Idiopathic glomerular disease : a prospective regional study

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IDIOPATHIC GLOMERULAR DISEASE

A Prospective Regional Study

IDIOPATHIC GLOMERULAR DISEASE

A PROSPECTIVE REGIONAL STUDY

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde aan de Rijks-
universiteit Limburg te Maastricht, op gezag van de Rector Magnificus,
Prof. Dr. F. I. M. Bonke, volgens het besluit van het College van
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Antonius T. M. G. Tiebosch

geboren te Maastricht

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Prof. Dr. J. A. Flendrig, Maastricht

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*Perhaps love is like an ocean
Full of conflict, full of pain
Like a fire when it is cold outside
Thunder when it rains
If I shall live forever
And all my dreams come true
My memories of love will be of you*

In herinnering aan Jasper en Mama

Aan Pauline en Marjolein

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INTRODUCTION

This thesis was designed to chart the epidemiology of idiopathic, immunologically mediated, glomerular disease in the Netherlands and is the result of a unique set of circumstances. First in 1976 a medical school was created in Maastricht as part of the University of Limburg; the Medical Faculty had a regional policy which implied that resources were available for long term health care projects. Next none of the major city hospitals in Limburg had facilities for the work up of renal biopsies, specifically ultrastructural analysis, since the number of biopsies taken at each hospital was not sufficient in terms of cost-benefit ratio to warrant such expenditure. Finally all of these city hospitals had chronic hemodialysis units and were consequently the reference centers of the local general practitioners.

The regional study group on renal disease was created in 1977 in order to execute a prospective study on the epidemiology and natural history of idiopathic, immunologically mediated, glomerular disease in adults.

The study was considered opportune because, apart from the fact that it created facilities which were none existing, the incidence rates of the various idiopathic glomerular diseases in this country or elsewhere were not known. Such a data base together with an identification of risk factors associated with a poor prognosis early in the natural history of disease is essential in designing policies aimed at prevention of renal insufficiency as is pointed out in chapter I which consists of a review of the literature, using idiopathic IgA nephropathy and membranous glomerulopathy as exemplary. It is pointed out in this chapter that optimal use of cadaveric donor kidneys and expansion of center and home dialysis are effective short term measures for treating end-stage renal insufficiency. Long-term, cost effective measures, should include increased funding of basic research and study of the epidemiology and natural history of chronic, idiopathic glomerular disease.

In chapter II the epidemiology, in terms of estimated incidence rates of idiopathic glomerular disease and incidence related to the indication for renal biopsy, is given for the period 1978 through 1984; in this period renal biopsies were taken on the basis of agreed indications and contra-indications. These indications were: recent onset uremia, recent onset nephrotic syndrome, persistent microscopic erythrocyturia in the absence or presence of proteinuria, recurrent macroscopic hematuria and chronic proteinuria.

In chapter III the relationship of sex and age to the incidence and severity of idiopathic glomerular disease is given. Although the literature holds that older age is associated with more severe lesions in the renal biopsy, it is not clear

whether this relates to duration of disease, age in itself or both. Since renal biopsies presented in chapter II were taken from patients at specific points early in the natural history of disease, the effect of age, not duration of disease could be assessed.

Chapter IV deals with the use of quantitative electron microscopy in the differential diagnosis of recurrent macroscopic hematuria and persistent microscopic erythrocyturia and uses the material - 81 patients - from the prospective epidemiologic study. Since renal biopsies were taken very early in the natural history of disease it became clear during the study that a number of patients with recurrent macroscopic hematuria and persistent microscopic erythrocyturia had normal renal tissue by light microscopy. Systematic analysis by means of quantitative electron microscopy revealed a subset of patients with glomerular basement membrane thinning in the absence of a familial history of hereditary renal disease. This disease entity is called 'thin basement membrane nephropathy' and is described in terms of differential diagnosis in this chapter.

Chapter V finally, deals with the pathogenesis of idiopathic IgA nephropathy, the most common idiopathic glomerular disease in this country as is shown in chapter II. The question asked here was whether or not immunologic studies conducted early in the natural history of disease, specifically measurements of circulating immune complexes, might identify patients with idiopathic IgA nephropathy thus making immunopathologic analysis of renal tissue redundant. These immunologic studies would in addition provide information on the pathogenetic significance of circulating immune complexes early in the natural history of disease by comparing patients with idiopathic IgA nephropathy to patients with hematuria not related to mesangial IgA deposits.

CHAPTER I

IDIOPATHIC GLOMERULAR DISEASE

The need for prospective epidemiologic studies on the natural history of disease

1.1 INTRODUCTION

In the affluent industrialized societies the last decade has witnessed a dramatic increase in the number of patients requiring renal replacement therapy. This increase is not caused by an increased incidence rate of end-stage renal disease, but is largely due to the fact that patients over 60 years of age and patients with renal failure secondary to systemic disease, particularly diabetic nephropathy, are nowadays eligible for renal replacement therapy.

At present the annual incidence rate of new patients requiring renal replacement therapy is estimated to be about 65 per million inhabitants (84) in Europe and between 77 and 100 per million inhabitants in the United States (13); a figure which will almost certainly increase in the near future.

At the moment, renal transplantation is the best alternative therapy for several reasons. First the reinstitution of blood transfusions to potential recipients of cadaveric renal allografts and advances in tissue typing (74) have decreased the incidence and severity of rejection episodes. Next the introduction of a new generation of immunosuppressive drugs, the cyclosporins, has decreased steroid associated morbidity and mortality and has increased graft survival (29), and finally the introduction in the last decade of monoclonal antibodies to T lymphocytes (58) and polyclonal rabbit anti-human thymocyte antisera (34) make rejection episodes more easily to handle. For the patient with end-stage renal disease this has resulted in a better rehabilitation when a successful cadaveric renal allograft is compared to chronic hemo- or peritoneal dialysis, whereas the major advantage for society are the ultimately lower costs of a successful cadaveric renal allograft compared to life-long chronic hemo- or peritoneal dialysis. (11)

A major logistic problem in treating patients with end-stage renal disease with cadaveric renal allografts is the shortage of donor kidneys. In The Netherlands in 1986, 471 new patients with end-stage renal failure were eligible for receiving a renal transplant whereas in the same year only 378 tissue typed cadaveric renal transplants were performed, and 1,078 patients waiting for renal transplantation at the end of 1986. (73) This results in rapidly increasing numbers of patients requiring renal replacement therapy from 2500 in 1985, the total number of patients on chronic hemodialysis inclusive those eligible for transplantations to 4,000 patients in 1990. (44) At best optimization of renal

transplant programs will slow down the increase to 3,500 patients requiring renal replacement therapy in 1990. (72)

Thus, apart from optimization of transplant programs, measures aimed at prevention of end-stage renal disease are opportune in order to control the costs implicit in expanding dialysis programs. To be most effective these measures should be tailored to the most frequent causes of end-stage renal insufficiency. At the time of the initiation in 1977 of the prospective study on idiopathic glomerular disease presented in this thesis, the most common cause of end-stage renal disease in patients being treated with chronic hemodialysis in The Netherlands and Europe was idiopathic glomerular disease. (43) (Table I) Since that time the proportion of idiopathic glomerular disease in patients with end-stage renal disease treated with renal replacement therapy has decreased in the United States (87), and a similar trend is observed in The Netherlands. (28) This is mainly due to an increase in the number of patients with end-stage renal disease due to nephrosclerosis and, above all diabetic nephropathy. (87) (Table II)

Idiopathic glomerular disease remains a major cause of end-stage renal disease however and long-term programs directed at prevention of end-stage renal disease due to idiopathic glomerular disease should include:

Intensification of basic research aimed at elucidating both the mechanisms involved in the pathogenesis of idiopathic glomerular disease – which in man are usually immunologically mediated (Table III) –, and the factors involved in progression to end-stage renal insufficiency. In conjunction with these studies, effective therapeutic strategies should be developed in order to prevent the development of end-stage renal failure. Such a strategy requires prospective

Table I

Cause of renal insufficiency in patients with end-stage renal disease entering dialysis and transplantation programs in 1977.

Renal disease	The Netherlands ¹	Europe ²
Glomerulonephritis	32.5	33.5
Pyelonephritis and interstitial nephritis	16.6	21.0
Drug induced nephropathy	3.8	3.5
Cystic renal disease	13.2	9.6
Other hereditary renal disease	2.6	2.8
Congenital abnormalities	2.1	–
Vascular disease (nephrosclerosis)	4.8	7.3
Others and unknown	22.4	22.3

Legend to Table I

Adapted from Gezondheidsraad. Advies inzake preventie nierinsufficiëntie. 1980

1. 379 new patients with end-stage renal disease in The Netherlands.

2. 9,873 new patients with end-stage renal disease in Europe.

Table II

Cause of renal insufficiency in patients with end-stage renal disease entering dialysis and transplantation programs. Michigan, United States, over the period 1974-1981.¹

	1974	1975	1976	1977	1978	1979	1980	1981
Glomerulonephritis	30.6	27.4	28.4	26.4	23.7	20.9	17.6	20.9
Hypertension	19.8	26.5	27.6	26.4	28.5	26.1	26	24.2
Diabetes Mellitus	11.8	13.8	11.3	13.6	15.5	21.2	22.6	24.5
Others ²	37.8	32.3	32.8	33.7	32.3	31.9	33.8	30.4
Total number of new patients with Esrd*	434	412	497	523	579	656	769	807

Legend to Table II

1. Adapted from Weller JM, Wu SCH, Ferguson CW, Hawthorne VM. End stage renal disease in Michigan, Am J Nephrol 5: 84-95, 1985.
2. Over 1974 through 1981, the other diseases include: polycystic disease (6.0), systemic lupus erythematosus (1.7%), malignancies (1.1%), Alport's syndrome (1.0%) and all others plus unspecified (13.9%); this was constant over the period of observation.

* Esrd: End-stage renal disease.

Table III

Characteristic patterns of glomerular immunoglobulin and complement deposits in idiopathic renal disease.

Histological diagnosis	No biopsies	No with diffuse deposits of immunoglobulin and complement
Glomerular inflammatory disease	69	47
Focal segm. glomerulosclerosis	14	0
Interstitial disease	7	0
Normal renal tissue ¹	66	11
Total	156	58

Legend to Table III

Renal biopsies originating from the prospective regional study in chapter II.

1. Includes ultrastructurally defined glomerular pathology, specifically membranous glomerulopathy stage I (6x), idiopathic IgA nephropathy (5x), minimal change glomerulopathy (9x), and thin basement membrane nephropathy (19x).
(see also figure 1 en 2)

studies on the epidemiology and natural history of chronic idiopathic glomerular disease in order to identify risk factors related to progression towards end-stage renal insufficiency.

In addition, timely immunohistological diagnosis of patients with idiopathic glomerular disease presenting with rapidly progressive renal insufficiency, usually characterized by diffuse extracapillary glomerulonephritis, will in some prevent or delay the development of end-stage renal insufficiency by institution of immunosuppressive therapy and plasmapheresis at an early stage of disease. (46, 60, 62) In addition, a renal biopsy diagnosis will reveal patients with – occasionally – potentially reversible renal insufficiency requiring altogether different medication. (Table IV)

In this chapter, the changing concepts on the mechanisms involved in the pathogenesis of immune complex mediated glomerular disease and the recent contributions to the understanding of hemodynamic mechanisms involved in the progression to end-stage renal insufficiency and its respective therapeutic consequences will be used as exemplary for the achievements of basic research in the past. Furthermore, this chapter will illustrate the need for prospective studies on the epidemiology and natural history of idiopathic glomerular diseases in order to identify risk factors associated with progressive renal disease and to evaluate the effects of therapeutic strategies in patients with chronic glomerular disease. Idiopathic IgA nephropathy and membranous glomerulopathy will be used as exemplary.

Table IV
Incidence of primary renal disease and systemic disease in patients clinically suspected of having idiopathic rapidly progressive glomerulonephritis.

Primary renal disease	No	Systemic disease	No
Diffuse and focal segmental extracapillary glomerulonephritis	10 ¹	Fibrinoid necrosis of the afferent arterioles ²	2
Diffuse sclerosing glomerulonephritis	5	Light Chain disease	2
Interstitial nephritis	1	Subacute bacterial endocarditis	1

Legend to Table IV
Patients originating from the prospective regional study presented in chapter II.
1. Polyarteritis nodosa (1x)
2. Recent onset hypertension in the absence of papilledema on fundoscopy.

1.2 PATHOGENESIS OF IDIOPATHIC, IMMUNOLOGICALLY MEDIATED, GLOMERULAR DISEASE

1.2.1 Introduction

Introduction of the percutaneous renal biopsy technique (55,57) in conjunction with new techniques like immunofluorescence (16) and electron microscopy (30) in the period 1950 to 1960, have established that in the majority of patients with glomerulonephritis, immunoglobulins and complement components frequently associated with electron-dense deposits by transmission electron microscopy, are demonstrable in the glomeruli. This observation agrees with our own experience, presented in chapter II of this thesis, and is illustrated in table III. Usually these glomerular immunoglobulin deposits together with complement components occur in characteristic patterns, either linear along the glomerular basement membrane or granular in the mesangium and/or capillary wall. (Figure I and figure II).

By 1970, work in animal models of immunologically mediated glomerulonephritis had shown or suggested that these characteristic patterns of glomerular immunoglobulin deposits were due to anti-glomerular basement membrane antibodies (59,65,80) or to the deposition of immune complexes from the circulation. (20,42) The linear deposits of immunoglobulins and complement were due to antibodies reacting with glomerular basement membrane antigens, whereas the granular type of immunoglobulin deposits was thought to arise from the deposition of circulating immune complexes from the blood.

1.2.2 Deposition of circulating immune complexes

The question of how these immune complexes were deposited in the glomeruli was addressed in the period 1960 to 1970, particularly by workers from Scripps Clinic and Research Foundation (La Jolla, California), notably Cochrane and Dixon, in rabbits and was summarized in 1971 in a supplement to the Journal of Experimental Medicine. (82) Because of this work it was widely believed at that time that all glomerular immune complexes were deposited from the circulation into the glomerular capillary wall and the mesangium, provided they were of a critical size, were formed in relative antigen excess and were capable of releasing vasoactive amines. That (preformed) circulating immune complexes can deposit in the glomerular capillary wall and mesangium has been demonstrated ever and again. In such studies an important determinant for glomerular deposition was immune complex size, more specifically the lattice or antigen-antibody ratio. Using human serum albumen as antigen (Ag) and antibodies directed to it (Ab) in a mouse model, large-lattice immune complexes (greater than Ag2Ab2) deposited in the mesangium and subendothelially in the capillary wall, whereas small-sized immune complexes in

Figure 1
Immunofluorescence microscopy of idiopathic, immunologically mediated, glomerular disease

Legend to figure 1

Clockwise, starting at the upper left corner: idiopathic IgA nephropathy, anti-glomerular basement membrane glomerulonephritis, endocapillary proliferative glomerulonephritis, and membranous glomerulopathy. These glomerular diseases have characteristic, different patterns of glomerular deposits of immunoglobulins and complement. Diffuse, granular, mesangial in idiopathic IgA nephropathy (IgA1); diffuse, linear along the glomerular basement membrane in anti-glomerular basement membrane glomerulonephritis (IgG); diffuse, granular along the endothelial site of the glomerular basement membrane in endocapillary proliferative glomerulonephritis (C3); and diffuse, granular in or at the epithelial site of the glomerular basement membrane in membranous glomerulopathy (IgG).

Figure II

Immunofluorescence microscopy of secondary, non-immunologically mediated, glomerular disease

Legend to figure II

Clockwise, starting at the upper left corner: K light chain disease – presenting histologically as nodular glomerulosclerosis –, diabetic nephropathy, primary amyloidosis, and immunotactoid glomerulopathy. In these systemic diseases immunoglobulin is demonstrated in the glomeruli in the absence of complement. Notice the diffuse, nodular mesangial staining in light chain nephropathy with nodular glomerulosclerosis (K light chain); the diffuse, linear staining along the glomerular basement membrane in diabetic nephropathy (IgG); the diffuse, granular, mesangial staining in primary amyloidosis (polyclonal IgG); and the diffuse, granular, mesangial and capillary staining in immunotactoid glomerulopathy (monoclonal IgG).

considerable antigen excess, did not deposit at all. (47,48) In later years the glomeruli itself, notably charge were studied in terms of factors governing immune complex deposition. Glomerular charge was shown to play an important role in the glomerular deposition of both circulating immune complexes and uncombined antigens and antibodies. The glomerular basement membrane contains fixed negative charges which represent anionic sialoproteins, located on endothelial and visceral epithelial cell membranes (68), and heparan sulfate located mainly along the laminae rarae of the glomerular basement membrane. (66,81) Interaction of these negative charges with positive charges on antigens or antibodies within immune complexes contributes to the glomerular binding of the circulating immune complexes (37), particularly during early stages. (39) These charge interactions and undoubtedly other factors, lead to increased local concentrations of immune complexes in the glomerular basement membrane or mesangium, resulting in concentration of these immune complexes into larger deposits; electron-dense by electron microscopy. These deposits could be demonstrated in the mesangium and glomerular basement membrane, specifically in the subendothelial region but rarely if ever at a subepithelial localization. (40,41,63) Thus glomerular deposition of circulating immune complexes does occur but probably not at a subepithelial localization.

1.2.3 *In situ* immune complex formation

This concept, revolutionary in terms of the view held by 1968 (21) owes a great deal to Dutch and American workers notably the Groningen and Boston City hospital groups, studying the so-called Heymann nephritis. (50) In this model of experimental renal disease, rats immunized with crude fractions of renal proximal tubular cells in Complete Freund Adjuvant will develop a nephrotic syndrome with glomerular subepithelial immune complex formation analogous to the membranous glomerulopathy in humans. (3) In 1968 Dixon and co-workers isolated 3 renal tubular epithelial (RTE) antigens from the brush border of the rat proximal tubules one of which, RTE α 5 was highly immunogenic and capable of inducing an immune complex glomerulonephritis in rats with as little as 20 μ g administered in Complete Freund Adjuvant. (23) They next suggested, erroneously, on the basis of a series of elegant experiments that the subepithelial deposits were due to deposition of circulating immune complexes containing autologous (RTE α 5) antigen and antibody. (45) The evidence in favour of this hypothesis was persuasive, for in the subepithelial deposits of nephrotic rats both RTE α 5 antigen, rat immunoglobulins and complement were demonstrable, whereas the RTE α 5 antigen could not be demonstrated with fluoresceinated antibodies directed against this antigen in the glomeruli of normal rats. Since passive transfer of Iodine-125 labelled rabbit IgG anti-rat RTE α 5 to normal rats yielded a very shortened half life they concluded that small amounts of RTE α 5 must circulate in the blood of normal rats. The inevitable conclusion, in the

context of those days, was that rats must be tolerant to the RTE α 5 antigen and that immunization breaks this tolerance, the consequence being (chronic) glomerular deposition of circulating autologous RTE α 5 antigen and antibody in the form of immune complexes. (45)

That this concept might be wrong was first observed by van Es in Leiden. (27) He infused rabbit antibodies to rat tubular antigens into rats and observed the development of subepithelial immune complexes, even after transplanting kidneys prior to the onset of proteinuria. Since in his experiment tolerance could not be broken and a continuing supply of rabbit antibody was excluded by transplanting the kidney within 3 days after infusion of rabbit antibody, he concluded that the glomerular immune complexes had to be formed locally in the kidney.

Next Hoedemaeker and associates in Groningen studied this model using passive infusions of rabbit IgG antibodies against rat proximal tubular antigens. They first showed that rabbit IgG was deposited granularly in the glomerular capillary wall within five minutes after infusion and that subepithelial electron-dense deposits were already demonstrable three hours after intravenous administration. These immune complexes were thought to be deposited from the circulation. (31) Subsequently, they showed the granular membranous glomerular deposits of rabbit IgG, usually observed five minutes after infusion of rabbit antibodies, to disappear or become of a mesangial localization when antigen, a crude rat proximal tubular fraction, was infused directly after the antibody administration. (33) Thus for subepithelial deposition of immune complexes to occur a condition of antibody excess had to be present, which went against the theory of circulating immune complex deposition and therefore favoured *in situ* immune complex formation. The antigen involved remained obscure however until it was demonstrated by the work of van Damme et al. using *ex vivo* renal perfusion in conjunction with electron microscopy. (19) Perfusion of normal rat kidneys with rabbit antibodies to rat renal tubular brush border antigens showed the IgG antibodies to deposit along the foot processes and the slit pores of the glomerular epithelial cells, thereby demonstrating the antigen at the site of immune complex deposition, suggesting strongly *in situ* immune complex formation.

These observations were confirmed by Boston City hospital workers in the same year (17) using the same reagents in the isolated perfused rat kidney model; they described the *in situ* formation of subepithelial immune complexes. The Groningen workers next showed that these observations were not specific for the Heymann nephritis. Subepithelial immune complex deposits could also be generated by alternating *ex vivo* perfusion of kidneys with foreign protein antigens (lysozyme and bovine serum albumen) and antibody. (35) This concept, that immune complexes may be generated locally in the glomerulus due to an interaction of autologous or 'planted' antigen and precipitating antibody, was accepted in 1980 as an alternative mechanism for glomerular immune complex formation in man, specifically membranous glomerulopathy. (18)

This focussed attention on the molecular, anatomical and functional properties of the glomerular basement membrane, notably charge in terms of immune complex mediated glomerular injury. It has been shown for example that the negative charge on the bovine serum albumen protein is required for 'planting' this molecule in the glomerular capillary wall by *ex vivo* infusion. Anionic bovine serum albumen will not be 'planted'. (2) Generally, highly cationized proteins ($pI > 8.5$) can be 'planted' in the glomerular capillary wall after intravenous infusion (85) or infusion into the renal artery, (71) and subsequent infusion of antibody may lead to local immune complex formation. These local interactions within the glomerular basement membrane will obviously be influenced by the permselective properties of this membrane (56) as related to charge, size and shape of antigen or antibody. This is well illustrated by a recent study (32) in which sheep IgG antibodies to rat proximal tubular antigens or glomerular basement membrane antigens were first separated on the basis of charge in $\gamma 2$ (cationic) and $\gamma 1$ (anionic) subclasses, and next 'planted' into rat kidneys by intravenous infusions. These kidneys with 'planted' sheep antibody were transplanted to syngeneic rats which received rat IgG antibodies to sheep IgG immunoglobulins. These rat antibodies were also separated into an anionic and a cationic fraction and pair labelled with Iodine 125 and iodine 131. This study showed that the negative (anionic) charged glomerular filtration barrier retards the diffusion of anionic antibodies directed against antigens located subepithelially, whereas antigen-antibody charge interactions predominate in the formation of subendothelial immune complex formation.

1.2.4 Clinical and therapeutic consequences

It is clear that the pathogenetic concepts described above have clinical and therapeutic consequences. First in patients suspected of having *in situ* glomerular immune complex disease, specifically membranous glomerulopathy, an extrarenal source of a 'planted' heterologous antigen eg. neoplastic, as in bronchial carcinoma (4), pharmacologic, as in gold therapy (83), bacterial, as in syphilis (38) viral, as in hepatitis B (54) or parasitic, as in malaria (49) should be looked for before the disease can be classified as idiopathic. Second therapeutic manoeuvres designed to remove circulating immune complexes like plasmapheresis are entirely useless in conditions of chronic *in situ* immune complex formation. Finally, the clinician is forced to try to determine the pathogenetic mechanism involved in patients with chronic and acute glomerular immune complex deposition and try to tailor therapy accordingly. For instance at this moment, no convincing evidence exists that circulating immune complexes play a pathogenetic role in idiopathic IgA nephropathy, a problem which is further addressed to in chapter V. If the pathogenetic mechanism in idiopathic IgA nephropathy is *in situ* immune complex formation, manoeuvres designed to eliminate circulating immune complexes by plasmapheresis or attempts to create

conditions of antigen excess by lowering the serum immunoglobulin A concentration (phenytoin therapy) are conceptually wrong and will in practice be ineffective. (14,24)

1.3 PROGRESSION OF GLOMERULAR DISEASE

The frequently observed slow progression to renal insufficiency in patients with idiopathic glomerular disease does not necessarily imply the continuous presence of the immune mechanisms initially responsible for the immunologically mediated glomerular damage. It has become clear that other mechanisms, superimposed on the original injury with the histological lesions of focal and segmental glomerulosclerosis as the common denominator, form the common pathway leading to end-stage renal disease.

1.3.1 Focal and segmental glomerulosclerosis

One of the most recent contributions of basic research towards understanding of mechanisms operating in progressive glomerular disease derives from experimental studies on focal and segmental glomerulosclerosis in rats.

These experimental studies all stress the importance of intraglomerular hemodynamic factors in the genesis of the lesions of focal and segmental glomerulosclerosis. Brenner and co-workers showed the development of proteinuria and focal glomerulosclerosis-like lesions to be associated with increased glomerular flows and pressures. (51) They concluded that the changes develop as a consequence of chronic vasodilatation of the afferent arterioles with consequently intraglomerular hypertension and hyperfiltration. Subsequent damage to the capillary wall and mesangial accumulation of macromolecules leads to proteinuria and glomerular sclerosis respectively, aggravated or even induced by a high protein intake. (10) It follows, and clinical evidence supports this view that any decrease in renal (glomerular) mass, regardless of the original process, eg. congenital disorder, surgical removal, (26) immunologically mediated glomerular disease or physiologic due to ageing (7) especially in male sex, older age and, at least in rats, genetically determined risk factors (86), leads to the development of glomerular capillary hypertension and hyperperfusion (51), damage to the capillary wall with proteinuria, resulting in focal glomerulosclerosis, thus creating a vicious circle, inevitably leading to renal insufficiency.

The observation that a high protein intake elevates the glomerular transcapillary pressure in man (9) and in animals (67), aggravating the lesions of focal glomerulosclerosis, led to investigations on the effects of dietary protein restriction. In virtually all models of experimental renal disease dietary protein restriction decreases renal injury. (22,36,52,69) Also decreasing of the glomerular transcapillary pressure pharmacologically by angiotensin I converting enzyme

inhibition has been shown to decrease proteinuria and to inhibit the development of glomerular sclerosis. (6)

1.3.2. Clinical and therapeutic consequences

This experimental work in rats has consequences clinically, for it follows that any reduction of functional renal mass will predispose for the development of focal and segmental glomerulosclerosis in conditions of a high protein diet, and possibly genetic susceptibility. The therapeutic implications in the treatment of patients with chronic idiopathic renal disease are obvious. Dietary protein restriction in humans with renal insufficiency has already been shown to reduce proteinuria and the rate of progression towards end-stage renal insufficiency. (64,78) Whether dietary protein restriction established early in the natural history of chronic idiopathic glomerular disease will prevent the development of azotemia and eventually end-stage renal failure remains to be established but even postponement of terminal renal insufficiency in these patients by dietary protein restriction will lead to considerable savings. (1)

1.4 EPIDEMIOLOGY AND NATURAL HISTORY OF IDIOPATHIC GLOMERULAR DISEASE

1.4.1 Introduction

There is a virtual absence of population based data on the incidence of idiopathic glomerular disease in adults. Most figures known derive from autopsy studies, dialysis and transplantation centres (43) or concern well defined groups, eg. military recruits. (79) To study the epidemiology of idiopathic glomerular disease in the population, identification of patients with signs and symptoms of glomerular disease followed by characterization of the immunopathologic defined substrate by means of a percutaneous renal biopsy is necessary. The often asymptomatic course of glomerular disease and the specific referral of prognostically unfavourable patients to nephrologic centres make unbiased epidemiologic studies extremely difficult to conduct.

