

# The renin-angiotensin-aldosterone system and its blockade in diabetic nephropathy

## Main focus on the role of aldosterone

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### THIS THESIS IS BASED UPON THE FOLLOWING ORIGINAL PAPERS:

1. Schjoedt KJ, Andersen S, Rossing P, Tarnow L, Parving H-H. Aldosterone escape during blockade of the renin-angiotensin-aldosterone system in diabetic nephropathy is associated with enhanced decline in glomerular filtration rate. *Diabetologia*, 2004;47:1936-1939
2. Schjoedt KJ, Jacobsen P, Rossing K, Boomsma F, Parving H-H. Dual blockade of the renin-angiotensin-aldosterone system in diabetic nephropathy: the role of aldosterone. *Horm Metab Res*. 2005;37 Suppl 1:4-8.
3. Schjoedt KJ, Rossing K, Juhl TR, Boomsma F, Rossing P, Tarnow L, Parving H-H. Beneficial impact of spironolactone in Diabetic Nephropathy. *Kidney Int*. 2005;68:2829-2836.
4. Schjoedt KJ, Lajer M, Andersen S, Tarnow L, Rossing P, Parving H-H. Aldosterone synthase (CYP11B2)-344T/C polymorphism and renoprotective response to losartan treatment in diabetic nephropathy. *Scand J Clin Lab Invest*. 2006;66:173-180.
5. Schjoedt KJ, Rossing K, Juhl TR, Boomsma F, Tarnow L, Rossing P, Parving H-H. Beneficial impact of spironolactone on nephrotic range albuminuria in diabetic nephropathy. *Kidney Int*. 2006;70:536-542.
6. Schjoedt KJ, Hansen HP, Tarnow L, Rossing P, Parving H-H. Long-term prevention of diabetic nephropathy: an audit. *Diabetologia*. 2008;51:956-961.
7. Schjoedt KJ, Astrup AS, Persson F, Frandsen E, Boomsma F, Rossing K, Tarnow L, Rossing P, Parving H-H. Optimal Dose of Lisinopril for Renoprotection in Type 1 Diabetic Patients with Diabetic Nephropathy. *Diabetologia*, 2009;52:46-49.
8. Schjoedt KJ, Christensen PK, Jorsal A, Boomsma F, Rossing P, Parving H-H. Autoregulation of glomerular filtration rate during spironolactone treatment in hypertensive patients with type 1 diabetes: a randomized crossover trial. *Nephrol Dial Transplant* 2009;24:3343-3349.

### ABBREVIATIONS

ABP, ambulatory blood pressure  
ACEI, ACE-inhibitor  
AngI, angiotensin I  
AngII, angiotensin II  
ARB, angiotensin II receptor blocker  
EH, essential hypertension  
ESRD, end-stage renal disease  
GFR, glomerular filtration rate  
RAAS, renin-angiotensin-aldosterone system  
RPF, renal plasma flow  
PA, primary aldosteronism  
PRA, plasma renin activity  
UACR, urinary albumin:creatinine ratio  
UAER, urinary albumin excretion rate

### 1. BACKGROUND

Diabetic nephropathy develops in as many as 25-40% of diabetic patients after 25 years of diabetes. This makes diabetic nephropathy the most common cause of end-stage renal disease (ESRD) in the western world (9) where it accounts for approximately 22% of patients starting dialysis in Denmark (10) and 44% in the U.S. (11). Diabetic nephropathy is characterised clinically by the occurrence of albuminuria, elevated blood pressure and a progressive decline in kidney function (9) and is associated with a marked increase in cardiovascular morbidity (12) and mortality (13). Before the introduction of renoprotective treatment, the median survival was 5-7 years after the onset of persistent albuminuria (9). However, within the last 25 years, intensive research has dramatically improved the treatment and thereby prognosis in diabetic nephropathy, as reviewed by Parving et al (14). A recent paper reported a median survival of more than 21 years from the onset of diabetic nephropathy in type 1 diabetic patients, mainly due to good blood pressure control (15) and decline in the incidence of ESRD has been reported in type 1 diabetic patients (16). Similar long-term observational data on survival in type 2 diabetic patients with diabetic nephropathy are not available, however it has been shown that reductions in proteinuria/albuminuria are associated with reduced risk for ESRD (17) and cardiovascular morbidity (18) and associated with improved survival (19) in type 2 diabetic patients.

Despite improvement in the prognosis large interindividual differences in response to therapy exist, thus the renoprotective effect is not complete and there are still patients with unacceptable fast disease progression. Therefore evaluations of

current treatment strategies, identification of new risk factors or risk markers as well as development of new treatment strategies are required.

The renin-angiotensin-aldosterone system (RAAS), figure 1, has long been known to play an important role in the initiation and progression of diabetic nephropathy (9). So far, focus of investigation has been mainly on the effects of angiotensin II (AngII). Blockade of RAAS by ACE-inhibitors (ACEI) and angiotensin II receptor blockers (ARB) has been shown to delay the initiation and progression of diabetic nephropathy in type 1 and type 2 diabetic patients (20-24). Consequently, ACEIs and ARBs are considered first line therapy for kidney protection in patients with diabetic nephropathy (22,24-28) but unfortunately this intervention can not prevent development of ESRD in all patients, so additional treatment options are needed. In recent years it has become clear, that aldosterone is not only responsible for the maintenance of fluid and electrolyte balance, rather it should be considered a hormone with widespread effects on the vasculature, the heart, and the kidneys.

## 2. AIMS

The main aim of this thesis was to evaluate the role of aldosterone in diabetic nephropathy and to evaluate the potential additional renoprotective effect of aldosterone antagonism with spironolactone on top of existing recommended treatment in diabetic nephropathy as reflected by short term changes in albuminuria and blood pressure. Furthermore, to evaluate whether spironolactone affects the ability to autoregulate GFR. In addition, some aspects of the existing guidelines recommending ACEIs for preventing and treating diabetic nephropathy in type 1 diabetic patients have been evaluated, including long-term effect of ACEI treatment in patients with microalbuminuria and optimal renoprotective dosing of ACEI in patients with diabetic nephropathy.

## 3. PATIENTS, DESIGNS AND METHODS

### 3.1 PATIENTS

All patients participating in the studies came from the Steno Diabetes Center. Except for the 'nephrotic range albuminuria' study (5) all patients had type 1 diabetes as defined by the World Health Organisation (WHO) (29). All patients had been insulin dependent from the time of diagnosis and all patients received at least two daily injections of insulin. In the study dealing with spironolactone treatment in nephrotic range albuminuria also patients with type 2 diabetes were included due to a very low number of type 1 diabetic patients with this condition at Steno Diabetes Center. Type 2 diabetes was diagnosed according to WHO criteria (29). The renal structural changes in type 1 and type 2 diabetic patients with diabetic nephropathy has been shown to be similar in previous biopsy studies if diabetic retinopathy is present (9), and there appear to be no substantial difference with respect to progression and treatment of diabetic nephropathy between type 1 and type 2 diabetic patients (30).

Studies are carried out in patients with persistent normoalbuminuria (8), microalbuminuria (6), macroalbuminuria (diabetic nephropathy) (1-4,7) and nephrotic range albuminuria (5) defined as follows:

- Normoalbuminuria, persistent urinary albumin excretion rate (UAER) < 30 mg/24-hour.
- Microalbuminuria, UAER between 30 and 300 mg/24-hour in at least 2 of 3 consecutive 24-hour urine collections.

- Macroalbuminuria, UAER higher than 300 mg/24-hour in at least 2 of 3 consecutive 24-hour urine collections.
- Nephrotic range albuminuria, UAER higher than >2500 mg/24-hour, corresponding to nephrotic range proteinuria >3500 mg/24-hour (31).

Diabetic nephropathy was diagnosed clinically if the following criteria were fulfilled: persistent macroalbuminuria, presence of diabetic retinopathy, and absence of any clinical or laboratory signs of other kidney or renal tract disease (9).

### 3.2 DESIGNS AND METHODS

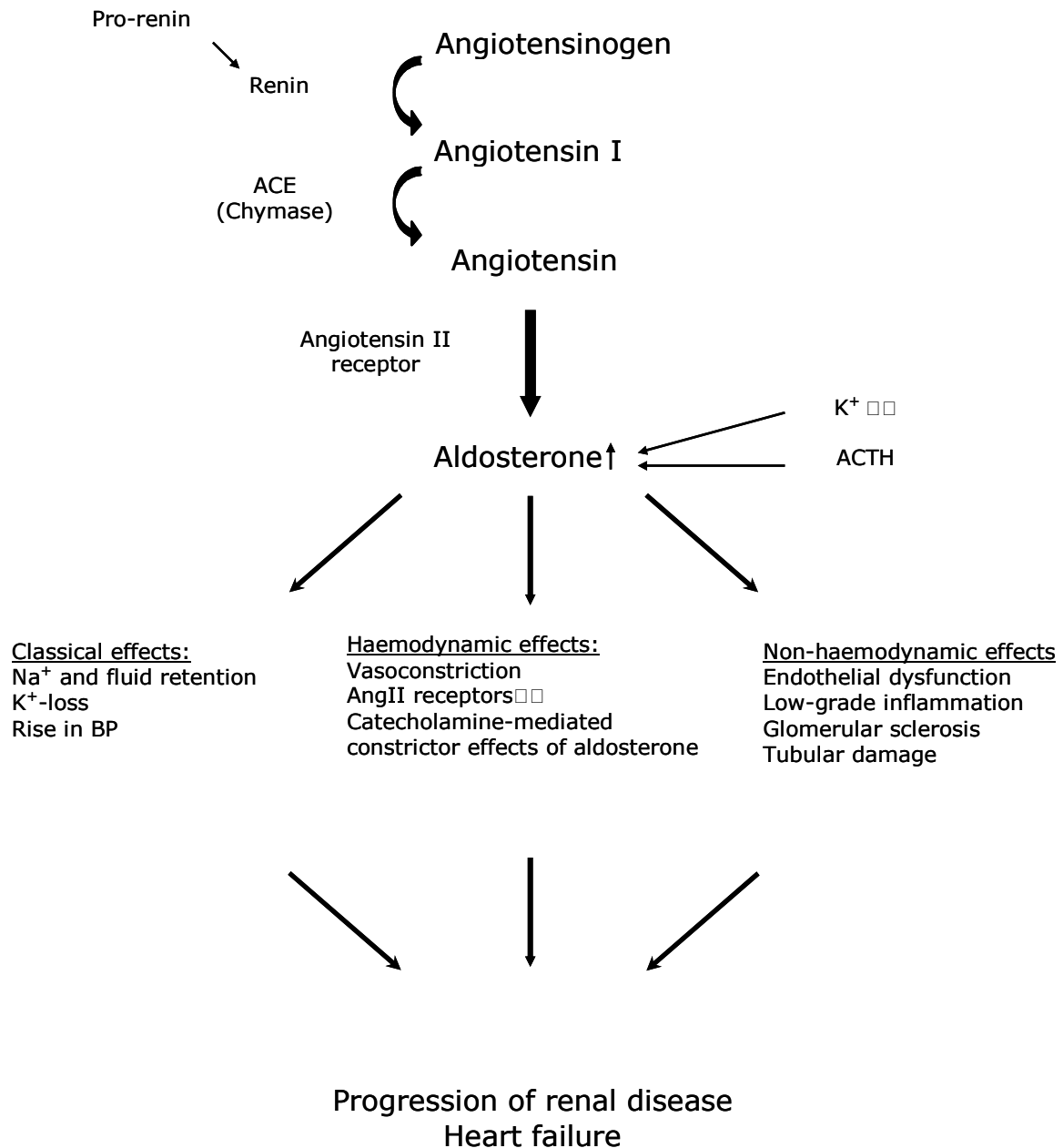
Three different types of designs were used:

1. Randomised, double-masked, crossover trials were used in the studies evaluating the renoprotective effect of spironolactone (3,5), the effect of spironolactone on renal auto-regulation (8) and in the lisinopril dose-titration study (7). In the spironolactone studies active treatment was compared with placebo whereas three different doses of active treatment were compared in the lisinopril study. The primary end-point was changes in albuminuria which has been shown to predict long-term renal and cardiovascular protection (17,18,32-35). All patients received both (all three) treatments and randomisation is used to determine the order in which the treatments are received. The results from crossover trials carries the risk of being influenced by a treatment-period interaction, i.e. a carry-over of treatment effect from one period to the next period, and by a period effect, i.e. a systematic difference between the two periods (36). In the spironolactone studies data were tested for a period effect and a treatment-period interaction with a two-sample t-test comparing the mean difference and the mean average, respectively, when patients were grouped according to order of treatment period as described by Altman (36). In the lisinopril dose-titration study, statistical software able to correct for treatment-period interaction and period effect was used (37).
2. The impact of aldosterone escape (1) and the role of CYP11B2 -344T/C polymorphism (4) in diabetic nephropathy during ARB treatment was evaluated in a prospective intervention trial, designed to investigate the long-term renoprotective effects of losartan in type 1 diabetic patients with diabetic nephropathy according to ACE/ID genotypes, which has been published previously (38). Samples for measuring plasma aldosterone were available in 63 patients and CYP11B2 -344T/C genotypes were available in 57 patients.
3. The audit, evaluating the long-term effect of blocking the RAAS with an ACEI or an ARB was an observational follow-up study (6). All type 1 diabetic patients with microalbuminuria were identified at Steno Diabetes Center in 1995 and followed until death, emigration or until the end of follow-up after 11 years in 2005.

Laboratory methods are described in detail elsewhere (1-8).

