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Article

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The occurrence of hyperactivated platelets and fibrinaloid microclots in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

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ABSTRACT

We have previously demonstrated that platelet poor plasma (PPP) obtained from patients with LongCovid/Post-Acute Sequelae of COVID-19 (PASC) is characterized by a hypercoagulable state reflected by hyperactivated platelets and the presence of considerable numbers of fibrin(ogen) amyloid microclots or fibrinaloid microclots. Due to substantial overlap in symptoms and aetiology between LongCovid/PASC and ME/CFS, we investigated whether coagulopathies – fibrinaloid microclots, platelet hyperactivation and/or fibrin amyloid formation – differed between individuals exhibiting ME/CFS and gender- and age-matched healthy controls. ME/CFS patients were statistically far more hypercoagulable as judged by thromboelastography of both whole blood and platelet-poor plasma. The area of plasma images containing fibrinaloid microclots was commonly more than 10-fold greater in untreated platelet-poor plasma from individuals with ME/CFS than in that of healthy controls. A similar difference was found when the plasma samples were treated with thrombin. Using fluorescently labelled PAC-1, which recognizes glycoprotein IIb/IIIa, and CD62P, which binds P-selectin, we observed massive hyperactivation and spreading of platelets in samples from individuals with ME/CFS. Using a quantitative scoring system, this was found to have a score of 2.72 ± 1.24 vs 1.00 (activation with pseudopodia formation) for healthy controls. We conclude that ME/CFS is accompanied by substantial and measurable changes in coagulability, platelet hyperactivation, and fibrinaloid microclot formation. However, fibrinaloid microclot load was not as prevalent as was previously noted in LongCovid/PASC. Fibrinaloid microclots in particular can provide a ready explanation, via (temporary) blockage of microcapillaries and hence ischaemia, for many of the symptoms, such as fatigue, seen in patients with ME/CFS. The discovery of these biomarkers pointing to significant and systemic endothelial inflammation, represents an important development in ME/CFS research. It also points at possible uses for treatment strategies using known drugs and/or nutraceuticals that target systemic vascular pathology and endothelial inflammation.

KEYWORDS: Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS); platelets; fibrinaloid microclots; hypercoagulability

INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating, multisystem disease that currently lacks a definitive diagnostic biomarker, an effective treatment, and a clear and widely accepted aetiological explanation. Symptoms of the condition include pathological fatigue (not influenced by exertion) that goes unresolved with rest and sleep; other symptoms include sleep perturbations, joint and muscle pain, intolerance towards exercise, headaches, gastrointestinal issues, flu-like symptoms, and cognitive impairments (Bested and Marshall, 2015, Clayton, 2015). Furthermore, the prevalence figures of ME/CFS are blurred by inconsistent case definitions, self-reporting assessments, and a large proportion of undiagnosed patients (Johnston et al., 2013, Clayton, 2015, Lim et al., 2020). Regardless of the inconsistent definitions, it has been estimated that there are 1.5 million individuals suffering from ME/CFS (low-end value) in the USA alone (Jason and Mirin, 2021). Alarmingly, the latest predictions suggest that the prevalence of ME/CFS might face a period of rapid growth, possibly by as much as a factor of six (Mirin et al., 2022).

Most cases of ME/CFS begin with a viral infection or involve multiple exposures to viral and/or bacterial pathogens over time (Chia et al., 2010, Kerr, 2020, Shikova et al., 2020, Ariza, 2021). Viruses implicated in initiating or exacerbating the ME/CFS disease process include human herpes virus (HHV)-6, HHV-7, Epstein-Barr virus (EBV), cytomegalovirus (CMV), enteroviruses, human parvovirus B19, and coxsackie B virus (Rasa et al., 2018). With regards to bacteria, gut dysbiosis and gram-negative LPS have been suggested to play a role in ME/CFS pathology (Proal and Marshall, 2018, Maes et al., 2012, Giloteaux et al., 2016). Patients have also met the diagnostic criteria for ME/CFS after infection with bacterial pathogens including *Coxiella burnetii* (Q fever) or bacteria in the genus *Brucella* (brucellosis) (Hickie et al., 2006, Raijmakers et al., 2020, Keijmel et al., 2015).

More recently, 10-30% of patients infected with the SARS-CoV-2 virus driving the COVID-19 pandemic are developing chronic symptoms that overlap greatly with those of ME/CFS. These patients are being given the diagnosis LongCovid or Post-Acute Sequelae of COVID-19 (PASC) (Al-Aly et al., 2022). The overlap between LongCovid/PASC and ME/CFS symptoms is so profound that many LongCovid/PASC patients meet the diagnostic criteria for ME/CFS after 6 months of ongoing symptoms (Wong and Weitzer, 2021, Hunt et al., 2022, Morrow et al., 2022, Siberry and Rowe, 2022, Kedor et al., 2021, Proal and VanElzakker, 2021).

We have demonstrated that LongCovid/PASC platelet poor plasma (PPP) contains large anomalous fibrin/amyloid deposits (fibrinaloid microclots) and hyperactivated platelets (Pretorius et al., 2021). The fibrinaloid microclots are relatively resistant to fibrinolysis even

after trypsinization. When solubilized in the laboratory via a second trypsinization step, it was shown that they contain inflammatory molecules and clotting factors including α(2)-antiplasmin (α2AP), various fibrinogen chains, and serum amyloid A (SAA). This pathology may lead to significant endothelial inflammation, (temporary) capillary blockage and hypoxia (Kell et al., 2022).

Due to the substantial overlap in LongCovid/PASC and ME/CFS symptoms and aetiology, it is very likely that coagulation-based pathology may also contribute to non-SARS-CoV-2 onset ME/CFS (cases initiated or exacerbated by other pathogens). Despite some inconsistencies in the literature (Kennedy et al., 2006, Brenu et al., 2010), there is precedent for hypercoagulability and platelet activation in ME/CFS. Brewer e*t al.* found that ME/CFS individuals presenting with active HHV-6 infection exhibited a state of hypercoagubility (Brewer and Berg, 2001), although over 80% of subjects involved in the study possessed hereditary risk factors for thrombosis, thereby skewing the precision of this interpretation. Another study demonstrated a hypercoagulable state with platelet activation, and suggested that fibrin deposits in microcirculatory vessels (perhaps fibrinaloid microclots?) can adhere to the endothelial lining and play a part in the manifestation of ME/CFS symptoms, possibly by impairing oxygen and nutrient delivery to tissues (Berg et al., 1999). More recently, platelet activity and markers have been implicated in ME/CFS, although the authors indicated a loss of significance after statistical corrections (Bonilla et al., 2022). Endothelial abnormalities have also been noted in ME/CFS (Newton et al., 2012, Scherbakov et al., 2020). For example, endothelial cells exposed to plasma from ME/CFS individuals exhibited functional defects (Bertinat et al., 2022).

There are clear molecular mechanisms by which viral/bacterial infection and/or chronic inflammation may contribute to coagulation-based sequelae in ME/CFS. Platelets can sense and bind to both viruses and bacteria via a variety of platelet receptors, and dozens of viral and bacterial products stimulate platelets, modulating their function (Lopes Pires et al., 2017, Li et al., 2020, Page and Pretorius, 2020, Antoniak and Mackman, 2021). If these infections or associated inflammation do not resolve, perpetual platelet stimulation by pathogens or their molecular products may lead to platelet hyperactivation and clotting sequelae. Pro-inflammatory processes and chronic inflammation alone can also prompt the coagulation system to take on a hypercoagulable state (Branchford and Carpenter, 2018, Pretorius et al., 2018, Aksu et al., 2012, Kell and Pretorius, 2015, Bester et al., 2015, Nunes et al., 2020, Abou-Ismail et al., 2020).

