Original Article

Phenotypes of B-Lymphocytes as a Predictor of Steroid Response in Idiopathic Nephrotic Syndrome.

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Abstract

Introduction: The pathogenesis of Idiopathic nephrotic syndrome (INS) is not clear, where the role of B-cells is not yet illustrated.

Aim of the study: To evaluate B- cells subsets as a predictor of steroid therapy sensitivity among INS at initial onset.

Methods: 45 children with idiopathic nephrotic syndrome were recruited in a follow- up study, patients were divided into steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS) groups based on their sensitivities to steroids. Venous blood samples were drawn from all children for assessment of memory B- cell population before and after one year of steroid therapy.

Results: We found statistically significant relation between steroid response and total memory CD19+ CD27+B cell at disease onset where it was higher in SSNS group. Our results showed The best cut-off of total CD19+ B cells in predicting steroid response was ≥32.7 with an area under curve (AUC) of 0.445, a sensitivity of 43.9%, a specificity of 50%, a positive predictive value (PPV) of 90%, a negative predictive value (NPV) of 8% and an accuracy of 44.4% ($p>0.05$) We also found that The best cut-off of CD19+ CD 27+ total memory B cells in predicting steroid response was ≥32 with an AUC of 0.817, a sensitivity of 78%, a specificity of 75%, a PPV of 97%, a NPV of 25% and an accuracy of 77.8% ($p<0.05$)

Conclusion: CD19+ CD 27+ total memory B cells could help in predicting steroid response among INS patients with a cut-off of total CD 19+B cells above 32.7 and PPV of 90% , a cut-off above 32 in CD19+ CD +27 total memory cells and PPV 97%.

Key words: Nephrotic syndrome, B-lymphocyte, children, flow cytometry, steroid.

Running title: Lymphocyte Subsets in Children with Nephrotic Syndrome

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Introduction

Idiopathic nephrotic syndrome (INS) is a frequent glomerular disease in children. It is a clinical syndrome characterized by massive proteinuria, hypoalbuminemia, hyperlipidemia, and edema [1] Current therapy is based on a standardized steroid regimen, which induces remission in approximately 80% of patients, However, more than half of these patients relapse frequently, requiring immunosuppression with a steroid-sparing agent [2, 3]. The pathogenesis of Steroid-resistant nephrotic syndrome (SRNS) and steroid-sensitive nephrotic syndrome (SSNS) might have different pathogenic factors and is not elucidated, especially SRNS is currently recognized as a genetic disease [4, 5].

Although, disordered immune system is responsible for the pathogenesis in NS [5], the role of B cells in the pathogenesis of INS is still being debated [6]. The best supporting evidence for a role of B cells in INS comes from rituximab, a B-cell-specific antibody, which has been successfully used to treat patients with steroid-dependent or frequent relapses [6 - 9].

Increased circulating levels of total B cells have already been described in SSNS pediatric patients at onset [10]. Elevated levels of memory B-cell subsets were considered to be closely related to the onset of INS [3], although altered B-cell homeostasis in children with INS is reported [6], few studies have investigated the role of total and memory B-cells subpopulation in predicting steroid therapy sensitivity in INS.

Therefore, we aimed to evaluate the role of B-cell subsets as a biomarker for predicting the sensitivity to steroid therapy in INS at initial onset, through comparing the distribution of B-cell subsets in the peripheral blood of SSNS and SRNS patients, as well as in healthy controls and in INS patients in relapse and remission.

Methods

Study design and setting: We conducted a prospective follow up cohort study from March 2019 to May 2020 in the nephrology outpatient clinic, Pediatric department, Clinical Pathology Department, Medical Microbiology and Immunology Department, at Our University, Egypt. The study included 45 newly diagnosed children with INS selected by systematic random sampling from children attending the nephrology outpatient clinic at our University, Egypt.

