Long COVID: a central role for SARS-CoV-2 spike protein in a multifaceted pathogenesis

Written by: Dani Holla
Master program: Infection and Immunity (Graduate School of Life Sciences, University Utrecht)
Student number: 6453813

Supervisor: Jeroen den Dunnen

Examiner: Niels Eijkelkamp

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Layman summary

This review will discuss the origins of long COVID, a syndrome that can affect individuals following SARS-CoV-2 infection. Typically, it is described as the continuation, worsening or even presentation of new symptoms that remain at least 3 months after the initial infection. Symptoms which cannot be explained by any other underlying disease in the affected individual. Long COVID can present with many different symptoms in multiple organ systems, however, the most common symptoms include fatigue, general incapability of brain and muscle functions, exercise intolerance, elevated heart rate after a postural change and mood affectations like depression or anxiety. As there is a broad presentation of symptoms, it is also thought that multiple organ systems can be affected in long COVID, where research currently focusses on muscle, vascular and nervous system tissues. Researchers and clinicians made compelling efforts to understand the syndrome and have identified multiple systems to act differently in long COVID patients versus non-long COVID patients. Research showed difference in blood components and how these clot together, immune reactions that target the patient itself, and dysfunctionality in the mitochondria, which are smaller parts of a human cell mostly responsible for energy production and the cell's metabolism. It is not unthinkable that these affected systems can lead to the observed symptoms in patients, but researchers are still uncertain on how these differences are established and can continue in the long COVID patient population. Therefore, this review will summarize and explain in detail what these systems are and how these are impacted during long COVID. Furthermore, it will be discussed how the SARS-CoV-2 spike protein, which is possibly present in many sick individuals, may lead to these dysfunctionalities in long COVID patients. This protein is found on the outside layer of the virus and is important for the virus to infect human cells. Finally, a scheme will illustrate how an individual goes from initial infection with SARS-CoV-2 to the most common symptoms observed in long COVID (**Figure 1**).

Abstract

Globally, it is estimated that over 400 million people suffer from Long COVID, a syndrome that originates from an earlier experienced SARS-CoV-2 infection. This syndrome presents with symptoms such as fatigue, post-exertional malaise, postural orthostatic tachycardia syndrome and mood disorders, persisting for months or years after initial infection. Although research is progressing, the syndrome pathogenesis remains to be fully understood, resulting in inadequate diagnostic tools and treatment strategies, and ultimately insufficient patient care. Investigations have provided evidence that patients with long COVID can exhibit multiple affected physiological systems. However, an explanatory mechanism resulting in these pathophysiological processes is lacking and, in addition, it remains unclear how these processes invoke the broad symptomology observed. In this review, these pathophysiological processes will be discussed and how they could originate from persistent presence of SARS-CoV-2 spike protein. Moreover, a schematic structure will be presented to illustrate the causality and chronology of events, and how these eventually could result in the observed symptoms in long COVID (**Figure 1**).

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic greatly impacted the modern-day society by halting economic growth, severely straining health-care infrastructures and dysregulating communitive interactions, resulting in a substantial disturbance peoples standard of living[1]. This virus, in its many variant forms, induced coronavirus disease 2019 (COVID-19), which differently affected human hosts from asymptomatic infection to severe respiratory disease, even with fatal outcome[2]. Currently, years after the pandemic onset, a subset of people infected with SARS-CoV-2 suffer from heterogenous, multisystemic manifestations of disease that can affect many organ systems. Commonly reported symptoms include general neurocognitive impairment (also described as 'brain fog'), fatigue, post-exertional malaise (PEM), and postural orthostatic tachycardia syndrome (POTS)[3]. PEM is the worsening of symptoms in patients after physical or mental exertion and POTS is characterized by abnormal increase in heart rate after postural changes like standing up, where this elevated heart rate could lead to many symptoms such as dizziness, fatigue and shortness of breath. Many of the long COVID symptoms seemingly resemble the symptomology of other known syndromes following infection, which are termed as post-acute infection syndromes (PAIS)[4]. These include, for example, post-treatment Lyme disease syndrome (PTLDS)[5] and Q fever fatigue syndrome (QFS)[6]. Moreover, there seems to be considerable overlap of long COVID with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS)[7].

Post-COVID manifestations are diversly defined across literature, with descriptions as post-COVID-19 syndrome/conditions, post-acute sequalae of SARS-CoV-2 (PASC) and long COVID[8,9]. This complicates direct literature comparisons, however, an overarching feature between the definitions is that it describes persistent (multisystem) symptoms following SARS-CoV-2 infection, after a minimum of 4- to 12-weeks following infection[10]. This review will focus on and adopt the term 'long COVID' described by Peluso et al.[10]. It is described as 'new or worsened symptoms following SARS-CoV-2 infection that cannot be explained by a known medical condition'. By following this definition, this review excludes the patients with exacerbated symptoms of previous comorbidities and hospitalized patients due to severe COVID-19 disease, two groups that are also subject to continued affectations following SARS-CoV-2 infection. However, when these groups are included in the mentioned articles, as there is overlap between these patient groups, it will be specifically annotated.

Although risk of long COVID can increase with severe COVID-19, the majority of affected patients (90%) presented with mild-to-moderate COVID-19 symptoms[11,12], as this group represents a much bigger proportion of the total infected population. This review will emphasize this subset of patients. Prevalence of long COVID varies and one nationwide survey estimated between 6.6–10.3% of the population to be affected[13]. Globally, the predictions amount to at least 400 million people suffering from this syndrome[12]. The actual number can be higher, as there might be underrepresentation of people reporting their symptoms following asymptomatic COVID-19 or of people without access to adequate health care. As mentioned, the incidence of long COVID, after SARS-CoV-2 infection, is dependent on the severity of COVID-19. One study showed that 6.2% of people, after symptomatic SARS-CoV-2 infection, exhibit at least one symptom associated with long COVID (fatigue, cognitive problems, ongoing respiratory problems)[14]. Important risk factors contributing to long COVID development are age, the female sex and severity of COVID-19 disease[10,15].

Long COVID can drastically impact the individual. Large proportions report severe impact on mental health and well-being, with patients being bedridden and unable to continue working, having combined results as social- and communitive seclusion of these patients[16]. Moreover, the complex, heterogenous clinical course of long COVID often requires medical care with multiple specialists involved, straining health-care infrastructures that are without clear treatment strategies to tackle this syndrome. Due to the combination of health care expenses and the negative impact of long COVID on the available workforce, a conservative estimate has been made that long COVID annually burdens the 'global' economy approximately 1 trillion US dollars or 1% of worldwide GDP 2024[12]. An important consideration is that, so far, research has shown that full recovery of long COVID is low at 7-

10% after 2 years of syndrome onset[12], where parallels with other syndromes as ME/CFS could indicate long COVID to have chronic tendencies.

