Section A -Research paper

Platelets Activation in COVID-19 associated with Chronic Viral Hepatitis Patients

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ABSTRACT

Background: COVID-19-associated coagulopathy is a life-threatening complication of SARS-CoV-2 infection with increased mortality and morbidity rate, especially in patients with severe or underlying chronic disease. The prevalence of chronic viral hepatitis in Egypt is much higher than other countries. Chronic viral hepatitis in association with covid disease can increase the risk of severe complications. Since Coagulopathy in patients with liver disease results from impairment in the clotting and fibrinolytic systems, as well as reduced number and function of platelets, understanding the effect of viral hepatitis on the coagulation state of covid 19 patients is of scientific and clinical importance if novel strategies are to be developed that can help in the management of the patients. **Aim of the Work:** To investigate the effect of chronic hepatitis in the coagulation state of COVID -19 among a sample of Egyptian patients and trying to demonstrate the platelets activation and its potential role in associated coagulopathy through determination of platelet activation marker (PF4) and other laboratory tests. **Patients and Methods:** The present study was conducted on 20 diagnosed cases of COVID- 19 associated with Chronic Viral Hepatitis from outpatient and inpatient clinics, Ain Shams University Hospitals. 20 age and sex matched healthy individuals served as control group. The study was carried out at the Clinical Pathology Department of Ain Shams University Hospitals and Electron Microscopy Department, Theodor Bilharz Research Institute. Assessment of PF4 level was done using ELISA technique and results from patients and controls were compared in addition to estimation of other lab tests as D dimer, serum ferritin, CRP and platelet count. **Results:** Patients with COVID 19 associated with chronic viral hepatitis were compared to healthy control group. Platelet factor 4, D-Dimer, Serum ferritin, and CRP were found significantly higher in COVID 19 associated with chronic hepatitis (P=<.001) for all mentioned variables. Platelet count was significantly lower in COVID 19 associated with chronic hepatitis than in the control group (P=<.001). Moreover, there was a significant positive correlation between PF4 level and D dimer, CRP, serum ferritin …and a negative correlation with platelet count. **Conclusion:** We can conclude that platelets may have a potential role in associated coagulopathy and risk of thrombotic tendency in COVID-19 associated with viral hepatitis patients

Keywords: Platelets, COVID-19, Chronic Viral Hepatitis

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INTRODUCTION:

In late December 2019 Coronavirus designated the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged from a cluster of pneumonia cases in Wuhan, China. The disease, now known as COVID-19, and spreads rapidly leading to a worldwide pandemic. SARS-CoV-2 is an enveloped RNA virus, and belongs to the beta-coronavirus genus. SARS-CoV-2 shares the same cellular receptor as SARS-CoV which is the angiotensin-converting enzyme 2 (ACE2) receptor. ACE2 receptors are enriched in alveolar epithelial type II cells of lung tissues, as well as extrapulmonary tissues

such as the heart, kidneys, and intestines, which might play a role in the multi-organ effects of COVID-19 ***(Hatmal al., 2020).***

Critically ill patients diagnosed with Covid 19 develop a pro-thrombotic state and eventually may develop thrombosis. The mechanisms underlining thrombosis in COVID-19 patients are not known and likely due to multiple processes including inflammation, oxygen demand injury, and plaque rupture triggered by the infection. Platelets mediate thrombotic vascular occlusion but are also increasingly recognized to have immunomodulatory activity ***(Koupenova et al., 2019).***

Elevated fibrinogen and D-dimer are among coagulation laboratory changes in patients with covid 19. These changes also correlate with a worse outcome, and increased mortality in those with hyperactive platelets ***(Meaghan and Yogendra, 2020).***

In Egypt an estimated 8–10 million people suffer from viral hepatitis. Hepatitis A virus (HAV) and hepatitis E virus (HEV) are the major causes of viral hepatitis in Egypt. Hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) are the main causes of chronic hepatitis, liver cirrhosis, and liver cancer (hepatocellular carcinoma [HCC]) in Egypt. Globally, Egypt had the highest age-standardized death rate due to cirrhosis from 1990 to 2017 ***(ELbahrawy et al., 2021).***

Coagulopathy in patients with liver disease results from impairment in the clotting and fibrinolytic systems, as well as reduced number and function of platelets ***(Kaul and Munoz, 2000).***

AIM OF THE WORK:

The aim of the study was to investigate the effect of chronic hepatitis on the coagulation state in covid 19 patients, and trying to demonstrate platelets activation and its potential role in associated coagulopathy.

