Original Research Article

Clinical and Apoptotic Factors Defining and Predicting Steroid Resistance in Nephrotic Syndrome in Children

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Abstract

Introduction. Nephrotic syndrome (NS) is a kidney disease characterized by albuminuria, hyperlipidemia, edema, and hypoalbuminemia. Above 20 % of nephrotic children do not show response to steroid treatment. Molecular markers controlling apoptosis have not been studied as a predictors of steroid resistant NS (SRNS) and steroid sensitive NS (SSNS) in children. *Aim of the Study.* To identify clinical and molecular markers which define and predict the steroid-resistance phenomenon in children with NS. *Methods.* Fifty-six clinical cases of children hospitalized in Pediatric Hospital No. 7 (Kyiv, Ukraine) with NS (26 SSNS and 30 SRNS) studied. Stepwise logistic regression models used to analyze data. Data processed using GraphPad Prism 9.0 Software for Windows (USA, San Diego, CA). *Results.* Arterial hypertension, WBC and RBC count, serum creatinine, serum urea, serum cholesterol found to be factors defining and predicting SRNS. Apoptosis regulating BcL-xL, Bax but not caspase-8 found to be those defining SRNS. Among transcriptional factors HIF-1alfa selected as a factor predicting steroid resistance phenomenon. For SSNS group significant negative correlation observed between BcL-xL and Bax, BcL-xL and caspase-3, significant positive correlation observed between marker of cellular hypoxia HIF-1alfa and proapoptotic factor caspase-3. For SRNS group significant negative correlation observed between BcL-xL and Bax, BcL-xL and caspase-3 level, significant positive correlation observed between HIF-1alfa and proapoptotic factor caspase-3. *Conclusions.* Arterial hypertension, serum creatinine level, serum urea level, serum cholesterol level, WBC and RBC count, BcL-xL, Bax, caspase-3, and HIF-1alfa identified as candidate biomarkers to predict and define SRNS in pediatric NS.

Keywords

steroid resistant nephrotic syndrome, apoptosis, hypoxia, predictors

Introduction

Nephrotic syndrome (NS) is the most common glomerular disease in childhood. Nephrotic syndrome is a syndrome characterized by increased glomerular filtration barrier permeability. It classically presents with heavy proteinuria (Urine protein excretion >50 mg/kg/day or a spot urine sample with albumin/creatinine ratio (UACR) greater than 3 g/g creatinine), hypoalbuminemia (serum albumin <3 g/dL), edema, and hyperlipidemia.[1]

The prevalence is 12 to 16 per 100 000 children aged under 16 years. The reported incidence in children varies between 1.2 and 3.5 per 100 000 per year in Western Europe, 4.7 per 100 000 per year worldwide.[2] Although more than 85% of children with nephrotic syndrome respond to corticosteroids, approximately 10% to 15% remain unresponsive or later become steroid-resistant. Steroid-resistant nephrotic syndrome (SRNS) has been associated with negative renal prognosis. Approximately 36% to 50% of patients progressing to end-stage kidney disease within 10 years.[3]

Steroid-resistant nephrotic syndrome is not a single entity.[4] Different factors discussed in term of steroid-resistance formation, that is, pathomorphological type of disease (the most dominant lesion is focal segmental glomerulosclerosis (FSGS),[3,4] genetic markers,[5] metabolic factors.[6]

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This study aimed to investigate basic clinical (age, gender, blood pressure), basic clinical (serum creatinine, serum urea, serum cholesterol, complete blood count data, GFR, proteinuria), markers of apoptosis (BcL-xL, Bax, caspase-3, caspase-8), transcriptional factors (HIF-1alfa, NF-kappa B, Mn-SOD) as possible predictors of steroid resistance in children with NS.

Material and Methods

Patients

This case control study was carried out in the duration from June 2018 to August 2020. The study was conducted on 56 selected patients with NS collected from the nephrology department, Padiatric Clinical Hospital No. 7 (Kyiv, Ukraine) which is a clinical base of the Bogomolets National Medical University. An informed written consent was obtained from the parents of all participants. The study was approved by the local ethical committee of Bogomolets National Medical University and the research is complied with Helsinki Declaration.

Patients were also subjected to physical examination to document fever, edema, ascites, nutritional status, volume status, and hypertension. The laboratory data including; complete blood count, serum creatinine (Scr), serum urea, serum cholesterol, eGFR using Schwartz formula, 24 hours urine protein, urine analysis.

Patients Characteristics

NS was diagnosed by the triad of heavy proteinuria (>3 g/ day), hypoalbuminemia, and edema. The steroid-sensitive nephrotic syndrome (SSNS) group included 26 patients who responded to steroid treatment within 8 weeks. Steroid-resistant nephrotic syndrome was defined as a failure to respond to standard steroid treatment (2 mg/kg/day) for at least 6 weeks. All patients received the same dose of corticosteroid for 4 weeks (prednisolone 2 mg/kg/day), ACE inhibitors were only given to patients with hypertension. The patients were selected upon the outcome of 6 weeks of corticosteroid treatment. Patients with history of infection, high ASOT, hematuria, fever, protein energy malnutrition, and autoimmune diseases were excluded.

Immunoblotting for Detection of BcL-xL, Bax, NF-κB, Caspase-3, Caspase-8, NF-κB, HIF-1 alfa, Mn-SOD

Tissue samples used to measure markers of apoptosis, intracellular hypoxia, anti-oxidative response. Proteins solubilized in Laemmli sample buffer were resolved in polyacrylamide gels by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were then blocked in 5% non-fat milk in TBS-T (136 mM NaCl, 10 mM Tris, 0.05% Tween 20) and immunoblotted using the Bcl-xL, Bax, NF-κB and HIF-1α Ab (Cell Signaling Technology, Danvers, MA USA) the caspase-3 and caspase-8 Ab (Cell Signaling Technology, Danvers, MA USA), MnSOD Ab (Abcam, Cambridge, Cambs, United Kingdom) for 1 hour at room temperature. The actin mouse mAb was used as a loading control. After 3 washes with TBS-T, the membranes were incubated with secondary anti-rabbit or anti-mouse antibodies labeled with horseradish peroxidase for 1 hour at room temperature. Membranes were washed 3 times with TBS-T. The protein bands were visualized by chemiluminescent substrate ECL. Quantification of the protein content was done by densitometric analysis.

