437

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Determination of the cut-off score of Human platelets membrane glycoprotein IIb/IIIa in COVID-19 patients: A novel biomarker and a therapeutic target

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Abstract

Abstract: Although many vaccines have been approved for COVID-19, the continuing high incidence motivates the importance of drug repositioning. Among COVID-19 multiple complications, severe pneumonia and thromboembolism-related death are the most aggressive. We aimed to monitor the mechanisms by which SARS-CoV-2 led to severe illness and death to explore targets for therapies. Our objective was achieved by determining the cut-off of the novel biomarker human platelet membrane glycoproteins IIb/IIIa (CD41/CD61) (GPIIb/IIIa) in microparticle free plasma separated from peripheral blood samples of COVID-19 patients and healthy subjects using the ELISA technique. In comparison with the control, we observed an elevated level of GPIIb/IIIa in COVID-19 patients, especially those with severe illness including severe pneumonia and pulmonary embolism. Our data indicate the relevance of determination of GPIIb/IIIa as a diagnostic and prognostic biomarker for haematological complications including platelet aggregation and pulmonary embolism in COVID-19 patients suffering from severe illness. We conclude that determination of GPIIb/IIIa level by an easy, reliable, and low-cost ELISA kit helps in early diagnosis of haematological complications in COVID-19 patients and may help in improving the clinical outcomes and treatment of COVID-19 patients. Adding GPIIb/IIIa inhibitors (synthetic or natural products) to the treatment protocol of COVID-19 patients may add benefit in improving the clinical outcomes of COVID-19 patients.

Keywords: drug repositioning, SARS-CoV-2, pulmonary embolism (PE), COVID-19, GPIIb/IIIa inhibitors.

1. Introduction

COVID-19 is still actively spreading, even though many efficient vaccines and treatment protocols have been developed that counteract the related mortality rate [1]. To improve the efficacy of both, there must be continued research efforts to evaluate the mechanism of action of SARS-CoV-2-induced infectivity and severity. human beings need all efforts of the World Health Organization and scientific research to evaluate the mechanism of action of SARS-CoV-2-induced infectivity and severity. Since the beginning of COVID-19 outbreak, the implication of the cytokines storm due to

overexpression of pro-inflammatory cytokines such as TNF-alpha, TGF-Beta and interleukins in severe illness of COVID-19 was confirmed. For example, SARS-CoV-2 elicits the inflammatory process via enhancing the NF-κB and STAT3 pathways. Subsequently, treatment of critical cases with immunomodulatory therapy was recommended [2]. For instance, treatment with Actemra (a monoclonal Ab that inhibits binding of IL-6 to its receptor) depended on the determination of IL-6 by ELISA kit in certain Lab. in Alexandria. Within few months, it was integrated as a routine pro-inflammatory marker that helped in relieving the critical cases, the data

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published in Aug. 2020 [3] is consistent with an earlier clinical trial in China that was published in March 2020 [4]. To date, more studies support the relevance of determination of IL-6 and efficacy of Actemra (Tocilizumab) in treatment of COVID-19 [5]. However, others suffered from severe illness but did not show high level of IL-6. Thus, we need to search and apply new diagnostic and prognostic biomarkers as well as markers helping in monitoring therapy.

Among COVID-19 different symptoms and consequences, the most severe complications are severe pneumonia, cytokine storm, thrombosis, and pulmonary embolism (PE) [6-8]. Dysregulation of platelet differentiation plays an essential role in all of these mechanisms. The essential role of platelets in SARS-CoV-2 pathogenicity directed us to search for a novel marker which may help to discriminate severe cases from mild and moderated cases, as well as to serve as a therapy target. Upon infection stress, platelets release vesicles called platelet-derived extracellular vesicles (PEVs) which are characterized by the presence of the human platelet membrane glycoprotein IIb/IIIa complex (CD41/CD61). GPIIb/IIIa is a marker for the megakaryocyte-platelet lineage essential for platelet aggregation and the platelet adherence to endothelial cells that enhances thrombosis [9,10]. We hypothesized that measuring GPIIb/IIIa by a simple method in peripheral blood (microparticle-free plasma) would allow us to make clear and cost-effective diagnostic evaluations of COVID-19 severity in patients. The widely applicable method for measuring GPIIb/IIIa is flow cytometry, but that method requires fresh samples and is costly [11,12]. However, Periayah et al. have also mentioned determining GPIIb/IIIa through the simpler enzyme-linked immunosorbent assay (ELISA) [13]. We found a novel ELISA kit to determine GPIIb/IIIa level in plasma, and we used it to study the correlation between GPIIb/IIIa levels and the frequency of severe pneumonia and PE in COVID-19 patients.

