Long COVID Patient Information

Heparin-induced Extracorporeal LDL Precipitation (H.E.L.P.) apheresis

A high percentage of our Long COVID patients have experienced improvement of symptoms from a treatment protocol that combines H.E.L.P. apheresis and targeted anticoagulant therapy (3) (4). H.E.L.P. apheresis is a well-established last-resort treatment for patients with vascular diseases. It mechanically filters blood serum using a heparin filter to eliminate cholesterol, clotting factors, autoantibodies, bacterial toxins, and inflammatory mediators such as cytokines and tumor necrosis factor-α toxins. Additionally, H.E.L.P. apheresis potentially removes the SARS-CoV-2 spike protein and microclots present in Long COVID patients (5). By doing this, the treatment improves organ perfusion, endothelial function, and microcirculation. Furthermore, H.E.L.P. apheresis has anti-inflammatory and anticoagulant effects (5).

In patients who respond to this treatment protocol, somewhere between 3-8 apheresis treatments are usually needed in order to see significant improvements. For some patients, however, H.E.L.P. apheresis does not seem to be sufficient. Early treatment seems to increase the chances of a successful outcome, as those who receive treatment earlier seem to require fewer H.E.L.P. apheresis sessions. For patients who have been sick for a longer duration, more treatments might be necessary. Further, patients who have become unwell, or whose Long COVID symptoms have worsened by the vaccine, also seem to respond to H.E.L.P. apheresis. However, these patients might require additional interventions (for example if autoimmunity is found).

Priority: Critically ill patients

Currently we are limited in our treatment capacity and we are prioritising the critically ill.

- If you consider your situation to be critical, contact Dr. Jaeger's clinic by email: drjaegerpatients@gmail.com
- In the "Subject" field of your email, please start with "CRITICAL:", so that incoming emails can be sorted automatically.
- Please provide important details such as: your age, when you got ill, to what extent you can work, to what extent you are bed bound, your main symptoms, if you got sick from the vaccine, phone number, country of residence, etc. This will help the clinic process incoming requests.

Other clinics offering H.E.L.P. apheresis

Other clinics are also able to provide H.E.L.P. apheresis or plasmapheresis. Please see this list for contact details.

Anticoagulant therapy

Microclotting and resulting hypoxia (oxygen deprivation) seem to be central in the pathology of Long COVID (1). Microclots are small blood clots in capillaries that are resistant to fibrinolysis (the body's own mechanism

of breaking down clots) (1). These microclots are hypothesised to decrease organ perfusion (a decreased oxygen supply to organs), resulting in such Long COVID symptoms. Additionally, platelet hyperactivation is frequently observed in Long COVID patients, further promoting hypercoagulation in patients.

Such abnormal microclots and platelet hyperactivation have been identified in majority of the Long COVID patients that we have tested at the clinic. For patients with a confirmed Long COVID diagnosis, microclot presence, platelet hyperactivation, and symptoms correlating with hypoxia, targeted anticoagulant therapy is recommended to prevent further clotting and to promote the breakdown of existing microclots. This anti-coagulant protocol was developed by Dr. Jaco Laubscher (Stellenbosch University) and has been proven effective in many patients with Long COVID, even without combined H.E.L.P. apheresis (2).

Note: Under no circumstance should anticoagulants be taken without the supervision of a qualified doctor.

Note: For severe patients, the anticoagulant medication needs to be combined with H.E.L.P. apheresis treat-ment to mechanically filter out circulating microclots.

Prescription for anticoagulant medication from Dr. Jaeger

Dr. Jaeger prescribes anticoagulant medication by consultation ONLY. This is prescribed to Long COVID patients with confirmed microclot and platelet hyperactivation presence resulting in subsequent hypoxia. We are not able to send any prescriptions - patients have to collect their prescriptions at the clinic. We are also not able to ship any medication.

Your local doctor or cardiologist might also be willing to prescribe the recommended anticoagulants, given the evidence now available with respect to hypercoagulability in Long COVID (1). In such cases, ask your doctor to call the clinic to get access to the targeted anticoagulant protocol that we use.

- Contact the clinic by emailing frederika.montpetit@clinicum-stgeorg.de to book a consultation for anticoagulant therapy only.
- In the "Subject" field of the email, please begin with "ANTICOAGULANTS:", so that incoming requests can be automatically sorted.
- The consultation with Dr Jaeger is held in the clinic.

Note: A German prescription should be valid in other EU countries, including the EEA countries (Iceland, Liechtenstein and Norway).

Laboratory services: Assessment of microclots and platelet activation

We offer a laboratory service for assessment of microclots and platelet activation. Blood analyses are done by appointment only. Proof of abnormal clotting activity is needed by Dr. Jaeger to guide your treatment plan. It will also hopefully contribute to convincing your local health services that your condition is real and needs to be treated.

- To make an appointment for this testing, please email drjaegerpatients@gmail.com.

- In the "Subject" field of the email, please begin with "MICROSCOPY:", so that incoming requests can be automatically sorted.

On the day of your appointment, please be at the clinic between 08:00-09:00 to have a blood sample taken. Payment for the analysis is made upfront at the clinic. Blood analyses will usually be complete from 14:00 onwards. The results will then be available to Dr. Jaeger to discuss via consultation.

Note: It is essential to make an additional appointment for a consultation with Dr. Jaeger, as this is a separate from the blood analysis. This consultation can occur the same afternoon as your blood withdrawal, or the following day depending on Dr. Jaeger's availability. To organize this appointment, please call the clinic's reception (0049 8061 398 0).

Note: Microscope analysis bookings are not available on Fridays.

Other interventions for Long COVID

Primary care physicia
Please consult with your doctor regarding other treatment options for Long COVID, including medication to reduce symptoms related to immune activation, Mast Cell Activation Syndrome (MCAS), tachycardia, Postural orthostatic tachycardia syndrome (PoTS), and dysautonomia for example.

Advice from Long COVID patients

Exercise
In Long COVID patients, exertion seems to worsen symptoms, so pacing is critical when in recovery. Strenuous exercise might mobilise clots and thus contribute to increased hypoxia symptoms. It seems that exercise and other stressors might also increase platelet activation.

Stress and "sympathetic activation" of the autonomic nervous system (ANS)

Sympathetic activation of the ANS is commonly seen in Long COVID. Typical symptoms of sympathetic activation include: excitation, tension, muscle cramping, an increased sense of anxiety, increased heart rate, sleep disturbances and more. Sympathetic activation increases stress hormones like cortisol and stimulates the adrenergic receptors, creating a "fight or flight" response (constant alertness). Many Long COVID patients find it useful to try encourage parasympathetic activation of the ANS to promote healing and rejuvenation - the "rest and digest" state. Any measures that might reduce stress are encouraged.

Some techniques for stress reduction and relaxation include:

- Breathing exercises (calm the ANS)
- Meditation/mindfulness
- Yoga (For example, Hatha yoga is usually slow-paced and seems to be well tolerated)
- Massage
- Vagus nerve stimulation (using a TENS device with an ear clip has been shown beneficial for many with Long COVID)

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PoTS (Postural Orthostatic Tachycardia Syndrome)

PoTS is commonly present in Long COVID, causing symptoms such as lightheadedness and palpitations upon standing, dizziness, fatigue, brain fog, fainting, and gastrointestinal upset. Patients also often experience an abnormal increase in heart rate upon standing up, whereas these symptoms are improved when the patient sits of lies down. More information can be found here.

Referenzen / Papers

1 Pretorius, E., Vlok, M., Venter, C., Bezuidenhout, J. A., Laubscher, G. J., Steenkamp, J., & Kell, D. B. (2021). Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovascular diabetology, 20(1), 172. https://doi.org/10.1186/s12933-021-01359-7

2 Pretorius, E., Venter, C., Laubscher, G. J., Kotze, M. J., Oladejo, S. O., Watson, L. R., Rajaratnam, K., Watson, B. W., & Kell, D. B. (2022). Prevalence of symptoms, comorbidities, fibrin amyloid microclots and platelet pathology in individuals with Long COVID/Post-Acute Sequelae of COVID-19 (PASC). Cardiovascular diabetology, 21(1), 148. https://doi.org/10.1186/s12933-022-01579-5

3 "Long COVID Patients Benefit from the Use of HELP apheresis - Proof of Principle" Download PDF by Dr. Jaeger et. al, 2021-04-07. Unpublished paper.

4 Article DEUTSCHES ÄRZTEBLATT. (2021-07-25). "Extrakorporale Verfahren für COVID-19-Patienten bisher wenig genutzt". Interview with Dr. Jaeger. https://www.aerzteblatt.de/nachrichten/114029/Extrakorporale-Verfahren-fuer-COVID-19-Patienten-bisher-wenig-genutzt Download PDF: ENGLISH: "Extracorporeal procedures for COVID-19 patients have so far been little used" . English translation (From Google Translate) / Download PDF

5 Kell, D. B., Laubscher, G. J., & Pretorius, E. (2022). A central role for amyloid fibrin microclots in long COVID/PASC: origins and therapeutic implications. The Biochemical journal, 479(4), 537–559. https://doi.org/10.1042/BCJ20220016

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Fluorescent microskopy

What analyses are available?

1. Microclot analysis: Microclots are microscopic blood clots that are often found in various diseases, including Long COVID, ME/CFS, and type II diabetes (Pretorius et al., 2020). These microclots resist breakdown and remain circulating in the blood.

2. Platelet pathology analysis: Platelets are the smallest component in the blood and they are essential in blood clotting (Periayah et al., 2017). In patients with pathological clotting, platelets are often hyper-activated.

Why are these analyses helpful?

These analyses help doctors visualise and understand clotting in a patient and helps them decide whether their clotting is pathological. Based on this, doctors are able to prescribe treatment to patients.

Time needed for analysis

Checking for the presence of microclots and assessing the platelet pathology takes roughly two hours each, as the samples need time to incubate before the analysis.

Frequently Asked Questions

1. Do I have to have both my microclots and platelets analysed?
   You can decide to analyse only your microclots, only your platelets OR you can have both analysed. However, it is usually recommended to have both analysed, as it gives doctors a better understanding and overall picture of what is happening in your blood.

2. How do I make a booking to have my blood analysed?
   For now, you can make a booking my emailing frederika.montpetit@clinicum-stgeorg.de. Please make the subject of the email "MICROSCOPY". In the email, please briefly state which microscope analyses you would like and we will confirm what date you are booked in for. In future, we hope to set up an online booking system that is more convenient.
   Note: Blood analyses will not be performed unless a booking has been made.

3. How does the process work?
   On the day of your appointment, please arrive at the clinic St. Georg (Rosenheimerstr. 6-8, 83043 Bad Aibling, Germany) between 08:00 and 09:00. You will be asked to sign a consent form, pay for the analysis upfront, and your blood sample will be withdrawn.

4. How much blood is drawn?
   Only one tube of blood is taken.

5. How long does the analysis take?
   The results of the blood analyses should be ready from 14:00 onwards and will be made available to Dr. Jaeger once completed.

6. What do the results include?
   It will include 12 example microclot images and/or 12 example platelet images from your blood sample. The laboratory staP will also classify the results based on our classification system. This helps patients understand the severity of their results.
   Moreover, example template images are also attached for patients to compare their results to. Lastly, a brief explanation of the analyses is provided.

7. Am I able to send my blood sample overseas for the analysis?
   Ideally, no. When blood samples are sent overseas, it often takes longer than expected to arrive. Therefore, the blood samples are usually too old when they arrive, and we are unable to perform the analysis. Only in exceptional cases can we organize sending blood samples, and this is still done by appointment only.

8. After the blood analysis, am I able to make an appointment with Dr. Jaeger?
   To discuss your results with Dr. Jaeger, a separate appointment must be made by calling the clinic's reception (0049 8061 398 234). You can organize this appointment to be the same day as your blood with- drawal or the following day depending on Dr. Jaeger's availability that week.

9. Does insurance cover the cost of the blood analyses?
   For now, many insurance companies do not cover the cost of the blood analyses.

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H. E. L. P. Apheresis in Long COVID: Effect on Microclots, Inflammatory Mediators, and Patient Function: A Case Series

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Abstract

Mounting evidence suggests that the plasma of individuals with acute COVID-19 or Post-Acute Sequelae of COVID-19 (PASC-19, or 'Long COVID') contains fibrin amyloid microclots comparatively resistant to fibrinolysis. A biologically plausible explanation links the presence of such microclots to the blockage of capillaries, with the inhibition of oxygen transport to tissues. This may contribute to many of the symptoms of Long COVID such as breathlessness, fatigue, cognitive dysfunction, post-exertional symptom exacerbation, and autonomic dysfunction. Here we report the presence of microclots in the blood of all 19 Long COVID patients included in this preliminary investigation. The presence of these microclots was confirmed using both fluorescence microscopy and microfluidics.

Heparin-induced Extracorporeal LDL Precipitation (H.E.L.P.) apheresis is an established last-resort treatment for patients with advanced vascular diseases. It uses heparin to eliminate cholesterol, clotting factors, inflammatory mediators such as cytokines and tumor necrosis factor-α toxins, and possibly the SARS-CoV-2 spike protein and microclots present in Long COVID patients. By doing this, the treatment improves organ perfusion, endothelial function, and microcirculation. Therefore, H.E.L.P. apheresis is a possible safe approach to treat acute or Long COVID patients. We report on the effects of one H.E.L.P. apheresis treatment on these 19 patients. The baseline clot burden assessed by fluorescence microscopy correlated significantly with the severity of the overall cognitive deficit ($r=0.69$; $p=0.001$). A single H.E.L.P. treatment resulted in a significant reduction in clot burden ($p<0.01$), and also in serum levels of inflammatory and clotting mediators including fibrinogen, α2-antiplasmin, and plasminogen activity. A significant reduction in clot burden persisted for at least 24 hours ($p<0.05$). Treatment with H.E.L.P. apheresis was also accompanied by statistically significant improvements in cognition ($p=0.03$) and gait speed ($p<0.001$). These improvements in functional markers highlight the benefits of H.E.L.P. apheresis as an effective treatment for Long COVID and signal an urgent need for larger controlled studies into this treatment.

Keywords

Long COVID; Post-Acute Sequelae of COVID-19; H.E.L.P. apheresis; fibrin amyloid microclots; platelet activation; cognitive dysfunction; dysautonomia; real-time deformability cytometry; fluorescence microscopy; postural tachycardia syndrome; POTS; orthostatic intolerance; heart rate variability; 10-meter walk speed.

Introduction

Long COVID/Post-Acute Sequelae of COVID-19 (hereafter referred to by the patient-defined term 'Long COVID') (1, 2) is a growing public health emergency, with an estimated 100 million people affected worldwide (3). Current data from the United Kingdom's of National Statistics demonstrate that an estimated 1.3 million people (give actual number now) in the UK alone (2% of the population) are living with Long COVID (4). In the United States, economists have estimated that Long COVID will incur cumulative future costs of more than 4 trillion USD (5).

As many as 30% of those affected by acute COVID-19 continue to experience significant symptoms nine months after the initial infection (6). Up to 203 symptoms have been identified, with a few of the most common being fatigue, post-exertional symptom exacerbation (PESE), cognitive dysfunction, headache, and sleep disturbance (7-10). A meta-analysis has revealed that at 12 or more weeks following COVID-19 diagnosis, 32% of individuals still experienced fatigue and 22% reported cognitive impairment (11).

Along with such symptoms, altered coagulability has been noted in individuals suffering from Long COVID (cite – Resia and Jaco). The cascade of events leading to coagulopathy and endothelial dysfunction with acute COVID-19 infection is likely to include both direct (viral infection of cells) and indirect (immune cells and mediators) mechanisms. SARS-CoV-2 has an affinity for angiotensin converting enzyme-2 (ACE-2) and transmembrane serine protease 2 (TMPRSS2) which are present on both endothelial cells and platelets (12-18). In addition, platelets contain a number of other receptors (19) which may be involved in sensing the virus, viral proteins (20), and potentially even SARS-CoV-2 specific antibodies (21). Hence, there are several potential ways through which platelets may trigger the immune-thrombotic cascade (22).

Whether coagulopathy is triggered via direct infection of endothelial cells (23) or indirectly following platelet-driven cell-cell interactions (24) remains unclear. In any case, the consequent clotting dysregulation results in pathologically raised levels of thrombin, fibrinogen, von Willebrand Factor, alpha-2-antiplasmin, plasminogen activator inhibitor-1 (PAI-1), and P-selectin (23, 25-31). The formation of neutrophil extracellular traps (NETs) (32-34) and macrophage infiltrates (35) further promotes a hypercoagulable state. The ensuing activation of the enzymatic clotting pathway, hyperactivation of platelets, and fibrinolysis shutdown leads to a vicious cycle of thrombosis and endothelial dysfunction (14, 36-44). This breakdown of normal clotting physiology induced by the virus culminates in tissue ischemia and hypoxia, which can cause symptoms affecting any organ system.

There have been multiple reports of thromboembolic phenomena well after the acute phase of the illness (45-48). It is now abundantly clear that endothelial dysfunction, platelet activation, and hypercoagulability play a significant role in the pathogenesis of Long COVID (49-51). We have previously shown that in certain chronic inflammatory disease states including type 2 diabetes, Parkinson's disease, and Alzheimer's disease (52, 53), fibrinogen can polymerize into an anomalous, amyloid form that is much more resistant to fibrinolysis than normal clots (52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54)(52, 54). We have identified these circulating fibrin amyloid microclots in the plasma of patients with acute COVID-19 (55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56)(55, 56) and, more recently, in Long COVID additionally (25). These clots have been found to be inflammatory and immunogenic (57). We hypothesize that removal of these clots may improve the symptoms of Long COVID, and that this may be achieved by Heparin-induced Extracorporeal LDL Precipitation (H.E.L.P.) apheresis (cite Arteblatt 2020).

Heparin-induced Extracorporeal LDL Precipitation (H.E.L.P.) apheresis

H.E.L.P. apheresis was first developed in 1984 by Seidel and Wieland, primarily for patients with severe hyperlipidemia or familial homozygous hypercholesterolemia (58-65). It has been shown to improve long-term outcomes in coronary artery disease (66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)(66, 67)66, 67) and can help prevent and treat graft vessel disease following cardiac transplantation (68-72) (69). Other conditions in which it has been used successfully include cerebrovascular disease (73-76), acute coronary syndrome (77), and pre-eclampsia (78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79)(78, 79). H.E.L.P. apheresis has also been tested in septic multi-organ failure in pilot studies (80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81)(80, 81). The procedure has been found to be very safe, with adverse events being rare (75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84)(75, 82-84).

