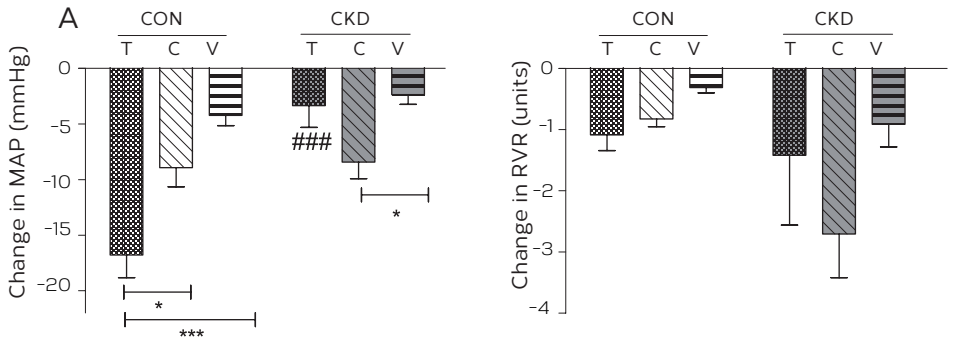


Figure 7. *Changes in MAP (panel A) and RVR (panel B) in CON (white bars) and CKD rats (grey bars) during infusion of Tempol (T, bars with squares), PEG-catalase (C, bars with stripes) and vehicle (V, bars with horizontal lines). Mean ± SEM. P CKD vs. CON =0.0011, P Interventions = 0.0028, P Interaction =0.0014, for panel A; P CKD vs. CON = NS, P Interventions = NS, P Interaction = NS for panel B). Tukey post-hoc test for comparison between groups: ### P<0.01 vs. CON. Between groups: *P<0.05. **P<0.01 ***P<0.001.*

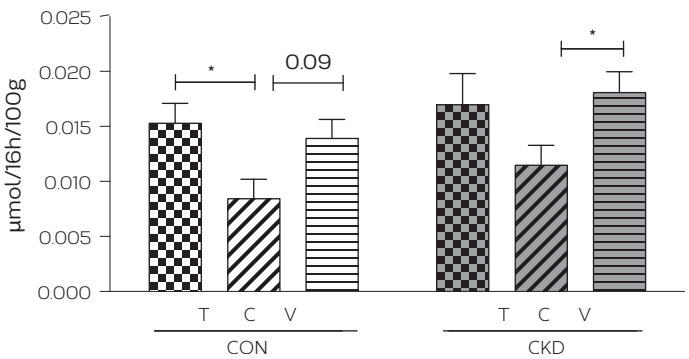


Figure 8. *16h TBARS excretion in CON rats (white bars) and CKD rats (grey bars) after intravenous administration of Tempol (T, bars with squares), PEG-catalase (C, bars with stripes) or vehicle (V, bars with horizontal lines). Mean ± SEM. P CON vs. CKD = NS; P Interventions = 0.003; P Interaction =NS. Tukey post hoc test for comparison between groups: *P<0.05.*

Comparison of TBARS excretion induced by Tempol, PEG-catalase or vehicle

Intravenous administration of Tempol did not affect excretion of TBARS in CON and CKD groups compared to vehicle, whereas PEG-catalase decreased TBARS excretion in CKD group ($P<0.05$) and showed a trend to decrease in CON group compared to vehicle ($P=0.09$) (Fig. 8).

Discussion

The main novel finding of this study is that in established CKD, MAP and RVR do not depend on ROS. This was demonstrated by the failure to alter MAP in CKD rats by acute scavenging of superoxide with Tempol. Reducing H_2O_2 with PEG-catalase did not normalize MAP in CKD rats. Furthermore, in CKD rats, Tempol had no effect on TBARS excretion while PEG-catalase reduced it.

Parameters of oxidative stress are increased and antioxidant enzyme activities are decreased in patients with various degrees of CKD [38-40]. Important endogenous antioxidant enzymes are SOD(s) that convert superoxide to H_2O_2, which is in turn disposed of by two other enzymes, catalase and glutathione peroxidase. In experimental CKD a marked down-regulation of hepatic and renal cytoplasmic and mitochondrial SOD was found as well as down-regulation of renal catalase and glutathione peroxidase protein abundance and catalase activity [41,42].

Effect of Tempol and PEG-catalase on MAP

In CKD rat models chronic Tempol administration only ameliorated hypertension for 10-14 days after nephrectomy [20,21]. Our data suggests that in long-term experimental CKD, once hypertension is established, other mechanisms contribute to its maintenance. Because Tempol caused a marked decrease in MAP in CON but not in CKD rats, maintenance of hypertension in our model of CKD appears not to depend on superoxide. Although Tempol infusion reduces superoxide levels, it results in accumulation of H_2O_2 that might serve as an important hypertensive factor [7] and has been reported to induce renal vasoconstriction [43,44]. The lack of antihypertensive effects of Tempol might be explained by the need of a fully functional system of other (non-SOD) antioxidant enzymes to drive the H_2O_2 generated from superoxide dismutation to CO_2 and H_2O. In contrast to Tempol, we found that acute administration of PEG-catalase did decrease MAP in CKD. However, MAP was not normalized to control levels in response to PEG-catalase, suggesting that H_2O_2 is not solely responsible for hypertension in established CKD. Increased production of ROS can reduce the availability of vasodilators such as nitric oxide (NO), which can lead to functional NO deficiency and thus contribute to

maintenance of hypertension. Indeed, CKD rats in the present study showed a tendency to lower levels of urinary NOx excretion vs. CON rats. However, VEGF-A gene expression and endothelial cell staining, although both clearly reduced in CKD rats, were not affected acutely by Tempol and PEG-catalase. Other factors than oxidative stress that can affect the blood pressure are RAS and the sympathetic nervous system. We found no changes in either gene expression of AT1, ACE1 or renin (Supplemental Table 1) or in detection of sympathetic nerves between treatment groups (Supplemental Figure 1). Thus, at least these levels of expression, these known regulators of blood pressure and renal perfusion were not acutely affected by Tempol and PEG-catalase.

Effect of Tempol and PEG-catalase on RVR

Tempol and PEG-catalase had limited effects on RVR in CKD suggesting that renal resistance vessels are not sensitive to renal vasoconstrictor effects of ROS in this model. We found no other reports on renal hemodynamics during acute treatment with either Tempol or PEG-catalase in rats with established CKD. Because we chose for a systemic intravenous rather than renal intra-arterial administration of Tempol and PEG-catalase we cannot evaluate their direct effects on the kidney. One might hypothesize that ROS-mediated vasoconstriction in the extrarenal circulation contributes to hypertension in established, long-term CKD. Although increased myogenic tone preceded structural vascular changes and hypertension in rats with CKD induced by renal mass reduction [45], ultimately, loss of myogenic response of the mesenteric arteries was observed [46]. Moreover, segments of the mesenteric arteries from CKD rats incubated with Tempol and PEG-catalase showed a significant increase rather than decrease in myogenic constriction suggesting that superoxide and H_2O_2 may be involved in pathological loss of the myogenic response [47].

Effect of Tempol and PEG-catalase on TBARS excretion

Tempol showed no effect on urinary TBARS excretion in neither CON nor CKD rats suggesting that it failed to reduce oxidative stress in both groups. Similar to the effect on MAP in the acute experiment, PEG-catalase reduced TBARS excretion in both CON and CKD. This once again suggests that oxidative stress is not the main force driving maintenance of hypertension in this established model of CKD.

Effect of Tempol and PEG-catalase on FE Na

A striking finding in this study is that FE Na in CKD rats was increased by both Tempol and PEG-catalase in comparison to CON rats suggesting that

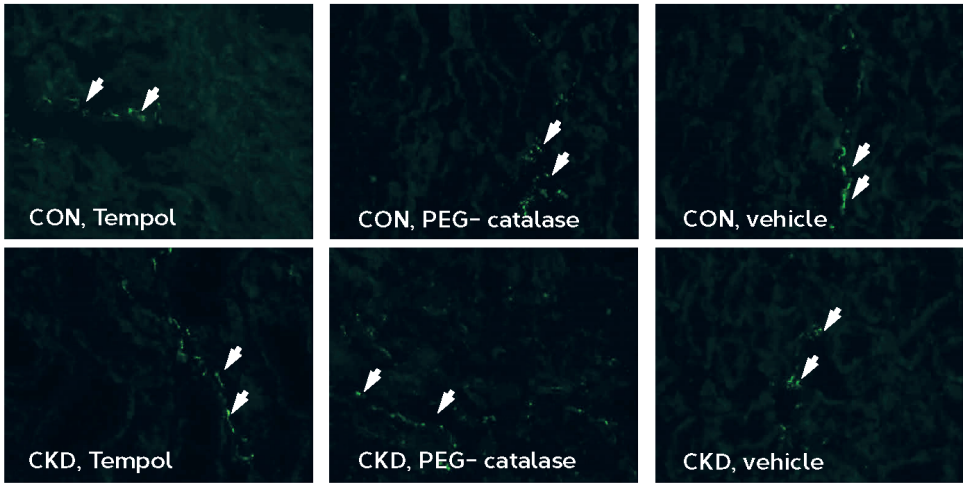
excessive ROS modulate natriuresis. In agreement with our observation, it has been demonstrated that ROS decreases sodium excretion [48]. It has been shown that ROS have multiple anti-natriuretic tubular actions [49]. Our data suggests, as indicated by the increase of FE Na, that Tempol and PEG-catalase decreased tubular reabsorption. The observation that both Tempol and PEG-catalase had no effects on MAP and RBF suggests that, in this model of CKD, they acted mainly via tubular mechanisms and thus can only affect BP indirectly and hence slowly. We observed a time-dependent reduction of GFR in all groups. However, relative to baseline, the reduction in the vehicle control group was smaller than the one observed in the Tempol and PEG-catalase control groups. Moreover, no significant difference was observed between the baseline and vehicle measurements in the CKD groups.

In conclusion, in the current study we show that in established CKD MAP and RVR did not depend more on ROS than in CON. Our findings suggest that anti-oxidant therapy in experimental CKD, although it can prevent the increase in BP in early stages, might not be effective in reducing BP once CKD is established.

Acknowledgements

We thank Paula Martens, Adele Dijk, Krista den Ouden, Jan Willem de Groot and Petra de Bree for their expert laboratory assistance.

Supplement



Supplemental Figure 1.

Immunohistochemical labeling of renal tissue for tyrosine hydroxylase (TH) in CON rats (first row) and CKD rats (second row) to detect sympathetic nerves (green, white arrows).

Supplemental Table 1

Gene expression of renin, AT1, ACE1 and VEGF-A in CON and CKD rats (first cohort), after intravenous infusion of with Tempol, PEG-catalase or vehicle in terminal setting.

	CON			CKD		
	Tempol	PEG-catalase	Vehicle	Tempol	PEG-catalase	Vehicle
renin	0.36± 0.47	-0.04 ±0.32	0.0 ± 0.37	0.08 ± 0.38	0.88 ± 0.31	0.0 ± 0.35
AT1	-0.27± 0.14	0.0 ±0.26	0.0 ± 0.15	0.19 ± 0.24	0.47 ± 0.16	0.0 ± 0.24
ACE1	0.02 ±0.29	0.12± 0.24	0.0 ± 0.17	-0.47 ± 0.38	-0.29 ± 0.30	0.0 ± 0.59
VEGF-A	-0.43± 0.14	-0.05 ± 0.25	0.0 ± 0.10	0.62 ± 0.15	0.92 ± 0.28	0.0 ± 0.46

Data are presented as log fold change relative to the calibrator (vehicle treated animals in CON and CKD groups). Means ± SEM.

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4

Arrhythmogenic remodeling in murine models of deoxycorticosterone acetate-salt-induced and 5/6-subtotal nephrectomy-salt-induced cardiorenal disease

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Published in CardioRenal Medicine 2015;5:208-218

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Abstract

Background

Renal failure is associated with adverse cardiac remodeling and sudden cardiac death. The mechanism leading to enhanced arrhythmogenicity in the cardiorenal syndrome is unclear. The aim of this study was to characterize electrophysiological and tissue alterations correlated with enhanced arrhythmogenicity in two distinct mouse models of renal failure.

Methods

Thirty-week-old 129Sv mice received a high-salt diet and deoxycorticosterone acetate (DOCA) for 8 weeks, followed by an additional period of high-salt diet for 27 weeks (DOCA-salt aged model). Adult CD-1 mice were submitted to 5/6-subtotal nephrectomy (SNx) and treated for 11 weeks with a high-salt diet (SNx-salt adult model). Vulnerability to arrhythmia as well as conduction velocities (CVs) of the hearts were determined *ex vivo* with epicardial mapping. Subsequently, the hearts were characterized for Connexin43 (Cx43) and fibrosis.

Results

DOCA-salt and SNx-salt mice developed renal dysfunction characterized by albuminuria. Heart, lung and kidney weights were increased in DOCA-salt mice. Both DOCA-salt and SNx-salt mice were highly susceptible to ventricular arrhythmias. DOCA-salt mice had a significant decrease in both longitudinal and transversal CV in the left ventricle. Histological analysis revealed a significant reduction in Cx43 expression as well as an increase in interstitial fibrosis in both DOCA-salt and SNx-salt mice.

Conclusion

DOCA-salt and SNx-salt treatment induced renal dysfunction, which resulted in structural and electrical cardiac remodeling and enhanced arrhythmogenicity. The reduced Cx43 expression and increased fibrosis levels in these hearts are likely candidates for the formation of the arrhythmogenic substrate.

Introduction

The cardiorenal syndrome is a condition characterized by the influence of a diseased kidney on the heart and vice versa, which can lead to progression of failure of both organs [1]. Patients with renal failure have an increased mortality risk due to cardiovascular disease[2]. Around 25% of all mortality of dialysis patients is caused by sudden cardiac death, mostly arising from arrhythmias such as ventricular tachycardia or fibrillation[3-5]. The mechanism leading to enhanced arrhythmogenicity in the cardiorenal syndrome is unclear.

Cardiac arrhythmias can be caused by three basic mechanisms: enhanced automaticity, triggered activity or reentry[6]. Reentry-based arrhythmias are responsible for the majority of ventricular arrhythmias and are often observed in dialysis patients[3,6]. Typical electrophysiological characteristics of reentry are slow impulse conduction, conduction block and a lower effective refractory period. Hearts prone to reentrant arrhythmias typically show reduced levels and a heterogeneous distribution of the gap junction protein Connexin43 (Cx43) as well as the presence of fibrotic tissue[6-8].

The aim of this study was to characterize electrophysiological alterations and cardiac remodeling correlated with enhanced arrhythmogenicity on the background of renal failure. Therefore, two mouse models of renal dysfunction with different etiologies were tested: (1) an aldosterone-induced hypertension model using deoxycorticosterone acetate (DOCA) in combination with a high-salt diet in aged mice (DOCA-salt aged model), and (2) a more severe model using 5/6-subtotal nephrectomy (SNx) in combination with a high-salt diet in adult mice (SNx-salt adult model).

Methods

Ethics statement and animal housing

All the experimental protocols were performed in accordance with the national guidelines and approved by the local Ethical Animal Experimental Committee of the University of Utrecht, The Netherlands (approval No. 2010.II.11.201 and 2012.II.10.154). All animals were housed under standard conditions in a light-, temperature- and humidity-controlled environment.

Animal models of renal dysfunction

DOCA-salt aged mice

Thirty-week-old male 129Sv mice were used (Harlan Laboratories, Horst, The Netherlands). Renal dysfunction was induced in mice (experimental week 0) by the combination of a DOCA pellet (3.3 mg/day) and a high-salt diet (chow

containing 3% NaCl) for a period of 8 weeks (*n*=7). At experimental week 8, the DOCA pellet was removed and the high-salt diet withdrawn. At experimental week 11, the high-salt diet (6% NaCl) was continued until termination at experimental week 38 (68-week-old mice). Untreated age-matched mice were used as controls (*n*=4).

SNx-salt adult mice

Adult (8-week-old) male CD-1 mice were used (Charles River Laboratories, Sulzfeld, Germany). SNx was performed in two steps. At week -1, the right kidney was surgically removed (uninephrectomy), followed by removal of the poles from the left kidney at week 0. To the SNx-salt group, a high-salt diet (chow containing 6% NaCl) was given from week 1 until sacrifice at week 11 (*n*=5), whereas 9 mice underwent only SNx (SNx group).

Renal function and arterial pressure evaluation

To collect 16-hour urine, mice were placed in metabolic cages with food and water; values are expressed as per 24 h. Albumin was measured with a mouse albumin ELISA kit (Bethyl Laboratories Inc., Montgomery, USA). Systolic blood pressure and mean arterial pressure were measured using tail-cuff plethysmography and a catheter directly inserted into the femoral artery, respectively. Blood samples were collected by cheek puncture. Plasma urea was determined by DiaSys Urea CT FS (DiaSys Diagnostic Systems, Holzheim, Germany).

Echocardiography and epicardial mapping of Langendorff perfused hearts

Echocardiography was performed to determine cardiac function using a Vevo 2100 device (VisualSonics Inc., Toronto, Canada) with an MS550D transducer. Before termination, the mice were anesthetized with 4-5% isoflurane in oxygen and air. Next, the heart was excised and attached to a Langendorff retrograde perfusion setup. The heart was continuously perfused with a carbogen-gassed buffer at 37°C, composed of (in mM): NaCl 116, KCl 5, $MgSO_4$ 1.1, NaH_2PO_4 0.35, $NaHCO_3$ 27, glucose 10, mannitol 16 and $CaCl_2$ 1.8. Extracellular electrograms were recorded during stimulation (2x stimulation threshold) from the center of a 19x13 multielectrode grid, both from the left ventricle (LV) and right ventricle (RV), as described previously [9]. The conduction velocity was determined off-line from the recorded electrograms, as described previously [7], using custom-made software based on MATLAB (The MathWorks Inc., Natick, USA). Susceptibility to arrhythmia was provoked by programmed electrical stimulation using a standardized protocol published earlier [7].

Immunohistochemistry and histology

After the epicardial mapping procedure, the hearts were snap frozen in liquid nitrogen. For immunohistochemistry and fibrosis detection, cryosections of the heart (10 μm thickness) were prepared. Immunolabeling was performed to assess the subcellular distribution of Cx43, as described previously[9], using rabbit polyclonal anti-Cx43 (71-0700; Invitrogen, Carlsbad, USA) and mouse monoclonal anti-N-cadherin (C1821; Sigma-Aldrich, Saint Louis, USA) antibodies. Cx43 levels were expressed as the area immunolabeled for Cx43 normalized to control. For cardiac fibrosis detection, cryosections were fixed with 4% paraformaldehyde and stained with Picrosirius red as described previously [10]. The cryosections were visualized by light microscopy (Nikon Eclipse 80i; Nikon Europe B.V., Amstelveen, The Netherlands) and digitally analyzed using ImageJ software. The percentage of fibrosis was calculated as the area stained by Picrosirius red relative to the total tissue area.

Statistical analysis

Data are presented as means ± standard errors of the mean and analyzed by Student's t test or Fisher's exact test using GraphPad Prism 6 software (La Jolla, California, USA). Differences were considered statistically significant if $p<0.05$. Animals that did not complete the experiment were excluded from analysis.

Results

Arrhythmogenic remodeling in DOCA-salt aged mice

Renal dysfunction, morphological and echocardiographic data

DOCA-salt treatment of aged mice resulted in renal dysfunction confirmed by a significant increase of albumin in the urine (4.8±1.4 vs. 0.1±0.02mg/24h, $p<0.05$, Figure 1A). DOCA-salt mice developed hypertension, detected by an increase in systolic blood pressure as compared with aged control mice (135±5 vs. 88±8mmHg, $p<0.01$, Figure 1B). Plasma urea was not elevated or different between the two groups of mice (11.1±1.0 vs. 11.0±0.9mmol/L, *n.s.*, Figure 1C).
DOCA-salt mice presented with cardiac hypertrophy, backward failure (indicated by increased lung weight) and enlargement of the kidneys, as shown in Figures 1D-F. Secondly, cardiac function was impaired in DOCA-salt mice as assessed by echocardiography, which showed a decrease in fractional shortening (22.5±1.2 vs. 29.9±1.6%, $p<0.01$, Figure 1G).

Arrhythmia induction and conduction velocity

Isolated Langendorff perfused DOCA-salt mouse hearts were highly arrhythmogenic. An example of a polymorphic tachyarrhythmia is shown in Figure

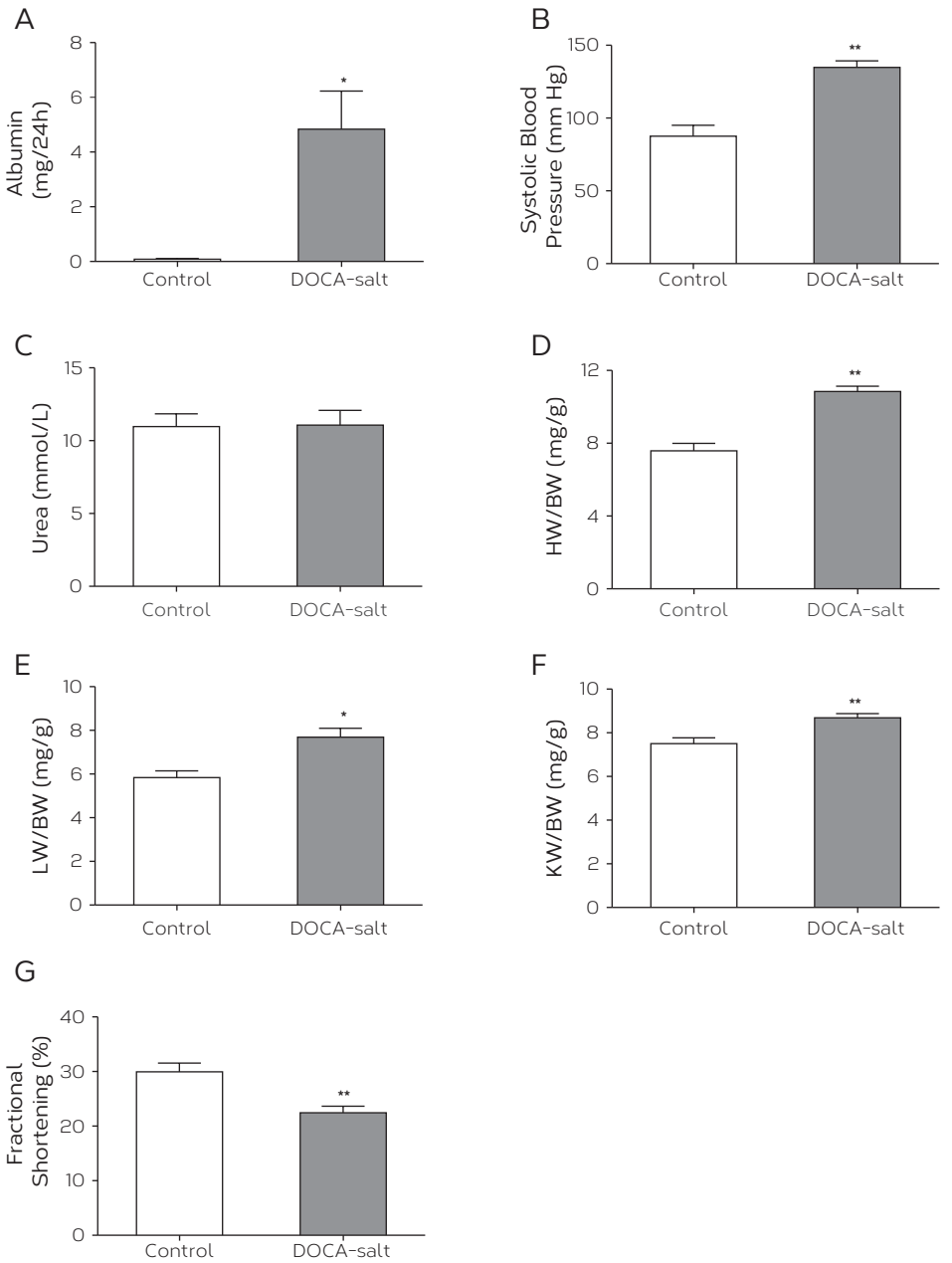

Figure 1. *Urinary albumin, systolic blood pressure, plasma urea, as well as tissue and echocardiographic parameters in control (n=4) and DOCA-salt (n=7) aged mice.* *p<0.05, **p<0.01 vs. control. *A*: Albumin measured in 24-hour urine samples. *B*: Systolic blood pressure measured by tail-cuff plethysmography. *C*: Urea measured in plasma samples. *D*: Heart weight-to-body weight ratio (HW/BW). *E*: Lung weight-to-body weight ratio (LW/BW). *F*: Kidney weight-to-body weight ratio (KW/BW; average of both kidneys). *G*: Fractional shortening.

2A. Arrhythmias were induced in 86% (6/7) of the DOCA-salt hearts as compared with 0% of control hearts ($p<0.05$, Figure 2B). Epicardial activation maps generated during ventricular tachyarrhythmias (VT) showed no signs of reentry activity (Figure 2C).

Conduction velocities (CVs) were obtained from paced epicardial activation maps for the LVs and RVs (Figure 2D). Longitudinal and transverse CVs in the RV were similar between control and DOCA-salt mice. Interestingly, DOCA-salt mice showed a significant decrease in both longitudinal (-32%) and transverse (-22%) CVs in the LV ($p<0.05$, Figure 2D).

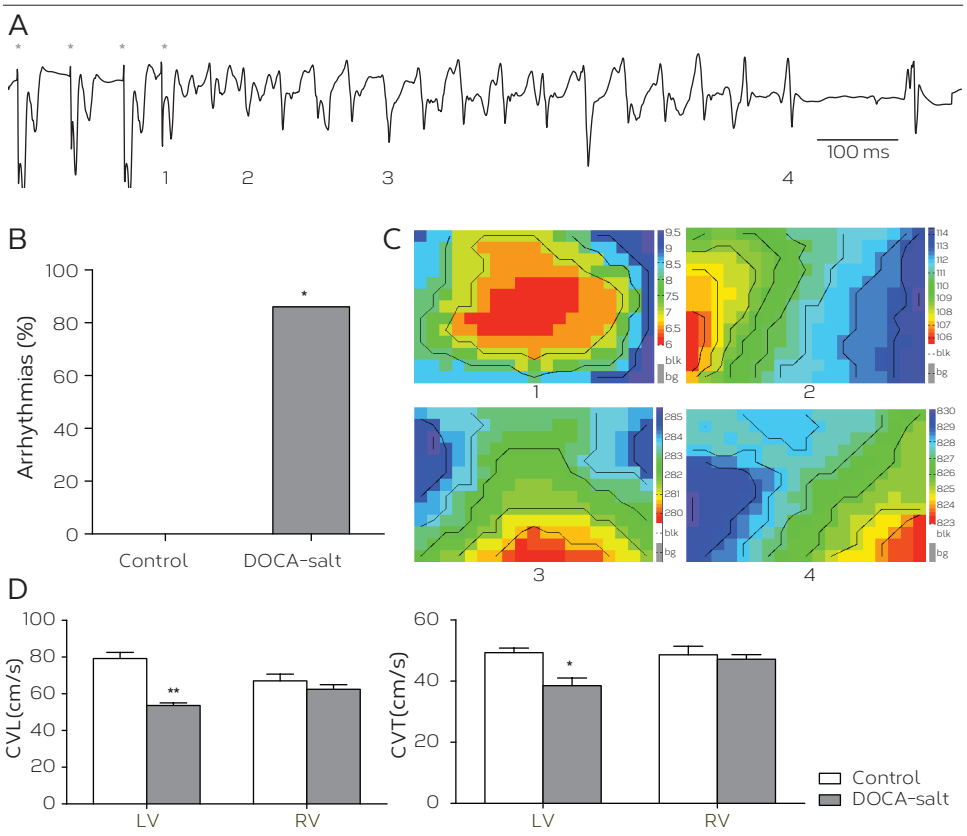

Figure 2. ***Arrhythmias and CV induced in perfused control and DOCA-salt aged mice. A**: Representative epicardial electrogram of a stimulation-induced polymorphic VT in DOCA-salt mice. Asterisks (*) indicate the last 4 burst-paced (60 ms) complexes. **B**: Incidence of arrhythmias in control (n=4) and DOCA-salt (n=7) mice. *p<0.05 vs. control **C**: Activation maps from the 4 numbered VT complexes indicated in the electrogram in **A**. The black isochronal lines of activation are 1 ms apart. Red represents the earliest activation time and blue the latest. **D**: CV measured by epicardial mapping on the LV (control: n=4; DOCA-salt: n=5) and RV (control: n=4; DOCA-salt: n=7) in longitudinal (CV_L; left) and transverse (CV_T; right) directions. *p<0.05, **p<0.01 vs. control.*

Cx43 expression and fibrosis

Cx43 expression was significantly reduced in DOCA-salt mice compared with control mice as assessed by immunohistochemistry (0.6±0.05 vs. 1.0±0.05, $p<0.01$, Figure 3A). Cardiac fibrosis as assessed by Picrosirius Red staining was significantly increased (3.7-fold) in DOCA-salt aged mice as compared with control mice of similar age ($p<0.01$, Figure 3B).

Arrhythmogenic remodeling in SNx-salt adult mice

Renal dysfunction and morphological data

SNx alone was not sufficient to develop renal dysfunction in adult mice of this strain (data not shown). Therefore, an additional SNx group was created in which salt was given to the food for 10 weeks as an extra renal challenge (SNx-salt).

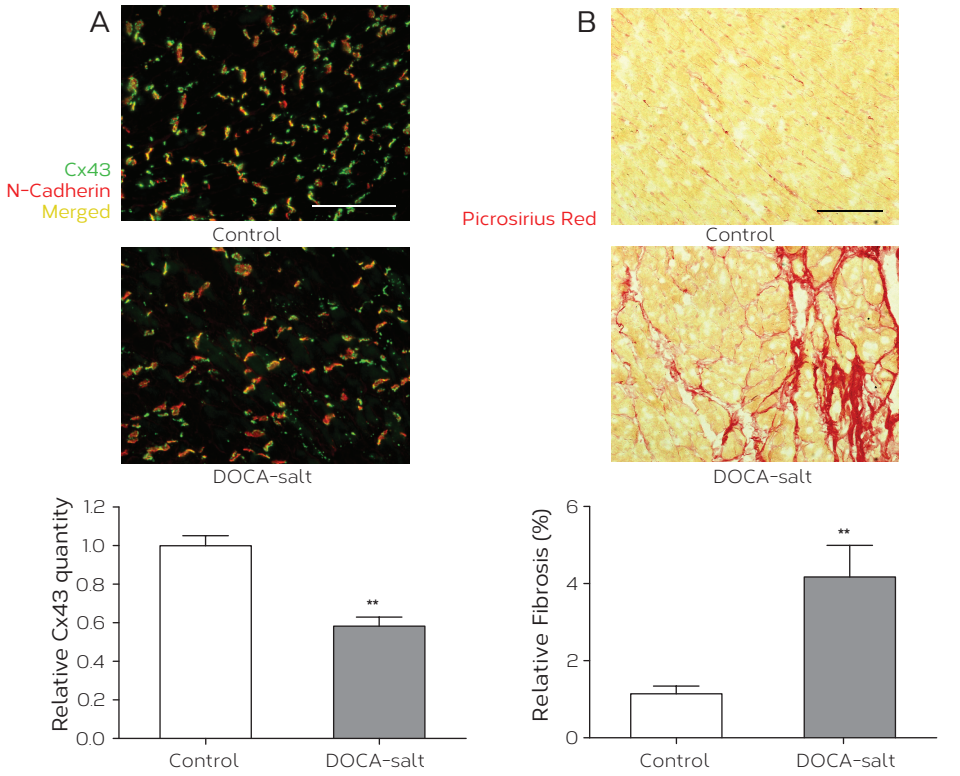

Figure 3. *Cx43 expression and fibrosis in isolated control (n=4) and DOCA-salt (n=7) aged mouse hearts.* ***p<0.01 vs. control.* ***A****: Representative pictures of Cx43 (green) and N-cadherin (red) expression in control and DOCA-salt hearts (top). N-cadherin was used as a marker for intercalated disks. Scale bar = 100 µm. Bottom: quantification of Cx43 immunolabeling.* ***B****: Representative pictures of fibrosis in control and DOCA-salt hearts (top). Scale bar = 100 µm. Bottom: quantification of fibrosis staining.*

Renal dysfunction was clearly present in SNx-salt compared to SNx, as indicated by a significant increase in albumin in the urine (186±25 vs. 8±6mg/24h, $p<0.01$, Figure 4A). The mean arterial pressure tended to be higher in SNx-salt mice (97±10 vs. 82±4mmHg, $p=0.16$, Figure 4B). Plasma urea was elevated in both the SNx-salt and the SNx group (21±2 vs. 22±2 mmol/L, *n.s.*, Figure 4C). Furthermore, there was a tendency towards increased heart, lung and kidney weights in the SNx-salt group (Figures 4D-F).

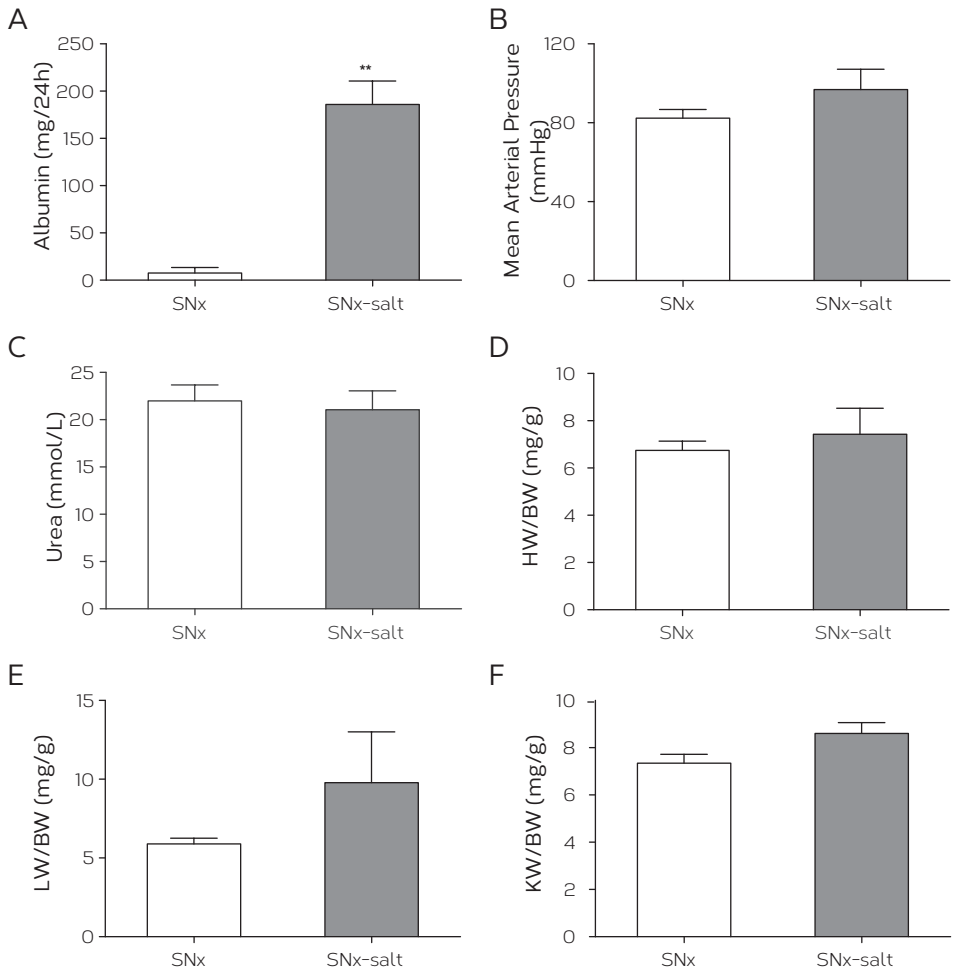

Figure 4. ***Urinary albumin, mean arterial pressure, plasma urea and tissue parameters in SNx and SNx-salt adult mice.*** ***p<0.01 vs. SNx.* ***A****: Albumin measured in 24-hour urine samples (SNx: n=5; SNx-salt: n=5).* ***B****: Mean arterial pressure measured using a catheter inserted into the femoral artery (SNx: n=9; SNx-salt: n=5).* ***C****: Urea measured in plasma samples (SNx: n=9; SNx-salt: n=5).* ***D****: Heart weight-to-body weight ratio (HW/BW).* ***E****: Lung weight-to-body weight ratio (LW/BW).* ***F****: Kidney (remnant) weight-to-body weight ratio (KW/BW).* ***D-F****: SNx: n=8; SNx-salt: n=5.*

Arrhythmia induction and conduction velocity

SNx-salt hearts were highly susceptible to polymorphic ventricular arrhythmias (Figure 5A). In the SNx-salt group, 75% of the hearts showed arrhythmias, as compared with 25% of the SNx hearts (*n.s.*, Figure 5B). The activation maps obtained during the VT showed no signs of reentry activity (Figure 5C). Epicardial CVs were similar in SNx and SNx-salt hearts (Figure 5D).

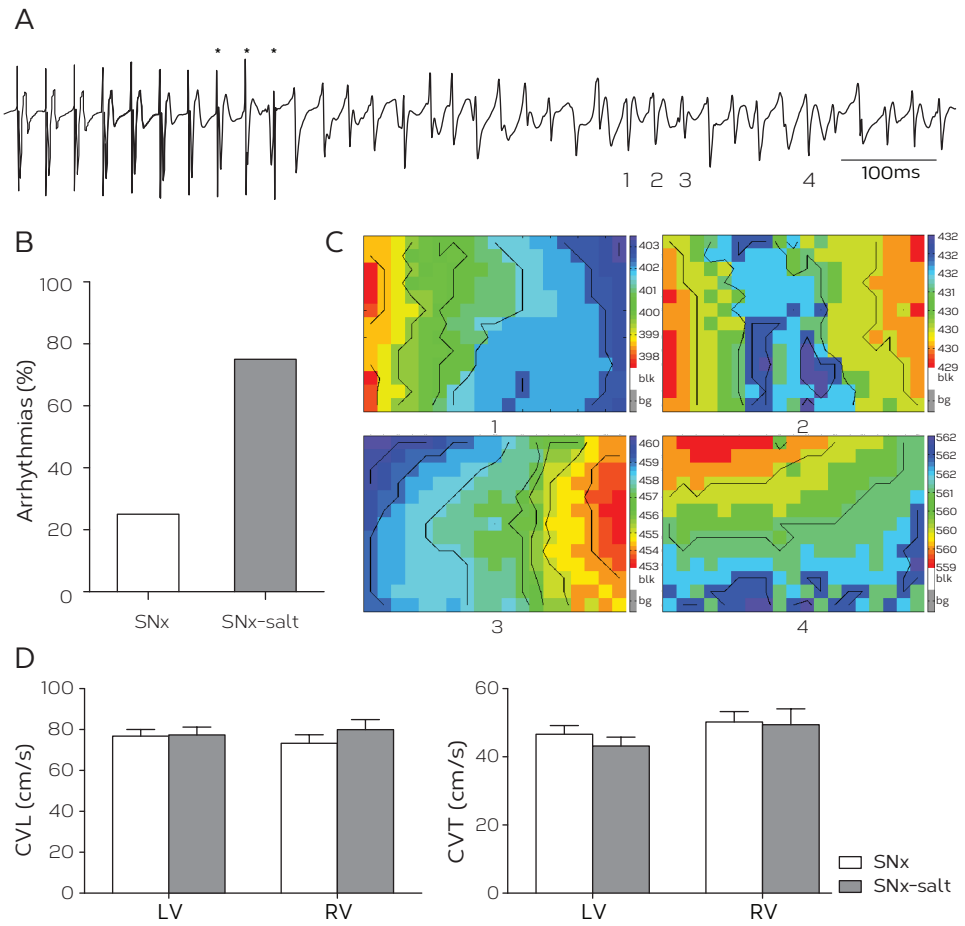

Figure 5. ***Arrhythmias and CVs induced in perfused SNx and SNx-salt adult mice.*** ***A****: Representative epicardial electrogram of a stimulation-induced polymorphic VT in SNx-salt mice. Asterisks (*) indicate the last 3 burst-paced (30 ms) complexes.* ***B****: Incidence of arrhythmias in SNx (n=8) and SNx-salt mice (n=4).* ***C****: Activation maps from the 4 numbered VT complexes indicated in the electrogram in* ***A****. The black isochronal lines of activation are 1 ms apart. Red represents the earliest activation time and blue the latest.* ***D****: CV measured by epicardial mapping on the LV (SNx: n=8; SNx-salt: n=3) and RV (SNx: n=8; SNx-salt: n=2) in longitudinal (CV_L: left) and transverse (CV_T: right) directions.*

Cx43 expression and fibrosis

Cx43 expression was significantly decreased upon administration of salt to SNx mice (0.6±0.08 vs. 1.0±0.06, $p<0.01$, Figure 6A). Additionally, SNx-salt hearts presented with significantly higher levels (1.4-fold) of cardiac fibrosis than SNx hearts ($p<0.05$, Figure 6B).

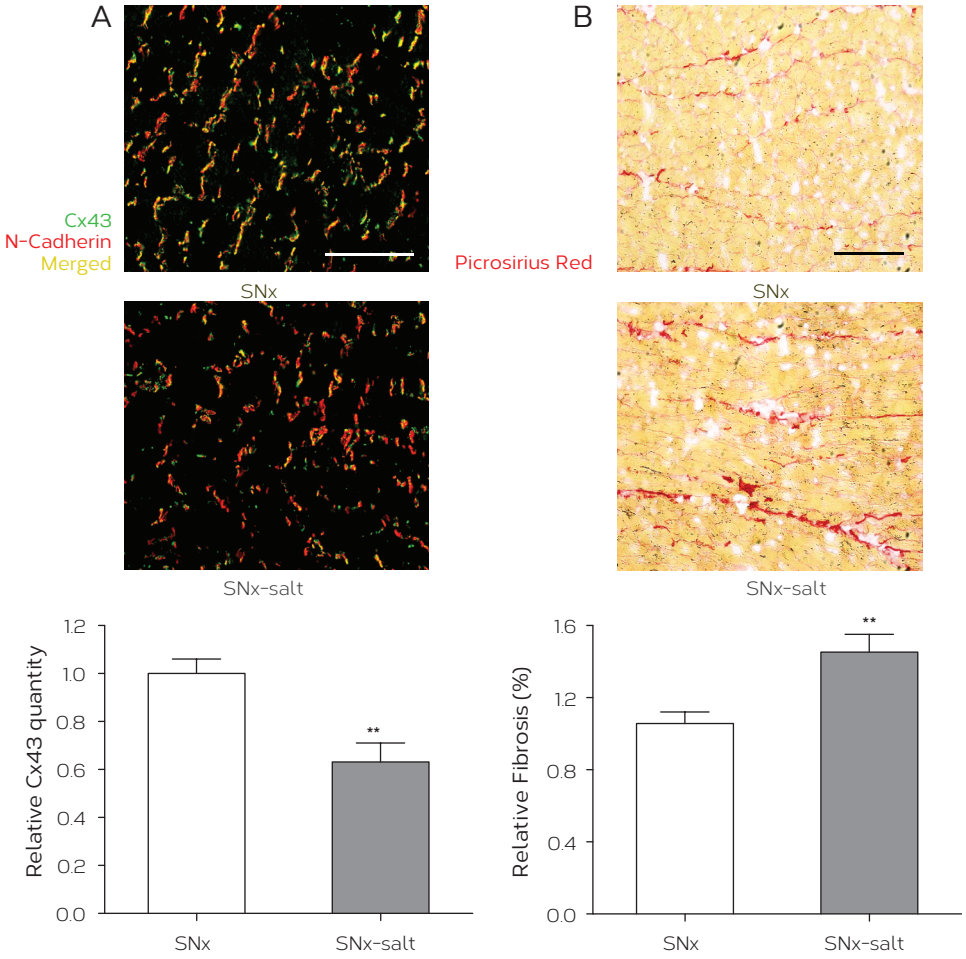

Figure 6. *Cx43 expression and fibrosis in isolated SNx (n=9) and SNx-salt (n=5) adult mouse hearts.* ***p<0.01 vs. SNx.* ***A****. Representative pictures of Cx43 (green) and N-cadherin (red) expression in SNx and SNx-salt hearts (top). N-cadherin was used as a marker for intercalated disks. Scale bar = 100 µm. Bottom: quantification of Cx43 immunolabeling.* ***B****. Representative pictures of fibrosis in SNx and SNx-salt hearts (top). Scale bar = 100 µm. Bottom: quantification of fibrosis staining*

Discussion

In this study, the relation between cardiac remodeling and arrhythmogenicity was investigated in two different mouse models of renal dysfunction: aged mice subjected to DOCA and salt (DOCA-salt) and adult mice subjected to SNx and salt (SNx-salt). The main findings of this study are the following: (1) The cardiorenal syndrome was present in both mouse models; renal failure was established with albuminuria in both mouse models, although DOCA-salt, but not SNx-salt, resulted in hypertension, cardiac hypertrophy, and decreased cardiac contractility; (2) Both models presented with a high incidence of arrhythmias accompanied by increased interstitial fibrosis and decreased Cx43 expression in the heart.

Induction of the cardiorenal syndrome in mice

Mice are known to be very resistant to induction of renal failure [11,12], usually requiring removal of large parts of the kidneys, therefore leaving little renal tissue for analysis. In order to retain both kidneys, we opted for the DOCA-salt mouse model and combined it with aging as an alternative trigger to the commonly used uninephrectomy [13], as aging increases the susceptibility for development of hypertension as well as renal and cardiac failure [14-16]. In our model, treating mice with DOCA-salt caused hypertension and albuminuria, confirming renal dysfunction. Besides renal injury, the combination of DOCA (which mimics aldosterone) and a high-salt diet induced cardiac remodeling as evidenced by hypertrophy, decreased fractional shortening, cardiac fibrosis, and reduced Cx43 expression. Several studies suggested that the development of cardiac hypertrophy and fibrosis in the DOCA-salt model is at least partly independent of the extent of hypertension [17-19]. Therefore, renal dysfunction but not hypertension may be the key factor causing cardiac remodeling.

The second model of renal failure was based on SNx in CD-1 mice. Although it is known that the susceptibility to developing renal failure is dependent on the strain of mouse used,[11,12] a study has shown that both CD-1 mice and 129S3 mice (a substrain of 129Sv) developed renal failure after SNx [20]. In our mouse model, however, SNx alone was not sufficient to result in hypertension or renal dysfunction, requiring the addition of another trigger. Therefore, we used a combination of SNx with a high-salt diet. The follow-up time of 11 weeks was limited by the progressive worsening of the clinical condition of the animals. The addition of a high-salt diet did not induce significant hypertension, but as the mean arterial pressure was determined under anesthesia, blood pressure might have been underestimated. Although no significant differences were found between SNx and SNx-salt mice with respect to

heart, lung or kidney weights, SNx-salt mice were more susceptible to arrhythmias than SNx mice. The low percentage of arrhythmias in the SNx group suggests that these mice were in an early stage of cardiac remodeling, which was further enhanced in the SNx-salt group.
Both mouse models used in this study showed renal dysfunction and cardiac remodeling. In addition, they were highly susceptible to arrhythmias; therefore, they at least phenotypically reflect the cardiorenal syndrome in patients. Furthermore, the presence of similar and severe arrhythmias in both models without significant hypertension, left ventricular hypertrophy and uremia in the SNx-salt model suggests that hypertension, left ventricular hypertrophy and uremia per se are presumably not prerequisites for arrhythmias in the cardiorenal syndrome. More subtle changes within the heart are probably required, as discussed below.

Development of the arrhythmogenic substrate in the cardiorenal syndrome

Both models of the cardiorenal syndrome exhibited high levels of arrhythmias. The high susceptibility to cardiac arrhythmias in our mouse models was accompanied by cardiac remodeling with reduced Cx43 expression and increased fibrosis. Previous studies have shown that reduced or abnormal Cx43 expression and/or increased fibrosis are strongly associated with arrhythmias in patients [21-23], as well as in dog [24,25] and mouse models of cardiac remodeling and failure [7,21,26]. Furthermore, in a mouse model of cardiac pressure overload, the involvement of abnormal Cx43 expression in the arrhythmogenic substrate was associated with dispersion in CV, rather than conduction slowing, suggesting a triggered activity mechanism [21]. In the LV of DOCA-salt aged hearts, CV was slowed both along and parallel to the fiber orientation, which may have contributed to the arrhythmogenic substrate. In SNx-salt hearts, however, arrhythmogenicity was also high, with comparable polymorphic VTs and activation patterns, albeit without conduction slowing. It seems therefore that conduction slowing is not a prerequisite for arrhythmias in this model. Triggered activity as an arrhythmia mechanism was shown earlier in a chronic kidney disease rat model [27]. This is well supported by the high-resolution epicardial activation mapping in our study, which did not show evident signs of reentry, making a triggered activity a very likely mechanism for the arrhythmias.

