TITLE: "Detection of recombinant spike protein in plasma from vaccinated against SARS-CoV-2 individuals".

Simone Cristoni[1+], Carlo Brogna[2*+], Aquilino Frongillo[2+], Giuliano Marino[3], Luigi Montano[4], Marina Piscopo[5*]

[1] ISB - Ion Source & Biotechnologies Srl- Bresso Milano; simone.cristoni@gmail.com (S.C.);
[2] Department of research, Craniomed group facility Srl., Montemiletto (Av), Italy; dir.brogna@craniomed.it (C.B.);
[3] Marsanconsulting Srl. Public Health Company; Via dei Fiorentini, 80133 – Napoli; Italy; marino@marsanconsulting.it (M.G.);
[4] Andrology Unit and Service of LifeStyle Medicine in Uro-Andrology, Local Health Authority (ASL) Salerno; luigimontano@gmail.com (L.M.);
[5] Department of Biology, University of Naples Federico II, 80126 Napoli, Italy; marina.prisco@unina.it (M.P.);

*** Correspondence:** marina.piscopo@unina.it; dir.brogna@craniomed.it
+ equal contributor

Keywords: SARS-CoV-2, vaccine mRNA, Spike protein, mass spectrometry, COVID-19

Abstract:

The unfortunate advent of the SARS-CoV-2 pandemic has given a new push to the development and use of a next-generation of vaccines. Among them, mRNA-based vaccines consist of injectable solutions of mRNA encoding for a recombinant spike, which is distinguishable from the wild-type protein by the presence of specific amino acid variations introduced to maintain the protein in a prefusion state. Here, we present a proteomic approach to reveal the recombinant spike protein in vaccinated subjects to detect its presence independently of antibody titer. This approach is of valuable support to complement antibody level monitoring and represents the first proteomic detection of the recombinant spike in the vaccinated individuals.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the coronavirus that causes the respiratory illness COVID-19 (coronavirus disease 2019), the ongoing pandemic keeping the whole world in check.
The RNA genome of SARS-CoV-2 consists of approximately 30,000 nucleotides and contains 11 major coding genes. From the structural point of view, SARS-CoV-2 is characterized by a great number of the glycosylated spike (S) proteins covering its surface, which facilitate binding to the angiotensin-converting enzyme 2 (ACE2) receptor of the host cell, mediating viral cell entry [1]. The S protein of SARS-CoV-2 is highly conserved among all human coronaviruses (HCoV) and is involved in receptor recognition, viral attachment, and entry into the host cells. For this reason, it

represents one of the most important targets for the development of COVID-19 vaccines and therapeutic approaches. Among the COVID-19 vaccines developed and tested, vaccines that have demonstrated the most promising results in preventing COVID-19 infection are a novel class of vaccine products composed of messenger ribonucleic acid (mRNA) filaments encapsulated in lipid nanoparticles (LNPs). Two of them received "emergency use authorization" (from the FDA) and "conditional approval" from the EMA.
Both of them consist of a recombinant mRNA to be inoculated as a vaccine, encoding for a recombinant SARS-CoV-2 spike protein. Although the mRNAs are different, they both encode for the same recombinant spike protein (here called PP-spike) that differs from the natural one (wt-spike) for a double amino acid variation at position 986 and 987 (K986P and V987P, i.e., the amino acids lysine and valine both replaced by two proline amino acids) [2,3], in order to stabilize the spike conformation into an inactive prefusion state.
The introduced double amino acid variation abolishes a tryptic digestion site. Consequently, it is possible to discriminate, through tryptic digestion [4], followed by mass spectrometry analysis [5-8], the synthetic spike proteins originated from the translation of the mRNA vaccines from the natural spike circulating in the biological fluids.
Here we present a methodological approach that can specifically detect PP-spike's presence in the biological fluids of the human and animal organisms, such as blood, urine, saliva, and bronchoalveolar lavage fluids.

