STUDIES ON THE MECHANISMS OF PROTEINURIA IN KIDNEY DISEASES OF CHILDHOOD

by

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SUMMARY

The set of studies documented here were undertaken primarily to verify the mechanisms of proteinuria in children with kidney diseases. This objective was achieved by a number of observations which, both extended previous information on the pathogenesis of proteinuria and added some new findings on this subject. However, an unexpected and potentially more valuable result was that, in studying the pattern of excreted urinary proteins, a correspondence between the latter and the underlying renal histopathological changes and response to steroid therapy was detected. This finding is of relevance in clinical diagnosis and management of children with some renal diseases.

The degree of proteinuria varies in the different glomerulonephritides, being most severe in nephrotic syndrome (NS). For this reason, and because NS is a major kidney disease of childhood, these studies concentrated this disease syndrome. Nephrotic syndrome is characterised by the presence of proteinuria, oedema and hypoalbuminaemia. Renal biopsy, an invasive procedure, is the definitive investigation in the assessment of underlying kidney disorders including NS. A histological diagnosis assists the clinician with regard to appropriate therapy and prognosis. Non- invasive methods, such as clinical features, biochemical criteria and immunological tests, have been previously utilised to predict the underlying kidney disorders and therefore prognosis, with limited success.

As proteinuria is a significant marker of renal disease, attention has been focused on the methods of assessing the mechanism of protein loss and the role of the glomerular capillary as a filter. The glomerular capillary recognises protein molecules on the basis of charge and size, and therefore acts as a charge- size-selective barrier.

The main objective of this research programme was to document the relative contributions to proteinuria made by the loss of anionic charge and defects in the size-selective barrier of the glomerular capillary in the NS of childhood.

The anionic charge on the glomerular basement membrane (GBM) is due to presence of negatively charged heparan sulphate proteoglycan molecules which present a negative barrier to the surface.

A study (n = 33) was conducted to examine this charge-selective barrier of the GBM in a group of nephrotic children with different types of renal histological lesions, (e.g. minimal change nephrotic syndrome [MCNS], focal glomerulosclerosis [FGS], membranous nephropathy [MGN] and membranoproliferative glomerulonephritis [MPGN]) by using a cationic probe polyethyleneimine (PEI) which binds on to the negatively charged heparan sulphate molecules. These are the common histological groups seen in our setting. These studies revealed that there was a significant loss of anionic sites in the nephrotic group as a whole compared to controls ($p < 0.05$). There was also a significant decrease of anionic sites in each histological group (MCNS, FGS, MGN and MPGN) when compared to their controls. An inverse correlation between anionic site number and proteinuria (estimated by urinary protein creatinine ratio) was detected in MCNS ($r = -0.6$), but not in other groups.

Charge-selectivity was further examined by a non-invasive procedure: binding of the cationic dye alcian blue to red blood cells (ABRBC) in a group of children (n = 58) and adults (n = 29) with NS. ABRBC was significantly reduced in the nephrotic group as a whole compared to controls ($p < 0.001$). A similar reduction was observed in children and adults in each of the different histological groups (MCNS, MGN, MPGN and FGS) when compared with controls. A moderate correlation between the degree of proteinuria and ABRBC was detected in nephrotic children ($r = 0.43, p < 0.001$) but not in nephrotic adults. ABRBC was also found to be age-dependent ($p < 0.02$) (age range 16yrs - 44 yrs).

The charge-selective barrier was also studied indirectly by a third method. This involved examining the ionic charge on the protein molecules lost in the urine by isoelectric focusing (IEF). The charge on the protein molecules in MCNS was restricted to the anionic albumin band, whereas in other groups of NS, (FGS, MGN and MPGN), both cationic and anionic protein bands were seen.

The size-selectivity of the GBM was examined indirectly by assessing the size of the protein molecules lost in the urine. The method employed was sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

SDS PAGE was used in children with MCNS and FGS. These studies revealed that the steroid sensitive children (presumed MCNS) had protein bands which were restricted to albumin and transferrin (< 80 kDa), whereas, the steroid resistant children with FGS exhibited additional excretion of IgG and low molecular weight proteins (RBP, lysozyme). The SDS PAGE method was able to distinguish MCNS from FGS. These diseases pose an intractable management problem in paediatric nephrology. The former being steroid responsive is easier to manage and therefore has a better outcome; the latter is steroid resistant, is difficult to manage and has a poor outcome.

Comparison of the SDS PAGE technique with the conventional Selectivity Index (SI) for predicting steroid response in newly diagnosed children with nephrotic syndrome (n = 34) indicated that the SDS PAGE was a more efficient (100%) method than SI (79%).

In children with MCNS, SDS PAGE was more reliable than SI for predicting steroid response, whereas in steroid resistant cases such as FGS, both methods were equally suitable in predicting steroid sensitivity.

The SDS PAGE technique was then applied to the prediction of a wide range histological lesions of childhood kidney diseases.

The study revealed that proteinuria was restricted to albumin and/or transferrin in MCNS and Poststreptococcal glomerulonephritis (PSGN), but was more extensive with additional excretion of high molecular weight glomerular proteins such as haptoglobulin and IgG in HbsAg positive membranous nephropathy (MGN) and FGS.

Direct (PEI) and indirect (ABRBC, isoelectric focusing [IEF]) methods of measuring fixed anionic sites were assessed in a group of nephrotic children with varying histological lesions (MCNS, FGS, MGN, MPGN). These results revealed a correlation between these two types of tests; the correlation between

PEI and ABRBC was strongest in MCNS, moderate in FGS and absent in other histological groups (MGN, MPGN).

In conclusion this study amplifies the mechanism by which the loss of protein may occur in various kidney diseases of childhood. The results lend further support to the hypothesis that MCNS may be the only histological group where pertubations of negative charge of the GBM may be the main cause for proteinuria. It appears to be a disease in which there is a generalised disorder of membrane negative charge affecting other cellular membranes, including the red blood cell membrane. In contrast to MCNS, nephritides (FGS, MGN, MPGN), where structural renal damage is obvious, the size-selective barrier of the glomerular polyanion, appears to play a greater role in the manifestation of proteinuria. The lack of correlation between direct and indirect mechanisms of charge selectivity in groups other than MCNS suggest that electrostatic defects may be mainly confined to the kidney in these diseases. However, the matter does not appear to be so clear cut, both processes may operate to differing degrees in individual diseases.

The SDS PAGE technique appears to be a valuable tool in the management of renal diseases of childhood. It can narrow the indication for renal biopsy (which is in accordance with developments in this field over the past two decades) but obviously cannot replace microscopic examination of kidney tissue.

PREFACE

The major part of the work reported in this thesis was performed in the Department of Paediatrics and Child Health, University of Natal, Durban, under the supervision of Professor M Adhikari and Professor Y K Seedat, Isoelectric focusing was performed in the Department of Neurosurgery Wentworth Hospital under the supervision of Professor Bighjee.

These studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

One study reported in this thesis has been published in a scientific journal, whilst others are submitted to respective journals for peer review. Research workers who are closely associated in these studies are co-authors in these publications.

PUBLICATION

One paper is in press whilst others listed below are with their respective journals for peer review.

1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of urinary proteins in steroid - responsive and steroid - resistant nephrotic syndrome in children. In Press. Paediatr Nephrol 1994.

2. SDS PAGE of urinary proteins predicts histology and prognosis of glomerular diseases. Submitted to Paediatr Nephrol 1994.

3. Depletion of glomerular anionic sites and proteinuria in nephrotic syndrome of children. Submitted to Am J Pathol 1994.

4. Direct and indirect tests of pore-size and charge-selectivity in nephrotic syndrome. Submitted to J Lab Clin Med 1994.

5. Anionic charge abnormalities of red blood cells and proteinuria in glomerulonephritides. Submitted to Paediatr Nephrol 1994.

PRESENTATIONS AT CONGRESS/ SCIENTIFIC MEETINGS

1. Ramjee G, Coovadia HM, Adhikari M. Charge-selection of the glomerular basement membrane. Paediatric Congress. Wild Coast, Natal. June 1992.

2. Ramjee G, Coovadia HM, Adhikari M. SDS PAGE of urinary proteins differentiates MCNS from FGS, predicts steroid responsiveness. Faculty Research Day September 1993.

3. Ramjee G, Coovadia HM, Adhikari M. SDS PAGE of urinary proteins: A valuable ancillary test in predicting histology, steroid responsiveness and prognosis in renal diseases of chidhood. Faculty Research Day September 1993. **PRIZE FOR BEST PAPER.**

4. Ramjee G, Adhikari M, Coovadia HM. SDS PAGE of urinary Proteins: A valuable ancillary test in Renal disease of childhood. Paediatric Congress. Eastern Transvaal, 14 - 18 August 1994.

5. Ramjee G, Coovadia HM, Adhikari M. Direct and indirect tests of pore-size and charge selectivity in nephrotic syndrome. Paediatric Congress. Eastern Transvaal, 14 - 18 August 1994.

6. Ramjee G, Coovadia HM, Adhikari M. SDS PAGE of urinary proteins differentiates MCNS from FGS. Paediatric Congress. Eastern Transvaal, 14 -18 August 1994.

7. Ramjee G, Coovadia HM, Adhikari M. Direct and indirect tests of pore-size and charge selectivity in nephrotic syndrome. Faculty Research Day, 7 September 1994, University of Natal.

8. Ramjee G, Coovadia HM, Adhikari M. Comparison of SDS PAGE of urinary proteins with the conventional selectivity Index (SI) for predicting steroid response in nephrotic syndrome. Faculty Research Day, University of Natal, 7 September 1994. **PRIZE FOR BEST PAPER IN A LABORATORY BASED STUDY.**

9. Ramjee G, Adhikari M, Coovadia HM. SDS PAGE of urinary proteins: A valuable ancillary test in Renal disease of childhood. Renal Congress. Pretoria, September 1994.

10. Ramjee G, Coovadia HM, Adhikari M. Direct and indirect tests of pore-size and charge selectivity in nephrotic syndrome. Renal Congress. Pretoria 11 - 15 September 1994.

11. Ramjee G, Coovadia HM, Adhikari M. Comparison of SDS PAGE of urinary proteins with the conventional selectivity Index (SI) for predicting steroid response in nephrotic syndrome. Renal Congress. Pretoria, 11-15 September 1994.

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LIST OF ABBREVIATIONS

NS	-	nephrotic syndrome
MCNS	-	Minimal change nephrotic syndrome
FGS	-	Focal glomerulosclerosis
MGN	-	Membranous nephropathy
MPGN	-	Mesangiocapillary glomerulonephritis
ALB	-	Albumin
Trans	-	Transferrin
HAP	-	Haptoglobulin
IgG	-	Immunoglobulin G
RBP	-	Retinol binding protein
$ß_2M$	-	$ß_2$Microglobulin
Lyso	-	Lysozyme
GFR	-	Glomerular filtration rate
MW	-	Molecular weight
kDa	-	Kilo daltons
GCW	-	Glomerular capillary wall
GBM	-	Glomerular basement membrane
LRI	-	Lamina rara interna
LRE	-	Lamina rara externa
LD	-	Lamina densa
PEI	-	Polyethyleneimine
AB	-	Alcian blue
ABRBC	-	Alcian blue binding to red blood cells
SDS PAGE	-	Sodium dodecyl sulphate polyacrylaminde gel electrophoresis
IEF	-	Isoelectric focusing

DEFINITIONS

1. **NEPHROTIC SYNDROME (NS):** is defined as heavy proteinuria (40mg/hr/m2) oedema and hypoalbuminaemia.

2. **STEROID RESPONSIVE NEPHROTIC SYNDROME (SRNS):** Patients who respond to conventional doses and duration of steroid therapy (Prednisone 2mg/kg/day for one month followed by decrease in doses over the next two months). Response was defined as clearing of proteinuria for at least one week. The clinical course was characterised by remissions and relapses with the latter responding to a course of steroids.

3. **STEROID RESISTANT NEPHROTIC SYNDROME:** Persistance of features of NS despite single or multiple doses of steroids as given in (2).

4. **RELAPSE:** Presence of the three diagnostic features of the NS

5. **REMISSION:** Absence of all criteria of NS while not on any specific drug therapy.

6. **FREQUENT RELAPSERS:** Patients who experience 3 or more relapses in 1 year or 2 episodes in 6 months.

7. **HISTOLOGICAL CLASSIFICATION:** Renal biopsy (light, electron and immunofluorescence) were classified according to Heptinstall 1992.

8. **NORMAL RENAL FUNCTION:** Normal serum creatinine, urea and glomerular filtration rate.

9. **GLOMERULAR PROTEINURIA:** Proteins of molecular mass of 60 kilodaltons (kDa) or greater. These are albumin (66 kDa), haptoglobulin (86 kDa), IgG (160 kDa) and transferrin (80 kDa).

10. **TUBULAR PROTEINURIA:** Proteins of molecular mass less than 60kDa. These include $ß_2$ Microglobulin ($ß_2M$) (11.3 kDa) and lysozyme (14 kDa).

PURPOSE

Precutaneous renal biopsy is a well established but an invasive technique, and remains as the definitive investigation in kidney diseases. Research in the past decade has been focussed on the development of alternative non-invasive methods which can narrow the indication for renal biopsy. Proteinuria is a significant marker of parenchymal renal disease. In this study we set out to develop non-invasive methods to study the mechanisms of glomerular injury in a spectrum of kidney disease of childhood. Our purpose was to correlate the findings of non-invasive tests to clinical and histological findings in these children and to determine if underlying renal parenchymal injury could be predicted.

OBJECTIVES OF THE STUDY

1. To assess the charge- selectivity of the glomerular basement membrane by use of cationic probe polyethyleneimine (PEI) in renal biopsy tissue.

2. Develop non-invasive techniques of measuring surface negative charge using alcian blue binding of anionic sites on red blood cell membranes.

3. Correlate the invasive and non-invasive techniques with proteinuria (assessed by urinary protein/creatinine [Up/Ucr] ratio) in glomerulonephritides.

4. Establish non-invasive method of measuring protein charge - Isoelectric focussing (IEF).

5. To establish a relationship between specific urinary proteins and various pathological states of the kidney to assess size-selectivity of the glomerular basement membrane.

6. Establish the relative charge- size- selective abnormalities of the glomerular basement membrane in a wide range of kidney diseases of childhood.

CHAPTER 1

INTRODUCTION

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1.1 BACKGROUND

Proteinuria as a marker of kidney disease was first discovered in the late 18th century by Richard Bright who correlated serum and urine abnormalities of oedematous patients, with autopsy observations of the kidney. After this period many attempts were made to categorise the pathologic features of Bright's findings. However, conflicting reports by many workers did not improve the understanding of pathological features of glomerular lesions (Widal et al 1912, Volhard and Fahr 1914).

Better clarification of Bright's disease came about with the findings of Longscope (1936) and Ellis (1942) who suggested that there were two distinct types of disease. An acute stage (type 1) in which patients had haematuria, proteinuria, some oedema and transient hypertension at onset and in which the symptons were preceded by a throat infection in the majority of patients. The general course of the disease was towards recovery. In the type II, the patients had severe persistent proteinuria and oedema and in whom the clinical course was generally fatal. It is clear today that the latter form was describing the Nephrotic Syndrome (NS) a term used to describe clinical states of multiple origin and varying histology. It represents a stage in a variety of disorders in which increased permeability of the glomerular basement membrane causes heavy proteinuria. The loss of proteins results in hypoproteinaemia with excessive salt and water retention resulting in oedema.

1.2 PROTEINURIA

Proteinuria as mentioned above is an important finding in the evaluation of patients with primary renal disease, as well as those with systemic disorders that affect the kidney, including hypertension, vascular disease and diabetes mellitus (Moore and Carome 1993).

The mechanism of protein loss can be understood once the structure and function of the kidney are known.

1.2.1 The Nephron (Figure 1)

Each kidney normally contains over 1 million nephrons, each of which is composed of a glomerulus and a tubule. The first barrier for fluid removal from the capillary lumen is composed of the endothelial cells which contain large holes in their cytoplasm known as fenestra.

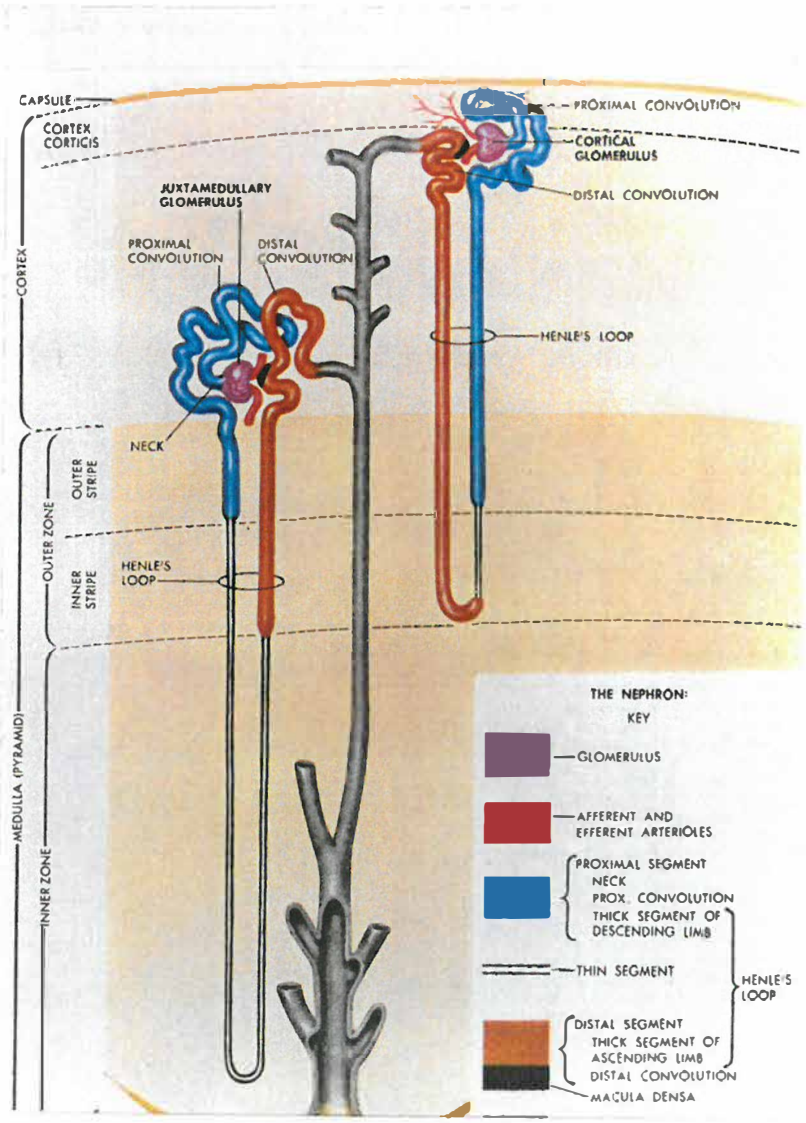

FIGURE 1 : THE NEPHRON From F Netter 1979

The middle layer is referred to as the basement membrane. This layer, made up of central lamina densa (LD) and a lamina rara (LR) on each side, restricts the movements of all blood cells and most proteins from the blood stream. The final layer before entering the tubular lamina is the epithelial cells which have foot processes. The spaces between the foot processes (FP) (slit pores) are lined with specialised proteins, the cell coat, and are connected at the filteration slits by thin slit diaphragm which further retards the movement of plasma proteins (molecular weight > 15,000 daltons (kDa) into the urinary space (Figure 2).

FIGURE 2 : The Glomerular Polyanion

The three layers thus make up the glomerular polyanion, a structure responsible for the passage of macromolecules such as proteins across the glomerular wall. The capillary structure is supported by specialised network of cells (mesangium) that form the structural stalk of the glomerular pole. The urine formed is an ultrafilterate of plasma, containing roughly 10 mg/L of albumin compared with 40g/L in plasma (Preuss 1993).

1.2.2 The Glomerulus as a Filter

1n 1843 Ludwig proposed that the glomerulus was a filtering mechanism (Morgan 1979). This idea was confirmed by the development of micropuncture techniques by Richards, who was able to reinforce Ludwig's proposal using amphibians (Morgan 1979).

The glomerular capillary wall is permeable to water and small solutes , however, it poses an extremely efficient barrier to the passage of plasma proteins (Brenner et al 1978). Infusion of inulin in rats indicated that inulin, and substances smaller than inulin, appear in the glomerular filtrate in the same concentration as plasma water but serum albumin was filtered to a lesser extent (Galaske et al 1979). In vivo studies in animals and man have contributed to the understanding of the sieving properties of the glomerular wall (Myers et al 1982, Kaysen et al 1986).

1.3 THE MECHANISM OF PROTEINURIA

The mechanism, whereby the normal glomerular capillary wall (GCW) restricts the passage of large plasma proteins while offering little resistance to the filtration of water and small solutes, has been explored extensively in recent years (Kaysen et al 1986, Cameron 1990, Myers 1994). It is now well established that the glomerular filter recognises molecules to be filtered on the bases of size and charge (Brenner et al 1978, Myers and Guash 1994). Glomerular permselectivity has been extensively studied in rabbits and rats by fractional clearance techniques using non-protein polymers such as dextran and polyvinylpyrrolidone (PVP) as neither are absorbed nor secreted by the tubules (Brenner et al 1978, Chang et al 1975).

The relative restriction of trans glomerular albumin was due to presence of negative charge on the glomerular polyanion was first suggested by Chang et al (1975) who in his experiments with an anionic molecule dextran sulphate showed that the passage of this molecule across the glomerulus was reduced relative to that of uncharged dextrans of equivalent size. These findings were consolidated by Rennke et al (1977) who employed a

morphologic approach which utilised the electron dense protein ferritin as a tracer. They found that native ferritin was completely restricted from entry into the glomerular filter at the level of the endothelial cell. By contrast, cationised derivatives of ferritin permeated through the glomerular capillary wall in direct proportion to the isoelectric point of each tracer. Moreover, the uptake of cationic stains by all three layers of the glomerular capillary attested to the presence of fixed negative charges throughout the structure (Blau and Haas 1973, Latta and Johnson 1976).

1.3.1 Size selectivity of the glomerular capillary wall

Size selective properties of the GCW have been demonstrated by using uncharged polydisperse preparations of dextran and PVP (Kaysen et al 1986). It has been shown that in many mammalian species such as rats, dogs and rabbits, excretion of dextran molecules was restricted with increasing molecular size (Hardwick et al 1968 Galaske et al 1979, Christensen et al 1981).

Recent studies hypothesised that the GCW is represented by two heteroporous membrane models (Deen et al 1985); an isoporous plus shunt model, which assumes that the GCW is perforated by cylindrical restrictive pores of uniform radius, and another model, of a set of larger pores which are non-discriminatory to molecules up to 6 nm. It has been suggested that both the models serve as parallel shunt pathways. A third model suggest that the membrane is perforated by cylindrical pores with uniform radii. Former models are thought to be more useful for assessing size selectivity in nephrotic humans more accurately whereas the latter model is useful in assessing sieving curves in non-nephrotic humans (Myers et al 1994). All the methods mentioned require infusion of graded series of dextran and PVP.

Alternate methods of measuring the size selectivity of the GCW is by characterisation of urinary proteins by ultracentrifugation (Lewis & Page 1953) or gel filteration on sephadex (Laurent & Killander 1964). However the methods were too time consuming for routine laboratory use.

Cameron and Blandford (1966) and Adeniyi et al (1970) used immunological approach to separate individual proteins. They studied differential clearance of various types of plasma proteins in patients with NS in an attempt to obtain correlations between urinary protein loss and underlying histological lesion, and also to predict the prognosis of the disease and its likely response to treatment. Although more easily applicable, these methods have the disadvantages of measuring only a limited number of proteins, being time consuming and do not give a full picture of glomerular and tubular involvement.

Recent studies have attempted to separate proteins by electrophoresis which has proved to be more successful (Shapiro & Maizel 1967, Jackson et al 1988, Brockelbank et al 1991).

Selectivity of proteinuria in NS

Joachim et al (1964) studied the selectivity patterns of protein extraction and compared the results with response to steroid therapy and renal pathology in patients with NS. They showed that patients with primary renal disease and nephrotic proteinuria displayed a high selectivity of proteinuria whereas those with advanced histological changes in the glomerulus showed least selectivity of proteinuria. Moreover those with selective proteinuria responded to steroid therapy. These results were confirmed by other studies (Cameron and Blandford 1966, Adeniyi et al 1970).

1.3.2 Charge selectivity

Numerous studies using experimental animals have shown that the normal GCW is also a charge selective filter (Bertolatus and Hunsicker 1985, Rennke and Venkatachalam 1979, Chang et al 1975).

Histochemical studies of kidney from rats with proteinuria induced by aminonucleoside of puromycin revealed a partial loss of negative charge on the surface of glomerular epithelial cells (Kanwar and Jabowski 1984). These negatively charged sites were shown to consist of sialic acid- rich

glycoprotein coat (Chang 1975, Farquar and Kanwar 1980). Cytochemical and biochemical studies have demonstrated that the charge barrier (glomerular polyanion) within the rat GCW consists of heparan sulphate as a major glycosoaminoglycan, a high molecular weight, negatively charged, polysaccharide side chain of proteoglycan with large molecular domain. Enzymatic digestion of heparan sulphate increased permeability of the GCW to ferritin and 131 I albumin (Kanwar et al 1980, Rosenweig and Kanwar 1982).

The functional basis of the glomerular polyanion to the glomerular filtration of macromolecules has been established in rats by the relative reduced glomerular clearance of negatively charged macromolecules, and increased clearance of neutral or positively charged macromolecules of the same size (Rennke et al 1979, Bohr et al 1978, Deen et al 1978, Carrie et al 1981).

The glomerular polyanion can be detected histochemically by colloidal iron, horse radish peroxidase and alcian blue (Rennke et al 1969, Mohos and Skoza 1969). These studies reported that the layers close to the capillary lumen i.e. the endothelium and the innermost layer of the GBM (lamina rara interna) are likely to provide the primary functional barrier to polycations (Latta and Johnson 1975). Vernier et al (1983) quantitatively demonstrated negatively charged sites within the LRE of normal human GBM by a new cytochemical technique using polyethyleneimine (PEI) a cationic probe. The technique is now widely used to demonstrate loss of electrostatic charge barrier in a variety of diseases (Okada et al 1986, Kitano et al 1993).

1.4 CLASSIFICATION OF PROTEINURIAS

Clinically important proteinuria is usually caused by glomerular disease however, there are certain conditions where proteinuria occurs in the absence of renal disease, such as exercise, standing, high altitude and febrile illness (Reuben et al 1982).

Proteinuria of pathological origin may result from the following abnormalities:

1.4.1 Proteinuria due to disease of the lower urinary tract

A small degree of proteinuria is commonly found in diseases involving the lower urinary tract, especially acute and chronic inflammation such as that caused by bacterial infections.

1.4.2 Proteinuria due to tubular damage

A variety of disorders involving damage to the renal tubule, result in increased protein excretion. These include acute tubular necrosis, toxic nephropathies and potassium depletion. Butler and Flynn (1958) reported that renal tubular disorders were associated with a particular type of proteinuria. They demonstrated that proteinuria in tubular disorders consisted of predominantly low molecular weight (LMW) proteins. These LMW proteins are proteins which pass relatively freely through the molecular sieve and are generally considered to have MW below 40 kDa. Under normal circumstances LMW proteins are reabsorbed within the proximal tubule by endocytosis and are subsequently catabolised within the lysosomes of the proximal tubular cells to their constituent amino acids (Tomilson 1992). Tubular reabsorption of LMW has been estimated to be about 99% complete in health. In disease states involving the proximal tubule, there is reduced reabsorption of LMW proteins and consequently an increased spillage of these proteins in the urine is detected. Increase in urinary concentration of LMW proteins is the basis for their use in diagnosis of renal tubular diseases (Tomilson 1992). The common marker proteins for tubular damage are α^1 Microglobulin (AIM), Retinol binding protein (RBP), lysozyme and β_2 Microglobulin (β_2M).

1.4.3 Proteinuria caused by increased glomerular permeability

The commonest form of proteinuria results from functional or structural alterations of the glomerular filtration barrier which lead to increased permeability of plasma proteins and their subsequent loss in the urine.

Differentiation of proteinuria by Peterson et al (1969) revealed that proteinuria in general glomerular disorder consists primarily of albumin. It has now been well documented that urinary proteins of glomerular origin are of molecular mass 60kDa or greater. These are largely albumin, transferrin, haptoglobulin and IgG (Balant et al 1974).

1.4.4 Immune Complexes

Antigen-antibody (Ag-Ab) reactions can take place locally in the glomerulus and result in the formation of Ag-Ab immune complexes (Wilson and Dixon 1971). Factors affecting glomerular permeability facilitate formation of these complexes which when localised cause structural damage to the GBM. This chain of events allows further deposition of more immune complexes. Some studies suggest that non-immune, cationic proteins bind on to anionic sites of the GBM which then localise antigens on the basis of charge, to produce immune complexes (Border et al 1982). Such cationic proteins may include neutrophil cationic protein, cationic products of complement activation and platelet activating factor (Couser et al 1978, Gallo 1981, Gautheir et al 1982, Couser et al 1985).

1.4.5 Complement

The role of complement in glomerular injury is an indirect one which links antibody deposition, via chemotactic and immune adherence mechanisms, to neutrophils which release proteolytic enzymes causing renal damage (Cochrane 1969).

1.4.6 Coagulation factors

Coagulation factors such as fibrin, factor VIII, platelets and polymorphs, are frequent features of nephritides and may have a role as indirect mediators of glomerular injury (Vassalli and Mc Cluskey 1964, Hoyer et al 1972, Peters and William 1975, Peters and Lachman 1979).

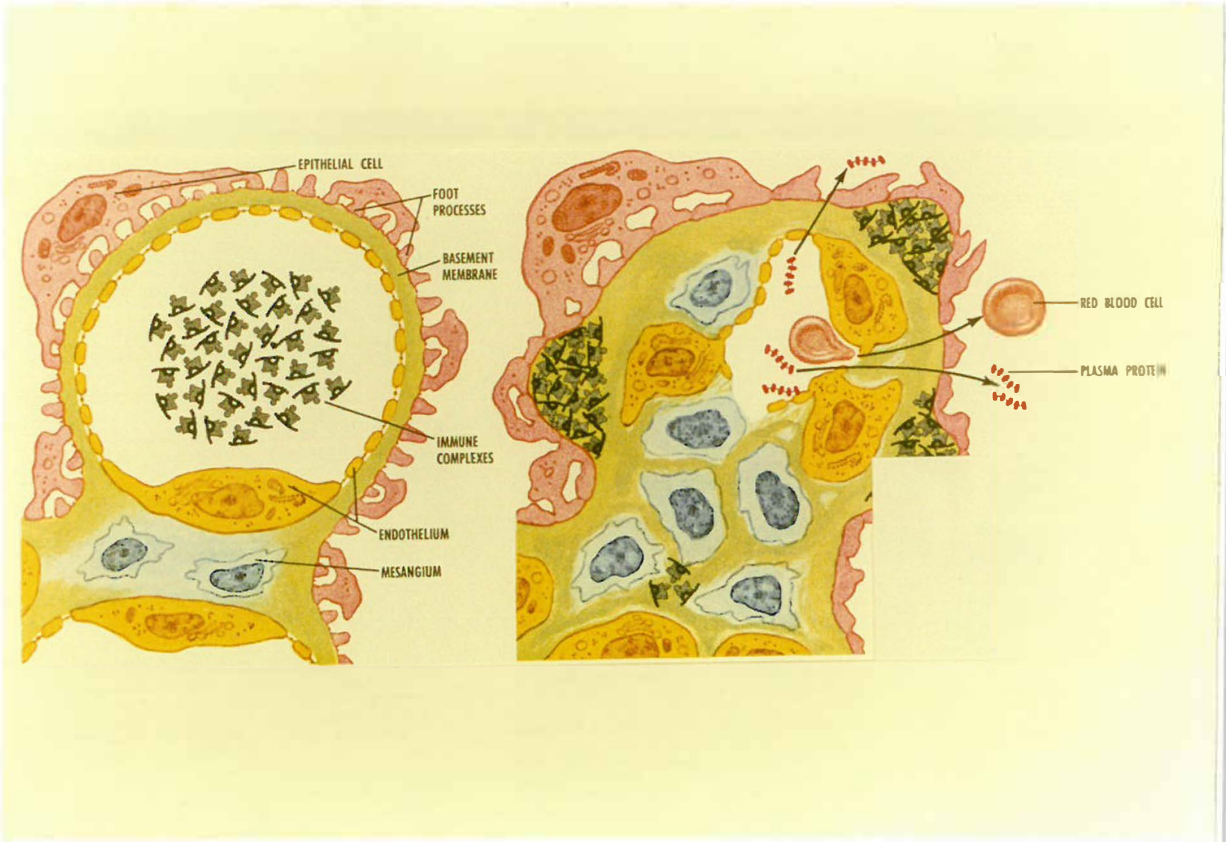

FIGURE 3 : A B

A : Immune complexes formed in the body deposited in the glomerular capillary

B : Complexes form deposits; increased porosity of capillary walls permits escape of plasma proteins and blood cells, causing proteinuria and haematuria

Netter F M - 1974

1.5 ROLE OF RENAL BIOPSY IN THE ASSESSMENT OF PROTEINURIA

Renal biopsies are performed in patients with kidney diseases when less invasive procedures such as urine analysis and radiological studies are not able to establish the correct diagnosis and prognosis. Iversen and Brun (1951) were the first to use them systematically in kidney diseases. Over the years the availabilty of fresh renal tissue and development of 3 successive histological techniques led to more precise diagnosis of renal glomerular disease. First, improved histological technique and application of electron microscopy (EM) revealed a number of previously unrecognised structural features. Secondly, the use of immunofluorescence microscopy (IF) introduced in the late 1960's, made possible the identification of immunoglobulins and complement deposition in various patterns within the renal tissue. The importance of this latter technique has been such that the post- renal biopsy period can be divided into two, before IF and the other after. Third, there was a possibility of examining graft biopsies in transplant patients with or without glomerular disease (Habib 1993).
The greatest value of renal biopsy has been in the NS (Muhorcke and Pirani 1975) as it is the only way of making exact morphological evaluation of renal tissue.

Renal biopsy is now a safe and essential part of the management of patients with renal diseases. The technique is invaluable in the diagnosis and has contributed to the understanding of the causes, pathogenesis, natural history and forms of treatment of many kidney diseases. Bleeding is the major complication of percutaneous renal biopsy, however, the amount of blood lost is relatively small (Dodge et al 1962).
A number of classifications have developed since renal biopsies became standard practice. Each pathological abnormality underlying the NS represents different glomerular responses to glomerular injury. Their causes may be known or unknown, but each represents a unique clinicopathological entity defined by it's morphology, characteristic

presentation and natural history (Moore and Carome 1993).

Heptinstall (1966) reviewed the etiology and the pathological changes of the nephrotic syndrome. Other reports by Churg et al (1970) and by ISKDC (Abramowicz et al 1970) examined the relative frequencies of the histological groups and the clinical course.

Each disorder of the NS is now classified under different headings; primary or idiopathic NS and secondary NS (Heptinstall 1992).

1.6 CATEGORIES OF NEPHROTIC SYNDROME

A brief description of the categories of NS encountered in this study will be discussed under the following specific headings of glomerular diseases.

