Changing profile of platelet activity and turnover indices during treatment response of immune thrombocytopenia

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Abstract

Both platelet count and function change after treatment of immune thrombocytopenia (ITP). Platelet function can be measured by plasma markers, including platelet activity (e.g. soluble P-selectin [sP-selectin] and soluble CD40 ligand [sCD40L]) and platelet turnover markers (e.g. glycocalicin [GC]). Patients were classified into no response (NR, including new diagnosis), partial response (PR) and complete response (CR). One hundred and sixteen samples (29 CR, 32 PR, and 55 NR) from 79 patients were collected. Plasma markers (sP-selectin, sCD40L and GC) were measured by ELISA. Platelet counts and mean platelet volume (MPV) were obtained in the clinical laboratory using GenS System-2. The results showed that responsive patients (PR + CR) had higher levels of sP-selectin ($P = 0.026$) and sCD40L ($P = 0.001$). Although there was no difference in MPV ($P = 0.077$) or GC ($P = 0.078$), there was a marked decrease of GC index ($P < 0.001$) in responsive patients. Paired sample analysis showed no difference in sP-selectin, sCD40L, MPV or GC but significant difference in GC index ($P = 0.017$) between NR and PR. Another paired sample analysis showed no difference in sP-selectin, sCD40L, MPV or GC but significant difference in GC index ($P = 0.029$) between PR and CR. Patients with refractory and newly diagnosed disease had significant difference in GC ($P = 0.020$) and sCD40L ($P = 0.001$), despite similarly low platelet counts. In conclusion, platelet activity markers (sP-selectin and sCD40L) and GC indices change in parallel with treatment response. Plasma levels of GC and sCD40L may be predictors of treatment response.

Introduction

Severity of bleeding symptoms is highly variable among patients with immune thrombocytopenia (ITP). Such variability in bleeding manifestations may result from platelet functions. The classical platelet function assay, light transmission platelet aggregometry, cannot be reliably performed in ITP due to its relatively low platelet counts [1]. Alternative assays, such as impedance aggregometry [1], flow cytometry [2,3] and plasma markers of platelet activity (e.g. soluble P-selectin [sP-selectin] and soluble CD40 ligand[sCD40L]) may be used [4,5]. Platelet activities may change along with treatment response [5,6]. In addition to platelet activity, ITP is also characterized by high turnover of platelets. Platelet turnover can be directly measured by the radioisotope-labeling method [7,8]. It can also be indirectly measured by flow cytometry or plasma markers (e.g. glycocalicin [GC]) [9,10].

It is unclear whether change of platelet activity and turnover markers may predict change of platelet counts in ITP. Herein, we presented our data of evolutional profiles of plasma sP-selectin, sCD40L, and GC in various phases of ITP treatment.

Materials And Methods

Patients were screened in the outpatient clinic of hematology department, Chang Gung Memorial Hospital at Linkou. The diagnosis of ITP was made by exclusion of other etiologies of thrombocytopenia. Only patients requiring treatment were included in this study. Treatment response of ITP was classified

according to the international consensus criteria [11]. The categories included no response (NR, including new diagnosis), partial response (PR) and complete response (CR).

After the diagnosis of ITP was established, an informed consent was obtained from each patient. Blood samples were collected from antecubital veins into tubes containing EDTA. The sample tubes were centrifuged for 15 minutes at 1710 g. The supernatant was retrieved and cryopreserved at -70℃ until assays. Plasma levels of sP-selectin and sCD40L, GC, were measured by enzyme-linked immunosorbant assays (ELISA) using commercial kits according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA). A GC index was calculated by a formula [GC×250× 10^6/mL]/[individual platelet count], as described in previous literature [9] .Platelet counts and mean platelet volume (MPV) were obtained in the clinical laboratory using GenS System-2.

Statistical analyses were performed using GraphPad Prism version 7.00 for Windows (San Diego, California, USA). All data are expressed as mean ± standard error of the mean (SEM). The Student's t-test was used to determine statistical significance when two groups of data were compared. A paired t-test was used when results of paired samples were compared between NR, PR and between PR, CR. The difference was considered significantly different if P <0.05. This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Chang Gung Memorial Hospital.

Results

Comparison of ITP and bone marrow disease

One hundred and sixteen samples (29 CR, 32 PR, and 55 NR) from 79 patients were collected for this study. For control, 15 subjects with severe thrombocytopenia due to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) were recruited and samples collected in the same manner. To compare the platelet activity and turnover indexes, data of ITP patients with NR (N=55, including new diagnosis) were compared to patients with MDS/AML (N=15). The platelet counts were similar between ITP and MDS/AML groups (19.0±1.6×10^9 *vs* 11.0±4.1×10^9 per liter, P=0.980). Patients with ITP and patients with MDS/AML were not significantly different in sCD40L (127.0±29.2 *vs* 132.0±22.9 ng/mL, P=0.5332), MPV (9.20±0.27 *vs* 8.25±0.28 famtoliter, P=0.2613), GC (0.402±0.042 *vs* 0.167±0.102 ug/mL, P=0.5864) and GC index (19.000±1.661 *vs* 11.000±4.103, P=0.6179). However, in ITP patients, plasma levels of sP-selectin were significantly different from subjects with MDS and AML (24.87±1.20 *vs* 24.90±3.91 ng/mL, mean level 24.80 *vs* 31.80 ng/mL, P=0.0271). The results were presented in Fig. 1.

