

Thesis for doctoral degree (Ph.D.)
2008

DIABETIC GLOMERULOPATHY - NATURAL HISTORY, INTERVENTION AND PREDICTION OF COMPLICATIONS



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Stockholm 2008

Cover: “In the kidney forest”, Dan Perrin, 2008.

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ISBN 978-91-7357-513-3

Printed by



www.reproprint.se

Gårdsvägen 4, 169 70 Solna

To my family, the small and the large

ABSTRACT

Diabetes mellitus is one of the most common chronic diseases in childhood. Diabetic nephropathy (DN) is one of the threatening complications of diabetes that occurs in 30-40% of patients after 20 years duration of disease. Before the introduction of antihypertensive treatment, patients with DN progressed to renal insufficiency within 5-7 years and had very high overall mortality.

The aim of this thesis was to study the natural history of the renal morphology and the effect of intervention with antihypertensive treatment in a well defined, unselected group of normoalbuminuric and normotensive adolescents with type 1 diabetes. Forty-six patients underwent their first renal biopsy between 1992 and 1994 when they were 17 years old and had diabetes for 10 years. Six years after the first renal biopsy, 29 patients underwent a second renal biopsy. During the follow-up period ten of the 29 patients developed microalbuminuria and/or hypertension and 6 of the patients had received antihypertensive treatment for at least two years.

In the 19 patients who were still normoalbuminuric and normotensive at the time of the second renal biopsy, progression of the diabetic glomerulopathy from the first renal biopsy was observed with further increases in mesangial matrix and mesangial volume fraction. The females had higher values of mesangial matrix, mesangial volume fraction and glomerular volume at the time of the first renal biopsy compared to the males. In males the mesangial matrix and mesangial volume fraction, but not glomerular volume, increased to the time of the second renal biopsy reaching values similar to the females.

The six patients who had received antihypertensive treatment during the follow-up period showed higher values in basement membrane thickness, mesangial matrix and mesangial volume fraction at the first renal biopsy but with antihypertensive treatment and better metabolic control the morphological parameters remained stable or even regressed.

The basement membrane thickness and mesangial matrix volume fraction at the time of the second renal biopsy correlated well with the degree of long-term metabolic control since onset and with male gender.

When predicting the outcome of microalbuminuria and hypertension after 17 years duration in the 46 patients who had a first renal biopsy, the basement membrane thickness, the day systolic blood pressure and the metabolic control, especially from puberty, were risk-factors. GFR or hyperfiltration could not be identified as a risk factor.

Following the second renal biopsy, thirteen normoalbuminuric and normotensive patients participated in a placebo controlled treatment study with an angiotensin II receptor blocker, candesartan. After 5 years of treatment, the candesartan group had a lower blood pressure compared to the placebo group as well as a significant regression in morphology on a third renal biopsy performed after 6 years. The placebo group

showed a tendency (not significant) to regression in morphology. Two patients in the placebo group developed hypertension and/or microalbuminuria and needed antihypertensive treatment but no patient in the candesartan group did.

It is concluded that it is possible to favorably affect renal morphological changes in as much as the previously observed deterioration in renal morphology was attenuated. The importance of regular follow-up and good metabolic control cannot be overestimated.

LIST OF PUBLICATIONS

- I. PERRIN NESS, Torbjörnsdotter TB, Jaremko GA and Berg UB
Follow-up of kidney biopsies in normoalbuminuric patients with type 1 diabetes.
Pediatric Nephrology (2004) 19:1004-1013
- II. PERRIN NESS, Torbjörnsdotter TB, Jaremko GA and Berg UB
The course of diabetic glomerulopathy in patients with type 1 diabetes: A 6-year follow-up with serial biopsies
Kidney International (2006) 69, 699 – 705
- III. PERRIN NESS, Torbjörnsdotter TB, Jaremko GA and Berg UB
Predicting the risk for future microalbuminuria and hypertension from clinical and morphological parameters in young adolescents with type 1 diabetes
Submitted
- IV. PERRIN NESS, Jaremko GA and Berg UB
The effects of candesartan on diabetes glomerulopathy - a double-blind placebo-controlled trial
Pediatric Nephrology, <http://dx.doi.org/10.1007/s00467-008-0745-x>

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LIST OF ABBREVIATIONS

24-h ABPM	24-hour ambulatory blood pressure measurement
ACEi	Angiotensin-Converting Enzyme inhibitor
AHT	Antihypertensive treatment
ARB	Angiotensin receptor blocker
BP	Blood pressure
DG	Diabetic glomerulopathy
DN	Diabetic nephropathy
BMT	Basement membrane thickness
BSA	Body surface area
ERPF	Effective renal plasma flow
FF	Filtration fraction
GFR	Glomerular filtration rate
GV	Glomerular volume
HbA _{1c}	Hemoglobin A _{1c} , glycosylated Hemoglobin A _{1c}
MA	Microalbuminuria
NA	Normoalbuminuria
PAH	Para-aminohippuric acid
UA	Urinary albumin concentration
UAE	Urinary albumin excretion rate
V _V (mes/glom)	Mesangial volume fraction/glomerulus
V _V (matrix/glom)	Mesangial matrix volume fraction/glomerulus
Δ BMT	Difference in BMT between two time points
Δ V _V (matrix/glom)	Difference in V _V (matrix/glom) between two time points
Δ V _V (mes/glom)	Difference in V _V (mes/glom) between two time points

1 PREFACE

During a sunny lunch outside Södertörns Högskola in the summer of 1997 Ulla Berg and Torun Torbjörnsdotter asked me what sort of pediatrician I wanted to be (a nephrologist of course!) and if I wanted to join them in their research about diabetic nephropathy in adolescents with type 1 diabetes.

It was very easy to accept the offer and since then, we have always had an enjoyable time. Even so, I have many times wished it could have been easier and envied those researchers that do short projects with immediate answers. The biopsy, the first part of the project, took more than 3 years to complete and after that the treatment study went on for 5 additional years. It was meant that the treatment study should be reported after this thesis but it turned out otherwise and here it is.

However, the project has surprised us over the years and by digging deeper into the data we found even more interesting results. In the beginning it was so interesting to see how the morphological changes in the kidney correlated with the clinical findings and how a therapeutic intervention could change this relationship. During the last year there have been many calculations -and even more calculations- and statistical analyses in an attempt to identify possible risk factors and patients at risk.

Clinical research is never easy and demands patience, as it is the art of the possible. All the time, it has felt meaningful to find answers about how diabetic glomerulopathy develops and progresses and if it is possible to prevent the patients with type 1 diabetes from progressing to renal failure. After meeting young 20-30 year old men and women at the adult nephrology department with kidney failure due to diabetic nephropathy, I became even more convinced of how important this is.

I started this project when my youngest daughter was a toddler and soon she is becoming an adolescent. Time to finish this book. Enjoy reading it!

Nina Perrin, April 2008

2 BACKGROUND

2.1 Introduction

Diabetes mellitus is one of the most common chronic diseases in childhood and it is life-long. Type 1 diabetes that affects children and adolescents is characterized by lack of insulin production from the pancreas whereas type 2 diabetes is characterized by insulin resistance. In type 1 diabetes, the insulin producing β -cells, in the Langerhan's cell islets in the pancreas, are gradually destroyed after an autoimmune attack. Insulin auto-antibodies (IAA), islet cell cytoplasm antibodies (ICA) and glutamic acid decarboxylase (GAD) are produced in response to, for example, an infection.

The incidence of type 1 diabetes is increasing throughout the world at 3-4% / year and this is occurring even in Sweden and Finland that have the highest incidence (Sweden 30 cases/100.000 in 0-14 year old children and Finland 41/100.000)¹. The incidence varies widely throughout the world from countries with very low incidence: Venezuela and Pakistan 0.1-0.5/100.000 to intermediate incidence in Italy, Bulgaria and Libya (9-10/100.000) to high incidence in USA (18/100.000) and very high incidence in Kuwait, Sardinia (22-38/100.000), Sweden and Finland.

Boys and girls are equally affected. The characteristic age of onset was previously prepubertal (5-9 years) but during the last 10 years the most frequent age of onset has increased to 10-14 years². The youngest children (< 5 years) have the greatest increase in incidence world wide and in Sweden^{1,2}. There is even a greater challenge for both parents and health personnel to take care of a small child with diabetes. They are often, compared to older children, more severely affected at time of onset as the metabolic disturbances are greater. The normal child as well as the child with diabetes will experience many infections during early childhood, with the difference being that every infection will have implications for the diabetic treatment.

The incidence of type 2 diabetes is increasing more rapidly than that of type 1 diabetes because of the global increase in the prevalence of obesity^{3,4}. While type 2 diabetes patients are even more burdened with complications, this thesis will concentrate on type 1 diabetes patients.

2.2 Natural history of the development

2.2.1 The "traditional" natural history

The natural history of kidney function, glomerular pathology and clinical changes in diabetes has been described by Mogensen⁵⁻⁷ as a well-defined step-by step process (see table 1, below).

Table 1

Stage	Designation	Glomerular Filtration	Urinary albumin	Blood Pressure	Morphological changes
I	Hyperfunction/hypertrophy	Increased	May be increased	Normal	Glomerular hypertrophy
II	Normo-albuminuria	Hyperfiltration	Normal	Normal	Increased BMT
II-III	Transitional	Hyperfiltration	High normal	Increasing circa 3 mm Hg per year	Increased BMT and mesangium
III	Incipient DN	Still increased but starting to decrease	20-200 ug/min	10-15% increase in BP levels	Changes correlate to UAE
IV	Overt DN	Normal to decreasing	>200 ug/min	Often frank hypertension +5% of BP levels yearly	Advanced glomerular lesion
V	End stage renal disease (ESRD)	<20 ml/min	>200 ug/min or decreasing	Hypertension	Glomerulosclerosis

After ^{6,7}

The Mogensen stages describe the progression to diabetic nephropathy but only 30-40 % of the patients reach stage V. Most patients reach stage III but can spontaneously ⁸ or by improved metabolic control ^{9,10} revert to stage II.

Stage I

Directly after diagnosis, increases in renal mass and glomerular hyperfiltration occur. The insulin treatment may somewhat reduce kidney size and glomerular hyperfiltration ^{11,12} but for the most part, patients will continue with hyperfiltration over the next 10 - 20 years.

Stage II

After about two years, thickening of the basement membrane can be seen. During the following years, depending on the metabolic control, the mesangial matrix and mesangial volume fraction increase.

Stage III

Intermittent microalbuminuria (urinary albumin excretion rate of 20-200 µg/min) may develop during periods of worse metabolic control and during puberty. It may regress to normoalbuminuria with improved metabolic control but it may progress to persistent microalbuminuria. Normally, it takes about ten years to develop persistent microalbuminuria but in some cases, especially during puberty, it takes less. Persistent microalbuminuria can regress to normoalbuminuria in 30 % of the patients ⁸ while

another 30 % continue with microalbuminuria and the remaining 30 % progress to macroalbuminuria.

The blood pressure gradually increases and is seen as (a) slightly higher but still within normal BP range or (b) raised night-time blood pressure and absence of night time BP dipping (non-dippers)¹³⁻¹⁵.

Some patients develop microalbuminuria and hypertension as parallel processes but others develop either hypertension or microalbuminuria.

The GFR starts to decline from hyperfiltration to upper normal levels.

Stage IV

The overt diabetic nephropathy shows proteinuria and macroalbuminuria with UAE > 200 µg/min and can be observed on an urine dipstick. The proteinuria is not selective for albumin and other proteins are also excreted. The mesangium volume now exceeds 30 % and the glomerular filtration rate declines at about 10 ml/min/1.73 m² /year^{16,17}. It takes approximately 15-25 years to reach stage IV and 5-7 more years to progress to stage V.

Stage V

30-40% of the patients reach stage V with end stage renal disease (ESRD) after around 25 years^{18,19}. The patient is in need of renal replacement therapy such as dialysis or renal transplantation. The morphology is dominated by sclerosed glomeruli that neither filter the blood nor permit passage of much protein. The mortality in stage V is very high and in the 1970's the median survival was 6-7 years^{19,20} with a ten year survival of 30-50%^{18,19}. The causes of death were renal failure and cardiovascular diseases.

2.2.2 The natural history development over time

The development of the diabetic nephropathy over time can be described as in figure 1. The normal text signifies changes that occur in all patients and the cursive text for changes in part of patients.

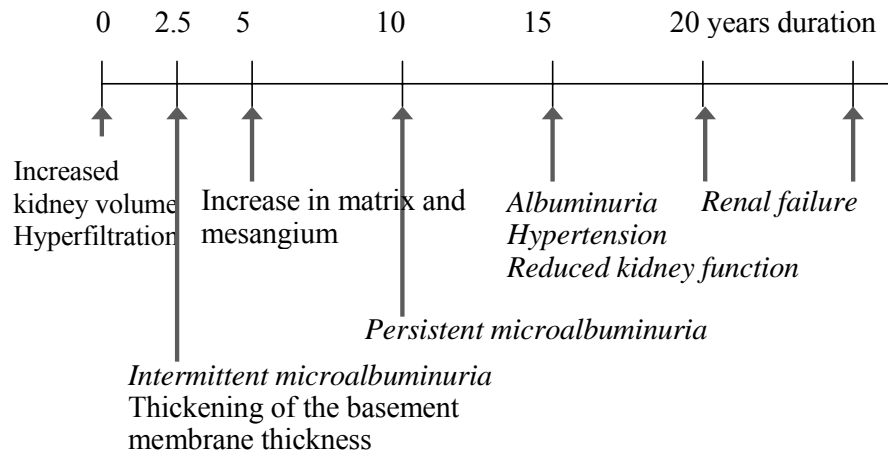


Figure 1

2.2.3 The changing natural history

Since the introduction of antihypertensive treatment (AHT) in the 1980's the natural history of the course of diabetic nephropathy has changed¹⁶. It is difficult to compare prevalence of DN before and after the introduction of AHT.

Prior to the introduction of AHT, the Linköping group showed reduced incidence of DN after 25 years from 30 % in the cohort of patients that were diagnosed between 1961-1965 to 8.9% DN in the 1966-1970 cohort⁹. Others could not show the same improvement, probably because of worse metabolic control and higher frequency of smokers²¹. The possibility of reducing the frequency of microalbuminuria and retinopathy by better metabolic control has since then been convincingly demonstrated by the DCCT study^{10,22}.

UAE can decrease either spontaneously⁸ or with better metabolic control^{9,10} and AHT¹⁶. The fall in GFR is also diminished by AHT²³. AHT reduces and postpones the frequency of DN^{6,22,24-29} and recently a frequency of 8-14% of DN after 20-25 years duration has been reported in Scandinavia³⁰⁻³².

