
Original Article**Urinary Nephryn as a Marker of Glomerular Diseases in Pediatric Patients.****Aziza A. El-Sebai ¹, Mervat S. Yousef ¹, Menat Allah A. Shaaban ¹, Mohamed S. El-Farsy ²****1-** Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt.**2-** Department of Pediatrics, Faculty of Medicine, Ain Shams University, Cairo, Egypt.**Abstract:**

Introduction: Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, present for more than 3 months with implications for health. Children with CKD have higher mortality rate, which is at least 30-fold higher than their age-matched peers. Glomerular diseases are one of the leading causes of CKD in children.

Aim of the study: To assess the clinical utility of urinary nephryn as a diagnostic tool in pediatric patients with glomerular disease (GD) and to evaluate urinary nephryn in assessing the activity and severity of GD in children.

Methods: This study was conducted on fifty (50) patients with GD in Pediatric Nephrology Clinic, Children's Hospital, at Ain Shams University, were further divided into five (5) subgroups according to their diagnosis by renal biopsy. The 5 subgroups included focal segmental glomerulosclerosis (FSGS), minimal change nephrotic syndrome (MCNS), membranoproliferative glomerulonephritis (MPGN), lupus nephritis (LN) and IgA nephropathy. In addition, twenty (20) apparently healthy age and sex matched subjects served as control group. All individuals were subjected to assay of urinary nephryn, kidney function tests, urinary protein/creatinine ratio and complete urine analysis.

Results: By comparing the different types of GD, a statistically significant difference was found regarding the urinary nephryn concentration in MCNS subgroup in comparison to FSGS ($p < 0.05$) and to MPGN ($p < 0.05$) subgroups. However, no significant difference was revealed between urinary Protein/Creatinine ratio in MCNS subgroup in comparison to FSGS and to MPGN subgroups. Based on the results of the study, urinary Nephryn has the potential to be a crucial sensitive marker for detection of glomerular injury. Nephryn can discriminate between steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS), so it can be used as a noninvasive diagnostic ($p < 0.01$) marker for SSNS and SRNS in children. Furthermore, urinary nephryn assessment can be of clinical utility in assessing the activity and severity of GD in children.

Conclusion: The present study indicates that determination of urinary nephryn can be used as a potential non-invasive marker for diagnosis of GD. Further improvement of its diagnostic efficiency might be reached using nephryn together with protein/creatinine ratio. Moreover, nephryn testing have" the potential to be a promising diagnostic marker since it can discriminate between the steroid-sensitive and steroid-resistant nephrotic syndrome, and thus, nephryn can avoid the non-beneficial usage of steroids -with its adverse effects- in such resistant cases. The best diagnostic cut off level for Nephryn was 5.5 ng/dL.

Keywords: Urinary Nephryn, Glomerular Disease, Pediatric Patients.

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INTRODUCTION

Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function, present for more than 3 months with implications for health. Children with CKD have higher mortality rate, which is at least 30-fold higher than their age-matched peers [1].

The early stages of CKD in the pediatric population are in most cases asymptomatic, and therefore many cases are not diagnosed at early stages of the disease [2]. End Stage Kidney Diseases (ESKD) represents the "Tip of the iceberg" of CKD and suggests that patients with earlier stages of disease are likely to exceed those reaching ESRD by as much as 50 times [3].

In contrast to the increasing availability of information pertaining to the care of children with CKD from large scale observational and interventional studies, epidemiological information on the incidence and prevalence of pediatric CKD is currently limited. Therefore, the diagnostic and therapeutic approach to CKD must emphasize primary prevention, early detection, and aggressive management [4]. Initiating therapy early is essential to improve health outcomes in children with CKD [5].

The histopathological findings in kidney diseases determined through percutaneous renal biopsy are of great importance in determining the accurate diagnosis and long-term prognosis [6].

Proteinuria is widely used for noninvasive assessment of kidney diseases. However, proteinuria is a nonspecific marker of diverse forms of kidney injury [7]. Thus, studies are concerned with evaluation of new clinically useful markers of glomerular injury [8].