However it should be mentioned that in all of the intervention trials in diabetic nephropathy patients (1-5,7) endpoints were evaluated on the last day of each treatment period: i.e. UAER was measured in three consecutive 24-hour urine collections completed immediately before the end of each treatment period due to a large day-to-day variation, 24-hour ambulatory blood pressure (ABP) was measured using the A&D TM 2420/1 device and GFR was determined using <sup>51</sup>Cr-EDTA-plasma-clearance as

## The Renin-Angiotensin-Aldosterone System RAAS



**Figure 1**  
The renin-angiotensin-aldosterone system (RAAS).

described by Bröchner-Mortensen (39). All blood samples for determination of components of the RAAS were drawn after the patients had been resting in the supine position for at least 15 minutes (30 minutes in all spironolactone studies) at approximately 8.30 a.m. to avoid the influence of circadian rhythms and orthostatic changes. In the autoregulation study (8) another set of samples were drawn in the afternoon after a new period of 30 minutes of supine rest in order to construct similar circumstances although circadian rhythm could not be compensated for.

#### 4. THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

The renin-angiotensin-aldosterone system (RAAS), figure 1, has long been known to be involved in the initiation and progression of diabetic nephropathy as reviewed previously (40-42).

Renin is synthesized and released by the juxtaglomerular cells in the afferent arteriole of the kidney in response to a decrease in intravascular volume detected by baroreceptors (mediated by  $\beta$ -adrenoreceptor activation) and by a reduced sodium concentration at the macula densa. Renin catalyses the hydrolysis of angiotensinogen to angiotensin I (AngI) which is then converted to AngII by angiotensin-converting enzyme (ACE), present in the lungs and vascular tissue. AngII acts on vascular smooth muscle to cause vasoconstriction, and on the adrenal zona glomerulosa to stimulate aldosterone production. The adrenal response to AngII occurs within minutes, a time course that implies that no new protein synthesis is required. Chronic stimulation by AngII results in zona glomerulosa hypertrophy and hyperplasia, increased CYP11B2 expression and subsequent aldosterone secretion.

Conflicting data has been reported regarding RAAS activity and aldosterone levels in diabetic patients with and without diabetic nephropathy. The RAAS has been shown to be activated in type 1 diabetic patients (43) whereas results from type 2 diabetic patients varies between suppressed and activated, but with evidence of activated intrarenal RAAS (44-46). Both low/normal (47-49) and high (43) plasma aldosterone concentrations have been reported in type 1 diabetic patients with and without nephropathy. In non-diabetic kidney disease, Hene et al (50) found levels of plasma aldosterone elevated proportionally to the degree of renal failure in 28 patients with creatinine clearances below 50 ml/min, whereas Bianchi et al (51) found a highly significant association between plasma aldosterone levels and proteinuria in 165 patients with chronic glomerulonephritis. In 63 type 1 diabetic patients with diabetic nephropathy and well preserved kidney function (GFR > 60 ml/min/1.73m<sup>2</sup>), we found that plasma aldosterone levels were neither related to GFR levels nor to albuminuria (1).

##### 4.1 ALDOSTERONE: CLASSICAL AND NON-CLASSICAL ACTIONS

Aldosterone is a steroid hormone secreted primarily by the glomerulosa cells of the adrenal cortex. A number of factors have been shown to stimulate or inhibit aldosterone production, including sympathetic activation, vasoactive intestinal polypeptide, serotonin, atrial natriuretic peptide, dopamine and adrenomedullin (52). However, the principal regulators of aldosterone synthesis and secretion of aldosterone are AngII, the concentration of extracellular potassium and ACTH.

Classically, aldosterone acts on epithelial cells, particularly in the renal collecting duct, but also in the parotid gland, sweat glands, and colon, where it regulates the transport of Na<sup>+</sup>, K<sup>+</sup> and water. Aldosterone-responsive epithelial cell monolayers act as barriers separating the internal and external environment; they

also permit the reabsorption of Na<sup>+</sup> and water. These functions are facilitated by the lipid composition of the apical membrane and by the formation of high-resistance tight junctions. Transport through these cells is facilitated by an electrochemical potential across the apical membrane from urine to cell and by an active-transport mechanism across the basolateral membrane from cell to interstitium. Sodium reabsorption across the apical membrane is mediated by the luminal amiloride-sensitive epithelial Na<sup>+</sup>-channel (ENaC). Transport across the basolateral membrane is driven by the ouabain-sensitive Na<sup>+</sup>/K<sup>+</sup>-ATPase which drives the entry of sodium and the excretion of potassium from the cell to the lumen through the luminal K<sup>+</sup> channel. Water follows the movement of Na<sup>+</sup> across the monolayer. These are considered to be the principal mediators of aldosterone action in epithelial cells. However, other protein targets in the apical membrane have been identified, e.g. the luminal thiazide-sensitive Na<sup>+</sup>/Cl<sup>-</sup> co-transporter in the distal convolute tubule, which appear to mediate sodium reabsorption in response to volume depletion (53). While the overall action of aldosterone on electrolyte transport is clear, the exact mechanism by which it exerts these effects is unknown. Apical channel activity is the limiting step in the transport process, and it is likely that aldosterone ultimately acts to increase the open time of the existing ion channels or by increasing the total number of channels; current evidence suggests that it can do both (52).

Apart from regulating electrolyte and fluid homeostasis and thereby contributing to the blood pressure control, aldosterone has been shown to have non-epithelial effects, also referred to as non-classical effects. In experimental studies, it has been shown that circulating aldosterone per se has a nephrotoxic effect by inducing vascular and glomerular sclerosis, inflammation and tubular damage independently of AngII (54-60). Greene et al (57) found that introduction of hyperaldosteronism in ARB and ACEI treated rats, by exogenous infusion of aldosterone, restored most of the arterial hypertension, proteinuria and glomerulosclerosis observed in the untreated, subtotaly ablated kidney model. Furthermore, Rocha (58,59) found that AngII infusion in stroke prone spontaneously hypertensive rats treated with captopril and eplerenone (a selective aldosterone receptor antagonist) resulted in a modest degree of nephrosclerosis compared to animals treated with captopril alone, suggesting that the harmful effect of AngII, at least in part, is mediated by the stimulation of increased aldosterone release.

Hemodynamic and non-hemodynamic actions of aldosterone have been suggested to contribute the progressive renal injury (57). These actions can be mediated through genomic mechanisms via the intracellular mineralocorticoid receptor or through fast non-genomic actions characterized by rapid onset, and insensitivity to spironolactone and agents inhibiting transcription and protein synthesis; e.g. aldosterone has been shown to induce a fast non-genomic vasoconstriction (61,62), as well as having a slower hemodynamic action, including upregulation of AngII receptors (63,64) and increased vasoconstrictive effects of catecholamines (65). Recently, highly significant correlations between aldosterone-to-renin ratios and measures of arterial stiffness were reported from the Framingham Heart Study (66). In experimental studies, it has been demonstrated that aldosterone induces vasoconstriction of both afferent and efferent arterioles with a higher sensitivity of the efferent arteriole (61), i.e. aldosterone has the potential of increasing the intraglomerular pressure, which is an important

**Table 1.** Changes in circulating components of the RAAS during treatment with different RAS blocking agents.

\*Data from various studies as indicated; \*\* Ref. 228 reported no change, ref. 229 reported a reduction in urinary aldosterone excretion, see text (section 5.7); \*\*\* Aldosterone escape during long-term ACEI or ARB treatment in a proportion of patients, see text (section 4.2.1).

	Renin inhibitor (228,229)*	ACE inhibitor (7)*	Angiotensin II receptor blocker (38)*	Spirolactone (3,5)*
Prorenin	↑↑			
Renin conc.	↑↑	↑↑	↑↑	
Renin activity	↓↓	↑↑	↑↑	↑↑
Angiotensin I	↓↓	↑↑	↑↑	
ACE-activity	↓↓	↓↓		
Angiotensin II	↓↓	↓↓	↑↑	↑↑
Aldosterone	↓**	↓(↑***)	↓(↑***)	↑↑

factor in the development and progression of diabetic and non-diabetic glomerulopathies as demonstrated in experimental settings (67). Aldosterone excess has also been shown to be associated with endothelial dysfunction in non-diabetic patients with hypertension (68-70), and in human endothelial cell monolayer cultures, Oberleithner et al (71) demonstrated that increasing the extracellular sodium concentration above a threshold of 135 mmol/l (i.e. within the physiological range) increased endothelial cell stiffness when aldosterone was present, but not in the absence of aldosterone. Overall, there is evidence for extrarenal mechanisms whereby aldosterone produces hypertension, primarily by its direct vasoconstrictor effects and by altering vascular compliance as reviewed by Epstein and Calhoun (72).

The non-hemodynamic effects of aldosterone have been suggested to include upregulation of the pro-sclerotic growth factors PAI-1 and TGF-β1, as well as promotion of macrophage infiltration, consequently leading to renal fibrosis (73). In adriamycin induced nephrosis, van den Hoven et al recently demonstrated that aldosterone induces glomerular heparanase expression leading to decreased expression of heparan sulphate (74). It has been proposed that decreased heparan sulphate content of the glomerular basement membrane (as observed in diabetic nephropathy) causes decreased permeability to negatively charged macromolecules such as albumin, allowing this protein to leak into the urinary space (75). In the study by van den Hoven et al, administration of spironolactone restored heparan sulphate expression in the glomerular basement membrane and reduced glomerular heparanase expression, which did not however lead to a reduction in proteinuria in this rat model (74).

Taken together, aldosterone should be considered a hormone that in addition to regulating electrolyte and fluid homeostasis has widespread actions through genomic and non-genomic effects in tissues not originally considered target tissue for aldosterone, such as vasculature, CNS and heart (72,76).

#### 4.1.1 Primary hyperaldosteronism

The classical features of primary aldosteronism (PA), i.e. hypertension, hypokalemia and metabolic alkalosis were first described by J. Conn in the mid-fifties of the last century. Already at that time, Conn reported proteinuria in 85%, and decreased concentrating ability in 80%, but otherwise normal kidney function in more than 60% of patients with PA (77). More recent studies have suggested that PA is associated with excessive urinary albumin excretion compared to patients with essential hypertension matched for duration and degree of hypertension (78,79). Furthermore, GFR has been suggested to be influenced by aldosterone excess. In a small study in patients with PA and a control group with essential hypertension, well matched for blood pressure level and duration of hypertension, baseline GFR was higher in PA patients than in patients with essential hypertension. However, after surgical removal of the aldosterone pro-

ducing adenoma, GFR declined by 15 ml/min/1.73m<sup>2</sup> and effective renal plasma flow (ERPF) by 54 ml/min/1.73m<sup>2</sup> (80). This relatively increased GFR during aldosterone excess followed by a marked reduction after treatment was suggested to reflect hyperfiltration due to elevated intraglomerular hydrostatic pressure during the state of aldosterone excess (80), as discussed further in section 5.3.2.

Primary aldosteronism is now considered one of the most common causes of secondary hypertension with a prevalence as high as 5-20% in patients with resistant hypertension (81-83). Although a validated and standardized diagnostic protocol for this entity is still missing, recent studies established the aldosterone to renin ratio as a useful screening test (82,84), and a straightforward three phase diagnostic approach has been suggested: case-finding tests, confirmatory tests and subtype evaluation tests as described by Young et al (84).

Patients in our studies do not fulfil the criteria for PA, i.e. we are treating a 'relative hyperaldosteronism'.

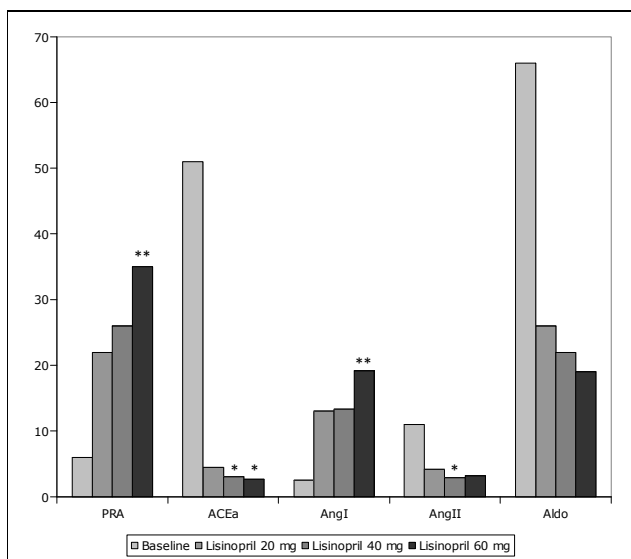
#### 4.2 ALDOSTERONE DURING BLOCKADE OF THE RAAS

As the name says, RAAS-blocking treatment reduces the activity of the RAAS downstream from the blockade. Furthermore, a compensatory increase is observed in RAAS components upstream from the blockade due to the tight feedback mechanisms, as depicted in table 1. The degree, to which e.g. PRA is increased, is widely recognised as a marker for the degree of RAAS-blockade.

Because AngII is probably the most important stimulus for aldosterone secretion, it has been assumed that RAAS blockade by an ACEI, ARB or the combination of both would suppress the downstream secretion of aldosterone. However, aldosterone levels have been reported to increase during long-term RAAS blocking treatment, a phenomenon known as 'aldosterone escape' or 'aldosterone breakthrough', as reviewed recently (85). For the present review the term 'aldosterone escape' will be used.

##### 4.2.1 Aldosterone escape: Definition and incidence

Aldosterone escape has been defined in somewhat different ways in the literature, e.g. some groups have defined aldosterone escape as a rise in plasma aldosterone during long-term ACEI therapy compared to pre-treatment levels (86,87), whereas others have defined aldosterone escape as aldosterone levels exceeding normal range after long-term RAAS blockade (88-90). We defined aldosterone escape as an increase in plasma aldosterone levels during long-term RAAS blockade, not compared to pre-treatment levels but to aldosterone levels after 2 months treatment (1), i.e. 'escape' from the initial treatment response, in accordance with others (91). Finally, aldosterone escape has been defined as aldosterone levels incidentally exceeding 80 pg/ml (mean value in a group of healthy subjects)



**Figure 2**  
Circulating RAAS components at baseline and during treatment with lisinopril 20, 40, and 60 mg daily, in 49 type 1 diabetic patients with diabetic nephropathy. Units on ordinat: Plasma levels of PRA (ngAl/ml/hour), ACE-activity(units), Angiotensin I x 10<sup>-1</sup> (pmol/l), Angiotensin II (pmol/l) and aldosterone (pg/ml). All treatment values were significantly different from baseline. \*P < 0.05 vs. 20 mg, \*\*P < 0.05 vs. 20 and 40 mg

one or more times during 18 months of ACEI treatment irrespective later suppression of aldosterone (92).