Comparison of new diagnosis and refractory disease

Patients with NR was subdivided into new diagnosis and refractory ITP patients. Platelet counts were different between these two groups (25.0±2.7×10^9 *vs* 15.6±1.8×10^9 per liter, P=0.004). Patients with new diagnosis of ITP have higher levels of sCD40L (127±56.7 *vs* 5.52±9.8 ng/mL, P=0.001) and lower levels of GC (0.150±0.053 *vs* 0.480±0.063 ug/mL, P=0.020) compared with patients with refractory ITP. Patients

with new diagnosis and refractory ITP were not different in blood levels of sP-selectin (29.2±2.2 *vs* 23.5±1.2 ng/mL, P=0.253), GC index (2.421±2.774 *vs* 7.881±1.777 , P=0.369) or MPV (9.35±0.46 *vs* 9.20±0.35 famtoliter, P=0.832). The results were presented in Fig. 2.

Comparison of patients with and without response

To characterize platelet activity and turnover indexes in different phases of ITP, patients were divided into no response (NR) and response (PR+CR) groups. The platelet counts were significantly lower in the no response group ($18.0\pm1.7\times10^9$ *vs* $95.0\pm13.7\times10^9$ per liter, P<0.0001). Patients with no response had lower levels of sP-selectin (24.9±1.23 *vs* 31.4±2.15 ng/mL, P=0.0008) and sCD40L (126.7±29.2 *vs* 238.2±84.9 ng/mL, P=0.0014) than those with PR or CR. There was a trend towards difference in platelet turnover markers MPV (9.0±0.27 *vs* 9.58±0.19 famtoliter, P=0.0771) and GC (0.404±0.418 *vs* 0.159±0.033 ug/mL, P=0.0784). After correction of GC by platelet counts, the GC index of the NR group was markedly higher (5.186±1.573 *vs* 0.430±0.122, P<0.0001), compared to patients with PR and CR. The data was presented in Fig. 3.

To evaluate the plasma platelet activity marker level contributed by each individual platelet on the average, we analyzed the sP-selectin/platelet count and sCD40L/platelet count ratios to evaluate the average platelet function of each individual platelet. The results showed both P-selectin/platelet count (1.36 ± 0.683 *vs* 0.24±0.029, P=0.0003) and sCD40L/platelet count ratios (6.45 ± 4.99 *vs* 1.82±0.631, P=0.0032) were significantly higher in patients with NR than patients with CR or PR.

The data was presented in Fig. 4.

Comparison between complete, partial and no response

The patients were divided into three groups (NR, PR, CR) and the platelet counts were different (NR: $18.0\pm1.7\times10^9$,PR: $66.0\pm2.5\times10^9$,CR: $201.0\pm20.6\times10^9$per liter, P<0.001). In comparing platelet activity markers, the 3 groups of patients had significantly different levels of sP-selectin (NR: 24.9±1.23, PR: 26.0±2.87, CR: 35.6.0±3.1 ng/mL, P=0.002) and sCD40L (NR: 126.7±29.2, PR: 123.4±66.5, CR: 469.8±151.4 ng/mL, P<0.0001). In comparison of platelet turnover markers, the 3 groups of patients were not significantly different in plasma GC levels (NR: 0.403±0.042, PR: 0.191±0.050, CR 0.157±0.044 ug/mL, P=0.2399) and MPV (NR: 9.2±0.27, PR: 9.6±0.27, CR 9.05±0.25 ng/mL, P=0.1675). After correction of platelet count, the GC indexes were significantly different among the 3 groups (NR: 5.186±1.573, PR: 0.698±0.203, CR 0.241±0.057, P<0.0001).

The data was presented in Fig. 5.

The P-selectin/platelet count ratio (NR: 1.36±0.68, PR: 0.39±0.04, CR 0.17±0.014, P<0.0001) were significantly different among NR, PR and CR patients. However, for the sCD40L/platelet count ratio, despite the significant overall difference (NR: 6.45±4.99, PR: 1.69±1.10, CR 2.50±0.56, P<0.0001) and

difference between NR and PR (P<0.001), there was no significant difference between patients with PR and CR. The data were presented in Fig. 6.

Analysis of paired samples

Thirteen patients had paired samples in NR and PR. Paired analysis showed no difference in sP-selectin (26.15±4.48 *vs* 26.75±3.81 ng/mL, P=0.234), sCD40L (121.4±47.9 *vs* 159.8±125.1 ng/mL, P=0.081), MPV (10.1±0.59 *vs* 9.8±0.32 famtoliter, P=0.256) or GC (0.148±0.118 *vs* 0.554±0.118 ug/mL, P=0.091) but significant difference in GC index (4.957±1.407 *vs* 1.676±0.529, P=0.017) (Fig. 7). Twelve patients had paired samples in PR and CR. Analysis showed no difference in sP-selectin (28.09±2.39 *vs* 28.67±4.14 ng/mL, P=0.234), sCD40L (258.6±99.8 *vs* 476.0±326.7 ng/mL, P=0.071), MPV (9.55±0.45 *vs* 9.00±0.39 famtoliter, P=0.148) or GC (1.005±0.146 *vs* 1.019±0.119 ng/mL, P=0.230) but significant difference in GC index (3.036±0.601 *vs* 1.717±0.217, P=0.037) (Fig. 7).

Discussion

ITP is characterized by increased platelet activity and rapid platelet turnover. Platelet activities can be measured by aggregation tests [12,1], flow cytometry [13-15] or representative plasma markers [4]. Platelet turnover can be measured by kinetic studies [9], flow cytometry [16,17] or representative plasma markers [18-20]. Compared to other laboratory methods, ELISA measurement of plasma markers is an easy, less labor-intensive and relatively standardized method.