Clinical implications

Our data using cardiorenal syndrome mouse models show that under conditions of excess mineralocorticoid hormone and high salt, or upon the combination of SNx and high salt, there is an increased susceptibility to arrhythmias.

Previously, we demonstrated that chronic treatment of aged arrhythmogenic mice with the aldosterone antagonist eplerenone significantly decreased cardiac fibrosis, restored normal levels of Cx43 in the heart and, most importantly, reduced the amount of arrhythmias [8]. A similar result was obtained in the context of pressure-overloaded mouse hearts treated with spironolactone, which normalized Cx43 expression, reduced fibrosis and restored normal impulse conduction [28]. Inhibiting the aldosterone pathway may therefore be a therapeutic avenue to suppress the development of the arrhythmogenic substrate in cardiorenal syndrome patients.

Conclusion

In conclusion, our data provide evidence that renal dysfunction in the high-salt DOCA and SNx models causes pronounced structural and electrical cardiac remodeling and a markedly enhanced susceptibility to arrhythmias. The reduced Cx43 expression and increased fibrosis levels in these hearts are likely candidates for the formation of the arrhythmogenic substrate.

Acknowledgments

We thank Krista den Ouden, Paula Martens and Myrthe den Toom for their excellent technical assistance.

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Cell-based therapies for experimental chronic kidney disease

a systematic review and meta-analysis

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Published in Disease Models and Mechanisms 2015 8:281-293

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Abstract

Cell-based therapy is a promising strategy for treating chronic kidney disease (CKD) and is currently the focus of preclinical studies. We performed a systematic review and meta-analysis to evaluate the efficacy of cell-based therapy in preclinical (animal) studies of CKD, and determined factors affecting cell-based therapy efficacy in order to guide future clinical trials. In total, 71 articles met the inclusion criteria. Standardized mean differences (SMD) and 95% confidence intervals (CI) were calculated for outcome parameters including plasma urea, plasma creatinine, urinary protein, blood pressure, glomerular filtration rate, glomerulosclerosis and interstitial fibrosis. Sub-analysis for each outcome measure was performed for model-related factors (species, gender, model and timing of therapy) and cell-related factors (cell type, condition and origin, administration route and regime of therapy). Overall, meta-analysis showed that cell-based therapy reduced the development and progression of CKD. This was most prominent for urinary protein (SMD, 1.34; 95% CI, 1.00–1.68) and urea (1.09; 0.66–1.51), both $P<0.001$. Changes in plasma urea were associated with changes in both glomerulosclerosis and interstitial fibrosis. Sub-analysis showed that cell type (bone-marrow-derived progenitors and mesenchymal stromal cells being most effective) and administration route (intravenous or renal artery injection) were significant predictors of therapeutic efficacy. The timing of therapy in relation to clinical manifestation of disease, and cell origin and dose, were not associated with efficacy. Our meta-analysis confirms that cell-based therapies improve impaired renal function and morphology in preclinical models of CKD. Our analyses can be used to optimise experimental interventions and thus support both improved preclinical research and development of cell-based therapeutic interventions in a clinical setting.

Introduction

Worldwide, the number of individuals with chronic kidney disease (CKD) is rising, mainly owing to a dramatic increase in atherosclerosis and type-2 diabetes [1]. CKD is a progressive condition causing significant morbidity and mortality. The ensuing end-stage renal disease and associated increase in cardiovascular risk represent a significant socio-economic burden. Slowing CKD progression is therefore a major health priority.

Cell-based therapy has proven to be a promising clinical approach for several pathological conditions and might represent a novel therapeutic strategy to slow the progression of kidney disease [2]. Preclinical studies have demonstrated beneficial effects of various (stem) cell populations and cell-derived factors – secreted growth factors, microvesicles and exosomes – in acute kidney injury models, suggesting a renal regenerative effect of cell-based therapies. Importantly, these preclinical observations have already translated into pioneering clinical trials. Recently, a phase I clinical trial showed that administration of allogeneic mesenchymal stem cells (MSCs) to open-heart surgery patients at high risk of acute renal failure was feasible and safe [2]. Furthermore, MSCs are being used in several clinical trials in kidney transplant recipients with the aim of increased immunosuppression and improved regeneration [3,4].

CKD is characterized by reduced renal regenerative capacity. Several studies suggest beneficial regenerative effects of cell-based therapies in animal models of CKD. However, it is unclear which cell types or cell products improve renal function and morphology most effectively in experimental CKD. The design of preclinical studies is very diverse, varying in terms of models of CKD, timing of interventions, cell type or cell product, number of cells, administration route and read-out of kidney function and morphology, which makes translation to the clinic difficult. A meta-analysis and systematic review of existing animal studies will facilitate the design of future clinical studies. Moreover, the information obtained can be used to optimise existing experimental animal models and interventions and thus to improve preclinical research in the future. We have performed a systematic review and meta-analysis in order to evaluate the effect of cell-based therapy on kidney function and morphology outcome parameters, and we have analysed cell- and model-related aspects. To identify potential bona fide markers of target organ injury in the setting of cell-based therapy, we performed a correlation analysis between functional data (blood pressure and blood and urinary markers) and morphological data [glomerulosclerosis (GS) and tubular interstitial fibrosis (IF)]

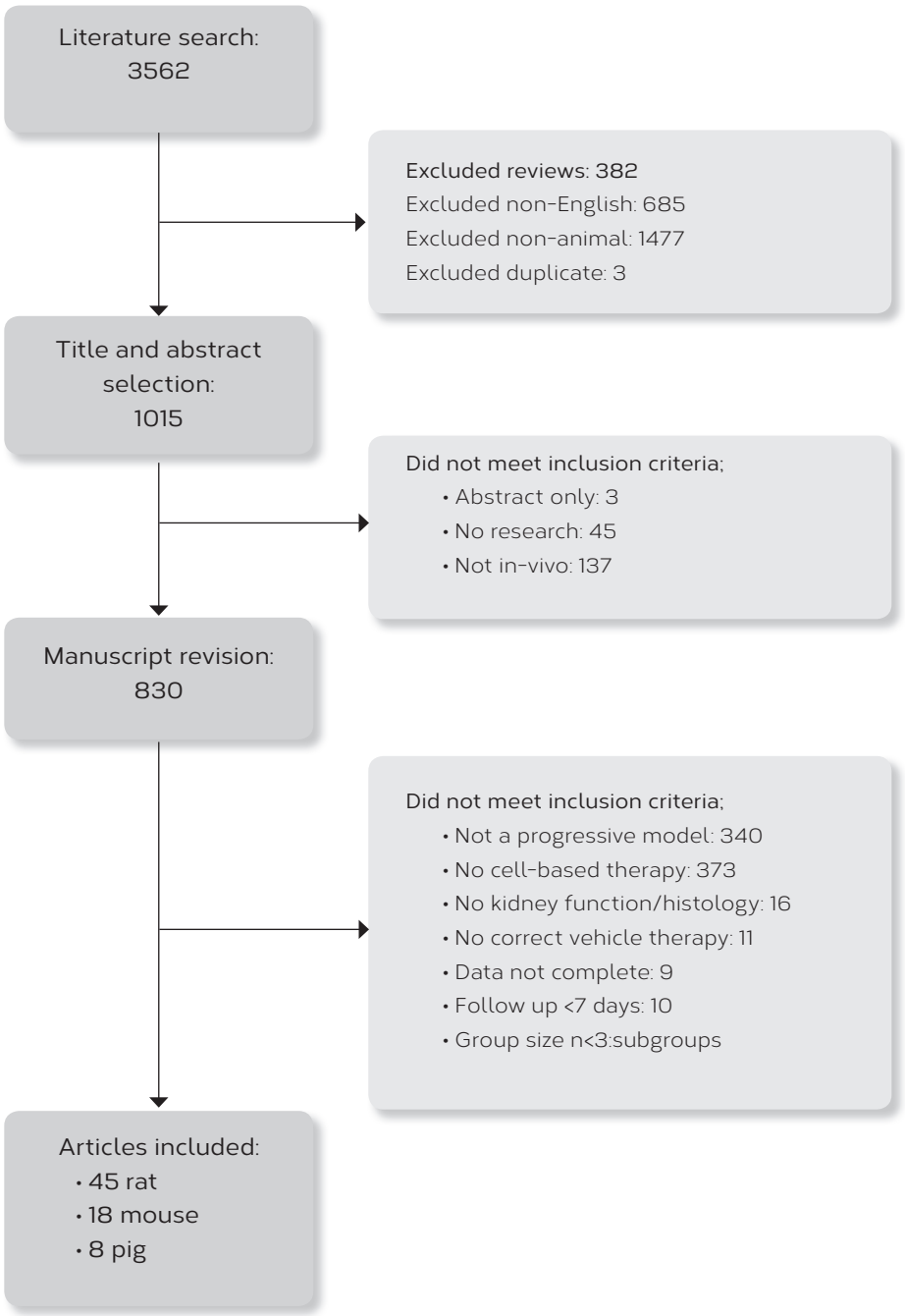

Figure 1. *Flow chart of study selection.*

Results

Study selection and characteristics

Our electronic search strategy delivered 1015 articles from PubMed database, 944 of which were excluded because inclusion criteria were not met. Data were extracted from 71 articles (Fig. 1). A large variation in study characteristics was observed (supplementary material Table S1). Cell types and products were pooled to facilitate analysis (supplementary material Tables S2, S3). Most studies used MSCs (58%) to evaluate their therapeutic efficacy on CKD. Only three studies evaluated both preventive as well as rescue cell-based interventions, whereas 38% studied only preventive and 58% only rescue interventions. Most studies used single administration (68%), 23% used multiple administrations (two to eight times) and 9% of the studies investigated both. Of all five cell-delivery routes (renal artery, intra-arterial non-renal, intravenous, parenchymal or subcapsular, and intraperitoneal), intravenous cell administration was used in the majority of studies (68%). A total of 1813 animals were used to investigate the effect of cell-based therapies on CKD – 442 mice, 1244 rats and 127 pigs, representing 1056 male and 585 female animals. Ten studies did not report the gender of the animals. In rats and mice, CKD was induced using 19 different models that we first pooled into the following groups: subtotal nephrectomy (SNX), diabetic nephropathy (DN), ischemia-reperfusion injury, genetic non-diabetes and hypertension (supplementary material Table S3). Studies in pigs all used renal artery stenosis to induce kidney injury.

Meta-analysis of outcome measures

The efficacy of cell-based therapy in treating CKD was assessed using the following functional parameters: plasma creatinine, plasma urea, glomerular filtration rate (GFR), blood pressure (BP) and urinary protein. We analysed 51 studies that measured plasma creatinine (mice, 10; pigs, 5; rats, 36) and 27 studies that measured plasma urea (mice, 8; rats, 19). The GFR analysis contained studies that reported inulin (rats, 6) or creatinine clearance (15 studies: mice, 2; rats, 13) and 8 studies with multi-detector computed tomography (MDCT, all in pigs). BP analysis included 10 studies in rats and 8 in pigs, and urinary protein analysis included 46 studies (mice, 10; pigs, 3; rats, 33). We also analysed the histological parameters GS [28 studies (mice, 8; pigs, 3; rats, 17)] and IF [27 studies (mice, 2; pigs, 8; rats, 17)].

Our meta-analysis showed that treatment of CKD with cells or cell products significantly improved functional and histological parameters. Results for the effect of cell-based therapy on plasma creatinine are summarised insupplementary material Fig. S1.

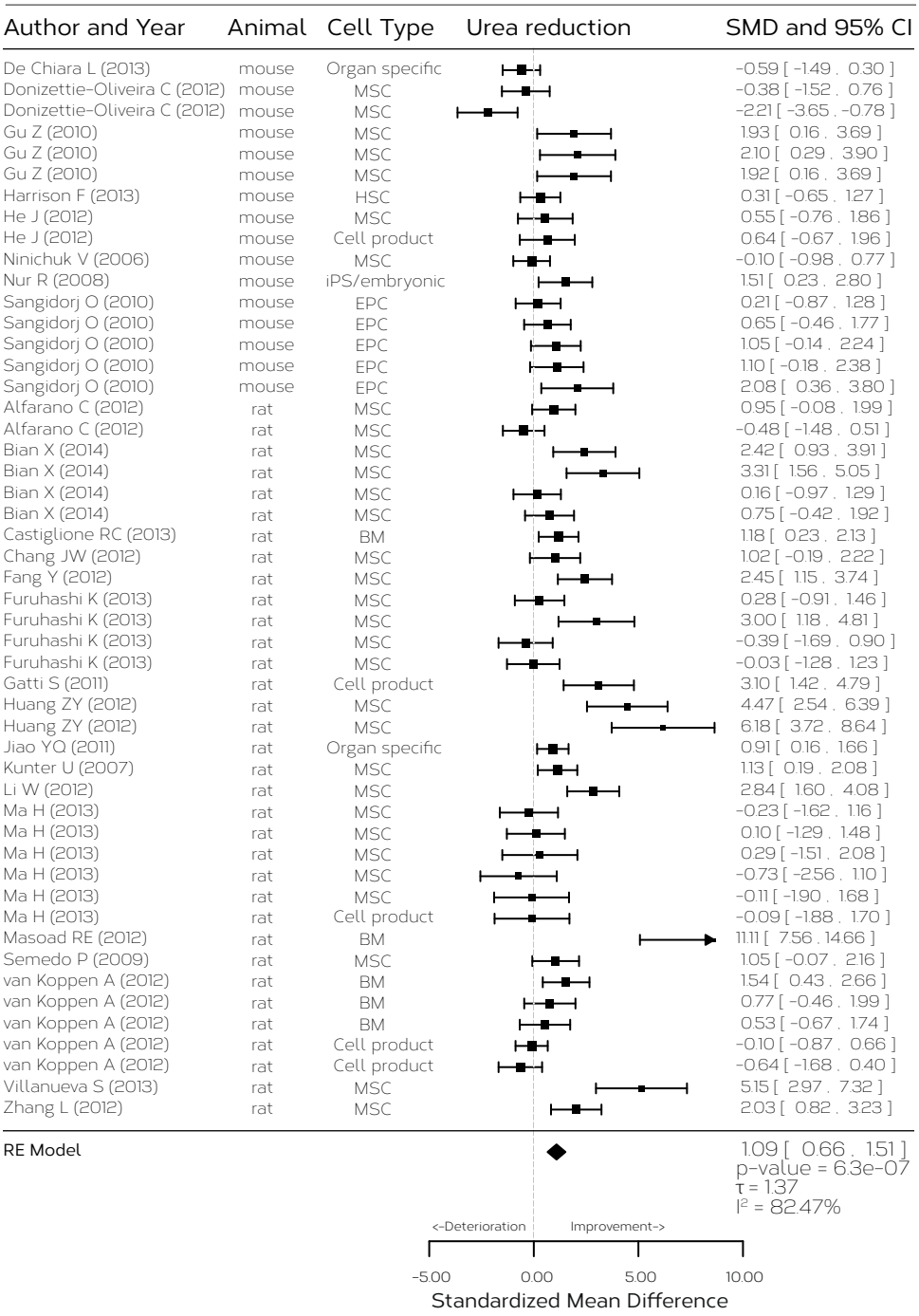

Figure 2. ***Effect of cell-based treatment in CKD on plasma urea.*** *Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI. Only the first author of each paper is shown. iPS, induced pluripotent stem cell; RE, random effects.*

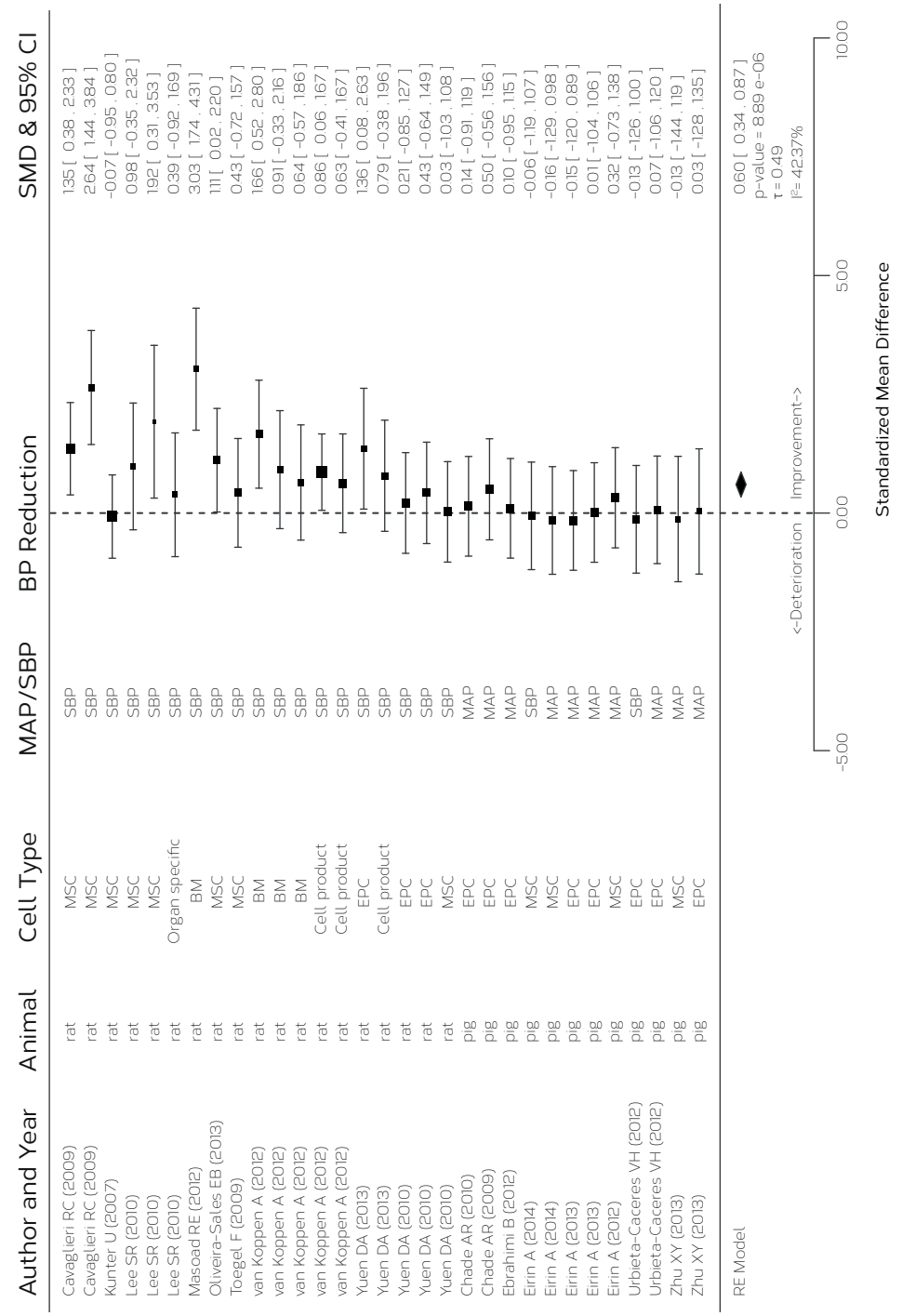

Figure 3. *Effect of cell-based treatment in CKD on blood pressure.* Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

Author and Year	Animal	Cell Type	Measurement	Urinary Protein Reduction	SMD & 95% CI
Cao Q (2013)	mouse	Other	Proteinuria		0.36 [-1.31 , 2.02]
Cao Q (2013)	mouse	Other	Proteinuria		0.73 [-0.96 , 2.42]
Cao Q (2013)	mouse	Other	Proteinuria		0.17 [-1.49 , 1.82]
Cao Q (2013)	mouse	Other	Proteinuria		4.05 [1.53 , 6.56]
Challen GA (2006)	mouse	Organ specific	Alb/Creat Ratio		0.50 [-1.48 , 2.49]
Challen GA (2006)	mouse	Organ specific	Alb/Creat Ratio		3.68 [0.58 , 6.79]
Chen J (2009)	mouse	BM	Alb/Creat Ratio		2.41 [0.54 , 4.28]
Chen J (2009)	mouse	BM	Alb/Creat Ratio		1.31 [-0.29 , 2.91]
Chen J (2009)	mouse	BM	Alb/Creat Ratio		0.96 [-0.76 , 2.67]
Chen J (2009)	mouse	BM	Alb/Creat Ratio		2.10 [0.12 , 4.07]
Ezquer F (2009)	mouse	MSC	Alb/Creat Ratio		1.44 [0.34 , 2.54]
Gu Z (2010)	mouse	MSC	Proteinuria		1.57 [-0.13 , 3.26]
Gu Z (2010)	mouse	MSC	Proteinuria		2.17 [0.35 , 3.98]
Gu Z (2010)	mouse	MSC	Proteinuria		1.45 [-0.23 , 3.12]
He J (2012)	mouse	MSC	Proteinuria		4.69 [2.47 , 6.91]
He J (2012)	mouse	Cell product	Proteinuria		6.59 [3.75 , 9.44]
Hyun YY (2012)	mouse	MSC	Prot/Creat Ratio		1.99 [0.13 , 3.85]
Hyun YY (2012)	mouse	MSC	Prot/Creat Ratio		4.21 [1.69 , 6.74]
Hyun YY (2012)	mouse	MSC	Prot/Creat Ratio		4.24 [1.90 , 6.58]
Ratliff BB (2010)	mouse	EPC	Prot/Creat Ratio		2.14 [0.72 , 3.56]
Sangidorj O (2010)	mouse	EPC	Prot/Creat Ratio		8.10 [3.95 , 12.45]
Zhang Y (2012)	mouse	EPC	Albuminuria		0.95 [-0.08 , 1.98]
Zhang Y (2012)	mouse	EPC	Albuminuria		5.00 [3.10 , 6.91]
Alexandre CS (2009)	rat	HSC	Proteinuria		1.12 [-0.16 , 2.40]
Alexandre CS (2009)	rat	HSC	Proteinuria		1.46 [0.12 , 2.79]
Caldas HC (2011)	rat	MSC	Proteinuria		2.10 [0.25 , 3.96]
Caldas HC (2011)	rat	BM	Proteinuria		1.88 [0.08 , 3.67]
Caldas HC (2011)	rat	MSC	Proteinuria		1.39 [-0.29 , 3.06]
Caldas HC (2011)	rat	BM	Proteinuria		0.95 [-0.64 , 2.54]
Caldas HC (2008)	rat	BM	Proteinuria		0.34 [-1.18 , 1.87]
Caldas HC (2008)	rat	MSC	Proteinuria		1.36 [-0.31 , 3.02]
Castiglione RC (2013)	rat	BM	Proteinuria		0.65 [-0.25 , 1.55]
Cavaglieri RC (2009)	rat	MSC	Proteinuria		-0.03 [-0.91 , 0.85]
Cavaglieri RC (2009)	rat	MSC	Proteinuria		5.81 [3.80 , 7.81]
Cavaglieri RC (2009)	rat	MSC	Albuminuria		0.49 [-0.40 , 1.38]
Cavaglieri RC (2009)	rat	MSC	Albuminuria		1.21 [0.26 , 2.17]
Chang JW (2012)	rat	MSC	Prot/Creat Ratio		-0.07 [-1.21 , 1.06]
Franquesa M (2012)	rat	BM	Proteinuria		0.59 [-0.80 , 1.99]
Franquesa M (2012)	rat	MSC	Proteinuria		0.94 [-0.40 , 2.28]
Furuhashi K (2013)	rat	MSC	Proteinuria		-0.34 [-1.63 , 0.95]
Furuhashi K (2013)	rat	MSC	Proteinuria		-0.14 [-1.40 , 1.12]
Gatti S (2011)	rat	Cell product	Proteinuria		1.59 [0.30 , 2.89]
Gatti S (2011)	rat	Cell product	Prot/Creat Ratio		2.32 [0.86 , 3.78]
Guo J (2013)	rat	MSC	Proteinuria		0.87 [0.34 , 1.40]
Huang ZY (2012)	rat	MSC	Proteinuria		4.09 [2.27 , 5.90]
Huang ZY (2012)	rat	MSC	Proteinuria		4.37 [2.48 , 6.27]
Jiao YQ (2011)	rat	Organ specific	Proteinuria		0.61 [-0.12 , 1.35]
Jiao YQ (2011)	rat	Organ specific	Albuminuria		-0.94 [-1.69 , -0.18]
Kunter U (2007)	rat	MSC	Proteinuria		1.44 [0.46 , 2.43]
Kunter U (2007)	rat	MSC	Prot/Creat Ratio		1.23 [0.27 , 2.19]
Lee SR (2010)	rat	MSC	Proteinuria		0.49 [-0.81 , 1.80]
Lee SR (2010)	rat	MSC	Proteinuria		0.90 [-0.54 , 2.34]
Lee SR (2010)	rat	Organ specific	Proteinuria		-0.16 [-1.46 , 1.13]
Li W (2012)	rat	MSC	Proteinuria		6.64 [4.40 , 8.87]
Lv SS (2013)	rat	MSC	Albuminuria		2.78 [1.81 , 3.76]
Ma H (2013)	rat	MSC	Proteinuria		-0.46 [-1.86 , 0.94]
Ma H (2013)	rat	MSC	Proteinuria		-0.32 [-1.71 , 1.07]
Ma H (2013)	rat	MSC	Proteinuria		3.58 [1.03 , 6.13]
Ma H (2013)	rat	MSC	Proteinuria		3.92 [1.25 , 6.59]
Ma H (2013)	rat	MSC	Proteinuria		3.95 [1.27 , 6.63]
Ma H (2013)	rat	Cell product	Proteinuria		0.40 [-1.40 , 2.21]
Ma H (2013)	rat	MSC	Albuminuria		-0.70 [-2.53 , 1.12]
Ma H (2013)	rat	MSC	Albuminuria		-1.21 [-3.10 , 0.68]
Ma H (2013)	rat	MSC	Albuminuria		-1.14 [-3.02 , 0.74]
Ma H (2013)	rat	Cell product	Albuminuria		-4.75 [-7.75 , -1.75]
Masoad RE (2012)	rat	BM	Alb/Creat Ratio		22.59 [15.54 , 29.65]
Oliveira-Sales EB (2013)	rat	MSC	Proteinuria		0.25 [-0.77 , 1.26]
Park JH (2012)	rat	MSC	Proteinuria		0.66 [-0.42 , 1.73]
Park JH (2012)	rat	MSC	Prot/Creat Ratio		1.06 [-0.06 , 2.18]
Semedo P (2009)	rat	MSC	Prot/Creat Ratio		0.28 [-0.77 , 1.34]
Semedo P (2009)	rat	MSC	Prot/Creat Ratio		1.16 [0.03 , 2.29]
Shuai L (2012)	rat	EPC	Proteinuria		-0.82 [-2.24 , 0.61]
Shuai L (2012)	rat	EPC	Proteinuria		-0.55 [-1.96 , 0.85]
Shuai L (2012)	rat	EPC	Proteinuria		-0.85 [-2.28 , 0.58]
Shuai L (2012)	rat	EPC	Proteinuria		-0.31 [-1.70 , 1.09]
Shuai L (2012)	rat	EPC	Proteinuria		-0.69 [-2.10 , 0.73]
Shuai L (2012)	rat	EPC	Proteinuria		-0.38 [-1.77 , 1.02]
Shuai L (2012)	rat	EPC	Proteinuria		0.60 [-0.81 , 2.01]
Shuai L (2012)	rat	EPC	Proteinuria		0.75 [-0.67 , 2.17]
Shuai L (2012)	rat	EPC	Proteinuria		0.86 [-0.57 , 2.30]
van Koppen A (2012)	rat	BM	Proteinuria		0.78 [-0.23 , 1.80]
van Koppen A (2012)	rat	BM	Proteinuria		1.29 [0.00 , 2.59]
van Koppen A (2012)	rat	BM	Proteinuria		1.03 [-0.22 , 2.29]
van Koppen A (2012)	rat	Cell product	Proteinuria		0.61 [-0.17 , 1.40]
van Koppen A (2012)	rat	Cell product	Proteinuria		-0.41 [-1.43 , 0.62]
Wang S (2013)	rat	MSC	Alb/Creat Ratio		3.06 [1.66 , 4.46]

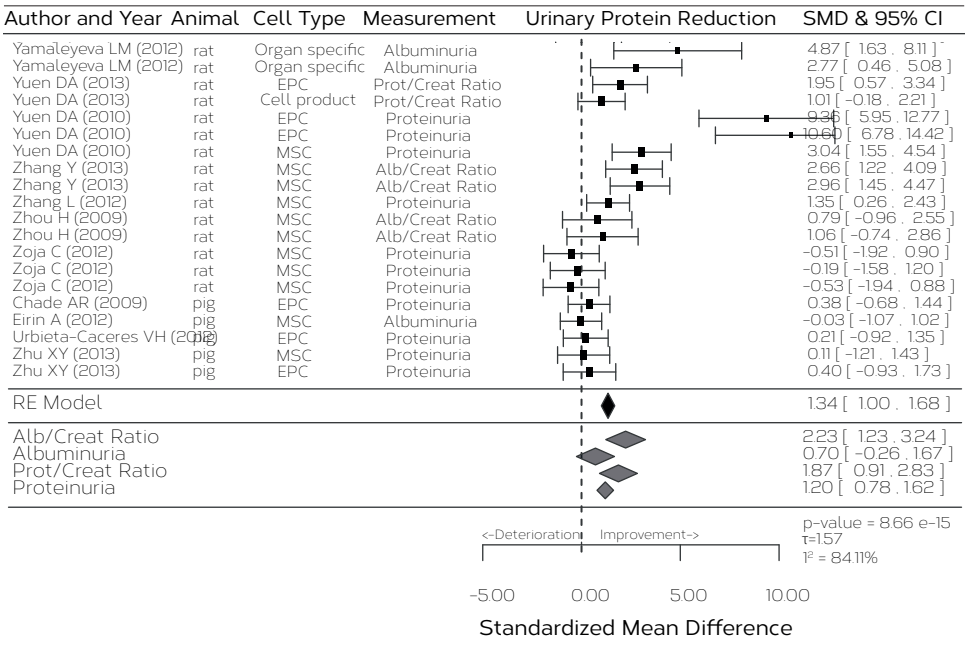

Figure 4. ***Effect of cell-based treatment in CKD on urinary protein.*** *Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI. Only the first author of each paper is shown. RE, random effects.*

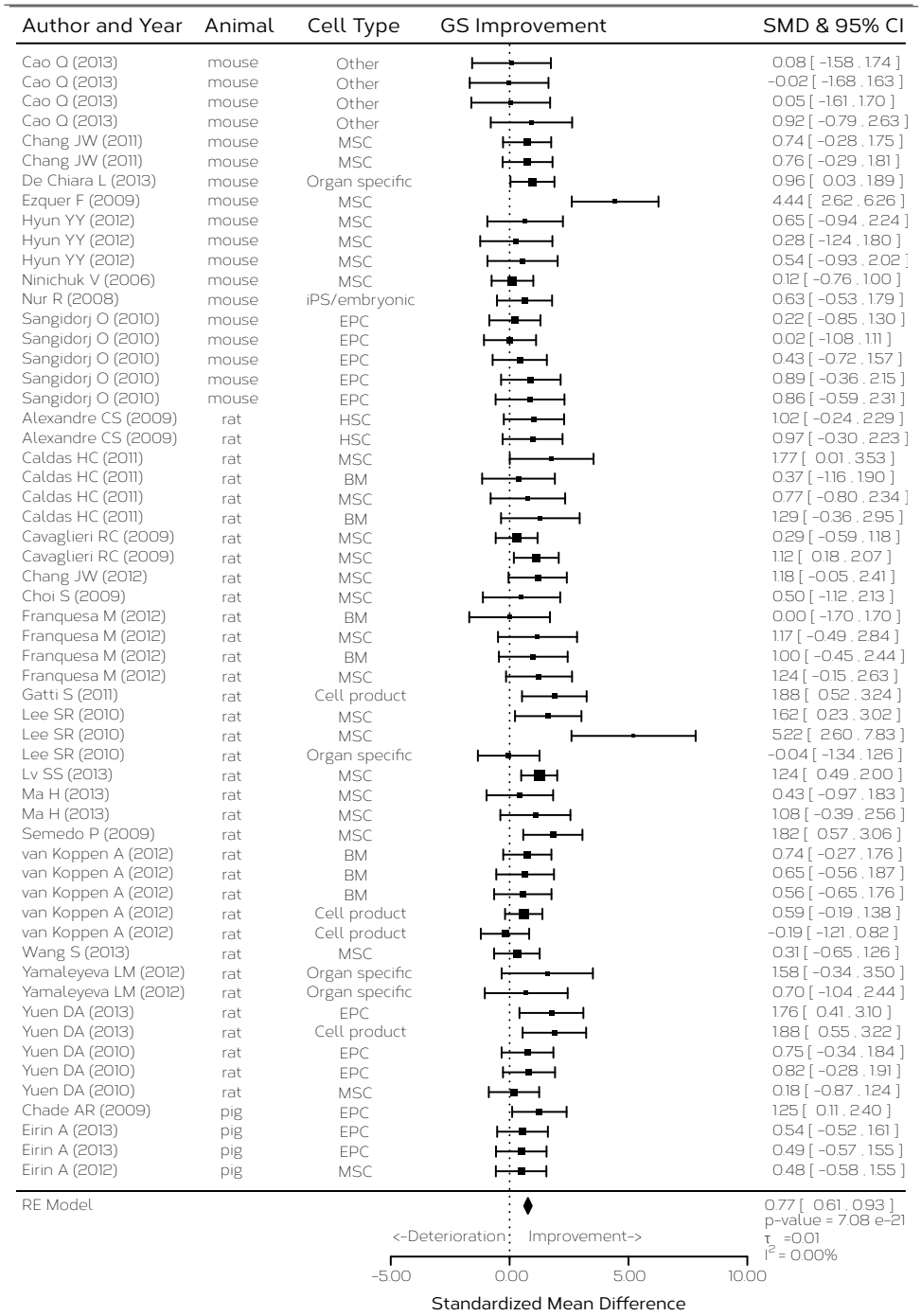

Figure 5. ***Effect of cell-based treatment in CKD on glomerulosclerosis.*** *Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI. Only the first author of each paper is shown. iPS, induced pluripotent stem cell; RE, random effects.*

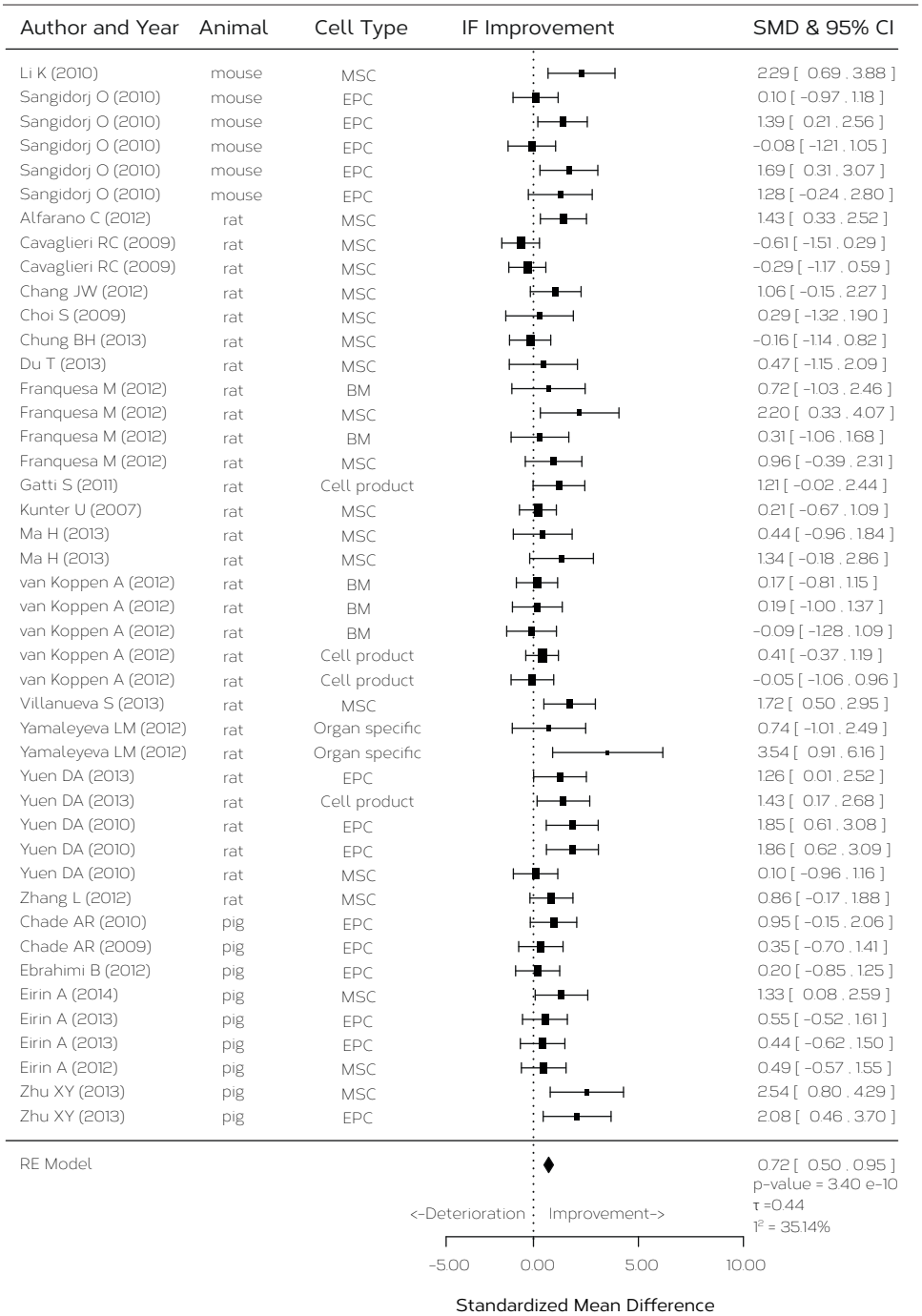

Figure 6. *Effect of cell-based treatment in CKD on interstitial fibrosis. Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI. Only the first author of each paper is shown. RE, random effects.*

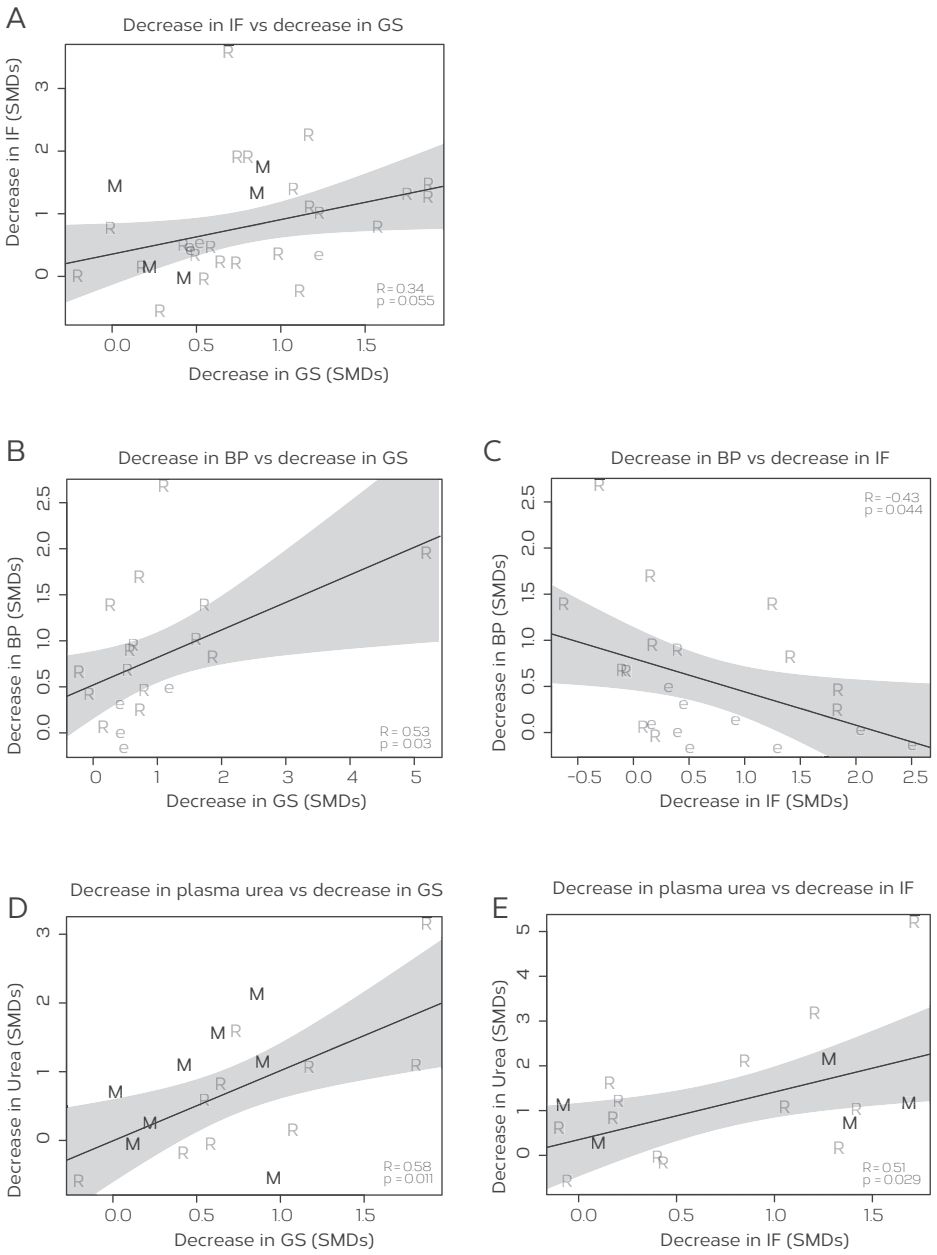

Figure 7. *Correlations between renal functional parameters and tissue injury parameters*

(A) Correlation between decrease in interstitial fibrosis (IF) versus decrease in glomerulosclerosis (GS). ***(B)*** *Correlation between decrease in blood pressure (BP) versus decrease in GS.* ***(C)*** *Correlation between decrease in BP versus decrease in IF.* ***(D)*** *Correlation between decrease in plasma urea versus decrease in GS.* ***(E)*** *Correlation between decrease in plasma urea versus decrease in GS. M. mouse; r. rat.; e. pig.*

Plasma creatinine decreased after cell-based therapy compared with that of vehicle-treated or control animals (SMD, 0.98; 95% CI, 0.73, 1.24; *P*<0.001). Similarly, cell-based therapy reduced plasma urea in experimental CKD (SMD, 1.09; 95% CI, 0.66, 1.51; *P*<0.001; Fig. 2). Cell-based therapy increased GFR (SMD, 1.05; 95% CI, 0.67, 1.43; *P*<0.001; supplementary material Fig. S2) and decreased BP (SMD, 0.60; 95% CI, 0.34, 0.87; *P*<0.001; Fig. 3) compared with that of control animals. The outcome measure most amenable to improvement by cell-based therapy was urinary protein (SMD, 1.34; 95% CI, 1.00, 1.68; *P*<0.001; Fig. 4). Reductions in GS (SMD, 0.77; 95% CI, 0.61, 0.93; *P*<0.001;Fig. 5) and IF (SMD, 0.72; 95% CI 0.50, 0.95; *P*<0.001; Fig. 6) were observed after cell-based therapy. Heterogeneity in effect size between studies was high (>72%) for all functional outcome parameters except for BP, where it was moderate (42%). No heterogeneity was detected between studies that measured GS. Heterogeneity between studies that measured IF was moderate (35%).

Correlations between functional measurements and renal tissue injury

No significant correlation was found between reductions in GS and IF (*R*=0.34,*P*=0.055, Fig. 7A). Therefore, we hypothesised that correlations between functional outcome measures and structural outcome measures would be different for GS and IF. Indeed, reduction in BP correlated positively with reduction in GS (*R*=0.53, *P*=0.030, Fig. 7B), but negatively with reduction in IF (*R*=–0.43, *P*=0.044,Fig. 7C). Urinary protein, the marker that was most strongly affected by cell-based therapy (Fig. 4) did not correlate with either reduction in GS (*R*=0.12, *P*=0.41,supplementary material Fig. S3A) or reduction in IF (*R*=0.28, *P*=0.13,supplementary material Fig. S3B). Reduction in plasma urea correlated positively with reduction in both GS (*R*=0.58, *P*=0.011, Fig. 7D) and IF (*R*=0.51, *P*=0.029,Fig. 7E). Reduction in plasma creatinine correlated with GS (*R*=0.48, *P*=0.003,supplementary material Fig. S3C), but not with IF (*R*=0.32, *P*=0.069, supplementary material Fig. S3D) and increased GFR did not correlate with GS (*R*=0.35, *P*=0.086, supplementary material Fig. S3E), but correlated strongly with IF (*R*=0.69, *P*<0.001, supplementary material Fig. S3F).

Subgroup-analysis and meta-regression

Cell-based-treatment-related factors

Cell type

Meta-regression showed that for most outcome measures, differences in the administered cell type did not explain variations in treatment effect. The most evidence currently supports MSC treatment, as MSC treatment consistently improved all functional and histological parameters except GFR (supplemen-

tary material Fig. S4A–G), the largest decrease being observed for urinary protein (SMD, 1.49; 95% CI, 0.97, 2.02; *P*<0.001; supplementary material Fig. S4E). Bone marrow cells (BM) had beneficial effects on urea, BP, urinary protein and GS (supplementary material Fig. S4B,D–F). Only GFR showed a significant difference between cell types, with endothelial progenitor cells (EPCs) causing significantly more improvement in GFR than other cell types (*P*<0.001, supplementary material Fig. S4C).

Delivery route

When delivered intravenously, cell treatment improved all functional and histological parameters, with the greatest increase observed on GFR (SMD, 1.51; 95% CI, 0.85, 2.18; *P*<0.001; supplementary material Fig. S4C). Cell administration directly via the renal artery was also effective on all functional parameters except GFR (supplementary material Fig. S4C). All other cell types or products [hematopoietic stem cells (HSCs), embryonic and organ specific] and delivery routes (intraperitoneal, intra-arterial non-renal and parenchyma or subcapsular) were applied too infrequently to be reliably interpreted.

Regime

We observed no difference between single and multiple cell administration regimes, except for GFR, where multiple administrations showed no effect (supplementary material Fig. S4C). Both regimes reduced the development and progression of CKD as shown by reduced creatinine, urea, BP and urinary protein and less GS and IF (supplementary material Fig. S4A,B,D–G). For none of the outcome parameters was there a significant relationship between administered cell number and effect size, even though administered cell number between studies differed by more than four orders of magnitude (data not shown).

Cell origin and condition

Both xenogeneic (human cells administered to rodents) and allogeneic (animals receiving cells from the same species) transplantation of cells were effective at improving outcome parameters, except for GFR, where xenotransplantation failed to improve this parameter (supplementary material Fig. S4A–G). The majority of studies used cells from healthy donors and 'healthy cells' consistently improved functional and histological outcome parameters.

Model-related factors (species, gender, model and timing of therapy)

All studies that used rats to study the efficacy of cell-based treatment in CKD showed improvement in all functional and histological parameters (supplementary material Fig. S5A–G). Cell therapy did not influence urea and GFR in

mice (supplementary material Fig. S5B,C), and did not influence creatinine, BP and urinary protein in pigs (supplementary material Fig. S5A,D,E). Gender did not affect the outcome of cell therapy except in the case of GFR, which was not improved in males (supplementary material Fig. S5C). Heterogeneity in design was too substantial to identify differential treatment effects between different disease models. The most commonly used model, SNX, showed consistent improvements in all functional and histological parameters, with the biggest increase for GFR (SMD, 1.78; 95% CI, 1.20, 2.36; $P<0.001$; supplementary material Fig. S5C). Diabetes models generally seem to show greater improvements than other models (except for GFR), although the power is too limited to achieve statistical significance (supplementary material Fig. S5A–G). Both preventive and rescue cell-based treatments improved development and progression of CKD, shown by all outcome measures (supplementary material Fig. S5). The timing of therapy in relation to clinical manifestation of disease (prevention or rescue treatment) was not associated with efficacy.

Quality assessment

The results of methodological quality assessment are shown in supplementary material Fig. S6. All included studies were assessed using 13 characteristics. Of the assessed characteristics, 70% were scored positive. Eight articles were of high quality (>80% positive characteristics) and two articles were of low quality (<50% positive characteristics). Numbers of animals, CKD model, follow-up, dosage, administration route, timing of intervention and outcome measures were all adequately reported. Animal characteristics were often only partly described (52 out of 71 articles), with strain, age, body weight and/or gender sometimes not being mentioned. Randomisation of animals was only reported in 40% of all studies. Scoring and analysis of histology parameters was only performed blindly by the researchers in a third of all studies.