This also holds for the study of the natural history of idiopathic glomerular disease. It also requires careful clinical and immunopathologic characterization of patients early after onset of signs and symptoms of disease. These premisses can only be fulfilled in prospective studies with a long follow up period, which will eventually yield clinical and/or pathologic characteristics demonstrable early in the natural history, which presence will identify patients at risk of progression to end-stage renal failure. In these patients, aggressive therapy inclusive immunosuppression may be given despite the often serious side effects and morbidity.

1.4.2 Idiopathic IgA nephropathy

The incidence rate of this disease, which frequently follows an asymptomatic course, in the open population is not known, nor is it known precisely which risk factors detectable early in the natural history of disease are associated with a poor prognosis. Since a renal biopsy is required to diagnose idiopathic IgA nephropathy, open population studies which determine the true incidence rate do not exist and will probably never be conducted.

The available data on incidence of disease and natural history consequently derive from retrospective studies conducted in nephrologic centres. In a recent large Italian study risk factors, present at the time of renal biopsy, of progressive renal disease were determined retrospectively. These include an older age at disease onset; absence of recurrent macroscopic hematuria; daily protein excretion over one gram; the presence of arterial hypertension; the extent of glomerular obsolescence and the presence of focal and segmental glomerulosclerosis, interstitial fibrosis, diffuse mesangial proliferation, the presence of extracapillary glomerular proliferation and the extension of the IgA immunoglobulin deposits from the mesangium to the peripheral capillary loops. (5) Several of these risk factors have been confirmed in another retrospective study. (8) It is however clear from these studies, specifically the Italian study, that renal biopsies were taken at very different times in the natural history of disease; therefore it is likely that several of these risk factors carrying a poor prognosis relate more to the duration of disease than to the signs and symptoms of disease at onset and are not useful in predicting the prognosis of idiopathic IgA nephropathy at an early stage in the natural history.

1.4.3 Idiopathic membranous glomerulopathy

The natural history of idiopathic membranous glomerulopathy is a topic of debate, apart from the problem of the complications of the nephrotic syndrome notably hypercoagulability with venous thrombosis and thromboembolism. (61) This chapter will focus on the natural history of the glomerular abnormalities itself.

In terms of natural history of membranous glomerulopathy the retrospective study reported from the Hospital Nêcker (70) is unique in the sense that a large number of patients, 116 from which a renal biopsy was taken between 1958 and 1975, were followed for a mean period of 4,5 years without any immunosuppressive or inflammatory therapy given. In this study nephrotic syndrome was defined as proteinuria in excess of three grams per day, and renal biopsies were classified very straight forward by light microscopic evaluation of silver impregnated sections into three types; type 1: normal glomerular basement membrane (22 patients), type 2: presence of subepithelial silver positive spikes in a diffuse pattern (79 patients), and type 3: complex glomerular basement

membrane changes (15 patients). This classification is comparable to, although not identical with, the most widely used ultrastructural staging of Ehrenreich and Churg (25), type 3 of the French study being the composite of stages III and IV of Ehrenreich and Churg. The findings of this French study were as follows: from patients with type 1 lesions a renal biopsy was usually taken within one year after onset of disease, they presented with nephrotic syndrome ($15 \times$) or proteinuria ($7 \times$) and had a favourable prognosis with complete clinical remission or decrease in proteinuria in 50 percent; none of the patients developed renal insufficiency. Patients with type 2 lesions (renal biopsies also performed within one year after detection) usually presented with a nephrotic syndrome (63 out of 79), but they had a less favourable prognosis since only 39 percent of these patients went into clinical remission or showed a decrease in proteinuria. Finally, in the patients with type 3 lesions the renal biopsy was taken late after onset of symptoms with a mean of three years after detection; 11 out of 15 patients showed stable disease with persistent nephrotic syndrome, one patient developed end-stage renal failure and only two patients showed complete clinical remission with resolution of the capillary wall lesions.

This French study raises three important points. First, about 40 percent of the patients, usually those with type 1 and 2 lesions, will show complete remission or at least a spontaneous decrease in proteinuria. Second, it is impossible to predict on clinical or histological grounds early in the natural history which of the patients with type 2 lesions, the most frequently observed type, will improve, show stable disease or progress to end-stage renal insufficiency. Finally, it can be concluded that type 3 lesions are mostly seen late in the natural history of disease. Preliminary results of the prospective regional study described in chapter II of this thesis are in agreement with these observations, for we observed a complete clinical remission of nephrotic syndrome or a decrease in proteinuria in 40 percent of our patients within four years of follow up, whereas the few patients with Churg stage III/IV membranous glomerulopathy all turned out to have longstanding disease. (88) In addition, all patients with idiopathic membranous glomerulopathy from which a renal biopsy was taken within 6 months after onset of disease, had proteinuria in excess of 5 grams per day and showed type 1 or 2 lesions according to the Hospital Nêcker classification or stage I or II by the Churg classification, whereas none of our patients with membranous glomerulopathy presented with chronic proteinuria. Probably, the patients with type I or less frequently type II lesions in the French study from which a renal biopsy was taken because of chronic proteinuria (70) represent those patients already going into remission at the time of renal biopsy. If this reasoning is correct, studies on the effects of immunosuppressive therapy of membranous glomerulopathy should not only be conducted in a double-blind fashion but should either use patients with stage I or II very early in the natural history of disease, or use patients with stage III/IV at late and specified points in the natural history of disease.

Most studies on the effect of corticosteroids or immunosuppressive-drugs on the natural history of adult idiopathic membranous glomerulopathy derive from patients with idiopathic membranous glomerulopathy in different stages in the natural history. (15,53,77) The only study known to us, in which patients in the treated and control group were equally divided in terms of duration of disease and histological staging into Churg stage I, II and III has been described by Ponticelli et al. (75) They treated the adult nephrotic syndrome with monthly alternations of methylprednisolone and chlorambucil for 6 months. This study shows unequivocally that treated patients fare better (22 complete or partial remissions, 9 persistent disease), compared to untreated patients (11 complete or partial remission, 13 persisting and 8 progressive disease) when followed for 37 months. Of the treated patients, those with stage I (remission in 8 out of 10) or stage II (remission in 13 out of 16) responded well in contrast to those with stage III/IV lesions (remission in 2 out of 6). The problem this study raises is clear: patients with stage I/II lesions respond best to this type of immunosuppressive therapy but precisely these patients show a high incidence of spontaneous remission. Moreover, there is no information on the risk factors of progressive renal disease which might allow the clinician to predict whether a patient with stage II lesions will go into clinical remission spontaneously or not. This is illustrated in a recent state of the art review on prognosis and treatment of idiopathic membranous glomerulopathy. (76) Here five generally accepted risk factors are mentioned; the prognosis is better in children compared to adults and better in females compared to males; the prognosis is better with slight compared to heavy proteinuria; patients with normal renal function fare better than those with renal insufficiency at the time of renal biopsy; and stage I and II lesions have more frequent remissions compared to stage III/IV although this point is contested. Less clear are the influences of HLA matching on prognosis. The enumeration of these risk factors is not very relevant in terms of prognosis and treatment of the individual patient with recent onset membranous glomerulopathy, since clinically this patient will usually be a male adult with heavy proteinuria and Churg stage I or II lesions on the renal biopsy. It is precisely in this group of patients that knowledge about risk factors in terms of prognosis is lacking. Institution of immunosuppressive therapy with chlorambucil and prednison, or cyclosporin and prednison (12) is inevitably associated with side effects, potentially serious, and requires the timely identification of the prognostically unfavourable patient.

1.5 CONCLUSIONS

On the basis of the literature reviewed in this chapter, it is evident that programs aimed at prevention of end-stage renal disease are opportune in order to slow down and eventually control the increase in numbers of patients requiring renal replacement therapy. With respect to idiopathic glomerular disease it has been illustrated that basic research programs aimed at elucidating pathogenetic mechanisms of idiopathic glomerular disease have been rewarding and have changed concepts on the pathogenesis of immunologically mediated glomerular disease. In addition, basic research has shown that aspecific mechanisms, with the histological lesions of focal and segmental glomerulosclerosis as common denominator, may be superimposed on the original immunologic injury and may ultimately bring about renal insufficiency.

In order to translate the findings of basic research into effective therapeutic strategies, prospective clinical studies on epidemiology and natural history of idiopathic glomerular disease are required. No such studies exist, not even of the common glomerular diseases: idiopathic IgA nephropathy and membranous glomerulopathy as is shown in this chapter. Moreover not even the frequency of occurrence of the divers idiopathic glomerular diseases is known today.

The prospective regional study on idiopathic glomerular disease in a circumscribed area in the Netherlands presented in this thesis was designed to provide such information. Careful long-term follow up of the patients presented in this thesis will ultimately reveal whether the presence of certain clinical, serologic or immunohistological parameters present early in the natural history of disease have prognostic significance in the individual patient; these patients can next be treated on the basis of the pathogenetic mechanisms elucidated by basic research and confirmed in a clinical context.

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CHAPTER II

EPIDEMIOLOGY OF IDIOPATHIC GLOMERULAR DISEASE

A prospective study

2.1 INTRODUCTION

For the differential diagnosis of acute onset uremia, nephrotic syndrome or hematuria of unknown origin, knowledge of incidence and mode of presentation of idiopathic, usually immunologically mediated, glomerular disease is required.

Renal biopsy studies suggest wide differences in the incidence of immunologically mediated idiopathic glomerular disease between various human races, in Caucasians between continents, or within one continent between various countries. (1,16,29) These observed differences in incidence should be interpreted with caution, since they may be influenced by screening procedures for detecting asymptomatic patients, selective referral of patients with progressive renal disease to nephrologic centers and different indications for renal biopsy. End-stage renal disease eg. the histological end-stage of various idiopathic glomerular diseases, represents a variable, though important percentage of patients in renal biopsy studies giving incidence of glomerular disease. (8)

In this study the incidence of idiopathic glomerular disease in adults was determined by means of renal biopsies, taken early in the natural history of disease in a prospective regional study. Renal biopsies were performed on strict indications and contraindications. The design of the study avoided further referral to a nephrologic centre, while the organization of the local primary health care insured stable populations serviced by the three participating city hospitals in an adjoining circumscribed area. This enabled us to estimate the incidence rates of various idiopathic glomerular diseases related to the presenting sign or symptom in the open population.

2.2 PATIENTS AND METHODS

2.2.1 Indications and contraindications for renal biopsy

In this study (absolute) indications for percutaneous renal biopsy were: recent onset uremia (serum creatinine concentration over 300 $\mu\text{mol/l}$), and recent onset nephrotic syndrome (24 hours proteinuria over five grams in the absence of Bence Jones proteinuria) of unknown origin. In the context of this study recent onset implied that the renal biopsy had to be taken within six months after

patient identification. Other indications were persistent microscopic erythrocyturia or recurrent macroscopic hematuria with or without significant proteinuria (24 hour protein excretion over 0.5 gram but less than five gram) of at least half a year duration in the presence of a normal urologic workup. Finally, chronic proteinuria (24 hours protein excretion over 0.5 gram) of at least half year duration in the absence of chronic hypertension was an indication for renal biopsy. Indications were arranged in the following hierarchic order: recent onset uremia, recent onset nephrotic syndrome, persistent microscopic erythrocyturia with proteinuria, persistent microscopic erythrocyturia without proteinuria, recurrent macroscopic hematuria, and chronic proteinuria. This hierarchy implied that a patient presenting with a serum creatinine over 300 $\mu\text{mol/l}$ and a nephrotic syndrome entered the study as uremia. Prior to renal biopsy, 24 hour urine was collected and protein excretion quantitated by the Biuret method; adequacy of the urine collection was verified by measuring the creatinine excretion.

Contraindications for percutaneous renal biopsy included the absence of one kidney, coagulation disorders, angiographically proven polyarteritis nodosa and chronic uremia (serum creatinine over 300 $\mu\text{mol/l}$ in patients with longstanding renal disease). Relative contraindications for renal biopsy were acute tubular necrosis, urinary tract infections or urologic abnormalities. Acute postinfectious glomerulonephritis was no indication for renal biopsy unless proteinuria and hematuria persisted.

2.2.2 Patients

Excluded from the study were patients with known systemic disease from which a renal biopsy was taken because of signs of renal disease (systemic lupus erythematosus, Henoch-Schönlein nephritis, diabetes mellitus, cirrhosis of the liver or chronic hepatitis, ankylosing spondylitis, multiple myeloma and generalized atherosclerosis), patients with drug-associated disease, patients with chronic hypertension, patients with urologic abnormalities and patients which originated from families with known hereditary renal disease. Also excluded were patients from which a renal biopsy was taken because of chronic hematuria or proteinuria known over two years of duration and patients with a nephrotic syndrome or uremia known over six months duration. The study was restricted to adults between 16 and 65 years of age.

Routine laboratory tests at the time of the renal biopsy included anti-nuclear factor and rheumatoid factor, cryoglobulines, anti-streptolysin titer, classical hemolytic complement activity and liver function tests. Nephritic factor was determined by Dr. M.H. Daha, Renal Unit, Leiden University hospital, The Netherlands. Circulating antibodies directed against glomerular basement membranes were determined by dr. P. Mahieu, Renal Unit, University of Liege, Belgium.

2.2.3 The renal biopsies

Renal biopsy specimens were processed and stained for light microscopy, transmission electron microscopy and immunohistochemistry, using polyclonal antisera directed against human IgG, IgM, IgA, C3, C1q, kappa and lambda light chains as described previously. (23) All biopsies from patients with idiopathic IgA nephropathy stained in the indirect immunofluorescence with monoclonal anti-IgA1-, and in two also with monoclonal anti-IgA2 (kindly donated by dr. J. Haayman, Department of Gerontology, TNO, Rijswijk, The Netherlands).

In membranous glomerulopathy, all sections were stained in the indirect immunoperoxidase technique with a polyclonal anti-Hbe antiserum (kindly donated by dr. G. Wolters, Organon, Oss, The Netherlands). Hepatitis Be antigen had to be absent from the glomeruli for the membranous glomerulopathy to be called idiopathic.

All biopsies taken because of erythrocyturia, hematuria and nephrotic syndrome were examined ultrastructurally. Quantitative measurements of glomerular basement membrane thickness were made by using the orthogonal intercept method as described by others. (17, 30) (see also addendum to chapter IV). Normal adult glomerular basement membrane thickness in our laboratory measures 354 ± 55 nanometers; a thickness less than 245 nanometers, was considered diagnostic for the presence of thin basement membrane nephropathy. (12) Biopsies containing less than five glomeruli per two micron section were classified as technical failures. Renal biopsies were classified according to the WHO criteria. (10,11) Renal disease was considered idiopathic when extrarenal systemic disease was absent at the time of renal biopsy and did not develop within one year of follow up.

2.2.4 Estimation of incidence rates of disease

Renal biopsies were taken in the city hospitals of Heerlen, Maastricht and Sittard, all direct referral hospitals for the general practitioners, with an adherent population of 135,000, 85,000 and 90,000 adults (16 to 65 years of age) respectively. The adherent population of a hospital was defined as the population served by the general practitioners, who refer their patients to that hospital. The adherent population for each hospital was determined by the 'Medisch en Maatschappelijk Informatie Centrum' at Maastricht halfway the study (1980) for each participating hospital. This adherent population was worked with since this part of the Netherlands has a stable population in terms of migratory behaviour and referral pattern. By multiplying the number of years of participation in the study with its adherent population, the total population from which the patients originated was calculated for each hospital. Renal biopsies were taken in each hospital for the number of years required to reach a total adherent population of about 500,000.

2.2.5 Statistical analysis

Statistical inferences were made by using the normal approximation to the binomial distribution; a probability value smaller than 0.01 was assumed to be significant.

2.3 RESULTS

2.3.1 Indications for renal biopsy and adherent population

From 1978 to 1984, 214 renal biopsies were taken in the city hospitals of Heerlen, Sittard and Maastricht, during four, five and six years respectively, because of signs and symptoms of suspected idiopathic renal disease.

From twelve patients a renal biopsy was taken because of chronic hematuria and from five patients because of chronic proteinuria, although these symptoms of renal disease were known for over two years. Likewise in four patients with a nephrotic syndrome and three with uremia renal biopsies were taken well over six months after patient identification. These 24 patients were excluded from the present study.

From these 24 patients, renal biopsies were taken during the first two years of the study in 20 patients. The twelve patients with chronic hematuria known for over two years showed idiopathic IgA nephropathy (5 ×), glomerular basement membrane pathology (4 ×), local interstitial nephritis (1 ×), focal global sclerosis (1 ×) and idiopathic focal glomerulonephritis (1 ×). The five patients with chronic proteinuria known for over two years showed focal segmental

Table I
Indications for renal biopsy per hospital

City Hospital	No of years	No biopsies	Indication for renal biopsy ¹						Total adherent population per hospital ²
			H	E	EP	P	NS	U	
Heerlen	4	52	4	9	8	7	16	8	540,000
Maastricht	6	78	15	12	19	6	15	11	510,000
Sittard	5	60	11	11	11	10	15	2	450,000
Total		190	30	32	38	23	46	21	1,500,000

Legend to table I

1. Indication for renal biopsy: H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia; EP: persistent microscopic erythrocyturia with proteinuria; P: chronic proteinuria; NS: nephrotic syndrome; U: uremia (serum creatinine over 300 $\mu\text{mol/l}$).
2. Number of adult patients between 16 and 65 years of age serviced by a given city hospital x the number of years of participation in the study.

glomerulosclerosis (2 ×) and renal vascular hyalinosis (3 ×). In the patients with nephrotic syndrome known for over six months a diagnosis of idiopathic membranous glomerulopathy, stage III/IV of the Ehrenreich, Churg classification (15) (2 ×), minimal change glomerulopathy (1 ×) and focal segmental glomerulosclerosis (1 ×) was made. The three patients with uremia known for over six months showed end-stage renal disease on renal biopsy caused by unclassifiable glomerulonephritis (2 ×) and membranous glomerulopathy stage IV (1 ×).

In Table I the indication for renal biopsy is given in a hierarchic order: uremia, nephrotic syndrome, persistent microscopic erythrocyturia with or without proteinuria, recurrent macroscopic hematuria and chronic proteinuria. The total adherent population from which these biopsies originated was 1.5 million adults.

For the duration of the study the number of patients entering annually was constant for each hospital and no change in indication was observed. In patients with uremia or nephrotic syndrome, the renal biopsy was performed within three months (mean) after detection of disease. In patients with chronic hematuria or proteinuria a renal biopsy was performed one year (mean) after referral to the nephrologists.

2.3.2 Incidence of idiopathic glomerular disease

The incidence of idiopathic glomerular disease related to the indication for renal biopsy is given in table II. Of the 190 adults clinically suspected of having idiopathic glomerular disease, 13 were shown to have systemic disease either at the time of renal biopsy (11 ×) or during follow up (2 ×); bronchial carcinoma associated with membranous glomerulopathy and IgA nephropathy preceding cirrhosis of the liver), seven showed renal vascular hyalinosis and from 27 adults normal renal tissue was obtained. From 14 patients material was insufficient for diagnosis. Thus 129 adults with recent onset idiopathic renal disease were identified out of a total population at risk of 1.5 million adults. When the hospitals were compared with one another, significantly less renal biopsies were taken because of recurrent macroscopic hematuria and persistent microscopic erythrocyturia associated with proteinuria at the city hospital of Heerlen. This was reflected in a significantly greater incidence of thin basement membrane nephropathy in the adherent populations of the city hospitals of Maastricht and Sittard. In these hospitals too, a greater number of renal biopsies were taken from patients because of recurrent macroscopic hematuria, which showed normal renal tissue by light microscopy, immunohistochemistry and electron microscopy. The incidence of idiopathic IgA nephropathy – usually associated with erythrocyturia and proteinuria or recurrent macroscopic hematuria – in the adherent population of the three hospitals did

not differ however. Clinically, the 29 patients with idiopathic IgA nephropathy could be divided into two subsets; those with recurrent macroscopic hematuria ($10\times$) and those with persisting microscopic erythrocyturia ($18\times$); in one patient a renal biopsy was taken because of recent onset nephrotic syndrome. The incidence rate of idiopathic IgA nephropathy was 20 per million adults per year;

Table II

Incidence of idiopathic renal disease related to the indication for renal biopsy

	No. biop- sies	Incidence rate per 10 ⁶ per year	Indication for renal biopsy ¹						
			H	E	EP	EPU	P	NS	U
Idiopathic renal disease									
– Idiopathic IgA nephropathy	29	20	10	5	12	1	–	1	–
– Foc. segm. extracap. prolif. glomerulonephritis ²	3		–	–	–	–	–	1	2
– Diff. segm. extracap. prolif. glomerulonephritis ³	7		–	–	–	–	–	–	7
– Membranoproliferative GN type I	2		–	–	–	–	–	2	–
– Membranous glomerulopathy	14	9	–	–	–	–	–	14	–
– Diffuse sclerosing GN	5		–	–	–	–	–	–	5
– Other proliferative GN									
– idiopathic	9		2	–	3	1	–	3	–
– post-infectious ⁴	8		–	–	2	2	–	4	–
– Minimal change glomerulo- pathy	9	6	–	–	–	–	–	9	–
– Thin basement membrane nephropathy	19	13	1	11	7	–	–	–	–
– Interstitial nephritis	7		–	3	–	2*	–	1*	1
– Focal segmental glomerular sclerosis and hyalinosis	14	9	–	–	1	–	7**	6	–
– Focal global glomerular sclerosis	3		–	1	2	–	–	–	–
Secondary renal disease									
– Systemic disease not suscep- ted or known prior to renal biopsy ⁵	13		1	–	–	–	2	4	6
– Renal vascular hyalinosis: no clinical evidence of systemic atherosclerosis or hypertension	7		–	–	–	–	7	–	–
Normal renal tissue	27		13	9	2	–	3	–	–
Technical failures	14		3	3	2	1	4	1	–

the incidence of this disease in patients with idiopathic recurrent macroscopic or persistent microscopic hematuria was 36 and 29 percent respectively. No differences were observed between the hospitals in the number of renal biopsies taken because of chronic proteinuria or recent onset nephrotic syndrome, and the various subsets were equally distributed over the hospitals. All patients with membranous glomerulopathy by light microscopy and immunohistochemistry could be classified ultrastructurally as stage I (6 ×) or II (8 ×) of the Ehrenreich-Churg classification (15), the indication for renal biopsy being a recent onset nephrotic syndrome in all instances. Morphologically, characteristic lesions of membranoproliferative glomerulonephritis were seen in only two patients with a recent onset nephrotic syndrome as the indication for renal biopsy. The incidence rate of this disease was less than 2 per million per year.

In addition, three patients admitted with acute azotemic glomerulonephritis after an upper respiratory tract infection, judged to be of viral origin on clinical grounds, remained proteinuric after remission of the acute episode. Subsequently, they developed a nephrotic syndrome with normalization of renal function. Renal biopsies taken at this time showed morphologic changes of acute glomerulonephritis, with diffuse endocapillary cell proliferation, margination of esterase positive cells in the glomerular capillaries without basement membrane changes, and ultrastructurally scattered subepithelial electron-dense deposits (Figure I and III).

After two years of follow up renal biopsies taken in these three patients because of persisting nephrotic syndrome showed changes of membrano-proliferative glomerulonephritis (type I in two and type II in one) (Figure II and IV).

Legend to table II

1. Indication for renal biopsy: H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia; EP: persistent microscopic erythrocyturia with proteinuria; EPU: persistent microscopic erythrocyturia with proteinuria and serum creatinine over 120 $\mu\text{mol/l}$; P: chronic proteinuria; NS: nephrotic syndrome; U: uremia (serum creatinine over 300 $\mu\text{mol/l}$).
 2. Focal (less than 70 percent of all glomeruli), segmental extracapillary proliferative lesions.
 3. Diffuse (over 70 percent of all glomeruli), segmental extracapillary proliferative lesions; anti-glomerular basement membrane glomerulonephritis (4 ×).
 4. Persisting symptoms of acute glomerulonephritis after post-streptococcal (1 ×) and post viral (7 ×) upper respiratory tract infections.
 5. Fibrinoid necrosis of the afferent arterioles (essential hypertension) (2 ×), polyarteritis nodosa (1 ×), light chain disease (1 ×), benign monoclonal gammopathy (1 ×), rapidly progressive glomerulonephritis in subacute bacterial endocarditis (1 ×), membranous glomerulopathy associated with hepatitis B (e-antigen) (1 ×) and bronchial carcinoma (1 ×), IgA nephropathy in cirrhosis of the liver (1 ×) and amyloid (1 ×), minimal change glomerulopathy after gold therapy for rheumatoid arthritis (1 ×) and diclofenac medication (1 ×), nephrosclerosis (1 ×).
- * Chronic interstitial nephritis associated with focal segmental glomerulosclerosis and hyalinosis.
 ** Situs inversus in 1 patient.

None of these five patients with membranoproliferative glomerulonephritis showed hypocomplementemia or nephritic factor at the time of the first renal biopsy or during two years of follow up.

Of the 21 patients with recent onset uremia significantly less patients derived from the city hospital of Sittard compared with Maastricht, for reasons unknown. The incidence of focal or diffuse segmental extracapillary (crescentic) glomerulonephritis in these patients was high, 43 percent, as was the incidence of diffuse sclerosing glomerulonephritis of unknown origin, 24 percent, representing one third of all patients presenting with recent onset uremia not related to systemic disease.

2.4 DISCUSSION

The main findings of this study are fourfold.

First, of the idiopathic renal diseases the incidence rate of idiopathic IgA nephropathy is the highest, 20 per million adults per year as is the incidence of this disease in patients from which a renal biopsy was taken because of persistent non-azotemic microscopic erythrocyturia or recurrent macroscopic hematuria. Second, the incidence of focal segmental glomerulosclerosis and hyalinosis is high, as is the incidence in patients presenting with chronic proteinuria and nephrotic syndrome. Third the incidence of membranoproliferative glomerulonephritis is low as is the incidence in patients with recently discovered nephrotic syndrome. Finally the incidence of extracapillary, (crescentic) glomerulonephritis and chronic sclerosing glomerulonephritis in patients with recent onset uremia is high, although its incidence rate is low.

