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Original Article

Apoptotic Tendency in Nephrotic Syndrome and Acute Post Streptococcal Glomerulonephritis

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ABSTRACT

Background: Renal diseases are in many cases associated with the presence of an increased number of apoptotic cells in the kidney and its potential role ranges from induction and progression to repair of renal injury.

Objective: This study was designed to assess the level of the apoptotic marker anti-annexin V IgG antibodies as an indicator of severity and a prognostic marker in both nephrotic syndrome (NS) and acute post streptococcal glomerulonephritis (APSG) patients.

Methods: The current study included 50 children and adolescents. Twenty were diagnosed as NS and 15 of them were diagnosed as APSGN based on their full medical history taking and relevant laboratory investigations. They were compared to 15 clinically healthy, age- and sex-matched children and adolescents as a control group. Each enrolled child or adolescent was subjected to detailed history taking and thorough clinical examination. Routine laboratory work up was done including full urine analysis, 24 hour urinary protein, complete blood count and serum protein, albumin, cholesterol, creatinine, blood urea nitrogen and complement (C3). Plasma anti-annexin V IgG was estimated for all patients and controls by ELISA technique. The patients were followed up until initial improvement then they were resubjected to the previous clinical and laboratory evaluation.

Results: There was significantly higher anti-annexin V IgG levels in NS and APSGN patients compared to the controls (p values are < 0.05 and < 0.01 respectively). There was also significantly higher anti-annexin V IgG levels in APSGN patients compared to NS patients before treatment ($p < 0.05$). There was no significant difference between each studied group and the controls or between each other after treatment. Anti-annexin V IgG level showed positive correlation with urinary protein and a negative correlation with serum albumin in NS patients and serum creatinine of APSGN patients showed positive correlation with the rate of change of anti-annexin V IgG level, however, these correlations were of no statistical significance.

Conclusions: From the present study we can conclude that anti-annexin V IgG antibodies can be used as a marker of renal affection indicating the severity of underlying pathology and it could be of prognostic value since its normalization couples the clinical improvement. Anti-annexin V IgG antibodies can be also used to differentiate between the underlying pathologies since it is significantly higher in patients with APSGN patients compared to NS patients further proving that apoptosis is the major cell clearance mechanism counterbalancing cell division, thereby mediating resolution of glomerular hypercellularity in APSGN.

INTRODUCTION

Apoptosis is defined as the main method of getting rid of cells which are in excess. This has been a subject of many previous studies in different pathological conditions[1]. Application of annexin V is one of the recent laboratory tests to detect early apoptosis. Cell membrane asymmetry in early apoptosis exposes phosphatidylserine on the cell surface. This exposure of phosphatidylserine can be detected by staining with annexin V[2]. Annexin V is a member of a family of calcium-binding proteins (annexin I-XIII) that binds with acidic phospholipids. Annexin V in the presence of Ca^{+} binds to phosphatidylserine

that composes the inner layer of the cell membrane and causes calcium-ion channel activity[3].

Renal diseases are in many cases associated with the presence of increased numbers of apoptotic cells in the kidney[4]. Ortiz et al.[5], previously summed up the potential role of apoptosis in renal diseases by saying that it ranges from induction and progression to repair of renal injury.

Apoptosis is a crucial process in patients with nephrotic syndrome (NS). It has been a useful indicator of acute renal injury related to urinary protein level in such patients[3]. In addition, Chesney[6] reported that novel therapies in NS will modulate the cell cycle, tyrosine kinase signaling and apoptosis. Moreover, apoptosis is of fundamental importance and plays a key role in determining the outcome of glomerulonephritis (GN). Under ideal circumstances, apoptosis deletes infiltrating leukocytes and excess numbers of resident cells that are surplus to requirements, thereby facilitating tissue remodeling and the restoration of normal tissue architecture. Apoptosis also has a darker side, however, and may be responsible for the deletion of critically important resident glomerular cells, resulting in hypocellular scarring and loss of renal function[7].

AIM OF THE WORK

This study was designed to assess the level of plasma anti-annexin V IgG antibodies as an apoptotic marker in both NS and acute post streptococcal glomerulonephritis (APSG) patients and determine its relationship to the severity of renal affection. Follow up of the detected changes, after therapy was done, as well, to determine the role of anti-annexin V IgG antibodies as a prognostic tool in such diseases.

