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CORRELATION OF URINARY ALPHA 2 MACROGLOBULIN WITH THE INCIDENCE OF ACUTE KIDNEY INJURY IN CHILDREN WITH IDIOPATHIC NEPHROTIC SYNDROME

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Abstract

Background: Acute kidney injury (AKI) is a known complication of idiopathic nephrotic syndrome (INS), though the exact mechanism still unclear, correlates and risk factors for the development and outcome of this problem is being studied in recent decades.
Objective: to determine the correlation of urinary alpha 2 macroglobulin (α2M) with the development and progression of AKI in children with INS
Methods: A cohort study of children 2-16 years with INS, admitted to Cairo University Children Hospital. AKI was diagnosed and categorized using pRIFLE criteria. Urinary (α2M) was measured for all admitted children using Human Alpha 2 macroglobulin ELISA kit
Results: of 90 hospitalisations, AKI was diagnosed in 48.9% of them. AKI detected in a total of 44 (48.9%) hospitalizations of 36 patients. The gender specific prevalence was in favor of males 27 (61.4%) against 17 (38.6%) for females but the difference was not statistically significant, $P = 0.296$. eGFR had significant negative correlation with α2M level ($r = -0.390$, $P < 0.05$), while no significant correlation found with eGFR and Albumin/Cr ratio.
Conclusion: AKI of varying severity is a more common complication in hospitalized children with INS, representing 48.9% of studied sample. The high molecular weight protein α2M was associated with reduced eGFR in the hospitalized children with AKI

Keywords: Idiopathic nephrotic syndrome, Acute kidney injury, alpha 2 macroglobulin.

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most prevalent form of NS in children. Children with INS develop a variety of acute life-threatening complications including infections, venous thromboembolism (TE), hypovolemia and AKI **(Menon, 2019)**.

Proteinuria is a key marker of glomerular disease severity, a factor responsible for further renal damage both at the glomerular and tubulointerstitial level, and an outcome predictor **(Cravedi and Remuzzi, 2013)**. Besides, proteinuria reduction following renin angiotensin system inhibition is associated with an improvement in kidney survival and a decreased risk of progression to renal failure **(van den Belt et al., 2018)**. Proteinuria is primarily caused by glomerular filtration barrier alterations that result in the loss of various molecular weight proteins. High molecular weight proteins [IgG 150 kD, α2-macroglobulin (α2M) 720 kD] and medium molecular weight proteins (albumin 69 kD, and transferrin 78 kD) are markers of glomerular filtration barrier alterations. However, the pathophysiology of high and medium molecular weight proteins is distinct for filtration and tubular reabsorption mechanisms; high molecular weight proteins have significantly lower tubular reabsorption than middle ones **(Bazzi, 2016)**. The low molecular weight proteins [α1-microglobulin (α1M) 26 kD, β2-microglobulin (β2M) 11 kDa], as well as the lysosomal enzyme β-NAG, are all markers of tubulo-interstitial damage. Protein accumulation in tubular cells promotes the expression of a variety of pro-inflammatory and pro-fibrogenic cytokines, resulting in interstitial inflammation, fibroblast proliferation, increased extracellular matrix production, and the development of interstitial fibrosis. Thus, proteinuria is a complex mixture of proteins with a varying of molecular weights, pathophysiological determinants, and clinical relevance **(Bazzi, 2016)**.

Considering the significance of proteinuria in glomerular diseases, few studies have examined its predictive value for functional outcome and therapeutic response. protein/creatinine and albumin/creatinine ratios have been shown to be crucial for early diagnosis, progression, cardiovascular events, and death in clinically silent chronic nephropathies **(Astor et al., 2011, Gansevoort et al., 2011)**. Number of studies have examined whether these proteins and this type of proteinuria are the most powerful predictors of renal and cardio-vascular outcomes in glomerular diseases and discovered that high molecular weight proteins (IgG, IgM, α2M) have a higher predictive value than protein/creatinine and albumin/creatinine ratios **(Bazzi et al., 2009, Tofik et al., 2011, Bazzi et al., 2014)**.

Remarkably, fractional excretion of a protein as well is more predictive of outcome than the protein/creatinine ratio, and a new method for indexing proteinuria for GFR better reflects the tubular load of proteins, which is one of the main causes of tubulo-interstitial damage and renal function decline. This method is more accurate than the protein/creatinine ratio **(Bazzi, 2016)**.

