Diagnostic and prognostic value of proteinuria in chronic renal diseases

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Clinical Studies

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Cover figure. Scanning electron micrograph demonstrating the endothelial surface of a glomerular capillary from the kidney of a normal rat. Numerous endothelial pores, or fenestrae, are evident (Magnification × 21,400.).

In the name of the God, Most Gracious, Most Merciful

وَمَا أُوتِيتُم مِّن الْعِلْمِ إِلاَّ قَلِيلاً

"Of Knowledge it is only A little that is communicated To you"

(From Holly Quran- Surat Al Israa, Ayat 85)

To all of you, who have made my life worth living

My parents

My sister and brothers

My teachers

My close friends

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LIST OF PUBLICATIONS

I. **Bakoush O, Torffvit O, Rippe B, Tencer J:** High proteinuria selectivity index based upon IgM is a strong predictor of poor renal survival in glomerular diseases. Nephrol Dial Transpl. 16 (7):1357-1363, 2001.

II. **Bakoush O, Torffvit O, Rippe B, Tencer J:** Renal function in proteinuric glomerular diseases correlates to changes in urine IgM excretion but not to the degree of albuminuria. Clin Nephrol. (59): 345-352.2003.

III. **Bakoush O, Grubb A, Rippe B, Tencer J:** Urine excretion of protein HC in proteinuric glomerular diseases correlates to urine IgG but not to albuminuria. Kidney Int. (60): 1904-1909, 2001.

IV. **Bakoush O, Tencer J, Tapia J, Rippe B, Torffvit O**: Higher urinary IgM excretion in type II diabetic nephropathy compared to type I diabetic nephropathy. Kidney Int (61): 203-208, 2002.

V. **Bakoush O, Tencer J, Torffvit O, Tenstad O, Skogvall I, Rippe B:** Characterization of increased glomerular albumin permeability in old spontaneously hypertensive rats (SHR), submitted.

ABBREVIATIONS

ACI	Albumin creatinine index
α_1-M	alpha1-microglobulin
Cr-EDTA	chromium ethylene diamine tetra-acetic acid
DN	Diabetic Nephropathy
ESRD	End Stage Renal Disease
GBM	Glomerular Basement membrane
GCW	Glomerular Capillary Wall
GFR	Glomerular Filtration Rate
GN	Glomerulonephritis
θ	"Theta". Glomerular sieving coefficient or fractional clearance
θ_{alb}	Theta of native albumin
$\theta_{n\text{-}alb}$	Theta of neutral albumin
HMW	High Molecular Weight
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG_2	Immunoglobulin G2
IgG_4	Immunoglobulin G4
IgM	Immunoglobulin M
kDa	Kilo Dalton
LMW	Low Molecular Weight
MW	Molecular Weight
PCI	Protein Creatinine Index
Å	Ångström

ABSTRACT

To the extent that increased urinary protein excretion is an indicator of alterations of the glomerular capillary wall (GCW) and appearance of tubulointerstitial damage, proteinuria can be a good marker of the overall severity of the glomerular and tubulointerstitial damage, and therefore, the prognosis of glomerular diseases. Studies I, II, and III show that it is the type of proteinuria, rather than the degree of albuminuria, that predicts the progression in renal, proteinuric diseases. For instance, we found that the quantity of urinary IgM correlated to the decrease of glomerular filtration rate (GFR) in primary glomerular diseases, irrespective of the degree of albuminuria. 21% of patients with initial proteinuria with high IgM content developed end-stage renal failure compared to none of the patients with proteinuria with low IgM content. Patients who maintained high urinary IgM excretion during the course of glomerular disease showed a more rapid GFR decline over time compared to patients with maintained low IgM excretion despite persistent high degree of albuminuria (study II). Changes in urinary IgG, but not in albumin excretion, during the course of the glomerular disease, correlated to changes in urinary protein HC excretion (study III). Protein HC is a marker of impairment of the proximal tubular function.

In study IV, we observed that patients with type 2 DN had a higher urinary excretion of high molecular weight proteins (IgG and IgM) than patients with type 1 DN, despite similar degree of albuminuria. This suggests partly different patho-physiological mechanisms in diabetic nephropathy (DN) in type 1 and type 2 diabetes mellitus. Patients with type 2 DN have a better preserved ratio of urinary excretion of IgG2/IgG4 than type 1 DN patients, indicating that the charge selectivity is less impaired in type 2 DN.

Finally, old but not young hypertensive rats (study V) develop proteinuria as a result of a dysfunction of the glomerular capillary filter, affecting primarily its size-selectivity. The

changes are functionally compatible with the appearance in the glomerular barrier of an increased number of unselective pores.

Conclusions: During the course of glomerular diseases a maintained low urinary excretion of IgG or IgM indicates a salutary prognosis. Different patho-physiological mechanisms of albuminuria in type 1 and type 2 diabetes have been found, and hypertension induced proteinuria is primarily a size-selective disorder.

INTRODUCTION

The process of urine formation begins with the filtration of nearly protein-free fluid across the glomerular capillary wall (GCW), as first experimentally demonstrated by Wearn and Richards in 1924 (1). In 1842, William Bowman described the structure of the glomerulus, and later on, Ludwig argued that the initial event in the process of urine formation involves separation from the plasma of a protein-free ultrafiltrate by the walls of the glomerular capillaries (2, 3). In 1896, Starling described the mechanisms responsible for the ultrafiltrate formation, namely the magnitude and direction of the hydraulic and colloid osmotic pressures across capillary walls (4). Since 1970's, tracer macromolecules of well defined size and shape such as dextrans, Ficoll, and proteins have been extensively used to characterize the permeability of the GCW. The fractional clearances of such test probes have proven to be determined by the size- and charge-selectivity of the capillary wall, and by the charge, shape, deformability and size of the transported macromolecules (5, 6). Glomerular diseases are characterized by defects in both size- and charge selectivity of the GCW and result in so-called "glomerular proteinuria" (6). The main interest of this thesis, and the studies it is based on, is the diagnostic and prognostic value of the urinary excretion of endogenous proteins in glomerular diseases.

The kidneys

The kidneys are situated on both sides of the posterior part of the abdomen, behind the peritoneum. Each kidney is about 11-12 cm long and weighs about 150 gram. The kidneys contains of a total of two millions glomerulae (7, 8). The glomerulus is a lobulated network of convoluted capillary blood vessels surrounded by the Bowman's capsule, Fig.1 (7, 8). The total length of the, 9-12 μm in diameter capillaries, in a single glomerulus is 9.5 mm, giving

an overall capillary length of 19 km, and a glomerular surface area of ≈ 1 m^2 in the kidneys (7-10).

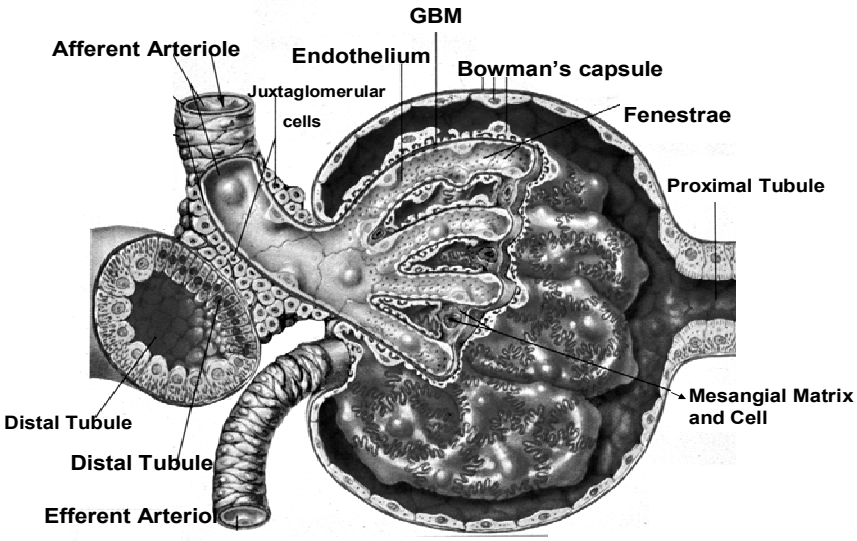


Figure 1. Schematic diagram of the glomerulus.