There are several mechanisms through which H.E.L.P. apheresis may be effective in treating Long COVID. First, H.E.L.P. apheresis uses unfractionated heparin (400,000 units) in the extracorporeal circuit for precipitation (cite) (58, 75). Heparin binds the spike protein with high efficiency (cite-West) and thereby enables removal from blood circulation (cite)(85-90). Heparin has also been demonstrated to have anti-inflammatory effects (91). Secondly, heparin binds to the ACE-2 receptor, potentially hindering cellular invasion by the virus (cite) (85-90). Therefore, heparin as used in H.E.L.P. apheresis may act through antiviral and anti-inflammatory mechanisms. Third, H.E.L.P. apheresis reduces hypercoagulability by lowering plasma fibrinogen by 50-60% and other clotting factors by 35-50% (58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75)(58, 75). Antithrombin III is only reduced by 25%, thereby attenuating the risk of bleeding (92). By reducing fibrinogen, H.E.L.P. apheresis confers rheological benefits: plasma viscosity is reduced by roughly 20% (cite) and erythrocyte aggregation is lowered by 66% (add cite of %s) (65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 93, 94)(65, 67, 68, 86, 87), improving myocardial (65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 94)(65, 87) and cerebral (95) blood flow rates, as well as coronary flow reserve (94). This has the effect of improving capillary gas exchange (96). Lastly, cytokines including interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α (97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(97, 98)(90, 91), as well as C-reactive Protein (CRP) (99) have been shown to be elevated in Long COVID. H.E.L.P. apheresis removes cytokines and reduces CRP levels by more than 50% (79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 100)(79-81, 93). Hence, H.E.L.P. apheresis is beneficial in removing these inflammatory molecules present in Long COVID.

19 Long COVID patients were included in this pilot study, where we investigate whether H.E.L.P. apheresis improves functional outcomes and reduces clot burden, and whether it is a beneficial treatment for Long COVID patients.

MATERIALS AND METHODS

Ethical Approval

The study was conducted between 15 and 19 November 2021 at the Lipidzentrum Nordrhein, Mülheim an der Ruhr, Germany. All experimental objectives, risks, and details were explained to the volunteers, and written informed consent was obtained prior to blood collection. Strict compliance to the ethical guidelines and prin-

ciples of the Declaration of Helsinki and Guidelines for Good Clinical Practice was maintained for the duration of the study and for all research protocols. Ethical approval for blood collection before and after best clinical treatment for Long COVID patients, was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University (South Africa) (N19/03/043, project ID 9521- amended November 2021). The research team that performed the blood analysis had no role in the planning of the clinical treatments or clinical measurements related to the patient well-being. For H.E.L.P. apheresis, separate written informed consent was obtained by the treating physician (BRJ) from all patients explaining the risks and benefits. Pharmacotherapy was instituted along the lines of BRJ's usual established clinical practice. Ethics must be adapted.

Long COVID diagnosis

The 19 patients included in the study were a combination of self-referred individuals and those referred by their physicians. The history of acute COVID-19 infection was either confirmed by the presence of a positive PCR or antibody test in the patient's medical history, or diagnosed as a case of probable COVID-19 infection based on symptom presentation at the time of acute illness using World Health Organization (WHO) recommendations (101). All patients had either received a formal diagnosis of Long COVID from their primary care practitioner or from a clinician experienced in the condition (BRJ) at the apheresis center. Participants were thoroughly pre-screened with a detailed intake form sent out prior to the study. This form was also used as a means of gathering baseline characteristics.

H.E.L.P. apheresis

The H.E.L.P apheresis technique is explained in Figure 1. Blood cells are separated from plasma in an extracorporeal circuit, 400,000 units of unfractionated heparin are added to the plasma, and the pH is lowered to 5.12 using an acetate buCer (cite?). This is the isoelectric point for optimal precipitation of apolipoproteins from low-density lipoprotein (LDL) cholesterol, lipoprotein(a) [Lp(a)], and very-low density lipoprotein (VLDL). These molecules are removed along with 60% of plasma fibrinogen. Excess heparin is adsorbed, and bicarbonate dialysis balances the pH to counter any risk of haemorrhage. The blood cells are then reinfused in parallel with a saline solution. The duration of the procedure is usually 1.5 to 3 hours, depending on flow rate achieved, and between 2.5 and 4 litres of blood are treated per session (58,75). Additionally, treatment options can be varied according to the patient's need (cite).

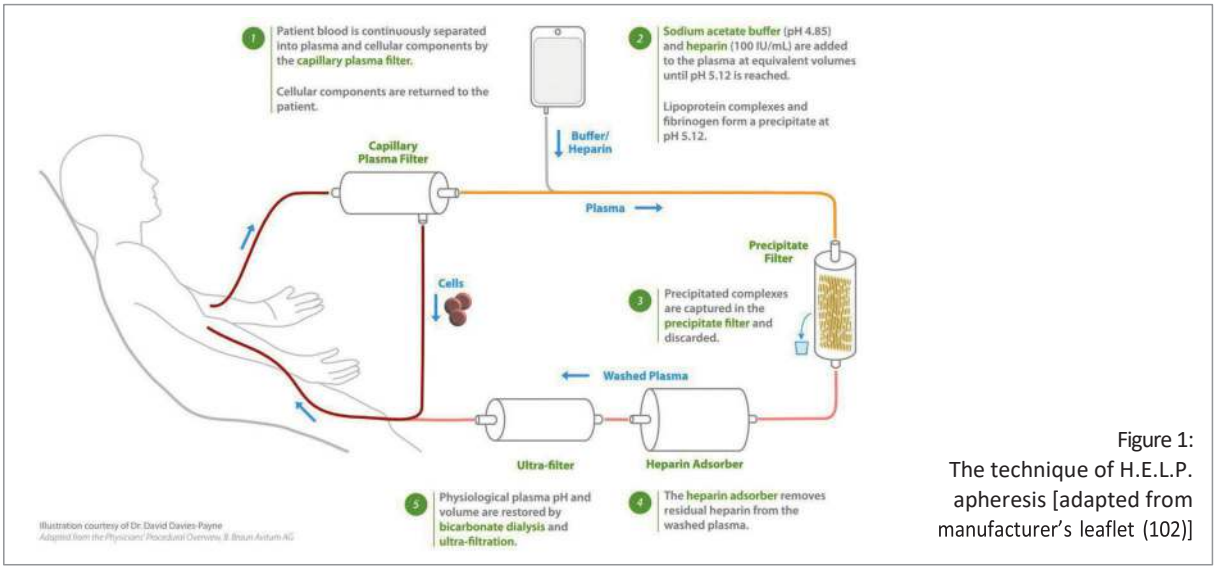

Figure 1:
The technique of H.E.L.P. apheresis [adapted from manufacturer's leaflet (102)]

Clinical testing before and after H.E.L.P. apheresis

All 19 participants underwent a program of functional and physiological testing before and within 24 hours of H.E.L.P. apheresis. The clinical evaluations that were performed and their frequency are listed in Table 1. The tests were performed by trained volunteers assisting the treating physician (BRJ) and the nursing staC at the center.

In the week prior to arrival, patients completed the following assessments remotely:

- A detailed symptom and function checklist based on the modified COVID-19 Yorkshire Rehabilitation Screen (C-19 YRS) (103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104)(103, 104).

- A baseline cognitive assessment was performed using a computerized cognitive battery (Brain Check; Hous- ton, Texas). The six measures that made up the cognitive battery are listed in Table 1. Each of the tasks were scored independently, and an overall score was automatically produced by the software, which has been validated in cases of sudden onset cognitive impairment (Yang et al., 2017).

The remaining functional and physiological testing and data collection was carried out face to face:

- Functional mobility testing was performed using the 10 meter walk test (10 MWT), which is a well-validated measure that can be completed with relatively low exertion on the part of the participant (105).

Two autonomic measures were completed:

- Root mean square of successive RR interval differences (RMSSD), which is known to correlate with autonomically-mediated changes in heart rate variability (HRV) (106) was collected using mobile app-based photoplethysmography technology developed by Happitech (Happitech, Amsterdam, The Netherlands).

- Postural orthostasis was assessed by monitoring blood pressure and heart rate after three minutes in the supine position and then again after three minutes in the upright position (107). This made use of the guidelines for heart rate changes (D30 beats per minute increase) set out by the American Autonomic Society (AAS; Freeman et al., 2011).

Spirometry and peripheral venous oxygen saturation tests were also completed:

- Spirometry with flow volume loop (FVL) and diffusing capacity for carbon monoxide (DLCO) were performed and analyzed following standards approved by the American Thoracic Society and European Respiratory Society (108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109)(108, 109) using the EasyOne Pro® lung function equipment (NDD Medical Technologies; Zurich, Switzerland). Global Lung Function Initiative (GLI) reference values for spirometry and DLCO were employed (110, 111).

- Peripheral venous oxygen saturation (SpvO2) was measured in a sample of venous blood drawn into a heparinized syringe at the time of cannulation of the antecubital vein for apheresis, and again after the procedure. The samples were processed using the GEM Premier 5000 blood gas analyzer (Werfen; Bedford, Maine, USA).

Table 1: Functional testing schedule

Measure	Domain	Pre-Aphresis	Post-Aphresis
10-Meter Walk Speed (10 MWT)	Functional Mobility	Yes	Yes
Symptom and Function Checklist	Symptom self-report	Yes	No
Heart Rate Variability (HRV)	Autonomic	Yes	No
Postural orthostasis	Autonomic	Yes	Yes
Spirometry with flow volume loop (FVL)	Respiratory	Yes	Yes
DiCusing Capacity for Carbon Monoxide (DLCO)	Respiratory	Yes	Yes
Peripheral Venous Oxygen Saturation (SpvO2)	Respiratory	Yes	Yes
Trails A (Attention)	Cognition	Yes	Yes
Trails B (Mental Flexibility)	Cognition	Yes	Yes
Digital Symbol Substitution			
(Executive Function)	Cognition	Yes	Yes
Stroop (Executive Function)	Cognition	Yes	Yes
Immediate Recognition (Memory)	Cognition	Yes	Yes
Delayed Recognition (Memory)	Cognition	Yes	Yes

Blood collection: before and after H.E.L.P. apheresis

Blood was collected into citrated and EDTA blood collection tubes (S-Monovette; Sarstedt, AG & Co. KG, Nümbrecht, Germany). All 19 patients contributed blood samples prior to H.E.L.P. apheresis. For fluorescence microscopy, seven patients contributed blood samples immediately post-apheresis, 15 patients contributed blood samples 24 hours later, and three patients did so at all three time points. For microfluidics analysis, 16 patients provided blood samples immediately before and after apheresis. Blood was analysed for inflammatory mediators and clotting factors at Labor Mönchengladbach, MVZ Dr. Stein und Kollegen, Mönchengladbach, Germany. The measurements for clotting factors were performed on the soluble part of the plasma. For each of the timed collections, whole blood (WB) was used for microfluidic analysis and centrifuged at 3000xg for 15 minutes at room temperature. The supernatant platelet poor plasma (PPP) was used for microclot analysis, and the haematocrit was used to study platelet pathology.

High-throughput microfluidic imaging of whole blood samples

Real-time deformability cytometry (RT-DC) on WB was performed as described in detail elsewhere (112, 113)). Briefly, apolydimethylsiloxane (PDMS)-made microfluidic chip with two inlets (sheath- and sample-flow) and an outlet connected by a channel constriction of 20 x 20 µm was mounted onto an inverted microscope (Axiovert- 200, Zeiss, Germany) equipped with an LED (CBT-120, Luminus Devices, USA) and a high-speed camera (Eo- Sens CL MC1362, Mikrotron, Germany). Two syringe pumps (NemeSyS, Cetoni, Germany) were used to deliver blood cell suspension (sample) and sheath-flow through the microfluidic chip at a sample flow rate of 0.015 µL sE1 and a sheath flow rate of 0.045 µL sE1, resulting in a total flow rate of 0.06 µL sE1. The sample consisted of WB and measurement buffer (MB) in a 1 to 20 ratio, respectively. The MB was based on phosphate buffered saline (PBS, Mg2+-, and Ca2+-free) and 0.6% w/w methylcellulose (4,000 cPs; Sigma Aldrich, USA). Viscosity and osmolarity were adjusted to 26 mPa s at room temperature and 280 – 290 mOsm/kg, respectively. At the end of the channel constriction (the region of interest), an image (250 x 80 pixels) of every cell

was captured at a frame rate of 3600 fps using ShapeIn Software (Zellmechanik; Dresden, Germany). In total, 0.45 µL blood was measured. Analysis of microclot-like structures was done using the freeware ShapeOut2 version 2.9.2 (114).

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

A fluorescent amyloid dye, Thioflavin T (ThT) (final concentration: 0,005mM) (Sigma-Aldrich, St. Louis, MO, USA) was used to identify anomalous (amyloid) microclotting in PPP (49). The excitation wavelength for ThT was set at 450nm to 488nm and the emission at 499nm to 529nm. PPP was exposed for 30 minutes (protected from light) at room temperature, whereafter 3 µL PPP was placed on a glass slide and covered with a coverslip. 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). Micrograph analysis was done using ImageJ (version 2.0.0-rc-34/1.5a), as discussed in our previous publication (55). The percentage (%) area of microclot and cellular debris signal was calculated using the thresholding method. The RGB images are opened in ImageJ and calibrated by setting the scale (calculated using the image pixel size and the known size of the scale bar). The images were converted to black and white (8 bit), followed by thresholding (Huang setting in ImageJ) adjusting the background intensity to white (255) thresholding the area signal to between 11 and 15. We chose the Huang setting as it finds the optimal threshold value and minimises fuzziness. The 'analyze particle' setting was used to determine particle size from 1 to infinity.

Platelet pathology

Haematocrit samples were used to study platelet activation. Samples (20µL) were exposed to 4µL of CD62P (PE-conjugated) (platelet surface P-selectin) (IM1759U, Beckman Coulter, Brea, CA, USA) and 4µL of PAC-1 (FITC-conjugated) (340507, BD Biosciences, San Jose, CA, USA). Samples were incubated for 30 minutes (protected from light) at room temperature, whereafter 10µL was placed on a microscope slide and covered with a coverslip. The excitation wavelength band for PAC-1 was set at 450 to 488nm and the emission at 499 to 529nm. For CD62P excitation was set at 540nm to 570nm and the emission was set at 577nm to 607nm. Samples were viewed using the Zeiss Axio Observer 7 fluorescent microscope (63x/1.4 Oil DIC M27 objective).

Statistics

Statistical analysis was performed using Graphpad Prism 8 (version 8.4.3). Shapiro-Wilks normality tests were performed and unpaired t-tests were performed on parametric data with the data expressed as mean ± standard deviation, whereas Mann-Whitney U tests were performed on unpaired non-parametric data and the data expressed as median [Q1 – Q3] (all two-tailed). Statistical analysis for microfluidic imaging was undertaken using Graphpad Prism 8 (version 8.4.3) by performing paired t-tests on parametric data with the data expressed as mean ± standard error of mean. Statistical analysis for clinical variables were performed in MATLAB, R2020B (Mathworks, Natick, Massachusetts). Paired t-tests were used to evaluate pre- and post-apheresis metrics and to compare baseline metrics to normative data. Where appropriate, Bonferroni Correction was used to adjust for multiple comparisons. Correlations between relevant variables were evaluated by computing Pearson's Correlations.

RESULTS

Baseline characteristics

A total of 19 [13 males and six females; age mean/standard deviation (SD) 40 ± 12; 18 Caucasian and 1 South Asian] H.E.L.P. apheresis-naïve Long COVID patients participated in the study. The mean/SD in months of disease was 15.2 months ± 5.8 months. A summary of patient responses to their initial symptom and functional inventories are detailed in Figure 2, showing the overall frequency of different symptom domains and functional limitations.

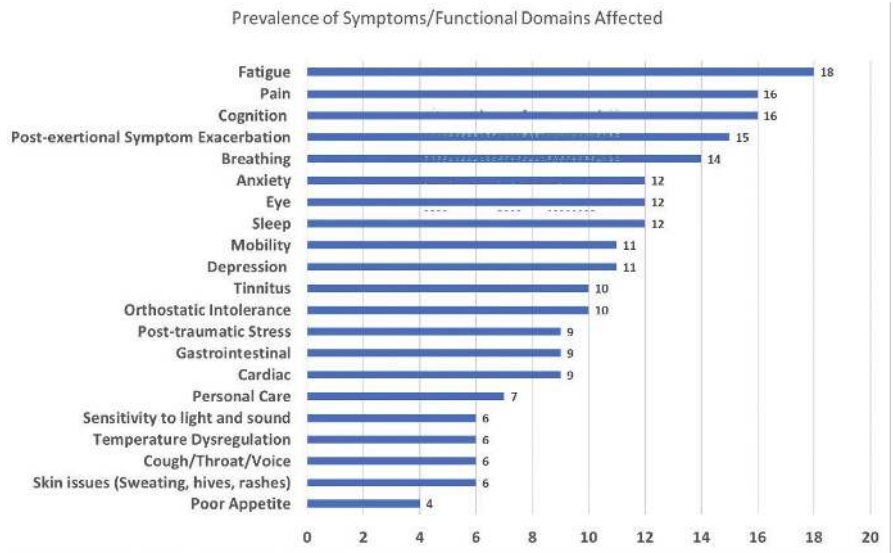

Figure 2: Summary of symptom and functional inventories at intake: Total number of patients who reported issues in each symptom domain or functional category at initial intake. Format? Patients were asked about the impact of the illness on their work or education. Three had lost their jobs completely, eight were on sickness absence, five had required either a reduction in hours or a significant adjustment to their working arrangements, and two did not provide an answer. Only one participant had experienced no change in status.

Clinical testing results

A total of 19 patients (six females) completed baseline clinical testing prior to their first apheresis session. All 19 patients also completed the post-apheresis evaluation 24 hours after treatment. One participant was unable to complete the 10 MWT both pre- and 24 hours post-apheresis due to limitations imposed by their condition.

