

HEPATITIS B VIRUS- ASSOCIATED MEMBRANOUS NEPHROPATHY

by

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DECLARATION

This thesis is the author's own work and has not been submitted previously to this or any other university.

R. Bhimma

DEDICATION

*For my wife, Caronisha, my mother Sonmati, my late dad
Nundlall and the rest of my family*

SUMMARY

Glomerulonephritis as an extra hepatic manifestation of chronic HBV infection has now been well documented [1,2,3,4,5]. HBV-associated nephropathy has been described in areas of both high and low endemicity [6]. In Africa HBV-associated nephropathy has been reported from the southern, central and northern regions [7,8,9,10,11]. In the southern African continent the prevalence of HBV-associated nephropathy appears to be higher than the rest of the continent [12]. In KwaZulu/Natal, South Africa, the prevalence of hepatitis B surface antigenaemia (HbsAg) in urban, rural and institutionalised children was reported to be 6.3%, 18.5% and 35.4% and the HBV exposure rates, as shown by the presence of any marker of HBV infection, 19.5%, 65.1% and 70.1% respectively amongst black children [13]. Prior experience of nephrotic syndrome (NS) and its association with HBV in black children, already published in a series of reports, showed HBV-associated nephropathy to be the commonest form of nephrotic syndrome among black patients in KwaZulu/Natal; membranous nephropathy (MN) being the commonest histological type reported [7,14]. The only other large series of HBV-associated nephropathy in southern Africa was from Cape Town of a large cohort of children, mainly of mixed ancestry (coloured), with a small number of black children [8]. There have been no other large studies of this condition amongst black children in Africa.

We therefore undertook a series of studies to delineate the spectrum of this disease in black children with regard to the following: clinical presentation, laboratory

findings, natural history, biosocial background, genetics (using HLA Class I and II antigens) as well as the impact of treatment and prevention by immunisation.

We commenced these studies by reviewing our 20-year experience of 636 children with NS in Durban, South Africa for the period 1976- - 1995. Three hundred and six (48.2%) were blacks, 307 (48.2%) Indians and 23 (3.6%) were a mixed group (coloured); 91 (14.3%) could not be categorised and were excluded from the analysis. In black children, membranous nephropathy accounted for 43% of all cases of NS; 86.2% of these 306 children were associated with hepatitis B virus antigens [15]. This contrasts with the 2% - 5% prevalence of idiopathic membranous nephropathy reported in western countries [16].

We then proceeded to document the clinical features of this disease in black children. One hundred and thirty-three children with NS positive for HBV carriage were studied. In 70 patients the histological type was membranous; 46 of these 70 patients were followed up for a mean of 3.4 years (range 1-11). Spontaneous elimination of both HBsAg and HBeAg occurred in 10 (21.7%) of the 46 patients; 16 (34.8%) cleared HBeAg alone. Co-existing liver disease occurred in 18 (25.7%); hypocomplementaemia (low C3 and C4) in 22 (47.8%) and 5 (10.9%) of these 46 children respectively. Sixty-five (92.9%) of the 70 patients had normal renal function; 1(1.4%) impaired renal function; 3 (4.3%) chronic renal insufficiency and 1(1.4%) end stage renal disease at last hospital visit. Twelve (17.1%) of the 70 patients were in remission; all having cleared HBeAg. HBVMN was clinically indistinguishable from

24 children with idiopathic MN although biochemical characteristics were different. There were 23 patients with histological lesions other than MN. Forty patients with clinical, biochemical and serological findings similar to those with HBVMN and the other histological types, were unbiopsied. This report delineates the natural history of HBV infection in black South African children with NS, the majority of whom have MN. Disease remission in HBVMN parallels elimination of HBV antigens, particularly HBeAg. Comparison of HBVMN with idiopathic MN revealed clinically indistinguishable characteristics but unexplained biochemical differences [14].

Little is understood of the biosocial context in which HBV-associated nephropathy (particularly MN) develops. In the next two studies we evaluated HBV status and proteinuria in family members and household contacts of index children with HBVMN to test the hypothesis that HBV carriage and asymptomatic proteinuria are closely linked and may be causally associated.

In the first of these two studies, thirty-one black children with biopsy-proven HBVMN were the index cases. One hundred and fifty-two family members and 43 black household contacts were the subjects of this study. We assessed HBV carrier status by testing for HBV antigens and antibodies using enzyme-linked immunosorbent assays (ELISA) and for HBV DNA by using slot-blot hybridisation and nested polymerase chain reaction. Sequencing of the precore HBV region of HBV was done in a subset of both index cases and subjects. Proteinuria was assessed by measuring the urinary protein: creatinine ratio.

Seventy-two (37%) of the 197 family members and household contacts were HBV carriers, and 53 (27%) had a protein: creatinine ratio greater than the physiological limit (protein: creatinine ratio <0.2). Abnormal proteinuria was defined by a protein: creatinine ratio ≥ 0.2 . Continuous data was compared using analysis of variance. Categorical data were compared using Chi-square test or Fisher's exact test where appropriate. A probability of <0.05 was considered significant.

The frequency of abnormal proteinuria was not significantly different in those with [22 (30.5%) of 72] or without [33 (32%) of 104] HBV carriage. This lack of association remained when carriers were classified into those who were HBsAg positive only and those with active viral replication (HBsAg and/or HBeAg and/or HBV DNA; $p = 0.01$). Family members were more predisposed to HBV carriage than household contacts, but abnormal proteinuria was present with equal frequency ($p = 0.48$). Age had a significant impact on proteinuria, with children less than five years being more likely to have abnormal proteinuria ($p = 0.008$). The prevalence of abnormal proteinuria in family members and household contacts of the index cases was more than in community-based controls. The 10 index HBVMN cases and 14 family members and household contacts that were tested all had HBV of genotype A.

The results suggest that the family members and household contacts of children with HBVMN are at very high risk of HBV carriage; they also have asymptomatic proteinuria at a significantly higher rate than community-based controls. The HBV carrier status was not associated with proteinuria. This lack of association was a

finding supported by peak prevalences of proteinuria in those under five years but no corresponding peak of HBV carriage. Proteinuria may indicate glomerular basement membrane dysfunction. Environmental and social factors may underpin development of these two disorders, but are insufficient to account for the index cases of HBVMN. The emergence of children with HBVMN from such households additionally depends on unidentified and possibly genetic factors [17].

In the second study of the biosocial background in which the HBV carrier-state with MN develops, we used the same subjects. One hundred and twenty-three unrelated individuals from the communities of the index cases, negative for HBV, served as controls. In this study, proteinuria was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein: creatinine ratios. Patterns of proteinuria on SDS-PAGE were classified as glomerular, tubular or mixed; IgG and haptoglobin were suggestive of MN. Seventy-two (36.9%) of the 195 family members and household contacts were HBV carriers; 21 (29.2%) of these carriers had evidence of proteinuria using SDS-PAGE. Twenty-eight (41.2%) of the sixty-eight members of the study group who were HBV negative and 26.8% of the controls also showed proteinuria on SDS-PAGE. This lack of association between HBV carriage and proteinuria remained when controlled for gender and family relationship. Also, HBV was not protective against the development of proteinuria. Age was associated directly with a glomerular pattern of proteinuria ($p = 0.007$). Those having a pattern of proteinuria suggestive of MN were more likely to have an abnormal protein: creatinine ratio ($p = 0.001$). Ten (59%) subjects with a membranous pattern of proteinuria and 19 (47.5%) with a non-membranous pattern

of proteinuria had microscopic haematuria. Such a pattern of proteinuria was not significantly different between subjects and community based controls (8.7% vs. 6.5%, $p = 0.5$). Environmental exposures in these subjects may be responsible for the proteinuria, which probably reflects underlying glomerular basement membrane damage. Discordance between the HBV carrier-state and patterns of proteinuria in the study group suggest that interaction between specifically vulnerable individuals and HBV group suggest HBV and MN may not be causally related or that it reflects exceptional interaction between specifically vulnerable individuals and HBV [18].

From the above two studies we inferred that the pathogenetic mechanisms by which individuals with chronic HBV infection develop MN are probably dependent on interactions between viral, host and environmental factors; some evidence suggests a genetic predisposition. We therefore undertook another two studies to explore HLA associations in black children with HBVMN.

In the first of these two studies, thirty black children, age range 2 to 16 years, with biopsy-proven HBVMN, were the subjects of this study. HLA A, B and C antigens were determined using a two-stage lymphocytotoxic test. HLA DRB1* and DQB1* typing was done using sequence-specific primers. HLA class I and II antigen frequencies of the study subjects were compared to controls that were randomly chosen healthy blood donors from the same population.

HLA DQB1*0603 was increased in patients with HBVMN compared to controls (chi-square 13.65, RR 4.3). DRB1*07 and DQB1*02 were increased in frequency in the

study subjects but failed to reach statistical significance. There was no significant difference in the frequencies of class I antigens in the study group compared to controls.

This study is the first report of HLA associations in black patients with HBVMN in whom Class I and II antigens were determined using molecular methodology. It shows a high frequency of DQB1*0603 in black children with HBVMN compared to controls suggesting a possible genetic predisposition to the development of HBVMN [19].

Following our findings of an HLA Class II association in black children with HBVMN, we proceeded to determine if HLA DQB1*0603 predisposes to HBV carriage and development of abnormal proteinuria in the second study. We studied 70 family members of 14 children with HBVMN positive for HLA DQB1*0603 selected from the first study. Associations of HLA DQB1*0603 to HBV carriage and abnormal proteinuria were determined using the mean probability ratio (LOD scores).

Forty-seven of the 70 (67%) family members were positive for HBV infection. Nineteen (27%) had abnormal range proteinuria. LOD scores in the study subjects with DQB1*0603 who were HBV negative vs. those with DQB1*0603 who were HBV positive was not significant (anti-log sum = 2.0559 and average 0.23). When a

similar calculation was done for abnormal proteinuria, there were no significant findings (anti-log sum = 3.8587 and average 0.43).

This lack of association between HLA DQB1*0603 with either HBV carriage or abnormal proteinuria in family members suggests that additional factors may play a role in predisposing children to chronic HBV carriage and the development of MN. We therefore conclude that the main effect of HLA DQB1*0603 which distinguishes HBVMN from family members is the degree of proteinuria which is a reflection of the severity of glomerular basement membrane damage in the latter [20].

In the next study we proceed to investigate the efficacy of Interferon alpha 2b (INTRON A ®) in the treatment of HBV-associated nephropathy in black children. Twenty-four black children with biopsy-proven HBV-associated nephropathy were recruited into the study during the period April 1997 to June 1999. Five defaulted treatment and were excluded from the primary analysis. IFN α 2b was administered for 16 weeks. Response to treatment was defined as loss of HBeAg, decrease in proteinuria, and prevention of deterioration in renal and liver function. A control group of 20 patients was followed up for the same period.

Ten (52.6%) of the treated children responded with clearance of HBeAg by 40 weeks. None cleared HBsAg. All responders showed remission of proteinuria, 90% maintained normal renal function and 1 (10%) showed improvement of renal

function. HBV DNA levels decreased in this group. Nine patients did not clear HBeAg; none showed remission of proteinuria, 2 showed deterioration of renal function. Liver enzymes rose during treatment but subsequently declined irrespective of response to therapy. No serious side effects were encountered. Only 5% of controls showed spontaneous clearance of HBeAg, and none had remission of proteinuria.

Black children with HBV-associated nephropathy show accelerated clearance of HBeAg with remission of proteinuria following treatment with IFN α 2b. IFN α 2b was well-tolerated [21].

We then went on to investigate the impact of HBV vaccination in South Africa over 6 years on HBV-associated MN. HBV vaccine has resulted in a decline in the incidence of HBV carriage and hepatocellular carcinoma in South East Asia. Vaccine efficacy in Africa has not been adequately assessed.

King Edward VIII Hospital, Durban, South Africa, is the only tertiary referral centre for the province of KwaZulu/Natal for children with renal diseases. HBV vaccine was introduced into the Extended Programme on Immunisation (EPI) in April 1995; vaccine coverage rates between 1995-2001 for children for the first, second and third doses were 85.4%, 78.2% and 62.0% respectively. HBV status was determined using radioimmunoassay (1984 – 1991) or ELISA. MN was confirmed on renal biopsy. The hospital average annual incidence of HBVMN was compared pre and post-vaccination, and according to age groups.

Between 1984 and 2001 there were 119 children with HBVMN; the mean age was 7 years (range 1 to 14 years) and 101(85%) were males. The average annual rate ratio (aRR) per 10^5 child population was 0.25. The aRR of 0.03 for the years 2000-2001, was significantly lower than the aRR of 0.22 during the pre-immunisation period (1984 – 1994) [$p = 0.003$; RR = 0.12 (95% CI: 0.03 – 0.5)]. The aRR in 2000-2001 for children 0 – 4 years (0.00) and 5 – 10 years (0.09) were significantly lower than in the pre-vaccination years (0.16 and 0.46, $p = 0.01$ and 0.02 respectively). Thus, HBV vaccine, even at low coverage for the full EPI schedule, reduced the hospital incidence of HBVMN by six years [22].

From this series of studies we concluded that prior to the introduction of the HBV vaccine into the Expanded Programme on Immunisation in Children, HBV-associated nephropathy, particularly MN was the commonest form of NS in black children. Several studies have suggested on the basis of epidemiological, clinical and immunological evidence a causal association between chronic HBV carriage and the development of nephropathy. In our present series of studies we have findings that lend further support to the causal association between HBV carriage and development of nephropathy, particularly MN, in black children. We have shown that genetic and other environmental factors may also play a role in determining the degree of proteinuria. Those children with abnormal range proteinuria less than the nephrotic range show no association with HBV carriage or genetic factors with regard to HLA linkage. The efficacy of interferon treatment in elimination of the HBV and abrogation of proteinuria following clearance of the virus (particularly the HBeAg) as well as the impact of routine HBV immunisation in preventing HBV

carriage and subsequent development of nephropathy lends further support to our findings. The impact of viral load has yet to be investigated.

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PREFACE

The major part of the work reported in this thesis was performed in the Department of Paediatrics and Child Health, Nelson R Mandela School of Medicine, University of Natal, Durban, under the supervision of Professor HM Coovadia. Professor M Adhikari also provided support and guidance with the studies and part of the clinical care of the patients. Dr Anna Kramvis and Professor MC Kew did slot-blot hybridization and polymerase chain reaction for Hepatitis B virus at the MRC/CANSA/Molecular Hepatology Research Unit, Department of Medicine, University of Witwatersrand. Dr Mike G Hammond from the South African National Blood Service did the human leukocyte antigen assays.

Ethical approval for all studies was obtained from the Ethics and Professional Standards sub-committee (Faculty of Medicine), Nelson R Mandela School of Medicine, University of Natal. In the case of minors, informed written consent was obtained from the parent or guardian before entry into the study.

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 other authors, this has been duly acknowledged in the text.

Seven studies reported in this thesis have been published in peer-reviewed scientific journals; one has been submitted for peer review. One of the articles published in a

peer-reviewed scientific journal but not comprising part of this thesis used the clinical data and serum samples of the subjects used in this study. Research workers who are closely associated in these studies are co-authors in these publications.

PUBLICATIONS

One paper has been submitted for publication. All others have been published in peer-reviewed journal or have been accepted for publication.

1. Bhimma R, Coovadia HM, Adhikari M (1997) Nephrotic Syndrome in South African children: changing perspectives over 20 years. *Pediatr Nephrol* 11(4): 429-434.
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11. Bhimma R, Adhikari M (2003) Review: A review of Hepatitis B virus-associated Membranous Nephropathy. *The Specialist Forum* 3(6); 45-53

The Hepatology Research Unit of the University of Witwatersrand published No. 9, but the clinical data and histology were from the patients used in this thesis.

PRESENTATION AT CONGRESSES AND SCIENTIFIC MEETINGS

Bhimma R, Coovadia HM, Adhikari. Nephrotic Syndrome in South Africa Children revisited - changing perspectives over 20 years (1976 - 1995). XIVth International Congress of Nephrology, Sydney, Australia 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Nephrotic Syndrome in South Africa Children revisited - changing perspectives over 20 years (1976 - 1995). XIVth International Congress of Nephrology, Sydney, Australia, 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Hepatitis B virus associated nephropathy in black children – a 20 year review of our experience in South Africa. XIVth International Congress of Nephrology, Sydney, Australia, 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Hepatitis B virus associated nephropathy in black children – a 20 year review of our experience in South Africa. The 31st Annual Meeting of the European Society for Paediatric Nephrology September 27-30, 1997, Athens, Greece. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari M. Ecology of Hepatitis B virus associated membranous Nephropathy. The Eleventh Congress of the International Pediatric Nephrology Association, London, UK, 12-16 September 1998. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari M. Hepatitis B virus associated nephropathy in black South African children in KwaZulu/Natal. The Eleventh Congress of the International Pediatric Nephrology Association, London, UK, 12-16 September 1998 (Poster Presentation)

Bhimma R, Coovadia HM, Ramjee G, Kramvis A, Adhkari M, Kew MC. Fine Urinary Protein analysis using SDS-PAGE in Family Members and Household Contacts of Children with Hepatitis B Virus Associated Membranous Nephropathy. Paediatric Nephrology in Asia: Challenges in the New Millennium. The Mandarin Singapore, November 4- 6, 2000. (Poster Presentation)

Bhimma R, Coovadia HM, Kramvis A, Adhikari M, Kew MC, Connolly CA. Characterisation of Patterns of Proteinuria in Households of Children with Hepatitis B virus associated Membranous Nephropathy. 12th International IPNA Congress – Seattle, 1 – 5 September 2001. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari M. The Spectrum of Hepatitis B associated Nephropathy in Black Children in KwaZulu/ Natal. African Association of Nephrology (AFRAN), South African Renal Society (SARS), Renal Care Society of

South Africa (RCSSA) Joint Congress: 7- 10 September 1997, Durban, SA. (Oral Presentation)

Bhimma R, Coovadia HM, Kramvis A, Adhikari M, Kew MC, Connolly CA. The Ecology of Hepatitis B virus-associated membranous nephropathy. Faculty Research Day, University of Natal, 10 September 1997. (Oral Presentation)

Bhimma R, Coovadia HM, Kramvis A, Adhikari M, Kew MC, Connolly CA. Clustering of Hepatitis B virus and prevalence of proteinuria in asymptomatic family members and household contacts of children with Hepatitis B virus associated membranous nephropathy. Faculty Research Day, University of Natal, 26 August 1998. (Oral Presentation)

Bhimma R, Coovadia HM, Ramjee G, Kramvis A, Kew MC, Adhikari M, Connolly CA. Characterisation of Proteinuria in asymptomatic family members and household contacts of Children with Hepatitis B Virus associated Membranous Nephropathy. MilliPaed 2000 Congress (17 - 21 May 22, 2000) - 24th Biennial Conference of the South African Paediatric Association and South African Association of Paediatric Surgeons. (Oral Presentation)

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LIST OF ABBREVIATIONS

ALT	Alanine amino transferase
aRR	Average annual rate ratio
ASOT	Anti-streptolysin O titre
Au	Australian Antigen
C3	Complement fraction 3
C4	Complement fraction 4
CRF	Chronic renal failure
CRI	Chronic renal insufficiency
ELISA	Enzym linked immunosorbent assay
EPI	Expanded Programme on Immunisation
ESRD	End-stage renal disease
FSGS	Focal segmental glomerulosclerosis
GGT	Gamma glutamyl transferase
HBV	Hepatitis B virus
HBVMN	Hepatitis B virus-associated membranous nephropathy
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus

HLA	Human leukocyte antigen
IEF	Isoelectric focusing
IFN α 2 β	Interferon alpha 2 β (INTRON A®)
IMN	Idiopathic membranous nephropathy
INS	Idiopathic nephrotic syndrome
LOD Scores	Mean probability ratio scores
MCNS	Minimal change nephrotic syndrome
MN	Membranous nephropathy
NS	Nephrotic Syndrome
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
SBH	Slot-blot hybridization
SDS – PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SR	Steroid Resistant
SS	Steroid sensitive

DEFINITIONS

Nephrotic syndrome - oedema, plasma albumin <25 g/l; proteinuria >40 mg/m²/hr or protein (mg/dl) / creatinine (mg/dl) ratio >2.0.

Histological features on renal biopsy to diagnose membranous nephropathy (light and electron microscopy and immunofluorescence) according to the criteria used by Morel-Maroger: (Fig. 1,2) characterised by subepithelial immune deposits, often associated with subendothelial and mesangial deposits. The immunofluorescence criteria included the presence of IgG and the C3 component of complement in a diffuse granular pattern with IgM and IgA detected occasionally (Fig.3).

Urinary sediment - The presence of blood and protein on urinary dipstick analysis.

Frequent relapses - A patient with two or more relapses within 6 months of initial response or four or more relapses within any 12-month period.

Steroid sensitive - The absence of oedema and urinary sediment (Albustix = O/trace) following four weeks of **corticosteroid** therapy.

Steroid dependence - Two consecutive relapses occurring during cortico-steroid treatment or within 14 days of its cessation.

Steroid resistance - Failure to achieve response despite corticosteroid therapy.

Quantitation of urinary protein excretion by measurement of urinary protein (mg/ dl) / urinary creatinine (mg/dl).

Normal = <0.2 in children ≥ 2 years and
<0,5 in children <2 years

Abnormal proteinuria = ≥ 0.2 in children ≥ 2 years and
 ≥ 0.5 in children <2 years (*mild*
+ moderate + severe)

mild proteinuria = ≥ 0.2 and
<0.5 in children ≥ 2 years

moderate proteinuria = ≥ 0.5
and <2.0 in children ≥ 2 years

severe proteinuria (nephrotic
range) = ≥ 2.0 in children
 ≥ 2 years

Glomerular proteinuria - Proteins of molecular mass of 60 kd or greater. These include albumin (66 kd), transferrin (80 kd), haptoglobin (86 kd) and IgG (160 kd).

Tubular proteinuria - Proteins of molecular mass less than 60 kd. These include β_2 M (11.3 kd) and lysozyme (14 kd).

Mixed proteinuria - Combination of glomerular and tubular proteins other than IgG and/or haptoglobin.

Pattern of proteinuria suggestive of membranous nephropathy - albumin and IgG and/or haptoglobin in combination with any other proteins in the urine on SDS-PAGE.

Pattern of proteinuria suggestive of non-membranous nephropathy - absence of IgG and/or haptoglobin in the urine on SDS-PAGE in subjects having glomerular, tubular or mixed proteinuria.

Hypertension in Children – Blood pressure higher than the 90th percentile for age as defined by the Second Task Force on Blood Pressure Control in Children on three separate occasions.

Hypertension in Adults – Defined according to the Sixth Report of the Joint National Committee on Presentation, Detection, Evaluation and Treatment of High Blood Pressure.

Hypocomplementaemia - Defined as a C3 value of < 0.83 g/l.

Chronic renal insufficiency - GFR 25 - 40% of normal corrected for age (patient asymptomatic, may have short stature on physical examination, and laboratory findings may show increased PTH).

Chronic renal failure - GFR 10% - 25% of normal corrected for age (patient may be acidotic, anaemic, hypertensive with lethargy etc on physical examination).

End-stage renal disease - GFR usually <10% (dialysis needed to maintain quality of life).

Family members - included parents, full-siblings, half-siblings, aunts, uncles and grandparents.

A household contact - a person whose residence, at least for the last two years, was the same as the index child. Children younger than 2 years old were included if they had been living with the index child since birth and were genetically related.

Categorisation of Index Case, Family Members and household contacts (subjects):

categorised by HBV status using Elisa, SBH and PCR as follows:

Category A: HBsAg positive only = HBV carriers

Category B: HBsAg and/or HBeAg and/or SBH and/or PCR positive
= HBV carriers: active replication of HBV

Category C: anti-HBc IgG positive only or all markers negative by Elisa but SBH or PCR positive for HBV DNA = carrier

Category D: anti-HBc IgG positive with SBH and PCR negative
= exposed

Category E: Negative by all markers of HBV

= unexposed

Category F: Elisa Negative, SBH and PCR not done

= indeterminate

Chronic Liver Disease - patients with clinical evidence of liver disease (firm hepatomegaly \pm signs of portal hypertension; growth retardation; metabolic bone disease) who had normal or raised liver enzymes.

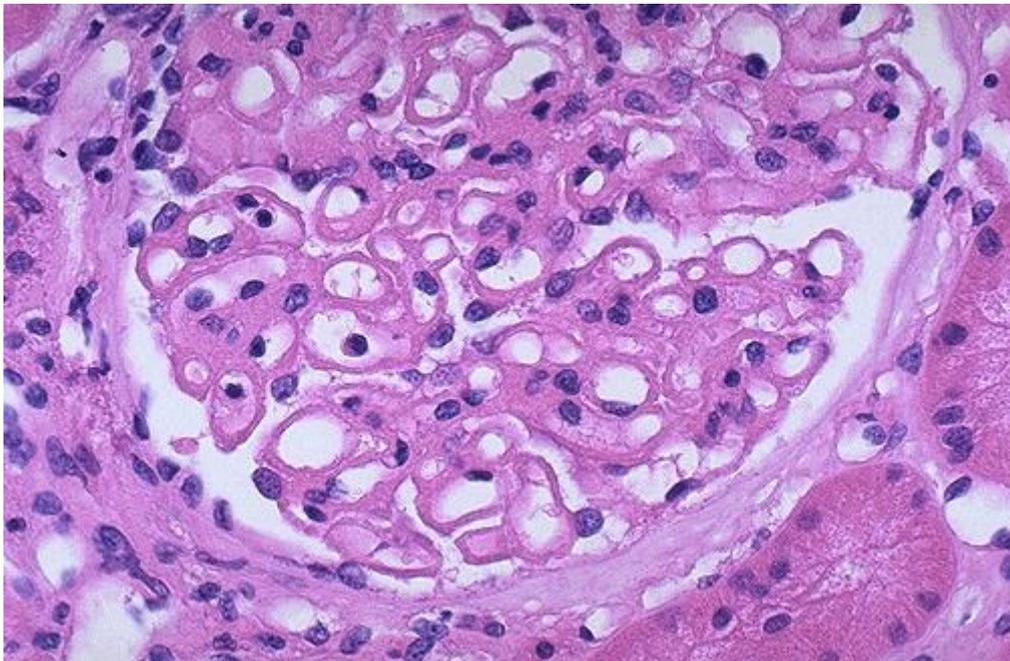


Figure 1: Haematoxylin and Eosin stain of light microscopic appearance of hepatitis B virus-associated membranous nephropathy in which the capillary loops are thickened and prominent, but the cellularity is not increased (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

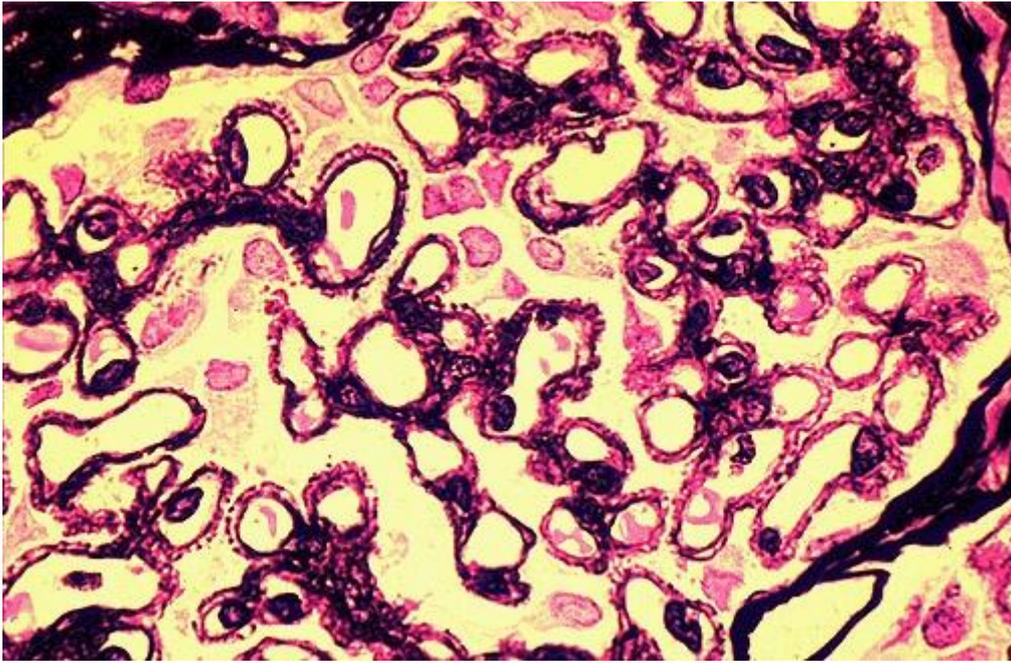


Figure 2: Periodic acid-schiff methenamine silver stain of the glomerulus highlights the proteinaceous basement membranes in black. There are characteristic "spikes" seen with hepatitis B virus-associated membranous nephropathy seen here in which the black basement membrane material appears as projections around the capillary loops (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

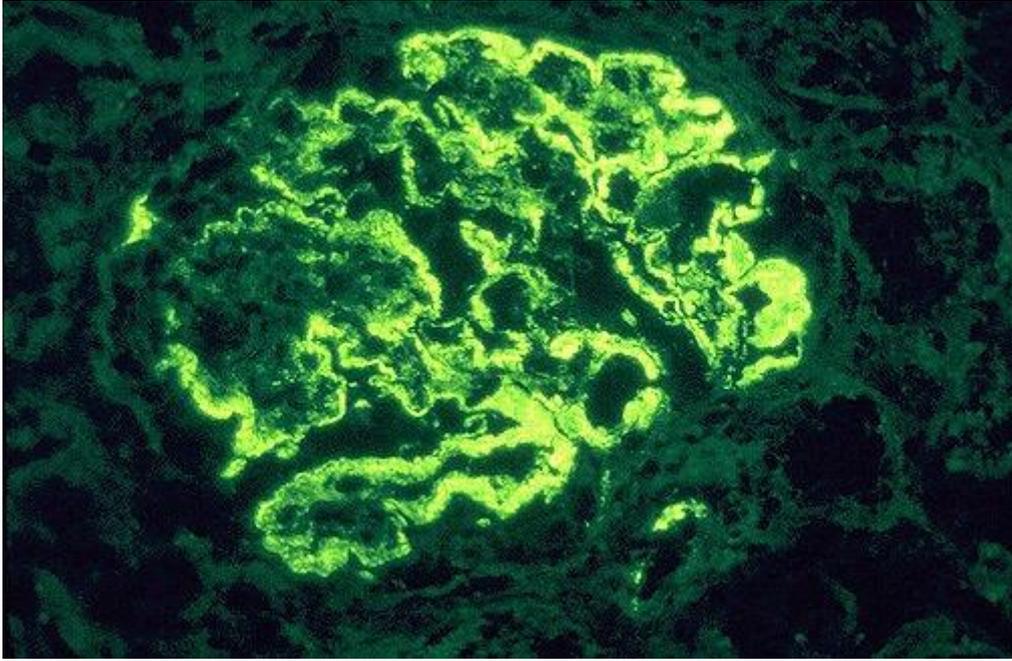


Figure 3: Hepatitis B virus-associated membranous nephropathy showing deposits of mainly IgG and complement in the basement membrane and appear in a diffuse granular pattern by immunofluorescence, as seen here (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

PURPOSE

One of the most common extra-hepatic manifestations of hepatitis B virus infection in black children is nephropathy, particularly membranous nephropathy. This set of studies explored various aspects of hepatitis B virus-associated membranous nephropathy in black children in South Africa in order to establish the following characteristics of the disease: natural history, clinical manifestations, pathogenesis (especially the biosocial background in which the disease develops and possible genetic predisposition), treatment and prevention.

HYPOTHESES

1. The clinical findings, biochemical features and the outcome of black children with hepatitis B virus-associated membranous nephropathy differ from that of children with idiopathic membranous nephropathy.
2. In the households of black children with hepatitis B virus-associated membranous nephropathy there will be a high level of clustering of hepatitis B virus infection.
3. In those individuals within these households infected with the virus, there will be a higher range of abnormal proteinuria resulting from glomerular basement membrane damage, compared to those individuals who are uninfected.
4. There is a genetic basis for the development of hepatitis B virus-associated membranous nephropathy.
5. Treatment with interferon alpha 2b will result in clearance of the virus and abrogation of proteinuria in black children with hepatitis B virus-associated nephropathy.
6. Childhood vaccinations programmes will result in a decline in the incidence of hepatitis B virus-associated nephropathy.

HEPATITIS B VIRUS- ASSOCIATED MEMBRANOUS NEPHROPATHY

by

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Submitted in partial fulfillment of the requirements for the degree of

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in the

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University of Natal, Nelson R Mandela School of Medicine
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DECLARATION

This thesis is the author's own work and has not been submitted previously to this or any other university.

R. Bhimma

DEDICATION

*For my wife, Caronisha, my mother Sonmati, my late dad
Nundlall and the rest of my family*

SUMMARY

Glomerulonephritis as an extra hepatic manifestation of chronic HBV infection has now been well documented [1,2,3,4,5]. HBV-associated nephropathy has been described in areas of both high and low endemicity [6]. In Africa HBV-associated nephropathy has been reported from the southern, central and northern regions [7,8,9,10,11]. In the southern African continent the prevalence of HBV-associated nephropathy appears to be higher than the rest of the continent [12]. In KwaZulu/Natal, South Africa, the prevalence of hepatitis B surface antigenaemia (HbsAg) in urban, rural and institutionalised children was reported to be 6.3%, 18.5% and 35.4% and the HBV exposure rates, as shown by the presence of any marker of HBV infection, 19.5%, 65.1% and 70.1% respectively amongst black children [13]. Prior experience of nephrotic syndrome (NS) and its association with HBV in black children, already published in a series of reports, showed HBV-associated nephropathy to be the commonest form of nephrotic syndrome among black patients in KwaZulu/Natal; membranous nephropathy (MN) being the commonest histological type reported [7,14]. The only other large series of HBV-associated nephropathy in southern Africa was from Cape Town of a large cohort of children, mainly of mixed ancestry (coloured), with a small number of black children [8]. There have been no other large studies of this condition amongst black children in Africa.

We therefore undertook a series of studies to delineate the spectrum of this disease in black children with regard to the following: clinical presentation, laboratory

findings, natural history, biosocial background, genetics (using HLA Class I and II antigens) as well as the impact of treatment and prevention by immunisation.

We commenced these studies by reviewing our 20-year experience of 636 children with NS in Durban, South Africa for the period 1976- - 1995. Three hundred and six (48.2%) were blacks, 307 (48.2%) Indians and 23 (3.6%) were a mixed group (coloured); 91 (14.3%) could not be categorised and were excluded from the analysis. In black children, membranous nephropathy accounted for 43% of all cases of NS; 86.2% of these 306 children were associated with hepatitis B virus antigens [15]. This contrasts with the 2% - 5% prevalence of idiopathic membranous nephropathy reported in western countries [16].

We then proceeded to document the clinical features of this disease in black children. One hundred and thirty-three children with NS positive for HBV carriage were studied. In 70 patients the histological type was membranous; 46 of these 70 patients were followed up for a mean of 3.4 years (range 1-11). Spontaneous elimination of both HBsAg and HBeAg occurred in 10 (21.7%) of the 46 patients; 16 (34.8%) cleared HBeAg alone. Co-existing liver disease occurred in 18 (25.7%); hypocomplementaemia (low C3 and C4) in 22 (47.8%) and 5 (10.9%) of these 46 children respectively. Sixty-five (92.9%) of the 70 patients had normal renal function; 1(1.4%) impaired renal function; 3 (4.3%) chronic renal insufficiency and 1(1.4%) end stage renal disease at last hospital visit. Twelve (17.1%) of the 70 patients were in remission; all having cleared HBeAg. HBVMN was clinically indistinguishable from

24 children with idiopathic MN although biochemical characteristics were different. There were 23 patients with histological lesions other than MN. Forty patients with clinical, biochemical and serological findings similar to those with HBVMN and the other histological types, were unbiopsied. This report delineates the natural history of HBV infection in black South African children with NS, the majority of whom have MN. Disease remission in HBVMN parallels elimination of HBV antigens, particularly HBeAg. Comparison of HBVMN with idiopathic MN revealed clinically indistinguishable characteristics but unexplained biochemical differences [14].

Little is understood of the biosocial context in which HBV-associated nephropathy (particularly MN) develops. In the next two studies we evaluated HBV status and proteinuria in family members and household contacts of index children with HBVMN to test the hypothesis that HBV carriage and asymptomatic proteinuria are closely linked and may be causally associated.

In the first of these two studies, thirty-one black children with biopsy-proven HBVMN were the index cases. One hundred and fifty-two family members and 43 black household contacts were the subjects of this study. We assessed HBV carrier status by testing for HBV antigens and antibodies using enzyme-linked immunosorbent assays (ELISA) and for HBV DNA by using slot-blot hybridisation and nested polymerase chain reaction. Sequencing of the precore HBV region of HBV was done in a subset of both index cases and subjects. Proteinuria was assessed by measuring the urinary protein: creatinine ratio.

Seventy-two (37%) of the 197 family members and household contacts were HBV carriers, and 53 (27%) had a protein: creatinine ratio greater than the physiological limit (protein: creatinine ratio <0.2). Abnormal proteinuria was defined by a protein: creatinine ratio ≥ 0.2 . Continuous data was compared using analysis of variance. Categorical data were compared using Chi-square test or Fisher's exact test where appropriate. A probability of <0.05 was considered significant.

The frequency of abnormal proteinuria was not significantly different in those with [22 (30.5%) of 72] or without [33 (32%) of 104] HBV carriage. This lack of association remained when carriers were classified into those who were HBsAg positive only and those with active viral replication (HBsAg and/or HBeAg and/or HBV DNA; $p = 0.01$). Family members were more predisposed to HBV carriage than household contacts, but abnormal proteinuria was present with equal frequency ($p = 0.48$). Age had a significant impact on proteinuria, with children less than five years being more likely to have abnormal proteinuria ($p = 0.008$). The prevalence of abnormal proteinuria in family members and household contacts of the index cases was more than in community-based controls. The 10 index HBVMN cases and 14 family members and household contacts that were tested all had HBV of genotype A.

The results suggest that the family members and household contacts of children with HBVMN are at very high risk of HBV carriage; they also have asymptomatic proteinuria at a significantly higher rate than community-based controls. The HBV carrier status was not associated with proteinuria. This lack of association was a

finding supported by peak prevalences of proteinuria in those under five years but no corresponding peak of HBV carriage. Proteinuria may indicate glomerular basement membrane dysfunction. Environmental and social factors may underpin development of these two disorders, but are insufficient to account for the index cases of HBVMN. The emergence of children with HBVMN from such households additionally depends on unidentified and possibly genetic factors [17].

In the second study of the biosocial background in which the HBV carrier-state with MN develops, we used the same subjects. One hundred and twenty-three unrelated individuals from the communities of the index cases, negative for HBV, served as controls. In this study, proteinuria was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein: creatinine ratios. Patterns of proteinuria on SDS-PAGE were classified as glomerular, tubular or mixed; IgG and haptoglobin were suggestive of MN. Seventy-two (36.9%) of the 195 family members and household contacts were HBV carriers; 21 (29.2%) of these carriers had evidence of proteinuria using SDS-PAGE. Twenty-eight (41.2%) of the sixty-eight members of the study group who were HBV negative and 26.8% of the controls also showed proteinuria on SDS-PAGE. This lack of association between HBV carriage and proteinuria remained when controlled for gender and family relationship. Also, HBV was not protective against the development of proteinuria. Age was associated directly with a glomerular pattern of proteinuria ($p = 0.007$). Those having a pattern of proteinuria suggestive of MN were more likely to have an abnormal protein: creatinine ratio ($p = 0.001$). Ten (59%) subjects with a membranous pattern of proteinuria and 19 (47.5%) with a non-membranous pattern

of proteinuria had microscopic haematuria. Such a pattern of proteinuria was not significantly different between subjects and community based controls (8.7% vs. 6.5%, $p = 0.5$). Environmental exposures in these subjects may be responsible for the proteinuria, which probably reflects underlying glomerular basement membrane damage. Discordance between the HBV carrier-state and patterns of proteinuria in the study group suggest that interaction between specifically vulnerable individuals and HBV group suggest HBV and MN may not be causally related or that it reflects exceptional interaction between specifically vulnerable individuals and HBV [18].

From the above two studies we inferred that the pathogenetic mechanisms by which individuals with chronic HBV infection develop MN are probably dependent on interactions between viral, host and environmental factors; some evidence suggests a genetic predisposition. We therefore undertook another two studies to explore HLA associations in black children with HBVMN.

In the first of these two studies, thirty black children, age range 2 to 16 years, with biopsy-proven HBVMN, were the subjects of this study. HLA A, B and C antigens were determined using a two-stage lymphocytotoxic test. HLA DRB1* and DQB1* typing was done using sequence-specific primers. HLA class I and II antigen frequencies of the study subjects were compared to controls that were randomly chosen healthy blood donors from the same population.

HLA DQB1*0603 was increased in patients with HBVMN compared to controls (chi-square 13.65, RR 4.3). DRB1*07 and DQB1*02 were increased in frequency in the

study subjects but failed to reach statistical significance. There was no significant difference in the frequencies of class I antigens in the study group compared to controls.

This study is the first report of HLA associations in black patients with HBVMN in whom Class I and II antigens were determined using molecular methodology. It shows a high frequency of DQB1*0603 in black children with HBVMN compared to controls suggesting a possible genetic predisposition to the development of HBVMN [19].

Following our findings of an HLA Class II association in black children with HBVMN, we proceeded to determine if HLA DQB1*0603 predisposes to HBV carriage and development of abnormal proteinuria in the second study. We studied 70 family members of 14 children with HBVMN positive for HLA DQB1*0603 selected from the first study. Associations of HLA DQB1*0603 to HBV carriage and abnormal proteinuria were determined using the mean probability ratio (LOD scores).

Forty-seven of the 70 (67%) family members were positive for HBV infection. Nineteen (27%) had abnormal range proteinuria. LOD scores in the study subjects with DQB1*0603 who were HBV negative vs. those with DQB1*0603 who were HBV positive was not significant (anti-log sum = 2.0559 and average 0.23). When a

similar calculation was done for abnormal proteinuria, there were no significant findings (anti-log sum = 3.8587 and average 0.43).

This lack of association between HLA DQB1*0603 with either HBV carriage or abnormal proteinuria in family members suggests that additional factors may play a role in predisposing children to chronic HBV carriage and the development of MN. We therefore conclude that the main effect of HLA DQB1*0603 which distinguishes HBVMN from family members is the degree of proteinuria which is a reflection of the severity of glomerular basement membrane damage in the latter [20].

In the next study we proceed to investigate the efficacy of Interferon alpha 2b (INTRON A ®) in the treatment of HBV-associated nephropathy in black children. Twenty-four black children with biopsy-proven HBV-associated nephropathy were recruited into the study during the period April 1997 to June 1999. Five defaulted treatment and were excluded from the primary analysis. IFN α 2b was administered for 16 weeks. Response to treatment was defined as loss of HBeAg, decrease in proteinuria, and prevention of deterioration in renal and liver function. A control group of 20 patients was followed up for the same period.

Ten (52.6%) of the treated children responded with clearance of HBeAg by 40 weeks. None cleared HBsAg. All responders showed remission of proteinuria, 90% maintained normal renal function and 1 (10%) showed improvement of renal

function. HBV DNA levels decreased in this group. Nine patients did not clear HBeAg; none showed remission of proteinuria, 2 showed deterioration of renal function. Liver enzymes rose during treatment but subsequently declined irrespective of response to therapy. No serious side effects were encountered. Only 5% of controls showed spontaneous clearance of HBeAg, and none had remission of proteinuria.

Black children with HBV-associated nephropathy show accelerated clearance of HBeAg with remission of proteinuria following treatment with IFN α 2b. IFN α 2b was well-tolerated [21].

We then went on to investigate the impact of HBV vaccination in South Africa over 6 years on HBV-associated MN. HBV vaccine has resulted in a decline in the incidence of HBV carriage and hepatocellular carcinoma in South East Asia. Vaccine efficacy in Africa has not been adequately assessed.

King Edward VIII Hospital, Durban, South Africa, is the only tertiary referral centre for the province of KwaZulu/Natal for children with renal diseases. HBV vaccine was introduced into the Extended Programme on Immunisation (EPI) in April 1995; vaccine coverage rates between 1995-2001 for children for the first, second and third doses were 85.4%, 78.2% and 62.0% respectively. HBV status was determined using radioimmunoassay (1984 – 1991) or ELISA. MN was confirmed on renal biopsy. The hospital average annual incidence of HBVMN was compared pre and post-vaccination, and according to age groups.

Between 1984 and 2001 there were 119 children with HBVMN; the mean age was 7 years (range 1 to 14 years) and 101(85%) were males. The average annual rate ratio (aRR) per 10^5 child population was 0.25. The aRR of 0.03 for the years 2000-2001, was significantly lower than the aRR of 0.22 during the pre-immunisation period (1984 – 1994) [$p = 0.003$; RR = 0.12 (95% CI: 0.03 – 0.5)]. The aRR in 2000-2001 for children 0 – 4 years (0.00) and 5 – 10 years (0.09) were significantly lower than in the pre-vaccination years (0.16 and 0.46, $p = 0.01$ and 0.02 respectively). Thus, HBV vaccine, even at low coverage for the full EPI schedule, reduced the hospital incidence of HBVMN by six years [22].

From this series of studies we concluded that prior to the introduction of the HBV vaccine into the Expanded Programme on Immunisation in Children, HBV-associated nephropathy, particularly MN was the commonest form of NS in black children. Several studies have suggested on the basis of epidemiological, clinical and immunological evidence a causal association between chronic HBV carriage and the development of nephropathy. In our present series of studies we have findings that lend further support to the causal association between HBV carriage and development of nephropathy, particularly MN, in black children. We have shown that genetic and other environmental factors may also play a role in determining the degree of proteinuria. Those children with abnormal range proteinuria less than the nephrotic range show no association with HBV carriage or genetic factors with regard to HLA linkage. The efficacy of interferon treatment in elimination of the HBV and abrogation of proteinuria following clearance of the virus (particularly the HBeAg) as well as the impact of routine HBV immunisation in preventing HBV

carriage and subsequent development of nephropathy lends further support to our findings. The impact of viral load has yet to be investigated.

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PREFACE

The major part of the work reported in this thesis was performed in the Department of Paediatrics and Child Health, Nelson R Mandela School of Medicine, University of Natal, Durban, under the supervision of Professor HM Coovadia. Professor M Adhikari also provided support and guidance with the studies and part of the clinical care of the patients. Dr Anna Kramvis and Professor MC Kew did slot-blot hybridization and polymerase chain reaction for Hepatitis B virus at the MRC/CANSA/Molecular Hepatology Research Unit, Department of Medicine, University of Witwatersrand. Dr Mike G Hammond from the South African National Blood Service did the human leukocyte antigen assays.

Ethical approval for all studies was obtained from the Ethics and Professional Standards sub-committee (Faculty of Medicine), Nelson R Mandela School of Medicine, University of Natal. In the case of minors, informed written consent was obtained from the parent or guardian before entry into the study.

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 other authors, this has been duly acknowledged in the text.

Seven studies reported in this thesis have been published in peer-reviewed scientific journals; one has been submitted for peer review. One of the articles published in a

peer-reviewed scientific journal but not comprising part of this thesis used the clinical data and serum samples of the subjects used in this study. Research workers who are closely associated in these studies are co-authors in these publications.

PUBLICATIONS

One paper has been submitted for publication. All others have been published in peer-reviewed journal or have been accepted for publication.

1. Bhimma R, Coovadia HM, Adhikari M (1997) Nephrotic Syndrome in South African children: changing perspectives over 20 years. *Pediatr Nephrol* 11(4): 429-434.
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11. Bhimma R, Adhikari M (2003) Review: A review of Hepatitis B virus-associated Membranous Nephropathy. *The Specialist Forum* 3(6); 45-53

The Hepatology Research Unit of the University of Witwatersrand published No. 9, but the clinical data and histology were from the patients used in this thesis.

PRESENTATION AT CONGRESSES AND SCIENTIFIC MEETINGS

Bhimma R, Coovadia HM, Adhikari. Nephrotic Syndrome in South Africa Children revisited - changing perspectives over 20 years (1976 - 1995). XIVth International Congress of Nephrology, Sydney, Australia 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Nephrotic Syndrome in South Africa Children revisited - changing perspectives over 20 years (1976 - 1995). XIVth International Congress of Nephrology, Sydney, Australia, 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Hepatitis B virus associated nephropathy in black children – a 20 year review of our experience in South Africa. XIVth International Congress of Nephrology, Sydney, Australia, 25 - 29 May 1997. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari. Hepatitis B virus associated nephropathy in black children – a 20 year review of our experience in South Africa. The 31st Annual Meeting of the European Society for Paediatric Nephrology September 27-30, 1997, Athens, Greece. (Poster Presentation)

Bhimma R, Coovadia HM, Adhikari M. Ecology of Hepatitis B virus associated membranous Nephropathy. The Eleventh Congress of the International Pediatric Nephrology Association, London, UK, 12-16 September 1998. (Poster Presentation)

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LIST OF ABBREVIATIONS

ALT	Alanine amino transferase
aRR	Average annual rate ratio
ASOT	Anti-streptolysin O titre
Au	Austarlian Antigen
C3	Complement fraction 3
C4	Complement fraction 4
CRF	Chronic renal failure
CRI	Chronic renal insufficiency
ELISA	Enzym linked immunosorbet assay
EPI	Expanded Programme on Immunisation
ESRD	End-stage renal disease
FSGS	Focal segmental glomerulosclerosis
GGT	Gamma glutamyl tranferase
HBV	Hepatitis B virus
HBVMN	Hepatitis B virus-associated membranous nephropathy
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus

HLA	Human leukocyte antigen
IEF	Isoelectric focusing
IFN α 2 β	Interferon alpha 2 β (INTRON A®)
IMN	Idiopathic membranous nephropathy
INS	Idiopathic nephrotic syndrome
LOD Scores	Mean probability ratio scores
MCNS	Minimal change nephrotic syndrome
MN	Membranous nephropathy
NS	Nephrotic Syndrome
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
SBH	Slot-blot hybridization
SDS – PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SR	Steroid Resistant
SS	Steroid sensitive

DEFINITIONS

Nephrotic syndrome - oedema, plasma albumin <25 g/l; proteinuria >40 mg/m²/hr or protein (mg/dl) / creatinine (mg/dl) ratio >2.0.

Histological features on renal biopsy to diagnose membranous nephropathy (light and electron microscopy and immunofluorescence) according to the criteria used by Morel-Maroger: (Fig. 1,2) characterised by subepithelial immune deposits, often associated with subendothelial and mesangial deposits. The immunofluorescence criteria included the presence of IgG and the C3 component of complement in a diffuse granular pattern with IgM and IgA detected occasionally (Fig.3).

Urinary sediment - The presence of blood and protein on urinary dipstick analysis.

Frequent relapses - A patient with two or more relapses within 6 months of initial response or four or more relapses within any 12-month period.

Steroid sensitive - The absence of oedema and urinary sediment (Albustix = O/trace) following four weeks of **corticosteroid** therapy.

Steroid dependence - Two consecutive relapses occurring during cortico-steroid treatment or within 14 days of its cessation.