Publication bias and sensitivity analysis

Publication bias was assessed for all outcome measures. Visual assessment of funnel plots showed no publication bias for plasma urea, BP, GS and IF (supplementary material Fig. S7A,E,F). Small negative studies appeared to be underrepresented for plasma creatinine, urinary protein excretion and GFR (supplementary material Fig. S7B–D) and Egger's test showed asymmetry for these three outcome measures ($P<0.001$). The 'trim and fill' method showed that there are seven hypothetical studies missing for creatinine, three for urinary protein and one for GFR (imputed in supplementary material Fig. S8). A re-run of the meta-analysis for those three outcome measures, including computed studies, showed very similar effects to the original results.

Discussion

Our systematic review and meta-analysis showed that cell-based therapy reduced the development and progression of experimental CKD, as measured by several commonly and clinically used measures of renal function (creatinine, urea, GFR, BP and urinary protein) and for common experimentally used measures of renal damage (GS and IF). This finding proved to be consistent despite considerable differences between studies in the selection and preparation of cells, administration route and choice of disease model and model species.

Before considering cell- and model-related factors that might influence the efficacy of cell-based therapy, we analysed whether correlation analysis could be of use in identifying potential bona fide markers of target organ injury. The rationale for this approach was that for ethical reasons only animal studies allow systematic quantitative analysis of target organ injury. Thus, correlation between functional and structural data in animal studies might affect our perspective of commonly used functional markers in the setting of cell-based therapy. Upfront, we need to acknowledge that, owing to limited availability of raw data, this analysis was performed with SMDs and was not weighted for study precision. Nevertheless, the outcome was illuminative.

First, we analysed whether reductions in GS and IF were correlated. Surprisingly, reductions in GS and IF did not correlate significantly, suggesting that, at least partly, correlations with functional data would be different for GS and IF. Indeed, we observed a positive correlation between reductions in GS and BP and a negative correlation between IF and BP. The positive correlation between GS and BP is in line with the results found in the SNX model in rats [5], suggesting that BP-dependent mechanisms are important for the development of GS. However, the negative correlation between change in BP and change in IF was unexpected. Importantly, changes in urinary protein do not correlate significantly with changes in either GS or IF. This is quite different from the results of multiple studies with blockade of the renin angiotensin system [6] suggesting that a different mechanism might be involved in cell-based treatment. In the correlation analysis, we found that, generally, changes in plasma markers and direct measurements of GFR correlate differently with changes in GS than with changes in IF. The notable exception is that a change in plasma urea shows consistent correlations with changes in both GS and IF. That changes in urea predict changes in IF is well known from biopsy studies [7]; however, for GS this is less well documented. All in all, changes in BP, plasma urea and plasma creatinine appear to be good predictors of changes in GS, whereas, for IF, plasma urea and measured GFR are the most important. The lack of significant correlations with urinary protein for either GS or IF

suggests that changes in this outcome predict functional rather than structural changes. However, it should be noted that we could not study temporal relations in our meta-analysis.

We performed subgroup analyses and meta-regression to investigate predefined factors that we hypothesised would modify the efficacy of cell-based treatment in CKD – cell-related factors (cell type, regime, condition, origin and delivery route) and model-related factors (species, gender, model and timing of intervention). Our meta-analysis most strongly supports the use of MSCs as therapy for CKD, although studies using BM and EPC seem to achieve similar results. MSCs are currently under investigation for a wide range of clinical applications, as they possess anti-inflammatory, anti-fibrotic and proangiogenic properties [8]. Clinical trials with MSCs have been initiated for acute kidney injury and transplantation [9], but application of MSCs in the setting of CKD has not yet taken place. There remain important issues in MSC therapy that need to be addressed before the translation to clinical studies can be made. The majority of the studies in our meta-analysis were performed with MSCs from donors free of kidney disease, with only one study testing cells that originated from uremic donors [10]. Uremia has been suggested to induce functional incompetence in BM-MSCs [11-13] but neither subcutaneous-adipose-tissue-derived MSCs nor bone-marrow-derived MSCs obtained from individuals with renal disease showed persistent dysfunction in *in vitro* assays after expansion in culture [14,15] Similarly, *in vivo* studies show no persistent dysfunction in pro-angiogenic effects of MSCs obtained from diseased individuals [16]. Whether the uremic environment is detrimental for cell-based therapy requires further investigation. Importantly, the low antigenicity and immunomodulatory properties of MSCs allow allogeneic transplantation, which could lead to an 'off-the-shelf' therapy. In general, the use of human cells in ~25% of all the experimental animal studies resulted in favourable results, even though the recipients were usually immune competent.

Cell products are also attractive candidates for off-the-shelf therapy. However, only six studies using cell products were available for our meta-analysis, prohibiting definitive conclusions. One might speculate that, in the chronic situation, multiple administrations of cells or cell products would confer benefits over single administration because paracrine actions might decrease over time. Similar considerations could be held regarding the number of administered cells. However, our meta-analysis showed no dose dependency, either in the number of cells or cell product administrations or in cell or cell product dose. Lack of dose dependency is a common finding in cell-based therapy [17], possibly suggesting that cell-based therapy acts primarily by switching on endogenous repair rather than as a persistent

source of exogenous cells or growth factors. Indeed, multiple clinical and experimental studies fail to find substantial numbers of exogenous cells in the kidney after their administration [18].

Systemic intravenous delivery (through the tail vein in most rodent studies) was the route that was most supported by evidence, despite the fact that the majority of administered cells appear to be trapped in the lungs [19]. This also suggests that even relatively few cells passing the pulmonary circulation are sufficient to switch on endogenous repair. This delivery route is feasible for patients, because injecting intravenously is relatively easy and minimally invasive. In patients, intravenous infusions of MSCs were well tolerated and no treatment-related serious adverse events are reported [3]. Direct intrarenal delivery was applied in 17 articles in our meta-analysis – five using subcapsular or parenchymal administration and 12 using delivery by injection in the renal artery. These studies generally show improved outcome measures, although findings were less consistent than with intravenous administration. In conjunction with their more invasive character, this makes these approaches less attractive, although theoretically combination with other common endovascular treatments of the renal artery (denervation or stenting) is attractive. Intraperitoneal delivery was only used in three studies, none of which showed a significantly improved outcome. Based on these limited findings, intraperitoneal delivery of cell-based therapy in CKD does not appear to be useful.

Our meta-analysis suggests differences in the efficacy of cell-based therapy between species in urinary protein and BP, but not in other outcome measures. Partly, such differences might be due to methodological limitations; for instance, BP measurements were practically absent in the mouse studies included in our meta-analysis. However, importantly, cell-based therapy improved GS and IF in all three species. Thus, for structural changes, all three species appear to be useful, although in pigs protective effects on GS were limited, albeit significant.

We did not observe consistent effects of gender on the outcome of cell therapy, except for improvements in GFR, which only occurred in studies using female animals. Mechanisms underlying gender-specific differences in outcome measures of cell therapy are obscure, and cannot be clarified by a meta-analysis. Nevertheless, the possibility that this is also the case in humans should be taken into account when designing cell-therapy studies in patients, and gender balance should be considered. Furthermore, differences in the functional efficacy of cell-based therapy in CKD appeared to be model dependent, perhaps reflecting the different pathogenesis of CKD when initiated by subtotal nephrectomy versus, for instance, toxic injury. However, for

structural efficacy, most models showed improvement of all outcome variables, perhaps reflecting the common pathway to end-stage kidney disease. Such differences might also be relevant when designing cell-therapy studies in specific patient populations.

Preventive and rescue interventions are both effective. However, the staging of CKD in animals is not as clearly defined as in humans, and therapy was never instituted at a late stage that clinically would be equivalent to pre-dialysis. Moreover, preventive therapy in animal models is often initiated directly after (or even before) renal ablation or administration of toxins, well before any GS or IF can be expected. Thus, the relevance of this finding to the clinical situation is, perhaps, limited. Nevertheless, it could be that in clinical studies very tight categorisation of CKD stage is not required to find significant effects of cell-based therapy.

Clearly, both human and animal studies should be performed according to the highest standards. Although randomisation and blinding for inclusion is often not feasible in the setting of an experimental study, blinding for analysis of both functional and structural parameters is readily achievable. Moreover, there is no excuse for not reporting all relevant animal characteristics as well as the number of drop-outs due to technical failures or premature (non-scheduled) death or sacrifice. Omitting such data is very common in animal studies, including many studies in our meta-analysis, and has been reported in other meta-analyses [17,20]. It is crucial that authors report these details, as they will likely influence outcome parameters.

Our meta-analysis confirms that cell-based therapies improve impaired renal function and structure in preclinical models of CKD. Animal studies are often regarded with scepticism because of large variation in study design and outcome measures. However, our meta-analysis demonstrates that perhaps because of this very variation they can be perceived as a rich source of useful information that could be helpful in designing clinical trials in the (near) future.

Materials and Methods

Literature search

We conducted a systematic review and meta-analysis of studies that investigated the effects of cell- and cell-based therapies on kidney function and structure in animal models of CKD. The PubMed database was searched for published articles up to 21 January 2014 using the following terms: 'kidney damage' [Tiab] OR 'kidney injury' [Tiab] OR 'kidney disease' [Tiab] OR 'renal injury' [Tiab] OR 'renal failure' [Tiab] OR 'kidney failure' [Tiab] OR 'nephropathy' [Tiab] OR 'renal disease' [Tiab] OR 'renal function' [Tiab] OR 'renovas-

cular' [Tiab] AND 'stem cells' [Tiab] OR 'cell therapy' [Tiab] OR 'progenitor cells' [Tiab] OR 'bone marrow' [Tiab] OR EPC [Tiab] OR 'endothelial progenitor cells' [Tiab] OR 'MSC' [Tiab] OR 'mesenchymal' [Tiab] OR 'conditioned medium' [Tiab] OR 'microvesicles' [Tiab] OR 'exosomes' [Tiab] OR 'microparticles' [Tiab]. Search results were filtered by PubMed filters to exclude reviews and non-English articles, and a custom-made filter [21] was used to select for studies containing laboratory animals. Articles were selected by reading the title and abstract; when these were not informative enough, the complete article was screened by two independent researchers (D.A.P. and N.R.O.). Articles were discussed with the other authors before inclusion or exclusion.

Inclusion criteria

The criteria for inclusion were as follows: (1) animal models of CKD were used to study the effects of cell-based therapy on kidney function and structure; (2) the therapy contained or consisted of cells or cell-derived products (conditioned medium or exosomes or microvesicles) and (3) the article was an original paper presenting unique data.

Exclusion criteria

The criteria for exclusion were as follows: (1) animal models of acute renal failure showing spontaneous recovery of kidney injury under the untreated condition. These models included ischemia-reperfusion injury, anti-Thy1 and various toxic models (cisplatin, glycerol, gentamicin, mercuric chloride, folic acid, carbon tetrachloride and lipopolysaccharide; (2) follow-up was less than seven days after disease induction; (3) group size smaller than n=3; (4) incomplete data (no untreated or vehicle-treated diseased control group present; missing data at last measurement point before or at termination; data from figures or tables with unclear captions; unknown group size and/or standard deviations or errors of mean); (5) no full-text article available.

Data extraction

Study characteristics extracted from selected articles included species, strain, gender, injury model, administered cell type or cell-derived product, number of cells, administration route, time-point of administration, follow-up duration. Data analysis for kidney function included plasma creatinine, plasma urea, GFR, BP and urinary protein [proteinuria, albuminuria, urine protein:creatinine (prot:creat) and urine albumin:creatinine (alb:creat) ratio]. Data analysis for renal structure included GS and IF. For longitudinal measurements, data from the last measurement (function) or at termination (morphology) were used. When one control group was used for comparison with multiple treatment

groups, control sample size was divided by the number of treatment groups to equalise the weight of each group in our analysis [22]. Plasma and serum urea, blood urea nitrogen (BUN) and plasma and serum creatinine levels shown as mg/dl were converted to mmol/l (mmol/l= BUN mg/dl×0.357 or mmol/l=plasma urea mg/dl×0.1665) and μmol/l (plasma/serum creatinine μmol/l=mg/dl×88.4), respectively. Both plasma creatinine and creatinine clearance were analyzed when reported in one article. BP measurements (systolic blood pressure and mean arterial pressure) of conscious animals were included to avoid the effects of anaesthesia. Standard error of the mean (s.e.m.) was converted to standard deviation (s.d., s.d.= n×s.e.m.). When necessary, GetData Graph Digitizer (version 2.25) was used to extract values from graphs. Data were extracted by two independent researchers (D.A.P. and N.R.O.). Missing data were requested from authors.

Data analysis

Data were analyzed with R software (version 3.1.0; R Foundation for Statistical Computing, Vienna, Austria) using the metafor package[23]. In order to correct for the different units and scales arising from the abovementioned considerations, all data are presented as standardized mean differences (SMDs). SMDs and accompanying variance were calculated for the following outcome parameters: plasma urea, plasma creatinine, urinary protein, GFR, BP, GS and IF. Random- and mixed-effects models were fitted using restricted maximum likelihood estimation (REML). The estimated average effect (μ), heterogeneity in effects (τ^2) and the estimated percentage of variability attributable to heterogeneity (I^2) are given. Heterogeneity was considered to be low, moderate or high at 25, 50 and 75%, respectively [24]. In order to study the relationship between improvements in renal morphology (GS and IF) with different outcome measures related to renal function, we calculated pair-wise correlations between the SMDs. Pearson product-moment correlation coefficients are supplied, with bands indicating 95% confidence intervals.

We studied the influence of several moderators on treatment effect using meta-regression. All models only employ a single moderator variable, as study designs were too diverse to get full models without empty cells in case of multiple factor models. Moderators were grouped in cell treatment-related moderators (cell type, administration regime, condition of cells, origin of cells, administration route) and model-related (animal model species, gender, model, timing of intervention) factors. These factors were pooled in classes for sub-analysis because of the wide variety in cell types and animal models (supplementary material Tables S2, S3). Sub-analysis for each outcome variable was performed for all cell treatment- and model-related factors. In

rodents, hypertension-induced kidney injury was used in one study; owing to this low number, data are only shown in figures, and not taken into account when interpreting results. Porcine studies were only included for species sub-analysis because in all porcine studies the same model (renal artery stenosis) was applied. All cell-based therapies were categorised as preventive or rescue. A treatment was defined as preventive when cell-based therapy was applied before clinical manifestation of disease (for induced models, between day 0 and day 6 after induction of kidney disease; for knockout models, before clinical manifestation of disease). Therapies were categorised as rescue when therapy was started after clinical manifestation of disease.

Quality assessment

Methodological quality of the included studies was assessed by a scoring system adapted from Wever et al. [20]. Publication bias was assessed by visually evaluating asymmetry in funnel plots for each outcome parameter. In case of visual asymmetry, Egger's test was used [25]. When Egger's test indicated asymmetry, the 'trim and fill' method [26] was used to correct for this.

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Supplementary material

Cell-based therapies for experimental chronic kidney disease

a systematic review and meta-analysis

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Table S1. *Study characteristics (A- animal strain; G-gender; NV – number of animals in vehicle group; NC – number animals in cell-based treated group). Other abbreviations are listed in the legends of Tables S1 and S2.*

Author, year	A Strain		G	NV	NC	Model
(Alexandre et al., 2009)	rat	Fischer 344	M	8	8	5/6 Nx
(Alexandre et al., 2009)	rat	Fischer 344	M		8	5/6 Nx
(Alexandre et al., 2009)	rat	Fischer 344	M	12	7	5/6 Nx
(Alfarano et al., 2012)	rat	Lewis	F	8	8	Unx + IRI + CsA
(Alfarano et al., 2012)	rat	Lewis	F	8	8	Unx + IRI + CsA
(Bian et al., 2014)	rat	Sprague-Dawley	M	6	6	5/6 Nx
(Bian et al., 2014)	rat	Sprague-Dawley	M	6	6	5/6 Nx
(Bian et al., 2014)	rat	Sprague-Dawley	M	6	6	5/6 Nx
(Bian et al., 2014)	rat	Sprague-Dawley	M	6	6	5/6 Nx
(Burst et al., 2013)	rat	Lewis	F	7	7	Unx + IRI
(Caldas et al., 2011)	rat	Wistar	F	5	5	2/3 Nx
(Caldas et al., 2011)	rat	Wistar	F		5	2/3 Nx
(Caldas et al., 2011)	rat	Wistar	F	5	5	5/6 Nx
(Caldas et al., 2011)	rat	Wistar	F		5	5/6 Nx
(Caldas et al., 2008)	rat	Wistar	M	5	5	5/6 Nx
(Caldas et al., 2008)	rat	Wistar	M		5	5/6 Nx
(Cantaluppi et al., 2012)	rat	Wistar	M	6	6	Unx + IRI
(Cao et al., 2013)	mouse	Balb/c	M	7	7	Adriamycin
(Cao et al., 2013)	mouse	Balb/c	M		7	Adriamycin
(Cao et al., 2013)	mouse	Balb/c	M		7	Adriamycin
(Cao et al., 2013)	mouse	Balb/c	M		7	Adriamycin
(Castiglione et al., 2013)	rat	Wistar	M	10	10	DN
(Cavaglieri et al., 2009)	rat	Wistar	M	10	10	5/6 Nx
(Cavaglieri et al., 2009)	rat	Wistar	M	10	10	5/6 Nx
(Chade et al., 2010)	pig	domestic	?	7	7	RAS
(Chade et al., 2009)	pig	domestic	?	7	7	RAS
(Challen et al., 2006)	mouse	BALB/c	?	3	3	Adriamycin

				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
Lin- BM	$2*10^6$	Intravenous	Rescue	15	60
Lin- BM	$2*10^6$	Intravenous	Rescue	15, 30, 45	60
Lin- BM	$2*10^6$	Intravenous	Rescue	15	120
MSC melatonin pretreated	$3*10^6$	Parenchymal	Rescue	7	28
MSC melatonin pretreated	$3*10^6$	Parenchymal	Rescue	14	28
MSC	$10*10^6$	Intravenous	Rescue	28	56
MSC	$10*10^6$	Intravenous	Rescue	56	84
MSC	$10*10^6$	Intravenous	Rescue	84	112
MSC	$10*10^6$	Intravenous	Rescue	112	140
Lin-CD90+HSC	$1*10^6$	Intra-arterial	Prevention	0	7
MSC	$2.5*10^6$	Scaffold kidney	Prevention	0	90
BMMNC	$5*10^6$	Scaffold kidney	Prevention	0	90
MSC	$2.5*10^6$	Scaffold kidney	Prevention	0	90
BMMNC	$5*10^6$	Scaffold kidney	Prevention	0	90
BMMNC	$1.5*10^6$	Parenchymal	Prevention	0	119
MSC	$1.5*10^6$	Parenchymal	Prevention	0	119
EPC MV	30ug	Intravenous	Prevention	0	180
BM-M0	$1*10^6$	Intravenous	Prevention	5	28
BM-M2	$1*10^6$	Intravenous	Prevention	5	28
SP-M0	$1*10^6$	Intravenous	Prevention	5	28
SP-M2	$1*10^6$	Intravenous	Prevention	5	28
BMMNC	$20*10^6$	Intravenous	Rescue	28	112
MSC	$2*10^5$	Subcapsular	Prevention	0	15
MSC	$2*10^5$	Subcapsular	Prevention	0	30
EPC	$10*10^6$	Intrarenal artery	Rescue	42	70
EPC	$10*10^6$	Intrarenal artery	Rescue	42	70
MP	$3*10^5$	Parenchymal/ intrarenal artery	Prevention	0	7

Author, year	A Strain		G	NV	NC	Model
(Challen et al., 2006)	mouse	BALB/c	?	3	3	Adriamycin
(Chang et al., 2012)	rat	Sprague-Dawley	M	6	6	5/6 Nx
(Chang et al., 2011)	mouse	NZB/W F1	F	8	8	Lupus nephritis
(Chang et al., 2011)	mouse	NZB/W F1	F	8	7	Lupus nephritis
(Chen et al., 2009)	mouse	Db/Db	M	5	6	DN
(Chen et al., 2009)	mouse	Db/Db	M		6	DN
(Chen et al., 2009)	mouse	Db/Db	M	4	5	DN
(Chen et al., 2009)	mouse	Db/Db	M		5	DN
(Choi et al., 2009)	rat	Sprague-Dawley	F	3	3	5/6 Nx
(Chung et al., 2013)	rat	Sprague-Dawley	M	8	8	CsA
(De Chiara et al., 2014)	mouse	129sv/C57	F	10	10	Unx + IRI
(Donizetti-Oliveira et al., 2012)	mouse	C57BL/6J	F	6	6	IRI
(Donizetti-Oliveira et al., 2012)	mouse	C57BL/6J	F	6	6	IRI
(Du et al., 2013)	rat	Sprague-Dawley	M	3	3	IRI
(Ebrahimi et al., 2012)	pig	Domestic	F	7	7	RAS
(Eirin et al., 2014)	pig	Domestic	F	6	6	RAS
(Eirin et al., 2013)	pig	Domestic	?	7	7	RAS
(Eirin et al., 2013)	pig	Domestic	?	7	7	RAS + PTRA
(Eirin et al., 2012)	pig	Domestic	F	7	7	RAS
(Ezquer et al., 2009)	mouse	C57BL/6J	M	8	8	DN
(Fang et al., 2012)	rat	Sprague-Dawley	M	8	8	DN
(Franquesa et al., 2012)	rat	Lewis	M	4	4	CAN
(Franquesa et al., 2012)	rat	Lewis	M	4	7	CAN
(Franquesa et al., 2012)	rat	Lewis	M	7	5	CAN
(Franquesa et al., 2012)	rat	Lewis	M	7	7	CAN

				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
SP	$3*10^5$	Parenchymal/ intrarenal artery	Prevention	0	7
chMSC	$1*10^6$	Intravenous	Prevention	0, 14, 28, 42, 56, 70, 84, 98	112
hU-MSC	$1*10^6$	Intravenous	Prevention	61	244
hU-MSC	$1*10^6$	Intravenous	Rescue	183	244
BMMNC db/m	$1*10^6$	Intravenous	Rescue	112, 122, 132, 142	161
BMMNC db/db	$1*10^6$	Intravenous	Rescue	112, 122, 132, 142	161
BMMNC db/db ex vivo Ebselen	$1*10^6$	Intravenous	Rescue	112, 122, 132, 142	161
BMMNC db/db in vivo Ebselen	$1*10^6$	Intravenous	Rescue	112, 122, 132, 142	161
MSC	$1*10^6$	Intravenous	Prevention	1	60
hADMSC	$3*10^6$	Intravenous	Prevention	0, 7, 14, 21	28
GTC	$2.5*10^5$	Intravenous	Prevention	0	42
ADSC	$2*10^5$	Intraperitoneal	Prevention	0	42
ADSC	$2*10^5$	Intraperitoneal	Rescue	42	70
hWJ-MSC	$2*10^6$	Intravenous	Prevention	2	42
EPC	$1*10^6$	Intrarenal artery	Rescue	42	70
ADMSC	$10*10^6$	Intrarenal artery	Rescue	42	77
EOC	$10*10^6$	Intrarenal artery	Rescue	42	70
EOC	$10*10^6$	Intrarenal artery	Rescue	42	70
ADMSC	$10*10^6$	Intrarenal artery	Rescue	42	70
MSC	$0.5*10^6$	Intravenous	Rescue	30, 51	119
ADMSC	$10*10^6$	Intravenous	Rescue	28	84
BM	$0.5*10^6$	Intravenous	Rescue	77	84
MSC	$10*10^6$	Intravenous	Rescue	77	84
BM	$0.5*10^6$	Intravenous	Rescue	77	168
MSC	$10*10^6$	Intravenous	Rescue	77	168

Author, year	A Strain		G	NV	NC	Model
(Furuhashi et al., 2013)	rat	WKY/NCrj	F	7	13	anti-GBM GN
(Furuhashi et al., 2013)	rat	WKY/NCrj	F		7	anti-GBM GN
(Furuhashi et al., 2013)	rat	WKY/NCrj	F	7	7	anti-GBM GN
(Furuhashi et al., 2013)	rat	WKY/NCrj	F		8	anti-GBM GN
(Gatti et al., 2011)	rat	Sprague-Dawley	M	6	6	UNX + IRI
(Gu et al., 2010)	mouse	MRL/lpr	F	6	8	Lupus nephritis
(Gu et al., 2010)	mouse	MRL/lpr	F		8	Lupus nephritis
(Gu et al., 2010)	mouse	MRL/lpr	F		8	Lupus nephritis
(Guo et al., 2014)	rat	Sprague-Dawley	M	30	30	Adriamycin
(Harrison et al., 2013)	mouse	Ctns(-/-)	M	9	8	Cystinosis
(He et al., 2012)	mouse	C57BL6/J	?	6	10	5/6 Nx
(He et al., 2012)	mouse	C57BL6/J	?		10	5/6 Nx
(Huang et al., 2012)	rat	?	?	10	10	NSN
(Huang et al., 2012)	rat	?	?		10	NSN
(Hyun et al., 2012)	mouse	HIGA	F	7	5	IgAN
(Hyun et al., 2012)	mouse	HIGA	F		6	IgAN
(Hyun et al., 2012)	mouse	HIGA	F		8	IgAN
(Jiao et al., 2011)	rat	Sprague-Dawley	F	15	15	Adriamycin
(Kunter et al., 2007)	rat	Lewis	M	10	10	Unx + aThy
(Lee et al., 2010)	rat	Sprague-Dawley	F	8	16	5/6 Nx
(Lee et al., 2010)	rat	Sprague-Dawley	F		8	5/6 Nx
(Lee et al., 2010)	rat	Sprague-Dawley	F		16	5/6 Nx
(Li et al., 2012)	rat	Wistar	F	10	10	CAAN
(Li et al., 2010)	mouse	C57BL/6	?	5	5	IRI
(Lv et al., 2013)	rat	Wistar	F	16	16	DN
(Ma, H. et al., 2013)	rat	Sprague-Dawley	M	6	6	Adriamycin
(Ma, H. et al., 2013)	rat	Sprague-Dawley	M		6	Adriamycin

				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
MSC	$2*10^6$	Intravenous	Prevention	0, 1, 2, 3, 4, 5	7
HASC	$2*10^6$	Intravenous	Prevention	0, 1, 2, 3, 4, 5	7
HASC	$2*10^6$	Intravenous	Rescue	0, 1, 2, 3, 4, 5	14
LASC	$2*10^6$	Intravenous	Rescue	0, 1, 2, 3, 4, 5	14
hMSC MV	30ug	Intravenous	Prevention	0	182
hU-MSC	$1*10^6$	Intravenous	Rescue	126	203
hU-MSC	$1*10^6$	Intravenous	Rescue	126, 133, 140	203
MSC	$1*10^6$	Intravenous	Rescue	126	203
MSC	$2*10^6$	Intravenous	Rescue	14, 21	70
HSPC	$1*10^6$	Intravenous	Rescue	77	365
MSC	$1*10^6$	Intravenous	Prevention	2	9
MSC MV	30ug	Intravenous	Prevention	2, 3, 5	9
MSC	$0.3*10^5$	Intrarenal artery	Prevention	6	21
GDNF-MSC	$0.3*10^5$	Intrarenal artery	Prevention	6	21
ADSC preonset disease	$5*10^6$	Intravenous	Rescue	168, 182, 196, 210, 224, 238	252
ADSC postonset disease	$5*10^6$	Intravenous	Rescue	168, 182, 196, 210, 224, 238	252
hADSC	$5*10^6$	Intravenous	Rescue	168, 182, 196, 210, 224, 238	252
MMC	$5\text{-}7*10^6$	Intravenous	Rescue	56	112
MSC	$2*10^6$	Intrarenal artery	Prevention	2	60
MSC	$3*10^6$	Intravenous	Prevention	1	56
MSC	$3*10^6$	Intravenous	Prevention	1, 7, 14, 21, 28	56
MC	$3*10^6$	Intravenous	Prevention	1	56
MSC	$20*10^6$	Intravenous	Rescue	28	84
hADMSC	$5*10^5$	Intravenous	Prevention	0	183
MSC	$2*10^6$	Intravenous	Rescue	56	112
hU-MSC	$2*10^6$	Intravenous	Prevention	1	84
hU-MSC	$2*10^6$	Intravenous	Prevention	1, 8, 15, 22	84

Author, year	A	Strain	G	NV	NC	Model
(Ma, Hualin et al., 2013)	rat	Sprague-Dawley	M	6	6	Adriamycin
(Ma, Hualin et al., 2013)	rat	Sprague-Dawley	M		6	Adriamycin
(Ma, Hualin et al., 2013)	rat	Sprague-Dawley	M		6	Adriamycin
(Ma, Hualin et al., 2013)	rat	Sprague-Dawley	M		6	Adriamycin
(Masoad et al., 2012)	rat	?	M	10	10	DN
(Ninichuk et al., 2006)	mouse	COL4A3 KO	?	10	10	Alport syndrome
(Nur et al., 2008)	mouse	TC KO	M	6	6	HSV
(Oliveira-Sales et al., 2013)	rat	Wistar	M	8	7	2K-1C
(Park, J. H. et al., 2012)	rat	Sprague-Dawley	M	7	7	DN
(Park, Jong Hee et al., 2012)	rat	Sprague-Dawley	M	7	7	DN
(Ratliff et al., 2010)	mouse	Balb/c	?		6	Adriamycin
(Sangidorj et al., 2010)	mouse	C57BL/6	M	4	20	5/6 Nx
(Sangidorj et al., 2010)	mouse	C57BL/6	M	4	16	5/6 Nx
(Sangidorj et al., 2010)	mouse	C57BL/6	M	4	12	5/6 Nx
(Sangidorj et al., 2010)	mouse	C57BL/6	M	4	8	5/6 Nx
(Sangidorj et al., 2010)	mouse	C57BL/6	M	4	4	5/6 Nx
(Semedo et al., 2010)	mouse	C57BL/6	F	5	5	IRI
(Semedo et al., 2009)	rat	Wistar	F	7	7	5/6 Nx
(Semedo et al., 2009)	rat	Wistar	F	7	7	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F	8	8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F	8	8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F	8	8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Shuai et al., 2012)	rat	Sprague-Dawley	F		8	5/6 Nx
(Togel et al., 2009)	rat	Sprague-Dawley	M	6	6	IRI

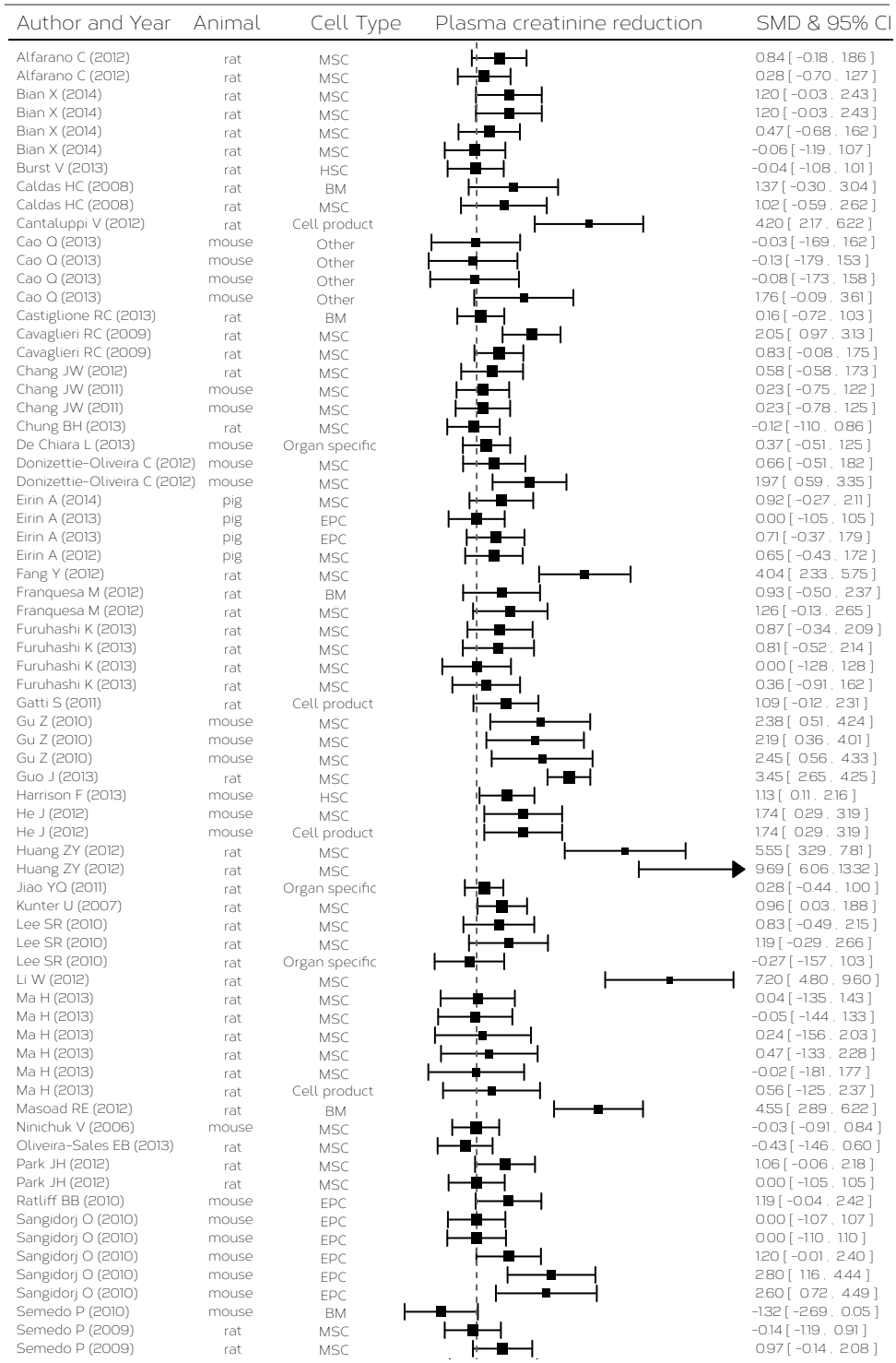
				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
hU-MSC	$2*10^6$	Intravenous	Prevention	1	28
hU-MSC	$2*10^6$	Intravenous	Prevention	1, 8, 15, 22	28
hU-MSC	$2*10^6$	Intraperitoneal	Prevention	1, 8, 15, 22	28
supernatant hU-MSC	$2*10^6$	Intravenous	Prevention	1, 8, 15, 22	28
hUB-MNC	$150*10^6$	Intravenous	?	?	8
MSC	$1*10^6$	Intravenous	Rescue	42, 49, 56, 63	66
DFAT	$5*10^6$	Intravenous	Prevention	0	21
MSC	$2.5*10^5$	Intravenous	Rescue	21, 35	42
hUB-SC	$1*10^6$	Intravenous	Rescue	28	70
hUB-MSC	$0.5*10^6$	Intravenous	Prevention	2	28
EPC	$5*10^5$	Intravenous	Prevention	0	21
EPC	$1*10^6$	Intravenous	Prevention	1, 7	28
EPC	$1*10^6$	Intravenous	Prevention	1, 7	56
EPC	$1*10^6$	Intravenous	Prevention	1, 7	84
EPC	$1*10^6$	Intravenous	Prevention	1, 7	112
EPC	$1*10^6$	Intravenous	Prevention	1, 7	140
BM-MNC	$1*10^6$	Intraperitoneal	Prevention	0	42
MSC	$2*10^5$	Intravenous	Rescue	14	84
MSC	$2*10^5$	Intravenous	Rescue	14, 28, 42	56
EPC	$1*10^5$	Intravenous	Rescue	7	28
empty plasmid-EPC	$1*10^5$	Intravenous	Rescue	7	28
TERT-EPC	$1*10^5$	Intravenous	Rescue	7	28
EPC	$1*10^5$	Intravenous	Rescue	7	56
empty plasmid-EPC	$1*10^5$	Intravenous	Rescue	7	56
TERT-EPC	$1*10^5$	Intravenous	Rescue	7	56
EPC	$1*10^5$	Intravenous	Rescue	7	84
empty plasmid-EPC	$1*10^5$	Intravenous	Rescue	7	84
TERT-EPC	$1*10^5$	Intravenous	Rescue	7	84
MSC	$1.5*10^6$	Intra-arterial	Prevention	0	90

Author, year	A Strain		G	NV	NC	Model
(Togel et al., 2009)	rat	Sprague-Dawley	M	6	6	IRI
(Togel et al., 2009)	rat	Sprague-Dawley	M		6	IRI
(Togel et al., 2009)	rat	Sprague-Dawley	M		6	IRI
(Togel et al., 2009)	rat	F334	M	6	6	IRI
(Togel et al., 2009)	rat	F334	M		6	IRI
(Togel et al., 2009)	rat	F334	M		6	IRI
(Urbieta-Caceres et al., 2012)	pig	Domestic	?	6	6	RAS
(van Koppen et al., 2012b)	rat	Lewis	M	8	8	5/6 Nx
(van Koppen et al., 2012b)	rat	Lewis	M	9	7	5/6 Nx
(van Koppen et al., 2012b)	rat	Lewis	M		7	5/6 Nx
(van Koppen et al., 2012a)	rat	Lewis	M	13	13	5/6 Nx
(van Koppen et al., 2012a)	rat	Lewis	M	7	8	5/6 Nx
(Villanueva et al., 2013)	rat	Sprague-Dawley	M	7	7	5/6 Nx
(Villanueva et al., 2011)	rat	Sprague-Dawley	M	7	7	5/6 Nx
(Wang et al., 2013)	rat	Sprague-Dawley	M	8	9	DN
(Yamaleyeva et al., 2012)	rat	RH-Foxn1rnu	M	2	4	IRI + gentamicin
(Yamaleyeva et al., 2012)	rat	RH-Foxn1rnu	M	2	4	IRI + gentamicin
(Yuen et al., 2013)	rat	F344	M	9	8	5/6 Nx
(Yuen et al., 2013)	rat	F344	M		9	5/6 Nx
(Yuen et al., 2010)	rat	F334	M	15	11	5/6 Nx
(Yuen et al., 2010)	rat	F334	M		11	5/6 Nx
(Yuen et al., 2010)	rat	F334	M		11	5/6 Nx
(Zhang, Y. et al., 2013)	rat	Sprague-Dawley	M	10	10	DN
(Zhang, Y. et al., 2013)	rat	Sprague-Dawley	M		10	DN
(Zhang, L. et al., 2013)	rat	Sprague-Dawley	M	8	8	DN
(Zhang et al., 2012)	mouse	Db/Db	M	12	12	DN
(Zhang et al., 2012)	mouse	Db/Db	M		12	DN

				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
MSC	$0.5*10^6$	Intra-arterial	Prevention	0	90
MSC	$2*10^6$	Intra-arterial	Prevention	0	90
MSC	$5*10^6$	Intra-arterial	Prevention	0	90
MSC	$0.5*10^6$	Intra-arterial	Prevention	0	90
MSC	$2*10^6$	Intra-arterial	Prevention	0	90
MSC	$5*10^6$	Intra-arterial	Prevention	0	90
EPC	$10*10^6$	Intrarenal artery	Rescue	42	70
BM	$50*10^6$	Intrarenal artery	Rescue	42	84
BM	$50*10^6$	Intrarenal artery	Rescue	42	140
CKD BM	$50*10^6$	Intrarenal artery	Rescue	42	140
hMSC CM	50 µg/ 250 µl	Intravenous	Rescue	42, 43, 44, 45	84
hMSC Exo	7µg/ 250 µl	Intravenous	Rescue	42, 43, 44, 45	84
hADMSC	$0.5*10^6$	Intravenous	Prevention	0	35
MSC	$0.5*10^6$	Intravenous	Rescue	35	42
MSC	$2*10^6$	Intrarenal artery	Rescue	28	56
hPKC	$5*10^6$	Parenchymal	Rescue	126	210
F+hPKC	$5*10^6$	Parenchymal	Rescue	126	210
EOC	$1*10^6$	Intravenous	Rescue	28	56
EOC CM	0.5 ml	Intravenous	Rescue	28, 30, 32, 35, 37, 39	56
BM CMC	$1*10^6$	Intra-arterial	Rescue	28	56
BM CMC	$1*10^6$	Intravenous	Rescue	28	56
SC	$1*10^6$	Intravenous	Rescue	28	56
MSC	$1*10^6$	Intravenous	Rescue	28	56
microbubble MSC	$1*10^6$	Intravenous	Rescue	28	56
hASC	$5*10^6$	Intravenous	Rescue	84, 112, 140, 168, 196	224
EOC db/m	$0.5*10^6$	Intravenous	Rescue	56	84
EOC db/db	$0.5*10^6$	Intravenous	Rescue	56	84

Author, year	A Strain		G	NV	NC	Model
(Zhou et al., 2009)	rat	Sprague-Dawley	M	4	4	DN
(Zhou et al., 2009)	rat	Sprague-Dawley	M		4	DN
(Zhu et al., 2013)	pig	Domestic	F	7	6	RAS
(Zhu et al., 2013)	pig	Domestic	F		6	RAS
(Zoja et al., 2012)	rat	Lewis	M	4	4	Adriamycin
(Zoja et al., 2012)	rat	Lewis	M	4	4	Adriamycin
(Zoja et al., 2012)	rat	Lewis	M	4	4	Adriamycin

				Days after induction	
Cell type	# cells	Route	Timing	Start therapy	End study
MSC	$2*10^6$	Cardiac infusion	Rescue	28	84
MSC + CsA	$2*10^6$	Cardiac infusion	Rescue	28	84
MSC	$10*10^6$	Intrarenal artery	Rescue	42	70
EPC	$10*10^6$	Intrarenal artery	Rescue	42	70
MSC	$2*10^6$	Intravenous	Prevention	1, 2, 3, 5, 7	9
MSC	$2*10^6$	Intravenous	Prevention	1, 2, 3, 5, 7, 14	16
MSC	$2*10^6$	Intravenous	Prevention	1, 2, 3, 5, 7, 14, 21	30



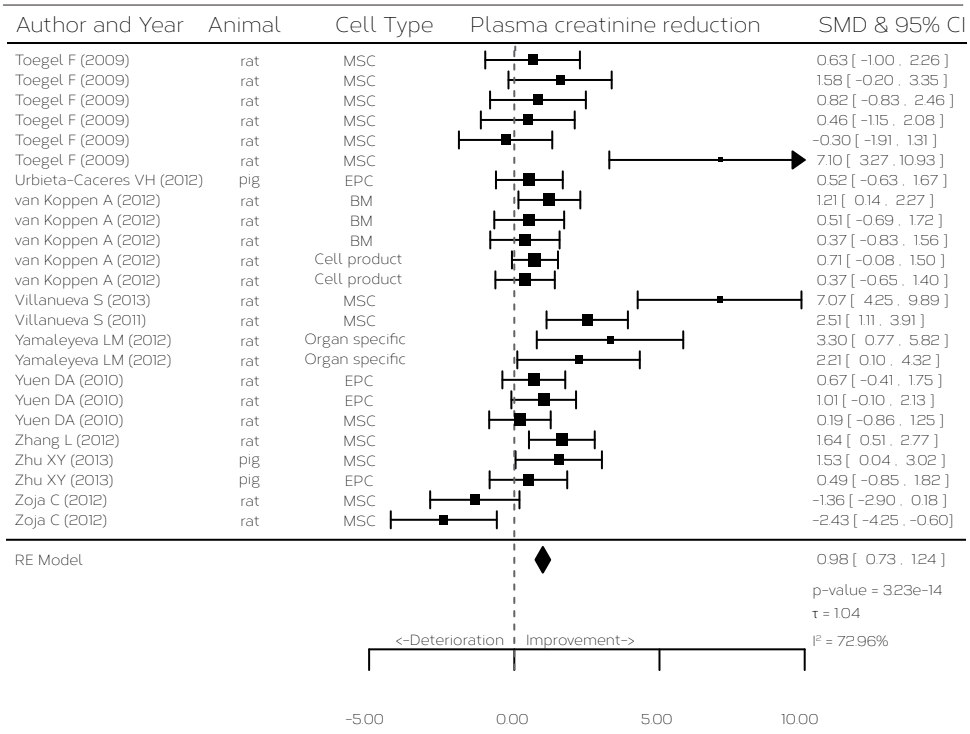
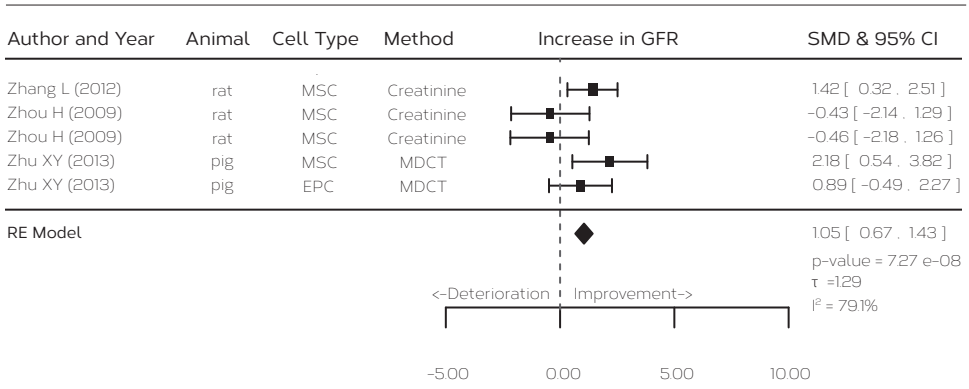


Fig. S1. *Effect of cell-based treatment in CKD on plasma creatinine.*

Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

Author and Year	Animal	Cell Type	Method	Increase in GFR	SMD & 95% CI
Alexandre CS (2009)	rat	HSC	Inulin		0.20 [-1.00 , 1.41]
Alexandre CS (2009)	rat	HSC	Inulin		0.00 [-1.20 , 1.20]
Alexandre CS (2009)	rat	HSC	Inulin		1.80 [0.71 , 2.89]
Caldas HC (2011)	rat	MSC	Creatinine		1.42 [-0.26 , 3.10]
Caldas HC (2011)	rat	BM	Creatinine		0.79 [-0.78 , 2.36]
Caldas HC (2011)	rat	MSC	Creatinine		1.16 [-0.47 , 2.79]
Caldas HC (2011)	rat	BM	Creatinine		1.51 [-0.19 , 3.21]
Caldas HC (2008)	rat	BM	Creatinine		1.07 [-0.54 , 2.68]
Caldas HC (2008)	rat	MSC	Creatinine		0.11 [-1.41 , 1.62]
Cao Q (2013)	mouse	Other	Creatinine		0.20 [-1.46 , 1.86]
Cao Q (2013)	mouse	Other	Creatinine		0.00 [-1.66 , 1.66]
Cao Q (2013)	mouse	Other	Creatinine		0.07 [-1.59 , 1.72]
Cao Q (2013)	mouse	Other	Creatinine		1.40 [-0.38 , 3.18]
Castiglione RC (2013)	rat	BM	Creatinine		-0.59 [-1.49 , 0.31]
Chade AR (2010)	pig	EPC	MDCT		1.25 [0.10 , 2.39]
Chade AR (2009)	pig	EPC	MDCT		0.73 [-0.36 , 1.81]
Chang JW (2012)	rat	MSC	Creatinine		2.08 [0.67 , 3.48]
Chung BH (2013)	rat	MSC	Creatinine		-0.08 [-1.06 , 0.90]
Ebrahimi B (2012)	pig	EPC	MDCT		0.33 [-0.72 , 1.39]
Eirin A (2014)	pig	MSC	MDCT		1.89 [0.53 , 3.25]
Eirin A (2013)	pig	EPC	MDCT		0.93 [-0.17 , 2.03]
Eirin A (2013)	pig	EPC	MDCT		0.51 [-0.56 , 1.57]
Eirin A (2012)	pig	MSC	MDCT		0.53 [-0.53 , 1.60]
Harrison F (2013)	mouse	HSC	Creatinine		0.23 [-0.72 , 1.19]
Huang ZY (2012)	rat	MSC	Creatinine		2.23 [0.89 , 3.56]
Huang ZY (2012)	rat	MSC	Creatinine		4.38 [2.48 , 6.28]
Jiao YQ (2011)	rat	Organ specific	Creatinine		1.17 [0.39 , 1.94]
Kunter U (2007)	rat	MSC	Creatinine		-0.36 [-1.25 , 0.52]
Lv SS (2013)	rat	MSC	Creatinine		-1.82 [-2.64 , -1.00]
Semedo P (2009)	rat	MSC	Inulin		1.10 [-0.03 , 2.22]
Shuai L (2012)	rat	EPC	Creatinine		2.08 [0.44 , 3.73]
Shuai L (2012)	rat	EPC	Creatinine		3.99 [1.80 , 6.18]
Shuai L (2012)	rat	EPC	Creatinine		4.74 [2.30 , 7.18]
Shuai L (2012)	rat	EPC	Creatinine		3.23 [1.28 , 5.18]
Shuai L (2012)	rat	EPC	Creatinine		2.88 [1.03 , 4.72]
Shuai L (2012)	rat	EPC	Creatinine		6.00 [3.10 , 8.90]
Shuai L (2012)	rat	EPC	Creatinine		7.43 [3.98 , 10.87]
Shuai L (2012)	rat	EPC	Creatinine		4.75 [2.30 , 7.20]
Shuai L (2012)	rat	EPC	Creatinine		10.76 [5.99 , 15.53]
Toegel F (2009)	rat	MSC	Creatinine		0.81 [-0.37 , 1.99]
Toegel F (2009)	rat	MSC	Creatinine		0.43 [-1.19 , 2.04]
Toegel F (2009)	rat	MSC	Creatinine		0.27 [-1.34 , 1.87]
Toegel F (2009)	rat	MSC	Creatinine		0.11 [-1.49 , 1.71]
Toegel F (2009)	rat	MSC	Creatinine		0.41 [-1.20 , 2.03]
Toegel F (2009)	rat	MSC	Creatinine		-0.48 [-2.09 , 1.14]
Toegel F (2009)	rat	MSC	Creatinine		2.89 [0.76 , 5.03]
Urbieta-Caceres VH (2012)	pig	EPC	MDCT		0.91 [-0.28 , 2.09]
van Koppen A (2012)	rat	BM	Inulin		1.38 [0.29 , 2.47]
van Koppen A (2012)	rat	BM	Inulin		1.10 [-0.16 , 2.37]
van Koppen A (2012)	rat	BM	Inulin		0.13 [-1.05 , 1.32]
van Koppen A (2012)	rat	Cell product	Inulin		1.08 [0.25 , 1.90]
van Koppen A (2012)	rat	Cell product	Inulin		-0.09 [-1.11 , 0.92]
Wang S (2013)	rat	MSC	Creatinine		-4.48 [-6.26 , -2.70
Yuen DA (2013)	rat	EPC	Inulin		1.08 [-0.15 , 2.31]
Yuen DA (2013)	rat	Cell product	Inulin		1.15 [-0.06 , 2.36]



