

**THE ROLE OF ALBUMIN, β_2 M AND CYSTATIN C AS URINARY
BIOMARKERS IN IDOPATHIC AND HIV-ASSOCIATED FOCAL
SEGMENTAL GLOMERULOSCLEROSIS IN CHILDREN.**

By

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Optics & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T Naicker and Professor R Bhimma.



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DECLARATION

I, **Kalindi Persadh** declare that:

- (i) The research reported in this dissertation, except where otherwise indicated is my original work.
- (ii) This dissertation has not been submitted for any degree or examination at any other university.
- (iii) This dissertation does not contain other person's data, pictures, graphs or other information,
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 - a) Their words have been rewritten but the general information attributed by them has been referenced.
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Signed: _____  _____

Date: _____08-01-2017_____

DEDICATION

This thesis is dedicated to my father, Dr H.K. Persadh

To others you are God sent

To me you are God

Thank you for the love and blessings always

“Happy 50th Birthday”

I humbly thank you for being an incredible role model and father. I have acquired my passion for science from you. This dedication to you is my expression of gratitude for instilling in me the value of respect, love and education. I pray to follow in your remarkable footsteps someday. I am grateful for your unwavering support and assistance in all that I do. Without your inspiration, gentle encouragement and unconditional love, I would not be the woman I am today.

Hare Krishna

PUBLICATIONS

Persadh K, Bhimma R and Naicker T (2017). The role of β -2-microglobulin and cystatin C as urinary biomarkers of focal segmental glomerulosclerosis in the setting of paediatric HIV infection. Submitted to the *J of Pediatric Nephrology*.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ARVS	Antiretroviral Drugs
β_2 M	beta-2-Microglobulin
cART	Combined Antiretroviral Treatment
CELL	Cellular
CKD	Chronic Kidney Disease
COLL	Collapsing
CVD	Coronary Artery Disease
CYS C	Cystatin C
eGFR	Estimated Glomerular Filtration Rate
ESKD	End Stage Kidney Disease
FSGS	Focal Segmental Glomerulosclerosis
GFR	Glomerular Filtration Rate
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency Virus
HIVAN	HIV- associated Nephropathy
HLA	Human Leukocyte Antigen
KZN	Kwa-Zulu Natal

LPV	Lopinavir
NOS	Not Otherwise Specified
NS	Nephrotic Syndrome
PH	Perihilar
RTV	Ritonavir
TDF	Tenovir Disproxil Fumerate

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CHAPTER 2

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ABSTRACT

Background: Africa has the highest rate of Human Immunodeficiency Virus (HIV) infection and HIV associated nephropathy (HIVAN) is currently one of the most frequent paediatric kidney diseases observed in children. The most common form of glomerular damage exhibited by children is focal segmental glomerular sclerosis (FSGS) not otherwise specified according to the Columbia classification. HIVAN in children commonly presents as a form of nephrotic syndrome that often leads to end stage kidney disease (ESKD). Due to pathology of the kidney, proteins are excreted out into urine. The objective of this study was to determine the relationship between the urinary concentrations of albumin, β -2-Microglobulin (β_2 M) and Cystatin C proteins in children with HIVAN and idiopathic FSGS compared to HIV infected children with no kidney disease and healthy controls (HIV negative with no kidney disease).

Methods: Urine samples from 74 black South African paediatric subjects were analysed. The study group consisted of 34 children; HIVAN (n=14) and idiopathic FSGS (n=20). The control group consisted of 40 children; HIV positive (n= 20) and HIV negative (n=20) children - both with no kidney disease. Urine samples collected from all these children were stored at -80°C . The urine samples were analysed for albumin, β_2 M and Cystatin C using the Bio-Plex ProTM RBM kidney toxicity assay. Statistical analysis was performed using GraphPad Prism version 5.

Results: The expression of urinary albumin was significantly different between idiopathic FSGS vs HIV positive and negative controls ($p < 0.0001$ each). Similarly, urinary albumin was significantly dysregulated between HIVAN vs HIV positive and negative control groups ($p = 0.0206$ and $p = 0.0056$ respectively).

The excretion of urinary β_2 M proteins was significantly different in the HIVAN group compared to the HIV negative group ($p = 0.0240$). Urinary β_2 M levels were elevated albeit non-significantly in both HIVAN and idiopathic FSGS compared to control groups.

Urinary Cystatin C displayed a statistically significant increase in the idiopathic FSGS in comparison to both HIV negative ($p= 0.0041$) and HIV positive controls ($p= 0.0256$). Urinary Cystatin C expression was significantly low in the HIVAN group compared to idiopathic FSGS ($p= 0.0150$). However, no significance of Cystatin C was noted in the HIVAN group compared to HIV negative ($p= 0.9860$) and HIV positive control groups ($p= 0.4311$).

Positive correlations were observed between albumin and Cystatin C ($r = 0.6761$), β_2 M and Cystatin C ($r = 0.6596$), albumin and β eta-2-microglobulin ($r = 0.4220$).

Conclusion: Albumin significantly correlated with Cystatin C and β_2 M. Urinary β_2 M was significant between the HIVAN and HIV negative control groups. Urinary Cystatin C expression is significantly elevated in children with HIVAN and idiopathic FSGS. Therefore it may be considered as a useful biomarker to detect kidney disease children.

Key Words: HIV, FSGS, children, urinary proteins, albumin, biomarkers, Cystatin C, β_2 M.

CHAPTER ONE

BACKGROUND AND LITRATURE REVIEW

1.1 HIV infection and chronic kidney diseases

The association of Human Immunodeficiency Virus (HIV) infection and kidney syndromes was initially established in the 1980's (Kopp and Winkler, 2003). Globally, chronic kidney disease (CKD) is of great concern to public health, with an incidence of 1.5 - 3.0 million occurring in both children and adults (Fogo, 2007). Patients that are HIV positive generally have a higher risk of proteinuria, albuminuria and low glomerular filtration rate (GRF) compared to those that are HIV negative (Shinha et al., 2015).

The human immunodeficiency virus is a retrovirus, due to the viral RNA genome being reversely transcribed into DNA within targeted cells by using the reverse transcriptase viral enzymes. HIV-1 belongs to the genus *Lentivirus* of the *Retroviridae* family (Ray, 2009).

In 1982, 18 months after the incidence of the first case of HIV infection in adults, paediatric acquired immunodeficiency syndromes (AIDS) were established. Paediatric HIV infections are the consequence of mother to child transmission occurring either *in utero*, via delivery or breast-feeding (Luzuriaga and Mofenson, 2016).

The histopathological changes in HIVAN may differ between children and adults. And in studies among in paediatric populations it has been shown that the proportion of children with HIV-associated nephropathy (HIVAN), that have collapsing glomerulopathy with FSGS, was approximate 14% and 32.5% respectively. Further, it has been shown in biopsies, that with HIVAN in children, collapsing glomerulopathy may not always be present (Bhimma et al., 2013).

By the end of 2011, it was estimated that 3.4 million children were infected with HIV and almost 91% of them were from Africa. Africa has the highest rate - 70% of HIV infection compared to the rest of the globe. Even though Africa is considered to have 10% of the world's population, it has the highest prevalence of HIV/AIDS in both paediatrics and adults (Bhimma et al., 2013).

By the end of 2013, 3.2 million children under 15 years of age globally had HIV infection, and 240000 children were newly infected by this time (UNAIDS, 2014, Perazzo et al., 2015). Whereas in 2010, the rate of HIV diseases displayed a slow decline in paediatric patients due to parents or children having greater access to treatment, in 2015 there were nearly 150 000 new children infected with with HIV (Jindal et al., 2017).

By 2016, an UNAIDS program had evaluated and overseen approximately 36.7 million people living with HIV in Africa. And, in the Eastern and Southern African regions, of an estimated 64% of them living with HIV infection, 160000 of these were children that were newly infected (UNAIDS, 2016).

In an observation in 1987 amongst 8 perinatally HIV infected babies who exhibited proteinuria and FSGS, it was highlighted that the observation of kidney dysfunction in this population of babies could not have been due to iatrogenic causes (Pardo et al., 1987, Jindal et al., 2017).

In-vitro studies have established the role of HIV-1 infection in kidneys. Efficient transfer of viral HIV-1 nucleic acids from the T cells to the kidney tubular epithelial cells has been shown (Bhimma et al., 2013). (Ramsuran et al., 2012) ultrastructurally demonstrated HIV virions within podocytes implicating them as latent HIV reservoir. Moreover, injured glomerular podocytes may undergo proliferation and apoptosis and the remainder of podocytes enlarge in size, leaving segments of the basement membrane - and this supports the growth of sclerotic lesions that is characterized as HIVAN (Bhimma et al., 2013).

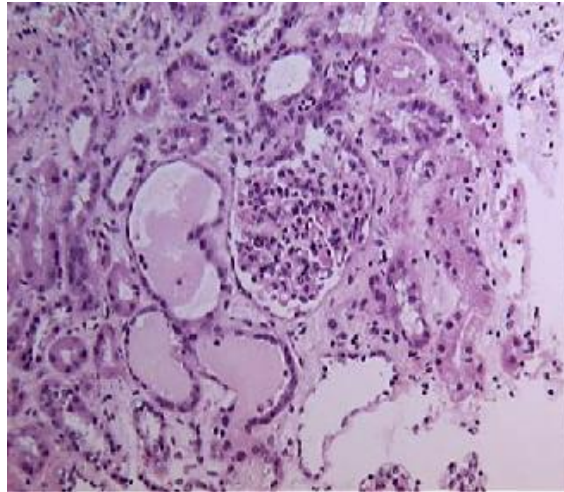


Figure 1: Renal Biopsy, stained with hematoxylin and eosin dye, taken from a child with HIVAN (X150). Taken from (Ray, 2009).