Steroid resistance - Failure to achieve response despite corticosteroid therapy.

Quantitation of urinary protein excretion by measurement of urinary protein (mg/ dl) / urinary creatinine (mg/dl).

Normal = <0.2 in children ≥ 2 years and
<0,5 in children <2 years

Abnormal proteinuria = ≥ 0.2 in children ≥ 2 years and
 ≥ 0.5 in children <2 years (*mild*
+ moderate + severe)

mild proteinuria = ≥ 0.2 and
<0.5 in children ≥ 2 years

moderate proteinuria = ≥ 0.5
and <2.0 in children ≥ 2 years

severe proteinuria (nephrotic
range) = ≥ 2.0 in children
 ≥ 2 years

Glomerular proteinuria - Proteins of molecular mass of 60 kd or greater. These include albumin (66 kd), transferrin (80 kd), haptoglobin (86 kd) and IgG (160 kd).

Tubular proteinuria - Proteins of molecular mass less than 60 kd. These include β_2 M (11.3 kd) and lysozyme (14 kd).

Mixed proteinuria - Combination of glomerular and tubular proteins other than IgG and/or haptoglobin.

Pattern of proteinuria suggestive of membranous nephropathy - albumin and IgG and/or haptoglobin in combination with any other proteins in the urine on SDS-PAGE.

Pattern of proteinuria suggestive of non-membranous nephropathy - absence of IgG and/or haptoglobin in the urine on SDS-PAGE in subjects having glomerular, tubular or mixed proteinuria.

Hypertension in Children – Blood pressure higher than the 90th percentile for age as defined by the Second Task Force on Blood Pressure Control in Children on three separate occasions.

Hypertension in Adults – Defined according to the Sixth Report of the Joint National Committee on Presentation, Detection, Evaluation and Treatment of High Blood Pressure.

Hypocomplemetaemia - Defined as a C3 value of < 0.83 g/l.

Chronic renal insufficiency - GFR 25 - 40% of normal corrected for age (patient asymptomatic, may have short stature on physical examination, and laboratory findings may show increased PTH).

Chronic renal failure - GFR 10% - 25% of normal corrected for age (patient may be acidotic, anaemic, hypertensive with lethargy etc on physical examination).

End-stage renal disease - GFR usually <10% (dialysis needed to maintain quality of life).

Family members - included parents, full-siblings, half-siblings, aunts, uncles and grandparents.

A household contact - a person whose residence, at least for the last two years, was the same as the index child. Children younger than 2 years old were included if they had been living with the index child since birth and were genetically related.

Categorisation of Index Case, Family Members and household contacts (subjects):

categorised by HBV status using Elisa, SBH and PCR as follows:

Category A: HBsAg positive only = HBV carriers

Category B: HBsAg and/or HBeAg and/or SBH and/or PCR positive
= HBV carriers: active replication of HBV

Category C: anti-HBc IgG positive only or all markers negative by Elisa but SBH or PCR positive for HBV DNA = carrier

Category D: anti-HBc IgG positive with SBH and PCR negative
= exposed

Category E: Negative by all markers of HBV

= unexposed

Category F: Elisa Negative, SBH and PCR not done

= indeterminate

Chronic Liver Disease - patients with clinical evidence of liver disease (firm hepatomegaly \pm signs of portal hypertension; growth retardation; metabolic bone disease) who had normal or raised liver enzymes.

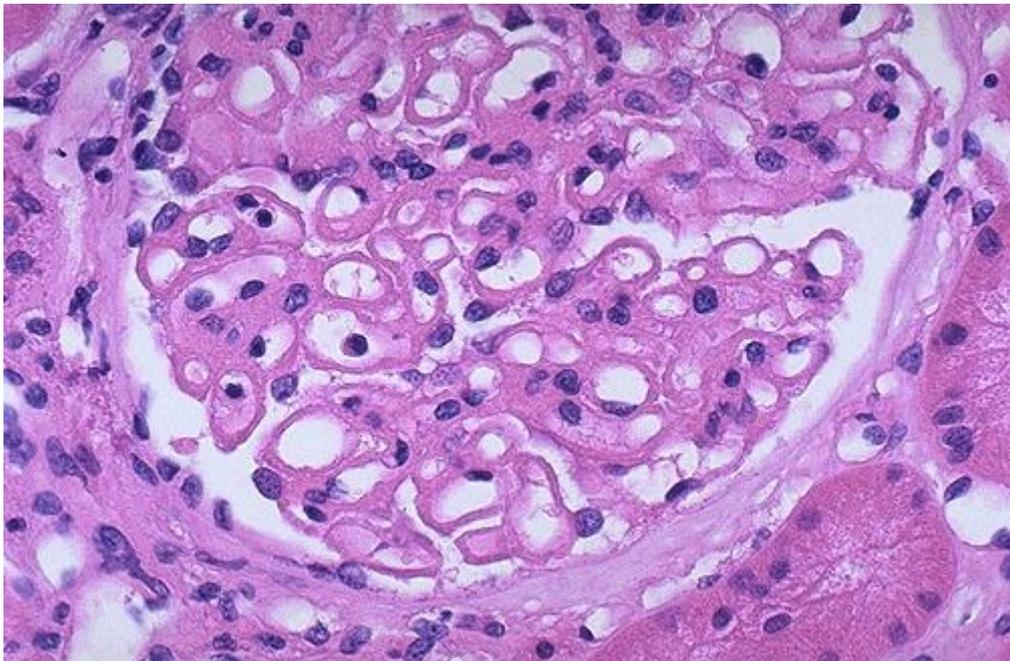


Figure 1: Haematoxylin and Eosin stain of light microscopic appearance of hepatitis B virus-associated membranous nephropathy in which the capillary loops are thickened and prominent, but the cellularity is not increased (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

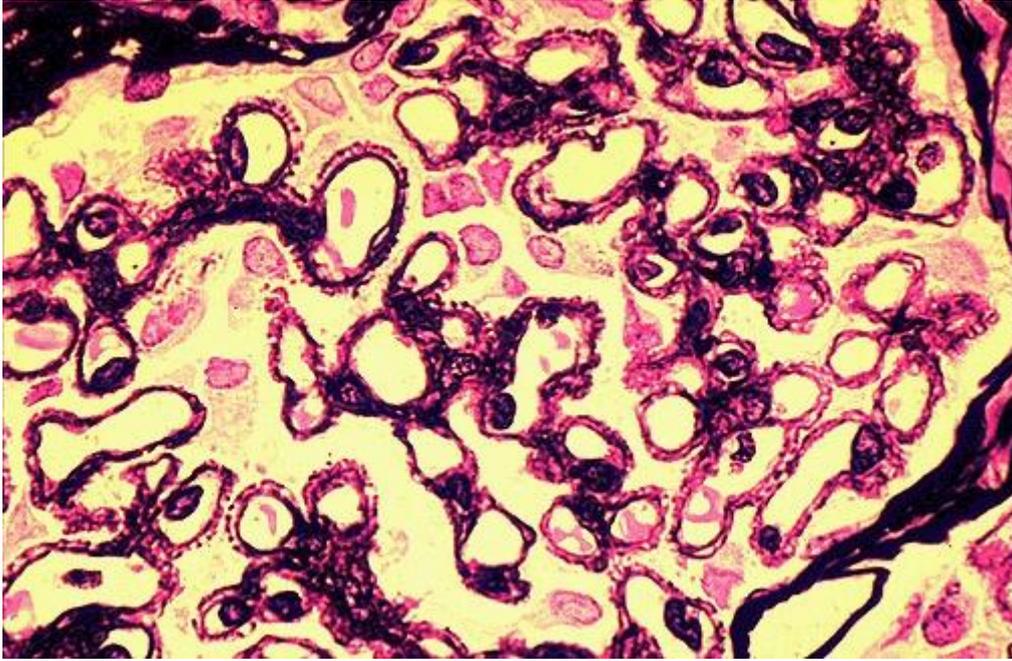


Figure 2: Periodic acid-schiff methenamine silver stain of the glomerulus highlights the proteinaceous basement membranes in black. There are characteristic "spikes" seen with hepatitis B virus-associated membranous nephropathy seen here in which the black basement membrane material appears as projections around the capillary loops (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

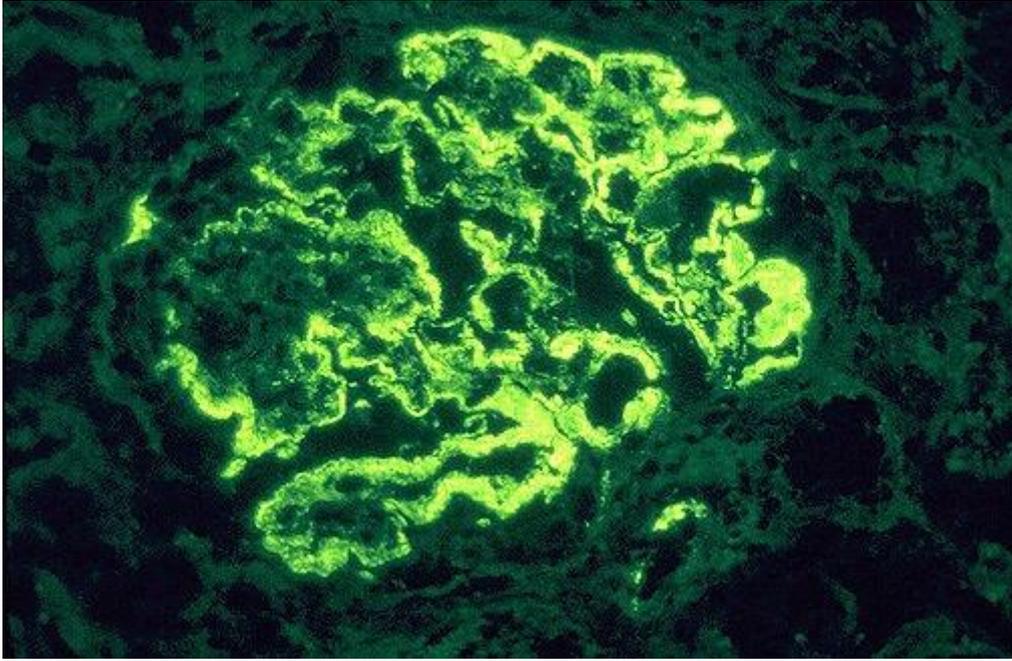


Figure 3: Hepatitis B virus-associated membranous nephropathy showing deposits of mainly IgG and complement in the basement membrane and appear in a diffuse granular pattern by immunofluorescence, as seen here (Magnification X 400, modified on computer software for best quality appearance). Permission of Medical Superintendent of King Edward VIII Hospital, Department of Pathology.

PURPOSE

One of the most common extra-hepatic manifestations of hepatitis B virus infection in black children is nephropathy, particularly membranous nephropathy. This set of studies explored various aspects of hepatitis B virus-associated membranous nephropathy in black children in South Africa in order to establish the following characteristics of the disease: natural history, clinical manifestations, pathogenesis (especially the biosocial background in which the disease develops and possible genetic predisposition), treatment and prevention.

HYPOTHESES

1. The clinical findings, biochemical features and the outcome of black children with hepatitis B virus-associated membranous nephropathy differ from that of children with idiopathic membranous nephropathy.
2. In the households of black children with hepatitis B virus-associated membranous nephropathy there will be a high level of clustering of hepatitis B virus infection.
3. In those individuals within these households infected with the virus, there will be a higher range of abnormal proteinuria resulting from glomerular basement membrane damage, compared to those individuals who are uninfected.
4. There is a genetic basis for the development of hepatitis B virus-associated membranous nephropathy.
5. Treatment with interferon alpha 2b will result in clearance of the virus and abrogation of proteinuria in black children with hepatitis B virus-associated nephropathy.
6. Childhood vaccinations programmes will result in a decline in the incidence of hepatitis B virus-associated nephropathy.

CHAPTER 1

HISTORY AND CLASSIFICATION OF NEPHROTIC SYNDROME

CHAPTER 1

HISTORY AND CLASSIFICATION OF NEPHROTIC SYNDROME

1. 1. History

The classical features of the disease now called the idiopathic nephrotic syndrome of childhood (INS) must have been known since before the days of Hippocrates, but the earliest written description seems to be that of Roelans in 1484. By 1722 (a century before Richard Bright clearly defined the link between serum and urine abnormalities of oedematous patients, with autopsy observations of the kidney), Zuinger had linked the natural history of INS from the oedematous nephrotic through to the prodigious thirst of terminal renal failure [1].

As the 20th century entered its third decade, an ever-increasing interest arose in various aspects of the disorders of the urinary tract. Edehhol's bilateral decapsulation of the kidney for nephrotic syndrome became the state of the art treatment in the 1920s. In 1929 Campbell published figures on the outcome of children with gross oedema who had bilateral decapsulation of the kidneys for nephrotic syndrome. He showed a 2-year recovery rate in 13 of 26 children. It is

interesting that even then, Campbell realised that the stress of the operation was the likely cause of improvement and not the decapsulation.

A term prevalent in the first half of the 20th century to denote a pale, enlarged, and puffy kidney from hypoproteinemia was *nephrosis*. As popularized by F. Volhard and T. Fahr, and C. Munck, nephrosis was largely replaced by the more descriptive term *nephrotic syndrome*, which indicates the features of hypoproteinemia, edema, and hypocholesterolemia [2]. Thomas Addis [3], a Scotsman who thought at Stanford, was highly critical of the term nephrosis as being “at best a nominal interim diagnosis, a convenience for the moment.” Although the term nephrosis persisted into the 1950s and 1960s in individual papers [4], by 1960 it was largely gone [5,6]. Another common term was Bright’s disease [7,8,9], which denoted chronic renal failure, proteinuria, edema, hypertension, and fatal prognosis [3,7,10].

Better clarification of Bright’s disease came about with the findings of Longscope (1936) [11] and Ellis (1942) [12], who suggested that there were two distinct types of disease. An acute stage (type I) in which patients had haematuria, proteinuria, some oedema and transient hypertension at onset and in which symptoms were preceded by a throat infection in the majority of patients. The general course of the disease was towards recovery. In the type II disease, the patients had severe

persistent proteinuria and oedema and the course was usually fatal. It is clear today that the latter form was describing the **Nephrotic Syndrome**.

During the period 1939 – 1945, the Second World War brought about a sharp change in scientific medicine and paediatric nephrology became established as a self-standing discipline. It was now the United States and not Europe that was the leader in the field. In the early 1950's renal biopsy was reported as a safe and relatively simple clinical procedure [13,14]. The first report of the use in nephrotic children was in 1957 [15]. The safety and value for both clinical and research purposes has subsequently been firmly established [16]. In 1966, Harmen Tiddens of Utrecht established the European Society for Paediatric Nephrology. Twenty-three European countries were invited and sent representatives to Glasgow where the inaugural meeting was held in September 1967. One year later, the American Society of Paediatric Nephrology was formed followed by the Japanese Paediatric Nephrology Society. In parallel with these events another International Group was developing. In 1965, Henry Barnett was invited to give a lecture to the British Paediatric Association. In discussion afterwards the idea of the International Collaborative Study of Kidney Disease in Children (ICSKDC) sprang to being. A central office was set up with Drs C. Edelmann, I. Grier and M. Abramowicz at the Albert Einstein College of Medicine, New York, USA. All clinics participating in the collaboration were asked to provide retrospective data from their records, and 541 cases were accepted.

A controlled double-blinded prospective study of azathioprine treatment of children with INS was commenced. The criteria, management and treatment were laid down for further trials, including cyclophosphamide treatment of INS. Each biopsy specimen was read 'blindly' by pathologists in France, England and USA (2). Four hundred children with INS were followed up prospectively to correlate histology with response to treatment. The prognostic significance of focal segmental glomerulosclerosis was recognised by the ICSKDC in 1970 and was re-discovered very rapidly at many centres. When cyclophosphamide gonadal toxicity became a problem the framework of ICSKDC together with the National Kidney Research Foundation of New York rapidly set up a register of cyclophosphamide gonadal toxicity for children [1].

1.2. Classification of the Nephrotic Syndrome

The nephrotic syndrome describes a constellation of features that have many different causes and histopathological appearances. 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.

The disease is presently classified into 2 major categories:

- | | | |
|----------------------|---|-------------------|
| Primary (Idiopathic) | - | aetiology unknown |
| Secondary | - | aetiology known |

The major histopathological categories of the primary nephrotic syndrome correspond to the World Health Organisation classification, which is the accepted world standard (Table I) [17].

One of the most well documented secondary forms of the nephrotic syndrome, particularly in children, is that associated with chronic hepatitis B virus carriage, particularly in regions endemic for hepatitis B virus.

Table I: Morphologic heterogeneity of glomerular lesions within the primary nephrotic syndrome of childhood

Idiopathic nephrotic syndrome with

- Minimal change disease
- Diffuse mesangial hypercellularity
- Focal segmental glomerular sclerosis

Mesangiocapillary glomerulonephritis with

- Subendothelial deposit (type 1)
- Dense deposit (type 2)
- Transmembranous deposit (type 3)

Membranous glomerulonephritis

Proliferative glomerulonephritis

- Focal
- Diffuse

Diffuse mesangial sclerosis in congenital forms of nephrotic syndrome: Denys-Drash syndrome, Idiopathic and recessive diffuse mesangial sclerosis, and Galloway-Mowat syndrome

Glomerular obsolescence and sclerosis (chronic glomerulonephritis)

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CHAPTER 2

PROTEINURIA

CHAPTER 2

PROTEINURIA

2.1. Introduction

Proteinuria is a common laboratory finding in children and is the hallmark of renal disease. It can be identified as either a transient or a persistent finding and can represent a benign condition or a serious disease. Most healthy children excrete small amounts of protein in their urine, representing so-called physiological proteinuria [1]. The protein is heterogenous in type and source, comprising plasma proteins, renal tissue enzymes and antigens, and renal secretions [2]. When corrected for body surface area, the protein excretion is highest in the newborn infants, decreases with age until adolescence, when adult levels are reached (Table II). The relatively high protein excretion observed in newborns represents tubular proteinuria, reflecting the immaturity of their renal function [3]. Asymptomatic or isolated proteinuria is defined as proteinuria not associated with any signs or symptoms of disease. Increased protein excretion is associated with renal pathology, but the qualitative composition of the protein is of most diagnostic value. To interpret urinary protein data, it is essential to know how the kidney handles proteins.

Table II: Normal urinary protein excretion in infants and children

Age group	Total protein (mg per 24 hours)	Total protein (mg per m² per 24 hours)	95 percent confidence limits (mg per m² per 24 hours)
5 - 30 days (premature)	29	182	88 to 377
7 - 30 days (full term)	32	145	68 to 309
2 to 12 months (infant)	38	109	48 to 244
2 to 4 years (child)	49	91	37 to 223
4 to 10 years	71	85	31 to 234
10 to 16 years	83	63	22 to 181

Adapted with permission from: Adham ML (1998) Evaluating Proteinuria in Children. Am Fam Phys 58: 1145-1152

2.2. Mechanisms of Proteinuria

Proteinuria can result from a structural lesion of the glomerular basement membrane, a disturbance in charges on the glomerular basement membrane or an abnormality in filtration haemodynamics. The glomerular capillary wall and its adjacent structures constitute the main barrier to the passage of macromolecules, including globulins and albumin. The barrier consists of the endothelial cells lining the capillary loops, the glomerular basement membrane and the visceral epithelial cells (Fig 4). The passage of macromolecules across the glomerular capillary wall is inversely proportional to their size.

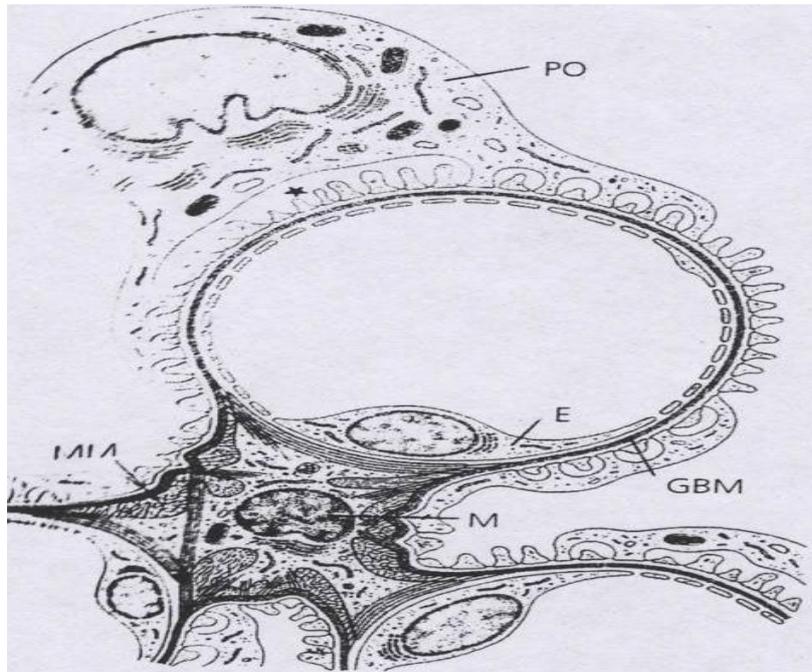


Figure 4: Structure of glomerular tuft, showing components of the glomerular capillary wall. (PO= epithelial cell with its foot processes forming the external aspect of the filtration barrier; MM= mesangial matrix; E= endothelial cell with fenestrae along the internal aspect of filtration barrier; GBM= glomerular basement membrane; M= mesangial cell). Adapted with permission from: Adham ML (1998) Evaluating Proteinuria in Children. Am Fam Phys 58: 1145-1152

2.2.1. Size Selectivity

In the initial observations of size selectivity, it was noted that there was a decline of fractional clearance with increasing molecular size [4]. The first model of the filtration barrier was isoporous, demonstrating pores of uniform size. This model failed to explain the appearance of small amounts of large test molecules in the glomerular filtrate. Subsequently a heteroporous model was proposed featuring two distinct populations of pores of small and large radii. This is the currently held concept of size selectivity on which the selectivity index is based. This index is a ratio of the clearance of transferrin to IgG. A ratio of more than 0.20 denotes non-selectivity and one of less than 0.20 denotes selective proteinuria predicting a good response to therapy with steroids or cyclophosphamide [5,6]. Alterations in size selectivity occur in Minimal Change Disease, diabetic nephropathy and IgA nephropathy [7]. Classically patients with minimal change disease have selective proteinuria while those with focal segmental glomerulosclerosis and membranous nephropathy (MN) have non-selective proteinuria and are unlikely to respond to therapy. Those with selective proteinuria like minimal change disease will respond to therapy. Patients with diffuse mesangial proliferation including those with IgA nephropathy, can have selective or non-selective proteinuria. Response to therapy would depend on the selectivity in patients with diffuse proliferative glomerulonephritis.

2.2.2. Charge Selectivity

In addition to the size barrier, the glomerular capillary wall also contains negative charges because of the presence of glycosaminoglycans such as heparan sulphate and sialic acid around the epithelial slits that maintain permselectivity and constitute a filtration barrier [8]. Thus the glomerulus acts as a size-and charge-dependent ultrafilter for plasma proteins and is almost impermeable to albumin (66, 000 Dalton) and higher-molecular weight proteins [8,9]. Significant amounts of albumin appear in the glomerular ultrafiltrate because plasma albumin concentrations are high. However, the actual amount of albumin excreted is considerably lower than what appears in the ultrafiltrate because most is actively reabsorbed in the proximal tubule and catabolized in tubular lysosomes. As a consequence of these two processes, filtration and reabsorption, increased albumin excretion may result from increased glomerular permeability, reduced proximal tubular reabsorption, or a combination of both (Fig 5). Most inflammatory glomerular diseases result in alterations of the size barrier and loss of anionic charges, leading to proteinuria. Large increases in urinary albumin therefore reflect increased glomerular permeability. This is not necessarily the case for albuminuria of lesser degree.

Low-molecular-weight proteins (molecular weight: less than 40 000 Daltons) are freely filtered through the glomerulus and subsequently absorbed and catabolized by the proximal tubule [10]. They include β_2 microglobulin, retinol binding protein, α_1 microglobulin and hormones such as vasopressin, insulin and parathyroid

hormone [10,11]. Injury to the proximal tubular epithelium leads to inability of the tubule to reabsorb low-molecular weight proteins and thus to their loss in urine [10].

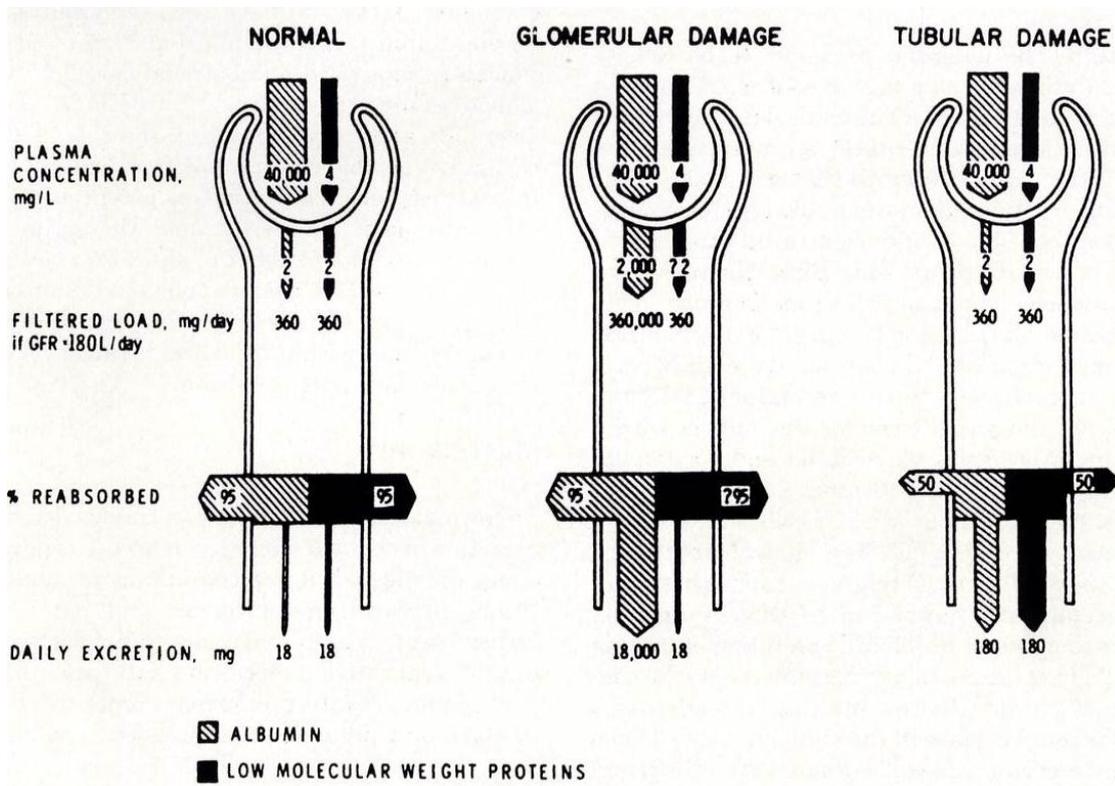


Figure 5: Schematic diagram illustrating two basic mechanisms that can cause proteinuria. Adapted with permission from: Heinemann HO, Maack TM, Sherman RL (1974) Proteinuria. Am J Med 56: 71-82

2.2.3 Renal Haemodynamics

Haemodynamic alterations in glomerular blood flow can also result in proteinuria. Any condition that causes systemic hypertension or increase in intraglomerular blood pressure can cause proteinuria because of the elevation of transcapillary ultrafiltration pressure [11]. It is believed that the mechanism is due to change in size selectivity (increase in number of large pores) or “stretch”. This is also referred to as “shunt pathway (P/W)” [12]. These large pores or shunt P/W can be decreased by treatment with angiotensin converting enzyme inhibitors, e.g. in patients with diabetic nephropathy, the shunt pathway is decreased by such therapy with decreasing proteinuria. Angiotensin converting enzyme inhibitors are believed to cause efferent arteriolar vasodilatation that counters the vasoconstrictive effect of angiotensin II. Angiotensin II is one of the most powerful vasoconstrictors in the human body and it causes efferent arteriolar vasoconstriction of the glomerulus with increase in intraglomerular blood pressure and elevation of transcapillary ultrafiltration pressure resulting in proteinuria [12].

A reduced number of functioning nephrons, as occurs in chronic renal failure, leads to increased filtration of proteins in the remaining nephrons and to proteinuria. Other conditions that cause proteinuria by altering glomerular haemodynamics include exercise [13], fever, seizures, epinephrine use and emotional stress (Table III).

Table III: Aetiologic classification of proteinuria in children and adolescents

Transient proteinuria

Fever

Strenuous cold exposure

Epinephrine administration

Emotional stress

Congestive heart failure

Abdominal surgery

Seizures

Isolated asymptomatic proteinuria

Orthostatic proteinuria

Persistent fixed proteinuria

Proteinuria secondary to renal disease

Minimal change nephrotic syndrome

Acute post-infectious glomerulonephritis

Focal segmental glomerulonephritis

Membranous glomerulonephritis

Lupus glomerulonephritis

Henoch-Schonlein purpura nephritis

HIV-associated nephropathy

Chronic interstitial nephritis

Congenital and acquired urinary tract abnormalities

Hydronephrosis

Polycystic kidney disease

Reflux nephropathy

Renal dysplasia

HIV = human immunodeficiency virus**Adapted with permission from: Adham ML (1998) Evaluating Proteinuria in Children. Am****Fam Phys 58: 1145-1152**

2.3 Epidemiology of Proteinuria

The prevalence of isolated asymptomatic proteinuria in children has been estimated to be between 0.6 and 6.3 percent [15,16,17,18]. Proteinuria is usually transient and intermittent, so that much higher prevalences are observed when a single urine specimen is tested. In a study of 8 954-school children in Finland [9], proteinuria was detected in one of four specimens in 10.7 percent of the children and in at least two of four specimens in 2.5 percent of the children. In both sexes, the prevalence of proteinuria increased with age. Most children who test positive for proteinuria on initial evaluation “lose” the proteinuria at follow-up. Only about 10 percent of children have persistent proteinuria after six to 12 months.

2.4. Measurement of Proteinuria

2.4.1. Qualitative Methods

The dipsticks method (e.g. Albustix, Multistix) provides an estimate of urinary protein concentration and is widely used in both Physician offices and clinical laboratories. Proteins in solution cause a change in the colour of the reagent strip impregnated with tetrabromophenol blue. Because tetrabromophenol is a pH indicator, the dipstick is buffered to prevent the influence of normal variations in urine pH on colour change. False-positive results can be obtained when the urine is alkaline (pH greater than 7) or when it contains heavy mucus, blood, pus, semen or vaginal secretions. The strips react preferentially to albumin and are relatively insensitive to other proteins such as gamma globulins. The amount of

protein in the urine is assessed as 1+ (30 mg per dl), 2+ (100 mg per dl), 3+ (300 mg per dl) or 4+ (1 000 mg or more per dl) [19].

It is common practice to verify the results of a positive dipstick test using sulphosalicylic acid turbidometry. In the latter test, three drops of a 20 percent solution of sulfosalicylic acid are added to 5mls of urine to cause an acidic pH and precipitation of protein. The turbidity of the urine is noted by visual inspection. This test is more accurate than the dipstick method because all classes of proteins are detected. False-positive results can occur in the presence of radiographic contrast material and in samples from children receiving high doses of penicillin, cephalosporins, or sulphonamides. Because dipstick and sulphosalicylic acid are sensitive to the concentration of protein in the urine, they can underestimate proteinuria or give false-negative results in the presence of a dilute urine (i.e. specific gravity less than 1.010). Urine with a specific gravity greater than 1015 is necessary for reliable results [19].

Other qualitative methods of assessing urinary proteins are by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Isoelectric Focusing (IEF). IEF can predict response to therapy in patients with nephrotic syndrome [6].

2.4.2. Quantitative Methods

Several colorimetric laboratory methods are available to quantitate protein concentrations in urine [20]. The benzethonium chloride, the Ponceau-S and the Coumassie Brilliant Blue dye-binding methods are the most commonly used. Urinary protein electrophoresis and direct measurements of low-molecular weight proteins such as β_2 microglobulin may be performed in special circumstances but are not part of the routine evaluation of a child with proteinuria. Similarly, the determination of microalbuminuria in diabetic children requires the use of more sensitive methods such as radioimmunoassay or enzyme-linked immunosorbent assay.

In adults, a protein excretion of less than 150 mg per 24 hours is considered normal. In children, however, physiological proteinuria varies with age and size of the child (Table II). After the first year of life, daily protein excretion in children, expressed in mg per metre squared per 24 hours, is relatively constant. The traditional way of quantifying urinary protein excretion has been to measure protein in a urine sample collected over a 12 to 24 hour period.

The patient is instructed to void when waking up in the morning and to discard that urine and mark the time. The urine from each subsequent voiding is then collected for the next 24 hours; the final urine sample is to be voided precisely 24 hours after the time collection was begun. In practice, however, the collection of

24-hour urine samples is fraught with error, and the collection often has to be repeated. Furthermore, timed urine collections are impractical in young children and impossible in infants without subjecting them to bladder catheterization.

Determining the amount of excreted creatinine in the same 24-hour urine sample may be helpful in evaluating the accuracy of the collection. Steady-state daily creatinine excretion is 20 mg per kg in children from one to 12 years of age and 22 to 25 mg per kg in older children, with the lower value corresponding with creatinine excretion in girls.

In a study of adults [21], a strong correlation was found between the urine protein-to-creatinine ratio (Upr/Cr, measured in mg per dl), obtained in random urine samples, and the 24-hour urinary protein excretion, corrected for body surface area. Other studies have confirmed this observation [22]. The usefulness of urinary protein-to-creatinine ratios has been documented in normal children [23] and in children with renal disease [24].

In adults and children over two years of age, a Upr/Cr of less than 0.2mg per dl on a random urine specimen obtained during the day is considered normal. In children aged six months to two years, the upper limit of normal should be

extended to 0.5mg per dl [23]. A Upr/Cr above 2.0mg per dl is consistent with nephrotic-range proteinuria.

Because serum and urine creatinine levels depend on muscle mass, the ratio is not valid in children with severe malnutrition. More-over, in the presence of significant reductions in the glomerular filtration rate, tubular secretion of creatinine increases, and this may result in artificially low Upr/Cr values. Nevertheless, the Upr/Cr ratio is more reliable than 24-hour urinary protein measurements. In one study [24], a collection error was found in 57 percent of 24-hour urine samples, as assessed by a high or low urinary creatinine content. The actual 24-hour protein excretion can be calculated from the UPr/Cr ratio at all levels of proteinuria, using a simple formula derived by log-log regression analysis [24].

$$\textit{The total protein (g per m}^2 \textit{ per day) = 0.63 X (UPr/Cr)}$$

This ratio circumvents the need for urine collection, allowing the result to be obtained more expeditiously. Furthermore, serial UPr/Cr ratios can be obtained over time to monitor the progression of proteinuria.

2.5. Aetiology of Proteinuria

2.5.1. Transient or Functional Proteinuria

As many as 30 to 50 percent of children with proteinuria have transient, non-repetitive proteinuria [25]. Transient proteinuria can occur with strenuous exercise, emotional stress, and exposure to extreme cold, epinephrine administration, abdominal surgery or congestive heart failure. It also occurs during febrile illnesses and after seizures. In all of these circumstances, proteinuria resolves spontaneously after the cessation of the causal factor, and an extensive work-up is usually not recommended.

2.5.2. Persistent Proteinuria

In general, the finding of proteinuria during a routine examination does not warrant an extensive work-up. The finding must be confirmed on two or three more occasions. The finding of at least two positive urine tests out of the three specimens would suggest persistent proteinuria and warrants a work-up. In addition to urinalysis with microscopic examination, a test for quantitation of urinary protein excretion should also be performed. Traditionally this has been done by collecting a 24-hour urine sample to measure the amount of protein excreted in mg per 24 hours. However, in children, 24-hour collections of urine are fraught with problems, as sample collection is often incomplete, or inadequate. The measurement of urinary protein and creatinine concentrations (in mg per dl) in a random daytime urine sample and calculation of the urinary protein: creatinine ratio, is a facile method of quantifying urinary protein excretion.

2.5.3. Orthostatic (Postural) Proteinuria

Orthostatic proteinuria accounts for up to 60 percent of all cases of asymptomatic proteinuria reported in children, with an even higher incidence in adolescents [26]. Variations in the quantity of daily protein excretion have been observed. If all laboratory tests are normal except for persistently elevated protein excretion, the possibility of orthostatic proteinuria should be investigated, particularly in children older than six years. This is done by collection of “supine” and “ambulant” urinary samples for protein estimation. In patients with orthostatic proteinuria, the supine sample will be free of protein, but the active sample will contain protein [14]. As a rule of thumb, children with orthostatic proteinuria excrete less than 1 gram of protein in 24 hours (Upr/Cr less than 1.0). The long-term prognosis for young adults with orthostatic proteinuria is excellent, even after 20 to 50 years of follow-up [26,27]. It is believed that the prognosis in children and adolescents is equally good. Yearly follow-up is recommended for children diagnosed with this condition.

2.5.4. Proteinuria Secondary to Glomerular Diseases

Many glomerular diseases result in proteinuria (Table III). The prognosis is usually less benign when haematuria is also present. Heavy or “nephrotic range” proteinuria is defined as protein excretion greater than 40mg per metre squared per hour or a Upr/Cr greater than 2.0.

2.5.5. Tubulointerstitial Nephropathies

Proteinuria, with or without haematuria, occurs in patients with tubulointerstitial disease of diverse origin. When the glomerular filtration rate is normal, the proteinuria is usually of tubular origin [19]. As the glomerular filtration rate decreases as a result of significant nephron loss, haemodynamic mechanisms contribute to glomerular proteinuria. In general, the proteinuria of tubulointerstitial disease is mild (less than 1.0 grams per 24 hours).

2.5.6. Prognosis

While patients with orthostatic proteinuria have an excellent prognosis, the long-term prognosis for children with isolated fixed proteinuria remains unknown. It is generally believed that children with isolated proteinuria not exceeding 1g per 24 hours have a better prognosis than those with higher amounts of protein in their urine. In a six-year retrospective study of 31 children with proteinuria of 10 months duration, renal biopsies were performed in 17 children [29]. Of these children, 12 had pathologic findings on biopsy, including eight with focal and segmental glomerulosclerosis. Among 12 of the 14 patients who were not biopsied and four of the five patients with normal histology, proteinuria completely resolved in 11(69%) patients.

In a study of 53 Japanese children with asymptomatic proteinuria, significant glomerular changes were observed on renal biopsy in 25(47%) patients [30]. Fifteen had focal glomerulosclerosis. Seven patients with abnormal glomerular histology developed renal insufficiency; none of the patients with normal histology developed renal insufficiency. In a survey of paediatric nephrologists [31], 36 percent of the physicians surveyed would perform a renal biopsy in a nine-year-old child with moderate proteinuria (600mg per day). Until more information is available, the decision to perform a renal biopsy in a child with isolated proteinuria will depend on factors such as parental or physician anxiety and the availability of effective therapy.

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CHAPTER 3

HEPATITIS B VIRUS

CHAPTER 3

HEPATITIS B VIRUS

3.1. Discovery of the Hepatitis B Virus

The discovery of Hepatitis B Virus (HBV) by Blumberg et al in 1965 followed the finding of a precipitin protein reaction unlike any seen before between the serum from an Australian aborigine and that of a frequently transfused hemophilia patient from New York City [1]. The unusual precipitin protein in this reaction from the aborigine was termed the “Australia antigen” (abbreviated Au).

The Australia antigen (Au) was stable in sera kept in a frozen state and on testing large collections of sera it was found that Au was rare in the United States population but common (about 5-10%) in some African, Asian and Oceanic groups [2]. It was also discovered by Blumberg and his co-workers that Au was common in leukaemia patients, most of whom had been transfused. In addition, Au was more frequent in institutionalised children with Down’s syndrome (~30%) compared to that in controls. These researchers also learned that the presence or absence of Au appeared to be a persistent trait; if Au was present at first testing, then it was likely to be present in all subsequent tests. Similarly, if Au was absent on first testing, then it was absent on subsequent testing. Early in 1966 however

there was an exception to this finding. One of the Down's syndrome patients, who previously did not have Au, did have it on subsequent testing. What appeared to be a "new" protein had appeared in his serum, and since most serum proteins are manufactured in the liver, a series of "liver function" tests were performed. It was shown that between the testing in which Au was not found in this patient's blood and the "positive" test, he had developed a form of chronic anicteric hepatitis, which could be detected only by measurement of the changes in his blood [2].

This observation, then, generated the hypothesis that Au was associated with "viral hepatitis". For many years this clinical syndrome was assumed to be of viral aetiology. The discovery of the virus followed the observation that there was a higher frequency of Au in the hepatitis patients, both acute and chronic, than in the controls. Also, Down's syndrome patients with Au had much higher levels of liver enzymes than those without Au [3]. Electron microscopy studies revealed the presence of particles with the appearance of a virus (Fig 6a and b); and a variety of other studies indicated that these particles, which reacted with the antibody against Au, were hepatitis viruses [4]. It was found that these particles did not have nucleic acid and it was later discovered that these particles were part of a virus which consisted only of the surface antigen. In 1969 the whole virus particle was identified by Dane and his colleagues [5].

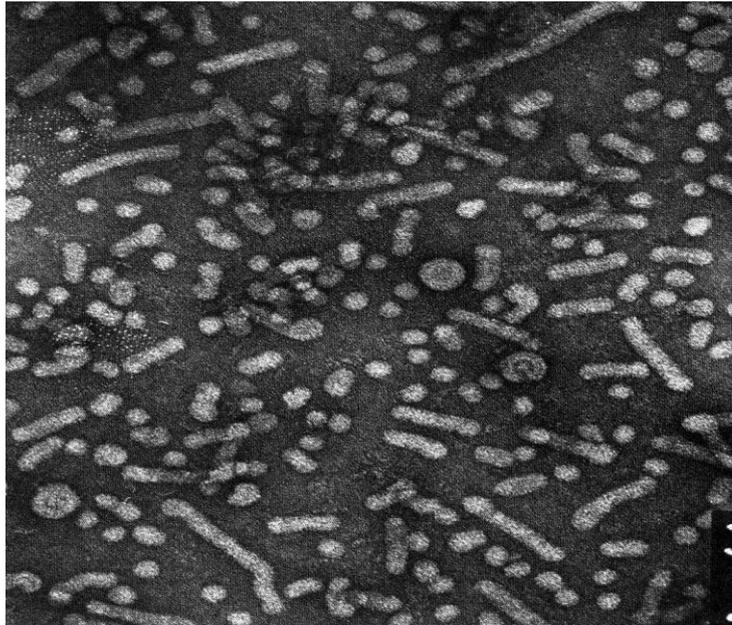


Figure 6 (a): Electron micrograph of hepatitis B virus showing whole viruses (the large spheres), surface antigen particles (the small spheres), and elongated surface antigen particles (magnification approximately 300,000 x). Adapted with permission from: *Hepatitis B: The Virus, the Disease, and the Vaccine*, eds. Millman I, Eisenstein TK, Blumberg BS 1984 pp 10.

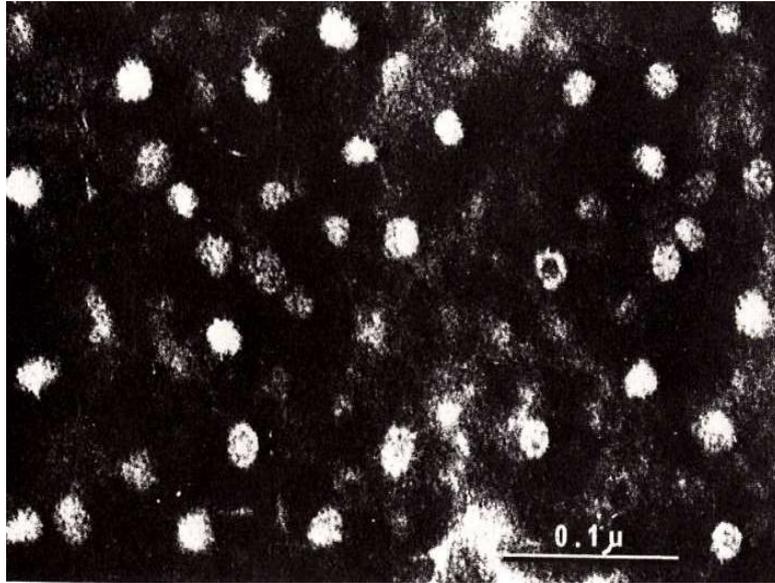


Figure 6 (b): Electron micrograph showing only the surface antigen particles from which the vaccine is prepared. Adapted with permission from: *Hepatitis B: The Virus, the Disease, and the Vaccine*, eds. Millman I, Eisenstein TK, Blumberg BS 1984 pp 11.

3.2. Hepatitis B Particle Types

HBV is a small DNA-containing virus that causes persistent noncytopathic infections of the liver. It is the first representative to be described out of a group of viruses that have received the status of a family, the Hepadnaviridae [6]. Infected hepatocytes continually secrete viral specific particles that accumulate to high levels (10^{13} /ml) in the blood. These particles (Fig 7) are of two types: (1) non-infectious particles consisting of excess viral coat protein (HBsAg) and containing no nucleic acid, and (2) lower amounts (10^{10} /ml) of infectious, DNA containing particles (Dane particles) consisting of a 27nm nucleocapsid core (HBcAg) around which is assembled an envelop (7nm thick) containing the major viral coat protein, carbohydrate, and lipid. The Dane particle has a diameter around 42nm. The outer envelop which contains high amounts of HBV surface proteins surrounds the inner nucleocapsid which is comprised of 180 hepatitis B core proteins arranged in an icosahedral arrangement with T=3 and T=4 symmetry [7,8,9]. The nucleocapsid also contains at least one hepatitis B polymerase protein as well as the HBV genome [10].

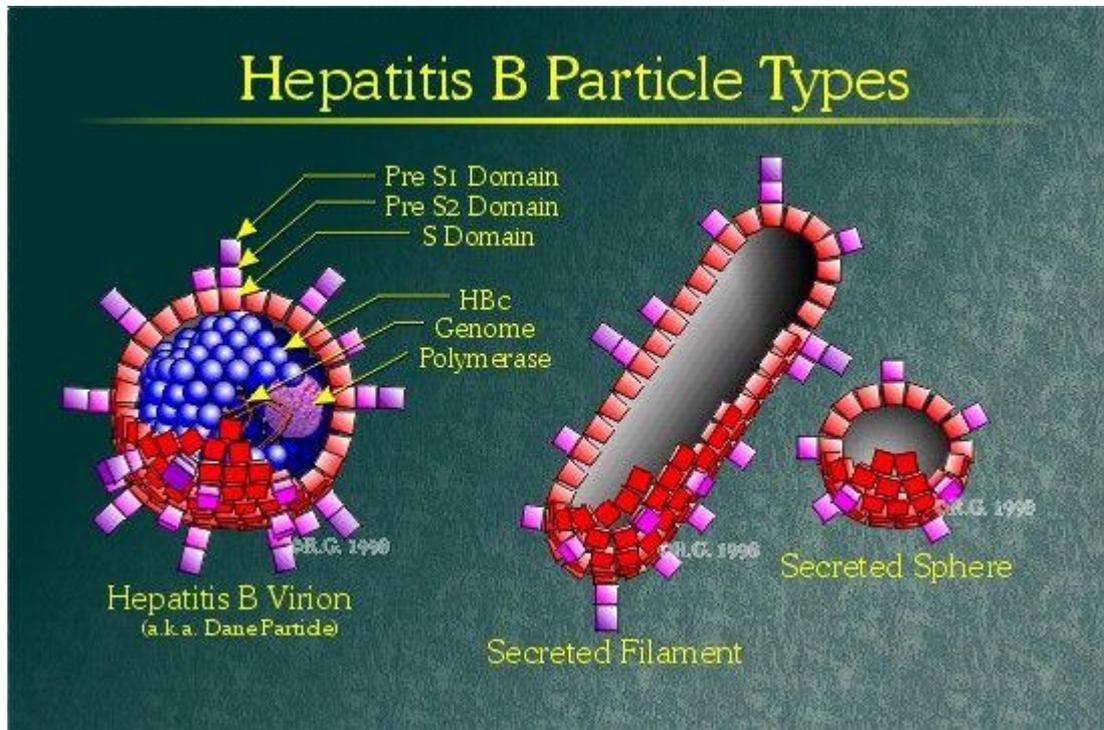


Figure 7: Hepatitis B virus particle types.

Adapted with permission from Robert's HBV page.

<http://www.globalserve.net/~harlequin/HBV/hbvparts.htm>

3.3 The Hepatitis B Genome

In virions, the genome appears to be circular, yet only partially double-stranded [11]. The genome is approximately 3200 nucleotides in length, but does not abide by the usual classification criteria for viruses [12]. Numbering of base pairs on the HBV genome is based on the cleavage site for the restriction enzyme *EcoR1* or at homologous sites, if the *EcoR1* site is absent. However, other methods of numbering are also used, based on the start codon of the core protein or on the first base of the RNA pregenome. There are at least seven major subtypes of HBV, distinguished by sequence differences in the surface antigen gene [13].

Unlike others, HBV virions contain both DNA and RNA. Moreover, some regions of the packaged genome can be single stranded, double stranded, or even triple stranded. These peculiar features are a direct result of the HBV genome replication, mechanism. The incomplete plus strand is complexed with a DNA polymerase in the virion, which can elongate using the complete minus strand as the template. These morphological and structural features distinguish HBV from all known classes of DNA-containing viruses.

There are four defined overlapping open reading frames (ORFs) in the genome which result in the transcription and expression of seven different hepatitis B proteins. Every base pair in the HBV genome is involved in encoding at least one of the HBV protein. However, the genome also contains genetic elements which

regulate levels of transcription, determine the site of polyadenylation, and even mark a specific transcript for encapsidation into the nucleocapsid.

The four ORFs lead to the transcription and translation of seven different HBV proteins through use of varying in-frame start codons. For example, the small hepatitis B surface protein is generated when a ribosome begins translation at the ATG at position 155 of the *adw* genome. The middle hepatitis B surface protein is generated when a ribosome begins at an upstream ATG at position 3211, resulting in the addition of 55 amino acids onto the 5' end of the protein. ORF P occupies the majority of the genome and encodes for the hepatitis B polymerase protein. ORF S encodes the three surface proteins [14]. ORF C encodes both the hepatitis e and core protein [15]. ORF X encodes the hepatitis B X protein [16,17].

Promoters and Signal Regions

The HBV genome contains many important promoter and signal regions necessary for viral replication to occur.

The four ORFs transcription are controlled by four promoter elements (preS1, preS2, core and X), and two enhancer elements (Enh I and Enh II). All HBV transcripts share a common adenylation signal located in the region spanning 1916-1921 in the genome. Resulting transcripts range from 3.5 nucleotides to 0.9 nucleotides in length. Due to the location of the core/pregenomic promoter, the

polyadenylation site is differentially utilized [18]. The polyadenylation site is a hexanucleotide sequence (TATAAA) as opposed to the canonical eukaryotic polyadenylation signal sequence (AATAAA). The TATAAA is known to work inefficiently [19], suitable for differential use by HBV.

