Progression and remission of nephropathy in type 2 diabetes: new strategies of treatment and monitoring

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1. BACKGROUND

Diabetes currently affects more than 170 million people world-wide and projections for the future are alarming. According to the World Health Organization, it is expected that the number of patients with diabetes will double within the next 20 years due to an epidemic rise in the prevalence of type 2 diabetes (10). This estimate is conservative and based only on the expected population increase and the rising proportion of elderly people. In addition, changes towards a more sedentary lifestyle with decreased physical activity and a rapid increase in the prevalence of obesity is likely to increase the future burden of type 2 diabetes and its associated micro- and macrovascular complications even further.

Without specific intervention, 20 to 40% of all diabetic patients will develop diabetic nephropathy characterized clinically by hypertension, a progressive increase in albuminuria, a high cardiovascular risk, and a relentless decline in GFR leading towards ESRD (11).

In the past, diabetic nephropathy was progressive and irreversible (12, 13). During the past decades, however, substantial improvements have been achieved in the prevention and treatment of the disease primarily through early and aggressive lowering of blood pressure (14). Antihypertensive treatment – in particular with agents that block the renin-angiotensin-aldosterone (RAAS) system – reduces albuminuria, slows progression of diabetic nephropathy and lowers the risk of ESRD and cardiovascular disease. Even remission of diabetic nephropathy at its most advanced stage as defined by the presence of nephrotic range albuminuria has been reported during aggressive antihypertensive treatment in patients with type 1 diabetes (15-17). Similar studies have, however, been lacking in type 2 diabetic patients.

Despite improved prognosis over the last decades, diabetic nephropathy remains a major health problem, and many patients still progress to ESRD. Today nephropathy due to type 2 diabetes has become the single most common cause of ESRD in the Western world accounting for 45% of all patients on dialysis in the US (18) and 22% in Denmark (19). It is therefore of uttermost importance to identify early modifiable risk factors for progression of diabetic nephropathy for prompt treatment of high risk individuals and for identifying new targets for intervention. Furthermore, new and more effective strategies for prevention and treatment of diabetic nephropathy are urgently needed. In this respect, reduction of albuminuria has emerged as a key therapeutic goal for both reno- and cardiovascular protection (20-26). Thus, the efficacy of new treatment modalities may be evaluated by their short-term antiproteinuric effects before long-term clinical studies are conducted with principal renal and cardiovascular end-points.

2. AIMS

The overall aim of the present review has been to evaluate progression and potential remission of nephropathy in type 2 diabetic patients and to assess new strategies for treatment and monitoring of these patients. More specifically the aims have been to evaluate:

1. determinants of loss of renal function and increased mortality among type 2 diabetic patients with nephropathy during conventional antihypertensive treatment
2. if remission of nephropatic range albuminuria is feasible and associated with improved outcome in type 2 diabetic patients during conventional antihypertensive treatment
3. new treatment modalities and strategies by enhanced blockade of the RAAS
4. new strategies for monitoring diabetic renal disease by the use of proteomics

3. PATIENTS, DESIGNS AND METHODS

3.1 PATIENTS

All study populations were composed of type 2 diabetic patients recruited from the Steno Diabetes Center. Type 2 diabetes was diagnosed according to WHO criteria (27).

Definitions of levels of albuminuria, nephropathy and diabetic nephropathy:

- *Normoalbuminuria* is defined as a urinary albumin excretion below 30 mg/24 hours.
- *Microalbuminuria* is urinary albumin excretion between 30 and 300 mg/24-hours.
- *Macroalbuminuria (nephropathy)* as a urinary albumin excretion greater than 300 mg/24 hours, and
- *Nephrotic range albuminuria* is a urinary albumin excretion greater than 2500 mg/24-hours. Remission of nephrotic range albuminuria is a sustained reduction for at least one year of albuminuria from nephrotic range albuminuria to below 600 mg/24-hour (15-17).

These terms only refer to conditions where the urinary albumin excretion were within the respective limits in at least two out of three consecutive 24-hour urine samples (11, 28).

Whereas the term *macroalbuminuria* or *nephropathy* only refers to the degree of albuminuria and not the underlying cause, *diabetic nephropathy* is a clinical diagnosis which can be established when persistent macroalbuminuria occurs in the presence of diabetic retinopathy and when there is no clinical or laboratory evidence of a urinary tract disease or a non-diabetic nephropathy (29). If diabetic retinopathy is absent a renal biopsy showing diabetic glomerulosclerosis is diagnostic (11). These criteria were met for all patients in three (1, 6, 9) out of seven of the studies which included macroalbuminuric diabetic patients. However, in the remaining four studies (3-5, 7), diabetic retinopathy was absent in approximately 10% of the patients with macroalbuminuria. As we did not have renal biopsies in all of these patients to confirm the presence of diabetic glomerulosclerosis we cannot exclude that a minor fraction of these patients have suffered from non-diabetic renal disease. A previous biopsy study of unselected type 2 diabetic patients with persistent macroalbuminuria and absence of diabetic retinopathy has shown that approximately two thirds have diabetic nephropathy as evidenced by a renal biopsy showing diabetic glomerulosclerosis whereas the remaining one third will have a renal biopsy consistent with a non-diabetic nephropathy as the underlying cause of albuminuria (30).

3.2 DESIGNS AND METHODS

Progression and remission of diabetic nephropathy were investigated in observational follow-up studies (5, 9). At the Steno Diabetes Center all patients with nephropathy are monitored on a routine basis with annual measurements of GFR (4-hour plasma clearance of 51 Cr-EDTA (31)) and with quarterly visits in the diabetes clinic for assessment of laboratory and clinical parameters such as albuminuria, HbA1c and blood pressure and for adjustment of the treatment when needed. The cohort for the study of progression consisted of all type 2 diabetic patients with nephropathy in whom GFR was monitored annually for at least three years at the Steno Diabetes Center (n=227). In this cohort we identified the patients with nephrotic range albuminuria (n=79) to assess the proportion who obtained remission and the impact of such remission on renal outcome and mortality.

Progression of diabetic nephropathy was assessed by the rate of decline in annually measured GFR (linear regression analysis). In accordance with recommended guidelines rate of decline in GFR was only assed in patients with repeated measurements over at least three years (32). In addition end-points included survival analysis of time to; doubling of baseline serum creatinine (corresponds to an approximate 50% reduction in GFR), ESRD and all cause mortality.

Short-term interventional studies dealing with new treatment strategies for enhanced RAAS blockade were all conducted as randomized double-masked cross-over studies. Treatment periods were at least 8 weeks in all studies. The primary end-point was albuminuria which due to the large day-to-day variation was assessed by collection of three consecutive 24-hours urinary collections to increase the statistical power. Major secondary end-points were changes in 24-h ambulatory blood pressure and GFR. Three different strategies of improved RAAS blockade were evaluated: 1) Two studies aimed to determine the antiproteinuric dose-response curve of the angiotensin II receptor blockers candesartan (4) and irbesartan (8) in type 2 diabetic patients with nephropathy and microalbuminuria, respectively. 2) Two studies measured the effect of dual RAAS blockade in type 2 diabetic patients with nephropathy (1, 3), and 3) one study evaluated the effect of adding spironolactone to recommended antihypertensive treatment including ACE-I and angiotensin II receptor blocker (ARB) (7).

Twenty-four hour ambulatory blood pressure profiles during long term (–2 years) treatment with irbesartan 150 and 300 mg daily were determined among microalbuminuric type 2 diabetic patients as a sub-study (2) of patients recruited at the Steno Diabetes Center for inclusion in the large multicenter study Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA2) (33). This study was a randomized double-blind parallel trial where patients were randomized to treatment with either placebo, irbesartan 150 or 300 mg o.d. in order to evaluate the impact of treatment on progression from microalbuminuria to overt nephropathy.

The study dealing with urinary polypeptide patterns (6) in diabetic nephropathy was a cross-sectional study comparing urinary polypeptides in type 2 diabetic patients with normoalbuminuria, microalbuminuria and diabetic nephropathy, respectively. We also evaluated changes in the urinary polypeptide pattern during treatment with increasing doses of candesartan using urine samples collected in the dose-response study of candesartan (4). Urinary polypeptide patterns were assessed by a proteomic approach using capillary electrophoresis (CE) coupled online to a mass spectrometer (MS) as described in more detail in section 7.

4. PROGRESSION OF DIABETIC NEPHROPATHY

Diabetic nephropathy develops through an initial period of diabetes usually exceeding 10 to 15 years in type 1 diabetic patients and a less clearly defined interval in type 2 diabetic patients because onset of diabetes is less precisely defined. Various degree of renal structural damage may be present at the initial low-grade elevation of urinary albumin excretion termed microalbuminuria but GFR is usually within or above normal levels. The onset of microalbuminuria is associated with other microvascular complications such as diabetic retinopathy and neuropathy as well as an increased risk of macrovascular disease (34-37). Approximately 5 to 10% of patients with microalbuminuria progress to overt diabetic nephropathy each year and then GFR starts to decline (33, 38-41). If no specific intervention is provided to slow the progression, the mean rate of decline in GFR is on average between 10 to 15 ml/min/year (42-44).

The decline in GFR during progression of diabetic nephropathy results from accumulation of extracellular material resulting in basement membrane thickening and mesangial expansion eventually leading to glomerular closure and thereby loss of filtration capacity (45-47). In addition, progression of diabetic nephropathy leads to size- and charge defects of the glomerular filtration barrier, podocyte loss and alterations in handling of proteins in tubular cells all leading to occurrence of elevated urinary albumin excretion. Renal structural changes during progression of diabetic nephropathy occur due to an imbalance in degenerative and reparative processes leading to excess cell death and extracellular matrix turnover in the kidneys (48). Renal morphological abnormalities can involve all renal compartments and include glomerular basement membrane thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis (49) The renal lesions are generally more heterogeneous in type 2 as compared to type 1 diabetic patients (49) which may in part be age related. The classical Kimmerstiel Wilson lesions with nodular glomerulosclerosis may be present but often this is not the case (50).

So far the single most successful treatment strategy to prevent the initiation and progression of diabetic nephropathy is aggressive antihypertensive treatment which has dramatically improved renal outcome and survival (51, 52). Seminal studies by Mogensen (53) and Parving (29) in the early 1980's demonstrated that treatment with diuretics and beta-blockers lowered albuminuria and reduced the rate of decline in GFR from >10 to <5 ml/min/year in type 1 diabetic patients with nephropathy. Since then, numerous studies have confirmed that antihypertensive improves renal outcome in diabetic nephropathy (14). A particular renoprotective benefit has been demonstrated for antihypertensive agents that block the RAAS either by ACE-I (54-56) or ARB (33, 57-59) and this important topic will be dealt with separately in chapter 6.

Nevertheless, in spite of antihypertensive treatment, the individual rate of decline in GFR remains highly variable ranging from ~ 0 to 20 ml/min/year (5, 60-66). This implies that some patients with diabetic nephropathy will preserve a stable renal function whereas others progress to ESRD within few years after onset of nephropathy. Therefore, it is of key importance to identify risk factors for enhanced renal function loss early in the course of nephropathy, in order to identify and treat high risk individuals at an early stage. Identification of such markers could also lead to a better understanding of the pathophysiologic processes and could help in the search for better treatment strategies.

Risk factors for progression of diabetic nephropathy have been extensively studied in a large observational follow-up study of patients with type 1 diabetes (66). In contrast, previous data from observational studies in type 2 diabetic patients has been restricted to relatively small numbers of patients (60-65) and studies of specific ethnic groups (Pima Indians (39) and Asians (67, 68)) which have led to rather conflicting results regarding putative progression promoters of diabetic nephropathy.

