Chapter 8

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Summary

Progression of renal disease is a worldwide increasing problem. Over the last decades, important progress has been made to optimize treatment strategies for patients with chronic renal disease. In many patients blockade of the RAAS, by Angiotensin Converting Enzyme-inhibition (ACEi) and Angiotensin II type 1 antagonists (AT1-A) is effective in reducing blood pressure and proteinuria, thereby ameliorating the rate of renal function loss. However, in many patients progressive loss of renal function still occurs, eventually leading to end-stage renal damage, reflecting resistance of the renal condition to the therapeutic effects of RAAS-blockade. Therefore, it is crucial to elucidate the mechanisms underlying the resistance to therapy, as identification of these factors allows designing novel renoprotective treatment regimen.

Unlike, for instance, the field of oncology, in nephrology the mechanisms of resistance to pharmacological intervention have not been a major research topic, and consequently, the determinants of response or resistance to renoprotective intervention are not well understood, although it has been shown consistently that dietary sodium restriction can potentiate the response to RAAS-blockade. Yet, the observation that the reduction of proteinuria during therapy predicts the long-term renal prognosis provides a useful starting point. Moreover, it would be logical to assume that intrarenal factors are involved in the response to therapy, but there is hardly any data on this, because in renal patients renal tissue is only available for investigation when the clinical condition and the therapeutic options justify a renal biopsy. Interestingly, however, a retrospective study in transplant recipients showed that the antiproteinuric efficacy of fosinopril was determined by the extent of tubulointerstitial lesions [1].

This report raised the hypothesis that tubulointerstitial lesions are a determinant of renal responsiveness or resistance to RAAS-blockade. This hypothesis is difficult to test in man, however, as it requires a pretreatment renal biopsy in all patients, preferably all with a similar renal condition. Therefore, to prospectively test the hypothesis that interstitial lesions determine the antiproteinuric response to RAAS-blockade, we used a model of chronic proteinuria-induced renal damage in rats, namely adriamycin nephropathy. In chapter 2 we showed that the extent of renal damage present before the start of treatment is indeed prognostic for antiproteinuric efficacy of RAAS-blockade. The early interstitial lesions were positively correlated with proteinuria at time of biopsy, and moreover, they predicted the antiproteinuric response after 2 weeks and 6 weeks of treatment. Thus, in animals with more pronounced early interstitial lesions, the antiproteinuric response was suboptimal compared to animals with less early interstitial lesions. The renal damage prior to treatment with ACEi was prognostic for both the short-term antiproteinuric responses as well as for the severity of structural glomerular damage, i.e. focal glomerulosclerosis after longer follow up. These prospective findings thus are consistent with the prior report in transplant recipients [1]. For ethical reasons, the data cannot easily be confirmed.
in patients. Nevertheless, some clinical studies have been performed that used protocol biopsies, and for the future it would be interesting to study whether the tubulointerstitial lesions in these protocol-biopsies predict the response to RAAS-blockade. The prognostic impact of the early tubulointerstitial lesions for the antiproteinuric response is all the more remarkable as the early reduction in proteinuria is considered to be primarily mediated by therapeutic effects on the glomerulus, i.e. the direct reduction of glomerular pressure by efferent vasodilatation, and the indirect reduction of glomerular pressure by the lower blood pressure.

The prognostic impact of the intrarenal lesions present at onset of therapy for the response to therapy is of great clinical impact. Renal damage can go largely unnoticed, and consequently, most renal patients come under medical attention not until some extent of established renal damage is present. So, this observation provided the starting point for the further studies in this thesis, that addressed the prognostic impact of tubulointerstitial as well as glomerular lesions at onset of treatment for the renoprotective effects of RAAS-blockade, the possible reversibility of these intrarenal factors during RAAS-blockade to determine whether their persistence during therapy might be a factor in therapy resistance, and finally, strategies to overcome resistance to RAAS-blockade.