The core/pregenome promoter controls the transcription of multiple RNAs which have 5' heterogeneity. These include the core antigen, e antigen, polymerase, and pgRNA. These transcripts are also regulated by both viral enhancer elements and negative regulatory element (NRE). The core/pregenome promoter has yet to be clearly defined, but has been mapped loosely in a region spanning nucleotides 1591 to 1851 [20,21]. Positive regulation of this region involves many transacting cellular factors. CCAAT/enhancer binding protein (C/EBP) [22], HNF-3 [23], HNF-4 [24], and an ubiquitous cellular factor, Sp 1 [25], appear assist in activation. Studies have also indicated that members of the nuclear receptor family may be involved in down regulation of the core/pregenome promoter [26,27]. The polymerase coding region does not appear to have a promoter element directly upstream. Its expression is believed to be the result of ribosome scanning of the pgRNA transcript.

ORF S contains three in-frame start sites which direct synthesis of the three distinct hepatitis B surface proteins [28,29,30]. There are two promoter regions which control the expression of these proteins, namely the preS1 promoter and

preS2 promoter. The preS1 promoter controls transcription of a single 2.4kb RNA molecule which includes the entire ORF S region. However, the preS2m promoter controls transcription of a family of transcripts of 2.1kb in length. The preS2 promoters has been shown to be stronger than the preS1 promoter [31,32,33]. This results in more middle and small surface proteins being expressed as compared to the large surface protein. It has been shown that over expression of the large surface protein results in retention of surface proteins in the endoplasmic reticulum [34,35,36]. Transcription factors such as Oct 1 [37], HNF-1 and HNF-3 [38,39,40] appear to be involved in the activation of the preS1 promoter. HBV enhancer elements also appear to be involved in preS1 promoter upregulation [41,42]. Downregulation of the preS1 promoter appears to be dependent on a region within the preS2 promoter (nucleotides 3160 to 3221) [43]. However the mechanism is poorly understood. The preS2 promoter appears to be upregulated by a CCAAT element which is also involved in the downregulation of the preS1 promoter. NF-Y, the CCAAT-binding factor, appears to be involved in the preS2 activation [44] as well as both HBV enhancers [45,46]. Also, stress on the endoplasmic reticulum due to the presence of hepatitis B large surface protein appears to result in increased activation [47].

ORF X, which encodes a 17kd protein known as the hepatitis B X Protein (HBx), has its own promoter controlling the transcription of a 0.9kb RNA. However, due to the proximity of the X promoter to enhancer I region, the precise borders remain

controversial. The promoter is believed to lie within the region spanning nucleotides 1230 to 1376 [48,49].

The enhancer I element spans a region from nucleotides 970-1240 [50,51]. Many transcription factors are believed to interact with this region of the HBV genome. These molecules include HNF-3 [52], HNF-4 [53], EF-C [54] and NF-1, to name a few. Both enhancer I and enhancer II can activate heterologous promoters despite their position or orientation [55,56]. Deletion of either enhancer region results in a strong reduction of viral transcripts [57].

The last region which will be mentioned in this section is known as the epsilon-stem loop (- stem loop). The region spans nucleotides 1847-1907 in the HBV genome and plays a key role in HBV DNA encapsidation. The location of the stem-loop was determined by fusing heterologous genes to various regions of the HBV genome and by looking for encapsidation of these foreign genes [58,59,60]. Despite the terminal redundancy of the pgRNA, only the 5'-stem loop retains functionality [61]. Because of this, only pgRNAs are encapsidated despite the fact that all HBV transcripts have the -stem loop coding region at their 3' ends. Sequence analysis of the -stem loop shows a series of inverted repeats that are predicted to fold into a three-dimensional stem-loop structure. This stem-loop is conserved among all hepadnaviruses despite differences in the primary sequence [62]. It is believed that the polymerase protein recognizes and interacts directly

with the stem-loop structure [63]. Interaction of the hepatitis B polymerase and the ω -stem-loop initiates both encapsidation as well as reverse transcription of the HBV pgRNA.

The genomic arrangement of the hepatitis B virus family makes it unique among viruses. The unusual nature of the packaged genome gives an indication that the method of replication employed by HBV is not that of conserved DNA replication [64,65] (Fig 8).

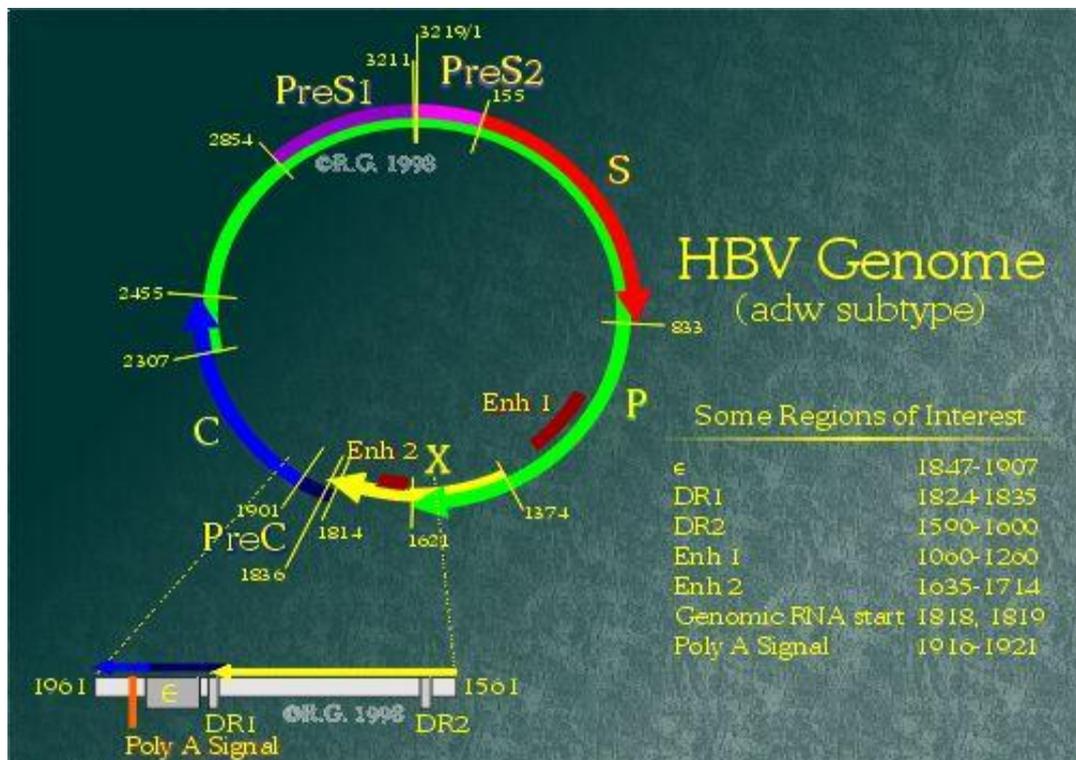


Figure 8: Hepatitis B virus genome.

Adapted with permission from Robert's HBV page.

<http://www.globalserve.net/~harlequin/HBV/genome.htm>

Two other subviral particles can be found in an infected individual's serum, namely the hepatitis B filament and hepatitis B sphere. Both particles have a diameter of 22nm and are composed solely of hepatitis B surface proteins. The absence of the hepatitis B core, polymerase, and genome reflects these particles' non-infectious nature. The sphere is composed of the small and middle hepatitis B surface proteins whereas the filament also includes the large hepatitis B surface protein (Fig 9).

High levels of these non-infectious particles can be found during the acute phase of infection [66]. Non-infectious particles present the same antigenic sites as the virion. High levels of these surface antigens induce a significant immune response from most who are infected. However, it is believed that the presence of such high levels of non-infectious particles may allow the infectious viral particles to traverse the blood stream undetected by neutralizing antibodies [67]. In addition, non-infectious particles have been reported to enhance HBV infection in the duck hepatitis B system [68]. Binding of non-infectious particles to potential host cells appears to activate the cells, possibly to prime the cells for infection. Whether this is true for humans, remains to be proven.

Purified virions possess the HBc protein, which aggregates to form the core particle (Fig. 10). This, in turn, results in the encapsulation of the viral genome and polymerase. In the mature virion, this core particle ends up enveloped by the

various hepatitis B surface proteins. In addition, there appears to be a protein kinase encapsulated within this core particle.

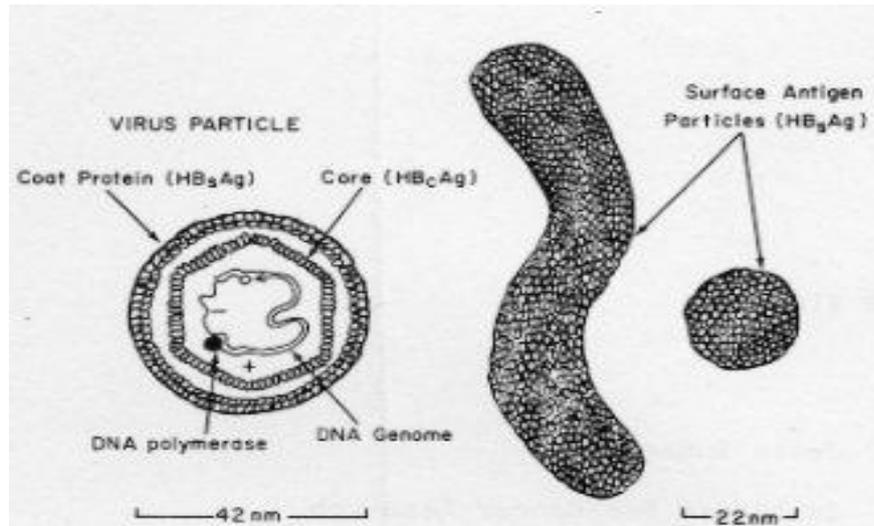


Figure 9: Virus specific particles and the genome structure of hepatitis B virus. Both infectious and non-infectious particles are produced by infected hepatocytes. Infectious particles contain a small circular DNA genome and a DNA polymerase. Adapted with permission from: Hepatitis B: The Virus, the Disease, and the Vaccine, eds. Millman I, Eisenstein TK, Blumberg BS 1984 pp 34.

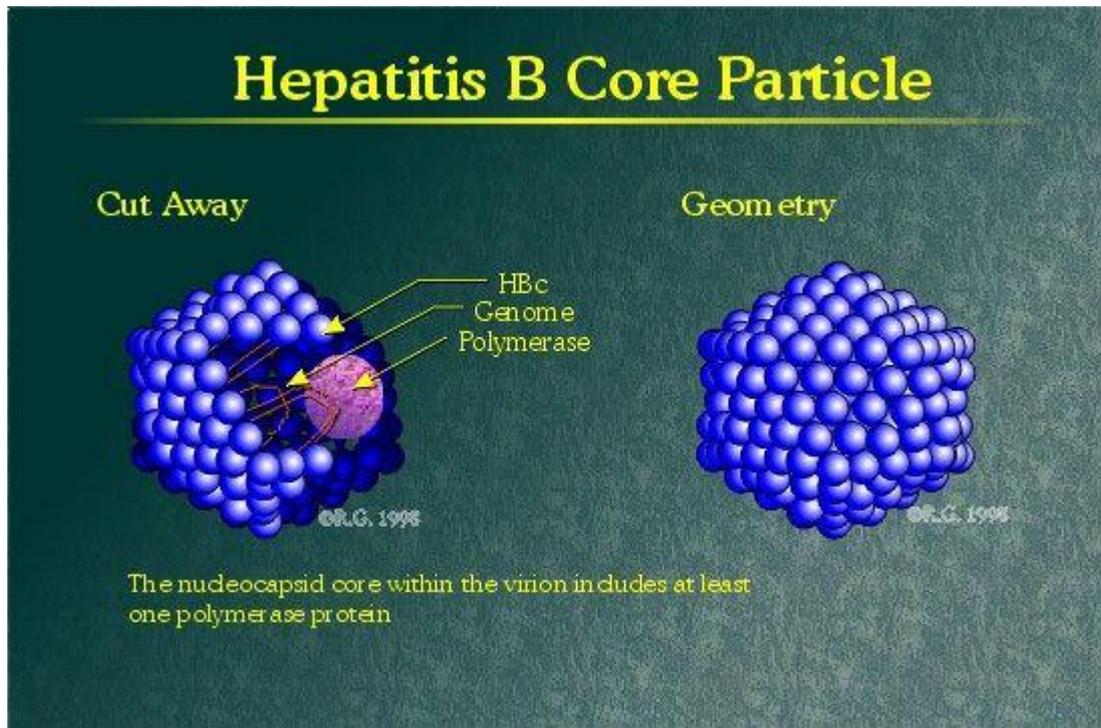


Figure 10: Hepatitis B virus core particles.

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<http://www.globalserve.net/~harlequin/HBV/hbvparts.htm>

There appears to be a critical concentration of HBc required for particle assembly. Dimers of HBc protein are initially formed, assembling at 0.8 μ M concentration into isometric particles of T3 symmetry (180 HBc subunits form one core particle). The core particle appears made up of T-shaped dimers of the core protein that form outward spikes. The N-terminal of the two core proteins in the dimer are at the tip of the spike according to electron microscope analysis. The assembled core particles are then stabilised through formation of disulphide bonds [69].

It has been more recently shown that the Pre-S1 and S domain of the envelop proteins interact with the core particles. This is not surprising as the core particle must become specifically packaged by membrane enriched for HBV surface proteins in order to generate the mature virion [69].

3.4. The Replication Cycle

The replication cycle of the HBV is strikingly different from other DNA-containing viruses; the main unusual feature is the use of an RNA copy of the genome as an intermediate in the replication of the DNA genome (Fig. 11a and b) [70]. Infected DNA genomes are converted to a double-stranded form(s), which serves as a template for transcription for RNA. Multiple RNA transcripts are synthesised from each infecting genome, and these transcripts either have messenger function or DNA replication function. The latter, termed “pre-genomes” are precursors of the progeny DNA genomes because they are assembled into nucleocapsid cores and reverse-transcribed into DNA before coating and export from the cell. Thus each mature virion contains a DNA copy of the RNA pre-genome and a DNA polymerase.

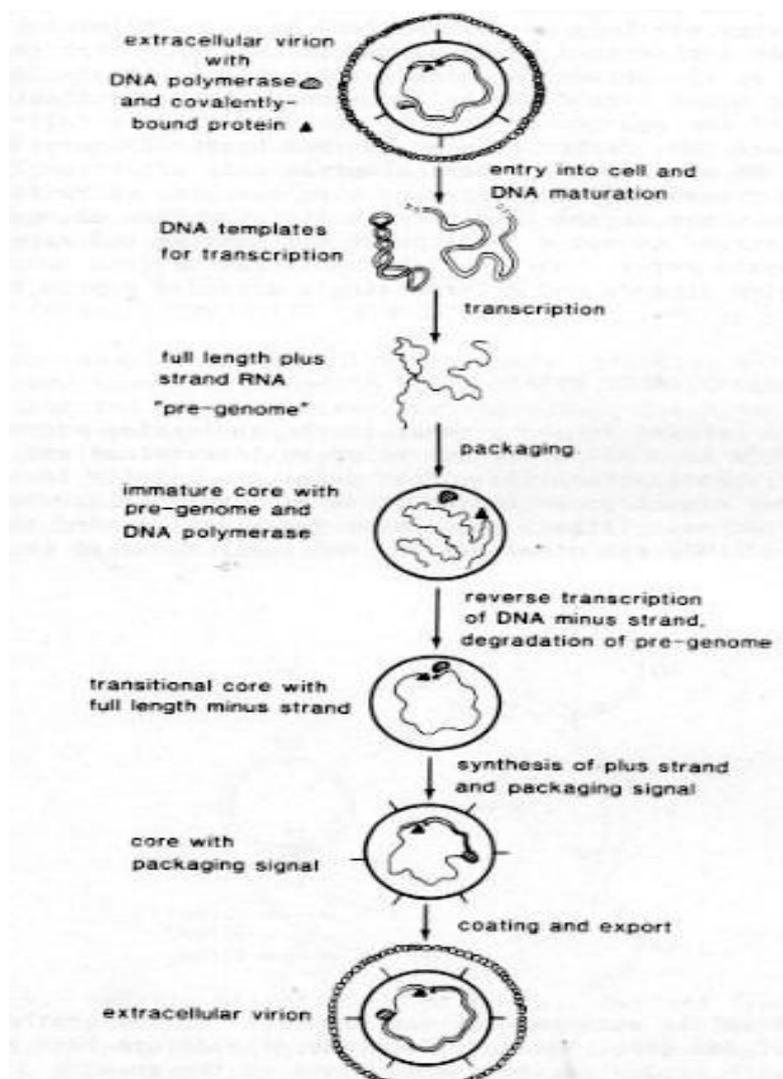


Figure 11 (a): Proposed replication cycle of the hepatitis B viruses. Infecting DNA genomes are converted to double-stranded template for transcription by a cellular RNA polymerase. This "proviral" DNA form(s) is responsible for the production of mRNA and pre-genomic RNA's. Pre-genomic RNA's are packaged into nucleocapsid cores, reverse-transcribed into DNA and matured into enveloped virions. Adapted with permission from Hepatitis B: The Virus, the Disease, and the Vaccine, eds. Millman I, Eisenstein

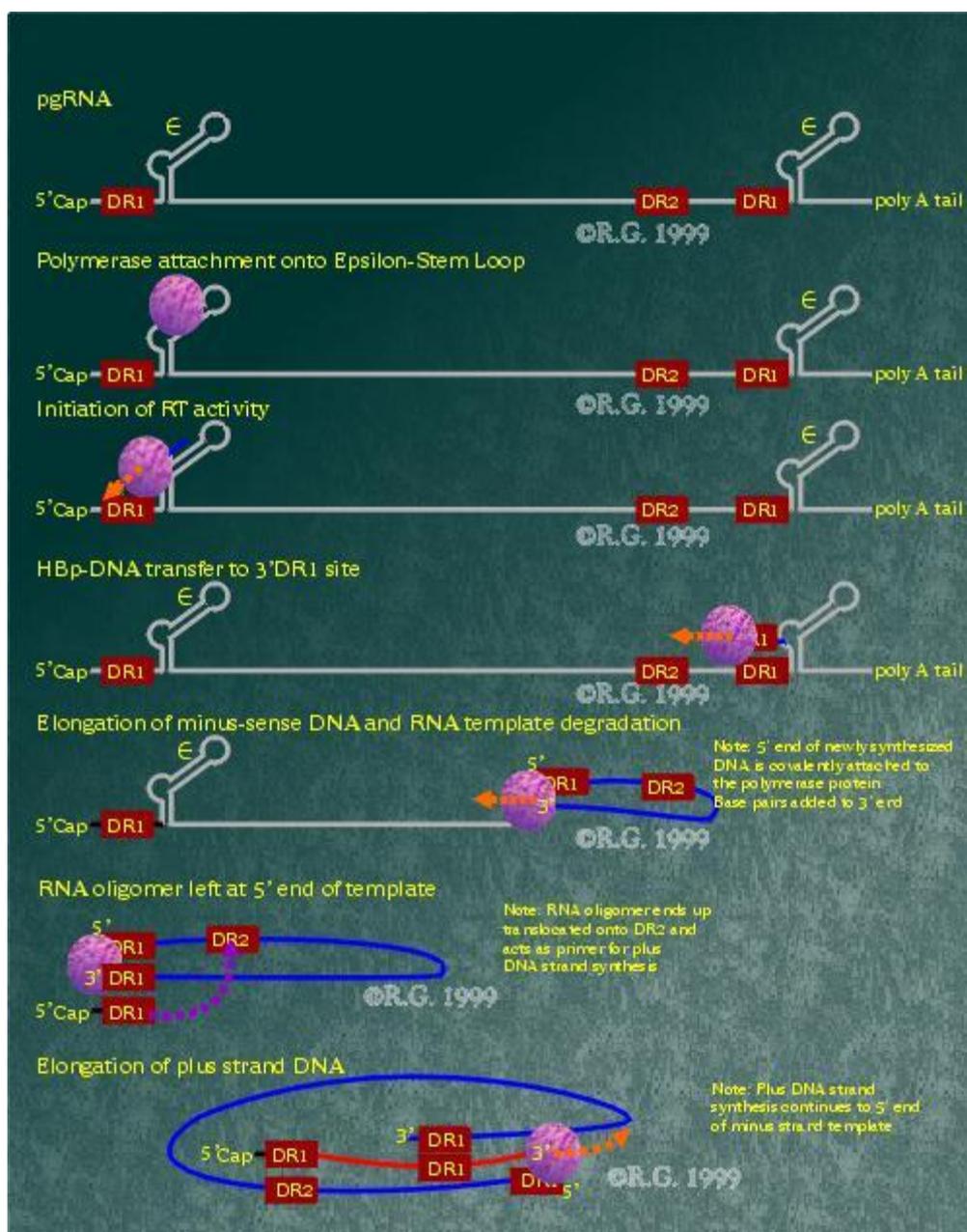


Figure 11 (b): Replication cycle of hepatitis B virus infection.

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<http://www.globalserve.net/~harlequin/HBV/genome.htm>

3.5. Serotypes

The small, circular genome of the HBV is highly compact, with its entire DNA encoding one or more proteins. Therefore, the regulatory elements are also located with the protein encoding regions [71]. The genomes of all mammalian hepadnaviruses show a similar organisation, with four open reading frames (ORFs). These are core (C), surface (S), polymerase (P), and X. The primary structure of the known viral proteins can be deduced from the transcriptional start sites of the unspliced mRNAs, which are all co-terminal [72].

There are nine different subtypes of the HBsAg reflecting genetic variability of HBV. These are *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4*, *sdrq⁺*, and *adrq⁻* [73].

3.6. Hepatitis B Virus Genotypes

A number of variants or mutant forms of the HBV structural proteins have been identified following nucleotide sequencing of the HBV genomes by using the polymerase chain reaction [74]. Genetic heterogeneity has been shown to represent an important element in viral pathogenesis [75]. Amino acid substitution may give rise to variations of viral epitopes that are relevant for virus neutralization [76]. Substitution in the primary structure of the HBV-encoded structural proteins may thus represent immune-selected mutations or naturally selected variants not yet selected for.

The entire nucleotide sequences of 18 human HBV genomes of various subtypes have been classified into four genetic groups designated A to D based on an inter-group divergence of 8% or greater or the complete nucleotide sequence [76]. Two new genomic groups, designated E and F were recently identified on the basis of the variability in the S gene of genomes on the basis of the variability of the S gene and genomes encoding the subtypes *ayw4* and *adw4* [77].

Accumulated S-gene sequences may be used to assess the worldwide molecular epidemiology of HBV [6]. In general, genotype A is most common in North-western Europe and sub-Saharan Africa. The genotypes B and C are confined to the original populations of the Far East. Genotype D is the most widespread and

is the predominating genotype of the Mediterranean area of the Near East as far as India. This genotype is also found in the aboriginal populations in Asia all the way from Indonesia and Papua to Alaska. Genotype E has so far only been found in West Africa. Genotype F is found in aboriginal populations of the Americas suggesting its roots in the New World. Some important features of the geographical distribution of subtypes and genotypes are summarised in Table IV [6].

Table IV: Geographical distribution of HBV genotypes and subtypes

Genomic group	Subtypes	Areas of Prevalence
A	adw2	Northwestern Europe
	ayw1	Central Africa
B	adw2	Indonesia, China
	ayw1	Vietnam
C	adw2	East Asia
	adrq+	Korea, China, Japan
	adrq-	Polynesia
	ayr	Vietnam
D	ayw2	Mediterranean area
	ayw3	India
E	ayw4	West Africa
F	adw4q-	American natives, Polynesia

Adapted with permission from: Magnius LO, Norder H (1995) Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 38:24-34

3.7. Blood tests for Diagnosing Hepatitis B Virus Infection

A variety of serological assays may be employed to differentiate the type of viral infection as well as discriminate between chronic and acute HBV infection. The most sensitive and specific methods used commercially in diagnosis are radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) (Table V). Both assays make use of specific antibodies against various HBV proteins and can detect HBsAg protein as low as 0.25 ng/ml and anti-HBs antibodies at levels of 1mIU/ml. Slot-blot hybridisation and polymerase chain reaction have been used in detecting low levels of HBV DNA present in both blood and liver tissue samples.

Table V: Interpretation of ELISA testing

Presence of HbsAg	Presence of Anti-HBs	Presence of Anti-HBc	Interpretation of Results
Positive	Negative	Negative	Characteristics of acute HBV infection.
Positive	Positive or Negative	Positive	Acute or chronic HBV infection which may be differentiated with respect to IgM anti-HBc.
Negative	Negative	Positive	No clear interpretation. They could be due to HBV infection in the remote past, low-level HBV infection, or false-positive/non-specific reactions. If present, anti-HBs helps validate anti-HBc reactivity.
Negative	Negative	Negative	Absent HBV infection. N.B. PCR or SBH Which are more sensitive may be positive.
Negative	Positive	Negative	Results are typical of a vaccinated individual.

3.8. Epidemiology and Transmission of Hepatitis B Virus

The HBV is globally distributed among humans. HBV alone is estimated to have infected 350 million people throughout the globe, making it one of the most common human pathogens [78]. The various strains of HBV are quite species specific. Although HBs has been found in other primates, humans remain the principal reservoir (Fig. 12).

Global Distribution of Chronic Hepatitis B Infection

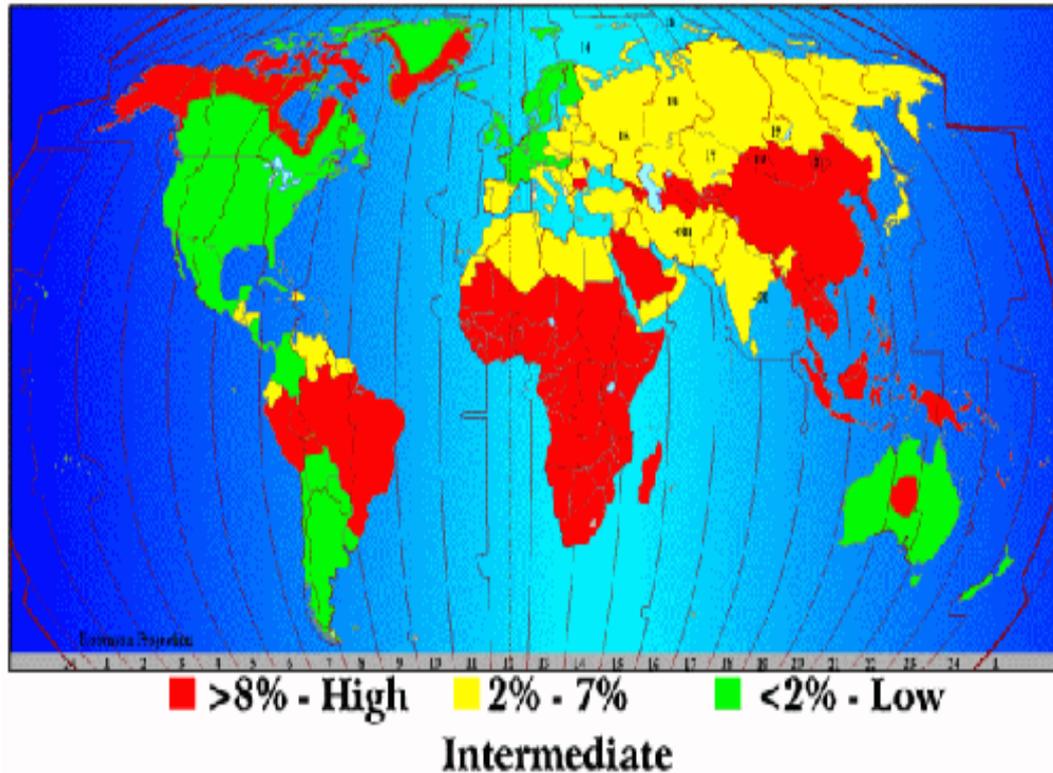


Figure 12: Epidemiology of hepatitis B virus.

Adapted with permission from Robert's HBV page.

<http://www.globalserve.net/~harlequin/HBV/epidem.htm>

The prevalence of HBV infections is highest in developing countries in Africa, Asia, and the Pacific Islands and the lowest in developed countries in North America, Western Europe, and Australasia (Table VI). The method of transmission i.e. vertical or horizontal varies in different regions of the world and is generally related to the incidence of the infection. In areas of high HBV incidence, transmission is usually vertical from infected mother to child, or horizontal within families (Fig 13). In intermediate areas of prevalence, HBV is spread horizontally, with the highest rate of infection occurring among older children, adolescents, and adults. In areas of low prevalence, HBV is primarily a disease of adolescents and young adults and is transmitted sexually or parentally [79].

Table VI: Worldwide distribution of hepatitis B virus infection

	Endemic status		
	Low	Intermediate	High
Prevalence;			
Chronic infection	< 2%	2-7%	8-15%
Total infection	< 20%	20-60%	> 60%
Distribution	North America	Eastern Europe	Southeast Asia
		Southern Europe	China
	Western Europe	Soviet Union	Philippines
		Central Asia	Indonesia
	Australia	Japan	Middle East
	New Zealand	Israel	Africa
	southern -	northern -	Amazon Basin
	South America	South America	Pacific Islands
			Arctic (Eskimo)

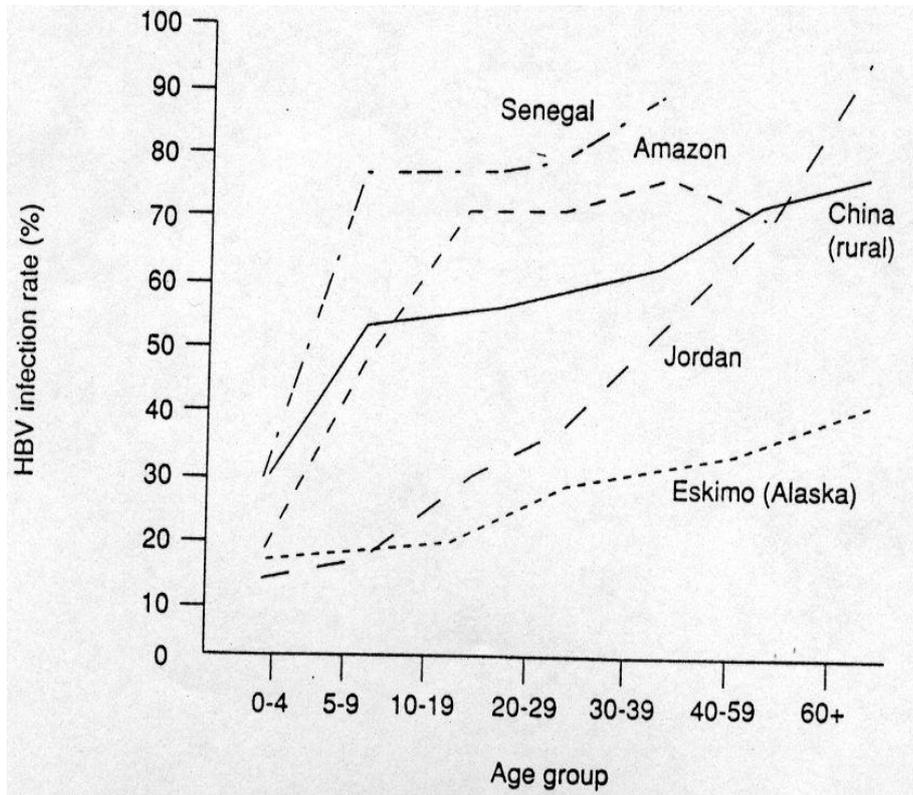


Figure 13: Age specific seroprevalence of hepatitis B virus (HBV) infection in selected populations with a high endemicity of infection. Adapted with permission from: Perrilli RP (1993) Hepatitis B: transmission and natural history. Gut 34: suppl S48-S49

HBV transmission occurs through exchange of body fluids and blood contact. This occurs in the following exposure settings: (1) perinatal infection (pregnancy or childbirth); (2) sexual contact (heterosexual or homosexual); (3) inadvertent transmission of contaminated blood via exchange of blood products (for example, blood transfusion, dialysis), shared needles in intravenous drug abusers, and needlestick type and other open wound injuries (for example, accidents, violence, tradition healer scarification marks).

Approximately 98% of the 470 million inhabitants of Africa are infected with this virus at some time during their lives and about 10% develop chronic infections [80,81,82]. In a study by Vardas et al of children of 0-6 years of age from the Eastern Cape Province of South Africa, 10.4% (95% CI, 9.2-11.7) of children tested were HbsAg-positive [80]

In South Africa, HBV infection rates have until 1995 been hyperendemic in the black population. Following the introduction of the HBV vaccine into the Expanded Programme for Immunisation of Children, prevalence rates are expected to decrease as has been demonstrated in two studies [81, 82]. To date, some 3.3 million black people are chronically infected with HBV. Prevalence rates in the remainder of the population comprising other ethnic groups such as whites, Asians, mixed race and Chinese, are low [83].

Rural black populations in southern Africa have HBV carrier rates of 9.6-14% and total exposure rates of 76-98%. The development of persistent HBV infection seems to be determined by host immune response rather than variations in the virulence of the virus. Lifetime exposure rates to HBV in black Africans are similar in the both sexes [79]. Despite this, HBV carriage is almost always more common in men, with ratios ranging from 1.1: 1 to 3.2: 1 (although figures of 1.4 – 2.5:1 are usual) [79]. In Southern Africa, ratios for HBsAg positivity vary from 1.5:1 to 3.2:1 [85,86,87]. In South Africa, as is the situation in some other countries in Africa, there appears to be rural-urban differences in HBV prevalence rates. For example, along the east coast of South Africa, chronic HBV infection is present in 15.4% of a rural population in the Transkei but in only 7.4% of the urban black population living in Durban [87].

HBV carriage in black Africans is largely established in early childhood with the predominant route of infection being horizontal transmission of the virus [85]. Familial clustering of HBV infection has been extensively documented in South Africa [88] although the precise mechanisms of transmission are not known [85,89]. A study undertaken in Umlazi in 1985, a dormitory black township south of metropolitan Durban in South Africa, the prevalences of HBsAg and HBV infection in children were 6.3% and 19.5% respectively [89]. In this study HBV infection was found to be more common ($p < 0.01$) in the household contacts of children who were HBV carriers than in households of contacts of children with

past HBV infection. In turn HBV carriers had a higher prevalence ($p < 0.01$) of HBV infection than the household contacts of children with no evidence of past or current HBV infection [89]. This is in contrast to what occurs in the Far East, where maternal-infant perinatal transmission is the predominant early route of infection [90]. The major sources of infection to black African children in early childhood are siblings less than 5 years of age and to a lesser extent, other family members and unrelated young playmates [91,92]. Perinatal infections occur to a much lesser extent than the Far East [93,94]. Babies born to replicative carrier mothers are at very high risk and substantially the same as that in the Far East [95]. However, far fewer black than Asian HBV carriers of childbearing age have replicative infection [96]. The first (and largest) wave of HBV infection in black Africans begins during the latter half of the first year of life and high carrier rates are already present by the age of 3-5 years [85,86,95,96]. A further small peak occurs at entry into school and another at the commencement of sexual activity. This general pattern is similar to that in the Far East

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CHAPTER 4

HEPATITIS B VIRUS-ASSOCIATED NEPHROPATHY

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4.1 Role of Hepatitis B Virus in the Development of Nephropathy

Following the discovery of the HBV, it became increasingly evident that a variety of extrahepatic disorders may appear in persons who are infected [1,2,3]. Immune complexes mediate most of these injuries [4].

In persons who carry HBV, various antibodies wax and wane in the circulation, and depending on the phase of infection, immune complexes involving different antigens are formed [5,6]. Therefore, tissue injury in patients with HBV infection has to be correlated with individual HBV-associated antigens, as well as with the phase of infection at the time studied.

In 1971, Coombes et al investigated a patient with membranous nephropathy (MN) apparently due to glomerular deposition of Australia (Au)-antigen-containing

immune complexes [1]. The patient was a 53-year old man, who was in good health until he was injured in a car accident in November 1968. Four months later, in March 1969, after a satisfactory convalescence, acute hepatitis developed, confirmed by needle biopsy of the liver, as an apparent complication of the earlier transfusions. He was given prednisolone and this was continued as indicated when the liver transaminase (S.G.O.T.) values did not return to normal, suggesting continuing hepatic activity. Pedal oedema developed in April 1970, and ascites developed three months later. The patient's serum was found to contain Au antigen which persisted in subsequent determinations. In November 1970, the patient underwent percutaneous renal and liver biopsies. Renal biopsy revealed glomeruli of normal size with patent capillaries. Focal mesangial hypercellularity and sclerosis were present. Glomerular capillary basement membranes were diffusely thickened. There was slight dilation of occasional tubules and a scattering of chronic inflammatory cells in the interstitium. Small blood vessels were normal. Electron microscopy of the renal-biopsy specimen revealed the changes of diffuse MN (Fig. 14). Electron dense deposits were distributed widely along the sub-epithelial surface of the glomerular capillary basement membranes. In some areas the deposits were completely incorporated within the more lucent basement-membrane material. There was diffuse fusion of the epithelial foot processes. Percutaneous liver biopsy revealed mild peri-portal fibrosis with proliferation of bile ducts; a mild cellular infiltrate, predominantly mononuclear, in portal triads and some Kupffer cell hyperplasia; no parenchymal-cell necrosis

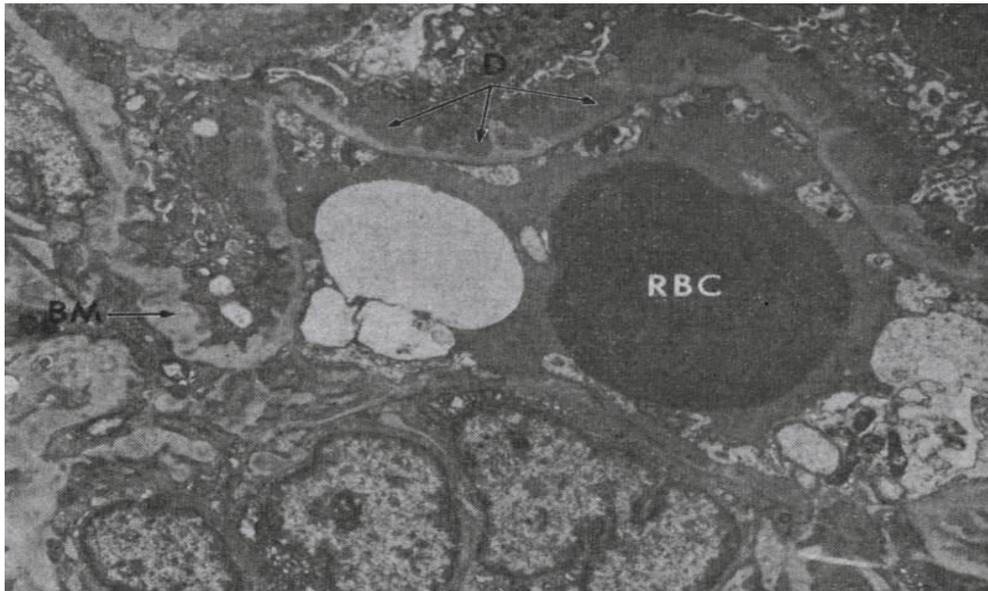


Figure 14: Electron micrograph showing part of a glomerulus. The lumen of a glomerular capillary is occupied by a red blood-cell (R.B.C). Electron dense deposits (D) are distributed widely along the subepithelial surface of the glomerular capillary basement membrane (BM). (Reduced to two-thirds of magnification on original.) Adapted with permission from: Combes B, Stastny P, Shorey J, Eigenbrodt EH, Barrera A, Hull AR, Carter NW (1971) Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. *Lancet* ii: 234-237

Three types of control data indicated that the finding of Au antigen in glomerular tissue of the patient was significant. Firstly, the indirect immunofluorescent technique for demonstrating Au antigen in tissue was specific for Au antigen; secondly, Au antigenaemia (with antigen present simultaneously in the liver) did not of itself result in detectable Au antigen in the glomerular basement membrane; and, thirdly, injured glomeruli and glomerular deposits in the absence of Au antigenaemia did not yield positive immunofluorescence for Au antigen. Coombes et al surmised that the presence of Au antigen, IgG, and complement C3, similarly distributed in renal glomeruli, represented the deposition of Au-antigen-containing immune complexes. In this report the authors stated that insufficient tissue was present in the renal biopsy to elute Au IgG antibodies from glomeruli, a finding that would have strengthened the association of HBV with glomerulonephritis.

Further reports by Takekoshi et al in two children who carried HBV with MN demonstrated the deposition of HBeAg together with IgG and β 1C along glomerular capillary walls [7]. These children had hepatomegaly without detectable hepatic dysfunction. HBeAg was identified in the serum of both patients at the time when they showed symptoms and signs of a full-blown nephrotic syndrome. Furthermore, two forms of HBeAg were found in their sera: small HBeAg and large HBeAg in association with IgG. The presence of HBeAg in both circulation and kidney, most notably in association with immunoglobulins, implicated the HBeAg as the aetiological antigen in the development of HBV-associated nephropathy [7].

4.2 Hepatitis B Virus-Associated Glomerulonephritis

Following the report by Coombes et al in 1971, there were several other studies in series of patients from South Africa [8], France [9,10], Poland [11], Spain [12], Japan [13] and Hungary [14]. It was shown that HBV infection was detected more frequently in patients with glomerular disease than in controls. In addition, the demonstration of glomerular lesions in patients presenting with HBV-related hepatic diseases was another argument in favour of an association between HBV and glomerulonephritis [15].

To date, almost all types of glomerular disease have been described in association with HBV carriage. One could argue that the co-existence of glomerulonephritis and HBV infection may be coincidental, given the high incidence of HBV infection in some endemic areas with prevalence rates >10%. However, the association of MN with HBV infection is generally well accepted and seems to be well established [16]. Reports from several centres showing parallel evolution of HBV markers and MN are additional arguments in favour of a significant association [16,17,18].

Nevertheless, the immune mechanisms leading to the development of MN remain unknown. Johnson and Couser [19] suggested three possible mechanisms:

- a. Sub-epithelial deposits in HBVMN may be due to passive trapping of HBV antigen containing immune complexes or a local immune complex formation. Although a glomerular deposition of all three major HBV antigens has been demonstrated in renal biopsies from some patients, a definite causal role of HBV in the pathogenesis remains uncertain. The potential importance of HBeAg was first advanced by Takekoshi et al, who demonstrated its presence within the glomerular deposits [7].
- b. HBV virus may cause the disease by induction of autoantibodies to intrinsic glomerular antigens.
- c. Glomerular deposits of HBV antigens in HBVMN are not pathogenic and the immune deposits develop by another viral induced mechanism
- d. MN and HBV infection are associated but are not pathogenically related

Other histological forms of glomerulonephritis commonly associated with HBV infection include membranoproliferative (mesangiocapillary) glomerulonephritis and IgA nephropathy.

Immune mechanisms leading to the development of HBVMN:

- a. *Subepithelial immune deposit formation secondary to passive trapping of circulating immune complexes containing HBV antigens.*

Circulating immune complexes (CICs) containing HBV antigens have been demonstrated in many but not all patients with HBV-MN [20,7,21]. However, CICs rarely result in subepithelial deposits unless the CICs are very small and cationic

[22]. HBsAg and HBcAg are large ($>10^6$ daltons) and anionic (Table VII), and CICs containing HBsAg have been identified in HBV-MN with a molecular wt of 2.5 to 3.6×10^6 daltons [21]. These are of a size that is unlikely to penetrate the basement membrane into the subepithelial space. In contrast HBeAg, which is also anionic, induces anti-HBe antibodies that are largely cationic (isoelectric point [pI] 5.8 to 10.2) [23] that can shift the pI of HBeAg from 4.3 to 4.8 (that is, unbound) to the 6.4 to 8.4 range (that is, bound to anti-HBe; Table VII) [24]. HBeAg CICs are also small (2.5×10^5 daltons) [21] and thus might be capable of localizing in the subepithelial space [22]. HBeAg CICs have also been identified in 44% of children with HBVMN, and appear to correlate with disease activity [21]. The potential importance of HBeAg in HBVMN is further supported by the observations that circulating HBeAg frequently correlates with the activity of the disease [21,25,26], is the major antigen in the immune deposits [4,7,21,25,27-31] and that those biopsies negative for HBeAg occur very late in the disease [4].

Thus, it is conceivable that the subepithelial deposits of HBeAg and antibody could result from passive trapping of small, cationic preformed CICs. However, there is currently little experimental evidence to support a preformed CIC trapping mechanism in the pathogenesis of MN [32,33], although this mechanism might account for the mesangial and subendothelial immune deposits seen frequently in HBVMN.

Table VII: HBV associated antigens

Antigen	Gene	Molecular weight	pI	Reference
HBsAg	S	$3.7-4.6 \times 10^6$	3.9-4.9	[34] ^a
HBcAg	C	$8.5-9 \times 10^{6b}$	3.7-4.0	[35]
HBeAg	C	$3 \times 10^4-5.4 \times 10^5$	4.3-8.4	[24]
DNA pol	P	9×10^4	“basic”	[36]
X Protein	X	1.7×10^{4c}	unknown	[37]

^a Properties of adw and ayw subtypes of HBsAg

^b Molecular weight of liver derived HBcAg

^c Molecular weight of synthesized X protein polypeptide component

pI isoelectric point

Adapted with permission from: Johnson RJ, Couser WG (1990) Hepatitis B infection and renal disease: Clinical, immunopathogenetic and therapeutic considerations. Kid Int 37: 663-676

b. Subepithelial immune deposits resulting from local IC formation

In contrast to CIC trapping, local formation of antigen-antibody complexes has been well established to induce the diffuse subepithelial immune deposits and proteinuria characteristic of HBVMN [33]. This process is usually initiated by a charge interaction between a cationic antigen or antibody and the highly anionic heparan sulphate proteoglycan present in the glomerular capillary wall [33]. As discussed above, all three major HBV antigens (HBsAg, HBcAg and HBeAg) are anionic, and only free HBeAg is of a size (molecular wt 3 to 9 X 10⁴ daltons) that would be expected to penetrate the basement membrane. However, as anti-HBe antibodies are cationic (pI 5.8 to 10.2) [23], sequential localization of cationic antibody followed by anionic HBeAg could result in subepithelial immune complex formation. This sequence has been shown experimentally to induce typical subepithelial immune complex deposits when cationic antibodies directed against various anionic antigens were studied [38].

A local mechanism of deposit formation would be favored by conditions which lead to persistence of free antigen or antibody in the circulation, rather than large lattice immune complex formation that results in rapid antigen elimination. In that regard, MN develops in a setting where antibody production is diminished or antibody avidity is low - circumstances also characteristics of idiopathic and lupus MN [39,40], of rabbits with MN secondary to chronic serum sickness [41,42], and of mice that develop MN following infection *in-utero* with the lymphochorimeningitis virus [43,44]. The development of MN in patients with chronic HBsAg antigenemia suggest that conditions which favour persistence of free antigen and antibody may

also prevail in these patients. Thus, if HBV antigens are pathogenic in HBVMN a local process of immune deposit formation involving HBeAg and antibody would seem the most likely mechanism.

c. Glomerular deposits of HBV antigens in HBVMN are not pathogenic and the immune deposits develop by another viral induced mechanism

The possibility that glomerular staining of HBV antigens may be artifact is raised by the observation that many studies [45,46,47,11] have not been controlled by the presence of rheumatoid factors within glomeruli that may bind anti-HBs and anti-HBe antibodies non-specifically [48]. Also, studies in which HBsAg is detected by indirect immunofluorescence with anti-HBs followed by fluorescein-labeled anti-IgG antibody must exclude potential cross-reactivity of the anti-IgG antibody with human IgG present in the lesion. Even when HBV antigens are unequivocally identified in glomeruli, their presence may represent non-specific trapping of antigens in injured glomerular capillaries, as suggested by the occasional observation of HBsAg in glomerular diseases not believed to be due to immune complex deposition, such as focal sclerosis [49] and minimal change disease [50,29]. Non-specific trapping may also account for the glomerular deposition of retroviral protein gp70 that can be frequently demonstrated in murine lupus nephritis, as cross-hybrid studies have demonstrated that immune complex GN can be induced in mice in which glomerular deposition of gp70 was either not detected or localized to areas of the glomerulus independent of the immune complex deposits [51].

An alternative pathogenic mechanism for HBVMN could involve the induction of autoantibodies to intrinsic glomerular antigens. In passive Heymann nephritis, an experimental model of MN, glomerulonephritis is induced by an antibody directed against a glomerular epithelial cell antigen [52]. Recent studies implicate a similar mechanism involving autoantibodies directed against intrinsic glomerular antigens in idiopathic MN in man [33]. A potential autoimmune aetiology for HBVMN is further supported by the demonstration of autoantibodies in patients with chronic HBV infection [53], including antibodies directed against DNA [26,54], cytoskeleton components [55], smooth muscle [26,53], liver membrane and liver-specific membrane lipoprotein antigens [56,57] and rat renal tubular brush border [58]. Autoantibodies may contribute to some of the liver injury in HBsAg-positive chronic active hepatitis [59]. The mechanism responsible for the induction of autoantibodies in patients with chronic HBV infection is unknown. Viruses may induce autoimmunity by a variety of mechanisms, including cross-reactions between viral and self antigens ("molecular mimicry"), release of sequestered self-antigens, alteration of self-antigens to induce an immune response, interference with T-cell tolerance, and generalized polyclonal B cell activation [reviewed in 60]. Alternatively, chronic liver disease may induce autoimmunity independently of HBV infection. Hypergammaglobulinemia, a sign of polyclonal B cell activation, has been observed in both HBsAg negative and HBsAg positive patients with chronic active hepatitis [53]. Chronic active hepatitis of other aetiologies has also been associated with MN [61], especially in patients with autoimmune or "lupoid"

chronic active hepatitis [62,63]. An autoantibody to glomerular antigens has been identified in some patients with lupoid chronic active hepatitis [64], including one

patient with MN [case 10, ref 65]. Renal eluates from two patients with MN and lupoid hepatitis were concentrated 42- to 69-fold for anti-U1-RNP antibody, and autoantibody that is directed against a small ribonucleoprotein [66]. Thus, given the autoimmune nature of experimental and probably human idiopathic MN, and the presence of autoantibodies in chronic HBV infection, an autoimmune etiology for HBVMN should not be excluded.

d. *MN and HBV infection are associated but are not pathogenetically related*

Finally, the increased frequency of MN in HBV infected patients may not be caused by either HBV or secondary liver disease, but rather may result from underlying immunologic abnormality or genetic predisposition that increases the likelihood of these patients to develop both diseases independently of each other. Conditions associated with defective cell-mediated immunity do predispose HBV infected patients to become chronic HBsAg carriers [59,67,68]. European adults with chronic HBV infection but no obvious condition associated with impaired immunity nevertheless can be shown to have suboptimal *in-vitro* interferon production [69]. Defects in cell-mediated immunity have also been reported in idiopathic MN [70,71], but it is not known whether these defects are the consequence of the nephrotic syndrome or a predisposing factor for the development of the disease. Immunogenetic studies have demonstrated an increased incidence of HLA B8 and DR3 in Europeans with idiopathic and drug –

induced MN, and an increase in DR2 in the Japanese population [72-74]. Patients with lupoid chronic active hepatitis also have an increased incidence of HLA B8 and DR3 [75], but these HLA patterns are not typically seen in the chronic HBV

carrier [75]. However, studies of the HLA patterns of patients with HBVMN have not been reported. Thus, the possibility that MN and chronic HBV infection are not causally related but rather are due to an inherent susceptibility of a subset of patients to develop both diseases seems unlikely but has not been definitively excluded.