Cognitive and Functional Outcomes

The results of all pre- and post-apheresis clinical and functional measures are presented in Tables 2-3 and Figure 2. Notably, patients showed significantly improved scores in walking speed measured during the 10 MWT (mean difference: 0.27m/s, $p<0.001$; Figure 2) and the Digit Symbol Substitution (DSS) cognitive task (mean difference: 13.3, $p<0.001$) post-apheresis. Post-apheresis improvements in the DSS were significantly correlated with improvements in the 10 MWT ($r=0.55$, $p<0.02$). Mean scores on the Trails A, Trails B, Stroop, Immediate Recognition, and Delayed Recognition domains did not show significant changes post-apheresis in comparison to pre-apheresis baseline. However, for the Trails B ($r=-0.68$, $p<0.002$), Stroop ($r= -0.91$, $p<0.0001$), and Delayed Recognition ($r = -0.75$, $p<0.0001$) tests, strong negative correlations were observed between the baseline pre-apheresis score and the change in score post-apheresis, indicating that any post-apheresis improvements in these domains were of greater magnitude in those who scored below average prior to the procedure.

Table 2: Cognitive function tests before and after H.E.L.P. apheresis.

COGNITIVE DOMAIN	PRE (MEAN ± SD)	POST (MEAN ± SD)	P-value
TRAILS A	97.9 ± 17.1	103.3 ± 11.4	46
TRAILS B	97.4 ± 15.6	102.2 ± 11.9	23
DIGIT SYMBOL SUBSTITUTION	94.3 ± 19.6	107.3 ± 9.5**	1
STROOP	88.7 ± 26.5	100.8 ± 10.6	6
IMMEDIATE RECALL	103.2 ± 12.1	105.3 ± 12.8	48
DELAYED RECALL	96.8 ± 18.9	98.1 ± 14.6	78
OVERALL SCORE	98.0 ± 16.3	105.4 ± 13.0*	3

*p<0.05, **p<0.002

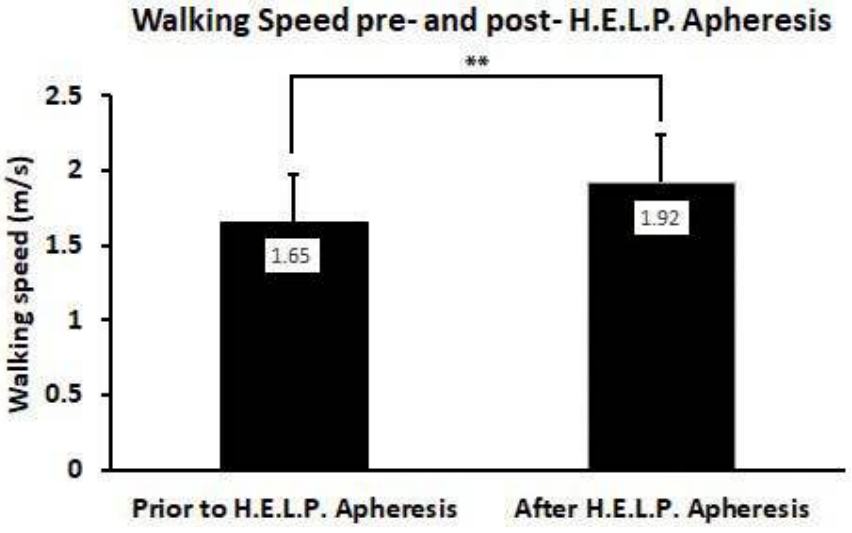

Figure 2: Change in walking speed (meters/second) immediately following one apheresis session compared with baseline. **p<0.001

Autonomic Outcomes

Heart rate variability was only collected at baseline. Patients had an RMSSD that was, on average, 80% lower than the median expected RMSSD for age- and gender-matched controls. Most patients did not show physiological signs consistent with an objective diagnosis of postural orthostatic tachycardia syndrome (POTS), with the average heart rate change after assuming the upright position being 13.2 (±15.0) beats per minute (bpm). Post-apheresis, the average positional heart rate change was 14.2 (±11.0) bpm. However, three patients presented with orthostatic changes that met the AAS criteria for POTS, with an average positional change of 39 (±7.6) bpm; this improved to 14.7 (±9.0) bpm after the H.E.L.P. apheresis procedure. Following apheresis, no patients in the cohort met AAS criteria for POTS.

Respiratory Outcomes

Mean dynamic lung volumes- both forced expiratory volume in one second (FEV1) and forced vital capacity (FVC)- were normal across the cohort both before and after apheresis. The mean FEV1/FVC ratio (using a cut-oC of <70% to define airflow obstruction) (115) was unchanged pre- and post-apheresis. Of note, no patient had a significant smoking history (defined as D20 pack-years). Similarly, mean DLCO expressed as a percentage of the predicted value was normal and remained unchanged after the procedure. Only one patient had

a low DLCO of 70% predicted pre-apheresis, and post-apheresis this remained virtually unchanged at 71% predicted. There was no significant difference in mean SpvO2 measured before and after apheresis. We elaborate on the interpretation and significance of the SpvO2 findings in the discussion.

Table 3: Respiratory function tests before and after H.E.L.P. apheresis.

RESPIRATORY DOMAIN	PRE (MEAN ± SD)	POST (MEAN ± SD)	P-value
FEV1	3.7 ± 0.9	3.8 ± 0.8	19
FEV1 % Predicted	97.3 ± 13.5	98.8 ± 13.2	14
FVC	5.0 ± 1.1	5.0 ± 1.	36
FVC % Predicted	104.6 ± 12.0	105.4 ± 12.0	41
IFEV1/FVC Ratio	0.8 ± 0.09	0.8 ± 0.08	46
DLCO % Predicted	106.9 ± 13.2	106.2 ± 13.8	93
SpvO2	56.3 ± 19.5	62.0 ± 15.1	30

Inflammatory mediators, coagulation parameters and lipids

Table 4 shows the analysis of selected inflammatory mediators and coagulation parameters for the 19 patients pre- and post H.E.L.P. apheresis. Even before apheresis, the mean values of these molecules in serum were within normal limits. This potentially coincides with our previous finding that perhaps majority of the inflammatory molecule load is trapped within the microclots, thereby escaping detection by standard lab assays (25). Apheresis further lowered serum levels of these molecules, which is consistent with previous work and demonstrates that they are temporarily removed or reduced by H.E.L.P. apheresis (85, 89, 93)check references that the order hasn't changed.

Table 4: Selected serum inflammatory mediators, clotting parameters, and lipid data before and after the first H.E.L.P. apheresis treatment.

Biomarker	Before	After	P value	Normal value/range	Unit
Alpha-2-antiplasmin	1012	828	****p<0.0001	80-120	%
Fibrinogen	2415	882	****p<0.0001	180-350	mg/dl
Plasminogen activity	9212	398	****p<0.0001	75-150	%
D-Dimer	3823	1777	****p<0.0001	<500	ng/ml
INR	11	19	****p<0.0001	0.9-1.3	INR
aPTT	258	654	***p<0.001	25.6-32	sec
Antithrombin III	958	N/A*	N/A*	79-120	%
CRP	2	N/A*	N/A*	<0.5	mg/dl
LDL Cholesterol	1283	528	****p<0.0001	0-160	mg/dl
HDL Cholesterol	558	522	****p<0.0001	>35	mg/dl
Triglycerides	1356	498	****p<0.0001	<200	mg/dl
HbA1c	54	54	p>0.05	4.2-6.0	%

*Antithrombin III and CRP were not measured after H.E.L.P apheresis and therefore no P-value was calculated. This is because it has been demonstrated consistently that CRP is reduced by 60% (79-81, 100) and antithrombin III by 25% (92) after H.E.L.P apheresis

High-throughput microfluidic imaging of whole blood samples

Real-time deformability cytometry was used to assess microclot-like structures in WB of Long COVID patients before and after H.E.L.P. apheresis. RT-DC is a microfluidic-based imaging technology used to acquire the physical properties of particles in flow at a speed of up to 1000 events per second and has been used previously to document changes in the physical phenotypes of blood cells during acute COVID-19 infection (116). We used a simple gating strategy to identify microclot-like structures by plotting 100,000 of the approximately 1.2 million acquired events for each patient, according to their projected area and deformation. We then applied a polygon gate to detected events as shown in Figure 5A. Representative appearances of gated microclot-like structures are illustrated in Figure 5B, whilst Table 5 displays the overall analysis of the microclot-like structures. A significant reduction in the overall count of microclot-like structures and a reduction in the percentage of microclot-like structures to all measured events in a blood sample was noted immediately after apheresis ($p<0.05$; Figure 5A and 5B). Clot size (projected area) and the standard deviation of the projected area did not change (Figure 5C and 5D). However, it should be kept in mind that not all events are necessary clots, as damaged endothelial cells or cellular debris generated by the apheresis process may have been present and therefore recognised in the analysis.blood samples

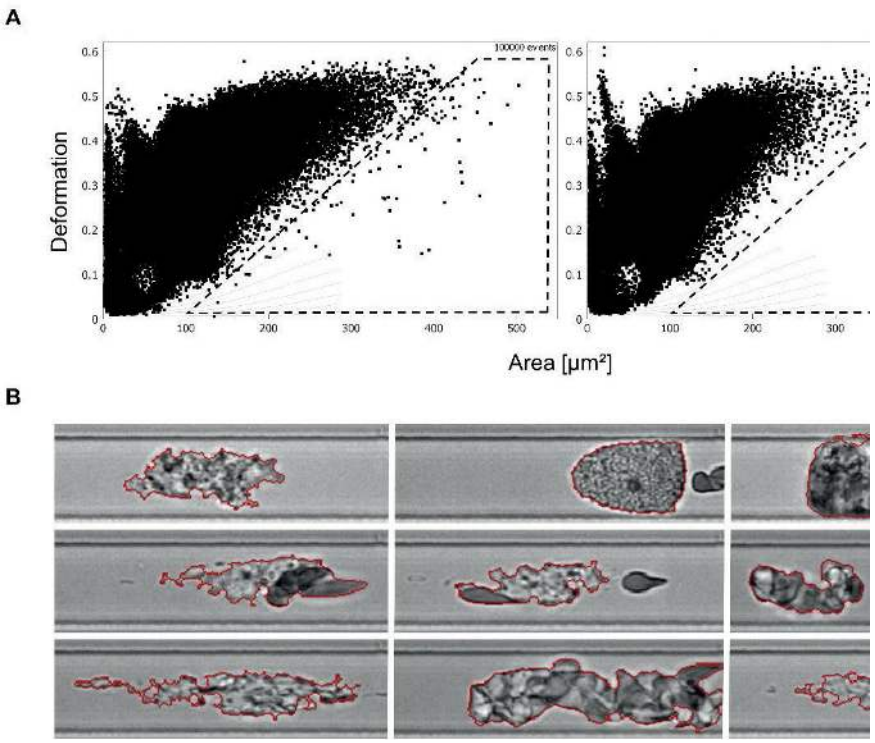

Figure 4:
Representative RT-DC measurements of whole blood (approximately 1.2 million event images per participant were acquired).
Lots of commas and then fullstops for numbers – maybe make it consistent

A) Gating strategy to identify microclot-like structures from WB before and directly after H.E.L.P. apheresis. Scatter plots of area and deformation show representative RT-DC measurements. Every dot is a measured event ranging from normal blood cells or cell agglomerates to microclot-like structures (100,000 events are shown). The events outside the gate (dashed polygon) are blood cells or cell agglomerates.

B) Representative images of microclot-like structures in RT-DC measurement channel. Red lines outline the event contour, which automatically gets assigned to the event in real-time and is the basis for size analysis (area within the contour in µmF).

Table 5: Overall analysis of n = 16 patients of microclot-like structures derived from RT-DC measurements. A: count of microclot-like structures per 0.45 µl of blood, B: microclot-like structures expressed as a percentage of all measured events (blood cells and cell agglomerates), C: projected area (µmF) of microclot-like structures; and D: standard deviation of the projected area (µmF) of microclot-like structures before and directly after H.E.L.P apheresis.

	Measurement	Before apheresis (Mean ± SEM)	Directly after apheresis (Mean ± SEM)	P-value
A	Count [per 0.45µl]	13 ± 1.9	7.86 ± 1.44	136
B	% [of all events]	0.00081 ± 0.00015	0.00053 ± 0.0001	769
C	Area [µmF]	420.1 ± 24.8	385.4 ± 15.6	2514
D	Area std [µmF]	129.7 ± 9.6	104.3 ± 11.4	101

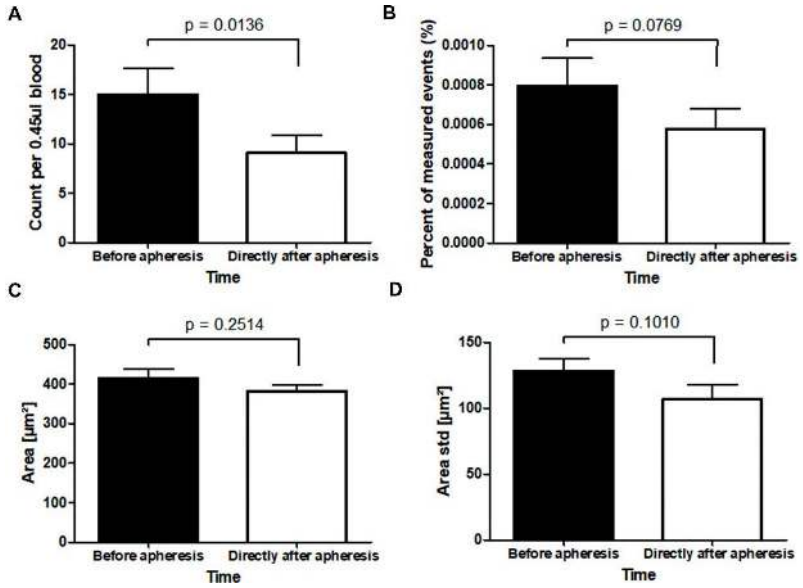

Figure 5:
Analysis of microclot-like structures from RT-DC measurements of 0.45 µl of whole blood. I thought graph headings went below graph? Make consistent throughout

A-D) Bar plots of n = 16 patients (repeat samples were not received for three patients):

A) Count of microclot-like structures per 0.45 µl of blood.

B) Microclot-like structures expressed as a percentage of all measured events (blood cells and cell agglomerates).

C) Projected area (µmF) of microclot-like structures.

D) Standard deviation of the projected area (µmF) of microclot-like structures.

Platelet poor plasma (PPP) and the detection of amyloid fibrin(ogen) protein microclots and cellular debris

Figure 6 shows micrographs of microclots before and 24 hours after H.E.L.P. apheresis, and Figure 7 shows graphs of the percentage area of microclot analysis before, immediately after, and 24 hours after the treatment. Every patient had evidence of microclots and activated platelets pre-treatment. Immediately after a

single H.E.L.P. apheresis session, there was a ~95% reduction in fluorescence area (**p<0.01), but at 24 hours there was a partial return to pre-treatment levels. The fluorescence area at 24 hours still displayed a ~50% reduction compared to pre-treatment (*p<0.05). This pattern suggests at least a short-term benefit of H.E.L.P. apheresis in these patients on the background of an ongoing thrombotic process likely to require further treatment such as anticoagulation with or without further apheresis.

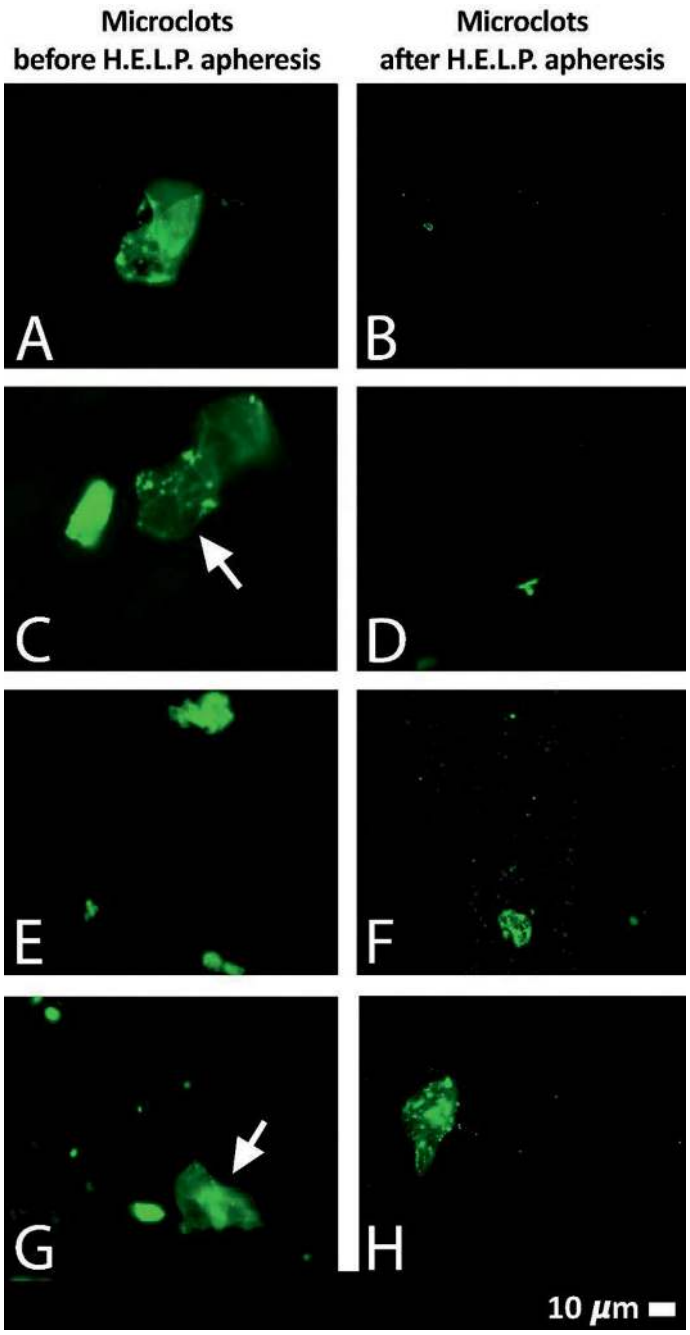

Figure 6:
Representative fluorescence micrographs of microclot and endothelial debris, present in platelet poor plasma before (A, C, E and G) and 24 hours after (B, D, F and H) first H.E.L.P. apheresis. Microclots (white arrows) were seen in the baseline blood samples whereas after apheresis most of the microclots were removed.

