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Autoantibodies in patients with post-COVID syndrome: a possible link with severity?

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Abstract. – OBJECTIVE: Coronavirus disease 2019 is an infectious disease associated with the respiratory system caused by the SARS-CoV-2 virus. Right now, an increasing number of patients with post-COVID Syndrome show, without clear evidence of organ dysfunction, a plethora of severe symptoms, such as fatigue, pain, shortness of breath, cognitive impairment, and sleep disturbance. It has already been demonstrated that SARS-CoV-2 virus can disrupt the self-tolerance mechanism of the immune system, thus triggering autoimmune conditions. Several studies have recently documented the presence of autoantibodies in the sera of post-COVID patients, but until now, it is unclear whether the persistence of symptoms could be directly correlated with the presence of autoantibodies.

PATIENTS AND METHODS: In this study, serum autoantibodies (AAbs) levels against four G protein-coupled receptors in 78 patients with post-COVID syndrome have been analyzed. The AAbs investigated are clustered in two groups: adrenergic receptors (α1 and β2) and muscarinic acetylcholine receptors (M3 and M4).

RESULTS: At least one or more AAbs were detected in 60.3% (47/78) of patients diagnosed with post-COVID syndrome, whereas 37.2% (29/78) of patients were positive for all receptors investigated. Interestingly, a strong correlation has been found between AAbs and pain intensity feeling by the patients measured by Visual Analogic Scale. A significant association was also obtained with insomnia and AAbs-positive patients.

CONCLUSIONS: The identification of AAbs and their correlation with pathological symptoms seriousness underly the possible role of AAbs as future therapeutic targets.

Key Words:
SARS-CoV-2, COVID-19, Post-COVID Syndrome, Long-COVID, Autoantibody, AAbs, Adrenergic receptors, Muscarinic cholinergic receptors, Diagnosis, Pain, Fatigue, Insomnia, Anxiety, Treatment.

Introduction

Respiratory syndrome caused by coronavirus (SARS-CoV)-2, sadly renowned as COVID-19[1], reached a worldwide pandemic level by March 2020[2] and led to 5.8 million deaths with over 518 million cases, according to the World Health Organization (WHO) report available on February 2022[3]. Before anti-COVID vaccination campaign, a huge number of medical devices that shield against the virus and thus protect from the infection have been patented. A lot of these innovative devices based on zinc[4], α-cyclodextrin and hydroxytyrosol[5,6], Iota-Carrageenan, a natural sulfate polysaccharide synthesized by red algae[7], and others led to promising results.

The real worldwide problem nowadays is that after the acute phase of SARS-CoV-2 infection, a plethora of persistent complications have been registered[8]. At the end of the first wave of infection, in an Italian study conducted by Carfi et al[9], only 12.6% of investigated patients stayed free of persistent symptoms, and similar results were obtained in a German study by Puntmann et al[10] in which 22% of patients did not develop

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any persistent pathological traits. Additional studies registered that 10-20% of patient complained severe symptoms persisting after the acute phase caused by viral infection[11,12] even after six months[13]. This phenomenon, namely post-COVID syndrome (PCS) or long-COVID[14], describes the clinical manifestation and symptoms that persist beyond 12 weeks after SARS-CoV-2 recovery and can affect all genders and ages independently of the severity of the acute disease[15]. Patients with PCS may exhibit more than 50 distinct symptoms[16], including fatigue, post-exertional malaise, loss of concentration, cognitive impairment (known as "brain fog"), shortness of breath, pain, sleep disturbance, and other neurological and cardiovascular symptoms[17-20]. The American Academic of Physical Medicine and Rehabilitation (AAPMR&R) estimates that there are more than 24 million cases of PCS as of May 2022[21].

