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Tubular Injury in Children with Steroid-resistant Nephrotic Syndrome

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Abstract Tubular lesion is frequently found in steroid-resistant nephrotic syndrome (SRNS) with massive proteinuria, which produces proximal tubular dysfunction. The presence of tubulointerstitial injury will assist the selection of therapy in nephrotic syndrome (NS) patients. Tubular injury can be diagnosed using tubular function test such as fractional excretion of magnesium (FE Mg) and urinary β2-microglobulin (β2M) in addition to kidney biopsy. The aim of this study was to compare the FE Mg and urinary β2M in children with SRNS and steroid-sensitive nephrotic syndrome (SSNS) in order to be able to detect the presence of tubular injury on SRNS. A cross-sectional study was conducted on children aged 2-15 years with SRNS and SSNS in remission. The urinary β2M and FE Mg were examined on 31 subjects of SRNS and SSNS in remission respectively. The urinary β2M was measured using *competitive binding enzyme immunoassay*. Serum and urinary magnesium and creatinine were measured using colorimetric and enzymatic colorimetry method respectively, afterwards FE Mg calculated with the formula: $\text{FE Mg} = \frac{\text{Urinary magnesium} / \text{Plasma magnesium}}{\text{Urinary creatinine} / \text{Plasma creatinine}} \text{x}100\%$. The mean of FE Mg in SRNS (2.34 (SD 1.37) %) was significantly higher than the SSNS in remission (1.59 (SD 0.85) %; p = 0.0065), as well the median of urinary β2M level in SRNS (0.50 (0.30-11.80) µg/mL) was higher than the SSNS in remission (0.40 (0.30-0.50) µg/mL; p <0.001). The urinary β2M was increased significantly in SRNS compared to SSNS (26/31 subjects *vs* 16/31 subjects, respectively; p = 0.007). The increase of FE Mg in SRNS is significantly higher than in SSNS in remission with a cut off point of 1.64% (21/31 subjects *vs* 11/31 subjects, respectively; p = 0.022). The FE Mg and urinary β2M level are higher in SRNS than SSNS in remission that indicate tubular injury in SRNS.

Keywords: *fractional excretion of magnesium, steroid-resistant nephrotic syndrome, tubular injury, urinary β2-microglobulin*

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1. Introduction

Resistance to steroid therapy happens in 10-20% of children with idiopathic nephrotic syndrome (NS) [1,2,3]. The main difference between the sensitive and resistant to steroids is that steroid-resistant NS (SRNS) is prone to develop a chronic kidney disease with higher risk of extra renal complication and adverse-effects from therapy [4]. Due to the poor prognosis, SRNS treatment remains a challenge in pediatric nephrology [2].

Steroid-resistant NS also affects the child's development. Research shows that exposure to glucocorticoids in the NS associated with impaired linear growth and suppression of the bone formation [5]. Another study showed SRNS children are frequently found with symptoms of emotional, behavioral and relationship problems with the same-age peers, and hyperactivity, when compared to other NS groups [6].

Tubular abnormalities have been reported in patients with NS [7]. Tubular lesions more often found in SRNS [8]. A study reported that 10 of the 12 SRNS pediatric patients had a tubulointerstitial injury in various levels histopathologically [9].

Proteinuria is toxic to the kidney tubular cells and causes tubulointerstitial lesions [10]. Furthermore, it is associated with tubular cell transdifferentiation into miofibroblast — a stage of renal fibrosis [11]. The increasing of proteinuria correlates with the tubular cell injury [12].

Clinical data indicates a prolonged and non-selective proteinuria induces tubulointerstitial injury [11]. Focal segmental glomerulosclerosis (FSGS) is the most common cause of SRNS, i.e. 58.8% of children with NS onset in all age groups [13]. In FSGS, tubulointerstitial abnormalities and proximal tubular dysfunction are common [14]. Proximal tubular dysfunction was also reported in SRNS with massive proteinuria [9]. The determination of the presence of tubulointerstitial injury will assist the selection of therapy in NS patients [3,15].

