Identification of SARS-CoV-2 Proteins from Nasopharyngeal Swabs Probed by Multiple Reaction Monitoring Tandem Mass Spectrometry

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ABSTRACT: Numerous reverse transcription polymerase chain reaction (RT-PCR) tests have emerged over the past year as the gold standard for detecting millions of cases of SARS-CoV-2 reported daily worldwide. However, problems with critical shortages of key reagents such as PCR primers and RNA extraction kits and unpredictable test reliability related to high viral replication cycles have triggered the need for alternative methodologies to PCR to detect specific COVID-19 proteins. Several authors have developed methods based on liquid chromatography with tandem mass spectrometry (LC−MS/MS) to confirm the potential of the technique to detect two major proteins, the spike and the nucleoprotein, of COVID-19. In the present work, an S-Trap mini spin column digestion protocol was used for sample preparation prodromal to LC−MS/MS analysis in multiple reactions monitoring ion mode (MRM) to obtain a comprehensive method capable of detecting different viral proteins. The developed method was applied to n. 81 oro/nasopharyngeal swabs submitted in parallel to quantitative reverse transcription PCR (RT-qPCR) assays to detect RdRP, the S and N genes specific for COVID-19, and the E gene for all Sarbecoviruses, including SARS-CoV-2 (with cycle negativity threshold set to 40). A total of 23 peptides representative of the six specific viral proteins were detected in the monitoring of 128 transitions found to have good ionic currents extracted in clinical samples that reacted differently to the PCR assay. The best instrumental response came from the FLPFQFGR sequence of spike [558−566] peptide used to test the analytical performance of the method that has good sensitivity with a low false-negative rate. Transition monitoring using a targeted MS approach has the great potential to detect the fragmentation reactions of any peptide molecularly defined by a specific amino acid sequence, offering the extensibility of the approach to any viral sequence including derived variants and thus providing insights into the development of new types of clinical diagnostics.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to the family of betacoronaviruses and comprises a single-stranded RNA about 30 kb in length.1,2 Coronavirus infectious disease 2019 (COVID-19) has spread in the world, causing the collapse of the healthcare system and economic crisis.3 The scientific community around the world has made huge efforts to counteract this pandemic event. However, specific treatments are still lacking and the goal of the scientific community is to develop new therapeutic drugs and effective vaccines.

It is worth mentioning here that mass spectrometry (MS)-based proteomics has emerged as a valuable tool to investigate and understand the pathogenesis of COVID-19, identify potential therapeutic targets, and develop rapid and effective diagnostic tests.4 Several proteomic strategies have been used to identify potential biomarkers of COVID-19.5 The 30 kb genome of SARS-CoV-2 encodes for 14 major open reading frames (ORFs) that are further transformed into 28 proteins: (i) glycoprotein spike (S), membrane (M), envelope (E), and nucleocapsid (N) structural proteins, (ii) accessory proteins (ORF10, ORF9a, ORF9b, ORF8, ORF7a, ORF7b, ORF6, and ORF3a), and (iii) 16 nonstructural proteins (nsp1−nsp16),
which are involved with host proteins and influence different cellular processes such as DNA replication, host gene translation, protein folding, and degradation pathways.\textsuperscript{2,6}