A recent study by Lin et al set out to elucidate why only some individuals with HBV infection developed MN. They did this by measuring serum HBeAg circulating immune complexes during the acute nephrotic phase of HBVMN and in the carrier stage of HBV [76]. They found that the level of circulating immune complexes was low in the HBVMN patients, and absent in HBsAg+/HBeAg+ patients without HBVMN, or HBsAg+/HBeAg- asymptomatic carriers. In addition, they found that HBVMN patients had lower cytotoxic T cell activity than did HBV carriers, HBsAg-/HBsAb+ subjects or, HBeAg-/HBeAb+ children, using autologous HBcAg-expressing Epstein-Barr-Virus-immortalised lymphoblastoid cell lines as stimulator/target cells. From the in-vitro cytokine production study of peripheral blood T cells after stimulation with HBcAg, they found that T-helper-cell-1-related IL-2 and IFN- γ production were very low in HBVMN patients but T-helper-cell-2-related IL-10 production was higher in HBsAg+/HBeAg+ patients with HBVMN

than in those without HBVMN. Based on these findings, they concluded that HBVMN children seem to have an inadequate cellular immune response to HBcAg.

A study by Xin et al from China investigated the impact and significance of HBV DNA in the pathogenesis of HBV-associated nephropathy [77]. Renal tissue from 43 children with HBV-associated glomerulonephritis were examined for HBV DNA by in-situ hybridisation assay. HBV DNA was identified in 41 of the 43 cases (95.3%) and was distributed generally in the nucleus and cytoplasm of epithelial cells and mesangial cells of glomeruli, and epithelial cells of renal tubules. HBV DNA also existed simultaneously in renal interstitial tissue in some of the cases. The positive results from HBV DNA in-situ hybridisation correlated well with HBV antigen assays. The duration of proteinuria in case of HBV DNA in renal tubules was much longer than in those with no HBV DNA in renal tubules. The analysis implied that the more extensive the existence of HBV DNA in the nephron unit and interstitial tissue, the more severe the clinical manifestation. Zhou et al [78] also reported that the existence of HBV DNA usually coincides with the presence of HBV antigens. This implied that HBV antigens, particularly HBcAg and its immunocomplex, could be derived from local expression of the viral genome by the infected glomerular cells, in addition to conventionally recognised blood-derived HBV antigens. The authors concluded that the persistence of the HBV genome or genes in the kidney could lead to the expression of viral antigens in renal tissue and might cause cellular pathological alterations and chronic immunological trauma.

4.3. Worldwide Perspective of Hepatitis B Virus-Associated Nephropathy

4.3.1. Africa

There have been no recent reports of HBV associated nephropathy in Africa apart from studies from South Africa [17,18]. The most recent report from Durban, South Africa forms part of the basis of this thesis (v. i.).

In the 1980s there were two studies that reported on the relatively high incidence of HBVMN infection in South African black children with nephrotic syndrome. Sixty-three of 388 children (16%), followed between 1969 – 1985 in Cape Town, were found to have MN. There were of either black or mixed race, that is, Cape Coloured and Malay, but not Caucasian or Asian [16,79]. Forty-six (86.7%) of these patients were HBsAg positive and 80% of these were HBeAg positive whereas the prevalence of HBsAg in patients with glomerular disease other than MN was 10.8%. In Johannesburg, 14 of 59 (24%) black children with nephrotic syndrome, seen between 1981 and 1985, were HBsAg and HBeAg positive and one half had circulating HBV DNA. All of these 14 children had MN.

In Zimbabwe, Seggie et al [80] reviewed all cases of glomerulonephritis and concluded that the major patterns were post-streptococcal glomerulonephritis in young adults and HBVMN in childhood. In their 18-month study, 8 (4 boys and 4 girls) of 23 children with nephrotic syndrome, had MN and all were HBsAg positive [81]. HBeAg was not found in any of the cases.

Results from Nigeria, where HBV infection is highly endemic, were different. In contrast with their earlier findings, Abdurrahman et al [82] did not find any increase of the percentage of HBsAg in nephrotic children (36%) compared with control patients (45.9%). Furthermore, in none of the HBsAg positive cases was MN demonstrated, but renal biopsies showed various glomerular patterns (membranoproliferative, Quartan malarial nephropathy, proliferative glomerulonephritis, chronic glomerulonephritis and minimal change disease).

4.3.2. Middle East

Saudi Arabia is among the countries in the Middle East where there is a high prevalence of HBV infection. Horizontal transmission of virus with intra-familial spread of infection are the most probable routes of contamination. In a series of 25 children with nephrotic syndrome, Elidrissy et al [83] found a 28% prevalence of HBsAg. Five of the 7 children (all males) were also HBeAg positive. Renal biopsy performed in 5 of the 7 children showed MN in 3, membranoproliferative glomerulonephritis (1), and minimal change disease (1). All parents were either carriers (3 fathers and 1 mother) or had been exposed to HBV infection. In

addition, screening for HBV markers in three families revealed that most siblings were also carriers.

In Israel, one Caucasian woman hospitalised with nephrotic syndrome and MN was HBsAg positive and HBeAg negative. A year prior to admission her daughter had had serologically proven HBV infection [84].

4.3.3 Asia

In Japan, the prevalence of HBsAg is presumed to be 1.5% in healthy children and 3% in healthy adults. In a series of 1250 children who had renal biopsies between 1972 and 1982 in Kobe, 16 (57%) of 28 children (13 boys and 3 girls) were found to have MN in association with HBV infection. Different Japanese centres [25,27,30,31,84-90] have reported other children with HBVMN, usually in boys. Most were HBeAg positive. When data were available, family history revealed HBsAg positivity for either mother [88], or mother and sister [89], or sister with parents being negative [86]. Membranoproliferative glomerulonephritis was rarely described in these children except in 4 cases of HBV infection associated with membranoproliferative glomerulonephritis type III [25,83].

The prevalence of IgA nephropathy in Japan is high and in a series of 130 patients reported by Iida et al [92], HBV infection was only detected in four of these cases.

Accordingly, these results did not suggest that HBV infection plays a role in the pathogenesis of IgA nephropathy. However in a subsequent study conducted by

Lai et al on paraffin sections of kidney biopsies from 40 chronic HBsAg carriers with MN, membranoproliferative glomerulonephritis or IgA nephropathy, HBcAg DNA was found in 31 biopsies (78%) using polymerase chain reaction (PCR). HBV DNA was detected mainly in the cytoplasm of proximal tubular epithelia but not in glomerular cells. HBsAg and/or HBcAg mRNA were detected by reverse transcriptase PCR in extracted RNA from 13 biopsies (33%). Based on these findings, the authors surmised that HBV DNA in renal tubules represented endocytosis of HBV DNA in the urinary filtrate and that HBV RNA extracted from kidney biopsies could derive from infiltrating cells bearing HBV RNA. Further analysis using in-situ hybridisation with specific HBV core gene RNA probes, showed HBcAg RNA in the nuclei and cytoplasm of glomerular and tubular cells in 56%, 20% and 36% of renal biopsies in chronic HBsAg carriers with MN, membranoproliferative glomerulonephritis and IgA nephropathy, respectively. These findings indicated the presence of viral transcription in glomerular cells and renal tubular epithelia, supporting an aetiological role of HBV in some chronic HBsAg carriers who developed coexisting glomerulonephritis [93].

In Taiwan, the HBsAg carrier rate of 20% of the general population is one of the highest in the world. Hsu et al [94] diagnosed MN in 14 of 63 children with primary glomerular disease seen during the period 1976 – 1981. Among the 14 patients, all 13 tested were HBsAg positive. In 1988, 54 (11.7%) of 463 children with glomerular disease had MN. Of these, 52(96.3%) children were positive for HBsAg and HBeAg was detected in 93% [95]. Lin [96] studied 34 cases (25 boys and 9 girls) with HBVMN. Of the 32 mothers tested, only 2 were HBsAg positive in contrast to 8 of the 31 fathers and 15 of the 42 siblings. Though vertical transmission during the perinatal period prevails in Taiwan, these data suggest that in these MN children HBV was transmitted horizontally, and it is likely that their playmates infected these children.

China is also a country highly endemic for HBV infection. In two different reports, 32 of 106(30%) [97] and 46 (17%) of 275 children [98] were HBV positive. Unfortunately histological examination was not done in the majority of cases. On the contrary, Wang et al [99] in 1987 did not find a high incidence of HBV infection in the 244 adult patients with nephrotic syndrome or proteinuria/haematuria they had studied.

A similar finding was reported by Sham et al [100] from Hong Kong where HBV is endemic, with a HBsAg carrier rate of nearly 10%. These authors showed an

association between MN and HBV (33% of patients with MN being HBsAg positive), but they concluded that the carrier HBsAg rate was comparable in patients with other types of glomerulonephritis and in the general population. Lai et al [50] suggested that HBV might play a role in the development of different forms of glomerulonephritis such as MN, mesangial proliferative glomerulonephritis and IgA nephropathy. In 311 patients (adults and children) with primary glomerulonephritis who fulfilled the following criteria: no history of jaundice

or liver disease, no history of blood transfusion, no history of intravenous drug abuse and normal liver function, 21% of patients were HBsAg positive and 34% of these were also HBeAg positive. These HBsAg positive patients showed a large variety of glomerular lesions. The incidence of HBV infection, however, was higher in patients with MN (64%), IgA nephropathy (17%) and membranoproliferative glomerulonephritis (42%), than in the general population (9.5%). HBV infection was the single most important aetiological agent for MN in the paediatric population in Hong Kong since two thirds of the patients with HBVMN were under 15 years of age; the majority were males. A subsequent study by Lai et al further supported an aetiological role of HBV in some chronic HBsAg carriers who develop coexisting glomerulonephritis [93]. There were other similar reports of HBVMN from Hong Kong [101,102].

4.3.4. North America

There have been few reports in adult patients with HBV associated nephropathy from the United States. Most often, their patients have co-existing hepatitis or belong to one of the high-risk groups [16]. Until 1985, there were only two reports of HBV associated glomerulonephritis in North American children. Both were black males aged 9 and 13 years. The Southwest Paediatric Group of Texas [103] reported 1 case of HBV infection among 47 patients with MN between 1969 – 1979. Subsequently, between 1979 – 1984 they reported 11 North American children with HBVMN, representing 20% of all cases of MN diagnosed during this period. All 11 children were boys and seven were black. Only one child had had clinically evident hepatitis with jaundice four months prior to onset of urinary abnormalities. None of the others had received blood products.

4.3.5. Europe

Europe can be divided into different areas of HBV prevalence. There has been no report of HBVMN in Northern Europe, where the prevalence of HBV infection is very low and HBsAg carrier rate is less than 0.1%. HBV associated nephropathy is rare in Britain [104]. In Switzerland [105] and in the Netherlands [106], two boys, aged seven and nine, having HBVMN (both HBsAg and HBeAg positive), have been reported. Interestingly, the last child was born in the Cape Verde Islands.

In France, prevalence of HBV infection is 5% to 10% and the carrier rate is under 0.5%. In the early 80s, 15 of 35 children with MN had HBV infection. Between 1983 to 1989, 14 children were found to have MN. Of these, eight children were HBV negative, two had anti-HBsAg; four were HBV carriers. Of these 4 children, two were Algerian boys aged two and four, one a black boy from Congo aged nine, and the last was a French Caucasian girl aged six. The latter child had chronic hepatitis secondary to perinatal transmission of HBV. It would appear that in this region, the majority of patients who are HBV carriers are immigrants [10,17,107].

In Italy and Spain, the prevalence is much higher (10% to 50%) and the HBsAg carrier rate is 1% - 5%. In both countries, several cases of HBV associated nephropathy have been reported from different centres. Between 1977 and 1982, HBsAg positivity was found in 19 of 60 children (32%) with glomerulonephritis studied in Naples [108]. Nine of the 14 children with MN in this series were HBV carriers; the majority was boys. On the contrary, in another series of 60 children with nephrotic syndrome studied in Turin, only two children aged four and five years, had MN and both had HBsAg and HBeAg [109]. In Padova, 6 cases of HBVMN had been previously reported [28]. In Madrid, 4 of 34 children with nephrotic syndrome were HBV carriers. Three had MN; two were males. Several Spanish patients had both symptomatic hepatic disease and renal disease [110,111]. In a series of 109 children with HBV chronic hepatitis studied between

1978 and 1987 in Madrid, eight children (7 boys and 1 girl), aged one to nine years showed glomerulonephritis [112]. Five had MN, one had membranoproliferative glomerulonephritis and another had mesangial proliferative glomerulonephritis. All were HBsAg positive, two were positive for HBeAg and six for anti-HBeAg. Two of these children had previously received injections, one had had a neonatal transfusion and in another there was a history of familial HBV infection.

In Bulgaria, 8.3% of adult patients with chronic glomerulonephritis, studied between 1983 – 1985 were HBsAg positive [113]. This frequency is similar to that observed in the general population. Eleven patients were biopsied, nine had IgA nephropathy and two had mesangial proliferative glomerulonephritis. None had MN.

In Russia HBsAg has been detected in 21.1% of cases of glomerulonephritis [114]. HBV is hyperendemic in Poland. In 387 children with nephrotic syndrome studied over 11 years, 49 showed HBV infection. Most were boys under six years of age. MN was demonstrated in 34 patients, whereas membranoproliferative glomerulonephritis was noted in 8 and mesangial proliferative glomerulonephritis in two other patients. In another series of 575 paediatric patients, 51 of 54 patients with MN were found to be HBV carriers [115]. This data clearly shows that HBVMN is a major form of nephropathy in children in Poland.

4.4. Clinical Presentation of Hepatitis B Virus-Associated Membranous Nephropathy

The clinical manifestations of HBVMN in paediatric and adults patients tend to be different (Table VIII). Paediatric chronic HBV carriers, with infection acquired through vertical transmission from infected mothers or through horizontal transmission from infected siblings, are not infrequently asymptomatic, and HBV-associated nephropathy is detected by routine urine and serological screening [16,54,116]. The other common clinical presentation in children is the nephrotic syndrome, not infrequently relapsing, which may mimic minimal change disease. There is a strong male predominance [4,116-118]. In adults, proteinuria and the nephrotic syndrome are the most common manifestations of HBVMN. The male predominance of the nephritis in the adult population is less prominent than that in children [11,23,119,120]. HBVMN from Southeast Asia in adult patients often have no known exposure and the disease is probably a sequel of chronic HBV infection since childhood [50]. Adults with HBVMN from non-endemic areas are more likely than children to have a history of acute hepatitis which could be related to intravenous drug abuse [121], homosexuality [122], and acquired immune deficiency [123].

Table VIII: Differences in clinical presentation of HBV-associated nephropathy between children and adults

Children	Adults
Mode of transmission	
Vertical transmission in the Far East	Often unknown
Horizontal transmission in USA, Africa and Europe	Horizontal transmission in areas of high endemicity. In areas of low endemicity often associated with drug abuse or sexual transmission
Clinical Presentation:	
Asymptomatic – detected by routine urine and serologic screening	Nephrotic syndrome and proteinuria
Nephrotic syndrome	
Gender:	
Strong male dominance (>80%)	Less pronounced male dominance
Mean age at presentation:	
Horizontal transmission: 5-7 years	Any age group
Vertical transmission:	
Acute hepatitis:	
Low incidence	Often present in adults from non-endemic areas where HBVMN associated with intravenous drug abuse, homosexually and acquired immune deficiency syndrome
Histology:	
Membranous (>85%)	Usually membranous
	Often associated with IgA nephropathy
Renal function:	
Preservation of renal function in over 95% of children	Progression to renal failure in 25%

4.5. Clinical Course and Prognosis

Several studies have documented the natural history of HBVMN, including reports from Africa [17,18,79,80,81,82]. Spontaneous regression of the nephrotic syndrome was reported in 30 to 60 percent of cases of HBVMN, and these patients usually remained asymptomatic for 12 months or longer [4,20,50,117]. The remaining patients had persistent proteinuria with fluid retention [20,50,117]. Seroconversion to anti-HBeAg is associated with remission of proteinuria [4,120]. The majority of children have a benign course. There have also been reports of progression to renal insufficiency in those patients who do not clear the virus [20,30,50,117]. In a study by van Buuren et al of 29 Namibian children with HBV-associated nephropathy, 26 had HBVMN, 1 membranoproliferative glomerulonephritis, 1 focal segmental glomerulosclerosis, and 1 child with advanced renal failure was not biopsied. Three of these children were reported to have progressed to end-stage renal failure [124].

4.6. Treatment of Hepatitis B Virus Membranous Nephropathy

Animal experiments and observations on human subjects have demonstrated that HBV-containing immune complexes may be formed in the course of HBV-induced acute and chronic hepatitis [125]. Immune complex-mediated glomerulonephritis requires a continuous supply of antigen and a maintained antibody response and, apparently, these requirements seem to be met in chronic HBV carriers. Hence, the rational approach of treatment of HBVMN should aim at eradication of the chronic HBV infection.

Attempts have been made to treat HBVMN for the following reasons:

- a) Spontaneous resolution does not necessarily occur in all nephrotic patients and 40% to 70% of patients remain symptomatic. Complications related to overt nephrotic syndrome such as hyperlipidaemia, oedema, and venous thrombosis have been observed in these patients.
- b) There is improvement of the liver disease and renal involvement following clearance of HBsAg from the blood [125].
- c) The disease may progress in a small percentage of patients and result in chronic renal insufficiency [30,117,124].

4.6.1. Corticosteroids

Corticosteroid therapy used in primary MN has been administered to some patients with HBV associated nephropathy as a therapeutic trial for symptomatic relief of proteinuria [13,20]. Furthermore, it is not uncommon for corticosteroids to

be given to nephrotic children suspected of having steroid-responsive minimal change nephropathy, and for subsequent serology and pathological examination to reveal the diagnosis of HBV-associated nephropathy [116,117]. However corticosteroids given at the onset of nephrotic syndrome in HBVMN, do not seem to have an ameliorative effect on the nephrotic state or lead to clearance of the virus [13].

A deleterious effect of corticosteroids with exacerbation of liver impairment following abrupt withdrawal of corticosteroids has been reported in patients with chronic HBV hepatitis [126]. In contrast to patients with chronic active hepatitis, patients with HBVMN may not have evidence of hepatic dysfunction and their liver biopsies may even be normal [50,118]. A prospective trial (compared with historic controls) of corticosteroids in nephrotic patients with HBVMN was conducted by Lai et al [127]. Serial serological measurements suggested that corticosteroid therapy was associated with active viral replication with increased serum concentrations of alanine aminotransferase, HBeAg and HBV DNA, although symptomatic liver dysfunction was not detected. Histopathological examination of post-treatment renal biopsy in a single patient revealed histological progression, which did not support a protective value of corticosteroid therapy [127].

Furthermore, the appearance of virus-like particles in the glomeruli after corticosteroid therapy supported the serological evidence of active viral replication.

Hence, these studies reveal corticosteroids should not be used in HBV-associated nephropathy.

4.6.2 Alternative Therapies

A major priority in HBV research is the development of safe and effective anti-viral therapies. Alpha-interferon (INF) is the only effective therapy for children with chronic hepatitis B. In most controlled studies, performed in Caucasian children, INF therapy achieves clearance of HBV-DNA and HBeAg in 30-50% of treated patients [128,129]. While this treatment is fairly well tolerated, better therapies are needed for higher rates of viral eradication. Furthermore, the long-term impact of this therapy in those children who exhibited an initial response needs to be clarified. Clearly more effective therapies for chronic HBV in children need to be defined by well-designed, large scale, placebo-controlled multi-centre trials, and for each therapy tested there is a need to identify factors predicting response. Candidate drugs include lamivudine, famcyclovir, pegylated interferon, lobucavir, and adepovir. Novel strategies, such as immunization of chronically infected children with adjuvant vaccines, should be also be tested.

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CHAPTER 5

PATIENTS AND METHODS

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5.1 Patients

The patients used in these studies were recruited from the Renal Clinic at King Edward VIII Hospital, Durban, that is the tertiary referral centre for the Province of KwaZulu/Natal, South Africa. All were black children aged between 0-16 years (Table IX). A total of 116 children with biopsy-proven hepatitis B virus (HBV)-associated membranous nephropathy (MN) were included in these studies. Cohorts of this group of patients were used in the various studies undertaken. All had nephrotic syndrome diagnosed in accordance with criteria used by the International Study for Kidney Diseases in Children [1]. Hepatitis B virus infection was diagnosed using ELISA (Auszyme ® Monoclonal; Abbott Laboratories, North Chicago, IL, USA) and in a subset of patients this was confirmed by Slot-blot hybridisation and nested polymerase chain reaction. Clinical examination and appropriate investigations were done to exclude other secondary causes of nephrotic syndrome.

Table IX: Cohorts of subjects used in the various studies:

Study	Year	Subjects
Retrospective review of Nephrotic Syndrome in Durban, KwaZulu/Natal, South Africa	1976 – 1996	636 children with Nephrotic Syndrome
HBV associated nephropathy in black South Africa children	1976 – 1996	133 black children with HBV-associated nephropathy
Clustering of HBV in households of children with HBVMN	1995 – 1997	31 black children with biopsy-proven HBVMN as index cases. 177 family members and household contacts
HBV and proteinuria in relatives and contacts of children with HBVMN	1995 – 1997	Same as above
Characterisation of proteinuria in asymptomatic family members and household contacts of children with HBVMN	1995 – 1997	Same as above
HLA class I and II in black children with HBVMN	1995 – 1998	30 black children with biopsy-proven HBVMN
HLA associations with HBV carriage and proteinuria in families with HBVMN	1995 – 1997	14 black children with biopsy-proven HBV for HLA QB1*0603 70 family members (parents and siblings) comprise the study group
Interferon Alpha 2b treatment of HBV-associated nephropathy in black children.	1997 – June 1999	24 black children with HBV-associated nephropathy

Table IX: Cohorts of subjects used in the various studies:

Study	Year	Subjects
The impact of HBV vaccine	1984 – 2001	116 children with biopsy-
On the incidence of HBVMN		proven HBVMN

5.2. Hepatitis B Virus Detection

5.2.1. Serum

Hepatitis B status was determined using third generation enzyme-linked immunosorbent assay (ELISA) (Auszyme^R Monoclonal, Abbott Laboratories) and HBV DNA was determined by slot-blot hybridisation (SBH) and nested polymerase chain reaction (PCR).

5.2.2 Method of DNA Extraction from Serum Samples

The QIAamp blood kit (QIAGEN GmbH, Hilden, Germany), a method for nucleic acid purification and removal of amplification inhibitors, was used according to the manufacturer's instructions. A 200 µl aliquot of serum was incubated with QIAGEN protease and buffer AL at 70 °C for 10 minutes. Potentially infectious agents were inactivated by incubation at 95 °C for 15 minutes following cell lysis. The lysate was applied to a QIA-amp spin column, spun, washed 3 times with buffer AW, and finally eluted with 50 ml best quality water (BQW). Known positive and negative sera and BQW were used as controls for the extraction procedure.

5.2.3. Polymerase Chain Reaction (PCR)

Primers specific for the precore/core region were used for nested PCR (Table 1) [2]. The final PCR reaction mixture contained: 0,02 Uml⁻¹ Taq DNA polymerase (Promega, Madison, WI), 200 µM each of the dNTPs, 1 µM of each primer, 50 mM potassium chloride, 10 mM Tris HCl (pH 8,8), 1,5mM magnesium chloride and 0,1% triton-X 100. 2,5 microlitres of target DNA was added to 22,5ml reaction mixture.

The first round PCR was performed in a programmable thermal cycler (Perkin Elmer, USA) with the following 3 step cycling profile:

94⁰C for 30 seconds - denaturation

62⁰C for 50 seconds - annealing

72⁰C for 50 seconds - polymerization

for a total of 40 cycles followed by a final extension of 10 minutes at 72⁰C. For the second round PCR, a 5 µl aliquot of the first round amplification product was re-amplified using inner primers in the cycling profile shown above except that the annealing temperature was decreased to 58⁰C.

All amplifications of serum and urine samples were performed with HBV-positive and HBV-negative controls. Sera positive for HBsAg, HBeAg and HBV DNA (by SBH) were used as positive controls. To avoid cross-contamination and false positive results the precautions and procedures suggested by Kwok and Higuchi were strictly adhered to [3]. DNA extraction, PCR amplification and electrophoresis were performed in physically separated venues. PCR controls to detect contamination consisted of BQW added to the PCR mixture instead of DNA; a water control interspersed between serum or urine samples.

5.2.4. Detection of Amplified Product

A 15 µl aliquot of the amplified product was electrophoresed on a 2% agarose gel. Bands of the appropriate size (314 base pairs with single PCR or 204 base pairs

with nested PCR) were visualized under ultraviolet light after ethidium bromide staining. Each sample was tested in duplicate and read as positive or negative only if the two results agreed. Positive and negative controls were run in parallel with every assay. The specificity of the amplified band was confirmed by Southern blot hybridization using ^{32}P labelled HBV DNA probe (HBV DNA in pBV325 vector, adr subtype, purified and supplied by Professor C. Bréchet, INSERM U 370, Institut Pasteur, Paris) (Fig 15).

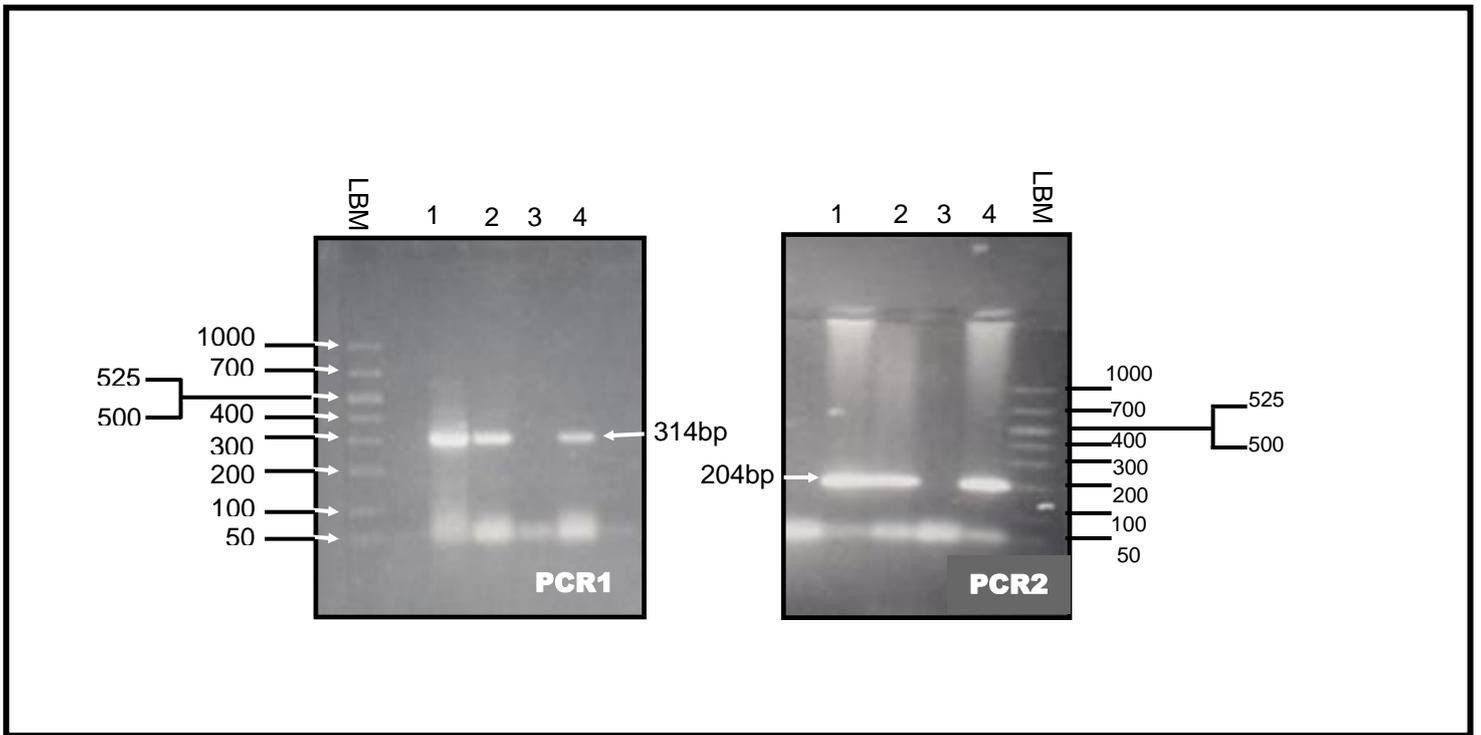


Figure 15: Ethidium bromide stained 2% agarose gel showing HBV DNA amplified from serum samples. PCR 1: single round amplification using outer primers 1730(+) and 2043(-). PCR 2: nested PCR using outer primers followed by amplification with inner primers 1763(+) and 1966(-).

LBM: low biomarker.

Lane 1: 23F

Lane 2: 24C

Lane 3: negative control

Lane 4: positive control

5.2.5. Slot-blot Hybridisation Assay for HBV DNA Detection

HBV DNA in serum or urine was detected using a SBH assay using the method of Zaaijer et al [4]. Briefly, 50 μ l of serum was denatured in an equal volume of denaturing solution (200mM EDTA, 10% sodium dodecyl sulphate, 20 mg ml⁻¹ proteinase K, 12,5mM Tris hydrochloride) at 56⁰C for 2 hours. The samples were heated to 95⁰C for 10 minutes and chilled on ice. 200 μ l 20 x SSC was added to the denatured samples and centrifuged at 13 000 rpm for 15 minutes. The clarified samples were blotted onto Hybond-N nylon membrane (Amersham Life Science, Buckinghamshire, UK) using the Bio-Dot R[®] SF microfiltration apparatus (BioLab Laboratories, South Richmond, CA, USA). The blots were treated with 0.4 molL⁻¹ NaOH, dried in an 80⁰C oven for 2 hours, and hybridized using ³²P-labelled HBV DNA probe (Megaprime DNA labelling system, Amersham). Blots were hybridized using QuikHyb[®] Hybridisation solution (Stratagene, La Jolla, CA). Autoradiography was carried out at -70⁰C with intensifying screens and Kodak film (X-omat, Sigma, St Louis, MO) for four days (Fig 16).

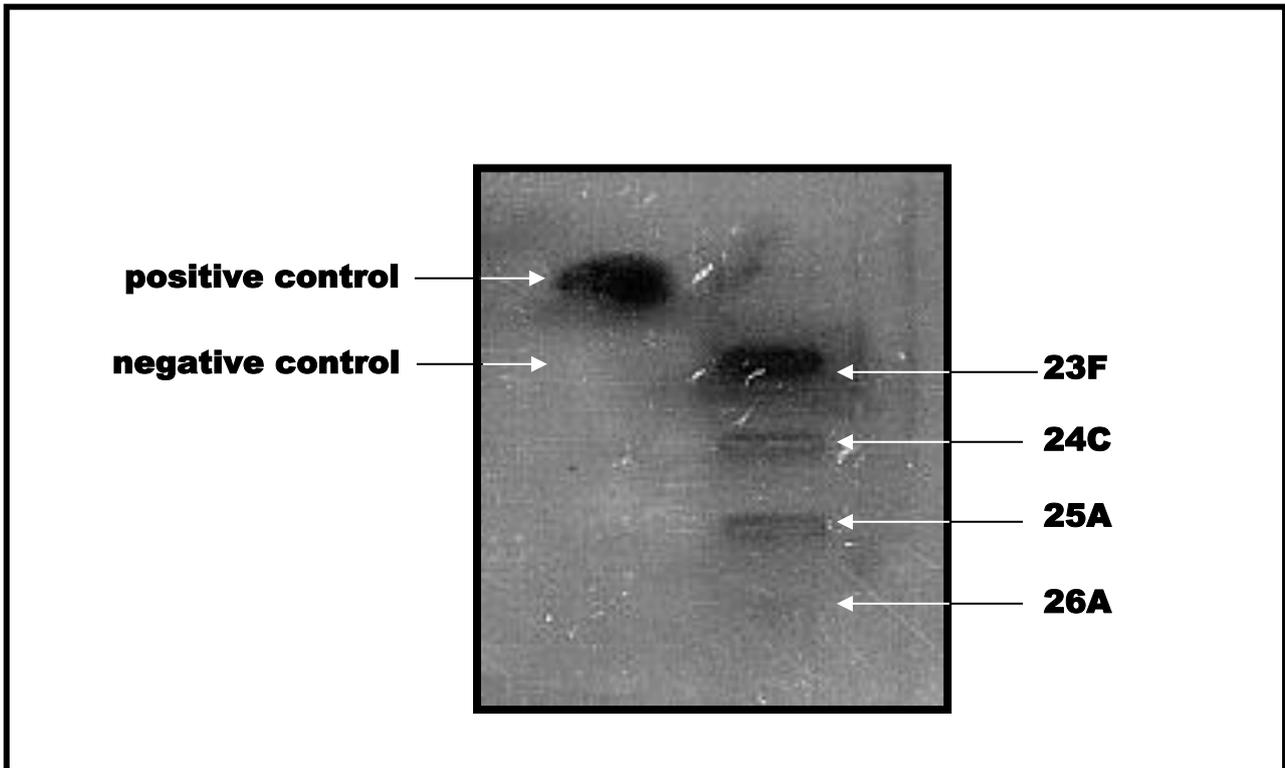


Figure 16: Autoradiograph of slot blot hybridization probed with ^{32}P labelled HBV DNA – 24 hour exposure.

5.3. Urine

5.3.1. Method of DNA Extraction from Urine Samples

Prior to DNA extraction, urine samples were concentrated 25 times using an Urifil-10 concentrator (Millipore, Bedford, MA, USA). DNA was extracted from 140 µl of concentrated urine using the QIAamp viral RNA purification protocol (QIAGEN). 140µl of urine was mixed with 560 µl Buffer AVL containing carrier RNA and incubated at room temperature for 10 min, and 560 µl of ethanol was added to the sample. The solution was applied to a QIAamp spin column and centrifuged at 8 000 rpm for one minute. The last step was repeated with the remaining solution. The QIAamp spin column was washed twice with Buffer AW. The DNA was finally eluted in 50 µl BQW.

5.4. Determination of Genotype

In order to determine the genotype, the pre-core region of HBV DNA extracted from serum samples was sequenced directly from PCR products. The PCR amplification mixture (5 –10 µl) was pre-treated with two hydrolytic enzymes: exonuclease (10.0 U/ml) to remove sticky ends and primers, and shrimp alkaline phosphatase (2.0 U/ml) to remove nucleotides (Sequenase PCR product sequencing kit; United States Biochemicals, Cleveland, OH, USA). Enzymatic pre-treatment was carried out at 37⁰C for 15 minutes and enzymes were inactivated by heating to 80⁰C for an additional 15 minutes in a thermal cycler.

Sequencing was carried out using the Sequenase PCR kit, but with modifications of the methods by Casanova et al [4] and Galibert et al [6]. The annealing mixture contained 0.5 pmol DNA (10% to 90% of the amplified sample, depending on the PCR yield), 10 pmol primer (1763 and 1966R), (Table X) and BQW in final 10 μ l volume. The sample was denatured at 99^oC and then immediately placed in an ice/water bath for 5 minutes. The labelling reaction was carried out using 10⁻¹ dilution of labelling mix and 5 μ Ci - ³⁵S-dATP for two minutes. Termination was carried out at 40^oC for five minutes. The sequences were analysed on an 8% polyacrylamide gel in glycerol tolerant buffer and autoradiographed using Kodak film (X-omat; Sigma, St. Louis, MO, USA). Mutations were recorded only when they were detected in both the forward and reverse sequences.

Table X - Oligonucleotide primers used in the study

	Primer	Sequence	Position^a	Size^b
Outer primers	1730 (+)	5'CTGGGAGGAGTTGGGGGAGG	1730-1747	314bp
	2043 (+)	5'CAATGCTCAGGAGACTCTAAGG	2043-2021	
Inner Primers	1763 (+)	5'GGTCTTTGTAAGGAGGCTG	1763-1783	204bp
	1966 (-)	5'GTCAGAAGGCAAAAACGAGAG	1966-1946	

Symbols are: (+), sense; (-), antisense

^a **Denotes nucleotide position of hepatitis B virus ayw (GenBank accession no. V00866)**

where the EcoRI cleavage site is position 1[6]

^b **Size of the polymerase chain reaction product in base pairs**

5.5. Urinary Protein: Creatinine ratio ($U_{p/cr}$)

5.5.1. Background

Urinary proteins were detected by dipsticks, 24 hour urinary protein estimation and analysis of a random urine sample. Quantitation of the urine samples can be carried out on timed urine collection (usually 24 hours) or by analysis of a spot urine sample. Timed urine collections however are cumbersome and not always possible in children. First morning urine samples were used for determination of protein by the method of Pesce and Strande [7] and creatinine concentrations were measured with the Beckman creatinine analyser [8]. With this method, a spot 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 correlates with the quantity of proteins excreted in 24 hours (Table XI).

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

Patients	Urine protein: creatinine ratio	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 = abnormal range

Ratio < 0.2 = normal range

5.5.2. Method

Early morning samples of urine and 24 hour timed samples from 18 patients were sent to laboratory at King Edward VIII Hospital for estimation of protein and creatinine respectively. Protein was measured by using Coomassie blue technique [9] and creatinine by the Jaffe method [10]. Urinary protein: creatinine ratios were then calculated [11,12].

5.5.3. Results

The correlation between 24-hour urine sample and random assessment of urine protein: creatinine ratio was significant ($r = 0.727$; $p < 0.116$). The regression equation being:

$$24 \text{ hour} = 1.24 + 2.926 (\text{urine protein: creatinine})$$

Urine protein: creatinine ratio of > 0.2 corresponded to significant proteinuria and value of < 0.2 was considered as abnormal range proteinuria.

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 the urine protein: creatinine ratio. The specificity was 100%.

Sensitivity

The sensitivity of the urine protein: creatinine ratio in assessing the degree of proteinuria, was calculated as a percentage of 24 hour urine sample with proteinuria at either normal range or abnormal 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	

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

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

5.6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS Page)

5.6.1. Background

One dimensional gel electrophoresis is a high-resolution separation technique for separating protein for 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 [13]. 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. In non-denaturing conditions (in absence of SDS) separation of protein is based on a combination of molecular properties including size, shape and charge [14].

5.6.2. 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 (Fig 17) the molecular weight of an unknown protein may be determined by comparison with known protein standards.

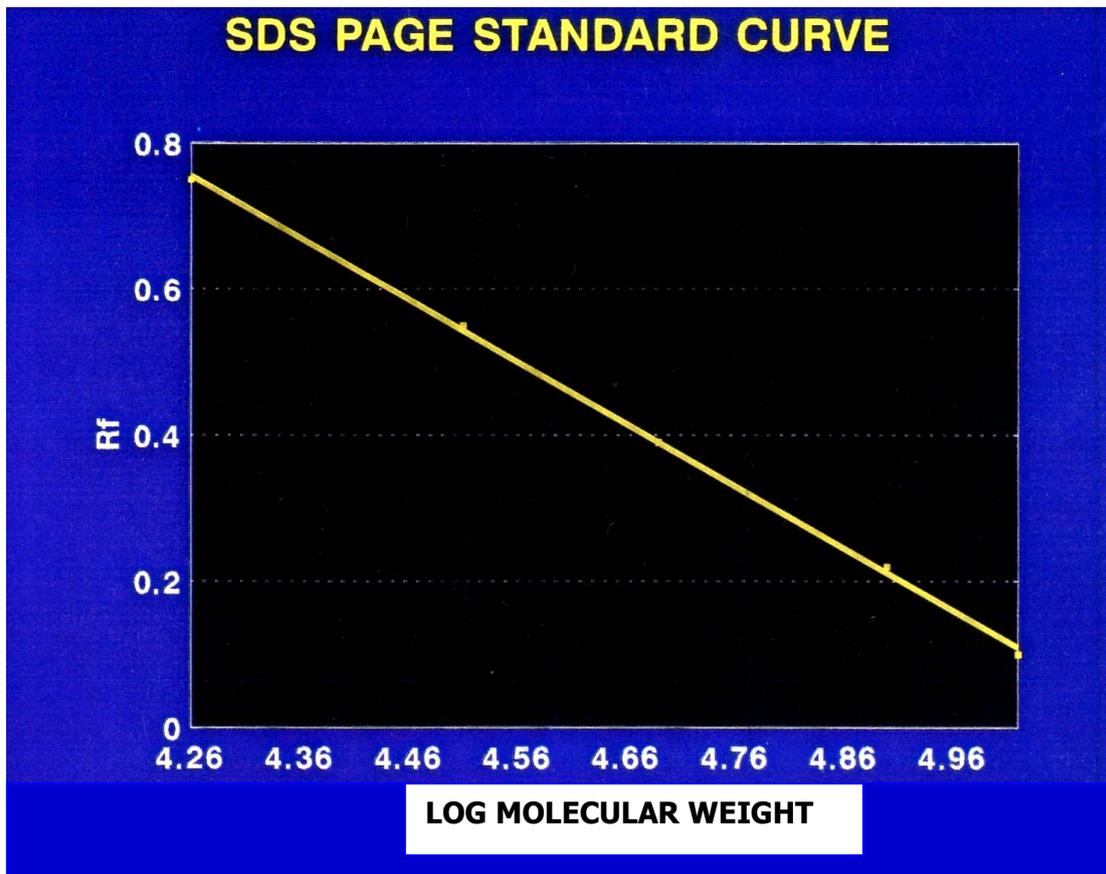


Figure 17: SDS PAGE standard curve

5.6.3. Material and Solutions

The following were obtained from Sigma Chemicals (St Louis Missouri)

- A. 1. Pure standards of albumin, transferrin, haptoglobin, immunoglobulin B (IgG), β_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.
- 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 might – 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	(SE211A/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

SDS (10%) – 20ml

Made up to 2 L 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 Jahansson:

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

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

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	25% Running Gel
	(mls)	(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	10 μ l	20 μ l
Ammonium persulphate	40 μ l	60 μ l

2. STACKING GEL

4% stacking gel	mls
Stock acrylamide (30%)	0.53
10% SDS	0.4

Stacking gel buffer pH 8.8	0.5
Water	2.57
Persulphate	60 μ l
Temed	20 μ l

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 7.5 μ l (0.5 μ g)

Haptoglobin 10mg/ml 15.0 μ l (0.5 μ g)

Transferrin 1.0mg/ml 5.0 μ l (0.5 μ g)

Albumin 2.0mg/ml 5.0 μ l (0.5 μ g)

Lysozyme (0.62-1.25mg/ml) 3.0 μ l (0.5 μ g)

β_2 Microglobulin (1.0mg/ml) 2.5 μ l (0.5 μ g)

Water 2.0 μ l

Sample buffer 10.0 μ l

The concentrations of the standards were adjusted to give a visible band with Coomassie blue stain. 5 μ l of protein standard (5-10 μ g) were applied to the gel.

5.6.4. 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 Coomassie blue method [9] and creatinine by Jaffe method [8].

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

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

Electrophoresis [15,16]

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 periplastic pump, with one end of the 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. 5 μ l (15-20 μ g/ μ l protein) of sample was underlayered in each well with a Hamilton syringe.
9. The unit was connected to the power pack supply and current was passed for 15 minutes at 85MA 100 volts and the increased to 200V for the remainder of the run. Once the dye-front reached the bottom, the power supply was turned off after \approx 90 minutes.

5.6.5. 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 (*R_f*) calculated (Fig. 18) as follows:

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

Choice of stain

Silver staining is reported to be more sensitive than Coomassie 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 Coomassie blue for all our staining purposes.

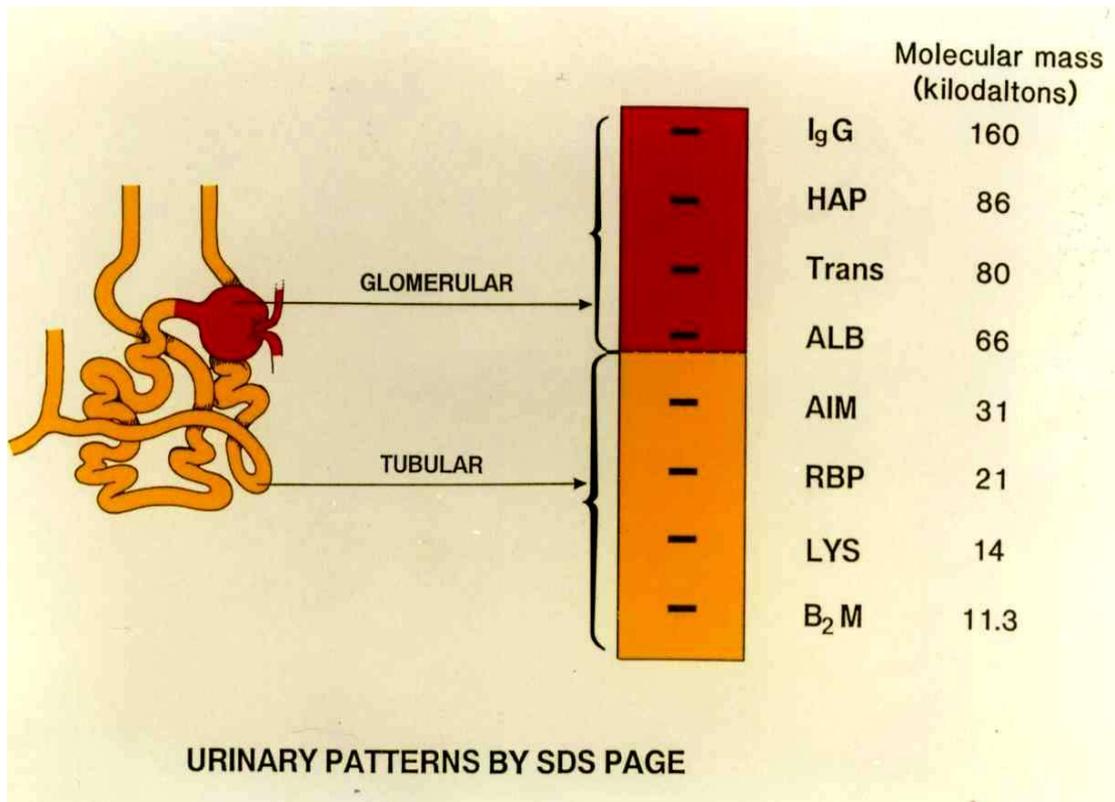


Figure 18: SDS PAGE separation of protein molecules according to size (molecular weight)

IgG	-	immunoglobulin G
HAP	-	haptoglobin
Trans	-	transferrin
ALB	-	albumin
AIM	-	alpha 1 microglobulin
RBP	-	retinol binding protein
LYS	-	lysozyme
B₂M	-	beta 2 microglobulin

5.6.6. 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.

5.6.7. Intra-reader Reproducibility

In order to assess intra-reader reproducibility of interpretation of protein bands, 20 gels which have various visible bands of all proteins interpretations were selected at random to be 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 [17] this test is defined by the formula: -

$$k = \frac{2(ad - bc)}{p_1q_1 + p_2q_2}$$

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 XII: Results of kappa test for protein bands

Protein bands	k
Albumin	0.80
Transferrin	0.72
Haptoglobulin	0.75
IgG	0.85
β_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 β_2 M and lysozyme.

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%.

5.6.8. Detection limit

Minimum detection limits were investigated for albumin, transferrin, haptoglobin, IgG, β_2 M and lysozyme. The minimum detection limit was found to be 0.1mg/ml for albumin, transferrin and IgG. β_2 M and lysozyme were visible at 0.4mg/ml.

5.6.9. 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. Changes in the pH of the buffer are detected by sudden increase in the current of the electrophoresis tank.
3. The de-stained 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 refrigerator for at least one week if carefully packed in foil and plastic.

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CHAPTER 6

NEPHROTIC SYNDROME IN SOUTH AFRICAN CHILDREN

CHAPTER 6

NEPHROTIC SYNDROME IN CHILDREN OF COLOUR IN KWA/ZULU/NATAL

6.1. Introduction

For about a quarter of a century we have known that the nephrotic syndrome (NS) among black children does not conform to the model established in other continents. In most parts of the world, this disorder is dominated by minimal change nephrotic syndrome (MCNS), with a predictable and gratifying response to steroids and an excellent long-term prognosis [1]. The conspicuous features of NS among black children, which differ from the pattern described elsewhere, are the paucity of MCNS, steroid resistance (SR) in the majority, a less satisfactory outcome and an identifiable causative agent in many [2-4]. The epidemiology of infectious agents differ substantially as one traverses Africa from the northern state of Tunisia to the most southern state of South Africa [5]. Often there is a strong correlation between renal histology and microbial aetiology, and thus the pathology of NS has a strong regional bias (Fig 19). The early work from West Africa by Hendrickse et al. [6,7] established the key features of malarial nephropathy. In the other regions of Africa, organisms such as streptococci, viruses (especially hepatitis B virus), onchocerca and schistosoma account for the majority of nephrotic patients [8,9].

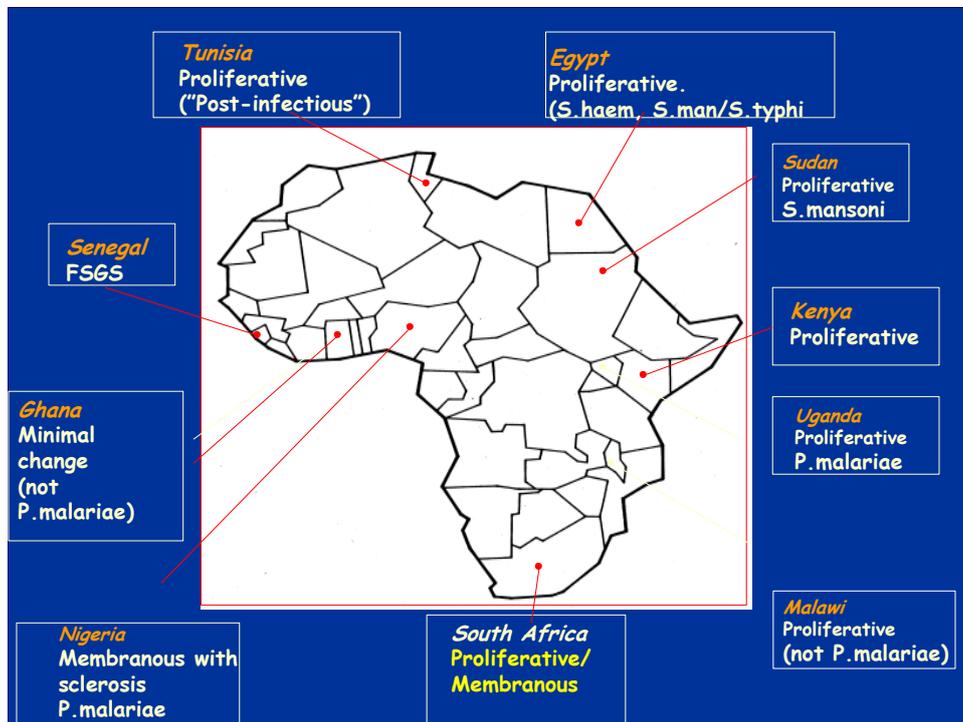


Figure 19: Predominant forms of Nephrotic Syndrome seen in different regions in Africa. Adapted from Seggie J, Davies PG, Ninin D, Henry J (1984) Patterns of glomerulonephritis in Zimbabwe; survey of disease characterized

There is possibly no other country beside South Africa where population groups have, up to recently, been segregated so distinctly and in which socio-economic differences rest so clearly in colour. During the late 1970s we sketched the profile of childhood NS in South Africa, where the malaria prevalence was very low. We pointed out that although the pattern of this disease in Indian, white and coloured¹ children was similar to that of children in the Western world, that in black children was different, not only with regard to histological findings, but also with respect to clinical behavior and response to therapy [2-4,10]. Glomerular proliferative change was the commonest lesion among black South Africans and consequently steroids were usually ineffective. In the intervening two decades, we have added much to this basic information; we have analysed our current data, which constitute one of the largest series in Africa, and now present the findings here.