With the various definitions the incidence of aldosterone escape has been reported to vary between 10-38% in chronic heart failure (88-90,92). Data on aldosterone escape in kidney disease are limited, but Sato et al (86) found that plasma aldosterone levels increased in 40% of type 2 diabetic patients with micro- or macroalbuminuria and creatinine clearance >60 ml/min, during 40 weeks of ACEI therapy. In our study in 63 type 1 diabetic patients with diabetic nephropathy, aldosterone escape developed in 26 patients (41%) during long-term ARB treatment as described more detailed in section 4.3. Subsequently, an incidence of aldosterone escape of 22% has been reported in type 2 diabetic patients with micro- and macroalbuminuria (93) and 53% in IgA nephropathy (87), altogether suggesting, that aldosterone escape is not a rare phenomenon.

#### 4.2.2 Aldosterone levels during dual blockade of the RAAS or ultra high doses of RAAS blocking agents

Incomplete blockade of RAAS with ACEI or ARB treatment has been suggested as a mechanism behind aldosterone escape. We therefore evaluated plasma aldosterone levels during dual blockade of the RAAS and during treatment with RAAS-blocking agents at doses exceeding the current recommendations. Plasma aldosterone levels were evaluated

in a combined analysis of three randomised, double-masked, crossover trials where a total of 51 type 1 diabetic patients with diabetic nephropathy received 8 weeks of dual blockade using an ARB in combination with ACEI and 8 weeks of monotherapy with the same ACEI (2). Plasma aldosterone levels were measured at the end of each treatment period. The study showed that dual blockade of RAAS induced a further reduction in plasma aldosterone levels of 28% (95% CI: 11 to 42%, P<0.01) compared to ACEI monotherapy (2). In a multiple linear regression analysis changes in aldosterone, diastolic blood pressure, GFR and ACE/ID genotypes were associated with changes in albuminuria. Fifteen pa-

tients had an increase in plasma aldosterone levels during dual RAAS blockade compared to ACEI monotherapy whereas 36 patients had unchanged or reduced levels of plasma aldosterone. However, the duration of dual blockade treatment was only 2 months in our study; i.e. according to our definition of aldosterone escape, we could only evaluate the initial response to dual blockade and there is a possibility that aldosterone levels would increase during long-term treatment. In fact, a similar reduction was observed after 17 weeks of dual RAAS blockade compared to ACEI monotherapy in 768 patients with congestive heart failure (94); however after 43 weeks of treatment aldosterone levels were identical in the two groups; indicating that dual RAAS blockade only offers temporary further suppression of circulating aldosterone levels. This is further supported by a study reporting a similar incidence of aldosterone escape during dual blockade of RAAS compared to ACEI and ARB monotherapy after one year of treatment in 43 patients with non-diabetic nephropathy (87).

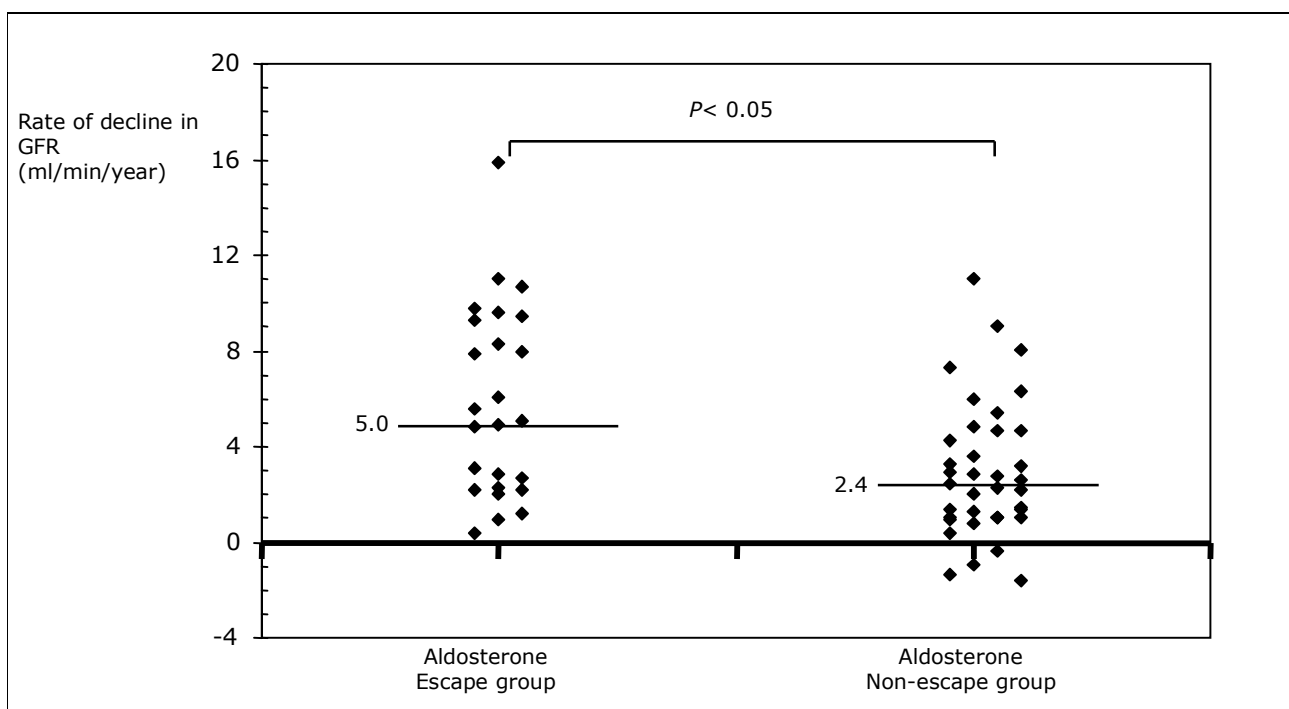
Ultra-high doses of ACEIs or ARBs constitute another strategy for overcoming incomplete RAAS blockade in diabetic nephropathy as discussed in section 5.2.1. We evaluated plasma aldosterone levels during treatment with high doses of lisinopril (20, 40, and 60 mg once daily) in 49 type 1 diabetic patients with diabetic nephropathy, in a randomised, double-masked crossover trial (7). All doses of lisinopril induced a reduction in plasma aldosterone compared to baseline, with no statistically significant difference between the three doses, figure 2. Changes in plasma aldosterone levels were not associated with changes in UAER (7). Presence and absence of aldosterone escape could not be determined due to the short duration of treatment periods. In a dose-titration study of similar design in 52 type 2 diabetic patients with microalbuminuria, we found that the currently recommended dose of irbesartan 300 mg daily did not induce a suppression of aldosterone levels, whereas the two higher doses (irbesartan 600 and 900 mg) induced a statistically significant reduction in plasma aldosterone of approximately 30% compared to baseline (95). Again long-term suppression of aldosterone and presence or absence of aldosterone escape could not be determined due to short treatment periods (2 months).

#### 4.2.3 Aldosterone during spironolactone treatment

During treatment with aldosterone antagonists there is a compensatory increase in circulating levels of aldosterone (3,5). These increased aldosterone levels could potentially be harmful if the role of aldosterone in initiation and progression of diabetic nephropathy was predominated by the non-genomic actions of the hormone which are not blocked by aldosterone antagonists. So far clinical trials in diabetic and non-diabetic nephropathies, as well as in heart failure studies, have shown that aldosterone antagonism with spironolactone or eplerenone is associated with improved clinical outcome as discussed below.

#### 4.3 CLINICAL IMPLICATIONS OF ALDOSTERONE ESCAPE IN DIABETIC NEPHROPATHY

Knowledge about the implication of aldosterone escape is limited in diabetic nephropathy. Originally, Walker (96) demonstrated that systolic blood pressure, hyperangiotensinaemia, and hyperaldosteronaemia in a longitudinal observational study acted as independent predictors of more rapidly declining kidney function (reciprocal creatinine slope) in a heterogeneous group of type 1 and type 2 diabetic patients suffering from microalbuminuria. Sato et al found that aldosterone escape in type 2 diabetic patients with micro- or macroalbuminuria was associated with higher UAER than patients



**Figure 3**  
Rate of decline in GFR during long-term losartan treatment, in type 1 diabetic patients with diabetic nephropathy, according to presence or absence of aldosterone escape.

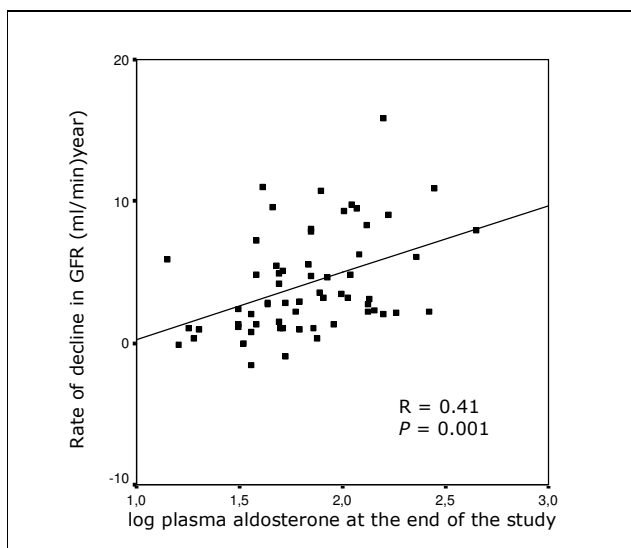
without aldosterone escape (86). No data were available in type 1 diabetic patients with overt nephropathy and more importantly; no data were available on the effect of aldosterone escape on progression of diabetic nephropathy. We therefore investigated the incidence and impact of aldosterone escape in 63 hypertensive type 1 diabetic patients with diabetic nephropathy during long-term treatment with the ARB losartan (1). Aldosterone levels were evaluated at baseline (after a 4-week washout period where all antihypertensive medication was withdrawn), after 2 months losartan treatment, and finally after a mean follow-up of 35 months treatment with losartan 100 mg daily. Additional antihypertensive treatment was allowed after 4 months losartan treatment in order to achieve a target blood pressure of 135/85. Overall, there was no change in plasma aldosterone levels during losartan treatment, neither after 2 months treatment, nor after 35 months treatment (1). However, 26 patients (41%) developed aldosterone escape during long-term losartan treatment defined as higher aldosterone levels at the end of follow-up compared to levels after 2 months treatment. To evaluate the clinical implication of aldosterone escape in patients with diabetic nephropathy, we compared rate of decline in GFR, albuminuria and blood pressure between patients with aldosterone escape ('escapers') and without aldosterone escape ('non-escapers'). Escapers had a significantly faster rate of decline in GFR than non-escapers as depicted in figure 3. Furthermore, changes in aldosterone and end-of-study values of aldosterone correlated significantly with rate of decline in GFR; i.e. the greater the increase in aldosterone and the higher the end-of-study value during long-term losartan treatment the faster the rate of decline in GFR, figure 4 and 5. There were no statistically significant differences in blood pressure and albuminuria between the two groups. A multiple regression analysis revealed that systemic blood pressure and aldosterone escape independently contributed to the enhanced rate of

decline in GFR, whereas albuminuria, HbA1c, baseline GFR and ACE/ID genotypes did not. It should be mentioned that if we applied the escape definition proposed by Sato et al (86) (i.e. rise in plasma aldosterone during long-term ACEI therapy compared to pre-treatment levels), we still found a significant correlation between changes in plasma aldosterone and rate of decline in GFR. Only three patients (5%) in our study had aldosterone levels exceeding normal range after long-term losartan treatment.

In 43 patients with IgA nephropathy, Horita et al (87) found that patients with aldosterone escape had significantly higher urinary protein excretion than patients without aldosterone escape during monotherapy with either temocapril or losartan, whereas dual blockade with the combination of the two eliminated this difference between escapers and non-escapers. In contrast they found no difference in kidney function between patients with and without aldosterone escape.

#### 4.3.1 Mechanism of aldosterone escape

The mechanisms involved in the aldosterone escape phenomenon are poorly understood. Incomplete RAAS blockade, lack of treatment compliance, variation in sodium intake, potassium homeostasis, pharmacogenetics, differences in the angiotensin II production at tissue level, and the sensitivity of the adrenal gland to angiotensin II may be involved. In our study (1) the two groups were alike with respect to demographic, clinical and laboratory data except for a lower plasma renin concentration in the escape group at baseline. A lower circulating level of renin has been reported to reflect increased intrarenal syntheses of angiotensin II by Hollenberg and his group (97). The enhanced initial reduction in aldosterone with later escape to pretreatment levels is fitting this concept. Since the circulating levels of renin and angiotensin II were similar in escapers and non-escapers during ARB treatment in our study, we can rule out major differences in compliance to losartan treatment.