Combining the patients' well-being and substantial societal implications originating from this condition, long COVID could be deemed as a major health crisis. Research is progressing towards elucidating underlying biological mechanisms, however, the multisystem complexity of this syndrome and heterogenous presentation of symptoms complicate uniform syndrome definition and diagnosis. Insufficient knowledge on long COVID pathogenesis and a diverse clinical presentation have resulted in a lack of a treatment/ intervention strategies to help patients, as there are currently no proven treatments for long COVID and management of it can vary widely on the between clinical practitioners[12,17]. There are signs that individual symptoms could improve with proper guidance, such as pacing protocols for PEM[18], but, overall, a clear treatment strategy is lacking which results in continued debilitation for long COVID patients. Therefore, when combining the impact on patients and the consequential ramifications on society, there is need for a better understanding of the pathogenesis of this syndrome towards adequate diagnosis and new treatment strategies.

This review will summarize the pathophysiological processes thought to be of importance in the development of long COVID. A more in-depth focus will be applied to describe how the processes in this pathophysiology are connected through the spike (S) protein and might result in the observed syndrome phenotype and symptom aggravation. Finally, a hypothetical, chronological structure of initial infection towards symptomology will be suggested as an explanatory biological cascade for long COVID patients.

Current understanding of the long COVID pathophysiology

The clinical pathophysiology in long COVID patients can vary between patients, with multiple affected systems. Currently, a large collective of symptoms/biomedical findings have been noted which can be broadly divided in the following categories with exemplary conditions: organ/tissue damage[10,19,20] (pulmonary fibrosis, cardiac impairments, other organ damage), vascular dysfunction[21,22] (thromboinflammation, endotheliopathy, tissue hypoxia), immune system dysregulation[23–26], neurological and cognitive impairment[27,28] (motor- and sensory symptoms, dysautonomia, depression), musculoskeletal sequelae[29] (fatigue, myalgia, PEM), gastrointestinal (GI) sequelae[19,20,30] (gut-brain axis dysfunction). As this diversity of symptoms progressed, the question was raised whether long COVID patients could truly be defined by a shared underlying biological mechanism that could provide an explanation for a multitude of these symptoms. Or, if research and diagnosis should develop strategies to stratify patients in subcategories, each perhaps in need of a

different diagnostic tool and treatment regimen. The answer remains unknown, although research has started to elucidate on the possibility of multiple biological mechanisms that could be linked to the origin and progression of long COVID. The following section will cover the most common hypothesized biological mechanisms though to relate to long COVID emergence.

Acute SARS-CoV-2 infection, a persistent viral reservoir and herpesvirus reactivation

SARS-CoV-2 entry into host cells is predominantly mediated by the binding of the S protein to its receptor, angiotensin-converting enzyme 2 (ACE2)[31], which is thought to initially occur in the human airway epithelial cells[32]. After binding, SARS-CoV-2 can enter via cell surface membrane fusion, mediated by cleavage into the S2 subunit of S protein by transmembrane protease, serine 2 (TMPRSS2)[33]. Another route is via membrane fusion, after internalization in the endosome, mediated by cathepsin L. Subsequent viral replication can result in major infection of lung tissue cells and severe respiratory disease.

Although COVID-19 is typically presented as a respiratory-related disease, research suggests SARS-CoV-2 can infect a much wider spectrum of host cells. Studies investigating virus tropism and capacity for replication found SARS-CoV-2 RNA in 84 distinct anatomical tissues in a cohort of 44 autopsy patients with acute or persistent COVID-19[34]. Tissues included the vasculature, lymphoid tissue, the GI-tract, and the central nervous system (CNS). These findings could indicate a relation between acute/persistent SARS-CoV-2 infection in multiple organ systems, subsequent tissue damage, possibly correlating to a wide variety of the observed symptoms. The presence of viral components in the CNS[34] even suggests the possibility of SARS-CoV-2 crossing of the blood-brain barrier (BBB). Stool samples were collected from participants with confirmed SARS-CoV-2 infection[35]. From participants with resolved SARS-CoV-2 oropharyngeal shedding, 12.7% still showed SARS-CoV-2 RNA in feces 4 months after infection and 3.8% at 7-month time point, percentages that closely overlay with estimates of prevalence of long COVID in the general population (see **introduction**). Moreover, in participants with exclusive fecal SARS-CoV-2 RNA shedding, many associations were found between fecal shedding and persistent GI-tract symptoms, which could be interpreted as signs of a continued infection in the GI-tract. Others showed continued presence of SARS-CoV-2 S protein in 60% of plasma samples from long COVID up to 12 months post infection, while this was not observed in COVID-19 resolved patient group[36]. However, it should be noted that the time-points of measurement (range of 12 months post infection) between these patients differed, making direct comparison more difficult. Another investigation also found presence of S1 protein in a specific CD16+ monocyte subset in the blood up to 15 months post infection[37]. Whether there is active virus replication of SARS-CoV-2 in long COVID or for instance abrogated replication with circulating viral remnants remains elusive. A recent trial, where

patients with long COVID were administered with antivirals Nirmatrelvir and Ritonavir, showed no improvement of symptoms in the treatment group versus placebo group[38]. This could suggest symptomology of long COVID not to be due to persistent SARS-CoV-2. However, this might also indicate the SARS-CoV-2 reservoir localized where these antivirals could not penetrate the tissue.

Not only does it seem that components of SARS-CoV-2 can persist for longer time periods, but research has found evidence for the reactivation of other latent viruses after SARS-CoV-2 infection. A great majority of the population is infected with latent herpesviruses, for instance, >90% of the population exhibit Eppstein-Barr virus (EBV) infection[10]. These viruses are known to reactivate in immunocompromised conditions or during stress reactions as other active viral infections[10]. Multiple reports have found signs of herpesvirus reactivation associated with SARS-CoV-2 infection. One study, using rapid extra-cellular antigen profiling observed increased scores against the EBV antigen gp23, gp42, and the varicella zoster virus (VZV) glycoprotein[39]. Others also found elevated antibodies against EBV and cytomegalovirus (CMV) components[40], suggesting a reactivation after SARS-CoV-2 infection. To investigate whether these reactivations influence long COVID development, a cohort of 280 patients was analyzed for associations between reactivation and symptoms[41]. They identified a strong association between EBV-reactivation and reported fatigue in patients. Interestingly, they identified a negative association between CMV seroprevalence (indicative of reactivation) and the development of long COVID. The study confirmed long COVID patients without viral reactivation, suggesting it not to be the sole component driving symptoms. Connections to the other observed biological mechanisms driving long COVID might reveal a role for herpesvirus reactivation in the development and continuation of long COVID symptomology.

