

# Combined triple treatment of fibrin amyloid microclots and platelet pathology in individuals with Long COVID/ Post-Acute Sequelae of COVID-19 (PASC) can resolve their persistent symptoms

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## Article

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# Combined triple treatment of fibrin amyloid microclots and platelet pathology in individuals with Long COVID/ Post-Acute Sequelae of COVID-19 (PASC) can resolve their persistent symptoms

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## ABSTRACT

We recognise that fibrin(ogen) amyloid microclots and platelet hyperactivation, that we have previously observed in COVID-19 and Long COVID/Post-Acute Sequelae of COVID-19 (PASC) patients, might form a suitable set of foci for the clinical treatment of the symptoms of long COVID/PASC. We first report on the comorbidities and symptoms found in a cohort of 845 South African Long COVID/PASC patients who filled in the South African Long COVID/PASC registry, of which hypertension and high cholesterol levels (dyslipidaemia) were the most important comorbidities. The gender balance (70% female) and the most commonly reported Long COVID/PASC symptoms (fatigue, brain fog, loss of concentration and forgetfulness, shortness of breath, as well as joint and muscle pains) were comparable to those reported elsewhere. This suggests that our sample was not at all atypical. Using a previously published scoring system for fibrin amyloid microclots and platelet pathology, we analysed blood samples from 70 patients, and report the presence of significant fibrin amyloid microclots and platelet pathology in all cases; these were associated with Long COVID/PASC symptoms that persisted after the recovery from acute COVID-19. A subset of 24 patients was treated with one month of dual antiplatelet therapy (DAPT) (Clopidogrel 75mg/Aspirin 75mg) once a day, as well as a direct oral anticoagulant (DOAC) (Apixiban) 5 mg twice a day. A proton pump inhibitor (PPI) pantoprazole 40 mg/day was also prescribed for gastric protection. Such a regime must only be followed under strict and qualified medical guidance to obviate any dangers, especially haemorrhagic bleeding, and of the therapy as a whole. Thromboelastography (TEG<sup>®</sup>) was used to assist in determining their clotting status. Each of the 24 treated cases reported that their main symptoms were resolved and fatigue as the main symptom was relieved, and this was also reflected in a decrease of both the fibrin amyloid microclots and platelet pathology scores. Nine patients were genotyped for genetic variation in homocysteine metabolism implicated in hypertension, a common COVID-19 co-morbidity reported in both patients found to be homozygous for the risk-associated *MTHFR* 677 T-allele. Fibrin amyloid microclots that block capillaries and inhibit the transport of O<sub>2</sub> to tissues, accompanied by platelet hyperactivation, provide a ready explanation for the symptoms of Long COVID/PASC. The removal and reversal of these underlying epitheliopathies underlying this provide an important treatment option that seems to be highly efficacious, and warrants controlled clinical studies.

## KEYWORDS

Long COVID/PASC; Symptoms, Co-morbidities, Amyloid Fibrin(ogen); Hyperactivated platelets

## INTRODUCTION

As many of 30% of COVID-19 patients infected with the severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) continue, and in some cases begin, to suffer a variety of debilitating symptoms weeks or months after the acute phase of infection. The precise definition of this Long COVID/ Post-Acute Sequelae of COVID-19 (PASC) (here referred to as Long COVID/PASC) is rather unclear and in some instances even vague. This is because most pathophysiological mechanisms have not yet been fully identified, and many different symptoms have been reported. The most frequently reported symptoms persist for as much as 6 months or longer after acute infection <sup>1</sup>. COVID-19 survivors complain of recurring fatigue or muscle weakness, being out of breath, sleep difficulties, and suffer from anxiety or depression <sup>2</sup>. Symptoms noted in Long COVID/PASC patients show numerous similarities to those seen in chronic illnesses, including Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) <sup>3, 4, 5, 6, 7, 8</sup>, postural orthostatic tachycardia syndrome <sup>9</sup> and Mast Cell Activation Syndrome <sup>1, 10</sup>. In a large global study, a survey of 3,762 Long COVID/PASC patients from 56 countries found that nearly half still could not work full-time six months post-infection, due mainly to fatigue, post-exertional malaise, and cognitive dysfunction <sup>11</sup>.

An important component of severe COVID-19 disease is virus-induced endothelialitis. This leads to disruption of normal endothelial function, initiating a state of failing normal clotting physiology. Massively increased levels of von Willebrand Factor (VWF) lead to overwhelming platelet activation, as well as activation of the enzymatic (intrinsic) clotting pathway. We have previously found persistent circulating fibrin amyloid microclots, that are resistant to fibrinolysis, in samples from acute COVID-19 patients <sup>12, 13</sup>. Endothelial, microclot and platelet pathologies are also present in Long COVID/PASC patients <sup>14</sup>. In a recent study we identified numerous dysregulated molecules in circulation that might cause or reflect the lingering symptoms for those individuals with Long COVID/PASC <sup>14</sup>. We used proteomics to study the proteins present in both digested supernatant and trapped persistent pellet deposits (after protein digestion via trypsin). Dysregulated molecules include the acute phase inflammatory molecule Serum Amyloid A (SAA) and  $\alpha(2)$ -antiplasmin ( $\alpha 2AP$ ). We had previously discovered that in many chronic diseases fibrinogen can clot into an amyloid form that is resistant to fibrinolysis, and that these fibrin amyloid (micro)clots could be detected with a fluorogenic amyloid stain <sup>12, 15, 16, 17, 18, 19, 20, 21, 22</sup>. Thus, we also used fluorescence microscopy to report large amyloid microclots and hyperactivated platelets present in blood samples from Long COVID/PASC; we also showed that these deposits are highly resistant to fibrinolysis <sup>14, 23</sup>. The plasmin-antiplasmin system plays a key role in blood coagulation and fibrinolysis <sup>24</sup>. Plasmin and  $\alpha 2AP$  are primarily responsible for a controlled and regulated dissolution of the fibrin polymers into soluble fragments such as D-dimer <sup>24, 25</sup>. We also developed a platelet and

microclot grading system to classify platelet and microclot pathology <sup>26</sup>. The grading system should ideally be applied as part of a multi-pronged approach, which in addition to appropriate anticoagulation, may also include antiviral treatment to limit cell entry of SARS-CoV2.

Differentiation of platelet and microclot pathology due to Long COVID/PASC from cardiovascular disease (CVD), hypertension, hypercholesterolemia or diabetes as the main co-morbidities associated with SARS CoV2 infection, is important in our search for ways to influence the underlying pathogenesis prophylactically. We recently used a pathology-supported genetic testing approach (Kotze et al., 2013), for interpretative commenting on the potential clinical relevance of the cholesterol-raising apolipoprotein (*APOE*) e4 allele associated with COVID-19 infection and severity <sup>27</sup>. Other pathophysiological mechanisms involving at least 20 metabolites may be involved, including a possible genetic link between the development of Long COVID/PASC and homocysteine metabolism <sup>28</sup>. However, genetic variation may be difficult to interpret, where the presence of several other genetic abnormalities may be needed to have phenotypic expression. In the next paragraph, we consider genetic variation in the 5,10-methylenetetrahydrofolic acid reductase (*MTHFR*) gene in the one-carbon-homocysteine pathway as a diagnostic, and conceivably an actionable target, in the life-threatening course of COVID-19 <sup>29</sup>.

Homozygosity for the *MTHFR* 677C>T (rs1801133) variant or compound heterozygosity with 1298A>C (rs1801131) is the most common genetic cause of high homocysteine previously studied in COVID-19 patients <sup>30</sup>. The cut-off value of homocysteine to predict progression of pathological findings in chest CT-imaging of COVID-19 patients was 10.58  $\mu\text{mol/L}$ , compared to the usual definition of high values above 15  $\mu\text{mol/L}$  <sup>31</sup>. This is in line with the effect on platelet activation and the enzymatic clotting pathway <sup>32</sup>. *MTHFR* enzyme function remains largely preserved when dietary folate intake is sufficient and therefore supplementation with B vitamins is suggested here. In patients with an unhealthy lifestyle, e.g. patients with high body mass index (BMI) <sup>33</sup>, reduced enzyme activity leads to accumulation of homocysteine <sup>34</sup>). <sup>35</sup> highlighted the need for prospective intervention studies involving homocysteine as a marker of inflammation that could be targeted to prevent endothelial damage and vascular comorbidities. The value of a personalized approach based on randomized trials as previously conducted in hypertensive patients pre-screened for genetic variation in the *MTHFR* gene <sup>36</sup> warrants further studies to elucidate the potential role of homocysteine accumulation in Long COVID/PASC.

Many of the Long COVID/PASC symptoms that have been reported are related to symptoms that are cardio-pulmonary in nature. In this work we present results from a cohort of 845

patients who completed an online South African Long COVID/PASC registry. In parallel, blood samples of 70 patients who visited the clinical practice of our clinical co-author were collected to report on the presence of microclots and platelet pathology associated with persistent symptoms after recovery from acute COVID-19. Before contracting acute COVID-19, these patients did not suffer from fatigue and other symptoms that they subsequently reported, and which are typically associated with Long COVID/PASC. Thus, they were diagnosed as having Long COVID/PASC by means of eliminating all other common diseases, including heart failure. After diagnosis, 24 patients were treated according to the presence of microclots and platelet pathology, and patient feedback obtained before and after treatment. These patients experienced significant improvement, and even a return to their health at levels similar to those before acute COVID-19. We also report genetic results in nine patients based on the knowledge that oxidative stress and platelet activation may partly be caused by the deleterious effect of folate deficiency on *MTHFR* activity and homocysteine levels <sup>32</sup>. We conclude that treatments focused on the elimination of these fibrin amyloid microclots and of platelet hyperactivation, and – where genetics or lifestyle dictates – the additional use of folate/B-vitamin supplements, can provide a solution to the symptoms of long COVID, and that in consequence the amyloid microclots should be seen as largely responsible for them.

## **MATERIALS AND METHODS**

### **Ethical clearance**

Ethical clearance for the study was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (South Africa) (references: B21/03/001\_COVID-19, project ID: 21911 and N19/03/043, project ID 9521). Informed consent for generic studies and for collecting data from patients who completed the South Africa Long COVID/PASC registry online. 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. The layout the study design is shown in Figure 1.

### **Data collection and analysis of patients who filled in the South African Long COVID/PASC registry**

The South African Long COVID/PASC registry is an online platform where patients can report long COVID/PASC symptoms and previous comorbidities. Data were analysed for risk factors associated with developing Long COVID/PASC. All data were anonymised. The statistical analysis of the South African Long COVID registry data was carried out in a Jupyter notebook

environment <sup>37</sup> and the Pandas library <sup>38</sup> was employed for data manipulation and statistical analysis. With the aid of an interactive python data library, Plotly (<https://plot.ly>), visualisation of the statistical analysis was effected using Sankey plots.

We have also used lattices (a technique based in knowledge representation and artificial intelligence) to visually represent the data. Lattices for exploratory data-science and artificial intelligence are less common than other techniques, but often yield different insights and paths for further exploration <sup>39</sup>. An introduction to lattices in exploratory data-science is given in <sup>40</sup> among others. The “conexp” software package (freely available from [conexp.sourceforge.net](http://conexp.sourceforge.net)) was used for preparing the lattices in this paper. The data of the 845 participants in the cohort were condensed into a single matrix mapping comorbidities to symptoms, in preparation for drawing the lattices. The input was a comma-separated values (CSV) file containing one patient per row, with entries of 0 or 1 (absence or presence) in columns, where the comorbidities and symptoms appear as individual columns. First, the percentage prevalence for each symptom was calculated by traversing all rows; this was subsequently used as a threshold vector. Next, for each comorbidity, the comorbidity-implied percentage prevalence was calculated for each symptom, giving a matrix of comorbidity (rows) versus symptoms (columns) and percentage entries. Finally, in order to draw easily visualised lattices with 0 and 1 entries, this last matrix was normalised based on the initially calculated threshold vector.

