Reports of Biochemistry & Molecular Biology
Vol.13, No.1, Apr 2024

Original article

www.RBMB.net

Association of ABCB1(Rs10276036, C/T) Gene, IL-18, and TNFα as Risk Factors for Nephrotic Syndrome Incidence

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Abstract

Background: The most common cause of Nephrotic Syndrome (NS) in children is idiopathic NS, also called nephrosis. The most prominent clinical signs are hyperlipidemia, severe proteinuria, edema, swelling of body tissues, and an increased risk of infection. The object of this study was to examine the correlation of the ABCB1 gene (rs10276036, C > T), IL-18, and TNFα to the prevalence of NS among Egyptian children having NS.

Methods: This study included 100 participants with NS and 100 healthy controls. To analyze the ABCB1 gene (rs10276036 C >T) variant PCR technique was used. IL-18 and TNF levels were estimated using Enzyme-Linked Immunosorbent Assay (ELISA).

Results: Increased frequency of CT and TT genotypes of the ABCB1 gene (rs10276036 C / T) in NS patients compared to controls, with p-value = 0.001, OR = 2.270, CI = (1.550-3.327) for CT genotype and p-value = 0.001, OR = 5.070, CI = (2.463-10.438) for TT genotype. The frequencies of ABCB1 (rs10276036 C >T) genotypes were statistically significant in the dominant model (OR 2.560; $p < 0.001$) and in the recessive model OR, 3.231; $p = 0.001$). Significantly high levels of both IL-18 and TNFα were found in NS patients compared to controls.

C*onclusion:* The ABCB1gene (rs10276036 C/T), IL-18, and TNFα are associated with the prevalence of NS in Egyptian children and might be considered as independent risk factors for its incidence.

Keywords: ATP Binding Cassette Transporter, Interleukin-18, Polymerase Chain Reaction, Subfamily B, Tumor Necrosis Factor-alpha.

Introduction

Nephrotic syndrome (NS) or nephrosis is defined as a glomerular filtration barrier structural and functional impairment (1). It causes an increase in protein loss, resulting in significant proteinuria (2). Nephrotic syndrome (NS) is the second leading cause of chronic kidney disease (CKD) in people under the age of 25 (3). Boys are more susceptible to pediatric NS than girls with a ratio of 2:1. Nephrotic syndrome (NS) affects children of any age, from infancy through adolescence (4). Minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are both common causes of nephrotic syndrome in children and adults (5). Steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS) are the two forms of NS. Some people with SSNS may eventually acquire SRNS (6).

The ABCB1 gene (multi-drug resistance MDR1) located on chromosome 7q21, consists of 28 exons with sizes ranging from 49 to 209 bp. P-glycoprotein (P-gp), a

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Received: 1 Mar, 2024; Accepted: 7 Jul, 2024

[DOI: 10.61186/rbmb.13.1.67]

multidrug transporter, is encoded by this gene which functions as a transmembrane efflux pump, and required for drug absorption, tissue targeting, and excretion (7). ABCB1 mRNA expression in lymphocytes has been linked to a poor response to prednisone, cyclophosphamide, and cyclosporine. In children with NS, more than 50 single nucleotide polymorphisms (SNPs) in ABCB1 have been found. Previous studies found that children with SRNS have high ABCB1 expression and P-gp activity. Several studies have investigated the relationship between P-gp polymorphisms and the responsiveness of contradictory glucocorticoids (GCs) (8). The MDR-1 gene product P-glycoprotein is also suggested to be a risk factor for NS and/or steroid resistance (9). Some natural and synthetic GCs have xenobiotic accumulations in the leukocyte membrane cytoplasm. Cytokines promote the role of P-gp in chronic autoimmune disease inflammation (10, 11).

IL-18 is a member of the IL-1 family of cytokines, and is expressed by a wide range of cells, including monocytes, stromal cells, melanocytes, progenitor cells, and glial cells. Tubular epithelial cells are the primary source of IL-18 production in the kidney. IL-18 is involved in inflammation, which is thought to be the root cause of both acute and chronic kidney disease (12). Recently, researchers have investigated the biological and pathological role of IL-18 in different diseases (13).