The diagnosis of INS was based on the presence of generalized edema, proteinuria (urine protein (+++) or more by the heat-precipitation method and/or spot-urine protein/creatinine > 2 mg/mg), hypo-albuminemia (serum albumin<2.5 g/dl) and hypercholesterolemia (serum cholesterol > 250 mg/dl mmol/L) [11]. We excluded patients with other immunological diseases, positive family history of genetic NS or renal diseases, those aged <1 year or >10 years. No patients received immunosuppressive therapy (B-cell depleting agent) before enrolment in the study, also we excluded patients with active infection during sampling.

These patients were followed up for one year. Standard initial steroid therapy, consisting of daily dosages of prednisone 60 mg/m^2/ day until remission was achieved, followed by 40 mg/m^2/day given on alternate days, then tapering-off over the next 4 to 8 weeks. Patients who

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achieved remission with prednisone (60 mg/m^2/day) within 4 weeks of treatment were classified as having steroid sensitive nephrotic syndrome (SSNS), whereas those who did not respond were classified as having steroid resistant nephrotic syndrome (SRNS) according to the Improving Global Outcomes (KDIGO) guidelines of 2012 for kidney disease [12].

The study was approved by the institutional review board (IRB) no #5267/18-3-2019, Faculty of Medicine, at our University. An informed written consent was obtained from all parents at the time of recruitment. This study was conducted in accordance with the Declaration of Helsinki and its later amendments.

B cell subsets were done by drawing a peripheral venous blood samples from all children for evaluation where they were collected into EDTA Vacutainers (K2E) and processed within 4 h of being taken**.** To identify different B-cell subpopulations, blood samples were stained with the following fluorochrome-conjugated antibodies: CD19-FITC (Fluorescein isothiocyanate), CD27-PE (phycoerythrin) (BD, Biosciences, San Jose, CA, USA). The antibodies were then analyzed by a flow cytometer (FACS Calibur; BD, Biosciences, Salt Lake City, UT, USA).

Gated events (30,000) on living lymphocytes were analyzed for each sample. Two-color data acquisition was performed by FACS Calibur, and data were analyzed with Cell Quest analysis software (BD Biosciences, San Diego, CA, USA). Subsets of gated CD19+ (total) B cells were identified on the basis of the expression of CD 27+ surface markers as total memory B-cells (CD19+CD27+).

Statistical analysis

Data analysis was performed using the software SPSS (Statistical Package for the Social Sciences) version 20. Quantitative variables were described using their means and standard deviations. Categorical variables were described using their absolute frequencies and were compared using Chi square test when appropriate. Kolmogorov-Smirnov (distribution-type) and Levene (homogeneity of variances) tests were used to verify assumptions for use in parametric tests. Mann Whitney test (used with non-normally distributed data) was used to compare variables in two groups and independent sample t test (used with normally distributed data) was used to compare means in two groups. To compare the change in the same variable within the same group at two points of time, Wilcoxon signed rank test was used. ROC curve was used to determine the best cutoff of a certain marker for diagnosis of certain health problem. The level of statistical significance was set at 5% ($P < 0.05$). Highly significant difference was present if $P \leq 0.001$.

Results

Basic characteristics of study population: The demographic, clinical and laboratory characteristic of the studied group was illustrated in **(Table 1)**. All the studied INS patients had puffiness of eyelids. Fifty six percent, 57.8% and 75.6% of patients had scrotal edema, ascites and history of infection before first attack.

Distribution of B cells subpopulations: Patients treated with steroid according to the protocol and After completing one year of steroid therapy we assessed

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patients according to their steroid response; 41 INS patients were steroid responders, while only four patients were non- responders. At the disease onset, total memory CD19+ CD27+ total memory B cell were significantly higher in SSNS group. Within SRNS group, there was non-significant change in both of total CD19+B cell and total memory CD19+ CD27+B after therapy. Within SSNS group, there was significant change both of total CD19+B cell and total memory CD19+ CD27+B after therapy (P<0.001) **(Table2)**.

Total and total memory B cells as a Biomarker for SSNS

The total and total memory B cells differed significantly between patients with SSNS and SRNS at the initial diagnosis. Hence, we attempted to use total B cells as biomarkers to predict the outcome of steroid therapy. ROC curve was used to assess the potential utility of total B cells and total memory B-cell detection in predicting steroid response at the time of initial INS diagnosis.