5

Fig. S2. *Effect of cell-based treatment in CKD on glomerular filtration rate, GFR.*

Forest plot, right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

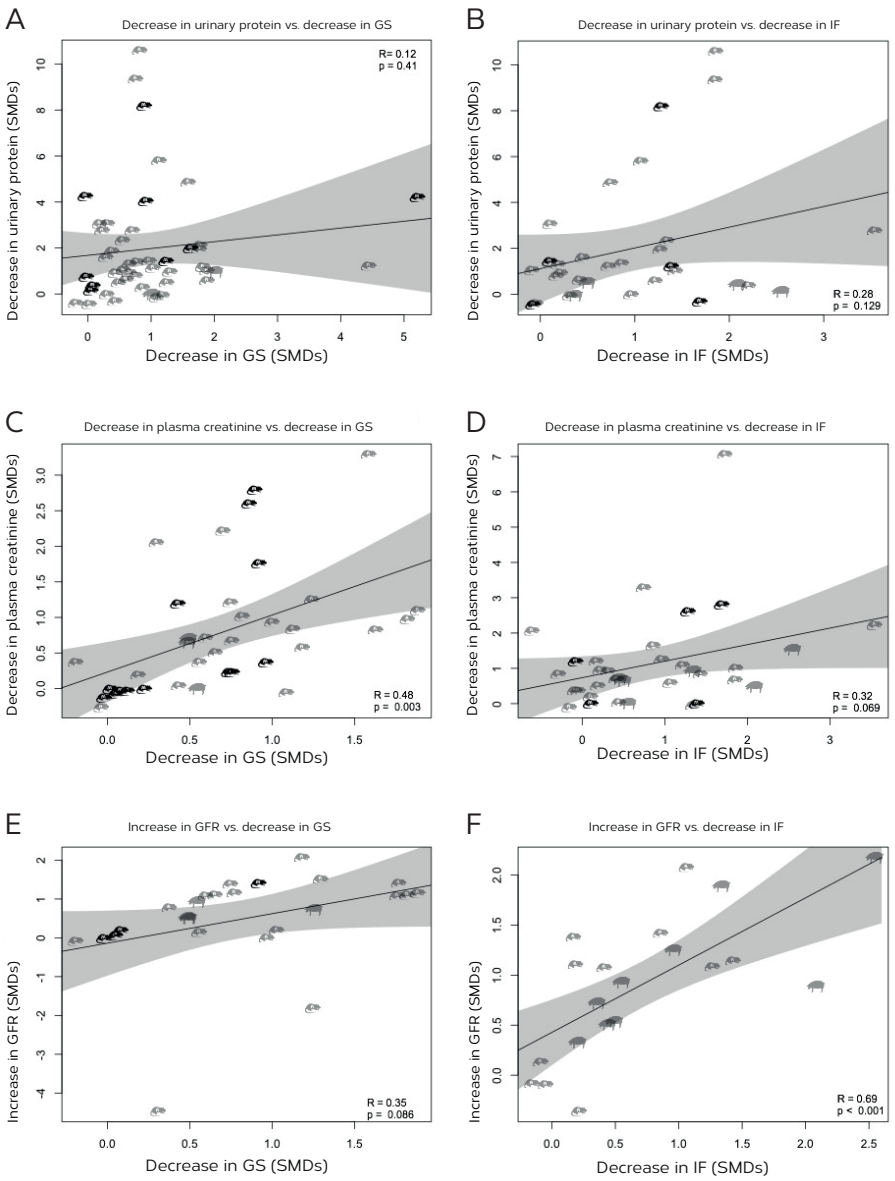


Fig. S3. *Correlations between renal functional parameters and tissue injury parameters.*
Decrease in urinary protein vs. decrease in glomerulosclerosis, GS ***(A)****, decrease in urinary protein vs. decrease in intersitial fibrosis, IF* ***(B)****; decrease in plasma creatinine vs. decrease in GS* ***(C)****, decrease in plasma creatinine vs. decrease in IF* ***(D)****, increase in glomerular filtration rate, GFR vs. decrease in GS* ***(E)****, increase in GFR vs. decrease in IF* ***(F)****.*

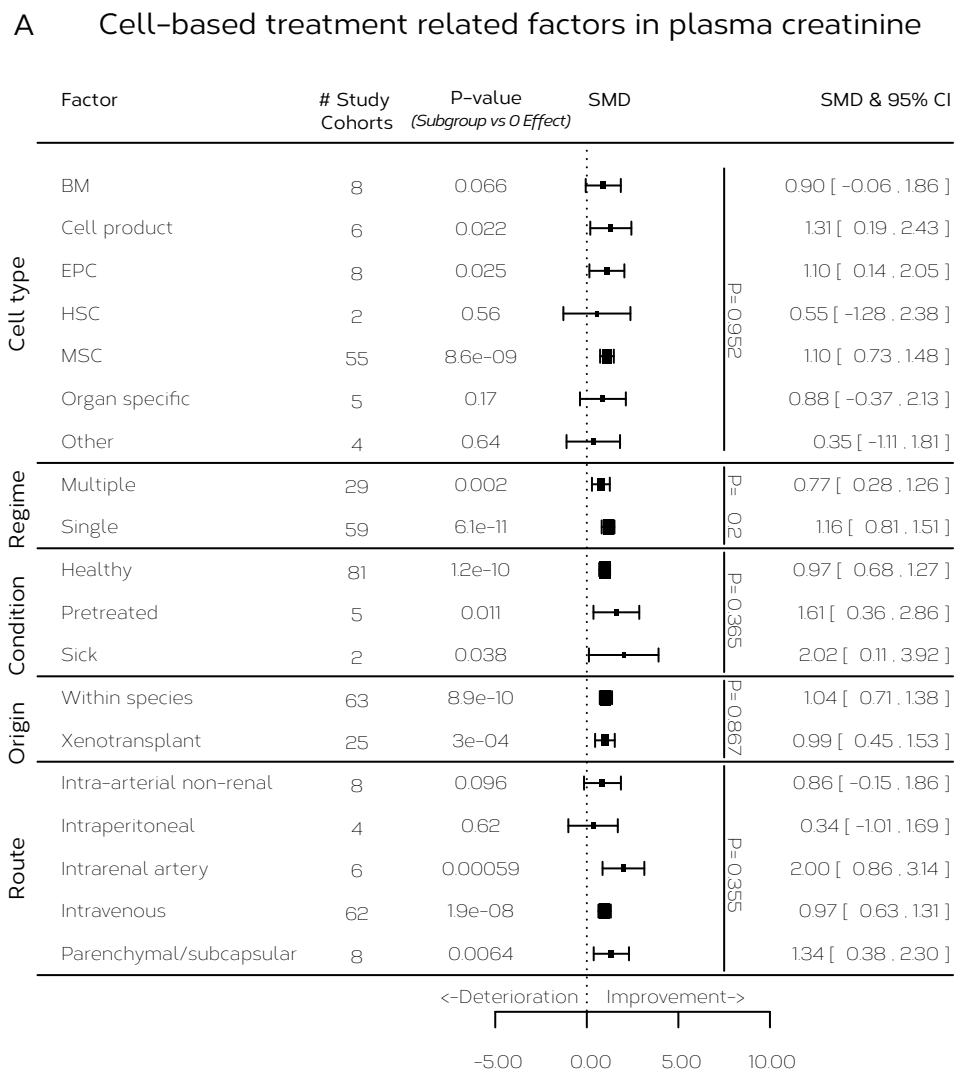


Fig. S4. *Subgroup analysis of cell-based treatment related factors. Plasma creatinine (A), Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.*

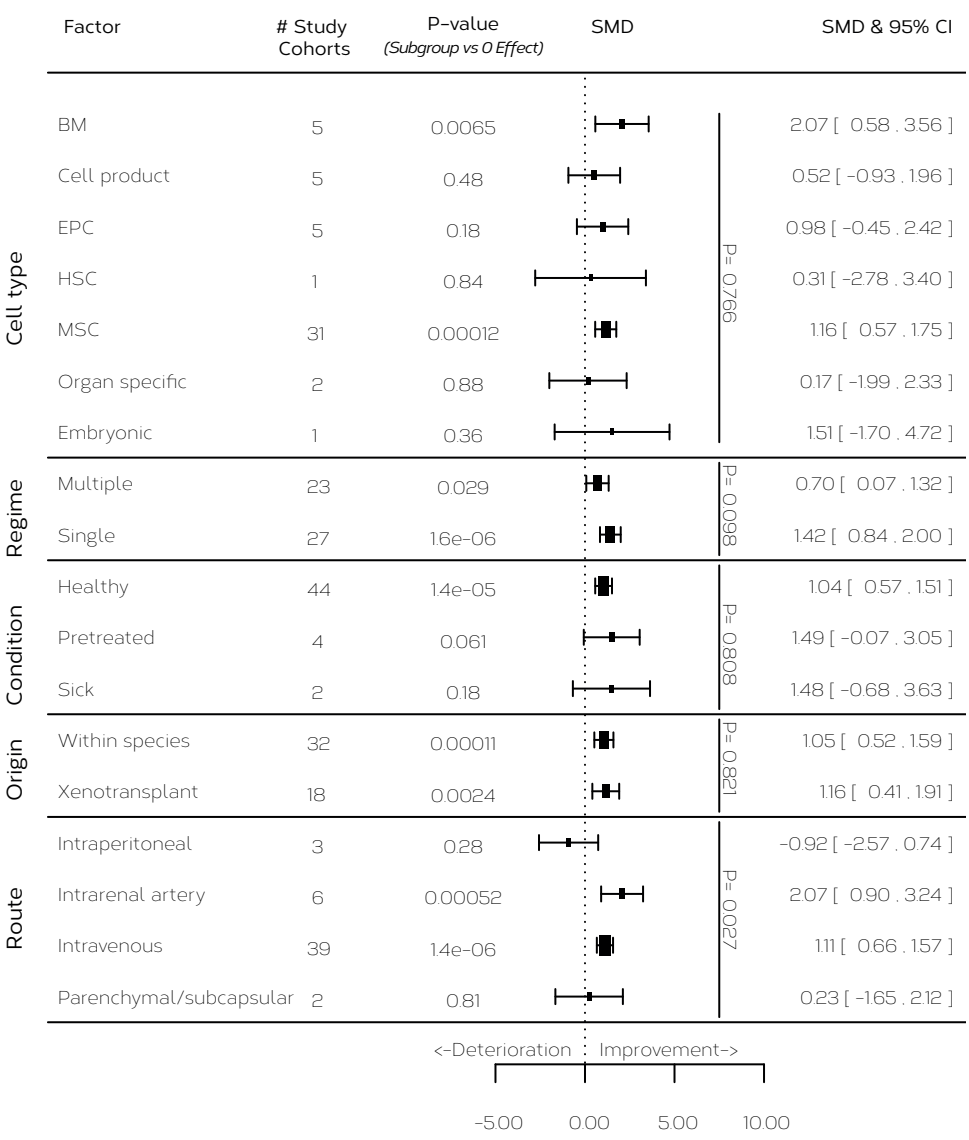


Fig. S4. *Subgroup analysis of cell-based treatment related factors. plasma urea (B), glomerular filtration rate, GFR (C), blood pressure, BP (D),*

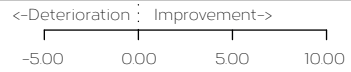
Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

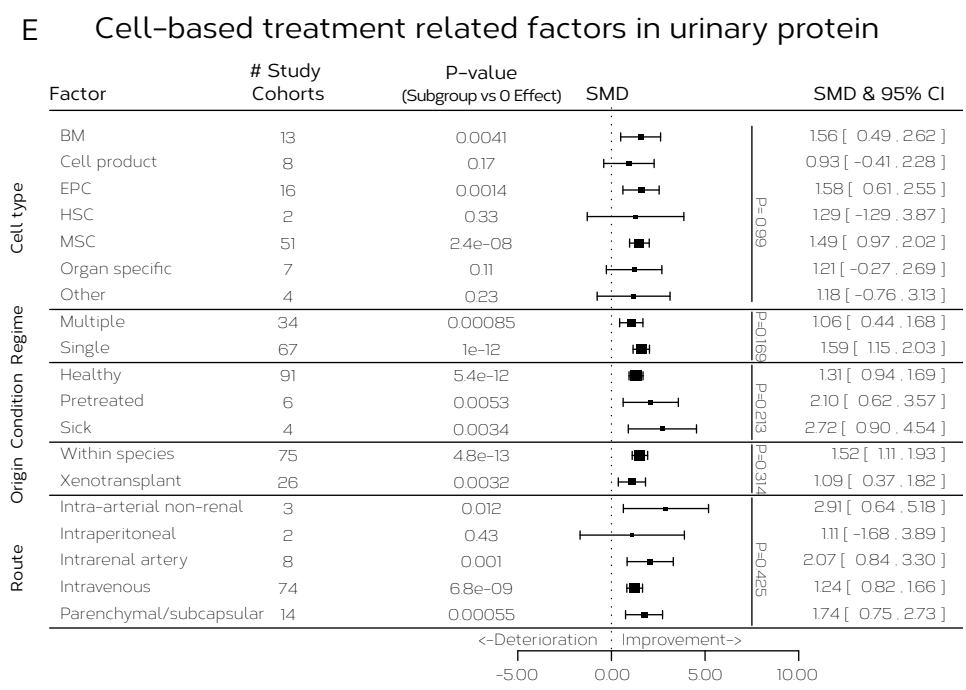
C Cell-based treatment related factors in GFR

	Factor	# Study Cohorts	P-value (Subgroup vs 0 Effect)	SMD & 95% CI	
Cell type	BM	7	0.17	0.73 [-0.31 , 1.77]	
	Cell product	3	0.36	0.71 [-0.80 , 2.22]	
	EPC	10	9.4e-13	3.86 [2.80 , 4.92]	P=1.47 e-05
	HSC	4	0.41	0.56 [-0.77 , 1.89]	
	MSC	21	0.14	0.47 [-0.15 , 1.08]	
	Organ specific	1	0.37	1.17 [-1.37 , 3.70]	
	Other	4	0.59	0.40 [-1.07 , 1.88]	
Regime	Multiple	8	0.17	0.82 [-0.34 , 1.98]	P=0.58
	Single	42	2e-05	1.18 [0.64 , 1.72]	
Condition	Healthy	47	3.1e-05	1.04 [0.55 , 1.53]	P=0.199
	Pretreated	2	0.0099	3.15 [0.76 , 5.54]	
	Sick	1	0.93	0.13 [-3.05 , 3.31]	
Origin	Within species	45	1.6e-05	1.15 [0.63 , 1.67]	P=0.714
	Xenotransplant	5	0.25	0.86 [-0.60 , 2.32]	
Route	Intra-arterial non-renal	9	0.54	0.37 [-0.81 , 1.54]	P=0.317
	Intrarenal artery	7	0.34	0.62 [-0.66 , 1.91]	
	Intravenous	28	8e-06	1.51 [0.85 , 2.18]	
	Parenchymal/subcapsular	6	0.17	1.00 [-0.43 , 2.43]	

D Cell-based treatment related factors in BP

	Factor	# Study Cohorts	P-value (Subgroup vs 0 Effect)	SMD & 95% CI	
Cell type	BM	4	0.00032	1.55 [0.70 , 2.39]	
	Cell product	3	0.09	0.76 [-0.12 , 1.65]	
	EPC	3	0.19	0.62 [-0.31 , 1.56]	P=0.564
	MSC	8	0.001	0.97 [0.39 , 1.54]	
	Organ specific	1	0.67	0.39 [-1.36 , 2.13]	
Regime	Multiple	5	0.007	0.99 [0.27 , 1.71]	P=0.937
	Single	14	1.7e-05	0.95 [0.52 , 1.39]	
Condition	Healthy	18	4.2e-07	0.98 [0.60 , 1.36]	P=0.701
	Sick	1	0.45	0.64 [-1.02 , 2.31]	
Origin	Within species	16	1.9e-05	0.88 [0.48 , 1.28]	P=0.328
	Xenotransplant	3	0.0023	1.36 [0.48 , 2.23]	
Route	Intra-arterial non-renal	2	0.55	0.31 [-0.71 , 1.34]	P=0.143
	Intrarenal artery	4	0.052	0.72 [0.00 , 1.44]	
	Intravenous	11	2.2e-05	0.97 [0.52 , 1.42]	
	Parenchymal/subcapsular	2	0.00019	1.92 [0.91 , 2.93]	





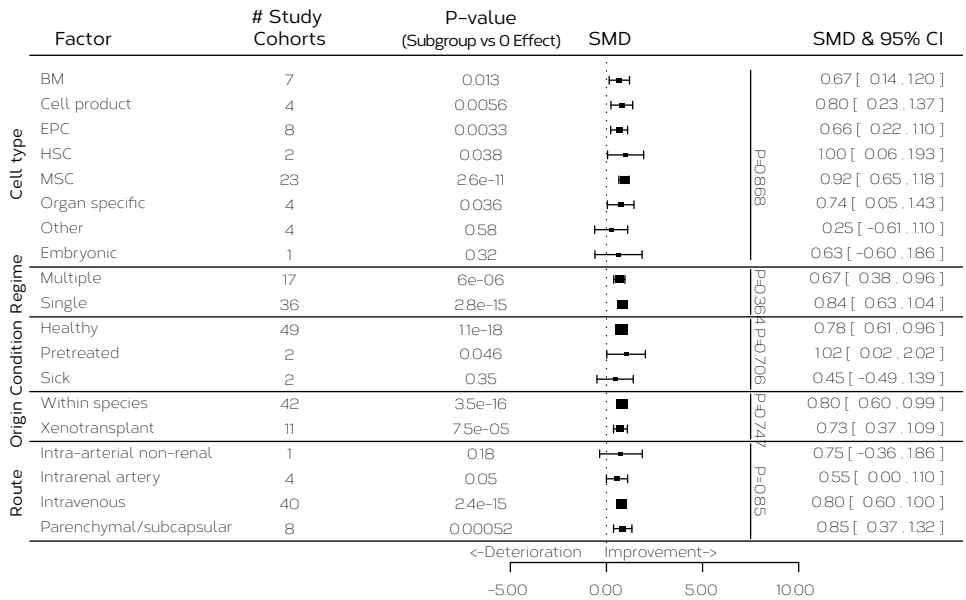
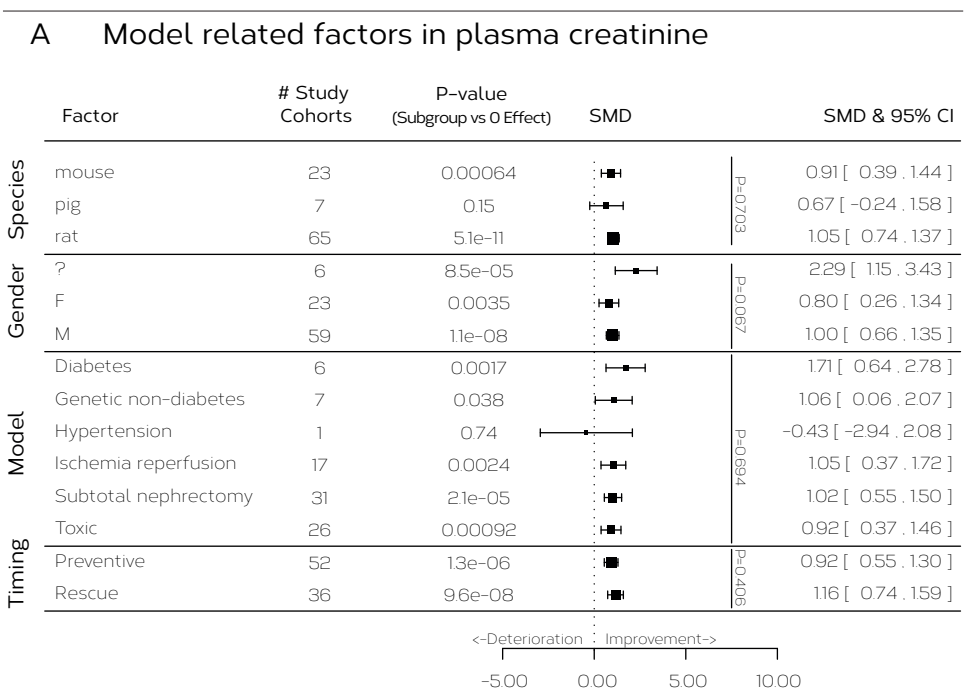


Fig. S4. *Subgroup analysis of cell-based treatment related factors. Urinary protein (E), glomerulosclerosis, GS (F) and interstitial fibrosis, IF (G).*

Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.



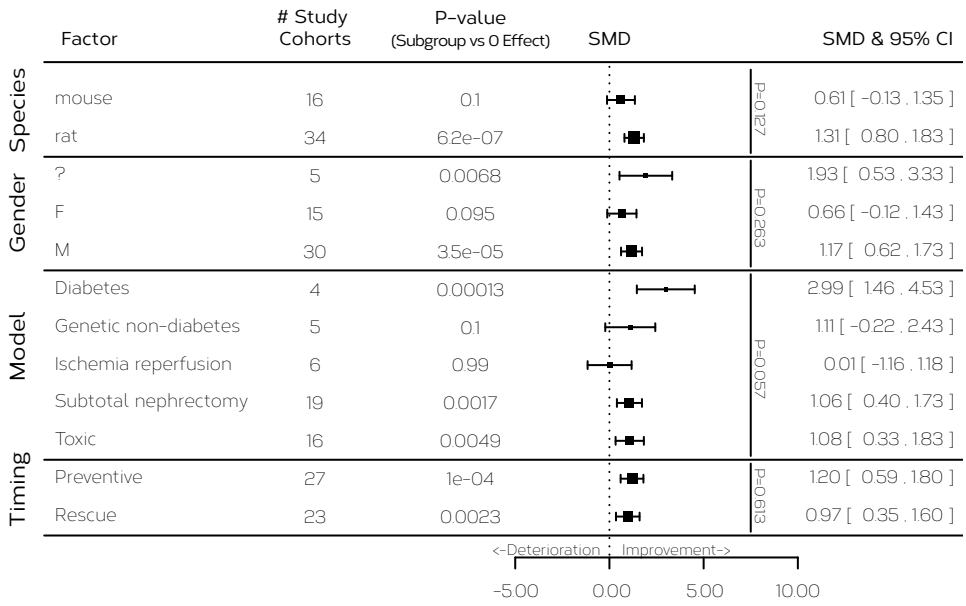
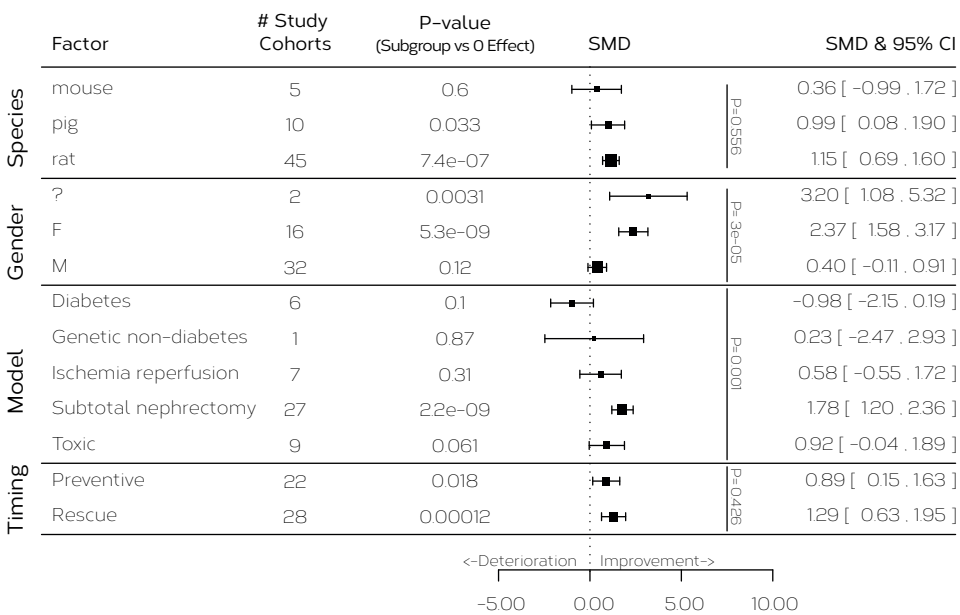
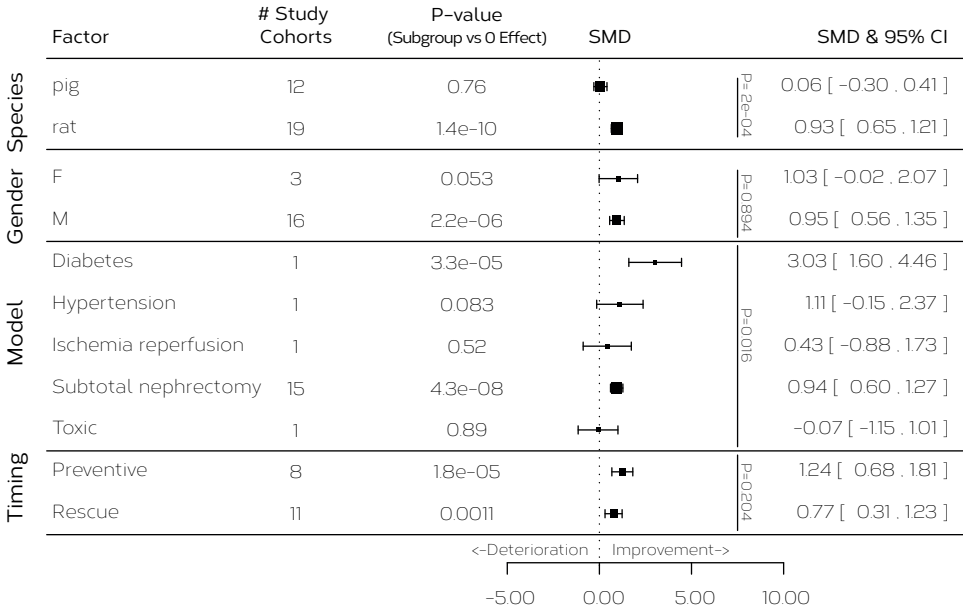


Fig. S5. *Subgroup analysis of model related factors. Plasma creatinine (A), plasma urea (B), Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.*

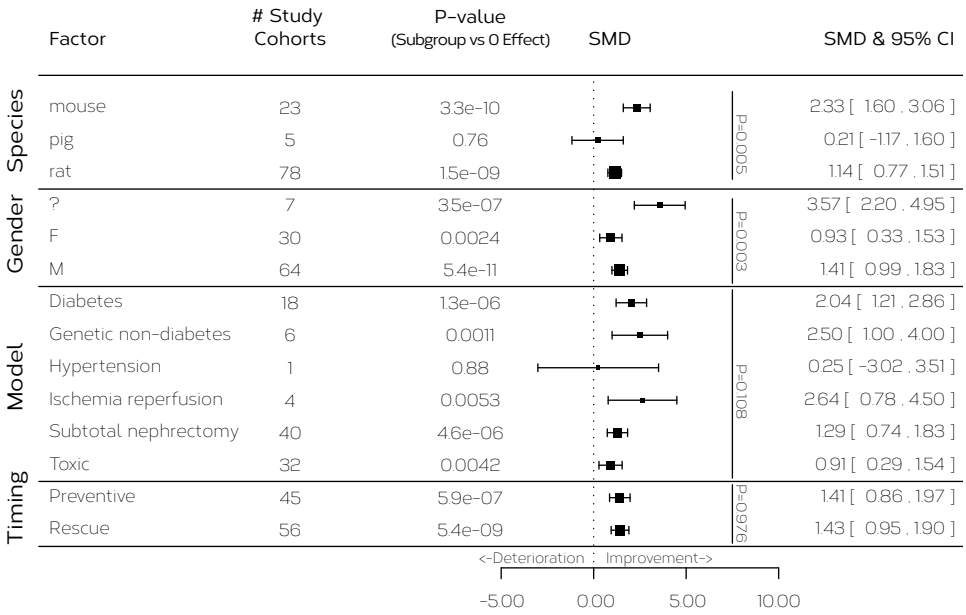
C Model related factors in GFR



D Model related factors in BP



E Model related factors in urinary protein



F Model related factors in GS

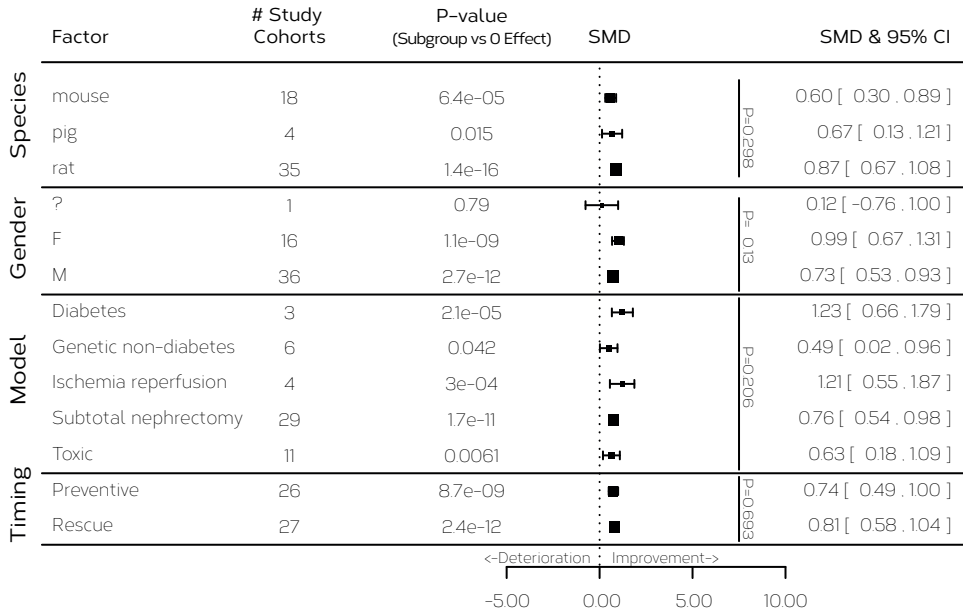


Fig. S5. *Subgroup analysis of model related factors. glomerular filtration rate, GFR (C), blood pressure, BP (D) Urinary protein (E), glomerulosclerosis, GS (F)*

Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

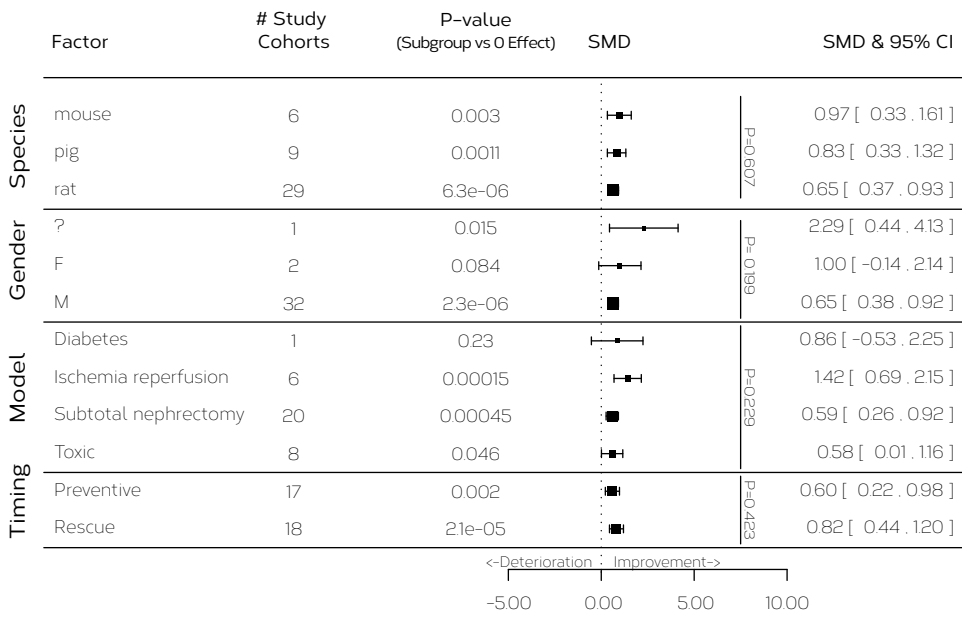


Fig. S5. *Subgroup analysis of model related factors. interstitial fibrosis, IF (G).*

Right side shows improvement by cell-based therapy. Data are presented as SMDs and 95% CI.

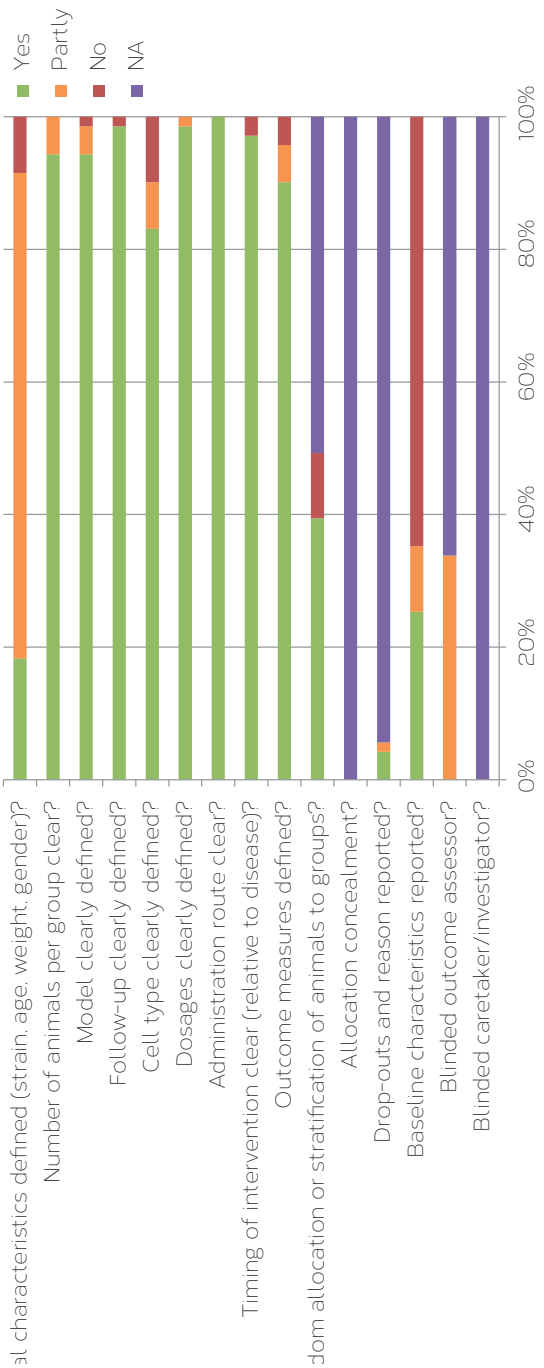


Fig. S6. *Quality assessment score.*

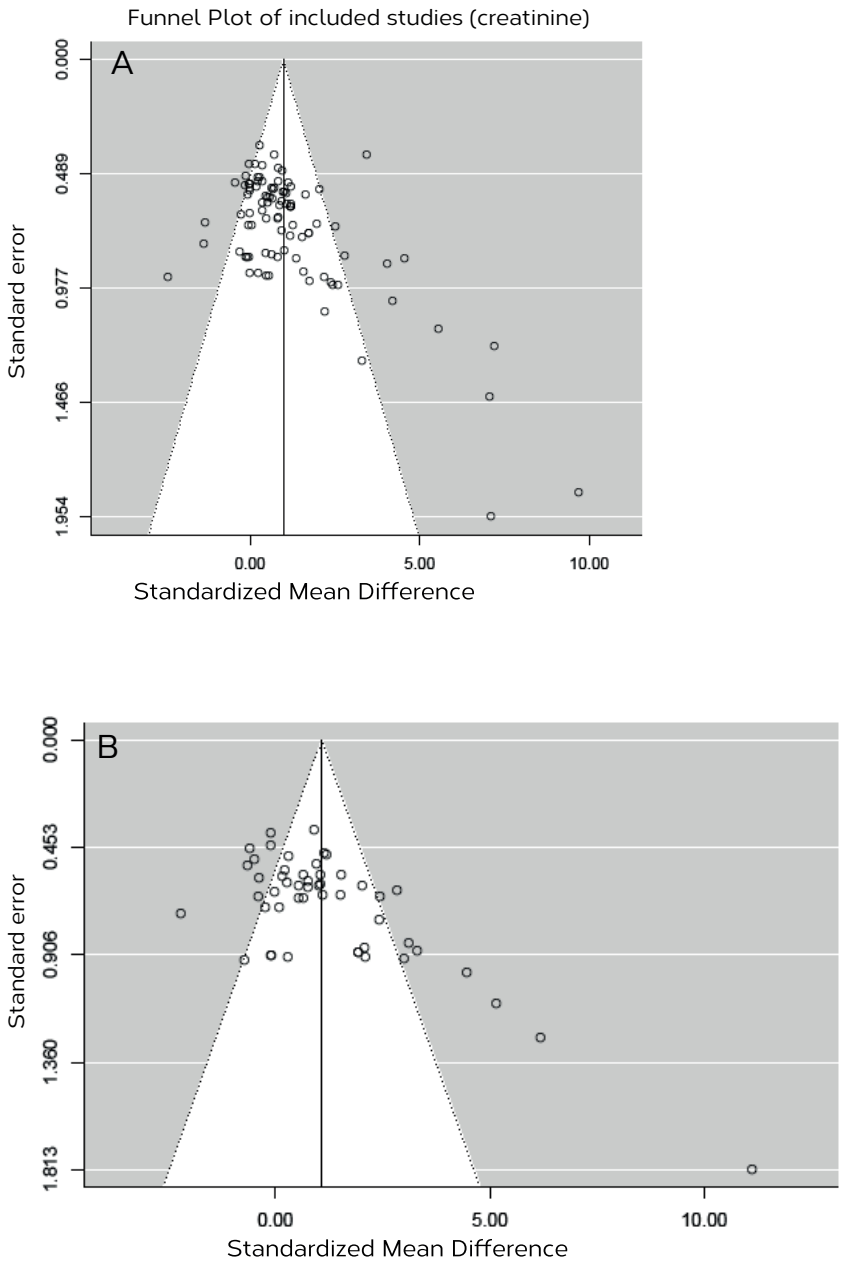


Fig. S7. *Funnel plots for plasma creatinine (A), plasma urea (B),*

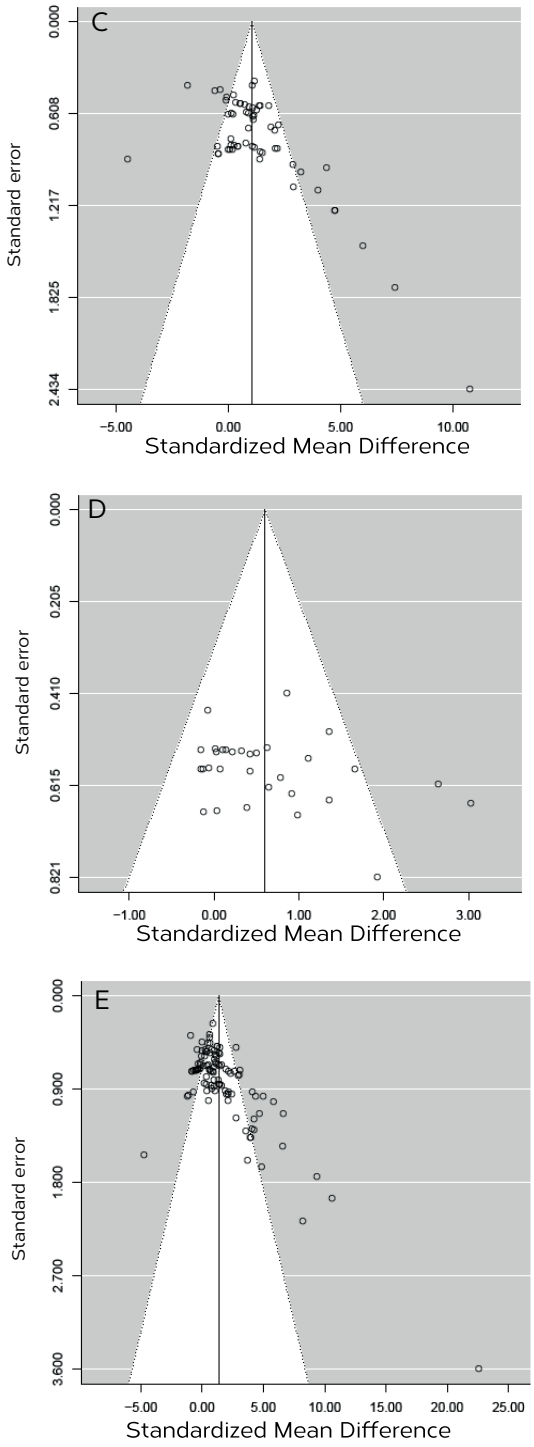


Fig. S7.*Funnel plots for GFR (C), BP (D), urinary protein (E), GS (F) and IF (G).*

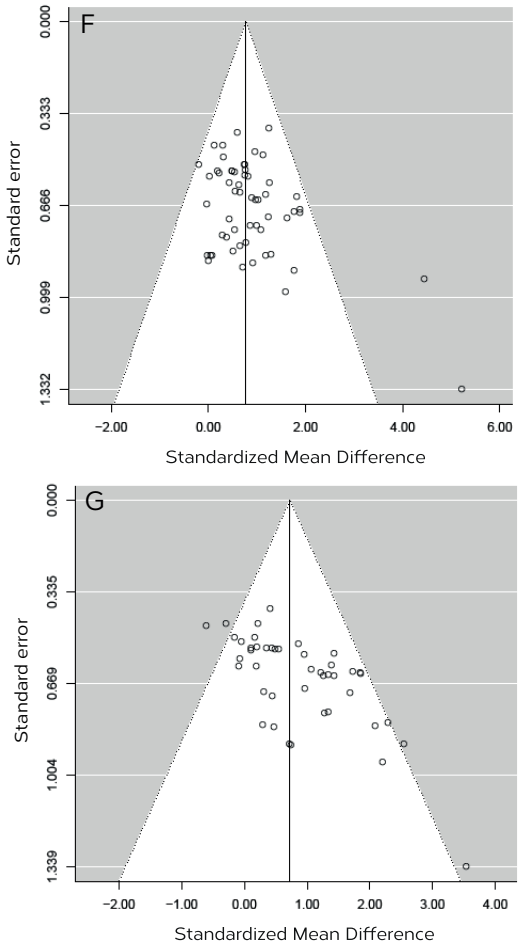


Fig. S7. *Funnel plots for GS (F) and IF (G).*

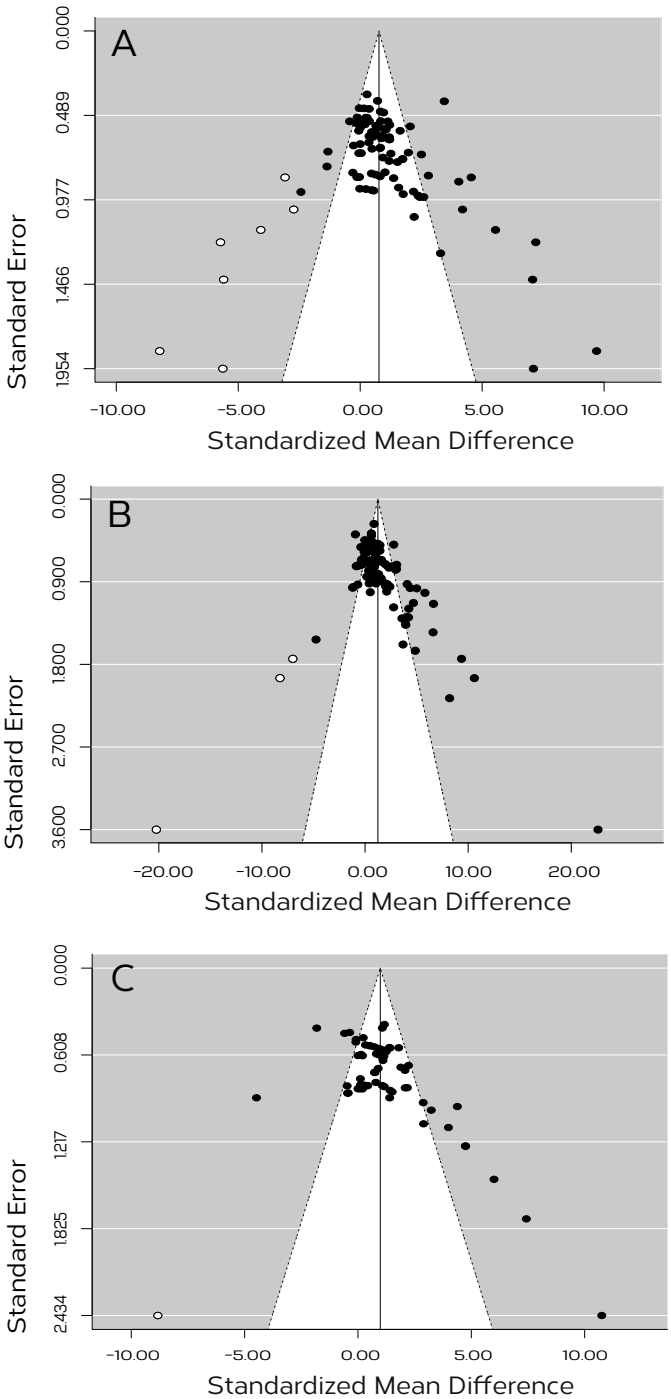


Fig. S8. *Imputed missing studies (open symbols) for plasma creatinine (A), urinary protein (B) and GFR (C).*

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Ex vivo exposure of bone marrow from chronic kidney disease donor rats to pravastatin limits renal damage in recipient rats with chronic kidney disease

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Published in Stem Cells Research and Therapy 2015 Apr 15;6:63

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Abstract

Introduction

Healthy bone marrow cell (BMC) infusion improves renal function and limits renal injury in a model of chronic kidney disease (CKD) in rats. However, BMCs derived from rats with CKD fail to retain beneficial effects, demonstrating limited therapeutic efficacy. Statins have been reported to improve cellular repair mechanisms.

Methods

We studied whether exposing CKD rat BMCs *ex vivo* to pravastatin improved their *in vivo* therapeutic efficacy in CKD and compared this to systemic *in vivo* treatment. Six weeks after CKD induction, healthy BMCs, healthy pravastatin-pretreated BMCs, CKD BMCs or CKD pravastatin-pretreated BMCs were injected into the renal artery of CKD rats.

Results

At 6 weeks after BMC injection renal injury was reduced in pravastatin-pretreated CKD BMC recipients vs. CKD BMC recipients. Effective renal plasma flow was lower and filtration fraction was higher in CKD BMC recipients compared to all groups whereas there was no difference between pravastatin-pretreated CKD BMC and healthy BMC recipients. Mean arterial pressure was higher in CKD BMC recipients compared to all other groups. In contrast, 6 weeks of systemic *in vivo* pravastatin treatment had no effect. *In vitro* results showed improved migration, decreased apoptosis and lower excretion of pro-inflammatory Chemokine (C-X-C Motif) Ligand 5 in pravastatin-pretreated CKD BMCs.

