

# Angiotensin II receptor blockade in diabetic nephropathy

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## 1. INTRODUCTION AND AIM

Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria, increasing arterial blood pressure, a relentless decline in GFR and highly elevated risk for cardiovascular morbidity (1) and mortality (2; 3). The cumulative incidence of 25-35% in type 1 and type 2 diabetic patients with diabetes duration of more than 20 years (4; 5) has made diabetic nephropathy the leading cause of end stage renal failure (3). The natural course of diabetic nephropathy is characterized by a mean rate of decline in GFR of 10-15 ml/min/year ranging from 0-25 ml/min/year (6-8) but aggressive antihypertensive treatment through the last decade at Steno Diabetes Center has reduced the mean rate of decline in GFR to 4.0 ml/min/year in type 1 diabetic patients (9).

Extensive investigation has documented the key role of the RAAS in the pathogenesis and pathophysiology of diabetic nephropathy (10). Accordingly, blockade of the RAAS is first-line therapy in the treatment of diabetic nephropathy (11). The primary effect of ACE inhibitors is mediated through reduced generation of angiotensin II, but additional effects, perhaps mediated through bradykinin accumulation, may also contribute to the beneficial effect. On the other hand, specific blockade of the AT1 receptor inhibits actions of angiotensin II generated by any enzyme pathway and may overcome the suggested interaction between ACE inhibition and the ACE/ID polymorphism. ACE inhibitors are currently first-line therapy in type 1 diabetic patients with diabetic nephropathy according to the Captopril Collaborative Study (12) but the effect of ARBs has not been investigated thoroughly.

Previous studies in diabetic (13) and non-diabetic albuminuric renal disease (14), have documented that an effective short-term response, i.e. reduction in albuminuria after initiation of antihypertensive treatment, is a strong predictor of subsequent long-term renoprotective capacity of slowing progression in kidney function. Furthermore, albuminuria is a predictor of progression of diabetic renal disease (15), but the mechanisms responsible for albuminuria are incompletely understood. Functional studies (16; 17) of the mechanisms of albuminuria and glomerular membrane permeability in diabetic nephropathy have demonstrated the presence of size-selective defects in advanced nephropathy that were partly repaired by ACE inhibitor treatment (17). However, changes in size-selective properties of the glomerular membrane have not been detectable in early nephropathy by previous available methods (16).

The aims of the present work were to investigate aspects of pathophysiological mechanisms responsible for albuminuria in early diabetic nephropathy, the effects of ARBs in treatment of diabetic nephropathy and the role of individual patient factors in response to renoprotective treatment including genes. Furthermore, expression of the RAAS in diabetic nephropathy, antihypertensive treatment

and dual blockade of the RAAS in diabetic renal disease will be reviewed.

## 2. DEFINITION OF DIABETIC NEPHROPATHY

Diabetic nephropathy was diagnosed clinically if the following criteria were fulfilled: persistent albuminuria  $\geq 300$  mg/24 hours in at least two out of three consecutive urine collections with presence of diabetic retinopathy and the absence of clinical or laboratory evidence of other kidney or urinary tract disease (18; 19). In case these criteria are not fulfilled, a kidney biopsy displaying diabetic glomerulosclerosis is required for the diagnosis of diabetic nephropathy.

## 3. ASPECTS OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM IN DIABETES

The status of the RAAS in diabetes has been extensively investigated. Suppression of the systemic RAAS is most often observed in diabetic nephropathy (20), but data of measurements of plasma renin are inconsistent, probably due to the numerous factors that influence the system (10; 21).

In recent years, the intrarenal RAAS has been focus of extensive studies. The intrarenal RAAS has a critical role in the paracrine regulation of renal function (22), as demonstrated by presence of mRNA and proteins of RAAS components in glomeruli and tubules in cultured cells from healthy rats and humans (23-27). Furthermore, recent immunohistochemical studies have demonstrated an abundance of AT1 receptors in afferent and efferent arteriolar vascular smooth muscle cells, mesangial cells of glomeruli and on the luminal surface of tubular cells (28). In the lumen of the proximal tubules, angiotensin II levels are approximately 1000-fold greater than levels in plasma, probably due to secretion from tubular cells to activate epithelial AT1 receptors and thereby stimulate sodium and water reabsorption (29).

The intrarenal RAAS may be activated early in the course of diabetes, despite normal (30) or suppressed (31) levels in plasma. A recent study in a model of early experimental diabetes suggested an interaction between activation of the intrarenal RAAS and hyperglycemia as demonstrated by significantly increased expression of renin mRNA in proximal tubules and whole kidney angiotensin II (32).

Activation of the intrarenal RAAS would be expected to generate increased levels of angiotensin II. In accordance, Ballermann et al (33) first reported a decrease in glomerular angiotensin II receptors in diabetic rats 3-4 weeks after induction of diabetes. Similarly, angiotensin II receptors were found to be reduced in the proximal tubules in streptozotocin diabetic rats 2 weeks after induction of disease (34). However, measurements of whole kidney angiotensin II concentrations in diabetic rats compared to control animals have revealed conflicting results (32; 35; 36).

Data from a clinical study in type 2 diabetic patients with diabetic nephropathy, demonstrated that despite the presence of a low plasma renin at baseline, renal vasodilation in response to ARB treatment was enhanced compared to control persons, which may reflect an increased intrarenal angiotensin II level in the diabetic patients (37). An indication of intrarenal RAAS activation in type 1 diabetes was demonstrated by Miller (38), who found that moderate hyperglycemia was associated with an increased renal vascular resistance and filtration fraction compared to euglycemia. Accordingly, renal haemodynamic responses to high blood glucose were blunted by administration of an ARB, whereas stimulatory effects of angiotensin II infusion were abolished during hyperglycemia compared to euglycemia.

Thus, increasing evidence suggest, that intrarenal RAASs may be activated early in the course of diabetes. Furthermore, the renoprotective effects of RAAS blockade in diabetic kidney disease support the hypothesis of an increased intrarenal angiotensin II production, which may exert feedback inhibition on systemic renin secretion.

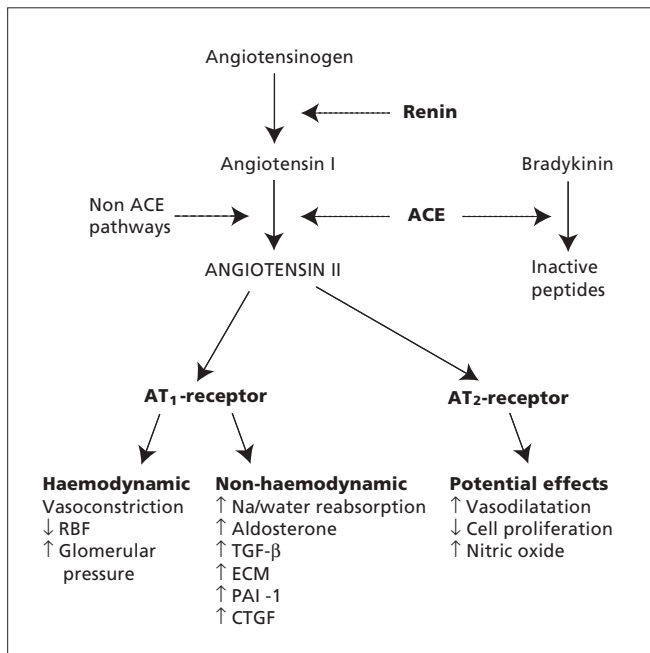


Figure 1. The renin angiotensin aldosterone system.

### 3.1 RENAL EFFECTS OF ANGIOTENSIN II IN DIABETIC NEPHROPATHY

Renal angiotensin II is delivered from the circulation or formed intrarenally from angiotensin I (22). Knowledge of the renal effects of angiotensin II have expanded considerably during the last three decades. Increasing evidence suggest that angiotensin II acts as a circulating vasoconstrictory hormone as well as a paracrine and autocrine peptide to modulate renal function (39) (Figure 1).

#### 3.1.1 Haemodynamic effects of angiotensin II

Although several vasoactive hormones exhibit effects upon renal haemodynamics, angiotensin II has been shown to be a major intrarenal hormone to regulate glomerular filtration rate (40). Early experimental studies of glomerular ultrafiltration with infusion of angiotensin II (41; 42) demonstrated by direct micropuncture measurements, that angiotensin II increases efferent and afferent arteriolar or at least preglomerular resistance, the latter primarily due to an autoregulatory response to an increased systemic blood pressure. As a consequence of a greater efferent than afferent arteriolar effect of angiotensin II, the glomerular capillary pressure increases.

In diabetic rats, glomerular capillary hypertension, proteinuria and renal structural abnormalities were found 14 months after induction of disease (43). However, ACE inhibitor treatment in a parallel group of diabetic rats, reduced glomerular capillary pressure to a level comparable to a non-diabetic control group which prevented development of proteinuria and structural abnormalities. These findings indicate that haemodynamic effects of angiotensin II are central in the pathogenesis of diabetic glomerulosclerosis and similar results have been found in non-diabetic rat models (44).

In type 2 diabetes, calculated glomerular pressure and efferent arteriolar resistance were found to be increased in patients with elevated urinary albumin excretion rate compared to normoalbuminuric patients (45). ACE inhibitor treatment lowered glomerular capillary pressure and albuminuria, in agreement with the experimental findings. Similar data in type 1 diabetic patients are presently not available, but our study of the time course of the antihypertensive and antiproteinuric effect of ARB treatment (46) discussed in section 6, demonstrated that systemic and renal haemodynamic mechanisms are of primary importance for the antiproteinuric effect. Hence, experimental and human data suggest that haemodynamic effects of angiotensin II are crucial in the pathogenesis of diabetic glomerulosclerosis.

#### 3.1.2 Non-haemodynamic effects of angiotensin II

The recognized role of angiotensin II in the pathogenesis of diabetic nephropathy can not exclusively be attributed to the haemodynamic effects of angiotensin II (10). Several studies have investigated the importance of non-haemodynamic effects of angiotensin II in the pathogenesis of diabetic nephropathy. Accumulating data support the hypothesis that angiotensin II exerts diverse actions such as growth stimulation, induction of fibrogenesis and modulation of endothelial function.

Angiotensin II induce expression of TGF- $\beta$ , collagen and fibronectin and stimulates mesangial cell proliferation resulting in increased synthesis of extracellular matrix (47; 48). Increased expression and synthesis of matrix proteins is not a direct effect of angiotensin II, but appear to be mediated through activation of PKC and p38 MAPK pathways which stimulate TGF- $\beta$  gene expression (49). Numerous studies have indicated that hyperglycemia induce an increase in TGF- $\beta$  protein and mRNA expression in experimental and human diabetes (50; 51) as well as in cell culture studies (51; 52). Other studies of mesangial cell cultures have disclosed striking similarities between the effects of high glucose-containing medium and incubation with angiotensin II on growth properties and induction of cytokines (53). Furthermore, increased gene expression of TGF- $\beta$  in cultured mesangial cells stimulated with angiotensin II and high glucose concentration appear to be mediated by the same PKC and p38 MAPK pathways (49). Hence, the effect of high glucose on TGF- $\beta$  gene expression may be mediated by increased angiotensin II production in mesangial cells. This hypothesis is supported by the observation that increased TGF- $\beta$  secretion induced by high glucose may be inhibited by addition of an ARB (53).

Numerous other cytokines are involved in mesangial matrix accumulation. Recent studies have demonstrated that CTGF is an important factor in the pathogenesis of mesangial matrix accumulation acting downstream from TGF- $\beta$  (54). VEGF is a family of potent cytokines which act to induce angiogenesis and markedly increase microvascular permeability, suggesting a key role in the pathogenesis of diabetic microvascular complications (55). VEGF is abundantly expressed in the renal glomerulus, especially in podocytes (56). Angiotensin II is a potent stimulator for VEGF mRNA expression and peptide production (55) and elevated plasma levels were recently demonstrated by Hovind et al (57) in type 1 diabetic patients with diabetic nephropathy. The exact role of VEGF on the pathogenesis of diabetic nephropathy is yet unclear, whereas evidence implicating VEGF in the pathogenesis of diabetic retinal neovascularization is much more substantial (58).

Plasmin is a key regulator of fibrinolysis and extracellular matrix turnover. Generation of plasmin from plasminogen by plasminogen activators are controlled by PAI-1. Angiotensin II has been shown to increase PAI-1 in cultured mesangial cells, which may lead to increased matrix accumulation (59).

Angiotensin II has been identified as a major modulator of endothelial function. Endothelial cells produce vasoconstrictive substances such as endothelin-1 and vasodilator substances such as nitric oxide (NO). NO serves as a functional antagonist for angiotensin II in the kidney, i.e. counterbalancing the constrictor actions of angiotensin II (60). Furthermore, NO has renal antiproliferative effects and decreases matrix protein synthesis (61). In vivo assessments of endothelial function by forearm pletysmography, has most often revealed an impaired state in diabetes (60). The NO system may be up-regulated in diabetic nephropathy, but according to Jaimes et al (62) angiotensin II also activates superoxide anion production. The superoxide anion interacts with NO, thus, the net effective NO availability in diabetic nephropathy appear to be reduced due to inactivation by superoxide anions. Angiotensin II has been identified as an important stimulator of the synthesis of the vasoconstrictor endothelin-1 in endothelial cells (63), vascular smooth muscle cells and mesangial cells (64). The interaction between RAAS and endothelin-1 is emphasized by demonstration of reduc-

tion of endothelin-1 by ACE inhibition in human and experimental studies (65). Furthermore, this effect appears to be independent of bradykinin accumulation (65). Thus, angiotensin II may induce an imbalance between expression of the vasodilator NO and superoxide anions or increased endothelin-1 expression, which may enhance progression of diabetic nephropathy.