Estimates on the frequency of occurrence of the different idiopathic glomerular diseases require morphologic characterization of signs or symptoms of renal disease. Three variables will influence these estimates: the chance of detection of the, frequently silent, signs of glomerular disease, a threshold between detection of disease and referral to urologist or nephrologist, and finally a threshold – eg. referral to a nephrologic centre – for performing a renal biopsy.

In this study the indication for renal biopsy was controlled and since most patients were directly referred to the participating nephrologists a centre effect was minimized. Detection and referral to the local nephrologist however was not controlled.

Most adults with recurrent macroscopic hematuria, symptomatic nephrotic syndrome or uremia will be detected and referred to the local hospital. This reasoning does not hold necessarily for asymptomatic adults with chronic erythrocyturia or proteinuria in the absence of (chronic) hypertension, as is illustrated by a report on Finnish military conscripts in which hematuria, hematuria and proteinuria and proteinuria were detected in 42, 66 and 87 percent respectively for the first time on routine medical examination (22); thus

the estimated incidence rates of some glomerular diseases, eg. thin basement membrane nephropathy, are probably too low.

The same reasoning holds a priori for idiopathic IgA nephropathy, particularly in those patients with asymptomatic erythrocyturia and proteinuria, but our results do not bear this out. The incidence rate of this disease was comparable for each hospital, although the incidence of idiopathic IgA nephropathy in patients with recurrent macroscopic hematuria and persistent microscopic erythrocyturia varied per hospital, from 29 to 41 percent. This observation suggests that patients with idiopathic IgA nephropathy, which are usually male (3) and present with hematuria and proteinuria (Table II), are both detected and referred for renal biopsy in contrast for example to patients with thin basement membrane nephropathy, a disease equally distributed in our study between the sexes and usually not associated with macroscopic hematuria or significant proteinuria. Interestingly, a comparable incidence of idiopathic IgA nephropathy, 25 per million inhabitants, was recently reported from a regional, not age-restricted, study in France. (26) These observations suggest that the huge differences in incidence of idiopathic IgA nephropathy between various countries, when expressed as percentages of the total biopsy material; 1.5 percent in the United States of America (20), 4 percent in the United Kingdom (28), 5 percent in the Netherlands (21) and 22 percent in France (14); do not reflect a differing incidence of idiopathic IgA nephropathy, but rather differences in referral and indication for renal biopsy. These are not specified in the mentioned studies. Whether the very high incidence of idiopathic IgA nephropathy in Japan (24) of 40 percent and Singapore of 56 percent (27), when expressed as percentages of total biopsy material, is caused by a true higher incidence rate of idiopathic IgA nephropathy remains to be determined.

Of the 19 patients with chronic proteinuria the incidence of renal vascular hyalinosis and focal segmental glomerulosclerosis and hyalinosis was equal. Others too have described renal vascular hyalinosis in normotensive patients with isolated proteinuria in the absence of diabetes mellitus and have suggested that these lesions may precede hypertension rather than be caused by it. (31) Of the seven patients with focal segmental glomerulosclerosis and hyalinosis and chronic proteinuria, a decreased renal function was observed in one patient with unilateral renal agenesis and situs inversus; the others had normal creatinine clearances and no hypertension or nephrotic syndrome in the past history. Several authors have described asymptomatic proteinuric patients with focal segmental glomerulosclerosis (2,6,19); they seem to have a more favourable prognosis compared to patients with a nephrotic syndrome associated focal segmental glomerulosclerosis. These observations suggest the existence of a specific form of focal segmental glomerulosclerosis and hyalinosis not related to an evident reduction of renal mass with subsequent perturbations in glomerular hemodynamics (5) and also distinct from the focal segmental glomerulosclerosis associated with the nephrotic syndrome, as observed in childhood. (9)

In this study the estimated annual incidence rate of idiopathic nephrotic syndrome was 27 per million adults. The incidence of idiopathic membranous glomerulopathy stage I to II, minimal change glomerulopathy, focal glomerulosclerosis and membranoproliferative glomerulonephritis type I in these patients with recent onset nephrotic syndrome was 34 percent, 22 percent, 15 percent and 5 percent respectively. This incidence of membranous glomerulopathy in patients with idiopathic nephrotic syndrome is comparable to that observed in the United States of America (4) or the United Kingdom (32) in patients with a nephrotic syndrome over the 1970-1978 period. The incidence of membranoproliferative glomerulonephritis of 5 percent in patients with a recent onset nephrotic syndrome however, is less than the 12 percent reported from the United States of America (4), the 8 percent reported from the United Kingdom (31) or the 18 percent reported from the Netherlands in the period 1957-1975. (25) The low incidence of membranoproliferative glomerulonephritis may be due to three, not mutually exclusive, causes. First, preliminary data from a multicenter study in Northern Italy suggest a significant decrease in the incidence rate of membranoproliferative glomerulonephritis type I. (13) A retrospective analysis of the renal biopsies from 1548 patients with a primary glomerulonephritis in the period 1972-1984 showed the percentage of membranoproliferative glomerulonephritis type I to decline from 21 percent (1972-1976) through 14 percent (1976-1980) to 6 percent (1980-1984), without an observed decrease in the numbers of renal biopsies taken because of a nephrotic syndrome. This decreased incidence rate in membranoproliferative glomerulonephritis has also been reported from France where it parallels the decrease in rheumatic heart disease. (18) A similar trend may hold for the Netherlands. Next classical membranoproliferative glomerulonephritis with hypocomplementemia and the presence of nephritic factor is mainly a disease of children and teenagers (7); the design of our study excluded these patients. Finally, it is a matter of personal philosophy whether or not one considers those patients, which developed membranoproliferative glomerulonephritis after an episode of acute glomerulonephritis to suffer from acute onset membranoproliferative glomerulonephritis or, as our clinical observations suggest, to suffer from an undefined reaction secondary to acute (post-viral) glomerulonephritis. In the former view the incidence of membranoproliferative glomerulonephritis in patients with the idiopathic nephrotic syndrome would be 12 percent.

Of the 16 patients presenting with recently discovered uremia, due to idiopathic renal disease, nine showed extracapillary, (crescentic), glomerulonephritis, of the focal ($2 \times$) or diffuse ($7 \times$) variety. The latter was in four patients mediated by antibodies directed against the glomerular basement membrane. A surprisingly high incidence, 25 percent, of diffuse sclerosing glomerulonephritis was found in patients with recent onset uremia. Whether these lesions represent chronic, clinically silent and undetected, glomerulonephritis or a

sclerosing variety of rapidly progressive glomerulonephritis remains uncertain.

This prospective regional study provides new and unique population based data on the frequency of occurrence of idiopathic glomerular disease. In addition, it provides figures on the incidence of various idiopathic, usually immunologically mediated, glomerular diseases in patients with chronic hematuria, chronic proteinuria, recent onset nephrotic syndrome and uremia, which contributes to the differential diagnosis of these symptoms.

2.5 SUMMARY

In this prospective regional study, the incidence rates of the various idiopathic glomerular diseases was estimated in a population of 1.5 million Dutch adults between 16 and 65 years of age; the incidence of these idiopathic glomerular diseases related to the presenting sign or symptom of disease was determined as well.

The study was conducted between 1978 and 1985; indications for renal biopsy in decreasing hierarchical order were recent onset idiopathic uremia, recent onset idiopathic nephrotic syndrome, chronic hematuria and chronic proteinuria of less than two years duration and of unknown origin.

The main findings are fourfold. The incidence rate of idiopathic IgA nephropathy and thin basement membrane nephropathy, was high with 20 and 13 per million adults per year respectively; their incidence in renal biopsies taken from patients because of non-azotemic chronic hematuria is 31 percent and 22 percent respectively. In the normotensive non-azotemic adults from which a renal biopsy was taken because of chronic mild proteinuria the incidence of focal segmental glomerular sclerosis and vascular hyalinosis was both 37 percent. Of the patients with nephrotic syndrome the incidence of membranous glomerulopathy stage I/II, minimal change glomerulopathy and focal and segmental glomerulosclerosis was 34, 22 and 15 percent respectively with an incidence rate per million adults per year of 9, 6 and 4 respectively. Both, the incidence, 5 percent, and incidence rate of less than 2 per million adults per year of membranoproliferative glomerulonephritis in patients with an idiopathic nephrotic syndrome was low. Finally, the incidence of extracapillary, crescentic glomerulonephritis and diffuse sclerosing glomerulonephritis in patients from which a renal biopsy was taken because of recent onset idiopathic uremia was high being 33 and 25 percent respectively.

This prospective regional study provides new population based data on the frequency of occurrence of idiopathic glomerular disease. The study provides in addition figures on the incidence of various idiopathic, usually immunologically mediated, glomerular diseases in patients with chronic hematuria, chronic proteinuria, recent onset nephrotic syndrome and recent onset uremia, which contributes to the differential diagnosis of these symptoms.

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Figure 1

*Light microscopy. Silvermethenamine, hematoxyline and eosin stain
($\times 46$)*

Legend to figure 1

Renal tissue from a patient with acute, severe but transient, azotemic glomerulonephritis after an upper respiratory tract infection, judged on clinical grounds to be of viral origin. 4 weeks after the acute episode and after remission of the azotemia, with persisting hematuria and proteinuria. Notice the diffuse endocapillary glomerulonephritis characterized by influx of mononuclear cells and loss of polyanionic charges of the glomerular basement membrane as shown by clearly diminished silver impregnation.

Figure II

*Light microscopy. Silvermethenamine, hematoxyline and eosin stain
($\times 46$)*

Legend to figure II

Renal tissue from the same patient as in figure 1, taken two years after the acute episode because of persistent microscopic erythrocyturia and proteinuria, the latter recently increasing to a nephrotic syndrome.

Notice the diffuse mesangial proliferation and the local thickening of the glomerular basement membrane, comparable with a membranoproliferative glomerulonephritis type I.

Figure III

Immunofluorescence microscopy. Stain for C3 ($\times 290$)

Transmission electron microscopy ($\times 10,500$)

Legend to figure III

Renal tissue from the first biopsy of the patient; histology shown in figure 1. Notice the granular, capillary deposits of C3 with the accompanying subendothelial and subepithelial electron-dense deposits on electron microscopy.

Figure IV

Immunofluorescence microscopy. Stain for C3 ($\times 185$)

Transmission electron microscopy ($\times 6,600$)

Legend to figure IV

Renal tissue from the second biopsy of the patient; histology shown in figure 2. Notice the granular, mesangial and capillary deposits on immunofluorescence with the accompanying mesangial, intramembranous, and subepithelial electron-dense deposits on electron microscopy.

CHAPTER III

THE RELATIONSHIP OF SEX AND AGE TO THE INCIDENCE AND SEVERITY OF IDIOPATHIC GLOMERULAR DISEASE

3.1 INTRODUCTION

It has been established that sex and age are related to immunologically mediated glomerular disease. Retrospective studies of large series of patients from nephrologic centers show systemic lupus erythematosus to have a female preponderance (6), and idiopathic IgA nephropathy (4), membranous glomerulopathy (21) and rapidly progressive glomerulonephritis (7) to be associated with males. Within one disease entity finally, eg. idiopathic IgA nephropathy (1,5), older age is associated with more severe histological lesions and carries a less favourable prognosis. These studies must be interpreted with caution because of a possible selection of prognostically unfavourable patients ('centre-effect') and because it is not clear to which extent duration of disease rather than age itself is associated with a poor prognosis, since renal biopsies were taken at varying moments in the natural history of disease.

In order to assess the relationship of sex and age to idiopathic glomerular disease, we studied the patients from a prospective regional study, in which renal biopsies were taken on strict indications and contra-indications early after onset or discovery of signs and symptoms of disease. A centre-effect was minimized. Results show sex to be related to various idiopathic glomerular diseases; in males age, not duration of disease, is shown to be related to the severity of the glomerular inflammatory lesions.

3.2 PATIENTS AND METHODS

3.2.1 Indications and contraindications for renal biopsy

Patients originated from a prospective regional study on idiopathic glomerular disease in adults between 16 and 65 years of age carried out between 1978 and 1986 in a circumscribed area of The Netherlands. The design of the study, inclusion criteria, and the methodology used has been described in chapter II. Briefly, indications for renal biopsy in hierarchical order were: recent onset uremia (serum creatinine concentration over 300 $\mu\text{mol/l}$), recent onset nephrotic syndrome (24 hours proteinuria over five grams in the absence of Bence Jones proteinuria), persistent microscopic erythrocyturia with or without significant proteinuria (24 hour protein excretion over 500 milligram),

recurrent macroscopic hematuria, and chronic proteinuria (24 hours proteinuria over one but less than five grams). Recent onset implied that renal biopsies were taken within half a year of patient identification. In the other patients, signs or symptoms of disease had to be present for at least half a year but renal biopsies had to be taken within two years of patient identification. The hierarchy implied that a patient presenting with serum creatinine over 300 $\mu\text{mol/l}$ and a nephrotic syndrome entered the study as uremia. Renal biopsies were classified according to the WHO criteria. (10,11)

Excluded from the present study were patients with known systemic disease at the time of renal biopsy (eg. systemic lupus erythematosus, Henoch-Schönlein nephritis, diabetes mellitus, liver disease, ankylosing spondylitis, multiple myeloma and generalized atherosclerosis), drug-associated disease, chronic hypertension, post-infectious glomerulonephritis, and patients originating from families with known hereditary renal disease. Also excluded were patients from which a renal biopsy was taken because of chronic hematuria or proteinuria known over two years of duration and patients with a nephrotic syndrome or uremia known over 6 months duration. All patients had normal liver function tests, negative anti-nuclear- and rheumatoid factor by Latex agglutination, no cryoglobulins, and a normal anti-streptolysin titer; patients with erythrocyturia or hematuria had a normal urologic workup including cystoscopy, intravenous urography and sometimes renal arteriography.

In membranous glomerulopathy, all sections were stained in the indirect immunoperoxidase technique with a polyclonal anti-Hbe antiserum (kindly donated by dr. G. Wolters, Organon, Oss, The Netherlands). Hepatitis Be antigen had to be absent from the glomerular basement membrane for the membranous glomerulopathy to be called idiopathic.

3.2.2 Patients

181 patients fulfilled the entry criteria. Of these adults 155 originated from a prospective regional epidemiologic study conducted over the period 1978 to 1985 and presented in chapter II; of these patients 27 had normal renal tissue and 128 idiopathic renal disease. From another 17 adults renal biopsies were taken at the city hospitals of Maastricht and Sittard in 1985; in these hospitals renal biopsies were consistently taken from patients with acute uremia, recent onset nephrotic syndrome and chronic hematuria with proteinuria according to the indications outlined above and therefore included. Finally nine renal biopsies, taken because of recent onset nephrotic syndrome or recent onset uremia in the city hospital of Heerlen in the period 1983 - 1985 are included, because these renal biopsies were consistently taken from patients presenting with recent onset nephrotic syndrome and recent onset uremia. Since renal biopsies were not consistently taken from patients with persistent erythrocyturia, recurrent macroscopic hematuria and chronic proteinuria at the city

hospital of Heerlen, all patients from which a renal biopsy was taken because of these indications in Heerlen in the period 1983-1985 were excluded.

3.2.3 Statistical analysis

Statistical inferences were made using Students-t-test. Statistical significance was accepted if the probability value was smaller than 0.05.

3.3 RESULTS

3.3.1 Sex, the indication for renal biopsy and immunopathology

Renal biopsies were taken from 181 patients of which 119 were male and 62 were female. Renal biopsies taken because of persistent microscopic erythrocyturia with proteinuria (35 ×), chronic proteinuria (17 ×) and recent onset uremia (19 ×) showed no sex preponderance. On the other hand, significantly more renal biopsies were taken from males because of recent onset nephrotic syndrome (52 ×; $p < 0.025$), recurrent macroscopic hematuria (28 ×; $p < 0.005$) and persistent microscopic erythrocyturia without significant proteinuria (29 ×; $p < 0.025$).

All but two patients, from which a renal biopsy was taken because of chronic hematuria and chronic proteinuria were normotensive at the time of renal biopsy. In patients from which a renal biopsy was taken because of recurrent hematuria, persistent microscopic erythrocyturia with or without proteinurias

Table I

Sex and age related to the indication for renal biopsy in adults with idiopathic renal disease

Indications for renal biopsy	No of biopsies			Median age	
	Male	Female	p-value *	Male	Female
Recurrent macroscopic hematuria	23	5	< 0.005	25	30
Persistent microscopic erythrocyturia without proteinuria	20	9	< 0.025	40	33
Persistent microscopic erythrocyturia with proteinuria	17	14	n.s.	38	25
Persistent microscopic erythrocyturia with proteinuria and azotemia ¹	3	2	n.s.	39	63
Persistent isolated proteinuria	9	8	n.s.	46	49
Recent onset nephrotic syndrome	34	18	< 0.025	46	50
Recent onset uremia	12	7	n.s.	55	38
Total	118	63			

Legend to Table I

1. Azotemia indicates serum creatinine over 120 $\mu\text{mol/l}$ but less than 150 $\mu\text{mol/l}$.

or chronic proteinuria, impaired renal function (serum creatinine concentration over $120\text{ }\mu\text{mol/l}$) was only noted in five patients, all presenting with persistent microscopic erythrocyturia and proteinuria. (Table I)

In patients with recent onset nephrotic syndrome a statistically significant male preponderance was found in patients with membranous glomerulopathy ($23\times$; $p < 0.025$); in minimal change glomerulopathy and focal segmental glomerulosclerosis no sex preponderance was seen. (Table II) In patients from which a renal biopsy was taken because of recent onset uremia ($19\times$), idiopathic diffuse extracapillary glomerulonephritis was male-associated ($5\times$; $p < 0.05$), whereas in chronic sclerosing glomerulonephritis ($5\times$), anti-glomerular basement membrane antibody mediated glomerulonephritis ($4\times$) and interstitial nephritis ($3\times$) no such association was apparent, although the figures were small. The renal biopsies of patients with chronic proteinuria ($17\times$) showed either focal and segmental glomerulosclerosis ($7\times$), which was middle aged male associated, or renal vascular hyalinosis ($7\times$) which lesion appeared to be associated with middle aged females; neither of these associations reached statistical significance. (Table II)

Idiopathic IgA nephropathy was the most striking male associated disease ($36\times$; $p < 0.005$). Of the 36 patients with idiopathic IgA nephropathy, a renal biopsy was taken in 31 patients because of non-azotemic, chronic hematuria. The male preponderance was found in recurrent macroscopic hematuria ($11\times$; $p < 0.025$), persistent microscopic erythrocyturia without proteinuria ($5\times$; $p < 0.05$) and persistent microscopic erythrocyturia with proteinuria ($16\times$, $p < 0.025$). When the patients with recurrent macroscopic hematuria and idiopathic IgA nephropathy were compared to those with recurrent macroscopic hematuria and normal renal tissue on renal biopsy, the male preponderance was identical, illustrating that the male preponderance in patients with idiopathic IgA nephropathy and recurrent macroscopic hematuria, is related to the indication for renal biopsy and cannot be attributed a priori to the immunopathologic disease. (Table II) In patients with idiopathic IgA nephropathy from which a renal biopsy was taken because of persistent microscopic erythrocyturia in the absence or presence of proteinuria, the male preponderance could not be related to the indication for renal biopsy, since no such association was demonstrable in patients with thin basement membrane nephropathy ($19\times$) or in patients with normal renal tissue ($11\times$) and persistent microscopic erythrocyturia in the absence or presence of proteinuria. (Table II)

3.3.2 Age, the indication for renal biopsy and immunopathology

In males, age was related to the indication for renal biopsy, for age increased significantly ($p < 0.025$, Table I) in patients from which a renal biopsy was

Table II

Sex related to the immunopathologic findings in renal biopsies of patients with signs and symptoms of idiopathic renal disease

Immunopathologic diagnosis	No	Indications for renal biopsy ¹						Male:Female	p-value ²
		H	E	EP	P	NS	U		
Idiopathic IgA nephropathy	36							31 : 5	< 0.005
		11						10 : 1	< 0.025
			5					5 : 0	< 0.05
				17*				13 : 4	< 0.025
						3		3 : 0	
Membranous glomerulopathy stage I	23	-	-	-	-	23	-	17 : 6	< 0.025
Membranoproliferative GN type I	2	-	-	-	-	2	-	1 : 1	
Minimal Change glomerulopathy	10	-	-	-	-	10	-	3 : 7	
Diffuse extracapillary GN	5	-	-	-	-	-	5	5 : 0	< 0.05
Diffuse extracapillary GN, anti-GBM antibody mediated	4	-	-	-	-	-	4	1 : 3	
Diffuse sclerosing GN	5	-	-	-	-	-	5	3 : 2	
Other proliferative GN	12	2	-	4**	-	4	2	6 : 6	
Focal global glomerulosclerosis	3	-	1	2	-	-	-	2 : 1	
Focal segmental glomerulosclerosis	16							12 : 4	
				1	7			6 : 2	
						8		6 : 2	
Renal vascular hyalinosis	7	-	-	-	7	-	-	1 : 6	
Interstitial nephritis	10	-	3	2*	-	2	3	5 : 5	
Thin basement membrane nephropathy	21							12 : 9	
		2						2 : 0	
			11					7 : 4	
				8				3 : 5	
Normal renal tissue	27							19 : 8	< 0.025
		13						10 : 3	< 0.05
			9					6 : 3	
				2				1 : 1	
					3			2 : 1	
Total	181	28	29	36	17	52	19		

Legend to Table II

1. Indication for renal biopsy: H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia without proteinuria; EP: persistent microscopic erythrocyturia with proteinuria; P: persistent isolated proteinuria; NS: recent onset nephrotic syndrome; U: recent onset uremia (serum creatinine over 300 $\mu\text{mol/l}$).

2. p-value, Students-t-test.

* 2 patients with azotemia (serum creatinine over 120 $\mu\text{mol/l}$ but less than 150 $\mu\text{mol/l}$).

** 1 patient with azotemia (serum creatinine over 120 $\mu\text{mol/l}$ but less than 150 $\mu\text{mol/l}$).

Table III

The relationship of age to the severity of the glomerular inflammatory reaction in adult males

Histological changes	No	Indication for renal biopsy ¹					Median age
		H	E	EP	NS	U	
Mesangial cell and matrix proliferation	11	3	2	6	–	–	31
Mesangial cell and matrix proliferation with extracapillary proliferation	20	4	2	9	5	–	44
Extracapillary proliferation	8	–	–	–	1	7	57

Legend to Table III

1. Indication for renal biopsy. H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia without proteinuria; EP: persistent microscopic erythrocyturia with proteinuria; P: persistent isolated proteinuria; NS: recent onset nephrotic syndrome; U: recent onset uremia (serum creatinine > 300 μ mol/l)

Statistical significance. Students-t-test.

Patients with extracapillary proliferation older than all others $p < 0.005$

Patients with mesangial cell and matrix proliferation with extracapillary proliferation, older than patients with only mesangial cell and matrix proliferation. $p < 0.025$

Table IV

The relationship of age to the severity of the glomerular inflammatory reaction in adult males with idiopathic IgA nephropathy.¹

Histological changes	No	Indication for renal biopsy ²					Median age
		H	E	EP	NS	U	
None	5	4	1	–	–	–	26
Mesangial cell and matrix proliferation	10	2	2	6	–	–	34
Mesangial cell and matrix proliferation with extracapillary proliferation	16	4	2	7	3	–	43

Legend to Table IV

1. Defined as diffuse mesangial granular deposits of polyclonal IgA1, C3 and C5b-9 in the absence of C1q deposits.
2. H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia without proteinuria; EP: persistent microscopic erythrocyturia with proteinuria; P: persistent isolated proteinuria NS: recent onset nephrotic syndrome; U: recent onset uremia (serum creatinine > 300 μ mol/l).

Statistical significance. Students-t-test.

Patients with mesangial cell and matrix proliferation with extracapillary proliferation, older than patients with only mesangial cell and matrix proliferation and those with no histological abnormalities $p < 0.025$.

taken because of recurrent macroscopic hematuria, persistent microscopic hematuria, recent onset nephrotic syndrome and recent onset uremia showed in this order. This was reflected in the immunopathologic diagnosis, for males with idiopathic IgA nephropathy, membranous glomerulopathy and idiopathic extracapillary glomerulonephritis were significantly older in this order. ($p < 0.025$). In males, regardless of diagnosis or indication for renal biopsy, the histological glomerular lesions were significantly age-associated. ($p < 0.025$, Table III); older age was associated with more severe glomerular inflammatory lesions. This also held within the immunopathologic entity of idiopathic IgA nephropathy. (Table IV)

3.4 DISCUSSION

The main findings of this prospective study on the relationship of sex and age to the type and severity of idiopathic glomerular disease in adults are threefold. First, some immunopathologic renal diseases, idiopathic IgA nephropathy and membranous glomerulopathy, are significantly male associated. Second, although no sex preponderance was observed in patients from which a renal biopsy was taken because of chronic proteinuria in the absence of longstanding hypertension, renal vascular hyalinosis was female associated, while focal segmental glomerulosclerosis with proteinuria was male associated. In males, finally, both the indication for renal biopsy as well as the glomerular inflammatory changes are related to age.

It is clear that the validity of data about the relationship of sex and age to the type and severity of idiopathic renal disease may be influenced by patient selection for renal biopsy. Although it is unlikely that the male preponderance observed in patients with recurrent macroscopic hematuria, recent onset nephrotic syndrome or rapidly progressive renal insufficiency is due to a more frequent detection of these symptoms in males, this is not necessarily so for persistent microscopic erythrocyturia and chronic proteinuria. More frequent medical examinations in adult life – eg. for compulsory military service, job applications and life insurance – might favour the detection of males with asymptomatic renal disease above females. Our data do not support this reasoning. Renal biopsies taken because of persistent microscopic erythrocyturia with proteinuria and chronic proteinuria were equally divided between the sexes; thus the Dutch draft screening program for asymptomatic renal disease which uses only the dipstick method for urinary protein excretion, did not contribute to a greater number of male referrals. (22) Also patients with persistent microscopic erythrocyturia and normal renal tissue on renal biopsy showed no male preponderance. (Table II)

Our results show that in adults with idiopathic IgA nephropathy renal biopsies were taken either because of recurrent macroscopic hematuria or because of

persistent microscopic erythrocyturia with or without significant proteinuria. It is a male associated disease, (ratio male : female = 31 : 5; $p < 0.005$) even when the indication for renal biopsy is corrected for, by excluding patients with recurrent macroscopic hematuria, which – in this study – is a male associated symptom (ratio male : female = 21 : 4; $p < 0.025$). Other European studies (3,4,24,26) have also reported that idiopathic IgA nephropathy is a male associated disease with male to female ratio's between 2.0 (4) to 6.0 (23). These studies differ from the present one in the sense that they were retrospective, and that in a greater percentage, varying from 37 percent (23) to 60 percent (24) of patients, the renal biopsy was taken because of recurrent macroscopic hematuria. In addition, renal biopsies were taken from patients with a less favourable prognosis or alternatively renal biopsies were taken late in the natural history of disease, judging from the percentage of patients with uremia (24 percent) and hypertension (36 percent) in these studies. (4) Why idiopathic IgA nephropathy is male associated is not known.

