Chapter 7

Effect of combining ACE inhibition with aldosterone blockade on proteinuria and renal damage in experimental nephrosis

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ABSTRACT
Aldosterone has pro-fibrotic properties and is therefore a potential target for additional intervention in patients with chronic renal disease that show therapy resistance during treatment with ACE-inhibitors (ACEi). Combining ACEi and aldosterone receptor blockade (aldoRB) in proteinuric renal disease reduces proteinuria, but the effects on proteinuria-induced renal damage are unknown. We studied the effect of ACEi/aldoRB on proteinuria and renal damage in adriamycin nephrosis, a model of chronic proteinuria-induced renal damage. Six weeks after injection of adriamycin in Wistar rats, randomized treatment with vehicle (n=8), aldoRB (n=12), ACEi (n=10) or a combination of ACEi/aldoRB (n=14) was given for 12 weeks. Healthy rats served as controls (n=6). Renal damage was quantified by markers of tubular injury (osteopontin and Kidney injury molecule-1 (Kim-1)), pre-fibrotic lesions (α-smooth muscle actin (α-SMA)), interstitial fibrosis and focal glomerulosclerosis (FGS). In all adriamycin animals proteinuria was increased compared to controls. ACEi and ACEi/aldoRB significantly reduced proteinuria compared to vehicle, whereas aldoRB monotherapy was without effect. Blood pressure was reduced in ACEi and ACEi/aldoRB compared to VEH and aldoRB. Osteopontin and Kim-1 were increased in adriamycin animals, but significantly reduced by ACEi/aldoRB only. Treatment with ACEi and ACEi/aldoRB prevented an increase of α-SMA, interstitial fibrosis and FGS. In conclusion, ACEi/aldoRB effectively reduced proteinuria, markers of tubular injury and prevented renal damage in this rat model of chronic proteinuria-induced renal damage. Whether the diuretic action of aldoRB added to ACEi, or a specific antifibrotic effect of spironolactone is responsible for the observed effects should be investigated in further studies.
INTRODUCTION

Blockade of the renin-angiotensin system (RAS) is currently the first choice therapy for chronic renal disease. However, in some patients RAS blockade by Angiotensin Converting Enzyme inhibition (ACEi) or Angiotensin II type 1 antagonists (AT1A) as such is not sufficiently effective in reducing proteinuria. This therapy resistance results in ongoing renal function loss and, consequently, a growing incidence of patients with end-stage renal disease[1;2]. This therapy resistance can partly be overcome by the combination of ACEi and AT1A or by adding diuretics or a low sodium diet to RAS blockade. These combination strategies aim at preventing the fibrotic actions of angiotensin II. However, angiotensin II is not the only effector hormone of the RAAS. The mineralocorticoid hormone aldosterone, that is stimulated by angiotensin II, is involved in sodium and potassium homeostasis, but has profibrotic properties as well [3;4]. Whereas ACEi can reduce aldosterone, however, the suppression is not complete, especially during low sodium intake [5], and during long term ACEi aldosterone escape may contribute to therapy resistance. [6].

In experimental models of hypertensive renal damage aldosterone receptor blockade (aldoRB) reduces glomerulosclerosis and proteinuria, supporting the importance of aldosterone in renal damage [7-9]. Not only hypertension, however, is important in chronic progressive renal function loss, proteinuria is crucially involved as well. No data, however, are available so far in models of proteinuria-induced renal damage. Studies in proteinuric patients showed that addition of the aldoRB spironolactone to an ACE inhibitor could further reduce proteinuria [10-12]. However, these were short-term studies, and no data on renal structural damage are available.

Therefore, we studied the effect of combining ACEi (lisinopril) and aldoRB (spironolactone) in adriamycin nephrosis, an experimental model of chronic proteinuria-induced renal damage on clinical parameters and on renal damage, such as tubular injury (osteopontin and Kidney injury molecule-1, (Kim-1)), interstitial pre-fibrotic changes (macrophage influx, α-smooth muscle actin), interstitial fibrosis and focal glomerulosclerosis.

