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Identification of Microclot by Fluorescent Microscopy in Patients with Long Covid-19 Infection

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ABSTRACT

The Corona virus disease 2019 (COVID-19) is characterized by acute clinical pathologies, including various coagulopathies that may be accompanied by hyper coagulation and platelet hyper activation. In this study, we collected and analyzed plasma samples from Long covid infection patients and healthy individuals and found out that the former has significant microclots in their blood compared to normal individuals. Also, other obvious variations between long-covid patient samples and normal ones were noted which is respiratory failure and hypoxia. People getting affected with covid-19 may or may not have life threatening prothrombotic problems. The technique of Fluorescence microscopy using thioflavin dye (ThT) was employed to study plasma samples obtained from long covid patients and healthy individuals. Plasma sample from both long covid patients and healthy individuals were found to contain microclots but long covid-19 patients had severe microclots. This may be due to ineffective lysis of the insoluble fibrin clot due to deviations from the normally functioning fibrinolytic system brought about by the dysregulated inflammatory molecules which inhibit the fibrinolytic system including up regulation of plasminogen activator inhibitor-1(PAI-1) which interferes with TPA function and results in dysregulated coagulation system. Detection of microclots can possibly help to identify patients with long COVID infection who might benefit from anticoagulant therapy. In addition to microclot formation, significant platelet dysfunction and a systemic endothelilitis drive systemic cellular hypoxia.

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Key Words
Variety of symptoms, including fatigue, post-exercise malaise (PEM), loss of focus, myalgias, endothelilitis, healthy fibrin

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INTRODUCTION

The SARS-CoV-2 virus-induced corona virus disease 2019 (Covid-19) has produced a rapid, significant rise in pneumonia hospitalizations with multi-organ issues. These symptoms can last for up to 6 months or more after an acute infection. Severe vascular problems, including thrombotic consequences, may be caused, in particular, by platelet and erythrocyte pathology[10]. This work uses the terms fibrin and fibrinogen to refer to healthy soluble fibrinogen and healthy fibrin, which is the term used to refer to healthy fibrin nets formed during normal physiological processes. The term fibrin (ogen) is used to refer to both soluble and polymerized fibrin/fibrinogen, viewed as unhealthy. Circulating inflammatory chemicals, including inflammagens from viruses and bacteria, may attach to fibrinogen, causing some of the proteins to polymerize into microclots[6-10]. Fibrin (ogen) could potentially be abnormal or amyloid in nature[5,6]. In the current study, we look for any deregulated molecules in the blood that might be responsible for the lingering symptoms in people with long COVID/PASC. These signs could be the result of lingering, fibrinolysis-resistant plasma clots that are circulating in the blood[1-4]. In-depth investigation is needed into the pathophysiology, clinical characteristics (phenotyping), and therapy strategies of this global health issue. Patients who have had acute coronavirus illness 2019 (COVID-19) run the chance of developing long COVID[11-15]. Long COVID [LC] can have an impact on people of any gender or age, regardless of how severe their acute COVID-19 condition is. Long-term conditions (LC) can cause a variety of symptoms, including fatigue, post-exercise malaise (PEM), loss of focus, myalgias, cognitive impairment (or "brain fog"), shortness of breath and neurological or cardiovascular symptoms (such as myocardial inflammation, palpitations and tachycardia that may manifest as postural orthostatic tachycardia syndrome (POTS)[11]. The virus itself, its interaction with the endothelium and subsequent endothelilitis and endothelial dysfunction, as well as the participation of humoral response and autoantibodies, appear to interact over time[11]. It is thought that microclots and neutrophils may be involved in reducing systemic blood circulation[11]. Breathlessness, exhaustion, chest pain, myalgia, cognitive impairment, innate immunological reactions linked to inflammatory cytokine release and a pro-coagulant state are just a few of the symptoms of protracted COVID[1]. Guidelines and recent consensus are available, but they are not specific enough to let the doctor decide whether to administer thromboprophylaxis to each of their particular COVID-19 patients who are recovering from an illness. Investigation continues into the long-term impact of COVID-19 infection[1]. We argue (with supporting data) that a significant portion of the long COVID an etiology can be attributable to the development of abnormal amyloid fibrin microclots, which are specifically induced by the SARS-Cov-2 spikes protein. These amyloid microclots are the primary cause of the numerous long COVID symptoms because they prevent erythrocyte transport to capillaries and, consequently, O2 transfer[1]. The main co-morbidities associated with SARS CoV-2 infection should be distinguished from platelet and microclot pathology caused by Long COVID/PASC, cardiovascular disease (CVD), high blood pressure, hypercholesterolemia, or diabetes[2]. In the initial period of the disease and, in some unfortunate individuals (those who experience the "Long-COVID" syndrome), for several weeks or months afterward, present microclots would be responsible for so many, if not the majority of the odd signs of COVID 19[7].

