Title

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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Short title:

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).

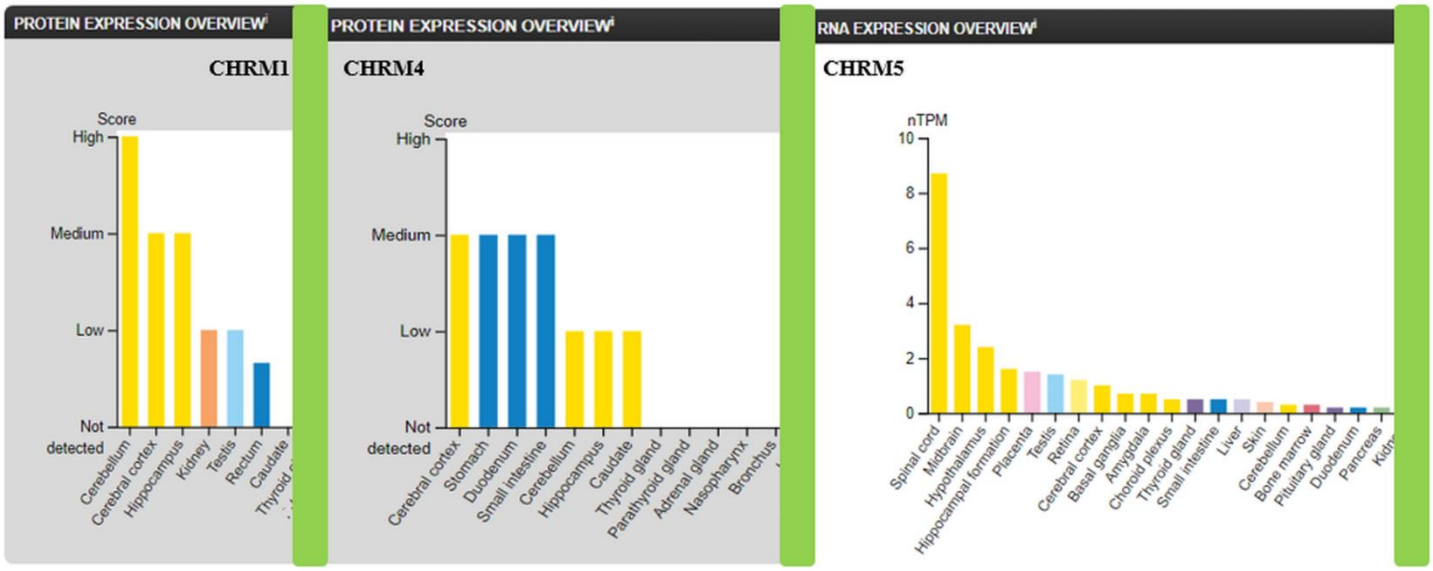


Figure 1. Bar chart showing expression levels of muscarinic receptor subtypes CHRM1, CHRM4 and CHRM5 in human organs and tissues. The AAbs against these receptors have been reported in patients with PASC. The effect of their interaction with AAbs has been reported to produce symptoms. The functional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