METHODS

Animals

Fifty male Wistar rats (HsdCpb: Wu; Harlan Inc, Zeist, The Netherlands) weighing 250 to 275 grams were used. All experimental procedures were approved by the Committee for Animal Experiments of the University of Groningen. The animals were housed in a temperature-controlled room with a 12 hour light-dark cycle. Animals were allowed to adjust 1 week after arrival and had free access to food and water during the entire study. All surgical procedures took place under isoflurane anesthesia in N₂O/oxygen (1:2).
Experimental design

Adriamycin nephrosis (AN) was induced by injection of 1.75 mg/kg adriamycin (Doxorubicin®) into the tail vein (n=44). After 6 weeks rats were stratified according to proteinuria in 4 groups and received a treatment for 12 weeks. The first group was a vehicle group, receiving normal drinking water (VEH, n=8). The second group was treated with the aldosterone-receptor blocker spironolactone (SPIR, n=12). The third group received the ACEi lisinopril (LIS, n=10). The fourth group received a combination of ACEi and aldoRB (LIS/SPIR, n=14). Healthy rats that did not receive adriamycin injection, served as time controls (CON, n=6). Spironolactone was provided by a 60-day slow release subcutaneous pellet (Innovative Research of America, Sarasota, FL, USA), dosing 3.3 mg/day. The pellet was placed in the lateral side of the neck in a pocket made with an incision and forceps. After 8 weeks (at week 14) a second pellet was placed for the remaining 4 weeks, average dosing of spironolactone was 8 mg/kg/day. Lisinopril was provided in drinking water (75 mg/L, Merck & Co, Rahway, NJ, USA), average lisinopril intake was 5 mg/kg/day. At the end of the study blood was collected by cannulation of the abdominal aorta, kidneys were perfused in situ with saline and removed. From the left kidney 2 sections were snap-frozen in liquid nitrogen and stored at -80°C for molecular analysis. From the right kidney one slice was fixed in 4% paraformaldehyde, another one was fixed in methacarn.

Clinical parameters

During the study systolic blood pressure (SBP) was measured weekly in trained conscious rats using tail-cuff plethysmography (Apollo 179, IITC Life Science, Woodland Hills, CA, USA). 24-Hour urine was collected 2-weekly in metabolic cages with measurement of food and water intake. Urinary protein excretion was measured by the pyrogallol red molybdate method [13]. Concentrations of creatinine, urea, sodium and potassium were all analyzed on a multi-test analyzer system (Merck Mega, Darmstadt, Germany) with Ecoline ® MEGA reagents (Diasys Diagnostic Systems, Holzheim, Germany). Creatinine concentrations in urine and serum were determined with the Jaffé method. Serum values of urea were determined with the urease-GLDH method and concentrations of potassium and sodium were measured with indirect potentiometry.

RNA isolation and quantitative PCR for osteopontin and KIM-1 expression

qPCR analysis based on the TaqMan methodology was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of the primers and probe for GAPDH mRNA: forward: 5' - GAA TAT CAT CCC TGC ATC CA-3'; reverse: 5' - CCA GTG AGC TTC CCG TTC A-3'; probe: 5' - CTT GCC CAC AGC CTT GGC AGC-3'. The Taqman probes were labelled at the 5'-end with a reporter fluorochrome (FAM) and at the 3'-end with a quencher fluorochrome (TAMRA). Osteopontin and KIM-1 gene-specific
Taqman probe and primer sets were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD IDs were: osteopontin (Spp1) Rn 00563571 m1 and Kim-1 Rn 00597703 m1.

Total RNA was extracted using the Trizol method (Invitrogen, Carlsbad, CA, USA). DNase treatment was performed using Turbo DNA-free (Ambion, Austin, TX, USA). cDNA was synthesized from 200 ng of total cellular RNA by First Strand cDNA Synthesis System and Superscript II RT (Invitrogen) using random hexamers in a volume of 20 µl and further diluted to a concentration of 2 ng/µL. The qPCR reaction mixture contained 5 µl cDNA, 10 µl 2X TaqMan Universal PCR Master Mix (Eurogentec, Seraing, Belgium). For GAPDH 900 nmol/L of each primer and 200 nmol/L probe in a total reaction volume of 20 µl was added. For osteopontin and Kim-1 1 µl 20* AOD Gene Expression Assay Mix was added to the 5 µl cDNA and 10 µl mastermix, nuclease free water was added to get a total reaction volume of 20 µl. All assays were performed in triplicate. Reaction tubes without template cDNA served as negative controls. The PCR plate was incubated for 2 minutes at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The Cₜ (threshold cycle) is inversely proportional to the logarithmic scale of the starting quantity of template cDNA. Consequently, the gene dosage was deduced by calculating the difference in Cₜ from the Cₜ of the reference gene GAPDH. The average Cₜ values for target genes were subtracted from the average housekeeping gene Cₜ values to yield the ΔCₜ. Results were finally expressed as 2⁻ΔCₜ which is an index of the relative amount of gene expression.

