Effects of interventions in the RAAS and sodium status on classical and non-classical outcome parameters in chronic kidney disease

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Stellingen

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Effects of interventions in the RAAS and sodium status on classical and non-classical outcome parameters in chronic kidney disease

1. Het streven naar adequate natriumbeperking vereist veel inspanningen van nierpatiënten en medische professionals maar is de moeite waard. (*Dit proefschrift*)
2. Het NT-proBNP gehalte zou richtinggevend kunnen zijn bij de intensivering van antihypertensieve en antiproteinurische therapie in patiënten met een chronische nierziekte. (*Dit proefschrift*)
3. De onveranderd hoge concentratie plasma CTGF is mogelijk een reflectie van de voortschrijdende cardiovasculaire schade in nierpatiënten ondanks antiproteinurische therapie. (*Dit proefschrift)*
4. De verlaging van de tubulaire natriumreabsorptie en het EPO gehalte suggereert een afname van de renale zuurstofconsumptie door natriumbeperking in nierpatiënten. (*Dit proefschrift*)
5. (Falen van) VEGFc-gemedieerde extrarenale natriumchloride homeostase speelt mogelijk een rol in zoutgevoelige proteinurische nierpatiënten. (*Dit proefschrift*)
6. Hoe behap je de wereld? Gewoon bij de lekkerste stukjes beginnen. (*Loesje*)
7. Angst is mar veur eben, spiet is veur altied. (*Daniël Lohues*)
8. Er is niemand van wie je niet iets zou kunnen leren. (*Dag H.A.C. Hammarskjöld*)
9. Vriendschap is één ziel in twee lichamen. (*Aristoteles*)
10. Where there is love there is life. (*Mahatma Gandhi*)

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General Introduction

CKD is a global health problem

Chronic kidney disease (CKD) affects several million people around the world, and with the rising prevalence of hypertension, obesity, and diabetes its occurrence is even increasing[1-3]. The prevalence of CKD stages 1 to 4 increased from 10.0% in 1988-1994 to 13.1% in 1999-2004[1]. In 2004 almost 2 million patients worldwide were reported to receive renal replacement therapy, i.e. dialysis or transplantation, because of end-stage renal disease (ESRD, CKD stage 5), and this number increases at a rate of 7% per year[2]. Although ESRD patients represent only a small part of the total population (0.06% in Europe, 0.15% in the United States), dialysis costs entail 0.7-1.8% of the health-service budget in European countries and approximate 40 billion dollars in the United States[3,4].

Complications of CKD

Besides a progressive decline in renal function towards ESRD, CKD entails an increased risk of cardiovascular events, hospitalization, and death[5-7]. The risk of these complications increases sharply as renal function decreases[5,6]. Compared to an estimated glomerular filtration rate (eGFR) of ≥60 ml/min/1.73m², the risk of a cardiovascular event is 1.4 times increased in case of an eGFR of 45-59 ml/min/1.73m² and 3.4 times increased in case of an eGFR of <15 ml/min/1.73m². Likewise, the risks of hospitalization and death from any cause are respectively 1.1 and 1.2 times increased when eGFR is 45-59 ml/min/1.73m² and respectively 3.1 times and 5.9 times increased when eGFR is <15 mL/min/1.73m², compared to when eGFR is ≥60 ml/min/1.73m²[7]

Furthermore, both renal and cardiovascular outcomes are strongly determined by the presence and severity of proteinuria[7-9]. The predictive value of proteinuria for these endpoints is not only independent of well-known risk factors, including hypertension and diabetes, but it is also independent of renal function. Individuals with urinary protein levels of 3-30 mg/dl or 30-300 mg/day (microalbuminuria) have 1.5 times higher risk of coronary heart disease, and individuals with urinary protein levels of >30 mg/dL or >300 mg/day (proteinuria) have 2.2. times higher risk, compared to individuals without these conditions[10]. Likewise, the risk of cardiovascular disease-related death is 1.6 times increased with urinary protein levels of 30-300 mg/dL (proteinuria) and is 1.8 times increased with urinary protein levels of >300 mg/dL (nephrotic range proteinuria), compared to urinary protein levels of <30 mg/dL[11].

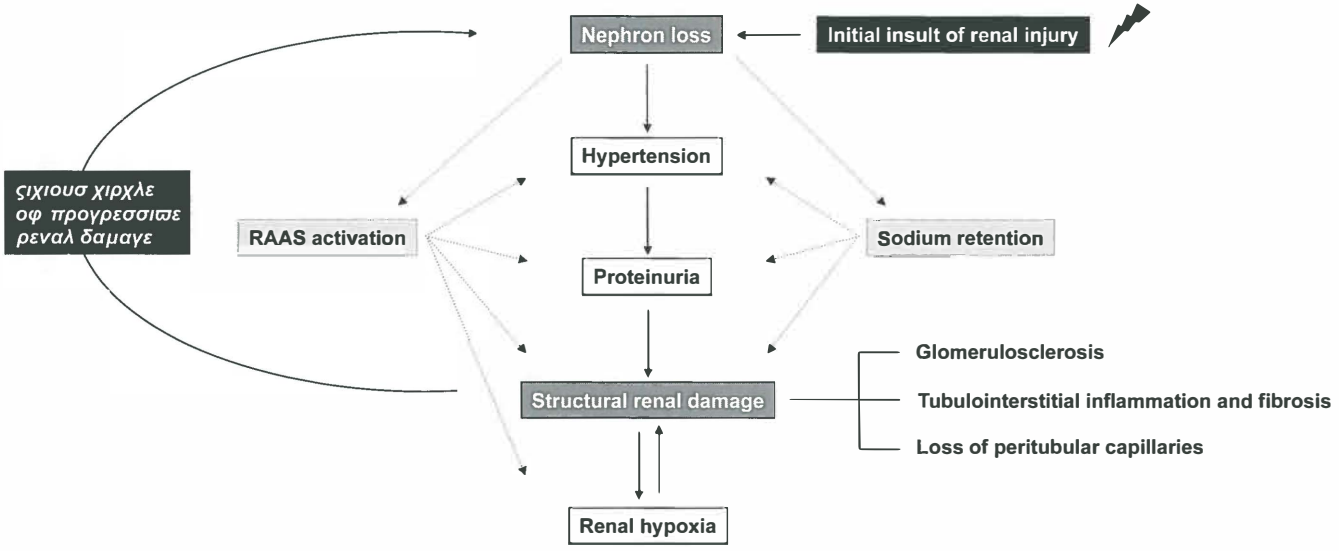
Pathophysiology of CKD

CKD is characterized by common pathways of progressive renal damage that can be initiated by various triggers, with the common long-term result of irreversible renal structural damage and function loss[12,13]. The final histological appearance involves glomerulosclerosis, interstitial fibrosis, and nephron loss[14,15]. Different factors are involved in the progression of renal injury after the initial insult. These include systemic and glomerular hypertension, hyperfiltration, proteinuria, tubulointerstitial inflammation and fibrosis, and intrarenal hypoxia (Figure 1).

Nephron loss, initiated by the primary insult of renal injury, results in glomerular hypertension and hyperfiltration of the remaining nephrons[12;16]. Hyperfiltration serves as a compensatory response to preserve renal filtration capacity in the short term, but induces increased glomerular permeability and protein loss, which contributes to glomerulosclerosis in the long term[17;18]. Additionally, the excessive protein filtration leads to tubular reabsorption of increased amounts of proteins, resulting in protein accumulation in lysosomes in proximal tubular cells, causing cell disruption and injury[19]. Filtered proteins may also incite a toxic response through stimulation of proinflammatory and profibrotic factors, which further contributes to tubulointerstitial inflammation and fibrosis[20]. Furthermore, loss of peritubular capillaries, together with renal fibrosis, diminishes tubulointerstitial perfusion. The resulting renal hypoxia favours release of proinflammatory and profibrotic factors, which further injures the renal tissue in a vicious circle of progressive renal damage[21;22].

Other consequences of renal injury are inappropriate activation of the renin angiotensin aldosterone system (RAAS), and sodium sensitivity (Figure 1). A main effector molecule of the RAAS is angiotensin II. Angiotensin II, by activating the angiotensin II type 1 receptor (AT1R), stimulates systemic vasoconstriction and tubular reabsorption of sodium and water (partly via induction of aldosterone), and can thereby induce extracellular volume excess and systemic hypertension[23;24]. Systemic hypertension in turn accelerates renal disease progression, likely due to the associated glomerular hypertension[3;25].
Furthermore, angiotensin II can also directly induce glomerular hypertension, hyperfiltration, and proteinuria, partly due to its stronger vasoconstrictive effect on efferent arterioles than on afferent arterioles[23;24]. In addition, angiotensin II can stimulate renal inflammation, fibrosis, and hypoxia, in a direct manner[26-29]. Hence, excessive RAAS activation contributes to the onset and progression of CKD via hemodynamic and non-hemodynamic mechanisms.

Figure 1 Pathophysiology of chronic kidney disease (simplified view)



The reduced renal ability to excrete sodium and the resulting sodium overload, that occurs with renal injury, also adds to progressive renal damage[30-32]. Sodium retention is particularly prominent in individuals with proteinuria or refractory hypertension, even when renal function is still normal[33-35]. Extracellular volume in these patients is expanded, due to larger body stores of osmotically active sodium[36-38]. Consequently, systemic and glomerular hypertension, hyperfiltration and proteinuria can occur. Futhermore, sodium excess also directly stimulates renal inflammation and fibrosis[39-41].
Recent data suggest that in patients with refractory hypertension, vascular endothelial growth factor C (VEGF-C) mediated extrarenal regulatory mechanisms might play a role in sodium homeostasis and blood pressure regulation[42]. Whether these recently discovered non-osmotic pathways of interstitial sodium storage are also active, or disturbed, in proteinuric patients, is so far unknown[42-44].

Treatment strategies

Treatment in CKD is aimed at deceleration of renal function loss as well as prevention of its (cardiovascular) complications. Given their pathophysiological role, the above-mentioned pathways are, at least theoretically, potential targets for intervention. In current clinical practice, therapy in CKD is multifactorial and involves prevention or treatment of risk factors (hypertension, proteinuria, diabetes, overweight, smoking, dyslipidemia) and complications (anemia, bone disease, neuropathy, malnutrition, decreased quality of life) of renal disease[45,46]. The principal measures in this approach are control of blood pressure and reduction of proteinuria (Table 1).

Blood pressure and proteinuria as treatment targets

Early reduction of hypertension and proteinuria significantly improve long-term renal and cardiovascular outcome in CKD[3,47,48]. Accordingly, blood pressure and proteinuria are regarded as the main modifiable risk factors, and hence key treatment targets, in CKD[3,49]. In this respect, reduction of proteinuria to below 1.0 g/day, reduction of blood pressure to below 130/80 mmHg, and reduction of blood pressure to below 125/75 mmHg in patients with proteinuria above 1.0 g/day, are currently recommended[9,46,49].

Research in the past few decades, testing diverse pharmacological strategies, showed that the largest protection against progression of CKD and its cardiovascular complications is achieved by pharmacological blockade of the RAAS by ACE inhibition (ACEi) or angiotensin receptor blockade (ARB)[46,50,51]. The long-term benefits of ACEi and ARB are thought to be mediated mainly by the reduction of systemic and glomerular

Table 1 Current recommendations on proteinuria and blood pressure in CKD

Treatment goals: · Deceleration of renal function loss · Prevention of cardiovascular complications
Treatment targets: · Reduction of proteinuria to <1.0 g/day · Reduction of blood pressure to <130/80 mmHg · Reduction of blood pressure to <125/75 mmHg if proteinuria >1.0 g/day
Treatment modalities: · ACE inhibition (ACEi) or angiotensin receptor blockade (ARB) · Addition of second RAAS blocker (i.e. combined ACEi and ARB) · Addition of diuretics · Institution of dietary sodium restriction of <100 mmol Na^+/day or <6 g NaCl/day · 'Supramaximal' dosing of ACEi or ARB · Addition of beta blockade or calcium channel blockade

blood pressure, and by a specific antiproteinuric effect that cannot be fully attributed to the reduction of blood pressure. By virtue of these effects, RAAS blockade is recommended as first-line therapy for the reduction of blood pressure and proteinuria in CKD patients[9,46,49].

Monotherapy with ACEi or ARB is often insufficient to achieve proteinuria and blood pressure targets, and the residual renal and cardiovascular risks remain high[52-55]. Hence, optimization of the efficacy of RAAS-based therapy is warranted. There are different strategies to improve the antiproteinuric and antihypertensive efficacy of treatment with ACEi or ARB[46]. These include addition of a second RAAS blocker (i.e. combined ACEi and ARB, known as 'dual RAAS blockade'), and addition of diuretics. For further reduction of blood pressure, beta blockade or calcium channel blockade can be added. Other effective but underexposed measures to enhance the antiproteinuric and antihypertensive response are institution of dietary sodium restriction, and increasing the dose of the single drug (ACEi or ARB) beyond the top of the dose-response-curve for blood pressure ('supramaximal dosing') as this can further reduce proteinuria[56-58].

Dual RAAS blockade with ACEi and ARB exerts a stronger antiproteinuric and antihypertensive effect than monotherapy, although it is uncertain whether this is more effective than optimal dosing of the single drug[52,57]. Importantly, the effect of dual RAAS blockade on hard renal and cardiovascular endpoints is not unequivocal[59,60].

Nevertheless, dual RAAS blockade is widely used in clinical practice. Addition of dietary sodium restriction and/or diuretics ('sodium targeting') has been consistently shown to enhance the antiproteinuric and antihypertensive efficacy of ACEi or ARB in short-term intervention studies[56;61-63]. Despite recommendations in current guidelines, dietary sodium intake remains excessively high in CKD patients, ranging from 160 to 200 mmol Na^+/day (i.e. 9.6 to 12 g NaCl/day)[64;65]. The impact of dietary sodium excess can be substantial, to the extent of virtual annihilation of the antiproteinuric and antihypertensive response to ACEi or ARB[61;66;67]. Of note, the effects of dietary sodium restriction or dual RAAS blockade, as a next step after insufficiently effective single RAAS blockade, have not been tested head-to-head so far.

Non-classical outcome parameters

Besides the clinical risk promotors hypertension and proteinuria, several intrarenal pathways of damage are involved in the progression of renal injury. These include among others tubulointerstitial inflammation and fibrosis, and intrarenal hypoxia (as was discussed above). These intrarenal pathways of damage are often initiated and perpetuated by hypertension and proteinuria, but can also persist rather independently. Their clinical relevance is substantial, as apparent from the fact that tubulointerstitial fibrosis is the most consistent predictor of progressive renal function decline, that is, in patients for whom data on renal morphology are available from a renal biopsy[68-70]. Biopsy data are not routinely available however, which hampers monitoring of renal injury during therapy. The latter could be of major importance, as animal data have convincingly shown that during treatment intrarenal pathways of damage can dissociate from hypertension and proteinuria, with satisfactory responses of blood pressure and proteinuria but ongoing or even aggravating renal structural damage, and vice versa[71;72].

The currently recommended therapy, based on RAAS blockade and sodium targeting (i.e. dietary sodium restriction and/or diuretics), influences intrarenal pathways of damage both downstream and independent of blood pressure and proteinuria[23;40;73]. Consequently, the benefits of blood pressure and proteinuria reduction can be augmented or counteracted by treatment effects on intrarenal pathways of damage. In line with the abovementioned animal data, recent hard endpoint trials showed that reduction of blood pressure and even proteinuria does not necessarily improve long-term renal and cardiovascular outcome[49;60;74].

Given their (partially) independent nature, intrarenal inflammation, fibrosis, and hypoxia could be regarded as 'non-classical' intermediate outcome parameters, as distinct from the 'classical' intermediate outcome parameters blood pressure and proteinuria. In line with this, monitoring of therapy effects beyond blood pressure and proteinuria, better reflecting the impact of therapy on intrarenal pathways of damage, is warranted[75].

Moreover, it has been argued that more attention should be given to adjunct effects of treatment regimens as possible determinants of long-term outcome. Such adjunct effects may include for instance effects on serum potassium, uric acid, and also effects on erythropoietin and hemoglobin levels[76-78]. In particular, ACEi and ARB have been shown to reduce erythropoietin and hemoglobin levels[78-80]. Although reduced erythropoietin levels could theoretically blunt the benefits of ACEi or ARB[81], clinical data suggest the opposite; Reduced erythropoietin levels are associated with improved survival in renal transplant recipients, and correction of anemia (unless severe) with recombinant erythropoietin is not beneficial and may in fact worsen long-term renal and cardiovascular outcome in CKD patients[82-84]. Furthermore, the beneficial effects of ARB on the risk for ESRD and death were found to be maintained despite a simultaneous decrease in hemoglobin in CKD patients[79]. Effects of concomitant sodium targeting on erythropoietin and hemoglobin levels in renal patients have not been documented up to now.

Altogether, to improve long-term renal and cardiovascular protection in CKD patients, the antiproteinuric and antihypertensive efficacy of RAAS-based regimens should be optimized. Yet, in order to adjust and titrate therapy towards a treatment response that is most likely to improve long-term outcome, it might be useful to take into account treatment effects on non-classical intermediate outcome parameters.

Outline of the thesis

The general aim of this thesis is to systematically explore the effects of interventions in the RAAS and sodium status on classical and non-classical intermediate outcome parameters in non-diabetic CKD patients. These data should provide a rational basis for further improvement of renoprotective therapy, in order to reduce long-term renal and cardiovascular risk.

In **part I** of the thesis we investigate the effects of interventions in the RAAS and sodium status on classical outcome parameters in CKD patients.

In **chapter 1** we review the rationale and evidence for a beneficial effect of dual RAAS blockade on blood pressure, proteinuria, and hard renal and cardiovascular endpoints, as available in the literature.
Thus far, the comparative efficacy of dual RAAS blockade and single RAAS blockade combined with dietary sodium restriction is unknown. Therefore, in **chapter 2** we compare head-to-head the antiproteinuric and antihypertensive effects of the addition of ARB to ACEi and the addition of dietary sodium restriction to ACEi, as well as their combination, in a randomized controlled cross-over trial.
Sodium excess hampers the therapeutic response to RAAS blockade, but is notoriously difficult to assess accurately, even in CKD patients. Accordingly, both under- and overtitration of sodium targeting can easily occur. A simple test that predicts the anti-hypertensive and antiproteinuric benefits of sodium targeting for the individual patient at any point in the titration process would be useful, but is currently not available. In **chapter 3** we therefore evaluate N-terminal pro-brain natriuretic peptide (NT-proBNP), which is a cardiac marker of volume expansion, as a candidate marker in this respect.
When applying sodium targeting for renoprotection, usually an early reduction of glomerular filtration rate is observed along with the induction of a negative sodium balance. Its impact on long-term outcome is unknown and might well be favourable due to a reduction of hyperfiltration. Such an effect however, can be obscured when renal function as such is the read-out parameter for success of long-term intervention. In **chapter 4,** therefore, we investigate the short-term effects of (withdrawal and) addition of diuretics to RAAS blockade on renal function, and their impact on the interpretation of long-term renal function outcome.

In **part II** we investigate the effects of interventions in the RAAS and sodium status on non-classical outcome parameters in CKD patients.
In **chapter 5** we investigate whether intensified reduction of proteinuria by combinations of ACEi, ARB, and dietary sodium restriction is accompanied by a further decrease of urinary markers of proximal tubular injury (N-Acetyl-β-Glucosaminidase, NAG; Kidney Injury Molecule 1, KIM-1; β2-microglobulin, β2MG), distal tubular injury (Heart-type Fatty Acid-Binding Protein, H-FABP), and tubular inflammation (Neutrophil Gelatinase-Associated Lipocalin, NGAL; monocyte chemoattractant protein-1, MCP-1).
In **chapter 6** we assess plasma and urinary levels of connective tissue growth factor (CTGF), which is a mediator of fibrogenesis, and the effects of stepwise antiproteinuric intervention with dietary sodium restriction, ARB, and diuretics, on CTGF.
In **chapter 7** we explore the effects of ARB, add-on diuretics, and dietary sodium restriction on erythropoietin and hemoglobin levels, since these interventions might theoretically influence erythropoiesis.

In **chapter 8** we extend these explorations by investigating the effects of the addition of ARB to ACEi and the addition of dietary sodium restriction to ACEi, as well as their combination, on erythropoietin, hemoglobin, and tubular sodium reabsorption and by studying their interrelationships.

As apparent from the above, intervention in sodium status is important in renoprotection. Until recently, it was assumed that total body sodium and extracellular volume are closely related and mainly controlled by renal excretion and dietary intake. Recently, this paradigm was shattered by the discovery of VEGF-C mediated non-osmotic, i.e. waterfree, sodium storage in the interstitium. Dysfunction of this extrarenal regulatory mechanism for sodium homeostasis may be involved in refractory hypertension. Whether this mechanism responds to changes in dietary sodium intake in humans, and whether it is active or disturbed in renal patients, is still unknown. Therefore, in **chapter 9** we investigate VEGF-C, blood pressure and measures of extracellular volume during different sodium intakes in healthy subjects and proteinuric CKD patients.

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Part I

Effects of intervention in the RAAS and sodium status on classical intermediate outcome parameters in CKD patients

1

Dual renin angiotensin aldosterone system blockade in cardiac and renal disease

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Abstract

Purpose of review: Renin angiotensin aldosterone system (RAAS) blockade improves outcome in cardiovascular disease (CVD) and chronic kidney disease (CKD), but the residual risk during monotherapy RAAS blockade remains very high. This review discusses the place of dual RAAS blockade in improving these outcomes.

Recent findings: The combination of angiotensin-converting enzyme inhibitors (ACEi) with angiotensin II type 1 receptor blockers (ARB) generally had a better antihypertensive and antiproteinuric effect than monotherapy in many studies, but is also associated with more adverse effects. Unfortunately, the effect on hard renal and cardiovascular endpoints is not unequivocal. Combination of ACEi (or ARB) with aldosterone blockade has long-term benefits in heart failure, and an added effect on proteinuria in CKD, but data on hard renal endpoints are lacking. Dual blockade including renin inhibition has added antiproteinuric effects, but long term data are still under way. Available strategies to optimize the effect of monotherapy RAAS blockade include dose titration and correction of volume excess. Whether dual blockade has better efficacy and/or less adverse effects than optimized monotherapy has not been investigated.

Summary: Several options are available to increase the effect of monotherapy RAAS blockade. For proteinuric CKD, these can be combined in a stepwise approach aimed at maximal proteinuria reduction; this includes dual blockade for patients with persistent proteinuria during optimized monotherapy RAAS blockade. Long-term randomized studies, however, are needed to support the benefits of dual blockade for long-term renal and cardiovascular outcome in CKD.

Introduction

Renin angiotensin aldosterone system (RAAS) blockade is a cornerstone of treatment in chronic kidney disease (CKD) and cardiovascular disease (CVD)[1-3]. In particular, the angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II type 1 receptor blockers (ARB) have proven efficacy for intermediate parameters (systemic and glomerular hypertension, and proteinuria) as well as hard cardiovascular and renal endpoints[4-8], whereas the role of other agents, such as mineralocorticoid receptor blockers (MRB) and renin inhibitors (RI), is still emerging.

Despite this proven efficacy, data from landmark studies show that the residual cardiovascular and renal risks remain very high. For example, in the HOPE trial, which studied patients with high cardiovascular risk, ACEi reduced the risk for myocardial infarction, stroke, or death, only from 18% to 15% in five years of follow-up[4]. Likewise, in patients with diabetic nephropathy, as studied in the RENAAL trial, ARB reduced the risk for doubling of serum creatinine level, end-stage renal disease (ESRD), or death only from 47% to 44% in three years of follow-up[8].

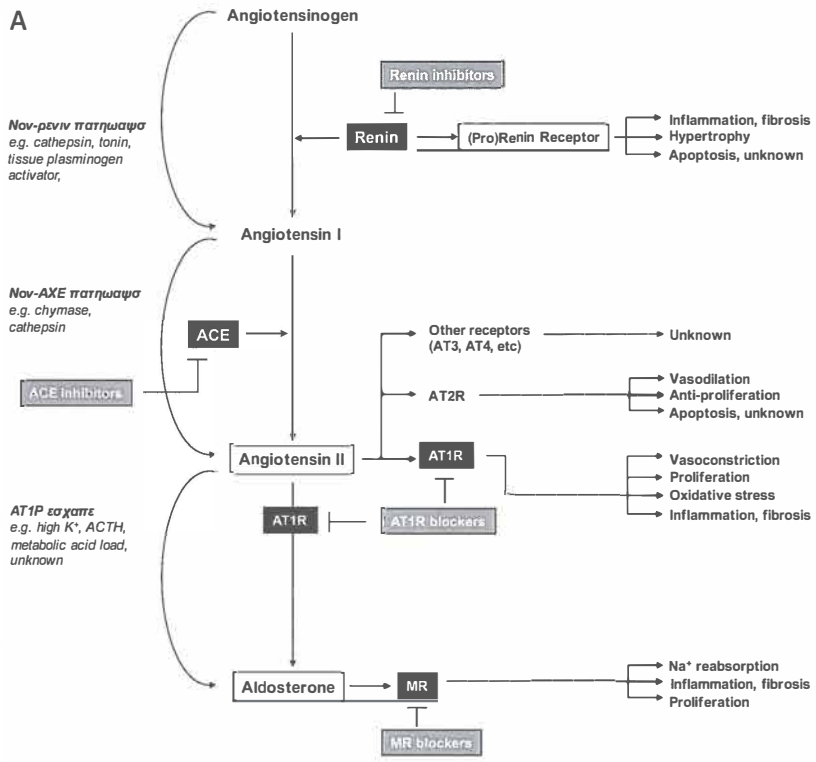
New strategies to improve long-term outcome in cardiovascular and renal disease are therefore of paramount importance. Simultaneous blockade of the RAAS at two levels ('dual blockade') has been advocated in this respect. This review discusses the rationale and the (lack of) evidence for the dual blockade combinations on intermediate and hard cardiovascular and renal endpoints.

Rationale for dual RAAS blockade

The protective effects of RAAS blockade on end-organ damage reflects the role of increased RAAS activity in CVD and CKD[9,10]. Why, however would it be beneficial to interfere in the same system at different levels?

The RAAS is an endocrine/autocrine cascade (Figure 1) with renin release as its first step, leading to cleavage of angiotensinogen into angiotensin I (ANG-I), which is converted into angiotensin II (ANG-II) by the angiotensin-converting enzyme (ACE). ANG-II activates AT1 receptors (AT1R), resulting in vasoconstriction, aldosterone production, and reabsorption of sodium and water[9;11]. ANG-II, aldosterone, and the recently discovered (pro-)renin receptor, are also pathophysiologically involved in cardiovascular and renal end-organ damage via local pro-inflammatory and profibrotic effects[12-15].

Figure 1 Renin Angiotensin Aldosterone System (RAAS)



General outline that shows where several drug classes interfere in the system.
Abbreviations: ACE, angiotensin-converting enzyme; AT1R, Angiotensin II Type 1 receptors; MR, mineralocorticoid receptor. Original figure.

B

	Plasma Renin Activity	Plasma Renin Concentration	Angiotensin I	Angiotensin II	Aldosterone
ACE Inhibition	⇧	⇧	⇧	⇩ ⇧[1]	⇩ ⇧[1]
Angiotensin Receptor Blockade	⇧	⇧	⇧	⇧	⇩ ⇧[2]
Mineralocorticoid Receptor Blockade	⇧	⇧	⇧	⇧	⇧
Renin inhibition	⇩	⇧	⇩ ⇧[3]	⇩ ⇧[3]	⇩ ⇧[3]

[1] *angiotensin II escape due to non-ACE pathways, and subsequent aldosterone escape*
[2] *aldosterone escape via non-AT1R mechanisms*
[3] *angiotensin I escape via non-renin pathways, and subsequent angiotensin II escape and aldosterone escape*

The RAAS blocking drug classes and the effect on circulatory RAAS components.
Due to the presence of feedback loops and alternative routes, inhibition of the RAAS at one level leads to compensatory activation at another level.

Due to multiple feedback loops and alternative routes within the RAAS, inhibition of the RAAS at one level leads to compensatory activation at another level (Figure 1B), which can blunt the pursued therapeutic efficacy.

Angiotensin-converting enzyme inhibitors inhibit ACE-mediated production of ANG-II, but ANG-I levels increase greatly, contributing to ANG-II generation via non-ACE pathways ("ANG-II escape"), which may result in incomplete blockade of RAAS activity[16;17]. During ARB, which blocks binding of ANG-II to AT1 receptors, ANG-II levels increase considerably, which may partly maintain AT1R signalling ("ANGII-escape") and stimulate non-AT1 receptors with uncertain effects[11,18]. With ACEi as well as ARB "aldosterone escape" occurs, that is, a secondary rise (after an initial fall) of aldosterone levels up to, or even beyond, pre-treatment values[19,20], irrespective sodium intake[21]. In addition, a reactive rise in renin occurs with probably undesired effects[22].

Mineralocorticoid receptor blockers inhibit binding of aldosterone to mineralocorticoid receptors on epithelial sites, thereby reducing sodium and water reabsorption, and on non-epithelial sites, thereby reducing cardiovascular and renal fibrotic injury[23]. During MRB a reactive rise in renin occurs.

RI can antagonize the ACEi, ARB or MRB induced renin increase by blocking renin activity [14;15], but renin concentration further increases[24], and the conversion of angiotensinogen into ANG-I via non-renin pathways continues ("ANG-I escape")[22], which may limit the effectiveness of RI.

In summary, blockade of the RAAS at any of the single levels does not provide full blockade of the cascade due to compensatory responses at other levels, which could be involved in suboptimal therapeutic efficacy.

Dual blockade may be useful for two different reasons. First, it could provide more complete blockade of the RAAS by limiting the compensatory responses of ANG-II, aldosterone, renin or their effects, thus maximizing blockade of the cascade. This "maximization approach", however, may induce adverse effects such as hyperkalemia, symptomatic hypotension, or hemodynamically mediated deterioration of renal function[25]. A different approach to dual blockade could therefore be to combine lower doses of the individual drugs to obtain a more favourable balance between increased efficacy and adverse effects. The latter approach, however, to the best of our knowledge, has not been explicitly pursued so far.

Dual RAAS blockade in hypertension

In essential hypertension (Table 1A) dual blockade with ACEi+ARB is more effective than monotherapy, but also has more side effects, including hyperkalemia[26-28]. MRB on top of ACEi or ARB resulted in a larger antihypertensive effect, at the expense however of hyperkalemia and gynaecomastia[29,30]. Aliskiren, the only renin inhibitor available so far, is a potent antihypertensive drug. In hypertensive patients, aliskiren combined with ACEi or ARB was more effective than monotherapy of one the three drugs[31-33]. Diarrhea, and not hyperkalemia, appears the main side effect in uncomplicated hypertension. Whether any of the dual RAAS blockade combinations has a beneficial effect on hard cardiovascular endpoints in uncomplicated hypertension has not been investigated so far. Hence, dual RAAS blockade is not preferred over other drug combinations in these patients[2].

Dual RAAS blockade in cardiovascular disease

In diverse cardiovascular diseases (Table 1B) dual blockade with ACEi+ARB resulted in a larger antihypertensive effect, but this did not translate in a reduction of cardiovascular events or mortality, whereas side effects leading to discontinuation of the medication were more prevalent with dual blockade[34;35]. In patients with heart failure, on one hand, studies were promising showing larger blood pressure reduction, less cardiac remodelling and less heart failure symptoms with ACEi+ARB compared with monotherapy[36-38]. However, data on long-term outcome are somewhat disappointing, since the benefits of dual blockade on mortality observed in the CHARM study[38] were not confirmed[36,37]. Unfortunately, the rate of adverse events, such as symptomatic hypotension, hyperkalemia, and renal function decline, has proven higher than anticipated, thus limiting clinical application of dual blockade with ACEi+ARB in heart failure[25,39]. Accordingly, combined ACEi+ARB treatment is not advocated in the CVD population[3].

In contrast, favourable outcomes were achieved by dual blockade with MRB+ACEi or MRB+ARB in patients with CVD. In heart failure, including post-myocardial infarction left-ventricular dysfunction, MRB on top of ACEi or ARB more effectively reduced mortality and hospitalizations for heart failure than ACEi or ARB monotherapy[40;41]. Dual therapy was associated with more hyperkalemia, renal function decline, and gynaecomastia with non-specific MRB (spironolactone)[40] but not with specific MRB (eplerenone)[41]. Altogether, these findings have led to the recommendation for dual blockade with low-

dose MRB in carefully selected patients with left-ventricular dysfunction post myocardial infarction or heart failure[3].

Data on dual blockade with RI and ACEi or ARB in CVD are still sparse. Aliskiren added to ACEi or ARB in heart failure improved intermediate endpoints (circulating NT-proBNP and urinary aldosterone) despite the absence of an effect on blood pressure, and dual therapy was well tolerated[42]. The effects of dual therapy with RI on top of ACEi or ARB in CVD patients remain to be verified in long-term large clinical trials.

Dual RAAS blockade in renal disease

In CKD patients dual blockade with ACEi+ARB has been shown to reduce blood pressure and proteinuria more effectively than either monotherapy[43-48], especially when baseline proteinuria was high. These were all small studies using proteinuria as an intermediate endpoint. Reliable extraction of the rate of adverse events is not feasible from these studies[46], which renders it difficult to weigh the overall benefit. The only long-term intervention trial on dual blockade with ACEi+ARB in proteinuric CKD (COOPERATE)[49] was recently retracted because of inconsistencies in the data and design, which were revealed when an attempt was made to include the data in a meta-analysis[50,51]. Thus, evidence for a benefit of ACEi+ARB combination on hard endpoints in CKD is lacking.

The ONTARGET study, a large (n=25,620) long-term (follow-up 56 months) clinical trial, recently reported the effects of dual blockade with ACEi+ARB on renal endpoints in patients with CVD[52]. Despite a beneficial effect on microalbuminuria, dual blockade was associated with a worse renal outcome, raising vigorous debate. On the one hand, the study was not well designed to study renal endpoints[53], i.e. the included patients were patients with low renal risk, there was a disputable choice of the composite endpoint (which included acute dialysis), and suboptimal methods were used to register the renal endpoints. On the other hand, the study illustrates that dual blockade with ACEi+ARB can be harmful if applied in CVD patients with low renal risk, with decreased glomerular pressure as a main candidate mechanism explaining the excess of acute renal failure, and that this adverse renal effect is not balanced by greater benefits.

