Original Research Article

Some Platelet parameters in patients with HCV-Associated Chronic Liver Diseases

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Abstract: Egypt has the highest hepatitis C virus (HCV) prevalence all over the world. Thrombocytopenia is a common finding in subjects with chronic hepatitis C. The aim of the study was to measure different platelet parameters in patients with chronic hepatitis C. This study included twenty age and sex-matched healthy volunteers as a control (Group I), 40 patients with chronic hepatitis B virus (HBV) (Group II), and 50 patients with chronic HCV (Group III). All subjects were subjected to: full history taking, thorough clinical examination, abdominal ultrasound, complete blood count, mean platelet volume (MPV), liver function tests, prothrombin time (PT), platelet aggregation in response to collagen and adenosine diphosphate (ADP), and IgG platelet antibodies (PA-IgG). The Patients showed significantly decreased platelet counts and increased MPV compared to controls. A statistically significant defective platelet aggregation function was recorded in patients with HCV versus those with HBV or the control group. PA-IgG was significantly more prevalent (62%) in patients with HCV than in HBV group (35%). There was a significant positive correlation between Child Pugh score and MPV, platelet aggregation response to collagen and ADP. Thrombocytopenia and qualitative platelet defects were common findings in chronic virus hepatitis patients especially in those with HCV and may play a role in further aggravating the condition. The clinical progression of the liver disease was associated with more thrombocytopenia and qualitative platelet dysfunctions.

Keywords: Chronic Hepatitis, HCV, HBV, Platelet Dysfunction.

INTRODUCTION

HCV infection is one of the most important health problems worldwide. Egypt has the highest HCV prevalence all over the world, with an estimated overall prevalence of 21.9% among adults [1]. Thrombocytopenia is a common finding in subjects with chronic hepatitis. Chronic HCV infection leads to cirrhosis in about 10 to 20 percent of patients, increasing the risk of complications of chronic liver disease, including portal hypertension, ascites, hemorrhage, and hepatocellular carcinoma [2, 3]. Cirrhotic patients are known to have acquired platelet dysfunction, which may be implicated in the development of variceal bleeding [4]. The mechanism of thrombocytopenia is still controversial. A variety of mechanisms may underlie this phenomenon as hyperglobulinemia, the presence of autoantibodies, circulating immune complexes and inhibitory effect of

platelet factor 4 (PF4), beta thromboglobulin (β-TG) and other inhibitory factors on thrombopoiesis [5]. Platelet specific proteins (PSP), PF4, and β-TG are known to have an inhibitory effect on megakaryocytic growth [6]. HCV-induced inflammatory and immunological phenomena in the liver tissues are assumed to be directly responsible for in-vivo platelet activation [7]. Accordingly, activated platelets are considered to play a significant role in the pathogenesis of liver damage by stimulating fibrogenesis and mitogenesis of Ito liver cells [8]. Many parameters are used to assess different platelet functions. The mean platelet volume (MPV) is the most commonly used measure of platelet size and is a potential marker of platelet reactivity. Larger platelets are metabolically and enzymatically more active and have greater prothrombotic potential [9]. On the other hand, lower MPV indicate smaller platelets, meaning that the person

is at risk for a bleeding disorder [10].

Platelet aggregation studies test the clumping response of platelets to various platelet activators (e.g., ADP, collagen, arachidonic acid, thrombin, epinephrine, and ristocetin) as continuously recorded by a light transmission aggregometer. With some aggregometers, the secretion of platelet granules, another indicator of platelet function, may also be evaluated simultaneously by measuring the release of ATP by the aggregating platelets. Decreased platelet aggregation may be associated with many conditions as autoimmune disorders that produce anti-platelet antibodies, fibrin degradation products, drugs that block platelet formation (e.g., thiazide diuretics, interferon and alcohol), myeloproliferative disorders, uremia, and von Willebrand disease [11, 12]. Immunoglobulin G (IgG) and albumin were initially described as platelet proteins localized within the secretory granules. However, the demonstration that idiopathic thrombocytopenic purpura (ITP) was caused by an anti-platelet antibody and that thrombocytopenic patient had abnormally high concentrations of platelet-associated IgG [4-6] led to an implicit assumption that platelet IgG was anti-platelet antibody. Most subsequent studies of platelet IgG focused on the potential value of IgG measurements in the diagnosis of thrombocytopenia [13].