1.6.1 Minimal Change NS (MCNS)

MCNS is the major cause of nephrotic syndrome in children accounting for 85% to 90% of cases under the age of 6 and more than 50% in older children (Churg et al 1970, White et al 1970, ISKDC 1981). Clinically MCNS is a disease with excellent prognosis and response to therapy (ISKDC 1981). The clinical course is characterised by relapses and remissions. Histologically it is a disease with minimal histological lesions with near normal appearance of glomeruli on LM. On EM the most evident feature being fusion of foot processes (Heptinstall 1992). The pathogenesis of MCNS is not clear although it has been suggested that a defect in the function of thymus derived lymphocytes (T cells) may be responsible (Moorthy et al 1976). Children presumed to have MCNS are rarely biopsied as diagnosis is based on clinical assessment and response to steroid therapy given for a period of up to 8 weeks (Blainey et al 1960). Response is assessed by clearing of proteinuria for at least one week during the treatment.

1.6.2 Focal Glomerulosclerosis (FGS)

FGS is a disease characterised by histological changes which are present in some glomeruli but not all. IF microscopy may reveal IgM and C3 mostly within the sclerotic areas (Heptinstall 1992). In addition tubular defects have been reported to be an early sign in patients presenting with FGS (McVicar et al 1980). Clinically patients present with signs of NS, however, some may present with proteinuria in the non-nephrotic range (Jenis et al 1974). In addition, haematuria is usually present. Hypertension and eleveted blood urea develop with time. Most patients do not respond to steroid therapy and the ISKDC has recommended renal biopsy in such patients. Prognosis in patients with FGS is poor with a few progressing to end stage renal disease (Cameron et al 1973, Tejani et al 1985).

1.6.3 MCNS vs FGS

The course of patients with MCNS who initially respond to steroid therapy is varied (ISKDC 1981). A number of children with MCNS may have multiple relapses at frequent intervals, requiring multiple courses of steroid therapy with it's attendent morbidity or infections, osteopenia, decreased growth, hypertension and cataracts (Avant et al 1982, Travis et al 1979). Serial follow up studies of relapsing patients show that a few initially steroid responsive patients later develop steroid resistance (Tejani 1985, Mcgovern 1964, Report from the Southwest Paediatric Association 1985). Renal biopsy in these steroid resistant case has been recommended, (ISKDC 1970) however, quite often sampling of renal tissue may give erroneous results. Renal tissue has to be obtained from the juxta-medullary region of the nephron in order to detect early signs of FGS. There are some who believe that a proportion of patients who were steroid responsive have FGS at onset and due to the progression of the disease become steroid resistant.

1.6.4 Membranous Nephropathy (MGN)

Membranous nephropathy is a common histological lesion dominated by the type associated with Hepatits B surface antigen (HbsAg) in parts of Africa and it is the dominant type of NS in Durban (Coovadia et al 1993). Electron microscopy reveals subepithelial deposits. Granular deposits in the capillary loop of IgG, C3, IgA and IgM on IF are diagnostic of MGN (Heptinstall 1992).

The major presenting features are oedema and nephrotic range proteinuria in 85% of patients with the remainder having non-nephrotic proteinuria (Row et al 1975, Davison et al 1984). The clinical course is variable. Complete recovery of the patient occures within weeks to 1 year when the offending agent is cleared. However, the clinical course is frequently not benign in idiopathic MN (Kleinknecht and Habib 1987). In children, approximately 50% have a spontaneous remission which is often permanent, 40% may have persistent proteinuria and 10% may progress to end stage renal failure (Habib and Kleinknecht 1971, Habib et al 1973).

1.6.5 Mesangiocapillary Glomerulonephritis (MPGN)

MPGN is an infrequent cause of glomerular disease in children (Cameron 1968, Churg et al 1970, Hayslett 1973). EM indicates marked increase in mesangial cells and matrix. LM reveals a characteristic double contour of the glomerular basement membrane (GBM). IF shows that the glomeruli typically contain granular deposits of C_3 in a peripheral lobular distribution. Some cases contain deposits of IgG, IgM, IgA, C_4 (Heptinstall 1992). The presenting features are NS, or asymtomatic proteinuria, or gross haematuria, or acute nephritic syndrome (Cameron 1970, 1973). The course is not affected by steroid therapy or immunosuppressive drugs (Kincaid-Smith 1972). The prognosis for MPGN is poor, with a slow progressive downhill course to renal failure, with recurrent episodes of gross hematuria, oedema and NS (Jenis and Lowenthal 1978).

1.6.6 Poststreptococcal Glomerulonephritis (PSGN)

PSGN is predominantly a disease of the children (Rodriguez-Iturbe 1984). The incidence of PSGN has declined in developed countries, although streptococcal infection remains the major cause (94%) in South Africa (Coovadia 1987). The characteristic clinical pattern is preceded by streptococcal pharyngitis, oedema, dark urine, moderate proteinuria and moderate hypertension (Jenis and Lowenthal 1978). Clinical course is short resulting in rapid recovery. Patients with PSGN are rarely biopsied, however, in the early 1970's widespread use of renal biopsy indicated the typical lesions to be hypercellularity, presence of polymorphonuclear leucocytes, monocytes and eosinophils within the capillary lumen (Heptinstall 1992).

1.6.7 Secondary Glomerular disease

These are diseases in which renal involvement is secondary to a primary systemic or a collagen vascular disease. Diseases include systemic lupus erythematosus (SLE) and amyloidosis.

1.7 Nephrotic Syndrome in South Africa

In South Africa there is an unusual distribution of histopathological types of NS (Adhikari et al 1976, Lewin et al 1979, Adhikari 1981, Coovadia 1987). In some communities the prevalence of membranous nephropathy associated with hepatitis B surface antigen is fairly high (Coovadia et al 1979, Wiggelinkhuizen et al 1987, Cameron 1990). Nephrotic syndrome behaves differently in African compared to most non-African children. Majority of African children have obvious structural lesions which are associated with unresponsiveness to steroid or immunosuppressive therapy while most Indians have MCNS which is steroid responsive. The prognosis appears to be less favourable with fewer remission rates in African children. Genetic predisposition, host factors and environmental influences, either singly or in combination, may influence the development

of obvious glomerular lesions (Adhikari 1981).

The constellation of pathophysiological changes leading to the development of NS result primarily from glomerular injury and attendent proteinuria.

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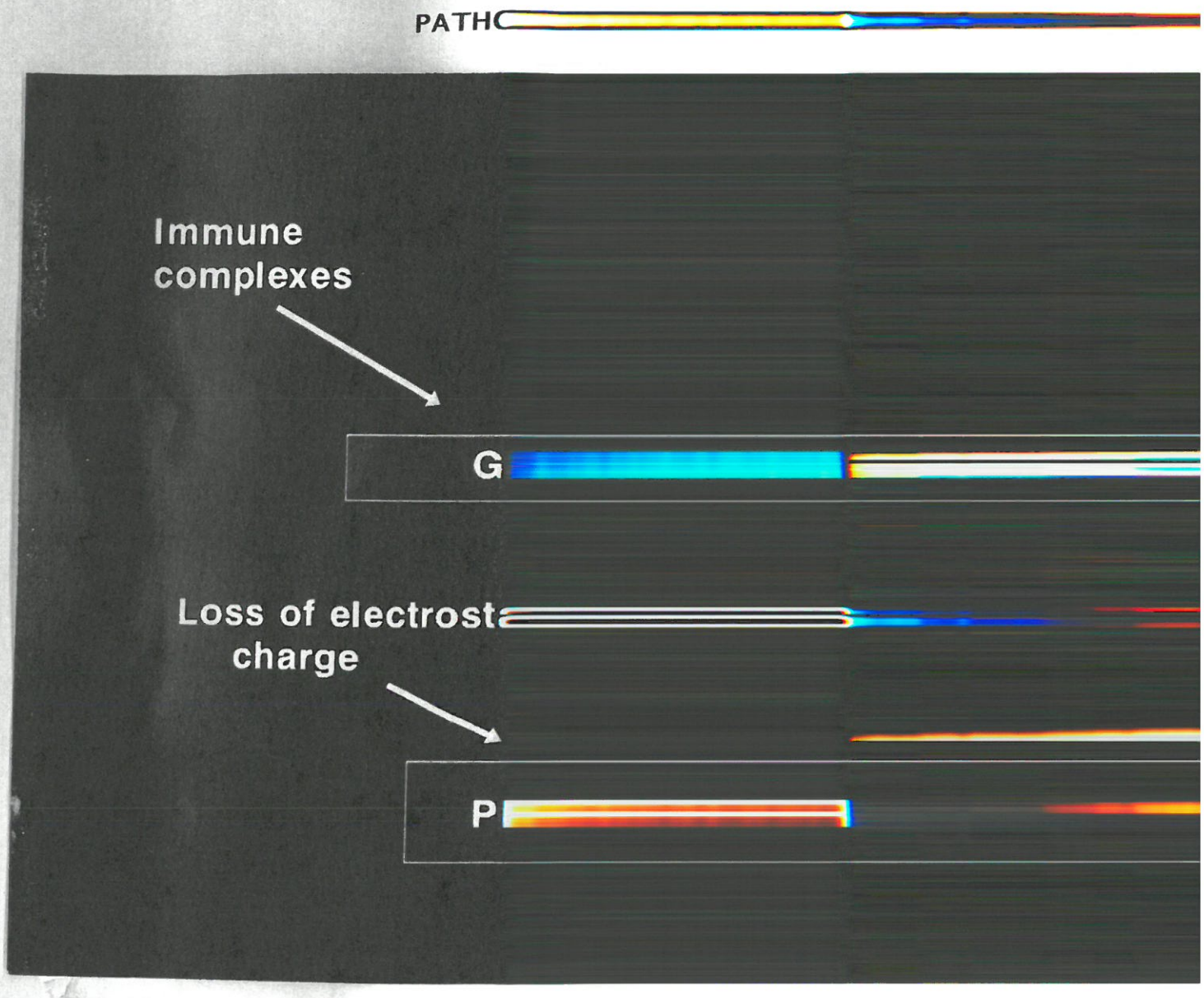
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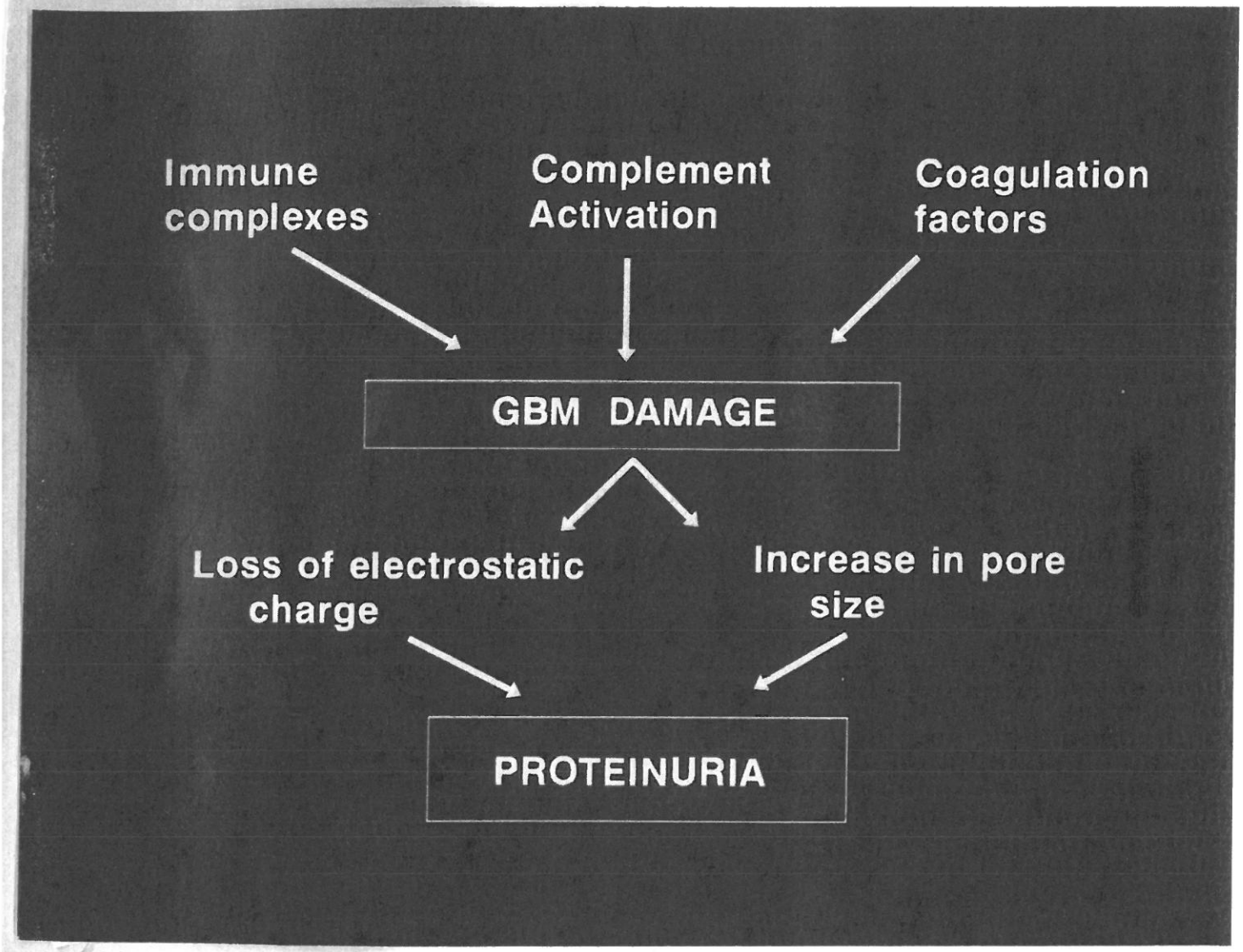
APPENDIX I

PATHOGENESIS OF PROTEINURIA



APPENDIX II

HISTOLOGICAL CLASSIFICATION OF GLOMERULONEPHRTIDES

Histological Groups	MCNS	FGS	MGN (HbsAg)	MPGN	PSGN
Pathogenesis	Immune	Immune	Immune complex	Complement activation	Streptococcal infection
Proteinuria	+	+	+	+	±
Haematuria	-	±	+	+	±
Serum Complement	N	N	N	decreased	decreased
Immune Complex	-	+	+	+	+
Response to Steroid Therapy	+	poor	-	-	-
Hypertension	-	+	+	±	±
Prognosis	excellent	poor	good	poor	excellent

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CHAPTER 2

METHODS

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METHODS

2.1 POLYETHYLENEIMINE BINDING TO ANIONIC SITES OF GBM

2.1.1 Background

Evidence in the past decade suggests that the glomerular capillaries act as both size and charge barrier to macromolecules. Heparan sulphate anionic sites are distributed in an orderly row in specific sites primarily within the lamina rara externa (LRE) and lamina rara interna (LRI) of the GBM. These high molecular weight, negatively charged proteoglycans are responsible for the charge barrier to glomerular filtration.

There are certain molecules available as electron-microscope tracers (cationic dye) in biological studies. The characteristic property of these molecules is that they can be detected against the ultrastructural background of the tissue. For this purpose ferritin and horseradish peroxidase are commonly used, however, the former being a larger molecule (12nm) cannot be transported across the cellular membrane, whereas the latter although smaller in size reacts with the fixing dye to give an area of low density making accurate localisation very difficult. Cationic probes such as ferritin and colloidal iron are insensitive to small changes in GBM proteoglycan network (Kitano et al 1993).

2.1.2 Principle of the method

Polyethyleneimine (PEI) has now been selected to overcome the above disadvantages due to its combination of properties; that are: easy

detectability; minimal non-specific binding; water solubility and inability to react with the fixative. PEI is a polymer which has a NH_2- group for conjugation purposes, complexing groups for contrast purposes and a cationic character to avoid an excessive binding of tissue component Suzuka et al 1984, Furness et al 1986). When PEI is contrasted with phosphotungstic acid and stained with uranyl acetate, particles with a diameter of about 10nm are seen on electronmicrographs.

In this study the cationic property of PEI is used as a direct measurement of anionic charge on the GBM of glomerulonephritides.

PEI MOLECULE

$$[- C - C - N - C - C - N -]_n$$

$$\begin{array}{cc} | & | \\ C & H \\ | & \\ C & \\ | & \\ NH_2 & \end{array}$$

2.1.3 MATERIALS

The following were purchased from Merck SA:-

- Sodium Cacodylate (CH_3) 2 As O_2 Na. 3 H_2O
- Hydrochloric acid (HCl)
- Gluteraldehyde
- Polyethyleneimine (PEI) MW 1500 from BDH Chemicals, Pool England.

2.1.4 SOLUTIONS

0.4M stock cacodylate

Sodium cacodylate	21.4g
Distilled water	250ml

Cacodylate Buffer (0.2M)

0.4M Sodium cacodylate	250ml
0.2M HCl (1.7ml HCl to 100ml H_2O)	40ml

This gives pH 7.2.

Distilled water to make up to 500mls.

0.1% gluteraldehyde/2% phosphotungstic acid (pH 7.4)

2g phosphotungstic acid

0.1g gluteraldehyde

8.5g sucrose

0.5% aqueous PEI

0.5ml PEI

100ml H_2O

pH adjusted to 7.4.

2.1.5 Method

Following the methods of (Schurer et al 1977, 1978) and (Okada et al 1986), fresh renal tissue obtained by percutanous renal biopsy were minced and immersed in 0.5% PEI (pH 7.4) solution for 35 - 40 minutes. The tissue blocks were then washed 4 times in sodium cacodylate buffer (pH 7.2) for 10 minutes each time and then stained for 1 hour in phosphotungstic acid/gluteraldehyde mixture (pH 7.4). Tissues were then fixed in 4% gluteraldehyde and left overnight at 4°C. Tissues were processed for electron microscopy as follows:- fixed in Karnovsky's fixture for 1 hour and then post fixed in 1% osmium tetroxide for 1 hour.

The specimens were dehydrated in a graded series of alcohols, and embedded in araldite resin. Ultra-thin sections were cut, counter-stained with uranyl acetate and lead citrate and viewed with a Jeol 100C electron microscope.

2.1.6 Calculation

The mean number of anionic sites per 1000nm length of LRE were calculated as follows:-

Print magnification x enlargement

eg. 20 000 x 1.45 = 29 000

therefore final print magnification was enlarged 29 times.

$$1\text{cm on print} = \frac{10 \times 10^3}{29\,000} = 344.8\text{nm}$$

1000nm = 2.9cm.

For a print magnification of 20k, 2.9cm were equal to 1000nm of LRE.

2.1.7 Effect of varying concentration of PEI

Concentration of 0.05%, 5% and 1% of PEI were tested as staining probes. The 0.05% solution indicated diffuse particles whereas 1%, solution was too concentrated and counting of the particles was not easy. The 0.5% was found to be ideal.

2.1.8 Effect of temperature on PEI binding

Treatment of biopsy tissue in PEI was carried out at 25°C (room temperature) and in ice at 4°C. Variation in temperature did not affect the PEI staining of anionic sites. Subsequent staining was carried out at 4°C.

2.1.9 Confirmation of the technique

Biopsy specimen from the same patients were processed in 2 ways:-

a. with PEI probe and

b. without PEI.

Hence each patient acted as their own control, making the detection of PEI particles very easy.

2.1.10 Intra-observer reproducibility

Intra-observer reproducibility in the counting of PEI-bound anionic sites was assessed by randomly selecting 3 electromicrographs from each histological types studied.

The coefficient of repeatability (CR) was calculated according to the method of Bland and Altman (1986).

Table I : Intra-observer reproducibility

Histological Group	Mean Initial Count	Mean Count (3 mths later)	CR	Interpretation
MCNS	16.44±1.6	16.77±2.3	1.587	Excellent
FGS	17.77±1.89	18.0 ±1.33	1.217	Excellent
MGN	12.22±2.14	13.0 ±3.2	2.34	Good
MPGN	21.5 ±2.34	21.7 ±1.7	1.217	Excellent
Control	34.22±1.5	34.5 ±1.0	1.154	Excellent

CR - Coefficient of Repeatability

VARIATION

According to Bland and Altman (1986) if the CR is less than the SD of the two tests then the 95% of the samples will fall in the range of CR.

2.2 ALCIAN BLUE BINDING TO RED BLOOD CELLS (ABRBC)

2.2.1 Principal of the method

In this method, red blood cell (RBC) surface charge is measured by binding of a cationic dye alcian blue (AB) to anionic sites on the RBC membrane (ABRBC) (Levin et al, 1985; Bernard et al, 1989). A known number of RBC's are mixed with AB in phosphate buffered saline. Identical volume of cell-free AB solution is taken as a blank control. The mixture is incubated at 37°C for a defined period of time, and the cells are precipitated by centrifugation. The optical density (OD) at 650nm of the supernatant is recorded and compared with that of the control. The amount of alcian blue bound to the cells, can therefore be calculated from the difference between OD of the blank and that of test supernatant.

2.2.2 Materials and apparatus

1. Alcian blue 8GX (76%) Lot 124-4375, was obtained from Sigma chemical, St Louis, USA.
2. Neuraminidase - Sigma Chemical, St Louis, USA.
3. Sodium chloride and magnesium chloride ($MgCl_2$) were obtained from Merck, South Africa.
4. Phosphate buffered saline (PBS) tablets - (Dulbecco A'), Unipath Ltd, Basingstoke Hampshire, United Kingdom.
5. Incubator
6. Spectrophotometer.

2.2.3 Preparation of solutions

PBS buffer

One tablet was dissolved in 100ml distilled water and pH adjusted to 7.4.

Alcian blue

One percent AB in PBS buffer was made up freshly on the day of the assay.

Initially AB solution was made up with 2M $MgCl_2$ and 0.15M NaCl, however, $MgCl_2$ and NaCl did not have any effect on AB binding, therefore, assays were performed with AB solution made up in PBS only.

2.2.4 Method

The method was carried out according to Bernard et al (1989) with the following modification.

1. 9ml of venous blood were collected in 3.8% trisodium citrate.
2. The tube was centrifuged at 1000g for 10 minutes.
3. The RBC's were removed from below the buffy coat and resuspended in twice their volume of PBS.
4. The cells were washed 4 times for 10 minutes each time in PBS and finally resuspended in fresh PBS (1ml).
5. The cells were counted diagonally in the centre square of the haemocytometer slide and the volume adjusted to give the final concentration of 1.2×10^9 RBC/ml. The cell count was adjusted by calculating the number of cells in the haemocytometer as follows:
   Number of cells x 5 x dilution x 10 x 1000 = number of cells/ml
6. 0.5ml of RBC's (1.2×10^6) were suspended in 0.5ml PBS and then mixed with equal volume of AB solution of pre-determined concentration (150mg/ml).
7. Blank controls were set up by mixing the cell-free AB solution with PBS.
8. The blank and the test samples were incubated in a water-bath at $37^{\circ}C$ for 30 minutes.
9. The tubes were centrifuged at 1000g for 5 minutes.
10. Supernatant of the blank and test samples were removed separately and optical densities (OD) were measured at a wavelength of 650nm.

11. The amount of AB bound was calculated as follows:

$$\text{AB bound} = \frac{[\text{AB}] \times \text{OD blank} - \text{OD test sample}}{\text{OD blank}}$$

12. The results were expressed as ng AB/10^6 cells by dividing the results by the appropriate number of cells.

2.2.5 Optimal AB concentration

The optimal concentration of AB binding to the RBC was determined by binding of AB solution of varying concentration ranging from 10 - 1000ug/ml to 1.2X10^9 RBC's. The AB bound at different concentration showed a sigmoid curve with the saturation a concentration above 500ug/ml. For this study AB concentration of 150ug/ml was selected as being in the mid-region of the binding curve.

2.2.6 Specificity of AB to charged groups (Figure 4)

The specificity of AB to negatively charged sialic acid residue of RBC was tested by removing the sialoprotein responsible for negative charge. This was done by treating the RBC with neuraminidase prior to incubation with AB. The effect of neuraminidase was assessed by comparing OD of ABRBC of neuraminidase treated cells to those of untreated cells.

2.2.6.1 Method

1. A known number of RBC's were incubated with neuraminidase in PBS (1mg/ml) at 37°C for 1½ hours.

2. Cells were washed in PBS and then resuspended in fresh buffer as described above.
3. The RBC's were incubated with AB at 37°C and procedure was carried out as mentioned previously.
4. ABRBC in treated and untreated cells was calculated by measuring OD of varying concentration of AB.
5. ABRBC was markedly reduced (by 60%)in neuraminidase treated cells.

2.2.7 Reproductibility

2.2.7.1 *Intra-assay*

Intra-assay reproducibility was assessed by measuring 1 unknown sample five times in the same assay. The result obtained was (mean ± SD) (62.28 ± 0.268) with a coefficient of variation (CV) of 4.3%.

2.2.7.2 *Inter-assay*

Inter-assay reproducibility was assessed by measuring OD of 5 samples five times in 5 consecutive assays. The coefficient of variation for the five samples ranged from 2.3% to 13.3%. The results are shown in Table II. To allow for variation a control sample was included in each assay of ABRBC study in Chapter 4.

Table II : Inter-assay reproducibility

Sample	Mean AB ng/ 10^6 RBC	± SD	C V - %
1	53.82	0.204	2.3
2	9.56	0.068	3.7
3	62.35	0.145	7.0
4	52.36	0.543	10.3
5	34.17	0.445	13.0

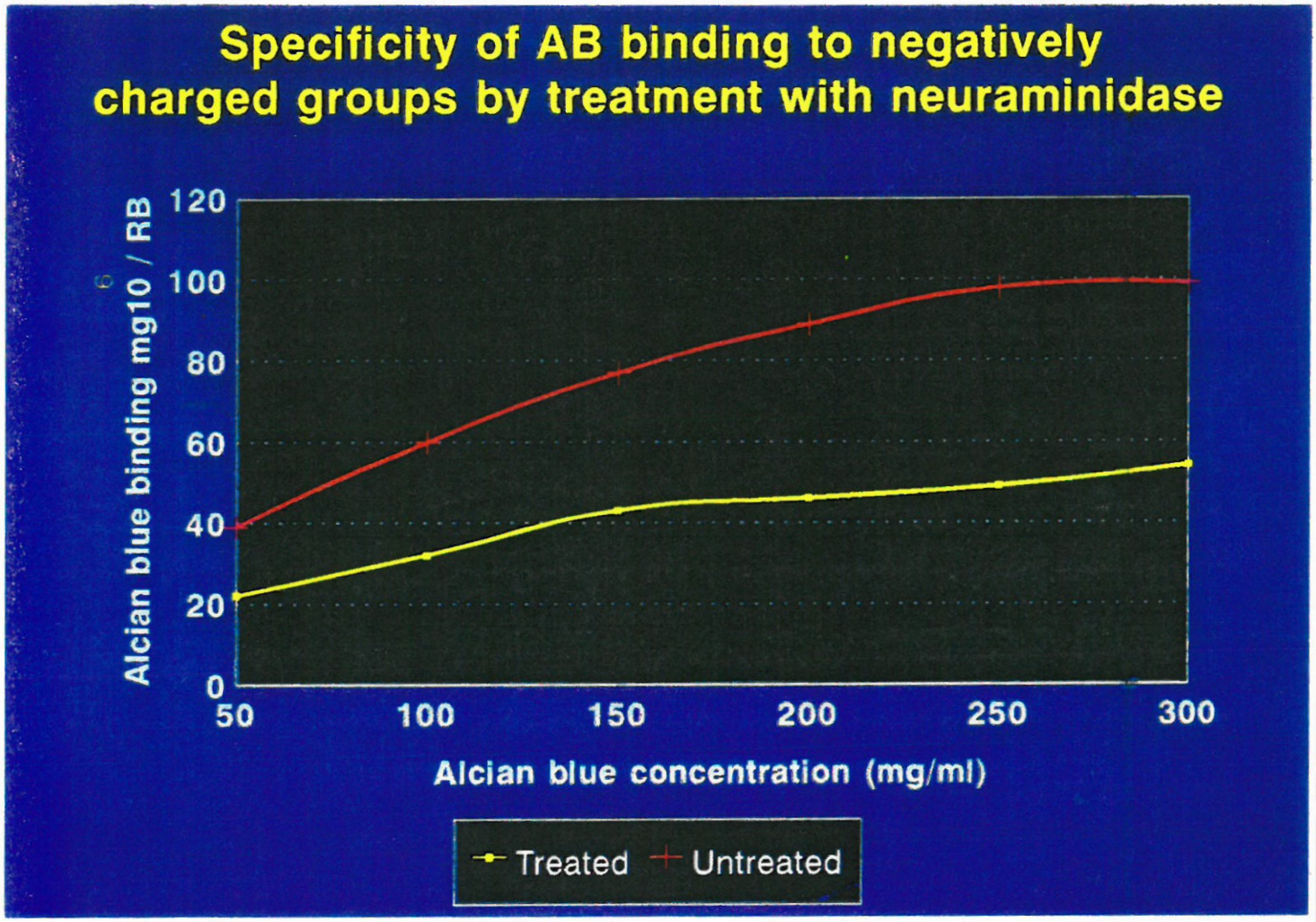


FIGURE 4 : Specificity of Alcian Blue (AB) binding to negatively charged groups

2.3 URINARY PROTEIN - CREATININE RATIO ($U_{p/cr}$)

2.3.1 Background

Urinary proteins are detected by dipstix, 24 hr urinary protein estimation and analysis of a random urine sample. Quantitation of the urine samples can be carried out on a timed urine collection (usually 24 hours) or by analysis of a random urine sample. First morning, urine samples are avoided due to overnight retention in the bladder. Second morning samples give a better estimation. However, for this study it does not matter whether first or second samples are used. Timed urine collection however are cumbersome and not always possible in children. Therefore total protein estimation was carried out in children using random urine samples (Ginsberg et al 1983). With this method, a random urine sample is analysed for both protein and creatinine with each result reported in mg/dl. A ratio of protein to creatinine is determined so the units of measurement cancel each other. The protein/creatinine ratio ($U_{p/cr}$) correlates with the quantity of proteins excreted in 24 hours.

2.3.2 Method

Second morning samples of urine and 24 hour timed samples from 18 patients were sent to routine laboratory at King Edward VIII Hospital for estimation of protein and creatinine respectively. Protein was measured by using the Coomasie blue technique (McIntosh 1977) and creatinine by the Jaffè method (Slot 1965). Urinary protein/creatinine ratios ($U_{p/cr}$) were then calculated (Ginsberg et al 1983, Arbitol et al 1990).

2.3.3 Results

The correlation between 24 hour urine sample and random assessment of ($U_{p/cr}$) ratio was significant ($r = 0.727$; $p < 0.006$). The regression equation being:

$$24 \text{ hour} = 1.24 + 2.926\ (U_{p/cr})$$

($U_{p/cr}$) ratio of > 0.2 corresponded to significant proteinuria and value of <0.2 was considered as non-nephrotic range proteinuria.

2.3.4 Specificity

Specificity of the test was measured as a percentage of 24 hour urine samples in each range of proteinuria that were properly designated by ($U_{p/cr}$). The specificity was 100%.

2.3.5 Sensitivity

The sensitivity of ($U_{p/cr}$) ratio in assessing the degree of proteinuria, was calculated as a percentage of 24 hour urine sample with proteinuria at either nephrotic range or non-nephrotic range. A two-way contingency table was constructed in the following way:-

		24 HOUR SAMPLE		
		+	-	
> 0.2	+	12	0	12
< 0.2	-	1	5	6
		13	5	

$$\text{Sensitivity} = \frac{12}{13} = 92.3\%$$

$$\text{Specificity} = \frac{5}{5} = 100\%$$

Table III: Values of urinary protein/creatinine ratios and 24 hour urine samples in patients

Patient	$U_{p/cr}$	24 hr (g/l)
1	0.8	3.5
2	5.3	16.6
3	4.3	51.1
4	0.1	1.8
5	3.2	10.3
6	0.1	1.6
7	0.1	1.5
8	2.1	7.2
9	5.7	17.8
10	4.8	15.2
11	1.1	4.4
12	3.3	10.7
13	2.4	8.2
14	1.3	4.8
15	1.6	5.8
16	0.6	2.7
17	1.2	4.6
18	0.1	1.2

Ratio > 0.2 = nephrotic range

Ratio < 0.2 = non - nephrotic range

2.4 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

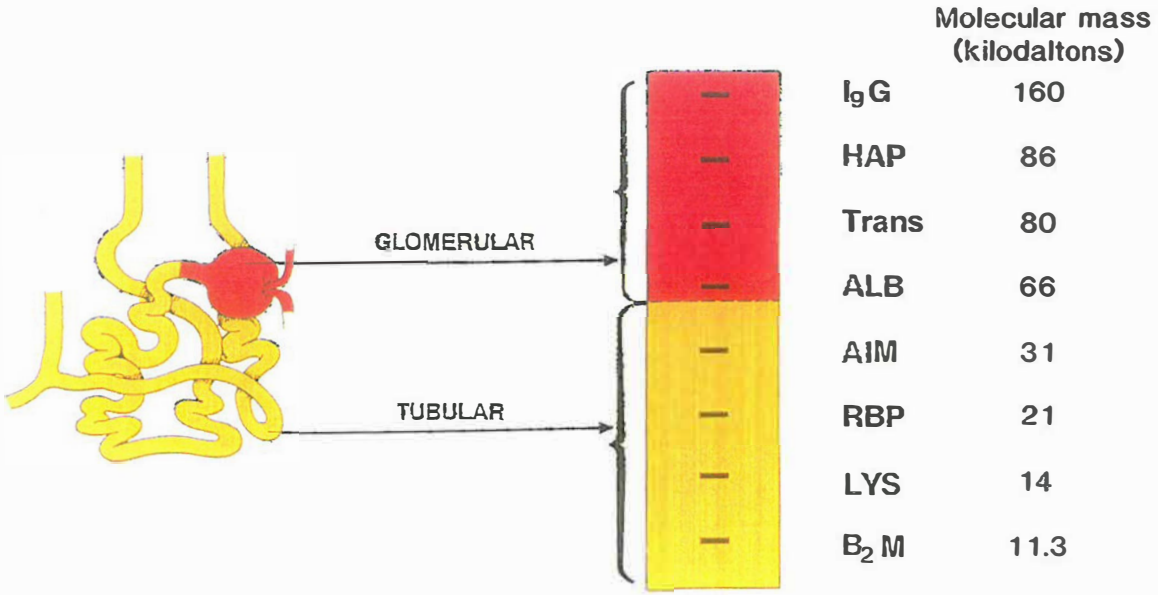
2.4.1 Background

One dimensional gel electrophoresis is a high-resolution separation technique for separating proteins from body fluids. In the presence of sodium dodecyl sulphate (SDS) (denaturing conditions) proteins separate according to their molecular size as they move through a polyacrylamide gel matrix towards the anode (Lamelli 1970). Most proteins bind SDS in a constant weight ratio, leading to identical charge densities for the denatured proteins. Thus allowing the protein to migrate in the polyacrylamide gel according to size not charge (Figure 5). In non-denaturing conditions (in absence of SDS) separation of protein is based on a combination of molecular properties including size, shape and charge (Brenner 1978).

2.4.2 Principle of the method

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric acrylamide chains and the cross-linking of these chains by N-N-methylene-bisacrylamide. The polymerization reaction is initiated by addition of ammonium persulphate.

The relationship between the relative mobility of $\log_{10}$ molecular weight is linear. With the use of standard plots (Figure 6) the molecular weight of an unknown protein may be determined by comparison with known protein standards.