PATIENTS AND METHODS

The present study was conducted on 20 diagnosed cases of COVID- 19 infected patients who also have chronic hepatitis chosen from Ain Shams University Hospitals. And 20 age and sex matched healthy individuals as a control group. The study was carried out at the Clinical Pathology Department of Ain Shams University Hospitals and Electron Microscopy department at Theodor Bilharz research Institute. Diagnosis of patients was based on history taking, complete clinical examination, laboratory and radiological investigations.

Subjects were divided into 2 groups as follows:

Group I: included twenty chronic hepatic patients who had COVID-19 as well and **Group II (Control)**: Included twenty age and gender matched healthy individuals.

Patients were recruited with ethics committee approval of Ain Shams University, under federal wide assurance No. FWA000017585. Written consent was granted in all cases.

The inclusion criteria were: Positive PCR for COVID-19 +/- chronic viral hepatitis confirmed by serological testing for HCV and HBV. While the Exclusion Criteria: Underlying coagulopathy related disease or platelet related disorders, Patients with Malignancies, Patients with Chronic disease as hypertension or diabetes mellitus or autoimmune disease and Chronic Chest diseases.

Patients under study were subjected to the following: Full history taking and clinical examination, **Imaging techniques:** computed

tomography (CT) chest, and **Laboratory investigations:** CBC, D dimer, Ferritin, CRP, and serological markers for HCV, HBV.

Special investigations: Estimation of the serum level of platelets factor 4 by ELISA technique).

Samples collection and handling:

Eight milliliters of venous blood were collected under complete aseptic conditions from patients and controls. They were divided into three tubes as follow: Two milliliters (2ml) were collected in a clean a sterile ethylenediamine tetra ascetic acid (EDTA) containing tube for performing CBC, three milliliters (4ml) of blood were collected in a sterile plain vacutainer and were left to clot for 30 minutes. Serum was separated by centrifugation at 3000 rpm for 10 minutes. The separated serum was used to assess CRP, serum ferritin, serological markers for HCV and HBV and serum platelets factor 4 levels by Enzyme Linked Immunosorbent Assay (ELISA) and Three milliliters (3ml) were collected in a clean a sterile citrate containing tube for performing D-Dimer

Equipment: EDTA, citrated and plain collection tubes (Vacutainer; BD Biosciences), 5ml polystyrene round-bottom test tube, Automatic pipettes (variable ranges), Centrifuge (Rotofix 32).

Reagents: Platelet factor 4 ELISA Kit (elk biotechnology (china) catalogue number (ElK 1230).

Serum platelet factor 4 assay by ELISA:

The kit was supplied by elk biotechnology (china) catalogue number (ElK 1230)

Principle of the assay: The test principle applied to this kit is sandwich enzyme immunoassay, The micro titer plate provided in this kit has been pre-coated with an antibody specific to Platelet Factor 4(PF4). Standards or samples are added to the appropriate micro titer plate wells then with a biotin-conjugated antibody specific to Platelet Factor 4(PF4). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each micro-plate well and incubated. After TMB substrate solution is added, only those wells that contains Platelet Factor 4(PF4), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of Platelet Factor 4(PF4) in the samples is then determined by comparing the OD of the samples to the standard.

Calculation of results: The readings were duplicated and the average were subtracted from zero standard optical density and A standard curve was constructed with platelets factor 4 on the vertical (Y) axis against its absorbance in pg./mL on horizontal (X) axis. Absorbance value of each sample was used to determine the corresponding concentration from the standard curve.

Kit specification: Lower limit detection of the kit is 1.56 pg./mL and Linearity of the kit is up to 100 pg./ml

RESULTS

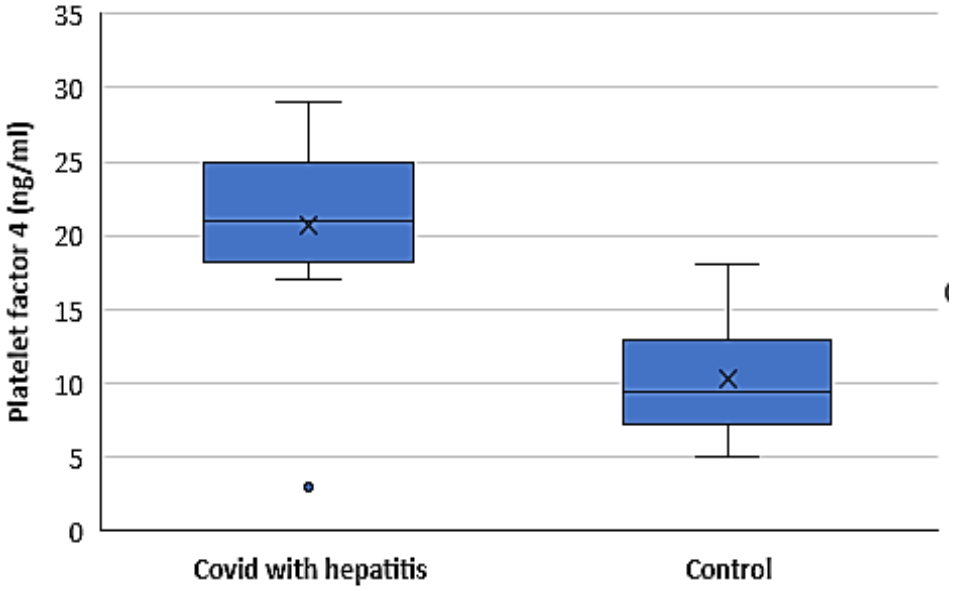
Table (1): Demographic data of participants:

			Groups	
			I (COVID-19 with hepatitis)	**II (Control)**
			N=20	**N=20**
Age (years) Mean ± SD			54.30±14.05 (34-80)	47.52±10.16 (18-68)
Sex	**Males n (%)**		11 (55%)	13(65%)
	Females n (%)		9 (45%)	7(35%)
Chest involvement for COVID 19	**No**		8 (40%)	-
	Mild or moderate		6 (30%)	-
	Severe		6 (20%)	-
GIT involvement	**Absent**		20 (100%)	-
	Present		0 (0%)	-
Hepatomegaly ± Splenomegaly	**Absent**		12 (60%)	-
	Present		8 (40%)	-
Liver Compensation for those with chronic hepatitis	**Not compensated**		5 (25%)	-
	Compensated	-	15 (75%)	

Table (2): Comparison between COVID-19 patients associated with hepatitis and control participants regarding measured laboratory variables:

	Groups		**P***
	I (Hepatitis with COVID 19)	**II (Control)**	
	N=20	**N=20**	
Platelet factor 4 (ng/ml) Mean ± SD	21.00±5.59	10.35±3.65	<.001 (HS)
Platelet count (x10^3/mcL) Mean ± SD	107.80±29.88	172.15±15.44	<.001 (HS)
D-Dimer (mg/ml) Mean ± SD	1.01±0.34	0.30±0.11	<.001 (HS)
Ferritin (ng/ml) Mean ± SD	471.60±372.47	154.05±36.11	<.001 (HS)
C-reactive protein (CRP)(mg/l) Mean ± SD	83.45±23.22	11.30±4.50	<.001 (HS)

*Student t-test was used to analyze the difference between the two groups. P <0.05 was defined as statistically significant. (HS): highly significant.



COVID 19 associated with hepatitis patients (group I) were compared to healthy control participants (group II). Platelet factor 4, D-Dimer, Serum ferritin, and CRP were found significantly higher in group I than in group II (P=<.001) for all mentioned variables While platelet count ($x10^3$/mcL) was significantly lower in group I than in group II (P=<.001).

DISCUSSION

Critically ill patients diagnosed with Covid 19 develop a pro-thrombotic state and eventually may develop thrombosis. The mechanisms underlining thrombosis in COVID-19 patients are not known ***(Koupenova et al., 2019).***

Studies have identified hypertension, diabetes, dementia, anemia, cardiovascular disease, and thyroid disease as comorbidities associated with worse COVID-19 outcomes ***(Gold et al., 2020; Putri et al., 2021; Hariyanto et al., 2021).*** Moreover, most chronic viral hepatitis infections, especially chronic hepatitis B and C infections, will develop into liver cirrhosis that can cause significant morbidity and mortality ***(Schuppan and Afdhal, 2008; Wiegand and Berg, 2013).***

Currently, there is little data regarding the association between viral hepatitis and COVID-19 outcomes and providing evidence of the association between viral hepatitis and severe outcomes of COVID-19 has to be elucidated ***(Hariyanto et al., 2022).***

Circulating platelets, the activity of which are central to hemostasis and thrombosis, are now understood to fulfil a much broader role in the pathophysiology of cancer, inflammatory diseases and infections such as that with severe acute respiratory syndrome coronavirus (SARS-CoV)-2, giving rise to COVID-19 ***(Weinreich et al., 2021).*** Platelet-mediated immune responses induced by viral infection have previously been reported, and a recent report has demonstrated altered platelet gene expression profiles and functional responses in patients infected with COVID-19 ***(Vuppalanchi et al., 2021; Efe et al., 2021).***

Based on the uncertainty which are currently exists with regards to the etiology of thrombotic risk in COVID-19, and the impact of coincidence of chronic viral hepatitis and COVID-19 infection on disease severity and risk of thrombotic tendency, this study was

designed with novelty to investigate the role of platelets in COVID -19 and viral hepatitis among a sample of Egyptian patients with COVID -19 and chronic viral hepatitis and trying to demonstrate the platelets activation and its potential role in associated coagulopathy .

