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PLASMA INTERLEUKIN-21 AND CXCL13 IN PRIMARY IMMUNE THROMBOCYTOPENIC PATIENTS RECEIVING CORTICOSTEROIDS AS A FIRST LINE THERAPY

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ABSTRACT

Background: This study aimed to assess the plasma interleukin-21 and CXCL-13 levels in adult patients with newly diagnosed immune thrombocytopenic purpura (ITP) as predictors of response to corticosteroids as first line therapy. **Methods:** 30 newly diagnosed adult ITP patients and 30 age and sex-matched controls were enrolled. Both groups were tested for IL-21 and CXCL-13 plasma levels by ELISA at diagnosis and after receiving 2-4 weeks of prednisolone 1-2 mg/kg/day. **Results**: Patients' age ranged from18 to 49 years with a mean of 33.70 ± 8.563 years; 76.67% were females. Clinically, 66.67% of patients complained of fatigue, 43.33% presented with menorrhagia, 36.67% of patients had ecchymosis, 20% complained of epistaxis, 10% presented with melena, 6.67% with gingival bleeding, 3.33% manifested by subconjunctival hemorrhage and 3.33% presented with intracranial hemorrhage. In addition, 10% of patients were accidentally diagnosed during routine laboratory work up. The platelet count of ITP patients at diagnosis (mean: 16.79±11.085X10^9/L) was significantly lower than the controls (mean: 239.80±43.245X10^9/L)), and it significantly increased after treatment (mean: 109.83 ±47.015 X10^9/L) compared to pre- treatment value [Z=4.783, p=0.000*]. Regarding response to treatment; 60% of patients showed a complete response (CR= platelet count ≥100 x 10^9/L and absence of bleeding), 33.33% showed a response (R= platelet count ≥30 x 10^9/L and at least 2-fold increase in the baseline count and absence of bleeding), and 6.66% of patients showed no response to treatment {NR= platelet count <30 x 10^9/L or less than 2-fold increase of baseline or bleeding). The mean plasma IL-21 level was 273.27± 226.962 pg/ml in ITP patients at diagnosis, which was significantly higher than the controls (mean: 208.82±100.636 pg/ml) [Z=2.524, p=0.012*]. After treatment; IL-21 level significantly decreased than before treatment with a mean of 171.78 ±49.759 pg/ml [Z=4.703, p=0.000]. Regarding CXCL-13; its mean value in ITP patients at diagnosis was 202.77± 147.337 pg/ml, which was significantly higher than the controls (mean: 172.43±72.568 pg/ml) [Z=2.392, p=0.017*]. After treatment; the CXCL-13 level significantly decreased compared to pre- treatment level (mean: 141.28 ±29.520 pg/ml [Z=4.788, p=0.000]. As predictors of response to corticosteroids, a cutoff point of 225 pg/ml for IL-21 at presentation could significantly define responders and non-responders (p=0.0157). However, plasma CXCL-13 level before treatment was not a statistically significant discriminator of response in our ITP patients. **Conclusion:** Plasma IL-21 and CXCL-13 levels were significantly elevated in treatment-naïve ITP patients and significantly decreased after corticosteroid therapy. IL-21, but not CXCL-13 was a significant predictor of response.

KEYWORDS: ITP, corticosteroids, CXCL-13, IL-21.

INTRODUCTION

Primary immune thrombocytopenia (ITP) is a common autoimmune disorder characterized by immune-mediated accelerated platelet destruction and suppressed platelet production and resulting in isolated thrombocytopenia.[1] It has a significant incidence of about 3.3 per 100,000 adults/year.[2] ITP is rarely fatal. Adult ITP typically has an insidious onset and rarely resolves spontaneously.[3] Concepts surrounding the mechanisms of thrombocytopenia in ITP have shifted from the traditional view of increased platelet destruction mediated by auto-antibodies to more complex mechanisms in which impaired platelet production, T-cell-mediated effects, and disturbed cytokine profiles play a role.[4]