The best cut-off of total CD19+ B cells in predicting steroid response was ≥32.7 with an area under curve (AUC) of 0.445, a sensitivity of 43.9%, a specificity of 50%, a positive predictive value (PPV) of 90%, a negative predictive value (NPV) of 8% and an accuracy of 44.4% (p>0.05) **(Figure1)**.

The best cut-off of CD19+ CD 27+ total memory B cells in predicting steroid response was ≥32 with an AUC of 0.817, a sensitivity of 78%, a specificity of 75%, a PPV of 97%, a NPV of 25% and an accuracy of 77.8% (p<0.05) **(Figure2)**.

Table 1 : Demographic, clinical and laboratory characteristics of the study population

Characteristics		(INS) group N = 45
Gender, no (%)	Male	25 (55.6)
Age (years)	Mean ± SD	4.34 ±2.1
Weight (kg)	Mean ± SD	19.08 ± 7.61
Consanguinity; no (%)	Positive	16 (35.6)
Systolic blood pressure	Mean ± SD	94.67 ± 9.44
Diastolic blood pressure	Mean ± SD	56.22 ± 7.16
Hemoglobin (g/dL)	Mean ± SD	11.16 ± 1.48
MCV	Mean ± SD	72.54 ± 5.88
Htc (%)	Mean ± SD	33.24 ± 2.58
Platelet count (10^3/$mm^{3)}$	Median (Range)	381 (246 – 897)
TLC (10^3/ul)	Mean ± SD	10.77 ± 2.31
Lymphocytes (10^3/µ l)	Mean ± SD	6.61 ± 1.16
Monocyte (10^3/ul)	Mean ± SD Range	1.58 ± 2.45 (0 – 13)
Neutrophil (10^3/ul)	Mean ± SD	2.52 ± 3
Total cholesterol (mg/dL)	Mean ± SD	399.72 ± 72.53
Triglycerides (mg/dL)	Mean ± SD	243.52 ± 72.78
HDL cholesterol (mg/dL)	Mean ± SD	54.72 ± 8.19
LDL cholesterol (m/dL)	Mean ± SD	289.76 ± 56.98
Serum creatinine (mg/dL)	Mean ± SD	0.85 ± 0.18
BUN (mg/dL)	Mean ± SD	7.6 ± 1.65
ALT (IU/L)	Mean ± SD	16.94 ± 6.56
AST (IU/L)	Mean ± SD	22.39 ± 5.64
T. Protein (g/dL)	Mean ± SD	3.61 ± 0.6

MCV (Mean corpuscular volume); Htc hematocrit; TLC (total leucocytic count);
HDL (high density lipoprotein) LDL (low density lipoprotein); PT (prothrombin time);
PTT (partial thromboplastin time); INR (international normalized ratio);
BUN (Blood urea nitrogen); ALT (Alanine aminotransferase); AST (aspartate aminotransferase

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Table 2 : Relation between steroid response and total CD19+ B cells and total memory CD19+CD27+ B cells before and after one year steroid therapy among INS patients.

	Response				Test	
	SSNS (n = 41)		SRNS (n = 4)			
	Mean ± SD			Median (range)	Mean ± SD	Median (range)
Total CD19+ B cells at disease onset	32.23 ± 16.91	22.4 (13.2-65.2)	33.96 ± 14.37	37.71 (15.2 – 45.21)	-0.359	0.719
Total CD19+ B cells after steroid	15.69 ± 5.19	8.5 –28.2	16.78 ± 4.93	11.1 – 20.9	-0.4	0.691
P-value (Wx)	<0.001**		0.066			
Total memory CD19+ CD27+ B cells at disease onset	48.65 ± 18.91	51.2 (8.31–86.44)	29.08 ± 5.79	31.8 (20.4–32.3)	-2.075	0.038*
Total memory CD19+ CD27+ B cells after steroid	22.19 ± 11.88	19.1 (6.2 – 51.2)	16.94 ± 7.08	19.85 (6.85 – 21.49)	-0.339	0.734
P- value (Wx)	<0.001**		0.066			

