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Regulatory T cell CD4 &CD25 expression and chemokine C-X-C ligand 13 level before and after corticosteroid therapy in pediatric ITP patients

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ABSTRACT

Childhood Idiopathic thrombocytopenic purpura (ITP) is one of the most commons autoimmune bleeding disorders characterized by isolated, immune-mediated low platelet count, the T-follicular helper (Tfh) cells are a subset of effector CD4 (+) T cells, that plays a pivotal role in maintaining self-tolerance, deregulation of Tfh activities has a key role in immune process taking place in ITP in which the production of platelet autoantibodies might be caused by cytokine network dysregulation. The objective of our study was to analyze the relationship of Tfh cells CD4&CD25 and C-X-C ligand 13 (CXCL13) expressions before and after steroid thereby in pediatric ITP. Material and method: the study included 45 pediatric patients with acute ITP and 20 healthy controls; we used flowcytometry to assess percentages of CD4 and CD25 cells as markers of regulatory T cells, also, the serum level of interleukin-CXCL13 was measured by ELISA at diagnosis and after 4 weeks receiving corticosteroid. Results: the expression of CD4 & CD25 markers were significantly reduced in ITP cases, who also showed an elevated CXCL13 level in comparison to controls, however, the level of CXCL13 declined after treatment, the CXCL13 optimum cut off point for predicting ITP response to therapy was determined to be 90 pg/ml with AUC 0.976, Sensitivity 88.89% and Specificity 100% P-value < 0.00. Conclusion: Serum CXCL-13 levels could be used as a significant predictor of response to therapy in ITP patients, CD4&CD25 expression has a role in the pathogenesis of childhood acute ITP principally linked to the level of platelet count drop.

1. Introduction

Idiopathic thrombocytopenic purpura (ITP), a heterogeneous disease described as reduced peripheral platelet count ($<100 \times 10^9$/L) and is initiated by a process of platelet destruction that is associated with different degrees and types of bleeding, ranging from mild bleeding as bleeding in skin and mucosal regions, to a more severe, life-threatening form, as gastrointestinal or intracranial bleeding that happens commonly if platelet count falls below 20×10^9/L [1],the diagnosis of ITP depends on clinical symptoms together with laboratory results, in addition to the ability to differentiate other conditions associated with thrombocytopenia [2]. The incidence of ITP is about 1.9–6.4 per 100,000 children/year and this number is growing [3].Normally, the human body can produce about 100 billion platelets per day ensuring a concentration of ~150,000–400,000 platelets per microliter blood this is the normal platelet life cycle, however, in ITP there is a major defect caused by autoantibodies mediated destruction of platelet, in fact, autoantibodies may interfere with various aspects of the platelet life cycle, including production and clearance resulting in thrombocytopenia [3] T-follicular helper (Tfh) cells are a subset of effector CD4 (+) T cells that are specialized in helping B cells generation of germinal center reactions and differentiation [4].B cells are involved in immune thrombocytopenia pathophysiology by generating auto-antibodies against platelet [5] Strict control of (Tfh) cells function is vital for the prevention of autoreactivity, deregulation of Tfh activities plays a key role in the immune response occurring with autoimmune disease [6]. When a microbial antigen mimic platelet autoantigens, or even the platelet

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antigens themselves, are presented to B cells, these B cells can develop into an autoantibody-producing plasma cells, Splenic macrophages and dendritic cells (DCs) may present platelet antigens to T helper (Th) cells which help B cells differentiate into autoantibody-producing plasma cells that are distributed both in peripheral blood and bone marrow [3]. Splenic macrophages produce more autoantibodies that can sequester more platelets, the autoantibodies increase platelet destruction via different routes that include; platelet removal via splenic macrophages, complement deposition, platelet apoptosis, or by inhibiting megakaryocytic platelet production [7].The CXC chemokine ligand-13 (CXCL13) is a cytokine that belongs to the CXC chemokine family, it is expressed by naïve B cells and T follicular helper (TFH) cells, and it controls the migration of these cells to the follicle [8].The CXCL13 level has been implicated in the pathogenesis of many autoimmune disorders and was correlated to its clinical outcomes, namely, due to dysregulated humoral responses [9–11].