During the past few decades, the percentage of patients with diabetic ESRD on renal replacement therapy (RRT) has increased³³ and today, DN is the most prevalent disease among new cases of RRT in Sweden and the western world^{34,35}.

If DN develops, it normally does so during the working age (< 65 years) in type 1 diabetes patients and the need for RRT has a major influence on the daily life of the patient. It also implies great costs for the community and the individual. The mortality rate among patients with DN³⁶ and on dialysis is still very high, at 82-86% 5-year mortality. However, a marked improvement in survival is seen when patients undergo renal transplantation with 20 % 5-year mortality^{35,37}.

2.3 Complications

The patient's family is very concerned about the acute complication of hypoglycemia with hypoglycemic convulsions and subsequent cerebral hypoxia and brain damage. This is an acute and threatening event but easily reversed and severe cerebral complications rarely occur. The much more common complications occur due to long-standing hyperglycemia which is a condition where neither the child nor the parents notice so many symptoms. The teenagers' fear of losing control during hypoglycemia make them prone to be hyperglycemic with the result that they think that hyperglycemia is their normal condition.

The diabetic angiopathy affects both the small and large vessels; the microangiopathy affects the arterioles in the kidney, the retina and the nervous system. The macroangiopathy affects large vessels with risk for acute myocardial infarction, cerebrovascular events and ischemia of the lower limbs requiring amputation.

2.3.2 Diabetic retinopathy

The first sign of diabetic retinopathy is a thickening of the basal membrane and loss of pericytes in the retina vessel wall. Long aggregated glucose-molecules, advanced glycated end-products (AGE) are closely involved in the process^{46,47}. Vascular occlusion occurs, resulting in retinal hypoxia. The response is a formation of microaneurysm, a widening of the arterioles, increased vascular permeability and with time neovascularisation. The new vessels are less resistant, grow uncontrolled and have increased permeability leading to edema and bleeding.

The diagnosis is made by direct visualization with the ophthalmoscope and fundus photography. The terminology of the retinal changes varies but mild/background retinopathy, moderate retinopathy and non-proliferative and proliferative diabetic retinopathy (DRP) are quite commonly used. The proliferative DRP requires treatment with laser photocoagulation to save vision. In a cohort from Linköping with type 1 diabetes patients, 27% of the diabetes patients showed retinopathy of any kind after 13 years duration⁴⁸ and after 25 years duration, 80% of the patients had background retinopathy³¹. The importance of metabolic control and the development of DRP has been clearly shown^{24,49}.

The incidence of diabetic retinopathy has been observed to decrease over recent time intervals in the Linköping cohort. At the end of 25 years of disease, patients with onset of diabetes in the 1960s had a 47% incidence of proliferative DRP while those with onset in the 1970s had a 24% incidence³¹. A similar decline was seen in Denmark, from 31 to 12.5% after 20 years duration³². Nevertheless, diabetes is the most common cause of blindness in individuals younger than 60 years of age⁵⁰ and the 14 year cumulative incidence of blindness was found to be 2.4% in a Wisconsin cohort⁵¹.

2.3.3 Diabetic neuropathy

During childhood and adolescence diabetic neuropathy seldom produces symptoms^{52,53}. The neuropathy includes dysfunctions in the autonomic nervous and peripheral nervous systems with sensory and motor dysfunction⁵⁴. The hyperglycemia and duration of diabetes correlate with the frequency of diabetic neuropathy^{55,56}. The pathogenesis of the diabetic neuropathy is multifactorial with storage of AGEs, fructose and sorbitol in the nerves, reductions in both nerve growth factor and insulin-like growth factor-1 (IGF-1), lack of c-peptide, oxidative stress and nerve hypoxia⁵⁴. The morphological changes of the nervous system are storage of AGEs, reduced myelinated fiber density, degeneration of the diabetic nerve nodula and neuroaxonal dystrophia⁵⁴.

The autonomic nervous system can be examined by continuous electrocardiogram during normal and forced breathing. A decrease in heart rate variability, R-R interval, is an early sign of diabetic neuropathy⁵⁷. In somewhat older diabetes patients, symptoms related to gastroparesis, impotence, disturbance of sudomotor and vasomotor thermoregulation can appear. Sudden deaths that occur among diabetes patients may be caused by abnormal cardiovascular reflexes.

The peripheral neuropathy involves sensory neuropathy with loss of sensation or dysesthesia and motor neuropathy with motor nerve impairment resulting in decreased motor nerve conduction and myopathic muscle weakness. The peripheral neuropathy contributes to diabetic foot ulcers with chronic infections which, together with macroangiopathy, increases the risk for amputation.

59 % of the Linköping cohort showed neuropathy defined as reduced nerve conduction velocity, affecting 2 or more nerves, when evaluated at 13 years duration⁴⁸. The R-R interval is significantly lower in patients with type 1 diabetes than in controls and the R-R interval is reduced proportional to the severity of nephropathy⁵⁸.

2.4 Pathophysiology of diabetes nephropathy

The development of diabetic nephropathy is the result of a complex of metabolic and hemodynamic factors. Figures 4 and 5 illustrate the multiple processes involved. The hyperglycemia is central with the non-enzymatic reaction between glucose and proteins, lipids or nucleic acids resulting in the formation of AGE. The proteins of choice for the glycation are long-lived proteins e.g. collagen IV, myelin, plasminogen activator 1 (PAI-1) and fibrinogen⁵⁹. The AGEs accumulate in the organs where the diabetic complications occur: kidneys, retina, atherosclerotic plaque^{46,60}. The AGE acts via a receptor for AGE (RAGE). The kidneys are responsible for the AGE clearance⁶¹ and the AGEs accumulate in the extracellular matrix, basement membrane and tubulointerstitium of the kidney⁶²⁻⁶⁴. The level of AGE correlates to the metabolic control and to the severity of the microangiopathy^{65,66}.

The hyperglycemia also enhances oxidative stress and reduces nitric oxide (NO)^{67,68}, both of which have a negative influence on endothelial function^{59,69,70}.

Hyperglycemia, AGE and endothelial dysfunction activate protein kinase C isoforms⁷¹ and further increase the transcription of different cytokines, transforming growth factor β (TGF β), vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF- α) and endothelin-1⁷¹⁻⁷³. The pro-sclerotic TGF β stimulates the production of collagen matrix components and also increases the vascular permeability which results in protein leakage^{59,74}. VEGF is up-regulated and stimulates glomerular cellular proliferation and neo-vascularisation and increases both glomerular and vascular permeability^{73,75}. The increase in PAI-1 leads to reduced extracellular matrix degradation⁷⁶.

The hemodynamic alterations in patients with diabetes are closely related to the renin-angiotensin-aldosterone system (RAAS) and especially the effects of Angiotensin II (ANG II)⁷⁷. Angiotensinogen is converted to Angiotensin I (ANG I) by renin from the juxtaglomerular apparatus in the kidney and angiotensin converting enzyme (ACE) in the lungs converts ANG I to ANG II, which stimulates the adrenal gland to secrete aldosterone. Also chymase can convert ANG I to II⁷⁷. ANG II can be further transformed to Angiotensin III and IV^{76,78}. ACE is also responsible for the degradation of bradykinin⁷⁶. Bradykinin has a vasodilator effect and reduces intraglomerular pressure and proteinuria and enhances extracellular matrix degradation and endothelial NO release^{76,77}.

Metabolic and hemodynamic effects

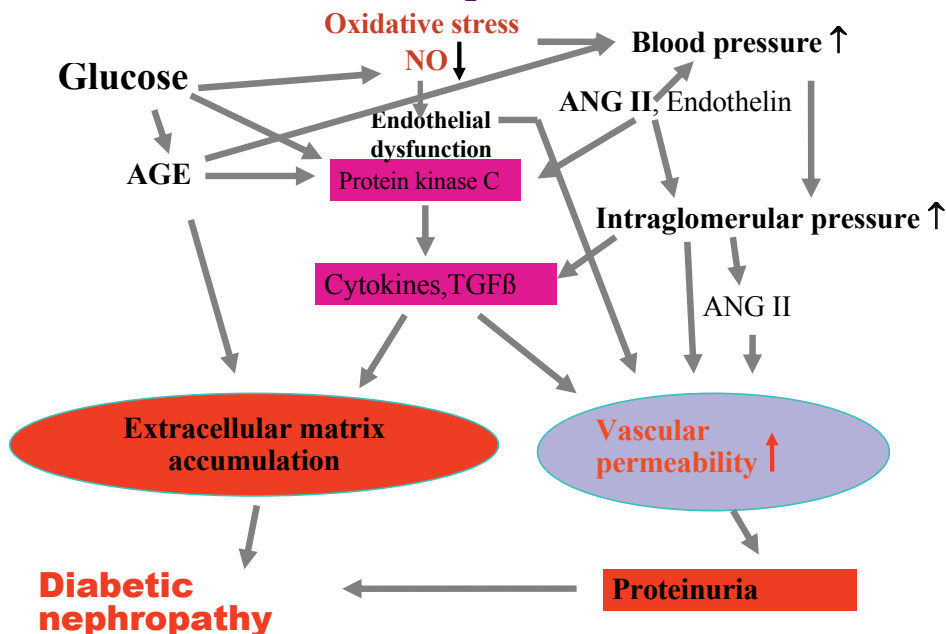


Figure 4

ANG II acts primarily on the angiotensin II receptors 1 (AT1 rec) and 2 (AT2 rec)^{76, 79}. ANG II actions via the AT1 receptor result in generalized vasoconstriction leading to decreased renal blood flow, local vasoconstriction of the afferent and the efferent glomerular arterioles resulting in an increased intraglomerular pressure, increased aldosterone and vasopressin release resulting in enhanced sodium reabsorption and decreased water excretion and cardiac and vascular hypertrophy^{76, 79, 80}. The AT1 receptors are present in the brain, heart, kidney, adrenal gland, liver, in the vascular and nervous system⁷⁹.

The ANG II actions mediated via the AT2 receptor are vasodilatation, increase in NO and bradykinin and an anti-proliferative effect⁸¹. AT2 receptors are found in fetal tissue and most of them are lost after birth. However, the AT2 receptor can be found in the brain, myocardium, adrenal gland, uterus, ovaries and endothelial cells⁷⁹. The AT2 receptor can be up-regulated during tissue repair e.g. after myocardial infarction⁷⁹.

The general action of ANG II is an increase in blood pressure and intraglomerular pressure^{76, 82}. ANG II increases vascular permeability resulting in protein leakage^{83, 84}. ANG II upregulates PKC which stimulates cytokines and TGF- β production which further enhance the matrix accumulation⁷⁶.

Other mechanisms of hypertension are the hyperglycemic reduction of NO which lead to vasoconstriction and hypertension⁸⁵ and arterial stiffness via the AGE-accumulation and collagen crosslinking⁸⁶. Vascular tone and structure is influenced by oxidative stress and ANG-II, catecholamines, free radicals and endothelin-1 act as

vasoconstrictors which also stimulate cellular proliferation. NO, bradykinin and prostacycline act as vasodilators and inhibit cellular proliferation^{59, 63, 68, 70, 77}.

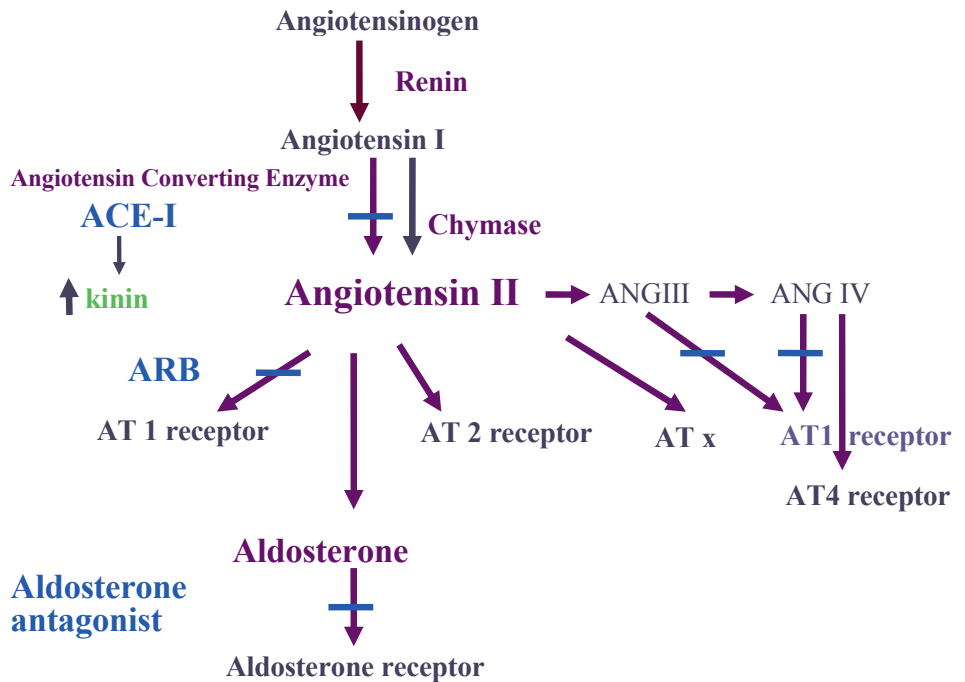


Figure 5 The Renin-Angiotensin-Aldosterone-Systeme, RAAS.

2.5 Intervention

Blockade of the renin-angiotensin-aldosterone-system (RAAS) has been shown to effectively inhibit progression of diabetic nephropathy (DN)^{16, 17, 87-90}. The angiotensin converting enzyme inhibitor (ACEi) blocks the conversion from ANG I to ANG II and reduces aldosterone levels (Figure 5). ACEi increases bradykinin level with the positive effect of vasodilation but the negative effects (clinical side effects) of cough, edema and skin rash⁸¹. The angiotensin receptor blocker (ARB) inhibits ANG II action on the AT1 receptor. The clinical effects of ACEi and ARB are lower blood pressure, lower intraglomerular pressure, reduced proteinuria and enhanced kidney perfusion^{76, 82, 91-95}.

An additional protective effect can be achieved by the combination of ACEi and ARB^{96, 97}. The effectiveness in lowering the urinary albumin excretion is enhanced by increasing doses and also by the addition of an aldosterone inhibitor^{98, 99}.

2.6 Survival

There is a need for long follow-up periods of 20-30 years to examine the morbidity and mortality rates in the type 1 diabetes population. The mortality rate should be analyzed both in absolute terms and in relative terms i.e., in comparison with the mortality in the general population at a similar age.