Tumor necrosis factor-alpha (TNF-α) is a cytokine that affects a variety of cell types (14). It is produced by activated macrophages, T-lymphocytes, and natural killer cells (15). TNF-α is regarded as the primary regulator of inflammatory responses (16). It plays a role in the development of various inflammatory and autoimmune disorders (17). Tumor necrosis factor receptors (TNFRs) are divided into two types (TNFR1 and TNFR2). TNFR1 is found in all cells. However, TNFR2 is found only in immune cells (18). It was observed that there is an increase in the TNF-α levels in NS children (19).

Materials and Methods

Study Population

This study included 200 children, with 100 diagnosed with nephrotic syndrome at Mansoura University's Urology and Nephrology Centre in Egypt, and 100 healthy volunteers visiting the hospital for routine check-ups. The two groups were matched in age and sex. Ethical approval for the study was granted by the ethical committee of Mansoura University (Code Number: MDP.20.12.51; Date: 12/01/2021). Nephrotic syndrome was diagnosed based on plasma albumin levels <2.1 g/dL, proteinuria ≥40 mg/m² per hour, and the presence of peripheral edema. Additionally, total cholesterol levels >350 mg/dL were indicative of nephrotic syndrome (Bagga and Mantan 2005). Cases with complications other than nephrotic syndrome were excluded from the study. Informed written consent was acquired from the legal guardians of all investigation participants with the declaration of data privacy. This study was performed by the "Declaration of Helsinki."

Sample collection and examination

Five ml of peripheral blood was collected from each participant. The blood sample was divided into two parts: two ml were deposited in EDTA-coated tubes for the analysis of both hematological markers and PCR, while the remaining part was drawn into ordinary vacutainer tubes for biochemical analysis.

Biochemical, hematological parameter estimation

A fully automated biochemical analyzer was utilized to measure creatinine, albumin, total cholesterol, triglycerides, Alanine Aminotransferase (ALT/GPT), and Aspartate Aminotransferase (AST/GOT) in both control and patient groups. Additionally, a complete blood count was performed for each participant using a cell analyzer.

[DOI: 10.61186/rbmb.13.1.67]

Extraction of whole genomic DNA and Genotyping of ABCB1 gene (rs10276036 C>T) variants

DNA was extracted from the whole blood samples using a GeneJET DNA extraction kit.

The ABCB1 (NCBI accession number: 5243) gene was genotyped using the T-ARMS-PCR technique, as described by Faraji et al. (20). The ABCB1 primers were used in this study and PCR condition are shown in Table 1.

Table 1. PCR primers and PCR condition for *ABCB1*.

Primer name	**Sequences (**5′-3′)		
ABCB1R	GAGCCCAGGAGGTAGAGGTT		
ABCB1WF	CCATCAGGCTACTGAGATAGTGTC		
ABCB1MF	CCATCAGGCTACTGAGATAGTGTTC		
PCR program			
Steps	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	5 min.	1
Denaturation	94	35 (s)	35
Annealing	59	35 (s)	35
Extension, Final extension	72,72	50 (s), 5 min	35, 1

The PCR reaction of the ABCB1 gene (C >T) was carried out by utilizing the technique described in detail in (20). The amplicons size was 260 bp.

Estimation of IL-18 and TNF-α

L-18 levels were assessed with the Human IL-18 ELISA Kit (Catalog No: EH0011, China) (21), while TNF-α levels were assessed using the TNF-α ELISA Kit (Catalog No: ELH-TNF-α, China).

Statistical Analysis

The collected data were revised, coded, and tabulated using the Statistical Package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Qualitative data were presented as frequencies. The selected study groups were assessed for Hardy-Weinberg equilibrium (HWE) to ensure suitability for genetic analysis of this SNP. Normality of data distribution was assessed using the Kolmogorov-Smirnov test. Mean (± SD) was reported for normally distributed numerical data, while median and range were reported for non-normally distributed numerical data. Non-numerical data were presented as frequency and percentage. The ROC Curve (receiver operating characteristic) was utilized to evaluate the sensitivity and specificity of quantitative diagnostic measures categorizing cases into two groups. Logistic regression analysis was employed to predict risk factors when dependent variables were categorical, utilizing generalized linear models (22).