**Part I: Intrarenal factors and therapy resistance**

To gain insight in the intrarenal mechanisms of resistance to RAAS-blockade we studied several intrarenal factors previously suggested to be involved in the pathogenesis of renal damage. As interstitial α-smooth muscle cell actin (α-SMA) expression - a marker of the pre-fibrotic myofibroblast transformation – was the best predictor of the antiproteinuric response in our first study, we studied two tubular cell related factors possibly upstream in this process, i.e. Osteopontin and Kidney injury molecule-1 (Kim-1). Moreover, we studied whether these proteins might be suitable as targets for intervention or monitoring of therapeutic efficacy by measurement in urine or blood. Second, as proteinuria is largely due to defects in the glomerular basement membrane (GBM), we also studied two glomerular factors involved in the maintenance of the integrity of the basement membrane, i.e. the expression of heparan sulfate and its association with heparanase in chronic proteinuria-induced renal damage and during treatment with an AT1-A.

Osteopontin is a molecule involved in the attraction of monocytes and macrophages, and is upregulated in several models of renal injury. In Chapter 3 we studied the time-course of renal osteopontin induction in relation to proteinuria, the influx of macrophages into the renal interstitium and the development of structural damage. Osteopontin - present in injured proximal tubular cells – increased progressively over time and was strongly correlated with proteinuria, suggesting that proteinuria itself induces osteopontin expression. In a second study animals underwent a biopsy before ACEi treatment was started. In this study the reduction of proteinuria was accompanied by a reduction of osteopontin protein and a stabilization of osteopontin...
mRNA, whereas in untreated animals osteopontin protein stabilized and osteopontin mRNA dramatically increased. So, by antiproteinuric treatment with ACEi, the upregulation of osteopontin was reversible, however without a corresponding reduction in fibrosis. Data in osteopontin knock-out mice and from treatment with anti-osteopontin antibodies [2;3] support a role of upregulation of osteopontin in the pathogenesis of proteinuria-induced interstitial fibrosis, so theoretically osteopontin could be an additional target for therapy. In other studies, however, osteopontin protected the interstitium from macrophage infiltration and interstitial fibrosis, suggesting protective effects as well. From the perspective of identifying targets for intervention it is important that our intervention with ACEi showed that reduction of osteopontin does not attenuate established fibrosis. Thus, osteopontin does not qualify as a suitable target for intervention in addition to RAAS blockade.

A new player in the tubulointerstitium, Kidney injury molecule-1 (Kim-1), is expressed in acute renal disease in human and experimental animal in injured tubules. In chapter 4 we showed that Kim-1 expression is induced in proteinuric renal disease, and that the Kim-1 ectodomain is shedded into urine. This is not unique to adriamycin nephrosis, but also to other models of chronic renal damage, such as protein-overload nephrosis and hypertensive renal damage [4;5]. Pharmacological intervention with ACE-i and AT1-A reduced both renal and urinary Kim-1 levels. Kim-1 is present at the apical membrane of tubules with mild to moderate damage, as indicated by slight dilatation, however it is not present when tubular cell damage is severe. The reduction of Kim-1 during RAAS-blockade along with proteinuria suggests that Kim-1, just like osteopontin is not a suitable target for intervention in the pathway of progressive tubulointerstitial fibrosis. However, since Kim-1 is closely associated with tubulointerstitial injury and shedded into urine, it might be a marker for activity of the cascade of proteinuria-induced tubulointerstitial damage – and thus a marker for the efficacy of therapy on the intrarenal processes downstream of proteinuria. If so, this would be of great clinical importance as it might be used to guide therapy. Unfortunately, we could not investigate this properly, as only urine of week 12 was available for measurement of Kim-1, and the value of urinary Kim-1 as marker for responsiveness or resistance to therapy (in addition to proteinuria) therefore remains to be studied. The functional role of Kim-1 would also be of interest for future studies. Kim-1 might be protective against toxic effects of excessive proteins and growth factors, but it might also have a harmful effect at the surrounding tubules and interstitium. The pattern of staining directs mostly towards a protective function.