Statistics

The data expressed as means $\pm$ SEM and as frequencies and percentages when appropriate. ANOVA followed by the post hoc test and nonparametric test (Mann-Whitney *U* test) were used to test significance of differences. Receiver operator characteristic (ROC) curves were constructed to assess sensitivity, specificity, and respective area under the curve. The optimum cutoff value was investigated by maximizing both sensitivity and specificity and minimizing the distance from the left upper corner of the ROC curves. Pearson correlation was run to study the correlation between factors regulating apoptosis. Regression analysis was performed to assess the studied parameter as potential predictors of steroid resistance. Data processed using GraphPad Prism 9.0 Software for Windows (USA, San Diego, CA). *P* values $<.05$ considered statistically significant.

Results

Patients

The study was carried out during the period over 24 months. 56 patients with NS were included in to the study. Twenty-six children had SSNS and 30 had SRNS. In the SSNS group the mean of patients ages was 11.2 ± 0.6, 53.3% of them were males, all of them had generalized edema, and 12 of them (46.2%) of them were hypertensive.

In the SRNS group the mean of patients' ages was 11.0 ± 0.6, 66.6% of them were males, all of them had generalized edema, and 70% of them were hypertensive. The comparison of SSNS and SRNS patients' clinical findings showed a statistically significant difference regarding the incidence of hypertension, which was evidently higher in the SRNS group (*P* value $<.01$). Major part of participants in SSNS and SRNS were boys (*P* $<.05$ and *P* $<.05$, respectively) (Table 1).

Table 1. Clinical Characteristics of Patients with SSNS ans SRNS included into the study.

Characteristics	SSNS (n = 26) M ± SEM or %	SRNS (n = 30) M ± SEM or %	P
Boys*	14 (53.3%)	20 (66.7%)	.05
Girls*	12 (46.2%)	10 (33.3%)	.05
Age, years	11.2 ± 0.6	11.0 ± 0.6	.05
Disease duration, years	1.5 ± 0.3	1.8 ± 0.3	.05
BMI	21.6 ± 0.6	22.1 ± 0.6	.05
Hypertension**	12 (46.2%)	21 (70%)	.01
Edema	26 (100%)	30 (100%)	.05

*$P < .05$, **$P < .01$. Data presented as means ± SEM and percentages. Statistical analysis done using chi-square test.
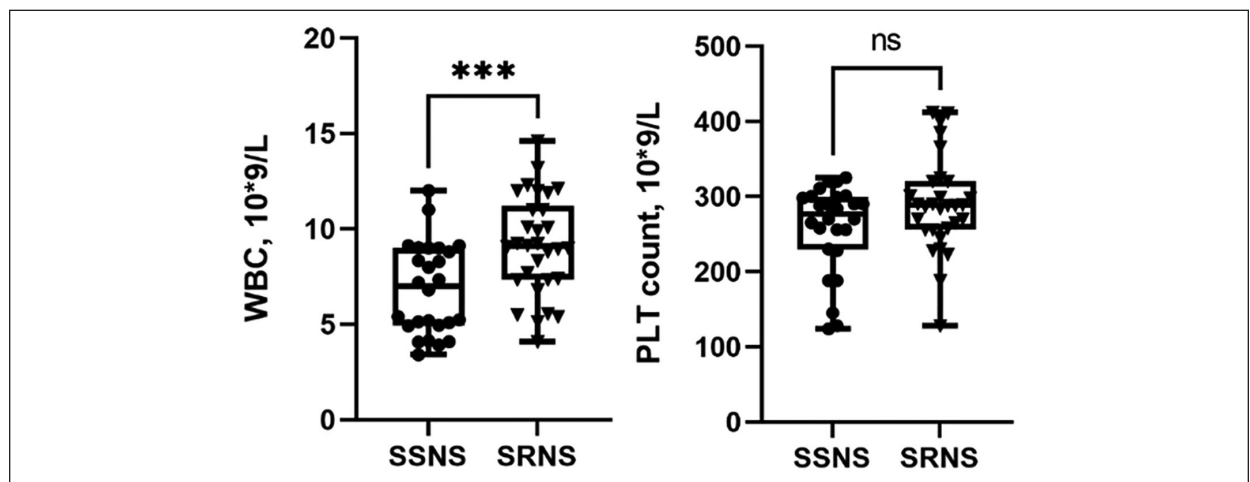

Figure 1. Complete blood count analysis in children with SSNS and SRNS. ***$P < .001$, ns: not significantly different. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

Basic Laboratory Biomarkers

Also, comparison of the laboratory findings showed statistically significant differences between SSNS and SRNS regarding WBC count—$6.87 \pm 0.47 \cdot 10^9$/L and $9.14 \pm 0.47 \cdot 10^9$/L, respectively ($P < .001$). No difference in level of platelets count found in SSNS and SRNS—$258.5 \pm 11.5 \cdot 10^9$/L and $289.0 \pm 11.5 \cdot 10^9$/L, respectively ($P > .05$) (Figure 1).

Biochemical markers, that is blood urea, serum creatinine, serum cholesterol measured in all children. Serum creatinine and serum cholesterol found to be significantly different between the SSNS and SRNS groups. Serum creatinine concentrations documented at level $68.38 \pm 3.46 \, \mu$mol/L in children with SSNS and $80.23 \pm 2.72 \, \mu$mol/L in children with SRNS ($P < .01$). Serum cholesterol levels were higher in SRSN 11.97 ± 0.38 mmol/L children as compared to $9.89 \pm 0.38 \, \mu$mol/L in children with SRNS ($P < .001$). Blood

urea levels were 9.99 ± 0.63 mmol/L and 11.08 ± 0.5 mmol/L ($P > .05$) (Figure 2).