**Histochemical staining and quantification**

Paraffin embedded methacarn-fixed sections were dewaxed with xylene and stained with Periodic-Acid-Schiff to evaluate focal glomerulosclerosis (FGS) and interstitial fibrosis (IF). FGS was scored semi-quantitatively on a scale of 0 to 4 in 50 glomeruli per kidney, FGS lesions were defined as glomerular areas with mesangial expansion and adhesion formation simultaneously present in one segment. The theoretical maximum score is 400. Interstitial fibrosis (IF) was scored similarly in 30 interstitial fields. A score of 0 was given when no interstitial fibrosis was present in a field, 1 for 0-25% with IF, 2 for 25-50%, 3 for 50-75% and 4 for 75-100% of the field showing IF. IF was defined as expansion of the interstitial space, with or without the presence of atrophied and dilated tubules and thickened tubular basement membranes.

**Immunohistochemistry and quantification**

To assess the extent of tubular and interstitial injury in the different groups, immunostaining for α-smooth muscle cell actin (pre-fibrotic changes; α-SMA, clone 1A4, Sigma, St. Louis, MO, USA), collagen type IV (interstitial fibrosis; cIV, Southern Biotech, Birmingham, AL, USA), osteopontin (marker of tubular injury; OPN, clone MPIIB10, Developmental Hybridoma Studies, Iowa City, IA, USA) macrophages (inflammation; ED1, Serotec, Oxford, UK) and Kim-1 (marker of
tubular injury; peptide 9, a kind gift from dr. V. Bailly, Biogen Inc, Boston, MA, USA) was performed. After dewaxing and blocking of endogenous peroxidase (PO) incubation with the primary antibodies was performed for 1 hour at room temperature. Subsequently, sections were incubated with appropriate secondary PO labelled antibodies for 30 min. Peroxidase activity was developed using 3,3’-diaminobenzidine tetrachloride (DAB) for 10 min. Interstitial staining of \( \alpha \)-smooth muscle actin, osteopontin, Kim-1 and collagen IV was measured by a blinded observer using computerized image analysis (Advanced QUIPS, Leica Imaging Systems, Cambridge, UK) in sections without counterstaining. Fifty cortical sections per rat were scored with exclusion of glomeruli and blood vessels. Macrophages were counted by a blinded observer in 50 interstitial fields per kidney.

**Statistical analysis**

Data were tested for normality and when non-parametric distribution was present differences between groups were detected by Kruskal-Wallis and Mann-Whitney U tests, otherwise ANOVA with Bonferroni post-hoc tests were used. We used ANOVA to test for linear trend. Spearman’s Rho correlation coefficients were calculated. Linear regression was performed to calculate the association between proteinuria, Kim-1 and OPN. Statistical analyses were performed using SPSS version 12.0.2 and GraphPad Prism version 3.02. Data are given as mean ± standard deviation (SD), except for Figure 1, where mean ± standard error of the mean (SEM) is given. Statistical significance was assumed at the 5% level.