Additionally, as Brenner et al. demonstrated in the five out of six nephrectomy model, the composition of proteinuria changes over time as certain pathophysiological mechanisms, such as decreased nephron mass, make the glomerular filtration barrier more permeable, particularly to high molecular weight proteins, and decrease tubular reabsorption **(Olson et al., 1982)**. Same conclusion reported by Bazzi et al. in IgA Nephropathy **(Bazzi et al., 2012)**. De Vriese and Fervenza suggested that the best biomarker should be logistically and financially feasible, reproducible, and able to predict clinical course and response to treatment **(De Vriese and Fervenza, 2015)**. Some carefully chosen protienuric biomarkers may meet these criteria now.

The clinical significance of protienuric biomarkers in INS has been studied only in five published studies: three in adult patients, and two in children with INS and histologic diagnoses of FSGS and MCD **(Bazzi, 2016)**. In idiopathic FSGS, the first study published on the outcome predictive value of protienuric markers was a pilot study **(Bazzi et al., 2003)**, followed by a larger study, Bazzi et al. evaluated 38 patients with idiopathic FSGS who underwent renal biopsy and had several GFR estimation, and protienuric markers measurements; 24 hour proteinuria , urinary protein/creatinine ratio, α2-macroglobulin/creatinine ratio (α2M/Cr), Fractional Excretion of IgG (FE IgG) and α1M **(Bazzi et al., 2013)**. All the patients received steroids alone or in combination with cyclophosphamide for the first episode of INS. Twenty- three patients (61%) went into remission and nine patients (24%) developed ESKD. When comparing low and high risk groups, only FE IgG and α2M/Cr predicted remission, while ESKD was predicted by FE IgG. Multiple regression analysis revealed that FE IgG as the only independent predictor of remission and α2M/Cr as the strongest predictor of ESKD. Combining FE IgG and α2M/Cr increased the prediction of remission and ESKD. After 147 months of follow-up, 21/23 patients had a long-term remission. nineteen of them had FE IgG levels below the cut off, while all patients with ESKD, patients who didn't respond to steroids, or cyclophosphamide had FE IgG levels above the cut off **(Bazzi et al., 2013)**.

PATIENTS AND METHODS

This is a prospective cohort study, conducted in Pediatric Nephrology Unit, Pediatric General Words and Pediatric ICU Units, Cairo University Children's Hospital. Ninety hospitalizations of 77 children diagnosed with INS were enrolled in the current study during the period starting from January 2021 to June 2022. Patients were classified into first presentation NS, frequent relapse NS (FRNS), steroid dependent NS (SDNS) and steroid resistant NS (SRNS). AKI was diagnosed and classified using pRIFLE criteria. GFR was estimated using standard Shwartz formula.

Sample collection and urine alpha 2 macroglobulin (Uα2M) measurement; each participant was issued with a well-labeled universal urine bottle for the collection of 10 ml of a random fresh urine sample on the day of appointment.

- Urine samples were centrifuged to separate cellular debris. Urine supernatants were stored at −80°C, until thawed for biomarker measurement.
- Uα2M was measured using Human Alpha-2-macroglobulin (α2M) ELISA Kit (Glory Science Co., Ltd USA).

a. Test principle:

After the samples were incubated in coated Eliza plate, addition of chromogen and stop solution allow the colour to change from blue to yellow and the intensity of the colour was measured at 450 nm using a spectrophotometer

The concentration of α2M in the samples was then determined by comparing the optic density (OD) of the samples to the standard curve. Which was obtained by plotting the OD of the standard against different standard concentration

b. Test procedure:

1. All reagents were prepared before starting assay procedure. all Standards and Samples were added in duplicate to the Microelisa Stripplate.
2. Standard 50µl was added to standard well.
3. Testing sample 10µl was added and Sample Diluent 40µl was added to testing sample well; Blank well did not add anything.
4. 100µl of HRP-conjugate reagent was added to each well, covered with an adhesive strip and incubated for 60 minutes at 37°C.
5. Each well was aspirated and washed; the process was repeating four times for a total of five washes. Washed by filling each well with Wash Solution (400µl) using a squirt bottle, manifold dispenser, or auto washer.

Complete removal of liquid at each step was essential to good performance. After the last wash, any remaining Wash Solution was removed by aspirating or decanting. The plate was inverted and blotted it against clean paper towels.