### **Blood sample collection from the cohort of 70 patients**

Blood was drawn from 70 patients (33 females and 37 males; (mean/SD age 51±17). Either a qualified phlebotomist or medical practitioner drew citrated blood into sample tubes (BD Vacutainer®, 369714), via venepuncture, adhering to standard sterile protocol. Whole blood (WB) was centrifuged at 3000xg for 15 minutes at room temperature and the supernatant platelet poor plasma (PPP) samples were collected and stored in 1.5mL Eppendorf tubes at -80°C, until the analysis was performed. Haematocrit samples were analysed on the day of collection.

### **Long COVID/PASC diagnosis**

Patients gave consent to study their blood samples, following clinical examination and/or after filling in the South African Long COVID/PASC registry. Symptoms must have been new and persistent symptoms noted after acute COVID-19. Initial patient diagnosis was the end result of exclusions, only after all other pathologies had been excluded. This was done by taking a history of previous symptoms (before and after acute COVID-19 infection), clinical examinations, and investigations including: full blood counts; N-terminal pro b-type natriuretic peptide (NTproBNP) levels (if raised it suggests cardiac damage); thyroid-stimulating



hormone (TSH); C-reactive protein levels; the ratio between the concentrations of the enzymes aspartate transaminase and alanine transaminase (AST/ALT ratio) and electrocardiogram (ECG) +/- stress testing. If the mentioned tests were in the normal ranges, the lingering symptoms that can be ascribed to Long COVID/PASC were then assessed and included shortness of breath; recurring chest pain; lingering low oxygen levels; heart rate dysfunction (heart palpitations); constant fatigue (more than usual); joint and muscle pain; brain fog; lack of concentration; forgetfulness; sleep disturbances and digestive and kidney problems. These symptoms should have been persistent and new symptoms that were not present before acute COVID-19 infection and persistent for at least two months after recovery from acute (infective) COVID-19.

#### [Patient treatment of a sub-group](#)

We also studied samples from a subgroup of 24 Long COVID/PASC patients before and after treatment. Clinical assessment identified Long COVID/PASC patients (as described above) that might also have coagulation and platelet pathologies. Because of the very wide variety of symptoms that may be present in Long COVID/PASC, a thorough clinical history and examination is required, supplemented by the appropriate specialized investigations/blood work. Caution should be used in deciding on the most cost-effective way to go about doing these tests seeing that most are virtually always normal in Long COVID/PASC patients. This may create the false impression that the patient has no organic disease process present. Platelet and microclot analysis were also performed on these samples. "Best clinical practice methods" were followed and hypercoagulation pathologies were treated using anticoagulation medication. Patients were informed that this is not yet standard treatment for Long COVID/PASC. Blood samples from patients were analysed before and after treatment, and thromboelastography (TEG<sup>®</sup>) was used to assist in determining the clotting status of the patients, to ensure that hypocoagulation or a likelihood of bleeding was not being induced <sup>26, 41</sup>. Once a diagnosis of Long COVID/PASC was confirmed, and microclots and platelet pathology were noted using fluorescence microscopy (see below), the patients were treated with one month of dual antiplatelet therapy (DAPT) (Clopidogrel 75mg/Aspirin 75mg) once a day before breakfast, as well as direct oral anticoagulants (DOAC) (Apixiban 5 mg twice a day (bd) (therapeutic dose). A proton pump inhibitor (PPI) e.g. pantoprazole 40 mg/day taken orally half an hour before main meal was added to this for gastric protection. After one month the blood analysis was repeated and together with symptomatology will indicate if further treatment is needed or not. Patients completed a checklist of symptoms, before and after treatment regimes. Anticoagulation is not needed on a long-term basis (unless there is an underlying alternative indication). Once platelet activation and microclot formation returns to

normal, indicating endothelial recovery, anticoagulation is stopped. The body is then able to return to managing normal clotting/physiology.

### Platelet pathology

Haematocrit samples of all 70 patients in the cohort were exposed to the two fluorescent markers, CD62P (PE-conjugated) (platelet surface P-selectin) (IM1759U, Beckman Coulter, Brea, CA, USA) and PAC-1 (FITC-conjugated) (340507, BD Biosciences, San Jose, CA, USA). CD62P is a marker for P-selectin that is either on the membrane of platelets or found inside them<sup>13,42</sup>. PAC-1 identifies platelets through marking the glycoprotein IIb/IIIa (gpIIb/IIIa) on the platelet membrane. To study platelet pathology, 4µL CD62P and 4µL PAC-1 was added to 20µL haematocrit, followed by incubation for 30 minutes (protected from light) at room temperature. The excitation wavelength band for PAC-1 was set at 450 to 488nm and the emission at 499 to 529nm and for the CD62P marker it was 540nm to 570nm and the emission 577nm to 607nm. Samples were viewed using a Zeiss Axio Observer 7 fluorescent microscope with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy, Munich, Germany).

### Platelet poor plasma (PPP) and the detection of amyloid (fibrin(ogen) protein and anomalous micro-clotting

Microclot formation in PPP samples from all 70 treatment-naïve Long COVID/PASC patients and the 24 patients in the treatment sub-group, after treatment were analysed. PPP were exposed to the fluorescent amyloid dye, Thioflavin T (ThT) (final concentration: 0,005mM) (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes (protected from light) at room temperature<sup>18, 19, 20, 21</sup>. After incubation, 3uL PPP was placed on a glass slide and covered with a coverslip. The excitation wavelength band for ThT was set at 450nm to 488nm and the emission at 499nm to 529nm and processed samples were viewed using a Zeiss Axio Observer 7 fluorescent microscope with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Carl Zeiss Microscopy, Munich, Germany)<sup>12, 13, 23</sup>.

### **Genetic studies**

Pathology-supported genetic testing as previously conceptualized for APOE genotyping<sup>27</sup> was used for assessment of clinically relevant non-communicable disease (NCD) pathways coincident with the SARS-CoV-2 viral disease. Genomic DNA was extracted from anti-coagulated whole blood samples using the Omega Bio-Tek E.Z.N.A<sup>®</sup> Blood DNA Mini Kit (Spin Protocol). Genotyping of two functional single nucleotide polymorphisms (SNPs) in the *MTHFR* (rs1801133, 677C>T and rs1801131, 1298A>C) gene was performed by high throughput real-time polymerase chain reaction (RT-PCR) on the Roche LightCycler<sup>®</sup> 480II

instrument, using the TaqMan® SNP Genotyping Assays (ThermoFisher Scientific). The accuracy of the high throughput genotyping was verified against direct DNA sequencing as the gold standard<sup>33</sup>. For the purpose of this study, *MTHFR* genotyping results of nine patients with known APOE 2/3/4 genotype were interpreted according to the matrix shown in Table 1<sup>43</sup>, which includes a questionnaire-based assessment as previously described by<sup>33</sup>.

**Table 1:** Matrix for data integration and semi-automated report generation using pathology-supported genetic testing of the coagulation pathway analysis in Long COVID patients.

<b>Disease pathway analysis</b>	<b>Family medical history and genetic susceptibility</b>	<b>Environmental factors and treatment response</b>
<b>Clinical risk profile</b> <ul style="list-style-type: none"> <li>• Deep vein thrombosis (DVT)</li> <li>• Pulmonary embolism</li> <li>• Recurrent pregnancy loss</li> </ul>	DNA screening for genetic variation in the <i>MTHFR</i> gene to determine clinical relevance based on the personal and family medical conditions documented	Questionnaire-based assessment of dietary folate status and other lifestyle factors known to interact with the genetic variants selected for this study
<b>Biochemical test results*</b> <ul style="list-style-type: none"> <li>• Lipid profile</li> <li>• Homocysteine*</li> <li>• Vitamin B12*</li> </ul>	Assessment of pathological indicators that may reflect gene-environment interactions as biological intermediates	Monitoring of relevant pathology/biochemical test results in relation to treatment response and potential side-effect profile

*Available in one case only*

## RESULTS

### South African Long COVID/PASC registry

In Figures 2 to 6, the distribution of the South African Long COVID/PASC registry participant data (845 participants) was analysed according to the patients' gender, comorbidities, age group, initial COVID-19 symptoms, and Long COVID/PASC symptoms, using Sankey plots. The same participant versus comorbidity versus symptom data were further manipulated to produce a mapping between comorbidities and symptoms, represented as a matrix with comorbidities as rows and symptoms as columns. This was used to draw a lattice, giving insight into the implications (simple binary implications for visualisation) from comorbidities to symptoms. The corresponding lattices with different components highlighted, correspond to the most prominent comorbidities emerging from the Sankey diagrams: high blood pressure, high cholesterol, Type 2 diabetes, auto-immune disease, and previous blood clots. In the following figures (Figures 2 to 6), we rehearse the implications in more detail.

Figure 2 gives a general overview of the South African Long COVID/PASC registry. About 10% (i.e. 87) of the participants were not initially tested for SARS-CoV2 using a PCR test, whereas in 90% (i.e. 758) of the patients, a COVID-19 positive test was reported. Moreover, patients were also categorised according to gender. Thus, 70% and 30% (i.e. 593 and 252) of the study cohort identified as female and male, respectively, in line with common

observations<sup>44, 45</sup>. The majority (i.e. 76%) of the participants were between ages 31-40, 41-50, and 51-60. We observed that participants with comorbidities such as high blood pressure, high cholesterol, type-2 diabetes, auto-immune disease, and previous blood clots were in the majority.

Figure 3 shows the gender distribution for the participants with the Long COVID/PASC symptoms in more detail. In a similar trend with Figure 2, the common Long COVID/PASC symptoms were noted as constant fatigue; brain fog, loss of concentration, and forgetfulness; shortness of breath, as well as joint and muscle pains. Interestingly, Long COVID/PASC symptoms such as kidney problems, digestive problems, and low oxygen levels were less commonly reported by the patients. Figure 4 shows the age versus Long COVID/PASC symptoms distribution of the participants. We note that the majority of participants were within the age ranges of 31-40, 41-50, and 51-60.

Figure 5 shows a Sankey plot that illustrates the population distribution of participants' comorbidities versus Long COVID/PASC while Figures 6A and B shows representative lattice plots of high blood pressure and high cholesterol levels, confirming in more detail the correlations already shown in Figures 4 and 7. Reading upwards in the lattice, for example in 8A, the "high blood pressure" node connects upwards through a highlighted network to a variety of symptoms, ranging from "digestive problems" (on the left) to "shortness of breath" on the right. The complexity/density of the blue highlighted network represents prevalence of the comorbidity amongst the patients, as well as implications of a variety of symptoms.

### **Blood analysis**

We studied blood samples from 70 diagnosed Long COVID/PASC patients (age median/SD 51±17) (33 females and 37 males). Microclot and platelet analysis showed presence of microclots and platelet pathologies in all 70 patients. We used a platelet grading system to identify platelet pathologies, that we have developed and described previously<sup>26</sup> see Figure 7 and 8 and Table 2.

**Table 2:** Platelet activation criteria showing level of spreading, as well as clumping in the haematocrit sample. (Taken from <sup>26</sup> with permission).