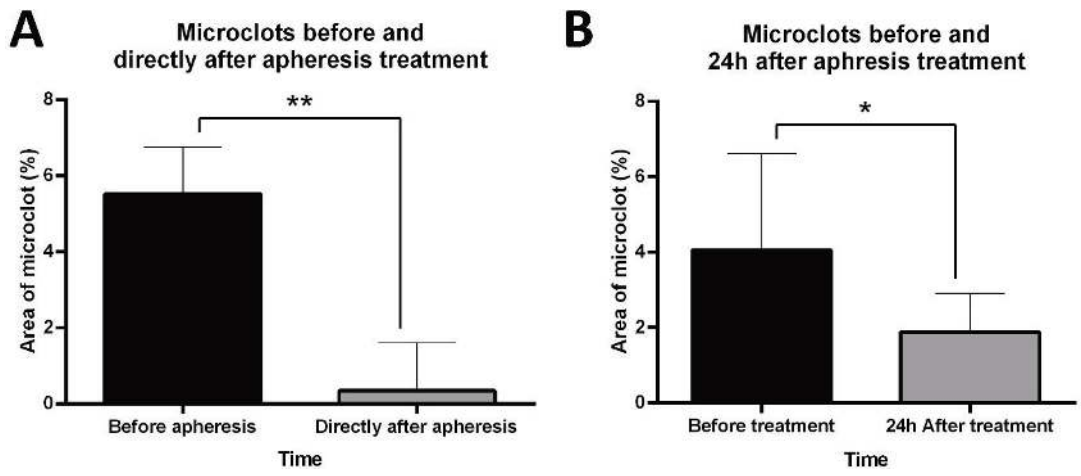

Figure 7:
Microclot and endothelial debris percentage area of fluorescence. (A) Before and directly after apheresis treatment (n=7) $p<0.01$. (B) Before and 24h after apheresis treatment (n=15) $p<0.05$.

Correlations between clinical and hematological measures

Strong (and negative) correlations were identified between baseline hematological and cognitive metrics: namely between the fluorescence area percentage and overall cognitive score ($r=-0.69$; $p=0.001$), and more strongly DSS ($r=-0.72$; $p<0.0001$). This indicates that the greater the evidence of micro-clotting on microscopy, the more severe the cognitive deficit at baseline. There was a strong trend towards significance for negative correlations between baseline fluorescence area percentage and both SpvO2 ($r=-0.45$; $p=0.05$) and 10MWT ($r=-0.45$; $p= 0.06$). This suggests that the greater the clot burden, the more likely it is for the patient to exhibit a low SpvO2 and slow 10-meter walk speed.

Discussion

In the current study we provide pilot evidence that even a single session of H.E.L.P. apheresis may produce a meaningful reduction in symptoms for patients with Long COVID. The patients in this study had a physician diagnosis of Long COVID and all displayed evidence of micro-clotting and platelet hyperactivation, in keeping with our previous published work (49, 117, 118). Hence, this study confirms the presence of microclots in the blood samples of all 19 Long COVID patients studied, using both fluorescence microscopy and microfluidics. This study further underlines the key role of hypercoagulation in Long COVID.

The present study identifies a significant correlation between clot burden and cognitive impairment in Long COVID. There were also strong trends towards significance for correlations between the severity of clotting pathology on the one hand, and SpvO2 and reduced 10-meter walk speed on the other. Post-apheresis, there were significant reductions in serum levels of inflammatory mediators, clotting factors, and lipids. There were also improvements in percentage area of clots measured by fluorescence microscopy, and also by detection of clot-like structures on microfluidic analysis. These changes may be contributing to the physiological improvements observed.

Cerebral hypoperfusion has been theorized as a potential mechanism for cognitive impairment in Long COVID (117, 118). It has previously been suggested that apheresis techniques may have a role in improving cerebral

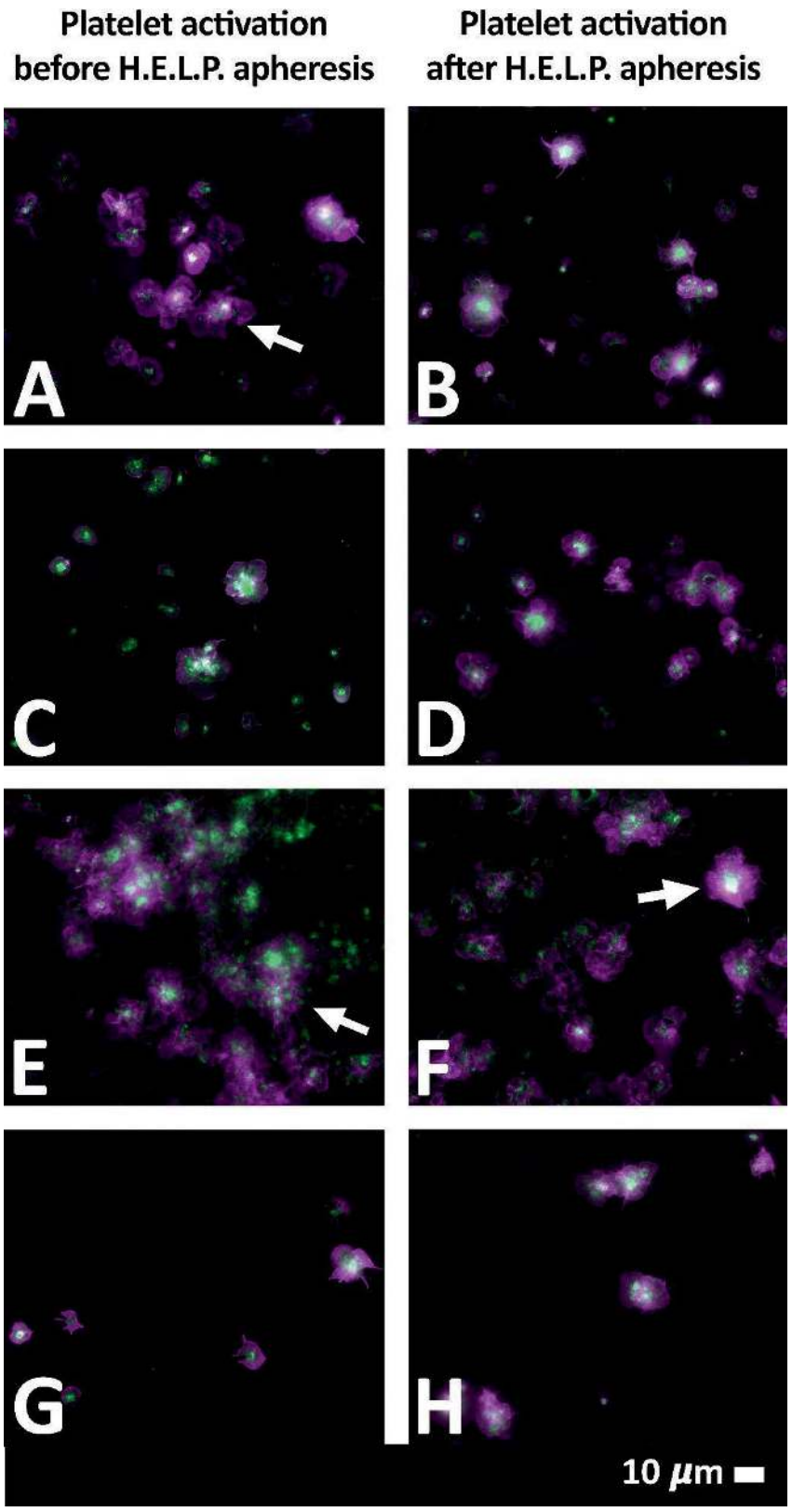

Figure 8:
shows micrographs of platelets before and 24 hours after H.E.L.P. apheresis. Individual platelets appeared more activated after H.E.L.P. apheresis. Activated and aggregated platelets are indicated by white arrows. This may be due to the stresses imposed on already fragile platelets by the apheresis process itself.

perfusion (59, 119)((59, 121).
In our patient group, cognition was the clinical domain that was most improved in patients within 24 hours of the first H.E.L.P. apheresis session, which is encouraging as cognitive deficits in Long COVID have been identified as particularly common and debilitating (9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 120, 121)(9, 104, 122, 123).

The DSS is a challenging test of executive function that has previously been used in conditions such as concussion and mild traumatic brain injury, where cognitive impairment can be subtle and diCicult to quantify (122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(122, 123)(124, 125). Our finding that the DSS was the most sensitive to change in this cohort should be of interest to those studying cognitive sequelae of Long COVID looking for a primary outcome measure that is sensitive to intervention. However, cognitive issues in Long COVID are known to be diverse and they vary between patients (124). At a within-patient level, many patients in our cohort showed improvement in certain cognitive domains but not in others. In these cases, improvement in these domains (Trails B, Stroop and Delayed Recognition) was strongly correlated with their initial baseline score. This suggests that in addition to the statistically significant improvement in DSS, the increased overall cognitive score was also driven by improvement in other impaired cognitive domains which varied from individual to individual.

Gait speed as measured by the 10MWT also showed a significant improvement post-apheresis of 0.27m/s, which is significantly larger than the minimal detectable change and the minimal clinically important diCerence of the 10MWT in other clinical populations (125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(125-127)(127-129). The correlation between the DSS and 10MWT improvements is of interest, since improvement in the DSS has been shown to correlate with improvements in gait speed in the past (128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(128, 129)(130, 131). It is therefore possible that the gait speed improvement was driven by the improvement in cognition.

Symptoms consistent with dysautonomia are commonly reported by patients with Long COVID, although the rate of formal diagnosis of autonomic dysfunction varies widely (130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(130-133)(132-135). Surveys of Long COVID reveal high rates of symptoms consistent with dysautonomia such as orthostatic intolerance, fatigue, palpitations, cognitive impairment, nausea, and temperature dysregulation (7, 9, 10). In our small cohort, these symptoms were commonly reported as well. Baseline median HRV measured as RMSSD was 80% lower than matched controls in our group; this is in keeping with other data on HRV abnormalities in Long COVID (134).

In the three patients who qualified for a formal POTS diagnosis as per AAS guidelines, the criteria for POTS were no longer met following the procedure. Interestingly, a recent series of nine Long COVID patients demonstrated that orthostatic intolerance, cerebral hypoperfusion (as measured by transcranial Doppler ultrasound), and dysautonomia [as measured by the Quantitative Scale for Grading of Cardiovascular Autonomic Reflex Tests and Small Fibers from Skin Biopsies (QASAT) score] were present in all patients regardless of whether they met criteria for POTS or orthostatic hypotension (OH) (118). This is consistent with studies of impaired cerebral perfusion in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) as well (135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(135-137)(137-139). Reliance on criteria for POTS and OH is therefore likely to miss many cases of dysautonomia in Long COVID. Given the disabling nature of the symptoms of dysautonomia, the huge number of patients with Long COVID, and the relatively small number of specialized testing facilities, there is a critical need for more practical, sensitive, and definitive measures of autonomic nervous system function.

It has been demonstrated that measures of lung function, in particular DLCO, can be persistently abnormal months after hospital discharge with acute severe COVID-19 (138). This finding would be consistent with a diagnosis of post-intensive care syndrome (PICS). With respect to patients suffering from Long COVID itself, there is insufficient data exploring lung function. However, persistent breathlessness in the presence of normal radiology and lung function tests has been reported (139). Our evaluation did not reveal any significant change in lung volumes and DLCO pre- and post-apheresis with a single treatment. This may be because most participants' measurements were within normal limits at baseline, in keeping with the aforementioned study (139). Breathlessness is, however, commonly reported in Long COVID and our cohort was no different. Recent work using hyperpolarized xenon 129 magnetic resonance imaging (XeMRI) has detected abnormalities of gas transfer in patients three months after hospital discharge despite normal lung function testing (140). Finally, autonomic nervous system dysfunction commonly manifests as dyspnea (133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(133, 141)(135, 143) and is a plausible explanation for this symptom in Long COVID patients with no gas transfer abnormality.

SpvO2 is not commonly used in clinical practice but has been evaluated in critically ill patients as an alternative to central venous oxygen saturation (ScvO2) in those who do not have central venous access (142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(142, 143)(144, 145). Whilst correlation between SpvO2 and ScvO2 is poor, an association with moderate trending ability has been noted (144). There is no widely accepted 'normal' range for SpvO2; the closest variable for which one exists is mixed venous oxygen saturation (SvO2) measured via a pulmonary artery catheter, for which a reference range of 65-75% is quoted (145). Whilst there was a wide range of SpvO2 values in our patient group, there was a low mean pre-apheresis SpvO2 of 56.3%. SpvO2 is a function of tissue oxygenation, which is required to preserve normal organ function. Hence, a reduced SpvO2 reflects a high tissue oxygen consumption/supply ratio, resulting in impaired tissue oxygenation. In the absence of anemia and arterial hypoxemia, potential causes for this include reduced cardiac output and hypovolemia (146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(146, 147)(148, 149). We argue that in the setting of Long COVID, a possible explanation for the reduced SpvO2 could be autonomic nervous system disturbance. Relative hypovolemia is a feature of both POTS and orthostatic hypotension, which causes a reduced stroke volume and cardiac output (148, 149)(150, 151), resulting in impaired tissue oxygen supply. The resultant compensatory sympathetic overdrive and tachycardia increase tissue oxygen demand, further disrupting the tissue oxygen consumption/supply ratio.

Small fiber neuropathy (SFN)- defined as 'preferential damage to unmyelinated or thinly myelinated group C or A nerve fibers' (148) has been documented in Long COVID (117, 149)(117, 149)(117, 149)(117, 149)(117, 149)(117, 149)(117, 149)(117, 149)(117, 149)(117, 151)(119, 153) and is a recognized cause of dysautonomia in the condition (118). We suggest that microclots may contribute to the development of SFN either due to ischemia or due to autoantibodies, which have previously been associated with SFN. In our patient group, greater fluorescence area percentage appeared to correlate with lower SpvO2, with a strong trend towards significance. This suggests that the greater the clot burden, the more severe the autonomic dysfunction, and the worse the tissue ischemia and hypoxia. Significantly, ischemic and hypoxic tissues switch to anerobic metabolism, with inefficient generation of ATP and increased lactic acid production. This ties in with studies of cardiopulmonary exercise testing in Long COVID, which demonstrate significant lowering of anerobic threshold (150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(150, 151)(152, 153)(154, 155). This could explain the effort intolerance and PESE noted in these patients (9, 152). Traditional rehabilitation models based on the principle of incremental loading are unhelpful in such patients and have the potential to result in worsening of symptoms.

Limitations and future directions

There are several limitations to this pilot report that must be addressed through a larger clinical trial. The lack of a control group undergoing a sham apheresis procedure means that we cannot exclude a placebo effect influencing some of the results. The patients described were a convenience sample attending a private clinic in Europe. As such, they were primarily Caucasian and male, which is not representative of the wider Long COVID population. Future studies by this group will aim to ensure a more diverse representation of Long COVID patients.

The finding of microthrombi was consistent in all 19 patients enrolled in the study; this is in keeping with the finding of microclots in all patients with physician diagnosed Long COVID in previous studies by this group: firstly in 11 subjects (25), and, more recently, in 70 individuals (153). Whilst microclots can accurately be identified by the methods described in this paper in a research setting, there is an urgent need to develop a standardized and more widely available clinical assay for the clinical setting given the scale of the problem of Long COVID.

Our sample size was small, but sufficient to detect within-patient changes in laboratory and physiological measures in this uncontrolled study. A larger study with an adequate control group is needed to understand the generalizability of these findings. We intend to collect longitudinal data on this and future cohorts, including measurements of immunological and inflammatory parameters such as cytokines both before and after apheresis, so that we can fully understand the mechanism of action of H.E.L.P. apheresis in Long COVID.

The partial return of microclots 24 hours after H.E.L.P. apheresis suggests that a single procedure is not sufficient to treat Long COVID. It is probable that further treatment designed to clear microclots permanently could result in a lasting improvement in symptoms. Such therapy could be comprised of a combination of antiplatelet and anticoagulant therapy, with or without further cycles of apheresis. A combination of drugs may be a more effective choice than one drug alone, as studies of single agent antiplatelet or anticoagulant therapy in both inpatient and outpatient acute COVID-19 disease have failed to demonstrate efficacy (154-159)(154-159)(154-159)(154-159)(154-159)(154-159)(154-159)(154-159)(154-159)(156-161)(156-161)(156-161)(156-161)(156-161)(156-161)(156-161)(157-162).

After completion of apheresis and post-procedure clinical testing, our 19 patients were commenced on a three-month course of anticoagulation with aspirin 100mg and clopidogrel 75mg once a day, plus a direct oral anticoagulant (DOAC- either dabigatran 150mg twice a day or apixaban 5mg twice a day) by the treating physician at the center (BRJ). Gastric protection was prescribed as routine with pantoprazole 20mg twice a day or famotidine 20mg twice a day. We chose dual antiplatelet blockade with aspirin and clopidogrel based on an understanding of the key role of platelet hyperactivation in COVID-19 disease, with the addition of a DOAC to inhibit the enzymatic pathway of coagulation. This regime has already been successfully used to treat Long COVID by two of the authors (BRJ and GJL) (153). We intend to repeat the functional and laboratory measures in all 19 patients in February 2022 after completion of 3 months of treatment. The results will be analyzed and submitted for publication.

It should also be noted that 'viral reservoir', or persistence of SARS-CoV-2 in patient tissue sites, is an active area of Long COVID research. Multiple research teams have identified SARS CoV-2 RNA or antigen in tissue samples and immune cells (160) collected months after the initial infection (161-164)(161-164)(161-164)(161-164)(161-164)(161-164)(161-164)(161-164)(161-164)(163-166)(164-167). In cases where the virus persists in immune cell or tissue reservoirs, spike protein may leak into the blood and perpetuate hypercoagulability. For example, in children with multisystem inflammatory syndrome (MIS-C), Fasano et al. demonstrated that the prolonged presence of SARS CoV-2 in the GI tract (viral reservoir) was associated with increased intestinal permeability and subsequent translocation of SARS CoV-2 antigens into the bloodstream (165). It follows

that in some patients with Long COVID, apheresis may need to be combined with therapeutics aimed at eliminating persistent SARS CoV-2 virus or antigen. It is also important to understand how hypercoagulation in Long COVID correlates with the abnormalities of immune function that are being documented in the condition (97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(97, 166, 167)(90, 168, 169)(90, 169, 170).

There is a significant overlap between the features of Long COVID and ME/CFS (7, 168-171). In both illnesses, patients present with multiple symptoms relating to different organ symptoms, and usual laboratory and radiological investigations are typically normal. There has been a strongly held belief amongst a section of the medical community that ME/CFS is psychosomatic, despite the large body of evidence pointing towards organic pathology (169, 172-178). Perhaps unsurprisingly, there have been attempts to label Long COVID as non-organic in nature as well (120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 171, 179-184)(120, 173, 181-186)(122, 174, 182-187). It is our hope that future work which replicates the clear abnormalities of coagulation demonstrated in this paper, along with the changes in laboratory and physiological indices after apheresis, will help dispel this misconception. Finally, defining the pathophysiology of Long COVID has a potentially enormous return on investment for global economies, on top of the urgent moral imperative to alleviate the burden of suCering.

