DISCUSSION AND FUTURE PERSPECTIVES
Aldosterone-escape-from-RAAS-blockade during intervention in sodium status: the next target?

In chapter 1 we found that sodium restriction reduces proteinuria and blood pressure. Moreover, we found that sodium restriction and diuretic therapy potentiate the antihypertensive and antiproteinuric effects of RAAS blockade. Sodium restriction not only reduces albuminuria,1 proteinuria and blood pressure (chapter 1), but it also reduces long term cardiovascular risk.2 Therefore, for renal patients, who are known to have a strongly elevated cardiovascular risk,3-5 it is of special importance to apply sodium restriction.

However, sodium restriction and diuretic treatment significantly increase plasma renin activity and aldosterone levels not only in the untreated condition, but also during RAAS-blockade by AT1 blockade (Figure 1, left panel, proteinuric patients from chapter 1)6 or by ACEi (data from prior study in healthy volunteers).7 ACEi therapy significantly decreases aldosterone during high and low sodium intake, due to decreased angiotensin II levels. AT1 receptor blockade, on the other hand, does not affect aldosterone levels during either diet in our nephrotic patients. Volume depletion increased plasma aldosterone during AT1 receptor blockade with the largest effect during the combination of sodium restriction and HCT, as well as during ACEi. A more or less similar pattern is shown for renin. Thus, during RAAS blockade, renin and aldosterone still respond to changes in volume status. Formerly, this preservation of the homeostatic response of the RAAS was considered neutral from the point of view of therapeutic efficacy, as angiotensin II was considered the main effector hormone of the RAAS. Currently, the pro-fibrotic effects of aldosterone are well-established,8-12 and recent studies suggest that renin can have direct, possibly pro-fibrotic effects, by the recently discovered (pro)renin receptor.13-16 Thus, better exploration of the role of the reactive increases in renin and aldosterone during volume depletion as a therapeutic measure is warranted.

The higher aldosterone-levels during volume-depletion measures are likely to be due to activation of the RAAS, considering the concomitant rise in renin. The decrease in aldosterone-to-renin ratio by AT1 receptor blockade and ACEi reflects the interference in the RAAS-cascade (Figure 1), but clearly during RAAS blockade volume depletion still induces a rise in renin as well as aldosterone.

During placebo, the aldosterone-to-renin-ratio is higher with a concomitant low sodium-
diet. This might well reflect the larger sensitivity of the adrenal cortex to angiotensin II during low sodium, but other factors like increased ACTH levels or a high metabolic acid load might also be involved.

During AT1 receptor blockade the rise in aldosterone due to low sodium was not associated with a change in the aldosterone-to-renin-ratio. The rise in aldosterone due to the addition of the diuretic however, was associated with a significant decrease in aldosterone-to-renin ratio, suggesting that the driving forces for aldosterone increase are not fully similar for low sodium diet and hydrochlorothiazide. Likely, the differences in serum potassium levels between the low sodium treatment periods and the diuretic treatment periods are involved.
What could be the relevance of the high aldosterone levels during RAAS blockade and volume depletion? As discussed extensively in our chapters 3 and 4, aldosterone can have deleterious effects on the kidney. Aldosterone is known to exert profibrotic effects through increased production of TGF-β, reactive oxygen species, PAI-1 and increased collagen gene expression and synthesis, which can be abolished by spironolactone.\(^8\)\(^\text{12}\) In addition, chapter 3 shows that aldosterone directly induces the heparan sulfate cleaving enzyme heparanase, exerting effects on tissue remodelling. Moreover chapter 4 shows that the aldosterone antagonist spironolactone attenuates neointima formation and glomerular fibrosis in an experimental model for CAN, suggesting that aldosterone plays a role in these processes. Thus, high aldosterone levels could contribute to progressive renal damage. In this respect, it should be mentioned that adverse renal effects of volume depletion on top of RAAS blockade were observed in healthy and proteinuric rats. In spite of a clear-cut antiproteinuric, and glomeruloprotective effect of volume depletion on top of RAAS blockade in the nephrotic rats, interstitial damage was considerably aggravated.\(^18\) Unfortunately, aldosterone levels were not available in this study.