¹ designated as those of mixed race who are descended from black, white and slaves from West and East Africa and from the Far East – the terms in South Africa is for the present used as a matter of convenience as it defines a group characterised by some common political, social, economic and cultural experiences and does not endorse racial classification

6.2 Patients and Methods

6.2.1. Patients

Six hundred and thirty-six children less than 16 years of age with NS presenting over a 20-year period at King Edward VIII Hospital, Durban, were studied. The mean age at presentation was 5.7 years, the youngest patient being 2 weeks old. There were 375 male and 261 females; 48,2% of patients were black, 48,2% Indian and 3,6% coloured. None of these patients was white as these patients previously did not attend our hospital. Ninety-one patients who were not biopsied, and whose responsiveness to steroids could not be assessed were excluded from the analysis. These include 71 black and 20 Indian children.

6.2.2. Methods

The following investigations were performed to exclude secondary causes of NS: blood culture, antistreptolysin O titre (ASOT), hepatitis B screen for 's' and 'e' antigens and antibodies, liver function test, Widal's test, Wasserman reaction: antinuclear factor and serum complement. Renal biopsy was undertaken in 480 patients and studied by light microscopy, immunofluorescence and electron microscopy. All black children were biopsied. During recent years biopsy was not undertaken in Indian and coloured children with steroid-sensitive (SS) disease, who had a clinical course strongly suggestive of SS-MCNS. In the 91 patients who were not biopsied and in whom the clinical course could not be confirmed as SSNS, biopsy was not undertaken for one of the following reasons: parental

refusal to give consent, prolongation of the bleeding time, coagulation defects or loss to follow-up.

6.3. Disease Categorisation

Patients are classified into ten categories based mainly on histological findings, but also by response to steroid therapy. Histopathological categories are based on the World Health Organisation classification [11].

6.4. Renal Function

This was assessed by measurement of serum electrolytes, blood urea and serum creatinine. The glomerular filtration rate was derived from the Schwartz formula as follows:

Estimated GFR = Height in cm x 0.5 (depending on age or sex) divided
by the serum creatinine concentration in $\mu\text{mol/l}$ [12]

6.5. Results

6.5.1. Overall Findings

Five hundred and forty-five patients were available for analysis: 384 patients were followed for more than a year, with a mean of 6,2 years and a maximum of 15 years: 197 of 636 (31%) patients at a time of evaluation were in remission, 158 of 636 (24.7%) in partial remission, 29 of 636 (4.5%) in relapse and 252 of 636 (39.8%) patients were SS, 194 (30.5%) SR, 10 (1.6%) steroid dependent and in 200 of 636 (31.4%) only symptomatic therapy was used for control of oedema or steroids were contraindicated. The histological classification of the various groups of NS in the different racial groups is shown in (Fig 20,21,22).

Peri-orbital and pedal oedema were the commonest modes of presentation, occurring in 74% of patients, whilst 22.7% of patients had ascites as the presenting complaint. The remaining patients presented with a clinical picture of acute glomerulonephritis with minimal oedema and only subsequently developed severe hypertension on initial presentation.

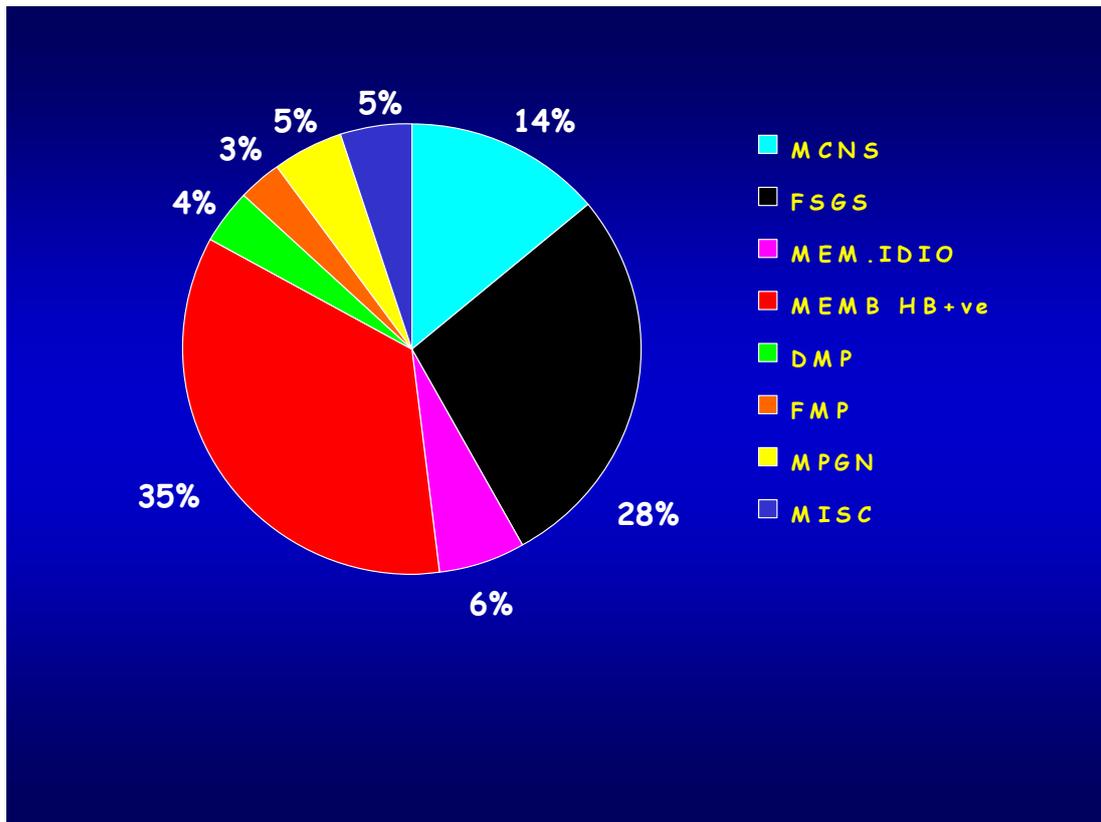


Figure 20: Histological classification of Nephrotic Syndrome in Black Patients in KwaZulu/Natal 1995

MCNS	-	minimal change nephrotic syndrome
FSGS	-	focal segmental glomerulosclerosis
Mem. Idio	-	idiopathic membranous nephropathy
Mem Hep +ve	-	hepatitis B virus-associated membranous nephropathy
DMP	-	diffuse mesangial proliferative glomerulonephritis
FMP	-	focal (mild) mesangial proliferative glomerulonephritis
MPGN	-	membranoproliferative glomerulonephritis
MISC	-	miscellaneous

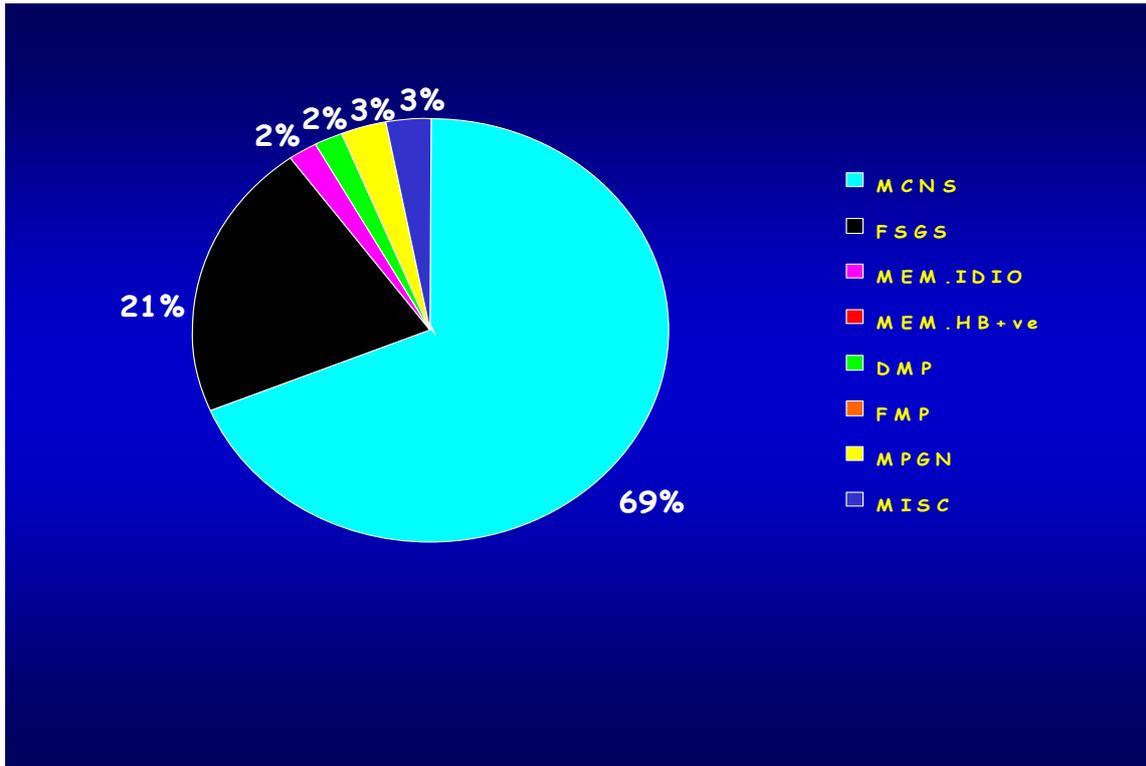


Figure 21: Histological classification of Nephrotic Syndrome in Indian Patients in KwaZulu/Natal 1995

MCNS	-	minimal change nephrotic syndrome
FSGS	-	focal segmental glomerulosclerosis
Mem. Idio	-	idiopathic membranous nephropathy
Mem Hep +ve	-	hepatitis B virus-associated membranous nephropathy
DMP	-	diffuse mesangial proliferative glomerulonephritis
FMP	-	focal (mild) mesangial proliferative glomerulonephritis
MPGN	-	membranoproliferative glomerulonephritis
MISC	-	miscellaneous

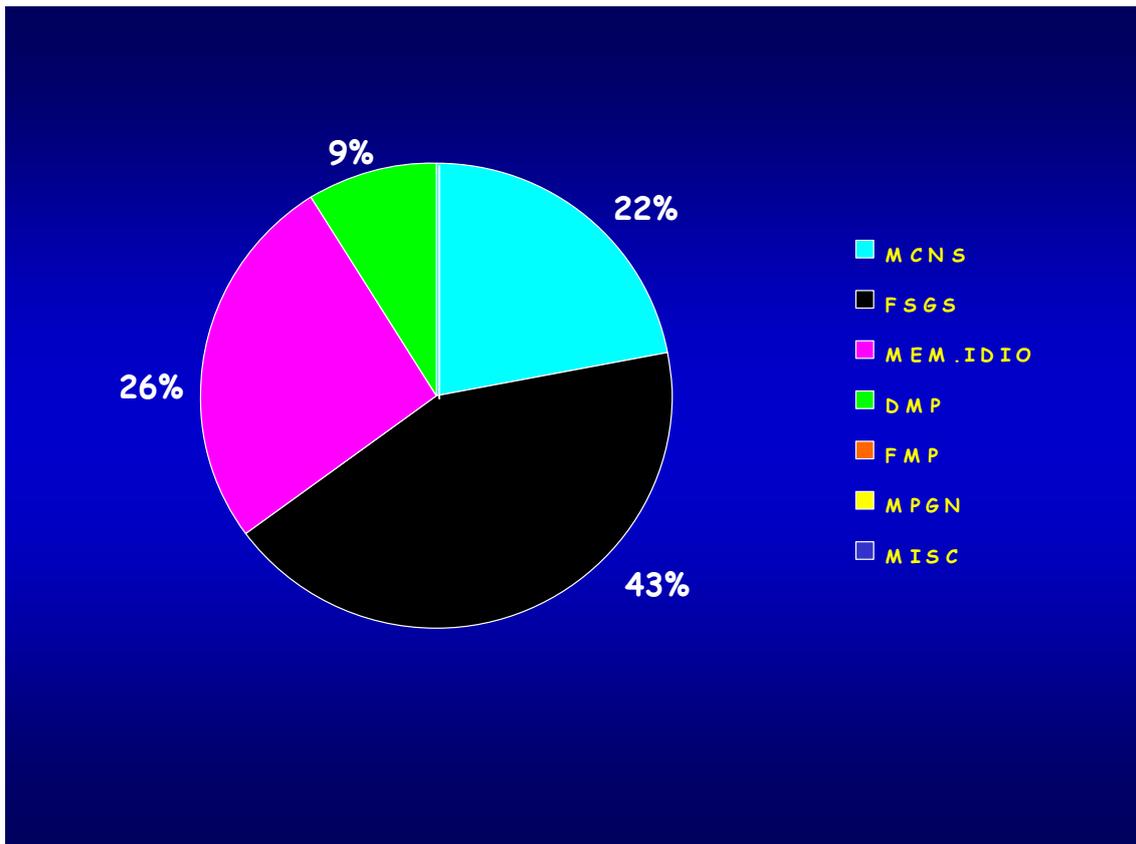


Figure 22: Histological classification of Nephrotic Syndrome in mixed Race Patients in KwaZulu/Natal 1995

MCNS	-	minimal change nephrotic syndrome
FSGS	-	focal segmental glomerulosclerosis
Mem. Idio	-	idiopathic membranous nephropathy
Mem Hep +ve	-	hepatitis B virus-associated membranous nephropathy
DMP	-	diffuse mesangial proliferative glomerulonephritis
FMP	-	focal (mild) mesangial proliferative glomerulonephritis
MPGN	-	membranoproliferative glomerulonephritis
MISC	-	miscellaneous

6.5.2. MCNS and SSNS

MCNS was detected on biopsy in 32 (13.5%) of the total 236 black children (Table XIII); however, only 12 of these were SS and 2 were steroid dependent. The remaining 18 were initially SS but subsequently developed steroid insensitivity; 2 black patients who were not biopsied were SS and followed a clinical course compatible with SS-MCNS (Table XIV). The ages of onset was between 6 and 8 years (Table XIII).

Of the 286 Indian children studied, 134 (46.8%) had biopsy-proven MCNS; 127 (94.8%) of the latter were SS. A further 60 had not biopsied SS disease (Table XIV). Accordingly, 187 (65.4%) Indian children had SSNS or biopsy-proven SS-MCNS; another 8 (2.8%) were steroid dependent; 32 patients were lost to follow-up after a year (Table XIV). The peak age of onset was 2 – 4 years (Table XIII). Two coloured children had biopsy-proven MCNS, both of whom were SS. Three were not biopsied and had SSNS. Accordingly, 5 (21.7%) patients had a clinical course compatible with typical MCNS (Table XIV). The peak age of onset in Indian and coloured children was 2-4 years (Table XIII).

Table XIII: Histological classification and clinical data

Category	No. Of Patients	Patients Details Peak age of onset (years)	Sex		Race			Outcome (last visit)		
			M	F	Black	Indian	Coloured	N	CRF	ESRD
Steroid-responsive Nephrotic syndrome	65	6-8 (blacks) 2-4 (Indian)	44	21	2	60	3	65	0	0
Minimal change nephrotic syndrome	168	6-8 (Black) 2-4 (Indian)	110	58	32	134	2	168	0	0
Focal segmental glomerulosclerosis	136	5-7	82	54	67	59	10	129	3	4
Membranous nephropathy (Hep B +ve)	81	6-8	51	30	81	0	0	80	1	0
Membranous nephropathy (Hep B -ve)	26	5-8	12	14	13	7	6	23	1	1
Membranoproliferative glomerulonephritis	21	6-8	10	11	12	9	0	17	3	1
Diffuse Mes. Prol. Glomerulonephritis	18	2-5	11	7	10	6	2	15	2	1
Mild Mes. Prol. hypercellularity	8	2-5	3	5	7	1	0	8	0	0
Miscellaneous	22	5-8	15	7	12	10	0	22	0	0
Total	545		375	261	236	283	23	527	11	7

N, Normal renal function; CRF, Chronic renal failure; ESRD, end-stage renal disease; Hep, Hepatitis (Hep B +ve) Mes. Prol.; Mesangial Proliferative

Table XIV: Minimal change disease and steroid-responsive nephrotic syndrome according to population group

Population group	Black		Indian		Coloured	
	Steroid sensitive	Steroid resistant	Steroid sensitive	Steroid resistant	Steroid sensitive	Steroid resistant
Minimal change nephrotic Syndrome biopsied	14	18	127	8	2	-
Steroid-responsive nephrotic Syndrome unbiopsied	2	-	60	-	3	-
Total	16	18	186	8	5	-

6.5.3. Focal segmental glomerulosclerosis

Sixty-seven (28.4%) of all black children, 59 (20.6%) of all Indian children and 10 (7.4%) of all coloured patients had focal segmental glomerulosclerosis (FSGS) (Table XIII). At the most recent assessment, 10 (7.4%) were in complete remission, 96 (70.6%) in partial remission, 25 (18.4%) had multiple relapses and 5 (3.6%) had unremitting relapse. Only six (4.4%) patients were SS. There were no differences in steroid response or outcome in the different population groups. The ages of onset was 5 – 7 years in all three races; 82 (60.3%) were males.

6.5.4. Hepatitis B virus-associated membranous nephropathy

Membranous nephropathy associated with hepatitis B virus infection was found exclusively in 81 (34.3%) black patients. None was given steroids or cytotoxics. All patients were in partial remission, with relapses occurring occasionally. Concomitant biochemical evidence of active hepatitis was found only in three (3.7%) patients. Hepatitis 'e' antigen was present in 50 (61.7%) patients. The ages of onset was 6 – 8 years; 51 (63%) were males.

6.5.5. Idiopathic membranous nephropathy

Idiopathic membranous nephropathy was found in 13 (5.5%) of the black, seven (2.4%) of the Indian and 6 (26.1%) of the coloured patients; 10 (38.5%) showed an initial response to steroids, but six of these patients subsequently relapsed and developed SR; 10 (38.5%) were SR ab initio and in six (23.1%) the response to steroids was unknown because of loss to follow-up. No differences were noted in

the different population groups. The ages of onset was 5 – 8 years; 12 (46.1%) were males.

6.5.6. Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis was found in 12 (5.1%) of the black and nine (3.1%) of the Indian patients; 19 (90.5%) were SR and 2 patients showed an initial response to steroids but subsequently relapsed and developed SR; 16 (76.2%) were in partial remission at the last clinic visit with five (23.8%) in unremitting relapse. No differences were noted between black and Indian patients. The ages of onset was 6 – 8 years; 10 (47.6%) were males.

6.5.7. Diffuse Mesangial Proliferative Glomerulonephritis

Diffuse mesangial proliferative glomerulonephritis was found in 10 (4.2%) of the black, six (2.1%) of the Indian and two (8.7%) of the coloured patient; four (22.2%) were SS and 14 (77.8%) SR. The ages of onset was 2 –5 years; 11 (61.1%) were males.

6.5.8. Mild (Focal) Mesangial Proliferative Glomerulonephritis

Seven (3.0%) of all black and one (0.3%) of the Indian patients had a mild mesangial proliferative glomerulonephritis; two were SS, four SR and in two the response was undetermined because of the loss to follow-up. At the last clinic visit, two patients were in complete remission and four were in partial remission. The ages of onset was 2 –5 years; three (37.5%) were males.

6.5.9. Miscellaneous

Twenty-two patients had histological lesions that were unclassified; 15 (68.2%) were males.

6.6. Management

All patients presenting in relapse were managed with salt restriction and diuretic therapy. Increased protein intake was encouraged when renal function was normal. Severe exacerbation of disease was managed by albumin infusions, together with concomitant administration of intravenous furosemide (2mg/kg) half way through the infusion. Those patients failing to respond to three daily doses of albumin infusions and diuretics were put into alternate-day infusions of plasma or dextran 70. Patients with compromised respiratory function had controlled abdominal and pleural paracentesis to improve breathing. All patients, except those with positive serology for hepatitis B or evidence of recent post-streptococcal glomerulonephritis, received a trial of steroids (prednisone 2mg/kg for 4 weeks), which were then tapered off gradually over 2 months. The steroid non-responders were managed with alternative therapy using various combinations of cyclophosphamide, methylprednisolone, chlorambucil, cyclosporin A and nitrogen mustard. Pneumococcal vaccine was administered to all patients over the age of 2 years.

6.7. Complications

These occurred with almost equal frequency in all three racial groups. One or more episodes of peritonitis occurred in 46 patients (7.2%). Cultures were positive in 16 patients, 9 having Gram-positive organisms and 7 Gram-negative organisms. Cushingoid features following steroid therapy developed in 44 patients with 12 patients having persistent hypertension requiring anti-hypertensive therapy. Six patients were suspected of having urinary tract infection on dipstick analysis; this was confirmed in only one patient in whom *Escherichia coli* was cultured. Additional complications included pulmonary tuberculosis (3 patients), bronchopneumonia (12 patients), hepatitis (3 patients), and asthma (4 patients). Other complications of therapy included leucopenia (8 patients), nail discoloration (3 patients), alopecia (4 patients), anaemia (4 patients), and early sub-capsular cataracts (2 patients); one patient had severe dyspepsia following prolonged steroid and cytotoxic therapy, but with no evidence of peptic ulceration on barium swallow or endoscopy.

6.8. Outcome

Twenty (3.1%) patients died: 17 deaths were due to overwhelming sepsis, two died from uncontrolled hypertension and cardiac failure, and one from unrelated cause. Four patients were transplanted, three with FSGS and one with membranoproliferative glomerulonephritis. Twenty-one patients had evidence of compromised renal function (6 requiring dialysis): seven of 136 (5.1%) FSGS, 1 of 81 (1.2%) hepatitis B virus-associated membranous nephropathy, three of 26 (11.5%) idiopathic membranous nephropathy, four of 21 (19%)

membranoproliferative glomerulonephritis and three of 18 (16.7%) diffuse mesangial proliferative glomerulonephritis which progressed to chronic renal failure or end-stage renal disease. Two patients had impaired renal function and one had chronic renal insufficiency.

6.9 Discussion

In this re-examination of NS among children in South Africa, a disorder whose broad outlines we have drawn 20 years ago, we now describe some new aspects which have an important bearing on diagnosis, management and pathogenesis. The main findings relate to differences in population-derived determinants of NS, age of onset, histological groups, response to steroids and outcome.

At the time we first reported on NS in South Africa, the prevalence of malaria was relatively low (1 –10 per 100,000 population) among the communities from which our patients come [14]. Malaria has been on the increase in South Africa and the current prevalence rate in our province is about 64 per 100,000. Despite this epidemic, we still do not see malarial nephropathy, a situation similar to that prevailing in the 1970s. In addition, human immunodeficiency virus (HIV) infection, following vertical transmission, has now reached epidemic proportions in KwaZulu/Natal. Nonetheless, HIV-associated nephropathy presenting as NS has not as yet been seen in our patients.

The NS in South Africa differs from that of other regions in the African continent. *Schistosoma haematobium* and *mansoni*, together with *Salmonella typhi*, have been implicated as the major cause of NS in Egypt, whilst a post-infectious type of proliferative nephropathy is seen in Tunisia (northern Africa) [15]. *Onchocerca* is considered the major causative agent in the Cameroons, whilst in the malaria-

infested regions of Kenya, Nigeria, Uganda and parts of Ghana, *Plasmodium malariae* is the most frequently encountered aetiological agent [16]. In Zimbabwe (southern Africa), a diffuse proliferative exudative glomerulonephritis is most commonly seen, following infection by group A β -haemolytic streptococci [5, 16]. In those countries or regions where there is a high incidence of hepatitis B chronic infection, there is a strong association between hepatitis B carriage and membranous nephropathy [17]. As there have been no further reports recently of any changing pattern of disease from these regions, one may assume that the clinicopathological picture has remained unchanged.

We have once again demonstrated that MCNS or SSNS in Indian patients resembles the disorder seen among children in most parts of the world. The lower percentage of 68.2% in this report is probably due to selection bias, as fewer patients with uncomplicated SS disease are now being referred to tertiary centres such as ours. MCNS remains an especially interesting and uncommon entity in black children: it accounted for 13.5% of all patients in this population group. However, there was discordance between histology and the prediction of steroid sensitivity. Of the 32 black children with biopsy-proven MCNS, 18 were SR; the remaining 14 children, together with another 2 unbiopsied patients who were characterised by a typical clinical course, were SS. Accordingly, only 6.8% of black children could be considered as having typical SS-MCNS; in our earlier reports we have doubted the existence of this type of NS. Among the small number of coloured children studied, 5 of 23 (21.7%) had SSNS or biopsy-proven

SS-MCNS. The therapeutic implications of this are that renal biopsy may be a pre-requisite for the proper management of NS in black and coloured patients.

From being a marginal therapeutic issue in our clinic, FSGS has now become the single most difficult management problem; this is in line with many other paediatric nephrology units, where it accounts for about 10% of children with NS [18-20]. It is the second commonest variety of NS, with a prevalence of about 25%, much higher than the previously reported figures of 3.7% (1976) and 3.9% (1981) from our center [2,3]. The increased incidence of FSGS may be real or an epiphenomenon. A recent review of FSGS supports the view that there has been an authentic increase in FSGS, as documented in two large series from geographically distinct practices, in Chicago and New York [21]. The same process may be occurring in South Africa. However the increase may be due to better pathological identification of biopsy tissue, more biopsies being performed, better access by blacks to health services, etc. FSGS in black African children in Johannesburg has been associated with tuberculosis, with deleterious effects on renal function [22]. Our results do not support the notion that tuberculosis is commonly associated with FSGS in black African children. The peak age of presentation was 5-7 years, with more females than males in all races, differing from data published previously [14]. Only 6 (4.4%) patients responded to the steroid therapy and were in remission for more than a year, much lower than the 23.3% steroid response reported by the World Health Organisation [11]. Seven

(5.1%) patients with FSGS have compromised renal function; all are steroid resistant. Steroid therapy consisted of prednisone in a dose of 2mg/kg body weight (maximum dose 60mg) given daily for six weeks, then alternate days for six weeks and tailed off over a period of two and half months.

Approximately 25-30% of patients with FSGS reach renal failure in five and 30-40% in ten years [23]. The prognosis been greatly improved in industrialised countries by the aggressive use of cytotoxics in combination with steroids. We have had some success using the regimen in the SR-FSGS with failing renal function (unpublished data).

Within a few years of embarking on this project we recognised that membranous nephropathy were frequent in black children; however, for a number of reasons, we failed to detect the close relationship with hepatitis B virus. This association has now been firmly established in many countries where the virus is endemic and our results add to this body of information [8,17]. Indeed because of the epidemiology of hepatitis B virus in KwaZulu/Natal and the rarity of idiopathic membranous nephropathy, we are able to predict fairly accurately the underlying nephropathy in any black child with NS who is a carrier of hepatitis B. Just over 86% of children with membranous nephropathy were hepatitis B carriers. Membranous nephropathy was the commonest histopathological type among

blacks, accounting for about 40% of all biopsied cases, this contrasts with the 2.4% prevalence in Indians (which is closer to 2%-5% reported in western countries) [24] and 26.1% coloureds. The incidence of hepatitis B urine carriage is extremely low in Indians, and therefore it is not surprising that hepatitis B-associated membranous nephropathy was not detected in these patients. Unlike the patients described here, children with hepatitis B-associated membranous nephropathy seen in Johannesburg, South Africa, have biochemical evidence of liver damage and hypocomplementaemia [25]. None of the patients with hepatitis B-associated disease was treated with steroids, and its use in the idiopathic form of the disease was less than encouraging. As reported in our other studies, the majority of patients had a benign course, however, in a small number of patients, there was progression to chronic renal failure [26].

Twenty-one (3.8%) of all patients had membranoproliferative glomerulonephritis on histology, with 15 (71.4%) of these being hypocomplementaemic, similar to findings reported from other regions. Hypocomplementaemia was noted in 77% of patients but was found to be unrelated to the clinical course or prognosis of this disease [27]. In our patients there was an almost equal sex incidence, which contrasts with children from temperate countries where there is a marked female preponderance. Although hypertension is a common complication of this condition, only 2 of our patients required specific antihypertensive therapy. Compromised renal function was found in 3 (14.3%) patients at presentation.

Hypertension and renal failure occur in about a third of patients from western countries [27]. The response to steroids and immunosuppressive therapy was unimpressive as has been reported by many others.

Mesangial proliferative glomerulonephritis occasionally occurs during the healing phase of acute post-streptococcal glomerulonephritis, and as the latter is one of the commonest renal problems in the paediatric wards at King Edward VIII Hospital, it might be expected to account for the majority of patients in this group. However, as a majority of such patients remit spontaneously, very few are subjected to renal biopsy. Biopsy was undertaken only in those who had persistence of disease for more than a year; it is this group that has been included in the present study. Although streptococcal infection was purportedly the offending agent in the majority cases, it is well known that several other infectious agents, IgA glomerulonephritis and systemic diseases may produce a similar histological pattern [4,5]. Of those patients with diffuse and mild mesangial proliferative glomerulonephritis, 22% and 25% were SS respectively: these are much lower than reported by the International Study of Kidney Diseases in Children, where 56.6% and 85.2% of patients were SS, respectively [11].

The juxta-position of different population groups in South Africa, each with its own background and environmental experiences, has made it possible for us to study the spectrum of NS in one centre. It is clear from the distribution of the various

histological types of NS within these population groups, that there are differences in frequency of clinico-pathological types and course of the disease when compared with other regions in Africa and the rest of the world. Whilst the disease in the Indian population parallels that of developed countries, with MCNS predominating, black children are different, with only a small percentage having MCNS. Not all of the latter respond to steroids. Nonetheless, typical SS-MCNS is now well documented in blacks and only a small percentage have a favorable outcome. Hepatitis B-associated membranous nephropathy is the commonest variety of NS in blacks, and the strong correlation between hepatitis B carriage and membranous nephropathy in our region makes prediction accurate and obviates the need for renal biopsy in these patients. FSGS is now the main management problem in both Indian and black children. Furthermore, the high prevalence of corticosteroid resistance in black children in all histological categories highlights the need for alternative efficacious therapy in the management of this disease. Coloured patients comprised only a small group of these patients and thus the pattern of NS seen may be poorly representative of this population group. However, it could appear they have a pattern of disease that is in intermediate between that seen in Indian and white patients and that seen in blacks.

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CHAPTER 7

NATURAL HISTORY OF HEPATITIS B ASSOCIATED NEPHROPATHY IN BLACK SOUTH AFRICAN CHILDREN

NATURAL HISTORY OF HEPATITIS B ASSOCIATED NEPHROPATHY IN BLACK SOUTH AFRICAN CHILDREN

7.1. Introduction

In the southern African continent the prevalence of HBV-associated nephropathy appears to be higher than the rest of the continent [1]. HBV-associated nephropathy is therefore, not unexpectedly, the commonest cause of nephrotic syndrome among black patients in KwaZulu/Natal. Membranous nephropathy is the commonest histological type reported [2]. There have been no other large studies of this condition amongst black children in Africa. This study delineates the spectrum of HBV nephropathy, as well as its clinical presentation, laboratory findings, and outcome.

7.2. Patients and Methods

7.2.1. Patients

One hundred and thirty-three black children with HBV-associated nephropathy attending the paediatric renal clinic at the King Edward VIII Hospital, Durban, over a twenty one-year period (1976 - 1996) were included in the study. Patients were classified into 3 HBV-related categories: biopsy-proven membranous nephrotic syndrome (HBVMN), those with histological findings other than MN, and those with HBV-positive serology but who were not biopsied. The patients with HBVMN were compared to a group of black patients with biopsy-proven idiopathic MN (IMN) prior to therapy.

7.2.2. Methods

All patients were investigated at initial presentation. Children with other secondary causes of nephrotic syndrome were excluded. In particular, HBV antigens (HBsAg and HBeAg) were measured using radioimmunoassay (Austria II Ansab Abbott HBeAg: Abbott Laboratories, Chicago, IL, USA) up to 1991 and subsequently by second-generation enzyme-linked immunosorbent (ELISA) assay (Abbott Laboratories, Chicago, IL, USA). None of the patients were tested for hepatitis C virus infection. In addition, full blood count, serum complement and protein electrophoresis, cholesterol and triglyceride levels, and liver function tests (including liver enzymes) were performed. Renal function was assessed by blood urea and creatinine levels, and estimation of the glomerular filtration rate using a creatinine transformation formula [3]. Renal ultrasound was normal in all patients.

Renal biopsy was undertaken in 93 patients with HBV-associated nephropathy and in all patients with IMN.

Light microscopy, electron microscopy, and immunofluorescence was used to evaluate biopsy material in the Department of Anatomical Pathology at the University of Natal, Durban, with all the clinical details available, using standard criteria as described previously.

7.2.3 Statistical analysis

Statistical analysis was done in consultation with the Institute of Biostatistics of the Medical Research Council of South Africa. Categorical data was compared using the Chi-squared test and Fishers exact test where appropriate. Quantitative data was compared using the Student's t-test, depending on the equality of the variances of the two groups being compared.

7.3. Treatment

During the period of study none of the patients with HBV-associated nephropathy was treated with steroids or interferon. Albumin infusions and diuretics were needed to control oedema and ascites. Patients with persistent oedema were maintained on thiazide diuretics and spironolactone. An angiotensin converting enzyme inhibitor (enalapril) was used for the control of proteinuria and hypertension. Patients with MN were all given a course of steroids for a minimum period of 8 weeks (2mg/kg up to a maximum of 60mg).

7.4. Results

The demographic data, clinical features, HBV serological status and biochemical findings of each of the 3 HBV related categories are shown in Table XV, XVI.

Table XV: Clinical features, hepatitis B virus status and biochemical findings of patients with hepatitis B virus-associated nephropathy.

	Hepatitis B virus membranous nephropathy	Hepatitis B virus associated non- membranous nephropathy
Number	70	23
<i>Demographic Data</i>		
Mean age of onset (years)	7.1 (1-8.8)	7.5 (0.75-10.1)
Mean period of follow-up (years)	3.4 (1-11)	2.6 (1-8)
Gender: Males	54 (77.1%)	17 (73.9%)
Females	16 (22.9%)	6 (26.1%)
<i>HBV status</i>		
HbsAg & HbeAg	49 (70.0%)	6 (26.1%)
HbsAg only	21 (30.0%)	17 (73.9%)
<i>Clinical Features</i>		
Hypertension on onset	7 (10.3%)	4 (17.4%)
Persistent hypertension	23 (32.9%)	2 (8.7%)
Liver disease		
CAH*	1 (1.4%)	-
CPH*	2 (2.8%)	-
Asymptomatic rise in liver enzymes	7 (10.0%)	1 (4.3%)

CAH = chronic active hepatitis

CPH = chronic persistent hepatitis

Table XVI: Biochemical findings of patients with HBV-associated nephropathy

	HBV membranous nephropathy	HBV-associated non-membranous nephropathy
<i>Low complement</i>		
C3 (0.77-1.43 g/l)	33 (47.1%)	6 (26.0%)
C4 (0.07-0.40 g/l)	8 (11.4%)	1 (4.3%)
<i>Lipid Profile</i>		
Raised cholesterol (3.20-4.40 mmol/l)	65 (92.9%)	23 (100.00%)
Raised triglycerides (0.4-1.3 mmol/l)	55 (78.6%)	23 (100.00%)

7.4.1. Hepatitis B Virus-associated Membranous Nephropathy

There were 70 patients in this group. Other diseases detected at presentation or during the course of the illness included bilharziasis (*Schistosoma haematobium*) 6(4.5%); chickenpox 1(0.7%), cholera 1(0.7%), pulmonary tuberculosis 3(2.3%), typhoid 1(0.7%), cardiomyopathy 1(0.7%), and cerebrovascular accident 1(0.7%). One patient had clinical peritonitis (culture-negative).

Eighteen patients, 3 with biopsy-proven liver disease, 7 with an asymptomatic rise in liver enzymes, and eight with clinical evidence of liver disease who were not biopsied, had evidence of the co-existence of liver disease and HBVMN. Clinical and histological evidence of liver disease was found in three patients; 23 had only an asymptomatic rise in their liver enzymes [alanine aminotransferase (ALT)].

The mean age of disease onset was 86 months; 46 patients were positive for both HBsAg and HBeAg; 33 patients had hypocomplementemia (low C3). At last hospital visit, 12 patients were in remission, 38 in partial remission, and 20 in relapse; 65 patients had normal renal function, one impaired renal function, three chronic renal insufficiency, and one patient had progressed to end-stage renal disease.

After a period of 12 months, 10 to 46 patients (21.7%) cleared both HBsAg and HBeAg, whilst 16(34.8%) had cleared only HBeAg. The mean period for the

clearance of both HBsAg and HBeAg in the ten patients was 32 months (range 12 to 72 months), whilst that for HBeAg in the 16 patients was 26 months (range 12 to 56 months). Twenty-four patients (34.3%) were lost to follow-up after 12 months.

At the last hospital visit 12 patients (17.1%) (10 of whom had cleared both HbsAg and HbeAg and 2 HbeAg only) were in remission. Of the remaining 58 patients (34 of whom were followed up for more than 12 months), all showed some degree of improvement (assessment by disappearance of oedema and decreasing proteinuria in dipstick), and at last visit 38 (54.3%) were in partial remission, and 20 (28.6%) had relapsed (Table XVII). Assessment of renal function at the last visit showed that 65 patients (92.9%) had normal renal function, one (1.4%) impaired renal function; 3 (4.3%) chronic renal insufficiency, and one (1.4%) end stage renal disease (Table XVIII).

Table XVII: Renal outcome in patients with HBV-associated membranous nephropathy at last hospital visit: proteinuria

	Remission	Partial Remission	Persistent nephrotic syndrome
Persistent HBsAg, HBeAg		19	17
Persistent HBsAg only		5	3
Cleared HBeAg	2	14	
Cleared HBsAg and HBsAg	10		

Table XVIII: Renal outcome in patients with HBV-associated membranous nephropathy at last hospital visit: renal function

	Normal	Impaired	CRF	ESRD
Persistent HBsAg, HBeAg	15	1	3	1
Persistent HBsAg only	24			
Cleared HBeAg	16			
Cleared HBsAg and HBsAg	10			

CRF = chronic renal failure

ESRD = end-stage renal disease

7.4.2. Hepatitis B Virus-Associated non-Membranous Nephropathy

There were 23 patients in this group. Renal histological findings in these patients were as follows: six (26.1%) had diffuse mesangial proliferative glomerulonephritis, five (21.7%) focal segmental glomerulosclerosis, five (21.7%) membranoproliferative glomerulonephritis, four (17.4%) focal mesangial proliferative glomerulonephritis, and three (13.0%) had indeterminate lesions (unclassified).

The mean age of disease onset was 78 months; six patients were positive for both HBsAg and HBeAg; eight patients had asymptomatic rise in their liver enzymes (ALT). Hypocomplementemia (low C3) was found in six patients. At their last hospital visit, eight patients were in remission, and three in relapse; 14 patients had normal renal function, three impaired renal function, and five chronic renal failure, and one patient had progressed to end-stage renal disease.

Of 11 patients followed for more than 12 months, four (36.4%) showed clearance of HBsAg and HBeAg, whilst for the clearance of HBsAg and HBeAg, whilst two (18.2%) had clearance of only HBeAg. Mean period for the clearance of HBsAg and HBeAg was 17 months (range 14-68 months), whilst that for HBeAg alone was 11 months (range 10-56 months). Twelve patients (52.2%) were lost to follow-up after 12 months.

At the last hospital visit 8 patients (34.8%) (two of whom cleared both HBsAg and HbeAg and one HbeAg only) went into remission. Of the remaining 15 patients, three (13.0%) were followed up for more than 12 months and showed improvement but subsequently relapsed. Twelve (52.1%) were in partial remission at last visit. Assessment of renal function at the last visit showed the following: 14 patients (60.9%) had normal renal function, three (13.0%) impaired renal function five (21.7%) chronic renal failure, and one (4.4%) end-stage renal disease.

7.4.3. Idiopathic and membranous nephropathy

There were 24 patients in this group. The mean age of the onset was 7.3 years (range 1.8-16.8 years) and follow-up was for a mean of 2.9 years (range 1.5-5.2 years). The clinical presentation and biochemical findings of patients with HBVMN were compared with those with IMN. The clinical presentation in the latter was similar to that of patients with HBVMN. Statistically significant biochemical and serological differences are shown in Table XIX. Serum gamma globulins, serum cholesterol, and liver enzyme aspartate aminotransferase (AST), ALT, γ -glutamyltransferase were similar in both groups. Renal status assessed at last visit showed 22 (91.7%) patients to have normal renal function and two patients (8.3%) with chronic renal failure.

Table XIX: Comparison of HBV membranous nephropathy with idiopathic membranous nephropathy

	HBV membranous nephropathy	Idiopathic membranous nephropathy	<i>P</i> value
<i>Serum lipids</i>			
Cholesterol (mmol/l)	11.07	11.71	NS
Triglycerides (mmol/l)	3.7±1.4	6.4±1.6	<i>P</i> <0.0001
<i>Serum compliment (g/l)^a</i>			
C3 (N = 0.77 – 1.43g/l)	0.85±0.28 (47.1%)	2.49±2.11 (nil)	<i>P</i> <0.007
C4 (N = 0.07 – 0.40g/l)	0.32±0.25 (11.4%)	1.87±1.5 (nil)	<i>P</i> <0.001
<i>Serum globulins (g/l)</i>			
Alpha 1	2.46±1.88	1.39±0.93	<i>P</i> <0.003
Alpha 2	14.11±5.36	7.90±5.86	<i>P</i> <0.0001
Beta	5.74±2.26	3.33±3.59	<i>P</i> <0.001
δ Globulins	6	7.22±2.65	NS
<i>Liver enzymes</i>			
AST	47.54±8.71	42.16±7.51	NS
GGT	57.85±13.21	53.77±10.10	NS

NS - not significant

AST - aspartate aminotransferase

GGT - δ glutamyl transferase

^a - percentage lower than normal in parentheses

7.4.4. Patients with Nephropathy Associated with Hepatitis B Virus Infection – not biopsied

There were 40 patients in this group. Twelve patients (30.0%) were lost to follow-up after 12 months. Eight (28.6%) of 28 patients followed up for more than 12 months showed clearance of HBsAg and HBeAg whilst five patients (17.9%) had clearance of HBeAg only. The mean period for the clearance of HBsAg and HBeAg was 17 months (range 11 to 9) whilst that for HBeAg alone was nine months (range 7 to 26).

At the last hospital visit 12 patients (23.0%) (four of whom cleared both HBsAg and HBeAg and two HBeAg only) went into remission. Of the remaining 38 patients (26 of whom were followed up for more than 12 months), at last hospital visit, 24 patients (46.1%) were in partial remission, and 16 patients (30.8%) relapsed. Assessment of renal function at the last visit showed the following: 33 patients (89.2%) had normal renal function, one (2.7%) impaired renal function, two (5.4%) chronic renal insufficiency, and one (2.7%) end-stage renal disease. The clinical and biochemical findings in the various histological groups of nephrotic patients associated with HBV infection is shown in Table XX.

Table XX: Clinical and Biochemical findings in the histological groups of nephrotic patients associated with hepatitis B virus infection

Histological Type	Membranous	Membrano-proliferative Glomerulo-Nephritis	Focal Segmental Glomerulo-sclerosis	Diffuse Mesangial Proliferative Glomerulonephritis	Focal Mesangial Proliferative Glomerulonephritis
Number	70	5	5	6	4
Mean age of onset in months	86	79	72	81	84
Chronic liver Disease	3				
Raised liver Enzymes (ALT) ^a	23	4	2	1	1
Hepatitis B status					
HbsAg & HbeAg	49	4	1	1	
HbsAg only	21	1	4	5	4
Complement					
Low C3 (0.77-1.43g/l)	33	4		1	
Low C4(0.07-0.40g/l)	8	1			

Table XX: continued

Histological Type	Membranous	Membrano-proliferative Glomerulo-Nephritis	Focal Segmental Glomerulo-sclerosis	Diffuse Mesangial Proliferative Glomerulonephritis	Focal Mesangial Proliferative Glomerulonephritis
Renal function at last visit					
Normal	65	2	1	5	4
Impaired	1	1	1	1	
CRI*	3				
CRF**		2	2		
ESRD***	1		1		
Disease status at last visit					
Remission	12	1	1	4	1
Partial Remission	38	3	3	2	2
Relapse	20	1	1		

* CRI = Chronic Renal Insufficiency

** CRF = Chronic Renal Failure

*** ESRD = End Stage Renal Disease

α ALT = Asparate alanine transferase

7.5. Discussion

The current evidence strongly suggests that MN is causally linked to HBV and the results obtained here are consistent with this interpretation [2,4]. HBV infection was detected with a variety of other forms of glomerular injury and occurred in 17.3% of our children with HBV and nephrotic syndrome. Histological types other than MN have been reported [5-7]. However the presence of HBV antigens in histological groups other than HBVMN is possibly fortuitous, especially in the non-immune complex group, given the low frequency of HBV in patients with histological types other than membranous seen in our unit, despite the high prevalence rates of HBV infection in the community. Studies from Africa have previously reported histological changes other than membranous in children with HBV, similar to that seen in our series; the strongest association being with membranoproliferative glomerulonephritis [5,6,8,9].

IMN comprises a small minority (1.0%) of nephrotic syndrome of childhood in industrialised countries [10]; in South African black children it accounts for 5.5% of cases [11]. It was of interest to compare the group with IMN to that with HBMV. Previous studies comparing these two groups showed no differences in clinical presentation except for age; patients with HBVMN presenting at a younger age [8]. However, significant biochemical differences in serum cholesterol, complement (C3), and liver enzymes (serum ALT and AST) levels were noted in a study from South Africa [12]. In our study, substantial biochemical differences between serum triglycerides, complement, and globulin levels were also noted,

although the clinical features were similar. The lower complement levels in children with HBVMN probably reflect the sequence of active replicating HBV, antibody production, formation of circulating immune complexes, and consequent consumption of complement proteins. Complement activation by circulating HBV containing immune complexes, with glomerular deposition, has been postulated as the pathogenetic mechanism resulting in accumulation of subepithelial deposits [13]. C3 and C4 levels may be low in patients with HBVMN, and is seen in up to 64% of cases in most studies [4,12]. In our study C3 and C4 levels were low in only 47.1% and 11,4% of patients respectively. However complement levels in HBV-associated nephropathy may vary due to fluctuations in the level of the various hepatitis B virus antigens, which combine with complement to form circulating immune complexes [4]. Fluctuating levels of C3, C4 and properdin factor B concentration have been reported in HBVMN and has also been postulated to be due to complement activation and degradation both by classic and alternative pathways [13]. The lower plasma triglycerides in the HBVMN group compared to those with IMN may represent sub-clinical chronic hepatitis in the former, restricting the hyperlipaemic response [14]. The higher serum globulins in HBVMN may represent ongoing immunological stimulation due to persistence of HBV infection.

The incidence of biopsy-proven liver disease or asymptomatic rise in liver enzymes in our patients was low. Underlying chronic hepatitis may be present despite virtually normal liver enzymes [9,10]. In most studies of HNVN, despite

the absence of clinical signs of hepatitis at presentation, liver function tests are usually mildly abnormal, in contrast to IMN, suggesting hepatocyte injury in HBV nephropathy [15-18]. In our patients, however, transaminases were similar in both HBVMN and IMN.

Amelioration of nephrotic syndrome is often accompanied by the spontaneous clearance of HBV antigens, especially HBeAg [8,10,15]. In the study from Cape Town, 37 of 71 patients (52.1%) with HBVMN went into remission, associated with HBeAg clearance in 33 (89.2%) after 90 months [8]. The average time of clearance of HBeAg to remission was five months. It is likely that the shorter the period of follow-up in our study accounts for the lower rate of HBV antigen clearance and improvement in their nephrotic syndrome, although the large number of patients lost to follow-up certainly limits such a conclusion. As the spontaneous elimination of HBeAg precedes that of HBsAg [19], it is possible that HBVMN patients with both HBsAg and HBeAg were at an earlier stage of HBV infection than those with only HBsAg. No significant chemical or biochemical differences were noted between patients having both HBsAg and HBeAg compared with those having only HBsAg.

In conclusion, this report describes the features of HBVMN in a large number of black South African children. Co-existing liver diseases was found in a quarter of

the patients with HBVMN and hypocomplementemia (low C3) in about half of the patients. The prognosis is generally favourable, with elimination of HBV antigens from the serum and spontaneous disappearance of proteinuria, similar to reports from other population groups. We also show that HBVMN is clinically indistinguishable from IMN, although there are unexplained biochemical differences between the two groups. Histological categories of nephrotic syndrome other than MN in which HBV antigens were present are reported, some of which may be fortuitous rather than casual.

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CHAPTER 8

CLUSTERING OF HEPATITIS B VIRUS IN HOUSEHOLDS OF CHILDREN WITH HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

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8.1 Introduction

The average prevalence of HBV carriage in Africa is 10.4%, although rates vary in different countries and in different ethnic groups within regions. [1-5]. It is estimated that 98% of black Africans are infected with the virus at some stage of their life, and in South Africa the disease is hyperendemic in the black population [2,3

Clustering of HBV infection among family members and household contacts has been recognised in many parts of the world where HBV is endemic [1,5,6,7,8,9,10]. In all these studies, household clustering was identified on investigation of the background of index-carriers recruited from the general population, patients with acute hepatitis, from institutions or from blood donor banks. [2,1,5,9,11]. In studies from the Far East carrier rates have been shown to be higher among siblings and offspring of index female carriers, with intense clustering within families, making vertical transmission the most likely mode of transmission. [12,13,14,15,16]. In a study conducted 12 years ago among black children in KwaZulu/Natal, the prevalence of HBV carriage in family members and household contacts of these index-carrier children was 20%. One of the most important findings from this study was that the degree of clustering was directly influenced by the choice of the index case: clustering was highest in homes of HBV carriers (20%) and least among uninfected children (3%) [5].

Two of the largest studies of HBVMN, including one from our clinic, have been reported from South Africa [17,18]. As HBVMN is a less frequent outcome compared to the other consequences of HBV infection, and as the development of this probably differs from the pathogenesis of liver disorders [19], we set out to investigate the clustering of HBV in the family members and household contacts of index HBVMN children. The hypothesis we wished to test was that the intensity of clustering in the homes of index children with HBVMN would be higher than previous rates where asymptomatic carriers of HBV were employed as index cases.

