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Presence of viral spike protein and vaccinal spike protein in the blood serum of patients with long-COVID syndrome

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Abstract. – OBJECTIVE: COVID-19 patients experience, in 10-20% of the cases, a prolonged long-COVID syndrome, defined as the persistence of symptoms for at least two months after the infection. The underlying biological mechanisms of this syndrome remain poorly understood. Several hypotheses have been proposed, among which are the potential autoimmunity resulting from molecular mimicry between viral spike protein and human proteins, the reservoir and viral reproduction hypothesis, and the viral integration hypothesis. Although official data state that vaccinal spike protein is harmless and remains at the site of infection, several studies proposed spike protein toxicity and found it in blood circulation several months after the vaccination.

To search for the presence of viral and vaccine spike protein in a cohort of long-COVID patients.

***PATIENTS AND METHODS:* In this study, we employed a proteomic-based approach utilizing mass spectrometry to analyze the serum of 81 patients with long-COVID syndrome. Moreover, viral integration in patients' leukocytes was assessed with a preliminary study, without further investigation.**

***RESULTS:* We identified the presence of the viral spike protein in one patient after infection clearance and negativity of COVID-19 test and the vaccine spike protein in two patients two months after the vaccination.**

***CONCLUSIONS:* This study, in agreement with other published investigations, demonstrates that both natural and vaccine spike protein may still be present in long-COVID patients, thus supporting the existence of a possible mechanism that causes the persistence of spike protein in the human body for much longer than predicted by early studies. According to these results, all patients with long-COVID syndrome should be analyzed for the presence of vaccinal and viral spike protein.**

Key Words:
Viral Spike Protein, Vaccinal Spike Protein, SARS-CoV-2, COVID-19, Long-COVID syndrome, Mass spectrometry, Viral reservoir, Viral integration.

Introduction

COronaVIrusDisease-2019 indicates the disease caused in humans by the SARS-CoV-2 virus, characterized by fever, cough, breathing difficulties, severe acute respiratory syndrome, and even death[1-4]. 10-20% of COVID-19 patients manifest long-COVID syndrome, defined as the persistence of symptoms two months after the infection[5-8]. The most common symptoms associated with long-COVID include fatigue, breathlessness and cognitive dysfunction[9,10]. Notably, even seven months after the initial infection, patients with long-COVID continue to experience cardiovascular and neural problems, indicating a prolonged and complex disease course, and highlighting the significant impact of long-COVID on individuals' health and quality of life[11]. Extensive research[12-22] has been conducted to elucidate the underlying mechanisms and pathophysiology of long-COVID. However,

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the exact cause of long-COVID and the factors contributing to its diverse symptomatology are still not fully understood. Several hypotheses have been proposed, among which potential autoimmunity resulting from molecular mimicry between viral spike protein and human proteins[12,13], the reservoir and viral reproduction hypothesis[14-17], and the viral integration hypothesis[18-22]. Finally, it has also been proposed that the spike protein, the primary antigen targeted by COVID-19 vaccines, could have a potential toxicity that is linked to the development of long-COVID symptoms[23-28].

The spike protein used in vaccines differs from the viral spike protein found in SARS-CoV-2 because it has been modified to enhance its stability and immunogenicity through prefusion stabilization with a double proline substitution[29]. Both the viral and the vaccine spike protein are considered harmless and are not expected to circulate freely in the bloodstream, this being one essential aspect of vaccine safety as official data report[30-32]. Indeed, the vaccine spike protein is synthesized by cells, it remains bound to the cellular membrane, and it is presented on the cell surface to the immune cells[33,34]. Moreover, as from official data, the spike protein should remain in the vicinity of the injection site and local lymph nodes, where the immune response is initiated[35], and it may persist up to a few weeks after vaccination[36,37]. The official data about spike protein have been challenged by recent studies[38,39] that proposed that the spike protein has inherent toxicity, acting as an inflammagen and thus stimulating inflammation and blood hypercoagulation. Moreover, viral and vaccine spike proteins have been found in the bloodstream of individuals even months after infection clearance and vaccination[40-45].