In the beginning HIVAN was initially viewed as a clinical and renal histopathological syndrome which was identified by the appearance of heavy proteinuria and speedy progression to ESKD (Ray, 2009). HIVAN generally develops in patients that have severe immune-suppression with advanced HIV infection, and the cause of death is rarely related to renal diseases (Steel-Duncan et al., 2008). However HIV renal disease can occur at any stage of the infection (Bhimma et al., 2013).

It is noted that HIV-1 and HIVAN in both adults and children are increasing over the years. However with the current treatment, using HAART, the survival rate in HIV infected persons has improved. As a consequence, in the long term, this treatment is causing secondary complications such as kidney and heart dysfunction and other long term complications (Soler-Palacín et al., 2011). Antiretroviral drugs that are frequently associated with renal toxicity are the following – Ritonavir (RTV), Lopinavir (LPV) and currently the Tenofovir disoproxil fumarate (TDF). Consequently the exposure to these drugs may be a contributing factor to complications of HIV - including kidney toxicity (Badiou et al., 2006, Soler-Palacín et al., 2011, Mocroft et al., 2010).

1.2 The structure of kidney

A human body consists of two kidneys that weigh approximately 150g each, located in the retroperitoneal space (Briggs et al., 2009).

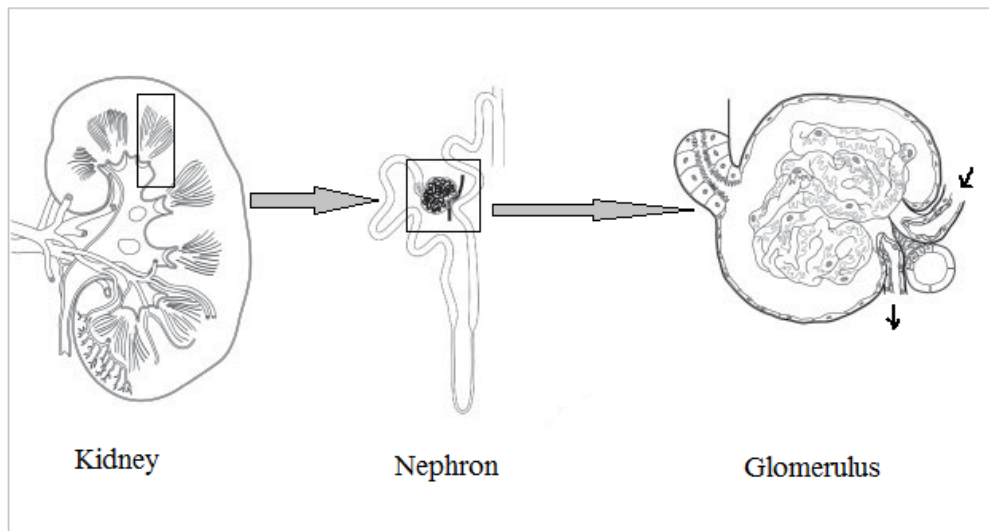


Figure 2: A diagram showing the structure of a kidney. Adapted from (Julian et al., 2009).

The nephron is the smallest structural and functional unit of the kidney, consisting of a renal corpuscle and a renal tubule. A kidney consists of approximately one million nephrons (Briggs et al., 2009).

The glomerulus is a tuft of capillaries, lying within the Bowman's capsule. Water and low molecular weight constituents of plasma are filtered by the glomerular filtration apparatus which then passes into the renal tubules.

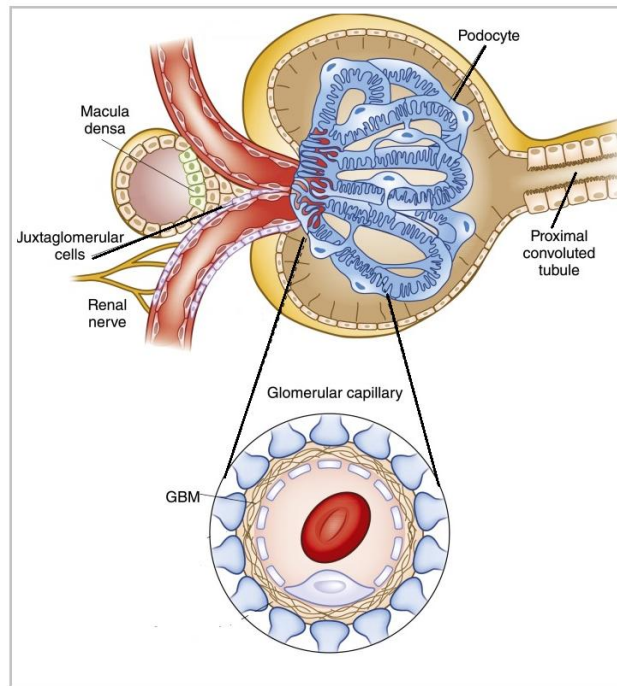


Figure 3: A diagram showing the structure of a glomerulus. Adapted from (Pollak et al., 2014).

The filtrate passes through 3 layers ie. fenestrated endothelium, glomerular basement membrane and the podocytes and epithelial cells which embrace the capillaries. Filtration is based on charge and size of molecules, and hydrostatic pressure gradient across the membrane. The glomerular filtrate then enters the renal tubule where a selective reabsorption of water, inorganic ions and other molecules occur.

The principal function of the kidney is to maintain water and electrolyte homeostasis. In most healthy patients these perturbations are rectified within hours, whereas in unhealthy patients, these perturbations and regulatory processes are disrupted, and may take longer to be rectified (Briggs et al., 2009).

Podocyte dysfunction is responsible for many renal diseases such as focal segmental glomerulosclerosis (FSGS), minimal change nephropathy, membranous glomerulonephritis and congenital nephrotic syndromes (Yu et al., 2016).

This abnormal functioning of the glomerulus often leads to chronic kidney diseases, the consequence which affects 5 to 10% of the population in many countries (Yu et al., 2016).

1.3 Focal segmental glomerulosclerosis

1.3.1 Definition

Focal segmental glomerulosclerosis is a cause of nephrotic syndrome in children and adolescents, as well as a leading cause of kidney failure in adults, and children (Sharma et al., 2004, Eddy and Symons, 2003). FSGS occurs due to podocyte injury from various causes like circulating factors, viral infections and iatrogenic means (Rosenberg and Kopp, 2017). It is the most common histopathological form of steroid resistant nephritic syndrome with a high propensity for progression to end stage kidney disease.

1.3.2 Epidemiology

The global incidence of FSGS across ethnicity has escalated over the past twenty years. In Africa the annual incidence of nephrotic syndromes (3.6) and FSGS (1.6) were considered to be significantly higher in Africans as compared to Caucasians (1.8 and 0.3 cases/10⁵ children per year) (Kiffel et al., 2011). A study by McGrogan et al demonstrated a high incident rate of FSGS, ie from 0.25/100000 in females to 1.8/100000 in males per year (McGrogan et al., 2010). Women generally have a 1.5 fold lower incidence rate of FSGS compared to men (Rosenberg and Kopp, 2017).

FSGS is an aggressive disease. About 30% - 40% of adult patients with FSGS progress to severe renal impairment within the first 10 years after onset of the disease (Fogo and Ichikawa, 1996, Silverstein and Craver, 2007). This however is different with children. According to Abrantes et al, the chances of progression to severe renal insufficiency in paediatric FSGS was 8% at 5years, 17% at 10yrs and 32% at 15yrs of age respectively (Abrantes et al., 2006). Another study showed a high rate of progression to severe kidney dysfunction – especially in Black participants (Sorof et al., 1998, Silverstein and Craver, 2007).

1.3.3 Classification

1.3.3.1 Primary/Secondary

This disease can be divided into two categories - primary (idiopathic) and secondary. In the history of the development of FSGS it is known that approximately 30% to 60% of patients that are diagnosed with primary FSGS are most likely to progress to ESKD, over a period of 5 to 10 years (Kiffel et al., 2011).

- a. Primary (idiopathic) FSGS occurs when there is no apparent etiology for renal histopathological result (Kiffel et al., 2011). It is generally known to be related to nephrotic-range proteinuria, low albumin levels and hyperlipidemia. In some cases, primary FSGS is thought to arise due to a circulatory factor such as cytokines which make certain patients susceptible to disease, as occurs after kidney transplants. (Rosenberg and Kopp, 2017).
- b. Secondary FSGS on the other hand arises due to several casual agents which include – drugs (e.g. pamidronate), infections (e.g. HIV, Hepatitis B virus, parvovirus), lifestyle (obesity) or genetic mutations in podocyte proteins (Dettmar and Oh, 2016). Kidney biopsy findings that support the evaluation of secondary FSGS are the following – enlarged glomeruli, large number of perihilar scars indicating sclerotic changes and partial foot process effacements (Rosenberg and Kopp, 2017).

1.3.3.2 Columbia classification of FSGS

FSGS can be subdivided into 5 pathological variants (Figure 4) – collapsing (COLL), cellular (CELL), perihilar, glomerular tip lesion and not otherwise specified (NOS)...For the diagnosis of FSGS, the Columbia classification proposed a hierarchy, where the observation of the higher- ranking lesion reveals the clinical course - collapsing variant > glomerular tip lesion > cellular variant > perihilar > not otherwise specified (Stokes and D'Agati, 2014, Rosenberg and Kopp, 2017). The collapsing variant of FSGS exhibit segmental or global mesangial consolidation including the loss of endocapillary patency in association with the extracapillary epithelial enlargement and with or

without proliferation. The glomerular tip lesion generally affects the part of the glomerular tuft which is juxtaposed to the tubular pole. The prognosis of the cellular variant is still uncertain (Stokes et al., 2006). The perihilar and not otherwise specified variants can be observed by either the segmental sclerosis or destruction of the capillary loops, with matrix increase involving the segment near the hilum (Rosenberg and Kopp, 2017).