Glomerular filtration

The major function of the glomerulus is to produce an ultrafiltrate from the blood using the GCW as a filter. The glomerular filtration process differs from the transcapillary exchange process as in other organs in two ways. First, the GCW almost completely excludes plasma proteins of the size of albumin (radius 36Å) or larger from the filtrate. Second, the glomeruli exhibit an extraordinary high permeability-surface area product (PS) to water and small solutes and also a very high capillary filtration capacity (6). Fluid movement across the glomerulus is, similar to the conditions in other capillaries, governed by the Starling forces, i.e. the effective hydrostatic pressure gradient minus the effective oncotic pressure gradient (4). The glomerular filtration rate (GFR) can thus be described by:

$$GFR = LpS \times (\Delta P - \Delta\Pi)$$

Where, Lp represent the hydraulic conductivity of the GCW, and S is the surface area available for filtration. ΔP denotes the hydrostatic pressure in the glomerular capillaries

minus the hydrostatic pressure in the Bowman's space, and $\Delta\Pi$ the effective oncotic pressure in the glomerular capillaries minus that in the Bowman's space. If LpS is 4 ml/min/mmHg/100g of kidney weight in humans, and $\Delta P \approx$ (52-15) mmHg, while $\Delta\Pi \approx$ (28-0) mmHg, then, the GFR in man equals 4 x 3 x [(52-15) - (28-0)] ≈ 120 ml/min. GFR can be measured clinically using molecules that are freely filtered across the glomerulus and that are not bound to plasma proteins nor are absorbed or secreted by the tubules, e.g. inulin or Cr EDTA. Normal GFR in females is 95±20 ml/min and in males 120±25 ml/min (11).

The size selective function of the GCW has been extensively investigated by measuring transglomerular filtration of tracer macromolecules (6). The filtrate-to-plasma concentration ratio of a test macromolecule (e.g. albumin) towards a reference solute such as Cr EDTA, which appears in Bowman's space in the same concentration as in plasma water, is denoted "fractional clearance" or "sieving coefficient" (θ) of the transported macromolecule through the GCW. It is a convenient way to measure permselectivity, varying from 0, when the test molecule is impermeable, to 1, when the molecule is not measurably restricted at normal GFR (6). Note that θ is not a constant, but varies with the GFR (12).

The permeability characteristics of the glomerular capillary wall: Despite the extremely low resistance to the flux of water, the human glomerular filter very efficiently restricts the passage of macromolecules from blood into Bowman's space, see table 1 (12-19). The passage of low molecular weight (LMW) proteins, e.g. proteins smaller than 30 kDa MW, and with a radius smaller than 25 Å, is almost completely unrestricted in normal individuals (6, 20, 21). The estimated albumin concentration in normal glomerular ultrafiltrate is only about 20 mg/L compared to approximately 40000 mg/L concentration of the protein in human plasma (19, 22). Thus, the glomerular sieving coefficient of albumin is 5-6 x 10^{-4} (12, 15-19).

In normal individuals the transport of negatively charged albumin is restricted by a factor of 7-10 compared to equally sized, uncharged macromolecules, table 1 (12, 14).

Table 1. Glomerular sieving coefficient (θ) of macromolecules of different size, charge and configuration in normal rats.

Protein	Radius	θ	References
Anionic HRP	32 Å	0.007	13
Native HRP	30 Å	0.06	13
Dextran	30 Å	0.37	13
Native albumin	36 Å	0.0006	12,15-19
Neutral albumin	36 Å	0.006	12,17
Ficoll	35 Å	0.008	14
IgG	55 Å	0.005	15,18
$\alpha_2$macroglobulin	90 Å	0.000029	18

This view of the glomerular filter as a highly size- and charge-selective barrier has been challenged recently by Comper and his associates (23). The authors found that θ for albumin was nearly a 100-fold higher than previously reported, and they found little evidence for charge-selectivity of the glomerular filter (23, 24). If this were correct, then no less than 600 g of albumin would pass the human glomerular filter every day! Therefore they had to postulate a non-degradative 'retrieval pathway' to account for the reabsorption to plasma of almost the entire filtered load of albumin. Furthermore, a substantial fraction of urinary proteins was reported to be degraded and excreted in the final urine as protein fragments. It was proposed that a reduced protein 'retrieval' to plasma, or reduced protein degradation, would be mainly responsible for the increased urinary protein excretion occurring in a number of proteinuric disorders. This concept was specifically tested by Ohlson et al in the isolated perfused kidney model. However, they found considerable evidence that the classical view is still the most acceptable (25).

The most widely used description of macromolecular transport across the GCW indicates that the glomerular filter is perforated by pores having either a continuous log-normal distribution of radii, or by of two discrete populations of pores (12, 18, 26, 27). This hypothetical description of GCW is not based on ultra-structural analyses but on a hydrodynamic theory of hindered solute transport through water filled pores, as first modelled by Pappenheimer et al (28). The two-pore theory of capillary permeability adequately describes the fractional clearance data obtained in experimental and in clinical studies (12, 18, 29-32). In the “two-pore with a shunt model” the vast majority of pores are "small pores". The small pores exhibit a radius of ≈29Å vis-à-vis negatively charged, rigid, spherical proteins, and a radius of 37-38Å vis-à-vis uncharged macromolecules (12, 18). The second pore population consists of a very small number of “large pores” of radius 90-115Å (18). The small pores are essentially impermeable to macromolecules the size of albumin or larger. Such molecules are normally transported by convection across the large pores (27). In addition to the two pores, the GCW may display “shunts”, which are very sporadic physiological “membrane defects”, large enough to allow the transport of very large proteins and even red blood cells (29, 33). Proteins such as IgM (radius 120Å) are able to pass the GCW only through these shunts (18). Conceivably, a repairing apparatus normally seals these shunts, and an increased transport of IgM indicates unsealing of the shunts and/or increased density of these defects in the GCW (29, 33).

The glomerular filter

The GCW consists of the glomerular basement membrane (GBM), an endothelial cell layer, and an epithelial cell layer (Fig.2). Both the cell layers are coated with a negatively charged surface coat (10-60 nm thick), called the glycocalyx (9). Furthermore, a much larger exclusion area extending from the endothelial surface for anionic macromolecules, possibly

composed of glycosylated macromolecules and adsorbed plasma proteins, has been described, denoted the "endothelial surface layer" (ESL) (34). The fenestrae between the endothelium cells are 50-60 nm in diameter, and also appear to be filled with plugs of glycocalyx or ESL up to 90 nm in height. They are thought to provide the GCW with size- and, most importantly, with charge-selectivity (9, 34, 35).