Glomerular diseases are one of the leading causes of CKD in children in several studies worldwide [9]. Podocytes injured in glomerular diseases collectively called podocytopathy, which is recognized as the common feature in minimal change disease, membranous glomerulopathy, and focal lupus nephritis through podocyte damage and dysfunction [10]. These changes lead to severe and progressive glomerular injuries and hence, early recognition of any podocyte injury is of clinical importance [11].

Nephrin is 180 KDa transmembrane protein expressed in glomerular podocytes. It forms an integral part of podocytes which together with endothelial cells and the glomerular basement membrane form the glomerular filtration barrier [12]. It was first identified in children with congenital nephrotic syndrome of the Finnish type (NPHS1). Nephrin has eight immunoglobulin-like domains and one fibronectin III-like domain in the extracellular region. Normally, it exists as complexes with other podocyte-specific proteins including podocin, and CD2-associated protein. Loss of nephrin fails to form functional complexes in the slit diaphragm, resulting in dysfunction of the filtration barrier [16].

Being an integral part of podocytes, urinary nephrin is being evaluated as a promising marker in pediatric patients with glomerular diseases, to assess its correlation with the type and the severity of the kidney disease in such patients [13].

Aim of the study: To assess the clinical utility of urinary nephrin as a diagnostic tool in pediatric GD and to evaluate urinary nephrin in assessing the activity and severity of GD in children.

Methods

It is a cross sectional observational study, this study was conducted on 50 pediatric patients with glomerular diseases who followed up at Pediatric Nephrology Clinic, Children's Hospital, at Ain Shams University, and 20 apparently healthy control subjects, age and sex matched. Sample size is calculated by clincalc.com and it is a stratified randomized sample. Informed consents were obtained from their parents before enrollment in the study.

Patients were classified into the following groups:

Group I: Patients with glomerular diseases (n=50): This group included fifty (50) pediatric patients with glomerular diseases who underwent renal biopsy, twenty-nine (29) females with mean age 9 years and twenty-one (21) males with mean age 10 years. They were further classified into 5 subgroups: **Subgroup Ia:** This subgroup included fourteen (14) pediatric patients diagnosed with focal segmental glomerulosclerosis (FSGS), **subgroup Ib:** This subgroup included nine (9) pediatric patients diagnosed with membranoproliferative glomerulonephritis (MPGN), **subgroup Ic:** This subgroup included nine (9) pediatric patients diagnosed with minimal change nephrotic syndrome (MCNS), **subgroup Id:** This subgroup included nine (9) pediatric patients diagnosed with IgA nephropathy, **subgroup Ie:** This subgroup included nine (9) pediatric patients diagnosed with lupus nephritis (LN).

All the patients were in active disease, with duration of the disease more than 1 year, with the following treatment: FSGS group are on oral steroids, cyclosporine A

and mycophenolate mofetil. MPGN are on oral steroids and cyclosporine A. MCNS group are on oral steroids only. IgA nephropathy group are on oral steroids and omega 3 fatty acids. LN group are on steroids, cyclophosphamide and/or cyclosporine A. Hematuria was present at the time of diagnosis with the patients in FSGS, MPGN and IgA nephropathy groups. Proteinuria was present at the time of diagnosis in MCNS group, but it was present at the time of the study in the other groups.

Group II: Healthy control group (n=20): This group included twenty (20) apparently healthy age and sex matched control subjects with normal renal function and no history of glomerular diseases who were coming with minimal medical condition (acute tonsillitis, gastroenteritis, ...etc.) or following up at outpatient clinic. They included 10 females and 10 males with a mean age of 9.5 years.

All individuals included in this study were subjected to the following: Full history including family history, general examination (weight, height, blood pressure, and Renal biopsy (patients only). Laboratory investigations include Kidney function tests (BUN and S. Creatinine), Urinary protein/creatinine ratio, Complete Urine analysis, Assay of urinary nephrin by enzyme-linked immunosorbent assay ELISA technique.

Sampling: Early morning urine samples were collected from all the subjects in sterile containers and centrifuged for 20 minutes at the speed of 2000 – 3000 r.p.m. The supernatant of the samples was separated in aliquots and assayed for urinary protein/creatinine ratio, and aliquots were stored at -20°C till

subsequent assay of nephrin. Repeated freezing and thawing were avoided.

Serum creatinine and BUN, and urinary protein and creatinine were assayed spectrophotometrically on the AU 480 auto-analyzer (Beckman Instruments Inc.)