The clinical relevance of platelet function in ITP has been well demonstrated. Most of such studies reported platelet function is correlated with bleeding manifestations and severity. The platelet function in the majority of such studies was measured by flow cytometry-based assays [13,21,14]. Clinical utility of plasma markers related to platelet activities, on the other hand, has been rarely reported. In some studies, platelet related plasma markers were used to distinguish ITP from hypoplastic etiologies of thrombocytopenia (e.g. acute leukemia, MDS and aplastic anemia) and thereby providing diagnostic values [4,5,22]. Such distinction was also found in the present study, which showed significantly different sP-selectin plasma levels between ITP and MDS/AML while their platelet counts were comparable. Despite the clear and reproducible difference of platelet function between ITP and hypoplastic thrombocytopenia, application of such assays appears to be limited in clinical medicine. The plasma marker of choice, the standard method of measurement and the optimal cutoff levels have not been well defined. On the other hand, such distinction by plasma markers is less important when bone marrow aspirations or biopsies are done and the morphological diagnosis is made definitively.

In addition to the utility in clinical differential diagnosis at the initial phase, the dynamic change of platelet function markers is interesting but relatively under-investigated. In general, ITP is characterized by high platelet activities (represented by sP-selectin) and increased turnover rates (represented by GC, reticulated platelets or immature platelet fractions) at initial presentation [4]. In previous studies, abnormalities of platelet function were reversed or normalized after treatment [23]. While such

normalization of platelet function seems to be reasonable in pathophysiology, persistent abnormality which is independent of platelet counts, has been reported [24].

In the present study, we performed multiple analyses to explore the change of platelet function during various stages of clinical course. In platelet activity markers, both sP-selectin and sCD40L increased after treatment response. The difference is significant in analysis of responder *vs*. non-responder and analysis of NR *vs*. PR *vs*. CR.

While it is certain that levels of platelet activity markers change with treatment response, the exact timing of change is not clear. If change of platelet activity markers precedes the change of platelet counts, such platelet activity markers may predict platelet count response in potential. The frequency of monitoring in the present study was insufficient to address this issue. However, in comparison of ITP patients with new diagnosis and refractory disease, we found significantly lower levels of sCD40L in patients with refractory disease. It suggests patients with refractory ITP and patients which later on responded to treatment may have different characteristics. In other words, a higher plasma level of sCD40L may predict treatment response. In literature, the significance of sCD40L had been reported. Nagahama et al. found that sCD40L levels were significantly higher in untreated ITP than the control group, the non-immune thrombocytopenia patients and the treated ITP patients [25]. However, to our knowledge, difference of sCD40L between refractory and newly diagnosed ITP is a novel finding that has not been reported before. The predictive power of sCD40L should be investigated in further prospective, large-scale studies. It should be noted that not all sCD40L is derived from platelets. Human T cells are a rich source of sCD40L and indeed, T cells are involved in the pathogenesis of ITP [26,25]. In fact, it had been shown that anti-CD40L antibody is a potential therapeutic agent for ITP [27]. As refractory and newly diagnosed ITP were distinguished by sCD40L but not sP-selectin, the possibility of immune mechanism, not platelet activity, should be considered.

Although platelet turnover markers are less frequently associated with bleeding symptoms, some studies suggested some markers, such as immature platelet fraction, is associated with bleeding risk [28]. On the other hand, platelet turnover markers are clinical relevant in their diagnostic value. For example, platelet kinetic studies revealed underproduction and relatively short half-lives of platelets in patients with ITP [7,29,8]. The unique features of immature platelet fractions and reticulated platelets measured by flow cytometry have been well demonstrated in ITP [23,17]. In addition, platelet turnover can be indirectly assessed by MPV [19]. GC is a fragment of platelet glycoprotein Ib which is shed into plasma after cleavage of the platelet membrane protein. It is a common method of measuring platelet turnover [10]. It was found plasma GC levels were correlated with MPV in previous studies, suggesting both markers can be used to evaluate platelet turnover [19]. In practice, GC level is often corrected by platelet counts. GC index, therefore, is more commonly used in clinical correlation. Barsam et al. had shown the difference of GC and GC index in ITP as opposed to controls. Such a difference is ameliorated at the time of platelet response of ITP treatment [23]. Such a phenomenon illustrated the high platelet turnover in ITP and the change of turnover during response to treatment.

In comparison of ITP and MDS/AML with thrombocytopenia, none of the platelet turnover markers (GC, GC index and MPV) was significantly different. On the other hand, in comparison of various groups divided according to disease status and treatment response, GC index explicitly distinguishes patients with and without response. In further analysis of paired samples, GC index again showed explicit difference between NR and PR and patients, as well as between PR and CR patients. Such findings suggested ITP patients with new diagnosis or refractory disease had high platelet turnover, as indicated by GC index. Such high turnover was ameliorated after treatment response.

Further comparison between new diagnosis and refractory ITP showed the GC index was not significantly different between the two groups. The difference, however, can be observed for the uncorrected blood GC levels. Whereas a plausible explanation of such disparity is difficult, the distinguished blood GC level may suggest a difference in the biological nature, especially platelet turnover, of these two groups of patients, with higher GC levels among the refractory disease patients. As a result, plasma GC level may be useful in predicting treatment response or outcome of ITP patients.

In summary, we have characterized the profile of platelet activity and turnover markers in patients with ITP. The sP-selectin levels are different from MDS/AML. Levels of sP-selectin, sCD40L and particularly GC indices, change with treatment response. Plasma levels of sCD40L and GC are difference between newly diagnosed and refractory ITP patients. It suggests sCD40L and GC may be useful in predicting treatment response of ITP.

Declarations

Funding

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Conflicts of interest/Competing interests

All authors declared they have no conflict of interest.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

Chewei Ou and Hung Chang designed the study and wrote this manuscript. Yu-Shin Hung, Ming-Chung Kuo and Tung-Liang Lin collected patient information and revised the manuscript. Pei-Ling Li conducted the experiment and analyzed the data. All authors reviewed and approved the contents of the manuscript.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Chang Gung Memorial Hospital.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Patients signed informed consent regarding publishing their data.

