Salt, Acid, and the Renin-Angiotensin System in Chronic Kidney Disease

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Salt, Acid, and the Renin-Angiotensin System in Chronic Kidney Disease

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CONTENTS

Chapter 1

Introduction and
aims of the thesis

KIDNEY REGULATION OF SALT AND ACID-BASE HOMEOSTASIS

Maintaining salt and acid-base homeostasis is essential to the survival of all species. In terrestrial mammals, the kidneys play a central role in maintaining this homeostasis. The kidneys regulate salt balance by adjusting sodium reabsorption along the kidney tubule to dietary intake. Kidney regulation of salt balance serves to maintain the extracellular fluid volume and blood pressure. Maintenance of acid-base balance is orchestrated by the urinary excretion of non-volatile acids and the reclamation of the filtered base bicarbonate. The normal regulation of salt and acid-base balance is disturbed in patients with kidney disease. The overarching term for any type of kidney disease that persists for more than three months is chronic kidney disease (CKD). CKD is classified based on the estimated glomerular filtration rate and the degree of albuminuria.[1] CKD impacts salt and acid-base homeostasis and may result in salt-sensitive hypertension and metabolic acidosis, two common complications of CKD.

SALT-SENSITIVE HYPERTENSION IN CKD

The prevalence of hypertension in CKD reaches nearly 100% when the stage of kidney failure is reached (eGFR < 15 ml/min/1.73 m^2).[2] Hypertension in CKD is associated with an increased risk of cardiovascular disease and further progression of CKD. A main limitation in the development of new treatment strategies for hypertension in CKD is the fact that its pathophysiology is still incompletely understood. Similar to hypertension in patients without CKD, the origin of hypertension of CKD is multifactorial. A key element of its pathophysiology is a rightward shift and flattening of the pressure-natriuresis curve.[3] In individuals with healthy kidneys, increased dietary salt intake affects blood pressure only minimally, because excess salt is excreted by increasing natriuresis. In CKD, however, a greater increase in blood pressure is necessary to excrete the same amount of salt. The reasons why CKD disturbs the pressure-natriuresis curve includes nephron loss with single-nephron hyperfiltration, disturbed glomerulotubular balance, and the presence of anti-natriuretic factors. Thus, CKD causes a salt-sensitive form of hypertension, indicating that blood pressure becomes sensitive to changes in dietary sodium intake. Of note, salt-sensitivity is associated with increased mortality even without the presence of hypertension or CKD.[4]

Fine-tuning of kidney sodium regulation takes place in the distal part of the nephron. Here, specific sodium transporters are present that are under the control of the renin-angiotensin and sympathetic nervous systems. More recently it has also become clear that changes in plasma potassium modify the activity of sodium transporters.[5-7] The main

apical sodium transporters in the distal nephron are the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC). Experimental studies in animals and patients indicate that dysregulation of the renin-angiotensin system (RAS), including increased plasma aldosterone and kidney angiotensin II, contributes to hypertension in CKD.[8-12] Other studies suggest increased activity of NCC and ENaC in CKD.[13-15] However, how the effects of angiotensin II, aldosterone and plasma potassium impact sodium and blood pressure regulation in CKD is incompletely understood. ***Chapter 2*** reviews the current understanding of the pathogenesis of salt-sensitive hypertension in CKD with a focus on distal tubular mechanisms. In ***Chapter 3***, a rat model of CKD and hypertension (the 5/6th nephrectomy model) is used to study the effect of dietary salt on RAS inhibition. A promising novel approach to target the RAS is the use of siRNA-based therapeutics against liver-derived angiotensinogen. This experimental treatment is reviewed in ***Chapter 4***. In ***Chapter 5***, siRNA targeting of angiotensinogen is used to analyze the effect on hypertension in a rat model of CKD (again using the 5/6th nephrectomy model).

Clinically, hypertension in CKD is often resistant to treatment with drugs or dietary interventions such as salt restriction. Possibly, non-adherence to medication and the difficulty to adhere to dietary restrictions for longer periods of time restricts treatment efficacy. In case of treatment with diuretics, limiting factors to treatment efficacy may also include reduced tubular secretion of diuretics during CKD.[16] However, anecdotal evidence suggests that thiazide-diuretics may actually still be effective for the treatment of hypertension in CKD.[17-22] Therefore, in ***Chapter 6***, we report the results of a randomized controlled trial in which we compared the effects of the distal diuretics hydrochlorothiazide and amiloride with dietary sodium restriction on blood pressure in patients with CKD.

A specific kidney disease in which the RAS may contribute to hypertension is autosomal dominant polycystic kidney disease (ADPKD). ADPKD leads to progressive cyst formation, early onset salt-sensitive hypertension, and CKD.[23] In the kidney, the progressive growth of cysts compresses normal kidney tissue, which in turn may cause ischemia and increase renin secretion.[24-26] Renin has also been measured in cyst fluid and previous data suggest that local production occurs by epithelial cells lining the cysts.[24, 25] However, measurements of plasma renin and aldosterone in patients with ADPKD did not consistently show elevated levels.[23, 27-37] Possibly, therefore, ADPKD may specifically promote intrarenal production of angiotensin II. Supporting this hypothesis, two studies showed that the urinary excretion of angiotensinogen, a precursor of angiotensin II, is increased in patients with ADPKD and hypertension.[36, 37] However, if the urinary excretion of RAS components truly reflects increased intrarenal angiotensin II activity remains uncertain.[38] Therefore, in ***Chapter 7***, blood and urinary parameters of the RAS

were analyzed in patients with ADPKD and compared to patients with CKD; in a number of ADPKD patients who underwent nephrectomy, renin was also measured in cyst fluid.

METABOLIC ACIDOSIS OF CKD

Metabolic acidosis is present in up to 40% of patients with advanced CKD.[39] Similar to hypertension, metabolic acidosis in CKD is associated with increased cardiovascular and all-cause mortality.[40] Moreover, clinical studies show that correction of metabolic acidosis in CKD slows disease progression, although the evidence-level was classified as low-to-moderate certainty in a recent meta-analysis and systematic review.[41] How metabolic acidosis contributes to adverse outcomes in CKD is incompletely understood. A prevailing hypothesis is that initial adaptive responses to increase ammoniagenesis may become maladaptive and contribute to kidney injury.[42] Data from animal studies suggest a role for angiotensin II, aldosterone and endothelin-1 in the pathogenesis of acidosis-induced kidney injury.[43-46] Furthermore, complement activation secondary to increased ammoniagenesis may contribute to inflammation and subsequent fibrosis.[47] However, at present, it is unclear if these mechanisms are also present in patients with CKD. To address this, we analyzed the response to an acute oral acid load in patients with CKD and healthy subjects (***Chapter 8***), and analyzed the effect of 4-week alkali treatment in patients with CKD and metabolic acidosis on the RAS (***Chapter 9***).

AIMS OF THE THESIS

To review the pathogenesis of salt-sensitive hypertension in chronic kidney disease (CKD) with a focus on distal tubular mechanisms (***Chapter 2***)

To analyze the role of dietary salt in the antihypertensive response of renin-angiotensin system (RAS) inhibition in a rat model of CKD and hypertension (***Chapter 3***)

To review a novel approach of RAS inhibition that targets angiotensinogen with RNA-based therapeutics (***Chapter 4***)

To test the antihypertensive effect of siRNA directed against angiotensinogen in a rat model of CKD and hypertension (***Chapter 5***)

To compare the antihypertensive effects of distal diuretics and dietary salt restriction in patients with CKD and hypertension (***Chapter 6***)

To compare blood and urinary parameters of the RAS in patients with polycystic kidney disease and patients with CKD (***Chapter 7***)

To characterize the differences between the responses to an acute oral acid load in patients with CKD and healthy subjects (***Chapter 8***)

To analyze the effect of alkali treatment with sodium bicarbonate on the RAS in patients with CKD and metabolic acidosis (***Chapter 9***)

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Chapter 2

Salt-sensitive hypertension in chronic kidney disease: distal tubular mechanisms

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ABSTRACT

Chronic kidney disease (CKD) causes salt-sensitive hypertension that is often resistant to treatment and contributes to the progression of kidney injury and cardiovascular disease. A better understanding of the mechanisms contributing to salt-sensitive hypertension in CKD is essential to improve these outcomes. This review critically explores these mechanisms by focusing on how CKD affects distal nephron sodium (Na^+) reabsorption. CKD causes glomerulotubular imbalance with reduced proximal Na^+ reabsorption and increased distal Na^+ delivery and reabsorption. Aldosterone secretion further contributes to distal Na^+ reabsorption in CKD and is not only mediated by renin and potassium, but also by metabolic acidosis, endothelin-1, and vasopressin. CKD also activates the intrarenal renin-angiotensin system (RAS) generating intratubular angiotensin II to promote distal Na^+ reabsorption. High dietary Na^+ intake in CKD contributes to Na^+ retention by an aldosterone-independent activation of the mineralocorticoid receptor mediated through Rac1. High dietary Na^+ also produces an inflammatory response mediated by T helper 17 cells and cytokines increasing distal Na^+ transport. CKD is often accompanied by proteinuria, which contains plasmin capable of activating the epithelial Na^+ channel. Thus, CKD causes both local and systemic changes that together promote distal nephron Na^+ reabsorption and salt-sensitive hypertension. Future studies should address remaining knowledge gaps, including the relative contribution of each mechanism, the influence of sex, differences between stages and etiologies of CKD, and the clinical relevance of experimentally identified mechanisms. Several pathways offer opportunities for intervention, including with dietary Na^+ reduction, distal diuretics, RAS-inhibitors, mineralocorticoid receptor antagonists, and potassium or hydrogen ion binders.

INTRODUCTION

The Global Burden of Disease Study recently reported that the 2017 global prevalence of chronic kidney disease (CKD) was 9.1%, a percentage that has increased by 29% since 1990 [1]. The death toll of CKD was 1.2 million, and CKD contributed to another 1.4 million cardiovascular disease-related deaths [1]. The disease burden of CKD was found to be highest in countries with a lower socio-demographic index [1]. There was also inequality between the sexes, with the age-standardized prevalence of CKD 1.29 times higher in females than males, yet, mortality was 1.39 times greater in males than females [1]. Thus, CKD represents an important global health issue that requires attention not only from public health and clinical medicine, but also from basic science, including the physiological sciences.

CKD is defined as kidney damage or a glomerular filtration rate (GFR) < 60 ml/min for three months or more, irrespective of cause [2]. The most common causes of CKD are type 2 diabetes and hypertension, while inherited and auto-immune diseases are less common causes of CKD. CKD reduces nephron number which often results in a progressive decline in GFR. CKD is also frequently accompanied by albuminuria, indicating damage to the glomerular filtration barrier. CKD disturbs normal kidney physiology and will therefore impact on the kidney's central role in sodium (Na^+) homeostasis and blood pressure regulation [3]. Indeed, CKD is not only a consequence of hypertension, but also contributes to hypertension. Accordingly, the prevalence of hypertension increases as CKD progresses, reaching nearly 100% in CKD stage G5 (GFR < 15 ml/min) [4]. Furthermore, hypertension in CKD is often uncontrolled and resistant to treatment meaning that blood pressure targets are not achieved despite three anti-hypertensive drugs including a diuretic [5]. Hypertension in CKD introduces a vicious cycle as it contributes to the progression of CKD and the high cardiovascular risk in this patient population. Therefore, a clearer understanding of the pathophysiology of hypertension in CKD is essential to improve treatment and outcomes.

In a large CKD cohort study higher extracellular water (measured by bioimpedance spectroscopy), older age, and higher albuminuria were identified as independent determinants of uncontrolled and resistant hypertension in patients with CKD [6]. The association between extracellular water and hypertension indicates that hypertension in CKD is accompanied by an increase in the extracellular fluid volume. Because extracellular fluid volume expansion implies Na^+ retention, hypertension in CKD can be classified as a salt-sensitive form of hypertension [7, 8]. In fact, some have argued that kidney dysfunction is a requirement for all forms of salt-sensitive hypertension [9]. In this view, kidney

dysfunction impairs pressure natriuresis and secondarily increases vascular resistance and therefore blood pressure.

In normal kidney physiology, glomerulotubular balance and tubuloglomerular feedback maintain a constant Na^+ delivery to the distal nephron [10]. Because the distal nephron fine-tunes Na^+ reabsorption, salt-sensitive hypertension is often caused by increased distal nephron Na^+ reabsorption [11]. The distal nephron is defined as the nephron segments originating after the macula densa and consists of the distal convoluted tubule, connecting tubule, and collecting duct [12]. The term "distal tubule" is used differently in different contexts, but here we use it to refer to the region between the macula densa and the initial collecting tubule [12]. The major Na^+ transport proteins in the distal nephron are the Na^+-chloride (Cl^-) cotransporter (NCC), the epithelial Na^+ channel (ENaC), Na^+-dependent bicarbonate exchanger, and Na^+-K^+-ATPase [11]. Importantly, there is a close interaction between ion transport in the distal nephron, with several interdependencies between Na^+, Cl^-, and potassium (K^+) transport [13, 14]. The majority of the Na^+ transport proteins in the distal nephron are regulated by angiotensin II and aldosterone, and this portion of the nephron is therefore sometimes referred to as the aldosterone-sensitive distal nephron [11, 15, 16]. In addition, it is becoming increasingly clear that basolateral channels in the distal nephron sense ambient Na^+ and K^+ concentrations and the accompanying change in membrane potential. In turn, these signals can be relayed to modify NCC and ENaC activity [13, 17, 18].

Although the mechanisms of salt-sensitive hypertension have been studied extensively in humans and animals with normal kidney function, remarkably little has been reported on the kidney mechanisms of salt-sensitive hypertension in CKD. In this review, we will discuss six mechanisms that focus on distal nephron Na^+ handling, including (1) CKD-induced changes in distal nephron Na^+ handling, (2) renin-independent aldosterone secretion, (3) activation of the intrarenal renin-angiotensin system (RAS), (4) the role of dietary salt, (5) metabolic acidosis, and (6) proteinuria-induced Na^+ reabsorption (**Figure 1**). Although the reviewed literature provides a relatively clear picture of the mechanisms contributing to salt-sensitive hypertension in CKD, including their interactions, several knowledge gaps remain and are to be addressed in future research (**Table 1**). While this review focuses on the distal tubular mechanisms of salt-sensitive hypertension in CKD, it is important to emphasize that other mechanisms also mediate hypertension in CKD, including reduced GFR, an activated central or sympathetic nervous system, and altered vascular reactivity [19].

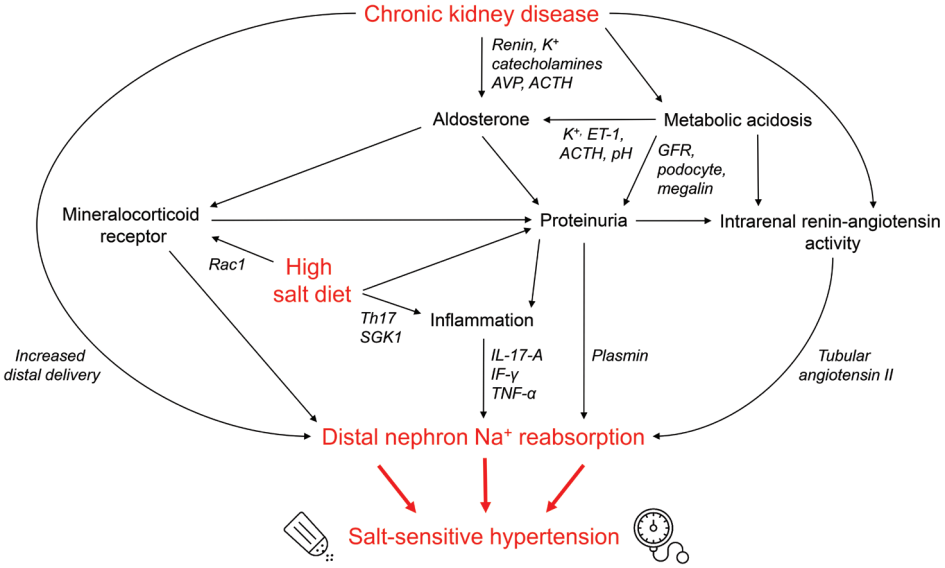

Figure 1. Working hypothesis for how chronic kidney disease increases distal nephron sodium (Na^+) reabsorption to cause salt-sensitive hypertension. The key elements are shown in red font. Arrows indicate a stimulatory effect. If known, mediators of the indicated effects are shown in italic font. See text for details. ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; ET-1, endothelin-1; GFR, glomerular filtration rate; IF-γ, interferon gamma; IL-17-A, interleukin-17-A; K^+, potassium; SGK1, serum and glucocorticoid-induced kinase 1; Th17, T helper 17 cells; TNF-α, tumor necrosis factor alpha.

CKD-INDUCED CHANGES IN DISTAL NEPHRON NA^+ HANDLING

Most experimental studies investigating the interactions between CKD, Na^+ handling, and blood pressure have relied on CKD models in rats or mice. Although several experimental models for CKD exist, a common model is 5/6th nephrectomy in which uninephrectomy is followed by polar excision or renal artery ligation of the remaining kidney (ablation or ligation model) [20, 21]. These so-called subtotal nephrectomy or remnant kidney models recapitulate human CKD, because they are characterized by low GFR, proteinuria, and hypertension. Indeed, incremental reductions in kidney mass increase the incidence and severity of hypertension in rats fed a high Na^+ diet [22, 23]. Conversely, a low Na^+ diet prevents hypertension in rats after 5/6th nephrectomy [24]. Of note, the method used to reduce kidney mass determines whether hypertension develops [25]. The ligation model typically generates hypertension, whereas this is not always the case for the ablation model. This suggests that the ablation model requires additional factors such as a high salt diet or further progression of CKD over time for hypertension to develop. CKD causes anatomical and functional adaptations in surviving nephrons, including elevations in single-nephron GFR and tubular hypertrophy [26, 27]. After 5/6th nephrectomy fractional Na^+

reabsorption in the proximal tubule is reduced [28]. In a model of anti-glomerular basement membrane glomerulonephritis, Na^+ delivery was higher at the end of the proximal tubule but lower at the end of the collecting duct [29]. Thus, CKD appears to cause glomerulotubular imbalance with a shift of Na^+ reabsorption from the proximal to the distal nephron. A modeling study indeed predicted that uninephrectomy or 5/6th nephrectomy increases the protein density of the Na^+-K^+-Cl^- cotransporter (NKCC2), NCC, ENaC, and Na^+-K^+-ATPase [26]. The three studies that have analyzed kidney Na^+ transporters in rats with CKD are largely in agreement with these predictions, although changes over time in individual transporters were observed [27, 30, 31]. When using other maneuvers to increase distal Na^+ delivery, such as loop diuretics, the distal nephron undergoes ultrastructural changes that coincide with a 4- to 5-fold increase in Na^+ reabsorption capacity [32, 33]. In this regard, increased Na^+ reabsorption by the distal nephron is a function of increased delivery. In addition, increased tubular flow may also directly increase transporter activity [34]. Although this is most evident for K^+ secretion (flow-mediated kaliuresis), a number of studies indicate that Na^+ reabsorption through ENaC can also be regulated by fluid shear stress and mechanosensory mechanisms [35-37]. Thus, from the viewpoint of balance, it seems logical that distal nephron Na^+ reabsorption increases in CKD to maintain Na^+ balance. However, this does not explain why CKD results in salt-sensitive hypertension. This raises the possibility that the changes in the distal nephron secondary to CKD are maladaptive or that CKD causes additional changes that further enhance distal nephron Na^+ reabsorption (**Figure 1**).

RENIN-INDEPENDENT ALDOSTERONE SECRETION

Angiotensin II and plasma K^+ are the two main secretagogues for aldosterone secretion by zona glomerulosa cells in the adrenal gland. Therefore, the presence of hyperreninemia or hyperkalemia in CKD could contribute to an increase in plasma aldosterone, and therefore Na^+ reabsorption (**Figure 1**). Indeed, CKD is often accompanied by hyperreninemia and hyperkalemia, although this varies depending on the underlying cause and stage of CKD. In patients with non-diabetic CKD and hypertension, plasma renin activity was approximately twice as high as in control subjects, despite the discontinuation of anti-hypertensive drugs [38]. The observation that plasma renin activity plays a role in hypertension in patients with CKD is also illustrated by older observations that bilateral nephrectomy in hemodialysis patients resolves resistant hypertension [39]. However, there are examples of kidney disease that exhibit a low-renin state and in which aldosterone still plays a role in hypertension and disease progression. The prime example is diabetic kidney disease in which not only the intrarenal RAS [40], but also aldosterone contributes to resistant hypertension [41]. The remnant kidney models of CKD

Table 1. Knowledge gaps in mechanisms of salt-sensitive hypertension in CKD

General questions
In salt-sensitive hypertension, does increased Na^+ reabsorption coincide with or precede the increase in vascular resistance? How does CKD cause glomerulotubular imbalance with increased distal Na^+ delivery? How do the mechanisms of salt-sensitive hypertension in CKD differ across CKD stage and CKD etiology and between the sexes? What is the role of vasopressin in salt-sensitive hypertension in CKD? Does inflammation play a primary or secondary role in salt-sensitive hypertension in CKD and could it be a therapeutic target?
Aldosterone
Does plasma K^+-induced aldosterone release contribute to hypertension in patients with CKD? Which plasma renin- and K^+-independent factors are most relevant in mediating aldosterone secretion in CKD? Why does CKD impair the aldosterone escape mechanism?
Intrarenal renin-angiotensin system
What are markers and therapeutic targets of intrarenal RAS activity in humans? Can intratubular angiotensin II promote Na^+ reabsorption through apical angiotensin receptors in distal nephron cells?
Dietary salt
Does the experimental evidence of high dietary Na^+ intake activating Rac1 and the mineralocorticoid receptor translate to patients with CKD?
Proteinuria
Is inhibition of ENaC or urinary serine protease activity an effective strategy for salt-sensitive hypertension in CKD?

also affect plasma renin differently with the ablation model reducing plasma renin and the ligation model increasing plasma renin [24]. The role of aldosterone in the remnant kidney model was clearly illustrated by showing that exogenous aldosterone nullified the protective effects of combined enalapril and losartan treatment on hypertension, proteinuria and glomerulosclerosis [42]. Hyperkalemia is the other recognized driver for aldosterone secretion and becomes more prevalent as CKD progresses, increasing from 2% with a measured GFR between 60 to 90 ml/min to 42% when GFR falls < 20 ml/min [43]. One study showed that lowering of plasma K^+ in CKD with the K^+ binder patiromer also reduced plasma aldosterone and blood pressure [44]. However, many normokalemic patients with advanced CKD were also shown to be in a state of hyperaldosteronism [45]. Similarly, in patients with CKD whose plasma K^+ and renin were within the normal range, plasma aldosterone was still usually elevated [46]. Patients with kidney failure treated with hemodialysis also had inappropriately high aldosterone levels for the degree of volume expansion, a finding that was not explained by plasma K^+ [47]. Patients with CKD, but not patients with essential hypertension, were found to have a positive correlation between the urinary excretion of Na^+ and the mineralocorticoid metabolite tetrahydroaldosterone [48]. Furthermore, patients with CKD failed to suppress aldosterone upon a Na^+ challenge, either as intravenous NaCl or dietary salt [49]. Together, these studies

suggest that (1) CKD changes the aldosterone-volume and aldosterone-Na^+ relationships and (2) factors other than hyperreninemia and hyperkalemia must contribute to the inappropriately high aldosterone levels in CKD. Several candidate secretagogues for inappropriate aldosterone secretion in CKD have been identified, including metabolic acidosis and endothelin-1 (discussed below), adrenocorticotropic hormone (ACTH) [50], catecholamines [51, 52], adipocyte-derived factors [53-55], and vasopressin (**Figure 1**). The role of vasopressin in CKD has recently gained interest, because plasma copeptin, a peptide derived from the pre-pro-hormone of vasopressin and used as a surrogate marker for vasopressin, is associated with the development and progression of CKD in the general population [56]. Whether this effect is mediated through hypertension is unclear, but it is clear that in animals the effects of vasopressin extend beyond water regulation and also includes the regulation of blood pressure and Na^+ homeostasis [57]. Vasopressin can stimulate aldosterone secretion through V1a receptor activation on cells of the adrenal cortex [58-60]. In addition, vasopressin can directly stimulate Na^+ reabsorption through effects on NKCC2 and NCC [61-63]. Of interest, pharmacological or genetic inhibition of the V1a receptor prevents salt-sensitive hypertension in mice after 5/6th nephrectomy [64, 65]. Whether these effects are explained by reduced aldosterone or tubular Na^+ and water handling is unknown. Thus, it is unclear whether the role of vasopressin on Na^+ reabsorption and hypertension translates from rodents to humans. Another consideration is that 5/6th nephrectomized animals but not humans with CKD develop a urinary concentrating defect, which will raise plasma vasopressin levels [66]. In a randomized clinical trial increased water intake in patients with CKD reduced plasma copeptin, but did not slow the decline in kidney function [67]. In summary, CKD may introduce several reasons for renin-independent aldosterone secretion. Under normal physiological circumstances, the kidneys are capable of 'escaping' persistent high levels of aldosterone and increase Na^+ excretion by downregulating NCC [68]. In CKD this aldosterone escape phenomenon may no longer operate effectively. In line with this, obese Zucker rats have reduced kidney function and an impaired response to aldosterone infusion, which may be mediated by increases in NKCC2 and ENaC or reduced nitric oxide bioavailability [69].

ACTIVATION OF THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM

Although the presence of an intrarenal RAS is generally accepted, its independence from the circulating RAS remains a subject of debate. Previous data suggested renin synthesis in the collecting duct being regulated in a manner opposite to renin regulation in the juxtaglomerular apparatus [70]. However, Tang *et al.* were unable to demonstrate migration of renin lineage cells to this site, and thus the presence of renin at this site likely reflects reabsorption rather than local synthesis [71]. Angiotensinogen can be synthesized

in the proximal tubule [72], and angiotensin converting enzyme (ACE) activity is present throughout the nephron with the highest activities in the proximal tubule and collecting duct [73]. Micropuncture studies have shown that the concentrations of angiotensin I and II in proximal tubule fluid exceed circulating levels by a factor of 1000, suggesting local release or generation [74, 75]. Similarly, the tissue concentrations of cortical and medullary angiotensin II are up to 100 and 60 times higher than in blood, respectively, suggesting compartmentalization through receptor-mediated binding to cells or endocytosis [76, 77]. However, the intrarenal RAS likely depends on the circulating RAS for filtration and reabsorption of renin and angiotensinogen via the endocytic receptor megalin in the proximal tubule [78, 79]. Indeed, liver-specific knock-out of angiotensinogen markedly reduced the levels of angiotensinogen and angiotensin II in the kidney, both under normal and pathological circumstances [80, 81]. There are, however, settings in which plasma renin is suppressed, while prorenin, renin, angiotensinogen, and angiotensin II are elevated in the kidney and urine [82]. Although this could be viewed as support for the independent operation of the intrarenal RAS, variations in megalin expression need to be considered [71, 79]. Such variations would allow a different degree of reuptake and thus non-parallel changes in circulating and intrarenal RAS activity. Several regulators of the intrarenal RAS have been postulated, including the (pro)renin receptor, Wnt/β-catenin signaling, and the prostaglandin E2 EP_4 receptor (positive regulators), and Klotho, the vitamin D receptor, and liver X receptors (negative regulators) [78, 83]. Increased intrarenal RAS activity increases angiotensin II in tubular fluid. In turn, angiotensin II in tubular fluid can activate amiloride-sensitive Na^+ transport in the distal nephron by activating the angiotensin II type 1 receptor localized at the apical plasma membrane [84].Using a cross-transplantation strategy, Crowley *et al.* showed that renal rather than extrarenal angiotensin II type 1 receptors mediate salt-sensitive hypertension [85]. In mice, overexpression of mouse angiotensinogen in the proximal tubule causes salt-sensitive hypertension without recruitment of the systemic RAS [86]. Conversely, in mice lacking kidney ACE, angiotensin II is no longer able to activate NKCC2, NCC, ENaC, pendrin and their regulating kinases SPAK and OSR1 [87]. However, the mouse model used for this study (so-called ACE 10/10 mice with ectopic ACE expression in myelomonocytic cells) does not rule out a role for other ACE-expressing tissues or for systemic renin upregulation [88, 89]. Thus, although the kidney expresses several RAS components, the independent contribution to Na^+ or blood pressure homeostasis remains uncertain. Comparison between studies is also complicated by the technical challenges of measuring angiotensin peptide concentrations in tissue. Despite these unanswered questions, it appears likely that the intrarenal RAS is also directly involved in distal nephron Na^+ reabsorption (**Figure 1**). Kidney disease may activate the intrarenal RAS and mediate progressive kidney injury. When kidney disease is induced experimentally by $5/6^{th}$ nephrectomy or by inhibition of nitric oxide synthesis, the number of interstitial cells expressing

angiotensin II increases [90, 91]. Losartan and the immunosuppressive drug mycophenolate mofetil prevent these changes and the related progression of kidney injury [90, 91]. Similarly, unilateral ureteral obstruction increases renin, ACE activity, and angiotensin II concentration in the obstructed kidney [92]. Studies using two different experimental CKD models – 5/6th nephrectomy and chronic nephritis induced with anti-thymocyte serum – showed that angiotensin II in the kidney cortex increased after high dietary salt intake, whereas they decreased in healthy rats [93, 94]. A high salt diet in 5/6-nephrectomized rats activated the intrarenal but not the systemic RAS, and increased NADPH oxidase, inflammation, blood pressure, and albuminuria [95]. Thus, a high salt diet can further augment activation of the intrarenal RAS in experimental CKD. Proteinuria may play an independent role in increasing kidney angiotensin II by providing the substrates for its local synthesis (**Figure 1**). In albumin-loaded uninephrectomized rats, proteinuria was accompanied by increased kidney angiotensin II in an NFKB-dependent manner with an increase in angiotensinogen and decrease in ACE2 [96]. A decrease in ACE2 is expected to lead to higher angiotensin II because ACE2 can convert angiotensin II to angiotensin 1-7 [97]. These pre-clinical studies suggest that the intrarenal RAS likely plays a role in human CKD. However, this has been difficult to confirm in the absence of established non-invasive read-outs for intrarenal RAS activity in humans [98]. Although urinary angiotensinogen has been proposed as marker for the intrarenal RAS in humans [99], its excretion pattern largely follows that of urinary albumin, and therefore likely reflects glomerular damage [100]. Urinary renin is filtered in larger amounts than albumin, and the presence of renin in urine reflects impaired reabsorption by the proximal tubule rather than local release by the kidney [71, 98]. In agreement, the elevated urinary renin observed in patients and mice with type 1 diabetes mellitus and diabetic kidney disease is likely attributed to altered glomerular filtration and impaired proximal tubular reabsorption [71]. Patients with type 1 diabetes who developed diabetic kidney disease had less change in renal vascular resistance in response to an angiotensin II infusion than the patients who did not develop diabetic kidney disease [101]. Thus, diabetic kidney disease exaggerates intrarenal RAS activity. This also explains the relative resistance to a rise in plasma renin upon RAS-inhibition in patients with diabetic kidney disease, and suggests higher dosing of RAS-inhibitors may be required [102].

THE ROLE OF DIETARY SALT

Salt-sensitive hypertension is unmasked with high dietary salt (NaCl) intake. Dietary Na^+ loading without Cl^- or dietary Cl^- loading without Na^+ does not increase blood pressure, illustrating that the anion accompanying Na^+ plays an important role in salt-sensitive hypertension [103, 104]. In salt-resistant subjects, high dietary salt intake induces pressure

natriuresis and suppresses the systemic RAS. In salt-sensitive subjects, however, high salt intake may promote Na^+ reabsorption independent of the RAS by direct activation of the mineralocorticoid receptor (**Figure 1**) [105, 106]. The link between dietary salt and the mineralocorticoid receptor is mediated by the small GTPase Rac1 [107]. In rodent models of salt-sensitive hypertension, a high salt diet activated Rac1, increased blood pressure, and caused kidney injury, despite suppressing aldosterone [105]. Conversely, in salt-resistant strains, high dietary salt reduced Rac1 activity. This suggests that either genetic differences in these strains mediates differential regulation of Rac1 or that paracrine factors, such as local angiotensin II synthesis, activate the mineralocorticoid receptor. In humans, genetic variants of Rac1 have been linked to increased salt sensitivity [108]. Rac1 is also expressed in the glomerulus and its aberrant activity in podocytes leads to proteinuria, which can also contribute to distal nephron Na^+ reabsorption (see 'proteinuria' section below) [109]. In addition to Rac1, high dietary salt can increase serum and glucocorticoid-induced kinase 1 (SGK1) in salt-sensitive Dahl rats independent of aldosterone [110]. Because SGK1 is a downstream effector of mineralocorticoid receptor signaling and regulates ENaC and NCC activity [111-114], one might postulate that this is another pathway through which dietary salt directly increases distal nephron Na^+ reabsorption. However, several other factors related to a high Na^+ diet may also explain the effects on Rac1 and SGK1. Both Rac1 and SGK1 can be activated by mechanical stretch [115, 116], while SGK1 is also regulated by changes in cell volume, transforming growth factor beta, diabetes and proteinuria [117-120]. Recent data suggest that the link between high dietary salt intake and hypertension could also be mediated through inflammation (**Figure 1**). The role of the immune system in hypertension was illustrated by studies showing that immune deficient mice are resistant to hypertensive stimuli such as angiotensin II or DOCA-salt [121, 122]. Similarly, a high salt diet increased blood pressure in immunocompetent mice after 5/6th nephrectomy but not in mice lacking T-cells suggesting that T-cells are required for salt-sensitive hypertension in CKD [123]. Dietary salt can activate a specific subtype of T cells, namely T helper 17 cells (Th17) through an effect on SGK1 [124, 125]. The clinical relevance of this effect was supported by the observation that a high salt diet exaggerated an experimental model of multiple sclerosis through SGK1-mediated Th17-induction [124, 125]. Conversely, patients with salt-losing tubulopathies (salt depletion) show reduced Th17 polarization, impaired interleukin 17 responses, and a higher incidence of mucosal infections and allergic disease compared to controls [126]. Th17 cells may also influence distal tubular Na^+ handling. Incubation of distal tubular epithelial cells with interleukin 17A, a cytokine produced by Th17 cells, increased NCC expression via SGK1 [127]. In mice with angiotensin II-induced hypertension, the pressor response and upregulation of NCC and ENaC were blunted by genetic interleukin 17A deficiency [127]. In mice with DOCA-salt hypertension $CD8^+$ T cells physically interacted with distal tubular cells to change the intracellular Cl^- concentration and subsequently activate

NCC [128]. Interferon gamma, a cytokine produced by $CD8^+$ T cells, can also activate NCC [129]. As $CD8^+$ T cells can be activated by Th17 cells, the interaction between these T cell subsets may increase distal nephron Na^+ reabsorption [130-132]. In clinical medicine, Th17 and γδ T cells play a pathogenic role in psoriasis and, indeed, patients with psoriasis more often have hypertension and antipsoriasis therapies reduce blood pressure [133]. Recently, tumor necrosis factor alpha (TNF-)alpha was also implicated in the pathogenesis of salt-sensitive hypertension in both the adenine and aristolochic acid nephropathy mouse models of CKD, but not in the $5/6^{th}$ nephrectomy model [31]. TNF-alpha was shown to increase distal nephron Na^+ reabsorption by increasing WNK1, possibly through an effect on the ubiquitin-protein ligase Nedd4-2, which subsequently promoted SPAK and NCC phosphorylation.

METABOLIC ACIDOSIS

A normal diet in developed countries typically generates a surplus of hydrogen ions (H^+). This H^+ is excreted by the kidneys as ammonium and titratable acid. In CKD, ammonium excretion is impaired and often results in metabolic acidosis once GFR falls below 40 ml/min [43, 134]. In response to metabolic acidosis, the renin-angiotensin and endothelin systems are activated to enhance urinary acidification [135]. Angiotensin II increases bicarbonate (HCO_3^-) reabsorption and ammonium production in the proximal tubules and increases H^+-ATPase activity in the distal nephron [136-140]. Aldosterone increases distal urinary acidification directly by activation of H^+-ATPases, and indirectly because ENaC-mediated Na^+ reabsorption increases the lumen-negative voltage and therefore stimulates H^+ secretion [141-144]. Although these actions of angiotensin II and aldosterone serve to restore acid-base homeostasis, a secondary effect may be increased distal nephron Na^+ reabsorption (**Figure 1**) [135]. Plasma levels of aldosterone increase in response to experimental acidosis in healthy humans [145-150]. Conversely, acute treatment with HCO_3^- lowers plasma aldosterone [151]. Two-thirds nephrectomy in rats, a model of early CKD, increases plasma aldosterone, which is reversed by supplementation of CaHCO3 [152]. In patients with CKD stage G2, 1-month treatment with oral $NaHCO_3$ reduced plasma and urinary aldosterone [153]. Previous studies indicate that the rise in plasma aldosterone during metabolic acidosis occurs independent of plasma renin [145, 148]. Four possible mechanisms for the renin-independent aldosterone secretion in metabolic acidosis have been proposed, including an effect of hyperkalemia, endothelin-1, ACTH, or interstitial pH. The first of these mechanisms, hyperkalemia, may be caused directly by metabolic acidosis. In healthy subjects, however, plasma aldosterone increased without a commensurate increase in plasma K^+ [145, 148]. Recently, we compared the response to an acute acid load in healthy subjects and patients with CKD [154]. In patients with CKD the

metabolic acidosis that followed after the acute acid load persisted for a longer time and did increase plasma K^+ as well as plasma aldosterone. The second mechanism for aldosterone secretion by acidosis is endothelin-1. Plasma levels of endothelin-1 increase in response to acidosis and decrease after oral alkali treatment [152, 153, 155]. Endothelin-1 directly promotes H^+ secretion through its action on the endothelin type-B receptors in the kidney [156-158]. Plasma levels of endothelin-1 are elevated in patients with CKD and gradually rise during progression of the disease [153, 159]. Endothelin-1 can directly stimulate the adrenal cortex and, additionally, increase its sensitivity to other hormones [160, 161]. The increase in plasma aldosterone required to excrete an acid load is dependent on endothelin-1 [162]. The third possibility is that ACTH increases plasma aldosterone. Plasma ACTH is increased in patients with kidney failure [50]. Although ACTH increases during acidosis [163], acute acid-loading in healthy volunteers does not increase plasma cortisol [145, 148]. Furthermore, the contribution of ACTH to aldosterone secretion in CKD-related metabolic acidosis has not been investigated. Finally, it has been proposed that intracellular pH might stimulate aldosterone secretion [145]. Interstitial H^+ is higher in CKD [152, 164] but this is not always reflected by changes in plasma pH, considering that most H^+ is buffered by HCO_3^- and intracellular buffers. In isolated perfused rabbit and canine adrenals a lower pH of the perfusate did not change adrenal aldosterone secretion rates [165, 166]. However, when the adrenals were stimulated with angiotensin II under low pH conditions, higher rates of aldosterone secretion where observed. Similarly, low pH conditions in a culture of adrenal zona glomerulosa cells increased angiotensin II, possibly through increased angiotensin receptor binding [167]. In the kidney, a lower interstitial pH increases sensitivity of proximal tubular cells to angiotensin II [168]. Mice treated with ammonium chloride (NH_4Cl) showed increased expression of the angiotensin II type 1 receptor in proximal tubules. Similar responses were observed in a proximal tubule cell line cultured in low pH medium [168]. Moreover, after stimulating the same cells with angiotensin II, higher production rates of ammonium were measured. In addition to these systemic effects, metabolic acidosis also increases the components of the intrarenal RAS (**Figure 1**). Subtotal nephrectomy in rats causes acid retention and increases kidney angiotensin II [140, 169]. In normal rats, one week of NH_4Cl increased angiotensinogen, ACE, and angiotensin II levels in the kidney [170]. The induction of chronic metabolic acidosis by NH_4Cl in mice was also accompanied by increased NCC activity, which was dependent on the binding of angiotensin II to the angiotensin II type 1 receptor [171]. Another pathway through which metabolic acidosis may contribute to increased distal nephron Na^+ reabsorption is proteinuria (**Figure 1**) [155, 170, 172, 173]. In healthy rats acute and chronic changes in acid-base balance correspond with urinary protein excretion [172, 173]. Acidosis may increase proteinuria by causing hyperfiltration [146, 174], affecting podocyte function [175, 176], or by facilitating recycling of the megalin receptor [177] (**Figure 1**). In turn, proteinuria may be aggravated by the secondary effects of acidosis on aldosterone and activation

of the complement system [42, 178, 179]. In animal models of CKD, complement activation leads to more proteinuria and higher concentrations of angiotensin II in the kidney [179, 180]. Finally, metabolic acidosis may be linked to activity of the prorenin receptor. Apart from its potential role in the intrarenal RAS, the prorenin-receptor is a functional subunit of the H^+-ATPase in the cortical collecting duct, and its expression is increased during metabolic acidosis [181, 182]. Previous studies showed that the prorenin-receptor is necessary for increased open probability of ENaC and the rise in blood pressure in response to angiotensin II [183, 184]. Although these findings were observed in healthy animals with normal kidney function, they do provide a potential link between CKD-related metabolic acidosis, distal nephron Na^+ reabsorption, and salt-sensitive hypertension.

PROTEINURIA-INDUCED NA^+ REABSORPTION

Kidney disease may also manifest as the nephrotic syndrome. The nephrotic syndrome is usually caused by a distinct etiology of kidney diseases called “podocytopathies”, which selectively affect the podocyte’s role in the glomerular filtration barrier and therefore cause massive proteinuria while often leaving GFR intact [185]. Clinically, the nephrotic syndrome is characterized by edema secondary to hypoalbuminemia and avid Na^+ retention by the kidneys. Na^+ retention in the nephrotic syndrome has a unique pathophysiology because it often develops in the context of a suppressed RAS. In those patients with nephrotic syndrome in whom the RAS is activated, treatment with an ACE-inhibitor or albumin suppresses the RAS, but does not improve Na^+ retention [186]. A breakthrough in the understanding of Na^+ retention in nephrotic syndrome was the demonstration that nephrotic urine can activate ENaC [187]. ENaC activation depended on urinary serine protease activity and mass spectrometry identified plasmin as the probable candidate. Plasmin increased the open probability of ENaC by cleavage and by releasing an inhibitory peptide from the gamma subunit [187]. Plasmin is not filtered by the glomerulus but rather converted in the tubular lumen from plasminogen and urokinase-type plasminogen activator (uPA) by the uPA-receptor [188]. Conditional knockout of podocin in mice mimics human nephrotic syndrome with nephrotic range proteinuria, weight gain, edema, and Na^+ accumulation [189]. These changes were reversed by treatment with the ENaC-blocker amiloride, which also prevented the conversion of plasminogen to plasmin. Similarly, treatment with an antibody targeting uPA also abolished urine activation of plasminogen, attenuated Na^+ accumulation, and prevented cleavage of γ-ENaC. Urinary serine protease activity in nephrotic urine can also be inhibited by the trypsin inhibitor aprotinin [190]. In mice with doxorubicin-induced nephrotic syndrome, treatment with either aprotinin or amiloride normalized urinary serine protease activity and prevented Na^+ accumulation. In *Xenopus laevis* oocytes expressing ENaC, aprotinin

had no direct effect on ENaC but did prevent proteolytic ENaC activation. Together, these studies show that proteinuria by itself can contribute to distal nephron Na^+ reabsorption through ENaC (**Figure 1**), which can be prevented by amiloride or the inhibition of urinary serine protease activity. Because nephrotic syndrome is characterized by massive proteinuria, a relevant question was whether the phenomenon of plasmin-induced Na^+ reabsorption was also present in more moderate stages of proteinuria as observed in CKD. This question was addressed in a cross-sectional study by Schork *et al.*, who analyzed fluid status and urinary plasmin in 117 patients with CKD [191]. Proteinuria was the strongest independent predictor for overhydration. The urinary excretion of plasmin correlated strongly with proteinuria and overhydration. In addition, the range of urinary plasmin concentrations was sufficient to activate ENaC currents *in vitro*. The relevance of this phenomenon has also been extended to other proteinuric conditions, including pre-eclampsia, diabetic kidney disease, and kidney transplantation [192-194]. Although these studies clearly indicate that proteinuria by itself contributes to Na^+ retention, the link with salt-sensitive hypertension has been less clear. One study did provide insight into this issue by analyzing the effect of adding amiloride to patients with type 2 diabetes and resistant hypertension [195]. The study showed that amiloride lowered blood pressure, albuminuria, and urine plasminogen excretion and activation.

INFLUENCE OF SEX

While it is generally accepted that the prevalence of CKD is greater in women than in men, the decline in kidney function occurs at a faster rate in men which coincides with the higher mortality rate attributable to CKD in men [1, 196, 197]. A caveat of this is that the estimated decline in kidney function depends on a number of factors including the population studied, diabetes mellitus, age, and body composition. Nevertheless, these sex differences in CKD likely reflect the longer life expectancy in women and the sex-and age-dependent decline in kidney function [198-201], with premenopausal women protected from the development of hypertension and CKD as compared to age-matched men and postmenopausal women [196, 202]. Plasma aldosterone levels and extracellular fluid volume are highest during the luteal phase of the menstrual cycle [203, 204]. However, compared to men, plasma aldosterone levels and extracellular fluid volume are lower in premenopausal women [205-207]. In women, salt-sensitivity of blood pressure is inversely associated with estrogens and progesterone [208]. In particular women with a history of preeclampsia have an increased risk for salt-sensitive hypertension, cardiovascular disease, and chronic kidney disease [209, 210]. Although the exact mechanisms remain incompletely understood, an enhanced vascular response to injury due to preeclampsia, immune system activation, and imbalance of NO:ET-1 might contribute [211-213]. Both surgical and

natural menopause increases salt-sensitivity, with the pressure-natriuresis curve of postmenopausal women shifted to the right [214-216]. In Dahl salt-sensitive rats fed a high salt diet, the increase in blood pressure is lower in ovary-intact females as compared to males and ovariectomized females [217], supporting an important role for ovarian hormones in regulation of salt-sensitivity of blood pressure. Similarly, with age and reproductive senescence, the chronic pressure-natriuresis in ovary-intact female mice is shifted rightward [218]. While animal models of CKD suggest that estrogen replacement [219, 220] and androgen deprivation [221] are protective against the development of CKD, the situation is less clear in humans [196]. Studies in rodents have demonstrated that females exhibit a lower abundance and activity of proximal transporters and a higher abundance and activity of distal transporters including, NKCC2, NCC and ENaC [222-224], indicating a greater role for the distal nephron in Na^+ reabsorption in females than males. Consequently, in the setting of salt-sensitive hypertension in CKD this pattern of increased Na^+ reabsorption in the distal nephron is likely to be enhanced in females. Certainly, this is the case in response to angiotensin II-induced hypertension in rodents [224]. As described above, in addition to CKD-induced changes in Na^+ handling, other mechanisms including renin-independent aldosterone secretion, activation of the intrarenal RAS, dietary salt, metabolic acidosis and proteinuria induced Na^+ reabsorption may all contribute to the development of salt-sensitive hypertension in CKD. Importantly, these mechanisms are likely to be influenced by sex. For example, the sex hormone milieu is known to modulate the synthesis and activity of nitric oxide, reactive oxygen species, endothelin-1, transforming growth factor beta, and the pressor:depressor balance of the RAS (particularly within the kidney) with estrogen downregulating these pathways and testosterone upregulating them [196, 199, 202]. Sex differences in the actions of vasopressin may account for a greater tendency for men to concentrate their urine [225], and may contribute to the faster progression of CKD in men than women. While experimental studies suggest that sex influences acid-base homeostasis, studies in humans are inconclusive [226-229]. A similar controversy exists regarding expression of sodium-glucose cotransporter-2 (SGLT2) [230, 231]. Further, experimental studies suggest that the T-cell profile differs between the sexes, particularly within the kidney with males having a higher proportion of pro-inflammatory T cells such as Th17 than females [232]. This may potentiate the effect of the higher dietary salt consumption in men [233] on the development of salt-sensitive hypertension in CKD. Evidence from spontaneously hypertensive rats and CKD patients suggest that ACE-inhibition may have a greater effect on reducing proteinuria in females than males [234, 235], however the effect on blood pressure, at least in rats, was greater in males. Finally, sex-specific risk factors e.g., complications of pregnancy and menopause [202], may also contribute sex differences in hypertension and CKD. Detailed mechanistic studies are required to dissect out the cellular and molecular mechanisms by which sex influences distal nephron Na^+ handling in salt-sensitive hypertension in CKD. Un-

derstanding sex-differences in salt-sensitive hypertension in CKD will inform whether treatment should be administered using a personalized medicine approach.

CLINICAL PERSPECTIVE

A relevant question is how the mechanisms discussed above translate to treatment options in clinical practice. The most obvious and straightforward approach to treat salt-sensitive hypertension in CKD is to reduce dietary salt intake. Worldwide people generally consume a high salt diet that exceeds recommendations by the World Health Organization and the Institute of Medicine, and patients with CKD are no exception [236, 237]. Approximately 80% of dietary salt consumed has been added by food manufacturers showing little to no reduction over time [238]. Dietary salt restriction may be even more challenging for patients with CKD as their taste threshold for salt is increased [239]. In clinical trials dietary Na^+ restriction consistently improves blood pressure, body weight, and proteinuria in patients with CKD [240-243]. With the proposed central role of distal nephron Na^+ reabsorption in CKD-related hypertension (**Figure 1**), another attractive strategy would be to employ distal diuretics. Diuretics have long been considered ineffective in CKD, especially with GFR < 30 ml/min, because they require tubular secretion [244, 245]. To address this, we recently performed a randomized cross-over trial to compare the anti-hypertensive effects of dietary Na^+ restriction to a combination of distal diuretics (hydrochlorothiazide and amiloride) [246]. We showed that distal diuretics actually produced a greater reduction in systolic blood pressure and extracellular water than dietary Na^+ restriction, and that diuretic sensitivity was maintained even at lower GFR [246]. It would be of interest to know if this effect was primarily due to NCC or ENaC inhibition, or both. The PATHWAY-trial showed that in patients with uncontrolled hypertension the combination of hydrochlorothiazide and amiloride was more effective than either treatment alone [247]. Especially patients with low-renin hypertension – which signals salt retention – may benefit from this combination of distal diuretics [248]. In patients with resistant hypertension, sequential diuretic therapy had a greater anti-hypertensive effect than sequential RAS inhibition [249]. As monotherapy, amiloride has been tested in patients with diabetic kidney disease in whom it effectively lowered blood pressure [250]. An ongoing trial is comparing chlorthalidone with placebo in patients with CKD stage G4 [251]. Another focus for future studies should be to explore whether amiloride could prevent plasmin-induced Na^+ reabsorption in patients with CKD (**Table 1**), as was previously shown in experimental nephrotic syndrome [190], and in patients with hypertension and diabetes [195]. Additional rationales for using a combination of diuretics is to prevent diuretic resistance and maintain plasma K^+ (when combining a K^+ sparing and non-K^+ sparing diuretic) [246, 252]. Because diuretics typically cause a hemodynamic reduction

in GFR, longer-term studies are necessary to evaluate if this is offset by the beneficial effects of diuretics on blood pressure and extracellular fluid volume. Of interest in this regard are the positive effects of SGLT2-inhibitors on kidney and cardiovascular outcomes in patients with CKD, because these drugs also have natriuretic and anti-hypertensive properties and reduce GFR initially [253-255]. SGLT2-inhibitors not only target the proximal tubule, but also the distal convoluted tubule by improving the dysregulation of kelch-like 3, WNK kinases, and NCC [256]. Because of the pre-clinical evidence that a high Na^+ diet activates the mineralocorticoid receptor through Rac1 (**Figure 1**), the application of mineralocorticoid receptor antagonists or Rac1-inhibitors (in development phase), may be another rational strategy for salt-sensitive hypertension in CKD [257]. A recent systematic review and meta-analysis showed that mineralocorticoid receptor antagonists reduce blood pressure in patients with diabetic and non-diabetic CKD as compared to placebo, but are not more effective than non-K^+ sparing diuretics [258]. The use of mineralocorticoid receptor antagonists in patients with CKD is often limited by the development of hyperkalemia. Of interest in this regard is the recent development of non-steroidal mineralocorticoid receptor antagonists, which cause hyperkalemia less frequently and will be tested in patients with CKD and hypertension [259, 260]. Instead of a pharmacological approach, another interesting dietary approach would be to address the effects of increasing dietary K^+ intake in patients with CKD. The general population as well as patients with CKD generally consume a low K^+ diet [261]. An increasing body of evidence suggests that higher urinary K^+ excretion (as proxy for dietary K^+ intake) is associated with better kidney outcomes [262]. A low K^+ diet activates NCC to reduce distal Na^+ delivery, but, in turn, contributes to Na^+ retention and hypertension [263, 264]. Indeed, dietary K^+ exerts anti-hypertensive effects, which in part may be attributable to NCC inactivation [265, 266]. In this regard, dietary K^+ could serve as a "natural diuretic". Whether supplementing dietary K^+ in patients with CKD reduces blood pressure and slows the progression of CKD is unknown, but this is currently being investigated in a randomized clinical trial [261]. A potential limitation of this approach is that a higher plasma K^+ increases plasma aldosterone, although K^+-induced NCC inactivation occurs despite an elevation in aldosterone [267]. An unresolved question is whether K^+-induced aldosterone release can contribute to hypertension in patients with CKD (**Table 1**). As noted previously, one study showed that the K^+ binder patiromer reduced aldosterone and blood pressure [44]. In salt-loaded uninephrectomized rats, high dietary K^+ suppressed the activity of NCC and blunted salt-sensitive hypertension [268]. A high K^+ diet may have the additional benefit that it is usually part of an alkaline diet. In patients with CKD, metabolic acidosis is common and contributes to the progression of CKD [152, 269-271]. Long-term alkali treatment slows CKD progression [270, 272, 273]. A diet rich in fruits and vegetables improves metabolic acidosis and reduces kidney injury to a similar degree as treatment with $NaHCO_3$ [271]. In addition to an alkaline diet and $NaHCO_3$, the non-absorbable H^+ and

Cl^- binder veverimer is under development for the treatment of metabolic acidosis in CKD [274]. Of interest, the K^+ binder sodium zirconium cyclosilicate also increases plasma HCO_3^-, likely through gastro-intestinal ammonium trapping [275]. Although kidney transplantation improves quality of life and mortality, salt-sensitive hypertension remains a challenge in the kidney transplant recipient and contributes to graft loss [276]. The pathogenesis of salt-sensitive hypertension in kidney transplant recipients is multifactorial [277], but calcineurin inhibitors play a prominent role due to their ability to activate NCC and thereby promote distal tubular Na^+ reabsorption [278, 279]. Which of the treatments, or combinations of treatments, is most effective for the individual patient with CKD and salt-sensitive hypertension, is difficult to recommend. Generally speaking, we believe that dietary approaches and distal diuretics deserve more emphasis, while the exact positioning of SGLT2-inhibitors will become clear in the next few years. In summary, ample opportunities are available to treat salt-sensitive hypertension in CKD, raising the expectation that this might improve long-term kidney and cardiovascular outcomes.