Research into the presence of SARS-CoV-2 after infection should determine whether these virus particles can persist and replicate in the host or show other signs of latency. Similarly, how SARS-CoV-2 triggers other latent viruses and their involvement in long COVID development should be explored further. If viral components themselves, such as the S protein, could trigger multisystemic manifestations, more investigations into the origins and survival times of these persistent antigens could reveal links to long COVID pathophysiology.

Autoimmunity

Evidence is coming up for abnormality in B cell populations after SARS-CoV-2 infection. One of the earlier reports, in a cohort of hospitalized patients with active COVID-19, observed naïve B cell dysregulation to result in autoreactivity, with findings of antinuclear antibodies (ANAs) and anticarbamylated protein responses found 100 days after symptom onset of long COVID[42]. Others found certain immune pathways, such as type 1 interferons (IFNs), to

be targeted by autoantibodies in patients with COVID-19[43], possibly mitigating adequate immune response and viral clearing during active infection. Another study included healthy controls, recovered COVID-19 patients, and a stratified cohort of long COVID into 3 groups with mild-moderate acute disease, hospitalized and intensive care patients[44]. They observed no difference in number of ANAs between recovered and healthy controls, but a significant increase when compared to the individual long COVID groups. Moreover, they found total ANAs to increase with severity of COVID-19 and that the total ANAs in all long COVID groups showed decline over 12-months. From these ANAs, they found Anti-U1-snRNP and anti-SS-B/La to correlate with long COVID symptoms fatigue and dyspnea. Another study did not observe differences in ANA titers based on severity of acute disease[45]. Interestingly, they observed that in females, and not in males, patients with higher ANA titers (≥1:160) to have significantly higher symptom frequency than in lower ANA titer group (≤1:160). This might link to the overrepresentation of females among long COVID patients. One investigation, in patients selected with higher ß2-adrenergic receptor autoantibodies, performed immunoadsorption to remove immunoglobulins[46]. They observed improvement of SARS-CoV-2-triggered ME/CFS symptoms assessed with short-form 36 physical function, although they did not identify correlation between decrease in autoantibodies with increase in symptom relieve. To infer on the possibility of autoantibodies instigating long COVID symptoms, a group transferred whole human immunoglobulin (Ig) G from patients with long COVID into mice [47]. They subdivided the long COVID cohort into three groups, one group showing elevated markers of neuronal damage and the other two groups characterized by discordance in type-1 IFNs. Interestingly, the transfer seemed to induce a mimicking of symptoms in the mice also observed in patients, suggesting that distinctive targeting of autoantibodies might provoke different symptoms. Similar findings were reported by others, where human IgG (autoantibodies) crossed the murine BBB, binding to tissues of the CNS and eliciting symptomology in the mice similar to the patients where the IgGs originated from[48].

In contrast to the findings above, a study on immune profiling did not observe significant differences in total number of autoreactivity reactions between COVID-19 recovered and long COVID patients, nor did there be a trend in specific autoantibodies more present in the long COVID patients[39]. Of importance, this investigation did not make subdivision in the long COVID cohort, also including a small percentage of hospitalized patients (13%). Another study found no difference in autoantibody seroprevalence between patients who developed and patients who did not develop long COVID in a cohort of 161 patients following SARS-CoV-2 infection[49]. It is entirely possible that only a subset of patients suffers from (exacerbated) symptomology originating from autoantibodies and that it is not the seroprevalence but unique autoantibodies to long COVID that elicit symptoms. In that sense,

use of more general long COVID cohorts in studies could skew results as non-significant, although a subset of patients might profit from drugs targeting autoimmunity. Discrepancies between literature entails the need for more research, as signs of autoimmunity are increasingly more reported related to long COVID.

Endothelial inflammation and microclots

Endothelial cells (ECs) can express ACE2, and studies have found SARS-CoV-2 viral particles in these cells[50], suggesting a mechanism of infection able to incur direct damage or by redirecting immune response towards the endothelium. Other studies found more indirect mechanisms of SARS-CoV-2 to affect the endothelium. For instance, elevated levels of complement factor C5bC6 and decrease of soluble complement factor C7 in long COVID patients[40], where this imbalance could be suggestive of increased presence of the terminal complement complex able to insert into cell membranes of ECs. Pathological responses of the complement system have been associated with endothelial activation and thromboinflammation[40].

The vascular abnormalities in long COVID are not exclusively retained to endothelial disruption, more research depicts irregularities in the coagulation system of patients. During active COVID-19, intensive care patients were discovered with fibrin clots denser, stiffer and more resistant to fibrinolysis than those of healthy donors[51]. Investigation into the mechanics revealed a central role for SARS-CoV-2 S protein, which seemed to be able to bind fibrinogen and fibrin, forming microclots[52]. This was corroborated by data, where addition of S protein added to healthy donor platelet poor plasma induced microclot formation more resistant to fibrinolysis[53]. These signs of microclot formation of fibrin and the changed kinetics of fibrinolysis were also confirmed in patients with long COVID[54]. In addition, research showed that these microclots are not exclusively polymerized structures of S protein and fibrin but are capable of entrapping other molecules[55]. They found, comparing deposits from long COVID versus healthy controls, enriched entrapment of molecules modulating coagulation as von Willebrand factor and platelet factor 4, pro-inflammatory molecules as galactin-3 binding protein, molecules involved in lipid metabolism and enrichment of certain immunoglobulins that could be related to autoimmunity. A recent study showed that increase in microclots in platelet poor plasma was associated with long COVID and recent COVID-19 infection[56]. Furthermore, the mean microclot count seemed significantly higher in females, who are at higher risk of developing long COVID after mild-moderate COVID-19, while there was no significant difference in mean microclot count between the male control and long COVID group.