Further cytokines and growth factors such as insulin-like growth factors, interleukin-6 and platelet-derived growth factor are involved as primary or downstream effects of angiotensin II in the pathogenesis of diabetic nephropathy (66). Comprehensive research is ongoing to clarify the field of non-haemodynamic effects of angiotensin II in diabetic nephropathy.

In summary, angiotensin II stimulate fibrogenic growth factors and cytokines in mesangial cell culture studies, however, studies of the effects of high glucose concentration reveal similar results. Activation of cytokines by hyperglycemia may be mediated by angiotensin II formation in mesangial cells and inhibited by ARBs. Interactions between hyperglycemia, angiotensin II and intrarenal cytokines should be further investigated.

### 3.2 ANGIOTENSIN II RECEPTORS

Angiotensin II mediates its main effects through binding to AT1 and AT2 receptors. Further receptor subtypes have been identified, but the physiological significance of these are unknown (67). AT1 and AT2 receptors belong to a superfamily of 7 transmembrane-domain G-protein coupled receptors, but differ significantly in distribution and expression in different tissues.

The AT1 receptors are responsible for the major actions of angiotensin II in adults. In the glomerulus, the presence of AT1 receptors has been demonstrated on mesangial cells (23), endothelial cells (68) and podocytes (69). AT1 receptor activation reduces renal blood flow and causes efferent arteriolar constriction (70). Activation of AT1 receptors in mesangial cells promote growth factors such as TGF- $\beta$  (47; 48) and decrease matrix degradation due to PAI-1 activation (59), as discussed previously. AT1 receptor function on podocytes is unclear, but may influence the glomerular filtration barrier through contraction of foot processes (71), as discussed in section 4. In proximal tubules, AT1 receptor activation stimulates transport of sodium and water and induce expression of TGF- $\beta$  (72) thereby stimulating the fibrogenic process.

The functions of AT2 receptors are incompletely understood. Experimental data suggest that activation of AT2 receptors oppose the actions mediated via the AT1 receptors, by endothelial cell-mediated renal vasodilation and exert antiproliferative effects (73; 74). Renal vasodilation induced by AT2 receptor activation may also be mediated through release of bradykinin and nitric oxide (75). The potential vasodilatory effect of AT2 receptor stimulation would be especially convenient during ARB treatment, since plasma concentrations of angiotensin II are increased. In contrast, recent data from experimental studies in non-diabetic kidney disease have suggested that that expression of VEGF may be mediated by AT2 receptors (76) and specific blockade of the AT2 receptor may confer a degree of renoprotection (77). Recently, a reduction of AT2 receptors was demonstrated in a model of early diabetes in streptozotocin diabetic rats (78) but the functional importance of possible downregulation of AT2 receptors in diabetes is unknown as is the state of the receptor in diabetic renal disease. Thus, evidence regarding the effect of AT2 receptor activation in diabetes is presently conflicting and limited.

### 3.3 PATHWAYS FOR GENERATION OF ANGIOTENSIN II AND ESCAPE OF ACE INHIBITION

Angiotensin II is primarily generated by conversion of angiotensin I through the ACE pathway, but alternative enzymes such as chymase, also contribute to formation of angiotensin II (79). Thus, inhibition of ACE will probably not induce a complete blockade of the RAAS. Angiotensin II levels in plasma are lowered after initiation of ACE

inhibitor treatment (80; 81), however, during prolonged treatment, the degree and duration of angiotensin II suppression may be reduced in agreement with partial escape of ACE inhibition (81). Incomplete RAAS blockade during chronic ACE inhibitor treatment due to ACE-escape and angiotensin II formation by alternative pathways may be overcome by inhibiting the action of angiotensin II at the site of the AT1 receptor by an ARB.

## 4. GLOMERULAR PERMSELECTIVITY IN DIABETIC NEPHROPATHY

The glomerular capillary walls behave as high-capacity ultrafiltration membranes. In healthy humans, a rate of ultrafiltration approximating 150 l/24 hours is achieved, despite an ultrafiltration pressure estimated to be < 20 mmHg (82). Despite their low resistance to water, glomerular capillary walls impose an extremely efficient barrier to the passage of proteins the size of albumin and larger (83). As demonstrated in animal micropuncture studies, the concentration of uncharged substances the size ( $r_s$ ) of inulin (16 Å) or smaller in the Bowman's Space fluid are essentially identical to those in plasma. In contrast, concentrations of albumin are approximately 3-4 orders of magnitude lower as compared to plasma (84-86). Furthermore, due to negative charge of the glomerular membrane, anionic proteins are probably more restricted than cationic proteins of equivalent size. Finally, molecular configuration has been demonstrated to influence the passage across the glomerular membrane (83; 87-90). Given the inaccessibility of Bowman's space in man, glomerular permeability has been most studied by determining fractional clearance to exogenous non-reabsorbable macromolecules. The fractional clearance of macromolecules is defined as the clearance of the molecule divided by the glomerular filtration rate of water determined by inulin clearance (82). Fractional clearance is then calculated from the urine (U) and plasma (P) concentrations of the macromolecule and inulin as  $(U/P)_M/(U/P)_I$ . If the probe macromolecule is neither reabsorbed nor secreted by the tubule, the fractional clearance of the macromolecule will be equal to the Bowman's space to plasma ratio. This sieving coefficient ( $\theta$ ) of a macromolecule of known radius and charge, provides the most direct functional measure of the barrier properties of the glomerular capillary wall in humans (91). Since endogenous proteins are heterogeneous regarding molecular charge and undergo variable rate of reabsorption in the tubules (84-86), they are not useful for quantifying the extent of barrier dysfunction. Therefore, numerous animal and human studies of diabetic and non-diabetic glomerulopathies, have investigated the permselective properties in the glomerular membrane by fractional clearance of exogenous non-reabsorbable macromolecules with dextran. When combined with analysis according to the models of hindered solute transport through water filled cylindrical pores, a description of the intrinsic properties of the glomerular capillary wall can be performed (92). By curve fitting techniques, three models of pore-size distribution have been found to successfully replicate the observed values of  $\theta$ . One is an isoporos plus shunt model, in which the major portion of the glomerular capillary wall is assumed to be perforated by cylindrical restrictive pores. A second population of large pores, which are non-discriminatory towards macromolecules of up to 60 Å, serves as a parallel shunt-pathway. A second model presumes that the glomerular capillary wall is perforated by a single population of cylindrical pores which have a continuous lognormal distribution of pore radii. The third model combines the foregoing distributions into a single heteroporous membrane model, the log-normal plus shunt model. It describes the glomerular capillary wall as a membrane with restrictive pores with a continuous lognormal distribution of radii and a parallel population of large shunt like pores (92).

Dextran, a polymer of glucopyranose, has been most widely employed as a test macromolecule to probe glomerular permselectivity (93; 94). Infusion of a polydisperse dextran mixture followed by gel chromatography to fractionate plasma and urine samples according

to dextran size, makes simultaneous determination of the sieving coefficients of chemically identical molecules of widely varying radii possible (91). However, a drawback of dextran is that the configuration becomes altered during transglomerular permeation (95). In solution, dextrans behave as random coils, whereas they become uncoiled under shear. Thus, their transport during transglomerular permeation is facilitated which may overestimate the effective pore radius presented to spherical proteins. To overcome this limitation of dextran, Ficoll, a polymer of sucrose, has been developed and employed as a probe of the glomerular capillary wall. Like dextran, Ficoll is filtered by the glomerulus and neither secreted nor absorbed by the tubules (96). However, during permeation of synthetic membranes Ficoll behaves like the spherical molecule envisioned in the theory of hindered transport through pores (95; 97). This has been supported by animal and human studies of transglomerular permeation (93; 94; 96). These studies demonstrated that glomerular sieving coefficients of Ficoll of a given radius are smaller than that of dextran of equivalent chromatographic radius. Thus, it is likely that the glomerular filtration of Ficoll, more closely approximates that of an ideal, uncharged globular protein.

#### 4.1 GLOMERULAR PERMSELECTIVITY IN ANIMAL MODELS

In normal rats, 90% of filtered albumin is reabsorbed in proximal tubular reabsorption, thus, measured albuminuria, approximates only 10% of the corresponding filtered load (98). In streptozotocin diabetic rats, filtered albumin load become significantly elevated 50-70 days after induction of diabetes (99). Tubular reabsorption is markedly depressed, suggesting that albuminuria is not simply related to a defect in the glomerular capillary wall, but also ascribed to a tubular dysfunction in this early phase of diabetic glomerulopathy (99). The size-selective properties of the glomerular capillary wall and effect of ARB treatment has been investigated in a rat model analogue to early nephropathy in humans as judged by the presence of normal glomerular filtration rate, two-fold increase in proteinuria and low prevalence of glomerulosclerosis (100). Three groups of rats were followed for 12 months: two streptozotocin diabetic groups, one treated with insulin only, and one treated with insulin + losartan and a non-diabetic control group. Analysis of the data after 12 months for evaluation of size-selectivity revealed that sieving coefficients of Ficoll for all molecular radii were significantly higher in diabetic, insulin treated animals as compared with the control group. However, in insulin + losartan treated diabetic animals, Ficoll sieving coefficients were significantly lower than the insulin treated rats and comparable to that measured in the non-diabetic control group. Proteinuria increased significantly in all groups compared to baseline but was even lower in the losartan group compared to the control animals (100). Computations based on the hydrodynamic theory of hindered solute transport (92) was carried out according to the model assuming that the glomerular membrane is perforated by two pore-size "populations", a log-normal restrictive distribution and a non-restrictive shunt pathway. From this model, it was found that in insulin treated animals, as compared with controls, pore-size distribution was shifted towards larger sizes for restrictive and non-restrictive pore populations. However, treatment of the diabetic animals with losartan induced a uniform significant reduction in membrane pore sizes. Hence, by Ficoll clearance, this rat model of early diabetic nephropathy revealed major modifications of size-selective functions of the glomerular capillary wall, whereas treatment with an ARB prevented the development of the size-selective dysfunction.

The influence of angiotensin II on glomerular capillary wall function has been investigated using Ficoll as a probe molecule in a model of isolated perfused kidney in non diabetic rats (101). Infusion of angiotensin II significantly increased proteinuria and fractional clearance of Ficoll molecules of sizes greater than 34 Å. However, pre-treatment with and ARB, completely prevented these

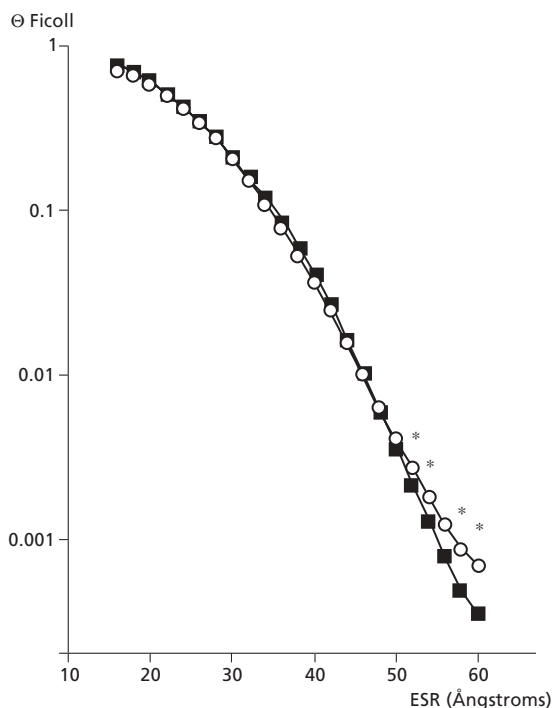
changes. An additional finding from this study was that angiotensin II infusion increased proteinuria by one order of magnitude more compared to clearance of Ficoll molecules corresponding to the size of albumin (36 Å). These observations indicated that changes in size-selectivity induced by angiotensin II, could not entirely explain the substantial proteinuria. Thus, angiotensin II may also affect other factors such as charge-selectivity or tubular function. However, caution should be taken before extrapolating data from an isolated kidney preparation to an intact human kidney.

#### 4.2 GLOMERULAR PERMSELECTIVITY IN DIABETIC NEPHROPATHY

Size-selectivity in type 1 diabetic patients with diabetic nephropathy and different levels of proteinuria have been extensively investigated by clearance of neutral dextran test molecules of graded size (30-60 Å) (16; 17; 82; 83; 102-104). Myers et al (83) originally demonstrated that advanced diabetic nephropathy with nephrotic range proteinuria is associated with elevated sieving coefficients above normal levels for large, nearly impermeant molecules >50 Å. Analysis of dextran sieving data according to the concept of hindered solute transport (92), suggested that the elevated  $\theta$  for the large molecules, could be explained by an expansion of a minor region of the glomerular capillary wall that behaves as a non-restrictive shunt pathway, whereas the radius of the restrictive pores ( $r_0$ ) in the major membrane component was indifferent from normal control persons. Furthermore, data have revealed that advanced diabetic glomerular disease is characterized by a graded increase in the fraction of the filtrate penetrating this shunt pathway ( $\omega_0$ ) (82; 102; 103). A relation between increasing proteinuria and the prominence of  $\omega_0$  is indicated by strong correlations between fractional clearance of albumin or IgG and  $\omega_0$  (82; 102; 103). Given the large size of immunoglobulins ( $r_0 = 55$  Å) it is likely, that IgG is filtrated exclusively through the large pores in the shunt pathway. Although the smaller size of albumin ( $r_0 = 36$  Å) it has been suggested that the major route of transcapillary escape of albumin could be via the non-restrictive pores in the shunt pathway rather than penetrating the smaller restrictive pores of the glomerular capillary wall because of the ellipsoid configuration and negatively charge of albumin (82; 102; 103). Similar results of size-selectivity in diabetic nephropathy have been found in type 2 diabetes (105) and by other research groups (104; 106; 107).