URINARY PATTERNS BY SDS PAGE

FIGURE 5 : SDS PAGE separation of protein molecules according to size (molecular weight)

2.4.3 Materials and solutions

The following were obtained from Sigma Chemical (St Louis Missouri).

A. 1. Pure standards of Albumin, transferrin, haptoglobulin, immunoglobulin B(IgG), $ß_2$ microglobulin and lysozyme.
   2. Acrylamide, N-N-methylene bisacrylamide, Temed ($N_1N_1N_1N$ tetramethylethylenediamine), ammonium persulphate, Coomassie blue.

B. High molecular weight pre-stained markers Br/161-0309 and low molecular weight pre-stained marker Br/161-0317 were obtained from Biorad Laboratories (U.K).

C. Ethanol, methanol, isobutanol, bromophenol blue were obtained from Merck S.A.

The following electrophoresis equipment was obtained from Hoefer Scientific Instruments, San Francisco, California.

Hoefer mighty - small	(SE 250)
Gel caster	(SE 245)
Gradient maker	(SE 30)
Plate washer plate mate	(SE 100)
0.75mm spacers	(SE)
Glass plates	(SE 202 PIO)
Alumina notched plates	(SE 202N-10)
10 well combs	(SE 211A/5/0.75)
Peristaltic pump	
Power supply	(PS 500 XT)
Red rotor orbital shaker	(PR 70)

Buffers

Running gel buffer (Tris-Cl) pH 8.8.

Tris -36.3g.

Made up to 200ml and pH adjusted to 8.8 with hydrochloric acid.

Tank - buffer

Tris-glycine pH 8.3

Tris - 6g

Glycine - 28.8g

SDS (10%) - 20ml

Made up to 2ℓ with distilled water.

Stacking gel buffer (pH 6.8)

Tris - 3.0g

Made up to 50ml with water and pH adjusted with hydrochloric acid.

Sample buffer

Tris (6.8) buffer 2.5ml

SDS 4.0ml

Glycerol 2.0ml

Made up to 10ml with water and bromophenol blue added for colour.

Stock Acrylamide

	30%	**50%**
acrylamide	58.4	23.7g
bisacrylamide	1.6	1.38g

Percent acrylamide was calculated as follows according to Karl-Erik Johansson:

$$T = \frac{a + b}{v} . \ 100\% \ (w/v)$$

$$C = \frac{b}{a + b} . \ 100\% \ (w/w)$$

T = total concentration of acrylamide and Bis

C = cross linking i.e. fraction of Bis

a = acrylamide

b = bis acrylamide

1. **REAGENT**

	7.5% Running Gel (mls)	**25% Running Gel (mls)**
Stock acrylamide (30%)	1.25	-
Stock acrylamide (50%)	-	5.0
Running gel buffer pH 8.8	1.88	3.76
Water	1.37	0.24
SDS	0.5	1.0
Temed	10μl	20μl
Ammonium persulphate	40μl	60μl

2. **STACKING GEL**

4% stacking gel	**mls**
Stock acrylamide (30%)	0.53
10% SDS	0.4
Stacking gel buffer pH 6.8	0.5
Water	2.57
Persulphate	60μl
Temed	20μl

Water saturated butanol

N-butanol	50ml
Water	5ml

Staining solution

Stain stock

1% Coomassie blue R250

Coomasie blue	- 2.0g
Water	- 200ml

Stir and filter.

Stain

Stain stock	62.5ml
Methanol	250ml
Acetic acid	50ml

Made up to 500ml with distilled water.

De-stain I

(50% methanol, 10% acetic acid)

Methanol	500ml
Acetic acid	100ml

Made up to 1L with distilled water.

De-stain II

Acetic acid	700ml
Methanol	500ml

Made up to 10L with distilled water.

Additional protein standards

IgG 1.0mg/ml	7.5µl (0.5µg)
Haptoglobulin 10mg/ml	15.0µl (0.5µg)
Transferrin 1.0mg/ml	5.0µl (0.5µg)
Albumin 2.0mg/ml	5.0µl (0.5µg)
Lysozyme (0.62-1.25mg/ml)	3.0µl (0.5µg)
β_2 Microglobulin (1.0mg/ml)	2.5µl (0.5µg)

Water	2.0μl
Sample buffer	10.0μl

The concentration of the standards were adjusted to give a visible band with Coomasie blue stain. 5μl of protein standard (5-10μg) were applied to the gel.

2.4.4 Method

Urine collection

A second voided morning sample of urine was collected and preserved with a few drops of 0.05% sodium azide. One aliquot was sent to the routine laboratory at King Edward VIII Hospital for total protein and creatinine estimation.

Total protein was estimated by Coomasie blue method (McIntosh 1972) and creatinine by the Jaffe' method Slot 1965). Urine samples of protein concentration of 3.0g/l were diluted to 100-300 mg protein. Samples of protein less than 3.0g/l were used without dilution.

Sample preparation

100μl of urine was mixed with 20μl of sample buffer and placed in boiling water (90°C) for 5 minutes and immediately placed in the freezer.

Electrophoresis (Shapiro et al 1967; Schiwara et al 1986)

1. 8cm x 7cm gels were cast using the mighty small gel caster. Gel-sandwich consisted of a glass plate, two spacers and a notched alumina plate, which were held in place on the unit with spring clamps. Two gels were cast at the same time.

2. Gradient gels were poured using the gradient maker and a peristaltic pump, with one end of a rubber tubing attached to the gradient maker and the other to a needle. The needle was placed on top of the sandwich to pour the gel.

The heavier gel (25%) was gradually mixed with a magnetic stirrer. The outlet tap was opened for the gel to pass into the rubber tubing. The tap between the two tanks was opened simultaneously so the 7.5% gradually mixed with the 25%. Hence a gradient was formed.

3. Once the gels were poured a layer of water-saturated butanol was overlaid in order to prevent the formation of a meniscus which could cause a distortion of the banding pattern.

4. Polymerization was complete within 30 minutes. The water-saturated butanol was poured off and the top layer washed several times with distilled water.

5. The stacking gel was poured on top of the separating gel and the 10-well comb was inserted. The gel was then allowed to polymerize for 30 minutes.

6. Once polymerization was complete the combs were removed and the wells formed were washed with distilled water.

7. The gels were placed in the electrophoresis tank which was filled with buffer.

8. 5μl (15-20μg/μl protein) of sample was underlayed in each well with a Hamilton syringe.

9. The unit was connected to the power supply and current was passed for 15 minutes at 85MA 100 volts and then increased to 200V for the remainder of the run. Once the dye-front reached the bottom, the power supply was turned off after ≈90 minutes.

2.4.4.1 Staining

1. The gels were removed from between the sandwich plates and placed in the fixative for 1 hour.

2. They were then placed into stain and gently shaken on the rotor for 2-3 hours.

3. On staining the gels were removed and placed in De-staining Solution I for 1 hour.

4. The stained gels were then transferred to De-stain II and left overnight. The relative mobility of the samples was measured and the corresponding MW was then extrapolated as follows: The migration distance of the sample protein standards and molecular weight markers were measured and their relative migration values (Rf) calculated (Figure 6) as follow:

$$Rf = \frac{\text{distance protein has migrated from the origin}}{\text{distance from origin to the end of gel}}$$

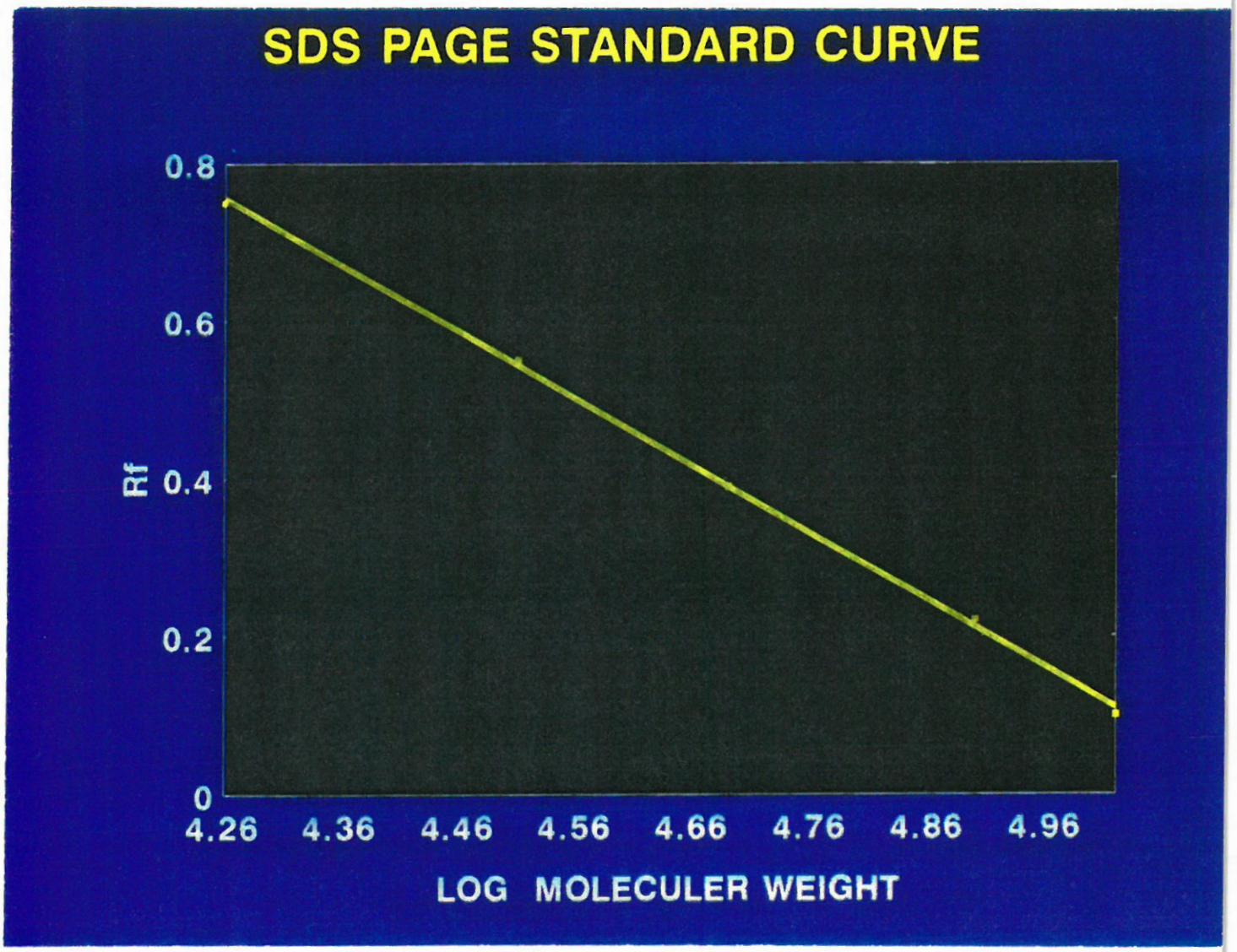


FIGURE 6 : SDS PAGE standard curve

Choice of stain

Silver staining is reported to be more sensitive than Coomasie blue stain. Silver-staining was not utilised because in heavy proteinuria the silver stain would identify bands which have not been identified previously. As we were looking for specific proteins, we decided to use Coomasie blue for all our staining purposes.

2.4.5 Reproducibility of separation

Urine samples were electrophoresed after a period of one, two, three and four weeks respectively to assess effect of storage on electrophoretic patterns. The bands did not change on storage.

2.4.5.1 Intra-reader reproducibility

In order to assess intra-reader reproducibility of interpretation of protein bands, 20 gels which had various visible bands of all proteins interpretations in Chapters 5 & 6 were selected at random to be re-interpreted by the same reader. In order to minimize recall bias this second interpretation was carried out 3 months after the original interpretation, using a blind procedure. The kappa statistic (k) was used to assess the intra-observer variability in these interpretations. According to Fleiss (1981) this test is defined by the formula:-

$$k = \frac{2\,(ad - bc)}{p_1 q_1 + p_2 q_2}$$

where a, b, c, d are proportions of the grand total of a 2-way contingency table and;

$$p_1 = a+c,\ p_2 = a+b,\ q_1 = b+d,\ q_2 = c+d$$

The two-way contingency table is constructed in the following way:

	Normal (N)	**Abnormal (Ab)**	
Normal (N)	a	b	p_1
Abnormal (Ab)	c	d	q_1
	p_2	q_2	

A kappa value greater than 0.75 may be taken to represent excellent agreement beyond chance, and values below 0.40 may be taken to represent poor agreement.

The kappa test for protein bands were as follows:-

Table IV : Results of kappa test for protein bands

Protein bands	**k**
Albumin	0.80
Transferrin	0.72
Haptoglobulin	0.75
IgG	0.85
$ß_2M$	0.615
Lysozyme	0.615

The kappa test showed an excellent agreement between tests for albumin, transferrin, haptoglobulin and IgG. A moderate agreement was found for $ß_2M$ and Lysozyme.

2.4.5.2 Sensitivity of the test

The sensitivity of the test was measured by comparing the interpretations of protein bands to the gold standard which was renal biopsy or response to steroid therapy. Sensitivity was 100%.

2.4.5.3 DETECTION LIMIT

Minimum detection limits were investigated for albumin, transferrin haptoglobulin, IgG, β_2M and lysozyme. The minimum detection limit was found to be 0.1mg/ml for albumin, transferrin and IgG. β_2M and lysozyme were visible at 0.4mg/ml.

2.4.6 USEFUL SUGGESTIONS

1. One of the common problems encountered in pouring the gel is that of leakage. In our experience if the rubber base of the gel caster was left overnight to dry, then the problem of leakage was solved. Also, additional padding with tissue was necessary.

2. The tank buffer can be used repeatedly. However, the one way of detecting that the pH of the tank buffer has changed is that the current of the electrophoresis tank would suddenly increase indicating that new buffer has to be used.

3. The de-stain solution II is also used to preserve the gels. However, the solution does evaporate with time so regular top up is necessary if the gels are to be preserved for a long time.

4. The gel sandwich can be left in the fridge for at least one week if carefully packed in foil and plastic.

2.5 ISOELECTRIC FOCUSING (IEF)

2.5.1 Principle of the method

The IEF technique separates protein in a pH gradient formed by addition of synthetic ampholytes (molecules with positive and negative charge which align themselves according to the pH gradient of the gel) within the gel. Proteins placed in a pH gradient migrate electrophoretically until they reach their isoelectric point, a point where the net charge of the protein molecule will be zero. According to Catsimpoolas (1973), protein molecules will be positively charged at the acidic end of its isoelectric point and negatively charged at the alkaline end (Righetti 1983, Walker et al 1983, Savans 1979, Vesterberg 1978). In combination with SDS PAGE parameters that may be studied by these analytical means include isoelectric point, molecular weight, sub-unit size, charge characteristics and immunological properties.

2.5.2 Materials

1. Sorbitol, gelbond, IEF markers pH 3-10, and ampholytes (pH3-10) obtained from Sigma Chemicals, Poole Dorset.
2. Methanol, ethanol, sulphosalycylic acid, coomassie blue, were obtained from Merck S.A.
3. Agarose for IEF (isogel) was obtained from FMC Bioproducts Rockland ME.

2.5.3 METHOD

1. 36g d-sorbitol was dissolved in 27ml aqueous glycerol (3ml in 27ml water).
2. 0.3g Agarose IEF was added to (1) and the mixture was dissolved in the microwave at medium power for 2 minutes.
3. The mixture was allowed to equilibrate in a water bath at 65°C and 2ml of ampholytes were added and mixed until well dispersed.

4. The gel mixture was poured into a pre-warmed perspex casting frame (220mm x 110mm) on a pre-warmed sheet of gelbond film which was sealed to a glass plate with 50% aqueous methanol (a small amount of methanol is placed on the glass plate and the gel bond is rolled on it with its hydrophobic side down.
5. The gel was allowed to set and placed in a damp chamber for 1 hour or overnight at 4°C.

IEF RUN (Figure 7)

1. The gel was allowed to equilibrate to room temperature in the damp chamber.
2. The perspex frame was removed and the casting meniscus was trimmed by cutting the outermost 2mm from the gel using a surgical blade.
3. The gel was placed on to the cooling platten of the tank using 50% aqueous methanol. Excess water on the gelbond film was wiped off with a tissue.
4. The surface fluid from the gel was removed from the gel using a sheet of dry nitrocellulose membrane (NCM). The NCM was placed carefully on the gel starting at one of the short ends and allowing the membrane to gently roll on to the surface. Air bubbles were avoided from being trapped underneath the membrane.

   Gloved fingers were used to gently smooth out any air bubbles. The NCM was left on the gel for few seconds and then removed.
5. The sample application foil (Pharmacia) was placed such that outermost edge was 2.5cm from the anodic (+) edge of the gel (see diagram).
6. Sample (5-10mg protein) were loaded using a 25ml Hamilton syringe with a blunt needle.
7. Filter paper wicks were soaked in electrolytes, 0.05M sulphuric acid for the anode and 1M sodium hydroxide for the cathode. The wicks were positioned at an interelectrode distance of 7cm.
8. Paper towel soakways were placed to absorb any excess water.
9. The voltage limit was set to 1200 and amperage of 150 milliamps for a total of 1000V/hr. Initial gel condition are typically 520V, 40mA at 2010. The running time for the gel was typically 1 hour.

10. Sample application foil was removed after 300V/hr or 20 minutes.
11. At the end of the run the soakways were used to dry excess fluid from the electrode wicks which were then removed and discarded.

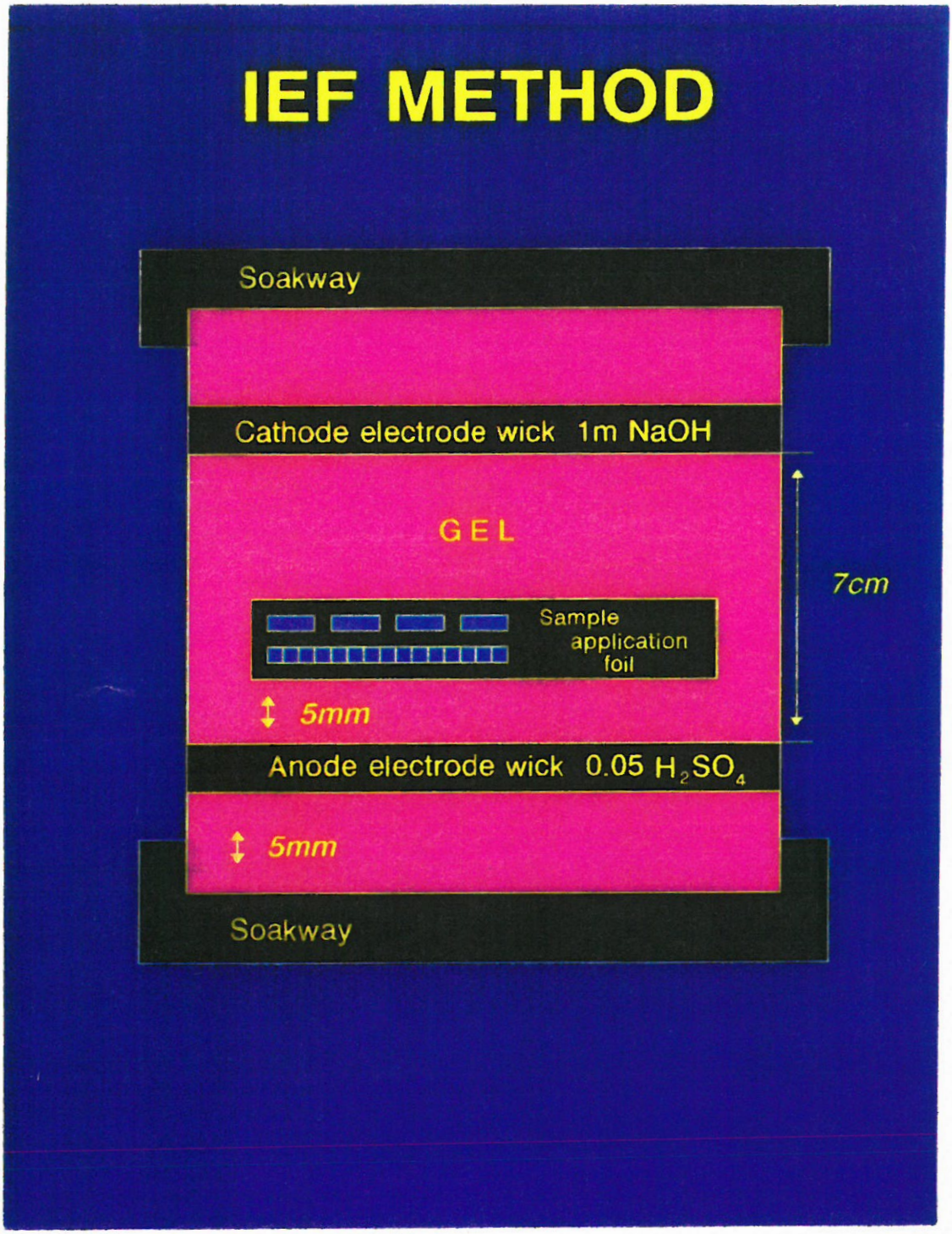


FIGURE 7 : IEF gel setup for isoelectric focusing

2.5.4 Fixing staining and de-staining

1. **Fixative solution**
   180ml methanol
   30g trichloroacetic acid
   18g sulfosalicylic acid
   Fill to 500ml with distilled water.

2. **Coomassie stain**
   1.0g Coomassie Brilliant Blue R-250
   250ml ethanol
   90ml glacial acetic acid
   Fill to 1 litre with distilled water.

3. **De-staining solution**
   250ml ethanol
   90ml glacial acetic acid
   Fill to 1 litre with distilled water.

METHOD

1. Place the gel in fixative for 10 minutes.
2. Grasping the edge of the film support with forceps the gel is lifted from the fixative.
3. The gel surface was rinsed with distilled water and the excess solution drained.
4. The gel was then placed on a paper towel with the gel side up.
5. A single sheet of Whatmann 3mm blotting paper wetted with distilled water was placed on the gel surface. The blotting paper was overlaid with six layers of absorbent paper towelling. A heavy glass plate was placed on top of the paper towel for 10 minutes.
6. The glass plate was removed and the blotting paper was rewet thoroughly with distilled water and gently lifted off the gel surface.

7. The gel was washed in distilled water for 15 minutes to remove residual fixative and ampholytes.
8. The gel was allowed to dry completely at room temperature overnight.
9. Staining was carried out for 1 - 2 hours.
10. On staining the gel was rinsed with distilled water.
11. The gel was placed in de-stain solution for 3 minutes and then rinsed briefly with distilled water.
12. Drying of the gel was carried out at room temperature overnight. The gels were then read and photographed without any further treatment.

Isoelectric points (PI) of the proteins of interest in this study are as follows:-

Protein	***PI***
Albumin	4.7
Haptoglobulin	4.1
Transferrin	5.9
IgG	5.8-7.3
Lysozyme	10.5 - 11.0
$ß_2$Microglobulin	5.56

2.6 USEFUL HINTS

1. If the sample tracts are too intense then excessive sample may be loaded on to the gel.
2. If the sample tracks are distorted then it could be due to one of the following reasons:-
   a. inadequate cooling during IEF
   b. condensation on gel surface during run
   c. sample volumes >15μl may cause 'ballooning' into adjacent tracks
   d. electrolyte wicks positioned at wrong electrodes
3. The appearance of ridges in the gel parallel to the two electrode wicks is an essential sign that focusing is occurring, failure of ridges

to appear within 10 - 15 minutes implies that there is a serious failure in either electrical power getting to the gel or through the gel.

2.7 REPRODUCIBILITY OF THE METHOD

Reproducibility of the method was assessed by 5 replicate runs of the same sample on separate occasions. All runs indicated identical bands.

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CHAPTER 3

DEPLETION OF GLOMERULAR ANIONIC SITES AND PROTEINURIA IN NEPHROTIC SYNDROME OF CHILDREN

CHAPTER 3

DEPLETION OF GLOMERULAR ANIONIC SITES AND PROTEINURIA IN NEPHROTIC SYNDROME OF CHILDREN

3.1 INTRODUCTION

Altered electrostatic charge and distortions to size and shape of the slits in the glomerular basement membrane (GBM) have been shown to be the most likely reasons for proteinuria although the relative contributions of each of these abnormalities in different renal diseases is not known (Chang et al 1975, Brenner et al 1978).

Fixed anionic sites consisting of heparan sulphate glycosoaminoglycans are responsible for the charge-selective property of the GBM. They present a negative charge to the surface, repelling negatively charged macromolecules from passing into the capillary lumen. Loss of these sites may allow negatively charged proteins to pass easily through the capillary wall (Chang et al 1975, Brenner et al 1978, Kanwar and Farquar 1979, Kanwar et al 1980). Polyethyleneimine (PEI) which detects this loss of anionic sites along the glomerular capillary (Schurer et al 1977, Vernier et al 1983) has been used to study proteinuria in some kidney diseases; there are few such studies and these have included only small numbers of children.

The present study was therefore undertaken to address unresolved issues by analysing quantitatively the presence of anionic sites on the GBM in different types of NS in children using PEI, by comparing the magnitude of change in the electrostatic charge barrier in various histological groups, and by seeking an inverse correlation between the loss of anionic sites and the degree of proteinuria.

3.2 PATIENTS AND METHODS

3.2.1 Patients

Thirty three children with NS (31 African and 2 Indian) were selected for the study. The following groups of children were studied: 4 children with minimal change disease (MCNS) 10 with focal glomerulosclerosis (FGS), 14 with membranous nephropathy (MGN), 12 HbsAg positive and 5 with mesangiocapillary glomerulonephritis Type I (MPGN). The diagnostic criteria for these conditions have been previously reported (Coovadia 1987).

3.2.2 Methods

Tissue samples were obtained by percutaneous renal biopsy. Control biopsies were obtained from 8 traumatic partial nephrectomy patients. The histological diagnosis was determined by conventional light and electron microscopy and by immunoflourescence and classified according to Heptinstall (1992).

3.2.2.1 **Tissue processing with PEI** (Figure 8)

Unfixed renal biopsy specimens were placed in 0.5% aqueous PEI solution (MW 1500) (pH 7.3) containing 8.5% sucrose according to the method of Schurer et al (1977) and Okada et al (1986). The blocks were washed 3 times for 10 minutes in cacodylate buffer containing 8.5% sucrose (pH 7.4) and then stained by immersion for 1 hour in 2% phosphotungstic acid/0.1% gluteraldehyde mixture containing 8.5% sucrose (pH 7.3). All washing and staining procedures were carried out at 4 ^{0}C. The tissue block was then prepared for transmission electron microscopy by conventional techniques.

The number of anionic sites in the lamina rara externa were counted over a distance of GBM in the photomicrograph per glomerulus (at least 2 were counted) and was expressed as mean number (± SD)

of LRE stained anionic sites per 1000 nm length of GBM. Stained sites in the lamina rara interna (LRI) and the lamina densa (LD) were not counted as they were scattered randomly. Detailed account of the method is given in Chapter 2.

3.2.2.2 Quantitation of proteinuria

Second morning samples of urine were collected in children for the estimation of proteins. Total protein was measured by the biuret method (McIntosh 1977) and creatinine was measured by the Jaffé method (Slot 1965). Urinary protein/creatinine ratio (Up/c) were then calculated (Abitol et al 1990). Detailed account of the method is given in Chapter 2.

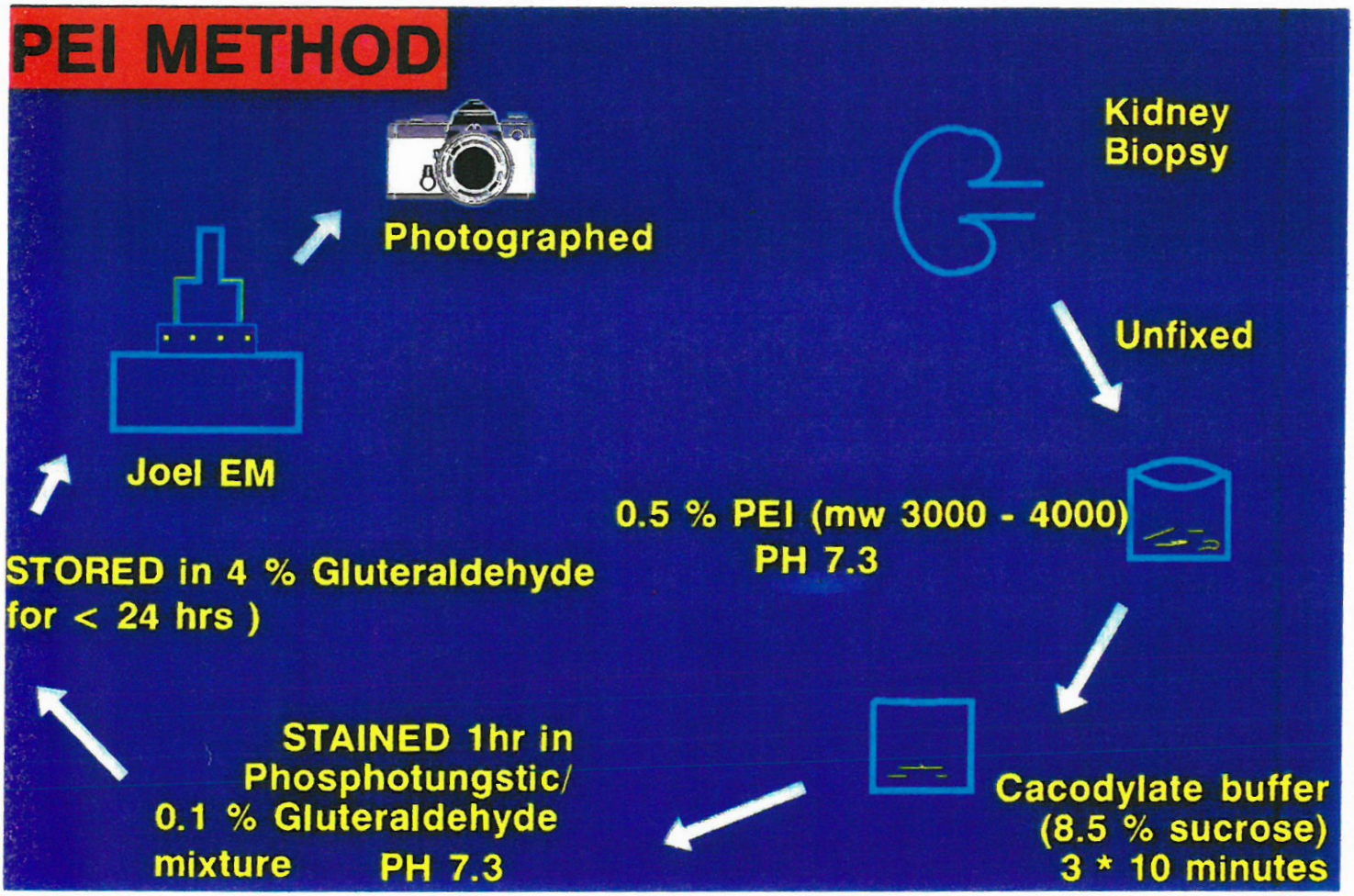

FIGURE 8 : Binding of GBM anionic sites by cationic probe PEI

3.3 STATISTICAL ANALYSIS

The mean number of anionic sites in all histological groups were compared using Kruskal-Wallis test (the non-parametric equivalent of analysis of variance) for all differences. If the overall result was statistically significant, pairwise comparisons were made using Wilcoxon 2-sample test to determine between which groups the differences were.

3.4 RESULTS

Control group (Figure 9A)

Glomerular anionic sites in the control kidneys were visible as a continuous array of particles along the LRE and numbered about (Mean ± SD) 25 ± 1.43 per 1000nm length of the LRE. Fewer irregularly spaced PEI particles were also observed along the LRI and LD.

All patients (Figure 10) (Table V)

There was a significant reduction in the number of GBM anionic sites in patients as a whole ($p<0.05$) compared to controls. There was also a significant difference between each group and the controls. No differences were noted between the groups.

PEI labelling of anionic sites revealed clearly demarcated electron dense particles (10nm diameter) within the LRE of the GBM, fewer randomly distributed sites were observed in the LD and the LRI.

TABLE V: PEI labelled anionic sites in the lamina rara externa of the GBM in different histological groups

HISTOLOGICAL GROUP	MCNS (N=4)	FGS (N=10)	MGN (N=14)	MPGN (N=5)	CONTROL (N=3)
Anionic sites Mean ± SD	15.25±2.98	16.0±5.1	14.18±3.83	20.04±1.69	25.0±1.49
p values#	$p < 0.05$	$p < 0.014$	$p < 0.005$	$p < 0.036$	

No difference between individual groups

\# difference from controls

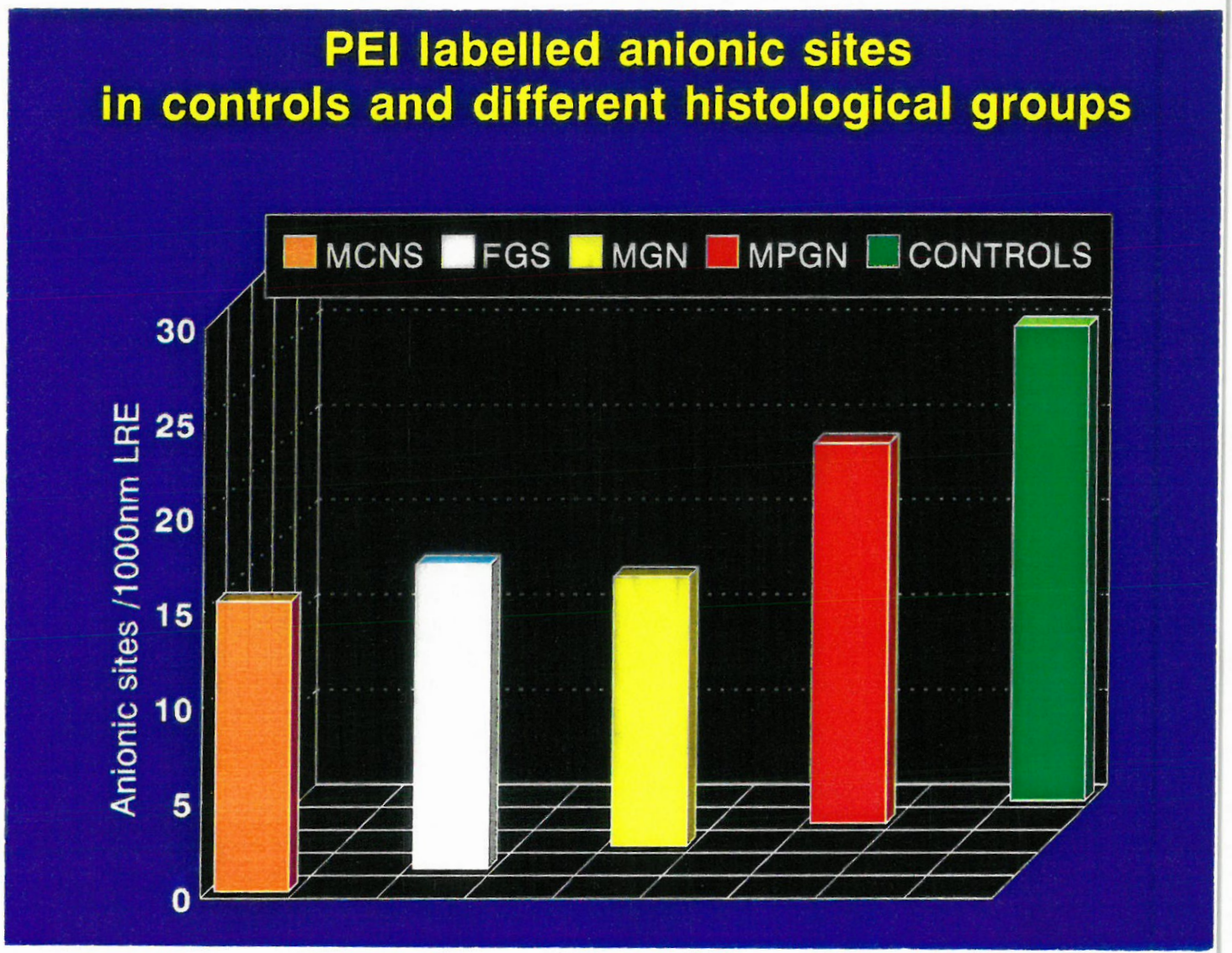

FIGURE 10 : PEI labelled anionic sites in controls and different histological groups of nephrotic syndrome

MCNS (Figure 9B)

There was a significant reduction in the number of labelled anionic sites on the LRE in the 4 children with MCNS. The mean number of anionic sites per 1000 nm length of GBM was 15.25 ± 2.98 ($p<0.05$).