There is plausibility for a role of microclots in developing long COVID. However, there is discussion in the scientific community about the validity of some of the data and importance

of microclots in the long COVID pathophysiology[57,58]. Furthermore, some research seems to lack important control groups, e.g. the paper describing protein entrapment did not compare long COVID patients to recovered, but to healthy controls, which could lead to an exaggeration of proteins entrapped which have clinical relevance[55]. With the knowledge that microclots are not exclusive to long COVID, as for instance in males there was no significant increase in microclots comparing long COVID to healthy controls[56], the use of proper control groups and experimental read-outs seem increasingly important. Perhaps this latter notion suggests that not only the amount or presence of microclots plays a role in long COVID, but more importantly the content trapped withing these.

Altogether, it seems that long COVID patients experience irregularities in complement activation, endothelial damage and microclot formation. These abnormalities could have widespread effects on the body. For example, vascular obstruction due to microclots could result in hypoxia and damage across organ systems. Entrapment of inflammatory molecules in microclots could induce local and systemic inflammation. Furthermore, microclot components and endothelial activation permeabilize the BBB, possibly granting accessibility to the CNS for toxins, autoantibodies and immune cells. For better understanding of the role of these microclots in long COVID, there should be more research into the circulating levels and persistence of microclots, and how these result in downstream manifestations.

Mitochondrial dysfunction

The high prevalence of exercise intolerance and fatigue in long COVID patients is suggestive of pathophysiology in muscle tissue. A study observed long COVID patients, in whom PEM was induced through exercise, to have a lower oxidative phosphorylation capacity in skeletal muscle[29]. They also found lower concentration of tricarboxylic acid (TCA) cycle metabolites in long COVID patients at baseline, although these did not change after PEM induction. In contrast, venous blood glycolytic metabolites were elevated at baseline and during PEM but decreased a week after PEM induction. Another long COVID cohort, with patients who had mild-moderate-severe COVID-19, showed an increase in blood lactate levels and a decreased rate of β-oxidation of fatty acids during exercise compared to control cohorts[59]. These changes in metabolism suggested SARS-CoV-2 infection to inflict abnormal functioning of the mitochondria that could persist beyond active infection.

During active COVID-19, SARS-CoV-2 dsRNA was found to localize in mitochondria, indicating the possibility of direct replication sites in these mitochondria[60]. Furthermore, SARS-CoV-2 seemed to disrupt mitochondrial membrane potential, resulting in an opened conformation of the mitochondrial permeability transition pore, which was observed to be beneficial for SARS-CoV-2 replication. Another study investigated the concentration of five

neuronal mitochondrial proteins, involved in Ca++ homeostasis, in patients with long COVID who had mild-moderate-severe COVID-19[61]. These mitochondrial proteins were at significantly lower levels in plasma neuron-derived extracellular vesicles in long COVID versus healthy control, suggesting possible mitochondrial dysfunction in neuronal tissue. Furthermore, morphological examination of the mitochondria in long COVID patients revealed dilated and washed-out cristae and enlarged mitochondria, demonstrating mitochondrial deformations induced by SARS-CoV-2[62].

Considering the evidence of mitochondrial dysfunction in long COVID, a recent phase 2a pilot study enrolled patients with fatigue-dominant long COVID for possible treatment testing. They received AXA1125, an endogenous metabolic modulator that increases mitochondrial β-oxidation[63]. No observed improvement was found in post-exercise skeletal muscle recovery rate time when comparing treatment versus placebo, although there was significant improvement in the phosphocreatine response within the treatment group's responders versus non-responders. Additionally, relief of physical and mental physique, assessed by the Chalder Fatigue Questionnaire, was reported by a subset of patients in the treatment group. This could indicate that targeting mitochondrial improvement could help a subset of patients. However, as this trial illustrated, proper assessment of physical improvement in long COVID patients requires more tools suited for the heterogenous patient population.

More research showed abnormalities in the concentrations of downstream metabolites of tryptophan in long COVID patients. One group discovered indoleamine 2,3-dioxygenase-2 (IDO2) expression, normally low or not expressed, in PBMCs and brain tissue in long COVID patients[64]. In these patients, PBMCs had enhanced concentration of kynurenine metabolites and lower tryptophan levels. Another study found serotonin levels in long COVID patients to be lower compared to healthy controls[65]. They observed abrogated intestinal absorption of tryptophan, serotonin storage impacted by platelet hyperactivation, and enhanced serotonin turnover through increased expression of MAO genes. Serotonin is another downstream product of tryptophan, where synthesis is mediated by other enzymes than IDO2. Both of these studies indicate a shift in the presence of certain metabolites, where long COVID patients have increased kynurenine metabolites and decreased serotonin.

Overall, the research shows that SARS-CoV-2 infection can lead to disturbances in mitochondrial functioning. This results in changed tissue metabolic states and energy production impairment that could be ground for observed symptoms such as fatigue and PEM. Moreover, the changes in downstream tryptophan products, such as lower levels of

neurotransmitter serotonin, could be explanations for more neurological impairments and mood changes such as depression and anxiety.

SARS-CoV-2 spike protein connects the pathophysiological processes in long COVID

All the pathophysiological processes discussed could have a role in the development of long COVID. Although these processes are often investigated and mentioned separately in literature, there remains the possibility of interactions between the affected systems or a commonality leading to multisystemic dysregulation. While this shared origin remains to be elucidated in long COVID, I suspect a bridging role in the persistent presence of SARS-CoV-2 S protein.

As discussed previously, the presence of S protein was detected in plasma, stool samples and in the CNS long after the initial SARS-CoV-2 infection. A study that found persistence of circulating S protein in patient plasma, also found S protein in the membrane of circulating extracellular vesicles, although a limitation is the inclusion of hospitalized patients and patients with co-morbidities in their long COVID cohort[66]. Others found circulating antibodies targeting S protein to decrease less than antibodies targeting nucleocapsid protein, adjusted for time since vaccination[45]. A recent study investigated the presence of S protein in skull and brain tissues[67]. They discovered presence of S protein in 10/34 autopsy skull samples from patients who had died due to non-COVID-19 related causes, suggesting the S protein to persist in CNS niches long after initial infection, also in non-long COVID patients. Continuing these findings, they demonstrated that the S protein, with the N501Y mutation, had a multi-organ dissemination and could even cross the BBB in mice models, whereas original Wuhan strain S protein retained mostly in the mice liver. This mutation was first found in the SARS-CoV-2 alpha strain, where it was observed to significantly increase binding affinity to ACE2[68]. Due to this, the mutation has been described as a major factor for increased SARS-CoV-2 transmission through ACE2 and is present in most prominent strains as alpha, beta, gamma and omicron[68,69]. Although they found N501Y S protein crossing over the BBB, the S protein seemed to colocalize with pericytes and not neurons. An *in vitro* study showed that presence of S protein resulted in pericyte activation in human pancreatic islets, where the loss of ACE2 activity, through S protein binding and internalization, resulted in vasoconstriction of the cappilaries[70]. These results could indicate that the neurovascularity might be targeted and affected more by S protein than neuronal tissue itself. These studies all indicate a possible role for S protein presence in the persistent pathophysiology of long COVID.