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Figures

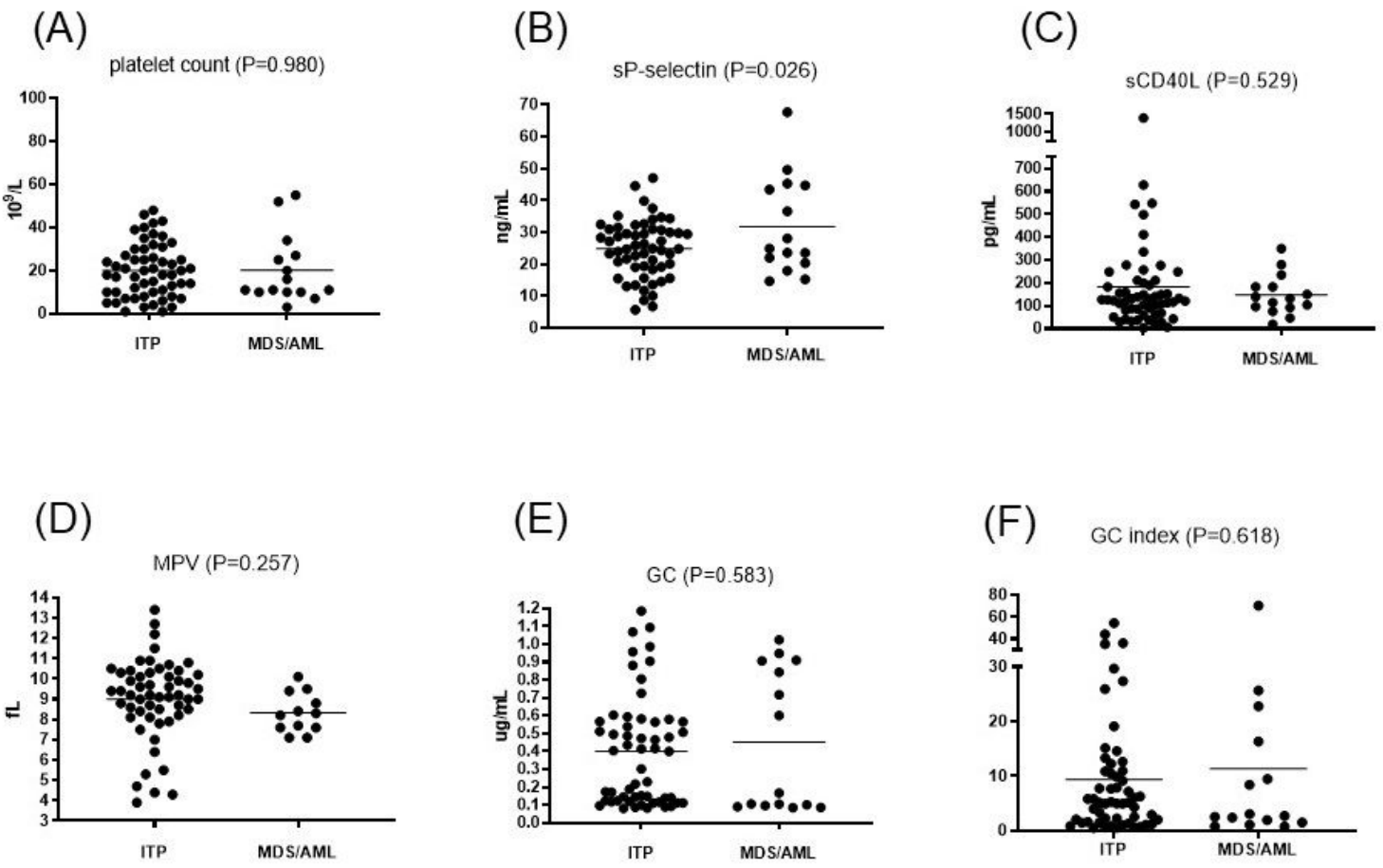

Figure 1

Comparison of platelet count, platelet activity and turnover markers between ITP and MDS/AML. The platelet counts were comparable (A). Plasma sP-selectin was significantly different between ITP and AML/MDS (B). However, no significant difference was found in plasma levels of sCD40L (C), MPV (D), GC(E), or GC index (F).