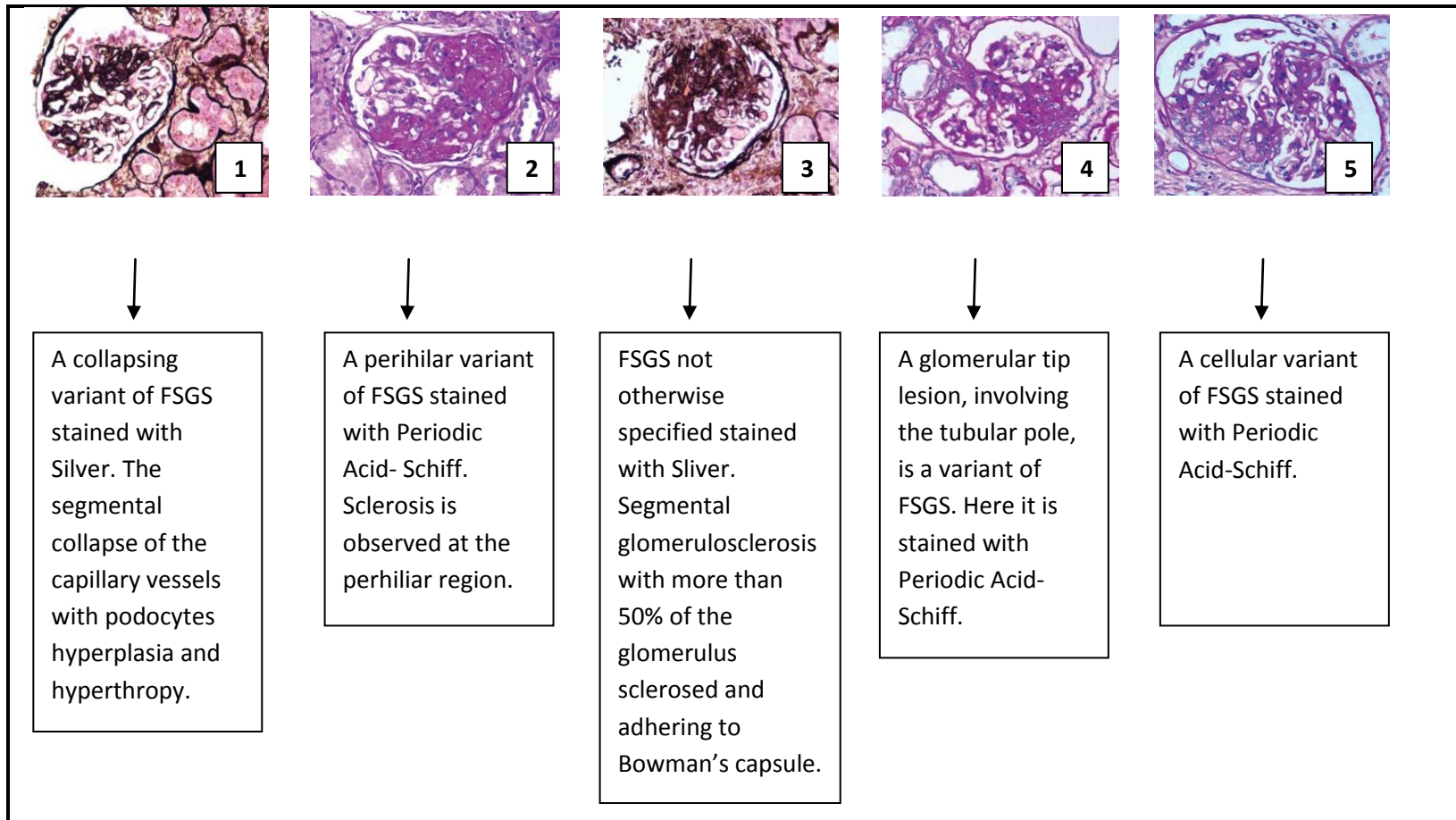


Figure 4: The 5 histopathological variants of paediatric FSGS viewed at X200, adapted from (Shakeel et al., 2014).

1.4 Diagnosis and Evaluation of FSGS

The examination of whether a patient has FSGS solely depends on kidney biopsies. The use of immunofluorescence analysis, electron microscopy and various staining techniques are crucial in the diagnosis, understanding and determining FSGS and other renal disorders such as minimal change disease (Rosenberg and Kopp, 2017).

FSGS is diagnosed by kidney biopsies which provides information on diseases and histopathological abnormalities (Giorgi, 2016). The biopsy can be conducted either surgically or percutaneously. This procedure may have several limitations - it is invasive, and has the possibility of internal damage to adjacent organs or tissue or even the kidney itself. There is also a danger of bleeding in certain patients which could be fatal - with a frequency of approximately 0.1% (Zollinger and Mihatsch, 2012). Another serious complication is infection. Any one of the above would definitely drive up the cost of the procedure – which is in itself is a limiting factor in a resource limited setting.

Therefore non-invasive techniques such as biomarkers should be investigated as an alternative for the diagnosis of kidney diseases including FSGS.

1.5 Biological Biomarkers

A biomarker is generally described as a characteristic that can be identified, measured and evaluated as an indicator of biological processes in response to different therapeutic interventions (Al-Ismaili et al., 2011).

Biomarkers can also possibly be used as surrogate end-points. However in paediatric studies and research, end-points are usually difficult or take extensive periods of time to confirm findings. The use of biomarkers are therefore viable alternatives. The expression and observations of biomarkers may result from either down-regulated or up-regulated genes or proteins in the kidney due to different mechanisms of renal tubular cell damage (Al-Ismaili et al., 2011).

Sensitivity and specificity are used to determine accuracy of results. The advantage of using urine as a biomarker is due to its easy accessibility. It is also non-invasive and correlates with kidney damage, and may also reflect certain aspects of the renal cycle (Tony et al., 2016).

Overall a good biomarker should have the following properties – easy accessibility, non-invasiveness and be measured using standardized assays with rapid results, including an affordable cost for experiments to be executed (Al-Ismaili et al., 2011). They should also have high sensitivity and high specificity.

1.5.1 Albumin Proteins

Albumin is a water soluble protein, anionic and is heart shaped molecule with a molecular weight of 65 kDa, that is synthesised in the liver (Friedman and Fadem, 2010). In humans, 170mg to 9g of albumin is synthesised daily (Birn and Christensen, 2006).

It plays a role in the transport of a variety of circulating molecules, as a carrier of metabolites, hormones, drugs, vitamins, as an acid and base buffer, antioxidant functions and also by maintaining the oncotic pressure and volume of blood plasma (Birn and Christensen, 2006).

Several studies have confirmed that albumin and its ligands causes the expression of inflammatory and fibrogenic mediators. It is assumed that the escalated filtration of albumin may result in excessive tubular reabsorbtion leading to inflammation and fibrosis, eventuating in a loss of renal function (Birn and Christensen, 2006).

The loss of albumin in the urine is considered to be an outcome of the imbalance between both glomerular filtration and tubular reabsorbtion. However both serum an urinary albumin levels are crucial prognostic indicators in many renal diseases (Birn and Christensen, 2006).

The integrity of the glomerular membrane determines whether albumin or other proteins will be excreted (Eknoyan et al., 2003, Abitbol et al., 2006). Albuminuria arises when higher than normal

amounts of albumin is excreted in both children and adults, and it is confirmed if it persists up to 3 months (Julian et al., 2009).

Because there may be false positives as well as false negatives on the dipstix test, a more accurate means of testing would be to analyse a 24hour urine sample for the quantification of albumin excretion by calculating the protein/creatinine ratio (Julian et al., 2009). The ratio above 3.5 (mg/mg) may be considered albuminuria. A ratio below 0.2 is considered normal in healthy people (Ginsberg et al., 1983). Patients that have albuminuria generally excrete 30 – 300 mg of albumin daily (Mudi et al., 2014), and in severe cases, urinary albumin up to 3g is excreted (Julian et al., 2009).

Albuminuria has been investigated in many diseases like - sickle cell, hypertension, HIV and diabetic nephropathy (McKie et al., 2007, Mudi et al., 2014). Albuminuria is considered to be precursor of HIV associated kidney diseases (Fabian, 2007, Mudi et al., 2014).

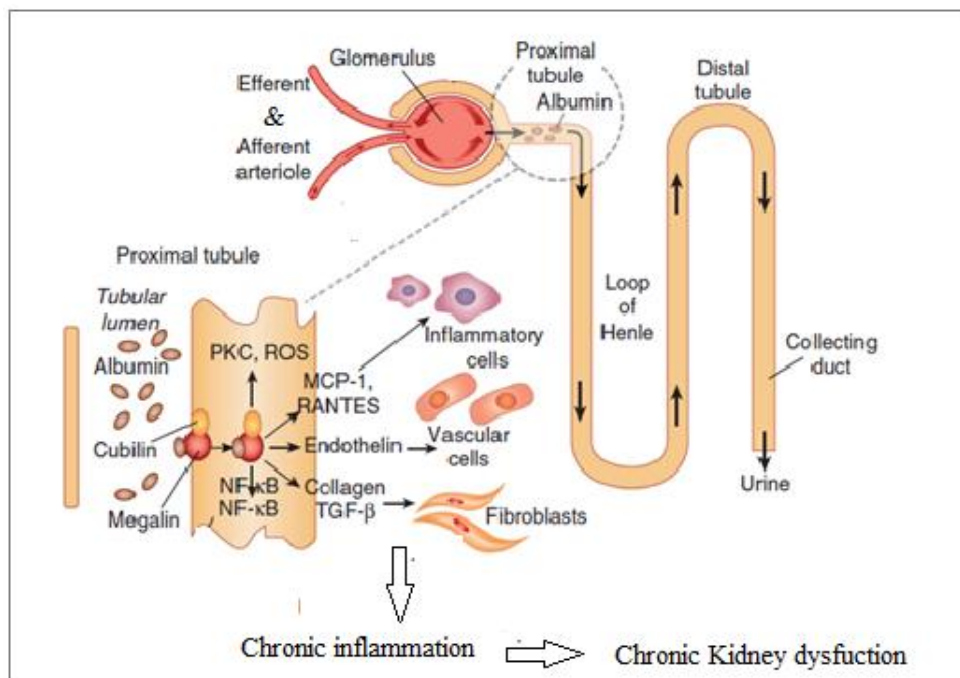


Figure 5: Mechanisms of albumin induced progression to the loss of renal function, taken from

(Roscioni et al., 2014).