Sample size was determined using the Quanto calculator software program (version 1.2.4) for Sample Size or Power for Association Studies (23).

Results

Our study comprised two groups: the first group consisted of one hundred children with nephrotic syndrome, and the second group included one hundred healthy children. Age and gender were matched between the two groups (p = 0.660 and p = 0.74, respectively (Table 2).

[DOI: 10.61186/rbmb.13.1.67]

Table 2. Basic clinical characteristics of NS patients and controls.

Parameter	Control group (n: 100)	Nephrotic Syndrome group (n: 100)	P-value
Age (y), mean ± SD	9.791 ± 0.411	9.550 ± 0.381	0.66
Sex (boys / girls), N (%) / N (%)	76(76%) / 24 (24%)	76 (76%) / 24 (24%)	0.7400
SDNS number (%)		*24.0 (24%)*	
SRNS number (%)		*76.0(76%)*	
Biopsy of the kidney n (%)			
MCD	*25 (25)*		
FSGS	*16 (16)*		
MPGN	*7 (7)*		
No -biopsy	*52 (52)*		

NS: Nephrotic Syndrome, SDNS: Steroid dependent nephrotic syndrome, SRNS: Steroid resistant nephrotic syndrome, MCD: Minimal change disease, FSGS: Focal segmental glomerulosclerosis, MPGN: Membranoproliferative glomerulonephritis (MPGN). The statistics were presented as mean ± standard deviation (SD), statistically significant if $p < 0.05$. () number (%)

The results indicate a significant increase in the weight and body mass index of the nephrotic syndrome (NS) patients compared to the healthy volunteers. However, there was no significant difference observed in either the hemoglobin (Hb%), GOT, or GPT levels between the two study groups (Fig. 1).

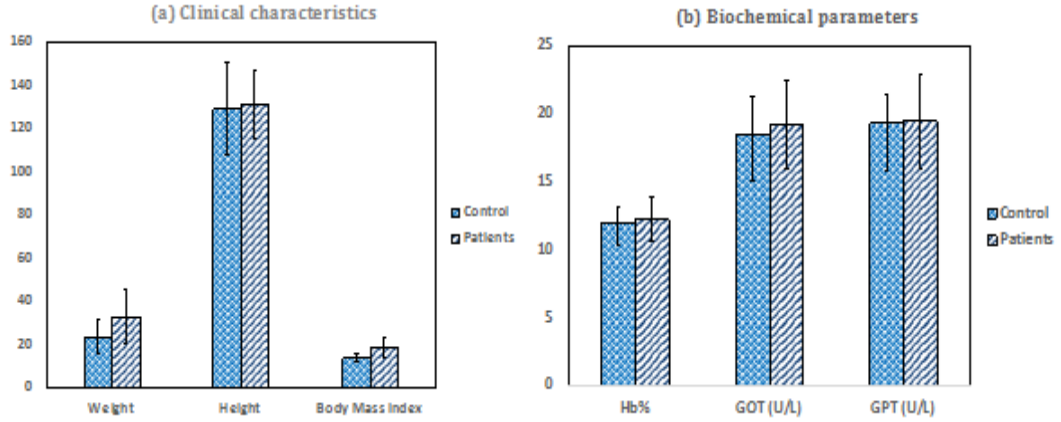

Fig. 1. Some clinical characteristics and biochemical parameters for both healthy volunteer and nephrotic syndrome groups. A: weight, height, and body mass. B: hemoglobin (Hb%), GOT, and GPT comparisons between NS patients and healthy controls.

The results revealed significantly higher total leucocyte blood counts (TLC) levels in the nephrotic syndrome (NS) group compared to the normal group ($p < 0.001$). However, there were no significant differences observed in either creatinine levels or platelet counts between the two groups. Albumin, cholesterol, triglycerides, sodium (Na+) levels, and potassium (K+) levels were also measured. Additionally, the study showed significant increases in cholesterol and triglyceride levels in the NS group ($p < 0.001$), while albumin levels were significantly lower in NS patients ($p < 0.001$) compared to the controls (Table 3).