We evaluated potential risk factors associated with enhanced renal function loss and increased mortality in an observational follow-up study of 227 Caucasian type 2 diabetic patients with nephropathy followed over a mean period of 6.5 (range: 3 to 17) years (5). Patients were followed from early in the course of the disease with the majority of patients having normal baseline levels of GFR and all

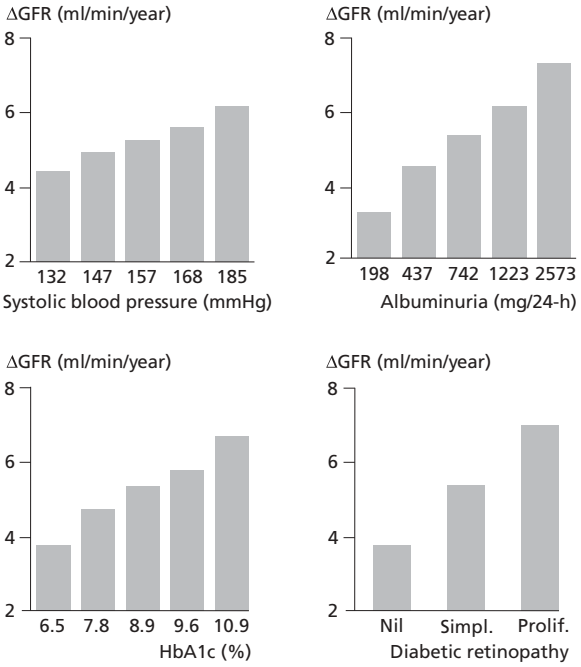

Figure 1. Impact of baseline parameters: level of systolic blood pressure, albuminuria diabetic retinopathy, hemoglobin A1c, on the rate of decline in GFR (continuous variables are separated into quintiles). Adjustment has been made for the other variables significantly associated with rate of decline in GFR.

patients received early and aggressive conventional antihypertensive treatment with on average 3 drugs at the end of the observation period. In most patients this included treatment with either an ACE-I or an ARB. During follow-up 63 (28%) of the 227 reached the composite renal end-point of doubling in serum creatinine or progression to ESRD, and 79 (35%) patients died. The most frequent cause of death was cardiovascular disease (n=55) followed by ESRD (n=14). The mean rate of decline in GFR was 5.2 ml/min/year, which confirms the beneficial effects of aggressive blood pressure lowering treatment when compared with the mean rate of decline of more than 10 ml/min/year reported in the previously mentioned studies of type 1 diabetic patients without blood pressure lowering treatment (42-44). In spite of aggressive antihypertensive we also observed a wide inter-individual variation in the rate of decline in GFR ranging from 0 to approximately 20 ml/min/year. In our study of type 2 diabetic patients followed early in the course of renal disease we identified several modifiable and non-modifiable risks for increased rate of progression of nephropathy (Figure 1 and Table 1) and mortality (Table 2). These will be discussed in detail in the following sections which will also cover some of the recent insights on risk factors for progression of diabetic nephropathy that have come from several post-hoc analysis of type 2 diabetic patients with nephropathy and impaired renal function included in two recent major clinical trials documenting a renoprotective effect of ARB treatment – the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study (57) and the Irbesartan in Diabetic nephropathy (IDNT) study (58). Genetic factors will only be briefly commented on.

4.1 MODIFIABLE RISK FACTORS

4.1.1 Blood pressure

Hypertension plays a major role in the onset and progression of diabetic nephropathy as well as in the development of macrovascular lesions. The importance of elevated blood pressure for development of diabetic nephropathy was emphasized more than thirty years ago in a case report of a type 2 diabetic patient with unilateral renal artery stenosis in whom diabetic glomerulosclerosis was present in the non-ischemic kidney while the other kidney was protected (69). Patients with diabetic nephropathy are particularly vulnerable to increased systemic blood pressure due to impaired autoregulation of blood pressure leading to pressure induced damage in various end-organs such as the kidney (70, 71). Furthermore, a large proportion of type 2 diabetic patients with nephropathy have elevated nocturnal arterial blood pressure and are therefore not relieved by the normal drop in systemic blood pressure during the night (72).

The importance of blood pressure was confirmed in our study of 227 type 2 diabetic patients with nephropathy (5) demonstrating a significant association between elevated systolic blood pressure and increased progression of diabetic nephropathy (Figure 1 and Table 1) and mortality (Table 2). There was no significant impact of diastolic blood pressure on progression of nephropathy or mortality which is probably due to the fact that type 2 diabetic patients often have isolated systolic hypertension.

As already discussed, the introduction of antihypertensive treatment has dramatically improved the prognosis of patients with diabetic nephropathy and it is now generally approved that aggressive lowering of blood pressure, irrespective of the agents used, is of key importance to improve renal and cardiovascular outcome. In addition a specific renoprotective benefit above and beyond the blood pressure lowering effect is obtained by blocking the RAAS as discussed in detail in chapter 6.

Today, guidelines suggest that blood pressure should be treated to ≤130/80 mmHg in diabetic patients (73) and even lower in those with overt nephropathy.

In the cohort of 227 type 2 diabetic patients with nephropathy there was no lower threshold in the linear correlation between mean systolic blood pressure during follow-up and rate of decline in GFR (the lower, the better) suggesting that currently recommended blood pressure goals for optimal renoprotection are arbitrary.

In cardiovascular disease it has been intensively debated if too vigorous reduction in blood pressure may be associated with increased cardiovascular risk (the so-called J-curve concept). A recent post-hoc analysis of 1590 hypertensive patients with type 2 diabetic patients with renal impairment included in the IDNT-study (74) showed that progressive lowering of systolic blood pressure to 120

Table 1. Baseline predictors of time to doubling of baseline serum creatinine (to at least 177 µmol/l) or end-stage renal disease in 227 type 2 diabetic patients with nephropathy followed for 6.5 years (Cox proportional hazard model).

Baseline	Hazard ratio (95%CI)	p-value
Albuminuria *log10*	7.35 (3.35 to 15.70)	< 0.001
Systolic blood pressure *per 10 mmHg*	1.23 (1.07 to 1.38)	0.001
HbA1c *per 1%*	1.48 (1.21 to 1.80)	< 0.001
Hemoglobin *per 1 mmol/l*	0.75 (0.57 to 0.98)	0.030
Baseline GFR *per 10 ml/min*	0.86 (0.74 to 0.96)	< 0.010

During follow-up, 63 (28%) of the patients doubled their baseline serum creatinine and 15 (7%) patients developed ESRD. The following baseline variables were excluded due to lack of statistical significance: age, gender, diabetes duration, diastolic blood pressure, BMI, serum cholesterol.

Table 2. Baseline predictors of time to death (all-cause mortality) in 227 type 2 diabetic patients with nephropathy followed for 6.5 years (Cox proportional hazard model).

Baseline	Hazard ratio (95% CI)	p-value
Age *per 10 years*	1.82 (1.32 to 2.63)	< 0.001
Albuminuria *log10*	2.56 (1.34 to 4.88)	< 0.01
Systolic blood pressure *per 10 mmHg*	1.14 (1.00 to 1.29)	0.049
HbA1c *per 1%*	1.24 (1.05 to 1.47)	< 0.01

During follow-up a total 79 (35%) patients died. Causes of death included cardiovascular disease in 55 patients, ESRD in 14, cancer in 6 and other various causes in 4 patients. The following baseline variables were excluded due to lack of statistical significance: age, gender, diabetes duration, diastolic blood pressure, BMI, serum cholesterol, GFR, degree of retinopathy, smoking, hemoglobin.

mmHg was associated with improved renal outcome (time to doubling of s-creatinine or ESRD) and patient survival. However, below that threshold all-cause mortality increased. The reasons for the increased mortality at follow-up systolic blood pressure below 120 mmHg were not clear. The authors suggested that it could reflect severe preexisting intrinsic cardiac disease primarily heart failure leading to hypotension, adverse effects of multiple antihypertensive agents, a tendency to orthostatic hypotension, or some combination of these factors. Furthermore, this observation was based on only 53 of 1590 patients included in the study, having mean systolic blood pressure values below 120 mmHg. In the vast majority of the patients (70%), the 135 mmHg-systolic blood pressure goal was not reached in spite of very intensive blood pressure lowering treatment with on average 3 to 4 different antihypertensive agents. This emphasizes the urgent need for new strategies for more effective lowering of blood pressure in order to reach current blood pressure targets.

4.1.2 Albuminuria

An elevated urinary albumin excretion rate (UAE) throughout the entire range from normoalbuminuria to severely increased albuminuria is a well-established powerful predictor of poor renal and cardiovascular outcome in type 2 diabetes (22, 23, 75-77). In agreement, elevated baseline albuminuria was highly predictive of the rate of renal function loss (Figure 1 and Table 1) and of increased mortality (Table 2) in our cohort of type 2 diabetic patients (5).

Moreover several recent studies in patients with diabetic and non-diabetic nephropathy have firmly documented that the short-term reduction in albuminuria upon initiation of antihypertensive treatment is a strong predictor of the long-term renal and cardiovascular outcome i.e *the greater the reduction in albuminuria, the lower the long-term cardiovascular risk and the slower the progression of renal disease* (20-26). This suggests that albuminuria is not only a marker of glomerular lesions and wide-spread microangiopathy, but also that albuminuria *per se* is a modifiable progression promoter that should be maximally reduced (78).

A recent analysis of 1496 type 2 diabetic patients with nephropathy included in the IDNT-study demonstrated that for every 50% reduction in proteinuria during the first year of the study, the risk for kidney failure was reduced by more than half (56%, 95% CI: 51 to 60%) (26). This is comparable to what as originally reported in a post-hoc analysis of 1512 type 2 diabetic patient included in the RENAAL study (23). Another analysis of the RENAAL study demonstrated that for every 50% reduction in albuminuria, there was an 18% reduction in cardiovascular risk, and a 27% reduction in the risk of heart failure (22). In both studies both baseline albuminuria and the short-term reduction in albuminuria were independent from other risk factors including arterial blood pressure the strongest predictors of long-term renal and cardiovascular outcome.

The close correlation between albuminuria and progression of renal disease is in agreement with experimental data suggesting that albuminuria *per se* may have direct toxic renal effects as filtered proteins are reabsorbed in the tubular system causing release of vasoactive substances and inflammation leading to tubulo-interstitial damage and further progression of renal function loss (78). Furthermore, in the experimental setting proteinuria has been demonstrated to activate tubular RAAS activity, thereby pointing towards a self-perpetuating process with proteinuria increasing intrarenal RAAS activity which in turns increases intraglomerular capillary pressure leading to more proteinuria (79, 80).

The fact that albuminuria can be regarded as a surrogate endpoint has important clinical implications as the degree of albuminuria can be used to monitor treatment efficacy in the individual patient and short-term changes in albuminuria can serve as a surrogate end-point in clinical trials.

4.1.3 Hyperglycemia

Hyperglycemia is a well established risk factor for both development and progression of diabetic nephropathy in type 1 diabetic patients (66, 81). Previous smaller studies of type 2 diabetic patients with nephropathy have, however, found conflicting results regarding the impact of hyperglycemia with some studies reporting an increased rate of decline in kidney function with poor metabolic control (64, 82, 83) whereas other studies have not found that association (60, 65, 67, 84, 85). In our long-term study of 227 patients followed early in the course or renal disease, poor glycemic control was associated with a faster rate of decline in renal function (Figure 1 and Table 1) and of increased mortality (Table 2). A recent post hoc analysis of baseline predictors in the RENAAL trial (86), did not find any impact of baseline hemoglobin A1c on the time to reach the composite renal end-point of doubling in baseline serum creatinine or development of ESRD. As patients included in the RENAAL trial all had advanced nephropathy with reduced renal function and severe proteinuria it can be suggested that hyperglycemia accelerates renal progression early in the course but the impact decreases over time with deteriorating renal function when other risk factors for progression, such as albuminuria and hypertension takes on a greater impact on renal outcome.

4.1.4 Dyslipidemia

An observational study of 301 type 1 diabetic patients with diabetic nephropathy followed at Steno Diabetes Center demonstrated that total cholesterol correlated to the rate of decline in GRF (66). This agrees with Moorhead's (87) more than 20 year old hypothesis that hyperlipidemia promotes progression of renal disease once an initial event had hit the glomerular capillary wall, thereby allowing lipoproteins to accumulate in mesangial cells and stimulate them to produce excess basement membrane material. In one study of type 2 diabetic patients increased total cholesterol has been associated with increased risk of development of nephropathy (88) and elevated triglyceride with development of ESRD (89). However, we and others (60, 67, 68, 84, 85) have not been able to demonstrate an independent association between total cholesterol and progression of renal disease in type 2 diabetic patients with nephropathy. A meta-analysis of several smaller studies of patients with various forms of renal diseases concluded that lipid-lowering agents can reduce the rate of decline in by 1.9 (0.3 to 3.4) ml/min/year (90) but large clinical trials are warranted. Due to the now well proven benefit on cardiovascular morbidity and mortality, all type 2 diabetic patients with nephropathy should now receive lipid-lowering treatment particularly statins (91-93) and thus this issue can not be addressed in clinical trials.

4.1.5 Hemoglobin

It is becoming increasingly clear that anemia occurs at a high frequency early in the course of diabetic renal disease even before GFR is severely reduced. Decreased erythropoietin (EPO) production from peritubular fibroblasts due to renal interstitial damage and autonomic neuropathy seems to be a major factor causing anemia in diabetes (94-97). More importantly reduced hemoglobin levels predict adverse renal outcome. This was first demonstrated in the previously mentioned post-hoc analysis of patients included in the RENAAL trial (86, 98). In this study of patients with advanced nephropathy (baseline GFR 40 ml/min/1.73 m^2 and hemoglobin 7.7 mmol/l), a decrease in baseline hemoglobin of 1 mmol/l was associated with an 11% increased risk of reaching doubling of serum creatinine or development of ESRD. Our study extended these findings by showing that hemoglobin levels even within the normal range predicts renal outcome much earlier in the course of the disease (baseline GFR 70 ml/min/1.73 m^2 and baseline hemoglobin 8.8 mmol/l). In our study for every 1 mmol/l decrease in hemoglobin there was a 25% greater risk of reaching the composite renal endpoint (Table 1). Since baseline GFR is both correlated to the baseline

level of hemoglobin concentration and the risk of developing ESRD it should be emphasized, that the predictive power of hemoglobin for development of ESRD remained after correction for baseline GFR both in our study in the RENAAL trial. However, we did not find a correlation between baseline hemoglobin and the rate of decline in GFR. This may be due to the fact that when the rate of decline in GFR is relatively low as in our study, a time-to-event analysis has greater statistical power than an analysis based on the slope of GFR (99).

