Proteinuria-associated renal injury and the effects of intervention in the renin-angiotensin-aldosterone system
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Chapter 1

General introduction
**Chronic renal disease**

Chronic renal disease (CRD) is a worldwide public health problem affecting an increasing number of patients. A substantial part of the patients with CRD progress to end-stage renal disease (ESRD) and need renal replacement therapy, such as dialysis or renal transplantation [2]. CRD is also associated with high morbidity and mortality, mainly due to cardio-vascular disease. CRD can result from a wide variety of causes, which eventually lead to progressive renal function loss by a final common pathway towards ESRD. In many patients blockade of the renin-angiotensin-aldosterone system provides renoprotection, apparent from reduction of proteinuria and blood pressure as intermediate parameters, and amelioration of the rate of renal function loss as eventual renal benefit. However, in many patients progressive loss of renal function still occurs, finally leading to end stage renal damage. Therefore, it is important to elucidate the mechanisms underlying the resistance to therapy, as identification of factors involved in therapy resistance may allow to design more effective treatment strategies to protect against end stage renal damage. As possible mediators of therapy resistance, factors pathogenetically involved in the progression of renal damage in the natural course of the disease would be logical candidates.

The rate of renal function decline shows a substantial variation between different patients, which allows identification of risk factors for progressive renal damage. A decline in renal function is predicted by clinical risk factors such as proteinuria [4,5], elevated blood pressure, low high density lipoprotein (HDL) and race [7], but cigarette smoking, obesity and poor diabetic control can enhance progression of CRD as well [2]. Hypertension and proteinuria are the main modifiable risk factors at which the current pharmacological treatment of CRD is targeted. To this purpose blockade of the RAAS, by angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II subtype 1 receptor blockers (AT1A) is currently therapy of choice in both diabetic and non-diabetic proteinuric chronic renal disease [8-11]. Studies on the effect of lipid modification are currently under way [12]. For obvious reasons data on renal histomorphological predictors are sparse in man. Nevertheless, the available data are consistent, and show that presence and severity of tubulointerstitial lesions predict the subsequent decline in renal function. Remarkably, in several studies the decline in renal function correlated more closely with interstitial fibrosis than with glomerular damage [13-16].

**Renin-angiotensin-aldosterone system (RAAS)**

Angiotensin II and aldosterone are considered the main effector molecules of the RAAS, a main regulator of blood pressure, renal hemodynamics and sodium balance (Figure 1). Activation of the cascade starts with renin release that catalyzes the conversion of angiotensinogen into angiotensin I. Angiotensin I is converted into angiotensin II by Angiotensin converting enzyme (ACE). Angiotensin II induces aldosterone release and acts via the angiotensin II subtype 1 (AT1) and type 2 receptors to induce systemic and renal vasoconstriction.
Treatment with ACE-inhibitors (ACEi) or AT1-antagonist antagonists (AT1A) induces vasodilatation of the efferent arteriole of the glomerulus resulting in reduction of glomerular pressure and systemic blood pressure. ACEi also reduces proteinuria mainly via a reduction of the glomerular pressure [1], preserved integrity of the slit diaphragm [3] and ameliorated podocyte foot process broadening [6], but other mechanisms may be involved too. Aldosterone is traditionally known for its effect on sodium and potassium balance, by its effects on the mineralocorticoid receptors in the distal nephron, leading to retention of sodium and water and excretion of potassium. In addition, aldosterone has profibrotic effects. RAAS-blockade by ACEi or AT1A reduces aldosterone, albeit not always permanently. Moreover, specific antagonists of aldosterone are available, such as spironolactone, that act as potassium sparing diuretics. In recent years, other components of the RAAS were identified that may have biological relevance as well. Smaller angiotensins, like angiotensin (1-7), that can be formed from angiotensin I as well as angiotensin II, have an antiproliferative and vasodilator effect that can counteract angiotensin II. Moreover, a recently discovered homologue of ACE - ACE2 - may act as the natural counterpart of ACE, by promoting breakdown of angiotensin II and formation of angiotensin (1-7). Thus, RAAS-action appears to involve a balance between vasoconstrictor and vasodilator effects, as well as a balance between pro- and anti-fibrotic effects.