Considering the patient selection in the ONTARGET study, it may be unwise for the moment to extrapolate these findings to CKD patients with overt proteinuria, where the benefit/risk ratio may be very different, considering the antiproteinuric potential of dual

Table 1A Dual blockade in hypertensive patients

Dual therapy	Trial	Design	Population	Intervention
ACEi+ARB	Azizi[26] 2000	RCT, 6 wk	Primary HT, 177 patients	• Enalapril 10 mg/d + Losartan 50 mg/d • Enalapril 10 mg/d • Losartan 50 mg/d
ACEi+ARB	Doulton[27] 2005	Meta-analysis, ±8 wk	Primary HT, 434 patients	• ACEi+ARB combinations • ACEi or ARB
ACEi+ARB	Scaglione[28] 2007	RCT, 24 wk	Primary HT, 57 patients	• Losartan 50 mg/d + Ramipril 5 mg/d • Losartan 50 mg/d • Ramipril 5 mg/d
MRB+ACEi, MRB+ARB	Nishizaka[29] 2003	Open label trial, 26 wk	Primary HT, 76 patients	• Spironolactone 12.5-50 mg/d + ACEi or ARB • ACEi or ARB
MRB+ACEi, MRB+ARB	Mahmud[30] 2005	Open label trial, 14 wk	Primary HT, 39 patients	• Spironolactone 50 mg/d + ACEi or ARB • ACEi or ARB
RI+ARB	Oparil[31] 2007	RCT, 8 wk	Primary HT, 1797 patients	• Aliskiren 150-300 mg/d + Valsartan 160-320 mg/d • Aliskiren 150-300 mg/d • Valsartan 160-320 mg/d
RI+ACEi, RI+ARB	O'Brien[32] 2007	Open label trial, 6 wk	Primary HT, 44 patients	• Aliskiren 75-150 mg/d + Ramipril 5 mg/d • Aliskiren 75-150 mg/d + Irbesartan 150 mg/d • Ramipril 5 mg/d • Irbesartan 150 mg/d
RI+ARB	Geiger[33] 2009	RCT, 8 wk	Primary HT, 641 patients	• Aliskiren 150-300 mg/d + Valsartan 160-320 mg/d • Aliskiren 150-300 mg/d • Valsartan 160-320 mg/d

Randomized controlled trials (RCT) and meta-analyses comparing dual RAAS blockade with combinations of ACE inhibitors (ACEi), angiotensin receptor blockers (ARB), mineralocorticoid receptor blockers (MRB), and renin inhibitors (RI), versus monotherapy, in patients with primary hypertension (HT).
Abbreviations: BP, blood pressure; K, potassium; LV, left-ventricular; TGF-beta, transforming growth factor beta.

Efficacy of dual RAAS blockade	Safety of dual RAAS blockade	Remarks
• Larger decrease of BP	• No difference in adverse events • No hypotension • No change in serum K • No change in renal function	• Submaximal doses of ACEi and ARB
• Larger decrease of BP	• More hyperkalemia	• Meta-analysis of 14 RCT • Submaximal doses of ACEi and ARB
• Similar decrease of BP • Larger decrease of LV mass • Larger decrease of circulating TGF-beta	• No difference in adverse events • No discontinuations	• Submaximal doses of ACEi and ARB • Better outcome apparently independent of BP
• Larger decrease of BP	• More gynaecomastia • More renal function decline	• Not blinded, no placebo • Cross-over design • Titration of spironolactone to optimal BP levels • ACEi and ARB type and dose not specified
• Larger decrease of BP	• More gynaecomastia • Higher serum K levels • Larger renal function decline	• Not blinded, no placebo • Cross-over design • ACEi and ARB type and dose not specified
• Larger decrease of BP	• No difference in adverse events • No difference in discontinuations	• Forced titration to maximum doses • Superiority of RI+ARB combination over RI monotherapy
• Larger decrease of daytime and nighttime BP	• More diarrhea • No difference in hyperkalemia	• Forced titration of aliskiren • Submaximal dose of ARB • 24h ambulatory BP measurements
• Larger decrease of BP	• No difference in adverse events • No difference in hyperkalemia • No difference in discontinuations	• Forced titration of aliskiren • Superiority of RI+ARB combination over RI monotherapy

Table 1B Dual blockade in cardiovascular patients

Dual therapy	Trial	Design	Population	Intervention
ACEi+ARB	RESOLVD[36] 1999	RCT, 43 wk	Heart failure, 768 patients	• Enalapril 20 mg/d + Candesartan 4-8 mg/d • Enalapril 20 mg/d • Candesartan 4-16 mg/d
ACEi+ARB	ValHeFT[37] 2001	RCT, 23 mo	Heart failure, 3034 patients	• Valsartan 320 mg/d + ACEi • ACEi
ACEi+ARB	CHARM-ADDED[38] 2003	RCT, 41 mo	Heart failure, 2548 patients	• Candesartan 32 mg/d + ACEi • ACEi
ACEi+ARB	VALIANT[34] 2003	RCT, 25 mo	Post-MI LV dysfunction, 14703 patients	• Captopril 150 mg/d + Valsartan 160 mg/d • Valsartan 320 mg/d • Captopril 150 mg/d
ACEi+ARB	Philips[25] 2007	Meta-analysis, ±25 mo	Heart failure, or post-MI LV dysfunction, 17337 patients	• ACEi+ARB combinations • ACEi
ACEi+ARB	Lakhdar[39] 2008	Meta-analysis, ±11 mo	Heart failure, or post-MI LV dysfunction, 18160 patients	• ACEi+ARB combinations • ACEi
ACEi+ARB	ONTARGET[35] 2008	RCT, 56 mo	Vascular disease and/or high risk DM, without heart failure, 25620 patients	• Ramipril 10 mg/d + Telmisartan 80 mg/d • Ramipril 10 mg/d • Telmisartan 80 mg/d
MRB+ACEi	RALES[40] 1999	RCT, 24 mo	Heart failure, 1663 patients	• Spironolactone 25 mg/d + ACEi • ACEi
MRB+ACEi, MRB+ARB	EPHESUS[41] 2003	RCT, 16 mo	Post-MI LV dysfunction, or heart failure, 6632 patients	• Eplerenone ±43 mg/d + ACEi or ARB • ACEi or ARB

Efficacy of dual RAAS blockade	Safety of dual RAAS blockade	Remarks
• No difference in mortality, or hospitalizations • Less increase in end-diastolic and -systolic volume • Larger decrease of BP	• No difference in hypotension • No difference in hyperkalemia • No difference in renal function decline • No difference in discontinuations	• Lower dose of ARB with dual blockade
• No difference in mortality • Less hospitalizations for HF • Improvement of HF symptoms and ejection fraction • Larger decrease of BP and LV diameter	• More hypotension • More hyperkalemia • More discontinuations	• No comparison of renal function decline between dual blockade and ACEi monotherapy
• Lower CV-caused mortality • Less hospitalizations for HF • Larger decrease of BP	• More hyperkalemia • More renal function decline • More discontinuations	• ACEi dose in some patients submaximal, however additional benefit of ARB preserved with all ACEi doses
• No difference in mortality • No difference in CV events • Larger decrease of BP	• More hypotension • No difference in hyperkalemia • More discontinuations	• Half dose of ARB with dual blockade
• Not studied	• More hypotension • More renal function decline • HF subgroup: more hyperkalemia • More discontinuations	• Meta-analysis of 4 RCT • Study into safety, no information about efficacy of dual blockade
• Not studied	• More hypotension • More hyperkalemia • More renal function decline • More discontinuations	• Meta-analysis of 9 RCT • Study into safety, no information about efficacy of dual blockade
• No difference in CV-caused death, MI, stroke, or hospitalizations for HF • Larger decrease of BP	• More hypotension • More hyperkaliemia • More renal function decline and renal dysfunction • More discontinuations	• Comparison of dual blockade versus ACEi, not versus ARB • No specific definition for renal dysfunction
• Lower all-cause mortality • Less hospitalizations for HF • Improvement of HF symptoms • Similar decrease of BP	• More gynaecomastia • Larger increase in serum K • Larger renal function decline • More discontinuations	• ACEi type and dose not specified • Better outcome apparently independent of BP
• Lower mortality (both all-cause and CV-caused) • Less hospitalizations for HF • Lower increase in BP	• More hyperkalemia • More renal function decline • No difference in endocrine disorders	• ACEi and ARB type and dose not specified

Table 1B Continued

Dual therapy	Trial	Design	Population	Intervention
RI+ACEi, RI+ARB	ALOFT[42] 2008	RCT, 13 wk	Heart failure, 302 patients	• Aliskiren 150 mg/d + ACEi or ARB • ACEi or ARB

Randomized controlled trials (RCT) and meta-analyses comparing dual RAAS blockade with combinations of ACE inhibitors (ACEi), angiotensin receptor blockers (ARB), mineralocorticoid receptor blockers (MRB), and renin inhibitors (RI), versus monotherapy, in patients with cardiovascular (CV) disease.

blockade. In general, it would be logical to assume that the potential benefits of dual blockade with ACEi+ARB depend on the specific risk profile of the population under study, as also suggested by subgroup analysis in the ONTARGET, showing that dual blockade was only harmful in individuals with a low renal risk (i.e without diabetes and hypertension, or without albuminuria), whereas in patients with albuminuria a trend towards a better renal outcome was observed[52]. The current data indicate absence of benefits (or even increased risk) in patients with no or little proteinuria and low renal risk, that is, a population one cannot expect to benefit from the added effects of dual blockade on overt proteinuria.

The results of studies on ACEi+ARB combination on hard endpoints in patients with overt proteinuria, such as LIRICO[54] and VA NEPHRON-D[55], are being awaited. In summary, dual blockade with ACEi+ARB may be useful in CKD patients with overt proteinuria despite monotherapy RAAS blockade, but not a standard approach in the management of CKD.

Notwithstanding the risk of hyperkalemia, several small studies have addressed the added effect of MRB on top of ACEi or ARB in CKD patients[56-59]. A stronger effect on proteinuria and blood pressure was found, whereas the effect on renal function was similar, in line with experimental data showing a beneficial effect on renal morphological damage[60]. The incidence of hyperkalemia and gynaecomastia was increased only with the non-selective MRB spironolactone, and not with the selective MRB eplerenone.
The effects of triple blockade with MRB+ACEi+ARB have also been studied in proteinuric CKD patients: Triple blockade reduced proteinuria to the same extent as MRB+ACEi, but to a greater extent than ACEi+ARB[61]. The superiority of MRB+ACEi+ARB over ACEi+ARB was confirmed by two other studies[62;63]. With triple blockade, more

Efficacy of dual RAAS blockade	Safety of dual RAAS blockade	Remarks
• Larger decrease of serum (NT-pro)BNP and urinary aldosterone levels • Similar decrease of BP	• No difference in hypotension, hyperkalemia, or renal dysfunction	• ACEi and ARB type and dose not specified

Abbreviations: BP, blood pressure; HF, heart failure; LV, left-ventricular; MI, myocardial infarction; DM, diabetes mellitus; K, potassium; (NT-pro)BNP, (N-terminal pro) brain natriuretic peptide.

hyperkalemia and a larger (initial) renal function decline arose, possibly related to the diuretic effect of MRB.
Long-term effects on renal outcome, mortality, and safety, of dual (or triple) blockade with MRB in CKD patients, however remain to be determined. Yet, in CKD patients with eGFR >30 mL/min/1.73 m2 with persistent proteinuria despite maximal doses of ACEi or ARB, low-dose MRB could be added to reduce proteinuria, with close monitoring for hyperkalemia[1,58].

Up to now, only very limited clinical evidence is available addressing the issue of RI on top of ACEi or ARB in CKD patients. In diabetic nephropathy, RI+ARB reduced proteinuria more effectively than monotherapy ARB, independent of the effect on blood pressure[64] (Table 1C). The incidence of hyperkalemia and adverse events were similar in both groups. Ongoing studies evaluate the role of RI in the prevention of cardiovascular events and hard renal endpoints in CKD (e.g. ALTITUDE)[65].

Optimization of efficacy of RAAS blockade: strategies other than dual blockade

In the vigorous debate on dual RAAS blockade it is important to keep in mind the therapeutic goals: the improvement of end-organ protection, i.e. delay of progression to ESRD, and prevention of CV events. As supported by current guidelines, monotherapy RAAS blockade is first line therapy in CKD and heart failure[1,3]. Before considering dual blockade, the effect of monotherapy drug should be optimal. This can be achieved by sufficient dosing of the single drug, and by correction of (subclinical) volume overload in the patient.

Table 1C Dual blockade in renal patients

Dual therapy	Trial	Design	Population	Intervention
ACEi+ARB	CALM[43] 2000	RCT, 12 wk	DM2 with micro-albuminuria, 199 patients	• Lisinopril 20 mg/d + Candesartan 16 mg/d • Lisinopril 20 mg/d • Candesartan 16 mg/d
ACEi+ARB	COOPERATE[49] 2003 **RETRACTED**	RCT, 36 mo	Non-DM CKD with proteinuria, 263 patients	• Trandolapril 3 mg/d + Losartan 100 mg/d • Trandolapril 3 mg/d • Losartan 100 mg/d
ACEi+ARB	MacKinnon[44] 2006	Meta-analysis, ±12 wk	CKD with proteinuria, 654 patients	• ACEi + ARB combinations • ACEi
ACEi+ARB	IMPROVE[45] 2007	RCT, 20 wk	HT with albuminuria (100%), DM and/or CV disease, 405 patients	• Ramipril 10 mg/d + Irbesartan 150-300 mg/d • Ramipril 10 mg/d
ACEi+ARB	Kunz[46] 2008	Meta-analysis, ±4 mo	CKD with albuminuria, 752 patients	• ACEi + ARB combinations • ACEi • ARB
ACEi+ARB	VALERIA[47] 2008	RCT, 30 wk	HT with micro-albuminuria, 133 patients	• Lisinopril 20 mg/d + Valsartan 320 mg/d • Lisinopril 40 mg/d • Valsartan 320 mg/d
ACEi+ARB	Catapano[48] 2008	Meta-analysis, ±4 mo	Primary GN with proteinuria, 425 patients	• ACEi + ARB combinations • ACEi • ARB
MRB+ACEi, MRB+ARB	Schjoedt[56] 2006	RCT, 8 wk	DM with macro-albuminuria, 20 patients	• Spironolactone 25 mg/d + ACEi or ARB • ACEi or ARB
MRB+ACEi	Epstein[57] 2006	RCT, 12 wk	DM with micro-albuminuria, 268 patients	• Eplerenone 50 or 100 mg/d + Enalapril 20 mg/d • Enalapril 20 mg/d

Efficacy of dual RAAS blockade	Safety of dual RAAS blockade	Remarks
• Larger decrease of UAE • Larger decrease of BP	• No difference in adverse events • Larger increase in serum K^+ • Larger renal function decline • No difference in discontinuations	• Submaximal ARB dose
• Slower progression towards ESRD • Larger decrease of UPE	• No difference in adverse events • No difference in hyperkalemia • No difference in discontinuations	• Retracted because of large inconsistencies in the design and data
• Larger decrease of UPE • Larger decrease of BP	• Larger increase in serum K^+ • No difference in renal function decline	• Meta-analysis of 21 RCT (incl. COOPERATE) • ACEi and ARB dose in some studies submaximal • Benefit present in both DM and non-DM CKD
• Larger decrease of UAE in macroalbuminuria and/or DM2 • Similar decrease of UAE in microalbuminuria without DM2 • Larger decrease of BP	• No difference in adverse events • No difference in hyperkalemia • Similar renal function decline • No difference in discontinuations	• Benefit dependent on baseline UAE and the presence of DM2
• Larger decrease of UAE in both micro- and macroalbuminuria, and in both non-DM and DM CKD • Effects on BP not specified	• More discontinuations • Insufficient safety data	• Meta-analysis of 23 RCT [excl. COOPERATE) • Benefit independent of baseline UAE, presence of DM, or time of follow-up (1-4 vs. 5-12 mo) • ACEi and ARB dose in some studies submaximal
• Larger decrease of UAE • Similar decrease of BP	• More adverse events • More hypotension • More hyperkalemia	• Half dose of ACEi with dual blockade
• Larger decrease of UPE • Similar decrease of BP	• No difference in adverse events • Larger increase in serum K^+ • No change in renal function • No difference in discontinuations	• Meta-analysis of 13 RCT • ACEi and ARB dose in some studies submaximal
• Larger decrease of UAE • Larger decrease of daytime but not nighttime BP	• No difference in adverse events • No difference in hyperkalemia • No difference in discontinuations	• 24 hour blood pressure measurements
• Larger decrease of UAE • Similar decrease of BP	• No difference in hyperkalemia • No difference in adverse events • No gynaecomastia	• Similar benefit with eplerenone 50 mg/d vs. 100 mg/d.

Table 1C Continued

Dual therapy	Trial	Design	Population	Intervention
MRB+ACEi, MRB+ARB	Navaneethan[58] 2009	Meta-analysis, ±18 wk	CKD with albuminuria, 845 patients	• Spironolactone 25-50 mg/d + ACEi or ARB • Eplerenone 50-200 mg/d + ACEi • ACEi or ARB
MRB+ACEi, ARB+ACEi	Mehdi 2009	RCT, 48 wk	DM with macro-albuminuria, 81 patients	• Spironolactone 25 mg/d + Lisinopril 80 mg/d • Losartan 100 mg/d + Lisinopril 80 mg/d + • Lisinopril 80 mg/d
RI+ARB	AVOID[63] 2008	RCT, 6 mo	DM with macro-albuminuria, 599 patients	• Aliskiren 150-300 mg/d + Losartan 100 mg/d • Losartan 100 mg/d

Randomized controlled trials (RCT) and meta-analyses comparing dual RAAS blockade with combinations of ACE inhibitors (ACEi), angiotensin receptor blockers (ARB), mineralocorticoid receptor blockers (MRB), and renin inhibitors (RI), versus monotherapy, in patients with chronic kidney disease (CKD). Abbreviations: DM2, diabetes mellitus type 2; UAE, urinary albumin excretion; BP, blood pressure; K+, potassium; non-DM, non-diabetic; ESRD, end-stage renal disease; UPE, urinary protein excretion; HT, hypertension; CV, cardiovascular; GN, glomerulonephritis.

First, optimal dosing for proteinuria may require higher doses than for correction of blood pressure, as shown for ACEi as well as ARB monotherapy[66-69]. Interestingly, all but one[29] of the dual RAAS blockade studies described above applied fixed dose combinations, rather than applying dose-finding prior to adding the second RAAS blocker. Accordingly, the observed benefit of combined therapy might relate to the submaximal dose of monotherapy, and monotherapy at higher doses could theoretically be as effective.

Several lines of evidence support the potential of adopting an individualized approach to optimize RAAS blockade based renoprotective therapy. First, the response to monotherapy RAAS blockade varies considerably between patients, and the between-patient differences by far exceed between-drug differences[70]. Next, there is evidence

	Efficacy of dual RAAS blockade	Safety of dual RAAS blockade	Remarks
	• Larger decrease of UAE • Larger decrease of BP	• More gynaecomastia with spironolactone but not with eplerenone • More hyperkalemia with spironolactone but not with eplerenone • No difference in renal function decline	• Meta-analysis of 10 RCT • Benefit present in both DM and non-DM CKD
	• Larger decrease of UAE with MRB+ACEi than with ACEi • Similar decrease of UAE with ARB+ACEi as with ACEi • Similar decrease of BP	• More discontinuations with MRB+ACEi • Larger increase in serum K^+ with MRB+ACEi and ARB+ACEi • Similar renal function decline	• Both clinic and ambulatory blood pressure measurements • Better outcome independent of BP
	• Larger decrease of UAE • Larger decrease of BP	• No difference in adverse events • No difference in hyperkalemia • Larger renal function decline with placebo	• Forced titration of aliskiren

that the ACEi dose and ARB dose required for a maximum antiproteinuric effect varies between patients[66,71], supporting a policy of individual dose titration. Differences in dose response may be due to differences in renal pathology, genetic background or different pharmacokinetics related to differences in renal function[70-72]. In a study from our own department, the antiproteinuric effect of combined ACEi+ARB was investigated for the individually established maximally effective dose[66]. Proteinuria decreased from 4.5 g/day at baseline to 1.0 g/day during optimally titrated monotherapy. With combined therapy, there was a further 30% decrease compared with monotherapy. Positive results from studies testing ARB doses much higher than generally recommended justify further exploration of this concept and data on safety are encouraging[67,69].

Second, volume status is a main determinant of the efficacy of RAAS blockade in hypertension, CVD and CKD[1-3], which unfortunately is often neglected[73]. Retention of sodium and water is central in the pathophysiology of heart failure[74], hypertension[75] (in particular in association with weight excess)[76] and proteinuric CKD[77,78]. Volume excess is consistently associated with a blunted response to RAAS blockade[79;80]. Volume intervention by diuretic therapy[80;81], or dietary sodium restriction[79;82;83] restores or enhances the effects on blood pressure and proteinuria of RAAS blockade. In proteinuric patients it has unequivocally been shown that combination of both sodium restriction and diuretic therapy is required for an optimal response to RAAS blockade[84].

Unfortunately, sodium status is seldom monitored, let alone optimized, not even in clinical trials on CVD or CKD, as illustrated by lack of data on 24 hour urinary sodium excretion in many trials[64 85 91], as recently reviewed[73]. Dietary sodium restriction and/or diuretic therapy are included in official guidelines for treatment of hypertension, heart failure, and CKD[1 3]. In heart failure, diuretics are standard care to control fluid overload, but not with the aim to improve therapeutic response of RAAS blockade.

In line with the lack of interest in volume status as a target for intervention, dietary sodium restriction and/or diuretic therapy have not been systematically implemented in the renoprotective regimens prior to establishing dual RAAS blockade. In this respect it should be noted that the additional benefit on blood pressure and proteinuria of volume correction measures may be in the same range, or larger, than found for dual blockade in most studies[66;84;92 98] (Figure 3). Whether combined ACEi+ARB results in better therapeutic effects than single RAAS blockade with volume correction measures, is currently under study (DUAAAL study, Dutch Trial Register, NTR675).

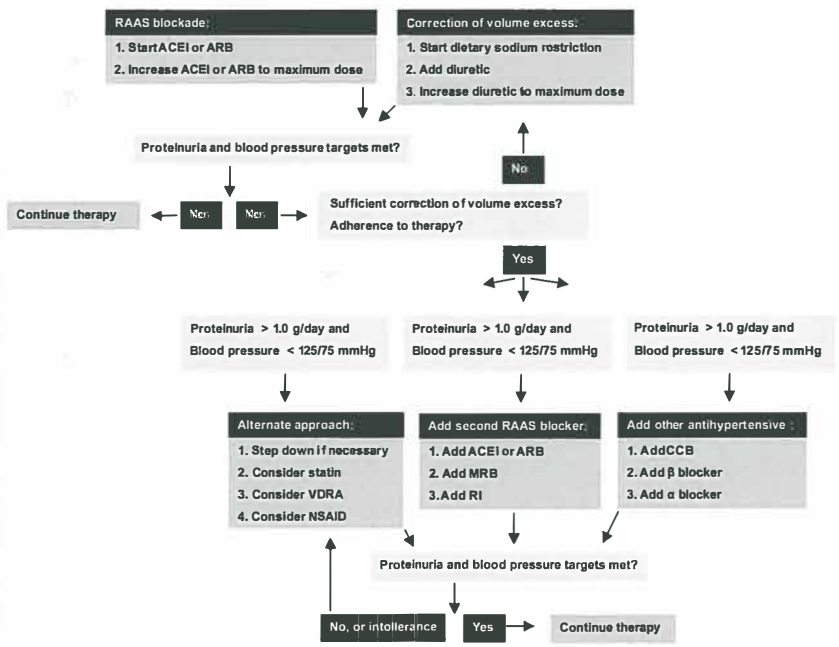
Towards rational RAAS blockade based treatment schedules

Residual proteinuria during RAAS inhibition predicts the subsequent course of renal function decline[99]. This is consistent with experimental data showing that proteinuria induces tubulo-interstitial inflammation and progressive fibrosis[100]. These considerations, supporting a pathogenetic role of proteinuria in progressive renal damage, provide the rationale to target renoprotective therapy to maximum reduction of proteinuria[101].

In spite of the successes of antiproteinuric intervention[102], and in spite of its general acceptance as a therapeutic target, we should be aware that titration for proteinuria has never prospectively been tested in a randomized controlled trial. Prospective animal

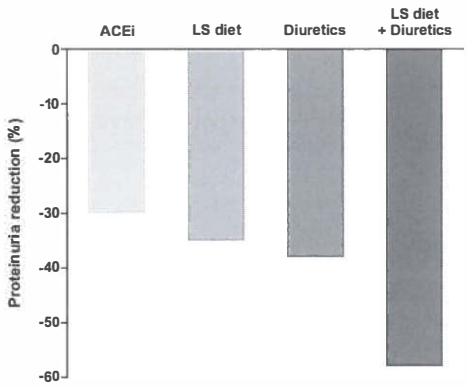
data[103] as well as retrospective human data[104] suggest that a poor antiproteinuric response to ACEi reflects more advanced interstitial fibrosis, and hence an intrinsically worse long term prognosis. If so, a policy of intensifying therapy to reduce proteinuria might not universally improve long-term outcome, despite further reduction of proteinuria. These considerations are supported by animal data, showing that aggressive antiproteinuric treatment can improve blood pressure and proteinuria, without however improvement[105] or even worsening[106] of renal structural damage.

Figure 2 Suggested treatment scheme for optimization of RAAS blockade in proteinuric CKD



Initiate therapy with ACE inhibitor (ACEi) or Angiotensin II Receptor Blocker type I (ARB), titrated to the optimal antiproteinuric effect. Correction of (subclinical) volume excess -which is almost universally present- is usually necessary. The current treatment targets are proteinuria <1.0 g/day and blood pressure <125/75 mmHg. Abbreviations: MRB, Mineralocorticoid Receptor Blocker; RI, Renin Inhibitor; VDRA, Vitamin D Receptor Activator; NSAID, Non Steroidal Anti-inflammatory Drug; CCB, Calcium Channel Blocker.

Figure 3 Comparison between the added effect of ACEi and volume intervention, on top of ARB



Antiproteinuric effect of the addition of ACE inhibitors (ACEi), low sodium (LS) diet, diuretics, or LS diet and diuretics, respectively, on top of angiotensin receptor blockers (ARB), in chronic kidney disease patients. The largest antiproteinuric effect is achieved by combined volume intervention (LS diet plus diuretics) on top of ARB monotherapy. Data derived from: Russo, Berger, Ferrari, Kincaid-Smith, Campbell, Song, Rutkowski, Laverman66;92-98 and Vogt84.

The possibility of dissociation between improved proteinuria and worse renal structural tubulo-interstitial damage is a matter of concern, as renal structural damage is not usually monitored in the clinical setting, as this would require repeated renal biopsies. Non-invasive biomarkers to monitor the severity and course of renal tubulo-interstitial damage therefore, are badly needed[107]. Urinary Kidney Injury Molecule-1 (KIM-1), a marker for tubular damage, might be useful in this respect[108] although its long term prognostic impact in CKD remains to be demonstrated.

On the other hand, in clinical practice residual proteinuria during RAAS blockade, or more precisely, a poor treatment response, is often at least partly due to a state of volume excess[73] even in the absence of overt edema[109]. This can be corrected by sodium restriction combined with diuretic therapy[80;84]. Whereas 24h urine provides a measure of intake, it does not reflect the extent of volume excess as such. It would be highly useful, therefore, to have an index of excess volume, indicating whether further volume-directed correction (i.e higher dose diuretic, more strict dietary measures) can be expected to be of benefit. In proteinuric patients uncontrolled blood pressure during RAAS blockade can

be considered to indicate persistent volume excess, as a non-RAAS dependent blood pressure indicates volume repletion. Moreover, NT-proBNP is a promising marker in this respect (Slagman MC, unpublished data).

Thus, in such circumstances on monotherapy RAAS blockade the rational approach is to first correct volume excess rather than to apply dual RAAS blockade. Practical translation of this approach, shown in Figure 2 ("HONEST-1"), has been proposed by the HONEST (HOlland NEphrology STudy) Group. Animal studies provide support for this approach, as dietary sodium restriction could overcome the adverse impact of (relatively mild) pre-existent interstitial damage on the response to ACEi[110] in proteinuria-associated renal damage, whereas dual blockade could not overcome treatment resistance to mono-therapy ACEi[111].

Conclusion

Combinations of RAAS blocking drugs have been tested in various CVD and CKD conditions, generally enhancing the therapeutic effects on intermediate endpoints such as blood pressure and proteinuria, but the long term benefit is not clear and may differ between patient groups.

We propose that more attention should be paid to gain optimal effect of single drug RAAS blockade prior to considering dual blockade. This implies individual dose titration and explicit correction of volume excess. Whether dual blockade will further improve the therapeutic response on top of sodium restriction and/or diuretic therapy remains to be proven. In CKD, dual RAAS blockade should be restricted in patients with residual proteinuria despite maximal monotherapy RAAS blockade and adequate volume control, although long-term benefit remains to be proven. The combination of MRB with ACEi or ARB may be used in selected patients with heart failure, whereas in CVD there is virtually no place for the ACEi+ARB combination according to the available literature. Results of ongoing studies evaluating the effects of dual blockade with RI and ACEi or ARB on hard cardiovascular and renal endpoints are expected with great interest.

In our opinion, future trials should be designed to test strategies aimed at maximum proteinuria reduction, accompanied by proper monitoring for possible adverse effects, rather than testing drug combinations per se.

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2

Moderate dietary sodium restriction added to angiotensin converting enzyme inhibition compared with dual blockade in lowering proteinuria and blood pressure: randomized controlled trial

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Abstract

Objective: To compare the effects on proteinuria and blood pressure of addition of dietary sodium restriction or angiotensin receptor blockade on maximum dose, or their combination, in patients with non-diabetic nephropathy who receive a background treatment of angiotensin converting enzyme (ACE) inhibition on maximum dose.

Design: Multicentre crossover randomized controlled trial.
Setting: Outpatient clinics in the Netherlands.
Participants: 52 patients with non-diabetic nephropathy.
Interventions: All patients were treated during four 6 week periods, in random order, with angiotensin receptor blockade (valsartan 320 mg/day) or placebo, each combined with, consecutively, a low sodium diet (target 50 mmol Na+/day) and a regular sodium diet (target 200 mmol Na+/day), with a background of ACE inhibition (lisinopril 40 mg/day) during the entire study. The drug interventions were double blind; the dietary interventions were open label.
Main outcome measures: The primary outcome measure was proteinuria; the secondary outcome measure was blood pressure.

Results: Mean urinary sodium excretion, a measure of dietary sodium intake, was 106 (SE 5) mmol Na+/day during a low sodium diet and 184 (6) mmol Na+/day during a regular sodium diet ($P<0.001$). Geometric mean residual proteinuria was 1.68 (95% confidence interval 1.31 to 2.14) g/day during ACE inhibition plus a regular sodium diet. Addition of angiotensin receptor blockade to ACE inhibition reduced proteinuria to 1.44 (1.07 to 1.93) g/day ($P=0.003$), addition of a low sodium diet reduced it to 0.85 (0.66 to 1.10) g/day ($P<0.001$), and addition of angiotensin receptor blockade plus a low sodium diet reduced it to 0.67 (0.50 to 0.91) g/day ($P<0.001$). The reduction of proteinuria by the addition of a low sodium diet to ACE inhibition (51%, 95% confidence interval 43% to 58%) was significantly larger ($P<0.001$) than the reduction of proteinuria by the addition of angiotensin receptor blockade to ACE inhibition (21%, (8% to 32%) and was comparable ($P=0.009$, not significant after Bonferroni correction) to the reduction of proteinuria by the addition of both angiotensin receptor blockade and a low sodium diet to ACE inhibition (62%, 53% to 70%).

Mean systolic blood pressure was 134 (3) mmHg during ACE inhibition plus a regular sodium diet. Mean systolic blood pressure was not significantly altered by the addition of angiotensin receptor blockade (131 (3) mmHg; $P=0.12$) but was reduced by the addition of a low sodium diet (123 (2) mmHg; $P<0.001$) and angiotensin receptor

blockade plus a low sodium diet (121 (3) mmHg; P<0.001), to ACE inhibition. The reduction of systolic blood pressure by the addition of low sodium diet (7 (SE 1)) % was significantly larger (P=0.003) than the reduction of systolic blood pressure by the addition of angiotensin receptor blockade (2% (1)) and was similar (P=0.14) to the reduction of systolic blood pressure by the addition of both angiotensin receptor blockade and low sodium diet (9% (1)), to ACE inhibition.

Conclusions: Dietary sodium restriction to a level recommended in guidelines was more effective than dual blockade for reduction of proteinuria and blood pressure in non-diabetic nephropathy. The findings support the combined endeavours of patients and health professionals to reduce sodium intake.

Introduction

In patients with chronic kidney disease, blockade of the renin-angiotensin-aldosterone system with an angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker provides long term renal and cardiovascular protection, apparently through the effects on blood pressure and proteinuria[1-3]. On the basis of the finding that outcome is related to the achieved blood pressure and proteinuria[4-6], guidelines recommend a blood pressure below 125/75 mm Hg in patients with residual proteinuria exceeding 1.0 g/day, with reduction of proteinuria to below 1.0 g/day as an independent target[4;7].

Blockade of the renin angiotensin aldosterone system with monotherapy seems to be insufficiently effective for a large proportion of patients[8-9]. Several potential strategies aim to lower blood pressure and proteinuria on top of ACE inhibition or angiotensin receptor blockade[7,10;11]. These include increasing the dose to higher than the top of the dose-response curve for blood pressure for a better antiproteinuric response[12-14], addition of a second renin-angiotensin-aldosterone system blocker (dual blockade)[8,14;15], and correction of excess extracellular volume by dietary sodium restriction, diuretics, or both[16-24].

Several studies in chronic kidney disease have shown an added effect of dual blockade on blood pressure and proteinuria[8,14,15], but this effect is very modest if dose titration of the single drugs was sufficient[25], and the long term effect is still unclear[26,27]. Addition of dietary sodium restriction might be more effective than dual blockade and is rational because dietary sodium intake in patients with renal disease is usually considerable above the recommended values[28-30].

The effects of dietary sodium restriction and dual blockade have not been tested head to head so far. Therefore, we tested head to head which of the two additional interventions -dietary sodium restriction and angiotensin receptor blockade- is more effective in reaching the treatment targets for proteinuria and blood pressure in patients with renal disease already treated with ACE inhibition at the maximum recommended dose. We also evaluated the efficacy of combining dietary sodium restriction and dual blockade.