In addition to tubulointerstitial lesions we also studied the role of integrity of the glomerular basement membrane (GBM) - which is a main determinant of proteinuria - in the response to therapy. Heparan sulfates are important constituents of the slit diaphragm and loss of heparan sulfates is associated with an altered charge-dependent permeability of the glomerular basement membrane (GBM). In an earlier study it was shown that loss of GBM heparan sulfate was due to reactive oxygen species (ROS), and ROS scavengers - such as dimethylthiourea
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(DMTU) - could partially prevent the loss of GBM heparan sulfate and partially reduce albuminuria. Thus, ROS cannot completely account for the loss of GBM HS and the induction of albuminuria. Recently, heparanase – an enzyme capable of degrading heparan sulfate polysaccharide side chains - was found to be increased in diabetic nephropathy [6]. In chapter 5 we studied whether heparanase is responsible for loss of GBM heparan sulfate and induction of proteinuria in adriamycin nephropathy. Second, the effect of the ROS scavenger DMTU on heparanase expression was studied. Finally, we studied the effect of RAS blockade on heparanase expression and its relevance for therapy resistance. We found that loss of heparan sulfate was strongly associated with increased heparanase-activity. Treatment with ROS-scavengers during induction of adriamycin nephrosis reduced heparanase expression compared saline treated adriamycin animals. Finally, in established adriamycin nephroisis, antiproteinuric treatment with AT1A reduced the expression of heparanase with a subsequent increase in heparan sulfates. Thus, the reduced heparanase expression after treatment with DMTU supports the involvement of hydroxyl radicals in the induction of heparanase expression, which subsequently leads to loss of GBM HS and development of proteinuria in AN. Moreover, the induction of heparanase in AN was reversible by AT1A. This suggests that angiotensin II has a role in heparanase induction – and that amelioration of heparanase induction, and the subsequent restoration of GBM HS, contributes to the beneficial effects of RAS-blockade. However, in contrast to tubulo-interstitial lesions in the previous chapters, GBM heparan sulfate and glomerular heparanase expression did not predict antiproteinuric response and interstitial fibrosis during follow up, suggesting that HS and heparanase activity are not limiting factors for therapy response. This might be due to the short follow-up, however in earlier studies tubulointerstitial markers already predicted antiproteinuric effect after 2 weeks.

HS and heparanase were not restored to normal levels, suggesting they could provide a target for additional intervention. Recent studies show that anti-heparanase treatment in experimental nephrosis reduces proteinuria [7;8]. Also studies using heparin or low molecular weight (LMW) heparin showed a reduction of albuminuria in diabetic patients [9-12]. These heparins and LMW heparins consist a mixture of glycosaminoglycans, mainly heparan sulfate. Thus, together, the above studies suggest that intervention directed at restoration of heparan sulfates in the glomerular basement membrane might result in an anti-proteinuric effect, however whether it is has additional effects on top of RAS blockade and improves long-term outcome, needs to be studied.
Part II: Strategies to overcome the renal resistance to RAAS blockade

Our finding that early interstitial damage is prognostic for the anti-proteinuric efficacy of ACEi, could raise the impression that the prognosis is already set by the time treatment is started. This would be particularly disquieting, as renal disease goes often unnoticed in man, and most patients have at least some degree of renal structural damage by the time they come to medical attention (with type I diabetes as the main exception). However, the prognostic value of pretreatment renal damage should not be a reason for therapeutic nihilism, but rather prompt the search for treatment strategies to overcome the renal therapy resistance.

In chapter 6 we demonstrated that modification of sodium status could overcome the prognostic value of early renal damage on antiproteinuric efficacy. Adriamycin nephrotic animals were instituted on different oral sodium intakes, and underwent a biopsy before treatment with ACEi was started. During high sodium diet the antiproteinuric effect was absent, but during normal and low sodium diet proteinuria was reduced to normal values. However, during normal and high sodium diet extensive early interstitial lesions were still associated with worse antiproteinuric efficacy after 3 or 6 weeks of treatment and structural damage after 6 weeks of treatment. On the opposite, this prognostic value was absent in rats treated with low sodium and ACEi. Thus, the prognostic value of interstitial lesions is modifiable by intervention strategies. Importantly, especially the individuals with high residual proteinuria (i.e. those with a poor prognosis) benefit from additional treatment, such as sodium restriction, to ACEi.