Glomerular filtration rate (GFR) and 24-h urine protein excretion were selected as markers of kidney function impairment. GFR found to be significantly lower in SRNS children as compared to children with SSNS—68.38 ± 2.72 mL/min/$1.73 \, \text{m}^2$ and 82.84 ± 4.3 mL/min/$1.73 \, \text{m}^2$ ($P < .01$). No difference found in proteinuria level when comparing SSNS group and SRNS group— 9.1 ± 0.71 g/24 h and 8.73 ± 0.59 g/24 h ($P > .05$) (Figure 3).

Apoptotic Markers in Children With Nephrotic Syndrome

The expression of proapoptotic and antiapoptotic factors were studied using immunoblotting. Bax, caspase-3 and caspase-8 were chosen as apoptotic markers, Bacl-xL as antiapoptotic.
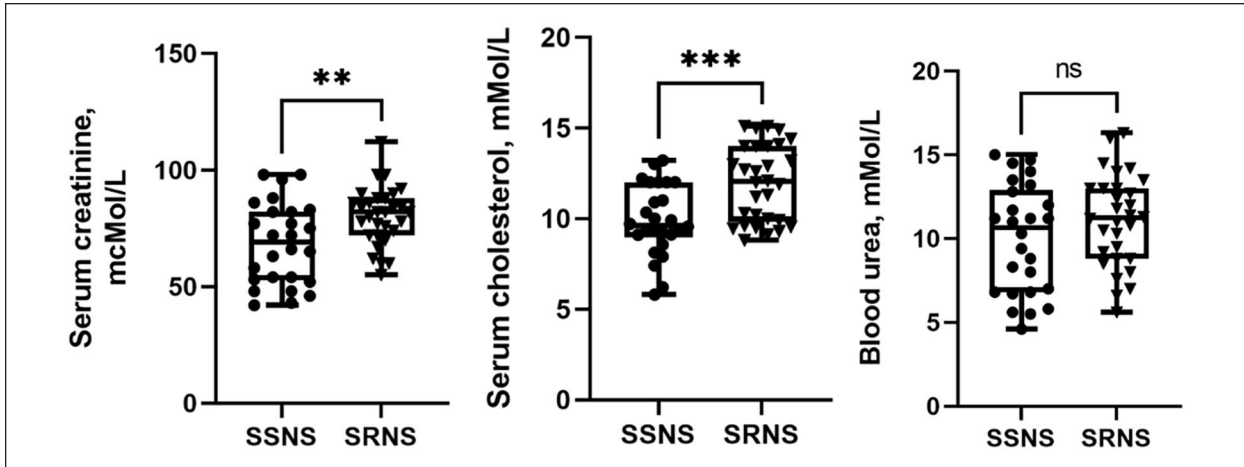
Figure 2. Biochemical markers in children with SSNS and SRNS. **$P < .01$, ***$P < .001$, ns: not significantly different. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test.
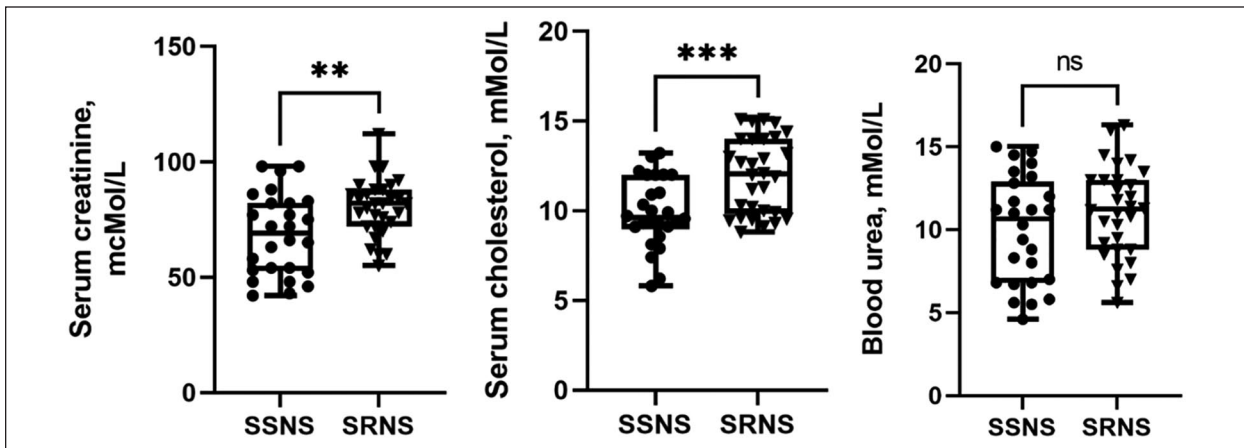

Figure 3. GFR and proteinuria levels in children with SSNS and SRNS. **$P < .01$, ns: not significantly different. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

Increased level of Bax expression in SRNS in comparison to SSNS documented—90.15 ± 2.0 mmol/L and 104.5 ± 3.o a.u., respectively ($P < .01$). Bax can initiate a cascade of events that eventually involves cleavage of caspase-3. Caspase-3 which is a final effector in mitochondrial level of apoptosis measured at level 131.1 ± 1.45 a.u. in children with SSNS and 141.4 ± 1.59 a.u. in children with SRNS ($P < .001$).

Caspase-8 selected as a proapoptotic marker and effector of the extrinsic apoptotic pathway. No difference in caspase-8 level found between SSNS and SRNS—37.3 ± 1.5 a.u. 139.6 ± 1.88 a.u., respectively ($P > .05$).

Significantly lower value of antiapoptotic factor BcL-xL found in children with SRNS in comparison to SSNS—SSNS and SRNS—110.5 ± 1.84 a.u. 92.88 ± 2.74 a.u., respectively ($P < .001$). (Figure 4).