Since aldosterone has adverse effects on the kidney, overcoming the aldosterone-escape-from-RAAS blockade induced by sodium restriction and/or diuretic treatment could be important for long term renoprotection. Several studies have shown that in proteinuric patients the addition of aldosterone receptor blockade to ACEi or AT1 receptor blockade leads to further reduction of proteinuria.\(^19\)\(^\text{22}\) However, from these clinical studies it remains largely unknown whether the benefits of aldosterone receptor blockade lie in its diuretic effects or in its ability to modulate non-volume-mediated effects of aldosterone. Moreover, it would be of interest to investigate whether spironolactone is specifically beneficial during sodium restriction. Therefore, future studies should investigate renal hemodynamics and extracellular fluid volume in a cross-over design with and without spironolactone during a high and a low sodium diet in proteinuric patients on conventional RAAS blockade.

The increase in renin during RAAS blockade may also be pathophysiologically relevant. (Pro)renin can directly contribute to tissue remodelling and renal damage through its profibrotic actions.\(^13\)\(^\text{16}\) Renin inhibition/ blockade may offer therapeutical options here. Recently, the ALTITUDE study started which investigates the renoprotective potential of renin inhibition by aliskiren on top of RAAS blockade with either ACEi or AT1 receptor blockade on hard end points in diabetic renal patients.\(^23\) For future studies it would be of
interest to also investigate this in non-diabetic proteinuric patients and whether renin inhibition/blockade is specifically beneficial during intervention in sodium status, when renin levels are higher.

So, it might be that future RAAS blockade based regimens may consist more and more of blockade at different levels of the RAAS-cascade simultaneously. So far, only for dual blockade, i.e. the combination of ACEi and AT1 receptor blockade, long term renoprotective benefits have been shown. It would be of interest to see whether volume depletion measures on top of dual blockade offers additional benefit. To this purpose, the investigator-driven DUAAAL study is currently ongoing. This randomized controlled cross-over trial investigates the antiproteinuric efficacy of sodium restriction on top of dual blockade; the results are keenly awaited.

Heparan sulfate and heparanase, evolving concepts in their role in renal physiology and pathophysiology

The questioned role in glomerular filtration

Glomerular filtration is not only dependent on renal hemodynamic factors (systemic blood pressure and glomerular pressure) and oncotic pressure defined by the protein concentration in the blood, but structural integrity of the glomerular basement membrane (GBM) and its size- and charge selective properties is also of major importance for glomerular filtration. The main structural components of the GBM include type IV collagens, laminin, fibronectin and heparan sulfate proteoglycans (HSPGs). Loss of heparan sulfate in the GBM is commonly present in glomerular diseases, especially during proteinuric conditions. This can be attributed to increased expression of the heparan sulfate cleaving enzyme heparanase.

In chapter 6 we showed that the effect of spironolactone monotherapy on proteinuria in adriamycin-induced nephropathy were modest and did not reach statistical significance, despite significantly reduced glomerular heparanase expression and a partially restored heparan sulfate expression in the GBM. This observation is in line with recent data that question the primary role of heparan sulfate in charge-selective glomerular filtration. Podocyte-specific agrin knock out mice, that have no agrin in the GBM which leads to the loss of heparan sulfate and anionic charge, did not have a compromised glomerular filtration.
tion barrier, even when challenged with an overload of bovine serum albumin. Moreover, the loss of heparan sulfate in the GBM in a mouse model with transgenic overexpression of heparanase did not lead to severe proteinuria. This was also shown by infusion of the bacterial heparan sulfate-degrading enzyme heparinase III, which did not result in proteinuria, despite severe charge disruption. However, it should be noted that these studies do not exclude a role for heparanase and/or heparanase-mediated heparan sulfate loss in the complex pathogenesis of proteinuria. Chronic loss of bioactive heparan sulfate fragments, heparan sulfate-bound factors, such as growth factors, cytokines and chemokines, the chronic disturbance of heparan sulfate-dependent glomerular cell-glomerular basement membrane interactions, and cellular responses induced by direct binding of the heparanase protein to glomerular cells, still may be involved in the development of proteinuria and its downstream effects on renal damage.