In this study we investigate if amyloid fibrinaloid microclots are present in ME/CFS plasma, and to what extent. We also measure platelet activity and characterize the constituent nature of identified clots, as well as the viscoelastic properties of blood, which can determine the state of coagulability (hypocoagulable vs normocoagulable vs hypercoagulable) (Pretorius et al., 2017b, Laubscher et al., 2021).

METHODS

Ethical Statement

Ethical clearance for the study was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (South Africa) (N19/03/043, project ID #9521). For the volunteers who provided blood samples, the experimental objectives, risks, and details were explained to volunteers and informed consent were obtained prior to blood collection. Strict compliance to ethical guidelines and principles of the Declaration of Helsinki, South African Guidelines for Good Clinical Practice, and Medical Research Council Ethical Guidelines for Research were kept for the duration of the study and for all research protocols.

Sample Demographics and Blood Collection

Blood samples were obtained from healthy individuals (n=15; 9 females, 6 males) to serve as controls for comparison. Healthy volunteers were only included if they did not smoke, did not suffer from cardiovascular disease or any coagulopathies, not pregnant or taking contraceptives, or did not suffer from Long COVID. ME/CFS patients (n=25; 20 females; 5 males) were recruited via the ME/CFS Foundation of South Africa, and only included in this study if they had not previously been infected with the COVID-19 virus. Participants had to have been diagnosed for longer than 6 months, and were still asked to complete the International Consensus Criteria (ICC) questionnaire (Carruthers et al., 2011) to gain an understanding of their perspective of disease severity. Blood was collected in citrated tubes. Whole blood (WB) was used for viscoelastic studies, after which the samples were centrifuged at 3000×g for 15 min at room temperature to collect platelet poor plasma (PPP). Platelets were identified in the hematocrit, after PPP was removed and a stored at −80 °C for later analysis.

Viscoelastic Analysis

Clotting properties of both WB and PPP samples were measured by using the Thrombelastograph® (TEG®) 5000 Hemostasis Analyzer (Haemoscope Corp). Analysing WB can allow for the detection of clotting abnormalities influenced by blood as a whole, while TEG® of PPP allows one to assess the contribution of only the clotting proteins without cellular components (such as erythrocytes and platelets) (Varin et al., 2013). 20µL of 0.01M calcium chloride (required to initiate coagulation in blood drawn within citrate tubes) was added to the

TEG® cup, followed by 340µL of either WB or PPP. The test was promptly started and left to run until the maximal amplitude of the clot had been reached.

Fibrinaloid Microclot Analysis inside Platelet Poor Plasma (PPP)

PPP was used to study microclots presence in participants with ME/CFS, and compared to those present in healthy participants. PPP samples were incubated with the fluorescent probe, Thioflavin T (ThT) (Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 0,005 mM and for a period of 30 min, prior to viewing with a fluorescence microscope. ThT binds to open hydrophobic areas on fibrinogen that is indicative of amyloid protein changes (Gade Malmos et al., 2017, Kell and Pretorius, 2017, Pretorius et al., 2017a, Pretorius et al., 2016), Samples were viewed on the Zeiss Axio Observer 7 fluorescent microscope with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy, Munich, Germany), with the excitation wavelength set at 450-488nm and the emission wavelength set at 499-529nm. The % area of the fibrinaloid microclot presence was determined by analysing micrographs using ImageJ 1.53e. After setting the scale, images are converted to 8-bit. The threshold, using the Huang setting, was then set by increasing the white background intensity to 255 and the black (fluorescence) signal intensity to 13-17. Next, we used the 'analyse particles' assessment and set particle size at 1-infinity. Three representative images were chosen per subject. The data generated were then analysed with GraphPad Prism 8.4.3.

Fibrinaloid Microclot Analysis after addition of thrombin to create extensive fibrin clots

ThT was used again to identify amyloid presence and load within clot networks. 49µL of PPP was incubated with ThT (again at a final exposure concentration of 0,005 mM) for 30 minutes at room temperature. 5µL of the sample was then transferred to a glass slide, followed by 2,5µL of thrombin (7 $U.ml^{-1}$, South African National Blood Service). The sample was left to stand for 2 minutes in order for fibrin networks to form, after which a coverslip was placed on top of the clot. Samples were viewed on the Zeiss Axio Observer 7 fluorescent microscope with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy, Munich, Germany), with ThT's excitation wavelength set at 450-488nm and the emission wavelength set at 499-529nm. Fluorescent intensity of fibrin clot micrographs was calculated using ImageJ 1.53e. 'Mean Gray Value' and 'area' were chosen as measurement settings.

Platelet Assessment Using Fluorescence Microscopy

Two fluorescent markers, PAC-1 (FITC-conjugated) (340507, BD Biosciences, San Jose, CA, USA) and CD62P (PE-conjugated) (IM1759U, Beckman Coulter, Brea, CA, USA), were obtained to assess the state of platelets by using the Zeiss Axio Observer 7 fluorescent microscope with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy,

Munich, Germany). After centrifugation of the blood tubes and removal of the plasma, 20µL of haematocrit was slowly (due to its viscosity) pipetted and transferred to an Eppendorf microcentrifuge tube. After allowing the fluorescent markers to reach room temperature, 4µL of both PAC-1 and CD62P was added to the microcentrifuge tube containing 20µL of haematocrit. The incubation period lasted for 30 minutes, in a dark room, at room temperature. The excitation wavelength for PAC-1 was set at 450 to 488 nm and the emission wavelength at 499 to 529 nm; and the excitation wavelength for CD62P was set at 540 to 570 nm and the emission wavelength at 577 nm to 607 nm. Platelet phenotype is assessed with the grading system we have recently implemented (Laubscher et al., 2021), where platelet spreading and clumping scores were used and allocated a score of 1 to 4 for severity of spreading and clumping.

Statistical Analysis

Statistics were completed on GraphPad Prism 9.3.1. Data were subjected normality tests (Sharpiro-Wilks). Parametric data were then subject to unpaired t-tests, and non-parametric data were analysed with the unpaired Mann-Whitney test. Data are represented as mean ± standard deviation, or median [Q1-Q3]. Graphical data is represented as mean ± SEM.

RESULTS

Table 1 shows the demographics and the disease scoring of the ME/CFS cohort using the International Consensus Criteria (ICC) questionnaire for ME/CFS patients (Carruthers et al., 2011). Both healthy and ME/CFS population age data were normally distributed, and did not yield significant differences when analysed with an unpaired t-test ($p=0.54$). Comorbidities are present within the ME/CFS population: 40% of subjects are afflicted with leaky gut/gut dysbiosis; 16% afflicted with POTS, psoriasis, fibromyalgia, gingivitis/periodontitis, hypercholesterolemia, and hypertension, 12% afflicted with rheumatoid arthritis, and cardiovascular disease; and 4% afflicted with orthostatic hypotension, mast cell activation syndrome, rosacea, and dysautonomia. The ICC questionnaire results indicate that this study's ME/CFS population predominantly experiences symptoms related to post-exertional neuroimmune exhaustion (7.8 ± 1.6), with subjects scoring the least in the 'immuno, gastrointestinal, and genitourinary impairments' section (5.9 ± 2.7). Scores that are registered as severe (i.e. with a score of 8-10) are also depicted. 60% of the ME/CFS subjects experience post-exertional neuroimmune exhaustion in a severe manner; 44% and 45% experience severe neurological and energy production/transportation symptoms, respectively; and 35% report enduring severe immuno, gastrointestinal, and genitourinary impairments. Our ME/CFS population therefore constitutes a sub-population experiencing predominantly post-exertion-related symptoms.

Table 1: Demographics and scoring analysis of the ME/CFS cohort using the International Consensus Criteria (ICC) questionnaire for ME/CFS patients. Score averages per ICC questionnaire section are given in bold face. Statistical significance was recorded at $p<0.05$.