The origins of persistent S protein remain unclear. Suggestions that have been made consist of a lingering SARS-CoV-2 infection or to S protein to remain present in immune-secluded tissues evading proper clearance. It should also be noted that most of the vaccines, including the newer mRNA vaccines, include or produce the full-sequence S protein, which could add to the effect of persistent S protein. In this line of thought, it seems to occur, albeit less prevalent than long COVID, that people can suffer from a similar syndrome as long COVID after vaccination[71,72]. This might serve as a sign of S protein involvement into pathophysiology of long COVID. However, the role of vaccines on long COVID development and progression requires more research. It is known that vaccination has a protective effect of developing long COVID, irrespective of the SARS-CoV-2 variant[73]. As severe COVID-19 patients have higher risk of developing long COVID, preventing this with vaccination could directly result in this protective effect. However, a mouse vaccination model administering BioNTech/Pfizer (Comirnaty), an mRNA vaccine based on S protein sequence, tested S protein dissemination after SARS-CoV-2 omicron variant infection[67]. They observed less accumulation of S protein, also into CNS areas, in vaccinated versus unvaccinated mice, possibly suggesting that vaccination prevents the wide spread of S protein throughout the body. Interestingly, a systematic review found lowering of odds ratio of having long COVID when getting vaccination after being diagnosed with long COVID, suggestive of vaccination as a possible treatment strategy for long COVID[74]. However, there is more research needed to depict the exact risks of vaccination and/or SARS-CoV-2 reinfection after long COVID diagnosis.

There are also signs of original antigenic sin during SARS-CoV-2 infection[75]. This phenomenon is described as an inadequate immune response towards a pathogen, due to the recalling of a memory response that cannot sufficiently clear the infection. For instance, an early-pandemic SARS-CoV-2 strain generates a specific immune response against S protein. This memory response is triggered by re-infection; however, clearance is insufficient as S protein has mutated beyond adequate binding capacity of the antibodies. A study showed that vaccination resulted in an impaired capability of the immune system to generate de novo response against SARS-CoV-2 omicron variant[76]. Others found an association between having humoral immunity generated against certain seasonal alphacoronaviruses and worse clinical outcome of COVID-19, suggestive of original antigenic sin[77]. Original antigenic sin could interfere in proper viral clearance. However, it is not unthinkable for this phenomenon to also contribute to improper response against S protein and subsequent clearance of viral components as S protein.

The mechanisms surrounding S protein persistence and how this is influenced by vaccination or original antigenic sin is not completely clear. There are signs of S protein involvement in the dysregulated systems during active SARS-CoV-2 infection and in long

COVID patients. This chapter will expand on how the S protein might persist. Additionally, S protein will be linked to the previously described pathophysiological processes, including possible feedback between systems and how this links to the symptoms observed in long COVID patients.

Spike protein induces microclot formation and subsequent effects on endothelial and neuronal tissues

One of the potential mechanisms for persistence of S protein is in an entrapped/coagulated state with microclots. As described before, these microclots are deposits of fibrin which seemed to have formed under the influence of S protein. These microclots have been found to be increasingly resistant to fibrinolysis and, therefore, could lead to the inability of physiological clearance of S protein in the body. Furthermore, the possibility of microclot entrapment of proteins, including S protein, autoantibodies and inflammatory molecules, could form a link to the observed persistence of symptoms and multiple affected organ systems in long COVID. For instance, it has been demonstrated that S protein can induce endothelial inflammation via ACE2 binding, independent of continued viral replication[78]. Earlier 2- and 3D *in vitro* models also showed S protein addition to result in loss of integrity of the endothelial layer, resembling a leaky BBB[79]. Therefore, S protein itself (or in complex) seems capable of activating the endothelium, including subsequent BBB permeabilization.

Research focused on combinatorial effects of S protein and fibrin, identified that S protein in clot with fibrin increased fibrin-induced release of ROS in bone-marrow-derived macrophages, where the S protein alone did not amount to a similar effect[52]. Additionally, they found S protein with fibrin to increase fibrin-induced microglial reactivity in a mouse model of fibrinogen-induced encephalomyelitis, suggesting these microclots to affect neuronal tissue. A mouse model, infected with HIV virions pseudotyped with an S protein incapable of binding to mouse ACE2, still observed extensive fibrin deposition (microclot formation) in the lungs accompanied by oxidative stress, where antibody targeting of the fibrin partially resolved inflammation and oxidative stress[52]. This suggests microclot formation to be possible solely with S protein, however, that ACE2 binding could be important for downstream endothelial and neuronal manifestations. More research has found links between long COVID (and S protein presence), BBB permeabilization and resulting neuroinflammation, suggestive for the symptoms of brain fog and other cognitive impairments[67,80,81]. Interestingly, pre-COVID-era research already observed fibrinogen injection into the CNS white matter in mice to result in demyelination, microglial activation with possible promotion of T-cell mediated autoimmunity[82], suggesting that more components of the microclots can affect neuronal tissue.

Although the microclots are small in nature (1-200 µm), an opinion article proposed these could potentially clog microcapillaries[83]. They suggested that clogging-induced tissue hypoxia, which would be sensed by the body, resulted in tachycardia. Consequently, this tachycardia would explain POTS and the fatigue observed in long COVID patients. By deeming long COVID as a syndrome with dysfunctional coagulation, clinicians have tried multiple ways to treat patients for this clinical phenotype. For instance, by using heparin-induced extracorporeal LDL/fibrinogen precipitation apheresis, which can bind and remove both S protein and fibrinogen from the circulation[84]. The authors state this technique has helped hundreds of patients, although patient data is not publicly available. A cohort of 91 patients with long COVID were treated with 'triple' therapy, including an anticoagulant regime of DAPT-Clopidogrel and a DOAC-Apixaban combined with a proton pump inhibitor pantoprazole[85]. Based on self-reporting of patients, the majority had symptom improvement. The patients were also presented with microclots in platelet poor plasma, and treatment seemed to remove these deposits from their plasma. It should be noted that this study lacked a control placebo group for comparison, which makes the real observed effect of this treatment less interpretable.