Methods

Study design

The HOlland NEphrology Study (HONEST) Group did a randomized, double blind, placebo controlled, crossover trial between April 2006 and October 2009 in three medical centres. The primary outcome measure of the trial was proteinuria, and the secondary outcome measure was blood pressure. All participants gave written informed consent. The study sponsor provided trial drugs at no cost.

Participants

We screened consecutive patients with renal disease who visited the nephrology outpatient clinics for the presence of non-diabetic nephropathy, as confirmed by analysis of blood and urine or renal biopsy. Inclusion criteria were blood pressure above 125/75 mmHg in combination with residual proteinuria above 1.0 g/day during ACE inhibition at maximal dose (lisinopril 40 mg/day), creatinine clearance of 30 mL/min or above, and age over 18 years.

For safety reasons, we excluded patients with systolic blood pressure of 180 mmHg or above, diastolic blood pressure of 110 mmHg or above, or both. Other exclusion criteria were diabetes mellitus, renovascular hypertension, decrease of creatinine clearance by at least 6 mL/min in the previous year, a cardiovascular event in the previous six months, immunosuppressive treatment, regular use (>1 day/week) of non-steroidal anti-inflammatory drugs, pregnancy, or breast feeding.

Treatment

During a run-in period of at least six weeks, patients received ACE inhibition at maximal dose (lisinopril 40 mg/day) and stopped all other renin-angiotensin-aldosterone system blockers. Additional antihypertensive drugs such as β blockers, α blockers, calcium channel blockers, and diuretics were allowed and kept stable during the study (Table 1). No dietary intervention took place during the run-in period.

Table 1 Baseline characteristics

Treatment sequence	I	II	III	IV
Number of participants	14	11	13	14
Age – yr	53 (3)	55 (3)	51 (4)	47 (4)
Male sex – %	64	82	92	93
Caucasian race – %	100	100	100	100
Body mass index – kg/m²	27 (1)	28 (1)	28 (1)	28 (1)
Renal diagnosis:				
IgA NP – %	21	18	31	44
FSGS – %	21	64	23	21
Membranous NP – %	14	0	15	21
Hypertensive NP – %	8	18	23	0
Other / inconclusive – %	36	0	8	14
Use of non-study medication:				
Betablocker – %	29	18	31	14
Calciumchannelblocker – %	7	36	23	14
Alphablocker – %	0	9	23	7
Diuretic – %	21	9	23	43
Lipid lowering agent – %	50	64	54	29
Systolic blood pressure – mmHg	131 (4)	135 (4)	135 (7)	123 (4)
Diastolic blood pressure – mmHg	78 (2)	78 (3)	78 (4)	71 (3)
Proteinuria – g/24h	1.5 (0.9-2.4)	2.0 (1.3-3.0)	1.5 (0.9-2.6)	1.5 (0.8-2.6)
Creatinine clearance – ml/min	70 (56-88)	60 (41-89)	74 (56-98)	78 (56-107)
Urinary sodium excretion – mmol/4h	166 (23)	161 (14)	197 (20)	182 (22)

Abbreviations: FSGS, focal segmental glomerulosclerosis; NP, nephropathy. Baseline data were compared between the four different treatment sequences, being I) placebo+LS → valsartan+LS → valsartan+RS → placebo+RS, II) placebo+RS → valsartan+RS → valsartan+LS → placebo+LS, III) valsartan+RS → placebo+RS → placebo+LS → valsartan+LS, and IV) valsartan+LS → placebo+LS → placebo+RS → valsartan+RS. No significant differences were found.

After the run-in period, patients were treated during four treatment periods of six weeks with, consecutively, ACE inhibition at maximal dose (lisinopril 40 mg/day) plus placebo and ACE inhibition plus angiotensin receptor blockade at maximal dose (lisinopril 40 mg/day plus valsartan 320 mg/day). Both treatments were combined with, consecutively, a low sodium diet (target sodium intake 50 mmol Na^+/day; approximately 1200 mg Na^+/day or 3 g NaCl/day) and a regular sodium diet (target sodium intake 200 mmol Na^+/day; 4800 mg Na^+/day or 12 g NaCl/day). The drug interventions were double blind, whereas the dietary interventions were open label.

To prevent systematic errors resulting from the crossover design, the different treatment periods were done in random order. Because of this randomisation and the rather short half life of the interventions (lisinopril 12.6 hours, valsartan 9 hours, low sodium diet <1 week[31]), the protocol did not include wash-out periods.
We defined four different treatment sequences as follows. (1) Placebo plus low sodium diet, valsartan plus low sodium diet, valsartan plus regular sodium diet, placebo plus regular sodium diet. (2) Placebo plus regular sodium diet, valsartan plus regular sodium diet, valsartan plus low sodium diet, placebo plus low sodium diet. (3) Valsartan plus regular sodium diet, placebo plus regular sodium diet, placebo plus low sodium diet, valsartan plus low sodium diet. (4) Valsartan plus low sodium diet, placebo plus low sodium diet, placebo plus regular sodium diet, valsartan plus regular sodium diet.
An independent pharmacist randomized these sequences, using a computer program. We implemented the random allocation sequence by means of sequentially numbered containers of study drug. Physicians enrolled patients, and the pharmacist allocated the study drug sequentially to consecutive participants. The randomization code remained secret during the entire study; all participants, investigators, and care providers were blinded, except for the pharmacist.

Physicians gave the participants a list of food products that are commonly consumed in the Netherlands, together with their sodium content, at the time of inclusion. Diverse professional dietitians gave further dietary counselling in various autonomous dietary practices in the community. Except for a request to achieve the particular sodium targets (that is, 50 mmol Na^+/day during the low sodium diet and 200 mmol Na^+/day during the regular sodium diet), dietitians did not receive extra training or a script for this study.
Each patient had two to four dietary counselling sessions. Individualized counselling used the general principle of remaining as close as possible to the patients' preferences and nutritional habits, to increase feasibility and compliance, taking into account adequacy of nutritional requirements as well as sodium content. For the periods on the regular sodium diet, the patients were advised to maintain their habits regarding sodium intake. For the periods on the low sodium diet, patients were advised not to add any salt to their food and to replace sodium rich products with sodium poor products. We monitored compliance by 24 hour urinary sodium excretion and informed the physician, patients, and dietitians of these results.

Measurements and calculations

At the end of each six week treatment period, patients collected 24 hour urine samples and blood pressure was measured and blood sampled after an overnight fast.

Additionally, in the middle of every six week treatment period, patients collected 24 hour urine samples to monitor dietary compliance.

We measured proteinuria in 24 hour urine samples with a turbidimetric assay using benzethonium chloride (Modular, Roche Diagnostics, Mannheim, Germany). We measured blood pressure at one minute intervals with an automatic device (Dinamap, G E Medical Systems, Milwaukee, WI, USA) with the patient in a supine position. After 15 minutes of measurements, we used the mean of the last three readings for further analysis. We determined blood electrolytes, lipids, proteins, and urinary electrolytes by using an automated multianalyser (Modular, Roche Diagnostics, Mannheim, Germany). We assessed dietary sodium intake from urinary sodium excretion. We calculated creatinine clearance from creatinine concentrations in plasma and in 24 hour urine samples. We used the Maroni formula to assess dietary protein intake from urinary urea excretion[32,33]. We assessed peripheral pitting oedema at the pretibial area of both legs by visual and manual examination and scored it as absent or present.

Statistical analysis

We expected that patients would present with a mean proteinuria of approximately 2 g/day during ACE inhibition. On the basis of previous studies, we assumed a 35% reduction in proteinuria by addition of a low sodium diet on top of ACE inhibition plus angiotensin receptor blockade and a standard deviation of 0.75 in log transformed proteinuria response [8,16;17,20,25]. From these numbers, we estimated that 51 patients had to complete the crossover design to provide 90% power to detect a statistically significant difference. We used a significance level of α=0.0083 (rather than α=0.05) to adjust for six primary comparisons of interest. To account for a 10% dropout rate during the trial, we would need to include 56 patients (PASS 10, NCCS, East Kaysville, UT, USA). Of note, the sample size is smaller than would have been needed in a non-crossover study, as the same patient provides data for each treatment group and this increases power, owing to the smaller within patient variability than between group variability[34,35].

We analysed data for the 52 patients who completed the trial, and we present these data here. Additionally, we analysed the data for all 54 patients who were included (intention to treat). As the effect estimates and confidence intervals were very similar and the statistical and clinical conclusions did not change, we have not shown these data.

Before statistical testing, we natural log transformed skewed variables to obtain normality. We determined differences between the four different treatment sequences

by using one way analysis of variance with Bonferroni's post hoc tests and Pearson's ^{2} tests. We used paired *t* tests (which account for the same patients providing data for both treatment groups) to determine effects of treatment.

We did six comparisons for each parameter: ACE inhibition versus ACE inhibition plus angiotensin receptor blockade, ACE inhibition versus ACE inhibition plus low sodium diet, ACE inhibition versus ACE inhibition plus angiotensin receptor blockade plus low sodium diet, ACE inhibition plus angiotensin receptor blockade versus ACE inhibition plus low sodium diet, ACE inhibition plus angiotensin receptor blockade versus ACE inhibition plus angiotensin receptor blockade plus low sodium diet, and ACE inhibition plus low sodium diet versus ACE inhibition plus angiotensin receptor blockade plus low sodium diet.

To allow for multiple testing, we set the type I error (α) at 0.0083 (Bonferroni correction) for analyses of the primary outcome (proteinuria). Furthermore, we did a linear mixed model analysis to check for carryover effects, with log transformed proteinuria as a dependent variable, participants as a random factor, and treatment and sequence as well as their interaction (treatment*sequence) as fixed factors.

We give data as mean with standard error (SE) when normally distributed or as geometric mean with 95% confidence interval when skewed. We report only unadjusted P values. We used SPSS 16.0 for Windows for all analyses.

Results

Study population

We assessed 71 patients for eligibility. Of these, 13 patients declined to participate and 58 patients gave informed consent and started the run-in period. During the run-in period, two patients discontinued because of symptomatic hypotension and two patients were withdrawn because of complete reduction of proteinuria. Of the 54 patients who were randomized, one patient was withdrawn because of a rash after starting valsartan and one patient discontinued because of lack of motivation to adhere to the low sodium diet. Finally, 52 patients completed the study and were included in the analyses.

Table 1 shows baseline characteristics. Before entry into the study, 14 of the 52 patients used a β blocker, whereas 12 patients were using a β blocker at the end of the run-in period (and during the rest of the study). The equivalent numbers were 10 versus 10 patients for calcium channel blockers, 5 versus 5 for α blockers, 24 versus 8 for thiazide

diuretics, 9 versus 5 for loop diuretics, 36 versus 52 for ACE inhibitors, and 29 versus 0 for angiotensin receptor blockers. Non-study drugs were kept stable during the study.

Compliance and efficacy

We assessed compliance from 24 hour urine samples and from pill counts. Urinary creatinine excretion was comparable during all treatment periods, indicating accurate 24 hour urine sample collection (table 2[t2]). Mean dietary sodium intake, as assessed from urinary sodium excretion, was 106 (SE 5) mmol Na^+/day (approximately 2500 mg

Table 2 Clinical parameters during four treatment periods

	Regular sodium diet		Low sodium diet	
	ACEi	ACEi+ARB	ACEi	ACEi+ARB
Plasma:				
Sodium - mmol/L	140.7±0.4	140.8±0.4	139.5±0.4 *†	139.1±0.4 *†
Potassium - mmol/L	4.6±0.1	4.6±0.1	4.7±0.1 *	5.0±0.1 *† #
Creatinine - umol/L	137±8	137±8	149±9 *	157±9 *†
Urea - mmol/L	9.8±0.7	10.2±0.7	11.8±0.8 *†	12.9±0.8 *†
Albumin - g/L	38±1	39±1	40±1 *†	40±1 *†
Total protein - g/L	68±1	69±1	71±1 *	72±1 *†
Total cholesterol - mmol/L	5.1±0.2	5.0±0.2	4.8±0.1	4.9±0.2
Urine:				
Creatinine - mmol/24h	13.8±0.6	14.0±0.5	13.5±0.6	13.4±0.6
Sodium - mmol/24h	189±8	180±9	106±7 *†	105±8 *†
Urea - mmol/24h	395±18	403±19	359±17 *†	352±19 *†
Potassium - mmol/24h	78±3	76±4	76±4	73±3
Calcium - mmol/24h	1.2 [0.9-1.5]	1.0 [0.7-1.3] *	0.7 [0.6-0.9] *	0.7 [0.5-0.9] *†
Creatinine clearance - mL/min	72 [62-84]	74 [65-84]	66 [57-76] *†	61 [53-70] *†
Protein/creatinine ratio - mg/mg	1.2 [0.9-1.5]	0.9 [0.7-1.3] *	0.6 [0.4-0.8] *†	0.5 [0.3-0.7] *† #
Other:				
Body weight - kg	89±3	89±2	87±2 *†	87±2 *†
Edema - no. (%)	35±8	38±8	15±6 †	8±4 *†
Symptomatic hypotension - no. (%)	-	-	3 (6)	4 (8)
Dry cough - no. (%)	1 (2)	1 (2)	1 (2)	1 (2)

Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; *p<0.01 vs. ACEi on regular sodium diet, †p<0.01 vs. ACEi-ARB on regular sodium diet, #p<0.01 vs. ACEi on low sodium diet

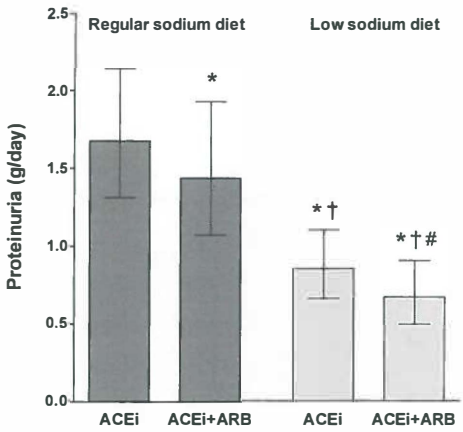
Na^+/day or 6 g NaCl/day) during the periods on a low sodium diet and 184 (6) mmol Na^+/day (4400 mg Na^+/day or 11 g NaCl/day; P<0.001 *v* low sodium diet) during the periods on the regular sodium diet. All patients adhered to the pharmaceutical intervention (>85% of valsartan and placebo capsules taken during each study period), except for two patients who took only 60-70% of the blinded study drug during the four different treatment periods. We included all 52 patients in the analyses.

The addition of a low sodium diet to ACE inhibition decreased body weight (from mean 89 (SE 3) kg to 87 (2) kg; P<0.001) and plasma sodium (from 140.7 (SE 0.4) mmol/L to 139.5 (0.4) mmol/L; P=0.001) and the prevalence of peripheral oedema (from 18 patients (35%) to 8 patients (15%), and increased plasma albumin (from 38 (1) g/L to 40 (1) g/L; P<0.001) and total protein (from 68 (1) g/L to 71 (1) g/L; P<0.001), consistent with a negative sodium balance. Addition of angiotensin receptor blockade to ACE inhibition did not affect these parameters, whereas addition of angiotensin receptor blockade plus a low sodium diet to ACE inhibition had approximately the same effect as addition of a low sodium diet alone (Table 2). Dietary protein intake, as assessed from urinary urea excretion, was 1.02 (0.04) g/kg/day during ACE inhibition. It was not altered by the addition of angiotensin receptor blockade (1.01 (0.04) g/kg/day; P=0.99) but was slightly reduced by the addition of a low sodium diet (0.96 (0.04) g/kg/day; P=0.004) or a low sodium diet plus angiotensin receptor blockade (0.91 (0.03) g/kg/day; P<0.001) to ACE inhibition.

Proteinuria (primary outcome)

During ACE inhibition combined with the regular sodium diet, geometric mean residual proteinuria was 1.68 (95% confidence interval 1.31 to 2.14) g/day (Figure 1). Addition of angiotensin receptor blockade reduced proteinuria to 1.44 (1.07 to 1.93) g/day (P=0.003), and addition of a low sodium diet reduced it to 0.85 (0.66 to 1.10) g/day (P<0.001). The lowest level of residual proteinuria (0.67 (0.50 to 0.91) g/day; P<0.001) was achieved by the addition of angiotensin receptor blockade plus a low sodium diet. Moreover, the reduction of proteinuria by the addition of a low sodium diet to ACE inhibition (reduction of 51% (95% confidence interval 43% to 58%) was significantly larger (P<0.001) than the reduction of proteinuria by the addition of angiotensin receptor blockade to ACE inhibition (reduction of 21% (8% to 32%). However, the reduction of proteinuria by the addition of both a low sodium diet and angiotensin receptor blockade to ACE inhibition (reduction of 62% (53% to 70%) was not significantly larger (P=0.009, not significant after Bonferroni correction) than the reduction of proteinuria by the addition of only a low sodium diet to ACE inhibition.

Figure 1 Additional effect of low sodium diet, ARB, or both on proteinuria during ACEi



Data are geometric mean with 95% confidence interval. Abbreviations: ARB, angiotensin receptor blockade; ACEi, angiotensin converting enzyme inhibition; * p<0.05 versus ACE inhibition on regular sodium diet; † p<0.05 versus ACEi plus ARB on regular sodium diet; # p<0.05 versus ACEi on low sodium diet.

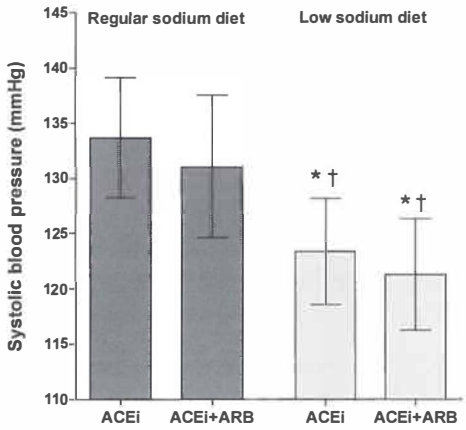
In an additional linear mixed model analysis, we verified the absence of carryover effects. Treatment was a significant determinant of residual proteinuria (P<0.001), whereas sequence (P=0.52) and treatment*sequence (P=0.98) were not. We found similar results for urinary protein/creatinine ratio (Table 2).

Secondary outcomes

Systolic and diastolic blood pressure were above the target of 125/75 mm Hg during ACE inhibition combined with the regular sodium diet. Mean systolic blood pressure was 134 (SE 3) mm Hg during ACE inhibition (Figure 2). Addition of angiotensin receptor blockade did not significantly alter systolic blood pressure (131 (3) mm Hg; P=0.12), whereas addition of a low sodium diet reduced systolic blood pressure to 123 (2) mm Hg (P<0.001) and addition of both angiotensin receptor blockade and a low sodium diet reduced systolic blood pressure to 121 (3) mm Hg (P<0.001).

Moreover, the reduction of systolic blood pressure by the addition of a low sodium diet to ACE inhibition (reduction of 7% (SE 1%) was significantly larger (P=0.003) than that achieved by the addition of angiotensin receptor blockade to ACE inhibition (reduction of 2% (1%)). However, the reduction of systolic blood pressure by the addition of both a

Figure 2 Additional effect of low sodium diet, ARB, or both on systolic blood pressure during ACEi



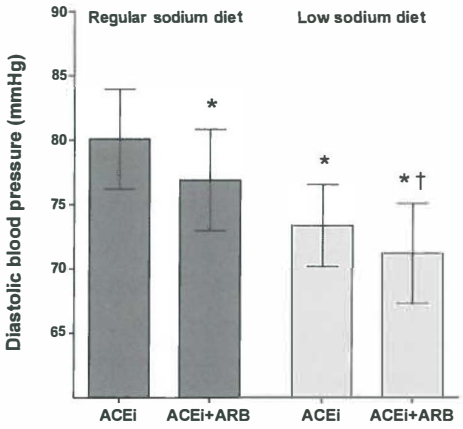
Data are mean with 95% confidence interval. Abbreviations: ARB, angiotensin receptor blockade; ACEi, angiotensin converting enzyme inhibition; * $p<0.05$ versus ACEi on regular sodium diet; † $p<0.05$ versus ACEi plus ARB on regular sodium diet.

low sodium diet and angiotensin receptor blockade to ACE inhibition (reduction of 9% (1%)) was not significantly larger ($P=0.14$) than that achieved by the addition of only a low sodium diet to ACE inhibition (reduction of 7% (1%)).

Mean diastolic blood pressure was 80 (SE 2) mm Hg during ACE inhibition combined with the regular sodium diet (Figure 3). Diastolic blood pressure was slightly reduced by the addition of angiotensin receptor blockade (77 (2) mm Hg; decrease of 4% (2%); $P=0.02$) and was considerably reduced by addition of a low sodium diet (73 (2) mm Hg; decrease of 8% (2%); $P<0.001$) and by the addition of angiotensin receptor blockade plus a low sodium diet (71 (2) mm Hg; decrease of 11% (2%); $P<0.001$).

Renal function was relatively preserved during ACE inhibition combined with the regular sodium diet (geometric mean creatinine clearance 72 (62 to 84) mL/min; mean plasma creatinine 137 (8) μmol/L). Renal function was not significantly altered by the addition of angiotensin receptor blockade (creatinine clearance 74 (65 to 84) mL/min; $P=0.65$), but decreased when a low sodium diet (66 (57 to 76) mL/min; $P=0.002$) or angiotensin receptor blockade plus a low sodium diet (61 (53 to 70) mL/min; $P<0.001$) was added

Figure 3 Additional effect of low sodium diet, ARB, or both on diastolic blood pressure during ACEi



Data are mean with 95% confidence interval. Abbreviations: ARB, angiotensin receptor blockade; ACEi, angiotensin converting enzyme inhibition; * $p<0.05$ versus ACEi on regular sodium diet; † $p<0.05$ versus ACEi plus ARB on regular sodium diet.

to ACE inhibition; this effect was reversible on withdrawal of the low sodium diet and angiotensin receptor blockade (not shown).

Mean plasma potassium was 4.6 (0.1) mmol/L during ACE inhibition combined with the regular sodium diet and was not significantly changed by the addition of angiotensin receptor blockade (4.6 (0.1) mmol/L; P=0.09), whereas addition of a low sodium diet (4.7 (0.1) mmol/L; P=0.03) or angiotensin receptor blockade plus a low sodium diet (5.0 (0.1) mmol/L; P<0.001) increased plasma potassium concentrations.
Potassium concentrations in the lower range (<4.0 mmol/L) were present in eight patients during ACE inhibition combined with the regular sodium diet and in two patients during ACE inhibition plus angiotensin receptor blockade plus a low sodium diet. Potassium concentrations in the higher range (>5.5 mmol/L) were present in three patients during ACE inhibition combined with the regular sodium diet and in ten patients during ACE inhibition plus angiotensin receptor blockade plus a low sodium diet.

Severe orthostatic complaints occurred in two patients during the first treatment period, which was ACE inhibition plus angiotensin receptor blockade plus low sodium diet for

one patient and ACE inhibition plus low sodium diet for the other. The complaints were resolved by tapering of lisinopril to 20 mg/day. In these patients, the dose of lisinopril was kept stable at 20 mg/day during the rest of the treatment periods. In five other patients, mild orthostatic complaints not necessitating drug withdrawal occurred: in three patients during ACE inhibition plus angiotensin receptor blockade plus low sodium diet and in two patients during ACE inhibition plus low sodium diet.
Dry cough occurred in one patient and was present during all study periods. These complaints resolved on tapering of ACE inhibition after the last study period.

Discussion

This study provides the first head to head comparison of moderate dietary sodium restriction, add-on angiotensin receptor blockade, and their combination, as measures to improve the therapeutic effect of angiotensin converting enzyme (ACE) inhibition. In patients with non-diabetic renal disease with insufficient control of proteinuria and blood pressure despite maximally dosed ACE inhibition monotherapy, addition of maximally dosed angiotensin receptor blockade had a modest added effect on proteinuria, without effects on systolic blood pressure. Addition of a low sodium diet to ACE inhibition induced a considerable reduction of proteinuria and blood pressure, and a slight additional reduction of proteinuria occurred during a low sodium diet combined with dual blockade.
These data show, firstly, that moderate dietary sodium restriction added to ACE inhibition is more effective to reach proteinuria and blood pressure targets than is dual blockade and, secondly, that a low sodium diet also improves proteinuria and blood pressure during dual blockade.

Comparison with other studies

Our findings on dual blockade and a low sodium diet are in line with previous studies in chronic kidney disease. A meta-analysis found no differences in systolic and diastolic blood pressure between ACE inhibition plus angiotensin receptor blockade and ACE inhibition alone[15]. In another meta-analysis, proteinuria was on average 22% (16% to 28%) lower during ACE inhibition plus angiotensin receptor blockade than during ACE inhibition[8]. In previous studies of dietary sodium intervention, blood pressure was on average 3% to 9% lower during ACE inhibition (or angiotensin receptor blockade) combined with a low sodium diet than during ACE inhibition (or angiotensin receptor blockade) combined with a regular sodium diet, and proteinuria was 31% to 40% lower[16,17;20;21]. In these studies, the urinary sodium excretion, reflecting dietary sodium

intake, was in the same range as in our study, supporting the generalizability of our findings in renal populations.

Other outcome measures

Plasma potassium concentrations were unaffected by addition of angiotensin receptor blockade to ACE inhibition but increased by addition of a low sodium diet or angiotensin receptor blockade plus a low sodium diet. This may be relevant given the previously shown U shaped relation between plasma potassium and outcome in patients with renal disease, with a higher risk of end stage renal disease and death at potassium concentrations below 4.0 mmol/L and a higher risk of cardiovascular events and death at concentrations exceeding 5.5 mmol/L[36,37]. Increases in potassium might be beneficial in patients with initial plasma potassium in the lower range (15% of our patients) and a potential threat in patients with initial plasma potassium in the higher range (6% of our patients) and would require careful monitoring.

Renal function was not significantly altered by addition of angiotensin receptor blockade to ACE inhibition but decreased by addition of a low sodium diet or angiotensin receptor blockade plus a low sodium diet. This decline in renal function was reversible and probably reflects a fall in glomerular pressure. No evidence suggests that such an effect is harmful; in contrast, it has been associated with a slower subsequent decline in renal function[38-41]. This relation between a treatment induced short term decline in renal function and long term preservation of renal function seems to hold for increases in plasma creatinine of up to 30% in people with creatinine exceeding 124 μmol/L (1.4 mg/dL)[40], which was the case in our patients.

Orthostatic complaints occurred in seven of our 52 patients, during the regimens with the strongest antihypertensive effect (that is, during dual or single blockade combined with the low sodium diet but not during the regular sodium diet). Only two patients needed tapering of ACE inhibition.

Diuretic treatment

The effect of a low sodium diet added to ACE inhibition is probably due to a correction of excess extracellular volume. An alternative approach is diuretic treatment or combination treatment[16,17,19,21]. Interestingly, up-titration of diuretic combined with half doses of ACE inhibition plus angiotensin receptor blockade was recently found to reduce proteinuria better than ACE inhibition plus up-titration of angiotensin receptor blockade to full dose[42]. Moreover, we previously showed that the combination of a low sodium diet and diuretics is more effective than either alone[16].

In the current study, we applied only the low sodium diet, but patients who needed diuretics during the run-in period to control oedema continued this treatment at a fixed dose. For the treatment protocol, we refrained from combining a low sodium diet and diuretics to avoid excessive volume depletion, and associated adverse effects on blood pressure and renal function, during the maximal pharmacological blockade of the renin-angiotensin-aldosterone system.
Thus, in individual patients with insufficient response to dual blockade plus a low sodium diet, the response could probably be improved by adding a diuretic, with monitoring of tolerability.

Strengths and limitations of study

This study provides the first head to head comparison of moderate dietary sodium restriction, add-on angiotensin receptor blockade, and their combination, as measures to improve the therapeutic effect of ACE inhibition. We selected patients with high residual risk during ACE inhibition monotherapy, which is precisely the target population for added measures and thus clinically relevant[8;9]. Reduction of blood pressure and proteinuria in the range seen here has previously been shown to predict a better renal and cardiovascular outcome[2;3;6].

We aimed to optimize the applicability of our results to clinical practice by doing the dietary intervention in an outpatient setting that reflects the usual nephrology care, with relatively simple dietary measures, replacing sodium rich food components with sodium poor products. In line with previous studies, regular sodium intake was well above the recommended intake[28-30]. Our dietary intervention policy did not result in the target of 50 mmol Na^+/day, but a substantial reduction in sodium intake to values in line with the guidelines for renal patients was nevertheless achieved[43]. This supports the applicability of our results to clinical practice.

Furthermore, we used maximal doses of ACE inhibition and angiotensin receptor blockade to ensure a maximal effect of the dual blockade on both blood pressure and proteinuria, because sub-maximal dosing hampers interpretation of many studies on dual blockade. Thus, the stronger effect of the low sodium diet cannot be attributed to a suboptimal dual blockade regimen.

The main limitation of the study is that it provides only short term data and no hard end points. Also, the population was relatively small, although this is the largest study of sodium intervention in proteinuric patients so far. Furthermore, we excluded patients with diabetes because of possible heterogeneity in the renal response to sodium

restriction[44]. These characteristics limit the generalizability of our data. Of note, a separate study in patients with diabetic proteinuria is ongoing (trial number NTR2366).

The sodium intervention was done in a way that closely mimics clinical care in the outpatient setting. For the periods on the regular sodium diet, the patients were advised to maintain their habits regarding sodium intake. For the periods on the low sodium diet, patients were advised not to add any salt to their food and to replace sodium rich products with sodium poor products. Accordingly, the lower proteinuria and blood pressure cannot specifically be attributed to the lower sodium intake, as inadvertent changes in other food components might be involved. However, such changes are likely to occur in clinical practice as well.
From the relevant food components that could be documented in 24 hour urine samples, potassium intake did not change. Urinary urea excretion was reduced during the low sodium diet, suggesting a somewhat lower protein intake. Hence, a lower protein intake may have contributed to the beneficial effect on proteinuria[45,46], although the direct effect of the low sodium diet on blood pressure, during both monotherapy and dual blockade, seems likely to be the main driving force for reduction in proteinuria. Urinary calcium excretion was lower during low sodium periods. We cannot exclude a lower calcium intake, but the lower calciuria is in line with corresponding findings in other populations, where it is attributed to altered renal calcium handling[47]. At any rate, the lower blood pressure during low sodium periods is not likely to be due to an inadvertent higher calcium intake.

Finally, as the study was not powered on blood pressure, we cannot exclude the possibility that the absence of a significant effect of add-on angiotensin receptor blockade on blood pressure may be due to a lack of power.

Policy implications

Our data clearly show that a moderate restriction of dietary sodium intake, which is feasible in routine nephrology care, is more effective than dual renin-angiotensin-aldosterone system blockade for control of blood pressure and proteinuria in chronic kidney disease, with an acceptable rate of adverse effects. Whether this translates into improved outcome in chronic kidney disease should be investigated in a well powered study with sufficiently long term follow-up. This is all the more relevant because long term results of dual blockade have turned out to be unreliable or controversial[26;27;48], whereas the long term benefits of dietary sodium restriction are increasingly appreciated[11;49-52].

Of note, the range of sodium intake associated with a more favourable long term health outcome in the literature is not excessively low, with respect to both spontaneous intake and after intervention[28,49;51], and corresponds to level of sodium restriction obtained in our study. This implies that general efforts to implement guidelines for sodium intake, as recently emphasised for the general population[51], will have the potential to greatly improve health outcomes in patients with chronic renal disease.
Furthermore, as renin-angiotensin-aldosterone system blockade is also a mainstay of treatment in essential hypertension, diabetic nephropathy, and heart failure, investigating the potential of sodium restriction to enhance the efficacy of such blockade in these populations as well would be of great interest.

Conclusions

Moderate dietary sodium restriction was more effective than the addition of maximal dose angiotensin receptor blockade for control of proteinuria and blood pressure in patients with renal disease on a maximal dose of ACE inhibition. Dual blockade should not be instituted in the absence of adequate dietary sodium restriction. Confirmation studies with hard end points are necessary, but in the meantime a coordinated effort to implement the guidelines on sodium intake is warranted. Our findings support the combined endeavours of patients and health professionals to accomplish persistent sodium restriction to improve the efficacy of renoprotective treatment.

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3

Elevated N-terminal pro-brain natriuretic peptide (NT-proBNP) levels predict an enhanced antihypertensive and antiproteinuric benefit of dietary sodium restriction and diuretics, but not angiotensin receptor blockade, in proteinuric renal patients

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Abstract

Background: Renin angiotensin aldosterone system (RAAS) blockade only partly reduces blood pressure, proteinuria, and renal and cardiovascular risk in chronic kidney disease (CKD), but often requires sodium targeting (i.e. low sodium diet (LS) and/or diuretics) for optimal efficacy. However, both under- and overtitration of sodium targeting can easily occur. We evaluated whether N-terminal pro-brain natriuretic peptide (NT-proBNP), a biomarker of volume expansion, predicts the benefits of sodium targeting in CKD patients.

Methods: In a cross-over randomized controlled trial 33 non-diabetic CKD patients (proteinuria 3.8±0.4 g/day, blood pressure 143/86±3/2 mmHg, creatinine clearance 89±5 mL/min) were treated during 6-week periods with placebo, angiotensin receptor blockade (ARB; losartan 100 mg/day), and ARB plus diuretics (losartan 100 mg/day plus hydrochlorothiazide 25 mg/day), combined with LS (93±52 mmol Na^+/day) and regular sodium diet (RS; 193±62 mmol Na^+/day, $p<0.001$ vs. LS), in random order. As controls, 27 healthy volunteers were studied.

Results: NT-proBNP was elevated in patients during placebo+RS (90 (60-137) vs. 35 (27-45) pg/mL in healthy controls, $p=0.001$). NT-proBNP was lowered by LS, ARB, and diuretics, and was normalized by ARB+diuretics+LS (39 (26-59) pg/mL, $p=0.65$ vs. controls). NT-proBNP levels above the upper limit of normal (>125 pg/mL) predicted a larger reduction of blood pressure and proteinuria by LS and diuretics, but not by ARB, during all steps of the titration regimen.

Conclusions: Elevated NT-proBNP levels predict an enhanced antihypertensive and antiproteinuric benefit of sodium targeting, but not RAAS blockade, in proteinuric CKD patients. Importantly, this applies to the untreated condition, as well as to the subsequent treatment steps, consisting of RAAS blockade and even RAAS blockade combined with diuretics. NT-proBNP can be a useful tool to identify CKD patients in whom sodium targeting can improve blood pressure and proteinuria.