SSNS (steroid sensitive nephrotic syndrome); SRNS (steroid resistant nephrotic syndrome); Z (Mann Whitney test); Wx (Wilcoxon signed rank test)

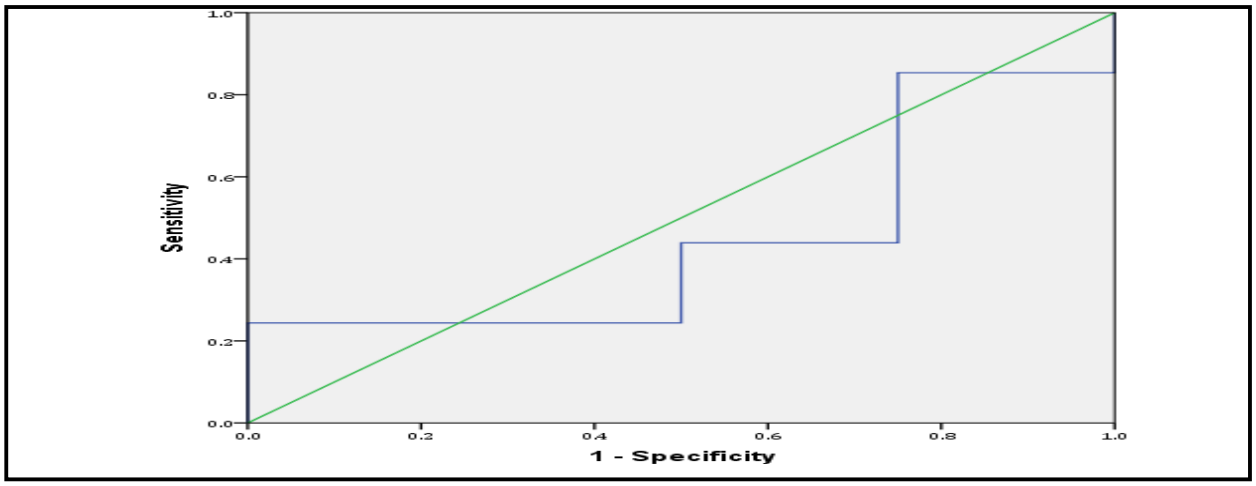

Figure 1 : ROC curve showing performance of CD19+ B cells in predicting steroid response among the studied INS patients.

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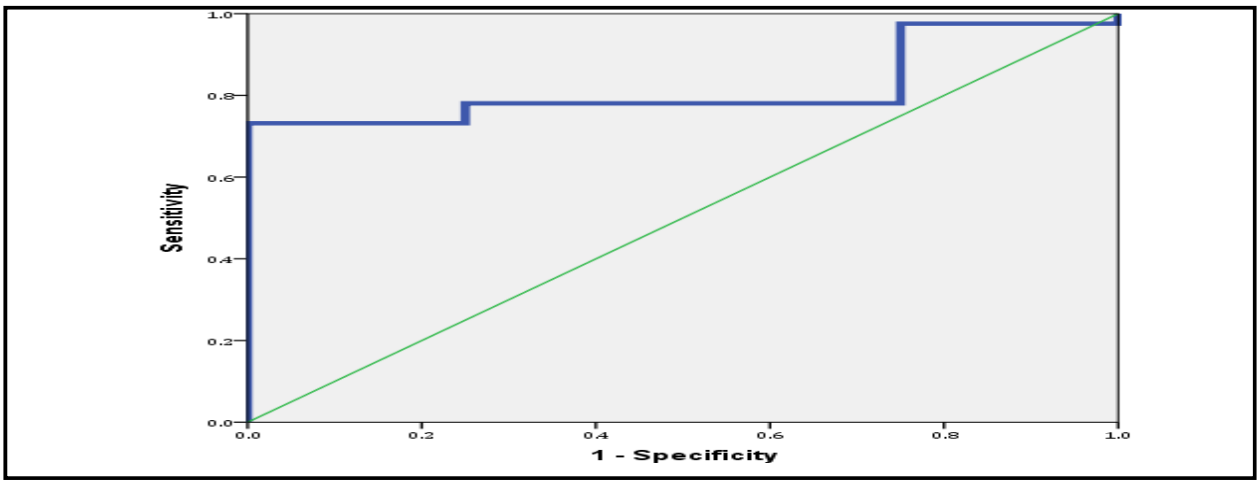

Figure 2 : ROC curve showing performance of CD19+CD27+ B cells in predicting steroid response among the studied INS patients.