To conclude, it seems that S protein in microclot formation with fibrin is capable of inducing endothelial inflammation and subsequent leakiness. Moreover, this loss of endothelial integrity has resulted in BBB permeabilization, making the CNS accessible to targeting. Although microclots have not been confirmed to enter the neuronal compartment by biopsy/autopsy, the individual components of S protein and fibrinogen driving the formation have both been found in the CNS. Therefore, it is not unthinkable that formation can still occur past the BBB, where these components can inflict damage on the tissue. Moreover, these microclots potentially hold more molecules able to induce inflammatory responses. More intervention studies, in properly controlled clinical trials, on apheresis and anticoagulant therapy could help long COVID patients.

Autoantibody formation through molecular mimicry of spike protein capable of targeting the central nervous system

As discussed in the section **autoimmunity**, there has been inconsistency in the reports on whether autoantibodies are relevant for long COVID development. However, the two independent studies of IgG transfer into mice[47,48], which resulted in mimicking of symptoms of long COVID patients by the mice, are suggestive of autoantibody capability of (in)directly inducing pathophysiology. The studies that did not observe involvement of autoantibodies in long COVID were predominantly focused on total autoantibodies or looked at trends in presence of a specific autoantibody in long COVID versus recovered controls, which did not yield results in favor of autoimmunity[39,86]. However, when taking in the half-life of autoantibody producing plasma cells[87], it might not be an unexpected discovery that there

is little change in the autoimmune signature between long COVID and recovered individuals, when there is no ongoing infection to trigger new autoimmunity. Instead, I hypothesize that not solely the autoantibody signature of long COVID patients, but, more importantly, the accessibility of their target autoantigens is required to induce the symptoms observed in long COVID. To clarify, the distinguishment between recovered and long COVID individuals might not result from a change in autoantibody production, but by possible restoration of the endothelial lining of the BBB in recovered patients, removing CNS tissues from autoantibody targeting.

A possible mechanism for inducing autoimmunity is by molecular mimicry between host and pathogen proteins. Studies found homology between SARS-CoV-2 S protein and proteins of the human CNS[88] and other tissues[89,90]. Following this, multiple studies have identified autoantibody production against targets in the CNS during COVID-19 and long COVID[43,47,48]. These studies suggest a possible mechanism of induced autoimmunity by molecular mimicry to S protein, capable of targeting host CNS proteins. This autoimmune signature might be exacerbated by S protein in microclot formation, resulting in continued immune stimulation of self- and non-self-antigens in close proximity.

As mentioned in the previous section (**Spike protein induces microclot formation and subsequent effects on endothelial and neuronal tissues**), it seems that there is evidence of loss of integrity of the BBB and EC activation in long COVID patients. This could pose as an initial step in CNS exposure to autoantibodies produced due to SARS-CoV-2 infection. Similarly, the mice model studies with IgG transfer, found the human IgGs to cross the BBB and targets several CNS compartments[47,48]. Another study observed cognitive impairment, as measured by Montreal Cognitive Assessment, to be worse in long COVID patients with presence of ANAs in their cerebrospinal fluid compared to patients with either presence or absence of ANAs in their serum[91]. It should be noted that this study did not have a healthy control group due to invasiveness of lumbar punction to retrieve cerebrospinal fluid. Moreover, a recent study found, in a long COVID cohort (including 2/12 hospitalized), significant increase of neuroinflammation in long COVID patients across a variety of brain regions[92]. Interestingly, they found significant positive correlations between neuroinflammation, and the concentration of different plasma analytes involved in vascular hemostasis, where the strongest correlation was with analyte fibrinogen. These findings strengthen that dysfunctionality in the vascularity, perhaps by S protein interference, can aid in autoreactivity towards the CNS.

An important question that remains is how these autoantibodies can elicit the effector mechanisms resulting in observed long COVID symptoms. A direct mechanism would involve the antibody targeting a receptor and activating it or by blocking its function due to

interrupted ligand-receptor binding. However, indirect effector functions through Fc-tail mediated mechanisms are perhaps more evident in long COVID. During severe, active COVID-19, there was evidence of immune complexes of IgG, IgM and complement components on ECs, platelets, glial cells and neurons[93]. Whether this can happen in long COVID remains unclear, but a recent investigation reported on persistent complement dysregulation to result in neurovascular injury in long COVID[40]. Another study found autoantibodies from long COVID patients to target atypical chemokine receptor 1 on ECs, where addition of patient peripheral blood mononuclear cells (PBMCs) and purified patient IgG resulted in significantly increased antibody-dependent cellular cytotoxicity towards ECs[94]. One limitation in the two recently mentioned studies is that these effects were not observed across the whole cohort and the cohorts included hospitalized patients, where autoimmunity might have a more significant role in severe COVID-19 survivors. Another study, only including long COVID patients that experienced mild COVID-19, observed increase of adherence of patient PBMCs to brain ECs[80]. They also found both patient serum and recombinant S protein to induce upregulation of ICAM1, VCAM1 and TNF mRNA in ECs, which are important for leukocyte adherence and extravasation.

To conclude, I propose that there could be a role for autoantibodies in long COVID development through (in)direct mechanisms as complement docking with membrane insertion and cellular cytotoxicity. This autoimmunity could potentially be induced through S protein molecular mimicry. The most notable autoantigens are localized in vicinity of the neurovascularity, including both CNS and endothelial targets. The availability of targets on the CNS should be preceded by loss of BBB integrity by S protein induced mechanisms. This combination of endotheliopathy and neuroinflammation could support many of the symptoms observed in long COVID patients as general neuronal and cognitive impairment (brain fog), depression/anxiety, fatigue and tissue damage resulting from local inflammatory response.