The concentration of nephrin in the sample was determined using a standard curve plotted on logarithmic graph paper. The standard curve was constructed by plotting the absorbance (Y-axis) of standards against log of the known concentration (X-axis) of standards. The concentration of the samples was read directly from this standard curve by using their average optical density. The concentration of nephrin was proportional to the color intensity of the test sample.

Statistical Analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical Package for Social Science (SPSS 15.0.1 for Windows; SPSS Inc., Chicago, IL, 2001). Data was presented and suitable analysis was done according to the type of data obtained for each parameter.

Descriptive statistics:

- Mean and Standard deviation for quantitative parametric data.
- Median and Interquartile range for quantitative non-parametric data.
- Frequency and percentage was used for presenting qualitative data.

Analytical statistics:

- Student T Test was used to assess the statistical significance of the difference between the study groups means.
- Chi-Square test was used to examine the relationship between two qualitative variables. P-value > 0.05

will be considered statistically significant.

Correlation analysis (using Pearson's method): To assess the strength of association between two quantitative variables. The correlation coefficient denoted symbolically "r" defines the strength (magnitude) and direction (positive or negative) of the linear relationship between two variables.

- $r = 0-0.19$ is regarded as very weak correlation.
- $r = 0.2-0.39$ as weak correlation.
- $r = 0.40-0.59$ as moderate correlation.
- $r = 0.6-0.79$ as strong correlation.
- $r = 0.8-1$ as very strong correlation.

ROC curve (receiver operating characteristic curve)

To assess the performance of a classification model at all classification thresholds.

To measure AUC and cut off value.

AUC: Area Under the Curve,

PPV: Positive Predictive Value,

NPV: Negative Predictive Value.

RESULTS

Table 1 shows descriptive and comparative statistics of the various studied parameters in the different studied groups; pediatric patients with GD and control group. Nephrin and Protein/Creatinine ratio showed high statistically significant difference between the two groups ($p < 0.01$). There is no statistically significant difference in weight, height and blood pressure between all groups of patients.

Table 2 shows descriptive and comparative statistics between the studied patient subgroups. It revealed a statistically significant difference in

Nephrin and Protein/Creatinine ratio ($p < 0.05$) between the 5 subgroups. The difference of Protein/Creatinine in FSGS, IgA nephropathy and MCNS is due to that all of the patients are in active disease with different degrees of proteinuria.

Table 3 shows a highly significant difference ($p < 0.01$) in Nephrin between FSGS and IgA nephropathy, significant difference ($p < 0.05$) in Nephrin between FSGS and MCD, and non-significant difference ($p > 0.05$) in Nephrin between FSGS with MPGN and LN respectively.

Table 4 shows a significant difference ($p < 0.05$) in Nephrin between MPGN with MCD and IgA nephropathy respectively, and non-significant difference ($p > 0.05$) in Nephrin between MPGN and LN.

Table 5 shows a highly significant positive correlation ($p < 0.01$) between

Nephrin and Protein/Creatinine ratio among patients' group. Also, it shows a non-significant correlation ($p > 0.05$) between Nephrin with KFTs and age respectively.

Table 6 shows a non-significant correlation ($p > 0.05$) between Nephrin with Protein/Creatinine ratio and KFTs respectively, among control group.

Receiver operating characteristic (ROC) curve analysis was applied to assess the diagnostic performance of Nephrin in discriminating patients from control group. The best diagnostic cut off level for Nephrin was 5.5 ng/dL, with a diagnostic sensitivity of 92 %, diagnostic specificity 85%, positive predictive value (PPV) 93.9 %, negative predictive value (NPV) 81%, diagnostic efficacy 90% and area under the curve (AUC) of 0.772 as shown in **Table 7 & Figure 1**.

Table 1: Descriptive and comparative statistics of various studied parameters in the different studied groups.