Several authors demonstrated the presence of autoantibodies (AAbs) in PCS patients' sera[22-24]. AAbs against G protein-coupled receptors (GPCRs) are part of normal human physiology, but when the production becomes abnormal, it leads to a persistent immune-activation with serious complications[25]. A possible explanation for the chronic inflammatory response caused by COVID-19 has been proposed by Lyons-Weiler[26], who found a high homology between the immunogenic peptides sequence of SARS-CoV-2 virus and different human proteins.

Even if it appears that PCS has an autoimmune involvement[27,28], its pathogenesis and molecular mechanisms are still under investigation. Recent works[29,30] have highlighted important links between PCS pathophysiological traits and myalgic encephalomyelitis/chronic fatigue syndrome (ME-CFS). In fact, an abnormal accumulation of agonistic receptor AAbs that target adrenergic (such as β2) and muscarinic (in particular M3 and M4) neurotransmitter receptors may play a crucial role in the onset and persistence of symptoms in both above-mentioned conditions[31-33].

Possible therapy for PCS patients is until available, but similar to other auto-immunological diseases the AAbs removal could be efficacious in the reduction of the symptoms. Immunoadsorption (IA) and plasmapheresis have both been proposed to treat patients with PCS; likewise, it is used for ME-CFS[34]. To date, unfortunately, there is not a well-defined scientific validation of these approaches, but Giszas et al[35] tried to shed light on this possible treatment with transient benefits in two patients.

During acute phase, the presence of AAbs has been associated with a more severe course of COVID-19[24], but it is contradictory for PCS persistence and clinical manifestations[36]. The aim of this work was to correlate the presence of AAbs in 78 well-characterized patients with the persistence of clinical manifestations of PCS, and to propose the apheresis as the starting point for a new, promising and effective treatment for PCS patients.

Patients and Methods

Study Design and Participants Recruitment

Adult patients who presented serious outpatient clinic manifestations and severe post-COVID syndrome have been enrolled. All patients were unrelated and were of Caucasian origin. They were recruited consecutively at Azienda Socio Sanitaria Territoriale degli Spedali Civili di Brescia (Department of Cardiology) between April 2021 and October 2022.

Blood samples were collected from each patient while visiting the outpatient clinic for the first visit. Patients were diagnosed at least six months following SARS-CoV-2 recovery.

Ethical Statement and Inclusion Criteria

The study was approved by the Ethics Committee of Brescia (Italy) Prot. No. NP4588. All research process was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

For all patients, a written informed consensus was obtained, and to each of them was assigned a unique alphanumeric code to protect their anonymity.

Exclusion criteria included: significant mental retardation or severe disabilities or in any case subjects unable to provide informed consent; subjects with an oropharyngeal swab still positive for COVID-19 at the time of screening; subjects presenting negative serological from COVID-19; underage patients (under 18 years old); subjects suffering from pathologies that can alter the normal functioning of the nervous system (e.g., serious heart disease, hereditary or acquired neuropathies, eating disorders, psychiatric disorders).

Clinical Manifestations and Questionaries

During recruitment, all patients filled out different questionnaires to assess their general mental status. The intensity of asthenia, headache, muscle pain, dyspnea, and cough during the acute phase and after restoration was measured with a classical scale from 0 (absent) to 10 (severe). Moreover, they

delivered clinical data and declared all therapies underway. The severity of pain, fatigue, sleeplessness, and anxiety was assessed using specific questionaries: VAS (Visual Analogic Scale), CFS (Chalder Fatigue Scale), ISI (Insomnia Severity Index), HAM-A (Hamilton Anxiety Rating Scale).

The VAS is a unidimensional and simple measure of pain intensity, used for the first time in 1921 by Hayes and Patterson, and for 100 years still reliable[37]. This test is a straight horizontal line of fixed length (100 mm). The left end indicated "no exhaustion at all" while the right end indicated "complete exhaustion", and the value was then determined by measuring the length (mm) from the left end of the line. The cut-off for no pain was assessed as less than 10, mild to moderate from 10 to 50 and severe >50.