[DOI: 10.61186/rbmb.13.1.67]

Table 3. Biochemical data of NS cases and healthy volunteers.

Parameter (unit)	Control group (n: 100), M (R)	Nephrotic Syndrome group (n: 100)	P-value
TLCs (× 10^9/L)	7.001 (4.011- 9.651)	14.091 (5.51 - 28.21)	*p< 0.001**
Platelet (× 10^9/L)	335.51 (206.01 – 480.0)	318.51 (191.01 – 645.01)	*p = 0.521*
Creatinine (mg/dl)	0.581 (0.421 - 0.751)	0.601 (0.301 - 7.801)	*p = 0.704*
Albumin (g/dl)	4.221 (3.901 - 5.101)	2.101 (1.201 - 5.201)	*p< 0.001**
Cholesterol (mg/dl)	154.01 (120.01 – 187.01)	574.51 (100.0 – 917.01)	*p< 0.001**
Triglycerides (mg/dl)	85.01 (50.01 - 135.01)	319.01 (68.01 - 710.01)	*p< 0.001**
Sodium Level (mmol/L)	139.561 (135.21 – 149.01)	138.91 (130.80 – 152.01)	*p = 0.0912*
Potassium Level (mmol/L)	4.11 (3.61 - 4.91)	4.081 (2.471 – 5.01)	*p = 0.4811*

The statistics were presented as mean ± standard deviation (SD), M = median, R = Range, statistically significant if p< 0.05.

ABCB1 gene analysis

The fit of goodness between both observed and expected genotype frequencies of the ABCB1 gene (rs10276036, C to T) genotype for NS and control groups was in alignment with the Hardy-Weinberg equilibrium (HWE) (Table 4).

Table 4. Assessment of Hardy Weinberg equilibrium (HWE) for studied SNPs.

	Genotype	Nephrotic syndrome cases n = 100		Control cases n = 100	
		Observed	Expected	Observed	Expected
ABCB1	CC	*24*	*28.09*	*59*	*60.84*
	CT	*58*	*49.82*	*38*	*34.32*
	TT	*18*	*22.09*	*3*	*4.84*
	P	0.101		0.284	

Using Tri ARMS-PCR, the ABCB1 gene (rs10276036) was studied and electrophoresed giving CC, CT, and TT genotypes, (Fig. 2).

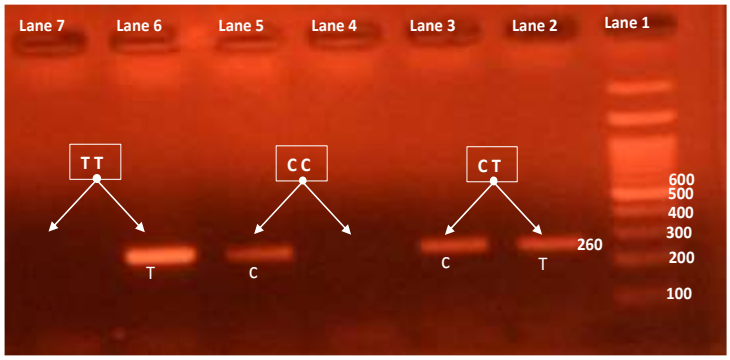

Fig. 2. The PCR products after electrophoresis on a 3% agarose gel. Lane (1) represents the DNA marker (100 bp), lanes (2, 3) show a CT genotype. Lanes (4, 5) show CC genotype. Lanes (6, 7) are TT genotypes.

The frequency of the minor T-allele was significantly higher in NS patients than in the control group. However, the frequency of the common allele (C-allele) was lower in NS cases than in the control group. The most prevalent genotype CC was significantly lower in the NS patients than in the control group (Table 5).

[DOI: 10.61186/rbmb.13.1.67]

Table 5. ABCB1(rs10276036, C>T) genotypic and allelic frequencies in NS and normal groups.