A causative role of anemia for progression of renal disease has been suggested in smaller interventional studies where reversal of anemia by EPO treatment has been suggested to greatly improve renal outcome in the pre-dialysis state among patients with various forms of advanced chronic renal disease (GFR between 20 to 30 ml/min) (100, 101). Several larger studies including patients with diabetic nephropathy are now being conducted to evaluate if EPO treatment earlier in the course of renal disease may have beneficial effects primarily on cardiovascular end-points (102-105). In light of the importance of reduced hemoglobin levels for progression of renal disease it seems somewhat paradoxical that first line therapy with ACE-I and ARB in diabetic nephropathy may actually aggravate anemia as these agents reduce hemoglobin levels by approximately 0.3 to 0.5 mmol/l (4, 8) possibly due to blockade of angiotensin II stimulated EPO production (4) However, this should not restrict the use of these agents in diabetic nephropathy because of the overall renoprotective effects of ACE-Is and ARBs.

4.1.6 Smoking

In both type 1 and type 2 diabetes smoking increases the risk of developing microalbuminuria and diabetic nephropathy (106-108). We found that heavy smoking (>20 cigarettes per day) increased the rate of decline in GFR by 1.3 ml/min/year when adjusting for other risk factors of progression. Several mechanisms may be involved as reviewed in detail by Orth (109).

4.2 NON-MODIFIABLE RISK MARKERS

4.2.1 Diabetic retinopathy

In our cohort of 227 type 2 diabetic patients with nephropathy the presence and severity of diabetic retinopathy at baseline was associated with the subsequent rate of decline in GFR (Figure 1). Such an association has also been reported in other studies of type 2 diabetic patients with nephropathy (65, 110). In particular, a recent sub-analysis of 1456 type 2 diabetic patients with nephropathy included in the RENAAL study extended our findings by showing that the presence of diabetic retinopathy at baseline was associated with a higher risk for ESRD and death in type 2 diabetic patients (110).

Two major factors play a role in the poor outcome for type 2 diabetic patients with nephropathy and presence of retinopathy. Firstly, retinopathy is the clinical hallmark of generalized microangiopathy in diabetes and the severity of diabetic retinal lesions correlates with the extent of renal lesions which in turns correlate with enhanced albuminuria and an accelerated rate of decline in GFR (49, 50). Secondly, the better prognosis in patients without diabetic retinopathy may in part be due to the fact that approximately 30% of patients without diabetic retinopathy suffer from non-diabetic renal diseases (30), which are generally characterized by a slower rate of decline in GFR (111).

4.2.2 Genetic factors

The hypothesis that hereditary factors are involved in both development and progression of diabetic nephropathy is strongly supported by ethnic differences and family clustering of diabetic nephropathy together with the apparent inability of currently known clinical variables to fully account for the incidence rates and progression of diabetic nephropathy. However, the genetic aspects of the disease have not been the subject of the present series of studies and have recently been extensively reviewed (112).

5. REMISSION OF DIABETIC NEPHROPATHY

During the last two to three decades substantial improvements have been achieved in the prevention and treatment of diabetic nephropathy primarily through early and aggressive antihypertensive treatment which reduces the risk of ESRD and cardiovascular disease as discussed previously. Furthermore, there is now increasing evidence suggesting that aggressive lowering of blood pressure not only slows progression of renal disease but can even in some cases reverse the course of disease and induce remission of renal structural and functional impairment.

5.1 REMISSION OF STRUCTURAL LESIONS

In various animal models of chronic renal disease it has been shown that tight blood pressure control in particular by ACE inhibitors and/or ARBs can induce remodeling and remission of vascular sclerosis, tubulointerstitial fibrosis, and glomerulosclerosis (113-116). Evidence that remission of glomerulosclerosis can occur emerges also from clinical observations. A renal biopsy study in type 2 diabetic patients with nephropathy demonstrated regression of renal structural abnormalities which correlated with a reduction of albuminuria following two years of treatment with an ACE-I or a beta-blocker (117). In type 1 diabetic patients with increased albuminuria repeated renal biopsies after normalization of blood glucose following pancreatic transplantation showed regression of mesangial expansion, more patent glomerular loops, and a proportional decrease in tubulointerstitial fibrosis over a 10-year period (118). Casuistic reports of transplantation of kidneys with diabetic structural lesions into non diabetic recipients have also shown subsequent resolution of mesangial expansion (119).

These important findings clearly demonstrate that not only can mechanisms of progression be dampened but remodeling of the existing renal sclerosis is possible. A conceptual parallel is seen in studies demonstrating that remission of vascular and myocardial sclerotic lesions can be accomplished in cardiovascular disease by inhibition of the renin-angiotensin-aldosterone system (120) and by lowering of cholesterol (121).

5.2 REMISSION OF FUNCTIONAL IMPAIRMENT

Clinical studies in which renal biopsies are most often not readily available have approached the concept of reversing the progressive nature of diabetic nephropathy by measuring remission of clinical markers of renal structural and functional damage such as albuminuria.

Recent studies in type 1 diabetic patients have demonstrated that sustained remission of very advanced stages of diabetic nephropathy as defined by nephrotic range albuminuria can be accomplished in a substantial proportion of patients by aggressive antihypertensive treatment and that such remission is associated with a significant slower rate of decline in GFR and an improved survival (15-17). These positive findings were not foreseen by the initial studies conducted before the introduction of antihypertensive treatment (13). In the early 1970's Watkins determined that the subset of type 1 diabetic patients with proteinuria greater than 3000 mg/24-hours all died within 2 to 6 years of follow-up (12).

It was previously unknown if similar beneficial effects of remission is obtainable in type 2 diabetic patients with NRA. In the study of the previously described cohort of 227 type 2 diabetic patients with nephropathy, NRA was a frequent phenomenon occurring in 79 (35%) of the 227 patients (9). During 6.5 years of observation, remission of NRA was induced in 20 (25%) of the patients by aggressive antihypertensive medication. Also, remission was associated with significantly improved renal outcome and survival as only 6 of the 20 patients (30%) with remission reached the composite end point of ESRD or death (2 patients developed ESRD and 4 patients died) as compared with 39 (66%) of the 59 patients without remission (16 patients developed ESRD and 23 patients died) ($p < 0.01$). Patients who obtained remission also tended to have a slower rate of

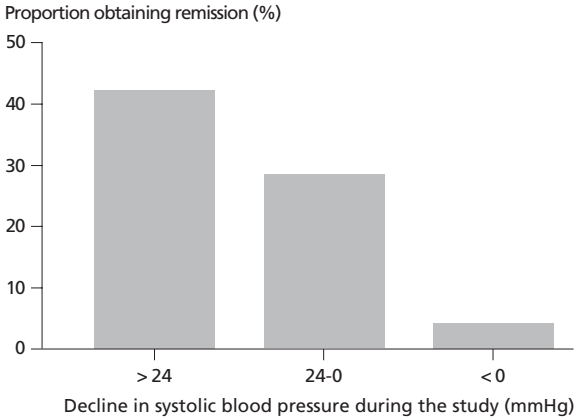

Figure 2. Proportion of patients obtaining remission among 79 type 2 diabetic patients with NRA according to tertiles of the systolic blood pressure reduction during the study from baseline to end of follow-up ($p < 0.05$).

decline in GFR as compared to those who did not remit (5.4 (1.0) vs. 8.4 (1.1) ml/min/year, respectively, $p = 0.08$).

All patients with remission received treatment with ACE-I or ARB and remission was not observed in any of the patients not receiving these compounds. Aside from antihypertensive treatment no other major treatment was introduced early enough during the study period to have a significant impact on remission rates. No significant changes were seen in HbA1c over the study period. There was no sodium or protein restriction and lipid-lowering drugs and low-dose aspirin were not introduced on a routine basis until the end of the observation period in 2002. We were not able to identify baseline predictors of remission as known risk factors for progression of diabetic nephropathy were similar at baseline among patients with and without remission. No differences were present in demographic, clinical or laboratory data suggesting underlying differences in genetic susceptibility to treatment (pharmacogenetics). Arterial blood pressure was reduced significantly more during the observation period in patients with remission as compared with patients who did not remit. It is evident from Figure 2 that the greater the reduction in systolic blood pressure over the study period, the greater the likelihood of obtaining remission. In addition to lower blood pressure levels, remission of NRA was associated with an improved cardiovascular risk profile with a substantial decline in cholesterol of 1 mmol/l probably as a direct consequence of the considerable reduction in albuminuria (122).

It should also be noted that a previous observational study of type 1 diabetic patients has shown that remission of diabetic nephropathy to microalbuminuria was possible by aggressive antihypertensive treatment in approximately 30% of the patients and such remission was associated with a 50% lower rate of decline in renal function (123). Furthermore, regression of diabetic nephropathy defined as a normalization of the rate of decline in GFR (equal to or less than 1 ml/min/year, which corresponds to the decline in healthy subjects due to ageing) could be obtained in approximately 20%. At lower grades of albuminuria recent interventional trials of type 2 diabetic patients demonstrated that remission from micro- to normoalbuminuria is feasible in approximately 25% of the patients during antihypertensive treatment and is associated with a lower rate of decline in GFR as compared to patients who remain microalbuminuric and even more so when compared to patients who progress to diabetic nephropathy (33, 124).

In general the above mentioned clinical studies dealing with remission of diabetic renal disease at its various stages are in close agreement with recent studies which have demonstrated that baseline level of albuminuria predicts the subsequent cardiovascular and renal risk, but also that the obtained level of albuminuria during treatment is related to outcome and the greater the reduction in albuminuria upon initiation of antihypertensive treatment the better the long-term renal and cardiovascular outcome (20-26).

To further improve prognosis and treatment of diabetic renal disease it is essential to identify factors explaining why some patients respond well to treatment and obtain remission, whereas others are less responsive and do not remit. Such factors may among others include genetic differences affecting response to antihypertensive treatment (pharmacogenomics) e.g. polymorphisms within the RAAS (112), as well as a wide range of physiological, psychological and behavioral factors. In particular insufficient treatment response may be due to poor adherence to prescribed medications (125, 126). Finally it can be due to inadequate dosing or combinations of blood pressure lowering agents. If these confounding factors can be corrected it may be possible to further decrease the proportion of patients reaching ESRD and furthermore reduce cardiovascular morbidity and mortality.

6. NEW STRATEGIES OF TREATMENT IN DIABETIC NEPHROPATHY

6.1 THE RAAS AND THE BASIS FOR ITS BLOCKADE

The RAAS (Figure 3) is activated in the kidneys of patients with diabetic nephropathy and plays an important role in both hemodynamic and nonhemodynamic pathogenetic mechanisms in renal disease (127). The importance of the RAAS in progressive diabetic renal injury is most firmly evidenced by the numerous large randomized double blind clinical studies which have demonstrated specific renoprotective effects by blocking the RAAS either by ACE-I in type 1 (54, 55, 128) and ARB in type 2 (33, 58, 129) diabetic patients with microalbuminuria or overt nephropathy. In these studies the renoprotective effect of RAAS blockade has at the same level of blood pressure reduction been superior to other antihypertensive agents including diuretics, beta-blockers and calcium channel blockers. Consequently RAAS blocking agents are now recommended as first line therapy in the prevention and treatment of diabetic nephropathy (130).

The observation that ACE-I and ARB offer renoprotective effects above and beyond what can be attributed to lowering of systemic arterial blood pressure alone may in part be attributed to specific reduction of intraglomerular capillary pressure independent from systemic blood pressure by dilatation preferentially of the efferent arteriole as originally demonstrated in animal models (131) and subsequently confirmed in diabetic patients by estimation of intraglomerular blood pressure using arterial blood pressure and urinary sodium clearance (pressure natriuresis curves) (132, 133). In addition, RAAS activation induces a series of non-hemodynamic effects as illustrated on Figure 3 and reviewed in detail by others (134-136). Briefly, blocking these non-hemodynamic effects leads to reduction

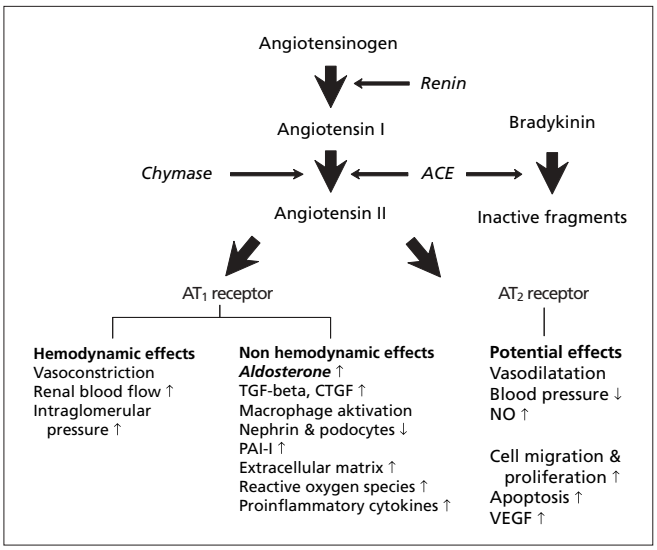

Figure 3. The renin-angiotensin-aldosterone system and the effects of angiotensin II type 1 (AT_1) and type 2 (AT_2) receptor activation.

of prosclerotic cytokines leading to reduced extracellular matrix turnover and reduction of proinflammatory cytokines leading to reduced macrophage infiltration and less fibrosis in the renal tissue. Furthermore RAAS blockade leads to improved permselective properties of the glomerular membrane (137) and reduction of the loss of glomerular nephrin and podocytes (138).

