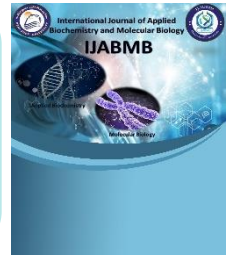




**International Journal of Applied  
Biochemistry and Molecular Biology  
(IJABMB)**



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## **Serum level of BLC/CXCL13 as biomarker in pediatric Immune Thrombocytopenic Purpura.**

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**Running Title: CXCL13 in children with ITP.**

**Abstract:**

**Background:** In children, immune thrombocytopenia (ITP) constitutes one of the most acquired bleeding disorders. There is abundant proof that T cells and their release of cytokines are essential for regulating anti-platelet autoantibodies.

**Objective:** This study set out to determine whether the chemokine C-X-C motif ligand 13 (CXCL13) was expressed in the sera in children with ITP, whether this biomarker had any therapeutic value, and whether it correlated with laboratory and clinical parameters related to the degree, course, and response to treatment of ITP disease.

**Patients and Methods:** Forty healthy children and sixty ITP patients participated in the study. subgroups: Acute ITP patients before steroid treatment, Acute ITP patients after steroid treatment, and Chronic ITP patients. All the study's patients underwent the following procedures: a thorough history taking that focused on demographic information, age, and sex, bleeding symptoms, a full clinical examination, a complete blood picture with differential count, a bone marrow aspiration, and enzyme-linked immunosorbent assays (ELISA) for the detection of the serum protein CXCL13.

**Results:** Compared to controls, ITP patients' serum CXCL13 level was significantly higher. Acute ITP was found to have greater serum CXCL13 levels than chronic ITP. Following therapy, ITP's serum CXCL13 level dropped noticeably and was discovered to be close to the serum control level.

**Conclusion:** the study concluded that serum CXCL13 was suggested to have a role in acute ITP pathogenesis and may be used as a biomarker creating a new target for ITP therapy with high significant sensitivity, and specificity in early detection of acute ITP.

**Keywords:** ITP, CXCL13, steroids

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## **Introduction**

Among the most common bleeding disorders that children get is immune thrombocytopenia (ITP). It is distinguished by the reticuloendothelial system's elimination of the antibody-sensitized platelets and the occurrence of isolated thrombocytopenia without splenomegaly (1).

ITP's pathophysiology is still not entirely understood. Nonetheless, anomalies of the humoral immune system are thought to be the primary cause of thrombocytopenia. These anomalies cause B lymphocytes to produce antiplatelet autoantibodies, which destroy platelets (2).

Although autoantibody-mediated immunological pathology underlies ITP, the exact mechanism of immune failure remains unclear. Furthermore, a large body of research indicates that T cells and the cytokines they release are essential for the regulation of anti-platelet autoantibodies (3, 4).

The main roles of the CXC cytokine family are to activate leukocytes in various immune responses and to recruit chemokines (5,6).

The chemokine C-X-C motif ligand 13 (CXCL13), which is also called either the Chemoattractant of B Lymphocyte or the attractive chemokine 1 of B lymphocyte (BCA-1), and its G-protein coupled receptor, CXCR5, are known to play essential roles in immunological, inflammatory, and infectious responses. According to available data, the CXCL13: CXCR5 axis also coordinates cell-to-cell interactions that control lymphocyte infiltration in the cancer's microenvironment, which controls the tumor's reactivity to immune- and cytotoxic-targeted treatments (7).

The primary secretors of CXCL13, a tiny molecule of the CXC chemokine family, are serum follicular dendritic cells, lymph glands, and secondary lymphoid tissue (8).

The major receptor gene, CXCR, is mapped on chromosomal segment 11q23,3, whereas the CXCL13 gene is found on 4q21. A 7-transmembrane G-protein with 372 amino acids, produced by osteoblasts, podocytes, B lymphocytes, dendritic cells of the skin, and follicular B helper T cells make up CXCR 5. B-cell migration into the spleen's B-cell follicles and the gut's Peyer's patches is facilitated by CXCR 5 (9).