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Part 1

Salt

Chapter 3

Dietary salt modifies the blood pressure response to renin-angiotensin inhibition in experimental chronic kidney disease

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ABSTRACT

Chronic kidney disease (CKD) contributes to hypertension, but the mechanisms are incompletely understood. To address this, we applied the 5/6th nephrectomy rat model to characterize hypertension and the response to dietary salt and renin-angiotensin inhibition. 5/6th nephrectomy caused low-renin, salt-sensitive hypertension with hyperkalemia and unsuppressed aldosterone. Compared to sham, 5/6N$_x$ rats had lower NHE3, NKCC2, NCC, α-ENaC and Kir4.1, but higher SKG1, prostasin, γ-ENaC, and Kir5.1. These differences correlated with plasma renin, aldosterone, and/or potassium. On a normal salt diet, adrenalectomy (0 ± 9 mmHg) and spironolactone (-11 ± 10 mmHg) prevented a progressive rise in blood pressure (10 ± 8 mmHg), and this was enhanced in combination with losartan (-41 ± 12 mmHg and -43 ± 9 mmHg). A high salt diet caused skin sodium and water accumulation and aggravated hypertension that could only be attenuated by spironolactone (-16 ± 7 mmHg) and in which the additive effect of losartan was lost. Spironolactone also increased natriuresis, reduced skin water accumulation and restored vasorelaxation. In summary, in the 5/6th nephrectomy rat CKD model, salt-sensitive hypertension develops with a selective increase in γ-ENaC and despite appropriate transporter adaptations to low renin and hyperkalemia. With a normal salt diet, hypertension in 5/6th nephrectomy depends on angiotensin II and aldosterone, while a high salt diet causes more severe hypertension mediated through the mineralocorticoid receptor.

New & Noteworthy

Chronic kidney disease (CKD) causes salt-sensitive hypertension, but the interactions between dietary salt and the renin-angiotensin system (RAS) are incompletely understood. In rats with CKD on a normal salt diet targeting aldosterone, the mineralocorticoid receptor (MR) and especially angiotensin II reduced blood pressure. On a high salt diet, however, only MR-blockade attenuated hypertension. These results reiterate the importance of dietary salt restriction to maintain RAS-inhibitor efficacy and specify the MR as target in CKD.

INTRODUCTION

Hypertension is a major complication of chronic kidney disease (CKD) and contributes to progression of CKD and cardiovascular morbidity and mortality [1]. With decreasing glomerular filtration rate (GFR), the incidence of hypertension increases to >90% for CKD stage G5 [2]. Hypertension in CKD is a salt-sensitive form of hypertension [3], but it is incompletely understood how CKD conveys salt-sensitivity, and how the various systems that govern sodium balance and blood pressure interact in CKD [4]. A reduction in nephron number will limit the capacity to excrete salt and water and the ensuing pressure natriuresis will contribute to hypertension in CKD [5]. A number of anti-natriuretic factors may be also be at play in CKD to further enhance salt-sensitive hypertension [4]. For example, in CKD, plasma aldosterone is often elevated without a rise in plasma renin, which may be caused by hyperkalemia or metabolic acidosis [6-9]. In addition, CKD may activate the intrarenal renin-angiotensin system (RAS) with local synthesis of angiotensin II in the kidney [10]. It has also been reported that high dietary salt can activate the mineralocorticoid receptor (MR) in an aldosterone-independent fashion [11]. Angiotensin II, aldosterone, and the mineralocorticoid receptor activate sodium transport proteins in the kidney, including the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC) [12-14].

Therefore, we hypothesize that aldosterone, angiotensin II, and the mineralocorticoid receptor, contribute to hypertension in CKD. Furthermore, we hypothesize that dietary salt interacts with these three pathways and therefore modifies their contributions to hypertension. To address our hypothesis, we used the 5/6th nephrectomy model in rats to mimic human CKD. Using this model, we first characterized hypertension by analyzing the RAS, kidney sodium and potassium transport proteins, and salt-sensitivity. Subsequently, we targeted the three pathways central to our hypothesis using adrenalectomy, losartan, and spironolactone. To analyze the role of dietary salt, all treatments were given both during a normal salt diet and a high salt diet. Our results show that dietary salt modifies the blood pressure response to RAS-inhibition and indicates that a switch occurs from predominantly angiotensin II-dependent hypertension during a normal salt diet to MR-dependent hypertension during a high salt diet.

MATERIALS AND METHODS

Animals and interventions

All animal experiments were approved by the Animal Welfare Committee of the Erasmus Medical Center (protocol number 16-790-02). Male Sprague Dawley rats (6

weeks old, average weight 200 g) were obtained from Envigo (United Kingdom). 5/6th nephrectomy was performed in two steps, including right uninephrectomy (combined with telemetry implantation and, in selected animals, right adrenalectomy) followed by resection of the left kidney poles ten days later (**Figure 1**) [15]. After a recovery period of five weeks, osmotic minipumps were implanted and, in selected animals, left adrenalectomy was performed. Rats were allocated to treatment groups on the basis of blood pressure and glomerular filtration rate (GFR) to ensure similar values between treatment groups (**Figure S1**). Rats were subsequently treated for three weeks with vehicle, spironolactone, losartan, or spironolactone + losartan (**Table S1 and S2**). These treatments were combined with a normal salt diet (0.4% NaCl) or a high salt diet (4.0% NaCl; both Envigo). During the 3-week treatment period, vehicle (10% ethanol, 15% DMSO, 75% PEG), dexamethasone (12 µg/kg/day, as glucocorticoid replacement after adrenalectomy) [16] or losartan (30 mg/kg/day) [17] were administered by osmotic minipump (Alzet, USA), while spironolactone (80 mg/kg/day) [18] was given by daily subcutaneous injection. The primary outcome parameter of the treatments was blood pressure, which was analyzed for 13 days Secondary outcome parameters were skin sodium and water content (to analyze the interstitium), heart weight (normalized for tibia length), and vasoreactivity.

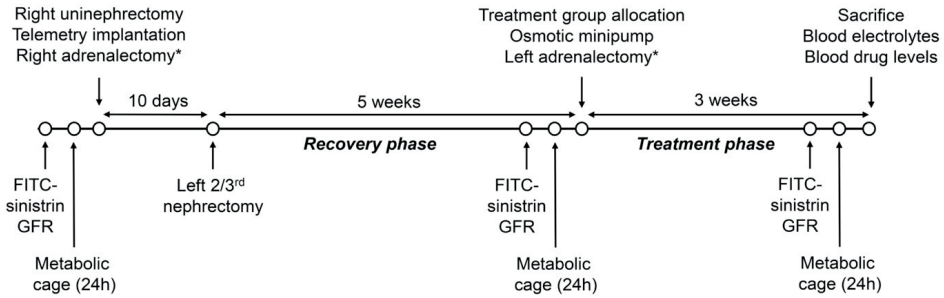

Figure 1. Overview of experimental design and maneuvers. Telemetry was recorded until the start of the final FITC-sinistrin GFR measurements. FITC, fluorescein isothiocyanate; * In selected animals.

Measurements

Blood pressure was recorded continuously via radiotelemetry transmitters (Data Sciences, USA). Because the GFR measurements and housing in metabolic cages interferes with blood pressure recordings, telemetry data were analyzed until the start of the final GFR measurements (**Figure 1**). GFR was measured using transcutaneous measurement of injected fluorescein isothiocyanate-labeled sinistrin [19]. Urine total protein was measured by the clinical chemistry laboratory of the Erasmus Medical Center. Plasma aldosterone was measured by radioimmunoassay (Demeditec, Germany), and plasma renin by an in-house enzyme-kinetic assay. The latter assay yields a value expressed in

µg Ang I/L.hr, which was converted ng renin based on the observation that 1 µg Ang I/L.hr equals 2.6 ng of renin. Plasma concentrations of losartan-carboxylic acid (the active metabolite of losartan, also called EXP3174) and canrenone (the active metabolite of spironolactone) were measured by liquid chromatography coupled to mass spectrometry (Thermo Fisher, USA) [20]. For measurement of skin sodium (in mmol/mL water) and skin water (in mL/gram dry weight), skin samples (1x1 cm) were freeze-dried, dissolved in nitric acid and hydrogen peroxide, centrifuged and filtered, after which sodium was measured by flame photometry.

Immunoblot

To compare transporter abundance between the intact healthy kidney and the remnant kidney we developed a method to normalize by estimated nephron number (**Figure S2**). Using this method, the weight of the remnant kidney at the time of the uninephrectomy can be estimated by the following equation: weight kidney poles at 2/3rd nephrectomy ("A") * 0.65 * (weight right kidney at the time of uninephrectomy * 0.9908 / (A + [A*0.65]). Subsequently, 20-70 µg of protein (protein assay Bio-Rad, USA) was separated by SDS-PAGE and transferred to a membrane with a trans-blot turbo system (Bio-Rad). Membranes were blocked in 5% milk or bovine serum albumin and incubated overnight at 4 °C with the primary antibody (**Table S3**).

Myograph studies

Immediately after sacrifice, mesenteric arteries were dissected, placed in a cold, oxygenated buffer solution, cut into 2-mm segments and mounted on myographs (Danish Myo Technology, Denmark). Subsequent experiments were performed as reported previously [21]. Briefly, contractile capacity was examined by adding KCl (30 and 100 mM), and concentration-response curves were constructed for the vasodilatory response to acetylcholine (after pre-contraction with the thromboxane A_2 analogue U46619 at 10 nM), and acetylcholine in combination with the nitric oxide synthase inhibitor L-N^G-Nitroarginine Methyl Ester (L-NAME, 100 µM).

Statistics

Results are presented as the mean ± standard error of the mean for normally distributed data and median with interquartile range for non-normally distributed data. Non-normally distributed data were log-transformed for statistical analysis. Group comparisons were performed with one-way ANOVA and two-way ANOVA for repeated measurements. If significant, selected post-hoc analyses were performed between individual groups by controlling for a false-discovery rate of 5% [22]. Correlations were analyzed using Pearson's correlation coefficient. If a correlation was present between normally distributed data and log-transformed data, nonlinear regression using a linear-logarithm model

was used to fit the original data. For myograph data, pEC_{50} (negative log of the agonist concentration needed to reach half of its maximal effect) and E_{max} (maximal response) between control and treated groups were analyzed using a paired T-test. Data were analyzed using GraphPad Prism (version 8.2.0, USA). $P \leq 0.05$ was considered statistically significant.

RESULTS

5/6th nephrectomy causes low-renin hypertension

The 5/6th nephrectomy model was first characterized by comparing blood pressure, GFR, proteinuria, and the RAS before 5/6th nephrectomy, 5 weeks after 5/6th nephrectomy (start of treatment) and 8 weeks after 5/6th nephrectomy (end of treatment). 5/6th nephrectomy reduced GFR by 71% (**Figure 2C**) and was accompanied by a progressive rise in systolic and diastolic blood pressure (**Figure 2A and 2B**) and proteinuria (**Figure 2D**), and a decrease in plasma renin (**Figure 2E**). The decrease in plasma renin was not accompanied by a decrease in plasma aldosterone (**Figure 2F**), and therefore the aldosterone-to-renin ratio increased over time (**Figure 2G**). A lower GFR correlated with a higher aldosterone-to-renin ratio (**Figure 2H**). 5/6th nephrectomy caused hyperkalemia with higher urinary sodium and potassium excretion (**Table S4, Figure S3**).

5/6th nephrectomy causes salt-sensitive hypertension with cardiac hypertrophy

Compared to the normal salt diet, 5/6th nephrectomy with a high salt diet resulted in a significantly higher urinary sodium excretion and higher blood pressure, confirming salt-sensitivity of the model (**Figure 3A and B, 4A, and S3**). 5/6th nephrectomy with the normal salt diet increased heart weight, and this was increased further by high salt (**Figure 3C**). Plasma renin was reduced by the high salt diet in sham rats and by 5/6th nephrectomy (**Figure 3D**). Plasma aldosterone was significantly higher in 5/6th nephrectomy rats on high salt compared with sham-operated rats on high salt (**Figure 3E**). A high salt diet or 5/6th nephrectomy alone did not lead to sodium or water accumulation in skin, whereas the combination did (**Figure 3F and G**).

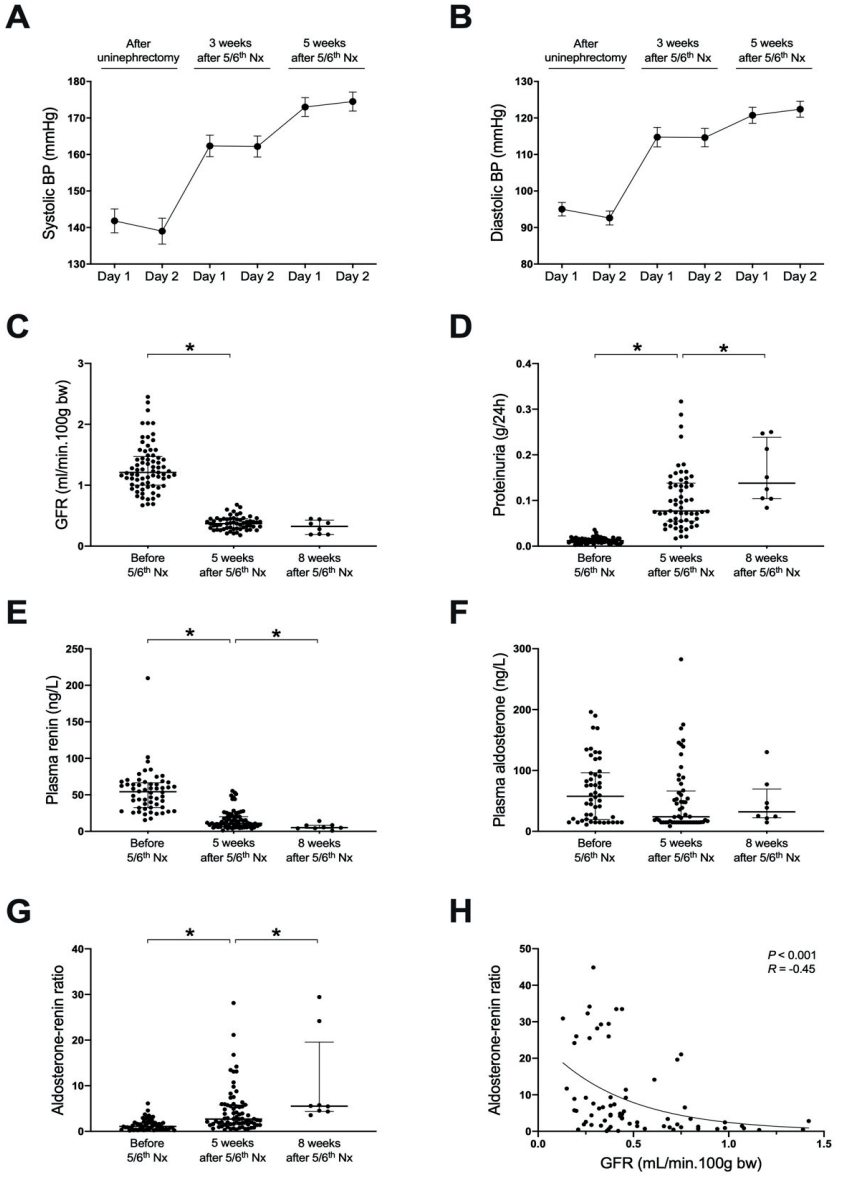

Figure 2. 5/6th nephrectomy reduces glomerular filtration rate and plasma renin, and increases proteinuria and blood pressure. (**A-B**) The average systolic and diastolic blood pressure is shown on two consecutive days after uninephrectomy (n = 27), and three (n = 37) and five weeks (n = 65) after 5/6th nephrectomy. (**C-G**) Glomerular filtration rate (GFR), proteinuria, plasma renin, plasma aldosterone were measured before uninephrectomy (n = 78), 5 weeks after 5/6th nephrectomy (5/6th Nx, n = 59) and 8 weeks after 5/6th Nx (n = 8). (**H**) Correlation between GFR and aldosterone-to-renin ratio (n = 70). * $P \leq 0.05$

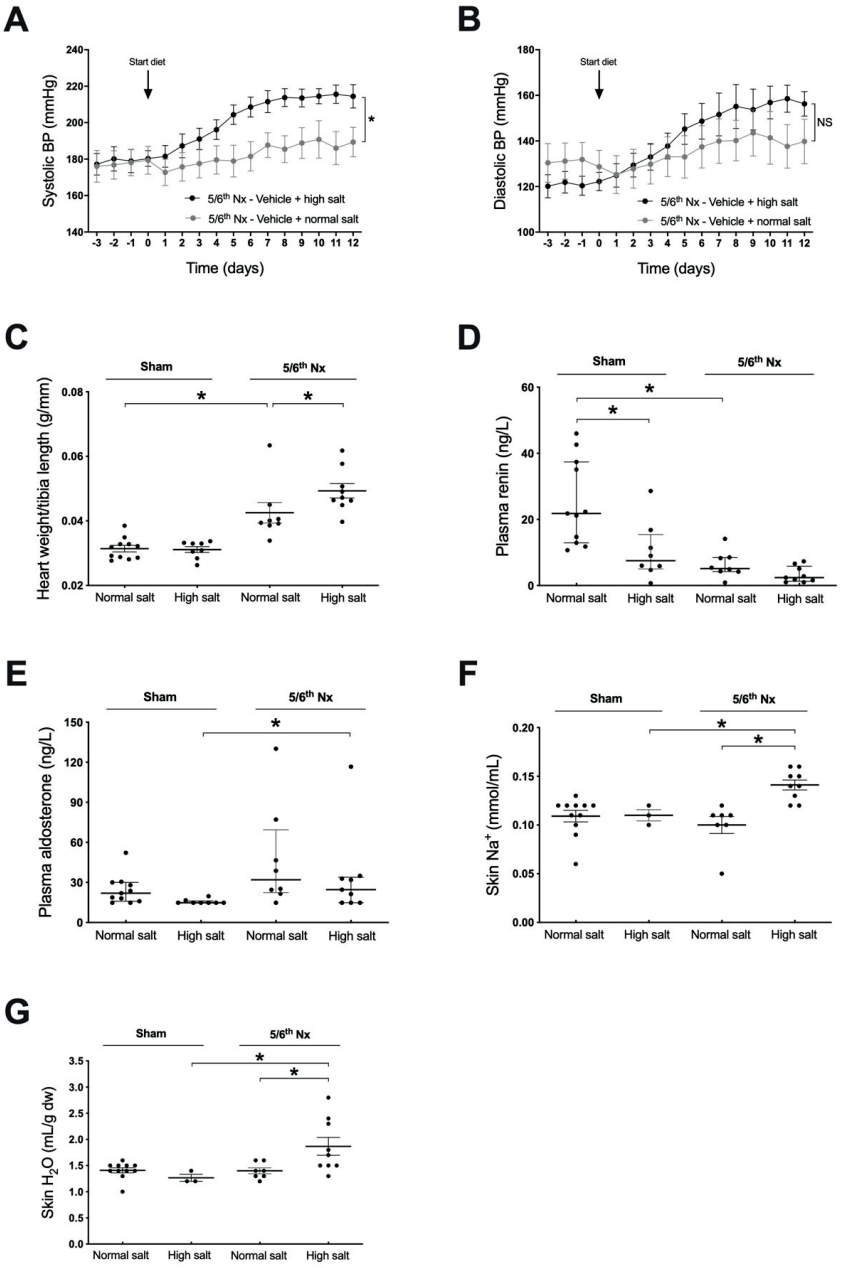

Figure 3. High salt exacerbates hypertension and cardiac hypertrophy, and increases skin sodium and water in rats after 5/6th nephrectomy (Nx). (**A-G**) Effects of normal and high salt diet on systolic and diastolic blood pressure, heart weight, plasma renin, plasma aldosterone, skin sodium (Na^+) concentration, and skin water (H_2O) content (n = 7 vs. 8 in panels A-B, n = 3–11 in panels C-G). * $P \leq 0.05$.

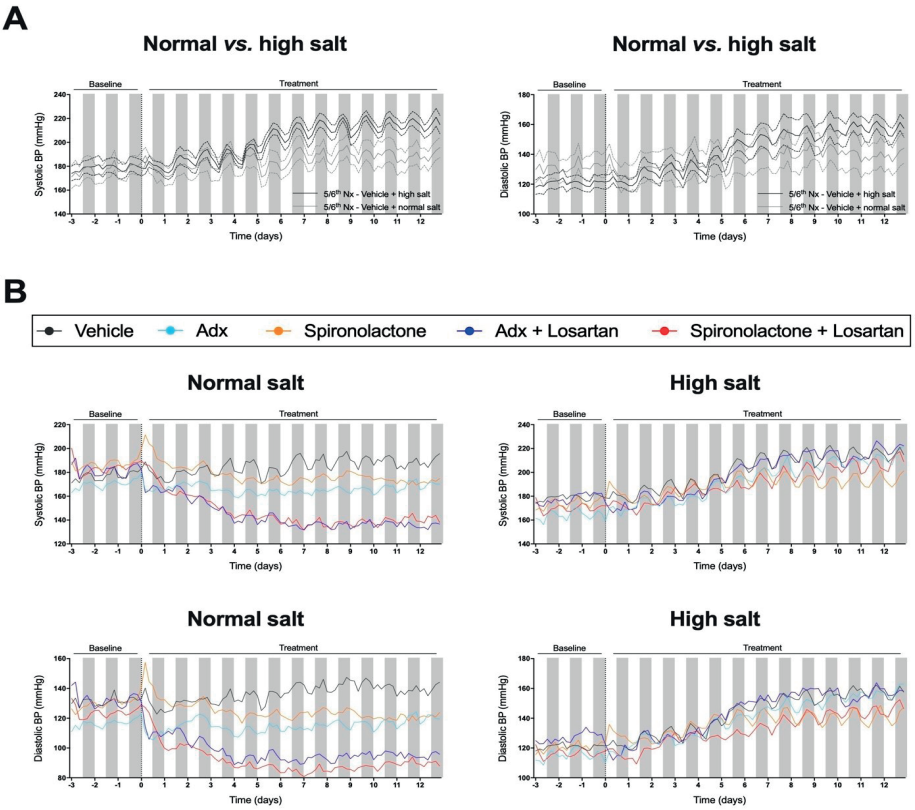

Figure 4. Telemetry data showing the effects on blood pressure of the high salt diet (**A**) and the various treatments (**B**). The dashed lines represent the SEMs (**A**). Telemetry was recorded during 13 days of the 3-week treatment (until the start of the final FITC-sinistrin measurements, see **Figure 1**). BP, blood pressure; Adx, adrenalectomy.

Kidney transporter profile of 5/6th nephrectomy

Compared to sham, 5/6th nephrectomy reduced the abundances of NHE3, total and phosphorylated NKCC2, total NCC, α-ENaC, and Kir5.1 regardless of the diet (**Figure 5A and B**). The decrease in phosphorylated NCC was statistically significant only for the high salt diet. Conversely, on both diets, 5/6th nephrectomy increased the abundances of SGK1, prostasin, γ-ENaC, and Kir4.1 compared to sham (**Figure 5A and B**). Of note, no shift in the molecular weight of γ-ENaC was observed. Next, we analyzed if these changes in transporter abundances correlated with known regulators, including the RAS and serum potassium. This analysis showed that higher plasma aldosterone correlated with higher γ-ENaC; a trend for a correlation with blood pressure was also found (**Figure 6A**). Lower plasma renin correlated with lower NHE3, total NCC, and α-ENaC, but higher SGK1 and prostasin (**Figure 6B**). Finally, higher serum potassium correlated with lower NHE3, total NCC, and phosphorylated NCC, but higher Kir4.1; a trend for a correlation with lower Kir5.1 was also found (**Figure 6C**).

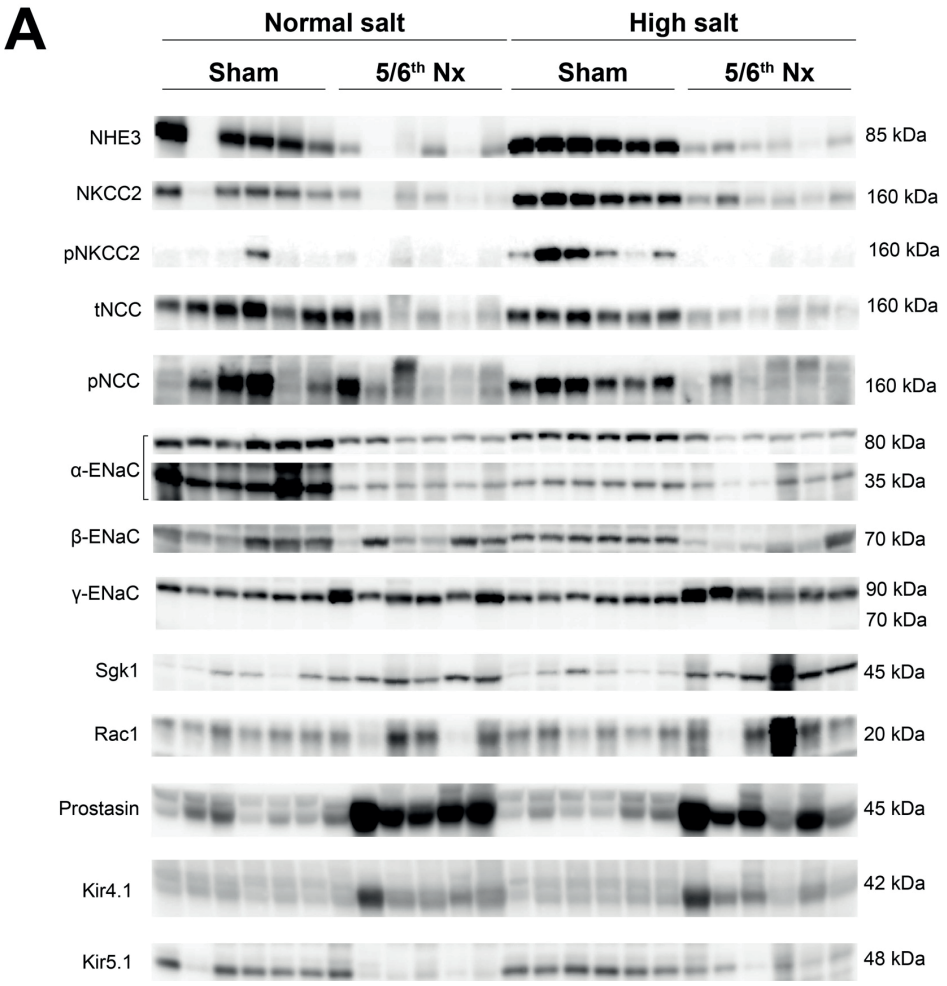

Figure 5. Rat kidney transporter profile after sham or 5/6th nephrectomy and on normal or high salt diets. (**A**) Immunoblots of transporters and regulatory proteins in kidney homogenates from sham rats and 5/6th nephrectomy (5/6th Nx) rats on a normal and high salt diet (n = 6/group).

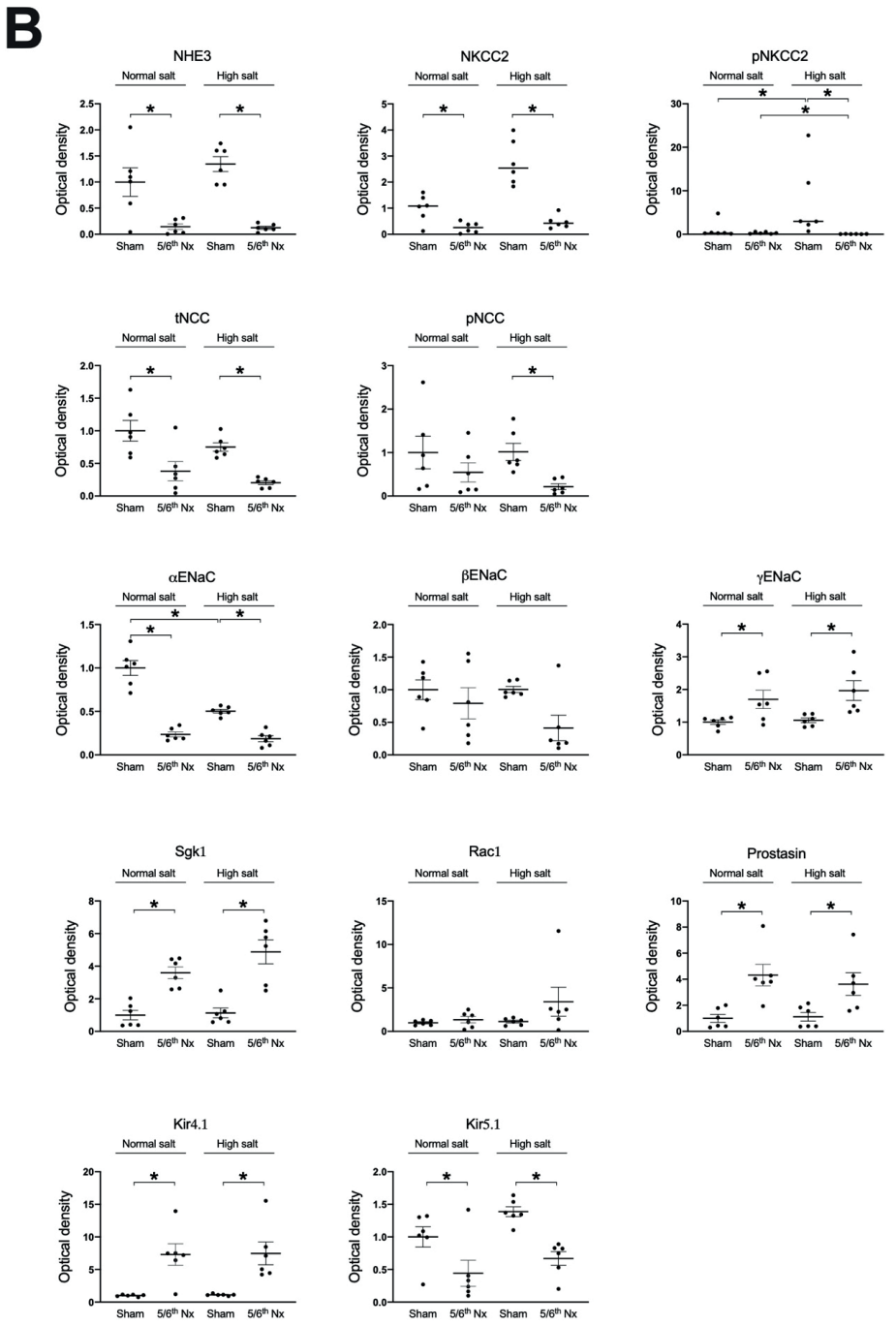

Figure 5. Rat kidney transporter profile after sham or 5/6th nephrectomy and on normal or high salt diets. (**B**) Densitometry was analyzed by unpaired t-test. See **Figure S2** for normalization method. * $P \leq 0.05$. NHE3, sodium-hydrogen exchanger type 3; NKCC2, sodium potassium chloride cotransporter 2; pNKCC2, phosphorylated NKCC2; NCC, sodium chloride cotransporter; pNCC, phosphorylated NCC at threonine 53/58; ENaC, epithelial sodium channel; Sgk1, serum and glucocorticoid-regulated kinase 1; Rac1, Ras-related C3 botulinum toxin substrate 1; Kir, inwardly rectifying potassium channel.

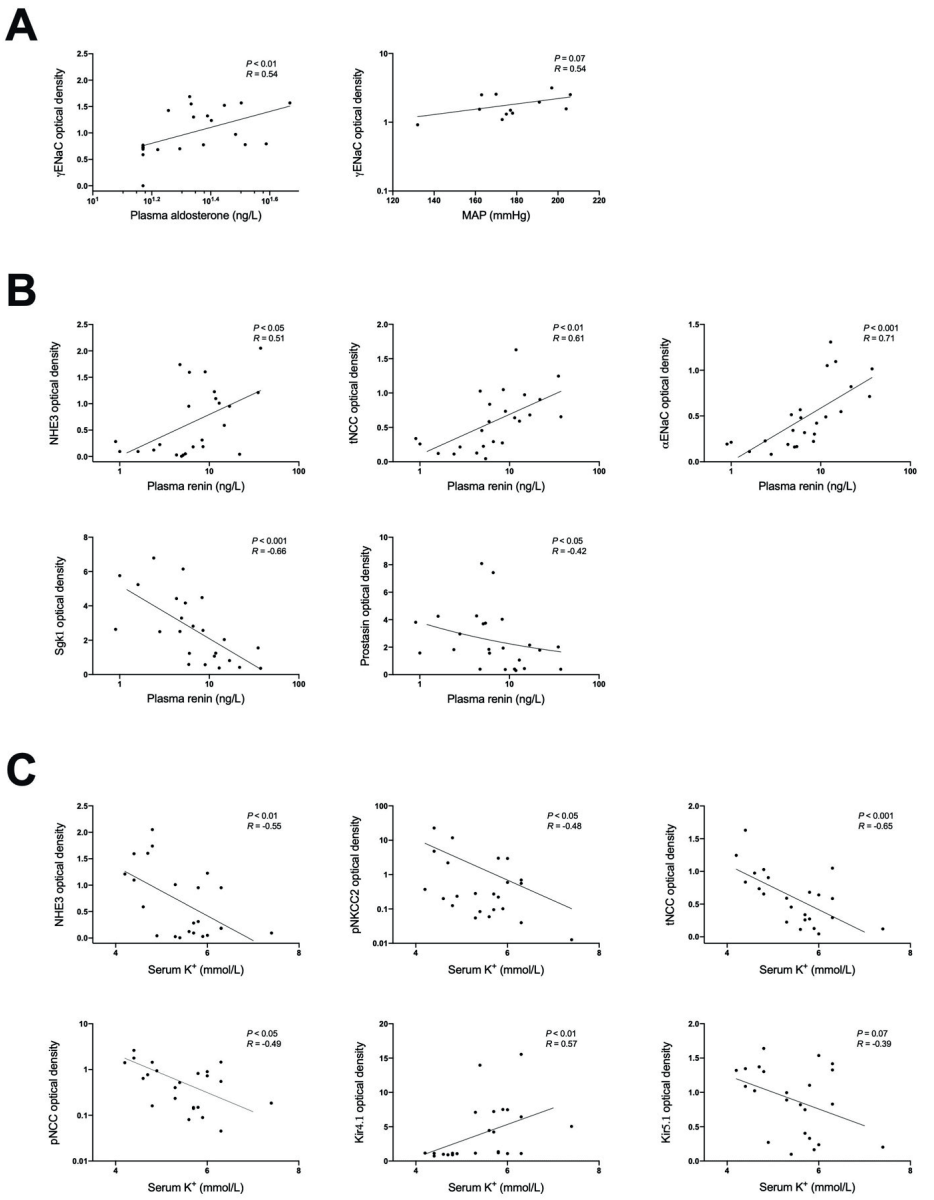

Figure 6. Correlations between the renin-angiotensin system, serum potassium, blood pressure, transporters, and regulatory proteins. (**A**) Correlations between plasma aldosterone, mean arterial pressure (MAP), and γ-ENaC (n = 24 and n = 12; MAP was not measured in sham). (**B**) Correlations between plasma renin, NHE3, total NCC (tNCC), α-ENaC, serum and glucocorticoid-regulated kinase 1 (Sgk1), and prostasin (n = 24). (**C**) Correlations between serum potassium (K+), NHE3, phosphorylated NKCC2 (pNKCC2), tNCC, phosphorylated NCC (pNCC), Kir4.1, and Kir5.1 (n = 24).

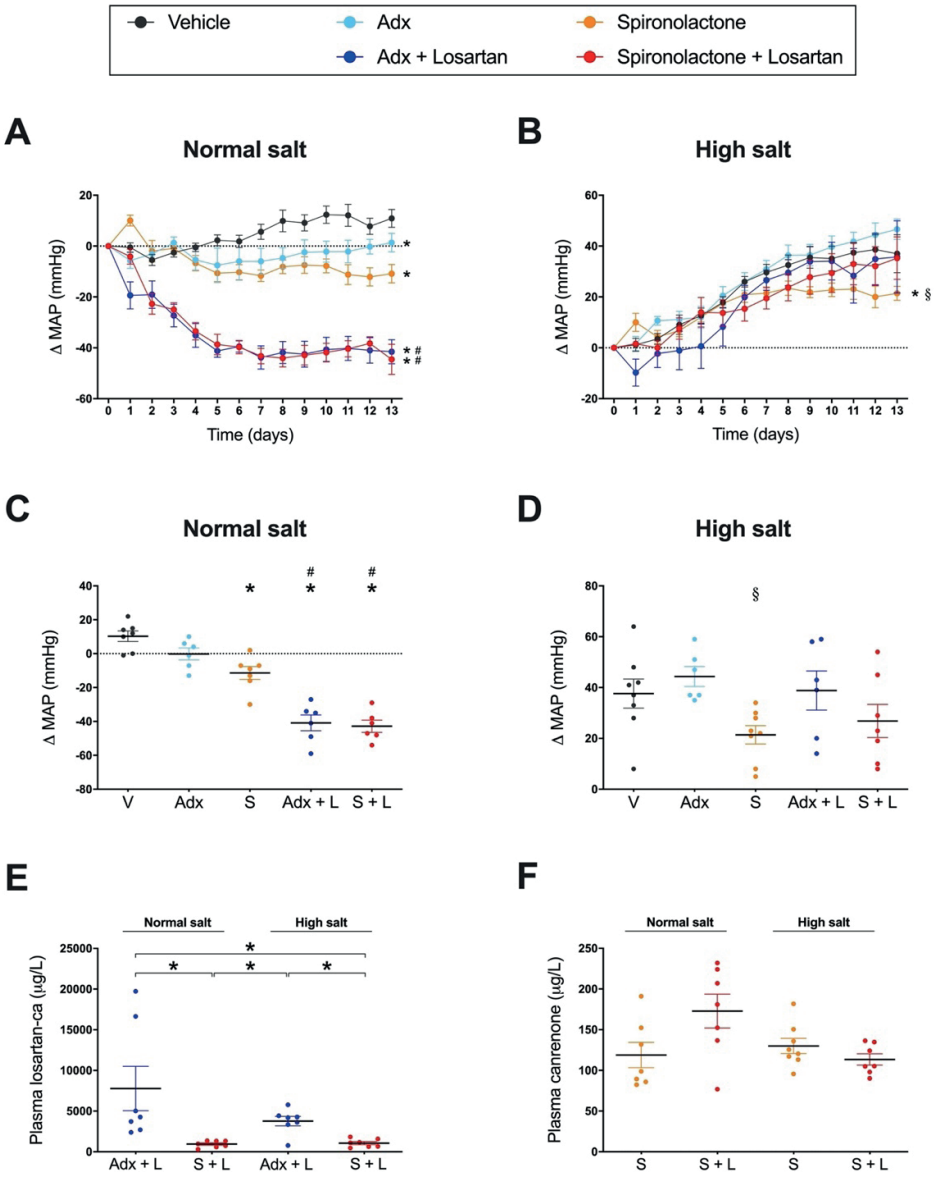

Figure 7. Blood pressure response to renin-angiotensin interventions and dietary salt. (**A-D**) The changes in systolic and diastolic blood pressure are shown on a normal salt diet (**A, C**) and a high salt diet (**B, D**). Rats (n = 6-8/group) were treated with a normal salt (0.4% NaCl) or high salt (4.0% NaCl) diet and with vehicle (V), adrenalectomy (Adx), spironolactone (S), adrenalectomy and losartan (Adx + L), or spironolactone and losartan (S + L). (**E, F**) Losartan carboxyl acetate (losartan-ca) and canrenone concentrations were measured at the day of sacrifice in rats (n = 7/group) receiving losartan and/or spironolactone. * $P \leq 0.05$ versus vehicle, # $P \leq 0.05$ versus spironolactone, § $P \leq 0.05$ versus adrenalectomy.

High salt diet modifies treatment response

First, we compared the anti-hypertensive effect of the RAS-interventions on a normal salt diet (**Figure 4B, 7A and C**). Blood pressure increased with vehicle (10 ± 8 mmHg), and was stabilized by adrenalectomy (0 ± 9 mmHg, not significant vs. vehicle) and reduced by spironolactone (-11 ± 10 mmHg, $P < 0.05$ vs. vehicle). The combinations of losartan with adrenalectomy or losartan with spironolactone had the strongest anti-hypertensive effects (-41 ± 12 and -43 ± 9 mmHg, respectively). Next, the anti-hypertensive effects of the same RAS-interventions were compared on a high salt diet. The high salt diet caused a progressive rise in blood pressure of 38 ± 16 mmHg that was resistant to most interventions (**Figure 4B, 7B and D**). Spironolactone was the only treatment that significantly attenuated this rise in blood pressure (-16 ± 7 mmHg). The lack of effect of the treatments with losartan on high salt was not explained by lower drug exposure, because no significant differences between the blood concentrations of losartan carboxylic acid on normal and high salt diet were observed (**Figure 7E**). Of note, the lower losartan carboxylic acid concentrations were likely due to spironolactone increasing the metabolism of losartan through induction of cytochrome P450 3A4 [23]. In both the normal and high salt groups, plasma aldosterone levels after adrenalectomy were below the limit of detection.

Treatment effects on skin sodium, skin water, and heart weight

None of the interventions reduced skin sodium concentration (**Figure 8A**), but some interventions did reduce skin water content (**Figure 8B**). Spironolactone with losartan significantly lowered skin water content on both diets; spironolactone monotherapy also had this effect with the high salt diet. On the high salt diet, adrenalectomy, adrenalectomy with losartan, and spironolactone with losartan reduced heart weight; spironolactone with losartan also reduced heart weight on the normal salt diet (**Figure 8C**). When combining the data of all rats, we identified positive correlations between blood pressure and heart weight (**Figure 8D**), skin water and blood pressure (**Figure 8E**), and skin water and heart weight (**Figures 8F**). On a normal salt diet, the interventions with losartan increased plasma renin, and the interventions with spironolactone increased plasma aldosterone; these effects, however, did not occur in rats fed a high salt diet (**Figure S4**). On a normal salt diet, all interventions prevented an increase in proteinuria; a high salt diet increased proteinuria despite the interventions (**Figure S4**).

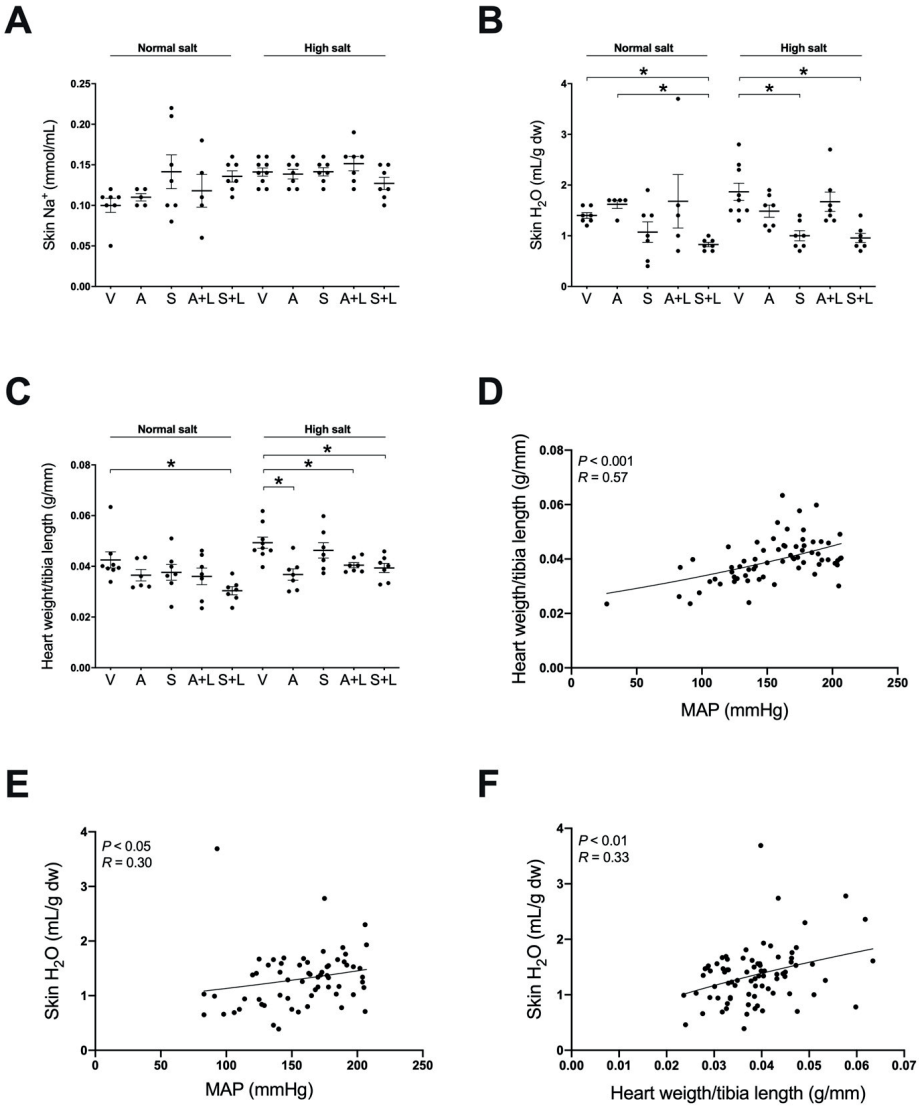

Figure 8. Effects of treatments on skin sodium, skin water, and heart weight, and relation with blood pressure. (**A-C**) Skin sodium (Na^+) concentration, skin water (H_2O) content, and heart weight were measured in the different treatment groups receiving the normal or high salt diet (n = 6–8/group). (**D-F**) Correlations between mean arterial pressure (MAP), heart weight, and skin H_2O content were calculated including rats from all experimental groups (n = 63–82). * $P \leq 0.05$. A, adrenalectomy; A+L, adrenalectomy + losartan; S, spironolactone; S+L, spironolactone + losartan; V, vehicle.

High salt impairs vasodilation which is relieved by spironolactone

On normal salt diets, the maximal responses to acetylcholine were unaffected by 5/6th nephrectomy; in contrast, on high salt intake, nephrectomized rats exhibited a suppressed maximal dilation (E_{max} 93 ± 5% *vs.* 73 ± 23%, $P < 0.05$, **Figure 9A**). The calculated

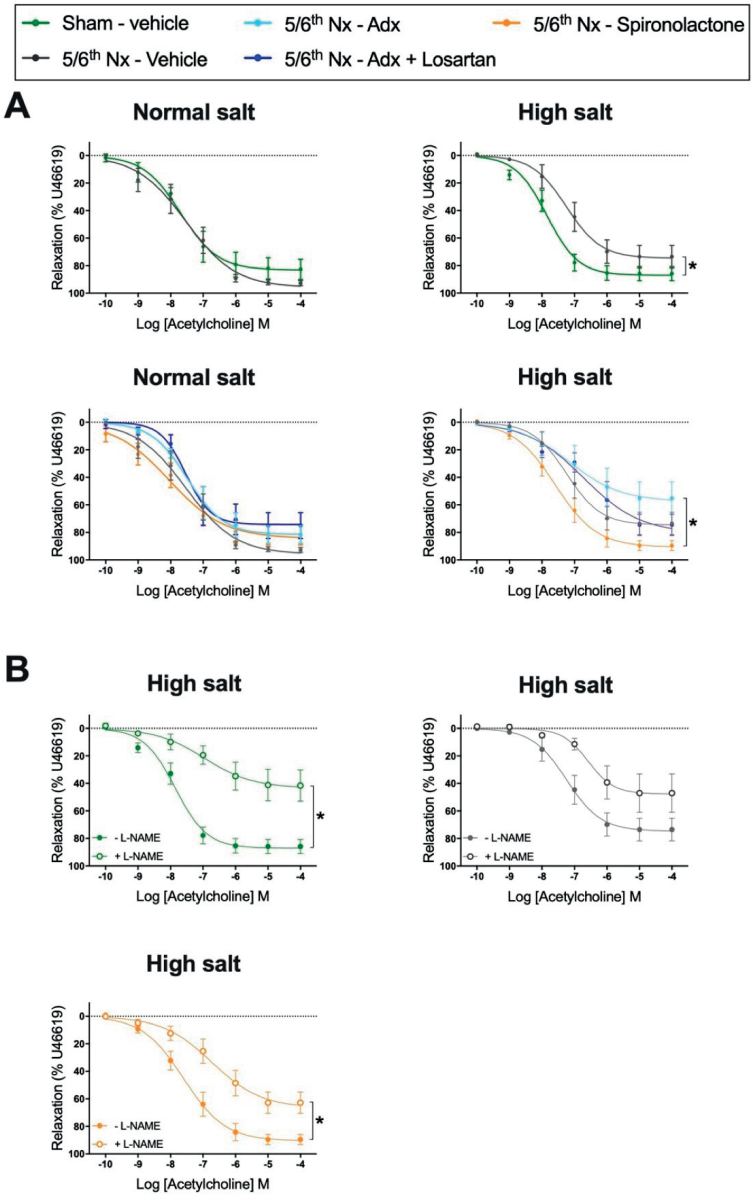

Figure 9. In 5/6th nephrectomy rats, high salt impairs vasodilation which is relieved by spironolactone. (**A**) The vasodilatory responses are shown in sham-operated rats and 5/6th nephrectomy rats on a normal salt or high salt diet (upper panels) and in 5/6th nephrectomy rats after treatment with vehicle, adrenalectomy (Adx), adrenalectomy with losartan, or spironolactone (lower panels). *n* = 4–8/group. (**B**) Vasodilatory responses to acetylcholine with or without L-N^G-Nitroarginine Methyl Ester (L-NAME) in sham-operated rats (*n* = 6) and 5/6th nephrectomy (5/6th Nx) rats treated with vehicle or spironolactone on a high salt diet (*n* =8/group). The three curves without L-NAME are also shown in panel A. Vasodilatory responses of mesenteric arteries are expressed as percentage of the pre-contraction induced by U46619. Curves covering the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain the pEC_{50} value (negative log of the molar concentration of an agonist needed to reach half of its maximal effect). The relaxation obtained at the highest concentration of an agonist was considered as E_{max} (maximal response). Data are expressed as mean ± SEM. * $P \leq 0.05$.

pEC_{50} values were also lower in 5/6th nephrectomy rats on high salt compared with sham-operated rats on high salt (pEC_{50} 7.2 ± 0.62 *vs.* 7.9 ± 0.31, $P < 0.05$). In 5/6th nephrectomy rats on high salt, spironolactone improved the vasorelaxation to acetylcholine, although this was only statistically significant when compared to adrenalectomy (E_{max} 90 ± 10% *vs.* 55 ± 31%, $P < 0.05$). Furthermore, spironolactone also restored the contribution of nitric oxide to the vasodilatory response of acetylcholine (**Figure 9B**). Vasoreactivity was not tested in 5/6th nephrectomy rats treated with spironolactone and losartan.

DISCUSSION

Here, we used the 5/6th nephrectomy rat model to analyze hypertension in chronic kidney disease (CKD) and the response to dietary salt and renin-angiotensin system (RAS) interventions. Our study produced three novel findings. First, hypertension after 5/6th nephrectomy was characterized as a low-renin, salt-sensitive form of hypertension with hyperkalemia. This was accompanied by a selective increase in ɣ-ENaC and its regulatory proteins SGK1 and prostasin, possibly contributing to salt retention. In turn, low renin and hyperkalemia were linked to appropriate changes in other kidney sodium and potassium transporters. Second, on a normal salt diet, especially angiotensin II contributed to hypertension, whereas the roles of aldosterone and the mineralocorticoid receptor were less prominent. A high salt diet completely changed this picture with spironolactone being the only treatment that attenuated the rise in blood pressure. Third, 5/6th nephrectomy with a high salt diet increased skin sodium and water content, likely reflecting interstitial accumulation. Skin water correlated with blood pressure and heart weight, and was reduced by spironolactone; spironolactone also increased natriuresis and restored vasorelaxation.