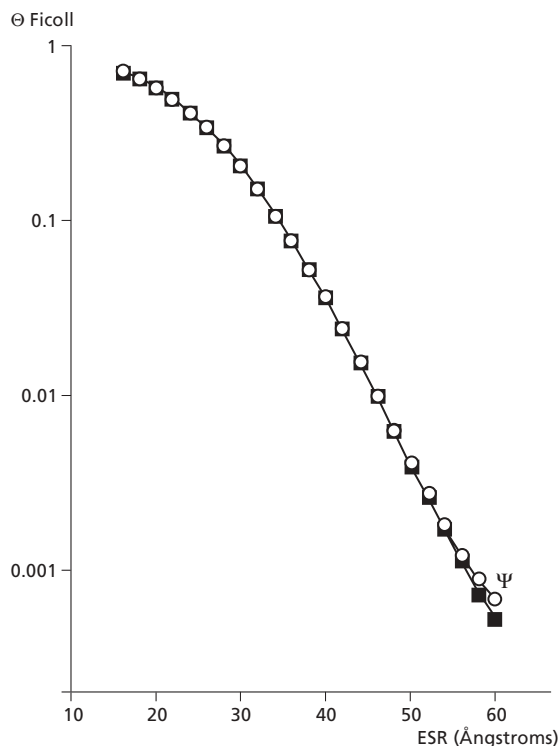
A comparable size-selective defect has been difficult to demonstrate in patients with lower grade proteinuria. In diabetic patients with sub-nephrotic range proteinuria or early nephropathy, significant elevations of  $\theta$  compared to control persons could not be demonstrated, even for large dextran molecules (16; 103-105). A number of studies have demonstrated that the urinary clearance of the most anionic species of either albumin or IgG may be elevated relative to that of less anionic or cationic type of the same protein, which may suggest impairment of charge-selectivity (103; 104; 108-111). However, given the unknown contribution of tubular function, evaluation of endogenous protein clearance are difficult to interpret. An enhanced binding of cationic molecules in the tubules, which may favour clearance of anionic proteins into the urine, has been suggested as an alternative explanation for the changes in charge selectivity (112; 113).

Previous investigations of size-selectivity in early nephropathy were performed with dextran to probe the glomerular capillary wall. To circumvent the limitations with this method, we investigated whether early diabetic nephropathy, defined as a serum creatinine within normal range, is associated with impairment of barrier size selectivity, using Ficoll 70 characterized by the superior rigid structure compared to dextran to probe the glomerular membrane (114). In addition, we investigated the influence of the ARB losartan on size-selectivity and albuminuria. The double-blind cross-over study consisted of two treatment periods each lasting 2 months in which the patients received losartan 50 mg o.d. and placebo. Twelve



**Figure 2.** Sieving coefficients ( $\theta$ ) of Ficoll plotted as a function of Einstein-Stokes molecular radius (ESR) in placebo-treated type 1 diabetic patients with diabetic nephropathy (○) and controls (■); \* =  $p < 0.05$  (114).

normotensive, previously untreated type 1 diabetic patients with diabetic nephropathy and normal kidney function were included in the study. Ficoll clearances were performed in the end of each treatment period during water diuresis. Twelve healthy volunteers provided control values for glomerular function. Bolus injections of inulin and para-aminohippurate for determination of GFR and RPF, was followed by continuous infusion of each marker. After a 60 minute equilibration period, a bolus injection of 1 mCi of tritiated Ficoll 70 ( $^3\text{H}$ -Ficoll) was given. Thereafter, four timed urine collections were made at 20 minutes interval. Plasma was sampled to bracket each collection. The average renal clearance of inulin was equated with the GFR. The molecules of Ficoll 70 in infusate spanned a radius range of 10–90 Å with the peak radius at 30 Å. The mean Ficoll sieving curve in placebo treated patients compared with healthy control persons is illustrated in Figure 2. As shown, diabetic nephropathy is associated with a selective elevation of  $\theta$  for large nearly impermeant molecules in the 50–60 Å interval. Membrane parameters computed from the Ficoll sieving curves, revealed that neither the mean radius nor the breadth of the lower distribution of restrictive pores in placebo treated patients differed from control values. However, the upper distribution of shunt-like pores was one order of magnitude more prominent in placebo treated type 1 diabetic patients as compared to control persons ( $p < 0.05$ ). Fractional IgG clearance was elevated by two orders of magnitude, whereas the corresponding elevation of  $\theta$  for Ficoll of equivalent radius (56 Å) was approximately 50%. However, a sieving coefficient of IgG comparable to that of Ficoll of 56 Å radius combined with an expected reduction in reabsorption of the enhanced load of filtered IgG in diabetic nephropathy, could justify this disparity. Thus, we conclude that barrier size defects could account for the elevated level of immunoglobulinuria in early diabetic nephropathy. Comparison of fractional clearances of albumin and Ficoll molecules of equivalent radius (36 Å) failed to reveal similar relations between albuminuria and glomerular barrier function. As shown in Figure 2,  $\theta$  of Ficoll of 36 Å radius is similar in diabetic nephropathy compared to the value in control persons. Furthermore, in healthy controls,  $\theta$  of Ficoll of 36 Å radius exceeds the estimated sieving coefficient of albumin by three orders of magnitude. As discussed below, losartan treatment lowered albuminuria significantly, whereas no correspond-



**Figure 3.** Sieving coefficients ( $\theta$ ) of Ficoll in type 1 diabetic patients with early diabetic nephropathy plotted as a function of Einstein-Stokes molecular radius (ESR) in placebo treatment (○) and losartan treatment (■).  $\Psi = 0.06$  (114).

ing change in  $\theta$  of Ficoll of 36 Å radius was observed (Figure 3). These disparities imply that the reduction in barrier size-selectivity in diabetic nephropathy probably have a minor influence on relatively small macromolecules such as albumin, at least in early diabetic renal disease. Thus, the glomerular filtration barrier may restrict albumin in addition to size, probably by charge- or shape selectivity as suggested by previous studies (93; 94; 96; 104; 115). Administration of losartan 50 mg o.d. significantly lowered mean arterial blood pressure, fractional clearance of albumin, IgG and the anionic subclass IgG<sub>4</sub>, whereas GFR and renal plasma flow remained unchanged (Table 1). As illustrated in Figure 3, losartan lowered  $\theta$  for the large Ficoll molecules towards normal values, though only approaching statistical significance for the molecule of 60 Å radius

**Table 1.** Mean arterial blood pressure and kidney function in healthy control persons and 12 type 1 diabetic patients with diabetic nephropathy (114).

Parameter	Controls	Diabetic nephropathy Placebo	Diabetic nephropathy Losartan 50 mg
Mean arterial blood pressure (mmHg) <sup>a</sup>	85 (3)	107 (2) <sup>c</sup>	104 (2) <sup>cd</sup>
Glomerular filtration rate (ml/min/1,73 m <sup>2</sup> ) <sup>a</sup>	104 (7)	79 (7) <sup>c</sup>	81 (6) <sup>c</sup>
Renal plasma flow (ml/min/1,73 m <sup>2</sup> ) <sup>a</sup>	623 (58)	459 (21) <sup>c</sup>	441 (23) <sup>c</sup>
Fractional albumin clearance (10 <sup>-5</sup> ) <sup>b</sup>	0.22 (0.13-0.36)	35.4 <sup>c</sup> (17.6-71.3)	26.7 <sup>cd</sup> (14.4-49.4)
Fractional IgG clearance (10 <sup>-5</sup> ) <sup>b</sup>	0.21 (0.15-0.29)	6.7 <sup>†</sup> (2.8-16.1)	5.3 <sup>cd</sup> (2.6-10.6)
Fractional IgG4 clearance (10 <sup>-5</sup> ) <sup>b</sup>	0.14 (0.11-1.17)	11.8 <sup>c</sup> (5.5-25.4)	9.1 <sup>cd</sup> (4.7-17.4)

a) Mean (SE); b) Geometric mean (95% CI); c)  $p < 0.01$  vs. controls; d)  $p < 0.05$  vs. placebo.

( $p = 0.06$ ). Computations of membrane parameters according to the heteroporous model of size-selectivity (92), revealed that the partly repair in the size-selective defect during losartan therapy may contribute to the observed reduction in proteinuria by a reduction in the fraction of the filtrate permeating the shunt-like pores ( $\omega_0$ ). Furthermore, losartan treatment induced a minor reduction in mean radius of restrictive pores. However, a more pronounced effect may be expected from a higher dose of losartan, i.e. 100 mg o.d. as suggested by our other studies (80; 116; 117). The effect of blockade of the RAAS by ACE inhibition on size-selectivity has been investigated by Morelli et al in type 1 diabetic patients with diabetic nephropathy (17). Data indicated that treatment with enalapril 10 mg, shifted both restrictive pores in the glomerular membrane and non-restrictive pores of the shunt pathway toward smaller sizes in keeping with our data. Similar results were found later by Remuzzi et al in patients with advanced diabetic renal disease (118). Effects of RAAS blockade on charge selectivity in microalbuminuric type 1 diabetic patients were investigated by Hansen et al (119) but a favourable influence of ACE inhibition could not be demonstrated. Two studies in non-diabetic kidney disease similarly suggested that the reduction in proteinuria induced by ARB treatment was associated with reductions of glomerular shunt volume (120; 121). Furthermore, comparison of ACE inhibition and ARB treatment in one of the studies (121) demonstrated similar effects on membrane parameters. Hence, the beneficial effect of blockade of the RAAS on glomerular size-selective function is likely to be a consequence of inhibition of the action of angiotensin II.

In summary, functional studies of size selectivity in type 1 diabetic patients have demonstrated the existence of a defect in the large shunt-like pores in early and advanced diabetic nephropathy which may be partly restored by ARB or ACE inhibitor treatment. However, loss of barrier size selectivity may be of minor importance in the genesis of modest albuminuria, thus, contributions from charge or shape selectivity, and the influence of RAAS blockade on these should be further investigated.

#### 4.3 STRUCTURAL AND MOLECULAR RELATIONSHIPS OF GLOMERULAR PERMELECTIVITY

Recently, studies of glomerular permeability have been performed to relate the functional properties of the glomerular membrane to the structural and molecular features at the cellular level as reviewed by Deen et al (90). Although it is generally acknowledged that the glomerular basement membrane restricts large plasma proteins, there is increasing evidence indicating that the ultimate barriers for proteins larger than albumin are the cellular layers and slit diaphragms (90; 122; 123). Foot processes are joined laterally by slit diaphragms. However, knowledge of structure and molecular configuration of the slit diaphragm has been rather limited. It represents a tiny membrane bridging the 30-40 nanometer filtration slit. The most frequently cited structure is that of Rodewald and Karnovsky (124) who described a slit diaphragm as made up of rod like units connected in center to a linear bar forming a "zipper-like" appearance. Discovery of the transmembrane protein nephrin as a major component of the slit diaphragm complex, provided a decisive progress in podocyte biology and pathophysiology (125-127). Lack of proper expression of the nephrin gene *NPHS1* has been shown by Tryggvason et al (123), to be linked to the congenital nephrotic syndrome of the Finnish type, a glomerular disorder that leads to severe proteinuria, loss of podocyte foot processes and slit diaphragms (128). Similarly, homozygous knock-out mice generated by inactivation of the nephrin gene, are nephrotic and fail to develop foot processes (129). Thus, the massive proteinuria associated with mutation or inactivation of the *NPHS1* gene suggests a key role for nephrin in the function of the glomerular filtration barrier. However, the function of nephrin is unclear. It has been hypothesized that nephrin molecules, which extend from adjacent podocytes, may interact in a homophillic manner, to form the possible

"zipper-like" structure discussed above (123), but this remain speculative.

Human and experimental studies of diabetic glomerulopathy have demonstrated podocyte injury and loss, along with broadening of podocyte foot processes (130; 131). Bonnet et al (132) demonstrated that expression of the nephrin gene and protein was significantly reduced in spontaneously hypertensive streptozotocin-diabetic rats compared to control animals. However, whether the decrease in nephrin expression is the cause of proteinuria or a consequence of advanced renal injury is unknown, but changes in nephrin expression were blunted by ARB treatment, which also prevented proteinuria.

Similar results have been found in animal studies of non-diabetic kidney disease, in which changes in nephrin expression were abolished by ARB and ACE inhibitor treatment (133). Furthermore, ACE inhibition has been shown to be associated with preservation of slit diaphragm function and protein zonula occludens-1, a component of the slit diaphragm (134). Changes observed during RAAS blockade in nephrin expression and slit diaphragm function could be related to the reduction in intraglomerular blood pressure rather than a direct action on podocytes. On the other hand, podocytes contribute significantly to the permeability properties of the glomerulus (71) and because of their elaborate cytoskeleton, suggestive of a contractile function, they are likely to participate in regulation of glomerular filtration. Furthermore, the demonstration of AT1 receptors on the podocyte surface (69), suggest that angiotensin II may influence the permselective properties of the glomerular filtration barrier through contraction of foot processes (71).