1.5.2 beta-2-microglobulin

Beta-2-microglobulin (β_2M) proteins were first isolated in 1964 from urine of patients with Wilson's disease. These proteins are indicators of kidney diseases, and they are also non-specific markers for other diseases such as autoimmune diseases, malignancies and especially in Acquired Immuno-deficiency Syndromes (AIDS) (Bethea and Forman, 1990).

β_2M is a polypeptide which forms the beta chain of the human leukocyte antigen (HLA) class 1 molecules, and its structure is 7-stranded β -pleated (Drüeke and Massy, 2009). Since these β_2M proteins have a low molecular weight (11 kDa), they pass through the glomerular membrane. These occur on most surfaces of nucleated cells and in greater parts of biological fluids, urine, synovial fluids and including serum (Drüeke and Massy, 2009).

Serum levels of β_2M are enhanced in several chronic inflammatory conditions, including rheumatoid arthritis, systemic lupus, in viral infections such as infectious mononucleosis, non A non B hepatitis, cytomegalovirus and AIDS (Bethea and Forman, 1990).

The standard β_2M serum concentration in the human body is approximately 1.5 – 3 mg/l, whereas the average normal production rate is estimated to be 2.4mg/kg/day. However in unhealthy patients that have ESKD, the β_2M levels are generally higher than normal concentration [20-50mg/l to 100mg/l] (Drüeke and Massy, 2009).

It is endocytosed by the proximal convoluted tubular cells and then catabolised into amino acids. These proteins are thus useful predictors of renal function especially in kidney transplant recipients and in those that are thought to have tubule-interstitial diseases (Bethea and Forman, 1990).

Serum β_2M is used to monitor changes in GFR. The urinary β_2M excretion of greater than 370 μ g/24hours reflects tubular dysfunction. However urinary assays are challenging since β_2M molecules degrade easily at pH < 6 (Bethea and Forman, 1990).

In healthy neonates, these molecules are filtered by the glomeruli, of which 99.9% is reabsorbed by the proximal convoluted tubules. The presence of increased plasma levels of β_2M can be observed in

those with kidney failure or tumours. Therefore a high concentration of β_2 M molecules may serve as a sensitive biomarker for kidney disease (Chaudhary et al., 2016).

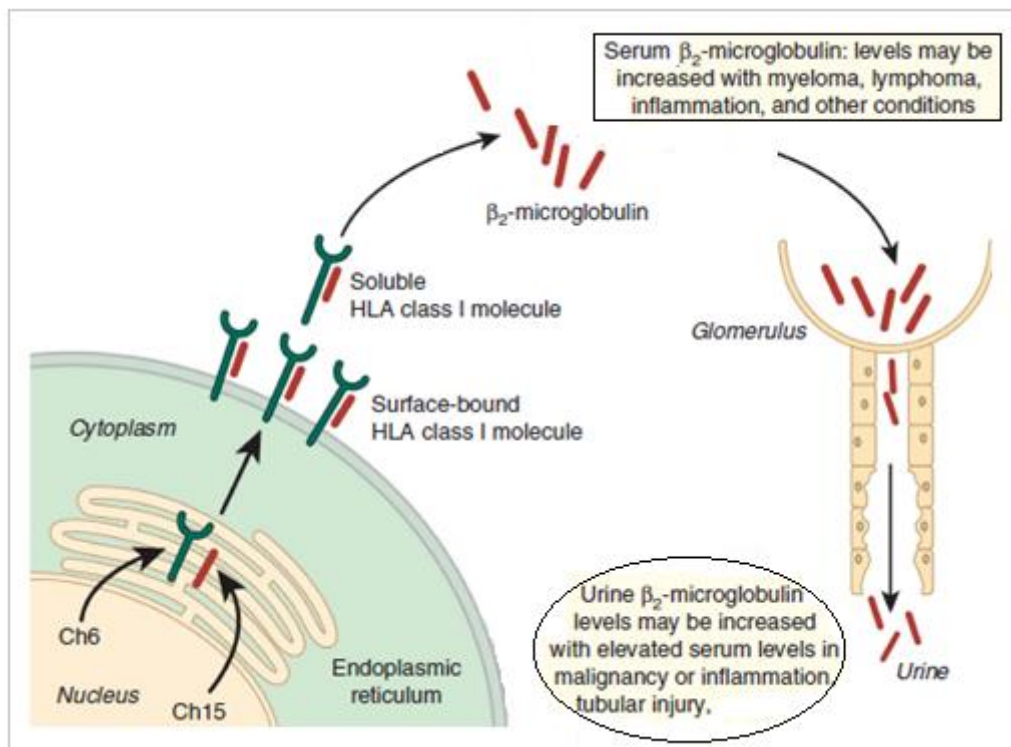


Figure 6: Diagram showing the production and excretion of β_2 M, adapted from (Keown, 2013).

1.5.3 Cystatin C

Cystatin C is a plasma protein (13 kDa) that is produced by all nucleated cells in the human body. It functions as the cysteine protease inhibitor (Dajak et al., 2010). The production of cystatin in the body is independent of age, gender and muscle mass. Some studies indicate that the changes in cystatin levels may contribute in the detection of renal failure (Bang et al., 2017).

These molecules are non-glycosylated, belonging to the type II cystatin gene family and is secreted after synthesis (Robles et al., 2017). Cystatin C molecules are filtered and entirely reabsorbed which is then broken down in the proximal tubules, and its levels are low in the urine whilst the levels remain relatively unchanged in the blood (Deyà-Martínez et al., 2016). In patients with kidney transplant, diabetes and CKD Cystatin C is found much earlier in the urine than creatinine. These

plasma proteins are known to protect the connective tissue from injury of intracellular enzymes emanating from cell death or secreted by malignant cells (Deyà-Martínez et al., 2016).

The estimation of glomerular filtration rates (GFR) is vital for the examination and follow up of patients with kidney diseases (Alberer et al., 2017). The measurements of Cystatin C is believed to be of better prognostic value than to creatinine for GFR assessment, especially in patients with advanced kidney disease who have a GFR below 60 ml/min/1.73 m². And it has been successfully demonstrated in children with kidney disorders caused by various conditions (Deyà-Martínez et al., 2016). The level of Cystatin C remains persistent after the first few years of life, from the age of 4 months to 70 years (Beegum et al., 2017).

Studies have shown that these molecules can be used as sensitive biomarkers for acute kidney failure and diseases. Urinary and serum Cystatin C concentrations are closely associated to kidney functions. However when kidney injuries occur these proteins are elevated. It is considered that in tubular disorders, these proteins would be degraded and consequently be observed in the urine (Robles et al., 2017).

There are multiple studies with evidence indicating that the analyses of cystatin could be used as a tool for the detection of HIV infected patients who are at risk for kidney disorders, coronary artery diseases (CVD) and cancer (Yanagisawa et al., 2015).

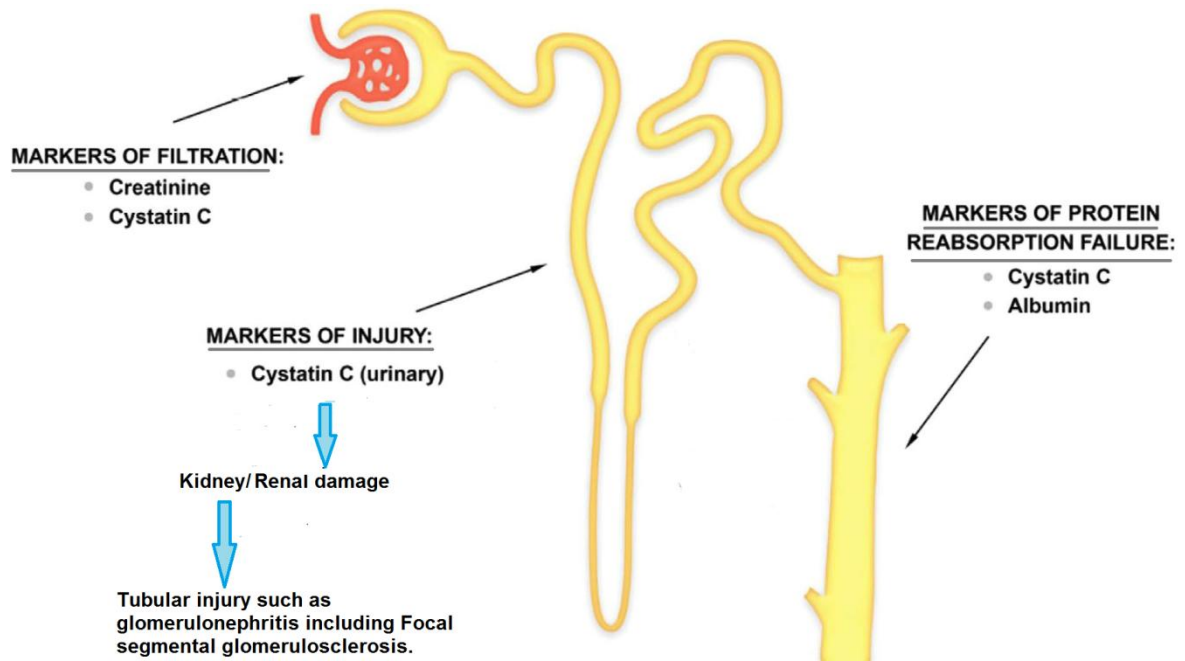


Figure 7: The manifestation of Cystatin C in urine may possibly be an indication of renal damage, adapted from (Malyszko et al., 2015).

1.6 Aim and Objectives

Aim

The aim of this study was to determine if albumin, β_2 Microglobulin and Cystatin C could be used as urinary biomarkers for the detection of focal segmental glomerulosclerosis in children.