The male preponderance of idiopathic adult nephrotic syndrome observed by us agrees with observations of others and is due to a high incidence of idiopathic membranous glomerulopathy which is usually reported to be male associated, (16, 17, 21, 26) although not invariably. (8, 14) The idiopathic nature of the membranous glomerulopathy has to be stressed, since membranous glomerulopathy has been described in several diseases, eg. carcinoma, – specifically bronchial – (3) and chronic hepatitis B infections (12), all of which have a male preponderance. Why idiopathic membranous glomerulopathy is male associated is not known.

The second finding of interest in this study is the observation that of the non-hypertensive patients with chronic proteinuria, normal serum creatinine concentration and no evidence of pre-existing renal disease (on renal biopsy or clinically), females usually show renal vascular hyalinosis (ratio male : female 1 : 6) and males focal segmental glomerulosclerosis and hyalinosis (ratio male : female 6 : 2). Of the women with renal vascular hyalinosis, one had been taken oral contraceptive drugs for a long period.

The patients with focal and segmental glomerulosclerosis associated chronic proteinuria reported in this chapter do not unequivocally fit into the known clinical categories associated with focal and segmental glomerulosclerosis; i.e. decreased renal mass (15) or nephrotic syndrome (9). In an Italian study, on the development of focal and segmental glomerulosclerosis in patients ($24 \times$) nephrectomized because of unilateral renal disease, a similar sex preponderance was found; 7 out of 24 patients developed proteinuria 12 years after a unilateral nephrectomy performed at 22 (median) years of age whereas 17 did not develop proteinuria. All of the patients with proteinuria were males, all had

normal renal function and a normal blood pressure at the time of renal biopsy which showed focal and segmental glomerulosclerosis. Interestingly, only 4 out of these 17 non-proteinuric patients were male. (28) Also in a retrospective study on focal segmental glomerulosclerosis from the United States, patients with proteinuria were usually males, 12 out of 17, whereas in patients with nephrotic syndrome no sex preponderance was noted. (19) We therefore suggest that in middle-aged man, males are more susceptible to the development of focal and segmental glomerulosclerosis and hyalinosis compared to females. Possibly, this is related to a high protein intake and a genetic susceptibility to disease. The latter has been described in rodents (15,20), in which specific strains are more susceptible to development of focal segmental glomerulosclerosis than others, (27) independent of the presence of arterial hypertension. It is not clear why renal vascular hyalinosis is specifically seen in females with chronic proteinuria in the absence of focal and segmental glomerulosclerosis. Similar lesions have been described by others and have been suggested to be pre-hypertensive lesions. (25)

The third finding of interest is the observation in males that the severity of glomerular inflammatory reactions and its symptoms – recurrent macroscopic hematuria, persistent microscopic erythrocyturia with proteinuria and rapidly progressive renal insufficiency –, increase with age. Others too have reported that patients with rapidly progressive uremia caused by a diffuse segmental extracapillary glomerulonephritis without characteristic deposits of immunoglobulins and complement are almost exclusively of older age. (2,7) The observed age difference between patients presenting with persistent microscopic erythrocyturia and proteinuria, and patients with recurrent macroscopic hematuria as well as the associated glomerular inflammatory reactions, confirms and extends observations of others who show older age to be associated with a relatively poor prognosis. (5)

This observation raises the question whether or not a standard glomerular injury, eg. of an immunologic nature, gives more severe glomerular injury in older males. Our data on patients with idiopathic IgA nephropathy appears to answer this question affirmative. First, one patient with monoclonal IgA1 deposits excepted, all of our patients with idiopathic IgA nephropathy had diffuse granular mesangial or mesangiocapillary deposits of IgA1 subclass in conjunction with the third component of complement, C3, and the membrane attack complex of complement, C5b-C9, (see chapter V) suggesting in terms of working hypothesis that comparable mechanisms of immunologically mediated injury were operative. Next, the absence of focal and segmental glomerulosclerosis, significant global glomerular sclerosis and interstitial fibrosis from all biopsies in contrast to other studies (4,5) suggest strongly, that renal biopsies were taken early in the natural history of disease in nearly all patients. This is also reflected by the fact that at the time of renal biopsy only 2

patients showed an increased serum creatinine concentration (over 120 but less than 150 $\mu\text{mol/l}$) which was associated histologically with interstitial abnormalities in one. Precisely these 2 patients showed mild arterial hypertension. This low incidence of hypertension and impaired renal function are well below that observed in the literature (4,5) and is compatible with renal biopsies taken early in the natural history of disease. If one accepts this reasoning then it is clear from our data that older males show more severe glomerular inflammatory (specifically extracapillary proliferation) reactions compared to young adults. The presence of focal and segmental extracapillary proliferative lesions will inevitably cause varying degrees of nephron loss which, superimposed on the age related glomerulosclerosis apparent over 40 years of age and progressive thereafter (19), will predispose for the development of focal and segmental glomerulosclerosis and will thus be associated with a relatively poor prognosis. That the presence of extracapillary proliferative glomerular lesions is a risk factor for progressive renal disease in idiopathic IgA nephropathy was found by others. (1,5) Why older males compared to young develop more severe glomerular injury remains to be determined.

3.5 SUMMARY

The relationship of sex and age to the incidence of idiopathic glomerular disease and the severity of the histological inflammatory lesions in adults between 16 and 65 years of age was determined in a prospective regional study in the Netherlands, in which renal biopsies were taken because of recent onset uremia (19 $\times$), recent onset nephrotic syndrome (52 $\times$), chronic hematuria (93 $\times$) and chronic proteinuria (17 $\times$) from 181 patients early in the natural history of disease.

The indications for renal biopsy, recurrent macroscopic hematuria, persistent microscopic erythrocyturia in the absence of proteinuria and recent onset nephrotic syndrome were male associated, whereas persistent microscopic erythrocyturia in the presence of proteinuria, chronic proteinuria and recent onset uremia were not. Idiopathic IgA nephropathy was significantly male associated ($p < 0.005$); both in patients presenting with recurrent macroscopic hematuria (11 $\times$) and with persistent microscopic erythrocyturia (22 $\times$). The male preponderance of idiopathic IgA nephropathy with persistent microscopic erythrocyturia could not be related to the indication for renal biopsy, since patients with thin basement membrane nephropathy also associated with persistent microscopic erythrocyturia, were equally distributed over the sexes. Patients from which a renal biopsy was taken because of chronic proteinuria were equally divided between the sexes. Males usually showed focal segmental glomerulosclerosis, and females renal vascular hyalinosis. Patients from which a renal biopsy was taken because of nephrotic syndrome were usually males ($p < 0.025$); membranous glomerulopathy was male associated ($p < 0.025$) in

contrast to minimal change glomerulopathy and focal segmental glomerulosclerosis. Patients with recent onset uremia showed no sex preponderance, although idiopathic extracapillary glomerulonephritis was male associated ($p < 0.05$).

In males the severity of the proliferative glomerular inflammatory reaction was related to age and not duration of disease. This also held for proliferative glomerular lesions within the immunopathologic disease entity of idiopathic IgA nephropathy.

This prospective study shows the type of idiopathic glomerular disease as well as the severity of the proliferative inflammatory changes to be related to sex and age respectively.

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THIN BASEMENT MEMBRANE NEPHROPATHY IN ADULTS

The use of quantitative electron microscopy in the differential diagnosis of persistent microscopic erythrocyturia

4.1 INTRODUCTION

In the differential diagnosis of idiopathic recurrent macroscopic hematuria or persistent microscopic erythrocyturia of non-urollogic origin in adults, mesangial deposits of IgA (4), IgM (15) or isolated mesangial deposits of the third component of complement (14) with or without glomerular inflammatory changes, hereditary or familial glomerular disease and interstitial nephritis should be considered. Histological and immunohistochemical analysis of renal tissue is required for the correct diagnosis. However, in a subset of patients, renal biopsy will show no abnormalities by light microscopy or immunohistochemistry. In some of these patients ultrastructural examination will reveal glomerular basement membrane changes, specifically diffuse thinning of the glomerular basement membrane. (1, 7)

The aim of the study presented in this chapter was to determine in a prospective study the incidence of the various, histological, immunohistochemical and ultrastructural defined glomerular abnormalities in adults with chronic hematuria of apparent non-urollogic origin, and to establish the usefulness of quantitative electron microscopy in the differential diagnosis of patients with recurrent macroscopic hematuria and persistent microscopic erythrocyturia. Incidence of disease was determined in a prospective regional study in which renal biopsies were taken from adults with chronic idiopathic hematuria on the basis of strict indications and contraindications, early after patient identification.

4.2 PATIENTS AND METHODS

4.2.1 Patients

Patients originated from a prospective regional study on idiopathic glomerular disease in adults, 16 to 65 years of age. In this study, renal biopsies were taken from patients referred to the nephrology departments of the three main city hospitals in a circumscribed area of The Netherlands according to strict indications and contraindications as described in Chapter II.

Included in the present study are all patients from which a renal biopsy was taken because of recurrent macroscopic hematuria or persistent microscopic erythrocyturia (defined as the repeated presence of more than five erythrocytes in a centrifuged urine specimen per high-power field) with evident proteinuria (albustix positive, 24 hours protein excretion over 500 mg but less than five gram determined by means of a modified Biuret method), with slight proteinuria (albustix trace positive, 24 hours protein excretion less than 500 mg but over 180 mg) or without significant proteinuria (albustix negative, 24 hours protein excretion less than 180 mg). Patients had to have a normal serum creatinine concentration (less than 120 $\mu\text{mol/l}$), the hematuria or microscopic erythrocyturia had to be of unknown origin, and of at least six months duration. In all patients the renal biopsy was taken within two years after patient identification.

None of the patients were consanguineous. All patients had normal liver function tests, a negative rheumatoid factor and negative anti-nuclear factor at the time of renal biopsy. Urologic workup including intravenous urography, ultrasonography, cystoscopy and occasionally renal arteriography did not reveal abnormalities in any of these patients.

Excluded were patients with systemic disease (eg. systemic lupus erythematosus, Henoch-Schönlein nephritis, liver disease, ankylosing spondylitis), patients with known chronic hypertension or drug-associated renal disease, patients originating from families with known hereditary renal disease and patients with acute postinfectious glomerulonephritis.

4.2.2 Morphologic studies

Renal biopsies were processed for light microscopy and immunohistochemistry as described before. (17) (see also chapter II) Renal biopsies were classified according to the World Health Organization criteria. (6)

For transmission electron microscopy, renal tissue was fixed for at least two hours in 2.5 percent glutaraldehyde in 0.1 M sodium phosphate buffer (pH : 7.2), postfixed in 1 percent osmium tetroxide in 0.1 M phosphate buffer (pH: 7.2) for 60 minutes at room temperature, dehydrated and embedded in Epon 812. From one non-ischemic glomerulus, ultrathin silvergray sections were cut on a Reichert ultramicrotome, and collected on formvar coated copper grids. Sections were contrasted with saturated uranylacetate in 50 percent ethanol for ten minutes and 10 percent leadcitrate for five minutes. Transmission electron microscopy was done using a Philips EM 400; evenly spaced electron-micrographs were obtained at a magnification of 31,000, from which glomerular basement membrane thickness was measured using the orthogonal intercept method, first described by Jensen et al. (13) and modified by Steffes et al. (21) A mean of 130 ± 21 intercepts was measured from one glomerulus of each biopsy.

The theoretical considerations relevant to the orthogonal intercept method

and the exact methodology of the glomerular basement membrane measurements are described in the Addendum to chapter IV.

4.3 RESULTS

4.3.1 Indications for renal biopsy, histology and immunohistochemistry

In the period 1978 to 1984, renal biopsies were taken from 89 patients because of recurrent macroscopic hematuria ($30\times$) or persistent microscopic erythrocyturia ($59\times$). The biopsies of eight patients contained less than five glomeruli per two micron section; these were considered insufficient material for proper histological diagnosis. From the remaining 81 patients, renal biopsies were taken in 26 because of recurrent macroscopic hematuria and in 55 because of persistent microscopic erythrocyturia. Of these 55 patients presenting with persistent microscopic erythrocyturia, 27 had significant proteinuria (24 hours urinary protein excretion over 500 mg), 12 had slight proteinuria (24 hours protein excretion less than 500 mg but over 180 mg), and 16 patients had a proteinuria within physiologic limits (24 hours protein excretion less than 180 mg).

Table I

The renal immunopathology of patients with chronic idiopathic non-urolgic hematuria.

Immunopathologic diagnosis	No	Indication for biopsy ¹		
		H	E	EP
Idiopathic IgA nephropathy	27	10	5	12
Idiopathic mesangioproliferative glomerulonephritis	5	2	—	3
Focal global glomerulosclerosis	3	—	1	2
Interstitial nephritis	3	—	3	—
Thin basement membrane nephropathy with mesangial C3 deposits	8	—	4	4
without mesangial C3 deposits	11	1	7	3
Normal renal tissue	23	13	8	2
with mesangial C3 deposits	1	—	1	—

Legend to Table I

1. Indication for renal biopsy. H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia without proteinuria; EP: persistent microscopic erythrocyturia with significant proteinuria (24 hours proteinuria > 500 mg).

In 33 of the 81 patients abnormalities were seen by light microscopy; in 22 of these patients, diffuse mesangial deposits of the IgA1 subclass were demonstrable immunohistochemically. The histological abnormalities in these patients with idiopathic IgA nephropathy were focal (20 ×) or diffuse (2 ×) segmental mesangial proliferation. The other eleven patients showed idiopathic mesangioproliferative glomerulonephritis (5 ×), focal global glomerulosclerosis (3 ×) and interstitial nephritis (3 ×).

In the remaining 48 patients no abnormalities were seen by light microscopy. In five of these patients, diffuse granular mesangial deposits of IgA1 subclass immunoglobulins associated with the third component of complement was shown by immunohistochemistry. In nine other patients, immunohistochemistry showed diffuse mesangial granular deposits of the third component of complement, without immunoglobulin deposits. Table I and II show the clinical and immunopathologic findings related to the indication for renal biopsy.

Table II

Clinical and renal biopsy findings in 81 patients with recurrent macroscopic hematuria or persistent microscopic erythrocyturia.

Clinical Findings	Immunopathologic diagnosis			
	Idiopathic IgA nephropathy	Thin basement membrane nephropathy	Normal renal Tissue	Others ¹
Total number of patients	27	19	24	11
Ratio Male : Female	22:5	10:9	17:7	5:6
Mean age in years	31	36	32	41
Thickness of glomerular basement membrane in nanometers	354 ± 55	190 ± 25	361 ± 69	327 ± 37
At the time of renal biopsy				
No with macroscopic hematuria	10	1	13	2
No with microscopic erythrocyturia				
– proteinuria > 500 mg < 5 g/24 hours	12	7	2	5
– proteinuria > 180 < 500 mg/24 hours		1	5	42
– proteinuria < 180 mg/24 hours	4	6	5	2
At the end of follow up (50 months mean)				
No with macroscopic hematuria	10	–	–	–
No with microscopic erythrocyturia	17	19	6 ²	11

1. Idiopathic mesangioproliferative glomerulonephritis (5 ×), interstitial nephritis (3 ×), focal global glomerulosclerosis (3 ×).

2. In 3 patients a urologic cause of hematuria was found during follow up. Urolithiasis (2 ×) and *in situ* papillary carcinoma of the bladder (1 ×).

4.3.2 Glomerular basement membrane thickness

The mean glomerular basement membrane thickness in the 27 patients with idiopathic IgA nephropathy was 354 ± 55 nanometers, which does not differ from the measurements obtained by others of normal adult kidney donors (males 373 ± 42 nanometers and females 326 ± 45 nanometers) using the same method. (21) The patients with non IgA deposits associated mesangioproliferative glomerulonephritis, focal and global glomerulosclerosis and interstitial nephritis also had a glomerular basement membrane thickness of 326 ± 36 nanometers, which is within normal limits.

The 43 biopsies which showed a normal histology and no deposits of immunoglobulins by immunohistochemistry, could be divided into two different populations in terms of glomerular basement membrane thickness.

One subset ($19 \times$) showed unequivocal diffuse thinning of the glomerular basement membrane; the thickness of the glomerular basement membrane in these patients was 190 ± 25 nanometers. Retrospectively, a positive family history for erythrocyturia could be obtained in six of the patients with this thin basement membrane nephropathy; none of the patients complained of hearing loss, but audiograms were not performed. The presence of thin basement membrane nephropathy was associated with diffuse granular mesangial deposits of the third component of complement in eight patients.

The other subset ($24 \times$) had a glomerular basement membrane thickness within normal limits (mean 361 ± 69 nanometers); only one of these patients showed trace mesangial deposits of the third component of complement.

Figure I shows the distribution of the glomerular basement membrane thickness in the patients and figure II is an exemplary electronmicrograph of patients with a normal and a thin glomerular basement membrane thickness.

4.3.3 Follow up

During follow up, which lasted 50 months (median), erythrocyturia persisted in all of the patients with thin basement membrane nephropathy, idiopathic IgA nephropathy, idiopathic mesangial proliferative glomerulonephritis, interstitial nephritis and focal global glomerulosclerosis. In contrast the macroscopic hematuria or microscopic erythrocyturia disappeared in 18 out of 24 patients with normal glomerular basement membrane thickness and normal glomerular light microscopy. Of the other six patients in which the hematuria persisted, one had isolated mesangial deposits of the third component of complement; while urolithiasis in two, and *in situ* papillary carcinoma of the bladder in one, were demonstrable in three others during the follow up period.

4.3.4 Incidence

The incidence of idiopathic IgA nephropathy and thin basement membrane nephropathy in patients with persistent microscopic erythrocyturia was identical, 32 percent. In patients with recurrent macroscopic hematuria idiopathic IgA nephropathy had an incidence of 39 percent and thin basement membrane nephropathy was observed in one out of 26 patients only. Since in all 13 patients without abnormalities on renal biopsy the recurrent macroscopic hematuria disappeared within two years of follow up, the incidence of idiopathic IgA nephropathy in patients with longstanding recurrent macroscopic hematuria is almost 100 percent when renal biopsies are taken three years after patient identification.

Figure I

Glomerular basement membrane thickness (nanometers) in patients with recurrent macroscopic hematuria and persistent microscopic erythrocyturia

Legend to figure I

1. Idiopathic mesangioproliferative glomerulonephritis (5 ×), interstitial nephritis (3 ×), and focal global glomerulosclerosis (3 ×).

Figure II
Transmission electron microscopy ($\times 13,500$)

Legend to figure II
Normal glomerular basement membrane thickness (1) of 324 nanometers as determined by the orthogonal intercept method and thin basement membrane nephropathy (2) of 215 nanometers as determined by the orthogonal intercept method.

4.4 DISCUSSION

The main findings of this study are twofold.

This study demonstrates that the incidence of thin basement membrane nephropathy in normotensive, non-azotemic adults with persistent idiopathic erythrocyturia, in the presence or absence of proteinuria is high, being identical to the incidence of idiopathic IgA nephropathy. Therefore, morphometric analysis of the glomerular basement membrane thickness of apparently normal renal tissue should be included in the workup of adult patients with persistent idiopathic erythrocyturia.

Furthermore, it is clear from this study that renal biopsies taken from patients with recurrent macroscopic hematuria usually show either idiopathic IgA nephropathy or normal renal tissue. Since in patients with normal renal tissue on renal biopsy, the recurrent hematuria disappears within three years, it is legitimate to monitor the renal function in these patients without resorting to early renal biopsy.

It is clear from this study that the presence of slight or definite proteinuria (24 hours protein excretion over 180 and 500 mg respectively) is highly suggestive of a glomerular origin of the microscopic erythrocyturia, since only six out of 38 patients with erythrocyturia and slight or definite proteinuria showed normal renal (glomerular) morphology. The absence of proteinuria however is not incompatible with a glomerular origin of the erythrocyturia, since 12 out of 17 patients with erythrocyturia and physiologic proteinuria showed glomerular or interstitial renal abnormalities.

The presence of erythrocyte casts was rarely reported in the present study in patients with glomerular erythrocyturia, inclusive patients with idiopathic IgA nephropathy with definite proteinuria. Since our patients had normal renal function and were thus able to produce a concentrated and acid urine which prevents the dissolution of casts, the absence of erythrocyte casts suggests that not enough attention was given to their detection. The presence of abnormal, dysmorphic, erythrocytes by phase contrast microscopy which has been reported to be indicative of a glomerular origin of the erythrocyturia (9) was not determined systematically; our limited experience with this screening method has been disappointing however and this has been confirmed by observations made by others. (18)

The frequent finding of normal renal tissue in patients with recurrent macroscopic hematuria is surprising. These patients do not differ in clinical presentation from the patients with idiopathic IgA nephropathy, since in some it is also associated with upper respiratory tract infections or strenuous exercise. In contrast to the recurrent macroscopic hematuria in patients with

idiopathic IgA nephropathy, the hematuria usually disappears within three years after the first onset of symptoms. The reason for the recurrent macroscopic hematuria in these patients remains obscure. The disappearance of symptoms in patients with normal renal tissue on renal biopsy, together with the observation that the presence of recurrent macroscopic hematuria in patients with idiopathic IgA nephropathy is associated with a favourable prognosis (3), makes it legitimate to monitor renal function and protein excretion in all patients with recurrent macroscopic hematuria without resorting to an early renal biopsy.

Renal biopsy studies of patients with chronic hematuria have shown an array of glomerular lesions and varying mesangial deposits of immunoglobulins and complement components. Usually idiopathic IgA nephropathy – characterized by focal or diffuse mesangium proliferation with mesangial deposits of IgA1 subclass immunoglobulins – is found, which is the most frequent type of glomerulonephritis observed in Europe. (2) Occasionally, focal glomerulonephritis with mesangial deposits of IgM (15), the third component of complement (14) or no deposits at all (15) are found in patients with chronic hematuria. However in many patients, renal biopsies fail to show abnormalities by light microscopy and immunohistochemistry, despite a chronic nature of the hematuria. Van de Putte et al (17) eg. could not demonstrate renal abnormalities in 12 out of 47 patients with recurrent or persistent hematuria and a 24 hour proteinuria less than one gram; in six of these patients the hematuria persisted for over five years. Petterson et al. (16) found normal renal tissue in 29 out of 143 young Finnish men from which renal biopsies were taken because of hematuria with or without proteinuria. In both studies systematic ultrastructural studies, either qualitative or morphometric, were not mentioned. The importance of morphometric measurements of glomerular basement membrane thickness was recently demonstrated by Coleman et al. (7) They observed in four cases with apparently normal glomeruli, glomerular basement membrane abnormalities which were demonstrable only by morphometric analysis. The present results confirm their findings and stress the importance of routine ultrastructural investigations inclusive morphometric analysis of the glomerular basement membrane thickness in patients with chronic idiopathic hematuria early in the natural history of disease, notably in patients with persistent microscopic erythrocyturia.

That abnormalities of the glomerular basement membrane are associated with hematuria is well known. Usually, glomerular basement membrane abnormalities are the main findings in a variety of familial nephropathies, notably in the early stages of Alport's syndrome (6), the nail patella syndrome (6) and the so-called benign familial hematuria. The latter condition is typically characterized ultrastructurally by diffuse thinning of the glomerular basement membrane and clinically by a non-progressive nephropathy usually inherited with

recurrent hematuria as the only symptom of disease. (22, 23, 24) Most reports on thinning of the glomerular basement membrane concern children. (22, 23, 24) Recently, two retrospective studies on thin basement membrane nephropathy in adults were published. In an Australian study 41 cases out of 480 consecutive renal biopsies were identified in which ultrastructural thinning of the glomerular basement membrane was the major lesion; from all patients a renal biopsy was taken because of idiopathic hematuria. (7) In a Japanese study, only 8 cases out of 998 renal biopsies showed these lesions and all patients presented with persistent microscopic erythrocyturia. (1) Like in our patients attenuation of the glomerular basement membrane was suspected on subjective routine electron microscopic examination in most patients, but morphometry revealed another few with significantly thinner glomerular basement membranes compared to normal controls. Since none of our patients with thin basement membrane nephropathy originated from families with known hereditary progressive renal disease or were known with hearing disorders, we probably detected patients whose ultrastructural abnormalities should be defined as benign hematuria, possibly familial in some, since a positive family history for erythrocyturia was obtained in six out of 19 patients, retrospectively.

Whether or not benign familial hematuria can be distinguished ultrastructurally from progressive hereditary nephritis as seen in Alport's syndrome remains to be determined. In contrast to the extensive attenuation of the glomerular basement membrane in the so-called familial benign hematuria, different electron microscopic findings are considered pathognomonic of progressive hereditary nephritis, eg. Alport's syndrome. (5, 12, 20) These include the presence of an irregular thinning as well as marked thickening of the glomerular basement membrane – due to lamellation, reticulation and fragmentation of the lamina densa –, and the development of lacunar networks in the membranes surrounding intramembranous particles, each in turn encircled by a lucent area. The specificity of the latter glomerular basement membrane lesions has been questioned since identical lesions, although not diffusely present, were observed in poststreptococcal glomerulonephritis and focal segmental glomerulosclerosis. (11) Several authors have reported patients who met all the classic criteria for Alport's syndrome, ie. the familial occurrence in successive generations of progressive nephritis with signs indicative of glomerular damage and of neural hearing loss, who showed by electron microscopy widespread thinning of the glomerular basement membrane. (10, 19, 25) Hereditary nephritis can thus be considered to be the composite of a number of genetically distinct syndromes (11) with varying rates of progression and non-uniformly glomerular basement membrane alterations. Thin basement membrane nephropathy may therefore well represent an expression of the same type of disease as Alport's syndrome and need not be benign in terms of ultimate prognosis.

The need for caution in predicting prognosis is reinforced by the recent description of twelve patients with thin basement membrane nephropathy, three of which developed progressive renal disease with end stage renal failure in one. (8) Moreover, three out of our patients with thin basement membrane nephropathy initially presenting with proteinuria over 500 mg a day, developed arterial hypertension within the follow up period.

Careful follow up of these patients, and their relatives is required to determine whether or not thin basement membrane nephropathy can be considered a benign glomerular disorder.