Beside apoptotic markers, studying of the NF-kB, HIF-1alfa, Mn-SOD done. NF-kB NF-κB is expressed in nearly all mammalian cells. Under physiologic (resting) conditions, NF-κB exists as an inactive form composed of a dimeric form of the transcription factor and its binding partner, IκB, which maintains NF-κB in an inactive form. NF-κB is activated in response to a various types of stress, including chronic inflamation.[7] We found that SRNS children have lower NF-kB level (107.3 ± 1.98 a.u) as compared to SSNS children (97.42 ± 3.34 a.u) ($P < .05$).

In mammalian cells, manganese superoxide dismutase (MnSOD) is the essential mitochondrial antioxidant enzyme that detoxifies the free radical superoxide, the major by-product of mitochondrial respiration.[8] Mitocholdrial respiration changes have place under
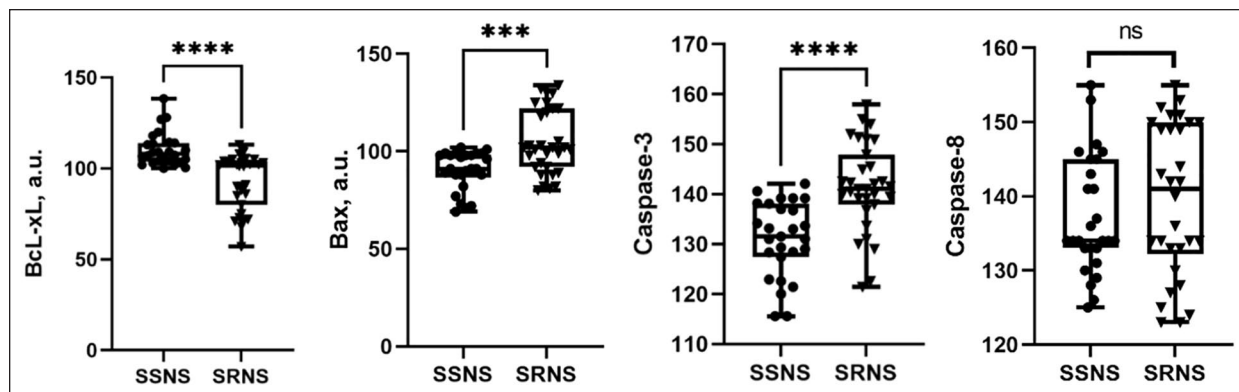
Figure 4. Levels of proapoptotic and antiapoptotic markers in children with SSNS and SRNS.
***$P < .001$, ns: not significantly different. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

Figure 5. Levels of NF-kB, Mn-SOD and HIF-1alfa in children with SSNS and SRNS. *$P < .05$, **$P < .01$, ns: not significantly different. Histograms represent means ± SEM. Statistical analysis was performed using the Mann-Whitney U test.
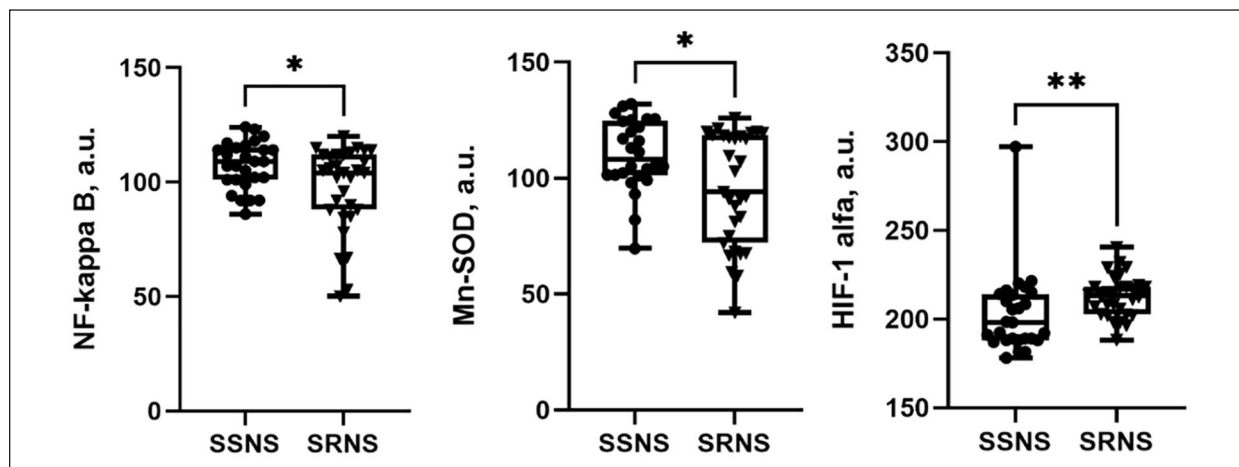
inflammatory conditions. Level of Mn-SOD was significantly lower in children with SRNS and compared to SSNS—109.9 ± 3.0 and 95.16 ± 4.33, respectively ($P < .05$).

Finally, levels of HIF-1alfa as a marker of intracellular hypoxia studied in nephrotic children.[9] Significantly higher HIF-1alfa in children with SRNS in comparison to group with SSNS found—202.8 ± 4.44 a.u and 212.9 ± 2.14 a.u, respectively ($P < .01$) (Figure 5).

Identification of Clinical and Metabolic Factors Defining and Predicting Steroid Resistance in Nephrotic Children

Multivariate linear regression analysis used to determine the clinical predictors of steroid resistance in nephrotic children among categorical factors. Demographic parameter, that is, age, gender and presence of arterial hypertension were included into analysis. Factor predicting SRNS these were identified arterial hypertension presence (β—0.31, SE 0.15, 95% CI −0.6 to −0.007, $P < .05$). None of other studied factors found as a predictors of SRNS—age (β—0.02, SE 0.03, 95% CI −0.07 to 0.03, $P > .05$), gender—(β 0.11, SE 0.13, 95% CI −0.16 to 0.39, $P > .05$) (Table 2).