ABCB1		NS N = 100		Control n = 100		P value	OR (95 % CI)		
		N	%	N.	%				
Genotypes	**CC**	24	24	59	59		Reference		
	CT	58	58	38	38	<0.001*	2.270	1.550	3.327
	TT	18	18	3	3	<0.001*	5.070	2.463	10.438
Dominant model	**CC**	24	24	59	59		Reference		
	CT+TT	76	76	41	41	<0.001*	2.560	1.771	3.701
Recessive model	**CC+CT**	82	82	97	97		Reference		
	TT	18	18	3	3	0.001*	3.231	1.623	6.431
Alleles	**C**	106	53	156	78		Reference		
	T	94	47	44	22	<0.001*	2.008	1.547	2.605

C, cysteine; T, Thymine; OR odds ratio; CI, confidence interval; OR>1 is considered risky; OR<1 is considered protective.

The polymorphism of *ABCB1* (rs10276036 C>T) in NS patients

A significant association of *ABCB1* (rs10276036 C>T) polymorphism with the steroid resistance utilizing multiple genetic association models including co-dominant, dominant, recessive, and allelic models had been revealed (Table 6). The TT genotype is significantly increased in SRNS cases in comparison with SDNS cases. In the dominant model, *CT+TT* genotypes were significantly increased in SRNS cases than in SDNS cases.

Table 6. ABCB1 among SDNS and SRNS patients.

ABCB1		*SDNS N = 26*		*SRNS n = 74*		*P value*	*OR (95 % CI)*		
		N.	*%*	*N.*	*%*				
Genotypes	***CC***	*18*	*69.2*	*6*	*8.1*		*Reference*		
	CT	*7*	*26.9*	*51*	*68.9*	*<0.001**	*6.335*	*3.188*	*12.587*
	TT	*1*	*3.8*	*17*	*23.0*	*0.001**	*9.657*	*3.247*	*28.720*
Dominant model	***CC***	*18*	*69.2*	*6*	*8.1*		*Reference*		
	CT+TT	*8*	*30.8*	*68*	*91.9*	*<0.001**	*6.866*	*3.535*	*13.335*
Recessive model	***CC+CT***	*25*	*96.2*	*57*	*77.0*		*Reference*		
	TT	*1*	*3.8*	*17*	*23.0*	*0.031**	*2.953*	*1.102*	*7.913*
Alleles	***C***	*43*	*82.7*	*63*	*42.6*		*Reference*		
	T	*9*	*17.3*	*85*	*57.4*	*<0.001**	3.094	1.972	4.853

C, cysteine; T, Thymine; OR odds ratio; CI, confidence interval; OR>1 is considered risky; OR<1 is considered protective.

TNF-α and IL-18 levels

The level of TNF-α was estimated in 54 patients with NS and 29 healthy individuals, while the serum level of IL-18 was estimated in 57 patients with NS and 32 healthy volunteers (Table 7). The levels of both TNF-α and IL-18 were significantly increased in NS patients than in controls.

[DOI: 10.61186/rbmb.13.1.67]

Table 7. The levels of TNF-α and IL-18 in patients with NS and control groups.

	NS	Control	p-value
TNF-α	**N = 54**	**N = 29**	
Mean ± SE.	0.32 ± 0.013	0.21 ± 0.005	U=53.5 P< 0.001*
Median	0.29	0.21	
Range	0.22-0.61	0.14-0.25	
IL-18	**N = 57**	**N = 32**	
Mean ± SE	0.42 ± 0.022	0.26 ± 0.005	U=1 P< 0.001*
Median	0.36	0.26	
Range	0.30-0.93	0.18-0.30	

The results were described as mean ± S.E and median (range.). * = Statistically significant (*p< 0.050*), TNF-α = Tumor necrosis factor-alpha. IL-18= Interleukin-18, NS= Nephrotic syndrome.

The sensitivity and specificity of the elevation of both IL-6 and TNF-α at the diagnosis of nephrotic syndrome were determined by the receiver operating characteristic (ROC) curve which is a plotting of sensitivity versus (1-specificity = false positive results). The accuracy was measured by the area under the ROC curve (AUC). Results of ROC curves of TNF-α and IL-18 showed high AUC values (0.966 and 0.999 respectively) which reflects a perfect accuracy of the test. The appropriate cut-off value at which the sensitivity and specificity have the highest values was > 0.25 for TNF-α, and > 0.296 for IL-18 (Fig. 3).

