Original Article

Is There Correlation between CD19, CD20, and CD25 Expressions with Platelet Changes within 6 Months in Children with Immune Thrombocytopenic Purpura?

Ali Amin Asnafi, Mostafa Moghtadaei, Masumeh Maleki Behzad, Najmaldin Saki

Thalassemia and Hemoglobinopathy Research Center, Research Institute of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Abstract

Background: Immune thrombocytopenic purpura (ITP) is a bleeding disorder in which the defects of immune system cells play a vital role. The aim of this study was to explore the possible correlation between independent CD markers' expressions and platelet counts in children with ITP. **Materials and Methods:** The frequency of CD19, CD20, and CD25 markers in the peripheral blood of twenty children with ITP was investigated by flow cytometry, and the possible correlation between the expressions of these markers with platelet counts was analyzed. **Results:** A significant negative correlation was found between CD20 expression with platelet counts before (P = 0.024) and 10 days after treatment (P = 0.016). There was no significant correlation between the expressions of CD19 and CD25 with platelet counts at different times of follow-up. Moreover, CD20 expression was higher in patients with no response compared to those having complete response to first-line therapies. **Conclusion:** We found that the expressions of these markers could not be considered as a prognostic factor independent of other contributors involved in ITP pathogenesis. It is important that future studies should focus on the potential effects of other factors involved in ITP pathogenesis and their impact on response to therapy, as well as evaluating CD markers during ITP progression.

Keywords: *CD19, CD20, CD25, immune thrombocytopenic purpura, prognosis*

Introduction

Immune thrombocytopenic purpura (ITP) is an immune bleeding disorder characterized by platelet destruction in peripheral blood (PB),[1,2] which is a function of antiplatelet antibodies causing platelet clearance through reticuloendothelial system.[3] In addition to platelet clearance in PB, impaired maturation of megakaryocytes can be associated with reduced platelet counts in ITP.[4] Based on its duration, ITP is classified into the following three phases: newly diagnosed, ITP within 3 months from diagnosis, persistent, ITP lasting between 3 and 12 months from diagnosis, and chronic, ITP lasting for more than 12 months after diagnosis.[5] In children, ITP usually occurs as an acute disease after viral and bacterial infections or following vaccination.[6,7] This type of disease resolves spontaneously and often does not take longer than 6 months.[8,9] Several abnormalities, including a defect in cellular immune mechanism, as well as several abnormalities in B- and T-cell subsets, can play a central role in this disease.[10,11] Patients with ITP due to loss of peripheral tolerance possess autoreactive B- and T-cells.[12,13] The possible mechanism for loss of tolerance in ITP is a defect in the circulation and function of regulatory cells such as CD19+CD24h^{i}CD38^{hi} regulatory B-cells (Breg) and CD4+CD25+FOXP3+ regulatory T-cells (Treg). Autoreactive B-cells have been demonstrated to be involved in ITP pathogenesis through the production of autoantibodies.[14] Furthermore, the depletion of B-cells after treatment with rituximab (RTX), a chimeric monoclonal antibody against CD20 on B-cells, is characterized by the modulation of B-cell subsets, which indicates the pivotal role of B-cells in ITP pathogenesis.[15,16]

In spite of abnormalities in the function and distribution of various immune cells in ITP, there are no studies on the correlation between CD markers' expressions in these cells with prognosis in ITP. In the present study, we investigated CD19, CD20, and

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Address for correspondence:
Dr. Najmaldin Saki, Thalassemia & Hemoglobinopathy Research Center, Research Institute of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Department of Clinical Laboratory, Allied Health Sciences School, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.
Golestan Hospital, Ahwaz, Iran.
E-mail: najmaldinsaki@gmail.com

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CD25 expressions in ITP patients, and for the first time examined the possible correlation between the expression level of these markers and platelet changes within 6 months of follow-up. The aim of this study was to determine whether the expression level of these markers could be used as a prognostic factor for platelet changes as well as a predictor for chronicity of ITP in children.