Although our transporter data are descriptive, the correlation analysis suggests that hyperkalemia decreased Kir4.1 and NCC, and increased Kir5.1, while aldosterone increased ɣ-ENaC. This is consistent with previous data from healthy animals [24, 25], but has not been analyzed previously in a CKD model. Kir4.1/5.1 forms heterotetramers which are crucial for excreting a dietary potassium load [26, 27]. This is the first study to analyze these channels in the context of hyperkalemia. It is not clear whether NHE3 and NKCC2 were also reduced secondary to hyperkalemia or other changes after 5/6th nephrectomy [28, 29]. Previous 5/6th nephrectomy studies in rats also showed reduced NHE3 and increased ENaC, whereas NKCC2 and NCC showed an initial increase but subsequent decrease [30, 31]. A recent study showed that inflammation increased NCC in both the aristolochic acid and adenine models of CKD, but not after 5/6th nephrectomy [32]. Our data suggest that the mechanisms of NCC dephosphorylation by serum potassium is

intact after 5/6th nephrectomy [24]. A selective effect on ENaC subunits has been observed previously in diabetic rats with high aldosterone [33]. Because the rats were proteinuric, urinary proteases such as plasmin may also have contributed to increased ɣ-ENaC [34]. Despite proteolytic processing prostasin-cleavage of ɣ-ENaC is not always detectable in the kidney [35]. Another relevant question is why 5/6th nephrectomy increases Sgk1. Although this could also be driven by aldosterone, other Sgk1 activating mechanisms pertaining to CKD may have contributed, including proteinuria, transforming growth factor β, mechanical stretch, and changes in cell volume [4]. Thus, these data suggest an important role for hyperkalemia and aldosterone. The clinical relevance of this interaction for hypertension was illustrated in a study in which the potassium binder patiromer reduced hyperkalemia, aldosterone, and blood pressure in patients with CKD [36]

With regard to the response to RAS-inhibition, a relevant question is why losartan loses its blood pressure lowering effect during a high salt diet. In rats with intact kidneys, high salt suppresses plasma and kidney angiotensin II, but increases tubular angiotensin II [37]. Therefore, we propose three possible explanations: (1) losartan is unable to inhibit tubular angiotensin II, (2) 5/6th nephrectomy with high salt requires a higher dose of losartan, or (3) high salt activates other hypertensive pathways. In a previous study losartan (similar dose as in this study) also failed to reduce blood pressure in normal rats on a high salt diet, but it did reduce kidney RAS components, and proteinuria [38]. Of note, previous studies showed that angiotensin receptor blockers can still lower blood pressure in 5/6th nephrectomized animals on a high salt diet [39-43]. However, the design of these studies differed from ours, including higher losartan dosing [42], intravenous dosing [39], the use of spontaneously hypertensive rats [40], Dahl salt-sensitive rats [44], or C57BL/6 mice [43]. Of note, in mice RAS activity [45] and response to 5/6th nephrectomy [46] are different. A strength of our study is that we used a dose of losartan that produced similar concentrations of the active metabolite as it does in patients [47].

The observation that spironolactone but not adrenalectomy attenuated the rise in blood pressure with high salt suggests activation of the mineralocorticoid receptor during a high salt diet. Alternatively, glucocorticoid replacement may have limited treatment response, despite the fact that a physiological replacement dose was used [48]. Aldosterone-independent activation of the mineralocorticoid receptor can occur through activation of Rac1 [49]. However, we observed no clear change in Rac1, although Rac1-inhibitors would be required to address this more conclusively. Another possibility is that the mineralocorticoid receptor in endothelial cells is involved [50]. CKD causes endothelial dysfunction that is characterized by reduced nitric oxide mediated vasodilation [51, 52]. Mineralocorticoid receptor blockade increases the bioavailability of nitric oxide and improves endothelial function [53]. Consistent with this observation, we

show that after 5/6th nephrectomy, the high salt diet reduced the vasodilatory response of mesenteric arteries to acetylcholine, and that spironolactone treatment restored the contribution of nitric oxide to this response. A related mechanism that could contribute is β-adrenergic overstimulation, which can be caused by both CKD and a high salt diet [54, 55]. Indeed, vasoconstriction induced by β-adrenergic overstimulation in rats was blunted by spironolactone but not by losartan and this occurred through restoration of nitric oxide bioavailability [56].

Although the primary focus of this study was on blood pressure, we also analyzed effects on heart weight (as measure of left ventricular hypertrophy) and skin sodium and water content (as measure of interstitial accumulation). Our data confirm previous findings that 5/6th nephrectomy increases heart weight, and that the addition of a high salt diet increases this even further [57]; this relationship is likely explained by hypertension. Recent data indicate that the phenomenon of skin sodium accumulation is associated with left ventricular hypertrophy in patients with CKD [58]. However, our interventions did not reduce skin sodium but rather skin water content, which also correlated with heart weight. A higher skin water content likely reflects interstitial fluid accumulation and might signal extracellular fluid volume expansion, which is an independent determinant of resistant hypertension in patients with CKD [59].

Although RAS-interventions have been extensively studied in rat CKD models, the strength of this study was that it dissected the roles of individual RAS-components on a normal and high salt diet. However, a number of limitations should also be mentioned. First, no CKD model fully translates to human CKD, although we do believe the characteristics of the 5/6th nephrectomy model are appropriate to study CKD-related hypertension [3, 7]. Second, we only performed the analysis in male rats, because we already studied several variables (multiple RAS-interventions, normal and high salt diet). Because sex differences in tubular transport and salt-sensitive hypertension are increasingly recognized [60, 61], future studies should address this topic in female rats. Third, no cumulative sodium balance measurements were performed, because we did not obtain approval for continuous housing in metabolic cages. Finally, the transporter profile was descriptive and future intervention studies should address which sodium reabsorptive pathways contribute to hypertension in CKD.

If our data allow translation, they reiterate the importance of restricting dietary sodium in CKD to maintain the efficacy of RAS-inhibitors. Previous clinical trials have shown that a low sodium diet enhances RAS-inhibitor efficacy in CKD [62, 63], again emphasizing the close interaction between dietary salt and the RAS in CKD. We recently showed that dietary sodium restriction and distal diuretics effectively lowered blood pressure in

patients with CKD in the absence of RAS-inhibitors [64]. Thus, RAS-inhibitors and mineralocorticoid receptor antagonists are not required for blood pressure lowering in CKD, but have been shown to provide blood pressure independent end-organ protection [10, 65-67]. Although the use of mineralocorticoid receptor antagonists in more advanced CKD is often limited by hyperkalemia, the non-steroidal mineralocorticoid receptor antagonist finerenone causes hyperkalemia less frequently, and reduces the surrogate outcome markers albuminuria and NT-pro-BNP [68, 69]. Future studies should address whether salt-induced activation of the mineralocorticoid receptor also occurs in human CKD and further explore the signaling pathways involved [70].

In summary, in the 5/6th nephrectomy rat CKD model, salt-sensitive hypertension develops with a selective increase in γ-ENaC and despite appropriate transporter adaptations to volume expansion and hyperkalemia. With normal salt, hypertension after 5/6th nephrectomy depends on angiotensin II and aldosterone, while high salt aggravates hypertension with enhanced sensitivity of the mineralocorticoid receptor.

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SUPPLEMENTAL MATERIAL

Table S1. Experimental groups

Group	Surgery kidney	Surgery adrenal gland	Dexa-methasone	Drug treatment
Normal salt (0.4% NaCl)				
Sham – vehicle	Sham	Sham	No	Vehicle
5/6Nx – vehicle	5/6th Nx	Sham	No	Vehicle
5/6Nx –Adx	5/6th Nx	Adx	Yes	Vehicle
5/6Nx – Spiro	5/6th Nx	Sham	No	Spiro
5/6Nx – Adx+Los	5/6th Nx	Adx	Yes	Los
5/6Nx – Spiro+Los	5/6th Nx	Sham	No	Spiro+Los
High salt (4.0% NaCl)				
Sham – vehicle	Sham	Sham	No	Vehicle
5/6Nx – vehicle	5/6th Nx	Sham	No	Vehicle
5/6Nx –Adx	5/6th Nx	Adx	Yes	Vehicle
5/6Nx – Spiro	5/6th Nx	Sham	No	Spiro
5/6Nx – Adx+Los	5/6th Nx	Adx	Yes	Los
5/6Nx – Spiro+Los	5/6th Nx	Sham	No	Spiro+Los

Adx, adrenalectomy; CKD, chronic kidney disease; Los, losartan; Nx, nephrectomy; Spiro, spironolactone.

Table S2. Body weight, food and water intake

	Body weight (g)	Δ Body weight (g)	Food intake (g/day)	Water intake (mL/day)
Normal salt (0.4% NaCl)				
Sham - vehicle	407 ± 24	40 ± 5	15 ± 3	19 ± 4*
5/6th Nx – vehicle	413 ± 24	42 ± 14	22 ± 4	54 ± 15‡
5/6th Nx – Adx	360 ± 28*‡	-6 ± 15*‡	20 ± 6	48 ± 12‡
5/6th Nx – Spiro	355 ± 35*‡	15 ± 35	19 ± 2	48 ± 5‡
5/6th Nx – Adx + Los	333 ± 48*‡	-32 ± 44*‡	24 ± 9	52 ± 28‡
5/6th Nx – Spiro + Los	393 ± 31	32 ± 20	27 ± 9‡	48 ± 26‡
High salt (4.0% NaCl)				
Sham - vehicle	431 ± 23*	29 ± 8	14 ± 3	33 ± 4
5/6th Nx – vehicle	370 ± 33‡	15 ± 36	15 ± 8	78 ± 43‡
5/6th Nx – Adx	343 ± 35‡	8 ± 57	16 ± 4	83 ± 20‡
5/6th Nx – Spiro	380 ± 31‡	11 ± 21	22 ± 6	113 ± 27‡
5/6th Nx – Adx + Los	352 ± 33‡	4 ± 34	14 ± 6	86 ± 35‡
5/6th Nx – Spiro + Los	409 ± 10	43 ± 10	21 ± 6	107 ± 31‡

Data are shown as mean ± standard deviation. Body weight was measured at the day of sacrifice, while the difference in body weight was between start of the interventions and sacrifice (3 weeks). Food and water intake were derived from metabolic cage housing during the 24 hours before sacrifice. Groups were analyzed separately for the normal and high salt diets which was performed by one-way ANOVA with post-hoc corrections according to Dunnett. * $P \leq 0.05$ versus 5/6th nephrectomy (5/6th Nx)-vehicle treatment, ‡ $P \leq 0.05$ versus sham-vehicle treatment.

Table S3. Antibodies

Antibody	Source	Dilution
NHE3	StressMarq	1:1000
NKCC2	StressMarq	1:1000
pNKCC2-Thr96/101	Dr. Kerim Mutig[1]	1:500
NCC	StressMarq	1:500
pNCC-Thr53/58	Dr. Robert Fenton[2]	1:500
α-ENaC	Dr. Jan Loffing[3]	1:1000
β-ENaC	Dr. Jan Loffing[3]	1:20000
ɣ-ENaC	Dr. Jan Loffing[3]	1:20000
SGK1	Millipore	1:1000
Rac1	Millipore	1:500
Prostasin	BD Biosciences	1:500
Secondary	Sigma-Aldrich	1:3000

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Table S4. Plasma sodium (Na^+), serum potassium (K^+), and urinary Na^+ and K^+ excretions

	Plasma Na^+ (mmol/L)	Serum K^+ (mmol/L)	Urine Na^+ (mmol/24h)	Urine K^+ (mmol/24h)
Normal salt (0.4% NaCl)				
Sham - vehicle	129 ± 8	4.5 ± 0.5	0.9 ± 0.3	1.6 ± 0.3
5/6th Nx – vehicle	134 ± 7	6.0 ± 0.4‡	2.0 ± 1.0‡	2.2 ± 0.6‡
5/6th Nx – Adx	133 ± 4	6.0 ± 1.0‡	1.5 ± 0.6	2.2 ± 0.8
5/6th Nx – Spiro	131 ± 11	6.1 ± 1.0‡	1.6 ± 0.2‡	1.8 ± 0.3
5/6th Nx – Adx + Los	136 ± 3	6.6 ± 0.4‡	1.6 ± 0.3	2.1 ± 0.8
5/6th Nx – Spiro + Los	127 ± 4	N.M.	1.9 ± 0.2‡	2.7 ± 0.2‡
High salt (4.0% NaCl)				
Sham - vehicle	135 ± 3	5.3 ± 0.7	9.7 ± 1.7	2.0 ± 0.2
5/6th Nx – vehicle	139 ± 7	6.8 ± 1.6‡	9.5 ± 4.1	2.0 ± 0.5
5/6th Nx – Adx	132 ± 13	6.2 ± 1.1	9.3 ± 4.2	2.1 ± 0.5
5/6th Nx – Spiro	131 ± 11	6.0 ± 0.6	14.8 ± 3.2*‡	2.5 ± 0.3
5/6th Nx – Adx + Los	140 ± 4	6.7 ± 1.2	8.8 ± 3.3	2.3 ± 0.4
5/6th Nx – Spiro + Los	129 ± 6	N.M.	14.3 ± 2.3	2.6 ± 0.3

Data are shown as mean ± standard deviation. Blood for measurements was collected at the day of sacrifice. Urine collections for sodium and potassium excretion were performed using metabolic cage housing during the 24 hours before sacrifice. Groups were analyzed separately for the normal and high salt diets which was performed by one-way ANOVA with post-hoc corrections according to Dunnett. N.M. Not measured. * $P \leq 0.05$ *versus* 5/6th nephrectomy (5/6th Nx)-vehicle treatment, ‡ $P \leq 0.05$ *versus* sham-vehicle treatment.

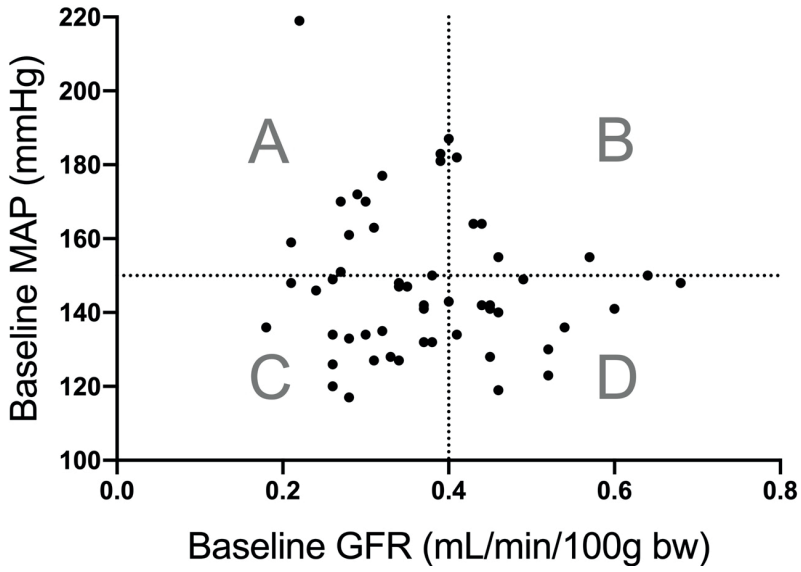

Figure S1. Group allocations
Figure shows the mean arterial pressure (MAP) and glomerular filtration rate (GFR) five weeks after 5/6th nephrectomy. These data were used to allocate equal numbers of each quadrant (A–D) to the treatment groups to ensure similar MAP and GFR prior to treatment between groups.

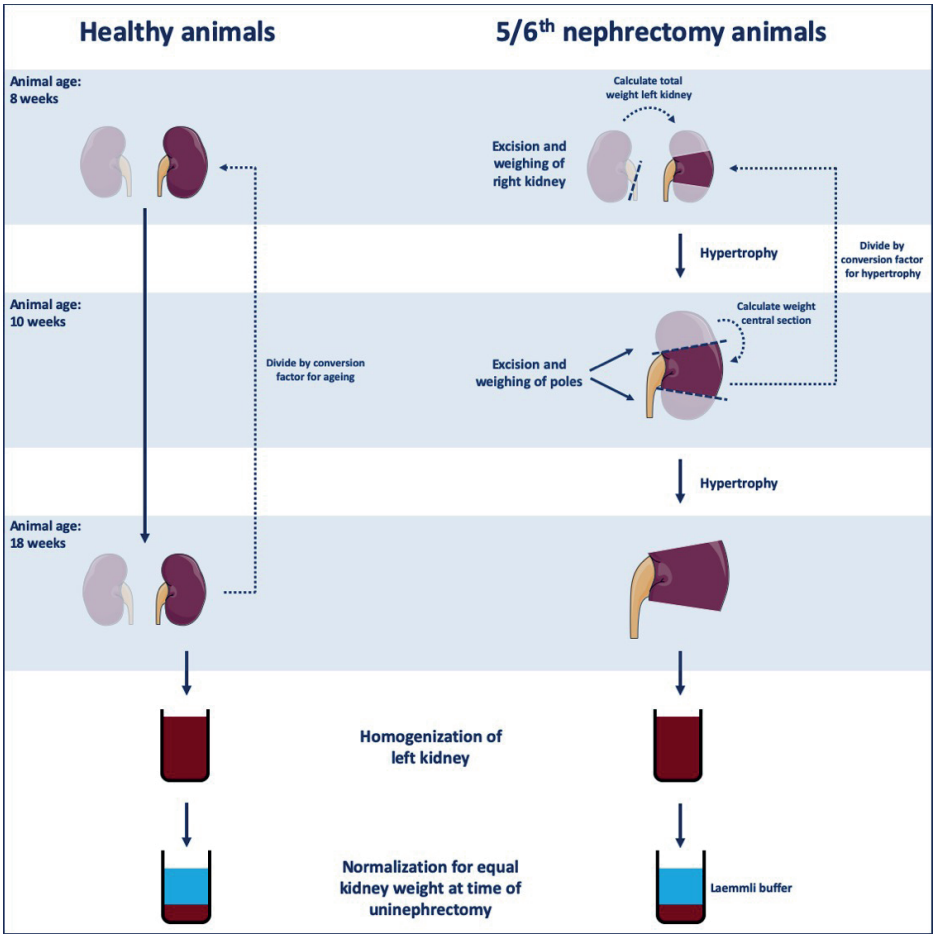

Figure S2. Overview of normalization method for kidney weight

After uninephrectomy the remaining kidney hypertrophies and gains weight, but not nephrons. To allow normalization by nephron number, we developed a method to estimate the weight of 1/3rd of remnant kidney of central section (i.e., before hypertrophy). By doing so we assume that kidney weight correlates with nephron number and that the nephrons are distributed homogeneously in the kidney.[1,2] First, we measured the weight of the right kidney directly after uninephrectomy. This weight was used to calculate the expected weight of the left kidney based on the established weight differences between the right and left male rat kidney at the age of 7 weeks (correction factor 0.9908).[3] Second, we performed a pilot study in which we weighed the two poles and the remnant kidney to establish the fractions of each segment. We established that the remnant kidney weight was on average 65% of the combined weight of the two poles. Third, we weighed the two poles after the 2/3rd nephrectomy and calculated the weight of the remnant kidney by multiplying it by 0.65. Subsequently, we added the actual weights of the poles to the projected weight of the remnant kidney to estimate the weight of the left kidney at the time of 2/3rd nephrectomy. Fourth, we calculated a correction factor by dividing the estimated weight of the left kidney at 2/3rd nephrectomy by the left kidney at uninephrectomy. This correction factor was used to deduce the weight of 1/3rd of the left kidney prior to uninephrectomy. In the healthy rats, there was a slight difference between the actual weight of the left kidneys at sacrifice and the estimated weight of the left kidney at uninephrectomy. Therefore, we also used a correction factor (mean left kidney weight at sacrifice/mean of estimated left kidney weight at uninephrectomy) to deduce the weights of the left kidneys of the healthy control animals at uninephrectomy.

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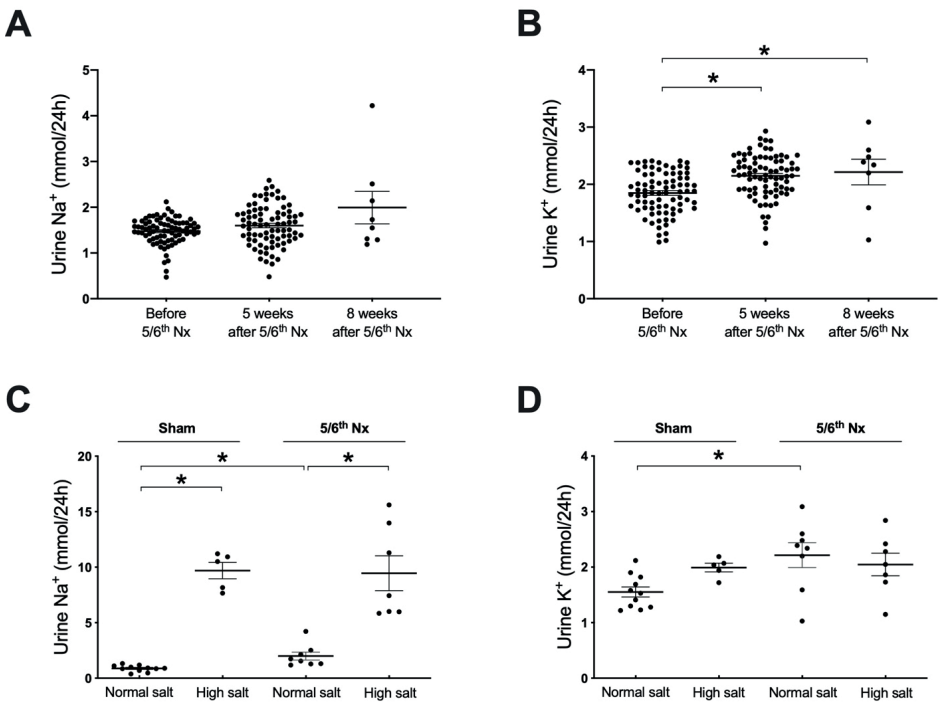

Figure S3. Urine sodium and potassium excretions

Urine sodium (Na^+) and potassium (K^+) excretion are shown before 5/6th nephrectomy (Nx) and 5 or 8 weeks thereafter (A, B). Furthermore, urine Na^+ and K^+ excretions are shown for rats after sham or 5/6th nephrectomy on normal and high salt diets (C, D).

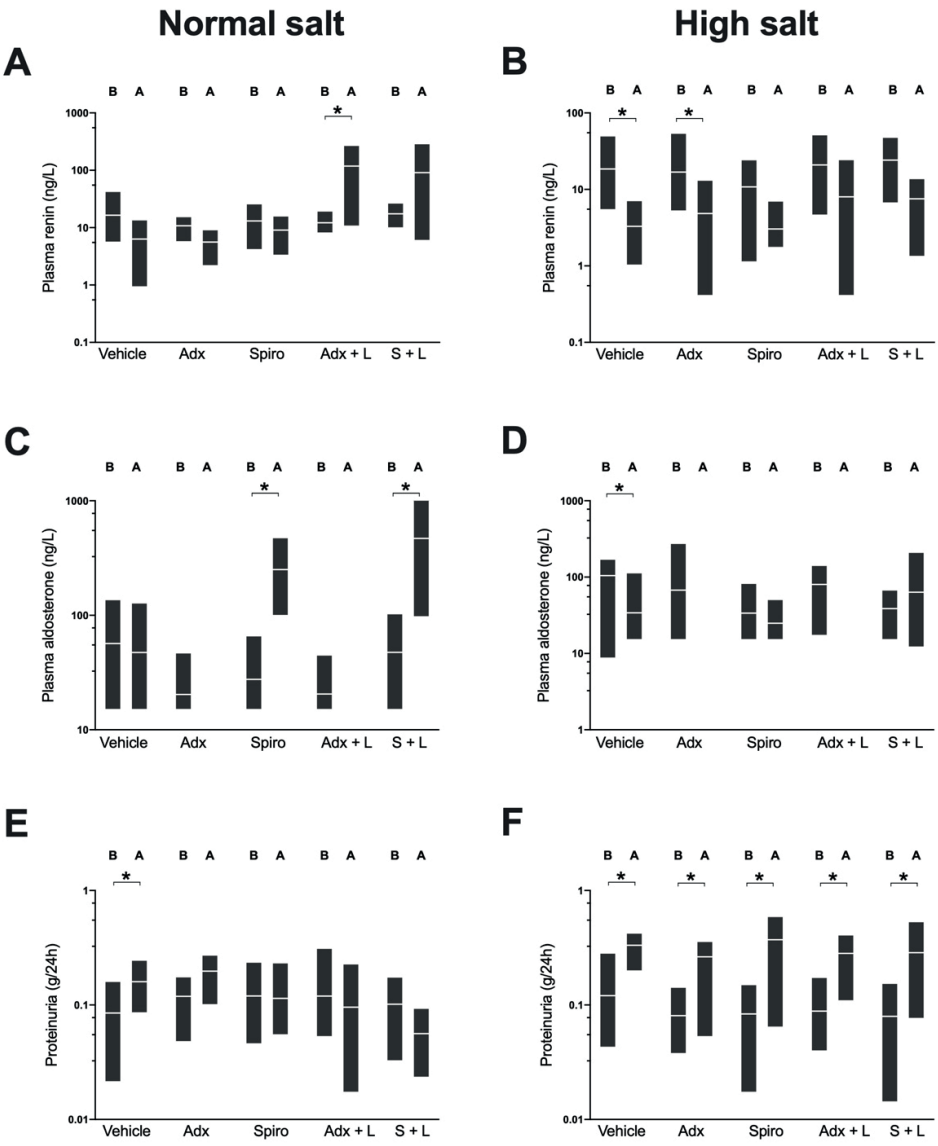

Figure S4. Treatment responses in plasma renin, plasma aldosterone, and proteinuria

Responses in plasma renin, plasma aldosterone, and proteinuria after treatment with vehicle, adrenalectomy (Adx), spironolactone (Spiro), adrenalectomy with losartan (Adx + L), or spironolactone with losartan (S + L). Data are shown before (B) and after (A) treatment. In addition, the data are shown for the normal (A, C, E) and high salt diet (B, D, F). * $P \leq 0.05$.

Chapter 4

Targeting angiotensinogen with RNA-based therapeutics

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ABSTRACT

Purpose of review: To summarize all available data on targeting angiotensinogen with RNA-based therapeutics as a new tool to combat cardiovascular diseases.

Recent findings: Liver-targeted, stable antisense oligonucleotides and small interfering RNA targeting angiotensinogen are now available, and may allow treatment with at most a few injections per year, thereby improving adherence. Promising results have been obtained in hypertensive animal models, as well as in rodent models of atherosclerosis, polycystic kidney disease and pulmonary fibrosis. The next step will be to evaluate the optimal degree of suppression, synergy with existing renin-angiotensin-aldosterone system blockers, and to determine harmful effects of suppressing angiotensinogen in the context of common comorbidities such as heart failure and chronic kidney disease.

Summary: Targeting angiotensinogen with RNA-based therapeutics is a promising new tool to treat hypertension and diseases beyond. Their long-lasting effects are particularly exciting, and if translated to a clinical application of at most a few administrations per year, may help to eliminate non-adherence.

INTRODUCTION

Although more than 100 commercial drugs and drug combinations are available for the treatment of hypertension, a substantial proportion of the hypertensive population remains uncontrolled or sub-optimally controlled. This could relate to non-adherence and/or drug ineffectiveness. The latter might be due to counterregulatory mechanisms (like a rise in renin [1]) that eliminate or diminish the initial blood pressure-lowering effect. Hence, given the deleterious consequences of uncontrolled blood pressure, there still is a need for novel treatment options that preferably are not counterbalanced by the upregulation of contractile mechanisms and circumvent non-adherence. An attractive option is targeting angiotensinogen (AGT) with RNA-based therapeutics. Since all angiotensins stem from AGT, deleting AGT will suppress angiotensin (Ang) formation, even when renin levels rise. Furthermore, the dosing frequency with this approach might go down to a few times per year, thereby potentially reducing the clinical and economic burden of non-adherence.

RNA THERAPEUTIC APPROACHES

RNA-based therapies bind to RNA and change the expression of any protein, even those not amenable to traditional approaches involving small-molecule drugs. A number of RNA therapeutic approaches have been developed [2, 3], with antisense oligonucleotides (ASO) that inhibit RNA translation and oligonucleotides that function via the RNA interference (RNAi) pathway being the most clinically relevant. ASO-based therapies utilize Watson-Crick's base-pairing rules and single-stranded DNA containing 15 to 30 nucleotides, which are designed in antisense orientation to the pre-mRNA and mRNA of interest. Mature mRNA is formed in the nucleus by splicing the introns of pre-mRNA. ASOs are able to modulate alternative splicing by binding to the pre-mRNA producing different protein variants and changing the prevalence of one variant. When ASOs bind to their target mRNA, RNase H1 cleaves the RNA in an RNA-DNA duplex in both the cytoplasm and nucleus [4] destroying the mRNA and inhibiting the translation of the target protein (**Figure 1A**). Chemically modified ASOs with phosphorothioate linkages can trigger RNA cleavage even in the absence of transfection reagent [5, 6]. In contrast to the ASO approach, RNAi-based therapies utilize double-stranded RNA and exploit the RNAi process, an evolutionary conserved mechanism for the regulation of gene expression. At present, the majority of RNAi-based therapies employ non-coding small interfering RNA (siRNA) that are 21 to 23 bases in length and work naturally in the cell as a part of the RNA-Induced Silencing Complex (RISC). Unlike ASOs, when siRNA enters the cell it stays inactive until loaded by transactivation responsive RNA-binding protein into

Argonaute where the passenger strand is removed and the remaining antisense strand binds complementary mRNA targets, leaving Argonaute endoribonuclease to cleave the mRNA, thus preventing protein translation [7] (**Figure 1B**). In mammals, Argonaute contains an RNase H domain, but cleaves RNA in an RNA-RNA duplex [8]. The majority of RNA-based therapies in clinical pipeline utilize the RNAi approach (Table 1). This is unsurprising given that a major advantage of RNAi over ASO for therapeutic applications is that RNAi is much more potent and has a longer inhibitory effect [9].

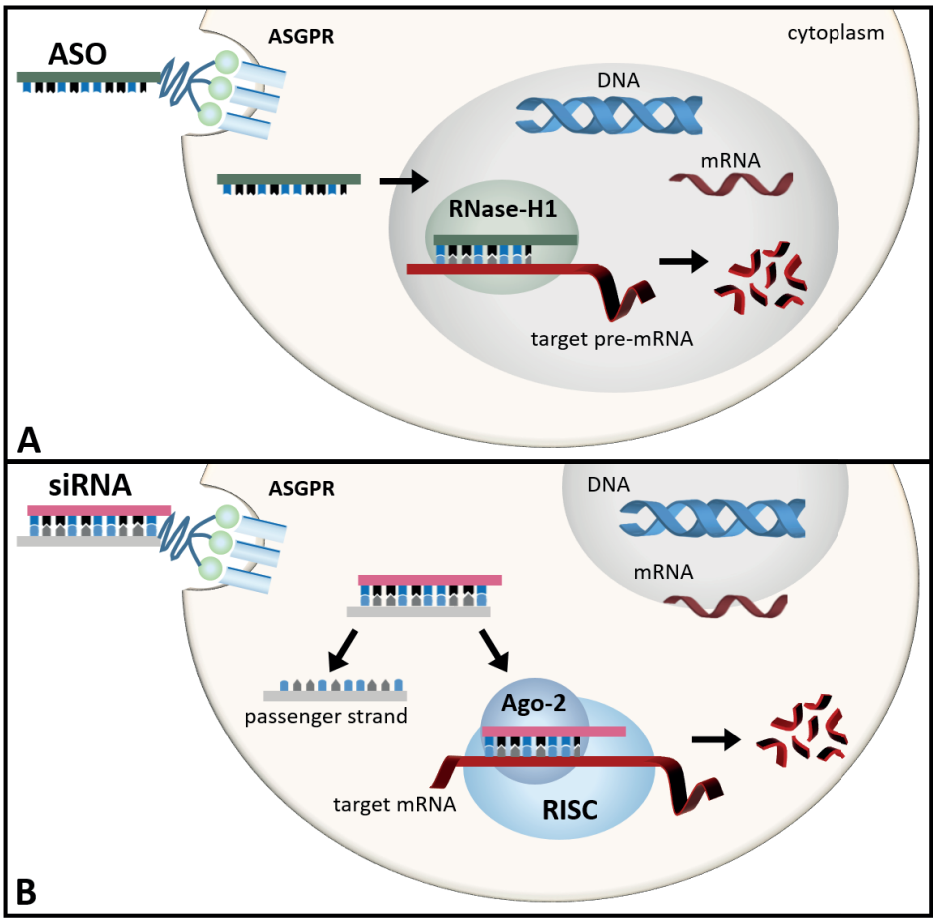

Figure 1. Inhibition of RNA translation by A) antisense oligonucleotide (ASO) or B) RNA interference. See text for further explanation. ASGPR, asialoglycoprotein receptor; Ago-2, argonaute-2; RISC, RNA-induced silencing complex.

Table 1. RNA-based therapies currently in clinical development.

Disease target	Pharmaceutical company	Drug (alternate name)	Therapeutic approach	Stage of development
CANCER				
Blood cancers				
miR-155	miRagen	Cobomarsen (MRG-106)	RNAi (miRNA)	Phase 1/2
Clear cell renal cell carcinoma				
HIF-2α	Arrowhead	ARO-HIF2	RNAi (siRNA)	Pre-IND
Cholangiocarcinoma				
TGF-β1 and COX2	Sirnaomics	STP705L	RNAi (siRNA)	Phase 1
Non-melanoma skin cancer				
TGF-β1 and COX2	Sirnaomics	STP705	RNAi (siRNA)	Phase 2
Pancreatic cancer				
KRAS G12D	Silenseed	KRAS-LODER	RNAi (siRNA)	Phase 2 [10]
Prostate cancer				
Androgen receptors	Ionis	ARRx (IONIS-AR-2.5Rx)	ASO	Phase 1/2
Various cancers				
STAT3	Ionis-AstraZeneca	Danvatirsen (ISIS481464)	ASO	Phase 1/2
CARDIO-METABOLIC				
Clotting disorders				
Hepatic Factor XI	Ionis-Bayer	IONIS-FXIRx	ASO	Phase 2 [11]
	Ionis-Bayer	IONIS-FXI-LRx	ASO	Phase 1
Dyslipidemias				
Hepatic PCSK9	Alnylam-The Medicines Company	Inclisiran (ALN-PCSSC)	RNAi (siRNA)	Phase 3 [12-16]
Hepatic Apo C-III	Arrowhead	ARO-APOC3	RNAi (siRNA)	Phase 1
	Ionis-Akcea/Novartis	AKCEA-APOCIII-LRx	ASO	Phase 2
	Ionis-Akcea	Waylivra (Volanesorsen)	ASO	Phase 3 [17-23]
Hepatic ANGPTL3	Arrowhead	ARO-ANG3	RNAi (siRNA)	Phase 1
	Ionis-Akcea	AKCEA-ANGPTL3-LRx	ASO	Phase 2
Hepatic Apo A	Arrowhead-Amgen	AMG 890	RNAi (siRNA)	Phase 1
	Ionis-Akcea/Novartis	AKCEA-APO(a)-LRx	ASO	Phase [24]
	Silence Therapeutics	SLN360		IND/CTA due in 2020

Salt

Table 1. RNA-based therapies currently in clinical development. (*continued*)

Disease target	Pharmaceutical company	Drug (alternate name)	Therapeutic approach	Stage of development
Undisclosed target	Ionis-AstraZeneca	AZD8233 (IONIS-AZ4-2.5LRx)	ASO	Phase 1
Hypertension				
Hepatic AGT	Alnylam	ALN-AGT	RNAi (siRNA)	Phase 1
	Ionis	IONIS-AGT-LRx	ASO	Phase 2
Ischemia				
miR-92a	miRagen-Servier	MRG-110	RNAi (miRNA)	Phase 1
Type 2 diabetes				
Hepatic glucagon receptors	Ionis	IONIS-GCGR(Rx)	ASO	Phase 2 [25]
ENDOCRINE DISORDERS				
Acromegaly				
Growth hormone receptor	Ionis	IONIS-GHR-LRx	ASO	Phase 2
FIBROSIS				
Hypertrophic scar reduction				
TGF-β1 and COX2	Sirnaomics	STP705L	RNAi (siRNA)	Phase 2
Cutaneous fibrosis				
miR-29	miRagen	Remlarsen (MRG-201)	RNAi (miRNA)	Phase 2
GENETIC				
β-***Thalassemia***				
Hepatic TMPRSS6	Ionis	IONIS-TMPRSS6-LRx	ASO	Phase 2
	Silence Therapeutics	SLN124	RNAi (siRNA)	CTA approved
Complement-mediated diseases				
Hepatic C5	Alnylam	Cemdisiran (ALN-CC5)	RNAi (siRNA)	Phase 2
	Alnylam-Regeneron	Cemdisiran/ Pozelimab Combo	RNAi (siRNA)	Phase 1/2
Hepatic complement factor B	Ionis-Roche	IONIS-FB-LRx	ASO	Phase 2
Cystic Fibrosis				
Pulmonary αENaC	Arrowhead	ARO-ENaC	RNAi (siRNA)	Pre-IND
	Ionis	IONIS-ENAC-2.5Rx	ASO	Phase 2
Hemophilia and are bleeding disorders				
Hepatic antithrombin	Alnylam- Sanofi Genzyme	Fitusiran (ALN-AT3)	RNAi (siRNA)	Phase 3

Table 1. RNA-based therapies currently in clinical development. (*continued*)

Disease target	Pharmaceutical company	Drug (alternate name)	Therapeutic approach	Stage of development
Hereditary angioedema				
Prekallikrein	Ionis	IONIS-PKK-LRx	ASO	Phase 2
Primary hyperoxaluria				
Hepatic glycolate oxidase type 1	Alnylam	Lumasiran (ALN-GO1)	RNAi (siRNA)	Phase 3
	Dicerna	DCR-PHXC	RNAi (siRNA)	Phase 1
Transthyretin-mediated (ATTR) amyloidosis				
Hepatic transthyretin	Alnylam	Vutrisiran (ALN-TTRsc02)	RNAi (siRNA)	Phase 3
	Ionis-Akcea	AKCEA-TTR-LRx	ASO	Phase 1
KIDNEY DISEASES				
Delayed graft function				
Pro-apoptotic gene p53	Quark	QRK306 (QPI-1002)	RNAi (siRNA)	Phase 3
AKI following cardiac surgery				
Pro-apoptotic gene p53	Quark	QRK209 (QPI-1002)	RNAi (siRNA)	Phase 2
LIVER DISORDERS				
AATD-associated liver disease				
Hepatic mutant AAT	Arrowhead	ARO-AAT	RNAi (siRNA)	Phase 2
	Alnylam	ALN-AAT02	RNAi (siRNA)	Phase 1/2
	Dicerna	DCR-A1AT	RNAi (siRNA)	Pre-IND
Acute hepatic porphyria				
Hepatic ALAS1	Alnylam	Givosiran (ALN-AS1)	RNAi (siRNA)	Phase 3 [26]
ARLD/NAFLD				
HSD17B13	Arrowhead	ARO-HSD	RNAi (siRNA)	Pre-IND
HBV				
HBV gene products	Arrowhead-Janssen	JNJ-3989 (ARO-HBV)	RNAi (siRNA)	Phase 2
	Arbutus	AB-729	RNAi (siRNA)	Phase 1
	Alnylam	VIR-2218 (ALN-HBV02)	RNAi (siRNA)	Phase 1/2
	Ionis-GSK	IONIS-HBV-LRx	ASO	Phase 2
	Ionis-GSK	IONIS-HBVRx	ASO	Phase 2
	Dicerna	DCR-HBVS	RNAi (siRNA)	Phase 1
NEUROLOGICAL DISORDERS				
Huntington's disease				
Huntingtin protein	Ionis-Roche	IONIS-HTTRx	ASO	Phase 3 [27]

Salt

Table 1. RNA-based therapies currently in clinical development. (*continued*)

Disease target	Pharmaceutical company	Drug (alternate name)	Therapeutic approach	Stage of development
Amyotrophic Lateral Sclerosis				
SOD1	Ionis-Biogen	Tofersen (BIIB067)	ASO	Phase 3 [28]
Mutated CORF72 gene	Ionis-Biogen	IONIS-C9Rx (BIIB078)	ASO	Phase 2
Alzheimer's disease & frontotemporal degeneration				
MAPT	Ionis-Biogen	IONIS-MAPTRx (BIIB080)	ASO	Phase 2
Centronuclear Myopathy				
Dynamin 2 protein	Ionis-Dynacure	IONIS-DNM2-2.5Rx	ASO	Phase 1
OCULAR DISORDERS				
Dry eye syndrome				
TRPV1	Sylentis	Tivanisiran (SYL1001)	RNAi (siRNA)	Phase 3
Glaucoma				
β-adrenergic receptor 2	Sylentis	Bamosiran (SLY040012)	RNAi (siRNA)	Phase 2 [29, 30]

αENaC, epithelial sodium channel alpha subunit; AAT, Alpha-1 antitrypsin; AATD, Alpha-1 antitrypsin deficiency; AGT, angiotensinogen; AKI, acute kidney injury; ALAS1, aminolevulinic acid synthase 1; ANGPTL3, angiopoietin-like protein 3; Apo A, apolipoprotein A; Apo C-III, apolipoprotein C-III; ARLD/NAFLD, Alcohol-related liver disease and non-alcoholic fatty liver disease; ASO, antisense oligonucleotide; C5, complement component 5; CORF72, chromosome 9 open reading frame 72; CTA, clinical trial authorization; HBV, hepatitis B virus; HIF-2α, hypoxia inducible factor 2α; HSD17B13, 17β-Hydroxysteroid dehydrogenase type 13; IND, United States Food and Drug Administration investigational new drug application; MAPT, microtubule-associated protein tau; PCSK9, proprotein convertase subtilisin kexin type 9; miRNA, microRNA; RNAi, RNA interference; siRNA, small interfering RNA; SOD1, superoxide dismutase 1; STAT3, signal transducer and activator of transcription factor 3; TMPRSS6, Transmembrane protease serine 6; TRPV1, Transient receptor potential vanilloid-1.

The clinical translation of RNAi-based therapies has faced a number of challenges including off-target effects, siRNA delivery, immune reactions and toxicity [2]. The biggest hurdle has been delivery since siRNAs do not readily cross the cell membrane. Various approaches have been proposed to solve the problem of siRNA delivery *in vivo* e.g., viruses, cationic lipids, polymers, nanoparticles [31, 32]. One of the most promising approaches to improve the delivery and safety of siRNA is bioconjugation, the covalent connection of siRNAs with biogenic molecules such as lipophilic molecules, antibodies, aptamers, ligands, peptides, or polymers which also cause less immunoreaction [33, 34]. The development of trivalent N-acetylgalactosamine (GalNAc)-siRNA conjugates for targeted delivery of siRNA to the liver is the most successful. GalNAc binds to the asialoglycoprotein receptor that is highly expressed on hepatocytes resulting in rapid endocytosis [35]. The GalNAc approach has also been employed to enhance ASO delivery to hepatocytes by ~10-fold versus free ASOs in preclinical models, resulting in a significant dose reduction [36]. In a

phase 2 clinical trial, hepatocyte-directed GalNAc-siRNA molecules have been shown to yield incredible results with stable suppression of proprotein convertase subtilisin/kexin type 9 (PCSK9) achieved for at least 6 months [16]. RNA-based therapies will not only fundamentally change the way we treat diseases, but may also provide treatments for diseases with unmet clinical need. This has led to RNA-based therapeutics becoming one of the most rapidly advancing fields in drug discovery.

CURRENT APPLICATION OF RNA-BASED THERAPIES

To date, four ASOs and one siRNA-based therapy have been approved for clinical use. Fomivirsen, an intravitreally injected inhibition ASO indicated for the treatment of ocular cytomegalovirus retinitis (CMV) in acquired immunodeficiency syndrome (AIDS) individuals, was the first ever RNA-based therapy to be approved by the U.S. Food and Drug Administration (FDA) in 1998 and by the European Medical Association (EMA) in 1999 [37]. However, in the early 2000's, fomivirsen was withdrawn from the market as the introduction of highly active antiretroviral therapy (HAART) dramatically reduced the number of cases of CMV. A second inhibition ASO, mipomersen, which is injected subcutaneously and targets mRNA encoding apolipoprotein B for the treatment of homozygous familial hypercholesterolemia, was approved for clinical use in 2014 [38]. Two splice modulating ASOs, nusinersen for the treatment of spinal muscular atrophy (2017) [39, 40] and eteplirsen for the treatment of Duchenne muscular dystrophy (2016) [41], are also approved for clinical use. In 2018, patisiran, an siRNA encapsulated in a lipid nanoparticle for delivery to hepatocytes, became the first globally (FDA and EMA) approved RNAi therapeutic. Patisiran is indicated for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR) in adults [42]. In addition, >50 RNA-based therapies for diseases as varied as cancer, neurodegenerative disease and cardiovascular disease are currently in clinical development with several investigational new drug and clinical trial applications expected to be filed within the next 2 years (Table 1). Interestingly, a number of ASO and RNAi therapies are being developed for the same disease targets (Table 1), including hepatic AGT for the treatment of hypertension which are in phase 2 and phase 1 respectively.

AGT RNA-BASED THERAPY IN HYPERTENSION

Since AGT is the sole precursor of the potent vasoconstrictor Ang II, it is a promising target for gene silencing. At present, inhibition of the renin-Ang-aldosterone system (RAAS) is the mainstay for the treatment of hypertension, heart failure and chronic kidney disease [43, 44]. Yet, the long-term management of these conditions remains complicated

by RAAS escape phenomena, during which a counterregulatory rise in plasma renin often restores Ang II to its original pre-treatment values [45-47]. As a consequence, blood pressure control commonly requires multiple antihypertensive drugs, but is impeded by a decline in therapy adherence with every drug added to the treatment regimen [48, 49].

The clinical need for an antihypertensive therapeutic which prevents Ang reactivation has long been recognized and may be achieved by blocking the RAAS at the AGT level. As opposed to classical RAAS inhibitors, a compensatory rise in renin could even potentiate blood pressure lowering as it would facilitate depletion of the remaining AGT (**Figure 2A**). This can be explained as follows. Normally, AGT is present at levels that exceed Ang levels by many orders of magnitude. Under such conditions, blocking renin, Ang I-converting enzyme (ACE) or the Ang receptor results in a renin rise which either (partially) restores Ang II, or increases Ang II above its original level (in order to overcome blockade of its receptor). This is easily achievable, simply because there is ample AGT around to allow this. Even a 100-fold rise in Ang I generation (sufficient to overcome 99% renin inhibition!) is feasible. Yet, if the renin rises are substantial, it is possible that at a certain moment AGT levels start to decrease. During AGT suppression by RNA-based therapy, AGT levels are down already, and now a rise in renin might be sufficient to result in complete AGT depletion, particularly when simultaneously introducing other types of RAAS blockade, which induce further rises in renin. Restoration of Ang II levels is then no longer possible.

While initial ASO constructs targeting *Agt* lowered circulating AGT, Ang II and blood pressure in parallel in spontaneously hypertensive rats, these methods lacked potency and specificity: AGT levels were halved at most, and the effects were short-lived (<1 week) [51-53]. Similar problems also impeded the use of *Agt*-directed siRNA. The introduction of lipid nanoparticle delivery enhanced AGT downregulation, although stable suppression still necessitated weekly intravenous dosing [54]. Additionally, treatment with lipid delivery vehicles causes an inflammatory reponse and must therefore be given with histamine receptor antagonists, non-steroidal anti-inflammatory drugs and relatively high doses of glucocorticoids [55].

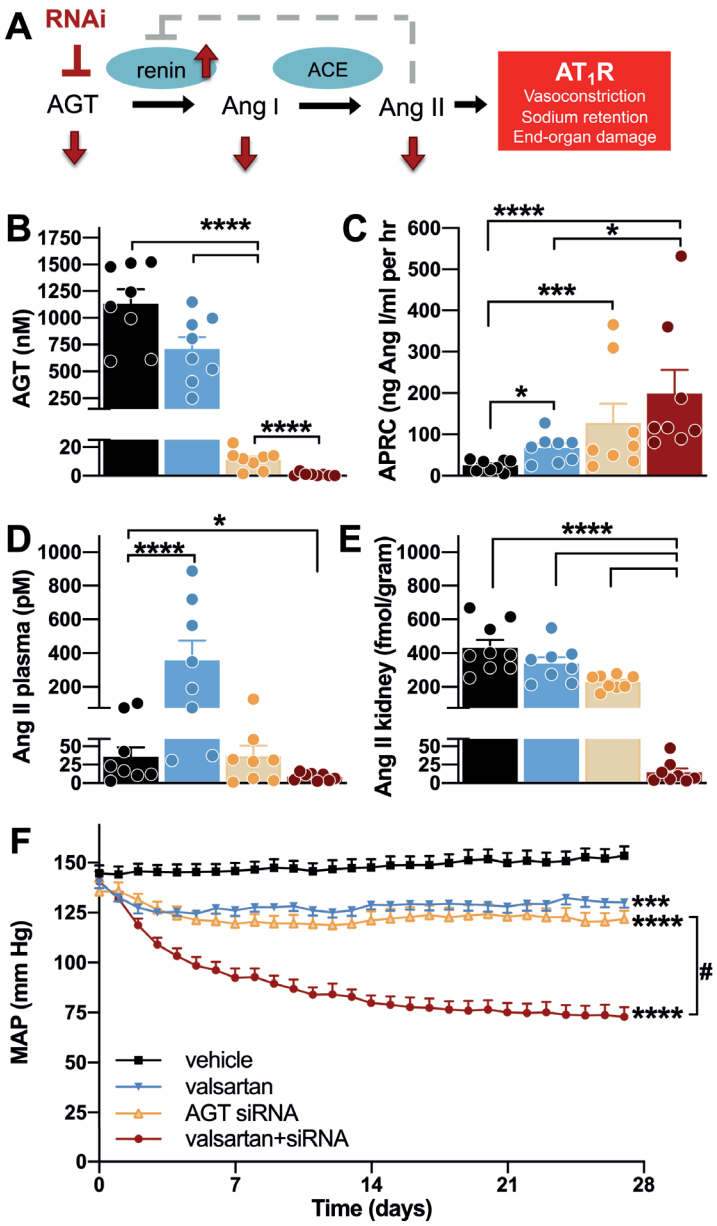

Figure 2. Near elimination of AGT is required for lowering of Ang II.
A) Inhibition of the renin-angiotensin-aldosterone-system (RAAS) at the angiotensinogen (AGT) level may prevent RAAS reactivation, because a rise in plasma renin levels, due to a lack of negative feedback exerted by angiotensin (Ang) II on renin production, could now facilitate depletion of remaining AGT. **B)** Plasma AGT, **C)** active plasma renin concentration (APRC), **D)** plasma Ang II, **E)** renal Ang II and **F)** mean arterial pressure (MAP) of spontaneously hypertensive rats after four weeks of vehicle, valsartan, AGT siRNA or valsartan+siRNA treatment (all groups n=8). Data, modified from Uijl *et al.* [50], are represented as means ± SEM and analyzed using two-way ANOVA and post-hoc Bonferroni (MAP) or transformed to natural logarithms before analysis by one-way ANOVA and post-hoc Bonferroni (RAS parameters). **P*<0.05, *** *P*≤0.001, *****P*≤0.0001 vs. vehicle or otherwise indicated; # *P*≤0.0001.

Conjugating GalNAc to ASO and RNAi molecules enabled general therapeutic application. Subsequent selective accumulation in the liver permitted minimal invasive delivery via subcutaneous injection and optimization of AGT downregulation, since circulating AGT levels are determined by hepatic synthesis [56, 57]. Indeed, by improving liver activity 8-fold, GalNAc-conjugation potentiated AGT inhibition of ASOs to maximally 88% in a dose-dependent manner [58]. Interestingly, a reduction of at least 75% was required to simultaneously lower blood pressure, which then remained low for a period of 7-10 days after a single dose. This relates to the above-mentioned upregulation of renin. In fact, in a study employing GalNAc-siRNA to target *Agt* [50], even when AGT was decreased by 97.9%, circulating Ang II remained intact due to renin upregulation. Only a 99.8% reduction, achieved by combining siRNA with valsartan (which further upregulates renin, thereby additionally lowering AGT), was required to deplete both systemic and renal Ang II (**Figure 2B-E**). The latter observation implies that renal Ang generation relies on AGT of hepatic origin. Consequently, AGT siRNA monotherapy provided similar antihypertensive and cardioprotective efficacy as conventional RAAS inhibition, whereas near elimination of AGT by addition of valsartan yielded a synergistic reduction in blood pressure and cardiac hypertrophy, without adversely affecting renal function (**Figure 2F**). Unique to GalNAc-siRNA is its long-lasting effectiveness [42], as opposed to an ASO liver half-life of 2 to 4 weeks [58]. These findings suggest that, in clinical practice, AGT inhibition mediated by RNAi may not only prevent RAAS reactivation but also improve cardiovascular outcome and therapy adherence due to a sustained and stable single-dose efficacy lasting weeks to months [5]

AGT RNA-BASED THERAPY IN OTHER DISEASES

While the potential for clinical translation is currently being investigated in patients, preclinical studies now focus on safety and efficacy in models of chronic kidney disease and heart failure. However, the application of AGT inhibition is not limited to hypertension and related end-organ damage. Polycystic kidney disease (PKD) appears an especially promising target, with multiple recent studies showing beneficial effects in animal models of this disease [59-61]. PKD is caused by mutations in the Pkd1 and Pkd2 gene. In affected patients, these mutations lead to early onset hypertension, the progressive growth of renal cysts and kidney failure. Conventional treatment with ACE inhibitors or Ang receptor blockers (ARBs) effectively lowers blood pressure in PKD, but whether this treatment also reduces the decline in kidney function is unclear [62, 63]. Experimentally, PKD may be studied in mice with targeted knockout of the Pkd1 or Pkd2 gene, with both models developing progressive intrarenal cysts and kidney failure [64, 65]. In Pkd2 knockout mice, treatment with AGT ASO resulted in a reduction in kidney size, lower total cyst

volume and improved kidney function compared to control treatment (scrambled ASO) [59]. Pkd1 knockout mice displayed similar reductions in kidney size and cyst volume after treatment with AGT ASO [60, 61]. Interestingly, in the latter model, the effects of AGT ASO on cyst growth were independent of changes in systolic blood pressure. In contrast, ACE inhibition with lisinopril significantly lowered systolic blood pressure, but did not reduce kidney size to a similar degree as AGT ASO and had no effect on cyst volume [60].