Conclusion

I think this could use a good conclusion to highlight the main points. The paper seems to end abruptly.

Contributions

MAK: clinical study concept and design, data collection, manuscript dictation and writing, editing, bibliography; DP: clinical study design, data analysis, manuscript writing, transcription of MAK's voice notes; MK: microfluidics data collection and analysis, manuscript writing, bibliography; ST: data collection and analysis; EBu: microscopy data collection and analysis; CV: laboratory study administration, microscopy data collection and analysis; GJL: manuscript editing; EBl: laboratory data collection and analysis; MF: infection control, data collection, pastoral care; EJK: clinical study administration, data collection; HL: laboratory study administration, data collection; AMcC: laboratory study coordination, data protection, procurement and transport of study materials; NP: data collection; AESB: manuscript editing and writing, bibliography; DCL: manuscript editing; SL: manuscript editing; AM: manuscript editing; PM: manuscript editing; AP: manuscript editing and writing, bibliography; IR: manuscript editing; WW: manuscript editing; AAW: manuscript editing and writing, proofreading; DBK: manuscript editing, abstract writing, data analysis; BRJ: clinical concept and design, payment of study, patient selection, treatment protocol and follow-up, manuscript editing, bibliography; EP: data collection, analysis, manuscript writing, bibliography.

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Competing interests

MAK, EB, MF, EK, HL and AMcC have Long COVID and are fee-paying patients of BRJ at Lipidzentrum Nordrhein. They have all received H.E.L.P. apheresis.

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Supplementary materials

Appendix 1: Results of pulmonary function testing and SpvO2 measurements pre- and post-H.E.L.P. Apheresis

	FEV1		FVC		DLCO % Predicted		SpvO2	
ID	Pre	Post	Pre	Post	Pre	Post	Pre	Post
GLC4	481	468	596	596	115	108	798	697
GLC3	307	328	368	396	DNC	91	864	569
GLC5	333	363	493	528	109	108	338	368
GLC17	514	526	583	581	112	118	305	784
GLC16	217	213	331	306	70	71	143	49
GLC24	458	46	632	637	130	136	487	403
GLC35	368	394	605	599	112	118	599	825
GLC45	325	335	582	561	103	104	715	462
GLC46	265	288	343	355	116	120	396	744
GLC48	383	359	479	459	124	114	601	756
GLC47	333	347	399	422	96	90	797	618
GLC52	439	418	587	59	99	107	169	DNC
GLC51	3	3	359	359	92	94	63	66,6
GLC59	313	313	459	459	106	101	645	826
GLC60	426	448	543	558	108	111	374	582
GLC61	296	297	388	395	114	109	657	819
GLC71	517	521	661	67	99	100	739	521
GLC72	407	402	511	502	109	108	50	579
GLC62	437	431	52	531	111	110	551	452
MEAN	37	38	50	50	1069	1062	543	620

DNC: Did not complete

Appendix 2: Results of cognitive testing pre- and post-H.E.L.P. Apheresis

	TRAILS B		DSS		STROOP		DR		AGGREGATE	
ID	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
GLC4	109	104	106	117	121	111	100	115	110	121
GLC3	DNC	110	97	111	29	106	107	107	107	111
GLC5	102	86	94	100	108	94	69	69	89	87
GLC17	85	107	88	95	99	85	117	83	105	89
GLC16	67	91	83	94	35	92	72	106	67	80
GLC24	117	117	114	119	113	115	77	107	103	118
GLC35	111	89	88	93	102	91	117	83	95	93
GLC45	110	93	115	118	100	112	107	115	107	119
GLC46	75	110	89	114	85	106	87	107	77	116
GLC48	95	107	109	114	97	108	107	115	115	118
GLC47	95	107	101	115	87	106	115	107	111	115
GLC52	129	131	113	121	113	94	115	100	122	119
GLC51	85	90	45	102	51	96	52	83	59	97
GLC59	102	100	104	108	70	98	117	106	113	106
GLC60	111	116	103	109	119	110	95	72	103	93
GLC61	84	96	98	104	82	103	107	100	103	106
GLC71	87	91	92	99	86	100	95	83	99	89
GLC72	94	90	46	93	83	75	100	100	88	100
GLC62	96	106	107	113	106	114	83	106	99	115
MEAN	974	1022	943	107.3*	887	1008	968	981	985	104.8*

*p<0.05; DNC: Did not complete

Appendix 3: HRV testing compared to controls; functional testing pre- and post-H.E.L.P. Apheresis

	RMSSD (only measured pre-apheresis)		Postural Orthostasis (beats/min change)		10 MWT (m/s)	
ID	Baseline	Age/Gender Corrected (%)	Pre	Post	Pre	Post
GLC4	26	0,69	-6	21	155	162
GLC3	9	0,25	27	23	142	156
GLC5	74	2,47	-13	-9	138	144
GLC17	17	0,33	5	7	208	196
GLC16	50	0,78	3	22	116	132
GLC24	DNC	DNC	7	28	179	167
GLC35	37	0,98	20	26	162	192
GLC45	23	0,77	48	14	205	242
GLC46	22	0,81	16	10	116	168
GLC48	25	0,84	8	-6	21	26
GLC47	51	1,42	16	8	DNC	DNC
GLC52	22	0,74	2	12	199	235
GLC51	33	0,69	8	24	155	241
GLC59	30	0,63	18	22	175	182
GLC60	50	0,96	10	26	178	191
GLC61	38	1,39	10	10	147	169
GLC71	29	0,56	36	24	156	232
GLC72	14	0,47	2	2	136	174
GLC62	13	0,34	34	6	188	204
MEAN	313	0.8*	132	142	165	1.92**

DNC: Did Not Complete; *p<0.05, **p<0.001

Appendix 4: Patient Intake Form · Patient Intake-Form-Update.pdf

Appendix 5: Function and Symptom Questionnaire · Functionality and Symptom Questions

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Sequence similarities in SARS-CoV-2 Spike Protein and Human Muscarinic receptors as the basis of Autoimmunity and Symptomology in Post-Acute Sequelae COVID-19

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Unveiling Autoimmunity in Long Covid

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Abstract

Of the complex and diverse syndromic picture in Post Acute Sequelae of SARS-CoV-2 (PASC), commonly known as Long-COVID, many if not all symptoms are caused by autoimmune GPCR antibodies (AAbs). GPCRs which include Cholinergic Human Receptors Muscarinic (CHRMs) and Alpha and Beta Adrenergic Receptors, are expressed in many organs and tissues of the human body which is the reason behind symptom diversity in LC. Molecular mimicry has been suggested as the reason for the AAbs but details of the reason for their development at the molecular level and the way they react on interacting with the CHRMs and adrenoceptors has not been discovered. The cross-reactivity between CHRMs and segments of spike (S) protein of SARS-CoV-2 was investigated and antigenicity was determined to explain the reason behind molecular mimicry. The functional effects of AAbs with CHRMs was investigated by studying the binding sites of known agonists and antagonists within the molecular structure of CHRM. We establish here that segments of S protein that are capable of evoking an antibody response with strong bonds (SB) form the basis of AAb production due to the similarity between them and certain segments of CHRM. A correlation between functional effects of AAbs and symptom complex in PASC was made considering their effects on CHRM. The effect of AAbs on the cholinergic nervous system directed against CHRM shows the spectrum of resultant effects and provides the explanation for conditions such as blood pressure changes, postural orthostatic tachycardia syndrome (POTS), GI complaints and neurological deficits reported in PASC. We are optimistic that the methodology reported here is equally applicable to the Adrenergic binding AAbs, an ongoing project to be detailed in the near future.

A. Introduction

Characterized by a wide range of symptoms continuing after the acute phase of COVID-19 and often by the onset of new symptoms months or years later, PASC poses a complex challenge to the medical community, with research into its pathophysiology still largely underfunded and only beginning to be understood. Autoimmunity directed against G Protein-Coupled Receptors (GPCRs) has been widely suggested as a mechanism underlying the chronic and diverse symptoms of PASC (1). However, the specific triggers and targets of these autoimmune processes in the context of SARS-CoV-2 infection have not been clearly identified.

This paper aims to bridge this knowledge gap by uncovering the molecular basis of potential autoimmune responses in PASC. We propose that the origin of autoantibodies (AAbs) in PASC can be attributed to the enduring presence of the virus's spike (S) protein within the body. This fosters a state of chronic inflammation and together with low cortisol stemming from adrenal cortical damage from the S protein (2), drfdgbhhfg.1creates an ideal setting for AAb formation.

Fragments of the S protein cleaved by white blood cell enzymes, such as neutrophil elastase, can prompt the immune system to produce antibodies against these fragments, which bear resemblance to sequences found in human proteins (3).

We hypothesize that sequence similarities between fragments of amino acids in the SARS-CoV-2 spike protein and human muscarinic receptors (CHRMs) may lead to the development of cross-reactive autoimmune antibodies and can explain the multi-systemic nature of PASC symptoms. We show that these sequences have regions of both identical as well as chemically similar amino acids that can be identified as sufficiently antigenic to provoke an antibody response with strong bonds (SB). Our approach first investigates the similarity between sequences of amino acids, then once found, identifies those sequences in the S protein capable of mounting an immune response. These sequences are then compared and examined for their potential for cross-reactivity and capacity to act as antigens that provoke an antibody response. CHRMs are GPCRs distributed widely in the body and expressed by tissues (Figure 1 and 2) where they play important roles in physiological functions (4).

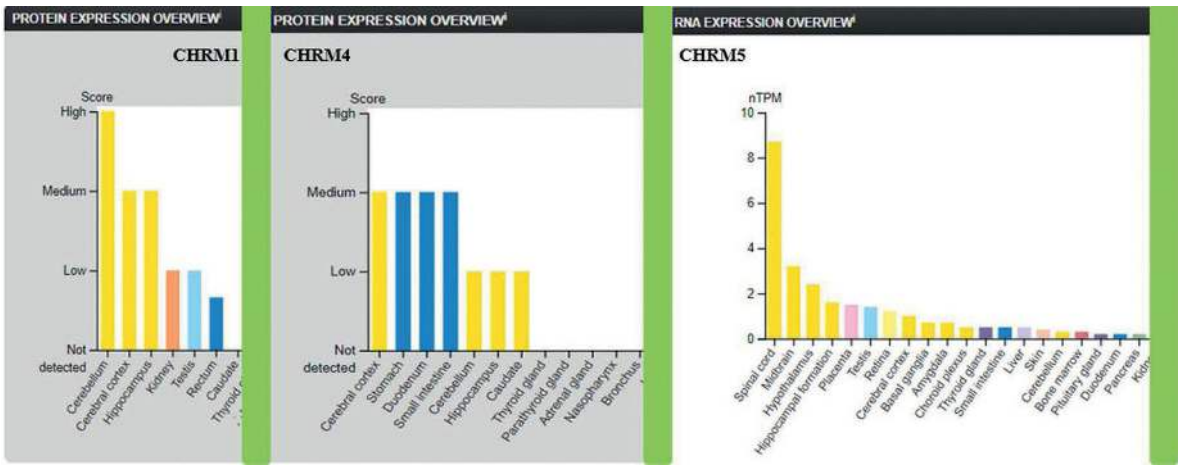

Figure 1:
Bar chart showing expression levels of muscarinic receptor subtypes CHRM1, CHRM4 and CHRM5 in human organs and tissues. The AAbs against these receptors have been reported in patients with PASC. The eHect of their interaction with AAbs has been reported to produce symptoms. The functional eHect of binding the AAbs could be agonistic, antagonistic or destructive. Data retrieved from https://www.proteinatlas.org/search/CHRM.

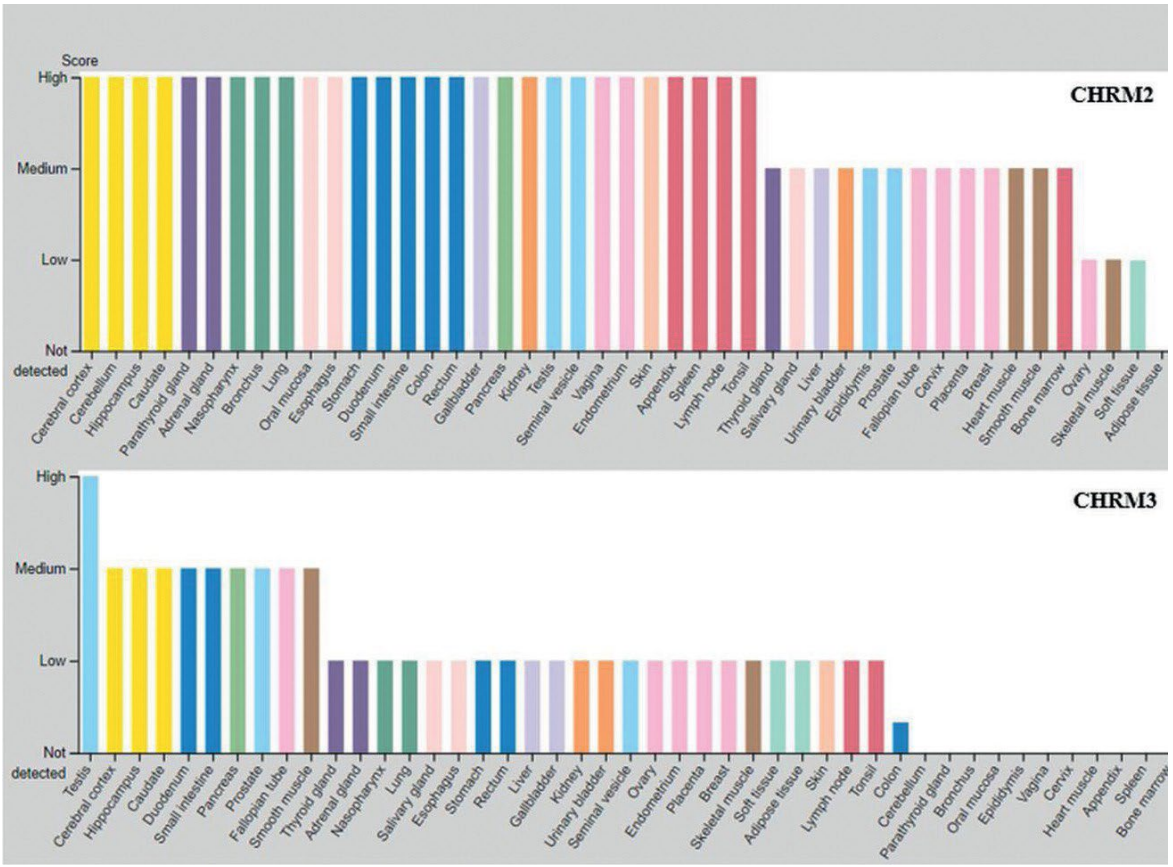

Figure 2:
Bar chart showing expression levels of muscarinic receptor subtypes CHRM2 and CHRM3 in human organs and tissues. The AAbs against these receptors have been reported in patients with PASC. The eHect of their interaction with AAbs has been reported to produce symptoms. The functional eHect of binding the AAbs could be agonistic, antagonistic or destructive. Data retrieved from https://www.proteinatlas.org/search/CHRM.

How the CHRM autoantibodies relate to the clinical symptoms observed in patients with PASC is described in detail. The subtypes of these CHRMs that the autoantibodies target and where there are expressed in the body may account for the range of symptoms associated with PASC given their wide expression and functional importance in various physiological systems (4).

Complex symptoms such as gut dysbiosis (CHRM3), POTS (CHRM1), brain fog (CHRM1), endothelial dysfunction (CHRM3), hypercoagulability (CHRM3), breathing difficulties (CHRM2) and many more are thought to be due to diverse etiologies but the AAbs alone can explain a significant number of these symptoms.

B. Composition of S protein and CHRMs: docking pockets, sequences, and crystal structure

B1. S Protein

Studded all over the surface of SARS-CoV-2 are host cell entry enabling glycoprotein molecules, the protein part of which is composed of 1273 amino acids called the S protein (5).
The sequence can be divided into two subunits: S1 which is responsible for receptor binding and S2 which mediates membrane fusion (5).

Located within the S1 subunit, the Receptor Binding Domain (RBD) is crucial for binding to the human ACE2 receptor (Figure 3) (6). Mutations in these regions, as seen in various variants, can alter binding aHinity and immune evasion properties. The Receptor Binding Motif (RBM) is a part of the larger RBD located within the S1 subunit of the spike protein (Figure 3) and is the segment that interacts specifically with ACE2 in the host cell (6).