PATIENTS AND METHODS

Patients

This study included 50 children and adolescents, 15 of them were diagnosed as APSGN and 20 were diagnosed as NS based on their full medical history taking and relevant laboratory investigations. Fifteen clinically healthy, age- and sex-matched children and adolescents (6 males and 9 females with an age range of 4-18 years) were included as a control group.

The APSGN group included 15 children and adolescents (6 males and 9 females) their ages ranged between 4-17 years. The NS group included 20 children and adolescents (8 males and 12 females) their ages ranged between 4-18 years.

After obtaining the approval of the ethical committee of the pediatrics board of Ain Shams University, an informed written consent for participation in the study was signed by the parents or the legal guardians. Each enrolled child or adolescent was then subjected to detailed history taking laying stress on the history of preceding upper respiratory tract or skin infections as well as the detailed family history. Thorough clinical examination was done as well as the laboratory investigations.

Follow up

The patients were followed up in hospital while receiving the proper treatment. After initial improvement which is achieved in the NS, according to Bergstein[8], as five days with the urine

protein free (negative, trace or +1 on the dipstick) and in the APSGN by the disappearance of hypertension and gross hematuria[9], the studied patients were then reevaluated by the previous clinical examination and laboratory investigations.

Sampling

Urine samples were collected over 24 hours in aseptic containers. Venous blood samples were collected and divided into two tubes. The first tube was a plain tube from which serum was separated and used for routine investigations. The second tube contained EDTA and was further divided into two parts. The first part was used for complete blood count. The other part was centrifuged and plasma was separated, aliquoted and stored at -20°C for the assay of anti-annexin V IgG.

Analytical methods

1. Full urine analysis was carried out according to Ringsrud and Linne[10].
2. Serum total protein, serum albumin, serum cholesterol, serum creatinine, blood urea nitrogen and urinary protein were assayed on the Synchron CX-7 auto-analyzer (Beckman Inst. Inc., CA, USA).
3. Complete blood count count (CBC) using T-450 coulter cell counter (Coulter, Florida, USA).
4. Complement (C_3) was determined by radial immunodiffusion (RID) using the commercially available kit provided by BIND A RID – TM [The Binding Site Limited, P.O. box 4073 Birmingham B 296 AT , England].
5. Estimation of plasma anti-annexin V, IgG using the Zymutest anti-annexin V, IgG kit supplied by Hyphen BioMed France (Hyphen BioMed: 95000 Neuville-sur-Oise-France) according to the principal of Reutelinspurger and Van Heerde[11].

Anti-annexin V IgG was estimated in plasma in order to avoid inhibition by Annexin V released from blood cells during clotting. Estimation of plasma anti-annexin V IgG was carried out by a solid phase enzyme linked immunosorbent technique (ELISA) in which highly purified human recombinant annexin V coated onto a microELISA plate wells is incubated with the diluted samples (dilution 1:100 by sample diluent). Following 5 washing steps, bound auto-antibodies of the IgG isotype are revealed with a goat antihuman IgG (Fc_γ specific)-peroxidase conjugate. This is followed by a second 5 washing steps, and then the bound enzyme activity was detected by the addition of the substrate tetramethyl-benzidine whose color intensity is directly proportional to the concentration of anti-annexin V auto-antibodies of the IgG isotype. The absorbance of each well is measured at 450 nm. The absorbance of standards was then plotted against standard concentration on linear graph paper, and the concentrations of unknown samples were read from the curve. The assay time is about two hours and quarters of an hour.

Statistical analysis

Statistical analysis of the results was done via the standard computer programs SPSS (version 10) and Statistical Software Package version 5 (Statsoft, Tulsa, OK, USA). Non-parametric data were detected by the Shapiro Wilk test. Central tendency and dispersion were assessed by mean ± SD in parametric variables and median (inter-quartile range) in non parametric variables.

Student-t and Paired-t tests were used for parametric quantitative data and Mann-Whitney U and Wilcoxon matched pairs tests for non-parametric quantitative data in addition to the correlation studies. The differences were considered significant if the probability (p) values were less than 0.05. The smaller the p-value obtained, the more significant is the result; the p-value being the probability of error of the conclusion.