Parameter	Patients group (n = 50) Median (Q1-Q3)	Control group (n = 20) Median (Q1-Q3)	Patients versus Controls	
			z	p
Age (years)	9 (7-12)	9.5 (6.25-12)	-0.026	>0.05
Serum Creatinine (mg/dL)	0.65 (0.5-1.1)	0.7 (0.5-0.9)	-0.033	>0.05
Protein/Creatinine ratio	1.2 (0.4-3.2)	0.12 (0.03-0.19)	-5.481	<0.01
Nephrin (ng/dL)	10.75 (6-16.25)	1.5 (1-4.75)	-3.714	<0.01

Q1: 25th percentile; Q3: 75th percentile; $p < 0.01$ highly significant; $p < 0.05$ significant; $p > 0.05$ non-significant

Table 2: Descriptive and comparative statistics between the studied patient subgroups.

Parameter	FSGS (n=14) Median (Q1-Q3)	MPGN (n=9) Median (Q1-Q3)	MCD (n=9) Median (Q1-Q3)	IgA Nephro (n=9) Median (Q1-Q3)	LN (n=9) Median (Q1-Q3)	p
Age (years)	8.5(6.75-11.25)	9 (9-12)	9 (5.25-10.75)	7.5 (6-11.5)	13 (11.75-15)	<0.05
BUN	27 (23.8-31)	23 (21.5-34.5)	25 (17.5-34.75)	35 (22.3-39.5)	35 (22.8-49.1)	>0.05
Serum Creatinine (mg/dL)	0.7 (0.5-1.35)	0.6 (0.35-0.95)	0.6 (0.5-0.7)	0.7 (0.5-1.03)	0.8 (0.6-1.2)	>0.05
Protein/Creatinine ratio	0.84 (0.5-2.4)	1.1 (0.8-3.1)	2.9 (1.3-6.7)	0.3 (0.2-0.77)	4.4 (2.5-7.1)	<0.05
Nephrin (ng/dL)	12 (9.3-16.2)	16 (6-22)	5.5 (2-7.9)	6 (3.25-9)	15.5 (4.6-38.8)	<0.05

FSGS: Focal Segmental Glomerulosclerosis; MPGN: Membranoproliferative Glomerulonephritis; MCD: Minimal Change Disease; IgA Nephro: IgA Nephropathy; LN: Lupus Nephritis; Q1: 25th percentile; Q3: 75th percentile; $p < 0.05$ significant; $p > 0.05$ non-significant.

Table 3: Statistical comparison between FSGS and the other Subgroups of patient's group regarding the various studied parameters.

Parameter	FSGS versus MPGN		FSGS versus MCD Steriod Sensitive		FSGS versus IgA Nephropathy		FSGS versus LN	
	z	p	z	p	z	p	z	p
Age (years)	-1.02	> 0.05	-0.34	> 0.05	-0.44	> 0.05	-2.7	< 0.01
Protein/Creatinine ratio	-0.75	> 0.05	-2.04	> 0.05	-2.08	< 0.05	-2.8	< 0.01
Nephryn (ng/dL)	-0.25	> 0.05	-2.73	< 0.05	-2.96	< 0.01	-0.45	> 0.05

FSGS: Focal Segmental Glomerulosclerosis; MPGN: Membranoproliferative Glomerulonephritis; MCD: Minimal Change Disease; LN: Lupus Nephritis; p <0.01 highly significant; p <0.05 significant; p >0.05 non-significant.

Table 4: Statistical comparison between MPGN and the other subgroups of group I regarding the various studied parameters.

Parameter	MPGN versus MCD Steriod Resistant		MPGN versus IgA Nephropathy		MPGN versus LN	
	z	p	z	p	z	p
Age (years)	-1.03	> 0.05	-1.11	> 0.05	-2.46	< 0.01
Protein/Creatinine ratio	-1.73	> 0.05	-2.35	> 0.05	-2.35	< 0.05
Nephryn (ng/dL)	-2.27	< 0.05	-2.24	< 0.05	0	> 0.05

MPGN: Membranoproliferative Glomerulonephritis; MCD: Minimal Change Disease; LN: Lupus Nephritis; p <0.01 highly significant; p <0.05 significant; p >0.05 non-significant.

Table 5: Correlation between Nephryn and other studied parameters among patients.

	Nephryn	
	R	p
Age	0.144	> 0.05
BUN	0.249	> 0.05
Serum Creatinine	0.239	> 0.05
Protein/Creatinine ratio	0.404	< 0.01

p <0.01 highly significant; p >0.05 non-significant.

Table 6: Correlation between Nephryn and other studied parameters among control group.