Discussion

This follow-up study confirmed that B-cell subsets are disturbed in an SSNS. We tested 45 newly diagnosed INS children before any immunosuppressive treatment, and all patients were followed up for one year. According to the efficacy of prednisone, patients were divided into SSNS and SRNS groups.

In the current study, the total memory CD19+CD27+ B cells at disease onset were significantly higher among SSNS group. These results agreed with [3, 13] but conflicting results are also existing [14,15]. This is most likely due to differences in the immunosuppressive treatment, whether used or not and what type of immunosuppressive used which can directly or indirectly affect the homeostasis and function of B lymphocytes by suppressing more the de novo production of transitional B cells and the accumulation of mature/naïve B cells compared to memory B cells [4] . In order to overcome that confounding effect, we evaluated the patients at disease onset, before any immunosuppressive treatment. Other studies evaluated SSNS patients who were receiving immunosuppression and observed that levels of transitional and mature B cells were affected in both relapse and remission with normal circulating levels of total memory and switched memory B cells [3].

The precise clinical implication of this observation requires further study. Hypothetically, it suggests that therapies specifically targeting the memory B cell compartment could be more efficient in maintaining remission in children with SSNS. Consistent with this finding, rituximab treatment efficiently depletes all B cell subpopulations, and the recovery of switched memory B cells, rather than of total B cells, is predictive of the risk of relapse, despite tapering of concomitant immunosuppression [15]. Moreover, a recent trial comparing tacrolimus to rituximab as first-line steroid-sparing therapy in children with SSNS indicates a superiority of rituximab in maintaining remission [16]. In parallel with their ability to produce antibodies, B cells exert several important functions, such as antigen-presentation to activate T cells and cytokine production to modulate inflammatory responses [17].

We reported that the best cut off of CD19 +CD27+ total memory cells in

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prediction of steroid response was ≥32 with an AUC of 0.817. Our results were supported by the study of Ling and his colleagues [18]; as they reported that when using a cut-off value of 2.05 transitional B cells allowed observers to distinguish SSNS from SRNS with an AUC of 0.907. This suggests that transitional memory B cells may be a biomarker for early prediction of steroid response for SSNS.

Memory B cells are one of the focuses of current research on children with primary nephrotic syndrome. The first report by Colucci et al. showed that delayed reconstitution of memory B cells was protective against relapse after rituximab [15]. This finding was partly confirmed by a study from India [19]. However, Bhatia et al [19] observed that total B cells and memory B cells recovered earlier in relapsers, but Colucci et al [15] considered that this is related to the prolonged depletion of memory B cells rather than of total B cells**.** In our current study, memory B cells were significantly elevated in the in the SSNS group at the disease onset. Overall, these phenomena may imply that memory B cells are involved in the pathogenesis of SSNS [20]**.**

Conclusion

In summary, memory B-cell count can be a predictor of steroid response among INS patients where it showed high levels in SSNS. This may be a promising marker for predicting successful immunosuppressive therapy during the initial onset of INS. Further research is needed to determine the function of memory B cells in the pathogenesis of INS.

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Statements

Ethics approval and consent to participate
This study protocol and the consents were approved and deemed sufficient by the Ethical Committee of Pediatric nephrology unit, Children Hospital, Zagazig University and informed written consent was obtained in every case from their legal guardians.

Consent for publication
The contents and material of the manuscript have not been previously reported at any length or being considered for publishing elsewhere.

Availability of data and material
"Not applicable"

Conflict of interest
The authors declare no conflict of interest.

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