Introduction

Blockade of the renin-angiotensin aldosterone system (RAAS) reduces blood pressure and proteinuria, improves long-term renal and cardiovascular outcome, and is first choice therapy, in chronic kidney disease (CKD)[1-3]. Despite RAAS blockade, blood pressure and proteinuria exceed the treatment target in many CKD patients and the residual risk remains high[4-6].

Previous research showed that inappropriate sodium retention is a main determinant of poor blood pressure control in CKD patients[7-9]. Furthermore, excessive dietary sodium intake blunts the antihypertensive and antiproteinuric response to RAAS blockade in hypertensive[10] and CKD patients[11-13]. Vice versa, sodium targeting (i.e. dietary sodium restriction and/or diuretics) can reduce blood pressure and proteinuria when instituted as monotherapy and, moreover, can potentiate the therapeutic efficacy of RAAS blockade[14-17].

However, the responses of blood pressure and proteinuria to sodium targeting are different between individuals[18-20] and in the absence of overt signs of volume-overload or -deficit it can be cumbersome to assess whether or not further sodium targeting is required for optimizing the therapy response[7,21]. Accordingly, both under- and overtitration of sodium targeting can easily occur[22-24]. A simple test that predicts the antihypertensive and antiproteinuric benefits of dietary sodium restriction and/or diuretics would be useful, but is currently not available.

For this reason we aimed to evaluate N-terminal pro-brain natriuretic peptide (NT-proBNP), a biomarker of the cardiac response to volume expansion, as a candidate marker in this respect[25-27]. To this purpose, we performed a post-hoc analysis on the responses of blood pressure and proteinuria to sodium targeting, in a previously published study in patients with proteinuric CKD, that underwent a treatment schedule including sodium targeting measures in the untreated condition as well as during RAAS inhibition by angiotensin receptor blockade (ARB)[14], specifically investigating the prognostic impact of elevated NT-proBNP for the responses of blood pressure and proteinuria to sodium intervention with sodium restricted diet, diuretic treatment, or their combination, during ARB.

Methods

Participants and protocol

This is a post-hoc analysis of a randomized double-blind placebo-controlled cross-over trial. The protocol was described in detail elsewhere[14]. In short, all patients (n=33) had stable proteinuria (>2 and <10 g/day) due to non-diabetic CKD, were middle-aged (18-70 years) and had stable creatinine clearance (>30 mL/min, <6 mL/min/yr decline). Only three patients had a history of cardiovascular disease, namely myocardial infarction (all >5 years ago).

Patients were randomized to a low sodium diet (LS; average sodium intake 92±8 mmol Na^+/day) or a regular sodium diet (average sodium intake 196±9 mmol Na^+/day, $p<0.001$). They remained on the assigned diet for 18 weeks, consisting of three 6-week treatment periods with consecutively placebo, angiotensin receptor blockade (ARB; losartan 100 mg/day) and ARB plus diuretics (losartan 100 mg/day plus hydrochlorothiazide 25 mg/day), in random order (Figure 1). After 18 weeks, patients changed diet and the three 6-week periods (placebo, ARB, ARB+diuretics) were repeated, again in random order. Additional antihypertensive drugs were allowed for blood pressure control (except for RAAS blockers or diuretics) and were kept stable during the study.

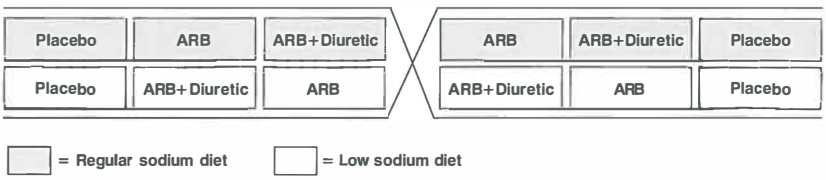
Healthy volunteers (n=27) on an unrestricted sodium intake served as controls. By definition, healthy subjects had no diabetes mellitus, renal function impairment, or history of cardiovascular disease.

Measurements

Proteinuria was measured by the pyrogallol red-molybdate method in 24h-urine samples. Blood pressure was measured at 1-minute intervals by an automatic device (Dinamap®; GE Medical Systems, Milwaukee, WI), with the patient in supine position. After fifteen minutes of measurements, the mean of the last four readings was used for further analysis. Dietary sodium intake was assessed from urinary sodium excretion. Peripheral pitting edema was assessed at the pretibial area of both legs by visual and manual examination, and scored as absent or present.

Peripheral blood was drawn by venipuncture, and aliquots from serum were stored (-80°C) until NT-proBNP analysis. NT-proBNP quantification was performed using electrochemiluminescent sandwich immunoassay (Elecsys ProBNP, Roche diagnostics, Mannheim, Germany). The intra- and interassay coefficients of variation were 1.2-1.5% and 4.4-5.0% respectively, with an analytical range of 5-35,000 pg/mL[28]. According to

Figure 1 Study design



In this cross-over study, non-diabetic proteinuric CKD patients were treated during six 6-week treatment periods with placebo, angiotensin receptor blockade (ARB; losartan 100 mg/day), and ARB plus diuretics (losartan 100 mg/day plus hydrochlorothiazide 25 mg/day). Regular sodium diet and low sodium diet (intake 196±9 versus 92±8 mmol Na+/day, p<0.001) were in random order.

local laboratory reference values, NT-proBNP levels ≤125 pg/mL were considered as within the normal range.

Data analysis

Before statistical testing, skewed variables were natural-log transformed to obtain normality. Associations between variables in patients were evaluated with Pearson's Correlation tests. Drug effects in patients were determined using Paired T-tests. Variables in patients versus healthy controls were compared using Unpaired T-tests. In this post-hoc exploratory analysis no Bonferroni correction for multiple comparisons was used.

Data are given as mean±standard error when normally distributed, or geometric mean (95%-confidence interval) if skewed. P<0.05 was considered statistically significant. SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Results

Baseline characteristics

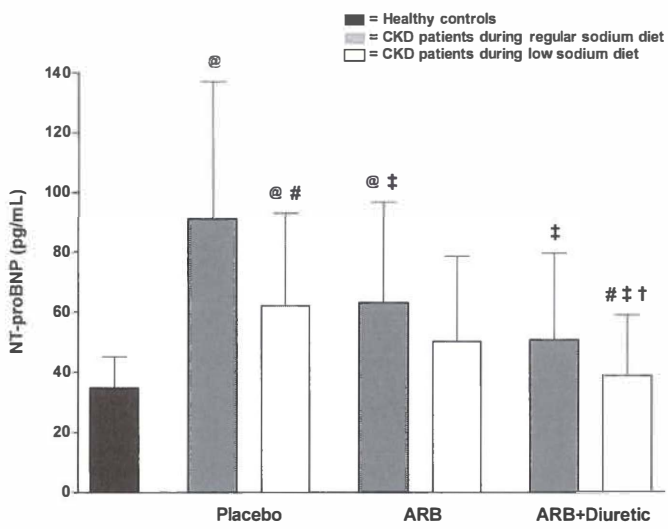
Data obtained during placebo combined with a regular sodium diet were taken as baseline values in CKD patients. CKD patients and controls were well matched for age (50±2 vs. 51±3 years, p=0.98), gender (73% vs. 59% male, p=0.28) and race (all Caucasian). At baseline, patients had overt proteinuria (3.8±0.4 g/day), on average a

blood pressure slightly above the treatment target (systolic and diastolic blood pressure 143/86 ± 3/2 mmHg), and a mildly impaired creatinine clearance (CrCl; 89±5 mL/min). As expected, control subjects had normal blood pressure (123/72 ± 3/2 mmHg, $p<0.001$ vs. CKD) and renal function (CrCl 114±6 mL/min, $p=0.001$ vs. CKD) and no proteinuria (0.15±0.02 g/day, $p<0.001$ vs. CKD). Dietary sodium intake, as reflected by urinary sodium excretion, was comparable in patients at baseline and controls (199±10 vs. 177±14 mmol Na^+/day, $p=0.17$).

NT-proBNP level in proteinuric CKD, and its response to LS, ARB, diuretics, and their combination

At baseline, the NT-proBNP level in the proteinuric CKD patients was approximately twofold higher than in healthy controls (91 (60-137) vs. 35 (27-45) pg/mL, $p<0.001$; Figure 2). LS reduced NT-proBNP up to 62 (41-93) pg/mL ($p=0.001$ vs. baseline), in these patients. ARB lowered NT-proBNP up to 63 (41-97) pg/mL ($p=0.005$ vs. baseline). Addition of LS plus diuretics to ARB further reduced NT-proBNP, up to levels comparable

Figure 2 NT-proBNP levels at baseline and during (combinations of) LS, ARB, and diuretics



Abbreviations: ARB, angiotensin receptor blockade; @ $p<0.05$ vs. healthy controls; # $p<0.05$ vs. same medication on a regular sodium diet in CKD patients (effect of low sodium diet); ‡ $p<0.05$ vs. placebo on same diet in CKD patients; † $p<0.05$ vs. ARB on same diet in CKD patients (effect of diuretics).

to controls (39 (26-59) pg/mL, p=0.002 vs. ARB, p=0.65 vs. controls). In line with this, body weight (91±3 kg at baseline) was significantly reduced by the addition of LS (89±3 kg, p=0.013, RS+placebo vs. LS+placebo), diuretics (89±3, p=0.003, RS+ARB vs. RS+ARB+diuretics) and LS+diuretics (88±3 kg, p<0.001, RS+ARB vs. LS+ARB+ diuretics), but not by ARB as such (90±3 kg, p=0.46,RS+placebo vs. RS+ARB), consistent with a negative fluid balance during LS and/or diuretics.

Baseline NT-proBNP and its association with the subsequent effect of LS, ARB, diuretics and their combination on blood pressure and proteinuria

The baseline NT-proBNP level exceeded the laboratory reference value of 125 pg/mL in 39% (13/33) of patients. These patients could not be identified by the clinical assessment of volume or sodium status (peripheral pitting edema, serum albumin, urinary sodium excretion; Table 1), but systolic and diastolic blood pressure were higher (p=0.002 and p=0.047), creatinine clearance was lower (p<0.001), and proteinuria tended to be higher (4.6±0.6 vs. 3.3±0.5 g/day p=0.13), in patients with baseline NT-proBNP >125 pg/mL than in patients with baseline NT-proBNP ≤ 125 pg/mL.

Figure 3 shows the responses of blood pressure and proteinuria to LS, ARB, and diuretics, compared between patients with NT-proBNP >125 pg/mL and patients with NT-proBNP ≤ 125 pg/mL. The differences in blood pressure and proteinuria between both patient groups gets progressively less during the subsequent treatment steps and is eventually annihilated, both groups achieving a similar maximum response for blood pressure and proteinuria during ARB+diuretics+LS.

Interestingly, institution of LS, the addition of LS on top of ARB, and the addition of diuretics on top of ARB+LS, induced an additional reduction of blood pressure in patients with NT-proBNP >125 pg/mL (LS vs. RS: p=0.001; ARB+LS vs. ARB: p=0.002; ARB+LS+diuretics vs. ARB+LS: p=0.002) but not in patients with NT-proBNP ≤125 pg/mL (LS vs. RS: p=0.10; ARB+LS vs. ARB: p=0.60; ARB+LS+diuretics vs. ARB+LS p=0.12). This is consistent with sodium-sensitivity of blood pressure in patients with NT-proBNP >125 pg/mL, whereas blood pressure in patients with NT-proBNP ≤125 pg/mL seems rather sodium-resistant. In contrast, ARB reduced blood pressure both in patients with NT-proBNP >125 pg/mL (p=0.001 vs. baseline) and patients with NT-proBNP ≤ 125 pg/mL (p=0.007 vs. baseline).

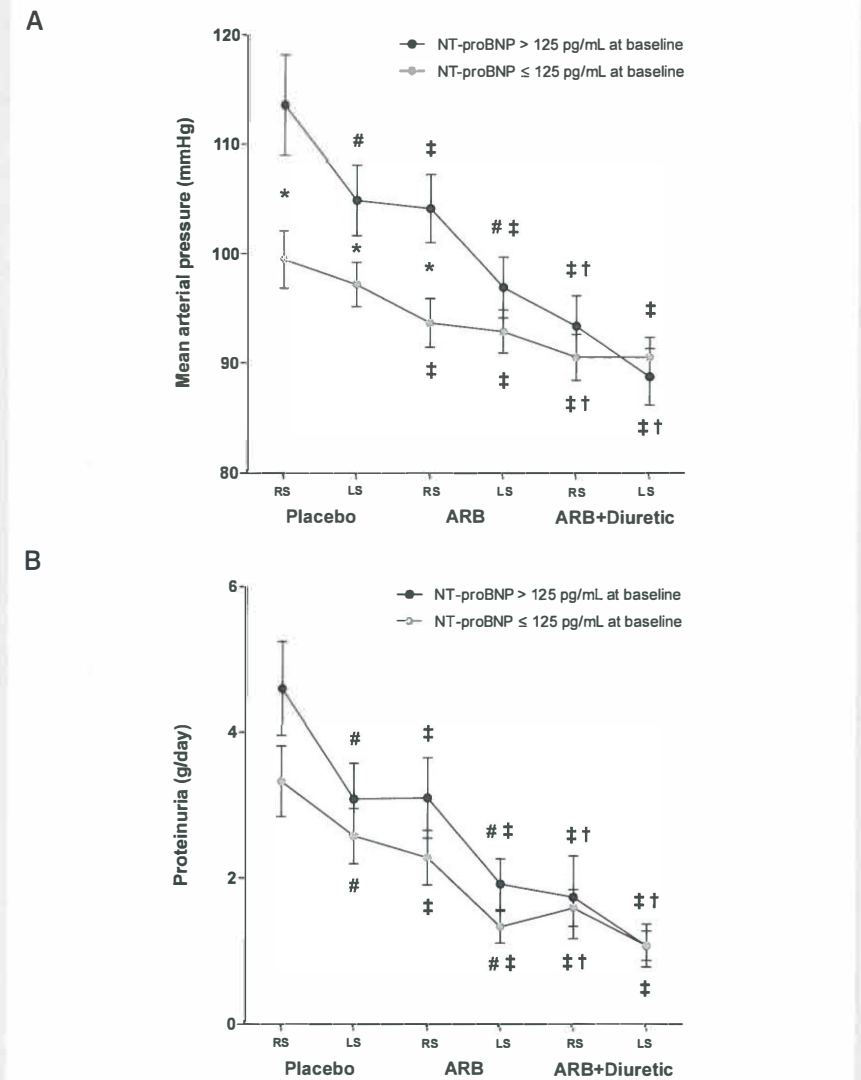
Proteinuria was reduced by all interventions in both patient groups, except for the addition of diuretics on top of ARB+LS which did not induce an additional reduction of proteinuria in patients with NT-proBNP ≤125 pg/mL (ARB+LS+diuretics vs. ARB+LS: p=0.15), consistent with a larger sodium-sensitivity of proteinuria in patients with NT-proBNP >125 pg/mL than in patients with NT-proBNP ≤125 pg/mL.

Table 1 Characteristics of patients with NT-proBNP ≤ 125 and > 125 pg/mL

	Baseline		ARB		ARB+Diuretics	
	NT-proBNP ≤125 pg/mL	NT-proBNP >125 pg/mL	NT-proBNP ≤125 pg/mL	NT-proBNP >125 pg/mL	NT-proBNP ≤125 pg/mL	NT-proBNP >125 pg/mL
Number of patients (n)	20	13	24	9	25	8
NT-proBNP (pg/mL)	42 [31-57]	285 [200-406] *	37 [25-55]	260 [196-346] *	30 [20-45]	254 [171-376] *
Edema prevalence (%)	42	31	35	33	14	19
Serum albumin (g/L)	39±1	38±1	39±1	39±1	41±1	39±1
Urinary Na^+ excretion (mmol/day)	205±13	194±17	200±14	188±14	175±14	198±13
Proteinuria (g/day)	3.3±0.5	4.6 ±0.6	2.3±0.3	3.5±0.7	1.4±0.2	2.4±0.9
Systolic blood pressure (mmHg)	133±4	158±7 *	131±4	146±6 *	124±3	128±5
Diastolic blood pressure (mmHg)	83±2	91±4 *	77±1	87±4 *	75±1	77±3
Creatinine clearance (mL/min)	102±5	69±6 *	108±6	59±4 *	93±7	64±5 *

Abbreviations: Baseline, placebo combined with a regular sodium diet; ARB, angiotensin receptor blockade combined with a regular sodium diet; ARB+Diuretics, angiotensin receptor blockade plus diuretics combined with a regular sodium diet; * $p<0.05$ vs. patients with NT-proBNP ≤125 pg/mL

Figure 3 Predictive value of baseline NT-proBNP on the benefit of (combinations of) LS, ARB, and diuretics



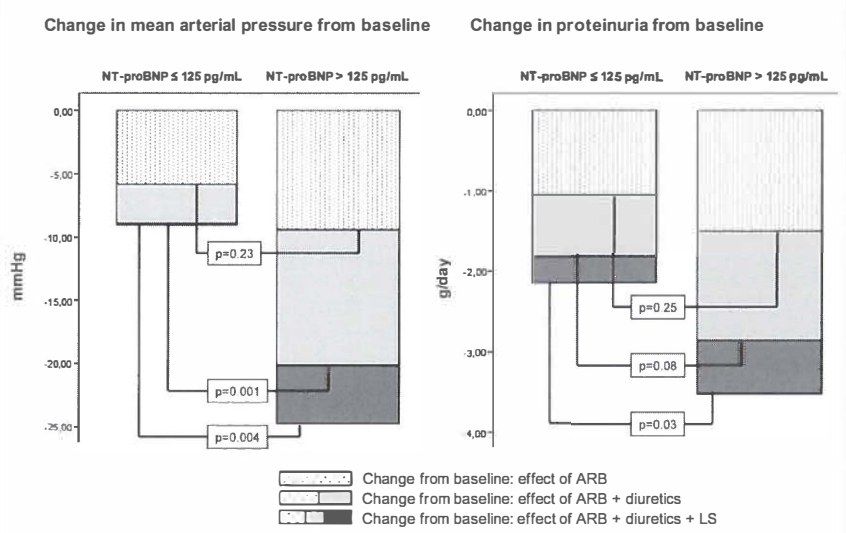
Blood pressure and proteinuria at baseline, and during (combinations of) LS, ARB, and diuretics, compared between patients with NT-proBNP >125 pg/mL and patients with NT-proBNP ≤ 125 pg/mL. Abbreviations: RS, regular sodium diet; LS, low sodium diet; ARB, angiotensin receptor blockade; # $p<0.05$ vs. same medication on RS (effect of LS); ‡ $p<0.05$ vs. placebo on same diet; † $p<0.05$ vs. ARB on same diet (effect of diuretics); * $p<0.05$ vs. patients with NT-proBNP ≤125 on same treatment.

Figure 4 shows the change in blood pressure and proteinuria from baseline, induced by the different steps of the titration regimen as performed in clinical practice, compared between patients with NT-proBNP >125 pg/mL and patients with NT-proBNP ≤ 125 pg/mL. The change of blood pressure (p=0.23) and proteinuria (p=0.25) by ARB was similar in both patient groups. However, the change in blood pressure (p=0.001 and p=0.004) and proteinuria (p=0.08 and p=0.03) from baseline by ARB+diuretics and by ARB+diuretics+LS tended to be larger in patients with NT-proBNP >125 pg/mL than in patients with NT-proBNP ≤125 pg/mL, consistent with increased sodium-sensitivity of blood pressure and proteinuria in patients with NT-proBNP >125 pg/mL.

NT-proBNP during ARB and its association with the subsequent effect of LS, diuretics and their combination on blood pressure and proteinuria

During ARB, 27% (9/33) of patients had NT-proBNP >125 pg/mL. These patients could not be identified by the clinical assessment of volume or sodium status (Table 1),

Figure 4 Predictive value of baseline NT-proBNP on the benefit of ARB, diuretics and LS as titrated in clinical practice

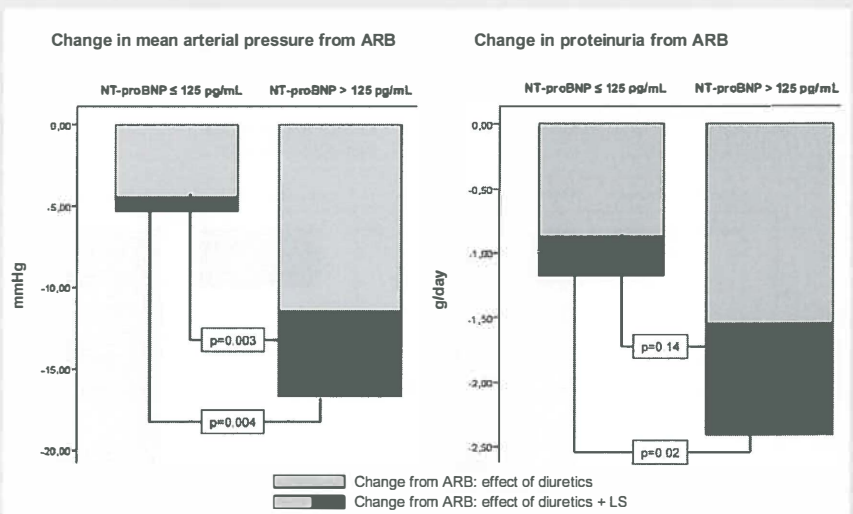


Change in blood pressure and proteinuria from baseline, induced by the different steps of the titration regimen as usually performed in clinical practice, compared between patients with baseline NT-proBNP >125 pg/mL and patients with baseline NT-proBNP ≤ 125 pg/mL. Abbreviations: ARB, angiotensin receptor blockade; LS, low sodium diet.

although systolic and diastolic blood pressure were higher (p=0.029 and p=0.003, respectively), creatinine clearance was lower (p<0.001), and proteinuria tended to be higher (3.5±0.7 vs. 2.3±0.3 g/day, p=0.10) in patients with baseline NT-proBNP >125 pg/mL than in patients with baseline NT-proBNP ≤ 125 pg/mL.

Figure 5 shows the change in blood pressure and proteinuria from ARB, induced by the different steps of the titration regimen as usually performed in clinical practice, compared between patients with NT-proBNP >125 pg/mL during ARB and patients with NT-proBNP ≤ 125 pg/mL during ARB. The change in blood pressure by diuretics (p=0.003) and by diuretics+LS (p=0.004) was larger in patients with NT-proBNP >125 pg/mL than in patients with NT-proBNP ≤125 pg/mL, consistent with increased sodium-sensitivity of blood pressure in patients with NT-proBNP >125 pg/mL. The change in proteinuria by diuretics was not significantly different (p=0.14) between both patient groups, whereas the change in proteinuria by diuretics+LS was larger in patients with

Figure 5 Predictive value of NT-proBNP during ARB on the benefit of diuretics and LS as titrated in clinical practice



Change in blood pressure and proteinuria from ARB, induced by the different steps of the titration regimen as usually performed in clinical practice, compared between patients with NT-proBNP >125 pg/mL during ARB and patients with NT-proBNP ≤125 pg/mL during ARB. Abbreviations: ARB, angiotensin receptor blockade; LS, low sodium diet.

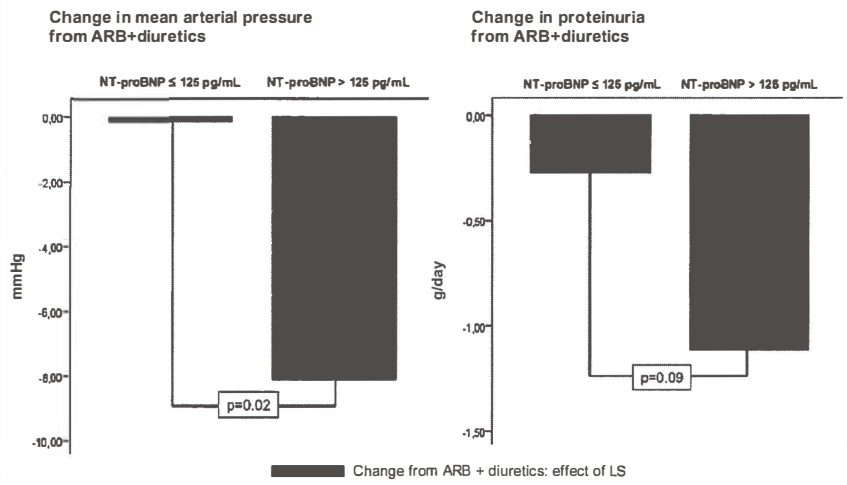
NT-proBNP >125 pg/mL than in patients with NT-proBNP ≤125 pg/mL (p=0.02), consistent with increased sodium-sensitivity of proteinuria in patients with NT-proBNP >125 pg/mL.

NT-proBNP during ARB+diuretics and its association with the subsequent effect of LS on blood pressure and proteinuria

During ARB+diuretics, 24% (8/33) of patients had NT-proBNP >125 pg/mL. It was not possible to identify these patients by clinical assessment of volume and sodium status, and the small numerical differences in systolic and diastolic blood pressure (p=0.48 and p=0.56) and proteinuria (p=0.34) between patients with NT-proBNP >125 pg/mL and patients with NT-proBNP ≤125 pg/mL were not statistically significant (Table 1). However renal function was significantly lower in patients with NTproBNP >125 pg/mL than in patients with NT-proBNP ≤125 pg/mL (p=0.024).

Figure 6 shows the change in blood pressure and proteinuria from ARB+diuretics, induced by LS, compared between patients with NT-proBNP >125 pg/mL during ARB+diuretics and patients with NT-proBNP ≤ 125 pg/mL during ARB+diuretics. In

Figure 6 Predictive value of NT-proBNP during ARB+diuretics on the benefit of LS



Change in blood pressure and proteinuria from ARB+diuretics, induced by LS, compared between patients with NT-proBNP >125 pg/mL during ARB+diuretics and patients with NT-proBNP ≤125 pg/mL during ARB+diuretics. Abbreviations: ARB, angiotensin receptor blockade; LS, low sodium diet.

patients with NT-proBNP >125 pg/mL LS induced a further fall in mean arterial pressure of approximately 8 mmHg whereas it was without effect in patients with NT-proBNP ≤ 125 pg/mL (p=0.02), consistent with increased sodium-sensitivity of blood pressure in patients with NT-proBNP >125 pg/mL and sodium-sensitivity of blood pressure in patients with NT-proBNP ≤ 125 pg/mL. This tended to be associated with a further reduction in proteinuria of approximately 1 g/day in patients with NT-proBNP >125 pg/mL, as compared to approximately 0.3 g/day patients with NT-proBNP ≤ 125 pg/mL (p=0.09), consistent with increased sodium-sensitivity of proteinuria in patients with NT-proBNP >125 pg/mL.

Discussion

In this study in non-diabetic proteinuric CKD patients NT-proBNP levels were elevated compared to age-matched healthy controls. The NT-proBNP levels were reduced by sodium targeting (i.e. dietary sodium restriction and/or diuretics), and RAAS blockade (i.e. angiotensin receptor blockade), and were normalized by combining these intervention. The main finding is that NT-proBNP levels exceeding the upper limit of normal (i.e. >125 pg/mL) predict an enhanced antihypertensive and antiproteinuric benefit of sodium targeting, but not RAAS blockade, in proteinuric patients. This predictive effect was observed during the untreated condition (placebo), as well as during the subsequent treatment steps consisting of RAAS blockade and even RAAS blockade combined with diuretics.
Hence, elevated NT-proBNP appears to reflect the sensitivity of blood pressure and proteinuria to sodium intervention, and can be a useful adjunct tool to identify patients that will effectively respond to sodium targeting with lowering of blood pressure and proteinuria.

The observation of elevated NT-proBNP levels in non-diabetic CKD patients with a relatively preserved renal function, but overt proteinuria, is a novel finding. In advanced renal disease, elevated NT-proBNP levels are associated with a faster progression to end-stage renal disease, a larger burden of cardiovascular disease, and increased mortality[29-32]. In our proteinuric patients with a relatively preserved renal function NT-proBNP levels were only mildly elevated, and substantially lower than in patients with advanced renal disease. Yet, similar mild increases of NT-proBNP have been found to independently predict cardiovascular outcome and mortality in the general population, suggesting that such mild elevations can be associated with clinical consequences [33,34].

The reduction of NT-proBNP levels by diuretics and RAAS blockade in our proteinuric CKD patients is line with previous findings in cardiac patients, and is probably explained by a reduction of cardiac volume- and pressure overload by diuretics and RAAS blockade through natriuresis and vasodilation[25-27].

The main finding of the current study is that elevated NT-proBNP levels predict a stronger reduction of blood pressure and proteinuria by sodium targeting, both in the untreated condition and during subsequent treatment steps. As RAAS blockade as a single intervention often insufficiently reduces blood pressure, proteinuria, and renal and cardiovascular risk in CKD, optimization of its efficacy is warranted[4-6]. Sodium targeting (dietary sodium restriction and/or diuretics) can potentiate the effects of RAAS blockade, but can easily be under- or overtitrated. A simple test that predicts the antihypertensive and antiproteinuric benefits of dietary sodium restriction and/or diuretics would be useful, but was currently not available.

Interestingly, the predictive value of NT-proBNP on the antihypertensive and antiproteinuric benefits of sodium targeting applies to the untreated condition (placebo), as well as to the subsequent treatment steps consisting of RAAS blockade and even RAAS blockade combined with diuretics. Hence, NT-proBNP appears to reflect the sodium-sensitivity of blood pressure and proteinuria in this patient population, which is in agreement with a previous study in healthy volunteers, showing that the degree of salt-sensitivity is related to baseline concentrations of N-terminal atrial natriuretic peptide levels[35].

A limitation of our study is the lack of information on the isolated effect of diuretics (i.e. without angiotensin receptor blockade). Also, the post-hoc nature of the study dictates that the predictive properties of NT-proBNP need to be prospectively tested as a next step. One question to be resolved is whether a lower limit value of NT-proBNP can be defined below which (additional) sodium intervention is unwarranted. NT-proBNP might then be useful to prevent the adverse events associated with too intensive sodium intervention, such as symptomatic hypotension, renal ischemia, and gout. Finally, the interpretation of our study in terms of mechanisms would have benefited from direct measurements of volume status, but no such data were available for this post-hoc study.

With respect to the diet, the 'regular sodium diet' very well reflected the average sodium intake in CKD and general populations, ranging from 150 to 200 mmol/day[36-38]. The 'low sodium diet' was well in excess of physiological needs (i.e. >10-20 mmol Na^+/day[38]) and corresponded with the recommendations in current guidelines[3].

To summarize, NT-proBNP levels are mildly elevated in non-diabetic CKD patients with overt proteinuria and a relatively preserved renal function, and are reduced by sodium targeting, and RAAS blockade. NT-proBNP levels exceeding the upper limit of normal predict the antihypertensive and antiproteinuric benefit of dietary sodium restriction and/or diuretics, but not RAAS blockade, during the different steps of the titration regimen. Hence, NT-proBNP can be a useful adjunct tool to identify proteinuric patients in whom (additional) sodium targeting can improve blood pressure and proteinuria.

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4

Reversible effects of diuretics added to renin angiotensin aldosterone system blockade: impact on interpretation of long-term kidney function outcome

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Letter to the Editor

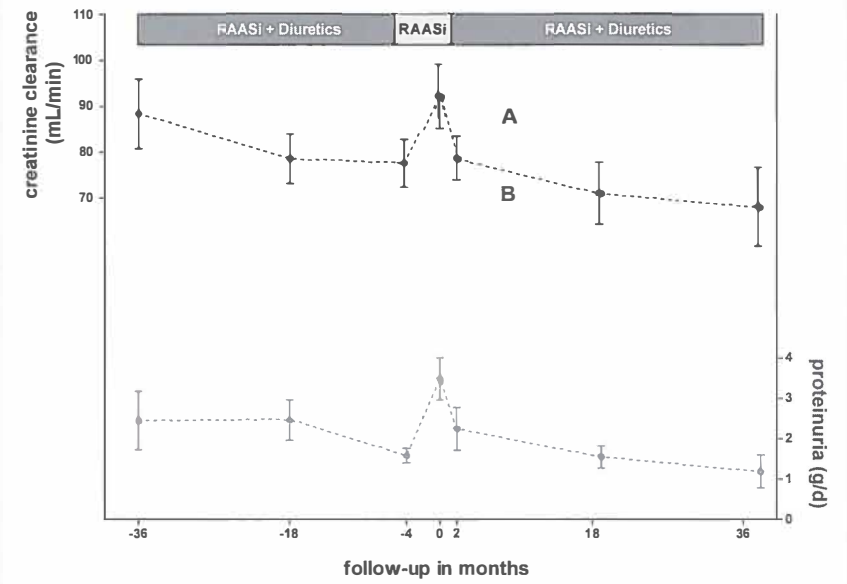
In a prespecified secondary analysis of the ACCOMPLISH (Avoiding Cardiovascular events through COMbination therapy in Patients Living with Systolic Hypertension) trial, Bakris et al conclude that benazepril/amlodipine therapy reduces chronic kidney disease (CKD) progression more effectively than benazepril/hydrochlorothiazide therapy in hypertensive patients at high cardiovascular risk[1]. We believe that the data as provided do not allow this conclusion.

Doubling of serum creatinine level accounted for the difference in kidney disease endpoints, without differences in treated kidney failure (end-stage renal disease). However, drug-induced changes in serum creatinine level must be interpreted carefully. At the onset of renin angiotensin aldosterone system (RAAS) blockade, an early decrease in glomerular filtration rate (GFR) often occurs. This is hypothesized to reflect a decrease in glomerular pressure, often is followed by a slower decrease in GFR thereafter, and is reversible upon withdrawal[2,3]. Heerspink and de Zeeuw note that in ACCOMPLISH a marked short-term decrease in GFR occurred with hydrochlorothiazide, but not with amlodipine therapy, suggesting hemodynamic changes rather than progression of CKD[4].

We analyzed 6-year follow-up data from a study on the effect of hydrochlorothiazide added to RAAS blockade in patients with CKD[5]. We selected all patients who used long-term diuretic therapy before study entry, who thus experienced systematic withdrawal of diuretic therapy and re-institution of diuretic therapy during the study (n=17). Withdrawal of hydrochlorothiazide therapy led to a distinct increase in creatinine clearance, mirrored by a decrease at re-institution (Figure). Including the early decrease (curve A) gives a significantly steeper long-term decrease in creatinine clearance than omitting it (curve B). Including the early decrease, 18% of patients had a ≥50% increase in serum creatinine level, compared with 0% of patients if the early decrease is omitted.

Not taking into account reversible renal hemodynamic drug effects may lead to misinterpretation of true long-term effects. Whether amlodipine or hydrochlorothiazide is preferable as add-on therapy to RAAS blockade to slow CKD progression is still unanswered.