Objectives

1. To compare the levels of albumin, β_2 M and Cystatin C in children with biopsy proven FSGS to healthy controls.
2. To measure and compare the levels of albumin, β_2 M and Cystatin C in FSGS according to HIV status.
3. To compare the levels of albumin, β_2 M and Cystatin C in FSGS and HIV- associated FSGS to HIV positive children with no kidney disease and healthy controls.

CHAPTER TWO

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The role of β eta-2-microglobulin and cystatin C as urinary biomarkers of focal segmental glomerulosclerosis in the setting of paediatric HIV infection

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Abstract

Background: Africa has the highest rate of Human Immunodeficiency Virus (HIV) infection and HIV associated nephropathy (HIVAN) is currently one of the most frequent paediatric kidney diseases observed in children. The most common form of glomerular damage exhibited by children is focal segmental glomerular sclerosis (FSGS) not otherwise specified according to the Columbia classification. HIVAN in children usually presents as a form of nephrotic syndrome that often leads to end stage kidney disease (ESKD). Due to abnormal kidney functioning, proteins are excreted out into urine. The objective of this study was to determine the urinary concentrations of β -2-Microglobulin (β_2 M) and cystatin C proteins in children with HIVAN and idiopathic FSGS compared to HIV infected children with no kidney disease and healthy controls (HIV negative with no kidney disease).

Methods: The study group comprised of 34 children; 14 with HIVAN and 20 with idiopathic FSGS. The control groups were 20 HIV positive and 20 HIV negative children with no kidney disease. Urine samples collected from these 74 children were stored at -80°C . Bio-Plex technology was used to analyse urinary protein concentration of cystatin C and β_2 M.

Results: A significant increase in urinary β_2 M levels was observed in the HIVAN group compared to the HIV negative group ($p= 0.0240$). High statistical significance was noted in urinary cystatin C excretion in the idiopathic FSGS compared to both HIV negative ($p= 0.0041$) and HIV positive controls ($p= 0.0256$). Urinary cystatin C displayed a significant decrease in the HIVAN group compared to idiopathic FSGS ($p= 0.0150$). However, no significance of cystatin C was noted in the HIVAN group compared to HIV negative ($p= 0.9860$) and HIV positive control groups ($p= 0.4311$).

Conclusion: Urinary cystatin C levels are significantly elevated in children with HIVAN and idiopathic FSGS. It may be a useful biomarker to detect kidney disease in children.

Key Words: HIV, FSGS, children, urinary proteins, biomarkers.

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Introduction

Africa has the highest incidence of Human Immunodeficiency Virus (HIV) infection, affecting approximately 36.7 million people [1]. Moreover, 2.1 million children (<15years) are HIV infected with a further 160 000 being newly infected annually [1]. Currently 3.4 million children are infected with HIV with 91% of these infections occurring in Africa [2]. Notably, a 50% decrease in new paediatric HIV infection are reported due to access to antiretroviral therapy (ARV) [3]. Globally kidney disease is rapidly becoming a major public health concern as a cause of morbidity and mortality in children. Kidney disease in the setting of HIV that is untreated often leads to rapid progression to end stage kidney disease [4].

In one the largest paediatric studies in Africa, Ramsuran *et al*, reported that nephrotic syndrome due to HIV-associated nephropathy is the commonest form of kidney disease in the setting of HIV seen in childhood [4]. The prevalence of HIVAN has increased in both adults and children [5]. (Soler-Palacín et al., 2011). Previous studies in paediatric populations have indicated that the proportion of children with HIVAN, was approximately 32.5% [2, 4].

Focal segmental glomerulosclerosis is a cause of nephrotic syndrome and the most common histopathological form of steroid resistant nephrotic syndrome in children and adolescents, with a high propensity for progression to ESKD [6]. Approximately 30% to 60% of patients that are diagnosed with primary FSGS are most likely to progress to ESKD, over a period of 5 to 10 years [7].

Whilst a kidney biopsy is the gold standard for providing histopathological assessment of the type of pattern of kidney disease, it is an invasive procedure with attendant complications such as bleeding, infection, visceral perforation or fistula formation. Currently, non-invasive strategies for detection and monitoring the effect of kidney diseases in children include the utility of creatinine levels [8, 9]. However, several factors are posed against it, as these values are influenced by protein intake, nutritional status, muscle mass and body weight, all of which are affected in HIV-infected children and nephrotic syndromes. Hence the use of a non-invasive biomarker to detect kidney disease is urgently warranted [10].

β_2 M proteins are freely filtered in the glomeruli and are reabsorbed and metabolized in the proximal tubule. Although urinary excretion of β_2 M is an indicator of underlying kidney disease, it is non-specific as increased urinary excretion may occur in other diseases such as autoimmune diseases, malignancies and especially in Acquired Immuno-deficiency Syndromes (AIDS) [11]. There is however a lack of data on the efficiency of β_2 M as a biomarker for various forms of kidney diseases.

Cystatin C is a 13kDa plasma protein that functions as a cysteine protease inhibitor [12], and its dysregulation has been implicated in the detection of impaired kidney function [13]. In patients with kidney transplant, diabetes and CKD Cystatin C is found much earlier in the urine than creatinine. The measurements of Cystatin C is believed to be of better prognostic value than to creatinine for GFR assessment, especially in patients with advanced kidney disease who have a GFR below 60 ml/min/1.73. This has successfully been demonstrated in children with kidney disorders caused by various conditions [14].

In an attempt to evaluate the accuracy of β_2 M and Cystatin C as predictors of kidney disease in HIV-infected children, notably HIVAN, we compared and contrasted the urinary levels of β_2 M and Cystatin C in children with HIVAN and idiopathic FSGS to a control group of children (HIV positive and HIV negative with no kidney disease).

Methods

Study design

Ethical permission to conduct this study was obtained from the Biomedical Research Ethics Committee of the College of Health Sciences, University of KwaZulu-Natal, (BREC reference: BE202/17). Urine samples were collected from children attending the Inkosi Albert Luthuli Central Hospital and King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa. Informed consent was obtained from the parent or guardian and assent (where applicable) from the patient prior to collection of urine samples. Samples were collected 2-4 years after the kidney biopsy was done, aliquoted and stored in cryovials at -80°C for a period of approximately 3 months until analysed.

Study population

Seventy four Black South African children aged 1 - 16 years were recruited. The study group (n = 34) consisted of children with biopsy proven HIVAN (n = 14) and idiopathic FSGS (n = 20). At the time of sample collection none of the children had fever or any other evidence of secondary infections. In children with HIVAN co-morbidities included cardiomyopathy (n = 6), chronic lung disease (n = 4) and stunting (n = 4). The control group (n = 40) consisted of children who were HIV positive with no kidney disease (n = 20) and HIV negative with no kidney disease (n = 20). The HIV negative children with no kidney disease were recruited from follow-up clinics e.g. neurology, endocrine and respiratory clinics.

Prior to recruitment, all 14 children with HIVAN were on cART and angiotension converting enzyme antagonists for a minimum of 2 years. At the time of sample collection the 20 children with idiopathic FSGS were on low dose steroids, angiotensin converting enzyme inhibitors as well as additional immunosuppressants such as calcineurin inhibitors.

Diagnosis of HIVAN

The classification of HIVAN was based on the confirmation of HIV- 1 infection and presence of persistent proteinuria of $\geq 1+$ on urinary dipsticks examination with one or more of the following (i)

abnormal urinary sediment; (ii) presence of enlarged echogenic kidney by renal ultrasound; (iii) histological findings of FSGS; (iv) microcystic dilation, a childhood variant of HIVAN in the absence of significant podocyte lesions [4, 15].

BioPlex Multiplex

The urine samples were analysed for β_2 M and cystatin C using the Bio-Plex Pro™ RBM kidney toxicity assay (panel 2) (Bio-Rad Laboratories, Inc., USA) according to manufacturer's instructions [16].

The detection of reaction was carried out using the Bio-Plex® MAGPIX™ 200 reader system (Bio Rad Laboratories Inc, 2017). Raw data was collated using a Bio-Plex Manager™ software version 4.1. A standard curve was generated using the known concentration (ng/ml) of each analyte by plotting the median fluorescent intensity (MEI) signal against concentration. These standards were used to interpolate the concentration of the unknown samples. Intra-plate variability were determined with CV <20% and (X100) between 70-130% ($r=0.8$, $p=0.05$). All data was imported to an Excel spreadsheet for statistical analysis.

Statistical Analysis

Non parametric tests (Mann-Whitney U) were performed for statistical analysis using GraphPad Prism version 5 (GraphPad software version 5, San Diego California, USA). One-way ANOVA and the Dunns post hoc multiple comparison test were used. Spearman's coefficients were used to evaluate correlations. The level of statistical significance was considered as $p < 0.05$. Graphical data represented as median and interquartile range.

Results

Fourteen children (19%) were HIV positive and were confirmed paediatric HIVAN and 20 children (27%) had idiopathic FSGS, all with FSGS had histopathological pattern of FSGS not otherwise specified. The mean age for HIVAN and idiopathic FSGS was 10 ± 3.62 (range: 6.00 – 19.00) and 9 ± 3.11 (range: 4.00 – 13.00) years respectively (Table 1). The control groups consisted of 40 children with no kidney disease; 20 (27%) children were HIV negative with a mean \pm SD age of 7 ± 3.87 (range: 2.00 – 14.00) years and 20 (27%) children HIV positive with a mean of 11 ± 3.52 (range: 5.00 – 15.00) years.

The patients with known FSGS had stages 1 and 4 CKD according to the KDIGO classification [9]. In the idiopathic FSGS group 11 patients had CKD stage 1; 4 stage 2; 2 stage 3; and 3 stage 4. In the HIVAN group 8 patients were CKD stage 1, 1 stage 2, 3 stage 3 and 2 stage 4. These patients were diagnosed with FSGS for a mean of 2.8 years with a range of 2.1-4.3 years prior to study entry. Kidney biopsy showed FSGS (not otherwise specified) in all patients with over 80% of glomeruli having more than 50% sclerosis.