2. Material and method

2.1. Patients and healthy volunteers

A total of 45 newly diagnosed pediatric ITP patients were enrolled in the study, there were 22 female patients (49%) and 23 male patients (51%), aged 1–16 years, only patients presented by bleeding tendency and diagnosed as primary ITP who fit diagnosis as primary ITP using the criterion of the International Working Group of ITP defined in 2009 [7] were included in the present study, exclusion criteria comprised cases previously treated with steroid alone or with immune suppressive drugs, patients underwent splenectomy, secondary ITP, and history of other hematologic diseases. Bleeding manifestation were grouped into three major domains: skin bleeding (mild),visible mucosae bleeding (moderate) and organs bleeding (severe) [12].

Steroid therapy comprised of; in cases of acute ITP when there was severe bleeding treatment start with Methylprednisolone 10 mglkg and increasing dose up to 30 mglkg can be used [13]. In case of mild to moderate bleeding patients received oral prednisolone 2mglkg,cases not responding to steroid and with active bleeding,they were given IV Immunoglobulin,few cases received platelets transfusion,the Steroid Non-responders were excluded from The study. A total of 20 healthy controls were enrolled in the study too, they matched with patients in terms of age and sex, and they have no history of hematologic or autoimmune diseases or history of blood transfusions. Patients were recruited from the Pediatric Hematology Clinic; during the period from January to June 2018.Informed consent was obtained from the parents of study cases. The study design was approved by the Research Ethics Committee of the Faculty of Medicine at our University.

All patients and controls were subjected to history, physical examination and lab investigations:

1 Complete blood count (CBC) using Cell-Dyn 3700, automated cell counter (Abbott diagnostic, Dallas, USA).
2 Leishman-stained peripheral blood and bone marrow aspirates smears microscopic assessment.
3 Immunophenotyping (IPT) with the assessment of CD4&CD25 expressions on peripheral blood.
4 Serum CXCL-13 levels were measured at diagnosis and after 4 weeks of corticosteroid treatment.

2.2. Flowcytometry assessment

Blood sample consists of 3 mL fresh peripheral venous blood was drawn into Ethylenediaminetetraacetic acid tube for flowcytometry detection of Regulatory T Cells CD4&CD25.Antihuman monoclonal antibodies conjugated with fluorochromes and appropriate isotype controls were used: fluoroisothiocyanate (FITC)-conjugated CD4 and phycoerythrin (PE)-conjugated CD25 (Becton Dickinson Bioscience, San Jose, California) according to the manufacturer's instructions; one hundred microliters of peripheral blood sample were incubated with 10 mL of CD4 and CD25 for 15 min at room temperature in the dark, following incubation; red blood cell (RBC) removed by a simple lysis step, then, washed with phosphate-buffered saline (PBS) also used as a suspension buffer. The flow cytometric analysis was done by fluorescence-activated cell sorter Calibur flowcytometry, data were obtained and analyzed using CellQuest software (Becton Dickinson Biosciences). An isotype-matched negative control was used for each sample. Forward and side scatter histogram was used to define the lymphocyte population.

2.3. Determination of levels of CXCL13

Blood sample consists of 3 mL of peripheral venous blood was drawn into plain tubes and allowed to clot; the serum is collected into Wassermann tubes for quantitative determination of CXCL13 serum level by quantitative sandwich enzyme-linked immunosorbent assay (R&D Co, USA, catalog number MAB470) at time of diagnosis and after 4 weeks of receiving corticosteroid treatment.

2.4. Data analysis

Data were analyzed using IBM SPSS Statistics for Windows version 24 and Medcalc version 15.8.0. Quantitative data were expressed as means ± standard deviation. Qualitative data were expressed as number and percentage. Quantitative data were tested for normality by Shapiro–Wilk test. Mann–Whitney *U* test, Wilcoxon Signed Ranks test and Spearman's correlation were used for data which wasn't normally distributed. Independent Samples T-test and Pearson's correlation were used for normally distributed data. Chi-square ($\chi 2$) test was used for comparison of qualitative variables as appropriate. Receiver operating characteristic (ROC) curve was constructed to detect CXCL3 optimum cut off point in predicting ITP, and the area under the ROC curve value with 95% CI was calculated. The optimal cut-off value was determined; sensitivity, specificity, positive predictive value and negative predictive value were calculated. A 5% level was chosen as a level of significance in all statistical tests used in the study.