Thus, renoprotective effects by RAAS blockade may be partly related to podocyte and slit diaphragm function mediated directly via AT1 receptors located on podocytes on glomeruli.

In summary, podocytes and slit diaphragms may be key determinants of the glomerular filtration barrier. Discovery of nephrin as a component of the slit diaphragm and the association with proteinuria has provided seminal progress in research of podocyte biology. Furthermore, data suggest that ARB's and ACE inhibitors ameliorate the reduction of nephrin expression simultaneously with prevention of proteinuria.

#### 5. ANTIHYPERTENSIVE TREATMENT IN INCIPIENT AND OVERT DIABETIC NEPHROPATHY

Blood pressure targets in treatment of hypertension in diabetes have been extensively debated throughout the last decade. Guidelines from Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC VI) (135) from 1997 recommended blood pressure levels below 130/85 mmHg. Recent guidelines from American Diabetes Association 2002 (11) based on a meta-analysis of clinical trials by Bakris et al (136) recommend slightly more aggressive treatment goals, i.e. blood pressure levels below 130/80 mmHg in all diabetic patients and 125/75 mmHg in proteinuric patients.

Reduction in proteinuria represents an additional titration parameter in antihypertensive treatment (137). The initial reduction in proteinuria after initiation of antihypertensive treatment is predictive of the long-term efficacy of subsequent renoprotection in diabetic and non-diabetic renal disease (13; 14). Furthermore, residual proteinuria during treatment predicts rate of decline in GFR (9). Therefore, in addition to blood pressure reduction, antihypertensive therapy should be titrated upon maximal antiproteinuric effect.

##### 5.1 ANTIHYPERTENSIVE TREATMENT IN INCIPIENT NEPHROPATHY

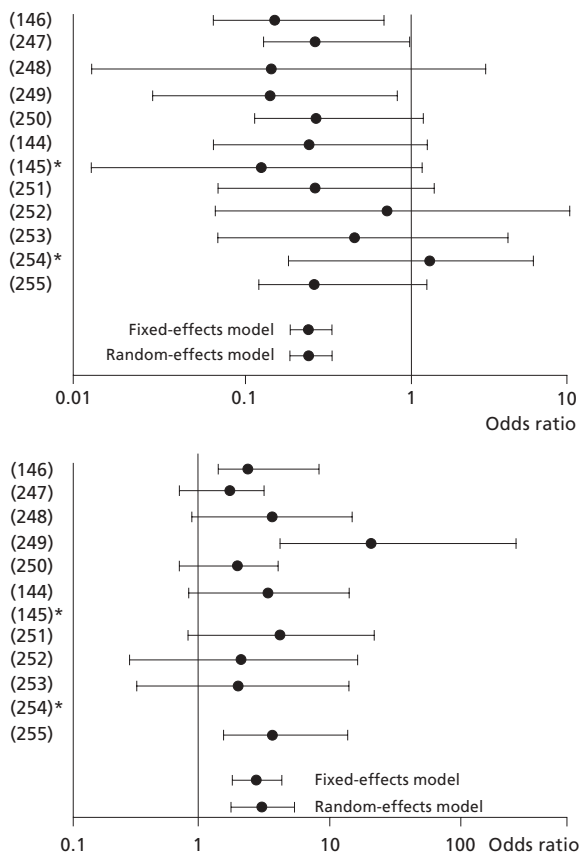
The concept of incipient nephropathy or microalbuminuria is based upon the observation that elevated urinary albumin excretion, defined as persistent urinary albumin excretion rate between 30-300 mg/24 hours or 20-200  $\mu\text{g}/\text{min}$ , predicts progression to overt dia-

betic nephropathy in type 1 and type 2 diabetes (138-141). Furthermore, microalbuminuria is associated with increasing blood pressure and hyperfiltration (140). Early intervention studies in normotensive type 1 diabetic patients demonstrated beneficial effects of antihypertensive therapy with beta blockers (142) on regression of microalbuminuria. Numerous subsequent studies in type 1 as well as type 2 diabetes have confirmed the renoprotective effects of antihypertensive treatment in microalbuminuria, even though the drug of choice has been debated (143).

### 5.1.1 Type 1 diabetes mellitus

How should we treat microalbuminuric type 1 diabetic patients in 2002? ACE inhibitors have been recommended as renoprotective therapy for all type 1 diabetic patients regardless of blood pressure based upon studies by Mathiesen et al (144), Marre et al (145), Viberti et al (146). A recent meta-analysis of individual patient data from 12 trials with 698 patients, addressed the question whether all type 1 diabetic patients with microalbuminuria should receive an ACE-inhibitor (143). Selected studies included at least 10 patients, had a placebo or conventional treatment group and at least 1 year of follow up. As illustrated in Figure 4, data showed that ACE inhibitors reduced the risk of progression to macroalbuminuria to approximately one third of that in the placebo group. Furthermore, regression to normoalbuminuria was three times greater as compared to the placebo group. At two years, the urinary albumin excretion rate was approximately 70% lower on average in the patients receiving ACE inhibitors weighed against the placebo group. According to Mathiesen et al (147), the beneficial effect of ACE inhibition in prevention of progression from microalbuminuria to overt nephropathy is long lasting and associated with preservation of normal kidney function.

One small study investigated the short-term antiproteinuric effect of losartan in 9 microalbuminuric type 1 diabetic patients (148).



**Figure 4.** Risk of progression to macroalbuminuria (top) and regression to normoalbuminuria (bottom) (143). Error bars represent 95% CI. \*: Zero events in the placebo group. (With permission from the publisher).

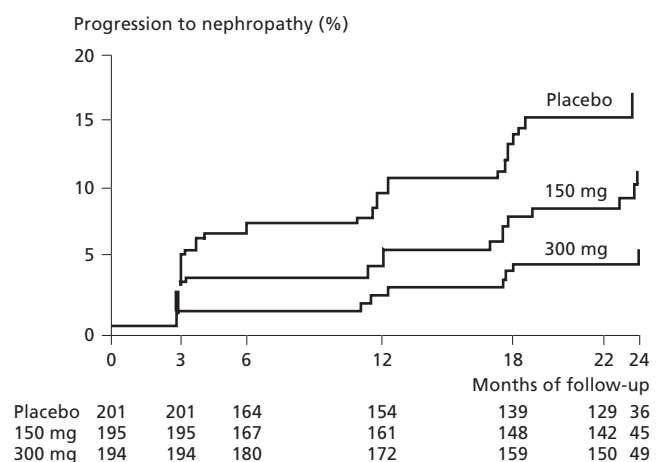
Proteinuria and blood pressure were significantly reduced after few days of treatment, suggesting a renoprotective effect.

Thus, accumulating data shows that ACE inhibitors prevent progression of microalbuminuria to overt nephropathy and preserves kidney function during long-term treatment. Consequently, ACE inhibitors must be regarded as first-line therapy for all type 1 diabetic patients with microalbuminuria regardless of blood pressure. Dual blockade of the RAAS with ACE inhibition and ARB may apply an additional beneficial effect and should be investigated in a long-term study in microalbuminuric type 1 diabetic patients.

### 5.1.2 Type 2 diabetes mellitus

Several studies have demonstrated beneficial renoprotective effects of antihypertensive treatment in type 2 diabetic patients with microalbuminuria (149-157). Originally, Ravid et al showed that normotensive patients treated with enalapril for a 7 year period, experienced an absolute risk reduction in progression from microalbuminuria to macroalbuminuria by 42 percentage points (149). Evidence has been conflicting in hypertensive patients with microalbuminuria regarding the existence of a specific renoprotective effect beyond the hypotensive effect of agents such as ACE inhibitors (150-158). Superior renoprotective effects of ACE inhibitors compared to conventional treatment has been reported by Lebovitz et al (153), Trevisan et al (157) and Chan et al (158), whereas similar effects of were found in the FACET (150), the ABCD (152) and the UKPDS (151) studies. The reasons for these conflicting results may in part be explained by short duration of antihypertensive treatment, blood pressure differences between treatment groups and small sample sizes. An exception from this is the long lasting UKPDS study suggesting equal effect of ACE inhibition and beta blockade.

Due to the inconclusive nature of the evidence, a multicenter study, IRMA-2 (159) was conducted. The study aimed to investigate the effect of the ARB irbesartan in hypertensive patients with type 2 diabetes and microalbuminuria. We included 50 patients at Steno Diabetes Center of 590 patients who were randomized to treatment with either irbesartan 300 mg, 150 mg or placebo in combination with conventional treatment for two years. The primary endpoint was time to progression to diabetic nephropathy defined as a urinary albumin excretion rate above 200 µg/min and at least a 30% increase from baseline. During the 24 months of follow up, diabetic nephropathy developed in 30 patients in the placebo group as compared to 19 patients in the irbesartan 150 mg group ( $p = 0.08$  vs. placebo) and 10 patients in the 300 mg group ( $p < 0.001$  vs. placebo) (Figure 5). The blood pressure levels were similar in the three groups, 144/83 mmHg on average throughout the study in the placebo group, 143/83 mmHg in the 150 mg group and 141/83 mmHg



**Figure 5.** Incidence of progression to diabetic nephropathy during treatment with Irbesartan or placebo (159). (With permission from the publisher).

in the 300 mg group. The hazard ratio for progression to diabetic nephropathy was 0.56 in the 150 mg group ( $p = 0.05$ ) and 0.32 in the 300 mg group ( $p < 0.001$  vs. placebo) after adjustment for a small difference in baseline albuminuria and systolic blood pressure during the study. Albuminuria remained unchanged in the placebo group, but was significantly decreased by 24% and 38% during treatment with 150 mg and 300 mg, respectively. Furthermore, regression to normoalbuminuria was more frequent in the 300 mg group as compared to placebo treatment, 34% vs. 21% ( $p = 0.006$ ). Thus, the IRMA-2 study demonstrated that irbesartan 300 mg is renoprotective beyond that attributable to lowering blood pressure in hypertensive type 2 diabetic patients with microalbuminuria. Recent interventions trials in type 2 diabetic patients with microalbuminuria have further established this conclusion (160; 161). Whether blockade of the RAAS by ACE inhibitors induce similar beneficial effects, will require a head-to-head comparative study to investigate. Consequently, ARBs must be considered first-line therapy in the treatment of hypertensive type 2 diabetic patients with microalbuminuria.

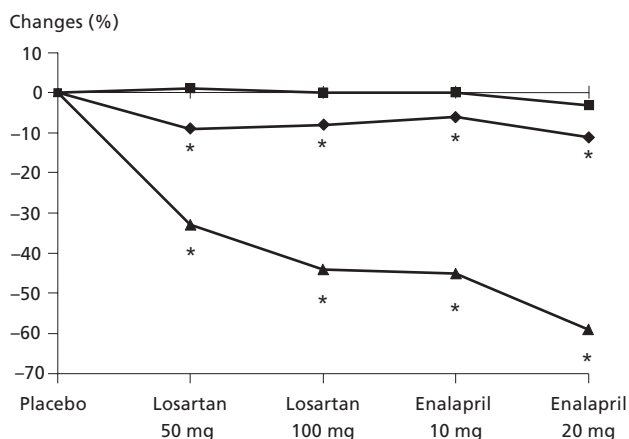
## 5.2 ANTIHYPERTENSIVE TREATMENT IN DIABETIC NEPHROPATHY

### 5.2.1 Type 1 diabetes mellitus

Antihypertensive treatment in diabetic nephropathy has become most successful since the first studies by Mogensen et al (162) and Parving et al (18) demonstrated that aggressive antihypertensive treatment with beta blockers and diuretics lowered rate of decline in GFR from more than 10 ml/min/year to less than 5 ml/min/year. Original studies dealing with ACE inhibition were reported in 1986 by Björck et al (163) followed by Parving et al (164) suggesting beneficial effects of ACE inhibition on rate of decline in GFR and albuminuria. In 1992, Björck et al (165) reported an open prospective study with 40 type 1 diabetic patients treated with either enalapril or metoprolol for a mean of 2.2 years. GFR declined by 5.6 ml/min/year in the metoprolol group, but only 2.0 ml/min/year in the enalapril group ( $p = 0.02$ ). These data indicated a specific renoprotective effect of ACE inhibition on kidney function in addition to what might be expected from blood pressure reduction alone. A similar conclusion was made by the Captopril Collaborative Study Group (12) who randomized 409 type 1 diabetic patients with diabetic nephropathy to treatment with either captopril or placebo in combination with conventional antihypertensive treatment for three years. A significant risk reduction by captopril treatment of 68% (39-83; 95% CI) ( $p < 0.001$ ) in time to doubling of serum creatinine was found in 102 patients with a baseline serum creatinine above 133  $\mu\text{mol/l}$ , whereas the risk reduction for the 307 patients with baseline serum creatinine below 133  $\mu\text{mol/l}$  of 33% (-44-69) did not reach statistical significance ( $p = 0.31$ ). The risk reduction for advanced nephropathy remained significant after adjustment for a difference in mean arterial blood pressure of 4 mmHg in favour of the captopril group.

A similar conclusion was made in a recent review (166) of randomized placebo controlled trials investigating the effect of ACE inhibitors in diabetic and non-diabetic kidney disease. In the ACE inhibitor groups, 115 of 700 patients developed ESRD or doubling of serum creatinine, as compared to 193 of 689 individuals in placebo groups. The aggregate relative risk for these endpoints was 0.60 (0.49-0.70; 95% CI) for individuals treated with an ACE inhibitor compared with placebo. Thus, ACE inhibition may have renoprotective effects in type 1 diabetic patients with diabetic nephropathy in addition to the benefit from lowering systemic blood pressure, at least in advanced nephropathy.