RESULTS

Tables 1 and 2 summarize the clinical data of both groups of studied patients upon enrollment and after achieving remission. It is clear that the marked improvement in the puffiness of eye lids and the edema of lower limbs in NS patients (Table 1) and in the hematuria, oliguria, puffiness of eye lids and hypertension in APSGN patients (Table 2) signify the proper treatment results achieved.

As regards Table 3 it demonstrates the significantly higher weight of NS patients compared to both the controls ($p < 0.01$) and APSGN ($p < 0.001$) which almost normalized after treatment. As for the systolic and diastolic blood pressure, both were significantly higher in APSGN patients compared to the controls and NS patients ($p < 0.001$ respectively) and the same table shows that these changes were normalized after treatment.

On comparing the laboratory parameters of both groups of patients, the current study revealed significantly higher serum creatinine and blood urea nitrogen in APSGN patients compared to the NS ones ($p < 0.01$ respectively) and this finding improved after treatment (Table 4). Table 4 also demonstrates the significantly higher urinary protein levels and the significantly lower serum albumin levels in NS patients compared to the APSGN patients ($p < 0.001$ respectively), a finding which nearly reversed after treatment.

The current study also revealed significantly higher plasma anti-annexin V IgG levels in NS and APSGN patients compared to the controls (p values are < 0.05 and < 0.01 respectively) (Table 5). There was also significantly higher plasma anti-annexin V IgG levels in APSGN patients compared to NS patients before treatment ($p < 0.05$). As regards the statistical comparisons after treatment, there was no significant difference between each studied group and the controls or between each other ($p > 0.05$ respectively).

Figure 1 shows the significant decrease in plasma anti-annexin V IgG level in APSGN patients on treatment ($p < 0.01$). On the other hand the same figure did not demonstrate such finding in the NS patients ($p > 0.05$).

Regarding the correlation studies, there is no significant correlation between plasma anti-annexin V IgG level or its rate of change and the studied clinical or laboratory parameters in either NS or APSGN patients. Nevertheless, there is negative correlation between the serum albumin and the plasma anti-annexin V IgG level ($r = -0.27$) and positive correlation between the urinary protein and the plasma anti-annexin V IgG level ($r = 0.34$) in NS patients yet these correlations are non-significant ($p > 0.05$). In addition, APSGN patients show positive correlation between serum creatinine and

the rate of change of plasma anti-annexin V IgG level (r = -0.25) which is also of no statistical significance (p > 0.05).

In the current study anti-annexin V IgG antibodies are of a high level (according to the cut off level calculated from the control values) in eight of the 35 patients (22.9%) (3 NS and 5 APSGN patients) and the remaining 27 patients (77.1%) show normal values (10 NS and 17 APSGN patients).

Table 1: Data of the clinical examination of nephrotic syndrome patients in the acute stage and on remission.

	Acute stage				Remission			
Frequency / Studied parameters	Positive		Negative		Positive		Negative	
	No.	Freq.	No.	Freq.	No.	Freq.	No.	Freq.
Hematuria	2	10%	18	90%	1	5%	19	95%
Puffiness of eye lids	20	100%	0	0%	1	5%	19	95%
Edema of lower limbs	20	100%	0	0%	0	0%	20	100%
History of URT infection	2	10%	18	90%	0	0%	20	100%
Recurrent attacks	12	60%	8	40%	12	60%	8	40%
Hypertension	4	20%	16	80%	1	5%	19	95%
Polyuria	12	60%	8	40%	8	40%	12	60%
Oliguria	2	10%	18	90%	1	5%	19	95%
Dysuria	7	35%	13	65%	0	0%	20	100%

Table 2: Data of the clinical examination of acute poststreptococcal glomerulonephritis patients in the acute stage and after treatment.

	Acute stage				Remission			
Frequency / Studied parameters	Positive		Negative		Positive		Negative	
	No.	Freq.	No.	Freq.	No.	Freq.	No.	Freq.
Hematuria	15	100%	0	0%	0	0%	15	100%
Puffiness of eye lids	15	100%	0	0%	0	0%	15	100%
Edema of lower limbs	5	30%	10	70%	0	0%	15	100%
History of URT infection	15	100%	0	0%	15	100%	0	0%
Recurrent attacks	1	6%	14	94%	1	6%	14	94%
Hypertension	12	80%	3	20%	0	0%	15	100%
Polyuria	0	0%	15	100%	12	80%	3	20%
Oliguria	15	100%	0	0%	0	0%	0	0%
Dysuria	3	20%	12	80%	0	0%	0	0%

Table 3: Comparison between nephrotic syndrome patients, acute poststreptococcal glomerulonephritis patients and the controls as regards weight and blood pressure.