3. Results

Clinical and laboratory data of study groups were shown in Table 1.

The CD 4 and CD25 expression were significantly decreased in ITP patients compared to healthy controls ($\boldsymbol{p} = < 0.001$).As indicated in Figure (1) plasma CXCL13 level demonstrated a significant increase in ITP patients compared to controls.The clinical data of ITP patients showed a range of variation as shown in Table 2.

Comparison between pre and post-treatment CXCL13 level among the studied ITP cases showed that CXCL13 level was significantly decreased in ITP after treatment Table 3 & Figure (2).

The correlation of plasma CXCL13 level, CD4 and CD25 expression with patients and controls parameters were analyzed in Table 4.

The results showed that CXCL13 level before treatment was correlated with WBCS, and platelet count in ITP patients, however, the CXCL13 level after treatment was only correlated with age of ITP patients.

Table 1
comparison between cases and control groups regarding age, gender, clinical and laboratory criteria Mean ± S.D. P-value is calculated by Mann Whitney test.

P-value	Control group (N = 20)	Cases group (N = 45)	Parameter
0 .102	7 ± 4.09 7 (2–14)	4.99 ± 3.16 4 (2–15)	Age (years) Mean ± S.D. Median (Range)
0.772**	9 (45%) 11 (55%)	22 (48.9%) 23 (51.1%)	Sex Female Male
0.319	7.25 ± 2.02 6.9 (4.2–11.2)	7.98 ± 2.62 7.8 (4.1–12.4)	WBCS (x1,000/mm3) Mean ± S.D. Median (Range)
0.591*	11.38 ± 0.83 11.45 (9.9–12.7)	11.22 ± 1.18 10.9 (8.6–13.2)	Hemoglobin (g/dl) Mean ± S.D. Median (Range)
<0.001	287.75 ± 100.21 261 (146–480)	42.02 ± 21.77 37 (13–97)	Platelets before treatment (x1,000/mm3) Mean ± S.D. Median (Range)
NA	–	161.88 ± 107.26 180 (8–420)	Platelets after treatment (x1,000/mm3) Mean ± S.D. Median (Range)
0.097	11.81 ± 16.31 8.25 (6.6–81)	8.67 ± 0.95 8.8 (6.4–10.1)	MPV Mean ± S.D. Median (Range)
0.834 *	41.82 ± 8.82 40 (28–55)	41.23 ± 11.01 40 (20–58)	Total lymphocytic count Mean ± S.D. Median (Range)
<0.001	11.3 ± 3.42 11 (7–20)	21.44 ± 8.27 20 (11–45)	ESR Mean ± S.D. Median (Range)
<0.001	38.58 ± 4.76 39 (27.3–48)	31.03 ± 1.81 30.5 (28–34.6)	CD4 Mean ± S.D. Median (Range)
<0.001*	5.89 ± 1.25 5.65 (4.1–8.5)	4.34 ± 1.15 4.5 (2–6.3)	CD25 Mean ± S.D. Median (Range)
<0.001*	62.18 ± 18.54 65.15 (20.2–90)	158.18 ± 49.05 150.9 (71.3–246.4)	CXCL13 Mean ± S.D. Median (Range)
NA	20 (100%) 0 (0.0%)	45 (100%) 0 (0.0%)	Splenomegaly No Yes

* P-value is calculated by Independent-Samples T test.
** P-value is calculated by Chi-Square test.
P-value < 0.05 is statistically significant.
NA (Not applicable).

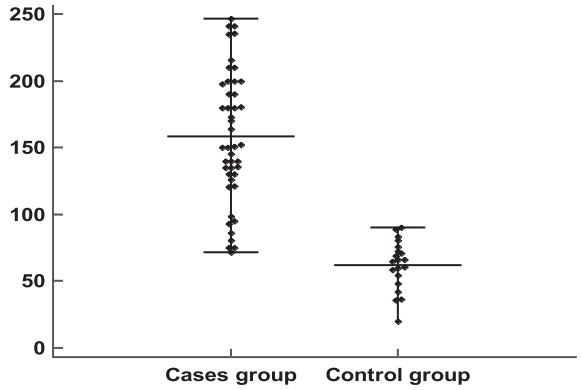

Fig. 1. comparison between cases and control groups regarding CXCL13 plasma level (pg/ml).