Follicle helper T cells (TFH), a subgroup of CXCR5+ T lymphocytes, can be stimulated to traffic by CXCL 13. These cells are especially prevalent in the B cell follicles of the tonsils, spleen, and lymph nodes, which are examples of secondary lymphoid organs (SLO) (10).

Only immature B cells and T cells are host to SLO. High endothelial venules (HEV) constitute specialized postcapillary venules that serve as the main point of entry for blood-borne lymphocytes into peripheral and mesenteric lymph node systems (PLN and MLN) as well as Payer's patches (PP). Selective integrin activation is necessary for lymphocyte adherence on HEV and is dependent upon CXCR 5 and its associated ligand, CXCL 13 (11).

CXCL13 plays a pivotal role in recruitment of B cells and T-cell subsets in pathological conditions and is a therapeutic target in various immune diseases (12).

According to a study, ITP patients have higher levels of CXCL13. Nevertheless, it is still unclear how CXCL13, the severity of ITP, and the reaction to treatment are related (13).

Therefore, the objective of the study was to ascertain if CXCL13 was expressed in the serum of children with ITP, as well as the therapeutic utility of this biomarker and its correlation to laboratory and clinical factors regarding the severity, activity, or response to therapy of ITP disease.

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**Patients and methods:**

Forty healthy children and sixty patients with immune thrombocytopenic purpura were the subjects of the current case-control research. From November 2016 and May 2017, they were referred to the Beni-Suef University Hospital's hematology clinic. According to clinical and analytical results, the diagnosis is made. All children's parents or legal guardians gave their approval for the study.

The American Society of Hematology (ASH) criteria were followed for the diagnosis and treatment of immune thrombocytopenia (ITP). Acute ITP has been defined as thrombocytopenia that disappeared within six months of start, while chronic ITP was described as thrombocytopenia that persisted for more than twelve months (14).

Clinical and laboratory studies randomly obtained from patients who routinely attended the clinic on various treatment protocols were used to diagnose sixty pediatric patients, aged three to fifteen, with ITP. There were 34 women and 26 men. For the control group, forty healthy people of same age and sex who were free of any visible disease were selected. There were 18 women and 22 men.

Next, we sorted group I into its subsequent subgroups: Individuals with acute ITP before steroid therapy (Group A): There were ten female and twelve male newly diagnosed youngsters with ITP. Individuals with acute ITP following steroid therapy (Group B): 20 recently identified children with ITP were included after two to six months of treatment. Ten of them were men and ten were women. Children who have chronic ITP (Group C): Of these, 18 were in the chronic phase (lasting more than 12 months). They ranged in age from 3 to 16 years old, with four males and Fourteen females.

Children between the ages of 3 and 15 who have been diagnosed with ITP based on clinical and laboratory studies meet the inclusion criteria.

Children under the age of three, children over the age of fifteen, children who have additional bleeding diseases, children suspected of having cancer, and children who have any autoimmune disorder are the exclusion criteria.

Steroids were the primary line of treatment for each patient. First-line treatment consisted of 3–7 days of prednisone at a dose of 3-5 mg/kg. (The platelet count, and the presence of active bleeding dictate the dosage and mode of administration; in less serious cases, taking prednisone by mouth or prednisolone may be sufficient, but in situations of emergency, infusion of dexamethasone or a medication called may be utilized). The steroid dose is gradually decreased as soon as the platelet count rises, and the likelihood of a relapse is monitored.

Platelets more than  $100 \times 10^9/L$  were in the complete response (CR) category. A platelet count in the range of  $30 \times 10^9/L$  to  $100 \times 10^9/L$  was considered a response, as was doubling the baseline value. "No response" was used to characterize any platelet counts that were below  $30 \times 10^9/L$  or were less than twice the baseline value. Patients classified as "refractory" were those who had either a splenectomy that had failed or severe ITP or an elevated risk of bleeding that required ongoing therapy (14).

All the study's patients underwent the following procedures: a thorough history taking that focused on gathering information about age, sex, demographics, bleeding symptoms, a full clinical examination, a complete blood picture with differential count, a bone marrow aspiration, and the use of enzyme-linked immunosorbent assays (ELISA) to detect the serum protein CXCL13 (Bioassay Technology Laboratory, Cat. No E0062Hu).