To determine the association with the variability, we compared urinary protein concentration of β_2 M and Cystatin C with age, weight, creatinine, urine creatinine, eGFR, urea, albumin and cholesterol in the four groups of children. No statistical significant correlation was observed in β_2 M and Cystatin C when compared to the above clinical and biochemical findings. This indicates that these factors had no major impact on the concentration of these urinary proteins in children.

Urinary concentrations β_2 M and Cystatin C

The urinary concentration of β_2 M and Cystatin C as displayed in Figures 1a and 1b, respectively.

A statistical significant increase was observed in urinary β_2 M excretion in the HIVAN (mean = 169.7 ng/ml; 95% CI: 272.3-67.15) group compared to the HIV negative control (mean = 52.15ng/ml; 95% CI: 75.19-29.10) (Mann Whitney $U= 75.00$; $p= 0.0240$). No other statistical significant differences of urinary β_2 M concentrations were noted across the study groups (Table 2).

There was a significant increase of Cystatin C in the idiopathic FSGS group (mean = 987.7 ng/ml; 95% CI: 1689-286.7) compared to HIV negative control group (mean = 87.49 ng/ml; 95% CI: 144.0-30.97) (Mann-Whitney $U= 93.50$; $p= 0.0041$). There was also a significant decrease in the HIVAN group (mean = 203.5 ng/ml; 95% CI: 400.5-6.522) compared to the idiopathic FSGS group (mean = 987.7 ng/ml; 95% CI: 1689-286.7) (Mann-Whitney $U= 70.00$; $p=0.0150$). Once again a statistical significant increase in idiopathic FSGS groups (mean = 987.7 ng/ml; 95% CI: 1689-286.7) compared to the HIV positive control (mean = 104.5 ng/ml; 95% CI: 152.5-56.41) (Mann Whitney $U= 117.0$; $p = 0.0256$).

Cystatin C levels were down-regulated in the HIVAN group (mean = 203.5 ng/ml; 95% CI: 400.5-6.522) compared to the HIV negative control group (mean = 87.49 ng/ml; 95% CI: 144.0-30.97) however, this did not reach a statistical significance (Mann-Whitney $U= 139.0$; $p= 0.9860$). A non-significant decrease (Mann Whitney $U= 117.0$; $p= 0.4311$) was observed in the HIVAN group (mean = 203.5 ng/ml; 95% CI: 400.5-6.522) compared to the HIV positive control group (mean = 104.5 ng/ml; 95% CI: 152.5-56.41).

The expression of urinary albumin was significantly different between idiopathic FSGS (mean= 135400 ng/ml; 95%CI: 170700-100100) compared to HIV negative (mean = 15380 ng/ml; 95%CI: 35860- -470.6; $p < 0.0001$). A statistical significance of albumin was observed in idiopathic FSGS (mean =135400 ng/ml; 95% CI: 170700-100100) compared to HIV positive (mean = 17690 ng/ml; 95% CI: 35860- -470.6; $p < 0.0001$). Urinary albumin was also significant in HIVAN (mean = 86530 ng/ml; 95% CI: 135500-37600) compared to HIV positive control (mean = 17690 ng/ml; 95% CI: 35860- -470.6; $p = 0.0206$). Similarly, urinary albumin was significantly dysregulated between HIVAN (mean = 17690 ng/ml; 95% CI: 35860- -470.6) compared to negative control groups negative (mean = 15380 ng/ml; 95%CI: 35860- -470.6; $p = 0.0056$) (Figure 2).

a)

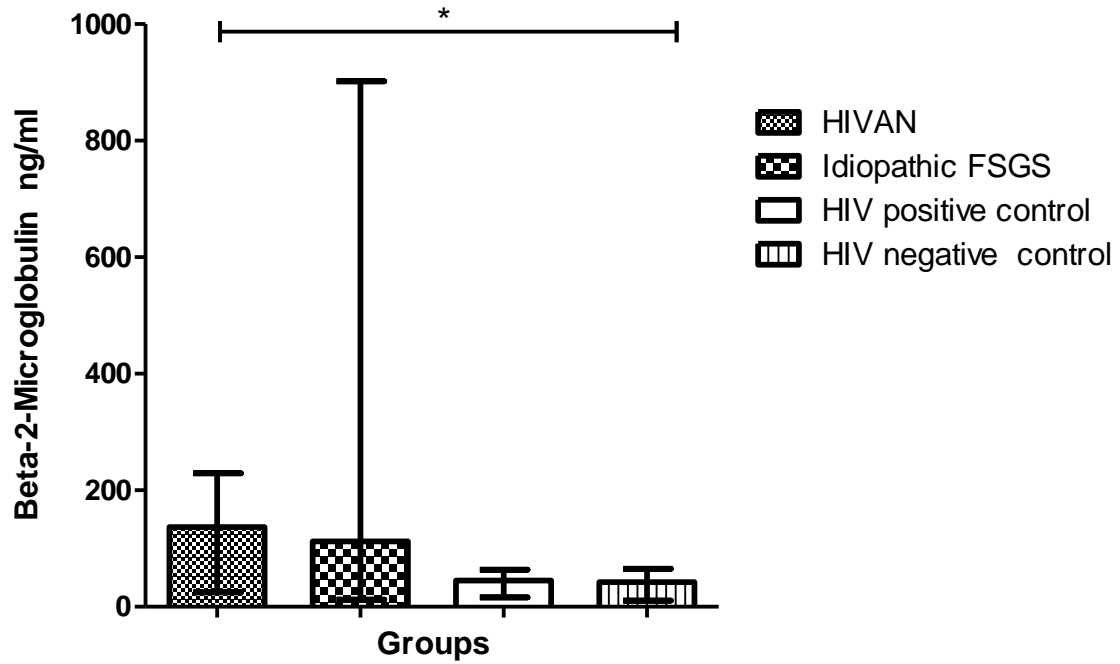


Figure 1(a): The urinary concentration of β_2M in HIVAN, idiopathic FSGS, HIV negative and HIV positive control groups. Results are represented by median and interquartile range. *The concentration of β_2M is significantly different between HIVAN and HIV negative controls, $p=0.0240$.

(b)

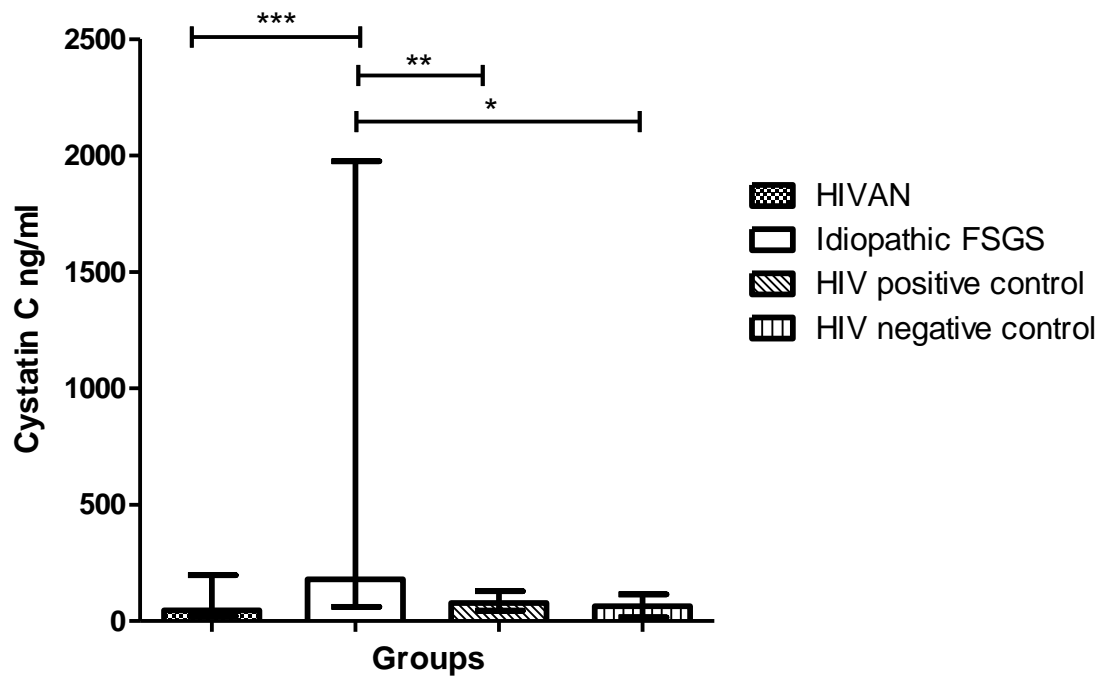


Figure 1(b): The urinary concentration of Cystatin C in HIVAN, idiopathic FSGS, HIV positive, and HIV negative control groups. Results are represented by median and interquartile range. *Urinary concentration of Cystatin C is significantly different between idiopathic FSGS and HIV negative controls, $p=0.0131$; **idiopathic FSGS and HIV positive controls, $p= 0.0256$; and *** HIVAN and idiopathic FSGS, $p= 0.0150$.

