

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

K Persad,¹ MMedSci; L Nandlal,¹ PhD; R Bhimma,² PhD, MD; T Naicker,¹ PhD

¹Discipline of Optics and Imaging, College of Health Sciences, Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa

²Department of Paediatrics and Child Health, Nelson R. Mandela School of Medicine, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

Corresponding author: T Naicker (naickera@ukzn.ac.za)

Background. Africa has the highest rate of HIV infection, and HIV-associated nephropathy (HIVAN) is one of the most frequent kidney diseases observed in children. HIVAN in children usually presents as a form of nephrotic syndrome, predominantly focal segmental glomerulosclerosis (FSGS) on histopathology, that often leads to chronic kidney failure.

Objective. This study determined the urinary concentrations of β -2-microglobulin (β 2M) and cystatin C proteins in children with HIVAN and primary FSGS.

Methods. The study group comprised 34 children; 14 with HIVAN and 20 with primary 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 the urinary protein concentration of cystatin C and β 2M.

Results. A significant increase in urinary β 2M levels was observed in the HIVAN group compared with the HIV-negative group ($p=0.0240$). No other statistically significant differences in urinary β 2M concentrations were noted across the study groups. Urinary cystatin C levels were significantly increased in primary FSGS children compared with both HIV-negative ($p=0.0041$) and HIV-positive controls ($p=0.0256$). Urinary cystatin C displayed a significant increase in the primary FSGS compared with the HIVAN group ($p=0.0150$). No significant differences in urinary cystatin C levels were noted in the HIVAN group compared with the HIV-negative and HIV-positive control groups.

Conclusion. Urinary cystatin C has promising prognostic value to predict primary FSGS from HIVAN.

Keywords. Biomarkers, paediatric, FSGS, HIV, urinary proteins.

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Africa has the highest incidence of HIV infection, affecting ~37.6 million people.^[1] Moreover, 1.7 million children (<14 years old) are HIV infected, with a further 160 000 being newly infected annually.^[1] Currently, 1.7 million children (<15 years old) are infected with HIV, most of these infections occurring in Africa.^[2] Notably, a 50% decrease in new paediatric HIV infections has been reported owing to access to antiretroviral therapy (ART).^[3] Globally, kidney disease is rapidly becoming a major public health concern as a cause of morbidity and mortality in children.^[3] Kidney disease in the setting of HIV that is untreated often leads to rapid progression to chronic kidney failure (CKF).^[4]

In one of the largest studies of HIV-related kidney diseases in children in Africa, Ramsuran *et al.*^[4] reported that nephrotic syndrome owing to HIV-associated nephropathy (HIVAN) is the most common form of kidney disease in the setting of HIV. The prevalence of HIVAN has increased in both adults and children.^[5] Previous studies have indicated that the proportion of children with HIVAN was 10 - 15%.^[2,6]

Focal segmental glomerulosclerosis (FSGS) is a common histopathological form of primary steroid-resistant nephrotic syndrome with a high propensity for progression to CKF in children and adolescents.^[7] Approximately 30% to 60% of patients who are

diagnosed with primary FSGS are likely to progress to CKF over a period of 5 - 10 years.^[8] While a kidney biopsy is the gold standard for providing a 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 arterio-venous fistula formation. The current strategy for detection and monitoring the effect of treatment in kidney diseases in children and adults includes the utilisation of serum creatinine levels.^[9,10] However, several factors limit its use, as serum creatinine values are influenced by protein intake, nutritional status, muscle mass and body weight, all of which are affected in HIV-infected children and primary nephrotic syndrome. Hence, the use of a non-invasive biomarker to detect kidney disease is urgently warranted.^[11]

β -2-microglobulin (β 2M) is an 11kDa polypeptide protein that is freely filtered in the glomeruli and reabsorbed and metabolised in the proximal tubule. Although urinary excretion of β 2M is an indicator of underlying kidney disease, it is nonspecific as increased urinary excretion may occur in other diseases such as autoimmune diseases, malignancies and especially in AIDS.^[12] There is, however, a lack of data on the reliability of β 2M as a biomarker to differentiate between various forms of kidney disease.