The final version of CFS test, revised in 2010[38], with an 11-item questionnaire was used to measure the severity of physical fatigue (e.g., weakness, lack of energy, reduced muscle strength) and mental fatigue (e.g., concentration, memory) on two separate subscales. Seven items represent physical fatigue (items 1-7) and 4 represent mental fatigue (items 8-11). Each item is scored 0-3: less than usual (0), no more than usual (1), more than usual (2) and much more than usual (3). The CFS can be used in two different ways: Bi-modal scoring and 4-point Likert scoring. In the bi-modal model, all 11 items were loaded onto a general fatigue factor. Lower scores indicate a low level of fatigue, whereas high scores represent high levels of fatigue[39].

A cut-off score of $\geq$ 18 has been used as previously reported to determine in adolescent subjects who have chronic fatigue[40].

In order to detect cases of insomnia in the population, a reliable and valid method is the ISI questionnaire. Consisting of seven questions, it is a simple test that can be used to identify cases of sleeplessness[41]. A total score of 0-7 indicates "no clinically significant insomnia" 8-14 means "subthreshold insomnia" 15-21 is "clinical insomnia (moderate)" and 22-28 means "clinical insomnia (severe)".

Finally, psychological and somatic symptoms of anxious mood were assessed by HAM-A scale[42]. This questionnaire is a clinician-based questionnaire consisting of 14 items[43]. Each item has a score ranging from 0 (no symptomatology) to 4 (severe anxiety), with a total sum of scores of 56. The cut-off was settled on 17. As a consequence, a total score $\leq$ 17 identifies mild anxiety symptoms, while a score ranging from 18 to 24 indicates moderate anxiety symptoms. A score between 25 and 30 indicates severe anxiety.

Quantification of AAbs by ELISA

We determined AAb-levels (anti-α1-adrenergic, anti-β2-adrenergic and anti-muscarinic cholinergic 3/4 receptors) in the serum of 78 patients who were still suffering from PCS. Levels of IgG targeting α1- and β2- adrenergic receptors (ARs) or targeting M3- and M4- muscarinic acetylcholine receptors (mAchRs) were determined by ELISA (CellTrend GmbH, Luckenwalde, Germany) according to the manufacturer's instructions.

Statistical Analysis

Data were checked for normality, outliers, and missing data. No imputation of missing data was performed.

Chi-square test was used to compare categorical variables. Correlations between variables were identified with Pearson correlation analysis and partial correlation analysis.

Statistical data analyses and figures were done using the software GraphPad Prism 9.2.0.332 (GraphPad software, La Jolla, CA, USA). t-test statistical methods were used.

A two-tailed p-value was considered statistically significant with the following threshold: $*p<0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Results

Sociodemographic characteristics, such as gender, age, Body Max Index (BMI), clinical manifestations, and severity score during and after acute infection, are shown in Table I. The cohort was composed of 78 PCS patients: 47 positives (60.3% of the total) for the presence of at least one AAb in the serum and 31 negatives (39.7%).

Women were 52, whereas men were 26, and they were distributed homogeneously in the two groups: positive (AAbs+) and negative (AAbs–). Thus, it means that there is no correlation between gender and the presence of AAbs (p=0.9998). The median age for all patients was 52.9 [minimum 26 and maximum 81 years], and also in this case, there is not a positive link with the AAbs levels. In fact, the positive and negative groups were similar, with a median age of 53.4 and 52.3, respectively (p=0.9084).

BMI mean value was 26.7 (minimum 17.7 and maximum 40.6), with only one patient presenting a grade III obesity (BMI > 40.0), five patients with a grade II obesity (35.0 < BMI > 39.9) and thirteen with grade I obesity (30.0 < BMI > 34.9). An association between BMI and at least one AAb was analyzed but with without a link (p=0.7984).

Table I. Demographics of post-COVID syndrome patients were reported as a total cohort (n=78) on the first column and divided in relation with autoantibodies presence (AAbs+ *vs*. AAbs-).