Tubulointerstitial injury is generally diagnosed based on the histopathological approach which has clinical limitation due to the disability to obtain kidney biopsy in every single case [16]. Recently, tubular function tests such as fractional excretion of magnesium (FE Mg) has been used to detect initial tubulointerstitial injury [16,17], as well as urinary β2-microglobulin (β2M) which is a method to identify tubular injury in renal disease [18]. Due to the limitation of renal biopsy, the increase in β2M excretion has been used to assess tubular dysfunction in glomerulonephritis [16,18]. Urinary β2M is a sensitive approach to screen kidney injury, especially tubular injury accompanied by glomerular disease [19]. A study shows that in SRNS, a significant increase in urinary β2M demonstrated proximal tubular cells dysfunction, which influenced by massive albuminuria [9]. A tubular function test with the mean of FE Mg reflects the tubular abilities to reabsorp filtered magnesium and to retain intraselular magnesium [17]. A normal FE Mg confirms an intact tubular function and is usually associated with an intact tubulointerstitial structure [15,16]. Fractional excretion of magnesium is a sensitive index to distinguish early lesions of tubulointerstitial fibrosis [17].

Among other tubular function tests, β2M has a higher specificity than any low molecular weight type of protein [18]. Moreover, the urinary β2M has a high sensitivity (86.3%) for detecting tubular injury [19]. Beta 2-microglobulin can be detected in the urine (proteinuria tubular) when there are an injury and a tubular reabsorption malfunction [20]. A study obtained a significant increase in the urinary β2M excretion in SRNS compared to steroid-sensitive NS (SSNS) [7]. However, urinary β2M measurement cannot be done in any laboratory and it is expensive. Tubular function test with a mean of FE Mg is sensitive to detect abnormalities in early structural and tubular function [16]. Each intracellular structural and epithelial tubules functional disorder will affect the reabsorption and loss of intracellular magnesium, thus increasing the FE Mg [21]. A high mean of FE Mg is reported to be directly correlated with the extent of the tubulointerstitial fibrosis [17]. The mean FE Mg on SRNS is significantly higher than the SSNS. The cost of FE Mg examination is not expensive and can be performed in any laboratory that can check magnesium and creatinine levels, so that the expected increase in the value of FE Mg can detect the earliest tubulointerstitial damage on SRNS [15].

The purpose of this study was to compare the FE Mg and urinary β2M in SRNS and SSNS in remission in order to be able to detect the presence of tubular injury on SRNS.

2. Methods

A cross-sectional study was conducted in the Child Health Department at Dr. Cipto Mangunkusumo National Public Hospital Jakarta, Ulin Regional Public Hospital Banjarmasin, Fatmawati Hospital and Harapan Kita Women and Children Hospital Jakarta in July to December 2015. The subjects were selected from all SRNS patients and SSNS in remission who were eligible for inclusion and exclusion criterias. The inclusion criterias were patients between the age of 2 to 15 year who had no previous consumption of medication which induced magnesium loss from urine, such as loop diuretics, thiazide, aminoglycoside, amphotericin B, cyclosporine and tacrolimus before taking urine sample, with informed consent from the subject or their parents. The exclusion criterias were patients with secondary NS, magnesium loss inducing conditions (diarrhea and burn) and magnesium supplementation.

A minimum sample size of 30 child for each group of SRNS and SSNS was calculated from the sample size formulation for hypothesis testing of the mean difference between two independent populations. Subjects were consecutively selected from in- and outpatients of the participating hospitals until the minimum sample size was reached. This study was ethically approved by the Ethical Committee of Faculty of Medicine Universitas Indonesia.

Patients' selection were based on the result of history taking, physical examination and traced medical records. A written informed consent was obtained from their parents. Patients who were eligible would had blood and urine sample collected for serum magnesium and creatinine, urinary magnesium and creatinine level, and also urinary β2M measurement.

From each patient, 3 mL of blood and 10 mL of spot urine was collected. The levels of magnesium in blood and urine were then measured using colorimetric method at 505 nm using ADVIA 1800 machine. The serum and urine creatinine levels were measured with enzymatic colorimetry method at 545 nm using ADVIA 1800 machine. The β2M in urine was measured using *competitive binding enzyme immunoassay* with *Microplate reader 680 series (Biorad®)*. The FE Mg in percentage will be calculated with the following formula [15,17]:

$$\text{FE Mg} = \frac{\text{Urinary magnesium} / \text{Plasma magnesium}}{\text{Urinary creatinine} / \text{Plasma creatinine}} \text{x100\%.}$$

Variable definition: Sensitive steroid NS in remission is NS patient with remission (receiving prednisone therapy of 2 mg/kg/day for maximum of 4 weeks) who are receiving alternating prednisone therapy or finishing their therapy, with no proteinuria or trace proteinuria (negative with dipstick or trace) for more than four consecutive weeks diagnosed by Pediatrician in Department of Child Health at the participating hospitals. Steroid-resistant NS is NS patients with no remission (receiving prednisone therapy of 2 mg/kg/day for 4 weeks), with proteinuria ≥ +2 (dipstick urine ≥ +2) for more than four consecutive weeks. Hipomagnesemia: Magnesium plasma level < 1.68 mg/dL. Normal FE Mg: ≤ 2.2%, increased FE Mg: >2.2%. Urinary β2M normal level: ≤ 0.3 µg/mL, increased urinary β2M level: > 0.3 µg/mL.