Score	Spreading	Score	Clumping
1	Activation with pseudopodia	1	None
2	Mild	2	Mild
3	Moderate	3	Moderate
4	Severe	4	Severe

We again used a clotting grading system that we have developed and have published on previously <sup>26</sup>, see Figure 9 and Table 3. Both the scoring of the platelet pathology and PPP microclots were combined and given a final score to determine the severity of the disease (Table 4).

**Table 3:** Microclot criteria to determine the amount of microclots in the platelet poor plasma sample. (Taken from <sup>26</sup> with permission).

Score	Analysis criteria
1	Very few areas of plasma protein misfolding ( $\leq 1\mu\text{m}$ ) visible with a few $\leq 10\mu\text{m}$ microclots
2	Very few areas of plasma protein misfolding ( $\leq 1\mu\text{m}$ ) visible with scattered/mild $\leq 10\mu\text{m}$ microclots
3	Moderate areas of plasma protein misfolding visible as microclots $\geq 15\mu\text{m}$
4	Severe areas of plasma protein misfolding visible as large microclots

**Table 4:** Overall microclot and platelet activation score results.

Scoring results			
Control / Healthy	Mild	Moderate	Severe
=3	4-7	8-10	11-12

Figure 10 shows an example of platelet activation and the presence of fibrin amyloid microclots in two of the Long Covid/PASC patients. Figure 10A to C shows representative micrographs of a patient suffering from Long COVID/PASC for 11 months, where Figure 10C shows a tile scan of cellular debris present in the haematocrit. Figure 10D and E shows representative microclots and platelets where the patients have been using Aspirin only, before sample collection. As anticipated, significant microclot formation were still seen, but platelets were not significantly activated.

We also followed a sub-population of 24 patients through an anticoagulation regime, and nine cases were also screened for the *MTHFR* rs1801133 (677C>T) and rs1801131 (1298A>C) SNPs. Table 5 shows patient demographics and disease status of this sub-group of 24 patients. Table 6 shows MTHFR genotyping for the nine patients. High cholesterol and high blood pressure were amongst the main co-morbidities that were noted in 25% and 33% of these, respectively. However, overall this cohort was quite healthy before being diagnosed with acute COVID-19. Their symptoms during acute COVID-19 varied from mild (33.3%) medium (33.3%) to severe (33.3%) and 33% were hospitalized, but no-one was ventilated.

Shortness of breath (70.8%), constant fatigue (87.5%) and brain fog, concentration and forgetfulness (83.3%), were the main long COVID/PASC symptoms that were reported. After treatment, every one of the 24 patients reported that their main symptoms were resolved and fatigue as main symptom, relieved. Their platelets were also calmed and microclotting scores were within healthy levels (see clotting and grading system).

Using the platelet mapping and plasma criteria <sup>26</sup> the patients displayed moderate platelet spreading and mild platelet clumping (white arrows) before treatment (Figure 11 A, C, E, G, I) with a reduction in platelet activity after treatment with mild spreading and no clumping (Figure 11 B, D, F, H, J). In the plasma samples a difference could be seen before and after treatment, with moderate plasma microclots (white arrows) found in the before treatment patients (Figure 12 A, C, E, G, I) and a small number of microclots present in the plasma after treatment (Figure 12 B, D, F, H, J). Similar platelet hyperactivation and plasma clotting were seen in acute COVID-19 and Long COVID/PASC patients in previous studies done on naïve COVID-19 and Long COVID/PASC samples <sup>12, 13</sup>.

**Table 5:** Patient demographics and disease status of 24 Long COVID/PASC patients before and after treatment.

<b>Demographics of sub-group of 24 Long COVID/PASC patients</b>			
<b>Age</b>	51±15.5		
<b>Gender</b>	<b>Females</b>	12	<b>Males</b> 12
<b>Comorbidities</b>			
<b>Comorbidities</b>	<b>Yes (%)</b>		<b>No (%)</b>
Type 1 diabetes	0%		100%
Type 2 diabetes	4%		96%
Cardiovascular disease	8%		92%
High blood pressure	33%		67%
High cholesterol	41.7%		58.3%
Previous blood clots	17%		83%
Previous stroke	4%		96%
Previous heart attack	0%		100%
Psoriasis	8%		92%
Rheumatoid arthritis	13%		88%
Lupus	0%		100%
Auto-immune disease not listed	0%		100%
Gingivitis and/or Periodontitis	0%		100%
Rosacea	0%		100%
<b>Acute COVID-19 data of cohort of 24 treated Long COVID/PASC patients</b>			
<b>Acute phase disease severity</b>	<b>Light</b>	<b>Medium</b>	<b>Severe</b>
Severity of acute COVID-19 or Long COVID	33.3%	33.3%	33.3%
<b>Hospitalization during acute COVID-19</b>	<b>Yes (%)</b>		<b>No (%)</b>
Were you hospitalized?	33%		67%
Did you receive oxygen?	33%		67%
Were you ventilated	0%		100%
<b>Long COVID Symptoms</b>			
<b>Questioner questions</b>	<b>Yes (%)</b>		<b>No (%)</b>
Shortness of breath	70.8%		29.2%
Recurring chest pain	37.5%		62.5%

Low oxygen levels	54.2%	45.8%
Heart rate dysfunction (heart palpitations)	41.7%	58.3%
Constant fatigue (more than usual)	87.5%	12.5%
Joint and muscle pain	62.5%	37.5%
Brain fog, concentration, forgetfulness	83.3%	16.7%
Sleep disturbances (more than before COVID-19)	58.3%	41.7%
Depression, anxiety (more than before COVID-19)	29.2%	70.8%
Digestive problems	16.7%	83.3%
Kidney problems	8.3%	91.7%
Did your main symptoms listed above resolve?	100%	0%
Fluorescence microscopy		
Scoring	Before treatment	After treatment
Platelet activation and clumping score	4.4 ± 0.9	3.6 ± 1.2
Microclotting score	2.7 ± 0.8	1.7 ± 0.6
Overall score for platelets and plasma micrographs based on platelet and microclot scoring system	7.0 ± 1.3	5.2 ± 1.3

Table 6 shows the clinical characteristics and *MTHFR* genotypes of nine Long COVID/PASC patients, of whom eight (89%) tested positive for at least one risk-associated allele. Homozygosity for the *MTHFR* 677 T-allele associated with the most severe effect on enzyme function was detected in two of the three patients with hypertension reported as a co-morbidity, prior to the diagnosis of Long COVID/PASC. In the majority of patients folate intake was inadequate, considered to be low when the score is less than 11, moderate between 11 and 13, and high above 13 (see Table 6). The negative effect of a low folate score was evaluated in relation to body mass index (BMI), as an indication of being overweight (>24.9 kg/m<sup>2</sup>) or obese (>30 kg/m<sup>2</sup>). Detection of the APOE polymorphism (data not shown) and/or a high BMI are known risk factors for development of dyslipidaemia frequently detected in our study cohort. A family history of DVTs was reported by both patients with this condition prior to their diagnosis of Long COVID/PASC, which warrants extended genetic testing to confirm or exclude a more severe genetic cause of inherited thrombophilia.

**Table 6:** Clinical characteristics and *MTHFR* genotyping results of nine Long COVID/PASC patients. Folate score should be evaluated in relation to body mass index. Ages were not added to assist with de-identification: age range was from 40 – 60.

Gender	BMI (kg.m <sup>-2</sup> )	MTHFR	Folate score	Comorbidities*
Female	23.7	High -14	1298 A > C (++)	Deep vein thrombosis Dyslipidaemia
Male	27.1	Very Low - 3	677 C > T (-)	Dyslipidaemia
Female	19.7	Low - 9	677 C > T (-)	Not reported
Male	33.4	High -15	1298 A>C (+)	Dyslipidaemia
Female	24.7	Low - 9	677 C > T (-) 1298 A > C (-)	Not reported
Female	36.3	Low - 8	677 C > T (++)	Dyslipidaemia Hypertension
Female	34.9	Low - 10	677 C > T (-)	Not reported
Male	28.1	Very Low - 4	677 C > T (++)	Deep vein thrombosis Hypertension

\*Prior to the diagnosis of Long COVID/PASC +- heterozygous and ++ homozygous

## DISCUSSION

Here we report on the comorbidities and symptoms identified in a cohort of 845 South African Long COVID/PASC patients who filled in the South African Long COVID/PASC registry. We show that hypertension and high cholesterol levels (dyslipidemia) are important comorbidities that may play a significant role in the development of Long COVID/PASC in this cohort. (We also recognise that other comorbidities, such as previous viral infections, are also very important, but may only manifest only in a larger cohort.) It is well-documented that impaired endothelial function is associated with increased cholesterol and hypertension (which may be underpinned by genetic variation) due to increased vascular oxidative stress and inflammation<sup>46, 47</sup>. Here we also showed the presence of fibrin amyloid microclotting and platelet pathologies in another cohort of 70 patients that visited a clinical practice complaining of persistent symptoms, where these patients were diagnosed with Long COVID/PASC. We found that in this cohort, all of the patients did indeed have both increased amyloid microclotting, as well as platelet pathologies, as assessed by a platelet and clotting grading system that we have developed previously<sup>26</sup>. In addition, we followed 24 of these patients through their anticoagulation treatment regimens and demonstrated sufficient endothelium recovery over a three to four week period to lower their microclot and platelet scores by almost two full units (7.1 → 5.2). While this approach may not yet be widely accepted, it is based on best clinical practice and GJL's experience in managing affected patients. We included a minor genetic component to determine the prevalence of two extensively studied *MTHFR* SNPs in 9 of the 24 patients. Detection of genetic variation in this *MTHFR* gene in the presence of clinically relevant biochemical findings may therefore also facilitate risk stratification for improved personalized patient management<sup>48</sup>.

Normal blood clotting goes through a variety of established mechanisms<sup>42</sup>, a major step being the cleavage by thrombin of the complex fibrinogen molecule (roughly cylindrical, with a 5 x 45nm size). This releases two fibrinopeptides, and causes the thermodynamically favourable formation of fibrin macrofibres, typically 50-100nm in diameter. They may be crosslinked by Factor XIII. The clots are usually removed by fibrinolysis, leading to the residual formation of D-dimer; its normally low background levels reflect this background activity. It was always assumed that the normal conformation of a protein is that of its lowest free energy, as per Christian Anfinsen's famous protein refolding experiments. However, this is not the case. Many proteins can fold into a form of lower free energy but retain the identical sequence. Some of these forms, containing ordered beta-sheet structures, are generically referred to as amyloids, and many are well known to be associated with certain diseases. Ab in Alzheimer's disease and synuclein in Parkinson's disease are examples. Over 50 are recognised<sup>18</sup>. Note however that almost any proteins can form amyloid structures (e.g. recombinant insulin will do



it over time, as will lysozyme held at an acid pH). Another well-known example of a class of proteins that can exist in two conformations of identical sequence is represented by prion proteins. The normal form with alpha-helices is called PrP<sup>c</sup> and the amyloid one PrP<sup>Sc</sup>, the latter being of lower free energy i.e. more thermodynamically stable. It is also highly resistant to proteolysis. This transition between the two forms can itself be catalysed by the PrP<sup>Sc</sup>. The key point of importance in microclot formation in Long COVID/PASC is that fibrin(ogen) too can, in the presence of various trigger substances, fold into an amyloid form that has a very different macrostructure characterised by different fibre diameters and pore sizes<sup>49</sup>. We noted that e.g. in type 2 Diabetes Mellitus (T2DM) clots have a netlike appearance<sup>50, 51, 52, 53</sup> while in Alzheimer's disease<sup>16, 54, 55, 56</sup> and Parkinson's disease the fibres may be larger in size<sup>15, 22</sup>; they are also much more resistant to proteolysis<sup>17</sup>, and so are much more prevalent in the steady state.