In chapter 7 we tested the efficacy of the aldosterone receptor blocker (aldoRB) spironolactone, a diuretic with anti-fibrotic effects, in combination with ACEi on proteinuria and renal damage. Aldosterone has - in addition to its role in sodium conservation - pro-fibrotic properties [13;14], and moreover, it is increased in a substantial part of the patients as an escape-mechanism during ACEi [15]. In our study, treatment with ACEi and the combination of ACEi with aldoRB reduced proteinuria and blood pressure, whereas aldoRB alone could not reduce proteinuria and blood pressure, which was comparable with other studies [16;17]. Although no significant difference between ACEi and ACEi/aldoRB for proteinuria and blood pressure were detected, tubular damage on the other hand- quantified by measurement of osteopontin and Kim-1 mRNA and protein – was reduced in the ACEi/aldoRB group only. Thus, combining ACEi and aldoRB was effective, however, there was no additional effect compared to ACEi alone. Future studies need to explore whether combining ACEi with aldoRB (eventually with the selective aldosterone blocker eplerenone) has additive effects compared to combining ACEi with low sodium. A recent paper showed that treatment with aldoRB by spironolactone reduced glomerulosclerosis in rats with 5/6 nephrectomy. When combined with antihypertensive treatment even regression of glomerulosclerosis was observed [18]. Thus, the effects of aldoRB in renal disease are promising, but the efficacy needs to be further studied.
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Future perspectives

In this thesis we demonstrate that the extent of renal damage present before start of treatment determines the antiproteinuric efficacy of RAAS-blockade. This might seem intuitively obvious, but on closer screening, it is a puzzling finding, with great potential relevance for the clinical setting as in man structural renal damage is often already present by the time of start of treatment. Moreover, it is intriguing that the relatively mild changes, that are not even apparent at routine morphology, interfere with therapeutic efficacy. How can we put these findings into effect to design better renoprotective strategies? Various strategies present themselves here, namely: first, pursue earlier detection of renal disease and start of treatment, and second, identify the specific pathways of therapy resistance and ongoing renal damage during RAAS-blockade, and finally, design additional modes of intervention.

The concept of early detection and early start of treatment, even before overt renal damage has developed is under investigation currently, and has proven to be successful in diabetic patients, as for instance in the recent BENEDICT trial, that showed that treatment with ACEi retarded the onset of microalbuminuria in hypertensive patients with type 2 diabetes [19]. Proof of concept for the long-term benefit of intervention during the stages that precede renal damage was provided by Nagai et al showed that in type 2 diabetic rats, temporary angiotensin II blockade at the pre-diabetic stage attenuated the development of renal injury [20].

How to identify patients with ongoing renal damage during RAAS-blockade?

The predictive effect of proteinuria reduction for long-term renoprotection is well-established in many conditions, and it is generally assumed that proteinuria closely reflects the mechanisms of ongoing renal damage. If so, proteinuria (along with blood pressure) would be the only factor needed to identify patients with ongoing renal damage. However, proteinuria and renal damage can be dissociated – thus hampering the predictive power of proteinuria, and requiring more direct measures of the intrarenal processes of tubulointerstitial damage downstream of proteinuria. In this respect we want to emphasize the finding in chapter 6, where we found that sodium restriction could overcome the negative effects of pre-treatment renal damage on the anti-proteinuric response, without, however, a benefit on interstitial damage. In recent other studies from our group, in fact, we found that in proteinuric rats the combination of ACEi and sodium restriction elicits pronounced interstitial lesions despite a significant reduction of proteinuria. The same abnormalities were found in healthy rats on the same regimen [21], strongly suggesting a link to this specific therapeutic regimen. Whereas the mechanism underlying the dissociation between proteinuria and renal damage requires further investigation, it also strongly underlines the need for non-invasive markers other than proteinuria that reflect the intrarenal pathways of damage. Studies that have to establish whether urinary Kim-1 is such a marker reflecting early tubulo-interstitial damage are currently under way. In addition to non-invasive markers of early tubulointerstitial damage, biomarkers of more advanced
tubulointerstitial damage, such as urinary collagen type IV, should be studied for their prognostic value for therapy response, and for their value in titrating therapy to improve long-term outcome. Urinary type IV collagen is a candidate marker for the detection of advanced renal injury, as it is significantly increased in various types of renal disease compared to healthy controls [22]. Moreover, urinary type IV collagen is correlated with renal injury in IgA nephropathy [23].