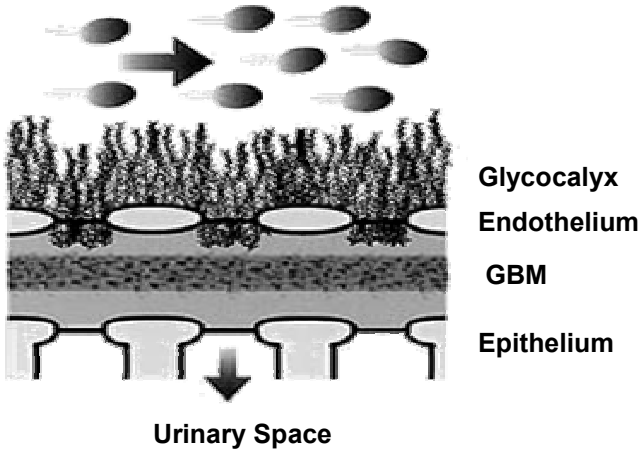


Figure 2. A schematic diagram of the glomerular capillary wall showing the luminal surface coat lining the endothelium and filling out the fenestrae. The ultrafiltrate passes through the downstream layers into the urinary space (Bowman's capsule).

The GBM is a gel like material, 200 nm in width in rats, and 300-400 nm in humans, and is composed of tightly cross-linked extracellular matrix proteins, such as type IV collagen, laminin, nidogen and proteoglycans (36). Type IV collagen and laminin provide strength and flexibility to the GBM and also an adhesion surface for endothelial cells and podocytes. The heparan sulfate proteoglycans, perlecan and agrin, may contribute to the charge-selective permeability of the GBM (37), although this was recently questioned (38). The epithelial cells, the podocytes, cover the external surface of the GBM. They are highly specialized cells forming multiple interdigitating foot processes leaving in between them filtration slits, spanned by a "slit diaphragm", 30 to 40 nm in width (39). The foot processes stabilize the glomerular architecture by counteracting the distension of the GBM (40). Nephrin is a major

structural component of the slit diaphragm, and the absence of nephrin, or any other slit diaphragm associated proteins, e.g. podocin, CD2-associated protein, Neph1, or alpha-actinin-4 leads to proteinuria and the nephrotic syndrome (41-45).

It is still not recognized which of the substructures of the GCW represents the ultimate permeability barrier, serving to retain plasma proteins in the circulation (46, 47). The blood flow is of great importance to maintain the GFR, and hence, the glomerular barrier function and, under normal hemodynamic conditions, albumin may be restricted already at the endothelial level, Fig.2 (33, 34, 47-49). In addition, the absence of concentration polarization within the GBM raises the possibility that the glycocalyx-filled fenestrae play a greater role in the size-selectivity than the GBM or the slit-diaphragm (9, 12, 50). The charge selectivity of the GCW is attributed mainly to the glycocalyx and/or ESL and to the heparan sulphate proteoglycans of the GBM (35, 37). Orosomucoid and albumin are among serum proteins that are thought to have a role in determining capillary permeability by maintaining and reinforcing the charge barrier (51-53). In all proteinuric diseases, whether immune complex mediated or not, the podocytes show structural changes with effaced foot processes, and occasionally separation from the GBM (40). Because of limited capacity of podocytes to proliferate, even after injury, podocyte loss or low podocyte number per glomerulus, may contribute to the development and progression of glomerulosclerosis and proteinuria (40, 54-57).

Proteinuria, pathogenesis and sequelae

An abnormal excretion of proteins in the urine is the hallmark of experimental and clinical glomerular diseases. Proteinuria is an indicator of alterations in the GCW and in tubular

protein uptake. It can be a good marker of the overall severity of the glomerular and tubulointerstitial damage, and therefore, of the prognosis of glomerular diseases.

The proteins filtered into the primary urine are normally reabsorbed via receptor-mediated endocytosis in the proximal convoluted tubules (19, 58). Megalin and cubilin are the two receptors known to be important for normal tubular reabsorption of filtered proteins (58, 59). The absorbed proteins are completely hydrolysed within the lysosomes and their resulting amino-acids cross the basolateral membrane to be returned to the circulation (58). Normally, the proximal tubules reabsorb approximately 90 –95% of the filtered albumin while LMW proteins, such as protein HC, or light chains, are reabsorbed almost completely (19). Tubulointerstitial injury causes an impairment of proximal tubular uptake of filtered proteins which leads to increased urinary excretion of LMW proteins, the characteristic feature of tubular proteinuria (20, 21). The normal upper limit of the total daily urine protein excretion is less than 150 mg, and normal urine consists of 20-30 mg albumin, 10-20 mg of LMW proteins, and 40-60 mg of secretory proteins, such as Tamm-Horsfall protein and IgA (60-62). In glomerular injuries, altered size- and charge-selective properties of the GCW result in increased filtered load of albumin and HMW proteins (63). When the reabsorptive capacity of the proximal tubules is exceeded, these proteins appear in the final urine, a phenomenon called “glomerular proteinuria”, although it also includes a component of “tubular” proteinuria.

Proteinuria may in itself contribute to ongoing renal injury by causing mesangial and tubulointerstitial damage (64, 65). In fact, proteinuria is the major determinant of progressive renal failure (66-68). The most widely proposed cause of tubular injury in proteinuric glomerular disease is the extensive tubular uptake of filtered plasma proteins, including

growth factors and complement factors, cytokines and protein bound substances, such as fatty acids, carried by the filtered albumin. These factors may induce tubular production of vasoactive and inflammatory cytokines, causing an invasion of the interstitium by inflammatory cells (69-75). In highly selective proteinuria, i.e. an almost pure urinary loss of albumin, the tubulointerstitial damage is infrequent, and almost all patients retain normal kidney function (76-78). With non-selective proteinuria, an increasing number of patients develop tubulointerstitial damage and progress to renal failure (79-81). It is generally believed that impairment of the charge-selectivity of the glomerular filter is the predominant lesion in selective proteinuria (82). The predominant lesion in non-selective proteinuria is a size-selectivity dysfunction, with the functional appearance of unselective "large pores" and or "shunts" in the glomerular filter (63).

Assessment of proteinuria

The clinical indication for proteinuria assessment is the screening for, and follow-up of renal diseases. During the screening, albuminuria is usually detected by dipstick methods, e.g. Albustix®, in which the albumin concentration may be underestimated in diluted urine. The amount of protein in urine collected over a 24-hour period is used as the "golden standard" for measurement of proteinuria. However, because of the high risk of collection errors in a 24-hour sampling, and for practical and economic purposes, many investigators prefer to use urinary protein to creatinine index (PCI) instead (83-93). In pathological conditions, the high correlation to the 24 hour urine protein excretion, makes PCI in a single voided urine specimens accepted in clinical routine practice (68, 85, 86, 89-94). Reference values for PCI are: < 3.8 mg/mmol for albumin, < 0.8 mg/mmol for IgG, and <0.7 mg/mmol for protein HC in random urine specimen (61, 62, 91, 94).

The selectivity of proteinuria, assessed by the selectivity index (SI), is measured by the comparison of the clearance of high molecular weight proteins, e.g. IgG or IgM, with the clearance of transferrin or albumin as markers of intermediate size proteins (29, 95). SI based on α_2-macroglobuin or IgM is of better predictive value than SI based on IgG (29).

Albumin is the most abundant circulating plasma protein, (69 kDa MW, serum concentration 38-40g/L) and has a variety of functions (96). These include maintenance of the oncotic pressure, buffering of acid-base changes, and transport of multiple bioactive substances such as fatty acids, steroid hormones and vitamins. Liver is the major site of synthesis of albumin, and its breakdown mainly occurs in the endothelial cells (96). The absolute level of serum albumin reflects not only the level of its synthesis and breakdown, but also its volume of distribution, the availability of amino acid precursors, and the albumin loss into urine, intestinal lumen and from the skin (97).