	Characteristics	Total cohort (n=78)	Post-COVID with AAbs (n=47) AAbs+	Post-COVID without AAbs (n=31) AAbs-
Particulars	Female/male	52/26 (66.7% / 33.3%)	31/16 (66% / 34%)	21/10 (67.7% / 32.3%)
	Age, in years	52.9 (26-81)	53.4 (32 - 72)	52.3 (26 - 81)
	Weight (Kg)	75.6 (43 - 120)	73.6 (43 - 120)	78.6 (50 - 110)
	Height (cm)	167.4 (150 - 190)	166.7 (150 - 180)	168.5 (153- 190)
	Body Mass Index (BMI)	26.7 (17.7 - 40.6)	26.4 (17.7 - 39.6)	27.4 (19.7 - 39.6)
Severity Score during infection	Out-/In- patient	47/31 (60.3% / 39.7%)	30/17 (63.8% / 36.2%)	17 / 14 (54.8% / 45.2%)
	Asymptomatic	2 (2.6%)	1 (2.1%)	1 (3.2%)
	Mild symptoms	35 (44.9%)	23 (48.9%)	12 (38.7%)
	Severe symptoms	40 (51.3%)	23 (48.9%)	17 (54.8%)
	Intensive care	1 (1.3%)	0	1 (3.2%)
	Asthenia Intensity	7.23	7.27	7.18
	Headache (during infection)	4.42	4.59	4.14
	Muscular pain	6.58	6.66	6.46
	Dyspnea	5.93	5.89	6.00
	Cough	4.74	4.31	5.43
Symptoms after infection	Asthenia Intensity	4.33	4.20	4.54
	Headache	2.03	2.04	2.00
	Muscular pain	3.14	2.89	3.54
	Dyspnea	2.78	2.64	3.00
	Cough	0.59	0.51	0.71
Questionnaries	VAS	32.9 (0-100)	44.8 (0-100)	22.3 (0-70)
	CFS	20.1 (11-31)	20.5 (11-31)	19.5 (11-27)
	ISI	9.9 (0-28)	12.4 (1-27)	7.5 (0-20)
	HAM-A	17.1 (3-47)	19.4 (4-47)	14.8 (3-32)

Thirty-one patients (39.7%) had required hospitalization during acute infection. Only 2 patients were asymptomatic, while 35 had mild symptoms and 40 severe symptoms. A sporadic case had needed an intensive care. The presence of AAbs was not correlated with severity during acute phase.

No differences were registered, as show in Table I, comparing positive or negative subjects in terms of asthenia, headache, muscle pain, dyspnea and cough both during acute phase of infection and after restored.

Hematological data during and after infection, such as glycemia, hemoglobin, white blood cells, red blood cells, platelet, creatinine, TSH and others were in the range of normality for all patients.

Forty-seven PCS patients (61.5%) presented at least one AAbs in the serum detected by ELISA, moreover twenty-nine (37.2 %) were positive for all four different AAbs analyzed simultaneously and four of them had out of range values.

AAbs results taken individually were presented in Figure 1, where the median and cut-off were reported to a better understanding. In detail:

- Anti-α1adrenergic receptor AAbs were detected in 36 patients (46.2%),

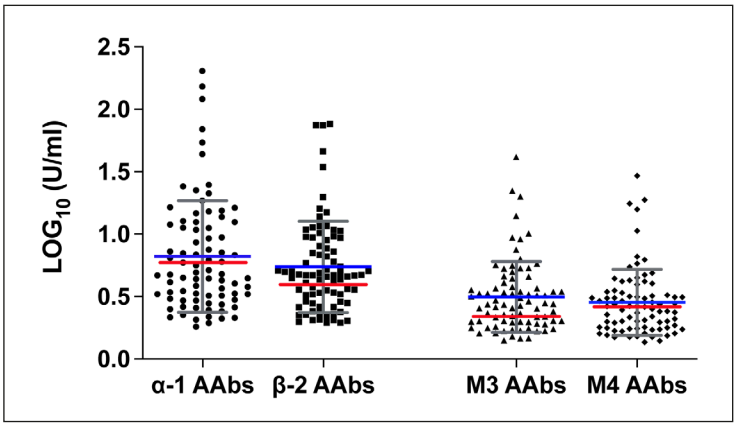

Figure 1. Scatter dot plots documenting autoantibodies (AAbs) levels in patient with post-COVID syndrome (n=78). α1 (*circles*) and β2 (*square*) adrenergic receptor together with M3 (*triangles*) and M4 (*rhombus*) were reported. Grey lines represent error bars. In blue line is reported the median, while in red is indicated the cut-off for each receptor.