We have been observing fibrin(ogen) changes generally for many years, initially via electron microscopy (e.g.<sup>57, 58, 59, 60, 61, 62</sup>, and many others). Since 2011 we have also studied the effect of fibrin(ogen) folding in the presence of various inflammatory molecules, such as iron ions<sup>63</sup>, that can stimulate the anomalous fibrin form<sup>64</sup>. These anomalous structures could also be found in a variety of disease states<sup>17</sup>, such as T2DM<sup>51</sup> and Alzheimer's disease<sup>65</sup>. In this earlier literature we often referred to these anomalous clots as 'dense matted deposits'. In 2016, we showed that this anomalous resistance to fibrinolysis was because the anomalous structures were in fact amyloid in nature<sup>49</sup>. Such structures are easily observed under the optical microscope, and in particular may be stained with the fluorogenic dye thioflavin T and by the more recently developed oligothiophene dyes marketed by Ebba Biotech as Amytrackers<sup>15, 19, 21</sup>.

Most recently, we have demonstrated this explicitly in COVID-19 patients<sup>12, 13</sup>. Here, these microclots were observed without the addition of clotting agents, i.e. thrombin, and therefore, these amyloid microclots were there in the plasma of the individuals at the time of sampling. In particular, it was found not only that the clots contained fibrin (fibrinogen after all being one of the most concentrated proteins in plasma) but that these microclots had entrapped many other proteins such as alpha-2-antiplasmin<sup>14</sup> and a variety of other proteins and even antibodies, which therefore were not observed in plasma from which the microclots had been removed (so did not appear as biomarkers, even though they were there). Because these clots are insoluble and effectively inert they do not contribute to plasma viscosity as determined via TEG<sup>®</sup>, whose values can thus appear normal in Long COVID/PASC (unpublished data). Another characteristic of COVID-19 is the extremely high levels of activation of platelets<sup>13</sup>. Together with platelet pathology and the presence of microclots in

the circulation, endothelial damage may be key drivers of persistent Long COVID/PASC symptoms <sup>14</sup>. See Figure 13 for a snapshot of the interactions that platelets have with circulating blood cells and the various complexes they form (for a detailed review, see <sup>66</sup>.

The intention of the treatment regime used in this study was to use best clinical practice methods to return the patient's clotting pathology to that of a normal clotting physiology and not to make them hypo-coagulable. It is well-known that DOACs act upon various parts of the clotting pathways <sup>67</sup>, while platelet hyperactivation is prevented with DAPT <sup>68, 69</sup>. This 'triple therapy' treatment therefore prevents platelet hyperactivation and also prevents new microclots from forming, while it allows the body's own fibrinolysis pathways to clear existing microclots. Figure 14 shows the various routes of action of the medication targeting the enzymatic pathway and platelet hyperactivation <sup>67, 68, 69</sup>. See Figure 15 for a concluding visual on our understanding of the most relevant physiological processes.

## CONCLUSION

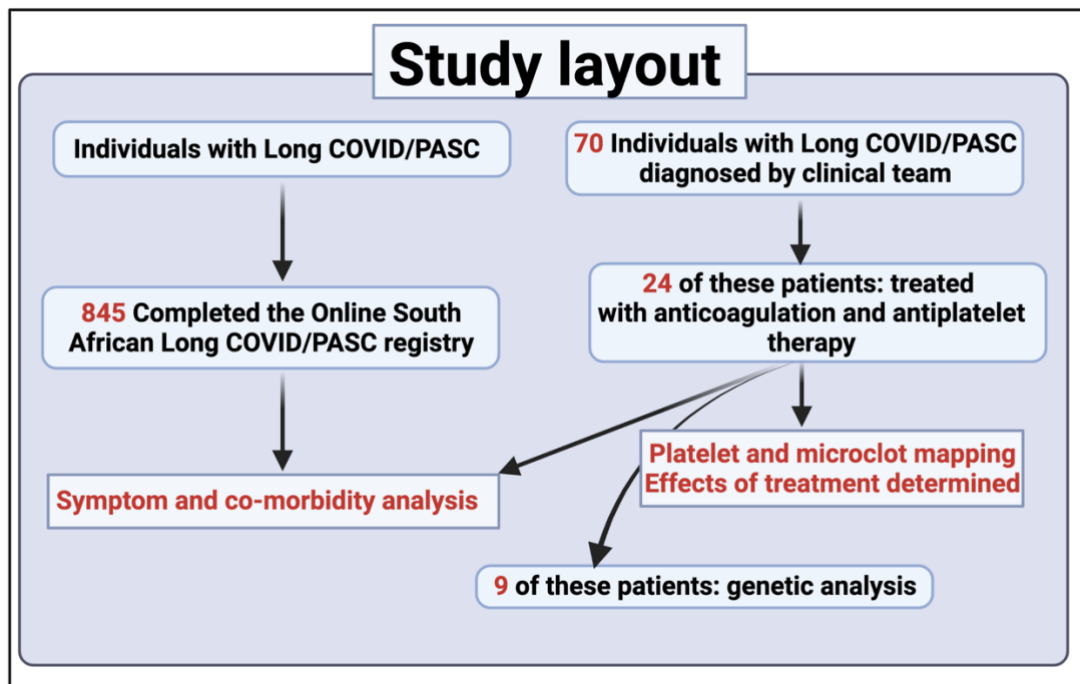
In the current study, it was noted that each of 70 patients diagnosed with Long COVID/PASC and who provided blood samples showed platelet hyperactivation and microclot formation. We used a triple therapy to treat both the microclots and the platelet hyperactivation. During this medication regime, where 24 patients were followed, the endothelium layers recover sufficiently over a three-to-four week period, and this allows for a normal clotting physiology and endothelium function to return. In the treated cohort, significant microclots were removed and platelet hyperactivation returned to more normal levels. All patients in this cohort, reported a significant reduction in their Long COVID/PASC symptoms. We suggest that a platelet and clotting grading system should be used as a simple and cost-effective diagnostic method for the early identification of long COVID/PASC. Diagnosis of Long COVID/PASC requires a diagnosis that excludes other pathologies, including using the duration of symptoms (>two months after acute infection). If a bleeding tendency (not seen commonly) is a concern, a TEG<sup>®</sup> can be used to manage that, its role being as a safety-net to not overtreat the patient. The exact combination of treatment and duration for mild cases probably needs further investigation and refinement. Patients need to be informed that the above approach and treatment is not yet widely accepted but is based on best clinical practice and experience in managing these patients.

Our literature study on the effect of homocysteine on platelet and clotting pathway activation underpinned by variation in the *MTHFR* gene is relevant to the COVID-19 era. Ponti and co-workers in 2021 <sup>70</sup>, correlated infection patterns with the *MTHFR* 677 T-allele frequency which could relate to studies we performed previously in different ethnic groups in South Africa <sup>71</sup>.

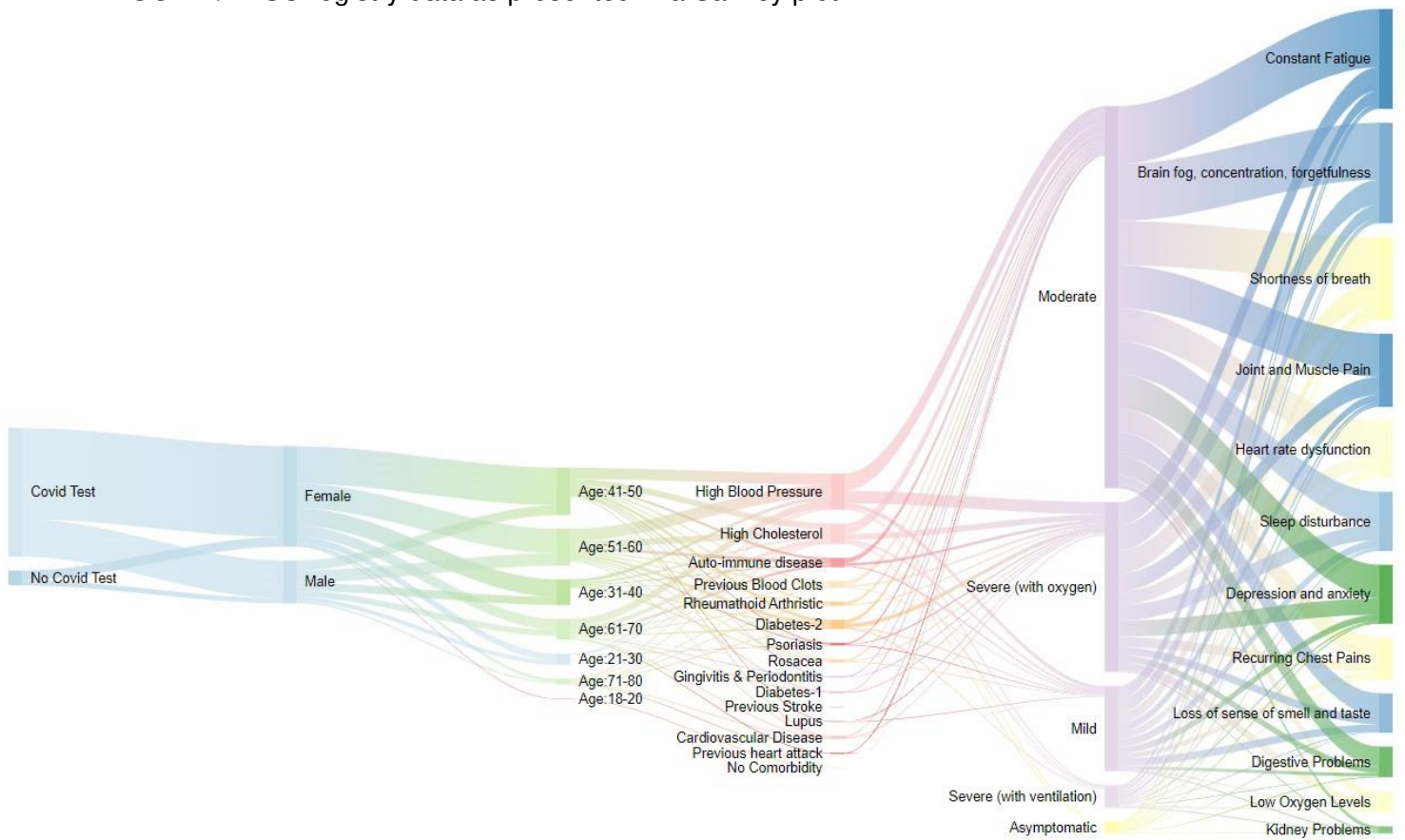
We also provided convincing evidence that homocysteine levels are mediated by the effects of *MTHFR* 677 C > T and the diet on BMI, which increases with a low folate score<sup>33,72</sup>. There a low folate score correlates with a high BMI. Detection of this low-penetrance variation therefore reinforces the importance of high intake of folate and other B-vitamins in the diet to prevent or restore dysfunction of the methylation pathway. There are no controlled trials done yet; however, this will be the important next steps to urgently consider in relation to clinical monitoring using a multi-modal pathology-supported genetic testing approach<sup>27</sup>.