**Figure 1.** Schematic representation of the RAAS. ACE: Angiotensin Converting Enzyme; NEP: neurtral endopeptidase; PEP: prolylendopeptidase

**Prevention of progressive renal function loss: current status and limits**
Over the last decade, the availability of effective antihypertensive and antiproteinuric intervention has afforded considerable progress in the protection against chronic progressive renal function loss. Blockade of RAAS by ACEi or AT1A has been proven particularly effective to that purpose. It has become increasingly clear that - in addition to effective blood pressure control - reduction of proteinuria is a prerequisite for effective long-term renoprotection. In spite of the recent progress, however, inter-individual differences in efficacy of renoprotective intervention
remain large, with significant residual proteinuria - and consequently long-term renal function loss - in many patients. Considering the prognostic impact of proteinuria reduction it is currently assumed - albeit not proven- that titration for maximal antiproteinuric effect will have the potential to improve long term renal prognosis. In this respect, treatment with ACEi or AT1A would be a logical first step, considering their proven antiproteinuric potential. Somewhat surprisingly, however, relatively few data are available on the appropriate dosing for optimal antiproteinuric effect - which may reflect their original introduction as antihypertensives. Usually doses of ACEi and AT1A are based on the dose-response for blood pressure. Yet, data on the antiproteinuric effect of non-hypotensive doses of ACEi demonstrated that responses of blood pressure and proteinuria are not necessarily concordant [17;18]. Taken together with the prognostic impact of antiproteinuric effect for long term renoprotection, these data prompt for exploration of the specific antiproteinuric potential of doses of ACEi higher than needed for maximal blood pressure reduction. This rationale is reinforced by animal data indicating a specific protective effect against renal fibrosis of very high doses ACEi or AT1A [19]. Moreover, data in heart failure patients showed that titration towards high dose (32,55-35 mg/day) lisinopril resulted in a slightly better survival than low dose (2,5-5 mg/day) [20].

Controversially, there is also data that titration on blood pressure of the ACEi spirapril strongly reduces proteinuria, however, doubling of the doses did not further reduce proteinuria. Does this mean that it is useless to increase the dose of an ACEi once blood pressure has stabilized? As noted above, in normotensive patients ACEi can reduce glomerular protein leakage in the absence of a fall in blood pressure [17;21], and a similar dissociation between reduction of blood pressure and proteinuria was recently also reported for the AT1A losartan [22]. Previously, a progressive antiproteinuric effect with doses up to 20 mg/day lisinopril was reported in normotensive subjects with IgA nephropathy [21] - in whom the maximum reduction of blood pressure was already obtained at 5 mg/day. Also, Laverman found a progressive reduction in proteinuria (and blood pressure) with increasing doses of 10, 20 and 40 mg/day lisinopril, in a mixed population of subjects with non-diabetic nephropathy [23]. Taken together, these data illustrate that the relative antihypertensive and antiproteinuric potency of increasing doses ACEi are apparently not similar across different studies and may depend on specific population characteristics, such as baseline blood pressure and proteinuria, which refutes straightforward generalizability of the present results. As to AT1A, the relative antihypertensive and antiproteinuric potency of increasing doses is also under study. Recent data on losartan indicate that the optimal antiproteinuric effect was obtained at a higher dose than the maximal blood pressure response in non-diabetic as well as diabetic nephropathy [24;25]. In a recent, uncontrolled study with candesartan, increasing the dose up to 96 (!) mg - independent of blood pressure control- was associated with a progressive reduction of proteinuria [26]. Thus, whereas there is data showing that supra-maximal dose ACEi does not provide a fit-for-all solution for

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better antiproteinuric efficacy, other studies strongly suggest that the therapeutic potential of increasing doses ACEi and AT1A has not been explored to the full.