This kit is designed for sandwich technique. The human BLC-1/CXCL13 antibody has been pre-coated onto the plate. When introduced, the sample's BLC-1/CXCL13 binds to the coated antibodies on the wells.

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Subsequently, biotinylated human BLC-1/CXCL13 Antibody is introduced into the sample, where it binds to BLC-1/CXCL13. Following the addition of streptavidin-HRP, the biotinylated BLC-1/CXCL13 antibody binds to it. During a washing phase, unbound streptavidin-HRP is removed following incubation. After adding the substrate solution, color changes in response to the concentration of human BLC-1/CXCL13. The addition of an acidic stop solution ends the process, and the amount of absorption is determined at 450 nm.

Serum was separated and stored at  $-20^{\circ}\text{C}$  until use. For each patient, 3-5 ml of venous blood were collected on a serum separator tube (SST), which was then allowed to clot for 10–20 minutes at room temperature before centrifugation for 20 minutes at 2000–3000 r.p.m. Curve construction was done using computer-based curve-fitting software, which plotted the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis.

The statistical evaluation was carried out with SPSS for Windows (Statistical Program for Social Science Analysis, Edition 23.0, SSPS Inc., Chicago, IL, USA).

## **Results**

Comparative studies were done between patients, controls and patients' subgroups as regards clinical, demographic, and laboratory data, as well as treatment outcome (Table1). The comparison between ITP patients and control as regarding age and sex was not significant statistically ( $p=0.493$ ,  $p=0.908$  respectively). There was a statistical significance between ITP patients' level and that of the control group ( $p=0.028$ ).

**Table (1): Comparison between ITP Patient and control as regarding age, sex and laboratory data**

	<b>ITP Patient (n=60)</b>	<b>Control (n=40)</b>	<b>P value</b>
<b>Age (Year)</b>			
Range	3-15	3-15	0.493
Mean $\pm$ SD	6.9 $\pm$ 3.4	7.6 $\pm$ 3.4	
<b>Sex, no. (%)</b>			
Male	26(43.3)	18(45)	0.908
Female	34(56.7)	22(55)	
<b>Hemoglobin</b>			
Range	8-14.6	10.9-14.2	<b>0.028*</b>
Mean $\pm$ SD	11.6 $\pm$ 1.3	12.4 $\pm$ 1.1	
<b>Platelets</b>			
Range	9-492	295-492	<b>0.001**</b>
Mean $\pm$ SD	118 $\pm$ 118.7	413.9 $\pm$ 53.3	
<b>CXCL13</b>			
Range	233.6-1620	235.5-396.6	<b>0.001**</b>
Mean $\pm$ SD	760.6 $\pm$ 353	283.5 $\pm$ 46.0	

**n**; number of cases, **ITP**; immune thrombocytopenia, **Mean  $\pm$ SD**; Mean $\pm$  Standard deviation, **CXCL13**; chemokine C-X-C motif ligand 13, \*: P-value < 0.05 (Significant), \*\*: P-value < 0.01 (High significant).

Regarding platelets, ITP patients have statistically significant low platelets counts compared with control children which have normal levels of platelets (p=0.001). Regarding CXCL13, ITP patients have statistically significant high serum levels of this marker compared with control (p=0.001).



Comparing the subgroups of ITP patients (acute before treatment, acute after treatment, and chronic) to the controls, there was no statistical significance as regards the Hb level. Meanwhile, there was statistical significance concerning the platelet's count and the CXCL13 (Table2).