Conclusions

Short *ex vivo* exposure of CKD BMC to pravastatin improves CKD BMC function and their subsequent therapeutic efficacy in a CKD setting, whereas systemic statin treatment did not provide renal protection

Introduction

The rapidly rising number of patients with chronic kidney disease (CKD) worldwide urgently calls for new interventions. Bone marrow (BM)-derived stem and progenitor cell-based therapies have been proposed as promising approach for the treatment of acute and chronic kidney disease. We recently demonstrated that administration of healthy donor BM cells (BMCs) in an established rat model of CKD reduced progression of CKD[1]. However, for clinical application of BMC therapy in CKD, the use of autologous BMCs would be preferred; the challenge is that BMCs derived from CKD donors are unable to recapitulate therapeutic efficacy [1]. Therefore, to optimize autologous BMC therapy for treatment of CKD, we aim to counteract the functional impairment of CKD BMCs.

everal investigators have reported strategies to improve function of autologous BM-derived progenitor cell populations. Systemic treatment with lipid-lowering 3-hydroxy-3-methyl-glutaryl Coenzyme A reductase (HMG-CoA) inhibitors (statins) has been shown to augment endothelial progenitor cell (EPC) and mesenchymal stem cell (MSC) number and function in several disease models including cardiovascular diseases[2-4] and hypertension[5;6]. *In vitro* statin incubation has been reported to improve cell differentiation, proliferation, migration, angiogenesis, adhesion and decrease senescence, apoptosis and inflammation[7;8], possibly by pleiotropic effects such as increased nitric oxide (NO) bioavailability, anti-inflammatory and anti-oxidant effects[2;3;8;9] and prevention of cellular senescence via regulation of cell cycle proteins[10]. However, few studies have investigated the effect of *in vitro* or *ex vivo* statin treatment on cells obtained from diseased cell source[11-16] and to the best of our knowledge this is the first study to report the effects of statin treatment on cells in the context of CKD. We hypothesized that exposing CKD BMCs to pravastatin *ex vivo* would improve their subsequent *in vivo* therapeutic efficacy, ameliorating the progression of renal failure in a rat model of established CKD. To this end, we studied long-term effects of intra-arterial delivery of vehicle- or pravastatin-pretreated healthy and CKD BMCs on renal hemodynamics and injury. Our data conclude that while systemic *in vivo* treatment with pravastatin does not influence the course of CKD, a short *ex vivo* pulse of pravastatin significantly ameliorates progression of CKD in an established rat model.

Table 1. *Donor characteristics and stratification of donor and recipient rats.*

In vitro pravastatin treatment	
	CKD (n=10)
SNX (%)	66±2
Wk 5 SBP (mmHg)	169±28
Wk 5 Urea (mmol/L)	11±2
Wk 5 Proteinuria (mg/24h)	18±28

Ex vivo pravastatin treatment experiment				
Donor rats	Healthy+ DMEM BMC (n=2)	Healthy+ Pravastatin BMC (n=2)	CKD+DMEM BMC (n=4)	CKD + Pravastatin BMC (n=5)
SNX (%)	-	-	64.0±6.0	69.0±5.5
Wk 5 SBP (mmHg)	-	-	148±34	152±8
Wk 5 Urea (mmol/L)	4.6±0.01	4.1±0.9	15.0±4.4	15.9±6.9
Wk 5 Proteinuria (mg/24h)	1.4±0.4	1.8±0.7	24±8	49±46
Recipient rats	Healthy+ DMEM BMC (n=5)	Healthy + Pravastatin BMC (n=5)	CKD+DMEM BMC (n=10)	CKD +Pravastatin BMC (n=9)
SNX (%)	66.3±1.6	66.7±6.4	66.5±3.5	67.6±7.1
Wk 5 SBP (mmHg)	156±15	155±16	159±25	155±19
Wk 5 Urea (mmol/L)	10.1±0.9	10.3±1.5	12.1±3.2	10.6±2.3
Wk 5 Proteinuria (mg/24h)	21.6±5.6	21.9±6.8	23.7±17.3	22.7±8.4

Systemic in vivo pravastatin treatment		
Recipient rats	CKD+pravastatin (n=5)	CKD (n=6)
SNX (%)	69±2	66±3
Wk 5 SBP (mmHg)	158±19	159±10
Wk 5 Urea (mmol/L)	12.7±1.1	12.8±1.4
Wk 5 Proteinuria (mg/24h)	14.6±2.7	16.5±6.6

At 1 wk before BMC or in vivo pravastatin administration (wk 5 after subtotal nephrectomy (SNX)) rats were stratified based on plasma urea and systolic blood pressure (SBP). Mean±SD.

Methods

In vitro experiments

BMCs were harvested at six weeks after CKD induction from CKD Lewis rats (characteristics, see Table 1) and were incubated with or without 1 mmol/L pravastatin in DMEM for 2h at 37°C. Directly after incubation, cells were centrifuged and conditioned medium was stored for further analysis. Cells were washed, resuspended in 1 ml Dulbecco's modified Eagle medium (DMEM) and used for assessment of *in vitro* migration and apoptosis. $1x10^6$ cells were stored in Trizol (Invitrogen, NY) for RNA extraction.

Bone marrow cell characteristics

Viability and proportions of myeloid and lymphoid cell precursors were studied by flow cytometry.

Migration assay

Migration of pretreated CKD BMCs was determined using a modified Boyden chamber assay. 300,000 living cells were loaded above a 5 μm polycarbonate membrane (Transwell permeable support system, Corning, NY) and below the wells contained 200 ng/ml stromal derived factor 1α (SDF1α), a strong BMC attractant[17]; vehicle (no SDF1α) was used as negative control. After 180 min, transwells were removed horizontally and 1 ml of 2 mmol/L PBS-EDTA was added to the bottom well and incubated for 15 min on ice. Cell suspensions were collected and counted by flow cytometry. Percentage of migrated DMEM-treated BMCs towards 200 ng/ml SDF1α was set at 100% and compared to migration of pravastatin-treated cells.

Apoptosis

50 ul of cell suspension was used to create a cell smear, air-dried, fixed with formalin and stored at -20°C. TUNEL-staining (Apoptag Plus in situ Peroxidase kit, Millipore, Temecula, CA) was performed according to manufacturer guidelines. The number of apoptotic cells was determined as the number of TUNEL-positive cells in the images of 20 randomly selected fields (x200 magnification).

qPCR

We performed quantitative real-time PCR to determine the effects of pretreatment of CKD BMCs with pravastatin on the mRNA expression of endothelial NO synthase (eNOS), protein kinase B (PKB), monocyte chemotactic protein 1 (MCP-1), tumor necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF) (ABiPRiSM 790Sequence Detection SYStem, Applied Biosys-

tems, Foster City, CA). The following TaqMan® Gene Expression Assays (Applied Biosystems) were used: eNOS: Rn02132634_s1, PKB: Rn00583646_m1, MCP-1: Rn00580555_m1, TNFa: Rn99999017_m1, VEGF: Rn00582935_m1, ß-actin: Rn00667869_m1, and calnexin: Rn00596877_m1. Reactions were carried out in duplicate. Cycle time (Ct) values for genes of interest were normalized for mean Ct-values of Calnexin and β-actin, which we previously determined to be the two most stable housekeeping genes across all groups using the geNorm-program (http://medgen.ugent.be/~jvdesomp/genorm/), and expressed relative to a calibrator (the DMEM group), using the Ct-method. Hence, steady state mRNA levels were expressed as *n*-fold difference relative to the calibrator.

Cytokine array

A rat cytokine array (R&D Systems) of 27 cytokines was performed according to manufacturer's instructions on conditioned medium of BMC samples obtained from six CKD rats pretreated in DMEM with or without 1 mmol/L pravastatin as reported before[1;18]. Equal amounts of protein were loaded on the blots. Each spot on the blot is represented in duplicate and averages of the two pixel densities were used to calculate the average pixel density with Image J software. Background staining and spot size were analysed as recommended by the manufacturer. In brief, images were converted to 8-bit inverted tagged image file format files and spots were circled. Per blot, equal spot sizes were analysed.

ELISA

An ELISA for CXCL5 (Sigma) was performed according to manufacturer's instruction on conditioned medium of BMC samples obtained from six CKD rats pretreated in DMEM with or without 1 mmol/L pravastatin to validate cytokine array results.

In vivo experiments

CKD induction

The study protocol was approved by the Utrecht University Committee of Animal Experiments. CKD was induced in 8-week-old inbred male Lewis rats (recipients) and enhanced green fluorescent protein (eGFP)$^+$ Lewis rats (donors) by two-stage subtotal nephrectomy (SNX) as described (t=0)[1;18]. At week 5, CKD was confirmed (plasma urea >9 mmol/L).

Table 2. *Bone marrow cell characteristics measured by FACS analysis.*

In vitro pravastatin treated cells		
Bone marrow samples	DMEM (n=10)	Pravastatin (n=10)
Granulocytes	31.5±2.6	30.8±2.2
Lymphocytes	37.4±2.6	38.6±2.5
Monocytes	10.3±1.9	10.9±1.9
Stromalcells	7.9±3.6	7.5±3.5
Viability	96.6±3.4	97.0±2.5
Systemic in vivo pravastatin treatment recipients		
Bone marrow samples	CKD (n=6)	CKD+pravastatin (n=5)
Granulocytes	23.6±1.5	23.8±1.3
Lymphocytes	31.76±2.8	31.8±2.5
Monocytes	21.3±0.77	16.9±1.3*
Stromal cells	6.04±0.4	7.76±0.50*
Viability	96.8±0.4	97.7±0.01*

Mean ± SD. P<0.05 vs CKD.*

Experimental design

Effects of *ex vivo* pravastatin-pretreated BMCs in established CKD

At week 5 after CKD induction recipient rats (n=29) were stratified based on plasma urea and systolic blood pressure (Table 1). At six weeks after SNX BMCs were harvested from femur and tibia of healthy or CKD eGPF^{+} donor rats (for donor characteristics, see Table 1) and suspended in DMEM. The cell suspension was filtered (100 μm sieve) and counted (Abbott Cell-Dyn 1800). BMCs were incubated with or without 1 mmol/L pravastatin in DMEM for 2 hours at 5% CO_2 in a humidified incubator at 37^{0}C. Cells were washed to remove pravastatin and resuspended in 500 μl DMEM. 50*10^{6} *ex vivo* pretreated BMC cells were injected directly into the remnant kidney via the renal artery as follows: **healthy+DMEM BMC** recipients (n=5), CKD rats injected with healthy eGFP^{+}BMC exposed to DMEM; **healthy+pravastatin BMC** recipients (n=5), CKD rats injected with healthy eGFP^{+}BMC exposed to pravastatin; **CKD+DMEM BMC** recipients (n=10), CKD rats injected with CKD eGFP^{+}BMC exposed to DMEM;

CKD+pravastatin BMC recipients (n=9). CKD rats injected with CKD eGFP^{+}BMC exposed to pravastatin. Longitudinal measurements were performed at week 7, 9 and 11 after SNX and at week 12 (six weeks after BMC injection) terminal kidney function was measured (see below). Directly thereafter, rats were sacrificed and tissues were collected and either frozen or fixed in 4% paraformaldehyde (PFA) for renal morphology measurements.

Effects of systemic in vivo pravastatin treatment in established CKD

At week 6 after CKD induction, CKD rats were divided into two groups: CKD (n=6), no supplement in drinking water; CKD+pravastatin (n=5), 50 mg/kg/day pravastatin added to drinking water. Longitudinal measurements were performed at weeks 7, 9 and 11 and at week 12 terminal kidney function was measured (see below). Directly thereafter, rats were sacrificed and tissues were collected and either frozen or fixed in 4% PFA for renal morphology measurements.

Longitudinal chronic kidney disease evaluation

Rats were weighed weekly and at regular intervals 24h urine and blood samples were collected and systolic blood pressure (SBP) was measured by tail cuff sphygmomanometry at in weeks 5, 9 and 11. To collect 24h urine, rats were placedin metabolism cages without food for 24h, but with free accessto water with 2% glucose. For the systemic *in vivo* pravastatin treatment studies, pravastatin was also supplemented to the drinking water during urine collection. Urine was collected in antibiotic/antimycotic solution(Sigma, St. Louis, MO; A5955) and stored at −80°C. Blood samples were collected from the tail vein. Urinary protein levels weremeasured with Coomassie blue. Sodium and potassium levels were determined by flame photometry. Nitric oxide metabolites were measured (Cayman Chemical, MI). Plasma urea and plasma and urinary creatinine levels were determined by DiaSys Urea CT FS (DiaSys Diagnostic Systems, Holzheim, Germany). Creatinine clearance was calculated by dividing urine creatinine excretion (µMol/min/100 g BW) by plasma creatinine (Mol/µL). Cholesterol and triglycerides were determined by DiaSys Cholesterol FS and DiaSys Triglycerides FS (DiaSys Diagnostic Systems, Holzheim, Germany).

Terminal kidney function

Kidney function was assessed by inulin clearance to determine glomerular filtration rate (GFR) and para-amino hippuric acid (PAH) clearance to determine the effective renal plasma flow (ERPF) as described[19]. Briefly, rats were anesthetized with isoflurane and placed on a servo-controlled surgical table to maintain body temperature at 37°C. A polyethylene (PE) 90 catheter

was placed in the left jugular vein for infusion of solutions. The left femoral artery was cannulated with PE-50 tubing for measurement of mean arterial pressure (MAP) and blood sampling. A PE-90 catheter was placed in the bladder for urine collection. During surgery, animals received an intravenous infusion of a 150 mmol/L NaCl solution containing 6% bovine serum albumin (BSA). Following surgery, the infusion was switched to a 150 mmol/L NaCl solution with 1% BSA at the same infusion rate. This infusion was maintained throughout the experiment. The solution also contained inulin and PAH for clearance measurements. A 60-minute equilibration period was observed before the start of the 60-minute clearance measurements. During this clearance measurement urine was sampled for 15 minute periods and before and after the clearance measurement blood was sampled. Clearance and fractional excretions were calculated by standard formulas. Renal blood flow was calculated from ERPF and hematocrit.

Renal and cardiac morphology

Focal glomerulosclerosis (FGS) and tubular interstitial damage were scored on 3 μm periodic acid Schiff (PAS)-stained paraffin-embedded slides[1;18]. Glomerular influx of donor BMCs (eGFP$^+$) and presence T-cells (CD3$^+$), monocytes/macrophages (ED-1$^+$), proliferating cells (Ki67$^+$), apoptotic cells (TUNEL$^+$), cells undergoing DNA damage repair (γH2AX$^+$), and endothelial cells (JG12$^+$) was counted in 50 glomeruli[20]. Tubular number of T-cells (CD3$^+$), apoptotic cells (TUNEL$^+$), cells undergoing DNA damage repair (γH2AX$^+$) and influx of donor BMCs (GFP$^+$) was determined in 20 tubular fields as described[1;18]. Cardiac collagen I and III contents was stained with Sirius red, visualized with circular polarized light and digitally analysed using ImageJ software[21]. The percentage of collagen area was calculated by dividing the Sirius red stained area by the total image area.

Blood and bone marrow characteristics

Blood and bone marrow were incubated with the cell-permeable DNA binding dye Vybrant® DyeCycle™ Violet (Invitrogen/Life, Bleiswijk, NL) for 30 min at 37°C in order to allow separation of nucleated cells from debris. Red blood cells (RBC) were then lysed by incubation with a 0.8% ammoniumchloride lysis solution and samples were washed with phosphate buffered saline (PBS) prior to flow cytometry. During flow cytometry, debris and dead cells were excluded by lack of DyeCycle binding and 7-Aminoactinomycin D (7-AAD, Invitrogen) staining, respectively. Remaining cells were divided in major leukocyte subpopulations (i.e. lymphocytes, monocytes, granulocytes) on the basis of low angle forward scatter and orthogonal ('side') scatter properties. Gates

for the fluorescent stains and eGFP were set on fluorescence-minus-one controls, gates for leukocyte subpopulations were set for each population center on the contour plot. A volumetric estimate of leukocyte subpopulation proportions was furthermore obtained using an automated haematology analyzer (Abbott Cell-Dyn 1800).

qPCR

To ascertain that the dose of pravastatin was sufficient, the production of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) was evaluated. cDNA was isolated from frozen liver tissue and expression of HMGCR (Rn00565598_m1) was determined using quantitative real-time RT-PCR as described for the *in vitro* experiments.

Statistical analyses

Data are presented as mean±SD and analysed by analysis of variance (One-way ANOVA with a Student-Newman-Keuls post-test, Two-way ANOVA with a Bonferroni post-test, and Student's T-test) with Graphpad Prism Software, La Jolla, CA) P<0.05 was considered significant.

Results

In vitro experiments

In vitro pravastatin pre-treatment of BMCs improves BMC function

Pravastastin treatment of CKD BMC did not induce differences in myeloid and lymphoid precursor cell composition (Table 2). CKD+pravastatin BMCs showed increased migration towards 200 ng/ml SDF1α as compared to CKD+DMEM BMC in 8 out of 10 rats (p=0.014; Figure 1). Fewer apoptotic cells were found in CKD+pravastatin BMCs vs. CKD+DMEM BMC in 8 out of 10 rats (p=0.16; Figure 2). Pravastatin treatment of CKD BMC did not alter mRNA expression of *TNF*α, *eNOS*, *PKB*, *MCP-1* or *VEGF* (Table 3). Of the 27 cytokines tested on a cytokine array, we could only detect expression of 4 cytokines. Pravastatin treatment of CKD BMC decreased the secretion of pro-inflammatory Chemokine (C-X-C Motif) Ligand 5 (CXCL5) and increased chemokine (C-X-C Motif) Ligand 7 (CXCL7), whereas secretion of L-Selectin and soluble intracellular adhesion molecule (ICAM) was not different between DMEM- and pravastatin-treated CKD BMC (Figure 3). Decreased CXCL5 secretion by CKD+pravastatin BMCs compared to DMEM-treated CKD BMC was confirmed by ELISA (60±12 vs. 98±31 pg/ml; p=0.0045).

Table 3. *Gene expression in CKD BMCs was not changed after in vitro treatment with pravastatin (expressed as fold change).*

	DMEM (n=10)	Pravastatin (n=10)	p-value
TNFα	1.000±1.290	0.868±0.969	0.7504
eNOS	1.000±4.609	2.161±2.874	0.5422
PKB	1.000±0.889	0.905±0.5512	0.6906
MCP-1	1.000±1.254	1.191±0.6933	0.6101
VEGF	1.000±0.566	0.687±0.464	0.0807

Mean ± SD.

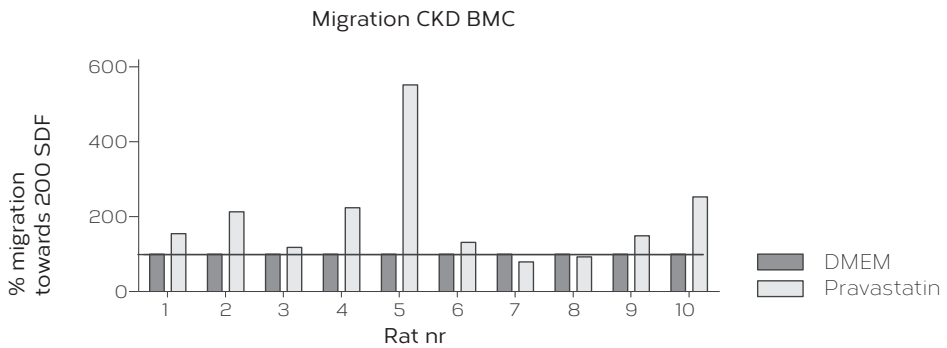

Figure 1. *Effect of in vitro pravastatin treatment on migration capacity of CKD BMCs. DMEM (n=10); Pravastatin (n=10).*

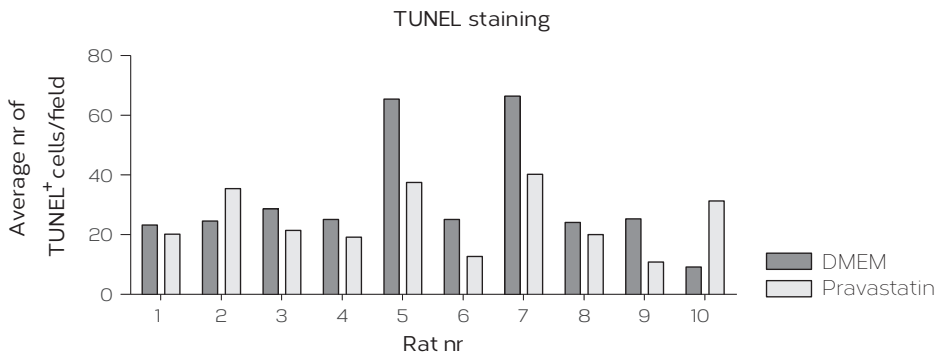

Figure 2. *Effect of in vitro pravastatin treatment on apoptosis in CKD BMCs. DMEM (n=10); Pravastatin (n=10).*

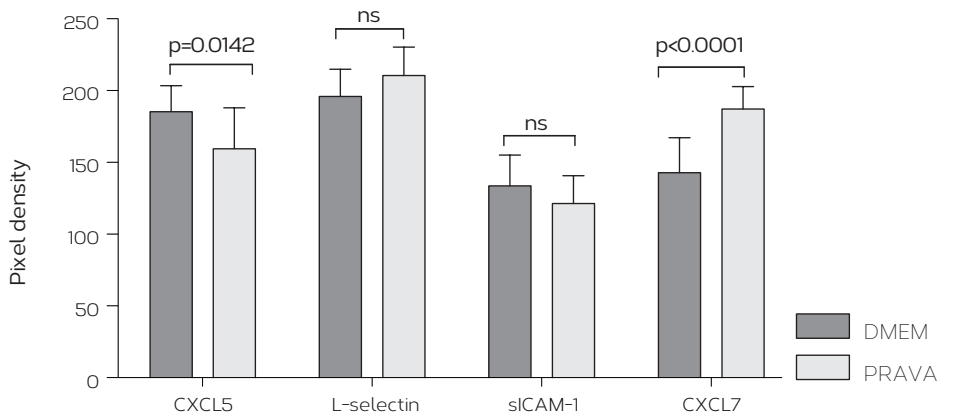

Figure 3. *Cytokine expression in CKD BMCs after exposure to vehicle (DMEM) or pravastatin in vitro. DMEM (n=6); Pravastatin (n=6).*

In vivo experiments

Ex vivo pravastatin pre-treatment of CKD BMC improves in vivo therapeutic efficacy

As compared to healthy+DMEM BMC recipients, CKD+DMEM BMC recipients had a 38% lower GFR and 45% lower ERPF at week 12. However, in CKD+pravastatin BMC recipients GFR and ERPF were not significantly different compared to healthy+DMEM BMC recipients (Table 4). Terminal mean arterial pressure (MAP) was 20 mmHg lower in CKD+pravastatin BMC recipients compared to CKD+DMEM BMC recipients and not different from either healthy+DMEM BMC recipients or healthy+pravastatin BMC recipients (Table 4). Hematocrit was lower in CKD+DMEM BMC recipients vs. healthy+DMEM BMC recipients, but higher in CKD+pravastatin BMC recipients vs. CKD BMC recipients (Table 4). Filtration fraction (FF) was higher in CKD BMC recipients vs. healthy BMC recipients whereas in CKD+pravastatin BMC recipients filtration fraction was not significantly different vs. healthy+DMEM BMC recipients (Table 4). Terminal body, kidney and heart weight did not differ (Table 4).

At week 11, plasma urea was higher in CKD+DMEM BMC recipients compared to healthy+DMEM BMC recipients (16.1±7.9 vs. 10.7±1.7 mmol/L $p<0.05$) whereas CKD+pravastatin BMC recipients (12.9±3.2 mmol/L) were not significantly different from healthy+DMEM BMC recipients (10.7±1.7 mmol/L) (Figure 4a). Diuresis, proteinuria, creatinine clearance and excretion of NO metabolites were not significantly different between groups, but from weeks 7 to 11 natriuresis was higher in CKD+pravastatin BMC recipients vs. CKD+DMEM BMC recipients (1680±473 vs. 1176±233; $p<0.05$; Figure 4b-f).

Table 4. *Terminal measurements of bone marrow cell recipients with posthoc p-values.*

Terminal measurements of bone marrow cell recipients						
Week 12	Healthy + DMEM BMC recipients (n=5)	Helthy + Pravastatin BMC recipients (n=5)	CKD + DMEM BMC recipients (n=10)	CKD + Pravastatin BMC recipients (n=9)	CKD + DMEM BMC vs. Healthy + DMEM BMC	CKD + DMEM BMC vs. CKD + Pravastatin BMC
Body weight (g)	376±23	379±26	359±38	373±22	ns	ns
Heart weight (g/100 g BW)	0.44±0.07	0.44±0.03	0.47±0.08	0.44±0.05	ns	ns
Kidney weight (g/100 g BW)	0.59±0.07	0.56±0.05	0.53±0.06	0.55±0.04	0.063	ns
MAP (mmHg)	149±28	146±35	173±21	153±21	0.095	0.091
GFR (µl/min/100g)	371±68	352±119	237±159	261±149	0.057	ns
ERPF (µl/min/100g)	1538±224	1388±502	844±626*	1157±359	<0.05	ns
Hema-tocrit	0.45±0.01	0.44±0.01	0.42±0.03*	0.46±0.02†	<0.05	<0.05
FF (%)	24.1±2.4	26.2±4.7	29.9±4.5*	26.4±3.7	<0.05	0.071

*Mean±SD. * p<0.05 compared to healthy BMC recipients, † p<0.05 compared to CKD BMC recipients. Trends (p<0.1) are only indicated with the exact p-value.*
MAP=mean arterial pressure; GFR=glomerular filtration rate; ERPF=effective renal plasma flow; FF= filtration fraction.

In all CKD groups, only 10% of glomeruli were completely normal, confirming severe kidney injury. The number of totally sclerotic glomeruli was higher in CKD+DMEM BMC recipients vs. healthy+DMEM BMC recipients (p<0.05). Comparing the number of partly and totally sclerotic glomeruli between CKD+DMEM BMC recipients and CKD+pravastatin BMC recipients revealed a favourable shift to better preserved glomeruli in CKD+pravastatin BMC recipients (p<0.05; Figure 5a).

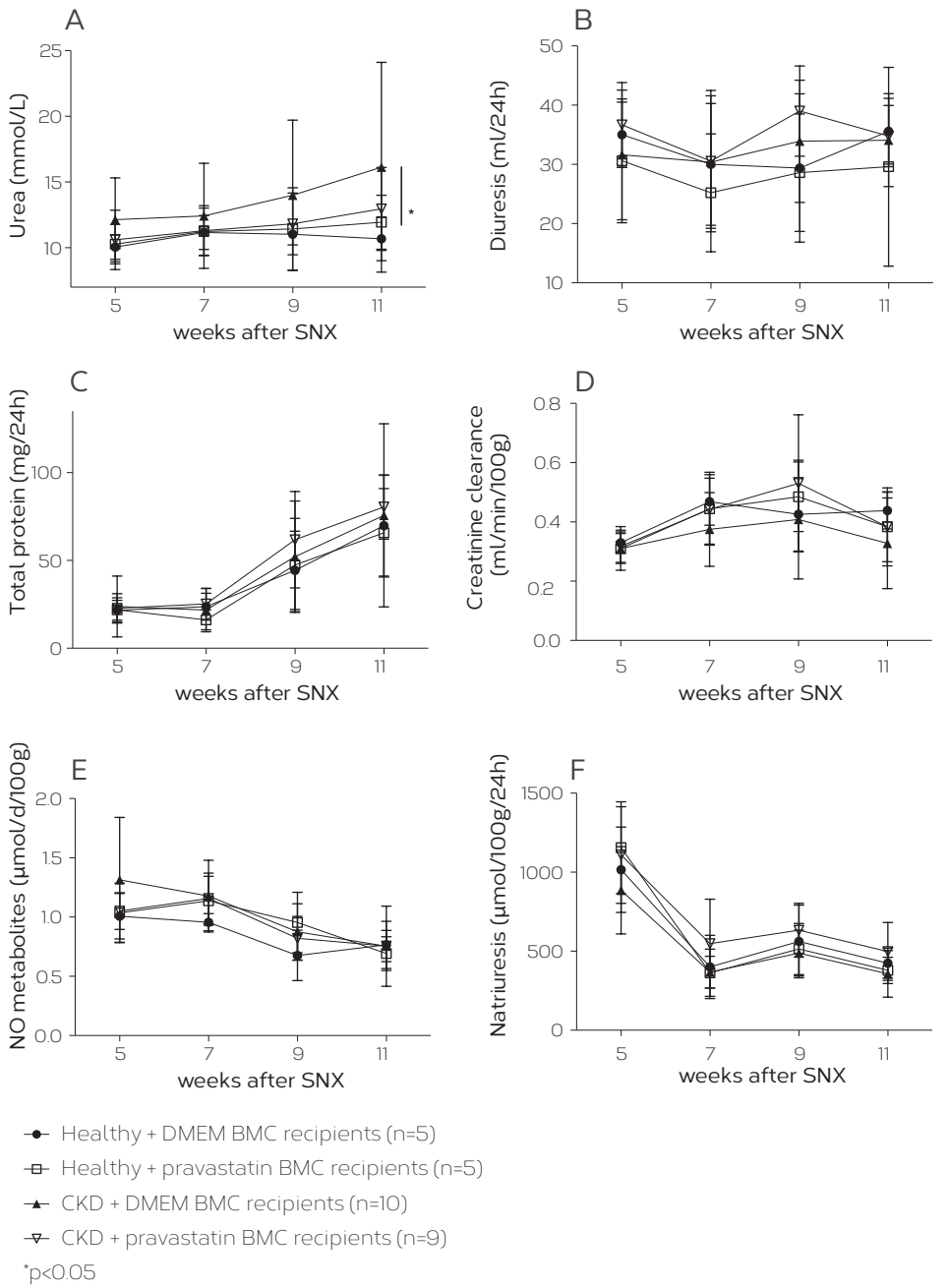

Figure 4. *Effects of ex vivo exposure of healthy and CKD BMCs to pravastatin or DMEM on longitudinal plasma and urinary variables of CKD recipients. **A**: urea; **B**: diuresis; **C**: proteinuria; **D**: creatinine clearance; **E**: nitric oxide metabolites (NOx); **F**: natriuresis. Week 5 represents one week before BMC injection. Mean±SD.*

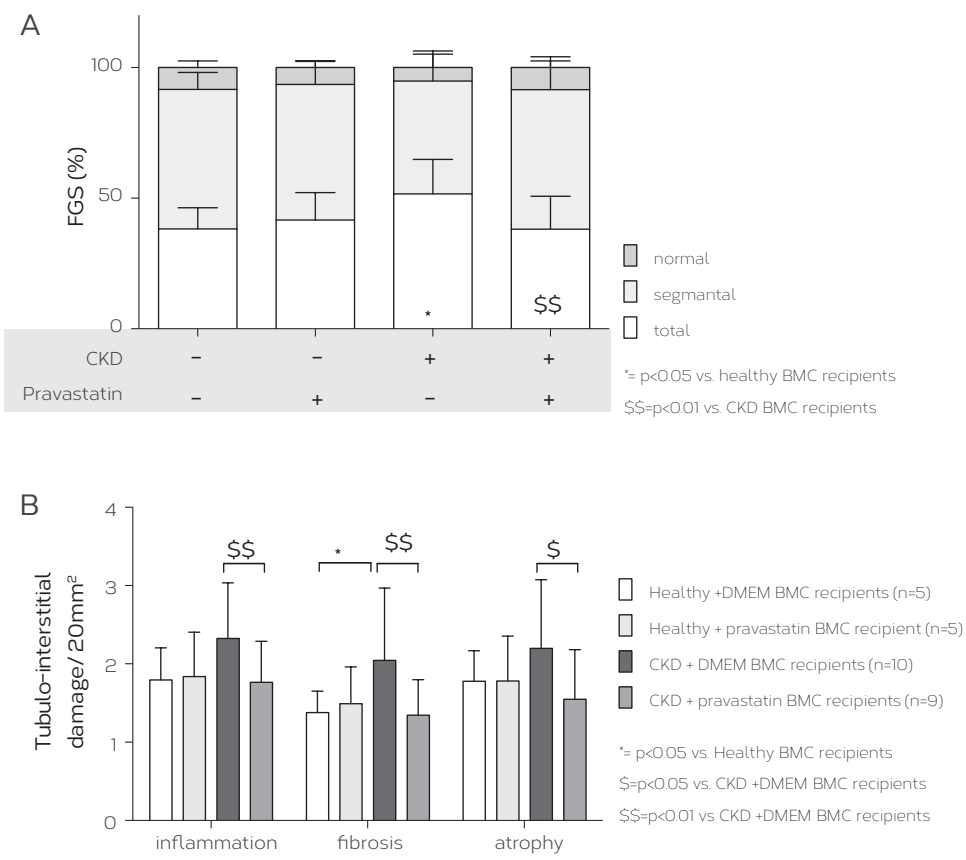

Figure 5. *Effects of ex vivo exposure of healthy and CKD BMCs to pravastatin on renal morphology in CKD recipients. A: Focal glomerulosclerosis; B: Tubulo-interstitial damage. CKD rats received healthy+DMEM BMCs (n=5); healthy+pravastatin BMCs (n=5); CKD+DMEM BMCs (n=10) or CKD+pravastatin BMCs (n=9). *p<0.1 vs. healthy BMC recipients, $p<0.1; $$p<0.05 vs. CKD+DMEM BMC recipients.*

Tubular interstitial inflammation, tubular atrophy and interstitial fibrosis were all lower in CKD+pravastatin BMC recipients vs. CKD+DMEM BMC recipients (Figure 5b). The number of glomerular inflammatory cells ($ED1^+$ and $CD3^+$) tended to be higher in CKD+DMEM BMC recipients compared to all other groups. Tubular $CD3^+$ influx was most abundant in CKD+DMEM BMC recipients (Table 5). Cardiac fibrosis tended to be more abundant in CKD+DMEM BMC recipients compared to healthy BMC+DMEM recipients as reported before[22]. In CKD+pravastatin BMC recipients cardiac fibrosis tended to be reduced compared to CKD+DMEM BMC recipients (15.3±7.6 vs. 20.5±4.7%; p=0.085). Healthy+pravastatin BMC did not influence cardiac fibrosis (healthy+DMEM BMC recipients: 16.7±6.5 vs. healthy+pravastatin BMC recipients: 16.8±6.7%).

Table 5. *Glomerular and tubular histological characteristics after ex vivo pravastatin pre-treatment.*

histological characteristics after ex vivo pravastatin pre-treatment.				
	Healthy + DMEM BMC recipients (n=5)	Healthy + Pravastatin BMC recipients (n=5)	CKD + DMEM BMC recipients (n=10)	CKD + Pravastatin BMC recipients (n=9)
Glomerular				
CD3	0.68±0.34	0.72±0.33	0.76±0.19	0.67±0.27
ED-1	7.4±1.0	5.0±2.9	10.5±5.5	7.9±1.9
Ki67	8.0±0.76	6.4±1.7	7.8±1.8	7.8±2.3
TUNEL	2.6±1.3	2.1±1.3	7.0±4.6	4.4±3.7
γH2AX	1.5±1.6	0.6±0.4	1.2±0.8	1.4±0.8
GFP$^+$	7.0±6.6	2.5±0.7	2.3±1.9	5.2±2.9
JG12	42.5±10	44.4±9.9	42.8±12.5	48.1±9.0
Tubular				
CD3	81±11	96±45	122±59	98±27
TUNEL	35±24	65±59	147±116	95±79
γH2AX	5.0±1.6	4.3±2.0	6.1±2.8	5.3±1.5
GFP$^+$	83±82	31±24	23±20	50±38

Representative images of stainings in glomeruli (A) and tubuli (B) are shown. Mean±SD per 50 glomeruli or 20 tubular fields.

The number of proliferating glomerular cells (Ki67$^+$), endothelial cells (JG12$^+$), and apoptotic cells (TUNEL$^+$) were not significantly different between groups (Table 5). No difference was found in the number of tubular apoptotic (TUNEL$^+$) cells, cells expressing DNA damage repair marker γH2AX$^+$ (Table 5), or the number of eGFP$^+$ cells. As shown previously, eGFP$^+$ cells were detected in the remnant kidney and heart but only in low numbers. No integration of these cells was observed and most were attached to the endothelial lining of small vessels[1]. No differences were observed in blood counts (Table 6).

Table 6. *Blood cell counts.*

pravastatin pretreatment experiment recipients				
Blood samples	Healthy + DMEM BMC recipients (n=5)	Healthy + Pravastatin BMC recipients (n=5)	CKD+ DMEM BMC recipients (n=10)	CKD + Pravastatin BMC recipients (n=9)
White blood cells	5.86±1.20	5.52±0.93	4.63±1.05	5.42±1.18
Lymphocytes	3.30±1.35	2.54±0.29	2.60±0.86	2.94±0.51
Midpopulation	1.58±0.29	1.56±0.31	1.18±0.28	1.59±0.45
Granulocytes	0.98±0.45	1.38±0.53	0.84±0.35	1.08±0.52
Red blood cells	6.66±0.40	6.59±0.36	6.24±0.59	6.63±0.23
Hemoglobin	8.06±0.47	7.90±0.42	7.63±0.72	7.86±0.29
Hematocrit	0.33±0.05	0.33±0.019	0.31±0.029	0.33±0.010
Mcv	49.08±0.83	50.08±1.08	50.10±2.09	49.35±0.78
Mchc	24.38±0.22	23.94±0.38	24.18±0.43	24.08±0.36
Rdw	15.44±0.43	15.22±0.29	15.63±1.125	15.151±0.818
Platelets	736±68	697±49	634±99	699±55

Systemic in vivo pravastatin treatment		
Blood samples	CKD (n=6)	CKD+pravastatin (n=5)
White blood cells	4.81±0.89	4.50±1.16
Lymphocytes	2.87±0.59	2.90±0.97
Midpopulation	1.27±0.39	0.90±0.19
Granulocytes	0.65±0.16	0.68±0.13
Red blood cells	6.38±0.22	6.25±0.15
Hemoglobin	7.58±0.32	7.52±0.18
Hematocrit	0.32±0.01	0.31±0.01
Mcv	49.37±0.79	49.96±0.32
Mchc	24.05±0.25	24.14±0.23
Rdw	15.13±0.63	15.14±0.68
Platelets	681±42	666±41

Mean ± SD.Ex vivo

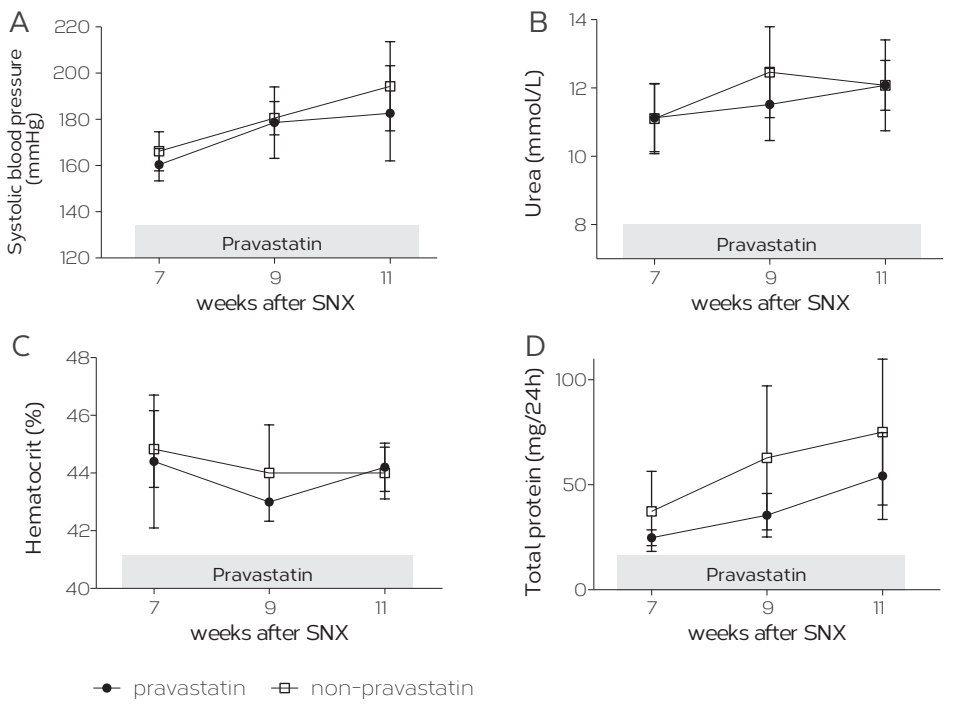

Figure 6. *Effects of systemic in vivo exposure to pravastatin on longitudinal variables in CKD recipients. **A:** systolic blood pressure; **B:** urea; **C:** hematocrit; **D:** proteinuria.*

Table 7. *Terminal measurements after systemic in vivo pravastatin treatment.*

Week 12	CKD (n=6)	CKD+ pravastatin (n=5)
Body weight (g)	357±24	369±16
Heart weight (g/100 g BW)	0.45±0.02	0.42±0.03*
Kidney weight (g/100 g BW)	0.57±0.02	0.57±0.04
MAP (mmHg)	168±19	164±19
GFR (µl/min/100g)	340±60	332±41
ERPF(µl/min/100g)	1335±291	1221±189
Hematocrit	0.41±0.02	0.40±0.02
FF (%)	28±3	26±2
Cholesterol (mmol/L)	2.89±0.53	2.73±0.46
Triglycerides (mmol/L)	1.42±0.38	0.70±0.37*
Fold change hepatic HMGCR mRNA expression	1.000±0.56	1.584±0.65

*Mean±SD. *p<0.05 compared to CKD.; MAP= mean arterial pressure; GFR= glomerular filtration rate; ERPF= effective renal plasma flow; FF= filtration fraction; HMGCR =3-hydroxy-3-methyl-glutaryl-CoA reductase.*

Systemic in vivo treatment of CKD rats with pravastatin does not reduce CKD progression

CKD rats developed hypertension, mild uremia, anemia and proteinuria (Figure 6). Systemic 6-weeks *in vivo* pravastatin treatment did not influence body weight, GFR, ERPF, FF or hematocrit (Table 7). Furthermore, no differences in blood counts (Table 6), FGS and tubulo-interstitial damage (Table 8), cardiac fibrosis (3.54±2.20 vs. 4.79±2.71%), or nitric oxide metabolite excretion (Figure 6) were observed between CKD and CKD+pravastatin rats. Cholesterol did not differ between CKD+pravastatin treated and CKD rats; however, triglycerides were significantly lower in CKD+pravastastin treated rats (Table 7). Systemic pravastatin decreased the number of monocytes and increased the stromal cell population in the bone marrow (Table 2) and increased hepatic HMGCR mRNA expression (Table 7).

Table 8. *Longitudinal, terminal and histological measurements after in vivo pravastatin treatment.*

	CKD (n=6)	*CKD+pravastatin (n=5)*
FGS (%)		
Normal	32.9±7.6	23.4±13.1
Partial	48.1±10.6	62.7±10.4
Total	19.0±6.7	14.0±15.8
Tubulo-interstitial damage		
Fibrosis	0.78±0.31	0.85±0.14
Inflammation	1.72±0.54	1.66±0.22
Atrophy	1.09±0.65	0.56±0.09

Mean ± SD.

Discussion

The present study demonstrates for the first time that BMC dysfunction in CKD can be reversed by short-term (2 hours) pretreatment with pravastatin outside the CKD environment and that this effect persists when the cells are returned to the CKD environment, providing augmented therapeutic efficacy *in vivo*.

Our recent studies have shown that a single injection of healthy BMCs in rats with established CKD slowed progression of the disease, probably via paracrine actions. Less disease progression was characterized by increased

glomerular capillary density and less sclerosis. Injection of BMCs derived from CKD rats was less effective[1] suggesting that CKD induces alterations in (paracrine) functions of BMC which reduce endothelial regenerative capacity and efficacy of CKD BMC therapy in rats. Previously, statins have been reported to exert beneficial effects on endothelial as well as on BM-derived endothelial progenitor cell (EPC) and mesenchymal stem cell (MSC) function both after *in vitro* incubation as after systemic *in vivo* treatment[5;7-9]. Here we show that short term *ex vivo* pretreatment with pravastatin reverses paracrine dysfunction in BMCs obtained from rats with established CKD resulting in preserved renal morphology in recipient rats with CKD.

The role of trans-differentiation and incorporation of BMCs in enhancing tissue regeneration has been questioned. BMCs appear to have a supportive function, secreting growth factors and cytokines, thereby stimulating resident cells to engage in regeneration[23]. Using a cytokine array we showed that short-term pravastatin pretreatment influences paracrine factor secretion by BMCs. CKD+Pravastatin BMCs significantly decreased expression of the pro-inflammatory chemokine CXCL5, which was shown to be involved in the recruitment and activation of polymorphonuclear neutrophils and in stimulation of local production of cytokines that have pro-apoptotic effects[24]. Our findings are in accordance with previous reports that atorvastatin dose-dependently inhibits basal CXCL5 production in human umbilical vein endothelial cells[25] and that simvastatin inhibited CXCL5 release from peripheral blood mononuclear cells[26]. In our experiments, renal influx of inflammatory cells was lowered in CKD+pravastatin BMC recipients versus CKD+DMEM BMC recipients. In glomeruli, there was a trend towards more ED-1$^+$ macrophages and CD3$^+$ T-cells in CKD+DMEM BMC recipients and the influx in CKD+pravastatin BMC recipients was lower and comparable with both healthy+DMEM BMC recipients and healthy+pravastatin BMC recipients suggesting an *in vivo* anti-inflammatory effect of *in vitro* pravastatin-pretreated BMCs. Increased paracrine function can explain why we did not observe an increase in the number of eGFP$^+$ cells in the remnant kidney. Our observations that few eGFP$^+$ BMCs were found in kidney sections of all recipients, and that those found were in close proximity to the microvasculature but did not differentiate into endothelial cells, are consistent with paracrine actions of BMCs as reported by others [29;30].

Impaired BMC function in CKD rats is consistent with clinical studies reporting impaired function of BM-derived EPCs obtained from CKD patients[31;32]. We previously showed that culturing healthy BM mononuclear cells in uremic serum caused reduced outgrowth of EPCs, suggesting that uremic serum contains either impairing toxins or lacks essential stimulants to support EPC

function[32]. Indeed, better *in vivo* removal of uremic toxins in CKD patients has been shown to improve EPC function[33;34]. However, culturing of CKD BMCs in non-uremic conditions *in vitro* could not reverse the impairment in outgrowth towards EPCs or EPC function[32;35]. In our study, two hours of incubation in DMEM outside of a CKD environment did not reverse BMC dysfunction. Importantly, our experiments show that 2h incubation with pravastatin improved rat CKD BMC function, which may have important clinical consequences if confirmed in human CKD. Induction of improvement in CKD BMC function by HMGCoA reductase inhibition within 2 hours is remarkable but seems consistent with previous reports showing that short-term statin incubation (< 10 minutes) induces a rapid elevation of NO production in endothelial cells[36] and rapid (<30 minutes) induction of Akt-mediated phosphorylation of endothelial nitric oxide synthase leading to nitric oxide production[37]. Filtration fraction was increased in CKD+DMEM BMC recipients vs. healthy BMC recipients ($p<0.05$), indicating a less well preserved glomerular structure. Mean arterial pressure was 20 mmHg lower in rats that received CKD+pravastatin CKD BMCs or healthy+DMEM BMCs compared to recipients that received CKD+DMEM BMC, and healthy+DMEM BMC, healthy+pravastatin BMC and CKD+pravastatin BMC recipients tended to have a higher natriuresis per 24h vs. CKD+DMEM BMC recipients. Blood pressure lowering of 20 mmHg has major renal and cardiovascular implications such as decreased risk of stroke[38], myocardial infarction, cardiac failure[39], peripheral arterial disease and an increased life expectancy[40]. Consistently, we observed significantly less glomerulosclerosis, tubular inflammation, atrophy and fibrosis in remnant kidneys of rats that received CKD+pravastatin BMCs compared to CKD+DMEM BMC recipients. Cardiac fibrosis tended to be increased in CKD+DMEM BMC recipients compared to CKD+pravastatin BMC recipients. *Ex vivo* pravastatin treatment did not further improve renal function or structure or decrease cardiac fibrosis in healthy+pravastatin BMC recipients compared to healthy+DMEM BMC recipients, whereas in CKD+pravastatin BMC recipients cardiac fibrosis was significantly decreased compared to CKD+DMEM BMC recipients, indicating that pravastatin specifically corrected CKD BMC function. We have not compared different statins in our experimental set-up, therefore our study does not allow conclusions as to whether this phenomenon is a specific effect of pravastatin or a generic statin effect.