Interleukin-21 is an IL-2 family cytokine produced by activated T cells to regulate immune responses.[5] IL-21 is strongly linked with inflammation and autoimmunity. Elevated amounts of IL-21 have subsequently been reported in many autoimmune diseases, including type 1

diabetes, systemic lupus erythematosus (SLE), and inflammatory bowel diseases.[6-8] The chemokine C-X-C motif chemokine 13 (CXCL-13) is necessary for follicle formation and is constitutively expressed in secondary lymphoid tissue, primarily by follicular dendritic cells (FDCs).[9] C-X-C chemokine receptor type 5 (CXCR5), the only known receptor for CXCL13, is expressed by naïve B cells and T follicular helper (TFH) cells, and it controls the migration of these cells to the follicle.[10] Recently, CXCL-13 has risen to be a possible new marker of disease and inflammation in rheumatoid arthritis and is suggested to be connected with both disease activity and rheumatoid factor.[11-13]

Few data is available about IL-21 and CXCL-13 in adult patients with ITP. The aim of the present study is to assess the plasma interleukin-21 and CXCL-13 levels in adult patients with newly diagnosed ITP as predictors of response to corticosteroids as first line therapy.

PATIENTS AND METHODS

This study comprised two groups of subjects; Group (I): 30 newly diagnosed adult primary ITP patients aged ≥ 18 years old and Group (II): 30 healthy adult age and sex-matched controls. The patients were selected from those attending the Hematology Outpatient Clinic and those admitted in the Hematology Unit, Internal Medicine Department, Alexandria Main University Hospital**.** Exclusion criteria included patients aged <18 years, preganant females, infectious causes of thrombocytopenia, collagenic causes of thrombocytopenia, lymphoproliferative disorders, acute leukemia, antiphospholipid syndrome, Evans' syndrome, bone marrow failure syndromes, patients on medications causing thrombocytopenia or platelet dysfunction, prior or ongoing therapy for ITP, previous chemotherapy or radiotherapy, patients with hematological or non-hematological (solid) malignancies and patients with chronic conditions associated with thrombocytopenia or platelet dysfunction e.g. renal or hepatic insufficiency. All patients were subjected to thorough history taking, full clinical examination and laboratory investigations including CBC, direct antiglobulin test to exclude associated autoimmune hemolytic anemia (Evans' syndrome), HCV Abs, HIV Abs and HBsAg, antinuclear antibody and anti-double stranded DNA, stool analysis for H pylori antigen and bone marrow examination in selected patients. Plasma IL-21 (Glory Science Co., Ltd. China, catalogue number 12749) and CXCL-13 (Glory Science Co., Ltd. China, catalogue number 95203) levels were measured by ELISA technique at diagnosis and after 2-4 weeks of receiving 1-2 mg/kg prednisolone as first line therapy.

The study was approved by the Research Ethics Committee of Alexandria Faculty of Medicine and informed consent was obtained from all subjects before enrollment.

Statistical Analysis

Data were collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis (ver 21). Data were entered as numerical or categorical, as appropriate. When Kolmogorov-Smirnov test revealed no significance in the distribution of variables, parametric statistics was carried out, while in the not-normally distributed data the non-parametric statistics was carried out.[14]

RESULTS

The results are presented in tables (I and II) and figures (1-5). The patients' age ranged from18 to 49 years with a mean of 33.70 ± 8.563 years; females were 76.67% and males were 23.33%. No statistically significant difference was found between patients and controls regarding age and sex. Figure (1) illustrates the clinical data in ITP patients at presentation.

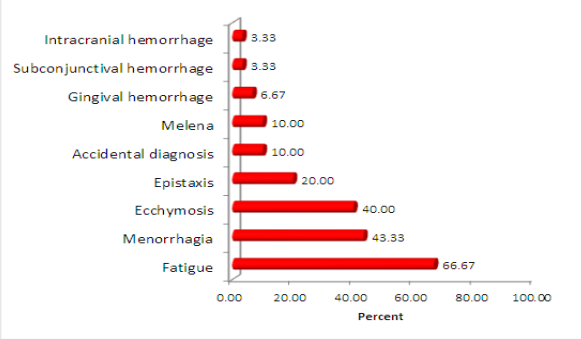

Figure 1: Clinical data in ITP patients at presentation.