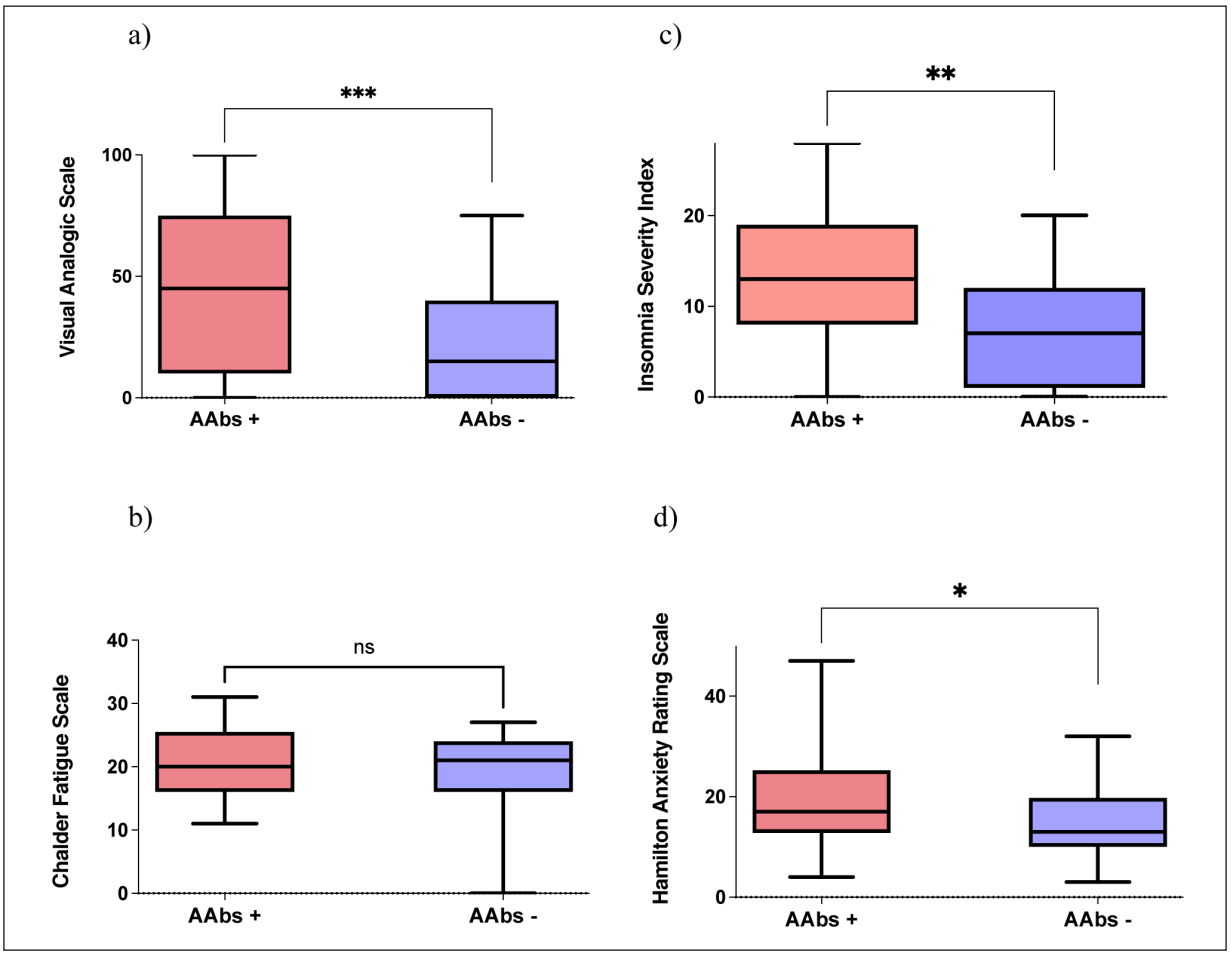

Figure 2. Boxplots showed the correlation between autoantibodies presence (AAbs+) or absence (AAbs-) in the serum of patients diagnosed with post-COVID Syndrome and different clinical manifestations, measuring by Visual Analogic Scale (**a**), Chalder Fatigue Scale (**b**), Insomnia Severity Index (**c**), and Hamilton Anxiety Rating Scale (**d**).

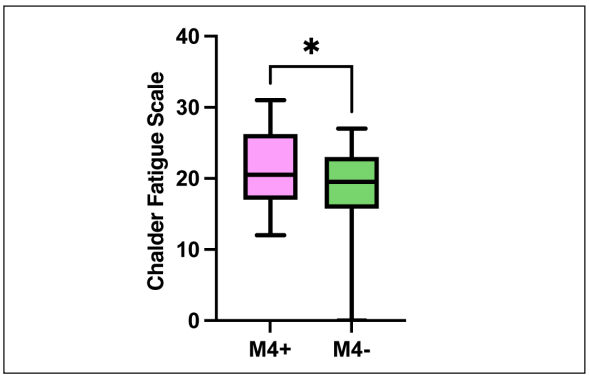

Figure 3. Boxplot showed the correlation between auto-M4 muscarinic cholinergic receptor autoantibodies (M4+) in the serum of and unidimensional score of Fatigue (CFS).

- Anti-β2 adrenergic receptor AAbs were found in 41 patients (52.6%),
- Anti-M3 muscarinic cholinergic receptor AAbs were present in 42 patients (53.8%),
- Anti-M4 muscarinic cholinergic receptor AAbs were discovered in 31 patients (39.7%).

The association between AAbs and symptoms is reported in Figure 2. In details, the presence of AAbs is linked respectively with unidimensional measure of pain intensity (Figure 2a), unidimensional score of persistence fatigue (Figure 2b), unidimensional sum of clinical insomnia (Figure 2c), and rating score of anxiety (Figure 2d).

Our analysis revealed a strong correlation between the levels of AAbs and the persistent pain symptoms registered by VAS test (p=0.0009). The severity and persistence fatigue did not match with the presence of AAbs. A positive correlation was also found between AAbs presence and insomnia (p=0.003) and anxiety (p=0.023).

In addition, a significant match was found between anti-M4 muscarinic cholinergic receptor AAbs and persistence fatigue (Figure 3).

Discussion

In according with previous results[35] our study verified and deeply confirmed the presence of AAbs in PCS patients with more than half of them resulting positive (61.5%). In the healthy population, the presence of AAbs is around 3.7-12.8%[44-45]. This data suggested that autoimmunity is associated not only with the SARS-CoV-2 acute phase, but also with PCS[46].

The possible explanation for this evidence could be the persistence of high levels in PCS patients of naive B cells, which are known to be a source of autoantibodies[47].

Sotzny et al[48] recently hypothesized in a cohort of 80 PCS patients *vs*. 78 healthy individuals that the presence of AAbs could be a reliable biomarker for screening PCS[48].