Materials and Methods

In accordance with applicable territorial regulations, human biological samples were collected with the express, free, and informed consent for collection and use. Candidates had not previously had COVID-19 and were negative for molecular testing by nasopharyngeal swab.

Rationale: Trypsin [4] is an enzyme belonging to the class of hydrolases capable of reducing proteins to smaller polypeptides by proteolytic cuts with specificity for arginine (R) and lysine (K). Therefore, the synthetic spike protein and the natural spike protein can be discriminated against as they produce different tryptic digestion products:
- the PP-spike encoded by mRNA vaccines, when digested by trypsin, produces an LDPPEAEVQIDR fragment (PP-spike marker).
- SAR-CoV-2 wild-type protein, when digested by trypsin, produces two smaller fragments, namely LDK + VEAEVQIDR.
More than 6,600,000 SARS-CoV-2 genomes have been sequenced, and it appears that none of them have any of the K986P and V987P mutations, including the Omicron variant [9].
Buffers used: Double distilled water (VWR); Ammonium bicarbonate (NH_4HCO_3) (Sigma Aldrich).
Reagents: Trypsin from Promega.
Reagent Preparation: Trypsin solution 25 ng/µL.
Preparation procedure: Resuspend, in a vial, 20 µg of solid trypsin in 800 µL of 50 mM NH_4HCO_3 solution. Vortex the vial until the trypsin is completely dissolved.

Enzymatic Digestion: The blood drop enzymatic digestion procedure is performed under a fume hood in the rationale of minimizing operator exposure to any form of chemical biohazard. The following method was used for each blood sample. Place 2 µL of capillary blood in a labeled Eppendorf; Add 40 µL of modified trypsin (Promega, Italy) solubilized into an NH_4HCO_3 50 mmoL solution on the blood sample; Vortex for 30 seconds; Test the pH, which must be between 7-8; Transfer the Eppendorf into the thermoblock; Incubate for two days at 37°C; Add 40 µL of NH_4HCO_3 50 mmoL. Collect 40 µL of the supernatant obtained after centrifugation at 13000 G for 10 minutes and transfer it to an Eppendorf. Add 2 µL of pure formic acid and transfer the solution to an injection vial; The

vial is inserted into the autosampler coupled to the mass spectrometer, and 15 µL are injected into the chromatographic column.

LC-SACI-MS Instrumentation: Analysis was performed using a Surveyor MS HPLC (Thermofisher, USA). The column used was a Kinetex 50 x 4.6 mm 2.6 µm. Analysis was performed using a two-phase gradient: Phase A (H2O+0.2 % Formic Acid (HCOOH)) and Phase C (CH3CN) (table1). The chromatographic gradient used is shown in Table 1. The volume of the sample injected is 5 µL. The ionization source used is a SACI-ESI. A surface potential of 0 V, a nebulizer gas pressure of 75 Psi, and a dry gas parameter flow of 1.0 L/min were used. The dry gas temperature is at 320 °C [5-8].

Results

The specific PP-spike fragment was found in 50% of the biological samples tested (figure 1 and Table 2). This presence is independent of the IgG SARS-CoV-2 antibody titer. Antibody titers had a geometric mean of 629.86 BAU/ml (table 3). The minimum time of presence PP-spike found was 69 days after vaccination, the maximum time 187 days. All controls, samples from unvaccinated individuals, were negative.

Discussion and conclusions

Some studies [10] have observed the presence of the vaccinal spike protein immediately after injection.
Using mass spectrometry examination of biological samples, we detect the presence of specific fragments of recombinant spike protein in about 50% of individuals vaccinated with mRNA-based vaccines. In some cases, we found the rec-spike marker in individuals vaccinated after more than 30 days to indicate that it is possible to detect vaccinal "spike" protein even at a certain distance from the vaccination and in any organic tissue (data in preparation). This finding opens new scenarios for monitoring the presence and half-life of vaccinal spike protein in vaccinated individuals.

Funding: "This research received no external funding"

Institutional Review Board Statement: the study did not require ethical approval.

Informed Consent Statement: "Informed consent was obtained from all subjects involved in the study."

