CHAPTER 1

Introduction

Prologue

Diabetic nephropathy, the renal disease complicating diabetes mellitus in 20 - 40% of cases, is one of the serious long-term sequelae threatening these patients. Diabetic nephropathy has a large impact for the individual patient in terms of associated morbidity and mortality and for health care and society in terms of costs (1). According to the RENINE registry, ± 16% of the patients starting with dialysis in the Netherlands have diabetic nephropathy as underlying kidney disease (2).

The emergence of microalbuminuria, is not only a strong predictor for overt diabetic nephropathy, it is also strongly related with a high cardiovascular morbidity and mortality concerning both patients with diabetes mellitus type 1 and type 2 (3-11). Diabetic nephropathy is incipient, when a persistent microalbuminuria (albumin excretion rate (AER) 20 - 200 µg/min or 30 - 300 mg/24 hours) is documented in a diabetic patient without other urinary abnormalities or evidence of urinary tract infection or heart failure (12,13). The term overt diabetic nephropathy is used for the syndrome of persistent macroalbuminuria (AER > 200 µg/min or > 300 mg/24 hours), high blood pressure, a progressive decline in glomerular filtration rate, and diabetic retinopathy (12).

The last two decades considerable progress has been made with respect to important aspects of diabetic nephropathy: natural history, classification of stages of clinically diagnosed nephropathy based on the albumin excretion rate, pathology studies using morphometric methods, genetic susceptibility for the development of diabetic nephropathy, and new treatment strategies have all been the subject of studies contributing to the progress of the current understanding of diabetes mellitus and diabetic nephropathy (13-21).

Traditionally, most studies on diabetic nephropathy have dealt with type 1 diabetes mellitus. However, the changing epidemiological figures in Europe and the USA with a large increase in patients with type 2 diabetes mellitus and a subsequent rise of numbers of patients with diabetic nephropathy will encourage more investigators to study these patients and their specific disease problems (9,11,22). There is a large heterogeneity in diabetes mellitus type 2 and consequently, the interpretation of data derived from studies in patients with type 2 diabetes mellitus and diabetic nephropathy are often difficult to interpret.

The Steno hypothesis, first formulated by Deckert *et al.*, proposes that a genetic defect in the regulation of the production of heparan sulfate by endothelial and glomerular cells determines the susceptibility and hence the development of proteinuria and angiopathy with its associated cardiovascular mortality risk (16,23). This hypothesis, which has been described originally for diabetes mellitus type 1, postulates that a single mechanism may explain the complexity of the pathogenesis of diabetic nephropathy. The attraction of such a hypothesis comes from its generation of ideas for further research and study to

understand and treat diabetic nephropathy aiming specifically at putative changes in heparan sulfate metabolism. In this thesis we will approach the problem of diabetic nephropathy by using this hypothesis as a starting point.

Classification of the stages of diabetic nephropathy by the degree of albuminuria

As of the 1980's, many studies have contributed to the concept of a clinical classification system of diabetic nephropathy based on the degree of albuminuria (3,4,13,14,24). This criterion can only be applied in the absence of urinary abnormalities, urinary tract infection, or heart failure. Furthermore diabetic retinopathy should be present, preferably proliferative (12). Based on the knowledge of the predictive power of microalbuminuria for diabetic nephropathy and the development of diabetic nephropathy in time with the pathophysiological changes of glomerular hyperfiltration and hypertrophy, the following stages have been proposed by Mogensen and are now generally accepted for both research and clinical purposes (13).

Stage 1. In the first stage glomerular hypertension and hypertrophy are often already present at the time of diagnosis of diabetes and are in general associated with a poor metabolic control. No urinary abnormalities can be detected.

Stage 2. The silent stage with normoalbuminuria, but with structural glomerular changes. Microalbuminuria might be observed during stress or concurrent disease.

Stage 3. Incipient diabetic nephropathy is defined by microalbuminuria (AER 20 - 200 μg/min or 30 - 300 mg/24 hours). These patients have clear structural changes and have a high risk of progression to overt diabetic nephropathy. However, interventions may result in clinical reversibility of the albuminuria and a stabilization of the symptoms.

Stage 4. Overt diabetic nephropathy is defined by proteinuria (AER > 200 μg/min or > 300 mg/24 hours, hypertension, retinopathy, and a relentless decline of the glomerular filtration rate.

Stage 5. Finally diabetic nephropathy will progress to end-stage renal failure.

These clinically diagnosed stages of diabetic nephropathy have at first been developed for the diabetic nephropathy complicating type 1 diabetes mellitus (12). Because type 2 diabetes mellitus is associated with a very heterogeneous renal histology, there has been some reluctance to use the similar classification system for type 2 diabetes (25,26). However, a recent review on the aspects of type 2 diabetic nephropathy stated that proteinuria in combination with diabetic retinopathy would suffice in general to clinically diagnose diabetic nephropathy (11).

The basement membrane and heparan sulfate proteoglycan

Basement membranes

Basement membranes are sheets of extracellular matrix located between cell and the interstitial space, but in the glomerulus and the retina this membrane is sandwiched between endothelial and epithelial cells. Diabetic microangiopathy is characterized ultrastructurally by thickening of the basement membrane. Basement membranes perform three major functions. First, they provide physical support, second they participate in cell attachment and growth and third, basement membranes participate in the ultrafiltration of plasma (27,28). The basement membrane is composed of collagenous, non-collagenous, and carbohydrate moieties. The non-collagenous components of the basement membrane comprise laminin, nidogen, and heparan sulfate proteoglycans.

Collagen

Type IV collagen is the major collagenous component of the glomerular basement membrane and the mesangial matrix and it consists of a triple helix of ∀(IV) chains with a non-collagenous (NC) globular domain at its carboxyl terminus. At least five distinct types of IV collagens chains have been identified (29). Recently other collagen types as type VI and type XVIII have been identified as components of basement membranes (27,30). Collagen XVIII is, next to perlecan and agrin, the third basal lamina heparan sulfate proteoglycan and the first collagen/proteoglycan with heparan sulfate side chains. Collagen XVIII has a molecular mass of 300 kDa. Collagen XVIII has typical features of a collagen and it also has characteristics of a heparan sulfate proteoglycan, such as long heparitinase-sensitive carbohydrate chains and a highly negative net charge (30).

Heparan sulfate proteoglycan

Heparan sulfate proteoglycan consists of a core protein with heparan sulfate glycosaminoglycan side chains (31-33). Heparan sulfate may be attached to the different core proteins in the basement membrane extracellular matrix. The core protein can either

be perlecan, agrin, or collagen XVIII (30,32,34-37). Perlecan is an ubiquitous heparan sulfate proteoglycan with angiogenic and growth-promoting properties. The name comes from its appearance which suggests a string of pearls. There are three glycosaminoglycan side chains located at one end of the molecule. It is a very complex molecule with five domains which harbor protein modules for different functions such as lipid uptake, cell adhesion and growth regulation (32).