Spike protein results in structural changes in mitochondrial functioning with subsequent affected cell metabolism in musculoskeletal tissue

The previous sections primarily focused on the longer persistence of extracellular S protein (in complex) and how this can induce a variety of responses, mainly affecting CNS and endothelium. However, after initial binding to ACE2, SARS-CoV-2 (and S protein) enter the intracellular compartment. As previously described in the section **mitochondrial dysfunction**, there was evidence of SARS-CoV-2 infection to induce structural changes in morphology and important mitochondrial processes such as mitophagy, membrane potential and an altered metabolic state. Theoretically, many different cell types expressing ACE2 might bind and internalize S protein, possibly resulting in changes in mitochondrial functions across multiple tissues. However, this would require S protein to have effector

mechanisms after lysosomal degradation or evade this process completely. One study into cardiomyocytes *in vitro*, which have relatively high ACE2 expression[95], showed that S1 subunit treatment resulted in mitochondrial dysfunction, disrupted mitochondrial membrane potential, and lowered expression of translocase of the outer membrane 20[96]. The subsequently observed increase in ROS production could have the potential to evoke cellular damage and apoptosis. This seemingly resembles the mitochondrial dysfunction occurring during active SARS-CoV-2 infection and long COVID. Stable S protein expression in a neuroblastoma cell line yielded similar results in impaired mitochondrial systems, where they found S protein to interact and enhance activity of MAO-B, a protein that can enhance mitochondrial dysfunction through ROS production[97]. As previously mentioned, SARS-CoV-2 infection also increased MAO mRNA expression, which is important in serotonin production[65].

Although long COVID patients could suffer from mitochondrial dysfunction and metabolic changes, it remains unknown whether these changes persist solely due to structural changes after initial SARS-CoV-2 infection or, if persistent S protein can result in this continued disbalance. As ACE2 is widely expressed in human tissues[95], it might be of equal importance to find out in which organs these mitochondrial dysfunctions persist. Mitochondria are not only important in cell energy production but also occupy a role in inducing cellular inflammation and apoptosis[98], where dysfunction might have damaging effects on the specific cell and its surroundings. Myalgia, PEM and fatigue are often reported in long COVID, which could suggest general metabolic disbalance in skeletal muscle related to mitochondrial dysfunction. The study, where PEM was induced long COVID patients[29], showed lowered mitochondrial function, metabolic dysfunction and even tissue necrosis in muscle biopsies after exercise. Interestingly, they found microclots (potentially harboring S protein) to be more abundantly present in muscle biopsies in long COVID patients compared to healthy control. They identified these deposits to be localized in the extracellular matrix in muscles and not in the capillaries, suggesting that PEM could not be explained by tissue hypoxia due to capillary blood flow obstruction. S protein presence in the microclots might have induced structural changes in muscle cell mitochondria resulting in dysfunction, ROS production, inflammation and subsequent tissue damage. Additionally, they found SARS-CoV-2 nucleocapsid protein in the extracellular matrix in both healthy controls and long COVID patients with unchanged concentration after PEM induction. This is suggestive of a low possibility of local active SARS-CoV-2 replication and no influence of nucleocapsid protein presence on PEM. Muscle biopsies showed, in long COVID patients with fatigue and myalgia, mild histopathological changes ranging from fiber atrophy/damage, mitochondrial pathology, inflammation and cell damage[99]. In another

cohort it was observed that, not only muscle fiber, but also the terminal nerves and the motor endplate in the muscle tissue to be damaged in long COVID patients [100]. Together, this data suggests general myopathy to have a role in long COVID observed symptoms such as fatigue, myalgia and PEM. Where research also implies a prominent role for mitochondrial/ metabolic dysfunction in long COVID patient musculoskeletal tissues.

There is evidence for mitochondrial dysfunction and consequent altered cell metabolism in long COVID patients. However, the exact mechanisms leading to this persistent phenotype are uncertain. Some research provides a context for the S protein capable of inducing these dysfunctions, but more studies should validate these results. Although muscle tissue is emphasized in this section, this dysfunction also occurs in the other SARS-CoV-2/ S protein host cells/tissues such as ECs and CNS tissue. Here, mitochondrial dysfunction was linked to symptoms such as PEM, fatigue and myalgia. Most likely, there are more factors that play a role. For example, interrupted/changed neurotransmission of nerve endings into muscle tissue, due to ongoing neuroinflammation and lower serotonin levels, could also affect muscle tissue. The disbalance in metabolite production due to mitochondrial dysfunction could have many affectations, including the many observed psychological manifestations.

Conclusion

S protein presence seems to persist throughout a substantial number of long COVID patients. Whether this persistence is due to continued infection, microclot formation, vaccination or general incapability of S protein clearance by the immune system should be investigated. The pathophysiological processes discussed can all be linked, at least in initial state, to the presence of S protein. Although this review focused on the commonality of S protein in the pathophysiology, the many resulting imbalances in signaling cascades, with varying effector mechanisms in different tissues, can also result in exacerbation of symptoms. There is also the possibility of interplay between these pathophysiological processes, therefore, a schematic representation is given to elucidate on how S protein persistence might lead to the most prevalent observed symptoms in long COVID patients through the multiple pathophysiological processes (**Figure 1**).

In future research, it might be worthwhile for studies to assess persistence of S protein in the patient cohorts, although this could prove difficult and inconsistent. To elaborate, S protein presence could for instance be determined by presence in the plasma of patients. However, it has been observed that not all long COVID patients have S protein in plasma samples[36]. Furthermore, S protein might be opsonized in microclots, which should be accounted for in the used detection methods for assessing presence. For instance, one study observed immunoadsorption of IgG to almost diminish circulating S protein in long

COVID patients, suggesting most S protein to be in opsonized form with antibodies and possibly in microclot formation[101]. Notably, this also demonstrated the incapability of physiological clearing of S protein by patients. In addition, they did not observe improvement of their patients after diminished circulating S protein. This could indicate that it is not solely the presence of S protein, but also the tissue where S protein persists to be important for factor to result in long COVID symptomology. A recent study demonstrated this in a mice model, where S protein injection into the brain resulting in long-term cognitive dysfunction, suggestive of long COVID[102]. As discussed extensively, many of the symptoms observed could result from damaged neuronal and neurovascular tissue, where S protein also has been observed to persist in many patients. Therefore, it could be that S protein persistence in CNS areas is a prominent indicator for long COVID development and continuation. This could pose difficulties in future research, diagnosis and treatment strategies. Investigation into these tissues by biopsies in living patients or healthy controls is ethically complicated and can pose risk to the subjects involved. Moreover, treatments to remove S protein presence in the CNS can come with challenges such as accessibility to these regions and to not invoke more damage than the syndrome itself. More preliminary research should elucidate on the role of S protein persistence in these regions in long COVID. After S protein is more evidently linked to long COVID pathogenesis, there should be a focus on safe and effective diagnostic and treatment strategies for these patients.