Cystatin C is a 13kDa plasma protein that functions as a cysteine protease inhibitor,^[13] and its dysregulation has been implicated in the detection of impaired kidney function.^[14] In patients with a kidney transplant, diabetes mellitus and chronic kidney disease (CKD), cystatin C is found much earlier in the urine than creatinine. The measurement of blood cystatin C is believed to be of better prognostic value than creatinine for estimating the glomerular filtration rate (GFR), especially in patients with more advanced kidney disease who have a GFR <60 mL/min/1.73 m². This has successfully been demonstrated in children with kidney disorders caused by various conditions.^[15]

To evaluate the accuracy of β 2M and cystatin C as predictors of kidney disease in HIV-infected children, notably, HIVAN, we compared the urinary levels of β 2M and cystatin C in children with HIVAN and primary FSGS with 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 (SA). 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 ~3 months until analysed.

Study population

Seventy-four black SA children aged 1 - 16 years were recruited. The study group ($N=34$) consisted of children with biopsy-proven HIVAN ($n=14$) and primary FSGS ($n=20$). At the time of sample collection, none of the children had a fever or any other evidence of secondary infections. In children with HIVAN, comorbidities 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 combined ART and angiotensin-converting enzyme antagonists for a minimum of 2 years. At the time of sample collection, the 20 children with primary FSGS were on low-dose steroids, angiotensin-converting enzyme inhibitors as well as additional immunosuppressants such as calcineurin inhibitors (cyclosporin or tacrolimus) and/or mycophenolate mofetil.

Diagnosis of HIVAN

The classification of HIVAN was based on the confirmation of HIV-1 infection and presence of persistent proteinuria $\geq 1+$ on urinary dipstick examination with histopathological findings of FSGS and one or more of the following: (i) abnormal urinary sediment; (ii) presence of enlarged echogenic kidney on ultrasound; and (iii) microcystic tubular dilation.^[14,15]

BioPlex Multiplex

The urine samples were analysed for β 2M and cystatin C using the Bio-Plex Pro RBM kidney toxicity assay (panel 2) (Bio-Rad Laboratories, USA) according to the manufacturer's instructions.^[17]

The detection of reaction was carried out using the Bio-Plex MAGPIX 200 reader system (Bio Rad Laboratories, 2017). Raw data were collated using 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 (MFI) signal against concentration. These standards were used to interpolate the concentration of the unknown samples. Intra-plate variability was determined with CV <20% and (X100) between 70% and 130% ($r=0.8$, $p=0.05$). All data were imported to a Microsoft Excel (version 2018; Microsoft Corp., USA) 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, USA). One-way ANOVA and Dunn's 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 were represented as median and interquartile range.

Results

The study group comprised 34 children with biopsy-proven FSGS with a histopathological pattern not otherwise specified based on the Columbia classification.^[18] Fourteen children (41%) were HIV-positive and were confirmed to have paediatric HIVAN, and 20 children (59%) had primary FSGS. The mean age (standard deviation (SD)) for HIVAN and primary FSGS was 10 (3.62) years (range 6.00 - 19.00) and 9 (3.11) years (range 4.00 - 13.00), respectively (Table 1). The control groups consisted of 40 children with no kidney disease; 20 (50%) children were HIV-negative with a mean (SD) age of 7 (3.87) years (range 2.00 - 14.00) and 20 (50%) HIV-positive children with a mean age of 11 (3.52) years (range 5.00 - 15.00).

The patients with known FSGS had stages 1 to 4 CKD. In the primary FSGS group, 11 patients had CKD stage 1, 4 stage 2, 2 stage 3, and 3 stage 4, according to the KDIGO classification of CKD.^[10] 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 >80% of glomeruli having more than 50% sclerosis.

To determine the associations with the variability, we compared the urinary protein concentration of β 2M and cystatin C with age, weight, creatinine, urine creatinine, eGFR, urea, albumin and cholesterol in the four groups of children. No statistically significant correlation was observed in β 2M and cystatin C when compared with 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 2M and cystatin C

The urinary concentrations of β 2M and cystatin C are displayed in Figs 1a and 1b, respectively.