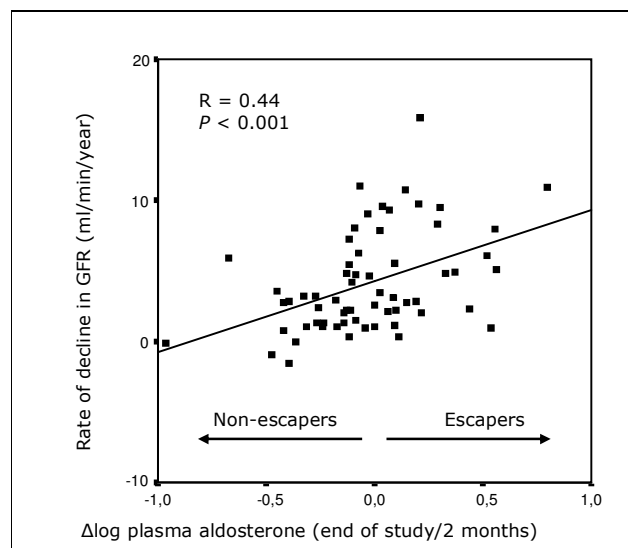


**Figure 4**  
Linear regression analysis between plasma aldosterone levels at the end of long-term losartan treatment and rate of decline in GFR during the same period, in 63 type 1 diabetic patients with diabetic nephropathy. A higher plasma aldosterone level corresponds to a faster rate of decline in GFR.

Furthermore the pattern of additional antihypertensive treatment, including diuretics, was similar in the two groups, as was plasma potassium levels and urinary K/Na-ratios, thus indicating, that the salt intake did not differ between groups. The insertion/deletion polymorphism of the ACE gene has been suggested to play a role for the aldosterone escape phenomenon (88), but since the distribution of the I/D alleles in the two groups did not differ in our study, this is hardly the explanation for our finding (1). There was a tendency that patients carrying the T-allele of the -344T/C polymorphism of the CYP11B2 (aldosterone synthase) gene (discussed below) were more likely to develop aldosterone escape, figure 6. In studies using ACEIs as RAAS blocking treatment, AngII reactivation (also known as AngII breakthrough or ACE-escape) has been proposed as a mechanism for aldosterone escape. However, in 22 patients with heart failure, aldosterone escape and reactivation of AngII were found not to occur simultaneously (92).

#### 4.4 ROLE OF THE ALDOSTERONE SYNTHASE GENE IN HYPERTENSION AND DIABETIC NEPHROPATHY

Aldosterone is synthesized from cholesterol in the zona glomerulosa of the adrenal cortex by a series of enzymatic reactions catalysed by dehydrogenases and mixed-function oxidases, many of which belong to the cytochrome P450 (CYP) superfamily, as reviewed by Connell (52). Of these, the CYP11B2 encodes for the aldosterone synthase which catalyses the last three steps of the synthesis from 11-deoxycorticosterone to aldosterone. Thus, the role of the CYP11B2 locus in hypertension and cardiovascular disease has been extensively evaluated. In humans, several frequent polymorphisms have been described. In particular the -344T/C polymorphism located in the 5' distal promoter region of the CYP11B2 gene. Conflicting data on the impact of this polymorphism on hypertension and aldosterone levels has been published (98-101), as reviewed in a large meta-analysis by Sookoian et al (102). The main finding from the meta-analysis was a lower risk of hypertension in patients homozygous for the C-allele (CC patients). Furthermore, they found a significantly lower PRA in CC patients, whereas there was no



**Figure 5**  
Linear regression analysis between changes in plasma aldosterone levels and rate of decline in GFR during long-term treatment with losartan, in 63 type 1 diabetic patients with diabetic nephropathy. A larger increase in aldosterone levels corresponds to a faster rate of decline in GFR.

difference in plasma aldosterone levels between genotypes (102). One should however be cautious in interpreting these results due to massive heterogeneity between included studies, exclusion of heterozygous patients, problems with turning blood pressure into a dichotomous variable (with a rather high limit for hypertension), and choice of statistical methods as discussed by Staessen et al (103).

In studies in hypertensive patients, no difference in the severity of hypertension was seen between genotypes (102,104). Correspondingly, blood pressure at baseline was the same in all three genotype groups in our study where we evaluated baseline (no antihypertensive treatment) levels of blood pressure, albuminuria, GFR, and plasma aldosterone in 57 hypertensive type 1 diabetic patients with diabetic nephropathy (4). No statistically significant differences between genotypes were found. Patients with the TT genotype had a higher GFR at baseline compared to patients with CT and CC genotypes, which was however most likely due to insignificant differences in age, duration of diabetes, duration of nephropathy and in blood pressure (4). The lack of significant differences in blood pressure may have been due to small numbers, i.e. in a larger long-term observational follow-up study in 163 type 1 diabetic patients with diabetic nephropathy treated with an ACEI, we observed a higher systolic and diastolic blood pressure during follow-up and at baseline respectively, in patients carrying the T-allele of the gene compared to patients homozygous for the C-allele (105). There was no difference in rate of decline in GFR between the genotype groups during 6 years of follow up (105).

In a study by Lovati et al (106) including 32 patients with ESRD due to diabetic nephropathy and 37 diabetic controls, the -344T/C polymorphism of the CYP11B2 gene was not associated with progression of diabetic nephropathy. We performed a large case-control study comparing 422 type 1 diabetic patients with overt diabetic nephropathy and 479 type 1 diabetic patients with persistent normoalbuminuria and long-standing diabetes and found no significant differences between cases and controls in either genotype distributions (cases TT 0.33, TC 0.48, CC 0.19; controls



TT 0.32, TC 0.48, CC 0.20) or allele frequencies (cases T/C 0.57/0.43; controls T/C 0.56/0.44) (105). In another study evaluating the impact of the same polymorphism in type 2 diabetic patients and healthy controls, there was no difference in genotypes between patients with normo-, micro- or macroalbuminuria (107). However, a higher frequency of the TT genotype and T allele was observed in patients with diabetes, than in healthy controls. Furthermore, they found that the TT genotype was associated with higher blood pressure and higher aldosterone levels (108).

The antihypertensive response to ARB treatment in patients with different -344T/C genotypes, has previously been investigated in studies of non-diabetic hypertensive patients (104). In 43 non-diabetic patients with primary mild-to-moderate hypertension and left ventricular hypertrophy, Kurland et al (104) found that patients homozygous for the T allele of the -344T/C polymorphism had a more pronounced reduction in systolic blood pressure after 3 months ARB treatment than TC and CC genotypes. In our study in 57 type 1 diabetic patients with diabetic nephropathy, we found no such difference in response to treatment with the ARB, losartan (4), neither when we looked at the three genotypes, nor when we compared patients homozygous for the T allele with patients carrying the C allele, as suggested previously (104).

Overall, there is conflicting evidence for the role of the -344T/C polymorphism in relation to aldosterone levels and hypertension. Our studies support the possibility of higher blood pressure in patients carrying the T-allele of the gene. However, we can rule out a major contribution of the polymorphism on development and progression of diabetic nephropathy.

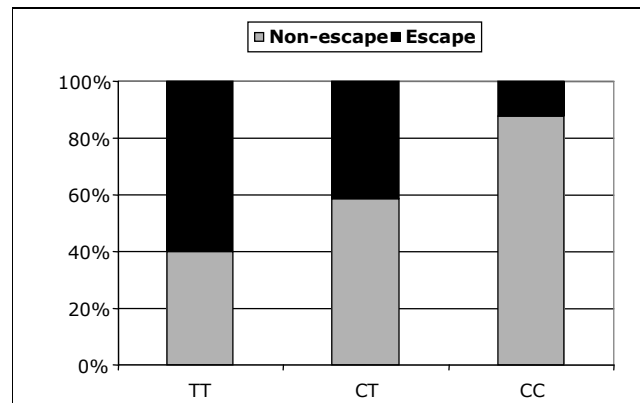
## 5. RAAS BLOCKADE IN INCIPIENT AND OVERT DIABETIC NEPHROPATHY

RAAS blocking treatment in diabetic patients with incipient or overt nephropathy serves several goals: 1) antihypertensive treatment and 2) 'renoprotective treatment' as reflected by a reduction in albuminuria/proteinuria above and beyond the effect of blood pressure reduction; which in turn protects against 3) ESRD and 4) cardiovascular morbidity and mortality.

The initial reduction in proteinuria after initiation of antihypertensive treatment predicts the long-term renoprotective effect of the treatment in diabetic and non-diabetic renal disease (32,33); i.e. a large initial reduction in albuminuria is associated with a slower rate of decline in kidney function. Furthermore, a reduction in albuminuria/proteinuria is associated with a reduced risk of progressing to ESRD and a reduction in cardiovascular endpoints (17,18,34) even in patients with nephrotic range albuminuria (19,109). RAAS blocking treatment in diabetic patients has until recently been synonymous with ACEI and ARB therapy, but increasing evidence justifies the treatment with other agents blocking the RAAS i.e. aldosterone antagonism (spironolactone and eplerenone) and renin inhibition (aliskirin) in patients with diabetic nephropathy, as discussed below.

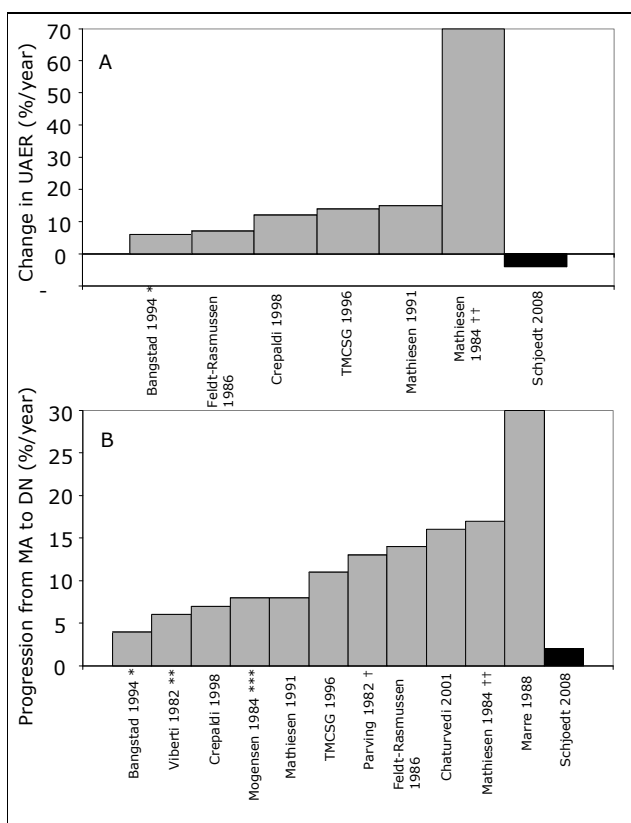
### 5.1 ACE-INHIBITORS AND ANGIOTENSIN II RECEPTOR BLOCKERS IN INCIPIENT DIABETIC NEPHROPATHY

Incipient nephropathy, also known as microalbuminuria in diabetic patients, has been demonstrated to precede and predict development of diabetic nephropathy. In fact, an increase in urinary albumin excretion rate (UAER) of 6% to 14%/year and a risk of developing diabetic nephropathy of 3% to 30%/year have previously been reported in type 1 diabetic patients with



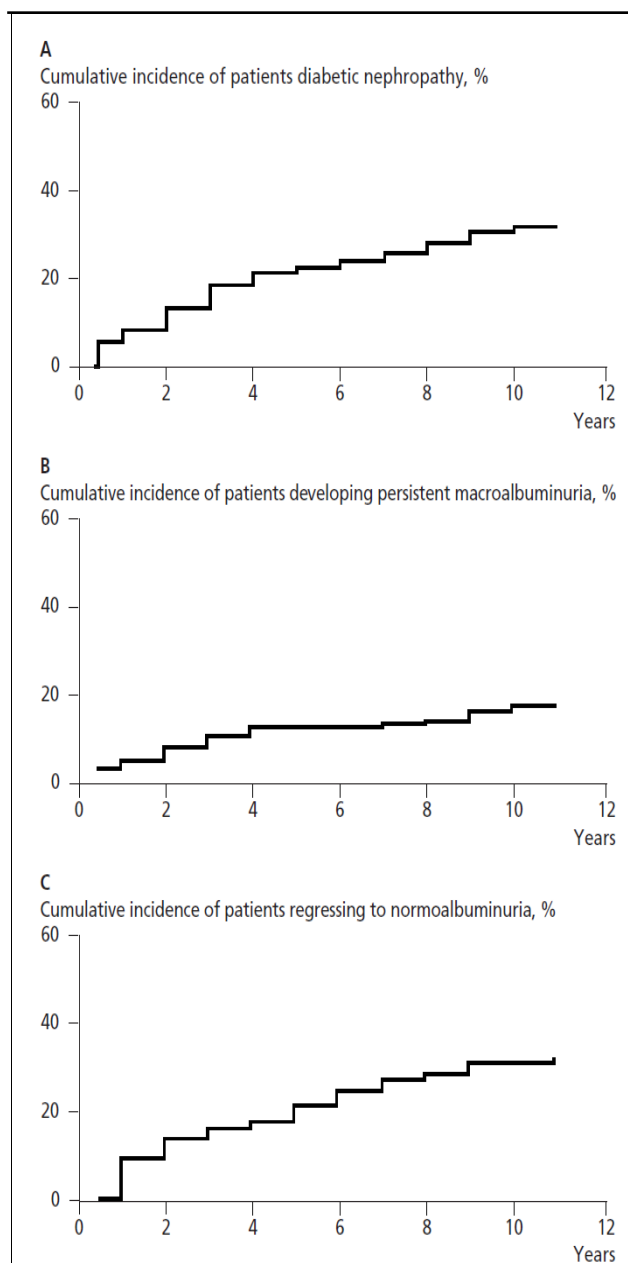
**Figure 6**  
Relative number of patients with and without aldosterone escape in patients with TT, CT, and CC genotypes of the *CYP11B2* -344T/C polymorphism, respectively, Pearson chi-square,  $P=0.09$ .