Data Availability Statement: "Not applicable".

Acknowledgments: we thank all the patients that have participated to our studies.

Conflicts of Interest: "The authors declare no conflict of interest."

References

[1] Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nat Microbiol. 2020 Apr;5(4):562-569. doi: 10.1038/s41564-020-0688-y. Epub 2020 Feb 24. PMID: 32094589; PMCID: PMC7095430.

[2] Corbett KS, Edwards DK, Leist SR, Abiona OM, Boyoglu-Barnum S, Gillespie RA, Himansu S, Schäfer A, Ziwawo CT, DiPiazza AT, Dinnon KH, Elbashir SM, Shaw CA, Woods A, Fritch EJ, Martinez DR, Bock KW, Minai M, Nagata BM, Hutchinson GB, Wu K, Henry C, Bahl K, Garcia-Dominguez D, Ma L, Renzi I, Kong WP, Schmidt SD, Wang L, Zhang Y, Phung E, Chang LA, Loomis RJ, Altaras NE, Narayanan E, Metkar M, Presnyak V, Liu C, Louder MK, Shi W, Leung K, Yang ES, West A, Gully KL, Stevens LJ, Wang N, Wrapp D, Doria-Rose NA, Stewart-Jones G, Bennett H, Alvarado GS, Nason MC, Ruckwardt TJ, McLellan JS, Denison MR, Chappell JD, Moore IN, Morabito KM, Mascola JR, Baric RS, Carfi A, Graham BS. SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. Nature. 2020 Oct;586(7830):567-571. doi: 10.1038/s41586-020-2622-0. Epub 2020 Aug 5. PMID: 32756549; PMCID: PMC7581537.

[3] Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020 Mar 13;367(6483):1260-1263. doi: 10.1126/science.abb2507. Epub 2020 Feb 19. PMID: 32075877; PMCID: PMC7164637

[4] Olsen JV, Ong SE, Mann M. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics. 2004 Jun;3(6):608-14. doi: 10.1074/mcp.T400003-MCP200. Epub 2004 Mar 19. PMID: 15034119.

[5] Cristoni S, Bernardi LR. Bioinformatics in mass spectrometry data analysis for proteomics studies. Expert Rev Proteomics. 2004 Dec;1(4):469-83. doi: 10.1586/14789450.1.4.469. PMID: 15966842. Expert Rev Proteomics. 2004 Dec;1(4):469-83.

[6] Lundgren DH, Hwang SI, Wu L, Han DK. Role of spectral counting in quantitative proteomics. Expert Rev Proteomics. 2010 Feb;7(1):39-53. doi: 10.1586/epr.09.69. PMID: 20121475.

[7] Cristoni S, Bernardi LR, Biunno I, Tubaro M, Guidugli F. Surface-activated no-discharge atmospheric pressure chemical ionization. Rapid Commun Mass Spectrom. 2003;17(17):1973-81. doi: 10.1002/rcm.1141. PMID: 12913861.

[8] Okada P, Buathong R, Phuygun S, Thanadachakul T, Parnmen S, Wongboot W, Waicharoen S, Wacharapluesadee S, Uttayamakul S, Vachiraphan A, Chittaganpitch M, Mekha N, Janejai N, Iamsirithaworn S, Lee RT, Maurer-Stroh S. Early transmission patterns of coronavirus disease 2019 (COVID-19) in travellers from Wuhan to Thailand, January 2020. Euro Surveill. 2020 Feb;25(8):2000097. doi: 10.2807/1560-7917.ES.2020.25.8.2000097. PMID: 32127124; PMCID: PMC7055038.