Today, the gold standard of diagnostics is based on the use of reverse transcription polymerase chain reaction (RT-PCR) leading to the amplification and measurement of viral RNA. This approach is highly specific, rapid, and sensitive.\textsuperscript{7,8} However, although widely used, this method suffers from some drawbacks due to the high demand for quantitative polymerase chain reaction (qPCR)-based assays, RNA extraction kits, and qPCR reagents especially during the first phases of the pandemic outbreak. Furthermore, nucleic acid variations in the target regions might affect the amplification rate.\textsuperscript{9} Therefore, alternative detection strategies are needed to complement the above tools. Methods based on immuno-assays,\textsuperscript{10,11} whole-genome sequencing,\textsuperscript{1} and mass spectrometry-based proteomics\textsuperscript{12,13} have been successfully applied. Among these, mass spectrometry in multiple reaction monitoring (MRM) ion mode offers a highly sensitive technology that allows the rapid identification of hundreds of peptides representing dozens of proteins in a single chromatographic sample.\textsuperscript{14} High-performance automated liquid chromatography with tandem mass spectrometry (LC−MS/MS) has become the technique of choice for various metabolite and protein analyses due to its ability to simultaneously separate and determine mixtures of multiple components. MRM targeted metabolomics and proteomic approaches, which focus on a specific subset of known representative of metabolites or proteins biologically relevant metabolic pathways, are valuable research tools in this field.\textsuperscript{15−17} Key advantages of the MRM methodology are the ability to target specific peptide sequences, including protein variants and modified forms, and the possibility of multiplexing, as hundreds of peptides can be analyzed simultaneously after injection of a single complex sample. The targeted approach of MRM experiments guarantees very high sensitivity, selectivity, and specificity while avoiding the problems associated with the availability of antibodies for all target molecules to be analyzed and the cross-reactivity typical of enzyme immunoassays. Recently, several works have been published to develop a robust LC−MS/MS method due to the urgent need to monitor COVID-19 in clinical samples. Currently, the availability of MS instruments from companies, such as Waters and AB Sciex, has prompted scientists to meet the demand for a method capable of detecting viral spike and nuclear proteins at the molecular level through LC-MRM/MS analysis.\textsuperscript{18,19} The Atlas platform is also collecting data on already observed metabolites or proteins biologically relevant metabolic pathways,\textsuperscript{1} and e−TRAP digital patient registry for the broader panel of proteins to directly identify the presence of SARS-CoV-2 in clinical swabs through the highly specific, highly sensitive, and unambiguous determination of a specific set of viral proteins.

### EXPERIMENTAL SECTION

#### Materials and Methods

Ammonium bicarbonate (AMBIC), acetonitrile (ACN), formic acid (HCOOH), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), and iodoacetamide (IAM) were purchased from Sigma-Aldrich (Milan, Italy). Recombinant human coronavirus SARS-CoV-2 spike glycoprotein S1 (Active) was supplied by Abcam (code ab273068). S-Trap mini columns (100−300 μg) were from ProtiFi (Huntington NY). Oro/nasoasopharyngeal swabs were provided by the Ceinge Institute. A code list identified each swab sample collected based on the qPCR test result into 81 positives and 20 negatives for COVID-19 (Supporting Information Table ST1).

#### Virus Inactivation and Protein Precipitation.

One hundred nasopharyngeal swabs (500 μL) collected in viral transport media (VTM) were inactivated by diluting each sample with lysis buffer (50% AMBIC and 5% SDS) at a 1:1 ratio and incubating them on a rotary platform for 20 min at room temperature.\textsuperscript{13} Proteins were extracted from oro/nasopharyngeal swabs of n. 81 RT-PCR-confirmed COVID-19 patients as follows. A 50% w/v trichloroacetic acid (TCA, Sigma–Aldrich) solution (250 μL) was added to each swab to obtain a final concentration of 10% TCA for protein precipitation. After stirring, each sample was centrifuged at 12000 rpm at 4 °C for 10 min, the supernatant was discarded, and the resulting pellet was rinsed with cold acetone three times. The washed pellet was used and subjected to an in-solution digestion using S-trap columns.

#### S-Trap Mini Spin Digestion Column.

S-Trap digestion was performed according to the manufacturer’s protocol. Proteins were solubilized in the 25 μL of lysis buffer (5% SDS, 50 mM AMBIC), reduced with 100 mM DTT to a final concentration of 20 mM, and incubated for 10 min at 95 °C. After cooling the protein solution to room temperature, the cysteines were alkylated by the addition of IAM to a final concentration of 40 mM, followed by incubation in the dark for 30 min. Aqueous phosphoric acid was added as a final concentration of 1.2% to block the alkylation reaction. Colloidal protein particulate was formed by adding 150 μL of S-Trap binding buffer (90% aqueous methanol, 100 mM AMBIC, pH 7.1) to the protein solution. The mixture was placed on 2 μL micro S-Trap columns and centrifuged at 4000g for 30 s. The columns were washed three times with 200 μL of S-Trap binding buffer and discarded after each wash step and centrifuged at 4000g for 30 s. Each sample was subjected to enzymatic digestion using a trypsin (Sigma, Milan, Italy) solution (0.12 μg/μL) at an enzyme-to-substrate ratio of 1:20 (w/w). The digestion was performed for 1 h at 47 °C. Peptides were eluted with 80 μL of 50 mM AMBIC, followed by 80 μL of 0.2% aqueous formic acid, 40 μL of 50% acetonitrile, and finally 40 μL of 70% acetonitrile both containing 0.2% HCOOH. The peptides were dried under vacuum and finally resuspended in 50 μL of 0.1% HCOOH for a further LC−MS/MS analysis.