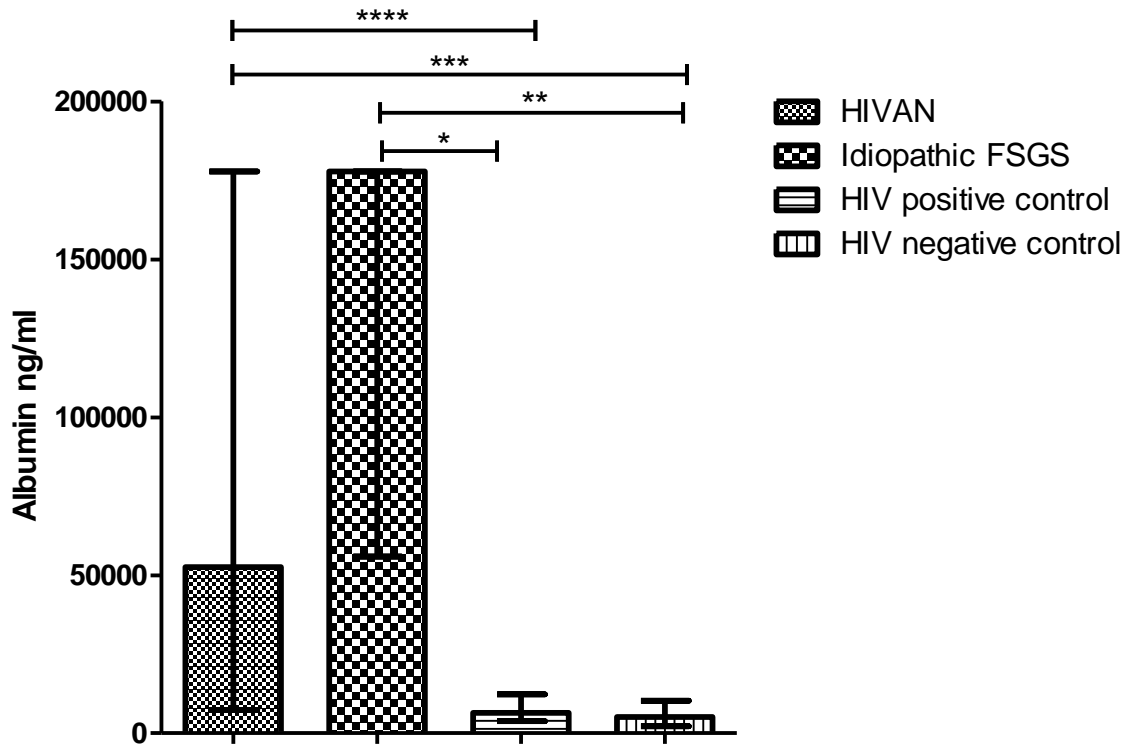


Figure 2: The urinary concentration of albumin in HIVAN, idiopathic FSGS, HIV positive, and HIV negative control groups. Results are represented by median and interquartile range. *Urinary concentration of albumin is significantly different between idiopathic FSGS and HIV positive $p < 0.0001$; **idiopathic FSGS and HIV negative controls, $p < 0.0001$; ***HIVAN and HIV negative $p = 0.0056$; ****HIVAN and HIV positive controls, $p = 0.0206$.

Table 1: Clinical and laboratory demographics of patients, expressed as mean \pm standard deviations.

	Sample Groups			
	HIV Negative Control n = 20	HIV Positive Control n = 20	Idiopathic FSGS n = 20	HIVAN n = 14
Sex (Male/Female)	Male	Male	Male	Male
Age (years)	7 \pm 3.87	11 \pm 3.25	9 \pm 3.11	10 \pm 3.62
Weight (kg)	18.20 \pm 10.55	37.25 \pm 13.73	29.04 \pm 10.33	31.13 \pm 11.57
Creatinine (mol/l)	29.14 \pm 9.25	40.10 \pm 9.25	45.32 \pm 15.44	82.83 \pm 62.36
Urine Creatinine (mmol/l)	2.38 \pm 0.42	-	6.71 \pm 4.50	4.05 \pm 2.68
Protein	1.85 \pm 0.67	-	4.22 \pm 4.21	3.07 \pm 2.04
eGFR (ml/ min /1.73m)	218.00 \pm 122.01	207.13 \pm 58.02	125.09 \pm 102.53	119.98 \pm 57.84
Urea blood (mol/l)	2.71 \pm 1.58	5.34 \pm 9.81	7.64 \pm 7.02	5.30 \pm 4.26
Albumin (mg/dl)	29.91 \pm 14.76	28.96 \pm 7.96	30.77 \pm 10.94	37.30 \pm 9.25
Cholesterol (mmol/l)	-	3.75 \pm 0.68	8.21 \pm 4.80	4.82 \pm 2.36
CD4 count		900.60 \pm 620.00	-	820.20 \pm 642.10

Table 2: The mean concentration of β_2 M and cystatin C in study groups versus controls.

	Comparison			Mean(ng/ml) (95% CI: upper and lower)			Mann Whitney U	P value
β_2 M	HIVAN	vs	HIV negative control	169.7(272.3- 67.15)	vs	52.15 (75.19- 29.10)	75.00	0.0240*
	HIVAN	vs	HIV positive control	169.7(272.3- 67.15)	vs	85.94(143.5-24.34)	88.0	0.0715
	Idiopathic FSGS	vs	HIV negative control	383.1 (605.3-161.0)	vs	52.15(75.19- 29.10)	138.0	0.0962
	Idiopathic FSGS	vs	HIV positive control	383.1 (605.3-161.0)	vs	85.94(143.5-24.34)	144.5	0.1367
	HIVAN	vs	Idiopathic FSGS	169.7 (272.3- 67.15)	vs	383.1(605.3-161.0)	140.0	0.9860
Cystatin C	HIVAN	vs	HIV negative control	203.5 (400.5-6.522)	vs	87.49 (1440- 30.97)	139.0	0.9860
	HIVAN	vs	HIV positive control	203.5 (400.5- 6.522)	vs	104.5 (152.5-56.41)	122,0	0,1600
	Idiopathic FSGS	vs	HIV negative control	987.7 (1689-286.7)	vs	87.49 (144.0- 30.97)	93.50	0.0041*
	Idiopathic FSGS	vs	HIV positive control	987.7 (1689-286.7)	vs	104.5 (152.5-56.41)	117.0	0.0256*
	HIVAN	vs	Idiopathic FSGS	203.5 (400.5-6.522)	vs	987.7(1689-286.7)	70.00	0.0150*

Discussion

In this study, we report on two candidate biomarkers ($\beta_2\text{M}$ and Cystatin C) in HIVAN (all presenting as classical variants of FSGS on histopathology) and idiopathic FSGS compared to HIV positive and negative controls.

The expression of urinary $\beta_2\text{M}$ was significantly upregulated in HIVAN as compared to HIV negative control groups but was not so when comparing HIVAN to HIV positive controls or idiopathic FSGS. A study by Nishijima *et al*, reported high levels of $\beta_2\text{M}$ and $\alpha_1\text{M}$ as biomarkers in the detection of kidney tubulopathy in patients with HIV-1 infection [17]. The latter study however, was not FSGS related. A study conducted by Garcia *et al*, reported an increase in $\beta_2\text{M}$ in urine of children with HIVAN [18].

Of note, in kidney disease, urinary $\beta_2\text{M}$ is generally elevated, reflecting tubular dysfunction [19]. In healthy individuals, due to the low molecular mass of these proteins, they are easily filtered through the glomerular filtration apparatus, and are reabsorbed in the proximal convoluted tubules [19]. These results indicate that the upregulation of $\beta_2\text{M}$ noted in our study may be attributed to either abnormal glomerular filtration, or tubular dysfunction in children with HIVAN. In idiopathic FSGS there is also tubular involvement to varying degrees [20]. But it is possible that the degree of tubular dysfunction may not have been sufficient in our group of patients to show significant differences between this group and healthy controls. A study by Kim and Lim, reported higher levels of $\beta_2\text{M}$ in children with FSGS [21]. This finding may be attributed to proximal convoluted tubular pathology where they are unable to absorb and transfer $\beta_2\text{M}$ back into the interstitial capillaries and into the general circulation.

Donadio, reported a significant elevation of urinary $\beta_2\text{M}$ concentrations in patients with chronic kidney disease at stage 4 and 5 [22]. It is also documented that high $\beta_2\text{M}$ is evident in kidney infection, chronic kidney failure and various connective tissue diseases [19]. Also, this outcome in our study may be due to the small sample size that was used, making it difficult to detect significant differences across the groups.

In our study we also report a statistical significant increase of urinary Cystatin C concentration in idiopathic FSGS group compared to the HIV negative control group, as well as the idiopathic FSGS compared to the HIV positive control group.

In a study on lupus nephritis, a condition that causes glomerular injury similar to FSGS, Tony *et al*, observed a significant increase of urinary Cystatin C excretion in patients with lupus nephritis compared to controls [23]. Further, Donadio reported a significant elevation of urinary Cystatin C concentrations in patients with CKD at stage 4 and 5, compared to individuals with normal GFR [22]. These findings on elevated Cystatin C excretion were similar to our study. Urinary Cystatin C proteins are known to protect tissues and cells from damage due to intracellular enzymes released from apoptosis or malignancy. However, when glomerular sclerosis is present, the levels of Cystatin C may increase [14], as was observed in our study.

We report a dysregulation, specifically a significant down regulation of urinary Cystatin C levels in the HIVAN group compared to the idiopathic FSGS group. In contrast to our study, elevated levels of Cystatin C in the urine of HIV-infected children with proteinuria have been reported by Garcia *et al*, suggesting a compromised capacity of the proximal tubular epithelial cells to reabsorb and metabolize Cystatin C in these patients [24, 25]. This apparent contradiction with our study may also be explained by a discordance between immunological and clinical stage of the HIV disease [26].

Nonetheless, in one study, serum Cystatin C, which reflects renal dysfunction directly correlated with HIV viral load [26]. On the other hand, patients with a very low viral load, including those receiving kidney transplants, may also develop HIVAN [25]. Further, even though Cystatin C is a potent marker for inflammation and kidney disease, it has also been shown to have antiviral activity [27, 28]. It is plausible, that in HIV infected patients, as in our cohort, there may have been a downregulation of serum Cystatin C due to its interaction with the virus, and hence low urinary Cystatin C excretion.

The limitations of our study were sample size and absence of viral load, hence we were not able to correlate our data with the severity of HIV infection. Future investigations should also consider an assessment of nutritional state.

Conclusion

This study demonstrates highly significant levels of urinary Cystatin C expression. However, β_2M was only significantly different between HIVAN and the HIV negative controls. Therefore only urinary Cystatin C proteins may be considered a suitable biomarker for the non invasive testing in the early detection of idiopathic and HIV – associated FSGS in paediatric subjects.