microalbuminuria not receiving antihypertensive treatment (110-119). Several clinical trials of short- or medium- term duration have shown a beneficial effect of blocking the RAAS with an ACEI on progression of UAER, development of diabetic nephropathy and decline in kidney function (115,116,118-124). In 1995, a consensus report on the detection, prevention and treatment of diabetic nephropathy with special reference to microalbuminuria was published, recommending treatment with ACEI and improved glycaemic control (HbA1c below 7.5-8.0%) in diabetic patients with microalbuminuria (125). A slightly modified version of these guidelines was implemented in our outpatient clinic at Steno Diabetes Center in 1995. This gave us the opportunity to prospectively evaluate the renoprotective effect of long-term RAAS-blockade in microalbuminuric type 1 diabetic patients in a clinical setting, which has not previously been done. We performed an observational follow-up study to audit 1) how successful we have been on implementing the new treatment regimen and to audit 2) the effect of long-term RAAS-blocking treatment in microalbuminuric type 1 diabetic patients on progression of UAER and development of diabetic nephropathy in a clinical setting in our outpatient clinic at Steno Diabetes Center (6). All patients with type 1 diabetes and persistent microalbuminuria were identified ( $n=227$ ) at Steno Diabetes Center in 1995 and followed until death, relocation or end of follow-up on 31 December 2005 with a median follow-up of 11 (range 0.5-11) years. The guidelines implemented at Steno Diabetes Center has been described previously (126). In short the guidelines included prescription of an ACEI in a predefined 'high-risk' group (defined as  $UAER \geq 100$  mg/24-hours and/or  $\Delta UAER > 6\%/year$ ) among the microalbuminuric patients. Furthermore, high-risk patients with a haemoglobin A1c (HbA1c)  $>8\%$  were offered intensive nurse guidance in order to improve glycaemic control. ACEI was only prescribed in 'low-risk' ( $UAER < 100$  mg/24-hours and  $\Delta UAER \leq 6\%/year$ ) microalbuminuric patients if considered appropriate by the individual physician. In 2002, the recommendations were extended to include ACEI treatment in all patients with microalbuminuria (independent of high- and low-risk status) and furthermore statins and low-dose aspirin (75 mg daily) were recommended for all these patients. Patients who did not tolerate ACEIs were prescribed ARBs, and additional antihypertensive treatment was prescribed as needed. During follow-up 79% were treated with an ACEI or ARB. Of patients who were still attending Steno Diabetes Center in December 2005,



**Figure 7**  
**A:** Progression in UAER in type 1 diabetic patients with microalbuminuria before introduction of ACEI or ARB treatment (refs. 110,111,113-116). **B:** Progression rates from micro- to macroalbuminuria in type 1 diabetic patients before introduction of ACEI or ARB treatment (refs. 107-116). Microalbuminuria was defined as UAER 20-200 µg/min or 30-300 mg/24-h except in \* (15-200 µg/min), \*\* (30-140 µg/min), \*\*\* (15-150 µg/min), † (40-300 mg/24-hour), and †† (71-200 µg/min). Gray bars: Data from the literature. Black bars: Data from our observational follow-up study after introduction of RAAS-blockade.  
 TMCSG: The Microalbuminuria Captopril Study Group.

85% received an ACEI or ARB. The remaining 15%, i.e. n = 29 patients, were not receiving RAAS-blocking treatment: 10 patients did not comply with the treatment, mostly because of adverse effects, the remaining 19 patients had spontaneously regressed to persistent normoalbuminuria early during follow-up, without receiving RAAS-blocking treatment. In our study, there was a mean decrease in UAER of 4%/year. This should be compared to previous studies reporting an annual increase in UAER of 6-14% in patients without RAAS-blocking treatment, figure 7A (113,114,116-119). Furthermore, only 65 patients (29%) progressed to overt diabetic nephropathy, corresponding to 3.1%/year and it should be noted that 45% of these patients, subsequently regressed to micro- or normoalbuminuria on intensified antihypertensive treatment; i.e. only 1.7%/year progressed to persistent macroalbuminuria after 11 years of follow-up as compared to progression rates of 3-30%/year as reported previously (110-119), figure 7B. The cumulative incidence (based on survival analysis) of patients developing A) diabetic nephropathy, B) persistent macroalbuminuria despite intensified antihypertensive treatment, and C) persistent normoalbuminuria (defined as UAER < 30 mg/24-hours in at least the last three consecutive urine samples) are shown in figures 8A-C. Despite determined effort, glycaemic control and blood



**Figure 8**  
 Cumulative incidence (based on survival analysis) of A. progression to diabetic nephropathy (albuminuria ≥ 300 mg/24-hour in 2 of 3 consecutive urine samples), B. progression to persistent macroalbuminuria despite intensified antihypertensive treatment, and C. regression to normoalbuminuria in 227 type 1 diabetic patients with microalbuminuria during 11 years of follow up after introduction of a new treatment strategy at Steno Diabetes Center.

pressure remained nearly unchanged during follow-up. From the audit, we concluded that by introducing new treatment guidelines including RAAS-blocking treatment in type 1 diabetic microalbuminuric patients, it is possible to keep the long-term rate of progression to overt diabetic nephropathy in a clinical setting as low as in clinical intervention trials of shorter duration (6). Suissa et al (127) previously suggested that long-term use of ACEI increases the risk of renal failure. The study was a population-based cohort study in all diabetic patients (mainly type 2 diabetes) selected from a database registering all prescription medicine in a Canadian province. The risk of developing renal failure was evalu-

ated according to prescribed antihypertensive treatment – leaving a risk of confounding by indication, even though the authors claim to have tried to minimize this (127). Our long-term follow-up data clearly demonstrate that implementation of RAAS-blocking treatment in microalbuminuric type 1 diabetic patients delay or even prevent progression from microalbuminuria to macroalbuminuria with a lower progression rate and a lower cumulative incidence after 11 years of follow-up (6).

In type 2 diabetic patients, RAAS blocking treatment has also been shown to effectively reduce the progression from micro- to macroalbuminuria (128-131). Evidence of a beneficial effect of ARB treatment above and beyond the antihypertensive effect of the drug was shown in the multicenter study IRMA-2 (132), where the renoprotective effect of the ARB irbesartan was evaluated in 590 hypertensive type 2 diabetic patients with microalbuminuria. Patients were randomised to treatment with placebo, irbesartan 150 mg or irbesartan 300 mg on top of conventional treatment for a study period of two years. The primary endpoint, i.e. time to progression to diabetic nephropathy, occurred in 15% of patients in the placebo group, 10% of patients in the irbesartan 150 mg group, and 5% of patients in the irbesartan 300 mg group. After adjustment for the baseline level of microalbuminuria and the blood pressure achieved during the study, the hazard ratio for diabetic nephropathy was 0.56 in the 150-mg group and 0.32 in the 300-mg group.

## 5.2 ACE-INHIBITORS AND ANGIOTENSIN II RECEPTOR BLOCKERS IN OVERT DIABETIC NEPHROPATHY

In large randomised, double-masked, clinical trials a specific renoprotective effect has been demonstrated with ACEIs in type 1 diabetic patients with diabetic nephropathy (26,133,134) and with ARBs in type 2 diabetic patients with diabetic nephropathy (22,24). As demonstrated in these studies, RAAS blockade has been superior to other antihypertensive agents in reducing albuminuria and slowing rate of decline in GFR despite similar blood pressure levels, i.e. renoprotection. Therefore ACEIs and ARBs are now considered first-line therapy in diabetic nephropathy as previously reviewed (41) and shall not be discussed in detail here. However, despite the uplifting results with ACEIs and ARBs in diabetic nephropathy, patients still progress towards ESRD. Part of the reason for this may be due to incomplete blockade of AngII. In the following sections, different approaches to overcome this problem will be discussed.

### 5.2.1 Dosing of RAAS blocking agents for optimal renoprotection

Incomplete blockade of the RAAS may be due to the administration of inadequate doses of the RAAS-blocking agent used. Dose titration studies of ACEIs and ARBs are traditionally conducted according to blood pressure lowering effect in essential hypertension (135,136). The optimal blood pressure lowering dose is however not necessarily the same as the optimal dose for renoprotection.

We previously demonstrated the importance of finding the optimal renoprotective dose of the ARB irbesartan in 52 type 2 diabetic patients with microalbuminuria (95). Patients with an insufficient response, i.e. UAER above the median during treatment with the standard dose (300 mg) of irbesartan, had a further reduction in UAER by increasing the dose to 900 mg despite similar blood pressure reductions (95). Similarly, other groups have found additional antiproteinuric effects of ARBs with and without concurrent blood pressure reductions, with doses exceeding standard maximally recommended doses (137-140).

One dose titration study has been evaluating the antiproteinuric effect of the commonly used ACEI lisinopril in 9 patients with

non-diabetic nephropathies (141). The study demonstrated that lisinopril 40 mg was more effective in reducing proteinuria than lisinopril 10 and 20 mg. There was no statistically significant difference in blood pressure between 20 and 40 mg of lisinopril. No higher doses of lisinopril were tested (141). Thus the optimal renoprotective dose of lisinopril, as evaluated by short-term changes in albuminuria was yet to be determined. We therefore performed a randomised, double-masked, crossover trial in 49 type 1 diabetic patients with diabetic nephropathy in order to evaluate the optimal renoprotective dose of lisinopril (7). After an initial two month wash-out period where previous antihypertensive treatment was withdrawn and slow-release furosemide was titrated to an individual but fixed dose, all patients received lisinopril 20, 40 and 60 mg in random order, each treatment period lasting two months. With increasing doses of lisinopril UAER was reduced from baseline by 63% (95% CI: 55 to 69%), 71% (66 to 76%), and 70% (64 to 75%) ( $P < 0.001$ ). Compared to lisinopril 20 mg there was a further reduction in UAER of 23% (8 to 35%) with lisinopril 40 mg with no further reduction with 60 mg daily. ABP was reduced by 10/5, 13/7, and 12/7 mm Hg from baseline ( $P < 0.001$  vs. baseline,  $P < 0.05$  for diastolic ABP 20 vs. 40 mg, otherwise NS between doses). The beneficial effects of lisinopril 40 mg were obtained without any additional adverse effects as compared to 20 mg (7). Our study showed a wide inter-individual variation in the effect of lisinopril doses on albuminuria and blood pressure. In contrast to our previous study, where the response to ultra-high doses of irbesartan was predicted by the response to standard doses (95), patients responding better to higher doses of lisinopril could neither be predicted by levels of albuminuria or blood pressure, nor by plasma renin activity or aldosterone levels at baseline or on lisinopril 20 mg.

The obtained beneficial effect on UAER in our dose escalation study (7) is in the same order of magnitude as adding on an ARB to lisinopril 20 mg; i.e. dual blockade (142-146). On the contrary, our data suggest that the beneficial effect of adding an ARB to lisinopril 40 mg (141,147,148) could not be obtained by increasing the dose of lisinopril further.

Even though the effect on albuminuria and ABP was maximal on 40 mg of lisinopril (7); the most pronounced changes in the components of the RAAS were induced by lisinopril 60 mg as reflected by further increases in PRA and Ang I levels (figure 2) – suggesting that the clinical effect of increasing the dose of ACEI reaches a level before the RAAS is fully blocked as reflected by changes in the RAAS components. A similar finding has been reported by Tylicki et al (149). This is in contrast to previous findings, demonstrating that further blocking the RAAS (reflected by compensatory increases in RAAS components) with an ARB (148) or an aldosterone antagonist (3,5) on top of an ACEI provide further renoprotection as reflected by reduction in albuminuria and blood pressure as compared to monotherapy with an ACEI in maximum recommended doses. The reason for this discrepancy is unclear and needs further study in the future; but it may emphasise the beneficial effect of blocking the RAAS with two or more agents.

### 5.2.2 Dual blockade of the RAAS in diabetic nephropathy

The rationale for dual blockade of the RAAS with an ACEI and an ARB is that compensatory mechanisms occur during long-term treatment with either drug alone. During long-term ACEI treatment AngII levels tend to increase, most likely as a result of incomplete enzyme inhibition and AngII generation through non-ACE-dependent pathways such as chymase and other serine proteases (150), figure 1. This so called “ACE-escape” phenomenon is overcome by treatment with an ARB. During long-term

monotherapy with an ARB however, there tend to be a compensatory increase in renin and AngII levels of 2-4(-6) fold (38,151), thereby increasing the competition at the angiotensin II type 1 receptor level. Furthermore increased levels of AngII leads to increased stimulation of other AngII receptor subtypes which may or may not induce untoward effects (152-155). The increase in AngII levels during ARB treatment is again minimized with addition of an ACEI; i.e. the combination of an ACEI and an ARB has the potential of reinforcing the effect of the other drug.

As reviewed by Rossing (156), dual blockade of the RAAS has been shown to effectively reduce albuminuria with or without concurrent blood pressure reduction in several clinic trials in type 1 and type 2 diabetic patients with incipient or overt nephropathy (142,144-148,151,157-159) although few trials have also shown lack of effect of dual blockade compared to monotherapy (143,160-163); most often in microalbuminuric patients, or due to small sample-size or addition of low-dose ARB. A recent meta-analysis including 49 randomised controlled trials involving 6181 patients evaluated the anti-proteinuric effect of ACEI and ARB monotherapy and combination therapy in diabetic (including diabetic microalbuminuria) and non-diabetic renal disease (164). The conclusion was that ARBs and ACEIs reduce proteinuria to a similar degree and that their combination is more effective than either drug alone (164). As described above, increasing the dose of lisinopril from 40 to 60 mg did not result in further reductions in UAER or blood pressure (7) whereas this has been demonstrated in several studies adding an ARB to lisinopril 40 mg (141,147,148). Long-term studies evaluating the efficacy of dual blockade in patients with diabetic nephropathy are missing. One previous trial reported improved renal outcome with combination therapy compared to monotherapy with ARB or ACEI in advanced proteinuric non-diabetic nephropathy (165). Serious concerns about the study has however been raised (166,167). The ONTARGET study comparing the effect of ramipril, telmisartan and the combination of both agents in 25620 patients with vascular disease or high-risk diabetes (38%) with a median follow-up of 56 months, was recently published (168,169). Overall, dual blockade did not reduce the risk of reaching the primary composite end-point of death from cardiovascular causes, myocardial infarction, stroke, or hospitalisation for heart failure (168). The primary composite renal end-point of death, dialysis and doubling of serum creatinine occurred in more patients in the combination group in the whole study population of patients with vascular disease or diabetes (169). A subgroup analysis showed that the point estimate for the primary outcome in those with overt diabetic nephropathy (more than 700) was in favour of combination therapy, with a relative risk reduction by 8% with a CI including a risk reduction of 24%, but it did not reach statistical significance (169). Detailed information on albuminuria, kidney function at baseline, rate of decline in kidney function and cause of ESRD were not presented, and the long-term efficacy and safety of dual blockade in diabetic nephropathy remains uncertain, as discussed in the comment by Bakris (170).