4.5 SUMMARY

In a prospective regional study on the incidence of idiopathic renal disease renal biopsies were taken from 81 normotensive adults between 16 and 65 years of age, because of apparent non-urologic recurrent macroscopic hematuria ($26\times$) or persistent microscopic erythrocyturia ($55\times$) of at least half a year duration. Renal biopsies were taken within two years after patient identification; all patients had a normal serum creatinine concentration. Follow up was 50 months median.

Idiopathic IgA nephropathy was present in 27 patients and associated with persistent microscopic erythrocyturia in 17. Histological abnormalities comparable with idiopathic mesangioproliferative glomerulonephritis ($5\times$), focal global glomerulosclerosis ($3\times$) and interstitial nephritis ($3\times$) were seen in another eleven patients. Normal renal tissue by light microscopy and absence of glomerular immunoglobulin deposits was present in 43 patients. In these patients morphometric analysis of the glomerular basement membrane thickness with electron microscopy revealed two subsets. Thin glomerular basement membranes (190 ± 25 nanometers) were present in 19 patients, 7 of which showed mesangial deposits of C3. Eighteen of these patients presented with persistent microscopic erythrocyturia which persisted during follow up in all. The glomerular basement membrane thickness of the other 24 patients was normal (361 ± 69 nanometers); during follow up, the macroscopic hematuria ($13\times$) disappeared in all, whereas the microscopic erythrocyturia disappeared in five, persisted in three, and was shown to be of urologic origin in another three patients.

This study shows the incidence of thin basement membrane nephropathy in patients with persistent microscopic erythrocyturia in the presence or absence of proteinuria to be high and identical to the incidence of idiopathic IgA nephropathy. Morphometric analysis of the glomerular basement membrane thickness of apparently normal renal tissue should therefore be included in the work up of adults with persistent microscopic erythrocyturia.

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IDIOPATHIC IGA NEPHROPATHY: AN *IN SITU* IMMUNE COMPLEX DISEASE ?

The incidence of circulating IgA immune complexes in the serums of patients with recurrent macroscopic and persistent microscopic hematuria early in the natural history of disease

5.1 INTRODUCTION

Idiopathic IgA nephropathy is characterized by mesangial deposits of IgA, usually the IgA1 subclass, together with the third component of complement, C3, and frequently with deposits of small quantities of other immunoglobulin classes as well. (1) Histologically, varying degrees of glomerular inflammatory reactions, especially mesangial cell and matrix proliferation are seen, and ultrastructurally the presence of electron-dense deposits in the mesangial matrix is characteristic. In order to make the diagnosis, secondary IgA nephropathies – of which Henoch-Schönlein nephritis, systemic lupus erythematosus glomerulonephritis and liver disease associated glomerular disease are the most important – have to be ruled out. (2) Idiopathic IgA nephropathy is usually a disease of young adults and has a strong male preponderance. (see also chapter III) The clinical presentation of most patients with idiopathic IgA nephropathy is recurrent macroscopic hematuria or persistent microscopic erythrocyturia (2), whereas the nephrotic syndrome and rapidly progressive renal insufficiency are uncommon. (11)

The mechanisms involved in the appearance of the glomerular IgA deposits are a topic of debate; indirect evidence suggests that the lesions are due to the mesangial deposition of circulating immune complexes or IgA polymers, although *in situ* immune complex formation due to an interaction of IgA antibodies with heterologous or autologous, 'planted' or intrinsic, antigens cannot be ruled out. The evidence which supports the circulating immune complex hypothesis derives from the following observations. First, circulating IgA immune complexes can be demonstrated in the serums of patients with idiopathic IgA nephropathy using various assays, including an anti-IgA inhibition binding assay (44), a Raji cell assay (18), a conglutinin binding assay (12), and a solid phase anti-C3 assay (13). Second, IgA and C3 deposits are frequently demonstrable in the skin capillaries of patients with idiopathic IgA nephropathy (3) and finally, IgA deposits may reoccur after renal transplantation of a normal cadaveric kidney into patients with idiopathic IgA nephropathy. (5) Why patients with idiopathic IgA nephropathy frequently have increased concentrations of serum

IgA (46), IgA polymers (26), IgA rheumatoid factor (38) and circulating IgA immune complexes is not clear, but *in vitro* studies suggest that these patients have disturbed immunologic functions, as evidenced by decreased numbers of T α suppressor cells (36) and increased numbers of T α helper cells (37), resulting in an enhanced production of immunoglobulin A both spontaneously and upon mitogen stimulation. (17) Since none of the studies cited above were prospective and because healthy volunteers were invariably used as controls for immunologic studies, there is no convincing unbiased evidence which relates the immunologic abnormalities described above to pathogenesis, inclusive the presence of circulating IgA immune complexes.

In the present chapter this issue was addressed by comparing serum IgA concentrations, including dimeric IgA, and the presence of circulating IgA immune complexes in the serum of patients with biopsy proven idiopathic IgA nephropathys to those in the serum of patients with biopsy proven thin basement membrane nephropathy or normal renal tissue of comparable age, sex and clinical presentation. All patients originated from a prospective regional study in which renal biopsies were taken early after patient identification.

5.2 PATIENTS AND RENAL BIOPSIES

5.2.1 Patients

Patients originated from a prospective regional study carried out between 1978 and 1984 on idiopathic glomerular disease in adults between 16 and 65 years of age as described in chapter II. The renal biopsies were taken because of recurrent macroscopic hematuria or persistent microscopic erythrocyturia (defined as the repeated presence of more than five red cells per high-power field in a centrifuged urinespecimen) of unknown origin and of at least six months duration in the absence of systemic or urological disease, with or without significant proteinuria (24 hours proteinuria less than 500 mg measured by a modified Biuret method). From all patients renal biopsies were taken within two years after patient identification.

Of the 82 patients from which a renal biopsy was taken, 81 showed a normal serum creatinine concentration (less than 120 $\mu\text{mol/l}$); one patient with idiopathic IgA nephropathy had a serum creatinine concentration of 149 $\mu\text{mol/l}$. Routine laboratory tests at the time of renal biopsy included anti nuclear and rheumatoid factors (Latex fixation and Roose-Waaler tests), anti-streptolysin titer, liver function tests and classic hemolytic complement activity (CH_{50}). These tests were negative or within normal limits in all patients. In one patient with monoclonal IgA1 lambda mesangial deposits, the presence of an IgA myeloma was ruled out. (No monoclonal IgA1 or lambda spike was demonstrable in the serum or in concentrated urine with polyacrylamide gradient gel electrophoresis and immunoblotting).

5.2.2 Renal biopsies

From all patients sufficient (over five glomeruli per two micron section) material was obtained. Biopsies were processed and stained for light microscopy, immunohistochemistry and electron microscopy as described before. (35) (see also chapter II) In addition unfixed frozen two micron sections were stained for the presence of IgA1 and IgA2 subclasses using monoclonal mouse IgG1 antibodies (kindly donated by Dr. J. Haayman, Dept. of Gerontology, TNO, Rijswijk, The Netherlands) in the indirect immunofluorescence with an affinity purified and fluorescein labelled goat IgG anti-mouse IgG (Cappel Worthington Biochemicals, Malvern, Pennsylvania, USA) after absorption with insolubilized human serum. The presence of the membrane attack complex of human complement (C5b - C9) was verified by indirect immunofluorescence using polyclonal and monoclonal mouse antisera directed against neoantigens of the C5b-C9 membrane attack complex (20) (kindly donated by Dr. S. Bhakdi, Institute of Microbiology, University of Giessen, West-Germany). Renal biopsies were classified according to the World Health Organization criteria. (10)

Of the renal biopsies a diagnosis of idiopathic IgA nephropathy was made in 28 patients; this required the presence of diffuse granular mesangial deposits of IgA immunoglobulins in the absence of systemic disease. In all patients electron-dense deposits in the mesangium could be easily demonstrated by transmission electron microscopy. (Figure I). In 19 patients a diagnosis of thin basement membrane nephropathy was made (see also chapter IV) and 24 patients showed normal renal tissue by light microscopy, immunohistochemistry and electron microscopy. The remaining 11 patients showed miscellaneous lesions including idiopathic mesangioproliferative glomerulonephritis (5 ×), focal global glomerulosclerosis (3 ×) and local interstitial nephritis (3 ×).

5.3 SEROLOGIC STUDIES

For serologic studies, serums of three groups of patients, ie. idiopathic IgA nephropathy, thin basement membrane nephropathy and normal renal tissue, all of comparable numbers and median age were used. As positive controls serums of patients with secondary IgA nephropathy associated with cirrhosis of the liver (2 ×) and Henoch-Schönlein nephritis (9 ×) were used from which renal biopsies were taken because of erythrocyturia and proteinuria. Finally, sixteen healthy volunteers (seven female, nine male) from laboratory and medical staff without erythrocyturia or proteinuria served as negative controls. Blood samples were obtained from all patients at the time of renal biopsy. For cryoglobulin studies, blood was transported and allowed to clot at 37 °C. Next serum was harvested by centrifugation at 2,000 g for 20 minutes at 37 °C. For all other serologic studies blood was allowed to clot for one hour at room

Figure 1
Transmission electron microscopy
($\times 6,700$)

Legend to figure 1
Idiopathic IgA nephropathy. Characteristic electron-dense deposits in the mesangium, indicative of immune complex formation.

* electron-dense deposit

temperature and one hour on ice, centrifuged at 2,000 g for 20 minutes, immediately frozen in liquid nitrogen and stored at -70 °C. Serum samples were thawed just before testing and used only once.

5.3.1 Reagents

Goat F(ab)₂ anti-human IgA (α -chain specific), goat F(ab)₂ anti-human IgG (γ -chain specific) and goat anti-human IgM (μ -chain specific) were purchased. (Cappel, Worthington Biochemicals. Malvern. Pennsylvania. USA). These polyclonal antisera were labelled with Horse Radish Peroxidase (HRP type VI Sigma, St. Louis. USA) according to standard procedures. (6) Monoclonal mouse IgG1 anti-human C3 (monoclonal antibody 130) was kindly donated by Dr. J.D. Tamerius. This monoclonal antibody 130 has been shown in ELISA to bind circulating immune complexes by virtue of its specificity for iC3b and C3d fragments. (1) Monoclonal mouse IgG1 anti-human IgA1 and IgA2 were kindly donated by Dr. J. Haayman (Department of Gerontology, TNO, Rijswijk, The Netherlands). Monoclonal antibodies to IgA1 and IgA2 were pair labelled with Iodine-125 and Iodine-131 (Radiochemical Centre, Amersham, United Kingdom) respectively, by means of the Chloramine-T method (29) resulting in specific activities of 63 kilo becquerel per microgram and 20 kilo becquerel per microgram of protein respectively. Goat F(ab)₂ anti-human complement component C3 was isolated as described by Tack and Prahl (39). Human IgG was isolated from serum by standard methods. (35) Human secretory IgA and human secretory component were isolated from human colostrum (kindly obtained through Prof. Dr. G. Essed, Department of Obstetrics and Gynaecology, Academic Hospital, Maastricht. The Netherlands) as described by Khayam-Bashi et al. and Underdown et al respectively. (24, 43) C1q was isolated from fresh frozen human plasma as described by Tenner and Cooper. (40) C1q was radiolabelled with Iodine-125 by means of a lactoperoxidase method (19) resulting in a specific activity of 82 kilo becquerel per microgram. The standard for normal human serum derived from the Central Laboratory of the Dutch Red Cross Bloodtransfusion (Amsterdam). As standards for immune complex assays heat aggregated immunoglobulins of restricted size, prepared according to Kauffmann et al. (22) and Nydegger et al. (33) were used. Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) were performed in flexible microtitre plates.(Dynatech).

Isolation procedures, purity and specificity controls are described in detail, in addition to the methodology of the assays in the addendum to chapter V.

5.3.2 Serum immunoglobulin concentrations

Serum immunoglobulin concentrations were determined by means of an ELISA. The results for the test sera were correlated with that of known concentra-

tions of the standard serum described above, run in the same test. The immunoglobulin concentration was considered increased when it exceeded the 95 percentile of the sixteen normal healthy controls, using non-parametric techniques. (IgG: 16.1 mg/ml, IgA: 3.6 mg/ml, and IgM: 2.0 mg/ml).

5.3.3 IgA dimers

IgA dimers were measured by a solid phase RIA by means of a secretory component binding assay. The binding of dimeric IgA1 or IgA2 to human secretory component was considered increased when it exceeded the 95 percentile of the sixteen normal healthy controls (188.7 ng/ml binding of monoclonal mouse anti-human IgA1 and 74.3 ng/ml binding of monoclonal mouse anti-human IgA2 determined using non-parametric techniques). A binding of dimeric IgA1 and IgA2 in serums of patients with secondary IgA nephropathy associated with cirrhosis of the liver of 538 ng/ml and 320 ng/ml respectively indicated that binding capacity for both IgA1 and IgA2 dimers was well in excess of the concentrations present in the serums of patients with idiopathic hematuria.

5.3.4 IgA rheumatoid factor

The presence of rheumatoid factor of the IgA class was determined by an ELISA essentially as described by Sinico et al. (38) Serum samples were considered positive when the optical density was two fold higher than that of a pool of 16 normal healthy controls in at least three dilutions. All positive serums failed to bind to the solid phase when the polyclonal IgG was substituted with human F(ab)2 fragments prepared from this IgG.

5.3.5 Circulating immune complexes

A solid phase goat F(ab)2 anti-human C3 or mouse IgG1 anti-human C3 monoclonal antibodies (monoclonal antibody 130) RIA or ELISA was performed essentially as described by Pereira et al. (34) For IgG, IgA and IgM immune complexes goat F(ab)2 anti-human IgG HRP, anti-human IgA HRP and goat anti-human IgM HRP respectively, appropriately diluted in Tris buffer were added and the assay was continued as described above. In addition, to detect circulating IgA immune complexes, 50 ng of pair labelled mouse monoclonal anti-human IgA1 and IgA2 in 100 μ l Tris buffer was incubated at 37 °C for 3 hours and after extensive washing the individual wells were cut out and counted in a gamma-counter.

In this immune complex assay coating of the polyclonal anti-human C3 and the monoclonal antibody 130 yielded identical results, both in sensitivity as well as specificity. Therefore only the goat F(ab)2 anti-human C3 solid phase was

used to determine the presence of circulating immune complexes. Immune complexes were considered present when anti-human IgG, anti-human IgA or anti-human IgM binding exceeded the 95 percentile of normal healthy controls; this was for IgG immune complexes the equivalent of 10 μ g heat aggregated IgG, and for IgA immune complexes the equivalent of 3 μ g heat aggregated IgA. In addition, the presence of circulating immune complexes was determined by means of a fluid phase C1q binding assay as described by Zubler et al. (47).

The solid phase anti-C3 circulating immune complex assay was tested for clinical specificity using 28 serums of patients with membranous glomerulopathy from which a renal biopsy was taken because of a nephrotic syndrome within half a year after onset of disease, 9 serums of patients with Henoch-Schönlein nephritis, 2 serums of patients with IgA nephropathy associated with cirrhosis of the liver and 4 serums of patients with streptococcus viridans associated subacute bacterial endocarditis in the absence of rheumatoid factor. IgA1 circulating immune complexes were absent from serums of patients with membranous glomerulopathy and subacute bacterial endocarditis; conversely 6 out of 9 patients with Henoch-Schönlein nephritis and both patients with cirrhosis of the liver had IgA1 immune complexes. IgG and IgM immune complexes were absent in all patients but for subacute bacterial endocarditis; in these patients IgG and IgM immune complexes were usually demonstrable, the latter in quantities up to 14 times normal serum controls.

5.3.6 Sucrose gradient ultracentrifugation

The size of the circulating IgA immune complexes was determined using 10 - 30 percent sucrose (w/v) gradient centrifugation. IgG, C1q and IgM were run along as markers for 7, 11 and 19 S respectively. Fractions were collected and tested for IgA content by ELISA, as described above, and for reactivity in the anti-C3 immune complex assay by RIA with radiolabelled monoclonal anti-human IgA1. Results were expressed as optical density readings at 492 nanometer and counts per minute respectively.

5.3.7 Statistical methods

Non-parametric tests have been used throughout this study: specifically the Mann-Whitney U-test for unpaired data. A value of $2\alpha < 0.05$ was considered significant. Correlation coefficients were calculated using the Spearman rank test.

5.4 RESULTS

5.4.1 Clinical presentation

Twenty-four out of 71 patients showed recurrent macroscopic hematuria which was male associated and almost equally distributed between patients with idiopathic IgA nephropathy (10 ×) and patients without glomerular pathology (13 ×). In idiopathic IgA nephropathy, the hematuria was associated with upper respiratory tract infections, so-called 'synpharyngitic' hematuria (5 ×), exercise (1 ×), and diarrhea (1 ×). In two patients with normal renal tissue the macroscopic hematuria was 'synpharyngitic' and in four patients there was an association with exercise. Therefore the clinical presentation was indistinguishable. The remaining 47 patients presented with persistent microscopic erythrocyturia with or without proteinuria. (Table I).

5.4.2 Serum immunoglobulin concentrations

Patients with idiopathic IgA nephropathy had significantly increased concentrations of serum IgA (1.02 - 7.17 mg/ml) compared to patients with thin basement membrane nephropathy (0.87 - 4.09 mg/ml), patients with normal renal tissue (0.49 - 5.90 mg/ml) and healthy volunteers (0.89 - 3.60 mg/ml). ($2\alpha < 0.005$, Mann-Whitney U test). An increased concentration of serum IgA, as was found in 10 out of 28 patients with idiopathic IgA nephropathy, did not identify positively patients with idiopathic IgA nephropathy however, since increased concentrations of serum IgA were also found in two out of 19 patients with thin basement membrane nephropathy and in three out of 24 patients

Table I

The immunopathologic findings in renal biopsies taken because of chronic idiopathic non-urologic hematuria.

Immunopathologic findings	No	Sex M:F	Age	Indication for biopsy ¹		
				H	E	EP
Idiopathic IgA nephropathy	28	23:5	33	10	5	13
Thin basement membrane nephropathy	19	10:9	36	1	11	7
Normal renal tissue	24	17:7	32	13	9	2

Legend to Table I

1. Indication for renal biopsy:

H: recurrent macroscopic hematuria; E: persistent microscopic erythrocyturia without proteinuria; EP: persistent microscopic erythrocyturia with proteinuria (24 hours proteinuria > 500 mg).

Figure II
Serum IgA concentration in patients with recurrent macroscopic hematuria, persistent microscopic erythrocyturia, and healthy controls

Table II.
Concentration of serum IgA in patients with recurrent macroscopic hematuria or persistent microscopic erythrocyturia.

Immunopathologic findings	No	Range (mg/ml)	Mean	No increased ¹
Idiopathic IgA nephropathy	28	1.02-7.17	3.30	10
Thin basement membrane nephropathy	19	0.87-4.09	2.12	2
Normal renal tissue	24	0.49-5.90	2.11	3
Normal healthy controls	16	0.89-3.60	1.77	–

Legend to Table II
 1. No of patients with serum IgA concentration over 95 percentile of normal healthy controls (3.6 mg/ml nonparametric).

with normal renal tissue. (Table II and Figure II). Increased concentrations of serum IgA in patients with idiopathic IgA nephropathy was not associated with a specific clinical presentation, eg. recurrent macroscopic hematuria.

With respect to the serum concentrations of IgG and IgM, we found no differences between the patients with idiopathic IgA nephropathy, thin basement membrane nephropathy or normal renal tissue: only a few patients had serum concentrations exceeding the 95 percentile of normal healthy controls. All but one of these patients had normal IgA concentrations. (Table IV)

5.4.3 IgA dimers

The amount of dimeric human IgA1 in the serums of patients with idiopathic IgA nephropathy (66.4 - 336.2 ng/ml) did not differ from those in patients with thin basement membrane nephropathy (83.9 - 291.7 ng/ml) or normal renal tissue associated hematuria (52.8 - 336.9). An increased binding of dimeric IgA1 to human secretory component was found in 9 out of 28 patients with idiopathic IgA nephropathy, in 5 out of 19 patients with thin basement membrane nephropathy and in 5 out of 24 patients with normal renal tissue associated hematuria. Thus, dimeric IgA1 is not discriminative for the diagnosis of idiopathic IgA nephropathy. (Table III and Figure III). Similarly, no difference was observed when the binding of dimeric IgA2 to human secretory

Table III

The secretory component binding capacity of dimeric IgA subclasses in serum of patients with recurrent macroscopic hematuria or persistent microscopic erythrocyturia.

Immunopathologic findings	No	anti-human IgA1 bound		anti-human IgA2 bound	
		Range (ng/ml)	Mean (.) ¹	Range (ng/ml)	Mean (.) ²
Idiopathic IgA nephropathy	28	66.4-336.2	162.4 (9)	21.4- 68.2	39.9 (0)
Thin basement membrane nephropathy	19	83.9-291.7	163.2 (5)	31.6-108.3	53.9 (2)
Normal renal tissue	24	52.8-336.9	157.2 (5)	20.2- 96.1	50.7 (3)
Normal healthy controls	16	79.8-188.0	124.2 (0)	16.9- 74.5	38.5 (0)

Legend to Table III

1. No in parentheses is the number of patients with anti-human IgA1 binding over 95 percentile of normal healthy controls (188.0 ng/ml, nonparametric).
2. No in parentheses is the number of patients with anti-human IgA2 binding over 95 percentile of normal healthy controls (74.5 ng/ml, nonparametric).

Secretory component binding capacity of dimeric IgA in patients with recurrent macroscopic hematuria, persistent microscopic erythrocyturia, and healthy controls

Figure III
dimeric IgA1

Legend to figure III
95 percentile of normal healthy controls:
188 ng/ml (Mann-Whitney U-test)

Figure IV
dimeric IgA2

Legend to figure IV
95 percentile of normal healthy controls:
74.5 ng/ml (Mann-Whitney U-test)

component was measured. (Table III and Figure IV). Binding of dimeric IgA1 or IgA2 to human secretory component did not correlate with the serum concentration of IgA, IgG or IgM. ($2\alpha > 0.05$ Spearman rank test). These correlations were also absent in the different clinical and immunohistological subsets.

5.4.4 IgA rheumatoid factor

IgA rheumatoid factor was positive in 3 out of 28 patients with idiopathic IgA nephropathy, whereas none of the patients with thin basement membrane nephropathy or normal renal tissue associated hematuria had detectable IgA rheumatoid factor.

5.4.5 Circulating immune complexes

Cryoprecipitable immune complexes were not found in any of the serums. Similarly, in the fluid phase C1q binding assay, all serum samples were negative. Also no IgG and IgM containing immune complexes could be demonstrated in the solid phase anti-C3 assay in the patient serums studied. Using the solid phase anti-C3 assay circulating immune complexes containing IgA1 were detected in 4 out of 28 patients with idiopathic IgA nephropathy, in 4 out of 24 patients with normal renal tissue associated hematuria, and in 1 patient with thin basement membrane nephropathy. (Table IV and Figure V). None of the positive serums showed any binding when goat F(ab)2 anti-human C3 – used for coating of the ELISA plates – was substituted with human F(ab)2 fragments, which demonstrated the absence of an aspecific binding of IgA1. With the exception of one patient with idiopathic IgA nephropathy, all patients with circulating IgA1 immune complexes had normal serum concentrations of IgA and a normal dimeric IgA1 binding to human secretory component.

IgA2 containing immune complexes were demonstrated with the solid phase anti-C3 assay in only one patient. This patient was shown to have thin basement membrane nephropathy on renal biopsy, a normal serum concentration of IgA, and an increased binding of dimeric IgA2 to human secretory component.

In 4 patients with idiopathic IgA nephropathy and 4 patients with normal renal tissue associated hematuria, the size of the IgA1 containing circulating immune complexes was determined using sucrose gradient ultracentrifugation; five patients with Henoch-Schönlein nephritis and two patients with IgA nephropathy associated with cirrhosis of the liver and positive IgA1 immune complexes, served as positive controls. The size of the circulating IgA1 immune complexes in patients with idiopathic IgA nephropathy and patients with normal renal

Figure V
Anti-C3 solid phase binding of IgA1 circulating immune complexes in patients with recurrent macroscopic hematuria, persistent microscopic erythrocyturia, secondary IgA nephropathy, and healthy controls

Legend to figure V

1. Henoch-Schönlein nephritis, ● (9 ×), and IgA nephropathy in cirrhosis of the liver, ○ (2 ×) 95 percentile of normal healthy controls: 43 ng/ml (Mann-Whitney U-test)

Table IV

Serologic findings in patients with recurrent macroscopic hematuria or persistent microscopic erythrocyturia.

	Idiopathic IgA nephropathy	Thin basement membrane nephropathy	Normal renal tissue
Total number of patients	27	19	24
– Increased serum IgA	10	2	3
– Increased serum IgG	2	1	2
– Increased serum IgM	0	1	2
– Increased dimeric IgA1	9	5	5
– Increased dimeric IgA2	0	2	3
IgA rheumatoid factor	3	0	0
Presence of circulating immune complexes			
– C1q binding assay	0	0	0
– anti-C3 assay IgA1	4	1	4
– anti-C3 assay IgA2	0	1	0
– anti-C3 assay IgG	0	0	0
– anti-C3 assay IgM	0	0	0

tissue were identical, 11 S, (Figure VI) which coincided with the sedimentation of dimeric IgA1. In contrast, large sized IgA1 immune complexes were

Figure VI

Sucrose density ultracentrifugation of serum from patients with idiopathic IgA nephropathy and normal renal tissue associated hematuria

Legend to figure VI

- Anti-C3 solid phase binding of IgA1 circulating immune complexes; counts per minute bound mouse anti-human IgA1-I 125 labelled
- IgA concentration; optical density readings at 492 nanometers in an enzyme-linked immunosorbent assay

demonstrable in patients with cirrhosis of the liver, whereas in patients with Henoch-Schönlein nephritis large, intermediate and small immune complexes were seen. (Figure VII).

Figure VII

Sucrose density ultracentrifugation of serum from patients with Henoch-Schönlein nephritis and IgA nephropathy in cirrhosis of the liver

Legend to figure VII

- Anti-C3 solid phase binding of IgA1 circulating immune complexes; counts per minute bound mouse anti-human IgA1-I 125 labelled
- IgA concentration; optical density readings at 492 nanometers in an enzyme-linked immunosorbent assay

5.4.6 Immunohistological findings in idiopathic IgA nephropathy

Both the histological and immunohistochemical findings in the renal biopsies of patients with idiopathic IgA nephropathy were heterogeneous varying from no abnormalities on light microscopy ($5\times$), or focal segmental mesangioproliferative glomerulonephritis ($20\times$), to diffuse mesangioproliferative glomerulonephritis ($2\times$). The mesangial or – less frequently – mesangiocapillary deposits of IgA1 were always associated with deposits of C3 and the C5b-C9 membrane attack complex of complement and occasionally accompanied with deposits of IgA2, IgM or IgG. (Table V).

