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Serum proteomic profiling reveals potential inflammatory biomarkers in long-COVID patients: a comparative analysis with healthy controls

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Abstract. – OBJECTIVE: The highly transmissible severe acute respiratory syndrome-Coronavirus-2 was responsible for the 2020 COVID-19 pandemic. COVID-19 mostly affects the respiratory system; however, this infection also affects several other organs. In addition, the sequelae of this disease affect patients for several months after recovery, resulting in long-COVID syndrome.

PATIENTS AND METHODS: In order to characterize the differences between healthy control individuals and long-COVID patients, proteomic profiling of the serum of both groups was performed by mass spectrometry. The obtained data were analyzed with multivariate and univariate statistical analyses.

RESULTS: Initially, performing a partial latent square discriminant analysis (PLS-DA) made it possible to identify thirty-three proteins of interest, which were then subjected to a receiver operating characteristic (ROC) analysis. Four proteins were identified as potential standalone biomarkers: Sirtuin 1, Natriuretic Peptide B, Hemopexin, and Arachidonate 5-Lipoxygenase. Moreover, a multivariate ROC analysis identified a panel of biomarkers composed of Natriuretic Peptide B, Anterior Gradient 2 Protein, Adiponectin, Endothelin Converting Enzyme 1, Interferon Induced Transmembrane Protein 1, Mannose Binding Lectin 2, Prostaglandin-Endoperoxide Synthase 2, Pirin, Prostaglandin Reductase 1 and Cystatin C.

CONCLUSIONS: The identified biomarkers are associated with inflammatory processes, corroborating literature evidence that long-COVID patients develop an inflammatory state that damages many tissues. Nevertheless, these data should be validated in a larger cohort.

Key Words:
COVID-19, SARS-CoV-2, Sirtuin 1, Natriuretic Peptide B, Hemopexin, Arachidonate 5-Lipoxygenase, Transmembrane Protein 1, Mannose Binding Lectin 2, long-COVID syndrome.

Introduction

In 2020, an outbreak of COVID-19 was identified in Wuhan; the virus, designated as severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2),

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was easily transmitted from human to human, turning into a worldwide pandemic in just a few months[1]. Since this virus can replicate within the epithelial mucosa of the respiratory tract and move into the lungs, the most affected organ system is the respiratory one. However, it is known that this viral infection triggers extensive immune responses throughout the body, which are not restricted to the respiratory system. Recent studies[2] indicate that SARS-COV-2 triggers an exacerbated and uncontrolled inflammatory response: after the initial innate immune response, the adaptative immune response is compromised and hyperinflammation becomes chronic. In fact, COVID-19 infection has been associated[3,4] with the development of multisystem inflammatory syndrome or the triggering of autoimmune diseases.

After discharge, patients who have been subjected to the intensive care unit (ICU) have an increased probability of readmission to nursing care and usually suffer from chronic pain[5]; moreover, some of their symptoms overlap with those of myalgic encephalomyelitis or chronic fatigue syndrome[6]. In addition, other organs that may be affected by this disease include the liver, kidney(s), or even the brain[7]. Particularly, in the first three months post-infection, most of the patients experienced neurological disturbances[8], as part of a condition that was named post-COVID or long-COVID syndrome[6-8]. Therefore, it is crucial to identify the chronic peripheral changes triggered by COVID-19 infection. Here, we present a proteomic profile of the serum proteome of or long-COVID patients compared to those of healthy controls. This approach could allow a comprehensive characterization of the alterations induced by the infection.

Patients and Methods

Study Cohort

Adult patients who presented serious outpatient clinic manifestations and severe or long-COVID syndrome have been enrolled. This case-control study involved 80 patients and 50 normal controls. 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 after 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 processes were conducted according to the ethical guidelines of the Declaration of Helsinki (1975). A written informed consensus was obtained from all patients at the time of enrollment, and each of them was assigned a unique alphanumeric code to protect their anonymity.

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

Clinical Manifestations and Questionnaries

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 recovery were measured with a classical scale, from 0 (absent) to 10 (severe). Moreover, patients handed in their clinical data and declared all therapies underway. The severity of pain, fatigue, sleeplessness, and anxiety was assessed using specific questionaries: Visual Analogic Scale (VAS), Chalder Fatigue Scale (CFS), Insomnia Severity Index (ISI), Hamilton Anxiety Rating Scale (HAM-A).

VAS is a unidimensional and simple measure of pain intensity, used for the first time in 1921 by Hayes and Patterson, and it has been reliable since then[9]. This test is visually represented by a straight horizontal line of fixed length (100 mm), with the left end indicating “no exhaustion at all” and the right end indicating “complete exhaustion”. The value is 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 mm, mild to moderate from 10 mm to 50 mm, severe >50 mm.

The final version of the CFS test, revised in 2010[10], was used to measure *via* an 11-item questionnaire 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, with items 1-7 representing physical fatigue and items 8-11 representing mental fatigue. 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). CFS can be used in two different ways: Biomodal scoring and 4-point Likert scoring. In the bimodal model, all 11 items are loaded onto a general fatigue factor. Lower scores indicate a low level of fatigue, whereas high scores represent high levels of fatigue[11].