## FIGURES

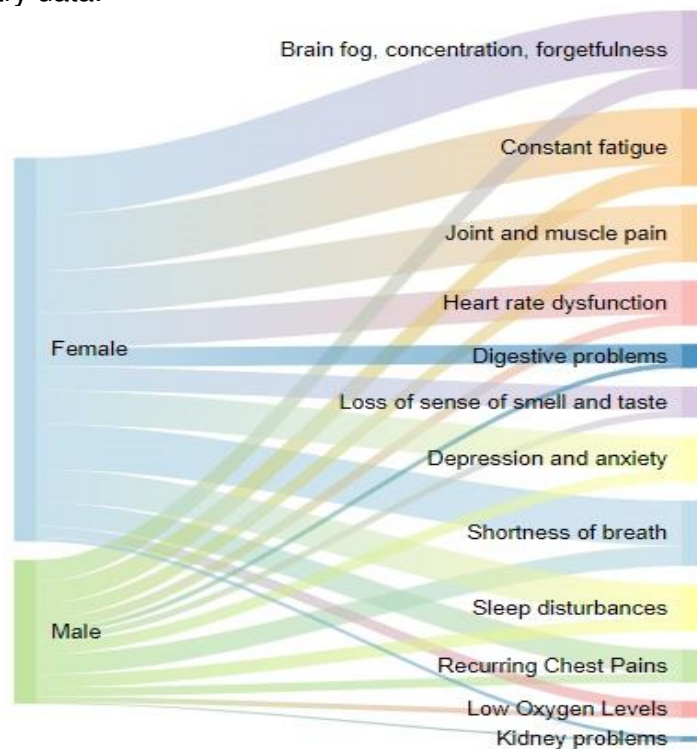
**Figure 1:** Study layout of the current paper.



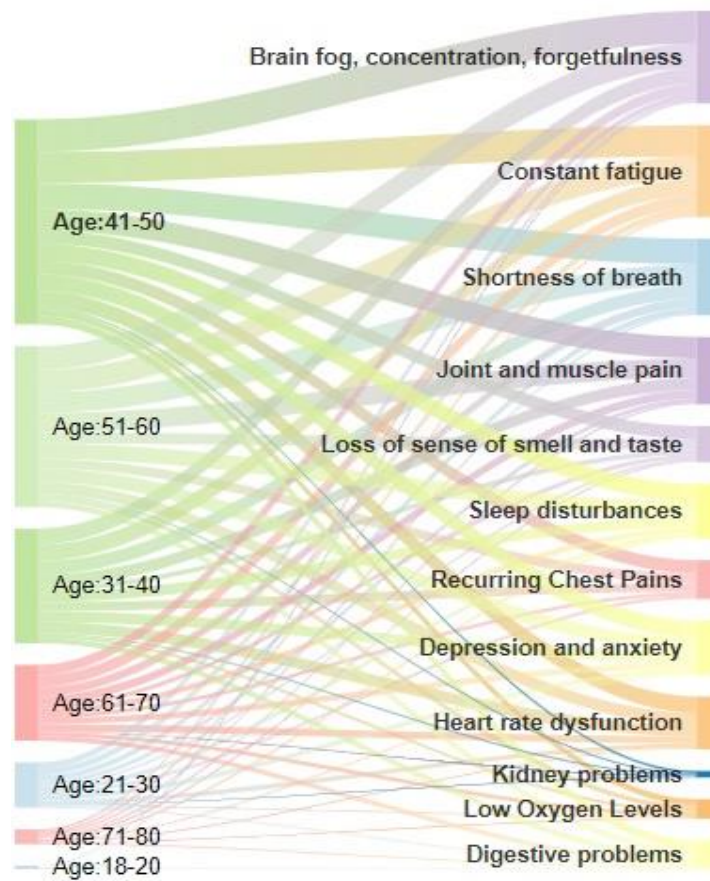
**Figure 2:** General Overview of the population distribution of the South African Long COVID/PASC registry data as presented in a Sankey plot.



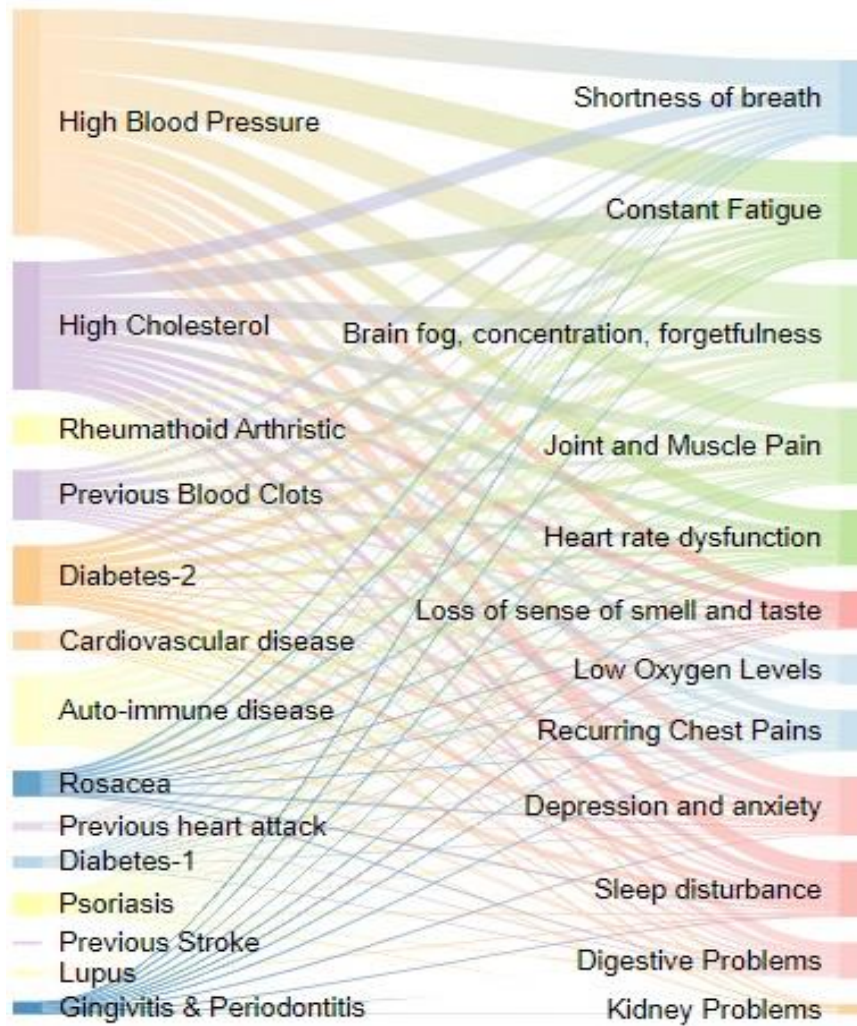
**Figure 3:** Gender versus Long COVID symptoms population distribution of the South African Long COVID registry data.



**Figure 4:** Age - Long COVID/PASC symptoms participant distribution of the South African Long COVID/PASC registry data.

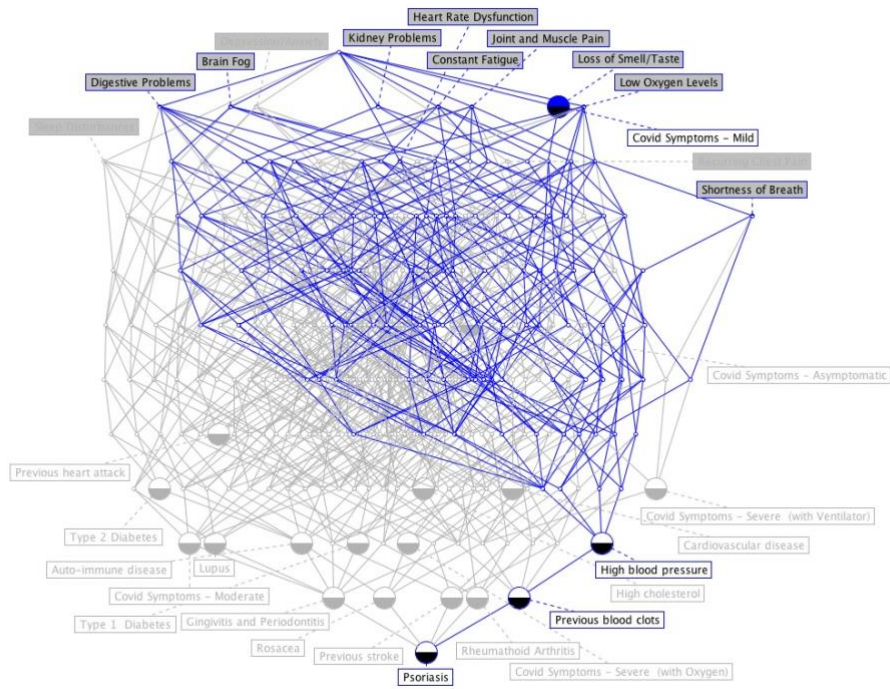


**FIGURE 5:** A Sankey plot showing participants comorbidities versus Long COVID/PASC symptoms population distribution of the South African Long COVID registry data.

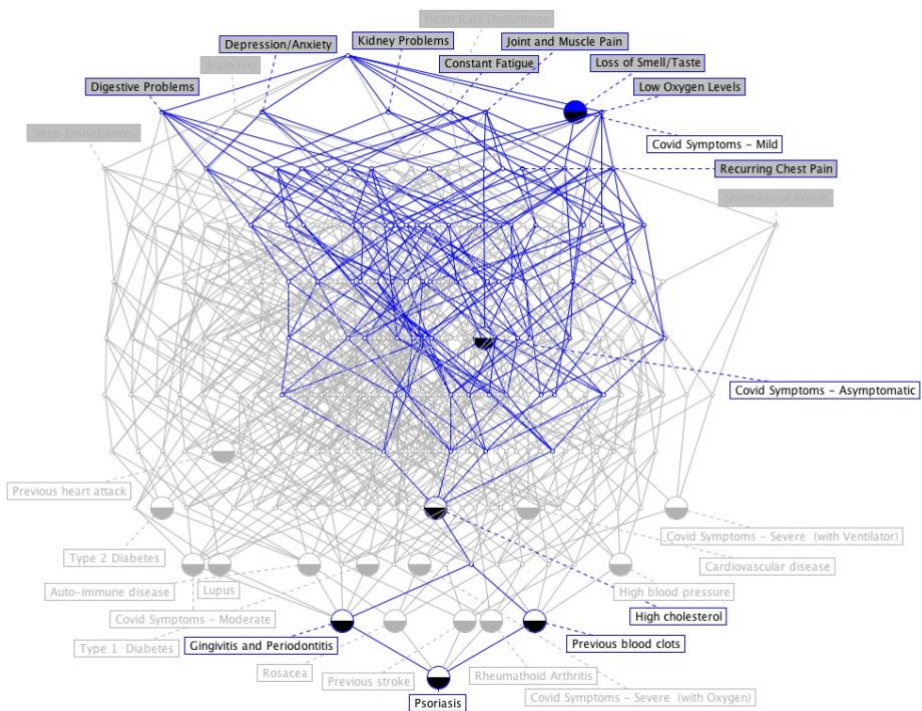


**Figure 6A and B:** Lattice plots, showing participants comorbidities versus Long COVID/PASC symptoms lattice, highlighting the high blood pressure and high cholesterol comorbidities.