Table (I) shows the platelet count in the two studied groups at presentation and in ITP patients after corticosteroid therapy. The platelet count of ITP patients at presentation was significantly lower than the controls (p_2=0.003). After treatment, it significantly increased as compared to pre-treatment value (p_1=0.000*).

Table I: Platelet count in the two studied groups at presentation and in ITP patients after corticosteroid therapy.

Platelet count ($x10^9$/L)	Group I before treatment	Group I after treatment
Min-max	4-50	15-215
Mean± SD	16.79±11.085	109.83±47.015
Median	15.00	110.00
Wilcoxon Signed Ranks Test	Z=4.783 P_1=0.000*	
	Group II	
Min-max	175-323	
Mean± SD	239.80±43.245	
Median	235.00	
Mann-Whitney U test	Z=6.655 P_2=0.003*	

Table (II) shows the response of ITP patients to corticosteroid therapy: The criteria for assessing the patient response to ITP treatment based on the platelet count was guided by the International Working Group Criteria (2009):[15]

1. Complete response (CR): platelet count ≥100 x 10^9/L and absence of bleeding.
2. Response (R): platelet count ≥30 x 10^9/L and at least 2-fold increase in the baseline count and absence of bleeding.
3. No response (NR): platelet count <30 x 10^9/L or less than 2-fold increase of baseline count or bleeding.

Table II: The response of ITP patients to corticosteroid therapy.

Response to CS therapy	n	%
Complete response (CR)	18	60.00%
Response (R)	10	33.33%
No response (NR)	2	6.66%
Total	30	100%

Figure (2) illustrates the plasma level of IL-21 in ITP patients at presentation and after receiving treatment compared to the controls, it was significantly higher in patients at presentation compared to controls (p=0.012). While after treatment the IL-21 level was significantly lower than its level at presentation and in controls (p=0.000, p=0.021; respectively). Figure (3) illustrates the plasma level of CXCL-13 in ITP patients at presentation compared to post-treatment levels and controls. It was significantly higher in patients at presentation compared to controls (p=0.017) while after treatment the CXCL-13 level was significantly lower than its level at presentation and in controls (p=0.000, p=0.004; respectively).

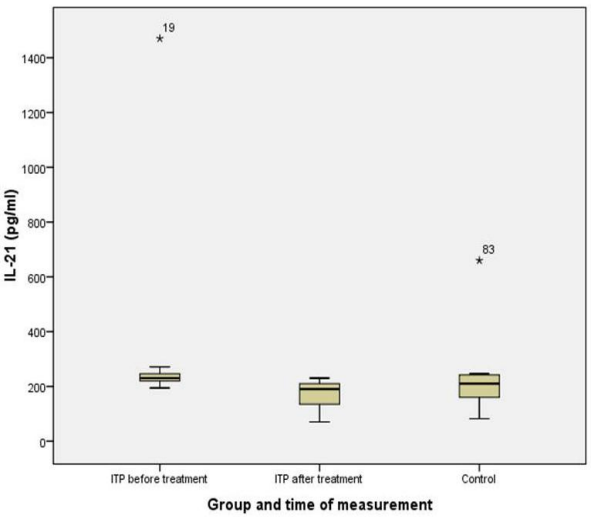

Figure 2: Box and whisker graph of IL-21 level (pg/ml) in ITP patients before treatment, after treatment and in controls.

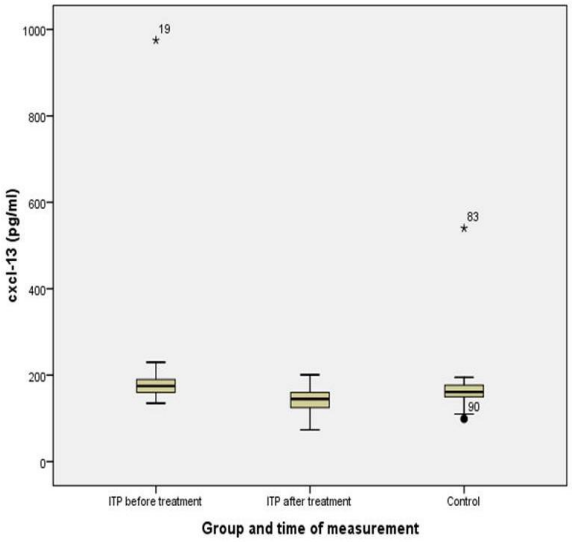

Figure 3: Box and whisker graph of CXCL-13 level (pg/ml) in ITP patients before treatment, after treatment and in controls.