Demographics		
P value (parametric analysis)	0.54	
Age of control population (n=15; 9 females; 6 males)	45.7 ± 6.9	
Age of ME/CFS population (n=25; 20 females; 5 males)	48.2 ± 14.1	
Comorbidities of ME/CFS Population		
Comorbidity	% Prevalence	
Leaky Gut/Gut Dysbiosis	40%	
POTS	16%	
Fibromyalgia	16%	
Psoriasis	16%	
Gingivitis/Periodontitis	16%	
Hypercholesterolemia	16%	
Hypertension	16%	
Rheumatoid Arthritis	12%	
Cardiovascular Disease	12%	
Orthostatic Hypotension	4%	
Mast Cell Activation Syndrome	4%	
Rosacea	4%	
Dysautonomia	4%	
ICC Questionnaire Results		
Parameter	Average Score (out of 10)	% of Subjects Experiencing Severely (Score of 8-10)
1. Post-Exertional Neuroimmune Exhaustion	**7.8 ± 1.6**	**60%**
a. Marked, rapid physical and/or cognitive fatigability in response to exertion	7.9 ± 1.6	64%
b. Post-exertional symptom exacerbation (worsening of other symptoms)	7.8 ± 1.4	56%
c. Post-exertional exhaustion	8.1 ± 1.3	64%
d. Recovery period is prolonged	7.4 ± 2.0	48%
e. Low threshold of physical and mental fatigability (lack of stamina)	8.1 ± 1.7	68%
2. Neurological Impairments	**6.6 ± 2.6**	**44%**
a. Difficulty processing information	6.52 ± 2.00	32%
b. Short-term memory loss	6.64 ± 2.04	44%
c. Headaches	6.00 ± 3.27	40%
d. Significant pain (in muscles, tendons, abdomen, or chest)	6.68 ± 2.84	52%
e. Disturbed sleep pattern (from the previous night, e.g., insomnia, sleeping most of the day and being awake most of the night)	6.72 ± 2.82	48%
f. Unrefreshing sleep (from the previous night)	7.92 ± 2.08	60%
g. Neurosensory and perceptual symptoms (e.g., inability to focus vision, sensitivity to light, noise, etc.)	7.20± 1.87	56%
h. Motor symptoms (e.g., twitching, poor coordination)	5.12 ± 2.65	16%
3. Immuno, Gastrointestinal, and Genitourinary Impairments	**5.9 ± 2.7**	**35%**
a. Flu-like symptoms (e.g., sore throat, sinusitis, enlarged or tender lymph nodes)	5.48 ± 1.94	16%
b. Gastrointestinal tract symptoms (e.g., nausea, abdominal pain, bloating, irritable bowel)	6.32 ± 2.66	44%
c. Genitourinary symptoms (e.g., urinary urgency, urinary frequency, nocturia or having to urinate two or more times a night)	5.76 ± 2.96	32%
d. Sensitivities to food, medications, odors, or chemicals	6.16 ± 3.05	48%
Energy Production/Transportation Impairments	**6.6 ± 2.6**	**45%**
a. Cardiovascular symptoms (e.g., orthostatic intolerance, postural orthostatic tachycardia syndrome or POTS, palpitations, light -headedness/dizziness)	7.00 ± 2.38	52%
b. Respiratory symptoms (e.g., air hunger, labored breathing, fatigue of chest wall muscles)	5.64 ± 2.98	32%
c. Loss of thermostatic stability (e.g., subnormal body temperature, sweating episodes, recurrent feelings of feverishness, cold extremities)	7.28 ± 1.54	52%
d. Intolerance of extremes of temperature	6.44 ± 2.89	44%
Data are represented as mean ± standard deviation.		

TEG® analysis of WB and PPP is shown in Table 2. Data for WB analysis from ME/CFS participants were assessed against a standard range of values for the TEG® analysis, as provided by TEG® guidelines. Data for PPP from ME/CFS participants were compared to those of the controls. With regards to the TEG® analysis of ME/CFS WB, no subjects fell outside of the normal range for TMRTG and TTG parameters, but our data suggests that WB from participants with ME/CFS fell outside the normal ranges for R, K, α angle, MA and MRTG parameters (see Table 2). R and K angles reflect time-dependent properties, which indicate that our ME/CFS population clots at a rate higher than what is considered normal. ME/CFS participants exhibited larger and stronger clots (MA), which formed at a rate higher than controls (MRTG).

In PPP, significant differences between the control and ME/CFS groups were only observed within the α angle (**) and MRTG (***) assessments, where the ME/CFS group exhibited higher clotting values, again pointing to a hypercoagulable state.

Table 2: TEG® analysis recorded in whole blood (WB) and platelet poor plasma (PPP) in healthy individuals and those with ME/CFS.

TEG® of WB			
Parameter	Standard WB Range	ME/CFS WB (n=25)	Out of Standard Range
R	9-27	7.73 ± 2.31	16 (below)
K	2-9	2.39 ± 0.82	9 (below)
α-Angle	22-58	56.99 ± 10.25	13 (above)
Maximum Amplitude	44-64	65.64 ± 7.02	14 (above)
MRTG	0-10	10,05 [7,18-12,12]	13 (above)
TMRTG	5-23	9.74 ± 2.73	0
TTG	251-1041	787.50 ± 84.07	0
Data are represented as mean ± standard deviation, or median [Q1-Q3]. Statistical significance was recorded at p<0.05 (*=p<0.05; **=p<0.01; ***=p<0.001). The standard WB range for the various parameters is as indicated by TEG® guidelines.			
TEG® of PPP			
Parameter	Control PPP (n=15)	ME/CFS PPP (n=25)	P value
R	10.25 ± 1.97	8.54 ± 3.51	0.09
K	3.10 [2.80-3.80]	1.90 [1.40-4.75]	0.05
α-Angle	51.70 [45.00-57.10]	61.80 [54.00-66.25]	**0.002 (**)**
Maximum Amplitude	25.90 [23.00-27.10]	29.30 [22.05-32.55]	0.20
MRTG	8.05 [6.80-8.93]	12.17 [9.13-15.77]	**0.0009 (***)**
TMRTG	11.30 ± 2.20	9.68 ± 3.71	0.13
TTG	306.40 [283.30-324.10]	350.90 [261.30-391.00]	0.22
Data are represented as mean ± standard deviation, or median [Q1-Q3]. Statistical significance was recorded at p<0.05 (*=p<0.05; **=p<0.01; ***=p<0.001)			

Figure 1A shows typical fluorescence micrographs of fibrinaloid presence in PPP (without added thrombin) from a cohort of 25 participants with ME/CFS and from 15 healthy participants. Figure 1B shows a micrograph plate of a clot grading system that we previously developed (Laubscher et al., 2021). PPP smears from the control group express little ThT signal, whereas the ME/CFS smears exhibit substantial fluorescence signals. Figure 2A represents the fluorescence signal of fibrinaloid micrographs as mean % area of amyloid signal, with those being vaccinated against COVID-19 highlighted in a specific colour. It is known that COVID-19 vaccines have the capability to influence fibrinaloid microclot formation/clearance (Grobbelaar et al., 2021) – due to spike protein S1 activity. We therefore included the vaccination status of participants to account for confounding results. A nonparametric Mann-Whitney test was performed, which indicated a significant difference ($p<0.0001$) between the control (0.10 ± 0.54) and ME/CFS (1.37 ± 3.05) mean % area amyloid. These qualitative and quantitative data suggests that fibrinaloid burden is notably greater in this study's ME/CFS population when compared to the controls. It is important to establish whether vaccine status is correlated or not with microclot formation. Fig 2A shows that this was not the case; while slightly fewer controls than ME/CFS patients had been vaccinated, mostly at least 3 weeks before the samples were taken, there was no discernible influence of vaccination status on the % area of microclots within the two groups.