5.4.7 Immunopathology and serologic parameters

The presence of circulating immune complexes, increased serum concentrations of IgA or increased dimeric IgA1 binding to human secretory component was neither related to the severity of the histological lesions nor to the severity of proteinuria or the presence of recurrent macroscopic hematuria. In 16 out of 27 patients with idiopathic IgA nephropathy both binding of dimeric IgA1

Table V.

The spectrum of histological and immunohistochemical findings in adult patients with idiopathic IgA nephropathy.

Histological findings	No	Immunohistochemical findings ¹							
		IgA1	IgA1 + IgA2	IgM	IgG	K	L	C3	C5b-C9
Normal renal tissue	5	5	–	1	1	5	5	5	5
Focal segmental mesangial proliferation ²	11	10	1	3	3	10	11	11	11
Focal segmental mesangial proliferation with a segmental extracapillary component ³	10	10	1	1	1	10	10	10	10
Diffuse segmental mesangial proliferation with a segmental extracapillary component	2	1	1	2	1	2	2	2	2

Legend to Table V

1. Glomerular deposits of IgA1 as the main immunoglobulin in a diffuse mesangial pattern ($23\times$) and a diffuse mesangiocapillary pattern ($5\times$).
2. Proliferation of mesangial matrix and mesangial cells.
3. Extracapillary component: focal segmental extracapillary proliferation.

and IgA2 to human secretory component, as well as the presence of IgA1 circulating immune complexes were within normal range or negative. The three patients with idiopathic IgA nephropathy and mesangial deposits of both IgA1 and IgA2 showed normal concentrations of dimeric IgA2 and no circulating immune complexes. Increased binding of dimeric IgA1 to human secretory component was observed in one of these three patients only.

5.5 DISCUSSION

The main findings of this study are two fold. First, although the concentrations of serum IgA and binding of dimeric IgA1 to secretory component were increased in many patients with idiopathic IgA nephropathy, they cannot be considered indicative of idiopathic IgA nephropathy in the individual patient. In addition, the presence of IgA1 circulating immune complexes is not specific for idiopathic IgA nephropathy, since IgA1 immune complexes are equally distributed over patients with idiopathic IgA nephropathy and patients with normal renal tissue associated hematuria.

These observations support two conclusions. Renal biopsies are required for the diagnosis of idiopathic IgA nephropathy and in addition there is no evidence for the hypothesis that idiopathic IgA nephropathy is caused by the mesangial deposition of circulating IgA1 immune complexes or macromolecular IgA1.

It is clear that evaluation of the perturbations of immune functions in patients with idiopathic IgA nephropathy require the presence of patients – of comparable age and sex – with hematuria of suspected glomerular origin without immunohistochemical characteristics of idiopathic IgA nephropathy, as clinical controls. However, most investigators used healthy persons as controls. In such studies the percentage of patients with idiopathic IgA nephropathy and increased serum concentrations of IgA varies from 39 percent (46) to 58 percent (31); these percentages are comparable with the 41 percent found in the present study. These increased concentrations of IgA are not a consequence of a polyclonal B-cell activation for the concentrations of IgG and IgM are within normal limits. However, the increased serum concentration of IgA is not diagnostic for idiopathic IgA nephropathy since it was also found in 2 out of 19 patients with thin basement membrane nephropathy and 3 out of 24 patients with normal renal tissue associated hematuria.

Compared to healthy volunteers, both increased (26) and normal (46) quantities of IgA polymers varying in size from 9 to 21 S have been demonstrated in serums of patients with idiopathic IgA nephropathy by means of sucrose gradient ultracentrifugation. The moment in the natural history of disease when the serums were obtained as well as the nature of disease – recurrent hematuria with upper respiratory tract infections versus persistent microscopic

erythrocyturia – may in part explain this discrepancy. For instance, using High Performance Liquid Chromatography, increased concentrations of IgA dimers and polymers could be demonstrated in 3 out of 6 patients during exacerbations of macroscopic hematuria in conjunction with acute upper respiratory tract infections. This returned to normal during remission. (15).

It is now well accepted that the mesangial IgA deposits consist of IgA dimers, because of the binding of free secretory component (4, 45) – which is known to bind only to dimeric IgA (or IgM) through their J chain (7) – and because of the demonstration of the J chain after 6 M urea treatment of frozen renal biopsy sections. (45) Consequently, considerable attention has been given to measuring concentrations of IgA dimers in the serum of patients with idiopathic IgA nephropathy. Using Iodine-125 labelled secretory component, increased binding was observed in 14 out of 30 patients with idiopathic IgA nephropathy, although no selective increase of the polymeric IgA fraction was noted. This increase appeared to be the consequence of a general increase of all IgA molecular forms. (32) Other investigators using exclusion chromatography followed by solid phase secretory component binding of IgA polymers, observed increased concentrations in 8 out of 15 patients when compared to 5 healthy volunteers. (45) The finding in the present study of increased binding of dimeric IgA1 to solid phase secretory component in patients with idiopathic IgA nephropathy therefore extends the observations made by others. The finding that only 9 out of 28 patients with idiopathic IgA nephropathy showed increased IgA dimer concentrations may be due to the fact that patients were both detected and renal biopsies taken early in the natural history of disease, and never during bouts of upper respiratory tract infections. Possibly, the assay used by us may be less sensitive compared to others because we used unfractionated serums. However, there is no reason to doubt its specificity, and since increased binding of dimeric IgA1 to secretory component was also observed in patients with recurrent macroscopic hematuria and persistent microscopic erythrocyturia without mesangial IgA1 deposits, an increased dimeric IgA1 concentration is not pathognomonic for idiopathic IgA nephropathy.

Only IgA rheumatoid factor was exclusively found in patients with idiopathic IgA nephropathy in this study, although at a very low frequency, 3 out of 28. IgA rheumatoid factor however, has been found in up to 41 percent of patients with idiopathic IgA nephropathy, late in the natural history. (38) Thus IgA rheumatoid factor is unlikely to be involved in the pathogenesis of disease.

The evidence which links circulating immune complexes to mesangial IgA deposits is circumstantial at best. Circulating immune complexes are usually not demonstrable by fluid phase or solid phase C1q binding (18, 42, 44) in contrast to assays designed to detect IgA specific immune complexes by virtue of the activated C3 complement components, iC3b or C3d, residing in the complexes. Using a solid phase conglutinin ELISA (8), which binds circulating

immune complexes by means of an early product of C3d (25), IgA and IgM circulating immune complexes were detected at least once during follow up in about 40 percent of 34 patients with idiopathic IgA nephropathy. Interestingly, IgA circulating immune complexes, but not IgM circulating immune complexes, increased markedly during bouts of pharyngitis associated with hematuria. (12) When, however, patients with IgA nephropathy were compared to patients with non-IgA associated glomerular hematuria, the conglutinin binding assay lacks specificity. (44) These discrepancies may in part be due to the moment in the natural history of disease at which serums were obtained; Hall et al eg. using the Raji cell assay found, compared to healthy controls, IgA1 circulating immune complexes in 6 out of 12 patients with idiopathic IgA nephropathy, provided serums were tested within 2 years after onset of symptoms. (18)

Using the solid phase anti-C3 immune complex assay (34) circulating immune complexes were frequently found in patients with idiopathic IgA nephropathy in the study of Czerkinsky et al. (13) They determined the presence of circulating immune complexes using an immune complex 'enriched' fraction – a redissolved 3.5 percent PEG-6,000 precipitate, according to Kauffmann (23) – which was tested for IgA1 circulating immune complexes in a solid phase anti-human C3 assay (34) and for both IgA1 and IgG containing circulating immune complexes using a solid phase anti-human IgG assay with radiolabelled monoclonal anti-human IgA1 or IgA2 for detection. Of the 25 patients with idiopathic IgA nephropathy, 11 had IgA1 circulating immune complexes whereas 12 out of 30 patients had both IgA1 and IgG circulating immune complexes, probably through binding of IgG and IgA rheumatoid factor. (13) There was no correlation between these 2 types of immune complexes however. These findings are in contrast with the infrequent finding of circulating IgA1 immune complexes with the solid phase anti-human C3 assay and of IgA rheumatoid factor in patients with idiopathic IgA nephropathy in the present study. In part this can be explained by methodologic differences. In the present study, the IgA1 circulating immune complexes could only be detected in whole serum and not in polyethylene glycol precipitated material (figures not shown), a finding consistent with the 11 S size of the circulating immune complexes as determined by sucrose gradient centrifugation (Figure VI) and the knowledge that 11 S IgA dimers are not precipitated by 3.5 percent polyethylene glycol. (44) The frequently observed IgA1 circulating immune complexes by Czerkinsky et al. may be due to *in vitro* artefacts, induced by polyethylene glycol precipitation of serum before testing. As has been suggested by Hack et al., polyethylene glycol may aggregate small circulating immune complexes which are subsequently precipitated, whereas it may also enhance the interaction between C1q and circulating immune complexes which in the absence of EDTA may lead to C3 activation and binding. (16)

In this study there is no clear evidence which relates IgA1 circulating immune complexes or increased dimeric IgA1 concentrations to mesangial IgA1 deposits. The observation that the cutaneous vascular deposits in IgA nephropathy are seen in only 25 percent of the patients and lack specificity when compared to clinical controls (44) is compatible with such a view. Hence, other mechanisms must be involved in the appearance of the mesangial deposits of dimeric IgA1. Non-specific deposition of dimeric IgA in the mesangium is unlikely given the invariable presence of electron-dense deposits known to originate from antibody-antigen interaction. (27)

An attractive hypothesis is that the immune complexes are formed *in situ*, the IgA dimers being directed against a 'planted' or intrinsic mesangial antigen. The finding that antibodies eluted from renal tissue of patients with IgA nephropathy recombined better with autologous than with heterologous renal tissue is compatible with such a view. (41) That *in situ* immune complex formation in the mesangium may give rise to immune deposits with characteristic electron-dense deposits has been shown in experimental models. Infusion of large IgG aggregates in the rat resulting in mesangial localization, followed by renal transplantation and subsequent passive transfer of anti-IgG antibodies resulted in mesangial electron-dense deposits similar to those observed in idiopathic IgA nephropathy. (28) The same holds for structural or intrinsic mesangial antigens; perfusion of a rat kidney with a specific monoclonal antibody directed against an intrinsic mesangial matrix antigen of the rat resulted in direct binding of the monoclonal antibody with subsequent development of immune- and electron-dense deposits in the mesangium. (30) The recently published observation that some patients with idiopathic IgA nephropathy have circulating IgA antibodies reactive with basement membrane antigens, specifically with antigenic structures common to collagen I, II and IV (9) favours the involvement of a circulating (auto- or cross-reactive) antibody of the dimeric IgA1 class, directed against an intrinsic mesangial antigen in *in situ* immune complex formation. The frequent presence of dimeric IgA1 rheumatoid factor in patients with idiopathic IgA nephropathy (38), the observation that idiopathic IgA nephropathy may be familial (21) and the finding that relatives of patients with idiopathic IgA nephropathy have significant increases in the percentage of polymeric IgA producing B-cells upon mitogen stimulation *in vitro* as well as aberrant concanavalin A induced generation of IgA specific suppressor cells (14), supports the involvement of a dimeric IgA auto-antibody directed against an intrinsic mesangial antigen.

5.6 SUMMARY

In a prospective epidemiologic study renal biopsies were taken from 82 patients with persisting microscopic erythrocyturia or recurrent macroscopic

hematuria within 2 years after patient identification. Of these patients those with idiopathic IgA nephropathy (28×), thin basement membrane nephropathy (19×) and normal renal tissue (24×) were of comparable age, sex and clinical presentation. In order to decide whether or not idiopathic IgA nephropathy was associated with circulating immune complexes these were measured using fluid phase C1q binding assay and a solid phase anti-human C3 assay using monoclonal and polyclonal antibodies to detect IgA1, IgA2, IgG and IgM respectively. IgA1 or IgA2 dimers were measured using solid phase secretory component binding.

In all patients C1q binding assay was negative. IgA1 but not IgM or IgG circulating immune complexes were present in 4 out of 28 patients with idiopathic IgA nephropathy, had a 11 S size on sucrose gradient centrifugation and were associated with the presence of increased concentrations of IgA1 dimers. Similar findings were observed in 4 out of 24 patients with normal renal tissue and 1 out of 19 patients with thin basement membrane nephropathy. Idiopathic IgA nephropathy differed from the other patients in terms of the number of patients with increased concentrations of serum IgA and IgA1 dimers, but only the presence of IgA rheumatoid factor was specific for idiopathic IgA nephropathy, although its frequency was low (3 out of 28). These observations are incompatible with the hypothesis that the mesangial IgA deposits in idiopathic IgA nephropathy originate from deposition of circulating immune complexes or macromolecular IgA. Other mechanisms eg. *in situ* immune complex formation caused by the interaction of dimeric IgA1 with a 'planted' or autologous mesangial antigen are more likely to be involved.

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SUMMARY AND CONCLUSIONS

This thesis originates from a prospective regional study which was designed in 1977 in order to chart the epidemiology and natural history of idiopathic glomerular disease. It deals with the epidemiology and differential diagnosis; the natural history of disease is touched upon occasionally since the median follow up of 50 months is not sufficient to allow definitive statements on the majority of the idiopathic glomerular diseases which are of a chronic nature.

It is pointed out in Chapter I, which is a review of the literature, that a prospective study on idiopathic glomerular disease was opportune because one third of all patients being treated because of terminal renal insufficiency in 1977 in Europe and this country suffered from glomerulonephritis. Yet prospective studies on the incidence of idiopathic glomerular disease were lacking and information on the natural history of disease derived entirely from retrospective centre-studies with an inevitable bias towards prognostically less favourable subsets. Since that time the number of patients being treated for end-stage renal disease has increased mainly because of more liberal admission policies; it is clear that optimal use of cadaveric donor kidneys alone cannot cope with this demand now or in the near future, thereby necessitating expansion of dialysis facilities. These increasing expenditures can only be reversed by policies designed at prevention of renal insufficiency. As far as idiopathic, usually immunologically mediated, glomerular disease is concerned, effective long-term strategies for prevention of renal insufficiency should include intensification basic studies aimed at elucidating the pathogenesis of disease in conjunction with clinical studies on epidemiology and natural history of disease.

It is shown in this chapter that basic research has been particularly rewarding in terms of new insights into the pathogenesis of idiopathic glomerular immune complex disease and in describing 'final common pathways' (eg. focal and segmental glomerulosclerosis) associated with the development of renal insufficiency, with potentially great clinical implications. In order to translate the findings of basic research into effective therapeutic strategies, prospective clinical studies on the epidemiology and natural history of idiopathic glomerular disease are required. Perusal of the literature shows such information to be lacking or circumstantial, this holds also for the most common forms of idiopathic glomerular disease, idiopathic IgA nephropathy and membranous glomerulopathy. The prospective regional study on idiopathic glomerular disease was designed to provide such information.

Careful long-term follow up of the patients presented in this thesis will reveal whether the presence of certain clinical, serologic or immunohistological

parameters early in the natural history of disease, have prognostic significance in the individual patient. These patients can next be treated on the basis of pathogenetic mechanisms of disease elucidated by basic studies and confirmed in a clinical context.

The prospective epidemiologic study is described in Chapter II. Three major city hospitals, all direct reference hospitals for the local general practitioners, participated in this study. In each hospital renal biopsies were taken because of clinically suspected idiopathic glomerular disease out of a local adult population at risk of half a million adults. The total population at risk was therefore 1.5 million. Indications for renal biopsy were: recent onset uremia ($21 \times$), recent onset nephrotic syndrome ($46 \times$), persistent microscopic erythrocyturia ($70 \times$), recurrent macroscopic hematuria ($30 \times$) and chronic proteinuria ($23 \times$). Recent onset implied that a renal biopsy had to be taken within six months after detection of signs or symptoms, whereas in patients with hematuria or proteinuria renal biopsies had to be taken within two years after detection of symptoms. That renal biopsies were taken early in the natural history of disease was suggested by the fact that of the 29 patients with idiopathic IgA nephropathy only one had disturbed renal function and by the finding that all patients with membranous glomerulopathy had Churg stage I or II. Of the 190 renal biopsies, 14 had insufficient material, 13 were shown to derive from patients with glomerular involvement in clinically unsuspected systemic disease, and in 27 patients – with recurrent macroscopic hematuria ($13 \times$), persistent microscopic erythrocyturia ($11 \times$), and chronic proteinuria ($3 \times$) – no renal disease was demonstrable. Thus 136 patients had idiopathic renal disease, which was glomerular in 122, interstitial in 7 and vascular 7. The main findings are fourfold. The estimated incidence rate of idiopathic IgA nephropathy and thin basement membrane nephropathy, was high with 20 and 13 per million adults per year respectively; their incidence in renal biopsies taken from patients because of chronic hematuria was 31 percent and 22 percent respectively. In the normotensive, non-azotemic adults from which a renal biopsy was taken because of chronic mild proteinuria the incidence of focal segmental glomerular sclerosis and vascular hyalinosis was both 37 percent. Of the patients with nephrotic syndrome, the incidence of membranous glomerulonephritis stage I/II, minimal change glomerulopathy and focal and segmental glomerulosclerosis was 34, 22 and 15 percent respectively with an estimated incidence per million adults per year of 9, 6 and 4 respectively. Both the incidence in patients with a nephrotic syndrome, 5 percent, and incidence rate of less than 2 per million adults per year of membranoproliferative glomerulonephritis was low. Finally, the incidence of extracapillary, glomerulonephritis and diffuse sclerosing glomerulonephritis in patients from which a renal biopsy was taken because of recent onset uremia was high being 33 and 25 percent respectively.

This prospective regional study provides new population based data on the frequency of occurrence of idiopathic glomerular disease. The study provides in addition data on the incidence of various idiopathic, usually immunologically mediated, glomerular diseases in patients with chronic hematuria, chronic proteinuria, recent onset nephrotic syndrome and uremia, which contribute to the differential diagnosis of these symptoms.

In chapter III the relationship of sex and age to the incidence of idiopathic glomerular disease and the severity of the glomerular lesions is assessed. The patients described derived mainly from the prospective epidemiologic study presented in Chapter II. From 181 patients renal biopsies were taken early in the natural history of disease because of recent onset uremia (19 ×), recent onset nephrotic syndrome (52 ×), persistent microscopic erythrocyturia (65 ×), recurrent macroscopic hematuria (28 ×) and chronic proteinuria (17 ×). Of these patients 27 had normal renal tissue, 10 had interstitial nephritis, 7 had renal vascular hyalinosis and 137 had idiopathic glomerular disease.

Results show recurrent macroscopic hematuria, persistent microscopic erythrocyturia in the absence of proteinuria and recent onset nephrotic syndrome to be male associated indications for renal biopsy. The other indications for renal biopsy, persistent microscopic erythrocyturia in the presence of proteinuria, chronic proteinuria and recent onset uremia showed no sex preponderance. Idiopathic IgA nephropathy was significantly male associated both for patients presenting with recurrent macroscopic hematuria (11 ×) and persistent erythrocyturia (22 ×). This male preponderance of idiopathic IgA nephropathy associated with recurrent macroscopic hematuria was related to the indication for renal biopsy. The male preponderance of idiopathic IgA nephropathy with persistent microscopic erythrocyturia however could not be related to the indication for renal biopsy since patients with thin basement membrane nephropathy, also presenting with persistent microscopic erythrocyturia, were equally distributed over the sexes. Males from which a renal biopsy was taken because of chronic proteinuria showed usually focal and segmental glomerulosclerosis, whereas the lesions of renal vascular hyalinosis were found in women with chronic proteinuria. Patients from which a renal biopsy was taken because of recent onset nephrotic syndrome were usually males; however only membranous glomerulopathy was male associated in contrast to minimal change glomerulopathy and focal segmental glomerulosclerosis. Patients with recent onset uremia showed no sex preponderance but diffuse extracapillary non anti-lomerular basement membrane antibody mediated, glomerulonephritis was male associated. In males the severity of the proliferative glomerular inflammatory reaction was related to age and not duration of disease. This also held for the severity of the proliferative glomerular lesions within the immunopathologic disease entity of idiopathic IgA nephropathy.

This prospective study shows, after correction for the indication for renal biopsy, the type of idiopathic, immunologically mediated, glomerular disease to be related to sex and in males the severity of the proliferative inflammatory lesions to be related to age.

In Chapter IV the use of quantitative electron microscopy in the diagnosis of thin basement membrane nephropathy is described. Quantitative electron microscopy was performed because a substantial number of renal biopsies taken from adults with recurrent macroscopic hematuria or persistent microscopic erythrocyturia failed to show evident glomerular disease by light microscopy and immunohistochemistry; in some however, poor silver staining of the glomerular basement membranes in the silver methenamine, hematoxylin and eosin stains was observed, suggesting the possibility of glomerular basement membrane pathology. Therefore quantitative measurements of the glomerular basement membranes were performed on all renal biopsies taken because of recurrent macroscopic hematuria ($26\times$) or persistent microscopic erythrocyturia ($55\times$) from normotensive, non-azotemic adults. These patients originated from the prospective epidemiologic study described in Chapter II; in all instances persistent erythrocyturia or recurrent macroscopic hematuria was of at least half a year duration and biopsies were taken within two years after patient identification. None of the patients originated from families with known hereditary renal disease. Follow up was 50 months median.

These 81 patients could be divided on the basis of the immunohistology of the renal biopsies into three subsets. Idiopathic IgA nephropathy ($27\times$), a subset with various histological abnormalities ($11\times$), including mesangioproliferative glomerulonephritis, focal and global glomerulosclerosis and interstitial nephritis, and finally patients ($43\times$) with normal renal tissue by light microscopy and absence of glomerular immunoglobulin deposits. The glomerular basement membrane thickness measured ultrastructurally, was normal in the patients with idiopathic IgA nephropathy (354 ± 55 nanometers) and those with divers histological lesions (327 ± 37 nanometers). The renal biopsies of the patients with histologically normal renal tissue were by morphometric analysis of the glomerular basement membrane thickness shown to consist of two different subsets. Those with a normal glomerular basement membrane thickness of 361 ± 69 nanometers ($27\times$) and those with diffuse thinning of the glomerular basement membrane as shown by a thickness of 190 ± 25 nanometers ($19\times$); this thinning of the glomerular basement membrane was associated with diffuse mesangial deposits of the third component of complement in eight. The patients with thin basement membrane nephropathy presented with persistent microscopic erythrocyturia ($18\times$) or recurrent macroscopic hematuria ($1\times$). The persistent microscopic erythrocyturia was associated with significant proteinuria in 7, with mild proteinuria in 5 and without proteinuria in 6.

During follow up, in all of the patients with thin basement membrane nephropathy the microscopic erythrocyturia persisted. Conversely in the 24 patients with normal renal tissue the macroscopic hematuria disappeared in all (13 ×) and the microscopic erythrocyturia disappeared in five, persisted in three, and was shown to be of urologic origin in another three.

This study shows the incidence of thin basement membrane nephropathy in adults presenting with persistent microscopic erythrocyturia in the presence or absence of proteinuria to be identical to the incidence of idiopathic IgA nephropathy. Renal biopsies taken early in the natural history including electron microscopy with quantitative measurements of the glomerular basement membrane thickness should therefore be included in the work up of adults with persistent microscopic erythrocyturia. Long-term follow up of patients with thin basement membrane nephropathy is required in order to determine whether or not thin basement membrane nephropathy is a benign disorder.

In Chapter V the issue was addressed whether or not idiopathic IgA nephropathy, the most common idiopathic glomerular disease in this country, is caused by deposition of circulating IgA immune complexes or macromolecular IgA into the glomerular mesangium. In the epidemiologic study described in Chapter II, serum and renal tissue was obtained from patients, early in the natural history, with idiopathic IgA nephropathy (28 ×) and from patients with thin basement membrane nephropathy (19 ×), and normal renal tissue (24 ×); the latter two being excellent controls for idiopathic IgA nephropathy in terms of age, sex and clinical presentation. Circulating immune complexes were measured in the serums of these patients using a fluid phase C1q binding assay and a solid phase anti-C3 assay with monoclonal antibodies to detect the presence of IgA1 and IgA2. In addition IgA1 and IgA2 dimers were measured using solid phase secretory component binding.

In all patients the C1q binding assay was negative. IgA1 circulating immune complexes but not IgM or IgG circulating immune complexes were present in 4 out of 28 patients with idiopathic IgA nephropathy, had a 11 S size on sucrose gradient ultracentrifugation and were associated with the presence of increased concentrations of IgA1 dimers. Similar findings were observed in 4 out of 24 patients with normal renal tissue and 1 out of 19 patients with thin basement membrane nephropathy. Idiopathic IgA nephropathy differed from the other patients in terms of the number of patients with increased concentrations of serum IgA and IgA1 dimers but only the presence of IgA rheumatoid factor was specific for idiopathic IgA nephropathy, although its frequency was low (3 out of 28).

These observations are incompatible with the hypothesis that the mesangial IgA deposits in idiopathic IgA nephropathy derive from deposition of circulating immune complexes. Other mechanisms eg. *in situ* immune complex formation caused by the interaction of dimeric IgA1 with a 'planted' or autologous antigen are more likely to be involved.

ADDENDUM TO CHAPTER IV

MEASUREMENT OF GLOMERULAR BASEMENT MEMBRANE THICKNESS

THEORETICAL CONSIDERATIONS

The thickness of a structure or barrier can be estimated by the arithmetic or harmonic mean of a series of measurements of its apparent thickness. In this way using geometric probabilities, Weibel and Knight, working on the alveolar basement membrane in the lung, arrived at the determination of the harmonic (reciprocal) mean thickness from the length of linear intercepts. (5) They showed that the harmonic mean thickness best approximates the barrier to diffusion between two separate regions, while determination of the harmonic mean corrects for an inordinate contribution of thick areas, representing only a small percentage of the measurements. Using the same geometric probabilities, Gundersen et al. showed that it was possible to obtain distributions of membrane thickness from the length of linear intercepts. (1) Shortly afterwards, Jensen et al. showed that by measuring orthogonal intercepts, estimation of the reciprocal and hence the true membrane thickness was far more efficient. (2)

THE ORTHOGONAL INTERCEPT METHOD

Since the orientation distribution of the glomerular basement membrane is not known, it is only possible to deduce the three-dimensionally defined thickness distribution from observations in two dimensions, based on the fact that once the sectioning is random – i.e. all orientations of the membrane surface relative to the sample plane must be equally likely to occur –, it is possible to derive the relationship between membrane thickness (T) and the length of linear intercepts with specified, non-uniform distribution of orientation relative to the trace of the cut membrane. By using an orthogonal intercept, complete control of orientation in the sample plane is accomplished which reduces the variation of the intercept length to that due to the distribution of the membrane thickness itself.