MATERIALS AND METHODS

Ethical Clearance: Ethical clearance for the study was obtained from the health research ethics committee (HREC) of Ramachandra medical university (reference: CSP/22/JUN/111/337). The experimental objectives, risk and details were explained to volunteers and informed consent were obtained prior to blood collection. The ethical guidelines for research were kept for the duration of the study and for all research protocols.

Sample Demographics and Considerations: Blood was collected from healthy volunteers (N=20., 13 males, 07 female) to serve as controls. Healthy volunteers did not smoke, or suffering from cardiovascular disease or coagulopathies and pregnancy, were exclusion criteria. Patients that affected from long COVID infection (N=20., 14 males, 06 females). These patients affected from long-COVID-19 symptoms for at least 2 months after they have recovered from acute COVID-19.

Blood Sample Collection: Either a qualified phlebotomist or medical practitioner drew the citrated blood samples [4.5mL sodium citrate tubes (BD vacationer)], via venipuncture, adhering to standard sterile protocol and drew the EDTA blood samples [4.5mL EDTA tubes for CBC values of normal healthy individuals and long covid infection patients. citrate tubes is centrifuged at 3000x g for 15min at room temperature.

Plasma Sample of Long Covid-19 and Healthy Individuals: Fluorescence Microcopy to Show Microclot Formation: To study microclot in plasma sample of long covid infection patients and normal individuals were exposed to the thioflavin (ThT) (final concentration: 0,005 mM) (sigma-Aldrich. St. Louis, Mo, USA) for 30 min at room temperature. this ThT method was developed to visualize microclots.

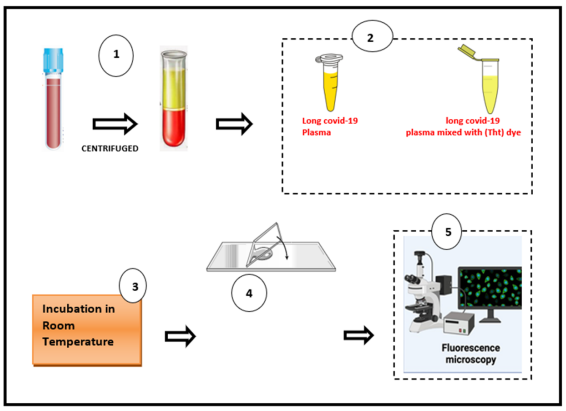

Fig. 1:Procedure: Collected Blood Samples in Citrate Tube from Long Covid-19 Infection Patients and Centrifugation
(2) Take 100 Microliter Pippete of Patient's Plasma Sample in Plastic test Tube and Add 100 Microliter of Thioflavin Dye (ThT)
(3) Incubated at Room Temperature for 30 Minutes
(4) After Incubation Keep a Drop on a Clean Glass Slide and Place a Cover Slip on It
(5) Finally View Under Fluorescent Microscope 450nm