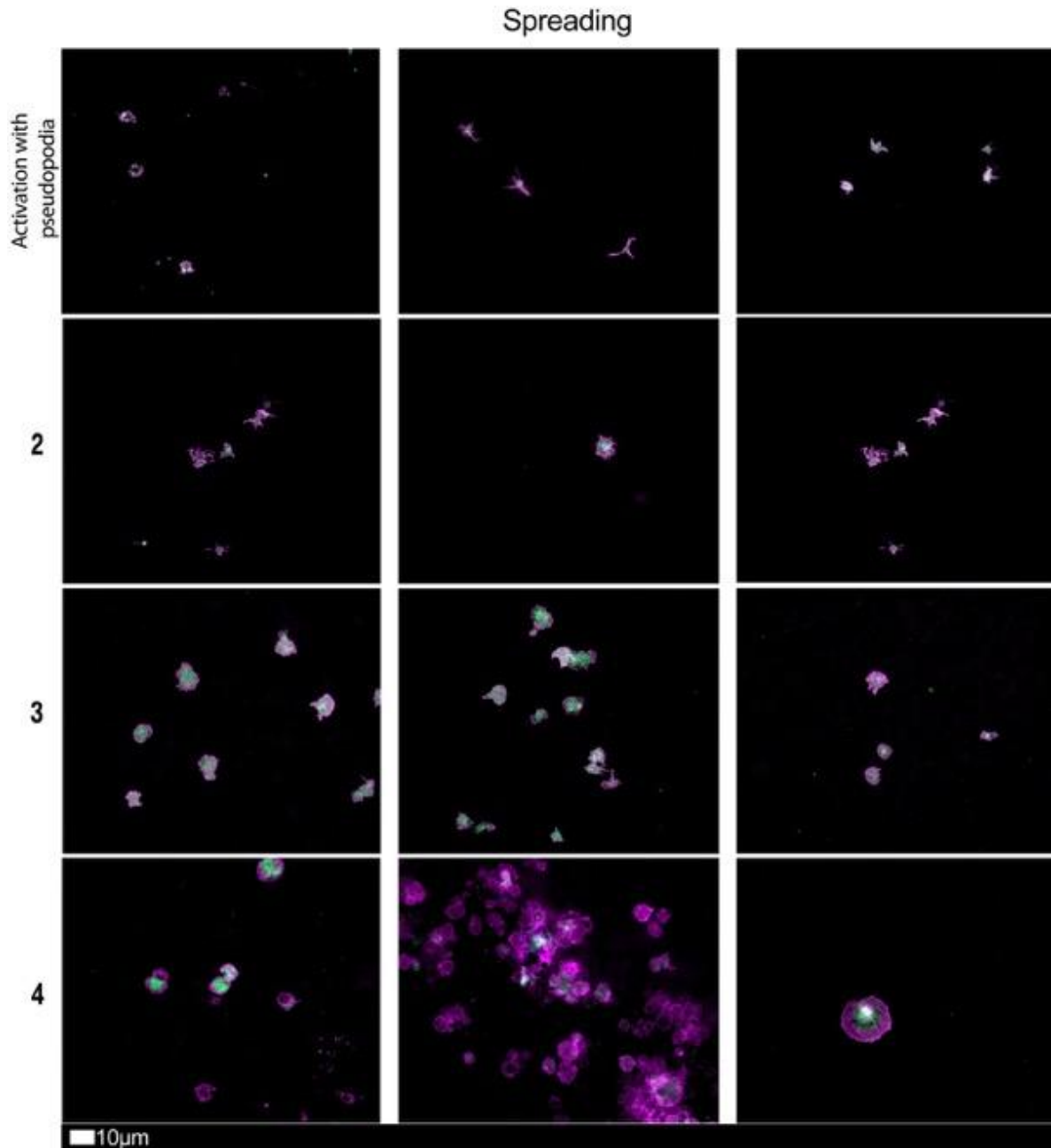
**A**



**B**

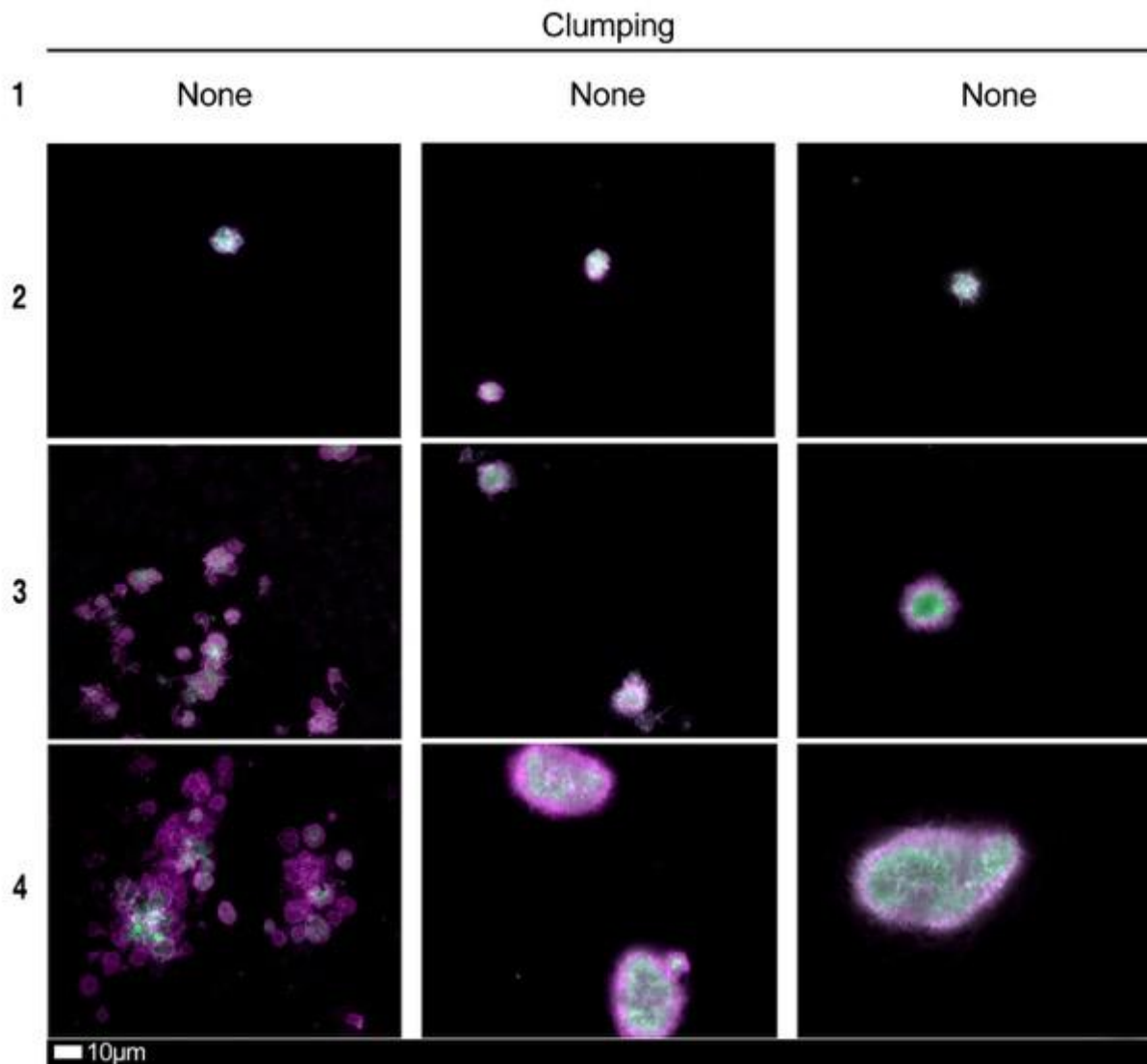


**Figure 7:** Fluorescence microscopy examples of the different stages of platelet activation and spreading, that was used to score the platelet activation in the Long COVID patients, with Stage 1, with minimally activated platelets, seen as small round platelets with a few pseudopodia, seen as healthy/control platelets that progresses to Stage 4, with egg-shaped platelets, indicative of spreading and the beginning of clumping. (Taken from <sup>26</sup> with permission).