Collagen XVIII has only very recently been reported to be present in basement membranes and to carry heparan sulfate (30). No extensive studies exists yet on the importance of collagen XVIII with respect to structure and function of the basement membranes.

Agrin is a protein with a molecular mass of 220 kDa that induces aggregation of acetylcholine receptors at the neuromuscular junction. It is thought that the expression of agrin is important for the development of axonal tracts and it regulates cell-cell interactions. Both lung and kidney tissue have high transcription levels of agrin mRNA (34). Agrin is a heparan sulfate proteoglycan (38) and the core protein of agrin is detected by the monoclonal antibody JM 72 (39). Agrin is expressed in the glomerular basement membrane where it is expressed in full length whereas in other renal basement membranes truncated isoforms are present (37). There is now evidence that agrin may be the major proteoglycan of the human glomerular basement membrane (36).

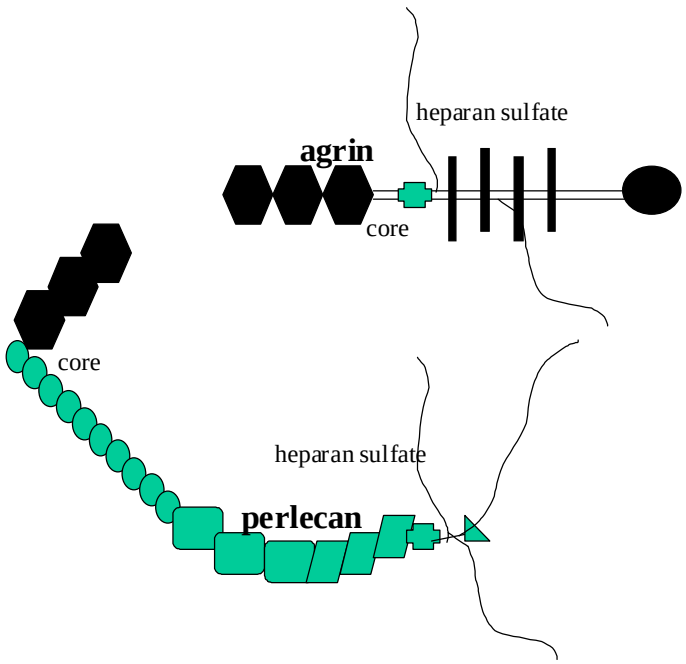

Figure 1. *A schematic view of the heparan sulfate proteoglycans agrin and perlecan. Note the localisation of heparan sulfate side chains in the middle of the agrin core and terminally at the perlecan core.*

Heparan sulfate

The glycosaminoglycan side chains are formed by repeating disaccharide units, which consist in the case of heparan sulfate of a hexuronate and a hexosamine moiety. Sulfate groups covalently linked to the repeating disaccharides of heparan sulfate and carboxyl groups give the glycosaminoglycan chains their high negative charge (31,33). Heparan sulfate and heparin may vary with respect to chain size, sulfation, and location of sulfate groups.

The biochemical processing has been described in detail elsewhere (32,33,40). Figures 1 and 2 show the schematic structure of proteoglycans (agrin and perlecan), and the biochemical processes involved in the sulfation of polysaccharide biosynthesis. Briefly, the heparan sulfate chains are modified post-transcriptionally by N-substitution of the glucosamine units, facilitated by the key enzyme glucosaminyl N-deacetylase/N-sulfotransferase, which has been demonstrated to be one enzyme (41). Several genes encoding for the N-deacetylase/N-sulfotransferase protein have now been identified (42,43) (44). The N-deacetylase/N-sulfotransferase protein is located in the Golgi network (45). No genetic polymorphisms for the genes of the N-deacetylase/N-sulfotransferase protein have been reported yet. A reduction of the activity of this enzyme in hepatocytes of diabetic rats has been found (46).

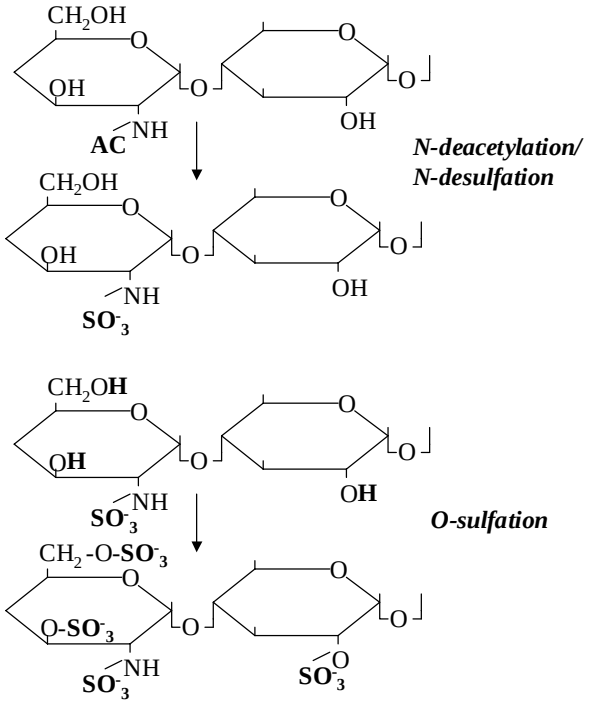

Figure 2. *Steps involved in the biosynthesis of heparan sulfate. Effects of glucosaminyl N-deacetylase/N-sulfotransferase and O-sulfotransferase are shown for glucuronic acid and N-acetylglucosamine.*

Heparan sulfate is thought to be important for the structure and function of the glomerular basement membrane; it prevents clogging and determines the sieving function (47-50). Heparan sulfate is mainly responsible for the negative charge barrier in the glomerular basement membrane, which prevents albumin passing through the capillary wall (50,51). It should be mentioned that there is still some debate on the role of heparan sulfate associated anionic sites with respect to the negative charge of the glomerular basement membrane (52). The key role that heparan sulfate has in the normal function of the glomerular basement membrane is illustrated by the *in vivo* finding that removal of heparan sulfate by heparinitase (50) or shielding of heparan sulfate by a monoclonal antibody (53) generates albuminuria.

Heparan sulfate exhibits also important actions in regulating growth and proliferation of mesangial cells (54-57). Because mesangial expansion is strongly correlated with decline of renal function in diabetic nephropathy this feature is of great importance (15,58,59).

The significance of microalbuminuria and macroalbuminuria with respect to the development of nephropathy and cardiovascular disease

The concept of microalbuminuria was first introduced by diabetologists long before it was applied for non-diabetic renal and vascular disease (13,60). Microalbuminuria is defined by the urinary loss of albumin (albumin excretion rate or AER) of 20 - 200 µg/min or 30 - 300 mg/24 hours and it has been shown clearly to be of great use for the study of diabetic nephropathy.

The mean albumin excretion rate in the normal population is around 5 µg/min, with a considerable range, but excretion only rarely exceeds 15 µg/min. In population studies among middle-aged and elderly individuals, higher values can be seen. Therefore an AER of 20 µg/min or 30 mg/24 hours is used as upper limit of normal.