Immunoglobulins, or antibodies, are a complex group of functionally and structurally related proteins that protect the organism from invasion by pathogenic microorganisms and their toxic products. The basic structure of the immunoglobulin molecule consist of a monomer that contains four polypeptide chains, two heavy chains (each of 50 kDa MW) and two light chains (kappa or lambda, each of 20 kDa MW) linked by disulfide bonds (98). There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM antibodies.

Immunoglobulin G (IgG) is a monomeric molecule of 150 kDa, which predominates in secondary or memory immune response against infectious organisms. It is found on surface of memory B cells and predominantly in the blood. It constitutes 75% of serum immunoglobulins. Normal serum concentration of IgG is 5-15g/l. There are four subclasses of IgG, differing in the number of disulfide bonds and in the length of the hinge region. IgG_1 has

the highest concentration in human serum (3-10g/l), followed in order by IgG_2 (1-3.5 g/l), IgG_3 (0.3-1g/l), and IgG_4 (0.2-0.5g/l) (99).
Since IgG_2 is neutral and IgG_4 is negatively charged, a low value of urine IgG_2/IgG_4 ratio reflects loss of charge selectivity of the glomerular capillary wall.

Immunoglobulin M (IgM) is the third most common serum Ig with serum concentration of 0.5-4g/l. IgM is composed of five complete Ig units linked by disulfide bonds to form a pentamer with a molecular weight of 950 kDa (98, 99). IgM is the first immunoglobulin to be made by the foetus and the first antibody made by a virgin B cells when it is stimulated by antigen. As a consequence of its pentameric structure, IgM is a good agglutinating and complement fixing immunoglobulin, very efficient in clumping and lysis of microorganisms.

Protein HC (alias α_1-microglobulin) is a human complex forming glycoprotein, heterogeneous in charge, and was fist described by Tejler and Grubb (100). It is composed of a single polypeptide chain and its physiological function is unknown (101). Complexes between protein HC and IgA and between protein HC and albumin are present in most human biological fluids but they are not present in normal urine and rarely in pathological urine (102). Only the free form of protein HC is filtered through the GCW, and thus, found in normal urine. Protein HC is relatively stable in urine and is also stable at low urinary pH values. The high sensitivity of increased urinary excretion of protein HC makes the determination of the urinary excretion of protein HC an ideal instrument for demonstration of proximal tubular disorders (101-106).

Glomerular diseases

Disorders of glomerular structure and function are the leading cause of end stage renal disease (ESRD) (107, 108). Glomerular diseases may be primary or secondary to systemic disease such as diabetes or SLE (109, 110). See table 2.

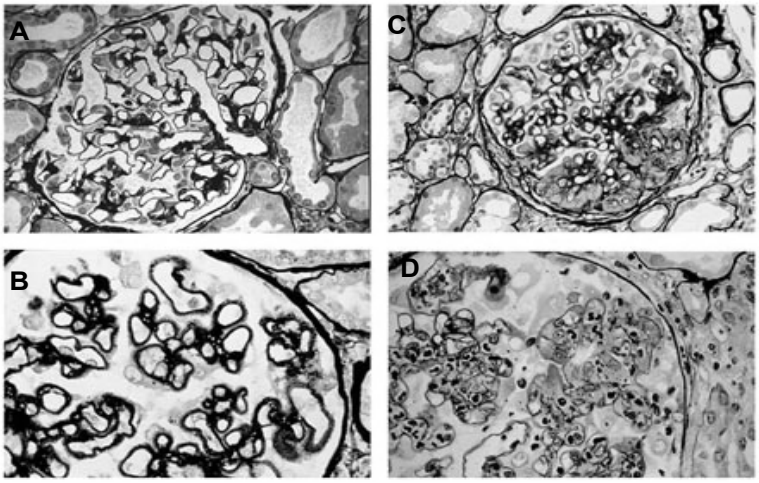


Figure 3. Four patterns of glomerulus histology. (A) minimal change, (B) membranous, (C) focal glomerulosclerosis and (D) Cresentic GN.

Table 2. Histological classification of glomerular diseases.

Non proliferative changes	Proliferative changes
•Primary renal disorder	
Minimal change Nephropathy	IgA nephropathy
Focal Segmental glomerulosclerosis	Mesangioproliferative glomerulonephritis
Membranous glomerulopathy	Mesangiocapillary glomerulonephritis
	Focal segmental glomerulonephritis
	Crescentic glomerulonephritis
•Secondary renal disorder	
Diabetic nephropathy	Lupus nephritis
Nephrosclerosis	Hepatitis induced GN

The term glomerulonephritis (GN) is used to denote diseases characterized by intraglomerular inflammation and cellular proliferation. Both humoral and cell-mediated immune mechanisms play a part in the pathogenesis of the glomerular inflammation (109-112). Non-proliferative forms of nephropathies, characterized by chronic non-inflammatory mechanisms of renal

injury, may also induce an increase in glomerular permeability and proteinuria without significant histological alterations in the glomeruli, Fig.3 (113, 114).

IgA nephropathy (IgA N) is a mesangioproliferative GN characterised by diffuse mesangial deposition of Immunoglobulin A. It is the most common form of GN worldwide (110). The disease occurs in all ages with a peak incidence in the second and third decades of life. Males are affected up to sex times more frequently than females. IgA N presents in 50-60% of cases with episodes of gross hematuria that is frequently associated with respiratory- or gastrointestinal tract infection. In 30% of cases it presents with persistent microscopic hematuria and proteinuria, and occasionally, in 10% of cases, with nephrotic syndrome or rapidly progressive nephritis (113). The disease progresses to ESRD in 20-40% of cases (110, 115, 116).

Mesangial proliferative glomerulonephritis is found in 5 to 10% of renal biopsies (117). The patho-histological findings are characterised by mesangial cell proliferation and increase in mesangial matrix. Circulating immune complexes are present in 50 to 70% of cases. The disease usually presents with proteinuria, often in the nephrotic range. It accounts for 3-5% of patients with idiopathic nephrotic syndrome (117). Corticosteroid treatment leads to remission in 20 to 60% of the cases. Frequent relapses, partial remissions and glucocorticoid dependence are not uncommon (113).

Membranous glomerulopathy is the most common cause of idiopathic nephrotic syndrome in adult Caucasians (118). It is characterised by immune deposits of IgG and complement components predominantly on the subepithelial surface of the GCW. The disease is usually idiopathic but may be secondary to drugs (gold, penicillamine), infections (hepatitis B virus),

autoimmune diseases (SLE), or malignancy (118). Patients present typically with the nephrotic syndrome, and in 20% of cases, with non-nephrotic range proteinuria. Spontaneous remission occurs in 40% of patients while ~30% develop progressive renal failure (118-120). Treatment with cytotoxic agents and prednisolone is indicated in progressive cases (118, 119).

Minimal change nephropathy is responsible for 90% of nephrotic syndromes in children and 20% of nephrotic syndromes in adults (121). Morphologic changes are apparent only on electron microscopy that shows diffuse effacement of foot processes of the podocytes. The pathogenesis is unknown, but probably linked to T-cell mediated immunity (122). Clinically, the disease is characterised by the abrupt onset of nephrotic syndrome, normal renal function, and normotension. Spontaneous remissions occur in 40% of cases, and progression to renal failure is very rare (121, 123).

