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Reticulated Platelets in Egyptian Patients with Chronic Hepatitis C Infection

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ABSTRACT

Background: *Thrombocytopenia, a frequent problem in patients with chronic hepatitis C, occurs due to several factors. The aim of this study is to evaluate reticulated platelets percent (RP%) in patients with chronic hepatitis C as an indicator of platelet production.*

Method: *Reticulated platelets were analyzed with flow cytometry using whole-blood, which is dual-labelled with monoclonal anti-glycoprotein (GP)-III antibody (CD61) for platelet identification and thiazole orange as platelet mRNA stain.*

Results: *Forty four patients with chronic hepatitis C were enrolled in this study, 32 of them were thrombocytopenic and 12 were non-thrombocytopenic. The median age was 50 years (range: 23 – 67 years), 59.1% were females and 40.9% were males. The RP% was significantly higher in thrombocytopenic than non-thrombocytopenic patients (P < 0.001). Highly significant inverse correlation was found between RP% and platelet count. Furthermore, Platelet count correlated directly with serum albumin levels and inversely with each of INR values and serum ALT levels. About 96.9% of the thrombocytopenic patients had splenomegaly, while non-thrombocytopenic patients had normal size of the spleen.*

Conclusion: *Our data demonstrates that measurement of RP% could be useful screening test in thrombocytopenic patients with chronic hepatitis C to evaluate platelet production.*

Keywords: *Egyptian, HCV, Low platelet count, Reticulated Platelet.*

1. INTRODUCTION

Hepatitis C virus (HCV) currently infects nearly 2% of the world's population [1], while it constitutes an epidemic in Egypt with the highest prevalence in the world amounting to 14-20% [2, 3]. Among the wide range of hematological alterations that affect the patients with chronic hepatitis C, thrombocytopenia is likely the most common [4-7]. In addition to the higher possibility of bleeding, thrombocytopenia has both diagnostic and prognostic impact [8,9]; moreover, it influences the management decisions such as interferon (IFN) therapy [10].

In fact, the etiology of HCV associated thrombocytopenia is multi-factorial, where both peripheral and central causes are implicated in the pathogenesis [11]. Platelet (PLT) sequestration with hypersplenism and immune-mediated platelet destruction due to antiplatelet autoantibodies or immune complexes are eventually the postulated peripheral causes, while the central causes include decreased platelet production within the bone marrow either due to reduced thrombopoietin (TPO) production by the liver, or direct viral suppression on megakaryocytes. Other likely cause of

thrombocytopenia in HCV patients is IFN therapy, which may represent an obstacle to antiviral treatment in some patients. Therefore, evaluating the possible mechanisms of thrombocytopenia that differ from one patient to another allows better management of HCV patients [12].

Reticulated platelets (RP), first described in 1969 [13], are the most immature platelets circulating in peripheral circulation. The RP contain residual messenger RNA (mRNA), they are functionally active and are equivalent to the reticulocytes of erythropoiesis. The RP are non-invasive indicator of recent megakaryopoiesis in the bone marrow, therefore, may be used to determine whether thrombocytopenia is due to decreased production or peripheral destruction of platelets [14].

Flow cytometric analysis had been considered the preferred method of measuring RP [14,15]. Thiazole orange (TO), a nucleic acid dye, [16-18] remains a good choice for RP staining [15]. However, there is an evident lack of analytical standardization as there is a significant variability in the various published protocols regarding the incubation time, the used substrate (whole blood or platelet-rich plasma), concentrations of the fluorescent dyes, and the use of a second antibody marker to distinguish platelets, which yielded much variation in the published reference intervals of reticulated platelets percent (RP%) [19]. Even so, numerous studies have suggested that analysis of RP represents a valuable estimate of the rate of platelet production in bone marrow [17, 19, 20- 24].

Automatic detection of reticulated platelets is available on some cell counters such as Sysmex and Abbott hematology analyzers, in which RP are expressed as an immature platelet fraction (IPF). However, the methods are essentially different and cannot be used interchangeably leading to slow adoption of the assay by physicians [25- 28].

The aim of our study was to measure RP% by flow cytometry as an indicator of platelet production in patients with chronic hepatitis C and to evaluate the relationship between the RP% and the platelet count in those patients.