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CHAPTER THREE

Synthesis

Children may have a range of chronic and acute diseases complicated by HIV-infection. Chronic kidney disease may be caused by the virus itself, and this may take a form of HIVAN or HIV – associated immune complex kidney disease (HIVICK) (Samarawickrama *et al.*, 2012). FSGS is a histopathological diagnosis that is made on kidney biopsy (Jindal *et al.*, 2017, Giorgi, 2016). With the progression of time, the immune system becomes further compromised in HIV individuals, many develop end stage kidney disease (ESKD), and other co-morbidities such as diabetes and hypertension (Samarawickrama *et al.*, 2012). It is very important that the correct diagnosis is made as early as possible so that appropriate treatment may be effected to halt the progression to ESKD (Herrero-Morín *et al.*, 2007). In a resource poor settings, biopsy of the kidney is often delayed (Ekulu *et al.*, 2016). Therefore, urinary biomarkers for FSGS may present an excellent opportunity to either guide the healthcare practitioner to motivate for a biopsy, or to offer the patient empirical treatment where the biopsy cannot be done.

Albumin excreted via the kidney glomeruli are generally reabsorbed back into the renal tubules. However, trace amounts of albumin may be found in the urine due to lack of tubular capacity (Samarawickrama *et al.*, 2012). In patients with HIVAN or HIVICK, the glomerular structure is damaged. This has a major impact on filtration and reabsorption of proteins. Therefore, higher concentrations of albumin are released in urine. This manifestation in children and adults is referred to as albuminuria (Samarawickrama *et al.*, 2012).

The upregulation of albumin proteins in our study demonstrates a significant difference between the FSGS in contrast to controls. A previous study, reported that albuminuria in school children was significantly associated with ESKD (Lin and Huang, 2016). Similarly, Fredrick (2012), also observed high albuminuria rates in children (Fredrick *et al.*, 2012). Despite varying methods, Ikpeme *et al.*, report that urine albumin/creatinine ratio, ($p = 0.03$), could be used for the early detection for HIVAN (Ikpeme *et al.*, 2012). Hence, in a resource restricted environment such as KwaZulu-Natal, such a test could be useful for HAART initiation.

Notwithstanding, albuminuria being an established finding in nephrotic syndrome, we demonstrated a correlation across the proteins where $\beta_2\text{M}$ and Cystatin C accurately predict the same diagnosis.

In relation to albumin and $\beta_2\text{M}$ we found that there was a significant correlation ($r = 0.4220$, $p < 0.0129$). This implies, that where albumin predicts FSGS, $\beta_2\text{M}$ confirms the presence of FSGS. Other studies indicated, both urinary albumin and $\beta_2\text{M}$ excretion may be used as markers for kidney or tubular diseases (Peterson et al., 1969, Tomlinson et al., 1997).

In relation to albumin and Cystatin C we found that there was a significant correlation ($r = 0.6761$, $p < 0.0001$). This implies, that where albumin predicts FSGS, Cystatin C confirmed the presence of FSGS. Similar findings by Aygun *et al*, reported that as GFR decreases, there would be an increase in both Cystatin C and albumin. However, this study was conducted in children with sickle nephropathy (Aygun et al., 2011). Similarly, Nejat *et al* observed an inverse relationship between albumin & Cystatin C with eGFR (Nejat et al., 2011, Thielemans et al., 1994).

Under normal physiological conditions, $\beta_2\text{M}$ is continuously produced by the body, and is eliminated mainly by the kidney (Wibell, 1978). After filtering through the glomerulus, these low molecular weight proteins are almost totally reabsorbed into the renal tubules where they are broken down by lysosomes of the renal tubular cells, and finally excreted into the urine (Wibell, 1978, Hong and Lim, 2012). In tubular disorders this process is impaired, and urinary excretion of $\beta_2\text{M}$ is increased (Hong and Lim, 2012). An increased serum creatinine level, indicating kidney impairment, is a measurement of the function of different compartments of the kidney. But an increase in $\beta_2\text{M}$ in the urine reflects proximal tubular injury (Zeng *et al.*, 2014). The presence of $\beta_2\text{M}$ proteins in the urine demonstrates an essential defect in proximal tubules, and it may manifest in conditions of parenchymal damage or lack of capacity to reabsorb due to overload (Ferguson *et al.*, 2008).

In our study, $\beta_2\text{M}$ expression was significantly different, being upregulated in the HIVAN group compared to HIV negative control group. This observation concurred with previous reports of an increase in $\beta_2\text{M}$ of children diagnosed with HIVAN and HIV – Hemolytic Uremic Syndrome (Soler-García *et al.*, 2009a). On the other hand, elevated concentrations of $\beta_2\text{M}$ was noted in HIV positive

children who were on a treatment regimen containing Tenofovir (Papaleo *et al.*, 2007). Individuals on HAART, particularly those that include Tenofovir, for extensive periods of time, may encounter renal complications such as tubular dysfunction (Gatanaga *et al.*, 2006).

Urinary β_2 M concentrations, in our study were elevated in children with idiopathic FSGS compared to HIV positive and negative controls, albeit non significant. Moreover, as early as 1986, an increase in urinary β_2 M was shown in children with FSGS (Portman *et al.*, 1986). Likewise, a study by Kim and Lim, reported similar findings of higher levels of β_2 M in children with FSGS (Kim and Lim, 2007). Also high levels of urinary β_2 M occur in children with nephrotic syndrome in comparison with healthy controls (Çalışkan *et al.*, 1996). Elevated β_2 M strongly correlated with proximal tubular injury in adults, with a high sensitivity of 86.3% (Zeng *et al.*, 2014).

Overall, the β_2 M concentrations were high in urine of children with regards to HIV status and FSGS in comparison with controls. Despite a low molecular weight of β_2 M, glomerular damage impacts on the movement of β_2 M across the glomerular basement membrane. Glomerular damage may be as a consequence of kidney infection, chronic kidney failure and various connective tissue disorders. Since the kidney is unable to reabsorb β_2 M back into the blood stream, it is possible that they are excreted into the urine due to lack of tubular reabsorption capacity (Sonkar and Singh, 2008).

Like β_2 M, Cystatin C is a plasma protein with a low molecular weight that are reabsorbed and catabolised by the proximal tubules. Hence, under normal physiological conditions, low concentration of Cystatin C is observed in urine but it remains stable in the blood stream (Deyà-Martínez *et al.*, 2016). Cystatin C proteins are known to protect tissues and cells from damage due to intracellular enzymes released from cell apoptosis or malignant cells (Deyà-Martínez *et al.*, 2016). Studies show that Cystatin C is a better alternative marker to assess kidney function (Shlipak *et al.*, 2013).

In our study, a statistically significant increase of the expression of Cystatin C in urine was observed in idiopathic FSGS compared to HIV positive and negative controls. Similarly, study in adults by Conti *et al.*, reported a high significant difference ($p < 0.0001$) of urinary Cystatin C in patients with tubular disease (Conti *et al.*, 2006).

In our study, we reported a low significant urinary cystatin C expression in the HIVAN group compared to the idiopathic FSGS group. A study focusing on growth factors as biomarkers for HIVAN prediction in children reported a significant difference of cystatin C ($p < 0.05$) in children with renal disease (Soler-García *et al.*, 2009b). However, when kidney injury is present, the levels of cystatin C may increase (Deyà-Martínez *et al.*, 2016). In our study it is plausible that cystatin C levels may be affected by HAART regimen or other medication, viral load and other systemic infection. Hence a higher concentration of cystatin C was expected in the HIV positive study group.

Overall the idiopathic FSGS study population demonstrated elevated concentrations of cystatin C in comparison with the other groups. Irrespective of HIV status and FSGS, urinary and serum cystatin C concentrations are known to be closely linked to kidney function and, in individuals with kidney impairment, these proteins are elevated (Robles *et al.*, 2017).

Alternatively, and unrelated to our study, it has been suggested that serum cystatin C may be used an alternative biomarker for further assessment of kidney disease regardless of HIV status (Randers *et al.*, 1998). Herrero-Morin *et al.*, also affirm that serum cystatin C could possibly be used as a biomarker for GFR (Herrero-Morín *et al.*, 2007).

We found that there was a significant correlation between β_2 M and cystatin C in FSGS ($r = 0.6596$, $p < 0.0001$). This implies, that β_2 M may be used as a predictor of FSGS and cystatin C as a confirmatory test or vice versa. A previous study reported that increased urinary cystatin C and β_2 M could be diagnostic of glomerular injury, but was observed in rats with drug induced glomerular injury. (Dieterle *et al.*, 2010, Nejat *et al.*, 2011). Similarly Behairy *et al.*, (2017), observed cystatin C and β_2 M as specific and sensitive markers of screening tubular and glomerular dysfunction in children with beta thalassemia (Behairy *et al.*, 2017). Similarly, a correlation was found in a cohort of HIV infected children, by Deyaz *et al.*, where β_2 M was directly related to cystatin C and GFR was inversely related to cystatin C (Deyà-Martínez *et al.*, 2016).

There were several confounding factors in our study. Firstly, the small sample size may have prevented cystatin C reaching a statistical significance. Secondly viral load and the presence of

systemic infection was not documented in all children, hence we were not able to correlate our data with the severity of HIV infection or other systemic co-infection. Furthermore, since HAART is standard of care in South Africa, its nephrotoxic effects may have confounded protein expression in the urine. Also, further investigations should also consider an assessment of nutritional state.

Urinary biomarkers need to have certain characteristics that make them suitable for use in evaluating kidney disease. They should be cost effective, so that they may be easily available in a resource poor environment. Also these biomarkers should have high specificity and high sensitivity for accurate diagnostic purposes. Further, they should be able to be of prognostic value.

Finally, this study demonstrates significantly high concentrations of Cystatin C expression, however, β_2 M was significantly different between HIVAN in comparison with the healthy controls only. Therefore urinary Cystatin C proteins may be considered suitable biomarker for non invasive testing in the early detection of idiopathic and HIV – associated FSGS in paediatric subjects.

CHAPTER FOUR

References

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Appendix