Focal segmental glomerulosclerosis (FSGS) has characteristic pathological features of focal and segmental glomerular scars found on light microscopy of renal biopsy (121). The disease is a common cause of nephrotic syndrome in adults (20 to 30%), especially in black males (124). Renal failure occurs in more than 50% of patients within 10 years (121, 125). FSGS is idiopathic, but may associate with intravenous drug use and is found in 20% of heterosexual HIV-positive persons (126). A non-immunoglobulin circulating factor seems to account to cases that recur after kidney transplantation (127).

Diabetic nephropathy (DN) is a (microvascular) complication of both type 1 and type 2 diabetes, that is associated with ESRD and premature death from cardiovascular disease (128, 129). Histologically, DN is characterised by diffuse or nodular mesangial expansion with thickening of the GBM (130). In patients with type 2 DN, particularly those with

hypertension, renal biopsy shows significant nephrosclerosis as well. Hyperglycaemia and increased intraglomerular pressure cause an increased synthesis of several cytokines and growth factors, in particular transforming growth factor-β (TGF-β). The cytokines have been identified to stimulate matrix production or inhibit matrix degradation and thus, lead to glomerulosclerosis and proteinuria (131, 132). The earliest clinical evidence of DN is appearance of low degree albuminuria, referred to as microalbuminuria (>30mg/day) (133, 134). Untreated, 20-40% of type 1 diabetes patients with microalbuminuria will progress to overt nephropathy over a period of 10 years (135-138). Once overt nephropathy occurs the GFR starts to fall at an average rate of 4-6 ml/min/year (135, 139). DN has become the most common cause of ESRD in Europe and US (108). As the incidence of type 2 diabetes increases, and the age of onset declines, the burden of DN will further increase in the future. Improved glycemic control has proven to dramatically decrease the incidence of DN (135, 140-142). Treatment with renin angiotensin system blockers and certain calcium channel blockers can reduce the progression to ESRD (143-146).

Hypertensive nephrosclerosis is the characteristic renal lesion associated with essential hypertension. Hypertension is considered present when the systolic blood pressure (SBP) is 140 mm Hg or higher, the diastolic blood pressure (DBP) is 90 mm Hg or higher, or the patient is on antihypertensive medication (147). It is estimated that 24% of the adult population is hypertensive, and 6% of hypertensive patients are at risk for progression to ESRD (147, 148). Systolic hypertension is a powerful predictor of development of renal injury, and the uncontrolled hypertension accounts for 27% of all new cases in ESRD (147-151). Histologically, hypertensive nephrosclerosis is characterized by myointimal hyperplasia of interlobular and afferent arteriolar vessels, hyaline arteriolosclerosis especially of the latter, wrinkling collapse of the glomerular tuft and, commonly, global glomerulosclerosis (147).

Hypertension may cause renal damage as result of glomerular ischemia and hypoperfusion induced by progressive narrowing of the lumina of preglomerular arteries and arterioles. By contrast, afferent vasodilatation in remnant nephrons of individuals with hypertension, especially those with a low number of nephrons, transmit the systemic hypertension to glomerular capillaries, which may lead to progressive renal failure (152-154). Renal susceptibility genes play a role in the development of nephrosclerosis (153). Increased awareness of high blood pressure and treatment of hypertension has contributed to a dramatic reduction in morbidity and mortality attributable to hypertension (155).

AIMS OF THE PRESENT STUDIES

1. To study the urinary excretion of IgM and albumin as prognostic markers in a number of proteinuric glomerular diseases in order to examine whether pathophysiological and patho-morphological differences may influence the renal outcome (Study I).
2. To re-examine correlations between the renal function and alterations (regression or progression) of urinary excretion of IgM and albumin in the course of proteinuric glomerular diseases (Study II)
3. To study the effect on renal tubular function of urine excretion of large proteins by examining the correlation between the degree of tubular damage, assessed by the level of urine α_1-microglobulin (protein HC), on the one hand, and urinary levels of IgG and albumin on the other hand, in chronic glomerular diseases (Study III).
4. To investigate the potential difference in the patho-physiology between patients with diabetic nephropathy in type 1 diabetes mellitus (type 1 DN) and type 2 diabetes mellitus (type 2 DN) by comparing the patterns of urinary excretion of proteins of different size and charge (study IV).
5. To examine the mechanisms of albuminuria resulting from severe, longstanding hypertension by measuring the transglomerular transport of native and neutral albumin in spontaneously hypertensive rats (SHR) of various age.

MATERIALS AND METHODS

The characteristic of the patients in the studies I to IV are shown in tables 3 and 4.

Table 3. Number of patients (male/female) in studies I-IV

Diagnosis	I Male/Female	II Male/Female	III Male/Female	IV Male/Female
Mesangioproliferative GN	17/15	4/6	12/12	-
IgA nephropathy	14/2	3/2	4/3	-
Membranous GN	12/7	10/4	8/4	-
Minimal change nephropathy	5/3	3/3	3/3	-
Nephrosclerosis	8/1	1/1	6/0	12/1
Diabetes type 1	-	-	-	15/7
Diabetes type 2	-	-	-	18/2
Healthy subjects	-	-	-	14/2
Total	84	37	56	71

Table 4. Inclusion and Exclusion criteria in studies I-IV

Criteria		Studies
Inclusion criteria		
•Albumin creatinine index	>20 mg/mmol	I, III, IV
	>100 mg/mmol	II
•Serum creatinine	< 250 µmol/L	II, IV
	< 400 µmol/L	I, III
Exclusion criteria		
•Diabetic Nephropathy		I, II, III
•Systemic diseases		I-IV
•Rapidly progressive glomerulonephritis		I-IV

The patients in the first three studies were all participants in a large investigation program of glomerular diseases being conducted at the Nephrology Department, University Hospital of Lund, Sweden.

In study I we followed renal function in 84 patients with biopsy verified glomerular disease over a median of 41 (± 3) months. The patients were subdivided into groups with low (≤0.002) and high (>0.002) proteinuria selectivity index based upon the IgM / albumin clearance ratio (IgM-SI), and into groups with low (≤200 mg/mmol) and high (>200 mg/mmol) albumin creatinine index (ACI).

In study II we followed 37 proteinuric patients (21 males and 16 females) with glomerular disease and significant initial albuminuria for a mean of 44 (± 3.6) months. The comparisons between the patients were made according to the findings at the end of the study, by dividing them into three groups. One group had decreasing albuminuria (by more than 50%), one group had persisting albuminuria and low (<0.04mg/mmol creatinine), urinary IgM excretion and the last group had persisting albuminuria and high (≥ 0.04mg/mmol) urinary IgM excretion.

In study III, we studied the relationship between urinary excretion of IgG, albumin, and protein HC in 56 patients (33 males and 23 females) with glomerular disease at the time of the diagnostic renal biopsy and after a mean of 49 follow-up months.

In study IV urinary albumin, IgG2, IgG4 and IgM were assessed in 20 patients (18 males and 2 females) with albuminuria and biopsy verified diabetic nephropathy due to type 2 diabetes, along with 22 (15 males and 7 females) patients with type 1 diabetes and macroalbuminuria (Tables 3 and 4). The measurements were compared with those in a control group consisting

of 13 (12 males and one female) patients with nephrosclerosis due to systemic hypertension, and with a second control group consisting of 16 (14 males and 2 females) healthy controls.