6. Chromogen solution A 50µl and chromogen solution B 50µl were added to each well. Gently mixed and incubated for 15 minutes at 37°C. Protect from light.

7. 50µl Stop Solution was added to each well. The colour in the wells changed from blue to yellow.

8. The OD at 450 nm was read using a microtiter plate reader within 15 minutes.

c. Calculation of results:

1. This standard curve was used to determine the amount in an unknown sample. The standard curve was generated by plotting the average OD (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis.

2. First, the mean OD value for each standard and sample was calculated. All OD values were subtracted by the mean value of the zero standard before result interpretation. The standard curve was contrasted using graph paper or statistical software.

3. The amount in each sample was determined as following; first the OD value was located on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line was drawn to the X-axis and the corresponding concentration was read.

4. The sensitivity by this assay is 0.1 g/L

RESULTS

Ninety hospitalizations of 77 children diagnosed with INS have been reported and analyzed in this study. More than half of children enrolled were males (52 males and 25 females). The mean age of the study group was 6.5 ± 3.0 years, range (1.7 – 11.3 years) **(Table 1).**

Table 1. Age and sex distribution of INS children

	Male	**Female**	**P value**
Age in years Mean ± SD	6.9 ± 3.0	5.6 ± 3.0	0.101
Age groups,	**n (%)**	**n (%)**	
≤ 3	6 (7.8)	7 (9.1)	
4-7	24 (31.2)	13 (16.9)	0.155
8-11	17 (22.0)	4 (5.2)	
≥ 12	5 (6.5)	1 (1.3)	
Total	52 (67.5)	25 (32.5)	77 (100%)

Student t test, Chi-square (χ2) test, Fisher exact test.

Mean Hb level was 12.6 ± 2.5, most of patients (88.9%) had Hb level between 10-16 g/dl. Very low Hb levels < 6 g/dl was recorded in two patients necessitating blood transfusion meanwhile the highest Hb level was 24.9 g/dl found in one child. All participants had serum albumin values less than 3 g/dl with a mean of 1.6 ± 0.4 g/dl at time of admissions. Eight (8.9%) of children admitted with SCr above 1 mg/dl. CRP was positive in approximately one third hospitalizations and 10 (11.1%) children had very high values ≥ 96 mg/L. The overall results of blood and urine investigations are depicted in **Table 2.**

Table 2. Mean and range of blood and urine examination results at admission.

Category	Mean ± SD	Range
Blood		
Hb, g/dl	12.6 ± 2.5	5.2 – 24.9
WBC, mm^3	12616 ± 7328	4800 - 35800
Platelets, mm^3	474820 ± 160516	186000 - 984000
Albumin, g/dl	1.6 ± 0.4	0.9 – 2.8
SCr, mg/dl	0.6 ± 0.3	0.2 – 4.7
BUN, mg/dl	20 ± 4.2	6.0 – 132
Na, meq/L	136.2 ± 4.7	123.0 – 147.0
K, meq/L	4.4 ± 0.7	2.6 – 6.0
Ca, mg/dl	7.7 ± 0.9	4.0 – 10.2
*CRP, mg/L	0 [0-14]	0 - 211
Urine		
*Pus cells	5.0 [3.0 – 10]	0 - 60
Albumin, mg/L	332.2 ± 171.5	200 - 635
Albumin/Cr, mg/g	8062.2 ± 7124.2	1000.0 – 44400.0
α2M, g/L	3.3 ± 0.5	2.5 – 4.9
*α2M/Albumin, g/g	8.1 [6.9 - 22.4]	4.4 – 160
α2M/Cr, g/g	10.4 ± 8.6	1.3 – 37.0
Cultures, n (%)		
Blood		
Positive	6	(6.7)
Negative	21	(23.3)
Not done	63	(70.0)
Urine		
Positive	8	(8.9)
Negative	32	(35.6)
Not done	50	(55.6)

**Median [interquartile range]*
Hb: hemoglobin, WBC: white blood cells, mm^3: millimeter mercury, SCr: serum creatinine, Ca: calcium, CRP: C-reactive protein, α2M: alpha 2 macroglobulin.

AKI detected in a total of 44 (48.9%) hospitalizations of 36 patients. The gender specific prevalence was in favor of males 27 (61.4%) against 17 (38.6%) for females but the difference was not statistically significant, $P = 0.296$. eGFR had significant negative correlation with α2M level ($r = -0.390$, $P < 0.05$), while no significant correlation found with eGFR and Albumin/Cr ratio (Table 3).