2. Materials and Methods

2.1. Patients

The present study was performed in Armed Forces hospital in Alexandria, Egypt. Upon admission, patients who were diagnosed with COVID-19 by a real time polymerase chain reaction (RT-PCR), Chest CT scan was done for all inpatients while CT angiography scan was done for those expected to have thromboembolism. Also, blood samples were collected for haematological and biochemical investigations including pro-inflammatory biomarkers (C-reactive protein: CRP, ferritin, and D-dimer). Our study was conducted on 67 COVID-19 patients and 22 healthy control subjects who signed a written informed consent which was approved by the Ethics Committee of the Medical Research Institute (IORG 0008812). A

follow-up of patients via collecting data from registration form to monitor the clinical outcomes of COVID-19 patients involved in our study. Determination of Human platelets membrane glycoprotein IIb/IIIa (CD41/CD61) GPIIb/IIIa using ELISA technique was based on preparation of microparticle-free plasma. It was prepared by double centrifugation of anti-coagulated blood; first centrifugation was at 3000 xg for 15 min, then centrifuge the supernatant at 20000 xg for 30 min [14].

2.2. Quantitative determination of Human platelets membrane glycoprotein IIb/IIIa (CD41/CD61) GP IIb/IIIa receptor by ELISA(cat. #SG-15131)

To evaluate the level of Human platelets membrane glycoprotein IIb/IIIa (CD41/CD61) in the plasma samples of COVID-19 patients and controls, we used Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sinogene Clon Biotech, China). Briefly, 50 µl of diluted samples were added in ELISA plate wells which were coated with polyclonal anti-human GPIIb/IIIa antibody.After incubation for 30 min and washing, GPIIb/IIIa antibody was combined with labeled HRP to form antibody-antigen -enzyme-antibody complex. After washing, we added TMB substrate solution was added and forming blue color at HRP enzyme-catalyzed, then reaction is terminated by the addition of a 50 µl stop solution and the color change to yellow. Finally, the absorbance is measured at a wavelength of 450 nm. The concentration of CD41/CD61 in the samples was then determined by comparing the O.D. of the samples to the standard curve.

2.3. Statistical analysis:

All quantitative data are presented as the mean ± SE. Mann Whitney and Chi-square tests were used for analytic comparison. The paired comparison of Receiver operating characteristics (ROC) was performed using MedCalc version 18.11.3. ROC curve was constructed to discriminate COVID-19 patients from control subjects depending on GPIIb/IIIa level to establish its sensitivity-specificity relationship. The optimum cut-off value of GPIIb/IIIa was determined and expressed as the area under curve-receiver operating characteristic (AUC). AUC values were reported with the 95% confidence interval (95% CI). $P<0.05$ was considered significant. Statistical analysis was performed using SPSS 15.0 statistical software.

3. Results & Discussion

Human platelet glycoprotein IIb/IIIa (GPIIb/IIIa) is a heterodimeric integrin membrane associated protein which binds to fibrinogen, VWF and fibronectin. Pathophysiological stress including vascular injury, metabolic disease, viral infection, and cancers initiates the binding of GPIIb/IIIa with

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fibrinogen causing crosslinking of platelets with either platelets or endothelial cells resulting in formation of thrombus [15,16]. We aimed to determine the cut-off score for GPIIb/IIIa level in COVID-19 patients to early predict of PE and as a trial to highlight a new target for drug repositioning in the treatment of COVID-19 patients who are at high risk factor of thromboembolism.