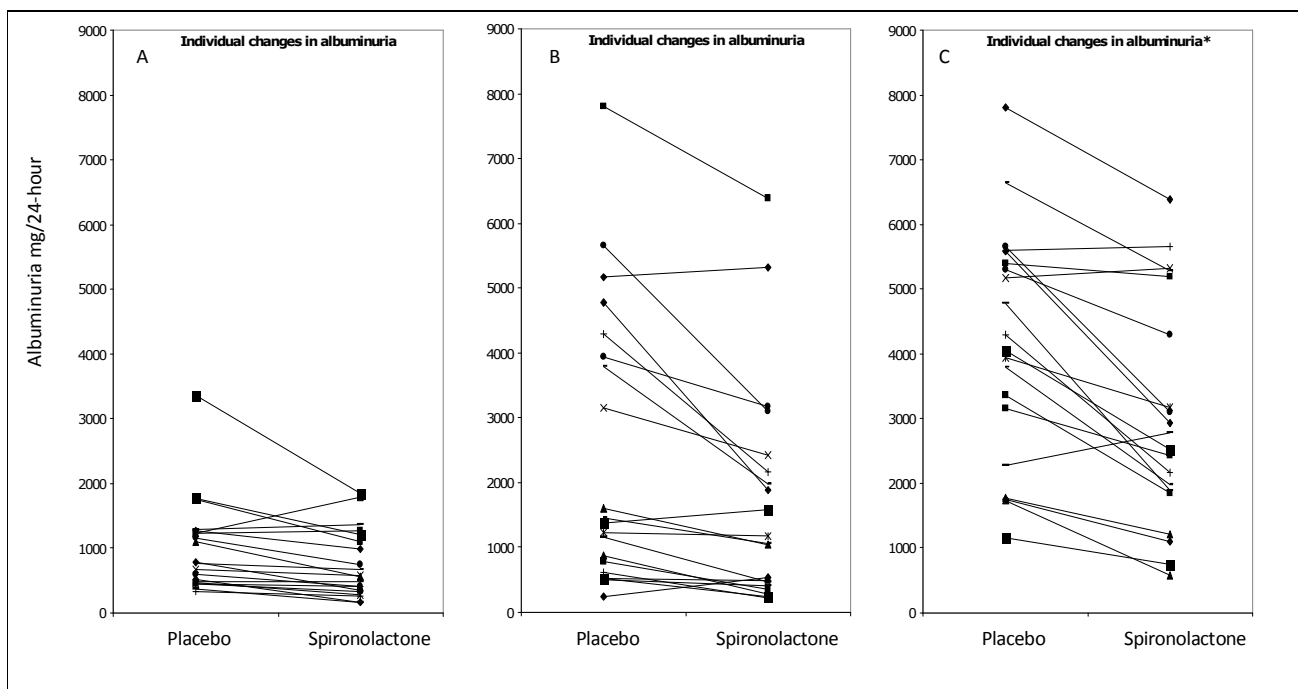
### 5.3 ALDOSTERONE ANTAGONISM IN INCIPIENT AND OVERT NEPHROPATHY

In the wake of the positive results from the Randomized Aldactone Evaluation Study (RALES) (171) showing reduced morbidity and mortality by the addition of low-dose spironolactone to standard treatment in patients with severe heart failure, Chrysostomou et al (172) performed an open-label study in 8 patients

with chronic proteinuric kidney disease of various causes. Spironolactone 25 mg added to ongoing antihypertensive treatment with an ACEI induced a reduction in proteinuria of 54% after 4 weeks treatment. Subsequently, Sato et al (86) reported a blood pressure independent reduction in albuminuria by adding spironolactone to ACEI in type 2 diabetic patients with micro- or macroalbuminuria and eGFR>60 ml/min/1.73m<sup>2</sup>, who developed aldosterone escape during ACEI treatment. Patients who did not develop aldosterone escape were not treated with spironolactone, so the effect in these patients was unknown. In the light of these encouraging results we performed a randomised, double-masked, crossover trial in 20 type 1 diabetic patients with diabetic nephropathy, GFR>30 ml/min/1.73m<sup>2</sup> and persistent macroalbuminuria despite recommended antihypertensive treatment (3). Patients were treated in random order with spironolactone 25 mg or matched placebo for 2 months. The study medication was added on top of ongoing antihypertensive treatment including an ACEI or ARB in all patients and a diuretic and/or other antihypertensive agents in most patients. At the end of each treatment period the primary end point albuminuria and the secondary end-points blood pressure and GFR were evaluated. Spironolactone on top of ongoing antihypertensive treatment induced a reduction in UAER of 30%, with an insignificant reversible decline in GFR of 3 ml/min/1.73m<sup>2</sup> (P=0.06). The reduction in 24-hour ABP of 8/3 mm Hg did not reach statistical significance (P=0.08). Day-time ABP was significantly reduced by 10/5 mm Hg (P<0.05) (3). In a study of identical design in 20 type 2 diabetic patients with nephropathy we found very similar results (173); i.e. UAER was reduced by 33% (P<0.001), 24-hour ABP by 6/4 mm Hg (P<0.01) with an insignificant reversible decline in GFR of 3 ml/min/1.73m<sup>2</sup>. Uplifted by these results we wanted to investigate whether spironolactone treatment is effective in patients with nephrotic range albuminuria; i.e. patients with a very poor kidney and cardiovascular disease prognosis. We therefore identified all patients with nephrotic range albuminuria at Steno Diabetes Center. Due to a low number of patients with this condition, both type 1 and type 2 diabetic patients were included. Twelve patients had already participated in the first studies and 8 additional patients completed the study. In total, 20 patients with diabetic nephropathy and nephrotic range albuminuria completed our randomised, double-masked crossover trial (5). In these patients spironolactone induced a reduction in albuminuria of 32% (P<0.001), a reduction in 24-hour ABP of 6/4 mm Hg (P<0.01) and in similarity to the other studies an insignificant reversible decline in GFR of 5 ml/min/1.73m<sup>2</sup>.

Overall, our 3 randomised, double-masked, placebo-controlled crossover trials showed that spironolactone 25 mg once daily reduced albuminuria 30-33% and blood pressure 6-8/4-5 mm Hg in patients with diabetic nephropathy who are already receiving a median of 3 (range: 1-5) antihypertensive agents including recommended RAAS-blockade with an ACEI or ARB.

The reduction in UAER and blood pressure did not correlate significantly with aldosterone levels at baseline or during spironolactone treatment in any of our studies (3,5,173). Absence or presence of aldosterone escape as defined previously (1) was not determined prior to the studies since all patients were on long-term stable RAAS-blocking treatment. All patients participating in the three studies had plasma aldosterone levels within or below the normal range both in the placebo and spironolactone (plus ongoing antihypertensive treatment including ACEI and/or ARB and diuretics) periods. Nevertheless, a



**Figure 9**  
Individual changes in albuminuria during spironolactone treatment compared to placebo treatment in A) Patients with type 1 diabetes and diabetic nephropathy (3), B) Type 2 diabetic patients with diabetic nephropathy (173), and C) diabetic patients with diabetic nephropathy and nephrotic range albuminuria (5). Study medication was added to ongoing antihypertensive treatment including an ACEI or ARB. \* Please note that 12 patients in panel C are also present in panel A (n=4) or panel B (n=8) as described in (5).

reduction in albuminuria was obtained in 40 of 48 patients (83%) having completed the study, figure 9A-C, indicating that the albuminuria- and blood pressure- lowering effect of aldosterone antagonism is not confined to patients with aldosterone escape, which occurred in approximately 40% of patients with diabetic nephropathy (1,86). This finding makes it easier in daily clinical practice since unreasonably long wash-out and run-in periods would be needed if presence of aldosterone escape should be determined before initiation of spironolactone treatment.

Other groups have subsequently confirmed a reduction in albuminuria/proteinuria of 30% or more during spironolactone (or eplerenone (174)) treatment in diabetic and non-diabetic renal disease (51,93,107,174-180) as recently reviewed by Bomback et al (181) and Navaneethan et al (182). No data are so far available on long-term efficacy and safety of spironolactone treatment in diabetic or non-diabetic nephropathies. The largest study with the longest follow-up so far, was conducted by Bianchi et al (51) as a prospective randomised open-label study in 165 patients with non-diabetic chronic kidney disease. Eighty-three patients received spironolactone 25 mg on top of conventional treatment and 82 patients continued conventional treatment without spironolactone. All patients were on long-term stable treatment with an ACEI and/or ARB in addition to other antihypertensive agents. Spironolactone induced a reduction in proteinuria of approximately 58% after one year of treatment, whereas proteinuria was unchanged in the control group (51). A smaller randomised, double-blind, placebo-controlled, parallel-group study with 1-year follow-up in type 2 diabetic nephropathy, also evaluated the effect on albuminuria, blood pressure and eGFR of spironolactone (25-50 mg) compared to placebo on top of ongoing antihypertensive treatment (179). They found that spironolactone induced a 41% reduction in albuminuria and a decrease in blood pressure of 7/3 mm Hg compared to placebo. In both studies (51,179), spiro-

nolactone induced an initial enhanced decline in kidney function where after the rate of decline levelled off. In contrast, rate of decline in kidney function was progressive in the groups receiving conventional treatment (51,179), and longer follow-up in a randomised, double-masked, placebo controlled study is needed in order to evaluate the long-term effect of aldosterone antagonism on renal outcomes, mortality, and safety.

### 5.3.1 Mechanisms for the effect of spironolactone

Several mechanisms may be involved in the antiproteinuric effect of spironolactone by antagonizing the harmful effects induced by aldosterone as described in section 4.1. This may in turn result in e.g. reversion of changes in the size and charge selective properties of the glomerular vascular barrier, increased tubular protein reabsorption, and finally, reduced glomerular capillary hydraulic pressure (183-185). Clinically significant changes in tubular protein reabsorption can be excluded since the process should already operate at maximum capacity in overt nephropathy. No data are available regarding the effect of spironolactone on alterations in the size and charge selective properties of the glomerular capillary wall. However, it has been suggested that RAAS blockade with an ACEI or ARB in incipient and overt diabetic nephropathy in man, can reduce the size selective abnormalities of the glomerular barrier (186). The reduction in albuminuria during spironolactone treatment was associated with a reversible decline in GFR (3,51,179), which may suggest that spironolactone induces a reduction in the intraglomerular hydraulic pressure, as discussed below. Attenuation of the intraglomerular pressure has been shown to limit renal damage in experimental studies (187,188) and in human (189).

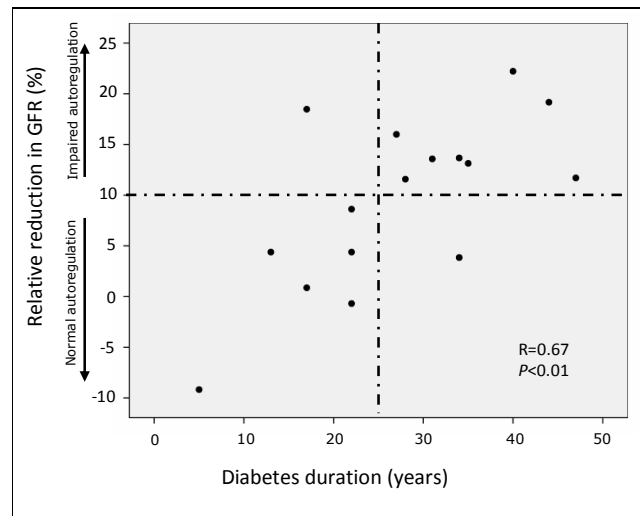
Even though the spironolactone induced reductions in albuminuria were independent of systemic blood pressure in our studies (3,5,173), other data suggest that the observed blood pressure reduction might contribute to diminished albuminuria (9).

Furthermore, spironolactone has been shown to reduce markers of oxidative stress (190), markers of inflammation (191), markers of tubular involvement (N-acetyl- $\beta$ -D-glucosaminidase) (180) and a marker of fibrosis (amino-terminal propeptide of type III procollagen) (180) in clinical trials; and in addition spironolactone has been shown in experimental settings to reduce growth factors (192), proinflammatory cytokines and markers of inflammation (193) and markers of fibrosis (73) all of which were associated with reductions in UAER and amelioration of glomerulosclerosis independently of blood pressure reductions, altogether suggesting a non-haemodynamic effect of spironolactone in addition to hemodynamic actions. These findings await confirmation in clinical settings.