The distribution of anionic sites in the LRI and LD appeared to be similar to the controls.

FGS (Figure 9C)

Indians (N=2)

The mean binding was lower compared to controls (19.19 ± 0.15). The PEI staining in LRE showed a pattern similar to those in MCNS. However in some parts of the GBM where the podocytes were not fused, the anionic sites were similar to controls and seen as a continuous array along the LRE and between the fenestrations. Part of the LRI was not visualised and very few anionic sites were noted in the LRI and the LD.

African (N=6)

The mean number of anionic sites was decreased compared to controls (14.19 ± 4.32) The GBM appeared to be thinner and complete fusion of the podocytes was noted along the GBM. The labelling was more diffuse, although an array of anionic sites was noted along the LRE. The LRI was not visualised in some samples and very few anionic sites were noted. A few scattered labelling spots were seen in the LD.

The two ethnic groups taken together showed a significant difference in the number of anionic sites compared to controls ($p<0.014$).

MGN (Figure 9D)

HbsAg positive (n = 12)

The mean number of sites in the LRE were reduced compared to controls (15.98 ± 2.61). Scattered labelling was noted in the region of LRI but very little in the LD. Glomerular anionic sites were absent in the area around the deposits.

HbsAg negative (n = 2) (Figure 8E)

The mean number of anionic sites were reduced compared to controls (13.06 ± 2.9). The membrane was thickened and the LRE was not clear. No labelling was seen in and around the epithelial deposits and very few were seen in the LD.

In the MGN group taken as a whole there was a significant reduction of PEI binding to anionic sites compared to controls (14.18 ± 3.83 $p<0.005$).

MPGN (Figure 9F)

There was a significant loss in the number of anionic sites in the 5 patients compared to controls (20.04 ± 1.69 $p< 0.046$). The GBM was thinner and the podocytes were not fused. Clear distinct labelling of anionic sites was noted along the LRE and between the fenestrations. Very little labelling was noted in the LRI and LD.

CORRELATION WITH PROTEINURIA

PEI labelling to anionic sites and Up/c ratio correlated inversely and moderately only in children with the MCNS ($r= - 0.6$) suggesting that proteinuria in this disease may be significantly due to loss of these anionic sites. No such correlation was found in the group as a whole or in the other groups.

Electron Micrographs of Glomerular Basement Membrane (GBM) stained with PEI in different histological groups

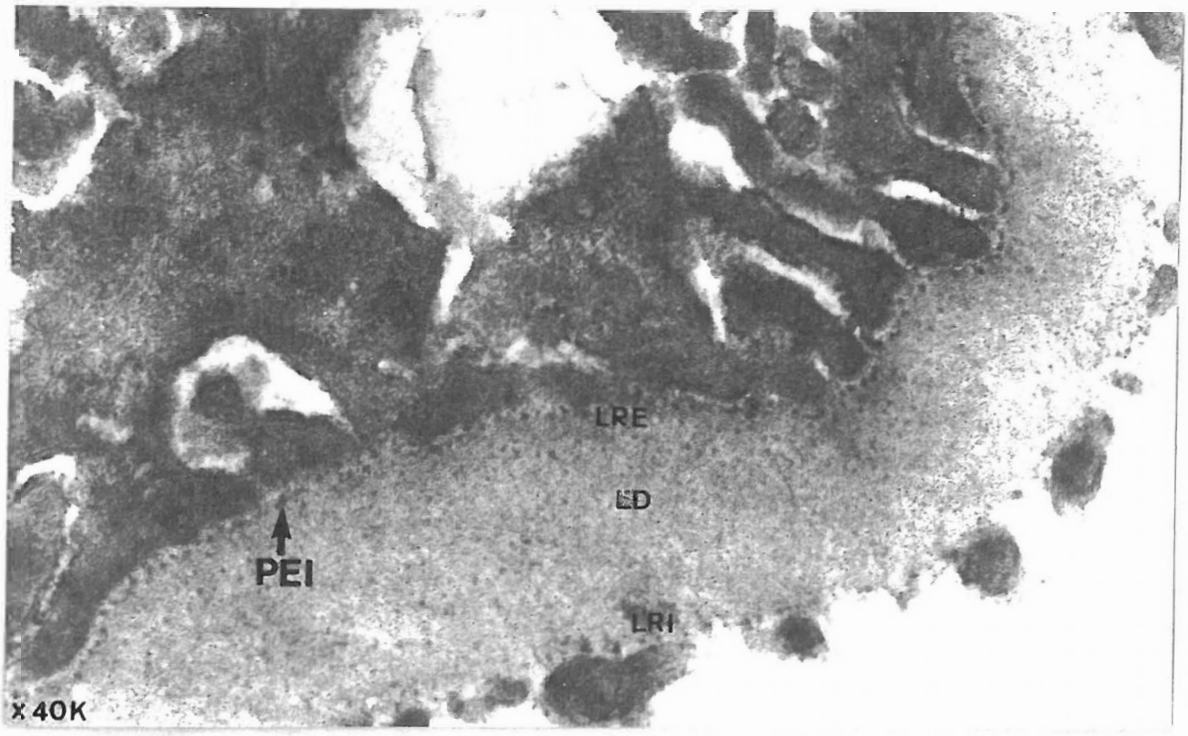

FIGURE 9A : Control

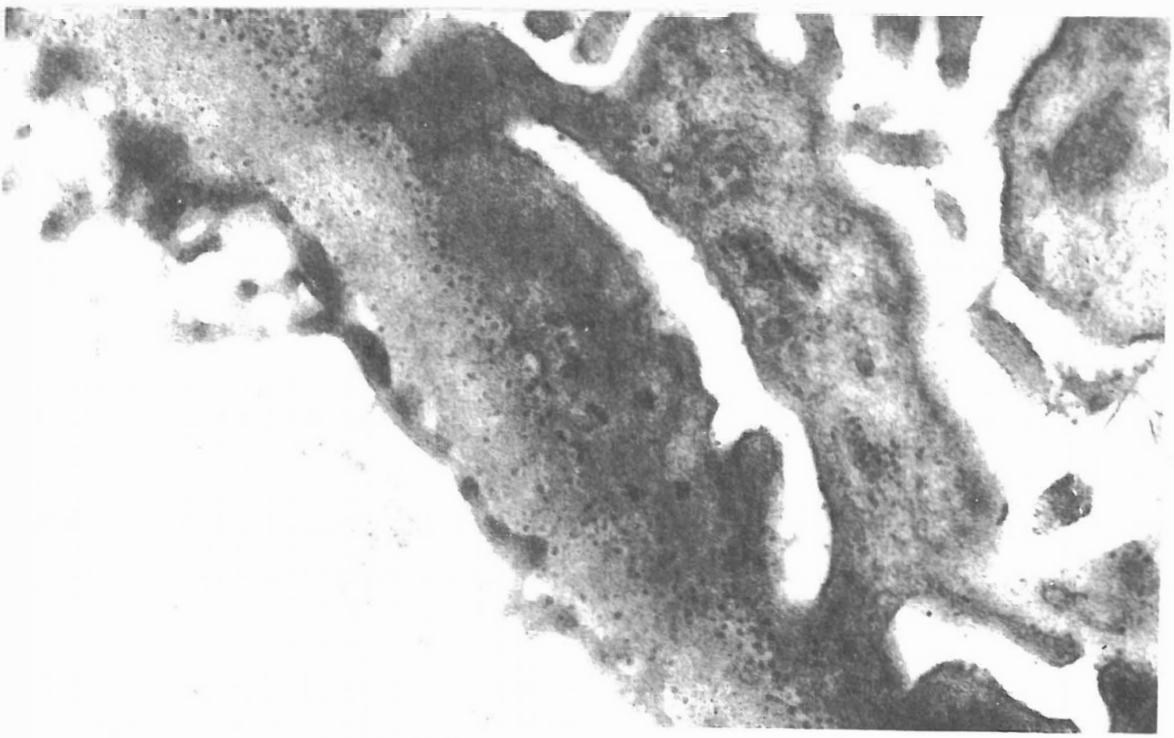

FIGURE 9B : MCNS

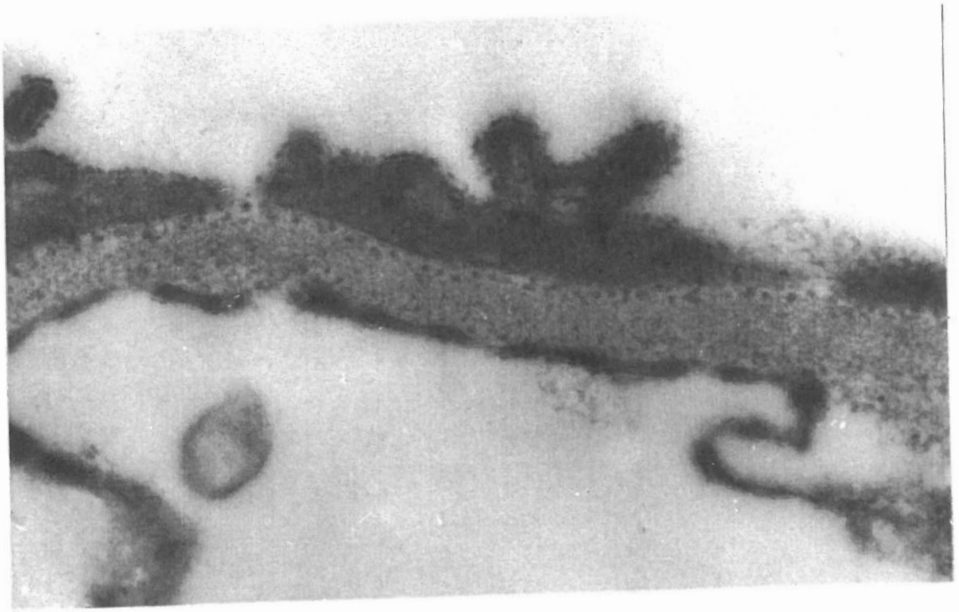

FIGURE 9C : FGS

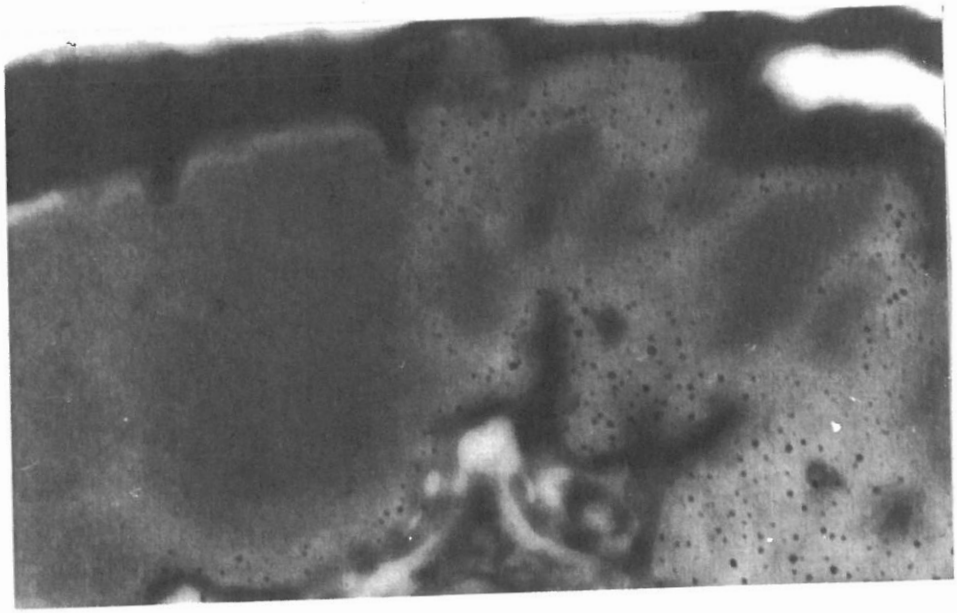

FIGURE 9D : MGN (HBsAg)

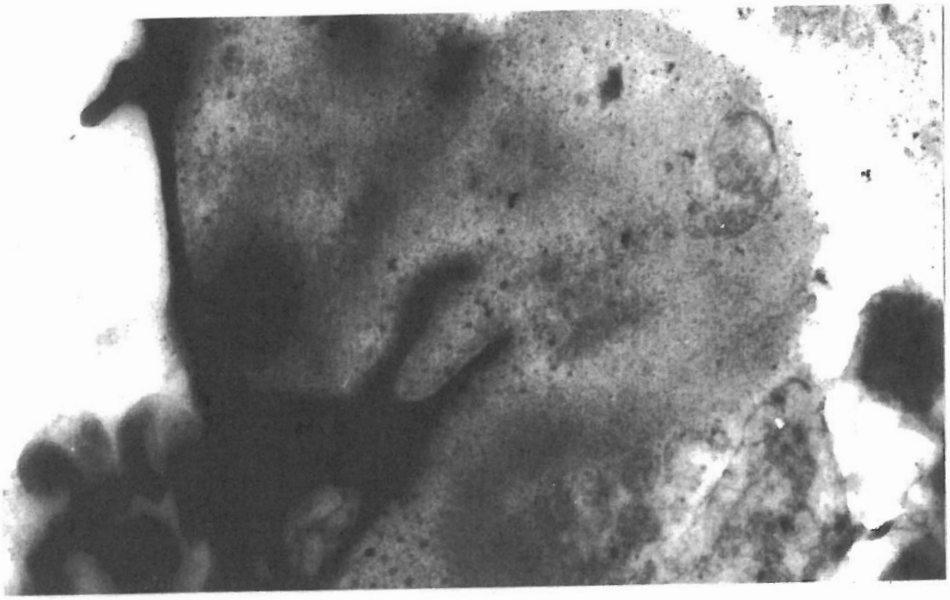

FIGURE 9E : MGN (HBsAg negative)

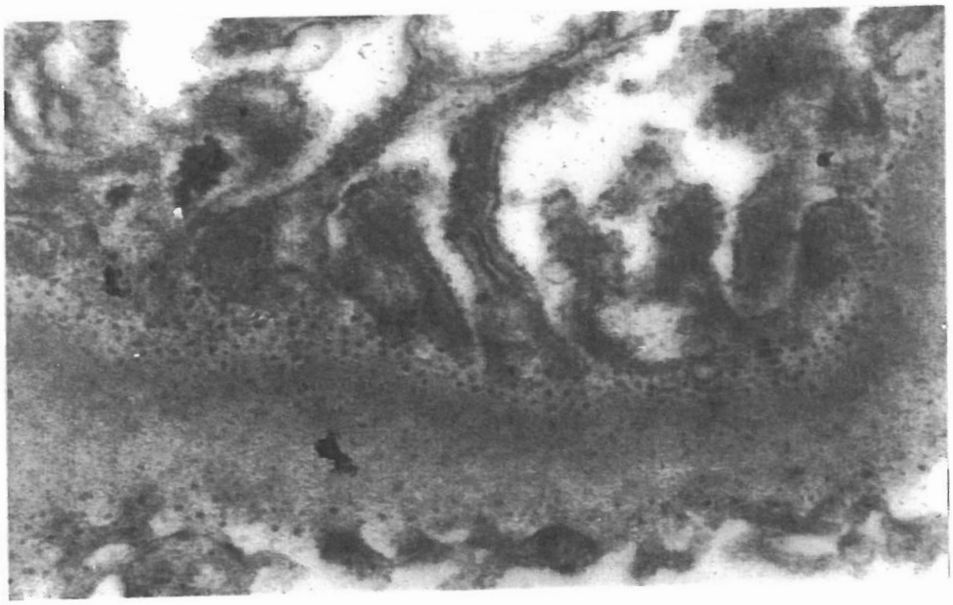

FIGURE 9F : MPGN

3.5 DISCUSSION

The main findings in this study are firstly, a diminution of anionic sites on the GBM which is common to different histological types of childhood NS, secondly, roughly similar degrees of reduction in anionic sites in MCNS, FGS, MGN and MPGN, and finally, an inverse correlation between anionic site number and the extent of proteinuria in MCNS only.

Experimental evidence and work in human subjects suggest that loss of negative charge of the GBM leads to proteinuria (Vernier et al 1983, Michael et al 1970). Therefore the results from the present report can be interpreted to mean that a decrease in the electrostatic charge of the GBM in the diseases studied may account in part for the escape of serum proteins across the glomerular capillary wall into the urine. This correlation is closest, albeit still modest, for MCNS, indicating that the reduction of anionic charge in the GBM contributes significantly to proteinuria in this disease. The absence of this association in the other histological categories of NS suggests that the decrease in anionic sites is not a major factor in the pathogenesis of proteinuria. However an alternative explanation for the results obtained is that the methods for detecting changes to the electrostatic properties of the GBM are not sufficiently sensitive to uncover the links with proteinuria. These findings are consistent with other studies which have concluded that the loss of glomerular electrostatic charge is the prime mechanism responsible for proteinuria in MCNS, whereas size selectivity plays a major role in the other varieties of NS (Cameron and Blandford 1966, Michael et al 1970, Carrie et al 1981). The wider implications of the above findings are interesting but remain speculative. The many characteristics which distinguish MCNS from other types of childhood NS are well known and the findings given above on electrostatic charge of the GBM in MCNS may simply be another such feature, or may reflect a more fundamental difference. It would be important, for example, to investigate the effect of steroids on restoring anionic balance in basement membranes.

As MCNS is rarely biopsied in most centres, only 4 such patients were

available; findings in these were uniform. In most patients in this study the depletion of anionic sites was pronounced in the LRE; differences were less marked in the LRI and LD. However in patients with MPGN there was a considerable loss of anionic sites in the LRI and LD; findings which are similar to those reported by Wada et al (1990). The LRI was also markedly abnormal in FGS where it was found to be interrupted and sclerosed with very few anionic sites in 7 of the 8 patients studied. One hypothesis (Kitano et al 1993) postulates that the changes detected in the LRI might alter the structure of the GBM thereby impairing its capacity to act as a - size- and shape- barrier to circulating proteins. The findings in MPGN, by this reasoning, may account for some adverse clinical features of the disease. Our findings in MGN are similar to those reported by Okada et al (1986). The anionic sites were markedly diminished in areas of sub-epithelial deposits, but were seen where these deposits were absent. Schneeberger et al (1993) suggested that immune complexes are formed "in situ" by the interaction of antibodies with epithelial surface glycoproteins. These immune complexes accumulate in the LRE and destroy or mask the heparan sulphate anionic sites.

The factors which precipitate relapse or maintain proteinuria in nephrosis are not known. Infections often precede an attack of oedema and proteinuria. There is a gap in our understanding of the connections (if any) between these events and disturbances in the electrostatic charge properties of the GBM. Levin et al (1989) reported the presence of a circulating macromolecule in the plasma of patients with steroid responsive NS responsible for neutralising the glomerular anionic sites. Tanaka et al (1992) and Yoshizawa et al (1989) suggested that it is the lymphocyte in MCNS which produces this cationic factor. The initial triggering event could be an interaction between the precipitating agent and immune cells.

This study has provided further evidence that depletion of anionic sites on the GBM is a common feature in the different histological types of NS

of childhood and may account to a limited extent for proteinuria in these diseases, except for MCNS where this abnormality probably plays a larger role. The lack of correlation between GBM charge and protein loss in the urine makes it likely (from other lines of evidence) that the major cause of proteinuria in FGS, MGN and MPGN is the failure of the barrier function of the GBM to circulating proteins on the basis of their size.

3.6 SUMMARY

The relative contributions to proteinuria made by the loss of ionic charge and defects in the size-selective barrier of the glomerular basement membrane (GBM) are not known. The number and distribution of glomerular anionic sites using polyethyleneimine (PEI) was measured and the ultrastructural changes in the adjacent GBM of 33 children with nephrotic syndrome studied.

Compared to the number of PEI labelled anionic sites in the lamina rara externa per 1000nm length of the GBM in 8 controls Mean ± SD; 25.0 ± 1.49; there was a significant decrease in 4 patients with minimal change nephrotic syndrome (15.25 ± 2.98 $p<0.05$); 10 patients with focal glomerulosclerosis (16.0 ± 5.1 $p<0.014$); 14 patients with membranous nephropathy (14.18 ± 3.83 $p<0.005$) and 5 patients with membranoproliferative glomerulonephritis (20.04 ± 1.69 $p<0.036$). Loss of labelled anionic sites in the lamina rara interna were seen in patients with FGS and MPGN only. A moderate inverse correlation between anionic site numbers and proteinuria (estimated by urinary protein creatinine ratio) was found in MCNS only ($r = -0.6$).

These findings suggest that a reduction in the glomerular anionic sites may be only partly responsible for proteinuria in the different types of childhood nephrosis, except for MCNS where it probably plays a major role. Altered size and shape selectivities due to structural changes in the GBM are likely to account for most of the proteinuria in NS with obvious histological lesions.

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CHAPTER 4

ANIONIC CHARGE ABNORMALITIES OF RED BLOOD CELLS AND PROTEINURIA IN GLOMERULONEPHRITIDES

CHAPTER 4

ANIONIC CHARGE ABNORMALITIES OF RED BLOOD CELLS AND PROTEINURIA IN GLOMERULONEPHRITIDES

4.1 INTRODUCTION

In the previous chapter mention was made of an association between loss of electrostatic charge and proteinuria in NS by direct measurement of anionic sites on the GBM. It was noted that anionic sites numbered variably in the different histological groups. Due to lack of readily available renal tissue to measure surface negative charge, Levin et al (1985) devised a simple chemical test using the presence of anionic sites on Red Blood cell (RBC) membrane to measure cell-surface negative charge. The method is based on the binding of the cationic dye alcian blue (AB) to anionic groups on the RBC surface. Levin et al (1985) suggested that altered RBC binding of AB reflected changes at glomerular level and these changes were more pronounced in steroid responsive nephrotic syndrome. Moreover, he suggested that any change in GBM charge is likely to be part of a generalised disorder of negative charge affecting all membranes. Validity of the technique of AB binding to RBC has been the subject of some controversy (Feehally et al 1986, Sewell 1986), however, an improvement of the method by Bernard et al (1989) has now increased the reliability of the test. Moreover the available data is restricted to children with MCNS and there is little information on the differential effects of other glomerular diseases on the charge- and the size- selective barriers.

The present study was under taken to explore these issues by correlating the degree of proteinuria (assessed by measuring urinary protein/creatinine ratio or 24 hour proteinuria), histopathological abnormalities and the effects of age, individually, with loss of anionic binding sites on RBC in a spectrum of glomerular diseases encountered in children and adults.

4.2 PATIENTS

Fifty eight children (aged 3-14 years) and 29 adults (aged 16-44 years) were studied. The reason adult patients were included in this study was because it has been reported that anionic sites on the RBC membrane decrease with age (Levin pers.comm) and it was important for this study to assess this correlation of AB binding with age. The second morning sample of urine was collected for the estimation of urinary protein and creatinine in children whereas in adults a 24 hour urine sample was taken for quantification of proteinuria.

Similar age-matched healthy children and adults were taken as controls. There were 58 children and 29 adult controls.

For the purpose of this study the patients were divided into the following groups.

GROUP I: STEROID RESPONSIVE NEPHROTIC SYNDROME (MCNS)

There were 2 African adult males and 30 children (22 males, 15 females) of whom 27 were Indians and 3 Africans. The two adults and three african children were biopsied.

GROUP II: FOCAL GLOMERULOSCLEROSIS (FGS)

There were 7 children (6 males,1 female) and 2 adult males. Two children were Indian the rest African. All were biopsied.

GROUP III: POST STREPTOCOCCAL GLOMERULONEPHRITIS (PSGN)

There were 4 children (3 males 1 female) and 1 adult male. All were Africans. Only the adult was biopsied. The diagnostic criteria for this condition have been previously described (Coovadia 1987).

GROUP IV: MEMBRANOUS NEPHROPATHY (MGN)

In this group there were 16 children (11 males, 5 females) and 1 adult (female), all were African and all were biopsied. Fourteen children were HBs antigen positive.

GROUP V: MESANGIOCAPILLARY GLOMERULONEPHRITIS (MPGN)

There were 11 adults and 1 child (female). All were African, biopsied and all were Type I MPGN.

GROUP VI: MISCELLANEOUS

There were 14 African adults (7 males,4 females) of whom 4 had SLE, 1 had Amyloidosis, and 5 had chronic glomerulonephritis. All were biopsied.

4.3 METHODS (Figure 11)

4.3.1 Alcian blue binding to RBC (ABRBC)

The AB technique has been previously described (Levin et al 1985, Bernard et al 1989). The technique was used with the following modification: 10ml of citrated blood was centrifuged and the RBC's were separated from below the buffy coat and mixed with twice it's volume of phosphate buffered saline (PBS). The cells were washed in PBS four times for 10 minutes each time.

AB 8GX (76%, lot 12h-4375) was obtained from Sigma chemicals St Louis, MO,USA. 1% of AB was made up in PBS on the day of the assay, filtered and the final concentration adjusted to 250ug/ml. In our laboratory AB concentration was found to be suitable at 150ug/ml for all assays.
RBC's were resuspended in AB solution and incubated at 37°C for 30 minutes, after which the suspension was centrifuged for 5 minutes. The supernatant was measured in a spectrophotometer at 650nm. Each determinant represents mean of three assays. The quantity of ABRBC was expressed as nanograms of AB bound to RBC's.

In the experimental conditions used in this study, the reproducibility of the method was acceptable because intra-assay variation of ABRBC was quite low (MEAN ± SD) 0.056 ± O.78 ngAB/10 RBC's. The technique is described in detail in Chapter 2.

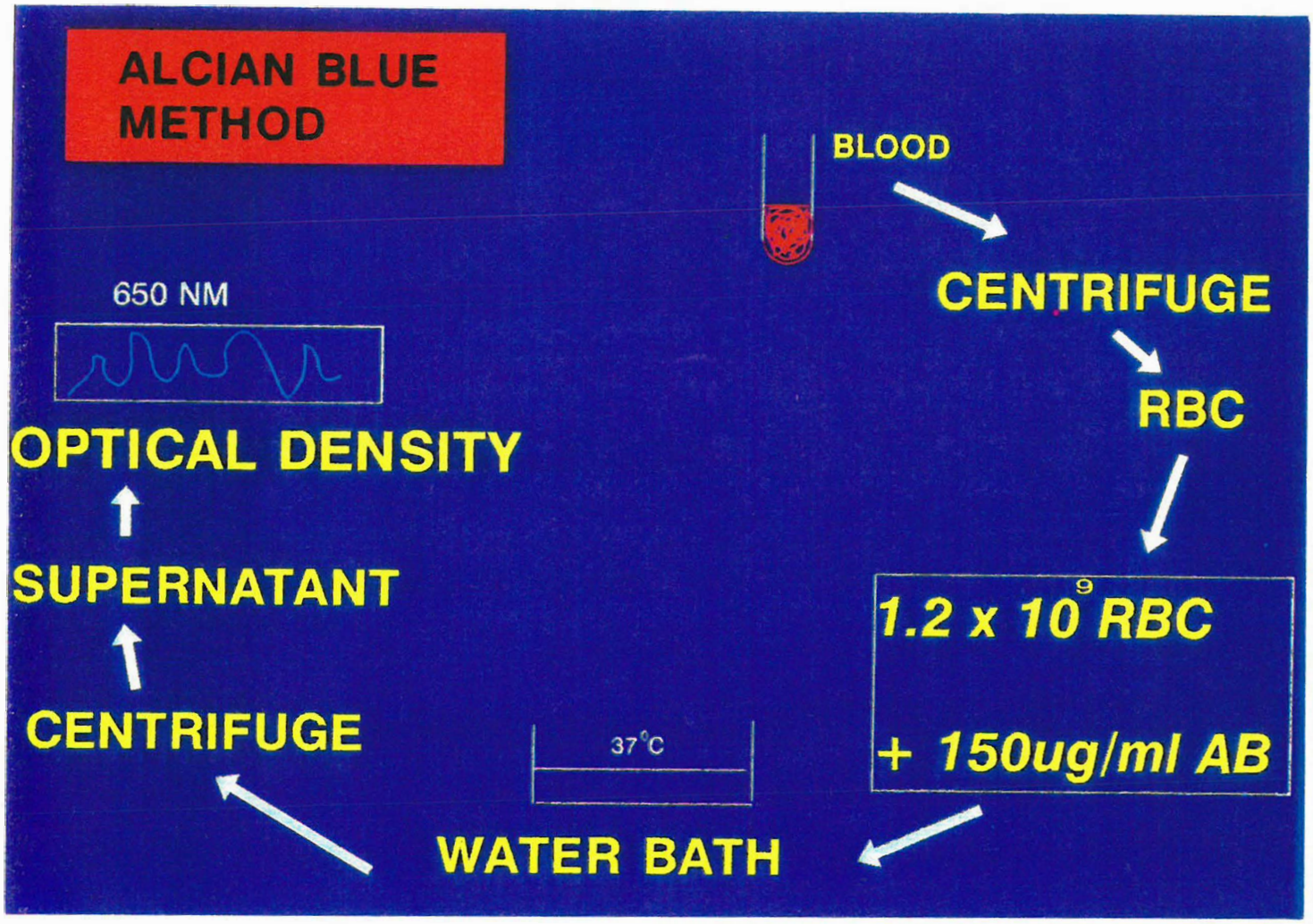

FIGURE 11 : **Binding of cationic dye Alcian Blue (AB) to anionic sites on red blood cell membranes**

4.3.1 Quantification of proteinuria

Second morning samples of urine were collected in children for estimation of proteins. Estimation of urinary albumin (isoelectric point 5.2) would have been ideal. However, the data was unavailable, therefore, estimation of total protein was carried out to assess proteinuria.

Total protein was measured by the biuret method (McIntosh 1977) and creatinine was measured by the Jaffé method (Slot 1965). Urinary protein/creatinine ratio (Up/c) was then calculated (Abitol et al 1990). Detailed account of the method is given in Chapter 2.

In adults total protein estimation was done on a collection of a 24 hour urine sample.

4.3.3 Statistical analysis

SAS package was used for all statistical analysis. Spearman correlation coefficient were calculated for association between AB/Up/c. Histological groups were compared using the Wilcoxin two sample test and analysis of variance. In addition regression analysis was performed to describe the above relationships.

4.4 RESULTS (Table VI,VII)(Figures 12,13,14)

A statistical significant reduction of ABRBC was observed in all children with renal disease, groups (I-V), compared to their controls (41.3 ± 21.5 vs 75.68 ± 8.64, $p<0.001$). This decrease, from their respective controls, was greatest for SRNS (40.57 ± 16.69 vs 75.09 ± 7.813, p=0,0001) but was also detected in the remainder of the paediatric patients (groups II,III,IV,V) (52.315 ± 26.07 vs 79.44 ± 5.88 p=0,001). There was a similar reduction in adult patients (groups I - VI) (23.5 ± 15.14 vs 72.85 ± 21.55, $p<0.001$).

ABRBC correlated moderately ($r = 0.4$, $p<0.001$) with Up/c ratio in the children with renal disease in all groups (Figure 1); correlation with

proteinuria was not seen in adult patients. ABRBC in children and adults was not significantly different between histological groups although there was a trend towards lower mean binding in childhood MCNS ($p = 0.0518$).

A moderate negative correlation ($r = -0.399$, $p < 0.02$) was detected between ABRBC and age in all patient groups; this finding was reinforced by the detection of lower AB binding in adults compared to children with glomerulonephritis (Groups I-V) (23.55 ± 15.14 vs 41.3 ± 21.55, $p < 0.001$). No such correlation was noted between ABRBC and age within the control subjects.