Experimental evidence indicates that AGT ASO may also be effective in the treatment of atherosclerosis. Increased activity of the RAAS promotes atherosclerosis through activation of AT_{1A} receptors [66-68]. Treatment of spontaneous hypertensive rats on a diet enriched with high fat and vitamin D3 (i.e., to induce atherosclerosis) with Gal-PEG-Et (GPE) nanoparticles carrying short hairpin RNA (shRNA) that specifically targets *Agt* in the liver decreased systolic blood pressure and attenuated the development of atherosclerotic lesions compared with control animals [69]. Similar effects were observed in another animal model for atherosclerosis and obesity, i.e., LDL receptor knockout mice fed with a saturated fat-enriched diet [70]. In these mice, treatment with AGT ASO resulted in decreased blood pressure, less atherosclerosis, and diminished body weight gain. Correspondingly, previous clinical studies also showed beneficial effects of ACE inhibitors and ARBs on the progression of atherosclerotic plaques [71, 72]. However, whether AGT ASO is effective in the treatment of atherosclerosis in patients has yet to be investigated.

Finally, AGT ASO treatment has been used as a therapeutic strategy against experimental pulmonary fibrosis. Currently, treatment options for idiopathic pulmonary fibrosis are limited, leaving most patients with a poor prognosis – the median survival rate is 2–5 years. Several studies in animals (i.e., mice and rats) with bleomycin-induced lung injury showed that Ang II contributes to pulmonary fibrosis, whereas treatment with renin inhibitors, ACE inhibitors and ARBs attenuates disease progression [73-77]. In this same animal model, intratracheal administration of AGT ASO was found to decrease pulmonary AGT concentrations and lung fibrosis [78], but no comparison was made to conventional RAAS inhibitors.

CONCLUSION

Targeting AGT with RNA-based therapeutics is a promising new tool to treat hypertension and diseases beyond, including heart failure, atherosclerosis, diabetic nephropathy, polycystic kidney disease and pulmonary fibrosis. The long-lasting effects of RNA-based therapeutics are particularly exciting, and if translated to a clinical application of at most a few administrations per year, may help to eliminate non-adherence. To what degree

the effects of such therapeutics are fully identical to those of other RAAS blockers (given the fact that they eliminate all Ang metabolites) remains to be determined. It will also be important to determine to what level AGT should be suppressed to induce meaningful effects, and whether there is a role for AGT synthesized outside the liver [79]. Synergy in combination with other RAAS blockers needs to be evaluated, as well as the potential harmful effects of suppressing AGT in the context of common comorbidities such as heart failure and chronic kidney disease, where AGT might already be suppressed.

KEY POINTS

Liver-targeted, stable antisense oligonucleotides and small interfering RNA targeting angiotensinogen, capable of suppressing angiotensinogen in a dose-dependent manner, are now available.

Suppressing angiotensinogen exerts similar effects as classical blockers of the renin-angiotensin-aldosterone system (RAAS) in hypertensive animal models, as well as in rodent models for atherosclerosis, polycystic kidney disease and pulmonary fibrosis.

Future studies should evaluate their safety, e.g. in the context of common comorbidities such as heart failure and chronic kidney disease, and synergy with existing RAAS blockers.

The long-lasting effects of this approach are particularly exciting, and if translated to a clinical application of at most a few administrations per year, may help to eliminate non-adherence.

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Bulleted References (12-18 month old)

Ref 2 (2019) ** Excellent review on the design and development of RNAi drugs

Ref 3 (2019) ** Overview of therapeutic oligonucleotides at this moment

Ref 36 (2019) * First evaluation of angiotensinogen antisense oligonucleotide in atherosclerosis

Ref 38 (2019) ** Extensive biochemical evaluation of angiotensinogen siRNA in hypertensive rats

Ref 41 (2018) * Evaluation of angiotensinogen antisense oligonucleotide in a polycystic kidney disease mouse model.

Chapter 5

Blood pressure-independent renoprotective effects of small interfering RNA targeting liver angiotensinogen in experimental chronic kidney disease

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ABSTRACT

Small interfering RNA targeting liver angiotensinogen (AGT siRNA) lower blood pressure, but their effectiveness in hypertensive chronic kidney disease (CKD) is unknown. Considering that the kidney may generate its own AGT, we assessed the effectiveness of liver-targeted AGT siRNA in the 5/6th nephrectomy (5/6Nx) rat, a hypertensive CKD model. Five weeks after 5/6Nx (baseline), rats were subjected to vehicle, AGT siRNA, AGT siRNA + losartan, losartan, or losartan + captopril. Baseline MAP was 160±6 mm Hg. Over the course of 4 weeks, MAP increased further by ≈15 mm Hg during vehicle treatment. This rise was prevented by AGT siRNA. Losartan reduced MAP by 37±6 mm Hg, and increased plasma angiotensin II. Both AGT siRNA and captopril suppressed these effect of losartan, suggesting that its blood pressure-lowering effect relied on stimulation of vasodilator angiotensin II type 2 receptors by high angiotensin II levels. Proteinuria and cardiac hypertrophy increased with vehicle and these increases were similarly abrogated by all treatments. No intervention improved GFR, while siRNA and losartan equally diminished glomerulosclerosis. AGT siRNA ± losartan reduced plasma AGT by >95%, and this was accompanied by almost complete elimination of angiotensin II in kidney and heart, without decreasing renal AGT mRNA. Multiple linear regression confirmed both MAP and renal angiotensin II as independent determinants of proteinuria. In conclusion, AGT siRNA exerts renoprotection in the 5/6Nx model in a blood pressure-independent manner. This relies on the suppression of renal angiotensin II formation from liver-derived AGT. Consequently, AGT siRNA may prove beneficial in human CKD.

NOVELTY AND SIGNIFICANCE

What is new?

- Small interfering RNA targeting liver angiotensinogen (AGT) provide cardio- and renoprotection in a blood pressure-independent manner in the 5/6th nephrectomy rat, a hypertensive chronic kidney disease model.
- Multiple linear regression confirmed both blood pressure and renal angiotensin II as independent determinants of proteinuria.
- Renal angiotensin II formation in this model depends entirely on angiotensinogen of hepatic origin.

What is relevant?

- Given its stable and sustained efficacy, lasting weeks, RNA interference may prove beneficial in human chronic kidney disease.

SUMMARY

Small interfering RNA (siRNA) targeting liver angiotensinogen abrogated proteinuria, glomerulosclerosis and cardiac hypertrophy in the 5/6th nephrectomy rat, a hypertensive chronic kidney disease model, to the same degree as the angiotensin II type 1 receptor blocker losartan. Angiotensinogen siRNA reduced plasma angiotensinogen by >95%, and this was accompanied by almost complete elimination of angiotensin II in kidney and heart. Multiple linear regression confirmed both blood pressure and renal angiotensin II as independent determinants of proteinuria. Given its stable and sustained efficacy, lasting weeks, angiotensinogen siRNA may prove beneficial in human chronic kidney disease.

INTRODUCTION

Treatment of chronic kidney disease (CKD) often involves the use of renin-angiotensin system (RAS) blockers to treat hypertension and confer renoprotection. The latter is believed to be due to interference with either the generation or effects of angiotensin (Ang) II at renal tissue sites. Here it has often been argued that apart from renin synthesis in the kidney, angiotensinogen (AGT) is also synthesized at renal tissue sites, for instance in the proximal tubule.[1] Given the broad expression of ACE in the kidney,[2] this implies that all components required to synthesize Ang II are present in the kidney,[3] thereby allowing locally synthesized Ang II to exert its effects fully independently from circulating Ang II, i.e., by stimulating angiotensin II type 1 and 2 (AT_1, AT_2) receptors at renal tissue sites.

RAS blockade is hampered by counterbalancing mechanisms, the most important of which is renin upregulation.[4] As a consequence, the degree of RAS suppression may be less than anticipated, even more so if organs express their own AGT. Furthermore, particularly during AT_1 receptor blocker (ARB) treatment, the elevated Ang II levels might stimulate vasodilator AT_2 receptors, thereby lowering blood perssure.[5]

A novel approach of interfering with the RAS is the use of small interfering RNAs (siRNAs) targeting AGT.[6] Currently siRNA designs exist with hepatocyte-directed, N-acetylgalactosamine (GalNAc)-conjugated molecules which allow stable suppression of hepatic proteins like proprotein convertase subtilisin/kexin type 9, requiring only biannual dosing in humans.[7] Given the hepatic origin of circulating AGT, this approach could allow a similar suppression of AGT. Importantly, under such circumstances counterbalancing renin rises should no longer be able to restore Ang II in blood, simply because AGT is lacking. Indeed, a GalNAc-conjugated siRNA targeting AGT was highly effective in suppressing circulating AGT in spontaneously hypertensive rats (SHR),[6] thereby reducing blood pressure to the same degree as ACE inhibitors (ACEi) and ARB. Moreover, combining this AGT siRNA with an ARB virtually eliminated Ang II, because the accompanying further renin rise now cleaved any remaining AGT and thus exhausted the source of angiotensin peptides.

A remaining question is to what degree this approach also exerts beneficial effects at tissue sites, in particular in the kidney, given its own AGT synthesis. Here, it should be noted that RAS blockade has a limit, and that too much RAS blockade (e.g., by combining multiple RAS blockers at the same time) may result in hypotension, acute kidney injury and hyperkalemia. Disappointingly, a two-week treatment of the 5/6th nephrectomy (Nx) rat, a CKD model which is responsive to RAS blockade,[8-10] with AGT antisense oligonucleotides (liver-specific or non-specific) revealed no beneficial effect on proteinuria or renal

histology.[11] Non-specific AGT suppression even impaired renal function (evidenced by a reduced creatinine clearance) and worsened histology, while hepatic-specific suppression was indistinguishable from vehicle treatment. Based on this, the authors argued that non-specific AGT suppression is potentially deleterious, because it lowers renal Ang II too much. Yet, they did not report renal angiotensin levels. Moreover, their results were obtained while exposing the 5/6th Nx rats to a very low-salt diet (0.015 % NaCl). Since this will greatly upregulate the dependency of renal function on the RAS, it may explain why no beneficial effects of AGT suppression were observed.

Therefore, in the present study we set out to investigate the effects of liver-targeted AGT siRNA in the same CKD model (the 5/6th Nx rat) under normal salt conditions, applying treatment for a longer time period (4 weeks) and making a comparison versus the ARB losartan or dual RAS blockade (AGT siRNA + losartan and captopril + losartan). We focused on blood pressure, proteinuria, glomerular filtration rate (GFR) and renal histology, and additionally measured angiotensins in blood, kidney and heart, to obtain a complete insight into the consequences of hepatic AGT deletion in the circulation and at tissue sites in this CKD model.

METHODS

Animal studies

All animal experiments were approved by the Animal Welfare Committee of the Erasmus MC (protocol number 16-790-06). Male 6-weeks old Sprague-Dawley (SD) rats were obtained from Envigo (Huntingdon, United Kingdom) and maintained on a standard sodium diet. 5/6th Nx was performed in a two-step procedure as previously described.[12] Briefly, right uninephrectomy was performed under isoflurane anesthesia, followed by resection of the poles of the left kidney 10 days later. Right uninephrectomy was combined with telemetry device (HD-S10, Data Sciences International, St. Paul, USA) implantation as previously described.[13, 14] Animals were allowed to recover for 5 weeks, as this period is necessary for the remnant kidney to attain a new steady-state condition.[15] Subsequently, animals were treated for 4 weeks with vehicle (n=10), AGT siRNA (10-30 mg/kg fortnightly by subcutaneous injection, n=12; Alnylam Pharmaceuticals, Cambridge, MA, USA), AGT siRNA + losartan (30 mg/kg per day; n=7; Sigma Aldrich, Zwijndrecht, The Netherlands), losartan (n=8), or losartan + captopril (6 mg/kg per day, n=8; Sigma Aldrich). The siRNA consisted of a chemically modified antisense strand with sequence UUGAUUUUUGCCCAGGAUAGCUC, hybridized with a chemically modified sense strand of sequence GCUAUCCUGGGCAAAAAUCAA. Oligonucleotides were synthesized as previously described.[6] To ensure selective and efficient delivery to hepatocytes, a trianten-

nary N-acetylgalactosamine (GalNAc) – a high-affinity ligand for the hepatocyte-specific asialoglycoprotein receptor – was attached to the 3′ end of the sense strand.[16] Two doses of AGT siRNA were tested (10 and 30 mg/kg), but since the degree of AGT depletion was identical with both doses (97.3±1.4% versus 95.9±1.1%; **Figure S1**) data for both doses were combined. Losartan and captopril were administered subcutaneously by osmotic minipump (model 2ML4, Alzet, Cupertino, CA, USA). Four additional 5/6th Nx animals were sacrificed after the recovery period, to establish plasma hormone levels, proteinuria, renal histology and cardiac hypertrophy before the start of treatment ('baseline'). Eight Sprague-Dawley rats (18 weeks old, weight 407±24 g) were sacrificed to establish renal histology in healthy controls. Animals were allocated to treatment groups by stratification based on the 3-day average of mean arterial pressure (MAP) and the glomerular filtration rate (GFR) measured at baseline. For biochemical measurements, we collected 24-hour urine in metabolic cages and blood plasma by venipuncture from the lateral tail vein before treatment (baseline), after 2 weeks and after 4 weeks of treatment. Blood pressure, heart rate and animal activity were recorded continuously via radiotelemetry. At the end of the treatment period, rats were anaesthetized by inhalation of isoflurane and exsanguinated: 1 mL blood was collected in 10 mL of 4 mol/L guanidine thiocyanate[17] (Sigma Aldrich) and used for quantification of angiotensin metabolites; remaining blood was supplemented with EDTA and centrifuged at 16000 x *g* to obtain plasma. Kidneys and heart were harvested, weighed, divided into transverse segments, and fixated in 4% paraformaldehyde for histological analysis, or snap frozen in liquid nitrogen for gene - and protein expression analysis. Mesenteric arteries were isolated and used directly in myograph studies.

Biochemical measurements

In plasma, AGT was measured by enzyme kinetic assay as the maximum quantity of Ang I generated during incubation, at pH 7.4 and 37°C, with rat kidney renin in the presence of a mixture of ACE, angiotensinase, and serine protease inhibitors.[18] The lower limit of detection of this assay was 0.2 nmol/L. Plasma renin was measured by quantifying Ang I generation in the presence of excess porcine AGT (detection limit 0.17 ng Ang I/mL per hour).[19] In the cases that measurements were at or below the detection limit, this limit was applied to allow for statistical analysis. Ang metabolites in plasma, kidney, and heart tissue (left ventricle) were measured by LC-MS/MS analysis as described before.[20] Briefly, tissue samples were homogenized under liquid nitrogen and extracted with a guanidinium-based extraction buffer. Stabilized whole blood and tissue extracts were spiked with stable isotope labeled internal standards for each individual target analyte (Sigma Aldrich) before being subjected to C18 based solid phase extraction and subsequent LC-MS/MS analysis. **Table S1** specifies the lower limit of quantification for each metabolite. Plasma NT-proBNP was measured with a rat NT-proBNP ELISA kit (detec-

tion limit 15.6 pg/mL; Aviva Systems Biology, San Diego, USA). Urine total protein was measured by the clinical chemistry laboratory of the Erasmus MC. Urinary neutrophil gelatinase-associated lipocalin (NGAL) was measured with a rat NGAL ELISA kit (detection limit 0.1 pg/mL; Abcam, Cambridge, UK).

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from snap-frozen kidney using TRI Reagent (Sigma Aldrich) and reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). cDNA was amplified in triplicate in 40 cycles (denaturation at 95°C for 3 min; thermal cycling at 95°C for 3 sec, annealing/extension at 60°C for 20 sec) followed by a melt curve with a CFX384 (Bio-Rad, Veenendaal, The Netherlands) using Kapa SYBR® Fast (Kapa Biosystems). Intron-spanning oligonucleotide primers were designed with NCBI Primer-BLAST for AGT (forward CCAGCACGACTTCCTGACT, reverse GCAGGTTGTAGGATCCCCGA), renin (forward TGTGGTAACTGTGGGTGGAAT, reverse GCATGAAGGGTATCAGGGGC) and β_2-microglobulin (*B2M;* forward ATGGCTCGCTCGGTGACCG, reverse TGGGGAGTTTTCTGAATGGCAAGCA). The $\Delta\Delta$Ct method was used for relative quantification of mRNA expression levels, using the housekeeping gene *B2M* for normalization.

Kidney function

Glomerular filtration rate (GFR) was determined at baseline and at the end of treatment, by transcutaneous measurement of fluorescein isothiocyanate (FITC)-labeled sinistrin (Mannheim Pharma & Diagnostics GmbH, Mannheim, Germany), administered as a bolus injection (0.24 mg/kg dissolved in saline) via the tail vein. A non-invasive clearance (NIC)-kidney fluorescent detection device together with partner software (Mannheim Pharma & Diagnostics GmbH) were used to generate the elimination kinetics curve of FITC-sinistrin. GFR was derived from the excretion half-life ($t_{1/2}$) of FITC-sinistrin, using a conversion factor and formula validated for rats[21]:
GFR (mL/min per 100g body weight (BW)) = 31.26 (mL/100g BW) / $t_{1/2}$ FITC-sinistrin (minutes).

Histology

Kidney segments, fixed in 4% paraformaldehyde, were dehydrated and paraffin-embedded. Transversely sliced and deparaffinized kidney sections (2 mm) were stained with periodic acid–Schiff (PAS) and scored semiquantitatively in a blinded fashion by a renal pathologist (M.C.C.v.G.) as previously described.[22] Focal segmental glomerulosclerosis (FSGS) was assessed and graded in all glomeruli of one kidney section per rat, basing on an arbitrary scale wherein 0%, <25%, 25-50%, 50-75%, and >75% of glomerular sclerosis were represented by grade zero (n_0), 1 (n_1), 2 (n_2), 3 (n_3), and 4 (n_4), respectively. The

glomerulosclerosis index (GSI) was calculated with the formula: $[(1\chi n_1)+(2\chi n_2)+(3\chi n_3)+(4\chi n_4)]/(n_0+n_1+n_2+n_3+n_4)$. Tubular atrophy, interstitial fibrosis, and tubulointerstitial inflammation were scored in the same kidney section and summed to obtain the tubulointerstitial score (TIS). A score of 0-3 indicated that <25% of tubulointerstitial tissue was affected, a score of 4–6 indicated 25–50% and a score of 7–9 indicated >50%. Finally, observing dilated tubules and acute thrombotic microangiopathy was used as an indication of hypertensive kidney injury.

Myograph studies

Mesenteric arteries were carefully dissected and placed in a cold, Krebs bicarbonate solution (composed as follows [in mmol/L]: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25 and glucose, 8.3; pH = 7.4), aerated with 5% CO_2 in O_2 (carbogen). Arteries were cut into 2 mm segments and mounted in Mulvany myographs (Danish Myo Technology, Aarhus, Denmark) with 6-mL organ baths containing oxygenated Krebs buffer and maintained at 37°C. Changes in tissue tension were measured using a LabChart data acquisition system (AD Instruments Ltd, Oxford, UK). After equilibration for at least 30 min and a wash, the vessel segments were stretched to a tension normalized to 90% of 100 mm Hg. After reaching equilibrium, the contractile capacity of the mesenteric arteries was examined by adding 30 mmol/L KCl. After washout, the tissue was exposed to 100 mmol/L KCl to determine the maximal contraction. Endothelial function was checked by verifying relaxation to 10 mmol/L acetylcholine after preconstriction with the thromboxane A_2 analogue U46619 (10 nmol/L) to >70% of the maximal contraction. Next, segments were equilibrated in fresh Krebs buffer for 30 min, and preincubated for 30 min with the NO synthase inhibitor L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride; 100 µmol/L), the small- and intermediate-conductance Ca^{2+}-activated K^+-channel (SK_{Ca} , IK_{Ca}) inhibitors apamin (100 nmol/L) and TRAM34 (10 µmol/L), the endothelin type A (ET_A) receptor antagonist BQ123 (1 µmol/L), or the ET_B receptor antagonist BQ788 (1 µmol/L). Thereafter, concentration-response curves (CRCs) were constructed to ET-1. To construct CRCs to the endothelium-dependent dilator acetylcholine (ACh) arteries were preconstricted with U46619. All drugs were obtained from Sigma-Aldrich.

Statistics

Data are expressed as mean values ± SEM in case of normal distribution and median with interquartile range in case of non-normal distribution. Non-normally distributed data were log-transformed before statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) and mixed linear models, using treatment and time as fixed effects, if appropriate. If significant, selected post-hoc analyses were performed between individual groups by controlling for a false-discovery rate of 5%.[23] Relaxant responses to either ACh or SNAP are expressed as a percentage of the contraction to

U46619. Contractile responses to ET-1 are expressed as a percentage of the contraction to 100 mmol/L KCl. CRCs were analyzed as described before[24] to obtain pEC_{50} ($-^{10}logEC_{50}$) and E_{max} values. Data obtained at multiple points in time were analyzed using a repeated-measures two-way ANOVA, followed by post-hoc correction according to Dunnett or Dunn in case of multiple comparisons, if appropriate. Univariate linear associations were assessed by calculation of Pearson's coefficient of correlation. Two-tailed *P* values <0.05 were considered statistically significant. Multiple linear regression analysis was applied to determine the variables affecting proteinuria. All analyses were performed using Prism version 9.0.0 (GraphPad Software Inc., La Jolla, USA)

RESULTS

AGT siRNA halts the blood pressure rise in the 5/6th Nx model

We have reported earlier that MAP in healthy SD rats is 103±1 mm Hg (n=7).[25] At 5 weeks after 5/6th Nx ('baseline') MAP had increased to 160±6 mm Hg (n=47; **Figure 1A**). MAP increased further to 174±5 mm Hg during the subsequent 4 weeks of vehicle treatment. AGT siRNA treatment prevented this increase (P<0.05 versus vehicle; **Figures 1A and 1B**). Losartan lowered MAP by 37±6 mm Hg, *P*<0.001 versus vehicle). Adding either AGT siRNA or captopril on top of losartan diminished (P<0.05 versus losartan) the effect of losartan, yielding MAP decreases of 22±7 and 21±6 mm Hg, respectively (both *P*<0.001 versus vehicle). As a consequence, MAP was identical during treatment with AGT siRNA alone, AGT siRNA + losartan and losartan + captopril, while it was lower in the losartan alone group versus the AGT siRNA alone group (P<0.05). No treatment affected heart rate (**Figure 1C**), activity (**Figure 1D**), body weight, or food intake (**Table S2**).

AGT SIRNA SUPPRESSES THE CIRCULATING RAS

Plasma renin levels in healthy SD rats are 11.8±0.8 ng Ang I/mL per hour.[25] At 5 weeks after 5/6th Nx, renin had decreased to 2.6 (range 0.8-3.9) ng Ang I/mL per hour (**Figure 2A**). All treatments modestly increased renin, although significance (P<0.05 versus baseline) was reached for losartan only (at 2 weeks). Plasma renin correlated negatively with MAP (**Figure 2B**). As expected, AGT siRNA, either alone or with losartan, diminished plasma AGT by >95% (**Figure 2C**). No other treatment affected circulating AGT. Losartan increased plasma Ang I (P<0.001 versus vehicle) and II (P<0.01) (**Figures 2D and 2E**), while in combination with captopril only plasma Ang I increased (P<0.001). AGT siRNA reduced plasma Ang I (P=0.07) and II (P<0.05) in parallel, with the plasma Ang I levels becoming undetectable in most animals. Combining AGT siRNA with losartan yielded

virtually identical Ang I and II levels as AGT siRNA alone. Only captopril reduced the Ang II/I ratio (**Table S1**). Neither Ang-(1-7), nor Ang III or IV were detectable in blood of 5/6th Nx rats (**Table S1**), and only after losartan (with or without captopril) did these metabolites become detectable.

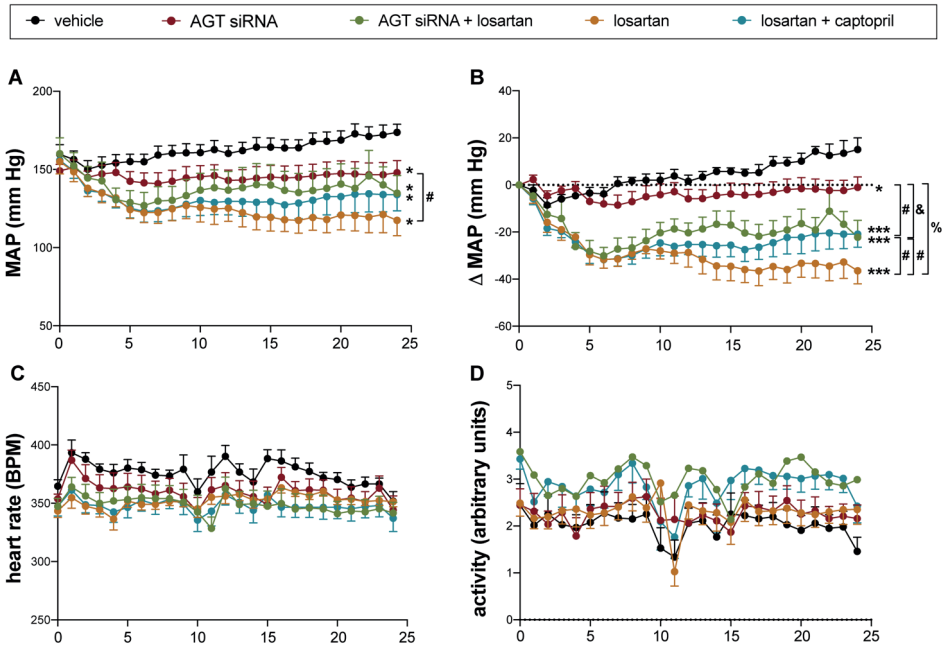

Figure 1. Mean arterial pressure (MAP; panel A), ΔMAP (panel B), heart rate (beats per minute, BPM; panel C), and locomotor activity (panel D) in Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery. Data are mean±SEM of n=7-12. *P<0.05, ***P<0.001 versus vehicle; #P<0.05, &P<0.01, %P<0.001 versus indicated group.

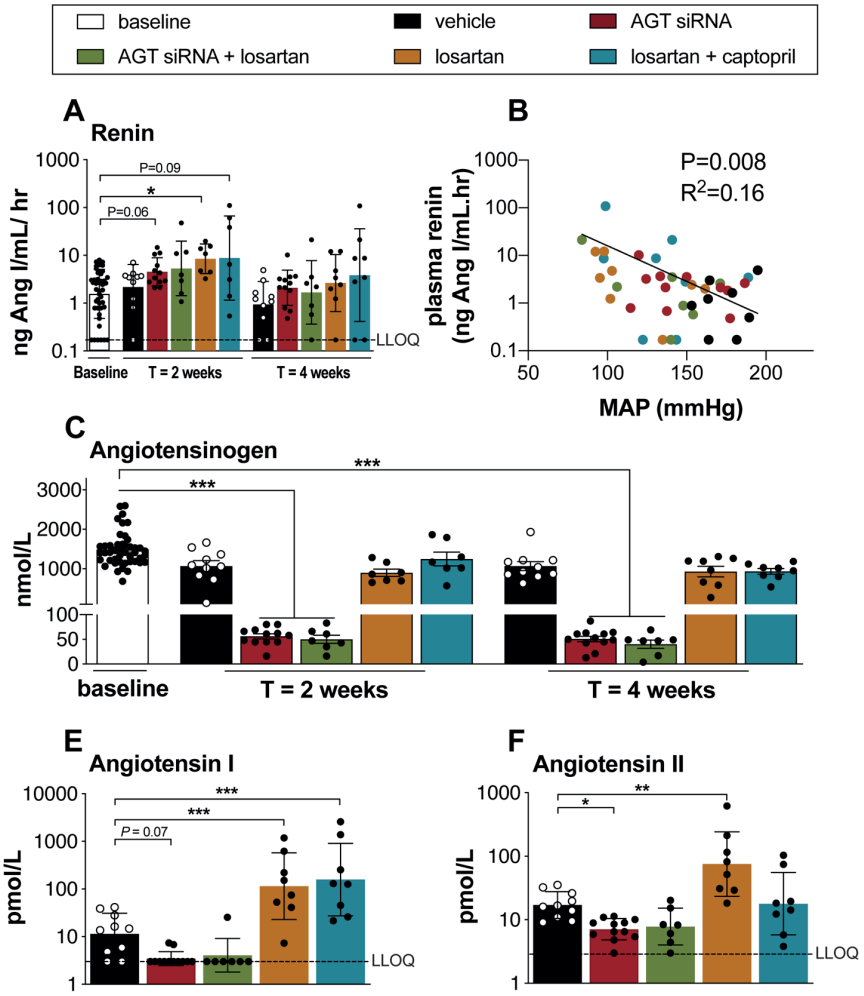

Figure 2. Renin (panel A), angiotensinogen (panel C), angiotensin I (panel D) and angiotensin II (panel E) in blood plasma of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery (=baseline). Data are mean±SEM of n=7-12. Panel B displays the relationship between plasma renin and mean arterial pressure (MAP) during the last 3 treatment days. *P<0.05, **P<0.01, ***P<0.001, ***P<0.001 versus indicated group. LLOQ, lower limit of quantification.

AGT SIRNA AND THE RENAL RAS

AGT siRNA, as well as the other treatments, tended to upregulate renal renin expression (**Figure 3A**), but no significance was reached. Renal renin expression correlated closely (P<0.001) with plasma renin levels (**Figure 3B**). AGT siRNA did not affect renal AGT expression (**Figure 3C**), in agreement with its liver-specificity, nor did any of the other

treatments affect this expression. Yet, AGT siRNA greatly suppressed renal Ang I and II levels (P<0.01 versus vehicle for both; (**Figures 3D and 3E**), while in combination with losartan, renal Ang I and II were virtually eliminated (P<0.0001 versus AGT siRNA alone). Losartan, with or without captopril, did not affect renal Ang I. Losartan when given alone, modestly reduced renal Ang II (P<0.05 versus vehicle), while in combination with captopril, it reduced renal Ang II more strongly (P<0.01). As a consequence, the renal Ang II/I ratio decreased during both losartan alone and losartan + captopril (P<0.001 versus

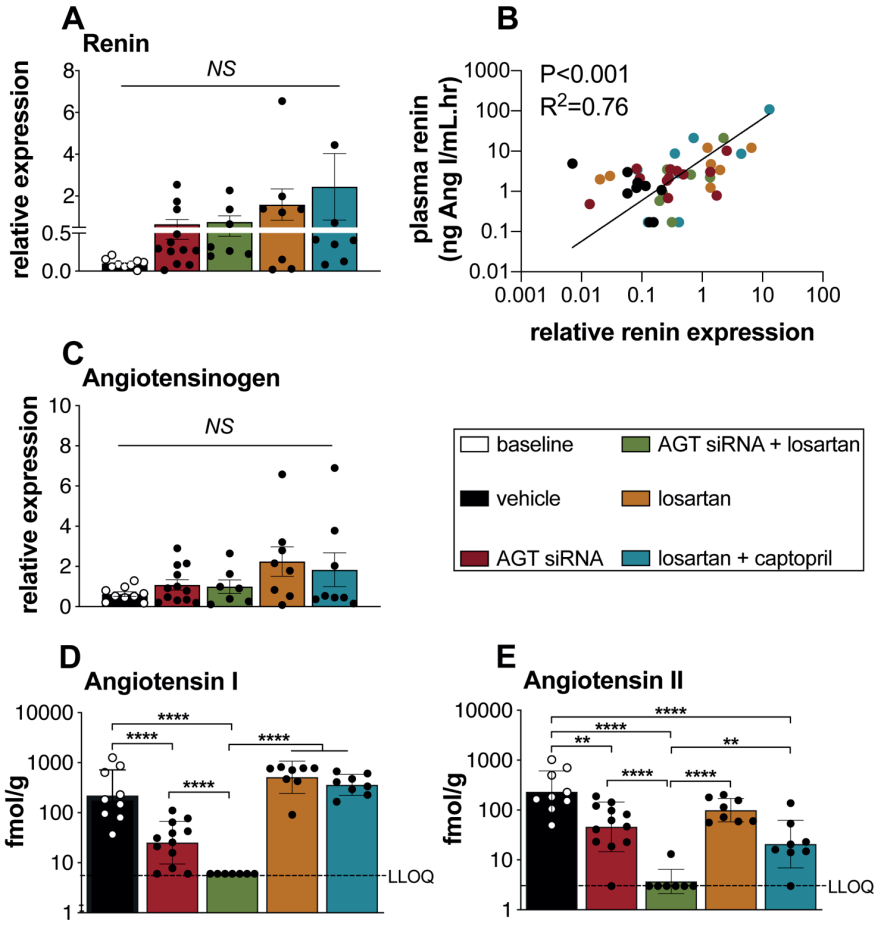

Figure 3. Renin expression (panel A), angiotensinogen expression (panel B), angiotensin I (panel D), and angiotensin II (panel E) in kidneys of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery. Data are mean±SEM of n=7-12. Panel B displays the relationship between plasma renin and renal renin expression. *P<0.05, **P<0.01, ***P<0.001, ***P<0.001 versus indicated group. LLOQ, lower limit of quantification.

vehicle; **Table S1**), but not during the other treatments. Renal Ang III and Ang IV levels were low in the 5/6th Nx model, and rapidly became undetectable after most treatments (**Table S1**). In contrast, renal Ang-(1-7) levels were of similar magnitude as the renal Ang II levels, and decreased in parallel with Ang I and II after siRNA (with or without losartan), but not after losartan alone or losartan + captopril

AGT SIRNA IS RENOPROTECTIVE

GFR in healthy SD rats is 1.0±0.04 mL/min per 100 g body weight.[25] At 5 weeks after the 5/6th Nx procedure, GFR had decreased to 0.38±0.10 mL/min per 100 g body weight. Neither vehicle, nor any treatment affected GFR over the next 4 weeks (**Figure 4A**). None of the treatments altered water intake (**Table S2**), urinary volume (**Table S2**) or urinary NGAL excretion (**Figure 4B**), although losartan, with or without AGT siRNA, did tend to reduce the latter (P=NS). Proteinuria (13 (range 8.5-16) mg/day in healthy SD rats (n=73, unpublished results) was 140 (range 92-170) mg/day at 5 weeks after 5/6th Nx. It rapidly increased further during vehicle treatment, and all treatments, whether given alone or in combination, fully prevented this rise (**Figure 4C**). Proteinuria correlated significantly with MAP (P<0.001; **Figure 4D**) and renal Ang II (P=0.03; **Figure 4E**). Multiple linear regression confirmed that both MAP (coefficient 0.693, P<0.0001) and renal Ang II (coefficient 0.246, P=0.023) were independent determinants of proteinuria (adjusted R^2=0.60).

FSGS, GSI and TIS all increased over the 8 week period after 5/6th Nx (**Figure 5**), although statistical significance for the latter was not reached (P=0.06). The significant increases in kidney injury scores versus healthy control disappeared after all treatments, except after the treatment with losartan + AGT siRNA. The number of rats with dilated tubules increased from 0 out of 8 (0/8) in the healthy control group, to 2/4 at baseline and 7/10 after 4 weeks of vehicle exposure. Acute thrombotic microangiopathy featured in these groups in 0/8, 2/4, and 5/10 rats, respectively, with arterial involvement in 1/10 of vehicle-treated rats. AGT siRNA, AGT siRNA + losartan, losartan, and losartan + captopril reduced the number of rats with dilated tubules to 5/12, 2/7, 2/8 and 4/8, and acute thrombotic microangiopathy to 4/12, 2/7, 1/8, and 2/8 rats (with arterial involvement in 2/12 AGT siRNA-treated rats and 1/8 losartan + captopril-treated rats) respectively. These data therefore fully agree with the histology scores.

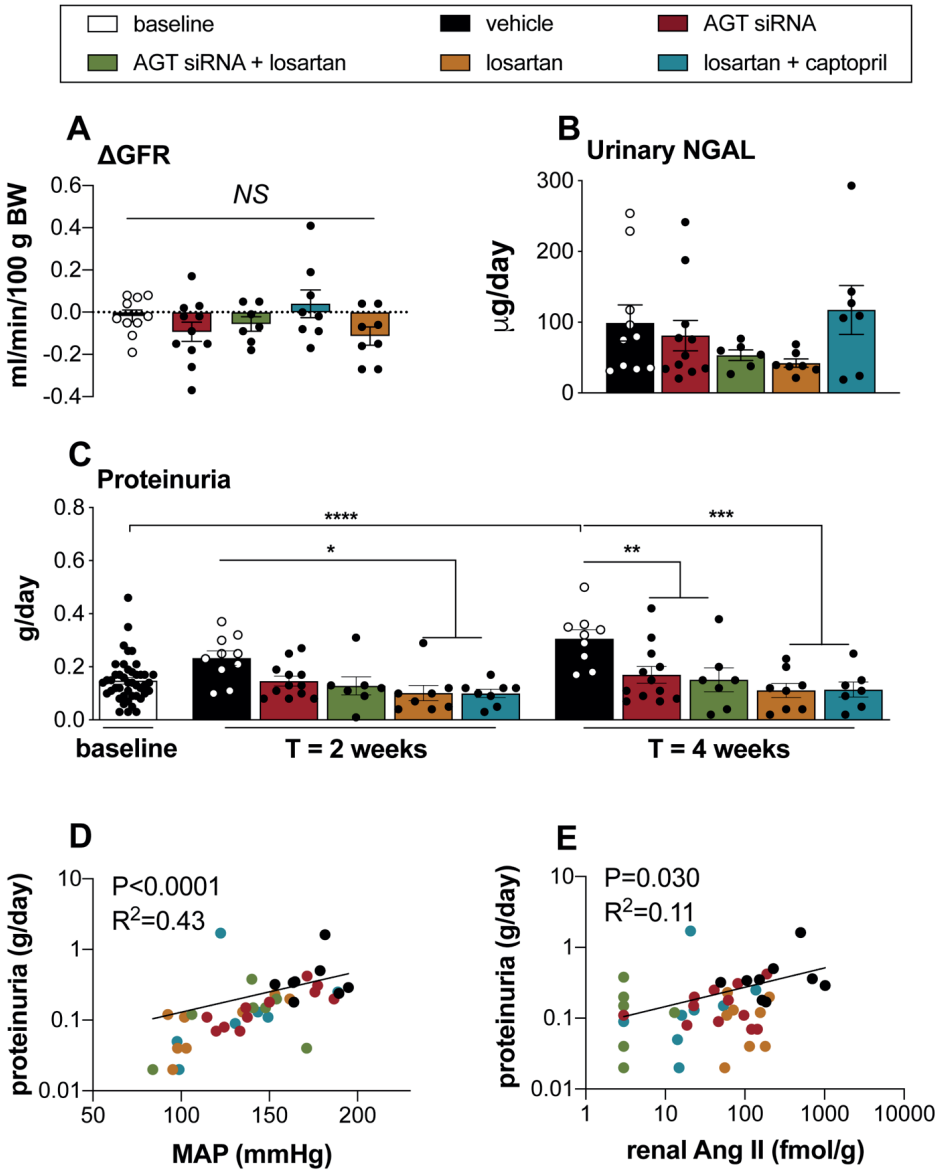

Figure 4. Glomerular filtration rate (GFR, expressed as change versus baseline; panel A), urinary NGAL excretion (panel B), and proteinuria (panel C) in kidneys of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery (=baseline). Data are mean±SEM of n=7-12. Panels D and E show the relationship between proteinuria at 4 weeks and mean arterial pressure (MAP) during the last 3 treatment days and the renal angiotensin II levels, respectively. *P<0.05, **P<0.01, ***P<0.001 versus indicated group.

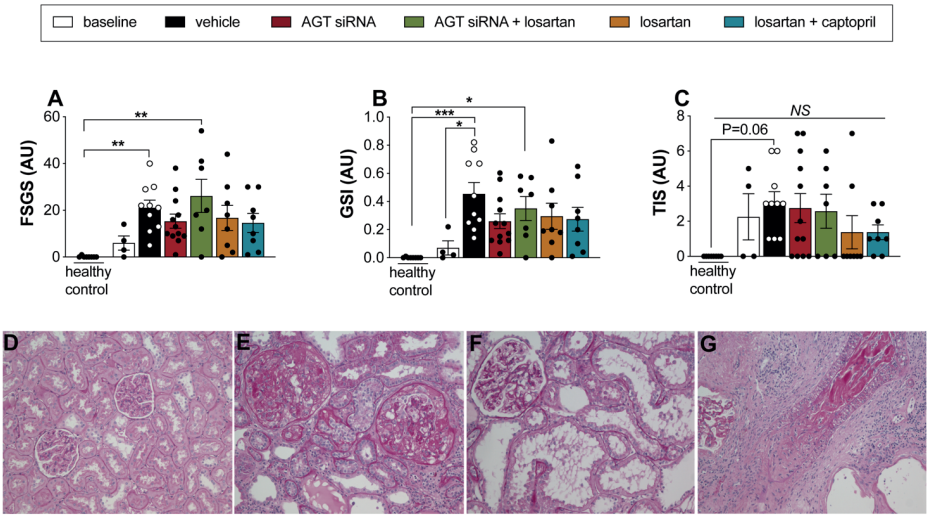

Figure 5. Focal segmental glomerulosclerosis (FSGS; panel A), glomerulosclerosis index (GSI; panel B), and tubulointerstitial score (TIS, panel C) in kidneys of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery (=baseline), and data in healthy rats have been added for comparison. Data are mean±SEM of n=7-12. Panels D-G show representative, PAS-stained renal histological images of different types of damage illustrating a normal kidney (D), dilated tubules (E), thrombotic microangiopathy of glomeruli and arteriole (F), and a thrombus in a main large vessel (G). NS, not significant, *P<0.05, **P<0.01, ***P<0.001 versus indicated group.

AGT SIRNA IS CARDIOPROTECTIVE

Cardiac Ang I and II levels in vehicle-treated 5/6th Nx rats were close to or below detection limit (**Figures 6A and 6B**). siRNA, with or without losartan, lowered these levels even further (P<0.05 vs. vehicle), while losartan alone and losartan + captopril upregulated cardiac Ang I (P<0.05). Losartan did not alter cardiac Ang II, while losartan + captopril lowered cardiac Ang II (P<0.05). As a consequence, the Ang II/I ratio decreased after both losartan and losartan + captopril (P<0.05; **Table S1**). All other angiotensin metabolites were undetectable in cardiac tissue of 5/6th Nx rats, and only after losartan and losartan + captopril did Ang-(1-7) occasionally rise above lower limit of quantification (P=NS). No treatment lowered NT-proBNP (**Figure 6C**). Yet, all treatments equally prevented the rise in HW/TL ratio that occurred over the 4 week period after starting therapy (**Figure 6D**). The HW/TL ratio correlated strongly with MAP (P<0.001; **Figure 6E**), but not with cardiac Ang II (data not shown).

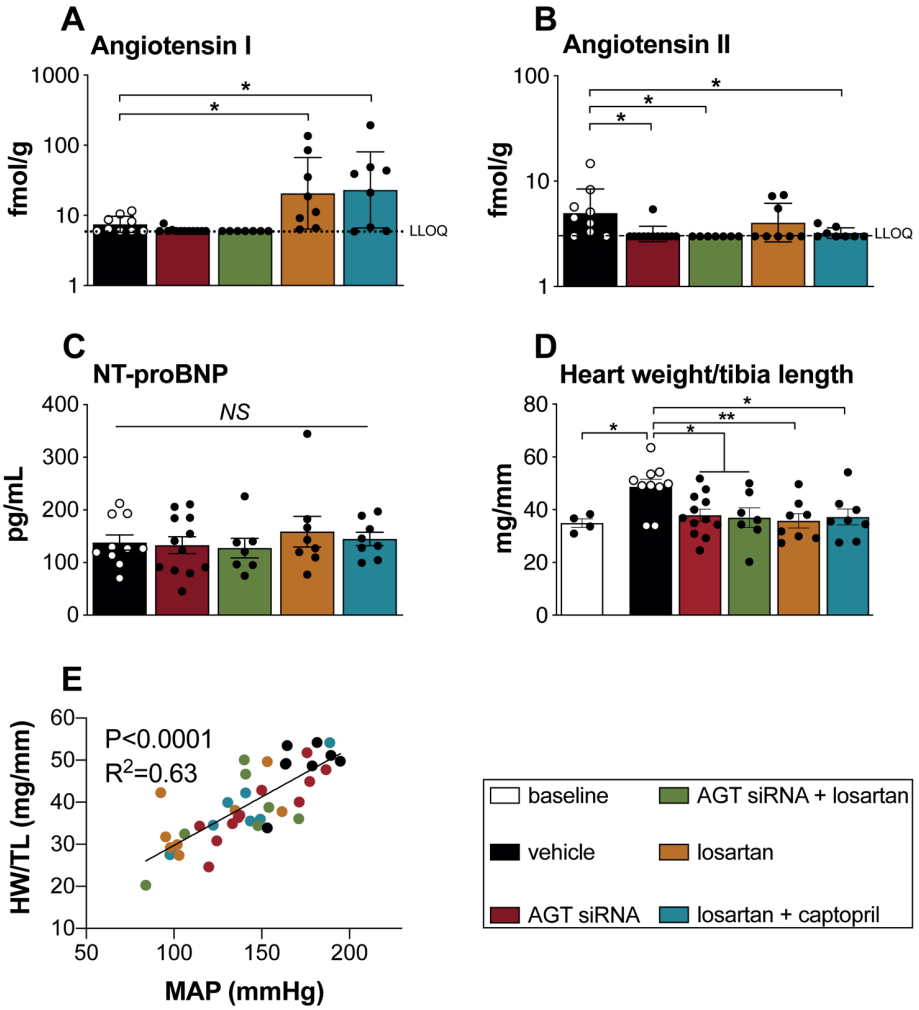

Figure 6. Cardiac angiotensin I (panel A), cardiac angiotensin II (panel B), plasma NT-proBNP (panel C), and heart weight/tibia length ratio (panel D) in Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started after 5 weeks of recovery (=baseline). Data are mean±SEM of n=7-12. Panel E shows the relationship between cardiac hypertrophy (heart weight/tibia length ratio) and mean arterial pressure (MAP) during the last 3 treatment days. NS, not significant, *P<0.05, **P<0.01 versus indicated group. LLOQ, lower limit of quantification.

5/6TH NX DOES NOT ALTER VASCULAR FUNCTION

Acetylcholine fully relaxed U46619-preconstricted mesenteric arteries of 5/6th Nx rats (**Figure S2 and Table S3**). Blocking NO (with L-NAME) or EDHF (with TRAM34+apamin) marginally prevented this relaxation, and only when combining L-NAME with TRAM34+

apamin, did the blockade become significant. This indicates that the ACH response depends on both NO and EDHF, and that the two pathways are interchangeable. No treatment altered this outcome. ET-1 strongly constricted mesenteric arteries of 5/6th Nx rats, and the ET_A receptor antagonist BQ123, but not the ET_B receptor antagonist BQ788, blocked this constriction, indicating that it depended entirely on ET_A receptor stimulation. No treatment altered this outcome.

DISCUSSION

This study is the first to demonstrate reno- and cardioprotection in the rat 5/6th Nx model after a 4-week treatment with liver-directed AGT siRNA. The model is characterized by hypertension, cardiac hypertrophy, proteinuria, reduced GFR, glomerulosclerosis and tubulointerstitial fibrosis, and therefore recapitulates important characteristics of CKD.[26] Although it is known to be responsive to RAS blockade,[8-10] we found circulating RAS activity in the 5/6th Nx rat to be greatly reduced, while renin upregulation during RAS blockade was barely detectable. This may simply reflect the fact that 5/6th of the kidneys was removed, reducing the capacity of the kidneys to release renin and/or to respond appropriately to RAS blockade.[27] In contrast, renin rises during RAS blockade (including angiotensinogen suppression) in salt-depleted humans[4] and spontaneously hypertensive rats[6] can easily be >100-fold, thereby allowing Ang II levels to stay in the normal range, even when blocking the system by >99%.

We compared the effect of AGT siRNA in the 5/6th Nx model with that of the ARB losartan and the ACE inhibitor captopril, two drugs that are commonly used in CKD. Treatment was started at 5 weeks after 5/6th Nx, when blood pressure had already increased by ≈≈60 mm Hg. Clearly, given the >75% reduction in circulating RAS activity, the Ang II-AT_1 receptor axis is unlikely to be a major mediator of this blood pressure rise. Nevertheless, losartan did lower blood pressure by 37 mm Hg. This was accompanied by renin and Ang II rises. Remarkably, when combining losartan with either AGT siRNA or captopril, its blood pressure-lowering effect was diminished, while AGT siRNA alone did not lower blood pressure, although it did prevent a further rise in blood pressure after the initial 5 week period. As a consequence, after 4 weeks of treatment MAP was lower in the losartan group than in the AGT siRNA group, while MAP in the losartan + AGT siRNA and losartan + captopril groups was identical to that in the AGT siRNA alone group (i.e., ≈40 mm Hg above that in healthy SD rats[25]). The most likely explanation for these observations is that losartan, by upregulating Ang II, allowed concomitant AT_2 receptor stimulation, which would result in more substantial blood pressure lowering (given that AT_2 receptors cause vasodilation[28-30]) than might be expected from blockade of the Ang II-AT_1

receptor axis alone. This is not possible in combination with either siRNA or captopril, since these drugs prevented such a rise in circulating Ang II. However, the relevance of AT2 signaling in humans is unclear. Despite the difference in blood pressure reduction, losartan, AGT siRNA and losartan + captopril reduced cardiac hypertrophy, proteinuria and glomerulosclerosis similarly, and a favorable trend was observed for the tubulointerstitial score and urinary NGAL. These protective effects in kidney and heart therefore must reflect the consequence of local RAS blockade. Indeed, AGT siRNA lowered the cardiac and renal Ang I and II levels more strongly than the circulating Ang I and II levels. Taken together, these data illustrate that cardiac and renal angiotensin generation depend on liver-derived AGT, accumulating at tissue sites either via diffusion or by active uptake mechanisms. Our data do not support the concept[11] that renal Ang II production in the 5/6th Nx rat depends on locally produced AGT and that liver-targeting of AGT siRNA would keep renal Ang II formation intact. We stress that AGT mRNA expression did occur in renal tissue of the 5/6th Nx rat. In agreement with the liver-specificity of our GalNAc-labeled siRNA, this expression was unaltered after AGT siRNA, nor was it altered by any of the other treatments. Yet, when treating the rats with AGT siRNA + losartan, renal Ang II entirely disappeared. The strong suppression of renal Ang II after AGT siRNA + losartan lowered proteinuria and cardiac hypertrophy to the same degree as single treatment, but no longer improved glomerulosclerosis. This observation supports the concept that complete elimination of the renal RAS is undesirable, and may underlie the renal side-effects of conventional dual and triple RAS blockade.[31, 32] However, the latter particularly concerns a reduction in GFR, and this was not observed in the present study. In fact, no treatment affected the ≈60% GFR reduction in our model, although GFR improvement has been observed previously in 5/6th Nx rats after RAS blockade.[10] This may relate to the larger (>75%) GFR reduction in those earlier studies.

Proteinuria correlated with MAP and the renal Ang II levels. A unifying concept to explain our data is therefore that both a decrease in blood pressure and a decrease in renal Ang II improve proteinuria, most likely in an additive manner, and involving a reduction in glomerulosclerosis. Multiple linear regression confirmed this view. Hence, losartan predominantly exerts its effects via blood pressure lowering (likely depending on AT_2 receptor stimulation), while siRNA rather acts by suppressing renal Ang II. A similar mechanism (blood pressure lowering and suppression of tissue Ang II generation[33]) may underlie the improvement of cardiac hypertrophy, although the heart weight/tibia length ratio correlated significantly with blood pressure only, and not with cardiac Ang II. The latter could relate to the fact that cardiac Ang II levels after treatment were often below detection limit. No effect of any treatment on NT-proBNP was observed, possibly because the blood pressure-lowering effects (with the exception of that of losartan

alone) were modest, so that atrial stretch (a major stimulant of BNP synthesis) would be reduced only mildly.

Both captopril and losartan suppressed the Ang II/I ratio in kidney and heart. In the case of captopril, this simply reflects the degree of ACE inhibition. In the case of losartan, like during ACE inhibition, there will be renin upregulation, resulting in enhanced Ang I generation. Ang II generation should increase in parallel. However, tissue Ang II reflects Ang II that has been internalized via AT_1 receptors,[33, 34] and consequently, given the fact that losartan inhibits this process, ARB treatment may indeed lower the tissue Ang II/I ratio, albeit without affecting the Ang II/I ratio in the circulation. Ang-(1-7) has been suggested to be responsible for the beneficial effects of RAS blockade in both kidney and heart, as it counteracts the Ang II-AT_1 receptor axis.[35] In the present study Ang-(1-7) was not detectable in either blood or cardiac tissue of 5/6th Nx rats, although it was present in the kidney. Nevertheless, AGT siRNA eliminated renal Ang-(1-7), thereby implying that its beneficial effects are unrelated to this peptide.