According to data published by the WHO, 2 million deaths were reported among twelve months in 2020 while approximately 2,770,000 deaths were reported among the first 9 months of 2021, even though many vaccines and treatment protocols were discovered [1]. This highlights the need for more scientific research to add more efficient therapies to improve the treatment protocols based on the novel SARS-CoV-2 variants, in addition to the wide varieties in immune response and ethnicity worldwide. Thus, we aimed to evaluate a novel marker for platelets dysregulation in COVID-19 patients who suffered from severe illness by simple and applicable methods such as ELISA in peripheral blood. Among numerous proteins which are present on platelets and PEVs, GPIIb/IIIa (CD41/CD61) is unique and plays an essential role in exerting a conformational change on activated platelets that allows them to adhere to each other [17].

3.1. Determination of the cut-off value for Glycoproteins IIb/IIIa (CD41/CD61

We found that the mean level of GPIIb/IIIa (55.30±91.89 ng/ml) in plasma of COVID19 patients was significantly higher than that in control subjects (5.03±4.32 ng/ml), P<0.001 as shown in table 1 and figure 1A. Also, our data showed that significant elevation of the mean level of GPIIb/IIIa 116.24 ± 129.8 ng/ml in COVID-19 patients suffered from severe illness compared to those with mild/moderate illness 19.03 ± 11.13 ng/ml (P<0.001) as shown in table (2)

This indicates that SARS-CoV-2 infection enhances the releasing of GPIIb/IIIa from the active PEVs. In such cases, shedding of these platelets microparticles release the pro-angiogenic factors and regenerate endothelial progenitors [8]. Also, it has been demonstrated that influenza viruses disrupt the haemostatic-inflammatory balance causing increasing of platelet monocytes aggregates and resulting in over-expression and releasing of GPIIb/IIIa [18]. Determining the cut-off of GPIIb/IIIa may be a prognostic biomarker for COVID-19 with high sensitivity and specificity, thus, it may improve the clinical outcomes in COVID-19 patients and helps in the early intervention to counteract the progress of severe pneumonia and PE, subsequently, limiting the related mortality rate.
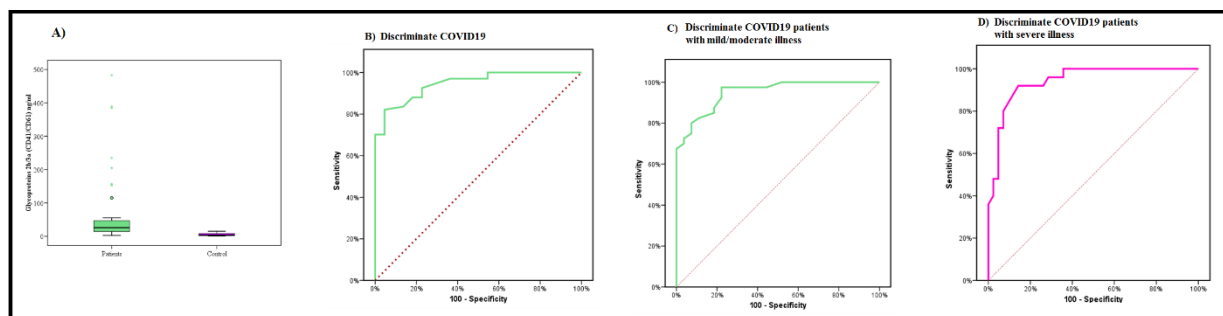

Figure 1. (A) Boxplot of plasma GPIIb/IIIa level in COVID19 patients and control healthy subjects, **(B)** Receiver operating characteristics (ROC) curve identifies the sensitivity and the specificity of the cut-off point of GPIIb/IIIa that discriminate the COVID19 patients **(C)** ROC curve identifies the sensitivity and the specificity of the cut-off point of GPIIb/IIIa that discriminate the COVID-19 patients with mid/moderate illness, **(D)** ROC curve identifies the sensitivity and the specificity of the cut-off point of GPIIb/IIIa that discriminate the COVID19 patients with severe illness. AUC: area under the curve.