**RESULTS**

**Clinical parameters (Table 1)**

The time course of proteinuria and blood pressure are shown in Figure 1. Six weeks after induction of nephrosis, mean proteinuria was 214±125 mg/24h. In vehicle rats proteinuria progressed to 641±287 mg/24h at wk 18. Treatment with monotherapy spironolactone did not reduce proteinuria (569±284 mg/24h), whereas lisinopril (289±218 mg/24h) and the combination of LIS/SPIRI (187±190 mg/24h) significantly reduced proteinuria compared to vehicle treated animals and to spironolactone alone. However, there was no significant difference between the lisinopril and LIS/SPIRI groups in proteinuria and blood pressure. There was a linear trend in proteinuria, with the highest values in vehicle and lowest in controls (p<0.01 for trend). LIS and LIS/SPIRI reduced blood pressure (118±25 resp 101±25 mmHg) compared to vehicle treated animals (169±33 mmHg) (both p<0.05), whereas spironolactone monotherapy was without effect. Blood pressure was also significantly reduced in the combination group compared to healthy controls (controls: 137±8 mmHg). Blood pressure also showed a linear trend with highest values in vehicle, and lowest in LIS/SPIRI (p<0.01 for trend).
ACEi/aldoRBI on renal damage in proteinuric rats

Figure 1 A) Proteinuria at wk 18 in the different adriamycin groups and healthy controls. B) Blood pressure at wk 18 measured by tail cuff plethysmography. Both graphs represent mean and SEM. Abbreviations: VEH: vehicle treated; SPIR: spironolactone treated; LIS: lisinopril treated; LIS/SPIR: combination of lisinopril and spironolactone; CON: healthy control rats.

Table 1. Clinical parameters

<table>
<thead>
<tr>
<th></th>
<th>VEH (n=8)</th>
<th>SPIR (n=12)</th>
<th>LIS (n=10)</th>
<th>LIS/SPIR (n=14)</th>
<th>CON (n=6)</th>
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<tr>
<td>Body Weight (gram)</td>
<td>474±28</td>
<td>455±28</td>
<td>451±28</td>
<td>464±30</td>
<td>504±52</td>
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<td>Water intake (ml)</td>
<td>13±6</td>
<td>12±4</td>
<td>22±7*</td>
<td>24±6**</td>
<td>15±9</td>
</tr>
<tr>
<td>S creatinine (µmol/L)</td>
<td>69±13</td>
<td>67±15</td>
<td>65±11</td>
<td>69±13</td>
<td>55±2</td>
</tr>
<tr>
<td>S urea (mmol/L)</td>
<td>8.8±2.7</td>
<td>10.4±4.9</td>
<td>11.2±3.7</td>
<td>16.6±5.7*</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>S potassium (mmol/L)</td>
<td>6.0±0.3*</td>
<td>5.6±0.5*</td>
<td>6.2±0.4*</td>
<td>5.7±0.4*</td>
<td>4.5±0.3</td>
</tr>
<tr>
<td>S sodium (mmol/L)</td>
<td>149±5</td>
<td>143±7</td>
<td>138±5</td>
<td>139±5</td>
<td>137±2</td>
</tr>
</tbody>
</table>

*p<0.05 vs CON, *p<0.05 vs VEH, *p<0.05 vs SPIR, @p<0.05 vs LIS/SPIR

Serum creatinine did not differ between the various groups; however, urea was increased in the combination group, compared to vehicles, reflecting the volume-depletion by LIS/SPIR. Plasma potassium was not increased in the LIS/SPIR group compared to the vehicle, spironolactone and lisinopril treated groups, and thus the combination of LIS/SPIR in this study did not induce hyperkalemia. Water intake, and therefore drug intake of lisinopril was comparable in all groups.
Markers of tubular injury

Osteopontin mRNA: In all AN animals osteopontin mRNA was increased compared to healthy controls (p<0.05). Monotherapy with spironolactone or lisinopril did not significantly reduce the amount of osteopontin mRNA compared to vehicle – although there was a stepwise numerical reduction. Only the combination of LIS/SPIR significantly reduced the amount of osteopontin mRNA induction compared to vehicle (Figure 2A).

![Figure 2](image)

Figure 2: Markers of tubular damage at termination, graphs represent mean and SD. A) Osteopontin mRNA (qPCR), B) Osteopontin protein expression (computer-assisted morphometry), C) Kidney injury molecule-1 (Kim-1) mRNA (qPCR), D) Kim-1 protein expression (computer-assisted morphometry). Mean and SD are given.