TABLE VI: MEAN ABRBC BINDING IN DIFFERENT HISTOLOGICAL GROUPS OF CHILDHOOD KIDNEY DISEASES

STUDY GROUP	Mean AB binding ng 10^6/RBC	
	PATIENTS	CONTROL
MCNS		
n = 30	40.57 ± 16.69	75.09 ± 7.81#
NON-MCNS*		
n = 27	52.31 ± 26.07	79.44 ± 5.88

p = 0.0518

* 7 FGS, 16 MGN, 4 PSGN

TABLE VII: MEAN ABRBC BINDING IN DIFFERENT HISTOLOGICAL GROUPS OF ADULT KIDNEY DISEASES

STUDY GROUP	Mean AB binding ng10^6/RBC		
	PATIENTS	CONTROLS	
MCNS n = 2	23.8 ± 5.68	80.18 ± 20.5	p = 0.0294
FGS n = 2	22.61 ± 3.55	37.4 ± 43.27	
MPGN n = 11	32.2 ± 15.49	73.25 ± 6.56	p = 0.001
MGN n = 3	34.12 ± 23.2	62.63 ± 36.4	

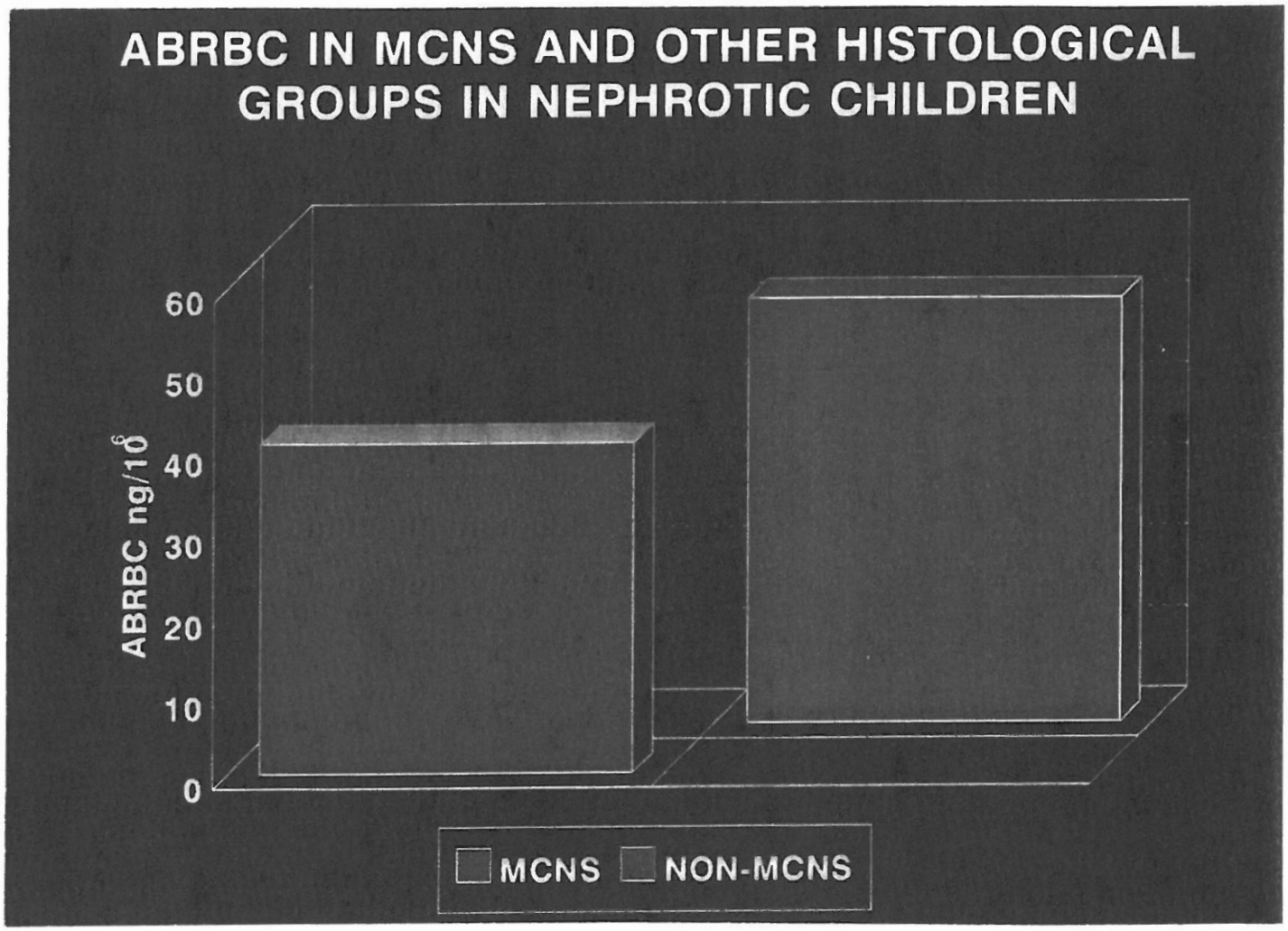

FIGURE 12 : Alcian Blue binding to red blood cells in MCNS and other histological groups of nephrotic syndrome in children

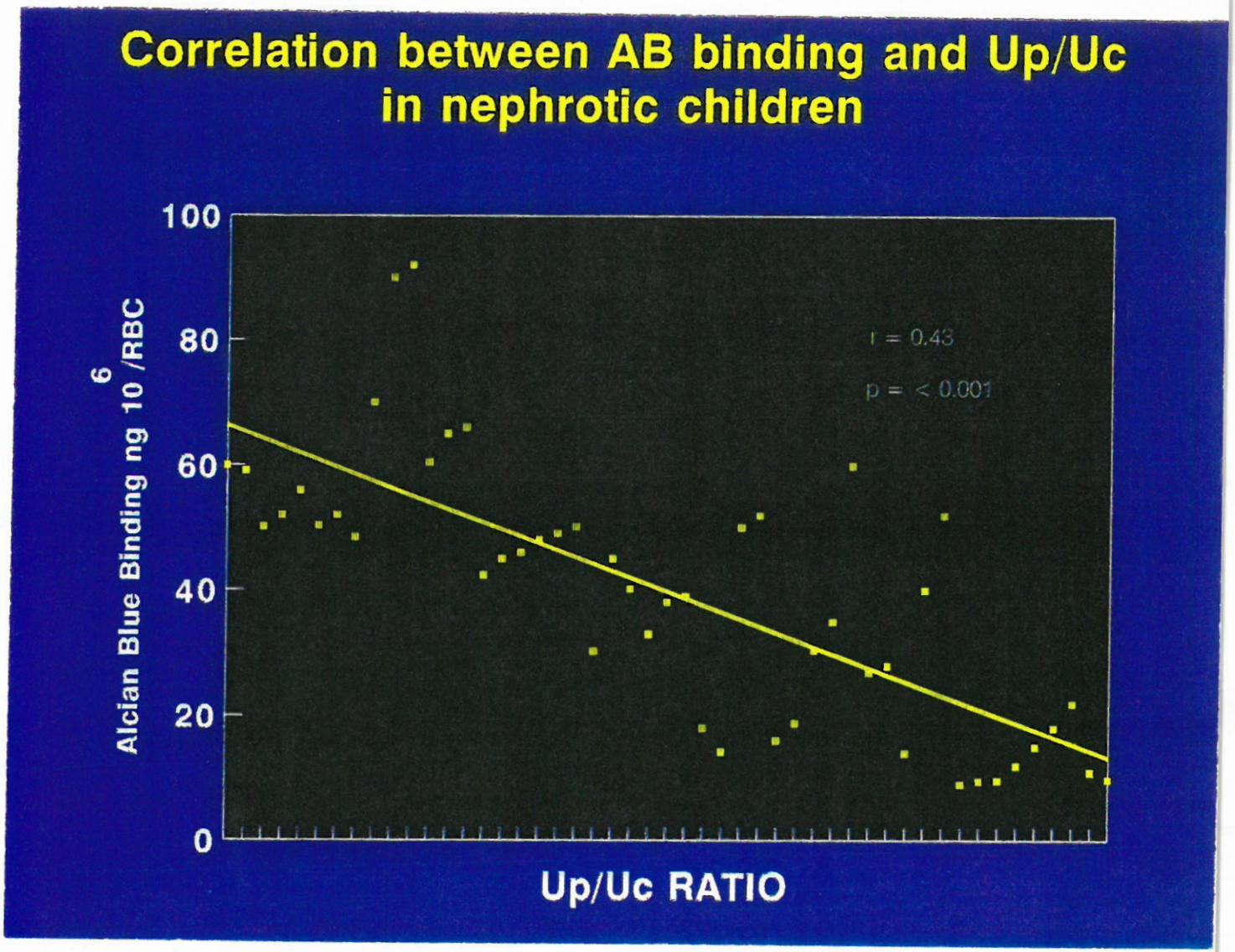

FIGURE 13 : Correlation between Alcian Blue binding to red blood cells and urinary protein/creatinine ratio

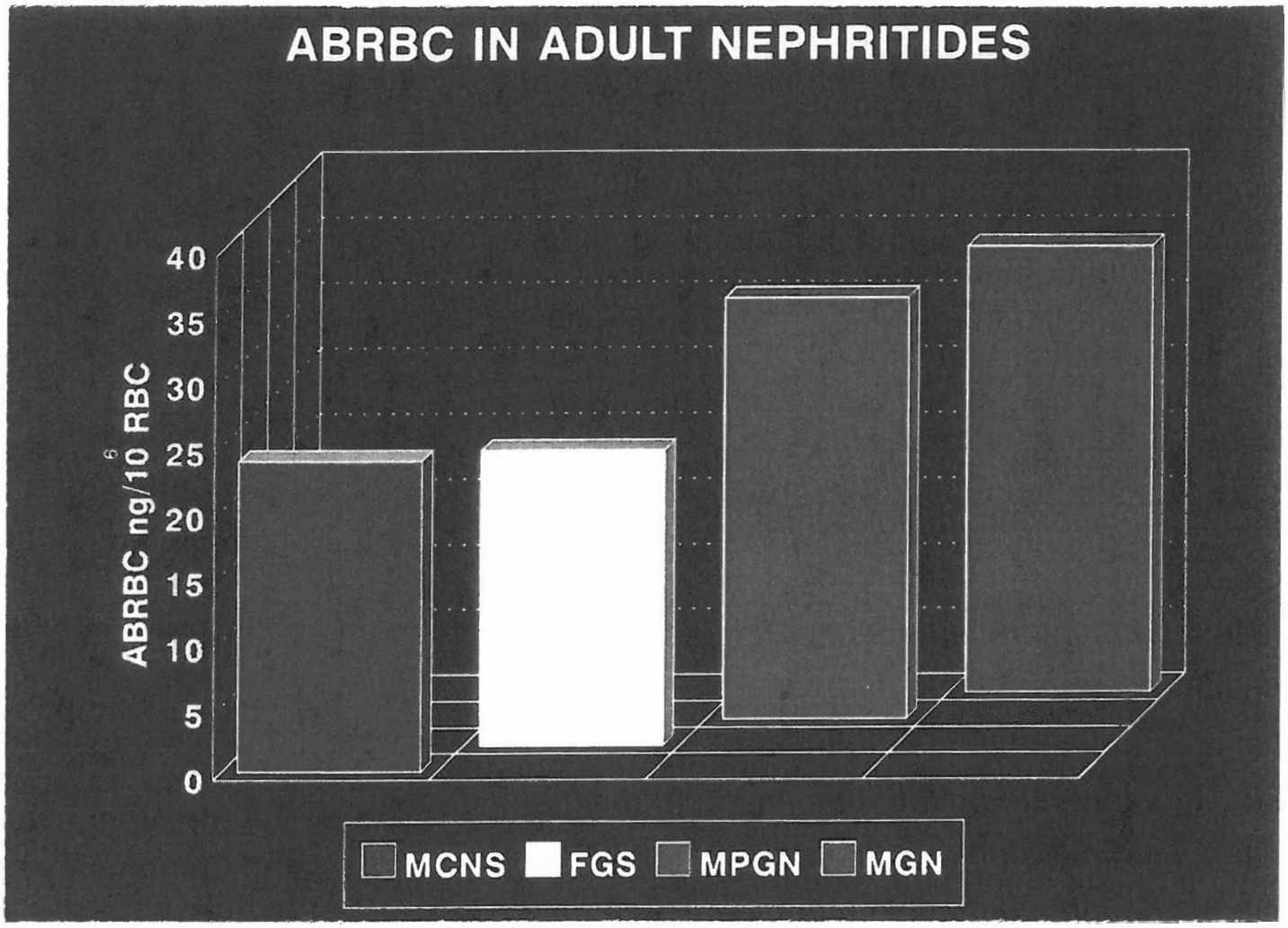

FIGURE 14 : **Alcian Blue binding to red blood cells in adult nephritides**

4.5 DISCUSSION

This study has shown that the extent of the electrostatic charge defects on RBC membrane as measured by AB binding correlated directly with the degree of proteinuria in some common glomerular diseases of childhood. This defect was also shown to be age- dependent, with higher ABRBC values present in children. Moreover, the diminution of anionic charge on the RBC membrane, previously described mainly in steroid responsive nephrotic children, has been shown to be a feature of other childhood and adult nephritides. These findings suggests that loss of anionic charge loss may be a generalised defect to a certain degree in all renal diseases and may be occurring secondary to biochemical abnormalities such as hyperlipidaemia, hypoproteinemia and platelet hyperaggregatibility (Cameron 1990). In addition other triggering agents such as immune complexes in HBs and HBe in MGN, possible T cell defects in MCNS and blood flow abnormalities in FGS may also have a contributory role in the initial injury to the glomerular polyanion.

The moderate degree of association observed between proteinuria and ABRBC in the children with renal diseases, considered as a whole, suggests that the electrostatic charge of the GBM may be only partially responsible for proteinuria; alterations to size and shape of the pores on the GBM may account for the rest of the protein loss. This is in accordance with the current understanding that proteinuria is due to abnormalities of pore-size and shape, and anionic charge on the GBM, but that there may be differences in their relative importance in a variety of renal diseases. Recent ultrastructural studies of the GBM suggest that loss of charged sites per se may induce structural abnormalities leading to an effective increase in pore size along the GBM (Washizawa et al 1993) (Chapter 3).

The marked reduction of ABRBC in childhood MCNS in contrast to other histological groups, probably reflects the major contribution to escape of proteins in the urine made by decreased anionic charge of the GBM in

MCNS and the lesser role of this mechanism in other histological types. This hypothesis is reinforced by the recognition that structural changes, which are slight in SRNS but often extensive in other glomerular diseases, are likely to underpin the increase in the pore size, thereby altering the size-pore selectivity of the GBM [Meyers and Guash 1994]. These findings reinforce the observations made in the previous chapter. However the matter may not be so clear cut; both processes may operate to differing degrees in individual diseases. For example, recent reports suggest that alterations of pore-size and density together with involvement of shunt pathways may also contribute to proteinuria in MCNS (Myers and Guash 1994). The results from children and adults reveal an age- dependent diminution of anionic charge on the RBC membrane in all patients. This may be due to the reduction of sialic acid content of cell membranes which occurs with age (Westberg and Michael 1973); findings which are supported by observations made by Levin et al (pers comm). No similar correlation was detected in the controls suggesting that this process is either absent or reduced during normal ageing and it is renal disease per se which is responsible for the differences in reduction of anionic charge between children and adults. The findings in this study of electrostatic change on RBC membrane are in accordance with direct examination of GBM using a variety of cationic probes (Kazuhiko et al 1993) (Chapter 3).

The reasons for the depressed levels of anionic sites are unclear; the systemic release of a polycation may be responsible for neutralising the negative charge on the RBC membrane in SRNS: it is likely that the same substance alters the charge or size barrier of the GBM (Barnes et al 1984, Levin et al 1989).

All the above findings are consistent with the hypothesis that loss of electrostatic charge in RBC mirrors that of GBM in kidney diseases. The study of an alternative tissues (RBC) to GBM is especially important in MCNS as biopsies are rarely undertaken and therefore glomerular tissues

often unavailable.

The pathogenesis of proteinuria in MCNS may be primarily, though not exclusively, due to loss of anionic charge, whereas in other histological groups, alteration of pore size and shape of the GBM is more likely to play a major role.

4.6 SUMMARY

Binding of cationic dye alcian blue to RBC membrane in NS has been shown to be an alternative non-invasive method of measuring GBM charge. Alcian blue (AB) binding to red blood cells (RBC) [ABRBC] was studied in 89 children and adults with a variety of glomerular diseases. ABRBC was significantly reduced in the group as a whole when compared with healthy controls ($p<0.001$). A moderate correlation between the degree of proteinuria and ABRBC was detected in the children with renal disease ($r=0.43$, $p<0.001$) but not in adult patients.

A significant reduction in ABRBC was detected in 30 of the above children who had steroid responsive nephrotic syndrome (MCNS) compared to their controls (40.57 ± 16.6 vs 75.09 ± 7.81, $p=0.001$). A similar decrease from normal values was observed in other childhood glomerular diseases: focal glomerulosclerosis, poststreptococcal glomerulonephritis, membranous nephropathy (52.31 ± 26.07 vs 79.44 ± 5.88, $p= 0.001$). Mean ABRBC was lower in MCNS than other histological groups ($p=0.0518$). ABRBC was age-dependent ($r= - 0.399$, $p<0.02$). These findings provide further indirect evidence of the relative roles of charge and size selective filters in GBM in renal diseases, and suggest that depletion of anionic charge may be a major cause of proteinuria in MCNS.

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CHAPTER 5

SDS PAGE OF URINARY PROTEINS IN STEROID RESPONSIVE AND STEROID RESISTANT NEPHROTIC SYNDROME IN CHILDREN

CHAPTER 5

SDS PAGE OF URINARY PROTEINS IN STEROID RESPONSIVE AND STEROID RESISTANT NEPHROTIC SYNDROME IN CHILDREN

5.1 INTRODUCTION

One of the central decisions to be made in the management of childhood nephrotic syndrome (NS) is whether the patient who is seen on first presentation has minimal change nephrotic syndrome (MCNS) or focal segmental glomerulosclerosis (FGS). The former is usually steroid sensitive, relatively easy to control and has an excellent prognosis while the latter is usually steroid resistant, difficult to manage and has a poor outcome (Figure 15). The best efforts of investigators have been unable to resolve the dilemma of whether MCNS and FGS are two distinct diseases or different histological positions on a continuous spectrum of a single disease entity (Churg et al 1970, Cameron et al 1978, Tejani et al 1983, Fogo et al 1990). If the latter is the case and the direction of progression is from MCNS to FGS then it will be exceedingly difficult to detect any subtle abnormalities which may distinguish the typical MCNS with an excellent prognosis, from MCNS which deteriorates to FGS. Indeed the results of a recent study illustrate precisely this point (Sesso et al 1992). Investigators have employed clinical, therapeutic, biochemical and histological criteria to differentiate one from the other but with only limited success, for even examination of renal biopsy material may fail to detect the scattered focal lesions (Churg et al 1970, Fogo et al 1990) and initially FGS may be steroid responsive (ISKDC 1981). As the success rate for the intensive regimens now being used for FGS substantially improves and the attendant side effects increase, it becomes especially important to distinguish this group from MCNS (Griswold et al 1987).

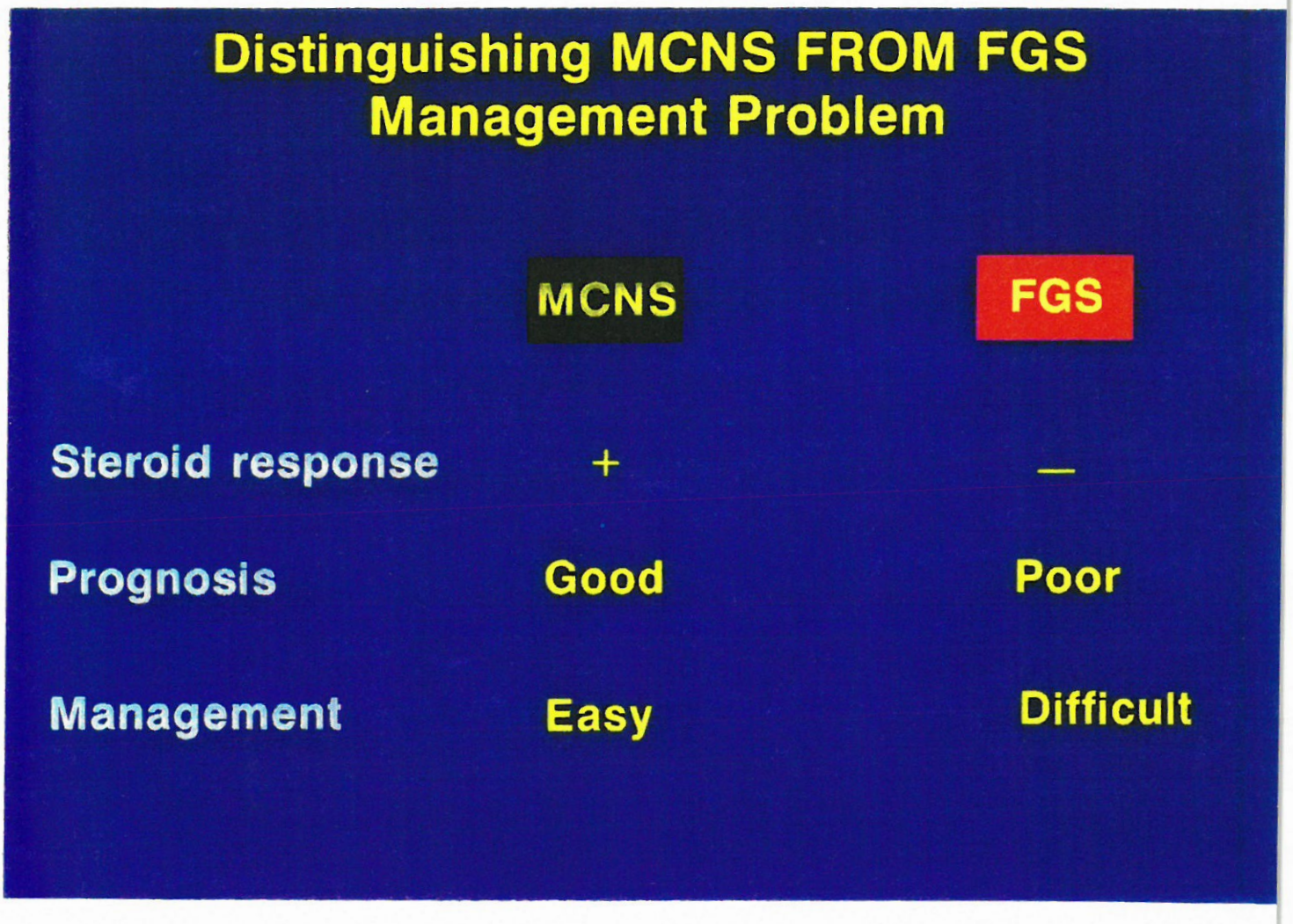

FIGURE 15 : Some diferences between MCNS and FGS

Greater accuracy in the identification of urinary proteins (glomerular or tubular) using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) (Brockelbank et al 1991) which separates protein molecules according to size, has led to better prediction of the underlying damage in kidney disorders (see diagram) although the separation of FGS from MCNS remains elusive.

This study reports the findings in Indian and African children which suggests that fine analysis of urine proteins with SDS/PAGE reveals different patterns in steroid responsive and steroid resistant patients and may therefore be useful in identifying FGS and MCNS.

5.2 PATIENTS

Fifty six children with NS (34 males, 22 females aged between 1-15 years) attending the renal clinic at King Edward VIII Hospital, Durban, were studied. A careful note was made of the relevant clinical and laboratory features and response to steroids, cyclophosphamide and other drugs during the course of the disease in each child. All children had routine investigations for renal function, including glomerular filtration rates.

Patients studied were divided by race (37 Indian and 19 African), and into 2 further groups, according to the time at which urine samples were collected. The one group is those whose urines were tested within 2 months of clinical presentation, and the other group comprises children who were first tested beyond this period. The 2 month cut-off period is used as nearly all steroid sensitive patients will have responded by this period.

Most (16 of 19) African children were biopsied since MCNS is unusual in these patients whereas Indian children underwent biopsy only if their clinical picture did not fit MCNS (Adhikari 1981; Coovadia 1987). Urine samples were taken from patients during relapse; in the steroid responsive children

at commencement of prednisone therapy. All steroid resistant patients were on a high protein diet and diuretics for the control of oedema. Some steroid responsive patients initially required diuretics if the oedema was severe.

Indian children

There were 37 children in this group: Mean age was 7,2 years and there were 27 males. All were given steroid therapy.

Patients at onset

Nineteen children had urine tested at the onset of NS (within two months). Seventeen patients were steroid responsive, and this, with their subsequent clinical course, suggested they had MCNS. The two children who did not respond to steroid therapy were shown to have FGS on renal biopsy.

Patients with established NS

Twelve of the patients tested later in the course of their disease; median of three years (range 6 months - 5 years) were steroid responsive and were presumed to have MCNS by the criteria given above; the remaining 6 patients were steroid resistant and renal biopsy in 4 patients indicated FGS: permission for biopsy was denied in the remaining 2 children. Two of the 6 steroid resistant patients who were given cyclophosphamide therapy did not respond to the treatment.

African children

There were 19 children. The mean age was 6.8 years and 6 were males.

Patients at onset

Twelve children had their urines tested at onset (within two months) and prior to steroid therapy. Three of these were steroid responsive and were presumed MCNS; the remaining 9 patients were steroid resistant and were biopsied (3 MCNS, 6 FGS). The steroid responsive patients had their urines tested prior to steroid therapy.

Patients with established NS

All 7 children tested later in the course of their disease; median two years (range 6 months - 2 years) were steroid resistant and had FGS on renal biopsy. One of these children had MCNS on initial biopsy, however the second biopsy after a period of 2 years showed FGS. One child was given cyclophosphamide but did not respond to treatment.

5.3 METHOD

SDS Page (Figure 16)

The second morning sample of urine was collected and preserved with 0,05% sodium azide, centrifuged, aliquoted and then stored at -20°C for future analysis.

Total protein in the urine was measured by the biuret method; samples with protein concentration of >3g/L were diluted to between 100-300mg protein/L prior to analysis,. 15-20 ug/ul protein was applied to the gel.

A vertical discontinuous electrophoresis system was used. Urinary electrophoresis was performed on a gradient gel of 7,5% -25% T acrylamide 4% C (constant) in the presence of SDS, for one and a half hours with the following electrical settings, 85 mA, 100V for half hour and 42 mA, 200V for remainder of the run. The gels were fixed in 4% acetic acid: 40% methanol prior to staining with coomassie blue. Technical details of the method are published elsewhere (Shapiro and Maizel 1967, Schiwara et al 1986) and are given in Chapter 2.

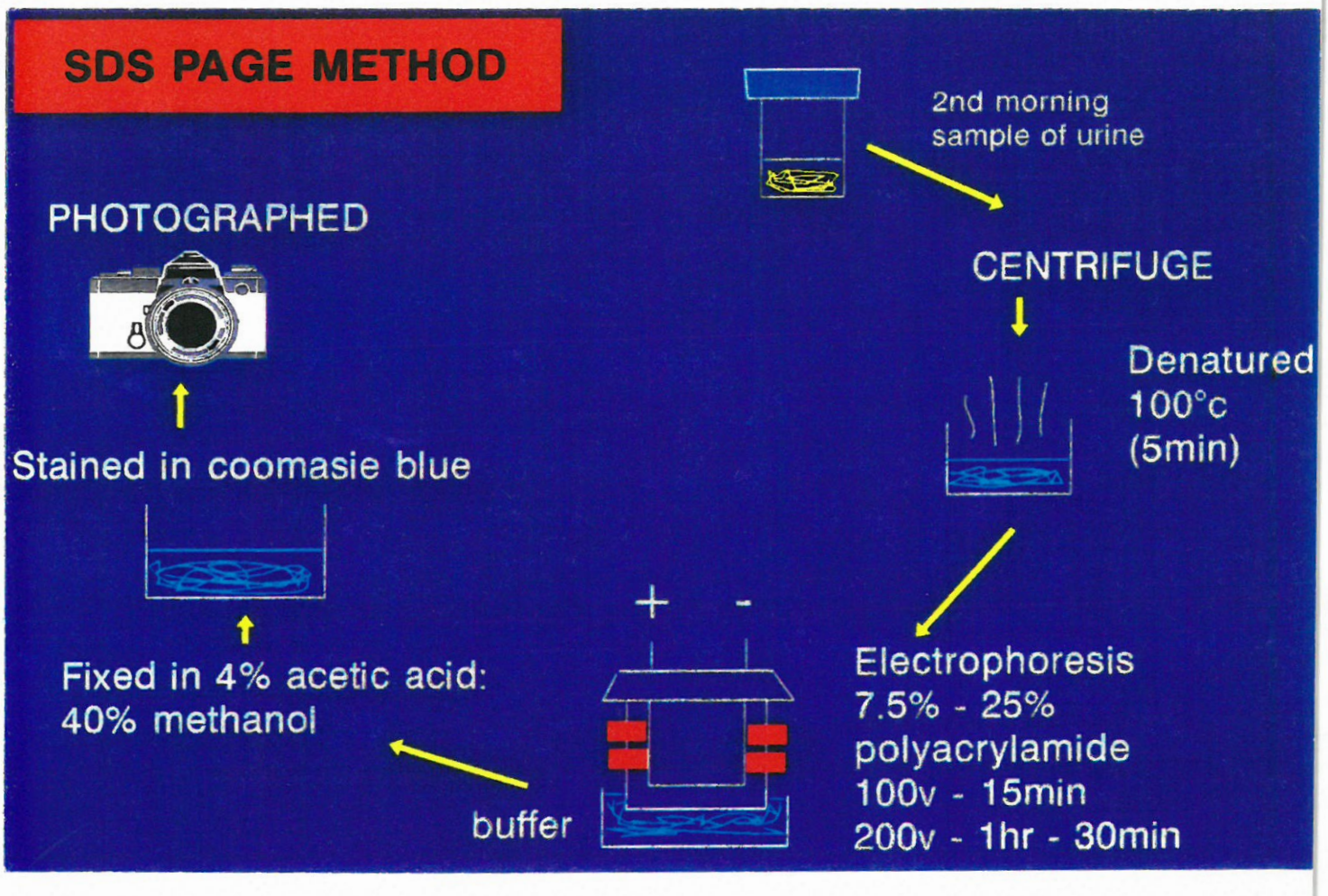

FIGURE 16 : SDS PAGE analysis of urinary proteins

Pure standards of albumin, transferrin, IgG, haptoglobulin, β_2M and Lysozyme (Sigma) together with high and low molecular markers (Biorad) were co-electrophoresed in order to identify specific proteins. These standards were co-electrophoresed for accurate quantification as binding of different protein molecules to the protein stain, (Coomassie blue) is non-stoichiomatic (Enrich and Wurster 1991).

Urine analysis was carried out without any prior knowledge of clinical or histological data. Correlations were then made between urinary electrophoresis outcome, clinical data and histology.

5.4 RESULTS (Table VIII) (Figure 17)

INDIAN CHILDREN

Patients at onset of NS

Glomerular proteinuria (i.e. bands of albumin and transferrin) was observed in 17 of the 19 children tested at onset. These children were steroid responsive and on two month follow up had gone into remission, no bands were detected on the SDS PAGE at this stage. Their subsequent clinical behaviour was typical of MCNS. The SDS PAGE pattern of proteinuria in the remaining 2 patients with FGS revealed presence of albumin,transferrin, IgG, β_2M and lysozyme. These patients were steroid resistant and the SDS PAGE pattern did not change on follow up.

Patients with established NS

The urinary pattern of proteinuria in 12 of the 18 children in this group showed presence of albumin and transferrin. All these patients were steroid responsive and on follow - up had the typical features of MCNS. A mixed pattern of (glomerular and tubular) of proteinuria (albumin, transferrin, IgG, β_2M and lysozyme) was detected in 6 children who were steroid resistant; renal biopsy in 4 of these patients indicated FGS. All 4 patients were initially steroid sensitive and their clinical behaviour was typical of MCNS.

Taken together, 29 children with glomerular proteinuria were steroid responsive and presumed MCNS, while 8 with glomerular and tubular proteinuria were steroid resistant. All 6 of the latter who were biopsied showed FGS. The pattern of proteinuria were identical in all the steroid resistant patients whether tested at onset or in the established stage was the same.

AFRICAN CHILDREN

Patients at onset of NS

In the 12 children studied at onset, glomerular proteinuria of albumin and transferrin was detected in 3 patients; the latter responded to steroid therapy and thus subsequent clinical behaviour was typical of MCNS. Bands of glomerular and tubular proteins (albumin, transferrin, haptoglobulin, IgG, β_2M and lysozyme) were detected in the remaining 9 patients who were steroid resistant (3 MCNS, 6 FGS).

Patients with established NS

The urinary protein pattern in the 7 children in this group, all of whom had FGS on renal biopsy revealed glomerular and tubular proteinuria on SDS PAGE. One child had MCNS on initial biopsy.

Diuretics did not have any effect on the pattern of proteinuria.

Table VIII: SDS patterns of urinary proteins in steroid responsive and steroid resistant nephrotic syndrome

	SDS PAGE					
	Alb	Trans	Hap	IgG	Lysozyme	β_2M
Steroid responsive (MCNS) N=31	+	+	-	-	-	-
Steroid resistant N=19 (FGS on biopsy)	+	+	+	+	+	+

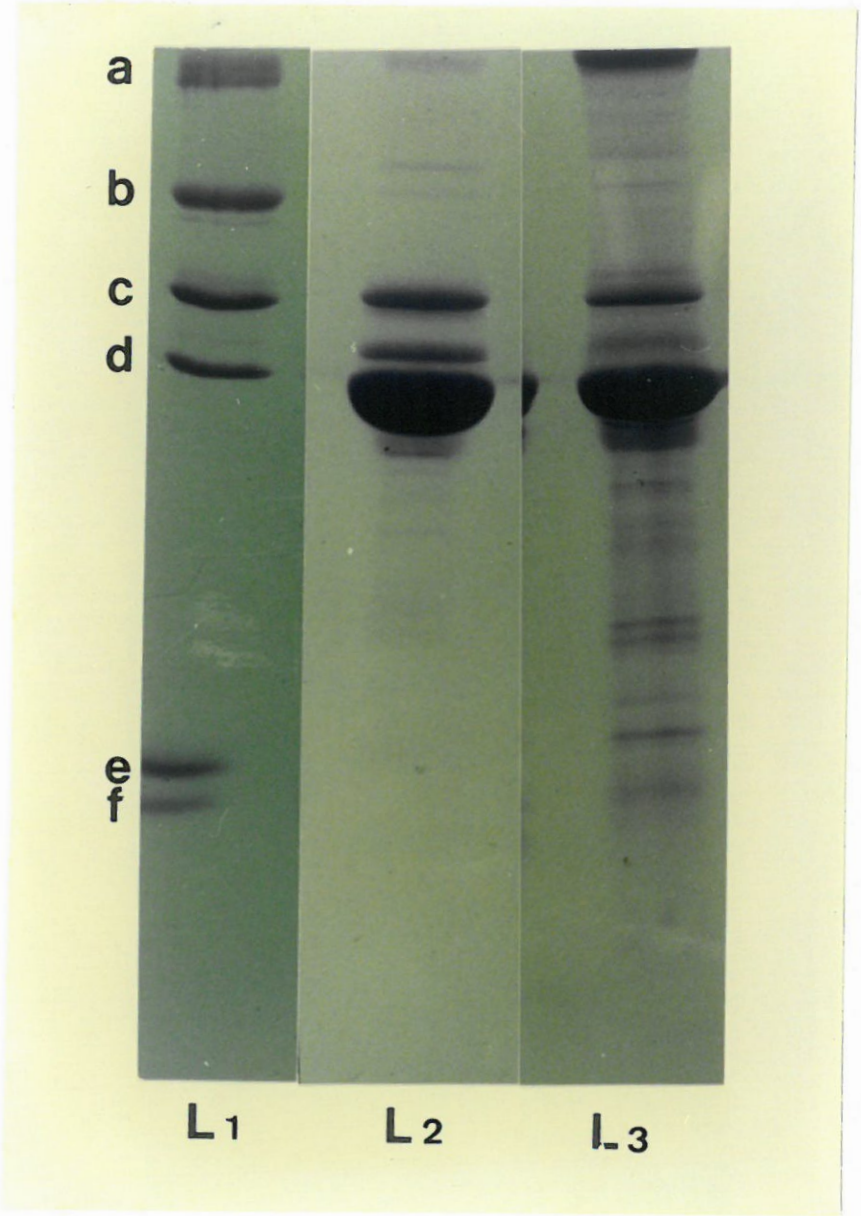

FIGURE 17 : Urinary patterns of proteinuria in MCNS and FGS
a = IgG; b = haptoglobulin; c = transferrin;
d = albumin; e = lysozyme; f = B_2M.
L1 = standards; L2 = MCNS; L3 = FGS

5.5. DISCUSSION

In this study we have shown that SDS-PAGE can be used to differentiate between steroid-responsive and steroid-resistant nephrotic syndrome. The former have selective while the latter have non-selective proteinuria. Other laboratory methods are available to measure selectivity, but require a blood sample and a more laborious procedure (Cameron and Blandford 1966). All the steroid responsive children are almost certainly MCNS whereas the steroid resistant children were found to have FGS. Presence of only glomerular proteinuria of albumin and transferrin in steroid responsive children and additional bands of haptoglobulin and IgG in steroid resistant cases confirm the findings reported by Brockelbank et al (1991) and Jackson et al (1992). However the difference is that all Brockelbank's patients were studied in the advanced stages of renal impairment whereas all our patients had normal GFR and were studied earlier in the course of the disease. The above findings could assist clinicians in the management of children with nephrotic syndrome: the decision to use steroids for presumed MCNS or other histological groups likely to benefit from such therapy can be made with some degree of confidence on the basis of urine protein analysis, therefore minimising the need for renal biopsy. It has been well documented that initially in FGS the biopsy tissue resembles that of MCNS (Churg et al 1970, Fogo et al 1990, Tejani et al 1983). This point is highlighted by the 5 children (4 Indian, 1 African) in this study whose urine indicated a mixed pattern of proteinuria which may suggest FGS; the initial biopsy in one African child showed MCNS but follow-up renal biopsy revealed FGS. Similar "progression" in the disease has been reported by other workers (Hayslett et al 1969, Tejani et al 1983, Tejani 1985).