Materials and Methods

Patients and samples

In this study, patients with ITP were selected from among 35 thrombocytopenic pediatric patients who were referred to Shafa Hospital, Ahvaz, from October 29, 2016, to June 21, 2017. All thrombocytopenic patients underwent an initial evaluation for the diagnosis of ITP. Diagnosis of ITP was based on patient's history; physical examination; platelet counts $<100 \times 10^3/\mu L$; a normal concentration of hemoglobin (Hb) and white blood cells (WBCs); PB smear examination; and the absence of other diseases causing thrombocytopenia, including human immunodeficiency virus infection, systemic lupus erythematosus, and lymphoproliferative disorders, which was confirmed by bone marrow aspiration assays according to the International Working Group guidelines for the investigation and management of ITP.[5] Then, twenty newly diagnosed children with ITP were selected. All newly diagnosed ITP patients in this study were treated with first-line therapy with Amp-intravenous immunoglobulin (IVIG) (400 mg/kg IV infusion per day) for 3 days, and the response to treatment was classified into three groups according to the International Working Group guideline.[5] Patients were followed up for at least 6 months to identify chronic ITP or any other hematologic disorders.

Sample collection and flow cytometric analysis

Blood samples from patients were collected in ethylenediaminetetraacetic acid-containing tubes at the time of examination for flow cytometric analysis and laboratory investigations such as platelet count, red blood cells (RBCs), WBCs, platelet distribution width (PDW), and mean platelet volume (MPV). For flow cytometric analysis, the samples were incubated with mouse monoclonal antibodies (Dako, Denmark) containing fluorescein isothiocyanate (FITC)-labeled anti-CD19 and anti-CD20, phycoerythrin (PE)-labeled anti-CD25, and peridinin-chlorophyll-protein (PerCP) anti-CD45. First, three flow cytometric tubes were considered for each sample. Then, according to the name of relevant tubes, 10 μL of conjugated monoclonal antibodies with FITC (CD19+ and CD20+) and 10 μL of conjugated antibodies with PE (CD25+) were added to the tubes, and 10 μL of PerCP (CD45+) was also poured into all the tubes. Afterward, 100 μL of the whole blood was added to all tubes and mixed using a small shaker for several seconds. The tubes were incubated for 15–20 min at 4°C in a dark place. After incubation, RBCs were lysed using RBC lysis buffer, and the remaining WBCs were twice washed with phosphate-buffered saline containing 0.2% bovine serum albumin. Immediately afterward, with the acquisition of 25,000 events in a lymphocyte gate, the expressions of CD19, CD20, and CD25 markers were analyzed by Partec Flow Cytometer (Partec PAS, Germany); the data were analyzed with FlowMax software (Partec PAS, Germany) and presented as proportions of antigen-expressing cells (%).

Statistical analysis

Quantitative data were expressed as mean ± standard deviation, and qualitative data were presented as frequency and percentage. Spearman's correlation analysis was performed for determining the correlation between CD markers' expressions and hematological parameters. Differences between the groups of patients were analyzed by ANOVA. All the tests were performed by SPSS software (IBM SPSS statistics version 22, IBM, New York, Armonk, USA). $P < 0.05$ was considered statistically significant.

Results

Twenty patients (9 boys and 11 girls) were enrolled in this study at the onset of their disease. The clinical and demographical data of all patients are shown in Table 1. Patients were followed up for at least 6 months, and mean platelet counts at different times of follow-up are presented in Table 1. Possible associations between the percentages of CD markers and platelet counts at different times of follow-up were analyzed to examine the expression effects of CD markers on platelet changes and disease duration. A significant negative correlation was found between the percentages of CD20 + lymphocytes and platelet counts before treatment and 10 days after treatment [Figure 1a and b]. Nevertheless, no significant correlation was observed between the percentages of CD19+ lymphocytes and platelet counts at different times of follow-up. Similar to the results of correlation between CD19 percentage and platelet counts at different times of follow-up, no significant correlation was detected between CD25 percentage with platelet counts at different times of follow-up [Table 2]. Within the studied population, six patients (30%) showed no response (NR), five (25%) had partial response (PR), and nine (45%) had complete response (CR) to first-line therapies within 3 days of treatment. We compared the frequency of CD markers' expressions among NR and CR patients [Figure 2a-c]. Upon the diagnosis of ITP, NR patients showed a lower expression level of CD19 in lymphocytes (19.80% ± 3.24%) compared to CR (25.41% ± 2.29%), but no significant difference was found in this regard ($P = 0.993$) [Figure 2aA4]. However, the higher expression level of CD20 in NR (27.30% ± 8.70%) showed a significant difference with CR (15.20% ± 8.46%) ($P = 0.015$) [Figure 2bB4].