Recent reports have shown a relatively low mortality among type 1 diabetes patients. In Norway, a mortality rate of 5.4% after 25 years duration was observed in diabetes patients with onset between 1973 and 1982¹⁰⁰. The most common cause of mortality was violent death, acute metabolic complications and cardiovascular disease. The first two causes were found especially among the younger patients and the last among those >30 years old¹⁰⁰. The overall mortality rate was 2.2/1000 person years, higher among males and among those with pubertal onset of diabetes. In Austria 13% of the type 1 diabetes patients had died at 35 years of duration (7.1/1000 person years)²⁸. The causes of death were vascular events, diabetes associated deaths and malignancy, and mortality was higher in those with worse metabolic control²⁸. The mortality was also greater in proportion to albumin excretion, being 14 % in microalbuminuric and 38% in macroalbuminuric patients in a 10 year observation study¹⁰¹.

The overall mortality rate in type 1 diabetes varies throughout the world. In Japan (6.1/1000 person years)¹⁰² and USA the mortality rate (23% after 30 years duration)¹⁰³ is much higher than in Finland (15% after 30 years duration or 3.5/1000 person years)¹⁰².

The mortality is higher among the type 1 diabetes population than among the general population but great variations are seen in different countries. In Sweden, Israel, Finland and Norway the diabetes population has a standard mortality rate 3-4.0 times^{100, 102, 104, 105} whereas USA have 5.2 times¹⁰⁶ and Japan 12.9 times higher mortality rate than the general population¹⁰².

Over the past decades there has been a favorable trend with reduction in mortality rates due to better diabetes treatment, teamwork and home bloodglucose monitoring. In a study in USA on type 1 diabetes patients followed for 30 years, a reduction in cumulative mortality from 39 to 23% was observed in the patients diagnosed between 1950-1959 compared to those diagnosed between 1965-1969¹⁰³. Similarly, in Japan the standard mortality rate declined from 15.7 to 6.9 comparing the 1965-1969 with 1975-1979 cohort¹⁰² and in Norway the overall mortality rate decreased from 10/1000 person years to 2.2/1000 person years from the 1950s to 2000¹⁰⁰. No such improvement was seen in Finland where the national diabetes care system was organized early in the 1960s and free insulin has been offered since 1965¹⁰².

In absolute terms pubertal onset of diabetes and male gender are associated with higher overall mortality rate than prepubertal onset or female gender but the standard mortality rate was the same reflecting higher mortality even in the background non-diabetic population of teenagers and males¹⁰².

3 AIMS

There are four studies presented in this thesis and the general aims were:

- to study the natural course of diabetic glomerulopathy in type 1 diabetes patients
- to study the effect of antihypertensive treatment with an angiotensin receptor antagonist on kidney morphology in a double-blind, placebo controlled, randomized trial extending over five years.

3.1 Specific aims

- How do the morphological changes in diabetic glomerulopathy develop in normoalbuminuric, normotensive type 1 diabetes patients?
- How do the morphological changes progress over time?
- How do the morphological changes correlate with metabolic control, microalbuminuria, 24 h ambulatory blood pressure and kidney function?
- What is the effect of early antihypertensive treatment on the morphological changes and clinical parameters such as kidney function, blood pressure or microalbuminuria?
- Which risk factors can predict future complications?

4 MATERIAL AND METHODS

4.1 Patients

In Sweden, all pediatric patients with type 1 diabetes attend pediatric diabetic clinics and the Children's Hospital, Karolinska University Hospital, Huddinge provides care for all patients less than 18 years of age from the south-west of Stockholm.

Since 1978, all patients with type 1 diabetes have been examined with regular kidney function tests every second-third year. In early 1992, all patients over 12 years of age who had had diabetes for more than five years were invited to undergo a kidney biopsy done in connection with their regular kidney function test. Forty-six unselected patients out of 61 eligible patients accepted and underwent a kidney biopsy. The biopsies were done between 1992 and 1994.

They were all normoalbuminuric and normotensive, based on office blood pressure, and had a mean age of 17.7 (2.8 SD) years and a disease duration of 10.6 (3.3) years. Six years after the initial kidney biopsy, they were asked to participate in a second kidney biopsy and 29 agreed and 17 declined. The second kidney biopsies were done between 1998 and 2002 and the 29 patients were 24 (lower quartile 21; upper quartile 26) years old and had had diabetes for 17 (14; 19) years. There were no differences in the clinical findings or morphological changes in the first biopsy or the frequency of hypertension and microalbuminuria during follow-up in patients who underwent a second biopsy as compared with those patients who did not. The rebiopsied patients underwent further kidney function tests during follow-up.

During the follow-up period of median six years, ten of the 29 patients had developed hypertension (HT) and/or microalbuminuria (MA) and 19 patients were still normoalbuminuric (NA) and normotensive (NT).

Fifteen of the total original cohort of 46 patients had developed MA and/or HT at 17 years duration. Seven had HT, 3 had MA and 5 had both MA and HT. Eight patients were on antihypertensive treatment (AHT), 6 on Enalapril (5-20 mg daily), one each on Metoprolol (100 mg daily) and Cilazapril (2.5 mg daily).

The 19 patients who were still NA and NT were invited after the second renal biopsy, to participate in a prospective, randomized, double-blind, placebo-controlled treatment trial for 5 years with an angiotensin receptor blocker (ARB); 13 patients accepted and 6 declined.

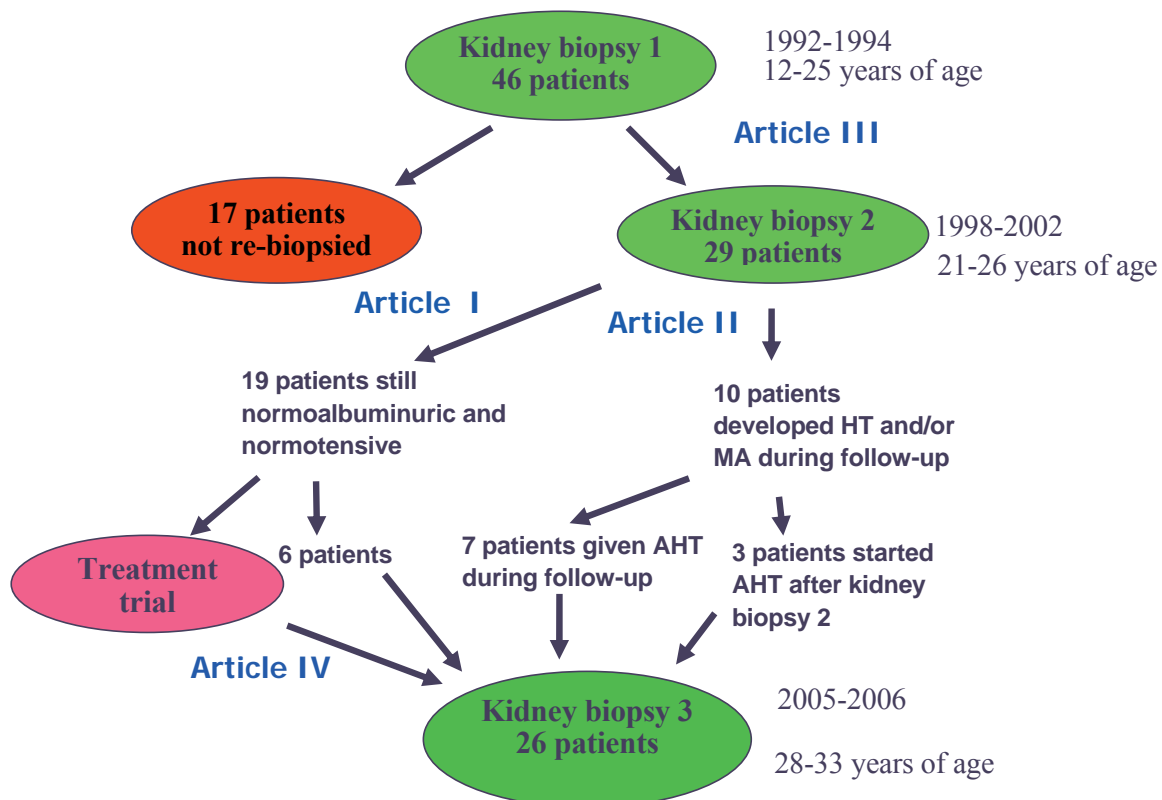
After 6 years all 29 rebiopsied patients were invited to an end-point examination including a third kidney biopsy and 26 patients participated.

Five patients had IgA deposits on the first biopsy but none of them showed clinical signs of IgA nephropathy and one girl had occasional microscopic haematuria.

4.1.1 Puberty status

The mean age at the onset of diabetes was 7.1 (3.7) years and all patients had onset before puberty. Start of puberty was defined as 11 years in females and 12 years in males since no accurate data on Tanner status for start of puberty were available.

4.1.2 Flow-chart of the cohort



4.2 Studies

The publications in this thesis are:

- I. The morphological changes between first and second renal biopsy (6 years interval) in 19 young type 1 diabetes patients who were still normoalbuminuric and normotensive at second biopsy.
- II. The morphological changes between first and second biopsy (6 years interval) in 29 rebiopsied patients of whom 10 developed microalbuminuria and/or hypertension and 6 had received antihypertensive treatment for more than 2 years.

- III. The natural history and the development of complications of microalbuminuria and hypertension in the cohort of 46 patients.
- IV. The morphological and clinical changes between second and third renal biopsy during a five year double-blind placebo-controlled treatment trial in 13 patients who were still normoalbuminuric and normotensive patients at second biopsy.

Table 2

The numbers of patients and investigations included in the different articles.

Article	I NANT	II NANT, AHT, MA/HT	III Natural History and Prediction of MA/HT	IV Treatment Trial
No of patients	19	29	46	13
Biopsy	Biopsy 1+2	Biopsy 1+2	Biopsy 1	Biopsy 2+3
Metabolic control	19 patients	29 patients	46 patients	13 patients
Urine Albumin Excretion Rate	17 patients (2 missing values at B1)	25 patients (4 missing values at B1)	41 patients (5 missing values at B1)	13 patients
Office blood pressure	19 patients	29 patients	46 patients	13 patients
24 h ambulatory blood pressure	16 patients (3 missing values at B1)	26 patients (3 missing values at B1)	40 patients (6 missing values at B1, 12 at B2)	13 patients
Kidney function	19 patients	29 patients	46 patients (5 missing at B2)	13 patients

4.2.1 Clinical follow-up

During the follow-up between first and second renal biopsy the adolescents had clinical check-ups every third month with HbA_{1c} and blood pressure (BP), once yearly urinary albumin excretion (UAE) and kidney function tests every second or third year.

When the patients reached 18-20 years of age, they were transferred to adult diabetic outpatient units with check-ups once every six months. The normal check-up for adult diabetes patients include HbA_{1c} and BP twice yearly, UAE and serum cholesterol once yearly. Kidney function and ABPM were planned to be done every second year from the Pediatric Nephrology Unit but this was not accomplished in the whole cohort. Some

patients continued at Karolinska University Hospital, Huddinge but some moved to other clinics in- or outside Stockholm.

Results of HbA_{1c} and UAE were collected during the follow-up period and results of cholesterol and triglycerides were collected at the endpoint. All kidney function tests were recorded from onset of diabetes while the results of ABPM were recorded at first and second renal biopsy (called baseline and the endpoint in paper III).

4.2.2 Group definitions

At the time of the second renal biopsy all patients' medical journals were reviewed concerning the development of MA, HT and if and when AHT was started. The patients were classified as: (a) patients who were normotensive (NA) and normoalbuminuric (NT) or (b1) treated patients i.e. those patients who developed complications of MA and/or HT and were on antihypertensive treatment during at least 2 years during the follow-up (AHT); (b2) untreated i.e., those patients who developed MA and/or HT during the follow-up, but had started AHT less than a year before the second renal biopsy (MA/HT).

4.2.3 Treatment trial

During the treatment study, the 13 patients underwent a physical examination every third month by the same person (NP) in the Children's Hospital, Karolinska University Hospital, Huddinge. The blood pressure, HbA_{1c}, UAE was determined and compliance was evaluated at every clinical check-up by counting the pills and giving the patient a new bottle of pills.

The pills, candesartan 16 mg or lactose 16 mg, were produced and packaged by Astra Zeneca and the pills could not be distinguished by the patients, medical or pharmacy personnel.

If the patients developed MA and/or HT during the study, they were given treatment with an ACE-inhibitor (enalapril), and continued the treatment study until its completion.

4.3 Methods

The investigations in connection with the first and second kidney biopsy were performed over a three-day period. On the first day, a renal function test was done. Overnight urine was collected on the first night to determine the urinary albumin excretion rate. On the second day, a renal biopsy was done. After the biopsy, the patients were kept supine for 24 hours and the pulse rate and blood pressure were checked at regular intervals. No severe complications occurred, apart from transient macroscopic hematuria in one patient and pain in the muscles overlying the biopsy site in another.

On the third biopsy a new protocol was tested. After the biopsy, the patients were observed for 8 h and if no complication occurred during the observation period and if

they did not have macroscopic hematuria, they were discharged. Of the 26 patients undergoing a third renal biopsy, 25 were discharged the same day. One patient had intraabdominal bleeding after the renal biopsy and was admitted to the hospital for two days. The probably cause of bleeding was puncture of a pool-artery in the kidney.

4.3.1 Metabolic control

The blood glucose provides instant information of how well the person with diabetes is controlled but the glycosylated hemoglobin, HbA_{1c} provides an averaged assessment of the metabolic control over the last three months. During the remission period and the prepubertal years, the children generally have good metabolic control (HbA_{1c} around 6-7 %) but during puberty, it is often very difficult to maintain good metabolic control (HbA_{1c} often around 8-9 %). The adolescents grow quickly with high levels of circulating metabolically active hormones (GH, IGF1, sex hormones), have greater insulin resistance and are subject to increased stress with enhanced release of cortisol and adrenaline, all of which influence glucose metabolism. They also develop more liberal life styles which depart from regularity, reflecting the difficulty that teenagers have with adjustments to the requirements of diabetic treatment.

Four different methods for determination of HbA_{1c} have been used since 1980. From 1980-1985, total HbA₁ was analyzed with ion exchange chromatography with a reference range of 5.5-8.5%. From 1986-1988, the FPLC method, (Fast Protein Liquid Chromatography; Pharmacia, Uppsala, Sweden) was used and after 1988 the HPLC method (1988-1996: KONTRON, Mono S kolonn, Pharmacia, Uppsala, Sweden; 1996-present: Variant, Bio Rad Laboratories, Hercules, CA, USA). The HbA₁ was assessed by converting to HbA_{1c}, using the equation $HbA_1 = 1.13 \times HbA_{1c} + 0.85$. In our laboratory, the correlation coefficient between the FPLC and HPLC methods was 0.957. The two methods were calibrated to show the same values and continuously checked with reference equipment. The normal reference range of HbA_{1c} were 3.4-5.0 %, which are about 0.7-1.0 % lower than those in the Diabetes Control and Complications Trial, DCCT study^{22, 107}.