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

There was a significant increase of cystatin C in the primary FSGS group (mean=987.7 ng/mL, 95% CI 1 689 - 286.7) compared with the HIV-negative control group (mean=87.49 ng/mL; 95%

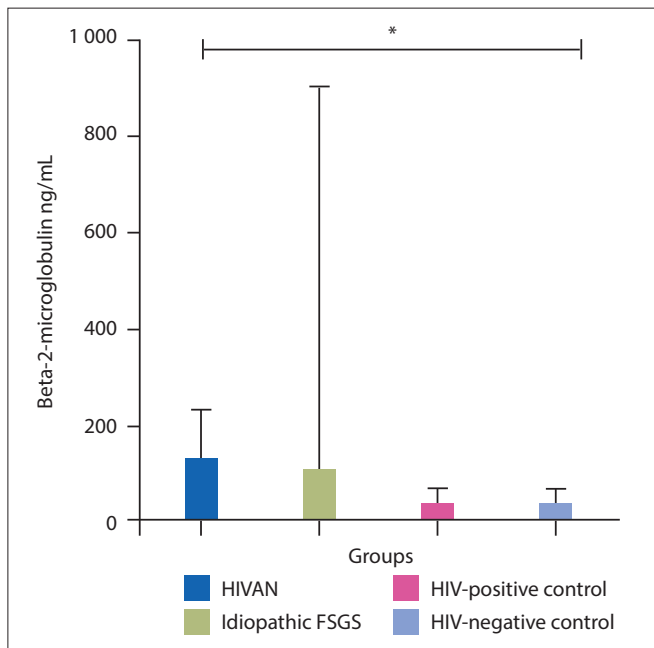


Fig. 1a. The urinary concentration of β 2M in HIVAN, primary FSGS, HIV-negative and HIV-positive control groups. Results are represented by median and interquartile range.

*The concentration of β 2M is significantly different between HIVAN and HIV-negative controls, $p=0.0240$.

(β 2M = β -2-microglobulin; HIVAN = HIV-associated nephropathy; FSGS = focal segmental glomerulosclerosis.)

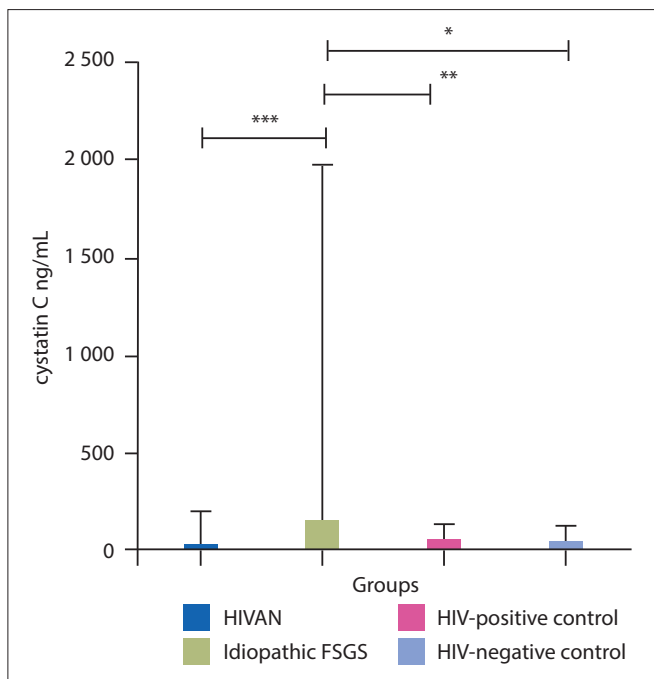


Fig. 1b. The urinary concentration of cystatin C in HIVAN, primary FSGS, HIV-positive and HIV-negative control groups. Results are represented by median and interquartile range.

*The urinary concentration of cystatin C is significantly different between primary FSGS and HIV-negative controls, $p=0.0131$.

**Primary FSGS and HIV-negative controls, $p=0.0256$.

***HIVAN and primary FSGS, $p=0.0150$.

(HIVAN = HIV-associated nephropathy; FSGS = focal segmental glomerulosclerosis.)