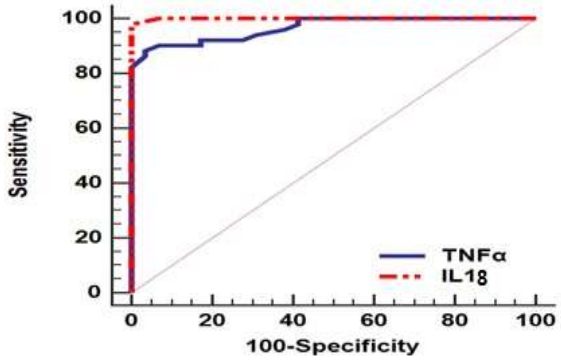

Fig. 3. ROC of TNF-α and IL-18 for diagnosis of NS.

When the levels of both TNF-α and IL-18 were compared between the steroid dependent patient group and the steroid resistant patient group, no significant differences were noticed (Table 8).

Table 8. TNF-α and IL-18 levels among SRNS and SDNS cases.

	SDNS	SRNS	P
TNF-α	**N = 26**	**N = 74**	
Mean ± SE.	0.34±0.036	0.32±0.014	U=167.5 P=0.242
Median	0.30	0.28	
Range	0.26-0.61	0.22-0.61	
IL-18	**N = 26**	**N = 74**	
Mean ± SE	0.43±0.048	0.41±0.026	U=0.283 P=0.954
Median	0.40	0.36	
Range	0.30-0.87	0.30-0.93	

Data were described by mean ± SE and median (range), U= Mann-Whitney U test, TNF-α= Tumor necrosis factor-alpha, IL-18= Interleukin-18, (SDNS)= steroid-dependent nephrotic syndrome, (SRNS)= steroid-dependent nephrotic syndrome.

[DOI: 10.61186/rbmb.13.1.67]

Discussion

Nephrotic syndrome in children and adolescents encompasses a spectrum of renal abnormalities, with minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) being the most prevalent causes, leading to glomerular sclerosis. Membranoproliferative glomerulonephritis (MPGN) represents another significant etiology of nephrotic syndrome. In our study, MCD accounted for 24% of nephrotic syndrome cases, FSGS for 14%, and MPGN for 5%, with the remaining 57% lacking kidney biopsy data. This distribution highlights MCD as the predominant histopathologic variant in our cohort, while MPGN was the least common. These findings are consistent with previous studies by Mubarak et al. (24) and with that of Arbus et al (25) which also noted a decline in MPGN prevalence in regions such as France, Italy, and Canada. Conversely, Ozkaya et al. (26) reported MPGN as the most frequent subtype among Turkish patients, underscoring regional variability in nephrotic syndrome etiology.

Our study found a higher incidence of nephrotic syndrome (NS) in boys compared to girls, consistent with findings reported by Arif et al. (27) and Siddiq et al (28), who similarly observed greater susceptibility to NS among males. Additionally, our study revealed a significant increase in the frequency of the TT genotype in NS patients compared to controls. These results align with studies by Moussa et al. (29) and Youssef et al (30) which highlighted higher TT genotype and T allele frequencies in NS patients compared to controls. Farhat et al. (31) also noted a higher prevalence of the TT genotype in Asian populations compared to European populations, further supporting our findings.

Furthermore, our study corroborates the findings of Wasilewska et al. (32), and Aziz and Islam (33), egarding the significant association of ABCB1 gene polymorphisms with NS, particularly in Asian and African populations. However, our results contrast with those presented by Han et al. (9), who reported a higher frequency of the CC genotype in European populations compared to Asian populations. Additionally, Cizmarikova et al. (34) found a non-significant difference in the distribution of ABCB1 genotypes between NS patients and controls in the Slovak population.

Our findings indicate significantly higher frequencies of the CT and TT genotypes in the steroid-resistant nephrotic syndrome (SRNS) subgroup compared to the steroid-dependent nephrotic syndrome (SDNS) subgroup. These results are consistent with the findings of Ganesan et al. (35) who similarly reported increased frequencies of CC, CT, and T alleles associated with SRNS in children compared to SSNS and control groups. Choi et al. (36) have suggested that the CC genotype and C allele in MDR1 may serve as predictors for better initial steroid responsiveness, contrasting with our findings.