Table 1: Baseline demographical and laboratory characteristics of the patients

Characteristics	ITP patients (*n*=20)
Sex, *n* (%)	
Boys	9 (44)
Girls	11 (55)
Age, (range)	5.6 (1 month-10 years)
RBCs ($\times 10^3$/L), mean±SD	4.06±0.063
WBCs ($\times 10^9$/L), mean±SD	9.65±3.50
Platelet counts at different times of follow-up ($\times 10^3$/μL), mean±SD	
Before treatment	20.05±6.67
3 days after treatment	94.4±4.89
Discharge	172.05±3.24
10 days after treatment	326.55±7.53
6 months after treatment	302.05±3.37
Presenting symptoms, number of patients (%)	
Petechial rash	11 (55)
Purpura	8 (40)
Ecchymosis	3 (15)
Mucosal bleeding	2 (10)
Gastrointestinal bleeding	0
Increased megakaryocytes	14 (70)
Normal BM smears	6 (30)
Splenomegaly	0
Hepatomegaly	0
Coombs test positive	0
Response therapy, number of patients (%)	
CR	9 (45)
PR	5 (25)
NR	6 (30)
Duration of thrombocytopenia, number of patients (%)	
<6 months	1 (5)
>6 months	19 (95)

RBCs: Red blood cells, WBCs: White blood cells, CR: Complete response, PR: Partial response, NR: No response, SD: Standard deviation, BM: Bone marrow, ITP, Immune thrombocytopenic purpura

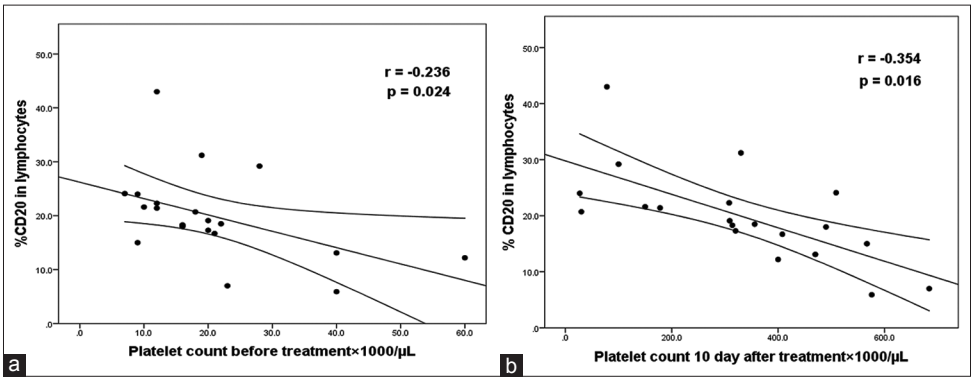

Figure 1: Correlation between CD20 expression levels and platelet counts at different times of follow-up in twenty patients. (a) Negative correlation between the percentage of CD20 expression and platelet counts before treatment. (b) Negative correlation between CD20 expressions and platelet counts 10 days after treatment

Similar to the results observed for CD19 percentage, the analysis did not reveal a significant difference between CR and NR patients concerning the percentages of CD25 cells (CR, mean, 4.86% ± 1.23%) (NR mean, 3.21% ± 2.81%) (P = 0.369) [Figure 2cC4].

Regarding the lymphocyte subsets, independent percentages of CD19, CD20, and CD25 expressions were assessed in PB of ITP patients. The frequency of independent CD markers' expressions in twenty patients is shown in Table 3. Since the follow-up of healthy controls was