Logistic regression models for the odds of steroid resistance among numerical factors in nephrotic children made using a step-wise selection procedure. Candidate predictors entering the model at $P < .05$. Three models created: (I) basic laboratory factors—RBC, WBC, PLT; (II) biochemical and factors: Serum creatinine, Serum urea, Serum cholesterol; Functional factors: GFR, proteinuria; (III) apoptosis controlling factors: Bax, BcL-xL, caspase-3, caspase-8; (IV)—transcriptional factors:

Table 2. Multivariate Linear Regression Analysis of Factors Predicting SRNS in Children.

Variable	β	SE	95% CI	P value
Intercept	0.8570	0.3440	0.1667 to 1.547	.0160
Age, years	−0.02374	0.02538	−0.07468 to 0.02719	.3538
Gender [male]	0.1120	0.1371	−0.1631 to 0.3871	.4176
Arterial hypertension	−0.3057	0.1487	−0.6042 to −0.007281	.0449

Table 3. Logistic Regression Analysis of Factors Defining and Predicting SRNS in Children.

Odds ratios	Variable	Estimate	95% CI (profile likelihood)
β0	Intercept	0.007103	3.102e-005 to 0.7313
β1	WBC	1.413	1.120 to 1.854
β2	PLT	1.000	0.9965 to 1.019
β3	RBC	1.149	0.3835 to 3.479
β0	Intercept	1.554	5.227e-006 to 435970
β1	Serum creatinine	1.039	0.9530 to 1.140
β2	Serum urea	1.033	0.8335 to 1.285
β3	Proteinuria	0.8281	0.6664 to 1.003
β4	GFR	0.9760	0.9028 to 1.051
β0	Intercept	4.726e-049	1.048e-093 to 4.771e-025
β1	BcL-xL	1.396	1.165 to 1.913
β2	Caspase-3	1.660	1.271 to 2.682
β3	Caspase-8	0.9505	0.8448 to 1.052
β4	Bax	1.152	1.035 to 1.367
β0	Intercept	753.3	9.386e-005 to 610212227
β1	HIF-1, alfa	1.016	0.9775 to 1.071
β2	NF-kappa B	0.9540	0.8959 to 1.010
β3	Mn-SOD	0.9531	0.9177 to 0.9831

NF-kB, HIF-1alfa, Mn-SOD. Receiver operating characteristics curve and area under the curve (AUC) analyses used to assess each model's ability to define clinical steroid resistance.

Logistic regression analysis (Table 3) of basic laboratory factors selected WBC level (OR: 1.4; OR 95% CI: 1.12-1.85) and RBC level (OR: 1.14; OR 95% CI: 0.38-3.48) but not PLT count (OR: 1.0; OR 95% CI: 1.0-1.02) as a markers able to define the steroid resistance in nephrotic children (AUC: 0.75; SE: 0.06; 95% CI: 0.63-0.88; $P < .001$) (Figure 6A).

Among biochemical factors and parameters showing kidney function impairment (Table 3) serum creatinine (OR: 1.04; OR 95% CI: 0.92-1.14), serum urea (OR: 1.14; OR 95% CI: 0.89-1.49), serum cholesterol (OR: 1.97; OR 95% CI: 1.37-3.18) were found to be a factors defining SRNS. GFR level (OR: 0.95; OR 95% CI: 0.87-1.04) and proteinuria level (OR: 0.85; OR 95% CI: 0.67-06) did not fit the model (AUC: 0.88; SE: 0.05; 95% CI: 0.78-0.97; $P < .001$) (Figure 6B).

Apoptosis regulating factors Bax, BcL-xL, caspase-3, caspase-8 (Table 3) were analyzed in terms of factors predicting SRNS. BcL-xL (OR: 1.4; OR 95% CI: 1.17-1.91), Bax (OR: 1.15; OR 95% CI: 1.04-1.37), caspase-3 (OR: 1.66; OR 95% CI: 1.27-2.68) but not caspase-8 (OR: 0.95; OR 95% CI: 0.84-1.05) (AUC: 0.93; SE: 0.03; 95% CI: 0.87-0.99; $P < .001$) fit the model (Figure 6C).