To date, to the best of our knowledge, no data are available about the pattern and the actual prevalence of platelet abnormalities and platelet antibodies among patients with chronic hepatitis C. Thus, the present study aimed to evaluate platelet function in patients with HCV by assessing some quantitative and qualitative platelet abnormalities in comparison to patients with HBV and healthy controls.

SUBJECTS AND METHODS

This study was carried out on 50 known chronic HCV patients (positive PCR "polymerase chain reaction" test for HCV-RNA) together with seronegativity for HBV (HBsAg negative test). The study also included 40 known chronic HBV patients infection (seropositive for HBsAg for more than 6-month period) together with negative PCR test for HCV-RNA). These cases were presented to the Tropical Medicine Clinic or admitted to Tropical Medicine Department, Alzhraa University Hospital, Faculty of Medicine for Girls, Alazhar University, Egypt from December 2014 to May 2015. Twenty ages and sex matched apparently healthy volunteers were enrolled as a control i.e., negative for markers of hepatitis virus infections (HBV and HCV).

Exclusion criteria:
1. Patient with evident liver cirrhosis: cirrhosis was documented by ultrasonographic, laboratory and endoscopic examination.
2. Other causes of chronic liver disease (CLD) as alcoholism, Wilson's disease, alpha-l antitrypsin deficiency, hemochromatosis, autoimmune hepatitis, and hepatotoxic drugs.
3. Patients with past history of schistosomiasis.
4. Patients treated with interferon, clinically overt thyroid disease, immunological disorders, acute illness, fever, or malignancy.
5. Patients with platelet counts of less than 100,000/ml or hematocrit values of less than 35%.
6. Medications that can interfere with a platelet aggregation test e.g., non-steroidal anti-inflammatory drugs (NSAID), acetylsalicylic acid, antihistamines, antibiotics as (penicillin, cephalosporins and nitrofurantoin), tricyclic antidepressants, anti-platelet drugs as (clopidogrel, dipyridamole, and ticlopidine), or theophylline.

Informed oral consent was obtained from all participants before starting the study. Participants were classified into three groups:
- Group I: included 20 ages and sex matched healthy volunteers enrolled as a control.
- Group II: included 40 patients with chronic HBV infection.
- Group III: included 50 patients with chronic HCV infection.

All study subjects were subjected to the following:
(i) Full history taking with special stress on age, sex, history of schistosomiasis, jaundice, blood transformation, operation, interferon therapy and immunological disorders.
(ii) Thorough clinical examination with special stress on the liver and spleen size, presence of ascites, encephalopathy, manifestations of a bleeding tendency, jaundice, edema of both lower limbs and thyroid diseases.
(iii) Abdominal ultrasound for the assessment of the liver texture, size, portal vein diameter, spleen size, and ascites.
(iv) Upper gastrointestinal tract endoscopy for exclusion the presence of esophageal varices.
(v) Laboratory tests:

All participants (controls and patients) fasted for 8 hours before sampling. All samples were taken at the laboratory and all analyses were done on the same day of collection without preservation or delay. Blood samples were drawn from the forearm of the subjects by venipuncture with a 21-gauge butterfly needle (to avoid both hemolysis and platelet activation) connected to a multiple sample Luer adapter and tube holder. Serum sample was collected first in a vacutainer tube without anticoagulant; plasma was then collected in vacutainer tubes that had solution of sodium citrate (3.2 g/dL, 1/9

citrate/blood) and ethylene-diamine-tetra-acetic acid (EDTA, 1 mg EDTA/1 mL blood) as anticoagulants. The serum-coagulated tube was put in the incubator at $37C^o$ for 20-30 minutes to allow for agglutination, and then centrifuged at 3000 revolutions/minute (RPM) for another 10-15 minutes, and then the supernatant serum was collected into another clean tube. Anticoagulated tubes were mixed both gently and soon after collection (to avoid both hemolysis and clotting, respectively).

(i) Liver function tests (total/direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins, and albumin) were done by the Hitachi-Roche Cobas C 311 autoanalyzer system using kits from Roche Company.

(ii) Prothrombin time (PT) was done from the citrated plasma with Biolabo Bio Solea 2 - Coagulometre 2 Canaux analyzer using a kit from the Biolabo Diagnostics Company.