Jensen et al. showed, using geometric probabilities, that the mean value of T is approximately 79 percent of the mean orthogonal intercept length, while for the harmonic mean the percentage is 85. (2) Moreover, they showed that the efficiency, i.e. the number of measurements required for a certain precision of the estimated membrane thickness, was the highest in using the harmonic mean of the orthogonal intercepts as an estimate of the harmonic mean membrane thickness. More precisely: $T^{-1} = 3/8 \times \pi \times L_0^{-1}$, in which T^{-1} is the harmonic mean of the membrane thickness, while L_0^{-1} is the harmonic mean of

the orthogonal intercepts. Through mathematical rearrangements, the distribution of the reciprocal membrane thickness is reconstructed from the distribution of the reciprocal orthogonal intercepts, by using grouped data of orthogonal intercepts. This, so-called unfolding procedure, results in the formula described below and corrects the number of orthogonal intercepts in the extreme upper classes, by giving them a low weight.

There are two major problems in the determination of the membrane thickness from orthogonal intercepts. First, that of sampling error, for the method used requires that all orientations of the membrane surface relative to the sample plane must be equally likely to occur. The relative random stereologic distribution of the glomerular basement membrane reduces the bias of sampling error but in other membranes, eg. those of the highly oriented striated muscle capillary basement membrane, proper sampling will be very difficult. The other problem concerns the glomerular basement membrane itself. The thickness distribution of the glomerular basement membrane in normal humans follows a strictly unimodal distribution. (3) When however in glomerular disease, eg. in Alport's syndrome, extensive local thickening in an otherwise uniformly glomerular basement membrane is present, it will yield a bimodal distribution of the glomerular basement membrane thickness. The harmonic mean, as estimator of the glomerular basement membrane thickness cannot be used in conditions with a bimodal thickness distribution.

METHODOLOGY OF THE DETERMINATION OF MEMBRANE THICKNESS FROM ORTHOGONAL INTERCEPTS

Glomerular basement membrane thickness was measured with the orthogonal intercept method described above, slightly modified by Steffes et al. (4) Briefly, a sampling line grid was superimposed on an electronmicrograph, magnified $31,000 \times$. At all points of intersection of the lines of the grid with the endothelial margin of the glomerular basement membrane, the thickness of the glomerular basement membrane was measured orthogonally to the point of intersection. The thickness between the endothelial and epithelial cell membranes apposed to the glomerular basement membrane was measured, regardless of the clarity of the cell membrane profiles (eg. tangential cuts). Only peripheral capillary loops were measured and the transition of the mesangium and peripheral capillary glomerular basement membranes was determined on the basis of widening of the distance between epithelial and endothelial cells as well as disappearance of the parallelism between the cell-membrane interfaces. A scale was calibrated in harmonic terms. According to Steffes et al. (3, 4) 0.75 was used as the common multiplier of the inverse or harmonic value of each division of the scale beginning with the smallest division. For example, the lowest division is 2 mm (in harmonic terms 0.5); the second division is

$1 / 0.75 \times 0.5 = 2.67$ from the origin; the third division is $1 / (0.75)^2 \times 0.5 = 3.56$ mm from the origin and so on. Similarly the harmonic midpoints of each region can be calculated. The length of each orthogonal intercept was classified into the divisions of the calibrated harmonic scale. The harmonic mean of all orthogonal intercepts lines was first calculated using the formula:

$$\begin{aligned} \text{Harmonic mean of orthogonal intercepts} &= \frac{n(t)}{\sum_{i=1}^j \frac{n(i)}{\text{midpoint } i} + \frac{n(2)}{\text{midpoint } 2} \dots + \frac{n(j)}{\text{midpoint } j}} \\ n(t) &= \sum_{i=1}^j n(i) \end{aligned}$$

To determine the actual mean glomerular basement membrane thickness, the calculated harmonic mean was multiplied by $8/3 \times \pi$, as mentioned above.

One of the advantages of this method is that it avoids the bias caused by tangential cuts and curvatures, which will occasionally occur and would influence an arithmetic mean dramatically. Since the longest intercepts are given the smallest weight in the distribution of the harmonic (reciprocal) membrane thickness, as is apparent from the formula, departures from the real membrane thickness caused by tangential cuts or curvatures, – which overestimate the real membrane thickness – will hardly influence the calibrated harmonic mean.

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ADDENDUM TO CHAPTER V

ISOLATION PROCEDURES AND SPECIFICITY CONTROLS

1.1 PURIFICATION OF THE THIRD COMPONENT OF HUMAN COMPLEMENT

Purification of the third component of human complement C3 from plasma was performed according to the method of Tack and Prahl with minor modifications. (9)

1.1.1 Reagents

A pool of fresh platelet-poor human plasma was obtained from 16 healthy volunteers.

DEAE-Cellulose (Whatman DE-52) and Sepharose-6B. Pharmacia biotechnology. Uppsala, Sweden.

Hydroxylapatite (Bio-Gel HT). Biorad laboratories. Richmond, USA.

Amicon XM-50 ultrafiltration membranes.

Polyclonal, xenogeneic antisera directed against human immunoglobulins G, A, and M. Dakopatts-A/S. Copenhagen, Denmark.

Polyclonal, xenogeneic antiserum directed against C3. Behring Diagnostics. La Jolla, USA.

Polyclonal, xenogeneic antiserum directed against C4. Nordic immunological laboratories. Tilburg, The Netherlands.

All other chemicals used were of the highest purity and purchased from Merck E. Darmstadt, Federal republic of Germany, unless stated otherwise.

1.1.2 Fractionation of plasma with PEG-4000

Fresh human plasma was chilled on ice and phenylmethylsulfonylfluoride (PMSF, Sigma. St. Louis, USA) was added to a final concentration of 0.5 mM using an anhydrous 2-propanol solution of 115 mM PMSF. The plasma was delipidated by centrifugation for 120 minutes at 4°C at 30,000 g using a JA-14 rotor in a Beckmann J-21B centrifuge. (Beckmann-Instruments Inc. Anaheim, California, USA). Next, in order to deplete the plasma of fibrinogen, a volume equivalent to half of the plasma volume of 15 percent PEG-4000 (w/v) in a buffer containing 100 mM sodium phosphate (pH: 7.4), 150 mM NaCl, 15 mM EDTA, 1 mM benzamidine and 0.5 mM PMSF, was slowly added with stirring resulting in a final polyethylen glycol-4000 (PEG-4000) concentration of 5

percent. After stirring for 30 minutes, the precipitate was removed by centrifugation at 5000 g for 15 minutes. The PEG concentration was then raised to 12 percent by slowly adding 26 percent PEG-4000 (w/v) in the same buffer as mentioned above, in order to remove the majority of the albumen and transferrin which remains soluble at 12 percent PEG concentration. After another 30 minutes, the precipitate was collected by centrifugation at 7000 g for 20 minutes and the supernatant was discarded. The precipitate was dissolved by gentle agitation in the phosphate buffer pH:7.4 mentioned above. The dissolved precipitate was then diluted with cold 5 mM EDTA to a specific conductance of 3 milliSiemens at 4°C, and the pH was adjusted to 7.0 (using 1 M NaOH or glacial acetic acid) and made 50 mM EACA by adding solid ϵ -aminocaproic acid (EACA).

1.1.3 DEAE-Cellulose chromatography

Mainly in order to remove the remaining albumen, transferrin and C4, the dissolved PEG-4000 12 percent precipitate was applied to a 9 × 45 cm column of DEAE-cellulose equilibrated with 25 mM potassium phosphate buffer (pH: 7.0) containing 5mM EDTA, at a flow of 140 ml/hour. Once the sample was loaded, the column was washed with one column volume (2.9 l) of 25 mM potassium phosphate buffer (pH: 7.0) containing 50 mM EACA, 5 mM EDTA, 1 mM benzamidine and 0.5 mM PMSF. Bound proteins were eluted using a linear NaCl gradient to a concentration of 300 mM NaCl in the potassium phosphate buffer described above. The flow rate was 125 ml/hour and 18 ml fractions were collected. The absorbance at 280 nm, the conductance of the fractions and their reactivity with different monospecific antisera were monitored. C3 and C5 eluted at a specific conductance of 6.5 milliSiemens; its main contaminants being IgG, IgM, IgA and α 2-macroglobulin. C4 eluted at 7.5 milliSiemens and was discarded. The fractions containing C3 were pooled and concentrated by precipitation with PEG-4000 by adding solid PEG to a final concentration of 16 percent (w/v). The precipitate was collected by centrifugation at 7000 g for 10 minutes and redissolved by gentle agitation in 100 mM potassium phosphate buffer (pH: 7.4) containing 150 mM NaCl, 50 mM EACA, 5 mM EDTA, 1 mM benzamidine and 0.5 mM PMSF.

1.1.4 Exclusion chromatography on Sepharose 6B

In order to remove the contaminating IgM and α 2-macroglobulin, the redissolved precipitate was applied to exclusion chromatography on a 3.5 × 150 cm column of Sepharose-6B equilibrated with 100 mM potassium phosphate buffer (pH: 7.4) containing 150 mM NaCl, 50 mM EACA, 5 mM EDTA, 1 mM benzamidine and 0.5 mM PMSF. Using ascending flow at a rate of 30 ml/hour, 12 ml fractions were collected monitoring absorbance at 280 nm

and reactivity with different monospecific antisera. The fractions containing C3 and C5 were pooled and concentrated to ± 8 mg/ml using ultrafiltration on Amicon XM-50 membrane under nitrogen pressure.

1.1.5 Hydroxylapatite chromatography

The concentrated C3/C5 peak was dialyzed against a 25 mM potassium phosphate buffer (pH: 7.4), containing 100 mM KCl and 50 mM EACA and diluted to 5 mg/ml in this buffer, and applied on a hydroxylapatite column of 5×30 cm, equilibrated in the same buffer. After application, the column was washed with 1.5 column volume (900 ml) of the mentioned 25 mM potassium phosphate buffer pH: 7.4 at a flow rate of 125 ml/hour. This buffer was followed by a 900 ml wash of 25 mM potassium phosphate buffer pH: 7.4 containing 2 M KCl and 50 mM EACA, in order to elute C5, IgG and IgA contaminants, and again washed with 900 ml of starting buffer; next C3 was eluted with a 125 mM potassium phosphate buffer (pH: 7.4), containing 100 mM KCl and 50 mM EACA. The appropriate C3 fractions were pooled, concentrated by ultrafiltration on Amicon XM-50 membranes and stored at -70°C .

1.1.6 Analysis of isolated C3

The isolated C3 preparation yielded one band on immunoelectrophoresis against xenogeneic anti-human serum and a line of identity with rabbit anti-human C3. Analysis of the C3 preparation on polyacrylamide gel electrophoresis (T = 10% and C = 2.7%), after denaturation with 0.2 percent sodium-dodecyl-sulphate and reduction by boiling for 10 minutes with 2-mercaptoethanol, consistently revealed two polypeptide chains.

Using low molecular weight markers (Pharmacia) these reduced chains were shown to be of 115,000 and 70,000 daltons in molecular weight, corresponding to the alpha and beta chains of human C3 respectively.

1.2 PRODUCTION OF POLYCLONAL GOAT F(AB)₂ ANTI-HUMAN C3

1.2.1 Reagents

DEAE-A50 Sephacel, and Sephadex G-200, Pharmacia.
Pepsin (Worthington, Freehold, New Jersey, USA).

1.2.2 Immunization

Goats were immunized intracutaneously with purified C3 in Complete Freund Adjuvant and boosted with C3 in Incomplete Freund Adjuvant given intra- and subcutaneously. Antibodies elicited were monitored by Ouchterlony double

diffusion against purified human C3 and normal human serum. Goats were bled when high titre antisera were elicited; these were monospecific in Ouchterlony double-diffusion and immunoelectrophoresis against normal human serum.

1.2.3 IgG purification

The hyperimmune serum was heat inactivated for 30 minutes at 56°C and delipidated by centrifugation for two hours at 30,000 g, in a JA-14 rotor in a Beckmann J-21B centrifuge. The heat inactivated serum was diluted 1:1 with cold phosphate buffered saline (pH: 7.3) and an equal volume of saturated ammonium sulphate solution (pH: 7.3) was added, and stirred for 30 minutes. The precipitate was collected by centrifugation at 5000 g for 20 minutes, redissolved in phosphate buffered saline, and a repeated ammonium sulphate precipitation was performed. The redissolved precipitate was dialyzed extensively against phosphate buffered saline to remove the ammonium sulphate and afterwards applied for ion-exchange chromatography to a 2.5 × 40 cm column of DEAE-A50 sephacel equilibrated in phosphate buffered saline. After application the column was washed with phosphate buffered saline and 5 ml fractions were collected, monitored for absorbance at 280 nanometer; the ascending part and top fractions of the eluted peak, containing pure IgG by immunoelectrophoresis, were pooled and concentrated by ultrafiltration on Amicon XM-50 membranes.

1.2.4 Specificity of the goat IgG anti-human C3

The goat IgG anti-human C3 was monospecific when tested in ouchterlony double-diffusion and immunoelectrophoresis reacting both with native C3 and activated C3b. Furthermore the goat IgG anti-human C3 did not bind to insolubilized human IgG, IgA and IgM when tested in enzyme-linked immunosorbent assays using rabbit anti-goat IgG labelled with horse radish peroxidase to detect goat IgG bound to insolubilized human immunoglobulins.

1.2.5 Peptic cleavage of goat IgG anti-human C3

The purified IgG was next digested with pepsin by adding 1 percent (w/w) of pepsin in 0.1 M sodium acetate buffer pH: 4.5. After incubation for 20 hours at 37°C, precipitate was removed by centrifugation for 10 minutes at 1500 g; the pH of the supernatant was raised to 8.0 and dialyzed against phosphate buffered saline. The F(ab)₂ fragments were separated from undigested IgG and smaller fragments by exclusion chromatography on a Sephadex G-200 column of 2.5 × 100 cm, equilibrated in phosphate buffered saline, collecting 5 ml fractions, monitoring the absorbance at 280 nanometers. The appropriate fractions were pooled, concentrated by ultrafiltration on Amicon XM-50 membranes, and stored at -70°C.

1.2.6 Analysis of the F(ab)2 fragments

Analysis of the F(ab)2 fragments on polyacrylamide gel electrophoresis (T = 10% and C = 2.7%) under denaturated conditions (0.2% sodium-dodecylsulphate) revealed one band with a molecular weight of $\pm 110,000$ daltons without any contaminating uncleaved IgG molecules.

1.3 PURIFICATION OF SECRETORY IGA FROM HUMAN MILK

Purification of human secretory IgA from human milk was performed according to the method of Khayam-Bashi, Blanken and Schwartz with minor modifications. (4)

1.3.1 Reagents

A pool of fresh human milk was obtained through the kind cooperation of Prof. Dr. G. Essed, Department of Gynaecology and Obstetrics, University of Limburg, Maastricht, The Netherlands.

DEAE-Cellulose (Whatman DE-52), and Sephadex G-200, Pharmacia.

Amicon XM-50 ultrafiltration membranes.

Polyclonal, xenogeneic antisera directed against IgA, lactoferrin and secretory component. Dakopatts.

All operations were performed at 4°C.

1.3.2 Preparation of whey from human milk

The milk was delipidated by ultracentrifugation at 105,000 g for two hours, Beckmann, type L8-70 ultracentrifuge. (Beckmann instruments Inc. Anaheim, California, USA). The milk was next diluted 1:1 with 0.15 M sodiumchloride, and the pH was adjusted to 4.6 using an 1 M acetate buffer (pH: 3.5), in order to precipitate the casein. The mixture was stirred for two hours at room temperature and again centrifuged for two hours at 105,000 g to remove the precipitated casein. The clear supernatant was adjusted to pH: 8.0 by 1 M Tris HCl buffer (pH: 8.5), and dialyzed against a 0.01 M Tris HCl buffer (pH: 8.0). The delipidated milk, void of casein, was concentrated by ultrafiltration on Amicon XM-50 membranes under nitrogen pressure in the cold room.

1.3.3 Chromatography on DEAE-Cellulose

The concentrated human whey was next applied to a DEAE-cellulose column 2,6 × 40 cm equilibrated with 0.01 M Tris HCl buffer (pH: 8.0). Afterwards the column was washed at a flow rate of 30 ml/hour. Proteins were eluted stepwise with Tris HCl buffers (pH: 8,0), containing 0.03 M, 0.05 M, and 0.1 M NaCl at a

flow rate of 30 ml/hour; 4 ml fractions were collected. The elution was monitored by measuring absorbance at 280 nanometer, and by reactivity with monospecific antisera directed against human IgA and secretory component. The 0.03 M containing Tris HCL buffer eluted among others lactoferrin, whereas the 0.05 M NaCl containing Tris HCL buffer eluted free secretory component. The secretory IgA was eluted with the 0.1 M NaCl containing 0.01 M Tris HCL buffer. The secretory IgA containing fractions were pooled, concentrated by ultrafiltration on Amicon XM-50 membranes and dialyzed against 0.15 M borate buffer (pH: 8.0) containing 0.1 M NaCl.

1.3.4 Exclusion chromatography on Sephadex G-200

The concentrated DEAE-cellulose eluate containing secretory IgA was applied to a 2.5×100 cm Sephadex G-200 column equilibrated in 0.15 M borate buffer (pH: 8.0) containing 0.1 M NaCl. The column was run in the same buffer with ascending flow, rate 20 ml/hour, and 5 ml fractions were collected and tested for their reactivity with monospecific anti-human secretory IgA antiserum. The positive fractions were pooled and concentrated by ultrafiltration and rerun on the same column. The peak containing secretory IgA was concentrated by ultrafiltration and dialyzed against phosphate buffered saline (pH: 7.3).

1.3.5 Analysis of the secretory IgA preparation

Analysis of the human secretory IgA, after the rerun on the Sephadex G-200 gel filtration column, by polyacrylamide gel electrophoresis (T = 7% and C = 2.7%) under denaturated conditions (0.2% sodium-dodecyl-sulphate) revealed one band with a molecular weight of $\pm 380,000$ daltons. After transfer to nitrocellulose membrane using Western blotting techniques, this band bound anti-human secretory component and anti-human IgA. After denaturation and reduction using 2-mercaptoethanol, analysis by polyacrylamide gradient gel electrophoresis (T = 3-25% and C = 2.7%) revealed one band with a molecular weight of $\pm 70,000$ daltons, which bound the anti-human secretory component, one band at 54,000 daltons, reactive with anti-human IgA (α -chain specific), and two other bands at $\pm 25,000$ and $\pm 16,000$ daltons.

1.4 PURIFICATION OF HUMAN SECRETORY COMPONENT FROM HUMAN MILK

Purification of secretory component from human milk was performed according to the method described by Underdown, DeRose, Koczekan, Socken, and Weicker with minor modifications. (11)

1.4.1 Reagents

A pool of fresh human milk was obtained through the kind cooperation of Prof. Dr. G. Essed, Department of Gynaecology and Obstetrics, University of Limburg, Maastricht, The Netherlands.

Serum from a patient with a monoclonal IgM gammopathy, M. Waldenström, was used as a source of human IgM.

Sephacryl 4B-CNBr activated, and Sephacryl S-300, Pharmacia.

High Performance Liquid Chromatography (LKB).

TSK-G-3000, 0.75 × 60 cm silicon column for gel permeation on HPLC and TSK-G-GWSP, 0.75 × 7.5 cm as guard column.

Amicon PM-10 ultrafiltration membranes.

Polyclonal xenogeneic antisera directed against IgA, IgM, lactoferrin and secretory component. Dakopatts.

1.4.2 Preparation of whey from human milk

The milk obtained was delipidated by centrifugation at 105,000 g for two hours, Beckmann type L8-70 ultracentrifuge.

1.4.3 Preparation of sepharose-4B coupled human monoclonal IgM

Human monoclonal IgM was isolated from serum of a patient with monoclonal, non-cryoprecipitable IgM gammopathy. First, a boric acid precipitate of the delipidated, heat-inactivated serum was obtained by adding 2 percent (v/v) of 0.1 M boric acid (pH: 7.4). The precipitate was obtained by centrifugation at 30,000 g for 30 minutes, and was dissolved in a Tris HCl buffer (pH: 7.3) containing 0.5 M NaCl. After dialyzing against this buffer, the sample was applied to a Sephacryl S-300 column (2.5 × 100 cm) equilibrated in this Tris buffer. The column was run with ascending flow using the same Tris HCl buffer containing 0.5 M NaCl, rate 20 ml/hour, and 5 ml fractions were obtained. The absorbance was monitored at 280 nanometer and the IgM containing fractions were pooled and concentrated on Amicon XM-50 membranes under nitrogen pressure. Coupling of the purified human monoclonal IgM to the CNBr-activated Sepharose-4B was performed as described by the manufacturer; approximately 20 mg of human monoclonal IgM was coupled to every millilitre of the swollen CNBr-activated Sepharose 4B.

1.4.4 Affinity chromatography of human secretory component on human IgM

Human whey was diluted 1 to 10 in 0.01 M citrate phosphate buffer (pH: 6.8) containing 500 mg ferrous ammonium sulphate, in order to diminish the binding of lactoferrin to the solid phase human monoclonal IgM. The diluted

they were stirred for two hours, after which the slurry of human monoclonal IgM coupled to Sepharose-4B (1:2 v/v) was added. This mixture was gently mixed overnight before pouring a column. The column was washed with 0.01 M citrate phosphate buffer (pH: 6.8) containing 500 mg ferrous ammonium sulphate to elute the unbound fractions. When the absorbance measured at 280 nanometer was less than 0.100, the column was eluted with 1 M KSCN in 0.01 M phosphate buffer (pH: 7.0). The eluted fractions were pooled and dialyzed against 0.01 M phosphate buffer (pH: 7.2) containing 0.5 M NaCl and concentrated by ultrafiltration on Amicon PM-10 membranes under nitrogen pressure.

1.4.5 Exclusion chromatography using High Performance Liquid Chromatography

The concentrated sample was next applied to a TSK-3000 silicon column equilibrated in a 0.1 M phosphate buffer (pH: 6.8) containing 0.5 M NaCl, in order to remove the contaminating lactoferrin; 1 ml fractions were obtained. The peak fractions containing human secretory component were pooled and concentrated by ultrafiltration on Amicon PM-10 membranes under nitrogen pressure.

1.4.6 Analysis of the secretory component preparation

Analysis of the purified human secretory component on polyacrylamide gel electrophoresis (T = 10% and C = 2.7%) under denaturing conditions (0.2% sodium-dodecyl-sulphate) revealed one band with a molecular weight of $\pm 70,000$ daltons, which bound anti-human secretory component but not anti-human IgA after transfer to a nitrocellulose membrane using Western blotting techniques. After denaturation and reduction using 2-mercaptoethanol, analysis on polyacrylamide gel electrophoresis (T = 10% and C = 2.7%) showed the same band.

1.5 ISOLATION OF HUMAN COMPLEMENT COMPONENT C1Q

Purification of human complement component C1q from fresh frozen human plasma was performed according to the method of Tenner, Lesavre and Cooper with minor modifications. (10)

1.5.1 Reagents

Fresh frozen human plasma was purchased from the Bloedbank Zuid-Limburg at Maastricht.

Biorex-70, (100-200 mesh), and Biogel A-5M, (200-400 mesh). Biorad.

Amicon XM-50 ultrafiltration membranes.

Polyclonal, xenogeneic antisera directed against C3, C4, IgG, IgA, IgM. Dakopatts.

1.5.2 Ion-exchange chromatography

Fresh frozen human plasma was made 5 mM EDTA, using a 200 mM EDTA solution (pH: 7.0). The plasma was delipidated by centrifugation for 120 minutes at 30,000 g (Beckmann ultracentrifuge). The delipidated human plasma was applied to a column of Biorex-70 equilibrated in 0.05 M phosphate buffer (pH: 7.3) containing 82 mM NaCl and 2 mM EDTA. The column was washed with the same buffer, at a flow rate of 80 ml/hour, until the absorbance at 280 nanometers was less than 0.100; over 99 percent of the serum proteins applied were eluted from the column. The bound C1q was eluted using a linear NaCl gradient from 82 mM to 500 mM NaCl in the 0.05 M phosphate buffer mentioned above, 5 ml fractions were obtained and the absorbance at 280 nanometers as well as the reactivity with anti-human C1q was monitored. The C1q positive fractions were pooled and concentrated by adding solid ammonium sulphate to a final concentration of 33 percent (w/v). The precipitate was obtained by centrifugation for 30 minutes at 5,000 g. The precipitate was redissolved in a 50 mM Tris HCl buffer (pH: 7.2) containing 2 mM EDTA and 0.5 M NaCl, and subsequently dialyzed against this buffer.

1.5.3 Exclusion chromatography on Biogel A-5M

The pooled and concentrated human C1q peak was applied to a Biogel A-5M column, (2 × 100 cm), equilibrated in 50 mM Tris HCl buffer (pH: 7.2) containing 2 mM EDTA and 0.5 M NaCl, in order to remove contaminating immunoglobulins. The column was eluted using the same buffer at a flow rate of 20 ml/hour using ascending flow, and 5 ml fractions were obtained. Absorbance at 280 nanometer and reactivity with anti-human C1q were monitored. The C1q containing fractions were pooled and concentrated by adding solid ammonium sulphate to a final concentration of 33 percent (w/v). The precipitate was redissolved in phosphate buffered saline (pH: 7.3) and dialyzed against this buffer. Subsequently it was radiolabelled with Iodine-125 by means of lactoperoxidase according to the method described by Heusser et al. (2) and stored at - 70 °C until used.

1.5.4 Analysis of the C1q preparation

Analysis in Ouchterlony double diffusion showed the absence of contaminating immunoglobulins and analysis by polyacrylamide gel electrophoresis (T = 7% and C = 2,7%) in 4M Urea and 1 percent SDS revealed two bands of 70,000 and 55,000 daltons respectively, which are comparable to the observations made by Reid and Porter on the behaviour of C1q in polyacrylamide gel electrophoresis. (8)

1.6 MONOCLONAL MOUSE IGG1 ANTIBODIES DIRECTED AGAINST-HUMAN IGA1 AND IGA2

Monoclonal mouse anti-human immunoglobulins A1 and A2 were kindly donated by Dr. J. Haayman (Department of Gerontology, TNO, Rijswijk, The Netherlands). They were pair labelled with Iodine-125 and Iodine-131 (Amersham) respectively by means of the chloramine-T method (5) to specific activities of 63 kilo becquerel per microgram and 20 kilo becquerel per microgram of protein respectively.

ENZYME-LINKED IMMUNOSORBENT ASSAYS AND RADIO IMMUNOASSAYS

Enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) were performed in flexible microtitre plates. (Dynatech) All proteins, diluted in 0.01 M sodiumbicarbonate buffer pH 9.6, were coated to the solid phase of the microtitre plate by passive adsorption overnight at 22 °C. For dilution and preincubation a Tris buffer pH 7.3 was used; 0.15 M Tris HCl, 0.9 percent NaCl, 0.5 percent Tween 20, 1 percent BSA. For washing a 0.5 percent Tween-20 solution was used. For all ELISA tests the enzyme substrate used was ortho-phenylene diamine 0.05 percent (w/v) in 0.2 M phosphate buffer containing 0.1 M citrate pH 5.0 and 0.03 percent (v/v) hydrogen peroxide. All tests were performed in triplicates and double blind.