Interestingly, six weeks of systemic *in vivo* treatment with pravastatin did not influence CKD progression or renal fibrosis in our model of established CKD. Although cholesterol-lowering effects of statins do not occur in rodents, pleiotropic effects have been reported[41] such as reduced inflammation and oxidative stress, enhanced endothelial function and increased mobilization

and function of endothelial progenitor cells. Some older studies showed beneficial effects of statins on renal function and morphology in experimental CKD, although others reported harmful effects such as induction of renal fibrosis[42][43]. Recently, Geng et al performed a meta-analysis on the effect of statins on renal function (eGFR and proteinuria) in patients with CKD. The analysis showed that the beneficial effect of statins on renal function may be dose- and time-dependent and that statins are well-tolerated in patients with KDOQi stages 1-3. However, the effect of statins on renal function in KDOQi stage 4 and 5 remains controversial[44]. The lack of effect of statin treatment in our *in vivo* study cannot be explained by insufficient dosing of pravastatin. Similar statin doses have previously been shown to increase EPC mobilization in mice[45]. Furthermore, we observed a significant decrease in triglycerides and increase in HMGCR mRNA expression after systemic pravastatin treatment which indicates that the dose was sufficient to affect the mevalonate pathway, and is in agreement with Zager et al[46].

Conclusions

Short-term pretreatment of CKD BMCs with pravastatin reversed CKD BMC dysfunction and improved their therapeutic efficacy *in vivo*. Our data suggest that this is due to an improvement in their paracrine profile. In contrast, systemic *in vivo* pravastatin treatment did not attenuate the progressive course of CKD. Our findings have relevance for potential clinical application of BMC therapy in patients with CKD as clinical application would involve autologous - and thus CKD - BMCs to avoid immunological reactions. If confirmed for human CKD BMCs, our findings will provide a basis for development of clinical trials and application of autologous BMC-based therapies in human CKD.

Acknowledgements

We thank Krista den Ouden, Adele Dijk, Nel Willekes, Maaike Meerlo and Paula Martens for their expert technical assistance.

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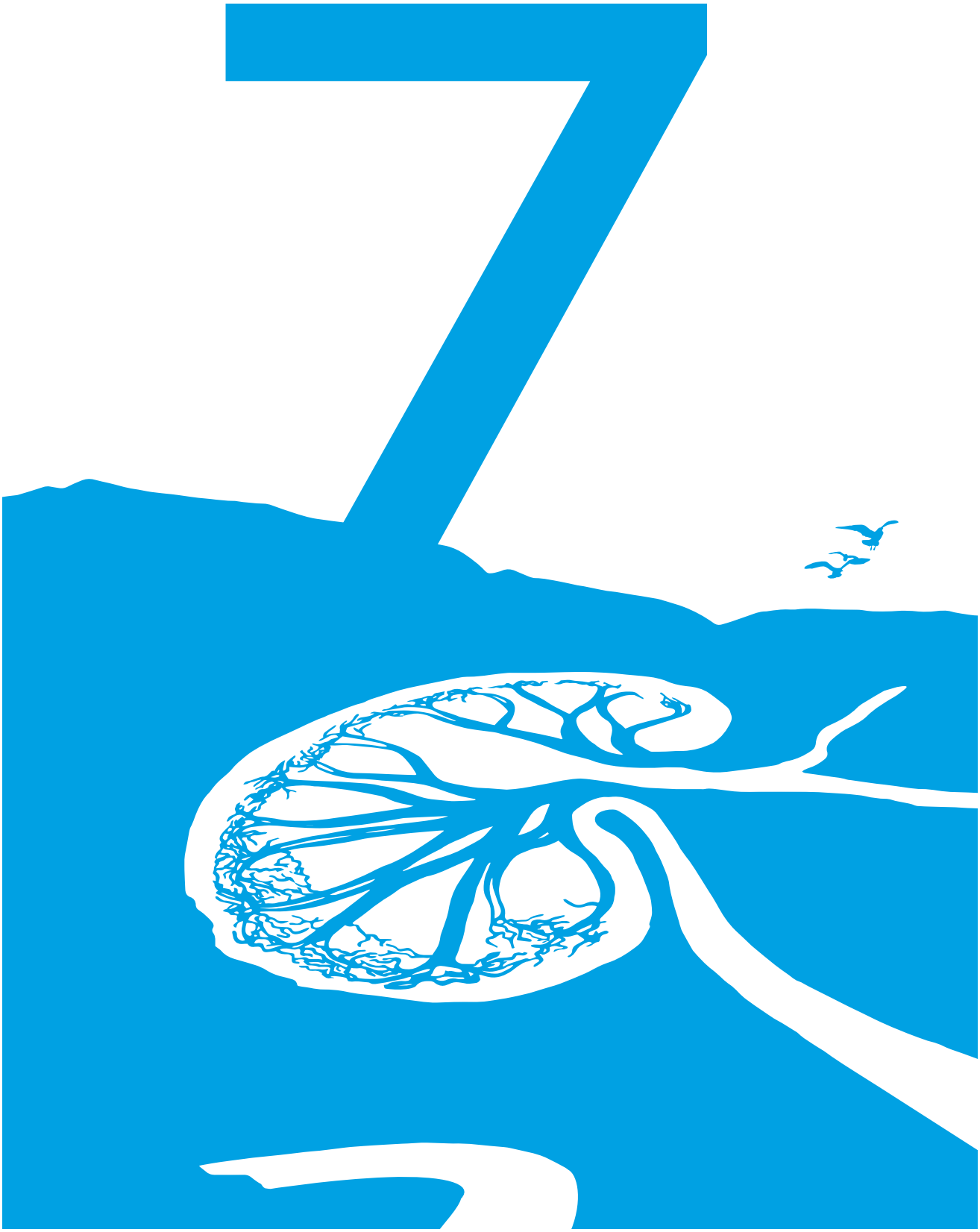

Renal transplantation induces mitochondrial uncoupling, increased kidney oxygen consumption, and decreased kidney oxygen tension

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Published in Am J Physiol Renal Physiol. 2015 Jan 1;308(1):F22-8.

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Abstract

Hypoxia is an acknowledged pathway to renal injury and ischemia-reperfusion (I/R) and is known to reduce renal oxygen tension (Po_2). We hypothesized that renal I/R increases oxidative damage and induces mitochondrial uncoupling, resulting in increased oxygen consumption and hence kidney hypoxia. Lewis rats underwent syngenic renal transplantation (TX) and contralateral nephrectomy. Controls were uninephrectomized (1K-CON) or left untreated (2K-CON). After 7 days, urinary excretion of protein and thiobarbituric acid-reactive substances were measured, and after 14 days glomerular filtration rate (GFR), renal blood flow, whole kidney Qo_2, cortical Po_2, kidney cortex mitochondrial uncoupling, renal oxidative damage, and tubulointerstitial injury were assessed. TX, compared with 1K-CON, resulted in mitochondrial uncoupling mediated via uncoupling protein-2 (16 ± 3.3 vs. 0.9 ± 0.4 pmol $O_2 \cdot s^{-1} \cdot mg\ protein^{-1}$, $P < 0.05$) and increased whole kidney Qo_2 (55 ± 16 vs. 33 ± 10 μmol O_2/min, $P < 0.05$). Corticomedullary Po_2 was lower in TX compared with 1K-CON (30 ± 13 vs. 47 ± 4 μM, $P < 0.05$) whereas no significant difference was observed between 2K-CON and 1K-CON rats. Proteinuria, oxidative damage, and the tubulointerstitial injury score were not significantly different in 1K-CON and TX. Treatment of donors for 5 days with mito-TEMPO reduced mitochondrial uncoupling but did not affect renal hemodynamics, Qo_2, Po_2, or injury. Collectively, our results demonstrate increased mitochondrial uncoupling as an early event after experimental renal transplantation associated with increased oxygen consumption and kidney hypoxia in the absence of increases in markers of damage.

Introduction

Kidney transplantation (TX) is the definitive treatment for end-stage renal disease. Even though most transplantations are successful, with a graft survival rate of 90% within 1 yr, delayed graft function (DGF) and interstitial fibrosis/tubular atrophy (IF/TA, previously denoted as chronic allograft nephropathy) [1] remain serious clinical problems [2-4]. Ischemia-reperfusion (I/R) is an inevitable event accompanying kidney TX and is considered a common cause for DGF and acute renal failure, ultimately resulting in IF/TA [5-8]. Mechanisms leading to DGF and IF/TA after renal TX are poorly understood, and, at present, we lack therapies to prevent I/R injury.

I/R is accompanied by an increased mitochondrial production of reactive oxygen species (ROS) [9], an event that is likely to induce mitochondrial uncoupling. Uncoupling proteins (UCPs) can be directly activated by superoxide radicals [10] to release protons independently of ATP production, thereby lowering the membrane potential and decreasing superoxide production. Indeed, mitochondrial uncoupling has been shown to occur in the kidneys of hypertensive and diabetic animal models [11-14].

In most tissues, mitochondrial uncoupling functions as an antioxidant mechanism. However, increased mitochondrial uncoupling is always accompanied by increased oxygen consumption to sustain ATP production, a side effect that is potentially detrimental for the kidney. Increased renal blood flow (RBF) does not correct the increase in oxygen consumption, as increased RBF will inevitably result in increased glomerular filtration rate (GFR) and therefore an increased tubular load of electrolytes destined for active reabsorption. Thus increased oxygen delivery is matched by increased demand. The inability of the kidney to compensate for increases in oxygen consumption renders it particularly sensitive to alterations in oxygen metabolism that result in decreased kidney oxygen tension (Po_2). In 1998, it was proposed by Fine et al.[15] that the kidney has a hypoxic threshold that, when overstepped, initiates mechanisms that cause nephropathy. Interestingly, a lower renal Po_2 is observed in rats with hypertension [16], diabetes [17], and after I/R [18], and chronic kidney hypoxia is now an acknowledged pathway to end-stage renal disease [19-21].

Increased mitochondrial fragmentation and production of ROS and oxidative damage have been shown to occur after I/R [9,22,23], but studies have yet to show the presence of kidney mitochondrial uncoupling or describe its connection to kidney oxygenation, function, and oxidative damage. We hypothesize that increased mitochondrial uncoupling and the associated increase in kidney oxygen consumption lead to kidney hypoxia early after experimental renal TX and that this increase precedes damage. Our aim was therefore to investigate mitochondrial function, in vivo kidney function, oxygen metabolism, and

markers of oxidative and structural damage in transplanted kidneys compared with one-kidney controls. In a follow-up study, we evaluated whether the scavenging of mitochondrial superoxide in the donor before the TX procedure could improve these variables.

Methods

Rats

Male inbred Lewis rats (LEW/Crl), 300–350 g, were purchased from Charles River and housed in a climate-controlled facility with a 12:12-h light-dark cycle under standard conditions. All rats had free access to standard rat chow and tap water. The study protocol was approved by the Utrecht University Committee on Animal Experiments, conformed to Dutch Law on Laboratory Animal Experiments (DEC number 2010.II.05.097) and the Uppsala Animal Ethics Committee [Ethics Statement (c143/12)], and was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

All chemicals were from Sigma-Aldrich (St. Louis. MO) unless otherwise stated.

Study 1

Age-matched rats of 8–10 wk were used as 2-kidney controls (2K-CON; n = 16), uninephrectomized rats as 1-kidney controls (1K-CON; n = 13), or underwent syngenic renal TX (TX; n = 12). Seven days after surgery, rats were placed in metabolic cages to collect 24-h urine for evaluation of excretion of protein (Bio-Rad Protein assay, Bradford, Bio-Rad, Hercules, CA) and thiobarbituric acid-reactive substances (TBARS; TBARS assay kit, Cayman Chemical, Ann Arbor, MI). Fourteen days after surgery, mitochondrial uncoupling and renal function, including arterial, venous, and direct tissue Po_2, were measured in separate cohorts. Directly after measurements of renal function, rats were euthanized and renal tissue was fixed in 4% paraformaldehyde for embedding in paraffin, or snap-frozen to measure protein carbonyl content in the kidney cortex. Protein carbonyls were determined with a kit (Cayman Chemical).

Study 2

Donors were treated intravenously with mito-TEMPO, a mitochondrial-targeted antioxidant, a specific scavenger of mitochondrial superoxide (200 μg/kg, TX-T, n = 11; Enzo Life Sciences, Farmingdale, NY) or vehicle (0.9% NaCl, TX-V, n = 10) daily for 5 days before TX. mito-TEMPO (100 μM) was added to an

organ-preserving Viaspan solution (Bristol-Myers Squibb) during cold storage of the kidneys from the TX-T group. All measurements of oxidative damage, mitochondrial uncoupling, and renal function and injury were determined as for *study 1*.

Transplantation Procedure

Renal TX was performed as described [24]. Briefly, via laparotomy, the left kidney of the donor rat was flushed with 0.9% NaCl followed by a flush with the Viaspan solution. Afterward, the donor kidney was kept in Viaspan on ice for 30 min (cold ischemia time). TX was performed with end-to-end anastomoses of vessels and the ureter, with 30- to 40-min warm ischemia time and subsequent removal of the contralateral kidney. Thus survival of the recipient was dependent on the function of the renal graft.

Mitochondrial Uncoupling

Mitochondria were isolated from kidney cortex as described [14]. Oxygen consumption in isolated mitochondria was measured with an Oroboros O2K (Oroboros Instruments, Innsbruck, Austria) with 10 mM glutamate (state 4) and 300 μM ADP (state 3), and the respiratory control ratio (RCR) was determined as state 3/state 4 respiration. Only mitochondria with an RCR >4 were used for experiments [25]. Mitochondrial uncoupling was evaluated in isolated mitochondria in the presence of glutamate (donates electrons to the electron transport chain), oligomycin (12 μg/mg protein; an ATP synthesis inhibitor), and sodium palmitate (48 μM; a fatty acid to enable the fatty acid cycling mechanism of UCP). This level of oxygen consumption is denoted as state 4 respiration in Table 3. The presence of an uncoupling mechanism, i.e., increased oxygen consumption that is not related to ATP production, will be evident as an increased state 4 respiration. If an increased state 4 respiration is mediated through UCPs, the sequential addition of the UCP-specific inhibitor GDP (500 μM) will inhibit oxygen consumption. The presence of mitochondrial uncoupling is calculated and presented as the decrease in oxygen consumption that occurs after UCP blockade. If no mitochondrial uncoupling mechanism is present, oxygen consumption will not be affected by GDP and the value presented will be close to zero. Mitochondrial membrane potential was determined by fluorescence of tetramethylrhodamine methyl ester (TMRM) as described[25]. Before the isolation process, some kidney cortical tissue was snap-frozen for analysis of protein carbonyls. All these measurements were corrected for protein concentration.

In Vivo Renal Function

Renal function was investigated under isoflurane anesthesia (Abbott Laboratories, Hoofddorp, The Netherlands) as described [26]. Renal function data are shown per kidney, and one-kidney function in the 2K-CON group was calculated as 50% of total renal function. At the end of the experimental period, blood-gas analysis was performed on arterial blood and on a sample obtained from the renal vein. Sampling from the renal vein was performed slowly to prevent backflow of blood from the vena cava. Blood oxygen content (O_2ct) was calculated as O_2ct = [hemoglobin] * oxygen saturation * 1.34 + blood Po_2 * 0.003, and kidney oxygen consumption was calculated as Qo_2 = (O_2ct artery – O_2ct renal vein) * total RBF. Carbon paste electrodes placed at 3-mm depth using a micromanipulator were used to determine Po_2 at the corticomedullary border (mean of at least 3 samplings/rat) [27]. In a pilot experiment, after measurement of kidney Po_2 with a carbon paste electrode, the kidneys were cut through the plane where the electrode was inserted. With a ruler we confirmed that this depth indeed corresponds to the corticomedullary border, as shown previously [28]. Plasma urea and plasma creatinine were determined by DiaSys Urea CT FS (DiaSysDiagnostic Systems, Holzheim, Germany). Sodium and potassium were measured by flame photometry. Fractional excretions of sodium and potassium (FE_{Na} and FE_K) were calculated using standard formulas. Tubulointerstitial injury (TI) was scored on periodic acid Schiff (PAS)-stained paraffin-embedded slides [29].

Statistics

Data were analyzed with one-way ANOVA with Dunnett's test as a post hoc test using 1K-CON for comparison with both 2K-CON and TX (*study 1*) or an unpaired Student's *t*-test for comparison of TX-T and TX-V (*study 2*). $P < 0.05$ was considered statistically significant and was two-tailed. All values are expressed as means ± SD.

Results

Kidney TX Led to Mitochondrial Uncoupling, Increased Oxygen Consumption, and Decreased Total Kidney Oxygenation (Study 1)

Seven days after surgery, excretion of TBARS and protein in urine was greater in 1K-CON rats compared with 2K-CON rats (Fig. 1A and Table 1). At 14 days after surgery, protein carbonyls from kidney cortex were increased in 1K-CON vs. 2K-CON (Fig. 1B) rats. TBARS and protein carbonyls were not significantly different in TX vs. 1K-CON groups. Mitochondria isolated from kidneys of TX rats displayed higher mitochondrial uncoupling via UCP-2

vs. kidneys of 1K-CON rats (Fig. 1D), which was associated with an elevated membrane potential after UCP-2 blockade by GDP (Fig. 1C). No significant differences were found in RCR in isolated mitochondria (Table 2). In TX and 1K-CON rats, no significant difference was found in urea, creatinine, GFR, RPF, and RBF. Urea, hematocrit, GFR/kidney, RPF/kidney, and RBF/kidney were lower in 2K-CON compared with 1K-CON rats. No significant differences were found in MAP (Table 1). Kidney oxygen consumption was higher and kidney Po_2 was lower in TX compared with 1K-CON rats (Fig. 1, E and *F*).

Table 1. *Clinical signs, renal function, and histology in age-matched healthy controls (2K-CON) and 2 wk after uninephrectomy (1K-CON) or renal transplantation (TX).*

age-matched healthy controls			
	2K-CON, N=10	1K-CON, N=7	TX, N=6
Body weight (BW), g	398 ± 42	346 ± 35	367 ± 25
Total renal mass (µg/100g BW)	701 ± 44	509 ± 51 **	545 ± 55 **
Plasma urea (mmol/L)	7.7 ± 1.2	9.5 ± 1.5 *	9.6 1.6 *
Plasma creatinine (µmol/L)	27.2 ± 6.3	37.5 ± 5.5	37.7 ± 3.8 *
Hematocrit (%)	43.7 ± 1.2	40.0 ± 1.4 *	38.2 ± 1.5 **
Mean arterial pressure (MAP, mmHg)	96 ± 6	97 ± 10	99 ± 11
Glomerular filtration rate (per kidney, ml/min/100g)	0.47 ± 0.05	0.61 ± 0.09 **	0.65 ± 0.07 **
Renal plasma flow (per kidney, ml/min/100g)	1.82 ± 0.22	2.45 ± 0.44 **	2.58 ± 0.23 **
Calculated renal blood flow (per kidney, ml/min/100g)	2.9 ± 0.3	3.9 ± 0.7 **	3.9 ± 0.4 **
Fractional excretion Na (%)	0.30 ± 0.20	0.17 ± 0.08	0.28 ± 0.18
Fractional excretion K (%)	37.5 ± 6.3	44.1 ± 5.7	37.9 ± 6.8
	2K-CON, N=13	1K-CON, N=13	TX, N=13
Proteinuria at day 7 (mg/d/100g)	1.0 ± 0.3	2.4 ± 0.5 **	2.1 ± 1.5 *
Tubulo-interstitial injury score	1.8 ± 0.9	2.6 ± 0.8 *	2.7 ± 0.8 *

*Values are means ± SD. *P <0.05, **P <0.01 vs. 1K-CON (1-way ANOVA, Dunnett's post hoc test).*

In comparison to 1K-CON, 2K-CON rats displayed less tubulointerstitial injury (Table 1). Tubulointerstitial injury was not significantly different in TX vs. 1K-CON rats.

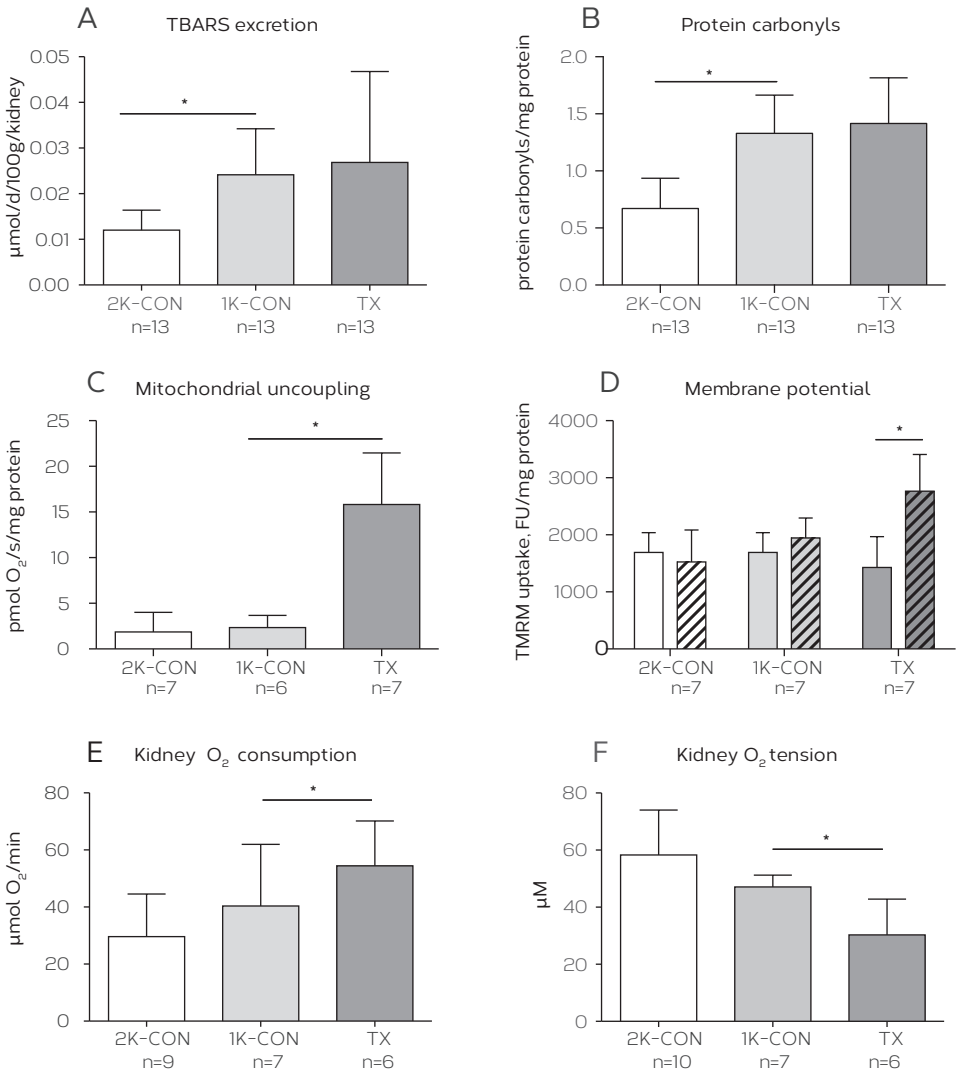

Figure 1. *Excretion of thiobarbituric acid-reactive substances (TBARS) in urine at day 7 (A), protein carbonyl content in the kidney cortex (B), mitochondrial uncoupling (C), mitochondrial membrane potential (D), kidney oxygen consumption (E), and kidney oxygen tension (F). B–F: 14 days after uninephrectomy (1K-CON) or transplantation (TX); 2K-CON are age matched. Values are means ± SD. *P < 0.05 vs. 1K-CON (1-way ANOVA, Dunnett's post hoc test) for panels A-C, E, and F; *P < 0.05 vs. baseline (unpaired Student's t-test) for D.*

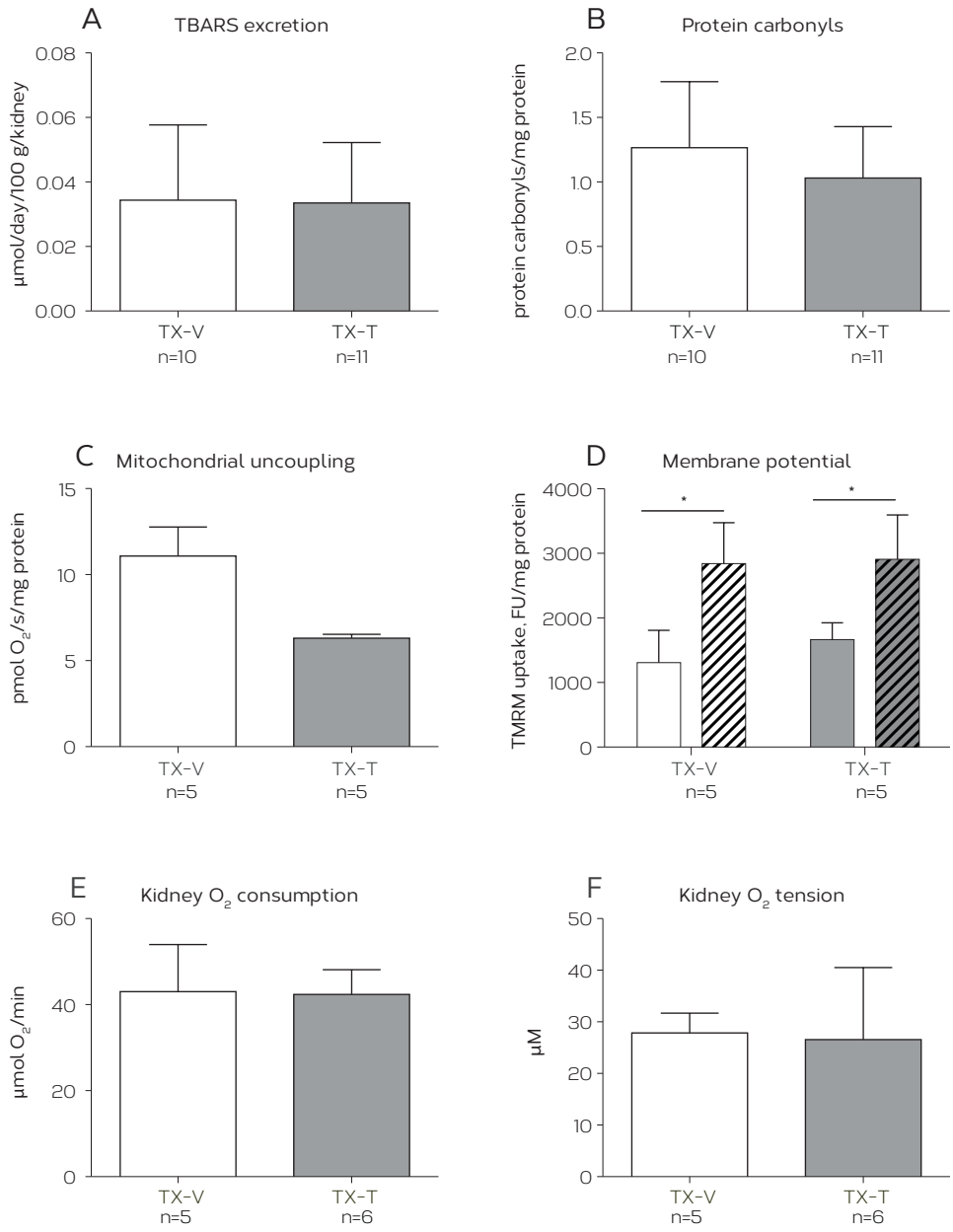

Figure 2. *Excretion of TBARS in urine at day 7 (A), protein carbonyl content in the kidney cortex (B), mitochondrial uncoupling (C), mitochondrial membrane potential (D), kidney oxygen consumption (E), and kidney oxygen tension (F) in vehicle (TX-V)- and mito-TEMPO (TX-T)-treated rats that underwent kidney TX. B-F: 14 days after TX. Values are means ± SD. *P < 0.01 vs. TX-V (A-C, E, and F) and vs. baseline (D); unpaired Student's t-test.*

Table 2. *Mitochondrial oxygen consumption during state 4 and state 3 respiration and the calculated respiratory control ratio (RCR).*

	Study 1			Study 2	
	CON, N=7	UNX, N=6	TX, N=7	TX-V, N=5	TX-T, N=5
State 4 (pmol O2/s/mg protein)	32 ± 6	32 ± 6	42 ± 8*	41 ± 10	39 ± 8
State 3 (pmol O2/s/mg protein)	194 ± 89	167 ± 48	170 ± 80	198 ± 47	193 ± 14
RCR	5.9 ± 1.8	5.3 ± 1.0	4.8 ± 1.5	4.8 ± 0.3	4.9 ± 0.3

*Values are means ± SD. *P <0.05 vs. 1K-CON (1-way ANOVA, Dunnett's post hoc test).*

Table 3. *Clinical signs, renal function, and histology*

	TX-V, N=6	TX-T, N=5
Body weight (BW), g	341 ± 19	325 ± 12
Total renal mass (µg/100g BW)	564 ± 53	584 ± 71
Plasma urea (mmol/L)	9.8 ± 0.5	9.9 ± 1.7
Plasma creatinine (µmol/L)	33.9 ± 11.6	33.6 ± 3.3
Hematocrit (%)	40 ± 2	41 ± 1
Mean arterial pressure (MAP. mmHg)	88 ± 7	96 ± 9
Glomerular filtration rate (per kidney. ml/min/100g)	0.62 ± 0.04	0.65 ± 0.06
Renal plasma flow (per kidney. ml/min/100g)	0.22 ± 0.02	0.23 ± 0.02
Calculated renal blood flow (per kidney. ml/min/100g)	12.1 ± 1.7	12.6 ±0.9
Fractional excretion Na (%)	0.11 ± 0.09	0.15 ± 0.13
Fractional excretion K (%)	45.8 ± 7.3	40.1 ± 8.3
	TX-V, N=11	TX-T, N=10
Proteinuria at day 7 (mg/d/100g)	2.3 ± 0.9	2.6 ± 1.1
Tubulo-interstitial injury score	3.4 ± 1.4	3.2 ± 0.8

2 wk after transplantation with daily vehicle (TX-V) or mito-TEMPO (TX-T) donor treatment for 5 days before donation for transplantation.
Values are means ± SD.

Donor Pretreatment with Mito-TEMPO Decreased Mitochondrial Uncoupling But Did Not Affect Oxygen Consumption or Total Oxygenation (Study 2)

Pretreatment of donor rats with mito-TEMPO and addition of mito-TEMPO to the organ-preserving solution did not affect excretion of TBARS in urine at *day 7 (*Fig. 2A) or protein carbonyls in tissue at *day* 14 (Fig. 2B). The degree of mitochondrial uncoupling via UCP-2 was lower compared with mitochondria isolated from vehicle-treated rats (Fig. 2C), and mitochondrial membrane potential after UCP-2 blockade by GDP was higher compared with baseline in both vehicle- and mito-TEMPO-treated rats (Fig. 2D), but no significant differences were observed in kidney oxygen consumption (Fig. 2E) or kidney Po_2 (Fig. 2F). RCR of isolated mitochondria was not affected (Table 2). Parameters of renal function and injury were not affected (Table 3).

Discussion

The present study shows that experimental renal TX induces mitochondrial uncoupling mediated via UCP-2. Furthermore, renal TX was accompanied by increased kidney oxygen consumption and decreased kidney Po_2. Interestingly, in other models of animals without underlying disease but with increased oxygen consumption due to mitochondrial uncoupling (from either pharmacological stimuli or hormonal stimuli), increased kidney oxygen consumption and decreased kidney Po_2 were also observed [30,31]. Kidney hypoxia is now an acknowledged pathway in the development of nephropathy [19]. However, in the present study we found no effect of TX on markers of oxidative damage or injury in the TX kidney compared with relevant 1K-CON, suggesting that the changes in renal oxygen handling induced by TX in the present study precede renal injury. Therefore, we speculated that mitochondrial uncoupling as an early event after renal TX may be a mechanism contributing to renal graft injury and DGF. However, it should be noted that the present study does not allow for definitive conclusions on causality.

Using blood oxygen level-dependent (BOLD)-MRI, several groups have displayed increased kidney oxygenation in renal allografts. No difference was observed in patients with normal allograft function compared with controls [32], while decreased deoxyhemoglobin levels that correspond to increased oxygenation were reported in patients with IF/TA [33]. This effect could be caused by decreased oxygen consumption that is observed in patients after inflammatory and immune-mediated kidney damage [34]. Impaired renal function with decreased GFR would also reduce the workload of electrolyte transport for the kidney and therefore contribute to a higher Po_2 in these patients. Interestingly, the BOLD-MRI-studies [34] contradict the observa-

tions of the present study of slightly decreased renal oxygenation after TX. However, these studies were performed once IF/TA was established. Importantly, the present study measured renal oxygenation 14 days after TX and will therefore reflect the early effects of renal TX that are likely to contribute to a later loss of kidney function and reduced workload, ultimately resulting in increased oxygenation. In addition, to avoid the confounding effects of immunosuppression we used a syngenic model of TX to specifically study the effect of I/R after TX. To our knowledge, we are the first to study the mechanism of altered oxygen handling as an early event after renal TX.

There are also other mechanisms besides mitochondrial uncoupling that could have influenced kidney Po_2 in the 1K-CON and TX groups in the present study. Kidney Po_2 represents the balance between oxygen delivery (represented by RBF) and oxygen consumption, but it can also be influenced by the arterial-to-venous (A-V) oxygen shunting in the renal cortex and medulla [35]. Studies have suggested that A-V shunting increases with increased RBF, and, indeed, increased RBF was observed after TX. However, RBF was similarly increased in 1K-CON, making increased shunting due to increased RBF an unlikely cause of decreased kidney Po_2 after TX. Moreover, there were no clear differences in gross morphology (evaluated as TI injury) between these two groups.

Studies have demonstrated that UCPs can directly be activated by superoxide radicals [10,36], and oxidative damage is known to induce mitochondrial uncoupling in the kidneys of diabetic animals [14]. As mitochondrial production of ROS is also increased after I/R [9], we hypothesized that mitochondrial uncoupling occurs after experimental renal TX. Indeed, we found an increase in mitochondrial uncoupling after experimental renal TX that was associated with increased levels of oxidative damage. The level of mitochondrial uncoupling that we observed was dependent on GDP, an inhibitor of UCPs. As UCP-2 is the only isoform of UCP present in the kidney [37], it can be assumed that the observed mitochondrial uncoupling was mediated via UCP-2. Interestingly, mitochondrial membrane potential was not different between groups without any incubation but was increased after UCP blockade in kidneys from TX rats. Thus in our TX model mitochondrial uncoupling does not decrease membrane potential below normal levels but only uncouples the membrane potential back to control levels, maintaining mitochondrial production of superoxide at physiological levels. In concordance with this, we observed no signs of oxidative damage at 7 and 14 days after TX vs. 1K-CON.

Studies have demonstrated a connection between oxidative stress and mitochondrial uncoupling [14,17], and it was therefore interesting to note that treating the donors with the mitochondrial-targeted antioxidant mito-TEMPO

resulted in reduced mitochondrial uncoupling compared with vehicle-treated TX rats. However, mito-TEMPO had no effects on renal function and oxygen handling. Mitochondria-targeted antioxidants are antioxidant molecules connected to tetraphenylphosphonium, a lipophilic cation that accumulates in the mitochondria due to the negative membrane potential. These molecules have been shown to be effective in the treatment of various pathologies such as acute pyelonephritis [23], hypertension [12], and cardiac I/R injury [38]. In the present approach, in which only donors are treated, it is evident that the degree of mitochondrial superoxide scavenging is not sufficient to completely prevent mitochondrial uncoupling. mito-TEMPO did reduce the level of mitochondrial uncoupling. However, levels of oxidative damage markers were not different between vehicle- and mito-TEMPO-treated TX rats. The failure of mito-TEMPO to scavenge all ROS could occur because the level of oxidative stress may continue to increase for some time after TX. Indeed, even the remaining degree of mitochondrial uncoupling was greater than what was previously observed in kidneys from untreated diabetic animals [13,14,25], and it is possible that the increased degree of mitochondrial uncoupling reflects the severity of insult and subsequent levels of oxidative stress. It is likely that for decreased mitochondrial uncoupling to be protective it must be completely prevented, as demonstrated in diabetic kidneys [14]. The concept of preventing mitochondrial uncoupling after experimental TX using antioxidants is an interesting approach for future studies. Although mitochondrial uncoupling was reduced after mito-TEMPO, the mitochondrial membrane potential was similar in mitochondria from both groups after UCP inhibition. Importantly, there is not a linear relationship between uncoupling and membrane potential change in mitochondria [39]. Thus the relatively small effect of mito-TEMPO on mitochondrial uncoupling may not be associated with a measurable change in mitochondrial membrane potential.

Kidney Po_2 was decreased after TX together with increased oxygen consumption. Although no cause-effect relationship can be established in this study, it is important to note that previous studies have shown that increased kidney oxygen consumption indeed results in kidney hypoxia and development of nephropathy [31,40]. Decreased kidney Po_2 has been observed in rat models of hypertension [16,41], diabetes [17,42,43], polycystic kidney disease [44], and after I/R injury [9,25]. Furthermore, decreased renal oxygenation was also observed with BOLD-MRI in patients with diabetes and chronic kidney disease [45]. Other human studies also support a role for kidney hypoxia in the development of kidney damage. Diabetic patients living at 1,700 m above sea levels have increased prevalence of diabetic nephropathy compared with similar patients living at sea level, but these to two groups were not different in terms

of mean arterial pressure, glycemia, or lipidemia status or prevalence of retinopathy [46]. Furthermore, an observational study correlated the degree of nocturnal hypoxemia with accelerated decline in GFR [47]. In summary, kidney hypoxia is now regarded as an independent causal pathway in the development of nephropathy [10-12,48-50]. The present study did not reveal a connection between hypoxia and development of nephropathy, possibly due to the early time point of analysis. We speculate that decreased Po_2 soon after experimental renal TX, as observed in the present study, could play a long-term role in DGF and the development of IF/TA.

Conclusion and Future Perspectives

This is the first study to demonstrate UCP-2-mediated mitochondrial uncoupling accompanied by increased kidney oxygen consumption and decreased kidney Po_2 as an early event after experimental renal TX. These early events may contribute to development of DGF and IF/TA after kidney TX.

Future studies should focus on investigating the role of UCP-2 mediated mitochondrial uncoupling and kidney hypoxia in the long-term outcome of experimental renal TX, the putative role of oxidative stress in inducing mitochondrial uncoupling, and how the damaging effects of mitochondrial uncoupling can be prevented.

Acknowledgements

We thank Adele Dijk and Krista den Ouden for expert technical assistance and Tim Klasson for carefully proofreading the manuscript.

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Environment and graft interaction after experimental kidney transplantation

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Abstract

At transplantation (TX) into a CKD recipient, the donor's endothelium is exposed to uremic and oxidative stress that may negatively influence graft function and structure. We hypothesized that in CKD milieu, graft endothelial damage is determined by both donor quality and exposition to uremic and oxidative stress. We also investigated whether healthy environment halts progressive endothelial loss in CKD graft.

Male inbred Lewis rats were used as donors and recipients. CKD developed in 24 rats after bilateral ablation of 2/3 of kidney mass. Control rats (n=24) were age-matched. Orthotopic TX was performed: healthy kidney to healthy rat (HD-HR); CKD kidney to healthy rat (CD-HR); healthy kidney to CKD rat (HD-CR); CKD kidney to CKD rat (CD-CR). Right donor kidney served as reference for left kidney (graft) injury at TX. Contralateral kidney of recipient was removed 10-14 days after TX. At wk 6, we evaluated graft function and morphology, and systemic oxidative (TBARS) and vascular damage (aorta calcification).

Graft function (inulin & PAH clearance) at wk 6 postTX confirmed marked impairment after ablation and was not affected by environment (CD-HR vs. CD-CR and HD-CR vs. HD-HR, all NS). TX of healthy vs. CKD graft did not influence oxidative and vascular damage at wk 6 postTX (CD-HR vs. HD-HR and HD-CR vs. CD-CR, both NS). Grafts from healthy donors developed more glomerulosclerosis (GS) and tubulointerstitial injury (TI) and a reduction in glomerular and interstitial endothelium (JG12 stain) compared to reference kidneys after TX in a CKD donor (all $P<0.05$). However, despite similar ischemia-reperfusion, TI and GS did not worsen in CKD grafts and TX of CKD grafts in healthy recipients did preserve glomerular and interstitial endothelium.

TX to CKD environment was less detrimental for CKD graft as shown by preserved endothelium compared to healthy graft, possibly due to preconditioning. In the reverse model, healthy environment halted progression of endothelial damage in CKD grafts.

Introduction

Although graft function and survival in kidney transplant patients have improved in the last years, graft function still deteriorates due to time-dependent immunologic and non-immunologic causes[1,2]. Recipient environment impacts long-term graft function and structure[3]. Isogenic experimental transplantation allows dissection of donor's and recipient's characteristics that could influence long-term graft function and structure.

Kidney transplantation is known to be the best treatment option for patients with end stage renal disease. The growing difference between decreasing supply of, and increasing need for, donors led to introducing marginal living donors as a strategy to provide more kidneys for transplantation. Organ shortage demands fundamental research on marginal living donors including accurate predictors of function and injury prior to experimental transplantation. However, most of the understanding in the pathophysiology in kidney transplantation comes from single insult animal models, which fail to reproduce the complexity of factors involved in the human situation, notably, the influence of the CKD environment and the graft on each other. The model we introduced[4] can be used in transplantation studies to develop strategies to improve graft survival after transplantation of marginal living donors. Furthermore, such a symmetrical ablation model, without uninephrectomy, will allow assessment of graft function and injury at the time-point of experimental transplantation by using one kidney as donor and the other one as a biopsy.

The quality of the donor kidney (age, hypertension, decreased GFR) is an important determinant of kidney transplant outcome [3]. Besides the condition of the donor kidney, general conditions (age, comorbidities) in the recipient are important for graft function and morphology[3]. Pre-emptive transplantation (before dialysis initiation) has been shown to confer benefit due to less dialysis-associated comorbidities, being associated with an allograft survival advantage[5].

Chronic kidney disease (CKD) is characterized by oxidative stress, endothelial dysfunction and uremia [6,7]. All these factors contribute to the progression of CKD and, at the moment of transplantation, could also be detrimental for the graft. Although endothelial function improves after successful renal transplantation, renal transplant recipients still have worse endothelial function in comparison to the healthy population [8]. Immunosuppressant drugs undoubtedly also contribute to this[9]. Dissecting the complex crosstalk between graft and recipient in a syngeneic model without immunosuppressant drugs allow analysis of factors contributing to graft endothelial integrity such as recipient-derived cells. Previous studies suggest incorporation of bone-marrow derived endothelial progenitor cells in renal grafts, but the data

is conflicting[10]. Availability of the green fluorescent protein (GFP)-positive Lewis rat[11,12] facilitates such analysis in experimental renal transplantation. We hypothesized that when the healthy graft is transplanted to a CKD environment, its endothelium is exposed to CKD (uremic) conditions and pre-existent oxidative stress, which could negatively influence endothelial regeneration, and the graft's structure and function in the long-term. However, due to chronic "preconditioning", the CKD graft might suffer less in comparison to the healthy graft when transplanted in CKD environment. As proof of concept, we further investigated whether the healthy environment could enhance endothelial regeneration and be beneficial for a chronically diseased kidney graft.

Materials and Methods

Animals

The study protocol was approved by the Utrecht University Committee on Animal Experiments (DEC number 2012.II.03.053), and conformed to Dutch Law on Laboratory Animal Experiments. Male inbred Lewis rats (Charles River, Germany) were used as donors (n=24) and green fluorescent protein (GFP) positive Lewis males (n=24; GG2861Uex rats, own breeding colony) as recipients. Rats were housed in a climate-controlled facility with a 12:12-hour light: dark cycle under standard conditions.

Experimental design and groups

The following groups (N=6/group) were used: HD-HR: healthy donor and healthy recipient; CD-HR: CKD donor and healthy recipient; HD-CR: healthy donor and CKD recipient; CD-CR: CKD donor and CKD recipient.

Models of CKD in rats

To develop established CKD in this strain, rats underwent 2/3 bilateral ablation (BA) of renal mass by coagulating branches of both renal arteries[4]. Subsequently, starting one week after surgery, development of CKD was accelerated with N(omega)-nitro-L-arginine (L-NNA), a NO-synthase inhibitor (200 mg/L) in drinking water[11] and animals were fed standard powdered chow (CRM (E) FG; Special Diet Services Ltd., Witham, Essex, UK),with phosphate content of 0.63% and NaCl content of 0.74%, supplemented with 6% NaCl. After reaching proteinuria of 200 mg/d (6-9 weeks), L-NNA was withdrawn and salt supplement was removed from the chow 2 weeks later. As described by Kang et al.[13], this resulted an immediate fall in systolic blood pressure and proteinuria, that both subsequently increased slowly (data not shown). CKD animals were included into transplantation protocol when proteinuria levels reached

a median of 111 mg/d [range 52 - 164] (without L-NNA and without salt) at a median age of 35 weeks [range 22-56]. At this stage, besides proteinuria, ablated rats show hypertension, high urea and a decrease in RBF, measured directly with Transonic flow probe[16]. Healthy rats with intact kidneys were used as controls at a median age of 25 weeks [range 20-42].

In a preparatory study (n=21) we established that in this bilateral model of CKD, injury is symmetrical: damage in the left kidney corresponds to damage in the right kidney (as quantified by both glomerulosclerosis (GS) and tubulointerstitial injury (TI) using correlation and Bland-Altman analyses. We therefore used injury in the right kidney as representative for injury in the left kidney (the isograft) at the moment of transplantation. As in this separate study proteinuria proved to be the best predictor for kidney damage all BA rats were screened and, where appropriate, matched with recipients on the basis of proteinuria before entering the transplantation protocol.

Kidney transplantation

We used orthotopic left kidney transplantation as described[14], with subsequent removal of the right native kidney 10-14 days after transplantation. Cold ischemia- and warm ischemia- times were 30 mins and all isografts were perfused and placed in organ preserving solution Viaspan (Bristol Meyers Squibb, Hoofddorp, Netherlands) prior to transplantation. Detailed description is provided in the supplement. The right kidney of the donor rat was preserved in formaldehyde and then embedded in paraffin to evaluate pre-transplantation histology.

Longitudinal measurements

We performed tail-cuff systolic blood pressure (SBP) registration and collected 24-h urine samples, for determination of protein excretion (Bio-Rad Protein assay: Bradford, Bio-Rad Laboratories, Hercules, CA, USA) with the rats in individual metabolic cages while fasting, as described [15] , at weeks 3 and 5 after transplantation. Blood samples were collected from the tail vein at the same time-points for determination of plasma urea (DiaSys Urea CT FS, DiaSys Diagnostic Systems, Holzheim, Germany).

Terminal protocol

Terminal measurements were performed six weeks after transplantation. Renal function was investigated under isoflurane anesthesia (Abbott Laboratories, Hoofddorp, Netherlands) as described[16]. We measured mean arterial pressure (MAP), glomerular filtration rate (GFR: inulin clearance), renal plasma flow (RPF:

PAH clearance), and excretion of sodium and potassium. Sodium and potassium were measured by flame photometry. Fractional excretions of sodium and potassium (FE_{Na} and FE_{K}) were calculated using standard formulae. At the end of the terminal protocol, rats were sacrificed by exsanguination via the aorta, perfused with 0.9% NaCl via the aorta at a perfusion pressure 10 mmHg above terminal MAP, and tissues were collected. Organs weights were noted. Furthermore, kidney samples were fixed in 4% paraformaldehyde for embedding in paraffin or snap-frozen for histological and immunohistochemical evaluation.

Renal morphology and immunohistochemistry

TI injury and GS were scored on periodic acid Schiff (PAS)-stained paraffin-embedded slides[17]. Peritubular and glomerular endothelial cells were stained with JG12 (mouse anti-JG12, BMS1104, 1:200, Bender Medsystems GmbH, Vienna, Austria). The endothelial (JG12+) area in at least 10 tubular fields and 50 glomeruli per kidney was determined using Adobe Photoshop CS5 Extended, version 12.0 x 32 (Adobe Systems; San Jose, CA). JG12+ area was corrected for glomerular area. ED-1 positive cells (monocyte/macrophage marker; mouse anti-rat CD68, ab31630, 1:250, Abcam, Cambridge, UK.) were counted in glomeruli and tubular fields. TI score, GS, glomerular area, JG12 staining and counting of ED1+ cells were performed in both donor (pre-transplantation) and recipient (post-transplantation). Anti-GFP (rabbit anti-GFP, Ab6556, 1:200, Abcam, Cambridge, UK) staining was performed on 5 μm snap-frozen sections in the recipients graft kidney, post-transplantation. GFP+ cells were counted in glomeruli, whereas in tubular fields we counted GFP+ pixels. All analyses were performed with ImageJ software, version1.46r (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD). Proliferative cells (Ki67 staining, rabbit anti-Ki67, RM-9106, 1:100, Fisher Scientific, Waltham, MA) were counted in glomeruli of the recipients graft kidney (post-transplantation).