The role of heparanase in proteinuria is thus doubtful. However, the role of heparanase may be more diverse than previously anticipated, and it may be relevant to renal conditions other than primary proteinuric renal disease.

**Heparanase in future studies in CAN**

There are several reasons to study the renal expression of heparan sulfate and its degrading enzyme heparanase in CAN. First, CKD and CAN share common mechanisms of progressive renal damage and consequent renal function loss. Chapter 3 demonstrates that heparanase is upregulated in CKD and decreased by renoprotective treatment. Therefore, it would be of interest to investigate whether this is also the case in CAN.

Second, in chapter 3 we found that aldosterone directly induces heparanase mRNA and protein expression *in vitro*, which could be prevented by the aldosterone receptor antagonist spironolactone. In chapter 4 in experimental CAN we found that spironolactone ameliorated the development of glomerulosclerosis and transplant arteriopathy (TA), providing evidence for the contributing role of aldosterone in the pathogenesis of CAN. TA is the hallmark of CAN and is characterized by neointima formation resulting from uncontrolled smooth muscle cell proliferation. In future research it would not only be interesting to investigate glomerular expression of heparan sulfate and heparanase, but also their vascular expression in this model with TA and the effects of spironolactone intervention. These studies are currently ongoing.

Heparanase is not only an enzyme involved in pathology, but may exert beneficial effects
under certain conditions. For instance, heparanase is involved in tissue repair processes such as wound healing.\textsuperscript{28} It is normally present in healing wounds and elevated levels of heparanase facilitate tissue repair, most likely through an enhanced angiogenic response.\textsuperscript{28} Heparanase can be induced in endothelial cells by TNF\textalpha{} and IL-18 during inflammation.\textsuperscript{29}

CAN is one of the important causes of late allograft loss after kidney transplantation. CAN is a multifactorial process influenced by both alloantigen-dependent and independent factors.\textsuperscript{30} Since heparanase plays an important role in the remodelling of basement membranes and extracellular matrices and in tissue repair during inflammation, we investigated glomerular heparan sulfate and heparanase expression in a pilot study in an experimental model for CAN, the Fisher to Lewis model (Figure 2). We hypothesize that heparanase through its wound healing properties may exert beneficial effects on the glomerulopathy, vasculopathy and interstitial fibrosis seen in CAN. Furthermore, there is some evidence that AGE intervention by pyridoxamine (PM) prevents the loss of glomerular heparan sulfate. In rats with daily intravenous injections with AGE-modified albumin, increased glomerular volume, increased glomerular AGE deposition and decreased glomerular heparan sulfate content was prevented by PM after 6 weeks of treatment.\textsuperscript{31} Therefore, we also investigated the effects of intervention by PM on the glomerular expression of heparan sulfate and heparanase in this pilot study in CAN (Figure 2). Remarkably, isograft transplanted kidneys already had a significantly lower expression of heparan sulfate in the GBM compared to the healthy control kidneys, which was further and significantly reduced in the allografted kidneys, without differences between the PM and vehicle treated rats. Heparanase was significantly increased in the isografted kid-
neys as compared to control and further but non-significantly increased in the allografted kidneys. Heparanase expression was similar in the PM and vehicle treated rats. This would suggest that the transplantation process itself or the concomitant ischemia/reperfusion injury already increases glomerular heparanase and decreases heparan sulfate expression. The superimposed inflammation in the allografts further increases heparanase and decreases heparan sulfate, suggesting that inflammatory mediators contribute to this process.

Future studies, for example in kidney transplantation models with heparanase overexpression or podocyte-specific heparanase knock-out, would be of conceptual interest to elucidate whether the increased heparanase expression is indeed a beneficial effect mediating tissue repair in CAN. However, since kidney transplantation in the mouse is unfortunately not a feasible option, one could make a start with an ischemia/reperfusion injury model. Inhibition of heparanase by heparinoids could possibly shed more light on the role of heparanase in CAN. It should be noted that it would then be difficult to dissect the heparanse inhibiting effects of heparinoids from the pleiotrophic effects. In human studies with timed protocol biopsies in transplant recipients it would be of interest to investigate renal heparanase and heparan sulfate expression in relation with the development of CAN.