The mean HbA_{1c} was determined for each year and the mean of all years was then calculated as a measure of long-term metabolic control since onset of diabetes. The metabolic control during a time period was calculated as the mean of the yearly mean HbA_{1c} during the time period. The patient's actual HbA_{1c} values at the time points have also been analyzed.

The metabolic control was also determined in different time periods to evaluate if the metabolic control in certain periods of the disease was more important than at other periods. We divided the time periods as: (a) early metabolic control (mean of all HbA_{1c} values 2 months - 2 years), (b) the prepubertal metabolic control (mean of all HbA_{1c} values from onset of diabetes to onset of puberty) and (c) postpubertal metabolic control (mean of all HbA_{1c} values from onset of puberty to 10 years duration).

4.3.2 Blood pressure

The blood pressure of children and adolescents varies with age, height and gender. Therefore the blood pressure (BP) must always be analyzed in relation to gender and

age/height¹⁰⁸. There are some limitations in the office BP wherein “white coat hypertension” is one of the most common problems, i.e., the patient gets nervous when visiting the physicians and the BP rises^{109,110}. A self measurement of the blood pressure at home can reveal white coat hypertension but the most appropriate way to differentiate true hypertension is to measure the blood pressure during 24 hours. In the adult setting, the hypertensive risk levels of 24 h ambulatory blood pressure (ABPM) correlates better to end organ damage, e.g., myocardial infarction, stroke, chronic kidney disease than office blood pressure¹¹¹. The ABPM is the most reproducible blood pressure measurement¹¹²⁻¹¹⁴. The average 24 h and day-time ABPM are generally 2-10 mm Hg lower than office BP^{115,116}. The night-time BP is normally 10-15 mm Hg lower than the day-time BP. The adult settings, target office BP for diabetes patients is < 130/80 and ABPM daytime < 135/85 and night-time 120/75¹¹⁷.

The office BP or casual BP was determined with a conventional mercury sphygmomanometer on the right arm after 5 min of rest in the sitting position. The 24-h ambulatory blood pressure was recorded with portable automatic Space Labs 90 207 equipment (Space Labs Inc., Wokingham, UK). The recordings were started at about 12.00 noon the day after the kidney biopsy when the patients were discharged. Daytime and nighttime BPs were based on standardized daytime (08.00 a.m. to 20.00 p.m.) and nighttime (00.00 a.m. to 06.00 a.m.) values, using the methods of Wuhl et al.¹¹⁸. Mean arterial blood pressure (MAP) was calculated as the diastolic BP plus one third of the difference between the systolic and diastolic BP. The daytime and nighttime systolic and diastolic BP and MAP were calculated using ABP PC Direct/Base Station Interface 90 219.

The percentage of the nighttime fall in BP (“dipping”) was calculated as: (daytime BP - nighttime BP) x 100/ daytime BP^{108,119}. Soergel et al.¹⁰⁸ found 13 (SD 6) % systolic and 23 (SD 9) % diastolic dipping in controls.

Since all patients had not reached final height at baseline, the standard deviation score (SDS BP) was calculated using the LMS method, taken from the formula by Cole and Green as adapted by Wuhl et al.¹¹⁸.

BP SDS = (BP (patient) / M (L(t)) - 1) / L (t) * S(t), where M is the median BP for gender and height, L is the measure of skewness and S is the coefficient of variation for given height (t) and gender.

The normal ABPM values for girls¹¹⁸ are below the adult levels (day-time 135/85 and night-time 120/75) but the boys exceed the BP limits at 14 years of age or at 165 cm’s height in day-time BP or 185 cm’s height in night-time BP (Wuhl personal communication). Therefore, also the SDS BP was calculated from the 14 years LMS’ values for the males. We have also used the ABPM percentiles (Wuhl, personal communication) and the categorization < and > 95th percentiles for given gender and height¹¹⁸.

Forty patients performed an ABPM at baseline (92% successful), 34 (94% successful) at 17 years duration and 31 patients (90% successful) at 23 years duration.

The diagnosis of hypertension was based either on repeated casual measurements > 130/85 or on ABPM > 95th percentile¹¹⁸.

4.3.3 Urine samples

One of the first clinical signs of diabetes nephropathy is the appearance of small amounts of albumin in the urine which is called microalbuminuria. Microalbuminuria is generally determined in a timed urine collection either as a 24 h collection with the reference value of $< 30 \mu\text{g}/\text{min}$ or as an overnight collection (reference value $< 15 \mu\text{g}/\text{min}$). During the recent years, spot urine samples have been collected for determination of the albumin to creatinine ratio (mg/mmol). The semi-quantitative test strip (Albustix) detects an albumin concentration above $300 \text{ mg}/\text{L}$.

We used the timed overnight-collected urine and analyzed for albumin with an automated immuno-nephelometric method (Behring Nephelometer Analyser; Behringwerke AG, Marburg, Germany) at first biopsy and immunoturbidometric method (during 2001-2003, Roche Regencies and Hitachi equipment 917, Roche, Boston, MA, USA; after 2003, Tinaquant Albumin, Modular, Roche) during follow-up. The different methods were calibrated to show the same values. The detection level of albumin concentration was $4 \text{ mg}/\text{L}$.

Persistent MA was defined as $\text{UAE} > 20 \mu\text{g}/\text{min}$ in two of three urine samples during a six-month period, determined in the overnight urine collections.

4.3.4 Renal function tests

To evaluate renal hemodynamics in a proper way, glomerular filtration rate and renal plasma flow should be determined. The ideal marker substance to determine the glomerular filtration rate (GFR) is one that is freely filtered through the glomerular capillary wall into the tubular fluid (not protein bound), and should not be secreted, reabsorbed or metabolised after glomerular filtration. Inulin and iohexol are suitable GFR marker substances. To determine effective renal plasma flow (ERPF), the marker should be filtered, secreted and completely cleared from the plasma. Para-aminohippuric acid (PAH) is a suitable ERPF marker.

We measured clearances of inulin and PAH during water diuresis using standard clearance technique. After a priming dose of inulin and PAH, to reach equilibrium, a continuous infusion of inulin and PAH is given over 3 h. Blood samples for plasma concentrations and urine collections for urine concentrations are obtained at timed intervals with the blood sample taken in the middle of the urine collection period.

The patients had performed a median number of 5 (lower quartile 4; upper quartile 5) kidney function tests since onset of diabetes before the first renal biopsy and an additional 2 (1; 3) kidney function tests during follow-up.

Until 1996, plasma and urine inulin concentrations were determined with the anthrone method and thereafter with an enzymatic method, Deltakonc¹²⁰. PAH was analysed photometrically with a modification of the Smith technique¹²¹. The filtration fraction, FF, was calculated as the ratio of GFR to ERPF, (%). As PAH was unavailable in two patients at the time of the second biopsy, ERPF and FF could not be determined.

Various analyses of GFR were made e.g., GFR at first biopsy and follow-up, the Δ GFR between first biopsy and follow-up, the mean of all previous GFR before first biopsy and follow-up, slope of GFR vs. time and presence of hyperfiltration in the individual patients (GFR > mean + 2 SD of the controls).

We compared the patients' renal function with that of healthy adolescents and young adults, matched for age and gender on a group level, and evaluated in our Pediatric Nephrology Unit. The controls at 17 and 24 years of age (corresponding to 10 and 17 years duration) had GFR 117 (11) and 114 (13), ERPF 639 (103) and 626 (113) ml/min/1.73 m² and FF 18.6 (2.6) and 18.8 (2.9) % at mean ages of 17.8 (3.4) years (34 controls) and 24.3 (5.2) years (46 controls), respectively. At 30 years of age the controls (15 controls) had GFR 113 (13) ml/min/1.73 m², ERPF 602 (106) ml/min/1.73 m² and FF 19.2 (3) %.

4.3.5 Renal biopsy

The patients were sedated with midazolam and morphine and the percutaneous renal biopsy was performed by a radiologist under lidocaine local anesthesia with ultrasound guidance (Acuson XP 10 and Acuson Sequoia 512, Mountain View, CA, USA) using an automatic biopsy device (Bard Magnum Biopsy Instrument, Urological, Covington, CA, USA) and a 16-gauge needle (Bard Magnum Core Tissue Biopsy Needle, Urological). The biopsy needle was inserted two or three times to obtain adequate histological material. The biopsies were directly fixed in 4% paraformaldehyde in phosphate buffer.

All biopsies were examined by a single nephropathologist (GAJ) at Karolinska Institutet without knowledge of the patient's history.

4.3.5.1 Light microscopy

The general features of the kidney were analyzed by light microscopy: numbers of glomeruli and numbers of sclerosed glomeruli, glomerular volume, presence of hyaline in the Bowmans capsula, mesangial matrix or mesangial volume expansion, the interstitium and the tubules, the presence of immune complexes, inflammation, fibrosis and presence of arteriolar intimal hyalinization.

The glomerular volume (GV) was estimated by Cavalieri's principle (see figure 6)¹²². The glucolmethacrylate-embedded tissue blocks were cut serially into 2.5- μ m thick sections and stained with periodic acid-Schiff. The glomeruli were sampled in the order in which they became visible in the sections and thereafter followed through at regular intervals (every 4th section). The number of sampled levels per glomerulus ranged from 13 to 21.

The area of the profiles was estimated by point counting using an ocular grid (see figure 6) and the section thickness (d) was estimated by the method based on confocal microscopy¹²³. The glomerular volume was then calculated as sum of points at all levels times d. In each biopsy, about 15 (12-17) glomeruli were measured and the mean GV calculated. In one patient, it was not possible to determine the glomerular volume in the second biopsy due to fibrotic sample area, possibly the scar after the first biopsy.

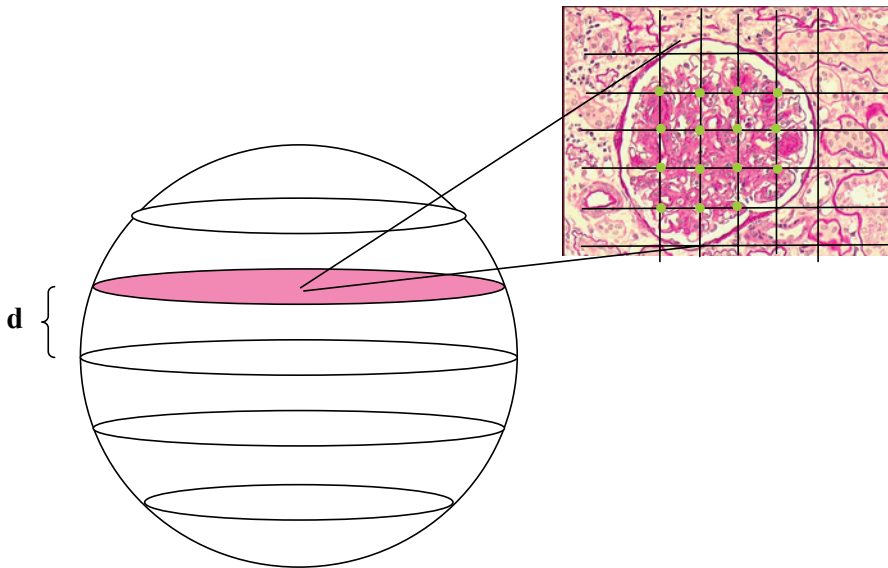


Figure 6 Glomerular volume according to Cavalieris method:
 Glomerular volume = sum of all • in all five sections x mean thickness **d**

4.3.5.2 *Electron microscopic quantitation*

The electron microscopy gives the possibility to analyze the changes observed in the light microscopy in greater detail and to quantify the morphological parameters in order to compare with controls or to compare changes over time in the same patients.

Small tissue blocks for electron microscopy were postfixed and embedded in Polybed 812. The biopsies were cut in ultra-thin sections, stained with uranyl acetate and lead citrate and four to six glomeruli from each biopsy were examined. As the whole glomerulus cannot be examined, glomerular profiles are sampled in a systematic random manner and 7 to 19 electron micrographs per glomerulus were examined which cover ca 40 % of the whole glomerulus. The micrographs, examined at a magnification of about 10 000X, were corrected using a grating grid (EF Fullan, Inc., 28 800 lines/inch). The reference space, called “glomerulus”, was defined as a minimal convex polygon enclosing the glomerular tuft.

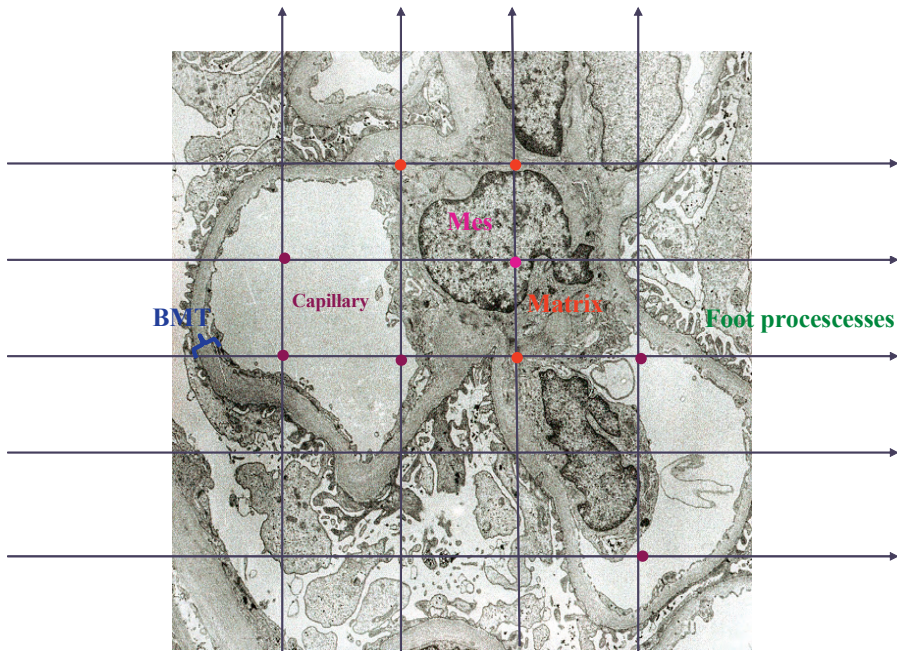


Figure 7

Mesangial and mesangial matrix volume fractions per glomerulus (V_V (mes/glom) (●+●) and V_V (matrix/glom)) (●) were estimated by point counting using a superimposed lattice square grid (Figure 7). The numbers of points contacting the mesangial matrix or the mesangium were related to the total number of points contacting the “glomerular” reference space. The horizontal lines in the grid, where they contact the epithelial aspect of the basement membrane, are used to measure the basement membrane thickness (BMT) using the orthogonal intercept method of Jensen and co-workers¹²⁴. The capillary volume is determined by the number of points contacting the open capillary (●). The mean foot process width is determined by the ratio of the total surface density of the peripheral capillary wall divided by the length density of filtration slits.