**Table (2): Comparison between all groups as regarding laboratory data**

	Acute before ttt (n=22)	Acute after ttt (n=20)	Chronic (n=18)	Control (n=40)	P value
<b>Hemoglobin</b> Range Mean $\pm$ SD	10.6-12.7 11.7 $\pm$ 0.7	9-12.7 11.6 $\pm$ 1.1	8-14.6 11.6 $\pm$ 2.0	10.9-14.2 12.4 $\pm$ 1.1	P <sub>1</sub> =0.978 P <sub>2</sub> =0.889 P <sub>3</sub> =0.106 P <sub>4</sub> =0.912 P <sub>5</sub> =0.110 P <sub>6</sub> =0.096
<b>Platelets</b> Range Mean $\pm$ SD	9-46 19.7 $\pm$ 11.8	56-260 120.3 $\pm$ 65.4	80-450 235.6 $\pm$ 131.4	295-492 413.9 $\pm$ 53.3	P <sub>1</sub> =0.002** P <sub>2</sub> =0.001** P <sub>3</sub> =0.001** P <sub>4</sub> =0.001** P <sub>5</sub> =0.001** P <sub>6</sub> =0.001**
<b>CXCL13</b> Range Mean $\pm$ SD	888.6-1620 1103.2 $\pm$ 246.8	643.4-881.1 763.7 $\pm$ 84.6	233.6-459.5 338.2 $\pm$ 74.4	235.5-396.6 283.5 $\pm$ 46.0	P <sub>1</sub> =0.001** P <sub>2</sub> =0.001** P <sub>3</sub> =0.001** P <sub>4</sub> =0.001** P <sub>5</sub> =0.001** P <sub>6</sub> =0.293

**ttt**; treatment, n; number of cases, **Mean  $\pm$ SD**; Mean $\pm$  Standard deviation, **CXCL13**; chemokine C-X-C motif ligand 13, **P1**: Comparison between acute before treatment group and acute after treatment group, **P2**: Comparison between acute before treatment group and chronic group, **P3**: Comparison between acute before treatment group and control group, **P4**: Comparison between acute after treatment group and chronic group, **P5**: Comparison between acute after treatment group and control group, **P6**: Comparison between chronic group and control group, SD: Standard deviation, \*: P-value < 0.05 (Significant), \*\*: P-value < 0.01 (High significant), P-value > 0.05 (Non-significant).

Correlation studies were done between Cxcl13 and the following parameters (age, Hb, and platelets count). They revealed negative correlation between platelet count and CXCL13 that means the lower the platelets, the higher level of this marker (r= -0.7, p=0.001) (Table3).

**Table (3): Correlation between CXCL13 and the following parameters (age, Hb and platelet count) in patient group.**

	CXCL13	
	r	P value
Age (Year)	<b>-0.2</b>	0.255
Hemoglobin	<b>0.1</b>	0.866
Platelet count	<b>-0.7</b>	0.001**

r: Correlation coefficient

Roc curve analysis of Cxcl13 as a test for early detection of acute cases showed significant sensitivity and specificity either before or after treatment while in early detection of chronic cases, it showed lower sensitivity and specificity as shown in Table 4.

**Table (4): Roc curve analysis of CXCL13 as a test for early detection of ITP**

CXCL13	Cutoff	Sensitivity (%)	Specificity (%)
Acute before ttt and control	> <b>396.6</b>	100	100
Acute after ttt and control	> <b>396.6</b>	100	100
Chronic and control	> <b>304.3</b>	66.7	85

ttt; treatment, CXCL13; chemokine C-X-C motif ligand 13.

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## **Discussion**

Primary immune thrombocytopenia (ITP) is a form of autoimmune disease defined by solitary thrombocytopenia in a lack of other causes (14).

ITP is mediated by antiplatelet autoantibodies. Antibody-coated platelets are phagocytosed by macrophages in the reticuloendothelial system, leading to accelerated platelet clearance. (15).

The cytokine receptor-ligand pairs CXC cytokine receptor-5 (CXCR5) and CXCL13 are important components in the immune system; CXCL13 is required for the recruitment of B and T cells. (10). In 2013, studies revealed that CXCL13 levels are higher in ITP patients (13).

As a result, this study was designed to investigate the level of B Lymphocyte Chemoattractant (BLC), also referred to as B cell attracting chemokine1 (BCA-1) or CXCL13, in the plasma of ITP children, as well as the clinical utility of such a biomarker and its relationship to clinical and laboratory tests variables concerning ITP disease severity, activity, and response to therapy.

The current study included 60 pediatric patients with immune thrombocytopenic purpura. They comprise 42 patients with acute ITP (22 before and 20 after steroids), as well as 18 patients with persistent ITP. The typical control group consisted of forty healthy children.