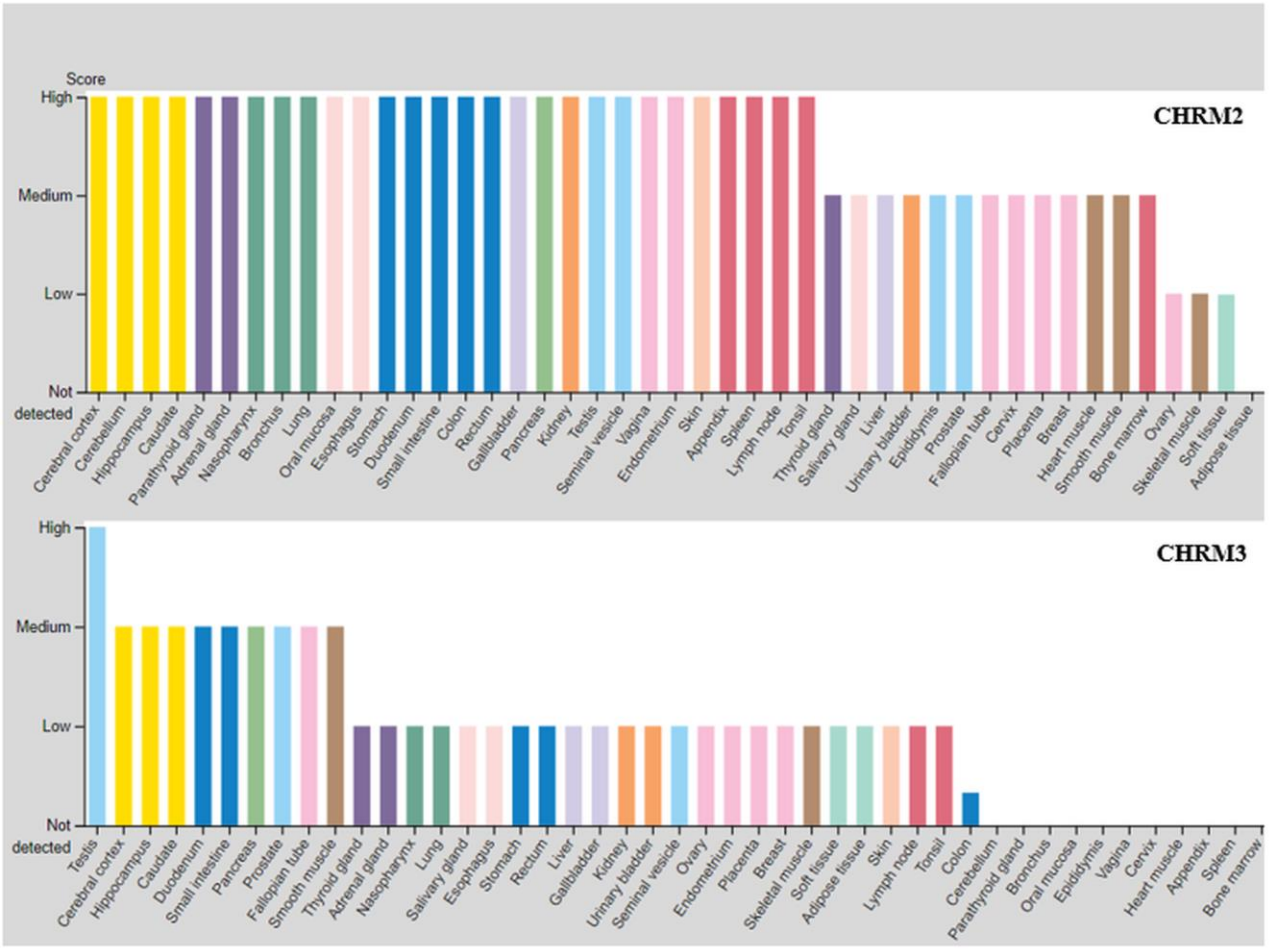


Figure 2. Bar chart showing expression levels of muscarinic receptor subtypes CHRM2 and CHRM3 in human organs and tissues. The AAbs against these receptors have been reported in patients with PASC. The effect of their interaction with AAbs has been reported to produce symptoms. The functional effect 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 affinity 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).