Table (1): Comparison between the mean of plasma level of GPIIb/IIIa (ng/ml) in COVID-19 patients and control subjects.

GPIIb/IIIa (D41/CD61) ng/ml	Patients (n = 67)	Control (n = 22)	U	p
Min.-Max.	2.68 –483.0	0.89 –15.18		
Mean ±SD.	55.30 ±91.89	5.03 ±4.32	79.0*	<0.001*
Median (IQR)	25.9(14.3 –46.9)	3.57 (1.8 –8.1)		

U: Mann Whitney test, p: p value for comparing between patients and control, *: Statistically significant at p ≤ 0.05

Table (2): Relationship between the severity of COVID-19 and plasma level of GPIIb/IIIa ng/ml

GPIIb/IIIa (D41/CD61) ng/ml	Severity of diseases		U	p
	Mild + Moderate (n = 42)	Severe (n = 25)		
Min. – Max.	2.68 – 55.35	21.43 – 483.0		
Mean ± SD.	19.03 ± 11.13	116.24 ± 129.8	60.50*	<0.001*
Median (IQR)	17.41 (10.7 – 25.9)	50 (33.03 – 156.3)		

U: Mann Whitney test, p: p value for comparing between the studied categories, *: Statistically significant at $p \leq 0.05$

3.2. Discrimination of COVID19 patients basing on the mean level of GPIIb/IIIa

Receiver operating characteristic (ROC) curve was used to represent the cut-off score for GPIIb/IIIa level to discriminate COVID19 patients from control. Figure 1B shows the ROC curve discriminating COVID-19 patients from control, Figure 1C shows discriminating COVID-19 patients with mild/moderate illness and Figure 1D shows discriminating COVID-19 patients with severe illness.

The sensitivity and specificity of GPIIb/IIIa test was plotted and the area under the curve (AUC), negative predicator value (NPV) and positive predicator value (PPV) were indicated as shown in figure 2(B-D). We found that the optimal cut-off point of GPIIb/IIIa was 9.82 ng/ml (AUC= 0.946; 95% confidence interval [CI] 0.903 – 0.990, P<0.001, with sensitivity of 88.06%, specificity of 81.82%, PPV of 93.7 % and NPV of 69.2%. Also, our data showed that the best cut-off point of GPIIb/IIIa to discriminate COVID-19 patients with mild/moderate ranged from 17.9 ng/ml (AUC =0.950, 95% CI: 0.904 – 0.995, P < 0.001) with sensitivity of 77.78%, specificity of 97.5%, PPV of 95.5%, NPV of 86.7%, while elevated level of GPIIb/IIIa more than 27.7 ng/ml (AUC = 0.942, 95% CI: 0.890 – 0.995, P < 0.001) predicted the worst clinical outcomes in COVID-19 patients with sensitivity of 92.0%, specificity of 85.71%, PPV of 79.3%, NPV of 94.7%.

3.3. Correlation between the Clinical-pathological features of COVID19 patients and level of GPIIb/IIIa

In relation to the clinical-pathological findings and pro-inflammatory markers, we found that the optimal cut-off for GPIIb/IIIa is 20 ng/ml. We therefore categorized the COVID19 patients who showed different clinical-pathological and biomarkers according to the level GPIIb/IIIa less or more 20 ng/ml as shown in table 4. Pulmonary embolism, the major deleterious consequence of COVID-19 and related to the high mortality rate, was more frequent in patients showing elevated level of GPIIb/IIIa more than 20 n Our data revealed that 44 out of 67 suffered from severe pneumonia ranging from CORAD4-CORAD6. Of them, ten patients developed pulmonary embolism (PE) as shown in table 4 and figure 2. CT scan image showed different clinical findings included ground-glass opacity (GGO), consolidations, bronchiectasis, and pleural effusion (Figure 2, 3). Axial and Coronal CT image for COVID-19 patients showed different stages of disease progress. Twenty-seven patients suffered from mild illness and showed appearance of residual GGO. Fifteen COVID-19 patients suffered from moderate illness and showed dense lesions, forming mixed pattern of GGO and consolidation. These lesions were accompanied with appearance of air bronchogram as a result of the exudation into the alveolar space, in addition to bronchovascular interstitial thickening. Twenty-five COVID-19 patients suffered from severe illness showed a diffuse consolidation due to fibrous exudates into the alveoli and accompanied with air bronchogram, subpleural lines and patchy GGO.

g/ml.