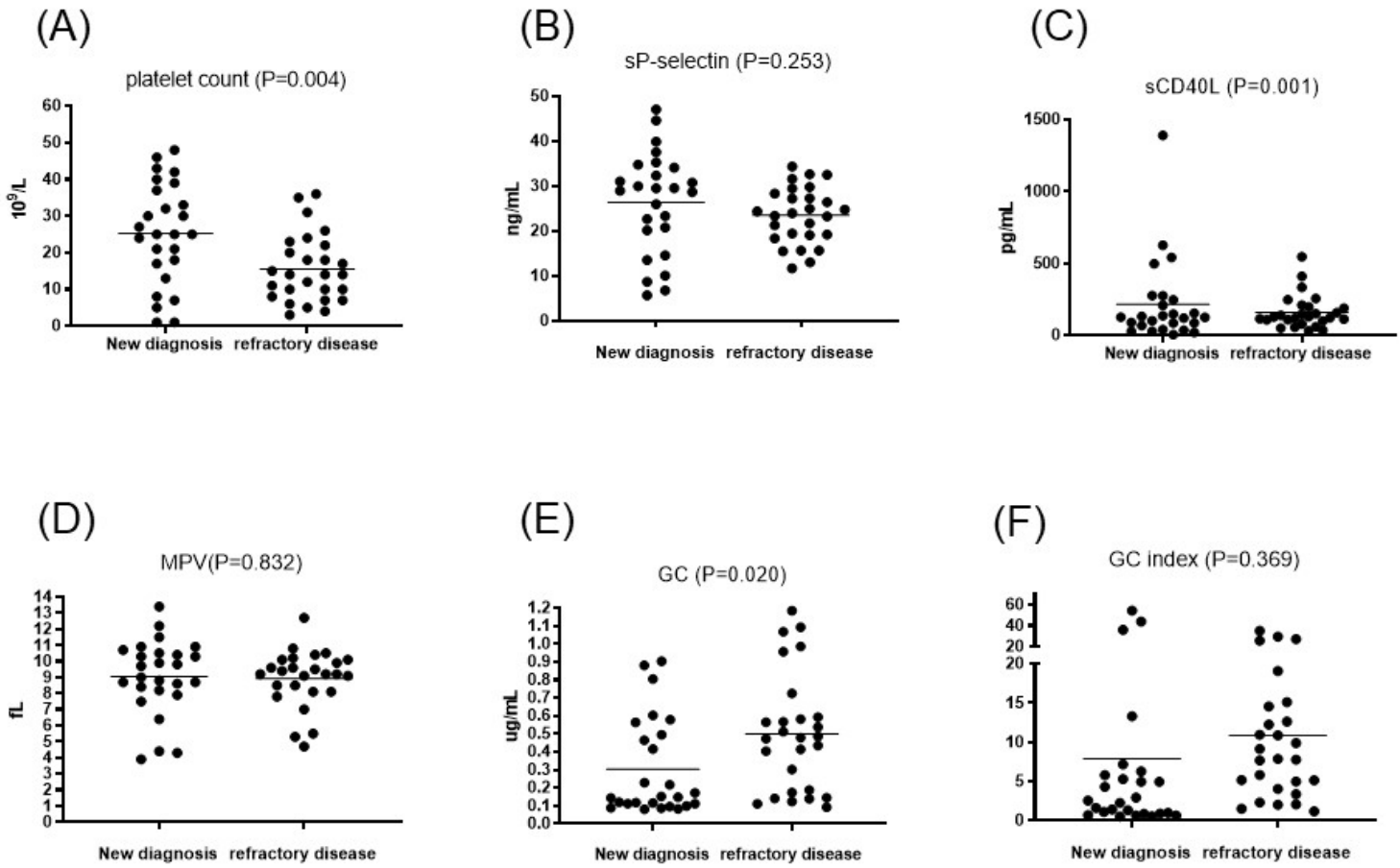

Figure 2

Comparison of platelet count, platelet activity and turnover markers between ITP at the time of new diagnosis and refractory disease. The platelet count was lower with refractory disease (A). Plasma sP-selectin levels were not significantly different (B). Levels of sCD40L were significantly lower in refractory disease (C). There was no difference in MPV (D). Plasma levels of GC were higher in refractory disease (E), and the GC indexes were not different (F).