**How to identify intrarenal pathways responsible for therapy resistance?**

The process of ongoing renal damage during therapy is complex and likely involved many factors. Identifying the key players is likely to be difficult, and may be like looking for a needle in a haystack. Our data show, that it is important to investigate the determinants of treatment response at tissue level, and not only monitor efficacy exclusively from clinical parameters like blood pressure and proteinuria. In this thesis we only tested only a small number of intrarenal factors, and by these examples we can illustrate a more general strategy to be used in screening the kidney for factors involved in therapy resistance. In our studies osteopontin and Kim-1 expression predicted therapy response, but despite the reduction of osteopontin and Kim-1 along with proteinuria during therapy, the interstitial fibrosis was not reversible. Therefore, osteopontin and Kim-1 are not suitable targets for additional intervention. Altered glomerular heparanase expression and GBM HS, on the other hand, did not predict short-term antiproteinuric efficacy neither progression of interstitial fibrosis, but were partly reversible upon treatment, possibly contributing to the antiproteinuric effect. So, intervention aimed at amelioration of the increased permeability of the GBM due to loss of heparan sulfate might be useful for further reduction of proteinuria, but is not likely to have direct effects on the progression of interstitial fibrosis. Finally, α-SMA was a consistent predictor of therapy response, that was not ameliorated at all during RAAS-blockade, and neither did collagen deposition. This suggests that fibroblasts and factors influencing the activation and proliferation of the fibroblast are important determinants of ongoing renal damage during RAAS blockage and may therefore by suitable targets for new modes of intervention in the progression of renal disease.

As mentioned above, the search for the factors responsible for the resistance to RAAS-blockade resembles looking for a needle in a haystack. However with new high throughput techniques, such as micro-arrays, proteomics, and kinomics the expression of hundreds of genes, and proteins, respectively, can be tested simultaneously. To select the ones with pathophysiological relevance for therapy response, the various response-patterns can be analysed by the scheme in Figure 1, that depicts the different possibilities of response to therapy. The schedule helps to indentify intermediate factors involved in therapy resistance as follows. First, in the situation that RAAS-blockade reduces proteinuria, the factor of interest (intermediate factor) is reduced and also long-term structural damage, there is no therapy resistance but a good response (situation A). However, when proteinuria is partly or completely reduced, and the intermediate factor is reduced while structural damage is not improved, the latter outcome should
be classified as therapy resistance. Whereas, in this situation (B), the intermediate factor may be a predictor of therapy resistance, it is not, however, a causal factor in therapy resistance, as it dissociates from the eventual outcome. In the third situation (C) the intermediate factor has not changed during RAAS-blockade (with reduction of proteinuria) but structural damage is reduced, which means that there is no therapy resistance and this intermediate factor is not relevant. In the final situation (D) both the intermediate factor and structural damage are not reduced by RAAS-blockade, indicating that therapy resistance has occurred and that the intermediate factor may well be involved in therapy resistance. The intermediate factors that follow the pattern from situation D are potential factors for intervention in addition to RAAS-blockade and when possible to measure in urine or serum, might also function to monitor the efficacy of treatment.

![Figure 1](image_url)

**Figure 1.** Theoretical model for the screening of potential factors of therapy resistance that are of interest for intervention and monitoring of proteinuric renal disease.

**New treatment strategies**

In addition to the screening for important factors, development of therapeutic strategies needs to go on. The alleged effect of pro-fibrotic changes in therapy resistance suggest that new strategies to prevent the progression of renal damage should be directed at profibrotic factors such as Angiotensin II and TGF-β1 or in pathways downstream of these factors. A promising novel approach to the treatment of renal disease is through inhibition of adamalysins [24]. These proteins, which are abundantly present in the kidney, are involved in shedding of fibrogenic growth factors and cytokines from the cell membrane. Furthermore, for the reversal of structural damage, proteases that degrade excessive matrix accumulation are of interest. However, there remains a risk that these proteases not only reduce the excessive matrix but also the normal matrix necessary for cell-cell adhesion and stability. Intervention directed towards TGF-β1 is already shown to be efficient in animals [25], and can also be directed at downstream pathways such as the smad-pathway [26]. Addition of statins to the regular treatment regime, is capable of reducing cardiovascular morbidity but experimental data showed that statins are also capable of reducing proteinuria [27]. Statins have also been reported to induce proteinuria; however, this is a mild tubular proteinuria, mediated by the reduction of receptor-mediated endocytosis in proximal tubular cells by statins [28] and therefore, we consider this mild proteinuria not likely to induce
tubulointerstitial injury. Finally the addition of aldosterone-blockade to current treatment strategies can be useful for long-term renoprotection, as we showed a protective value in this thesis and others recently found reversibility of glomerulosclerosis by combining antihypertensive treatment with aldosterone-blockade [18].