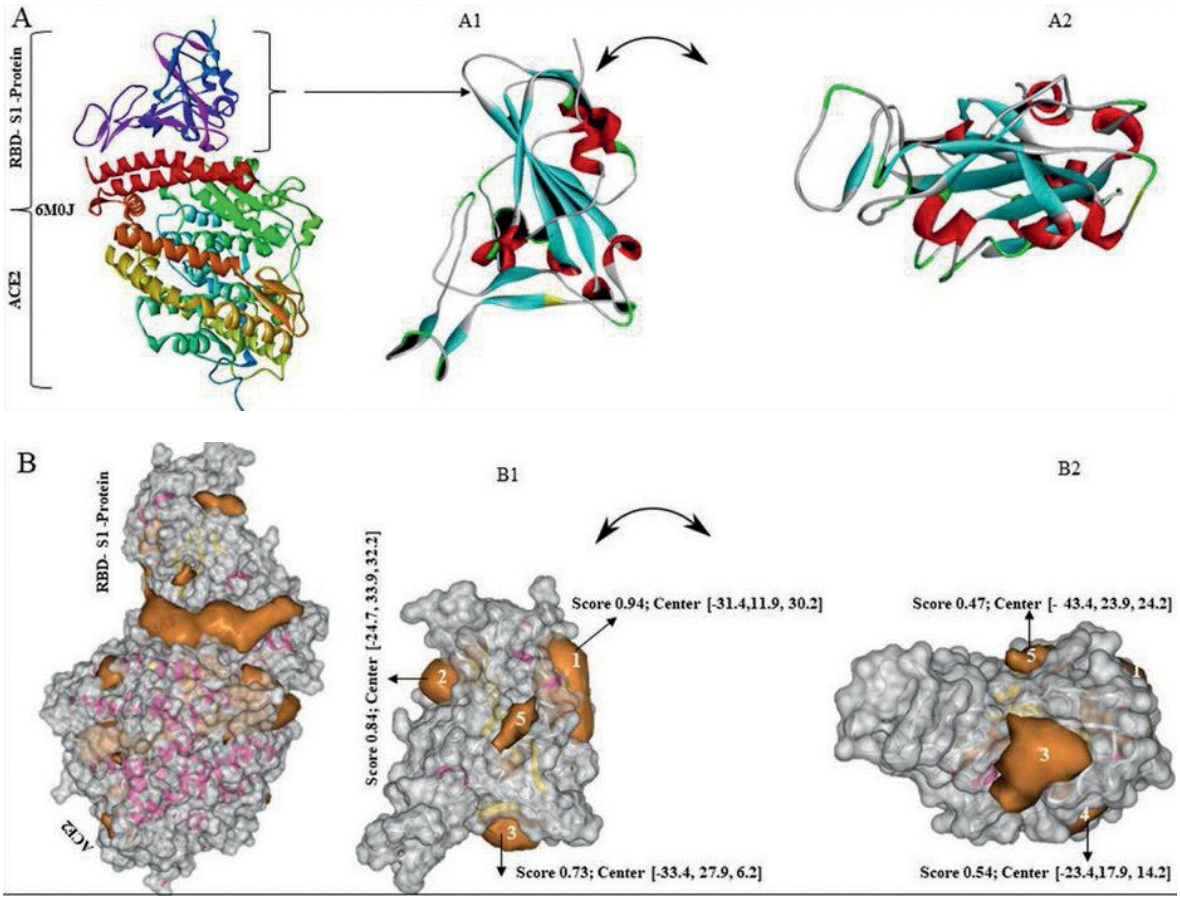

Figure 3:
Illustration showing S protein and ACE2 interaction (A). The RBD of the S protein alone (A1) with the RBM (A2) at the base of the RBD. Playmolecule server using DEEPSITE gemerated drug binding pockets on S protein RBD and ACE2 receptor (B). On the RBD alone, 5 pockets were detected (B1) of which Pocket 3 (B1, B2) is the location of the RBM which interacts with ACE2 receptor and many monoclonal antibodies. Data retrieved from https://www.playmolecule.com/deepsite/

Structurally, the RBM forms a loop-like structure that directly interacts with the peptidase domain (PD) of the ACE2 receptor on human cells (7). The RBM's specific amino acid sequence and structure enable it to fit precisely into a corresponding site on the ACE2 receptor (7). This interaction is the first critical step for the virus to gain entry into the host cell. For SARS-CoV-2, the RBM has evolved to eHectively bind to the human ACE2 receptor (Figure 3 B2 Pocket 3), a key factor in its transmissibility in humans (8). Mutations within the RBM can significantly aHect the virus's infectivity and immune evasion capabilities. Changes in key amino acids can alter the binding aHinity to the ACE2 receptor and can lead to either enhanced receptor recognition or reduced antibody neutralization, as seen in various SARS-CoV-2 variants (9). Due to its exposed position and essential role in receptor binding, the RBM is a major target for neutralizing antibodies. Many therapeutic antibodies and small molecule inhibitors are designed to bind to the RBM, blocking its interaction with ACE2 and thereby preventing viral entry (10). The spike protein is densely glycosylated, with N-linked glycosylation sites playing roles in protein folding, shielding from neutralizing antibodies, and receptor binding

(11). The distribution and composition of these glycans are critical for understanding immune evasion strategies. The binding of these molecules to the spike protein can prevent ACE2 interaction or obstruct the conformational changes required for membrane fusion. Advanced computational techniques, including molecular docking and dynamic simulations, are employed to identify potential inhibitors. These studies focus on the interaction energy and binding stability within identified docking pockets, considering the dynamic nature of the spike protein's conformation. Docking pockets are regions on the protein's surface where small molecules, like antiviral drugs, can bind (12). They are identified through structural analysis and computational modeling, looking for pockets where drugs could potentially bind and inhibit the protein's function.

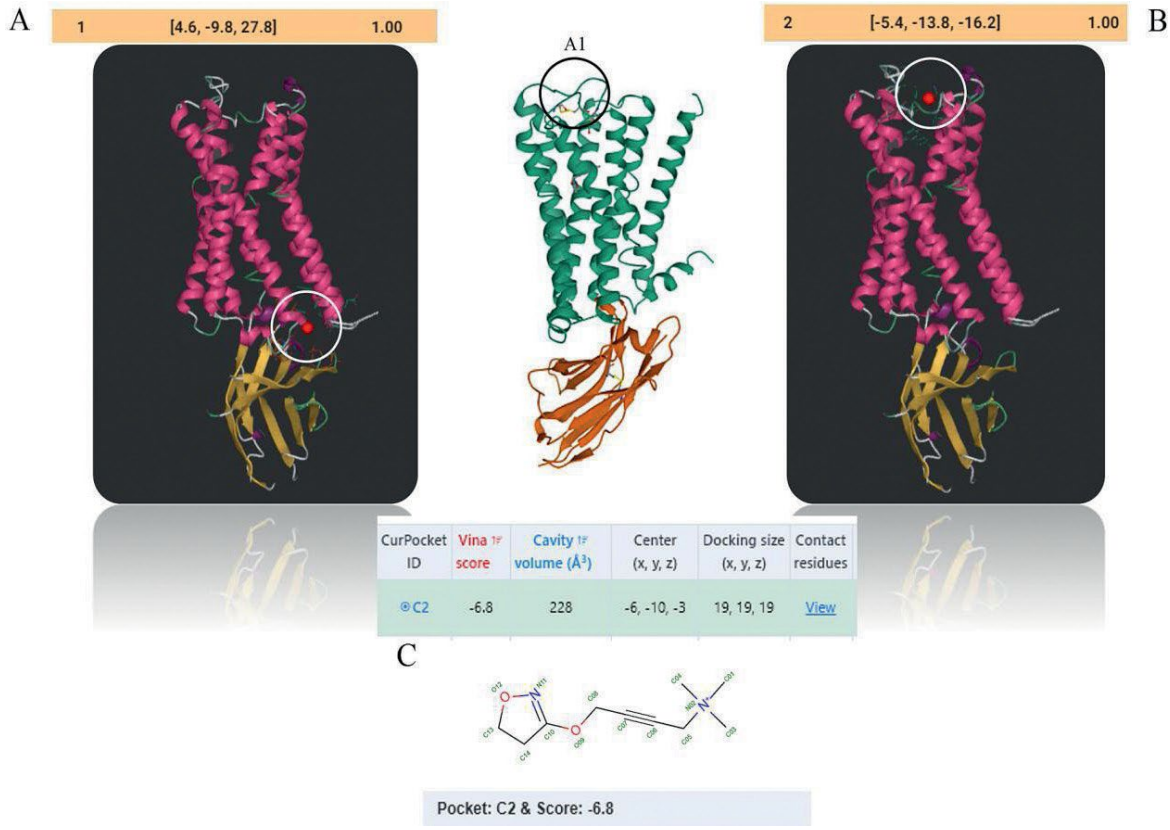

Figure 4:
DEEPSITE software at Playmolecule automated server showed 2 pockets of the CHRM2 protein (white circles, A and B). Coordinates are shown at the top (orange bars at top). An agonistic drug Iperoxo as docked onto the CHRM2 (which is the possible interacting site of AAbs against this receptor subtype). The binding pockets and Vina scores were calculated (C- green bar) with interacting amino acid of the binding pocket listed for chain A (CHRM2) also shown (3-letter amino acids with positions). Data retrieved from https://www.playmolecule.com/deepsite/

B2. CHRMs

The Cholinergic Receptors, Muscarinic (CHRMs), are a family of GPCRs responsive to the neurotransmitter acetylcholine (13). There are five subtypes of muscarinic receptors CHRM1 to CHRM5 (Figure 1 and 2), each with distinct functions, tissue distributions, and pharmacological properties (13). Understanding their crystal structures, docking pockets, and sequences is key in pharmacology and drug design. Like other GPCRs, muscarinic receptors have a characteristic seven-transmembrane helical structure (14). The extracellular portion includes the ligand-binding domain, while the intracellular part interacts with G-proteins, triggering intracellular signaling pathways (14). The orthosteric binding pocket, where acetylcholine binds, is located deep

within the transmembrane domain (14). Muscarinic receptors also possess allosteric sites, distinct from the orthosteric site (Figure 4), where allosteric modulators bind (14). These modulators can fine- tune receptor responses, offering a potential avenue for drug development with greater subtype selectivity and fewer side eHects. The docking pockets were detected in CHRMs with CHRM2 shown here as a model bound to a agonist iperxio (Figure 4).

B2a. Orthosteric Site

This is the primary site for acetylcholine binding. It's characterized by a conserved amino acid aspartate in the third transmembrane domain (critical for ligand interaction), along with other amino acids contributing to ligand specificity and binding affinity (Figure 4-A1-B) (15).

C. Sequence similarities in segments of S protein and CHRMs that are antigenic and capable of mounting an antibody response

In order to understand the mechanisms behind potential molecular mimicry, the complete sequences of individual CHRMs were aligned with the S protein by using the EMBOSS automated server, with CHRM3 modelled here (Figure 5A-A1) (18). The regions of significant amino acid identities and similarities were se-

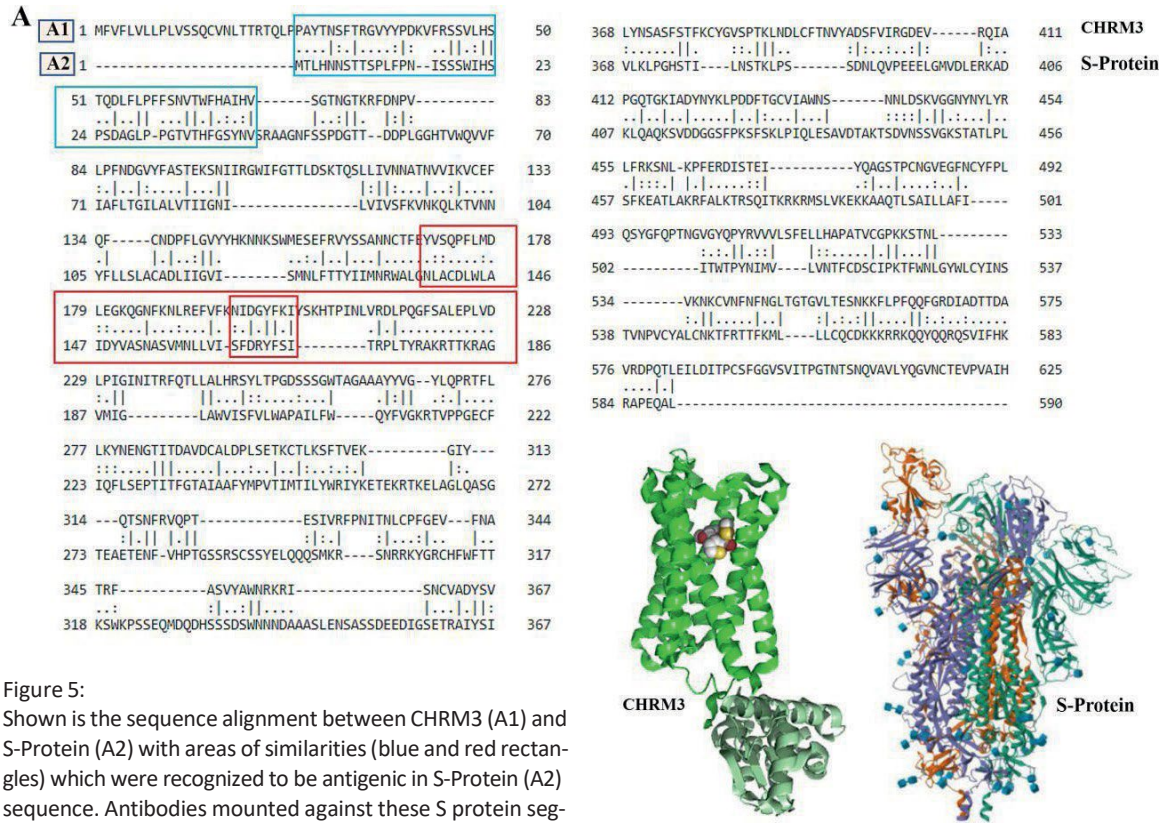

Figure 5:
Shown is the sequence alignment between CHRM3 (A1) and S-Protein (A2) with areas of similarities (blue and red rectangles) which were recognized to be antigenic in S-Protein (A2) sequence. Antibodies mounted against these S protein segments after being digested by endopeptidases like neutrophil elastase and presented as antigens by macrophages can produce antibodies which can cross-react with CHRM3 (as with other CHRM segments of similarity with fragments of S protein) resulting in antagonistic or destructive influence causing brain fog, memory loss, cognitive decline, decreased gut motility, oligospermia, reduced testosterone, endothelial injury, bladder hypomotility, thyroid hypofunction, new onset diabetes mellitus, hypocortisolenemia and fertility problems. . Data retrieved from https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle

lected (Figure 5A-Blue and red recangles). The DTU server was used to identify sequences of segments in S protein that were capable of mounting an antigenic response (Figure 5A-A2) (Supplementary file figure S1) (19). We identified regions in the S protein which were recognized as being antigenic and capable of mounting an antibody response and cross-reacting with similar segments of CHRMs (Figure 5A- A1-A2).

D. Docking pockets in CHRMs for agonists, antagonists and antibodies

In order to compare and predict functional effects of AAbs directed against CHRMs, the docking pockets in the crystal structures of CHRM for agonists and antagonists were studied and the effect of AAbs were computed (Figure 4C). As the crystal structures of AAbs over the CHRM could not be obtained from the PDB database, antagonists and agonists binding to specific segments of the CHRMs (Figure 4, A1, B and C) were used as control substitutes and studied (20). The docking pockets where CHRMs bind nown ligands is shown (Figure 6) are the sites targeted by agonostic and antagonistic AAbs.

Docking pockets of autoantibodies in CHRMs

As the crystal structures of AAbs are not known, the exact docking pockets of these AAbs can not be predicted with precision. Since the interaction of AAbs in PASC against CHRM2 is known to be agonistic (21) we used the PDB database model of 4MQS to detect the pocket of interaction of AAbs with CHRM2 (Figure 6A). The Iperoxo agonistic interaction with the CHRM2 takes place within a segment in this muscarinic receptor which appeared as a segment of similarity in sequence alignment with SARS-CoV-2 S protein (Figure 6B). It can be computed that if the body mounts an antibody response to the segment of the S protein that matches the antigenic segment of CHRM2 (a binding site of Iperoxo – agonist of CHRM2) the antibody being found as agonistic in nature is explained. Not only is cross-reactivity of S protein fragment directed antibodies with similar segments in CHRM receptors the basis of the disease, but also cross-reactivity between CHRMs AAbs among themselves due to their own segment similarities (Figure 6). This concept is of cardinal significance as one AAb directed against a particular CHRM can produce diverse symptoms by engaging other subtypes of CHRM.

Correlation of AAbs with symptom complex in PASC

In this study, we aim to explain the ultimate effects that occur when autoimmune antibodies (AAbs) interact with cholinergic receptors (CHRMs), resulting in the complex and sometimes surprising symptoms observed in patients with PASC. Whether the AAbs interact at the orthosteric or allosteric site determines whether they function as a receptor agonist or antagonist or are destructive for the cell. CHRM2 AAbs have been found to be agonists while the other CHRMs are either destructive or act as antagonists. This section details the variety of symptoms observed in PASC (Figure 5), which may arise from direct interaction of AAbs with CHRM receptors or indirectly through pathways not related to these receptors. It should also be noted that variations in these symptoms can occur in individuals with genetic differences in CHRMs that alter how AAbs engage with them.

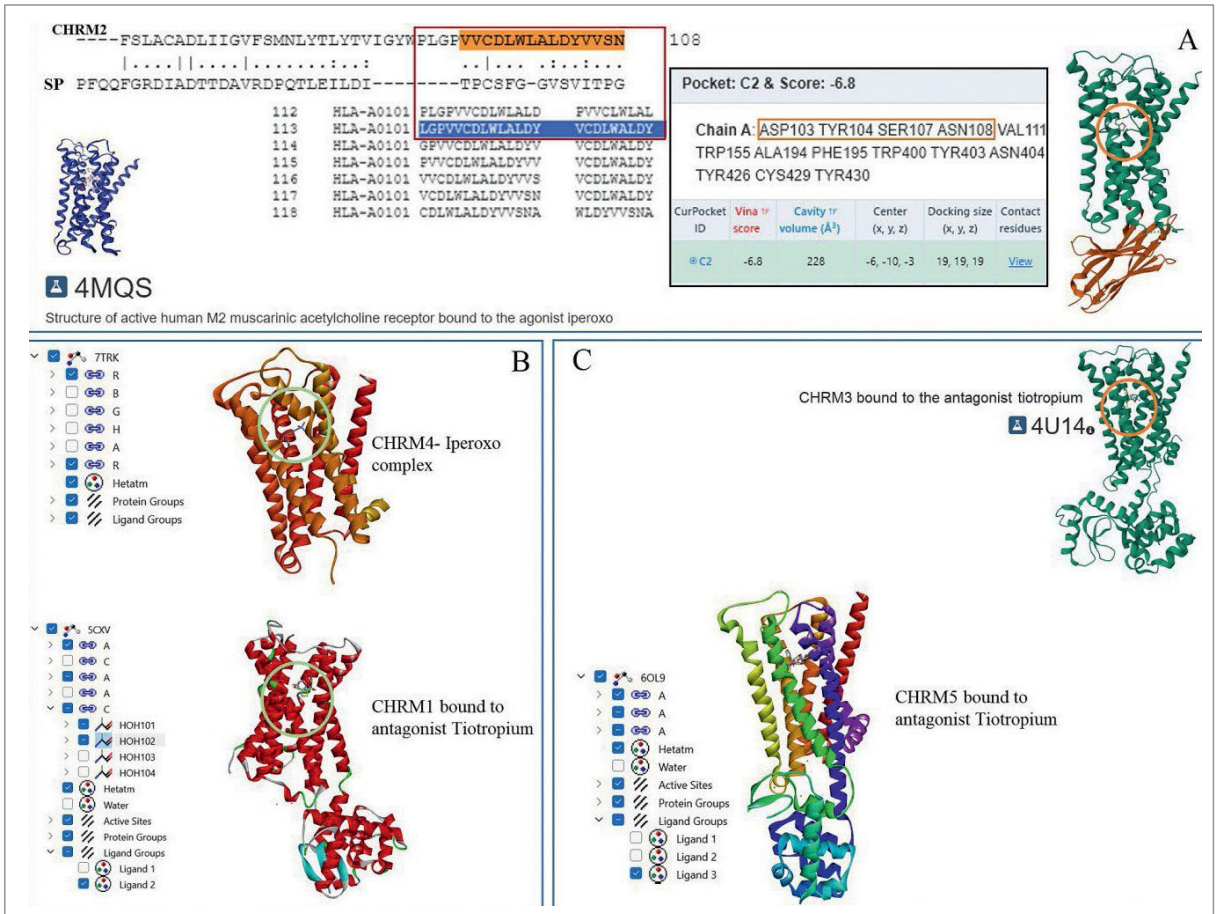

Figure 6:
Illustration showing possible origins of and reason behind AAbs targeting CHRM2
(A) Sequences highlighted in (A – red rectangle, top row) have similarities with amino acids in S protein fragment aligned below. An antibody response in the bottom row of the SP sequence within the red rectangle could excite antibody production which when compared with the model 4MQS identified the agonistic site of interaction of AAbs generated to interact with CHRM2. Being agonistic in nature the AAbs against CHRM2 are hypothesized to bind and stimulate the CHRM2 at a site known for Iperoxo (A - black rectangle) as AAbs for CHRM2 are known to stimulate this receptor (see text). Interaction of other types of CHRMs with ligands that stimulate or inhibit them are also shown (B and C). Given the fact that complement activation can result from the binding of AAbs to the CHRMs it is important to emphasize that at receptors other than CHRM2 the eHects could be destructive for the tissue and therefore leading to functional decline of the receptor function. Data retrieved from https://www.rcsb.org/

1. General Symptoms:

a. Exhaustion and Muscular Pain

Prevalent yet non-specific symptoms in PASC - fatigue, exhaustion and muscular pain - may predominantly stem from diminished tissue perfusion due to cardiovascular constraints, where cardiac factors could be partially linked to specific receptor interactions.