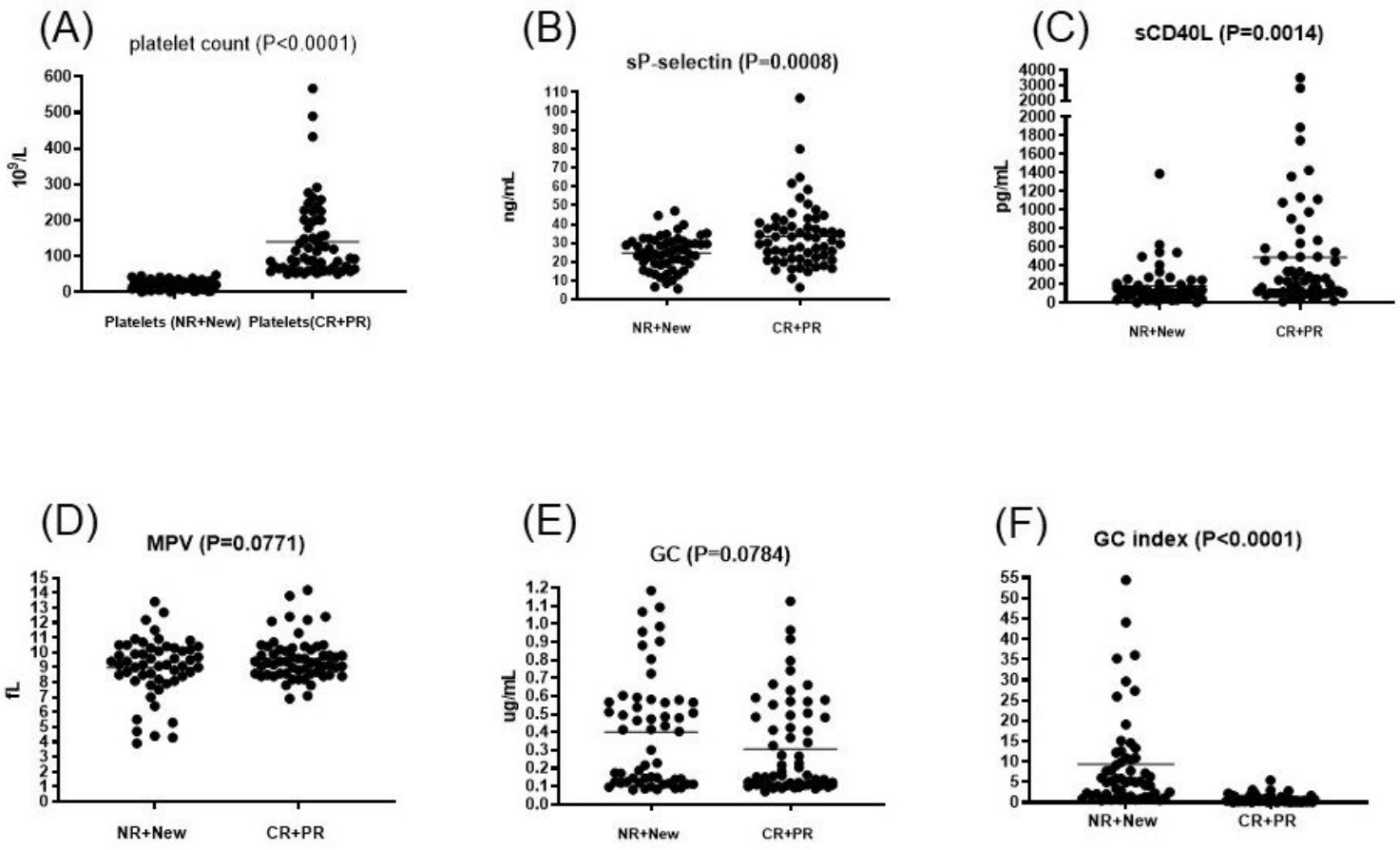

Figure 3

Platelet count, platelet activity and turnover markers of ITP patients at new diagnosis and refractory disease vs. patients with response to treatment. The platelet counts were significantly different (A). Both activity markers, sP-selectin and sCD40L were significantly different (B and C). There was no difference in MPV or plasma GC levels (D and E). The GC index was significantly lower after treatment response (F).

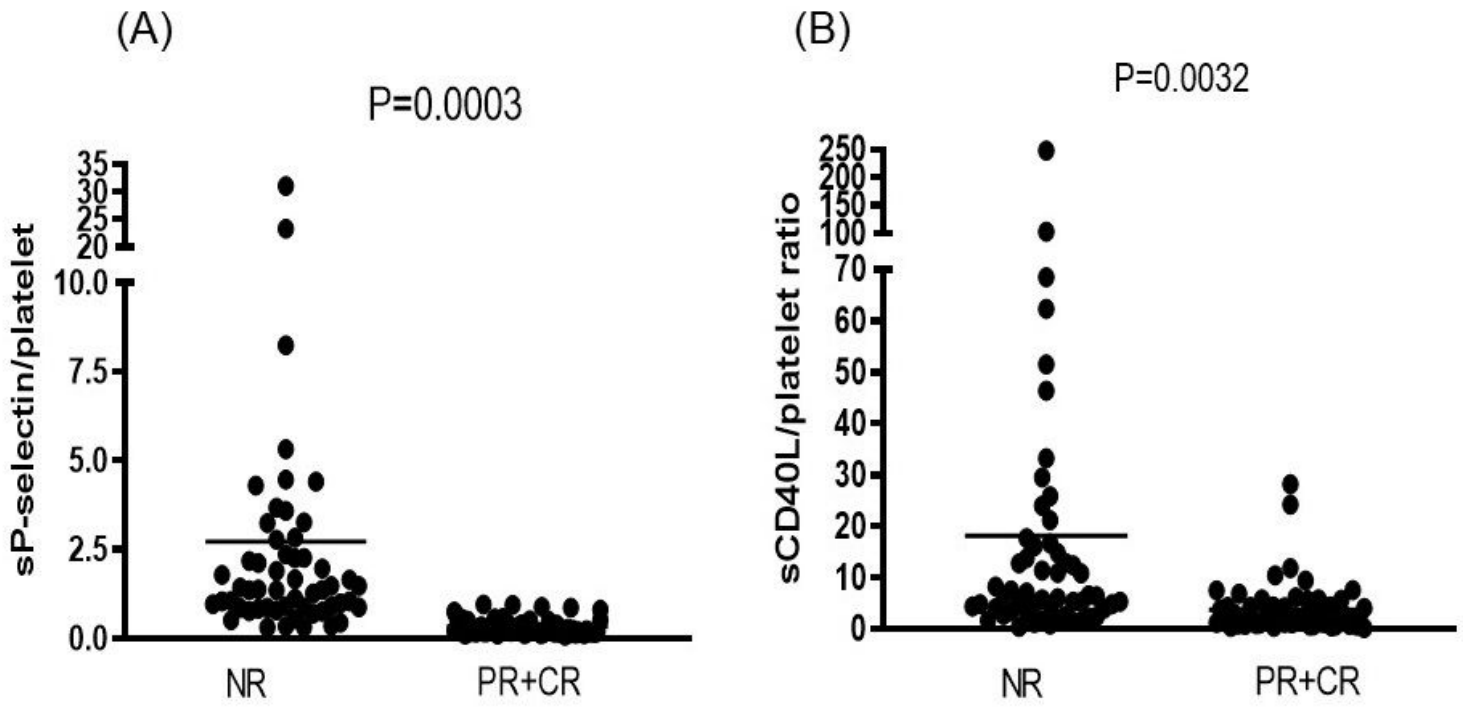

Figure 4

Platelet activity markers, sP-selectin and sCD40L were corrected by the platelet count. Both sP-selectin/platelet count (A) and sCD40L/platelet count (B) ratios were significantly different between patients with new diagnosis (NR) and patients with response to treatment (CR+PR).