Microalbuminuria is diagnosed when the albumin excretion rate exceeds the normal reference range, but the AER would still be undetectable for the classical dipstick method testing for urinary albumin or protein. The threshold for detection of albumin by the classical dipstick is around an AER of 200 µg/min or 300 mg of albumin per 24 hours urinary collection.

The EURODIAB IDDM Complications Study has reported that the prevalence of microalbuminuria was 31% in European patients with type 1 diabetes mellitus and that the prevalence of macroalbuminuria was 9% in the same cohort (61). A prevalence of 19% was found among patients having diabetes type 1 for 1 - 5 years in that large survey study demonstrating that microalbuminuria might be observed very early in the disease. In newly diagnosed type 2 diabetes patients, about 40% of them may show an excretion rate

above 15 - 20 µg/min. In the course of type 2 diabetes about 20 - 30% of all patients with diabetes type 2 will have developed microalbuminuria (8,62). Several arteriosclerosis related risk factors are seen more frequently in patients with microalbuminuria, such as hyperlipidemia, high blood pressure as well as other markers of cardiovascular disease (8).

The findings of microalbuminuria imply a seriously increased risk for the development of diabetic nephropathy and also an increased risk for the premature development of cardiovascular disease with subsequent high morbidity and mortality rates (3,4,24,63-65).

Although the predictive strength of microalbuminuria with regard to the development of diabetic nephropathy as suggested by the results from earlier studies has been questioned and has been put in perspective by the study of Almdal *et al.*, they showed nevertheless clearly that 19% of patients with microalbuminuria progressed to overt nephropathy (65). Similar results were obtained by Forsblom *et al.*, who found that 28% of the microalbuminuric patients developed macroalbuminuria during 10 year follow-up with 8% of the normoalbuminuric patients becoming macroalbuminuric (66).

Messent *et al.* showed on the other hand that the original findings demonstrating the value of microalbuminuria as a strong predictor for diabetic nephropathy are still valid. They found that compared with normoalbuminuric patients microalbuminuric patients had a relative risk of 9.3 developing clinical proteinuria and that these patients had a relative risk of 2.94 of dying from a cardiovascular cause (67).

Even small increases of (micro)albuminuria in type 1 diabetic patients have been shown to be significantly predictive for the development of atherosclerotic vascular disease and when clinical nephropathy with proteinuria is diagnosed, a more than ten-fold increase in mortality of cardiovascular diseases compared with diabetic patients without nephropathy can be found (5,6,68-73).

Figure 3 demonstrates the dismal prognosis of Dutch patients with type 1 diabetes mellitus who started with renal replacement therapy between 1985 and 1996.

With regard to the link between microalbuminuria and total and cardiovascular mortality in type 2 diabetes mellitus a large review has appeared recently on 11 cohort studies, representing a total of 2138 patients with a mean follow-up of 6.4 years. The reported prevalence of microalbuminuria ranged from 20% to 36% among these 2138 patients. A significant association between microalbuminuria and total mortality or cardiovascular disease (morbidity or mortality) resulting in an overall odds ratio for death of 2.4 and for cardiovascular disease of 2.0 was reported by all cited cohort studies (74).

Microalbuminuria has also been demonstrated to be an independent risk factor for cardiovascular disease in non-diabetic patients (60,75,76).

Diabetes type 1 patient survival on dialysis in the Netherlands 1985 - 1996

Figure 3. *Survival curve of type 1 diabetes patients on renal replacement therapy (dialysis), censored for transplantation.*

Diabetic retinopathy, a longterm microvascular complication with capillary leakage and its relationship with diabetic nephropathy

The large majority (> 97%) of patients with diabetes mellitus type 1 will eventually develop diabetic retinopathy (77,78). A population-based study from Minnesota found a cumulative incidence of retinopathy of 70% in type 1 and 36% in type 2 diabetes patients (79) and a large cross-sectional study, the EURODIAB IDDM complications study, showed an overall prevalence rate of retinopathy in 3250 European type 1 diabetic patients of 46% and a prevalence of 82% in those patients having more than 20 years of diabetes duration (61). Eventually 67% of the type 1 diabetic patients will have developed proliferative retinopathy after 30 years duration of diabetes (78). Patients with type 2 diabetes mellitus show a somewhat lower prevalence of diabetic retinopathy: 78% of these patients develop some degree of retinopathy and 15.5% of them will have proliferative retinopathy in the end (80). Interestingly, in a retrospective study similar incidences (45%) of retinopathy were found in diabetes patients with idiopathic or

pancreatic origin of their diabetes (81). A French epidemiological study showed that for type 2 diabetes patients the finding of nephropathy or neuropathy warrants an ophthalmologic examination, since 87% of the patients with either one of these complications had advanced diabetic retinopathy (82).

The very high cumulative incidence of diabetic retinopathy contrasts strikingly with the finding that not all type 1 diabetic patients (maximum estimates of 30 - 40%) develop diabetic nephropathy. Chavers *et al.* explored the relationship between retinal structural lesions and quantitative measures of glomerular structure in patients with type 1 diabetes mellitus (83). The retinopathy score correlated well with several structural glomerular parameters such as the glomerular basement membrane width, the mesangial volume fraction, the surface density of the peripheral capillary wall, and the degree of arteriolar hyalinosis. However, 27% of the patients with a normal or near-normal degree of albuminuria had advanced retinopathy but normal renal structural measures. The typical features of background diabetic retinopathy are early thickening of the basement membrane with loss of pericytes, the development of micro-aneurysms, and an increased permeability. The onset of proliferative retinopathy, the more severe form of retinal disease with neovascularization, is often preceded by background retinopathy. It is believed that biochemical alterations related to hyperglycemia such as the formation of advanced glycosylation end products, hemodynamic mechanisms, and endocrine factors contribute to the development of retinopathy (77).

Diabetic retinopathy can cause serious retinal damage and finally lead to blindness. Non-proliferative diabetic retinopathy may cause visual loss when associated with macular edema or macular ischemia (secondary to retinal capillary non-perfusion). Proliferative diabetic retinopathy may cause severe visual loss if complicated by vitreous hemorrhage or traction detachment of the macula. Epidemiological data show that in the United States 5000 patients and worldwide 30000 to 40000 become blind each year from diabetic retinopathy (77).

Regular retinal inspections and appropriate intervention with laser or vitrectomy help to preserve vision in patients with established macular edema or proliferative diabetic retinopathy. For diabetic maculopathy the standard treatment is focal laser treatment (84). However, areas of maculopathy close to the fovea are not amenable to laser treatment. Therefore, local laser treatment is not always able to improve visual acuity or to prevent further loss of vision. The natural history of hard exudates is one of progression in time, spontaneous improvement or stabilization occurs rarely (84-86).