(iii) Platelet count and MPV were performed by the Sysmex KX-21N automated hematology analyzer using Sysmex hematology reagent. This analyzer was calibrated and controlled by a standard laboratory quality control method provided by Sysmex Corporation.

(iv) In vitro platelet aggregation in response to collagen and ADP was done by a single channel platelet aggregometer [14] using kits provided from Sigma Diagnostica, St Louis Mo 63178, USA.

(v) Detection of serum IgG platelet antibody was done by ELISA [15] using kit provided by Immco Diagnostica, 936 Kenmore Avenue Buffalo.

Statistical Analysis:
Data were expressed as mean±SD and percentages. Continuous variables were compared between the two groups with Student's "t" test. Categorical variables were compared by X^2 test, and the Fisher exact test when appropriate. One-sided analysis of variance (ANOVA) was used to assess differences among the three studied groups. P-value of less than 0.05 was considered significant. Data analysis was performed with the SPSS Statistical Package, version 20.

RESULTS
One hundred and ten subjects were studied; twenty apparently healthy individuals were included as the control (Group I), forty patients with chronic HBV infection (Group II), and fifty patients with chronic HCV infection (Group III). Their ages were 44.9±7.9 (Group I), 43.7±8.5 (Group II), and 45.9±4.7 (Group III), with no statistically significant difference among the three studied groups. They were 11 males and 9 females (Group I), 23 males and 17 females (Group II), and 30 males and 20 females (Group III), with no statistically significant difference between them. Most

of the patients (groups II and III) were in Child-Pugh class A (55% in-group II and 58% in-group III), followed by Child-Pugh class B (32.5% in group II and 34% in group III), with only 12.5% in group II and 8% in group III who were in Child Pugh Class C liver status with no significance difference between groups II and III (tables 1).

Table 2 revealed liver function tests of the studied groups, where; highly significant differences (increases in total bilirubin, ALT, and AST and decreases in total proteins and albumin) were found between group I versus groups II and III, with no significant difference between CLD groups II and III. Serum total bilirubin (mg/dL) was 0.8±0.2, 1.9±0.5, and 1.8±0.6 in the control group I, group II, and group III, respectively. ALT (U/L) was 32.8±7.1, 61.3±13.5, and 61.8±13.6 in the control group I, group II, and group III, respectively. AST (U/L) was 42.8±5.9, 75.32±4.2, and 79.4±4.3 in the control group I, group II, and group III, respectively. The serum total protein (g/dL) was 6.8±0.6, 5.4±0.9, and 5.3±0.8 in the control group I, group II, and group III, respectively. Albumin level (g/dL) was 4.8±0.4; 3.6±0.5 and 3.8±0.7 in the control group I, group II, and group III, respectively. The prothrombin time (seconds) was 10.7±1.1, 14.3±1.6, and 14.1±1.5 in the control group I, group II, and group III, respectively.

Platelet count was 286,768±5568.7 mm^3, 183,093±5559.5 mm^3, and 176,537±5810.8 mm^3 in the group I, II and group III, respectively, with statistical significant difference between control group and hepatitis groups (significant decrease in hepatitis groups). No statistical significant difference between group II and group III. The MPV was 8.4±0.4 fL, 8.8±0.6 fL, and 8.9±0.6 fL, in groups I, II and III, respectively, with significantly increased MPV value in the hepatitis patients compared to the control group, with no significant difference between groups II and III.

Platelet aggregation percentage in response to collagen was 86.5±3.1, 73.5±6.6, and 59.4±7.7 in group I, II, and III, respectively. Platelet aggregation percentage in response to ADP was 74.9±5.3, 71.3±4.9, and 55.6 ± 8.4 in the group I, II, and III, respectively. There was a statistically significant defective platelet aggregation function in chronic hepatitis patients compared to control group. In addition, platelet aggregation function was significantly lower in patients with HCV compared to those with HBV induced by collagen or ADP (P<0.0001). PA-IgG were highly statistically significant (more prevalent) in group III patients (62%) compared to 35% of patients in group II (p<0.001). There was a significant positive correlation between Child Pugh score and MPV, platelet aggregation response to collagen and ADP (table 4, figure 1, 2).