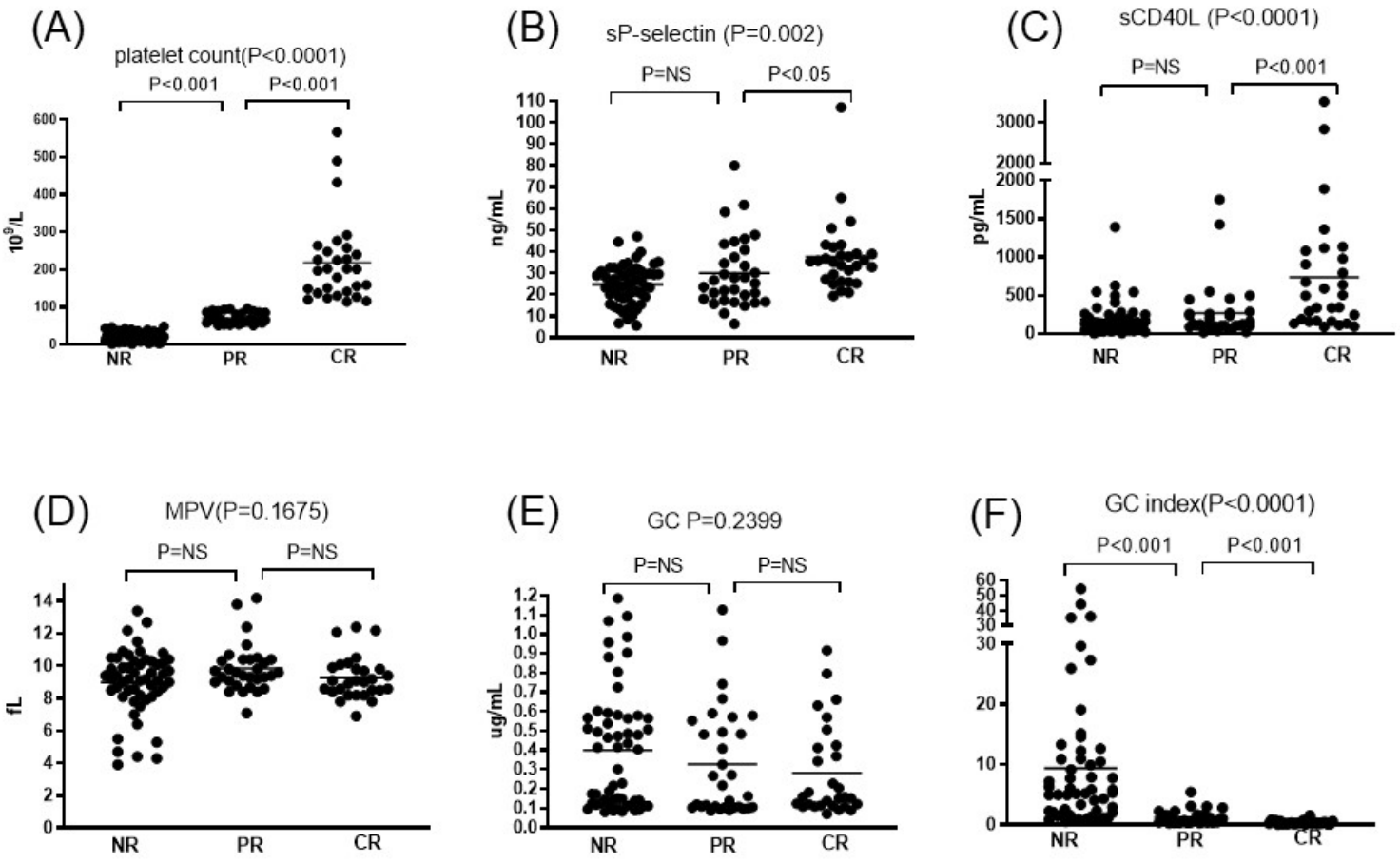

Figure 5

Comparison of platelet count, platelet activity and turnover markers among patient samples in NR, PR and CR. (A). Platelet counts were different among NR, PR, and CR. (B-C). Plasma sP-selectin and sCD40L levels were different among NR, PR, and CR with the main difference in the CR group. (D-E). MPV and GC levels were not significantly different among NR, PR, and CR. (F). Significant difference of GC index among NR, PR, and CR. The difference was also significant between NR and PR and between PR and CR.