Could high dose ACEi (or AT1A) have the potential to overcome therapy resistance in renal patients? Obviously, a conclusive answer would require long term data on the renoprotective effect of high dose ACEi - which are not available. However, it may be relevant to realize that between-patient differences in therapeutic efficacy of ACEi (and AT1A) by far exceed the differences in therapy response that so far have been obtained by using higher doses. Increasing the dose of enalapril from 10 to 20 mg, or losartan from 50 to 100 mg, slightly enhanced therapy response for the patient group as a whole, but failed to turn poor responses into good responses [27]. Moreover, the poor or good individual response persisted after switching from ACEi to AT1A or vice versa, and - in another subset of patients - after switching to NSAID. These data suggest that individual differences in responsiveness to antiproteinuric intervention are related to individual patient factors rather than to drug factors such as class of drug. An interesting, albeit retrospective, analysis in transplant recipients reported that the differences in antiproteinuric efficacy of a non-hypotensive dose fosinopril correlated to the severity of pre-existent vascular and interstitial renal lesions, providing a pathophysiological basis for individual differences in responsiveness to antiproteinuric therapy [18]. It would be relevant to know whether uptitration of ACEi or AT1A for antiproteinuric response could overcome such individual therapy resistance - and whether some patients would need a higher dose for optimal reduction of proteinuria than others. Alternatively, it would be interesting to see whether add-on therapy with spironolactone, which has antifibrotic effects in cardiac patients, might be able to overcome this type of renal resistance to RAAS-blockade, by a combination of diuretic and antifibrotic effects. Finally, animal data suggest that specific treatment targeting the renal interstitial inflammation, by for instance MMF, might be of use to overcome this type of resistance to RAAS-blockade [28], but so far no human data are available on this issue.

As to the effect of supramaximal dosing of RAAS-blockade, data in adriamycin nephrosis in rats indicate that neither a supramaximal ACEi nor dual blockade by ACEi plus AT1A could overcome the individual therapy resistance in animals with a poor antiproteinuric response to adequately dosed ACEi [29]. Thus, at least in this normotensive experimental model, the potential of rigourous blockade of the RAAS to overcome individual resistance to antiproteinuric therapy seems limited, and approaches combining RAAS-blockade with other modes of intervention (such as for instance statins [30]) may provide better perspectives.

In addition to blood pressure control, reduction of proteinuria is recognized as an independent and essential treatment target for renoprotection. Considering the prognostic impact of proteinuria reduction, perhaps the most promising strategy to improve renoprotection will be to titrate for antiproteinuric effect. Dose-response data for proteinuria may be helpful to this purpose, but it is important to recollect here that several measures have already been proven to be effective to enhance the antiproteinuric effect of ACEi. These include, in particular, control of
volume excess by dietary sodium restriction and/or diuretic - but also dietary protein restriction (and for selected patients co-treatment with NSAID). It is unlikely that use of high dose ACEi will alleviate the need for proper control of sodium status for optimal reduction of proteinuria. It should be noted, moreover, that adverse effects may limit the potential of a “maximal dose-maximal volume depletion” approach [31].

So, specific studies addressing the mechanisms of renal resistance to RAAS-blockade, the mechanisms of progressive renal damage during RAAS-blockade, and the development of additional strategies on top of RAAS-blockade for patients with a suboptimal response to RAAS-blockade are warranted. Considering its impact on long-term renal prognosis, the role of tubulointerstitial fibrosis deserves specific interest.

**Tubulointerstitial damage**

Tubulointerstitial damage can result from many different causes, and eventually occurs in almost any type of chronic renal damage. Whereas in some conditions (such as chronic interstitial nephritis) tubulointerstitial damage is the primary cause that initiates renal damage, in many instances tubulointerstitial damage occurs as a phenomenon secondary to glomerular diseases.