2. PATIENTS AND METHOD

2.1 Patients

This study was approved by the local ethical committee. Forty-four patients with chronic hepatitis C attending to Ain Shams University Hospitals had been enrolled in this study. The diagnosis of HCV infection was proved by HCV PCR assays. Fully informed consent was obtained from each patient. All the patients underwent full history taking including current medications, thorough clinical examination laying stress on splenomegaly along with imaging modalities such as abdominal ultrasound or CT scans when available. All medications taken by the patient at the time of sample collection were reviewed in determining eligibility. Medications that prompted exclusion were interferon, ribavirin, warfarin and any drug known to affect platelet count within six months of the time of venipuncture.

2.2 Method

a. Sample collection

Venous blood samples were collected into K3 EDTA (tripotassium ethylene diamine tetraacetate) vacutainer tubes (final concentration of 1.5 mg/mL) to obtain complete blood count (CBC) and flow cytometric assays of reticulated platelets. Also peripheral blood (PB) was collected on citrate vacutainer tubes and was used for coagulation profile including the international normalization ratio (INR). Two mL of PB were collected and used for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin measurements. The CBC was analyzed using Coulter LH750 cell counter (Coulter, Electronics, Hialeah, FL, USA) within 2 hours of collection. Thrombocytopenic samples were confirmed after review of Leishman stained PB smears. Coagulation profile was measured using Stago coagulation analyzer (STA compact). Serum levels of ALT, AST and albumin were measured on Synchron CX-9 autoanalyser (Beckman Instruments Inc.; Scientific Instruments Division, FL, USA).

b. Flow cytometric detection of reticulated platelets

Reticulated platelets were analyzed within 2 hours of collection and were identified following the previously described technique by Pons et al. [19] without sample manipulation, avoiding fixation and blood centrifugation. Double staining with thiazole orange for mRNA and CD61 Per CP was done for platelet identification. Such dual-color staining of unseparated whole blood avoids uncontrolled loss of platelet which may occur during preparation of platelet rich plasma by centrifugation and also helps for reliable platelet identification [29]. Briefly, two tubes were prepared for each sample, one was the test tube and the other was the control tube. The test tube prepared as follows: 5 µL of whole blood were added to 5 µL of PerCP®-labeled antiglycoprotein IIIa monoclonal antibody (CD61 PerCP® Becton Dickinson, San Jose, CA, USA) and 30 µL of phosphate-buffered saline, then incubated in the dark for 15 min at room temperature. The control tube was prepared with 5 µL of isotypic mouse control (IgG1-mouse PerCP® Becton Dickinson, San Jose, CA, USA). Then, 1000 µL thiazol orange (TO; Retic-count®, Becton Dickinson, San Jose, CA, USA) at 1 / 10 dilution in Isoton II® (Beckman-Coulter, Miami, FL, USA) was added to the test tube and 1000 µL Isoton II® (Beckman-Coulter, Miami, FL, USA) solution was added to the control tube. Incubation in the dark for 60 min at room temperature was done after which, they were immediately analyzed using with Navios flow cytometer (Beckman- Coulter, Miami, FL, USA). Platelets were identified by their logarithmic side scatter (SSC) and CD61 positivity expression. A dot plot cytogram of TO versus CD61 was generated and platelets were identified as CD61 positive excluding red blood cells which are CD61 negative. The RP rate was expressed as a percentage of TO and CD61-PerCP® double-positive cells in ten thousand identified platelets. The threshold of TO fluorescence was established; the level was more than 99% of CD61 PerCP® positive population was negative for TO in the control tube. A control with normal platelet count was tested in each assay.

Results of hepatitis C viral load (obtained within 3 months of study venipuncture) were recorded and expressed as a logarithm.

c. Statistical Analysis

Data analysis was conducted using the Statistical Package for Social Science (IBM SPSS) version 20 software. All descriptive qualitative data was presented as number and percentage. Comparisons for categorical data were performed using chi-square or Fisher exact tests, as appropriate. All descriptive quantitative data was presented as average (mean or median) and variation {standard deviation (SD) or interquartile range (IQR)}. Comparisons for quantitative data were performed using unpaired student t-test or Mann-Whitney test. Spearman correlation coefficients were used to determine the inter-correlations between parameters. P value $\leq$ 0.05 was considered statistically significant.

3. RESULTS

This study recruited 44 patients suffering from chronic hepatitis C viral infection, 26 (59.1%) were females and 18 (40.9%) were males. The ages ranged between 23 and 67 years with median age of 50 years. The biochemical and clinical parameters of the studied patients are shown in Table 1.