- The M2 GPCR-AAbs may induce a decrease in heart rate, thereby diminishing cardiac output and potentially leading to fatigue. This is consistent with the recognized agonistic nature of this GPCR and the known bradycardic outcome of M2 receptor activation by acetylcholine may contribute to fatigue, exhaustion and muscular pain.

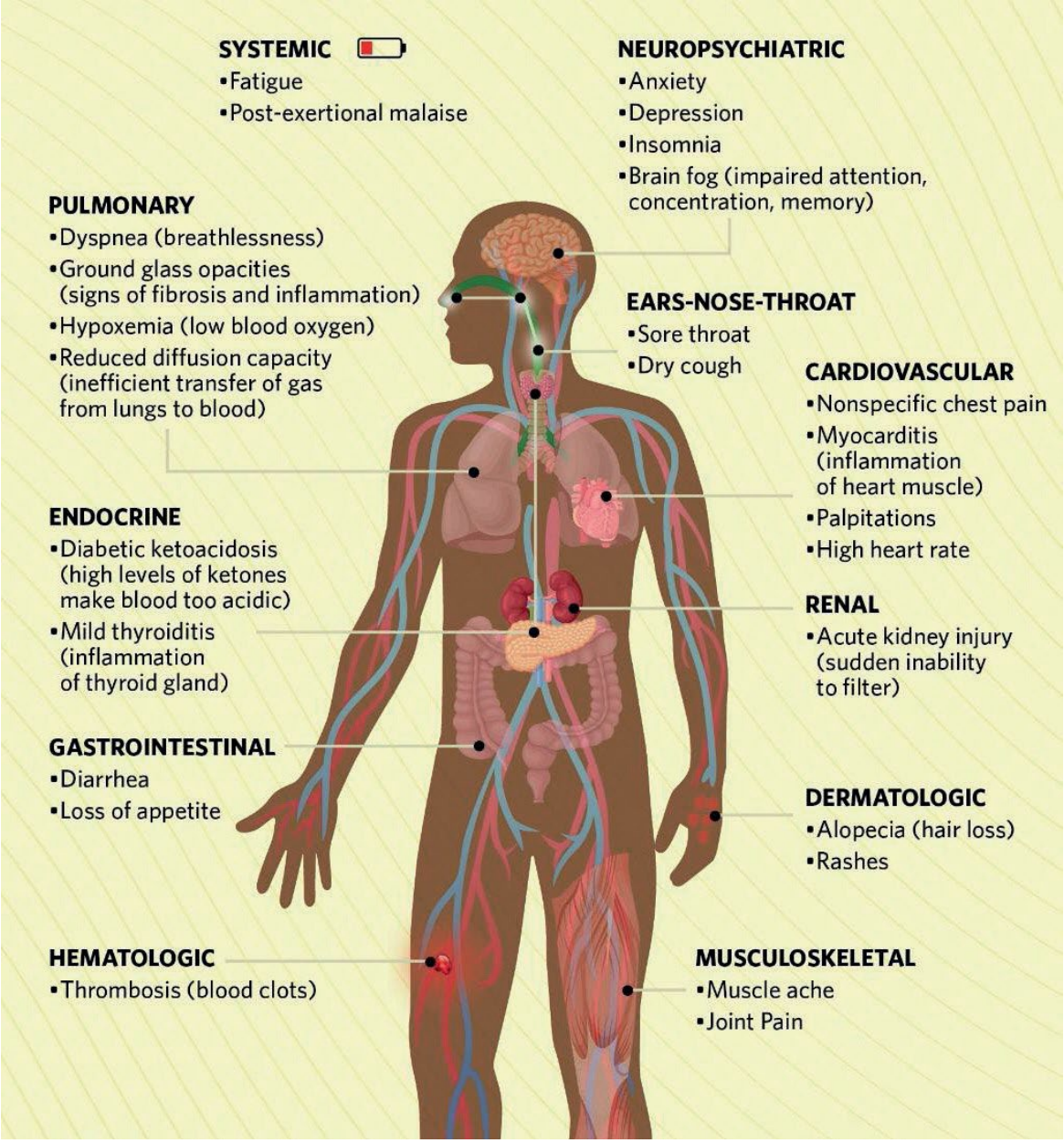

Figure 7:
A concise representation of organ eHects resulting from the interaction of AAbs with the CHRM type of GPCRs. Several symptoms that are experienced by the patient as a result of the above interaction has been detailed in the text.

- Vascular contributions to fatigue might include aberrant platelet clustering, possibly leading to thrombosis. Vasoconstrictive substances released by platelets are known to precipitate such outcomes leading to muscular aches, fatigue and exhaustion.
- Hypoperfusion, particularly in skeletal muscles due to M2 GPCR-AAbs-mediated vasodilation coupled with bradycardia, could be a significant contributor to fatigue.
- The antagonistic behavior of M3 GPCR-AAbs in PASC may lead to decreased gastrointestinal motility. This could contribute significantly to constipation which is reported in these patients. Consequently, this reduction could impair nutrient uptake, which logically contributes to the fatigue experienced by patients.

b. Fever

Fever may be linked to the activation of the hypothalamic centers in response to COVID-19. While cholinergic receptors within this center are not directly associated with fever induction, it's notable that anticholinergic agents like atropine are known to induce pyrexia (22). Given this information and the current findings, it can be posited that antagonistic CHRM3-targeting AAbs could mimic atropine's effects and elicit fever. The mechanisms by which CHRM AAbs might provoke fever could involve both central and peripheral pathways akin to atropine's mode of action.

c. Weight loss

Nutritional deficiencies could result in muscle atrophy and overall weight reduction. Impeded digestive motility and secretory functions, influenced by anti-CHRM AAbs, might contribute to such weight loss over time. While the direct impact of these AAbs on the hunger and satiety centers is still to be fully elucidated, their potential role in weight reduction should not be overlooked.

d. Respiratory Symptoms:

– Shortness of breath
– Cough
– Chest pain or tightness

Whereas some of the signs and symptoms of the respiratory system such as bronchioconstriction, hypoxia and dyspnea can be related to AAbs against CHRMs, particularly the M3 receptor subtype, not all of them can be explained by CHRM agonism or antagonism by AAbs. The action related to these symptoms can be direct or indirect effects of AAbs, which can be due to effects of hypoxia and hypercarbia (dyspnea), chest pain (cytokine release), and peripheral nerve effects and proinflammatory influence on leukocytes (cough).

Agonistic variants of AAbs have been reported recently, and if cross-reacting with CHRM3, can induce an opposite effect resulting in bronchial spasm and therefore enhanced airway hyperactivity. Considering AAbs against the CHRM3 are antagonistic, such an eHect like bronchiodilation can cause a reduction in the amount of the air reaching the exchange zone of the lung, therefore causing hypoxia. This explains dyspnea, mentioned above.

e. Cardiovascular Symptoms:

– Palpitations
– Chest pain
– Deep vein thrombosis (DVT)
– Stroke
– Pulmonary embolism
– myocarditis
– hypertension/hypotension
– POTS

Many features related to syndromic presentation regarding cardiovascular complaints are observed in patients with PASC (Figure 6) that can be explained by the AAbs against CHRM subtypes reported in these patients.

• Bradycardia ➔ Palpitations ➔ AAbs CHRM2 ➔ decreased heart rate

• Tachycardia ➔ Palpitations ➔ AAbs CHRM3 ➔ dominance of the sympathetic nerves to heart ➔ increased heart rate

• Chest pain ➔ myocarditis ➔ due to inflammatory cytokines generated during CHRM3 attack

• Thrombosis and embolism ➔ a) immobility resulting from prolonged bed rest
b) CHRM3 attack over many tissues and organs ➔ release of tissue factor ➔ coagulation cascade activation ➔ venous thrombus ➔ DVT ➔ pulmonary embolism

• Myocarditis:
Damage to myocardial cells by circulating spike protein has been reported (23, 24) but direct damage caused by AAbs against CHRM receptors is not yet established. An indirect effect that can lead to myocarditis involving AAbs against CHRM can be via endothelial injury and the consequent thrombus formation. Inflammatory cytokines released secondary to ischemic damage can form the basis of myocarditis.

• Hypertension/hypotension
As described above, CHRM2 agonistic AAbs are capable of causing decreased heart rate and, this decreased cardiac output can cause hypotension, while AAbs against CHRM3 can indirectly cause hypertension by increasing the peripheral vascular resistance secondary to vasculitis and vasoconstriction caused by endothelial injury and platelet release reaction.

f. Neurological Symptoms:

– Headache
– Sleep disturbances
– Dizziness
– memory loss
– dysautonomia and POTS
– Loss of taste and/or smell
– “Brain fog” or cognitive impairment
– Pins-and-needles
– Nerve pain

As neurological deficits encompass a wider range of etiological factors in PASC (Figure 7), discussion here is limited to the ones caused by the AAbs against CHRMs and causative agents like SARS-CoV-2. Neuroinflammation secondary to the virus and other factors causing neurological effects are thus avoided. As can be seen from protein expression slides, CHRMs are widely distributed on neurons and glial cells. Based on sequence similarities of CHRM subtypes, we show that CHRM3 and, to some degree CHRM2 have regions of similar amino acids capable of mounting AAbs against the CHRM2, CHRM3 and CHRM5 (but not CHRM1 and CHRM4). We therefore implicate the AAbs against CHRM2, CHRM3 and CHRM5 in the causation of neurological symptoms. The above mentioned AAbs could be contributing to:

• Cognitive deficits contributing to brain fog
• Sleep disturbances (insomnia or hypersomnia)
• Clouding of judgement
• Inability to concentrate

- Cerebral exhaustion secondary to concentration
- Demyelination
- Nerve palsies
- Premature aging with loss of cortical grey matter
- Problems with posture and balance
- Tinnitus and vertigo
- Visual disturbances

  Dysautonomia and Postural Orthostatic Tachycardia Syndrome (POTS) is an autonomic dysregulation and part of the the autonomic nervous system. CHRM terminal receptors and cells providing myelination over the vagus nerve could be attacked by AAbs against them. The commonly known vagus nerve stimulation maneuvers (25) which may temporarily increase the quantitative release of acetylcholine provides only transient relief. Inability to respond to pooling blood on postural changes, termed as POTS, while thought to be a cardiovascular defect is actually a failure of reflex sympathetic discharge failing to cause vasoconstriction that normally restores venous return to the heart. Although more a deficit of alpha-adregernic receptors, it can be a consequence of demyelination or neuronal CHRM attack in the spinal cord. The CHRM5 is known to dominate in the human spinal cord (26) and could be contributing to POTS through an attack by AAbs in the lateral horn neurons in the thoracolumbar region where the sympathetic neurons reside. Other complex mechanisms involved in POTS are more non- neural in origin and, therefore, are not discussed here.
- Movement disorders: motor movements of the limbs and skeletal muscles elsewhere in the body have been reported to be affected, which can be related to demyelination – as mentioned above
- Memory impairment: The temporal lobe of the brain is known to store memory and the neurons in this lobe are known to express CHRM1 and CHRM3 in particular. An attack on these receptors by AAbs and / or neuroinflammation secondary to the attack, can cloud this neurological function, and is widely reported in patients with PASC.
- Nerve pain: Small fiber neuropathy is now known to be a feature of PASC (27) and can be explained by myelinating nerves and cell bodies of the neurons that express CHRM receptors. Secondary neuroinflammation that follows can be the basis of the pain in these sets of patients
- Loss of smell and taste: Although more a feature of acute COVID, both anosmia and ageusia can continue in PASC (28) and, given the expression levels of CHRMs over neural and supporting cells in the olfactory mucosa and taste buds, an attack by AAbs can be the basis of these symptoms in PASC.

g. Psychological/Mental Health Symptoms:

– Depression
– Anxiety
– Mood changes

The regions of the brain that are concerned with behavior and involuntary functions express CHRMs, in particular CHRM1 and CHRM3. AAbs that attack these CHRMs can evoke depression, anxiety and mood changes. Associated neuroinflammation (29) in the limbic system, secondary to neuronal and glial cell damage, can also be the basis of the above-described conditions affecting mental health.

h. Gastrointestinal Symptoms:

– Diarrhea
– Abdominal pain
– Nausea
– Vomiting

–Gut motility

Gastrointestinal Symptoms in PASC are mainly due to viral persistence and bacteriophage behaviour of SARS-CoV-2 resulting in repeated injury to mucosal cells (30). However, given the high density expression of CHRM3, AAbs attacking the mucosal cells can contribute greatly to the gastrointestinal damage. Constipation, diarrhea, vomiting, malabsorption and erosion in the mucosal and sub mucosal regions can be expected and has been reported in patients with PASC. The parasympathetic nervous system that is composed of the vagus nerve and the pelvic splanchinic nerve function by releasing acetylcholine on CHRMs, in particular on CHRM3, with regards to promotion of gut motility. If attacked by AAbs, this can explain the reduced gut motility and constipation which is reported in patients with PASC. Agonistic activity of CHRM2 AAbs may also be, in part, contributing to constipation in these patients.

i. Dermatological Symptoms:

– Rash
– Hair loss

The CHRM expression in the epidermis and associated layers of the skin can subject them to an attack by AAbs directed against them, but vaso-occlusive components of PASC secondary toischemia are more at play than AAbs.

j. Ear, Nose, and Throat (ENT) Symptoms:

– Tinnitus (ringing in the ears)
– Earaches
– Sore throat

The symptoms related to ENT are more relevant in context of viral persistence and spike protein mediated damage rather than to AAbs attacking CHRMs.

k. Endothelial injury and hypercoagulability

CHRM expression, in particular the M3 type, is known over the endothelium; therefore, an attack by AAbs is expected to encourage platelet aggregation, vasoconstriction and activation of the coagulation cascade (Figure 8). The resultant ischemia in organs and tissues is expected to amplify (31), and result in diverse symptoms related to their reduced functional state.

l. Endocrinological eUects

Given the expression of CHRM receptor subtypes on endocrine organs (Figures 1 and 2) an aftermath of AAbs targeting and causing organ injury is emerging as new onset of diabetes mellitus, hypothyroidism, lowered testosterone levels, hypocortisolenemia, lower levels of circulating catecholemines with pituatury hormone abnormalities in patients with PASC.

H. Discussion

This study elucidates the molecular mechanisms underlying PASC. Basic to the understanding of the molecular mechanisms were considerations such as a) why the immune system launches an AAb attack on its own CHRM receptors, b) are some of the segments of CHRM antigenic or are the AAbs the result of immune response to segmented S protein portions which cross-react with CHRM receptors, c) do the AAbs mask, destroy, stimulate or antagonize the function of these receptors. Last but not least and possibly the most important aspect that we show is the correlation of AAbs with the symptom complex of PASC. The flow of the findings and the results shown here uncover answers to the above questions. We show that regarding a) above, there are segments of amino acids within the CHRMs protein molecules which are antigenic and can excite antibody production if exposed to the immune system secondary to tissue damage caused by COVID-19 and PASC. The discovery of sequence similarities and the resulting antibody responses provide a compelling explanation for the diverse symptomology observed in PASC patients. Persistence of SARS-CoV-2 with an ongoing low grade inflammation could be at play in these disease states. Most importantly we show that regions of sequence similarity between the S protein segments and CHRMs could be inducing crossreactivity of the AAbs with CHRMs (Figure 3 & 4). The cross-reactivity observed suggests a molecular mimicry mechanism, where the immune system, while targeting the viral spike protein, inadvertently generates autoantibodies that recognize and bind to CHRMs. This can potentially disrupt various physiological processes, contributing to the wide range of symptoms associated with PASC. Our research underscores the importance of understanding these molecular interactions to develop targeted therapeutic interventions.

The effects of AAbs on CHRM and whether they were exerting an agonistic and antagonistic action was determined by comparing the known binding sites of these molecules and correlating them with the clinical symptoms in COVID-19 and PASC. Organ related symptoms which point towards loss of function of the tissues appear to be the effects of AAbs where an inflammatory cytokine-mediated destruction is at play. The complement system activated products like C5b9 are known to cause antibody mediated cell damage while many other cytokines from WBCs can equally contribute to tissue injury. Acting as a biomarker these cytokines and complement products in patients with COVID-19 and PASC with positivity for AAbs has already been revealed.

Furthermore, the study opens avenues for exploring similar mechanisms in other post-viral syndromes and autoimmune conditions. While these findings are a significant step forward, they also highlight the need for continued research to fully understand the complexities of PASC and related conditions. Though the spectrum of expression of CHRMs (Figure 1 and 2) on organs and tissues appear to correlate with the symptoms of the patients, heterogenous patterns have emerged which do not seem to follow this. Many patients dominate in clinical features of organs and tissues which show low expression of CHRMs such as thyroid gland, adrenal gland and endocrine pancreas resulting in dysregulated thyroid function, altered adrenal cortical hormones and reports of new onset of diabetes mellitus. It is also important to keep in mind that clinical features not related to AAbs against CHRMs can dominate the symptomatology and blend with the symptoms related to AAbs. Pathogenetic factors like S protein mediated direct damage to the endothelium and myocardium can play a vital role in the symptoms of PASC independent of CHRM directed AAbs. AAbs directed against non CHRM GPCRs are also at play which presents a more complex symptomatic picture.