- CD4 and CD25 levels were in correlation to platelet count, MPV, and ESR in ITP patients.
- For detection of optimum CXCL13 cut off level that is needed for predicting ITP a ROC curve analysis was done that demonstrated that CXCL13 optimum cutoff is > 90 AUC 0.976 with a sensitivity of 88.89% and Specificity 100% P-value <0.001 Table 5 & Figure (3).

Table 2
Disease-related criteria of ITP patients.

Summary statistics	Parameter
	Type of bleeding
26 (57.8%)	Cutaneous
19 (42.2%)	Mucosal
	Previous treatment
30 (66.7%)	Steroid
15 (33.3%)	Steroid + Methotrexate

4. Discussion

Immune thrombocytopenia is an immune-mediated disorder characterized by acute or chronic reduced platelet count which is initiated by a platelet destruction process, and the platelet antibody-mediated platelet destruction is the major concern in the pathogenesis of ITP [14]. The T follicular helper (TFH) cells play a critical role in autoimmune diseases; its possible role in ITP patients is regulating the production of platelet antibodies [2]. In the present study, we aimed to evaluate the levels of regulatory T cell CD4, CD25 & CXCL-13 expression before and after corticosteroid therapy to assess its role in the pathogenesis of ITP patients compared to their age and sex-matched healthy counterparts. In the present

Table 3
comparison between pre and post treatment CXCL3 level among the studied ITP cases regarding response to steroid.

P-value	CXCL3 steroid non responder N (14) Mean ± S.D.	CXCL3 steroid responder N (31) Mean ± S.D.	Parameter
0.512	**183.77 ± 48.17**	**146.61 ± 45.59**	**Pre-treatment**
0.018*	**182.62 ± 44.79**	**49.27 ± 27.84**	**Post-treatment**

P-value is calculated by Wilcoxon Signed Ranks Test.
* Statistically significant.

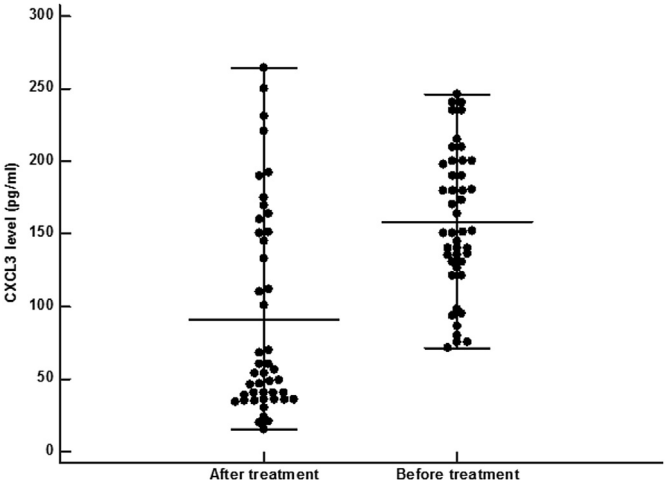

Fig. 2. Pre and post treatment CXCL3 level among ITP cases.

study, plasma CXCL-13 level among ITP patients before treatment was statistically significantly higher compared to the controls ($p = 0.001$) this is in agreement with [15](16) and [10,11] (17) who studied 30 ITP children and found that CXCL13 plasma level was markedly elevated in ITP patients than in controls suggesting that it is possibly involved in the pathological process of ITP, similarly,Nazir et al., in 2018(18) studied 30 ITP adult patients and found that the CXCL-13 plasma level among ITP patients before steroid treatment was statistically higher compared to controls.

Our results showed that the plasma CXCL-13 level in ITP patients decreased significantly after steroid treatment compared to the pre-treatment level ($\boldsymbol{p} = 0.001$) which is consistent with [10,11](17) & Nazir et al., 2018 (18) who noticed an elevated plasma CXCL13 level that was reduced after steroid treatment.