6.2 THE CONTINUOUS NEED FOR IMPROVED TREATMENT

The urgent need for improvements in the treatment strategies of diabetic nephropathy is emphasized by the fact that despite aggressive antihypertensive treatment including RAAS blockade with ACE-Is or ARBs, recommended blood pressure targets are often not reached, albuminuria frequently remains elevated and diabetic nephropathy accounts for an increasing proportion of patients reaching ESRD (139).

The lack of success with regard to reaching treatment goals and completely preventing ESRD may in part be due to insufficient blockade of the deleterious actions of the RAAS either because currently recommended doses of RAAS blocking agents are too low (as discussed in detail in 6.3) or because effective blockade may require a multiple drug approach with concomitant use of several drugs that target the RAAS at different sites (as discussed in detail in section 6.4 and 6.5). In a series of short-term studies we therefore assessed new strategies to block the RAAS more effectively including studies on the optimal dosing of ARBs and the effects of multiple RAAS blockade with ACE-I, ARBs and spironolactone. Such short-term studies of new treatment strategies should eventually lead to large clinical trials using rate of decline in GFR, ESRD or death as end-points which requires large groups of patients and several years of follow-up. However, short-term reduction of albuminuria has emerged as a key therapeutic goal for both reno- and cardiovascular protection as discussed previously in section 4.1.2. Therefore it is a sensible strategy to assess new treatment modalities by their short-term antiproteinuric effects before long-term clinical studies are conducted.

6.3 DOSING RAAS BLOCKING AGENTS BEYOND BLOOD PRESSURE CONTROL

Although ACE-I and ARB have been recommended for several years as first line agents to prevent and treat diabetic nephropathy the optimal dosing for renoprotection has not been evaluated in the past. Currently recommended doses of RAAS blocking agents for renoprotection are primarily based on studies of the blood pressure lowering effects in patients with essential hypertension. In these studies there has been no additional lowering of systemic blood pressure in doses above those currently recommended for ACE-Is (140-143) and ARBs (144-146).

Studies evaluating the optimal dosing for antiproteinuric and renoprotective effects have, however, been lacking and there are several reasons why higher doses may be needed for optimal renoprotection: 1) patients with diabetic nephropathy are characterized by low to normal circulatory levels of renin and yet increased RAAS activity locally in the kidney with up-regulation of chymase (147), increased angiotensin II concentration and increased angiotensin II receptor density (148, 149), 2) Reduced drug penetration in local tissue such as the kidney and perhaps in particular in ischemic lesions may require higher doses to obtain sufficient tissue concentration of ACE-I and ARBs, 3) Doses needed to maximally reduce intraglomerular hydraulic pressure may be different from those that affect systemic blood pressure (132), and 4) the beneficial non-hemodynamic actions of RAAS blockade such as inhibition of prosclerotic and pro-inflammatory cytokines may require higher doses than those needed to block the direct hemodynamic actions of the RAAS as demonstrated in animal models of renal disease (114, 150).

6.3.1 Renoprotective effects of ARBs within currently recommended dose levels

We initially performed a randomized double blind crossover study to evaluate the antiproteinuric and blood pressure lowering effects of candesartan cilexetil in doses of 8, 16 and 32 mg o.d. versus placebo in 23 hypertensive type 2 diabetic patients with nephropathy (4). Placebo tablets and each dose of candesartan were given for two months. All three doses of candesartan cilexetil significantly reduced albuminuria and 24-hour arterial blood pressure compared to placebo. Albuminuria was reduced by 33 (95% CI: 21 to 43)%, 59 (52 to 65) % and 52 (44 to 59) % relative to placebo with increasing doses of candesartan. Interestingly, albuminuria was reduced significantly more by the two highest doses as compared to the lowest dose. In contrast, 24-hour blood pressure was reduced to a similar extent by all three doses of candesartan (approximately 10 mmHg systolic and 5 mmHg diastolic). The study therefore suggests a dissociation between the dose response-curves for arterial blood pressure and albuminuria with higher doses needed to maximally reduce albuminuria. Systemic levels of renin and angiotensin II increased as could be expected as a compensatory mechanism during ARB treatment. However, in accordance with the blood pressure reductions there were no additional rise in circulatory concentrations of renin and angiotensin II when the dose was increased above 8 mg daily. This suggests that circulatory concentrations of angiotensin II contributing to the regulation of systemic blood pressure are inhibited at doses of ARB lower than those needed to block the deleterious effects of angiotensin II locally in the kidney.

The finding that high doses of ARB are needed for maximal reduction of albuminuria was also observed in a dose response study of losartan in 10 patients with non-diabetic nephropathies (151) where maximal reduction of blood pressure was achieved by losartan 50 mg daily whereas 100 mg daily was needed for maximal reduction of albuminuria. A study in 50 type 1 diabetic patients with diabetic nephropathy also demonstrated that the optimal dose of losartan for reduction of albuminuria is 100 mg (152). In both of these studies there was no additional antiproteinuric or blood pressure lowering effect by increasing the dose of losartan to 150 mg daily.

The Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA2) study (33) is so far the only major long-term clinical trial of the renoprotective effects of RAAS blockade in diabetic renal disease which has taken the importance of evaluating optimal dosing into account in the study design. In this study, 595 type 2 diabetic patients with microalbuminuria were randomized to placebo or treatment with either irbesartan 150 or 300 mg daily. The study medication was added to a background therapy of non-RAAS blocking antihypertensive agents to keep blood pressure below 135/85 mmHg. The study firmly demonstrated that treatment with the ARB irbesartan reduces UAE and the risk of progression to overt diabetic nephropathy, in a clearly dose-dependent manner. Progression to overt diabetic nephropathy over the two years study period was only 5% among patients receiving irbesartan 300 mg as compared to 10% and 15% among patients receiving respectively 150 mg and placebo. UAE was reduced by 24% and 38% by irbesartan 150 and 300 mg respectively whereas it remained unchanged in the placebo group. The importance of optimal dosing was further emphasized by a subsequent sub-study of the IRMA-2 trial demonstrating that after withdrawal of highest dose (irbesartan 300 mg/day), the reduction of albuminuria remained, although blood pressure values rose to initial increased values. In contrast patients assigned to irbesartan 150 mg daily had a subsequent increase to initial values of both blood pressure and albuminuria upon withdrawal. The authors therefore suggested that the prolonged benefit seen only by the highest dose of irbesartan could reflect reversal of renal structural/and or biochemical abnormalities.

A key issue in the IRMA-2 study as well as the other major clinical trials of ACE-I and ARB treatment in diabetic nephropathy has been the finding that the beneficial effects of RAAS blockade on preventing initiation and progression of diabetic nephropathy were apparently above and beyond what could be explained by the measured reduction in systemic blood pressure. However, in all of these trials

systemic blood pressure was measured at the end of the dosing interval of the study medication (trough blood pressure). Differences in diurnal blood pressures may therefore have been overlooked and consequently led to an underestimation of the true effects of systemic blood pressure. As a sub-study of the IRMA-2 trial we therefore evaluated 24-hours blood pressure patterns by ambulatory blood pressure measurements in 43 type 2 diabetic patients with microalbuminuria who took part in the IRMA-2 study at the Steno Diabetes Center (2). Patients included in the sub-study were comparable to the overall IRMA-2 population with respect to demographic, clinical and laboratory characteristics. We found that reductions in office trough blood pressure and 24-hour as well as night and day blood pressure patterns were comparable among patients randomized to placebo or irbesartan 150 or 300 mg. In agreement with the overall IRMA-2 study there was also a dose-dependent reduction of UAE by irbesartan treatment which were independent of reductions in 24-hour blood pressures and consequently the study supports a blood pressure independent effect of angiotensin II receptor blockade by irbesartan.

6.3.2 Renoprotective effects of ARBs above recommended dose levels

The optimal dose of irbesartan could not be established in the IRMA-2 trial as doses above 300 mg daily were not assessed. We therefore evaluated if further antiproteinuric effects is obtainable when exceeding the currently recommended dose of irbesartan 300 mg daily. In a randomized double-blind crossover study we included 52 type 2 diabetic patients with microalbuminuria at ongoing antihypertensive treatment (8). At entry to the study, all previous antihypertensive treatment was discontinued and replaced with bendroflumethiazide 5 mg o.d. control blood pressure and edema formation and to diminish the influence of varying dietary salt intake on the effects of irbesartan. Following two months wash-out (baseline), patients were treated randomly with irbesartan 300, 600 and 900 mg o.d. and each dose was given for two months. In this study we observed that all doses of irbesartan significantly reduced UAE and 24-hour arterial blood pressure.

There was a completely flat dose response curve for arterial blood pressure with reductions in 24-hour systolic blood pressure of 8 (4 to 12), 9 (5 to 13) and 9 (5 to 13) mmHg, and 24-hour diastolic blood pressure of 6 (4 to 7), 7 (6 to 9), and 7 (6 to 9) mmHg at increasing doses of irbesartan relative to baseline (Figure 4). The lack of additional blood pressure lowering effect by increasing the dose of irbesartan above 300 mg daily is in accordance with findings in a previous study of 2955 patients with mild to moderate essential hypertension (144).

In contrast, reductions in 24-hour UAE from baseline were 52 (95% CI: 46 to 57), 49 (43 to 54) and 59 (54 to 63) % with increasing doses of irbesartan ($p < 0.01$) and UAE was reduced significantly more by irbesartan 900 mg as compared with lower doses with an additional reduction in 24-hour UAE of 15 (2 to 26) % by irbesartan 900 mg compared with 300 mg (Figure 4). Similar reductions were obtained when evaluating UAE from samples collected during a four hour period in the morning at Steno Diabetes Center and when evaluating fractional clearance of albumin (Figure 4).

The study also demonstrated that patients with the highest UAE during conventional treatment with irbesartan 300 mg having the poorest cardiovascular and renal prognosis were more likely to benefit from increasing the dose of irbesartan to 900 mg. This is illustrated in Figure 5 which shows the positive correlation between the level of UAE during treatment with irbesartan 300 mg and the relative reduction of UAE when irbesartan was increased from 300 to 900 mg ($r = 0.66$, $p < 0.001$). It is also evident from Figure 5, that the majority of patients not having an additional reduction in UAE when irbesartan was increased from 300 to 900 mg were those who had UAE reduced to the normoalbuminuric range already on 300 mg irbesartan.

Based on these observations it is tempting to speculate that patients with overt diabetic nephropathy have even greater antiproteinuric effects by ultrahigh doses of irbesartan.

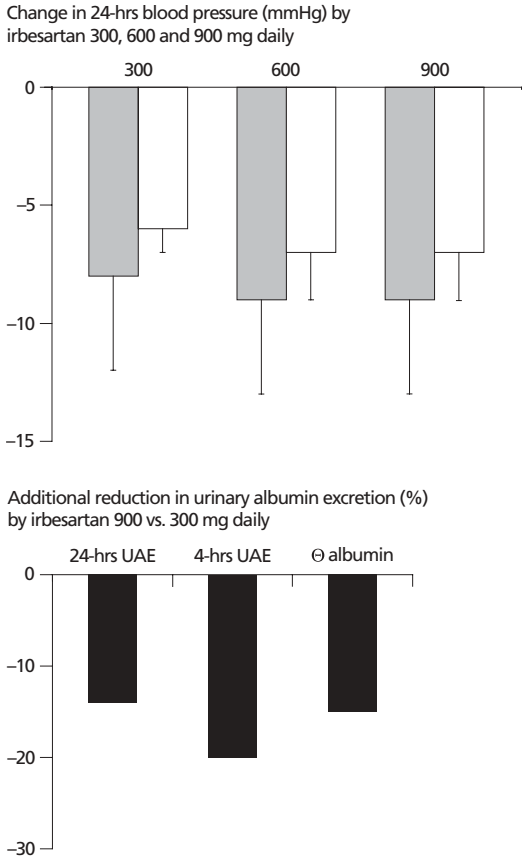

Figure 4. Randomized double blind dross-over study evaluating the efficacy and safety of irbesartan 300, 600 and 900 mg o.d. in 52 type 2 diabetic patients with microalbuminuria. *Upper panel:* effects on 24-hour blood pressure (gray bars: systolic, white bars: diastolic) of irbesartan 300, 600 and 900 mg daily. *Lower panel:* additional reduction in urinary albumin excretion (UAE – as determined in 24 hours urinary collections and 4-hour urinary collection) and fractional clearance of albumin (Θ albumin) of irbesartan 900 vs. 300 mg daily.