Table 1: The laboratory and clinical findings of the study patients

		Total no. = 44
WBC (×109/L)	Median (IQR) Range	7.15 (4 – 8.6) 1.1 – 22.4
Hb (g/dL)	Mean ± SD Range	11.00 ± 2.56 4.5 – 15.8
PLT (×109/L)	Mean ± SD Range	105.34 ± 83.36 20 – 325
ALT (IU/L)	Median (IQR) Range	6.75 (3.3 – 10.1) 10 – 101
AST (IU/L)	Median (IQR) Range	25 (14.5 – 54) 13 – 151
Albumin (g/dL)	Median (IQR) Range	22 (16 – 62) 1 – 4.6
INR	Median (IQR) Range	1.5 (1.1 – 2) 1 – 5.8
RP (%)	Mean ± SD Range	13.38 ±14.86 1.54 – 66.7
Spleen size	Enlarged Normal	31 (70.5%) 13 (29.5%)

IQR: Interquartile range; WBC: White blood cell count; Hb: Hemoglobin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalization ratio; RP: Reticulated platelets

Among the studied patients, 32 were thrombocytopenic (PLT < 150 x 109/L) while 12 were non-thrombocytopenic (PLT ≥ 150 x 109/L). Comparison between both groups revealed statistically significant differences as regards the median WBC count and mean serum albumin levels (P < 0.05). Moreover, highly significant differences were found regarding the median RP% (Figure 1), the median INR values and spleen size (P < 0.001). However, the log hepatitis C viral load was not significantly different between the thrombocyt-openic and non-thrombocytopenic groups (Table 2). Patients with splenomegaly had median RP% of 12.5% versus 3.7% in those with normal spleen size (P < 0.001).

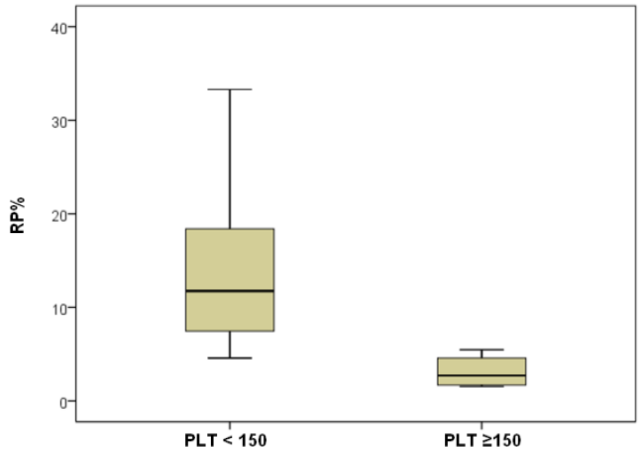


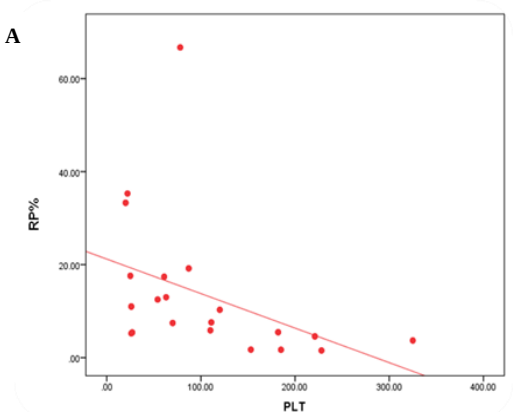
Figure 1: Reticulated platelets percent (RP%) in thrombocytopenic [platelet count (PLT) < 150 x 109/L] and non thrombocytopenic patients (PLT ≥ 150 x 109/L).

Table 2: Comparison between thrombocytopenic and non-thrombocytopenic patients as regards the different studied parameters