Oxidative and vascular damage

Thiobarbituric acid reactive substances (TBARS assay kit, Cayman Chemical, Ann Arbor, MI, USA) excretion was measured in urine collected prior to termination as described[4,14,18].Thoracic aortas from all rats were collected at termination and subsequently snap frozen. We performed von Kossa staining to detect abnormal calcium deposits [19,20].

Statistics

Data are presented as means ± SD. T-test, two-way ANOVA or two-way RM ANOVA and Newman-Keuls post-hoc test, were used when appropriate.

Results

Pre-transplantation data in donors and recipients

At week -1 (baseline) before donation/transplantation, all rats (n=24) that underwent BA on average had higher SBP (136 ± 21 vs. 110 ± 15 mmHg, P<0.001), proteinuria (111 ± 31 vs. 10 ± 3 mg/d, P<0.001) and plasma urea (9.9 1.8 vs. 5.8 ± 0.8 mmol/L, P<0.001) than all control rats (n=24). Pre-transplant data for all four donor and recipient combinations are shown in Table 1.

Table 1. *Development of CKD.*

	Donors (N=24; 6/combination)			Recipients (N=24; 6/combination)		
	SBP (mmHg)	Proteinuria (mg/d)	Urea (mmol/L)	SBP (mmHg)	Proteinuria (mg/d)	Urea (mmol/L)
HD-HR	106±10	7±2	6.1±0.4	116±17	8±1	5.8±0.8
CD-HR	144±21	84±24	10.5±1.9	113±14*	13±2*	5.3±1.0*
HD-CR	106±18	10±2	5.9±0.5	127±19	124±35*	10.2±1.9*
CD-CR	134±18	117±23	9.3±1.2	137±27	119±28	9.7±2.1

Baseline data (week -1) of donor and recipient combinations.

*Mean ± SD. Unpaired T-Test.*P<0.05 recipients vs. donors.*

Longitudinal data

Higher body weight was observed in CR than in HR corresponding to the age difference (P<0.05). The SBP of the recipient was influenced by the transplanted kidney at week 5 as shown in Table 2: HD-CR rats had lower SBP in comparison to CD-CR; CD-HR rats had higher SBP compared to HD-HR (both P<0.05). A comparable pattern was observed for urea and proteinuria. HD-CR rats had lower urea and proteinuria compared to CD-CR at week 3 and 5 (all P<0.05). CD-HR rats showed higher urea and proteinuria compared to HD-HR at week 3 and 5 but these levels in CD-HR were lower when compared to CD-CR at both time points (all P<0.05).

Terminal data

Terminal renal function data was not available for one rat (in the CD-CR group) that died of heart failure during the terminal experiment.

Table 2. *Longitudinal data after transplantation.*

	HD-HR N=6	CD-HR N=6	HD-CR N=6	CD-CR N=6
Body weight (g)				
Week 3	403 ± 40	332 ± 44#	461 ± 31*	487 ±45
Week 5	422 ± 38	355 ± 17*#	488 ± 20*	498 ±44
SBP (mmHg)				
Week 3	104 ± 15	113 ± 8	89 ± 12#	106 ± 17
Week 5	112 ± 15	136 ± 18*	89 ± 19*#	122 ± 9
Urea (mmol/L)				
Week 3	8.2 ± 1.8	18.9 ± 5.8*#	7.4 ± 3.7#	10.2 ± 1.9
Week 5	8.5 ± 2.1	18.5 ± 5.4*#	9.1 ± 4.8#	13.3 ± 0.9
Proteinuria (mg/d)				
Week 3	10 ± 3	28 ± 5*#	7 ± 5#	60 ± 17
Week 5	12 ± 1	51 ± 30*#	11 ± 4#	102 ± 32

*Mean± SD. Two-way ANOVA, Newman-Keuls post-hoc test. *P<0.05 vs. HD-HR. #P<0.05 vs. CD-CR.*

Environment-graft interaction in relation to renal function, GFP+ and Ki67+ cell count

Anemia (decreased hematocrit) and decreased renal function (increased plasma urea and decreased GFR measured by inulin clearance) at termination confirmed marked impairment after ablation and was not affected by environment (CD-HR vs. CD-CR and HD-HR vs. HD-CR; comparisons for all parameters, NS, Table 3). A comparable pattern was observed for renal hemodynamics (RPF, RBF: renal blood flow and RVR: renal vascular resistance) and FE_{Na} and FE_{K}. No significant differences were found in the kidneys of the recipients for proliferating cells expressing Ki67 in the glomeruli, GFP+ cells/glomerulus or GFP+ area/tubular field (Table 3).

Environment-graft interaction in relation to systemic damage and renal damage

TBARS excretion was higher in the recipients with CR than HR, irrespective of the transplanted kidney (P<0.05 for CR vs. HR, Fig 1A). A similar pattern was observed for vascular damage that we assessed by scoring aorta calcification (P<0.05 for CR vs. HR, Fig 2B, representative histology shown on Fig 1C,D). Note that on the low phosphate content of the standard rodent diet used in this

Table 3. *Terminal kidney function and immunohistochemistry post-trans plantation at week 6.*

	HD-HR	CD-HR	HD-CR	CD-CR
Renal function				
N	6	6	6	5
Hct (%)	41.7 ± 1.2	31.8 ± 4.7*	44.0 ± 1.4#	38.0 ± 2.9
Urea (mmol/L)	10.4 ± 1.5	19.0 ± 4.5*	8.4 ± 1.8#	15.8 ± 1.6
GFR (ml/min/100g)	0.56 ± 0.06	0.22 ± 0.07*	0.57 ± 0.04#	0.19 ± 0.05
RPF (ml/min/100g)	2.20 ± 0.26	0.87 ± 0.29*	2.31 ± 0.18#	0.83 ± 0.25
RBF (ml/min/100g)	3.59 ± 0.43	1.34 ± 0.48*	3.93 ± 0.36#	1.30 ± 0.40
RVR (MAP/RBF, units)	7.1 ± 1.0	38.8 ± 21.2*	5.9 ± 0.9#	25.0 ± 7.7
FE_{Na} (%)	0.20 ± 0.16	2.02 ± 2.45*	0.43 ± 0.17#	1.27 ± 0.67
FE_{K} (%)	37.6 ± 9.6	93.9 ± 26.3*	40.1 ± 5.3#	99.2 ± 17.7
Immunohistochemistry				
N	6	6	6	6
Ki67+ cells/glomerulus	2.3 ± 0.9	2.4 ± 0.7	4.2 ± 2.0	4.3 ± 3.3
GFP+ cells/glomerulus	3.8 ± 1.2	4.0 ± 2.4	4.1 ± 2.5	4.8 ± 1.4
GFP+ area/tubular field	7.8 ± 1.6	7.6 ± 2.1	8.2 ± 0.9	7.4 ± 1.2

study (0.63% w:w) calcification is restricted to the subendothelium.

Representative histology (PAS) and immunohistochemistry (JG12 and ED1) pre- and post- transplantation of a HD-CR combination (analogous to clinical transplantation with a living donor) are shown in Figures 2 and 3. At pre-transplantation (comparison between donors' reference kidneys), marked differences were observed between CD and HD for all histological parameters (Two-way RM ANOVA, HD/CD $P<0.05$, Figures 2 and 3). These differences remained significant when comparing grafts (CD vs. HD) 6 weeks post-transplantation for all histological parameters except for TI score in CR, where TI increased in HD but remained stable in CD (Figure 2A); GS score in all recipients, where GS increased in HD but remained stable in CD (Figure 2D); JG12+ area/tubular field in CR, because of the pronounced fall in HD-CR (Figure 3A); and ED1+ cells/glomerulus in HR, due to the large variation (Figure 3J). Note that TI and GS generally became more severe and glomerular endothelium was lost in HD kidneys, while further progression of injury and glomerular endothelial loss was not observed in CD kidneys (Figures 2 and 3, individual responses in Figure S1).

The strongest interaction was observed for glomerular endothelial area relative to glomerular area (Figure 3D, P<0.003). Less glomerular endothelium was observed at post-transplantation time-point in HD-CR vs. HD-HR groups and CD-CR vs. CD-HR groups (Figure 3A, D, P<0.05). Similarly, glomerular endothelial area decreased in post vs. pre transplantation in the HD-CR group. On the contrary, when compared in healthy environment, the glomerular endothelium, although at donation lower than in the healthy kidney, was preserved in the CKD kidney as shown by the comparison CD-HR vs. CD-CR (Figure 3D, P<0.005). More loss of peritubular capillaries was observed in healthy kidneys transplanted in CKD recipients (Figure 3A, P<0.05). At 6 weeks post-trans-

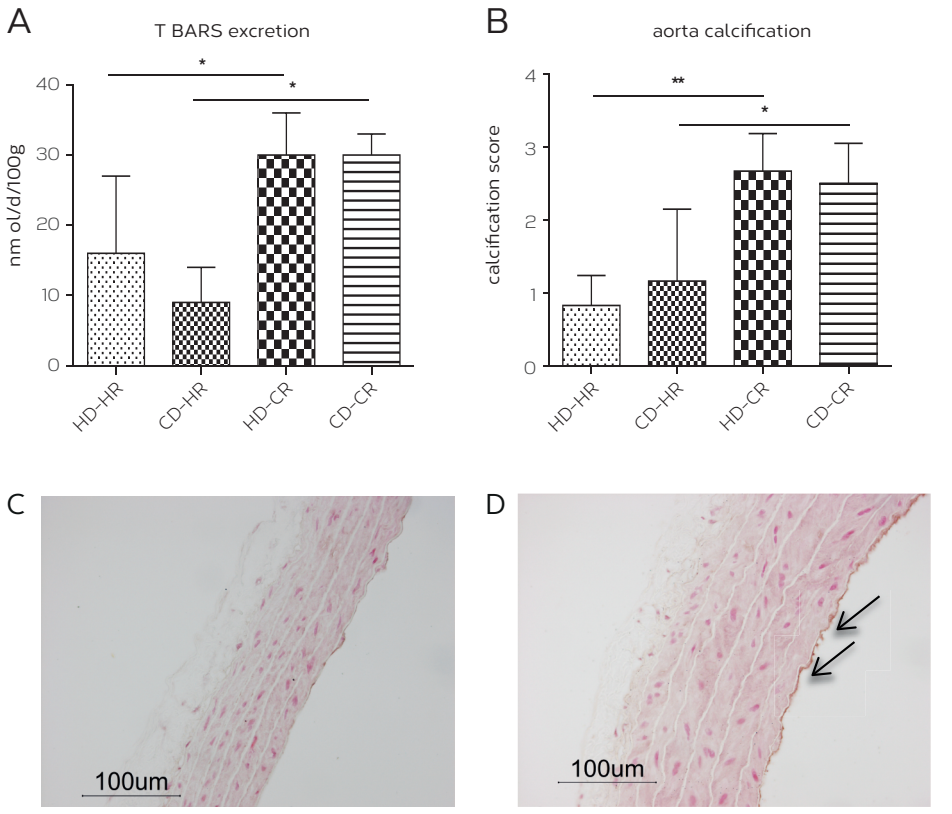

Figure 1. *Systemic oxidative damage (TBARS excretion, panel A) and vascular damage (aorta calcification, von Kossa stain, panel B).*

*H: Healthy, C: CKD, D: Donor (pre-transplantation), R: Recipient (post-transplantation). All N=6. Mean ± SD. Two-way ANOVA, Newman-Keuls post-hoc test. *P<0.05, **P<0.01. Post-transplantation CR vs. HR P<0.05 for both variables (not shown in graph). Representative histology post-transplantation of a HD-HR (panel C) and HD-CR (panel D) combinations is shown. Arrows show subendothelial calcification of aortas.*

plantation, we observed less ED1+ cells per tubular field and glomerulus in CD-HR compared to CD-CR (Figure 3E and 3F, both P<0.05). In HD-CR group 6 weeks post-transplantation we observed more TI damage, GS, larger glomerular area, and more ED1+cells/glomerulus in the graft than in the reference kidney (all P<0.05, Figure 3, panels A-D, F). Glomerular area was consistently increased in CD vs. HD kidneys and in post-transplantation vs. pre-transplantation kidneys (Figure 3K). Note that the size distribution within CD kidneys was much broader than in HD kidneys because of the absence of very large glomeruli in healthy kidneys (Figure S2).

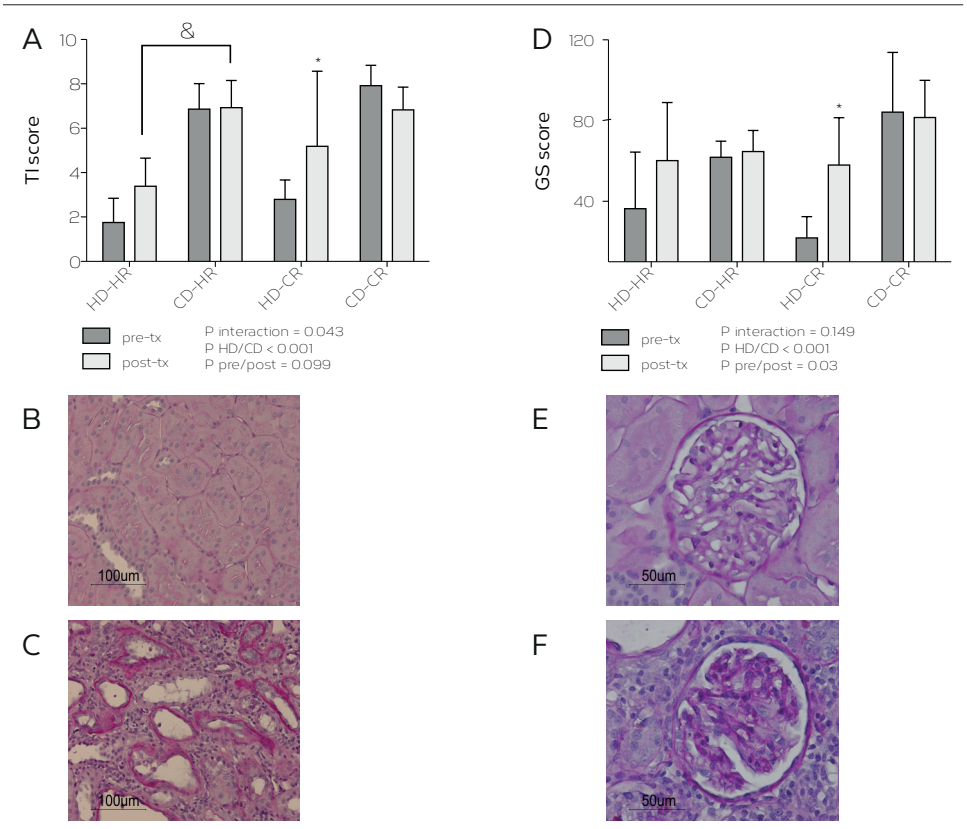

Figure 2. *Histology pre- and post-transplantation (PAS staining): Tubulo-interstitial score (TI score, panel A), Glomerulosclerosis (GS, panel D). Representative histology pre- (panels B and E) and post- transplantation (C and F) of a HD-CR combination is shown (analogous to clinical transplantation with a living donor).* H: Healthy, C: CKD, D: Donor (pre-transplantation), R: Recipient (post-transplantation). All N=6.Mean ± SD. Two-way RM ANOVA, Newman-Keuls post-hoc test. *P<0.05 vs. pre-transplantation. Interaction: &P<0.05 shows effect of graft in recipient. Pre-transplantation CD vs. HD P<0.05 for all variables (not shown in graph).

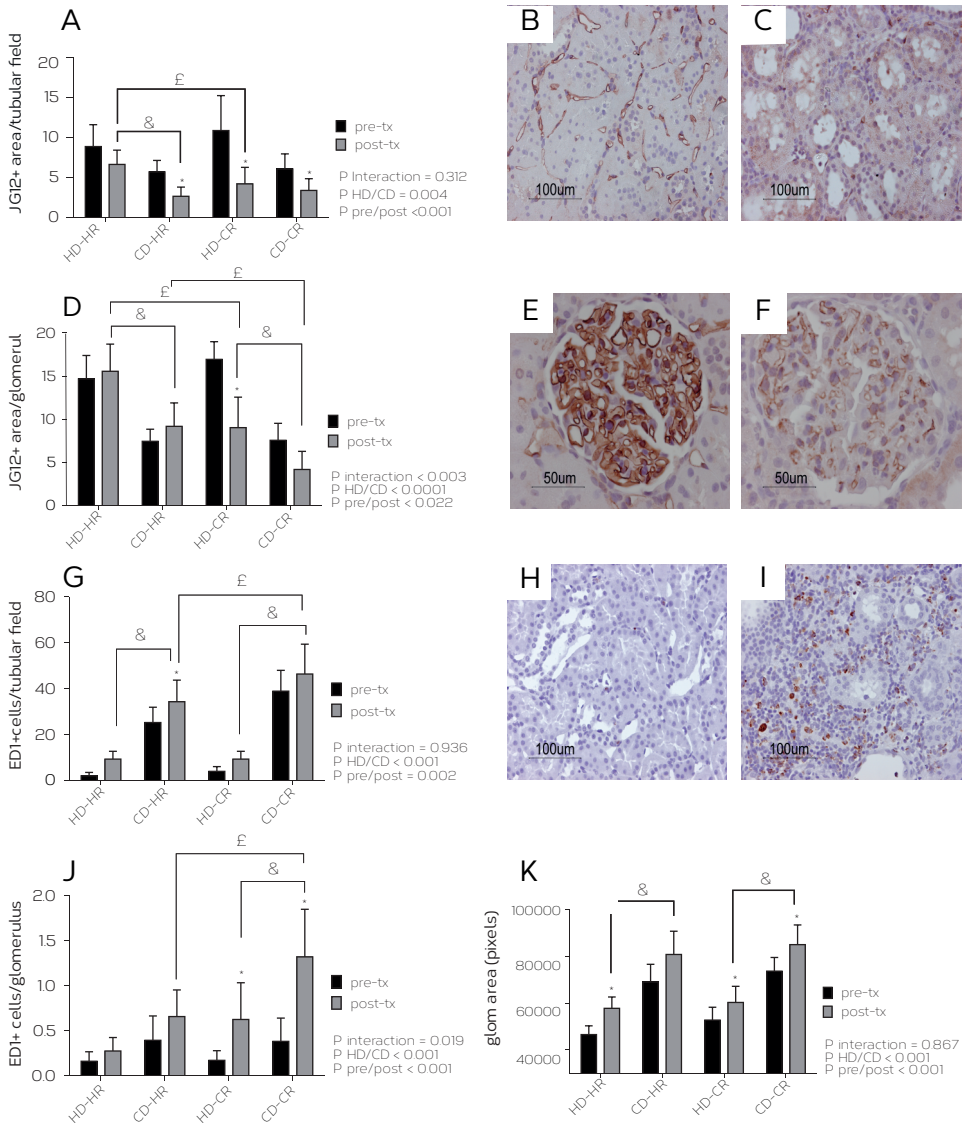

Figure 3. *Immunohistochemistry pre- and post-transplantation:*
*JG12+area/tubular fie**ld (panel A)**. JG12+area/glomerulus (**panel D**). ED1+ cells/tubular field **(panel G)** and ED1+ cells/glomerulus **(panel J)** and glomerular area (scored on slides stained with JG12, **panel K**). H: Healthy, C: CKD, D: Donor (pre-transplantation), R: Recipient (post-transplantation). All N=6. Representative histology pre- **(panels B, E, H)** and post-transplantation **(C, F, I)** of a HD-CR combination is shown (analogous to clinical transplantation with a living donor). Mean ± SD. Two-way RM ANOVA, Newman-Keuls post-hoc test. *P<0.05 vs. pre-transplantation. Interaction: &P<0.05 shows effect of graft in recipient; £P<0.05 shows effect of environment on graft. Pre-transplantation CD vs. HD P<0.05 for all variables (not shown in graph).*

Discussion

Our results demonstrate that transplantation of a healthy kidney to a CKD environment enhanced loss of glomerular endothelium and worsened TI injury and GS as shown by post vs. pre- transplantation histology and that the significant difference in glomerular endothelial integrity, GS and TI injury between healthy and CKD grafts at time-point of TX disappeared when comparing these at 6 weeks post-transplantation (HD-CR vs. CD-CR). Healthy environment, on the other hand, associated with preserved glomerular endothelium and decreased inflammation in the CKD graft but did not ameliorate TI damage and GS (CD-HR vs. CD-CR). Interestingly, preservation of glomerular endothelium was not associated with a difference in the number of GFP-positive cells or an increase in cell proliferation, suggesting that incorporation and proliferation of circulating recipient-derived cells is not a major factor in preservation of glomerular endothelium in CKD. This is consistent with our previous observations that renal artery injections with bone marrow cells protected against glomerular damage in experimental CKD but was not related to incorporation or transdifferentiation of these cells into glomerular endothelium [11]. Within the time-span of the experiment the differences in glomerular endothelial and inflammatory cell numbers and did not associate with corresponding changes in renal function, as pre-existent renal damage at the time of transplantation determined proteinuria and terminal renal function. Clearly, the ablated kidney used as a graft in our experimental study was far more severely injured than the kidneys from marginal living donors. Nevertheless, CKD environment persistently determined systemic injury, as reflected by TBARS excretion and aortic calcification, and this may have impacted on intrarenal endothelial integrity. Unexpectedly, despite ischemia-reperfusion injury, TI damage and GS did not worsen in the CKD kidney grafts.

Proteinuria is an important marker for kidney dysfunction. Patients with moderately reduced GFR without proteinuria have better clinical outcomes compared to patients with heavy proteinuria without abnormal GFR[21]. There are three components of the filtration barrier that could be responsible for development of proteinuria: fenestrated endothelium, glomerular basement membrane and podocytes. All three components are in constant two-way molecular and biochemical communication with each other. Although in most cases, podocyte effacement is associated with proteinuria and glomerular disease, it has been demonstrated that proteinuria can be observed without podocyte foot process effacement[22]. Thus, a loss of glomerular endothelium could be also responsible for proteinuria. However, our results show the opposite: healthy grafts transplanted in CKD rats showed increased loss of glomerular endothelium but with normal low levels of proteinuria (HD-CR: 12 mg/d,

HD-HR 11 mg/d) which means that, in this model, endothelial damage could be less important for the development of proteinuria within the follow-up period of six weeks. Furthermore, our study shows that both glomerular and peritubular endothelium are damaged when a healthy kidney is transplanted into a CKD rat in comparison to transplantation in a healthy recipient (HD-CR vs. HD-HR). This observation might be due to the concomitant existence of oxidative and inflammatory systemic injury in CKD recipients [23].

Endothelial damage could be due to systemic and/or local factors. The association between oxidative stress, inflammation and CKD is well established[24]. Systemic factors such as vascular calcification and oxidative injury (shown by increased TBARS excretion) might be the reason for the progression of TI and GS in healthy grafts when in CKD environment as both cause endothelial dysfunction [25] that appears to have persisted within the follow-up period of 6 weeks. This confirms our previous observation that reactive oxygen species appear not to be important direct determinants of hypertensive renal hemodynamics in CKD [4]. Our results show, that in this model of transplantation, proteinuria is probably driven by blood pressure as we observed a low level of proteinuria together with normal blood pressure in the HD-CR group despite an increase in TI and GS.

The importance of the systemic environment is confirmed by the observation that the diseased grafts showed no progression of glomerular endothelium damage after being transplanted to a healthy recipient with low levels of vascular oxidative and structural injury. Increased loss of endothelium in CD-CR group was associated with more inflammation as shown by higher number of macrophages in both glomeruli and tubular fields. Clustering of vascular calcification, oxidative stress and endothelial dysfunction is characteristic of CKD and may contribute to enhanced mortality [26].

In the current study, we observed that the presence of CKD inhibits further development of TI injury and GS after transplantation as shown by no pre vs. post TX difference in CD-CR group. Vercauteren et al. showed that the remnant kidney is less susceptible to ischemia-reperfusion injury (IRI) in comparison to a healthy kidney, possibly due to preconditioning [34]. In a subsequent study from the same group, when transplanting healthy kidneys into healthy rats or rats with CKD, they reported that a CKD environment causes resistance to IRI and that the regeneration capacity of the transplanted healthy kidney was not hampered by chronic uremia[35]. In the latter study, the authors argue that a CKD milieu is protective against IR partly due to less accumulation of inflammatory cells 10 days after transplantation. In our study at 6 weeks after transplantation, we observed the opposite: there was more TI damage, GS, more loss of peritubular and glomerular endothelium and more inflammation in

the HD-CR group as compared to changes in these variables in HD-HR. Our data suggest that, although in the first days after transplantation the CKD milieu might be protective, systemic injury induced by a history of CKD can be harmful for the healthy graft in the long term even though initially renal function is restored.

The current study shows that long-term quality of the healthy graft is dependent on the CKD history of the recipient as we observe more damage even though blood pressure, proteinuria and urea levels were normalized. However, for grafts from marginal living donors this may not hold, as TI and GS in CKD grafts did not progress after transplantation. This observation is quite provocative and hypothetically would implicate that the more frequent use of marginal living donors might be acceptable. Whether this unexpected pause in the progression of fibrosis lasts for a longer period remains to be seen.

In summary, our study stresses the importance of the systemic environment (oxidative stress and vascular injury) for graft outcome in renal transplantation in the absence of hypertension and raises the question how to improve the systemic environment in order to minimize long-term damage to the renal graft. Interventions to improve pre-existent vascular calcification, oxidative stress and inflammation prior to renal transplantation should be explored.

Acknowledgements

We would like to thank Adele Dijk, Krista den Ouden and Paula Martens for their expert technical assistance and Hendrik Gremmels for preparing figure S2.

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Supplement

Expanded methods

Kidney transplantation procedure

This procedure was performed as described[1,2]

Donor procedure

Donor rats were placed on an operating table with a heating pad keeping the body temperature at 37 C. A long abdominal incision was made from the sternum to the symphysis. The bowel was moved slightly to the left side, covered with moist gauze. The renal vessels and urether were dissected carefully using atraumatic technique. Heparin was administered directly into the spleen and five min after the left kidney was perfused with saline, the renal vessels cut close to their junction to the aorta and vena cava and the urether - about 2 cm distal to the kidney hilus. Finally the donor kidney was kept on ice with standardized cold ischemia time of 30 min.

Recipient procedure

The recipient rats were prepared in the same way as the donor with the difference that heparin was not given. The renal vessels were clamped using separate microvascular clamps and, including the urether, were cut close to the kidney hilus. The donor kidney was placed and the following anastomoses were performed: end-to-end arterial anastomosis using 8 to 10 separate stitches; end-to-end venous anastomosis using continuous suture; end-to end anastomosis of the urether with four separate stitches. All anastomoses were performed with 10-0 prolene sutures. After completing the anastomoses at standardized time of warm ischemia of 30 min the microvascular clamps were released. The immediate patency of anastomosis was checked 20 min after clamp removal. After 10-14 days, the animals were anaesthetized again, the contralateral kidney was removed, and the graft vessels were checked for late patency and long-term complications (infection, aneurysms) and the graft urether anastomosis for hydronephrosis. Only once case of hydronephrosis was observed, and this rat was excluded from the study.

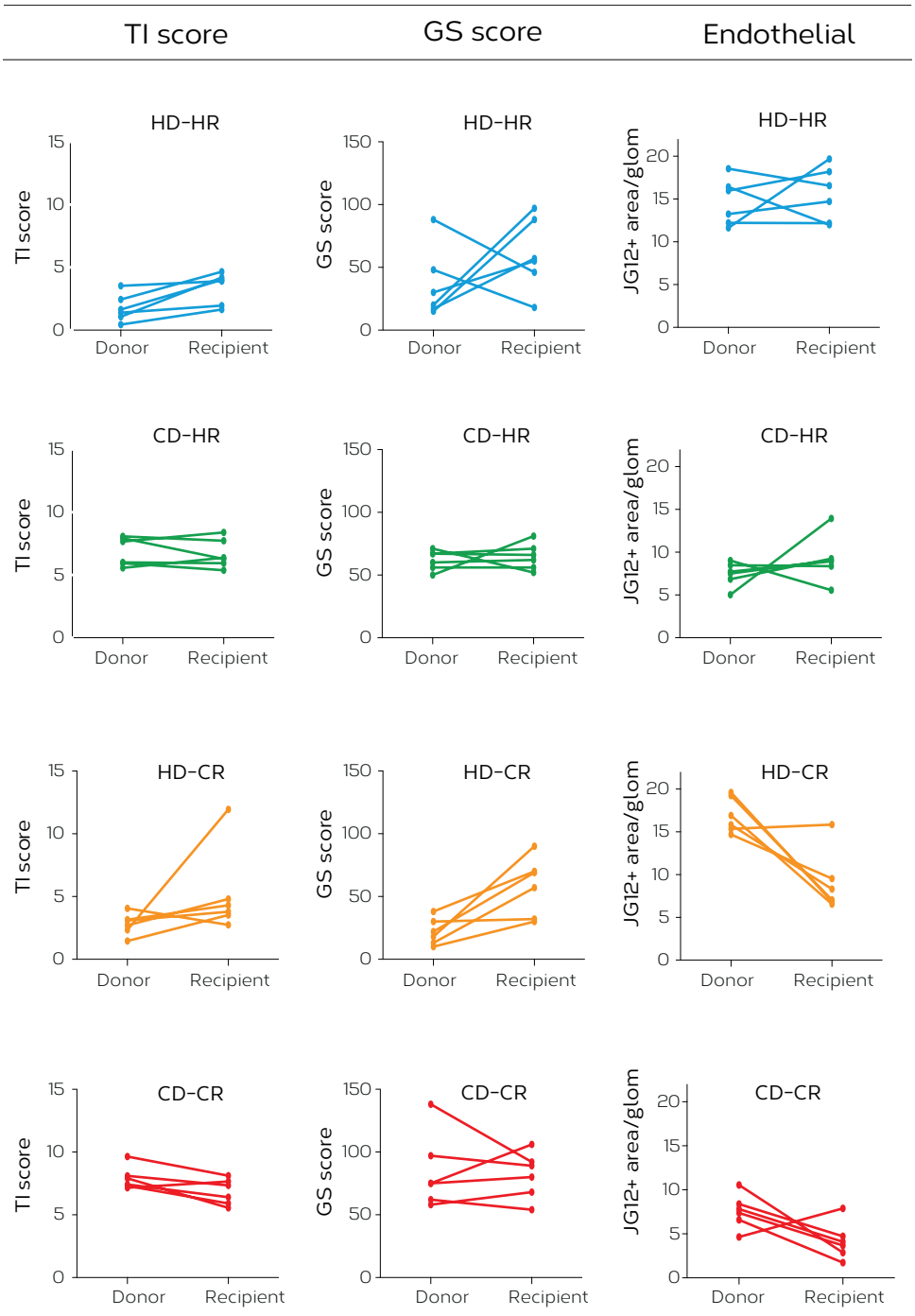

Figure S1. I*ndividual responses to transplantation (post- vs. pre-transplantation) for TI injury, GS and glomerular endothelium.*

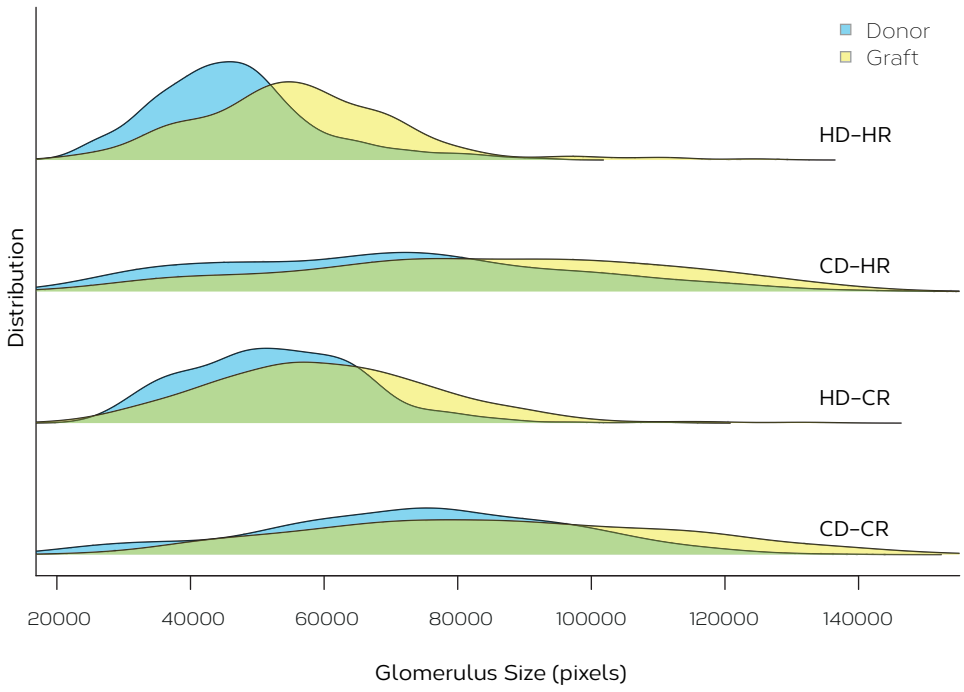

Figure S2. G*lomerular size distribution in both pre-transplantation (donor) and post-transplantation (graft) kidneys in all 4 groups*

n=300/distribution (50 glomeruli/kidney). Graph was made using R software (version 3.1.0: R Foundation for Statistical Computing, Vienna, Austria).

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9

Dissociation between left ventricular hypertrophy and cardiac fibrosis after experimental kidney transplantation

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(In preparation)

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Abstract

Left ventricular hypertrophy (LVH) is the most common cardiac alteration observed in patients with chronic kidney disease (CKD). Normalization of hypertension and correction of the uremic state in CKD patients receiving a healthy kidney allograft are known to reverse LVH. However, its effects on cardiac fibrosis, another characteristic of CKD, are unknown, as are effects of marginal living donor kidneys on recipient LVH and cardiac fibrosis. Aim of our study was to investigate the interaction between graft and environment on both LVH and cardiac fibrosis in kidney transplantation (TX).

CKD was induced in male Lewis rats by bilateral ablation of 2/3 of kidney mass and L-NNA (20 mg/L water) plus 6% salt diet. Orthotopic TX was performed: healthy kidney to healthy recipient (HD-HR); CKD kidney to healthy recipient (CD-HR); healthy kidney to CKD recipient (HD-CR); CKD kidney to CKD recipient (CD-CR); N= 6/group. Contralateral kidney was removed 10-14 days after TX. At wk 6, we measured glomerular filtration rate (GFR: inulin), mean arterial pressure (MAP) and LV mass (LVM) of all recipients and the hearts were stained for fibrosis (Sirius red). We evaluated the expression of selected genes in the LV apex.

At 6 wk after TX, similarly impaired GFR was observed in all CD vs. HD kidneys, irrespective of the recipient ($P<0.01$). MAP and LVM were higher in CD-HR vs. HD-HR (162±25 vs. 110±4 mmHg, $P<0.01$ and 0.32±0.04 vs. 0.24±0.01 mg/100g body weight (BW), $P<0.01$). In HD-CR, MAP and LVM were normalized to HD-HR levels, and improved vs. CD-CR (111±7 vs. 147±13 mmHg, $P<0.01$ and 0.26±0.02 vs. 0.32±0.06 mg/100g, $P<0.05$). Cardiac fibrosis was neither different in CD-HR vs. HD-HR (2.6 ± 2.9 % vs. 1.6 ± 1.6 %) nor in HD-CR vs. CD-CR (8.6 ± 5.2 % vs. 9.6 ± 7.0 %, NS). Gene expression of ANP, BNP, CTGF and GDF11was higher in CR rats vs. HR rats, irrespective of the kidney graft. However, AdrB2 was only increased in CD-CR vs. HD-CR

Diseased kidney grafts increased MAP and LVM whereas healthy kidney grafts normalized MAP and reversed pre-existent LVH. However, cardiac fibrosis and expression of CTGF, ANP, BNP and GDF11 were mainly determined by recipient history and barely influenced by the kidney graft. Thus, TX of either a healthy kidney in previously uremic subjects or a diseased kidney in previously healthy subjects dissociates LVH from cardiac fibrosis within 6 wks in our rodent model.

Introduction

Chronic kidney disease (CKD) is associated with high prevalence of cardiovascular diseases (CVD). Even in patients with mild-to-moderate chronic kidney disease (CKD), the estimated glomerular filtration rate (GFR) is inversely related to the development of CVD and mortality [1]. Left ventricular hypertrophy (LVH) is the most common cardiac alteration observed in patients with CKD[2], [3]. Another key finding in CKD population is "uremic cardiomyopathy", typical structural changes comprising fibrosis and a reduction in capillary supply per unit volume of cardiac tissue [4]. Around 25% of all mortality of dialysis patients is caused by sudden cardiac death, mostly arising from arrhythmias such as ventricular tachycardia or fibrillation[5]. Kidney transplantation can reverse some of the cardiac abnormalities but compared with the general population, renal transplant recipients are still at higher risk for cardiovascular morbidity and mortality [13-15], with cardiovascular disease being the leading cause of death with a functioning kidney graft [12].

Pathophysiological mechanisms involved in LVH and fibrosis in CKD are complex[6] and can be divided into three groups: afterload related, preload related, and neither preload nor afterload related[7]. The latter is due to various pathways with myocardial fibrosis as common final event that leads to disturbances in the electrical circuit of the heart and arrhythmogenesis caused by the superimposition of high-resistance pathways for ventricular electrical conductance and the occurrence of re-entry pathways[8,9].

Normalization of hypertension and correction of the uremic state in CKD patients receiving a healthy kidney allograft are known to reverse LVH [10]. LVH regression was found to continue during the first two years, and remained unchanged up to four years after renal transplantation[11]. The effects of renal transplantation on cardiac fibrosisare unknown, as are effects of (marginal) living donor kidneys on recipient LVH and cardiac fibrosis. We hypothesized that there would be concordant changes in LVH and cardiac fibrosis after kidney transplantation and that the healthy donor graft will ameliorate both LVH and cardiac fibrosis. Thus, we explored the effect of transplant kidney graft function and damage on recipient LVH and cardiac fibrosis.

Materials and Methods

Animals

The study protocol was approved by the Utrecht University Committee on Animal Experiments (DEC number 2012.II.03.053), and conformed to Dutch Law on Laboratory Animal Experiments. Male inbred Lewis rats (Charles River, Germany) were used. Rats were housed in a climate-controlled facility with a

12:12-hour light: dark cycle under standard conditions.

Groups

The following groups (N=6/group) were used: HD-HR: healthy donor and healthy recipient; CD-HR: CKD donor and healthy recipient; HD-CR: healthy donor and CKD recipient; CD-CR: CKD donor and CKD recipient. Thus, in all, 24 donors and 24 recipients were used.

Models of CKD in rats

To develop established CKD in this strain, rats underwent 2/3 bilateral ablation of renal mass by coagulating branches of both renal arteries[16].Subsequently, development of CKD was accelerated with L-NNA, a NO-synthase inhibitor (200 mg/L) in drinking water[17] and animals were fed standard powdered chow (CRM-FG; Special Diet Services Ltd., Witham, Essex, UK) supplemented with 6% NaCl, until proteinuria reached 200 mg/24h at which point L-NNA and 6% NaCl were discontinued, as previously described[16]. CKD animals were included into the transplantation protocol when proteinuria reached 100 mg/24h (without L-NNA and without 6% NaCl) and were matched with healthy control rats with intact kidneys.

Kidney transplantation

We used orthotopic left kidney transplantation, with subsequent removal of the right native kidney 10-14 days after transplantation, as described[18]. Cold ischemia - and warm ischemia- times were 30 mins each and all isografts were perfused and placed in organ preserving solution Viaspan (Bristol Meyers Squibb, Hoofddorp, Netherlands) prior to transplantation.

Longitudinal measurements

At week 3 and week 5 after transplantation, we performed tail-cuff systolic blood pressure (SBP) registration and blood was sampled for urea levels.

Acute protocol

Acute measurements were performed six weeks after transplantation. Renal function was investigated under isoflurane anesthesia (Abbott Laboratories, Hoofddorp, Netherlands) as described[19]. We measured mean arterial pressure (MAP), GFR(inulin clearance) and creatinine. At the end of the acute protocol, rats were sacrificed, perfused with 0.9% NaCl via the aorta, and

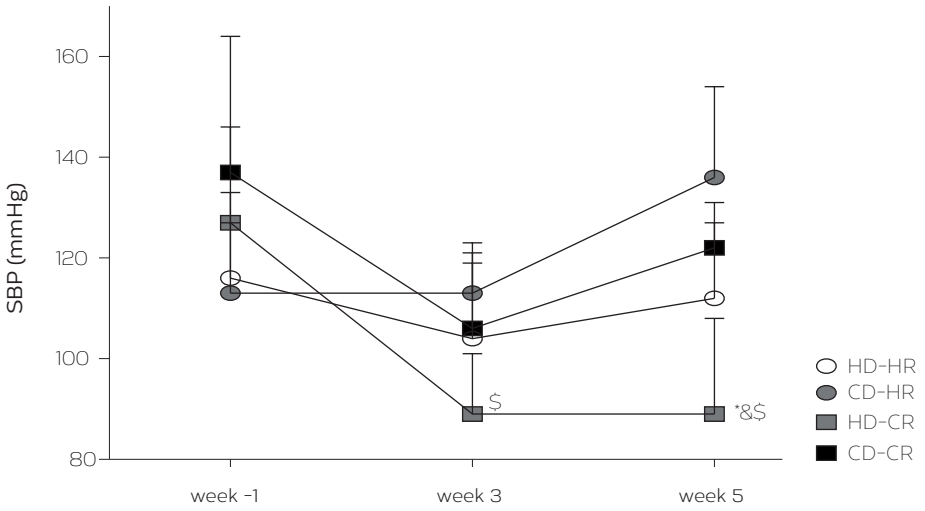

Figure 1. *Systolic blood pressure (SBP) in recipient before (baseline, week -1) and at week 3 and 5 after transplantation. H:healthy; C:CKD; D:donor; R:recipient. Two-way RM ANOVA (Pinteraction, Ptime and Pmodel all <0.01). Tukey post-hoc test. $P<0.01 vs. week -1; *P<0.05 vs. CD-HR; &P<0.01 vs. CD-CR.*

tissues were collected. Organs weights were noted. Furthermore, transverse mid LV samples were fixed in 4% paraformaldehyde for embedding in paraffin and the apexes were snap frozen.

Cardiac morphology

Heart tissue of all recipients was stained for collagen I and III content using Picro Sirius Red, visualized with circular polarized light. All analysis was performed with ImageJ software, version1.46r (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD).

Gene expression in heart tissue

Gene expression in hearts of recipients for atrial natriuretic peptide (ANP), connective tissue growth factor (CTGF), brain natriuretic peptide (BNP), bone morphogenic protein-6 (BMP6), growth differentiation factor 11 (GDF11), growth differentiation factor 15 (GDF15), and beta2 receptor was assessed by qPCR as described[17]. The following TaqMan Gene Expression Assays (Applied-Biosystems) were used: (CTGF: Rn00573960_g1), (ANP:Rn00561661_m1), (BNP:Rn00580641_m1), (GDF11: Rn01756258_m1), (GDF15:Rn00570083_m1), (AdrB2: Rn00560650_s1).Housekeeping genes were beta-actin (Rn00667869_m1) and calnexin (Rn00596877_m1). Cycle time (Ct) values for all genes were normalized for mean Ct-values of beta-actin and calnexin

which we previously determined to be the two most stable housekeeping genes for cardiac tissue in rats with CKD[20].

Statistics

Data are presented as means ± SD. Two-way ANOVA, Tukey post-hoc test and linear regression, were used when appropriate.

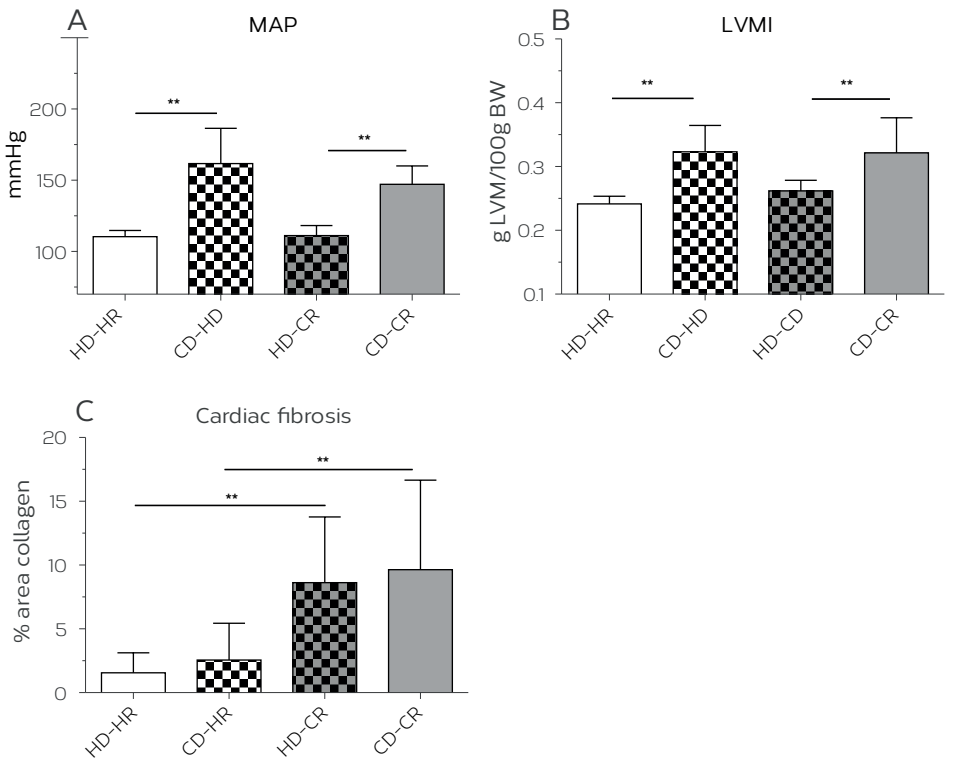

Figure 2. ***Mean arterial pressure (MAP, panel A), left ventricular mass index (LVMI, panel B) and cardiac fibrosis presented as %area collagen (panel C) at 6 weeks after TX.*** *Means ± SD. Two-way ANOVA, Newman-Keuls post-hoc test. **P<0.001, *P<0.05. H:healthy; C:CKD; D:donor; R:recipient. Open square represents HD-CR outlier.*

Results

Environment-graft interaction in relation to LVH and cardiac fibrosis

BP and LVM are related to kidney graft function

Systolic BP rapidly fell after TX of a HD graft in CR environment; conversely systolic BP rapidly rose after TX of a CD graft in HR environment (Figure 1). At 6 wk after TX, MAP and LVM were higher in CD-HR vs. HD-HR (162±25 vs. 110±4

mmHg, P<0.01 and 0.32±0.04 vs. 0.24±0.01 mg/100g body weight (BW), P<0.01, Figure 2A, B). In HD-CR, MAP and LVM were normalized to HD-HR levels, and improved vs. CD-CR (111±7 vs. 147±13 mmHg, P<0.01 and 0.26±0.02 vs. 0.32±0.06 mg/100g, P<0.05, Figure 2A,B).