Bivariate analysis with independent t-test will be conducted for normally distributed data, while Mann-Whitney test will be use for non-normally distributed data. Bivariate analysis for categorical data will be conducted with chi square test. Correlation analysis with Pearson test and Spearman test for normal and non-normal data distribution, respectively. Sensitivity and specificity of the cut off point measured with *receiver operator curve* (ROC) and *area under curve* (AUC). Statistical significance of 5% will be used in the analysis (p<0,05). All analysis were conducted with *Statistical Product and Service Solution* (SPSS) for Windows version 20.0.

3. Results

Sixty two patients were eligible for the study, 28 patients from Child Health Department at Dr. Cipto Mangunkusumo National Public Hospital Jakarta, 24 patients from Ulin Regional Public Hospital Banjarmasin, 5 patients from Fatmawati Hospital and 5 patients from Harapan Kita Women and Children Hospital Jakarta, consisted of 31 patients each of SRNS and SSNS in remission groups. The overall male/female ratio was 2.1:1 and 2.8:1 for SRNS group. All of SSNS remission group have normal magnesium level, however, 3 out of 31 subjects of SRNS found to have hypomagnesemia. Characteristics and distribution of the subjects could be seen in Table 1.

The mean FE Mg in SRNS group was significantly higher than SSNS remission group (p=0.0065). While the median urinary β2M level in SRNS group was found to be higher than SSNS remission group (p<0.001) as could be seen in Table 2.

Table 1. Subject characteristics

Parameter	N	SRNS (n = 31)	SSNS remission (n = 31)
Sex : Male	42	23	19
Female	20	8	12
Age (median, min-max),year	62	8 (2-15)	10 (3-15)
Onset (median,min-max), year	62	4 (1-14)	5 (2-12)
Plasma creatinine (median, min-max), mg/dL	62	0.4(0.13-1.30)	0.4(0.23-0.82)
GFR: ≤ 60 mL/mnt/1,73m^2	1	1	0
> 60 mL/mnt/1,73m^2	61	30	31
Plasma magnesium (Mean, SD), mg/dL	62	1.95 (0.26)	2.18 (0.19)
Hipomagnesemia	3	3	0
Normal	59	28	31

Note: SD: Standard Deviation, SRNS: steroid-resistant nephrotic syndrome, SSNS: steroid-sensitive nephrotic syndrome

Table 2. FE Mg and urinary β2M levels

Parameter	SRNS (n=31)	SSNS remission (n=31)	*p*
FE Mg (mean, SD), %	2.34 (1.37)	1.59 (0.85)	0.0065*
β2M (median, range), µg/mL	0.50 (0.30-11.80)	0.40 (0.30-0.50)	<0.001**

Note: * Independent T-test, ** Mann-Whitney Test. FE Mg: fractional excretion of magnesium, β2M: β2-microglobulin, SD: Standard Deviation.

Table 3. Comparison of FE Mg and urinary β2M level between SRNS and SSNS groups

Parameter	SRNS n	SSNS remission n	*p*
FE Mg : Increase	15	8	0.066
Normal	16	23	
β2M : Increase	26	16	0.007
Normal	5	15	

Note: FE Mg: fractional excretion of magnesium, β2M: β2-microglobulin, SRNS: steroid-resistant nephrotic syndrome, SSNS: steroid-sensitive nephrotic syndrome.

In this study, an increase in urinary β2M level was observed in 26 of 31 (83.9 %) subjects in SRNS group and 16 of 31 subjects in SSNS remission group. There was a statistically significant difference in the proportion of the increase in urinary β2M level between two groups (x^2, p=0.007) (Table 3).

The prevalence ratio (PR) of the increase in urinary β2M level between SRNS and SSNS remission groups was 4.875 with 95% Confidence interval between 1.485 and 15.998 (p=0.007) showed an association of the increase in urinary β2M level with the incident of SRNS.

Similarly, the increase in FE Mg was observed in 15/31 subjects of SRNS group and 8/31 subjects of SSNS group. However, there was no statistical difference for the increase in urine FE Mg between these groups (p=0.066) as seen in Table 3.