### 5.3.2 Autoregulation of GFR during spironolactone treatment

The ability to autoregulate GFR is a highly important mechanism to protect the kidney against fluctuations in systemic blood pressure (194). It designates the ability to maintain GFR constant despite variations in perfusion pressure. Impaired autoregulation of GFR implies disturbances in the downstream transmission of the systemic blood pressure into the glomerulus that lead to capillary hypertension or hypotension, depending on the level of systemic blood pressure, both of which are injurious to the kidney. Increased glomerular capillary pressure is an important factor in the development and progression of diabetic and non-diabetic glomerulopathies as demonstrated in experimental settings (67,194-196). Previous studies in hypertensive type 2 diabetic patients without nephropathy have shown that different antihypertensive drugs can affect the ability to autoregulate GFR (197,198). The ARB, candesartan, reduced blood pressure significantly without altering the preserved ability to autoregulate GFR (197). In contrast, the calcium channel blocker, isradipine, induced a variable response ranging from no impact to impaired or abolished autoregulation of GFR (198). In accordance with this, Delles et al (199) found that GFR and glomerular hydrostatic pressure was maintained during treatment with the ARB valsartan, whereas the calcium channel blocker (CCB) amlodipine induced hyperfiltration associated with increased glomerular hydrostatic pressure despite a reduction in systemic blood pressure in patients with essential hypertension. This ambiguous effect of CCBs is caused by the vasodilatory effect on the afferent arteriole of the drug which can result in an increase in intraglomerular pressure in particular when systemic arterial pressure is not adequately reduced. In contrast ACEIs and ARBs by blocking the effect of AngII, predominantly dilate the efferent but also the afferent arteriole, due to the higher sensitivity of the efferent arteriole than the afferent arteriole to AngII (200).

In our three spironolactone studies there tended to be a reversible decline in GFR during spironolactone treatment (3,5,173) which has also been described in type 2 diabetic patients and patients with non-diabetic nephropathy treated with spironolactone for one year (51,179). The mechanism behind the reversible decline in GFR is unknown, but could potentially be due to: A) a spironolactone induced impairment on the ability to autoregulate GFR, leaving the glomerulus vulnerable to systemic hypertension or hypotension; or B) a spironolactone induced beneficial reduction in the intraglomerular pressure with preserved ability to autoregulate GFR. An initial rapid decline in GFR during spironolactone treatment has recently been suggested to be followed by a stabilization of kidney function (51) as also suggested from experimental studies (187,201).



**Figure 10**  
Clonidine-induced changes in GFR during placebo treatment in 16 hypertensive type 1 diabetic patients with persistent normoalbuminuria. All patients with impaired autoregulation ( $\Delta$ GFR>10%), except for one, had diabetes duration > 25 years.

Aldosterone has been suggested to increase renal vascular resistance in the presence of endothelial dysfunction (202). In experimental studies in isolated perfused afferent and efferent arterioles from rabbit kidneys, it has been demonstrated that aldosterone induces vasoconstriction of both afferent and efferent arterioles with a higher sensitivity of the efferent arteriole (61), i.e. blocking aldosterone with spironolactone may reduce intraglomerular pressure, but also has a potential of affecting the ability to autoregulate GFR by altering the vascular tone.

Since autoregulation of GFR is often impaired or even abolished in type 1 (203) and type 2 (204) diabetic patients with diabetic nephropathy, the antihypertensive drugs used for renoprotection in these patients should not further impair the ability to autoregulate GFR which may render the kidney even more vulnerable to changes in systemic blood pressure (205). As a consequence, it is important to find out whether different antihypertensive drugs including spironolactone affect this ability.

We therefore conducted a randomised double-masked crossover trial designed to evaluate the influence of spironolactone treatment on the ability to autoregulate GFR after acute blood pressure reduction (8). In random order, patients received spironolactone 25 mg once daily for one month and matched placebo for one month. At the end of each treatment period, the ability to autoregulate GFR was determined by measuring GFR twice in the same day before and after injection of clonidine 75  $\mu$ g intravenously. Clonidine injected slowly induces a transient reduction in blood pressure, with no influence on renal plasma flow and GFR in healthy subjects and subjects with essential hypertension (203,206,207). In order to be able to identify a potential interfering effect of spironolactone on the ability to autoregulate GFR, we included type 1 diabetic patients with hypertension but without diabetic nephropathy, i.e. a group of patients in need for antihypertensive medication but with expected preserved autoregulation during placebo treatment.

In our study spironolactone did not change the overall ability to autoregulate GFR in 16 hypertensive type 1 diabetic patients with normoalbuminuria (8). Signs of impaired autoregulation ( $\Delta$ GFR >10%) were present in 9 patients during placebo and 9 patients during spironolactone treatment. Abolished autoregulation

tion, (where the relative reduction in GFR exceeds the reduction in systemic blood pressure, i.e.  $\Delta\text{GFR}\% > 10$  and  $\geq \Delta\text{MAP}\%$ ) was present in 4 patients during placebo and in 1 patient during spironolactone treatment. The reduction in GFR could be ascribed to a reduction in MAP below 80 mm Hg in 2 patients during placebo and 5 patients during spironolactone treatment; i.e. actual impaired autoregulation was present in 7 patients during placebo and 4 patients during spironolactone.

Nine patients in our study unexpectedly showed signs of impaired autoregulation during placebo treatment (8). In previous autoregulation studies performed in patients with type 1 and type 2 diabetes, impaired autoregulation was only observed in few patients without nephropathy (197,198,203,204). Looking further into this unexpected observation, we found that impaired autoregulation may be present as a result of long-standing diabetes. In the previous studies mean known diabetes duration was 7-14 years (197,198,203,204) as compared to our patients with mean diabetes duration of 27 years (8). In our study, clonidine-induced changes in GFR correlated positively with diabetes duration, figure 10. In fact all patients with impaired autoregulation during placebo in our study except for one, had a diabetes duration >25 years, whereas all patients with preserved autoregulation except for one, had diabetes duration <25 years, figure 10. There was no association between ability to autoregulate GFR and age or duration of hypertension. The association between diabetes duration and ability to autoregulate GFR has never previously been described. Our finding is however, supported by another recent study where impaired dynamic cerebral autoregulation was found to correlate closely to diabetes duration (208). Altogether suggesting, that long-standing diabetes per se, i.e. in the absence of microvascular complications, can affect renal and cerebral autoregulation, although acute changes in blood glucose do not influence the ability to autoregulate GFR (209) or the dynamic cerebral autoregulatory capacity (210).

Our study indicates that spironolactone treatment does not impair the ability to autoregulate GFR in hypertensive type 1 diabetic patients; however this finding should be confirmed in patients with preserved renal autoregulation. Moreover, future studies should further explore the impact of spironolactone on renal hemodynamics by measuring renal plasma flow in parallel to GFR before and after administration of the drug, e.g. in resemblance to a study previously performed with the angiotensin II receptor blocker irbesartan (44). Ultimately the long-term efficacy and safety of spironolactone treatment needs to be evaluated in patients with diabetic nephropathy.

## 5.4 DOSING, ADVERSE EVENTS AND SAFETY OF SPIRONOLACTONE

### 5.4.1 Dosing

Prior to the Randomized Aldactone Evaluation Study (RALES) (171), the RALES Investigators performed a dose-finding study (211) in 214 patients with symptomatic heart failure, comparing effect and side effects of spironolactone 12.5, 25, 50, and 75 mg daily to placebo. Spironolactone doses of 25, 50, and 75 mg induced significant reductions in blood pressure. Regarding adverse effects, they found a dose-dependant risk of hyperkalemia and concluded that the initial dose of spironolactone should not exceed 25 mg x 1 daily. Patients with type 1 diabetes or serum creatinine >180  $\mu\text{mol/l}$  were not included in the study. In our patients with diabetic nephropathy and normal or moderately reduced kidney function (3,5,173), one has to be even more cautious in order to avoid hyperkalemia. We chose to use the same dose, i.e. spironolactone 25 mg once daily,

- Use eGFR instead of creatinine concentration
- Only prescribe spironolactone if eGFR or GFR is > 30 ml/min/1.73 m<sup>2</sup>
- Use a dose of spironolactone 25 mg or lower
- Withdraw potassium supplement
- Prescribe a low-potassium diet, pay attention to salt substitutes containing potassium
- Prescribe thiazide (GFR > 40 -50 ml/min/1.73 m<sup>2</sup>) or loop diuretics in adequate doses
- Measure plasma potassium 1,2, and 4 weeks after initiation of spironolactone treatment and regularly thereafter
- If plasma potassium increases > 5.5 mmol/l; increase the dose of loop-diuretics and if necessary reduce dose of spironolactone and/or ACEI/ARB. Closely monitor plasma potassium until normal.

**Figure 11**

Points to consider before initiating spironolactone treatment.

where a clinically significant effect could be expected with an acceptable risk of hyperkalemia.

### 5.4.2 Hyperkalemia

To reduce the risk of hyperkalemia in our studies, most patients were treated with a loop or thiazide diuretic and all patients had their potassium supplement withdrawn and finally patients were educated to lower the dietary intake of potassium. For safety reasons, plasma potassium, sodium and creatinine were measured after 1, 2, and 4 weeks of treatment with the study medication. With this regimen, spironolactone treatment was generally well tolerated in our studies in patients with diabetic nephropathy, GFR > 30 ml/min/1.73 m<sup>2</sup> and plasma potassium  $\leq 4.5$  at inclusion (3,5,173). Withdrawal of spironolactone due to hyperkalemia was necessary in two of 51 randomized patients. Of these two patients, one had an increase in plasma potassium from 4.5 mmol/l to 5.4 after one week treatment, with a further increase to 7.1 after two weeks treatment which was reversed within hours by intravenous infusion of glucose and insulin with continuous electrocardiographic monitoring in a cardiology unit (173). The other patient developed hyperkalemia after 4 weeks treatment, plasma potassium 5.7 mmol/l, which was reversed by intensified treatment with loop diuretics. Further two patients, of whom both completed the study, developed mild-to-moderate hyperkalemia (5.0-5.4 mmol/l): one patient had transiently elevated plasma potassium (two measurements of plasma potassium 5.0-5.4 mmol/l) which was controlled by dietary advice and one patient had persistently elevated plasma potassium (5.0-5.4 mmol/l) in both treatment periods (placebo and spironolactone treatment) which was treated with kayexalate (a cation exchange resin).

In most other spironolactone studies in patients with nephropathy, a dose of spironolactone 25 mg has been used and hyperkalemia (p-potassium >5.0 mmol/l) has been reported to occur in 0-14% of the patients of whom only a few were excluded from the studies, whereas others had their hyperkalemia reversed by standard treatment (51,86,178). As in the dose-finding study by the RALES investigators (211), a higher dose of spironolactone was associated with a higher incidence of hyperkalemia, i.e. 17% (179) and 15% (212), in patients with type 2 diabetes and diabetic nephropathy who were treated with spironolactone 50 mg. The incidence of spironolactone-induced hyperkalemia in patients with nephropathy in clinical practice has not been described. However, after the publication of RALES (171) there has been several reports about increasing number of hospitalisation due to

hyperkalemia in patients with CHF (213-216), and the difficulties in transferring new treatment guidelines from closely controlled clinical trials to daily practice has been discussed (217). The main reasons for the discrepancy between numbers of adverse events in RALES and clinical practice, has been suggested to include prescription of higher doses of spironolactone than recommended from RALES, and lack of homogeneity between the RALES study population and patients in clinical practice who are generally older, have lower kidney function, include more diabetic patients, and receive higher doses of ACEIs and ARBs (217). Finally, there is more attention to safety and careful monitoring of patients in a clinical trial than in clinical practice. With careful monitoring, treatment with spironolactone has been shown to be well tolerated in CHF patients in a clinical setting (218). No audits reporting the incidence of hyperkalemia in spironolactone treated patients with diabetic nephropathy and impaired kidney function in a clinical setting are available. However in these patients the risk of developing hyperkalemia is even greater and it should be emphasised that the need for proper (i.e. low) dosing of spironolactone and close monitoring of kidney function and plasma potassium is crucial in these patients. However, to have the full benefit of a given treatment we must accept complex, carefully instituted and monitored polypharmacy as we do for other conditions (i.e. potassium supplement with non-potassium sparing diuretics, regular adjustment of oral vitamin K antagonists for anticoagulation, and folic acid with methotrexate etc., etc.), and consequently patients at risk of developing hyperkalemia must be identified and precautions to avoid development of hyperkalemia must be implemented, as listed in figure 11 and reviewed by Palmer (219). Only physicians with expert knowledge who have the opportunity to closely monitor their patients should initiate spironolactone treatment in patients with impaired kidney function.

#### 5.4.3 Gynecomastia

In our studies, no patients experienced gynecomastia (3,5,173) which was however present in 10% of patients in the spironolactone group in RALES compared to 1% in the placebo group (171). Although annoying, gynecomastia is a reversible and harmless adverse event and should not keep physicians from initiating spironolactone treatment. If gynecomastia should occur during spironolactone treatment, the patient may benefit from a switch to the selective aldosterone receptor antagonist, eplerenone, which is less likely to induce gynecomastia as discussed below.

#### 5.4.4 Orthostatic hypotension

In our studies (3,5,173), one patient withdrew consent due to severe symptoms of orthostatic hypotension (blood pressure was not measured since the patient discontinued the study medication before contacting the physician). In addition two patients who chose to complete the study, experienced acceptable symptoms of orthostatic hypotension during spironolactone treatment.

### 5.5 EPLERENONE

In our studies, we used spironolactone for aldosterone antagonism. Spironolactone is a generic, low-cost drug that has been on the market for 30 years, i.e. the profile for adverse effects is well described.

Eplerenone, a new selective aldosterone receptor antagonist, has been on the market since 2004. It has the advantage of having lower affinity of eplerenone for progesterone, androgen, and glucocorticoid receptors, i.e. the risk of gynecomastia is lower with eplerenone.

Pharmacological differences between spironolactone and eplerenone also include differences in nongenomic properties, and the presence of long-acting metabolites for spironolactone as reviewed by Struthers et al (220).

Eplerenone has been shown to be an effective antihypertensive agent (221-223), to prevent cardiovascular and renal end-organ damage in patients with essential hypertension (174,224,225), and to reduce morbidity and mortality in the short- and long-term in patients with heart failure and left ventricular systolic dysfunction after myocardial infarction (226,227).