Tighter control of blood glucose levels and lower blood pressure reduce the risk of progression of diabetic retinopathy (17,87). Interestingly, studies of primary prevention in type 1 diabetic patients predominantly with normoalbuminuria have demonstrated the putative role of the angiotensin conversing enzyme system by the fact that ACE inhibition

reduces not only significantly the rate of progression of the albumin excretion rate, but it had also a beneficial effect on the progression of retinopathy (88,89).
The retinal capillary basement membrane and the glomerular basement membrane contain heparan sulfate proteoglycan, which is important for the charge-selective barrier function (50,90). It has been demonstrated in diabetic retinopathy that the synthesis of heparan sulfate proteoglycan is decreased similarly as in diabetic nephropathy (91). After having induced diabetes in male Wistar rats by intraperitoneal injection of streptozotocin, these authors assessed, after isolation of the retinas, the heparan sulfate proteoglycan synthesis by incorporation of ^{35}S-sulfate into heparan sulfate and they quantified the expression of mRNA for perlecan. Both the synthesis of heparan sulfate and mRNA expression for perlecan were decreased in the retinas of diabetic rats compared to normal rats. The decrease in heparan sulfate synthesis may account for the decrease in retinal basement membrane anionic sites and the associated increased capillary permeability and leakiness observed in diabetic retinopathy (91). Interestingly, one study has reported that therapy with heparan sulfate (suleparoid) resulted in an effective inhibition of angiogenesis in a corneal neovascularization model (92). No formal clinical studies in humans have been performed yet to evaluate whether treatment with glycosaminoglycans such as heparan sulfate or heparin exerts any effects on diabetic retinopathy.

Epidemiology of diabetic nephropathy in diabetes mellitus type 1

Prevalence of diabetic nephropathy
Two large surveys have reported their findings from southern Europe. One study from Catalunya, Spain evaluated 936 diabetic patients starting renal replacement therapy during the period 1984-1994, 25% of these patients were classified as having diabetic nephropathy and type 1 diabetes mellitus, 66% as having diabetic nephropathy and type 2 diabetes mellitus (93). In France the prevalence of diabetes mellitus was only 1.4% for type 1 diabetes mellitus and 5.5% for type 2 diabetes mellitus among 12903 patients treated in 1989 for end-stage renal failure (94).

Cumulative incidence of diabetic nephropathy
One of the landmark studies on diabetic nephropathy came from Andersen *et al.* from the Steno Memorial Hospital (95). In a large cohort of 1475 type 1 diabetic patients 41% developed clinical diabetic nephropathy, while other causes of proteinuria were found in 3%, and 57% of the patients did not develop persistent proteinuria. Two incidence peaks of the onset of proteinuria could be discerned: one after 16 and another after 32 years duration of diabetes. After 35 years duration of diabetes mellitus only very few patients

developed diabetic nephropathy. The cumulative incidence was 45% after 40 years of diabetes. Patients with nephropathy had a much poorer survival than those without proteinuria; 40 years after onset of diabetes, only 10% of patients who developed nephropathy were alive, whereas the large majority (> 70%) of patients without nephropathy survived (95). Similar results have been reported from a cohort of American diabetic patients showing a 35% cumulative incidence (96). Two population-based cohorts consisting of 1374 patients with type 1 diabetes mellitus from Japan and 995 patients with diabetes mellitus type 1 from Allegheny County, Pennsylvania, USA, showed a cumulative incidence after 20 years of end-stage renal failure of 13% and 19% respectively (97). Unfortunately, this study did not give any results of the cumulative incidence after a diabetes duration longer than 20 years.

The definition of diabetic nephropathy as endpoint differs with respect to the cited studies, some use end-stage renal disease while persistent proteinuria is used in other studies. Furthermore, it should be noted that these figures on the incidence of nephropathy in both European and American patients are at variance with the incidence of other late complications of diabetes like neuropathy and retinopathy, which continue to occur even late after the onset of diabetes.

The effects of intensive glycemic control

Recently, a decrease of the incidence of diabetic nephropathy possibly due to a better glycemic control has been reported by various authors. The cumulative incidence of end-stage renal disease was 21% in a cohort of 142 white American patients with type 1 diabetes mellitus. The first case of end-stage renal disease occurred after 13 years of diabetes and a total of 25 cases had developed by 35 years of duration. The median survival after the diagnosis of end-stage renal disease for the 16 patients was only 3.5 years. The level of glycemic control during the first two decades of type 1 diabetes was a strong predictor of the development of end-stage renal disease: end-stage renal disease developed in 36% of patients in the worst tertile for glycemic control, but only in 14% and 9% of those in the middle and best tertiles (98). A decrease of the cumulative incidence of diabetic nephropathy was also found by Bojestig *et al.* (99). The incidences of diabetic nephropathy (defined as persistent proteinuria) after 25 years of diabetes dropped from 30% to 9% (99) and they showed furthermore that 19% of a cohort of initially microalbuminuric patients eventually developed macroalbuminuria and that the majority of patients had normalized AER values after improving their metabolic control (100). Finally, a study on 356 Caucasian patients should be mentioned, because this prospective study did not show any decrease of the cumulative incidence figures with respect to diabetic nephropathy. However, this study did confirm that the mean HbA_{1c} was significantly higher in patients with nephropathy (9.4%) and persistent

microalbuminuria (8.9%) than in patients with normoalbuminuria (8.5%). The prevalence of persistent microalbuminuria at time of follow-up was 24% in the group with onset of diabetes in 1965-1969, 28% with onset of diabetes in 1970-1974, and 19% with onset of diabetes in 1975-1979. The cumulative incidence of diabetic nephropathy (defined by AER > 300 mg/24 h) after 15 years of diabetes was ± 18% for the three cohorts. After 25 years of diabetes duration a cumulative incidence of diabetic nephropathy was reached of 35% which was not different for the three cohorts neither (101).

Histology of diabetic nephropathy in diabetes mellitus type 1

The histologic alterations observed in diabetic nephropathy include expansion of the mesangium, especially the mesangial matrix, and thickening of the glomerular basement membrane. These changes may regress to normal by a very strict glycemic control with pancreas transplantation (102).

Typical glomerular and periglomerular lesions are the diffuse and nodular lesions, the fibrinoid cap and the capsular drop. The round acellular bodies consisting of PAS positive material are called Kimmelstiel-Wilson nodules. Olsen has recently reviewed the history of knowledge on the lightmicroscopy findings of diabetic nephropathy, which started with the observations by Kimmelstiel and Wilson (103). Non-specific for diabetic nephropathy, but often observed is the arterial hyalinosis (12,103).

The severity of lesions may be estimated as the volume fraction of the solid substance by morphometry or described semiquantatively by use of a scoring system. Electron microscopy provides even more detailed information of the structures involved. The mesangial regions occupy a large proportion of the tuft and the basement membrane is thickened (12,15). A positive correlation has been obtained between AER and several of the glomerulopathy parameters such as basement membrane thickness, the mean volume fraction of mesangium per glomerulus and mean volume fraction of matrix per glomerulus. These results indicate a parallel course of mesangial and peripheral basement membrane changes (104).

The deterioration of glomerular function will be followed eventually by tubular degeneration. Thickening of the tubular basement membrane, a non-vascular basement membrane is a well known feature (12,105). There is still debate whether the interstitial abnormalities are more important than the glomerular changes with regard to the correlation with renal function and progression to end-stage renal failure (12).