The development of interstitial fibrosis can be divided in different stages (illustrated in Figure 2). First, tubular injury occurs which activates the tubular epithelial cell. Injury to the tubular epithelial cell can be induced by several factors. Proteinuria is an important factor in the initiation of the tubulointerstitial inflammation. Excessive reabsorption of albumin and larger proteins by proximal tubular epithelial cells (PTEC) induces a release of chemokines, cytokines and growth factors [32;33]. Incubation of PTEC with glucose or lipids also induces release of pro-inflammatory factors [34;35]. Another important profibrotic factor is angiotensin II. Whereas circulating angiotensin II is involved in the maintenance of arterial blood pressure and regulation of renal hemodynamics, it also acts as a local intrarenal hormone. These local intrarenal effects are assumed to be involved in the profibrotic actions of angiotensin II.

The activation of the tubular cells induces secretion of cytokines, chemokines and growth factors, which initiates an inflammatory cascade. Macrophages are attracted by chemokines such as RANTES (regulated on activation normal T-cell expression and secreted), monocyte chemotactic protein-1 (MCP-1), tumour necrosis factor-α (TNF-α) and osteopontin. Macrophages are multifunctional cells capable of production of several factors contributing to ongoing tissue injury. They secrete factors that regulate matrix production by proliferation and activation of fibroblasts, such as transforming growth factor-β1 (TGF-β1), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF). Second, they also secrete vasoactive peptides - such as endothelin-1 and angiotensin II – and products that impair extracellular matrix degradation, as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMP) [36;37]. Therefore, macrophages play an important role in the initiation and progression of interstitial damage.
The inflammatory reaction induces the tubular epithelial cell to undergo apoptosis or tubular atrophy, which is the flattening of the epithelial cells. Cytokines and growth factors stimulate local fibroblasts to proliferate, and become myofibroblasts, characterized by α-smooth muscle cell actin (α-SMA) expression. Activated fibroblasts and myofibroblasts produce excessive extracellular matrix, which ultimately leads to interstitial fibrosis. Myofibroblasts are therefore important in the progression of interstitial fibrosis. They can be derived from different cell types. First, as mentioned above, local proliferation of fibroblasts contributes to an increase in myofibroblasts. Second, bone-marrow derived cells can transform into myofibroblasts [38]. And finally, epithelial to mesenchymal transformation (EMT) also contributes to the increase in myofibroblasts during early stages of interstitial fibrosis. During EMT, epithelial cells dedifferentiate and migrate to the interstitial space and transform to myofibroblasts [39].

![Diagram](Image)

**Figure 2.** Schematic presentation of the stages of tubulointerstitial fibrosis. TBM = tubular basement membrane. ECM = extracellular matrix.
SCOPE OF THIS THESIS

This study will focus on the mechanisms of resistance to renoprotective therapy with RAAS-blockade by investigating factors involved in the progression of renal damage as candidate-mechanism for therapy-resistance. We will investigate the prognostic impact of tubulointerstitial lesions and glomerular protein leakage at onset of treatment for the renoprotective effects of RAAS-blockade. Second we will study whether these intrarenal factors are reversible during RAAS-blockade to determine whether their persistence during therapy may be a factor in therapy resistance, and finally, we will investigate strategies to overcome resistance to RAAS-blockade. To be able to focus on intrarenal tubulointerstitial and glomerular involvement we used an experimental model of chronic renal damage: adriamycin-induced proteinuria. In this model, a single injection of adriamycin induces a gradually progressive proteinuria, that stabilizes after approximately 6 weeks. During the early phase of proteinuria, morphological changes are not yet present, but later on the disease progresses to focal glomerulosclerosis associated with severe tubulointerstitial damage.

The use of the experimental model allows to study a standardized renal disorder and to obtain renal tissue to analyze for tubulointerstitial and glomerular damage in a standardized fashion before onset of therapy. Assessment of the severity of prevalent renal damage at onset of therapy as a possible factor in therapy resistance is of considerable potential relevance to human renal disease, as (with the exception of type I diabetes, where patients are usually under treatment before renal damage develops) usually patients come to medical attention only after there is well-established renal damage, as early renal damage usually goes without appreciable symptoms. For obvious reasons, this issue is difficult to address in human, as there is not always clinical justification for doing a renal biopsy before onset of treatment.