Figure 1A: Representative fluorescent micrographs of fibrinaloid microclot presence in platelet poor plasma from controls and individuals with ME/CFS. Images were taken at 63x machine magnification.

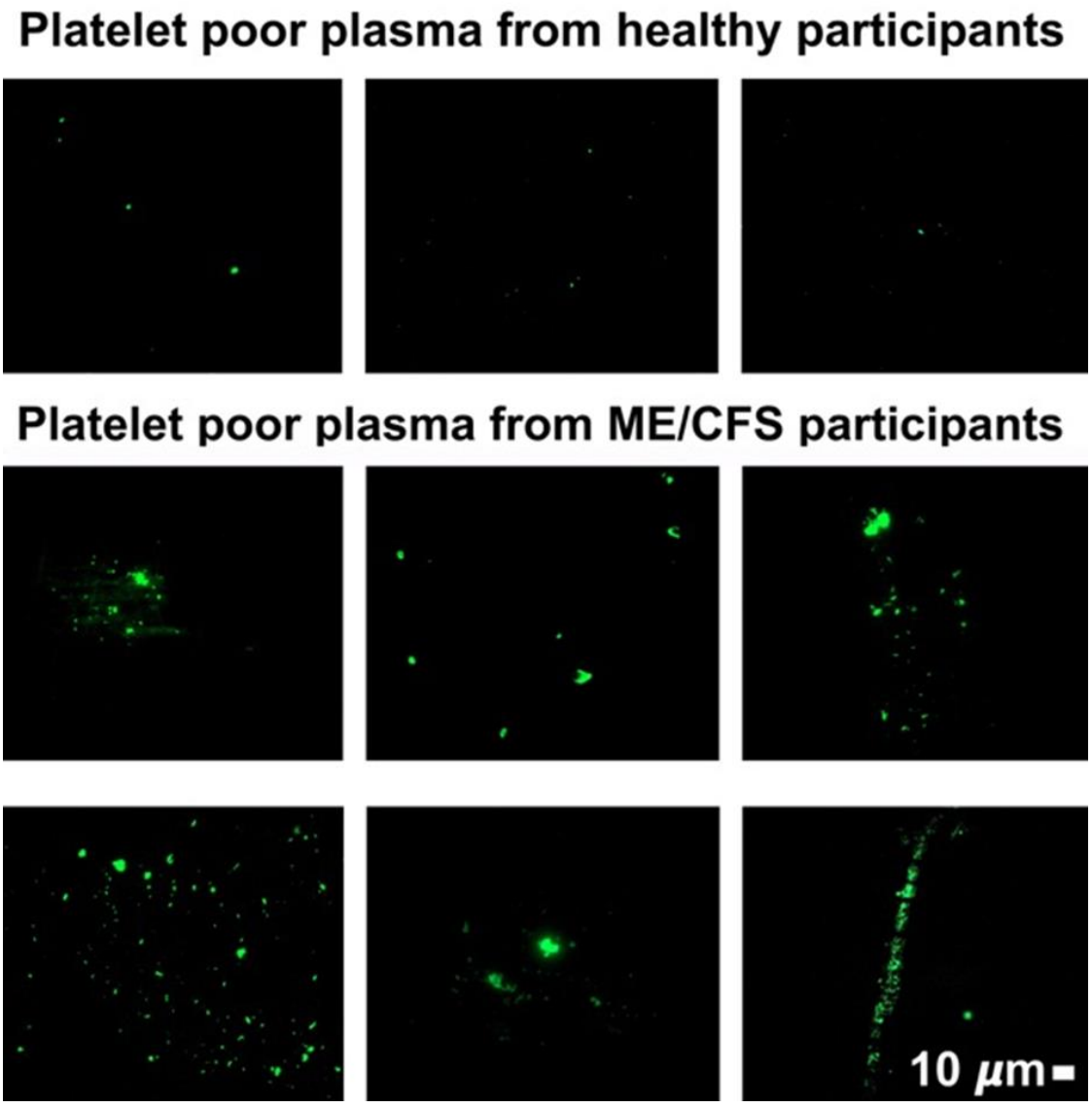


Figure 1B: Fluorescence microscopy showing fibrinaloid microclots in platelet poor plasma (PPP) with representative examples of the different stages of different stages of fibrinaloid microclot formation. Stage 1 shows minimal microclot formation in healthy/control PPP which progresses to the presence of the severe microclotting as seen in Stage 4. Bottom row represents examples of stage 4 microclots using (A) bright-field microscopy, (B) fluorescence microscopy, and (C) an overlay of fluorescence and bright-field microscopy (Laubscher et al., 2021).