Figures (4) and (5) demonstrate the ROC curve diagnostic test accuracy of IL-21 and CXCL-13 before treatment, respectively. Plasma IL-21 level before treatment was statistically significant discriminator of occurrence of response to corticosteroids with area under the ROC curve (AUC) =0.813 (95% CI 0.629 -0.931) (Z=2.417, p=0.0157). A cutoff point of 225 pg/ml for IL-21 at presentation could significantly define responders and non-responders (p=0.0157). However, plasma CXCL-13 level before treatment was not significant discriminator of occurrence of response with area under the ROC curve (AUC) =0.589 (95% CI 0.396 to 0.765) (Z=0.501, p=0.6167).

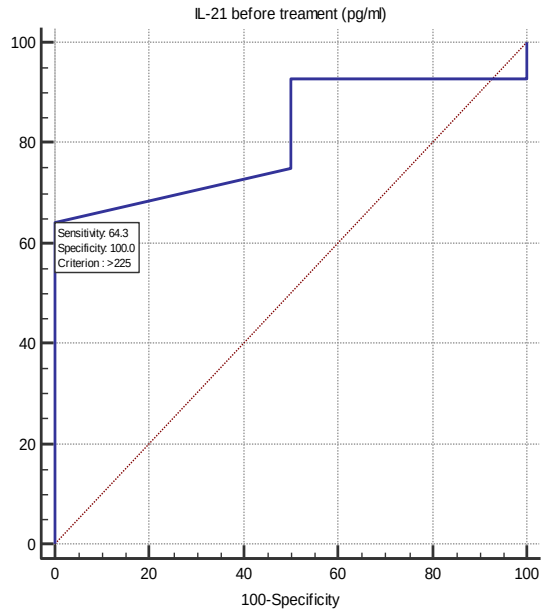

Figure 4: ROC curve diagnostic test accuracy of IL-21 before treatment.

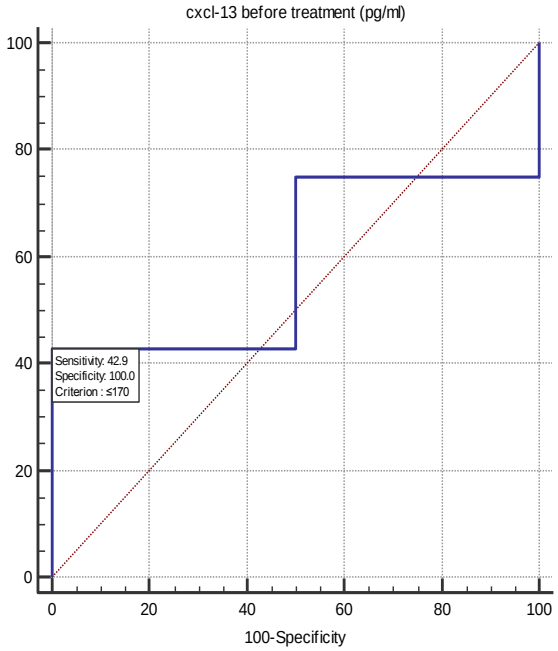

Figure 5: ROC curve diagnostic test accuracy of CXCL-13 before treatment.

DISCUSSION

In the present study, we aimed to monitor the plasma levels of IL-21 and CXCL-13 before and after corticosteroid treatment to see if they have any prognostic value in ITP patients compared to their age- and sex- matched healthy counterparts.

The age of ITP patients ranged from 18 to 49 years with a mean of 33.7 years. This was in accordance with Moulis et al (2015)[16] who performed a large scale nationwide population-based study of epidemiology of ITP in France.