Table 3. eGFR correlation with urine investigations

Parameter	Correlation coefficient (*r*)	*P* value
Albumin, mg/L	- 0.188	0.228
Creatinine, g/dl	- 0.150	0.337
α2M, g/L	- 0.390	**0.010**
Albumin/Cr, mg/g	- 0.047	0.767
α2M/Cr, g/g	-0.277	0.069
α2M/Albumin, g/g	-0.212	0.167
eGFR at admission, ml/min/1.73m^2	0.632	**<0.001**

Pearson`s correlation, Spearman`s correlation

α2M: alpha 2 macroglobulin, eGFR: estimated glomerular filtration rate.

DISCUSSION

Idiopathic nephrotic syndrome (INS) is one of the most prevalent childhood kidney diseases worldwide. The mean age of hospitalized children with INS in this study was 6.5 years, which was lower than the 7.8 years observed in Pakistani **(Yaseen et al., 2017)** and 8.2 years in Korean studies **(Kim et al., 2018)** but higher than the 5.3 and 4.7 years identified in two Indian studies **(Kushwah et al., 2019, Kumari et al., 2021)**. Males were predominant (67.0%), similar to other studies **(Rheault et al., 2015, Kushwah et al., 2019, Kumari et al., 2021)**

In this cohort the incidence of AKI according to pRIFLE definition was 48.9 % which was comparable to 50.9% reported by Rheault et al 2015., **(Rheault et al., 2015)** but higher than 32.2% reported by Kim et al. **(Kim et al., 2018)**.

Alpha 2 macroglobulin (α2M), is a 720 kD glycoprotein mediates complex physiological and pathological activities, including growth factor delivery, matrix degrading enzyme regulation, and fibrinolysis factors modulation, all of which are believed to involve in the pathogenesis of glomerular injury **(Yang and Chen, 1997)**. Plasma level of α2M is elevated in children with INS due to enhanced production, which maintains oncotic pressure. Urinary loss of α2M is restricted by its large size **(Suresh et al., 2016)**. In this study, patients with SRNS had the highest levels of urine α2M and the greatest α2M/creatinine ratio, although the difference with SSNS was not statistically significant. In an older study, Ellis et al. evaluated urine levels of α2M in INS and observed higher amounts in the FSGS group as compared to the MCD group **(Ellis and Buffone, 1981)**. It was assumed that the presence of this high-molecular-weight protein in urine was due to a disruption in non-discriminatory pores in the GBM

rather than other type-restrictive pores **(Suresh et al., 2016)**. A more recent study by Bazzi et al., revealed a strong association between urine α2M and adult FSGS patients. They concluded that elevated levels of α2M can predict SRNS-FSGS from SSNS **(Bazzi et al., 2013)**.

To the best of our knowledge, this is the first study to examine the association between urine α2M and the development of AKI. The ratio of α2M to albumin was significantly higher in children with AKI stage F. Moreover, α2M was associated with lower eGFR in the hospitalized children with AKI, even though it was not recognized as a risk factor for AKI complications. The impact of α2M in glomerular disease has not been thoroughly investigated. Among limited researches, glomerular deposition of α2M have been described in renal disease patients, particularly those with NS. They hypothesised that the highly selective deposition of α2M in certain glomerular disorders suggests that α2M may play an active role in the control of local inflammatory response and tissue repair in these glomerular diseases **(Asami et al., 1992, Yang and Chen, 1997)**. Bazzi et al., evaluated the predictive value of high-molecular-weight proteins excretion such α2M in the outcome of 38 nephrotic patients, 9 (24%) progressed to ESKD. α2M to creatinine ratio was identified as a predictor of functional outcome in their study. These findings revealed that severe alterations of the glomerular filtration barrier resulted in high excretion of high-molecular-weight proteins and an increased risk of progressive renal damage **(Bazzi et al., 2013)**. The predictive value of high-molecular-weight protein excretion for renal impairment and outcome has been shown in other types of glomerular diseases, in which IgG rather than α2M was investigated **(Tofik et al., 2011, McQuarrie et al., 2011)**. Recently, the highly predictive value of α2M for diabetic nephropathy was already established **(Trink et al., 2021)**.

CONCLUSION

AKI of varying severity is a more common complication in hospitalized children with INS, representing 48.9% of studied sample. The high molecular weight protein α2M was associated with reduced eGFR in the hospitalized children with AKI.

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