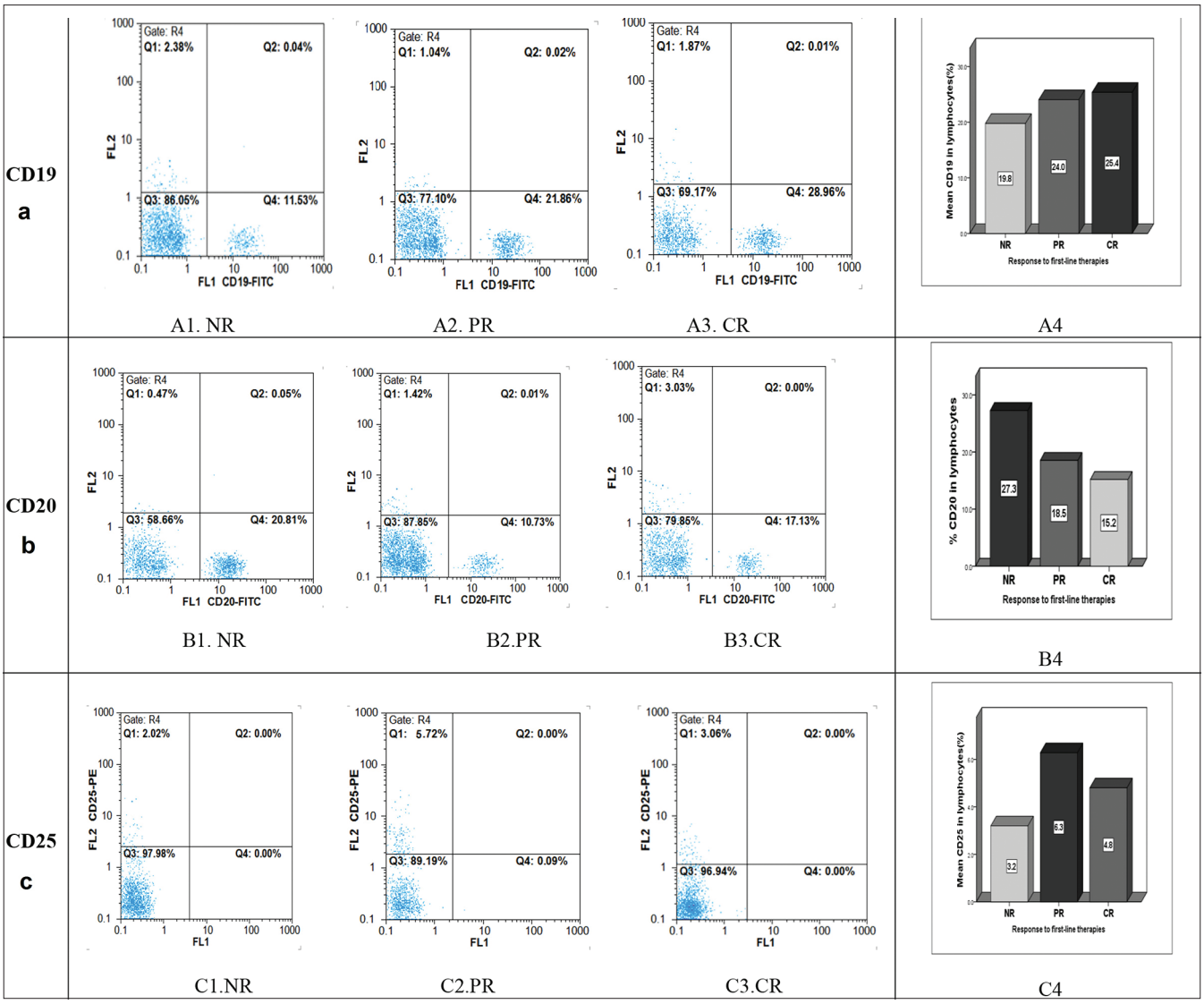

Figure 2: (a) (A1-A3) Representative dot plots of CD19 + cells in three patients with NR, PR, and CR. (A4) Lower expression percentage of CD19 in lymphocytes of NR (19.80 ± 3.24) with no significant difference (*P* = 0.993) compared with CR (25.41 ± 2.29) patients. (b) (B1-B3) Representative dot plots of CD20 + cells in three patients with NR, PR, and CR. (B4) Statistically significant expression of CD20 percentage in lymphocytes of NR (27.30 ± 8.70) compared with CR (15.20 ± 8.46) patients with significant difference (*P* = 0.015). (c) (C1-C3) Representative dot plots of CD25 + cells in three patients with NR, PR, and CR. (C4) CD25 expression in lymphocytes of NR patients is lower (3.21 ± 2.81) than CR (4.86 ± 1.23) with no significant difference (*P* = 0.369)

Table 2: Statistical analysis of correlation between CD markers' expression and hematological parameters