8.2 Patients and Methods

8.2.1. Patients (Index Cases)

Twenty-eight black patients, age range 2 to 13 years, with biopsy-proven HBVMN served as the index cases. One hundred and seventy-seven family members and household contacts of these index cases comprised the study group.

Index cases were recruited over a 3 year period (1995 to 1997) from the Renal Clinic at the King Edward VIII Hospital, Durban, which serves as the tertiary referral centre for the region of KwaZulu/Natal, South Africa. Nephrotic syndrome in the index cases was diagnosed in accordance with criteria used by the International Study for Kidney Diseases in Children [20]. Clinical examination and appropriate investigations were done to exclude other secondary causes of nephrotic syndrome. The features of these cases have been described in a previous publication [17].

8.2.1.1 Family Members and Household Contacts

The family members and household contacts in the household study group (family members and household contacts) were all black. A careful note was made of the relationship of each member of the study group to the index case. Hepatitis B status was determined using third generation enzyme linked immunosorbent assay ELISA (Auszyme^R Monoclonal, Abbott Laboratories, Chicago, IL, USA). In addition one hundred and seventy-five (89.7%), family members and household contacts had determination of HBV DNA by slot-blot hybridisation (SBH) and nested polymerase chain reaction (PCR).

8.2.1.2. Family Relationships

It was difficult to assess precisely the impact of household relationships on HBV status in index-carrier children with HBVMN because family units in black South African families in many instances are disrupted, often as a result of economic necessities and the socio-political policies in the apartheid era. Deviations from the usual approach to a family study were therefore essential. In several households, there were half-siblings with different biological fathers and the index child's present 'role father' was not his/her biological father. It is possible that the data collected will not truly reflect the extent of this phenomenon, although all attempts were made to be as accurate as possible.

8.2.2. Methods

The methods for HBV detection are discussed in chapter 5.

8.3. Classification of HBV Status of Family Members and Household Contacts

Family members and household contacts were classified as carriers, exposed, infected or negative according to their HBV status (see definitions on pages xlix-liii). The identification of the HBV status of family members and household contacts according to the different methods used is shown in Table XXI. There is increased sensitivity of detecting HBV carriage when all three tests are combined ($P = 0.001$).

Table XXI: HBV status of family members and household contacts of index carrier children with HBVMN using ELISA, SBH and nested PCR.

HBV Status	ELISA only	ELISA + SBH	ELISA + SBH + PCR	P value
HBV carriers	46 (26.0%)	54 (30.5%)	73 (41.2%)	0.001
HBV exposed	51 (28.8%)	45 (25.4%)	36 (20.3%)	0.13
HBV infection	97 (54.8%)	99 (55.9%)	109 (61.6%)	0.49
HBV negative	80 (45.2%)	78 (44.1)	68 (38.4%)	0.005

SBH - Slot-blot hybridisation

PCR - Polymerase chain reaction

8.4. Statistical Analysis

Continuous data (age) was compared using analysis of variance. Categorical data (hepatitis B status, gender and family relationships) were compared by χ^2 – test (Fisher’s exact test was used where appropriate). Strength of the associations was assessed using relative risks

8.5. Results

8.5.1. Characteristics of Index Cases

Seventeen (61%) of the 28 index cases were of rural origin. Twenty-four (86%) were males. The mean age at presentation was 8 ± 3.3 years (range 2 to 16). The average family size was 7 ± 3 family members (range 2 to 13). None of the index cases had a history of any other major illnesses. Three (11%) were found to be hypertensive and 12 (43%) to have hypocomplementaemia (low C3). Eight (29%) had an asymptomatic rise in the liver enzymes (alanine and aspartate amino transferase and gamma glutamyl transpeptidase). None had clinical evidence of chronic liver disease. All except one had normal renal function. Twenty-five (90%) of the index cases were HBeAg positive. All were positive for HBV DNA.

8.5.2. Characteristics of Family Members and Household Contacts

One hundred and forty-five (82%) of the 177 family members and household contacts were of rural origin. Eighty-eight (50%) were males. One hundred and thirty-five (76%) were family members and 42 (24%) were household contacts. The mean age at testing was 21.2 years ± 14.4 (range 0.5 to 71 years). Twenty-three (13.0%) had hypertension. One had well-controlled non-insulin dependent diabetes mellitus and one had a past history of tuberculosis. Twenty (11%) had an asymptomatic rise in the liver enzymes; none had clinical evidence of co-existing liver disease. All had normal renal function as assessed by blood urea and serum creatinine levels and estimation of the glomerular filtration rate using the creatinine transformation formula [21].

8.5.3. Categorisation of Family Members and Household Contacts according to their Hepatitis B Virus Status.

Seventy-three (41%) of 177 family members and household contacts were carriers; 36(20%) were exposed and 68(38%) were negative. Therefore 109 (62%) were infected. The gender distribution of family members and household contacts according to their HBV status is shown in Fig. 23.

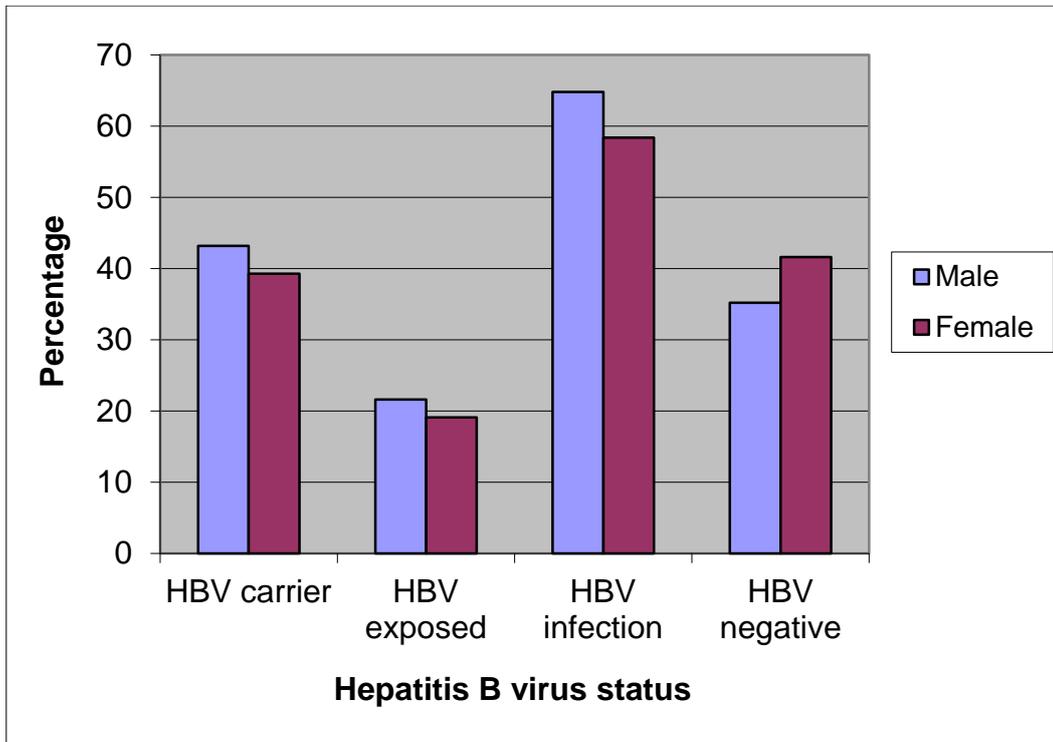


Figure 23: Gender distribution of family members and household contacts according to hepatitis B virus status

Carriers [n =73]

Thirty-eight (52.0%) were males. Sixty-five (89.0%) were family members and 8 (11%) were household contacts. Sixteen (22%) were HBeAg positive. Thirteen (81%) were males, fourteen of these (87%) were family members of whom 12 (86%) were nuclear family members. None of the parents was HBeAg positive. Of the 12 siblings who were HBeAg positive, ten (83%) were males.

Table XXII shows the HBV status of family members and household contacts according to age groups. In family members and household contacts with HBV carriage the mean age was 20 years (range 1 to 60). There was no peak age for HBV carriage in any of the age groups studied. The mean age of family members and household contacts with HBV carriage did not differ significantly from those who were HBV exposed or HBV negative. When HBV carriage in family members was compared to that in the household contacts, family members had a significantly higher rate of HBV carriage in the age groups over twenty years, RR 3.2 (95% confidence limits (CI), 1.1 to 9.3). In those below 20 years there were no significant differences in the prevalence of HBV carriage between family members and household contacts. However in each of the age groups, HBV carriage was higher in family members than in household contacts although this was not statistically significantly different ($P = 0.72$).

Table XXII: Categorisation of family members and household contacts of children with HBVMN by HBV status and age groups.

Age	Carrier	Exposed	Infected	Negative	Total
0 – 10	24 (39.3%)	5 (8.2%)	29 (47.5%)	32 (52.5%)	61
11 – 20	20 (44.4%)	15 (33.3%)	35 (77.8%)	10 (22.2%)	45
21 – 40	21 (42.0%)	8 (16.0%)	29 (58.0%)	21 (42.0%)	50
> 40	8 (38.1%)	8 (38.1%)	16 (76.2%)	5 (23.8%)	21
Total	73 (41.2%)	36 (20.3%)	109 (61.1%)	68 (38.4%)	177

Table XXIII shows the clustering effects of HBV status in the nuclear family, extended family members and household contacts of index-carrier children with HBVMN. HBV carriage in nuclear family members was not significantly different from extended family members ($P = 0.78$). However, HBV carriage rate in family members (48%) was significantly higher than in household contacts (19%), RR 2.5: (95% CI; 1.3 to 4.8, $P = 0.005$).

Exposed [n = 36]

In the group of family members and household contacts who were exposed 19 (53%) were males. Twenty-five (69%) were family members and 11 (31%) were household contacts. Of the 25 family members, 22 (88%) were nuclear family members (Table XXIII).

HBV exposed family members and household contacts had a mean age of 25 years (range 3 to 71). Twenty (56%) of exposed family members and household contacts were less than 20 years old. Family members and household contacts who were HBV exposed were significantly older than those who were HBV negative were (25 versus 17 years, $P = 0.006$). On chi-square analysis there was no significant differences in the proportion of exposed family members and household contacts who were family members (19%) or who were household contacts (26%) ($P = 0.28$), or between nuclear family members or extended family members ($P = 0.76$).

Table XXIII: Categorisation of family members and household contacts of children with HBVMN by HBV status and relationship to the index case

Relationship	Carrier	Exposed	Infected	Negative	Total
Mother	13 (52.0%)	3 (12.0%)	16 (64.0%)	9 (36.0%)	25
Father	7 (46.7%)	5 (33.3%)	12 (80.0%)	3 (20.0%)	15
Brother	21 (46.7%)	6 (13.3%)	27 (60.0%)	18 (40.0%)	45
Sister	14 (50.0%)	8 (28.6%)	22 (78.6%)	6 (21.4%)	28
Nuclear Family	55 (48.7%)	22 (19.5%)	77 (68.1%)	36 (31.9%)	113
Extended Family	10 (45.5%)	3 (13.6%)	13 (59.1%)	9 (40.9%)	22
Unrelated	8 (19.0%)	11 (26.2%)	19 (45.2%)	23 (54.8%)	42
Total	73 (41.2%)	36 (20.3%)	109 (61.1%)	68 (38.4%)	177

Infected [109]

One hundred and nine family members and household contacts had at least one marker of HBV. Fifty-seven (53%) were males. Ninety (83%) were family members and 19 (17%) were household contacts. Seventy-seven (85.6%) of the 90 family members and household contacts who had evidence of HBV infection were nuclear family members (Table XXIII).

The mean age of HBV infection was 23 years (range 1 to 60 years). Sixty-four (59%) were less than 20 years old. There were no statistically significant differences in HBV infection in the various age groups. The overall prevalence of HBV infection did not differ significantly among parents, siblings and extended family members, $P = 0.56$. However the HBV infection rate was greater in family members than in household contacts, 67% versus 45%, RR 1.5; (95% CI, 1.0 to 2.1).

Negative [n = 68]

Thirty-one (46%) of 68 family members and household contacts negative for all markers of HBV were males, 45 (66%) were family members and 23 (34%) were household contacts. Thirty-six (80%) of the 45 family members and household contacts negative for HBV were nuclear family members (Table XXIII).

HBV negative family members and household contacts had a mean age of 17 years (range 2 to 50). Forty-two (62%) of family members and household contacts were

less than 20 years old. There were no significant differences between the different age categories of HBV negative family members and household contacts. Also there was no significant differences between HBV negative family members and household contacts who were family members compared to those who were household contacts ($P = 0.12$) or between nuclear family members versus extended family members ($P = 0.41$). Brothers were more likely to be HBV negative than sisters (40% versus 21%) and mothers more likely to be negative than fathers (36 versus 20%)

8.5.4. Characteristics of Hepatitis B Virus: Genotyping

Genotyping was done in a subgroup of index cases with HBVMN and their family members and household contacts. The pre-core region of HBV DNA was extracted from 10 index cases and 14 family members and household contacts. All were genotype A and there were no major differences in this region between index cases and family members and/or household contacts.

8.5.5. Comparative studies on Household Clustering of Hepatitis B Virus

A comparison of the various studies of clustering of HBV within households is shown in Table XXIV.

Table XXIV: continued

HBV STATUS				HBV STATUS						
COUNTRY		INDEX SUBJECT			IN FAMILY MEMBERS					
HOUSEHOLD CONTACTS										
		N	TYPE	AGE	N	CAR	EXP	UNINF	N	
CAR	EXP	UNINF	TOT							
HONG KONG		240	Asympt-	9-75	731	207	315	209	N/A	
N/A	N/A	N/A	731							
1983-1984			omatic carriers							
INDIA		40	Acute	N/A	132	16	35	81	61	6
14	41	193								
			Hepatitis							
		27	HBV	N/A	89	4	13	72	14	0
			1	13	103					
			negative							
GREATER NEW		197	Blood	N/A	445	30	105	310	4	0
0	0	449								
YORK			donors							
METROPOLITAN										
			Controls	N/A	247	2	18	227	N/A	
N/A	N/A	N/A	247							
BALTIMORE		157	Asympt-	N/A	402	39	82	281	20	1
6	13	422								
1977-1978			omatic carriers							

N - number

UNINF - uninfected

CAR - carrier

N/A - not available

EXP - exposed

T - total

8.6. Discussion

We chose to study the home environment of children who had HBVMN. The advantage of preference for this type of index patient is that the factors that lead to kidney disease are probably different from those which underpin development of the other consequences of HBV infection (e.g. hepatitis, asymptomatic carrier state) [2,4]. The results obtained therefore add new information on the epidemiology of HBV infections, in particular the likely force of transmission within the homes of children with HBVMN. The most important fact to emerge from this investigation is that the intensity of clustering of HBV within the families and household contacts of index children with HBVMN is among the highest yet reported for any other types of index subject, in any other study, and in any other country. [1,2,5,6,11,22]. HBV carrier rate was almost equally high in all age-groups in the households studied and there was a significantly greater risk of HBV carriage among older family members as compared to age-group matched household contacts.

Results of the higher risk of HBV carriage among family members rather than other household contacts can be interpreted in two ways. Firstly, and probably more persuasively, the findings suggest an inborn vulnerability to carriage of HBV. While some studies have shown a genetic predisposition to the development of HBV carriage [8,9], other studies have refuted an autosomal recessive genetic susceptibility to the HBV carrier state [12,23,24]. Evidence for a genetic susceptibility

or resistance to HBV carriage and development of clinical manifestations of HBV infection has been shown in associations between different loci in the Human Leucocyte Antigen complex, although findings remain inconclusive. [25,26,27]. In a previous study of black children with NS (where the D locus antigen was not studied) from our region HLA Bw21 was strongly associated with HBVMN [26]. Therefore, although the reasons for the development of overt nephropathy in the index-carriers remain obscure, it would appear that there might be a genetic vulnerability to HBV carriage among family members of index-carrier children with HBVMN.

The second interpretation is that there are certain risk factors, behaviours or exposures, which predispose to HBV carriage, that are shared among family members and not with unrelated occupants of the same household. The majority of households in our study were of lower socioeconomic status with inadequate housing, overcrowding and close contact within households. Non-parenteral mechanisms of spread such as sharing of household items e.g. toothbrushes, razors and wash cloths have been implicated in the mechanical transmission of HBV [34]. It is possible that exposure to some of these household items is more common among family members than unrelated household contacts. Family members showed significantly higher rates of HBV carriage when compared to household contacts; whereas those who were HBV exposed or HBV negative were not more frequently represented among family members in comparison to household contacts. On balance therefore, we favour the view that heritable traits together with

environmental factors, and certain high-risk behaviors render some family members open to carriage of HBV.

A strength of this study is the advanced group of laboratory tests we employed to detect and define the HBV status of the family members and household contacts studied. To the best of our knowledge, such an array of sensitive and specific methods has not been previously used for community-based studies. We found an extra 27(15.3%) family members and household contacts to be HBV carriers, over and above the number detected with the use of conventional methods in similar projects. The observation we make that these are some of the highest rates of HBV carriage reported anywhere remains true when we compare findings using only ELISA results. Very high rates of HBV carrier state have also been reported from South East Asia, (excluding Australia, New Zealand and Japan) parts of Africa and the Middle East. [3,29,30].

Accordingly, it would appear that the development of the renal manifestations of HBV are contingent upon high density prevalence of the virus within households. This supports the notion of a marked facilitation of virus transmission and may be dependent on virus or host characteristics. In a small subset of family members and household contacts we showed that they all had genotype A. However there may be undetectable genotype variations which enhance transmissibility. Genotypic variation in the viruses which may change due to external pressure such as

treatment, may also influence viral transmissibility [31,33,32]. On the other hand host susceptibility to HBV carriage and development of clinical manifestations of HBV infection may be responsible and have been suggested above [27].

In the South African black population, the prevalence of HBV carriage is age-dependent. HBV infection and HBV carriage appear at about 0 to 2 years of age, peak at 6 to 14 years and decrease during adulthood. The figures we had in the 1988 study were 2% and 22% for HBV carriage at 0 to 2 years and 6 to 14 years respectively in urban black children, and 19% HBV carriage in the 6 to 14 year age group in rural black children. The prevalence of HBV carriage in adult blood donors was reported as 7.4%. [34]. The striking difference in this study is the stability of very high rates of HBV carriage in all age groups. HBV carriage rates in our study were comparable to that of institutionalized black children 0 to 2 year old and 6 to 14 year old who have been reported as having rates of 25% and 21% respectively. [34]. This suggests extremely vigorous HBV transmission dynamics within these households similar to that in overcrowded and unhygienic public institutions. It would be important to assess whether the capabilities of virus elimination, which occur in the general population, are compromised in these family members and household contacts.

The impact of gender on virus-host interactions in our study is minimal although reports have shown that males are more likely to be HBV carriers than females [17].

In Southern Africa, HBV carriage ratios in males compared to females vary from 1.5:1 to 3.2:1 [35,36,37]. In this study we found a much lower ratio of 1.08:1. This aspect is of more than marginal interest as HBVMN is more frequent in males than females. We have a ratio of 3.4: 1 in our clinic [17].

In brief, we have uncovered extremely high rates of HBV carriage and infection among family members and household contacts of children who develop HBVMN. There was a significantly greater risk of HBV carriage among older family members as compared to age-group matched household contacts. The observations on virus transmissibility and host susceptibility are likely to be key elements in the pathogenesis of HBVMN.

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CHAPTER 9

ASYMPTOMATIC PROTEINURIA IN RELATIVES AND CONTACTS OF CHILDREN WITH HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

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9.1. Introduction

The available information indicates that genetic, social, economic, infectious, nutritional and climatic factors make differing contributions to paediatric renal disorders [1]. Genetic causes may predominate in industrialised countries, whereas exposure to pathogens may be more important in the third world [1,2]. In order to explore this subject further we have chosen to study the settings in which hepatitis B virus associated membranous nephropathy (HBVMN) emerges.

Epidemiological, clinical and immunological evidence suggests a causal association between HBV carriage and the development of HBVMN [2,3]. The pathogenetic mechanisms by which individuals with chronic HBV infection develop MN remain enigmatic, although a number of immunological processes relating to immune complex deposition have been implicated [4,5]. Genetic factors may play a role [6]. Some evidence supports a genetic predilection for the persistence of HBV antigenaemia and for protection against HBV infection [6]. The reasons for

the diversity of clinical outcomes after HBV infection, some of which are subclinical, are not known. To date there have been no substantial reports showing strong associations between HLA types and HBVMN.

Asymptomatic proteinuria, without other evidence of renal disease, is not infrequent in adults and children [2]. The amount of protein excreted in the urine has been used as the most sensitive method to assess the significance and magnitude of renal damage [7]. Measurement of the protein: creatinine ratio on a single urine sample is accurate and provides physiologically relevant information, as it avoids collection errors [7], and above a certain threshold, the ratio is strongly indicative of glomerular basement membrane injury [2].

In this study, we report on the prevalence of asymptomatic proteinuria and the HBV carrier state among family members and household contacts of index children with HBVMN. The hypothesis we framed was that there would be a high level of clustering of HBV in the households of children with HBVMN, that HBV was causally related to the development of nephropathy, possibly on a genetic basis, and that those subjects infected with HBV would be more likely to have significant proteinuria resulting from more subtle damage to the glomerular basement membrane than that in the index cases.

9.2. Patients and Methods

9.2.1. Patients

The index cases and study groups were the same as that used in the previous study (Chapter 8). All subjects were assessed in particular for underlying renal disease by urinary dipstick analysis and culture, measurement of blood urea and serum creatinine levels and estimation of their glomerular filtration rate using the creatinine transformation formula [8]. In addition, all subjects had liver function tests (including liver enzymes) done at the time of evaluation. All were tested for hepatitis C virus, but none was screened for parasitic infections. Testing for human immunodeficiency virus was done only if there was clinical evidence of infection.

9.2.2. Methods

Urinary protein concentration in first morning urine samples was measured by the method of Pesce and Strande [9] and creatinine concentrations were measured with the Beckman creatinine analyser [10] to determine the value of the protein: creatinine ratio in first morning urine samples.

Hepatitis B status was determined as stated in Chapter 5.

9.3. Community Based Controls

One hundred and twenty-three control subjects, none of whom were family members or household contacts of children with HBVMN, had their protein: creatinine ratios determined. All controls were negative for HBV and retrovirus.

All were tested for hepatitis C virus and found to be negative for anti-HCV. None had evidence of any co-existing illnesses. None were screened for parasites.

9.4 Statistical Analysis

Continuous data was compared using analysis of variance. Categorical data were compared using Chi-square test or Fisher's exact test where appropriate. A probability of <0.05 was considered significant.

9.5. Results

9.5.1. Index Cases

Seventeen (54.8%) of the 31 index cases were of rural origin; 24 (74.2%) were males. The mean age at presentation was 8 years \pm 3.3 (range 2 to 16). The average family size was 7 \pm 3 family members (range 2 to 13). None of the patients had a history of any other major illnesses. Three (10%) were found to be hypertensive and 12 (39%) to have hypocomplementaemia (low C3). Eight (25.8%) had an asymptomatic rise in the liver enzymes (alanine and aspartate amino transferase and gamma glutamyl transpeptidase). None had evidence of acute hepatitis or chronic liver disease. All except one had normal renal function. All had a protein: creatinine ratio in the nephrotic range.

9.5.2. Classification of Index Cases into Categories of Hepatitis B Virus Status by Elisa; Slot-Blot Hybridisation and Polymerase Chain Reaction

One (3%) of the index cases was classified as category A, and 30 (97%) as category B. However, in 3 of the index cases in category B SBH and PCR were not done

9.5.3. Family Members and Household Contacts (Subjects)

One hundred and forty-five (74%) of the 195 family members and household contacts (subjects) were of rural origin; 97 (50%) were males. One hundred and fifty two (78%) were family members, and 43 (22%) were household contacts.

The mean age at testing was 20.3 years \pm 15.4 (range 0.5 to 71.2). Twenty-three (12%) had hypertension. One had well-controlled non-insulin-dependent diabetes mellitus and one a past history of tuberculosis. Twenty (10%) had an asymptomatic rise in the liver enzymes; none had evidence of acute hepatitis or chronic liver disease. All had normal renal function.

9.5.4. Classification of Family Members and Household Contacts (Subjects) by HBV Status using Elisa, Slot-Blot Hybridisation and Polymerase Chain Reaction.

Twelve (6%) subjects were classified as category A, 33 (17%) as category B, 27 (14%) as category C, 36 (19%) as category D, 68 (35%) as category E, and 19 (10%) as category F. Therefore, 72 (37%) subjects were HBV carriers (categories A, B and C). Thirty-six 36 (18%) were HBV exposed (category D). Sixty-eight

(35%) were unexposed (category E), and 19 (10%) were indeterminate for their HBV status category F; (Table XXV). Twenty-nine (93%) subjects in category B were HBV DNA positive, giving a total of 56 (29%) subjects positive for HBV DNA. SBH or PCR were not performed in two (6%) of the subjects in category B.

Table XXV: Classification of 30 subjects (family members, household contacts of index children with HBVMN) according to HBV status (using ELISA, Slot-Blot hybridization and polymerase chain reaction) and protein: creatinine ratio.

Quantitation of proteinuria by protein: creatinine ratio						
CATEGORY OF HBV STATUS	N	NORMAL	MILD	MODERATE	NEPHROTIC RANGE	NOT DONE
A (HbsAg positive only)	12 (6.2%)	8 (66.7%)	3 (25.0%)	1 (8.3%)	Nil	Nil
B (HbsA and/or Hbe and/or SBH and/or PCR positive)	33 (16.9%)	22 (66.7%)	9 (27.3%)	1 (3.0%)	1 (3.0%)	Nil
C (Anti-HBc IgG only or all markers negative by ELISA but SBH and/or PCR positive for HBVDNA)	27 (13.8%)	20 (74.1%)	1 (3.7%)	3 (11.1%)	2 (73.4%)	1 (3.7%)
D (Anti-HBc IgG positive with SBH and PCR negative)	36 (18.5%)	29 (80.6%)	2 (5.6%)	3 (8.3%)	Nil	2 (5.6%)

Table XXV: continued

Quantitation of proteinuria by protein: creatinine ratio						
CATEGORY OF HBV STATUS	N	NORMAL	MILD	MODERATE	SEVERE	NOT DONE
E (All Negative)	68 (35.0%)	43 (63.2%)	15 (22.1%)	7 (10.3%)	3 (4.4%)	Nil
F (ELISA Negative, SBH + PCR not done)	19 (9.7%)	16 (84.2%)	2 (10.5%)	Nil	Nil	1
TOTAL	195	138 (70.8%)	32 (16.8%)	15 (7.7%)	6 (3.1%)	4 (2.1%)

SBH = Slot – Blot Hybridisation

PCR = Polymerase Chain Reaction

Hepatitis B virus Carriers (Categories A, B and C)

The mean age was 20.1 (SD ± 15) years (range 2.5 to 60.0). Thirty-eight (53%) were males. Sixty four were family members, and eight were household contacts; these accounted for 42% of all family members and 19% of all household contacts ($P = 0.01$).

Hepatitis B virus Exposed (Categories C, D)

The mean age was 24,7 (SD $\pm 17\%$) years (range 3 to 71). Nineteen (53%) were males. Twenty-five were family members and 11 were household contacts; these represented 16% of all family members and 26% of all household contacts ($P = 0.25$).

Hepatitis B virus Unexposed (Category E)

The mean age was 17.1 (SD ± 14) years (range 2 to 50). Thirty-one (46%) were males. Forty-five were family members, and 23 were household contacts. These were 30% of all family members and 54% of all household contacts ($P = 0.006$).

Hepatitis B virus Indeterminate (Category F)

The mean age was 22 (SD ± 15) years (range 0.5 to 58 years). Nine (47.4%) were males. Eighteen were family members, and one was a household contact, accounting for 12% of the former and 2% of the latter ($P = 0.08$).

The odds ratios for HBV carrier status compared with HBV exposed status in the family members and household contacts was 3.5 (95% confidence limits (CL), 1.3 to 9.5) and to HBV unexposed, it was 4.1 (95% CL, 1.7 to 9.6), respectively. Family members were more predisposed to being HBV carriers and exposed than household contacts ($P = 0.01$ and $P = 0.01$) respectively.

9.5.5. Urinary Findings

Forty-five (23%) subjects had microscopic haematuria (maximum 2+ on dipstix analysis) in addition to proteinuria. Fourteen (31%) were carriers. Eight (18%) were exposed. Twenty (44%) were negative, and three (7%) had an indeterminate status for HBV. None had leucocytes, nitrites, or glucose in the urine on dipstix analysis, and all subjects had negative urinary culture.

9.5.6. Protein: Creatinine Ratio

Fifty-three (27%) subjects had abnormal proteinuria (Table XXVI). Their mean age was 14 years (range 2 to 51). Three of four children less than 2 years had a protein: creatinine ratio in the normal range, that is, less than 0.5. Twenty-seven (49%) were males. Thirty-nine were family members, and 14 were household contacts. These accounted for 26% of all family members and 33% of all household contacts. Six (11%) family members with abnormal proteinuria had an asymptomatic rise in the liver enzymes. Five (9%) family members with abnormal proteinuria were hypertensive.

9.5.7. Hepatitis B virus Status and Abnormal Proteinuria

We then analysed the results to detect any association between the different categories of HBV status and abnormal proteinuria (Table XXVI). Twenty-one (29%) of the HBV carriers, five (14%) HBV exposed, 25 (37%) HBV unexposed, and two (10%) HBV indeterminate subjects had abnormal proteinuria. There were no statistically significant differences between HBV carriers compared to HBV exposed ($P = 0.07$), HBV unexposed ($P = 0.44$) and HBV indeterminate subjects ($P = 0.14$).

Table XXVI: Comparison of normal and abnormal proteinuria according to the category of HBV Status in family members and household contacts of index children with HBVMN

Category of HBV status	Quantitation of proteinuria by protein: creatinine ratio				p value
	N	Normal	Abnormal	Not done	
CATEGORY A					
FM	11	8 (72.7%)	3 (27.3%)	Nil	p = 0.33
HC	1	Nil	1 (100%)		
TOTAL	12	8 (66.7%)	4 (33.3%)		
CATEGORY B					
FM	29	19 (65.5%)	10 (34.5%)		p = 0.9
HC	4	3 (75.0%)	1 (25.0%)	Nil	
TOTAL	33	22 (66.7%)	11 (33.3%)		
CATEGORY C					
FM	34	19 (79.2%)	5 (20.8%)	Nil	p = 0.41
HC	3	1 (33.3%)	1 (33.3%)	1 (33.3%)	
TOTAL	27	20 (74.1%)	6 (22.2%)	1 (3.7%)	
CATEGORY D					
FM	25	20 (80.0%)	3 (12.0%)	2 (8.0%)	p = 0.9
HC	11	9 (81.8%)	2 (18.2%)	Nil	
TOTAL	36	29 (80.5%)	5 (13.9%)	2 (5.6%)	
CATEGORY E					
FM	45	29 (64.4%)	16 (36.6%)		p = 0.85
HC	23	14 (60.9%)	9 (39.1%)	Nil	
TOTAL	68	43 (63.2%)	25 (36.8%)		
CATEGORY F					
FM	18	15 (83.8%)	2 (11.1%)	1 (5.6%)	p = 0.90
HC	1	1 (100%)	Nil	Nil	
TOTAL	19	16 (84.2%)	2 (10.5%)	1 (5.3%)	
GRAND TOTAL		195	138 (70.8%)	53 (27.2%)	4 (2.1%)

P Value = Comparison of abnormal proteinuria between family members and household contacts

Normal = Protein:creatinie ratio < 0.2

Abnormal = Protein:cratinine ratio > 0.2

HC = Household contact

FM = Family Member

9.5.8. Family Members and Household contacts, Hepatitis B virus Status and Abnormal Proteinuria

Analysis of subjects into those who were family members and those who were household contacts showed 39 (26%) of 152 family members and 14 (33%) of 43 household contacts to have abnormal proteinuria ($P = 0.48$).

These family members and household contacts with abnormal proteinuria were then classified into the various categories according to their HBV status (Table XXVII). Of the 39 family members with abnormal proteinuria, 18 (46%) were HBV carriers. Three (8%) were HBV exposed. Sixteen (41%) were HBV unexposed, and two (5%) were of indeterminate HBV status. Of the 14 household contacts with abnormal proteinuria three (21%) were HBV carriers, two (14%) were HBV exposed, nine (64%) were HBV unexposed and none were of HBV indeterminate status. Comparison between family members and household contacts with abnormal proteinuria among those who were HBV carriers to those who were HBV exposed ($P = 0.24$) and HBV unexposed ($P = 0.18$) showed no statistically significant differences. We next compared the different categories of HBV status according to whether they were family members with abnormal proteinuria or household contacts with abnormal proteinuria (Table XXVII). There were no statistically significant differences using chi-square analysis.

Table XXVII: Incidence of Proteinuria in Family members and Household Contacts compared to Community Based Controls.

Number	Family members and household controls contacts	Community based	P value
Normal Proteinuria			
< 5 years	16/34	30/38	p = 0.001
> 5 years	124/161	75/85	p = 0.03
Abnormal Proteinuria			
< 5 years	18/34	8/38	p = 0.01
> 5 years	37/161	10/85	p = 0.02

9.5.9. Gender, HBV Status and Abnormal Proteinuria

We compared gender differences in the various categories of HBV status for normal and abnormal proteinuria. Twenty-seven (51%) of those with abnormal proteinuria were males and 26 (49%) were females. A comparison of gender differences in subjects with abnormal proteinuria in each of the categories according to HBV status showed no statistically significant differences except in category B with females having a higher frequency of abnormal proteinuria than males ($P = 0.05$). In addition, gender had no impact on HBV status ($P = 0.44$).

9.5.10 Age, Hepatitis B virus Status, and Abnormal Proteinuria

We studied the impact of age on proteinuria in subjects in the various categories of HBV status. Children less than 5 years had a higher prevalence (53%) of abnormal proteinuria than those older than 5 years (23%) ($P = 0.008$). Age had no impact on HBV carriage ($P = 0.42$).

9.5.11. Hepatitis B virus Genotype

The precore region of HBV DNA was extracted from 10 index cases and 14 family members and household contacts. All were genotype A, and there were no major differences in this region between index cases and family members and/or household contacts.

9.5.12. Community Based Controls

One hundred and twenty-three control subjects had their protein: creatinine ratio done. All were black. Thirty-eight were less than 5 years old and 53 (43%) were

males. One hundred and five (85%) had a protein: creatinine ratio in the normal range; 13 (11%) had mild proteinuria and five (4%) moderate proteinuria. Children less than 5 years old had a higher incidence of abnormal proteinuria (21%) compared to older children and adults (12%) (Table XXV) but the difference was not statistically significant ($P = 0.22$) on a chi-square analysis (Table XXVII).

9.6. Discussion

The finding of clustering of HBV within families and household contacts in this study is not new [11]. What is new is the high prevalence rate of asymptomatic proteinuria (28%) in families and household contacts of index children with HBV nephropathy. Indeed, this is the highest prevalence of this abnormality yet reported [12-15]. The most intriguing observation, however, is the absence of any correlation among family members and household contacts between HBV markers and asymptomatic proteinuria. The dissociation between HBV carriage and proteinuria is further highlighted by the fact that although proteinuria was most frequent in children under five years, there were no significant differences in HBV carriage in either age range. These findings support a conclusion that is the converse of the hypothesis we set out to test. In the light of these unexpected results, we need to find a different explanation for the biosocial context and pathogenesis of HBVMN.

Although it is difficult to determine the exact source of infection in these children with HBVMN, it is possible that it was acquired from members within the households, given the high level of clustering. It is possible that there are certain risk factors, behaviours, or exposures within families and household contacts of children with HBVMN which predispose to the intensity of clustering of HBV in these households. As these households were from a lower socioeconomic group with inadequate facilities, this resulted in overcrowding and close contact between occupants. Risk

behaviours such as sharing of household items e.g. toothbrushes, razors and wash cloths are prevalent in these household and may contribute to mechanisms of HBV transmission [16].

In a previous study from our institution of clustering of HBV in the home backgrounds of children identified as HBV carriers, and HBV exposed and HBV unexposed individuals, the investigators detected prevalences of HBV carriage of 20%, 9% and 3% in the families and household contacts of these three groups, respectively [11]. In this study of children with HBVMN, we show that 16.9% were both HBsAg and HBeAg positive with or without HBV DNA, 6.2% had only HBsAg, and 13.8% were anti-HBc IgG positive or ELISA negative but SBH or PCR positive, that is, a total of 37% HBV carriage. Thus, the figures for HBsAg (with or without HBeAg) carriage in families and household contacts of the index HBsAg carrier children in the earlier study and the index children with HBVMN in this study are not significantly different. The population group studied is the same, but there is a 12-year interval between these reports. HBV vaccination given in the Extended Programme for Immunisation (EPI) was introduced into South Africa in April 1995 and cannot have influenced our current findings.

The techniques employed to detect HBV and proteinuria require comment. The two methods used to detect HBV DNA (SBH and PCR) enabled us to identify 15 family members and household contacts with HBsAg, only who had active viral replication

(category B), 12 children negative for HBsAg, HBeAg and anti-HBc IgG but SBH or PCR positive, and a further 15 children positive for anti-HBc IgG but SBH and/or PCR positive (category C). Measurement of protein: creatinine ratio in single urine samples is potentially more accurate than 24 hour urine collection because it avoids collection errors and may give more physiologically relevant information [2]. Thus, in the family members and household contacts of patients with HBVMN we used this non-invasive method to uncover subtle forms of underlying renal dysfunction. Renal biopsies would have been informative and would be indicated in subjects with severe proteinuria but are self-evidently unethical in these asymptomatic subjects with mild or moderate range proteinuria. Six of this study subjects had nephrotic range proteinuria, however renal biopsy was not undertaken because of failure to obtain parental consent. Ethical approval was refused for those with lesser degrees of proteinuria.

Abnormal proteinuria that is independent of HBV status in family members and household contacts is the most striking observation in this study and forms the core of the hypothesis we advance to explain our results. Central to our hypothesis is that the amounts of protein excreted in the urine of these asymptomatic subjects exceed the normal threshold and are surrogate manifestations of an underlying glomerular basement membrane disorder. Proteinuria is one of the main expressions of kidney disease [17]. Although proteinuria can occur as a result of glomerular or tubular disorders, proteinuria detected by urinary dipsticks analysis is usually indicative of disturbances in injury

to the glomerular basement membrane [18]. Physiological causes of proteinuria such as exercise, fever and high altitudes were excluded in all family members and household contacts. Durban is a seaport, and families were mostly from the coastal regions. The possibility remains that some of these family members and household contacts may have had orthostatic proteinuria. We believe this is unlikely in the majority, however, because protein excretion rates rarely exceed 1g per 24 hour in orthostatic proteinuria [19-21], and this level was detected in 70% of the family members and household contacts with proteinuria in this study. Manifestations of renal disease and renal insufficiency were not evident in any of the family members or household contacts. A number of studies have recorded the prevalence of asymptomatic proteinuria in children and adults but prevalences were much lower than that reported here. In a large study, isolated proteinuria was detected in 2.5% of children aged 8 to 15 years [22]; in adults, the incidence of isolated proteinuria in the absence of underlying renal disease varies from 0.4% to 5% in different studies [20]. Reports from the United States in children with similar amounts of protein excretion (< 99mg/dl) suggest that in the long term, although the majority of children with urinary abnormalities have either no renal disease or at most a self-limited condition, a small percentage (2%) have some form of underlying renal pathology [12]. A similar study in adults in Japan showed that about 10% of subjects with asymptomatic haematuria and/or proteinuria followed up for long periods had various forms of renal pathology [22]. Unfortunately, there are no comparable prevalence studies of proteinuria or determination of long-term outcome in the population group we studied.

The high prevalence of proteinuria in the African households studied and in community controls may be the result of exposure to common environmental antigens (helminths, bacteria, viruses, toxins, drugs) that produce mild degrees of glomerular basement membrane damage [2]. A recent hypothesis on the pathogenesis of MN suggests a predisposition in different individuals for vigorous IgG₄ responses to certain antigens (helminths, drugs etc), which may be genetic in nature [23]. It is possible that these factors may account for the increased proteinuria in household contacts although we did not specifically screen for these agents. HBV carriage and abnormal proteinuria rates were not significantly different between family members and household contacts. Accordingly, there does not appear to be a heritable component to the development of either of these two disorders. This finding corroborates the results of our earlier study [11] where HBV carriage was unrelated to family membership.

Associations between different loci on the HLA complex and susceptibility or resistance to the chronic HBV carrier state, chronic active hepatitis, and HBVMN have been reported, although the findings are not conclusive [6]. In a study of black children with nephrotic syndrome from our region, an association of MN with HLA Bw21 was detected [24]. The HLA D locus however was not studied in this group. We have done HLA studies in the index cases with HBVMN as well the family members and household contacts, which will form the basis of another report. The current evidence on genetic factors influencing outcome of HBV infection is inconclusive. Although some studies have shown a genetic

predisposition to the development of HBV carriage [25,26] other studies have refuted a genetic predisposition to the HBV carrier state [27-29]. Thus, on balance, there appears to be a complex interplay of heritable traits together with environmental factors and certain high-risk behaviours that render these children open to HBV carriage.

In brief, this study, which portrays one aspect of the biosocial context of HBVMN, describes a concentration of individuals with high prevalence of the HBV carrier state and abnormal proteinuria within the families and household contacts of children with HBVMN. We argue that this combination of findings implies an environmental or social vulnerability in the family members and household contacts of children with HBVMN to multiple antigen exposure, including HBV. Among young children exposure to multiple antigens within these households leads to glomerular basement membrane injury. Within households of the index cases of HBVMN, there is substantial further risk of HBV carriage. The operation of additional factors, especially genetic influences, on children within these settings may produce HBVMN. Given the unexpected outcome of our study, it is plausible that HBV infection is associated with HBV – induced MN, but not causally related to it.

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CHAPTER 10

CHARACTERISATION OF PROTEINURIA IN ASYMPTOMATIC FAMILY MEMBERS AND HOUSEHOLD CONTACTS OF CHILDREN WITH HEPATITIS B VIRUS ASSOCIATED MEMBRANOUS NEPHROPATHY

CHAPTER 10

CHARACTERISATION OF PROTEINURIA IN ASYMPTOMATIC FAMILY MEMBERS AND HOUSEHOLD CONTACTS OF CHILDREN WITH HEPATITIS B VIRUS ASSOCIATED MEMBRANOUS NEPHROPATHY

10.1. Introduction

Proteinuria has been recognised as a feature of renal disease for over a hundred years [1]. Although the amount of protein excreted in the urine has been used as the most sensitive method to assess the significance and magnitude of renal damage [1,2], substantial quantities of protein may be lost in asymptomatic, apparently normal subjects [3-5]. Precise urinary protein analysis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a valuable method to separate urinary proteins according to their molecular weight and provides evidence of renal disorders beyond those detected by quantitative assessment of proteinuria [6]. This method has been used to detect tubular and glomerular diseases, drug toxicity, reflux nephropathy, birth asphyxia, and insulin dependent diabetes mellitus and, in recent years, to determine the response to steroid therapy in primary nephropathies [7-9]. The presence of high molecular weight proteins such as Immunoglobulin G (IgG), transferrin, haptoglobin, and albumin suggests glomerular disease, whereas the presence of β_2 -microglobulin

(β_2M) and other low-molecular weight proteins indicates tubular dysfunction [10,11]. The speed and reproducibility of the method used to separate urinary proteins according to their molecular size has been shown to be useful even in routine clinical practice [6].

Ramjee et al found that excretion of high molecular weight proteins such as haptoglobulins and IgG served as a surrogate marker for glomerular basement damage, particularly that associated with membranous nephropathy [MN] [12].

This report explores the association between HBV carriage and patterns of proteinuria seen in family members and household contacts of children with HBVMN by using SDS-PAGE. The purpose of this study is to assess if HBV carriage in asymptomatic family members and household contacts of children with HBVMN correlates with a pattern of proteinuria suggestive of glomerular basement membrane damage, in particular, a pattern suggestive of MN.

10.2. Patients and Methods

10.2.1. Patients

The index cases and subjects (family members and household contacts) in this study were the same as those in the previous study (Chapter 8).

10.2.2. Methods

Renal disease was detected by urinary dipstick analysis and culture, measurement of blood urea and serum creatinine levels and estimation of their glomerular filtration rate using the creatinine transformation formula [13]. The methodology used to investigate the study subjects is the same as that in Chapter 9.

10.2.2.1 Sodium Dodecyl Sulphate Polyacrylamide Agarose Gel Electrophoresis (SDS Page)

All subjects had two samples of urine collected (first and second morning urine samples). The first morning sample was sent to the routine laboratory at King Edward VIII Hospital for total-protein and creatinine estimation. The second-voided morning sample of urine was collected and preserved with a few drops of 0.05% sodium azide for SDS-PAGE analysis (Chapter 5). Urine samples of protein concentration greater than 3.0 g/L were diluted to 100 to 300 mg of protein/L. Samples of protein less than 3.0 g/L were used without dilution. The SDS-PAGE method was carried out according to the method of Shapiro and Maizel [14] and Schiwara et al [15] and modified according to a previously described technique [12].

10.2.2.2. Protein: Creatinine Ratio

Urinary protein concentration in the first morning urine samples was measured by the method of Pesce and Strande [16] and creatinine concentrations were measured with the Beckman 360 creatinine analyser [17] (Beckman Coulter Incorporation, Mayville, Durban, South Africa) to determine the protein: creatinine ratio.

10.2.2.3. Determination of Subclasses of Urinary Proteins

All subjects had quantitative assessment of subclasses of urinary proteins (albumin, IgG, haptoglobin, transferrin, lysoszyme and β_2M) tested on SDS-PAGE measured using nephelometry (Beckman 360 analyser) [18,19].

10.3. Community Based Controls

One hundred and twenty-three control subjects randomly selected from the communities of the index cases, none family members or household contacts of children with HBVMN, had their protein: creatinine ratios and patterns of proteinuria determined using SDS-PAGE. In addition, all had quantitative assessments of their subclasses of urinary proteins measured by nephelometry. All controls were negative for HBV, hepatitis C virus, and retrovirus. Evidence of any co-existing illnesses was excluded on history, clinical examination, and appropriate laboratory investigations. All controls were assessed in particular for underlying renal disease by urinary dipstick analysis and culture, blood urea and serum creatinine level measurement and glomerular filtration rate estimation using the creatinine transformation formula [13].

10.4. Statistical Analysis

Statistical analysis was done in consultation with the Institute of Biostatistics of the Medical Research Council. Comparisons by HBV status and SDS-PAGE were made using a Chi-square analysis. Odds ratio and 95% confidence limits were used to report strengths or associations. Analysis was performed using SAS software (SAS version 6, SAS Institute, USA).

10.5. Results

10.5.1. Index Cases

Seventeen of the 31 index patients (54.8%) were of rural origin; 24 patients (74.2%) were boys. The mean age at presentation was 8 ± 3.3 years (range 2 to 16 years). The average family size was 7 ± 3 family members (range, 2 to 13 family members). None of the patients had a history of any other major illnesses. Three patients (9.7%) had hypertension and 12 patients (38.7%) to have hypocomplementaemia (low C3 level). Eight patients (25.8%) had an asymptomatic rise in their liver enzymes (alanine and aspartate amino transferase and δ -glutamyl transpeptidase); none had evidence of acute hepatitis or chronic liver disease. All except one index patient had normal renal function, and all index patients. All had a protein:creatinine ratio in the nephrotic range.

10.5.2. Family Members and Household Contacts (Subjects)

One hundred and forty-five of the 195 family members and household contacts (74.4%; subjects) were of rural origin; 97 subjects (49.7%) were men. One hundred and fifty-two subjects (77.9%) were family members and 43 subjects (22.1%) were household contacts. Mean age at testing was 20.3 ± 15.4 years (range 0.5 to 71.2 years). Twenty-three (11.8%) had hypertension; One subject had well-controlled non-insulin dependent diabetes mellitus, and one subject had a history of tuberculosis. Twenty subjects (10.3%) had an asymptomatic increase in liver enzyme levels; none had evidence of acute hepatitis or chronic liver disease.

All subjects had normal renal function, assessed by blood urea and serum creatinine levels and estimation of the glomerular filtration rate by means of the creatinine transformation formula. Forty-five had microscopic haematuria, and none had hypocomplementaemia. Based on their HBV status and patterns of urinary protein excretion, subjects were grouped into different categories. For definitions, see Definitions, page I.

10.5.3. Classification of Family Members and Household Contacts (Subjects) by HBV Status using Elisa, Slot-Blot Hybridisation and Polymerase Chain reaction.

10.5.3.1. Hepatitis B Virus Carriers

Seventy-two subjects (36.9%) were HBV carriers. The mean age was 20.1 ± 14.8 (SD) years (range, 2,5 to 60 years). Thirty-eight subjects 38 (52.8%) were males, 64 were family members, and 8 were household contacts. The proportion of carriers was greater in family members than in household contacts (42.1% versus 18.6%, OR; 3.2; 95% confidence interval [CI], 1.3 to 8.0; $P = 0.01$).

10.5.3.2. Hepatitis B Virus Exposed

Thirty-six subjects (18.5%) were exposed to HBV. The mean age was $24,7 \pm 16,8$ (SD) years (range 3 to 71 years); 19 subjects (52.8%) were males, 25 were family members, and 11 subjects were household contacts. The proportion of HBV-exposed subjects was similar in family members and household contacts, (16.4% versus 25.6%; OR; 0.6; 95% CI, 0.2 to 1.4; $P = 0.25$).

10.5.3.3. Hepatitis B Virus Negative

Sixty-eight subjects (34.9%) were negative for HBV. The mean age was 17.1 \pm 14.1 (SD) years (range, 2 to 50 years). 31 (45.6%) were males, 45 subjects were family members, and 23 were household contacts. The proportion of HBV negative subjects was significantly less in family members than in household contacts, (29.6% versus 53.5%; OR; 0.4, 95% CI, 0.2 to 0.8; $P = 0.006$).

10.5.3.4. Hepatitis B Virus Indeterminate

Nineteen subjects (9.7%) were indeterminate for their HBV status. The mean age was 22.4 \pm 15.1 (SD) years (range 0.5 to 58 years). 9 subjects (47.4%) were males, 18 were family members, and one subject was a household contact. There was no statistically significant difference in the proportion of subjects indeterminate for HBV between family members and household contacts, (11.6% versus 2.4% OR; 5.6, 95% CI, 0.8 to 116.7, $P = 0.08$).

10.5.4 Patterns of Proteinuria

Seventy-two of family members and household contacts (36.9%) had evidence of proteinuria using SDS-PAGE (Table XXVIII).

Table XXVIII: Patterns of proteinuria in family members and household contacts of children with HBVMN and controls

	Nonmembranous Pattern of Proteinuria			Membranous Pattern of Negative Proteinuria	
	Glomerular	Tubular	Mixed		
Total					
Subjects 195	40 (20.5%)	3 (1.5%)	12 (6.2%)	17 (8.7%)	123 (63.1%)
Controls 123	24 (19.5%)	1 (0.8%)	0 (0)	8 (6.5%)	90 (63.1%)

Glomerular Proteinuria

Fifty-seven subjects (29.2%) had a glomerular pattern of proteinuria. Seventeen subjects (8.7%) had albumin in combination with IgG, haptoglobin, and/or transferrin, suggestive of a membranous pattern of proteinuria. All 17 subjects with a membranous pattern of proteinuria had tubular proteinuria (β_2 M and lysozyme).