Future perspectives on monitoring tools for therapy response

A decade ago it was common practice to titrate the dose of RAAS blockade merely on blood pressure effects, but is has become more and more clear that for long-term renoprotection reduction of blood pressure and proteinuria should be independent treatment goals. Since proteinuria is a known predictor of subsequent renal function loss and a fall in blood pressure is not a prerequisite for a dose-dependent reduction of proteinuria, it is of special importance to titrate the dose of RAAS blockade on proteinuria. However, responders to antiproteinuric treatment may still show progressive renal function loss. Proteinuria generally reflects glomerular damage. Importantly, renal interstitial damage is consistently reported to be a major predictor of future renal function loss too. Experimental data in rats show that, in spite of a reduction in proteinuria, pronounced progression of renal interstitial damage can be present. So, therapy response to proteinuria and renal interstitial damage can dissociate. These data indicate that better or additional tools for the monitoring of therapy response are needed and that biomarkers for interstitial damage could be valuable for this purpose and for predicting renal outcome.

Based on chapter 2, urinary KIM-1 excretion, a biomarker for tubular damage, seems a
promising tool to monitor therapy response and target intervention. Future studies should investigate whether glomerular (proteinuria) and interstitial markers (KIM-1) should be independent treatment targets. Moreover, long-term follow-up studies should elucidate whether targeting treatment on KIM-1 can improve outcome in progressive renal function loss.

However, for future use in clinical practice it seems likely that next to targeting treatment on proteinuria and KIM-1 we will need other biomarkers to more precisely predict therapy response and renal outcome. For this purpose we will probably need “biomarker panels.” With the emergence of newer technologies, in particular mass spectrometry, it is now possible to study urinary protein excretion in more detail. Such proteomics-based approaches hold great promise. In our opinion combining interstitial markers, such as KIM-1 with glomerular markers as proteinuria and more specific with proteomics-based approaches could yield a valuable “biomarker panel” to facilitate earlier detection of renal disease, improve assessment of prognosis and allow closer monitoring of therapy response. Future studies should focus on validating specific “biomarker panels” in large renal patient populations.

**Systemic consequences of proteinuria: target for intervention?**

Not only proteinuria, but also the systemic abnormalities seen in non-diabetic renal damage can be part of the ongoing vicious circle of progressive renal function loss. Glomerular injury induces proteinuria. Proteinuria, in its turn then elicits a complex of systemic abnormalities, that intermingle with the abnormalities induced by the loss of glomerular filtration rate. Coagulation abnormalities, dyslipidaemia and accumulation of AGEs are all sequelae of proteinuria-induced renal damage that could also be involved in mediating further renal damage, and in the cardiovascular complications of renal disease. Those systemic factors are not yet a specific target for intervention in non-diabetic renal damage. Future research should investigate the role of those systemic factors as a target for intervention in non-diabetic proteinuria-induced renal damage.

*Coagulation abnormalities*

The association between the nephrotic syndrome and thromboembolic events has been known for decades; however, no data were available on the absolute risk of either venous
(VTE) or arterial thromboembolism (ATE). The reported risks of thromboembolism in patients with nephrotic syndrome were based on case reports and small studies, most with limited follow-up. Previous assessments of VTE risk have been based largely on asymptomatic cases of renal vein thrombosis, the clinical relevance of which is not clear. Therefore, we have assessed the absolute risks of symptomatic VTE and ATE in nephrotic range proteinuria in a large retrospective cohort study. Thromboembolism was many times higher than the estimated age- and sex-weighted absolute risks in the general population (about 10-fold for VTE and 7-fold for ATE), confirming the increased cardiovascular risk in proteinuric patients. The risk of thromboembolism was excessively high in the first 6 months of observation. Whereas the risk of VTE was related to the severity of nephrotic syndrome, the risk of ATE was related to estimated glomerular filtration rate and classic risk factors for atherosclerosis.