	NS	APSGN	Control	t/z* (p) test
Weight B	116.65 ± 13.55 (114.00 [19.75])	94.13 ± 14.41 (94.00 [20.00])	104.13 ± 10.02 (104.00 [7.00])	t1 = -3.01 (p ≤ 0.01) t2 = -2.21 (p ≤ 0.05) t3 = -4.73 (p < 0.001)
Weight A	110.00 ± 16.00 (104.00 [16.00])	93.00 ± 14.00 [93.00 (20.00)]	104.13 ± 10.02 (104.00 [7.00])	t1 = 2.91 (p < 0.05) t2 = 2.18 (p < 0.05) t3 = 1.17 (p > 0.05)
Systolic blood pressure B	101.93 ± 3.08 [102.00 (3.00)]	121.95 ± 6.15 (123.00 [6.75])	98.19 ± 3.90 (100.00 [6.00])	z1 = -2.58 (p < 0.05) z2 = -5.01 (p < 0.001) z3 = -5.01 (p < 0.001)
Systolic blood pressure A	100.00 ± 2.90 [102 (1.66)]	103 ± 250 [104 (311)]	98.19 ± 3.90 (100.00 [6.00])	z1 = 1.33 (p > 0.05) z2 = 1.99 (p > 0.05) z3 = 1.67 (p > 0.05)
Diastolic blood pressure B	106.87 ± 5.95 (106.00 [10.00])	125.30 ± 7.96 (124.00 [10.00])	100.80 ± 1.94 (100.00 [3.00])	z1 = -3.34 (p < 0.01) z2 = -5.05 (p < 0.001) z3 = -4.03 (p < 0.001)
Diastolic blood pressure A	101.00 ± 3.80 [103.00 (9.00)]	105.00 ± 4.57 [110.00 (9.00)]	100.80 ± 1.94 (100.00 [3.00])	z1 = 1.81 (p > 0.05) z2 = 1.81 (p > 0.05) z3 = 1.77 (p > 0.05)

* Non-parametric data are detected by **Shapiro-Wilk test**. The test of significance used here is Mann-Whitney test.

N.B.
- Weight and blood pressure are expressed as percent from mean for age
- Data are presented as mean ± SD [median (interquartile range)].
- t1/z1 compares NS patients to controls, t2/z2 compares APSGN to controls and t3/z3 compares NS to APSGN patients.
- p > 0.05 is not significant, p < 0.05 is significant, p < 0.01 is highly significant and p < 0.001 is very highly significant.
- Vs means versus, B means before treatment and A is after treatment

Table 4: Comparison between nephrotic syndrome and acute poststreptococcal glomerulonephritis patients as regards laboratory parameters before and after treatment.

	NS	APSGN	t/z* (p) test
S. Creatinine before	0.64 ± 0.23 (0.70 [0.40])	1.69 ± 1.33 (1.10 [1.48])	z = -2.99 (p < 0.01)
S. Creatinine after	0.61 ± 0.18 [0.63 (0.38)]	1.17 ± 1.12 (0.90 [1.14])	z = 2.71 (p < 0.05)
BUN before	17.13 ± 4.16 (16.00 [4.00])	35.80 ± 19.66 [26.50 (28.25)]	z = 3.44 (p < 0.01)
BUN after	15.09 ± 4.09 [14.15 (3.88)]	25.08 ± 16.73 [19.07 (21.97)]	z = 2.98 (p < 0.01)
Urine protein before	2773.75 ± 617.23 [2719.50 (743.00)]	1057.42 ± 373.420 [1126.00 (243.75)]	t = 8.69 (p < 0.001)
Urine protein after	1443.25 ± 519.00 [1412.70 (688.00)]	1002.13 ± 301.00 [1022.10 (202.00)]	t = 2.89 (p < 0.05)
S. Albumin before	1.38 ± 0.65 [1.10 (0.98)]	3.44 ± 0.50 [3.40 (0.50)]	z = -4.83 (p < 0.001)
S. Albumin after	3.81 ± 1.95 [3.71 (0.87)]	3.91 ± 0.40 [3.90 (0.60)]	z = 0.98 (p > 0.05)

* Non-parametric data are detected by **Shapiro-Wilk test**. The test of significance used here is Mann-Whitney test.