There is a broad range of extracellular matrix components in the developing lesions of diabetic nephropathy (106). In diabetic nephropathy both in type 1 and type 2 diabetes there is a separate and distinct alteration in the glomerular basement membrane and the

mesangium of a differential expression of collagen type IV components (29). An increase of the collagens type IV and type V, laminin and fibronectin in the enlarged mesangial matrix may be observed in diabetic nephropathy and diabetic nodular lesions can show accumulations of collagen type III (107-109).
In patients with type 1 diabetes mellitus and diabetic nephropathy, staining for heparan sulfate in the glomerular basement membrane is reduced proportionally to the amount of proteinuria, but staining for the agrin core protein is not altered (107,110).

Epidemiology of diabetic nephropathy in diabetes mellitus type 2

Prevalence
Figures for patients with type 2 diabetes mellitus vary greatly for the different countries and registries (22,111).
In France 5.5% of the 12903 patients in 1989 on renal replacement therapy had type 2 diabetes mellitus. In this study 37% of the patients with type 2 diabetes were supposed to have diabetic nephropathy. In contrast, in the French territories like the Caribean Islands and other overseas territories nearly 20% of patients on dialysis had type 2 diabetes and diabetic nephropathy (112).
A study from the lower Neckar (Germany) region on all patients admitted for renal replacement therapy in 1993 - 1994: 85 out of 225 (38%) patients had diabetes type 2 (113).
Data from the Registry of the European Dialysis and Transplant Association showed that at 31 December 1990 a total of 15197 diabetic patients were receiving renal replacement treatment with ± 35% type 2 diabetic patients (114).
Data from from a population study (Wadena, Minneota, USA) on 455 adults (Americans from Northern European origin)showed that 8% had an AER > 15 µg/min, and 1.7% had overt proteinuria. The mean AER in a stratified random sample of 374 adults was 3.6 µg/min and mean AER values for 277 subjects with normal glucose tolerance and for 80 subjects with impaired glucose tolerance were very similar (3.8 and 3.7 µg/min, respectively), whereas mean AER was 5.4 and 9.4 µg/min for patients with type 2 diabetes respectively without and with insulin (115).
A prospective study showed a prevalence of 14% of an AER > 300 mg/24 hours in 370 type 2 diabetic patients. A kidney biopsy was performed in 70% of these cases and revealed diffuse and nodular diabetic glomerulosclerosis in 77%, while the remaining 23% had non-diabetic renal disease. Diabetic retinopathy was present in 56% of patients with diabetic glomerulosclerosis, while none of the eight patients with a non-diabetic glomerulopathy had retinopathy. This study showed thus a rather high prevalence of

albuminuria complicating type 2 diabetes mellitus. Furthermore, this study showed also that diabetic retinopathy strongly suggests that a diabetic glomerulopathy is the cause of albuminuria and that albuminuric diabetic patients without retinopathy require a kidney biopsy (116).

Incidence

A German study showed a cumulative risk of proteinuria after 25 years of diabetes mellitus type 2 of 57% (117). Twenty-six albuminuric type 2 diabetic patients with biopsy-proven diabetic glomerulosclerosis were followed-up prospectively for a mean of 5.2 years. During the observation period the glomerular filtration rate decreased from 83 to 58 ml/min and the albuminuria doubled. In this prospective observational study ± 20% of the patients developed end-stage renal failure (118).

Gall *et al.* reported on incipient and overt diabetic nephropathy in 191 patients with type 2 diabetes mellitus in a prospective, observational study of a cohort of white patients followed for a median period of 5.8 years. Persistent microalbuminuria developed in 20% and macroalbuminuria in 2.8%. The five year cumulative incidence of incipient diabetic nephropathy was 23% among the 191 patients (119)

Histology of diabetic nephropathy in diabetes mellitus type 2

The histology of diabetic nephropathy associated with type 2 diabetes mellitus is rather heterogeneous. Renal biopsies from fourteen type 2 diabetic patients with microalbuminuria and control biopsies showed a significantly increased glomerular volume in diabetic as compared to control glomeruli and the scores for mesangial sclerosis and arteriolar hyalinosis were higher in diabetic patients than in control subjects. No significant differences between diabetic and control subjects were found with respect to globally sclerotic glomeruli or interstitial fibrosis, tubular atrophy and arteriosclerosis. The findings of this study point out that type 2 diabetic patients with microalbuminuria show histological findings consistent with diabetic nephropathy (glomerular hypertrophy, mesangial sclerosis and arteriolar hyalinosis) but changes are mild and appear less marked than in type 1 diabetic patients (120).

Fioretto *et al.* confirmed the heterogeneity of renal lesions in type 2 diabetic patients with microalbuminuria with a study of a cohort of 34 unselected patients. A normal or near normal renal structure was found in ± 30%; changes "typical" of diabetic nephropathy in type 1 diabetes (glomerular, tubulointerstitial and arteriolar changes occurring in parallel) in ± 30% and "atypical" patterns of injury, with absent or only mild diabetic glomerular changes associated with disproportionately severe renal structural changes in ± 40%. No

differences in demographic characteristics could be discerned by this study among the 3 classes of patients. Diabetic retinopathy was present in all patients with typical diabetic nephropathy. No proliferative retinopathy was found in the other two classes and only half of these patients had background retinopathy. Thus, in this study less than 1/3 of the microalbuminuric patients had typical diabetic nephropathy (25).

A postmortem study of 55 type 2 diabetic patients and matched non-diabetic subjects showed that 31% of the diabetic cases had nodular glomerulosclerosis, and another 47% showed suggestive changes while none of the controls showed nodular glomerulosclerosis (121). An autopsy-based German study on a series of 210 consecutive diabetic patients showed glomerulosclerosis in 80% (129 diffuse, 37 nodular) (122).

Gambara *et al.* studied renal biopsies from 52 patients affected by type 2 diabetes with overt, proteinuric nephropathy. Thirty-seven percent had typical changes of diabetic nephropathy (glomerulosclerosis, marked glomerular hypertrophy, and arteriolar hyalinosis). Chronic and aspecific changes were seen in 31%. As compared with those having typical changes, the 31% of patients had less glomerulosclerosis and less arteriolar hyalinosis but more severe ischemic glomerular lesions and arteriosclerosis. Glomerular disease superimposed on diabetic glomerulosclerosis was seen in 33%. No differences in demographic characteristics could be discerned by this study among the 3 classes of patients. The findings from this study indicate that renal lesions in proteinuric patients with type 2 diabetes manifest in a quite heterogeneous fashion, but in this study findings were not correlated to retinopathy (123).