Is the effect of ACE inhibitors mediated by interference in the renin-angiotensin system? ACE is not a specific enzyme for conversion of angiotensin I, but has other substrates such as bradykinin. Bradykinin is a potent vasodilator acting through the release of prostacyclin, nitric oxide and endothelial-derived factors (167).



**Figure 6.** Changes in GFR (black squares), MABP (black diamond) and albuminuria (black triangles) in 16 hypertensive type 1 diabetic with diabetic nephropathy. \*:  $p < 0.05$  vs. placebo (80).

Whereas animal studies have reached conflicting results (168-170), a human study suggested that bradykinin may play a role in the effects of ACE inhibitors on blood pressure and kidney function (171). A recent study investigated the intraindividual, intrarenal haemodynamic responses to ACE inhibition and ARB treatment in type 1 diabetic patients on a high-salt diet (172). High correlation between the individual responses to the two antihypertensive agents was reported, which may suggest, that reduced formation of angiotensin II is the primary action of ACE inhibition, rather than bradykinin accumulation.

Multiple studies have suggested the existence of alternative ACE independent pathways for angiotensin II generation, as reviewed by Hollenberg et al (79). Thus, angiotensin II formation may not be completely suppressed by ACE inhibition. On the other hand, specific blockade of the action of angiotensin II at the receptor level omits the possibility of contribution from bradykinin accumulation to the antihypertensive effect. We compared the short-term renoprotective effects of the ACE inhibitor enalapril to the effect of specific intervention in the RAAS by the ARB losartan in 16 hypertensive type 1 diabetic patients with diabetic nephropathy (80). The double blinded cross-over study consisted of five treatment periods each lasting two months. The patients received losartan 50 mg, 100 mg, enalapril 10 mg, 20 mg and placebo o.d. in random order. Both doses of the drugs significantly reduced albuminuria and 24 hour arterial blood pressure without significant differences between the high doses (Figure 6). Mean arterial blood pressure decreased from  $104 \pm 2$  mmHg (mean  $\pm$  SEM) in the placebo period to  $95 \pm 2$  and  $96 \pm 2$  mmHg during treatment with losartan 50 and 100 mg, and to  $98 \pm 3$  and  $93 \pm 3$  by treatment with enalapril 10 and 20 mg, respectively (all  $p$  values  $< 0.05$  vs. placebo). Albuminuria in the placebo period was 1156 (643-2080) mg/24 hours (geometric mean; 95% CI) and was significantly reduced by 33% (12-51; 95% CI) by losartan 50 mg, 44% (26-57) by losartan 100 mg, 45% (23-61) by enalapril 10 mg and 59% (39-72) during treatment with enalapril 20 mg. GFR remained unchanged from baseline ( $90 \pm 6$  ml/min/1.73 ml/min/m<sup>2</sup>) in all treatment periods. Thus, the results demonstrated that blockade of the RAAS at the angiotensin II receptor level by losartan, induce similar renoprotective effects as inhibition of ACE by enalapril as evaluated by reduction in albuminuria and blood pressure in hypertensive type 1 diabetic patients with diabetic nephropathy. Therefore, the study suggests that the primary action of ACE inhibition is mediated by interference in the RAAS, i.e. reduced formation of angiotensin II. A similar outcome was found in an analogous study in non-diabetic patients (173).

The long-term renoprotective effects of losartan 100 mg o.d. on a principal renal end-point, i. e.  $\Delta$  GFR, was investigated in our prospective study of 54 hypertensive type 1 diabetic patients with diabetic nephropathy homozygous for the I or D allele of the ACE/ID

polymorphism with an average follow-up time of 36 months (174). Considering the total cohort disregarding genotypes, the annual rate of decline in GFR was 3.2 (2.2-4.7) (95% CI) ml/min/year with a 24 hours mean arterial blood pressure of  $95 \pm 1$  mmHg (mean  $\pm$  SEM) during three years of treatment with losartan 100 mg o.d. in combination with conventional antihypertensive treatment other than ACE inhibition if required. The study was not designed to compare the renoprotective efficacy of different treatment modalities, but demonstrates the capacity of an ARB to reduce the rate of decline in GFR in hypertensive type 1 diabetic patients with diabetic nephropathy. A rate of decline in GFR of 3.2 ml/min/year is considerably lower compared to the outcome of the largest ACE inhibitor study, the Captopril Collaborative Study (12) in which the average rate of decline in creatinine clearance was 8.0 ml/min/year in the captopril group and 10.8 ml/min/year in the patients treated with antihypertensive drugs other than ACE-inhibitors or calcium-channel blockers. Mean arterial blood pressure level in the captopril group was similar to our study (174) but assessed by office measurements, which complicates the comparison.

What is the long term effect of calcium channel blockers on kidney function in type 1 diabetes? Our group compared the long-term renoprotective effect of the ACE inhibitor lisinopril with the long-acting dihydropyridine calcium channel blocker nisoldipine in 51 hypertensive type 1 diabetic patients with diabetic nephropathy (175). Patients were followed for 4 years in a randomised double-blinded (single-blinded after 12 months) design. During the study period, mean rate of decline in GFR was similar in treatment groups, 6.0 ml/min/1.73 m<sup>2</sup>, mean arterial blood pressure levels  $100 \pm 2$  mmHg (mean  $\pm$  SEM) and  $103 \pm 2$  mmHg in the ACE inhibitor and calcium channel blocker group, respectively. Albuminuria was lowered by approximately 50% by ACE inhibitor treatment, but remained unchanged in the calcium channel blocker group. However, levels of albuminuria were not significantly different in the two groups during the study.

In summary, ACE inhibition may confer renoprotective effects beyond the effect of lowering arterial blood pressure, however, blockade of RAAS by ARB treatment induce similar short-term renoprotective effects as inhibition of ACE. Long-term ARB treatment imply beneficial effects on preservation of GFR, but large-scale studies of ARBs in type 1 diabetes on a principal renal and cardiovascular endpoint should be performed. Based on the literature, ACE inhibitors must be considered the first-line drug in the treatment of diabetic nephropathy in type 1 diabetes. However, increasing evidence suggest that the effect of ACE inhibitors is mediated by interference in the RAAS which indicate that ARBs represent an effective alternative to ACE inhibition. Calcium channel blockers may represent an effective class of drugs and long-term studies, e.g. in supplementary treatment to RAAS blockade in diabetic nephropathy, should be performed.

### 5.2.2 Type 2 diabetes mellitus

A number of studies have been performed comparing the effect of antihypertensive treatment with and without ACE inhibition on rate of decline in GFR in type 2 diabetic patients with diabetic nephropathy (152; 153; 176-179). Except from one study (176), no significant differences between treatment groups were found (152; 153; 177-179). However, recent evidence from the RENAAL and IDNT studies have outlined the treatment of this group of patients (180; 181). Both studies were large multinational, placebo controlled trials, investigating the long term renoprotective effect of ARBs in hypertensive type 2 diabetic patients with diabetic nephropathy and elevated serum creatinine. In RENAAL, 1513 patients were randomized to treatment with losartan or placebo alone or in combination with conventional antihypertensive treatment except ACE inhibitors. Mean follow up time was 3.4 years and primary efficacy measure was the time to first event of the composite end point of a doubling of serum creatinine concentration, end-stage renal disease

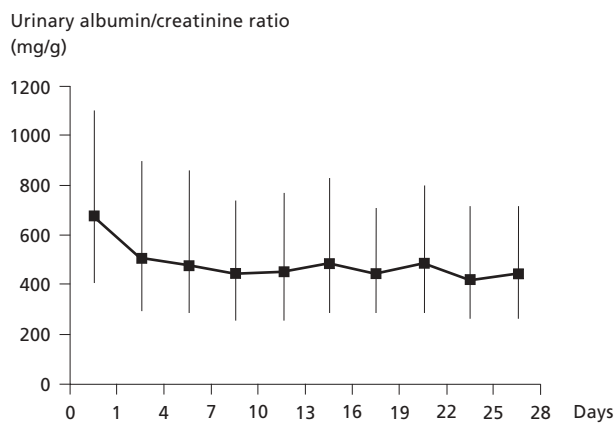
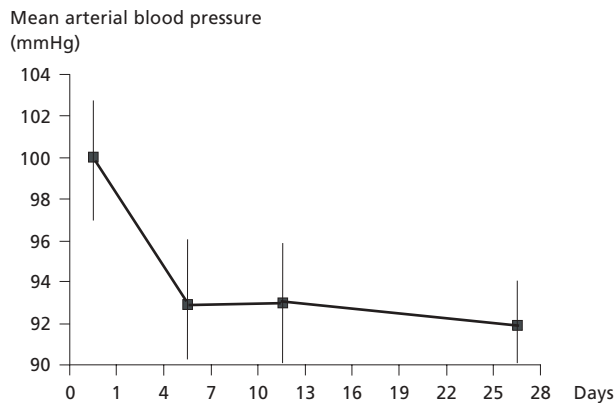
or death. Losartan treatment caused a 16% risk reduction of the primary composite end point ( $p = 0.02$ ). Analysis of individual components revealed a lowering of risk by losartan for doubling of serum creatinine of 25% ( $p = 0.006$ ), end-stage renal disease by 28% ( $p = 0.002$ ), whereas no differences in mortality were found between groups. The estimated rate of decline in GFR was 4.4 ml/min/1.73 m<sup>2</sup>/year in the Losartan group compared to 5.2 ml/min/1.73 m<sup>2</sup>/year in the conventional treated group ( $p = 0.01$ ). In the IDNT study 1715 patients were randomized to treatment with irbesartan, amlodipine or placebo, alone or in combination with conventional therapy except ACE inhibitors, for a mean of 2.6 years. The primary composite end point was similar as described for the RENAAL study. Treatment with irbesartan was associated with a 20% risk reduction for the primary composite end point compared with placebo ( $p = 0.02$ ) and 23% compared with amlodipine ( $p = 0.006$ ). The risk of a doubling of serum creatinine was 33% lower in the irbesartan group than in the placebo group ( $p = 0.003$ ) and 37% lower compared to the amlodipine group ( $p < 0.001$ ). In both studies there was a small difference in blood pressure control between groups, but statistical adjustment for the disparity had no impact on study conclusions.

Consequently, RENAAL and IDNT demonstrated renoprotective effects of treatment with ARBs on progression of diabetic nephropathy beyond that attributable to lowering blood pressure in type 2 diabetes. Similar results may be obtained by ACE inhibitors, but would require investigation in a large-scale head-to-head comparative study with an ARB. Therefore, first-line therapy of newly diagnosed hypertensive type 2 diabetic patients with diabetic nephropathy should be ARBs within the recommended range. However, blood pressure control in hypertensive type 2 diabetic patients is rarely obtained by monotherapy. In the IDNT study, patients received on average  $\geq 3$  non-study antihypertensive drugs to control the blood pressure.

## 6. TIME COURSE OF THE ANTIPROTEINURIC AND ANTIHYPERTENSIVE EFFECT OF ANGIOTENSIN II RECEPTOR BLOCKADE

Are haemodynamic mechanisms of primary importance in reduction of proteinuria during ARB treatment in diabetic nephropathy? Studies in patients with non-diabetic kidney disease (173; 182) have shown a dissociation between the time course of the antihypertensive and antiproteinuric effects after initiation of RAAS blockade by either ACE inhibitor or ARB treatment. Arterial blood pressure was lowered shortly after treatment was started, whereas a slow onset of the antiproteinuric effect of 3-4 weeks was observed, which may reflect contributions from non-haemodynamic mechanisms.

We investigated the time course of the antiproteinuric and antihypertensive effects of losartan in type 1 diabetic patients with diabetic nephropathy (46). In contrast to the studies in non-diabetic patients, our results demonstrated closely matched time courses of reductions in 24 hour arterial blood pressure and albuminuria as evaluated by daily urinary albumin/creatinine ratio samples (Figure 7). Maximal effects appeared within seven days and remained stable without trends for a second phase of protracted reductions during the study period of 28 days. Similarly, plasma renin (data not shown) was significantly increased and stabilized after 7 days ( $p < 0.05$ ). Thus, our data suggest that the antiproteinuric effect of losartan is primarily mediated by systemic and renal haemodynamic mechanisms. Similar results were recently found in microalbuminuric type 1 diabetic patients (148). As discussed in section 3, animal and human studies suggest that hyperglycemia activate the intrarenal RAAS, which may increase the reactivity to AT1 receptor blockade (32; 38). Hence, ARB treatment may induce an instant reduction in glomerular pressure and subsequently decline in albuminuria (43; 45). This hypothesis could partly explain the prompt antiproteinuric response observed in poorly controlled diabetes compared to data from non-diabetic kidney disease.



**Figure 7.** Time course of mean arterial blood pressure (top) and urinary albumin/creatinine ratio (bottom) during treatment with Losartan 100 mg o.d. All  $p < 0.05$  vs. baseline (46).