Osteopontin protein: Healthy control animals showed virtually no cortical osteopontin protein expression. As anticipated, osteopontin was present in the medulla and collecting ducts of these animals. In adriamycin animals osteopontin was found in the cytoplasm of injured and dilated tubules, medulla and collecting ducts. Treatment with spironolactone or lisinopril numerically reduced cortical interstitial osteopontin staining, without reaching statistical significance, however. Only the combination of LIS/SPIR significantly reduced cortical osteopontin expression compared to vehicle animals (Figure 2B + 3A-E).

Kidney injury molecule-1 mRNA: Kim-1 mRNA, which is induced in injured proximal tubules, was significantly induced in all adriamycin animals compared to healthy controls. None of the treatments reduced the expression of Kim-1 mRNA, although a stepwise decrease in Kim-1 mRNA from vehicle downwards to LIS/SPIR was apparent (Figure 2C).
Figure 3. Photomicrographs of osteopontin and Kim-1 immunohistochemistry (brown) with PAS counterstaining. Left panel: Osteopontin immunohistochemistry. Right panel: Kim-1 immunohistochemistry. A+B: Vehicle treated animals, C+D: spironolactone treated animals, E+F: lisinopril treated animals, G+H: LIS/SPIR treated animals, I+J: Healthy controls. In VEH, osteopontin and Kim-1 are abundantly present, during treatment osteopontin and Kim-1 staining decreases. In controls no osteopontin and Kim-1 staining is present in the cortex.
**Kim-1 protein:** In healthy control animals no renal Kim-1 protein expression was detected. However, Kim-1 was significantly induced in vehicle treated adriamycin animals, compared to controls. A numerical, but not statistically significant, reduction of Kim-1 was observed in spironolactone and lisinopril treated groups. However, a significant reduction was found in the LIS/SPIR treated rats compared to the vehicle treated rats (Figure 2D + 3F-J).

The induction of osteopontin and Kim-1 mRNA showed a strong positive correlation with proteinuria (Fig 4A and 4B). For higher levels of proteinuria, more tubular damage is present. Also, the expression of osteopontin and Kim-1 were strongly associated with each other (Fig 4C).

![Graph 4A](image1)

**Figure 4** Linear regression for OPN (A) and Kim-1 (B) with proteinuria at week 18. The more proteins passed the tubules, the more tubulointerstitial damage is present. Also OPN and Kim-1 show a strong correlation with each other (C).

**Interstitial inflammation, pre-fibrotic changes and structural damage**

**Macrophages:** Influx of peritubular interstitial macrophages was very low in healthy control animals, whereas in all adriamycin animals macrophage influx was increased. Treatment with spironolactone, lisinopril or LIS/SPIR did numerically reduce macrophage influx (Fig 5A).

**α-Smooth Muscle Cell Actin:** In healthy control animals α-SMA was present only in the vessel wall of intrarenal arteries. In adriamycin animals, α-SMA expression was found in arteries and in the interstitial space surrounding dilated tubules. In vehicle and spironolactone treated animals interstitial α-SMA was increased compared to controls. In lisinopril and LIS/SPIR treated rats α-SMA was significantly reduced compared to vehicle and values were comparable to healthy controls (Fig 5B).
Collagen type IV: In all groups, collagen IV deposition was observed surrounding all tubular basement membranes. Collagen deposition was significantly increased in the vehicle group compared to healthy controls. Treatment with lisinopril or LIS/SPIR reduced collagen IV deposition compared to vehicle, and LIS/SPIR also reduced collagen type IV compared to spironolactone treated animals (Fig 5C).

Focal glomerulosclerosis (FGS) and interstitial fibrosis (IF): There was a significant increase in FGS and IF in all adriamycin animals compared with controls, with highest values in vehicle, followed by spironolactone, lisinopril and with lowest scores in the LIS/SPIR group (p<0.01 for trend). IF was significantly reduced in the LIS/SPIR group compared with the spironolactone group.
Correlation between renal damage and proteinuria. In line with the association of proteinuria and markers of tubular injury in figure 5, also for the other markers of renal damage a strong association exists. When all animals (treated and untreated) are taken together, proteinuria shows the following Spearman rho coefficients at week 18 with markers of renal damage: macrophages: $r=0.73$, $\alpha$-SMA $r=0.84$, collagen type IV $r=0.50$, interstitial fibrosis $r=0.77$ and FGS $r=0.83$, all $p<0.001$.