In summary, liver-targeted AGT siRNA exerts beneficial renal and cardiac effects in the 5/6th Nx rat despite the fact that this is a low-renin CKD model. Its tissue effects are likely to represent the maximum effect of RAS blockade, since adding losartan on top of AGT siRNA (or combining losartan with captopril) yielded a similar degree of reno- and cardioprotection. These effects occur in a blood pressure-independent manner, and reflect the dependency of both renal and cardiac Ang II on liver-derived AGT. In view of the potential long-lasting effects of siRNA treatment (as with inclisiran, which is dosed every six months[7]) targeting hepatic AGT offers new possibilities for the treatment of CKD in humans, especially in non-adherent patients. Future studies will be needed to understand both the long-term risks and benefits of such long-term tissue RAS suppression, considering that too much renal Ang II suppression may not be desirable.

SOURCES OF FUNDING

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SUPPLEMENTAL MATERIAL

Angiotensin metabolite or ratio	Vehicle	AGT siRNA	AGT siRNA + Losartan	Losartan	Losartan + Captopril
Blood (pmol/L)					
Ang-(1-10) = Ang I	17 (4, 31)	4 (<3, 3)	6 (<3, 3)	295 (44, 522) &	572 (31, 1098) &
Ang-(1-8) = Ang II	19 (10, 27)	8 (5, 10) *	9 (4, 15)	145 (30, 189) #	32 (7, 61)
Ang-(1-7)	<8	<8	<8	9 (8, 8)	16 (8, 25) *
Ang-(2-8) = Ang III	<3	<3	<3	11 (<3, 14) #	3 (<3, 3)
Ang-(3-8) = Ang IV	<1	<1	<1	9 (<1, 14) #	2 (<1, 3)
Ang II/I ratio	2.31 (0.73, 2.77)	2.27 (1.43, 2.99)	2.48 (1.00, 2.80)	0.80 (0.52, 0.73)	0.22 (0.04, 0.49) $
Kidney (fmol/g)					
Ang-(1-10) = Ang I	394 (88, 755)	37 (10, 59) #	<6 #	604 (437, 777)	399 (243, 576)
Ang-(1-8) = Ang II	345 (129, 602)	71 (23, 115) #	4 (3, 3) &	113 (60, 174) *	35 (14, 46) &
Ang-(1-7)	261 (58, 486)	25 (<19, 19) $	<19$	235 (173, 273)	164 (107, 251)
Ang-(2-8) = Ang III	14 (<6, 23)	6 (<6, 14) #	<6 #	<6 #	<6 #
Ang-(3-8) = Ang IV	11 (<10, 10)	<10	<10	<10	<10
Ang II/I ratio	1.19 (0.7, 1.42)	2.36 (1.02, 3.59) *	0.74 (0.50, 0.50)	0.23 (0.13, 0.25) &	0.10 (0.02, 0.17) &
Heart (fmol/g)					
Ang-(1-10) = Ang I	8 (6, 10)	<6	<6	39 (7, 73) *	46 (6, 47) *
Ang-(1-8) = Ang II	5 (<3, 7)	3 (<3, 3) *	<3*	4 (<3, 7)	3 (<3, 4) *
Ang-(1-7)	<19	<19	<19	28 (<19, 31)	30 (<19, 31)
Ang-(2-8) = Ang III	<6	<6	<6	<6	<6
Ang-(3-8) = Ang IV	<10	<10	<10	<10	<10
Ang II/I ratio	0.80 (0.44, 0.87)	0.52(0.50, 0.50)	0.50(0.50, 0.50)	0.26 (0.08, 0.44) #	0.24 (0.07, 0.50) #

Table S1. Angiotensin (Ang) metabolites measured by LC-MS/MS in blood, kidney, and heart in Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started 5 weeks after 5/6th Nx. Data are mean and interquartile range of n=7-12. All data were log-transformed to perform one-way ANOVA, followed by post-hoc correction according to Bonferroni (*P<0.05, #P<0.01, &P<0.001, $P<0.0001 vs. vehicle).

Salt

parameter		vehicle	AGT siRNA	AGT siRNA + losartan	losartan	losartan + captopril
body weight (g)		379±11	378±8	388±18	390±14	411±9
Δtreatment		38±8	38±7	46±9	49±8	57±5
food intake (mg/day)	baseline	21±1	21±1	21±1	22±1	23±1
	T=2 weeks	19±1	21±1	21±1	21±2	21±1
	T=4 weeks	17±2	22±4	20±1	21±2	21±1
water intake (mL/day)	baseline	52±3	50±3	50±4	51±5	52±2
	T=2 weeks	55±3	62±3	56±4	51±6	56±4
	T=4 weeks	56±7	48±6	59±6	54±6	60±4
urine (mL/day)	baseline	39±3	34±3	36±4	37±4	37±3
	T=2 weeks	44±4	41±3	38±4	37±4	40±5
	T=4 weeks	40±3	39±2	39±6	37±4	41±3

Table S2. Growth, food and water intake, and 24 hour-urine parameters of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started 5 weeks after 5/6th Nx (=baseline), and measurements were performed at baseline, after 2 weeks and after 4 weeks of treatment. Data (mean ± SEM of n=7-12) were analyzed by paired t-test (baseline vs. 4 weeks) or one-way ANOVA followed by post-hoc correction according to Bonferroni.

pEC_{50}	control	AGT siRNA	siRNA+losartan	losartan	losartan+captopril
ACh	8.0±0.3	8.0±0.2	7.9±0.3	8.5±0.5	8.2±0.3
ACh+L-NAME	7.9±0.4	8.0±0.2	7.6±0.2	7.6±0.3	8.2±0.4
ACh+T+A	7.2±0.2	7.5±0.1	7.2±0.3	7.4±0.2	7.4±0.2
ACh+L-NAME+T+A	7.5±0.4	7.3±0.3	7.4±0.5	7.4±0.6	7.2±0.5
ET-1	8.2±0.1	8.3±0.1	8.3±0.1	8.1±0.2	8.2±0.1
ET-1+BQ123	7.0±0.1*	7.2±0.1 #	7.1±0.04 #	7.2±0.1*	7.0±0.03 &
ET-1+BQ788	8.3±0.5	8.3±0.3	8.4±0.4	8.3±0.3	8.2±0.1
E_{max}	control	AGT siRNA	siRNA+losartan	losartan	losartan+captopril
ACh	82.6±5.1	90.9±2.7	78.9±8.6	82.3±9.2	84.2±3.4
ACh+L-NAME	70.9±9.0	76.0±6.	65.1±7.9	58.1±11.6	66.9±7.2
ACh+T+A	63.8±11.7	84.8±3.7	68.0±10.3	79.0±7.9	77.0±6.2
ACh+L-NAME+T+A	29.0±6.9 &	43.6±7.0 &	36±7.8 #	45.7±11.7	30.2±5.4 &
ET-1	134.8±11.6	113.4±5.0	134.7±14.4	138.5±14.6	120.6±11.1
ET-1+BQ123	75.0±11.5*	105.6±11.1	110.1±9.2	117.7±20.9	94.4±8.3
ET-1+BQ788	129.7±18.4	107.1±9.6	141.8±19.3	131.1±7.1	114.1±12.4

Table S3. E_{max} (maximum effect) and pEC_{50} (the negative logarithm of the half-E_{max} concentration) for vascular response to acetylcholine (ACh) and endothelin-1 (ET-1) in mesenteric arteries of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started 5 weeks after 5/6th Nx. ACh responses were studied in the absence (control) or presence of the inhibitors L-NAME, TRAM34+apamin (T+A), or L-NAME+T+A. ET-1 responses were studied in the absence (control) or presence of the endothelin receptor blockers BQ123 and BQ788. Data (mean ± SEM of n=5-11) were analyzed by one-way ANOVA followed by post-hoc correction according to Bonferroni. *P<0.05, #P<0.01, &P<0.001, $P<0.0001 vs. no inhibitor.

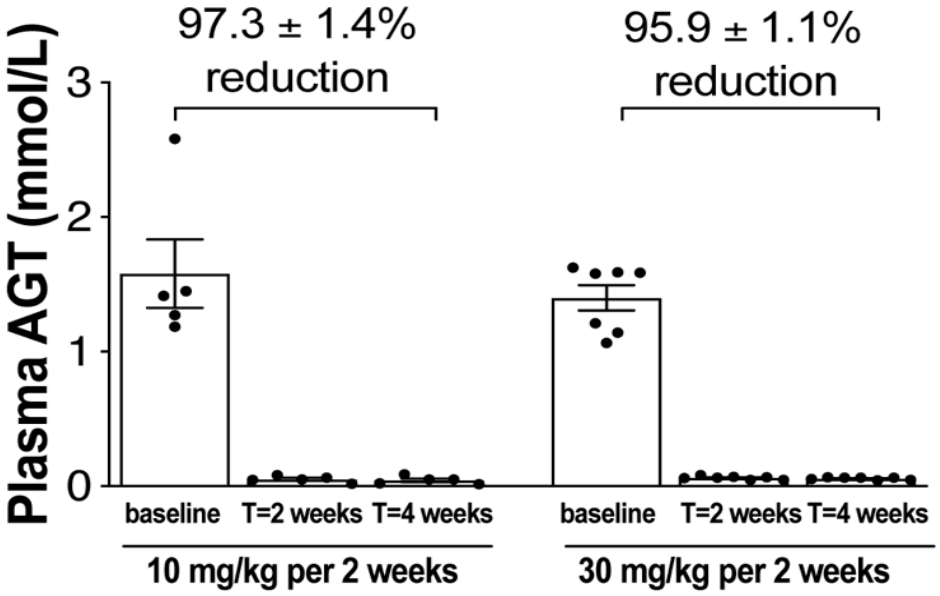

Figure S1. Plasma angiotensinogen (AGT) in Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with AGT siRNA 10 or 30 mg/kg biweekly. Treatment was started 5 weeks after 5/6th Nx (=baseline), and measurements were performed at baseline, after 2 weeks, and after 4 weeks of treatment. Data are mean ± SEM of n=5-7.

Salt

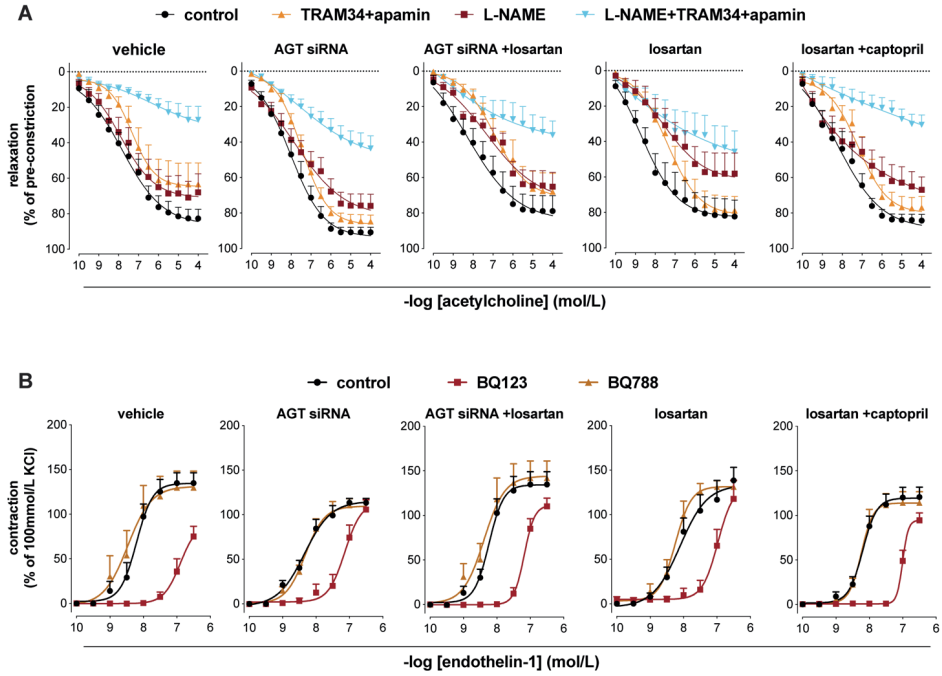

Figure S2. Vascular response to acetylcholine and endothelin-1 in mesenteric arteries of Sprague-Dawley rats subjected to 5/6th nephrectomy, and treated with either vehicle, angiotensinogen (AGT) siRNA, AGT siRNA + losartan, losartan, or losartan + captopril for 28 days. Treatment was started 5 weeks after 5/6th Nx. Panel A shows the relaxant responses to acetylcholine, expressed as a percentage of the U46619-induced preconstriction, in the absence (control) or absence the inhibitors TRAM34+apamin, L-NAME, and L-NAME+TRAM34+apamin. Panel B shows the responses to endothelin-1, expressed as a percentage of the response to 100 mmol/L KCl in the absence (control) or presence of the endothelin receptor blockers BQ123 and BQ788. Data are mean ± SEM of n=5-11, and the accompanying statistics are shown in supplemental Table S3.

Chapter 6

A randomized trial of distal diuretics versus dietary sodium restriction for hypertension in chronic kidney disease

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ABSTRACT

Background: Distal diuretics are considered less effective in CKD, but data to support this are limited. Here, we hypothesize that distal diuretics are non-inferior to dietary sodium restriction in reducing blood pressure in patients with CKD and hypertension.

Methods: In patients with CKD stage G3 or G4 and hypertension, we discontinued antihypertensive drugs and performed a 6-week randomized open-label cross-over trial to compare amiloride/hydrochlorothiazide (5/50 mg/day) with dietary sodium restriction (60 mmol/day). We analyzed effects on blood pressure, kidney function, and fluid balance and related this to renal clearance of diuretics.

Results: Twenty-six patients (eGFR 39 ± 13 ml/min/1.73m^2) completed both treatments. Dietary sodium restriction reduced sodium excretion from 160 to 64 mmol/day. Diuretics reduced 24-hour systolic blood pressure more than sodium restriction (138 to 124 vs. 134 to 129 mmHg, $P < 0.05$). Both interventions decreased body weight and NT-pro-BNP similarly, whereas diuretics had a significantly greater effect on extracellular water, eGFR, plasma renin and aldosterone. Neither intervention decreased albuminuria significantly, whereas diuretics did significantly reduce urinary angiotensinogen and β2-microglobulin excretion. Lower eGFR and higher plasma indoxyl sulfate correlated with lower diuretic clearance. However, the diuretic effects on body weight and blood pressure at lower eGFR were maintained. During diuretic treatment, higher prostaglandin E2 excretion correlated with lower free water clearance, and four patients developed mild hyponatremia.

Conclusions: Distal diuretics are non-inferior to dietary sodium restriction in reducing blood pressure and extracellular volume in CKD. Diuretic sensitivity in CKD is maintained despite lower diuretic clearance.

SIGNIFICANCE STATEMENT

Chronic kidney disease (CKD) is characterized by increased extracellular volume and salt-sensitive hypertension. It is unknown whether dietary or pharmacological approaches are preferable to reduce sodium in CKD and whether distal diuretics are still effective. To address this, we performed a randomized cross-over trial in patients with CKD stage G3 or G4 and hypertension to compare dietary sodium restriction with distal diuretics (hydrochlorothiazide and amiloride). Both interventions effectively lowered 24-hour blood pressure and extracellular volume, with diuretics exerting a stronger effect. Although the tubular secretion of diuretics was impaired at lower eGFR, the reductions in body weight and blood pressure effect were maintained. This shows that – even at lower eGFR – dietary sodium restriction and distal diuretics are effective therapies for hypertension and overhydration in CKD.

INTRODUCTION

Salt-sensitive hypertension and overhydration are hallmarks of chronic kidney disease (CKD) and are associated with adverse outcomes.[1-4] Dietary sodium restriction effectively lowers blood pressure, extracellular volume, and albuminuria in CKD.[5-8] However, given the high sodium content of most food products, long-term adherence to dietary sodium restriction remains a challenge.[9] Therefore, a pertinent question is whether other approaches to reduce sodium in CKD such as diuretics are similarly effective. In order to inhibit sodium reabsorption, diuretics first need to be secreted by the proximal tubule, a process that may be impaired in CKD.[10]

Although "high-ceiling" loop diuretics are commonly used in CKD stages G3-5, "low-ceiling" distal diuretics are considered less effective.[11-13] Whether these assumptions are justified is uncertain. Experimental data indicate that the pharmacological targets of thiazide diuretics and amiloride – the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC) – are upregulated in CKD.[14-16] Several small case series (n = 5–12)[17-23] and one larger study (n = 60)[24] analyzed the effects of thiazide or thiazide-like diuretics on blood pressure in patients with CKD stages G3 to G5D. The majority of these studies found that the antihypertensive effect of thiazide diuretics is preserved in CKD, except for three studies that included patients with CKD stage G5.[11, 22, 23] Two small studies analyzed amiloride in CKD and also observed a preservation of its natriuretic and anti-kaliuretic effects.[25, 26] These observations provide a rationale for a more systematic investigation of distal diuretics in CKD. Several investigators have previously called for such a study.[27-31] In designing this study, we considered it rational to combine diuretics to prevent diuretic resistance secondary to upregulation of the uninhibited transporter.[32-34] Although the efficacy of combining loop and thiazide diuretics has been shown previously in CKD[33], the effect of combining inhibitors of NCC and ENaC in CKD has not been analyzed. Advantages of a combination of distal diuretics could be to maintain potassium balance[35] and to prevent proteinuria-induced activation of ENaC.[36] Therefore, we set out to address the hypothesis that distal diuretics are non-inferior to dietary sodium restriction in reducing blood pressure in patients with CKD. To do so, we recruited patients with CKD stage G3 or G4 and hypertension, discontinued their anti-hypertensive drugs, and subsequently performed a randomized cross-over trial to compare the two sodium-reducing strategies. In addition to the effects of both interventions on clinical parameters, we also analyzed markers of fluid balance, the circulating and intra-renal renin-angiotensin system, and renal clearance of diuretics. We demonstrate that distal diuretics are at least as effective as dietary sodium restriction for the treatment of hypertension in CKD.

METHODS

Participants

We conducted a single-center, randomized, open-label, cross-over study (**Figure 1**). The study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2015-576) and registered at www.clinicaltrials.gov (NCT02875886). The Consolidated Standards of Reporting Trials (CONSORT) flow-diagram and checklist are available as **Supplemental Material**. Patients were recruited from the nephrology outpatient clinic of the Erasmus Medical Center between June 2016 and May 2017. Patients aged >18 years old with CKD stage G3 or G4 (eGFR 15-59 ml/min/1.73m^2) with hypertension were eligible for inclusion. Hypertension was defined as 1) current use of antihypertensive drug or 2) no use of antihypertensive drugs, but a mean systolic blood pressure >140 mmHg after 6 consecutive measurements with an oscillometric blood pressure monitor. Exclusion criteria were previous intolerance or allergy to thiazide diuretics or amiloride, pregnancy, the presence of certain diseases (nephrotic syndrome, salt-wasting nephropathy, liver cirrhosis with ascites, heart failure class III or IV), electrolyte disorders (serum sodium < 136 mmol/l, serum potassium < 3.5 or > 5.5 mmol/l), high likelihood of kidney replacement therapy < 4 months, and previous kidney transplantation or use of immunosuppressive drugs.

Study design

The study started with a 2-week run-in period during which all antihypertensive medication was discontinued, except for β-blockers (for cardiac reasons). Patients were provided with a home blood pressure monitor (Omron HBP-1300, Omron Healthcare, The Netherlands) and instructed to measure blood pressure twice daily. If the systolic blood pressure (SBP) was ≥ 160 mmHg during three consecutive measurements, amlodipine was started (5 mg once daily with possible uptitration to 10 mg once daily). Subsequently, patients were randomly assigned to start with sodium restriction (60 mmol/day) or amiloride/hydrochlorothiazide (combination preparation of 5/50 mg once daily). Allocation to treatment order was done by randomization using sequentially numbered, opaque, sealed envelopes. Treatment periods lasted for two weeks and were separated by a 2-week washout period (**Figure 1**). All patients received dietary counseling by a renal dietitian at the start of treatment with sodium restriction. In addition, salt-free bread was provided for the complete duration of the dietary intervention. After one week of treatment patients were called by the dietitian to increase adherence to the diet and provide additional counseling, if necessary. Compliance to sodium restriction was monitored with 24-hour urinary sodium excretion and adherence was defined as >10% reduction. Two patients repeated the 2-week period of dietary sodium restriction.

Adherence to diuretics was evaluated using drug accountability (counting pills) and the measurement of urinary diuretic concentrations.

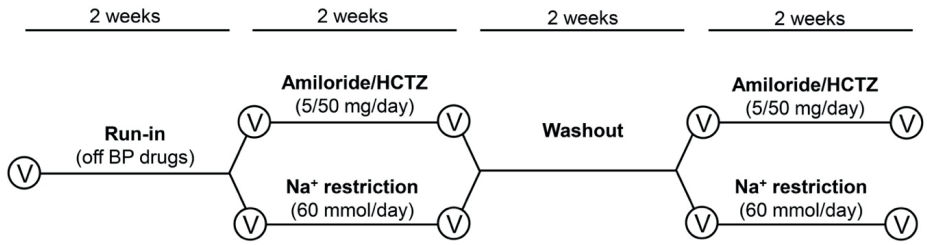

Figure 1. Overview of the study design. HCTZ, hydrochlorothiazide; Na^+, sodium; V, visit.

Measurements

Before and after each intervention blood pressure, body weight, and body composition were measured, and blood and urine were collected. 24-hour ambulatory blood pressure measurements (ABPM) were performed with the 90217A Ultralite (Spacelabs Healthcare, USA) with masked screen. Blood pressure was measured at 15-minute intervals during daytime (16 out of 24 hours) and at 30-minute intervals during nighttime (8 out of 24 hours). The starting time of daytime and nighttime measurements was set based on the patient's sleeping habits. An ABPM was considered successful when ≥ 70% of expected measurements were valid (45 valid awake, 11 valid asleep).[37] Extracellular water was measured using a bio-impedance spectroscopy monitor (Body Composition Monitor, Fresenius Medical Care, Germany). All urinary measurements were performed in 24-hour urine samples. Compliance of 24-hour urine collection was determined by creatinine excretion-to-weight-ratio.[38] Plasma and urine electrolytes, albumin, creatinine, and β2-microglobulin were measured at the Department of Clinical Chemistry. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation.[39] eGFR was also recorded up to 1 year after completion of the study. Plasma renin was measured using a radioimmunometric assay (Cisbio, France). Urinary renin and angiotensinogen were measured using an in-house enzyme-kinetic assay that quantifies angiotensin I generation in the presence of excess angiotensinogen and recombinant renin, respectively.[40, 41] Plasma and urine aldosterone were measured by radioimmunoassay (Demeditec, Germany). Prostaglandin E2 (PGE2) and its metabolite were measured using an enzyme-linked immusorbent assay (Cayman Chemicals, USA). Plasma and urine hydrochlorothiazide and amiloride concentrations were measured using liquid chromatography–mass spectrometry (LC-MS; Waters, USA), as previously described with minor modifications.[42] Renal clearance of hydrochlorothiazide and amiloride was calculated based on their concentration in 24-hour urine and plasma samples that were collected immediately after urine collection. Plasma indoxyl sulfate was measured using LC-MS (Agilent Technologies, Germany), as described.[43] During the

treatments, the following objective side-effects were monitored, including orthostatic hypotension (20 or 10 mmHg decrease in SBP or DBP within three minutes of standing after 5 minutes of supine rest[44]), gout, hyponatremia (plasma sodium < 136 mmol/L), hypo- and hyperkalemia (plasma potassium < 3.5 or > 5.5 mmol/L), and hyperuricemia (plasma uric acid > 7.1 mg/dL).

Statistics

The primary outcome was the change in mean 24-hour SBP from baseline. Secondary endpoints included change in extracellular volume, body weight, albuminuria and adverse effects. All endpoints were analyzed per protocol and intention to treat. A power calculation based on previous studies indicated that a minimum of 22 patients was required to establish non-inferiority of diuretics compared with sodium restriction (α = 0.05, β = 90%, expected effect of sodium restriction –8.75 ± 8.5 mmHg[5], expected effect of diuretics –10 ± 8.5 mHg[18], correlation coefficient between effects of both treatments 0.8, variance of difference in treatment effect 5.38, non-inferiority margin -2 mmHg). The omnibus K2 test was used to screen for normality. Results are presented as mean ± standard deviation for normally distributed data and median with interquartile range for non-normally distributed data. Non-normally distributed data were log-transformed for statistical analysis. Grubb's test was used to detect outliers. One outlier in the urinary PGE2 data was not included in the analysis because the result suggested the presence of semen in urine, a known cause of very high urinary PGE2 levels.[45] Primary and secondary outcomes were analyzed by two-way repeated measures ANOVA that also included treatment order as between-subject factor. A pretest was performed and indicated that the assumption of negligible carryover effects was met.[46] A paired T-test was performed to analyze if the effects of diuretics or dietary sodium restriction affected the baseline parameters after wash-out. The possibility of a period effect was analyzed and found to be absent.[47] Adverse events were analyzed by McNemar's test. Correlations were analyzed on normally distributed or log-transformed data using Pearson's correlation coefficient. If a correlation was present between normally distributed data and log-transformed data, non-linear regression using a linear-logarithmic model was used to fit the original data. Data were analyzed using SPSS Statistics (IBM, version 24.0) and Graphpad Prism (GraphPad Software, version 7, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics and Study Compliance

1563 patients were assessed for eligibility of whom 1274 did not meet inclusion criteria, and 262 declined to participate (**Supplemental Figure 1**). 27 patients entered the study protocol, of whom 1 patient discontinued during the run-in phase (because of study burden). Therefore, 26 patients finished both treatments; their baseline characteristics are shown in **Table 1**. In 7 out of 26 patients, SBP increased >160 mmHg during the run-in phase and amlodipine was given until the end of the study protocol (average dose 5.8 ± 2.0 mg). During the treatment phase with diuretics, there was 100% drug accountability and all patients had detectable plasma and urine diuretic concentrations. The response in urine electrolyte excretion confirmed study compliance to dietary sodium restriction in all patients (**Figure 2**). Urine sodium decreased from 160 to 64 mmol/day during dietary sodium restriction (mean difference -95.3 mmol, 95% CI, 67.6–123.1, $P < 0.01$), whereas it remained similar during treatment with amiloride/hydrochlorothiazide (154 to 153 mmol/24h, mean difference -0.8 mmol/24h, 95% CI, 26.9–28.6, $P = 1.0$). Urine calcium decreased significantly with both interventions, but more so with amiloride/hydrochlorothiazide. Finally, urine potassium did not change during both interventions, indicating stable dietary potassium intake during the study period. A subanalysis excluding patients who appeared non-compliant with the 24h urine collection (n = 4), did not change these findings.

Table 1. Baseline characteristics of study participants ($n = 26$)

Characteristic	Value
Age (years)	61 ± 14
Men	17 (65)
Diabetes mellitus	5 (19)
Number of antihypertensive medications	1.8 ± 1.1
Renin-angiotensin system blockade	23 (89)
β-blockers	8 (31)
Calcium channel blockers	8 (31)
Diuretic	7 (27)
Body mass index (kg/m^2)	28.0 ± 4.8
eGFR (mL/min/1.73m^2)	39 ± 13
Office systolic blood pressure (mmHg)	140 ± 17
Office diastolic blood pressure (mmHg)	88 ± 15
Albuminuria (mg/24 h)	145 (10, 1050)
Urine sodium (mmol/24 h)	135 (100, 207)

Data are presented as n (%), mean ± SD or median (interquartile range).

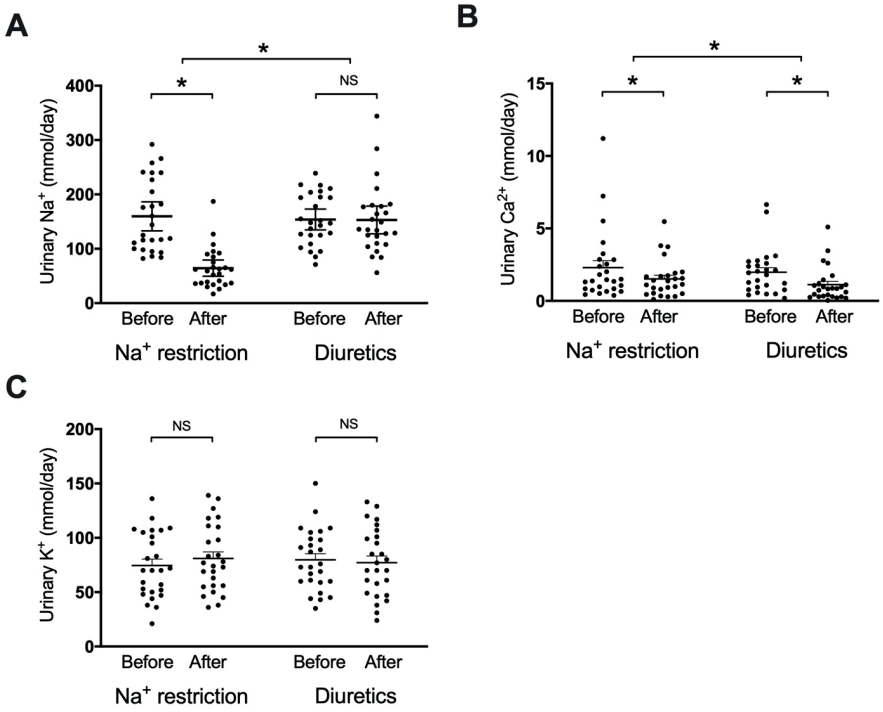

Figure 2. Effect of sodium (Na^+) restriction or diuretics on urinary electrolyte excretion. Two-way repeated measures ANOVA was used for analysis. Urine potassium (K^+) was normally distributed, while urine Na^+ and calcium (Ca^{2+}) were not. * $P < 0.05$ for difference before *versus* after treatment, and for difference between treatments. NS, not significant.

Effect on Blood Pressure and Kidney Function

Both treatments reduced mean 24-hour SBP from 134 to 129 mmHg for sodium restriction (mean difference -5 mmHg, 95% CI -1, -9, $P < 0.05$) and from 138 to 124 mmHg for amiloride/hydrochlorothiazide (mean difference -14 mmHg, 95% CI -10, -18, $P < 0.01$, **Figure 3**). The treatment effect of amiloride/hydrochlorothiazide on 24-hour SBP was significantly greater compared with sodium restriction ($P < 0.01$). Intention to treat analysis similarly showed that both treatments reduced mean 24-hour SBP (from 134 to 130 mmHg for sodium restriction (mean difference -4 mmHg, 95% CI 0, -9, $P < 0.05$; 138 to 124 mmHg for amiloride/hydrochlorothiazide, mean difference -14 mmHg, 95% CI -10, -18, $P < 0.01$). 24-hour DBP also decreased by both treatments, but this change was only significant for the diuretic treatment. SBP was reduced by diuretics in all patients and by dietary sodium restriction in 19 patients. The effects of both interventions on day and night blood pressure is shown in **Supplemental Table 2**. Albuminuria and plasma renin measured at the start of the first treatment period did not correlate with SBP responses to both treatments (data not shown). eGFR decreased with both treatments and this effect was significantly greater with diuretics compared with dietary sodium

restriction (**Figure 3**). Follow-up eGFR's obtained after the study showed that eGFR returned to baseline. No significant change in albuminuria was detected for both treatments. Plasma potassium remained constant with both interventions. No statistically

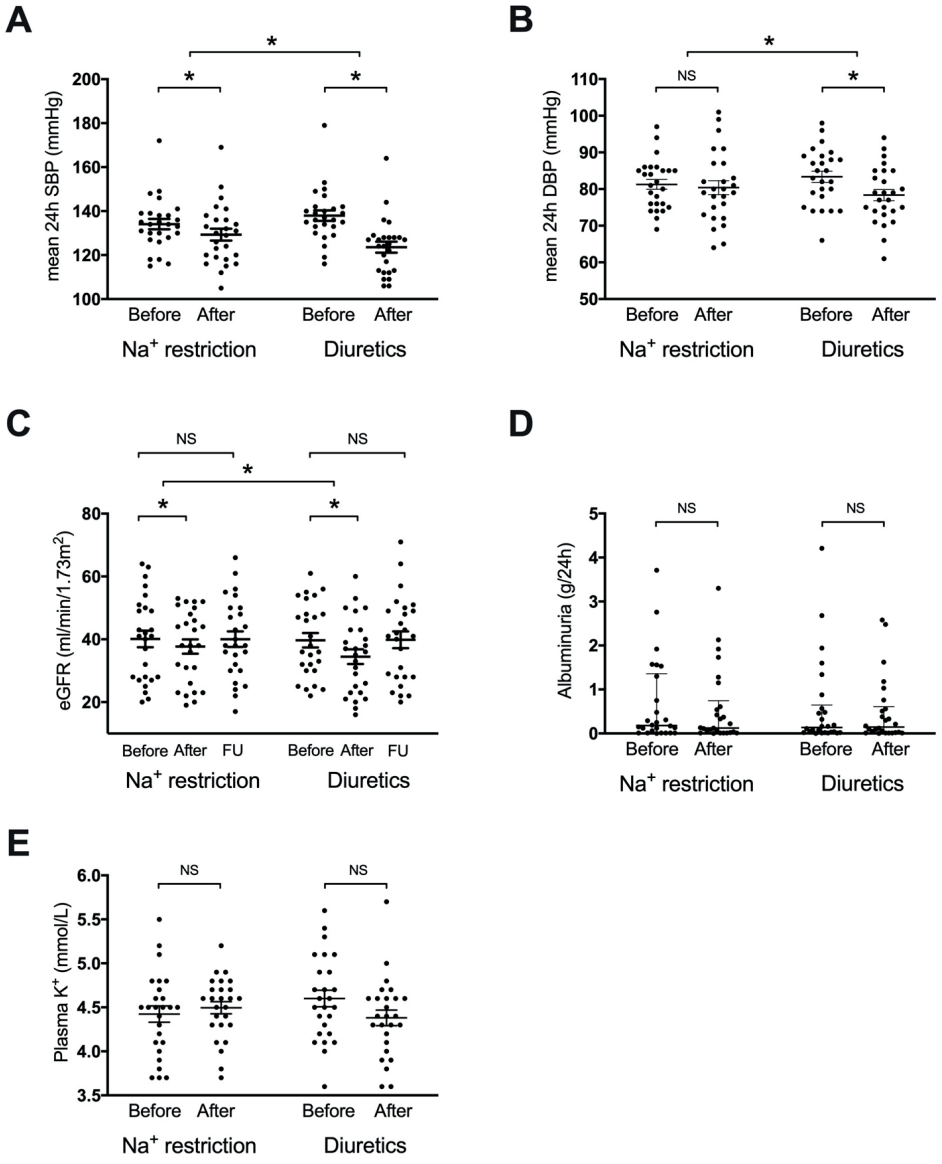

Figure 3. Effect of sodium (Na^+) restriction or diuretics on blood pressure, kidney function, and plasma potassium (K^+). Two-way repeated measures ANOVA was used for analysis. Systolic and diastolic blood pressure (SBP, DBP), estimated glomerular filtration (eGFR), and plasma K^+ were normally distributed, while albuminuria was not. * $P < 0.05$ for difference before *versus* after treatment, and for difference between treatments. FU, follow-up; NS, not significant.

significant carry-over effects of both treatments were present. However, a persisting effect of the diuretics on blood pressure and plasma potassium after wash-out was observed (**Figure 4**). A sensitivity analysis including patients that first received sodium restriction (n = 12) confirmed that diuretics had a stronger antihypertensive effect than sodium restriction (138 to 132 mmHg for sodium restriction, P = 0.06; 140 to 125 mmHg for amiloride/hydrochlorothiazide, P < 0.01; P < 0.05 for interaction).

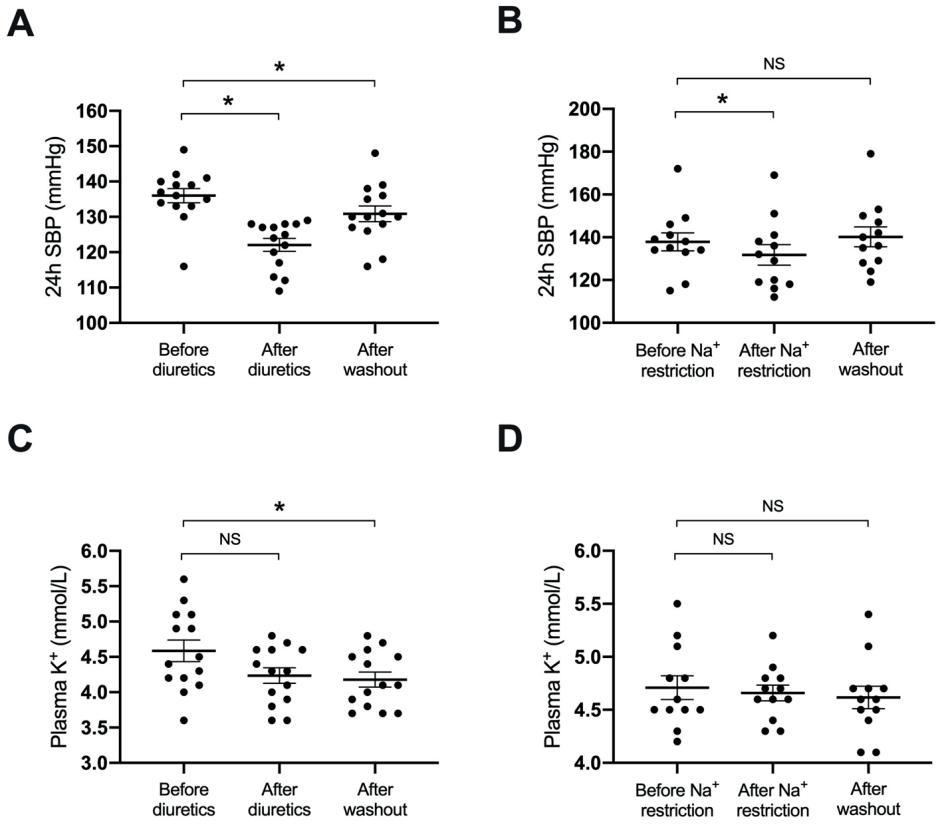

Figure 4. Systolic blood pressure (SBP) and plasma potassium (K^+) in patients who started with diuretics or dietary sodium (Na^+) restriction. The data show that the effect of diuretics but not Na^+ restriction persists after discontinuation of their use. A paired T-test was used for analysis. * P < 0.05 for difference before *versus* after treatment, or before treatment *versus* after washout. NS, not significant.

Effect on Fluid Balance and Volume Markers

Both interventions decreased body weight (-1.6 ± 1.1 kg for sodium restriction, P < 0.001; -1.9 ± 1.5 kg for amiloride/hydrochlorothiazide, P < 0.001) and NT-pro-BNP similarly (**Figure 5**).

Diuretics had a significantly greater effect on extracellular water, plasma renin, and plasma aldosterone compared with dietary sodium restriction. Fluid balance and volume markers did not correlate with the 24-hour SBP response to both treatments (data not shown).

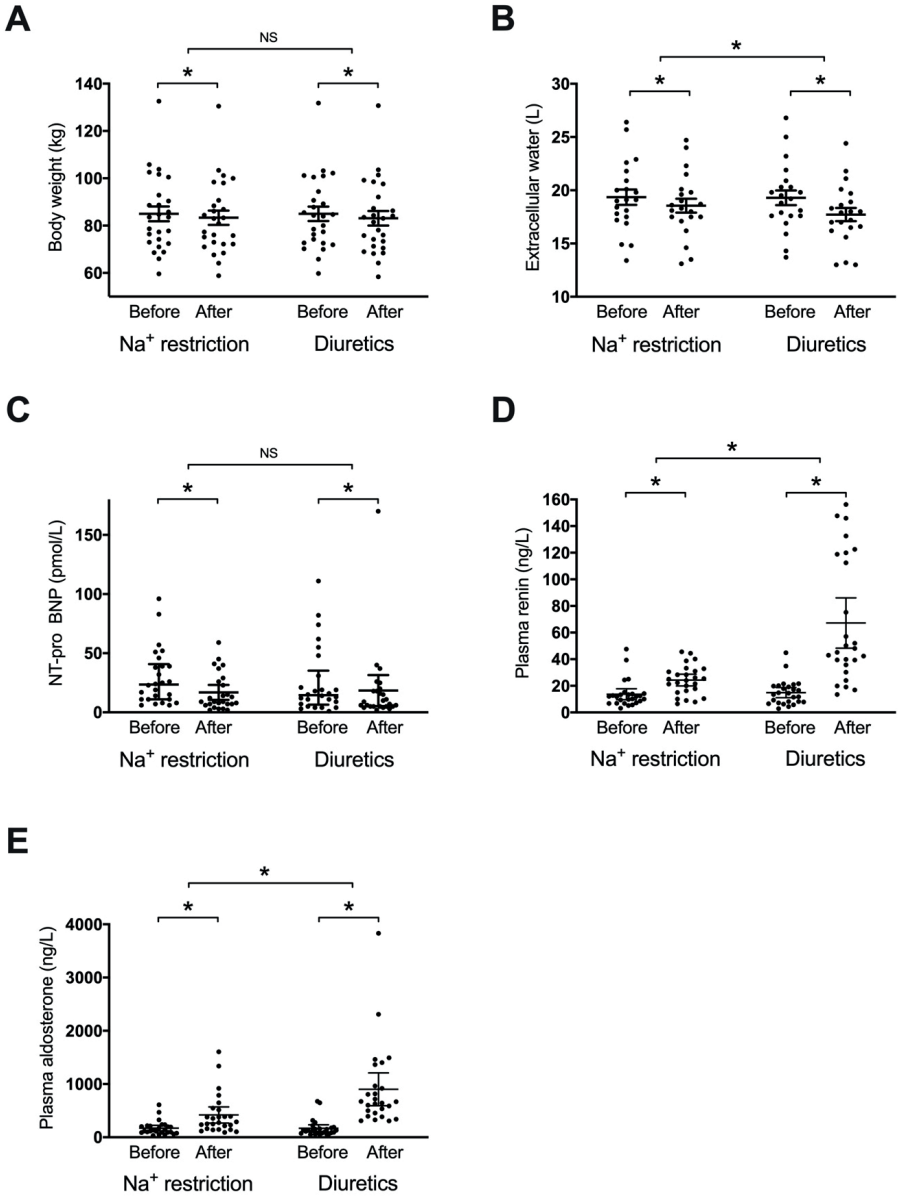

Figure 5. Effect of dietary sodium (Na^+) restriction or diuretics on fluid balance. All data were normally distributed. Two-way repeated measures ANOVA was used for analysis. * $P < 0.05$ for difference before *versus* after treatment, and for difference between treatments. NT-pro-BNP, N-terminal pro B-type natriuretic peptide.

Clearance of Distal Diuretics in CKD

Lower eGFR's were associated with lower diuretic clearance, in a non-linear manner, indicating reduced tubular secretion of diuretics at lower eGFR (**Figure 6**). To explore this further, plasma indoxyl sulfate concentrations were measured, based on previous data showing that this uremic toxin competes with the tubular secretion of diuretics in the proximal tubule.[10] Indeed, higher plasma indoxyl sulfate concentrations were as-

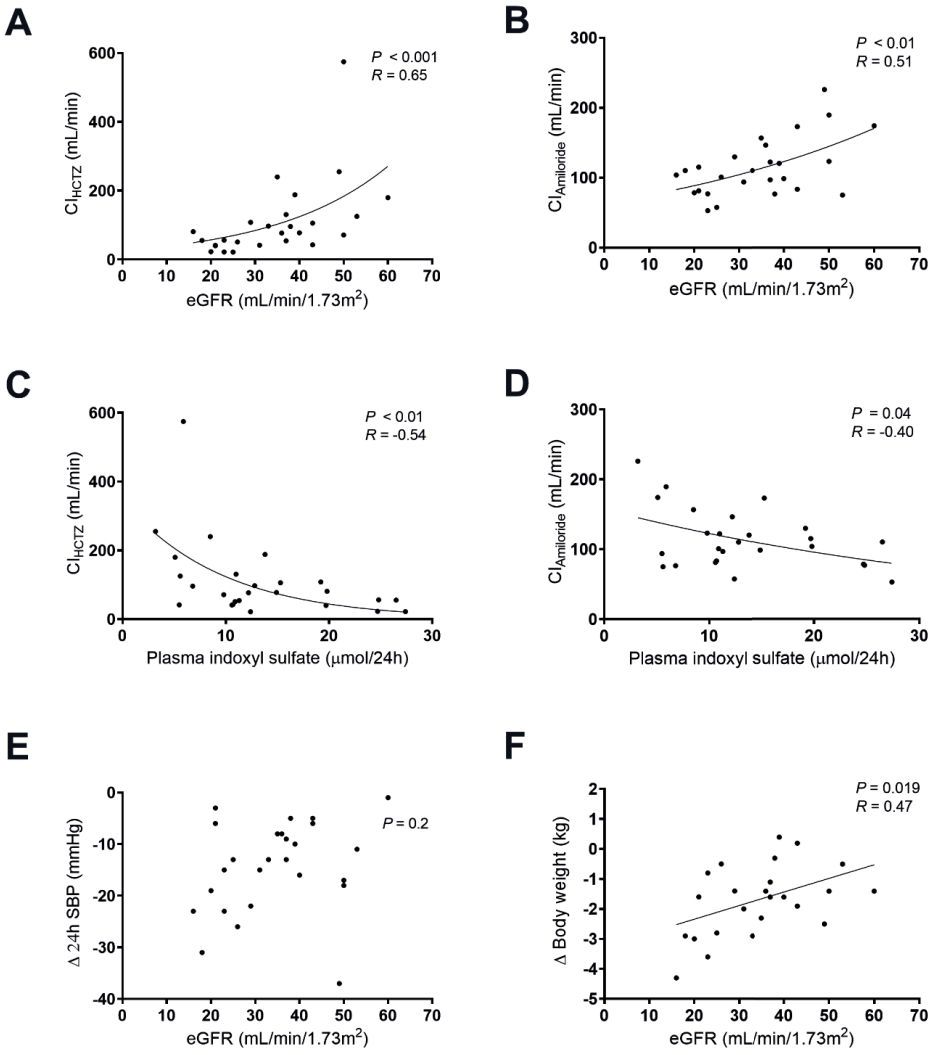

Figure 6. Correlations between diuretic clearance, eGFR, and plasma indoxyl sulfate and between eGFR and the diuretic response in body weight and blood pressure. Clearances were not normally distributed. Pearson's correlation coefficient was calculated. Cl_{HCTZ}, clearance of hydrochlorothiazide; $Cl_{Amiloride}$, clearance of amiloride; eGFR, estimated glomerular filtration rate.

sociated with significantly lower clearance of both diuretics (**Figure 6**). To analyze the whether these pharmacokinetic effects also had pharmacodynamic consequences, we analyzed the diuretic response on body weight and blood pressure across the different levels of eGFR. Of note, lower eGFR was associated with a greater reduction in body weight and a similar reduction in blood pressure. Prior to diuretic treatment, lower eGFR correlated with higher NT-pro-BNP ($P < 0.01$, r = -0.5), suggesting more fluid overload. In contrast to patients with hypertension and a normal kidney function[35], plasma renin and albuminuria at baseline did not predict the blood pressure response to diuretics (data not shown).

Thiazide-induced Hyponatremia

Diuretic treatment was generally well tolerated with a comparable incidence of adverse effects (**Table 2**). The only exception was mild hyponatremia, which developed in four patients after diuretic treatment (plasma sodium 135 $\pm$ 1 mmol/L). Because thiazide-induced hyponatremia was recently linked to PGE2[48], we measured the excretion of PGE2 and its metabolite (**Figure 7**). Diuretics but not dietary sodium restriction increased urine PGE2 excretion. Higher urine PGE2 excretion was associated with lower free water clearance.

Table 2. Adverse effects.

Side-effect	Dietary sodium restriction (*n* = 26)	Amiloride/hydrochlorothiazide (*n* = 26)
Orthostatic hypotension	4 (15)	6 (23)
Gout	0 (0)	1 (4)
Hyponatremia	0 (0)	4 (15)
Hypokalemia	0 (0)	0 (0)
Hyperkalemia	0 (0)	1 (4)
Hyperuricemia	17 (65)	22 (85)

Data are presented as n (%). No significant differences by McNemar's test. Hyponatremia was defined as plasma sodium < 136 mmol/L, hypo- and hyperkalemia as plasma potassium < 3.5 or > 5.5 mmol/L, and hyperuricemia as plasma uric acid > 7.1 mg/dL.

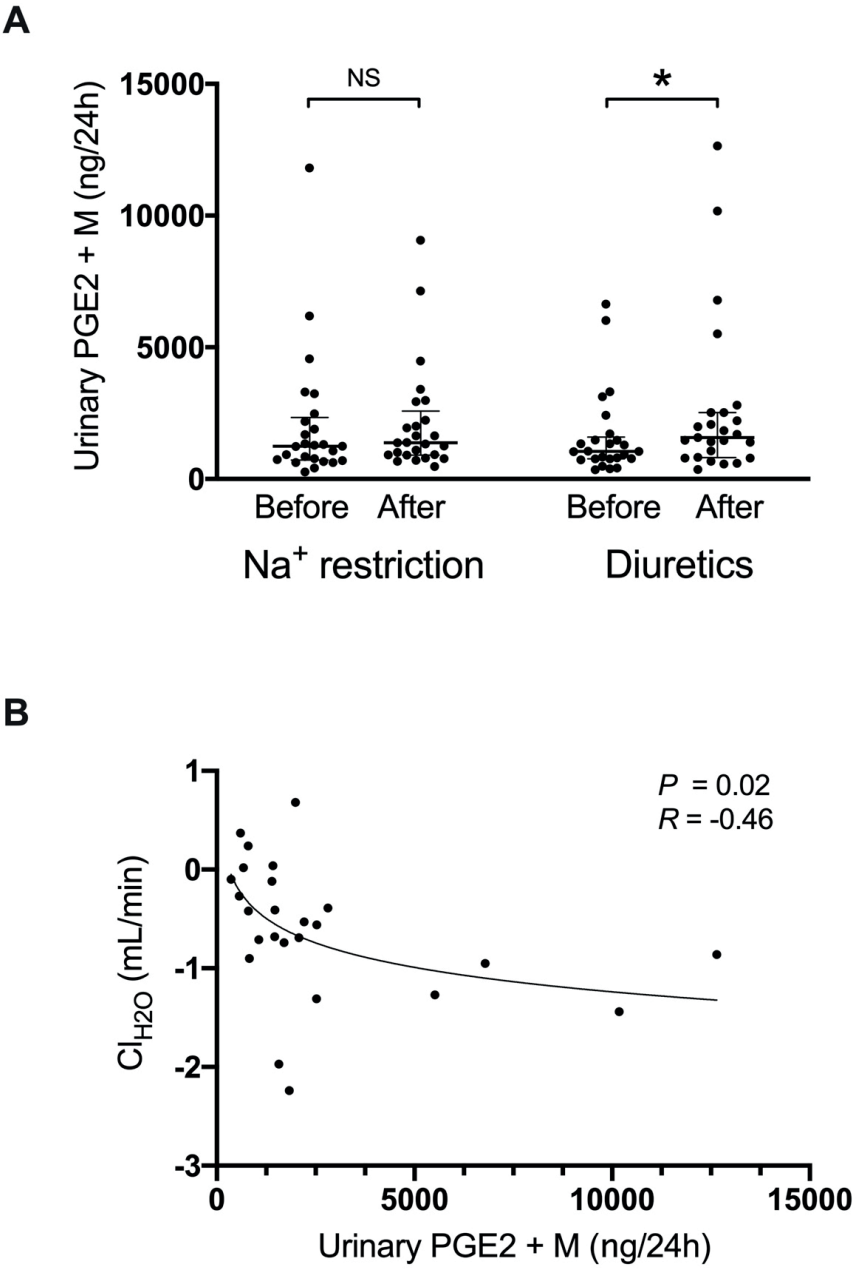

Figure 7. Effects of dietary sodium (Na^+) restriction and diuretics on the excretion of prostaglandin E2 and its metabolite (PGE2 + M, **panel A**) and correlation between urinary PGE2 + M excretion with free water clearance (Cl_{H2O}) in patients treated with diuretics (**panel B**). Cl_{H2O} was normally distributed, while PGE2 + M were not. Two-way repeated measures ANOVA and Pearson's correlation coefficient were used for analysis. One patient had 10- to 100-fold higher PGE2 values and this outlier was excluded from the analysis; we suspect that his urine was contaminated with semen, which contains high PGE2 levels.[45]

Effects on Urinary Renin, Angiotensinogen, and β2-microglobulin

CKD may activate the intra-renal renin-angiotensin system with urinary angiotensinogen and renin as potential markers for the activity of this system.[49, 50] Therefore, we analyzed whether our interventions changed these parameters. To account for changes in the tubular reabsorption of filtered proteins, we also measured β2-microglobulin. Of interest, dietary sodium restriction selectively increased urinary renin, whereas diuretics selectively decreased urinary angiotensinogen and β2-microglobulin (**Figure 8**). To account for the concurrent changes in plasma renin and eGFR, we also analyzed the change in the fractional excretions of renin. This analysis showed that diuretics selectively reduced the fractional excretion of renin.