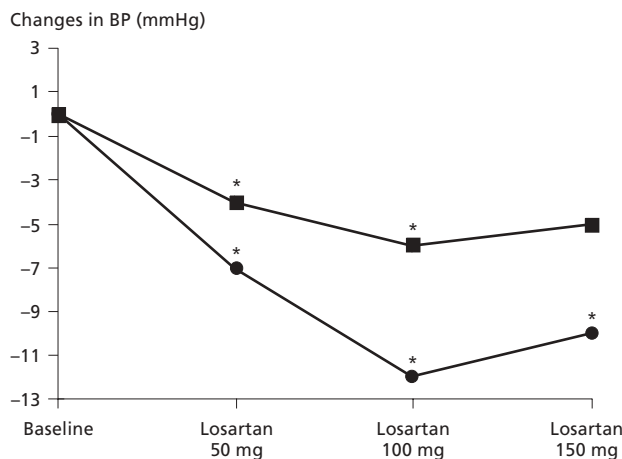
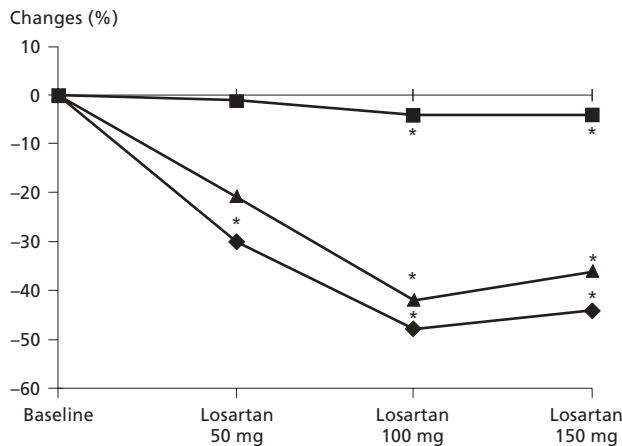
Even though haemodynamic mechanisms are of primary importance in the reduction of albuminuria, contributions from slowly on- setting non-haemodynamic effects of losartan are likely to appear.

In summary, the concordant time courses of the antihypertensive and antiproteinuric effects of ARB treatment suggest that systemic and renal haemodynamic mechanisms are of primary importance in the reduction of albuminuria.

## 7. DOSE TITRATION OF ANGIOTENSIN II RECEPTOR BLOCKADE FOR OPTIMAL RENOPROTECTION

Lowering of arterial blood pressure has been the main strategy for renoprotection during the past two decades. Reduction of albuminuria has been considered a secondary benefit from decreasing blood pressure, rather than a primary goal for treatment. Therefore, renoprotective therapy is administered in doses extrapolated from treatment of primary hypertension, which may be inadequate for optimal renoprotection. However, numerous studies in diabetic and non-diabetic kidney disease have documented that albuminuria is an important progression promoter as reviewed by Rossing (183) and Remuzzi (184). Furthermore, albuminuria is a putative predictor of progression during treatment, i.e. the more albuminuria is lowered, the better renal function outcome will be (13; 14).

In accordance with the Captopril Collaborative Study (12), ACE inhibition has been considered first-line therapy for renoprotection in treatment of type 1 diabetic patients with diabetic nephropathy through the last decade (11). In three recent trials in type 2 diabetic patients with early or advanced diabetic renal disease (159; 180; 181), the renoprotective effects of ARBs went beyond their blood pressure lowering effects and were superior to conventional anti-hypertensive agents in reducing albuminuria and postponing development of diabetic nephropathy and the composite end-points of doubling of serum creatinine, ESRD and death. Accordingly, ARBs



**Figure 8.** Top: reduction in GFR (squares), urinary IgG (triangles) and albuminuria (diamonds). Bottom: diastolic blood pressure (squares) and systolic blood pressure (circles) during treatment with increasing doses of losartan. \*:  $p < 0.05$  vs. placebo (117).

are now first-line treatment in type 2 diabetic patients with incipient and overt nephropathy according to American Diabetes Association (11). However, titration of doses of ACE inhibitors and ARBs as evaluated by reduction in albuminuria were not performed.

Dose-response studies of losartan in primary hypertension have demonstrated that doses above 50 mg o.d. provide no further anti-hypertensive effect (185). However, in non-diabetic renal disease, dose-response relationships of RAAS blockade for reduction of blood pressure and proteinuria may be different (173). Our initial study of the renoprotective effect of losartan in diabetic nephropathy (80) demonstrated a similar blood pressure reduction by losartan 50 and 100 mg, whereas high dose treatment was insignificantly more effective in lowering albuminuria. Therefore, we performed a dose-escalation study with the aim to determine the optimal dose of losartan for renoprotection and blood pressure reduction in 50 type 1 diabetic patients with diabetic nephropathy (117). Patients received increasing doses of losartan, initially 50 o.d. mg followed by 100 and 150 mg in three treatment periods each lasting two months. All doses of losartan significantly lowered arterial blood pressure and albuminuria (Figure 8). Albuminuria, assessed by at least two 24 hour urine collections, was significantly reduced from baseline by 30% (15-41) (95% CI) by losartan 50 mg compared to 48% (35-57) and 44% (32-56) by 100 and 150 mg, respectively. Hence, high dose treatment was significantly more effective in reducing albuminuria compared to 50 mg ( $p < 0.05$  losartan 50 vs. 100 mg), without differences between the beneficial effects of 100 and 150 mg. Moreover, analysis of data separated by the median albumin value at baseline, revealed a similar antialbuminuric efficacy of losartan in low and nephrotic range albuminuria. Losartan

100 mg was more effective in reducing 24 hours systolic blood pressure ( $p = 0.05$  losartan 50 vs. 100 mg) and diastolic blood pressure ( $p = 0.02$ ) as compared to 50 mg, whereas no difference was found between the blood pressure lowering effects of the two high doses. Separate analyses of day- and night-time blood pressures revealed a similar blood pressure lowering efficacy of the three doses of losartan during day and night. Hence, the beneficial effect of blood pressure reduction by losartan persists during the night even though the medication is administered once daily in the morning. These results are consistent with systemic levels of RAAS hormones during treatment, which indicated a maximum blockade of RAAS by losartan 100 mg. GFR declined by 4 ml/min/1.73 m<sup>2</sup> in the two high dose treatment periods, probably reflecting a haemodynamic reversible consequence of blood pressure reduction, as suggested previously (186). Thus, we suggest that the optimal dose of losartan for renoprotection is 100 mg o.d. in type 1 diabetic patients with diabetic nephropathy.

Although a study design with blinded randomized treatment periods is preferable, our previous five armed double-blind cross-over study (80) of the renoprotective effect of two doses of losartan and enalapril administered in random order, showed that the magnitude of the reduction of blood pressure and albuminuria was independent of treatment order. Furthermore, as discussed in section 6, we have recently demonstrated that the maximal antialbuminuric and antihypertensive effects of losartan 100 mg o.d. in hypertensive type 1 diabetic patients with diabetic nephropathy appear within 7 days after onset of treatment and remain stable thereafter (46). Similar results were found in microalbuminuric type 1 diabetic patients treated with losartan 50 mg (148). To avoid acute hypotension in patients with autonomic neuropathy starting directly on losartan 150 mg in a randomized design, an open study with increasing doses of losartan was chosen. However, we are confident that the superior effect of losartan 100 mg as compared to 50 mg o.d. demonstrated here is not simply a reflection of time on treatment.

A similar dose-escalation study of losartan in 10 patients with non-diabetic kidney disease (187) revealed in agreement with our study, that the optimal antiproteinuric effect was achieved by losartan 100 mg o.d. On the contrary, no additional antihypertensive effect was obtained by increasing the dose above 50 mg.

Urinary TGF- $\beta$  may represent a future supplementary target in treatment of diabetic nephropathy and titration of renoprotective therapy. Recent studies have suggested that overproduction of TGF- $\beta$  stimulated by factors such as angiotensin II and high glucose may be reduced by RAAS blockade (188-190). A dose-escalation study targeting TGF- $\beta$  expression in a rat model of non-diabetic kidney disease demonstrated that the maximal reduction in pathological glomerular TGF- $\beta$  expression was seen at doses of enalapril and losartan higher than those known to control blood pressure (191). A similar dissociation of doses of ARB and ACE inhibition were found in studies of regression of renal structural changes in another rat model of proteinuric non-diabetic kidney disease (192). Thus, doses of RAAS blockade required for maximal antifibrotic benefit may be higher than those needed for optimal blood pressure reduction. Future dose-response studies in diabetic nephropathy may include measurement of plasma or urinary TGF- $\beta$  or other indicators of renal fibrosis, as new surrogate markers for progression of kidney disease in titration of renoprotective therapy.

In summary, studies in diabetic and non-diabetic kidney disease have documented the importance of dose titration of ARBs and ACE inhibitors aimed at achieving the optimal renoprotective effect, assessed by reduction of albuminuria. Doses might be higher than those defined or required for optimal treatment of hypertension. Antifibrotic dose-response studies for renoprotection are awaited for ARBs and ACE inhibitors in diabetic renal disease since present recommended doses may be insufficient for optimal renoprotective therapy.

## 8. ASPECTS OF NON-HAEMODYNAMIC EFFECTS OF ANGIOTENSIN II RECEPTOR BLOCKADE IN DIABETIC NEPHROPATHY

### 8.1 EFFECT OF ANGIOTENSIN II RECEPTOR BLOCKADE ON TGF- $\beta$

Intrarenal angiotensin II stimulates mesangial cell proliferation and induces expression of TGF- $\beta$  independent of blood pressure, as discussed previously (47). Increased renal production of TGF- $\beta$  is a feature of diabetes expressed by elevated urinary TGF- $\beta$  excretion noted in patients with type 1 and type 2 diabetes and diabetic nephropathy (193-195). Therefore, reduced stimulation of TGF- $\beta$  expression may be part of the renoprotective effect of RAAS blockade in diabetic nephropathy.

Various *in vivo* experimental models of renal disease including diabetes have demonstrated that blockade of RAAS decrease expression of TGF- $\beta$  and matrix proteins. ARB treatment of streptozotocin diabetic rats in an experimental model with overexpression of angiotensin II, revealed marked reduction of renal TGF- $\beta$  and collagen IV mRNA compared to untreated animals 12 weeks after induction of disease (188). Furthermore, RAAS blockade by ACE inhibition in streptozotocin diabetic rats normalised expression of TGF- $\beta$  type II receptor mRNA and proteins compared to untreated animals 30 days after induction of diabetes mellitus (196). In consistence, a recent study in a rat model of non-diabetic kidney disease revealed similar beneficial effect of ARB treatment and ACE inhibition on lowering expression of renal TGF- $\beta$  mRNA (197).

Effects of ARB treatment on plasma levels of TGF- $\beta$  in humans were originally investigated in patients with chronic allograft nephropathy by Campistol et al, who found a significant reduction of plasma TGF- $\beta$  by losartan treatment for 8 weeks (198). Esmatjes et al (189) investigated fourteen type 2 diabetic patients with mild hypertension and microalbuminuria in an open study. Plasma TGF- $\beta$  was higher in diabetic subjects compared to healthy controls, but significantly lowered by losartan treatment in seven patients with TGF- $\beta$  levels at baseline above the mean. Levels remained unchanged in patients with TGF- $\beta$  levels below the mean value. In conflict with these data, plasma TGF- $\beta$  was unaffected by losartan in a recent study of a similar group of patients, whereas urinary TGF- $\beta$  was significantly reduced (190). Furthermore, urinary TGF- $\beta$  excretion at baseline correlated closely with indexes of glycemic control, possibly reflecting a relation between glucose concentration and TGF- $\beta$ . Effect of RAAS blockade on TGF- $\beta$  in diabetic nephropathy was investigated in serum samples of 58 type 1 diabetic patients from the Collaborative Study Group Captopril Trial (12; 199). Captopril significantly lowered serum TGF- $\beta$  compared to an increase in the placebo group six months after initiation of treatment. Furthermore, inverse correlations of percentage changes in serum TGF- $\beta$  during the six month period and the relative decline in GFR during the following two years of follow-up were found in both the captopril and placebo groups.

In summary, blockade of the RAAS is likely to reduce glomerular scarring, indicated by reductions in urinary TGF- $\beta$  levels, thus, TGF- $\beta$  may represent a supplementary therapeutic target in the treatment of diabetic nephropathy. Further studies evaluating effects of ARBs on urinary and blood levels of TGF- $\beta$  should be performed.

### 8.2 EFFECT OF ANGIOTENSIN II RECEPTOR BLOCKADE ON CIRCULATING ADHESION MOLECULES

Type 1 diabetic patients with diabetic nephropathy are at extremely high risk of atherothrombotic disease (200). Adherence of circulating leukocytes to endothelial cells and their subsequent migration into the arterial intima is an early feature in the formation of atherosclerotic lesions (201). The endothelial attachment of leukocytes is mediated by an increased expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and E-selectin. Soluble forms (e.g. sICAM-1) have been detected in plasma and are thought to reflect shedding

of membrane bound forms (202). Angiotensin II induces expression of VCAM-1 on endothelial cell surfaces in animal studies, which may be inhibited by ARB treatment, thus mediated by the AT1 receptor (203).

A potential mechanism for increased expression of sVCAM-1 and accelerated vasculopathy in diabetes may be related to interaction between advanced glycation endproducts and their endothelial receptors (202). A recent study in type 2 diabetes (201) demonstrated that increased levels of sVCAM-1 are independently associated with the risk of cardiovascular mortality. Elevated levels of circulating adhesion molecules in type 1 diabetic patients with diabetic nephropathy have recently been demonstrated (204; 205). However, data dealing with the effect of blockade of the RAAS on circulating adhesion molecules were not available.