Considering the proposed persistence of spike protein in long-COVID syndrome, this study aimed to specifically investigate the presence of viral and vaccine spike proteins in the blood serum of long-COVID syndrome patients using mass spectrometry analysis[46,47]. Secondly, polymerase chain reaction (PCR) was used for a preliminary study to check for SARS-CoV-2 integration in the long-COVID patients' leukocytes[18], without further investigation[48,49].

Patients and Methods

Patient Recruitment

Patient recruitment was conducted based on clinical history and symptoms. We aimed to include a diverse cohort of 81 long-COVID syndrome patients, ensuring representation across different age groups, genders, and disease severity. Informed consent was obtained from each participant, and ethical guidelines were strictly followed throughout the study. 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 Declaration of Helsinki. A written informed consensus was obtained from all patients at the time of enrollment, and each of them was anonymized.

Mass Spectrometry

Mass spectrometry analysis was performed on the serum samples obtained from the recruited long-COVID syndrome patients, with the aim of detecting and quantifying spike protein fragments present in the samples. To achieve this, trypsin digestion was employed, generating specific tryptic fragments for each spike protein variant. The distinct tryptic fragments identified in the samples allowed for discrimination between the vaccine spike protein and the viral spike protein. The analysis was conducted using an HPLC Surveyor system (Thermofisher, Waltham, MA, USA) equipped with a Halo Peptide ES-C18 column (2.1 x 50 mm, 2.7 µm). A two-phase gradient was utilized, with Phase A consisting of H_2O with 0.2% Formic Acid (HCOOH) and Phase C consisting of acetonitrile (CH_3CN). A volume of 5 µL of the sample was injected for analysis. Data acquisition was performed using a "SANIST" mass spectrometer, utilizing electrospray ionization (ESI) as the ionization source.

Data Analysis

Statistical analysis was not performed due to the descriptive design of our study. Data analysis was carried out to analyze the mass spectrometry data and draw meaningful conclusions. The analysis was processed by SANIST Hb software using a database containing the glycoprotein spike and other proteins randomly selected to increase accuracy. For the detection of the LDPPEAEVQIDR fragment, ion extractions of the child ion fragments at m/z 577 of the ions at m/z 979.4 and m/z 830.3 (MS3 technique) were performed.

Results

Patient Recruitment and Clinical Data Analysis

The study included a total of 81 patients with long-COVID syndrome. Clinical data were available for 70 patients (Table I).

Mass Spectrometry Analysis

Out of the 81 long-COVID patients analyzed, fragments of the vaccine spike protein were found in 2 patients, while fragments of the viral spike protein were found in 1 patient (Table II). Control samples from unvaccinated individuals were negative for spike protein. The areas of the identified fragments were quantified to assess the presence and abundance of the spike protein. Table III provides the areas of the standard and of the samples in which the vaccine protein was identified, as well as the corresponding percentages.

The samples in which vaccine spike protein was identified were collected at least two months after the administration of the second dose (Table IV).

Table I. Summary of clinical data for 70 patients.

Characteristics		Case subjects (n=70)
Sex	Male	35 (50%)
	Female	46 (65.71%)
Age (year)		52
BMI		26.1
Vaccine (YES)		51 (72.86%)
Severity score	Asymptomatic	0 (0%)
	Mild symptoms	34 (48.57%)
	Severe symptoms	35 (50%)
	Intensive care	1 (1.43%)
	Asthenia (during COVID)	7.8%
	Asthenia (long-COVID)	5.1%
	Headache (during COVID)	4.4%
	Headache (long-COVID)	2.1%
Reinfection	Yes	27 (38.57%)
	No	43 (61.43%)
Clinical data	Pneumonia (NO)	31 (44.29%)
	Pneumonia (YES)	39 (55.71%)
	Fever (NO)	16 (22.86%)
	Fever (YES)	54 (77.14%)
Serology	Not done	35 (50%)
	Negative	13 (18.57%)
	Doubtful	0 (0%)
	Positive	22 (31.43%)
Therapy	Paracetamol	50 (71.43%)
	Hydroxychloroquine	13 (18.57%)
	Antibiotics	42 (60%)
	Antivirals	18 (25.71%)
	Corticosteroids	23 (32.86%)
	Eparine	18 (25.71%)
	Ventilation	44 (62.86%)

BMI: body mass index.