We analyzed both the absolute values of GV and BMT and $GV/1.73 \text{ m}^2$ body surface area (BSA) and $BMT/1.73 \text{ m}^2$ BSA, denoted as GV/BSA and BMT/BSA , respectively, since we were studying growing adolescents.

Plastic embedded renal biopsy tissues from controls (donors) were obtained from Prof Michael Mauer, Dept. of Pediatrics, University of Minnesota, Minn, USA. The control biopsies were prepared by cutting the tissue blocks as previously described and then morphometrically analyzed. Since renal tissue prepared for light microscopic analysis and data on the patients’ height and weight were not obtained, glomerular volume was not determined. All patients’ and control biopsies were examined by a single nephropathologist (GAJ).

4.4 Statistical analyses

First the normality and skewness of the parameters were analyzed. The normally distributed parameters are presented as mean (standard deviation, SD) in describing the group and the Student's paired t-test in comparisons of the change between two time points. To compare two normally distributed groups at the same time point the t-test was used.

The skewed parameters were described as median (lower; upper quartiles) and comparison in paired variables were done with Wilcoxon's paired test and for comparison at same time point between groups, the Mann-Whitney U-test was used.

Multiple regression analysis was used to analyze the relation between morphological changes and clinical parameters. In comparison between the three groups (paper II) the one-way ANOVA was used followed by post-hoc t-tests with the Bonferroni adjustment for normally distributed variables. We used Kruskal-Wallis ANOVA by ranks followed by multiple comparisons of groups based on ranks with the Bonferroni adjustment for variables having a skewed distribution.

In the analyses of risk factors and the prediction of negative outcome logistic regression analysis were performed. Correlations and odds ratio were calculated both on continuous variables and categorical variables. For the stepwise logistic regression analyses the SAS system was used, otherwise the statistical program of Statistica 7.0 was used. $P < 0.05$ was considered significant.

4.5 Ethical consideration

All studies were approved by the Ethical Committee of Karolinska Institute at Karolinska University Hospital, Huddinge, former called Huddinge University Hospital and Huddinge Hospital. The patients had given their informed consent for each of the renal biopsies and the treatment trial.

5 RESULTS

The results in this thesis are based on the following publications:

- I. The morphological changes between first and second renal biopsies (6 year interval) in 19 normoalbuminuric and normotensive type 1 diabetes patients in relation to metabolic control, ambulatory blood pressure and renal function. We have analyzed differences between men and women and predictors from first to second renal biopsy.
- II. The morphological changes between first and second renal biopsies (6 year interval) in 29 patients of whom 10 developed microalbuminuria and/or hypertension and 6 had received antihypertensive treatment for more than 2 years. The morphological differences between normoalbuminuric, normotensive patients and those who developed complications, both treated and untreated, were compared.
- III. The natural history in the cohort of 46 patients (metabolic control, insulin use, blood pressure evolution, cholesterol) and the development of complications of microalbuminuria and hypertension. Clinical and morphological parameters from the first renal biopsy were evaluated as possible risk factors for the outcome of MA and/or HT.
- IV. The morphological and clinical changes between the second and third renal biopses during a five year double-blind placebo-controlled treatment trial in 13 of those 19 patients still normoalbuminuric and normotensive at the time of the second renal biopsy.

5.1 General description of the cohort

Tables 3 - 5 present the complete data for the 46 patients at the time of the first renal biopsy and at follow-up after 17 and 23 years duration. These results are not presented in the articles as the articles concern different portions of the cohort at the follow-up endpoint of 17 years duration (article I, II and III) and the treatment trial with the endpoint of 23 years duration (article IV). The frequency of hypertension (HT), microalbuminuria (MA) and antihypertensive treatment (AHT) at 23 years duration, has been determined after the third renal biopsy and after the completion of treatment trial. If the frequency of hypertension, microalbuminuria and AHT had been determined exactly at the time of the third renal biopsy certain patients in the treatment trial would have been regarded as normoalbuminuric and normotensive because of treatment and certain other patients would have been identified as treated patients even though they did not have clinical indications for treatment.

At 10 years duration the cohort was normoalbuminuric, normotensive and free of other diabetes complications. The frequency of complications increased during the follow-up period (Table 3). At 23 years duration, 2 patients have severe cardiovascular disease, 2 patients have rapidly declining kidney function and 18 patients have hypertension and/or microalbuminuria. Nine patients have severe diabetic retinopathy and received photocoagulation treatment and 7 patients have severe neuropathy with gastropareses, pain or impotence. Twenty three of the 46 patients do not have signs of hypertension, microalbuminuria, diabetic retinopathy or diabetic neuropathy.

During the follow-up the number of antihypertensive treated patients increased. At 17 years duration 8 patients had started on AHT. After the treatment trial, at around 23 years duration, 18 patients were on AHT. The need for insulin decreased and metabolic control improved when the patients left adolescence and entered adulthood (Table 3).

Table 3

Clinical data of 46 patients with type 1 diabetes at 10 (B1), 17 (B2) and 23 (B3) years duration, respectively. Results are given as number (%), mean (SD) or median (lower; upper quartiles). The p-value signifies if there are any changes during follow-up, either between 10 and 17 years duration or between 17 and 23 years duration.

	10 years duration (B1)	17 years duration (B2)	23 years duration (B3)	p-value
Age	17.7 (2.8)	24.1 (3.3)	30.1 (3.0)	< 0.0001
Duration	10.6(3.3)	17.0 (3.5)	23.0 (3.1)	< 0.0001
Weight (kg)	63.9 (11)	71.8 (10)	75.2 (13)	< 0.0001
BMI	22.3 (2.8)	24.2 (3.0)	25.4 (4)	< 0.0001
Clinical hypertension (BP>130/80 mm Hg)	0	12 (26 %)	16 (35 %)	0.00002 (0.0015 B1-B2)
Persistent microalbuminuria (MA) (>20 µg/min)	0	8 (17 %)	11(24%)	0.00015 (0.013 B1-B2)
Complication (HT+ MA)	0	15 (33 %)	18 (39 %)	<0.00001 (0.0003 B1-B2)
Antihypertensive treatment	0	8 (17 %)	18 (39 %)	<0.00001 (0.013 B1-B2) (0.009 B2-B3)
Severe Neuropathy	0	3 (7%)	7 (15%)	0.01
Proliferative Diabetic Retinopathy	0	6 (13%)	9 (20 %)	0.0009 (0.04 B1-B2)
Long-term yearly HbA _{1c} (%)	8.0 (7.4; 9.1)	8.0 (7.5; 8.6)	7.8 (7.4; 8.5)	0.0003 (0.027 B1-B2) (0.0007 B2-B3)
Insulin doses (U/kg)	0.98 (0.8; 1.0)	0.83 (0.7; 1.0)	0.64 (0.6; 0.9)	< 000.1 (0.012 B1-B2) (0.000005 B2-B3)
Insulin regime				
Pump	5/46 (11 %)	6/46 (13 %)	9/46 (20 %)	
3 doses	1/46 (2 %)	0	1/46 (2 %)	
4-doses	10/46 (22 %)	8/46 (17 %)	14/46 (30 %)	
5-doses	27/46 (59 %)	23/46 (50 %)	17/46 (37 %)	
6 doses	3/46 (6 %)	9/46 (20 %)	5/46 (11 %)	

The office systolic blood pressure increased from 10 to 17 years duration as did the night-time diastolic blood pressure (Table 4). The dipping changed during the follow-up concomitant with the introduction of AHT. The urinary albumin excretion did not change at the group level but 8 and 11 patients had developed microalbuminuria at 17 and 23 years duration, respectively. Most of the patients received treatment and therefore a reduction in UAE was anticipated.

In regard to kidney function, patients exhibited hyperfiltration through 20 years duration and thereafter GFR returned to normal at 23 years duration (Table 4). The fraction of hyperfiltering patients was gradually but not significantly decreasing from 34% to 21% at last follow-up. The filtration fraction (FF) decreased slightly during follow-up from 22% to 20%.

Table 4

Data from the investigations at 10 (B1), 17 (B2) and 23(B3) years duration. Day- and night-time blood pressure refers to 24 h ambulatory blood pressure measurement. BP is shown as mm Hg and GFR and ERPF are shown as ml/min/1.73 m². The normally distributed values are presented as mean (SD) and the skewed data as median (lower; upper quartiles). The p-value signifies if there are changes during follow-up, either between 10 and 17 years duration or between 17 and 23 years duration.

	10 years duration (B1)	17 years duration (B2)	23 years duration (B3)	p-value
Office blood pressure, syst.	122 (12)	128 (16)	128 (17)	0.016
Office blood pressure, diast.	73 (10)	76 (9)	73 (10)	Ns
Day syst. BP	129 (8) (n=40)	126 (10) (n=34)	126 (13) (n=32)	Ns
Day diast. BP	77 (6)	75 (7)	76 (7)	Ns
Night syst. BP	113 (10)	113 (10)	112 (13)	Ns
Night diast. BP	60 (7)	63 (7)	65 (8)	0.007
Syst. dipping	14 (11; 19)	12 (9; 15)	17 (10; 20)	0.026
Diast. dipping	17 (14; 21)	13 (8; 16)	14 (6; 18)	0.015
Syst. dip%	12 (9; 15) %	10 (7; 12) %	12 (9; 15) %	0.048
Diast. dip %	22 (18; 28) %	17 (11; 23) %	18 (9; 23) %	0.008
Cholesterol	4.5 (4.1; 5.0) (n=36)	4.2 (3.9; 4.7) (n=38)	4.3 (3.9; 4.8)	Ns
Triglyceride	1.1 (0.8; 1.4)	1.0(0.8; 1.6)	1.0 (0.6; 1.2)	0.009
UAE (µg/min)	6 (4; 13)	8.5 (4.5; 22)	10.0 (4; 47)	Ns
No of patients under median AER	22/41 (54 %)	29/43 (67 %)	33/45 (73 %)	Ns
UAE <4 µg/min	7/41 (17 %)	21/43 (49 %)	26/45 (58%)	
UAE 4-10 µg/min	24/41 (59 %)	11/43 (26 %)	7/45 (15%)	
UAE 10-20 µg/min	4/41 (10 %)	4/43 (9.5 %)	4/45 (9%)	
UAE >20 µg/min	6/41 (15 %)	7/43 (16.5 %)	8/45 (18%)	
Mean previous GFR	137(18)	134 (16)	130 (17)	0.007
Mean previous ERPF	630 (83)	624 (93)	625 (108)	Ns
Mean previous FF	22.1 (2.5)	21.9 (2.2)	21.4 (2.6)	Ns
GFR	133 (22)	130 (23)	116 (24)	0.002
GFR>2SD(% hyperfilterers)	35 %	34 %	21 %	Ns
ERPF	610 (130)	656 (166) (n=41)	580 (120) (n=34)	Ns
FF (%)	21.9 (19.7; 24.1)	20.9 (17.7; 22.4)	20.2 (17.5; 21.9)	0.006

Kidney biopsies were done in 46 patients at 10 years duration, 29 patients at 17 years duration and 26 patients at last follow-up (Table 5). Those 29 patients who had 2 renal biopsies were invited for a third renal biopsy; all but 3 (one of whom suffered from severe cardiovascular disease) accepted. The morphometric parameters show an increase in glomerular volume in absolute terms and in comparison to body surface area. The BMT was stable after the first biopsy. The mesangial matrix volume, the mesangial volume and the foot process width increased at the second biopsy but diminished at the third biopsy.

Table 5

Morphological data of kidney biopsies performed at 10, 17 and 23 years duration of diabetes. The normally distributed values are presented as mean (SD) and the skewed data as median (lower; upper quartiles). The p-value signifies if there are any changes during follow-up, either between first and second biopsy or between second and third biopsy.

	First biopsy	Second biopsy	Third biopsy	p-value
	n=46	n=29	n=26	
GV (M μ m)	2.6 (2.2; 3.0)	3.4 (3.0; 3.8)	3.6 (3.2; 4.2)	0.0001
GV/BSA (M μ m/1.73 m ²)	2.6 (2.2; 3.0)	3.2 (2.8; 3.7)	3.2 (2.9; 3.8)	0.0005
BMT (nm)	477 (427; 545)	514 (470; 592)	510 (392; 555)	Ns
BMT/BSA (nm/1.73 m ²)	487 (428; 564)	482 (446; 522)	440 (408; 517)	Ns
V _v (matrix/glom) (%)	10.1 (8.9; 11.7)	11.5 (10.7; 13.0)	9.1 (8.3; 10.7)	0.0005
V _v (mes/glom) (%)	19.3 (17.1; 20.9)	22.7 (20.5; 24.1)	18.8 (16.9; 22.1)	0.00024
Foot process width (nm)	416 (37)	458 (54)	449 (52)	0.0009

5.2 Results from the studies

We describe the changes in metabolic control, blood pressure, albumin excretion rate, renal function and morphology in the different groups: normoalbuminuric and normotensive (NANT) and microalbuminuric and/or hypertensive (MA/HT) as described in the various studies. The MA/HT classification was further analyzed in 29 patients with multiple renal biopsies as well as the total cohort. MA/HT has been stratified as MA/HT with AHT for at least 2 years (AHT) and MA/HT with AHT for less than a year at the time of the second renal biopsy (MA/HT). The number of NANT patients in study I (2 biopsies) is 19 and in study III (one biopsy) is 31. The patients with microalbuminuria and hypertension in study II (2 biopsies) are 10 (6 AHT and 4 MA/HT patients) and in study III are 15.

5.2.1 Metabolic control

5.2.1.1 *Normoalbuminuric and normotensive patients (NANT)*

Metabolic control did not change in the NANT patients (both male and female) between the first and second renal biopsy.

5.2.1.2 *Microalbuminuric, hypertensive and antihypertensive treated patients (MA/HT and AHT)*

In the entire cohort, the 15 MA/HT patients (study III) had poorer long-term metabolic control from onset to 10 years and 17 years duration compared to the NANT group.

The AHT-treated MA/HT patients showed a decrease in long-term HbA_{1c} during follow-up to the time of the second renal biopsy (study II). Before the first renal biopsy, the untreated MA/HT patients in study II had the same level of HbA_{1c} as the NANT group but their metabolic control became worse during the interval between the first and second renal biopsies.