Table 1: Clinical characteristics of the studied groups

Variable/group	Group I (No=20)	Group II (No=40)	Group III (No=50)	P
Age in years (M±SD)	44.9±7.9	43.7±8.5	45.9±4.7	NS
Male/Female number	11:9	23:17	30:20	NS
Liver status: Child Pugh Class A Child Pugh Class B Child Pugh Class C		22 (55%) 13 (32.5%) 5 (12.5%)	29 (58%) 17 (34%) 4 (8%)	NS

* NS= non-significant.

Table 2: Liver function tests of the studied groups

Variable/Group	Group I (Control) M±SD	Group II (HBV) M±SD	Group III (HCV) M±SD	P-value
Total bilirubin (mg/dL)	0.8±0.2 [a]	1.9±0.5 [c]	1.8±0.6	<0.0001
ALT (U/L) **UIL**	32.8±7.1 [a]	61.3±13.5 [c]	61.8±13.6	<0.0001
AST (U/L) **UIL**	42.8±5.9 [a]	75.32±4.2 [c]	79.4±4.3	<0.0001
T. protein (g/dL)	6.8±0.6 [b]	5.4±0.9 [c]	5.3±0.8	<0.0001
Albumin (g/dL)	4.8±0.4 [b]	3.6±0.5 [c]	3.8±0.7	<0.0001
PT (seconds)	10.7±1.1 [a]	14.3±1.6 [c]	14.1±1.5	<0.0001

a, a significant increase of groups II and III versus control group I. b, a significant decrease of groups II and III versus control group I. c, non-significant difference between group II versus group III.

Table 3: Platelet count, MPV, platelet aggregation, and in the studied groups

| Variable/Group	Group I (Control)	Group II (HBV)	Group III (HCV)	P-value
Platelet count mm³	286,768±5568.7	183,093±5559.5	176,537±5810.8	0.0001*
	No significant difference between group II and III			
MPV fL	8.4±0.4	8.8±0.6	8.9±0.6	0.0015*
	No significant difference between group II and III			
Platelet aggregation % response to:				
• Collagen	86.5±3.1	73.5±6.6	59.4±7.7	0.0001*
• ADP	74.9±5.3	71.3 ± 4.9	55.6 ± 8.4	0.0001*
PA-IgG	**No (%)**	**No (%)**	**No (%)**	0.001*
	0 (0)	14 (35%)	31 (62%)	

Table 4: Correlation between Child Pugh score and different platelet parameters

		Platelet count	MPV	Aggr/Collagen	Aggr/ADP
Child Pugh Score	r	- 0.1487	0.0136	0.2304	0.2300
	p	0.1620	0.0001*	0.0289*	0.0292*