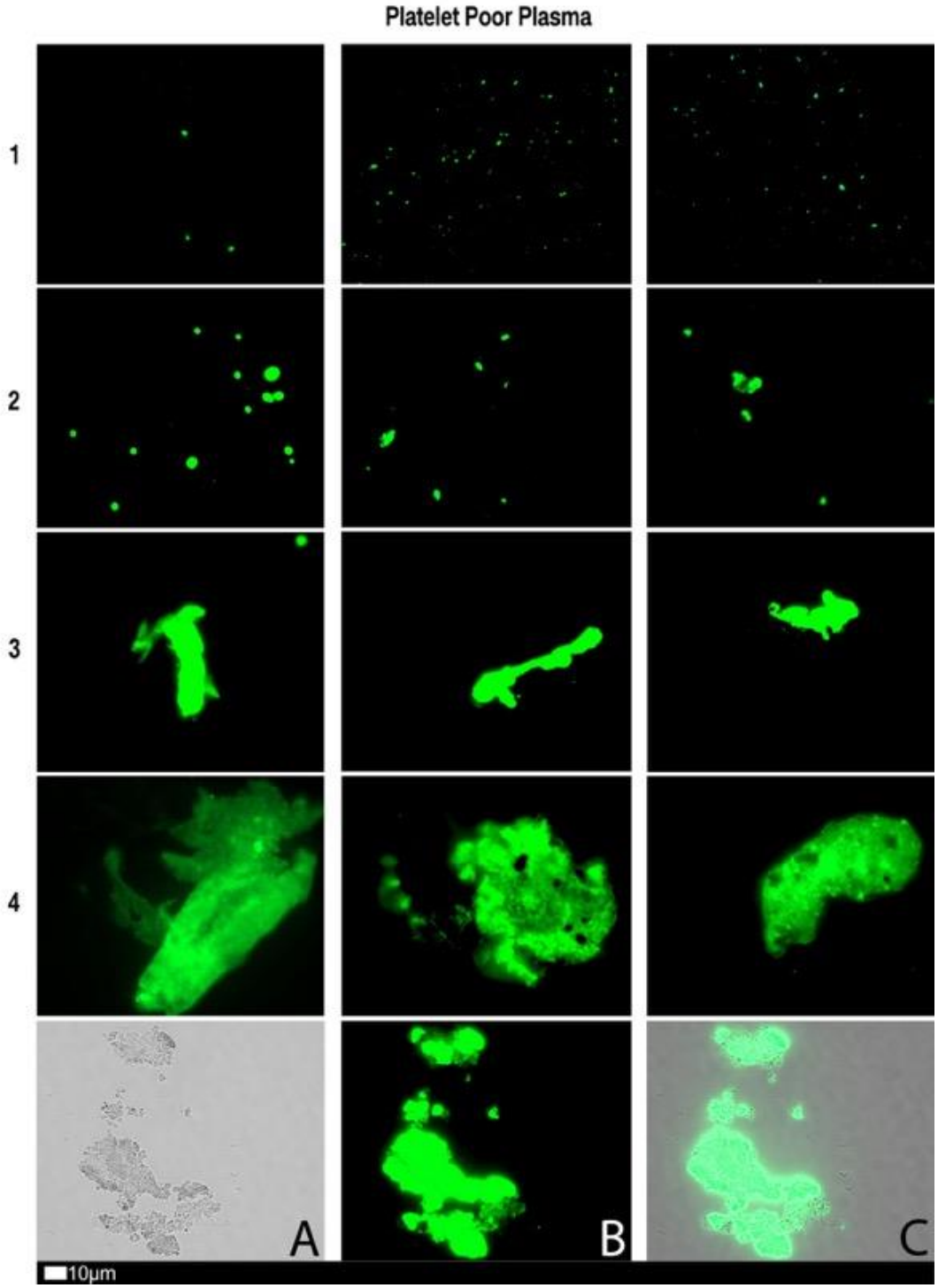
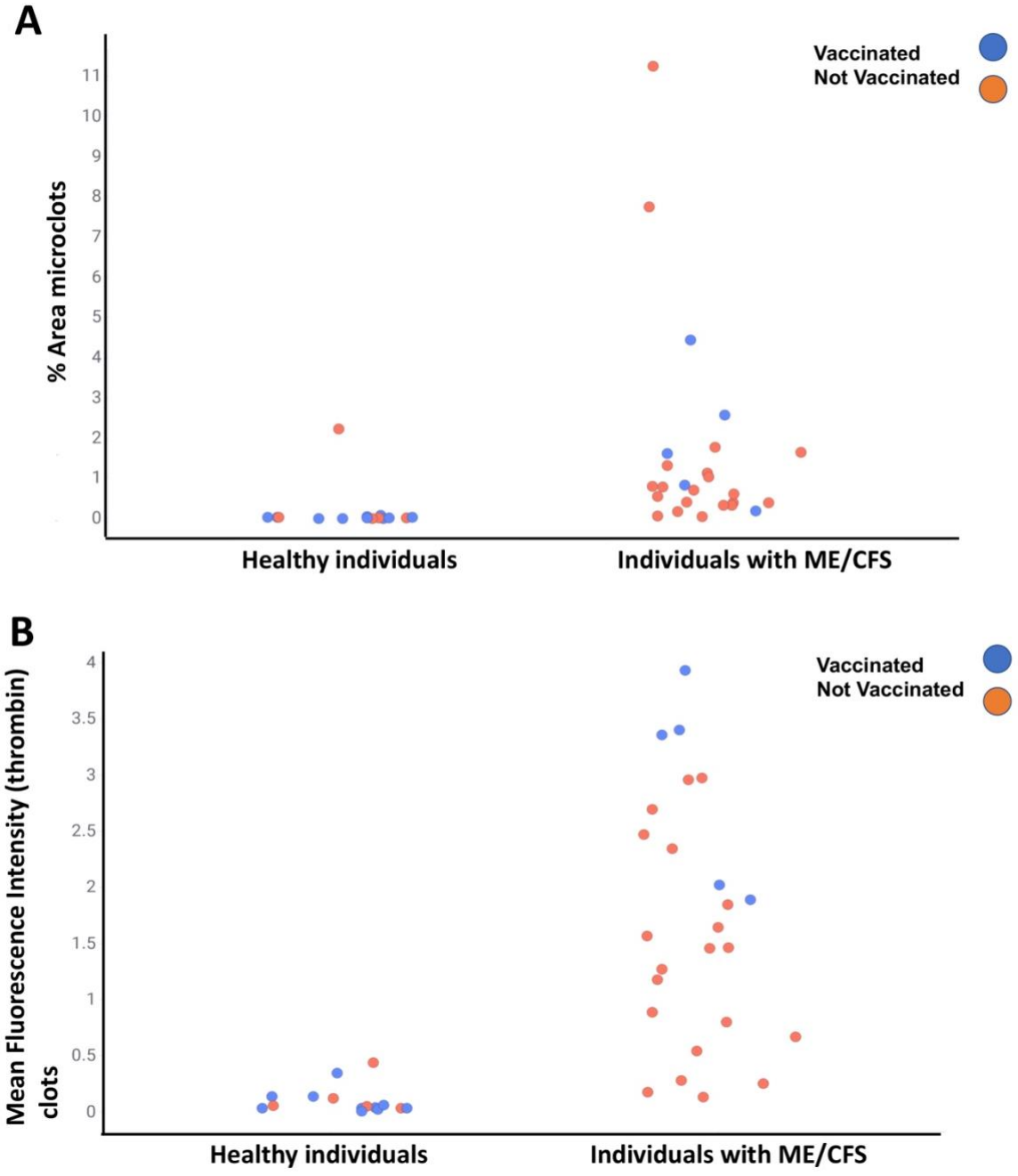
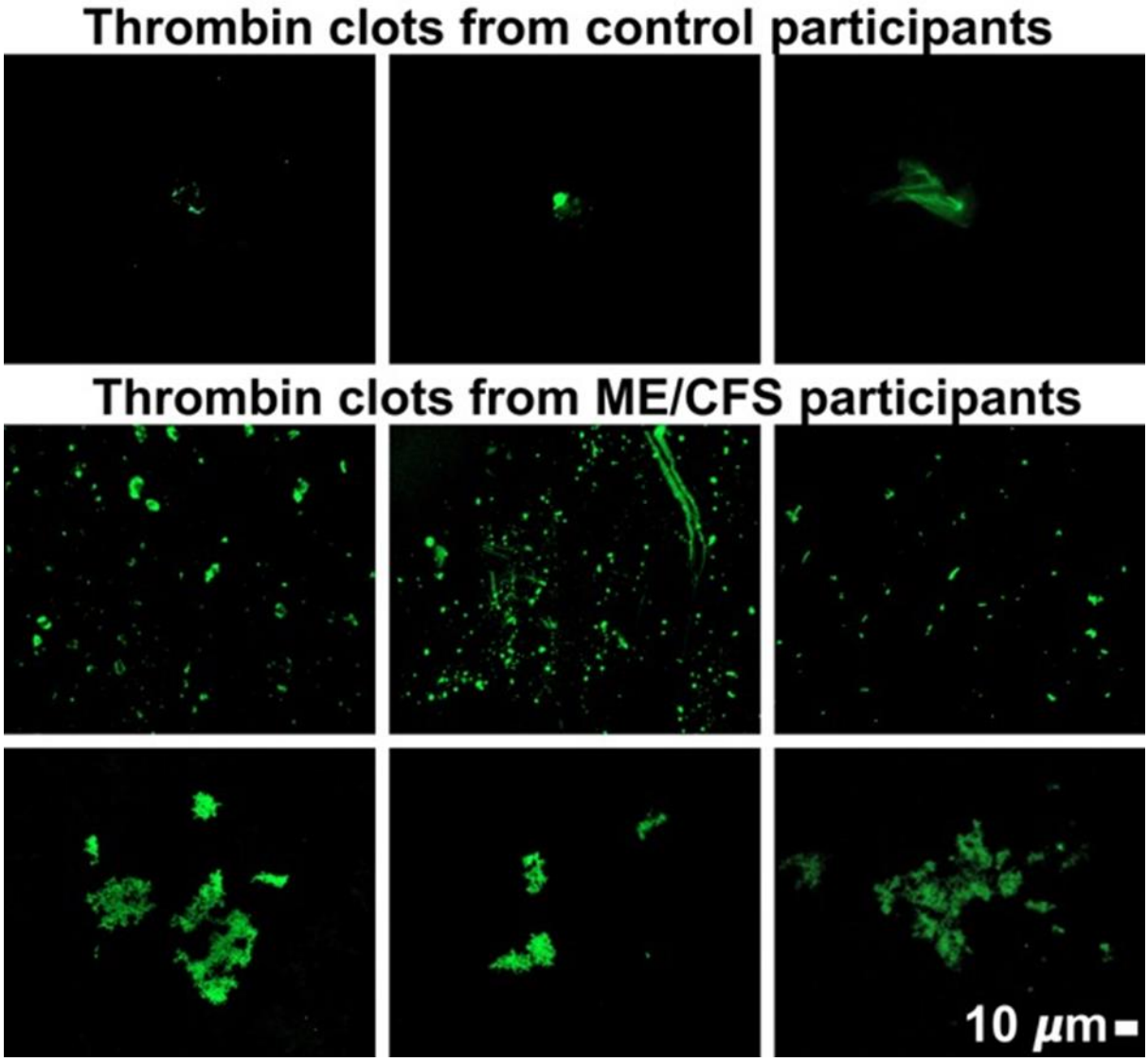