2.1 SERUM IMMUNOGLOBULIN CONCENTRATIONS

Serum immunoglobulin A, G or M concentrations were determined by means of a sandwich ELISA as follows. Goat (Fab)2 anti-human IgA (α -chain specific), goat F(ab)2 anti human IgG (γ -chain specific) and goat anti-human IgM (μ -chain specific), purchased from Cappel Laboratories, were coated at a concentration of 1 μ g in 50 μ l bicarbonate buffer. After extensive washing the plates were preincubated for 30 minutes at 22 °C with 150 μ l Tris buffer to cover free binding sites. Next 50 μ l per well of test serum, appropriately diluted in Tris buffer, was added and incubated for 60 minutes at 37 °C. After extensive washing, 50 μ l of goat F(ab)2 anti-human IgA, goat F(ab)2 anti-human IgG or goat anti-human IgM (Cappel laboratories) respectively, labelled with Horse Radish Peroxidase (HRP type VI Sigma. St. Louis, USA) according to standard procedures and appropriately diluted in Tris buffer, were added and incubated for 60 minutes at 37 °C. After further washing, 100 μ l of substrate solution was added, incubated for 30 minutes at 22 °C after which the reaction was stopped by adding 50 μ l of a 2 M sulfuric acid solution. The optical density was measured spectrophotometrically at 492 nanometers using a multiscan (Titertek).

2.2 BINDING OF DIMERIC IGA TO HUMAN SECRETORY COMPONENT

IgA subclass dimers were measured by a solid phase RIA as follows. Microtitre plates were coated with 1 μ g secretory component in 50 μ l bicarbonate buffer. After extensive washing and preincubation with Tris buffer plates were incubated with 50 μ l of 1 to 5 diluted test serum and incubated overnight at 4 °C. After extensive washing 50 μ l of the paired label containing 50 nanogram of both monoclonal anti-human IgA1 I-125 and IgA2 I-131 was added and incubated for 3 hours at 37°C. After extensive washing individual wells were cut out using a hot-wire and counted individually in a gamma counter (LKB).

2.3 DETERMINATION OF CIRCULATING IMMUNE COMPLEXES

As standards for immune complex assays heat aggregated immunoglobulins were used. For the C1q binding assay and anti-human C3 solid phase assay, heat aggregated human IgG immunoglobulins (A1gG) were prepared. A 30 mg/ml solution of DEAE-purified human IgG was aggregated by heating for 20 min. at 63 °C. Aggregates of restricted size without free monomeric IgG were obtained by centrifugation on a sucrose cushion and subsequent pooling of fractions with a refraction index of 1.366 - 1.389, according to Nydegger et al. (6). The aggregates were stabilized by adding bovine serum albumin (BSA) to a concentration of 1 percent and stored for up to one year at -70 °C, according to Kauffmann et al. (3). For the IgA standards, equimolar quantities of secretory IgA and IgG immunoglobulins were mixed and aggregates were prepared as described for A1gG. Using Iodine-131 trace labelled IgG and Iodine-125 trace labelled secretory IgA, the aggregates thus prepared were shown to be half IgG and half secretory IgA immunoglobulins.

2.3.1 Fluid phase C1q binding assay

Immune complexes were measured by means of a fluid phase C1q binding assay as described by Zubler et al. (12) Briefly, human C1q was radiolabelled with Iodine-125 by means of a lactoperoxidase method to a specific activity of 82 kilo becquerel per microgram and stored at -70 °C. Before use it was thawed, diluted in veronal buffered saline containing 1 percent bovine serum albumen and centrifuged at 18,000 g for 40 minutes. The supernatant was used for testing. 50 μ l of patient serum was mixed with 100 μ l of 0.2 M EDTA in order to block complement activation, and incubated for 30 minutes at 37 °C. The mixture was then immediately transferred to an ice-bath, after which 50 μ l of human C1q Iodine-125 labelled (diluted to 40,000 counts per minute) and 1 ml of 3 percent poly ethylene glycol 6000, dissolved in borate buffered saline pH: 8.3 was added. The mixture was incubated for 60 minutes on ice and centrifuged at 1500 g for 20 minutes. The supernatant was completely discarded

and the radioactivity precipitated counted in a gamma counter (LKB compugamma 1282).

2.3.2 Solid phase anti-human C3 assay

Goat F(ab)2 anti-human C3 produced as described above, was used in a solid phase RIA or ELISA and performed essentially as described by Pereira et al. (7) Briefly, 2.5 μ g goat F(ab)2 anti-human C3 or 1 μ g of mouse monoclonal IgG1 anti-human C3b neoantigens, kindly donated by Dr. J. Tamerius (monoclonal antibody 130) (1), in 100 μ l bicarbonate buffer were coated on microtitre plates overnight. After preincubation 100 μ l of test serum diluted 1:10 in Tris buffer containing 10 mM EDTA-disodium salt, was incubated at 37 °C for 60 minutes. On the same microtitre plate a dilution of standard aggregated IgA (AIgA) or standard aggregated IgG (AIgG) was tested. AIgA or AIgG was diluted in human pooled serum, incubated for 30 min at 37 °C in a water bath and diluted 1:10 in Tris buffer containing 10 mM EDTA-disodium salt. For IgG and IgM immune complexes goat F(ab)2 anti-human IgG HRP, anti-human IgA HRP and goat anti-human IgM HRP respectively, appropriately diluted in Tris buffer were added and the assay was continued as described above. In addition, to detect circulating IgA immune complexes 50 nanogram of pair labelled mouse monoclonal anti-human IgA1 and IgA2 in 100 μ l Tris buffer was incubated at 37 °C for 3 hours and after extensive washing, the individual wells were cut out and counted in a gamma-counter.

In this immune complex assay the polyclonal anti-human C3 and the monoclonal antibody 130 coated, yielded identical results, both in sensitivity as well as in specificity. Therefore only the goat F(ab)2 anti-human C3 was used to determine the presence of circulating immune complexes. Immune complexes were considered present when anti-human IgG, anti-human IgA or anti-human IgM binding exceeded the 95 percentile of normal healthy volunteers; which was for IgG immune complexes the equivalent of 10 μ gr heat aggregated IgG, for IgA immune complexes the equivalent of 3 μ gr heat aggregated IgA.

2.4 SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION

The size of the IgA immune complexes was determined by 10 - 30 percent sucrose gradient ultracentrifugation using a Beckman SW-40 rotor. 300 μ l of test serum was layered on top of the sucrose density gradient (saccharose in phosphate buffered saline pH: 7.4 containing 10 mM EDTA-disodium salt) and centrifuged at 4 °C for 16 hours at 160,000 g. 250 μ l fractions were collected from the bottom and tested in the solid phase goat F(ab)2 anti-human C3 immune complex assay for IgA immune complexes and for IgA immunoglobulin using the sandwich ELISA described above. As markers for 7, 11 and 19 S, monomeric human IgG, human C1q, and human IgM, respectively were run along with the test samples.

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SAMENVATTING EN CONCLUSIES

Deze dissertatie is voortgekomen uit een prospectief regionaal onderzoek naar de epidemiologie en het natuurlijk beloop van idiopathische glomerulonefritiden en glomerulopathieën, welke in 1977 in Zuid-Limburg werd geïnitieerd. Gezien het chronische karakter van de meerderheid van de idiopathische glomerulaire ziekten en de relatief korte follow up duur, valt een beschrijving van het natuurlijk beloop van deze ziekten buiten het kader van deze dissertatie en ligt de nadruk op de epidemiologie en de differentiaal diagnostiek van deze groep van idiopathische nierziekten.

In hoofdstuk I wordt aan de hand van een literatuur overzicht de importantie van prospectieve studies naar het natuurlijk beloop van idiopathische glomerulaire ziekten benadrukt. In het jaar 1977 was bij een derde van alle patiënten behandeld wegens een terminale nierinsufficiëntie, deze het gevolg van een idiopathische glomerulonefritis of glomerulopathie. Sinds 1977 is het aantal patiënten behandeld wegens een terminale nierinsufficiëntie sterk gestegen. Deze toename is voornamelijk het gevolg van een meer liberaal toelatingsbeleid tot niervervangende therapie met name van patiënten met nefrosclerose en systemische ziekten, met name diabetische nefropathie. Dit beleid leidt onvermijdelijk tot een sterke toename van het aantal, dure, dialyse mogelijkheden daar het steeds duidelijker wordt dat de, goedkopere, niertransplantatie niet in staat is de sterk gestegen vraag naar niervervangende therapie te doen stabiliseren dan wel afnemen. De toegenomen kosten van niervervangende therapie, met name de toename van het aantal dialyse mogelijkheden, kan slechts omgebogen worden door meer nadruk te leggen op preventie van nierinsufficiëntie.

Bij idiopathische, meestal door immunologische mechanismen veroorzaakte, glomerulaire ziekten zullen maatregelen ter preventie van nierinsufficiëntie op twee aandachtsvelden gericht moeten zijn. Ten eerste dient in toenemende mate onderzoek verricht te worden naar de pathogenese van idiopathische glomerulaire pathologie en naar de mechanismen die een rol spelen bij de progressie tot nierinsufficiëntie. Ten tweede is klinisch onderzoek naar epidemiologie en natuurlijk beloop van deze glomerulaire ziekten van groot belang.

In hoofdstuk I wordt aangetoond dat onderzoek naar de pathogenese van glomerulopathieën en glomerulonefritiden heeft geleid tot nieuwe inzichten in de pathogenese van immuuncomplex gemedieerde glomerulaire ziekten, *in situ* immuuncomplex vorming, en in het ontrafelen van de pathogenetische mechanismen, fokale en segmentale glomerulosclerose, welke een rol spelen in de progressie van nierziekten. Beide zijn potentieel van grote betekenis in de behandeling van patiënten met glomerulaire afwijkingen. Teneinde deze bevindingen te kunnen vertalen naar effectief therapeutisch ingrijpen in de

individuele patiënt, is prospectief onderzoek naar het natuurlijk beloop van de diverse vormen van glomerulaire pathologie noodzakelijk. Ondanks het frequent voorkomen van deze nierziekten laat inventarisatie van de literatuur zien dat, naast het ontbreken van epidemiologische gegevens, prospectieve studies naar het natuurlijk beloop niet voorhanden zijn, zelfs van de meest frequent voorkomende glomerulopathieën, idiopathische IgA nefropathie en membraneuze glomerulopathie. Derhalve is de meeste informatie omtrent het natuurlijk beloop van deze afwijkingen afkomstig van retrospectieve studies van patiëntengroepen in grote nefrologische centra, hetgeen onvermijdelijk leidt tot een concentratie van patiënten met een slechte prognose welke niet representatief zijn voor het natuurlijk beloop van de glomerulopathie in de open populatie.

Langdurige en zorgvuldige follow up van de in deze dissertatie gepresenteerde patiënten, zal bepalen of de aanwezigheid van bepaalde klinische, serologische of immunohistologische parameters vroeg in het natuurlijk beloop van de ziekte, prognostische betekenis hebben in de individuele patiënt.

Hoofdstuk II beschrijft een epidemiologische studie naar de frequentie van voorkomen van idiopathische glomerulaire ziekten zoals deze is uitgevoerd in de regio Zuid-Limburg van 1977 tot 1985. Alle nefrologen in de drie grootste ziekenhuizen in deze regio participeerden in deze studie. In elk ziekenhuis werd een nierbiopsie verricht op strikte indicaties en contra-indicaties uit een lokale populatie van een half miljoen patiënten, hierdoor was het mogelijk de incidentie van 'frequent' voorkomende idiopathische glomerulaire ziekten te schatten. Indicaties voor nierbiopsie waren recent ontstane uremie ($21 \times$), recent ontstaan nefrotisch syndroom ($46 \times$), persisterende microscopische erythrocyturie ($70 \times$), recidiverende macroscopische hematurie ($30 \times$) en chronische proteïnurie ($23 \times$). Bij patiënten met een nefrotisch syndroom of recent ontstane uremie diende binnen zes maanden na ontstaan van symptomen of afwijkingen een nierbiopsie verricht te worden. Bij patiënten met idiopathische hematurie en/of proteïnurie welke langer dan zes maanden persisteerde, diende de nierbiopsie binnen twee jaar na identificatie van de afwijkingen of symptomen verricht te worden. Dat in deze studie de nierbiopten vroegtijdig in het natuurlijk beloop werden genomen, wordt geïllustreerd door het feit dat, van de 29 patiënten met idiopathische IgA nefropathie, slechts een patiënt een licht gestoorde nierfunctie had.

In het kader van deze studie werd bij 190 patiënten een nierbiopsie verricht. Hiervan werd bij 14 patiënten onvoldoende materiaal verkregen, bij 13 patiënten bleken de glomerulaire afwijkingen te berusten op glomerulaire pathologie van een klinisch niet vermoede systemische afwijking, terwijl bij 27 patiënten met recidiverende macroscopische hematurie ($13 \times$), persisterende microscopische erythrocyturie ($11 \times$), of chronische proteïnurie ($3 \times$), het nierweefsel histologisch, immunohistochemisch en ultrastructureel geen af-

wijkingen vertoonde. Derhalve werd bij 136 patiënten een idiopathische nierziekte aangetroffen. Deze was primair glomerulair bij 122, interstitieel bij 7, en vasculair eveneens bij 7 patiënten.

De voornaamste bevindingen van deze studie zijn de volgende. Het geschatte incidentiegetal van idiopathische IgA nefropathie en dunne basaalmembraan nefropathie is relatief hoog, respectievelijk 20 en 13 per miljoen volwassenen per jaar. Het voorkomen van deze ziekten bij patiënten met chronische idiopathische hematurie is respectievelijk 31 en 22 procent. De incidentie van zowel fokale en segmentale glomerulosclerose als renale vasculaire hyalinose bij normotensieve patiënten met chronische proteïnurie is 37 procent. De incidentie van membraneuze glomerulopathie, 'minimal change' glomerulopathie en fokale en segmentale glomerulosclerose bij patiënten met een recent ontstaan nefrotisch syndroom is 34, 22 en 15 procent respectievelijk met incidenties per miljoen per jaar van 9,6 en 4 respectievelijk. Verrassend was de lage frequentie van voorkomen bij patiënten met een recent ontstaan nefrotisch syndroom, 5 procent, als ook de lage incidentie van minder dan 2 per miljoen per jaar van membranoproliferatieve glomerulonefritis. Tenslotte was het voorkomen van extracapillaire glomerulonefritis en diffuus scleroserende glomerulonefritis bij patiënten met recent ontstane uremie hoog, 33 en 25 procent respectievelijk.

Deze prospectieve regionale studie levert data over de incidentie van idiopathische glomerulaire ziekten in de populatie, gegevens welke tot op heden onbekend waren. Tevens voorziet ze in cijfers over het voorkomen van de verschillende idiopathische, meestal door immuunmechanismen veroorzaakte, glomerulaire ziekten bij patiënten met chronische hematurie, chronische proteïnurie, recent ontstaan nefrotisch syndroom en recent ontstane uremie, hetgeen bijdraagt aan de differentiaal diagnostiek van deze symptomen.

In Hoofdstuk III wordt nagegaan of er een verband bestaat tussen leeftijd en sekse van patiënten met een nierziekte en het type alsook de ernst van de glomerulaire pathologie. De meerderheid van deze patiënten is afkomstig van de epidemiologische studie beschreven in Hoofdstuk II.

Bij 181 patiënten werd een nierbiopsie verricht wegens recent ontstane uremie ($19 \times$), recent ontstaan nefrotisch syndroom ($52 \times$), persisterende microscopische erythrocyturie ($65 \times$), recidiverende macroscopische hematurie ($28 \times$) of persisterende proteïnurie ($17 \times$). Bij 27 van deze patiënten werd normaal nierweefsel aangetroffen, bij 10 een interstitiële nefritis, bij 7 renale vasculaire hyalinose, terwijl bij de resterende 137 patiënten het nierweefsel tekenen van een idiopathische glomerulonefritis danwel glomerulopathie vertoonde. Significant vaker werden mannen gebiopteerd wegens de symptomen recidiverende macroscopische hematurie, persisterende microscopische erythrocyturie zonder proteïnurie en recent ontstaan nefrotisch syndroom. Dit in tegenstelling tot de

overige indicaties, persisterende microscopische erythrocyturie met proteïnurie, persisterende proteïnurie en recent ontstane uremie, waarbij de gebiopteerde patiënten gelijkelijk verdeeld waren over beide seksen.

Bij mannen werd significant vaker de diagnose idiopathische IgA nefropathie gesteld dan bij vrouwen, zowel bij patiënten met recidiverende macroscopische hematurie ($11 \times$) als ook bij hen met persisterende microscopische erythrocyturie ($22 \times$). Aangezien meer mannen dan vrouwen werden gebiopteerd wegens recidiverende macroscopische hematurie kan bij deze indicatie dit niet zonder meer aan een sekse gebonden voorkomen van de idiopathische IgA nefropathie worden toegeschreven. Bij patiënten met idiopathische IgA nefropathie en persisterende microscopische erythrocyturie is het sekse gebonden voorkomen echter wel duidelijk, daar patiënten met persisterende microscopische erythrocyturie en dunne basaalmembraan nefropathie gelijkelijk over beide seksen waren verdeeld. Bij patiënten gebiopteerd wegens persisterende proteïnurie werd fokale en segmentale glomerulosclerose vaker bij mannen aangetroffen, terwijl de afwijkingen van renale vasculaire hyalinose vooral bij vrouwen, voorkwamen. Patiënten gebiopteerd wegens een recent ontstaan nefrotisch syndroom waren meestal mannen. Dit sekse gebonden voorkomen was uitsluitend gerelateerd aan de idiopathische membraneuze glomerulopathie en werd niet waargenomen bij de 'minimal change' glomerulopathie. Bij patiënten gebiopteerd wegens een recent ontstane uremie werd een sekse gebonden voorkomen alleen geobserveerd bij de diffuus extracapillaire glomerulonefritis, niet veroorzaakt door anti-glomerulaire basaalmembraan antilichamen, die vrijwel uitsluitend bij mannen voorkwam.

Bij mannelijke patiënten bleek de ernst van de proliferatieve glomerulaire ontstekingsreactie, met name het voorkomen van extracapillaire proliferatie, gerelateerd aan de leeftijd en niet aan de duur van het ziekteproces. Deze bevinding werd ook waargenomen binnen één glomerulaire afwijking, de idiopathische IgA nefropathie.

De bevindingen uit dit hoofdstuk tonen aan dat er een verband bestaat tussen sekse en het type van de glomerulaire pathologie, terwijl de ernst van de glomerulaire inflammatoire afwijkingen gerelateerd is aan de leeftijd van de patiënt.

Hoofdstuk IV beschrijft het gebruik van kwantitatieve elektronenmicroscopie in de diagnose van dunne basaalmembraan nefropathie. Routinematige ultrastructurele morfometrie van de glomerulaire basaalmembraan werd verricht toen bleek dat het nierweefsel van een substantieel deel van de patiënten, gebiopteerd wegens recidiverende macroscopische hematurie of persisterende microscopische erythrocyturie, lichtmicroscopisch en immunohistochemisch geen duidelijke afwijkingen toonden. Bij sommigen echter werd lichtmicroscopisch een slechte zilverimpregnatie van de glomerulaire basaalmembraan in de zilver methenamine, hematoxyline en eosine kleuring waargenomen, hetgeen

glomerulaire basaalmembraan pathologie deed vermoeden. Quantitatieve metingen van de glomerulaire basaalmembraan werden daarom uitgevoerd op alle normotensieve en niet azotemische patiënten waarbij een nierbiopsie was verricht in verband met recidiverende macroscopische hematurie ($26\times$) of persisterende microscopische erythrocyturie ($55\times$). Deze patiënten waren afkomstig van de epidemiologische studie beschreven in Hoofdstuk II; bij allen bestond de hematurie minstens een half jaar en de nierbiopsie werd verricht binnen twee jaar na herkenning van de hematurie. Geen van de patiënten had een voor hereditaire nierziekten belaste familieanamnese en allen werden gedurende 50 maanden (gemiddeld) vervolgd. De glomerulaire basaalmembraan dikte werd gemeten met behulp van de orthogonale intercept methode vanaf elektronenmicroscopische opnames.

De 81 patiënten konden verdeeld worden in drie groepen op basis van lichtmicroscopie en immunohistochemie; idiopathische IgA nefropathie ($27\times$), een groep met diverse histologische afwijkingen te weten: mesangio-proliferatieve glomerulonefritis, interstitiële nefritis en focale globale glomerulosclerose ($11\times$), en tenslotte een groep met lichtmicroscopisch normaal nierweefsel zonder glomerulaire afzetting van immuunglobulinen ($43\times$). De dikte van de glomerulaire basaalmembraan was normaal in de patiënten met idiopathische IgA nefropathie (354 ± 55 nanometer) en in de groep met de diverse histologische afwijkingen (327 ± 37 nanometer). Morfometrische analyse van de glomerulaire basaalmembraan dikte van de patiënten met lichtmicroscopisch normaal nierweefsel maakte een verdeling in twee groepen mogelijk. Bij een groep patiënten ($24\times$) was de dikte van de glomerulaire basaalmembraan normaal (361 ± 69 nanometer) terwijl in de andere groep ($19\times$) diffuus verdunde glomerulaire basaalmembranen werden aangetroffen (190 ± 25 nanometer). Bij acht van deze patiënten waren deze verdunde glomerulaire basaalmembranen geassocieerd met een diffuus mesangiale afzetting van complement factor C3. Bij de patiënten met deze dunne basaalmembraan nefropathie was de nierbiopsie verricht wegens persisterende microscopische erythrocyturie ($18\times$) of recidiverende macroscopische hematurie ($1\times$), welke gepaard ging met significante proteïnurie in 7, lichte proteïnurie in 5 en geen proteïnurie in 6 patiënten. Gedurende de 'follow up' periode persisteerde de erythrocyturie bij alle patiënten met dunne basaalmembraan nefropathie, dit in tegenstelling tot de 24 patiënten met normaal nierweefsel, waarin de macroscopische hematurie verdween bij allen ($13\times$) terwijl de microscopische erythrocyturie verdween bij 5 en persisteerde bij 6 patiënten. Bij 3 van de laatsten werd urologische pathologie evident gedurende de follow up periode. Deze studie toont aan dat de incidentie van dunne basaalmembraan nefropathie bij volwassenen met persisterende microscopische erythrocyturie met of zonder proteïnurie niet verschilt van idiopathische IgA nefropathie. Een nierbiopsie met elektronenmicroscopie en quantitatieve metingen van de glomerulaire basaalmembraan dikte dient bij het differentiaal diagnostisch onderzoek

van de volwassen patiënt met persisterende microscopische erythrocyturie te worden opgenomen. Langdurige follow up van patiënten en studies naar familiair voorkomen van de afwijkingen is gewenst teneinde te bepalen of dunne basaalmembraan nefropathie een variant van de hereditaire nierziekten is met een goede prognose.

In het onderzoek beschreven in Hoofdstuk V werd nagegaan of idiopathische IgA nefropathie, de meest frequent voorkomende idiopathische glomerulaire nierziekte in Nederland, veroorzaakt wordt door afzetting van circulerende IgA immuuncomplexen of macromoleculair IgA in het glomerulaire mesangium. In de epidemiologische studie beschreven in hoofdstuk II werd serum en nierweefsel verkregen van patiënten met recidiverende macroscopische hematurie of persisterende microscopische erythrocyturie in een vroeg stadium in het natuurlijk beloop van de ziekte. In het serum van patiënten met idiopathische IgA nefropathie (28 ×), dunne basaalmembraan nefropathie (19 ×) en normaal nierweefsel werden circulerende immuuncomplexen en IgA dimeer gemeten. De laatste twee patiënten groepen vormden excellente klinische controles gezien hun identieke leeftijd en klinische presentatie. Circulerende immuuncomplexen werden gemeten met behulp van een C1q 'binding assay' en een 'solid phase' anti-C3 assay. Macromoleculair IgA, dimeer, werd gemeten met behulp van een 'secretory component binding assay'. Monoclonale antilichamen werden gebruikt om binding van IgA1 en IgA2 aan te tonen.

Bij geen van de patiënten konden circulerende immuuncomplexen met behulp van de C1q 'binding assay' worden aangetoond. IgA1 circulerende immuuncomplexen (geen IgG of IgM) waren aantoonbaar bij 4 van de 28 patiënten met idiopathische IgA nefropathie. De grootte van deze circulerende immuuncomplexen werd door middel van ultracentrifugatie van sucrose dichtheids gradienten bepaald op 11 S en hun aanwezigheid was geassocieerd met verhoogde concentraties van IgA1 dimeer. Deze bevindingen verschilden niet van die in vier van de 24 patiënten met normaal nierweefsel en een van de 19 patiënten met dunne basaalmembraan nefropathie. Verhoogde concentraties van IgA en IgA1 dimeer werd bij patiënten met idiopathische IgA nefropathie frequenter waargenomen dan bij de overige patiënten. Slechts de aanwezigheid van IgA rheuma factor was specifiek voor IgA nefropathie maar had een lage frequentie van voorkomen (3 van de 28).

Deze observaties zijn niet in overeenstemming met de hypothese dat depositie van circulerende IgA immuuncomplexen of macromoleculair IgA verantwoordelijk is voor de mesangiale IgA afzettingen in idiopathische IgA nefropathie. Waarschijnlijk zijn andere pathogenetische mechanismen, bijvoorbeeld *in situ* immuuncomplex vorming door interactie van dimeer IgA1 met een heteroloog of autoloog mesangiaal antigeen, van meer belang.

CURRICULUM VITAE

A. Tiebosch werd op 19 mei 1959 geboren te Maastricht. In 1977 na behalen van het eindexamen Atheneum B aan het Bisschoppelijk College te Roermond studeerde hij Geneeskunde aan de Rijksuniversiteit te Maastricht en behaalde in juli 1982 en november 1983 achtereenvolgens het doctoraal examen en arts-examen. Vanaf 1983 was hij buiten bezwaar van 's lands schatkist werkzaam bij de vakgroep Immunologie (Hoofd: Prof. Dr. P.J.C. van Breda Vriesman), om vanaf december 1984 als wetenschappelijk assistent aan deze vakgroep verbonden te zijn. Sinds april 1987 is hij in opleiding tot patholoog anatoom bij de vakgroep Pathologie in het Academisch Ziekenhuis te Maastricht. (Hoofd: Prof. Dr. F.T. Bosman.)