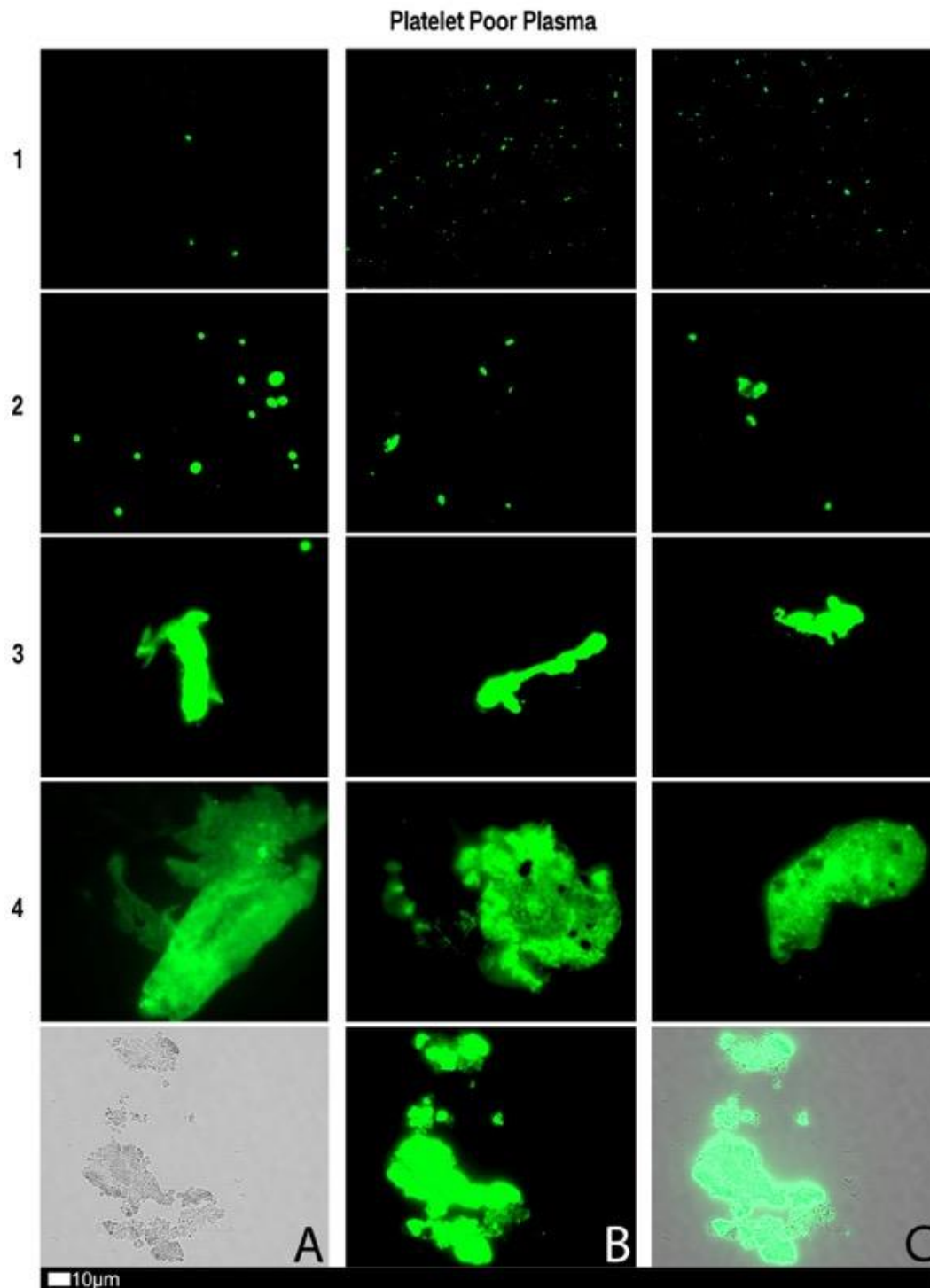




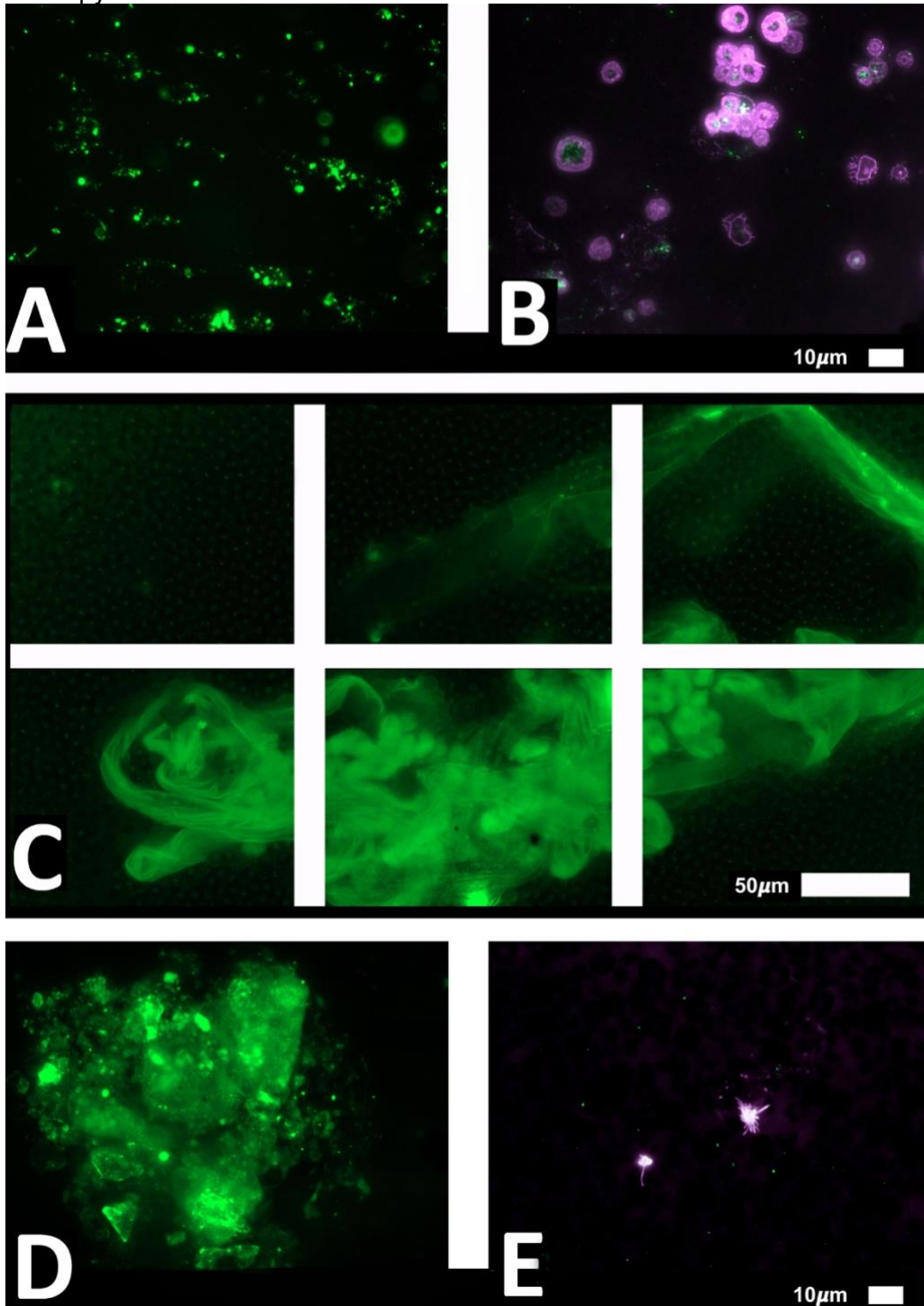
**Figure 8:** Fluorescence microscopy examples of the different stages of platelet clumping. With no clumping occurring in the healthy/control samples in Stage 1 (no figures shown), progressing to severe clumping of platelets as seen in Stage 4. (Taken from <sup>26</sup> with permission).



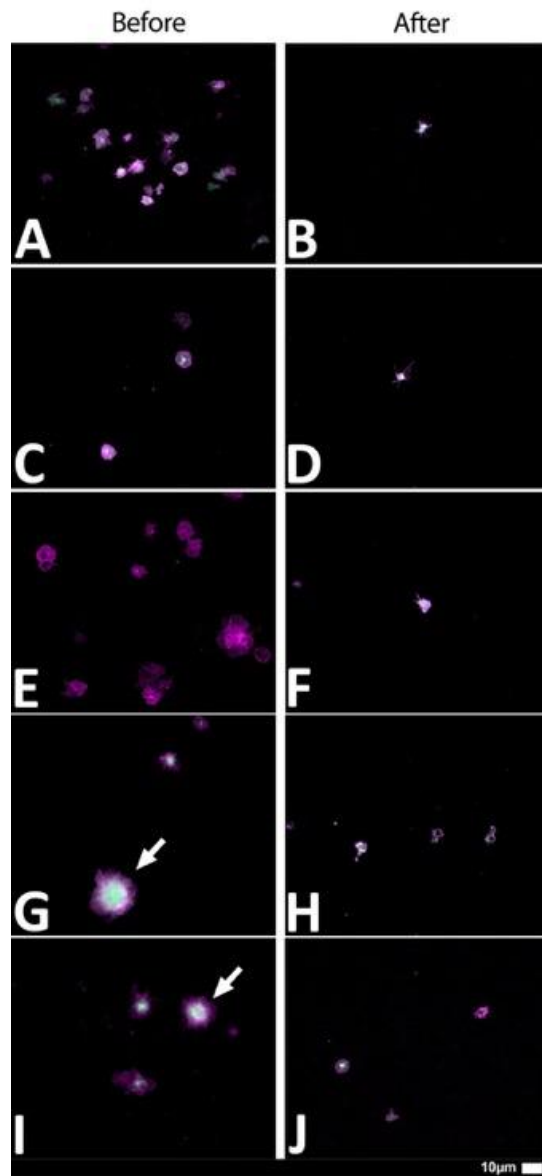
**Figure 9:** Fluorescence microscopy showing microclots in platelet poor plasma (PPP) with representative examples of the different stages of different stages of microclot formation. Stage 1 shows minimal microclot formation in healthy/control PPP which progresses to the presence of the severe microclotting 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. (Taken from <sup>26</sup> with permission).



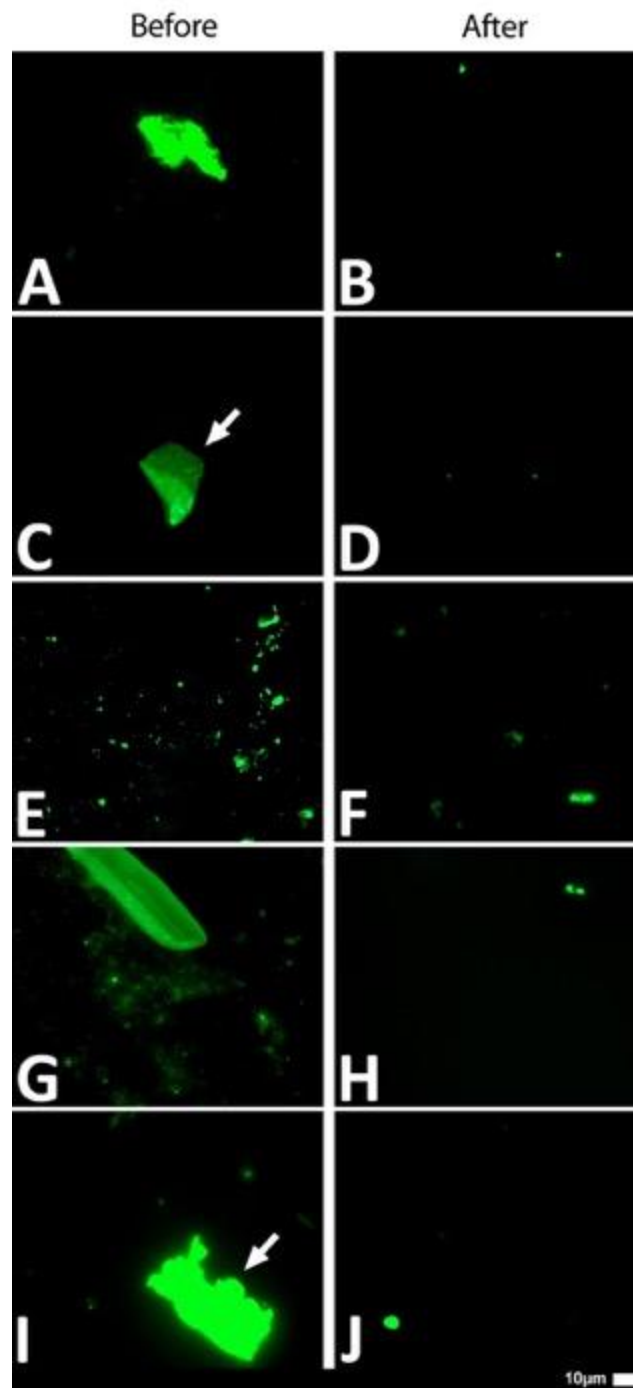
**Figure 10:** Microclots with platelets in 2 patients with Long COVID/PASC. **(A):** Representative micrographs of microclots in an untreated patient diagnosed with Long COVID/PASC, suffering from the condition for 11 months. The plasma was stained with thioflavin T (ThT); **(B)** Platelet hyperactivation of the same patient, where PAC-1 and CD62PE were used to mark platelets. **(C)** In this patient, cellular debris in the haematocrit was noted – here such cellular debris is shown in a tile scan. **(D and E):** Microclot presence and platelets from a patient who was on anti-platelet therapy before blood collection, where significant microclots were noted, but the platelets were not significantly hyperactivated, due to the use of anti-platelet therapy.



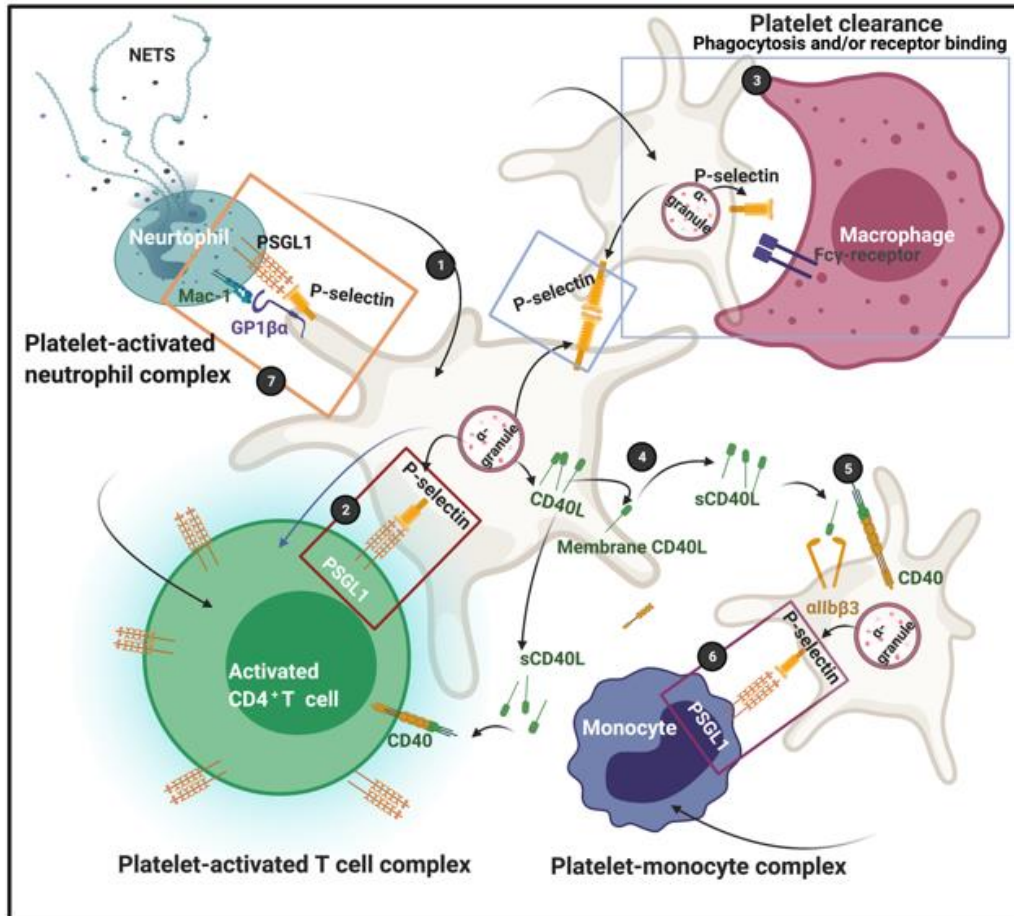
**Figure 11:** Representative fluorescence micrographs of platelet pathology before and after treatment. Moderate platelet spreading and mild platelet clumping (white arrows) was seen in the naïve patient's samples (Fig. A, C, E, G, I) that improved after treatment, with mild platelet spreading and no clumps (Fig. B, D, F, H, J).