Figure 2: A) Mean % area of amyloid signal between control and ME/CFS groups represented as a strip plot. The COVID vaccination status of individuals is colour-coded. A Mann-Whitney analysis yielded a significant difference ($p<0.0001$) with the ME/CFS group exhibiting a greater mean (1.37) than that of the controls (0.10). **B)** Strip plot showing the difference in mean fluorescence signal between control (0.11 ± 0.19) and ME/CFS (1.69 ± 1.69) PPP fibrin amyloid networks induced by exogenous thrombin. Again, the COVID vaccination status is colour-coded. A significant difference (****) was determined by a Mann-Whitney test.



We also studied amyloid clot formation where thrombin was added to citrated PPP exposed to ThT, to form an extensive fibrin network. Figure 3 shows representative micrographs of healthy and ME/CFS fibrin networks. We also calculated the mean fluorescence intensity of the fluorescent signal. Fibrin networks created form PPP of participants with ME/CFS exhibited a much increased fluorescence signal compared to that of healthy participants, with control and ME/CFS mean fluorescent intensity valued at 0.11 ± 0.19 and 1.69 ± 1.69 (****), respectively.

Figure 3: Representative micrographs showing thrombin-induced fibrin networks stained with ThT from healthy participants and participants with ME/CFS. Images were taken at 63x machine magnification.



Platelet morphology was also studied after adding two fluorescent platelet markers, namely PAC-1 (FITC-conjugated), which recognizes glycoprotein IIb/IIIa, and CD62P (PE-conjugated), which binds P-selectin (see Figure 4A). Platelet scoring was done as per our previously developed scoring system (see Figure 4B and C) (Laubscher et al., 2021). The platelet populations from participants with ME/CFS exhibited a hyperactivated phenotype with significant spreading and granule release (2.72 ± 1.24). Comparably, control platelets are scored with a value of 1.00. 80% of ME/CFS haematocrit samples exhibited this phenotype, with 48% scoring 3 or 4 (severe end of platelet scoring range). Platelet clumping was observed (2.04 ± 1.21) in 52% of subjects, with only 32% scoring 3 or 4.

Figure 4A: Representative fluorescent micrographs of hematocrit samples from ME/CFS individuals stained with PAC-1 (green fluorescence); CD62P-PE **(**purple fluorescence); white areas represent overlap of the two markers. Images were taken at 63x magnification.

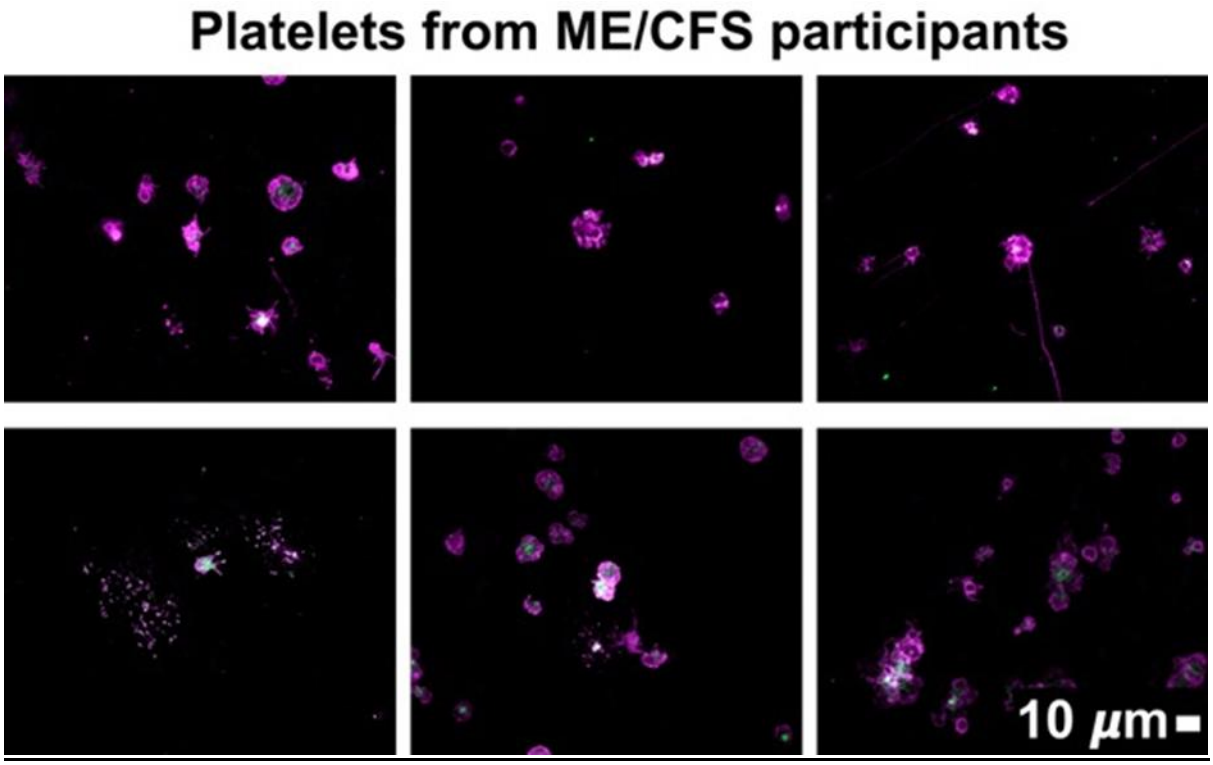


Figure 4B: Fluorescence microscopy examples of the different stages of platelet activation and spreading, that was used to score the platelet activation in the Long COVID patients, with Stage 1, with minimally activated platelets, seen as small round platelets with a few pseudopodia, seen as healthy/control platelets that progresses to Stage 4, with egg-shaped platelets, indicative of spreading and the beginning of clumping (Laubscher et al., 2021).