Parameters	Markers					
	CD19 in lymphocytes (%)		CD20 in lymphocytes (%)		CD25 in lymphocytes (%)	
	R	*P*	*R*	*P*	*R*	*P*
Platelet count before treatment	0.391	0.89	−0.236	0.024*	−0.236	0.316
PDW before treatment	0.078	0.742	0.212	0.370	0.401	0.079
MPV before treatment	−0.052	0.828	0.179	0.451	−0.175	0.460
WBCs before treatment	0.367	0.312	−0.133	0.576	−0.194	0.413
RBCs before treatment	0.359	0.120	−0.063	0.791	0.046	0.847
Hb before treatment	0.187	0.430	−0.069	0.772	0.067	0.779
Absolut count of lymphocyte before treatment	0.442	0.051	−0.167	0.483	−0.133	0.575
Platelet count 3 days after treatment	0.111	0.640	0.146	0.539	−0.240	0.307
Platelet count 10 days after treatment	0.135	0.569	−0.354	0.016*	−0.150	0.529
Platelet count 6 months after treatment	−0.135	0.571	0.072	0.762	0.047	0.845

*Significant correlation was found, *P* value calculated by Spearman's correlation test. PDW: Platelet distribution width, MPV: Mean platelet volume, WBCs: White blood cells, RBCs: Red blood cells, Hb: Hemoglobin

Table 3: The frequency and difference in mean platelet counts between the two groups with increased and decreased expressions of the studied CD markers

Independent CD marker expression in lymphocyte gate percentage, median±SD (range)	Difference in mean platelet counts between the two groups with increased and decreased expressions of the studied CD markers' Time of follow-up (mean±SD) Increased Decreased			
	Before treatment	3 days after treatment	10 days after treatment	6 months after treatment
CD19				
20.65±15.36 (13.3-84)	20.50±5.86	98.12±28.23	230.50±74.42	290±43.50
	22.11±19.91	90.44±19.91	388±99.55	280.88±23.49
P[a]	0.059	0.092	0.080	0.123
CD20				
18.80±8.30 (5.9-43)	22.66±12.92	90.66±66.44	297.11±50.90	289.88±10.18
	20.77±5.18	75±13.9	88.65±62.44	399.66±29.81
P[b]	0.905	0.638	0.002	0.014
CD25				
3.45±4.21 (1.8-21)	16±3.88	48.66±13.84	287.33±81.27	123.44±113.23
	22.66±15.27	109.33±75.76	327±178.53	309.50±21.86
P[c]	0.867	0.736	0.777	0.632

[a]Significant of difference of mean platelet counts between the two groups with increased and decreased expressions of CD19, [b]Significant difference of mean platelet counts between the two groups with increased and decreased expressions of CD20, [c]Significant difference of mean platelet counts between the two groups with increased and decreased expressions of CD. SD: Standard deviation

not possible, we analyzed the expressions of the studied markers in patients compared to healthy children in a study by Ikincioğullari *et al*.[17] Among the 20 evaluated patients, CD19 expression decreased in nine patients (45%) but increased in eight patients (40%), while it was normal in three patients (15%). Furthermore, CD25 expression decreased in nine patients (45%), increased in eight patients (40%), and was normal in three patients (15%). Nevertheless, there was no significant difference in mean platelet counts between the two groups with increased and decreased expressions of CD19 and CD25 at different times of follow-up [Table 3]. Moreover, CD20 expression decreased in seven patients (35%), increased in nine patients (45%), and was normal in four patients (20%). Interestingly, mean platelet counts were comparatively lower but without significant difference in the patients with increased CD20 expressions in comparison to patients with decreased CD20 expressions before and 3 days after treatment. On the other hand, 10 days after treatment, mean platelet counts in patients with increased CD20 expressions were higher than patients with decreased CD20 expressions with a significant difference. Furthermore, mean platelet counts in these patients were significantly higher than those with increased CD20 expression within 6 months after treatment [Table 3].

Eventually, after 6 months of follow-up, one of the patients showed progression toward chronic ITP; however, we did not observe any significant difference in the expression of the studied CD markers between the present study patients and those of other studies who did not progress toward chronic ITP. In addition, as shown in Figure 3, mean platelet counts in all patients were increased.