Finally, group of transcriptional factors NF-kB, HIF-1alfa, Mn-SOD included into the logistic analysis (Table 3). We found that HIF-1alfa, which is a marker of intracellular hypoxia selected as a factor defining steroid resistance phenomenon (OR: 1.02; OR 95% CI: 0.98-1.07). Nuclear transcriptional factor NF-kappaB (OR:
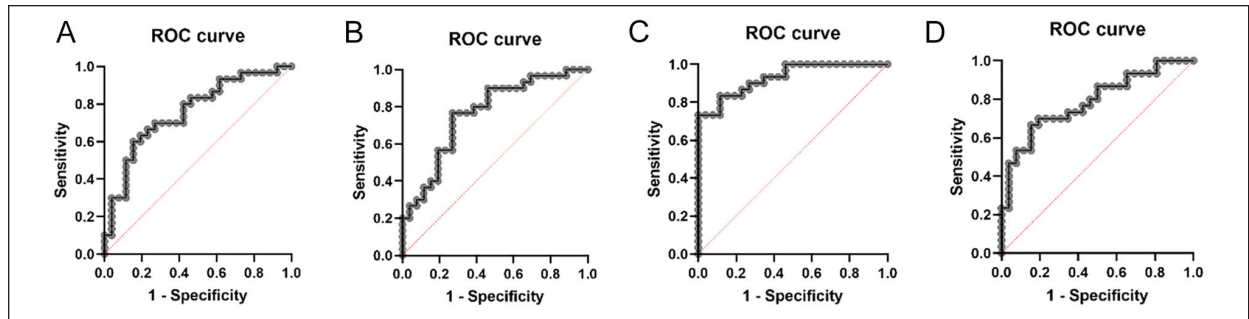
Figure 6. Receiver operating characteristics curve defining clinical steroid resistance. (A) basic laboratory factors—RBC, WBC, PTC; (B) biochemical and factors: Serum creatinine, Serum urea, Serum cholesterol; Functional factors: GFR, proteinuria; (C) apoptosis controlling factors: Bax, BcL-xL, caspase-3, caspase-8; (D)—transcriptional factors: NF-kB, HIF-1alfa, Mn-SOD.
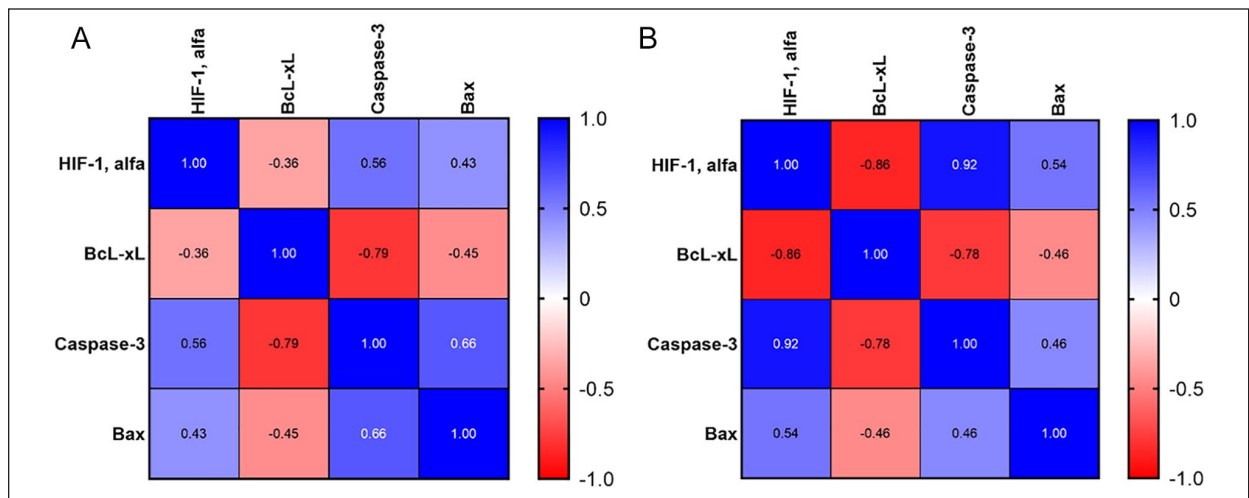

Figure 7. Heat map correlation between factors regulating apoptosis. (A)SSNS group and (B)SRNS group.

0.95; OR 95% CI: 0.9-1.01) and antioxidative factor Mn-SOD did not fit the model (OR: 0.95; OR 95% CI: 0.92-0.98)—(AUC: 0.78; SE: 0.06; 95% CI: 0.67-0.91; $P < .001$) (Figure 6D).

Pearson correlation was run to correlate principal factors found to be those defining steroid resistance in nephrotic children—Bax, BcL-xL, caspase-3, HIF-1alfa in both SSNS group (Figure 7A) and SRNS group (Figure 7B).

For SSNS group significant negative correlation was observed between antiapoptotic factor BcL-xL and proapoptotic factor Bax ($r = -0.45$, $P < .05$), significant negative correlation was observed between antiapoptotic factor BcL-xL and proapoptotic factor caspase-3 ($r = -.79$, $P < .05$), significant positive correlation was observed between marker of cellular hypoxia HIF-1alfa and proapoptotic factor caspase-3 ($r = .56$, $P < .01$) and proapoptotic factor Bax ($r = .43$, $P < .05$).

For SRNS group significant negative correlation was observed between antiapoptotic factor BcL-xL and proapoptotic factor Bax 1 ($r = -.46$, $P < .05$), significant negative correlation was observed between antiapoptotic factor BcL-xL and proapoptotic factor caspase-3 ($r = -.78$, $P < .01$), significant positive correlation was observed between marker of cellular hypoxia HIF-1alfa and proapoptotic factor caspase-3 ($r = .92$, $P < .05$) and proapoptotic factor Bax ($r = .53$, $P < .05$).

Discussion

Patients with SRNS have a more progressive disease course and poor outcomes when compared with SSNS patients. Different factors predicting and defining steroid resistance in nephrotic children discussed. Predictors of initial steroid resistance in study done by Kim et al[10] were age at onset, hypertension, and decreased kidney function.

Other research group identified creatinine concentration, malonate and glutamine concentration as 2 candidate biomarkers predictive of SRNS. In addition, paired-sample analyses done this study identified several candidate biomarkers with the potential to identify mechanistic molecular pathways/targets that regulate clinical steroid resistance, including lipoproteins, adipate, pyruvate, creatine, glucose, tyrosine, valine, glutamine, and sn-glycero-3-phosphcholine.

In study done by Bennett et al Vitamin D–binding protein (VDBP), alpha-1 acid glycoprotein 1 (AGP1), alpha-1 acid glycoprotein 2 (AGP2), alpha-1-B glycoprotein (A1BG), fetuin-A, prealbumin, thyroxine-binding globulin and hemopexin, and alpha-2 macroglobulin were measured and combined with urine neutrophil gelatinase–associated lipocalin (NGAL), which had been previously shown to distinguish patients with SRNS. A subset of 5 markers (including VDBP, NGAL, fetuin-A, prealbumin, and AGP2) showed significant associations with SRNS.[11]

In our study we focused on general clinical parameters, markers of apoptosis, cellular hypoxia and antioxidant effector. Previously it was shown that apoptosis is a hallmark of proteinuria kidney diseases. Albuminuria, a well-documented predictor of progressive loss of kidney function, is considered a cause of kidney damage and loss of function.[12-15]

And the BCl-2 family has long been identified for its role in apoptosis regulation. The members of the Bcl-2 family are designated as such due to their BCL-2 homology (BH) domains and involvement in apoptosis regulation. The BH domains facilitate the family members' interactions with each other and can indicate pro- or anti-apoptotic function.[16]

Wada et al[17] shown that puromycine-induced podocyte apoptosis is p53 dependent and associated with changes in Bcl-2-related proteins and AIF translocation. The protective effects of dexamethasone on PA-induced apoptosis were associated with decreasing p53, increasing Bcl-xL, and inhibition of AIF translocation.