	Nephryn	
	R	p
Age	-0.158	> 0.05
Serum Creatinine	0.105	> 0.05
Protein/Creatinine ratio	0.029	> 0.05

p >0.05 non-significant

Table 7: The diagnostic performance of Nephryn in discriminating patients from normal control group.

Parameter	Cutoff	AUC	Diagnostic Sensitivity %	Diagnostic Specificity %	PPV%	NPV%	Diagnostic Efficacy%
Nephryn (ng/dL)	5.5	0.772	92%	85%	81%	93.9%	90%

AUC: Area Under the Curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value.

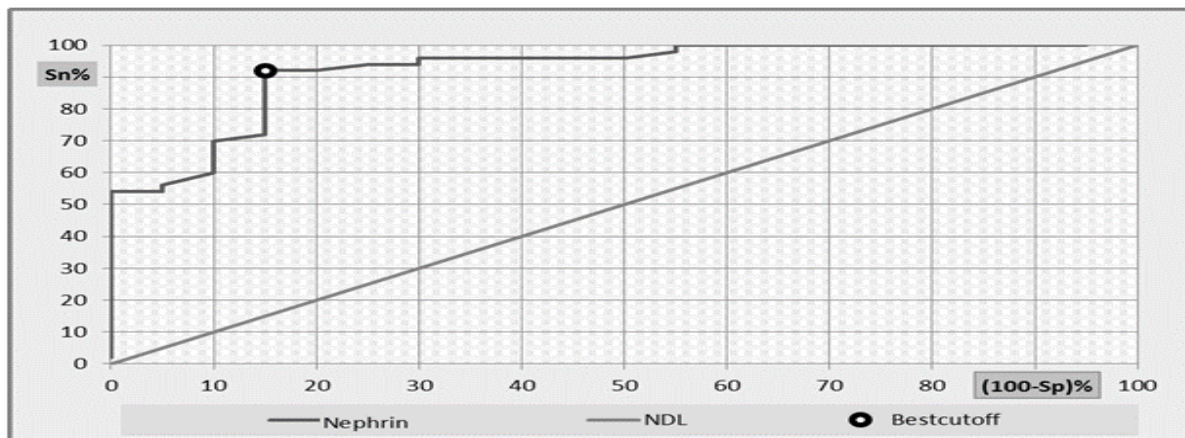


Figure 1: ROC curve analysis showing the diagnostic performance of Nephrin for discriminating patients from those controls.

DISCUSSION

CKD is a major public health problem where little information is available on the prevalence of earlier stages of CKD, as patients are often asymptomatic. Patients with earlier stages of disease are likely to exceed those reaching ESRD by as much as 50 times [4].

Many studies have focused on identifying the risk factors that determine the progression of CKD. Podocyte damage is the key factor of GD [13]. Proteinuria and/or albuminuria are widely used for noninvasive assessment of kidney diseases. However, proteinuria is a nonspecific marker of diverse forms of kidney injury whether glomerular or non-glomerular [7].

Rakesh et al. (2015) [14] demonstrated that random urine protein-creatinine ratio is highly reliable and rapid test for quantification of proteinuria in children. It reflects the amount of protein in a 24-hour collection. Thus, it avoids all the drawbacks which are associated with timed collection method of urine.

Urine testing for biomarkers could replace renal biopsy as a simple, safe, and accurate test that could be repeated to follow progression of the disease and monitor response to therapy [15]. Many investigations have focused on biomarkers of podocytes such as Nephrin can be readily identified via varying techniques, to evaluate the activity and severity of GD [13].

Results of the present study revealed that urinary Protein/Creatinine ratio and Nephrin showed a highly significant difference between the patients and controls. Moreover, there was a positive correlation between Nephrin and Protein/Creatinine ratio as supported by Proletov et al. (2014) [17], who found out that the more severe the degree of podocyte injury (as evidenced by increased urinary nephrin), the worse the level of proteinuria. However, there is no significant difference between the patients and controls regarding serum creatinine.

Steroids have been claimed as standard treatment for GD as it exerts therapeutic effect by alteration in the GD and thus improving the progression of the

disease and consequently proteinuria [18]. Thus, comparison of such individual groups was based on comparison of steroid sensitive versus steroid resistant groups.