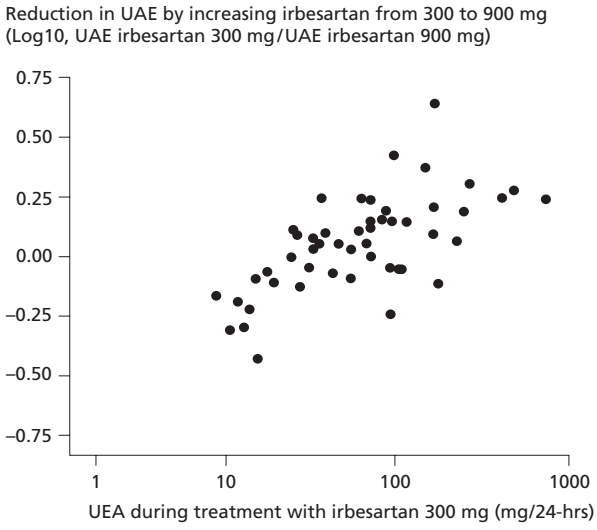

Figure 5. Randomized double blind dross-over study evaluating the efficacy and safety of irbesartan 300, 600 and 900 mg o.d. in 52 type 2 diabetic patients with microalbuminuria. Linear regression analysis between urinary albumin excretion rate (UAE) during conventional treatment with irbesartan 300 mg o.d., and the relative change in UAE (log 10) when irbesartan was increased from 300 to 900 mg o.d. ($r = 0.66$, $p < 0.001$). The figure illustrates that the beneficial impact on UAE of 900 mg irbesartan increases with higher levels of UAE during treatment with irbesartan 300 mg.

Additional benefits of the 900 vs. 300 mg included a more pronounced reduction of aldosterone of approximately 30%, which may contribute to the renoprotective effects as discussed in detail in section 7.5 and there was also a dose-independent reduction of cholesterol 0.3 of mmol/l during irbesartan treatment as also reported previously (153).

The use of high doses of irbesartan was found to be safe with no significant dose-related side effects. This is in accordance with previous studies of doses of up to 900 mg irbesartan in patients with essential hypertension (154).

A very recent study of patients with non-diabetic nephropathy demonstrated in accordance with our initial dose response study of candesartan that 32 mg did not have additional antiproteinuric effects as compared to the maximally recommended dose of candesartan 16 mg daily. However, when candesartan was increased to 64 mg there was a further decrease in proteinuria of 30% (155). This effect was also independent from effects on blood pressure. These results clearly indicate that it is time to reassess current recommended maximal doses of ARBs in order to reach the full renoprotective potential of these agents. Furthermore, studies evaluating optimal renoprotective doses of ACE-I are also urgently needed, as these has never been established.

6.4 DUAL RAAS BLOCKADE

Since ACE-Is and ARBs block the RAAS at different sites, concomitant treatment with both agents (the so-called dual RAAS blockade) can potentially lead to additive or even synergistic renoprotective effects not obtainable by either drug alone even if used at very high doses as discussed below.

6.4.1 The incomplete RAAS blockade by ACE-I

ACE-Is decrease the conversion of angiotensin I to angiotensin II through competitive binding to the ACE enzyme (Figure 3). In addition ACE-I lowers blood pressure by reducing the degradation of the potent vasodilator bradykinin (156). However, ACE-I leads only to an incomplete blockade of angiotensin II synthesis, possibly as a result of incomplete enzyme inhibition or generation of angiotensin II through non-ACE-dependent pathways such as chymase and other serine proteases (157). Chymase does not seem to be upregulated in the kidneys of uncomplicated diabetic patients as evidenced by a similar renal vascular response to ACE-I and ARB (158). The opposite is apparent in patients with advanced diabetic nephropathy where both chymase and ACE expression is up-regulated in renal tissue and the expression is closely correlated with both hypertension and extracellular matrix deposition (147). Non-ACE generating pathways and incomplete blockade of the ACE can explain the observation that plasma angiotensin II levels return to pretreatment levels after chronic ACE-I treatment, the so called "*ACE-escape*" phenomenon (159).

6.4.2 The incomplete RAAS blockade by angiotensin II receptor blockers

In comparison to ACE-I, ARBs have the advantage of blocking angiotensin II at the receptor level and the effect can therefore not be counteracted through non-ACE dependent pathways of the angiotensin II synthesis. However, during ARB treatment there is a compensatory increase in renin and angiotensin II which would tend to counteract the effect of ARB if non-competitive blockade of all AT_1 receptors is not achieved. The compensatory increase in angiotensin II during ARB treatment also leads to increased stimulation of other subtypes of the angiotensin II receptors including the type 2 and 4 receptor (AT2 and AT4). Stimulation of the AT2 receptor was initially viewed as being exclusively beneficial with effects opposing the binding of angiotensin II to the AT1 receptor (160, 161). However, recent animal studies have suggested that stimulation of the AT2 receptor can induce adverse effects including glomerular cell migration, tubular cell proliferation, apoptosis, increased vascular endothelial growth factor and development of elevated urinary protein excretion (162-164). Furthermore, enhanced stimulation of the AT4 receptor during ARB treatment can have adverse effects as it increases plasminogen activator-inhibitor-1 (PAI-1) expression in tubular cells which in turn reduce the extracellular matrix turnover leading to renal fibrosis, at least in the experimental setting (165).

6.4.3 Clinical effects of dual RAAS blockade in diabetic nephropathy

The first and presently also the largest study to demonstrate clinical benefits of dual RAAS blockade in diabetic patients was the CALM-study (166), which included 199 patients with type 2 diabetes, microalbuminuria and hypertension. The study demonstrated a greater reduction in systemic blood pressure by dual blockade (candesartan cilexetil 16 mg and lisinopril 20 mg) as compared with either agent alone (Table 3). The reduction in sitting systolic blood pressure on mono-therapy using either drug alone was approximately 15 mmHg. The additional effect of a combination therapy was a further reduction in systolic blood pressure of 10 mmHg. In the CALM study there was also a trend towards a more pronounced antiproteinuric effect of dual blockade. Inspired by these encouraging results we evaluated the short-term effect of dual RAAS blockade in a randomized double blind crossover study of 18 type 2 diabetic patients with diabetic nephropathy (1). All patients included in the study had hypertension and albuminuria above 1000 mg/24-hours. This was in spite of aggressive antihypertensive therapy with several different blood pressure lowering agents including ACE-I in doses corresponding to enalapril/lisinopril 20 mg or captopril 100 mg daily. By adding the ARB candesartan cilexetil in a dose of 8 mg o.d. for two months albuminuria was significantly reduced by 24% (Figure 6), the fractional clearance of albumin by 35% and 24-hour systolic/diastolic blood pressure by 10/3 mmHg (Figure 6). This demonstrated for the first time that dual RAAS blockade could lead to significant reductions in albuminuria in patients with overt nephropathy. Since then several short-term studies have been conducted in diabetic patients with microalbuminuria or overt nephropathy most of which have confirmed the beneficial effect of dual RAAS blockade (Table 3). Recent results from the CALM II study (167) which included both type 1 and type 2 diabetic patients with varying degrees of albuminuria most of whom had microalbuminuria demonstrated that dual blockade with lisinopril 20 mg and candesartan 16 mg were not superior to lisinopril 40 mg in reducing arterial blood pressure or albuminuria.

6.4.4 Clinical effects of ACE-I and ARB combined at maximally recommended doses

It has been unknown if dual RAAS blockade would also provide additional clinical renal benefits in diabetic patients up-titrated to maximal recommended doses of ACE-I and ARB. We therefore conducted a randomized double-blind crossover study of 20 type 2 diabetic patients with nephropathy (3) who all received maximally recommended doses of an ACE-I corresponding to 40 mg of enalapril/lisinopril and 150 mg of captopril daily. In this study the addition of 16 mg candesartan cilexetil induced a significant decline in albuminuria of 28 (95% CI: 17 to 38) % ($p < 0.05$) whereas there was no significant reduction of 24-hour arterial blood pressure (Figure 7). Since there was no correlation between individual changes in systemic blood pressure and albuminuria this study clearly indicated a blood pressure independent reduction of albuminuria upon dual RAAS blockade.

To explore potential mechanisms responsible for the additional reduction of albuminuria upon dual RAAS blockade in our study of patients titrated to maximal recommended doses of ACE-I we subsequently determined urinary concentrations of connective tissue growth factor (CTGF) which seems to be an important profibrotic growth factor implicated in the pathogenesis of diabetic nephropathy which acts downstream of transforming growth factor (TGF)

Table 3. Clinical trials of dual RAAS blockade in diabetic patients with microalbuminuria or overt nephropathy.

Authors	Patients	Design	Duration	Combination	Control	Results – dual vs. mono RAAS blockade
Mogensen et al, 2000 [166]	199 T2DM microalbuminuria	R, DB, P	12 wks	candesartan 16 mg & lisinopril 20 mg	candesartan 16 mg or lisinopril 20 mg	Alb: ↓34% vs. candesartan ↓18% vs. lisinopril (NS) BP: ↓11/6 mmHg vs. candesartan ↓9/6 mmHg vs. lisinopril
Tutuneu et al, 2001 [219]	34 T2DM microalbuminuria	R, P	12 mo	losartan 50 mg & enalapril 5 mg	losartan 50 mg or enalapril 5 mg	Albuminuria: – BP: –/–
Andersen et al, 2005 [167]	75 T1&T2DM microalbuminuria**	R, DB, P	12 mo	candesartan 16 mg & lisinopril 20 mg	lisinopril 40 mg	Alb: – BP: –/–
Sengul et al, 2005 [220]	192 T2DM microalbuminuria	R, OL, P	24 wks	telmisartan 80 mg & lisinopril 20 mg	telmisartan 80 mg or lisinopril 20 mg	Alb: ↓46% vs. telmisartan ↓32% vs. lisinopril BP: ↓10/5 vs. telmisartan ↓10/5 vs. lisinopril
Hebert et al, 1999 [221]	7 T1&T2DM nephropathy	NR, OL, CO	1 wk	losartan 50-100 mg & *lisinopril 10-40 mg	*lisinopril 10-40 mg	Alb: – BP: –
Agarwal et al, 2001 [222]	16 (12 with T2DM) nephropathy	R, PC, CO	4 wks	losartan 50 mg & lisinopril 40 mg	losartan 50 mg	Alb: – BP: –/–
Rossing et al, 2002 [1]	17 T2 DM Nephropathy	R, PC, DB, CO	8 wks	candesartan 8 mg & *lisinopril 20 mg	*lisinopril 20 mg	Alb: ↓25% 24-hrs BP: ↓10/– mmHg
Kuriyama et al, 2002 [223]	9 T2DM nephropathy	NR, OL, CO	2 wks	candesartan 4 mg & temocapril 2 mg	temocapril 2 mg	Alb: ↓50% BP: ↓9/– mmHg
Jacobsen et al, 2002 [224]	19 T1DM nephropathy	R, PC, DB, CO	8 wks	irbesartan 300 mg & *lisinopril 20 mg	*lisinopril 20 mg	Alb: ↓37% 24-hrs BP: ↓8/5 mmHg
Rossing et al, 2003 [3]	20 T2DM nephropathy	R, PC, DB, CO	8 wks	candesartan 16 mg & *lisinopril 40 mg	*lisinopril 40 mg	Alb: ↓28% 24-hrs BP: –/–
Jacobsen et al, 2003 [195]	18 T1DM nephropathy	R, PC, DB, CO	8 wks	valsartan 80 mg & benazepril 20 mg	valsartan 80 mg or benazepril 20 mg	Alb: ↓43% vs. valsartan ↓43% vs. benazepril 24-hrs BP: ↓7/7 mmHg vs. valsartan ↓7/7 mmHg vs. benazepril
Jacobsen et al, 2003 [225]	24 T1DM nephropathy	R, PC, DB, CO	8 wks	irbesartan 300 mg & *lisinopril 40 mg	*lisinopril 40 mg	Alb: ↓25% 24-hrs BP: ↓8/4
Song et al, 2003 [226]	18 T2 DM nephropathy	R, DB, PC, CO	16 wks	candesartan 4 mg & ramipril 5-7.5 mg	ramipril 5-7.5 mg	Alb: – BP: –/–
Cetinkaya et al, 2004 [227]	22 T2DM nephropathy	R, CO	12 wks	losartan 50 mg & enalapril 10 mg	losartan 50 or 100 mg or enalapril 10 or 20 mg	Alb: ↓68% vs. both losartan 50 and enalapril 10 mg Alb: ↓38% vs. both losartan 100 and enalapril 20 mg MAPBP: ↓4 mmHg vs. all doses of both enalapril and losartan
Fujisawa et al, 2005 [228]	27 T2DM nephropathy	OL, NR, CO	12 wks	candesartan 4 mg & imidapril 5 mg	candesartan 4 mg or imidapril 5 mg	Alb: ↓34% BP: –/–

T1/2DM = type 1/2 diabetes; R = randomized; NR = non randomized; DB = double blind; OL = open labelled; P = parallel; CO = crossover; alb = albuminuria; BP = blood pressure (x/y, x = systolic, y = diastolic); ? = reduction (all reductions given were statistically significant – p at least <0.05); – = not significantly changed; MAPBP = mean arterial blood pressure. * = If different ACE-I have been used equipotent doses of lisinopril is shown.

beta (168). Overall we found that urinary CTGF was reduced by 18% ($p=0.05$) upon dual RAAS blockade. Interestingly, there was a significant carry-over effect in our randomized crossover trial by dual blockade on U-CTGF as reflected by a 36% (17 to 51) ($p<0.001$) reduction in those 10 patients who received ACE-I alone in the first period and dual blockade in the second period, whereas there was an insignificant change in U-CTGF of –5% (–38 to 20) ($p=0.71$) in patients who received dual blockade in the first period and mono-blockade with ACE-I in the second period. There was no significant carry-over effect for albuminuria, arterial blood pressure or GFR. The carry-over effect on U-CTGF suggests a prolonged effect of dual RAAS blockade which may thus represent a previously unknown mechanism responsible for the beneficial effects of dual RAAS blockade. We have recently shown that long-term treatment (3 years) with losartan reduced urinary-CTGF in type 1 diabetic patients with nephropathy and the reduction was found to correlate with a lower rate of decline in GFR (169).