In the present study, COVID-19 patients had a mean age of 51.18 ± 14.0 years old. Of them, thirty-nine were males and twenty-eight were females. Of note, higher level of GPIIb/IIIa was more frequent in aging COVID-19 patients who were more than 50 years old (P=0.017) compared to those who were less than or 50 years old. Our data showed that higher level of GPIIb/IIIa more than 20 ng/ml was more frequent in COVID19 patients who suffered from severe pneumonia (P=0.001) and PE (P=0.005). Also, we observed that elevated level of GPIIb/IIIa > 20 ng/ml was more frequent in COVID-19 patients who had elevated level of CRP (> 12 mg/L, P=0.0413) and D-dimer (> 2 µg/ml, P=0.0178) compared to those who showed lower level of GPIIb/IIIa≤20 ng/ml, however, no significant correlation was observed according to the level of ferritin.

Table (3): Determination of the cut-off of GPIIb/IIIa (CD41/CD61) ng/ml in COVID-19 patients

GPIIb/IIIa (D41/CD61) ng/ml	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Discriminating of COVID19 patients from control	0.946*	<0.001*	0.903 – 0.990	>9.82	88.06	81.82	93.7	69.2
Discriminating of COVID19 patients with mild/moderate illness	0.950*	<0.001*	0.904 – 0.995	≤17.9	77.78	97.50	95.5	86.7
Discriminating of COVID19 patients with severe illness	0.942*	<0.001*	0.890 – 0.995	>27.7	92.0	85.71	79.3	94.7

AUC: Area Under a Curve, p value: Probability value, CI: Confidence Intervals, NPV: Negative predictive value, PPV: Positive predictive value, *: Statistically significant at p ≤ 0.05
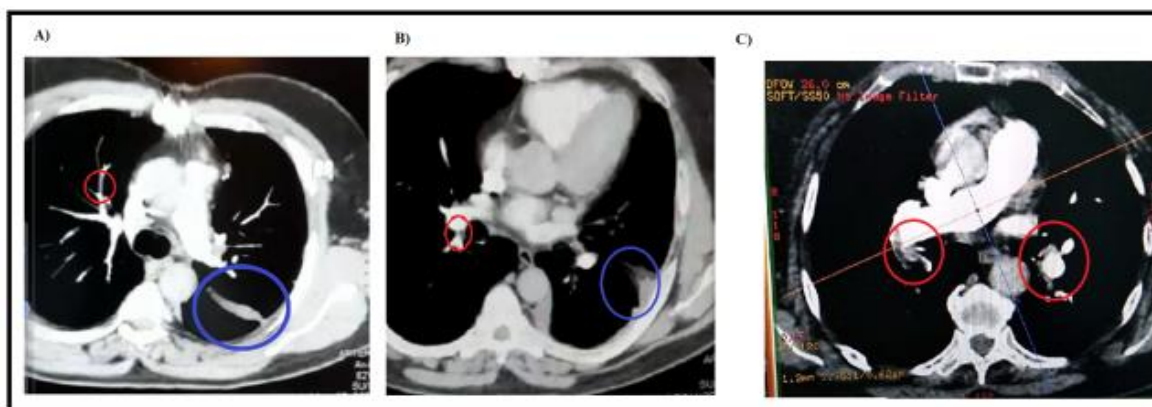