Thirty-eight of the 40 subjects (19.5%) with a nonmembranous pattern of proteinuria had had albumin only, and two subjects (1.0%) had albumin in combination with transferrin, 12 subjects (6.2%) had tubular proteins, as well. Ten of the 17 subjects (58.8%) with a membranous pattern of proteinuria and 19 of the 40 subjects (47.5%) with a nonmembranous pattern of proteinuria and microscopic haematuria. There were no significant differences between subjects with a nonmembranous glomerular pattern of proteinuria ($P = 0.43$). Thirteen subjects (32.5%) with a nonmembranous glomerular pattern of proteinuria and 10 subjects (58.9%) with a membranous pattern of proteinuria had mildly elevated blood pressure.

Tubular Proteinuria

Three subjects (1.5%) had a tubular pattern of proteinuria. The only tubular proteins found in the urine were β_2 M and lysozyme. These three subjects with only a tubular pattern of proteinuria had both proteins detected using SDS-PAGE. None had microscopic haematuria or hypertension.

Mixed Proteinuria

Twelve subjects (6.1%) a mixed pattern of proteinuria. Eleven subjects (5.6%) had only albumin with tubular proteins, and one (0.5%) had albumin with transferrin in combination with tubular proteins. One subject (8.3%) had microscopic haematuria; none were hypertensive.

10.5.5. Classification of Proteinuria according to Hepatitis B Status

Table XXIX shows the classification of patterns of proteinuria in relation to the HBV status of the family members, and household contacts. Patterns of proteinuria (membranous and non-membranous) were not associated with HBV status using chi-square analysis ($P = 0.06$ and 0.08 , respectively).

Table XXIX: Patterns of proteinuria and hepatitis B virus status in family members and household contacts of children with HBVMN.

Hepatitis B Status	Non – Membranous Pattern of proteinuria			Membranous pattern of proteinuria	Negative	Total
	Glomerular	Tubular	Mixed			
Carrier	10 (13.9%)	0	4 (5.5%)	7 (9.7%)	51 (70.8%)	72
Exposed	8 (21.1%)	0	1 (2.6%)	4 (10.5%)	23 (60.5%)	38
Negative	17 (25.0%)	3 (4.4%)	2 (2.9%)	6 (8.8%)	40 (58.8%)	68
Indeterminate	5 (26.3%)	0	5 (26.3%)	0	9 (47.4%)	19
Total	40	3	12	11	123	195

10.5.6. Age, Gender and Relationship

The impact of age, sex and household relationship to the index case on the patterns of proteinuria in family members and household contacts of children with HBVMN was determined. The only significant association was that of increasing age with glomerular proteinuria ($P = 0.007$). In the 0 to 10 year, 11 to 20 year, and older than 20 year age categories, a glomerular pattern of proteinuria was found in 13 (19.1%), 16 (32.6), and 24 subjects (30.8%) respectively. Family members and household contacts having a pattern of proteinuria suggestive of MN showed no association with age, sex, or relationship.

10.5.7. Pattern of Proteinuria on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis and Protein: Creatinine Ratio

Table XXX shows the relationship between the pattern of proteinuria and the protein: creatinine ratio. The 17 subjects having a pattern of proteinuria suggestive of MN were more likely to have an abnormal protein: creatinine ratio (OR; 8.3; 95% CI, 2.7 to 25.5, $P = 0.001$). Also, those who had no proteinuria on SDS-PAGE were more likely to have a normal protein: creatinine ratio compared to those having significant proteinuria (98 versus 25; OR; 2; 95% CI, 1.04 to 3.8), $P = 0.008$).

Table XXX: Protein: Creatinine ratio and patterns of proteinuria in family members and household contacts of children with HBVMN.

Protein: Creatinine ratio	Non – Membranous pattern of proteinuria			Membranous pattern of proteinuria	Negative	Total
	Glomerular	Tubular only	Mixed			
Normal	28 (19.7%)	2 (1.4%)	9 (6.3%)	5 (3.5%)	98 (69.0%)	142
Mild	6 (15.8%)	0	3 (7.9%)	4 (10.5%)	25 (59.4%)	38
Moderate	4 (40.0%)	1 (10.0%)	0	5 (50.0%)	0	10
Severe	2 (40.0%)	0	0	3 (60.0%)	0	5
Total	40 (20.5%)	3 (1.0%)	12 (6.2%)	17 (8.7%)	123 (63.1%)	195

10.5.8. Quantitative Assessment of Subclasses of Urinary Proteins and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Of the 25 subjects with a protein: creatinine ratio in the mild range (≥ 0.2 to < 0.5) but negative for SDS-PAGE, none had the proteins screened for by SDS-PAGE detectable by nephelometry. This suggests that the proteins detected by the protein: creatinine ratios were likely to be proteins not screened for by SDS-PAGE; none suggestive of a membranous pattern of proteinuria. All subjects having IgG and other glomerular proteins suggestive of a membranous pattern on SDS-PAGE also had these proteins detected using nephelometry.

10.5.9 Community based controls

One hundred and twenty-three randomly chosen control subjects from the community in which the index patients live had their protein: creatinine ratio and SDS-PAGE performed. The mean age was 19.2 ± 13.7 years (range, 1 to 67) and were age-and sex-matched to the study population ± 2 years. All were black; 85 controls were adults, and 53 controls (43.1%) were males. All had normal renal function, assessed by blood urea, serum creatinine levels and estimation of the glomerular filtration rate using the creatinine transformation formula [13]. Thirteen had microscopic haematuria. One hundred and five controls (85.3%) had a protein: creatinine ratio in the normal range, 13 controls (10.6%) had mild proteinuria, and 5 controls had (4.1%) moderate proteinuria. Thirty-three controls (26.8%) showed positive proteinuria on SDS-PAGE, 27 controls (21.9%) showed a positive glomerular pattern proteinuria on SDS-PAGE, one control (0.8%) had only a tubular pattern of proteinuria and 5 (4.1%) had a mixed pattern. Eight (6.5%)

had a pattern of proteinuria, and 5 control (4.1%) had a mixed pattern. Eight controls (6.5%) had a pattern of proteinuria on SDS-PAGE suggestive of MN, all had microscopic haematuria, and six were males. Six of the 8 controls were children. Four, with albumin only had an abnormal protein: creatinine ratio. Pattern of proteinuria on SDS-PAGE in controls and subjects is shown in Table XXVIII. On quantitative estimation of subclasses of proteins in the urine, only those controls having a pattern of proteinuria suggestive of MN had IgG and/or haptoglobin detected by nephelometry. In the 18 controls having an abnormal protein:creatinine ratio, 5 controls had no proteins detected by SDS-PAGE. None of these 5 had any proteins detected using nephelometry to subclasses of proteins tested by SDS-PAGE. Seven controls had microscopic haematuria.

10.6 Discussion

We have previously reported on the clustering of HBV and proteinuria in households of children with HBVMN by using a semi-quantitative method (protein:creatinine ratio) to determine abnormal levels of proteinuria. As expected, there was a high level of clustering of HBV in these households but contrary to our hypothesis, significant levels of asymptomatic proteinuria in family members and household contacts occurred independently of HBV status. Except for age, where children younger than 5 years showed significantly greater levels of abnormal proteinuria than those aged older than 5 years, detection of abnormal proteinuria was independent of sex or relationship to the index case [20]. There was a trend towards greater frequency of proteinuria in family members compared to household contacts; family members were also significantly more likely to be HBV carriers than household contacts [20]. We therefore postulated that HBV carriage was not the only determinant of such high levels of abnormal proteinuria within these households, but that other factors, such as environmental and infective agents (other than HBV), resulting in exposure to multiple antigens were responsible. Genetic factors may also play a role.

This study reaffirms these findings using SDS-PAGE and quantitative assessment of subclasses of proteins in the urine in the same cohort of households. We extend the previous findings by showing that a qualitative analysis of proteinuria suggestive of MN in asymptomatic family members and household contacts of children with HBVMN provides better evidence of likely underlying glomerular

basement membrane damage because it correlated with increasing protein: creatinine ratios. Moreover, the use of qualitative and quantitative assessments of proteinuria in the study reinforces the validity of our findings of unprecedentedly high levels of abnormal proteinuria in asymptomatic family members and household contacts of children with HBVMN and healthy individuals within the community. Discordance between the HBV carrier state and patterns of proteinuria in the study group suggests that the recognised association between HBV and MN may not be causally related or that this association reflects exceptional interaction between specifically vulnerable individuals and HBV.

In third-world countries, individuals are exposed to myriads of antigens and it is possible that such high levels of antigenic exposure may be responsible for glomerular basement membrane damage [21]. Excessive antigenic exposure may account for the subjects with abnormal proteinuria but is probably unrelated to those who had minor degrees of proteinuria. For example, the 26 subjects who excreted albumin only with protein: creatinine ratios in the normal range probably reflect normal individuals with physiological proteinuria as albumin may be normally lost in the urine because of its relatively high plasma concentration [22]. The excretion of small amounts of low-molecular-weight proteins may be totally asymptomatic and occur in the absence of structural tubular abnormality [23]. However, the excretion of increased amounts of low-molecular-weight proteins is often associated with perturbations in renal tubular function [23]. The two subjects with only tubular proteinuria and the eight subjects with albumin and tubular

proteins having normal protein: creatinine ratios probably reflect physiological proteinuria in healthy individuals. The one subject with only tubular proteinuria in the moderate range probably suggests tubular damage in the absence of a glomerular lesion.

Thirty-five subjects (18%) were considered to have abnormal proteinuria on SDS-PAGE, reflecting damage to the glomerular basement membrane [22,24]. This figure is less than that found with abnormal asymptomatic proteinuria using the protein: creatinine ratio in the same group of subjects. SDS-PAGE is more sensitive to detecting proteinuria than the conventional use of protein: creatinine ratio; the minimum detection limit for the former to detect protein in the urine is 1mg/mL for albumin, transferrin, IgG and haptoglobin and 0.4 mg/mL for β_2 M and lysozyme [12], and for the latter in the range 10 – 2500 mg/L (Cobas Integra, Roche, Johannesburg, South Africa). It is possible that some of the subjects with proteinuria by SDS-PAGE but normal protein: creatinine ratios may in fact have subtle forms of glomerular basement membrane damage.

The loss of IgG and haptoglobin, high molecular-weight proteins (> 80 kd) occurs as a result of increase in pore size caused by structural damage to the glomerular basement membrane; a pattern of proteinuria often associated with membranous nephropathy [12]. The 17 subjects (8.7%) with this pattern of proteinuria suggestive of MN were also more likely to have abnormal proteinuria by protein:

creatinine ratio ($P = 0.001$). This finding was further supported by quantitative assessment of subclasses of urinary proteins by nephelometry. However, loss of these proteins in the urine was independent of their HBV status. Also, the number of subjects with a pattern of proteinuria on SDS-PAGE suggestive of MN was not significantly different from that seen in community-based controls (8.7% versus 6.5%; $P = 0.5$). These findings therefore fail to support the hypothesis that HBV is causally related to the development of HBVMN.

The high prevalence of abnormal patterns of proteinuria detected in community-based controls was unexpected. However, using sensitive detection tests, this may not be unusual in some poor communities. In a recent report, the prevalence of abnormal proteinuria in asymptomatic individuals in an Australian aboriginal community using an albumin: creatinine ratio less than 1.1 was 17.9% [25]. Hoy et al [25] suggested that renal disease in these communities might be linked to a low birth weight. The subjects studied in this Australian report come from communities with a low socio-economic status, high incidence of low birth weights (similar to the population studied here, in which the incidence of low birth weight ranged from 19.1 to 20.7%) [26], and at especially greater risk of developing kidney disease.

There was an association between increasing prevalence of abnormal proteinuria with age using SDS-PAGE; this finding is consistent with other reports and is interpreted to mean the cumulative impact of antigen exposure [25]. HBVMN is

more frequent in males [27]. In this study, subjects with abnormal proteinuria showed no other significant associations: with HBV status, sex or family relationship to the index case. Also, the pattern of proteinuria suggestive of MN was not significantly different in subjects and community-based controls. These findings again fail to support the hypothesis that HBV is causally related to the development of glomerular basement membrane damage in subjects within these households. In addition, the absence of any difference in patterns of proteinuria between family members and households contacts makes genetic factors predisposing to glomerular basement membrane damage less likely in these subjects.

In conclusion, this report further supports our previous findings of a high frequency of proteinuria in family members and household contacts of index children with HBVMN. Discordance between the HBV carrier state and patterns of proteinuria in the study group and patterns of proteinuria between household subjects compared to community-based controls suggests that HBV is probably not directly related to the development of MN. Other factors, such as environmental and infective agents resulting in exposure to multiple antigens and a high prevalence of low birth weights may be responsible for glomerular basement membrane dysfunction. Genetic influences, although less likely, may also have a role.

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CHAPTER 11

HLA CLASS I AND II IN BLACK CHILDREN WITH HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

CHAPTER 11

HLA CLASS I AND II IN BLACK CHILDREN WITH HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

11.1 Introduction

Only a small minority of individuals who are HBV carriers develop nephropathy and the direct causal relationship of the virus in the development of nephropathy remains a controversial issue [1]. It appears that additional factors, such as genetic and environmental elements, may predispose vulnerable individuals, to develop nephropathy [1]. For example, although childhood minimal change nephrosis has a slight male predominance, MN due to HBV has a male preponderance of 80%, even higher than that reported in idiopathic MN [2,3]. This lends support to the role of a gender-related genetic predisposition to the development of MN.

The host immune response to HBV, which is under T lymphocyte control, is major histocompatibility-complex restricted; both in mice [4] and humans [5]. An association with non-responsiveness to the HBV vaccine with HLA-DR3 has been described in Caucasians [6,7]. There is evidence that HLA-DR3 in Caucasians with renal disease is associated with a MN phenotype, regardless of cause [8].

Studies of HLA-DRB and DQB1 alleles in Polish patients of Caucasian descent with HBVMN showed a significant increase in DRB1*09-DQB1*0303 when compared to healthy controls [9]. A study performed in twelve black South African children with MN, nine of whom were HBV carriers, showed a significantly increased frequency of HLA Bw21. The DR antigens in the latter group of patients were not typed [10].

We performed molecular phenotyping of the HLA complexes to investigate a possible genetic predisposition to the development of MN in black children who were HBV carriers, all of whom had nephrotic syndrome.

11.2. Patients and Method

11.2.1. Patients

Thirty children, age range 2 to 16 years, with biopsy proven HBVMN, were the subjects of this study conducted at King Edward VIII Hospital, Durban in KwaZulu/Natal, South Africa during the period 1995 – 1998. Clinical details of these subjects have been the subject of a previous publication [11]. All were black and came from semi-urban or rural communities. None were siblings or related in any way and none had received HBV vaccine.

11.2.2. Methods

Informed consent for phlebotomy was obtained from the child's parent or guardian. Fifteen millilitres of peripheral blood was obtained for HLA typing, viral studies and assessment of renal function. The control subjects were randomly chosen healthy blood donors from the same province in South Africa. All were black subjects, N = 3176 in the HLA A and B groups, N = 1739 in the HLA C group and N = 490 in the HLA DR and DQ groups. Fifty-two percent of these healthy controls were males.

11.2.2.1. HLA Typing

The HLA A, B, C antigens were determined using a two-staged lymphocytotoxic test [12]. These antigens were identified with 180 antisera, which consisted of local sera and sera requested for use by the International Histocompatibility Workshops and sera exchanged with other laboratories worldwide. The sera were verified by use in parallel with the International Workshop sera. DNA was

extracted from 10mls of ACD blood using a Genomix Kit. HLA DRB1* and DQB1* typing was done using sequence-specific primers (SSP) [13].

11.2.2.2. Viral Studies

Hepatitis B status was determined using third generation enzyme-linked immunosorbent assay (ELISA; Auszyme[®] Monoclonal; Abbott Laboratories, North Chicago, IL, USA) and viral genotyping was done according to the methods stated (Chapter 5). HCV was determined using a microparticle enzyme immunoassay (MEIA) for the detection of antibodies to the hepatitis C virus (Abbott Imx^R HCV version 3.0). HIV testing was done using an ELISA method (Abbott recombinant HIV1-2^R, 3rd generation EIA) and if positive, confirmation was done using an immunofluorescence assay (Virion^R).

11.2.2.3. Histology

HBVMN was defined by examination of light and electron microscopy, and by immunoflorescent staining of kidney biopsy specimens. HBVMN has classical subepithelial deposits, but with varying degrees of mesangial involvement that may include proliferation [14,15].

11.3. Statistical Analysis

Chi-square was used to determine the significance of both susceptibility and protective HLA alleles in the study group of patients and was compared to the frequencies in population based controls of black subjects. Corrected *P* values were obtained by multiplying by the number of alleles tested for at each locus.

Haplotypes were estimated according to the method of Mattiuz et al [16]. The significance of linkage disequilibrium was measured by the delta value (the difference between observed and expected frequencies) divided by the standard error of the estimated haplotype frequencies.

11.4. Results

All thirty patients had nephrotic syndrome diagnosed in accordance with criteria used by the International Study of Kidney Diseases in Children [17] and were in relapse at the time of the study. Twenty-eight of the thirty patients were both HbsAg and HbeAg positive; two were only HbsAg positive. Twenty-one (70%) were males. All were genotype A. Although ten had evidence of mildly raised liver enzymes (gamma glutamyl transferase and aspartamine alanine transferase), none had clinical evidence of chronic liver disease and liver biopsy was not performed in any of the patients. Twenty patients had mild hypertension at presentation [18] and all except one patient had normal renal function. None had serological evidence of hepatitis C virus or human immunodeficiency virus (HIV) infection.

The number and phenotypic frequencies of the HLA A, B, C, DRB and DQB1 alleles in children with HBVMN and population based controls are shown in Tables XXXI – XXXV. A statistically significant difference was detected in the Class II antigens: HLA DQB1*0603 was increased in patients with HBVMN compared to controls ($\chi^2=13.65$, $P<0.001$; RR 4.3). Six (28.6%) of 21 males and three (33.3%) of nine females were HLA DQB1*0603 positive. There were no significant

differences in frequency between males and females positive for DQB1*0603. HLA DRB1*07 and DQB1*02 were increased in frequency in the study subjects when compared to controls but failed to reach statistical significance. The HLA

DRB1*07 and DQB1*02 antigens were in linkage disequilibrium in both patients and in controls.

There were no significant differences in the frequencies of class I antigens in the study group compared to controls.

Table XXXI: HLA A antigen frequencies in subjects and controls

	Random		Patients		X ²	Relative
	N=3176	%	N=30	%		Risk
A1	238	7.5	0	0.00	2.43	0.00
A36	52	1	1	3.3	0.53	2.1
A2	756	24	8	26.6	0.12	1.1
A3	394	12.4	2	6.6	0.90	0.5
A11	2	0.0	0	0.0	0.02	0.0
A23	519	16.3	4	13.3	0.20	0.8
A24	198	6.2	2	6.6	0.01	1.1
A25	26	.08	0	0.0	0.25	0.0
A26	332	10.4	5	16.6	1.22	1.7
A34	385	12.1	7	23.3	3.48	2.2
A28	708	22.2	7	23.3	0.02	1.1
OA29	428	13.4	5	16.6	0.26	1.3
A74	41	1.2	2	6.6	6.49	5.5
A30	1005	31.6	13	43.3	1.87	1.7
A31	93	2.9	0	0.0	0.90	0.0
A32	71	2.2	0	0.0	0.69	0.0
A33	86	2.7	1	3.3	0.04	1.2
A43	19	0.6	0	0.0	0.18	0.0
A66	3	0.0	0	0.0	0.03	0.0
X ^a	987	31.0	3	10.0	6.19	0.2
Total	3176	100.00	30	100.00		

^a Only one antigen detected

Table XXXII: HLA B antigen frequencies in subjects and controls

	Controls		Patients		X ²	Relative
	N=3176	%	N=30	%		Risk
B7	685	21.5	9	30.0	1.25	1.6
B8	408	12.8	2	6.6	1.02	0.5
B13	133	4.1	1	3.3	0.05	0.8
B14	202	6.3	2	6.6	0.00	1.1
B18	150	4.7	2	6.6	0.25	1.4
B22	1	0.0	0	0.0	0.01	0.0
B27	16	0.5	0	0.0	0.15	0.0
B35	254	8.0	1	3.3	0.88	0.4
B37	2	0.0	0	0.0	0.02	0.0
B38	44	1.3	0	0.0	0.42	0.0
B39	66	2.0	1	3.3	0.23	1.6
B41	42	1.3	0	0.0	0.40	0.0
B42	595	18.7	7	23.3	0.41	1.3
B44	508	15.9	10	33.3	6.60	2.6
B45	269	8.4	2	6.6	0.12	0.8
B47	2	0.0	0	0.0	0.02	0.0
B48	3	0.0	0	0.0	0.03	0.0
B50	24	0.7	1	3.3	2.55	4.5
B51	45	1.4	2	6.6	5.67	5.0
B52	4	0.1	0	0.0	0.04	0.0
B53	51	1.6	0	0.0	0.49	0.0
B5I	0	0.0	0	0.0		
B57	260	8.1	3	10.0	0.13	1.2
B58	937	29.5	8	26.6	0.11	0.9

B60	6	0.1	0	0.0	0.06	0.0
B61	0	0.0	0	0.0		
B62	30	0.9	0	0.0	0.29	0.0
B63	29	0.9	0	0.0	0.28	0.0
B70	908	28.5	7	23.3	0.40	0.8
Y^a	632	19.90	2	6.6	3.28	0.3
TOTAL	3176	200.00	30	200.00		

^a Only one antigen detected

Table XXXIII: HLA C antigen frequencies in subjects and controls

	Controls		Patients		X ²	Relative Risk
	N=1739	%	N=30	%		
Cw1	12	0.6	0	0.0	0.21	0.0
Cw2	309	17.7	2	6.6	2.51	0.3
Cw3	260	14.9	2	6.6	1.60	0.4
Cw4	345	19.8	8	26.6	0.86	1.5
Cw5	16	0.9	0	0.0	0.28	0.0
Cw6	612	35.1	10	33.3	0.04	0.9
Cw7	721	41.4	15	50.0	0.89	1.4
Cw8	91	5.2	1	3.3	0.22	0.6
Cw17	312	17.9	6	20.0	0.08	1.1
Z	800	46.0	16	53.3	0.64	1.3
TOTAL	1739	200.00	30	200.00		

Table XXXIV: HLA DR antigen frequencies in subjects and controls

DRB	Controls		Patients		X ²	Relative Risk
	N=1739	%	N=30	%		
01	37	7.5	2	6.6	0.03	0.9
15	90	18.3	9	30.0	2.48	1.9
16	3	0.6	0	0.0	0.18	0.0
0301	93	18.9	4	13.3	0.59	0.7
0302	125	25.5	9	30.0	0.30	1.3
04	52	10.6	0	0.0	3.54	0.0
11	144	29.3	5	16.6	2.24	0.5
12	54	11.0	1	3.3	1.77	0.3
13	148	30.2	14	46.6	3.57	2.0
14	24	4.9	3	10.0	1.49	2.2
07	75	15.3	8	26.6	2.72	2.0
08	24	4.9	0	0.0	1.54	0.0
09	8	1.6	1	3.3	0.48	2.1
10	20	4.0	0	0.0	1.27	0.0
D	83	16.9	4	13.3	0.26	0.8
Total	490	100.00	30	200.00		

Table XXXV: HLA DQ antigen frequencies in subjects and controls

DQB	Controls		Patients		X ²	Relative Risk
	N=490	%	N=30	%		
05	127	25.9	3	10.0	3.82	0.3
06	22	4.4	1	3.3	0.09	0.7
0601	2	0.4	1	3.3	4.22	8.4
0602	140	28.5	9	30.0	0.03	1.1
0603	44	8.9	9	30.0	13.65	4.3
0604	77	15.7	2	6.6	1.80	0.4
02	153	31.2	13	43.3	1.91	1.7
0301	95	19.3	5	16.6	0.13	0.8
0302	26	5.3	0	0.0	1.68	0.0
0303	5	1.0	1	3.3	1.33	3.3
0304	24	4.9	1	3.3	0.15	0.7
03	19	3.8	1	3.3	0.02	0.9
04	102	20.8	6	20.0	0.01	1.0
'Q'	144	29.3	8	26.6	0.10	0.9
Total	490	100.00	30	100.00		

11.5. Discussion

Nephrotic syndrome in children has been shown previously to be associated with HLA class II alleles based on serological and DNA typing [19-26]. Such studies emphasise the role of genetic factors in the development of idiopathic NS in children of Caucasian, Chinese and Japanese descent. In children of Polish descent, HBVMN was associated with DQB1*0303 [9]. In the only study of HLA associations in HBVMN in black children in Durban, South Africa, the frequency of Bw21 in black children with MN compared to healthy controls was significantly increased (15% vs. 1%, respectively, $P_c < 0.04$; RR 22.1). However, only eleven of these children were tested for HBV; nine were positive. The number of children negative for HBsAg was too few to allow meaningful comparison in HLA frequencies between the two groups [10]. This small study did not evaluate the DR and DQ antigens. Therefore, this is most likely a gratuitous finding. Also when the results of the findings in the previous study [10] are combined with the present study, no statistically significant differences are noted (7% in study subjects vs. 2.7% in controls; $P > 0.05$). To our knowledge, this is the first report of HLA associations in black children with HBVMN in which both class I and II antigens have been studied using molecular methodology.

HLA DR7 has been frequently detected in relation to HBV immune reactions. DR7 is most often associated with DQ2. Earlier studies have shown that in Caucasians, there is a low responsiveness to HBV associated with DR7. The initial reports of an HLA genotype to hyporesponsiveness to HBV vaccine

demonstrate an increased incidence of DR7. However, none of these studies report on the DQ associations [27,28]. A report from Qatar in Arabs showed significantly increased levels of DR7 in subjects with persistent HBV infection [29], although again there were no reports of associated DQ antigens. Recently, DRB1*0701-DQB1*0202 in Caucasians has been shown to be associated with a response to the hepatitis coat antigen [30].

However, our study in black children with HBVMN failed to show any significant associations with DRB1*0701 or DQB1*0202. Although previous studies in black patients did not include the class II antigens, inferences can be made on the basis of linkage disequilibrium. These antigens are not in linkage disequilibrium with DQB1*0603 in blacks in South Africa, which suggests that more than one allele in the major histocompatibility group of genes may be involved in the development of HBVMN. In another study done in Caucasians, DQB1*0602 and DQB1*0603 were associated with a response to immunisation with HbsAg [31]. These findings suggest that ethnic differences may also play a role in HLA associations to HBV antigens.

The results of the current study reinforce the concept that within a specific ethnic community there is a group that is genetically susceptible to the development of MN following chronic infection by HBV. The degree of susceptibility might vary

between populations, susceptibility to HBVMN may be expected to be associated with antigens other than DRB1*0701 and/or DQB1*0202 in other populations, as reported [10,31].

To date, HLA DQB1*0603 has not been found to be associated with any other diseases in the black population. Thus, it is possible that the significant increase noted in the DQB1*0603 in HBVMN patients is secondary to the low incidence of the DQB1*0603 in our control population (8.98%). Alternatively, this may be a chance finding, but without further studies this remains a possible explanation.

The study subjects are patients with HBV carriage with associated MN, whereas the controls have neither. It is possible that DQB1*0603 may predispose to HBV carriage only, as opposed to predisposing to the development of HBVMN, as only a small percentage of subjects who are carriers of HBV develop MN (7). As part of our larger study (which is the subject of another report), we also screened unrelated household contacts of the study subjects. In the household contacts (N = 43), eight (18.6%) were HBV carriers but only 2 (4.6%) were DQB1*0603 positive. None had proteinuria. Thus, it appears that DQB1*0603 does not predispose to development of HBV carriage in black children, but may predispose specifically vulnerable individuals with HBV carriage to develop MN.

The most widely accepted mechanism in the pathogenesis of HBVMN is that there is tissue trapping and deposition of immune complexes of viral antigen and host antibody production. Other possible mechanisms include a cytopathic effect induced by virus infection of the cell; virus specific immunological effector mechanisms (specific T lymphocyte or antibody) and by indirect effects mediated via virus-induced cytokine or mediator release (such as tumour necrosis factor, interferons) [32].

The mechanisms through which the HLA class II antigens interact with the HBV to induce membranous nephropathy have been the subject of speculation [27]. It is possible that in black subjects, the DQB1*0603 molecule is unable to present the appropriate HBsAg epitope in a configuration that can be effectively recognised by the receptor of T helper cells. The result shows a weak stimulation of potentially reactive B-cell clones, leading to incomplete clearance of HBeAg, resulting in HBeAg deposition on the epithelial side of the glomerular basement membrane. Subsequent damage to the membrane by complement activation results in damage to the glomerular basement membrane with increased permeability to plasma proteins, resulting in the nephrotic syndrome. Clearance of the HBeAg by a heightened antibody response results in resolution of the nephrotic state [1,3].

All our patients were genotype A, in keeping with previous findings in South Africa of HBV isolates from patients with acute hepatitis B or chronic carriers of the virus

[33]. While it is possible that viral genotypic characteristics may play a role in HBVMN, to date there have been no reports of genotypic associations of HBV with particular disease manifestations. Thus it is unlikely that viral genotype plays a major role in the development of MN.

HLA association in black patients with HBVMN in whom class I and II antigens were determined using molecular methodology have not been reported previously. Our study shows a significantly high frequency of DQB1*0603 in children with HBVMN compared to controls and suggests a possible genetic predisposition to the development of this disease. However, the lack of association between DQB1*0603 and HLA DRB1*07 (which is associated with a low response to HBV in Caucasians) or any of the other immune response genes, together with the preponderance of HBVMN in males, suggests that genetic factors other than HLA may play a role in the development of HBVMN.

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CHAPTER 12

HLA ASSOCIATIONS WITH HEPATITIS B VIRUS CARRIAGE AND PROTEINURIA IN FAMILIES WITH HEPATITIS B VIRUS- ASSOCIATED NEPHROPATHY

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12.1. Introduction

Viruses have been implicated in the pathogenesis of human glomerular diseases with clinical manifestations ranging from transient proteinuria to overt nephritis or nephrotic syndrome [1]. Progress in human molecular genetics offers the prospects of unravelling the complex genetic basis of many common diseases [2]. Genetic factors may also play a role in the development of HBV infections [3] and studies of HLA associations have been reported in children with HBVMN [4].

In a previous study we tested the hypothesis that family members and household contacts of children with HBVMN would have a prevalence of HBV carriage higher than population controls and which would be associated with significant proteinuria. We showed a high prevalence of HBV carrier-state (37%) and

asymptomatic proteinuria (28%) in families and household contacts of children with HBVMN, but contrary to our expectations there was no correlation among

family members and household contacts between HBV markers and abnormal proteinuria [5]. We also showed that HLA DQB1*0603 was significantly increased in the index cases of these families i.e. in children with HBVMN compared to population based controls [6].

Thus it would appear that there is a complex interplay of heritable traits with environmental factors, which renders individuals susceptible to HBV carriage and development of proteinuria. Moreover, we concluded that a genetic basis probably existed for the development of HBVMN with nephrotic range proteinuria, but that environmental conditions influenced the acquisition of HBV and development of abnormal proteinuria [5]. In the light of these findings, we now report an investigation of the association between the HLA DQB1*0603, HBV carriage and the development of abnormal proteinuria using the mean probability ratio (LOD scores) in families of children with HBVMN and nephrotic range proteinuria. We postulated that family members who are HLA DQB1*0603 positive will have significantly higher rates of HBV carriage and abnormal proteinuria.

12.2. Patients and Methods

12.2.1. Patients

Thirty black children, age range 2 – 13 years, with biopsy proven HBVMN served as the index cases. These index cases were recruited over a 3 year period (1995 – 1997) from the Renal Clinic at the King Edward VIII Hospital, Durban, which serves as the tertiary referral centre for the KwaZulu/Natal region of South Africa. Nephrotic syndrome in the index cases was diagnosed in accordance with criteria used by the International Study for Kidney Diseases in Children [7]. Clinical examination and appropriate investigations were done to exclude other secondary causes of nephrotic syndrome. Clinical details of these children have been documented in Chapter 11.

12.2.2. Methods

For all cases HLA typing for Class I and II antigens done using the methodology stated below. Analysis of the phenotypic frequencies of the HLA Class I and II antigens in the index cases versus those in population- based controls showed a statistically significant increase in the Class II antigen HLA DQB1*0603 ($\chi^2=13.65$, $P_c < 0.001$, RR = 4.3) [6].

Study Subjects

The family members of 14 children with HBVMN and HLA DQB1*0603 comprised the study group. A careful note was made of the relationship of each member of the study group to the index case. Once the index cases were recruited, family members (study subjects) were evaluated once during the study period. History and clinical examination was used to detect concurrent illnesses in combination with appropriate laboratory testing. All subjects were assessed in particular for underlying renal disease by urinary dipstix analysis and culture, measurement of blood urea and serum creatinine levels and estimation of their glomerular filtration rate using the creatinine transformation formula [8]. In addition, all subjects had liver function tests (including liver enzymes) and serum complement levels measured at the time of evaluation. All were tested for hepatitis C virus using radioimmunoassay. None were screened for parasitic infections and testing for human immunodeficiency virus was undertaken only if there was clinical indication of disease.

12.2.2.1. Hepatitis B virus Detection

Hepatitis B status was determined using the methods described in Chapter 5. HBV genotype was determined using PCR (chapter 5)

12.2.2.2. Protein: Creatinine ratio

Urinary protein and creatinine concentrations in the first morning urine samples was measured to determine the value of the protein: creatinine ratio [9,10].

12.2.2.3. HLA Typing

The HLA A, B, C antigens were determined using a two-staged lymphocytotoxic test [11]. These antigens were identified with 180 antisera, which consisted of local sera and sera requested for use by the International Histocompatibility Workshops and sera exchanged with other laboratories worldwide. The sera were verified by use in parallel with the International Workshop sera. DNA was extracted from 10ml of acid-citrate-dextrose blood using a Genomix Kit. HLA DRB1* and DQB1* typing was done using sequence-specific primers [12,13]. LOD scores in siblings were calculated using the method described by Race and Sanger [14].

12.3. Statistical Analysis

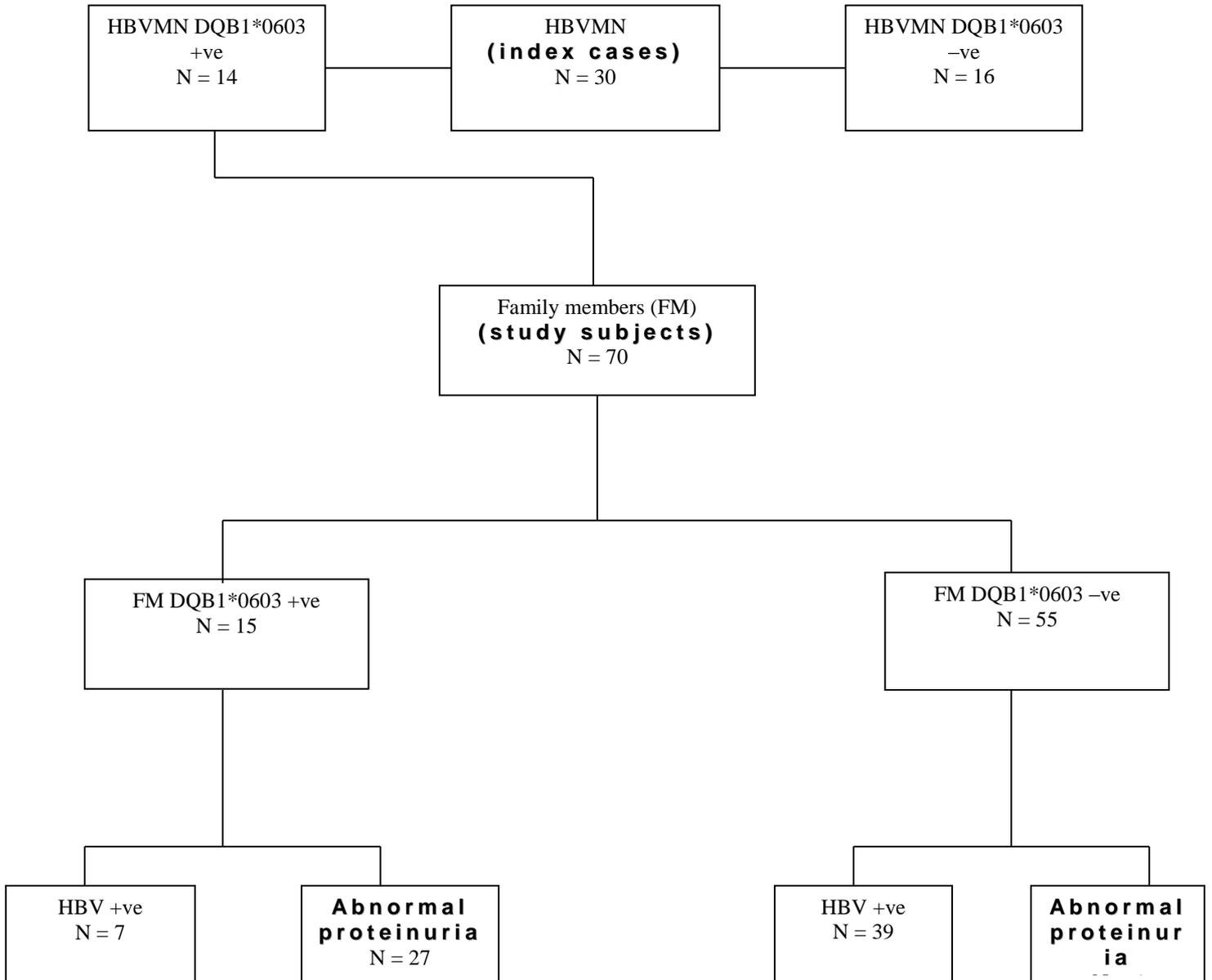
Statistical analysis was done in consultation with the Institute of Biostatistics of the Medical Research Council. Comparisons by HBV status and SDS-PAGE were made using a Chi-square analysis. Odds ratio and 95% confidence limits were used to report strengths or associations. Analysis was performed using SAS software (SAS version 6, SAS Institute, Cary, N.C., USA).

12.4. Results

12.4.1. Index Cases

Fourteen (47%) of the 30 index cases were positive for HLA DQB1*0603; 12 (86%) were males (Fig. 24). The mean age at presentation was 8 years \pm 3.3 (range 2 - 16). The average family size was 7 \pm 3 family members (range 2 - 13). The family members of these 14 index cases positive for HLA DQB1*0603 comprised the study group.

Figure 24: Family members of children with HBVMN and HLA DQB1*0603 screened for HBV carriage and proteinuria



12.4.2. Family Members (Study Subjects)

The study group comprised 70 family members (parents and siblings); 58 (83%) were of rural origin and 34 (49%) were males. The study subjects were all black. The mean age at testing for proteinuria HBV and HLA was 16.1 years (range 0.5 – 71). Fourteen subjects (20%) had hypertension and one a past history of tuberculosis. Thirty-three (47%) had an asymptomatic rise in their liver enzymes; none had clinical evidence of chronic liver disease. All had normal renal function as assessed by blood urea and serum creatinine levels and estimation of the glomerular filtration rate using the creatinine transformation formula [8]. Fourteen (20%) had microscopic haematuria; none had hypocomplementaemia (Table XXXVI).

Table XXXVI: Characteristics of family members of children with HBVMN and HLA DQB1*0603.

Characteristic	Number (%age)
Age: <15 years	35 (50%)
>15 years	35 (50%)
Gender: Males	34 (49%)
Females	36 (51%)
Hepatitis B virus status	
Positive	47 (67%)
Negative	23 (33%)
Proteinuria	
Abnormal (protein: creatinine ratio ≥ 0.2)	19 (27%)
Normal (protein: creatinine ratio < 0.2)	51 (73%)
Liver Enzymes	
Raised aspartamine alanine transferase (n = 10-45μl)	33 (47%)
Raised gamma glutamyl transferase (n = 10-60μl)	18 (26%)
Hypertension	14 (20%)
Hypocomplementaemia	0
Renal Function	
Normal	70 (100%)
Impaired	0

12.4.3. Hepatitis B Virus Status and Proteinuria

Forty-seven (67%) of the study group were positive for HBV infection. The subset of patients who had HBV genotyping done was infected with HBV belonging to genotype A. Nineteen (27%) had abnormal range proteinuria; all had mild to moderate range proteinuria (range of protein: creatinine ratio 0.2-1.99). In the subjects that were DQB1*0603 positive, two had abnormal range proteinuria and were HBV positive. In the subjects who were DQB1*0603 negative, 16 were HBV positive and had abnormal range proteinuria. As shown in the previous publication there was no significant association between HBV infection and abnormal proteinuria ($P=0.3$) [5]. On comparing age and gender for HBV status, no significant associations were found ($P=0.8$ and 0.6 , respectively). The only significant association, as reported previously, was between age and abnormal range proteinuria with 14 subjects (40%) <15 years having abnormal range proteinuria compared to 5(14%) >15 years ($P=0.02$) [6]. No significant gender differences with regard to abnormal range proteinuria were present ($P=0.9$).

12.4.4. LOD Scores

The calculation of the mean probability ratio in the study subjects with DQB1*0603 who were HBV negative versus those with HBV infection and DQB1*0603 was not significant (anti-log sum = 2.0559, average 0.23). When a similar calculation was done for abnormal range proteinuria, there were no significant differences either (anti-log sum = 3.8587, average 0.43).

12.5. Discussion

The results of the present study fail to support our hypothesis that the presence of HLA DQB1*0603 predisposes to the development of chronic HBV carriage or abnormal range proteinuria in families of children with HBVMN accompanied by nephrotic range proteinuria. In children with HBVMN and nephrotic range proteinuria, we have previously established that HLA DQB1*0603 is found in significantly higher incidence than in population-based controls [6]. The present findings in family members of children with HBVMN and nephrotic range proteinuria appear to lend support to the hypothesis we proposed previously, namely that additional factors are responsible for the development of HBVMN [5]. These factors could include environmental influences with exposure to multiple antigens and/or undetected genetic influences.

Previous studies have shown clustering of HBV in households of children with HBV carriage [15]. Clustering of HBV carriage was higher in families of children who were HBV carriers (20%) compared to those who were HBV exposed (9%) or HBV unexposed (3%) [15]. Furthermore, clustering of HBV carriage in families and household contacts of children with HBVMN was significantly higher than in population-based controls [5]. However, none of these studies found any association between HBV carriage and the relationship of the household member to the index case. These findings suggest that the causative factors for acquisition of HBV infection are not restricted to genetic similarities between household

members but operate between all members of the household. The results of the present study lend further support to this interpretation.

Some studies have shown a genetic predisposition to the HBV carriage [16,17], while others have refuted such findings [18-20]. The absence of an association between HBV carriage and the DQB1*0603 haplotype in the present study suggests that HLA does not appear to play a role in the development of HBV carriage. This discordance is in contrast to the development of HBVMN, which is HLA linked both in black and Caucasian children [4, 6].

Vigorous IgG₄ responses, which may be genetic in nature, to certain antigens (helminths, drugs, etc.), may lead to glomerular basement membrane damage and development of abnormal range proteinuria [21]. It is possible that these factors may account for the increased proteinuria in the study subjects who were HBV negative, although we did not specifically screen for these agents. The finding that abnormal range proteinuria was higher in those subjects <15 years compared to those >15 years ($P=0.02$) may result from the higher levels of antigen exposure during childhood.

All our study subjects were genotype A, in keeping with previous findings in South Africa of HBV isolates from patients with acute hepatitis B or chronic carriers of the

virus [22]. While it is possible that viral genotypic characteristics may play a role in the development of proteinuria, to date there have been no reports of genotypic associations of HBV with particular disease manifestations. Thus it is unlikely that viral genotype plays a major role in the development of proteinuria in the study subjects.

It is possible that a complex interplay of environmental factors and social vulnerability predisposes families of children with HBVMN to multiple antigen exposure, including HBV. This results in the development of abnormal proteinuria, which serves as a surrogate marker of glomerular basement membrane dysfunction, independent of HBV carriage and probably not directly linked to progression to HBVMN. It follows that the effect of HLA DQB1*0603 in the pathogenesis of HBVMN is not expressed directly through susceptibility to HBV or development of abnormal range proteinuria. On the other hand, nephrotic syndrome caused by HBVMN is likely to be linked to genetically determined factors, as it is significantly associated to HLA DQB1*0603.

We conclude from these findings that the essential difference between the asymptomatic carriers among family members who have abnormal range proteinuria and the index cases of HBVMN, is the degree of proteinuria. HLA DQB1*0603 probably influences the development of a sustained and massive proteinuric response in selected children with HBV, resulting in HBVMN. The

histological finding in HBVMN is characterised by sub-epithelial deposits of immune complexes along the glomerular capillary walls resulting in damage to the glomerular basement membrane with loss of anionic charge leading to development of proteinuria [23]. It follows therefore that HLA DQB1*0603 regulates inflammatory mechanisms, which have a direct impact on these anionic sites in the glomerular basement membrane. There is a critical interaction between these putative inflammatory mechanisms and HBV in the pathogenesis of HBVMN.

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CHAPTER 13

INTERFERON ALPHA 2b TREATMENT OF HEPATITIS B VIRUS- ASSOCIATED NEPHROPATHY IN BLACK CHILDREN

CHAPTER 13

INTERFERON ALPHA 2b TREATMENT OF HEPATITIS B VIRUS- ASSOCIATED NEPHROPATHY IN BLACK CHILDREN

13.1 Introduction

Several reports have documented progression of HBV-associated nephropathy to chronic renal failure in a small percentage of patients [1-5]. The risk of developing chronic renal failure and the protracted course of the disease with significant morbidity have encouraged clinicians to search for specific and effective therapy for this disease. Immunisation against HBV is the most effective protection against infection, and will over the long-term reduce the incidence of HBV-associated nephropathy. The pathogenesis of this disease most likely results from unexpected consequences of the immune response to HBV; these include passive trapping of HBV antigen-containing immune complexes or local immune complex formation [6]. Persistence of HBeAg correlates with continuation of disease. Accordingly, specific antiviral products and immunomodulating agents may be expected to produce beneficial effects.

In patients with established HBV infection and nephropathy, corticosteroids have been shown to be ineffective [7]; the most effective therapeutic agent available is interferon- α (IFN α), a naturally occurring cytokine primarily produced by B - lymphocytes, null lymphocytes, and macrophages [8-10]. IFN α has anti-viral, anti-proliferative and immunomodulatory effects [11]. In 1993, a meta-analysis of all studies in patients with chronic HBV carriage showed IFN α therapy was beneficial in HBeAg positive patients who were treated for 3 to 6 months [15]. Treated patients were much more likely than controls to seroconvert to anti-HBe and to show normalisation of liver enzymes (alanine amino transferase (ALT)) [12]. To date there has been only one report of a randomised, controlled trial of IFN α in Chinese children with HBV-associated nephropathy; other studies have been mainly in small numbers of adult patients [7,11,13,14]. There have been no similar reports from sub-Saharan Africa, where most countries are hyperendemic for HBV infection.

Based on the high morbidity and small but significant mortality associated with this disease, and the high prevalence of the disease in our region, we undertook a study to prospectively treat a cohort of these patients using IFN α 2b.

13.2. Patients and Methods

13.2.1. Patients

Twenty-four black patients (0 – 16 years) were recruited from the Renal Clinic at the King Edward VIII Hospital, Durban during the period April 1997 to June 1999. All had nephrotic syndrome diagnosed in accordance with standard criteria [15] and all were carriers of HBsAg and HBeAg for a minimum period of 6 months before entry into the study. Clinical examination and appropriate investigations were done to exclude other secondary causes of nephrotic syndrome. There was no formal randomization but only those patients with written consent and who were close to a health care facility for administration and monitoring of interferon therapy were entered into the study.

13.2.2. Methods

IFN α 2b (Intron A, Shering-Plough Co., USA) in a dose of 10 million units per metre squared (maximum 10 million units per dose) was administered subcutaneously 3 times per week by trained health care professionals to all study subjects. Before starting treatment, a test dose of 3 million units was given to all subjects in hospital with full resuscitation facilities available. Patients were treated for 16 weeks. Treatment dosage and adverse events were recorded by the health professional on a report card carried by the parent or guardian. Proteinuria, HBV status, liver function tests (including liver enzymes), and renal function were done and recorded on study entry and weeks

8, 16 and 40 on the record cards. The card was monitored by the principal investigator and returned to him at the end of treatment. IFN α 2b therapy was discontinued if the child developed severe neurological symptoms (convulsions, severe psychiatric symptoms) or haematological disturbances (white cell count < $1.5 \times 10^9/l$; haemoglobin < 7.0×10^9 g/l or platelets < $150 \times 10^9/l$).

None of the patients had been treated with steroids or with cytotoxic agents. Symptomatic oedema was treated with salt restriction and diuretics. In those patients unresponsive to these measures, albumin solution (20%) in combination with diuretics was used. Hypertension was controlled using angiotensin converting enzyme antagonists, thiazide diuretics, and calcium ion antagonists, singly or in combination.

13.2.2.1. Hepatitis B Virus Determination

HBV status determination using 3rd generation ELISA and assays of HBV DNA using Amplicor to determine HBV genomes/ml were undertaken at study entry, 8, 16 and 40 weeks. Using Amplicor, the upper threshold was defined as $>4 \times 10^7$ genomes/ml and the lower limit as 400 genomes/ml.

13.2.2.2. Hepatitis C virus and HIV-1 Determination

All subjects also had hepatitis C virus (HCV) testing using both microparticle enzyme immunoassay (MEIA) for detection of antibodies (Abbott Imx $\text{\textcircled{R}}$ HCC version 3.0) and reverse transcriptase polymerase chain reaction (RT-PCR) [16]

Human immunodeficiency virus (HIV) status was determined using enzyme linked immunosorbent assay (Abbott Laboratories recombinant HIV 1-2, third generation EIA) with confirmation by immunofluorescence assay (Viron^R).

13.2.2.3. Renal Function

This was determined by measuring blood urea and serum creatinine concentrations and estimating glomerular filtration rate using the creatinine transformation formula [17]. Impaired renal function was defined as a urea and creatinine level above the normal range for age and an estimated glomerular filtration rate of less than 80mls/m²/min corrected for age and gender. In addition, urinary protein excretion was measured by urinary dipsticks analysis (Combur^R 9 Dipsticks). The urine protein and creatinine concentrations in the first morning urine samples was measured to determine the value of the protein: creatinine ratio [18,19]. Based on the presence or absence of oedema and proteinuria, subjects were classified as relapse, partial remission, or remission. Relapse was defined as the presence of oedema and urinary sediment (Albustix = 2+ or more and a protein:creatinine ratio >2.0) for 3 consecutive days after a period of remission; partial remission as urinary sediment (Albustix = 1 - 2+ and a protein: creatinine ratio >0.2) in the absence of oedema, and remission as absence of urinary sediment on dipstick analysis (Albustix = 0/trace and a protein: creatinine ratio <0.2) and total subsidence of oedema.