		Thrombocytopenic group (PLT< 150 x 109/L)	Non-thrombocytopenic group (PLT ≥ 150 x 109/L)	Z/X² */t●	P-value
		n = 32	n = 12		
Age (years)	Median (IQR) Range	5 0 (47 – 55) 23 – 67	54 (28 – 66) 23 – 67	-0.476	0.634
Gender	Female Male	18 (56.3%) 14 (43.8%)	8 (66.7%) 4 (33.3%)	0.392 *	0.531
WBC (×109/L)	Median (IQR) Range	4.7 (3.85 – 7.9) 1.2 – 22.4	9.1 (8.1 – 10.2) 1.1 – 21.4	-2.402	0.016
Hb (g/dL)	Mean ± SD Range	10.87 ± 2.74 4.5 – 15.8	11.35 ± 2.07 8.9 – 14.6	0.547 ●	0.587
ALT (IU/L)	Median (IQR) Range	26 (16 – 50) 10 – 101	15.5 (11 – 70) 10 – 78	-1.346	
AST (IU/L)	Median (IQR) Range	31 (19 – 56) 13 – 150	16 (15 – 68) 14 – 151	-1.188	0.235
Albumin (g/dL)	Mean ± SD Range	2.49 ± 0.95 1 – 4.6	3.20 ± 0.74 2.2 – 4.2	2.311 ●	0.026
INR	Median (IQR) Range	1.6 (1.3 – 2.5) 1 – 5.8	1.1 (1 – 1.2) 1 – 1.83	-3.522	<0.001
RP (%)	Median (IQR) Range	11.75 (7.46 – 18.4) 4.58 – 66.7	2.72 (1.7 – 4.58) 1.54 – 5.48	-4.774	<0.001
Spleen size	Enlarged Normal	31 (96.9%) 1 (3.1%)	0 (0.0%) 12 (100.0%)	39.346*	<0.001
Log viral load	Mean ± SD Range	4.73 ± 2.19 1.7 – 6.7	3.65 ± 2.21 1.7 – 6.7	1.446 ●	0.155

*: Independent t-test
•: Chi-square test
Z: Mann-Whitney test; X2: Chi-square test; t: Student t test; IQR: Interquartile range; WBC: white blood cell count; Hb: Hemoglobin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalization ratio; RP: Reticulated platelets

The platelet count showed a significant inverse correlations with each of RP % (r = – 0.698; P < 0.001) (Figure 2A), serum ALT levels (r = – 0.390; P = 0.009) and INR values (r = – 0.694; P < 0.001). The platelet count also showed a significant direct correlation with serum albumin levels (r = 0.579; P < 0.001). While RP% correlated directly with each of INR values (r = 0.762; P < 0.001) (Figure 2B) and WBC (r = 0.329; P = 0.029) (Figure 2C). Significant inverse correlation was found between RP% and the Hb levels (r = – 0.433; P = 0.003) (Figure 2D). Log viral load showed a significant positive correlation with each of serum ALT levels (r = 0.514; P < 0.001), serum AST levels (r = 0.432; P = 0.003) and INR values (r = 0.326; P = 0.031), however, there was no correlation between log viral load and RP%.



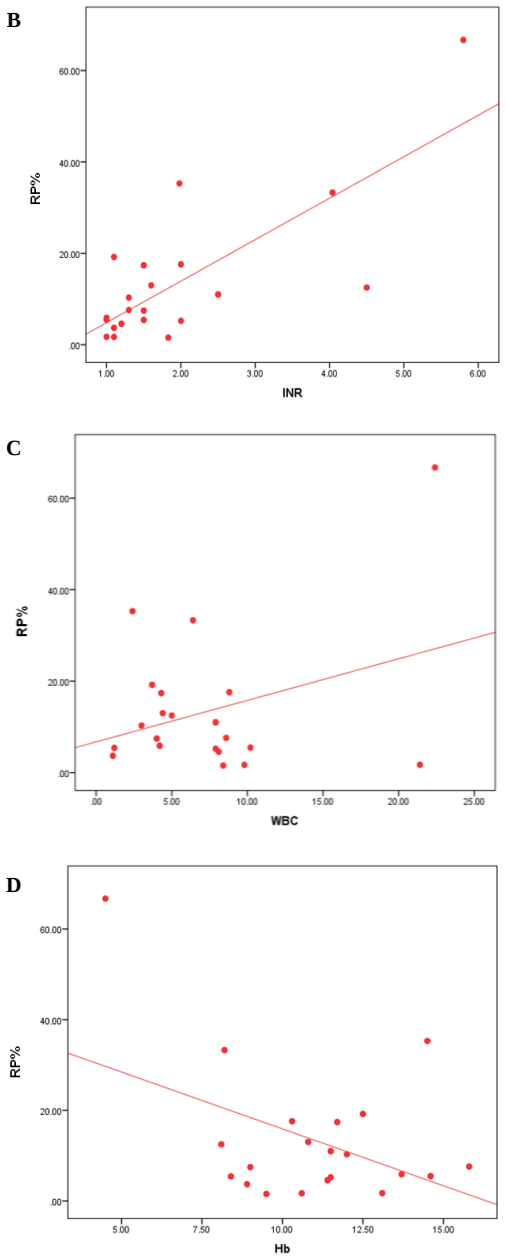


Figure 2: A) Inverse correlation between reticulated platelets percent (RP%) and platelet count (PLT). B) Direct correlation between reticulated platelets percent (RP%) and international normalization ratio (INR). C) Direct correlation between reticulated platelets percent (RP%) and white blood cell count (WBC). D) Inverse correlation between reticulated platelets percent (RP%) and hemoglobin level (Hb)