To date there is no long-term trial of dual RAAS blockade in patients with diabetic nephropathy. In non-diabetic patients, however, the long-term effect of dual RAAS blockade on principal renal end-points has been addressed in the COOPERATE trial (170). In this double-blind randomized study of 263 patients only 11% of patients on dual blockade (100 mg losartan daily and 3 mg of trandolapril daily) developed doubling of s-creatinine or reached end stage renal disease during a median of 3 years of follow-up, whereas 23% reached these primary end-points during treatment with either mono-therapies ($p=0.02$). According to the authors of this study the optimal dosage of trandolapril had been confirmed during the run-in period, where no further antiproteinuric effects were seen by doses above trandolapril 3 mg daily (increased to 6 mg daily).

In light of the recent findings regarding additional antiproteinuric effects of ultra-high doses of ARBs it is hard to completely rule out that the beneficial effect seen in the presently available studies of dual RAAS blockade could be due to the fact that currently licensed doses of ACE-I and ARB do not reach the top of the antiproteinuric

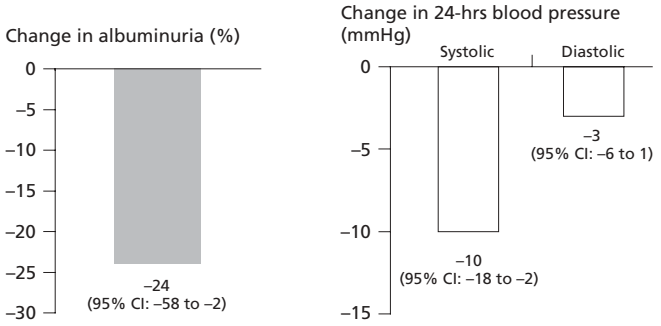

Figure 6. Randomized double-blind cross over study of 17 type 2 diabetic patients with nephropathy. Effect of adding candesartan 8 mg to lisinopril 20 mg daily.

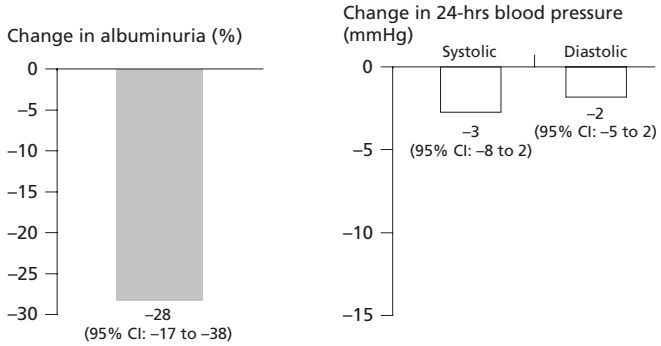

Figure 7. Randomized double-blind cross over study of 20 type 2 diabetic patients with nephropathy. Effect of adding candesartan 16 mg to lisinopril 40 mg daily.

dose response curve. When combining two agents working on the same hormone system a true additive effect can only be demonstrated when the compounds are combined at maximal effective doses. Despite its obvious importance, the issue of dose often escapes the medical community (171) and the maximal effective doses of ACE-Is and ARBs still needs to be established.

Nevertheless the studies dealing with dual RAAS blockade have put emphasis on the importance of high dosing of RAAS blocking agents for optimal renoprotection and since dual blockade has additional antiproteinuric effects as compared to mono RAAS blockade with the presently recommended maximally doses, it remains a promising strategy for reinforcing renoprotection in the clinical setting.

6.5 ALDOSTERONE BLOCKADE

6.5.1 The changing paradigm of aldosterone

For many years angiotensin II has been regarded as the main mediator of the pathophysiologic effects of the RAAS mainly because interventional strategies within the RAAS have been focused on blocking the actions of angiotensin II through ACE-I and/or ARB. In contrast, there has been little interest in the possible renoprotection by blocking aldosterone, the end-product of the RAAS, primarily because of concerns of hyperkalemia. However, aldosterone, has gained increasing attention as a key mediator of both renal and cardiovascular disease by inducing inflammation, fibrosis and necrosis in end-organ tissues such as the heart, brain and kidney (172-174). In particular, large clinical trials have now demonstrated greatly improved survival upon aldosterone blockade among patients with severe heart failure (175) and among patients with acute myocardial infarction complicated by left ventricular dysfunction and heart failure (176).

It was previously believed that aldosterone was only produced in the zone glomerulosa of the adrenal gland and acted almost exclusively on the renal tubular cells to modify sodium and water retention through the classical mineralocorticoid receptor. Recent studies have extensively broadened this view by demonstrating extra-adrenal synthesis of aldosterone and expression of mineralocorticoid receptors in endothelial and vascular smooth muscle cells in the heart, vessel and kidneys where aldosterone exerts direct auto- and paracrine effects (173, 174, 177). Moreover, it has become evident that in addition to the classic mineralocorticoid receptor dependent changes in gene expression, aldosterone also has non-genomic effects (178). They are characterized by their rapid onset of action (within minutes), and their insensitivity to inhibitors of transcription, protein synthesis, and to antagonists of the type 1 mineralocorticoid receptor such as spironolactone (179). It is also becoming increasingly clear that aldosterone exerts a vast array of both hemodynamic and non-hemodynamic actions in addition to the classical effects of salt and water retention. Direct effects on the vasculature of aldosterone includes vasoconstriction in resistance arteries leading to systemic hypertension and increase in glomerular capillary pressure by preferential constriction of the efferent renal arteriole (180). Furthermore, aldosterone exerts hemodynamic effects through up-regulation of angiotensin II receptors (181), increased vasoconstrictive effects of catecholamines (182) and impairment of endothelial function (183,184). From studies both in animals and humans several non-hemodynamic actions of aldosterone have been proposed to play a role for progressive cardiovascular and renal injuries, many of which resemble those previously ascribed entirely to angiotensin II. These include stimulation of prosclerotic and pro-inflammatory growth factors such as TGF-β1 and PAI-1, promotion of macrophage infiltration (185) and increased oxidative stress (186).

Studies in rats suggest that the harmful effect of angiotensin II, at least in part, is mediated by the stimulation of increased aldosterone release (187, 188).

Studies in humans have demonstrated that both ACE-I and ARB treatments initially suppress plasma aldosterone. Eventually however, plasma aldosterone may return to pre-treatment levels i.e. the aldosterone escape phenomenon. Aldosterone escape has been reported to occur in approximately 20% of patients with chronic heart failure (172) and up to 40% of patients with diabetic nephropathy (173, 174). Recently, such aldosterone escape was associated with enhanced proteinuria and a faster decline in renal function among patients with diabetic nephropathy (173, 174). Hence, unsuppressed actions of aldosterone contribute to progression in patients with diabetic nephropathy despite treatment with ACE-I or ARB-treatment and specific blockade of aldosterone might thus provide additional renoprotective benefit.

6.5.2 Clinical studies of aldosterone blockade in renal disease

The therapeutic benefit of spironolactone treatment in chronic renal disease was initially proposed in an open-labelled study of 8 patients with various renal diseases and severe proteinuria (above 1000 mg/24-hour) where the addition of 25 mg spironolactone to ongoing ACE-I treatment reduced proteinuria by 54% (189). A subsequent study of 13 Japanese type 2 diabetic patients with early nephropathy and aldosterone escape during long-term ACE-I treatment, showed that albuminuria was significantly reduced by 40% during addition of spironolactone 25 mg daily (173). However, the overall therapeutic potential could not be estimated in this study, since the antiproteinuric effects of spironolactone treatment was not evaluated in patients without aldosterone escape. The same group recently extended these findings in an open-labelled non-randomized study of patients with various renal diseases and proteinuria persistently greater than 500 mg/24-hour despite well controlled blood pressure during long-term ACE-I (190). Addition of spironolactone 25 mg reduced albuminuria by 46% among 17 diabetic patients and by 26% among 13 patients with non-diabetic renal disease (190). A recent study of 60 type 2 diabetic female patients with diabetic nephropathy compared the effect of spironolactone 100 mg and cilazapril 5 mg in a randomized parallel study followed by an open labelled period where all patients received a combination of

spironolactone and cilazapril at halved doses (191). In this study urinary albumin/creatinine ratio, was reduced by 52% and 34% upon spironolactone and cilazapril treatment, respectively, and a further reduction on the combination therapy on halved doses (191). In this study the antiproteinuric effect was isolated from the blood pressure lowering effect by keeping blood pressure stable below 135/85 mmHg through careful titration of atenolol and hydrochlorothiazide. Consequently, the blood pressure lowering effect of spironolactone could not be established. Since hydrochlorothiazide is known to potentate the antiproteinuric effects of ACE-I, differences in dosing of hydrochlorothiazide between treatment regiments could also affect the observed differences in proteinuria (192).

Limitations in study design of these previous open-labelled and non-randomized trials preclude an overall assessment of the clinical effects of spironolactone in diabetic nephropathy. We therefore conducted the first randomized double-blinded study to evaluate the short-term antiproteinuric and blood pressure lowering effect of spironolactone as add-on therapy in 21 type 2 diabetic patients with nephropathy (7). All patients received diuretics and were treated with maximal recommended doses of ACE-I and/or ARB. In addition the patients received on average two other types of antihypertensive agents primarily amlodipine and beta-blockers. Despite such aggressive antihypertensive treatment the patients had an average 24-hour blood pressure of 138/71 mmHg and a mean level of albuminuria of 1566 (interquartile range: 655 to 4208) mg/24-hour, thus representing a group of patients in need of additional therapeutic strategies. Patients were treated in random order with spironolactone 25 mg o.d. and matched placebo for eight weeks respectively, on top of ongoing antihypertensive treatment. In this study the addition of spironolactone 25 mg daily for two months reduced albuminuria by 33 (95% CI: 25 to 41) % (Figure 8) and fractional clearance of albumin by 40 (95% CI: 24 to 53) %. Spironolactone significantly reduced office morning blood pressure by 10 (95% CI: 5 to 16) mmHg systolic and 5 (1 to 9) mmHg diastolic and the 24-hour blood pressure was reduced by 6 (2 to 10) mmHg systolic and 4 (2 to 6) mmHg diastolic (Figure 8). Interestingly, the blood pressure reduction was not sustained during the night so administration of spironolactone twice daily may lead to a more persistent reduction of arterial blood pressure. We observed very similar reductions in albuminuria and arterial blood pressure in a study with identical design in patients with type 1 diabetes and nephropathy (193).

In both of our studies (7, 193) the reduction in albuminuria upon spironolactone treatment was found to be independent of changes in arterial blood pressure as there was no correlation between reductions in arterial blood pressure and albuminuria.

The aldosterone escape phenomenon could not be established as patients were on long-term (at least one year) RAAS blockade before entry to the trial and aldosterone concentration was not determined before initiation of RAAS blocking treatment. However, albuminuria was reduced in 17 out of 20 patients who completed the study and therefore our study suggests that the antiproteinuric effect is not restricted to the smaller fraction of approximately 40% of patients with aldosterone escape (173, 174).