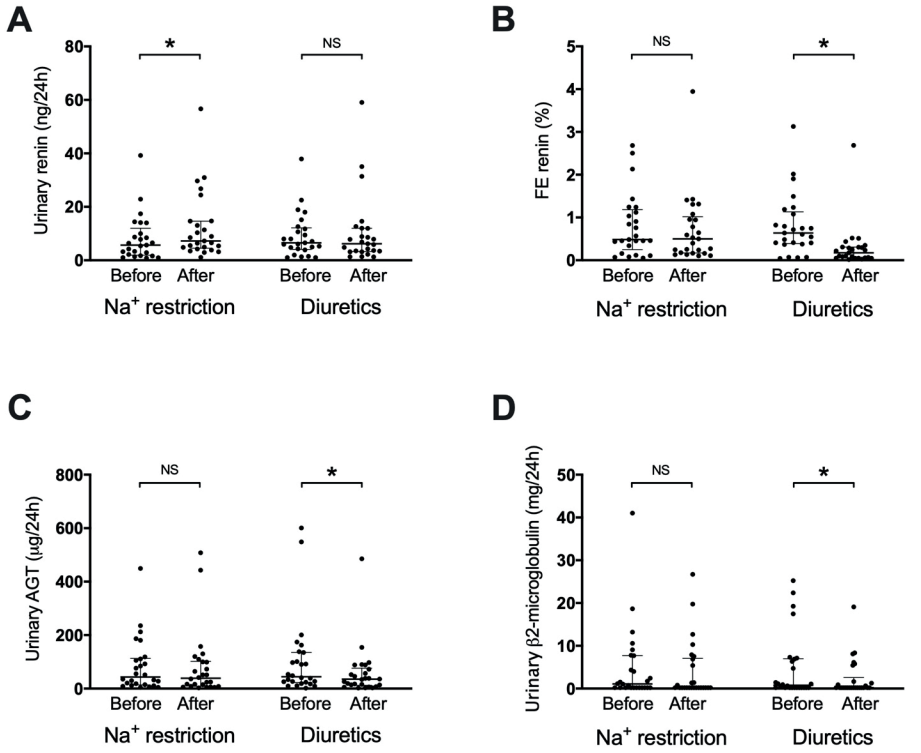

Figure 8. Effects of dietary sodium (Na^+) restriction or diuretics on urinary renin, angiotensinogen (AGT), and β2-microglobulin excretion. All data were normally distributed. Two-way repeated measures analysis was used for analysis. FE, fractional excretion.

DISCUSSION

This is the first study to investigate the effects of the distal diuretics hydrochlorothiazide and amiloride in patients with chronic kidney disease (CKD). The effects of distal diuretics on blood pressure and extracellular volume were analyzed in the absence of renin-angiotensin inhibition and compared with dietary sodium restriction as active comparator. We showed that distal diuretics are non-inferior to dietary sodium restriction in reducing blood pressure and extracellular volume. In fact, diuretics appear to exert a stronger antihypertensive effect than dietary sodium restriction, although the non-inferiority design of our study precludes a definitive conclusion. In addition, a longer treatment period than two weeks may be necessary to obtain the full response to dietary sodium restriction on blood pressure and total peripheral resistance.[8, 51]

The diuretic effects were preserved at lower eGFR despite a lower clearance of diuretics. Overall, both dietary sodium restriction and distal diuretics were well-tolerated, except for mild diuretic-induced hyponatremia in four patients.

Thiazide diuretics are often considered ineffective in CKD, especially with eGFR < 30 ml/min.[52] Several small case series and pilot studies have challenged this assumption by showing that thiazide diuretics can still lower blood pressure when added to other antihypertensive drugs.[17-19, 21, 53] A larger study by Cirillo *et al.* also showed that chlorthalidone effectively reduced blood pressure, but restricted inclusion to an eGFR between 30 and 60 ml/min/1.73m^2.[24] Our study confirms that thiazide diuretics in combination with amiloride are still effective in CKD across a wide eGFR-range. Bennett *et al.* did show that some residual kidney function is required, because thiazide diuretics had no effect in hemodialysis patients.[22] In an acute experiment, Reubi *et al.* showed that only at very low filtration rates (GFR < 15 ml/min) the saluretic effect of chlorothiazide was impaired.[11] It is difficult to directly compare the blood pressure response in our study to previous studies because we discontinued most other antihypertensive drugs. However, both interventions showed a clinically relevant blood pressure response.

A novel finding is that the antihypertensive effect of distal diuretics is maintained at lower eGFR. Diuretics are secreted by organic anion transporters (OAT) in the proximal tubule.[34] Renal clearance of diuretics is reduced in CKD, an observation that was also confirmed by our study. Several mechanisms can contribute to reduced diuretic clearance in CKD, including lower nephron number and competition for peritubular uptake through OATs.[10] One of the metabolites that can compete with diuretics for OAT is the uremic toxin and organic anion indoxyl sulfate.[54] We measured plasma indoxyl levels and indeed found a negative correlation with diuretic clearance. The observation that

the blood pressure response to diuretics was independent of eGFR may be explained by several mechanisms. First, at lower eGFR, the reduction in renal diuretic clearance may have been leveraged by increased diuretic sensitivity. Second, single-nephron diuretic concentrations may have been higher in patients with lower eGFR because of a lower nephron number. Third, non-renal mechanisms such as vasodilation may have contributed to the antihypertensive effects, although we did not measure vascular tone. The possibility that thiazide diuretics can cause vasodilation is supported by the demonstration of thiazide-induced vasodilation in patients with Gitelman syndrome, who lack functional NCC.[55] In one study, the vasodilatory effect of thiazide diuretics was observed only at high plasma concentrations.[56] This could imply that these vasodilatory mechanisms are more prominent in CKD, because it raises the plasma concentrations of diuretics. Whether amiloride can also cause vasodilation is less clear, although ENaC is expressed in endothelial cells and involved in vascular tone.[57] Endothelial ENaC can increase vascular stiffness and reduce nitric oxide.[58] Therefore, it would be interesting to analyze whether the diuretics increased the nitric oxide indices.

Both interventions decreased eGFR, and this has been a consistent finding in previous studies.[59, 60] Although this may be interpreted as progression of CKD, the effect on eGFR was reversible and is therefore most likely a hemodynamic effect. This is supported by the observed neurohumoral activation, and restoration of eGFR after follow-up. Bank *et al.* also observed an initial decrease in eGFR with thiazide diuretics in CKD, but showed that eGFR subsequently remained constant or rose toward pretreatment levels.[17] Cakalaroski *et al.* showed that long-term treatment with a thiazide diuretic (3 months) did not change eGFR in patients with CKD.[61] Longer-term studies powered for hard endpoints would be required to analyze if thiazide-induced reduction in blood pressure and extracellular volume offset the decrease in eGFR. The initial diuretic-induced eGFR decrease is reminiscent of the effects of ACE- and SGLT2-inhibitors.[62, 63] Because these drugs are renoprotective, this raises the possibility that thiazides may also help preserve eGFR in CKD. Several experimental and clinical studies suggest a possible renoprotective effect of thiazide diuretics in CKD, especially in combination with renin-angiotensin inhibition.[64-70] Clinical trials, however, are lacking.

In contrast to previous studies, we did not observe a significant decrease in albuminuria by distal diuretics or sodium restriction.[59, 60] However, previous studies usually combined dietary sodium restriction or diuretics with an inhibitor of the renin-angiotensin system.[8, 71-73]

Diuretics did decrease the excretion of angiotensinogen and β2-microglobulin. Because both proteins are reabsorbed in the proximal tubule, this suggests that diuretics increase

proximal tubular reabsorption. This would also be in agreement with the hypocalciuric effect of diuretics.[74] Of note, urinary renin increased after dietary sodium restriction. Some investigators consider urinary angiotensinogen and renin markers for the intrarenal renin-angiotensin system and postulate local production by the kidney. However, we previously showed that an increase in urinary renin reflects increased glomerular filtration or reduced reabsorption rather than conversion of prorenin to renin in the tubular fluid.[75] The data in this study also support this conclusion, because dietary sodium restriction did not change the fractional excretion of renin, while this was reduced by thiazide diuretics. Thus, we propose that the changes in urinary angiotensinogen and renin represent changes in renal tubular handling rather than changes in the activity of the intrarenal renin-angiotensin system.

Both interventions were generally well-tolerated, but diuretics did cause mild hyponatremia in four patients. Hyponatremia is a well-characterized side-effect of thiazide diuretics.[76] Ware *et al.* recently linked thiazide-induced hyponatremia to increased production of PGE2.[48] We also found a negative correlation between urinary PGE2 and free water clearance. A final observation is that patients who first received the diuretics had a significantly lower blood pressure and plasma potassium after the 2-week wash-out than the patients who first received dietary sodium restriction. This suggests that the effects of distal diuretics temporarily persist after their discontinuation. This “legacy” effect may be related to distal tubule remodeling that was described in mice lacking NCC phosphorylation.[77]

Our study has a number of limitations. First, we used a combination treatment, and therefore it is not clear if both diuretics equally contributed to the observed effects. Second, we excluded patients with CKD stage G5, and therefore we were not able to study the possibility that distal diuretics become ineffective at a certain level of eGFR. A sensitivity analysis did show that patients with an eGFR < 30 ml/min/1.73m^2 had a similar blood pressure response that occurred independent of eGFR. Finally, we did not specifically select patients on salt sensitivity or an expanded extracellular volume. However, sodium retention is a generally accepted hallmark of CKD.[1, 3, 4]

In conclusion, distal diuretics are at least as effective as dietary sodium restriction in reducing blood pressure and extracellular volume in CKD. These effects were preserved at lower eGFR despite a lower clearance of diuretics. Longer-term studies should determine which sodium-reducing strategy – or combinations thereof – optimally prevents complications from sodium retention in CKD.

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Urinary aldosterone measurements were performed using a commercial assay (Demeditec, Germany). The detection limit and mean coefficient of variation of the within-run and between-run variability of this assay are 1.44 pg/mL, 3.3 % and 8.4 %, respectively. Moreover, the antibody used in the immunoassay is highly specific for aldosterone. Extremely low cross-reactivities were obtained against other naturally occurring steroids (cortisone, corticosterone, DHEAs, etc.). Urinary renin and angiotensinogen measurements were performed using an in-house developed enzyme-kinetic assay. The detection limit and mean coefficient of variation of the within-run and between-run variability of this assay are 2 pg/mL, 2.9% and 12.6% for renin measurements and 0.12 ng/mL, 4% and 10% for angiotensinogen measurements. Please note that in every enzyme-kinetic assay, we measure a set of standard samples allowing us to determine the within- and between-run variability. Furthermore, we always include buffer samples to correct for background noise. As an example, when measuring renin in the presence of excess angiotensinogen, the same amount of excess angiotensinogen is incubated with buffer, and the generated amount of angiotensin I is subtracted from the amount of angiotensin I generated in the actual sample. This needs to be taken into account, particularly when measuring samples with low renin levels like urine.[78] In previous studies we have added fixed amounts of (pro)renin and angiotensinogen to urine samples, and found recovery to be >95%.[79, 80] This is not surprising, since the levels of degrading enzymes in urine are negligible. Finally, regarding cross-reactivity, it is important to note that the assays rely on the detection of angiotensin I making use of an angiotensin I-directed antibody. Cross-reactivity of this antibody with other angiotensin metabolites is <0.1%.[81] In this regard, the correction for background noise as explained above seems to be of greater importance, since the assay is performed in the presence of angiotensinase inhibitors, not allowing the generation of metabolites other than angiotensin I.

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Table S1. Consolidated Standards of Reporting Trials (CONSORT) 2010 checklist of information.

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomized trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4-5
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6-7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	6-7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7-8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	8
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomization:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomization; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	18
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	NA
	11b	If relevant, description of the similarity of interventions	4
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	8-9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	8-9

Salt

Table S1. Consolidated Standards of Reporting Trials (CONSORT) 2010 checklist of information. (*continued*)

Section/Topic	Item No	Checklist item	Reported on page No
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analyzed for the primary outcome	10
	13b	For each group, losses and exclusions after randomization, together with reasons	10
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	19
Numbers analyzed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	8 & 10
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	10-12
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	-
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	10-12
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	20
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	16
Generalizability	21	Generalizability (external validity, applicability) of the trial findings	13-17
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	13-17
Other information			
Registration	23	Registration number and name of trial registry	6
Protocol	24	Where the full trial protocol can be accessed, if available	NA
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	18

Table S2. Effect of sodium restriction and diuretics on day and night systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP).

	Na^+ restriction	*P* for treatment effect	Distal diuretics	*P* for treatment effect	*P* for interaction
Day SBP (mmHg)	−6 ± 2	*P* < 0.01	−14 ± 2	*P* < 0.01	*P* < 0.01
Day DBP (mmHg)	−2 ± 1	*P* = 0.2	−5 ± 1	*P* < 0.01	*P* = 0.09
Day MAP (mmHg)	−4 ± 1	*P* < 0.05	−8 ± 1	*P* < 0.01	*P* < 0.05
Night SBP (mmHg)	−3 ± 2	*P* = 0.4	−14 ± 2	*P* < 0.01	*P* < 0.01
Night DBP (mmHg)	0 ± 2	*P* = 1	−5 ± 2	*P* < 0.01	*P* < 0.05
Night MAP (mmHg)	−1 ± 2	*P* = 0.8	−8 ± 2	*P* < 0.01	*P* < 0.01
SBP dipping (%)	+2.1 ± 3.4	*P* = 1	+1.4 ± 3.4	*P* = 1	*P* = 1

Treatment effect and treatment interaction were analyzed by two-way repeated measures ANOVA that included treatment order as between-subject factor. DBP, diastolic blood pressure; MAP, mean arterial pressure; Na^+, sodium; SBP, systolic blood pressure.

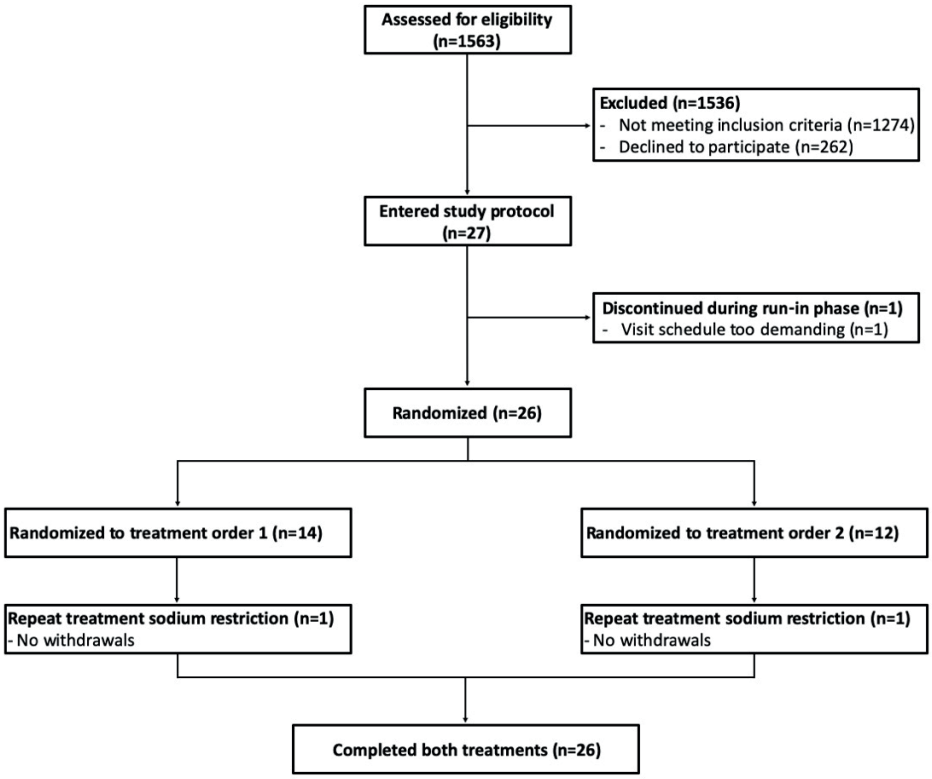

Figure S1. Consolidated Standards of Reporting Trials (CONSORT) diagram.
Treatment order 1: first treatment period diuretics, second treatment period dietary sodium restriction. Treatment order 2: first treatment period sodium restriction, second treatment period diuretics.

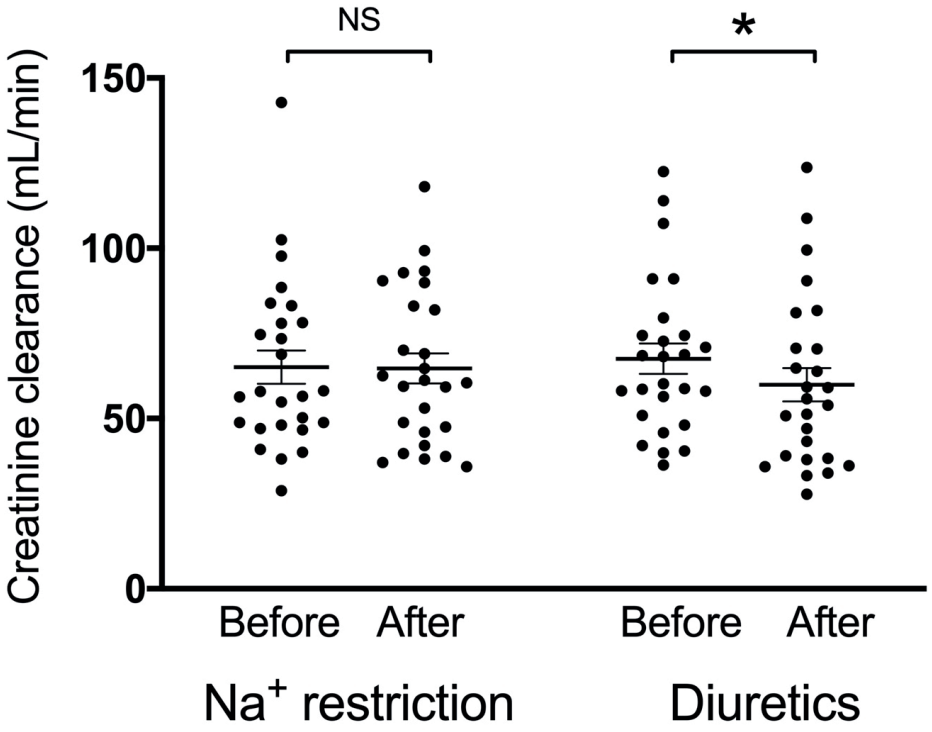

Figure S2. Effect of sodium (Na^+) restriction and diuretics on creatinine clearance.
Data are normally distributed and two-way repeated measures ANOVA was used for analysis. * $P < 0.05$ for difference before versus after treatment.

Chapter 7

Urinary renin-angiotensin markers in polycystic kidney disease

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ABSTRACT

In autosomal dominant polycystic kidney disease (ADPKD) activation of the renin-angiotensin aldosterone system (RAAS) may contribute to hypertension and disease progression. Although previous studies focused on circulating RAAS-components, preliminary evidence suggests APDKD may increase urinary RAAS-components. Therefore, our aim was to analyze circulating and urinary RAAS-components in ADPKD. We cross-sectionally compared 60 patients with ADPKD to 57 patients with non-ADPKD chronic kidney disease (CKD). The two groups were matched by gender, estimated glomerular filtration rate (eGFR), blood pressure, and RAAS-inhibitor use. Despite similar plasma levels of angiotensinogen and renin, urinary angiotensinogen and renin excretion were 5- to 6-fold higher in ADPKD (P<0.001). These differences persisted when adjusting for group differences, and were present regardless of RAAS-inhibitor use. In multivariable analyses, ADPKD, albuminuria, and the respective plasma concentrations were independent predictors for urinary angiotensinogen and renin excretion. In ADPKD, both plasma and urinary renin correlated negatively with eGFR. Total kidney volume correlated with plasma renin and albuminuria, but not with urinary renin or angiotensinogen excretions. Albuminuria correlated positively with urinary angiotensinogen and renin excretions in ADPKD and CKD. In three ADPKD patients who underwent nephrectomy, the concentrations of albumin and angiotensinogen were highest in plasma followed by cyst fluid and urine; urinary renin concentrations were higher than cyst fluid. In conclusion, this study shows that, despite similar circulating RAAS-component levels, higher urinary excretions of angiotensinogen and renin are a unique feature of ADPKD. Future studies should address the underlying mechanism and whether this may contribute to hypertension or disease progression in ADPKD.

INTRODUCTION

Hypertension develops early in autosomal dominant polycystic kidney disease (ADPKD), usually occurring before a reduction in glomerular filtration rate (GFR) with an average age of onset of 30 years [1, 2]. Increased activity of the renin-angiotensin-aldosterone system (RAAS) has been implicated in the pathogenesis of hypertension in ADPKD. One hypothesis is that cyst expansion results in areas of local renal ischemia which increases renin release [2, 3]. In addition, renin has also been suggested to be produced by the epithelial cells lining the cysts and active renin can be found within the cyst fluid [4, 5]. However, measurement of plasma renin and aldosterone in patients with ADPKD yielded equivocal results (**Table 1**). Several studies found that plasma renin and aldosterone concentrations were not higher in hypertensive ADPKD patients when compared to controls, even during specific interventions (low or high sodium diet, ACE-inhibition, angiotensin II infusion) [6-11]. Different control groups were used for these studies, including normotensive ADPKD patients, normotensive siblings without ADPKD, patients with essential hypertension, or healthy volunteers (**Table 1**). The observation that plasma renin activity was not consistently higher in hypertensive ADPKD patients is notable, because this is contrary to what would be expected if cysts caused local renal ischemia [2, 3]. Therefore, to further address the role of the RAAS in ADPKD, it may be informative to analyze RAAS-components in urine, as urinary angiotensinogen and renin have previously been used as markers of the intra-renal renin-angiotensin system [12]. Emerging data suggest that filtered or locally produced RAAS-components may activate this intra-renal renin-angiotensin system and thereby contribute to hypertension [13]. Two recent studies reported higher urinary angiotensinogen concentrations in hypertensive ADPKD patients [14, 15]. However, despite its postulated central role in the pathogenesis of hypertension, urinary renin has never been measured in patients with ADPKD. We recently showed that it is important to measure urinary renin with standardized assays, because commercial assays may produce ≥ 10-fold higher results [16]. Therefore, here, we measured urinary renin in patients with ADPKD using a validated renin immunoradiometric assay and an in-house enzyme kinetic assay. In addition, we also measured multiple other RAAS-components in plasma and urine, including plasma renin and aldosterone, and urine angiotensinogen, prorenin, and aldosterone. As a comparator, and for the first time, we used matched patients with non-ADPKD chronic kidney disease (CKD) to address whether the type of kidney injury affects the RAAS differently.

Salt

Table 1. Comparison of studies measuring RAAS-components in patients with ADPKD.

Study	Cases	CKD stage	Controls	Numbers	Measurement(s)	Difference
Valvo [10]	ADPKD + HT	1-3	ADPKD + NT	20 vs. 12	PRA	↔
Bell [3]	ADPKD + HT	1-2	ADPKD + NT	9 vs. 7	PRA during low/high Na^+ diet + ACEi	↔ but ↑ during ACEi + high Na^+ diet
Chapman [2]	ADPKD	1	Essential HT + healthy controls	14 + 11 vs. 9 + 13	PRA and aldo during ACEi	PRA and aldo ↑ in ADPKD + HT
Harrap [17]	ADPKD	1	Siblings	19 vs. 20	PRA and aldo	PRA and aldo ↑
Watson [11]	ADPKD	1-2	Siblings	13 vs. 10	PRA	↔
Barrett [6]	ADPKD	1-2	Siblings	21 vs. 12	PRA and aldo during low/high Na^+ diet, ACEi, Ang II infusion	↔
Martinez-Vea [8]	ADPKD + HT	2-3	Essential HT	20 vs. 20	PRA, aldosterone, ANP, Ang II	↔
Ramunni [9]	ADPKD + HT	1-2	ADPKD + NT	17 vs. 17	PRA	↔
Doulton [7]	ADPKD + HT	1-2	Essential HT	11 vs. 8	PRA during low/high Na^+ diet and ACEi	↔
Kurultak [18]	ADPKD	1	Healthy controls	20 vs. 20	Urinary AGT	↔
Kocyigit [14]	ADPKD + HT	1-2	ADPKD + NT, healthy controls	43 vs. 41 + 40	Plasma and urinary AGT	Urinary AGT ↑
Park [15]	ADPKD	1-5	None	186	Plasma renin+ aldo, urinary AGT	Correlation urinary AGT with eGFR, TKV, BP
Present study	ADPKD	3	CKD	69 vs. 58	Plasma + urinary AGT, renin, aldo	Urinary AGT + renin ↑

ADPKD, autosomal dominant polycystic kidney disease; ACEi, angiotensin converting enzyme inhibitor; AGT, angiotensinogen; aldo, aldosterone; Ang II, angiotensin II; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HT, hypertension; NT, normotension; PRA, plasma renin activity; BP, blood pressure.

MATERIALS AND METHODS

Patients

Patients with ADPKD were recruited from one of the centers (Erasmus Medical Center, Rotterdam, The Netherlands) participating in a national ADPKD consortium (DIPAK study, with inclusion criteria CKD stage 3 at entry into the study and age ≤ 60 years) [19]. Patients were matched to non-ADPKD CKD patients (referred to hereafter as 'CKD') from the PREVEND cohort (University Medical Center Groningen) [20]. The Medical Ethics Committees of the Erasmus Medical Center and University Medical Center Groningen approved the studies (MEC-2012-313 and METC-90/01/022). Patients with ADPKD and CKD were individually matched for gender, eGFR (using the Modification of Diet in Renal Dis-

ease equation [21]), use of RAAS-inhibitors (defined as the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers) and blood pressure (difference in systolic blood pressure ≤ 5 mmHg). Patients with a history of diabetes mellitus, or those using insulin or oral glucose lowering drugs were excluded, because diabetes mellitus may activate the intrarenal renin-angiotensin system [22]. From three ADPKD patients who underwent elective nephrectomy (not part of the DIPAK cohort), we collected plasma, cyst fluid, and urine samples. For this part of the study, a separate approval from the Medical Ethics Committee of the University Medical Center Groningen was obtained (METC 2014.396).

Data Collection

Detailed description of data collection for both the DIPAK and PREVEND studies has been described elsewhere [19, 20]. Briefly, participants of both studies collected 24-hour urine and visited the outpatient clinic for blood sampling and blood pressure measurements using an automatic oscillometric device. Hypertension was defined as a blood pressure > 140/90 mmHg or the use of anti-hypertensive medication. Participants were instructed to store urine at 4°C during collection. Upon arrival in the university medical center, blood and urine samples were immediately stored at -80°C until further use. We have previously shown that in 24-hour urine, prorenin is not converted into renin prior to freezing [22]. To determine adequate 24-hour urine collection, we calculated the expected 99% quantile of creatinine excretion based on previously defined criteria [23]. Patients who exceeded the expected range were excluded (8 patients with ADPKD and 1 patient with CKD). ADPKD patients underwent magnetic resonance imaging to determine total kidney volume [19]. The World Health Organization defined daily dose (DDD) was used to calculate daily RAAS-inhibitor use.

Measurements

Plasma and urine samples from the DIPAK and PREVEND cohorts were measured simultaneously. Renin in plasma was measured with a commercially available immunoradiometric kit (Renin III; Cisbio, Gif-sur-Yvette, France), making use of an active site-directed radiolabeled antibody [24]. Total plasma renin was determined simultaneously using the same kit after the induction of a conformational change in the prorenin molecule with aliskiren (10 µmol/l for 48 hours at 4°C), which enabled its recognition by the active site-directed radiolabeled antibodies applied in the Cisbio kit [25]. The detection limit of this assay is 1 pg/ml with intra- and inter-assay coefficients of variation (CVs) of 2.4% and 7.2%. Urinary renin and urinary total renin (after prorenin activation with trypsin) were measured with an in-house enzyme kinetic assay (EKA) [26]. This measurement involves the incubation of the urine sample with excess sheep angiotensinogen and angiotensinase inhibitors and the subsequent detection of the generated Ang I by radioimmunoassay.

The detection limit of the EKA is 0.05 ng Ang I/ml per hour with intra- and inter-assay CVs of 2.9 and 12.6%. Ang I–generating activities were converted to renin concentrations based on the fact that 1 ng Ang I/ml per hour corresponds with 2.6 pg human renin/ml [26]. Prorenin was determined by subtraction of renin from total renin. Angiotensinogen in plasma and urine was measured as the maximum quantity of Ang I that was generated during incubation with excess recombinant renin [27]. The detection limit of this assay is 0.50 pmol/ml with intra- and inter-assay CVs of 4 and 10%. Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, USA), with a detection limit of 25 pg/ml with intra- and inter-assay CVs of 3.3 and 8.4% [28].

Statistical Analyses

Results are expressed as mean and standard deviation or median and range, as appropriate. Data were logarithmically transformed before analysis in case of non-normal distribution. Levels that were below the detection limit were considered to be half the detection limit to allow for statistical analysis [29]. Analysis of variance (ANOVA) was used for group comparison (using log-transformed data as appropriate). Further analysis was performed using analysis of covariance (ANCOVA) to adjust for covariates. To analyze which parameters independently predicted urinary angiotensinogen or renin excretion, we performed multivariable linear regression. Finally, the Pearson correlation coefficient was analyzed for selected variables. A P-value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS (version 21, IBM).

RESULTS

ADPKD Increases Urinary Angiotensinogen and Renin Excretion

Table 2 shows the baseline characteristics and RAAS-component measurements for the ADPKD and CKD groups. Patients with ADPKD were younger (47 vs. 68 years), taller (175 vs. 169 cm), and used more RAAS-inhibitors. While plasma levels of angiotensinogen, renin, and aldosterone were similar between the two groups, 24-hour urine volume, and urinary albumin, angiotensinogen, renin, and aldosterone excretions were significantly higher in patients with ADPKD (P < 0.05 for all). Similarly, when expressed as ratio with creatinine, urinary angiotensinogen and renin were also significantly higher in ADPKD (urinary angiotensinogen 14.6 vs. 3.3 pmol/mol creatinine, urinary renin 204 vs. 44 pg/mol creatinine, p < 0.01 for both). Because of the group differences in age, height, DDD, and albuminuria, we also performed a second analysis adjusting for these factors (**Table 2**). This analysis showed that urinary angiotensinogen and renin excretion were still significantly higher in ADPKD than CKD (P < 0.001 for both). In addition, a subanalysis

was performed in patients (n = 17 vs. 11) with similar age, gender, height and a similar degree albuminuria, which also showed that urinary angiotensinogen (286.2 vs. 38.7 pmol/day) and renin (1874 vs. 398.6 pg/day) excretions were significantly higher in ADPKD compared to CKD ($P < 0.05$ for both).

Table 2. Patient characteristics and renin-angiotensin-aldosterone system measurements.

Category	Parameter	ADPKD (n = 60)	CKD (n = 57)	P-value*	P-value**
Clinical data	Age, years	47 ± 8	68 ± 8	< 0.001	
	Male gender, n (%)	25 (42)	25 (44)	N.T.¶	
	Height, cm	175 ± 10	169 ± 9	0.001	
	Weight, kg	81.6 ± 17.0	81.2 ± 11.9	0.9	
	Hypertension, n (%)†	56 (93)	49 (86)	N.T.	
	SBP, mmHg	131 ± 14	134 ± 14	N.T.	
	DBP, mmHg	79 ± 9	76 ± 7	N.T.	
	RAAS-inhibitors, n (%)	50 (83)	44 (77)	N.T.	
	DDD RAAS-inhibitors, n	1.9 ± 1.5	1.1 ± 0.9	< 0.001	
Plasma	Creatinine, mg/dL	1.5 ± 0.4	1.4 ± 0.4	0.6	
	eGFR, ml/min per 1.73 m^2	48 ± 11	46 ± 9	N.T.	
	Angiotensinogen, pmol/mL	1599 (313–8067)	1455 (474–3567)	0.3	
	Renin, pg/mL	81.3 (9.6–950.0)	68.3 (14.8–464.5)	0.3	
	Aldosterone, pg/mL	121.7 (16.5–470.1)	105.9 (16.6–546.4)	0.2	
Urine	Volume, mL/day	2233 (800–6500)	1652 (530–3140)	< 0.001	0.2
	Creatinine, mmol/day	13.3 (5.2–21.2)	10.9 (6.0–18.2)	< 0.001	0.8
	Albumin, mg/day	40.0 (3.1–266.4)	26.7 (3.4–293.2)	0.05	-
	Sodium, mmol/day	150 (40 – 354)	142 (60 – 371)	0.5	
	Angiotensinogen, pmol/day	194.4 (3.5–3384.0)	36.0 (2.3–1070)	< 0.001	< 0.001
	Renin, pg/day	2717 (375.7–69248.0)	485.5 (154.7–2293.0)	< 0.001	< 0.001
	Aldosterone, µg/day	4.6 (0.9–32.8)	3.5 (1.0–18.0)	0.02	0.2

ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; DBP, diastolic blood pressure; DDD, defined daily dose; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

Footnotes:

* Using analysis of variance (ANOVA) with log-transformed data as appropriate

** Using analysis of covariance (ANCOVA) with log-transformed data as appropriate and adjustments for age, height, defined daily dose, and albuminuria

¶ N.T., not tested (matching criteria). † Defined by use of antihypertensive drugs.

Effects of RAAS-Inhibitors

Because the use of RAAS-inhibitors increases plasma renin, this may also increase urinary renin. Therefore, we also report the plasma and urinary RAAS-components in patients with and without RAAS-inhibitor use (**Figure 1**). Plasma renin was indeed significantly higher in both ADPKD and CKD patients using RAAS-inhibitors. In patients *without* RAAS-inhibitors,

plasma renin was significantly *lower* in ADPKD than in CKD. Despite these differences in plasma renin, urinary renin excretion was consistently higher in the patients with ADPKD than in the patients with CKD regardless of RAAS-inhibitor use (**Figure 1**). Urinary angiotensinogen excretion was significantly higher only in patients with ADPKD and RAAS-inhibitor use, but this may be a power issue, as few patients were without RAAS-inhibitors.

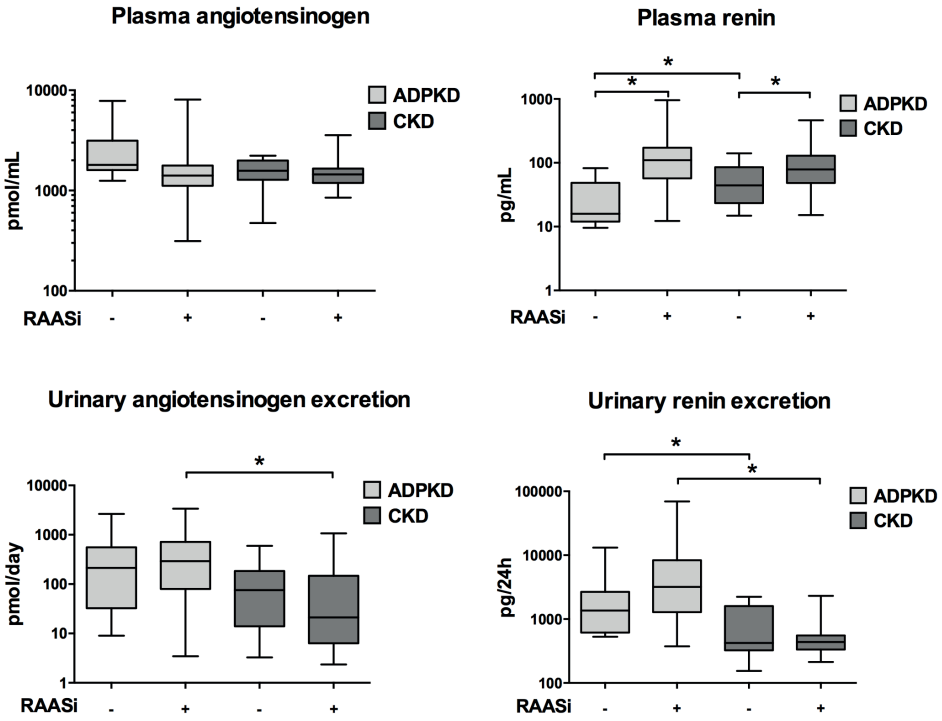

Figure 1. Plasma concentrations and urinary excretions of angiotensinogen and renin in patients with ADPKD or CKD and with or without RAAS-inhibitors. Box-and-whisker plots of plasma concentrations and urinary excretions of renin and angiotensinogen for ADPKD (light grey) and CKD (dark grey). Groups were subdivided into those with (+) and without (-) use of RAAS-inhibitors (RAASi). Of the 60 patients with ADPKD, 10 patients did not use RAASi; of the 57 CKD patients, 13 patients did not use RAASi. Boxes show the median, interquartile range and range. ANOVA was used for comparison with * $P < 0.05$.

Predictors of Urinary Angiotensinogen and Renin Excretion

Two multivariable linear regression analyses were performed to analyze which factors independently predict urinary angiotensinogen or urinary renin excretion (**Table 3**). In the model we included the presence of ADPKD, eGFR, age, DDD, plasma concentrations of angiotensinogen and renin, and urinary sodium and albumin excretion. For urinary angiotensinogen excretion, ADPKD, eGFR, and albuminuria were identified as independent predictors. For urinary renin excretion, ADPKD, plasma renin, and albuminuria were identified as independent predictors. When the analyses were restricted to patients with

ADPKD, only albuminuria predicted urinary angiotensinogen excretion, and only plasma renin predicted urinary renin excretion (data not shown).

Table 3. Multivariable analysis of factors predicting urinary angiotensinogen and renin excretion.

Variable	Urinary angiotensinogen excretion		Urinary renin excretion	
	β	*P*-value	β	*P*-value
Presence of ADPKD	9.6 (3.7 – 24.5)	< 0.001	4.9 (2.6 – 9.0)	< 0.001
eGFR, ml/min/1.73 m^2	0.96 (0.94 – 0.99)	0.002	0.99 (0.98 – 1.01)	0.3
Age, years	1.0 (0.9 – 1.1)	0.2	0.99 (0.97 – 1.02)	0.6
DDD RAAS-inhibitors, n	0.8 (0.7 – 1.0)	0.1	0.9 (0.8 – 1.1)	0.2
Plasma renin, pg/mL	0.5 (0.3 – 1.0)	0.05	1.9 (1.2 – 2.9)	0.007
Plasma AGT, pg/mL	3.0 (0.9 – 10.7)	0.08	1.1 (0.5 – 2.4)	0.9
Urinary sodium, mmol/day	1.6 (0.4 – 7.1)	0.5	1.0 (0.4 – 2.8)	0.9
Albuminuria, mg/day	6.2 (3.6 – 10.7)	< 0.001	1.6 (1.1 – 2.3)	0.01

ADPKD, autosomal dominant polycystic kidney disease; AGT, angiotensinogen; DDD, defined daily dose; eGFR, estimated glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system.

Correlations with Total Kidney Volume and Kidney Function

Within the ADPKD group, we analyzed whether circulating or urinary RAAS-components correlated with total kidney volume and kidney function (eGFR). A higher total kidney volume correlated with higher plasma renin, and more albuminuria, but not with urinary angiotensinogen or renin excretion (**Figure 2A**). Both higher plasma renin and higher urinary renin excretion correlated with lower eGFR (**Figure 2B**). To analyze the possible mechanism of urinary angiotensinogen and renin excretion, we analyzed in the ADPKD and CKD groups whether these two urinary RAAS-components correlated with albuminuria. Indeed, both in patients with ADPKD and CKD, a higher degree of albuminuria correlated with higher urinary angiotensinogen or renin excretion, although the strength of this correlation was modest (**Figure 2C**). For urinary renin excretion, this correlation was of borderline significance in patients with ADPKD ($P = 0.06$).

Comparison of Concentrations in Plasma, Cyst Fluid, and Urine

In three patients with ADPKD who underwent nephrectomy, we measured albumin, angiotensinogen, prorenin, and renin in plasma, cyst fluid (average concentration of five cysts) and urine. For all four parameters, the concentrations were highest in plasma followed by cyst fluid and urine (**Figure 3**). Of interest, urinary concentrations were lower than cyst concentrations except for renin. Urinary prorenin concentrations were close to or below the detection limit.

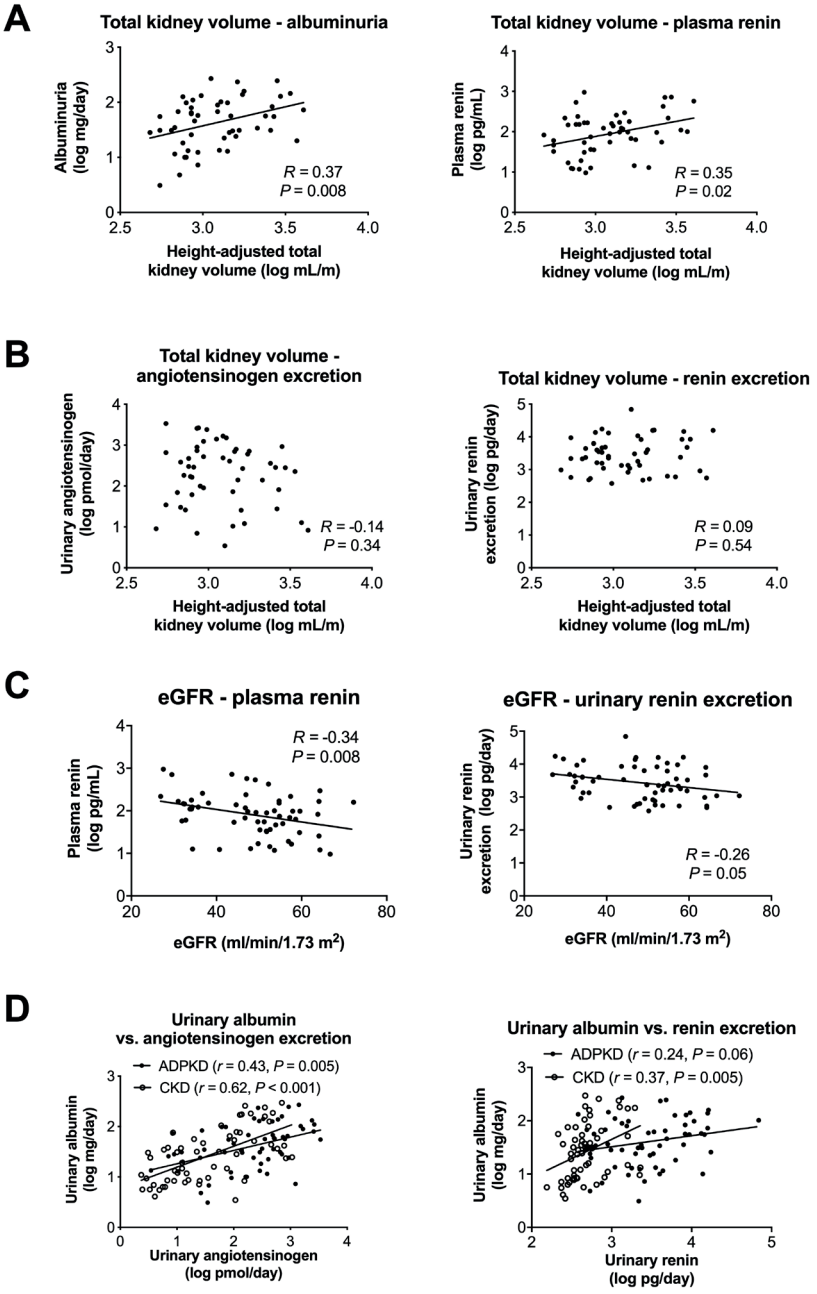

Figure 2. Correlations of RAAS-components with total kidney volume and kidney function. Pearson correlation coefficients were calculated using log-transformed data. Height-adjusted total kidney volume was available in 51 patients with ADPKD. Correlations between urinary albumin, angiotensinogen, and renin excretion are shown both for patients with ADPKD and CKD.

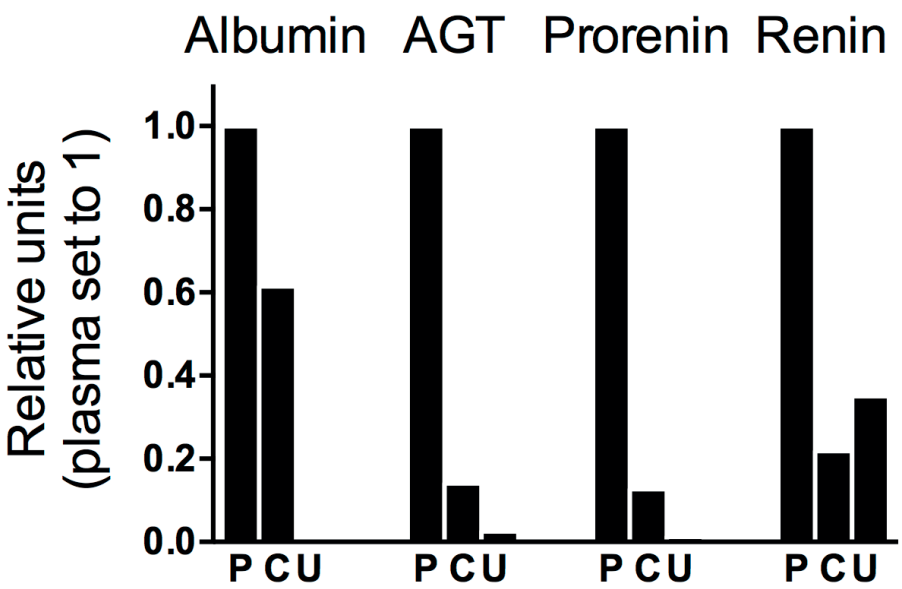

Figure 3. Concentrations of Albumin and RAAS-components in plasma, cyst fluid, and urine. With plasma values set to 1, this figure shows the relative mean concentrations of albumin, angiotensinogen (AGT), prorenin, and renin in plasma (P), cyst fluid (C) and urine (U). The actual mean plasma concentrations were 42 g/L, 1814 pmol/L, 573.8 pg/mL, and 81.3 pg/mL, respectively. Measurements were performed in three ADPKD patients, who underwent elective nephrectomy to create space for kidney transplantation (45 and 52 year-old males with eGFRs of 12 and 9 mL/min/1.73 m^2, respectively) or because of mechanical discomfort (71-year-old female, eGFR 18 mL/min/1.73 m^2).

DISCUSSION

This study reveals a unique feature of patients with ADPKD, namely a consistently higher urinary excretion of angiotensinogen and renin compared to patients with CKD. Urinary angiotensinogen and renin excretions were 5- to 6-fold higher in patients with ADPKD than in patients with CKD, who were matched by eGFR, blood pressure, and RAAS-inhibitor use (**Table 2**). ADPKD remained a significant predictor for urinary angiotensinogen and renin excretion in adjusted and in multivariable analyses (**Tables 2** and **3**), and regardless of RAAS-inhibitor use (**Figure 1**). Recent studies found higher urinary angiotensinogen to creatinine ratios in normotensive ADPKD patients compared to healthy controls [18], or higher levels within ADPKD patients in the presence of hypertension [14] or reduced kidney function [15]. The magnitude of the urinary angiotensinogen levels reported in these previous studies are comparable to our data. Our study is the first to analyze urinary renin and to use patients with CKD as control group (**Table 1**). Although our cross-sectional study cannot give definitive answers, our data give direc-

tions on the possible mechanisms and potential clinical implications of the increased urinary excretions.

In principle, urinary angiotensinogen and renin excretion can increase in ADPKD because of (1) damage to the glomerular filtration barrier, (2) reduced proximal tubular reabsorption, (3) enhanced tubular secretion by intact nephrons, (4) differences in degradation, or (5) ectopic production by cyst-lining epithelial cells.

When evaluating the first two possibilities, it is important to correct for differences in the plasma levels. Although the correlation between total kidney volume and plasma renin in ADPKD patients indeed suggests that renal ischemia by cysts can increase plasma renin (**Figure 2A**), patients with ADPKD in general do not have higher plasma renin concentrations than patients with CKD (**Table 2**). In fact, patients with ADPKD *without* RAAS-inhibitors had significantly *lower* plasma renin concentrations than patients with CKD (**Figure 1**). Thus, the higher urinary excretions of angiotensinogen and renin in ADPKD are not simply the consequence of elevated plasma RAAS concentrations exposed to the same degree of filtration and reabsorption as in CKD patients. In addition, increase of cysts (i.e. total kidney volume) did not correlate with increased excretion of urinary angiotensinogen or renin. Next, it is important to emphasize that ADPKD is primarily a tubular disorder that is less likely to damage the glomerular filtration barrier [30]. Indeed, previous studies have attributed albuminuria in animal models of ADPKD to disturbed endocytosis of albumin in the proximal tubule [30, 31]. In these studies, immunohistochemistry showed less expression of the chloride channel ClC-5 and megalin, which are both involved in the reabsorption of low-molecular weight proteins. Because albumin, angiotensinogen, and renin are all reabsorbed by a megalin-dependent pathway, a proximal tubular disorder should by definition result in higher urinary angiotensinogen and renin excretion, even in the face of identical or lower plasma RAAS-component levels [32, 33]. In agreement with this concept, we recently showed that patients with Dent's disease (who lack ClC-5) displayed a 20-40-fold rise in urinary angiotensinogen and renin levels, although their plasma RAAS levels were in the normal range [33]. Similarly, other urinary markers of proximal tubule damage, such as fetuin-A and β2-microglobulin, are increased in ADPKD [34, 35]. The ADPKD component that independently predicted urinary angiotensinogen and renin excretion in our multivariable regression analysis therefore possibly reflects a difference in tubular reabsorption. We showed that albuminuria correlated with total kidney volume (**Figure 2A**), as was shown previously [36]. In addition to ADPKD, albuminuria also independently predicted urinary renin and angiotensinogen excretion. This also suggests that the urinary excretions of albumin, angiotensinogen, and renin was at least in part due to similar mechanisms, and argues against selective tubular secretion of RAAS-components. This leaves the issue of altered degradation.

Reduced reabsorption would also be expected to result in elevated levels of RAAS-component degrading enzymes, leading to enhanced degradation. Yet, higher levels of urinary RAAS-components were found in ADPKD. Combined with data from previous studies showing no evidence for urinary degradation of renin or prorenin [22], and identifying all urinary angiotensinogen as intact (and not cleaved) [37], it appears that reduced degradation does not underlie the increased urinary excretion of angiotensinogen and renin in ADPKD.

The possibility of ectopic RAAS-component production by cyst-lining epithelial cells has been suggested by several investigators [38, 39]. Although we cannot entirely exclude this possibility, such local production should have resulted in angiotensinogen and renin concentrations in cyst fluid that would have been at least comparable to, if not far above, their plasma concentrations. Remarkably, this was not the case (**Figure 3**). In fact, relative to albumin, the concentrations of angiotensinogen, renin and prorenin were lower in cyst fluid (**Figure 3**). In other words, when using cyst albumin concentrations as a measure of blood-derived proteins, cyst RAAS-component concentrations can be entirely explained on the basis of leakage from blood plasma. Of interest, the urinary concentrations of albumin, angiotensinogen, and prorenin were lower than their concentrations in cyst fluid. This most likely reflects further dilution and/or tubular reabsorption. Surprisingly, this was not the case for renin: its concentrations in cyst fluid and urine were similar (**Figure 3**). Moreover, the correlation between albumin and renin excretion, although modest, showed a different pattern in ADPKD than in CKD (**Figure 2C**). Taken together, these data suggest that ADPKD affects the urinary excretion of renin differently than of albumin, angiotensinogen and prorenin. This difference between renin and prorenin excretion (resulting in urinary prorenin levels that are virtually undetectable), has been observed before [22, 33]. Of note, we did not measure the sodium concentration in cyst fluid; previously, more active renin was found in so-called “gradient cysts” in which the sodium concentration is low [5].

Many aspects of the intra-renal renin-angiotensin system remain unclear, although some groups have suggested that angiotensinogen and renin in tubular fluid may lead to high tubular angiotensin II levels [13, 38]. Such high local angiotensin II levels could promote renal sodium retention, hypertension, kidney damage, or even cystogenesis [38]. Of interest in this regard is a recent report where targeting the intra-renal renin-angiotensin system reduced cyst growth in an animal model of polycystic kidney disease [40]. Based on our cross-sectional data, we cannot conclude whether the higher urinary angiotensinogen and renin excretions are a cause or a consequence of the kidney damage in ADPKD. In other words, it is unclear if higher urinary angiotensinogen and renin excretion should be considered as a damage marker or as a potential contributor to kidney damage.

Given the experimental link between the intra-renal renin-angiotensin system and cystogenesis, this deserves further study.

A number of limitations of this study should be mentioned. First, RAAS-inhibitor use could have influenced our results. Ideally, urinary RAAS-components should be measured before and after starting a RAAS-inhibitor. Previous studies have shown a decrease of urinary angiotensinogen and no effects on urinary renin after initiation of an angiotensin receptor blocker in patients with chronic kidney disease [41, 42]. Therefore, if anything, our ADPKD group would be expected to have lower urinary angiotensinogen excretion, which was not the case. Second, despite matching of ADPKD with CKD patients by the most relevant parameters (eGFR, blood pressure, RAAS-inhibitors), several differences remained. The difference in age was inevitable, as eGFR decline occurs much earlier in ADPKD than in other forms of CKD. We addressed these differences by using an adjusted analysis, multivariable analyses, and a subanalysis (**Table 2, Table 3**). Finally, although significant, the strength of the correlations observed in this study were modest, suggesting high inter-individual variability or a multifactorial origin.

In conclusion, ADPKD uniquely increases urinary angiotensinogen and renin excretion despite their circulating levels being comparable to those in CKD. We believe these findings warrant further analysis in mechanistic or intervention studies.

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Part 2

Acid

Chapter 8

Acute acid load in chronic kidney disease increases plasma potassium, plasma aldosterone, and urinary renin

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Metabolic acidosis is common in patients with chronic kidney disease (CKD) and may contribute to progression of CKD and all-cause mortality [1]. However, little is known on how CKD changes the response to an acute acid load, and whether an altered response could contribute to adverse outcomes [2]. Therefore, our aim was to characterize the differences between the response to an acute oral acid load (to mimic the dietary acid load) in patients with CKD and healthy subjects.