Regarding sex of ITP patients, females significantly predominated over males with a ratio of 3:1. This was in accordance with Khan & Mikhael (2010)[17] in their review of ITP, where they stated that the female to male ratio is 1.7:1 in those aged less than 60 years, but it becomes equal among older age.

The clinical data of our ITP patients showed wide diversity at presentation; however, fatigue was the most common complaint present in 20 (66.67%) patients. Bleeding manifestations were seen in 27 out of our 30 ITP patients varying between minor skin purpura and ecchymosis in 16.67% to moderate mucous membrane bleeding including epistaxis, gum bleeding and some cases of menorrhagia (46.67%). Some patients (26.67%) presented with severe hemorrhages in the form of subconjunctival hemorrhage, melena, massive menorrhagia and one patient had intracranial hemorrhage. 3 patients had no complaints and were accidentally diagnosed.

Fatigue is one of the most common symptoms of ITP which increases with lower platelet counts. Although anemia due to blood loss was found in 14 (46.66 %) of our patients, yet this anemia was mild in 10 (33.33 %) and thus; could not entirely explain this fatigue which is seen in both anemic and non-anemic subjects. Kashiwagi & Tomiyama (2017)[18] stated that fatigue is one of the most common and distressful symptoms for patients with a chronic disease. It has been shown that a significant proportion of ITP patients suffer from fatigue, and many feel that they have less energy when the platelet count is low.

Of course, menorrhagia was the commonest bleeding presentation in our series since we had a significantly higher number of females in the reproductive age compared to male patients. Life threatening bleeding as internal hemorrhage or intracranial hemorrhage- which was encountered in one of our patients at presentation- is rare to occur in ITP. These data were in accordance with Liebman & Pullarkat[19] as presented in the annual ASH meeting 2011 regarding the diagnosis and management of ITP in the era of thrombopoietin mimetics.

Anemia in our patients was mostly normocytic normochromic anemia (33.33%), followed by microcytic hypochromic anemia (20%) due to iron deficiency and there was no evidence of autoimmune hemolytic anemia as all patients included in our study were Coombs' test - negative. Provan et al (2010)[20] stated - in the International Consensus Report on the Investigation and Management of Primary Immune Thrombocytopenia - that anemia from blood loss may be present, but it should be proportional to the amount and duration of bleeding and may result in iron deficiency.

In the present study, the platelet count in ITP patients at presentation ranged from 4 to 50 $X10^9$/L with a mean of 16.79±11.085 $X10^9$/L. After 2-4 weeks of corticosteroid treatment, it ranged from 15 to 215 $X10^9$/L with a mean of 109.83 ±47.015 $X10^9$/L. There was a statistically significant increase in platelet count (p=0.000) which is attributed to patients' response to corticosteroids given as first line therapy.

The response to steroid therapy among our ITP patients included a complete response (CR) to treatment in 18 (60%) patients, a response (R) to treatment in 10 (33.33%) patients and no response (NR) in 2 (6.66%) patients. Our results were in agreement with Barsam et al (2011)[21] who reported that, at least 80% of patients with ITP initially respond to corticosteroids.

Regarding plasma IL-21, it was significantly higher in our ITP patients at presentation compared to their controls (p=0.012). After first-line therapy with 1-2 mg/kg of prednisolone for 2-4 weeks, a statistically significant decrease in IL-21 level occurred compared to pre-treatment values (p=0.000) and even lower than in the controls (p=0.021). Similar findings were reported by

Zhang et al (2014)[22] who performed a study on 24 ITP patients and 9 healthy controls. They found that plasma IL-21 measurement with ELISA was significantly increased in active ITP patients compared to healthy controls. They also studied the expression of IL-21 mRNA on peripheral blood mononuclear cells (PBMCs) and found that the percentage of IL-21 mRNA was significantly increased in ITP patients compared to healthy controls. Moreover, real time - PCR showed that the frequencies of circulating IL-21- producing T cells were significantly higher in ITP patients than that in healthy controls.