Tubulo-interstitial damage is present in almost all cases of FGS, occurring in 87% in one series (McVicar et al 1980, South West

Paediatric Nephrology study group 1985) and is most probably the pathological basis of the tubular proteinuria. Furthermore tubular defects have been reported to be an early sign of FGS in nephrotic children (McVicar et al 1980). Our results have shown that in the 6 African and 2 Indian children with FGS where urine was analysed at onset of nephrotic syndrome, tubular damage was easily detected.

Although the numbers are small we suspect that the three African children classified as MCNS on histology but who were steroid-resistant and who had a glomerular and tubular urinary pattern, may have FGS. This conforms with our previous experience of such African children who on first biopsy had MCNS but on repeat biopsy indicated FGS. Urine analysis by SDS-PAGE is therefore of great importance in such patients. This interpretation is supported by studies of nephrotic syndrome carried out in Johannesburg (Lewin et al 1979) which have shown that the commonest histological lesion in African children is FGS. These arguments are only partly diminished by the criticism that the African children with minimal lesions on histology, may not have FGS, for the type of proteinuria will remain of value to the clinician who wishes to avoid the unnecessary and potentially harmful use of steroids in this situation.

Intensive therapy for FGS which is proving to be successful involves a 2 year course of methylprednisolone pulse therapy along with oral alkylating agent such as cyclophosphamide (Griswold et al 1987). In patients unsuitable for biopsy, due to azotaemia or a bleeding tendency, clinical findings correlated with urinary analysis by SDS PAGE, may be a useful adjunct in considering commencement of immunosuppressive therapy. In 6 steroid resistant Indian children with established NS mentioned previously who had persistent proteinuria and who were clinically unsuitable for biopsy, the SDS PAGE pattern revealed glomerular and tubular damage suggestive

of FGS. On commencement of pulse therapy, all patients improved and subsequent renal biopsy in 4 patients confirmed FGS. In the remaining patients lack of parental consent has precluded performing renal biopsy.

Ultra-thin layer SDS-PAGE in vertical/horizontal electrophoretic cell is easy to set up, provided all aspects of the technique are standardized. Total time from pouring the gel to complete staining is seven hours. Hence preliminary results can be obtained by the end of a working day. Automated commercial systems such as Phastsystem (Brockelbank et al 1991, Jackson et al 1992) (Pharmacia, Uppsala, Sweden) have been recommended recently for routine analysis of pathological urines; however costs need to be reduced if such systems are to be useful on a wider basis. One of the advantages of using the commercial system is that the final results are achieved in 2 hours. Immunoenzymometric assay in older patients has been shown to be helpful in identifying patients who are likely to be steroid responsive (Sesso et al 1992). However, the distinction between MCNS and FGS using the above technique was blurred.

SDS PAGE pattern of urinary protein makes the distinction between steroid responsive and steroid resistant nephrotic syndrome and may be useful in differentiating MCNS from FGS and other histological groups. However as we have been able to investigate only a small number of patients at about the time of onset of the disease, these preliminary findings need to be confirmed and consolidated by larger prospective studies.

5.6 SUMMARY

Distinguishing MCNS from FGS remains an intractable management problem in childhood NS. The former being steroid responsive whereas the latter steroid resistant.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of urinary proteins was performed in 56 children with nephrotic syndrome during relapse, of whom 31 had their urines tested within 2 months of the onset disease. All 32 steroid sensitive (presumed MCNS) patient's urines revealed albumin and transferrin bands only; whereas 19 steroid resistant children with focal glomerular sclerosis (FGS) showed additional excretion of IgG and low molecular weight proteins (lysozyme, $\beta_2$microglobulin). This mixed pattern of proteinuria was also detected in 5 other steroid resistant cases, 3 of whom were Africans with MCNS on biopsy and 2 were Indians who were not biopsied. Findings in patients studied within 2 months of presentation were identical to those investigated later in the course of the disease.

SDS-PAGE analysis of urine, which appears to distinguish steroid responsive from steroid resistant patients may provide a valuable adjunct to the management of childhood nephrotic syndrome.

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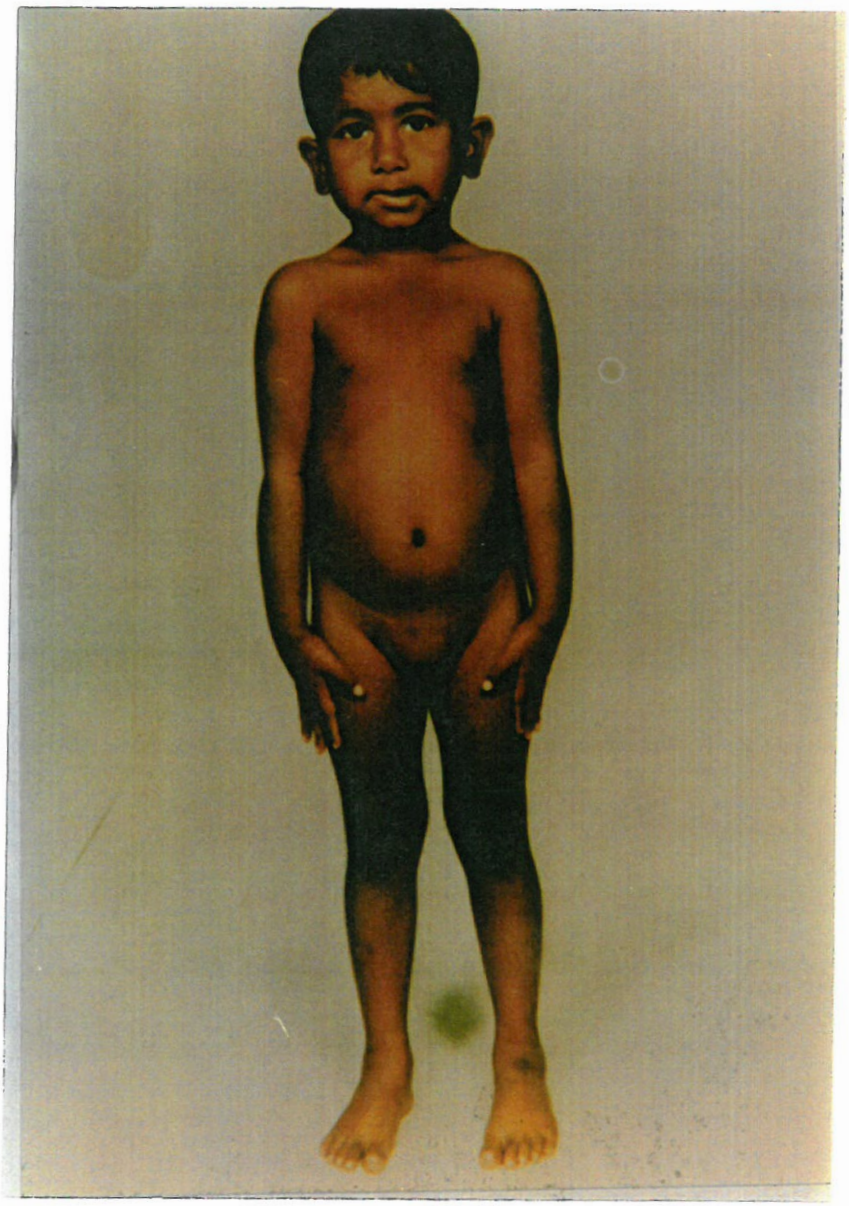
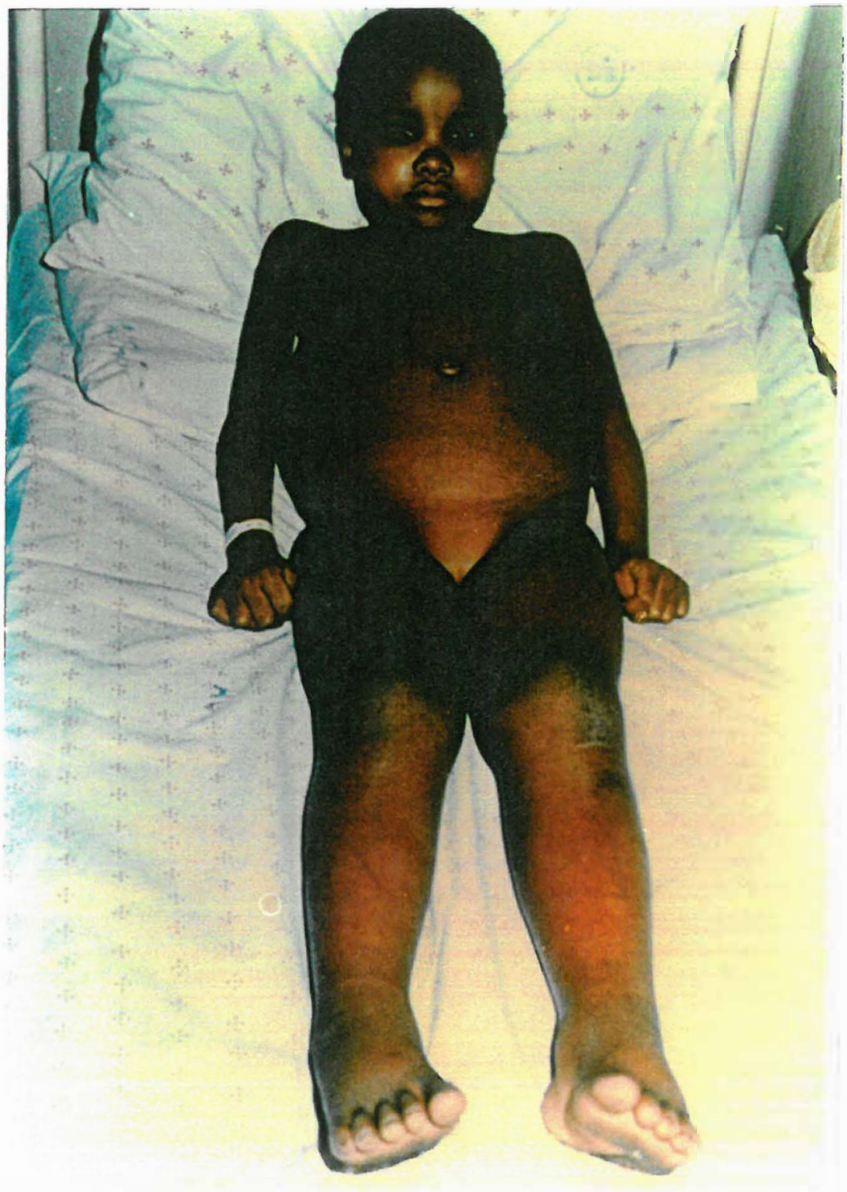
APPENDIX III



A CHILD WITH A TYPICAL STEROID RESPONSIVE NEPHROTIC SYNDROME (MCNS)

APPENDIX IV



CHILD WITH STEROID RESISTANT NEPHROTIC SYNDROME (FGS)

CHAPTER 6

COMPARISON OF SDS PAGE OF URINARY PROTEINS WITH THE CONVENTIONAL SELECTIVITY INDEX (SI) FOR PREDICTING STEROID RESPONSE IN NEPHROTIC SYNDROME

CHAPTER 6

COMPARISON OF SDS PAGE OF URINARY PROTEINS WITH THE CONVENTIONAL SELECTIVITY INDEX (SI) FOR PREDICTING STEROID RESPONSE IN NEPHROTIC SYNDROME

6.1 INTRODUCTION

In the previous chapter the SDS PAGE technique was used to differentiate steroid responsive from steroid resistant NS, and MCNS from FGS. However, for the past twenty seven years, nephrologists have relied on the relative clearance values of certain plasma proteins (IgG, transferrin, albumin) as a means of predicting steroid sensitivity and underlying histology. Further, these clearance values were also used to predict prognosis (Cameron and Blandford 1966, Soothill and Hendrickse 1967, Adeniyi and Hendrickse 1976).

The clearance ratio is termed as the selectivity index (SI); values of ratio < 0.1 was suggestive of good response whereas that of > 0.1 indicated poor response (Cameron and Blandford 1966).

However many researchers have found the SI to be unreliable (ISKDC 1978). In a recent study Laurent et al (1993) recommended that SI be reinstated as a means of assessing steroid response in NS. However, in his study an SI of <0.1 predicted only 76% of steroid sensitive cases, further 11% of steroid resistant patients had a low SI.

In a previous study (Ramjee et al 1994; Chapter 5) SDS PAGE was shown to be a useful test in predicting steroid response (Chapter 5).

In view of the findings reported by Laurent et al (1993) this study was designed to compare the SDS PAGE and SI methods in predicting steroid sensitivity in children with minimal change nephrotic syndrome (MCNS) and focal glomerulosclerosis (FGS).

6.2 PATIENTS

Thirty four newly diagnosed children aged between (2-10 years) were selected for the study. Of the 34 children 24 Indian children had presumed MCNS and were not biopsied (Adhikari 1981, Coovadia 1987). Of the 24 presumed MCNS there were 3 children who initially responded to steroid therapy but the presence of other infections such as viral upper respiratory tract infections and measles rendered them steroid resistant for the duration of the infection. Following clinical improvement, the children responded to steroid therapy.

In addition there were 10 children, 8 African and 2 Indian who were diagnosed as FGS on renal biopsy. Renal biopsies were classified according to Heptinstall (1992).

Blood and urine samples were collected simultaneously from all patients. Samples were collected during relapse and prior to the commencement of steroid therapy. Analysis of samples were carried out without any prior knowledge of clinical or histological outcome in these patients.

6.3 METHODS

6.3.1 SDS Page

A second morning sample of urine was collected and preserved with 0.05% sodium azide, centrifuged, aliquoted and stored at -20C for future analysis.

Total protein was measured by the coomassie blue technique (Slot 1992); samples with protein concentration of >3.0 g/l were diluted to between 100-300mg/l protein prior to analysis; 15-20 ug/ul protein was applied to the gel.

A vertical discontinuous electrophoresis system was used. Urinary electrophoresis was performed on 7.5% T acrylamide 4% C (constant) gel in presence of SDS for half an hour with the following electrical setting , 85 mA, 100V for half an hour and at 42mA 200V for remainder

of the run. The gels were fixed in 4% acetic and 40% methanol prior to staining with Coomassie blue.

Pure standard of albumin,transferrin and IgG were coelectrophoresed in order to identify specific proteins.

Detailed account of the method are given elsewhere. (Schiwara et al 1986, Shapiro & Maizel 1967).

6.3.2 IgG / Transferrin ratio (SI) (Figure 18) (Laurent el al 1993)

IgG and transferrin levels in serum and urine were measured by Nephelometry in the Chemical Pathology laboratory at King Edward VIII Hospital. The SI was calculated as follows:

$$SI = \frac{IgG\ (urine)}{IgG\ (Serum)} \times \frac{Transferrin\ (serum)}{Transferrin\ (urine)}$$

Ratio of <0.1 = good steroid response

Ratio of >0.1 = poor steroid response

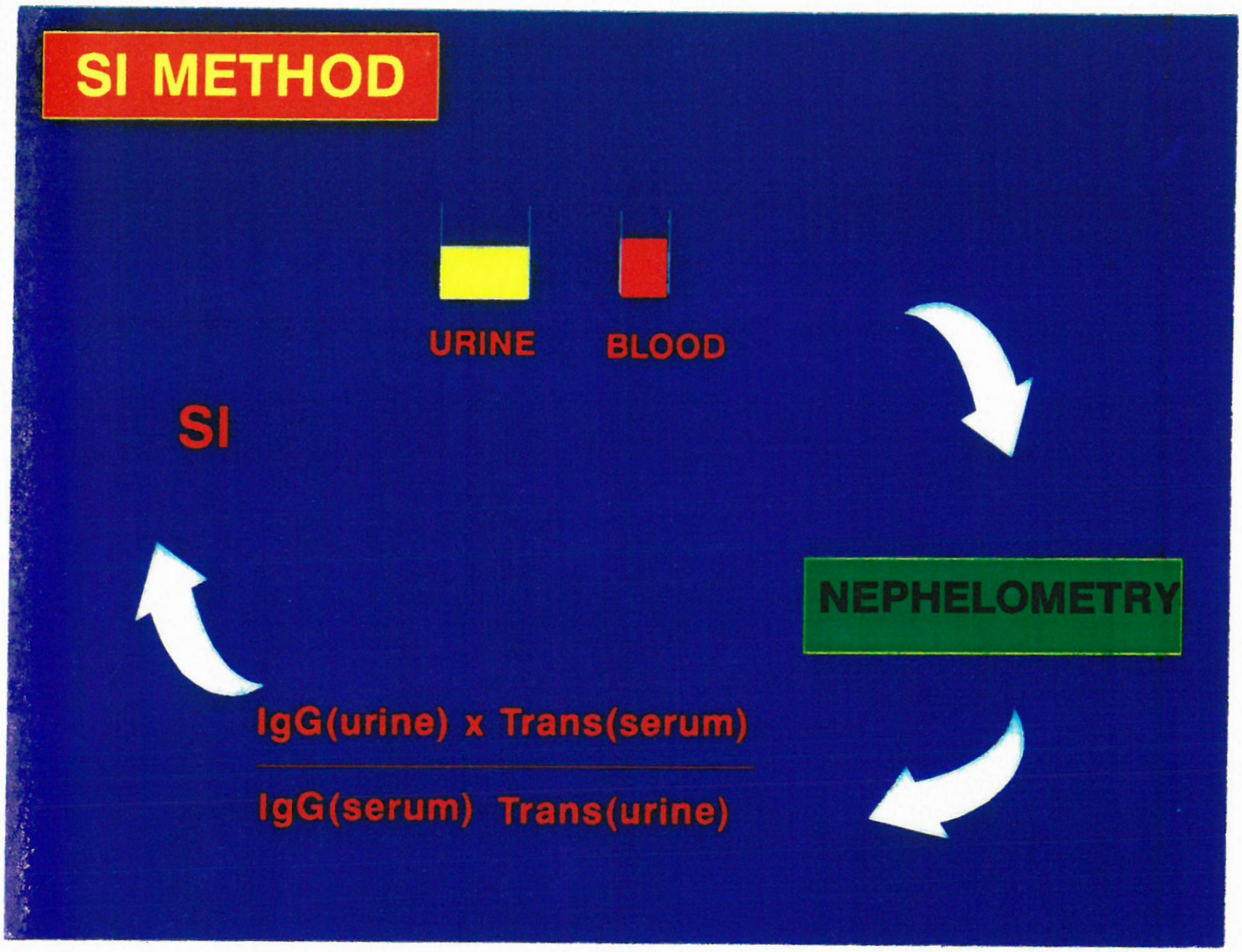


FIGURE 18 : Selectivity Index Method (SI)

6.4 RESULTS (Figure 19)

In the 24 patients with MCNS proteinuria ranged from 3-8 g/l and SI varied from 0.002 to 0.38. In the 10 patients with FGS proteinuria ranged from 3.5 - 19 g/l and SI varied from 0.189 - 0.937. Proteinuria was selective (SI<0.1) in 17 out of 24 (70%) children with MCNS. In the FGS group SI indicated poor response in all patients (100%), SI >0.1.

In patients with MCNS, SDS PAGE analysis of urinary proteins showed selective proteinuria restricted to albumin (n=10) or albumin and transferrin only (n=11) whereas in the FGS group there was unselective proteinuria with additional presence of haptoglobulin and IgG.

The positive predictive value for steroid sensitivity by the SI and the SDS PAGE technique was 100% . The negative predictive value was 56% by SI and 100% by SDS PAGE. This means that the SI method was predicting a false positivity of 44%. Sensitivity of the test was 100% by SDS PAGE whereas in the SI test it was only 67% sensitive. The specificity of the test was 100% by both methods (Table IX,X).

The three patients with MCNS who had transient steroid resistance were detected as steroid resistant by SDS PAGE but not the SI method.

6.4.1 Statistical Calculations

Sensitivity, specificity, predictive values and efficiency measurements were carried out using a two-way contingency table.

TABLE IX

	Steroid Sensitivity		
	Good (< 0.1)	**Poor (> 0.1)**	
Responsive	TP 16	FN 8	24
Resistant	FP 0	TN 10	10
	16	18	34

$$\textbf{Sensitivity} = \frac{TP}{TP + FP}$$

$$\textbf{Specificity} = \frac{TN}{FP + TN}$$

$$\textbf{Positive predictive value} = \frac{TP}{TP + FP}$$

$$\textbf{Negative predictive value} = \frac{TN}{TN + FN}$$

$$\text{Efficiency of test} = \frac{TP + TN}{TP + TN + FP + FN}$$

TP = **true positive**

FP = **false positive**

TN = **true negative**

FN = **false negative**

TABLE 10 : STATISTICAL ANALYSIS OF SDS PAGE AND SI METHODS

	SDS PAGE	SI
SENSITIVITY	100%	(16/24) 67%
SPECIFICITY	100%	100%
PREDICTIVE VALUE (+)	100%	100%
PREDICTIVE VALUE (-)	100%	(10/18) 56%

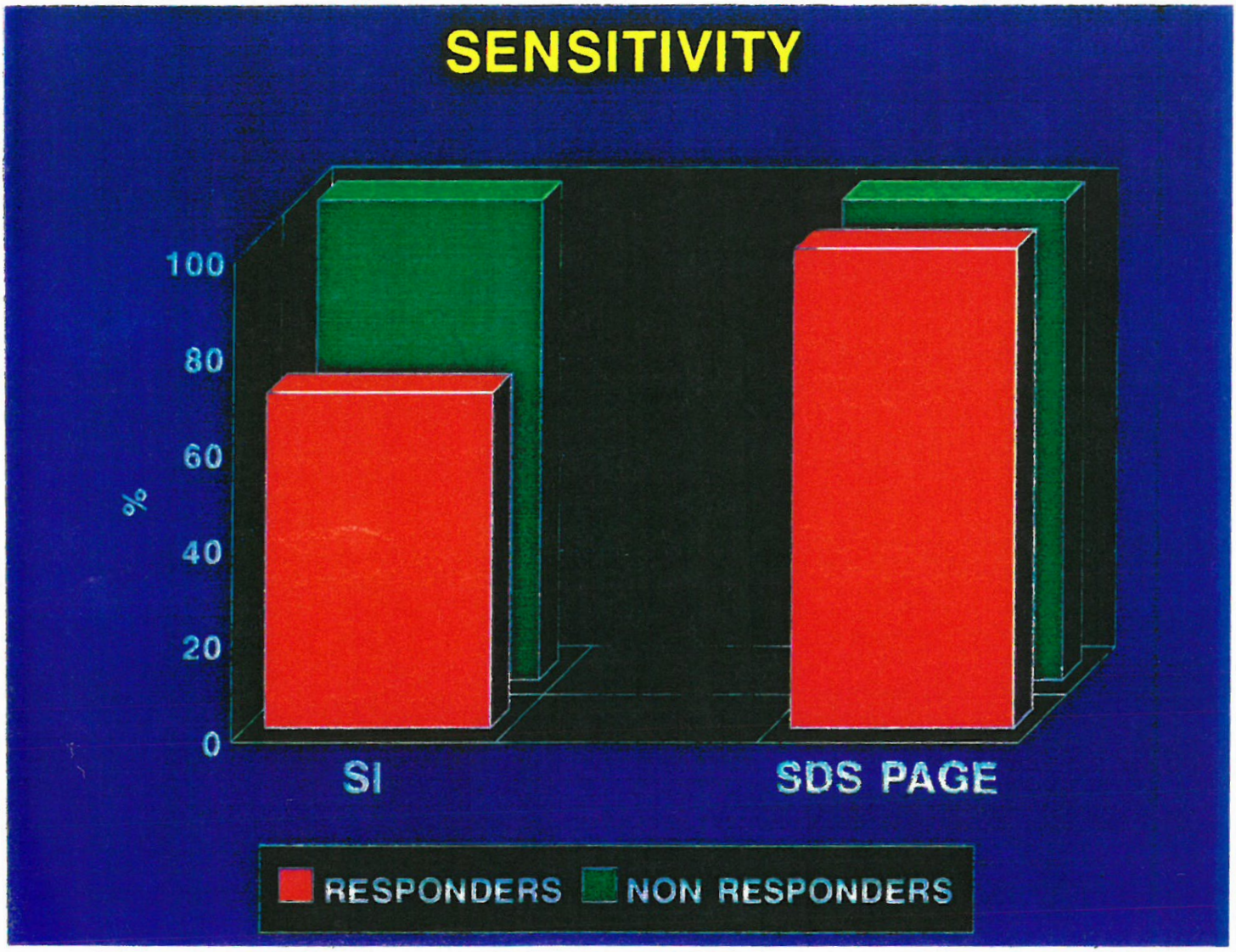


FIGURE 19 : Sensitivity of the SI and SDS PAGE methods in predicting steroid response and resistance in MCNS and FGS

6.5 DISCUSSION

This study has shown that selectivity of proteinuria can be appreciated in several ways. The SDS PAGE method appears to be a more sensitive ancillary test in the assessment of steroid response in newly diagnosed nephrotic patients. Therefore the urinary pattern of proteinuria correlated very well with the clinical outcome in patients.
The SI method appears to be less sensitive in the MCNS group but accurately predicts steroid resistance in the FGS group.

The initial information regarding steroid sensitivity is clinically very useful and provides an indication of the prognosis of the disease. Awareness of steroid sensitivity and a good prognosis minimise the use of all other tests used in the assessment of these patients. Further, in steroid resistant patients renal biopsy is clearly indicated. This information is crucial in the African children as only a small number respond to steroid therapy (Adhikari 1987), and a large proportion tend to experience steroid toxicity.

In steroid resistant cases serial SDS PAGE testing has the advantage of providing information suggesting "progression" to FGS (by presence of low molecular weight tubular proteins) (Ramjee et al 1994). In addition SDS PAGE predicts transient resistance due to presence of other infections as found in the three children with MCNS. With this knowledge, clinicians will be able to decide with confidence on alternative forms of therapy that can be offered to the patient.
In patients with features FGS, SDS PAGE permits the decision of commencing alternative aggressive therapy to be made with confidence especially in cases where renal biopsy is not possible.
The disadvantage of the SDS PAGE method is that quantitative assessment cannot be made. On the other hand SI method although quantitative, has several disadvantages; a) Presence in the urine of fragments of IgG would increase the value and therefore the ratio. b) Non specific precipitates of protein can occur in presence of high lipid serum concentration which are

seen in particularly severe and prolonged cases of MCNS. This is precisely the point emphasised by Laurent et al (1993) who recommended that any discrepancies in the results should be confirmed by electrophoresis.

In conclusion SDS PAGE is a more sensitive method in predicting steroid response in MCNS than the SI. In steroid resistant cases the SI and SDS PAGE are equally sensitive. In addition the SDS PAGE technique allows one to recognises those patients who have true steroid resistance from those who may have transient resistance.

6.6 SUMMARY

Steroids are the mainstay of treatment of NS and have profound influence on management and outcome. Renal biopsy in these diseases depicts changes which correspond roughly to steroid responsiveness . However biopsy is an invasive procedure and is not always reliable to judge therapeutic behaviour to steroids. Accordingly, other predictive methods have been employed and the most widely used since 1966 has been the Selectivity Index (SI) measured by the ratio of IgG/Transferrin in serum and urine. However, the validity of SI have been questioned in a number of studies and this has led to decrease in its use. We have shown in the previous chapter (5) that qualitative detection of a range of protein by SDS PAGE in the urine enables reliable prediction of steroid responsiveness.

In this study the predictive powers of SI and SDS PAGE for steroid responsiveness in children with newly diagnosed MCNS and FGS are compared.

Thirty four children with NS were studied. There were 24 children with MCNS and 10 children with biopsy proven FGS. MCNS patients responded to conventional doses and duration of steroid therapy and had a clinical course which was characterised by remissions and relapses, with the latter responding to a course of steroids. All FGS patients were steroid resistant. SDS PAGE was carried out on a second morning sample of urine. SI was measured by nephelometry using simultaneously collected blood and urine samples.

SDS PAGE was 100% sensitive whereas SI was 67% for predicting steroid responsiveness. Both methods were equally sensitive in predicting steroid resistance (100%). The positive predictive value was the same for both the methods (100%) whereas the negative predictive value was only 56% by SI but 100% by SDS PAGE. The efficiency of the test was 100% by SDS PAGE and 79% by SI.

This study has shown that SDS PAGE is a more efficient test than SI in predicting steroid responsiveness.

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CHAPTER 7

SDS PAGE OF URINARY PROTEINS PREDICTS HISTOLOGY AND PROGNOSIS OF GLOMERULAR DISEASES

CHAPTER 7

SDS PAGE OF URINARY PROTEINS PREDICTS HISTOLOGY AND PROGNOSIS OF GLOMERULAR DISEASES

7.1 INTRODUCTION

In Chapter 5 the SDS PAGE technique was utilised to distinguish steroid responsive from steroid resistant cases. In addition the method was shown to be a useful non - invasive ancillary test in differentiating MCNS from FGS.

Renal biopsy is the definitive investigation in classifying histological changes in kidney disorders. However, sampling errors occur with sparsely scattered lesions and frequently the underlying histological changes do not correlate with steroid response or outcome. There is also a minimal risk of the invasive procedure. Accordingly ancillary tests play a large part in paediatric nephrology. In many of the common renal disease where the clinical and biochemical features are reliable pointers to pathology and outcome, biopsies are in fact avoided by clinicians. These include minimal change nephrotic syndrome (MCNS), post-streptococcal glomerulonephritis (PSGN) (Tejani et al 1983) and Hbs membranous nephropathy (MGN) (Adhikari 1981, Coovadia 1987).

Selectivity of proteinuria for example has been used in nephrotic syndrome to identify patients who are likely to be minimal change and steroid responsive. However the method involves taking of blood samples and the results often have not been reliable (Adeniyi et al 1970, Adeniyi et al 1976, Cameron and Blandford 1966). In the previous chapter (6), our study also showed that the method used by the above authors was not very sensitive. The ISKDC has utilised clinical criteria and a range of laboratory tests to predict histopathology without much success (ISKDC 1978).

Fine urine analysis of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) is proving to be a valuable test for the interpretation of renal proteinuria (Schiwara 1986, Shapiro and Maizel 1967). Indeed early success has led to the development of automated SDS PAGE systems. Recent studies (Balant et al 1974, Brockelbank 1991, Jackson et al and Ramjee et al 1994) have suggested that SDS PAGE is particularly helpful in predicting steroid response (Chapters 5,6). Patterns of proteinuria detected by this technique have led to better prediction of the underlying kidney disorder, although the distinction between the different forms of glomerulonephritis remains blurred. Poor standardisation of the technique (Enrich and Wurster 1991), small numbers of patients and inclusion of children in advanced stages of renal impairment (Brockelbank et al 1991, Jackson et al 1988), have limited the usefulness of the data reported.

This study reports the findings of a study in Indian and Black South African children which suggest that fine urine analysis of proteins with SDS PAGE reveals distinct patterns of proteinuria in different histological groups of glomerulonephritis. The spectrum of renal problems encountered in our setting is unusual and therefore allows the study of both common and uncommon types of diseases. Indian children have the typical nephrotic syndrome of childhood, dominated by minimal change nephrotic syndrome (MCNS), whereas in Africans, focal glomerulosclerosis (FGS), MGN and PSGN are more frequent with MCNS being uncommon (Adhikari 1981, Coovadia 1987, Lewin et al 1979).

7.2 PATIENTS (Figure 20)

Ninety three children comprised of relatively homogeneous groups with nephrotic syndrome and/or glomerulonephritis were studied. In addition urine samples from another miscellaneous set of twenty children who had different clinical diagnoses and overlapping histology were also analysed. Serial analysis of urines were carried out in 30 of these patients to assess the patterns of proteinuria after therapeutic intervention.

A further 20 patients had their urines tested at the onset of the disease to assess the validity of the test in predicting steroid responsiveness, histology and prognosis.

Fifty seven males and thirty five females aged between 1-12 years were studied. Children were followed up in the clinic over a period of 2 months - 10 years. All African children were biopsied as "true" MCNS is uncommon in these children, Indian children underwent biopsy only if their clinical picture did not fit MCNS (Adhikari 1981, Coovadia 1987).

Seventeen out of 30 (56%) of the Indian patients and 40 out of 62 (64%) of the African children had their urines tested within two month after onset of proteinuria. Repeat samples of urines were taken to assess progression of disease.

For the purpose of this study the patients were divided into 4 groups:

GROUP I:STEROID RESPONSIVE NEPHROTIC SYNDROME (SRNS)

There were 28 children in this group: 26 Indian children (unbiopsied) and 2 African children (biopsied). Mean age was 9.1 years and there were 18 males. GFR was within the normal range. Urine samples were taken after a median of three years (range 1 month - 5 years) from the time of initial presentation of nephrotic syndrome. These children had presumed MCNS.

GROUP II: ACUTE POST-STREPTOCOCCAL GLOMERULONEPHRITIS (APSGN)

There were 31 children (29 African and 2 Indian) in this group 18 were males. All children had the typical features of PSGN with raised ASOT and reduced C_3 and 29 children were azotaemic. All children were on diuretics and antibiotics, were not biopsied and all recovered uneventfully. Urine samples were collected within a few days of the onset of the disease.

GROUP III(A): MEMBRANOUS GLOMERULONEPHRITIS (MGN) (Hbs HBeAg positive)

There were 20 African children in this group and 18 were males. Four children had initially presented with acute glomerular nephritis. With the exception of 2 children all had a normal GFR. Urine samples were collected after a median of 6 months (range 2-18 months) from the initial presentation of nephrotic syndrome.

GROUP III(B): MEMBRANOUS GLOMERULONEPHRITIS (MGN) (idiopathic)

There were four African children in this group and 3 were males. All children were given a course of steroids but were unresponsive. All four children had initially presented with acute glomerulonephritis. One child died due to uncontrollable oedema and complications.All children had a normal GFR. Urine samples were collected after a median of 6 months (range 3 months - 1 year) from the initial presentation of nephrotic syndrome.

GROUP IV: FOCAL GLOMERULAR SCLEROSIS (FGS)

There were 10 children (8 Africans and 2 Indians) who were steroid resistant and had FGS on renal biopsy. The two Indian children were initially steroid responsive and then became steroid resistant with one being cyclophosphamide resistant. One African child had MCNS on initial biopsy, however the second biopsy after a period of 2 years showed FGS. The mean age was 5.6 years and there were 5 males. GFR was within the normal range. Urine samples were collected after a median of two years (range 1 month - 4 years) from the initial presentation of nephrotic syndrome.

Miscellaneous patients

In the 20 children with overlapping clinical and histological features, 10 were males. The patients were categorised as follows:

a. Steroid resistant nephrotic syndrome (3 African MCNS, 6 unbiopsied Indian).
b. MPGN Type 1; 2 HBsAg positive (n=7).
c. Systemic lupus Erythematosus (SLE) Grades IV (patient M);(n=2).
d. IgA nephropathy (n=1).
e. Amyloidosis (n=1).

Urine samples were collected after a median of one year (range 6 months - 3 years) from the initial onset of the disease. All children in group (a), (c) and 3 in group (b) were given a course of steroids, in addition to diuretics. Four patients in group (b) and 1 in group (c) were initially azotaemic, but on follow-up all had a normal GFR except for the one child in (c).