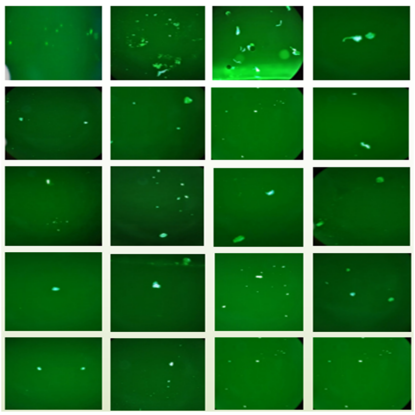

Fig. 2: Shows that Minimal Microclot of Normal Healthy Patients

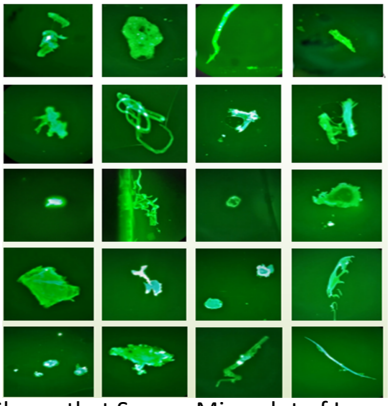

Fig. 3: Shows that Severe Microclot of Long COVID-19 Infection Patients

Statistical Analysis: Statistical analysis was performed using SPSS system software for windows. All data were subjected Shapiro-wilks normality tests. We measured the microclot by using imageJ1.53 (Wayne rasband and contributors national institutes of health, USA) application and calculated the mean, median, area of these data. (Fig. 4) shows mean median and area of clot formed in normal individuals, (fig 5) shows mean median and area of clots in patients with long covid infection. And two-sample independent t test was performed. After all of this calculation we found that result of this statistical analysis is microclot in long covid infection patients is significant. P value is (<0.0000001) Table (1).

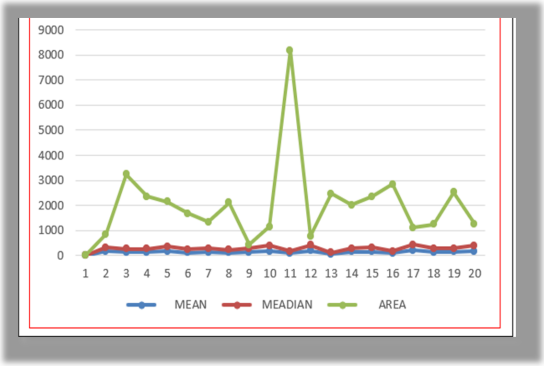

Fig. 4: Mean, Median, Area of the Clots in Normal Individuals

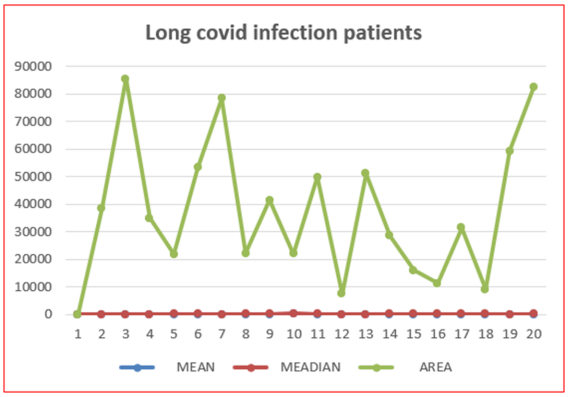

Fig. 5: Mean, Median and Area of Clot in Patients with Long COVID Infection

RESULTS AND DISCUSSIONS

Two Samples Independent t Test s: The severity of coronavirus illness 2019 (covid-19) is correlated with abnormal coagulation traits[15]. Disseminated intra vascular coagulation was the term used in the earliest accounts of covid-19 coagulopathy[11,15]. However, the majority of patients continue to have normal levels of clotting factors, fibrinogen and platelet, indicating that COVID-19 generates a distinct prothrombotic state that differs from conventional definitions of sepsis-induced

Table (1):Mean and Standard Deviation of Two Groups with Confidence Interval 95%

GROUP	Sample Size	Mean	Standard Devetion
Normal healthy individuals	20	1818.68	1705.1
Long covid infection patients	20	39025.63	24405.63

	T Statistics	DF	P-Value	Mean Different	Lower Limit	Upper Limit
Equal Variance	-6.8013	38	<0.0000001	-37206.9	-48281.5	26132.4
Unequal Variance	-6.8013	19	0.000001708	-37206.9	-48656.9	-25757