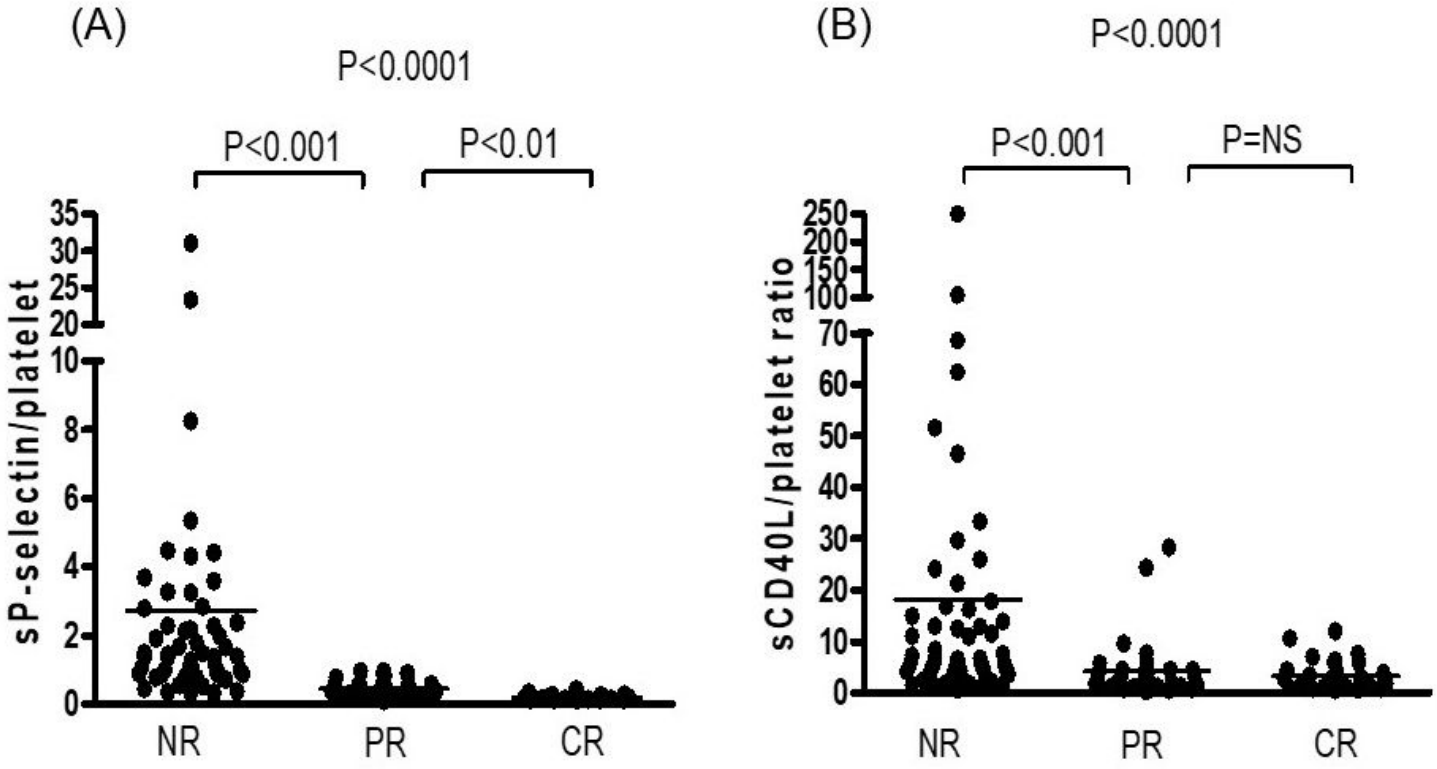

Figure 6

Platelet activity markers, sP-selectin and sCD40L were corrected by the platelet count and compared among NR, PR and CR. (A) sP-selectin/platelet count ratios were significantly different among NR, PR and CR. The difference was also significant between NR, PR and between PR, CR. (B) sCD40L/platelet count ratios were significantly different between NR and PR but not significantly different between PR and CR.

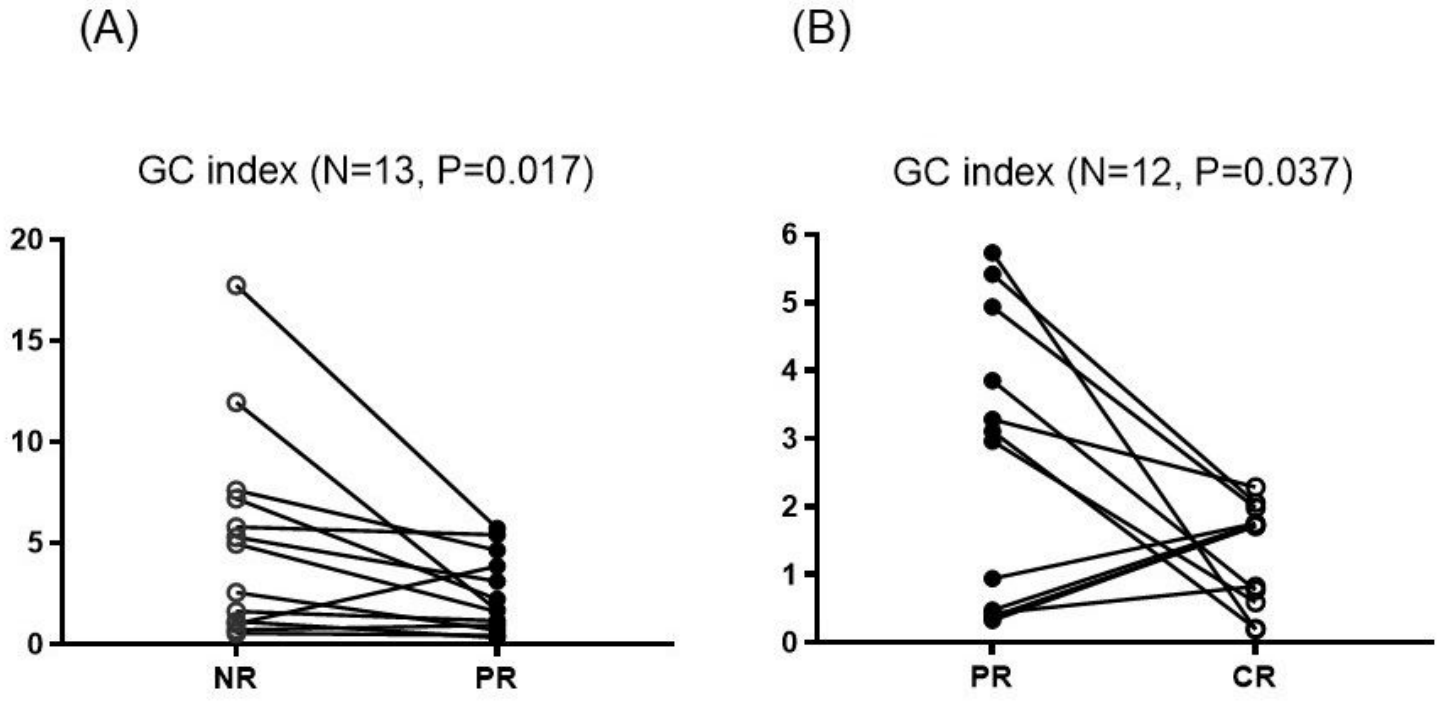

Figure 7

Samples paired for NR vs PR and for PR vs CR were compared to compare the change of GC and GC index during different phases of disease. (A) significantly lower GC during PR compared to NR. (B) significantly lower GC index during CR compared to PR.