European Review for Medical and Pharmacological Sciences 2023; 27: 13-19

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 persi of symptoms for at least two months afte fection. The underlying biological mechanis of this syndrome remain poorly understood. S al hypotheses have been proposed, among wh are the potential autoimmunity re ting from m lecular mimicry between viral tein an human proteins, the reservoi d vir roduction hypothesis, and the v integrat hypothesis. Although official tate spike protein is harml s a at th of infection, several ies p d spike protein toxicity and fo it in blood lation several months afte ccination.

To search for p e of viral a vaccine spike protein a coho ng-COVID patients.

PATIEN AND ME S: In this study, we emp d a proteomic- approach utilizing ss sp trometry to analyze the serum 1 pati with long-COVID syndrome. Mo vi integra on in patients' leukocytes essed n a preliminary study, out r inv gation.

ULT tified the presence of the vira ike pr n one patient after infection cle ce and n gativity of COVID-19 test and th ike protein in two patients two ths a the vaccination.

CONCLUSIONS: This study, in agreement other published investigations, demons that both natural and vaccine spike protein ay still be present in long-COVID patients, thus supporting the existence of a possible mechanism that causes the persistence of spike pr in the l an body for much longer than pred y studies. According to these results, all patients with long-COVID syndrome ould be analyzed for the presence of vaccinal 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 [illegible] the spike protein should remain in the vic[illegible] the injection site and local lymph nodes, [illegible]re the immune response is initiated[35], and it [illegible] persist up to a few weeks after vaccination[illegible] The official data about spike p[illegible] have bee[illegible] challenged by recent studie[illegible] roposed that the spike protein has i[illegible]nt tox[illegible], acting as an inflammagen and [illegible]stimula[illegible] mation and blood hy[illegible]erc[illegible]ti[illegible] viral and vaccine sp[illegible] protei[illegible] been found in the bloodstrea[illegible] individua[illegible]n months after infection [illegible] and vaccina[illegible]9-45].

Considerin[illegible] prop[illegible]ersistence of spike protein in lon[illegible]OVID syndr[illegible] this study aimed to specifica[illegible]vestigate the pre[illegible]of viral and vaccine s[illegible] protei[illegible] in the blood [illegible]rum of long-COVID [illegible]drome [illegible]ients using mass spectrometry anal[illegible]47]. [illegible]ondly, [illegible]merase chain reaction (PCR) w[illegible]d for a[illegible]minary study to check for [illegible]-Cov[illegible]g[illegible]n in the long-COVID patients [illegible]ocytes [illegible]ut further investigation[48,49].

Patients and Methods

[illegible]nt Recruitment

[illegible]ent 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 [illegible] study was approved by the Ethics Com[illegible]e of Bi[illegible]scia (Italy) Prot. No. NP4588. All r[illegible]ch process was conducted according to the e[illegible]guidelines of the Declaration of Helsinki. A wri[illegible]formed consensus was obtained fro[illegible]patients [illegible]ime of enrollment, and each of[illegible]n was anonym[illegible]

Mass Spectrometr[illegible]

Mass spectrom[illegible]y a[illegible]as perf[illegible]ed on the serum sam[illegible] obtain[illegible]m th[illegible]ecruited long-COVID [illegible]rome patie[illegible]ne aim of detecting a[illegible]ying spike p[illegible]n fragments present in[illegible] samp[illegible] To achieve this, trypsin digestion was employed[illegible]rating specific tryptic frag[illegible]r each spike p[illegible] variant. The distinct t[illegible]c fragments identified in the samples allowed [illegible]iscriminatio[illegible]tween the vaccine spike protein [illegible]the viral spi[illegible]rotein. The analysis was con[illegible]using an [illegible]LC Surveyor system (Thermofish[illegible]A, USA) equipped with a Halo Peptide [illegible] column (2.1 x 50 mm, 2.7 µm). A [illegible]wo-phase gradient was utilized, with Phase A con[illegible]f H_2O with 0.2% Formic Acid (HCOOH) [illegible]se 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 per

The samples in which vaccine spik tein w identified were collected at least tw onths after the administration of the second d able IV).

Table I. Summary of clinical data for 70 patients.

Characteristics		Case subjec =70)
Sex	Male	35 (50%)
	Female	46 (65.)
Age (year)		52
BMI		2
Vaccine (YES)		%)
Severity score	Asymptomatic	0%
	Mild symptoms	34 (48.5
	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 (3 %)
	No	43 3%)
Clinical data	Pneumonia (NO)	.29%)
	Pneumonia (Y	39 (55.71%)
	Fever (NO)	16 (22.86%)
	Fever (YES)	54 (77.14%)
Serology	Not done	35 (50%)
	Negative	13 (18.57%)
	Doub	0 (0%)
	R	22 (31.43%)
Therapy	aceta	50 (71.43%)
	ydroxy oquine	13 (18.57%)
	Antibio	42 (60%)
	tiv	18 (25.71%)
	teroids	23 (32.86%)
	E	18 (25.71%)
	Vent	44 (62.86%)

BMI: body mas x.

Table II. analysis processin ts.

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

N.D ot Dete

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

	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 protei
1	Pfizer	02/2021	26/04/2021	Yes	
37	Pfizer	02/2021	30/04/2021	Yes	

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 blood downstream even after infection clearance and several months after vaccination[40-42,45-49]. Furthermore, vaccine integration in patients' leukocytes was assessed via PCR in a preliminary study following the protocol of Merchant[18], without further investigation (**Supplementary Data**). Having detected the vaccine protein in two subjects and the viral protein in one subject in a cohort of 95 patients, this data has limited descriptive function. Nevertheless, these results are aligned with many other already published literature performed on other independent cohorts and conclude that considering the proposed toxicity of the spike protein and that official data sustain that it should not persist in blood circulation for 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 proteins can circulate and persist in blood circulation several months after viral clearance or vaccination and the negative effects.

Conclusions

This study, in accordance with other published investigations, shows the persistence in blood circulation of viral spike protein in some patients after infection clearance and the negative the COVID-19 test, vaccine spike protein in two patients two months after vaccination. This study underlines 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

All data are within the test or in the supplementary material document.

Funding

This research was funded by the Provincia Autonoma di Bolzano, in the framework of LP 14/2006.

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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