#### LC−MS/MS Analysis.

The peptide mixture was analyzed by LC−MS/MS analysis using a Xevo TQ-S (Waters) equipped with an IonKey coupled to an Acquity UPLC system (Waters, Milford, MA). For each run, 1 μL of peptide mixture was injected and separated on a BEH C18 peptide separation device (130 Å, 1.7 μm, 150 μm x 50 mm) at 45 °C with a flow rate of 3 μL/min using an aqueous solution (LC−MS grade) containing 2% ACN as buffer A and 95% ACN as
buffer B, both acidified with 0.1% HCOOH. The gradient for the MRM method started with 7% buffer B for 5 min, reached 50% buffer B from 5 to 40 min, to 95% buffer B during the next 2 min. The column was finally re-equilibrated to initial conditions for 4 min. The parameters of the MS source were as follows: 3900 V as ion spray voltage, 150 °C interface heater temperature, and 150 L/h gas flow with 7 bar nebulizer pressure.

MRM mass spectrometric analyses were performed in a positive-ion mode using an MRM time window of 0.4–1 min per peptide, 5 points per peak, and dwell times were 3 ms. The cone voltage was set to 35 V. A range of 300–1000 m/z was preferentially selected as a precursor or product ion.

The latest version of Skyline software (20.2–64 bit version MacCossLab Software, University of Washington) was used for in silico selection of peptides with a unique sequence for each selected protein. For each peptide, m/z precursor ion, m/z product ion, and relative collision energy were provided by Skyline and included in the Supporting Information (Table ST2). Tryptic peptides (preferably without missed cleavages) with a length between 7 and 30 amino acids and devoid of methionine and cysteine residues, if possible, were chosen for the development of MRM assays. Sequences with a proline (P) at the C-terminal side of R or K or showing the NXT/NXS motif were frequently identified by PCR and those occurring in the pep03 fragment ions.

Development of the MRM Method. Ten most suitable protein biomarkers of SARS-CoV-2 (P0DTC2, P0DTC3, P0DTC4, P0DTC5, P0DTC6, P0DTC7, P0DTC8, P0DTC9, P0DTD1, P0DTD2) were selected for the development of the LC-MRM/MS method on the basis of those more frequently identified by PCR and those occurring in the literature data.

RESULTS AND DISCUSSION

The high demand for qPCR assays caused an unexpected need for reagents or solvents especially during the first phases of the pandemic outbreak. An alternative method to the qPCR assay would have compensated for this lack above the detection of viral proteins to confirm qPCR-based molecular assay. Here, we propose the use of tandem mass spectrometry in MRM ion mode to detect the presence of SARS-CoV-2 virus in nasopharyngeal swabs through the direct detection of specific viral proteins. A protocol of S-Trap digestion was performed to trap, digest proteins, and clean up the peptides directly on columns to minimize the sample protein loss and optimize times of analysis (2 h) compatible with those for a qPCR assay. The developed MRM method was aimed at detecting the presence of selected target peptides belonging to specific viral proteins within the swab samples. In this study, a preliminary test was performed by analyzing SARS-CoV-2-positive swabs for the presence of selected target peptides belonging to specific viral proteins within the swab samples. In this study, a preliminary test was performed by analyzing SARS-CoV-2-positive swabs confirmed by qPCR. This step was critical to evaluate the performance of the specific LC-MRM/MS assay aimed at direct and simultaneous monitoring of relevant viral proteins of SARS-CoV-2 according to the designed workflow shown in Figure 1.

**Development of the MRM Method.** Ten most suitable protein biomarkers of SARS-CoV-2 (P0DTC2, P0DTC3, P0DTC4, P0DTC5, P0DTC6, P0DTC7, P0DTC8, P0DTC9, P0DTD1, P0DTD2) were selected for the development of the LC-MRM/MS method on the basis of those more frequently identified by PCR and those occurring in the literature data.
An attempt was made to construct a comprehensive method to broaden the spectrum of applicability of the method as much as possible to be developed taking into account the specific instrumental performance.