N.B
- Serum creatinine and BUN are measured in mg/dL, serum albumin in g/dL and urine protein in mg/24 hours
- Data are presented as mean ± SD [median (interquartile range)].
- p > 0.05 is not significant, p < 0.05 is significant, p < 0.01 is highly significant and p < 0.001 is very highly significant.

Table 5: Comparison between nephrotic syndrome patients, acute poststreptococcal glomerulonephritis patients and the controls as regards plasma anti-annexin V IgG antibodies before and after treatment.

	NS	APSGN	Control	z (p)
Antiannexin before	4.59 ± 2.55 (4.08 [3.84])	7.59 ± 4.73 (6.00 [5.80])	2.94 ± 2.17 (1.90 [3.70])	z1 = -2.25 **(p < 0.05)** z2 = -3.21 **(p < 0.01)** z3 = -2.14 **(p < 0.05)**
Antiannexin after	4.59 ± 4.03 [3.18 (3.25)]	3.32 ± 1.28 [2.90 (1.65)]	2.94 ± 2.17 (1.90 [3.70])	z1 = -1.44 (p > 0.05) z2 = -1.31 (p > 0.05) z3 = -0.30 (p > 0.05)

Data here is non-parametric as detected by **Shapiro-Wilk test**. The test of significance used here is Mann-Whitney test.

N.B.
- Plasma anti-annexin V is measured in U/ml (arbitrary units/ml).
- Data are presented as mean ± SD [median (interquartile range)].
- z1 compares NS patients to controls, z2 compares APSGN to controls and z3 compares NS to APSGN patients.
- $p > 0.05$ is not significant, $p < 0.05$ is significant, $p < 0.01$ is highly significant and $p < 0.001$ is very highly significant.

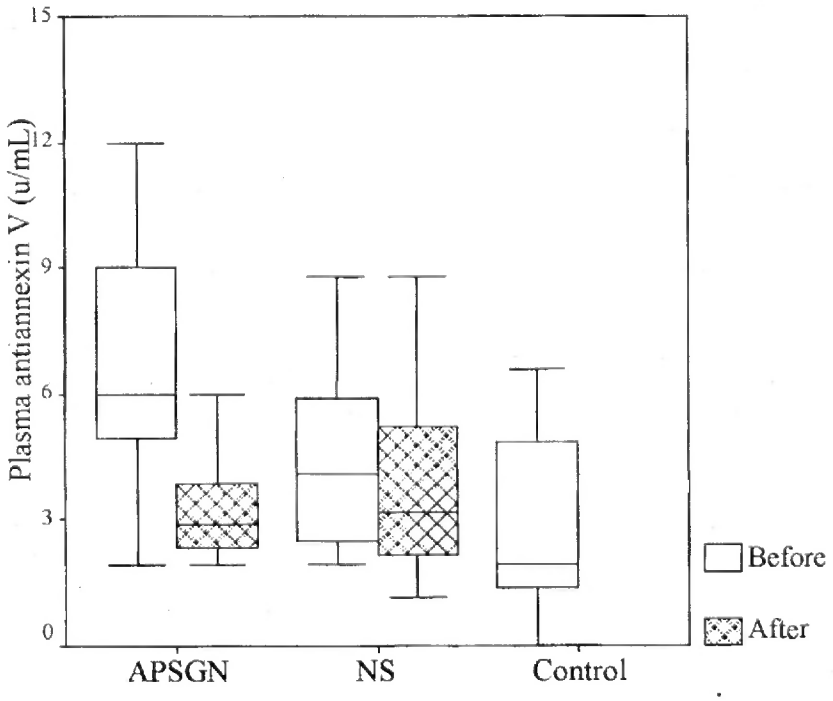

Fig. 1: Comparison between plasma anti-annexin V IgG anti-bodies before and after treatment in both APSGN and NS groups of patients.

DISCUSSION

In the present study, anti-annexin V IgG antibodies were significantly higher among NS and APSGN cases before treatment when compared to the control group.