CI 1 44.0 - 30.97) (Mann-Whitney $U=93.50$; $p=0.0041$). There was a statistically significant increase in the primary FSGS group (mean=987.7 ng/mL, 95% CI 1 689 - 286.7) compared with the HIV-positive control (mean=104.5 ng/mL, 95% CI 152.5 - 56.41) (Mann-Whitney $U=117.0$; $p=0.0256$). There was also a significant increase in the primary FSGS group (mean=987.7 ng/mL, 95% CI 1 689 - 286.7) compared with the HIVAN group (mean=203.5 ng/mL, 95% CI 400.5 - 6.522) (Mann-Whitney $U=70.00$; $p=0.0150$).

Cystatin C levels were down-regulated in the HIVAN group (mean=203.5 ng/mL, 95% CI 400.5 - 6.522) compared with 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 with the HIV-positive control group (mean=104.5 ng/mL, 95% CI 152.5 - 56.41).

Discussion

In this study, we report on two candidate urinary biomarkers (β 2M and cystatin C) in HIVAN and primary FSGS (all presenting as not-otherwise-specified variants of FSGS on histopathology) compared with HIV-positive and -negative controls.

The expression of urinary β 2M was only significantly upregulated in HIVAN compared with the HIV-negative control group. Our results are corroborated by previous studies by Nishijima *et al.* who reported high levels of β 2M and α ₁M as biomarkers in the detection of kidney tubulopathy in patients with HIV-1 infection.^[19] The latter study, however, was not on patients with HIVAN. A study conducted by Garcia *et al.* reported an increase in β 2M in urine of children with HIVAN.^[20]

Of note, in kidney disease, urinary β 2M is generally elevated, reflecting a dysfunction in proximal tubular reabsorption.^[21] In healthy individuals, owing 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.^[21] These results indicate that the upregulation of β 2M noted in our study may be attributed to either abnormal glomerular filtration or proximal tubular reabsorptive dysfunction in children with HIVAN. In primary FSGS, there is also tubular involvement to varying degrees.^[22] It is possible that the degree of proximal tubular dysfunction may not have been enough in our group of patients to show significant differences between this group and healthy controls. A study by Kim and Lim, however, reported higher urinary levels of β 2M in children with FSGS.^[23] This finding may be attributed to proximal convoluted tubular pathology where they are unable to absorb and transfer β 2M back into the interstitial capillaries and into the general circulation.

Donadio reported a significant elevation of urinary β 2M concentrations in patients with CKD at stage 4 and 5.^[24] It is also documented that high β 2M is evident in kidney infection, chronic kidney failure and various connective tissue diseases.^[21] 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 statistically significant increase of urinary cystatin C concentration in the primary FSGS group compared with the HIV-negative control group, as well as 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 with controls.^[25] Further, Donadio reported a significant elevation of urinary cystatin C concentrations

Table 1. Clinical and laboratory demographics of patients

Demographics	Sample groups			
	HIV-negative control, (mean (SD) n=20)	HIV-positive control, (mean (SD) n=20)	Primary FSGS, (mean (SD) n=20)	HIVAN, (mean (SD) n=14)
Sex (male/female)	Male	Male	Male	Male
Age (years)	7 (3.87)	11 (3.25)	9 (3.11)	10 (3.62)
Weight (kg)	18.20 (10.55)	37.25 (13.73)	29.04 (10.33)	31.13 (11.57)
Creatinine (mol/L)	29.14 (9.25)	40.10 (9.25)	45.32 (15.44)	82.83 (62.36)
Urine creatinine (mmol/L)	2.38 (0.42)	-	6.71 (4.50)	4.05 (2.68)
Protein	1.85 (0.67)	-	4.22 (4.21)	3.07 (2.04)
eGFR (mL/min/1.73 m ²)	218.00 (122.01)	207.13 (58.02)	125.09 (102.53)	119.98 (57.84)
Urea blood (mol/L)	2.71 (1.58)	5.34 (9.81)	7.64 (7.02)	5.30 (4.26)
Albumin (mg/dL)	29.91 (14.76)	28.96 (7.96)	30.77 (10.94)	37.30 (9.25)
Cholesterol (mmol/L)	-	3.75 (0.68)	8.21 (4.80)	4.82 (2.36)
CD4 count		900.60 (620.00)	-	820.20 (642.10)

eGFR = estimated glomerular filtration rate; FSGS = focal segmental glomerulosclerosis; HIVAN = HIV-associated nephropathy.