We analyzed levels of apoptotic family proteins in kidney material of children with NS. Our results show that level apoptotic factor Bax, apoptotic factor caspase-3 higher in children with SRNS and antiapoptotic factor BcL-xL level is lower in SRNS as compared to SSNS. No difference found in capse-8 level. Logistic regression model shows that BcL-xL, Bax and caspase-3 selected as factors predicting and defining SRNS.

Nuclear transcriptional factor NF-kB was downregulated in SRNS children as compared to SSNS group. NF-kB regulated Bax translocation to mitochondrial membrane and subsequently regulates apoptosis. Children with SRNS have lower NF-kB level as compared to SSNS.

We analyzed level of HIF-1alfa which is an important transcriptional factor regulating many cellular functions.[18] HIF-1 is a heterodimer composed of the rate limiting factor HIF1α and the constitutively expressed HIF-1β.[19]

Nephrotic syndrome associates with chronic inflammation under the persistent influence of proteinuria.[20] Hypoxia can induce apoptosis by causing hyperpermeability of the inner mitochondrial membrane, which leads to the release of cytochrome C. The most direct induction of hypoxia induced apoptosis is the inhibition of the electron transport chain at the inner membrane of the mitochondria. The lack of oxygen inhibits the transport of protons and thereby causes a decrease in the membrane potential. The reduction of mitochondrial derived ATP causes activation of Bax, leading to caspase-3 activation.[21]

Our results show that HIF-1alfa level was higher in SRNS children as compared to SSNS. At the same time children with SRNS have significantly lower level of Mn-SOD as compared to SSNS. Mn-SOD is an important player in apoptosis regulation via suppression of cytochrome c release.[22] We found that HIF-1alfa bur not NF-kB and Mn-SOD is a factor defining steroid resistance phenomenon in nephrotic children.

Correlation analysis shown significant negative correlation between antiapoptotic factor BcL-xL and pro-apoptotic factor Bax level, significant negative correlation between antiapoptotic factor BcL-xL and proapoptotic factor caspase-3, significant positive correlation was between marker of cellular hypoxia HIF-1alfa and proapoptotic factor caspase-3 and proapoptotic factor Bax. This relationship between principal factors regulating apoptosis observed in both SSNS and SRNS.

Finally, we hypothesize that apoptosis triggered by proteinuria in children with nephrotic syndrome depends on intrinsic mitochondrial pathway activation (Figure 8).

The intrinsic apoptotic pathway is controlled by the family of Bcl-2 proteins. The mitochondrial apoptotic pathway is initiated by activation of Bax, a prominent pro-apoptotic member of the Bcl-2 family, to the outer mitochondrial membrane, where it oligomerizes and penetrates the inner mitochondrial membrane. This results in release of cytochrome C and caspase activation, the apoptotic executors. Bcl-xL, a prominent antiapoptotic member of the Bcl-2 family, counteracts Bax accumulation on the mitochondria and Bax-induced permeabilization of the mitochondrial membranes.

Individual analysis of factors controlling apoptosis, that is, Bax, BcL-xL, caspase-3. HIF-1alfa, may have clinical significance in predicting and defining steroid resistance in nephrotic syndrome. Further investigations of other proapoptotic and antiapoptotic factors in children with NS is important in terms of finding new approaches of adequate treatment.
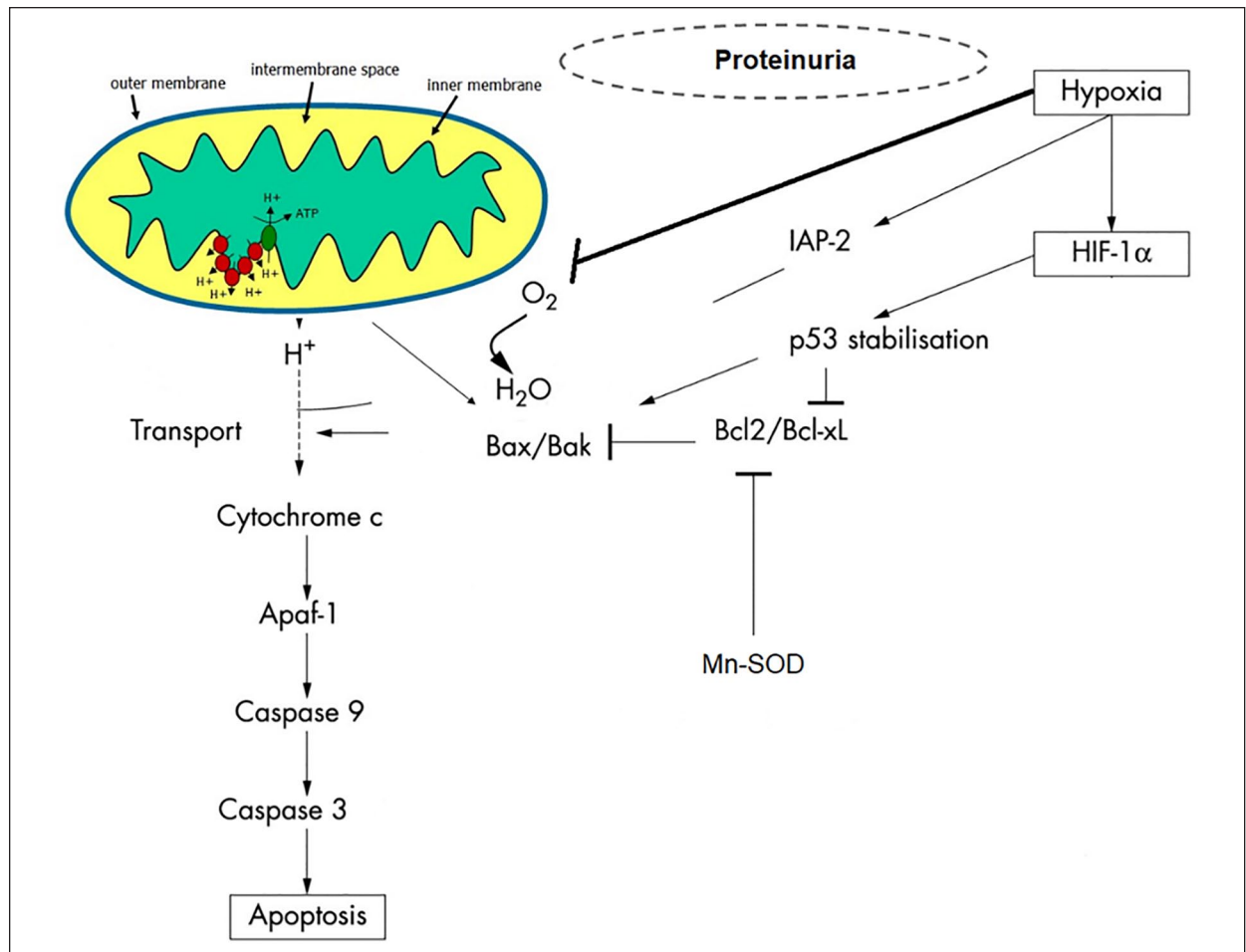
Figure 8. Signaling apoptotic pathway induced by proteinuria in nephrotic children.