As we showed earlier, fibroblasts are important in the progression of renal disease and therefore intervention should be directed on the proliferation and activation of fibroblasts. Fibroblasts are derived from several sources, which makes intervention less easy. It has been estimated that about 35% of the fibroblasts are derived from local epithelial to mesenchymal transition (EMT), which are tubular epithelial cells under (inflammatory) stress that transformed into fibroblasts. Second, circa 15% of the fibroblasts are derived from the bone marrow. And finally the rest is contributed by proliferating fibroblasts mainly from the interstitial space [29;30]. TGF-β1 induces EMT and also activates fibroblasts to proliferate. Interestingly, all these actions of TGF-β1 can be counteracted by bone morphogenic protein-7 (BMP-7) [31]. BMP-7 reduces interstitial fibrosis in a mouse model of progressive chronic renal injury (nephrotoxic serum nephritis) [32]. Together with BMP-7 hepatocyte growth factor is capable of reducing EMT as well, and thereby reducing interstitial fibrosis [33;34]. Intervention in the process of EMT may therefore be of interest for better prevention of long-term proteinuria-induced renal damage. Another innovative possibility for intervention would be modulation of stem cell function. Theoretically, improvement of endogenous stem cell function, or administration of exogenous stem cells could contribute to renal repair processes and reversibility of renal damage. As, however, stem cells in themselves can also contribute to fibrotic processes, in-depth studies aimed at purposeful modulation of stem cell function are needed [35].

**Implications for clinical practice**

This thesis shows that the extent of renal damage present before the start of treatment predicts the outcome of therapy with RAAS-blockers. Whether this also applies to human renal disease needs to be confirmed in further studies, which could be done in the protocol-biopsy studies that are available. It is important to note that, notwithstanding the predictive effect, the renal prognosis during therapy is still modifiable by optimizing therapeutic measures, as we showed for sodium restriction. Whether this also holds true for other measures that potentiate the effects of RAAS-blockade, such as dual blockade (combining ACEi with AII antagonist) or combining ACEi with aldosterone-antagonists, remains to be studied. At any rate, our data show that the prognostic impact of pre-treatment renal damage is not a fixed phenomenon, and that intensifying the therapeutic regimen can overcome the prognostic impact of pre-treatment damage on anti-proteinuric response. However, recent data from our group also direct that titration to low blood pressure values might be harmful as well, and induce interstitial lesions that dissociate from the further reduction of proteinuria [21]. Moreover, in man, the feasibility of aggressive titration of RAAS-blockade-based therapy may be limited by a poor tolerability [36].
Together, these findings indicate, first, that it would be important to design additional modes of intervention, and second, that it would be useful to have non-invasive biomarkers for monitoring renal tubulo-interstitial damage directly, as the prognostic impact of proteinuria, although important, is apparently not perfect. Further reduction of proteinuria by restoring the permeability characteristics of the GBM by low molecular weight heparin and added heparan sulfates could provide such an additional mode of intervention. Moreover, one could imagine that in the future, patients with chronic renal disease will be treated with the conventional treatment strategies such as RAAS-blockade and measures that optimize RAAS-blockade, as assessed from the reduction of proteinuria, and that based on increased levels of biomarkers, additional treatment will be given to prevent ongoing progression of renal damage. Such additional strategies could be aimed at directly interfering with the processes of tubulo-interstitial fibrosis downstream of proteinuria, for instance by targeting EMT.

In conclusion, this thesis showed that the glomerular lesions responsible for initial protein leakage and early tubular lesions are reversible during treatment with RAAS-blockade. However interstitial lesions are not reversible with the treatment regimens used in this thesis. Interestingly, these irreversible interstitial lesions are predictive for therapy resistance. Thus, with the current treatment strategies, the clue to resolution of resistance to the renoprotective effects of RAAS-blockade probably lies in the interstitium. Innovative therapies such as intervention in the EMT process and stem cell therapy are being developed currently. Until their feasibility in patients has been demonstrated, renoprotective treatment should be guided by the individual response to therapy, monitoring blood pressure and proteinuria as well as possible adverse effects during treatment schedules aimed at optimizing the response to RAAS blockade by dual blockade, and correction of volume excess.
References