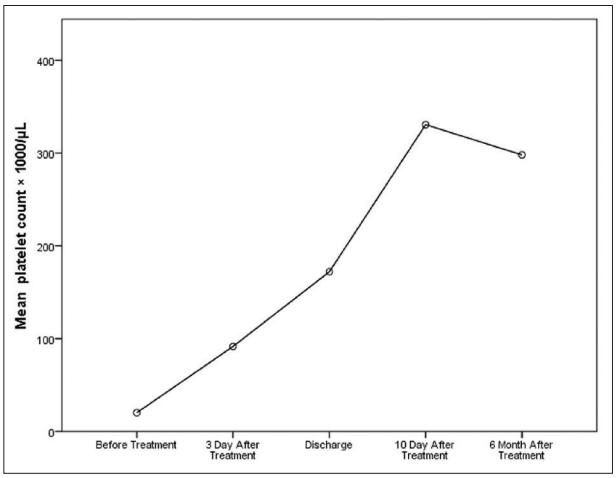

Figure 3: Platelet recovery in twenty patients with immune thrombocytopenic purpura within 6 months of follow-up

Discussion

ITP is a common autoimmune bleeding disorder characterized by the presence of autoantibodies against GPIIb/IIIa.[18] Multiple dysfunctions of immune system, genetic changes such as polymorphisms in immune system-related genes and aberrant expressions of microRNAs, chemokine receptors, and CD markers can play a vital role in the dysregulation of megakaryocytic maturation and decreased platelet counts in ITP.[19-23] As mentioned above, our data showed that there was a significant negative correlation between the percentage of CD20 expression and platelet counts before and 10 days after treatment. This result can be in line with Chen *et al*. study who showed that circulating B-cells secreting

anti-GPIIb/IIIa antibody were negatively associated with platelet counts in primary ITP.[14] Accordingly, negative correlation between CD20 expressions with platelet counts may have independent prognostic value for the percentage of autoreactive CD20+ B-lymphocytes producing antiplatelet autoantibodies in ITP patients. In contrast to CD20, no significant correlation was found between CD19 expressions with platelet counts at different times of follow-up, which was unexpected in our study since previous studies have reported that CD19 is a key marker of activated B-cells producing antiplatelet antibodies in ITP.[24] Similar to the results observed in CD19+ cells, we could not find any significant correlation between the expressions of CD25+ cells with platelet counts at different times of follow-up. This finding was not consistent with previous studies, indicating that the decrease in the percentage of CD4+CD25+ Tregs in patients with chronic and acute ITP is associated with the production of pathogenic autoantibodies in this disease.[25,26] Interestingly, similar to the correlations between CD20 expressions in lymphocytes with platelet counts before treatment, we found a negative significant correlation between total CD20 expression in whole blood cells and platelet counts before treatment [Figure 4a]. Nonetheless, no significant correlation was detected between total CD19 and CD25 expressions with platelet counts at different times of follow-up. With respect to response to first-line therapies, we found that CD20 expression was significantly higher in NR patients upon diagnosis of ITP. In contrast to this finding in our study, Zaja *et al*. demonstrated that there was no significant correlation between response to therapy and CD20+ lymphocyte counts in ITP patients receiving RTX.[27] Although few studies have investigated the impact of IVIG on CD20 expression in ITP, our hypothesis here is that since CD20+ B lymphocytes are ultimate producers of antiplatelet antibodies, increased CD20 expression in ITP is likely to indicate the extensive presence of this B-lymphocyte subset, which may be associated with increased platelet clearance, delayed platelet recovery, and unfavorable response to treatment. Since RTX has a crucial role in the depletion of B-cells, patients with an unfavorable response to IVIG may be candidates for treatment with RTX as second-line therapy. Moreover, in contrast to the higher frequency of CD20+ cells in NR patients, the frequency of CD19+ cells tended to be lower in NR patients compared to CR patients. Although we did not find significant differences in CD19+ cell expressions in CR compared with the NR group in our study, this result can contradict with Zhao *et al*. study who showed that CD19+ expression increased in responsive and nonresponsive ITP patients.[28] Furthermore, in contrast to our data, Lyu *et al*. reported that patients with ITP had a significantly higher frequency of naïve CD19+ and CD72+ B-cells compared with patients in remission.[29] Furthermore, we observed that CD25 expression was lower in NR than CR patients, although the difference was not statistically significant. This finding in our study may be in accordance with previous reports, indicating that ITP patients with active disease have a reduced percentage of CD4+CD25+ Treg cells.[26,30] Since previous studies have shown that the two cell subsets with CD19 and CD25 expressions play a critical role in ITP pathogenesis,[31] the imbalance between these subsets might result in impaired response to first-line therapies.