From the ROC analysis, the AUC for FE Mg in predicting SRNS was 0.679 (*p* = 0.015, 95% CI (0.546-0.812). The cut-off point of 1.64% showed 64.5% and 64.5% for sensitivity and specificity, respectively (Figure 1). While the cut-off point of 2.2% gave sensitivity and specificity of 48.4% and 74.2%, subsequently.

With the cut-off point of 1.64, the proportion of subjects with the increase in FE Mg level (>1.64%) in SRNS group was higher than SSNS remission. In contrast, higher proportion of normal FE Mg level (≤1.64 %) was found in SSNS group than SRNS group. The chi square analysis revealed that there was a significant difference in the proportion of FE Mg level with cut-off point of 1.64% (p=0.022) as shown in Table 4. The PR of FE Mg level in SRNS compare to SSNS remission was 3.306 (*p* = 0.022, 95% CI (1.168-9.357)).

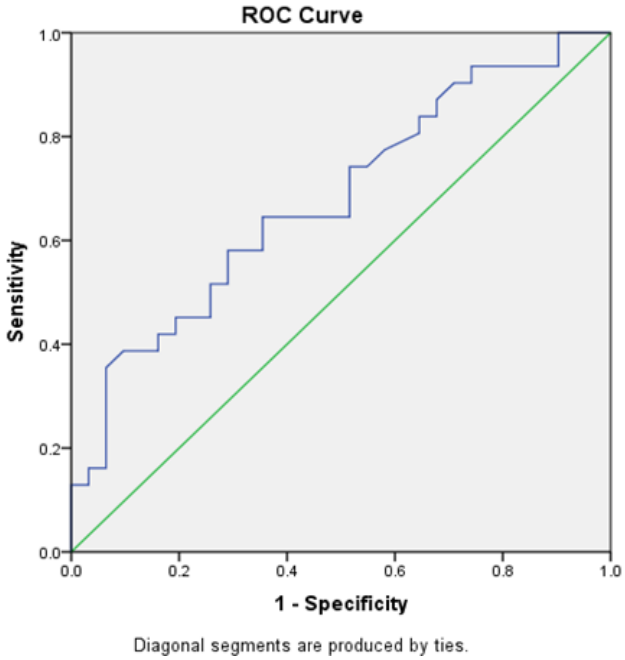

Figure 1. ROC Curve for FE Mg to predict SRNS

Table 4. Comparison of FE Mg level between SRNS and SSNS with cut-off point of 1.64%

Parameter	SRNS n	SSNS remission n	*p*
FE Mg : Increase	20	11	0.022
Normal	11	20	

Spearman correlation test indicated that there was no correlation between FE Mg and urine β2M with correlation coefficient of 0.169 (*p* = 0.188).

4. Discussion

The men to women ratio in this study was 2.1:1, while in the SRNS group was 2.8:1. These are consistent with Olowu et al [22] which obtained a men to women ratio of 2.3:1 in the SRNS group. In our study, the median age of onset of NS on SRNS group was 4 (1-14) year, which almost similar to Olowu et al [22] study (2-12.9 year). The age median at SRNS group was 8 (2-15) year, closely similar to the Olowu et al [22] report (2.1-13 year).

There was no difference in GFR decrease ($\leq$60 mL/min/1.73m^2) between groups SRNS and SSNS in this study. Rumana et al [15] obtain no significant difference in the mean plasma creatinine between groups SRNS with SSNS without the GFR data.

Any disruption of the intracellular structure and the function of epithelial tubules will affect the magnesium reabsorption and cause intracellular magnesium loss through urine [20]. A study reported a varying degrees of tubulointerstitial injury in children with SRNS [9]. This may explain hipomagnesemia in the 3/31 of the SRNS group, but not any in the SSNS.