We performed a double-blind five-armed cross-over study investigating the effect of ARB treatment by losartan 50 and 100 mg and ACE inhibition by enalapril 10 and 20 mg compared to placebo on circulating adhesion molecules (206). Furthermore, values were determined in 29 healthy control persons. Plasma levels of sVCAM-1, sE-selectin and vWF were elevated during the placebo period as compared to the control persons ( $p < 0.05$ ). General endothelial dysfunction was suggested by elevated vWF in all treatment periods. However, sVCAM-1 and sE-selectin were significantly decreased by RAAS blockade, even though lowering of sE-selectin did not reach statistical significance during losartan treatment ( $p = 0.08$ ). Thus, inactivation of angiotensin II by ARB or ACE inhibition may reduce the proatherogenic leukocyte-endothelial adhesion in type 1 diabetic patients with diabetic nephropathy. Evidence from previous intervention studies is limited, but elevated plasma levels of sVCAM-1, sICAM-1 and sE-selectin have been reported in hypertensive type 2 diabetic patients with microalbuminuria (207) and non diabetic obese patients (208). Similarly, plasma levels of sVCAM-1 were reduced by RAAS blockade.

If plasma concentrations of sVCAM-1 are independently associated with the risk for cardiovascular disease, as suggested by the Hoorn study (201) and potentially modifiable by RAAS blockade, should sVCAM-1 be measured in all diabetic patients? According to present data, RAAS blockade may have an antiatherogenic effect in diabetic patients with elevated albumin excretion rate. sVCAM-1 may be elevated even in normoalbuminuric patients (205), even though most studies report values within normal range (204; 209). Before routine assessments of adhesion molecules are considered, further prospective studies are required to investigate the pathophysiological significance of increased expression of adhesion molecules and the potential beneficial effect of RAAS blockade on the cardiovascular risk profile.

## 9. ROLE OF PATIENTS FACTORS IN THERAPY RESISTANCE TO ANTI-PROTEINURIC INTERVENTION IN DIABETIC AND NON-DIABETIC NEPHROPATHY

Large variability in individual response to renoprotective therapy is observed and individual patient factors may be relevant determinants of treatment response. Systemic and renal response to intervention may be consistent for a given individual, irrespective of treatment mode. We investigated this hypothesis by analysis of individual data from two studies in diabetic and non-diabetic nephropathy (80; 173). Group values from the study in diabetic patients are given in section 5 (80). The individual antiproteinuric responses to ACE inhibition and ARB were significantly correlated for both diabetic ( $r = 0.67$ ;  $p < 0.01$ ) and non-diabetic ( $r = 0.75$ ;  $p < 0.01$ ) subjects (210). Similar correlations were found for antihypertensive efficacy in diabetic ( $r = 0.73$ ;  $p < 0.001$ ) and non-diabetic ( $r = 0.55$ ;  $p < 0.05$ ) patients. Hence, patients responding favorably to one class of renoprotective drugs, perform with a similar positive outcome to other agents, whereas patients with a poor response to one class of drugs are likely not to benefit from changing to another agent. Analyses of the individual antiproteinuric responses to two different

doses of ACE inhibition revealed a significant positive correlation between responses to the two doses in both populations ( $p < 0.05$ ). Similar analyses for two doses of ARB demonstrated a similar significant correlation for non-diabetics, whereas statistical significance was not reached for the diabetic group.

These analyses support the hypothesis of individual patient related factors as determinants for response to renoprotective treatment. Similar results were found by Langsan et al (172), who investigated the individual renal haemodynamic responses to ACE inhibition and ARB in normoalbuminuric type 1 diabetic patients, as discussed previously.

Elucidation of individual patient related factors is of great importance for identification of high risk individuals and to improve efficacy of treatment regimens. Factors may relate to activation and responsiveness of the RAAS in the individual patient. High dietary sodium intake suppresses RAAS, thereby reducing the efficacy of RAAS blockade, which can be restored by addition of a diuretic (211). Severe glomerular structural changes reflected by higher baseline level of albuminuria, may be a factor that influence the individual response to treatment. However, no such relation was found in the present data set, though numbers were small.

Susceptibility to treatment may depend on genetic polymorphisms. The ACE/ID polymorphism has been suggested as a major determinant of therapy responsiveness to ACE inhibition (212; 213), i.e. type 1 diabetic patients with diabetic nephropathy homozygous for the D allele, have a reduced antialbuminuric response to ACE inhibitor treatment compared to patients homozygous for the I allele. However, we suggest in our pharmacogenetic study, that this specific interaction may be overcome by changing treatment to an ARB (116).

A number of patients with a poor response to the lowest doses enhanced the benefit of treatment by increasing the dose, whereas others tended to have a similar limited effect of higher doses (210). According to our dose-escalation study in diabetic nephropathy (117) and a similar study in non-diabetic kidney disease (187), the optimal dose of losartan for renoprotection is 100 mg o.d. without additional effects of further dose increments. However, a specific titration study for poor responders has not been performed.

In summary, individual patient related factors are involved in responsiveness or resistance to renoprotective treatment. Elucidation of individual determinants of responsiveness to therapy may provide a new concept for improvement of renal prognosis by individualization of renoprotective therapy.

## 10. ACE INSERTION/DELETION POLYMORPHISM AND DIABETIC NEPHROPATHY

The insertion (I)/(deletion (D) polymorphism within the human ACE gene that accounts for half of the observed variation in serum ACE between individuals was originally described by Rigat et al (214) in 1990. Based on the presence or absence of a 287 base pair sequence in intron 16, three genotypes are found, DD and II homozygotes and ID heterozygotes.

The association between the ACE/ID polymorphism and the development of diabetic nephropathy has been thoroughly investigated through the last decade and reviewed in four meta-analyses. Staessen et al (215) reported a general increased risk for diabetic nephropathy in individuals carrying the D allele. Fujisawa et al (216) suggested based on a meta-analysis of 18 studies that the D-allele was significantly associated with development of diabetic nephropathy in Caucasian and Asian type 1 and type 2 diabetic patients. In contrast, a meta-analysis by Kunz et al (217) failed to confirm this association, at least for Caucasian type 1 diabetics. In a meta-analysis from our group by Tarnow et al (218), a trend towards a protective effect of the II genotype on development of elevated urinary albumin excretion was found in Caucasian type 1 diabetic patients. In type 2 diabetes, this association was confined to Japanese populations. Thus, the D allele may be a risk factor for development of diabetic nephropathy, even though data are conflicting.

**Table 2.** Short-term effects of losartan on kidney function, blood pressure and RAAS in hypertensive type 1 diabetic patients with diabetic nephropathy and homozygosity for the I (n = 26) or D (n = 28) allele of the ACE/ID polymorphism (116).

ACE genotype	Baseline		Losartan 50		Losartan 100	
	II	DD	II	DD	II	DD
Albuminuria (mg/24 hr)	1123 (821-1537)	1210 (886-1655)	755 <sup>c</sup> (511-1115)	802 <sup>c</sup> (558-1152)	508 <sup>c</sup> (314-820)	645 <sup>c</sup> (434-959)
Fractional albumin clearance (10 <sup>-6</sup> )	309 (205-465)	332 (223-495)	214 <sup>c</sup> (133-345)	219 <sup>c</sup> (137-351)	162 <sup>c</sup> (94-281)	212 <sup>c</sup> (135-333)
GFR <sup>a</sup> (ml · min <sup>-1</sup> · 1,73 m <sup>-2</sup> )	86 (4)	88 (4)	84 (4) <sup>c</sup>	84 (4) <sup>c</sup>	81 (4) <sup>c</sup>	84 (4) <sup>c</sup>
24 hr systolic BP <sup>a</sup> (mmHg)	156 (3)	153 (3)	145 (3) <sup>c</sup>	145 (3) <sup>c</sup>	144 (4) <sup>c</sup>	143 (4) <sup>c</sup>
24 hr diastolic BP <sup>a</sup> (mmHg)	82 (2)	80 (2)	77 (2) <sup>c</sup>	76 (2) <sup>c</sup>	76 (2) <sup>c</sup>	76 (2) <sup>c</sup>
S-renin <sup>b</sup> (μU/ml)	40 (1)	38 (1)	49 (1) <sup>c</sup>	57 (1) <sup>c</sup>	69 (1) <sup>c</sup>	72 (1) <sup>c</sup>
S-ACE <sup>b</sup> (IU/L)	17 (1)	25 (1) <sup>d</sup>	16 (1)	24 (1) <sup>d</sup>	17 (1)	24 (1) <sup>d</sup>
P-angiotensin II <sup>b</sup> (pmol/l)	8 (1)	12 (1)	15 (1)	17 (1)	21 (1) <sup>c</sup>	22 (1) <sup>c</sup>

Geometric mean (95% CI); a) Mean (SE); b) Geometric mean (SE); c) p < 0.05 vs. baseline; d) p < 0.05 II vs. DD.

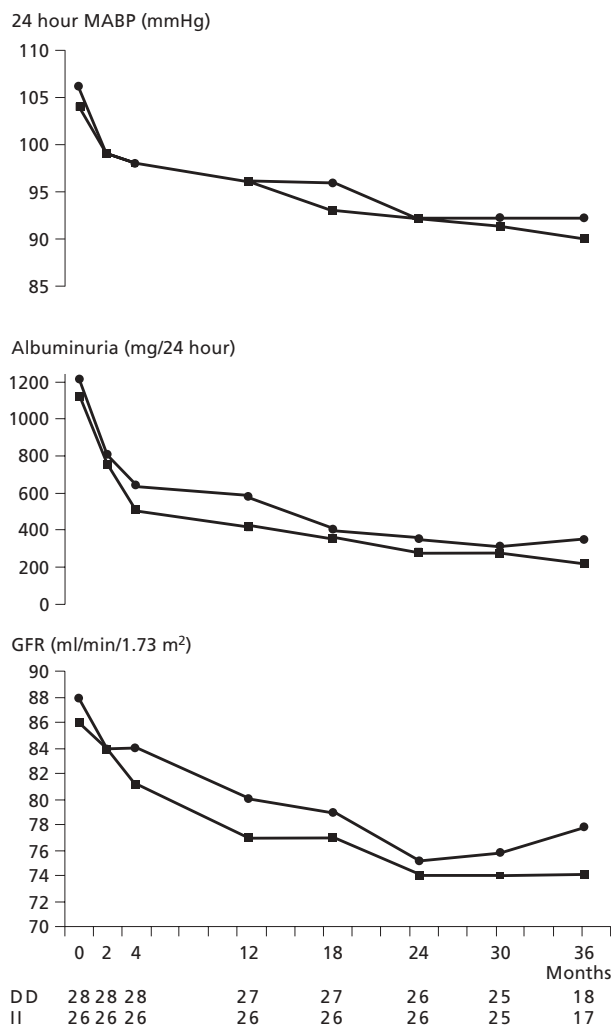
Several studies have demonstrated an association between the D allele and progression of diabetic kidney disease (212; 219-221) or a trend for a worse renal prognosis (222). Furthermore, Hadjadj et al (223) found, that a smaller proportion of the II genotype progressed from one stage of albuminuria to a higher level. Results from recent morphological studies in type 1 (224) and type 2 diabetes (225) support the hypothesis that the DD genotype is associated with progression of diabetic glomerulopathy. Similar associations between the D allele and decline in renal function have been described in non-diabetic renal disease although data are inconsistent (226-228).

Although ACE inhibitors may be renoprotective in type 1 diabetic patients with diabetic nephropathy beyond the effect of lowering blood pressure (12), there is considerably variation in response to treatment between individuals. Several factors may influence the response, however, the ACE/ID polymorphism has been suggested to play a key role in the individual variability (212; 229; 230). Individuals with the II genotype exhibit lower plasma levels of ACE compared to the DD genotype (231; 232), which obviously could influence the individual efficacy of ACE inhibitor treatment. The EUCLID trial tested the hypothesis that the ACE/ID polymorphism modulates the therapeutic efficacy of ACE inhibition in normoalbuminuric type 1 diabetic patients in a randomized prospective design (230). Progression from normoalbuminuria to microalbuminuria did not differ between II and DD genotypes, however, a beneficial effect of lisinopril on urinary albumin excretion rate was observed only in the II group. As a predictor of long-term renal function outcome we investigated in an observational study, the reduction in albuminuria after initiation of ACE inhibitor treatment in type 1 diabetic patients with diabetic nephropathy (229). In patients homozygous for the I allele, albuminuria was reduced by 61% as compared to only 31% in the DD patients (p < 0.01). Conflicting results regarding changes in urinary albumin excretion have been reported in non-diabetic renal disease (226; 228; 233; 234). Our initial study of the influence of the ACE/ID polymorphism on long-term efficacy of ACE inhibition on progression of renal disease in type 1 diabetic patients with diabetic nephropathy demonstrated that DD patients had an accelerated initial and sustained rate of decline in GFR during ACE inhibition compared to patients homozygous for the I allele (212). Hence, the beneficial effect of ACE inhibition may be lower in type 1 diabetic patients with diabetic nephropathy homozygous for the D allele compared to the I allele.