The mechanism explaining the increased risk for VTE and the hypercoagulability in nephrotic syndrome is incompletely understood. It is believed that the increased thrombotic activity is due to an imbalance between procoagulant/thrombotic and anticoagulant/antithrombotic factors in plasma. A variety of haemostatic abnormalities has been described, including increased levels of factor VIII, Von Willebrand Factor, α2 antiplasmin, high molecular weight fibrinogen moieties and decreased levels of protein S and antithrombin III. An increase in the protein C activity is thought to be one of the protective mechanisms. Low antithrombin III levels in nephrotic patients may both be due to urinary loss and/or intravascular consumption.

The findings from our retrospective cohort study highlight the need for prospective randomized trials to evaluate the coagulation abnormalities during nephrotic syndrome and the effect of current antiproteinuric treatment on these abnormalities and the risk of thromboembolism. We hypothesize that a reduction in proteinuria will ameliorate the systemic coagulation abnormalities seen in nephrotic syndrome, which would decrease the risk of thromboembolism. To make a start, it is interesting to investigate the effects of a stepwise reduction in proteinuria on the systemic coagulation abnormalities as a post hoc analysis in our non-diabetic proteinuric patients described in chapter 1. These studies are currently ongoing. Subsequently, a prospective randomized trial is needed to evaluate the effect of antiproteinuric treatment on the risk of thromboembolism. Furthermore, findings from our retrospective cohort study suggest that primary thromboprophylaxis could be beneficial, particularly during the first 6 months of nephrotic range proteinuria. Therefore, this should be evaluated prospectively in a randomized trial.
Lipid abnormalities
Dyslipidaemia (including increased total cholesterol, triglycerides and low density lipoprotein cholesterol concentrations and decreased high density lipoprotein cholesterol concentrations) is not only implicated in progressive renal function loss, but is also an important factor in the increased cardiovascular risk associated with CKD. Data from our own study described in chapter 1 show that a reduction in proteinuria with pharmacological (AT1 receptor blockade) and/or non-pharmacological measures (dietary sodium restriction) is accompanied by a decrease in plasma total cholesterol. Proteinuria reduction is indeed the main independent measure in the management of dyslipidaemia in CKD, which is associated with a decreased renal and cardiovascular risk.

Does specific management of dyslipidaemia have renal and cardiovascular benefits in CKD? A recent meta-analysis showed that statins are associated with lipid lowering, cardiovascular and antiproteinuric benefits in CKD in a setting of secondary prevention of cardiovascular events. At different stages of CKD, statin treatment reduced cardiovascular risk and mortality in a similar fashion to that seen in trials of statins in non-CKD populations. We should underline that Die Deutsche Diabetes Dialysis study (4D) is generally interpreted as a negative trial. This randomized controlled trial evaluated the effect of statin treatment on the primary endpoint (the composite of death from cardiac causes, non-fatal myocardial infarction and stroke) in haemodialysis patients. Except for the findings on stroke, the results from this trial are broadly in line with expectations from other trials. Statin treatment lowered the incidence of death due to coronary heart disease, but had no effect on sudden death. Since the incidence of death from non-atherosclerotic disease was very high and it is unlikely that statins prevent death from cardiac arrhythmias, statins did not reduce the incidence of the primary endpoint. The study of heart and renal protection (SHARP), which investigates whether simvastatin in combination with ezetimibe reduces renal and cardiovascular risk in a setting of primary prevention in patients with CKD or ESRD, is currently ongoing.