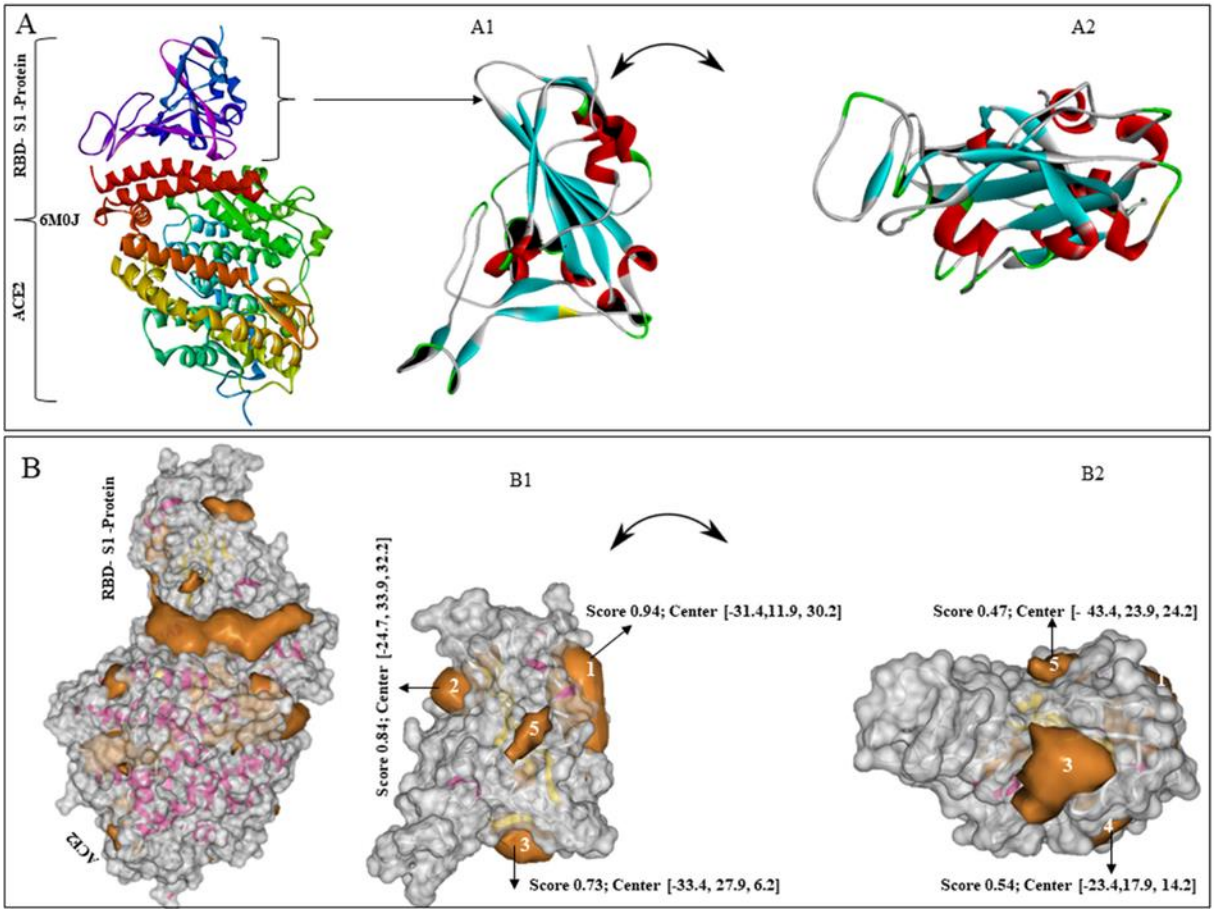


Figure 3. Illustration showing S protein and ACE2 interaction (A). The RBD of the S protein alone (A1) with the RBM (A2) at the base of the RBD. Playmolecule server using DEEPSITE gemerated drug binding pockets on S protein RBD and ACE2 receptor (B). On the RBD alone, 5 pockets were detected (B1) of 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 effectively 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 affect the virus's infectivity and immune evasion capabilities. Changes in key amino acids can alter the binding affinity 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.

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 effects. 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).