Glomerular and retinal pathology and clinical correlates were studied in 36 patients enrolled in a prospective clinical trial of patients with type 2 diabetes mellitus, proteinuria, renal insufficiency, and hypertension. Seventeen (47%) biopsies had diabetic glomerular sclerosis with Kimmelstiel-Wilson nodules; 15 (42%) biopsies had glomerular changes characteristic of the diabetic state including enlarged glomeruli and an increase in mesangial matrix without Kimmelstiel-Wilson nodules (mesangial sclerosis lesion); and two (6%) had non-diabetic glomerular disease. Six of seven patients with proliferative retinopathy had Kimmelstiel-Wilson nodules, and seven of the eight patients without retinopathy had mesangial sclerosis lesions. This study showed that in 94% of patients with type 2 diabetes mellitus diabetic glomerulosclerosis was the cause of the renal disease (124).

Olsen and Mogensen have reported a study on 33 consecutive biopsies from type 2 diabetes patients with proteinuria. In 4 (12%) patients evidence of non-diabetic lesions was found. In the remaining 29 (88%) patients typical diffuse (n = 9) or nodular (n = 20) diabetic lesions were found. They have put their findings in perspective with results from 9 other studies comprising 580 patients. In these non-biased reports the prevalence of non-diabetic renal disease was very similar (26). These findings were confirmed by the

prospective study on albuminuria in type 2 diabetic patients showing that 77% of the kidney biopsies revealed diffuse/nodular diabetic glomerulosclerosis, while 23% had a non-diabetic glomerulopathy (116).

Studies on extracellular matrix and specifically on heparan sulfate expression are rare in type 2 diabetes mellitus, but a reduced glomerular basement membrane expression of heparan sulfate glycosaminoglycan side chains was found in renal biopsies from patients with type 2 diabetes mellitus and proteinuria and typical diabetic nephropathy (107). The expansion of the mesangial matrix and the thickening of the glomerular basement membrane involves separate and distinct type IV collagen components and these site specific matrix changes are similar in type 1 and type 2 diabetes (29).

Etiology of diabetic nephropathy

Hyperglycemia and advanced glycated endproducts

Hyperglycemia and the subsequent development and accumulation of advanced glycated endproducts is the one of the major culprits of diabetic complications (17,125-127). Numerous clinical and animal studies have pointed out that hyperglycemia is associated with the development of diabetic complications (128). Intensive glycemic control of diabetes effectively delays the onset and slows the progression of diabetic retinopathy, nephropathy, and neuropathy in patients with type 1 and type 2 diabetes mellitus (17,129,130).

With respect to diabetic nephropathy the recent observations by Fioretto *et al.* should be mentioned. They found a complete normalization of the glomeruli after successful pancreas transplantation which had given euglycemia (102). At a cellular level hyperglycemia leads to activation of the polyol pathway and to activation of diacyl glycerol. The latter activates protein kinase C (reviewed by (27), (28), and (131)). A high glucose concentration induces a decrease in heparan sulfate production (both qualitatively and quantitatively) in cultured glomerular visceral epithelial and mesangial cells (132-134).

Hyperglycemia leads also to the accumulation of advanced glycosylation end products which can be found in glomeruli, arterioles, and in the skin collagen of diabetic patients (135). These accumulations increase as normal renal status advances to microalbuminuria and macroalbuminuria (127,136,137).

Advanced glycation endproducts are associated with premature arteriosclerosis and by crosslinking these products interfere with various cell and matrix components (126,127).

Blood pressure and renin angiotensin system

High blood pressure is a very important promotor of progression in diabetic nephropathy and a close correlation between bloodpressure and decline in glomerular filtration rate has been documented in type 1 and type 2 diabetes (138). It has been reported that angiotensin converting enzyme inhibition gives a better anti-proteinuric and renoprotective effect in comparison with other antihypertensive treatments with equal blood pressure effects in patients with type 1 diabetic nephropathy with several degrees of nephropathy (18,88,139). Some authors, however, found that angiotensin converting enzyme inhibition and other antihypertensive treatments resulted in equal effects (140). In diabetic nephropathy a disproportional activation of the intrarenal renin angiotensin system has been found in the glomeruli and renal vasculature, indicating at least at a glomerular level high levels of angiotensin II inducing the hemodynamic and structural changes (141).

This dissociation between hemodynamic and anti-proteinuric effects has been demonstrated also by the study by Gansevoort *et al.* in non-diabetic glomerular diseases (142). This suggests that the inhibition of the renin angiotensin system system not only influences the course of diabetic nephropathy through inhibition of glomerular hypertension and/or hyperfiltration, but also through direct effects on glomerular cell phenotype, thereby affecting glomerular structure and function. Evidence for this hypothesis also comes from *in vitro* studies. Mesangial cells were found to produce increased amounts of TGF-ß, biglycan, fibronectin, and collagen type I, in the presence of angiotensin II, while these effects could be blocked completely by an angiotensin II inhibitor (143,144). *In vivo* administration of angiotensin II augmented glomerular mRNA for TGF-ß and collagen type I with 70%. In mesangial cell cultures angiotensin II induced a decreased mRNA content for perlecan and strongly inhibited heparan sulfate proteoglycan production, which could be blocked by losartan (144). Thus the findings from clinical studies are supported by *in vitro* and *in vivo* experiments with angiotensin II and mesangial cells resulting in a decreased heparan sulfate proteoglycan production and an increased synthesis of matrix proteins associated with the development of glomerulosclerosis.

With regard to diabetic nephropathy, the findings of a reduced heparan sulfate expression might thus be explained by long-standing high glucose concentrations, non-enzymatic glycosylation and the functional changes of the renin-angiotensin system.

Optimizing the treatment of diabetes mellitus type 1 with the goal of slowing down the progression of vascular and renal damage has been the subject of many studies. In the past, intensified insulin treatment, antihypertensive treatment in general, and especially angiotensin converting enzyme inhibition have been shown to slow the progression of diabetic nephropathy (17-19,145). These treatment strategies have resulted in a dramatic

- but not complete - amelioration of the progressive decline of renal function. Nevertheless, there is still a need for a more complete treatment of diabetic nephropathy, that not only slows down progression, but may also be able to cure the disease and its extra-renal manifestations.

The Steno hypothesis (and the putative role of heparan sulfate proteoglycans in diabetic nephropathy)

In diabetes mellitus all tissues are exposed to hyperglycaemia, but only a subset (20 - 40%) of diabetic patients develop diabetic kidney disease (16,95). The development of microalbuminuria, the first sign of diabetic nephropathy, is strongly related with a very high cardiovascular morbidity and mortality. These findings suggest a genetic defect which explains that a specific patient with that defect is vulnerable for the development of diabetic nephropathy. In addition the systemic changes leading to micro- and macroangiopathy suggest a systemic change of extracellular matrix. These features were integrated to the Steno hypothesis by Deckert *et al.*. The Steno hypothesis, first formulated by Deckert *et al.* in 1988, proposes that a genetic defect in the regulation of the production of heparan sulfate by endothelial and mesangial cells determines the susceptibility and hence the development of proteinuria and angiopathy with its associated increased cardiovascular mortality (16,23). According to this hypothesis, albuminuria and associated complications result from a genetic polymorphism of enzymes involved in the metabolism of heparan sulfate proteoglycans (16,23). A genetic polymorphism of the N-deacetylase/N-sulfotransferase protein with subsequent changes in activity secondary to for example hyperglycemia and advanced glycated endproducts might thus lead to quantitative changes in heparan sulfate. The latter is a well known finding in diabetic nephropathy. As such the decreased tissue levels of heparan sulfate will contribute to the development of diabetic nephropathy and the associated systemic vasculopathy.