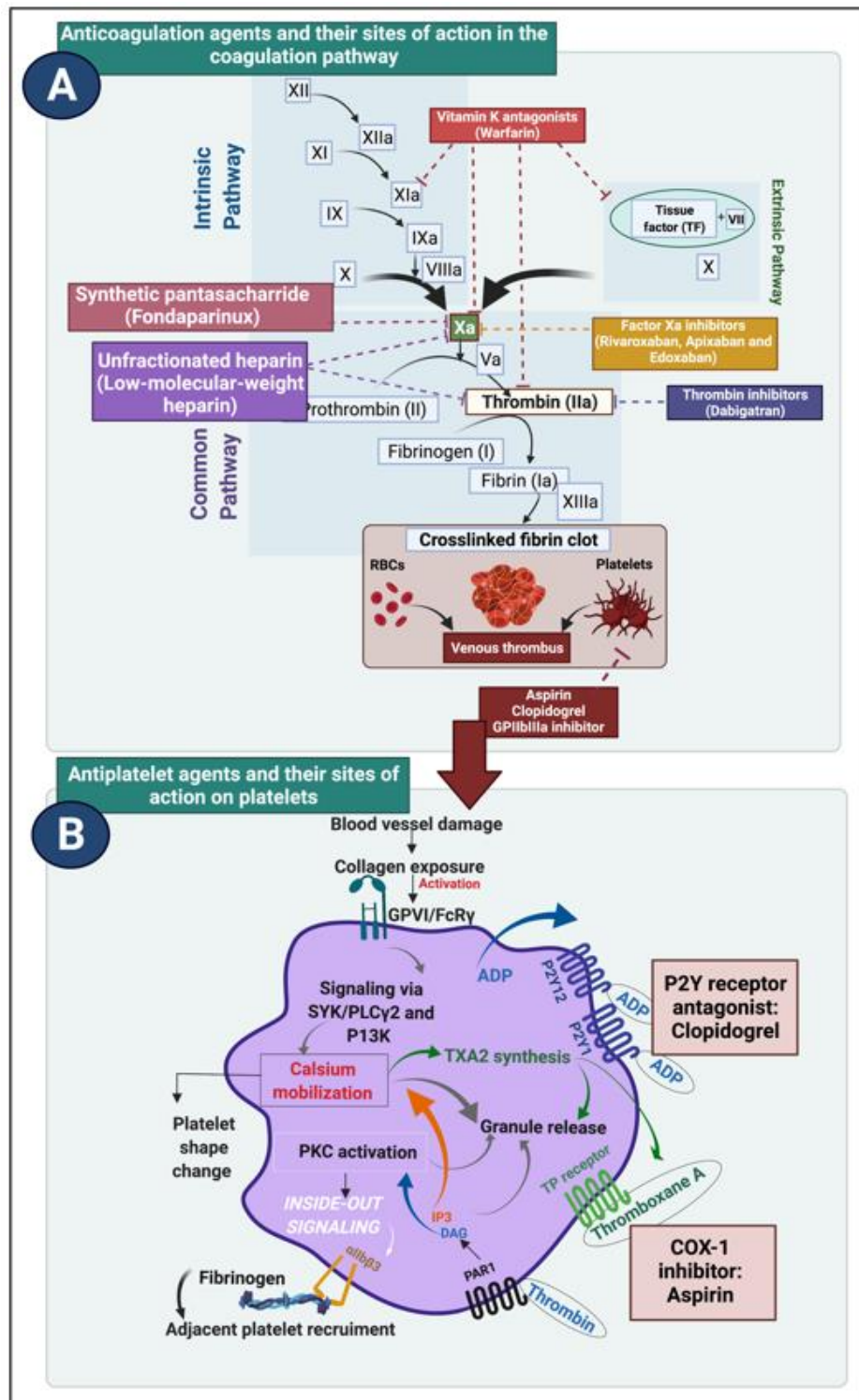
**Figure 12:** Representative PPP fluorescence micrographs with moderate areas of plasma protein misfolding forming microclots (some larger than 15 $\mu$ m; white arrows) before treatment (Fig. A, C, E, G, I), with a few microclots visible in the samples after treatment (Fig. B, D, F, H, J).



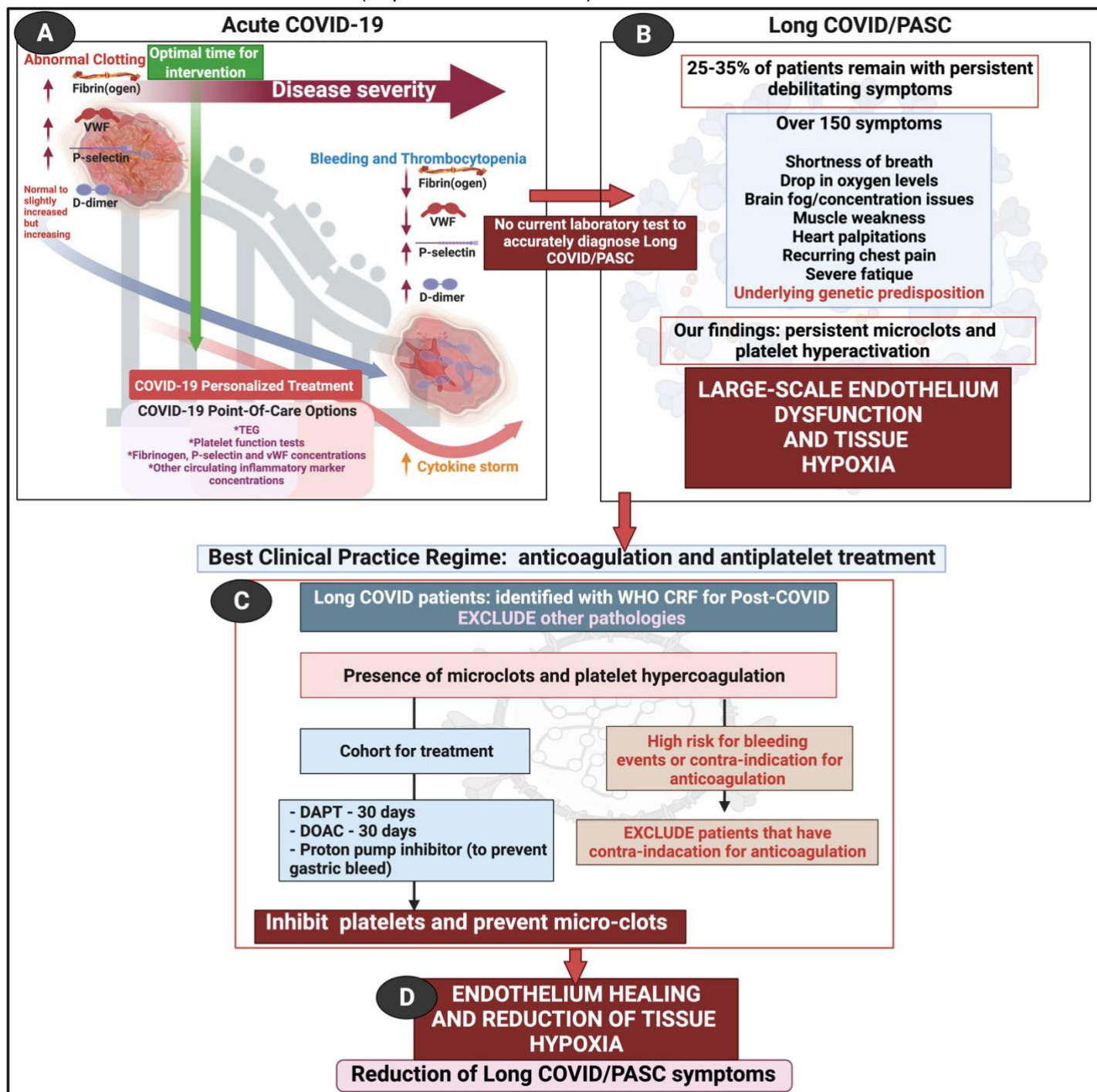
**Figure13:** (1) After activation, platelets express P-selectin on their membranes, followed by platelet-T cell complex formation (2); P-selectin on platelet membranes are also recognized by macrophages, possibly by the Fcγ-receptor; clearance may result due to either receptor binding or phagocytosis (3). CD40L is released from platelets and can migrate to membranes or shed as soluble (s)CD40L (4). sCD40L can bind to both the  $\alpha_{IIb}\beta_3$  or CD40 receptors (5). The P-selectin on the membranes of sCD40L-activated platelets can also form complexes with monocytes (6). Platelet-neutrophils also form complexes (7). Diagram created with BioRender (<https://biorender.com/>) and adapted from <sup>66</sup>.



**Figure 14:** Effects of selected direct oral anticoagulants (DOAC) and dual antiplatelet therapy (DAPT) medication on clotting and platelet function. Diagram created with BioRender (<https://biorender.com/>)



**Figure 15:** Understanding the pathological pathways of Long COVID/PASC. **A)** The rollercoaster vascular pathology in acute respiratory syndrome coronavirus 2 (COVID-19) [adapted from <sup>42</sup>]. **B)** Long COVID/PASC pathology resulting in persistent microclot formation and platelet hypercoagulation. **C)** Best clinical practice regime that reduces platelet hyperactivation and prevent microclot formation. **D)** Treatment should result in endothelium healing and reduction of tissue hypoxia and reduction of long COVID/PASC symptoms Image created with BioRender (<https://biorender.com/>).





## **ACKNOWLEDGMENTS AND DECLARATIONS**

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### **Consent for publication**

All authors approved submission of the paper.

### **Availability of data and materials**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. For data see:

<https://1drv.ms/u/s!AgoCOmY3bkKHi7Zrl0KftaiA7H35dg?e=sW8Q9L>

### **Competing interests**

MK is a non-executive director and shareholder of Gknowmix (Pty) Ltd. EP is the managing director of BioCODE Technologies. The other authors have no competing interests to declare.

### **Authors' contributions**

EP: Sample analysis, Long COVID/PASC registry curation, writing of the paper, editing the paper, co-corresponding author Study-leader; CV: Platelet analysis and data curation; GJL: sample collection, diagnoses and treatment of patients; MJK and KM: genetic analysis and editing of paper; LPW, KR, BWW, SO: Long COVID/PASC registry data analysis; DBK: Editing and writing of paper, co-corresponding author.

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