By comparing the different patients' subgroups, MPGN showed the highest nephrin concentration followed by FSGS while, MCD showed a relatively low nephrin concentration which is consistent with Wang et al. (2015) [13]. This is against Hingorani et al. (2004) [19] who stated that there was no difference in nephrin expression between different GD.

By comparing individually, the different types of GD, a statistically significant difference was found regarding the urinary nephrin concentration in MCD subgroup in comparison to FSGS and to MPGN subgroups. However, no significant difference was revealed between urinary Protein/Creatinine ratio in MCD subgroup in comparison to FSGS and to MPGN subgroups. This is in agreement with Camici (2008) [10], who reported that an increase in excretion of podocytes and podocyte associate proteins as nephrin, in FSGS compared to MCD provide the hope of easily distinguishing between diseases. Moreover, in concordance with Nakamura et al. (2000) [20], nephrin has been observed as a more sensitive indicator of GD damage than proteinuria.

Moreover, a significant difference was revealed between nephrin levels in FSGS and MPGN in comparison to IgA nephropathy. On the other hand, regarding urinary Protein/Creatinine ratio, a significant difference was observed between FSGS and IgA nephropathy, however, no significant difference was found between MPGN and IgA nephropathy. It is of importance to

mention that neither nephrin nor Protein/Creatinine ratio showed significant difference between FSGS and MPGN.

As for LN subgroup, no significant difference was obtained between nephrin levels in comparison to FSGS and MPGN subgroups. This is in comparable results with Wang et al. (2015) [13] which revealed that nephrin expression from the LN group were significantly higher than those of the other groups. Strikingly, Protein/Creatinine ratio showed highly significant difference between FSGS and MPGN in comparison to LN. In contrast to Wang et al. (2015) [13], who subclassified patients with LN to patients with active LN and patients in remission. Urinary nephrin detection through nephrin mRNA expression was used to determine urine nephrin levels, and they found that nephrin was significantly elevated in the active group in comparison to the remission group.

Based on the results of the study, urinary Nephrin has the potential to be a crucial sensitive marker for detection of glomerular injury. Nephrin can discriminate between steroid sensitive nephrotic syndrome -namely MCD- and steroid resistant nephrotic syndrome - namely FSGS and MPGN, (2nd column in table 3 and 1st column in table 4) So , it can be used as a non-invasive diagnostic marker for GD in children. Furthermore, urinary Nephrin assessment can be of clinical utility in assessing the activity of GD in children.

CONCLUSION

In conclusion, the present study indicates that determination of urinary nephrin can be used as a potential non-invasive marker for diagnosis of GD. Further improvement

of its diagnostic efficiency might be reached using nephrin together with protein/creatinine ratio. Moreover, nephrin testing have the potential to be a promising diagnostic marker since it can discriminate between the steroid-sensitive and steroid-resistant nephrotic syndrome and thus, nephrin can avoid the non-beneficial usage of steroids -with its adverse effects- in such resistant cases. The best diagnostic cut off level for Nephrin was 5.5 ng/dL.

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ABBREVIATIONS

BUN	Blood urea nitrogen
CKD	Chronic kidney disease
ELISA	Enzyme-linked immunosorbent assay technique
ESRD	End Stage Renal Diseases
ESKD	End Stage Kidney Disease
FSGS	Focal segmental glomerulosclerosis
GD	Glomerular disease
LN	Lupus nephritis
MCNS	Minimal change nephrotic syndrome
MPGN	Membranoproliferative glomerulonephritis
SRNS	Steroid resistant nephrotic syndrome
SSNS	Steroid sensitive nephrotic syndrome

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AUTHORS' CONTRIBUTIONS

All authors have read and approved the manuscript.

Study conception and design: 1st & 3rd author.

Data acquisition: 4th, 2nd & 3rd author.

Analysis and data interpretation: 2nd & 3rd author.

Drafting of the manuscript: 4th author.

Critical revision: 4th author.

STATEMENTS

Ethics approval and consent to participate

This study protocol and the consents were approved and deemed sufficient by the Ethical Committee of Ain Shams University and informed and written consent was obtained in every case from their legal guardians

Consents for publication

"Not applicable"

Availability of data and material

"Not applicable"

Conflict of interest

The authors declare no conflict of interest.

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