Table 2. The mean concentration of β 2M and cystatin C in study groups v. controls

Comparison		Mean (ng/mL) (95% CI upper and lower)		Mann-Whitney U	p-value
β 2M	HIVAN v. HIV-negative control	169.7 (272.3 - 67.15)	v. 52.15 (75.19 - 29.10)	75.00	0.0240*
	HIVAN v. HIV-positive control	169.7 (272.3 - 67.15)	v. 85.94 (143.5 - 24.34)	88.0	0.0715
	Primary FSGS v. HIV-negative control	383.1 (605.3 - 161.0)	v. 52.15 (75.19 - 29.10)	138.0	0.0962
	Primary FSGS v. HIV positive control	383.1 (605.3 - 161.0)	v. 85.94 (143.5 - 24.34)	144.5	0.1367
	HIVAN v. Primary FSGS	169.7 (272.3 - 67.15)	v. 383.1 (605.3 - 161.0)	140.0	0.9860
Cystatin C	HIVAN v. HIV-negative control	203.5 (400.5 - 6.522)	v. 87.49 (1440 - 30.97)	139.0	0.9860
	HIVAN v. HIV-positive control	203.5 (400.5 - 6.522)	v. 104.5 (152.5 - 56.41)	122.0	0.1600
	Primary FSGS v. HIV-negative control	987.7 (1 689 - 286.7)	v. 87.49 (144.0 - 30.97)	93.50	0.0041*
	Primary FSGS v. HIV-positive control	987.7 (1 689 - 286.7)	v. 104.5 (152.5 - 56.41)	117.0	0.0256*
	HIVAN v. Primary FSGS	203.5 (400.5 - 6.522)	v. 987.7 (1 689 - 286.7)	70.00	0.0150*

CI = confidence interval; FSGS = focal segmental glomerulosclerosis; HIVAN = HIV-associated nephropathy.

in patients with CKD at stage 4 and 5, compared with individuals with normal GFR.^[24] 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 owing to intracellular enzymes released from apoptosis or malignancy. However, when glomerular sclerosis is present, the levels of cystatin C may increase,^[15] as was observed in our study.

We demonstrated a significant down-regulation of urinary cystatin C levels in the HIVAN group compared with the primary 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 metabolise cystatin C in these patients.^[26,27] This apparent contradiction with our study results may also be explained by a discordance between the immunological and clinical stages of HIV disease as all patients were on ART.^[28]

Nevertheless, in one study, serum cystatin C, which reflects kidney proximal tubular dysfunction, directly correlated with HIV viral load.^[28] On the other hand, patients with a very low viral load, including those receiving kidney transplants, may also develop HIVAN.^[27] Further, even though cystatin C is a potent marker for inflammation and kidney disease, it has also been shown to have

antiviral activity.^[29,30] It is plausible that in HIV-infected patients, as in our cohort, there may have been a down-regulation of serum cystatin C owing to its interaction with the virus, and hence low urinary cystatin C excretion in the HIVAN group.

The limitations of our study were sample size and absence of viral load; hence we were not able to correlate our data with severity of HIV infection. The findings of this study may not apply to other population groups as this was a single-centre study in a homogeneous group of black African children. Also, all HIV-infected patients recruited into the study were on treatment, which could have affected the levels of urinary biomarkers studied. Future investigations should also consider an assessment of nutritional state.

Conclusion

This novel study demonstrates significant difference of urinary cystatin C expression in children with primary FSGS compared with HIVAN and with controls. Moreover, β 2M was significantly different between HIVAN v. HIV-negative controls. Larger prospective studies to determine the role of cystatin C in early detection of HIVAN, thus obviating the need for kidney biopsy, and allowing early institution of appropriate therapy, thereby improving clinical outcome and survival, are needed.

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