Zhang et al (2014)[22] treated their patients with high dose dexamethasone. Plasma IL-21 and IL-21 mRNA expression in patients with ITP significantly decreased suggesting IL-21might be important in ITP. Similar results were obtained in cultures of PBMCs derived from ITP patients, where dexamethasone reduced IL-21 secretion.[23]

In a genome study, IL-21 was identified as a target gene of the regulated microRNA in ITP, which supports the increased expression of IL-21.[24] Moreover, Zhu et al (2010)[25] examined the expression of IL- 21, IL-17, and interferon (IFN)-γ in ITP patients and controls by ELISA and flow cytometry. Their study demonstrated elevated IL-21 in ITP patients and its positive correlation to Th17 cells and Th1 cells, indicating a possible role of IL-21 in ITP.

CXC chemokine ligand-13 (CXCL13) is a small cytokine belonging to the CXC chemokine family, mainly secreted by secondary lymphoid tissue, lymph glands and serum FDCs.[26] The primary functions of CXC chemokine family are chemoattraction and activation of leukocytes in multiple immunological response.[27] CXCL13 is required for B1 cell homing, natural antibody production and body cavity immunity. In addition, it has been reported that CXCL13 plays a key role in recruitment of B cells and T- cell subsets in pathological conditions, and is considered to be a therapeutic target in various immune diseases.[28]

In the present study, plasma CXCL-13 level among ITP patients before treatment was statistically significantly higher when compared to the controls (p=0.017). Similar findings were reported by others.[24,29]

To investigate the role of microRNA in ITP, Jernas et al (2013)[24] performed a genome-wide expression analyses of mRNA and microRNA in T cells from ITP patients and controls. They identified 1915 regulated genes and 22 regulated microRNA that differed between ITP patients and controls. Seventeen of the 22 regulated micro RNA were linked to changes in target gene expression; 57 of these target genes were associated with the immune system, e.g., T-cell activation and regulation of immunoglobulin production. CXCL13 and IL-21 were two microRNA target genes significantly increased in ITP. They could demonstrate increased plasma levels of CXCL13 in ITP. They suggested that microRNA may be important regulatory molecules involved in the loss of tolerance in ITP.

More recently; Li et al (2015)[29] performed a study on 30 ITP patients and found that CXCL13 is the target gene of miR-125-5p, and its plasma level was markedly elevated in ITP patients than controls suggesting that it is possibly involved in the pathological process of ITP.

In the present study, the plasma CXCL-13 level in ITP patients after steroid treatment decreased significantly compared to pre-treatment level (p=0.000) and even became lower than the control group (p=0.004). Our findings were in line with Li et al (2015)[29] who also demonstrated significantly elevated plasma CXCL13, the concentration of which was reduced after treatment. Moreover, they performed in vitro experiments in which dexamethasone was added to CD4+ T lymphocytes isolated from PBMCs from healthy volunteers. They found that dexamethasone decreased CXCL13 level in a dose- dependent and in a time-dependent manner. They also treated the CD4+ T cells by miR-125-5p mimic / inhibitor, to observe the regulation of CXCL13. MiR-125-5p mimic decreased CXCL13 level and miR-125-5p inhibitor increased CXCL13 level in CD4+ T cells. CXCL13 was implied to be target gene of miR-125-5p. MiR-125-5p inhibitor also cancelled dexamethasone induced decrease of CXCL13.

In the present study, plasma IL-21 level before treatment was a significant predictor of occurrence of response to corticosteroids with AUC =0.813 (95% CI 0.629 -0.931) (Z=2.417, p=0.0157). A cutoff point of 225 pg/ml for IL-21 at presentation could significantly define responders and non- responders. However, plasma CXCL-13 level before treatment was not significant discriminator of occurrence of response with AUC =0.589 (95% CI 0.396 to 0.765) (Z=0.501, p=0.6167). The latter could be due to the relatively small number of studied cases and needs to be addressed in future studies.

To conclude, plasma IL-21 and CXCL-13 levels were significantly elevated in ITP patients at diagnosis and significantly decreased after receiving a course of 1-2 mg/kg of prednisolone. Pre-treatment IL-21 could be used as a significant predictor of response to steroid therapy. Further molecular studies on the regulation of CXCL-13 by miR-125-5p are warranted to elucidate its exact role in development of ITP. IL-21 and CXCL/ miR-125-5p are potential therapeutic targets for ITP.

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