4. DISCUSSION

Several possible mechanisms have been postulated in the pathogenesis of thrombocytopenia that occur frequently in chronic hepatitis C, including decrease bone marrow production and accelerated peripheral platelet destruction [30]. Many studies showed that RP measurement is a non-invasive test that represents a surrogate marker for megakaryocytic activity [13- 21]. Therefore, the current study aimed to evaluate the RP% in patients with chronic hepatitis C as an index of platelet production and to evaluate the relationship between the RP% and the platelet count in those patients.

We measured RP% using flow cytometry by a simple technique directly from whole-blood, avoiding extra manipulation, with a dual-labelling method for platelet identification. Monteagudo et al. [24] who used the same protocol for measuring RP% as the one used in our study found that RP% value in excess of 11% had a high sensitivity and good specificity for a diagnosis of thrombocytopenia with increased thrombopoietic activity which reflected that RP% determination is a reliable platelet turnover marker. In the present study, the median RP% was significantly higher in thrombocytopenic versus non-thrombocytopenic patients (11.75% versus 2.72% respectively, $P < 0.001$). Moreover, there was a highly significant inverse correlation between RP% and platelet count ($r = – 0.698$; $P < 0.001$). The same association was found between IPF% and platelet count in prior studies reported by Zucker et al. [30] and Tana et al. [31], this suggested that peripheral platelet destruction was the main cause of thrombocytopenia in chronic hepatitis C patients.

In agreement with a previously published study [32], which confirmed that decreased platelet count was a good indicator of the severity of chronic liver diseases, we found that serum albumin levels were significantly lower ($P = 0.026$) and INR values were significantly higher ($P <0.001$) in thrombocytopenic than non-thrombocytopenic patients. Furthermore, platelet count showed significant positive correla-tion with serum albumin levels ($r = 0.579$; $P < 0.001$) and negative correlation with INR values ($r = – 0.694$; $P < 0.001$). Similar results were found by prior report [30]. Additionally, we found a significant inverse correlation between platelet count and the serum ALT levels ($r = – 0.390$; $P = 0.009$) which was consistent with the findings of Olariu et al. [33].

The spleen normally contains one- third of the total platelets within the body however when the spleen

increases in size, percentage of sequestered platelets within the spleen increases. Hypersplenism is often implicated in thrombocytopenia in the portal hypertensive or cirrhotic patient. Another possible mechanism of splenic consumption of platelets occurs when platelets are opsonized by immunoglobulin and sequestered in the spleen [31]. Splenomegaly was significantly more common in thrombocytopenic patients than in those with normal platelet counts ($P < 0.001$). It is noteworthy that RP% was significantly higher in patient with splenomegaly than those with normal size of the spleen ($P < 0.001$). In contrast, Zucker et al. [30] found that IPF% was similarly elevated in thrombocytopenic patients with splenomegaly and those without splenomegaly. Our findings agreed previous literature [33,35] in showing that the traditional model of increased platelets pooling by an enlarged spleen, is still relevant in many patients and represent a considerable factor contributing to thrombocytopenia in our patients with chronic hepatitis C.

It had been suggested that high hepatitis C viral load in peripheral blood corresponds to elevated viral level in hepatocytes which are the major site of TPO synthesis [30]. A significant correlation was found between the level of viral load and the degree of thrombocytopenia in prior reports [30,33,36]. On the contrary, log viral load was not significantly different between thrombocytopenic and non-thrombocytopenic patients in the current study, and there was no correlation between the log viral load and the platelet count, however significant direct correlations were found between log viral load and each of serum ALT levels ($r = 0.514$; $P < 0.001$), serum AST levels ($r = 0.432$; $P = 0.003$) and INR values ($r = 0.326$; $P = 0.031$) which provide an evidence that direct inhibition of protein synthesis by the host that occur in the presence of an infection results in increased quantities of HCV inside hepatocytes.

In conclusion, we found that thrombocytopenic patients with chronic hepatitis C had predominantly higher RP% compared to those with normal platelet count suggesting that peripheral causes of thrombocytopenia represent relevant mechanism. Future studies including larger number of patients that focus on the effect of splenomegaly on platelets may shed more light on the mechanism of thrombocytopenia in patients with chronic hepatitis C.

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