To do so, we performed the short acid-loading test with ammonium chloride in 9 males with CKD stage G4 and in 16 healthy male subjects (see **Supplement** for complete methods and baseline characteristics) [3]. The study was approved by the Medical Ethics Committee (MEC-2016-329) and registered at ClinicalTrials.gov (NCT03293446). In the patients with CKD, all antihypertensive drugs (except beta-blockers) were discontinued for 2 weeks to avoid drug interference. The test started after an overnight fast by giving a 10% oral solution of ammonium chloride (100 mg/kg body weight) over a period of 1 hour together with a standardized meal (25 mmol sodium, 28 mmol potassium) and a water load (5 mL/kg followed by 2.5 mL/kg/hour). The response to the acid load was observed for 6 hours with repeated sampling of venous blood (at t = 0, 3, and 6 hours) and urine (hourly). Group comparisons were performed using repeated measures 2-way ANOVA.

At baseline, blood and urine pH and plasma bicarbonate of the patients with CKD was significantly lower (**Figure 1** and **Figure S1**). After three hours, ammonium chloride reduced blood pH and plasma bicarbonate and raised plasma potassium and plasma aldosterone similarly in both groups (**Figure 1** and **Figure S1**). At the end of the test, however, blood pH, plasma potassium, and plasma aldosterone were returning to normal in the healthy subjects, but not in the patients. Plasma renin did not change significantly during the test. The patients and healthy subjects adequately lowered urine pH (< 5.3 in all participants). The healthy subjects and the patients also increased urine ammonium excretion after one hour, but the increase in healthy subjects was significantly greater and persisted over time (**Figure 1**). This resulted in a significantly lower cumulative ammonium excretion in patients (**Figure S1**). Accordingly, net acid excretion was also significantly lower in patients (no difference in titratable acid, **Figure S1**). The same pattern as for urine ammonium excretion was also observed for urine sodium, chloride, and potassium excretion. The urine excretion of creatinine, albumin and the low-molecular weight proteins retinol-binding protein and renin also acutely increased after the acid load, with normalization thereafter (**Figure 1** and **Figure S2**). No differences between the groups were observed for the courses in creatinine and protein excretion, except for urinary renin. In patients with CKD, systolic blood pressure fell during the first two hours and increased thereafter, whereas it remained stable in the healthy subjects (**Figure S2**). Because patients were significantly older than healthy subjects, we also performed a subanalysis with older healthy subjects and found similar results (data not shown).

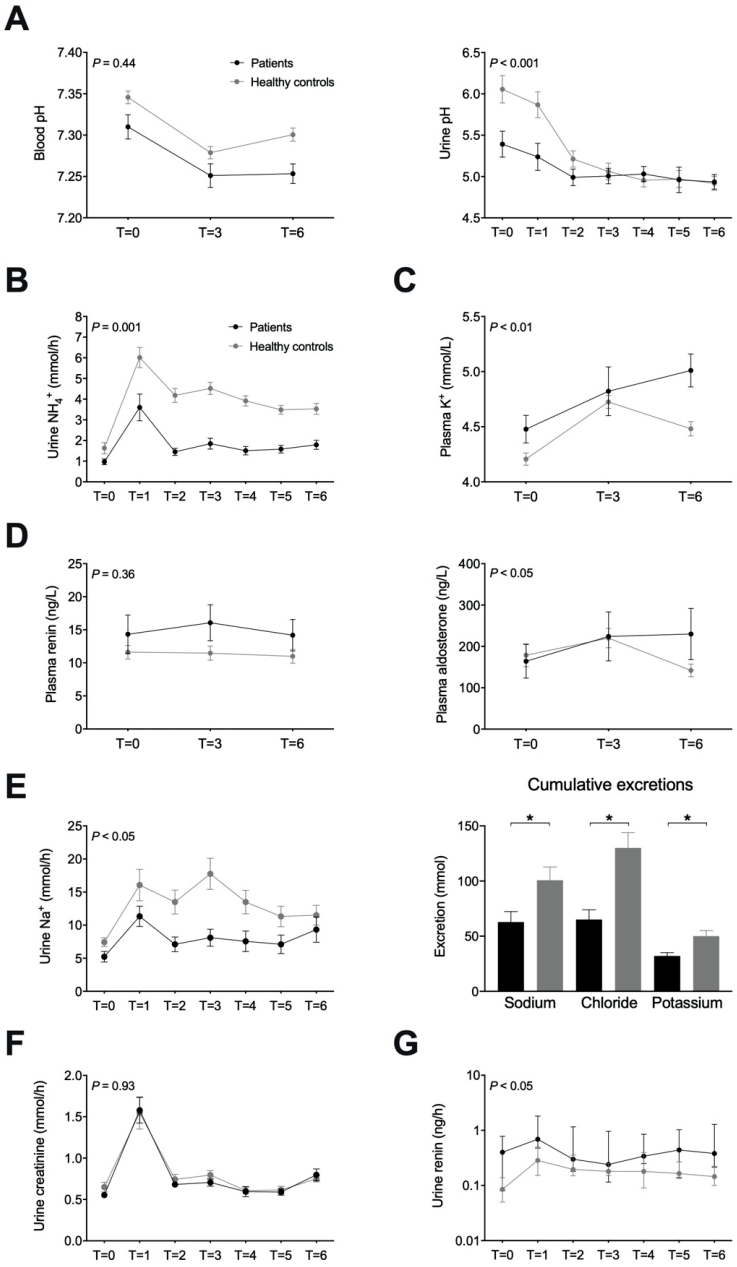

Figure 1. Effects of an acute acid load with ammonium chloride on venous blood pH and urine pH (**A**), urine ammonium excretion (**B**), plasma potassium (K^+, **C**), plasma renin and aldosterone (**D**), urine sodium excretion, and cumulative excretion of sodium, chloride, and potassium (**E**), urine creatinine excretion (**F**), and urine renin excretion (**G**). Group comparison was performed using repeated measures 2-way ANOVA reporting the *P*-value for interaction. Cumulative excretions were compared with unpaired T-tests. Urine renin was not normally distributed and therefore log-transformed for analysis.

Here, we characterized the response to an acute acid load on acid-base, electrolyte, creatinine, and protein handling by the kidney and addressed whether this response is altered in patients with CKD. We show that urinary ammonium excretion is reduced in patients with CKD increasing the duration of the acidosis. Of note, per-nephron ammonium excretion was likely higher in patients with CKD, although this was not sufficient to prevent the acidosis after acid-loading. Persisting acidemia may have contributed to the higher plasma potassium in patients with CKD by decreasing sodium-hydrogen exchange and sodium-potassium ATPase activity in cells [4]. Furthermore, cumulative potassium excretion was lower in patients with CKD, which may also have contributed to the rise in plasma potassium. Aldosterone was not a limiting factor for potassium secretion, as this increased in the patients with CKD. It is well characterized that metabolic acidosis induces natriuresis initially [5-7], and we also observed this. In patients with CKD sodium excretion was lower, although – similar to ammonium – per-nephron sodium excretion was likely higher. Patients with CKD developed higher plasma aldosterone levels after acid loading, with higher plasma potassium or acidosis as potential drivers [8]. Another interesting observation was that the acid load acutely increased the excretion of creatinine, albumin, and low-molecular weight proteins. This could be caused by glomerular hyperfiltration or an effect on the proximal tubule (decreased protein reabsorption, increased creatinine secretion). We favor hyperfiltration as explanation, because this was previously observed after ammonium chloride loading in children [7] and rats [9], and because a previous micropuncture study did not find evidence for inhibition of proximal tubular reabsorption [6]. However, measurement of glomerular filtration rate would have been necessary to differentiate between the two possibilities.

Our data add potential explanations why metabolic acidosis in patients with CKD may contribute to adverse outcomes [2]. First, longer lasting acidemia causes a greater rise in plasma potassium and will therefore increase susceptibility to hyperkalemia and its complications [10]. Second, the lower pH and higher plasma potassium raise plasma aldosterone, which may promote kidney fibrosis [11]. Third, acidosis increases proteinuria, which has been identified as risk factor for progression of CKD [12]. Although this change also occurred in healthy volunteers, the acid load exposes patients with CKD to a greater degree of proteinuria, which may contribute to further kidney injury. Moreover, the acid load did cause a greater increase in urinary renin in patients than in healthy subjects, indicating differences between individual proteins [13]. A limitation of this study is that we did not include measurements of glomerular hemodynamics. Future studies are required to evaluate if the identified differences in the acute response to acid loading also play a role in the long-term outcomes of CKD. In summary, we show that CKD alters the response to an acute acid load and we propose that this altered response may explain some of the associations between metabolic acidosis and outcomes in CKD.

ACKNOWLEDGMENTS

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SUPPLEMENTAL MATERIAL

Complete methods

Study participants

Patients with chronic kidney disease stage G4 were recruited from our nephrology outpatient clinic. Healthy volunteers were recruited using flyers on the university campus and the hospital. Exclusion criteria for patients were plasma bicarbonate level < 20.0 mEq/L, plasma potassium > 5.5 mmol/L, use of bicarbonate, citrate or acetazolamide in the month preceding the test, heart failure, liver cirrhosis, blood pressure >140/90 mmHg despite the use of 3 different anti-hypertensive drugs, history of kidney transplantation, use of calcineurin inhibitors, known urea cycle disorder, alcoholism or drug abuse, pregnancy, current use of antibiotics, non-steroidal anti-inflammatory drugs, inability to adhere to the study protocol (due to language barrier or intellectual disability).

Study procedure

The study started with a 2-week run-in period during which all antihypertensive medication was discontinued, except for beta-blockers. Patients were provided with a home blood pressure monitor (Omron HBP-1300, Omron Healthcare, The Netherlands) and instructed to measure blood pressure twice daily. If the systolic blood pressure (SBP) was ≥ 160 mmHg during three consecutive measurements, amlodipine was started (5 mg once daily with possible titration to 10 mg once daily). 24-hour urine was collected during the day prior to the test. Participants remained in supine position for at least 45 minutes before each measurement and sample collection.

Measurements

Plasma and urine electrolytes, albumin, and creatinine were measured at the Department of Clinical Chemistry of the Erasmus Medical Center. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation [14]. Venous blood gas analysis and the measurement of bicarbonate in urine was performed directly after sample collection on a blood gas analyzer on site (ABL90 Flex Plus, Radiometer, The Netherlands; RAPIDLab 1265, Siemens, Germany). Urine pH was measured by a HI991001 pH meter (Hannah Instruments, Nieuwegein, The Netherlands) immediately after collection. Urine ammonium was measured using the Berthelot-method as described previously [15, 16]. Urine titratable acid (expressed as mEq/L) was calculated by the following method: the molarity of NaOH (0.1) times the volume of NaOH that was needed to titrate a urine sample to pH 7.40 at 37° C, divided by the volume of the urine sample. Net acid excretion was calculated as the sum of urinary ammonium and titratable acid *minus* bicarbonate (all in mEq/L). Plasma renin was measured using a radioimmunometric assay (Cisbio,

Saclay, France). Urine renin was measured using an in-house enzyme-kinetic assay that quantifies angiotensin I generation in the presence of excess sheep angiotensinogen [17, 18]. In order to convert angiotensin I-generating activity to renin concentration, a conversion factor was used based on the fact that 1 ng Ang I/mL per hour corresponds with 2.6 pg human renin/mL. Plasma aldosterone was measured by radioimmunoassay (Demeditec, Kiel, Germany).

Calculations

Estimated protein intake was calculated using the following formula: [protein intake = 6.25 * (UUN + 0.031 * body weight) – urinary protein (g/d) if daily urine protein excretion ≥ 5 g.[19] UUN indicates urine urea nitrogen excretion (g/24 hour) and was calculated by multiplying the 24-hour urine urea excretion (mmol/day) times 2 (the number of nitrogen atoms in urea) and the molecular weight of nitrogen (14.0067 g/mol). Net endogenous acid production (NEAP) was calculated using the following formula: NEAP = −10.2 + 54.5 [protein intake (g/d) / potassium intake (mEq/d). Daily potassium intake was estimated using 24-hour urinary potassium excretion [20].

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Acid

Table S1. Baseline characteristics

	Healthy subjects (n = 16)	Patients with CKD (n = 9)
Age, years	39 ± 17	66 ± 15*
Body mass index, kg/m^2	24.7 ± 2.9	27.1 ± 2.9
eGFR, ml/min/1.73m^2	101 ± 16	26 ± 8*
Diabetes mellitus, n (%)	0 (0)	4 (44)
Albuminuria, g/day	0.01 (0.00, 0.01)	0.10 (0.01, 1.13)*
Venous pH	7.35 ± 0.008	7.31 ± 0.01*
Urine pH	6.1 ± 0.2	5.4 ± 0.2*
Plasma bicarbonate, mEq/L	27.7 ± 1.5	23.8 ± 2.1*
Estimated protein intake, g/day	93.1 ± 37.7	71.6 ± 18.2
NEAP, mEq/day	55.1 ± 24.6	55.2 ± 16.7

Data are presented as n (%), mean ± SD or median (interquartile range). * $P < 0.05$ for comparison to healthy controls by unpaired T-test or χ^2-test. CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; NEAP, net endogenous acid production.

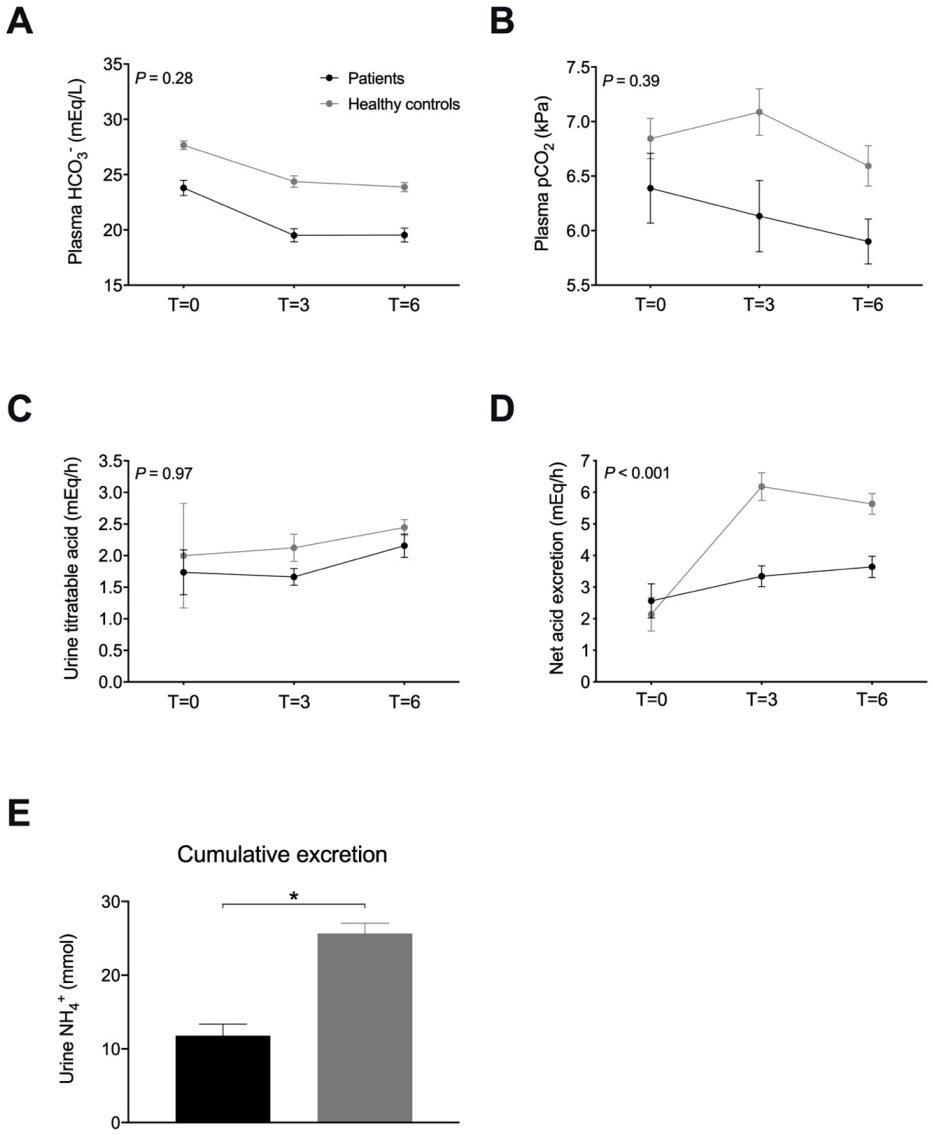

Figure S1. Effects of an acute acid load with ammonium chloride on plasma bicarbonate (HCO_3^-, **A**), venous pCO2 (**B**), urinary titratable acid excretion (C), net acid excretion (**D**), cumulative urine ammonium excretion (**E**). Group comparison was performed using repeated measures 2-way ANOVA reporting the *P*-value for interaction.

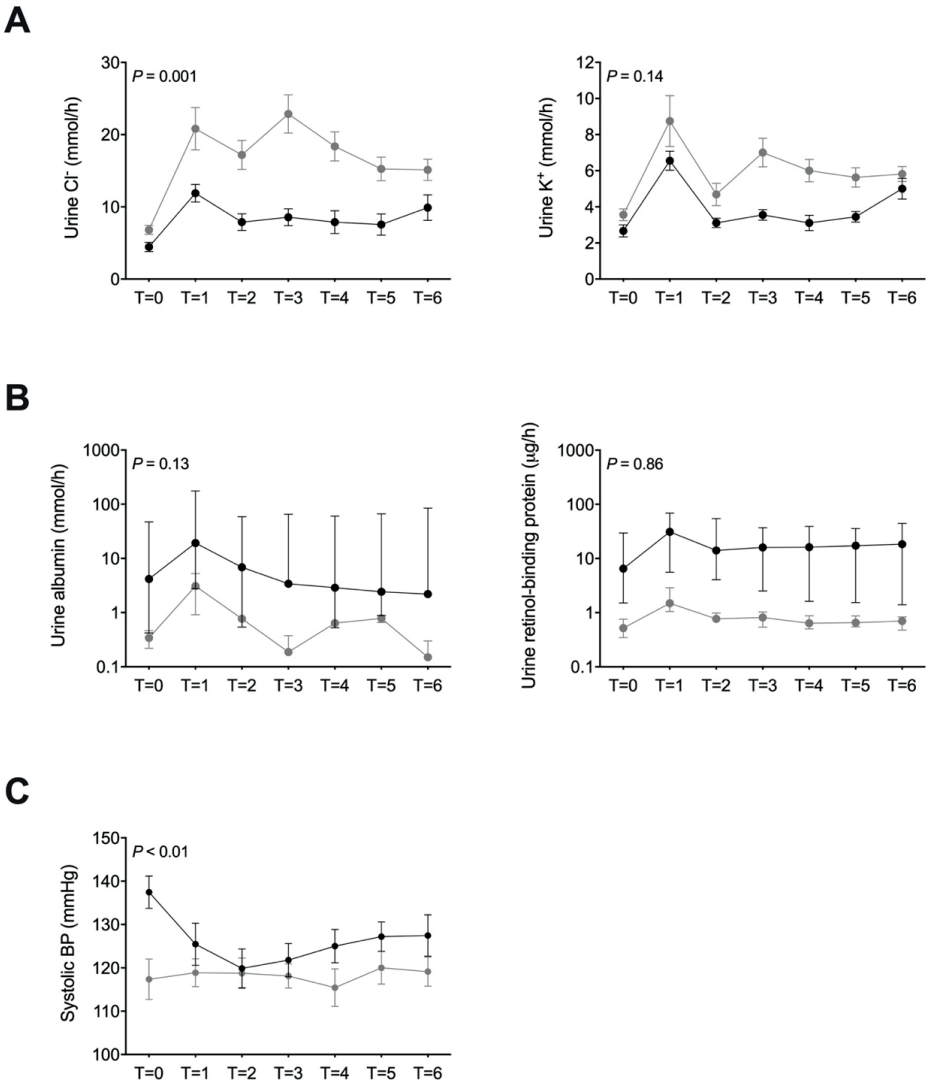

Figure S2. Effects of an acute acid load with ammonium chloride on the urine excretion of chloride and potassium (**A**), and urine excretion of albumin and retinol-binding protein (**B**), and systolic blood pressure (**C**) Group comparison was performed using repeated measures 2-way ANOVA reporting the *P*-value for interaction. Cumulative excretion was compared with unpaired T-tests. Urine albumin and retinol-binding protein were not normally distributed and therefore log-transformed for analysis.

Chapter 9

Effect of sodium bicarbonate supplementation on the renin-angiotensin system in patients with chronic kidney disease and acidosis: A randomized clinical trial

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ABSTRACT

Background: Acidosis-induced kidney injury is mediated by the intrarenal renin-angiotensin system, for which urinary renin is a potential marker. Therefore, we hypothesized that sodium bicarbonate supplementation reduces urinary renin excretion in patients with chronic kidney disease (CKD) and metabolic acidosis.

Methods: Patients with CKD stage G4 and plasma bicarbonate 15-24 mmol/l were randomized to receive sodium bicarbonate (3x1000 mg/day, ~0.5 mEq/kg), sodium chloride (2x1000 mg/day), or no treatment for 4 weeks (n=15/arm). The effects on urinary renin excretion (primary outcome), other plasma and urine parameters of the renin-angiotensin system, endothelin-1, and proteinuria were analyzed.

Results: Forty-five patients were included (62 ± 15 years, eGFR 21 ± 5 ml/min/1.73m^2, plasma bicarbonate 21.7 ± 3.3 mmol/l). Sodium bicarbonate supplementation increased plasma bicarbonate (20.8 to 23.8 mmol/l) and reduced urinary ammonium excretion (15 to 8 mmol/day, both P<0.05). Furthermore, a trend towards lower plasma aldosterone (291 to 204 ng/L, P=0.07) and potassium (5.1 to 4.8 mmol/l, P=0.06) was observed in patients receiving sodium bicarbonate. Sodium bicarbonate did not significantly change the urinary excretion of renin, angiotensinogen, aldosterone, endothelin-1, albumin, or α1-microglobulin. Sodium chloride supplementation reduced plasma renin (166 to 122 ng/L), and increased the urinary excretions of angiotensinogen, albumin, and α1-microglobulin (all P<0.05).

Conclusions: Despite correction of acidosis and reduction in urinary ammonium excretion, sodium bicarbonate supplementation did not improve urinary markers of the renin-angiotensin system, endothelin-1, or proteinuria. Possible explanations include bicarbonate dose, short treatment time, or the inability of urinary renin to reflect intrarenal renin-angiotensin system activity.

INTRODUCTION

Metabolic acidosis is a common complication in patients with chronic kidney disease (CKD). The prevalence of metabolic acidosis (usually defined as a plasma bicarbonate concentration <22 mmol/l) increases with higher CKD stage and is 26% and 47% for CKD stages G4 and G5, respectively.[1, 2] Metabolic acidosis in CKD is associated with a more rapid progression of CKD.[3-5] A recent systematic review showed that metabolic acidosis is a modifiable risk factor for CKD progression as interventions with oral alkali supplementation or an alkaline diet reduce this risk.[6] However, the mechanisms of acidosis-induced kidney injury are incompletely understood.

Current understanding of how acidosis contributes to kidney injury suggests that acid retention triggers an adaptive response to increase ammoniagenesis.[7] This process is orchestrated by activation of the circulating and intrarenal renin-angiotensin systems (RAS) and endothelin-1. However, in a chronic setting and at the single-nephron level, this adaptive response may become maladaptive with the RAS and endothelin-1 contributing to inflammation and fibrosis.[8] For example, it has been shown that locally produced ammonium can activate the complement system with subsequent tubulointerstitial inflammation and fibrosis.[9] In rats, induction of CKD with 2/3rd nephrectomy causes acid retention and higher levels of angiotensin II and aldosterone in the kidney; alkali treatment reverses these changes.[10] In clinical studies, alkali treatment reduced plasma and urinary aldosterone in patients with CKD stage G2 and G4.[11, 12] Similar findings have been reported for plasma and urinary endothelin-1.[10, 13-16] However, the activity of the intrarenal RAS is difficult to assess, because it is questionable to what degree urinary RAS components truly reflect intrarenal RAS activity.[17, 18]

Previous data suggest that the production of angiotensin II in the kidney depends on filtered (i.e., blood-derived) components of the RAS, including renin and angiotensinogen.[19-21] Accordingly, the modest alkali-induced lowering of urinary angiotensinogen in patients with CKD stage G3 could suggest reduced intrarenal angiotensin generation.[22] Alternatively, urinary angiotensinogen may simply follow the same urinary excretion pattern as albumin.[23] Since this is not the case for urinary renin,[23, 24] this marker may be a more attractive parameter to assess intrarenal RAS activity. Accordingly, we hypothesized that sodium bicarbonate supplementation reduces urinary renin excretion in patients with CKD and metabolic acidosis. To address this, we performed an open-label clinical trial in which patients were randomized to receive sodium bicarbonate or sodium chloride, or served as time-controls. In addition to the measurement of plasma and urinary RAS parameters, we also analyzed the effects on urinary endothelin-1, albumin, α1-microglobulin, and complement.

METHODS

Study design

We conducted an open-label randomized controlled trial at 4 study sites in The Netherlands, including Erasmus Medical Center, Rotterdam, Amsterdam University Medical Centers. University Medical Center Groningen and Leiden University Medical Center. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2013-332). The trial was registered at clinicaltrials.gov with registration number NCT02896309. Patients were recruited from outpatient nephrology clinics between April 2014 and December 2018. All patients with CKD stage G4 (eGFR 15-30 ml/min/1.73m^2) and with plasma bicarbonate levels between 15.0 and 24.0 mmol/l were eligible for inclusion. Exclusion criteria were sodium bicarbonate use in the month preceding the study, heart failure New York Heart Association class 3 or 4, liver cirrhosis with ascites and the inability to withdraw diuretics, systolic blood pressure > 140 mmHg despite the use of three different antihypertensive drugs, kidney transplantation, and use of calcineurin inhibitors. Patients were randomized for 4-week treatment with sodium bicarbonate (3 x 1000 mg/day, providing a sodium load of 36 mmol per day), sodium chloride (2 x 1000 mg/day, providing a sodium load of 34 mmol per day) or no treatment (time control). Allocation to treatment was done by randomization using sequentially numbered, opaque, sealed envelopes. Stratified randomization was used to ensure that a similar number of patients were allocated to each intervention at the different study sites.

Measurements

At baseline and after 2 and 4 weeks, blood and 24-hour urine samples were collected and office blood pressure was measured. Plasma and urine electrolytes, albumin, creatinine, and α1-microglobulin were measured at the Department of Clinical Chemistry of the Erasmus Medical Center. Venous blood gas analysis was performed directly after sample collection on a blood gas analyzer (ABL90 Flex Plus, Radiometer, The Netherlands; RAPIDLab 1265, Siemens, Germany). Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation.[25] Creatinine clearance was calculated based on plasma and urinary creatinine excretion. Urinary ammonium was measured using the Berthelot-method, as described previously.[26] Plasma renin was measured using a radioimmunometric assay (Cisbio, Saclay, France). Urinary renin was measured using an in-house enzyme-kinetic assay that quantifies angiotensin I generation in the presence of excess sheep angiotensinogen.[27] In order to convert angiotensin I-generating activity to renin concentration, a conversion factor was used, based on the fact that 1 ng Ang I/mL per hour corresponds with 2.6 pg renin/mL. Urinary angiotensinogen was measured as the maximum quantity of Ang I that was generated during incubation with excess recombinant renin using the same in-house assay.[27] Plasma and urinary aldosterone were

measured by radioimmunoassay (Demeditec, Kiel, Germany). Endothelin-1 was measured using a Quantikine enzyme-linked immunosorbent assay (ELISA; R&D systems, Minneapolis, USA). Urine soluble terminal complement complex sC5b-9 was measured by ELISA as previously described.[28] All urinary excretions were expressed as ratio with urine creatinine to correct for any incomplete collections, as reported previously.[29]

Statistics

Data are presented as frequencies (percentages), mean ± standard deviation and median with 10^{th}-90^{th} percentile, as appropriate. The primary outcome was the change in urinary renin-to-creatinine ratio. A power calculation based on previous data indicated that a minimum of 45 patients (15 per treatment arm) was required to show that sodium bicarbonate supplementation would reduce urinary renin excretion by 0.3 ng/L ($\alpha = 0.025$, $\beta = 0.8$, standard error 0.26).[23] Secondary outcomes included the urinary-to-creatinine ratios of angiotensinogen, endothelin-1, albumin and α1-microglobulin. An exploratory analysis was performed for the treatment effects on kidney function, blood pressure and plasma potassium. The omnibus K2 test was used to test for normality. Non-normally distributed data were log-transformed for statistical analysis. Primary and secondary outcomes were analyzed using mixed linear models that included treatment and period (time) as fixed effects. In case a significant interaction between treatment and period was found, post-hoc tests were performed with correction for multiple comparisons according to Dunnett. Data were analyzed using SPSS Statistics (IBM, version 24.0). $P < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

Forty-seven patients entered the study protocol, of whom two patients discontinued treatment due to adverse reactions to sodium chloride supplementation (1 patient with gastrointestinal symptoms, 1 patient with polydipsia). Forty-five patients completed the study protocol (15 patients/arm). All patients that finished the treatment period were included in the analysis of the primary and secondary outcomes. The average age was 62 ± 15 years, 78% were males, the average eGFR was 21 ± 15 ml/min/1.73m^2, and the average plasma bicarbonate 21.7 ± 3.3 mmol/l (**Table 1**).

Table 1. Baseline characteristics. Cr, creatinine; eGFR, estimated glomerular filtration rate; NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate; RAS renin-angiotensin system.

	Total (n = 45)	$NaHCO_3$ (n = 15)	NaCl (n = 15)	Time control (n = 15)	P-value
Demographics					
Age, years	62 ± 15	61 ± 17	61 ± 14	64 ± 14	0.9
Males, n (%)	35 (78)	11 (73)	14 (93)	10 (67)	0.2
European descent, n (%)	39 (87)	13 (87)	14 (93)	12 (80)	0.6
Comorbidities					
eGFR, mL/min/1.73m^2	21 ± 5	21 ± 6	22 ± 3	20 ± 4	0.6
Systolic blood pressure, mmHg	137 ± 16	134 ± 10	126 ± 13	135 ± 22	0.3
Use of RAS-inhibitors, n (%)	39 (87)	13 (87)	12 (80)	14 (93)	0.6
Diabetes mellitus, n (%)	7 (16)	2 (13)	2 (13)	3 (20)	0.8
Laboratory data					
Plasma bicarbonate, mmol/l	21.7 ± 3.3	20.8 ± 3.9	21.8 ± 2.9	22.4 ± 3.1	0.4
Plasma potassium, mmol/l	5.0 ± 0.6	5.1 ± 0.7	5.0 ± 0.6	4.9 ± 0.5	0.6
Plasma renin, ng/l	41.8 (20.3, 119.4)	31.0 (18.4, 64.0)	75.2 (22.1, 130.4)	41.8 (16.0, 126.1)	0.5
Plasma aldosterone, ng/l	301 (175, 449)	236 (188, 427)	349 (155, 604)	332 (169, 415)	0.5
Urine sodium, mmol/mmol Cr	10.4 (8.8, 12.3)	10.4 (10.1, 13.7)	10.6 (8.5, 11.3)	9.8 (7.8. 13.5)	0.2
Urine ammonium, mmol/mmol Cr	1.2 (1.0, 1.6)	1.1 (0.9, 1.6)	1.0 (0.9, 1.9)	1.4 (1.1, 1.6)	0.6
Urine renin, ng/mmol Cr	1.1 (0.7, 2.0)	0.7 (0.5, 1.5)	1.1 (0.7, 2.1)	1.4 (1.0, 2.1)	0.5
Urine aldosterone, ng/mmol Cr	352 (272, 492)	337 (267, 632)	335 (289, 443)	353 (269, 461)	0.8
Urine angiotensinogen, µg/mmol Cr	3.4 (0.9, 14.5)	2.0 (0.6, 26.8)	7.1 (1.5, 12.3)	2.0 (1.2, 12.6)	0.9
Urine endothelin-1, ng/mmol Cr	0.02 (0.01, 0.05)	0.02 (0.01, 0.13)	0.02 (0.01, 0.02)	0.02 (0.02, 0.05)	0.3
Urine albumin, mg/mmol Cr	30 (5, 102)	30 (7, 93)	39 (6, 87)	16 (4, 96)	0.8
Urine α1-microglobulin, mg/mmol Cr	3.6 (2.0, 5.3)	3.9 (1.9, 6.4)	2.8 (2.2, 4.0)	3.8 (1.9, 6.2)	0.5

Effects on acid-base status and the renin-angiotensin system

Sodium bicarbonate supplementation increased plasma bicarbonate (with 3.0 ± 0.7 and 2.9 ± 0.8 mmol/L after 2 and 4 weeks of treatment, respectively; $P < 0.01$ *versus* baseline) and lowered urinary ammonium excretion (with -7.0 ± 1.5 mmol/day and -3.6 ± 1.9 mmol/day, $P < 0.05$ *versus* baseline, **Figure 1**). No significant changes in plasma bicarbonate or urinary ammonium excretion occurred with sodium chloride supplementation and without treatment. Sodium chloride but not sodium bicarbonate supplementation significantly reduced plasma renin (with -9.5 and -7.9 ng/L, $P < 0.05$ *versus* baseline,

Figure 1). A trend towards a reduction in plasma aldosterone was observed with sodium bicarbonate supplementation after 4 weeks (-99 ng/L, $P = 0.07$). No changes in the aldosterone-to-renin ratio were observed with either treatment (data not shown).

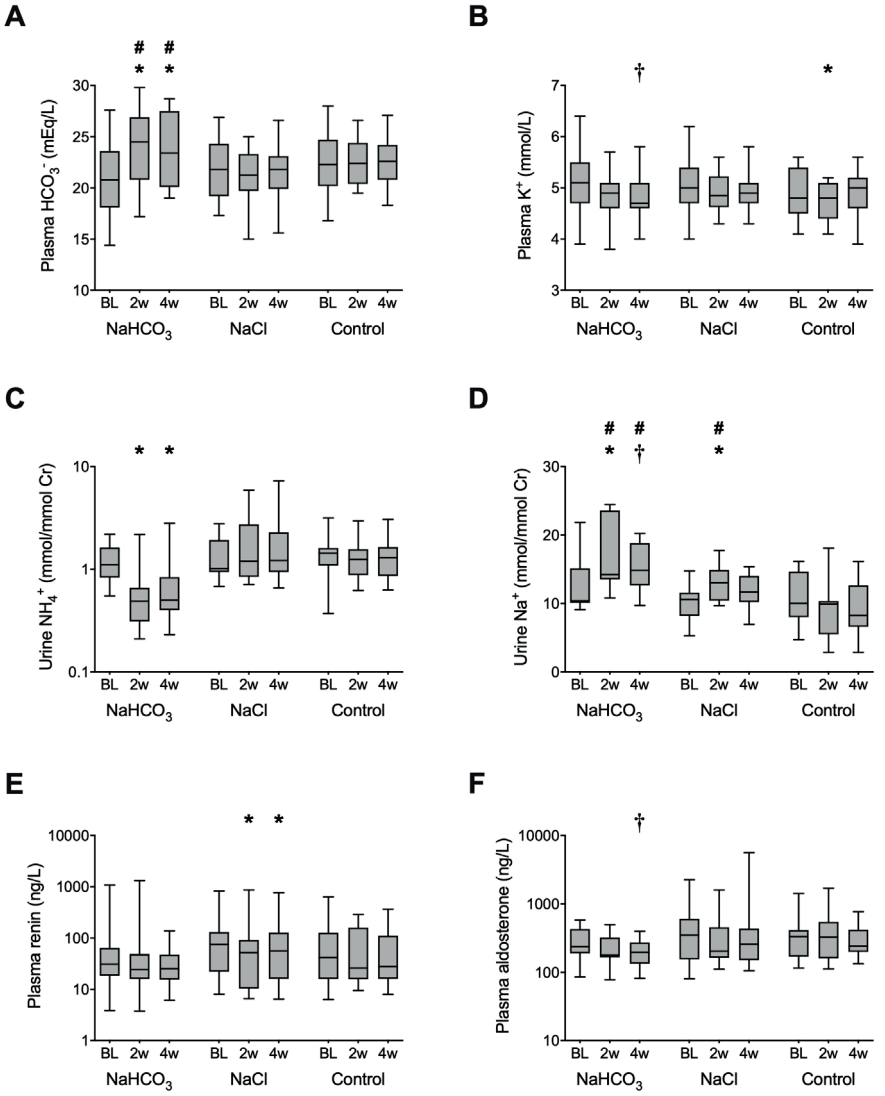

Figure 1. Sodium bicarbonate increased plasma bicarbonate and urinary sodium excretion and lowered urinary ammonium excretion, whereas sodium chloride treatment only increased urinary sodium excretion after 2 weeks of treatment. The horizontal black lines represent the median value; the lower and upper boundaries of the box represent the 25th and 75th percentiles; the whiskers represent the lowest and highest values. HCO_3^-, bicarbonate; K^+, potassium; Na^+, sodium; NaCl, sodium chloride; NH_4^+, ammonium; $NaHCO_3$, sodium bicarbonate. * $P < 0.05$ for the within-group difference from baseline; † $P = 0.07$ for the within-group difference *vs.* baseline; # P < 0.05 for the difference *vs.* control.

Primary and secondary outcomes

In all three treatment groups, no significant within-group differences were detected in the urinary renin-to-creatinine ratio after two or four weeks of treatment (**Table 2**). In addition, no between group differences were found. Similarly, sodium bicarbonate supplementation had no significant effect on any of the secondary outcome parameters (**Table 2**). In the within-group comparison, sodium chloride supplementation increased urinary angiotensinogen, albumin, and α1-microglobulin excretion; no between-group differences were shown for these outcomes (**Table 2**). Five patients did not use RAS-inhibitors. Two of these patients received sodium bicarbonate and this reduced urinary aldosterone (65% and 39% reduction after 2 and 4 weeks), an effect that was not observed with the other interventions. No effects on the other outcome parameters was observed. In addition, no differences were observed in a sensitivity analysis of the primary and secondary outcomes between patients with CKD stage G4a (eGFR 29-23 mL/min per 1.73m^2) and G4b (eGFR 22-16 mL/min per 1.73m^2). To determine whether correction of metabolic acidosis reduced the activity of the complement system, we also measured soluble terminal complement complex sC5b-9 in urine at the end of treatment. Urine sC5b-9 was undetectable in forty patients (< 0.05 U/ml). Of the five patients with detectable urinary complement, three had albuminuria > 1 gram/day.

Table 2. Treatment effects on urinary renin-angiotensin, endothelin-1, and protein excretions.

Measurement	Treatment	Baseline	2 weeks	4 weeks
Urine renin, ng/mmol Cr	$NaHCO_3$	0.7, 0.4-2.2	0.9, 0.3-3.3	0.9, 0.2-3.1
	NaCl	1.1, 0.3-3.7	1.1, 0.1-4.3	1.2, 0.2-5.4
	Time control	1.4, 0.5-2.8	1.6, 0.5-2.4	1.8, 0.3-3.1
Urine aldosterone, ng/mmol Cr	$NaHCO_3$	337, 171-1179	318, 125-513	356, 166-426
	NaCl	335, 142-787	**337, 126-627†**	317, 156-779
	Time control	353, 216-501	394, 142-676	293, 181-750
Urine angiotensinogen, µg/mmol Cr	$NaHCO_3$	2.0, 0.4-83.5	4.3, 0.4-84.7	4.3, 0.2-104.5
	NaCl	7.1, 0.5-22.9	**7.7, 0.4-30.9***	**6.2, 0.4-39.0***
	Time control	2.0, 0.8-61.5	2.9, 0.9-55.3	5.5, 0.8-78.5
Urine endothelin-1, ng/mmol Cr	$NaHCO_3$	0.02, 0.01-0.56	0.05, 0.01-0.31	0.02, 0.01-0.29
	NaCl	0.02, 0.01-0.07	0.02, 0.01-0.07	0.02, 0.01-0.07
	Time control	0.02, 0.01-0.06	0.02, 0.01-0.04	0.02, 0.01-0.05
Urine albumin, mg/mmol Cr	$NaHCO_3$	30.4, 3.7-208.3	36.7, 5.8-196.9	40.4, 5.1-189.6
	NaCl	38.7, 1.6-124.4	**46.4, 1.2-130.3***	**58.6, 1.4-177.1***
	Time control	15.5, 1.9-171.2	17.6, 1.7-156.3	21.6, 2.1-160.7
Urine α1-microglobulin, mg/mmol Cr	$NaHCO_3$	3.9, 1.4-10.5	5.6, 1.1-11.1	5.2, 1.3-11.1
	NaCl	2.8, 1.3-6.1	**3.6, 1.3-8.0***	**3.4, 1.4-8.1†**
	Time control	3.8, 1.0-9.3	6.2, 1.5-9.5	4.5, 1.0-10.8

Cr, creatinine; NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate. * $P < 0.05$ for the within-group difference *vs.* baseline; † $P = 0.06$ for the within-group difference *vs.* baseline.

Effects on kidney function, blood pressure and plasma potassium

Sodium bicarbonate or sodium chloride supplementation did not lead to significant changes in eGFR (**Table 3**). However, sodium bicarbonate did cause a small but statistically significant increase in urinary creatinine excretion after 4 weeks, which was not observed with sodium chloride treatment. No significant differences were identified for systolic and diastolic blood pressure within or between groups. After 4 weeks, there was a trend towards a reduction in plasma potassium with sodium bicarbonate ($P = 0.06$ for difference baseline *versus* 4 weeks), which was not observed with sodium chloride and without treatment.

Table 3. Effects of the sodium bicarbonate intervention on exploratory outcomes kidney function, blood pressure, and plasma potassium.

Measurement	Treatment	Baseline	2 weeks	4 weeks
eGFR, mL/min/1.73m^2	$NaHCO_3$	21 ± 6	21 ± 5	21 ± 5
	NaCl	22 ± 3	22 ± 4	22 ± 4
	Time control	20 ± 4	20 ± 4	20 ± 4
Creatinine clearance, mL/min	$NaHCO_3$	30 ± 10	29 ± 11	33 ± 12
	NaCl	39 ± 12	39 ± 14	39 ± 14
	Time control	32 ± 10	33 ± 10	30 ± 8
Creatinine excretion, mmol/day	$NaHCO_3$	10.7, 8.7-13.4	10.2, 8.0-11.9	**11.1, 10.3-14.0***
	NaCl	13.9, 10.9-17.0	12.5, 10.8-18.4	12.6, 10.7-16.6
	Time control	10.3, 8.1-17.7	12.9, 7.4-15.2	10.7, 8.0-15.1
Systolic blood pressure, mmHg	$NaHCO_3$	134 ± 10	132 ± 18	132 ± 16
	NaCl	126 ± 12	125 ± 13	123 ± 13
	Time control	135 ± 22	140 ± 24	134 ± 20
Diastolic blood pressure, mmHg	$NaHCO_3$	76 ± 10	75 ± 10	75 ± 10
	NaCl	78 ± 8	77 ± 9	78 ± 11
	Time control	81 ± 12	81 ± 10	78 ± 12
Plasma potassium, mmol/l	$NaHCO_3$	5.1 ± 0.7	4.8 ± 0.5	**4.8 ± 0.5†**
	NaCl	5.0 ± 0.6	4.9 ± 0.4	4.9 ± 0.4
	Time control	4.9 ± 0.5	**4.7 ± 0.4***	4.9 ± 0.5

eGFR, estimated glomerular filtration rate; NaCl, sodium chloride; $NaHCO_3$, sodium bicarbonate. * $P < 0.05$ for the within-group difference from baseline; † $P = 0.06$ for the within-group difference from baseline.

DISCUSSION

In this open-label, three-arm randomized controlled trial (RCT) we investigated if sodium bicarbonate supplementation in patients with CKD and metabolic acidosis lowers urinary renin, as a potential measure of the intrarenal RAS. Sodium bicarbonate supplementation corrected metabolic acidosis and lowered urinary ammonium excretion.

Despite these effects, we observed no within or between group differences for urinary renin. In addition, sodium bicarbonate had no significant effect on the urinary excretion of angiotensinogen, aldosterone, endothelin-1, albumin, or α1-microglobulin. Despite these negative findings, we believe our study adds three relevant aspects to the evolving field of metabolic acidosis in CKD.

First, several other clinical trials with sodium bicarbonate supplementation were also unable to show an effect on their primary endpoints. In this regard, our study is most comparable to the recent study by Raphael and colleagues who investigated the effect of sodium bicarbonate on urinary kidney injury markers.[29] In their placebo-controlled, double-blind RCT sodium bicarbonate was supplemented for six months in a dose of 0.5 mEq/kg to patients with type 1 or type 2 diabetes and an eGFR between 15 and 89 ml/min/1.73m^2. Sodium bicarbonate supplementation increased plasma bicarbonate and reduced urinary ammonium, but did not reduce urinary TGF-β1, KIM-1, fibronectin, NGAL, or albumin. The most likely explanation for a lack of effect is that the dose of sodium bicarbonate was too low. Indeed, most RCTs that were unable to show an effect on the primary endpoint used a dose of 0.3–0.5 mEq/kg[29-31], whereas positive RCTs used a higher dose of approximately 1.0 mEq/kg.[11, 16, 32] To address this issue, Raphael *et al.* recently published a dose-finding study confirming that a higher dose of sodium bicarbonate (0.8 mEq/kg) had a stronger effect on plasma bicarbonate and urinary ammonium compared with a lower dose (0.5 mEq/kg). Wesson *et al.* showed that 0.5 mEq/kg sodium bicarbonate supplementation for 30 days did reduce plasma aldosterone and endothelin-1 levels in patients with CKD stage G1 or G2.[12] In agreement, we also observed that sodium bicarbonate reduced plasma aldosterone, although this was of borderline significance. The effect of sodium bicarbonate on aldosterone may be mediated by lowering of plasma potassium, although this was also of borderline significance in our study. In the RCT by Melamed *et al.* sodium bicarbonate supplementation also increased plasma bicarbonate and reduced plasma potassium.[30] In contrast, three previous studies did find effects of a lower sodium bicarbonate dose (0.3 or 0.5 mEq/kg) on urinary aldosterone, endothelin-1, angiotensinogen, albumin, and NAG, although the effect sizes were modest.[12, 15, 16] Possible explanations for the discrepancy with our study is that previous studies applied a longer treatment time (up to five years) and included patients with earlier stages of CKD. Finally, the use of RAS-inhibitors (used by 88% of the patients in this study) may suppress the RAS to an extent that alkali has no further effect.

A second issue that is raised by our study is if urinary renin can truly be considered as a marker of the intrarenal RAS. Determinants of urinary renin excretion include glomerular filtration, proximal tubular reabsorption, local production in the collecting duct, and intratubular conversion of plasma-derived prorenin to renin. In a study including 101

patients with or without diabetes mellitus and hypertension, urinary renin did not correlate with plasma renin and especially dissociated in patients with diabetes mellitus or on RAS-inhibitors. Accordingly, we proposed urinary renin to be a marker for the intrarenal RAS.[33, 34] In a subsequent study, however, we showed that the glomerular sieving coefficient for renin is higher than for albumin and that variation in proximal tubular reabsorption explains the different urinary excretion patterns of renin and albumin.[34] We also showed that urinary renin does not reflect converted prorenin. A recent study in mice and humans with diabetes confirmed these concepts and did not find evidence for local production of renin.[35] Together these recent insights suggest that urinary renin excretion is mainly determined by variation in glomerular filtration and proximal tubular reabsorption and is therefore not a good marker for the intrarenal RAS. It would be of interest to explore whether renin mRNA or protein in urinary extracellular vesicles – which are mainly derived from tubular epithelial cells – is a better read-out of intrarenal RAS.[36] This also implies that positive effects of oral alkali may have been obscured by counteracting effects of the sodium load on filtration or reabsorption. We recently showed that an acid load increases albuminuria.[37] Therefore, correction of acidosis would be expected to reduce albuminuria, unless this effect is counterbalanced, for example by the sodium load. This could also explain why fruits and vegetables have more positive effects than sodium bicarbonate.[38] In this regard it would be interesting to assess the effect of oral alkali given with another cation. A clinical trial that compares the effects of potassium citrate with potassium chloride and placebo on kidney outcomes in CKD stage G3b and G4 is currently ongoing and may provide more insight in this matter.[39]

A third relevant finding in our study is that sodium chloride but not sodium bicarbonate increased albuminuria. This is relevant, because the dose-finding study by Raphael *et al.* observed an increase in albuminuria with the high dose (i.e., 0.8 mEq/kg per day) but not with the low dose (i.e., 0.5 mEq/kg per day) sodium bicarbonate.[40] The effect of alkali treatment on albuminuria is most likely the result of hemodynamic changes due to the sodium load given with bicarbonate. Another possibility is that a higher urine pH resulted in the detection of more intact albumin in the assay.[41] However, the results in our study and previous studies showing that sodium bicarbonate in both high and low doses also *lowers* albuminuria[11, 15, 22] suggest that assay characteristics do not fully explain the reported changes in albuminuria.

This is the first study to analyze the effect of sodium bicarbonate on urinary renin excretion. Another strength of this study is the inclusion of two control groups. However, this study also has a number of limitations. As discussed above, the dose of sodium bicarbonate or treatment time may explain why previously observed effects on aldosterone, endothelin-1, and proteinuria were not observed in this study. Although sample size was

also modest, we recently showed in a study with a similar sample size that an acute acid load caused significant differences in urinary renin excretion between healthy subjects and patients with CKD.[37] Again, these results suggested that glomerular hyperfiltration or reduced proximal tubular reabsorption caused these changes in urinary renin. Therefore, the lack of effect of sodium bicarbonate supplementation on urinary renin likely means that no net changes in filtration or reabsorption occurred.

In conclusion, despite correction of acidosis and reduction in urinary ammonium excretion, sodium bicarbonate supplementation did not improve urinary markers of the renin-angiotensin system, endothelin-1, or proteinuria. Explanations for the lack of effect include bicarbonate dose, treatment time, or the inability of urinary renin to reflect intrarenal renin-angiotensin system activity.

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Chapter 10

Summary and conclusions
Samenvatting en conclusies
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Dankwoord

SUMMARY AND CONCLUSIONS

Salt-sensitive hypertension and metabolic acidosis are two common complications of chronic kidney disease (CKD). The studies presented in this thesis aimed to provide more insight into the pathophysiology of salt-sensitive hypertension and metabolic acidosis in CKD. In particular, emphasis is placed on the role of the renin-angiotensin system (RAS). Furthermore, new treatment strategies for hypertension in CKD are explored.

Chapter 1 provides a brief introduction into the subject and outlines the aims of the thesis.

Chapter 2 consists of a comprehensive review on the distal tubular mechanisms of salt-sensitive hypertension in CKD. Apart from reduced GFR, activation of the central or sympathetic nervous system, altered vascular reactivity, and increased renal sodium reabsorption are important contributors to hypertension in CKD. Regarding the latter, six mechanisms are introduced that explain how CKD increases distal tubular sodium reabsorption, namely (1) changes in glomerulotubular balance and tubular remodeling, (2) renin-independent aldosterone secretion, (3) activation of the intrarenal RAS, (4) the role of dietary salt, (5) metabolic acidosis, and (6) proteinuria-induced sodium reabsorption. The role of dietary salt is discussed with regard to its effects on mineralocorticoid receptor signaling and its effects on the immune system. Metabolic acidosis is linked to activation of the RAS and proteinuria and is therefore discussed as separate entity contributing to salt-sensitive hypertension.

Chapter 3 describes rat studies aimed at dissecting the interaction of dietary salt and the RAS in an experimental model of CKD. For this purpose, 5/6th nephrectomies (Nx) were performed in rats. 5/6th Nx recapitulates the hallmarks of CKD, namely a reduced glomerular filtration rate (GFR), proteinuria and salt-sensitive hypertension. First, we showed that 5/6th Nx causes a low-renin salt-sensitive form of hypertension with hyperkalemia and unsuppressed aldosterone, mimicking human CKD. Although the expression of most kidney sodium transporters was reduced after 5/6th Nx, we found that the gamma subunit of the epithelial sodium channel (ENaC) and its regulatory proteins prostasin and serum and glucocorticoid-regulated kinase 1 (SGK1) were increased. Next, several interventions were introduced to selectively block the RAS under normal and high dietary salt conditions. We showed that on a normal salt diet hypertension of the 5/6th Nx model is dependent on aldosterone and angiotensin II. On a high salt diet, hypertension was more severe and the response to angiotensin II type 1 receptor blockade with losartan, either given alone or in combination with adrenalectomy, was reduced. The only intervention that attenuated hypertension on a high salt diet was treatment

with the mineralocorticoid receptor antagonist spironolactone. Moreover, spironolactone increased natriuresis, reduced skin water content, and restored vasorelaxation, suggesting that dietary salt increases blood pressure in the CKD-model through mineralocorticoid receptor-dependent kidney and vascular mechanisms.