5.2.1.3 *Correlations*

The long-term metabolic control correlated strongly with BMT, V_v (matrix/glom) and V_v (mes/glom) at the time of the second renal biopsy in the group of 29 rebiopsied patients (study II) and significantly but slightly weaker in the NANT group of 19 patients (study I).

The metabolic control between the first and second renal biopsies also correlated closely with the changes in BMT (Δ BMT), ΔV_v (matrix/glom) and ΔV_v (mes/glom) between first and second biopsy (study II).

5.2.1.4 *Prediction*

The long term metabolic control together with other factors such as night-time systolic BP and time interval between the renal biopsies were the strongest predictors for the evolution of the morphological changes between the first and second renal biopsy in the NANT group in study I.

5.2.2 Blood pressure

5.2.2.1 Normoalbuminuric and normotensive patients (NANT)

The night-time diastolic BP rose from first to second renal biopsy; this occurred only in the male patients. The males were still growing with an increase in height from 174 to 180 cm while the females did not exhibit further growth. The daytime systolic BP was also higher in males than in females.

5.2.2.2 Microalbuminuric, hypertensive and antihypertensive treated patients (MA/HT and AHT)

At 10 years duration the cohort of 15 patients that developed MA or HT during follow-up of 6 years had higher day-time systolic, night-time systolic and diastolic BP (absolute values) and in SDS BP than the 31 NANT patients (study III).

In the AHT treated MA/HT patients, the day-time SDS BP decreased and dipping was unchanged during the follow-up of 17 years duration (study III) compared to the untreated MA/HT patients who showed less systolic dipping.

Comparing the patients classified as MA with HT patients at 17 years duration, a greater tendency to higher office systolic BP was seen in the HT-patients compared to the MA-patients (study III).

5.2.2.3 Correlations

Day-time diastolic BP at second renal biopsy correlated with BMT and foot process width at second renal biopsy in study II within the entire rebiopsied group as well as in NANT patients in study I.

5.2.2.4 Prediction

The day-time systolic BP at first renal biopsy in the 19 NANT patients in study I predicted the BMT at second renal biopsy and the night-time systolic BP predicted the V_v (mes/glom) and foot process width at second renal biopsy.

5.2.3 Albumin excretion rate

5.2.3.1 Normoalbuminuric and normotensive patients (NANT)

The levels of urinary albumin excretion rate were similar at the first and second renal biopsies in both sexes.

5.2.3.2 Microalbuminuric, hypertensive and antihypertensive treated patients (MA/HT and AHT)

The UAE at the first renal biopsy did not differ between the patients that developed MA or HT during follow-up and those who remained NANT. At 17 years duration when 8 had developed MA, their UAE was 101 (26; 139) $\mu\text{g}/\text{min}$ and the UAE of the 7 HT patients was 7.5 (5.5; 18.5) $\mu\text{g}/\text{min}$. The MA patients differed from that of the NANT patients whose UAE was 5 (3; 9) $\mu\text{g}/\text{min}$, $p=0.0001$ and from HT patients,

$p=0.02$. The UAE at 17 years duration did not differ significantly between treated or untreated but the sample sizes were small.

5.2.3.3 Correlations

The patients with UAE above the median level at the first renal biopsy in the NANT group in study I had lower GV/BSA at first renal biopsy than those with UAE below the median level. The patients with UAE values above the median UAE level at the second renal biopsy had a larger foot process width at second renal biopsy than those whose UAE values were below the median UAE in study I.

Log UAE correlated most strongly with V_v (matrix/glom) and V_v (mes/glom) and inversely with slit pore length density in the entire group of rebiopsied patients in study II.

5.2.4 Kidney function

5.2.4.1 Normoalbuminuric and normotensive patients (NANT)

No change occurred in GFR, ERPF or FF from the first to the second renal biopsy. However, after 17 years' duration, the patients still showed hyperfiltration and FF was significantly higher as compared to controls. ERPF was similar at the time of both renal biopsies as in the controls. The males had higher ERPF and lower FF than the females at the second renal biopsy.

5.2.4.2 Microalbuminuric, hypertensive and antihypertensive treated patients (MA/HT and AHT)

In the time interval between 10 and 17 years duration, GFR decreased more (-2.3 ml/min/ 1.73 m²/year) in the 15 MA/HT patients than in the NANT patients (-0.6 ml/min/ 1.73 m²/year), $p=0.04$ (study III).

In study II, the five patients receiving ACE-inhibitor showed a tendency for GFR to decrease between the first and second renal biopsies, -1.5 (-4.7 ; -1.5) ml/min/ 1.73 m²/year, which was not significantly different from the values of $+0.3$ (-1.3 ; $+2.0$) ml/min/ 1.73 m²/year in the 19 NANT patients and -1.4 (-3.6 ; $+0.7$) in the 4 untreated MA/HT patients. FF did not change in the treated or untreated groups.

Comparing the MA patients with HT patients, the MA patients decreased their GFR during follow-up by 5.4 ml/min/ 1.73 m²/year compared to $+0.14$ ml/min/ 1.73 m²/year in HT patients (study III).

5.2.4.3 Correlations and prediction

Positive correlations were found between the mean FF before the first renal biopsy and BMT, V_v (matrix/glom), and V_v (mes/glom) at the first renal biopsy in the 29 rebiopsied patients in study II. However, at the second renal biopsy, correlations between kidney function and the morphometric parameters or in the changes in morphology between the two renal biopsies were not found.

5.2.5 Morphology

5.2.5.1 *Normoalbuminuric and normotensive patients (NANT)*

The NANT patients showed an increase in glomerular volume in absolute terms and in relation to BSA from the first to the second renal biopsy. The males had a tendency to smaller GV/BSA at first biopsy but it increased significantly to the second biopsy. The females, however, still had larger GV/BSA than males at the second biopsy. The BMT increased in absolute terms but not in relation to BSA. No gender difference was observed in BMT/BSA.

The V_V (matrix/glom) and V_V (mes/glom) increased significantly from the first to the second biopsy. Females had larger values at the first biopsy but the values of males increased to the level of the females at the second biopsy. The foot process width increased significantly between the two renal biopsies, seen only in the males.

5.2.5.2 *Microalbuminuric, hypertensive and antihypertensive treated patients (MA/HT and AHT)*

The MA/HT group had increased BMT, BMT/BSA and V_V (matrix/glom) at the first renal biopsy compared with NANT groups (study III).

In study II, in the patients who received AHT, a decrease in BMT and no further change in V_V (matrix/glom) and V_V (mes/glom) between the two renal biopsies was seen in contrast to the MA/HT patients without treatment, who showed a tendency to an increase in BMT and a significant increase in V_V (matrix/glom) and V_V (mes/glom) during follow-up.

5.2.5.3 *Multiple regression analysis with morphology at second renal biopsy as dependent variable*

BMT/BSA and V_V (matrix/glom) at the second renal biopsy were strongly related to long-term metabolic control and gender (males at risk) in the rebiopsied group in study II (adjusted $r^2=64\%$ and 24% , respectively). The long-term metabolic control and log UAE correlated strongly with V_V (matrix/glom) (adjusted $r^2 = 63\%$).

In the NANT group in study I, a similar relation between long-term metabolic control and gender was shown for BMT/BSA while V_V (matrix/glom) and V_V (mes/glom) correlated with long-term metabolic control and post-pubertal duration.

5.2.5.4 *Predictive factors for morphology outcome at second renal biopsy*

The strongest predictive factors for the morphological changes between the first and second renal biopsy in the NANT group in study I were the night-time systolic BP at first renal biopsy, long term HbA_{1c} and time interval between the two renal biopsies.

5.2.6 General predictors for the outcome of MA/HT

The investigations at 10 years duration have been scrutinized as possible risk factors for MA/HT outcome at 17 years duration. It was found that long-term metabolic control, day-time systolic BP and night-time systolic and diastolic BP and BMT/BSA could be used as risk factors.

Different time intervals of long term metabolic control were analyzed and it was found that the post-pubertal metabolic control was the strongest risk factor predicting the outcome of MA/HT at 17 years duration. A threshold of postpubertal HbA_{1c} > 8.2 % was observed.

From the ABPM at the time of the first renal biopsy, the day-time systolic BP, day-time systolic SDS BP and night-time systolic and diastolic BP were the best predictors for the outcome of MA/HT at 17 years duration. The threshold BP was determined to be day-systolic BP > 130 mmHg and night-time BP > 120/59 mm Hg.

None of the different GFR measurements (GFR at 10 years and 17 years duration, mean of all previously performed GFR, GFR-slopes, GFR-profile tendencies, presence of hyperfiltration) had predictive value for outcome of MA/HT at 17 years duration.

Concerning the morphometric parameters, the BMT/BSA and V_v (matrix/glom) at first renal biopsy predicted the outcome of MA/HT at 17 years duration. The threshold for BMT/BSA was 490 nm.

The combination of long-term post-pubertal HbA_{1c}, BMT/BSA and day-time systolic BP at 10 years duration accounted for 62% of the outcome of MA/HT at 17 years duration.

5.2.7 Treatment trial

In the treatment trial, 13 patients who were normoalbuminuric and normotensive at the second renal biopsy participated. After the second renal biopsy they began a 5 year double blind placebo controlled treatment trial study with an angiotensin receptor blocker, candesartan at 16 mg.

The thirteen patients (6 males) were 24 (22.6; 25.5) years of age at the start of the treatment study and 30.3 (28.1; 32.7) years at follow-up and had had diabetes for 16.4 and 21.9 years, respectively. During follow-up 2 patients developed hypertension and/or microalbuminuria and ACEi was added. Two patients dropped out, one because of side-effects.

After decoding the trial, it was found that 7 patients were treated with candesartan and 6 with placebo. The drop-outs and additional drug treatment occurred in the placebo group. Candesartan was well-tolerated.

A decline in mean yearly long-term HbA_{1c} from onset to follow-up was seen in the candesartan group but the median HbA_{1c} during the study was similar in the candesartan- and placebo groups.

At follow-up, the office blood pressure was significantly higher in the placebo group than in the candesartan group. We found no significant difference in the decline in GFR between the groups (median 1 ml/min/1.73 m² in the candesartan group vs. -2.5 ml/min/1.73 m² in the placebo group).

In regard to morphology, a significant decline in the V_V (mes/glom) and V_V (matrix/glom) was seen in the candesartan group and a tendency (not significant) to decline in the placebo group. However, we found no significant differences between the groups in the Δ GV, Δ BMT, ΔV_V (mes/glom) and ΔV_V (matrix/glom).

6 DISCUSSION

A cohort of normoalbuminuric and normotensive patients with type 1 diabetes has been studied from onset of diabetes to 23 years duration. In addition to examining the natural history of the disease prior to intervention, the change in the natural history of diabetic nephropathy in the presence or absence of intervention has also been characterized.

In the first renal biopsy performed 10 years after onset, changes typical of diabetic glomerulopathy (DG)⁴⁴ were observed in unselected normoalbuminuric and normotensive (NANT) patients. In a second renal biopsy, done 6 years later, the DG changes had become worse in stationary NANT patients¹²⁵. Males, who were still growing between the two renal biopsies, showed an increase in the V_V (mes/glom) and V_V (matrix/glom) to the levels of the females but the GV/BSA was not increased to the same extent. Those patients who developed microalbuminuria and/or hypertension between the two renal biopsies also exhibited progressive DG changes¹²⁶. However, those patients with MA and/or HT who improved their metabolic control and were treated with AHT for at least two years did not progress further or even exhibited renal morphological regression¹²⁶.

The predictive factors at 10 years duration for the outcome of MA/HT at 17 years duration were long-term metabolic control, day-time systolic or night-time blood pressure and increased BMT/BSA.

The patients who were still NANT after the second renal biopsy participated in an intervention study, a double-blind placebo-controlled long-term candesartan treatment trial. A lower blood pressure compared to placebo and a reduction in V_V (mes/glom) and V_V (matrix/glom) was seen in a third renal biopsy.

6.1 Clinical outcome

The cohort is an unselected population and may be considered as representative for all patients with type 1 diabetes in Sweden. In the cohort, 33% of the patients developed complications of MA/HT after 17 years duration but none had persistent proteinuria or macrovascular complications which are in accordance with recent reports^{29, 30, 32, 127}.

6.2 Morphological changes

Some studies have been done on the natural history of the early morphological changes in NA patients^{43-45, 128-132}. The DG changes are well described in patients with type 1 diabetes with varying amounts of albuminuria^{42, 43, 132-136}. Our NANT patients showed thicker BMT and larger V_V (matrix/glom) but normal V_V (mes/glom) at the time of the first renal biopsy compared to controls¹³⁷ which is in agreement with other reports^{42, 45, 135}.

The large GV both in NANT and in all rebiopsied patients agrees with the findings of others, generally in microalbuminuric patients or in relation to UAE^{41, 130, 138, 139}, but exceeds reported values of other groups^{45, 131, 133, 140}. The differences in GV are

presumably due to the level of UAE and the different methods used in handling and preparing the renal biopsy specimens. We used plastic embedment for light microscopy. Deformation of the glomerulus occurs to a greater extent with paraffin than with plastic embedment^{141, 142}.

The finding of an increase in glomerular volume between renal biopsies is in agreement with other reports^{131, 138, 139}, most of which concern patients with microalbuminuria.

In the studies, no significant change occurred in BMT when corrected for BSA in the NANT patients and in entire group of patients, a finding also reported by Fioretto¹³¹ who studied both NA and MA patients. Drummond et al. have also shown a gradual increase in BMT up to 15 years duration and no further increase thereafter¹⁴³.

The thicker BMT/BSA in the treated MA/HT group is in agreement with reports on microalbuminuric patients^{42, 144, 145}.

A significant increase in mesangial matrix volume and mesangial volume fraction was found in the NANT patients and in entire group of patients, between first and second renal biopsies with the greatest increase being seen in the mesangial volume fraction, as reported by Fioretto et al.¹³¹.

The increase in foot process width that was found between first and second renal biopsies has not been previously noted. The total slit pore length, as a product of slit pore length density and GV, was unchanged in contrast to a decrease in the slit pore length density. The total slit pore length is of more relevance in reflecting the total filtration area. With an increasing GV, the total slit pore length remains at the same level although a decrease is seen in the slit pore length density. Perhaps GFR remains stable as long as the total slit pore length is well maintained but if the slit pore length density continues to decline the GFR will decrease when the GV cannot further increase.

6.2.1 Morphology and BSA

The GV and BMT were normalized to BSA as our adolescents and young adults were still growing and increased their BSA from 1.74 m² to 1.86 m² between the first and second renal biopsies. GV has been shown to increase with BSA in children with the nephrotic syndrome¹⁴⁶ and Neugarten et al. also found a correlation between GV and BSA in autopsy studies of patients without diabetes¹⁴⁷.