In study V, we assessed the glomerular sieving coefficients (θ) for neutral albumin ($\theta_{n\text{-}alb}$) and for native (negatively charged) albumin (θ_{alb}) in spontaneously hypertensive rats (SHR) of age 3, 9, and 14 months in comparison with age matched normal control Wistar rats (NCR). The hypothesis was that increases in the glomerular permeability of both negatively charged and neutral albumin would indicate a preferential size-selective dysfunction of the glomerular capillary wall (GCW), while an increased permeability to negatively charged albumin, as compared to neutral albumin, predominantly would indicate a charge selectivity dysfunction of the GCW. The glomerular sieving coefficients (θ) was assessed using a tissue (renal) uptake technique together with urinary sampling, described at some length elsewhere. Renal tracer protein clearance was calculated from the amount of tracer radioactivity accumulated in the two kidney cortices + the TCA precipitable urine tracer activity (collected during the tracer infusion period) divided by the plasma tracer "area under curve" (AUC). Protein sieving coefficients θ were calculated by dividing the measured protein clearance by the GFR. The GFR was assessed using the plasma to urine clearance of ^{51}Cr-EDTA.

Calculations

The "gold standard" techniques for measurement of GFR using renal inulin or iothalamate clearances are time consuming and difficult to perform. However, given its simplicity, low cost, and widespread use, serum creatinine and creatinine clearance have been relied upon as the principal indicator of renal function in clinical and epidemiological studies (156-158). Various formulas for conversion of serum creatinine into creatinine clearance have been developed. The Cockcroft-Gault formula is probably the most used (159), where:

$$\text{Creatinine clearance (Ccr)} = \frac{(140 - \text{age}) \times \text{weight}}{\text{Serum creatinine } (\mu\text{mmol/L})} (\times 1.23 \text{ for men})$$

A more recently developed formula, based on data derived from the Modification of Diet in Renal Disease (MDRD) study, correlates well with measured GFR, and is of use in patients with mild to moderate renal insufficiency but is inaccurate in patients with normal or above normal GFR (160, 161), namely:

$$\text{GFR} = \{186 \text{ x (serum creatinine mg/dl)}^{-1.154} \times (\text{age})^{-0.203} (\times 0.742 \text{ if female}).$$

IgG2/IgG4 ratio was calculated as:

$$\frac{\text{Urine IgG2 concentration (mg/l)}}{\text{Urine IgG4 concentration (mg/l)}}$$

IgM-selectivity index (IgM-SI) was calculated according to the formula (29, 162):

$$\frac{\text{Urine IgM x Serum Albumin}}{\text{Serum IgM x Urine Albumin}}$$

Theoretical analysis

According to the two-pore model, the glomerular clearance of any protein larger than the assumed small-pore radius is determined by its convective transport across large pores. Thus, its large-pore clearance (Cl_L) is just the product of the large pore volume flow (Jv_L) and the (large pore) reflection coefficient (σ_L), or actually $(1-\sigma_L)$, of the solute:

$$Cl_L = Jv_L(1-\sigma_L) \qquad (1)$$

Since native albumin is negatively charged, it would be completely excluded from the small pore pathway in the glomerular filter, and thus be confined to convective transport through large pores (12). Based on that consideration, the large pore volume flow Jv_L could be

determined from the sieving coefficient of native albumin (θ_{alb})and from GFR assuming a fixed value (100Å) for the large pore radius (18).

$$\theta_{alb} = \frac{Jv_L}{GFR}(1-\sigma_L) \qquad (2)$$

Charge selectivity defect: If charge selectivity is lost, then native albumin, which is normally excluded from the small pore pathway, will be able to penetrate the small pores, leading to a selective increment in θ_{alb}, so that it will eventually approach the sieving coefficient of neutral albumin ($\theta_{n\text{-}alb}$).

Size selectivity defect: If enlarged, less selective pores are formed when permeability is increased, then charge and size selectivity will be changed in parallel, leading to increases in both θ_{alb} and $\theta_{n\text{-}alb}$. If new large pores are formed, then, because of the presence of negative charges in the large pores, the changes in θ_{alb} would be less pronounced than those in $\theta_{n\text{-}alb}$, according to the following equation:

$$\theta_{alb} \propto \frac{(1-\sigma_L)_{alb}}{(1-\sigma_L)_{n-alb}}\theta_{n-alb} \qquad (3)$$

For 100 Å large pores $(1-\sigma_L)_{alb}$ will be 0.398 (accounting for negative charges on albumin and on the pore walls according to the Debye Hückel theory of ion-ion interaction) and $(1-\sigma_L)_{n-alb}$ will be 0.593.

Statistical methods

The data in the tables are expressed as medians and ranges or means ± SE (study II and V). Statistical comparison between the patient groups was performed with non-parametric Mann-Whitney test**,** or Kruskal Wallis test when applicable. Correlation was tested using Spearman's correlation coefficient. $P < 0.05$ was selected as the level of significance. Urine

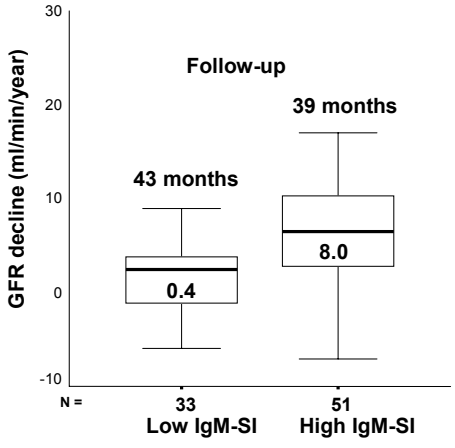
concentrations of IgM below the detection limit were set at 0.01 mg/mmol and urinary HC-CI and IgG-CI below the detection limit were set at 0.1 mg/mmol. The statistical package for social science (SPSS, version 10) was used. $P \leq 0.05$ (or when appreciate $P \leq 0.01$) was selected as the level of significance.

RESULTS AND DISCUSSION

Studies (I - III)

Clinical and experimental data indicate that glomerular proteinuria affects the progression of renal impairment in glomerular diseases by enhancing the formation of tubulointerstitial fibrosis (163). However, several recent reports suggest that it may not be albumin per se, but rather other factors associated with the enhanced urinary leakage of plasma proteins, such as complement factors or protein-bound inflammatory cytokines (e.g. TGF-β), that might cause these sequelae (70, 164).

Figure 4. GFR decline rate in study I patients with low respectively high values of IgM-selectivity index.



Studies I-II show that an increased urine IgM excretion predicts an unfavourable outcome, while a decreased urine excretion of this protein correlates to a more salutary prognosis in patients with primary glomerular diseases. This is true also when the total proteinuria, often presented as albuminuria, is persisting. In study I, patients with a high IgM based selectivity index (IgM-

SI) significantly decreased their renal function, by an average of 8 ml/min/year (Fig.4). Furthermore, 21% of the patients in this group developed end stage renal failure (ESRD) during the study time. In comparison, patients with low IgM-SI on average maintained their kidney function unaltered during observation time, although they had a higher degree of albuminuria (411 mg/mmol) than the patients in the high IgM-SI group (151 mg/mmol) ($p<0.001$). None of the patients in low IgM-SI group developed ESRD.

Observations of patients with glomerular diseases with long-time persistent albuminuria in study II showed that only patients with persistent high urinary IgM excretion decreased significantly in renal function, while those with reduced urine IgM excretion preserved their renal function (Fig.5). In both studies, an increased urine IgM excretion was the strongest single predictor of the GFR decline ($r=0.73$, $p<0.001$).