A number of unique peptides belonging to each target protein were then defined on the basis of their mass spectrometric behavior together with their specific precursor ion−product ion transitions as defined by their unique amino acid sequence by in silico analysis using the Skyline software. The in silico data of the selected peptides were then compared with the literature data.\textsuperscript{16,21,22,29} Peptides defined by Skyline analysis monitored for each protein are reported in the Supporting Information Table ST2. This method contained a total of 77 peptides and 407 precursor ion−daughter ions transitions associated with the peptides selected from the defined target proteins that constitute the protein signature of CoV-2 as reported in Table ST2.

A specific peptide from human albumin (AEFAEVSK, MRM transitions 440.72 → 809.40 m/z; 440.72 → 680.36 m/z; 440.72 → 533.29 m/z; 440.72 → 462.25 m/z; 440.72 → 333.21 m/z) was also chosen as an internal standard to monitor the performance of the sample treatment protocol according to Singh et al.\textsuperscript{27,30}

Figure 2. Extraction of ion chromatogram (EIC) of peptides from the S1 portion (f16-685) of the SARS-COVID-2 spike. The most abundant, e.g., 558−566, was monitored in negative swabs with standard proteins at different concentrations. A comparison of the extracted ion chromatogram (EIC) between the outer and inner standards was reported in the lower panel.
Preliminary experiments were performed on a recombinant sample of the CoV-2 spike protein after tryptic digestion in solution. Figure 2 shows the extracted ion chromatogram (EIC) of the eight most intense peptide signals (top panel). The most abundant peak was observed for the 558–566 peptide, which was then selected as a quantifier to define the limit of detection (LOD) and limit of quantification (LOQ). A comparison of the EIC between the 558–566 peptide and the internal standard in different samples is shown in the lower panel.

Matrix Effect and Analytical Parameters. The matrix effect was studied using 20 qPCR-negative swab samples with different amounts of the standard spike protein in the range of 5 ng/μL to 5 pg/μL and subjected to tryptic digestion. The resulting peptide mixtures were then analyzed by the MRM method and the 558–566 peptide was monitored, resulting in LOD and LOQ values of 5 pg/μL and 15 pg/μL, respectively. In fact, a LOD of 5 pg/μL (36 attomoles/μL) was in agreement with the previous data.25 The precision, reported as RSD %, was calculated on N = 30 replicates for each concentration point. The injections were repeated five times a day for each concentration for 6 days. RSD % was estimated as lower than 10% for each concentration of spike standard protein analyzed. Accuracy (% Accuracy) 87.4–96.4% was determined following three repeated analyses on three different quality control (QC) samples prepared by spiking known quantities of spike protein (50, 500, 2000 pg/μL) in a virus-negative matrix and comparing the obtained value with corresponding standard protein solution according to the following formula:

\[
\%\text{ accuracy} = \frac{c_{\text{exp}}}{c_{\text{std}}} \times 100
\]

Preliminary Trial of the MRM Method on Real Samples. The MRM procedure was then tested in a preliminary trial with the analysis of five qPCR-confirmed SARS-CoV-2-positive swabs to verify which of the selected peptides were indeed suitable for mass spectrometric analysis in a real sample matrix. Proteins were extracted from the swabs in lysis buffer, reduced and alkylated with iodoacetamide, and purified by the solid-phase extraction on S-Trap microcolumns. The samples were digested with trypsin directly on the columns, and the resulting peptide mixtures were eluted and analyzed by mass spectrometry using the developed MRM method.

As an example, Figure 3 shows MRM TIC chromatograms of four peptides from the spike protein, namely, 346–354, 444–453, 558–566, and 634–645, together with their precursor ion–daughter transitions (left panels). In the right panel, the same peptides were compared with the peptide albumin used as an external standard (Figure 3, right panel). The different transitions perfectly co-eluted at the same retention time, thus indicating that they belong to the same precursor ion.

An accurate selection of peptides was carefully carried out to make the method as robust as possible that enabled us to monitor six COVID-19 proteins in human nasopharyngeal swabs. An analysis of the CoV-2-positive samples demonstrated that 5 peptides from the spike protein, 12 peptides from nucleoproteins (N), and 2 from membrane protein (M) were detected together with 2 unique peptides belonging to the envelope (E) and with 1 peptide for the P0DTC3 and P0DTC7 proteins as summarized in Table 1. A total of 23 peptides representative of the selected proteins were chosen for the optimized MRM method toward the monitoring of 128 transitions in a single LC−MS/MS run. The optimized MRM method contained all precursor ion–daughter ion transitions associated with the selected peptides from the defined target proteins that constitute the specific protein signature of the SARS-CoV-2 virus. Analysis of the qPCR-positive SARS-CoV-2 swab samples by the optimized MRM method resulted in a...
Table 1. List of Peptides Recorded in MRM Analyses of qPCR-Positive SARS-CoV-2 Swab Samples Together with the Number of Precursor Ion—Daughter Ion Transitions Monitored