Currently, based on all the available evidence, the K/DOQI clinical practice guidelines for managing dyslipidaemias in kidney transplantation patients recommend to consider statin treatment if low density lipoprotein levels are >2.59 mmol/L. A recent meta-analysis showed no effect of statins on all cause mortality or cardiovascular mortality in renal transplant recipients. However, there is a trend towards a lower incidence of cardiovascular events with statin treatment. In line with this, the primary endpoint (cardiac death, non-
fatal myocardial infarction, or coronary revascularization) of the Assessment of Lescol in Renal Transplantation (ALERT) trial did not achieve statistical significance, but there were significant differences in cardiac death and nonfatal myocardial infarction favoring statin treatment. In renal transplant recipients the effects of statins on CAN have not been reported in large randomized trials. It is of interest that the pathogenesis of CAN shares many features with the pathogenesis of systemic atherosclerosis. Known risk factors for cardiovascular disease, like hypertension, proteinuria and hypercholesterolemia are independent risk factors for CAN. Future studies in renal transplant recipients should elucidate whether statins have beneficial effects on the development and progression of CAN.

In our current studies, we did not find renoprotective effects of PM in proteinuria induced renal damage. To the contrary, renal damage was aggravated when PM was given in combination with ACEi (chapter 8). Pronounced hypercholesterolemia which occurred in both PM treated groups, was accompanied by marked glomerular lipid deposition. In the combination group of PM with ACEi glomerular lipid accumulation was worse which was associated with more renal damage. The mechanism behind these renal effects needs further analysis.

We hypothesize that the liver X receptor (LXR) could play a role in the observed glomerular lipid deposition. The LXR is a nuclear hormone receptor, a ligand-activated transcription factor that regulates the expression of genes involved in lipid metabolism and plays an important role in cholesterol homeostasis. The LXR is not only expressed in the liver, but also in the kidney, in the proximal tubule and in the glomerular mesangium. In vitro, activation of LXR enhances ATP binding cassette transporter (ABCA)-1 expression, a membrane-associated transporter mediating cholesterol efflux. Recently an LXR agonist reduced glomerular and tubulo-interstitial lesions in a model for experimental glomerulonephritis, which was associated with increased ABCA-1 expression and reduced renal cholesteryl ester content. In two mice models of type 1 diabetes glomerulosclerosis was associated with increased renal triglyceride and cholesterol accumulation and decreased LXR and ABCA-1 expression. These results suggest that LXR is an important modulator in the regulation of lipid metabolism in the kidney, especially in maintaining intraglomerular lipid homeostasis. RAAS blockade with ACEi increases plasma renin levels (Figure 1). LXR is co-expressed with renin in juxtaglomerular cells in vivo. Moreover, through binding to the renin promoter, LXR enhances the transcription
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of renin. In future research it would be interesting to investigate whether PM downregulates renal LXR expression, impairing cholesterol efflux, which would explain the glomerular lipid accumulation in PM treated rats. We would expect that the increased plasma renin levels, as present during ACEi, would downregulate the LXR as a negative feedback mechanism. If so, then it is logical that most renal lipid accumulation occurs in the combined treatment group of PM and ACEi, since we expect this group to have the lowest LXR expression. Renal lipid accumulation as such explains the observed renal damage. Studies testing this assumption are currently ongoing.

Conclusion

Blockade of the RAAS by ACEi and/or AT1 receptor blockade is an effective tool for the reduction in blood pressure and proteinuria. This thesis shows advancements in the efficacy of renoprotective intervention of RAAS blockade by combined intervention in sodium status by sodium restriction and diuretic treatment. We also show that aldosterone receptor blockade by spironolactone is a promising tool to improve renoprotective intervention in CKD and CAN. In our opinion overcoming aldosteron-escape-from-RAAS blockade during intervention in sodium status should be the next topic of research in strategies for optimization of RAAS blockade based therapy in renal disease.

In addition to proteinuria lowering, correction of systemic derangements induced by proteinuria (coagulation abnormalities, dyslipidaemia and AGE accumulation), could have considerable impact on reducing renal and cardiovascular risk. However, the position of intervention in AGE formation in this respect is less clear. Based on the results of this thesis, we caution to study the AGE formation inhibitor pyridoxamine in renal patients, especially when combining pyridoxamine with ACEi. Coagulation abnormalities and dyslipidaemia, systemic factors that can modify the damaging effect of proteinuria, may guide the development of additional renoprotective strategies, such as anticoagulants or statins. At present, RAAS blockade remains the cornerstone of renoprotective therapy.
References


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