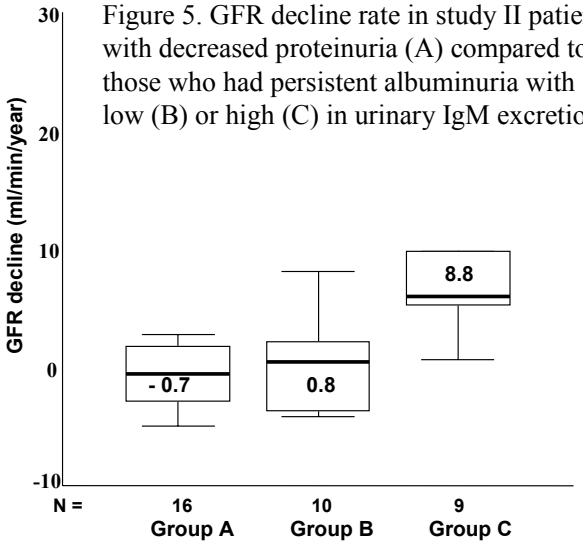


Figure 5. GFR decline rate in study II patients with decreased proteinuria (A) compared to those who had persistent albuminuria with low (B) or high (C) in urinary IgM excretion.

The pivotal role of albuminuria in the progression of renal tubular function impairment in glomerular diseases was further questioned in study III, where its relation to the urinary excretion of protein HC (reflecting the impairment of tubular protein uptake) was examined. We found that changes in urinary excretion of protein HC in a single patient during the follow-up time were much more strongly ($r^2=0.7$) associated with changes in the urine IgG excretion than with changes in the degree of albuminuria ($r^2=0.29$) (Fig.6). In consistency

with other recent investigations, these studies (I-III) shows a significant correlation between non-selective proteinuria and the degree of tubulointerstitial damage (77).

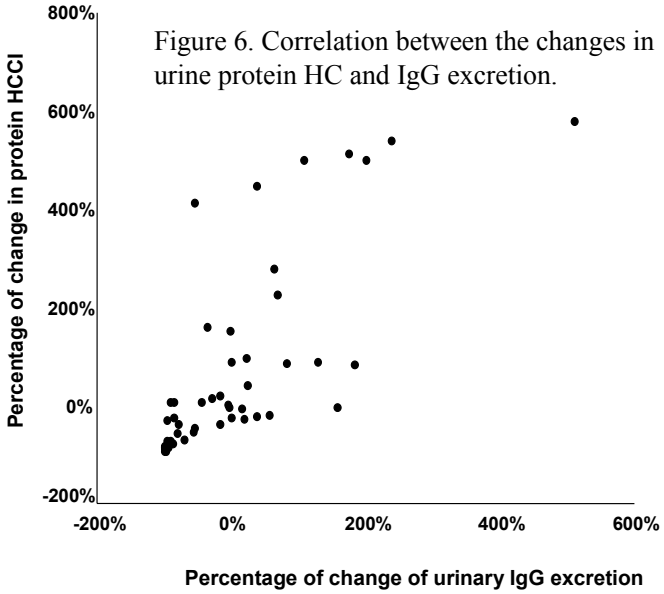
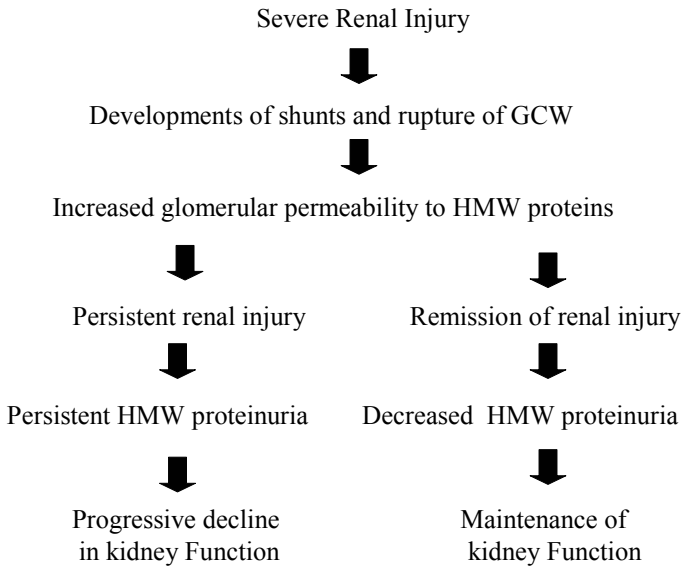


Figure 6. Correlation between the changes in urine protein HC and IgG excretion.

According to the "two-pore with a shunt" theory, macromolecules of the size of albumin or larger are normally transported through large pores of the GCW (26, 27). Since the population of these pores is, under normal conditions, relatively small, the transport of albumin across the GCW is usually low. Very large proteins, such as IgM, are able to pass the GCW only through the extremely rare shunts (18, 33). In case of loss of charge selectivity of the glomerular filter the "effective" small pore radius increases (by 8 Å) from ~29 to ~37 Å, thus enabling albumin to escape in large quantities through the small pores. Proteins larger than albumin are, however, still unable to pass through the small pore pathway. Thus, selective proteinuria is usually a consequence of a charge-selective defect rather than a size-selective disorder. This situation is conceivably the case in minimal charge nephropathy, but also in some other glomerular diseases (165). Once the glomerular disease produces alterations in the size-selective properties of the GCW, the urine contains an increased amount of large proteins

such as IgG and IgM. However, whereas the increased urinary excretion of IgG reflects increased density of "large pores" in the GCW, the occurrence of IgM in the final urine conceivably reflects a markedly increased population of highly unselective pathways, i.e. shunts. Recently, varying degrees of ultrastructural defects in the glomeruli, measuring 15-200 nm in diameter, were revealed in nephrotic patients by transmission electron microscopy using a tissue negative staining method. These ultrastructural defects were not seen in normal renal tissue (166). Thus, proteins of size of IgM could make a sensitive marker of such defects, and an increased urinary IgM excretion apparently would predict a more severe glomerular injury and poorer renal outcome in glomerular diseases (Fig. 7).

Figure 7. Pathophysiology of progressive nephropathy



Isolated albuminuria may represent alterations in either the charge- or size-selective properties of the GCW, or both. These alterations do not necessarily correlate to a gross damage of the GCW, which in turn, could explain the lack of correlation between albuminuria and the progress of the renal function impairment or development of interstitial fibrosis.

Study IV

Figure 8. Urinary IgM excretion in healthy individuals and in patients with diabetic nephropathy due to type 1 and type 2 diabetes.

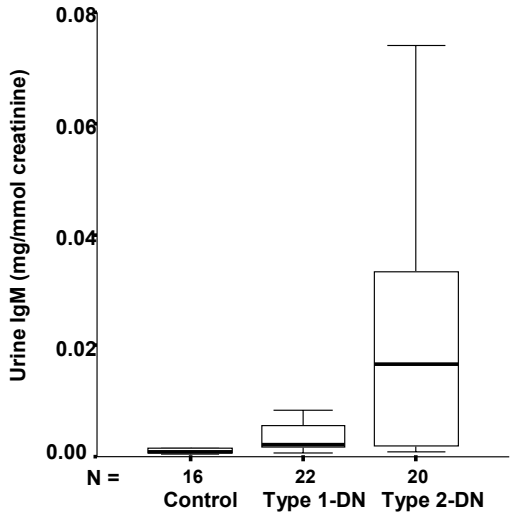
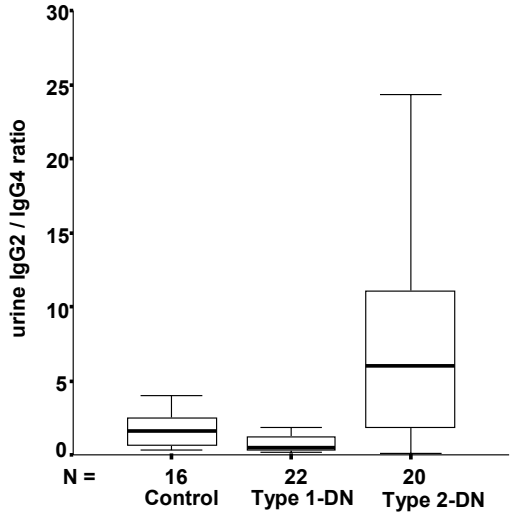


Figure 9. urine IgG2/IgG4 ratio in healthy individuals and patients with diabetic nephropathy due to diabetes mellitus type 1 and type 2.