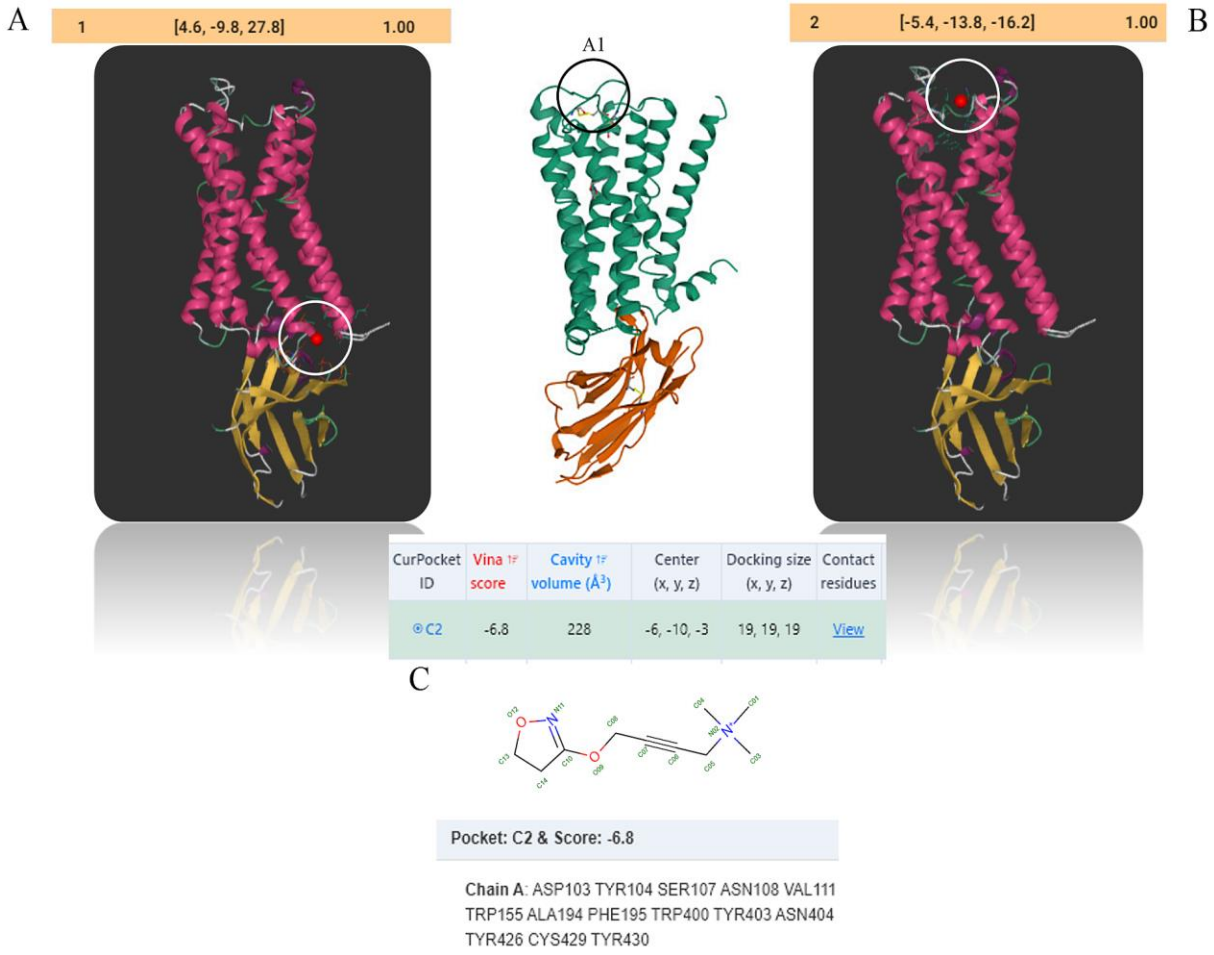


Figure 4. DEEPSITE software at Playmolecule automated server showed 2 pockets of the CHRM2 protein (white circles, A and B). Coordinates are shown at the top (orange bars at top). An agonistic drug Iperoxo as docked onto the CHRM2 (which is the possible interacting site of AAbs against this receptor 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/

B2b.Allosteric Sites: These sites can be exploited to develop drugs that modulate receptor activity without directly competing with acetylcholine at the orthosteric site (15). They offer a strategy for developing drugs with subtype selectivity, given the conservation of the orthosteric site across the CHRM family (15).

Each CHRM subtype has a distinct amino acid sequence, which underlies their different physiological roles and pharmacological properties (16).

In pharmacological research and drug development, understanding the crystal structures, docking pockets (Figure 4, A - B), and sequences of CHRMs is crucial for designing selective ligands, which can act as therapeutic agents in a variety of conditions related to the cholinergic system, such as Alzheimer's disease, schizophrenia, and various cardiovascular disorders (17).

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 selected (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).