In contrast, our results differ from those reported by Jafar et al. (10), who found no significant difference in mutant genotype frequencies between the two subgroups.

Inflammation is recognized as a pivotal factor in the pathogenesis of nephrotic syndrome (NS). Investigating certain cytokines as potential biomarkers could offer NS patients more effective therapeutic strategies and help mitigate glucocorticoid toxicity. Our study found significantly elevated levels of IL-18 in the NS group compared to the control group. This aligns with findings from Saleem et al. (11) suggested that inflammatory factors, such as interleukin-18, play vital roles in the pathological who proposed that inflammatory mediators, such as interleukin-18, play crucial roles in the pathological processes underlying nephrotic syndrome.

Additionally, VanderBrink et al. (37) demonstrated that inflammatory factors, including IL-18 and TNF-α, are closely associated with the progression of nephrotic syndrome and contribute to kidney cell

[DOI: 10.61186/rbmb.13.1.67]

damage. These insights underscore the potential of cytokines like IL-18 as important biomarkers in NS pathogenesis and their relevance in developing targeted therapeutic interventions.

These findings are corroborated by Kho et al. (38), who suggested that elevated IL-18 levels post-therapy may contribute to the development of steroid-resistant nephrotic syndrome (SRNS). Our results are consistent with Ahmadian et al. (16), who reported an association between high IL-18 levels and nephrotic syndrome (NS). Schachter (39) similarly observed significantly higher serum IL-18 concentrations in children with NS compared to controls. Additionally, Moharrerpour et al. (40) documented increased serum IL-18 levels in children with steroid-sensitive nephrotic syndrome (SSNS). Moreover, Attalla and Ahmed (41) found significantly elevated IL-18 levels in the acute kidney injury group compared to controls, further highlighting the role of IL-18 as a potential biomarker in kidney-related disorders. These studies collectively underscore the importance of IL-18 in the pathophysiology of NS and its potential implications for therapeutic strategies and monitoring disease progression.

TNF-α is widely recognized for its significant association with various inflammatory diseases, including glomerulonephritis. Our study reveals markedly elevated levels of TNF-α in patients with nephrotic syndrome (NS) compared to controls. However, we did not observe any significant difference in TNF-α levels between steroid-resistant nephrotic syndrome (SRNS) and steroid-sensitive nephrotic syndrome (SSNS) subgroups. This finding is consistent with the results reported by Al-Assadi et al. (42). Additionally, our findings align with those of Suranyi et al. (43), who observed elevated TNF-α levels in both plasma and urine of NS patients compared to healthy controls. Liang et al. (44) further suggested that TNF-α might interfere with glucocorticoid activity and directly impact glucocorticoid receptor function, potentially influencing treatment responses in NS patients. These studies collectively underscore the role of TNF-α in the pathophysiology of nephrotic syndrome and its implications for therapeutic strategies targeting inflammatory pathways.

Various inflammatory mediators can interfere with glucocorticoid binding to target cells, potentially impacting their therapeutic efficacy. The expression of P-glycoprotein (P-gp), encoded by the ABCB1 gene, plays a crucial role in modulating the response of nephrotic syndrome (NS) patients to pharmacological treatments. P-gp is involved in the transport of many drugs, including steroids, across cell membranes, influencing their bioavailability and therapeutic effects. Inflammatory processes can alter ABCB1 gene expression, thereby affecting drug responses in NS.

The ABCB1 gene, along with TNF-α and IL-18, are emerging as independent risk factors associated with the prevalence of NS and serve as potential biomarkers for early prediction of disease onset. These biomarkers not only aid in early diagnosis but also have the potential to mitigate complications associated with advanced stages of NS.

Understanding the interplay between inflammatory responses, ABCB1 gene expression, and cytokine levels such as TNF-α and IL-18 is crucial for developing targeted therapies and improving outcomes for NS patients, particularly in managing treatment resistance and reducing disease progression to end-stage complications.

Acknowledgments

We sincerely appreciate all participants in this study.

Funding

This work did not receive any external funding.

Conflict of interest

The authors declare that they have no conflict of interests.

[DOI: 10.61186/rbmb.13.1.67]

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