Figure 1 Plot of creatinine clearance over time



Curves A and B denote decreases in creatinine clearance that include or exclude the initial steeper decrease, respectively (mean slope -7.6±3.3 [SD] versus -3.5±1.0 mL/min/year, p=0.031). The diuretic-associated changes in creatinine clearance are paralleled by changes in proteinuria (lower plot), supporting the notion that the early decrease in creatinine clearance using diuretics reflects a hemodynamic effect, rather than progression of chronic kidney disease. Abbreviations: RAASi, renin angiotensin aldosterone system inhibition.

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Part II

Effects of intervention in the RAAS and sodium status on non-classical intermediate outcome parameters in CKD patients

5

Effects of intensified proteinuria reduction by dietary sodium restriction and dual renin angiotensin aldosterone system blockade on markers of tubular injury in patients with renal disease

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Abstract

Background: Amelioration of proteinuria-driven tubulointerstitial injury by renin angiotensin aldosterone system (RAAS) blockade and sodium reduction induces renoprotection, and can be monitored by tubular injury markers. Previously, we found that proteinuria reduction below 1 g/day lowers KIM-1 and NAG. Here we tested whether intensified proteinuria reduction, i.e. below 0.3 g/day, by sodium restriction and dual RAAS blockade, further decreases a broad panel of tubular markers.

Study design: Cross-over randomized controlled trial.

Setting and participants: 52 non-diabetic renal patients with proteinuria (median: 1.9 [0.9-3.4] g/day) and mildly impaired renal function (69 [50-110] mL/min), and 52 healthy subjects.

Intervention: Patients were treated with combinations of ACE inhibition (ACEi; lisinopril 40 mg/day), placebo (PLA), angiotensin receptor blockade (ARB; valsartan 320 mg/day), regular sodium diet (RS; 189±8 mmol Na^+/day), and low sodium diet (LS; 106±7 mmol Na^+/day, $p<0.001$): during four randomly-ordered six-week study periods: 1. ACEi+PLA+RS (baseline), 2. ACEi+ARB+RS, 3. ACEi+PLA+LS, 4. ACEi+ARB+LS.

Outcomes and measurements: 24-hour urinary excretion of markers of proximal (NAG, KIM-1, 2MG) and distal (H-FABP) tubular injury and tubular inflammation (MCP-1, NGAL).

Results: All tubular injury markers were elevated in the renal patients at baseline. NAG, KIM-1, 2MG, and H-FAPB correlated positively with proteinuria, and were reduced along with further proteinuria reduction by combinations of ACEi, ARB and LS. The lowest levels of NAG, 2MG, and H-FAPB were achieved when proteinuria fell below 0.3 g/day. In contrast, MCP-1 and NGAL did not correlate with proteinuria, and were not reduced during proteinuria reduction.

Conclusions: Markers of proximal and distal tubular injury and inflammation are elevated in proteinuric renal patients on ACE inhibition, consistent with ongoing renal injury. Intensified treatment with dietary sodium restriction and dual RAAS blockade reduces tubular injury markers in proportion to proteinuria, without improvement of tubular inflammation markers.

Introduction

Reduction of proteinuria and hypertension are the main treatment targets for renoprotection[1,2]. This can be achieved by blockade of the renin angiotensin aldosterone system (RAAS) with angiotensin converting enzyme inhibition (ACEi) or angiotensin receptor blockade (ARB) -the cornerstone of therapy in renal disease- and sodium reduction with dietary sodium restriction or diuretics[3-6].

One of the mechanisms allegedly contributing to the renoprotective effect of proteinuria reduction is amelioration of proteinuria-driven tubulointerstitial injury[7]. Tubulointerstitial injury is a main determinant of renal outcome but cannot be assessed directly on a routine basis, as this requires renal biopsy[8,9]. Urinary tubular injury markers might provide a useful non-invasive alternative, as these markers correlate with tubulointerstitial injury and predict renal outcome[10;11].

Recently we reported that reduction of proteinuria by combinations of ARB, dietary sodium restriction, and diuretics, is associated with reduction of the tubular injury markers N-Acetyl-β-glucosaminidase (NAG) and Kidney Injury Molecule 1 (KIM-1) in renal patients[12]. Interestingly, the lowest levels of NAG and KIM-1 were achieved when proteinuria fell below the current treatment target of below 1.0 g/day, although even in this condition NAG and KIM-1 remained substantially elevated[13]. This presumably reflects ongoing renal damage and, moreover, is in line with the notion that further reduction of proteinuria, i.e. below 0.3 g/day, may augment renoprotection[14].

In the current study therefore we investigated whether intensified proteinuria reduction to levels below 0.3 g/day by combinations of ACEi, ARB, and dietary sodium restriction, results in further reduction of a broad panel of urinary markers reflecting diverse aspects of tubular injury, in patients with chronic kidney disease (CKD).

Methods

Patients

This is a post-hoc analysis of a randomized double-blind placebo-controlled cross-over multicenter trial. The protocol was described in detail elsewhere[15]. In short, we studied 52 patients with non-diabetic nephropathy. Inclusion criteria were blood pressure above 125/75 mmHg in combination with residual proteinuria above 1.0 g/day during ACEi on maximal dose (lisinopril 40 mg/day), creatinine clearance of 30 mL/min or above, and

age over 18 years. Exclusion criteria were systolic blood pressure of 180 mmHg or above, diastolic blood pressure of 110 mmHg of above, diabetes mellitus, renovascular hypertension, decrease of creatinine clearance by at least 6 mL/min in the previous year, a cardiovascular event in the previous six months, immunosuppressive treatment, regular use (>1 day/week) of non-steroidal anti-inflammatory drugs, pregnancy, or breast feeding.

Protocol

During a run-in period of at least six weeks, patients received ACEi at maximal dose (lisinopril 40 mg/day) and stopped other RAAS blockers. Additional antihypertensives were allowed and kept stable during the study. No dietary intervention took place during the run-in period.

After the run-in period patients were treated with combinations of lisinopril 40 mg/day, placebo (PLA), ARB at maximal dose (valsartan 320 mg/day), regular sodium (RS; target 200 mmol Na^+/day), and low sodium diet (LS; target intake 50 mmol Na^+/day), during four randomly-ordered six-week study periods: 1. ACEi+PLA+RS, 2. ACEi+ARB+RS, 3. ACEi+PLA+LS, 4. ACEi+ARB+LS. The drug interventions were double blind, whereas the dietary interventions were open label.

Healthy controls

Fifty-two age and gender matched subjects that had no renal disease or diabetes served as controls. In these subjects no dietary intervention was performed.

Measurements and calculations

At the end of each 6-week treatment period, patients collected 24 hour urine samples, and blood pressure was measured and blood was sampled after an overnight fast. Additionally, in the middle of every period, patients collected 24 hour urine samples to monitor dietary compliance (sodium excretion).

We measured proteinuria in 24 hour urine samples with a turbidimetric assay using benzethonium chloride (Modular, Roche Diagnostics, Mannheim, Germany). We measured blood pressure for 15 minutes at one minute intervals with an automatic device (Dinamap, G E Medical Systems, Milwaukee, WI, USA)in the supine position and used the mean of the last three readings. We determined blood electrolytes, proteins, and urinary electrolytes by using an automated multianalyser (Modular, Roche Diagnostics, Mannheim, Germany). We assessed dietary sodium intake from urinary sodium excretion. We calculated creatinine clearance from creatinine concentrations in plasma and in 24 hour urine samples.

We stored (-80°C) aliquots from 24 hour urine until biomarker analysis. We vortexed and centrifuged (14.000 rpm) all urine samples after thawing. We used the supernatant for measurements. We diluted the samples to obtain the optimal concentration for measurement. All tubular markers were determined in one run. We measured urinary albumin levels by nephelometry (Dade Behring Nephelometer, intra-assay CV 2.7%). For quantification of neutrophil gelatinase-associated lipocalin (NGAL), β2-microglobulin (β2MG), monocyte chemoattractant protein-1 (MCP-1), and heart-type fatty acid-binding protein (H-FABP) we used direct sandwich-enzyme-linked immunosorbent assays using monoclonal coating antibodies and labeled polyclonal detection antibodies on a Maxisorp plate (Nunc, Denmark) in which is the concentration of the analyte was determined spectrophotometrically by conversion of o-phenylenediamine by Horse-Radish Peroxidase label. We obtained H-FABP, NGAL, β2MG, and MCP-1 antibodies from Hytest (Turku, Finland, intra-assay CV 9.3%) and R&D systems (Minneapolis, USA, intra-assay CV 6.8 %, 9.7 and 15.7 &, respectively). We measured KIM-1 using microbead based ELISA (microsphere-based Luminex xMAP technology (Luminex, Austin, TX), with polyclonal antibodies raised against the human KIM-1 ectodomain as described previously[16]. The intra-assay variability was less than 15%. We measured urinary concentration of N-acetyl-β-D-glucosaminidase (NAG) using a modified enzyme assay according to Lockwood and corrected for nonspecific conversion (HaemoScan, Groningen, The Netherlands, intra-assay CV 3.1%).

Statistical analysis

Data are presented as mean with standard error (SE) when normally distributed or otherwise as median with interquartile range (IQR). We used paired *t* tests, Wilcoxon signed rank tests, and Pearson's ^{2} tests (which account for the same patients providing data for both treatments) to determine effects of treatment. Independent *t* tests or Mann-Whitney U tests were used to determine differences between patients and healthy subjects. Multivariate models were employed to investigate which factors predict change in tubular injury marker excretion. To this purpose, the change in tubular injury marker excretion from baseline (ACEi+RS) was calculated for each treatment period and used as a dependant variable in the model. Change in proteinuria, change in blood pressure, salt intake and addition of ARB were added as covariates in the model. Alpha was set at $p<0.05$. SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Results

Participants characteristics

CKD patients and healthy controls were matched for age (mean: 51(2) vs. 53(2) years, p=0.49), gender (83% vs. 73% male, p=0.24) and race (all Caucasian). During ACEi combined with regular sodium diet (ACEi+RS), which was taken as the reference, or baseline, period, CKD patients had overt proteinuria (1.9 (0.9-3.4) g/day), high-normal blood pressure (SBP 134(3) mmHg, DBP 80(2) mmHg), and mildly impaired renal function (creatinine clearance 69 (50-110) mL/min). As expected, healthy controls had no relevant proteinuria (0.1 (0-0.2) g/day, p<0.001), normal renal function (CrCl 130(6) mL/min, p<0.001), a lower blood pressure (SBP 122(2) mmHg, p=0.002; DBP 74(1) mmHg, p=0.008) compared with CKD patients. Dietary sodium intake, as reflected by urinary sodium excretion, was comparable in CKD patients during ACEi+RS and in controls (189(8) vs. 198(12) mmol Na^+/day, p=0.51). Other patient characteristics are shown in Table 1.

Table 1 Patients' characteristics

Number of patients	**52**
Renal diagnosis:	
IgA NP – no. (%)	15 (29)
FSGS – no. (%)	16 (31)
Membranous NP – no. (%)	7 (13)
Hypertensive NP – no. (%)	6 (12)
Other / inconclusive – no. (%)	8 (15)
Use of non-study medication:	
Betablocker – no. (%)	12 (23)
Calciumchannelblocker – no. (%)	10 (19)
Alphablocker – no. (%)	5 (10)
Thiazide diuretic – no. (%)	8 (15)
Loop diuretic – no. (%)	5 (10)
Lipid lowering agent – no. (%)	25 (48)

Renal diagnoses, and non-study medication as used at the end of the run-in period. Non-study medication was kept stable during the study. Abbreviations: NP, nephropathy; FSGS, focal segmental glomerulosclerosis.

Clinical parameters during the four treatment regimens

In CKD patients, urinary creatinine excretion was comparable during all treatment periods, indicating accurate 24-hour urine collection (Table 2). Dietary sodium intake, as reflected by urinary sodium excretion, was considerably and consistently lower during the LS periods compared with the RS, thus reflecting dietary compliance.

Table 2 Clinical parameters during four treatment regimens

	Regular sodium diet		Low sodium diet	
	ACEi	ACEi+ARB	ACEi	ACEi+ARB
Urinary creatinine excretion - mmol/day	13.8 (0.6)	14.0 (0.5)	13.5 (0.6)	13.4 (0.6)
Urinary sodium excretion - mmol/day	189 (8)	180 (9)	106 (7) *†	105 (8) *†
Systolic blood pressure - mmHg	134 (3)	131 (3)	123 (2) *†	121 (3) *†
Diastolic blood pressure - mmHg	80 (2)	77 (2) *	73 (2) *	71 (2) *†
Creatinine clearance - mL/min	69 (50-110)	72 (54-105)	67 (43-93) *†	59 (42-81) *†‡
Plasma renin concentration - ng/L	54 (17-178)	77 (25-230) *	172 (43-460) *†	230 (49-1148) *†‡
Proteinuria - g/day	1.9 (0.9-3.4)	1.6 (0.6-3.4) *	0.9 (0.5-1.7) *†	0.7 (0.4-1.4) *†‡
Proteinuria >1.0 g/day - % (no.)	66 (34)	67 (35)	42 (22) *†	31 (16) *†
Proteinuria 0.3-1.0 g/day - % (no.)	33 (17)	25 (13)	44 (23) *†	45 (24) *†
Proteinuria <0.3 g/day - % (no.)	2 (1)	8 (4)	14 (7) *†	24 (12) *†

Abbreviations: ACEi, ACE inhibition; ARB, angiotensin receptor blockade; * $p<0.05$ vs. ACEi on regular sodium diet; † $p<0.05$ vs. ACEi-ARB on regular sodium diet; ‡ $p<0.05$ vs. ACEi on low sodium diet.

Addition of ARB to ACEi resulted in a modest decrease in proteinuria, but LS reduced proteinuria more effectively, and the lowest proteinuria was achieved by combined ARB and LS added to ACEi. ARB did not decrease systolic blood pressure, whereas addition of LS significantly reduced systolic blood pressure, with no further effect of combined ARB and LS. Likewise, ARB had no effect on creatinine clearance, whereas creatinine clearance was decreased by LS, and was further reduced by combined ARB and LS. The decrease in creatinine clearance was reversible upon withdrawal of LS and ARB. Plasma renin level was increased by ARB, and was more increased by LS added to ACEi. The highest level of plasma renin was found by combined ARB and LS added to ACEi. Plasma aldosterone levels were increased by LS, but not by ARB.

Tubular injury markers during the four treatment regimens

During ACEi+RS urinary levels of NAG, KIM-1, β2MG, H-FABP, NGAL and MCP-1, were all elevated in CKD patients compared to healthy controls (Table 3). The levels of NAG (rho=0.66, p<0.001), KIM-1 (rho= 0.46, p=0.001), β2MG (rho=0.42, p=0.003), and H-FABP (rho=0.58, p<0.001) positively correlated with proteinuria during ACEi+RS. In contrast, the levels of NGAL (rho=-0.12, p=0.40) and MCP-1 (rho=0.18, p=0.22) did not correlate with proteinuria during ACEi+RS.

NAG was not significantly altered by the addition of ARB, but was lowered by the addition of LS or ARB+LS, to ACEi (Table 3). Likewise, KIM-1 was reduced by addition of LS or ARB+LS, but not significantly altered by the addition of ARB as such. β2MG was reduced by addition of ARB, LS, and ARB+LS, to ACEi. H-FABP was reduced by the addition of ARB and further reduced by the addition of LS, with the lowest levels of H-FABP during the addition of ARB+LS to ACEi. NGAL was reduced by the addition of LS, but was not significantly altered by the addition of ARB or ARB+LS, to ACEi. MCP-1 was not altered by any of the regimens.

Tubular injury markers according to achieved proteinuria

The number of patients that reached the proteinuria target of below 0.3 g/day was largest during combined treatment with ACEi+ARB+LS (Table 2). Individual data for this treatment period are given in figure 1, providing the levels of the different tubular markers by a break-up by achieved proteinuria. For the proximal tubular injury markers NAG and β2MG and the distal tubular injury marker H-FABP, the levels were progressively lower in the patients that achieved proteinuria below 1.0 g/day and below 0.3 g/day respectively.

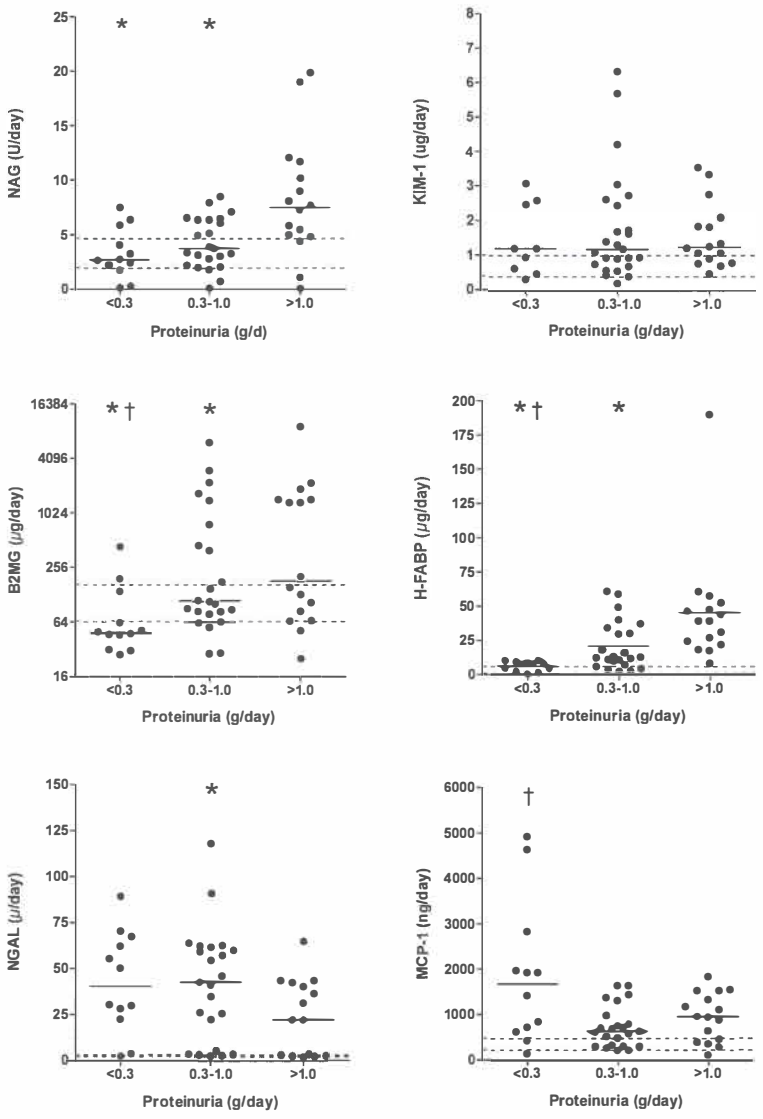
In contrast, the proximal tubular marker KIM-1 showed no differences for the different proteinuria categories, and the tubular inflammation markers NGAL and MCP-1 were not lower in patients with a lower achieved proteinuria, but, if anything, even somewhat higher. A similar trend was found during the other treatment regimens (i.e. ACEi+RS, ACEi+LS, and ACEi+ARB+RS; data not shown). Underlying renal diagnoses were not essentially different between the patients groups that achieved proteinuria below 0.3 and above 0.3 g/day. We further studied the subgroup of patients that reached proteinuria below 0.3 g/day during ACEi+ARB+LS, and found no differences in patient characteristics between patients in whom tubular inflammatory markers rose versus patients in whom tubular inflammatory markers decreased during ACEi+ARB+LS.

Table 3 Tubular injury markers during four treatment regimens

	Healthy subjects	CKD patients			
		Regular sodium diet		Low sodium diet	
		ACEi	ACEi+ARB	ACEi	ACEi+ARB
NAG – U/day	2.9 (2.0-4.7)	6.3 (3.0-10.9) #	5.0 (3.1-8.2) #	5.0 (3.1-8.0) # *	4.9 (2.5-7.1) # *
KIM-1 – ug/day	0.6 (0.4-1.0)	1.6 (1.1-2.7) #	1.5 (0.9-2.9) #	1.3 (0.7-2.3) # * †	1.2 (0.8-2.5) # * †
β2MG – ug/day	108 (65-166)	148 (78-2444) #	140 (62-712) *	136 (51-362) *	106 (57-760) *
H-FABP – ug/day	4 (1-6)	29 (17-94) #	31 (12-65) # *	18 (9-41) # * †	13 (8-39) # * † ‡
NGAL – ug/day	3 (2-3)	42 (3-65) #	35 (3-67) #	33 (4-63) # *	36 (4-60) #
MCP-1 – ng/day	334 (221-479)	804 (470-1276) #	717 (468-1069) #	810 (407-1200) #	763 (421-1448) #

Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; RS, regular sodium diet, LS, low sodium diet; # $p<0.05$ vs. healthy subjects; * $p<0.05$ vs. CKD on ACEi+RS; † $p<0.05$ vs. CKD on ACEI+ARB+RS; ‡ $p<0.05$ vs. CKD on ACEi+LS.

Figure 1 Individual values for urinary markers ranked by achieved proteinuria



Tubular injury markers ranked by achieved proteinuria during combined treatment with ACE inhibition plus angiotensin receptor blockade plus low sodium diet. * $p<0.05$ vs. proteinuria above 1.0 g/day, † $p<0.05$ vs. proteinuria between 0.3-1.0 g/day. The areas between dotted lines represent the interquartile range in healthy subjects.

Predictors of change in tubular markers

In a multivariate analysis we investigated whether the change in urinary tubular marker excretion is associated with the change in proteinuria, blood pressure, salt intake or addition of ARB. We found that for all markers, the change in tubular marker was positively correlated with the change in proteinuria, whereas for change in blood pressure the same was only true for NAG and MCP-1 excretion. In this model the mode of intervention per se (ARB or salt diet) was no significant predictor.

Discussion

We found that urinary markers of proximal tubular injury (NAG, KIM-1, β2MG), tubular inflammation (MCP-1, NGAL), and remarkably also distal tubular injury (H-FABP), are elevated in non-diabetic proteinuric CKD patients despite treatment with ACEi on maximally recommended dose. The proximal and distal tubular injury markers (NAG, KIM-1, β2MG, H-FABP) correlated with residual proteinuria, and were reduced along with reduction of proteinuria, irrespective the mode of treatment. The lowest levels of proximal and distal tubular injury markers were achieved when proteinuria fell below 0.3 g/day. In contrast, the tubular inflammation markers (MCP-1, NGAL) did not correlate with proteinuria, and remained roughly unaltered despite reduction of proteinuria.

Urinary markers of tubular injury are elevated in patients with tubulointerstitial injury and provide a potential non-invasive tool to monitor renal damage[10,11]. We studied a broad panel of tubular markers, that reflect injury in different renal compartments, and that are mediated by different processes. NAG, KIM-1 and ß2MG were measured as markers for proximal tubular damage, H-FABP was measured as a distal tubular marker, and NGAL and MCP-1 were measured as tubular inflammation markers.

NAG (N-Acetyl-β-glucosaminidase; 135 kDa) is a lysosymal enzyme that is predominantly produced in the proximal tubule, and released into urine upon cellular damage. Elevated urinary NAG predicts the subsequent occurrence of albuminuria in diabetic patients[17], and was found to predict CKD progression better than proteinuria in non-diabetic CKD[18].

KIM-1 (kidney injury molecule-1; 104 kDa) is a transmembrane glycoprotein that is abundantly expressed on proximal tubular cells and shed into urine, during acute or chronic renal injury[19-21]. No other organs express KIM-1 to a degree that would influence renal excretion of KIM-1[22]. Urinary KIM-1 predicts long-term renal outcome in acute renal injury and renal transplant recipients[23-25]. So far, long-term data on the prognostic significance of urinary KIM-1 in CKD are lacking.

ß2MG (β2-microglobulin; 12 kDa) is a component of MHC class 1 molecules, which are present on all nucleated cells. ß2MG is freely filtered through the glomerulus and subsequently reabsorbed by proximal tubular cells. Urinary ß2MG is a marker of proximal tubular reabsorption incapacity and predicts the rate of CKD progression[26-28].
H-FABP (heart-type fatty acid-binding protein; 15kDa) is an intracellular carrier protein present in cytoplasm of distal tubular cells[29,30]. Urinary H-FABP results from release by structurally damaged tubular cells. Elevated urinary H-FABP predicts prognosis in CKD[31].
NGAL (neutrophil gelatinase-associated lipocalin; 25 kDa) is expressed by neutrophils and a number of other epithelial and non epithelial cell types. NGAL was reported to reflect damage to glomeruli, and proximal and distal tubules[32-34]. Elevated urinary NGAL have been reported to predict CKD progression[35].
MCP-1 (monocyte chemoattractant protein-1; 13-30 kDa) is expressed by inflammatory cells such as monocytes, and also by resident renal cells, i.e. mesangial, endothelial, and tubular epithelial cells[36]. Renal cells produce MCP-1 in response to a variety of pro-inflammatory stimuli[37]. Elevated urinary MCP-1 predicts the rate of renal function loss in CKD[38,39].

All measured tubular markers were elevated in proteinuric CKD patients during monotherapy ACEi compared to healthy controls, suggesting ongoing proximal and distal tubular injury and tubular inflammation, and a worse renal prognosis. Indeed, despite the proven benefits of monotherapy RAAS blockade, the residual renal risk remains high in CKD patients[3;4]. The elevated tubular markers in our CKD patients probably reflect this ongoing renal injury.

The proximal and distal tubular injury markers correlated with proteinuria, and were reduced in proportion to the reduction of proteinuria irrespective the mode of intervention, suggesting a beneficial effect of intervention on tubular damage. Previous studies also found a tight relationship between proteinuria and proximal tubular injury markers[18;31;38;40-42], and a reduction of these markers by antiproteinuric therapy[18;40;43]. Our data are the first to demonstrate a similar association for H-FABP and proteinuria, including an effect of antiproteinuric therapy. This is remarkable, as the distal tubule is classically assumed to be less sensitive to the toxic effects of urinary proteins, but, in line with our current findings, recent data challenged this assumption[31,42]. It should be kept in mind though that the cascade could as well run vice versa, i.e. tubular injury causing proteinuria.

In contrast, the tubular inflammation markers (MCP-1, NGAL) remained roughly unaltered despite reduction of proteinuria by either therapy. This was unexpected, as reduction of proteinuria is assumed to protect the tubulointerstitium by amelioration of the proinflammatory effects of leaked proteins[44-46]. Whereas the latter assumption is supported by the findings on NAG, KIM-1, ß2MG, and H-FABP, their reductions dissociate from the lack of effect on tubular inflammation makers. It cannot be excluded that the interventions were not rigorous enough, or that six weeks of treatment was too short for an anti-inflammatory effect to become apparent. Alternatively, tubular inflammation as reflected by MCP-1 and NGAL may not have been exclusively proteinuria-driven, which is in line with the absence of a correlation between MCP-1, NGAL and proteinuria in these patients.

Others also reported absence of a correlation between MCP-1 and proteinuria in renal patients without (high-grade) inflammatory nephropathy treated with RAAS blockade[39;47], whereas a correlation between (a change in) MCP-1 and proteinuria was present in patients with inflammatory nephropathy and in renal patients that were treated with immunosuppressive therapy, antibiotics, or oral antidiabetics[48-50]. Hence, the presence of a relationship between (delta) MCP-1 and proteinuria seems to depend on renal diagnosis and the mode of treatment.

However, in the current study we could not find determinants of the urinary inflammatory markers. In particular no association with particular underlying disorders could be identified, rendering it unlikely that these results are due to the subset of patients with 'inflammatory' diagnose of renal disease. Finally, possible anti-inflammatory effects of a reduction in proteinuria per se may have been offset by reactive increases in renin or aldosterone, which can exert proinflammatory effects[46;51;52].

The levels of proximal and distal tubular injury markers were lowest in patients in whom proteinuria levels fell below 0.3 g/day. This is in line with previous, principally observational, data suggesting that proteinuria below 0.3 g/day is associated with a better renal outcome[6,53]. It can be considered to support the notion that the current treatment target for proteinuria to below 1.0 g/day is too liberal and that titration of proteinuria to levels below 0.3 g/day may be needed for optimal renoprotection.

However, such a conclusion should be taken with caution. First, the discrepancies between inflammatory markers and residual proteinuria indicate that ongoing tubular damage is a complex process. It remains to be proven by prospective intervention studies whether our short-term findings translate into long-term renal outcome, in other words, whether patients with proteinuria below 0.3 g/day and low levels of tubular injury markers have slower -or even absent- progression to end stage renal disease. It is also not known whether specific titration of residual proteinuria to below 0.3 g/day will

improve urinary tubular marker profile and outcome, or whether the better reduction of tubular injury markers in subjects in whom proteinuria fell below 0.3 g/day simply reflects a more benign phenotype.

The strengths of this study are that we measured a panel of tubular injury markers that reflect injury in different renal compartments and are mediated by different processes. Furthermore all samples were measured in one run, thus avoiding interassay variation. The major limitations of this study are, first, that it is a post-hoc analysis, and second, is that it provides short term data only, so the impact of our data for long-term outcome will require separate study.

In conclusion, urinary markers of proximal and distal tubular injury, and tubular inflammation are elevated in non-diabetic proteinuric CKD patients with persistent proteinuria despite ACEi, probably reflecting ongoing renal injury. Proximal and distal tubular injury markers are reduced along with intensified reduction of proteinuria by combinations of ACEi, ARB and low sodium diet. In contrast, markers of tubular inflammation remained largely unaffected and, if anything, increased in patients with the lowest proteinuria values. Long-term prospective intervention studies should investigate whether titration of proteinuria to levels below 0.3 g/day further improves renoprotection.

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6

Effects of antiproteinuric intervention on elevated Connective Tissue Growth Factor (CTGF/CCN-2) plasma and urine levels in nondiabetic nephropathy

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Abstract

Background and objectives: Connective Tissue Growth Factor (CTGF/CCN-2) is a key player in fibrosis. Plasma CTGF levels predict end-stage renal disease and mortality in diabetic chronic kidney disease (CKD), supporting a role in intra- and extrarenal fibrosis. Few data is available on CTGF in non-diabetic CKD. We investigated CTGF levels and the effects of antiproteinuric interventions in non-diabetic proteinuric CKD.

Design, setting, participants, and measurements: In a cross-over randomized controlled trial 33 non-diabetic CKD patients (proteinuria 3.2 [2.5-4.0] g/day) were treated during 6-week periods with placebo, ARB (losartan 100 mg/day), and ARB plus diuretics (losartan 100 mg/d plus hydrochlorothiazide 25 mg/day) combined with consecutively a regular and a low sodium diet (193±62 vs. 93±52 mmol Na^+/day, $p<0.001$).

Results: CTGF was elevated in plasma (464 [387-556] pmol/L) and urine (205 [135-311] pmol/day) of patients compared to healthy controls (n=21; 96 [86-108] pmol/L and 73 [55-98] pmol/day, $p<0.001$ and $p=0.001$). Urinary CTGF was lowered by antiproteinuric intervention, in proportion to the reduction of proteinuria, with normalization during triple therapy (CTGF 99 [67-146] in CKD vs. 73 [55-98] pmol/day in controls, $p=0.82$). In contrast, plasma CTGF was not affected.

Conclusions: Thus, urinary and plasma CTGF are elevated in non-diabetic CKD. Only urinary CTGF is normalized by antiproteinuric intervention, consistent with amelioration of tubular dysfunction. The lack of effect on plasma CTGF suggests that its driving force might be independent of proteinuria, and that short-term antiproteinuric interventions are not sufficient to correct the systemic pro-fibrotic state in CKD.

Introduction

Connective Tissue Growth Factor (CTGF/CCN-2) is a main mediator of fibrogenesis both downstream and independent of transforming growth factor β1[1-3]. CTGF was shown to be a key player in the development and progression of diabetic renal fibrosis. In experimental diabetic nephropathy, glomerular and tubulointerstitial CTGF over-expression induce glomerulosclerosis, tubulointerstitial fibrosis, and albuminuria[4-6].

Likewise, in human diabetic nephropathy, CTGF overexpression in renal biopsies is associated with tubulointerstitial fibrosis, proteinuria and renal function impairment[7,8], and urinary CTGF levels correlate with albuminuria and renal function impairment[9,10]. Plasma CTGF levels independently predict end-stage renal disease, intima-media thickness, and mortality in diabetic nephropathy[11,12], supporting a role in intra-renal as well as extrarenal fibrotic processes[13,14]. This is underscored by efficacy of CTGF inhibition in experimental models[15].

Few data is available, however, on the role of CTGF in non-diabetic chronic kidney disease (CKD) although intra- and extrarenal fibrosis are of well-recognized importance in this disease condition[16-19]. We therefore investigated plasma and urinary levels of CTGF, and the effects of antiproteinuric intervention in non-diabetic proteinuric CKD.

Methods

Patients and protocol

This is a post-hoc analysis of a randomized, double-blind, placebo-controlled cross-over trial. The protocol was described in detail elsewhere[20]. In short, all patients (n=33) had stable proteinuria (>2 and <10 g/day) due to non-diabetic CKD, were middle-aged (18-70 years) and had stable creatinine clearance (>30 mL/min, <6 mL/min/yr decline). Renal diagnoses were membranous nephropathy (n=7), focal segmental glomerulosclerosis (n=7), IgA nephropathy (n=5), hypertensive nephropathy (n=5), membranoproliferative glomerulonephritis (n=2), minimal-change disease with secondary glomerulosclerosis (n=2), Alport syndrome (n=1), non-conclusive diagnosis (n=4).

Patients were randomly assigned to one of 4 treatment sequences, namely I. RS+PLA > RS+ARB > RS+ARB+Diuretics > LS+ARB+Diuretics > LS+ARB > LS+PLA, II. RS+PLA > RS+ARB+Diuretics > RS+ARB > LS+ARB > LS+ARB+Diuretics > LS-PLA, III. LS+PLA > LS+ARB > LS+ARB+Diuretics > RS+ARB+Diuretics >

RS+ARB > RS+PLA, IV. LS+PLA > LS+ARB+Diuretics > LS+ARB > RS+ARB > RS+ARB+Diuretics > RS+PLA, with LS being low sodium diet (target: 50 mmol Na^+/day), RS being regular sodium diet (target: 200 mmol Na^+/day), ARB being angiotensin receptor blockade (losartan 100 mg/day), and Diuretics being hydrochlorothiazide 25 mg/day.