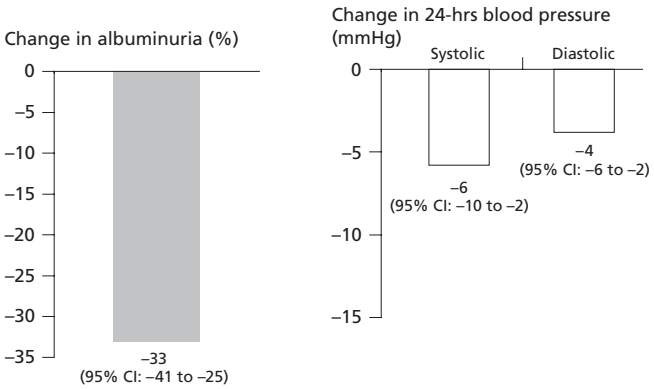

Figure 8. Randomized double-blind cross over study of 20 type 2 diabetic patients with nephropathy. Effects of adding spironolactone 25 mg to maximally recommended doses of ACE-I and/or ARB.

In relation to the studies dealing with dual RAAS blockade it is of particular interest that a subset of five of the patients in the study with spironolactone received concomitant treatment with an ACE-I and an ARB both given at maximally recommended doses. Among these patients spironolactone reduced albuminuria by 22 (22 (–4 to 42) % (p = 0.09), 24-hour systolic blood pressure by 8 (0.5 to 16) mmHg and 24-diastolic blood pressure by 5 (–1 to 10) mmHg ((p = 0.09). Although the number of patients was small in this subanalysis, the findings point towards a potential benefit of triple RAAS blockade as a new treatment strategy to effectively reduce the deleterious actions of both angiotensin II and aldosterone in diabetic nephropathy.

In light of the previously mentioned studies dealing with nephrotic range albuminuria it should be noted that a total of 8 patients in the study had nephrotic range albuminuria (4656 (range: 3162 to 7762) mg/24). Among those, albuminuria was reduced by 35% (15 to 50) during the two months of spironolactone. This clearly demonstrates that aldosterone blockade is effective even in advanced stages of diabetic nephropathy and may help to increase remission rates of NRA.

6.5.3 Side-effects

A primary concern with aldosterone blockade in patients with chronic renal disease is the risk of hyperkalemia in particularly when spironolactone is added to the treatment with other RAAS blocking agents blocking and when renal function is severely reduced. Addition of spironolactone was generally well tolerated in our short-term study of type 2 diabetic patients with nephropathy who generally had well-preserved kidney function. On average plasma potassium was increased by 0.3 (0.04 to 0.5) mmol/l during spironolactone treatment, which is similar to what is seen upon ACE-I and ARB treatment (4, 8, 194, 195). However, in our study one patient was excluded due to severe hyperkalemia during spironolactone treatment requiring admission to hospital. The patient was discharged from hospital the following day without complications. The patient had a moderately reduced GFR of 41 ml/min/1.73 m^2 and received a rather low dose of long-acting furosemide of 30 mg o.d. It is likely that the incident could have been prevented by increasing the relatively low dose of the loop diuretics. Nevertheless, it emphasizes that potassium should be monitored regularly during aldosterone treatment in particular when renal function is reduced.

A previous dose-response study of 214 patients with symptomatic heart failure, evaluated the effect of spironolactone 12.5, 25, 50, and 75 mg daily relative to placebo (175). In that study, the risk of hyperkalemia was approximately 5% at 25 mg of spironolactone and increased by roughly 5% for each 25 mg increase in the dose of spironolactone. The authors concluded that for safety reasons the initial dose of spironolactone should not exceed 25 mg daily.

Anti-androgen side-effects can be avoided by using the newer selective aldosterone receptor antagonist eplerenone, which has shown promising short-term effects in type 2 diabetic patients with microalbuminuria (196).

In relation to possible side-effects upon aldosterone blockade by spironolactone or eplerenone, it should be noted that these agents do not inhibit the non-genomic actions of aldosterone. In the microcirculation of isolated and micro-perfused rabbit glomeruli, the non-genomic actions of aldosterone have been demonstrated to include a dose-dependent elevated glomerular capillary pressure (197). Although this has not been confirmed in another similar study (198) such unopposed non-genomic actions of aldosterone are likely to reduce the therapeutic benefits of spironolactone and

may potentially cause unrecognized side-effects. However, so far the short-term clinical studies of patients with nephropathy have demonstrated clear net benefits of spironolactone treatment in terms of reducing albuminuria and blood pressure being the most important risk factors of progression of diabetic nephropathy (7, 173, 189-191, 193). Furthermore, the improved survival of patients with heart disease during spironolactone and eplerenone treatment suggests an overall benefit of reducing genomic actions of aldosterone even at the expense of an eventual increased non-genomic activity of aldosterone.

With respect to potential non-genomic side-effects during spironolactone treatment it is of interest that we found the compensatory increase in aldosterone during spironolactone treatment to be less pronounced in patients receiving dual RAAS blockade with both ACE-I and ARB as compared to patients receiving only single RAAS blockade (7).

7. NEW STRATEGIES FOR MONITORING DIABETIC NEPHROPATHY

Progression of diabetic nephropathy is dictated by several well-known risk factors such as urinary albumin excretion, arterial blood pressure and hyperglycemia as discussed previously. These factors can be used to establish the individual risk of progressing towards ESRD and changes in these factors can be used to monitor treatment efficacy. However, only a minor part (~25 to 50%) of the total variation in the rate of renal function loss can usually be explained by these risk factors and even when adding the most powerful known genetic risk factors, the major part of the total variation in the rate of renal function loss is left unexplained (199).

Assessment of renal structural impairment through renal biopsies may provide some additional prognostic information. Renal biopsies are not carried out on a routine basis, though, due to the invasive nature of the procedure and its associated complications.

The continuous search for new risk indicators has resulted in a series of markers indicative of pathophysiologic mechanisms for diabetic renal injury. They include markers of inflammation, endothelial dysfunction, oxidative stress, hemostasis/thrombosis, cellular adhesion molecules and prosclerotic growth factors as reviewed by Stuveling et al (200). Interestingly, many of these factors are associated not only with renal but also with cardiovascular disease and consequently they give important insights into mechanisms linking micro- and macrovascular complications. Still, knowledge of the molecular and pathophysiologic mechanisms that underlie the origin and progression of diabetic nephropathy remains limited, in part because conventional research tools have restricted investigators to focus on a single or relatively few risk markers at a time. However, recent advances in technologies within the fields of genomics and proteomics have resulted in novel methods by which a vast array of respectively genes or proteins can be screened in one process for a potential role in the development and progression of disease.

DNA microarrays use gene chip technology to simultaneously measure, several thousand genes in biological specimens at the level of mRNA. Because the human genome comprises approximately 30,000 to 40,000 genes, microarrays can monitor the entire genome in a single specimen. Thereby microarrays make it possible to investigate differential gene expression at different stages of disease all on a genomic scale (201). However, a shortcoming in relation to studies of gene expression is the fact that cellular function is governed by proteins and not by genes. Post-transcriptional modifications of proteins are important to determine differential functions of the same gene and these modifications may confound attempts to correlate gene activation, protein expression, changes in cellular functions and phenotypic expressions.

These limitations of genomic analysis may to some extend be overcome by proteomic analysis. The term "proteome" describes all the protein expressed by a given tissue or body fluid and proteomic analysis entails the characterization and quantification of these proteins and their post-translational modifications. Thereby proteomics can be used similar to genomics but at the protein level to investigate differential protein profiles in normal versus diseased tissue, in treated versus non-treated tissue and at different stages during the course of a disease.

Several methods have been established to find, monitor, and document pathological changes in the proteome including protein arrays, 2-dimensional gel electrophoresis, and mass spectroscopy as discussed in detail by others (99, 202, 203). The online-combination of capillary electrophoresis (CE) and electrospray mass spectrometry (MS) was recently developed to be a fast (one sample is analyzed in approximately 45 minutes), sensitive and automated measurement of different body fluids including urine. The basic principle of the method is shown in Figure 9 and has been described in detail elsewhere (204-206).

A recent clinical study demonstrated that CE-MS could be used to discriminate healthy individuals from patients suffering from various forms of non-diabetic renal disease based on differences in their urinary polypeptide patterns (206). Furthermore, it has recently been demonstrated that CE-MS analysis of urinary polypeptide patterns may be used to differentiate between patients with minimal change disease, focal segmental glomerulosclerosis and IgA nephritis (205, 207) thereby providing a non-invasive diagnostic tool which may help to avoid renal biopsies.

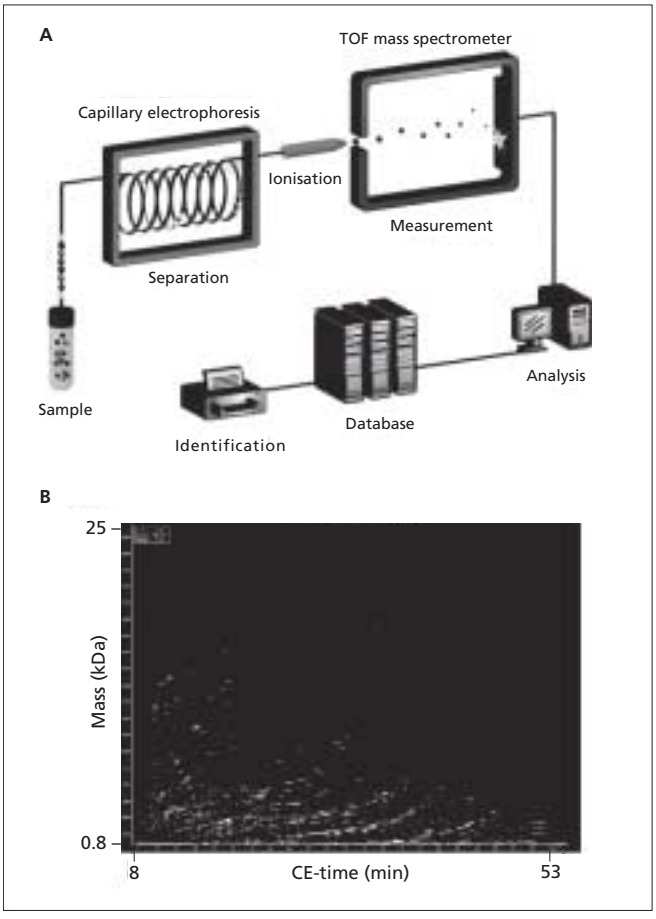

Figure 9. Schematic drawing of the on-line coupling of capillary electrophoresis (CE) to the mass spectrometer used to separate and identify proteins and polypeptides in body fluids by their charge and size (**A**). After electrophoretic separation, the polypeptides are ionized by the application of high voltage and analyzed in the electrospray ionization Time-of-Flight (TOF) mass spectrometer. The combination of the two instruments together with computer analysis of the raw data yields a protein plot with information on mass, capillary electrophoresis migration time (CE time) and signal intensity (color coded, not shown on figure) of each individual polypeptide detected in the each of the samples (**B**).

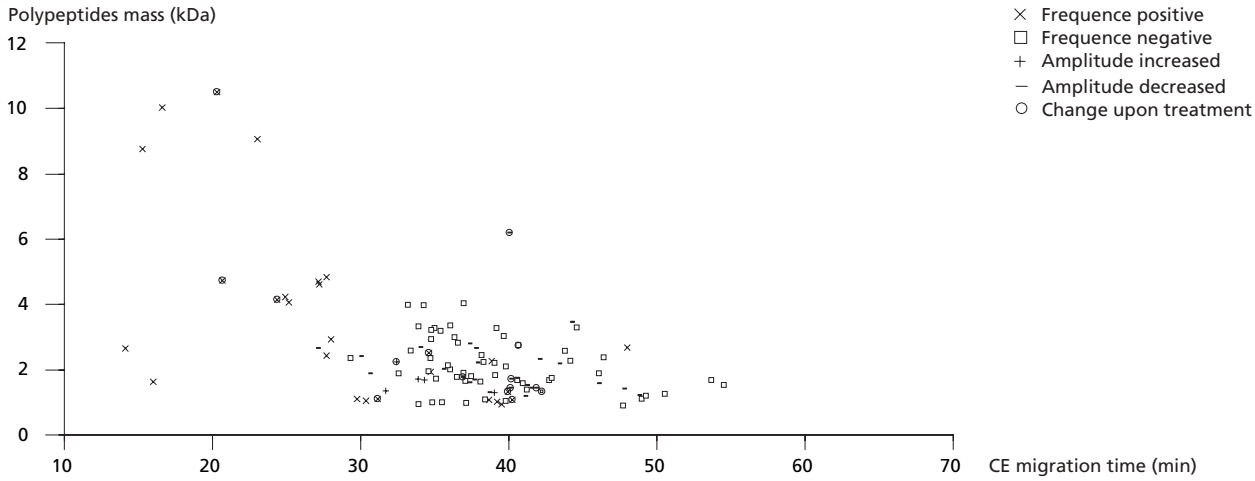

Figure 10. Diabetic renal damage marker polypeptides. A total of 113 polypeptides were found to be significant changed between normo- and macroalbuminuric patients. 55 were reduced (□) and 27 were increased (×) in frequency of occurrence by at least 70%. 31 Polypeptides were signific antly altered in their amplitude, 5 increased in mean amplitude at least two fold (+) and 26 decreased at least two fold (–). 15 of the marker polypeptides were distinctively changed by the candesartan treatment (○).