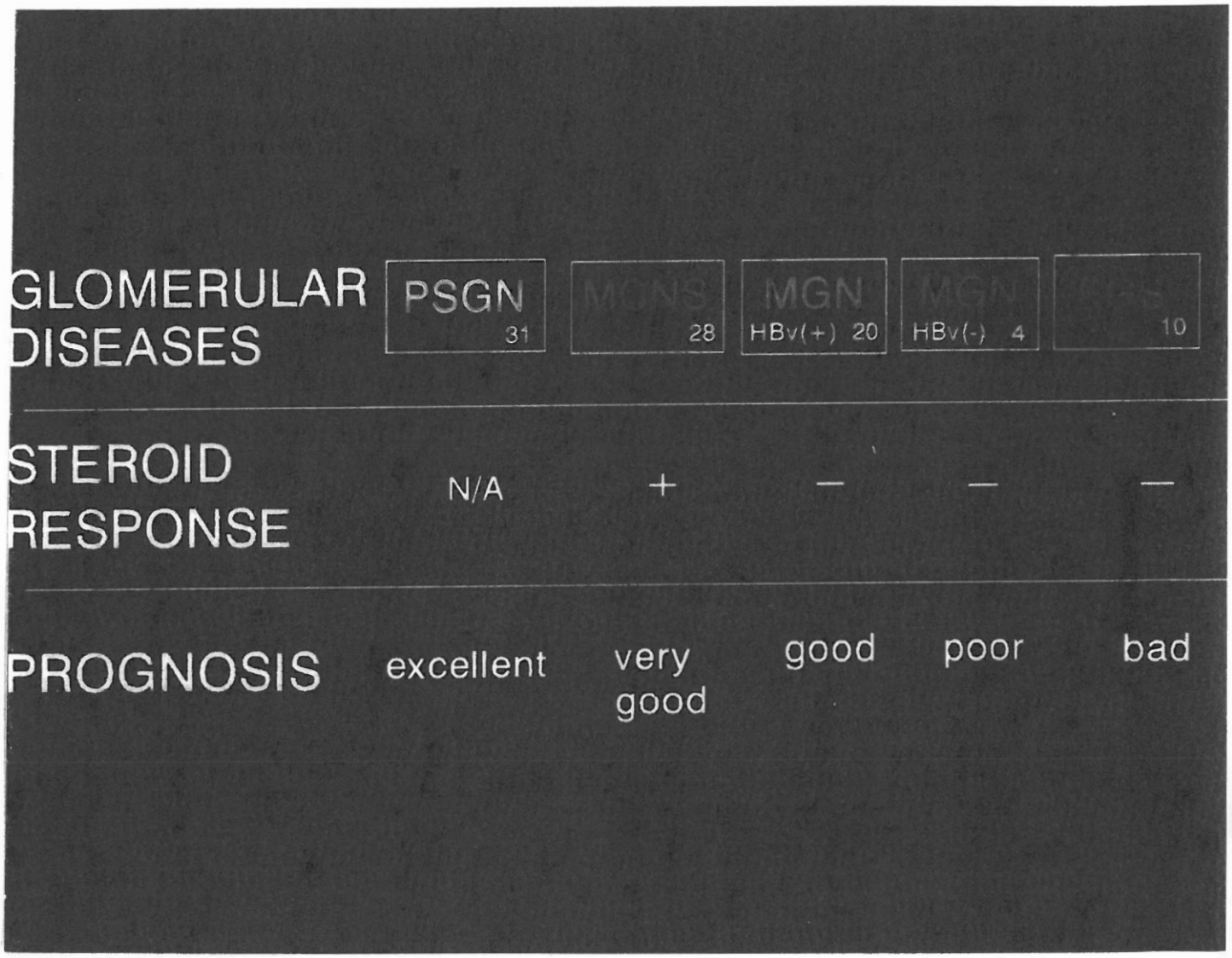

FIGURE 20 : Summary of patients studied

7.3 METHOD

SDS PAGE

Second morning sample of urine was collected and preserved with 0.05% sodium azide, centrifuged, aliquoted and then stored at -20°C for future analysis. Total protein in the urine was measured by the biuret method. All urine samples were diluted to a standard concentration of 15-20 ug/ul.

A vertical discontinuous electrophoresis system was used. Urinary electrophoresis was performed on a gradient gel of 7.5%-25% T acrylamide 4% C (constant) in presence of SDS, for one and a half hours with the following electrical settings, 85 Ma, 100 V for half hour and 42mA, 200V for remainder of the run. The gels were fixed in 4% acetic acid: 40% methanol prior to staining with coomasie blue. Technical details of the method are published elsewhere (Schiwara et al 1986, Shapiro and Maizel 1967) and are given in Chapter 2.

Pure standards of albumin, transferrin, IgG, haptoglobulin B_2M and lysozyme (Sigma) together with high and low molecular markers (Biorad) were co-electrophoresed in order to identify specific proteins. The minimum detection limit for each standard protein was calculated.

Urine analysis was carried out without any prior knowledge of clinical or histological data. Correlations were made between urinary electrophoresis, outcome, clinical data and histology.

7.4 RESULTS

Blinded analysis of the ninety three urine samples (group I-IV) for excretion of different proteins revealed a limited number of patterns. When these results were matched against histological and other features of the patients studied, it was found that a specific pattern corresponded to a particular group in groups I - IV. The pattern ran true for all the

patients within each group. The proteinurias were not as distinctive in the miscellaneous set of children who had overlapping patterns with those from groups I-IV. The results show clearly that some patients have glomerular proteinuria whereas others have glomerular and tubular proteinuria. Within the groups showing mainly glomerular proteins, some have selective excretion of proteins of MW≤80 kDa (albumin and transferrin) whilst others have in addition excretion of proteins of MW > 80kDA (haptoglobulin, IgG). A similar selectivity applies to the tubular proteinuria in Groups IV, MPGN and steroid resistant nephrotic syndrome. MPGN had tubular proteinuria restricted to AIM and RBP whereas the other two groups excreted all tubular proteins tested.

Groups I - IV (Table XI) (Figures 21,22,23)
In the two groups with only glomerular proteinuria; Group 1 (albumin and transferrin) and Group II (albumin), the former is a chronic, steroid responsive disease with an excellent prognosis whereas the latter is an acute self-limiting disease with a good outcome (Lewin et al 1979, Schiwara et al 1986). On the other hand the more severe diseases (group III and IV) have additional bands of haptoglobulin and IgG.

Group IIIa with its more restrictive glomerular proteins (albumin, transferrin, and IgG) and less tubular proteinuria (AIM) has a better outcome compared with group IIIb in which the whole range of glomerular proteins (albumin, transferrin, haptoglobulin and IgG) and some tubular proteins (AIM,RBP) was detected.
Importantly, in the FGS group which has the worst prognosis, the patients excrete the whole range of glomerular and tubular proteins.

Serial analysis for assessment of therapeutic response
Serial analysis of urinary proteins in 30 patients to determine the effect of therapeutic intervention on the patterns of proteinuria showed a gradual regression in the patterns from selective glomerular type in MCNS to

purely albumin which gradually faded with time. Children with FGS who were given immunosuppressive therapy, the pattern of proteinuria regressed from a mixed type to MCNS pattern and then to a faint albumin band on remission. Those patients who did not respond to steroids did not show any change in the urinary pattern.

Patients at onset

In group of 20 patients selected at the onset of disease the pattern of proteinuria correlated with steroid responsiveness and histology (Table XII).

Miscellaneous Patients

The position was less clear in the miscellaneous group of 20 patients. The 2 children (IgA nephropathy, SLE (S) with a good prognosis (as established at follow - up) had restricted glomerular proteinuria; however so does the child with amyloid who defaulted. The steroid resistant group of Indian children, the other child with SLE (M) and the MPGN group have glomerular and tubular proteinuria; they had generally a poor outcome with the SLE child having gone into renal failure and is awaiting transplantation.

TABLE X1 : SDS-PAGE of urinary proteins in - groups I-IV

Type of Proteinuria	Glomerular				Tubular			
Renal Disease Groups	**Alb**	**Tran**	**HAP**	**IgG**	**AIM**	**RBP**	**Lys**	**B_2M**
Group I (n = 27)								
S^+ N S	+	+	-	-	-	-	-	-
Group II (n = 31)								
PSGN	+	-	-	-	-	-	-	-
Group III (n = 24)								
a. MGN								
$(HbsAg)^+$	+	+	-	+	±	-	-	-
b. MGN								
$(HbsAg)^-$	+	+	+	+	+	+	-	-
Group IV (n = 10)								
FGS	+	+	-	+	+	+	+	+

Table X11: **SDS PAGE patterns of proteinuria at onset of nephrotic syndrome**

Patients	SDS patterns	Clinical/histological Outcome
n = 14	Albumin,transferrin	Steroid responsive MCNS
n = 6	All glomerular and tubular proteins	FGS on renal biopsy

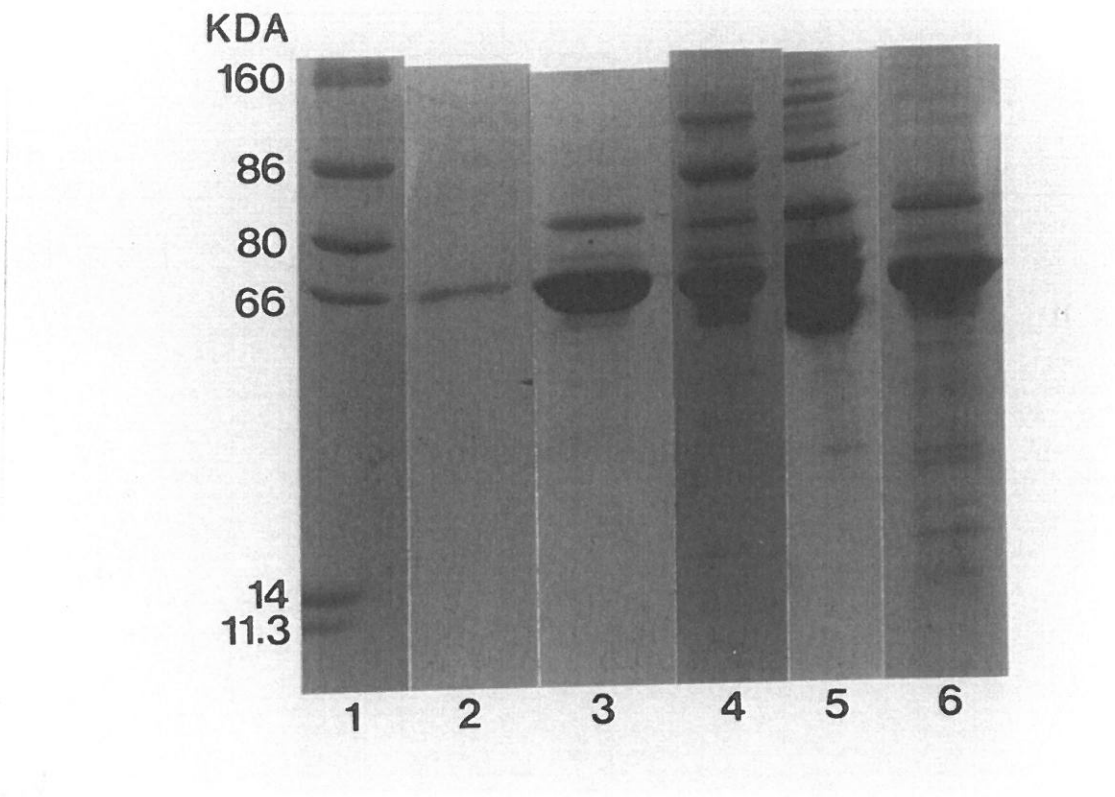

FIGURE 21 : Urinary patterns of proteinuria in different histological groups of childhood nephrotic syndrome

1 = STDS; 2 = PSGN; 3 = MCNS; 4 = MGN (HBsAg); 5 = MPGN; 6 = FGS

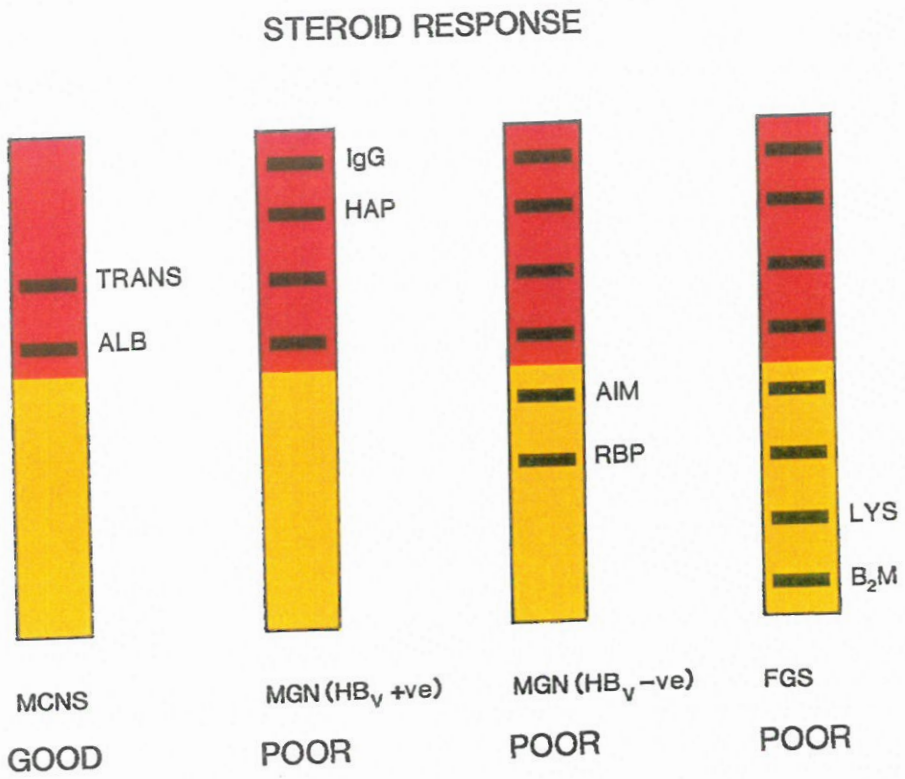

FIGURE 22 : **SDS PAGE of patterns of urinary proteins to predict steroid response in glomerulonephritides**

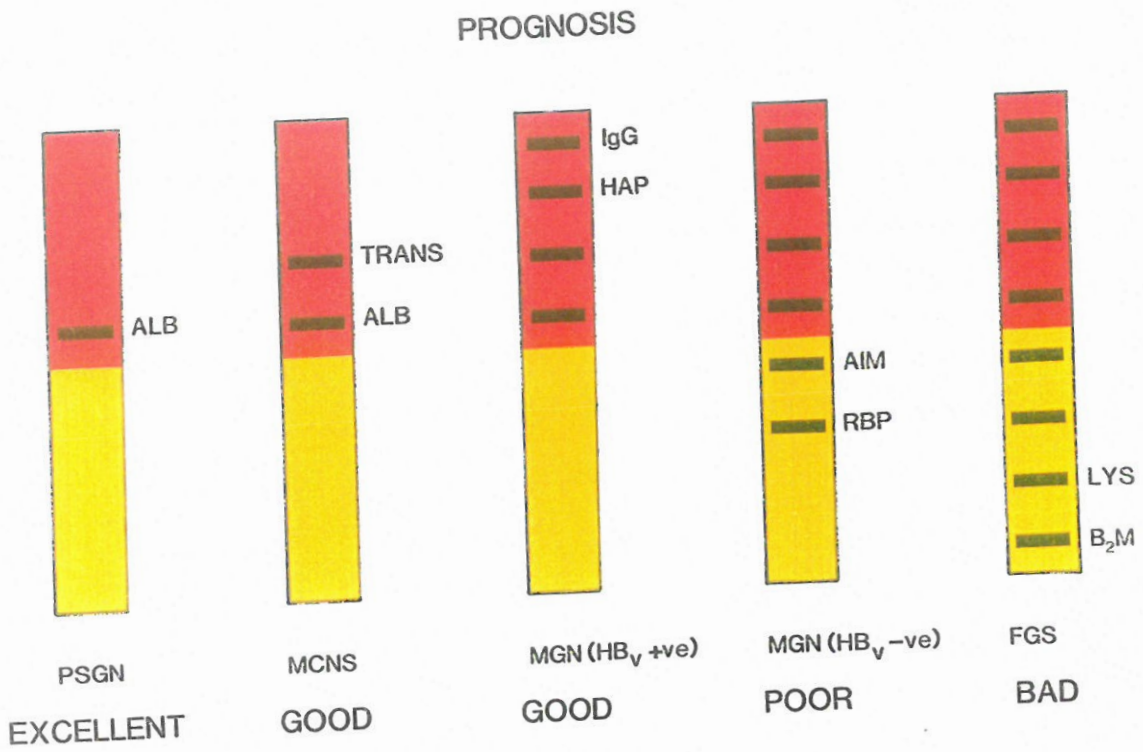

FIGURE 23 : **SDS PAGE patterns of urinary proteins to predict prognosis in glomerulonephritides**

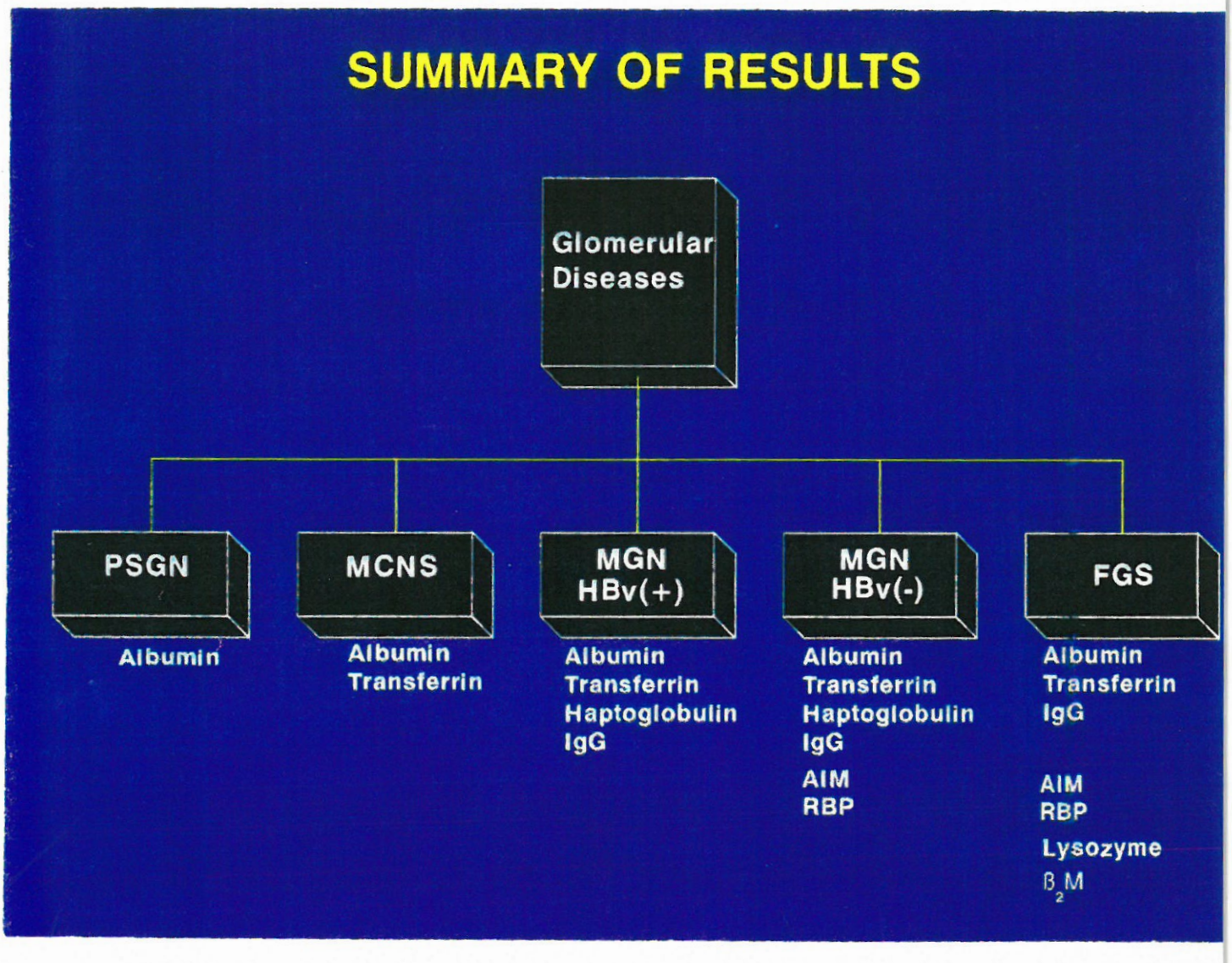

FIGURE 24 : Summary of urinary patterns of proteinuria in glomerulonephritides

7.5 DISCUSSION

This study of a sizeable number of children with different types of nephrotic syndrome and glomerulonephritis has shown that fine analysis of urinary proteins appears to correspond in most instances to specific histological groups, steroid responsiveness and to prognosis. The correlation is closest when the microscopic changes in renal diseases are uniform; SRNS, FGS, MGN (HBsAg positive), MGN (idiopathic) and PSGN, but less secure if the clinical disease is associated with several histopathological lesions: amyloid, IgA nephropathy, SLE, MPGN and steroid resistant nephrotic syndrome. These conclusions linking histology with electrophoretic patterns of urinary proteins based on their molecular size are new and taken together with other correlations, extend the findings reported recently by Brockelbank et al.

This technique has shown to be useful in discriminating between some kidney diseases which have similar clinical presentations. HBsAg antigen positive MGN had purely glomerular proteinuria whereas the idiopathic type had a mixed pattern. MCNS which closely mimics FGS at onset, can be distinguished from it by the same method (Chapter 5).

Overlapping patterns of proteinuria were observed between the miscellaneous set of chlidren and group I - IV.

The unbiopsied steroid - resistant Indian children had a mixed pattern of proteinuria identical to that in the FGS patients. Most of these children probably have FGS. We know from previous experience that 69% of steroid resistant Indian children have subsequently been shown to have FGS on biopsy (Adhikari 1981). This is a far higher proportion than that reported by the ISKDC (1978) where 25% of their steroid-resistant group had FGS. Although the patterns of proteinuria overlapped between other groups (viz. SLE (S), steroid responsive nephrotic syndrome, amyloid, IgA nephropathy and PSGN) the clinical context are so markedly different and simple tests so reliable that precise diagnosis is not difficult.

Steroid response in nephrotic syndrome is a reliable marker of MCNS; however prediction of histopathology is less secure in those who are steroid resistant and the ISKDC has recommended renal biopsy in such patients. SDS-PAGE of urinary proteins may be especially helpful in these steroid-resistant cases for decisions on alternative forms of therapy and prognosis. In particular, for those patient in whom consent for renal biopsy has not been obtained.
Serial analyses on 30 patients to assess the relationship between the patterns of proteinuria and therapeutic intervention clearly indicated that the patterns of proteinuria can predict response.

Single radial immunodiffusion of urinary proteins for selectivity indices has been utilised for predicting steroid response by Cameron, Blandford, Adenyi et al and the ISKDC study. In the study carried out by Adenyi et al in only 7 of the 19 patients were the authors able to predict steroid response. The ISKDC study also revealed lack of reliability of this technique. It has been suggested that the lack of reliability of the method was due to the circadiam rhythm involved in the excretion of proteins (Koopman et al 1985). However assessment of the circadian rhythm of proteins requires collection of timed 24 hour urine samples, which can be cumbersome, subject to errors and extremely difficult in children. Therefore most nephrologists use random samples which are more practical (Moore and Carome 1992).

Prognosis was similarly linked to degree and type of proteinuria: those with an excellent outcome (SRNS, PSGN) had excretion of only albumin and/or transferrin, whereas children with a generally poorer prognosis (FGS, steroid resistant nephrotic syndrome and severe SLE) had more extensive proteinuria.

In the limited number of previous studies available, the authors did not specifically set out to correlate urinary patterns of proteinuria with the

different histological groups of glomerulonephritis, but to examine the potential of an automated SDS PAGE system to distinguish tubular and glomerular patterns of proteinuria in various diseases with renal involvement. In their studies Brockelbank et al (1991) and Jackson et al, (1992) observed a mixed pattern of proteinuria in the four patients with FGS; results similar to ours. However their patients were in advanced stages of renal impairment, in contrast to ours who had normal GFR's. Attention has recently been drawn (Ehrich and Wurster 1991) to the major drawbacks of the studies carried out by Brockelbank's group: the numbers of patients studied were small, standardisation of the technique of urine collection was poor and the reliability of the technique was not tested by co- electrophoresis of a number of marker proteins.

The pathogenesis of specific patterns of proteinuria in different renal diseases is obscure. Clearly the immunological and pathological basis of the diseases studied here is varied; there is good evidence for immune complexes in SLE and PSGN, IgA complexes in IgA nephropathy, HBs and HBe as triggering antigens in membranous nephropathy, possible T cell defects in MCNS and glomerular blood flow abnormalities in FGS. Given the limited span of clinical manifestations of renal diseases it is highly probable that these different pathogenetic events terminate in a few final pathways of renal damage.

Loss of charge and size selectivity of the glomerular polyanion are the two mechanisms most likely to be responsible for proteinuria in glomerulonephritis. Excretion of purely albumin in the urine may be due to loss of charge selectivity, whereas loss of larger molecules (e.g.IgG) is probably due to loss of the size selectivity property of the glomerular polyanion (19,20). It can thus be inferred that in MCNS, mild SLE and PSGN loss of charge - selectivity is mainly affected, whereas in the more aggressive diseases such as FGS, HBs antigen negative MGN and MPGN there is an added loss of size - selectivity. This findings are in agreement

with our previous studies (Chapter 3 & 4). The tubular interstitial changes in FGS and extensive interstitial infiltration in the child with SLE (M) are likely to account for the low molecular weight proteinuria. Similar tubular interstitial changes were noted in the MPGN group on renal biopsy. However these changes were not observed in the HBsAg negative MGN group although tubular proteinuria was detected. This can therefore only be the first approximation of an explanation for the above findings and moreover does not clarify the link between glomerular polyanion damage and the pathogenetic mechanisms alluded to above.

Limitations of this study include analysis of urine samples of patients in different stages of similar renal diseases. The small number of patients studied at the onset, however, show that patterns of proteinuria do correlate with steroid response,prognosis and histology. As expected, the results do not correlate well in the miscellaneous set which embraced patients with a variety of disorders. It thus appears that the SDS PAGE method works best when a homogeneous group in the active phase of the disease, presumably with a restricted range of damaging effects, is studied.

Further studies are essential to confirm and consolidate these findings. Results of this study look promising for prediction of histology, steroid response and prognosis, and suggest that the SDS PAGE method is a valuable tool, in conjunction with clinical assessment and routine tests, in the management of renal diseases of childhood. It can narrow the indications for renal biopsy, which is in accordance with the development in this field over the past 3 decades, but obviously cannot replace microscopic examination of kidney tissue.

7.6 SUMMARY

Renal biopsy is often the definitive investigation in kidney disorders but is invasive and occasionally may fail to detect scattered lesions, correlate with steroid response, or predict outcome. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) of urinary proteins is helpful in predicting steroid response and is proving to be a valuable tool in interpretation of renal proteinuria.

SDS PAGE of urinary proteins was carried out in ninety three Indian and African children with kidney diseases, and correlations were sought by single blind analysis of the results. There were 5 such groups: I: steroid responsive nephrotic Syndrome (SRNS) (N = 27); II: acute post-streptococcal glomerulonephritis (PSGN) (N = 31); III: membranous nephropathy (MGN) a: HBsAg positive (N = 20); b: HBsAg negative (N = 4); IV: Focal glomerulosclerosis (FGS) (N = 10). Glomerular proteinurias were restricted (albumin and/or transferrin) in SRNS and PSGN but more extensive with additional excretion of high molecular weight proteins haptoglobulin and IgG in MGN and FGS. The additional excretion of tubular proteins were detected in MGN and FGS. Selective excretion of albumin and transferrin (group I) predicted steroid responsiveness. Restricted glomerular proteinuria was detected in those with a good prognosis (groups I, II), whereas added excretion of haptoglobulin and IgG indicated a poorer outcome (MGN). Presence of both glomerular and tubular proteins indicated the worst prognosis (FGS, HBsAg negative MGN). SDS PAGE patterns of proteinuria correlated closely with histology, steroid responsiveness and prognosis in children with homogeneous groups of kidney disorders. It is a useful ancillary test in management of renal diseases of children, narrowing the indications for biopsy even further.

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CHAPTER 8

MECHANISMS OF PROTEINURIA: CORRELATIONS OF THE DIRECT AND INDIRECT TESTS OF PORE-SIZE AND CHARGE SELECTIVITY IN THE NEPHROTIC SYNDROME

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MECHANISMS OF PROTEINURIA : CORRELATIONS OF THE DIRECT AND INDIRECT TESTS OF PORE-SIZE AND CHARGE SELECTIVITY IN NEPHROTIC SYNDROME

8.1 INTRODUCTION

One of the major impediments to research in the mechanism of proteinuria in NS is the difficulty in obtaining enough renal tissue to examine GBM at the ultrastructural level. This has led to the development of non-invasive methods of examining blood and urine (Levin et al 1985, Brockelbank et al 1991, Myers et al 1982).

In Chapter 7, it was shown that the increase in pore size of the GCW has been indirectly assessed by analysing the size of the protein molecules lost in the urines. SDS PAGE of urinary proteins separated protein molecules according to size. Excretion of protein molecules <80 kDa was shown to be due to loss of charge selectivity, whereas, in those with loss of larger proteins (>80 kDa) pore size appears to be the major contributory factor in the pathogenesis of proteinuria. Findings similar to those of Carrie et al 1981.

The negative film of the GBM has been assessed directly by visualisation of histochemical binding of the cationic dye polyethyleneimine (PEI) to anionic binding sites (Schurer et al 1977) (Chapter 3). Levin et al (1985) used a non-invasive method of alcian blue (AB) binding to negative sites on the RBC membrane and postulated that the loss of charge on the GBM in steroid responsive nephrotic syndrome is a generalised disorder affecting all membranes. Correlations between direct and indirect methods of GBM charge selectivity have however not been reported.

In Chapters 3 & 4 it was shown that depletion of AB and PEI binding sites correlated with the degree of proteinuria in minimal change nephrotic syndrome (MCNS) but not in other histological groups of NS.

An additional confirmatory test of indirectly measuring charge- selectivity

was also included. This test involves Isoelectric focusing (IEF) of urinary proteins, a method used to separate protein molecules according to their charge.

The present investigation was undertaken to correlate the direct and indirect methods of assessing GBM charge.

Presence of anionic protein molecule suggest that loss of GCW surface charge may be the main cause of protein loss whereas presence of molecules of heterogeneous charge indicate increase in pore-size (Wrigley 1976, Meyers 1982).

The lack of correlation between proteinuria and GBM surface charge in histological groups other than MCNS, prompted us to examine the role of glomerular pore size by detailed analysis of urinary proteins in some of these patients.

8.2 PATIENTS

Thirty three children aged between (2-11 years) were studied. There were 31 African and 2 Indian children.

The following groups of children were studied: 4 with MCNS; 10 with focal glomerulosclerosis (FGS); 14 with HbsAg Membranous Glomerulonephritis (MGN); and 5 with Mesangiocapillary Glomerulonephritis Type I (MPGN).

Urine samples for SDS PAGE were collected from 22 of these 33 children: 2 with MCNS; 5 with FGS; 11 with MGN; and 4 with MPGN.

Urine samples for IEF were collected from 20 patients with MCNS including the 4 patients from above, 10 with FGS, 15 with MGN and 5 with MPGN.

Controls

Control biopsies were obtained from 8 post trauma partial nephrectomy patients. Similarly 33 age matched healthy children were selected as controls for the AB binding to RBC study.

8.3 METHODS

8.3.1 Renal biopsies

Tissue samples were obtained by percutaneous renal biopsy.
The histological diagnosis was determined by conventional light and electron microscopy and by immunofluoresence and classified according to Heptinstall (1992).

PEI staining of anionic sites [direct method of measuring charge selectivity]

Unfixed renal biopsy specimens were immersed in 0.5 % PEI solution for thirty minutes and then washed 3 times for ten minutes each time in cacaodylate sucrose buffer according to the method of Okada et al (1986). The tissue was then stained in phosphotungstic acid/ gluteraldehyde mixture for one hour and then prepared for electron microscopy according to conventional methods. The number of anionic sites were counted over a distance of the GBM in the photomicrograph per glomerulus and expressed as Mean ± SD of the lamina rara externa (LRE) stained anionic sites per 1000nm length of GBM. Stained sites in the lamina rara interna (LRI) and lamina densa (LD) were not counted as they were scattered randomly. Detailed account of the method is given in Chapter 2.

8.3.2 AB binding to RBC (ABRBC) [indirect method of charge selectivity]

Nine millilitres of venous blood was mixed with 1 ml of 3.8% trisodium citrate. The binding of AB to RBC anionic sites was determined by the method of Levin et al (1985) and modified according to the method of Bernard et al (1986). Detailed accounts are given in Chapter 2. The results presented here were obtained with a saturating AB concentration of 150 ug/ml.

8.3.3 SDS PAGE of urinary proteins [indirect method for measurement of size selectivity]

Second morning sample of urine was collected and preserved with 0.05% sodium azide, centrifuged, aliquoted and then stored at -20°C for future analysis. All urines were diluted to a standard concentration of 15-20 ug/ul. A vertical discontinous electrophoresis system was used. Urinary electrophoresis was performed on a gradient gel of 7.5% - 25% T acrylamide 4% C (constant) in presence of SDS, for one and a half hours with the following electrical setting, 85 Ma, 100 V for half hour and 42 mA, 200V for remainder of the run. The gels were fixed in 4% acetic acid: 40% methanol prior to staining with coomasie blue. Pure standards of albumin, transferrin, IgG and haptoglobulin, (Sigma) together with high and low molecular weight markers (Biorad) were co-electrophoresed in order to identify specific proteins. Low molecular standards were also run to detect these proteins but the results are not provided here.

8.3.4 IEF of urinary proteins [indirect method for measurement of charge selectivity]

IEF was carried out on a flatbed Pharmacia apparatus. 0.3g of agarose was dissolved in a solution containing 3.6g d-sorbitol and 27ml aqueous glycerol. Two millilitres of ampholytes (pH 3-10) were added to the solution and the warmed solution was then cast into a casting frame onto a pre-warmed gel bond sheet. Two ul of urine (5-10ug) was applied to the sample applicator foil. Standard marker proteins of albumin and IgG were cofocused with the test samples. The voltage limit was set to 1200, power to 20 W and the amperage to 150 mAmps for a total of 1000V/hr. The initial gel conditions were typically 520V, 40mA at 20 W. The running time was typically one hour. The gels were than stained in coommasie blue stain. Detailed account of the method is given in Chapter 2.

8.3.5 Statistical analysis

Descriptive statistics were calculated for all the variables. Associations between PEI and ABRBC were analysed using Spearman correlation coefficients. They were calculated for overall associations as well as within groups separately. Correlation coefficient of > 0.7 is regarded as a strong association.

8.4 RESULTS

8.4.1 ABRBC and PEI in different histological groups (Figure 23)

ABRBC in the group as a whole was significantly reduced compared to controls (40.87 ± 11.7 vs 73.54 ± 13.14 $P < 0.05$). Similarly PEI staining of anionic sites on the LRE were significantly reduced compared to their controls (15.75 ± 4.3 vs 35.0 ± 1.2 $P < 0.005$) (Figure 25).