DISCUSSION

In this experiment in proteinuria-induced renal damage we demonstrate that combining an ACEi with an aldoRB is effective in reducing proteinuria and blood pressure. To our knowledge this is the first study that combines these two regimens in animals with proteinuria-induced renal damage to study the effects on renal damage. Our data on the clinical parameters are in line with reports in proteinuric patients, where the addition of aldoRB to ACEi treatment led to a further reduction of proteinuria [14-16]. Furthermore, we noted a remarkable reduction of FGS and tubular damage in the combination group providing further evidence for the beneficial effect of this treatment regimen.

The beneficial effect of combining ACEi and aldoRB has been demonstrated in radiation injury and hypertensive injury in rats [17;18]. In line with these studies - ACEi and ACEi/aldoRB in our study showed a strong reduction of proteinuria compared to untreated animals. Monotherapy with aldoRB, on the other hand, did not reduce proteinuria, which was also seen in other studies [19;20]. An important difference of our study compared with other studies is the timing of the onset of treatment. We used a curative setting, investigating the efficacy of intervention started at a time when proteinuria is well-established and when early lesions are already present, whereas others only studied the combination of ACEi and aldoRB in a preventive setting; namely starting treatment together with the induction of disease. Our curative setting more closely resembles the clinical situation, where patients usually come under medical attention after proteinuria has been present for some time already. Delayed-onset treatment has been used in a hypertensive rat model to compare losartan, eplerenone and placebo for cardiac and renal damage [21], where treatment was started either at disease induction or 4 weeks thereafter. No differences in renal damage were present between the different treatment regimens, neither for the timing of treatment, nor between losartan and eplerenone. Proteinuria was only prevented when treatment was started at disease induction, whereas in our study proteinuria and tubular damage were reduced and the development of structural damage was prevented with a delayed start of treatment with ACEi and ACEi+aldoRB.

However, our study has some limitations. We cannot discriminate whether the effective reduction of proteinuria and renal damage in our ACEi/aldoRB group relates to optimisation of
sodium status by the diuretic effect of spironolactone, or to specific antifibrotic properties of spironolactone. To this purpose, future studies should address the comparative efficacy of the ACEi + aldosterone regimen and control groups with ACEi + low sodium diet or ACEi + potassium sparing diuretic. Moreover, it would be of interest to address the specific aldosterone-antagonist eplerenone for its effectiveness in combination with ACEi on proteinuria-induced renal damage. Another limitation might be the dose of spironolactone. The dose was based on earlier studies, however - due to higher body weight of our animals - the dosing of spironolactone is slightly below the dose that inhibits 95% of the aldosterone binding in vivo, but far above the dose that inhibits 60% of the aldosterone binding [22].

The combination of ACEi and aldosterone effectively reduced markers of tubular damage compared to untreated animals, and prevented the interstitial and glomerular damage that developed in the spironolactone and vehicle treated animals. The therapeutic effect on renal damage can be due to the reduction of proteinuria by this regimen, which is supported by the strong correlation between proteinuria and all markers of renal structural damage. The therapeutic effect could also be due to the antifibrotic effect of spironolactone, which could exert its effect through a reduction in TGF-β levels [23]. Blockade of the mineralocorticoid receptor and reduced angiotensin II levels (by ACEi) both may have direct effects on osteopontin, as earlier studies showed in vivo and in vitro induction of OPN after treatment with aldosterone [24-27]. Reduction of markers of tubular damage, such as OPN and Kim-1, by ACEi/aldosterone as observed here are in line with those studies. Whether such a reduction in itself could play a role in the mechanism of prevention of (pro)fibrotic lesions such as expression of α-SMA and increased collagen type IV deposition, cannot be derived with certainty from our study and should be subject of further study.

In conclusion, combination of aldosterone and ACEi reduced proteinuria, blood pressure and tubular damage and prevented the development of interstitial fibrosis and FGS in a rat model of proteinuria-induced renal damage. Further studies are needed to investigate whether this effect is due to an optimized sodium status by the diuretic action of by a direct antifibrotic effect of aldosterone. These data support a potential role for adjunct aldosterone in proteinuric patients treated by RAAS-blockade, but long-term clinical trials are needed to substantiate this assumption.

References