Proteinuria is widely regarded as a hallmark of nephropathy in both type 1 and type 2 diabetes (128, 129). Until now, few studies have compared type 1 and type 2 DN (167).

Indeed, urinary IgM excretion as marker of the size-selective injury in type 2 DN has, to our knowledge, not been studied before at all. In study IV, the patients with type 1 DN and type 2 DN did not differ with regard to the degree of albuminuria, serum creatinine or urine protein HC concentration. However, compared to patients with type 1 DN, the type 2 DN patients showed an increased urine excretion of IgG, suggesting a more severe size-selective dysfunction in type 2 DN. The size-selective properties of the glomerular capillary wall were relatively intact in type 1 DN. The appearance of IgM in the urine in type 2 DN patients can be interpreted to reflect a markedly increased population of highly non-selective “shunt”-pathways (Fig.8). The urine IgG2/IgG4 ratio was high in type 2 DN and low in type 1 DN patients ($p<0.01$) (Fig.9). This suggests that the charge selectivity of the glomerular barrier in type 2 DN patients is preserved. Thus, while

an impairment of the charge selectivity of the GCW is probably the major cause of proteinuria in early type 1 diabetes, the proteinuria in type 2 DN is primarily due to a size-selective dysfunction. It is likely that the presence of hypertension and an increased vascular resistance in patients with type 2 DN may induce ischemia and structural changes in the glomeruli resulting in proteinuria mainly reflecting a size-selective dysfunction in type 2 DN (168, 169).

Study V

The major result from study V is that the glomerular sieving coefficient of native albumin (θ_{alb}) in SHR, was normal during the first 9 months of hypertension, but significantly increased in old animals, as compared to that in age matched NCR (Fig.10). The glomerular sieving coefficient of native albumin (θ_{alb}) in SHR increased from 5.0 (±0.5) x10^{-4} at 3 months, to 7.6(±0.8)x10^{-4} at 9 months, and to 12.9(±0.9)x10^{-4} at 14 months of age ($p<0.001$), while θ_{alb} did not change significantly with age in NCR, remaining at 7.0(±0.5)x10^{-4} at 3 and 9 months and at 7.2(±0.9)x10^{-4} at 14 months of age, respectively. Thus, in SHR, it takes more than half a lifetime of hypertension to develop proteinuria and kidney damage.

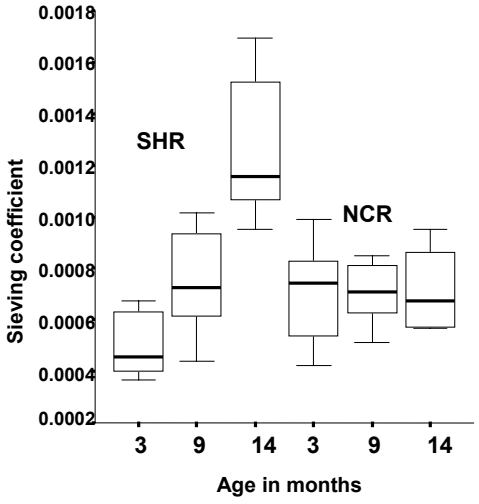


Figure 10. Sieving coefficient (θ_{alb}) of native albumin in animals studied (SHR and NCR) at 3, 9 and 14 months of age.

Furthermore, the glomerular disturbance developed during long-standing hypertension is of size-selective nature, and not represented by a primary charge-selective defect of the GCW. Thus, the ratio of neutral to negative albumin clearance was maintained high throughout the

life span of the hypertensive rats, and the increases θ_{alb} in old SHR was significantly correlated with increases in $\theta_{n\text{-}alb}$ ($r=0.86$, $p<0.001$). This may, according to pore theory, be explained by the creation of an increased number of rather unselective pores of intermediate radius (64.2 Å; *cf.* small and large pores). All the animals studied (NCR and SHR) thus showed a generally higher clearance of neutral albumin (7 fold) than of native (negatively charged) albumin, indicating a normally marked influence of charge on transglomerular protein transport.

The albumin sieving coefficient (0.0007) obtained by the present tissue renal uptake technique or earlier micropuncture studies (0.00062) (19) along with a recent report by Norden et al, (170) of the absence of a significant amount of albumin degradation products in normal urine, contradicts the recent reports that the glomerulus is normally highly leaky to albumin (23). Our data reconfirm the old concept of the GCW as a highly charge- and size-selective barrier to the passage of macromolecules of the size of albumin or larger.

CONCLUSIONS

- The findings of the present studies indicate that it is possible to predict the rate of progression in renal diseases in patients with non-diabetic glomerulopathies by the type of proteinuria rather than by the degree of albuminuria.
- High urinary IgM excretion correlates to a decreased GFR in primary glomerular diseases regardless of the degree of albuminuria. In parallel, low urinary IgM excretion indicates beneficial prognosis in these diseases.
- Since IgM can be predicted to pass the glomerular barrier entirely through large shunts or defects in the glomerular capillary wall, a decreasing urine content of IgM might be considered as a sign of recovery of the glomerular damage
- The difference in the proteinuria patterns in type 1 and type 2 diabetic nephropathy, in addition to the clinical and functional differences, suggests mutually different patho-physiological mechanisms of nephropathy in the two entities of diabetic renal disease.
- In old age, hypertensive rats develop proteinuria as a result of a dysfunction of the glomerular capillary wall, affecting primarily its size selectivity. This conceivably occurs by the appearance of an increased number of rather unselective pores in the glomerular filtration barrier in untreated, long-standing hypertension.

FUTURE PERSPECTIVES

An increased use of non-invasive diagnostic and prognostic approaches in glomerular diseases would be beneficial to the patients and also be cost-effective. The studies presented in this thesis illustrate the use of urinary proteins as markers of glomerular and tubular dysfunction and the prognosis of proteinuric renal diseases. The studies have raised several questions requiring further investigation. Identification, at early stages of glomerular disorders, of patients with poor renal prognosis suggests more intensive clinical follow-up of such patients and provides insights for the design of future therapeutic studies on e.g. urinary IgM excretion. An interesting issue would be to evaluate the effects of ACE-inhibitor treatment and/or immunosuppressive drug treatment of this group of patients. Further studies are needed to elucidate the association between urine IgM excretion and the urinary excretion of other biologically active proteins, such as TGF-β or complement factors, to further evaluate the mechanisms of glomerular disease and the progression to renal failure.

Finally, the permeability properties of the glomerular filter in healthy and diseased conditions might be further studied using proteins of different size and charge, e.g. albumin, IgG and IgM, in order to add further understanding to the function of the glomerular sieving process and the mechanisms of glomerular injury. Although, at present, this can only be made in animal models of glomerular diseases, this may help in improving the medical care of individuals with glomerular diseases.

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