Additional antihypertensive drugs were allowed for blood pressure control (except for renin angiotensin aldosterone system blocking agents or diuretics) and were kept stable during the study. At the end of each 6-week treatment period patients collected 24-hour urine during one day, and after an overnight fast blood pressure was measured and blood was sampled.

Healthy controls

Healthy volunteers (n=21) were kept on a regular sodium diet and, by definition, had no diabetes mellitus or renal function impairment.

Measurements and calculations

Proteinuria was measured by the pyrogallol red-molybdate method in 24-hour urine samples. Dietary sodium intake was assessed from urinary sodium excretion. Blood pressure was measured at 1-minute intervals by an automatic device (Dinamap; GE Medical Systems, Milwaukee, WI), with the patient in supine position. After fifteen minutes of measurements, the mean of the last four readings was used for further analysis.

Peripheral blood was drawn by venipuncture. Aliquots from blood and 24 hour urine were stored at -80°C until CTGF analysis. CTGF levels were determined by enzyme-linked immunosorbent assay, using monoclonal antibodies against two distinct epitopes on the NH_2-terminal part of human CTGF (FibroGen, San Francisco, CA), as previously described(9). This assay detects both CTGF NH_2-terminal fragments and full-length CTGF with similar efficiency. Recoveries of full length CTGF and CTGF-N fragment spiked in plasma were identical, but in urine, full length CTGF rapidly disappeared while detection of CTGF-N fragment remained stable. To avoid confusion due to differences in molecular mass of full-length CTGF and fragments, CTGF levels are expressed as picomoles (per mL or 24 hour) instead of milligrams.

Data analysis

Data are given as mean with standard error when normally distributed (i.e. gender, age, proteinuria, blood pressure, creatinine clearance, urinary sodium excretion, and body

weight), or geometric mean with 95%-confidence interval if skewed (i.e. plasma CTGF and urinary CTGF). Before statistical testing, skewed variables were natural-log transformed to obtain normality. Associations between variables in patients were evaluated with Pearson's Correlation tests or Spearman's Rank tests. Drug effects in patients were determined using Paired T-tests. Variables in patients versus healthy controls were compared using unpaired T-tests. $P<0.05$ was considered statistically significant. SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Results

Baseline characteristics

Data obtained during placebo combined with the regular sodium diet were taken as baseline values. CKD patients and healthy controls had same gender (73% vs. 76% male, NS, Table 1) and race (all Caucasian), but patients were slightly younger (50±2 vs. 58±1 years, $p=0.001$). At baseline, patients had overt proteinuria (3.2 [2.5-4.0] g/day), hypertension (systolic and diastolic blood pressure 143±3 and 86±2 mmHg), and a relatively preserved renal function (creatinine clearance [CrCl] 89±5 mL/min). As expected, healthy controls had no relevant proteinuria (0.2 [0.1-0.2] g/day, $p<0.001$ vs. CKD), a lower blood pressure (systolic and diastolic blood pressure 120±3 and 72± 2 mmHg, $p<0.001$ and $p<0.001$ vs. CKD) and better renal function (CrCl 111±6 mL/

Table 1 Participants' characteristics

	CKD patients	Healthy controls	p-value
Number	33	21	-
Age – yr	50±2	58±1	0.001
Male sex – no. (%)	24 (73)	16 (76)	0.78
Caucasian race – no. (%)	33 (100)	21 (100)	-
Systolic blood pressure – mmHg	143±3	120±3	<0.001
Diastolic blood pressure – mmHg	86±2	72±2	<0.001
Proteinuria– g/day	3.2 [2.5-4.0]	0.2 [0.1-0.2]	<0.001
Creatinine clearance – mL/min	89±5	111±6	0.006

Data are shown as mean±SEM or as geometric mean [95%-confidence interval].

min, p=0.006 vs. CKD), than patients. Dietary sodium intake, as reflected by urinary sodium excretion, was comparable in patients at baseline and controls (199±10 vs. 162±16 mmol Na^+/day, p=0.053).

Response of proteinuria and blood pressure to ARB, LS and diuretics

The average urinary sodium excretion was 196±9 mmol Na^+/day during the 3 periods on a regular sodium diet and 92±8 mmol Na^+/day during the 3 periods on LS (p<0.001), indicating an adequate dietary compliance (Table 2).

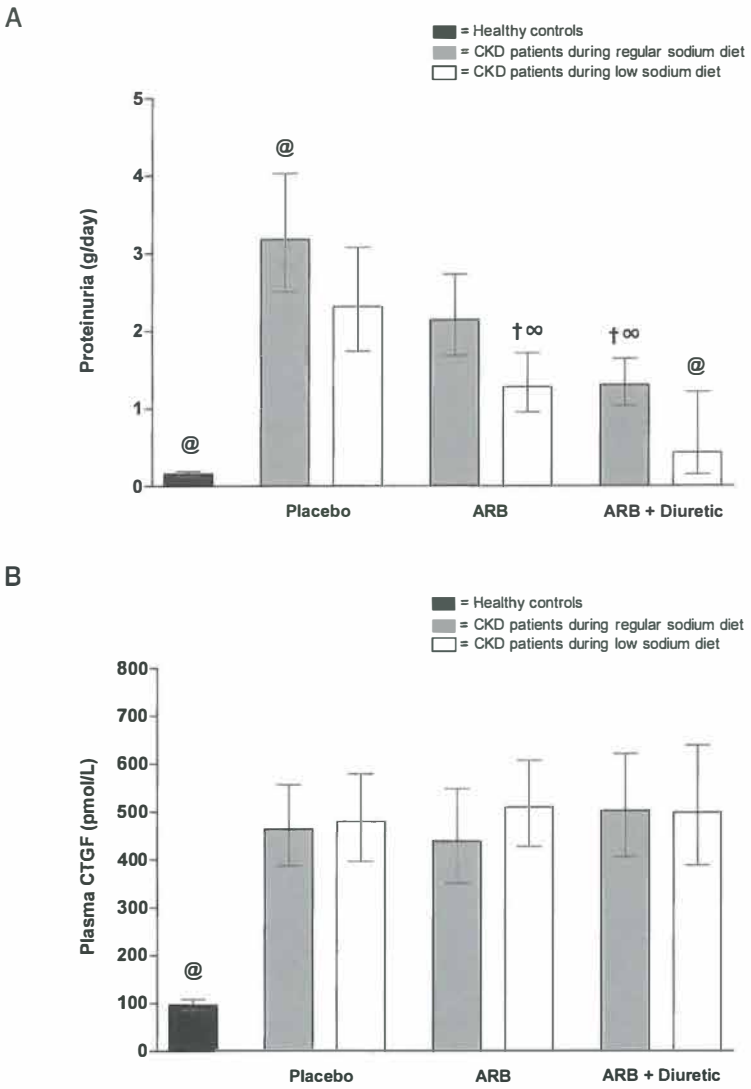
Table 2 Clinical parameters during ARB, LS, and diuretics

		Placebo	ARB	ARB+Diuretics
Urinary Na^+ excretion (mmol/day)	Regular sodium diet	200±10	197±11	193±11
	Low sodium diet	90±10 #	92±8 ‒	93±8 *
Systolic blood pressure (mmHg)	Regular sodium diet	143±4 @	135±3	125±3 † ‒ ‡
	Low sodium diet	137±3	128±3 † ‒	121±2 † ‒ ‡
Diastolic blood pressure (mmHg)	Regular sodium diet	86±2 @	80±2	75±1 ‒ ‡
	Low sodium diet	83±1 @	78±1	74±1 ‒ ‡
Body weight (kg)	Regular sodium diet	91±3	90±3 †	89±3 # ‡
	Low sodium diet	89±3 #	88±3 # †	88±3 # † *
Creatinine clearance (mL/min)	Regular sodium diet	89±5	94±6 †	86±6 ‒
	Low sodium diet	82±6	83±7 ‒	75±5 @

Data are shown as mean±SEM. Abbreviations: ARB, angiotensin receptor blockade; LS, low sodium diet; @ p<0.05 vs. all periods; # p<0.05 vs. placebo+RS; † p<0.05 vs. placebo+LS, ‒ p<0.05 vs. ARB+RS; ‡ p<0.05 vs. ARB+LS, * p<0.05 vs. ARB+diuretics+RS.

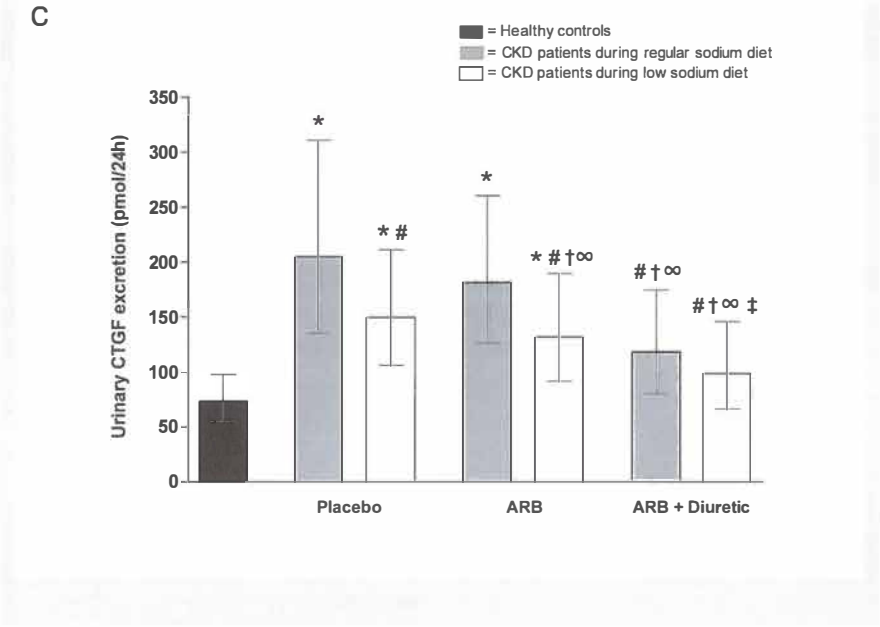
Proteinuria was significantly reduced by monotherapy with either LS (residual proteinuria 2.3 [1.7-3.1] g/day, p<0.001 vs. baseline; Figure 1A) or ARB (2.1 [1.7-2.7] g/day, p<0.001 vs. baseline). Proteinuria was further reduced by combination therapy with ARB+LS (1.3 [0.9-1.7] g/day, p<0.001 vs. ARB) or ARB+Diuretics (1.3 [1.0-1.6] g/day, p<0.001 vs. ARB). The maximal antiproteinuric effect was achieved by triple therapy with ARB+LS+Diuretics (0.4 [0.1-1.2] g/day, p=0.005 vs ARB+Diuretics, p<0.001 vs. ARB+LS). Blood pressure decreased accordingly (Table 2). Body weight and creatinine clearance decreased as well, consistent with a negative fluid balance during LS and/or diuretics.

Figure 1 Response of proteinuria and CTGF to ARB, LS and diuretics



Proteinuria and CTGF levels are shown as geometric mean with 95%-confidence interval. Figure 2A was adapted and modified from the original study[20]. Abbreviations: ARB, angiotensin receptor blockade; LS, low sodium diet; @ $p<0.05$ vs. all; * $p<0.05$ vs. healthy controls; # $p<0.05$ vs. placebo+RS in CKD patients; † $p<0.05$ vs. placebo+LS in CKD patients; ∞ $p<0.05$ vs. ARB+RS in CKD patients; ‡ $p<0.05$ vs. ARB+LS in CKD patients.

Figure 1 Continued

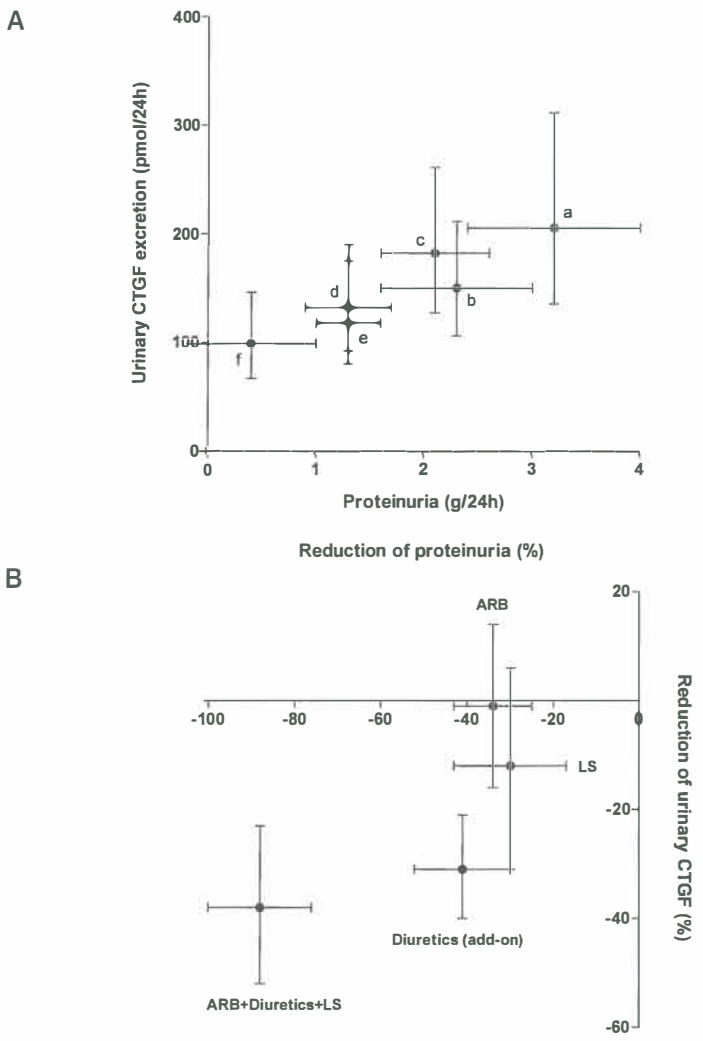


Response of CTGF to ARB, LS and diuretics

At baseline, plasma CTGF levels in CKD patients were approximately fivefold higher than in healthy controls (464 [387-556] vs. 96 [86-108] pmol/L, $p<0.001$; Figure 1B). Urinary CTGF excretion was approximately threefold higher than in controls (205 [135-311] vs. 73 [55-98] pmol/day, $p=0.001$; Figure 1C). Baseline urinary CTGF excretion correlated positively with baseline plasma CTGF levels ($r=0.41$, $p=0.027$) and inversely with baseline creatinine clearance ($r= -0.54$, $p=0.002$), but not with baseline proteinuria. Plasma CTGF was not correlated with proteinuria or renal function.

Plasma CTGF levels remained completely unaltered by ARB, LS, and/or diuretics. Urinary CTGF excretion was stepwise reduced by the antiproteinuric intervention, paralleling the reduction in proteinuria (Figure 2), resulting in values not significantly different from healthy controls during the treatment regimens with the lowest proteinuria, i.e. during triple therapy with ARB+LS+Diuretics (99 [67-146] in CKD vs. 73 [55-98] pmol/day in controls, $p=0.82$).

Figure 2 The reduction of urinary CTGF parallels the reduction in proteinuria



Data are shown as geometric mean with 95%-confidence interval.

Panel A: Stepwise concomitant reduction of proteinuria and urinary CTGF during the 6 different treatment periods. Symbol a: placebo plus regular sodium diet (RS), symbol b: placebo plus low sodium diet (LS), symbol c: angiotensin receptor blockade (ARB) plus RS, symbol d: ARB+LS, symbol e: ARB+RS+diuretics, symbol f: ARB+LS+diuretics.

Panel B: Percentage change in proteinuria and urinary CTGF by LS combined with placebo (change from placebo+RS), ARB combined with RS (change from placebo+RS), diuretics combined with ARB+RS (change from ARB+RS), and by ARB+Diuretics+LS (change from placebo+RS), respectively.

Discussion

Plasma and urinary levels of CTGF were significantly elevated in non-diabetic proteinuric CKD patients. Antiproteinuric intervention was associated with a stepwise reduction in urinary CTGF in proportion to the reduction in proteinuria, but did not affect the elevated plasma CTGF levels.
CTGF is strongly implicated in diabetic renal fibrosis and injury[4-12]. The elevated levels of CTGF in plasma and urine in our patients suggest that CTGF may also play a role in the pathophysiology of non-diabetic CKD and its extrarenal complications, as a biomarker and/or as a pathogenic factor.

The source of the urinary CTGF is of interest. Because of their small size (≤38 kDa) CTGF and the fragments thereof are predicted to be cleared from plasma by glomerular filtration[21,22]. Consequently, glomerular filtration of elevated plasma CTGF may be one of the causes of the elevated urinary CTGF levels in our patients, who had a relatively preserved renal function. Second, the elevated urinary CTGF in our proteinuric patients may result from proteinuria-induced proximal tubular saturation or dysfunction[22;23]. Accordingly, the reduction in urinary CTGF during antiproteinuric therapy could reflect amelioration of tubular dysfunction by reduction in proteinuria, as also observed for other proximal tubular markers like KIM-1[24;25]. Third, local production of CTGF in the kidney, e.g. downstream of angiotensin II[26;27] and high sodium intake[28-30], may be a determinant of the elevated urinary CTGF levels as well. Local CTGF production in the kidney has been observed in animal experiments and human biopsies[4-12;31;32].
In addition to renal CTGF production, also enhanced CTGF ultrafiltration and impaired tubular CTGF reabsorption may increase the exposure to CTGF of the proximal and distal nephron respectively, and thus contribute to a profibrotic microenvironment[33-35].

Urinary CTGF levels were reduced by antiproteinuric intervention, paralleling the reduction in proteinuria, with the lowest values of urinary CTGF during triple therapy. As proteinuria may reduce proximal tubular CTGF reabsorption[22;23], the reduction in urinary CTGF may reflect amelioration of proximal tubular dysfunction, which might be a consequence of proteinuria reduction. Such amelioration is plausible, from previously published data on this population, showing reduction of the urinary proximal tubular damage markers kidney injury molecule 1 (KIM-1) and N-acetyl-beta-D-glucosaminidase (NAG)[24].
More specifically, besides its antiproteinuric action, the reduction of urinary CTGF by ARB might also be independent of proteinuria, as angiotensin II can induce CTGF expression directly or through aldosterone[26;27,36]. Also dietary sodium restriction might

inhibit urinary CTGF independent of proteinuria reduction, because high sodium intake promotes CTGF and transforming growth factor β1 expression[28-30].

Plasma CTGF levels were elevated in our patients, consistent with previously observed increase of plasma CTGF levels in patients with diabetic nephropathy[11;12;37]. As CTGF can be expressed by vascular smooth muscles cells and endothelial cells of atherosclerotic lesions[38], and also by injured myocardium[39], circulating CTGF might also reflect fibrotic activity outside the kidney as a biomarker. In addition elevated circulating CTGF might generate a systemic profibrotic environment and contribute to the pathogenesis of e.g. cardiovascular complications[13;14]. Consistently, plasma CTGF was found to independently predict intima-media thickness, end-stage renal disease and overall mortality in diabetic CKD patients[11;12].

In contrast to urinary CTGF, plasma CTGF levels in our patients were not reduced by antiproteinuric intervention, although we cannot exclude the possibility that longer duration of treatment would have been required to reduced plasma CTGF. Due to its small size, glomerular proteinuria is not expected to affect clearance of plasma CTGF[22]. Our current observation suggests that proteinuria is also not directly associated with major determinants of plasma CTGF. Of note, despite the proven benefits of antiproteinuric intervention in CKD the residual risk for cardiovascular events remains high[40-41]. The increased level and therapy-resistance of plasma CTGF in our patients might be a reflection of the ongoing cardiovascular injury in CKD patients even under appropriate anti-proteinuric therapy. Therefore, it would be interesting to see the possible effects on cardiovascular outcome of emerging therapies that reduce plasma CTGF levels[42;43].

Our study has several limitations. First, it provides short term data only, so the impact of our data for long term outcome will require separate study. Another limitation is the lack of information on monotherapy with diuretics.

Conclusions

Plasma and urinary levels of CTGF are substantially elevated in non-diabetic proteinuric CKD patients, and antiproteinuric intervention lowers urinary CTGF in proportion to the reduction in proteinuria, but does not affect plasma CTGF levels. Hence, CTGF may play a role in the pathophysiology of non-diabetic CKD and its extrarenal complications. Long-term studies will be needed to determine the impact of urinary and plasma CTGF levels, and their response to therapy, on outcome in non-diabetic CKD.

Funding

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7

Erythropoietin is reduced by combination of diuretic therapy and RAAS blockade in proteinuric renal patients with preserved renal function

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Abstract

Background: Renin angiotensin aldosterone system (RAAS) blockade improves prognosis in renal patients, but usually requires diuretic co-treatment. RAAS blockade can decrease erythropoietin and/or hemoglobin levels. Diuretics decrease erythropoietin in rodents, but their effect on erythropoietin and hemoglobin in humans is unknown.

Methods: Proteinuric renal patients with preserved renal function were treated during 6-week periods with placebo (PLA), losartan 100 mg/day (LOS), and LOS plus hydrochlorothiazide 25 mg/day (LOS/HCT), in random order.

Results: Hemoglobin was inversely related to proteinuria, and erythropoietin levels were inappropriately low in relation to hemoglobin. Hemoglobin was lowered by LOS with and without HCT. Erythropoietin was decreased by LOS/HCT, but not by LOS.

Conclusions: Erythropoietin and hemoglobin are reduced by hydrochlorothiazide added to losartan in proteinuric renal patients with preserved renal function. We hypothesize that erythropoietin reduction by hydrochlorothiazide is caused by a decrease in renal oxygen requirement, which is the main stimulus for erythropoietin production, due to the inhibition of active tubular sodium reabsorption. Further studies should explore the exact mechanism of this phenomenon, and its clinical impact.

Introduction

Blockade of the renin angiotensin aldosterone system (RAAS) with angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB) reduces hypertension and proteinuria and improves renal and cardiovascular outcome in chronic kidney disease (CKD)[1,2]. For optimal therapeutic efficacy often co-treatment with diuretics is required[3,4]. ACEi and ARB decrease erythropoietin (EPO) and/or hemoglobin (Hb) levels in different populations[5-8], by blocking the effects of angiotensin-II on erythropoiesis[9;10]. Diuretics reduce EPO levels in rodents[11;12], but their effect on EPO and Hb in humans is unknown. We report the effects of the diuretic hydrochlorothiazide and the ARB losartan on EPO and Hb levels in proteinuric CKD patients with preserved renal function.

Methods

Patients and protocol

This is a post-hoc analysis of a randomized, double-blind, placebo-controlled cross-over study. The protocol was described in detail elsewhere[13]. In short, 33 non-diabetic CKD patients with overt proteinuria and preserved renal function (Table 1) were included. Patients were treated during 6-week periods with placebo (PLA), losartan 100 mg/day (LOS), and LOS plus hydrochlorothiazide 25 mg/day (LOS/HCT), combined with consecutively a low sodium diet (LS, 92±8 mmol/day) and a high sodium diet (HS, 196±9 mmol/day), in random order (Figure 1).

Measurements and calculations

EPO levels were measured by chemiluminescence immunoassay (Siemens, Los Angeles, CA, USA). To relate the EPO level to the actual Hb, the observed/predicted log EPO ratio (O/P_{EPO}) was calculated as proposed by Westenbrink et al[14]. O/P_{EPO} in healthy reference subjects (age 50±5 years) was 0.90±0.029.

Data analysis

Data obtained during placebo plus high sodium diet (HS/PLA) were taken as baseline values.

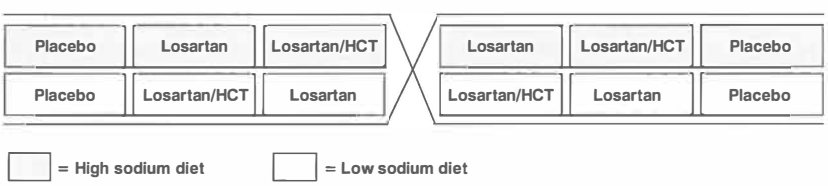
Data are given as mean±standard error, or geometric mean [interquartile range] when skewed. Before statistical testing, skewed variables were natural log-transformed to obtain normality. Associations between variables were evaluated with Pearson's Correlation tests. Therapy effects were determined using Paired T-tests, with a Bonferroni correction for multiple testing. $P<0.05$ was considered statistically significant. SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Table 1 Patients' characteristics

	Males	Females
General parameters:		
Number	24	9
Age (years)	52 ± 2	46 ± 5
Caucasian race (%)	100	100
Body mass index (kg/m^2)	28 ± 1	27 ± 2
Systolic blood pressure (mmHg)	144 ± 5	140 ± 10
Diastolic blood pressure (mmHg)	87 ± 3	85 ± 4
Creatinine clearance (mL/min)	92 ± 6	81 ± 9
Proteinuria (g/day)	3.6 ± 0.5	4.5 ± 0.8
Hematological parameters:		
Hemoglobin (mmol/L)	9.4 ± 0.2	8.6 ± 0.3 *
Hematocrit (L/L)	0.45 ± 0.01	0.41 ± 0.01 *
Ferritin (ug/L)	153 ± 22	76 ± 28
Erythropoietin (U/L)	14.8 [12.3 - 17.7]	12.9 [7.4 - 22.5]
Observed/predicted log EPO ratio	0.65 ± 0.02	0.59 ± 0.05

Characteristics of patients with chronic kidney disease during placebo plus high sodium diet. Abbreviations: EPO, erythropoietin; * $p<0.05$ versus males.

Figure 1 Study design



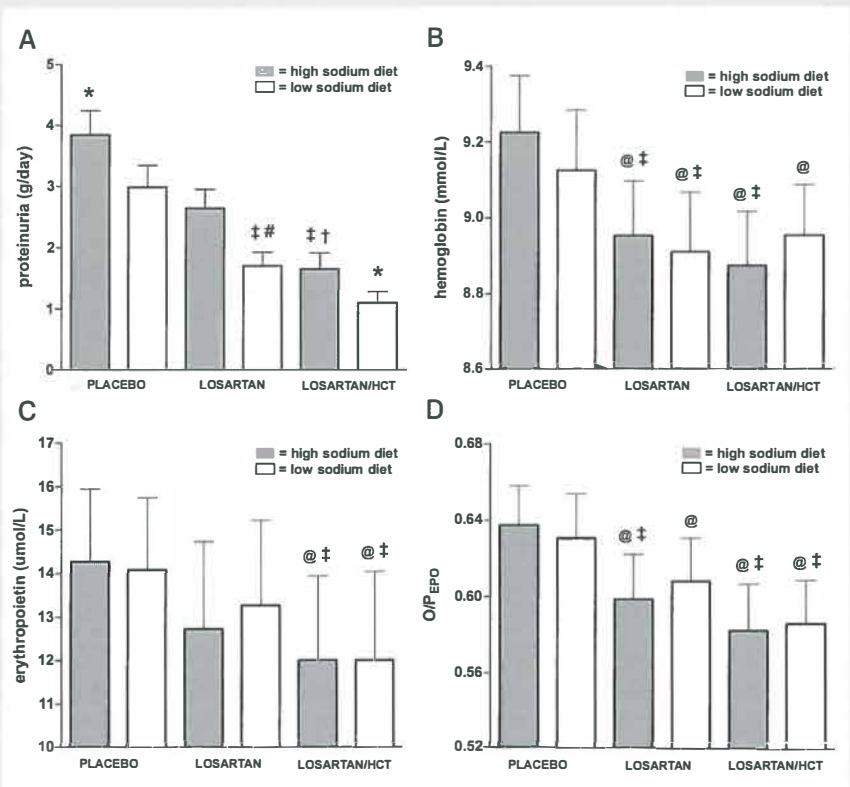
Proteinuric renal patients were treated during six 6-week periods with placebo, losartan 100 mg/day, and losartan 100 mg/day plus hydrochlorothiazide 25 mg/day (losartan/HCT), combined with a high and low sodium diet (intake 196±9 versus 92±8 mmol Na+/day, $p<0.001$), in random order.

Results

General parameters

Baseline characteristics are shown in Table 1. During the six different treatment periods proteinuria decreased from 3.8±0.4 g/day at baseline (HS/PLA) to 1.1±0.2 during RAAS blockade with maximal volume intervention (LS/LOS/HCT, p<0.001; Figure 2A). Mean arterial pressure decreased accordingly (105±3 at baseline versus 90±1 mmHg during LS/LOS/HCT, p<0.001), as previously described in more detail[13]. Creatinine clearance (89±5 at baseline versus 75±5 mL/min during LS/LOS/HCT, p=0.001) and

Figure 2 Individual values for urinary markers ranked by achieved proteinuria



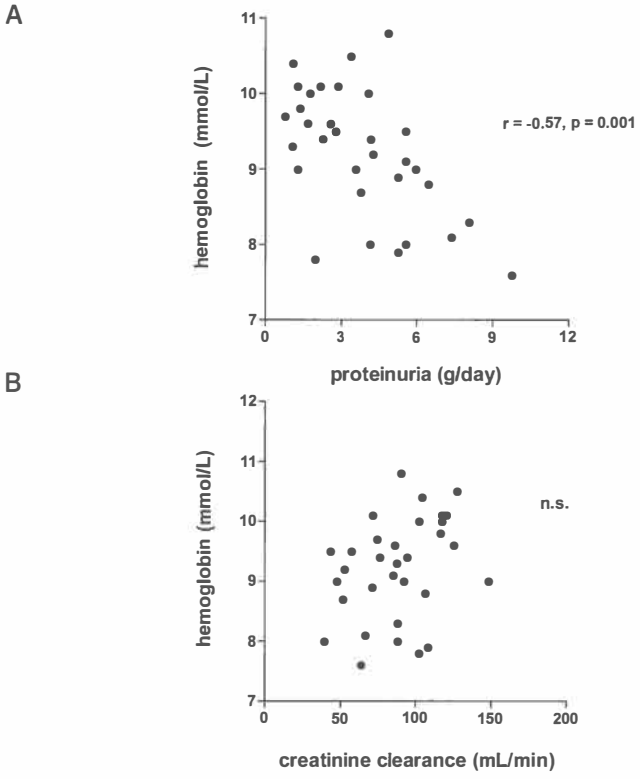
Tubular injury markers ranked by achieved proteinuria during combined treatment with ACE inhibition plus angiotensin receptor blockade plus low sodium diet. * p<0.05 vs. proteinuria above 1.0 g/day, ¹ p<0.05 vs. proteinuria between 0.3-1.0 g/day. The areas between dotted lines represent the interquartile range in healthy subjects.

body weight (91±3 at baseline versus 88±3 kg during LS/LOS/HCT, $p<0.001$) decreased as well, consistent with a negative fluid balance during low sodium diet and hydrochlorothiazide.

Hematological parameters

At baseline (HS/PLA) Hb was inversely related to proteinuria, but was not related to creatinine clearance (Figure 3). O/P_{EPO} was decreased (0.64±0.02 versus 0.90±0.029 in healthy reference subjects, $p<0.001$), indicating that EPO levels were inappropriately low in relation to Hb levels.

Figure 3 Relationship of hemoglobin with proteinuria and creatinine clearance in untreated renal patients



Hemoglobin is inversely related to proteinuria, but is not related to creatinine clearance, in renal patients during placebo plus high sodium diet.

Hb was decreased by losartan with and without hydrochlorothiazide (Figure 2B). EPO levels were reduced by the addition of hydrochlorothiazide on top of losartan, but not by losartan monotherapy, as compared to placebo (Figure 2C). There was no statistical difference, however, between the effect of losartan monotherapy and the effect of hydrochlorothiazide on top of losartan on EPO levels. O/P_{EPO} was further decreased by losartan with and without hydrochlorothiazide (Figure 2D). No clear-cut effect of low sodium diet on Hb, EPO, or O/P_{EPO} was observed.

Discussion

We found that Hb levels are inversely related to proteinuria, and EPO levels are inappropriately low in relation to Hb (low O/P_{EPO}), in untreated non-diabetic CKD patients with overt proteinuria and preserved renal function. Hydrochlorothiazide added to losartan decreases EPO, O/P_{EPO} and Hb in these patients.
Hb was inversely related to proteinuria, but was not related to renal function, which strongly suggests an effect of proteinuria as such on Hb levels. This is a new finding. Proteinuria can reduce circulating EPO levels through urinary EPO loss[15-17], which may explain the inappropriately low circulating EPO in our patients at baseline. No relationship was however found between circulating EPO and proteinuria in these patients, suggesting that other factors such as inflammation may be involved as well[18-20].

Remarkably, hydrochlorothiazide added to losartan, while reducing proteinuria, decreased EPO and O/P_{EPO} levels compared to placebo. Although RAAS blockade is known to reduce EPO levels[6-8], effects of (add-on) diuretics on EPO were not reported before, besides hydrochlorothiazide added to enalapril reducing hematocrit in hypertensive patients[21].
In rodents diuretics reduce renal EPO production[11;12], via the inhibition of tubular sodium reabsorption which reduces renal oxygen consumption and increases renal oxygen pressure[22;23], causing decreased EPO production[24]. This mechanism might also be involved in our patients.
Of note, the effects of low sodium diet added to losartan were similar to add-on hydrochlorothiazide, for proteinuria, blood pressure, renal function and body weight, but low sodium diet did not affect EPO levels, suggesting a direct pharmacological effect of hydrochlorothiazide on EPO rather than a volume-mediated effect. This notion is supported by the finding that the negative fluid balance, with an anticipated reduction of the distribution volume of EPO[16], during hydrochlorothiazide was associated with a decrease, instead of an increase, in EPO levels. At present, no direct effects of diuretics on erythroid precursor cells are known.

EPO production was compromised in our patients as shown by the low O/P_{EPO} at baseline. Whether hydrochlorothiazide can affect uncompromised EPO production cannot be ascertained. It would be relevant to explore this issue in other populations, as (combinations of) diuretics and RAAS blockade are widely used in non-renal conditions such as essential hypertension and heart failure[25;26].

Effects of RAAS blockade on EPO and Hb usually become evident 3-12 weeks after initiation of therapy[5]. Therefore, our treatment periods may have been too short to evaluate the full hematological effect of the treatment regimens. Other limitations are the small sample size, the overall small changes and large variation of EPO levels and the lack of information on hydrochlorothiazide monotherapy.

To conclude, Hb levels are inversely related to proteinuria, and EPO levels are inappropriately low in relation to Hb, in renal patients with overt proteinuria and preserved renal function. EPO and Hb levels are reduced by hydrochlorothiazide added to losartan in these patients. We hypothesize that EPO reduction by add-on hydrochlorothiazide is caused by a decrease in renal oxygen requirement, which is the main stimulus for EPO production, due to the inhibition of active tubular sodium reabsorption. Further studies should explore the exact mechanism of this phenomenon, and its clinical impact.