[9] Miller NL, Clark T, Raman R, Sasisekharan R. Insights on the mutational landscape of the SARS-CoV-2 Omicron variant. bioRxiv [Preprint]. 2021 Dec 8:2021.12.06.471499. doi: 10.1101/2021.12.06.471499. PMID: 34909771; PMCID: PMC8669838

[10] Cognetti,J.S.;Miller,B.L. Monitoring Serum Spike Protein with Disposable Photonic Biosensors Following SARS-CoV-2 Vaccination. *Sensors***2021**,*21*,5857. https:// doi.org/10.3390/s21175857

Figure and table 1 Captions

Figure 1: Panel A: shows the m/z (molecular ion) ratio of the SEQ ID NO:1 detention. It shows an example of a spectrum of the peptide zone marked by the failure to enzymatically cut the "PP" amino acids, of the "Spike" protein, induced by mRNA vaccines. **Panels B, C, D, E**: extraction mass chromatograms of the **(B)** vaccinated subjects and **(D)** control samples obtained by fragmenting the [M+3H]3+ion at m/z 461 and monitoring the singly-charged signal at m/z 851. The tandem mass spectra achieved analyzing **(C)** vaccinated subject and **(E)** control one are also reported. 5 microliters of sample digested sample were injected and the eluent flow rate was 250 microliter/min.

Table 1: Gradient elution chromatography conditions. The % of C (CH_3CN) eluent phase is reported together with the eluent flow rate at different gradient elution times.

Table 1

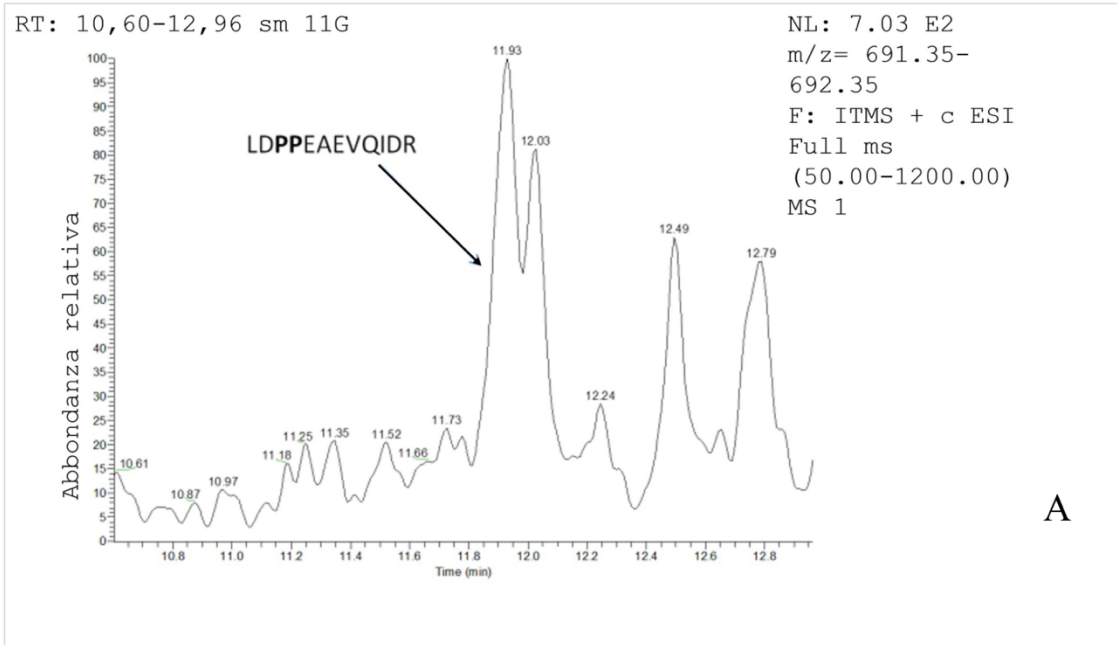
Time (min)	% C	Flow (mL/min)
0	2 %	0.250
2.5	2 %	0.250
3	80 %	0.250
7	80 %	0.250
8	2 %	0.250