The key findings revealed that serum CXCL13 levels were considerably higher in ITP children than in healthy controls ( $p=0.001$ ). The patients with acute ITP exhibited greater CXCL13 serum levels than those with chronic ITP ( $p=0.001$ ). Furthermore, individuals who had received steroids had lower serum CXCL13 levels than those who had not yet received steroids ( $p=0.001$ ).

Our findings were consistent with Jian et al. (16), who reported on a retrospective evaluation of children diagnosed with ITP at Soochow University Affiliated Children's Hospital. Medical records for all children with ITP have been gathered: (1) Demographic data: age and sex; (2) Clinical information: count of platelets, hemoglobin concentration, bleeding severity, and type of ITP at the time of previous presentation. IVIG, corticosteroids, splenectomy, immunological therapy, and chemotherapy were used in the treatment of ITP.

Jian et al. (16) study included 30 children who had been diagnosed with ITP. The male/female ratio was 1:1.3. The average age of children who suffered from ITP was  $5.4 \pm 3.7$  years. 56.7% of the 30 patients had nil or mild hemorrhage, 26.7% of ITP children suffered moderate hemorrhage, and 16.7% had severe hemorrhage. Of the 30 patients, 20 have been identified with acute ITP and 10 with chronic ITP.

In our study, 60 children were diagnosed with ITP, with a male-to-female ratio of 1:1.3. The average age of children who suffered from ITP was  $6.9 \pm 3.4$  years. Of the 60 patients, 86.7% suffered no or little bleeding, 13.3% of ITP children suffered moderate bleeding, and nobody had serious bleeding. Of the 60 patients, 42 had been diagnosed with acute ITP and 18 with chronic ITP.

Our findings were consistent with Jian et al. (16), which found that the concentration of CXCL13 in peripheral blood was considerably higher in ITP children than in healthy controls.

Furthermore, our study looked at the difference in CXCL13 serum concentrations between chronic and acute ITP. There was a substantial increase in acute versus chronic patients ( $p=0.001$ ). However, in the Jian et al. (16), investigation, the increase in CXCL13 serum levels between chronic and acute ITP was not considered statistically significant ( $P = 0.0561$ ).

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This discrepancy could be explained by the difference in patient's race and elevated platelets count in our chronic patients at the time of study.

Also, in the Jian et al. (16), trial, as well as in our investigation, patients who received steroid treatment had lower plasma CXCL13 levels than patients who had not yet received steroid treatment. ( $p=0.001$ ). In addition, in the Jian et al. (16), trial, as well as in our investigation, patients who received steroid treatment had lower plasma CXCL13 levels than patients who had not yet received steroid treatment. ( $p=0.001$ ).

Our study investigated the relationship between serum CXCL13 levels and disease activity indicators. There was no statistical association between CXCL13 levels and gender or age in ITP children. The findings revealed that plasma CXCL13 levels were favorably linked with platelet counts in ITP patients before treatment. However, following therapy, the relationships were missing, as in their previous study.

However, their results suggested that serum CXCL13 exhibited a positive statistical link with the level of hemoglobin in ITP patients, but our results revealed no statistically significant correlation between the level of CXCL13 and hemoglobin in patients with ITP ( $p= 0.866$ ). In our study, children had high Hb levels ranging from 8-14.6, with a mean SD of  $11.6\pm 1.3$ , due to their brief duration of hemorrhage before therapy.

Our findings align with a prior study by Margareta Jernås et al. (17), who discovered higher serum CXCL13 levels in ITP patients. They found 1915 modulated genes and 22 modulated microRNAs that changed among ITP patients and controls using genome-wide expression analysis in T cells. Furthermore, CXCL13 has been identified as one of the miRNAs' target genes for ITP regulation. T-cell activation and control of immunoglobulin synthesis. CXCL13 as well as IL-21 identified as two microRNA target genes that increased considerably during ITP.

## **Conclusion:**

The study concluded that serum CXCL13 was suggested to have a role in acute ITP pathogenesis and may be used as a biomarker creating a new target for ITP therapy with high significant sensitivity, and specificity in early detection of acute ITP.

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