The BMT correlated with BSA and the increase in BMT between the two renal biopsies was not seen BMT was normalized to BSA (BMT/BSA).

6.2.2 Metabolic control and morphology

Correlations were found between long-term metabolic control and BMT, V_v (matrix/glom), and V_v (mes/glom). The MA/HT group had significantly higher long-term HbA_{1c} at 10 years duration compared with the NANT group.

Metabolic control has convincingly been proven to be important for progression to MA or DN^{18, 24, 28-30, 42, 101, 125-127, 132, 139, 144, 148-153}. The correlation between metabolic control and renal morphological changes has been reported to be stronger^{42, 44, 132, 144} or weaker^{45, 131, 140} in relation to the different methods used to evaluate metabolic control. Some have determined a single HbA_{1c} at the time of the investigation^{43, 129, 132, 140}, while others have used the one-year or five-year mean HbA_{1c} before the renal biopsy^{42, 45, 144} or the mean HbA_{1c} between the renal biopsies¹⁵⁴. The importance of metabolic burden and metabolic long-lasting effect has also been demonstrated in recent years^{149, 155}. Therefore, it is more appropriate to analyze the total metabolic control since onset or since puberty.

6.2.3 Gender and morphology

Male and female patients were studied separately to assess gender differences and it was found that the females showed earlier changes in mesangial volume and mesangial matrix volume fraction than the males, as has recently been reported by Drummond and Mauer⁴⁵. However, during the six years between the renal biopsies, the mesangial matrix and mesangial volume fraction increased in male patients so as to reach the female levels. But the glomerular volume did not follow the same progression; GV/BSA increased in males between the first and second renal biopsy but did not reach the female level of GV/BSA.

The earlier maturation of the female patients, who had already reached their final height at the first renal biopsy unlike the males who were still growing, might explain why the females had more DG changes at the first renal biopsy. Another explanation is gender differences in DN. It is suggested that if the mesangial matrix increased in small or normal-sized glomeruli, the risk of DN might increase⁴⁴. This is in agreement with the views of others^{156, 157}, but not with those of Drummond and Mauer, who believe that large glomeruli are a sign of the severity of the disease⁴⁵. The larger GV/BSA in the female patients may adapt to mesangial expansion without a change in the intraglomerular pressure or filtration surface area.

In patients with type 2 diabetes, it is well known that males are more affected by DN than females¹⁵⁸. In type 1 diabetes, the importance of gender has been disputed. Kofoed-Enevoldsen et al. reported a higher incidence of proteinuria in male patients¹⁵⁹ and, in a recent study by Harvey et al., more males were in the MA and manifest DN groups¹⁰. Schultz et al., however, found that more female patients were in the MA group during adolescence¹⁶⁰, but the prevalence of MA increased among males after a longer duration of disease¹⁶¹. Rossing et al. did not regard gender as a risk factor for DN¹²⁷ in a 10-year follow-up but Hovind et al. reported male gender as strong predictors for development of MA after 20 years duration¹⁶².

6.2.4 Renal hemodynamics and morphology

Hyperfiltration was still present after diabetes of 17 years' duration, a finding reported to be a risk factor for development of DN¹⁶³. The mean FF prior to the first renal biopsy correlated with the BMT, V_v (matrix/glom), and V_v (mes/glom) at the time of the first renal biopsy¹²⁶. Thereafter, however, correlations between kidney function and

the morphometric parameters or changes in renal morphology were not found¹²⁶. Some authors have been unable to find correlations between kidney function and morphology^{33, 42, 45, 129, 132, 139, 154, 164}, while others do so^{43, 133, 134}.

Expansion of mesangial volume has been suggested to reduce the effective filtration area and, consequently, lead to a reduction in GFR^{43, 133}. We found an increase in mesangial volume and a decrease in slit pore length without a reduction in GFR probably due to the small increase in V_V (mes/glom) compared to that noted by others, 22% vs. > 30%¹³³.

6.2.5 UAE and morphology

The first renal biopsy showed an increase in foot process width and a decrease in the slit pore length density with increasing UAE⁴⁴. This was confirmed at the second renal biopsy, where wider foot processes in the group with UAE > 5 µg/min were found. An inverse correlation was observed between UAE and the slit pore length density but not with the total slit pore length.

The finding of an increase in foot process width and a decrease in slit pore length have been noted by others^{41, 128, 130, 145, 165, 166} but generally in patients with various degrees of albuminuria and not in NANT patients or in serial renal biopsies.

6.2.6 Blood pressure and morphology

On first renal biopsy correlations were found between the nighttime BP and BMT, V_V (matrix/glom) and the foot process width¹⁴. On the second renal biopsy, daytime diastolic BP correlated with BMT/BSA and slit pore length.

Non-dippers (systolic dipping < 7%, diastolic dipping < 14%) had more severe morphological changes at first renal biopsy¹⁵ and at the second renal biopsy less dipping was seen in the MA/HT group. In accordance with these results, Drummond et al. noted a correlation between the daytime diastolic BP and morphology⁴⁵. Other authors did not find any correlations between casual BP and morphology^{41, 43, 129, 139, 144, 145, 154}. ABPM seems therefore more informative and is considered to be the most appropriate BP measurement¹¹⁴.

6.2.7 AHT and morphology

In the AHT group, a decrease in BMT between the renal biopsies was observed. In the untreated NANT and MA/HT group, the V_V (matrix/glom) and V_V (mes/glom) increased during the six year follow-up, an observation which has been reported in follow-up studies of NA and MA patients^{131, 139, 154}. The stable V_V (matrix/glom) and V_V (mes/glom) in the AHT group may be the result of AHT or improved metabolic control or both.

In the ESPRIT study, where all patients were micro- or macroalbuminuric and had advanced renal morphological changes, no changes in renal morphology were seen in the AHT (enalapril or nifedipine) or placebo groups¹⁶⁷. The authors thought it might indicate irreversible renal morphological changes or too short a treatment period¹⁶⁷. However, we found either a regression or a stabilization of renal morphological

parameters after only 2 years of AHT in combination with improved metabolic control. This would suggest that the very early DG changes are reversible following a relatively short treatment period which is in contrast to the findings of Fioretto et al., who showed regression in renal morphology after 10 years of better metabolic control¹⁶⁸ in a group of DN patients who received pancreas transplantation.

6.2.8 Duration of disease and morphology

The consequences of prepubertal or postpubertal duration of diabetes were analyzed. Our use of a proxy age for puberty is similar to that of others¹⁶⁰. We found that the prepubertal duration had no significant effect on morphology. However, the postpubertal duration affected the V_v (mes/glom) and V_v (matrix/glom) on the second renal biopsy in our 19 NANT patients. Ellis et al. found that both the prepubertal and postpubertal durations affected the development of mesangial volume despite a normal UAE¹³⁰. Some authors report that pubertal onset (≥ 10 -14 years) of the disease is more strongly related to development of MA¹⁶⁹, to earlier onset of ERSD^{29,170} and to higher mortality¹⁰⁰ than prepubertal onset. Drummond et. al. could not find in a large study, any effect of either prepubertal or postpubertal onset of the disease on morphology¹⁴³. They found that the total duration of the disease was the most important time factor¹⁴³.

6.2.9 Prediction of morphometric variables

The nighttime systolic BP and MAP at the first renal biopsy predicted the ΔV_v (mes/glom), V_v (mes/glom), slit pore length and foot process width at the second renal biopsy in the NANT group¹²⁵. The predictive value of the ABPM in diabetic subjects in regard to the later development of morphological changes is not clear.

6.3 Kidney function

The patients were still hyperfiltering at the second renal biopsy, as compared to the controls.

The GFR showed no change between the first and second renal biopsies as noted by others¹³¹. However, Mauer et al., who studied patients with more severe morphological changes than our patients¹³³, reported a decrease in GFR in patients with mesangial expansion exceeding 30%. The ESPRIT study, which included micro- and macroalbuminuric patients, also showed a decline in GFR with time, age and the severity of albuminuria¹⁶⁷. Therefore, the change in GFR seems to occur only after DG has deteriorated to DN with marked mesangial expansion^{45,133}.

The hyperfiltration persisted from onset of diabetes and was reduced to normofiltration at 23 years duration. This is in contrast to an early decline in renal function that has been described in young normoalbuminuric and microalbuminuric patients with type 1 diabetes^{171,172}. Their GFR observations are based on measurement of Cystatin C which has poor correlation with the standard inulin technique¹⁷³.

6.4 Blood pressure

During the follow-up, the entire cohort showed an increase in nighttime diastolic BP. This first alteration in ABPM agrees with the findings of Lurbe et al.¹³.

Dipping at baseline was not identified as a significant predictor of future MA/HT although dipping correlated to more severe morphological changes at first renal biopsy¹⁵. Moreover, the MA/HT group also showed less dipping at the second renal biopsy¹²⁶.

6.5 Possible predictive risk factors

HbA_{1c} was observed to be a strong predictor for MA/HT which others have reported^{18, 24, 28-30, 42, 125-127, 144, 148-153}. Different predictive HbA_{1c} threshold values (>8.1-8.2% and >8.6-8.8%) have been reported^{30, 127, 152, 174} in good agreement with our threshold of 8.2%.

When analyzing the metabolic control in different time periods, it was found that the postpubertal metabolic control from start of puberty until 10 years duration was the strongest predictor while others have reported the first years' metabolic control to be the most important^{18, 175}. Other studies could not confirm this¹⁷⁶. The pubertal years often imply deterioration in metabolic control and perhaps the metabolic burden from that time period is more important for the development of complications than the age at onset.

The absolute ABPM risk values for macrovascular complications in adults has been determined as daytime BP of 135/85 mm Hg and nighttime BP of 120/75 mm Hg¹¹⁷, uncorrected for gender, age or height.

It was found that day systolic and night systolic and diastolic BP to be good predictors of MA/HT which is in accordance with others who have reported that different BP measurements relate to progression to DN, although not all have used the 24 h ABPM^{30, 150, 151, 177, 178}. Our threshold values were daytime systolic BP >130 mm Hg or a night-time systolic BP >120/70 mm Hg. This is quite near the limits of 120/70 mm Hg suggested by Shankar et al.¹⁷⁹. Our cut off levels are below the adult risk levels but BP risk levels for MA/HT could be lower than the BP risk levels for macrovascular disease.

According to morphology it was found that BMT/BSA was the strongest risk factor for future MA/HT and the threshold was 490 nm/1.73 m². The BMT of controls was not correlated with BSA (as BSA of controls was not obtained) but BMT of the controls was significantly lower than 490 nm, namely 320 (25) nm¹³⁷.

GFR was not identified as a predictor of MA/HT. The difficulties in finding correlations between GFR and complications agrees with some other reports^{153, 180, 181} but contrasts with earlier reports which were largely based on one GFR determination,^{178, 182, 183}. It might be that 17 years of follow-up is insufficient time in terms of making firm conclusions concerning hyperfiltration as a risk factor for DN.

UAE was not identified as a risk factor as others have reported^{30, 127, 183}. This might be caused by the very low UAE, often not measurable, found in our primarily NANT patients.

During recent years, several groups have reported on risk factors for development of complications and progression to DN. The level of UAE at baseline, male gender, baseline HbA_{1c}, MAP and short stature were reported as strong predictors for development of MA after 20 years duration¹⁶². Renal morphological variables were included as risk factors to see if they could improve the prediction of poor outcome. Additionally, there is a need for feasible cut off levels for various risk factors so that the patients at risk could be identified more easily and considered for therapeutic intervention.

A trilogy of postpubertal HbA_{1c}, daytime systolic BP and BMT/BSA was found to be a strong risk factor for future MA/HT. Understanding that glomerular morphological abnormalities^{44, 125, 126} are already present when MA develops, the goal must be to intervene at some prior time point.

It is suggested that if intervention should start before appearance of MA and HT, after 10 years duration of disease, ABPM and calculation of postpubertal HbA_{1c} and kidney biopsy should be done. If these measurements exceed the threshold values of risk factors, it is proposed that these patients should start AHT and be followed closely. It is very important to encourage compliance with clinic visits, improvements of metabolic control and AHT, especially in the adolescent age group.

6.6 The effect of candesartan

The intervention study, a double-blind placebo-controlled long-term candesartan treatment trial, was designed to investigate the effects of ARB on the clinical and morphological parameters in a NANT group of patients. The morphological effects of ACEi in microalbuminuric and proteinuric patients with diabetes^{167, 184-186} have been reported and several studies have been performed on the effects of ARB and ACEi on albuminuria and hypertension^{27, 94, 187-194}.

An ARB was chosen because the effect of ARB on reduction of albuminuria has been reported to be comparable with that of ACEi^{190, 191, 195, 196} but with fewer reported side-effects⁷⁶.

An interesting finding in our study was that 2 of 6 patients in the placebo group needed supplementary treatment but none (0/7) in the candesartan group. The placebo group had higher office blood pressure at follow-up than the candesartan group although the BP changes were similar between the groups.

Although candesartan had no apparent effect on UAE, most of our patients had very low urinary albumin excretion and at follow-up, the majority had no measurable UAE. In MA and proteinuric patients with diabetes, however, several authors have reported significant decline in UAE after treatment with ARB and ACEi^{27, 88, 94, 189-193, 197}.

GFR tended to decline but no significant difference was noted between the groups. In MA type 1 diabetes patients treated with ACEi or a beta-blocker during 3-5 years, GFR was not affected^{27, 185-187} although some have observed an initial fall in GFR during the first 6 months of treatment²⁷.

The increase in morphological changes between 10 and 16 years' duration in our NANT patients¹²⁵ did not progress during the 5-year trial observation period. Other studies of microalbuminuric or proteinuric patients have shown more advanced diabetic glomerulopathy changes at baseline^{42, 43, 126, 129, 145, 167, 185, 186, 198} than in normoalbuminuric patients^{43, 125, 129, 145}; in addition, renal morphological changes progress over time in the absence of treatment as observed in sequential biopsies^{126, 131, 167, 185, 186, 198}. This occurs to a greater extent than as observed in NANT patients¹²⁶.

The candesartan-treated patients showed a significant reduction in V_V (mes/glom) and V_V (matrix/glom) but no significant decrease in BMT. These morphological regresses were not seen in an ACEi/beta-blocker trial¹⁸⁵, an ACEi/Ca-channel blocker/placebo trial¹⁶⁷ or an enalapril/placebo trial¹⁸⁶ during an observation periods ranging from 2.5-5 years. This suggested that the effects of an ARB on morphology would not be inferior to the effects of ACEi in patients with type 1 diabetes.