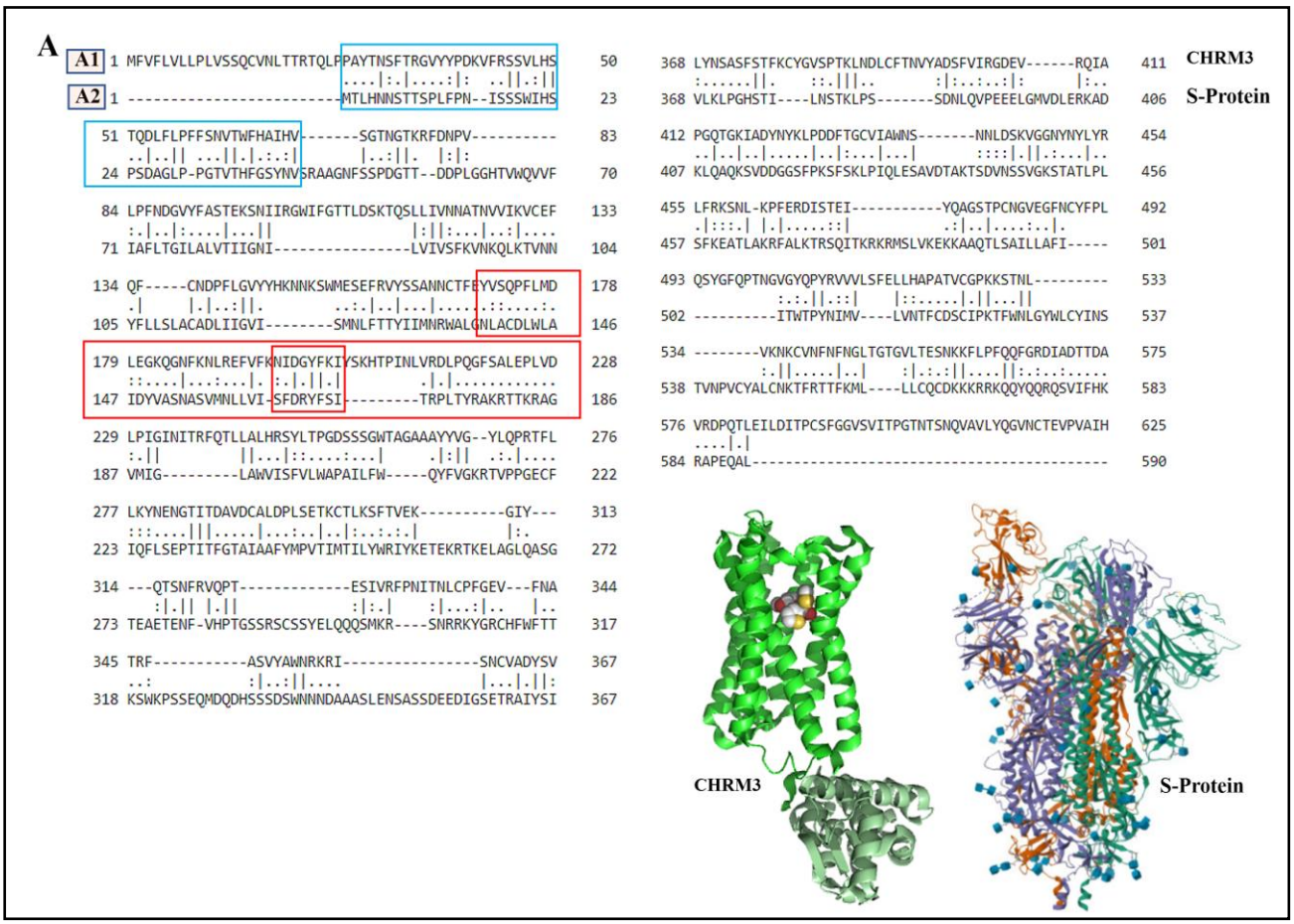


Figure 5. Shown is the sequence alignment between CHRM3 (A1) and S-Protein (A2) with areas of similarities (blue and red rectangles) which were recognized to be antigenic in S-Protein (A2) sequence. Antibodies mounted against these S protein segments after being digested by endopeptidases like neutrophil 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

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.