Table II. Spike analysis processing results.

ID	Viral Spike Protein	Vaccinal Spike Protein (PP)
1	N.D.	Low signal
8	Signal	N.D.
37	N.D.	Low signal

N.D. = Not Detected.

Table III. Areas of the samples in which vaccine protein was identified and area of the standard.

ID	Area m/z 830.3	Area m/z 979.4	830.3%	979.4%
1	12.42	35.27	26.04	73.96
37	15.94	13.05	45.02	54.98
Std	6554	3385	65.94	34.06

Table IV. Vaccine and sample data for patients

ID	Type of vaccine	Date of 2nd vaccine dose	Date of sample collection	Vaccine Spike protein	Viral Spike protein
1	Pfizer	02/2021	26/04/2021	Yes	No
37	Pfizer	02/2021	30/04/2021	Yes	No

Discussions

This study employed mass spectrometry analysis to investigate the presence of viral and vaccine spike proteins in the blood serum of patients with long-COVID syndrome. As reported in Table II, the mass spectrometry analysis revealed the presence of both viral and vaccine spike protein fragments in a subset of patients with long-COVID syndrome even two months after vaccination or after infection clearance and negativity of the COVID-19 test (Table IV). Official data sustain that the vaccine spike protein remains in the vicinity of the injection site and local lymph nodes and that it may persist in the body up to a few weeks after vaccination[20-24]. Our findings, in alignment with other studies and in contradiction with official data, show the presence of both the vaccine and the viral spike protein in the bloodstream even after infection clearance and several months after vaccination[40-42,45-49]. Furthermore, viral integration in patients' leukocytes was assessed with a preliminary study following the protocol of Merchant[18], without further investigation (**Supplementary Data**). Having detected the vaccinal protein in two subjects and the viral protein in one subject in a cohort of 95 patients, this study has mainly a descriptive function. Nevertheless, these results are aligned with many other already published literature performed on other independent cohorts. We conclude that considering the proposed toxicity of the spike protein and that official data sustain that it should not persist in blood circulation a few weeks after vaccination, blood samples of long-COVID patients should be routinely tested for the presence of vaccine and viral spike protein. Future research should focus on investigating the specific pathways and interactions through which viral and vaccine spike protein can circulate and persist in blood circulation several months after viral clearance or vaccination and on its possible negative effects.

Conclusions

This study, in accordance with other published investigations, shows the persistence in blood circulation of viral spike protein in one patient after infection clearance and the negativity of the COVID-19 test, of vaccine spike protein in two patients two months after vaccination. This study underscores the importance of mass spectrometry analysis of long-COVID patients to detect spike protein persistence. Further research is needed to understand the underlying mechanisms of spike protein persistence.

Availability of Data and Materials

The data are within the test or in the supplementary materials document.

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Authors' Contributions

Conceptualization, M.B.; Methodology, S.C.; Investigation, F.F., A.C., A.P., M.G.D.A., G.A., and S.N.; Writing- original draft preparation, K.D., and M.C.M.; Writing, review and editing, C.M., K.D., A.M., F.F., A.C., A.P., M.G.D.A., G.A., S.C., S.N.; Project administration, M.B.; Funding acquisition, M.B. All authors have read and agreed to the published version of the manuscript.

Ethics Approval

The study was approved on the 12th of January 2021 by the Ethics Committee of the University of Brescia (Italy), Prot. No. NP4588.

Informed Consent

A written informed consensus was obtained from all patients at the time of enrollment, and each of them was anonymized.

Conflicts of Interest

The authors declare no conflict of interest.

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