Table 2

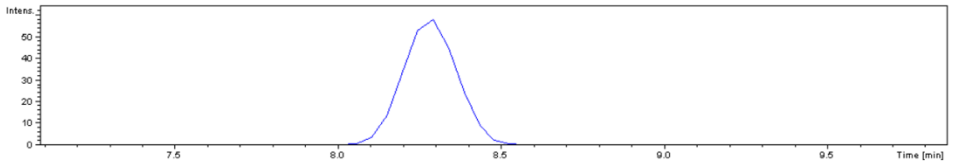
Subjects ID	Sample	Sequences RT (min) Positive per double aminoacids "**PP**" of mRNA spike vaccines	Area (counts/s)
1	Blood	11.93	6.0E+3
4	Blood	11.93	6.1E+2
6	Blood	11.93	9.9E+2
2	Blood	-	-
3	Blood	-	-
5	Blood	-	-
7	Blood	-	-
8	Blood	-	-
9	Blood	11.92	3.4E+2
10	Blood	11.94	3.1E+2
from 11 to 20 negative controls	Blood	-	-

Table 3

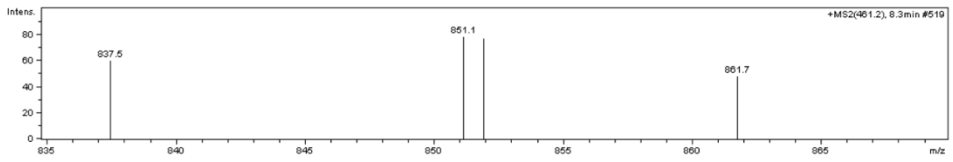
Code	Type of vaccine	Date of 2nd dose	SARS-CoV-2 IgG BAU/mL	Date of proteomic exam	Presence of Spike PP	Days of presence
V 01	mRNA	01/09/2021	1015	08/05/2021	YES	188
V 02	mRNA	01/24/2021	527	08/05/2021	----	
V 03	mRNA	01/23/2021	101	08/05/2021	-----	
V 04	mRNA	01/29/2021	930	08/05/2021	YES	187
V 05	mRNA	02/02/2021	1517	08/05/2021	-----	
V 06	mRNA	02/04/2021	1400	08/05/2021	YES	177
V 07	mRNA	03/04/2021	1104	08/05/2021	-----	
V 08	mRNA	03/30/2021	842	08/05/2021	----	
V 09	mRNA	04/21/2021	463	08/05/2021	YES	99
V 10	mRNA	05/21/2021	214	08/05/2021	YES	69


RT: 10,60-12,96 sm 11G
NL: 7.03 E2
m/z= 691.35-692.35
F: ITMS + c ESI Full ms (50.00-1200.00) MS 1
LDPPEAEVQIDR
Abbondanza relativa
Time (min)
10.61
10.87
10.97
11.18
11.25
11.35
11.52
11.66
11.73
11.93
12.03
12.24
12.49
12.79

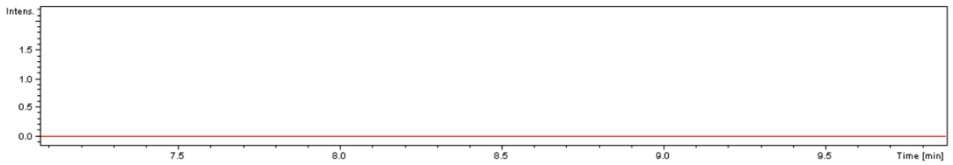
A


Intens.
Time [min]

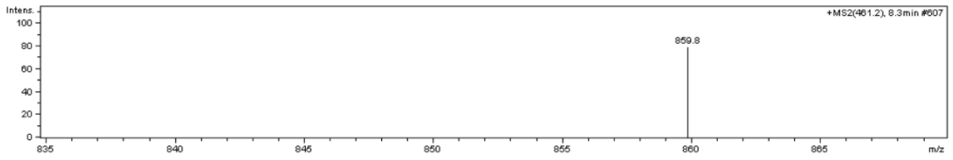
B


Intens.
+MS2(461.2), 8.3min #519
837.5
851.1
861.7
m/z

C


Intens.
Time [min]

D


Intens.
+MS2(461.2), 8.3min #607
859.8
m/z

E