Figure 2. (A) Computerized tomography (CT) angiography (axial image) of COVID19 patients showing pulmonary embolism (red circle) accompanied with a linear band in the left lower lobe (blue circle), **(B)** CT scan shows a pulmonary embolism that affect the right artery and present a peripheral wedge-shaped area (blue circle) that may be resulted from a pulmonary embolism-induced lung infarction, **(C)** CT scan shows a pulmonary embolus that affects the right main pulmonary artery (red ring) and nonocclusive pulmonary embolism in the left pulmonary artery (blue circle)
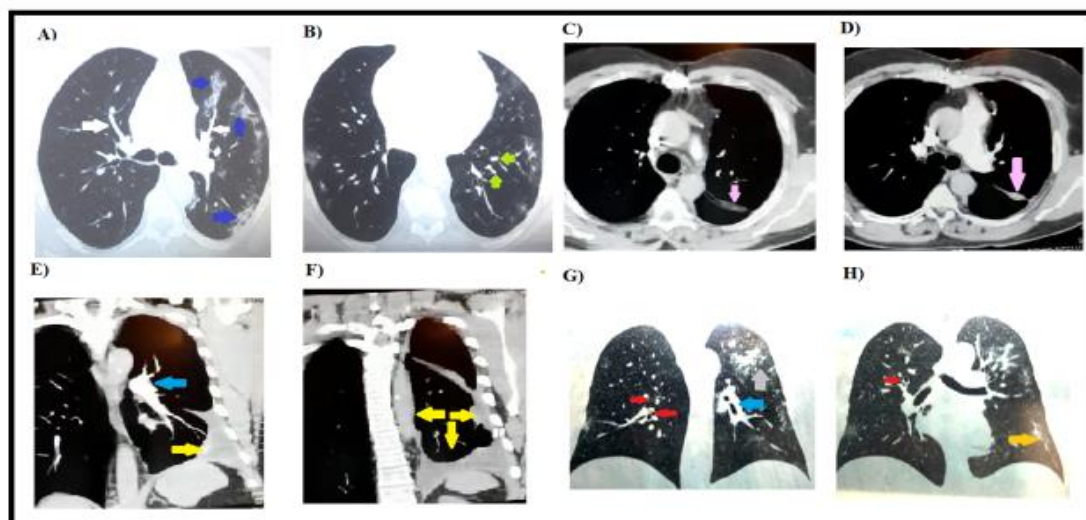

Figure 3. Computerized tomography (CT) angiography for lung of COVID-19 patients, axial image shows areas of consolidation with peripheral ground glass lesion (GGO) with broncovascular interstitial thickening (white arrow), multifocal consolidation (dark blue arrow) **(A)** and multiple centrilobular nodules with tree in bud pattern opacities (green arrow) **(B)**, Axial CT image shows presence of parenchymal bands (pink arrow) **(C, D)**, Coronal image showing scleroderma (blue arrow) with diffuse reticular interstitial pattern and a plural effusion (yellow arrow) **(E, F)**, and Coronal CT image shows GGO with air-bronchogram (red arrow), and consolidation lesion (grey arrow) **(G)**, appearance of scleroderma interstitial lung disease (blue arrow) and subplural lines (orange arrow) **(H)**.

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Table (4): Comparison between the Clinical-patholgical features of COVID19 patients according to plasma level of GPIIb/IIIa

Clinical- pathological parameter	No. of cases 67	Gp≤20 ng/ml (n=27)	Gp>20 ng/ml (n=40)	χ^2	p value
Sex					
Male	39	12	27	3.522	0.060
Female	28	15	13		
Age (years)					
≤50	28	16	12	5.673	0.017
>50	39	11	28		
PLR≤100	22	13	9	4.808	0.028
PLR>100	45	14	31		
CRP (mg/L)					
≤12	54	25	29	4.161	0.041
>12	13	2	11		
Ferritin (ng/ml)					
≤500	19	10	9	1.676	0.195
>500	48	17	31		
D-dimer (µg/ml)					
≤2	13	9	4	5.612	0.017
>2	54	18	36		
Severe Pneumonia	44	11	33	9.655	0.001
Moderate Pneumonia	23	16	7		
No Embolism	58	26	32	3.681	0.055
Pulmonary Embolism	9	1	8		

χ^2: Chi square test, p: p value for association between different categories, *: Statistically significant at p ≤ 0.05