| uniprot code and protein name | no. detected peptides | peptides | no. monitored transitions |
|-------------------------------|-----------------------|----------|---------------------------|
| P0DTC2 spike protein          | 5                     | 346−354  | 6                         |
|                               |                       | 444−453  | 5                         |
|                               |                       | 558−566  | 6                         |
|                               |                       | 634−645  | 6                         |
|                               |                       | 1000−1013| 6                         |
| P0DTC4 E protein              | 2                     | 38−60    | 6                         |
|                               |                       | 63−68    | 3                         |
| P0DTC5 M protein              | 2                     | 50−71    | 6                         |
|                               |                       | 72−100   | 6                         |
| P0DTC9 N protein              | 12                    | 14−31    | 6                         |
|                               |                       | 107−126  | 6                         |
|                               |                       | 127−142  | 6                         |
|                               |                       | 149−168  | 6                         |
|                               |                       | 169−176  | 6                         |
|                               |                       | 177−184  | 5                         |
|                               |                       | 209−225  | 5                         |
|                               |                       | 226−232  | 5                         |
|                               |                       | 237−247  | 6                         |
|                               |                       | 266−275  | 6                         |
|                               |                       | 361−368  | 5                         |
|                               |                       | 373−384  | 6                         |
| P0DTC3 ORF3a protein          | 1                     | 21−29    | 5                         |
| P0DTC7 ORF7                   | 1                     | 25−31    | 4                         |
| Total detected peptides       | 23                    | 128      |                           |

direct unequivocal assessment of the presence of CoV-2 virus in the different samples.

**Application of the MRM Method to Real Samples.** In addition to the development of MS methods using a classical LC–MS/MS approach, a nano-LC–MS/MS acquisition method in the MRM ion mode was developed for application to clinical swab samples that were also analyzed simultaneously by qPCR. The samples were prepared according to the procedure described above, and the entire protein content of the swabs was digested with trypsin on S-trap columns. The resulting mixtures of tryptic peptides were subjected to the developed MRM analytical procedure, and the results are summarized in Table ST3 where the peptides responding to MRM/MS analysis for each CoV-2 protein in each swab sample are shown.

More than 75% of the spike proteins reported on the ATLAS database were monitored for spike protein, but five putative peptides were selected due to the detection of some peptides marked as false-positive peptides, as they were also detected in negative swabs. The best instrumental response came from the FLPFQFPR sequence of spike [558−566] peptide used to test the analytical performance of the method (Figure 3). The detection of this peptide was shared with other studies to compare the concordance of the MS method with the current gold standard assay on swab samples previously tested as positive by positive quantitative reverse transcription PCR (RT-qPCR).24 A high percentage of N protein sequence peptides were similarly monitored as reported by others,21,16,20,21 while fewer authors monitored other proteins besides S and N proteins in real samples. In fact, lower sequence coverage has been reported in full scan experiments for the small membrane protein envelope (E), membrane protein (M), or ORF proteins in virus-enriched cells.25,31

A minimum number of six transitions were monitored for most peptides to increase the selectivity and specificity of the method. Indeed, two or four MRM transitions per peptide previously monitored22,21 might be inappropriate due to the complexity of the matrix to be analyzed.

When these data were compared to the relative qPCR results, MRM analysis was shown to unambiguously detect the specific viral protein components present in the different samples through their unique peptide signature, thus leading to direct identification of the presence of CoV-2 virus in the swabs. A perfect agreement with the qPCR data was observed: no peptide was detected in the qPCR-negative samples.

In addition, a positive correlation between the MRM results and the viral load of the different samples was also observed. Positive swab samples were grouped according to their cycle threshold (Ct) values. These values correspond to the number of RNA duplication cycles required to detect viral RNA in qPCR analyses and correlate with the viral load present in each sample. Table ST3 shows that almost all selected peptides and their respective transitions could be detected by the MRM method in swab samples with Ct values below 35, corresponding to a high-to-moderate viral load. In contrast, fewer peptides and mass transitions were observed in swab samples with higher Ct values representing mild to low viral load, with nondetection of three peptides from spike protein, five from nucleoprotein N, and one from P0DTC3_ ORF3a. Two peptides from protein E were successfully detected especially for subjects with Ct values below 35 (Table ST3).