A premature cell ageing was found in cells from patients with nephropathy. Since it is known that senescence modifies also the production of proteoglycans, this premature ageing might offer a clue in the development of diabetic nephropathy (146,147). The same cell type has been shown to be involved in patients with diabetic nephropathy in a different expression of collagen production and mRNA expression of integrin subunits (148,149). Although studies with skin fibroblasts did not reveal unequivocally that the N-deacetylase/N-sulfotransferase was changed (150-152), other studies found indeed a link between prematurely aged fibroblasts and the development of diabetic nephropathy (153,154). In diabetic rats a decrease of the N-deacetylase/N-sulfotransferase protein activity has been reported (46). No genetic polymorphisms for the genes of the N-

deacetylase/N-sulfotransferase protein have been reported yet.

In patients with diabetic nephropathy, staining for heparan sulfate in the glomerular basement membrane is reduced proportionally to the amount of proteinuria, but staining for the agrin core protein is not altered (107,110). Basement membranes of large vessels, such as the aorta may also show a decreased content of heparan sulfate in diabetes (155,156). In addition, similar findings have been observed in membranes of small vessels, such as muscle capillary basement membrane (157). In this study on heparan sulfate expression in muscle capillaries, microalbuminuria was associated with an undersulfation of heparan sulfate, while an absolute reduction in heparan sulfate staining was observed in type 1 diabetic patients with macroalbuminuria (157). Similar observations have been done in experimental diabetic nephropathy. In streptozotocin-diabetic rats with an increased urinary albumin excretion, a reduced heparan sulfate charge barrier/density was found at the lamina rara externa of the glomerular basement membrane in comparison to control rats (158).

The techniques used for detection of heparan sulfate merit a few comments. Reports on a decreased heparan sulfate expression by ^{35}S incorporation in diabetic nephropathy have given unequivocal results (reviewed by (159) and (33)). With biochemical techniques Parthasarathy and Spiro found that the glomerular basement membrane of diabetic patients contained less glycosaminoglycans than the kidneys from controls (160). These findings were reproduced by the same group a few years later (161). Another technique was used by Vernier *et al.* (110). They used cuprolinic blue and electron microscopy to assess the number of anionic sites in the basement membrane and confirmed that heparan sulfate glycosaminoglycans content in the lamina rara externa of the glomerular basement membrane was reduced in patients with macroalbuminuria. With recently developed monoclonal antibodies specifically directed against the agrin core protein (JM72) and directed against the heparan sulfate glycosaminoglycan side chains (JM403) several studies on the composition of the glomerular basement membrane have demonstrated that a specific decrease of the heparan sulfate glycosaminoglycan side chain can be found (53,107,162).

The JM403 binding to heparan sulfate depends on the presence of an N-unsubstituted glucosamine unit in heparan sulfate and the expression of the JM403 epitope in heparan sulfate preparations from various sources is inversely correlated with heparan sulfate sulfation (163). A reduced staining for JM403 can therefore be explained by a decreased synthesis and/or increased degradation of heparan sulfate, or an altered structure of heparan sulfate resulting in loss of the JM403 epitope.

Van den Born *et al.* has reported that the expression of heparan sulfate is reduced in several non-diabetic renal diseases such as lupus nephritis, crescentic nephritis and minimal change disease (162). Changed heparan sulfate expression may thus occur via

several possible pathways, and it is tempting to speculate that it is a relevant factor in proteinuria and/or the progression of renal insufficiency, in general, rather than a complete specific marker for the development of diabetic nephropathy.
Results from treatment strategies with heparin and heparan sulfate in animal models and humans with diabetic nephropathy underline the important role attributed to the heparan sulfate metabolism in diabetic nephropathy - as also suggested by the Steno Hypothesis (20,159,164-167).

Treatment of diabetic nephropathy with glycosaminoglycans

Heparan sulfate glycosaminoglycan side chains may have biological functions that could possibly be substituted by administration of similar molecules like heparin (33). Treatment strategies with heparin and heparan sulfate have indeed shown to be effective in animal models and humans with diabetic nephropathy and also in animal models with non-diabetic renal disease (20,159,164-167).
In studies using sulfated glycosaminoglycans, no differences were found for glomerular filtration rate, renal plasma flow, and filtration fraction thus suggesting that hemodynamic and flow related changes do not account for the observed antiproteinuric effects of glycosaminoglycan therapy (20,166,168).
As heparan sulfate is mainly cleared by the kidneys, both replacement of “faulty” heparan sulfate molecules in the glomerular basement membrane as well, as a beneficial effect on smooth muscle cell and mesangial cell proliferation, may have accounted for the observed effects on proteinuria. Heparins and heparan sulfate both exert anti-inflammatory actions as well as anti-proliferative effects. Many of these aspects have been subject of thorough recent reviews (57,159,167).
Transforming growth factor ß has been implicated to influence the production of matrix components (169,170). Heparin can reverse the increase in transforming growth factor ß induced by hyperglycemia (108,171,172). Basic fibroblast growth factor’s potent mitogen activities on various renal cells are modulated by heparan sulfate (33,173). Heparin prevents basic fibroblast growth factor induced proliferation and may also stop smooth muscle cell proliferation by releasing transforming growth factor ß from its binding protein (174,175). It also inhibits the synthesis and release of endothelin-1 by endothelial cells (176). The negative charge at the N position is thought to be required for the antiproliferative action (177). A possible way by which heparin inhibits the proliferation is by interfering with oncogene expression. It has been found that heparin inhibits a protein kinase C-dependent pathway for cell proliferation and that heparin suppresses the induction of c-fos and c-myc mRNA at a site distal to activation of the kinase (178,179).

Vascular permeability factor or vascular endothelial growth factor is a crucial mediator of blood vessel growth associated with diabetic retinopathy. Binding of vascular endothelial growth factor to extracellular matrix and expression of receptors of vascular endothelial growth factor may be influenced by an altered sulfation pattern of proteoglycans (170). Transforming growth factor ß is known to enhance the expression of vascular endothelial growth factor in the diabetic kidney (170). Both the expression of hyperglycemia induced transforming growth factor ß and the production of glomerular cell α1(IV) collagen are prevented by heparin treatment (108,171).

Heparin stimulates in a concentration-dependent manner, the synthesis of heparan sulfate secreted by cultured endothelial cells. This takes place immediately after exposure of the cells to heparin, affects only heparan sulfate, and is specific for the endothelial cell (180-182). Glycosaminoglycans can also induce perlecan expression in mesangial cells (171). In their model Gambaro *et al.* observed that glycosaminoglycan treated animals had normal glomerular basement membrane thickness with normal charge density as compared to non-treated control animals. They stated that the effect as observed on the visceral side of the glomerular membrane is caused by a more complex effect of glycosaminoglycans on cell matrix synthesis (159,167,183). Interestingly, a study was recently reported suggesting that unfractionated heparin might differ with low molecular heparins with respect to the effects on P- and L-selectin (184).