Our results as regards NS patients come in agreement with Matsuda et al.[3]. The increased apoptotic tendency in NS patients was also reported by Yamaguchi et al.[12] who demonstrated the presence of apoptotic cells in the tubulo-interstitium in the kidneys of an animal model of NS but not in that of controls. More recently, Zachwieja et al.[13,14] concluded that patients with NS showed increase in the apoptosis rate of circulating lymphocytes and attributed it to reduced antioxidant defense mainly lower glutathione reductase and peroxidase enzymes.

Regarding the enhanced apoptosis detected in the studied APSGN patients, Viera et al.[15] had previously delineated an additional pathway for the pathogenesis of APSGN related to the role of cationic streptococcal erythrogenic toxin type B or its precursor on the induction of apoptosis and proliferation during the course of the disease. The results of the current study are also reinforced by the findings of Goumenos et al.[16] who reported the presence of apoptotic bodies in the renal tissue of patients with GN suggesting that the apoptotic process is ongoing during the evolution of renal disease. In GN, apoptosis is a double weapon. It has been involved in the resolution of renal injury as well as in the development of scarring[8]. Yang et al.[17] found that reduction in renal apoptosis, by caspase inhibition, ameliorates inflammation and fibrosis, and improves proteinuria in experimental GN.

Anti-annexin V IgG antibodies decreased in all studied patients after treatment, which was significant in APSGN patients only, and it was no longer significantly different from the control values.

Similarly, Zachwieja et al.[13] reported that in NS the number of apoptotic cells is greater in children with an acute attack of the disease than in both children in remission and in controls. This comes in agreement with the present results except for the non-significance of the decrease in the current study which could be explained by the short period of enrollment after therapy. As regards APSGN cases, the values of anti-annexin V decreased significantly after two weeks of treatment probably because the course of NS takes several months[10], while that of APSGN is short term and takes several weeks. These results highlight the prognostic value of serum anti-annexin V IgG.

The current study demonstrated that anti-annexin V IgG antibodies were significantly lower in the NS group compared to the APSGN group. In agreement with this finding is the report of decreased apoptosis detection in glomeruli and tubulo-interstitial areas in renal biopsies obtained from patients with NS (minimal change disease) compared to patients with APSGN[18]. Similarly, Rodriguez-Iturbe[19] reported an increased number of apoptotic cells in glomeruli and tubulo-interstitial areas in patients with proliferative glomerulonephritis that are associated with a good prognosis, particularly APSGN. In fact, Baker et al.[20] reported that apoptosis is the

major cell clearance mechanism counter-balancing cell division, thereby mediating resolution of glomerular hypercellularity in animal models of nephritis. From these results we can deduce the diagnostic value of anti-annexin V IgG in different renal diseases.

In NS patients the current study revealed negative correlation between the serum albumin and the anti-annexin V IgG level and positive correlation between the urinary protein and the anti-annexin V IgG level denoting that this apoptotic marker is related to the severity of renal affection in such a disease. In addition, APSGN patients showed positive correlation between serum creatinine and the rate of change of anti-annexin V IgG level also denoting the relation of anti-annexin V IgG level to the severity of renal affection in APSGN. The absence of statistical significance in these results could be related to the small sample size. Worth mentioning here is the report of Soto et al.[18] who could not find any detectable correlation between the value of apoptosis and the evolution of the disease in patients with APSGN suggesting that factors other than natural history are involved in the development of augmented or suppressed apoptosis in this disease.

In the current study anti-annexin V IgG antibodies were of a high level in 8 of 35 patients (22.9%) (three nephrotic syndrome and five APSGN patients) and 27 patients (77.1%) were normal (10 nephrotic syndrome and 17 APSGN patients). This is close to the report of Kaburaki et al.[21] who reported that, anti-annexin V IgG antibodies were detected in 27 of 140 (19%) patients with systemic lupus erythrematosis.

From the present study we can conclude that anti-annexin V IgG antibodies can be used as a marker of renal affection indicating the severity of underlying pathology and it could be of prognostic value also since its normalization couples the clinical and laboratory improvement. Anti-annexin V IgG antibodies can be also used as an additive differentiating marker between the underlying pathologies since it is significantly higher in patients with APSGN patients compared to NS patients.

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