This study showed the mean FE Mg urine was significantly higher in SRNS 2.34% (SD 1.37), compared to 1.59% (SD 0.85) in SNSS remission group (p=0.0065). Rumana et al [15] also reported a significant mean difference between groups SRNS and SSNS. An increase of the value of FE Mg (> 2.2%) was found 15/31 in SRNS group. When the FE Mg cut-off point of 1.64% was used, 20/31 in SRNS had an increase in FE Mg. This result is similar to Rumana et al [15] in which all children with SRNS has a significantly higher mean value of FE Mg than the ones in the SSNS, and all children with SSNS remission has a normal value of mean FE Mg. An increase in FE Mg varies according to the histopathology of SRNS [15]. In our study, not all of the SRNS children had an increase in the FE Mg. This may be due to the minimal change lesion on the subjects. Rumana et al [15] reported 5 of 20 SRNS children showed a minimal change histopathological lesions. Madani et al [23] acquired 22.2% SRNS has minimal change lesions. Minimal change lesions are usually associated with an intact nephron structure, without glomerulosclerosis nor tubulofibrosis, accompanied by nomal FE Mg [16,24]. A normal FE Mg is usually associated with an intact tubulointerstitial structure and function, while an abnormal FE Mg with tubulointerstitial fibrosis [15,16]. The value of FE Mg reflects the extent of the damage to the tubules, the higher the value, the more extensive the tubulointerstitial fibrosis and can detect an early tubulointerstitial damage on SRNS [15,17]. Thus, the significantly higher mean FE Mg in SRNS compare to SSNS remission can indicate the presence of tubular injury in SRNS.

Our study obtained an increased of FE Mg in 8/31 of the SSNS remission group. More than 80% of children with an idiopathic nephrotic syndrome showed minimal change lesions were more than 95 % responsive to corticosteroid [25,26]. The increased FE Mg on SSNS can be explained that most FSGS initially resembles minimal change lesions that would later develop into full FSGS [15]. Tubulointerstitial fibrosis is significantly higher in FSGS [27].

We found that the proportion of increased FE Mg (>2.2%) has no significant association beetween the SRNS and the SSNS remission (p=0.066). This could be due to differences in population and the cut off point of FE Mg normal on this research compared to the literature. If we used a cut off point of 1.64%, we obtained a significantly higher proportion of increased FE Mg in SRNS than in the SSNS remission (p=0.022). Rumana et al [15] did not report a significant difference in the proportion of FE Mg in SRNS.

The SRNS urinary β2M was significantly higher than the SSNS remission (p <0.001). This result was consistent with Chavan et al [7] which showed significantly elevated urinary β2M in SRNS than in SSNS in total or partial remission. It reflects the existence of tubular dysfunction and cell damage [7]. Valles et al [9] found urinary β2M in the onset of SRNS significantly higher when compared with SSNS in remission. Proximal tubular cells dysfunction, influenced by the massive albuminuria, especially in the presence of interstitial fibrosis, can explain the higher urinary β2M in SRNS [9]. Urinary β2M has a higher tubular specificity [18]. In addition, the increased urinary β2M shows tubular dysfunction associated with proximal tubular injury [28]. In this study a significantly higher urinary β2M in SRNS indicates a tubular dysfunction and tubular injury.

There was a significant association between the elevated urinary β2M with SRNS events in this study (p=0.007). Urinary β2M was a sensitive approach to screen renal injury, especially in tubular injury patients with glomerular disease. Because of the urinary β2M high sensitivity (86.3 %) for detecting tubular injury, the elevated urinary β2M in this study can be used for SRNS early screening [19].

In this study, we found no correlation between FE Mg and urinary β2M, possibly due to the pathophysiological differences. FE Mg reflects the tubular reabsorption ability against the filtered magnesium, and the ability to maintain intracellular magnesium, and to determine whether the renal is handling Mg properly [17]. In normal condition, magnesium is filtered and reabsorbed mainly in the proximal tubule and the ascending loop of Henle [20]. Glomerulus partially filters the Mg plasma [29]. Beta-2 microglobulin is freely filtered through the glomerulus because of its small size [30], up to 99.9% of it is reabsorbed by endocytosis of the proximal tubule in healthy subjects, then be catabolized in the renal proximal tubular cell lysosomes, only small amount of β2M is excreted in urine [31]. Beta-2 microglobulin plasma levels showed the glomerular filtration function [9]. Meanwhile, the detected tubular proteinuria such as β2M in urine indicates tubular dysfunction [20]. No previous research reports the correlation between FE Mg and urinary β2M.

5. Conclusion

FE Mg and urinary β2M level is higher in SRNS group than SSNS remission group. There is a significant difference in the proportion of the increase in FE Mg between SRNS and SSNS remission groups with cut-off point of 1.64%. The proportion of the increase in urinary β2M level in SRNS group is higher than SSNS remission group. All of these findings indicate the presence of tubular injuries in SRNS.

Further research is required for idiopathic NS with serial FE Mg level to understand the pathophysiology and

the degree of tubular injury in order to deliver early treatment in this group of patients. It is recommended to measure FE Mg level in children with idiopathic NS to initiate imunosuppresive treatment other than corticosteroids if the FE Mg level is found to be elevated.

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Conflict of Interest

There is no conflict of interest.

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