As known, elevation of the pro-inflammatory proteins such as CRP, ferritin, D-dimer and the platelet/lymphocytes ratio (PLR) play an essential role in the severity of COVID-19, especially, in those who showed severe pneumonia, thromboembolism and PE. This substantiates previous findings in that under thrombin stimulation, increasing expression of the GpIIb/IIIa complex on the platelet membrane surface was observed [19]. Recent study supports our finding that vascular disorder was strongly associated with fibrinogen (a ligand of GPIIb/IIIa) and D-dimer levels in COVID-19 patients with thromboembolism [20] as well as CRP in pulmonary disease [21]. An explanation is that the proteolytic degradation of GPIIb/IIIa by plasmin produces D-dimer. In addition, elevated levels of pro-inflammatory biomarkers; CRP and D-dimer are strongly correlated with the progressive inflammatory process and coagulopathy in patients with acute aortic dissection, a fatal consequence of COVID-19 [22,23]. In this aspect, a previous study indicated that shedding of GPIIb/IIIa from platelets membrane upon stress in lung, cardiovascular and renal diseases and could a reliable early diagnostic marker [10]. In consistent, it has been demonstrated that the lung injury enhances the platelets aggregation-induced heatstroke and multi-organ failure [24]. Furthermore, a previous study indicated the role of platelet derived growth factor (PDGF) expression in mediating the neuro-inflammation action, blood–brain barrier and cerebrovascular disruption in HIV patients [25]. A recent study pointed to the strong association between pneumonia (14%) and thromboembolism (range from 3% in Italy to 80% in China and Brazil) with high mortality among COVID-19 patients [26].

3.4. GPIIb/IIa inhibitor therapy for COVID19 based on the assessment of its level

Assessing the GPIIb/IIIa level in COVID-19 patients is clearly essential because of its pivotal role in platelet aggregation and subsequent induction of thrombus formation, the major deleterious and lethal consequence of COVID-19. Experimental and clinical studies supported the efficacy of GPIIb/IIIa inhibitors either synthetic or natural products in counteracting the pulmonary inflammation and embolism [27]. In this aspect, GPIIb/IIIa inhibitors such as Tirofiban, Eptifibatide, and Abciximab were approved

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for intravenous administration to prevent platelet aggregation, therefore, interfering with thrombosis progress [28-31]. In addition, natural products such as Isomaltol and pentagalloyl glucose inhibit the gene expression of GPIIb/IIIa [32]. Surprisingly, one study mentioned to the benefit of GPIIb/IIIa inhibitor in the treatment of COVID-19 patients suffering from myocardial infarction [33], while another previous study detected a pulmonary haemorrhage among patients post percutaneous coronary intervention with synthetic inhibitor; Abciximab [34]. An explanation is the polymorphic variations among different populations [8, 20]. Thus, COVID-19 patients who showed elevated level of GPIIb/IIIa are the only target for GPIIb/IIIa inhibitors.

Since the beginning of COVID-19 pandemic, all events and research supported our findings in that there is no general or global treatment protocol showed efficacy in opposing SARS-CoV-2 infectivity. Subsequently, discrimination of COVID-19 patients according to the level of GPIIb/IIIa may not only predict the clinical outcomes but also improve it via targeting individual therapy, subsequently, limiting the adverse effects. To our knowledge, the current study is the first study that mentions the relevance of the determination of the cut-off score of GPIIb/IIIa to predict clinical outcomes of COVID-19 in addition to being a target for therapy. Our study may be a preliminary study for further clinical trials designed to examine the efficacy of GPIIb/IIa inhibitor therapy in improving the clinical outcomes of COVID-19 patients who have elevated levels of GPIIb/IIa as diagnosed using the ELISA technique. We further suggest that researchers strongly consider safe natural products for GPIIb/IIIa inhibitors rather than synthetic products to avoid adverse effects.

4. Conclusion

We conclude that using a simple, reliable and low-cost ELISA method compared to flow cytometry in detection of GPIIb/IIIa level may help in the predicating PE in COVID-19 patients. In addition, GPIIb/IIIa provides a novel target for more approved therapies either synthetic or natural products in the treatment of COVID-19.

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