In the context of such a pivotal role of glycosaminoglycans, putative benefits of supplementation of sulfated glycosaminoglycans might be expected in diabetic nephropathy. The development of diabetic nephropathy could be prevented by giving glycosaminoglycans to rats with streptozotocin-induced diabetes mellitus (164). These authors showed also that in the same model intervention could reverse diabetic abnormalities (165,183,185).

In the past Kincaid-Smith has claimed that for non-diabetic nephropathy therapy with anticoagulants could be effective (186). Indeed, numerous studies with heparins and heparan sulfate have been performed in animal models with non-diabetic renal disease. Unfractionated heparin gave less renal damage and less albuminuria and high blood pressure as compared with a low molecular weght fraction of heparin with a low anticoagulant activity in a remnant kidney model (187). Heparin treatment was evaluated in an experimental model with mesangioproliferative anti-Thy 1.1 nephritis by Floege *et al.* (188). They found that early and late heparin treatment inhibited mesangial matrix expansion for a variety of extracellular matrix proteins and that heparin inhibited mesangial proliferation (188). Effective inhibition of mesangial proliferation by heparin has also been reported for the habu-venom induced glomerulonephritis model (56). Finally, heparin treatment prevents also the binding of immune complexes with

nucleosomal antigens to the glomerular basement membrane and delays nephritis in lupus prone mice (189).

Studies in patients with diabetic nephropathy are limited. Myrup *et al*. found a reduction of microalbuminuria after treatment with low molecular weight heparin and with standard heparin (20). Also in type 1 diabetes, in macroalbuminuric patients treated with enoxaparin, a significant decline of the urinary albumin excretion rate was found, but significance was not reached in comparison with the placebo treated group (166).

Two controlled studies have been reported in type 2 diabetes (190,191). Nielsen *et al.* did a placebo-controlled study with low molecular weight heparin during 3 weeks in 44 patients with varying rates of albuminuria and they found no changes in the transcapillary escape and the urinary excretion rate of albumin (191). Solini, on the contrary, found a clear reduction of the albuminuria after long-term treatment with oral sulodexide (80% heparan sulfate and 20% dermatan sulfate) (190). Further, sulodexide has been studied in both type 1 and type 2 diabetes patients, but these studies were uncontrolled (192,193).

Thus both unfractionated heparin, various types of low molecular weight heparin (tinzaparin, enoxaparin), and a mixture of heparan sulfate and dermatan sulfate have been used for clinical studies in diabetic nephropathy with promising but unequivocal results.

Aims of this thesis

In this thesis we have further explored the role of glycosaminoglycans in clinical diabetic nephropathy. The first two studies, described in **chapter 2** and **chapter 3**, focus on the extracellular matrix components of the skin basement membrane.
The diabetic milieu affects all body tissues. It has been demonstrated that in diabetic nephropathy a generalized decrease of heparan sulfate can be found in basement membranes of small and large vessels. The skin has a non-vascular lining and easily accessible basement membrane, because tissue can be obtained with a simple skin punch biopsy technique. We therefore embarked on a cross-sectional study to evaluate the expression of extracellular matrix components of the epidermo-dermal junction basement membrane zone, a non-endothelial membrane, in patients with different stages of diabetic nephropathy, controls subjects, and patients with non-diabetic renal failure.
Question: Is the expression of heparan sulfate changed in the skin basement membrane of patients with diabetic nephropathy?

The encouraging findings from the study reported in **chapter 2** decided us to determine whether a successful pancreas-kidney or kidney transplantation can reverse extra-renal, extracellular matrix abnormalities. We therefore prospectively evaluated the basement membrane extracellular matrix in sequential skin biopsies from patients with diabetic or non-diabetic renal failure before and after successful pancreas-kidney or kidney transplantation.
Question: Is the decreased expression of heparan sulfate in the skin basement membrane of patients with diabetic nephropathy reversible after transplantation?

In the studies described in the following three chapters, we focussed on the treatment with glycosaminoglycans for diabetic nephropathy. Several studies have already investigated whether supplementation of sulfated glycosaminoglycans for diabetic nephropathy could be of any benefit. This was supported by experimental studies showing that the development of diabetic nephropathy in a streptozotocin model could be prevented by glycosaminoglycans. We had the opportunity to employ danaparoid sodium, a heparinoid. This mixture of sulfated glycosaminoglycans consists mainly of heparan sulfate with a small subfraction, which is highly sulfated. Several large studies have shown that danaparoid sodium is efficacious and relatively safe for the prevention and treatment of venous thromboembolism. Therefore and because danaparoid sodium consists to a large extent of heparan sulfate, we decided to study the effect of danaparoid sodium on proteinuria in type 1 diabetic patients with overt diabetic nephropathy (described in **chapter 4**).

Question: Does treatment with low dose danaparoid sodium decrease on albuminuria in type 1 diabetic patients with overt nephropathy?

Both retinopathy and nephropathy are parts of the spectrum of microvascular disease complicating diabetes mellitus. Increased capillary permeability in diabetic retinopathy gives foveal edema and hard exudates, while increased capillary permeability leads to albuminuria in diabetic nephropathy. The decrease of heparan sulfate in the diabetic state has been documented for both the glomerular basement membrane and the retinal capillary basement membrane. After completion of the study reported in **chapter 4** on the effect of danaparoid sodium on albumin excretion in type 1 diabetic patients, we hypothesised post-hoc that treatment with danaparoid sodium may also have an influence on retinal permeability. We reviewed therefore the fundus photographs, which had been taken as a safety measure before and at the end of the study, to look whether changes in hard exudates occurred. This study is decribed in **chapter 5**.
Question: Does treatment with low dose danaparoid sodium reduce the number and severity of hard exudates in type 1 diabetic patients with overt nephropathy?

The findings of **chapter 4** and **chapter 5** challenged us to test these questions also for patients with type 2 diabetes mellitus and proteinuria. Diabetes mellitus type 2 is a rather heterogenuous disease. The various mechanisms influencing and interacting with the heparan sulfate metabolism might thus evolve differently in type 1 and type 2 diabetic nephropathy. Consequently, the diabetic nephropathy complicating diabetes mellitus type 2 may behave differently. With respect to the treatment with glycosaminoglycans, only limited controlled studies have been reported and they show conflicting results. We therefore performed a prospective study to evaluate whether treatment with danaparoid sodium exerts also beneficial effects on the albumin excretion rate in type 2 diabetes patients with severe proteinuria and renal insufficiency. We looked furthermore at the endothelial dysfunction as estimated by plasma levels of von Willebrand factor, and at the retinopathy by evaluating the severity of hard exudates. This study is decribed in **chapter 6**.
Question: Does treatment with low dose danaparoid sodium influence on albuminuria, von Willebrand factor, and retinopathy in type 2 diabetic patients with overt nephropathy?

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