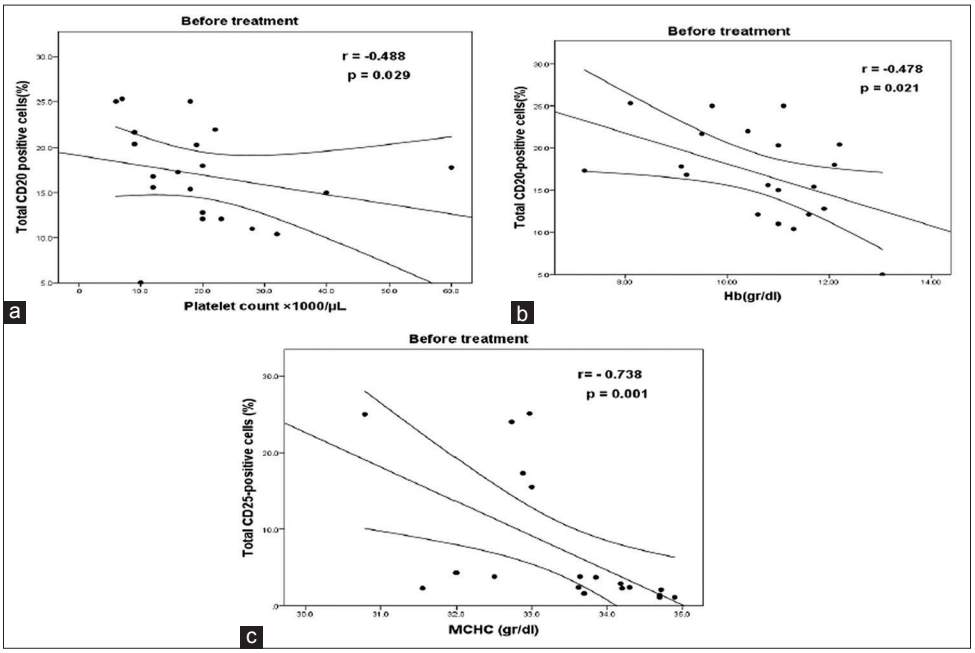

Figure 4: (a) Negative correlation between the percentage of total CD20-positive cells and platelet counts before treatment. (b) Negative correlation between the percentage of total CD20-positive cells and hemoglobin before treatment. (c) Negative correlation between the percentage of total CD25-positive cells and mean corpuscular hemoglobin concentrations before treatment

In addition, we analyzed the possible correlations between CD19, CD20, and CD25 positive lymphocytes with some hematological parameters such as WBC, RBC, PDW, MPV, Hb, and absolute lymphocyte counts before treatment. However, neither of these parameters showed a significant correlation with the studied markers. Interestingly, we found a negative correlation between total CD20 expressions with Hb concentrations, as well as between total CD25 expressions with mean corpuscular Hb concentrations (MCHC) before treatment [Figure 4b and c]. Although this result was quite unexpected for authors, it may be in accordance with the results of Fahim and Monir's study who reported a significant decrease in Hb in acute ITP due to bleeding as well as a significant increase in WBC and lymphocytes in this disease due to preceding viral infections.[32] However, it is unclear whether this correlation may be associated with the onset or severity of ITP. Thus, further studies are warranted to clarify these issues.

Conclusion

It can be concluded from this investigation that the high expression rate of CD20 may be associated with an unfavorable response to first-line therapies. However, we are aware that our research may have some limitations. First, it is a relatively small-scale study, which was designed to explore the possible correlation between CD markers' expressions and ITP prognosis. Second, the analysis of CD markers' expressions after treatment was not possible. Based on this limitation, we suggest that the assessment of CD markers' expressions only in the beginning of the diseases cannot be considered as a prognostic factor for chronicity of ITP. Therefore, further research with more patients is necessary for the detection of other factors involved in disease progression as well as assessment of CD markers' expressions during and after treatment or upon relapse in order to provide a reasonable answer to the prognostic value of CD markers in ITP.

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Conflicts of interest

There are no conflicts of interest.

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