The placebo-treated patients also had small, but not significant, decreases in BMT, V_V (mes/glom) and V_V (matrix/glom). This occurred even though the group had slighter worse metabolic control and higher blood pressure than the candesartan group. Unlike these findings, other placebo-treated microalbuminuric patients showed increases in the severity of the morphological changes^{139, 185, 186}. Our effects in the placebo group may be related to regular check-ups, encouragement and reminders by the same investigator, as has been shown in other studies¹⁹⁹⁻²⁰¹. Therefore, we suggest that the placebo effect is very important to analyze, even in trials studying the effects of treatment on morphology.

Generally, it seems difficult in treatment trials to show an improvement in morphology^{167, 185, 186}. This may be due to a multi-complex system with various interactions, small treatment groups, insufficient treatment duration, far advanced irreversible renal morphological changes in proteinuric patients or very minor morphological changes in NANT patients. Some morphological parameters can be used as predictors¹²⁵ but it seems more difficult to prove that treatment causes significant changes in morphology. The end-points of ESRD, dialysis, transplantation or mortality may be the ultimate end-points although in a young type 1 diabetes population, these end-points seem ominous and somewhat distant.

7 CONCLUSION

Normotensive and normoalbuminuric patients with type 1 diabetes have morphological changes of diabetic glomerulopathy at ten years duration and those changes progress over time. The initial alteration is thickening of BMT during the first decade of diabetes and in the second decade there is expansion of mesangial matrix and mesangial volume fraction.

Long-term metabolic control correlates with the histological changes and their progress over time. Female patients developed morphological changes earlier and had larger and progressively increasing glomerular volume. In males, the morphological changes developed later and progressed but the GV/BSA did not increase to the same extent as in females.

The continuous and progressive changes over time, even in NANT patients and even after 15 years duration, provide strong support for the importance of thorough and frequent follow-up of the patients with diabetes.

Assessment of the changes in renal morphology between first and second renal biopsy, it was noted that the diabetic glomerulopathy increased in both NANT and MA/HT patients. However, in the small group of MA/HT patients who received AHT treatment and achieved better metabolic control, further progression of diabetic glomerulopathy was not seen. This indicates the importance of good metabolic control, strict blood pressure surveillance and antihypertensive treatment early in the clinical course of diabetes.

In analyzing predictive factors, the patients with type 1 diabetes who develop MA/HT had worse metabolic control, higher day systolic and nighttime BP and more advanced glomerular morphological changes with increases in BMT at 10 years duration and could be identified as patients at risk. An earlier intervention in such patients will hopefully postpone and/or reduce the incidence of MA, HT, DN and ESRD in the future. There is a great need for pedagogical programs designed to maintain both metabolic control in adolescence and patient compliance to medication and regular clinical follow-up visits.

In the treatment trial, the previously observed progression in the renal morphological changes in normoalbuminuric and normotensive type 1 diabetes patients can be prevented or arrested by early intervention. Compared to placebo, candesartan attenuated renal morphological changes and lowered BP. These results support the use of candesartan as an alternative therapy for the treatment of diabetic glomerulopathy in patients with type 1 diabetes. However, the importance of regular frequent check-ups should not be underestimated.

8 THE DIRECTION OF FUTURE RESEARCH

The four articles presented in this thesis do not present all the results from the investigations that have been done during the last several years.

Twenty six patients have undergone a third biopsy and the results from those investigations and the long term follow up of 23 years of diabetes will be analyzed and presented. This will permit the description of the natural and renal morphological history of a small but well-studied cohort. An advantage relates to the unique information to be derived from a longitudinal study involving three consecutive renal biopsies.

Control renal biopsies have been obtained from Professor Michael Mauer; the ages of the control subjects correspond to the cohort ages at the time of the first and second renal biopsy. The plan is to analyze the control biopsies and to compare the changes in the diabetic cohort with those in the control population. This comparison will permit the separate identification of changes that reflect aging and changes which represent progression of diabetic glomerulopathy.

Recently, the use of cystatin C has become more frequent. This has resulted in various investigators reporting on renal function based on cystatin C levels. We have reported at the IPNA meeting in August 2007 that cystatin C does not correlate well with GFR as measured by inulin clearance in patients with diabetes and hyperfiltration. This abstract will be presented in an original article.

Most patients with diabetes have hyperfiltration for the first 15-20 years of their disease before a decline of GFR to more normal levels occurs. Some patients show normal GFR in the absence of perfect metabolic control and others continue with long-standing hyperfiltration without any signs of complications. Hyperfiltration has been regarded as a predictor of future diabetic nephropathy. This premise is not supported by the data presented in this thesis. Furthermore, in a long-term follow-up of kidney function and albuminuria in a portion of our total cohort of diabetes patients followed since 1978 (325 patients), it could not be confirmed that hyperfiltration was a predictor of DN. These patients will be recalled for kidney function test and measurement of albumin excretion rate and collect data on the frequency of DN as well as morbidity and mortality. This more complete long-term follow-up of kidney function and albumin excretion rate in patients with diabetes for 30-35 years should permit an accurate determination as to whether hyperfiltration is a predictor for DN.

Since 2004 all diabetes patients at our unit over 10 years of age and with 5 years duration of disease undergoes an ABPM every second year. This large body of information on ABPM requires analysis in regard to the progression of renal disease in the diabetic population.

When accomplished this five tasks, the patients who underwent their third biopsy have already start asking for their fourth biopsy.

9 SUMMARY IN SWEDISH-SVENSK SAMMANFATTNING

Typ 1 diabetes är en av de vanligaste kroniska sjukdomarna som drabbar barn och ungdomar. Diabetes nefropati med högt blodtryck, äggvite-utsöndring i urinen och sjunkande njurfunktion är en komplikation som drabbar 30-40 % av patienterna efter 20 års diabetesduration. Obehandlad leder diabetesnefropatin till njursvikt med behov av dialys/transplantation och även till hög dödlighet i hjärt-kärlsjukdomar. Med blodtrycksbehandling kan förloppet fördröjas och kanske förhindras.

Målet för detta avhandlingsarbete har varit att studera naturalförloppet av de njurförändringar som uppkommer tidigt i diabetessjukdomen, diabetesglomerulopati, samt att studera effekten av blodtrycksbehandling på diabetesglomerulopatin i en behandlingsstudie.

Mellan 1992 och 1994 genomfördes en första njurbiopsi på 46 patienter med typ 1 diabetes som inte hade någon äggviteutsöndring (normoalbuminuriska) eller högt blodtryck (normotensiva) och som då var 17 år gamla och hade haft diabetes i 10 år. Sex år senare genomfördes en andra njurbiopsi på 29 patienter. Mellan första och andra njurbiopsin hade 10 av de 29 patienterna utvecklat högt blodtryck (hypertoni) och äggviteutsöndring (mikroalbuminuri) och sex av dessa hade fått blodtrycksbehandling under minst 2 års tid. Efter den andra biopsin deltog 13 patienter, som fortfarande var normoalbuminuriska och normotensiva, i en behandlingsstudie som pågick under 5 år. Sex år senare gjordes en tredje njurbiopsi på 26 av de 29 rebiopserade patienterna.

I den första studien på 19 patienter som var normoalbuminuriska och normotensiva vid andra njurbiopsin, sågs att diabetesglomerulopatin progredierade i mesangium och mesangiellt matrix. De kvinnliga patienterna hade mer uttalade förändringar vid första biopsin men de manliga patienterna ökade sina förändringar i mesangium och mesangiellt matrix till andra biopsin. Dock ökade inte glomerulusvolymen hos männen till samma nivåer som hos de kvinnliga patienterna.

I andra studien med de 29 rebiopserade patienterna kunde man se att de patienter som utvecklat hypertoni och/eller mikroalbuminuri och fått blodtrycksbehandling under minst två år och hade förbättrat sin blodsockerkontroll (metabol kontroll), hade mindre progression eller t.o.m regression av de morfologiska förändringarna jämfört med de normoalbuminuriska och normotensiva patienterna samt de patienter som utvecklat hypertoni och mikroalbuminuri men ännu inte fått behandling. Njurförändringarna i basalmembranet och i mesangiellt matrix korrelerade väl med metabola kontrollen sedan diabetesdebuten och manligt kön.

I tredje studien studeras förekomsten av och prediktiva faktorer för uppkomst av hypertoni och mikroalbuminuri vid 17 års duration hos de 46 patienterna som genomförde en första njurbiopsi. Från undersökningarna vid tio års diabetesduration befanns den metabola kontrollen, framför allt från pubertetsstart, dagsystoliskt blodtryck och basalmembranet kunna prediktera 62 % av utfallet av hypertoni och

mikroalbuminuri vid 17 års duration. Njurfunktion kunde inte prediktera komplikationerna.

Behandlingsstudien var en dubbelblind, placebo kontrollerad studie med en angiotensin II receptorblockerare, candesartan 16 mg. I behandlingsstudien tredje njurbiopsi noterades ingen ytterligare progression i de njurförändringar som setts mellan första och andra njurbiopsin. I candesartangruppen sågs signifikant reduktion i mesangium och mesangiellt matrix och lägre blodtryck jämfört med placebogruppen. Placebogruppen visade tendens till, men ej signifikant, reduktion i njurförändringarna. Två patienter i placebogruppen utvecklade mikroalbuminuri och hypertoni och behövde få tillägg av blodtrycksmedicin men ingen i candesartangruppen utvecklade mikroalbuminuri eller hypertoni.

Tidig intervention och regelbunden uppföljning kan således påverka och förbättra den kliniska och njurmorfologiska utvecklingen vid diabetes typ 1.

10 ACKNOWLEDGEMENTS

I would like to express my gratitude to many people who have made this work come to pass, especially to:

All the rebiopsied patients that I came to know and especially the patients in the treatment group with whom I met every third month for five years. You taught me much about diabetes and how it is to live with a chronic disease. Thanks to Malin, Catharina, Ulf, Maria, Natalia, Theresa, Fredrik, Johan, Maria, Sanna, Michael, Mattias, Petri.

Ulla Berg, the leader of the gang, the head of our respected research group, always has sunshine in her outlook. Ulla always wants to dig deeper, to ask more questions or search for answers that you never knew you were looking for. Sometimes you have to completely circumnavigate the problem and return to the same result, but then you can feel both content and exhausted. With Ulla it is always fascinating to go on as you never know where you will end up.

Torun Torbjörnsdotter, my forerunner who taught me so much about diabetes, of how research work is done and how you become the queen of Excel spreadsheets. Her irreplaceable husband, Björn Sandberg, has computer technology under his skin and nothing is impossible; it will just take time and patience. You even managed the mystery of Soduko construction!

Georg Jaremko, our beloved pathologist who has been counting renal morphology for 15 years now. And Gorg, as we kindly proclaim, you know that we are not finished yet!

Agne Larsson, the former professor and head of the Division of Pediatrics at Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institutet for letting me work with Ulla although you thought she had enough PhD-students.

Claude Marcus, the current professor of the Division of Pediatrics, for all interesting and valuable discussions in your office.

Ann-Britt Bohlin, my adorable director of Childrens Hospital, Karolinska University Hospital, Huddinge and by now the substitute chief in the Childrens Division, the center for the care of all children in Karolinska University Hospital. You have always been the model of how to be a good director (the best in fact), a wonderful physician and a thorough and reliable mainstay in all sorts of weather.

Birgir Jakobsson who was the head of the Childrens Hospital when I restarted and currently is a much appreciated head of Karolinska University Hospital. Peter Graf, the former chief in the Childrens Division who encouraged me and taught me about “good enough”.

Elisabeth Berg, for helping in the statistical mystery world! Thank you for helping to establish results when they were to be found and to preventing me from seeing a connection when there was none.

Zoe Walsch and Jerry DiBona for the much needed correction of the English language and the changes required to convert the text into proper English language.

Ann-Britt Boman, nurse in the pediatric diabetes team, who assisted me with the second renal biopsies. Anna-Lena Sandström, my research nurse in the middle of the diabetes team work, who helped accomplish the third renal biopsies within a year! I know it cost blood, sweat and tears but I really enjoyed our trips with our patients at the ground floor of Karolinska Huddinge.

Kerstin Ekbohm and the rest of the DEMO-team for helping and encouraging me during the treatment trial. One is very lonely on Friday afternoon in the outpatient clinic. Kristina Lundin, Birgitta Almgren, Anette Andersson, Helena Öborn, Anki Lindahl, Gunilla Malm and the kidney team at the pediatric ward, B76. Thank you for managing so many kidney function tests over the years although the patients did not always appear when you had expected them to appear.

All my dear, interesting, individual and lovely colleagues; the general pediatricians, the colleagues on the seventh floor: gastro-hem and nephro-friends (I am still expecting to become a colleague), the brainists, the pulmonologists and the fabulous neoboys, my introducer Lasse, my tutor Antal, my former room-mate Maria, my stand-in Fredrika, the always so inspiring Ewa H, my BUP-friend Daiva, my archipelago-friends Maria M and Boubou, all pediatricians in training at the Childrens Hospital who have encouraged me and pushed me forward (“Nina, do you get any research done?”).

Thanks to all of you who during the last months really have tried to help to get this ship into the harbour; Mari, Britt, Birgit, Gunilla M and Ulla.

And Wouk, without you and what you have done instead of me doing it, this would never have been possible.

One’s research mates are very important and many thanks for all the good advice: Katarina, Kajsa, Gianni, Svante, Anne, Anna, Torun and Ricard.

My dear friends who have always been there as a nice wall, something to trust, to lean on or even to hide behind when the rest pushes hard. Sometimes there has not been such frequent contact but you have not run away.

Thanks to my mother and father, Åsa and Staffan, who have continuously encouraged me with statistical advice and a lucky pot when everything seemed impossible.

My big family with all my many cousins and second cousins who are always nearby and prepared to take action when it is needed.

Thanks to the not so numerous but lovely Perrin family who is never far away from a big laugh and has always supported me. Danne, the painting is really a smash-hit!

And finally my small family. Caj, my lovely husband, who has been enormously patient with me and all the mountains of paper that I have created during the last few months, on top of the already previously existing mountains. I have gratefully noticed your silence on this topic and I will improve. Your generosity even included silence when I crashed the car three days before our winterholiday! And I promise my freetime will from now on be in the cellar, in company with a wannabe nice armchair.

Thanks to you, my daughters, Vera and Agnes, who have done everything in their power to help me to finish my book!

This work was supported by grants from Karolinska University Hospital, Karolinska Institutet, the Freemasons in Stockholm Foundation for Children's Welfare, Sven Jerring, Samariten Foundation, Astra Zeneca and the regional agreement on medical training and research (ALF) between Stockholm county council and the Karolinska Institutet.

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