E. 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.

F. 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.

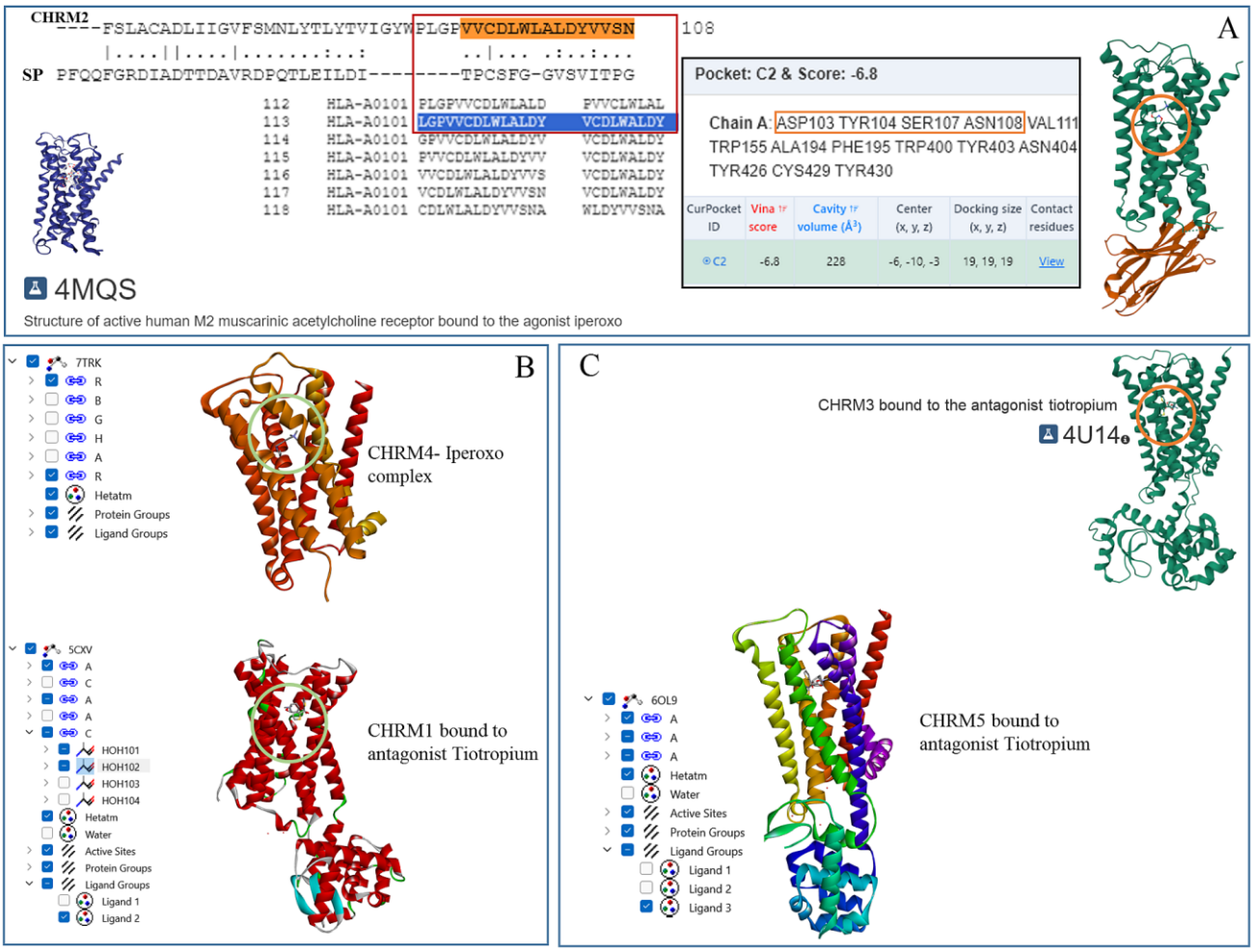
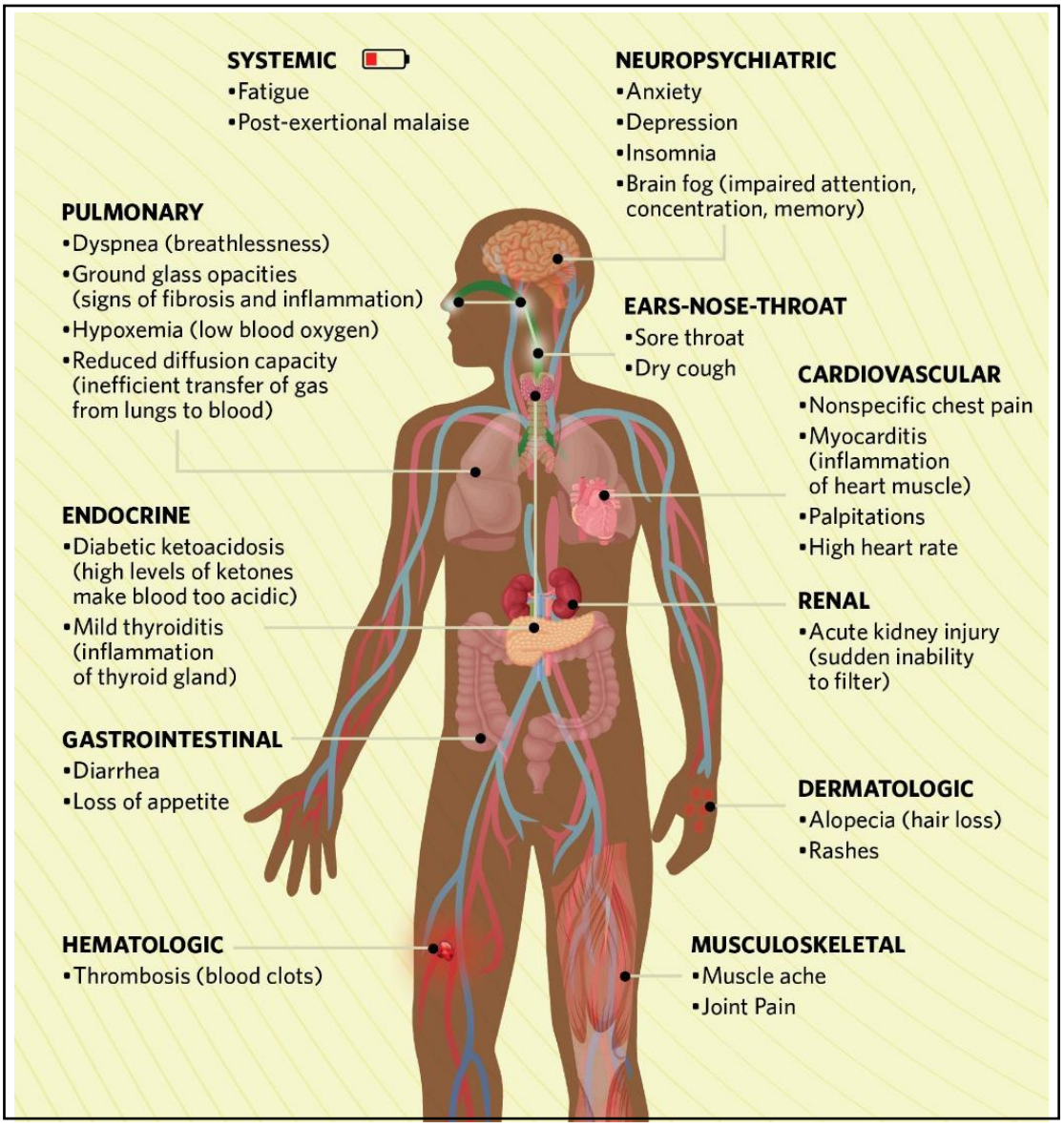


Figure 6. Illustration showing possible origins of and reason behind AAbs targeting CHRM2 (A). Sequences highlighted in (A – red rectangle, top row) have similarities with amino acids in S protein fragment aligned below. An antibody response in the bottom row of the SP sequence within the red rectangle 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 effects 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.



NAT MED, 27:601–15, 2021; NATURE, 594:259–64, 2021; MEDRXIV, DOI:10.1101/2020.12.24.20248802 2021

Figure 7. A concise representation of organ effects 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.

- 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.
- 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 effect 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 to ischemia 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 effects

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 suffering newer symtpoms raise the curiosity of something in effect 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 effective treatments 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
YTMSLGAENSVAYSNNSIAIPTNFTISVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGI
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.