ABRBC and PEI did not differ between the histological groups (Figures 26,27).

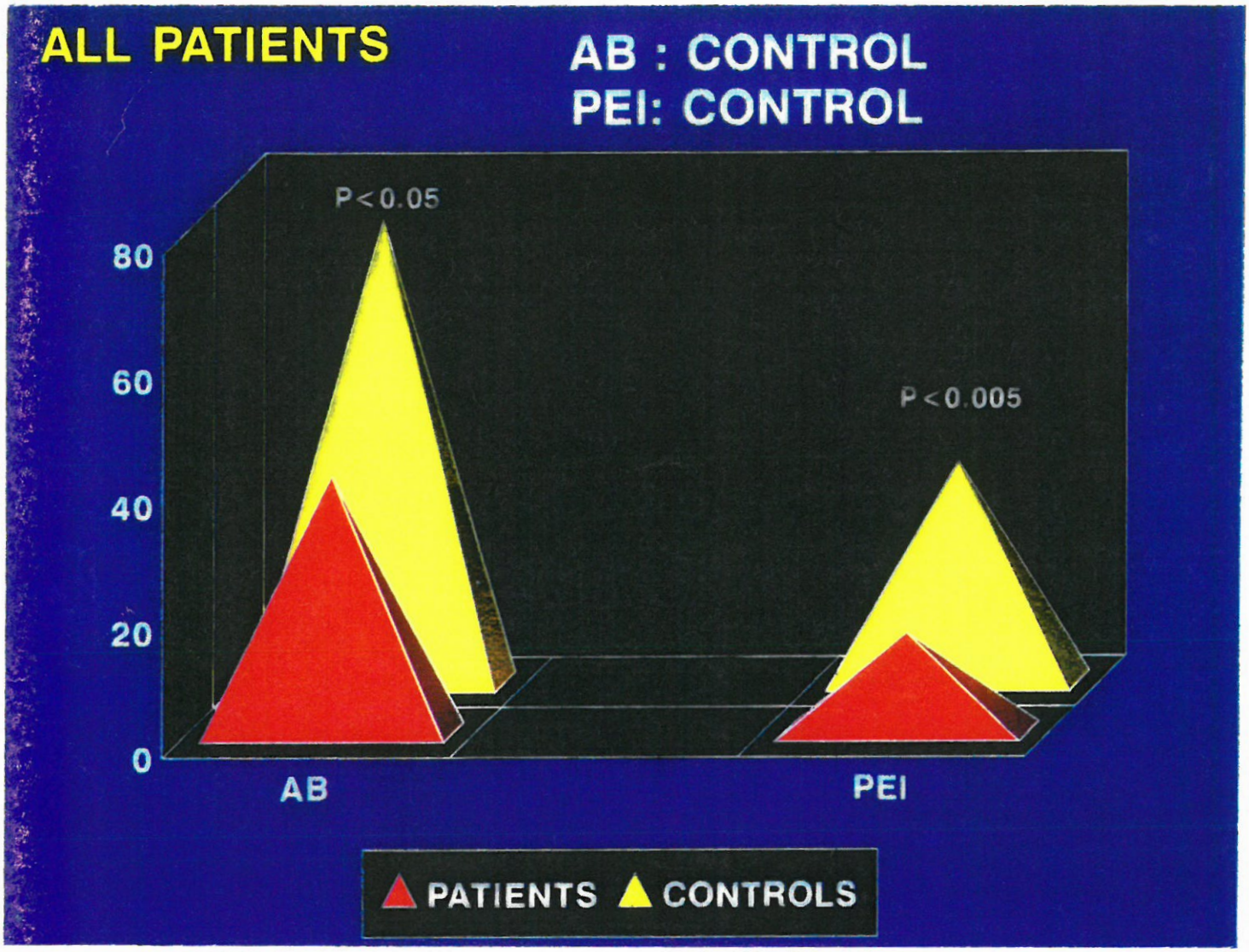

FIGURE 25 : Comparison of charge- selectivity by direct (PEI) and indirect (AB) in nephrotic and control children

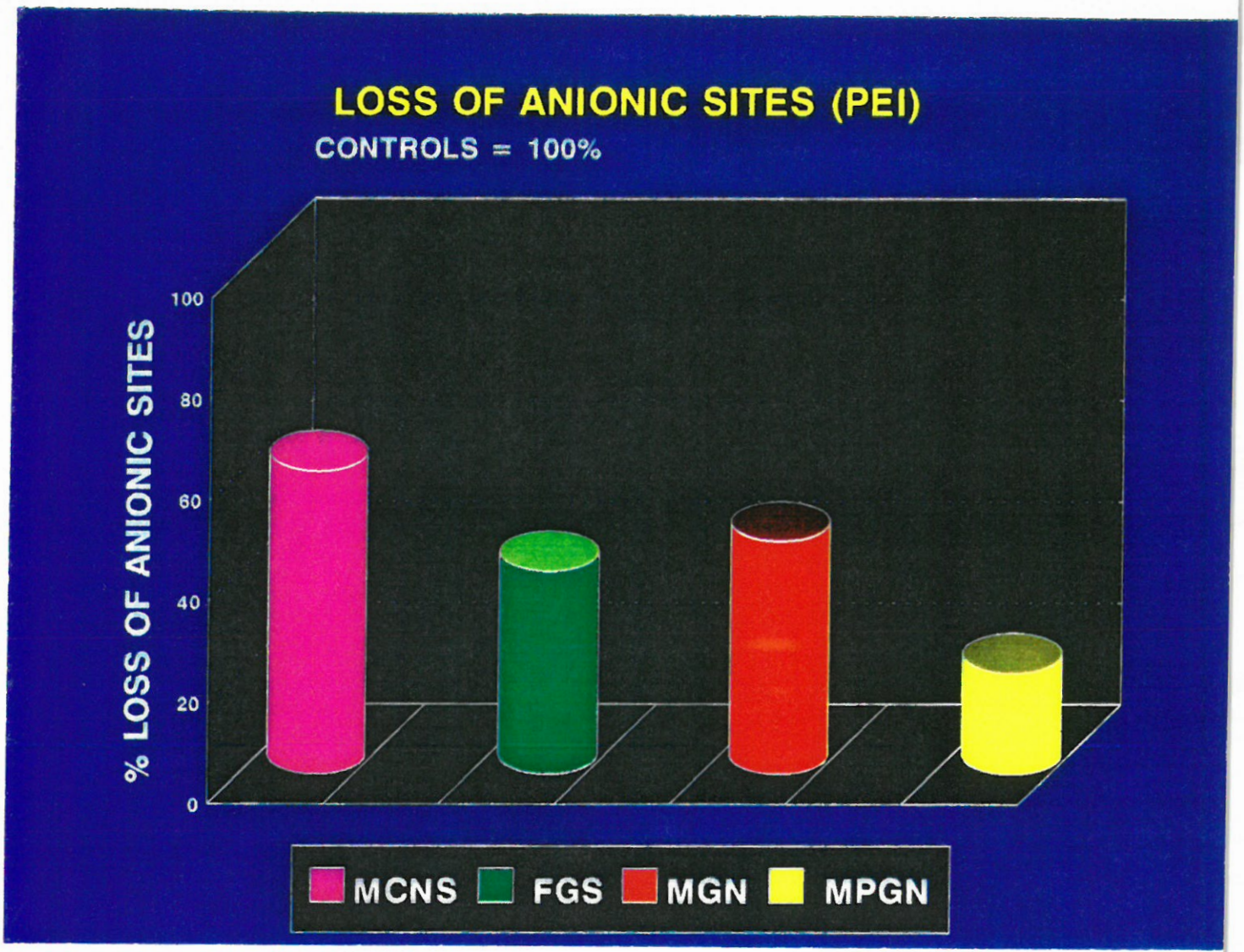

FIGURE 26 : Loss of glomerular anionic sites in different histological groups of nephrotic syndrome

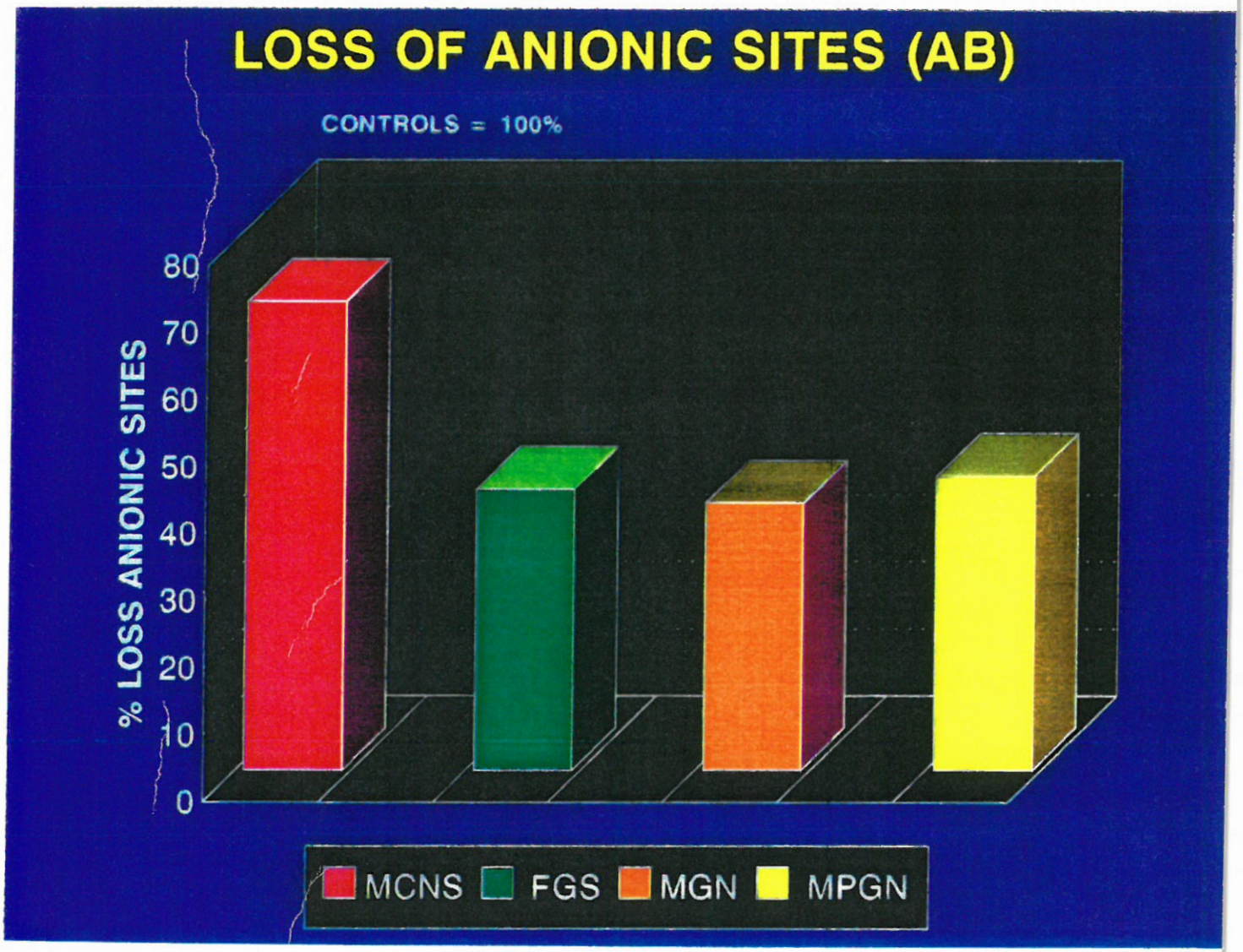

FIGURE 27 : Loss of red blood cell anionic sites in different histological groups of nephrotic syndrome

8.4.2 Correlation between ABRBC and PEI (Table XIII)

No correlation was detected between PEI and ABRBC in the group as a whole. A strong but not significant correlation (because of small numbers) was seen between ABRBC and PEI in the 4 children with MCNS (r= 0.8); a similar correlation, but to a modest degree, was detected in the 10 children with FGS (r= 0.316). No such relationship was found in MGN and MPGN.

8.4.3 Urine analysis by SDS PAGE (Figure 28)

Analysis of urines revealed distinct patterns for each histological group.

MCNS

In this group a pattern of restricted glomerular proteinuria, albumin and transferrin only (MW < 80 kDa) was seen.

MGN

This group had unrestricted glomerular proteinuria.
In addition to albumin and transferrin, bands of larger proteins (MW > 80 kDa) haptoglobulin and IgG, were observed.

MPGN, FGS

There was unrestricted proteinuria with all glomerular proteins (albumin, transferrin, haptoglobulin, IgG) being seen. In addition tubular proteinuria was also detected.

8.4.4 Urine analysis by IEF (Figure 29)

MCNS

Urine samples from these patients revealed restricted heterogeneity of charge displaying predominantly anionic species of protein which was

mainly albumin in the pH range of 5.2. The pattern ran true for all patients in this group with excretion of anionic proteins restricted to isoelectric point <6.0.

FGS, MGN, MPGN

Electrofocusing of urine samples revealed heterogeneity of charge showing protein bands with isoelectric points ranging from 4-10. This pattern revealed presence of anionic and cationic species of IgG.

TABLE XIII: Correlation between ABRBC and PEI staining in the histological groups of NS

HISTOLOGICAL GROUP	N	GBM ANIONIC SITES PEI/1000nm	ABRBC (ng 10^6 RBC)	r
MCNS	4	15.25 ± 2.9	23.85 ± 5.3	0.8
FGS	10	16.02 ± 5.1	43.46 ± 12.98	0.31
MGN	14	14.18 ± 3.8	43.8 ± 8.7	0.03
MPGN	5	20.04 ± 1.69	41.2 ± 10	0

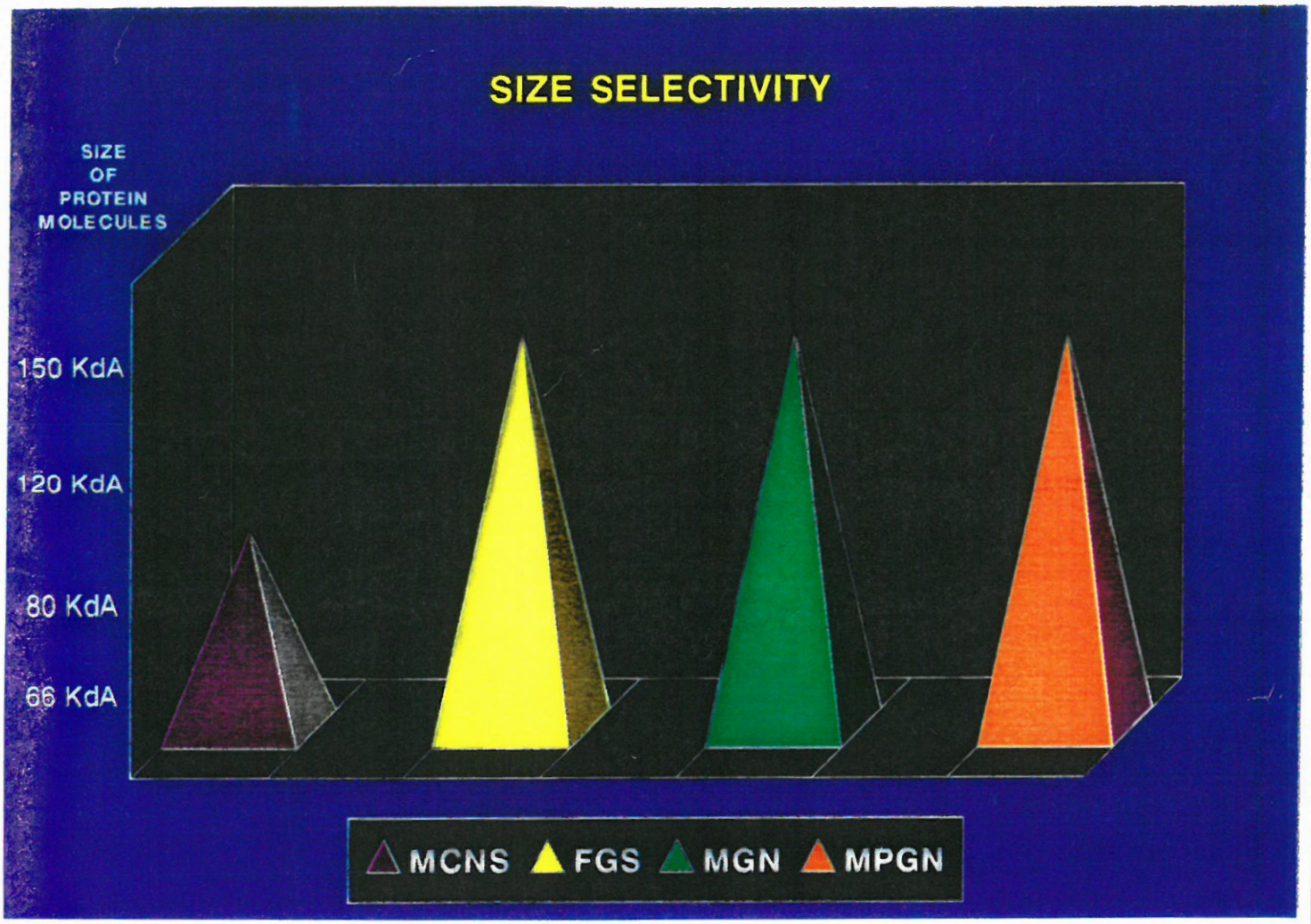

FIGURE 28 : Excretion of proteins of varying size in different histological groups of nephrotic syndrome

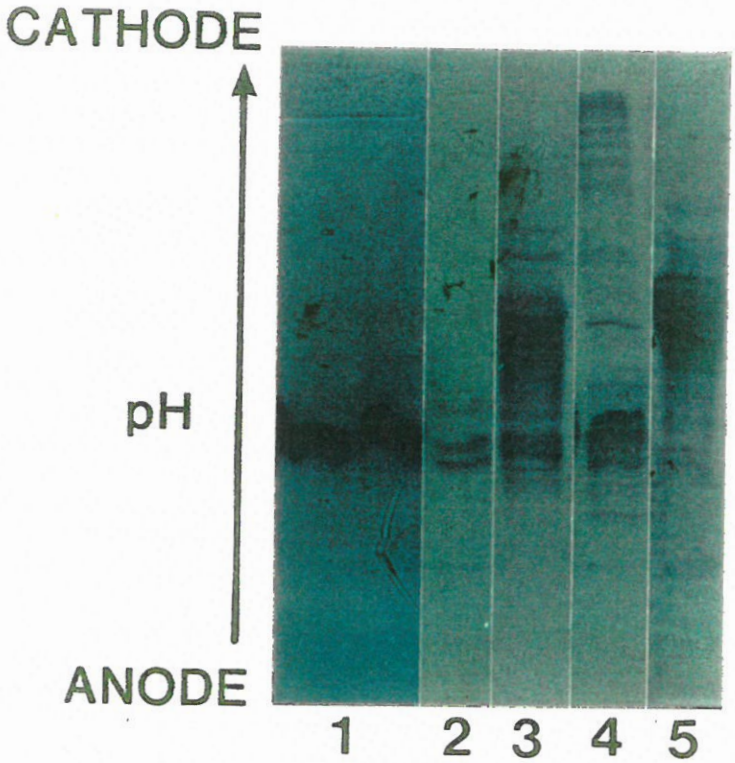

FIGURE 29 : Urine analysis by IEF in different histological groups of nephrotic syndrome
1, 2 = MCNS; 3 = MGN (HBsAg);
4 = MPGN; 5 = FGS

8.5 DISCUSSION

There are several findings in this study which amplify the mechanisms by which proteinuria occurs in glomerular diseases. The first of these is that a correlation exists between indirect and direct measurements of negative charge on the GBM in some common types of childhood nephrotic syndrome. In these diseases therefore, examination of the basement membrane of more easily accessible tissues such as the formed elements in blood (eg. the RBC used here) and urine obviates the need for a renal biopsy, thereby facilitating investigations into the role of electrostatic charge defects in causing protein loss across the glomerular capillary wall. In Levin et al's initial study that there was a depletion of anionic charge on the RBC membrane in MCNS, however, this was not related to direct assessment of electrical charge on the GBM. This correlation is closest in MCNS but can also be detected, albeit to a lesser degree, in FGS.

Available evidence suggest that MCNS is the only histological group where perturbations of negative charge of the GBM is the dominant mechanism of proteinuria (Carrie et al 1981, Yoshizawa et al 1989). Further the magnitude of anionic site reduction on the GBM is significantly associated with the extent of proteinuria in MCNS but not in the other types of nephrotic syndrome (Chapter 3). These findings are further supported by the IEF of urinary proteins in this study which revealed that the charge on the protein molecules in this disease was almost exclusively composed of anionic proteins. In diseases other than MCNS it is likely that GBM pore size and shape defects facilitates increased transglomerular passage of large protein molecules. This was demonstrated in MGN, MPGN and FGS where bands of large proteins (> 80 kDa), IgG and haptoglobulin, were detected on electrophoresis of urine samples. These findings were further confirmed by IEF of urinary proteins which revealed that proteins excreted in this group of patients were of heterogenous charge composition.

Structural renal damage may determine the relative roles of charge- and size-selectivity in producing proteinuria (Kanwar and Rozenweig 1982, Brenner et al 1978) In MCNS and early stages of FGS histological lesions are minimal,

whereas in MGN and MPGN there are obvious lesions. However the matter may not be so clear cut; both processes may operate to differing degrees in individual diseases. For example, recent reports suggest that alteration of pore size and density together with involvement of certain shunt pathways may contribute to proteinuria in MCNS (Meyers and Guash 1994).

In contrast to MCNS, nephritides with obvious glomerular lesions in which the major abnormality is impairment of size selectivity (e.g. MGN and MPGN) may have localised defects confined to the glomerular capillaries with obvious histological lesions. FGS appears to be one example in which lesser disorder of basement membranes in which both electrical charge and pore size abnormalities are responsible for proteinuria.

Is their any other data to reinforce the proposition that MCNS and FGS might be systemic disorders of negative charge of basement membrane? Current evidence suggests that a circulating cationic permeability factor may be neutralising anionic binding sites of the GBM in steroid responsive nephrotic syndrome and in patients with recurrent FGS (Levin et al 1985, Zimmerman 1984, Yoshizawa et al 1989). The systemic release of this macromolecule may affect charged sites on many tissue membranes including the capillary endothelium and formed elements of the blood.

The lack of correlation between ABRBC and PEI in MGN and MPGN could be due to the presence of cationic antigens, antibodies and immune complexes which bind only onto anionic sites on the GBM (Border et al 1982,Gautheir et al 1982, Couser et al 1985) but not on RBC's. In keeping with this, our previous studies (Chapter 3) have shown that in the GBM where the deposits were formed, a marked depletion of PEI bound anionic sites was noted; however, these findings did not correlate with proteinuria. The current results indicate loss of size- selectivity. This discordance might be due to the fact that anionic sites are not lost but merely masked by the proteins derived from immune reactions. Okada et al (1986) also reported a loss of PEI bound anionic sites in patients with MGN.

The indirect method of measuring charge-selectivity of basement membranes

is useful in the assessment of proteinuria in diseases which may be systemic and with little evidence of structural damage (MCNS), but not in those with obvious histological lesions. In the latter, basement membrane disorders appear to be confined to the kidney and urinary protein loss may be due to defects in shape and size of the apertures on the GBM. IEF of urinary proteins appears to be a better indirect test of measuring charge in all groups of glomerulonephritides.

SDS PAGE appears to be a better indirect test for the evaluation of proteinuria in these diseases. Moreover, it appears that even detection of loss of anionic sites by direct tests may not always accurately reflect the contributions made by alterations of glomerular electrostatic charge to proteinuria.

8.6 SUMMARY

Direct and indirect methods of measuring membrane charge were studied by detecting fixed anionic sites using Polyethyleneimine (PEI) on the glomerular basement membrane (GBM), Alcian blue on red blood cell (RBC) membrane [ABRBC] and Isoelectric focusing (IEF) of urinary proteins respectively in 33 children with nephrotic syndrome (NS). Size selectivity of the GBM was measured indirectly by fine analysis of urinary proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) in 22 of these children.

Correlation between ABRBC and PEI was strongest ($r = 0.8$) in 4 children with minimal change NS (MCNS); moderate ($r = 0.31$) in 10 children with focal glomerulosclerosis (FGS); and absent in 14 children with HBs membranous nephropathy (MGN) and 5 with mesangiocapillary glomerulonephritis (MPGN). ABRBC and PEI were reduced in the group as a whole compared to their controls (40.87 ± 11.7 vs 73.54 ± 13.14 $P < 0.05$; 15.75 ± 4.3 vs 35.0 ± 1.2 $P < 0.005$ respectively).

IEF of urinary proteins revealed restricted charge heterogeneity in MCNS, protein molecules were exclusively composed of anionic albumin. In groups other than MCNS both anionic and cationic protein bands were seen.

Excretion of glomerular proteins was restricted by size ($\leq$ 80 kDa) in MCNS, but unrestricted ($\leq$ 80 kDa plus >80kDa) in FGS, MGN and MPGN.

The main cause of proteinuria is likely to be depletion of negative charge on the GBM in MCNS, and distortion of capillary pore size in MGN and MPGN, with probable overlap of these mechanisms in each disease especially in FGS. Basement membrane injury appears widespread in MCNS but confined to the kidney in MGN and MPGN.

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OVERALL DISCUSSION

Implications for Pathogenesis of Proteinuria and for recognition of underlying histological patterns

This thesis has confirmed findings from other studies which have shown that the mechanism of protein loss in NS is due to loss of the charge- and size- selective barriers of the glomerular capillary wall (Brenner et al 1978, Galaske et al 1979, Kaysen et al 1986). The initial studies in the mechanism of proteinuria were limited to only a few histological groups (mainly MCNS and FGS). The range of renal disorders studied is widened so as to include different histological types predominant in the Third World. Moreover, the patients studied were investigated at an early stage of the disease process when renal function was well preserved.

Direct measurements of membrane negative charge by the cationic probe PEI had been previously reported. However, these studies included only a limited number of diseases (MCNS, FGS [Kitano et al 1993], MGN [Okada et al 1986]) and not most of the histological groups of NS. The generalisability of the conclusions drawn from these studies has been enhanced by including a wider spectrum of NS. Findings of a greater depletion of anionic sites in MCNS, and the simultaneous correlation of this with proteinuria in only this disease and not in any other histological groups of NS, has not been previously reported. Carrie et al (1981) used charged molecules of dextran to show that the main cause of proteinuria in MCNS was an electrochemical disturbance of the GBM. Findings using PEI therefore extend and support these.

Indirect measurements of the glomerular polyanion using the cationic dye Alcian Blue revealed a reduction in negative charge (Levin et al 1989). The results of this thesis have confirmed these findings and extended them to diseases predominant in the Third World. It is not known what causes the electrostatic abnormalities of the GBM . It may be a circulating factor responsible for neutralising membrane charge (Levin et al 1989). The biochemical changes occurring secondary to nephrotic syndrome may play some part. Immune complexes in HBs MGN, T cell

defects in MCNS, and blood flow abnormalities in FGS, may also play a contributary role in the initial injury to the glomerular capillary anions.

Correlations between direct and indirect methods of measuring membrane negative charge have not been previously reported. This correlation was detected in MCNS, suggesting that MCNS may be a disease in which there is a generalised disorder of membranes. Moreover the results obtained indicate that the indirect method (alcian blue) could be a valid measure of changes at glomerular level, in certain disorders. This may be helpful as using red cells is obviously easier than getting renal biopsy material.

Loss of size selectivity of the GBM as a cause of proteinuria in renal disorders has been reported by many workers (Hardwick et al 1968, Kaysen et al 1986, Myers and Guash 1994). In all these studies, dextran molecules of varying sizes were used to study size selectivity. Other studies have utilised the SDS PAGE technique for urinary proteins as an indirect method of measuring defects in the GBM pore size (Shapiro & Maizel 1967, Balant et al 1974, Schiwara et al 1986, Jackson et al 1988, Brockelbank et al 1991). In the present study the latter technique was employed. This study not only confirmed the pathogenetic role of pore-size in proteinuria but also predicted steroid response; these findings support those of Brockelbank et al (1991). In addition, this technique was utilised to assess the patterns of proteinuria in the common histological groups of NS. This led to the success in differentiating MCNS from FGS by this relatively simple non-invasive technique. No such findings have been reported previously. The absolute reliability of SDS-PAGE in making this distinction is both unexpected and unusual. It is therefore a matter of some concern. No test should be expected to be so reliable! This can be attributed to the fact that the sensivity and specificity of SDS PAGE of urine proteins was not checked in a much larger number of patients from many other centres. It is therefore important before the findings of this study can be adopted for use that others reproduce these results in their laboratories.

Moreover, the findings in this thesis contribute to resolving some of the uncertainities regarding FGS; is it due to progression of MCNS or does it ansi de novo? This study suggests that FGS can be distinguished from MCNS at the clinical onset of the nephrotic syndrome in these children, providing further evidence that the two may be different diseases and histological deterioration to FGS from MCNS may not always be the case.

SDS PAGE appears to predict therapeutic response to steroids in MCNS better than the conventional SI method. Comparison of the two techniques for the assessment of steroid response are new; however, they are in accordance with the findings by the ISKDC (1970) and Adeniyi et al (1970) that the SI was an unreliable test in steroid responsive nephrotic syndrome.

Use of SDS PAGE to assess the patterns of proteinuria in the common histological groups seen in Durban are new and extend the findings reported by Brockelbank et al (1991). The latter studied fewer patients in a limited histological groups of NS. Furthermore, the majority of their patients were studied in advanced stages of renal impairment, whereas the children reported here had normal GFR's.

This method of predicting steroid response can be easily set up at appropriate levels of the health services in underdeveloped countries at reasonable cost. Whereas the cost of renal biopsy is about 1000 rands, (including hospital stay, and laboratory analysis by light microscopy and immunofluoroscence but excluding electron microscopy) SDS PAGE analysis is about 40 rands per patient. An added advantage being that the patient can be treated at an outpatient level. Moreover, renal biopsy is an invasive technique with its attendent morbidity and complications, whereas SDS PAGE analysis is non-invasive with no discomfort to the patients. With the SDS PAGE the expense occurs with the initial setting up of the technique, however, once established, the running cost is very reasonable.

Most laboratories in health facilities above clinic and community health centre level in many countries, including some in the third World, are equipped for electrophoretic techniques, hence the method can be set up in most regional and tertiary hospitals.

MODIFICATION OF LABORATORY TECHNIQUES

The established techniques for alcian blue binding to red blood cells (ABRBC) and SDS PAGE as reported in this thesis did not prove entirely successful. It was therefore necessary to modify the described techniques for both the above laboratory investigations. These modified techniques were used on the patients studied which has resulted in a better understanding of the mechanism of proteinuria and accurate prediction of underlying histological changes in nephrotic children.

SDS PAGE

In the limited number of previous studies available, the authors did not specifically set out to correlate urinary patterns of proteinuria with the different histological groups of glomerulonephritis, but to examine the potential of an automated SDS PAGE system to distinguish glomerular and tubular proteinuria in various diseases with renal involvement.

In the previous studies (Brockelbank et al 1991, Jackson et al 1992) the authors were less successful in the interpretation of proteinuria in renal diseases, primarily due to poor standardisation of the SDS PAGE technique. In the present study, the technique was modified and standardised as follows:

1. Accurate and unbiased interpretation of urinary patterns of proteinuria was made by applying the same amount of urinary proteins (20 ug/ml) to the polyacrylamide gel for all groups of nephrotic syndrome studied.
2. Co-electrophoresis of specific standard marker proteins such as albumin, IgG, haptoglobulin etc.enabled accurate identification of glomerular and tubular proteinuria.
3. Timed collection of urine samples (second morning) avoided any bias in the interpretation which may have occurred due to the circadian rythm of proteinuria (Koopman et al 1985).

4. Whereas many studies have reported the use of silver staining as a more sensitive method of detecting urinary proteins, they fail to recognise that in heavy proteinuria, as seen in nephrotic syndrome, there are many proteins in the urine and sensitive techniques are likely to reveal protein bands that have not been previously identified. Therefore in this thesis, use was made of the less sensitive coomasie blue stain to avoid any confusion with any less commonly recognised protein bands.
5. Densitometry analysis of proteinuric bands on the electrophoretic gel for quantification has been recommended by many workers. This was avoided in this thesis as it is well known that the binding of protein molecules to dyes such as coomasie blue is not stoichiomatic (i.e. not all protein molecules present will bind to the dye). Following this, quantification of proteinuria was not carried out.

ALCIAN BLUE TECHNIQUE

Reproducibility of the method of alcian blue binding to red blood cells has been a subject of controversy for many years. In this thesis, the method was modified as mentioned in chapter 2. One of the main problems encountered by most groups is the instability of the AB dye in solution. This problem was overcome by making up fresh solution of AB prior to use in order to avoid any precipitation of the dye. In addition the entire alcian blue study was performed from the same batch of alcian blue to avoid any inter-batch variation which may alter the absorbance value of the dye. With these modifications the inter- and intra-assay variations were of acceptable range enabling us to complete the study without bias.

CLINICAL APPLICATION OF THE FINDINGS

From these studies the single most important finding is the practical application, of SDS PAGE of urine proteins to the management of NS of childhood. This method is more sensitive than the conventional SI for prediction of steroid response. Prediction of steroid response at the onset of NS is important for children in any part of the world with this disease, but is especially useful in Africa. Extensive studies by our group in the past (Adhikari 1976, 1981; Coovadia 1987) have established that the overwhelming majority of African children with NS do not respond to steroid therapy. Indeed during the earlier years it was believed (incorrectly) that MCNS was rare or simply did not exist among African children (Coovadia 1979). However, it is now known that a small proportion of these patients do in fact respond to conventional doses and duration of steroids and behave in most respects like typical MCNS. SDS PAGE analysis of urine in African children enables the clinician to decide with confidence on the use of steroid therapy. In majority of non-African children in SA, in whom MCNS is the dominant type of NS, this test is also very useful. Moreover, the ability of this method to detect subtle changes in renal function in the presence of infection has direct clinical relevance as such patients often become temporarily steroid resistant.

The patterns of proteinuria provide suggestive evidence of the underlying histology; the proviso here is that the histological group should be homogeneous and firmly linked to a single clinical syndrome e.g. HBs positive MGN, MCNS, FGS etc. The test does not appear to work where nephrotic syndrome is part and parcel of a systemic disease, especially where the latter is characterised by a range of renal histological changes e.g. SLE, amyloidosis. Renal biopsy in these systemic diseases is clearly still the gold standard for diagnosis of underlying

kidney pathology.

Improved clinical and biochemical criteria have already been employed to restrict the indications for renal biopsy in the majority of children with diseases such as MCNS (ISKDC 1970) and PSGN (Coovadia et al 1987). Depending on the community profile of HBs, renal biopsy is also not indicated in HBs induced MGN (Coovadia et al 1993). Our findings arising from the use of this non-invasive technique (SDS PAGE), narrow the indications for renal biopsy even further. It is especially useful in distinguishing MCNS from FGS. It is accepted that the appropriate long term management of FGS requires precise histological diagnosis and therefore renal biopsy remains an essential investigation. However, SDS PAGE of urine proteins may be an especially helpful ancillary test until such time that findings of this thesis are refuted or confirmed.

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