#### 10.1 ACE INSERTION/DELETION GENOTYPES AND ANGIOTENSIN II RECEPTOR BLOCKADE IN DIABETIC NEPHROPATHY

Specific blockade of the AT1 receptor may overcome this suggested interaction between ACE genotype and ACE inhibition and potentially induce similar renoprotective effects independent of geno-

types. We performed a study to evaluate the short-term renoprotective effect of ARB treatment by losartan assessed by reduction in albuminuria in hypertensive type 1 diabetic patients with diabetic nephropathy (116). Fifty-four patients homozygous for I (n = 26) or D (n = 28) allele of the ACE/ID polymorphism were investigated. Patients and the primary investigator were blinded to genotypes. The study consisted of two treatment periods each lasting two months. Patients received two doses of losartan starting with 50 mg followed by 100 mg o.d. in the second treatment period. No significant influence of the ACE/ID polymorphism on the antiproteinuric effect of losartan was found (Table 2). Albuminuria, assessed by at least two 24 hour urine collections, was lowered by 55% (35-68; 95% CI) (p < 0.01 vs. baseline) in the II group and 46% (28-61) (p < 0.01 vs. baseline) in the DD group by treatment with losartan 100 mg (p < 0.01) (NS between groups). Similarly, 24 hour arterial blood pressure was significantly reduced by both doses of losartan without significant differences between genotype groups. Levels of ACE in serum were as expected approximately 50% higher in DD patients compared to the II group at baseline and remained stable during treatment. In contrast, renin and angiotensin II increased similarly in both groups in keeping with blockade of the RAAS (Table 2). Thus, blockade of the action of angiotensin II at the AT1 receptor may be independent of ACE genotype, as evaluated by changes in albuminuria and blood pressure. However, to investigate the long-term beneficial effect of blockade of the AT1 receptor on a principal renal endpoint such as rate of decline in GFR, the patients continued in a prospective trial with an average follow-up time of 36 months (174). Patients continued treatment with losartan 100 mg o.d. whereas additional antihypertensive therapy was given to reach a target blood pressure below 135/85 mmHg. Twenty-four hour arterial blood pressure, GFR and albuminuria were measured every sixth month, as illustrated in Figure 9. Mean arterial blood pressure levels were significantly reduced to an average of 95 ± 1 mmHg in both genotype groups during follow up. We found no significant difference between rate of decline in GFR in the two genotype groups: GFR decreased by 2.9 (2.0-4.2) ml/min/year in the II group compared to 3.4 (2.3-5.1) ml/min/year in the DD group during follow-up (p = 0.4 II vs. DD). Thus, in contrast to previous studies dealing with renoprotection by ACE inhibitor, long-term treatment with an ARB conferred similar beneficial effects on progression of diabetic nephropathy in II and DD genotypes. Concentrations of angiotensin II in plasma were similar in both genotype groups throughout the study in accordance with previous data (235). Higher levels of ACE in the DD patients could be expected to result in elevated concentrations of angiotensin II. Enhanced pressor response to exogenous angiotensin I, probably caused by increased angiotensin II formation in individuals homozygous for the D allele as compared to II persons, has been reported in a number experi-



**Figure 9.** 24 hour mean arterial blood pressure (top), albuminuria (middle) and GFR (bottom) in II (black squares) and DD (black circles) patients (174).

mental and human studies (236-238). Systemic levels of angiotensin II do not specifically reflect RAAS activity at tissue level, as discussed previously. Possible differences in renal RAAS activity between II and DD patients can not be evaluated from systemic concentrations.

The studies discussed above are included in the recent defined concept of pharmacogenetics, i.e. how genetic differences influence the variability in individual patient responses to pharmacological intervention (239). Uncovering gene polymorphisms in patients with similar disease phenotypes may identify groups of patients who are most likely or unlikely to benefit from treatment of particular drugs. Treatment strategies and modalities may be individualized according to genotypes, which may also become a consequence of our studies if results are confirmed in large scale trials.

In summary, several studies have found an increased risk for renal function loss in DD patients compared to II patients, even during ACE inhibition. However, ARB treatment may confer similar long-term renoprotective effects in both genotypes. Large scale comparative trials with ACE inhibition should be performed before recommendations of selective therapy in different ACE/ID genotypes can be justified. Pharmacogenetic studies related to other RAAS polymorphisms may also be considered.

## 11. DUAL BLOCKADE OF THE RAAS

Despite maximal recommended doses of ACE inhibitors, several patients with diabetic nephropathy do not reach blood pressure target, have persistent albuminuria and rapid progression of renal disease. Insufficient response during sustained therapy may be partly related to under-dosing or the ACE-escape phenomenon as discussed in

section 3 (81), generation of angiotensin II through alternate enzyme pathways such as chymase (79) or individual factors. Blockade of the RAAS at AT1 receptor level by ARB treatment should overcome these drawbacks, but a potential benefit from bradykinin accumulation during ACE inhibitor treatment will then be missed. Furthermore, increased plasma concentrations of angiotensin II during ARB treatment (80; 240) could be unfavourable if AT2 receptor stimulation confers adverse renal effects as suggested by Cao et al (76; 77). Concomitant ACE inhibitor treatment reduces angiotensin II levels (240), thus, dual blockade of the RAAS by ACE inhibition and ARBs may offer additional renoprotective effect compared to monotherapy with either agent alone.

A recent study of combined treatment with ACE inhibition and ARB in healthy rats demonstrated that renal angiotensin II levels were lowered by monotherapy with either agent (241) in accordance with previous experimental studies of ARBs (22). Dual blockade of the RAAS caused a further reduction of kidney angiotensin II levels, whereas separate dose-escalation studies of both agents were unable to demonstrate similar degree of suppression of kidney angiotensin II levels. According to these experimental data, combined treatment with ACE inhibition and ARB may induce a supplementary effect on the intrarenal RAAS, unavailable by monotherapy with either agent.

### 11.1 EFFECT OF DUAL BLOCKADE OF RAAS IN INCIPIENT DIABETIC NEPHROPATHY

Dual blockade of RAAS was investigated by the CALM study group (242) in 199 type 2 diabetic patients with hypertension and microalbuminuria. Combination therapy with candesartan 16 mg o.d. and lisinopril 20 mg o.d. was significantly more effective in reducing both systolic and diastolic blood pressure compared to monotherapy with either agent. Reduction of microalbuminuria tended to be greater with combination therapy, though only reaching statistical significance compared to Candesartan monotherapy. As discussed previously, ARBs must be considered first-line therapy in hypertensive type 2 diabetic patients with microalbuminuria according to the IRMA-2 study (159), but additional antihypertensive and antiproteinuric effect may be achieved by dual blockade of RAAS. Data of dual blockade in incipient nephropathy in type 1 diabetes are not available.

### 11.2 EFFECT OF DUAL BLOCKADE OF RAAS IN DIABETIC NEPHROPATHY

Rossing et al (243) investigated hypertensive type 2 diabetic patients with diabetic nephropathy with an insufficient response to previous antihypertensive medication, i.e. albuminuria >1000 mg/24 h as well as arterial blood pressure >135/85 mmHg despite treatment with at least two antihypertensive agents including maximal recommended dose of ACE inhibition. Eighteen patients were randomized in a double-blind crossover design to treatment with Candesartan 8 mg o.d. and placebo for two months in addition to previous antihypertensive medication. Dual blockade of RAAS lowered systolic blood pressure by 10 mmHg compared to placebo, whereas albuminuria was reduced by 25%. A similar study in type 1 diabetic patients with diabetic nephropathy has been performed by Jacobsen et al (244). Irbesartan 300 mg o.d. lowered albuminuria and diastolic blood pressure were significantly lowered by 37% and 5 mmHg, respectively, as compared to the placebo period, when Irbesartan was added to previous antihypertensive medication. Furthermore, plasma renin increased significantly during Irbesartan treatment, indicating an additional blockade of RAAS. A similar alteration in plasma renin during dual blockade in diabetic nephropathy has previously been reported (245). Hyperkalaemia was observed in patients with GFR < 35 ml/min/1.73 m<sup>2</sup>, but were not seen in patients with higher GFR levels. Thus, dual blockade of the RAAS may induce additional beneficial effects in therapy resistant patients with diabetic nephropathy as compared to conventional treatment.

The COOPERATE study (246) investigated the effect of mono-

therapy with either ACE inhibition or ARB compared to dual blockade with combined treatment in 263 patients with non-diabetic kidney disease followed for 2.9 years. In the combination group, 11% of the patients reached the combined primary endpoint, time to doubling of serum creatinine or end-stage renal disease compared to 23% in both monotherapy arms ( $p \leq 0.018$ ).

Thus, long-term studies of dual blockade in diabetic kidney should be initiated, however, dose-response relationships for optimal renoprotective effects of monotherapy with both agents should be clarified before further action is taken.

## 12. SUMMARY AND FUTURE PERSPECTIVES

Diabetic nephropathy is a clinical syndrome characterized by a relentless decline in GFR, persistent albuminuria, arterial hypertension and highly elevated risk for cardiovascular morbidity and mortality. The RAAS is a key player in the progression of diabetic renal disease. The activity of the systemic RAAS is generally suppressed in diabetic nephropathy, whereas the intrarenal RAAS may be activated even early in the course of diabetes. Renal angiotensin II is a strong determinant of glomerular haemodynamics and intraglomerular pressure and stimulates mesangial cell proliferation by increased expression of TGF- $\beta$  and other cytokines and growth factors independent of blood pressure. Furthermore, pro-sclerotic effects of high glucose concentrations are most likely mediated by interaction with angiotensin II and autocrine production of TGF- $\beta$ .

Our studies in type 1 diabetic patients with diabetic nephropathy have investigated glomerular permselectivity by new and improved methods and the effects of ARB treatment in different settings. Glomerular permeability in early diabetic nephropathy is associated with a size-selective defect in the large shunt-like pores of the glomerular membrane that may account for immunoglobulinuria in early diabetic renal disease. In addition to the size-selective defect that was partly restored by ARB treatment, charge- or shape selectivity may also be affected to account for albuminuria. Recent evidence from experimental studies has documented the importance of the function of podocytes and expression of the transmembrane protein nephrin within the slit diaphragm that join the foot processes. Reduced nephrin expression is associated with severe proteinuria, but both may be prevented by ARB treatment as documented in animal studies. Further investigation combining functional, structural and molecular properties of the glomerular capillary wall may contribute to elucidate unresolved questions in the mechanisms of proteinuria in diabetic nephropathy, such as molecular structure of the slit diaphragm and podocyte function.

In accordance with large-scale trials in type 2 diabetes, our intervention studies in hypertensive type 1 diabetic patients with diabetic nephropathy demonstrated that ARBs represent an effective new class of drugs in treatment of diabetic renal disease with similar short-term renoprotective effects as ACE-inhibition in type 1 diabetic patients with diabetic nephropathy. We found that high dose ARB treatment, beyond the maximal dose required in treatment of primary hypertension, was needed for optimal renoprotection assessed by reduction in albuminuria. Furthermore, studies revealed that individual patient related factors are involved in responsiveness or resistance to renoprotective treatment. Elucidation of individual determinants of responsiveness to therapy may provide a new concept for improvement of renal prognosis by individualization of renoprotective therapy.

The time course of the antihypertensive and antialbuminuric effects after initiation of ARB treatment shows that the maximal antialbuminuric effect is present within 7 days and therefore primarily a consequence of systemic and renal haemodynamic changes. However, non-haemodynamic effects such as reduced renal expression of TGF- $\beta$  and other cytokines are likely to contribute to the long-term renoprotective effect.

Elevated levels of sVCAM-1 are independently associated with increased cardiovascular mortality in type 2 diabetes. We found that

blockade of the RAAS may have an antiatherogenic effect in diabetic patients with elevated albumin excretion as evaluated by a reduction in concentrations of circulating adhesion molecules. Measurements of adhesion molecules should be included in future prospective intervention studies to investigate the pathophysiological significance of potential long-term reduction by ARB treatment on renal and cardiovascular outcome.

A number of studies in diabetic renal disease have found an increased risk for renal function loss in patients homozygous for the D allele of the ACE/ID polymorphism compared to II patients, even during ACE inhibition. ARB treatment in hypertensive type 1 diabetic patients with diabetic nephropathy homozygous for I or D allele of the ACE/ID polymorphism conferred similar beneficial effect on changes in urinary albumin excretion and rate of decline in GFR in patients with different genotypes. A large-scale trial comparing the effects of ARB treatment and ACE inhibition should be performed to further investigate the pharmacogenetic possibilities of the ACE/ID polymorphism in treatment of diabetic renal disease.

Preliminary studies have suggested that dual blockade of the RAAS by combined treatment with ACE inhibition and ARBs may offer an additional effect compared to monotherapy. Provided that dual blockade is superior to treatment with optimal doses of monotherapy, long-term studies of dual blockade in incipient and overt diabetic nephropathy should be initiated.

Increasing evidence suggest an implication of aldosterone in the pathogenesis of progressive renal disease, which may suggest a beneficial effect of aldosterone blockade. Therefore, selective and non-selective aldosterone blockers are being investigated as future treatment options in diabetic nephropathy. The increased expression of renal TGF- $\beta$  and other cytokines in diabetic glomerulopathy may be lowered by RAAS blockade, but probably not completely abolished even by high dose treatment, according to animal studies. Therefore, agents that modulate other pathogenic pathways are currently being evaluated in preclinical and early clinical trials as potential treatment modalities in diabetic renal disease. These studies include agents that interfere in formation of advanced glycation end products, compounds targeting the activity of protein kinase C and antibodies/receptor blockers to cytokines such as TGF- $\beta$ .

## ABBREVIATIONS

ACE: angiotensin converting enzyme  
ACE/ID: insertion/deletion ACE gene polymorphism  
ARB: angiotensin II receptor blocker  
CTGF: connective tissue growth factor  
CRP: C reactive protein  
EDTA: ethylenediaminetetraacetic acid  
GFR: glomerular filtration rate  
ICAM-1: intercellular adhesion molecule-1  
NO: nitric oxide  
PAI-1: plasminogen activator inhibitor-1  
P 38 MAPK: p38 mitogen activated protein kinase  
PKC: protein kinase C  
RAAS: renin angiotensin aldosterone system  
RPF: renal plasma flow  
TGF- $\beta$ : transforming growth factor beta  
VCAM-1: vascular cellular adhesion molecule-1  
VEGF: vascular endothelial growth factor

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