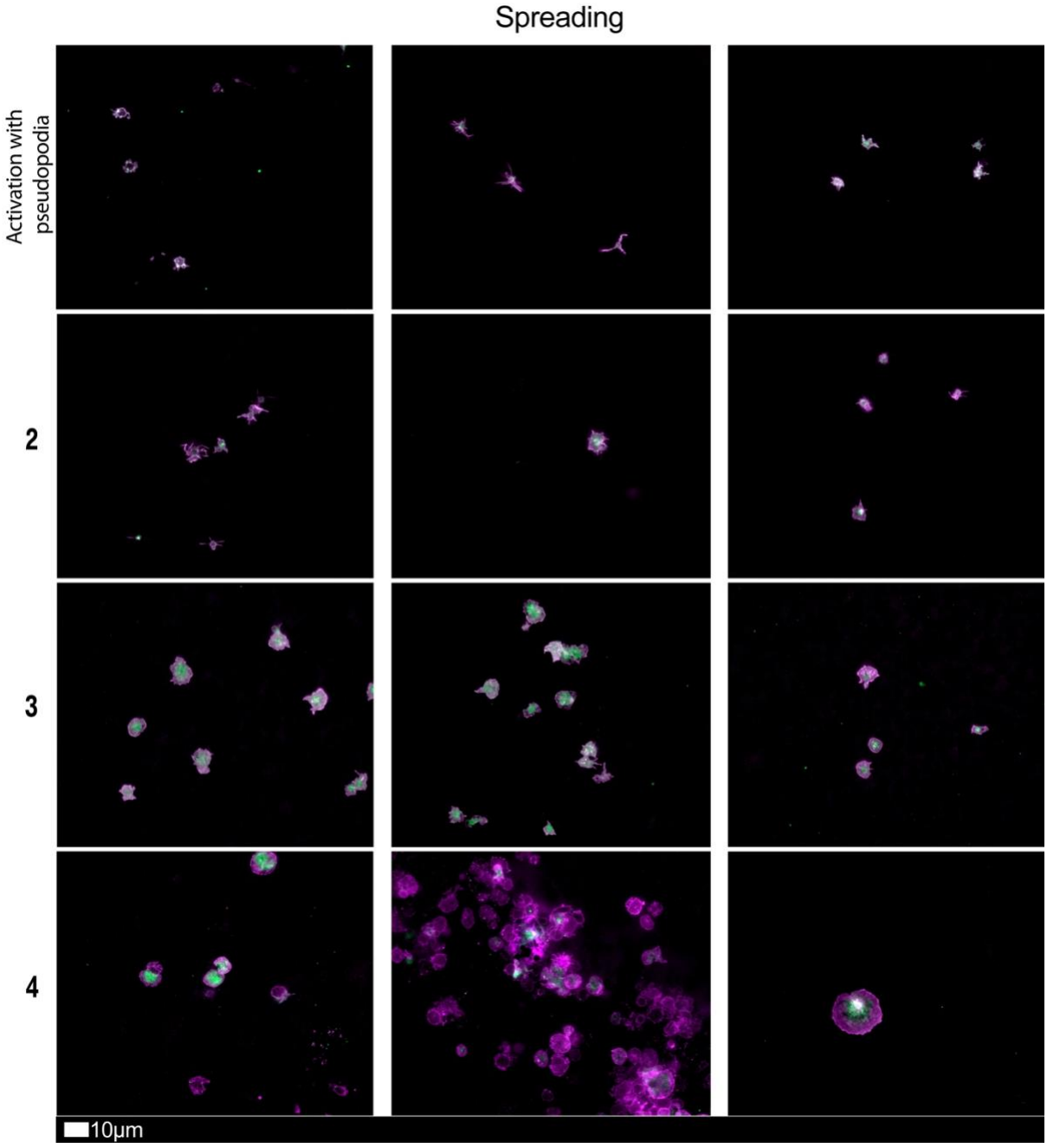
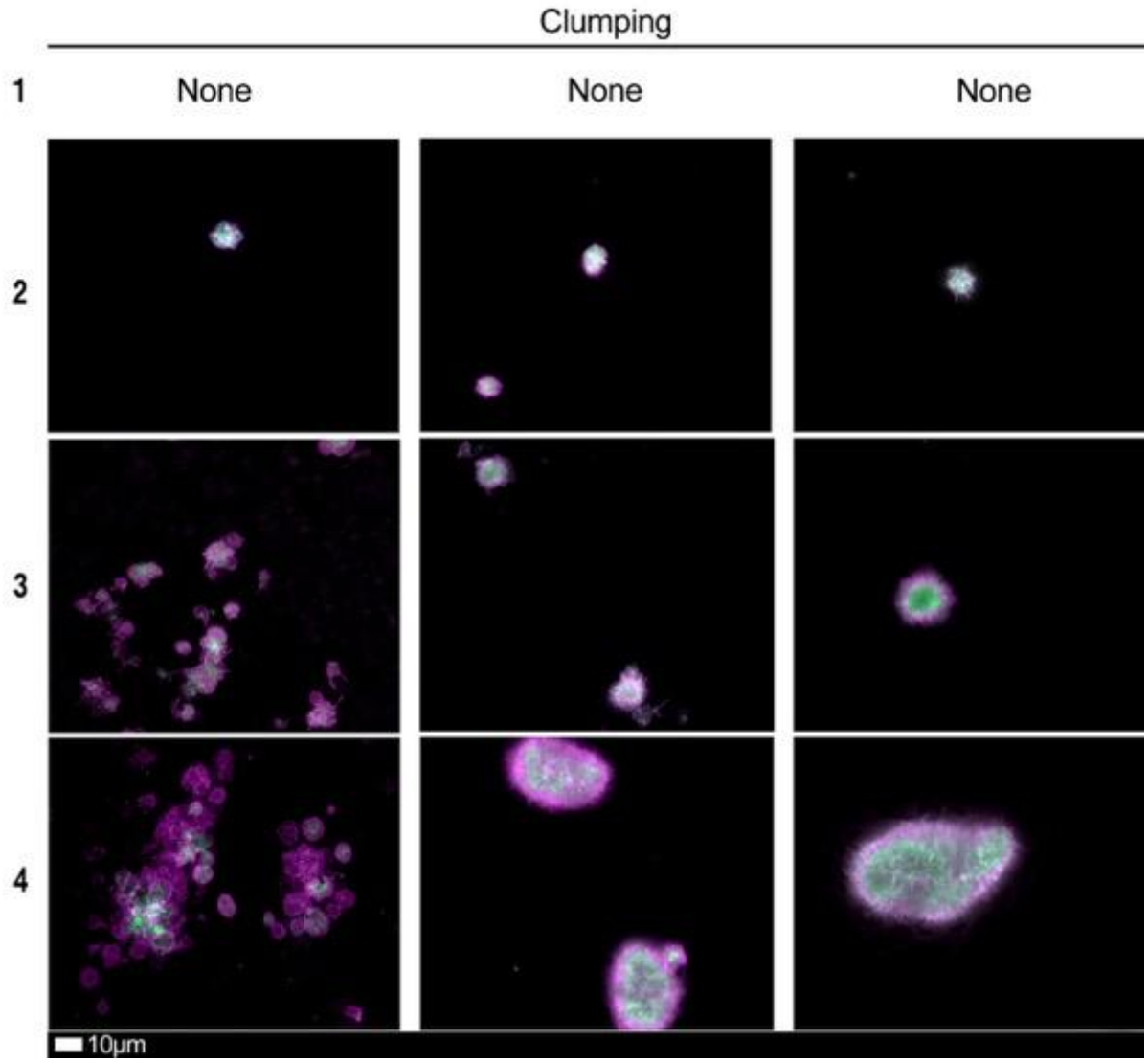


Figure 4C: Fluorescence microscopy examples of the different stages of platelet clumping. With no clumping occurring in the healthy/control samples in Stage 1 (no figures shown), progressing to severe clumping of platelets as seen in Stage 4 (Laubscher et al., 2021).



DISCUSSION

We have previously demonstrated that LongCovid/PASC platelet poor plasma (PPP) contains large anomalous fibrin/amyloid deposits (microclots) and hyperactivated platelets (Pretorius et al., 2021b). Due to the substantial overlap between LongCovid/PASC and ME/CFS symptoms and etiology, we performed a series of experiments to determine if fibrinaloid microclots are also present in ME/CFS plasma, and to what extent. We additionally characterized the constituent nature of identified ME/CFS fibrinaloid microclots, as well as the viscoelastic properties of participant blood, which can determine the state of coagubility (hypocoaguable vs normocoaguable vs hypercoagulable) (Pretorius et al., 2017b, Laubscher et al., 2021).