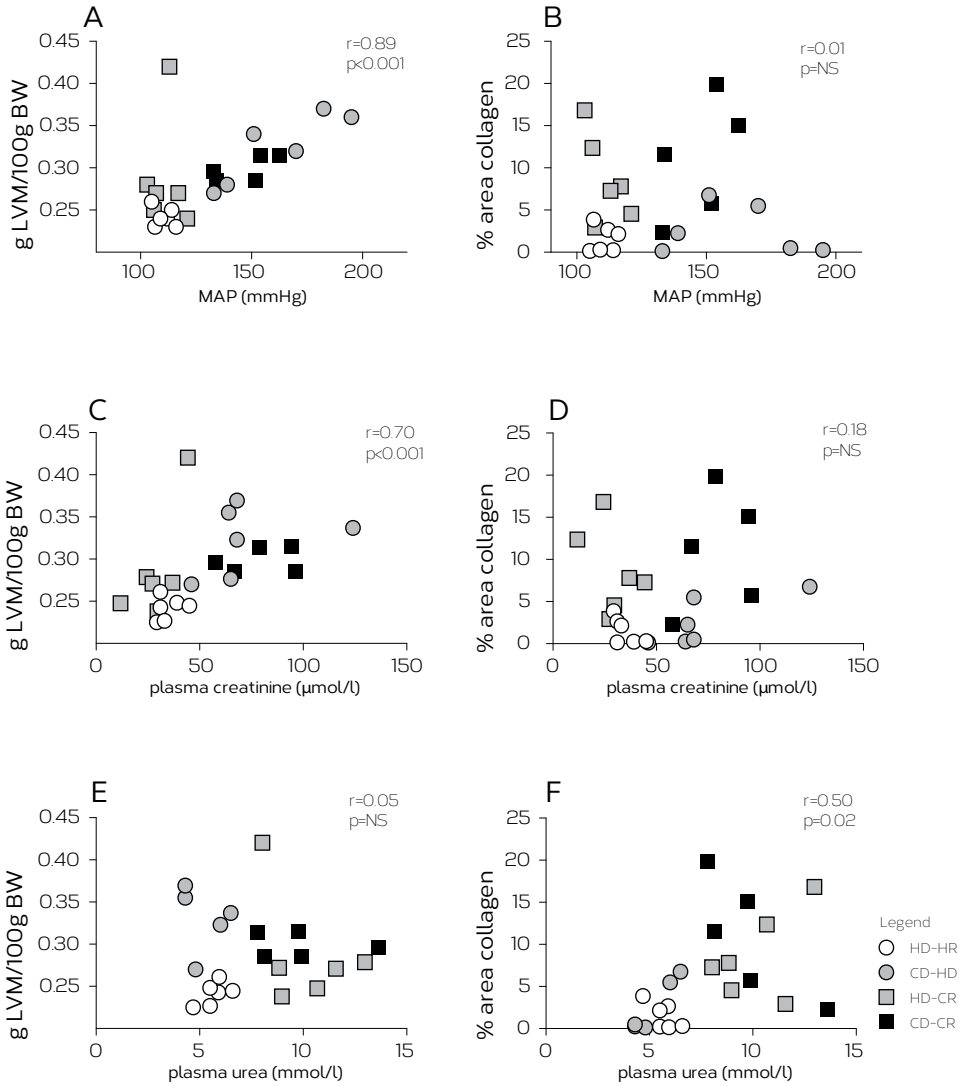

Figure 3. *Correlations at 6 weeks after TX between LVM and MAP (panel A), cardiac fibrosis and MAP (panel B), LVM and plasma creatinine (panel C) and cardiac fibrosis and plasma creatinine (panel D). Correlations at pre-transplantation between LVM and plasma urea (panel E) and cardiac fibrosis and urea (panel F).* *LVM: left ventricle mass; MAP: mean arterial pressure; GFR: glomerular filtration rate; H:healthy; C:CKD; D:donor; R:recipient. Open square represents HD-CR outlier.*

Cardiac fibrosis is related to recipient history but not to kidney graft function

Cardiac fibrosis was neither different in CD-HR vs. HD-HR (2.6 ± 2.9 % vs. 1.6 ± 1.6 %) nor in HD-CR vs. CD-CR (8.6 ± 5.2 % vs. 9.6 ± 7.0 %, NS), but there was a significant difference when comparing HD-CR vs. HD-HR and CD-HR vs. CD-CR (Figure 2C).

LVMI, but not cardiac fibrosis, correlates with MAP and creatinine

When correlating LVMI and cardiac fibrosis to MAP, we observed dissociation between LVMI and cardiac fibrosis in the HD-CR group (Figure 3A, B). A similar pattern was observed when correlating LVMI and cardiac fibrosis to creatinine (Figure 3C, D). When correlating the LVM against SBP at three different time-points (baseline at week 01, week 3 and week 5 after transplantation), we observed that the LVMI at week 6 after TX already showed a borderline correlation with SBP at week 3 after TX (P=0.08, Figure 4B), and correlated significantly with SBP at week 5 after TX (r=0.61, p<0.05, Figure 4C).

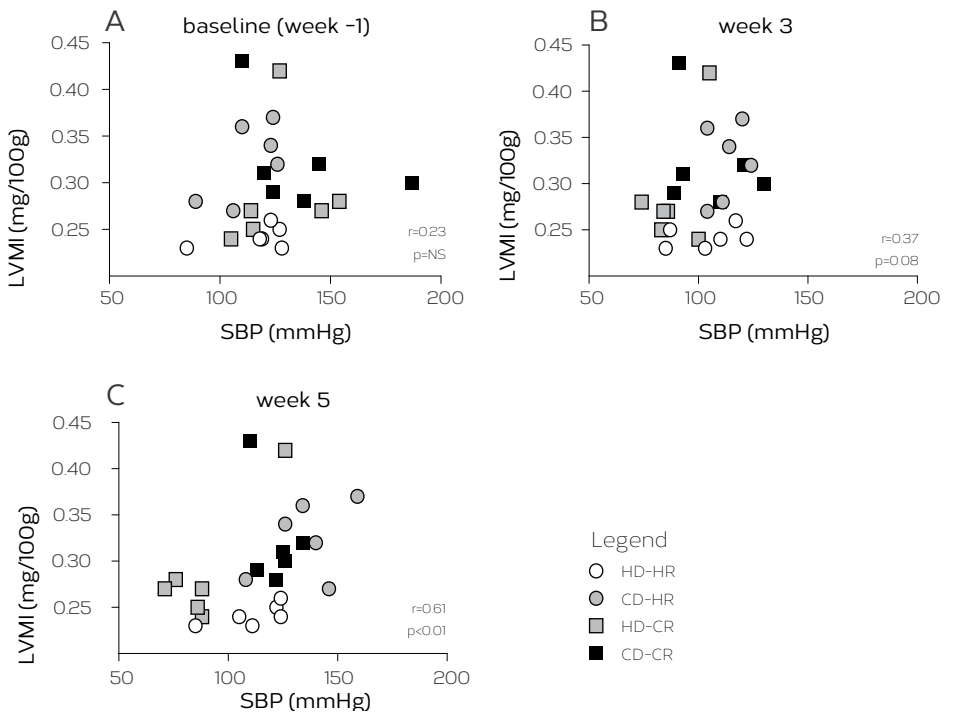

Figure 4. *Correlations between LVM at 6 weeks after TX and SBP at baseline (week -1 before transplantation, panel A) and at week 3 (panel B) and week 5 (panel C) after TX. LVM, left ventricle mass; SBP, systolic blood pressure; H:healthy; C:CKD; D:donor; R:recipient.*

Gene expression in cardiac tissue

Expression of ANP, BNP, GDF11 and CTGF was consistently higher in CKD recipients versus healthy recipients and in no case was significantly affected by transplantation of a healthy kidney (HD-CR vs. CD-CR, Figure 5A-D). GDF15 was not differentially expressed between groups (Fig 5E). AdrB2was only increased in CD-CR vs. HD-CR (Fig 5F).

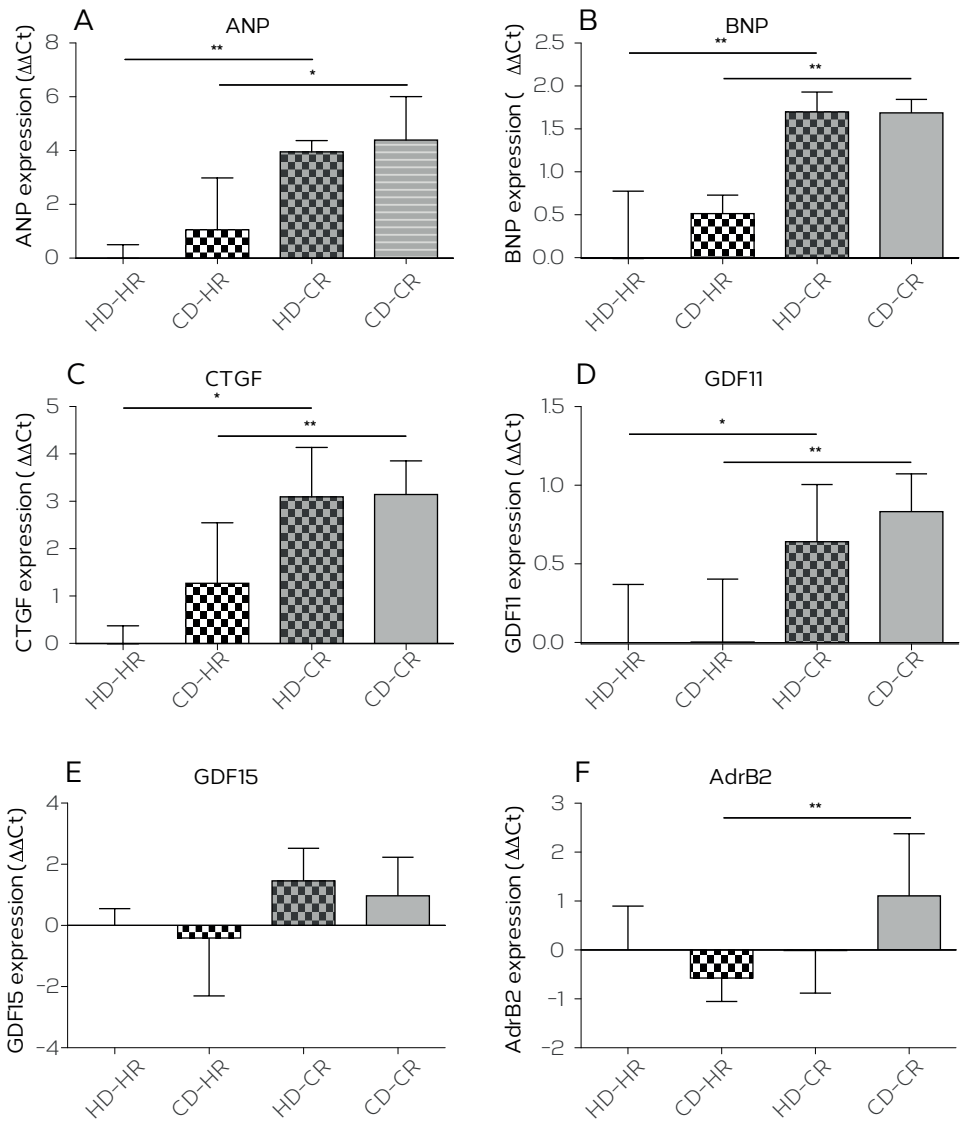

Figure 5. *Gene expression in heart tissue at 6 weeks after TX. Mean ± SD. Two-way ANOVA, Newman-Keuls post-hoc test. **P<0.001, *P<0.05. H:healthy; C:CKD; D:donor; R:recipien*

Discussion

The main finding of the present study is that diseased kidney grafts with impaired renal function increased MAP and LVM whereas healthy kidney grafts with normal renal function normalized MAP and reversed pre-existent LVHaftersix weeksin a model of experimental kidney transplantation. However, cardiac fibrosis and expression of CTGF, ANP, BNP and GFD11 were mainly determined by recipient history and barely influenced by the kidney graft. Thus, TX of either a healthy kidney in previously uremic subjects or a diseased kidney in previously healthy subjects dissociates LVH from cardiac fibrosis within 6 weeks in our rodent model.

In line with previous studies, our experiment shows that blood pressure follows kidney function. Furthermore, we show that normalization of blood pressure is an early event after transplantation as we observed a significant correlation between LVH at week 6 and SBP measured at week 5 and a trend towards correlation between LVH at week 6 and SBP measured at week 3.

Eventhough kidney transplantation is superior over dialysis treatment for ESKD patients, the survival of transplant recipients is significantly lower than age-matched controls in the general population [5, 6]. The higher mortality in renal transplant recipients is, partially, due to co-morbidity, such as CVD, which often has its origins prior to transplantation. In line with several clinical studies [10,11], our results show that LVH is reversed after transplantation of a healthy graft. However, the current study shows that eventhough LVH and MAP normalized, cardiac fibrosis persisted as shown by a higher percentage of the cross-sectional area occupied collagen. Cardiac fibrosis can mechanically impede electrical propagation which induces electrical instability leading to arrhythmias and sudden cardiac death[8,3]. Besides infection, myocardial infarction, and sudden death were the most common causes of mortality in patients with preserved graft function [21]. Considering that the transplant populationincreasingly includes older patients withcomorbidities, our findingcontributes to our understanding of the lower survival of transplant recipients compared to the general population.

CKD patients are not only exposed to traditional CV risk factors but also to non-traditional risk factors, such as aortic stiffness and oxidative stress. Oxidative stress could beresponsible for persistent myocardial injury after reversal of hypertension. In a previous study (chapter 7) we showed that, in the same model, transplantation of a healthy kidney to a CKD rat did not ameliorate systemic vascular or oxidative damage.

For most of the genes that we studiedexpression followed the distribution of cardiac fibrosis. However, for the adrenergic beta2 receptor gene a completely different pattern was observed, namely that in a CR environment, the graft

determined expression. This suggests interaction between LV injury and the environment on cardiac sympathetic innervation or activity. Currently we are staining the hearts with tyrosine hydroxylase in order to detect sympathetic fibers [16]. We are also investigating the cardiactranscriptome in all 24 recipients in order to identify patterns of gene expression and pathways that match interactions as found for the beta2 receptor gene or match the distribution of either LVH or LV fibrosis in our study.

In conclusion, our study shows reversal of LVH but persistent cardiac fibrosis after normalization of blood pressure after kidney transplantation. Our observation could partially explain the better outcomes after pre-emptive (before initiation of dialysis) kidney transplantation. This experimental model allows dissection of pathways contributing to either LV hypertrophy or LV fibrosis.

Acknowledgements

We would like to thank Petra de Bree for her excellent technical assistance.

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10

Summarizing discussion

Despite elucidation of major factors involved in the progression of chronic kidney disease (CKD) and development of better treatments, the number of individuals with end-stage kidney disease (ESKD) requiring renal replacement therapy is steadily increasing. The aim of this thesis was to investigate the interaction between the kidney and its environment in the settings of CKD and kidney transplantation.

To study the interaction between the kidney and its environment in the setting of CKD and kidney transplantation, we developed a novel, bilateral model. A detailed description of this model and its systemic predictors of function and injury are provided in **Chapter 2**. In this study we show that injury is symmetrical (judged by a similar degree of glomerulosclerosis (GS) and of tubulointerstitital injury (TI) in the left and right kidneys). Proteinuria proved to be the best predictor for injury that allowed us to use proteinuria in further studies (chapters 3, 8 and 9) to stage CKD. Moreover, this model allows us to assess kidney damage at the time-point of transplantation by using the contralateral kidney as a "protocol biopsy".

It is known that parameters of oxidative stress are increased and antioxidant enzyme activities are decreased in patients with CKD [1]. However, clinical studies showed no beneficial effects of antioxidants in the CKD population [2]. In **Chapter 3** we investigated whether blood pressure is dependent on reactive oxygen species (ROS: superoxide and H_2O_2) in the experimental CKD model described in Chapter 2. All CKD rats had higher levels of oxidative damage, shown as increased excretion of TBARS, H_2O_2 and 8-isoprostanes, and higher mean arterial pressure (MAP) compared to their healthy, age-matched controls. In series of acute terminal experiments we showed that scavenging superoxide by Tempol or H_2O_2 by catalase did not normalize MAP in CKD rats. Our findings suggest that antioxidant therapy in experimental CKD, although it can prevent the increase in BP in early stages, might not be effective in reducing BP once CKD is established.

In patients with CKD and end-stage kidney disease (ESKD) sudden cardiac death is the most common cause of mortality [3]. Sudden cardiac death occurs due to arrhythmia, for example ventricular fibrillation. In **Chapter 4** we investigated cardiac remodeling and arrhythmogenicity in two mouse models of cardiorenal disease: aged mice subjected to DOCA and salt and adult mice subjected to 5/6th nephrectomy and salt. Both models presented with a high incidence of arrhythmia accompanied by increased interstitial fibrosis and decreased Cx43 expression in the heart. The reduced Cx43 expression and increased fibrosis

levels in these hearts are likely candidates for the formation of the arrhythmogenic substrate. Both models had renal dysfunction as shown by albuminuria but only the DOCA-salt model showed significant hypertension and left ventricular hypertrophy (LVH). This means that the latter are not prerequisites for development of arrhythmias in these murine models of CKD. Thus, renal dysfunction in these models causes pronounced structural and electrical cardiac remodeling and a markedly enhanced susceptibility to arrhythmias.

Treatment for CKD is symptomatic and there are no specific therapies available to halt its progression. Preclinical studies have suggested beneficial effects of cell-based therapy. To facilitate the translation of this knowledge into therapies for individuals with CKD or even ESKD, we performed a systematic review and meta-analysis of animal studies of cell-based therapy in CKD, which is described in **Chapter 5.** In total, 71 articles met our inclusion criteria (briefly: a primary research article on effects of cell based therapy in an animal model of CKD). We used the following parameters as primary outcomes: renal function (creatinine, urea, GFR, blood pressure and urinary protein) and renal damage (GS and interstitial fibrosis). All these primary outcomes showed improvement when cell based therapy was used pointing out that this therapy is efficient in CKD. The next step was to identify the factors that modify the efficacy of cell based therapy. We studied cell-related factors (cell type, regime, condition, origin and delivery route) and model- related factors (species, gender, model and timing of intervention). Mesenchymal stromal cells, bone marrow progenitor cells and endothelial progenitor cells were the most effective cell-based therapies, and intravenous and intrarenal artery were the most effective delivery routes. Single and multiple administrations were equally effective and no dose response in delivered cell number was found. This systematic review and meta-analysis confirms that cell-based therapies can effectively improve impaired renal function and morphology in animal models of CKD. These results can be used to optimize experimental models and interventions and thus improve preclinical research and support development of cell-based therapeutic interventions in a clinical setting.

When translating cell-based therapy to the clinic, we should consider that it would involve autologous transplantation. In our meta-analysis (chapter 5), only a study from our group tested cells that originated from uremic donors [4]. The latter study showed that injection of bone marrow cells derived from CKD rats was less effective possibly because CKD induces alterations in (paracrine) functions of these cells, suggesting that the uremic environment is detrimental for these cells. Previously, statins have been reported to

exert beneficial effects on endothelial as well as on bone marrow-derived endothelial progenitor cell and mesenchymal stem cell function both after in vitro incubation as after systemic in vivo treatment[5] by increasing NO-bio-availability, and anti-inflammatory and anti-oxidant effects. In Chapter 6 we demonstrated for the first time that bone marrow cell dysfunction in CKD can be reversed by short-term (2 hours) pre-treatment with pravastatin outside the CKD environment and that this effect persists when the cells are returned to the CKD environment, providing augmented therapeutic efficacy in vivo. This was reflected by better renal function and less GS and TI of CKD rats treated with healthy or pravastatin-treated CKD bone marrow cells, which had comparable renal function and injury. The beneficial effect of pravastatin can be explained by enhanced excretion of effective paracrine factors (such as CXCL5 and CXCL7). In contrast, systemic in vivo pravastatin treatment did not attenuate the progressive course of CKD. If confirmed for human CKD bone marrow cells, our findings will provide a basis for development of clinical trials and application of autologous cell-based therapies in human CKD[6].

Kidney transplantation offers the best treatment for ESKD patients. However, long-term results from kidney transplantation have not improved in the last years. In **Chapter 7** we investigated the mitochondrial uncoupling and oxygen metabolism in a model of isogenic kidney transplantation, with minimal cold and warm ischemia time (30 min each). We chose this model as it focuses exclusively on effects of ischemia/reperfusion on graft function and injury. Our results demonstrate increased mitochondrial uncoupling as an early event after experimental renal transplantation associated with increased oxygen consumption and kidney hypoxia in the absence of increases in markers of damage. Uncoupling of mitochondrial respiration was assessed in vitro, as the decrease in oxygen consumption that occurred after inhibition of uncoupling protein with guanosine diphosphate. Moreover, treating the donors for five days with mito-TEMPO (superoxide scavenger working in the mitochondria) reduced mitochondrial uncoupling but did not affect renal hemodynamics, oxygen tension or kidney injury. Our interpretation of these findings was that treatment with mito-TEMPO may have had limited efficacy, so that the kidney remained hypoxic because of the residual level of mitochondrial uncoupling. The mitochondrial uncoupling was associated with decreased renal oxygen consumption in the whole kidney and hypoxia in the renal cortico-medullary border. Importantly, at this 2-wk time point there was no evidence of oxidative damage (as assessed by renal content of protein carbonyls and urinary excretion of thiobarbituric acid-reactive substances), proteinuria, or impaired function in the transplanted kidney.

The above model describes 'ideal' kidney transplantation: transplanting a healthy donor kidney into a healthy recipient environment. However, we should take into account that kidney recipients have CKD, characterized by oxidative stress, inflammation and uremia, all factors that could influence the graft outcome. That is why we further explored the interaction between the kidney and its environment in a cross transplantation study that included healthy and CKD donors and recipients using the model described in Chapter 2. This study is described in **Chapter 8.** Our results demonstrate that transplantation of a healthy kidney to a CKD environment enhanced loss of glomerular endothelium and worsened TI and GS. Despite ischemia-reperfusion injury, TI damage and GS did not worsen in the CKD kidney grafts, possibly due to preconditioning[8,9].Healthy environment, on the other hand, was associated with preserved glomerular endothelium and decreased inflammation in the CKD graft but did not ameliorate TI damage and GS. Interestingly, preservation of glomerular endothelium was not associated with a difference in the number of GFP-positive cells or an increase in cell proliferation, suggesting that incorporation and proliferation of circulating recipient-derived cells is not a major factor in preservation of glomerular endothelium in CKD. This is consistent with our previous observations [4]. Within the time-span of the experiment the differences in glomerular endothelial and inflammatory cell numbers did not associate with corresponding changes in renal function, as pre-existent renal damage at the time of transplantation determined proteinuria and terminal renal function. Clearly, the ablated kidney used as a graft in our experimental study was far more severely injured than the kidneys from marginal living donors. Nevertheless, CKD environment persistently determined systemic injury, as reflected by TBARS excretion and aortic calcification, and this may have impacted on intrarenal endothelial integrity. Interventions to improve pre-existent vascular calcification, oxidative stress and inflammation prior to renal transplantation should be explored.

In **Chapter 9** we used the same model as in Chapter 8 to explore the effects of transplantation of healthy or CKD kidney on the recipient's hearts, more specifically focusing on LVH and cardiac fibrosis. Our results showed that diseased kidney grafts with impaired renal function increased MAP and LVH whereas healthy kidney grafts with normal renal function normalized MAP and reversed pre-existent LVH, in line with several clinical studies[10,11]. However, cardiac fibrosis and expression of CTGF, ANP, BNP and GFD11 were mainly determined by recipient history and barely influenced by the kidney graft showing that there is persistent irreversible cardiac fibrosis even after-

normalization of blood pressure after kidney transplantation. This finding could explain the better outcome results after pre-emptive (before dialysis) kidney transplantation.

In conclusion, the results in this thesis show the complex interaction between the kidney and its environment in the setting of CKD and kidney transplantation. It stresses that improvement of the systemic environment may be the key factor needed to halt both the progression of CKD and to minimize long-term damage to the renal graft.

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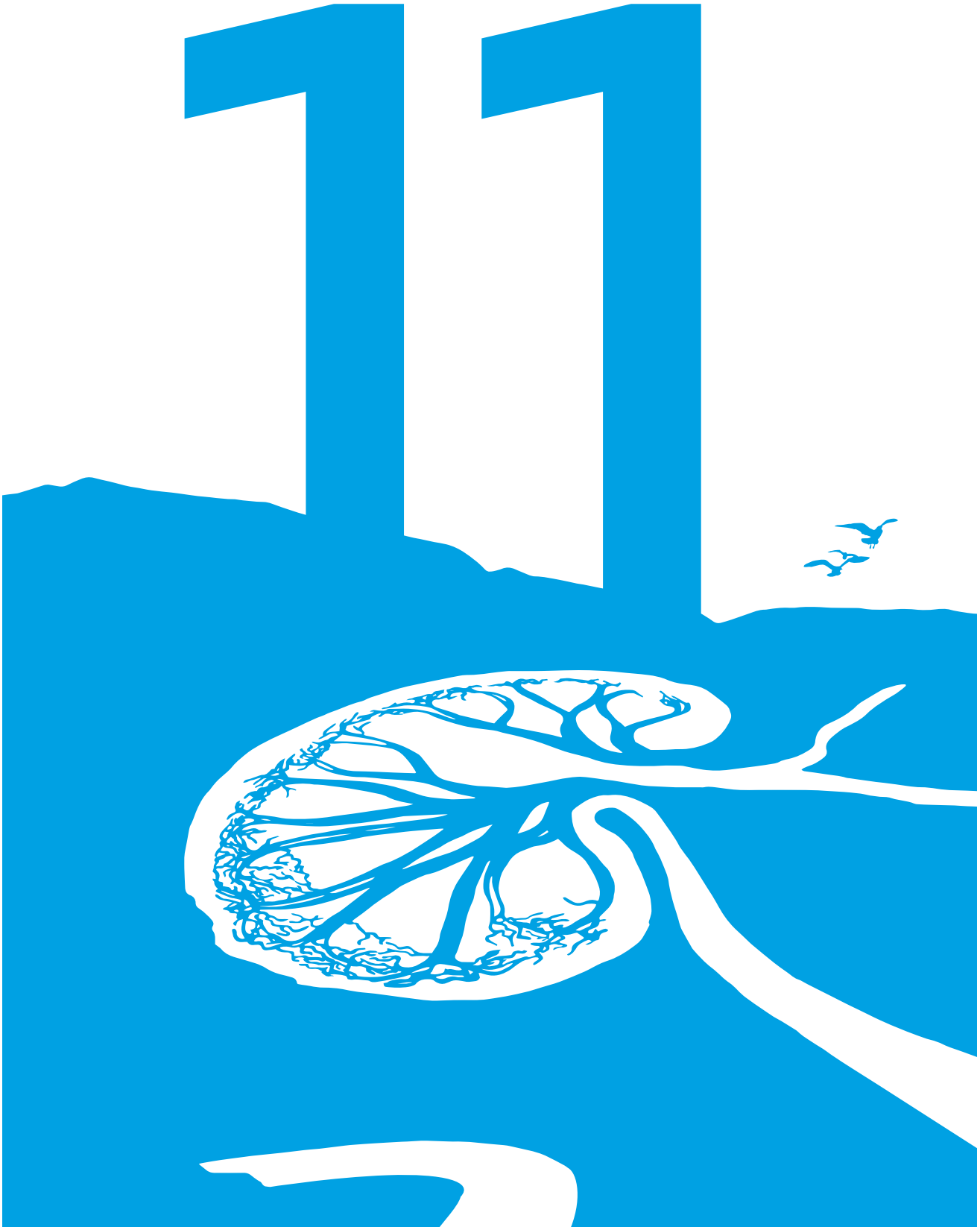


Appendix

Nederlandse samenvatting

Ondanks recente nieuwe inzichten omtrent de achterliggende oorzaken van chronische nierziekten (*Chronic Kidney Disease*, CKD) en de ontwikkeling van betere behandelingsmogelijkheden, neemt het aantal patiënten met nierfalen *(End Stage Kidney Disease, ESKD)* toe. Het doel van dit proefschrift is het onderzoeken van de interactie tussen de nier en zijn omgeving binnen CKD en niertransplantatie.

Om de interactie van de nier en de omgeving te kunnen bestuderen, zoals deze plaatsvindt in CKD en rondom niertransplantatie hebben we een nieuw diermodel ontwikkeld. Een gedetailleerde omschrijving van dit model wordt gegeven in **Hoofdstuk 2**. In dit model ontstaat in beide nieren tegelijk nierschade, als vervolgens een van de nieren getransplanteerd wordt, kan de andere nier vervolgens gebruikt worden als referentiekader voor weefselonderzoek.

In patiënten met CKD is het bekend dat er een toegenomen hoeveelheid schadelijke zuurstofradicalen (ook wel *Radical Oxygen Species*, ROS) in het lichaam aanwezig is, een toestand die oxidatieve stress genoemd wordt. Tevens is er een afname van anti-oxidatieve enzymen, de natuurlijke verdediging van het lichaam tegen oxidatieve stress. Hoewel het voor de hand zou liggen om patiënten met CKD antioxidanten, bijv. via het eten te geven, tonen klinische studies aan dat dit niet werkzaam is.
In **Hoofdstuk 3** onderzoeken we of bloeddruk afhankelijk is van ROS in het experimentele CKD model uit Hoofdstuk 2. In patiënten gaan hoge bloeddruk en CKD vaak samen, maar wat oorzaak en gevolg zijn, is onduidelijk. We hopen dat het diermodel opheldering kan geven.
Onze bevindingen laten zien dat behandeling met antioxidanten de bloeddruk verlaagt, maar alleen in gezonde dieren of dieren met milde nierschade. In dieren met bestaande CKD is er geen effect van anti-oxidanten op bloeddruk, en lijkt deze dus onafhankelijk van ROS

Bij patiënten met CKD en ESKD is plotselinge hartdood de voornaamste doodsoorzaak. Plotselinge hartdood doet zich voor vanwege hartritmestoornissen zoals bijvoorbeeld ventrikelfibrillatie, waarbij de spier van de hartkamers (ventrikels) niet meer gecoördineerd samentrekt. In **Hoofdstuk 4** onderzoeken we veranderingen in de ventrikels en het ontstaan van hartritmestoornissen in twee muismodellen van gecombineerd hart- en nierfalen, het cardiorenale syndroom. Eén model is in oudere muizen die worden behandeld met DOCA en zout, het andere in muizen met chirurgische nierschade (5/6de nefrectomie) en zout. We zien in beide diermodellen dat nierschade leidt tot struc-

turele afwijkingen in de hartkamers en tot elektrische geleidingsstoornissen, wat de dieren opvallend gevoelig maakt voor hartritmestoornissen.

De huidige behandeling voor CKD is symptomatisch en er bestaan momenteel geen specifieke therapieën tegen verdere toename van schade voorkomen. Dierstudies suggereren dat therapie met stamcellen de ontwikkeling van CKD kan afremmen.
Om de resultaten van vele dierstudies te bundelen opdat zij beter gebruikt kan worden voor het ontwikkeling van nieuwe patiënt- gerichte behandelingen voor CKD, hebben we een systematische review en meta-analyse uitgevoerd bij dierenstudies van cel gebaseerde therapieën. Dit staat beschreven in **Hoofdstuk 5**. Deze systematische review en meta-analyse bevestigt dat cel gebaseerde therapieën ef nierfunctie in verschillende dierenmodellen van CKD kunnen verbeteren. Eveneens hebben we onderzocht welk type stamcellen volgens de huidige stand van kennis het meest geschikt is voor een klinische vertaling.

Wanneer cel-gebaseerde therapie vertaald wordt naar de kliniek, zou ook rekening gehouden moeten worden met autologe transplantaties. Dit houdt in dat een patiënt behandeld wordt met zijn eigen stamcellen, om afstoting te voorkomen. Het is echter aangetoond dat het toedienen van beenmerg cellen van CKD ratten minder effectief is in het voorkomen van nierschade dan beenmerg cellen van gezonde ratten. Het is aannemelijk dat de in het lichaam opgehoopte afvalstoffen, de zgn. uremische toxines, schadelijk zijn voor de functie van stamcellen. In **Hoofdstuk 6** tonen we aan dat beenmerg cel stoornissen in CKD opgeheven kunnen worden middels korte termijn (2 uur) voorbehandeling van de cellen met pravastatine in een kweekschaal. Dit effect houdt vervolgens aan tot na de toediening van de cellen in het lichaam.

Niertransplantatie biedt de beste behandeling voor patiënten met nierfalen. Echter zijn de lange termijn resultaten van niertransplantatie de afgelopen jaren ondanks technische innovaties nauwelijks verbeterd. In **Hoofdstuk 7** onderzoeken we een mogelijk nieuw aangrijpingspunt om transplantaties te verbeteren door te kijken naar de rol van mitochondriën omtrent transplantatie. Mitochondriën zijn de 'kracht-centrales' van elke cel die voedingsstoffen en zuurstof omzetten in energie. Als de nier tijdens transplantatie wordt afgesneden van bloed en zuurstof, ontstaan er veel zuurstofradicalen die functie van mitochondriën verminderen. Omdat de mitochondriën minder efficiënt werken heeft de nier meer zuurstof nodig en ontstaat er een zuurstoftekort. Verder laten we zien dat we speciale mitochondriële antioxidanten (Mito-TEMPO) de

toegenomen zuurstofbehoefte van de nier kunnen beperken. Dit leidde in onze proef echter niet tot een verbeterde nierfunctie na transplantatie.

Het bovenstaande diermodel beschrijft een 'ideale' niertransplantatie: het transplanteren van een gezonde nier in een gezonde "patiënt". Echter moet er rekening gehouden worden met een ontvanger met CKD, met bijbehorende oxidatieve stress, infecties en uremische toxines. Al deze factoren kunnen de functie van de getransplanteerde nier beïnvloeden. In **Hoofdstuk 8** hebben we aangetoond dat de CKD omgeving (TBARS excretie en vasculaire verkalking) bepalend is voor de functie van de getransplanteerde nier. Interventies om de reeds bestaande vasculaire verkalking, oxidatieve stress en chronische inflammatie te verbeteren voorafgaand aan de renale transplantatie zouden verkend moeten worden.

In **Hoofdstuk 9** hebben we hetzelfde model toegepast als in Hoofdstuk 8 om de effecten van transplantatie van ofwel een gezonde of een CKD nier op het hart van de ontvanger te onderzoeken, specifiek gericht op hypertrofie van de linker ventrikel en hartfibrose. Onze resultaten laten zien dat transplantatie van een CKD nier leidt tot een toename van de bloeddruk en pathologische vergroting (hypertrofie) van de linker ventrikel, zelfs als de ontvanger voorheen gezond was. Transplantatie van een gezonde nier leidt juist tot een daling van de bloeddruk en kan linker ventrikel hypertrofie omkeren.

Samenvattend demonstreren de resultaten van dit proefschrift de complexe interactie tussen de nier en omgeving binnen de CKD en niertransplantatie. Het benadrukt dat verbetering van de systemische omgeving de sleutelfactor kan zijn voor het stoppen van het verslechteren van CKD en voor het minimaliseren van de lange termijn schade aan de renale graft.

Резюме на български

Въпреки изясняването на значими фактори за прогреса на хроничната бъбречна недостатъчност (ХБН) и разработването на нови терапии, броят на пациентите, нуждаещи се от диализа и/или трансплантация, значително нараства. Целта на тази дисертация е да се изследва взаимодействието между бъбреците и системната среда в условията на ХБН и ренална трансплантация.

За целта ние създадохме нов, билатерален експериментален модел на ХБН, който е описан подробно в **Глава 2**. Този модел позволява да се оцени степента на увреждане на бъбрека в момента на трансплантиране като контралатералният бъбрек служи за протоколен биопт.

При пациенти с ХБН, нивото на оксидативен стрес е повишено и активността на антиоксидантните ензими е занижена. Въпреки това, клинични проучвания в тази област не показват бенефициални ефекти от терапия с антиоксиданти сред пациентите с ХБН. В **Глава 3** изследвахме дали кръвното налягане е зависимо от свободни кислородни радикали (супероксиден анион и водороден пероксид) като за целта използвахме експерименталния модел на ХБН, описан в Глава 2. Въпреки, че в ранните фази на ХБН антиоксидантната терапия може да предотврати покачване на кръвното налягане, тя не е ефективна в късните статии на ХБН за неговото редуциране.

За пациентите с терминална ХБН внезапна сърдечна смърт е най-честата причина за леталитет. Внезапна сърдечна смърт настъпва поради аритмии, например камерно трептене. В **Глава 4** изследвахме сърдечното ремоделиране и възникването на аритмии в два модела на кардиоренално заболяване: при възрастни мишки, подложени на дезоксикортизонацетат и сол и мишки, подложени на 5/6-нефректомия и сол. Бъбречната дисфункция и в двата модела предизвика структурно ремоделиране на сърцето и значително повишена чувствителност към аритмии.

Терапиите за ХБН са симптоматични и досега няма специфични такива, целящи да спрат нейния прогрес. Преклинични проучвания показват бенефициални ефекти от клетъчна терапия. За да се улесни транслирането на резултатите от преклиничните проучвани в клиниката, проведохме систематично ревю и мета-анализ (**Глава 5**) на преклинични изследвания върху ефектите на клетучна терапия върху ХБН. Този мета-анализ потвърди твърдението, че клетъчната терапия подобрява бъбречната функция и морфология в различни преклинични модели на ХБН. Тези резултати могат да бъдат използвани за оптимизиране на експериментални модели и интервенции и

по този начин за подобряване на преклиничните проучвания и подпомагане развитието на клетъчни терапевтични интервенции в клинични условия.

При транслацията на клетъчната терапия в клинични условия, трябва да се вземе предвид, че трансплантацията на клетки ще бъде автоложна. Доказано е, че добавяне на клетки от костен мозък от плъхове с ХБН са по-малко ефективни в сравнение с костномозъчни клекти от здрави плъхове, най-вероятно поради факта, че ХБН индуцира промени в паракринните функции на тези клетки. В **Глава 6** показваме за първи път, че дисфункцията на костномозъчни клетки при ХБН може да бъде предотвратена чрез кратко (2 часа) третиране с правастатин ин витро и че този ефект персистира след аминистрация в среда на ХБН ин виво. От друга страна, терапия с правастатин ин виво не намалява прогресивния ход на ХБН.

Бъбречната трансплантация е най-ефективното лечение за пациенти с терминален стадий на ХБН. Въпреки това, дългосрочните резултати от бъбречна трансплантация не са се подобрили през последните години. В **Глава 7** изследвахме митохондриалната дисоциация (mitochondrial uncoupling) и кислородния метаболизъм в модел на изогенна бъбречна трансплантация, с минимални периоди на исхемия. Нашите резултати показват повишена митохондриална дисоциация като ранно събитие след бъбречна трансплантация, повишена консумация на кислород и бъбречна хипоксия при липсата на повишен оксидативен стрес. Терапия с mito-Tempo (препарат, който неутрализира супероксидните аниони в митохондриите) бе с ограничена ефикасност, което вероятно се дължи на остатъчното ниво на митохондриална дисоциация.

Гореописаният модел описва идеалната бъбречна трансплантация: трансплантиране на здрав бъбрек в здрав реципиент. Трябва да се вземе под внимание обаче, че реципиентите с ХБН се характеризират с повишено ниво на оксидативен стрес, хронично възпаление и уремия: фактори, които мога да повлияят върху структурата и функцията на графта. Резултатите от експериментите, описани в **Глава 8**, показват как системната среда (съдова калцификация, оксидативен стрес) при ХБН е определяща за евентуалното увреждане на графта, и че това има влияние върху интрареналния ендотел. Интервенциите за подобряване на съдова калцификация, оксидативен стрес и възпаление преди бъбречна трансплантация следва да бъдат проучени.

В **Глава 9** използвахме същия модел, описан в Глава 8, като се фокусирахме върху ефектите от трансплантазия на здрав или субоптимален бъбрек върху сърцето на реципиента, по-спефиално върху параметрите левокамерна

хипертрофия и сърдечна фиброза. Нашите резултати показват, че субоптималните бъбречни графтове с намалена функция водят до увеличено кръвно налягане и повишена левокамерна дисфункция, докато здрав бъбрек с нормална фукнция нормализира кръвното налягане и води до намаляване на вече съществуващата левокамерна хипертрофия.

В заключение, резултатите, описани в тази дисертация, показват взаимодействието между бъбреците и системната среда в условия на ХБН и ренална трансплантация. Това подчертава, че подобряването на системната среда може да бъде ключов фактор за спиране прогреса на ХБН и свеждане до минимум на дългосрочното увреждане на бъбречната присадка.

Acknowledgements

This thesis wouldn't have been possible without the help and support of many.

Prof. dr. Verhaar, beste Marianne, bedankt voor de mogelijkheid om promotieonderzoek in jouw lab te mogen doen. In de afgelopen paar jaren heb ik ontzettend veel van je geleerd. Bovendien, steeds wanneer ik een nieuw plan of experiment bedacht heb je mij altijd in de juiste richting geholpen. Bedankt voor je steun en vertrouwen in mij.

Dr. Joles, Jaap! Een betere copromotor zou ik niet kunnen wensen. Alles wat ik heb geleerd over fundamenteel onderzoek heb ik geleerd van jou. Ik heb ontzettend genoten van onze discussies, van pathofysiologie tot hiking.

Leden van de beoordelingscommissie. Prof. dr. Goldschmeding, prof. dr. Masereeuw, prof. dr. Van Rijen, prof. dr. Bleys, prof. dr. Ploeg: bedankt voor uw tijd en interesse in mijn proefschrift.

Co-authors. Malou Friederich-Persson, Magda Fontes, Arjan van Zuilen, Maarten Koeners and Maarten Jan Cramer, thank you for the fruitful discussions and your contributions to this thesis. Raechel Toorop, dankzij jou kon de beenmergstudie in niertransplantatie-patiënten vlot beginnen.

Dept. Pharmacology at Medical University – Sofia. Prof. dr. Krassimira Yakimova and dr. Dilyan Ferdinandov, thank you for introducing me to research. I spent three wonderful years in your lab during my studies and ever since I performed the first experiment I knew I wanted to continue working in science.

De **experimentele microchirurgen** van CDP in Groningen en in het bijzonder Annemieke Smit-van Oosten, bedankt voor de mogelijkheid om de niertransplantatie in ratten te mogen leren onder jullie begeleiding.

Paranimfen. Petra, alles wat ik hier zou schrijven, zou niet genoeg zijn. Jouw lach, vriendelijkheid en bezorgdheid hebben mij vanaf het begin gewonnen. Ik kon altijd naar je toe, no matter what, of het voor honderden albumine bepalingen was of gewoon kletsen en opluchten. De 'martenitsa' zal ik nooit vergeten! Hendrik, we entered the lab more or less at the same time and we have been friends ever since. It was great to have a partner in crime for a micaffe brunch before starting work, for a coffee break anytime and for a white russian or two while submitting a manuscript late at night.
Thank you both for being beside me on this day.

The lab. Maarten Rookmaaker, Rachel Giles, Caroline Cheng, Bas van Balkom en Joost Fledderus, bedankt voor de nuttige input tijdens werkbesprekingen. Adele en Nel, bedankt voor alle klaringen die jullie altijd snel bepaald hebben. Chantal en Marloes, ik denk met veel plezier terug aan de gezelligheid met jullie op het lab. Krista, bedankt voor alle kleuringen en hulp met de microscoop. Paula, met jouw geduld en nauwkeurigheid zijn alle dierproeven een succes geworden. Ik ben dankbaar dat je mij hebt geholpen tijdens deze jaren en dat jij de eerste was die met mij in het Nederlands bleef praten. Sebas, thanks for explaining Sigmaplot. Pradeep, the social activity organizer, seems that the party scene in UMCU moved with you to Switzerland.

GDL medewerkers: Helma, Suzane, Nico, Jan en veel anderen, bedankt voor de ondersteuning van mijn experimenten en de goede zorg voor mijn dieren. In het bijzonder wil ik Anja bedanken voor alle tips, hulp en reddende acties op de microchirurgische ok. De compliance officers, Romy en Ron, bedankt voor het zorgvuldig nakijken van alle protocollen, soms met spoed.

Alle mede-promotieonderzoekers: Nynke, bedankt voor alle histologie die je ontzettend zorgvuldig hebt verricht, succes met afronden van je boekje! Gisela, veel succes in Keulen! Tim, thanks for proofreading chapter 7. Femke, leuk dat je de beenmerg studie hebt overgenomen, ben benieuwd naar de resultaten. Eva, Ira, Claire, Margreet, Frans, Tonja, Rosa, Maarten W, congressen en etentjes waren een stuk gezelliger met jullie. Chris en alle nieuwe mensen, succes met afronden van jullie promotie onderzoek.

Studenten. Saskia Oosterbroek , Joost Jansen, Martijn Van Nieuwburg en Julie Mulders (allemaal geen studenten meer), bedankt voor jullie inzet, nieuwsgierigheid en bijdrage aan dit proefschrift. Ook wil ik Danny, Summeye, Frans, Robin en veel andere studenten bedanken die altijd klaar stonden om een extra kleuring of telling te doen.

The roomies. Arie, I missed you much since you left our small office. For you being a morning person and me an evening person we were an incredibly good combi during the nefrologiedagen. Thank you for all the help throughout my PhD. Martin, I admire your work attitude, you managed to focus and write even during busy conversations about shopping. Good luck with the surgical residency. Ollie, altijd klaar om je collega's te helpen of te brainstormen over rare resultaten. Je bent een zeer goede onderzoeker en ik wens je veel succes met de postdoc. Dimi, je kan altijd iets verzinnen om een dag op het lab vrolijker te maken, succes met de geneeskunde opleiding. Dries, you had the

honour to own the only cardio spot in our room, I enjoyed all our conversations about PhD-life.

Cardio people. Fre, always ready to help, talk and laugh (and drink more tequila), I appreciate your friendship and wish you best of luck in your career. Judith, feestbeest, we need to make more crapberries soon. Krijn, Alain, Sandra, Sanne and many more, thanks for all vrijdag middag borrels. Arjan, Sander and Loes, thank you for all your tips and the possibility to always borrow a bit of this and that. Roberto, the other foreigner, I enjoyed your dinners and all our conversations late in the empty lab.

Mijn huidige collega's en chirurgen van de **Thoraxchirurgie UMCG** wil ik bedanken voor alle leermomenten, interesse in mijn onderzoek en de ruimte om soms een vrije dag te nemen om dit boekje af te maken of een congres te bezoeken. En, natuurlijk, de gezellige vrimibo's!

Utrecht community. I have been very fortunate to meet wonderful people in Utrecht during my PhD period. Claudia, Frie, Judith & Antonis, Nacho, Babis, Jan, Giannis, Bettina, Eduardo and many others, thank you for all the summer reunion holidays, all the laatste rondes in café België and these truly amazing times in Utrecht and around. Looking forward to many more. The one and only Daniel Roberts, I have never met such fun- and life-loving person! Utrecht is not the same since you moved to Hong Kong. Francesca, my friend from the very first moment in the Netherlands, thank you for the support during these years, for sharing all the PhD drama, for laughing at our clashes with the Dutch culture and for bringing pizza in the GDL late in the evening.

The Hartveldts. Wat een avontuur was het om jullie huisgenoot te zijn! Sam, bedankt dat je me in de weekenden (letterlijk) uit bed gesleept hebt en mee naar feestjes nam. Stefan, het surfkamp in Mimizan was awesome! Veel succes met het afronden van je geneeskunde opleiding, je word zonder twijfel een geweldige arts.

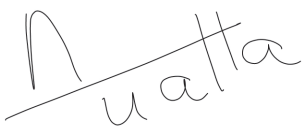
The Bulgarians. Kate, you were great support for me these years. Thank you, Ivaylo and your two lovely boys for the hospitality and great times in Brussels. Eli, I still can't believe that you moved temporarily to Utrecht and we were living even closer to each other than in Sofia! I hope you and Alex will return to the Netherlands some day. Kate and Asen, Desi (great job with proofreading the bulgarian summary!), Gale, Mime, Sonya, thank you for not giving up on me even though my time at home is getting less and less. I wish I can spend

more time with you in the future. Silvia, my roomie from med school and a true friend, I am very happy to have you in my life. I would love to revive the tradition of the Amaretto nights. Albena and Dragan, officially Amsterdammers now, Nate, Vasko, Hrisi, Yassar, Vera, Ivan and Deni, my friends from med school, it is always a pleasure.

My family in Bulgaria. Mishe, my niece, seems like yesterday you were a baby and now you are entering your first year at uni! I am proud of you. My youngest nieces, Mariya and Yoana, you are only 1 year old now, I am looking forward to see you growing. My sister Sashka, thank you for being a true friend, wish you and Plamen happiness and patience with the twins. My aunt Emiliya and uncle Valeri, thank you for your hospitality and kindness. Mariya and Atanas, my parents, I am grateful to you for raising me to be the person who I am now, for giving me unconditional love and teaching me to never ever give up. You always supported me even though I never quite listened to your advice 'don't move abroad' and you never quite understood what exactly I was doing in this lab in the Netherlands.
Мамо и татко, дано и вие се гордеете с мен така, както и аз се гордея с вас!
Липсвате ми.

Jurrian, zonder jouw steun en liefde was het afmaken van dit boekje niet gelukt. Ik ben benieuwd wat de toekomst ons brengt.

August 13th 2015
Gotse Delchev



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- Maintenance of Hypertensive Hemodynamics Does Not Depend on ROS in Established Experimental Chronic Kidney Disease.
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Submitted

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In preparation

- Dissociation between left ventricular hypertrophy and cardiac fibrosis after experimental kidney transplantation.
  Papazova D. A., Oosterhuis N. R., Cramer M.J., Joles J.A. and Verhaar M. C.
- The contribution of T-cells to hypertension and renal injury in mice with subtotal nephrectomy.
  Oosterhuis N. R., **Papazova D. A.,** Gremmels H., Joles J. A. and Verhaar M. C.

Curriculum Vitae

Diana Atanasova Papazova was born on December 1, 1984 in Gotse Delchev, Bulgaria. After completing her secondary education in 2003, she obtained her MD cum laude at the Medical University of Sofia in 2009. Her interest in pathophysiology and microsurgery brought her to UMC Utrecht, where she started a PhD project under the supervision of prof. dr. Marianne Verhaar and dr. Jaap Joles. The results of this project are described in this thesis.