Other authors have compared MS data with PCR data, pointing out that the MS test developed allowed detection of viral proteins in 200 positive patients with Ct below 2512,28 or even in negative patients,27 highlighting the higher sensitivity of the MS technique. Actually, the comparison between two techniques based on different analytical principles related to the specific molecular nature is reasonably debated, but several authors have attempted that due to the availability of mass spectrometry, which shows high sensitivity and specificity. Therefore, we observed a different response for each of the monitored peptides related to the specific ionization efficiency; however, at the end of the day, the data matched those of the qPCR tests if all of the monitored peptides were observed.

The main advantage of using the MS apparatus comes from the highly sensitive multiplexed detection of proteins in a single run, whereas qPCR-based assays detect three proteins. The targeted MS approach hides its selectivity in the great possibility of recording only specific pairs of mass-to-charge ratios selected as representative of the specific peptide sequence to be monitored for each protein. Therefore, the extensibility of the developed MRM method to each protein opens the way to detect any viral protein, even those showing genetic mutations compatible with the limits of instrumental sensitivity. Therefore, the unique flexibility of the targeted MRM approach provides a more complete picture of the viral peptidome including the monitoring of variant sequences that are currently required to follow the evolution of the pandemic.

Combining the results of both viral protein and RNA qPCR detection may make the detection of SARS-COV-2 more reliable by providing two orthogonal and complementary approaches and offering a valid, time-efficient viral diagnostic tool for the detection of virus-positive individuals. Of
importance, none of these n. 81 samples analyzed were found false negative for qRT-PCR with positive MRM results, thus suggesting that the method has a good sensitivity to give a low false-negative rate. Recently, PCR-MS has been proposed as a new technology to directly identify known pathogens from clinical samples and to identify genetic evidence of undiscovered pathogens. The requirement for miniaturization systems based on easily usable biosensor devices has been widely reviewed as a promising alternative to conventional diagnostic methods. New MS applications aligned with airport screening or rapid forensic scanning offer horizons of unexplored possibilities. Indeed, protein-based MS methods could offer a great opportunity to support routine qPCR-based assay analysis to be implemented in clinical practices. Furthermore, recent advances in sgN RNA transcript detection to focus on the highest viral load could be supported by MS analysis in the discovery of new markers of early and more severe infection.

**CONCLUSIONS**

The present work aims to offer a method based on LC−MS/MS in the MRM ion mode to detect six COVID-19 proteins in human gold/nasopharyngeal swabs for monitoring 128 transitions that define the molecular response of viral sequence portions, suggesting a more complete picture of the viral peptidome. Analyses performed in parallel on the same swabs allowed direct comparison of the two techniques based on viral and protein qPCR (MS data). Almost a hundred clinical samples were tested to validate the reliability of the optimized LC-MR by confirming the positivity detected by qPCR at different replication cycle values. The versatility of the LC-MRM/MS method offers valuable support to the RNA-based technique to also detect the newly arising variant and highlight new insights for the development of new types of clinical diagnostics including both RNA and protein expression from the same swab and other human samples with the aim of verifying active and replicative viruses during early and late infections to determine disease progression.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05587.

List of codes identifying each swab sample (81 were positive to SARS-COVID-2 and 20 negative). For each sample, values of cycle threshold (Ct) were recorded for E, RdR, and N genes by qPCR analysis (Table ST1) (XLSX).

MRM method reporting selected peptide sequence, m/z precursor, m/z product ions, collision energy (CE), and cone voltage (CV) for each selected protein (Table ST2) (XLSX).

Matching between qPCR and LC-MRM/MS data for each swab sample (Table ST3) (XLSX).

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**Author Contributions**

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**Author Contributions**

G.P., A.I., and C.F. performed the methodology, sample preparation, and MS analyses. V.F., F.Q., C.D.D., and B.I. were responsible for the methodology and experimental design of molecular gene evaluation. G.P., A.I., C.F., and A.A. performed the MS data interpretation. M.Z. provided critical evaluation and acquired grant support. P.P. and G.M. supervised the study. All authors contributed to the writing, editing, and revision of the manuscript.

**Notes**

The authors declare no competing financial interest.

The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of Federico II University (no. of protocol 000576 of 10 April 2020). Informed consent was obtained from all subjects involved in the study.

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