TEG® analysis demonstrated that a high proportion of ME/CFS participants present with a hypercoagulable state (Table 2). In the WB analysis, several participants fell out of the healthy range, tipping towards the hypercoagulable side of the scale. This was noted in all parameters except TMRTG and TGG. In PPP, significant differences were identified in only the α angle (**) and MRTG (***). However, mean ME/CFS PPP values for all TEG® parameters assessed also leaned towards the hypercoagulable end.

Fluorescence microscopy identified fibrinaloid microclots within the haematological system of ME/CFS individuals with a burden significantly greater than that of controls (Figure 1 and Figure 4A). However, we note that the extent of fibrinaloid microclot load as judged by % area is significantly lower than those seen in acute COVID-19 and LongCovid/PASC, and even in type 2 diabetes (Pretorius et al., 2021). A comparison of comorbidities, sensitivity and specificity is beyond the scope of the present pilot study, though, which was simply designed to assess whether those with ME/CFS differed in their coagulation behaviours from age- and gender-matched 'healthy' controls. These microclots were probed for without the addition of thrombin, thereby indicating that these clot particles are likely circulating through the blood of afflicted individuals. Furthermore, these fibrinaloid microclots are amyloid in nature (as inferred by ThT staining), and as such have been shown to resist degradation via fibrinolytic means (Grobbelaar et al., 2021, Pretorius et al., 2021, Kell and Pretorius, 2015, Kell and Pretorius, 2017). These amyloid microclots may contribute to poor blood circulation and perfusion, possibly by blocking microcapillaries (Kell et al., 2022), and impairing oxygen and nutrient delivery to various tissues. This provides a ready explanation for the fatigue and other symptoms experienced by individuals with ME/CFS. Furthermore, fibrinaloid microclots may contribute to further inflammation in the haematological system and at the endothelial linings of blood vessels in a feed-forward fashion.

Extensive fibrin clot networks (stained with ThT) were also induced where PPP samples were exposed to thrombin, and fibrin clots were assessed for the presence and load of amyloid fibrin(ogen). Note that this is different from the fibrinaloid analysis as we are adding thrombin to form a clot network instead of probing for fibrinaloid microclots in the absence of exogenous thrombin – this gives us insight into the molecular profile of fibrin networks mimicking the terminal stages of the coagulation cascade. Amyloid protein changes were markedly increased in thrombin-induced fibrin clots from the ME/CFS group compared to controls.

Platelet morphology in the ME/CFS population varied across subjects, with 48% and 32% scoring as severe (3 or 4) for spreading and clumping parameters, respectively.

Together, our results indicate that clotting abnormalities are present in the haematological system of ME/CFS individuals. However, it must be noted that not all participants presented with hypercoagulable values when measured by the TEG®, and not all participants possessed platelets indicative of a hyperactivated phenotype. Further study is therefore required to determine what factors, including possible coagulation-promoting genetic variants or lifestyle/ environmental issues, may contribute to clotting/platelet activity in potential ME/CFS subsets. It is possible that identified clotting and platelet abnormalities may contribute to ME/CFS symptoms. Overzealous clotting leads lead to hypoperfusion of the vascular system, resulting in hypoxia. Additionally, associated inflammation damages the vasculature, further exacerbating circulatory issues. This might contribute to ME/CFS symptoms including fatigue, exercise intolerance, and cognitive impairment.

Fibrinaloid microclots and associated coagulation issues are present in ME/CFS, and point to a systemic vascular pathology and endothelial inflammation. Targeted therapies to address vascular and endothelial pathology may benefit the relevant individuals. Treating apparent vascular pathology might result in the amelioration of fatigue and other ME/CFS symptoms, as it did in a LongCovid/PASC population where symptom alleviation coincided with a decrease in fibrinaloid microclot load (Kell et al., 2022, Laubscher et al., 2021). However, it is important to note that the fibrinaloid burden in ME/CFS seems to be less than that present in LongCovid/PASC (Pretorius et al., 2021), thus caution is required when coagulant therapy is to be considered in ME/CFS. Further research is required to determine if treatment targeted against vascular pathology, including hypercoagulability, significant fibrinaloid microclot loads, and hyperactivated platelets, could benefit individuals suffering from ME/CFS.

Declarations

Ethics approval and consent to participate

Ethical approval for blood collection and analysis of blood from participants with ME/CFS and healthy individuals, was given by the Health Research Ethics Committee (HREC) of Stellenbosch University (N19/03/043, project ID #9521). This laboratory study was carried out in strict adherence to the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the South African Medical Research Council (SAMRC), Ethical Guidelines for research. Consent was obtained from all participants. Participants or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Consent for publication

All authors approved submission of the paper.

Availability of data and materials

The datasets generated as well as figure micrographs analyzed during the current study are available on request.

Competing interests
The authors have no competing interests to declare.

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Authors' contributions
JMN: Sample and data analysis; wrote the paper; EP: Edited the paper, funding, co-corresponding author; study leader; AK: control participant identification, collection and screening: DBK: Edited the paper, funding, co-corresponding author. AP: Edit the paper.

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Supplementary data
Metadata for the ME/CFS and control groups used in this study.

TABLE AND FIGURE LEGENDS

Table 1: Demographics and scoring analysis of the ME/CFS cohort using the International Consensus Criteria (ICC) questionnaire for ME/CFS patients. Statistical significance was established at $p<0.05$.

Table 2: TEG® analysis recorded in whole blood (WB) and platelet poor plasma (PPP) in healthy individuals and those with ME/CFS.

Figure 1A: Representative fluorescent micrographs of fibrinaloid microclot presence in platelet poor plasma from controls and individuals with ME/CFS. Images were taken at 63x machine magnification.

Figure 1B: Fluorescence microscopy showing fibrinaloid microclots in platelet poor plasma (PPP) with representative examples of the different stages of different stages of fibrinaloid microclot formation. Stage 1shows minimal microclot formation in healthy/control PPP which progresses to the presence of the severe microclotting Stage 4. Bottom row represents examples of stage 4 microclots using (A) bright-field microscopy, (B) fluorescence microscopy, and (C) an overlay of fluorescence and bright-field microscopy (Laubscher et al., 2021).

Figure 2: A) Mean % area of amyloid signal between control and ME/CFS groups represented as a strip plot. The COVID vaccination status of individuals is colour-coded. A Mann-Whitney analysis yielded a significant difference ($p<0.0001$) with the ME/CFS group exhibiting a greater mean (1.37) than that of the controls (0.10). **B)** Strip plot showing the difference in mean fluorescence signal between control (0.11 ± 0.19) and ME/CFS (1.69 ± 1.69) PPP fibrin amyloid networks induced by exogenous thrombin. Again, the COVID vaccination status is colour-coded. A significant difference (****) was determined by a Mann-Whitney test.

Figure 3: Representative micrographs showing thrombin-induced fibrin networks stained with ThT from healthy participants and participants with ME/CFS. Images were taken at 63x machine magnification.

Figure 4A: Representative fluorescent micrographs of hematocrit samples from ME/CFS individuals stained with PAC-1 (green fluorescence); CD62P-PE **(**purple fluorescence); white areas represent overlap of the two markers. Images were taken at 63x magnification.

Figure 4B: Fluorescence microscopy examples of the different stages of platelet activation and spreading, that was used to score the platelet activation in the Long COVID patients, with Stage 1, with minimally activated platelets, seen as small round platelets with a few pseudopodia, seen as healthy/control platelets that progresses to Stage 4, with egg-shaped platelets, indicative of spreading and the beginning of clumping (Laubscher et al., 2021).

Figure 4C: Fluorescence microscopy examples of the different stages of platelet clumping. With no clumping occurring in the healthy/control samples in Stage 1 (no figures shown), progressing to severe clumping of platelets as seen in Stage 4 (Laubscher et al., 2021).

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- Table1.png
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