This study has certain limitations that must be presented. Our pilot study was cross-sectional, at a single center with somewhat limitation in patients. The strength is that enrolled patients were age-matched with no difference between SRNS patients and SSNS patients in disease duration. The strength of our panel to distinguish SRNS from SSNS (AUC 0.75-0.95; $P < .0001$) is high enough.

Author Contributions

IB: substantially contributed to conception or design; contributed to acquisition, analysis, or interpretation of data; drafted the manuscript; critically revised the manuscript for important intellectual content; gave final approval; Agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IM: substantially contributed to conception or design; contributed to acquisition, analysis, or interpretation of data; drafted the manuscript; critically revised the manuscript for important intellectual content; gave final approval; Agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IB: substantially contributed to conception or design; contributed to acquisition, analysis, or interpretation of data; drafted the manuscript; critically revised the manuscript for important intellectual content; gave final approval; Agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

The study conducted according to the guidelines of the Declaration of Helsinki. An informed written consent obtained from the parents of all participants. The study approved by the local ethical committee of Bogomolets National Medical University.

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Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Kanwal K, William SH, Larry GA. *Clinical Pediatric Nephrology*, 3rd ed. CRC Press; 2016:307.
2. Lee JM, Kronbichler A, Shin JI, Oh J. Current understandings in treating children with steroid-resistant nephrotic syndrome. *Pediatr Nephrol*. 2021;36:747-761.
3. Nourbakhsh N, Mak RH. Steroid-resistant nephrotic syndrome: past and current perspectives. *Pediatric Health Med Ther*. 2017;8:29-37.
4. Zaorska K, Zawierucha P, Świerczewska M, Ostalska-Nowicka D, Zachwieja J, Nowicki M. Prediction of steroid resistance and steroid dependence in nephrotic syndrome children. *J Transl Med*. 2021;19:130.
5. Lovric S, Ashraf S, Tan W, Hildebrandt F. Genetic testing in steroid-resistant nephrotic syndrome: when and how? *Nephrol Dial Transplant*. 2016;31:1802-1813.
6. Gooding JR, Agrawal S, McRitchie S, et al. Predicting and defining steroid resistance in pediatric nephrotic syndrome using plasma metabolomics. *Kidney Int Rep*. 2021;5(1):81-93.
7. Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol*. 2002;3:221-227.
8. Candas D, Li JJ. MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx. *Antioxid Redox Signal*. 2014;20(10):1599-1617.
9. Liu W, Shen S-M, Zhao X-Y, Chen G-Q. Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int J Biochem Mol Biol*. 2012;3(2):165-178.
10. Kim JS, Bellew CA, Silverstein DM, Aviles DH, Boineau FG, Vehaskari VM. High incidence of initial and late steroid resistance in childhood nephrotic syndrome. *Kidney Int*. 2005;68(3):1275-1281.
11. Bennett MR, Pleasant L, Haffner C, et al. A novel biomarker panel to identify steroid resistance in childhood idiopathic nephrotic syndrome. *Biomark Insights*. 2017;12:1177271917695832.
12. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell*. 2002;9(Suppl):S81-S96.
13. Tojo A, Kinugasa S. Mechanisms of glomerular albumin filtration and tubular reabsorption. *Int J Nephrol*. 2012;2012:481520.
14. Zoja C, Abbate M, Remuzzi G. Progression of renal injury toward interstitial inflammation and glomerular sclerosis is dependent on abnormal protein filtration. *Nephrol Dial Transplant*. 2015;30:706-712.
15. Birn H, Christensen EI. Renal albumin absorption in physiology and pathology. *Kidney Int*. 2006;69:440-449.
16. Warren CFA, Wong-Brown MW, Bowden NA. BCL-2 family isoforms in apoptosis and cancer. *Cell Death Discov*. 2019;10:177.
17. Wada T, Pippin JW, Marshall CB, Griffin SV, Shankland SJ. Dexamethasone prevents podocyte apoptosis induced by puromycin aminonucleoside: role of p53 and Bcl-2-related family proteins. *J Am Soc Nephrol*. 2005;16(9):2615-2625.
18. Ziello JE, Jovin IS, Huang Y. Hypoxia-inducible factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale J Biol Med*. 2007;80(2):51-60.
19. Park SJ, Shin JI. Complications of nephrotic syndrome [published correction appears in Korean J Pediatr. 2012 Apr;55(4):151]. *Korean J Pediatr*. 2011;54(8):322-328.
20. Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. *J Clin Pathol*. 2004;57(10):1009-1014.
21. Robbins D, Zhao Y. Oxidative stress induced by MnSOD-p53 interaction: pro- or anti-tumorigenic? *J Signal Transduct*. 2012;2012:101465.
22. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*. 2008;9:47-59.