A head-to-head comparison between spironolactone and eplerenone should be performed comparing safety and efficacy in patients with diabetic nephropathy as well as resistant hypertension and heart failure, especially with focus on the differences in the risk of developing hyperkalemia, which has been suggested to be lower with eplerenone in low doses (174,220) but not with higher doses (eplerenone 200 mg) (174,225). In a multicenter, randomized, double-blind, placebo-controlled parallel group trial including a total of 268 type 2 diabetic patients with urinary albumin:creatinine ratio (UACR) > 50 mg/g, Epstein et al (174) found a reduction in UACR of 7%, 41% and 48% after 12 weeks treatment with placebo, eplerenone 50 mg or eplerenone 100 mg daily, respectively, on top of enalapril 20 mg daily. Open-label amlodipine (2.5-10 mg) was added as needed after week 4 if blood pressure was > 130/80 mm Hg. The incidences of sustained (serum potassium > 5.5 mmol/l on two consecutive occasions) or severe (serum potassium > 6.0 mmol/l) hyperkalemia did not differ significantly between the three treatment arms (174). It was not specified in the article how hyperkalemia was treated, e.g. whether addition of thiazide or loop diuretics was allowed during the study. It is uncertain whether a similar reduction in UACR could have been obtained by using the maximal dose of enalapril in combination with a loop or thiazide diuretic, with an even lower risk of developing hyperkalemia in these type 2 diabetic patients with normal kidney function at baseline (calculated creatinine clearance > 70 ml/min based on Cockcroft-Gault formula). New clinical trials evaluating the effect of low-dose eplerenone on top of maximal recommended dose of an ACEI or ARB and a thiazide or loop diuretic on albuminuria and risk of hyperkalemia are needed.

### 5.6 TRIPLE BLOCKADE OF THE RAAS

The beneficial short-term effect of dual blockade with an ACEI and an ARB or the addition of an aldosterone antagonist, has of course raised the question: does triple blockade of the RAAS offer further beneficial effects in albuminuric kidney disease?

Chrysostomou et al (178) recently compared the anti-proteinuric effect of an ACEI alone, in combination with an ARB or spironolactone, or the combination of all three drugs for three months in a double-blind, placebo-controlled, parallel group study in patients with diabetic and non-diabetic nephropathies. They found that spironolactone-containing regimens were more effective in reducing proteinuria than ACEI alone or in combination with an ARB. Triple-blockade of the RAAS was more effective in reducing proteinuria compared to dual-blockade with an ACEI and an ARB, but not compared to the combination of an ACEI and spironolactone (178). Subsequent open-label treatment with spironolactone, showed sustained anti-proteinuric effect after 6 and 12 months. The results may be somewhat influenced by the addition of other antihypertensive drugs, in particular addition of diuretics which enhance the effect of RAAS blocking agents (228,229), and which were added if hyperkalemia occurred - a condition more likely to occur during spironolactone treatment.



Maximal recommended doses of ACEI and ARB were not used in the study and it is possible that combination treatment with ACEI and ARB in optimal doses can reduce proteinuria as efficiently as the combination of ACEI and spironolactone. Another study subsequently tested triple- against dual blockade of the RAAS in a randomized, open-label, multicenter, parallel group design in patients with non-diabetic renal disease and proteinuria exceeding 0.5 g/24-hour despite dual blockade for at least 12 weeks (107). In contrast to the study by Chrysostomou the dual blockade control group received a diuretic in a dose equivalent to spironolactone, and hyperkalemia was treated with addition of a potassium binder to avoid differences in diuretic treatment in the two groups. The study showed that proteinuria was reduced by 58% after 1 year of treatment with triple blockade, whereas proteinuria was unchanged in the dual blockade group. There was no significant difference between the two groups regarding changes in blood pressure and creatinine clearance (107). In a more recent study (180) in patients with non-diabetic chronic kidney disease; the anti-proteinuric effect of dual blockade with an ACEI and ARB in maximal recommended doses with addition of a fixed dose of hydrochlorothiazide, was compared to triple blockade with the same combination of ACEI, ARB and hydrochlorothiazide plus spironolactone, in a randomised, open-label, crossover trial. A target blood pressure of 130/80 was achieved by adding doxazosin during the run-in period if necessary. Triple blockade of the RAAS induced a 50-55% reduction in proteinuria after 8 weeks treatment compared to dual blockade (during run-in and treatment period respectively) independently of blood pressure. This means that even on top of dual blockade with ACEI and ARB in maximal recommended doses, addition of spironolactone induces a marked reduction in albuminuria. No long-term studies evaluating the renoprotective effect of triple blockade of the RAAS are available and it should be emphasized that triple blockade of the RAAS (in similarity to dual blockade) must be accompanied by close control of plasma potassium as discussed above.

### 5.7 RENIN INHIBITION

Within the last few years, a new RAAS blocking agent, namely the direct renin inhibitor aliskiren, has become available. Inhibition of the RAAS with direct renin inhibition was already targeted 30 years ago with compounds for intravenous use, but low bioavailability after oral intake and short duration of action of the first generations of renin inhibitors withheld their clinical success. With the new generation of non-peptide orally available renin inhibitors, a new substance to inhibit the RAAS is feasible.

Because most antihypertensive agents including ACEIs, ARBs, CCBs, diuretic therapy and aldosterone antagonists all lead to an increase in plasma renin activity which in turn tend to increase the downstream activity of the RAAS, direct renin inhibition offers an interesting new treatment modality as mono or combination therapy.

Aliskiren has been shown to effectively reduce blood pressure in essential hypertension (230,231). In the recent AVOID study (232), aliskiren was shown to reduce albuminuria in hypertensive, proteinuric, type 2 diabetic patients who were already receiving the optimal recommended renoprotective treatment with losartan and optimal antihypertensive therapy (232).

In a time course study of the antiproteinuric and antihypertensive effect of the renin inhibitor aliskiren in type 2 diabetic patients with micro- or macro- albuminuria, we found a 44% reduction in urinary albumin/creatinine ratio and a reduction in systolic blood pressure of 6-8 mm Hg (233). During renin inhibi-

tion with aliskiren, we found significant reductions in PRA, and circulating AngI and AngII levels, accompanied by a compensatory increase in plasma prorenin and renin concentrations (233). In contrast, no changes in plasma aldosterone levels were observed at the end of the 28 days treatment period (233). In healthy subjects, a reduction in urinary aldosterone excretion has been reported after short-term (1 and 8 days) treatment with aliskiren (234). The reduction was not reflected in circulating aldosterone levels (234). The possible lack of aldosterone suppression during renin inhibition needs further evaluation, and provides a new important treatment strategy, combining an aldosterone antagonist and a renin inhibitor.

The impact of the compensatory increase in prorenin and renin concentration induced by renin inhibition is unknown, but has been suggested to play a role in diabetic microangiopathy in the absence of renin inhibition (235,236). In an experimental setting, prorenin, when bound to the (pro)renin receptor, has been shown to display enzymatic activity comparable to that generated by fully active renin (237). In another experimental study however, aliskiren was shown to reduce *in vivo* gene expression for the (pro)renin receptor, furthermore aliskiren may also block prorenin-induced angiotensin generation (238).

### 6. SUMMARY, CONCLUSIONS, AND FUTURE PERSPECTIVES

Diabetic nephropathy is the most common cause of ESRD in the western world. Approximately one in three diabetic patients develops diabetic nephropathy, which manifests itself clinically by albuminuria, elevated blood pressure, and a persistent loss of renal function. Before the introduction of renoprotective therapy, the median survival was 5-7 years from the time of diagnosis of diabetic nephropathy.

Over the last approximately 30 years, persistent research efforts focusing on identification of high-risk patients, as well as prevention and treatment of diabetic nephropathy, has resulted in a reduced risk of developing diabetic nephropathy, slower disease progression, and significant improvements in survival among patients with diabetic nephropathy. In an 11-year observational follow-up study, we found that introduction of a new treatment strategy including an ACE inhibitor (ACEI) or an angiotensin II receptor blocker (ARB) in type 1 diabetic patients with microalbuminuria – i.e. patients at high risk of developing diabetic nephropathy - led to a decrease in urinary albumin excretion and a decrease in progression to diabetic nephropathy to the same extent as found in controlled clinical trials of shorter duration. In a recent follow-up study from the Steno Diabetes Center, more than half of the patients were still alive 21 years after diabetic nephropathy had been diagnosed. This improvement in prognosis can be particularly attributed to the introduction of aggressive antihypertensive treatment.

Agents that block the renin-angiotensin aldosterone system (RAAS) have been shown to play a particularly important role in both prevention and treatment of diabetic nephropathy. ACEIs and ARBs, which block the system at two different levels, are now considered the first line therapy in high-risk patients (patients with microalbuminuria) and patients with diabetic nephropathy. Despite major improvements in both prevention and treatment of diabetic nephropathy, a large interindividual variation in response to therapy exist and although renal function is stabilised in some patients with diabetic nephropathy, other patients show a rapid decline in renal function and develop renal failure after a few years. Therefore, there is a continuous need to improve identification and treatment of 'non-responders'.

In recent years, several experimental studies have shown that aldosterone plays a role in the development and progression of diabetic nephropathy, independent of angiotensin II and blood pressure levels. Angiotensin II is the main stimulus for aldosterone secretion and blocking the RAAS with an ACEI and/or ARB should theoretically inhibit the secretion of aldosterone. In heart failure studies, however, an increase in aldosterone during long-term treatment with ACEIs, so-called aldosterone escape, has previously been described. In a short-term study in type 2 diabetic patients with micro- or macroalbuminuria, patients who developed aldosterone escape during ACEI therapy, had higher albuminuria than patients without aldosterone escape. Addition of spironolactone treatment caused a further decrease in albuminuria in these patients.

We investigated the prevalence and significance of aldosterone escape in 63 type 1 diabetic patients with diabetic nephropathy during long-term treatment with the ARB losartan. During this treatment, aldosterone escape developed in 41% of patients and was associated with an approximately twice as rapid decline in renal function compared with patients without aldosterone escape.

Dual blockade of the RAAS with an ACEI and an ARB, has been shown to have a beneficial effect on reduction of albuminuria in type 1 and type 2 diabetic patients with diabetic nephropathy. To investigate whether this beneficial effect can be attributed to a further suppression of aldosterone levels, we measured aldosterone in 51 type 1 diabetic patients who had undergone a dual RAAS blockade study where the combination of an ACEI and an ARB was compared with single blockade with the same ACEI. The study showed that dual blockade of the RAAS reduced plasma aldosterone levels by 28% compared with ACEI therapy alone. In a multiple linear regression analysis, changes in aldosterone, diastolic blood pressure, GFR and ACE / ID genotype were associated with the observed changes in albuminuria. In contrast, we could not confirm that a known polymorphism in the gene encoding for aldosterone synthase played a role for aldosterone levels in type 1 diabetic patients with diabetic nephropathy.

Based on these studies we carried out a clinical, randomized, double-masked, placebo-controlled, crossover study where we examined the renoprotective effects of spironolactone in addition to the ongoing recommended antihypertensive treatment, including an ACEI or an ARB. The study showed that spironolactone in addition to standard treatment resulted in a decrease in albuminuria of 30% compared to standard treatment plus placebo. Moreover, there was a decrease in day blood pressure of 10 / 5 mm Hg in this group of patients who were already receiving a median of 3 antihypertensive drugs. We found similar results in an identical study conducted in type 2 diabetic patients. Finally, in a third study we examined the renoprotective effects of spironolactone in diabetic patients with albuminuria in the nephrotic range ( $\geq 2.5$  g / 24-hour). Even in these patients who have an extremely poor prognosis, we found a reduction in albuminuria of 32% and in blood pressure of 6 / 4 mm Hg. Two patients of a total of 51 patients enrolled in the spironolactone studies were excluded due to hyperkalemia (one severe) underlining the need for continuous monitoring of renal function and electrolyte status in patients with diabetic nephropathy during RAAS-blocking treatment. In our spironolactone studies, the decrease in albuminuria was associated with a reversible non-significant decrease in GFR. Our autoregulation study showed that spironolactone does not affect the ability to autoregulate GFR, indeed other studies indicate that changes in GFR could be due to a beneficial decrease in the intraglomerular pressure.

Suboptimal efficacy of ACEI therapy has been suggested to be due to under-dosing in some cases. Most dose-titration studies are based on the antihypertensive efficacy in patients with essential hypertension. The optimal dose for renoprotection is not necessarily the same as for hypertension, i.e. it has been shown in several studies that increasing the dose of ACEIs or ARBs above the recommended doses for antihypertensive treatment offer additional albuminuria-lowering effect independent of blood pressure reductions. In a randomised, double-masked, crossover trial, we found an additional reduction of albuminuria by doubling the lisinopril dose despite similar blood pressure reduction.

Evidence of a short-term beneficial effect of adding spironolactone to an ACEI or an ARB in diabetic nephropathy is piling up. In fact, no studies have reported no or negative effects of spironolactone in diabetic nephropathy.

As the short-term reduction in UAER has been shown to predict the long-term renal and cardiovascular outcome in other settings, there is well-founded hope for a long-term beneficial effect of adding spironolactone to recommended standard therapy including ACEIs, ARBs and diuretics.

The incidence of severe hyperkalemia in clinical trials has been low, probably due to close clinical observation, adjustment of dietary intake of potassium and dose of diuretics and should not be taken as an indication for liberalized use of spironolactone in patients with impaired kidney function. Rather, this potentially life threatening adverse event should be taken seriously and only physicians with expert knowledge who have the opportunity to closely monitor their patients should initiate spironolactone treatment in patients with impaired kidney function.

Future studies that could provide further important knowledge on the effects of aldosterone blockade includes: dual RAAS blockade combining a direct renin inhibitor and an aldosterone antagonist, studies on renal hemodynamics further exploring the effect of aldosterone antagonism on RPF and intraglomerular pressure. Finally, long-term randomized intervention trials with 'robust endpoints', i.e. ESRD, cardiovascular morbidity and mortality, are needed in order to evaluate safety and efficacy of spironolactone treatment in diabetic patients with diabetic nephropathy.

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