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8

Dietary sodium restriction added to single and dual RAAS blockade is associated with a reduction in circulating erythropoietin, proportional to changes in tubular sodium reabsorption

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Submitted

Abstract

Background and objectives: The renoprotective effects of ACE inhibition (ACEi) and angiotensin receptor blockade (ARB) can be potentiated by low sodium diet (LS) or diuretics. We previously reported that diuretics added to ARB reduce circulating erythropoietin in renal patients. Animal data suggest that this is caused by reduced renal oxygen requirement, the main stimulus for erythropoietin production, due to decreased active tubular sodium reabsorption (TNa^+R). Here, we investigated the effects of dual blockade with ACEi+ARB, and LS, on erythropoietin and TNa^+R in renal patients.

Design, setting, participants and measurements: Post-hoc analysis of a cross-over RCT. 49 renal patients (creatinine clearance 69 [50-110] mL/min, urinary protein excretion 1.9 [0.9-3.4] g/day) received a background treatment of ACEi (lisinopril 40 mg/day) during four six-week periods, that was combined with placebo or ARB (valsartan 320 mg/day), whereas dietary sodium intake was either regular (RS, 189±8 mmol Na^+/day) or low (LS, 106±7 mmol Na^+/day, $p<0.001$ vs. RS), in random order.

Results: From ACEi+RS as baseline (mean erythropoietin 13.4 (9.6-18.0) mIU/mL, Hb 8.7 (0.1) mmol/L, TNa^+R 14.9 (9.8-22.3) mol/day), TNa^+R, erythropoietin, and hemoglobin were reduced by the addition of LS (11.5 (8.1-17.0) mIU/mL, $p=0.09$; 8.5 (0.2) mmol/L, $p=0.04$; 14.6 (9.2-19.2) mol/day, $p=0.001$) and LS+ARB (10.6 (8.0-13.9) mIU/mL, $p=0.005$; 8.3 (0.1) mmol/L, $p=0.001$; 11.8 (8.8-16.5) mol/day, $p<0.001$), but not by the addition of ARB alone (13.9 (9.6-18.3) mIU/mL, $p=0.9$; 8.6 (0.1) mmol/L, $p=0.3$; 15.1 (10.9-20.9) mol/day, $p=1.0$), to ACEi. The reduction of erythropoietin was quantitatively related to the reduction of TNa^+R by these interventions ($r=0.28$, $p=0.05$ for add-on LS, $r=0.38$, $p=0.01$ for add-on LS+ARB).

Conclusions: Erythropoietin levels are reduced in proportion to the reduction in TNa^+R by addition of LS and LS+ARB to ACE in proteinuric renal patients. These findings suggest that reduction in TNa^+R by LS during RAAS blockade alleviates renal hypoxia, which might be involved in the benefits of LS during RAAS blockade in renal patients.

Introduction

Reduction of proteinuria and blood pressure is the cornerstone of renoprotective intervention[1-3]. Blockade of the renin angiotensin aldosterone system (RAAS) by angiotensin converting enzyme (ACE) inhibition or angiotensin receptor blockade (ARB) is first choice treatment to this purpose[4-6].

Usually, concomitant correction of volume excess, by dietary sodium restriction and/or diuretics is required to obtain the maximal effect on blood pressure and proteinura[7-9]. In patients with residual proteinuria during ACE inhibition, moderate dietary sodium restriction is more potent than addition of ARB, but for a maximal effect on proteinuria and blood pressure both dual blockade and sodium restriction are required, as was recently shown in the DUAAAL study by our group[10].

The adjunct effects of treatment regimens aimed primarily at proteinuria and blood pressure may be relevant as well[11;12]. Several studies have shown that both ACE inhibition or ARB can reduce erythropoietin and hence hemoglobin levels in renal patients[13-15]. The effect of their combination, and the combined effects of dual blockade with sodium restriction are unknown.

Therefore we investigated the effects of dual RAAS blockade and dual RAAS blockade combined with sodium restriction, as compared to monotherapy ACE inhibition, on erythropoietin and hemoglobin levels in a post-hoc analysis of the DUAAAL study.

Methods

Patients

This is a post-hoc analysis of a randomized double-blind placebo-controlled cross-over multicenter trial. The protocol was described in detail elsewhere[10]. We excluded 3 of the original 52 patients because they used eythropoiesis stimulating agents. Thus, 49 patients were investigated in the current study.

In short, inclusion criteria were blood pressure above 125/75 mmHg in combination with residual proteinuria above 1.0 g/day during ACE inhibition on maximal dose (lisinopril 40 mg/day), creatinine clearance of 30 mL/min or above, and age over 18 years. Exclusion criteria were systolic blood pressure of 180 mmHg or above, diastolic blood pressure of 110 mmHg or above, diabetes mellitus, renovascular hypertension, decrease of creatinine clearance by at least 6 mL/min in the previous year, a cardiovascular event

in the previous six months, immunosuppressive treatment, regular use (>1 day/week) of non-steroidal anti-inflammatory drugs, pregnancy, or breast feeding.

Protocol

During a run-in period of at least six weeks, patients received ACE inhibition at maximal dose (lisinopril 40 mg/day) and stopped all other RAAS blockers. Additional antihypertensive drugs such as β blockers, α blockers, calcium channel blockers, and diuretics were allowed and kept stable during the study. No dietary intervention took place during the run-in period.

After the run-in period patients received background treatment of ACE inhibition at maximal dose (ACEi; lisinopril 40 mg/day). This was randomly combined with placebo (PLA) or angiotensin receptor blockade at maximal dose (ARB; valsartan 320 mg/day) and dietary intervention by either a regular sodium diet (RS; target: 200 mmol Na^+/day) or a low sodium diet (LS; target: 50 mmol Na^+/d) in randomized order. So, the study protocol consisted of four six-week study periods: 1. ACEi+PLA+RS, 2. ACEi+ARB+RS, 3. ACEi+PLA+LS, 4. ACEi+ARB+LS, in random order.

Measurements and calculations

At the end of each six week treatment period, patients collected 24 hour urine samples, and blood pressure was measured and blood was sampled after an overnight fast. Additionally, in the middle of every six week treatment period, patients collected 24 hour urine samples to monitor dietary compliance.

Proteinuria was measured in 24 hour urine samples with a turbidimetric assay using benzethonium chloride (Modular, Roche Diagnostics, Mannheim, Germany). Blood pressure was measured at one minute intervals for 15 minutes by an automatic device (Dinamap, GE Medical Systems, Milwaukee, WI) with the patient in supine position, and we used the mean of the last 3 readings for further analysis. Blood and urinary electrolyte levels were determined with an automated multianalyzer (Roche Diagnostics, Mannheim, Germany). Dietary sodium intake was assessed from urinary sodium excretion. Creatinine clearance was calculated from creatinine concentrations in plasma and in 24 hour urine samples. Ferritin, vitamin B12, folic acid, and erythropoietin levels were measured by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany, and Siemens, Los Angeles, CA, USA).

Filtered sodium load (mmol/day) was calculated as plasma sodium concentration (mmol/L) times creatinine clearance (mL/min) times 1.44. We assessed absolute tubular

sodium reabsorption (mmol/day) by subtracting urinary sodium excretion (mmol/day) from filtered sodium load (mmol/day). Fractional sodium excretion (%) was calculated as urinary sodium excretion (mmol/day) divided by plasma sodium concentration (mmol/L) times creatinine clearance (mL/min) times 0.0144. We determined fractional tubular sodium reabsorption (%) by subtracting fractional sodium excretion (%) from 100%. To relate the erythropoietin level to the actual hemoglobin, we calculated the observed/predicted log erythropoietin ratio as proposed by Westenbrink[16], with predicted log erythropoietin calculated by 3.015 minus 0.130 times hemoglobin (mmol/L).

According to local laboratory reference ranges, anemia was defined as hemoglobin below 7.5 mmol/L in women and hemoglobin below 8.7 mmol/L in men. Ferritin was considered deficient if below 30 ug/L in males and if below 15 ug/L in females. Mean corpuscular volume, iron, vitamin B12, and folic acid levels were considered abnormally low when below 80 fl, below 10 umol/L, below 145 pmol/L, and below 4 nmol/L, respectively. Normal range for serum erythropoietin was defined as 4.5 to 19.6 U/L, based on local laboratory reference ranges. Normal range for observed/predicted log EPO ratio was defined as 0.84 to 0.96[16].

Statistical analysis

We give data as mean with standard error (SE) when normally distributed or otherwise as median with interquartile range (IQR). We used data during monotherapy ACE inhibition (ACEi+PLA+RS) as baseline values. Analogous to the primary analyses[10], we used Paired T-tests, Wilcoxon signed rank tests, and Pearson's ^{2} tests (which account for the same patients providing data for both treatments) to determine effects of treatment. We used Spearman correlation tests to determine associations between variables. Alpha was set at $P<0.05$. We used SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) for all analyses.

Results

Baseline characteristics

Baseline characteristics are shown in Table 1. By default patients had overt proteinuria, a high-normal blood pressure and a mildly impaired renal function. Anemia was present in 51% of males and in 25% of females. Mean corpuscular volume, vitamin B12, and folic acid levels were normal in all patients. One male had low ferritin levels (26 ug/L), with normal iron (10 umol/L), mean corpuscular volume (96 fl) and hemoglobin levels (9.8

Table 1 Patients' characteristics

	Males	Females
General parameters		
Number	41	8
Age – y	51±2	51±4
Body mass index – kg/m²	28±1	28±2
Systolic blood pressure – mmHg	134±3	127±6
Diastolic blood pressure – mmHg	81±2	77±3
Creatinine clearance – mL/min	75 (48-114)	72 (51-103)
Proteinuria – g/day	1.8 (0.8-3.4)	2.2 (1.5-3.3)
Urinary sodium excretion – mmol/day	196±10	163±10
Hematological parameters		
Hemoglobin – mmol/L	8.7±0.2	8.5±0.4
Anemia – %	51	25
Hematocrit – L/L	0.414±0.008	0.403±0.022
Mean corpuscular volume – fl	90.5±0.7	89.5±1.6
Ferritin – ug/L	141 (78-209)	59 (29-74) *
Iron – umol/L	17±1	12±1 *
Vitamin B12 – pmol/L	309 (247-389)	294 (269-363)
Folic acid – nmol/L	18 (14-23)	17 (12-26)
Erythropoietin – U/L	13.3 (9.3-18.0)	15.4 (10.3-22.0)

Abbreviations: EPO, erythropoietin; * $p<0.05$ versus males.

mmol/L). Three females had low iron levels (8±1 umol/L), with normal ferritin (69 (41-74 ug/L), mean corpuscular volume (90±3 fl) and hemoglobin levels (8.8±0.2 mmol/L). Six patients were using thiazide diuretics and five patients used loop diuretics.

General parameters during the different treatment regimens

The treatment period with monotherapy ACE inhibition (ACEi+PLA+RS) was considered as baseline. The effects of ARB, LS, and their combination, are described as change from baseline. Plasma renin level was increased stepwise by the addition of ARB, LS, and ARB+LS to ACEi (Table 2). Plasma aldosterone levels were increased by LS, but not by ARB. Data on proteinuria, blood pressure and creatinine clearance were extensively reported in the primary publication[10] and are summarized in Table 2.

Table 2 General parameters during four treatment regimens

	Regular sodium diet		Low sodium diet	
	ACEi	ACEi+ARB	ACEi	ACEi+ARB
General parameters:				
Proteinuria - g/day	1.9 (0.9-3.4)	1.3 (0.6-3.1) *	0.8 (0.5-1.5) *†	0.7 (0.4-1.3) *†‡
Systolic blood pressure - mmHg	133 (3)	130 (3)	123 (2) *†	121 (3) *†
Diastolic blood pressure - mmHg	80 (2)	77 (2) *	74 (2) *†	71 (2) *†
Creatinine clearance - mL/min	75 (50-113)	75 (54-106)	75 (46-95) *†	60 (44-81) *†‡
RAAS parameters:				
Plasma renin concentration - ng/L	54 (18-175)	83 (27-235) *	175 (45-426) *†	273 (50-1012) *†‡
Plasma aldosterone - nmol/L	0.20 (0.12-0.38)	0.20 (0.14-0.28)	0.34 (0.18-0.52) *†	0.30 (0.18-0.52) *†

Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; * $p<0.05$ vs. ACEi on regular sodium diet; † $p<0.05$ vs. ACEi-ARB on regular sodium diet; ‡ $p<0.05$ vs. ACEi on low sodium diet.

Hematological parameters during the different treatment regimens

Erythropoietin levels remained unchanged during addition of ARB, but were significantly reduced by adding LS and LS+ARB, respectively. This was paralleled by a reduction in the observed/predicted log EPO ratio. Likewise, hemoglobin levels were not significantly changed by the addition of ARB to ACEi, whereas the addition of LS reduced hemoglobin. A comparable reduction of hemoglobin was achieved when LS+ARB was added to ACEi. Similar results were found for hematocrit (Table 3).

Changes in tubular sodium reabsorption, and its association with changes in EPO during the different treatment regimens

Consequent to the reduction in creatinine clearance and plasma sodium levels (Table 2), filtered sodium load was decreased by the addition of LS, with a further decrease by the addition of LS+ARB, whereas it was not altered by the addition of ARB to ACEi (Table 3). Fractional tubular sodium reabsorption was increased by the addition of LS and LS+ARB, but not ARB, to ACEi, in agreement with the increase of aldosterone levels during the two LS conditions. Absolute tubular sodium reabsorption was reduced by adding LS, with a further reduction during LS+ARB, but was not affected by the addition of ARB as such.

Table 3 Sodium and hematological parameters and during four treatment regimens

	Regular sodium diet		Low sodium diet	
	ACEi	ACEi+ARB	ACEi	ACEi+ARB
Sodium parameters:				
Urinary sodium excretion - mmol/day	189 (8)	180 (9)	106 (7) *†	105 (8) *†
Plasma sodium - mmol/L	140.7 (0.4)	140.8 (0.4)	139.5 (0.4) *†	139.1 (0.4) *†
Filtered sodium load - mol/day	15.1 (10.1-22.5)	15.3 (11.1-21.1)	14.7 (9.3-19.3) *†	11.9 (8.8-16.6) *†‡
Fractional sodium reabsorption - %	98.9 (98.2-99.2)	98.9 (98.4-99.2)	99.3 (99.0-99.5) *†	99.3 (98.7-99.5) *†
Absolute sodium reabsorption - mol/day	14.9 (9.8-22.3)	15.1 (10.9-20.9)	14.6 (9.2-19.2) *†	11.8 (8.8-16.5) *†‡
Hematological parameters:				
Erythropoietin - mIU/mL	13.4 (9.6-18.0)	13.9 (9.6-18.3)	11.5 (8.1-17.0) †	10.6 (8.0-13.9) *†
Observed / predicted log EPO ratio	0.60 (0.52-0.68)	0.60 (0.51-0.67)	0.56 (0.51-0.64) *†	0.53 (0.47-0.61) *†‡
Hemoglobin - mmol/L	8.7 (0.1)	8.6 (0.1)	8.5 (0.2) *	8.3 (0.1) *†
Hematocrit - L/L	0.412 (0.007)	0.410 (0.007)	0.403 (0.007) *	0.400 (0.007) *†

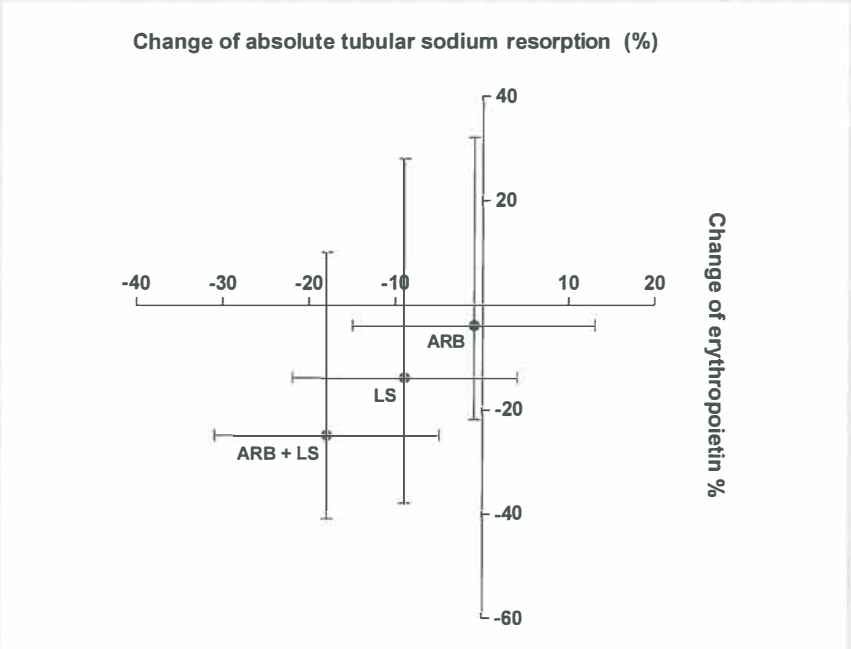
Abbreviations: ACEi, ACEi inhibition; ARB, angiotensin receptor blockade; EPO, erythropoietin; * $p<0.05$ vs. ACEi on regular sodium diet; † $p<0.05$ vs. ACEi-ARB on regular sodium diet; ‡ $p<0.05$ vs. ACEi on low sodium diet.

In figure 1, the change of erythropoietin is plotted against the change of absolute tubular sodium reabsorption, by the addition of ARB, LS, and ARB+LS, to ACEi. The change of erythropoietin by add-on LS and add-on LS+ARB, but not add-on ARB, was closely related to the change of absolute tubular sodium reabsorption by these interventions ($r=0.28$, $p=0.05$ for the addition of LS to ACEi, $r=0.38$, $p=0.01$ for the addition of LS+ARB to ACEi; $r=0.24$, $p=0.1$ for the addition of ARB to ACEi).

Discussion

This study has two main findings. First, erythropoietin and hence haemoglobin and hematocrit levels are reduced by the addition of dietary sodium restriction to monotherapy ACE inhibition (single RAAS blockade) and to combined ACE inhibition and ARB (dual RAAS blockade), but not by dual RAAS blockade as compared to ACE

Figure 1 Relationship between the change of erythropoietin levels and the change of tubular sodium reabsorption



Data are shown as median with IQR. ARB: change from ACEi+RS to ACEi+ARB+RS. LS: change from ACEi+RS to ACEi+LS. ARB+LS: change from ACEi+RS to ACEi+ARB+LS.

inhibition. Second, the reduction of erythropoietin during the LS conditions is quantitatively related to the reduction of absolute tubular sodium reabsorption, mainly elicited by the reduction in filtered sodium load.

Reduction of erythropoietin and hence hemoglobin levels by (add-on) dietary sodium restriction has not been described in chronic kidney disease previously. Although reduced erythropoietin levels could theoretically blunt the benefits of dietary sodium restriction in these patients, since erythropoietin might exert (non-hematological) tissue protective effects[17], clinical data suggest the opposite. Recently it has been found that reduced erythropoietin levels are associated with improved overall and cardiovascular survival in renal transplant recipient (submitted data by Sinkeler SJ, Bakker SJ and Navis G et al). In line with this, large cohort studies in chronic kidney disease showed that correcting anemia, unless severe, with recombinant erythropoietin is not beneficial

and may in fact worsen long-term renal and cardiovascular outcome[18-20]. Furthermore, in the RENAAL study the beneficial effects of ARB on the risk for end-stage renal disease and death were found to be maintained despite a simultaneous decrease in hemoglobin[14]. Hence, the decrease of erythropoietin and hemoglobin by dietary sodium restriction might reflect an improved condition in the kidney and may contribute to the long-term benefits of dietary sodium restriction on renal outcome in chronic renal disease[21].

What could be the mechanism of the reduction of erythropoietin by dietary sodium restriction in our renal patients? We found that dietary sodium restriction resulted in a reduction of absolute tubular sodium reabsorption, despite a rise in fractional tubular sodium reabsorption, due to a lower filtered sodium load by the combined effects of lower creatinine clearance and lower plasma sodium levels. Since tubular sodium reabsorption is the main determinant of tubular oxygen consumption, and the change of absolute tubular sodium reabsorption was paralleled by the change of erythropoietin during dietary sodium restriction, the reduction of erythropoietin presumably reflects an increase of renal oxygen tension by dietary sodium restriction[22-24]. Indeed, in other studies it has been shown that dietary sodium restriction reduces tubular oxygen consumption and increases renal oxygen tension[25,26].

Recent studies point to the role of renal hypoxia in the development and progression of both acute and chronic renal disease[23,27]. Hypoxia of tubulointerstitial cells leads to renal fibrosis with the loss of peritubular capillaries and subsequent chronic hypoxia, inducing a downward spiral that ultimately results in end-stage renal disease. It is tempting to speculate that this mechanism of a reduction in GFR leading to a reduction in filtered load, which subsequently causes a reduction in active tubular sodium reabsorption and hence a reduction in tubular work, may also explain why a short-term decrease in renal function at onset of therapy is associated with long-term preservation of renal function, during antihypertensive and antiproteinuric therapy with RAAS blockade or diuretics[28-30].

Our current finding that erythropoietin and hemoglobin levels are reduced by dietary sodium restriction during monotherapy ACE inhibition and during dual RAAS blockade, is somewhat at variance with our previous study where dietary sodium restriction added to ARB (losartan) did not affect erythropoietin or hemoglobin[31]. In the current study renal function was slightly more compromised than in the previous study, which might imply distinct sodium and oxygen handling and hence a distinct effect of interventions thereof. Apparently, the effect of sodium intake on erythropoietin levels is context dependent and cannot be generalized to other conditions.

As a limitation of our study it should be mentioned that we studied only net tubular sodium reabsorption and we do not have information on tubular sodium reabsorption of the separate tubular segments. Another limitation is that we did not directly measure renal oxygen tension: in this respect our study should be considered hypothesis generating.

To conclude, erythropoietin levels are reduced in proportion to absolute tubular sodium reabsorption by dietary sodium restriction on top of single and dual RAAS blockade in renal patients with mildly impaired renal function and overt proteinuria. These findings suggest that dietary sodium restriction may alleviate renal hypoxia in renal disease, which might partly explain the (long-term) benefits of dietary sodium restriction in renal patients.

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9

Vascular endothelial growth factor C levels are modulated by dietary salt intake in proteinuric chronic kidney disease patients and in healthy subjects

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Abstract

Background: Recent experimental findings demonstrate vascular endothelial growth factor C (VEGF-C) mediated water-free storage of salt in the interstitium, which prevents a salt-sensitive blood pressure state. It is unknown whether this mechanism plays a role in salt homeostasis and regulation of blood pressure in humans as well. Therefore, we investigated circulating VEGF-C levels and blood pressure during different well-controlled salt intakes in healthy subjects and in chronic kidney disease (CKD) patients.

Methods: In two cross-over studies, healthy subjects (n=31) and non-diabetic proteinuric CKD patients (n=32) were treated with consecutively a low sodium diet (LS, aim 50 mmol Na^+/d) and a high sodium diet (HS, aim 200 mmol Na^+/d) in random order, during two 1-week (healthy subjects) and two 6-week periods (CKD patients).

Results: We found that VEGF-C levels are higher during HS than during LS in CKD patients ($p=0.034$) with a trend towards higher VEGF-C in healthy subjects as well ($p=0.070$). In CKD patients, HS was associated with higher NT-proBNP levels ($p=0.005$) and body weight ($p=0.013$), consistent with ECV expansion, and with higher blood pressure ($p<0.001$), indicating salt-sensitivity. In healthy subjects, blood pressure was not affected by dietary salt ($p=0.14$), despite a rise in ECV ($p=0.023$).

Discussion: Our findings support a role for VEGF-C mediated salt homeostasis in humans. Considering the salt-sensitivity of blood pressure, this buffering mechanism appears to be insufficient in proteinuric CKD patients. Future studies are needed to proof causality, and to substantiate the clinical and therapeutic relevance of this VEGF-C mediated regulatory mechanism in humans.

Introduction

Classically, total body salt and extracellular volume (ECV) are thought to be closely linked and controlled by renal salt excretion and dietary salt intake only. Based on the assumption that extracellular body fluids are in equilibrium, excess interstitial salt is considered to be readily mobilized into the bloodstream for renal salt clearance. Blunted renal salt excretion in this concept results in ECV expansion, which can induce a rise in blood pressure, denoted as the salt-sensitivity of blood pressure[1;2]. In support of this concept we found that salt-sensitive healthy men have a higher ECV than salt-resistant men during high salt intake, but not during low salt intake[3].

However, recent experimental findings demonstrating water-free storage of salt, question our current understanding on internal environment composition and warrant novel insights into regulatory mechanisms for salt homeostasis[4-9]. Salt can be stored in a newly discovered subcutaneous interstitial compartment, by binding to polyanionic proteoglycans and glycosaminoglycans without commensurate water retention[10;11]. In response to salt-mediated interstitial osmotic stress, mononuclear phagocyte system cells secrete vascular endothelial growth factor C (VEGF-C), which stimulates lymphatic growth and endothelial nitric oxide synthase (eNOS) expression[12;13]. When this system is inhibited, high salt intake induces excess interstitial fluid and hypertension[4;5].

In patients with refractory hypertension, a condition which is eminently salt-sensitive[14;15], circulating VEGF-C levels were elevated compared to normotensive subjects[4], suggesting that this extrarenal regulatory mechanism might play a role in salt homeostasis and regulation of blood pressure in humans as well. If so, it can be hypothesized that circulating levels of VEGF-C respond to changes in salt intake, with higher VEGF-C levels during high salt intake. To test this hypothesis, we investigated circulating VEGF-C levels and blood pressure during steady state on different well-controlled salt intakes in two independent studies, in proteinuric chronic kidney disease (CKD) patients and in healthy volunteers, respectively.

Methods

This is a post-hoc analysis of two previous studies described in detail elsewhere [3;16].

CKD patients

For the current study, we used data and samples collected during placebo-treatment on high sodium (HS; target intake 200 mmol Na+/d) and low sodium diet (LS; target

intake 50 mmol Na+/d) from 32 non-diabetic proteinuric CKD patients (age 50±2 years, 73% men, all Caucasian, BMI 27±1 kg/m2). Mean achieved sodium intake was above target (90±10 mmol/d) and according to protocol (200±10 mmol/d) during LS and HS diet, respectively. Duration of the dietary interventions was two times 6 weeks, and the order was random. For 2 subjects from the original study, good quality samples were no longer available.

Healthy subjects

From the original 34 study subjects, samples of sufficient quality were available for 31 subjects (age 23±1, 100% men, all Caucasian, BMI 24±1 kg/m²). Data and samples were obtained after one week on a low sodium diet (LS; target intake 50 mmol Na^+/d) and after one week on a high sodium diet (HS; target intake 200 mmol Na^+/d), respectively, in random order. Mean achieved dietary sodium intake was below and above target values (34±11 mmol/d and 257±16 mmol/d, respectively) during LS and HS diet, respectively.

Measurements and calculations

At the end of each study period all participants collected 24h-urine and, after an overnight fast, blood pressure was measured and blood was sampled. Proteinuria was measured by the pyrogallol red-molybdate method. Dietary sodium intake was assessed from 24h urinary sodium excretion. Blood pressure was measured at 1-minute intervals by an automatic device (Dinamap®; GE Medical Systems, Milwaukee, WI), with the patient in semi-supine position. After fifteen minutes of measurements, the mean of the last four readings was used for further analysis. Plasma VEGF-C levels were measured by ELISA (R&D Systems, Germany). Intra- and interassay variation of the ELISA is 6.6% and 8.5%, respectively. The minimal detection level is 48.4 pg/mL. In the healthy subjects ECV was measured by the distribution volume of ^{125}-I-iothalamate as described previously[17].

Data analysis

Data are given as mean±SEM, or geometric mean [95%-confidence interval] when skewed. Before statistical testing, skewed variables were natural log-transformed to obtain normality. Comparisons between HS and LS were performed using paired T-tests. $P<0.05$ was considered statistically significant. SPSS 18.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Results

VEGF-C and general parameters in CKD patients

CKD patients had overt proteinuria, a slightly elevated blood pressure, and a rather preserved renal function (Table 1). Urinary sodium excretion, a measure of dietary sodium intake, was lower during LS than during HS. VEGF-C levels were significantly higher during HS than during LS (1228 [1024-1471] vs. 1004 [857-1177] pg/mL, respectively, p=0.034; Figure 1). NT-proBNP levels and body weight were also higher during HS than during LS, consistent with ECV expansion during HS. Blood pressure and proteinuria were higher during HS as well, indicating salt-sensitivity of blood pressure and proteinuria in CKD patients.

Table 1 General parameters in CKD patients

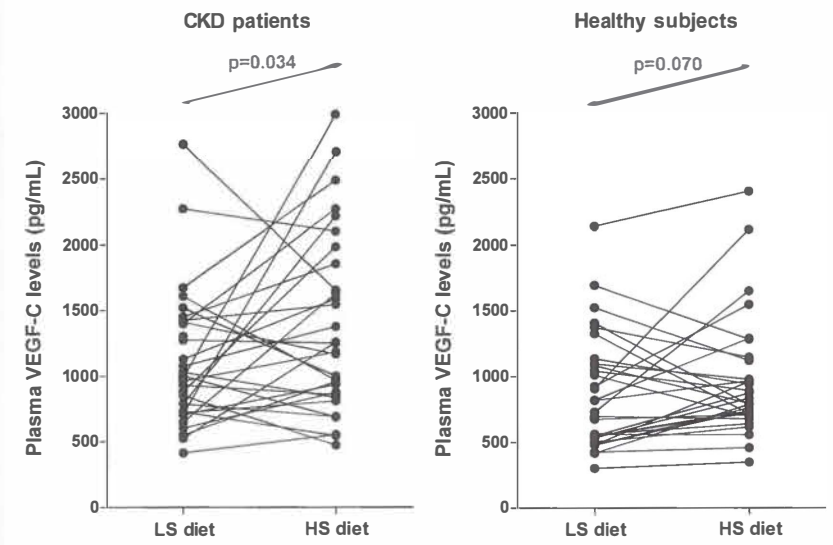
	LS	HS	P-value
Proteinuria – g/day	3.0±0.4	3.8±0.4	<0.001
Systolic blood pressure – mmHg	137±3	143±3	<0.001
Diastolic blood pressure – mmHg	83±1	86±2	0.004
Mean arterial pressure – mmHg	101±11	105±15	0.001
Creatinine clearance – mL/min	82±6	89±5	0.21
NT-proBNP – pg/mL	62 (41-93)	91 (60-137)	0.005
Body weight – kg	89±3	91±3	0.013
Plasma VEGF-C – pg/mL	1004 (857-1177)	1228 (1024-1471)	0.034
Plasma Na^+ – mmol/L	139.0±0.4	139.1±0.4	0.67
Urinary Na^+ excretion – mmol/day	90±10	200±10	<0.001

Abbreviations: LS, low sodium diet; HS, high sodium diet; VEGF-C, vascular endothelial growth factor C.

VEGF-C and general parameters in healthy subjects

As expected, the healthy subjects had normal blood pressure, normal renal function and no proteinuria (Table 2). Urinary sodium excretion was considerably lower during LS than during HS, indicating excellent dietary compliance. VEGF-C levels tended to be higher during HS than during LS, but the difference was not statistically significant (881 [758-1023] versus 773 [748-921] pg/mL, respectively, p=0.070; Figure 1). Assuming that VEGF-C distributes over the ECV, we calculated the total amount of VEGF-C as plasma VEGF-C levels times ECV. Total VEGF-C was higher during HS than during LS

Figure 1 VEGF-C levels in CKD patients and healthy subjects



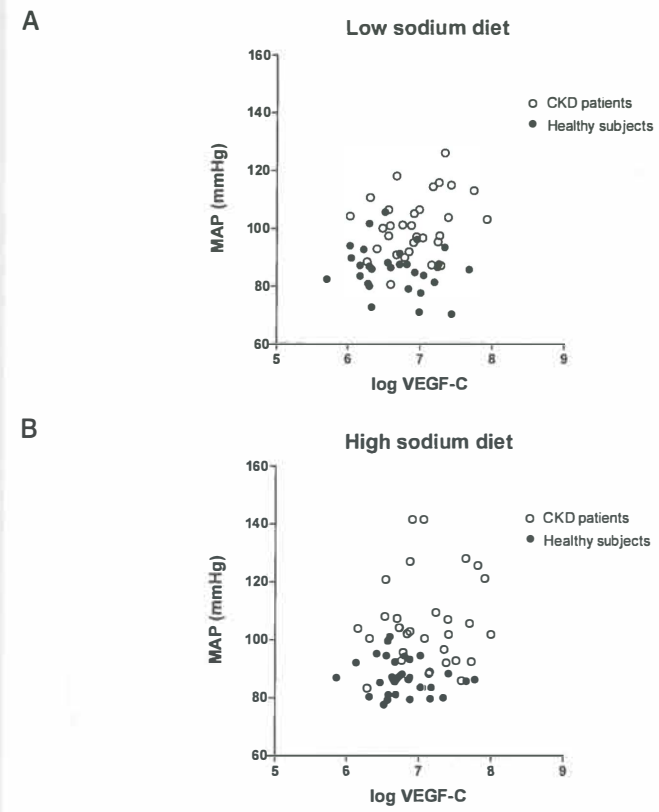
Abbreviations: VEGF-C, vascular endothelial growth factor C; LS, low sodium; HS, high sodium.

Table 2 General parameters in healthy subjects

	LS	HS	P-value
Proteinuria - g/day	<0.2	<0.2	-
Systolic blood pressure - mmHg	123±2	124±1	0.14
Diastolic blood pressure - mmHg	68±1	69±1	0.45
Mean arterial pressure - mmHg	86±8	87±7	0.25
Creatinine clearance - mL/min	103±5	123±5	0.003
NT-proBNP - pg/mL	14 (11-19)	26 (20-35)	0.002
Body weight - kg	80±2	82±2	<0.001
Extracellular volume - L	19.8±0.5	20.8±0.5	0.023
Plasma VEGF-C - pg/mL	773 (648-921)	881 (758-1023)	0.070
Total amount of VEGF-C - pg	14539 (1002-22751)	18176 (14320-26405)	0.016
Plasma Na^+ - mmol/L	138.5±0.4	139.8±0.4	0.001
Urinary Na^+ excretion - mmol/day	46±11	257±16	<0.001

Abbreviations: LS, low sodium diet; HS, high sodium diet; VEGF-C, vascular endothelial growth factor C.