In our study we used platelet factor 4 as a marker for platelet activation and we found that Platelet factor 4 were significantly higher in group I than in group II (P=<.001)

In agreement with our study, ***Comer et al.*** found that the circulating levels of the platelet activation markers PF4 and sP-selectin were highly elevated among COVID-19–positive patients compared to controls which further supports the hypothesis that COVID-19 is associated with enhanced platelet activation ***(Comer et al., 2021).***

There is evidence for an in vivo platelet activation in viral liver disease demonstrated by the increased concentration of beta thromboglobulin and PF4 in serum, P-selectin expression on resting platelets is also elevated in chronic hepatitis and cirrhosis compared to normal controls ***(Panasiuk et al., 2001).***

Also, plasma soluble P-selectin levels where markedly elevated in chronic hepatitis C. This correlated directly with serum hepatitis C virus-RNA and was significantly higher in patients with low platelet counts. This suggests that hepatitis C infection may be directly responsible for the in vivo platelet activation in patients with chronic hepatitis C ***(Ferroni et al., 2001).***

Furthermore, there is evidence supporting the platelet activation in vivo in patients with severe viral hepatitis where aggregation and release function of platelets and its ultrastructure was carried out. The results showed that the ultrastructural abnormalities of platelet suggested its renewal, denaturation and activation in vivo together with the abnormal platelet function are mainly related to the activation of platelet in vivo ***(Wang, 1990).***

This was confirmed in further study that demonstrate that there is increased platelet activation in patients with chronic hepatitis C, which may contribute to the occurrence of thrombocytopenia and liver fibrosis ***(Fusegawa et al., 2002).***

In our study, Platelet count in severe covid19 patients and in covid 19 with hepatitis patients were significantly lower than mild and moderate covid this agrees with

In agreement with our study, Liu et al. reported that the SARS-CoV-2 and HBV coinfected patients showed significantly lower platelet counts than the other group ***(Liu et al., 2021).***

Also, ***Ronderos et al.*** enrolled 1207 patients with confirmed COVID-19 and showed that patients with HCV had lower platelet count ***(Ronderos et al., 2021).***

Our results showed that, D-Dimer, Serum ferritin, and CRP were significantly higher in group I than in group II (P=<.001) for all mentioned variables

This agrees with ***Lagunas-Rangel*** found out In severe COVID-19 patients, CRP increased significantly at the initial stage and was highly associated with disease progression in the early stages of infection ***(Lagunas-Rangel, 2020, Boeckmans et al., 2020). Chen et al.*** consistently suggested that CRP was an independent risk factor for the progression of liver dysfunction in COVID-19 patients ***(Chen et al., 2021).*** in disagreement with our study, ***Chen et al.*** retrospectively enrolled 830 covid-19 patients (27.3%) had different

degrees of liver dysfunction on admission, specifically, mild lba (n = 195) and hepatic impairment (n = 32). they observed that elevated crp were also highly associated with liver abnormality (lba), elevated crp index higher were highly associated with increased odds of lba ***(Chen et al., 2021).*** this difference may be due to different severity of cases.

Yu et al. reported higher in-hospital mortality, more severe disease, and liver function abnormalities in covid-19 patients infected with hbv compared to covid-19 patients without hbv ***(Yu et al., 2023).***

A meta-analysis showed that patients with chronic liver disease (cld) were more likely to have severe or critical covid-19 than those without cld, and they were also more likely to have higher mortality and acute on chronic liver failure (aclf) can also occur in patients with compensated cld who can also occur in patients with compensated cld who had severe covid-19 ***(Kovalic et al., 2020).***

Yameny found a significant value with critically ill infection as ferritin level which increased in 71.4% ldh has a high level in 67.7% and d-dimer has positive results in 36.4% ***(Yameny, 2021).*** Also, the study by wool and miller, reported that, temporally increasing d-dimer levels indicate the progressive severity of covid-19 infection and can be used as a predictor that more aggressive critical care will be needed ***(Wool and miller, 2021).*** Also, the study by ***Yang et al.*** found that, crp, ferritin, tlc and creatinine levels were statistically significantly increased among critically ill group than other groups ***(Yang et al., 2020).*** Another study by helms et al. revealed that, d-dimers are significantly increased in covid-19 infection ***(Helms et al., 2020).***

Also, wool and miller, reported that temporally increasing d-dimer levels indicate the progressive severity of covid-19 infection and can be used as a predictor that more + aggressive critical care will BE NEEDED ***(Wool and Miller, 2021).*** The study by ***Sadeghi-Haddad-Zavareh et al.*** evaluated the association between crp and covid-19 infection, and the findings indicated that a patient with a crp level >64.75 mg/l was more likely to develop the severe form of the disease ***(Sadeghi-haddad-zavareh et al., 2021).***

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