The first section of this thesis addresses the role of tubulointerstitial and glomerular factors in therapy resistance to RAAS-blockade and the progression of proteinuric renal damage during RAAS-blockade. The second section of this thesis addresses the efficacy of therapeutic interventions adjunct to RAAS-blockade, to test whether these additional interventions can overcome resistance to RAAS-blockade and thus provide more effective renoprotection.

As noted above, retrospective data in man suggest that the extent of tubulointerstitial damage is a determinant of therapy resistance to RAAS-blockade. However, this hypothesis has never prospectively been tested. In chapter 2 we therefore prospectively investigate whether the extent of renal damage that is present at the start of renoprotective therapy is a determinant of the responsiveness or resistance to therapy with RAAS-blockade. To this purpose, in rats with established adriamycin-induced proteinuria, a renal biopsy was taken before the start of treatment with ACEi for assessment of histomorphological changes, and their predictive value for the antiproteinuric response to ACEi.
Osteopontin is one of the factors that attracts macrophages to the interstitium and may therefore well be involved in the pathogenesis of proteinuria-induced renal interstitial damage. In chapter 3 we studied whether osteopontin is involved in the development of proteinuria-induced renal interstitial fibrosis in a time-course study. To study whether osteopontin is involved in the therapy resistance and whether it might thus be a possible target for additional intervention on top of RAAS blockade, renal osteopontin expression was determined in a biopsy before treatment with ACEi, and after 6 weeks of treatment.

A recently discovered molecule, Kidney injury molecule-1 (Kim-1), is induced in proximal tubular epithelial cells after toxic and ischemic injury and shedded into the urine as well. Kim-1 has been suggested to play a role in the modulation of acute, and possibly chronic tubulointerstitial damage, but whether it is associated with proteinuria-induced chronic tubulointerstitial damage and resistance to RAAS-blockade has not been explored so far. The renal expression of Kim-1 in adriamycin nephrosis before and after treatment with ACEi or AT1A is therefore explored in chapter 4.

Proteinuria - reflecting glomerular leakage of proteins - is an important determinant of progression of renal diseases. The antiproteinuric effect of ACEi is mediated not only by reduced glomerular pressure, but likely also by a preserved integrity of the glomerular slit diaphragm. 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). A recent study in diabetic nephropathy suggests that increased expression of the catalysing enzyme heparanase is involved in loss of heparan sulfates. Whether loss of heparan sulfates, be it or not in relation to heparanase is of relevance as a determinant of therapy response to RAAS-blockade, has not been investigated. Therefore, we studied expression of heparan sulfate and its catalysing enzyme heparanase in adriamycin nephrosis before onset of RAAS-blockade, and during follow-up, in relation to therapy response, in chapter 5.

The second part of this thesis addresses the effect of interventions on top of RAAS-blockade on the reduction of proteinuria and glomerular and tubulointerstitial damage. As earlier mentioned, dietary sodium restriction enhances the efficacy of ACEi on intermediate parameters blood pressure and proteinuria. Therapy resistance often occurs, and sodium intake might be a factor to overcome this therapy resistance. Therefore, in chapter 6 we studied whether modification of dietary sodium intake can be used to overcome the negative prognostic value of renal damage prior to treatment with ACEi as found in chapter 2. We studied the effect of low, normal and high sodium intake combined with ACEi on proteinuria, blood pressure, glomerular and tubulointerstitial damage.

Another possible factor that might be involved in therapy resistance is aldosterone. Aldosterone, a determinant of the RAAS with profibrotic properties, is increased in a substantial part of the patients during treatment with ACEi, due to an escape mechanism. Blockade of
aldosterone resulted in additional reduction of proteinuria in two small studies in proteinuric patients already on ACEi treatment. The effect on renal damage however is not clear. Therefore, in chapter 7, we studied whether addition of the aldosterone-antagonist spironolactone to conventional treatment with ACEi leads to added efficacy in reducing proteinuria, blood pressure, glomerular and interstitial damage.

Finally, in chapter 8 the findings of the above stated chapters will be taken together and discussed in the perspective of studies by others. Based on this overview, recommendations for further research, as well as therapeutic implications in human disease are given.

References