In ***Chapter 4***, a novel RNA-based treatment strategy that targets angiotensinogen for the treatment of hypertension is reviewed. A rat study that investigated the effect of siRNA directed against angiotensinogen (AGT) on blood pressure and kidney outcomes is presented in ***Chapter 5***. Similar to the experiments described in ***Chapter 3***, 5/6th Nx was used to model human CKD. A comparison is made between treatment with AGT siRNA alone, angiotensin II type 1 receptor blockade with losartan, dual RAS-blockade with AGT siRNA and losartan, and dual RAS-blockade with losartan and the angiotensin-converting enzyme inhibitor captopril. Monotherapy with AGT siRNA prevented the ongoing progression of hypertension in the 5/6th Nx model, while losartan, either given as single treatment or combined with AGT siRNA or captopril, lowered blood pressure. Interestingly, monotherapy with losartan reduced blood pressure more than single treatment with AGT siRNA and both dual treatments, suggesting that its antihypertensive effect is dependent on stimulation of vasodilatory angiotensin II type 2 receptors. All treatments reduced proteinuria and heart weight (a measure for cardiac hypertrophy) to a similar degree, and no intervention improved GFR, while AGT siRNA and losartan reduced glomerulosclerosis similarly. AGT siRNA lowered plasma AGT by >95% resulting in depletion of angiotensin II at tissue level. Multivariable regression analysis identified blood pressure and intrarenal angiotensin II as independent predictors of proteinuria. We conclude that AGT siRNA is effective in the treatment of hypertension in experimental CKD and that it may offer cardiorenal protection. Moreover, its renoprotective effects are blood pressure-independent and rely on suppression of renal angiotensin II formation from liver-derived AGT.

Chapter 6 describes a randomized clinical trial comparing two treatments for hypertension in CKD. Previous studies showed that dietary salt restriction effectively lowers blood pressure in CKD, but for many patients long-term adherence remains challenging. Pharmacological interventions that lower blood pressure are therefore often necessary. The study presented here investigated whether the distal diuretics hydrochlorothiazide and amiloride are as effective as dietary salt restriction using an open-label crossover design. Twenty-six patients with CKD stage G3 or G4 (eGFR 15-60 mL/min per 1.73m^2) were treated with amiloride/hydrochlorothiazide (5/50 mg/day) and dietary salt restriction (60 mmol/day), with both treatment periods lasting 2 weeks. Dietary salt restriction lowered blood pressure, whereas the effect of diuretics on blood pressure was much stronger. Both treatments lowered indices of fluid overload (body weight, NT-proBNP

and overhydration assessed by bio-impedance analysis) and caused a trend towards a reduction in albuminuria. The reduction in blood pressure was accompanied by a decrease in eGFR with both interventions, that was again stronger with diuretics. After discontinuing treatment, eGFR returned to pretreatment levels. Considering that the decrease in eGFR was transient and was associated with increased plasma renin and aldosterone levels, we conclude that the effect on eGFR was most likely caused by hemodynamic changes. Furthermore, in light of the non-inferiority design of the study, we conclude that diuretic treatment was at least as effective as dietary salt restriction for the treatment of hypertension in CKD.

Chapter 7 focuses on the RAS in patients with autosomal dominant polycystic kidney disease (ADPKD). Results from previous studies suggest that certain components of the RAS are produced in or around renal cysts. Whether such local production occurs, and if these components also end up in plasma or urine and could therefore be used as biomarker for intrarenal RAS activation, has not yet been confirmed. To investigate this, plasma and urinary angiotensinogen and renin levels were determined in 60 ADPKD patients and compared to 57 CKD patients that were matched for sex, eGFR, blood pressure and RAS-blocker use. The results show that urinary excretion of angiotensinogen and renin is 5- to 6-fold higher in ADPKD compared to CKD, while plasma renin was lower in ADPKD (in patients without RAS-blockers). In a multivariable analysis, the presence of ADPKD, low GFR, plasma renin and albuminuria predicted angiotensinogen and renin excretion. However, the correlation between albuminuria and urinary renin showed a different pattern in ADPKD than in CKD. Additionally, RAS-measurements in cyst fluid from three ADPKD-patients that underwent nephrectomy showed that the concentrations of angiotensinogen, prorenin and renin are lower in cysts than in plasma. Angiotensinogen and prorenin concentrations in urine were also lower compared to cyst fluid. In case of renin, however, fluid and urine concentrations were similar. Overall, our results do not provide evidence for ectopic production of renin in the kidney and the changes in urinary excretion may be explained by differences in tubular reabsorption in ADPKD and CKD.

In ***Chapters 8 and 9***, the interaction between the RAS and acid-base balance in CKD is studied. When GFR decreases, the capacity of the kidneys to excrete acid is impaired and patients often develop metabolic acidosis. The presence of metabolic acidosis in CKD is associated with increased cardiovascular and all-cause mortality. Yet, how metabolic acidosis leads to adverse outcomes in CKD is incompletely understood. The currently available evidence points towards an important role of the RAS and endothelin-1. Two different approaches were used to investigate if and how the RAS responds to changes in acid-base homeostasis in CKD.

First, ***Chapter 8*** describes the response of patients with CKD stage G4 (eGFR 15-30 mL/min per 1.73m^2) and healthy subjects to an oral acid load. CKD reduced the capacity for acid excretion and increased the duration of acidosis. Moreover, induction of acidosis in CKD resulted in higher concentrations of plasma potassium and plasma aldosterone, more excretion of urinary albumin and renin, and a reduction of the cumulative excretion of sodium, chloride and potassium. Together, these findings provide potential mechanisms that explain the adverse effects of metabolic acidosis in CKD, including the deleterious effects of hyperkalemia, aldosterone and proteinuria induced by acidosis.

In ***Chapter 9***, we evaluated the effect of correction of metabolic acidosis on the RAS in CKD. Forty-five patients with CKD stage G4 were treated for 4 weeks with either sodium bicarbonate, sodium chloride (providing the same dose of sodium as given with sodium bicarbonate) or received no treatment. Sodium bicarbonate caused a trend towards a reduction in plasma aldosterone and plasma potassium, but did not lead to changes in plasma renin or the urinary excretion of renin, angiotensinogen or aldosterone. Furthermore, no changes were observed in urinary endothelin-1. The lack of effect on urinary outcomes may be explained by the prescribed dose of bicarbonate. Several earlier clinical trials that used a similar dose of sodium bicarbonate also failed to show a significant effect on the primary outcome, whereas three RCTs that used a higher dose were positive. Other explanations may include factors such as CKD stage and treatment time, considering that previous studies that showed a (modest) effect of alkali treatment on urinary RAS-components also included patients with earlier stages of CKD and had treatment times lasting up to five years. Moreover, RAS-inhibitors were used by most patients in our study (88%) and may have suppressed the RAS to a point that further suppression by alkali treatment was no longer possible. A final explanation for the lack of an effect on urinary renin is that urinary renin may not reflect intrarenal activity of the RAS. This notion has emerged from recent studies showing that urinary renin excretion is mainly determined by glomerular filtration and proximal tubular reabsorption, and not by local production (i.e., intratubular conversion from prorenin or tubular secretion).

SAMENVATTING EN CONCLUSIES

Zoutgevoelige hypertensie en metabole acidose zijn twee veel voorkomende complicaties van chronische nierinsufficiëntie. Het doel van de studies in dit proefschrift is om meer inzicht te verkrijgen in de pathofysiologie van zoutgevoelige hypertensie en metabole acidose bij patiënten met chronische nierinsufficiëntie. Er wordt een bijzondere nadruk gelegd op de rol van het renine-angiotensine systeem (RAS). Ook worden nieuwe behandelingsmogelijkheden voor hypertensie bij chronische nierinsufficiëntie onderzocht.

Hoofdstuk 1 geeft een korte introductie over het onderwerp en de doelstellingen van het proefschrift worden gepresenteerd.

Hoofdstuk 2 bestaat uit een uitgebreid review waarin de pathofysiologische mechanismes van zoutgevoelige hypertensie bij chronische nierinsufficiëntie worden uiteengezet. De nadruk ligt specifiek op mechanismes in de distale tubulus van de nier. Naast een verlaagde GFR zijn activatie van het centrale of sympathische zenuwstelsel, veranderde vasculaire reactiviteit en een verhoogde neiging tot natriumreabsorptie door de nier belangrijke bijdragers aan hypertensie bij chronische nierinsufficiëntie. In het review worden zes mechanismes geïntroduceerd die verklaren hoe chronische nierinsufficiëntie leidt tot meer natriumreabsorptie, namelijk 1) veranderingen in de glomerulotubulaire balans en remodelering van de tubulus, 2) renine-onafhankelijke aldosteron secretie, 3) activatie van het intrarenale RAS, 4) de rol van zout in het dieet, 5) metabole acidose, en 6) proteïnurie-geïnduceerde natriumreabsorptie. De rol van zout in het dieet wordt besproken in relatie tot de effecten van zout op activatie van de mineralocorticoïdreceptor en het immuunsysteem. Metabole acidose wordt gerelateerd aan activatie van het RAS en proteïnurie en wordt daardoor besproken als aparte factor die kan bijdragen aan zoutgevoelige hypertensie.

Hoofdstuk 3 beschrijft rattenstudies die als doel hebben om de interactie tussen zout in het dieet en het RAS te onderzoeken in een experimenteel model voor chronische nierinsufficiëntie. Hiervoor is bij ratten een 5/6de nefrectomie (Nx) uitgevoerd. Deze operatie resulteert in de belangrijkste kenmerken van chronische nierinsufficiëntie, te weten een verlaagde glomerulaire filtratie snelheid (in het Engels: glomerular filtration rate, ofwel GFR), proteïnurie en zoutgevoelige hypertensie. Als eerste laten we zien dat 5/6de Nx leidt tot een vorm van zoutgevoelige hypertensie die lijkt op chronische nierinsufficiëntie bij mensen, namelijk met een laag plasma renine, hyperkaliëmie en een niet-onderdrukt plasma aldosteron. Ondanks dat de expressie van de meeste natriumtransporters in de nier verlaagd waren na 5/6de Nx vonden we dat het gamma-onderdeel van het epitheliale

natriumkanaal ENaC, en de regulerende eiwitten prostasin en 'serum and glucocorticoid-regulated kinase 1' (SGK1) waren verhoogd. Daaropvolgend zijn verschillende behandelingen gegeven om selectief onderdelen van het RAS te blokkeren bij ratten die een dieet kregen toegediend met een normale hoeveelheid zout of een dieet met veel zout (i.e., 10x meer). We laten zien dat hypertensie in het 5/6de Nx model afhankelijk is van aldosteron en angiotensine II tijdens een normale zoutinname. Tijdens inname van het zoutrijke dieet nam de hypertensie toe en was het bloeddrukverlagende effect van angiotensine II type 1 receptor blokkade met losartan – alleen gegeven of in combinatie met een adrenalectomie – sterk verminderd. De enige interventie die de bloeddruk verlaagde bij de ratten op een zoutrijk dieet was behandeling met de mineralocorticoidreceptor antagonist spironolacton. Daarnaast leidde behandeling met spironolacton ook tot meer natriurese, minder water in de huid en herstel van de relaxatie van bloedvaten. Concluderend suggereren onze resultaten dat de bloeddrukstijging door zout in het 5/6de Nx model wordt veroorzaakt door mineralocorticoïdreceptor-afhankelijke mechanismes in de nier en de bloedvaten.

Hoofdstuk 4 bestaat uit een review waarin een nieuwe behandelmethode om angiotensinogeen (AGT) met RNA te onderdrukken wordt beschreven. Aanvullend wordt in ***Hoofdstuk 5*** een rattenstudie gepresenteerd waarin het effect van siRNA gericht tegen AGT op de bloeddruk en nier-gerelateerde uitkomsten wordt onderzocht. Net als in de experimenten die worden beschreven in ***Hoofdstuk 3*** wordt hier ook 5/6de Nx gebruikt als model voor chronische nierinsufficiëntie bij mensen. Er wordt een vergelijking gemaakt tussen behandeling met alleen AGT siRNA, alleen angiotensine II type 1 receptor blokkade met losartan, duale RAS-blokkade met AGT siRNA en losartan en duale RAS-blokkade met losartan en captopril, een remmer van het angiotensine-converterend-enzym. Behandeling met alleen AGT siRNA voorkwam de voortdurende progressie van hypertensie in het 5/6de Nx model, terwijl losartan, gegeven als enige behandeling of samen met AGT siRNA, de bloeddruk verlaagde. Een interessante uitkomst is dat monotherapie met losartan de bloeddruk sterker verlaagde dan monotherapie met AGT siRNA en beide behandelingen met duale RAS-blokkade. Deze resultaten doen vermoeden dat het bloeddrukverlagende effect van losartan afhankelijk is van activatie van de vaatverwijdende angiotensine II type 2 receptoren. Alle behandelingen leidden tot een gelijkwaardige verlaging van proteïnurie en hartgewicht (een maat voor cardiale hypertrofie) en geen enkele interventie resulteerde in verbetering van de GFR. AGT siRNA en losartan verminderden glomerulosclerose in gelijke mate. AGT siRNA verlaagde de plasma AGT concentratie met > 95%, wat resulteerde in depletie van angiotensine II op weefselniveau. Met een multivariabel regressiemodel werden bloeddruk en intrarenaal angiotensine II geïdentificeerd als onafhankelijke voorspellers van proteïnurie. We concluderen dat AGT siRNA effectief is voor de behandeling van hypertensie in een

experimenteel model voor chronische nierinsufficiëntie en dat deze behandeling bij kan dragen aan het voorkomen van hart- en nierschade. Daarnaast is het beschermende effect op de nieren niet afhankelijk van de bloeddruk en wel afhankelijk van onderdrukking van de productie van angiotensine II in de nier, dat afkomstig van angiotensinogeen in de lever.

Hoofdstuk 6 beschrijft een gerandomiseerde klinische studie waarin twee behandelingen voor hypertensie bij patiënten met chronische nierinsufficiëntie worden vergeleken. Eerdere studies laten zien dat een zoutbeperking effectief de bloeddruk verlaagd bij patiënten met chronische nierinsufficiëntie, maar het is voor patiënten vaak moeilijk om dit dieet voor langere tijd vol te houden. Farmacologische middelen om de bloeddruk te verlagen zijn daarom vaak nodig. In deze open-label cross-over studie is onderzocht of thiazidediuretica even effectief zijn als een zoutbeperking. Zesentwintig patiënten met chronische nierinsufficiëntie stadium G3 of G4 (eGFR 15-60 ml/min per 1.73m^2) zijn behandeld met amiloride/hydrochloorthiazide (5/50 mg/dag) en een zoutbeperking (60 mmol/dag). Beide behandelperiodes duurden 2 weken. De behandeling met een zoutbeperking verlaagde de bloeddruk, maar het effect van diuretica op de bloeddruk was veel sterker. Ook verlaagden beide behandelingen maten voor overvulling, namelijk lichaamsgewicht, plasma NT-proBNP en 'overhydratie' (geanalyseerd met bio-impedantiemetingen), en was er een trend naar vermindering van albuminurie. Bij beide behandelingen ging de verlaging van de bloeddruk gepaard met een verlaging van de eGFR, die ook weer sterker was met diuretica. Na het stoppen van de behandelingen normaliseerde de eGFR naar het niveau van voor de behandeling. Omdat de daling van de eGFR van tijdelijke aard was en gepaard ging met een stijging van plasma renine en aldosteron concentraties, concluderen we dat het effect op de eGFR meest waarschijnlijk werd veroorzaakt door hemodynamische veranderingen. Omdat de studie is opgezet als 'non-inferiority'-studie concluderen we daarnaast dat diuretica in ieder geval even effectief zijn als een zoutbeperking voor de behandeling van hypertensie bij chronische nierinsufficiëntie.

In ***Hoofdstuk 7*** staat de activatie van het RAS bij patiënten met autosomaal dominante polycysteuze nierziekte (ADPKD) centraal. Resultaten uit eerdere studies suggereren dat er in en rondom cysten in de nier bepaalde componenten van het RAS worden geproduceerd. Of dit werkelijk het geval is, en of deze componenten ook in de circulatie en in de urine terecht komen, en dus gebruikt zouden kunnen worden als biomarker voor activatie van het intrarenale RAS, is nog niet bekend. Om dit te onderzoeken is er bij 60 patiënten met ADPKD angiotensinogeen en renine bepaald in plasma en urine en werden deze uitkomsten vergeleken met angiotensinogeen- en reninewaarden van 57 patiënten met chronische nierinsufficiëntie die waren gematcht op geslacht, eGFR,

bloeddruk en gebruik van RAS-blokkers. De resultaten laten zien dat de uitscheiding van angiotensinogeen en renine in de urine 5 tot 6 keer hoger is bij ADPKD dan bij CKD, maar dat plasma renine lager is bij ADPKD (bij patiënten die geen RAS-blokkers gebruiken). In een multivariabel regressiemodel voorspelden de aanwezigheid van ADPKD, een lagere eGFR, plasma renine en albuminurie de excretie van angiotensinogeen en renine. Echter, de correlatie tussen albuminurie en urine renine liet een ander patroon zien bij ADPKD dan bij CKD. Daarnaast lieten RAS-metingen in cystevloeistof bij drie patiënten met ADPKD die een nefrectomie ondergingen zien dat de concentraties van angiotensinogeen, prorenine en renine lager zijn in cysten dan in plasma. Ook waren de concentraties van angiotensinogeen en prorenine in urine lager dan in cystevloeistof. De concentraties van renine waren daarentegen wel hetzelfde in cystevloeistof en in urine. Concluderend leveren onze resultaten geen bewijs voor ectopische productie van renine in de nier en de variaties in excretie kunnen worden verklaard door een verschillende mate van tubulaire reabsorptie bij ADPKD en CKD.

In ***Hoofdstuk 8 en 9*** wordt de interactie tussen het RAS en de zuur-base balans bij CKD onderzocht. De capaciteit van de nieren om zuur uit te scheiden neemt af als de GFR daalt en hierdoor ontwikkelen patiënten vaak een metabole acidose. De aanwezigheid van een metabole acidose bij chronische nierinsufficiëntie is geassocieerd met een hoger risico op sterfte (zowel cardiovasculair als niet-cardiovasculair). Hoe metabole acidose leidt tot schadelijke uitkomsten bij chronische nierinsufficiëntie is niet goed bekend. Het huidige beschikbare bewijs suggereert dat het RAS en endotheline-1 een belangrijke rol spelen in de pathofysiologie van metabole acidose. In deze hoofdstukken worden twee verschillende strategieën gebruikt om te onderzoeken of en hoe het RAS reageert op veranderingen in de zuur-base homeostase bij chronische nierinsufficiëntie.

Hoofdstuk 8 beschrijft hoe patiënten met chronische nierinsufficiëntie stadium G4 (eGFR 15-30 mL/min per 1.73m^2) en gezonde controles reageren op een orale zuurbelasting. De capaciteit om zuur uit te scheiden werd verminderd door de aanwezigheid van chronische nierinsufficiëntie en de duur van de acidose was langer bij de patiënten. Daarnaast resulteerde inductie van acidose bij chronische nierinsufficiëntie in hogere concentraties van plasma kalium en plasma aldosteron, meer excretie van urine albumine en renine, en een verminderde cumulatieve excretie van natrium, chloride en kalium. Samenvattend verschaffen deze resultaten potentiele mechanismes die de negatieve effecten van metabole acidose bij chronische nierinsufficiëntie kunnen verklaren, zoals de schadelijke acidose-geïnduceerde effecten van hyperkaliëmie, aldosteron en proteïnurie.

In ***Hoofdstuk 9*** wordt het effect van correctie van metabole acidose op het RAS geëvalueerd bij chronische nierinsufficiëntie. Vijfenveertig patiënten met chronische nierinsuf-

ficiëntie stadium G4 werden gedurende 4 weken behandeld met natriumbicarbonaat, natriumchloride (met een gelijkwaardige hoeveelheid natrium als werd gegeven met natriumbicarbonaat) of geen behandeling. Natriumbicarbonaat veroorzaakte een trend naar een verlaging van het plasma aldosteron en het plasma kalium, maar leidde niet tot veranderingen van plasma renine of de excretie van renine, angiotensinogeen en aldosteron in urine. Daarnaast werden geen veranderingen gezien in de urine excretie van endotheline-1. Een mogelijke verklaring voor het feit dat er geen effecten op urine-uitkomsten zijn gevonden is dat de voorgeschreven dosis van het bicarbonaat te laag was. Verschillende eerdere klinische studies waarin een soortgelijke dosering werd gebruikt lieten ook geen significante verschillen zien op de primaire uitkomstmaat. Drie andere RCT's waarin een hogere dosering werd gebruikt waren daarentegen wel positief. Overige factoren die het gebrek aan significante verschillen kunnen verklaren zijn het stadium van chronische nierinsufficiëntie en de behandelduur. In eerdere studies waarin een effect werd gezien van alkali-behandeling op urine RAS-markers werden ook patiënten met een vroeger stadium van chronische nierinsufficiëntie geïncludeerd en werden patiënten voor periodes tot 5 jaar behandeld. Daarnaast gebruikten de meeste patiënten in onze studie een RAS-blokker (88%) en mogelijk werd het RAS hierdoor onderdrukt tot een niveau waarbij geen verdere onderdrukking meer mogelijk was. Een laatste verklaring voor de observatie dat de uitscheiding van urine renine niet veranderd tijdens correctie van een metabole acidose is dat urine renine geen uitleesmaat is voor activiteit van het intrarenale RAS. Deze onderkenning komt voort uit recente studies die laten zien dat de excretie van renine in de urine vooral wordt bepaald door glomerulaire filtratie en proximale tubulaire reabsorptie, en niet door lokale productie in de nier (i.e., intratubulaire omzetting van prorenine of tubulaire secretie).

DISCUSSION AND FUTURE DIRECTIONS

Identifying determinants of hypertension in chronic kidney disease (CKD) is important to improve treatment strategies. To do so, a better understanding of the pathophysiology of hypertension in CKD is required.[1] An important characteristic of hypertension in CKD is an increased extracellular fluid volume.[2] In ***Chapter 2*** we propose that increased sodium reabsorption by the distal nephron is a main driving force for extracellular fluid expansion in CKD. Plasma aldosterone and plasma potassium are two important regulators of sodium reabsorption in the distal tubule and both are often elevated in CKD (***Chapter 2***). However, it is incompletely understood if and to what degree both factors contribute to hypertension in CKD. While aldosterone is an anti-natriuretic hormone that increases activity of the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC), plasma potassium acts as a natriuretic factor that suppresses activity of NCC. Moreover, plasma potassium also directly stimulates aldosterone secretion. In a clinical trial, the potassium binder patiromer reduced hyperkalemia, aldosterone, and blood pressure in patients with CKD, suggesting that potassium-induced aldosterone secretion contributes to hypertension in CKD.[3] How plasma potassium regulates NCC is becoming increasingly clear.[4] Recent studies indicate that extracellular potassium raises the membrane potential of the basolateral membrane of the distal convoluted tubule, which leads to an intracellular signaling cascade that dephosphorylates NCC.[4] Essential for this mechanism are the potassium channels Kir4.1 and Kir5.1 which 'sense' extracellular potassium at the basolateral membrane. In ***Chapter 3***, we analyzed how CKD changes the abundance of kidney sodium and potassium transporters in an animal model of CKD, namely the 5/6th nephrectomy (Nx) model. Previous experimental studies demonstrated that induction of CKD in either rats or mice increases the abundance of NCC, α-ENaC and ɣ-ENaC, but the relation between sodium transporter abundance and activity of the renin-angiotensin system (RAS) or plasma potassium has not yet been investigated.[5-7] In our study, 5/6th Nx *decreased* NCC and α-ENaC, whereas β-ENaC remained unchanged and ɣ-ENaC was increased. Moreover, 5/6th Nx increased Kir4.1, whereas it decreased Kir5.1. Further analysis showed that higher plasma renin was associated with higher protein abundances of NCC and α-ENaC, and higher serum potassium (potassium was measured in serum and not in plasma in our study) was associated with lower (p)NCC and Kir5.1, suggesting that both the RAS and potassium regulate sodium transporter activity in CKD. Several factors may explain the differences between the reported sodium transporter abundances in our study and previous studies, including different experimental models of CKD,[7] lower serum potassium in other CKD-models,[7] differences in the time-period between the measurement of protein abundances and 5/6th Nx,[5, 6] and alternative methods for protein normalization.[5-7] In previous studies, transporter abundances were normalized based on protein concentration in the kidney

homogenates of healthy and 5/6th Nx rats. However, protein composition changes considerably after 5/6th Nx with extracellular matrix expansion and fibrosis due to compensatory adaptations of remaining nephrons.[8] To account for these changes, we used a novel method for normalization based on kidney weight, which approximates normalization by nephron number.[9, 10]

The observation that sodium transporter activity is regulated by potassium suggests that increasing dietary potassium intake may be an effective strategy to lower blood pressure in CKD. Previous studies in hypertensive patients without CKD showed that a higher dietary potassium intake reduces blood pressure.[11, 12] Currently, a randomized clinical trial is ongoing that will evaluate if dietary potassium supplementation lowers blood pressure and is renoprotective in patients with CKD stage G3B and G4.[13] However, it is equally important to gain more insight in the mechanisms of how dietary potassium regulates blood pressure in CKD. At present, the most eligible model to study these mechanisms is the 5/6th Nx model, because this model is characterized by the hallmarks of CKD, including low GFR, proteinuria, hyperkalemia, and hypertension (***Chapter 3***). Moreover, the 5/6th Nx model shows high reproducibility (e.g., compared to the vascular ligation model)[14] and offers several practical advantages to models that require other species, have much shorter or much longer time courses, or are more time-consuming.[15] As with any animal model, however, a caveat is that 5/6th Nx is not fully representative of human CKD as it specifically causes hyperfiltration, which is not a characteristic of many CKD-etiologies.[8] In addition to determining if and how dietary potassium reduces blood pressure (i.e., through vascular or kidney mechanisms), a number of questions should be addressed, including whether potassium confers renoprotection (as previously suggested),[12] whether potassium can be combined with RAS blockers (i.e., this may lead to exaggerated hyperkalemia), and to what degree potassium-induced increases in aldosterone are deleterious in CKD. This last question is particularly relevant considering that potassium-induced aldosterone secretion may contribute to hypertension in CKD.[3, 16] If this mechanism exceeds the blood pressure-lowering effects of dietary potassium, hyperkalemia should be avoided in CKD. Indeed, data from previous studies indicates that plasma potassium concentrations between 4.0 and 5.0 mmol/L are associated with a lower rate of adverse outcomes in CKD, whereas higher or lower plasma potassium levels are associated with more adverse outcomes.[17] Therefore, another question that may need to be addressed is whether titrating plasma potassium to a certain concentration range (e.g., using dietary potassium or potassium binders) is beneficial in CKD.

In ***Chapter 6*** we show that targeting NCC and ENaC with the combination of hydrochlorothiazide and amiloride in patients with CKD stage G3 and G4 effectively lowers extracellular volume and blood pressure. While non-renal effects of hydrochlorothiazide on

blood pressure cannot be fully excluded, the pronounced reduction in extracellular volume suggests that NCC, ENaC, or both are either more active or insufficiently suppressed in patients with CKD. Of note, plasma potassium did not change during treatment with this combination of kaliuretic and potassium-sparing diuretics. Furthermore, in patients with a lower eGFR, diuretic clearance was reduced while the antihypertensive effect remained intact, suggesting that CKD limits pharmacokinetics but not pharmacodynamics of distal diuretics (***Chapter 6***).[18] Although several previous studies similarly showed that thiazides reduce blood pressure in CKD, large RCTs powered for hard endpoints are still missing. Therefore, an important question that needs to be addressed in future studies is if distal diuretics also confer cardiovascular- and renoprotection. Moreover, thiazides – either in combination with amiloride or given alone – reduce GFR, and long-term intervention studies are required to see whether the beneficial effects on extracellular fluid volume and blood pressure outweigh the hemodynamic effects on GFR. Another issue that should be addressed is whether distal diuretics are more effective in specific patient-categories, for instance in women. Data from preclinical studies suggest that NCC is more active in females than in males.[19-21] Also, several clinical studies in hypertensive patients without CKD indicate that women are more responsive to thiazides than men. A small randomized placebo-controlled crossover trial demonstrated that premenopausal women receiving hormone-replacement therapy show a greater antihypertensive response to thiazides than premenopausal women without such therapy.[22] Moreover, in a secondary analysis of the INCLUSIVE study that included 1005 patients with uncontrolled hypertension, female sex was identified as a predictor for the blood pressure response to irbesartan/hydrochlorothiazide.[23] Also, in a smaller randomized crossover trial in 52 hypertensive patients of African descent, the blood pressure response to hydrochlorothiazide was stronger in women than in men.[24] However, in a larger and more heterogeneous population, differences in thiazide-responsiveness between women and men were not reported.[25] Possibly, this is due to the fact that sex-differences in the blood pressure-response to thiazide diuretics are only present in certain subpopulations, such as older patients or black patients. Questions regarding thiazide-safety and effectiveness in CKD are currently addressed in the CLICK trial.[26] This study completed its inclusion phase last year and included 131 patients with CKD stage G4. The primary outcome of the trial is 24-hour systolic blood pressure and secondary outcomes include adverse events and markers of extracellular volume and kidney damage. Furthermore, it will be interesting to see how distal diuretics compare with other sodium reducing strategies, such as dietary potassium, which is currently under investigation in CKD stage G3B and G4[13], and sodium-glucose co-transporter 2 (SGLT2) inhibitors. SGLT2 inhibitors were initially designed with the aim to improve glycemic control in patients with diabetes mellitus. These drugs reduce plasma glucose levels by inhibition of sodium-glucose reabsorption in the proximal tubule. As a result, SGLT2 inhibitors increase glucosuria and

natriuresis.[27] Interestingly, a recent study showed that SGLT2 inhibition not only targets the proximal tubule, but also reverses diabetes-induced NCC activation through inhibition of Kelch-like 3.[28] Indeed, in patients with type 2 diabetes SGLT2 inhibition and NCC blockade with thiazide diuretics both reduced blood pressure, body weight and GFR.[29] However, the corresponding rise in plasma renin and plasma aldosterone that usually accompanies a reduction in blood pressure and extracellular volume, was much lower with SGLT2 inhibition than with thiazide treatment. In another study SGLT2 inhibition also lowered extracellular volume (measured by bioimpedance spectroscopy) in patients with diabetes. In this study, plasma renin and aldosterone were initially increased after the initiation of treatment, but the levels normalized after 3 months and were reduced after 6 months.[30] A similar effect of SGLT2 inhibitors on the RAS was reported in experimental CKD. When given to 5/6th Nx rats, the SGLT2 inhibitor TA-1887 increased urinary volume and reduced body weight, but plasma renin and kidney angiotensin II remained unchanged.[31] An explanation for the observation that renin is not consistently upregulated during SGLT2 inhibition may be that blockade of proximal tubular sodium reabsorption causes suppression of renin secretion due to increased sodium delivery to the macula densa.[32] Another explanation may be that SGLT2 inhibitors reduce activity of the sympathetic nervous system and thereby inhibit renin release.[33, 34] These 'off target' effects of SGLT2 inhibitors on NCC, the RAS and the sympathetic nervous system may contribute to the beneficial effects that SGLT2 inhibitors have on blood pressure and kidney outcomes and therefore deserve further investigation. Several large placebo-controlled trials demonstrated beneficial effects of SGLT2-inhibitors on cardiovascular endpoints, progression of kidney disease and mortality in patients with type 2 diabetes and CKD stage G3-5.[35-37] Recently, Heerspink *et al.* also showed that CKD-patients (eGFR 25 to 75 mL/min per 1.73 m^2) with and *without* diabetes benefit from treatment with SGLT2 inhibitors.[38] The SGLT2 inhibitor dapagliflozin reduced blood pressure, slowed CKD-progression and lowered the risk of mortality compared to placebo. The most frequently observed side-effect of SGLT2 inhibitor treatment was volume depletion and the previously reported higher risk of amputation was not found. Future clinical studies focusing on SGLT2 inhibitor treatment in CKD should explore if there are factors that determine the response to treatment (such as eGFR or the degree of albuminuria), identify additional groups of patients that may benefit from SGLT2 inhibitor treatment (such as kidney transplant recipients), and determine how SGLT2 inhibitors should be used in combination with other glucose-lowering agents.

Chapter 3 demonstrates that the mineralocorticoid receptor (MR) could be a promising treatment target for hypertension in CKD, especially in the context of high dietary sodium intake. Previous studies showed that MR antagonists are effective for resistant hypertension in patients with an eGFR > 45 mL/min per 1.73m^2.[39] Moreover, a recent

meta-analysis (n = 5745) concluded that MR antagonists given on top of RAS inhibitors may reduce blood pressure and proteinuria in CKD stage G2-3, although the effects on mortality, cardiovascular and kidney outcomes were inconclusive.[40] Other studies showed that spironolactone decreased cardiovascular morbidity and mortality in patients with end-stage kidney disease.[41, 42] Whether MR antagonists lower blood pressure and protect against long-term adverse outcomes in patients with an eGFR < 30 mL/min per 1.73m^2 was recently addressed in the FIDELIO trial. This was a large placebo-controlled trial that included diabetic patients with an eGFR between 25 and 60 mL/min per 1.73m^2.[43] Treatment with the novel non-steroidal MR antagonist finerenone prevented CKD progression and cardiovascular events. Finerenone was given on top of RAS inhibition and had a moderate effect on blood pressure, but lowered albuminuria significantly. Hyperkalemia, a common side effect of steroidal MR antagonists that often necessitates discontinuation of treatment was present in 11.8% of patients on finerenone and resulted in treatment discontinuation in 2.3% of patients (compared to 0.9% during placebo). No fatal events due to hyperkalemia were reported. Moreover, previous studies showed that the risk of hyperkalemia with finerenone is lower than with the older MR antagonists spironolactone and eplerenone, indicating that finerenone has a more favorable safety profile.[44-46] Taken together, the results from the recent finerenone trial are encouraging and more studies are necessary to clarify if finerenone is beneficial and safe in patients without type 2 diabetes, in patients with an eGFR < 25 mL/min per 1.73m^2, or in kidney transplant recipients. Finally, head-to-head studies between finerenone and the other MR antagonists are warranted to compare their renoprotective effects. Data from preclinical and smaller clinical studies comparing finerenone with spironolactone and eplerenone suggest that finerenone may be equally effective in preventing target organ damage in patients with CKD and heart failure.[46] Of note, other novel MR antagonists are in clinical development as well, such as the non-steroidal KBP-5074. A currently ongoing placebo-controlled trial will evaluate whether KBP-5074, when given on top of RAS blocker therapy, lowers blood pressure and prevents CKD progression in patients with CKD stage G3B-G4 and resistant hypertension.[47]

Another line of research investigates the use of drugs targeting Ras-related C3 botulinum toxin substrate 1 (Rac1). These drugs are still experimental and clinical studies have not been reported. Fujita *et al.* showed that dietary salt induces MR activation in animal models with normal kidney function and that this effect is mediated by Rac1.[48, 49] In the same studies they subsequently showed that the Rac1 inhibitor EHT1864 reduced blood pressure in animals receiving a high salt diet. A recent study also showed that increased activation of Rac1 in podocytes causes proteinuria and is important in the pathogenesis of proteinuric kidney disease.[50] Since proteinuria may increase sodium reabsorption (***Chapter 2***), Rac1 activation in podocytes may contribute indirectly to hypertension

in CKD. In ***Chapter 3*** we show that 5/6th Nx causes vascular dysfunction in rats on a high salt diet and that blood vessel function under these conditions is improved by spironolactone. Interestingly, previous investigators identified Rac1 in endothelial cells of the vasculature as a contributor to endothelial damage using isolated vessels from hypertensive mice and diabetic mice.[51, 52] In a follow-up study, the same investigators showed that pharmacological blockade of Rac1 with NSC23766 improved endothelial function in veins resected from patients that underwent surgery for venous or arterial insufficiency.[53] Taken together, these data may point towards a role for Rac1 in the pathophysiology of hypertension in CKD. Therefore, future experimental studies aimed at determining the relevance of Rac1 in blood pressure regulation in CKD are needed. In our study (***Chapter 3***) we did not observe any changes in total kidney expression of Rac1. Moreover, to date, no experimental studies have investigated the effects of Rac1 inhibitors in animal models of CKD. If Rac1 is identified as potential target in CKD, a relevant question is if Rac1 inhibitors reduce blood pressure in CKD, and, if so, how the effects on blood pressure, blood vessel reactivity and sodium transporter activity compare to MR antagonists.

Targeting the RAS is an effective strategy to lower blood pressure and slow the progressive loss of kidney function in CKD.[54] However, long-term adherence to hypertension treatment, whether this consists of dietary advice or pharmacological drug prescriptions, remains challenging for many patients.[55] ***Chapter 4*** describes the application of a novel RNA-based therapy that suppresses the production of liver-specific angiotensinogen (AGT) – the precursor of all angiotensins. The advantages of this new class of drugs are that it may be resistant to the counterregulatory rises of renin limiting the efficacy of 'typical' RAS inhibitors,[56] and that the dosing frequency may be reduced to a few times per year. At present, multiple RNA-based therapies have been approved for clinical use, including drugs for the treatment of familial hypercholesterolemia, spinal muscular atrophy, Duchenne muscular dystrophy, and hereditary transthyretin-mediated amyloidosis. Two types of RNA-based therapies suppressing AGT are currently available, namely AGT antisense oligonucleotides (ASOs) and AGT siRNA. In ***Chapter 5*** we describe the first preclinical study evaluating the antihypertensive effect of liver-specific AGT siRNA in an animal model of CKD (5/6th Nx). We showed that AGT siRNA reduced blood pressure, proteinuria and glomerulosclerosis and that the renoprotective effect is independent of blood pressure. Interestingly, monotherapy with losartan caused a greater reduction in blood pressure than AGT siRNA either given alone or in combination with losartan. In a previous study in spontaneously hypertensive rats (SHRs), AGT siRNA lowered blood pressure to a similar degree as valsartan and captopril, and the combination of AGT siRNA and valsartan reduced blood pressure even further.[57] Why the antihypertensive effect of AGT siRNA is less pronounced in the 5/6th Nx model is unclear.

Possibly, this difference is explained by the level of AGT knockdown, which was 97.9 ± 1.0% after AGT siRNA monotherapy in the SHRs *versus* 96.5 ± 1.4% in the 5/6th Nx rats. Small differences in AGT suppression may matter for the blood-pressure lowering effect, considering that 99.8 ± 0.1% knockdown of plasma AGT caused an even greater blood pressure-reduction in the SHRs. Another explanation may be that hypertension in the 5/6th Nx model is less dependent on the RAS than it is in SHRs. The data presented in ***Chapter 3*** suggest that hypertension in the 5/6th Nx model (during normal salt intake) depends largely on angiotensin II and aldosterone. However, the degree of renin upregulation during RAS blockade is much less pronounced compared to SHRs (approximately 10 times less), indicating that RAS responsiveness may be limited in this model. Finally, more activation of the vasodilatory angiotensin type 2 receptor by angiotensin II in the 5/6th Nx model may also explain inter-model differences. Previous studies showed that angiotensin type 2 receptor levels are elevated after 5/6th Nx and that treatment with losartan increases these levels even further.[58, 59] Taken together, the promising results of AGT siRNA in experimental studies merit further study aimed at increasing our understanding of the mechanisms of RNA-based RAS blockade. Currently, a randomized, double-blind, placebo-controlled trial evaluating the antihypertensive effect of a single injection of AGT siRNA in patients with mild to moderate hypertension is ongoing (www.clinicaltrials.gov, identification number: NCT03934307). Patients are randomized to treatment dosages ranging from 10 mg to 200 mg AGT siRNA. A recently presented interim analysis including the first 60 patients showed that a single dose of 200 mg AGT siRNA suppressed plasma angiotensinogen by 94.9 percent and reduced systolic blood pressure by 11 ± 2 mmHg at 8 weeks after injection.[60] Moreover, AGT siRNA was well tolerated and caused no major side effects, including no kidney function decline, hyperkalemia, or hypotension requiring intervention. If AGT siRNA keeps performing well in clinical trials, comparator studies will be required to position RNA-based RAS inhibitors in the broad landscape of hypertension treatment. First, studies are needed to define which patient categories benefit from AGT siRNA treatment. The results presented in ***Chapter 5*** show that AGT siRNA confers renoprotection in the 5/6th Nx model, suggesting that RNA-based RAS inhibition may be beneficial in CKD. Because RAS inhibitors are currently prescribed to virtually all patients with CKD, comparative studies between RAS inhibitors and AGT siRNA should be conducted. If similar or better outcomes with AGT siRNA are found, a major advantage contributing to clinical implementation will be practical applicability (i.e., long-lasting effects after a single injection). However, in case AGT siRNA proves inferior to current RAS inhibitors, adding AGT siRNA to other pharmacological treatment regimens, including other RAS inhibitors, may still be an option. A specific concern of the new RNA-based RAS inhibitor is its long-lasting effect. A single dose of AGT siRNA may have a sustained effect on blood pressure lasting weeks to months (***Chapter 4***). In patients that become hypovolemic during AGT siRNA treatment

maintaining adequate blood pressure levels may require specific treatments to reverse its effects (irrespective of the cause of hypovolemia). Current therapies for excessive RAS blockade (e.g., during intoxications with RAS blockers) include intravenous fluid infusion and treatment with vasopressors (e.g., dopamine, norepinephrine). If these agents are similarly effective in hypovolemic patients using AGT siRNA should be addressed in future studies. Similarly, studies should show how other possible adverse effects of AGT siRNA (i.e., hyperkalemia and kidney failure) should be treated. Of interest, the potential renal adverse effects of AGT ASOs were recently studied in a preclinical study by Mullick *et al.*.[61] Administration of a liver-specific AGT ASO to salt-deprived 5/6th Nx rats did not result in changes in creatinine clearance or proteinuria, suggesting that it has a more favorable safety profile than a non-liver specific AGT ASO that worsened kidney function and proteinuria under similar conditions.[61] Lastly, too much RAS blockade may be undesirable in CKD. Previous studies showed that hyperkalemia, hypotension, and kidney failure occur more frequently with dual RAS blockade than with single RAS blockade in CKD, without having an additional protective effect on mortality.[62] However, a recent meta-analysis concluded that dual RAS treatment may be safe in specific patients with CKD, specifically those with diabetic kidney disease and hypertension.[63] Therefore, dual RAS treatment including AGT siRNA may potentially prove beneficial in CKD and future studies are required to determine clinical safety.

In ***Chapter 8*** and ***Chapter 9*** we demonstrate that plasma aldosterone is increased during metabolic acidosis in CKD. Elevated levels of plasma aldosterone may contribute to fluid retention and hypertension in CKD (***Chapter 2***). However, whether metabolic acidosis leads to more fluid retention and hypertension in CKD is incompletely understood. Interestingly, a previous study showed that metabolic acidosis augments the blood pressure response to exercise in CKD, suggesting dysregulation of blood pressure during metabolic acidosis.[64] Studies in patients with hypertension and CKD, however, did not identify metabolic acidosis as an independent risk factor for hypertension.[65] If metabolic acidosis contributes to hypertension in CKD, a relevant question is whether correction of metabolic acidosis improves hypertension treatment and treatments aimed at fluid reduction in CKD. Another focus of research should address if sodium, potassium or the accompanying anion is important for antihypertensive and renoprotective effects of alkali-treatments in CKD. Multiple studies showed that sodium bicarbonate and dietary acid reduction with fruits and vegetables prevent the decline of kidney function in patients with CKD and metabolic acidosis.[65] However, dietary interventions reduced systolic blood pressure, whereas sodium bicarbonate had no antihypertensive effect.[66, 67] Possibly, the differences in blood pressure between both interventions is explained by the presence of the sodium ion that accompanies bicarbonate. Another explanation may be that potassium intake was higher in the diet group, although plasma potassium

did not change over time.[67] The ongoing 'K^+ in CKD study' will show whether the anion is important as this study evaluates the antihypertensive and renoprotective effects of potassium chloride and potassium citrate in CKD.[13] To date, only one large randomized placebo-controlled trial showed that alkali treatment prevents the decline in kidney function *and* reduces mortality in patients with CKD and metabolic acidosis.[68] The prescribed dose of sodium bicarbonate in this trial was titrated to reach a blood bicarbonate concentration between 24-28 mEq/L, with the final bicarbonate dose ranging from 0.8 to 1.1 mEq/kg per day. In contrast, another large randomized controlled trial including acidotic patients with CKD failed to show beneficial effects of sodium bicarbonate on physical function or renal function and used a lower dose, similar to the dose used in the clinical trial presented in ***Chapter 9*** (approximately 0.5 mEq/kg per day).[69] The lack of clinical effects with lower bicarbonate dosages and the positive outcomes with higher dosages suggest that the clinical effects of alkali treatment are dose-dependent. Indeed, in a recent dose-finding study, Raphael *et al.* showed that a sodium bicarbonate dose of 0.8 mEq/kg per day increased serum bicarbonate and lowered urinary ammonium more than a dose of 0.5 mEq/kg per day.[70] Therefore, in future trials that aim to evaluate the effects of bicarbonate in CKD, bicarbonate dosages of 0.8 mEq/kg per day or higher are recommended.

Chapter 8 shows that the excretion of urinary renin is increased after induction of metabolic acidosis in CKD. The increase in renin excretion occurred specifically in patients with CKD and not in healthy controls. We did not analyze by which mechanism urinary renin excretion increased, but the available evidence indicates that the higher excretion pattern was due to hyperfiltration rather than a reduction in proximal reabsorption of renin. Indeed, in a previous experimental study in healthy mice, induction of metabolic acidosis caused hyperfiltration, which was then followed by a progressive decline in kidney function.[71] However, whether hyperfiltration contributes to the adverse effects of metabolic acidosis in patients with CKD is still unknown. Hyperfiltration may promote proteinuria, which is a known risk factor for CKD progression. To what degree filtered renin itself contributes to kidney injury during metabolic acidosis is incompletely understood as well. Future studies are needed to address these questions.

Finally, in ***Chapter 7*** we show that the excretion of RAS components including renin and angiotensinogen is increased in patients with autosomal dominant polycystic kidney disease (ADPKD). This specific pattern of increased renin and angiotensinogen excretion was not observed in patients with CKD. In contrast to the mechanism of increased renin excretion in patients with CKD and metabolic acidosis (***Chapter 8***), the increase in renin and angiotensinogen excretion in ADPKD is most likely caused by a reduction in proximal tubular reabsorption. Whether an increase in urinary excretion of RAS components con-

tributes to hypertension and cyst formation in ADPKD is incompletely understood, but targeting the intrarenal RAS with siRNA did prevent cyst formation in an animal model of ADPKD.[72, 73] Increases in tubular renin and angiotensinogen concentrations may lead to (more) intratubular formation of angiotensin II. However, angiotensin II formation in tubule segments distal to the proximal tubule remains uncertain and should be examined in future studies.

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CURRICULUM VITAE

The author of this thesis was born on November 4th 1986. After graduating from secondary school at the Regionale Scholengemeenschap Goeree-Overflakkee in Middelharnis in 2005, he started studying biomedical sciences at the University of Amsterdam. Two years later, he began his medical training at the Erasmus University in Rotterdam. During a research elective at the Division of Endocrinology under the supervision of prof. dr. Aart-Jan van der Lelij, he developed a keen interest for internal medicine. Furthering this interest, he completed his fourth-year research project at the Division of Nephrology and Transplantation under the supervision of dr. Michiel Betjes, drs. Marcia Kho and dr. Nicole van Besouw. He received his medical degree in 2014 and subsequently worked for nine months at the IJsselland Hospital in Capelle aan den IJssel. In March 2015 he started his PhD trajectory under the supervision of prof. dr. Ewout Hoorn and prof. dr. Jan Danser at the Divisions of Nephrology and Transplantation and Pharmacology and Vascular Medicine. The work that he performed under their supervision resulted in this thesis. His PhD project was funded by the Dutch Kidney Foundation and had a translational character, including both clinical and experimental studies. In March 2020 he returned to the IJsselland Hospital and from January 1st 2021 he started his training in internal medicine at the Erasmus Medical Center in Rotterdam (program director dr. Adriënne Zandbergen).

PUBLICATIONS

Salih M, **Bovée DM**, Roksnoer LCW, Casteleijn NF, Bakker SJL, Gansevoort RT, Zietse R, Danser AHJ, Hoorn EJ. Urinary renin-angiotensin markers in polycystic kidney disease. Am J Physiol Renal Physiol. 2017 Oct 1;313(4):F874-F881.

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Hoorn EJ, **Bovée DM**, Geerse DA, Visser WJ. Diet-Exercise-Induced Hypokalemic Metabolic Alkalosis. Am J Med. 2020 Nov;133(11):e667-e669.

Bovée DM, Janssen JW, Zietse R, J Danser AH, Hoorn EJ. Acute acid load in chronic kidney disease increases plasma potassium, plasma aldosterone and urinary renin. Nephrol Dial Transplant. 2020 Oct 1;35(10):1821-1823.

Bovée DM, Cuevas CA, Zietse R, Danser AHJ, Mirabito Colafella KM, Hoorn EJ. Salt-sensitive hypertension in chronic kidney disease: distal tubular mechanisms. Am J Physiol Renal Physiol. 2020 Nov 1;319(5):F729-F745.

Bovée DM, Roksnoer LCW, van Kooten C, Rotmans JI, Vogt L, de Borst MH, Zietse R, Danser AHJ, Hoorn EJ. Effect of sodium bicarbonate supplementation on the renin-angiotensin system in patients with chronic kidney disease and acidosis: a randomized clinical trial. J Nephrol. 2020 Dec 31.

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Bovée DM, Uijl E, Severs D, Rubio-Beltrán E, van Veghel R, Maassen van den Brink A, Joles JA, Zietse R, Cuevas CA, Danser AHJ, Hoorn EJ. Dietary Salt Modifies the Blood Pressure Response to Renin-Angiotensin Inhibition in Experimental Chronic Kidney Disease. Am J Physiol Renal Physiol. 2021 Feb

Bovée DM, Ren L, Uijl E, Clahsen-van Groningen MC, van Veghel R, Garrelds IM, Domenig O, Poglitsch M, Zlatev I, Kim JB, Huang S, Melton L, Hoorn EJ, Foster D, Danser AHJ. Blood pressure-independent renoprotective effects of small interfering RNA targeting liver angiotensinogen in experimental chronic kidney disease. Hypertension. 2021 May

Kho MML, Roest S, **Bovée DM**, Metselaar HJ, Hoek RAS, Van der Eijk AA, Manintveld OC, Roodnat JI, Van Besouw N. Herpes Zoster in solid organ transplantation: incidence and risk factors. Frontiers Immunol. 2021 (accepted for publication).

PORTFOLIO

Candidate	Dominique M. Bovée
Erasmus MC Department	Internal Medicine
	Division of Nephrology and Transplantation
	Division of Pharmacology and Vascular Medicine
Postgraduate school	Molecular Medicine
Period	2015-2019
Promotors	Prof. dr. Ewout J. Hoorn
	Prof. dr. A.H. Jan Danser

Training activities		
Courses	**Year(s)**	**ECTS**
Laboratory animal science	2015	3.0
Basic course for clinical investigators (BROK)	2015	1.5
Basic course Pubmed and Endnote	2015	0.6
Winterschool Dutch Kidney Foundation	2016	2.0
Research management for PhD-students	2016	1.0
Electrolyte and acid-base disorders	2016, 2017	2.0
Presenting skills for researchers	2016	1.0
Research integrity	2016	0.3
Biostatistical methods I: Basic principles	2017	5.7
Workshop Negotiation	2018	0.3
Workshop Photoshop and Illustrator	2018	0.3
National and international conferences		
Dutch Nephrology Days	2015	0.6
New Kids on the Block*	2015	0.9
PLAN day	2015	0.3
Benelux Kidney Meeting	2015	0.3
ASN kidney week	2015	1.5
PLAN day	2016	0.3
Coeur PhD day	2016	0.3
New Kids on the Block	2016	0.3
Scientific Fall Symposium Dutch Federation of Nephrology	2016	0.6
Erasmus MC Science days	2017	0.6
Dutch Nephrology Days	2017	0.6
PLAN day	2017	0.3
Dutch Pharmacology Society yearly symposium	2017	0.3
Scientific Fall Symposium Dutch Federation of Nephrology*	2017	0.6
ASN Kidney week*/**	2017	2.1

Erasmus MC Science days**	2018	0.9
Spring Meeting Dutch Pharmacology Society*	2018	0.6
AHA Council on Hypertension*	2018	1.2
PLAN day	2018	0.3
Erasmus MC Science days	2019	0.6
Dutch Nephrology Days^	2019	0.6
New Kids on the Block	2019	0.3
BENELUX Kidney Meeting*	2019	0.6
ASN kidney week**	2019	1.8
Gordon Conference**	2019	1.8
^ Invited talk, * Oral presentation, ** Poster presentation		
Oral presentations at the Erasmus MC		
Research meetings Dep. of Pharmacology and Vascular Medicine	2015-2019	2.7
Research meetings Dep. of Nephrology and Transplantation	2015-2019	2.7
Internal Medicine research meeting	2016	0.3
Vascular Medicine research meeting	2018	0.3
Peer reviewed articles		
Laboratory animals (1)	2017	0.1
Journal of the American Society of Nephrology (1)	2018	0.1
Nature reviews (1)	2018	0.1
Teaching activities		
Supervising practicum first-year medical students	2015-2019	3.6
Supervising research project fourth-year medical students		
Dafsy Bouari - Metabolic complications of CKD	2016	1
Joost Janssen – Acute acid-loading in CKD	2017	1
Supervising research internship third-year laboratory technician student		
Linda de Rooij	2019	1
Other activities		
Board member and secretary of PLAN	2015-2019	2
Organizing PLAN-day in Rotterdam	2017	2
Grands and awards		
Erasmus Trustfonds conference grand	2017	
AHA AFHRE Travel Award for Patient-Oriented or Clinical Research in Hypertension	2018	
AHA Paul Dudley White International Scholar Award (highest ranked abstract from the Netherlands)	2020	
Total		52.9

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