The AAbs investigated in this study were four crucial GPCRs and they concerned a unique biological pathway: "fight or flight". The AAbs presence acts in an agonistic manner and as a consequence block the natural biological response and are associated with many clinical symptoms in different chronic diseases[45,49].

The alpha 1 (α1)-adrenergic receptor is a classic postsynaptic receptor found on vascular smooth muscle. This kind of receptor acts as a mediator of sympathetic nervous system *via* binding endogenous catecholamines (adrenaline and noradrenaline) and it regulates in particular blood pressure[50].

The beta 2 (β2)-andrenergic receptor is expressed by all the cells of the immune system, including T and B lymphocytes, dendritic cells (DCs) and macrophages. This receptor plays a pivotal role in macrophage activation and proinflammatory cytokine production and is already associated with ME-CFS and cancer[51].

AAbs against β2-andrenergic receptor, M3- and M4-muscarinic acetylcholine receptors were significantly elevated in patients diagnosed with chronic fatigue syndrome compared to healthy controls[31]. M3 and M4 receptors both play an important role in the regulation of nociception at the spinal level in both animals and humans[52].

The presence of four AAbs is not linked with gender, age, BMI (as expected) but it is strongly correlated with pain intensity, clinical insomnia, and anxiety (as reported in Figure 2). Dysregulation of AAbs against GPCRs may result in their alternated expression and/or signal and as a consequence in a loss of homeostatic response. It has been demonstrated that the post-COVID effects could carry on after the infection for long time and are due to persistent endothelial dysfunction[53].

The chronic fatigue is the only symptom linked to M3 AAbs presence, probably due to a systemic vasodilation mechanism[54].

Limitations of the Study

Our study has some limitations. First of all, healthy seronegative controls or asymptomatic post COVID-19 patients are missed. It could be important to improve this study with new data on a control group matched for gender and age. We propose to investigate the presence of the analyzed AAbs in a group of patients enrolled in other studies before March 2020, thus ensuring a free-contamination for COVID-19 both by direct infection and/or vaccine.

Conclusions

Our study, in conclusion, identified dysregulation of AAbs in PCS patients and their correlation with pathological traits, in particular with pain intensity. These results, together with previously association, underlines a possible role of AAbs in a future therapy.

To this end, in an isolate PCS case report, Hohberger et al[55] showed a successful result with BC 007 (Berlin Cures, Berlin, Germany), a DNA aptamer drug with a high affinity to GPCR-AAbs that neutralizes AAbs[55]. The removal of GPCR-AAb ameliorate the characteristics of PCS patient, such as capillary impairment, loss of taste, and chronic fatigue syndrome.

Likewise, Giszas et al[35] demonstrated that apheresis provides some relief in term of both depressive symptoms as well as the severity of fatigue in two PCS patient[35]. However, these results need to be confirmed in further wide cohorts.

In the end, the present work strengthens the idea that GPCR-AAb dysregulation is involved in PCS severity and probably this is due to both vaso- and immunoregulatory dysfunctions, typically associated with chronic diseases.

Conflict of Interest

The authors declare no conflict of interest.

Informed Consent

All subjects gave their informed consent for inclusion before they participated in the study.

Availability of Data and Materials
All data and materials are inside the manuscript.

Ethics Approval
The study was conducted in accordance with the 1975 Declaration of Helsinki and its latest amendments. The study was approved the 12th of January 2021 by Ethics Committee of Brescia (Italy) Prot. No. NP4588 with the following title: "Studio prospettico analitico per la valutazione degli effetti del COVID-19 sul sistema nervoso autonomo in pazienti con Sindrome PostCovid".

Authors' Contributions
MRC wrote the main manuscript text, tables, and figures. MB provided the resources. SN, GA, FF, AC, AP and MGDA conducted the investigation. MCM, Kristjana D, ST, GB and Kevin D, CM worked on data curation. PEM, SC, LL revised the manuscript. MB worked on the conceptualization of the study. MRC worked on statistical analysis. TB provided the supervision.

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