7.1 IDENTIFICATION OF DIABETIC RENAL DAMAGE USING PROTEOMICS

We performed a cross-sectional study using the combination of using the combination of CE and MS to evaluate urinary polypeptide patterns (UPP) in type 2 diabetic patients with varying degrees of renal disease (6). The study comprised four groups of type 2 diabetic patients matched for age, gender, diabetes duration, HbA1c and arterial blood pressure including; 20 normoalbuminuric patients with and 20 without diabetic retinopathy, 20 microalbuminuric patients with diabetic retinopathy and 18 with diabetic nephropathy who all had macroalbuminuria and presence of diabetic retinopathy (thus fulfilling the clinical criteria of diabetic nephropathy).

Overall CE-MS analysis identified a total of 4551 different polypeptides in the urine of the patients, illustrating the high sensitivity of the method and also the vast amount of potential bioinformation available from urine samples. However, many of these polypeptides occurred in only few of the samples and they appeared to contain little relevant information. As a consequence and to reduce the amount of data and potential signal "noise" due to random variation, only "relevant peptides" (peptides that appear in at more than half of the patients in at least one of the different patient groups) were examined further. This reduced the number of polypeptides to 758, which were used for further evaluation. The frequencies of occurrence of these 758 polypeptides were comparable in the groups of patients with normo- (with and without diabetic retinopathy) and microalbuminuria whereas distinct differences were found in the group of patients with diabetic nephropathy.

By comparing the UPP patterns between patients with normoalbuminuria and diabetic nephropathy we were able to determine a "diabetic renal damage" (DRD) pattern consisting of 113 polypeptides which differed significantly between the two groups either in frequency of occurrence or in amplitude (abundance) which is a measurement of concentration (Figure 10). Eleven of these 113 polypeptides had been sequenced and identified by a matrix assisted laser desorption/ionization (MALDI) target spotter and an offline combination to matrix assisted laser desorption/ionization time-of-flight MS/MS and included fragments of albumin, Tamm-Horsfall protein, esterase and collagen.

7.2 TREATMENT MONITORING WITH PROTEOMICS

As the second step we evaluated if CE-MS would allow detection of changes in the UPP among patients with diabetic nephropathy during treatment with the angiotensin II receptor blocker candesartan cilexetil. Samples from these patients were obtained from the previously mentioned randomized double-blind, cross-over trial evaluating the antiproteinuric effect of two months treatment with candesartan 8, 16 and 32 mg daily vs. placebo. We restricted the analysis of treatment effects to include only those 113 polypeptides constituting the diabetic renal damage pattern and for simplicity the statistical testing of treatment effects was performed by comparison of changes in frequencies and abundance between the placebo period where patients did not receive any antihypertensive treatment and during ARB treatment with candesartan 16 mg o.d. Among the 113 polypeptides in the diabetic renal damage pattern fifteen polypeptides were found to be significantly changed during treatment with candesartan. The changes upon candesartan treatment among these 15 polypeptides represented a combined reduction of disease-specific and an increase of normal-specific signals, and all polypeptides were found to converge towards the polypeptide pattern seen in normoalbuminuric diabetic patients.

The technically constituted limitation of CE-MS to the low and middle molecular weight proteome up to about 20 kDa allows the creation of diagnostic polypeptide maps depleted from high abundant large protein. The occurrence of polypeptides from high molecular weight proteins such as albumin supports the assumption that there is no implied need to display the complete proteome for diagnostic purpose. However, recent studies demonstrate that conventional immunoassays may underestimate albumin concentration, particularly in urine from diabetic patients, because of different, immuno-unreactive albumin isoforms (208, 209). Moreover, recent studies demonstrate the importance of a differential consideration of low molecular weight fragments of proteins, especially albumin. In healthy kidneys of rodents and humans, more than 90% of the filtered albumin is fragmented into small peptides (< 15 kDa) within minutes (210-213). In the diseased kidney this pathway appears to be impaired, leading to reduction of the fragmentation ratio (213). In agreement we found that not all fragments from albumin were increased in patients with diabetic renal damage, in fact, some were even decreased. This is likely best explained by changes (both increase as well as decrease) of the activity of certain proteases leading to impaired fragmentation of large proteins in the kidneys (214). Evidently, a more thorough investigation of these proteases and their physiological regulation is well justified and should give additional insights into the pathophysiology of chronic renal damage.

In addition to fragments of albumin other polypeptides in the diabetic renal damage pattern occurred more frequently or with a higher abundance among normoalbuminuric patients. So far most research has primarily been concerned with substances which are increased during disease. By evaluating substances which are decreased, new protective mechanisms may be identified. Indeed one

of the polypeptides which occurred with increased abundance among normoalbuminuric patients as compared to patients with diabetic nephropathy were fragments of Tamm-Horsfall protein. Previous studies using conventional immunohistochemical methods have in agreement with our findings showed reduced levels of Tamm-Horsfall protein in patients with renal disease (215-217). Tamm-Horsfall protein has been suggested to serve as an immunoregulatory molecule in the kidney involved in the urothelial defense against infections, and to regulate the water permeability in the thick ascending limb of Henle and the distal tubular reabsorption of sodium (218). Of particular interest we even observed that the fragment of Tamm-Horsfall increased in a dose-dependent matter during treatment with candesartan which may thus represent a previously unknown mechanism explaining the renoprotective effects of ARB treatment.

Our study clearly represents an early step towards the implementation of proteomics in the clinical setting and has several limitations. A particular problem is how to best analyze the huge amount of data that derives from sensitive techniques such as proteomics and genomics. Presently there is no consensus regarding optimal statistical testing. The high sensitivity of both genomics and proteomics will obviously lead to identification of false positive markers of disease both due to multiple statistical testing and because of random biologic variation which is particularly high when evaluating urinary polypeptides and proteins. Consequently findings from one study clearly need confirmation in other trials. Longitudinal studies are also needed to establish the diagnostic and prognostic value of proteomics in diabetic renal disease. In particular, long-term studies are warranted to evaluate if it is possible to identify specific urinary polypeptide patterns which at an early stage can identify those patients with normo- or microalbuminuric who are at a high risk of subsequent development of diabetic nephropathy. Likewise long-term studies with principal renal endpoints such as rate of decline in GFR or development of ESRD are needed to establish the prognostic value of CE-MS among patients with diabetic nephropathy.

Despite the current limitations in the use of CE-MS in the clinical setting, our study demonstrates that CE-MS is as a fast and sensitive tool for identification of biomarkers and urinary polypeptide patterns that can be used to discriminate between diabetic patients with and without diabetic nephropathy. The data also indicate that several potential biomarkers in addition to albumin can be defined using CE-MS and, if required, MS/MS sequenced. It is to be hoped for that these additional markers allow a more thorough and accurate characterization of the renal function, leading to a better understanding of the pathophysiology of diabetic renal damage and ultimately to the identification of new targets for intervention.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Type 2 diabetes is one of the fastest growing epidemics World-wide and diabetic nephropathy has become the single most common cause of ESRD in the Western world. Without specific intervention, 20 to 40% of all diabetic patients will develop diabetic nephropathy characterized clinically by hypertension, a progressive increase in albuminuria and a relentless decline in GFR leading towards ESRD. In addition diabetic nephropathy is associated with a greatly increased cardiovascular morbidity and mortality.

During the past decades substantial improvements have been achieved in the prevention and treatment of the diabetic nephropathy primarily through antihypertensive treatment which reduces the risk of ESRD and improves survival. Nevertheless, in spite of aggressive antihypertensive treatment some patients still rapidly progress to ESRD. Therefore it is essential to identify early risk factors for enhanced progression for prompt treatment of high risk individuals and for identifying new targets for intervention.

In a long-term observational follow-up study of a large cohort of type 2 diabetic followed early in the course of nephropathy several modifiable risk factors for enhanced renal function loss were identified. They include; albuminuria, elevated blood pressure, poor glycemic control and smoking. In addition and as a novel finding moderate reductions of hemoglobin even within the normal range were also predictive of an adverse renal outcome. It was also demonstrated that increased albuminuria, elevated blood pressure and poor glycemic control are associated with increased mortality.

In the past, diabetic nephropathy was progressive and irreversible and a particular poor prognosis was described for patients with the most advanced stages of the disease with albuminuria in the nephrotic range. Recent studies have, however, shown that aggressive antihypertensive treatment not only slows progression of renal disease but can even in some cases reverse the course of disease and induce remission of renal structural and functional impairment. Among patients in the previously described cohort of 227 type 2 diabetic patients with nephropathy it was found that nephrotic range albuminuria is still frequent, occurring in approximately 35% of our patients but aggressive lowering of blood pressure in particular with agents that block the RAAS results in sustained remission (albuminuria <600 mg/24-hour for at least one year) in a substantial proportion of the patients (25%). Such remission is associated with a greatly improved renal outcome and survival. These observations are in close agreement with recent studies demonstrating that albuminuria is not only a marker of glomerular lesions, but also a powerful predictor (surrogate endpoint) of the long-term beneficial effect of blood pressure-lowering therapy i.e. the more albuminuria is reduced the better the long-term renal and cardiovascular outcome.

Intrarenal RAAS activity is elevated in diabetic nephropathy and plays an important role in both hemo- and nonhemodynamic pathogenetic mechanisms. Numerous clinical trials have demonstrated specific renoprotective effects of treatment with an ACE-I or an ARB in diabetic nephropathy. In spite of such treatment many patients still progress to ESRD. In part, this can be due to incomplete RAAS blockade with the present use of ACE-I and ARB either because doses are too low or because effective blockade of the system requires combinations of several agents that block the system at different levels.

Currently used doses for ACE-I and ARB are based on dose-response studies of the blood pressure lowering effect in patients with essential hypertension whereas the optimal dosing for renoprotection have previously been unknown. In two dose-response studies with two different ARBs we found a clear dissociation between the optimal dose for blood pressure reduction and for lowering of albuminuria with higher doses needed to maximally reduce albuminuria. Moreover, additional antiproteinuric effects can be obtained without additional side-effects by increasing the dose of the ARB irbesartan to ultrahigh doses (900 mg o.d.) exceeding by far the currently recommended dose (300 mg o.d). Similar data suggesting that the full renoprotective effects are not reached within currently recommended doses are now emerging for other ARBs. Future studies are needed to define the optimal renoprotective doses of ACE-I, which has still not been established.

Two studies demonstrated that dual blockade of the RAAS using both an ACE-I and an ARB is safe and superior to mono RAAS blockade with an ACE-I. Finally, it was shown that blockade of aldosterone by adding spironolactone on top of conventional antihypertensive treatment including maximally recommended doses of an ACE-I and/or an ARB leads to additional reduction of both albuminuria and systemic blood pressure. Spironolactone was generally well tolerated but one patient developed severe hyperkalemia highlighting the need for carefully monitoring of plasma potassium.

Overall, three new strategies for improved renoprotection as assessed by short-term reductions of albuminuria can be proposed from these studies. They are: high dosing of an ARB, combination of an ACE-I and an ARB, and finally aldosterone blockade. Larger studies are needed to establish the long-term safety and efficacy but the continued lowering of albuminuria is a promising indication. A

fourth new strategy for improved renoprotection is the recently developed renin inhibitors which target the RAAS at its first and rate limiting step. The renoprotective effect of renin inhibitors is presently being evaluated in diabetic nephropathy.

Due to recent advances in technology, proteomics is now emerging as a new field in clinical research allowing a fast and sensitive method for detection of a vast array of protein and protein derivatives for discovering new pathophysiologic mechanisms for disease progression and for monitoring of treatment efficacy. The online-combination of capillary electrophoresis and electrospray mass spectrometry was used to establish a "diabetic renal damage" pattern consisting of 113 urinary polypeptides that differed significantly between normoalbuminuric patients and those with diabetic nephropathy. Twelve of these polypeptides had been identified and includes; fragments of albumin, Tamm-Horsfall protein and collagen. Furthermore it was shown that ARB treatment in patients with diabetic nephropathy significantly changed 15 of these towards levels more closely associated with normoalbuminuria. Future studies will have to further establish the prognostic value of proteomics in diabetic nephropathy.

ABBREVIATIONS

ACE-I:	angiotensin converting enzyme-inhibitor
ARB:	angiotensin II AT1 receptor blocker
AT(1-4):	angiotensin II type(1-4) receptor
CE:	capillary electrophoresis
CI:	confidence interval
EPO:	erythropoietin
ESRD:	end-stage renal disease
IDNT-study:	Irbesartan in Diabetic Nephropathy-study
IRMA2-study:	Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria-study
MALDI:	matrix assisted laser desorption/ionization
MS:	mass spectrometry
NRA:	nephrotic range albuminuria
RAAS:	renin-angiotensin-aldosterone system
RENAAL-study:	Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan-study
UAE:	urinary albumin excretion rate
UPP:	urinary polypeptide pattern

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