Investigating the plasma **CXCL13** level before treatment,the study showed that it can be used as a good predictor of response to corticosteroids with AUC = **0.976** (95% CI **0.903–0.998**), sensitivity of 88.89% and Specificity 100% P-value <0.001, suggesting a cutoff point of 90 pg/ml for **CXCL13** at presentation to be helpful in predicting treatment response. The response to treatment was assessed on basis of clinical signs as the disappearance of purpura and laboratory signs as elevated platelet count, this finding is in concurrence with [10,11](17) who found that Dexamethasone reduced CXCL13 level in a dose and time-dependent manner. In contrast, Nazir, et al., 2018 (18) reported that plasma CXCL-13 level before treatment was not a considerable discriminator of response to steroid with AUC = 0.589 (95% CI 0.396 to 0.765) ($Z = 0.501$, $p = 0.6167$). Mixed data from different studies could be due to a relatively small number of cases. In this study there was a correlation between CXCL13 level before treatment with the platelet count in ITP patients this is in agreement with [10,11] who reported that CXCL13 blood level was positively correlated to the hemorrhage severity, platelet count and hemoglobin concentration respectively in ITP patients before treatment, however, the correlations was not established after treatment.

As a possible immunologic predictors, Treg CD4 & CD25 expression was investigated in this study, they found to be significantly decreased in ITP patients compared to controls, and the CD4 & CD25% level is significantly correlated to platelet count suggesting that a decrease in T cell population is associated with a decline in immunotolerance which maintains a normal platelet

Table 4
Correlation between age, laboratory parameters and the studied markers among cases (n = 45) & control group (n = 20).

CXCL13 after treatment	CXCL13 before treatment		CD25		CD4		Parameter
P-value	P-value patient	P-value control	P-value patient	P-value control	P-value patient	P-value control	
0.001	0.148	0.057	0.967	0.771	0.963	0.476	**Age (years)**
0.737	**0.010**	0.301	0.021	0.727	0.522	0.936	**WBCS (x1,000/mm3)**
0.542	0.031	0.167	0.530	0.367	0.389	0.522	**Hemoglobin (g/dl)**
0.563	**0.041**	0.021	**0.021**	0.574	**0.043**	0.726	**Platelets (x1,000/mm3)**
0.189	0. 704	0.962	**0.026**	0.164	**0.049**	0.081	**MPV**
0.716	0.288	0.747	0.790	0.103	0.635	0.081	**Total lymphocytic count**
0.096	0.503	0.961	**0.049**	0.166	**0.035**	0.503	**ESR**

*Pearson correlation coefficient.
P-value <0.05 is statistically significant.

Table 5
Receiver operating characteristic (ROC) curve of CXCL3 for optimum cut off point in predicting ITP.

P-value	NPV	PPV	Specificity	Sensitivity	CI	AUC	Cutoff	Marker
<0.001*	80	100	100	88.89	0.903–0.998	0.976	>90	CXCL3

*Statistically significant.
- ***PPV = positive predictive value.***
- ***NPV = negative predictive value.***
- ***AUC = area under the curve.***
- ***CI = confidence interval.***

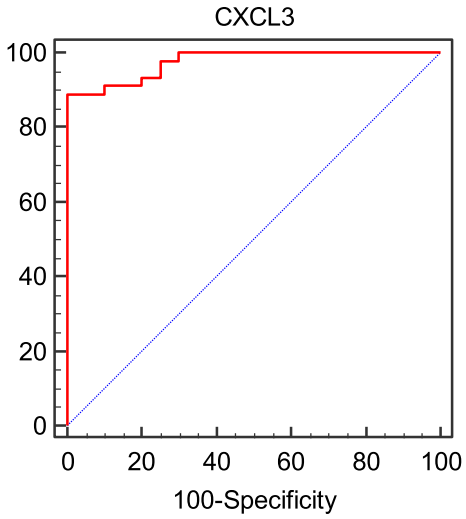

Fig. 3. Receiver operating characteristic (ROC) curve of CXCL13 for optimum cut off point in predicting ITP.

count,thus, proving the pivotal role of Tregs in the pathogenesis of childhood ITP principally linked to the level of platelet count drop, these findings are in agreement with relevant studies (19–21).

5. Conclusion

Plasma CXCL-13 level assessment in ITP patients at diagnosis and after receiving corticosteroid could be used as a significant predictor of response to therapy. CD4&CD25 expression has a role in the pathogenesis of childhood ITP principally linked to the level of platelet count drop.

Footnotes

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Conflicts of interest

The authors declare no conflicts of interest.

Authorship contributions

HA conceptualized the study collected the data, EF recruited the patients and prepared the samples, EN interpreted the data and wrote the manuscript. All Authors read, edited, and approved the final manuscript.

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