I. Conclusion and Future Direction

The toll of COVID-19 is itself enormous to the point that it can be fatal but the disabling capability of this disease gets reflected in its protracted state called PASC. More disabling than lethal, the patient suHering newer symtpoms raise the curiosity of something in eHect that yet covert needed to be researched. The discovery of AAbs in PASC provided an incomplete answer as to why the body launches an autoimmune state, to which we have added explanation in this paper. Overall, the study contributes substantially to the growing body of knowledge on the pathophysiology of PASC, emphasizing the role of autoimmunity and molecular mimicry in its symptomatology. This understanding is crucial for the development of eHective treat-

ments and management strategies for those suffering from this condition. Selective removal of these AAbs by immunoadsorption apheresis and modified H.E.L.P apheresis in the near future can help fight the diverse symptoms caused by AAbs. Immunosuppresive agents that may dilute AAb production can provide temporary relief from symptoms but may act as a double-edged sword as it can increase the chances of opportunistic infections. Pivotal to the concept of providing relief for the symptoms of PASC is the necessity to address the underlying cause and not the effect of AAbs against GPCRs. Targeting persistance of the virus as evidenced by serological testing that indicates viral replication should be the central approach which if eradicated can result in substantial gains over the disease symptoms and complications. The emergence of AAbs against GPCRs like CHRM are yet to be scaled in patients with vaccine induced adverse events who produce S protein for extended periods. Inhibition or inactivation of the enzymes involved in partial digestion of the S protein and therefore the generation of immunogenic peptides as shown in this study is also an additional mode of treatment that can be considered. Surprisingly, we were able to show antigenic segments within the CHRMs (Figure 6 – orange highlight) that when liberated during extensive cellular damage due to viral persistence and related low grade inflammation can themselves evoke AAbs and therefore produce related symptoms. This understanding is crucial for the development of effective treatments and management strategies for those suffering from this condition. Selective removal of these AAbs by modified H.E.L.P apheresis (32) and immunoadsorption apheresis like DNA230 (33) and Immunosorba® or GLOBAFFIN (34) which can be tailored with appropriate filters to selectively remove AAbs (33) in the near future can help fight the diverse symptoms caused by AAbs.

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Supplementary file

>QIH45093.1 spike protein [Severe acute respiratory syndrome coronavirus 2]

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFSNVTWFHAIHV
SGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSKTQSLLIVNNATNVVIKVCEFQFCNDPFLG
VYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLEGKQGNFKNLREFVFKNIDGYFKIYSKHTPI
NLVRDLPQGFSALEPLVDLPIGINITRFQTLLALHRSYLTPGDSSSGWTAGAAAYYVGYLQPRTFLLKYN
ENGTITDAVDCALDPLSETKCTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASV
YAWNRKRISNCVADYSVLYNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGKIAD
YNYKLPDDFTGCVIAWNSNNLDSKVGGNYNYLYRLFRKSNLKPFERDISTEIYQAGSTPCNGVEGFNCYF
PLQSYGFQPTNGVGYQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPF
QQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVPVAIHADQLT
PTWRVYSTGSNVFQTRAGCLIGAEHVNNSYECDIPIGAGICASYQTQTNSPRRARSVASQSIIA
YTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTG
AVEQDKNTQEVFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDC
LGDIAARDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAMQMAYRFNGIG
VTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDI
LSRLDKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLM
SFPQSAPHGVVFLHVTYVPAQEKNFTTAPAICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNT
FVSGNCDVVIGIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEVA
KNLNESLIDLQELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCGSCCKFDED
DSEPVLKGVKLHYT

Figure S1.
The image shows a sequence of S protein in SARS-CoV-2. The green highlighted areas are segments predicted to evoke an antibody response with strong bond and were aligned with CHRM3 which showed regions of similarities and fall within the green colored segments of the S protein.

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The potential of heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.)-apheresis for patients with severe acute or chronic COVID-19

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Patients with long COVID and acute COVID should benefit from treatment with H.E.L.P. apheresis, which is in clinical use for 37 years. COVID-19 can cause a severe acute multi-organ illness and, subsequently, in many patients the chronic illness long-COVID/PASC. The alveolar tissue and adjacent capillaries show inflammatory and procoagulatory activation with cell necrosis, thrombi, and massive fibrinoid deposits, namely, unsolvable microthrombi, which results in an obstructed gas exchange. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis solves these problems by helping the entire macro- and microcirculation extracorporeally. It uses unfractionated heparin, which binds the spike protein and thereby should remove the virus (debris). It dissolves the forming microthrombi without bleeding risk. It removes large amounts of fibrinogen (coagulation protein), which immediately improves the oxygen supply in the capillaries. In addition, it removes the precursors of both the procoagulatory and the fibrinolytic cascade, thus de-escalating the entire hemostaseological system. It increases myocardial, cerebral, and pulmonary blood flow rates, and coronary flow reserve, facilitating oxygen exchange in the capillaries, without bleeding risks. Another factor in COVID is the “cytokine storm” harming microcirculation in the lungs and other organs. Intervention by H.E.L.P. apheresis could prevent uncontrollable coagulation and inflammatory activity by removing cytokines such as interleukin (IL)-(, IL-8, and TNF-α, and reduces C-reactive protein, and eliminating endo- and ecto-toxins, without touching protective IgM/IgG antibodies, leukocyte, or platelet function. The therapy can be

used safely in combination with antiviral drugs, antibiotics, anticoagulants, or antihypertensive drugs. Long-term clinical experience with H.E.L.P. apheresis shows it cannot inflict harm upon patients with COVID-19.

KEYWORDS

H.E.L.P. apheresis, PASC, COVID-19, long COVID, SARS-CoV-2, heparin, fibrinogen, rheology

Introduction

In COVID-19 pandemic, the key question is: which therapeutic approach should be favored in order to save seriously sick patients? What kind of approach is suitable to prevent looming acute lung failure involving microthrombi and inflammation of the endothelium (1–5) as a result of an excessive immune response of the body when the host's first lines of defense have already failed? We know that SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor and the transmembrane serine protease 2 (TMPRSS2) as gateways (6–8) to infect cells of the alveolar epithelium (1–4) and endothelial cells in the lungs, heart, kidneys, intestines, and liver (5). This is why patients with coronary artery disease (9–12), hypertension (3, 13), diabetes (3, 13), or obesity (3, 13) exhibit a higher mortality risk as their receptor density is up-regulated (14). Moreover, the binding of the SARS-CoV-2 spike protein inhibits and down-regulates ACE2 function which in turn promotes the inflammatory response (6–8). Diabetes for instance increases thrombogenicity and hyperactivates platelets, and so does hypertension by increasing shear stress in the vessels (15–17).

Histological studies confirmed the presence of the virus in both cell types: alveolar epithelium and endothelial cells (1–5). Alveolar tissue and adjacent capillaries reveal massive inflammatory and procoagulatory activation together with cell necrosis, thrombi, and massive fibrinoid deposits (1–5, 18, 19). It results in the clinical picture of an obstructed gas exchange. The enlargement of the diffusion barrier limits the benefits of artificial ventilation and extracorporeal membrane oxygenation (ECMO) (20–23). In addition, the latter promotes the formation of radicals as a side effect (20–23).

The application of H.E.L.P. apheresis could significantly contribute to the restoration of microcirculation in the lungs and other affected organs. The method, developed by Seidel and Wieland in 1984, primarily for patients with severe hyperlipidemia or familial homozygous hypercholesterolemia (24–30), has not only been proven beneficial as an ultima ratio treatment of arteriosclerosis and its atherothrombotic sequelae, it also has been successfully applied in coronary heart disease (24–27, 31–33) to prevent and treat graft vessel disease following heart transplantation (33–39), acute thrombotic graft occlusion following aortocoronary bypass surgery (40), preeclampsia (41, 42), strokes (43–46), unstable angina pectoris (47), and hyperlipoproteinemia (a) (32). It exhibits anti-inflammatory effects in chronic, and also acute inflammatory processes of the endothelium in the micro- and macrocirculation (26–36, 40, 48, 49) and has anticoagulant and anti-inflammatory properties (25, 50, 51).

Methodology

During H.E.L.P. apheresis, blood cells are first separated from plasma in the extracorporeal circuit, then 400.000 units of unfractionated heparin are added to the plasma, and the pH is lowered to 5.12 using an acetate buffer. That is the isoelectric point for the optimal precipitation of the apolipoproteins from LDL cholesterol, lipoprotein (a) [Lp(a)], and VLDL, which are precipitated in the precipitation filter together with fibrinogen. The excess heparin is adsorbed, and bicarbonate dialysis balances the pH again. The blood cells of the patients are reinfused in parallel with a saline solution (24, 50). The duration of treatment-−2 h on average—can be shortened or extended to meet individual needs (50).

Rationale for H.E.L.P. apheresis

Patients with acute and long COVID-19 most probably will benefit from H.E.L.P. apheresis due to the following reasons:

1. It has no allocation problem and allows direct access to the entire macro- and micro-circulation owing to its extracorporeal access.
2. It uses 400.000 units of unfractionated heparin in the extracorporeal circuit, which was shown of being capable to bind SARS-CoV-2 spike protein (19, 52), and thereby could directly remove the virus and viral debris during viraemia.
3. The large quantity of unfractionated heparin allows the desolvation of forming microthrombi without a bleeding risk due to the heparin adsorber (50).
4. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis removes about 50–70% of fibrinogen, the most important coagulation protein, within 2–3 h, that in turn immediately improves oxygen supply in the capillaries (50, 51).
5. In addition, it partially removes the precursors of both the procoagulatory and the fibrinolytic cascade by 35–50%,

thus de-escalating the entire haemorheologic system (50). However, antithrombin III is only eliminated by 25% (50) ensuring minimized bleeding risk complications.

6. From the very beginning, H.E.L.P. apheresis is rheologically effective (30, 31, 33, 53): it increases myocardial (30, 53), cerebral (54), pulmonary blood flow rates, and coronary flow reserve (53). These effects facilitate oxygen exchange in the capillaries sustainably (51).
7. It removes cytokines such as interleukin (IL)-6, IL-8, and TNF-α, and reduces C-reactive protein (CRP) concentrations by more than 50% (41, 48, 49). The heparin adsorber completely eliminates endo- and ecto-toxins (48), so that the excessive inflammatory response, the so-called "cytokine storm", can calm down (18, 19, 48, 49).
8. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis has already been successfully applied for septic multi-organ failure in pilot studies by Bengsch et al. (48, 49). In modified form, it showed a successful outcome in the enterohaemorrhagic *E.coli* (EHEC) epidemic in patients suffering from the hemolytic-uraemic syndrome (HUS) (55).
9. Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis is an established, commercially available system (B. Braun AG, Melsungen, Germany) that has been in clinical use for 37 years. It is easy to handle and was shown to reduce complication rates in acute and chronic cardiac patients very effectively by 82–97% (27, 29, 30, 32, 34, 36). The long-term clinical experience with H.E.L.P. apheresis suggests, with a probability close to certainty, that it cannot inflict harm upon patients with COVID-19.
10. It does not remove protective IgM or IgG antibodies and does not affect leukocyte or platelet function. In the past, the therapy has been shown to be well-tolerated and safe during treatment with antiviral drugs, antibiotics, anticoagulants, or antihypertensive drugs.

Background

In patients who are suffering from severe COVID-19, the computed tomography (CT) scan of the lungs shows ground-glass-like interstitial thickening (5), (which presumably leads to acute respiratory distress syndrome (ARDS). As a result of an excessive immune response, it appears uncontrollable. The advanced disease stage develops after the initial antiviral defense lines of the innate immune system—such as protective effects of interferons and secretory IgA on alveolar epithelium—have failed to eliminate the virus. The presence of SARS-CoV-2 viraemia is the prerequisite for humoral antibody synthesis of IgM and IgG subtypes. They could lyse virus-infected cells in the presence of complement factors. As far as we know, the nature and extent of the cellular immune response to viral antigens are almost entirely dependent on T-lymphocytes (56). The cell-mediated antibody-dependent cytotoxicity is T-cell-dependent and, currently, is being the subject of intensive virological and cell biological research.

In principle, intervention in the inflammatory cascade takes place as early as possible before the onset of the "cytokine tsunami" in order to prevent uncontrollable coagulation and inflammatory activity (18, 19) harming microcirculation in the lungs and other organs. This may be the case in COVID, for example, as this cytokine storm likely results in the presence of microthrombi found in patients suffering from COVID-19 (57). These microthrombi have the ability to block microcapillaries and hence, inhibit oxygen exchange and supply at various organs, resulting in the various symptoms of long COVID such as muscle fatigue, breathlessness, sleep impairment, and anxiety or depression (58). The phenomenon of a "cytokine storm" was first described in 1973 in graft-vs.-host disease (GvHD) following organ transplantation, and later in ARDS, sepsis, Ebola, avian flu H5N1, smallpox, systemic inflammatory response syndrome (SIRS), and now in COVID-19 (59).

Cytokines are proteins that coordinate and modulate cellular immune responses: they guide and activate leukocytes–in particular, T-lymphocytes and monocytes–to the site of inflammation where cytokine secretion is regulated by positive feedback. During a "cytokine storm", leukocytes are activated to such an extent that the immune response seems inexorable. High concentrations of IL-1β, IL-6, and IL-8 are expressed (18, 19, 59–61). Furthermore, IL-1β and IL-6, together with TNF-α—the latter being mainly expressed by macrophages-direct systemic inflammatory effects such as the increase in body temperature and blood flow, capillary permeability, and leakage. Due to the complexity of regulation and orchestral functions, IL-6 plays a key role in the transition of mechanisms of innate to acquired immunity (60, 62). The CRP triggers IL-6 (61) and IL-6 links procoagulatory activation, especially triggering fibrinogen production in the liver [51]. Whenever the body's defense is not able to clear the virus from all sites, the inflammation may persist in macrophages, in vascular beds, or in the brain stem and chronify, as recently reviewed by Proal and VanElzakker (63) with the consequence of a wide range of long-lasting clinical symptoms and impaired host immunity. In recent years, Pretorius and Laubscher (64) proved the persistence of insoluble clots containing excess alpha2-Antiplasmin bound plasminogen fibrinogen and amyloid proteins, which results in hindered fibrinolysis in long COVID patients.

Discussion: E*ects of HELP apheresis

The anti-inflammatory effects of H.E.L.P. apheresis had been intensively investigated by Bengsch et al. (35, 36) in the nineties.

It has been applied by them in pilot studies to successfully treat sepsis and septic shock patients with multiple organ failures. In 2012, we were able to rescue a patient with EHEC-induced HUS from her comatose state within hours, and from kidney failure within 2 days (55).

In the case of COVID-19, H.E.L.P. apheresis could be of immediate benefit because this extracorporeal system can reduce the trigger and effector of the overwhelming immune response in a simultaneous manner. The SARS-CoV-2, circulating cytokines, CRP, on top fibrinogen are reduced drastically, the latter by 50% within 2 h. As a result, the rheology of the pulmonary microcirculation will immediately be relieved—without reduction of the erythrocyte concentration. Fibrinogen is the effector of plasmatic coagulation and decisive determinant in microcirculation, plasma viscosity, and erythrocyte aggregability (51). Owing to the use of unfractionated heparin, the antithrombotic effect is maximal.

Previous studies using positron emission tomography in heart transplant patients showed that the median coronary blood flow rate remains significantly increased by 17.5% for 24 h after a single 2-h apheresis procedure. It increases by 27% under simulated exposure to the administration of adenosine (33). In principle, the decreased fibrinogen concentration causes rheologically significant effects and facilitates oxygen exchange. Plasma viscosity is reduced by an average of 19%, and erythrocyte aggregability is significantly decreased by 60% (33). In addition, the vascular endothelial growth factor (VEGF) and nitric oxide (NO) release are favorably influenced (33). The improvements have also been demonstrated for cerebral blood flow in the cardiac patients, where they profit from a 63% increase in the CO_2 reserve capacity (54).

Heparin-induced extracorporeal LDL/fibrinogen precipitation (H.E.L.P.) apheresis reduces LDL cholesterol and Lp(a) concentrations with similar efficacy as fibrinogen (24, 25), thereby improving endothelial function (33, 53, 54). With regards to LDL reduction through apheresis, it remains unclear whether SARS-CoV-2 resembles delta coronavirus, which uses cholesterol as a vector due to its lipid envelope (65).

For practical reasons it is important to mention that H.E.L.P. apheresis is not restricted to a 2-h treatment time. The system can be recirculated for many hours—until the precipitate filter is saturated. The precipitate filter however can also be exchanged during the procedure, so the fibrinogen concentration theoretically could be reduced by up to 99.9999%. In-depth preliminary studies into the influence of H.E.L.P. apheresis on the kinetics of the procoagulation and fibrinolytic cascades have shown that the precursors of both cascades are also reduced by 35–50% within 2 h—with the exception of antithrombin III, which is reduced by 25% (50). Taking together, H.E.L.P. apheresis thus de-escalates the coagulation situation of both arms without any bleeding risk due to the complete adsorption of unfractionated heparin (50).

The heparin adsorber also has the ability to eliminate endo- and exo-toxins regardless of viral or bacterial origin (48, 49, 55). Recent data from Carlo Brogna indicate that the SARS-CoV-2 virus acts as a bacteriophage on the microbiome of the lungs and the guts of infected patients, thereby inducing the bacteria to produce neurotoxic "conotoxins". These so-called conotoxins might also be eliminated by means of H.E.L.P. apheresis (64).

The use of H.E.L.P. apheresis should be considered for the treatment of patients with acute and long COVID in order to restore the vascular homeostasis, remove inflammatory and thrombogenic mediators, and to avoid unnecessary suffering. Our first experiences with patients with long COVID are promising and summarized in the corresponding article. Meanwhile, we could successfully treat hundreds of patients with long COVID with this method.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

BJ created the working hypothesis and wrote the paper. HA helped in editing, proofreading, and discussing the theory. WK-M helped brainstorm, discuss the theory, and refine it. DS was the inventor of the HELP apheresis helped with the theoretical hypothesis and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors BJ and DS filed a patent of the use of HELP Apheresis for long COVID to avoid misuse.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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