* Significant, * Aggr. (Aggregation)
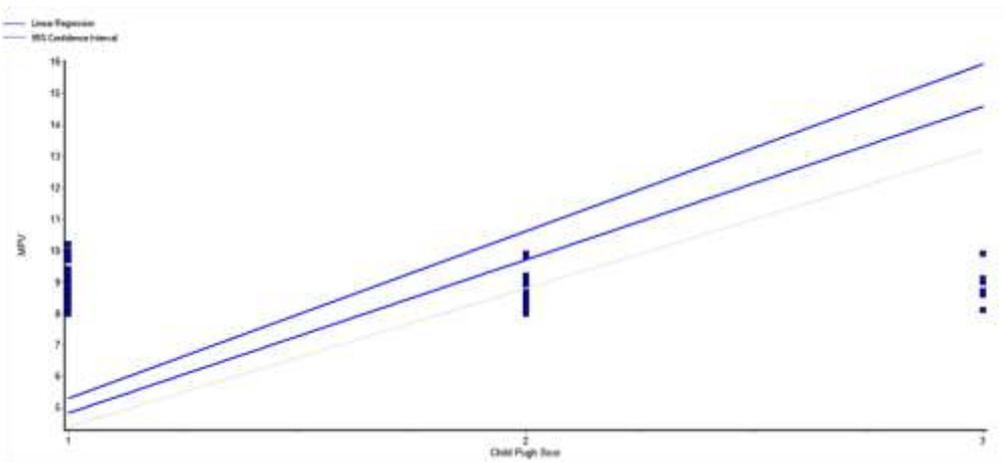
Fig 1: Correlation between MPV and Child Pugh Score
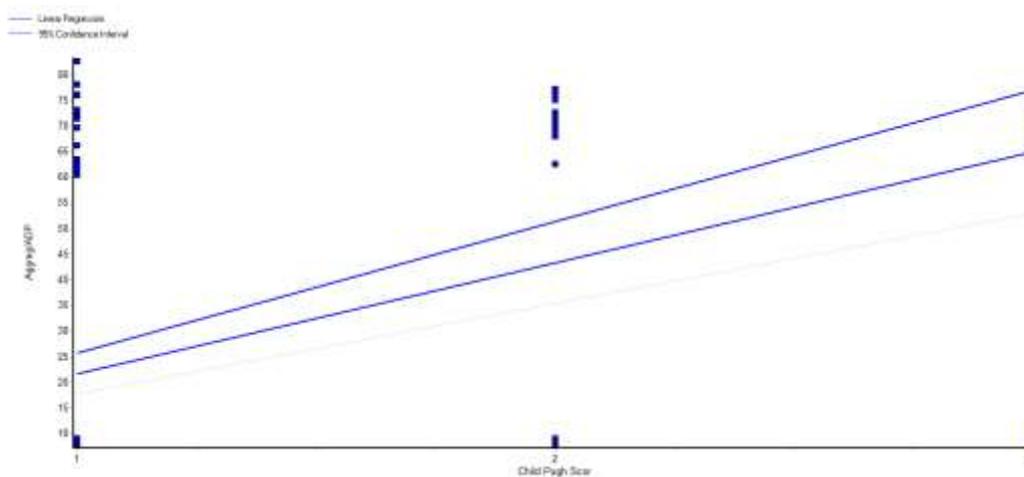
Fig 2: Correlation between Child Pugh Score and platelet aggregation percent to ADP

DISCUSSION

Approximately 120-130 million individuals are chronically infected with HCV worldwide. In adults, acute HCV infection leads to chronic infection in approximately 80% of cases. HCV was found to be responsible for the majority of chronic hepatitis and cryptogenetic liver diseases. Chronic HCV infection results in cirrhosis in approximately 20% of cases [16]. The present work revealed a significant decreased mean platelet counts in the patient groups (II and III) compared to the healthy subjects (p<0.0001). Thrombocytopenia is frequently encountered in patients with viral liver disease. The low platelet count is commonly independent of the cause of liver disease, but it seems to be related to the degree of liver pathology. Olariu *et al.;* [17] demonstrates that chronic hepatitis C may be associated with variable degrees of thrombocytopenia. In most cases, both central (bone marrow suppression) and peripheral (platelet antibodies) mechanisms are involved. There was a correlation between thrombocytopenia and the severity of liver disease (ALT and liver fibrosis stage) and the viral load (HCV-RNA).

A significant increase in MPV in the two patients groups with a positive correlation with severity of liver diseases was found. This may be attributed to increased thrombopoietic stimulation because of platelet over-destruction [18]. These findings were confirmed by increased megakariopoiesis on bone marrow examination, observed in patients with chronic liver disease [19]. MPV may be considered as an indicator of platelet function and activation. Large platelets have more granules, produce higher levels of thromboxane A2, and express more glycoprotein Ib and IIb/IIIa receptors than smaller ones [16].

MPV has been reported as a risk factor for atherothrombotic diseases like acute myocardial ischemia, acute myocardial infarction, and cerebrovascular events [20]. In addition, MPV has been shown to be a sign of inflammation in ulcerative colitis, Crohn's disease, and rheumatoid arthritis [21]. The MPV was reported to be independently associated with advanced fibrosis in patients with chronic hepatitis B. Karaman *et al.;* concluded that MPV levels (especially

those over 8.4 fL) may help to predict advanced fibrosis in patients with chronic hepatic cirrhosis (CHC) [16]. Eminler *et al.;* concluded that MPV values are more reliable in hepatitis C patients than hepatitis B for predicting the advanced damage in liver histology [10].

Additionally, there was a statistically significant defective platelet aggregation function in response to collagen and ADP in the patient groups compared to healthy subjects (p<0.0001). These abnormalities were significantly more pronounced in the HCV group, compared to the HBV group (P<0.0001). Federico A *et al.;* [22] in their study revealed a defect in platelet aggregation in patients with chronic HCV related liver disease. Caldwell *et al.;* [23] found that patients with both acute and chronic liver disease often show secondary aggregation defects manifested as blunted aggregation to collagen, thrombin, and ristocetin as well as absent secondary aggregation waves after primary aggregation with ADP and epinephrine.