Limitations

This review posed as an overarching hypothesis for the development of long COVID. However, there are several limitations. The broad definition of long COVID makes comparison difficult between literature. Symptoms could be more prominent in hospitalized patients during COVID-19, where inclusion into cohorts might skew results towards these patients. Moreover, the wide variety in symptoms observed might require the need for further stratification of long COVID patients into more than hospitalized versus non-hospitalized, e.g. based on the long COVID clinical presentation. Lastly, this review did not include all affected tissues and possible links to long COVID development. For instance, there has also been research focused on the gut[10], also expressing ACE2[95], where gut-brain axis distortions might also partially explain long COVID symptoms.

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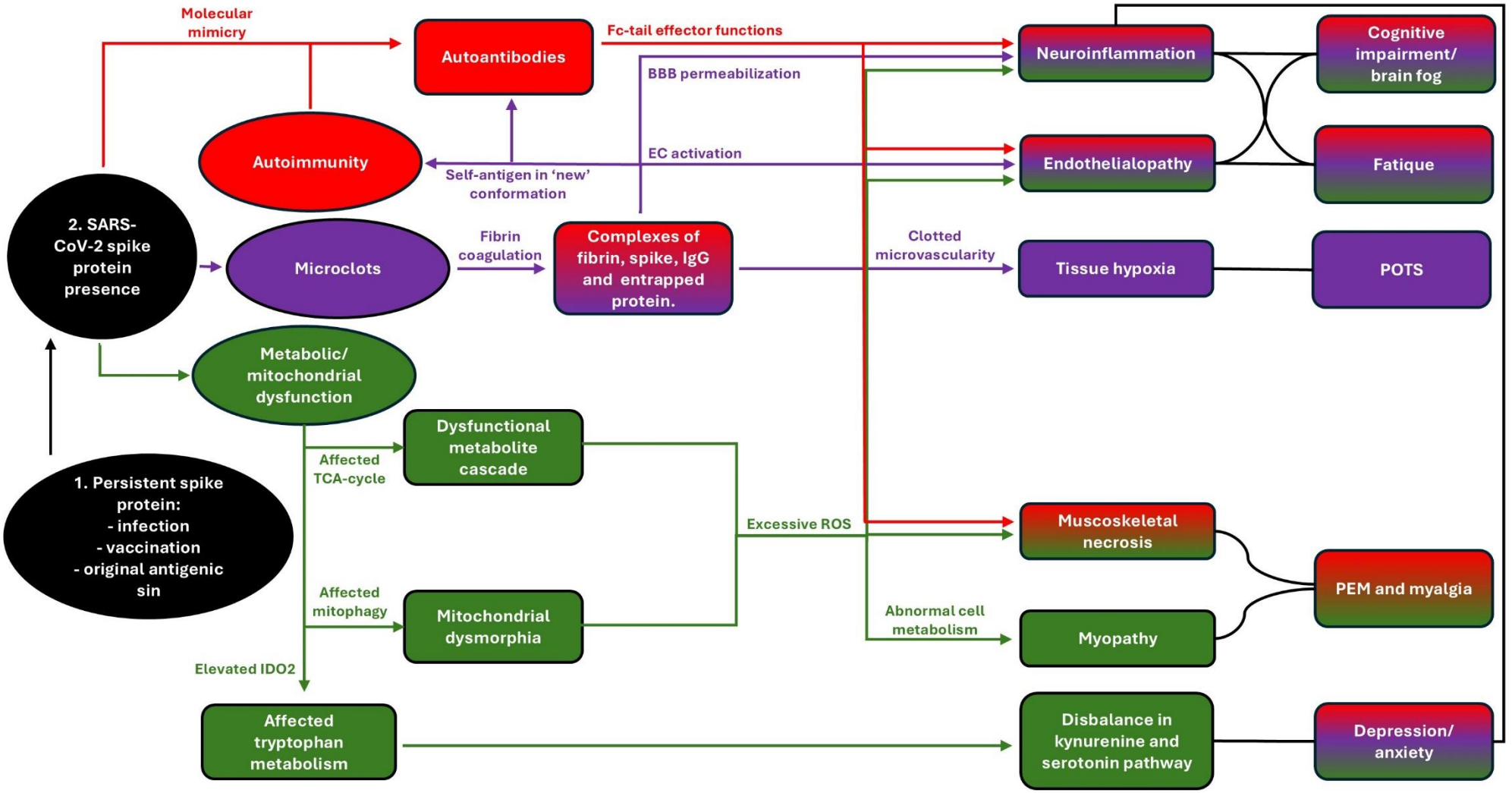

Figure 1: the chronology of the driving processes resulting in long COVID

Schematic representation of the hypothesis of S protein to induce various systemic dysfunctions leading to the broadly observed symptomology of long COVID. Colored paths and blocks resemble the color of the initial physiological dysfunction, where causality of symptoms shared between multiple physiological dysfunctions can be recognized by a multi-color gradient in the blocks.

BBB: Blood-brain-barrier; EC: Endothelial cell; IDO2: Indoleamine 2,3-dioxygenase-2; IgG: Immunoglobulin G; PEM: Post-exertional malaise; POTS: Postural orthostatic tachycardia syndrome; ROS: Reactive oxygen species; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TCA: Tricarboxylic acid

Abbreviation list

ACE2	Angiotensin-converting enzyme 2
ANA	Antinuclear antibody
CMV	Cytomegalovirus
CNS	Central nervous system
COVID-19	Coronavirus disease 2019
EBV	Eppstein-Barr virus
EC	Endothelial cell
GenAI	Generative artificial intelligence
GI	Gastrointestinal
IDO2	Indoleamine 2,3-dioxygenase-2
IFN	Interferon
Ig	Immunoglobulin
ME/CFS	Myalgic encephalomyelitis/chronic fatigue syndrome
PAIS	Post-acute infection syndromes
PASC	Post-acute sequalae of SARS-CoV-2
PBMC	Peripheral blood mononuclear cell
PEM	Post-exertional malaise
POTS	Postural orthostatic tachycardia syndrome
PTLDS	Post-treatment Lyme disease syndrome
QFS	Q fever fatigue syndrome
S protein	Spike protein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TCA	Tricarboxylic acid
TMPRSS2	Transmembrane protease, serine 2
VWF	Von Willebrand factor
VZV	Varicella zoster virus

Generative artificial intelligence disclosure

During this project, generative artificial intelligence (GenAI) was used in the primary stages for literature search. This was performed by using prompts including content-related search terms, e.g. long COVID, microclots, in the Perplexity GenAI.
No written content or illustrations were made using GenAI.

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