13.2.2.4. Assessment of Liver Disease

Liver biopsy was done in all subjects prior to commencement of IFN α 2b therapy. Liver function tests (including ALT and γ -glutamyl transpeptidase (GGT)) were done concurrently with HBV testing.

Other investigations done before starting IFN α 2b and again at 8, 16, and 40 weeks were a full blood count, serum complement, thyroid function tests, and antinuclear factor assays.

13.3. Controls

Twenty children from the same community with HBV carriage (HBsAg and HBeAg positive) for a minimum period of six months and biopsy-proven HBV- associated nephropathy during the period of the study were selected as controls and compared to the subjects in the study group. All were negative for HCV and HIV. None had HBV DNA levels measured. None had liver biopsies because of failure to obtain ethical consent from the Research Ethics Committee. Proteinuria, HBV status, liver function tests (including liver enzymes), and renal function were monitored at study entry, 8, 16 and 40 weeks. There were no significant differences between controls and those treated with IFN α 2b at study entry and controls received the same treatment for control of oedema and hypertension as study patients.

13.4. Outcome Measures

These included HBV status, clinical criteria, and biochemical assessment of renal and liver function. Clinical criteria assessed were oedema, hypertension, and proteinuria. Histological assessment of renal and hepatic status on follow-up renal and liver biopsies was not undertaken because of failure to obtain ethical consent for these procedures from the University of Natal Research Ethics Committee.

13.5. Study Design

This was a prospective, open-labeled, observational study of the use of IFN α 2b in black African children.

13.6. Statistical Analysis

Statistical comparison of the data was carried out using the unpaired Student's t-test and chi-square test between groups and paired Student's t-test within each group. When the variance was not normally distributed, the non-parametric t-test was used to compare the difference. Serial data were examined by analysis of variance.

13.7. Results

Nineteen patients completed the full course of therapy. The remaining 5 patients defaulted treatment, completing less than 80% of the course of treatment, and were excluded from the primary analysis. The mean age of the patients was 8.7 years (range 5-14 years), 22 were males. Sixteen children were from a rural, 4 from a peri-urban, and 4 from an urban region. Thirteen (68.4%) children were in relapse at the time of entry into the study and the remaining 31.6% were in partial remission. All had a protein: creatinine ratio in the nephrotic range (>2.0). Fifteen (78.9%) had hypertension, 8(42.1%) had hypocomplementaemia (low serum C3 levels), and 17(70.8%) had biochemical evidence of hepatitis (raised AST and GGT levels) (Table XXXVII).

Table XXXVII: Clinical, biochemical, histological and hepatitis B viral status of subjects and controls prior to commencement of IFN-alpha 2b therapy

Characteristics	Patients N = 19	Controls N = 20	P – value
CLINICAL			
Mean age (years)	8.7	9.2	
Gender: Male	17 (89.5%)	17 (85%)	
Female	2 (10.5%)	3 (15%)	
Hypertension	15 (78.9%)	10 (50%)	p = 0.86
Nephrotic Range:			
Proteinuria	19	20	
RENAL FUNCTION			
Mean estimated glomerular filtration			
Rate: Male	77.8 (±5.3)	82.3 (±6.2)	p = 0.6
Female	77.9 (±8.1)	157.2 (±10.3)	p = 0.83
LIVER ENZYMES			
↑ aspartate alanine tranferase	5 (26.3%)	3 (15%)	p = 0.70
↑ γ glutamyl transpetidase	2 (10.5%)	6 (30.%)	p = 0.20
LIVER HISTOLOGY			
Chronic active Hepatitis	6 (31.6%)	Not Done	
Chronic persistent Hepatitis	9 (47.4%)	Not Done	
Cirrhosis	1 (5.3%)	Not Done	
Normal	2 (10.5%)	Not Done	
RENAL HISTOLOGY			
Membranous Nephropathy	17 (89.4%)	19 (95%)	p = 0.93
Membranoproliferative glomerulonephritis	1 (5.3%)	1 (5%)	p = 0.49
Indeterminate or not done	1 (5.3%)	0	p = 0.98
COMPLEMENT			
Low C3	8 (42.1%)	12 (60%)	p = 0.5
Low C4	10 (52.6%)	13 (65%)	p = 0.9
HEPATITIS B VIRUS STATUS			
HbsAg and HbeAg positive	19 (100%)	20 (100%)	
HbsAg positive only	0	0	

13.7.1. Hepatitis B virus Status

At 40 weeks of follow-up, 10 patients (52.6%) had cleared HBeAg (responders), four (21.1%) had cleared HBeAg during treatment but then reverted to being HBeAg positive (reverters), and five patients (31.6%) failed to clear HBeAg (failures) (Table XXXVIII). In all responders clearance of HBeAg was associated with the presence of antiHBe. None cleared HBsAg. In the five children excluded from the primary analysis, all showed persistence of HBsAg and HBeAg. All children had high HBV DNA levels on commencement of treatment ($> 2 \times 10^6$ genomes/ml). In the 10 responders, eight (80%) showed a decline in HBV DNA levels at 40 weeks. HBV DNA levels in the remaining two children were unchanged. Four (80%) of the five children who were failures had no change in HBV DNA levels; one (33.3%) of these children showed a decrease in HBV DNA levels during treatment, but HBV DNA levels rose to pretreatment levels on follow-up at 40 weeks. In those who reverted, three (75%) had no change in their HBV DNA status, whilst one (25%) showed a decline in HBV DNA levels.

Table XXXVIII Clinical, biochemical and hepatitis B viral status of study subjects and controls at 40 weeks of follow-up after completion of IFN α 2b therapy

Charcteristics	Patients (40 weeks)	Controls (40 weeks)	P - value
CLINICAL FEATURES			
Hypertension	7 (36.8%)	10 (50%)	p = 0.06
NEPHROTIC STATUS			
Remission	10 (52.6%)	0	p = 0.001
Partial Remission	4 (21.1%)	5 (25%)	p = 0.93
Relapse	5 (26.3%)	15 (75%)	p = 0.01
RENAL FUNCTION			
Estimated glomerular filtration rate	180.2 (\pm 13.9%)	160.1 (11.2%)	p = 0.79
LIVER ENZYMES			
\uparrow aspartate alanine tranferase	4 (21.1%)	5 (25%)	p = 0.93
\uparrow γ glutamyl transpetidase	2 (10.5%)	6 (30.%)	p = 0.26
COMPLEMENT			
Low C3	2 (10.5%)	8 (40%)	p = 0.08
Low C4	3 (15.8%)	10 (50%)	p = 0.5
HEPATITIS B VIRUS STATUS			
HbsAg and HbeAg positive	9 (47.4%)	19 (95%)	p = 0.01
HbsAg positive	19 (100%)	20 (100%)	

13.7.2. Clinical Outcome

The ten children who responded all went into complete remission. Eight (80%) of these children had normal blood pressure at 40 weeks of follow-up following withdrawal of all anti-hypertensive therapy. In the five children who failed treatment, all remained in relapse at 40 weeks. The four children who reverted to being HBeAg positive were all in partial remission at 40 weeks of follow-up. All of those excluded from the primary analysis were in relapse during the course of the study period.

13.7.3. Renal Function

One (10%) of the children who responded to treatment had impaired renal function at commencement of treatment with return of renal function to normal at 40 weeks of follow-up. The remaining nine children maintained normal renal function throughout the course of treatment and follow-up. Two (40%) of the five children who failed treatment had impaired renal function at commencement of therapy. At 40 weeks of follow-up these two children showed further deterioration in their renal function. In the remaining three children who were failures, renal function remained normal for the period of the study. In the four children who reverted to being HBeAg positive after clearing the antigen during the course of treatment, renal function was normal at commencement of therapy, during the course of treatment, and at 40 weeks of follow-up.

13.7.4. Liver Enzymes

The change in the level of liver enzymes (ALT and GGT) is shown in figures 25 and 26, respectively. In the children who were responders, mean ALT and GGT levels initially rose, but declined during the later period of treatment and at 40 weeks of follow-up. Mean ALT levels remained slightly higher than normal values, whilst mean GGT levels returned to normal levels. The failures showed a similar rise in mean liver enzyme levels, but a decline to normal values at 40 weeks of follow-up. In the reverters, the mean liver enzyme levels remained elevated throughout the period of treatment and subsequently declined. In the five children who were excluded from the primary analysis, mean liver enzyme levels remained elevated during the period of the study and at 40 weeks of follow-up.

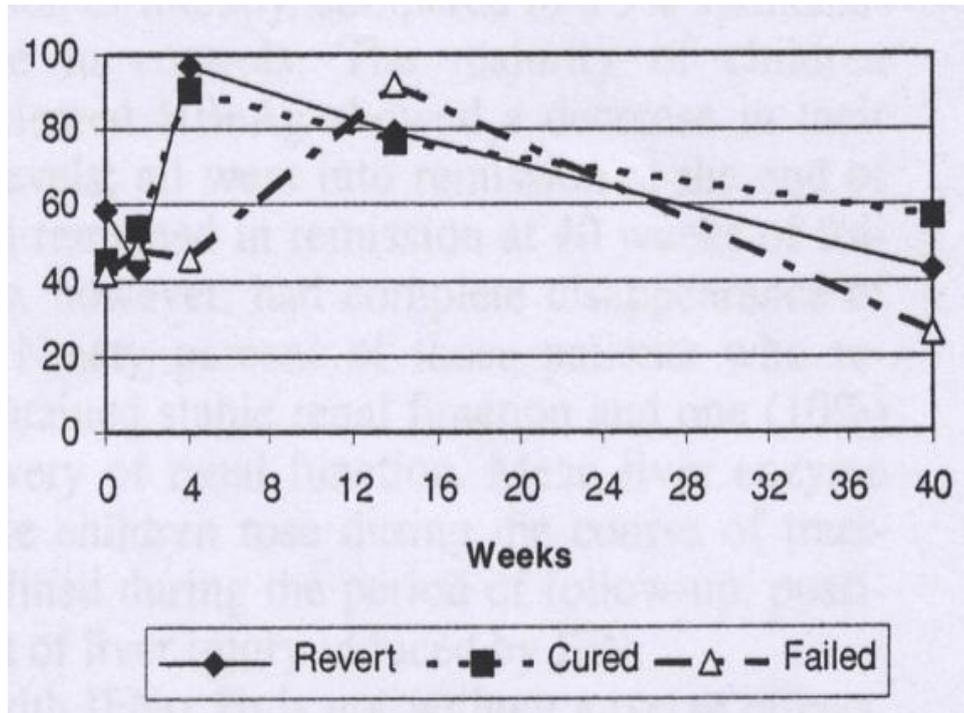


Figure 25: Change in mean alanine aminotransferase (ALT) levels during the course of treatment and follow-up.

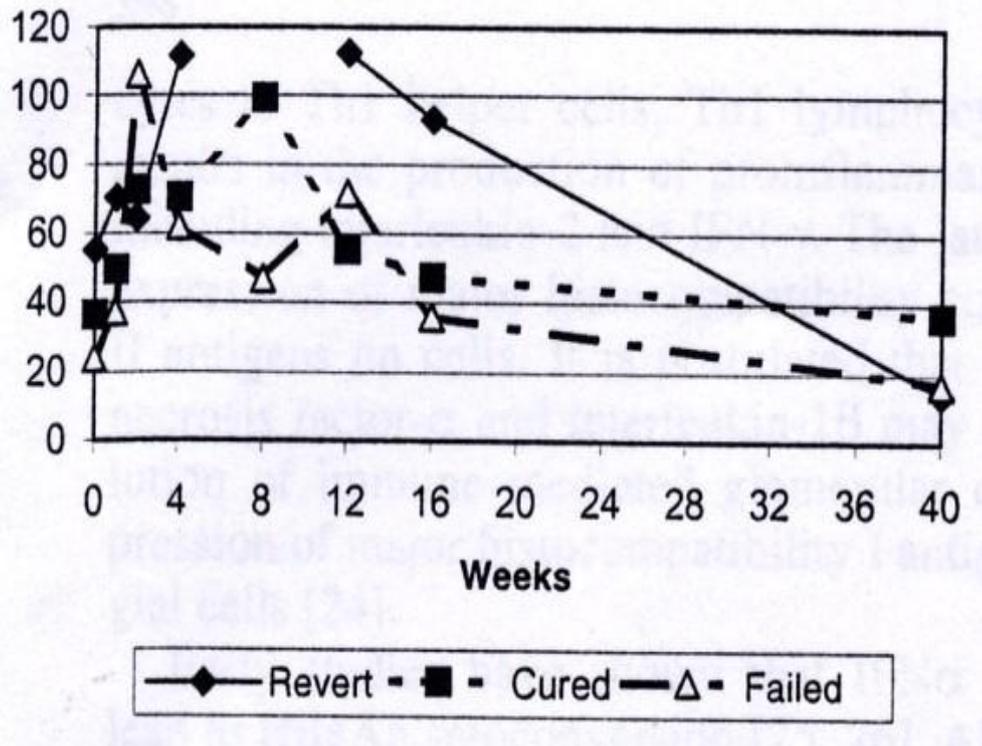


Figure 26: Change in mean γ -glutamyl transpeptidase (γ GT) levels during treatment and follow-up.

13.7.5. Renal and Liver histology

Renal biopsy showed 17(89.4%) children to have membranous nephropathy; ten of these children responded to therapy. One child (5.3%) had membranoproliferative glomerulonephritis and in one child (5.3%) the biopsy was unrepresentative; both failed treatment. Liver biopsy showed six (31.6%) to have chronic active hepatitis, 3 (50%) of whom were responders and three (50%) failed treatment. Nine (47.4%) children had chronic persistent hepatitis, six (66.7%) of whom were responders, 1 failed treatment, and two reverted to being HBeAg positive. One child who reverted had chronic active hepatitis with features of early cirrhosis. Two (10.5%) children had normal liver histology; one responded and one failed treatment. In one patient liver biopsy was not done because of failure to obtain consent. This child failed therapy. In the 5 children excluded from the primary analysis, all had membranous nephropathy (MN) on histology; two had chronic active hepatitis; 2 chronic persistent hepatitis, and one had normal liver histology.

13.7.6. Side Effects

No serious adverse events were encountered. Ten (52.6%) of the treated patients had a flu-like illness with fever, headaches and myalgia. This was noted during the first 4 weeks of therapy and responded to analgesics. One patient had transient thrombocytopenia of 2 weeks duration on commencement of therapy. Treatment was stopped for this period and on recommencing therapy, no further episodes of thrombocytopenia were noted. Other minor adverse effects include abdominal pain (two), insomnia (two) and weakness (five). All five children who

defaulted treatment did so because of socio-economic reasons and not because of adverse effects of IFN α 2b.

13.7.6. Controls

There were no significant differences between study subjects and controls on commencement of therapy (Table XXXVI). Only one of the control subjects cleared HBeAg; all remained in relapse at 40 weeks of follow-up. Nineteen (95%) had MN and one (5%) membranoproliferative glomerulonephritis on renal biopsy. All maintained normal renal function during treatment and follow-up and there were no significant changes in liver function (including liver enzymes).

13.8. Discussion

The results of this open-labeled study show the value of treatment of HBV-associated nephropathy with IFN α 2b, particularly in developing countries. Moreover, we provide evidence of the underlying hepatic disease in these chronic carriers of HBV with nephropathy, which has extended previous findings. Our study showed a 52.6% sustained clearance of HBeAg at 40 weeks of follow-up after completion of therapy, compared to a 5% spontaneous clearance in controls. The majority of children (80%) who cleared HBeAg showed a decrease in their HBV DNA levels; all went into remission at the end of treatment and remained in remission at 40 weeks of follow-up. None, however, had complete disappearance of HBV DNA. Ninety percent of those patients who responded maintained stable renal function and one (10%) showed recovery of renal function. Mean liver enzyme levels in these children rose during the course of treatment but declined during the period of follow-up, possibly as a result of liver injury induced by IFN.

Therapy with IFN α 2b is not without adverse effects, some of which may be serious. Despite the high dose used in treating our patients, the majority had only minor adverse effects. The majority of these adverse effects occurred early in the course of therapy and were responsive to analgesic therapy. None of our patients showed long-term side effects of IFN α 2b therapy, such as psychiatric disturbances, bone marrow suppression, or hypothyroidism.

In a previous study it was shown that the most important independent predictor of response to therapy was high HBV DNA levels [20]. As all the patients who were entered into our study had high HBV DNA levels, this could not be utilized to predict response to HBeAg or HBV DNA clearance. None of the patients or controls cleared HBsAg during the period of the study.

The mechanism by which IFN α results in clearance of HBV antigens has not been fully elucidated, but it is believed that the interaction of IFN α with the cytokine cascade and T-cell system is pivotal to its mechanism of action. IFN α has been shown to induce the proliferation of natural-killer cells, which are helpful in clearing the virally infected cells [8-10]. Stimulation by IFN α results in proliferation of cytotoxic CD8+ T cells [8-10] and induces the differentiation of T helper (Th) lymphocytes to Th1 helper cells. Th1 lymphocyte stimulation results in the production of proinflammatory cytokines, including interleukin-2 and IFN- γ . The latter induces the expression of major histocompatibility complexes I and II antigens on cells. It is postulated that IFN- γ , tumour necrosis factor- α and interleukin-1 β may cause the evolution of immune mediated glomerular disease by expression of major histocompatibility I antigens of mesangial cells [21].

Early studies have shown that IFN α therapy could lead to HBeAg seroconversion [22,23]. Also, it has been shown that high doses of IFN- α not only have an anti-

viral effect, but also an immunomodulatory effect [24,25]. Based on these findings, we chose a high dose of IFN α therapy for our study.

The majority of our patients had MN (70%), a disease with a variable course [2]. Ten to 45% of these patients progress to chronic renal failure or end-stage renal disease, and the response to treatment with steroids, cytotoxic agents, and cyclosporin is variable [26]. Clearance of HBeAg is associated with clearance of proteinuria and preservation of renal function [3,27,28]. Treatment with IFN α has been shown to hasten significantly seroconversion of HBeAg to anti-HBeAg, HBV DNA clearance, and to reduce proteinuria in these patients. In our study, the 10 children who responded to therapy, as evidenced by HBeAg clearance, all had MN. It is difficult to draw conclusions regarding the impact of histology on the outcome of IFN α treatment, as only one patient had non-MN. However, it appears that in those with HBVMN, about half will respond to treatment. At 40 weeks of follow-up, all responders had preservation of renal function with one patient showing amelioration of renal function following treatment. Thus IFN α does not appear to have any detrimental effects on renal function during treatment or on short-term follow-up; one patient showing improvement of renal function. One limitation of our study is the short period of follow-up, which does not allow us to draw conclusions regarding the impact of such therapy on renal function in these patients in the longterm.

In most studies of HBV-associated nephropathy, despite the absence of clinical signs of liver disease, liver function tests are usually mildly abnormal [4,29-31]. There are very few studies in the literature in which concomitant liver and renal biopsies were done in children with HBV-associated nephropathy treated with interferon to assess the degree of hepatic involvement [24,29,32,33]. All these studies were in adults. In our study, both study subjects and controls had mildly raised liver enzymes on entry, which rose during the course of treatment in the study subjects. However, on liver biopsy, 84.2% had histological evidence of liver disease. Unfortunately, because of failure to obtain ethical approval, follow-up liver biopsies were not undertaken to assess changes in hepatic response to therapy. Our findings indicate that liver enzymes have poor correlation with the degree of hepatic involvement based on histological findings and are independent of response to therapy. Even in the absence of significant biochemical features of hepatic involvement (raised liver enzymes), we have demonstrated that the majority of patients with HBV-associated nephropathy had evidence of hepatitis on histology.

One of the major limitations to the use of IFN therapy in developing countries is its prohibitive cost. In a cost-benefit analysis of IFN therapy in children with chronic active HBV infection, even with a latency period of 20 years prior to developing progressive liver disease, the cost per year of life saved was estimated to be less

than that for adults, with a latency period of 10 years [33]. The same would be true for children with HBV associated nephropathy, given the high morbidity of the disease and in the event of progression to end-stage renal disease, the cost of renal replacement therapy. The cost effectiveness of other agents e.g. *Lamivudin* for the treatment of these patients in our setting has not yet been evaluated.

In conclusion, IFN α therapy for HBV-associated nephropathy appears to be effective in black children and safe, even at high doses, with about 50% having clearance of HBeAg and resolution of proteinuria. Despite the high cost of therapy, given the high morbidity and small but significant mortality associated with this disease, we recommend that IFN α therapy be used, even in developing countries, especially in areas with a high prevalence of HBV infection.

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CHAPTER 14

THE IMPACT OF HBV VACCINE ON THE INCIDENCE OF HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

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THE IMPACT OF HBV VACCINE ON THE INCIDENCE OF HEPATITIS B VIRUS-ASSOCIATED MEMBRANOUS NEPHROPATHY

14.1. Introduction

Several reports of the impact of mass immunisation with hepatitis B virus (HBV) vaccination on HBV carriage [1,2,3,4,5,6,7,8] and hepatocellular carcinoma [9,10,11,12,13] have documented a significant reduction in the prevalence of HBsAg carriage and an accompanying decline in the annual incidence of hepatocellular carcinoma. After the introduction of immunisation against HBV in July 1984 in Taiwan, an area of hyperendemic prevalence of HBV infection, HBV carriage in six-year old children declined from about 10% in the period 1981 to 1986, to between 0.9 to 0.8 percent in the period from 1990 to 1994 [13]. Vaccination targets were progressively extended from newborns of HBsAg-positive mothers to all newborns in July 1986, [14] and in 1987 to preschool children as well [15].

HBV vaccine programmes have been implemented in most countries and in the USA has shown a decline in the total number of cases (Fig 27). HBV infections however continue to pose a major public health problem and are present on all continents and in almost every country with an estimated 320 million people being chronically infected [16]. In sub-Saharan Africa, rural communities appear to be at greater risk of exposure and chronic viral infection with carriage rates of between 8-15% [16]. In South Africa, HBV is the main cause of liver related diseases, especially among blacks [17]. More than 70% of the South African black population has been exposed to HBV, with an estimated 10% being carriers (i.e. HBsAg positive) of the virus [17,18]. The pattern of virus transmission contrasts with that in the Far East, with the predominant route being horizontal, especially between siblings [19,20,21,22]. The mechanism of horizontal transmission of HBV in sub-Saharan Africa remains elusive [17]. In the Far East, toddlers and young children are at highest risk of contracting HBV infection. The chronic carrier state declines rapidly with increasing age (82% in infants under 6 months old to 15% in children between 2 and 3 years), males being at higher risk for chronic carriage of HBV [23].

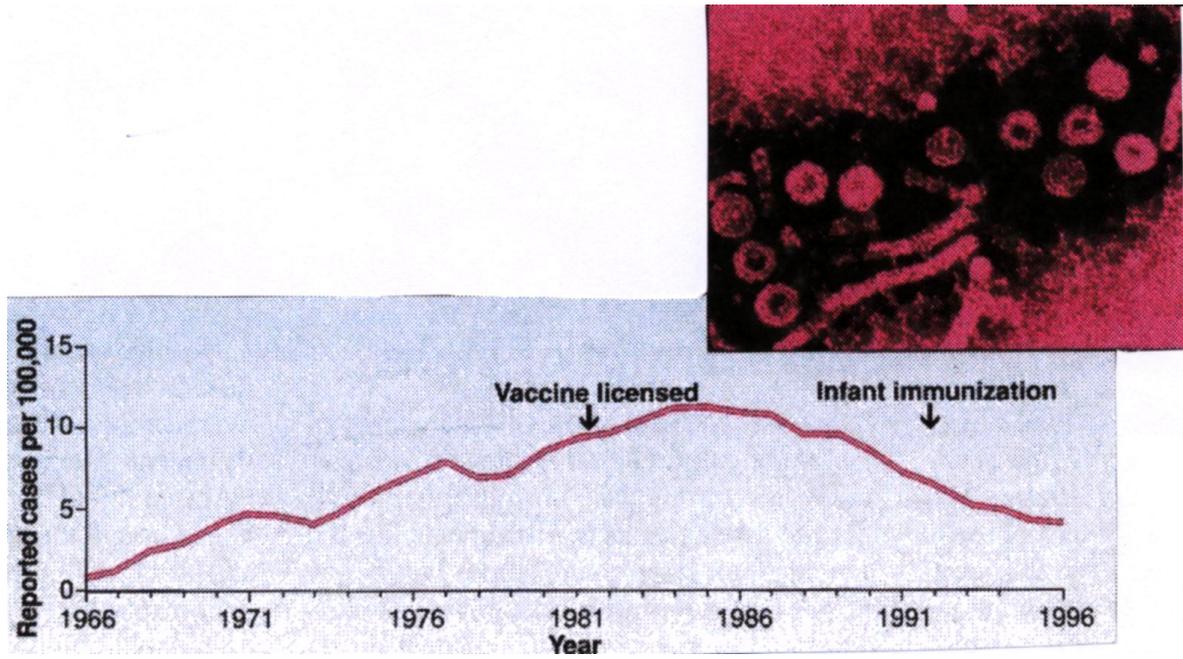


Figure 27: Hidden threat. The hepatitis B virus (inset) infects many more people than U.S. case counts indicate, but the total is in decline. Adapted with permission from: Marshall E (1998) *Science* 281: 630-631.

One of the major extra-hepatic manifestations of chronic HBV carriage in children is HBV-associated nephropathy, particularly membranous nephropathy (MN), [24,25,26] which develops in a small number of chronic carriers of HBV [27]. In Durban, South Africa, up until 1995, HBV-associated nephropathy accounted for 86% of all cases of nephrotic syndrome in black children [28]. HBVMN accounts for a sizeable proportion of childhood NS in other regions too e.g. Cape Province, Zambia, Taiwan [24,29,30]. The medium to long-term impact of HBV vaccine programmes, using an extra-hepatic manifestation of HBV, HBVMN, which is less frequent, as an endpoint, has not been assessed.

The HBV vaccine was incorporated into the South African Expanded Programme on Immunisation of Children in April 1995 [31]. This study examines the impact of HBV immunisation on the incidence of HBVMN, in the ensuing 6 years following the introduction of the HBV vaccine.

14.2. Patients and Methods

14.2.1. Patients

The province of KwaZulu/Natal is one of 9 provinces in South Africa with a total child population (0 – 14 years) of 2 985 708 based on the 1996 census figures [32]. The King Edward VIII Hospital is the tertiary referral centre for the province of KwaZulu/Natal and the only centre where tertiary nephrology services for children are available. All children with complex renal diseases, including nephrotic syndrome are referred to the hospital for assessment and management. Following the strong association with chronic HBV carriage and nephrotic syndrome in children in KwaZulu/Natal, particularly in the black African population, all cases of nephrotic syndrome were screened for HBV carriage as part of a standard protocol [33]. Thus the number of new cases of nephrotic syndrome associated with HBV carriage seen at the hospital is the nearest approximation available of the incidence of HBV associated nephropathy in the province. Over the last three decades, we reported our experience of HBV-associated nephropathy that is the largest series documented so far [25]. The predominant histological form of nephrotic syndrome in black children in Durban, South Africa, is MN, which accounts for over 86% of all cases of HBV-associated nephrotic syndrome [28]. HBVMN is the only histological type in which we assessed the impact of the HBV vaccine, as it is the lesion most clearly associated causally with chronic HBV infection. In the immediate post-vaccination period we extended our efforts to trace any residual cases of HBVMN not already referred and also for further studies. During the period 1996 – 1997 requests were sent to all health centres in the province to refer children 10 years or younger with HBV associated nephropathy to the tertiary centre as part of a study on the bio-social background

of this disease in households. In order to enhance the recruitment of index cases, in 1998 further requests were sent out for children 16 years or younger with HBV associated nephropathy to be referred to the tertiary centre.

14.2.2. Methods

14.2.2.1. Viral Studies

Hepatitis B status was determined using third generation enzyme-linked immunosorbent assay (ELISA) (Auszyme^R Monoclonal, Abbott Laboratories) from 1991. From 1984 to 1991 HBV status was determined using radioimmunoassay (Ausria II Ansab Abbott, Abbott Laboratories, Chicago, USA).

14.2.2.2. Histology

HBVMN was defined by examination of light and electron microscopy, and by immunoflorescent staining of kidney biopsy specimens. HBVMN has classical subepithelial deposits, but with varying degrees of mesangial involvement, that may include proliferation [34,35]. (see Definitions, page I).

14.2.2.3. Population Estimates

The only reliable population data available is the 1996 census. Populations for the remaining years of the study were estimated based on a 3% growth rate per year. Two further denominators were employed to assess the incidence of HBVMN. These were hospital admissions of all children aged 0-14 years, and of children 0-

14 years with nephrotic syndrome admitted to hospital during the period of the study. Information for this was obtained from the hospital registry.

14.2.2.4. Hepatitis B Virus Vaccine

From April 1995 all children born in South Africa since the beginning of that year were required to receive three doses of Hepatitis B vaccine as part of their routine immunisation schedule [31,36]. The vaccine used for immunisation is plasma-derived and contains a lower dose of HbsAg (1,5 µg/0.5ml/dose) than the other vaccines on the market. It is administered at 6, 10 and 14 weeks to infants in the antero-lateral thigh. The doses are administered concurrently with the routine oral polio vaccine (OPV) and diphtheria, pertussis and tetanus (DPT) immunisations [37]. The average immunisation coverage for the years 1995 – 2001 is 85.4%, 78.2% and 62.0% for the first, second and third doses respectively [37].

14.3. Statistical Analysis

Statistical analysis was done in consultation with the Institute of Biostatistics of the Medical Research Council using SAS software (SAS version 6, SAS Institute, USA).

The annual incidence of HBVMN was determined by dividing the annual number of cases in children by the year-end population of children of the same age in KwaZulu/Natal. The effect of the vaccination programme on the incidence of HBVMN was assessed by Poisson regression. The Poisson model included the

number of cases as the response variable, the log of the population as an offset, and the age group as a co-variate. Similar comparisons were made for hospital admissions of all children aged 0 – 14 and for all children with nephrotic syndrome in the same age category. A comparison between the number of cases occurring before and after the introduction of the vaccine was made using the Wilcoxon Rank Sum Test.

14.4. Results

One hundred and nineteen children, age 1 -14 years (mean 7 years), with HBVMN comprised the subjects of this study for the period 1984-2001; 101 (85%) were males. The average annual incidence of HBVMN (calculated as half the incidence over two years) during the study period was 0.25 per 10^5 children (range 0.03 – 0.33) (Table XXXIX). Following the introduction of the HBV vaccine in 1995, the average annual incidences in the immediate post-immunisation period showed no significant decline and were 0.43 per 10^5 for 1996 – 1997 and 0.25 per 10^5 for 1998 - 1999 [Incidence rate ratio (IRR) 1.3 (95% CI 0.9-1.9)] when compared to the pre-immunisation period 1984-1995. When the average annual rate ratio of HBVMN for the pre-immunisation period 1984-1995 (0.22) was compared to 2000-2001 (0.03), there is a sharp decline per 10^5 child population [IRR = 0.12 (95% CI: 0.03-0.5)] (Fig. 28).

Table XXXIX: Average annual incidence rates of HBVMN per 10⁵ of the population aged 0 – 14 years in KwaZulu/Natal, South Africa

Year	Population	Number of cases of HBVMN	Incidence rate / 10⁵
1984 – 1985	2 125 527	14	0.33
1986 – 1987	2 254 972	11	0.25
1988 – 1989	2 392 300	11	0.23
1990 – 1991	2 537 991	15	0.30
1992 – 1993	2 692 554	12	0.23
1994 – 1995	2 856 531	12	0.21
1996 – 1997	3 030 494	26	0.43
1998 – 1999	3 215 051	16	0.25
2000 – 2001	3 360 441	2	0.03

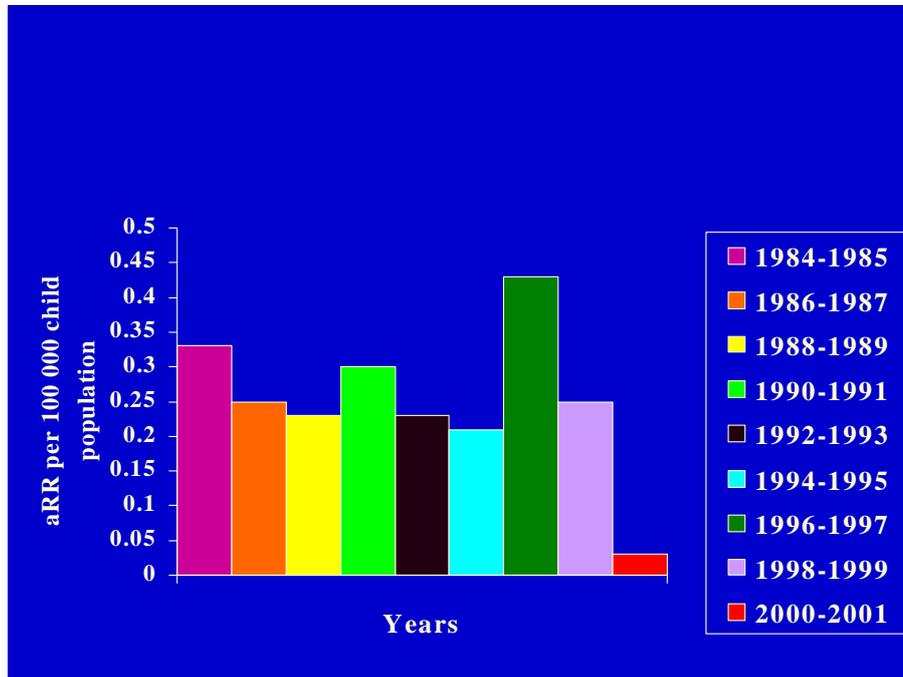


Figure 28: Average annual incidence rates of HBVMN per 10⁵ of population aged 0 – 14 years in KwaZulu/Natal, South Africa

14.4.1. Incidence of Hepatitis B Virus Membranous Nephropathy per Child Population (0 – 14 years) according to Age Categories

The change in rate ratios differed significantly by age categories (Table xl). In children 0 – 4 years, the average rate ratio was 0.16 per 10⁵ in the pre-immunisation period and 0.20 per 10⁵ in the years immediately thereafter, 1996-1999 [IRR = 1.3 (95% CI: 0.5-3.0)]. All cases of HBVMN occurred before 1997 and no cases occurred in the subsequent years 1998 – 2001. The probability of observing no cases in this period compared to the number of cases observed in the years prior to 1998 however was statistically significant, [$P=0.01$].

In the 5 – 9 year age category, the average rate ratios during the pre-immunisation period was 0.46 and during 1996 – 1999 was 0.55 [IRR = 1.2 (95% CI: 0.7-2.0)]. When the average rate ratio for the pre-immunisation period (0.46) was compared to the average rate ratio for the period 2000-2001 (0.09), there was a statistically significant decrease [IRR = 0.19 (95% CI: 0.05-0.8)].

In the 10 – 14 year age group, the average rate ratio during the pre-immunisation period was 0.14 and during the period 1996-1999 it was 0.26, [IRR = 1.9 (95% CI: 0.85-4.1)]. No cases were observed during the period 2000-2001. The probability of observing no cases in this period compared to the number of cases observed in the years prior to 1998 however was not statistically significant, [$P=0.08$].

The rise in the average rate ratios of HBVMN during 1996 was most likely due to the active recruitment drive embarked upon during this period for purposes of another study as described under Methods [38].

Table xl: Average annual incidence rates of HBVMN per 10⁵ of the population 0-14 years in KwaZulu/Natal, South Africa, in three age categories.

Age Groups		0 – 4 years		5 – 9 years			10 – 14 years		
Year	Population size	HBVMN	Average annual incidence	Population size	HBVMN	Average annual incidence	Population size	HBVMN	Average annual incidence
			Rate / 100 000			Rate / 100 000			Rate / 100 000
1984 – 85	686661	2	0.146	716133	7	0.489	722734	5	0.346
1986 – 87	728479	2	0.137	759745	6	0.395	766748	3	0.196
1988 – 89	772843	3	0.194	806014	7	0.434	813443	1	0.061
1990 – 91	819909	3	0.183	855100	9	1.526	862982	3	0.174
1992 – 93	869841	4	0.230	907176	7	0.386	915537	1	0.055
1994 – 95	922815	1	0.054	962423	10	1.520	971293	1	0.051
1996 – 97	979014	8	0.409	1021034	16	0.784	1030445	2	0.097
1998 – 99	1038636	0	0.000	1083215	7	0.323	1093199	9	0.411
2000 – 01	1085605	0	0.000	1132200	2	0.088	1142636	0	0.000
Total		23			71			25	

14.4.2. Incidence of Hepatitis B Virus Membranous Nephropathy per Paediatric Hospital Admissions (0 – 14 years)

The incidence rate of HBVMN as a proportion of hospital admissions showed a similar trend. There was no significant reduction in the incidence rate from 520.6 in 1996 to 226.2 in 1999 [IRR =0.43 (95% CI; 0.2-1.1)]. The decline in the incidence rates of HBVMN was statistically significant between the years 1996 - 1997 and 2000 -2001 [IRR = 0.14 (95% CI: 0.02-0.6)] (Table xli).

Table xli: Annual incidence rates of HBVMN per 10⁵ of all hospital admissions (0-14 years) and, per 10² children admitted with nephrotic syndrome.

Year	Number of cases of HBVMN	Paediatric hospital admissions (0 – 14 years)	Incidence rates/10⁵ of hospital admissions	Hospital admissions of Nephrotic Syndrome	Incidence rate/100 of Nephrotic Syndrome
1995	6	4003	148.7	50	12.0%
1996	16	3073	520.7	52	30.8%
1997	10	4190	238.7	72	13.9%
1998	9	3841	234.3	49	18.4%
1999	8	3537	226.2	60	13.3%
2000	2	2659	75.2	55	3.6%

14.4.3. Incidence of Hepatitis B Virus Membranous Nephropathy per Childhood Nephrotic Syndrome (0 – 14 years)

When the incidence rate of HBVMN as a proportion of the number of cases of children with all histological types of nephrotic syndrome admitted to the hospital was calculated, there was an unusual increase in incidence from a low level of 12.0% in 1995 to 30.8% during the period 1996. This was due to hospital industrial action in the latter half of 1995 when nurses went on strike and all elective referrals were cancelled. Again the incidence rate/100 showed a significant decline from 1999 to 2000 [IRR = (95% CI: 0.03-1.4)] (Table xli); no cases of HBVMN were seen in 2001.

14.4.4. Incidence of Unrelated Conditions

We compared the annual incidences of two diseases which are the commonest reasons for admission in children, and which are unlikely to have been affected by the HBV vaccine programme in 1995. The incidence of pneumonia and gastroenteritis in children aged 0-14 years admitted to hospital remained steady during the period 1995 – 2001 (data not shown). These two conditions accounted, on average, for 38% and 24% respectively, of all paediatric admissions.

The mortality rate in children with HBVMN during the period 1984 – 1999 was 2.5%; a total of 3 deaths due to progressive renal failure. Neither of the two children with HBVMN between 2000 – 2001 died.

14.5. Discussion

The results of this study show that over a period of 6 years following the introduction of the HBV vaccine into the routine childhood immunisation schedule in South Africa, which corresponds to the earliest period of susceptibility of children to HBVMN, the incidence of HBVMN declined significantly. We have assessed this trend by determining the proportion of HBVMN differences using three denominators viz. (a) childhood population in the province, (b) hospital admissions of children with any disease and (c) childhood nephrotic syndrome admitted to hospital. The decline in HBVMN detected by population size was reinforced by similar trends for the other two denominators. These findings are in-keeping with reports from South East Asia that have shown a decline in HBV carriage and hepatocellular carcinoma following the introduction of HBV immunisation after 6 to 10 years of follow-up [3,10,13,39].

In South Africa HBV carriage in black Africans is largely established in early childhood, the first (and largest) wave of HBV infection begins during the latter half of the first year of life and high carrier rates are already present by the age of 3-5 years [19,20,21,22]. The earliest age of onset of HBVMN is around 1 year while the peak age is around 7 years (range 1 – 11 years), in-keeping with reports from other centres [24,25,40]. Therefore we anticipated that there would be an intervening period of about 4 to 5 years before the effect of the vaccine on acquisition of new cases of HBVMN could be detected among those earliest infected and in the peak age group. We found that the incidence of HBVMN

remained relatively constant up to 1999, 5 years after the implementation of the HBV vaccine; a significant decline in the incidence of HBVMN was noted thereafter.

The success of the HBV vaccination in decreasing the incidence of HBVMN in South Africa may be due to the predominantly horizontal transmission of HBV, as seen in the rest of sub-Saharan Africa. Pre-immunisation HBsAg carriage rates in 3-year old black South African children is 12.8% [41]. Following the introduction of the HBV vaccine in April 1995, with coverage rates of 85.4%, 78.2% and 62.0% for the first, second and third doses in children aged 12 – 23 months, [32] the majority of children would have been already protected against HBV infection and therefore against HBVMN, as there is overlap between the age periods of high HBV carriage (3 - 5 years) and first appearance of HBVMN (1 – 14 years).

In the 0-4 year age group, which is the lowest age range at which HBVMN develops in susceptible individuals, there was a significant decline in the incidence of HBVMN ($p = 0.01$) following the implementation of the HBV vaccine in April 1995. It is likely that vaccination of this group directly protected from HBV infection and resulted in decreased incidence of HBVMN.

The 5 - 9 year age group (the group with greatest susceptibility to HBVMN) also showed a significant decline in the incidence of HBVMN when the average rate ratios for the pre-immunisation period of the study was compared to the period 2000-2001. Part of this decrease may have been due to the direct protective

effects of HBV vaccine on the youngest in this age group. However, there may be an indirect cause. Children in the age group 0 – 4 years are the primary source of HBV transmission to family members and household contacts, and there is intense clustering of HBV carriage within households of children with HBVMN [38,42]. It is likely that the HBV vaccination of children under 6 years (i.e. the group available for assessment in this study) led to a decrease in the pool of the most infectious cases in households and resulted in a sharp decline of HBV transmission to older siblings and household occupants. This would have resulted in a decline in the number of cases of HBVMN in the 5 – 9 year age group as the disease peaks around 7 years.

In the 10-14 year age group, the large number of cases of HBVMN seen during the period 1998-1999 probably reflects a change in the referral patterns during this period. During this period, all hospitals and clinics in KwaZulu-Natal were requested to refer patients with HBVMN to the tertiary centre for documenting all residual cases and for evaluation as part of a study [38]. Although there was a trend towards a decline in the incidence of HBVMN in this age group, this was not statistically significant, $p=0.7$.

Although full vaccine coverage for all three dose of HBV was only 62%, the herd immunity resulting from the mass vaccination of children in the first 6 months of life probably reduced the rate of horizontal HBV infection to unvaccinated peer groups and to older children [37]. A consequence of this was significant reduction in the

overall incidence of HBVMN among those children in age groups most susceptible to this disease (i.e. < 10 years). Similar findings are reported for other communicable diseases such as polio and measles with relatively low vaccine coverage. In KwaZulu Natal, the average immunisation rates for polio and measles are 59.6% and 82.5% respectively. There have been no cases of polio and only 19 cases of measles reported during the period 2000 to 2001 [43]. These figures reflect a significant reduction in the overall incidence of these disease compared to the pre-immunisation period.

A bias of the study is that the sample is an hospital based population and thus may not be fully representative of the incidence of HBVMN in community. In KwaZulu/Natal, King Edward VIII Hospital is the only tertiary referral centre for paediatric nephrology and all children with complex renal diseases, especially black Africans, including those with HBV-associated nephropathy, are referred to the hospital for assessment and management at some stage in their illness. Moreover we extended efforts to maximise recruitment of HBVMN in the post-immunisation period. Thus the number of new cases of HBVMN seen at the hospital is the best estimate of the incidence of HBVMN in the province.

Another shortfall of that study is that a sharp decline in the incidence of HBVMN was seen only during the period 2000-2001. It is possible that this reduction in referrals was an isolated event and not a real trend. Continued evaluation would clarify this issue. With the sharp decline in the incidence of HBVMN, there has to

be a rise in the incidence of focal segmental glomerulosclerosis in the local black population, with hospital admissions of nephrotic syndrome remaining stable. This rising incidence of focal segmental glomerulosclerosis has been documented [28,41].

The results of this study indicate that HBV vaccine given as part of routine immunisation, even with low full coverage rates, is highly effective within the framework of the South African Expanded Programme on Immunisation in reducing the rate of HBVMN.

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CHAPTER 15

OVERALL DISCUSSION

CHAPTER 15

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Following the first report of a case of nephrotic syndrome with membranous nephropathy (MN) and hepatitis B virus (HBV) infection by Coombes et al in 1971, several other reports of similar cases in series of patient from different regions around the world followed [1,2,3,4]. These include reports from series of patients from France [5,6], Poland [7], Spain [8], Japan [9] and Hungary [10]. These studies showed HBV infection to be detected more frequently in patients with glomerulonephritis than in controls. In addition, the demonstration of glomerular lesions in patients presenting with HBV-related hepatic disease was another argument in favour of an association between HBV and glomerulonephritis [11]. Also, reports of individuals showing parallel evolution of HBV markers and MN are additional arguments in favour of a significant association. The association between chronic HBV carriage and glomerulonephritis, particularly membranous nephropathy, is most striking in children as compared to adults. It has been proposed, although not proven, that the deposition of HBeAg and anti-HBe antibody is responsible for the formation of pathogenic subepithelial immune deposits [12,13,14,15].

In South Africa, Vos, Grobbelaar and Milner [16] were among the first to show a “relationship between persistent hepatitis B antigenaemia and renal disease in South African Bantu.” In subsequent studies from Cape Town, 46 (86.7%) of 63 children with HBsAg had MN. Eighty percent were also HBeAg positive; all were either black or of mixed ancestry, none were White or Asiatic [17]. In Johannesburg, 14 (24%) of 59 black children with nephrotic syndrome, seen between 1981 and 1985, were HBsAg and HBeAg positive and one half had circulating HBV DNA. All of these 14 children had MN [18]. Subsequent reports followed from KwaZulu/Natal which showed an association between HBV carriage and MN in black children [19,20].

This prompted us to embark on a series of studies to study the natural history, clinical characteristics, biochemical findings and pathogenesis of HBVMN. In addition, we studied the efficacy of interferon alpha 2b treatment in HBVMN with regards to viral clearance and amelioration of the nephrotic state in these children, as well as the impact of vaccination programmes of HBV on the incidence of HBVMN.

In the first of these studies we reviewed our 20-year experience of nephrotic syndrome in Durban, South Africa for the period 1976 – 1995. In black children, MN accounted for 43% of all cases of nephrotic syndrome,

86.2% of these were associated with HBV carriage. [21]. This contrasts with the 2.4% of idiopathic MN seen in Indian children, none of whom had HBVMN [21].

In the next study we proceeded to document the clinical features and natural history of this disease in black children. We showed that disease remission with HBVMN parallels elimination of HBV antigens, particularly HBeAg. Comparison of HBVMN with idiopathic MN showed both groups to have similar clinical characteristics but unexplained biochemical differences. Those children with HBVMN had a higher incidence of biochemical hepatitis and hypocomplementaemia. In both groups there was good preservation of renal function with less than five percent having some evidence of compromised renal function [22].

We next undertook studies to try and elucidate the pathogenesis of this disease by studying the biosocial background in which it develops. In the two studies that followed we evaluated HBV status and proteinuria in family members and household contacts of children with HBVMN to determine if HBV carriage correlates with abnormal range proteinuria and whether the pattern of proteinuria in these households correlated to that seen in HBVMN.

These studies showed strong clustering of HBV carriage in family members and household contacts of children with HBVMN and significantly higher rates of asymptomatic abnormal range proteinuria in these households as compared to community-based controls [23]. However HBV carrier status was not associated with abnormal range proteinuria [23]. This lack of association was also supported by peak prevalences of abnormal range proteinuria in those under five years in these households but no corresponding peak of HBV carriage. Discordance between the HBV carrier state and patterns of proteinuria in the study group, and the absence of significant differences in patterns of proteinuria between household subjects compared to community based controls, suggests that HBV may not be causally related to MN [24]. It is therefore plausible that HBV infection is only associated with HBV-induced MN, but not causally related to it or that it reflects exceptional interaction between specifically vulnerable individuals and HBV.

The results of these two studies led us to postulate that HBV carriage in itself was not sufficient for the development of HBVMN but that additional factors such as environmental and genetic influences may play a role. In order to determine the role of genetic influences we undertook a study to explore HLA associations in black children with HBVMN.

This study showed an association between HLA DQB1*0603 and HBVMN in black children (chi-square 13.65, RR 4.3) when compared to population based controls. DRB1*07 and DQB1*02 were also increased in frequency in the study subjects but failed to reach statistical significance. There was no significant difference in the frequencies of Class 1 antigens in the study group compared to controls [25]. We then proceeded to determine if HLA DQB1*0603 predisposes to HBV carriage and development of abnormal proteinuria in the households of those children with HBVMN positive for the above gene [25].

There was a lack of association between HLA DQB1*0603 with either HBV carriage or abnormal proteinuria in family members suggesting that factors other than HLA influences, (such as other genes yet undetected, environmental factors etc.), may play a role in predisposing children to chronic HBV carriage. We concluded that the main effect of HLA DQB1*0603 which distinguishes HBVMN from family members with abnormal range proteinuria is the degree of proteinuria which is a reflection of the severity of glomerular basement membrane damage [26].

In the next study we proceed to investigate the efficacy of interferon alpha 2b (INTRON A ®) treatment of HBVMN in black children. About half the children treated with INTRON A ® showed clearance of HBeAg with abrogation of proteinuria [27].

There was preservation of renal function and no major side effects were experienced.

Lastly we undertook a study to determine the impact of childhood vaccine programmes on the incidence of HBVMN. HBV vaccine was introduced in the Expanded Programme on Immunisation (EPI) in April 1995 in South Africa. We showed that even with low coverage rates for the full EPI schedule, there was a substantial reduction in the hospital incidence of HBVMN after six years, which is the peak age around which HBVMN develops [28].

In conclusion, this series of studies have added to the body of knowledge on the natural history, pathogenesis, treatment and prevention of HBVMN in black children. It would appear that genetic factors (HLA DQB1*0603) together with chronic HBV infection and environmental influences yet undetermined predisposes to the development of severe glomerular basement membrane damage with nephrotic range proteinuria. Interferon alpha 2b therapy is to date is effective in the treatment of HBVMN and childhood immunisation against HBV infection decreases the incidence of HBVMN. Further studies on the impact of viral load in the development of HBV-associated nephropathy have yet to be undertaken.

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CHAPTER 16

RECOMMENDATIONS

CHAPTER 16

RECOMMENDATIONS

The recommendations we make based on this series of studies include:

1. Assiduous testing for hepatitis B virus in patients with membranous nephropathy, particularly in the regions where vaccination programmes do not include hepatitis B virus immunisation as part of the routine immunisation schedule.
2. Active treatment of hepatitis B virus-associated nephropathy using interferon therapy, particularly in those patients who are HBeAg positive and have high viral loads.
3. In order to decrease the incidence of hepatitis B virus-associated nephropathy, inclusions of hepatitis B virus vaccination into routine childhood immunisation programmes, particularly in those regions endemic for the virus,
4. Further studies to determine the impact of environmental factors and genetic influences (other than HLA genes) on susceptibility of individuals to hepatitis B virus infection and subsequent development of nephropathy.