PA-IgG were highly statistically significant (more prevalent) in group III patients (62%) compared to 35% of patients in group II (p<0.001). In accordance with the findings of others [24], platelet antibodies were statistically more frequently encountered in HCV patients than HBV group (p<0.0001). Some authors reported that platelet associated IgG was significantly more prevalent and showed higher titres in HCV related CLD when compared to chronic hepatitis B patients and even the reported titres of these antibodies were strikingly inversely correlated with the corresponding platelet count. In chronic liver disease, there is a clear hypoaggregability, as demonstrated with in vitro aggregation tests and by flow cytometric analysis of stimulation dependent antigens. A decreased response to collagen, thrombin, arachidonic acid, ADP, epinephrine, and ristocetin has been reported in the literature [24]. These findings are also confirmed by thromboelastography. Crossover experiments (using platelet aggregometry) confirmed both an intrinsic platelet defect and a circulating plasma factor responsible for this hypofunction [25].In the HCV patients, the progression of the liver disease was associated with more frequent occurrence of platelet antibodies, and consequently disturbed platelet aggregation function. However, these effects were also noticed at lower degree in the HBV patients.

Nagamine *et al.;* [26] assumed that chronic infection with hepatitis C virus may produce a significant autoimmune reaction if compared to chronic liver diseases (CLD) states due to other etiological factors. To date, sufficient data about the platelet status in HCV infected patients is still insufficient. Different mechanisms have been proposed as patients with HCV associated CLD may have portal hypertension secondary to liver cirrhosis and increase in the splenic platelet pool due to accompanying splenomegaly [27], impaired platelet production in the bone marrow [28], immunological disturbance like hyperglobulinemia, presence of autoantibodies and circulating immune complexes are common features in chronic liver disease and these can cause thrombocytopenia, or that the inhibitory effect of PF4 and β-TG on thrombopoiesis may play a role.

Finally, viral infection may account for thrombocytopenia as Nagamine *et al.;* [26] isolated HCV-RNA from bone marrow megakaryocytes of patients with HCV infection, but not in patients with hepatitis B or control subjects. Pockros *et al.;* [29] reported that HCV-RNA was detected in platelets from patients with ITP associated HCV infection but not in those without ITP. Some authors demonstrated that HCV replicates in megakaryocytes causing reduced production of platelets by the infected megakaryocytes [30].

Impaired platelet production due to thrombopoietin (TPO) deficiency was proposed as another cause of thrombocytopenia in liver cirrhosis (LC). This theory assumes that the level of TPO, a principal regulator of megakaryogenesis and thrombopoiesis predominantly produced by the liver, is insufficient in the state of advanced liver disease. This is supported by the clinical observation that the reduced circulating TPO level in LC patients is restored with an increase in platelet count after orthotopic liver transplantation. However, it is still controversial as to whether TPO production is actually suppressed or maintained in LC patients [31]. Ghozlan *et al.;* [32] found that HCV-induced inflammatory and immunological phenomena in the liver tissues are assumed to be directly responsible for in-vivo platelet activation. Accordingly, activated platelets are considered to play a significant role in the pathogenesis of liver damage by stimulating fibrogenesis and mitogenesis of the liver cells.

CONCLUSION
Thrombocytopenias and qualitative platelet defects are common findings in chronic viral liver diseases. However, HCV infection has a more severe effect on platelet function that may play a role in further aggravation of the condition. This hazardous effect of HCV on platelets may be due to increased prevalence of platelet antibodies IgG initiating an inhibitory effect on thrombopoiesis.

STUDY LIMITATIONS
The relatively limited number of HCV patients included. Detection of HCV-RNA in the platelets was not performed.

RECOMMENDATIONS

Platelet abnormalities in liver disease are a growing area of investigation. The study of platelet numbers and functional changes in relation to different types and grades of chronic liver disease may have a considerable impact on our understanding of the pathophysiology of liver disease, portal hypertension, liver fibrosis, and therapy. Detection of HCV-RNA in the platelets may be needed to confirm the possible direct effect of HCV on platelets.

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