Test Foe Equality of Variance	F Statistics	df(numerator. denominator)	P-Value
	204.871	19.19	<0.0000001

coagulopathy. Venous thromboembolism in COVID-19 patients has been reported more frequently lately[11,19]. Acute respiratory distress syndrome, multi-organ failure and atypical interstitial bilateral lung are symptoms of severe disease in 20% of covid-19 patients who advance quickly[5,19]. The development of macro vascular thrombotic consequences, such as venous thromboembolism, stroke and cardiac injury/ infarction, was also demonstrated in one-third of patients hospitalized for severe COVID-19[5,19]. Right ventricular heart dilation affects patients as well. Additionally, autopsy of COVID-19 patients showed multi-organ damage patterns consistent with microvasculature injury. Additionally, tiny thrombi have been found throughout the lung according to autopsy findings[5,19]. The pulmonary arteries of COVID-19 patients exhibited extensive thrombosis with micro angiopathy, according to a paper by Ackermann and colleagues published in 2020. In addition, the researchers discovered that patients with COVID-19 had alveolar capillaries micro thrombi nine times more frequently than those with influenza[5,20]. An effective lysis of the insoluble fibrin clot depends on a fibrinolytic system that is functioning normally. Plasminogen activator inhibits the formation of insoluble fibrin clots in unhealthy fibrinolytic systems, preventing the lysis of these clots[4]. For this laboratory analysis, we collect 40 samples in total (plasma). 20 individuals with long-term COVID-19 infections and 20 controls (normal, healthy people). Each process sample was looked at under a fluorescence microscope. The obvious variations between long-covid patient samples and normal ones. While the micro clot in the sample of healthy participants was small, regular and unimportant, the micro clot in the sample of long-term covid patients was huge, irregular and important. We can aspirate some more particles that are on the Microclot as well. In samples from healthy patients, we can find a lot of little microclots, but in samples from COVID-19 patients, there were just a few large microclots that were absorbed. In 79% of the individuals receiving treatment, the microclots shrank along with clinical improvement[10]. Nine of the eleven individuals who still tested positive after therapy had relapsed within 1-2 months[20,9]. Blood from specific illnesses has previously been observed to have stellate microclots that stain for fibrin[19,10]. Treatment with warfarin quickly reduced the risk of microclot formation, but the psoriatic lesions showed little to no improvement. For a few hours, the development of microclots was prevented by high doses of strong corticosteroids administered while occluded[20,10]. Endotoxin release from severe psoriasis may be possible and it may be reduced by effective treatment[16-20]. Fragile microclot may momentarily occlude sporadic areas of the microvasculature as they form and dissolve throughout the circulation[7,10]. Repeated studies conducted over a period of days revealed that a transient release of endotoxins can take place in patients who initially do not produce microclots[20,6]. Two distinct but well-studied mechanisms are probably what govern how these fragile early microclots behave. The dynamics of protofibrils and early fibrin microclots are substantially greater than initially thought[7,12]. By fusing a platelet flow mechanical sensing platform based on collagen microtissue arrays with a microfluidic integrated microclot array elastometry (clotMAT) technology[14,10]. In 79% of patients, the microclots shrank as their clinical conditions improved with treatment. Nine of the eleven patients who had a positive test after therapy and who had a recurrence within a month or two showed up after treatment with a positive test[20,10] Treatment with warfarin quickly reduced the risk of microclot formation, but the psoriatic lesions showed little to no improvement. For a few hours, the development of microclots was prevented by high doses of strong corticosteroids administered while occluded[20,10] Endotoxin release from severe psoriasis may be possible and it may be reduced by effective treatment[20,8]. Inhibition of fibrinolysis furthers dysregulated hemostasis in COVID-19-associated disseminated intravascular coagulation, suggesting the plasminogen-plasmin-system as a viable target to stop thromboembolic consequences in COVID-19 patients[12,6]. Fragile microclots may momentarily occlude sporadic locations of the microvasculature throughout the body as a result of microclots developing and dissolving throughout the circulation[7]. Despite anticoagulant medication, autopsy results for COVID-19 patients revealed that respiratory failure with exudative widespread alveolar injury and severe capillary congestion was the primary factor in these patients' deaths.

CONCLUSION

In current study we have found that long covid patients have significant microclot in comparison to normal individuals. People getting affected by covid 19 have a may or may not be have a life threatening prothrombotic problems. In addition to microclot formation, significant platelet dysfunction and a systemic endothelilitis drive systemic cellular hypoxia. Presence of microclots can possibly help to identify patients with long COVID infection who might benefit from anticoagulant therapy.

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