Figure 2 Association between VEGF-C levels and MAP during low and high sodium diet



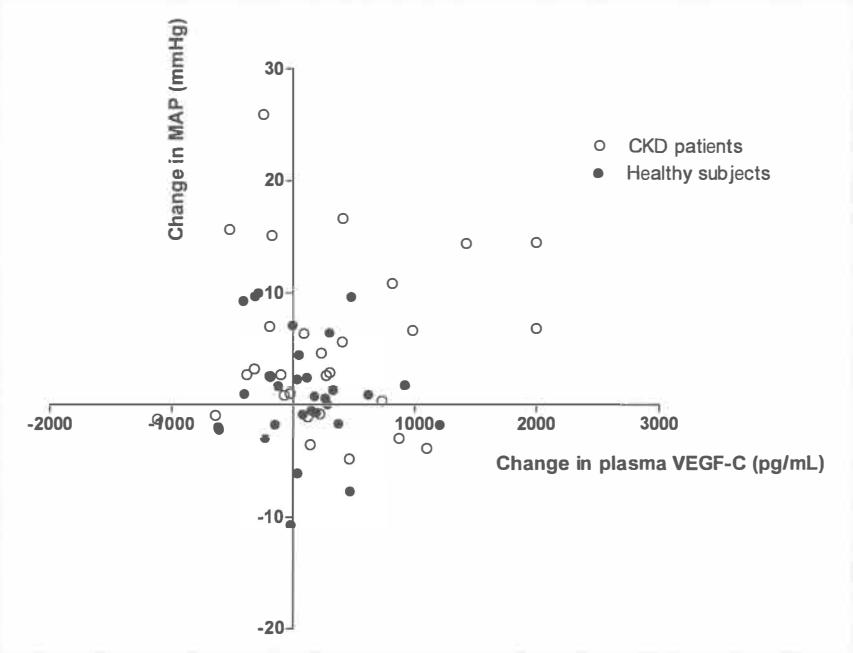
Abbreviations: LS, low sodium diet; HS, high sodium diet; VEGF-C, vascular endothelial growth factor C.

(18176 [14320-26405] versus 14539 [1002-22751] pg, respectively, p=0.016). In line with the higher ECV during HS, NT-proBNP levels, body weight, and creatinine clearance were also significantly higher during HS than during LS. Blood pressure in the healthy young men was not affected by dietary salt intake.

Individual values for blood pressure and VEGF-C during LS and HS in the CKD patients and the healthy subjects are given in figure 2. No significant correlation could be detected in the healthy subjects nor in the CKD patients. For the pooled data on either sodium intake, a borderline significant correlation was present (R^2=0.217, P=0.095 and

R^2=216, P=0.096 on LS and HS, respectively). However, the correlation disappeared after adjustment for population. The individual change in VEGF-C elicited by HS intake was not correlated with the change in MAP in either study population, separately or pooled (Figure 3). Furthermore, no significant associations were found between change in VEGF-C levels / total amount of VEGF-C and change in ECV, NT-proBNP, or body weight. VEGF-C levels, however, were significantly higher in CKD patients than in healthy subjects on either sodium intake (P=0.027 and P=0.006 on LS and HS, respectively).

Figure 3 Association between change in VEGF-C levels and change in MAP elicited by HS diet



Abbreviations: MAP, mean arterial pressure; VEGF-C, vascular endothelial growth factor C; HS, high sodium.

Discussion

We found that VEGF-C levels are modulated by salt intake in two different independent studies, with higher VEGF-C levels during high salt intake. First, in proteinuric CKD patients after two 6-week periods of dietary intervention, and second, in healthy subjects, after two 1-week periods of dietary intervention, albeit the latter of borderline statistical significance. In the CKD patients higher salt intake was associated with higher blood pressure, whereas in the healthy subjects the measured blood pressure was not affected by dietary salt, despite a rise in ECV.

Animal studies have found that during high salt diet the content and polyanionic character of glycosaminoglycans increase, accompanied by hypertonic salt storage in the ensuing reservoir tissue[7,18]. VEGF-C, which is secreted by mononuclear phagocyte system (MPS) cells in response to interstitial hypertonicity, induces eNOS expression by binding to VEGFR-2[12] and stimulates lymphangiogenesis by binding to VEGFR-3[13]. The resulting vasodilatory response and electrolyte removal from the interstitium prevents a salt-sensitive blood pressure state[4;5,19-21]. This non-osmotic VEGF-C-macrophage-lymphangiogenesis pathway may act alongside the osmotic storage of salt that translates into ECV excess. As currently no methods are established for investigation of salt storage in patient-oriented research, we can only speculate that dietary salt induces salt storage in specific reservoirs as well. However, the close association between changes in dietary salt intake followed by parallel changes in plasma VEGF-C levels supports the notion that changes in MPS-derived VEGF-C levels might serve as a clinical indicator for salt overload and salt storage in humans. We believe that this new research area warrants further investigation in patient-oriented research.

In our proteinuric CKD patients blood pressure increased during the high salt diet, in line with the well-established salt-sensitivity of blood pressure in CKD[22;23], and along with a rise in body weight and NT-proBNP, suggesting ECV expansion. Concomitantly, VEGF-C levels were increased, suggesting that high salt intake induces an extrarenal homeostatic pathway in these patients as well. This increase in VEGF-C was present despite the fact that during LS dietary sodium intake was substantially higher than the target of 50 mmol/d, thus limiting the difference with the HS period.

Animal data support a role for the VEGF-C-macrophage-lymphangiogenesis pathway in the protection against developing hypertension in response to a high sodium intake[5]. Furthermore, subjects with refractory hypertension show higher plasma VEGF-C levels than controls, suggesting that this pathway is relevant in the pathogenesis of human

hypertension as well[4]. In our study populations we did not find a between-individual correlation between levels of VEGF-C and blood pressure, or between the responses of VEGF-C and blood pressure to high sodium when analyzing for individual responses, neither in the separate populations, nor for pooled data. This could implicate either absence of an association, or complete protection against a sodium-induced rise in blood pressure by the adaptive response of the VEGF-C-macrophage-lymphangiogenesis pathway. Whereas we want to emphasize that a head-to-head comparison between the two populations should be interpreted with caution, due to differences in the experimental design and patient characteristics, nevertheless it is noteworthy that VEGF-C levels were higher in the CKD patients, i.e. in the population where blood pressure was sodium sensitive.

The mechanism for the higher VEGF-C levels in CKD patients is of interest, but cannot be derived with certainty from our data. The data are consistent with the assumption that in CKD patients VEGF-C is stimulated more than in healthy controls on a similar sodium intake, which can be hypothesized to reflect a less effective response to sodium intake and hence a persisting stimulus. Whether this is due to differences in osmotic storage, non-osmotic storage, or to blunted sodium excretion in CKD leading to difference in overall sodium balance cannot be ascertained from our data. However, the higher NT-proBNP levels in CKD on each sodium intake are consistent with a higher ECV and hence differences in overall balance and osmotically stored sodium in CKD patients.

The rise in blood pressure during high sodium in CKD patients suggests that the presumed extrarenal, MPS-driven regulatory mechanism is not sufficient to preclude a rise in blood pressure in response to high sodium. Of note, as VEGF-C reduces the permeability of the glomerular filtration barrier and promotes podocyte survival[24;25], an increase in VEGF-C is theoretically expected to reduce proteinuria, independently of blood pressure. At variance with this consideration, in our patients proteinuria increased during high salt, probably secondary to the rise in blood pressure.

In an independent study in healthy subjects VEGF-C levels were also increased by a 1-week period on high salt diet, with a concomitant rise in the extracellular volume and creatinine clearance, whereas blood pressure was salt-resistant. These data suggest that the MPS-driven VEGF-C-macrophage-lymphangiogenesis regulatory pathway, which is specific for local tissue salt storage, is stimulated by high salt intake, alongside the conventional renal osmotic pathways of salt homeostasis. The rise in creatinine clearance can be considered part of the integrative homeostatic response to high

sodium, and is considered instrumental in facilitating excretion of the excess sodium, and sodium resistance of blood pressure. This is consistent with our current observation of a rise in creatinine clearance in our, sodium resistant, healthy subjects, and a smaller, non-significant rise in creatinine clearance in our, sodium-sensitive, CKD patients. Of note, we previously demonstrated that the rise in GFR on high sodium closely corresponds to the rise in extracellular volume, i.e. the osmotic storage pathway, in healthy subjects[17].

Our data are the first to document an effect of salt intake on VEGF-C, a crucial step in the newly identified VEGF-c-macrophage-lymphangiogenesis pathway as an extrarenal homeostatic mechanism in the response to an increase in salt intake in humans, in a salt-sensitive as well as a salt-resistant condition. Unfortunately, we have no data on total body composition and salt content. Furthermore, it would be of great interest to directly monitor local interstitial changes in humans during dietary salt intervention in future research.

To conclude, VEGF-C levels are increased by high salt diet in proteinuric CKD patients and in healthy subjects, supporting a role for VEGF-C mediated interstitial regulatory mechanisms in salt homeostasis in humans. Considering the rise in blood pressure during high salt diet, this buffering mechanism for salt-sensitive hypertension appears to be insufficient in proteinuric CKD patients. Future studies should investigate the clinical relevance, the reasons for failure in CKD, and potential targets for intervention, of VEGF-C mediated interstitial electrolyte- and volume homeostasis in humans.

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Summary and General Discussion

In this thesis we aimed to systematically explore the effects of interventions in the RAAS and sodium status on classical and non-classical intermediate outcome parameters in non-diabetic CKD patients, to provide a rational basis for further improvement of renoprotective therapy in order to reduce long-term renal and cardiovascular risk.

Effects of interventions in the RAAS and sodium status on classical intermediate outcome parameters

Blockade of the RAAS by ACEi or ARB is currently recommended as first-line therapy for the reduction of blood pressure, proteinuria, and renal and cardiovascular risk in CKD patients[1-3]. To further reduce long-term renal and cardiovascular risk, the antiproteinuric and antihypertensive efficacy of RAAS-based regimens should be optimized. Different strategies are available that might improve the antiproteinuric and antihypertensive efficacy of treatment with ACEi or ARB[1].

Dual RAAS blockade with ACEi plus ARB reduces proteinuria and, sometimes, blood pressure more than ACEi or ARB monotherapy (**chapter 1 and 2**). Importantly, many dual blockade studies used doses of ACEi and ARB below the highest dose recommended for clinical practice[4,5]. Hence, the observed benefit of dual blockade might stem from the submaximal dose of monotherapy in these studies. Furthermore, increasing the drug dose beyond the top of the dose-response-curve for blood pressure can improve the antiproteinuric effect[6-8]. Applying dose-finding of ACEi or ARB monotherapy, therefore, might be as effective as combining ACEi with ARB. However, to our knowledge this approach has not been explicitly studied so far.

Moreover, the effects of dietary sodium restriction as compared to dual blockade as a tool to improve the therapeutic efficacy of RAAS blockade had not been investigated up to now. We found that addition of dietary sodium restriction to ACEi is considerably more effective than dual RAAS blockade with ACEi plus ARB for the reduction of proteinuria and blood pressure (**chapter 2**). Proteinuria is slightly further reduced when dietary sodium restriction is combined with dual RAAS blockade. Since ACEi and ARB were used in the highest dose recommended for clinical practice, the superiority of dietary sodium restriction cannot be attributed to submaximal dosing of dual RAAS blockade. Furthermore, as sodium intake was reduced from a level that equals the prevailing sodium intake in the renal and general population to a level conform current recommendations, the benefits of dietary sodium restriction in this study can be easily translated to clinical practice[1,9,10].

Not only dietary sodium restriction but also diuretics can potentiate the antiproteinuric and antihypertensive effect of ACEi or ARB, as was previously shown by us and others[11-13]. Interestingly, uptitration of diuretics on top of combined half doses of ACEi and ARB was recently found to reduce proteinuria more than uptitration of combined half doses of ACEi and ARB to combined full doses[14]. Furthermore, we previously

showed that the addition of combined dietary sodium restriction and diuretics to RAAS monotherapy is more effective than the addition of either alone[12]. Thus, in individual patients with insufficient antiproteinuric or antihypertensive response to dual RAAS blockade combined with dietary sodium restriction, the response could probably be improved by addition of diuretics.

Yet, we found that a stronger reduction of proteinuria and blood pressure by intensified RAAS-based therapy is accompanied by adjunct effects, including an increased prevalence of hyperkalemia, orthostatic complaints, and short-term renal function decline (**chapter 1, 2 and 4**). This could question the safety and tolerability of these regimens. However, these studies, investigating short-term treatment only, do not provide the most suitable set-up to assess side effects and tolerability, and it would be important in this respect to consider data from studies with long-term treatment.

So far, studies from the literature demonstrated an U-shaped relationship between plasma potassium and outcome in CKD patients, with a higher risk for ESRD and death at K^+<4.0 mmol/L and a higher risk for cardiovascular events and death at K^+>5.5 mmol/L[15,16]. Hence, an increase in potassium could be beneficial in patients with initial K^+<4.0 mmol/L (which was the case in 15% of our patients in **chapter 2**), but a potential threat in patients with initial K^+>5.5 mmol/L (which was the case in 6% of our patients), and at any rate requires careful monitoring.

A decline in renal function shortly after the institution of antihypertensive and antiproteinuric therapy is a well-recognized phenomenon in CKD patients[17,18]. It is reversible upon withdrawal of therapy, as was previously shown for ACEi, and probably reflects a (reversible) fall in glomerular pressure rather than (irreversible) structural renal damage[19]. In **chapter 4** we demonstrated that this also applies to the addition of diuretics to RAAS blockade. There is no evidence that such an effect is harmful. On the contrary, a therapy-induced short term fall in renal function has been consistently shown to predict a slower long-term renal function decline[17;18;20].

Orthostatic complaints can reduce patients' wellbeing and presumably also their overall compliance with therapy. We do not know whether orthostatic complaints predict a worse outcome. However, if they reflect symptomatic hypotension they might be associated with renal hypoperfusion. If so, it is conceivable that renal hypoxia and tubulointerstitial injury might occur despite a stronger reduction of proteinuria and blood pressure, as has been observed in experimental nephrosis[21]. Reduction of blood pressure up to very low levels appears to be paralleled by accelerated renal function

loss in CKD patients[3;22]. Likewise, results from a recent trial in patients with vascular disease or high-risk diabetes suggest that forced titration of dual RAAS blockade with ACEi plus ARB, which was paralleled by a further reduction of blood pressure and microalbuminuria but also by an increased prevalence of hypotensive symptoms, might be associated with a worse renal outcome[23;24]. Based on the data that are currently available we feel that orthostatic hypotension should be avoided, through careful uptitration and if required downtitration of RAAS blockade and sodium targeting.

All in all, to improve the antiproteinuric and antihypertensive effects of RAAS-based regimens the institution of dietary sodium restriction up to levels recommended in current guidelines should be prioritized. Probably this strategy is even more effective in patients with elevated NT-proBNP levels, since we found that elevated NT-proBNP levels predict a larger reduction of blood pressure and proteinuria by sodium targeting, but not by RAAS blockade, during the different steps of the titration regimen in CKD patients (**chapter 3**).

Effects of interventions in the RAAS and sodium status on non-classical intermediate outcome parameters

RAAS blockade and sodium targeting influence intrarenal pathways of damage both dependent and independent of blood pressure and proteinuria[25-27]. Consequently, the benefits of blood pressure and proteinuria reduction can be augmented or counteracted by treatment effects on intrarenal pathways of damage. In line with this, monitoring of therapy effects beyond blood pressure and proteinuria, better reflecting the impact of therapy on intrarenal pathways of damage, is warranted[28]. This could contribute to defining the optimal levels of reduction of blood pressure and proteinuria, as the latter are still a matter for investigation[22;29].

Urinary tubular injury markers correlate with tubulo-interstitial injury and predict renal outcome in CKD patients[30;31]. We found that proximal and distal tubular injury markers, but not tubular inflammation markers, positively correlate with proteinuria, and are reduced along with further proteinuria reduction by combinations of ACEi, ARB, and dietary sodium restriction (**chapter 5**).
Interestingly, we found that the lowest levels of proximal and distal tubular injury markers are achieved when proteinuria falls below 0.3 g/day. Although we cannot prove causality, these data may suggest that the current target of proteinuria reduction up to levels below 1.0 g/day might be too liberal, and that titration of proteinuria up to levels below

0.3 g/day is needed for optimal renoprotection. This is in line with previous data suggesting that proteinuria reduction up to levels below 0.3 g/day is associated with a better long-term renal outcome[32,33]. Long-term prospective intervention studies are however needed to confirm this.

For CTGF, a mediator of fibrogenesis[34;35], results comparable to those on tubular injury markers were found. Urinary CTGF is lowered by stepwise antiproteinuric intervention with dietary sodium restriction, ARB, and diuretics, in proportion to the reduction of proteinuria, with normalization of urinary CTGF during triple therapy (**chapter 6**). In contrast, plasma CTGF is not affected by these therapies.

Apparently, tubular inflammation and the systemic profibrotic state in (these) CKD patients may not be entirely proteinuria-driven, in line with the known residual renal and cardiovascular risk despite reduction of blood pressure and proteinuria[36-38], and a different (intensified, extended, or specifically anti-inflammatory) treatment regimen is needed.

Thus, the benefits of proteinuria and blood pressure reduction by RAAS blockade and sodium targeting are presumably mediated or augmented by an accompanying reduction of renal (fibrotic) injury, but their impact on long-term renal and cardiovascular outcome might be hampered by ongoing tubular inflammation and a systemic profibrotic state.

We found that diuretics added to ARB decrease erythropoietin and hemoglobin levels in CKD patients (**chapter 7**). In line with previous data, we hypothesized that erythropoietin reduction by add-on diuretics is caused by a decrease in renal oxygen requirement, which is the main stimulus for erythropoietin production, due to the inhibition of active tubular sodium reabsorption by diuretics[39-41].

Additionally we found that addition of dietary sodium restriction to ACEi reduces erythropoietin and hemoglobin levels, proportionate to the reduction of tubular sodium reabsorption by this intervention (**chapter 8**). Since tubular sodium reabsorption is the main determinant of tubular oxygen consumption, the reduction of erythropoietin might reflect an increase of renal oxygen tension by dietary sodium restriction. Indeed, other studies showed that dietary sodium restriction reduces tubular oxygen consumption and increases renal oxygen tension[42;43].

Addition of dietary sodium restriction or diuretics to RAAS blockade might therefore alleviate renal hypoxia by reducing the tubular work load that is elicited by active tubular sodium reabsorption.

So, together with the reduction of proteinuria and blood pressure, reduction of renal hypoxia might contribute to a favorable effect of sodium targeting on long-term renal and cardiovascular outcome.

Sodium excess blunts the antihypertensive and antiproteinuric response to ACEi and/or ARB (**chapter 2**)[11,12,44]. This warrants better understanding of the (patho)physiology of sodium status. Recently a VEGF-C mediated extrarenal mechanism of sodium and blood pressure homeostasis was discovered, involving subcutaneous non-osmotic sodium storage[45-47]. We carried this concept to humans, and found that excessive sodium intake enhances circulating VEGF-C levels, along with the conventional renal osmotic mechanisms of sodium homeostasis, in proteinuric CKD patients and healthy subjects (**chapter 9**). Considering the rise in blood pressure (and proteinuria) during excessive sodium intake in CKD patients but not in healthy subjects, the presumed VEGF-C mediated buffer mechanism appears to be insufficient to prevent a sodium-sensitive blood pressure state in CKD patients. It would be of great interest to further explore the contribution of (deficient) non-osmotic sodium storage to sodium excess, hypertension, and proteinuria in CKD patients.

Effects of interventions in the RAAS and sodium status on long-term outcome

In this thesis we compared the short-term effects of different strategies to improve the efficacy of ACEi or ARB, i.e. after 6 weeks of treatment. Although the intermediate outcome parameters that we studied are associated with long-term renal and cardiovascular outcome, they can by no means replace hard endpoints such as ESRD, cardiovascular events, or mortality. Hence, it would be important to substantiate the effects of improved short-term therapy response on hard endpoints as well.

Although dual RAAS blockade with ACEi plus ARB is widely used in clinical practice, the only study on the long-term effects of dual RAAS blockade in CKD patients has turned out unreliable[48,49]. The only data on the long-term renal effects of dual RAAS blockade with ACEi plus ARB that are currently available, come from a clinical trial in patients with vascular disease or high-risk diabetes, showing that forced titration of dual RAAS blockade is associated with a worse renal outcome[23,24]. In this trial additional reduction of blood pressure and microalbuminuria by dual RAAS blockade was found, but also an increased prevalence of hypotensive symptoms, which might explain (probably through renal hypoperfusion) the excess of acute renal failure. However, this trial was not suitable to study renal endpoints, as the included patients had a low renal risk and there was a disputable choice of the renal endpoint (which included acute dialysis). Clearly, the results cannot be generalized to (proteinuric) CKD patients[50]. The results of ongoing studies on the effects of ACEi plus ARB on hard endpoints in CKD patients, such as LIRICO[51] and VA NEPHRON-D[52], are being awaited.

Dietary sodium restriction has been shown to decrease renal function decline, cardiovascular events, and mortality[53-58]. Furthermore, a collaborative study by the REIN investigators[59] and our own group recently showed that during ACEi a lower dietary sodium intake is associated with lower proteinuria and less progression to ESRD in CKD patients (Vegter S et al, provisionally accepted for JASN, oral presentation during ASN Renal Week 2010). Likewise, we recently showed that during ARB a lower dietary sodium intake is associated with lower proteinuria, less progression to ESRD, and fewer cardiovascular events in CKD patients (Lambers Heerspink HJ et al, submitted to JASN, oral presentation during ASN Renal Week 2011). Albeit retrospective, these data are the first to document an association of sodium status with hard renal endpoints. Evidently, these findings provide a strong rationale for a prospective intervention study on dietary sodium intake, against a background of RAAS blockade, to improve long-term renal and cardiovascular outcome in terms of hard endpoints in CKD patients.
Of note, the amount of sodium intake associated with a more favourable long-term outcome is not excessively low, both with respect to spontaneous intake and after intervention, and corresponds to the level of sodium restriction that is currently recommended[1;10]. This implicates that general efforts to implement the current guidelines on sodium intake will have the potential to greatly improve long-term renal and cardiovascular outcome in CKD patients.

Diuretics have also been found to reduce ESRD, cardiovascular events, and mortality[60-64], although studies in true CKD populations are scarce. Although additional studies on the long-term benefits of (add-on) diuretics in CKD patients are required, the data as currently available favors diuretic therapy in CKD.

General conclusion: optimal treatment regimen

Based on our findings we conclude that the addition of sodium targeting, i.e. dietary sodium restriction with or without diuretics, to ACEi or ARB, appears the optimal strategy to further improve proteinuria, blood pressure, tubular (fibrotic) injury, and perhaps also renal hypoxia, in order to prevent progression of CKD and its complications in non-diabetic proteinuric CKD patients. Dual blockade with ACEi plus ARB should not be a standard approach in the management of CKD, but might perhaps be useful in patients in whom overt proteinuria persists despite monotherapy with ACEi or ARB combined with adequate sodium targeting, although the long-term benefits of this strategy remain to be proven.

Future perspectives

In spite of general awareness of the deleterious effects of dietary sodium excess over the past years[57,65,66], there seemed to be a lack of interest in sodium status as a target for intervention in clinical nephrology[67]. Sodium status was seldom monitored, as illustrated by the lack of data on 24h-urinary sodium excretion in many trials and by the observation that dietary sodium intake in CKD patients considerably exceeds the current recommendations[9,68]. Achievement of persistent dietary sodium restriction, even when moderate, requires substantial effort. Our findings support the combined endeavours of patients, health professionals, and governments to accomplish adequate sodium restriction for optimal renal and cardiovascular protection.

There is evidence that dietary sodium restriction might be relevant as a primary preventive measure. Experimental research showed that excessive sodium intake in early childhood induces alterations in proximal tubular sodium pumps, which is associated with increased sodium reabsorption and hypertension in adulthood[69]. Furthermore, maternal excessive sodium intake during pregnancy was found to induce fetal programming of hypertension and cardiovascular disease through among others increased expression of the angiotensin II receptor AT1R[70]. Hence, there may be a link between early life style and late cardiovascular (and perhaps renal) injury. Together with the rise in sodium intake in children that has been documented over the last few years[71,72], this suggests that early intervention in sodium intake is warranted for optimal cardiovascular (and renal) outcome. However, the long-term benefits of measures aimed at primary prevention are obviously difficult to substantiate.

Concerning the optimal intensity of blood pressure reduction, it was found that if blood pressure is reduced up to very low levels renal function loss may accelerate, even when a further reduction of proteinuria is achieved[3,21,22]. Hence, in patients with a relatively low blood pressure the intensification of antiproteinuric therapy may be limited by its antihypertensive effects, and strategies that reduce proteinuria with no/minor effect on blood pressure are required. Addition of oral vitamin D, on top of RAAS blockade and sodium targeting, might be a potential strategy in this respect, and is currently being studied by our group in the VIRTUE study (clinical trial number NTR2898).
The specific mode of RAAS blockade may also be relevant. It has been pointed out that aldosterone escape and a reactive rise in renin may limit the benefits of ACEi, ARB, and sodium targeting (**chapter 1 and 5**). Possibly, regimens including aldosterone blockade or renin inhibition might possess a more favourable profile in this respect[73-75]. This is currently being addressed for aldosterone blockade combined with ACEi and sodium

targeting in the ESCAPE study (clinical trial number NTR2133), and for renin inhibition combined with ACEi and sodium targeting in the ARIA study (clinical trial number NTR10325), by our group.

Finally, it would be of great interest to see whether the beneficial effects of sodium targeting on top of RAAS blockade also apply to patients with diabetic nephropathy, since diabetes mellitus is a main cause for CKD worldwide[76;77]. This issue is currently being studied by our group in the DINAMO study (clinical trial number NTR2366).

The results of these trials, which become available within the next two years, will contribute to further improvement of renoprotective therapy in CKD, optimizing outcome by a rational combination of pharmacological intervention and dietary measures.

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Nederlandse Samenvatting

Minder zout, minder pillen, minder dialyse

Achtergronden

Chronische nierziekte is een veel voorkomende aandoening (circa 13% van de totale bevolking), die door het stijgende aantal mensen met een hoge bloeddruk (*hypertensie*), overgewicht en/of suikerziekte bovendien verder toeneemt. Chronische nierziekte gaat gepaard met voortschrijdende achteruitgang van de nierfunctie waardoor patiënten afhankelijk kunnen worden van nierfunctievervangende therapie (dialyse of niertransplantatie). Daarnaast wordt chronische nierziekte gekenmerkt door een toegenomen ziektelast en oversterfte door hart- en vaatziekten.

Als gevolg van nierziekte ontstaan dikwijls hypertensie en eiwitverlies via de urine (*proteïnurie*), waardoor de nierschade verergert. Hypertensie en proteïnurie worden veelal onderhouden door buitensporige activatie van het RAAS hormoonsysteem (*Renine Angiotensine Aldosteron Systeem*) en door overmatig vasthouden van natriumchloride (*zoutretentie*).

De behandeling van chronische nierziekte is erop gericht voortschrijdend achteruitgang van de nierfunctie en hart- en vaatziekten te voorkomen. Centraal bij deze behandeling staan de reductie van proteïnurie (streefwaarde: minder dan 1.0 gram proteïnurie per dag) en hypertensie (streefwaarde: bloeddruk lager dan 130/80 mmHg, of lager dan 125/75 mmHg indien er meer dan 1.0 gram proteinurie per dag is).

De behandeling van eerste keuze is blokkade van het RAAS met behulp van bepaalde medicijnen, namelijk ACEi (*Angiotensine Converterend Enzym inhibitoren*) of ARB (*Angiotensine Receptor Blokkers*). Helaas blijven ondanks deze behandeling de proteïnurie en bloeddruk vaak ruim boven de streefwaarden en treden bij veel patiënten nog steeds voortschrijdende achteruitgang van de nierfunctie en hart- en vaatziekten op.

Om de lange termijn prognose van chronische nierpatiënten te kunnen verbeteren onderzochten wij in dit proefschrift of proteïnurie en hypertensie beter kunnen worden gereduceerd door intensievere RAAS blokkade en/of door het corrigeren van zoutretentie met behulp van een zoutbeperkt dieet en zoutafdrijvende medicatie (*diuretica*). Ook gingen we na of er verschillen zijn in effectiviteit tussen intensivering van RAAS blokkade en het corrigeren van zoutretentie.

Daarnaast onderzochten wij welke effect dergelijke geïntensiveerde behandelingen hebben op de beschadiging van de nierbuisjes (*tubuli*), de vorming van littekenweefsel

(*fibrose*) in de nieren en bloedvaten, en de aanmaak van e*rythropoietine* (een hormoon dat de vorming van rode bloedcellen stimuleert) door de nieren. Intensieve behandelingen zouden namelijk ook nierschade kunnen remmen of eventueel kunnen verergeren (als ongewenst bijwerking) via hun invloed op de hierboven genoemde processen.

Resultaten

Uit ons onderzoek blijkt dat geïntensiveerde behandeling bestaande uit het toevoegen van ARB aan ACEi (*duale RAAS blokkade*) proteïnurie wel enigszins verlaagt, maar geen effect heeft op de bloeddruk. Het toevoegen van een zoutbeperking daarentegen leidt tot een veel grotere afname van proteïnurie en ook een aanzienlijke daling van de bloeddruk. Het toevoegen van zowel ARB als een zoutbeperkt dieet aan ACEi verlaagt proteïnurie nog een klein beetje verder.

Opmerkelijk genoeg werden deze uitgesproken gunstige effecten bereikt met een relatief milde zoutbeperking, bestaande uit het gebruiken van de hoeveelheid zout die ook wordt aangeraden in de Richtlijn Gezonde Voeding voor de algemene bevolking, van het Ministerie voor Volksgezondheid, Welzijn en Sport (namelijk circa 6 gram keukenzout per dag). De 'beperking' bestaat dus in feite uit het vermijden van overmatig zoutgebruik.

Bij het voorschrijven van zoutbeperking en/of diuretica is het in de klinische praktijk nuttig om een schatting te kunnen maken van de mate van zoutretentie, om de intensiteit van de behandeling daarop te kunnen afstemmen. Uit ons onderzoek blijkt dat een verhoogde concentratie NT-proBNP (*N-terminal pro-Brain Natriuretic Peptide*; een merkerstof van onder andere de vochthuishouding in het lichaam) in het bloed voorspelt of patiënten extra baat hebben bij de toevoeging van een zoutbeperkt dieet en diuretica om de bloeddruk en proteïnurie te verlagen. Mogelijk kan het NT-proBNP gehalte dus behulpzaam zijn bij het instellen van therapie-op-maat bij de individuele patiënt.

Daarnaast vonden wij dat een geïntensiveerde behandeling een viertal merkerstoffen van beschadiging van tubuli (*N-Acetyl- -Glucosaminidase, Kidney Injury Molecule-1*, *2-Microglobuline* en *Heart-type Fatty Acid-Binding Protein*) verlaagt. Het gehalte van deze merkerstoffen is het laagst wanneer de proteïnurie daalt tot <0.3 gram/dag. Mogelijk zou de streefwaarde van proteïnurie moeten worden teruggebracht van <1.0 gram/dag naar <0.3 gram/dag om tubulaire schade zoveel mogelijk te voorkomen. Aanvullend onderzoek is echter nodig om deze theorie te bevestigen.

Een merkerstof die te maken heeft met de vorming van fibrose in de nieren (*Connective Tissue Growth Factor*) wordt eveneens verlaagd door een geïntensiveerde behandeling. Ook deze merkerstof neemt gradueel af naarmate de proteïnurie verder daalt.

Een tweetal merkerstoffen van ontsteking van tubuli (*Neutrophil Gelatinase-Associated Lipocalin* en *Monocyte Chemoattractant Protein-1*) blijkt niet noemenswaardig te worden geremd door een geïntensiveerde behandeling. Ook de concentratie van Connective Tissue Growth Factor in het bloed, als maat voor de vorming van fibrose in de bloedvaten, wordt niet verlaagd. Verder onderzoek is nodig om een behandelregime te kunnen identificeren dat tubulaire ontsteking en littekenvorming in de bloedvaten remt, bijvoorbeeld een behandelregime dat nog intensiever is, langer aanhoudt, of meer specifiek gericht is tegen ontsteking en littekenvorming.

Het gehalte aan erythropoietine blijkt te worden verlaagd door een geïntensiveerde behandeling. Waarschijnlijk wordt dit veroorzaakt door afname van zuurstoftekort in de nieren, en is de verlaging van erythropoietine door deze behandeling dus een gunstig teken. Een ander onderzoek van onze researchgroep laat ook zien dat een afgenomen erythropoietine gehalte in nierpatiënten gepaard gaat met een betere prognose.

Lange termijn effecten

Dit proefschrift omvat onderzoek naar de effecten van geïntensiveerde antiproteinurische en antihypertensieve behandelingen op korte termijn. Hieruit kwam onder andere naar voren dat het vermijden van overmatig zoutgebruik, als extra maatregel naast de standaardbehandeling met ACEi, de proteïnurie, hypertensie, merkerstoffen van tubulaire schade en nierfibrose, evenals het erythropoietine gehalte, beter verlaagt dan het toevoegen van ARB aan ACEi (*duale RAAS blokkade*). Andere recent onderzoek van onze researchgroep toonde dat het toevoegen van een zoutbeperkt dieet aan ACEi óók op de lange termijn de nieren beschermt, en daadwerkelijk leidt tot uitstel van de noodzaak tot dialyse. De lange termijn effecten van duale RAAS blokkade zijn nog onbekend.

Advies voor de praktijk

Op basis van bovenstaande bevindingen concluderen wij dat het vermijden van overmatig zoutgebruik, eventueel aangevuld met diuretica, de optimale strategie is om de standaardbehandeling met ACEi of ARB te intensiveren. Het toepassen van duale RAAS blokkade (ACEi plus ARB) is minder effectief, maar kan als aanvullende maatregel soms overwogen worden in individuele patiënten. Onze bevindingen laten zien dat maatregelen gericht op een gezonde leefstijl essentieel zijn voor het bereiken van een

adequaat behandeleffect bij nierpatiënten. Een relatief milde reductie van de zoutinname leidt al tot aanzienlijke gezondheidswinst. Dit rechtvaardigt extra inzet om nierpatiënten te ondersteunen bij het bereiken en volhouden van een gezonde leefstijl, ter bescherming tegen voortschrijdende schade aan nieren, hart en bloedvaten. Minder zout, minder pillen, minder dialyse!

Dankwoord

Dankwoord

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