As previously reported[12], a cut-off score of ≥18 has been used to diagnose chronic fatigue in adolescent subjects. A reliable and validated method for detecting cases of insomnia in the population is the ISI questionnaire, consisting of seven questions; it is a simple test that can be used to identify cases of sleeplessness[13]. A total score of 0-7 indicates "no clinically significant insomnia", 8-14 means "subthreshold insomnia," 15-21 means "clinical insomnia (moderate)," and 22-28 means "clinical insomnia (severe)". Finally, psychological and somatic symptoms of anxious mood were assessed by the HAM-A scale[14]. This questionnaire is a clinician-based questionnaire consisting of 14 items with a score ranging from 0 (no symptomatology) to 4 (severe anxiety), with a total sum of 56[15]; the cut-off was set at 17. As a consequence, a total score ≤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.

LC MS/MS Analysis

Mass spectrometry analysis was performed on the serum samples obtained from the recruited long-COVID syndrome patients. The analysis was conducted using a High-performance liquid chromatography (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 used, 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, (I.S.B. - Ion Source & Biotechnologies, Bresso, Milan, Italy) and electrospray ionization (ESI) as the ionization source.

Data Processing

The data was normalized, and proteins were selected in accordance with the literature; all the proteins processed in the analysis are represented in **Supplementary Table I**. Briefly, proteins that have been previously described to be associated with the disease were selected for statistical analysis.

Statistical Analysis

Proteins previously selected were subjected to further analysis. The normalized spectral counting values were imported to Metaboanalyst (available at: https://www.metaboanalyst.ca/) and data was scaled using the "autoscaling" method. Then, a multivariate analysis was performed, both unsupervised and supervised, respectively: principal component analysis (PCA) and partial latent squares discriminant analysis (PLS-DA), as well as a clustering analysis. In addition, proteins were also subjected to a univariate analysis (*t*-test). Proteins with a Visual Infusion Phlebitis (VIP) score >1, $p<0.05$ were selected for different receiver operating characteristic (ROC) curve analysis (AUC>0.98).

Results

A proteomic analysis was performed to identify potential biomarkers in the serum of long-COVID patients. To accomplish this, the proteomes of the two groups were compared. However, given the complexity of these samples and the high abundance of certain proteins (such as albumin), we conducted a literature search and identified proteins associated with metabolism, immune response, inflammation, or mitochondrial function for the subsequent statistical analysis.

The first step was to compare the levels of these proteins in both the control and long-COVID groups, using a multivariate and univariate analysis. Both PLS-DA and PCA analysis (Figure 1a, and **Supplementary Figure 1a**) showed a clear distinction between the two groups; in addition, a clustering analysis separated control and long-COVID groups (Figure 1b). Also, the heatmap profiling enhanced the differences between the two groups. Together, these analyses allowed the identification of relevant proteins that could be used as potential biomarkers.

Proteins were then filtered based on (a) statistical analysis, considering proteins with a VIP>1 (PLS-DA) and $p<0.05$ (*t*-test); (b) coefficient of variation (CV), where only proteins with CV<10 were considered; (c) and fold change (FC), for which proteins with FC<0.67 (total proteins=4) or FC>1.5 (total proteins=29) were selected for further analysis.

Next, a PCA model was built only with the proteins of interest (Figure 2a). This analysis demonstrates a clear separation between the two groups; moreover, it is possible to observe a group clustering along the PC2 axis within the long-COVID group. Additionally, these proteins were subjected

to an ROC curve analysis. Using a univariate approach, where each protein is studied individually, it was possible to identify antithrombin as a potential biomarker since it has an area under the curve (AUC) near 1, and its levels are significantly increased in long-COVID patients compared to normal controls (Table I and Figure 2c,d).

Other proteins, displaying an AUC>0.98, were also identified as potential biomarkers: Sirtuin 1, Natriuretic Peptide B, Hemopexin and Arachidonate 5-Lipoxygenase (**Supplementary Figure 2**).

In addition, a multivariate ROC method, using linear SVM as a classification method, identified that a combination of ten proteins would also result in an AUC close to 1 (Figure 2b). Except for Natriuretic Peptide B (previously identified as a potential stand-alone biomarker), a panel of biomarkers composed of Anterior Gradient 2 Protein, Adiponectin, Endothelin Converting Enzyme 1, Interferon Induced Transmembrane Protein 1, Mannose Binding Lectin 2, Prostaglandin-Endoperoxide Synthase 2, Pirin, Prostaglandin Reductase 1, and Cystatin C was identified (**Supplementary Figure 3**). Interestingly, most of these proteins did not have significant AUC values as a stand-alone biomarker.

Finally, a correlation between the proteins of interest and the clinical manifestations was performed. Information was available for 75 patients, concerning their symptomatology during COVID infection: age, patient type (outpatient or hospitalized), clinical manifestations (asymptomatic or mild or severe symptoms), Chest x-ray/CT (positive for COVID-19), pneumonia and myocarditis/pericarditis. Myocarditis/pericarditis and age were not correlated with any other symptomatology (**Supplementary Figure 4a**). Moreover, for 31 individuals, it was possible to obtain information about their long-COVID clinical manifestations. The patient type was positively correlated with concentration and memory deficits, as well as anxiety or depression (Figure 3a). Also, the severity of symptoms was correlated with the presence of dyspnea.

To further explore if there was any protein associated with this phenotype, a correlation analysis was performed. A positive correlation (above 0.5)

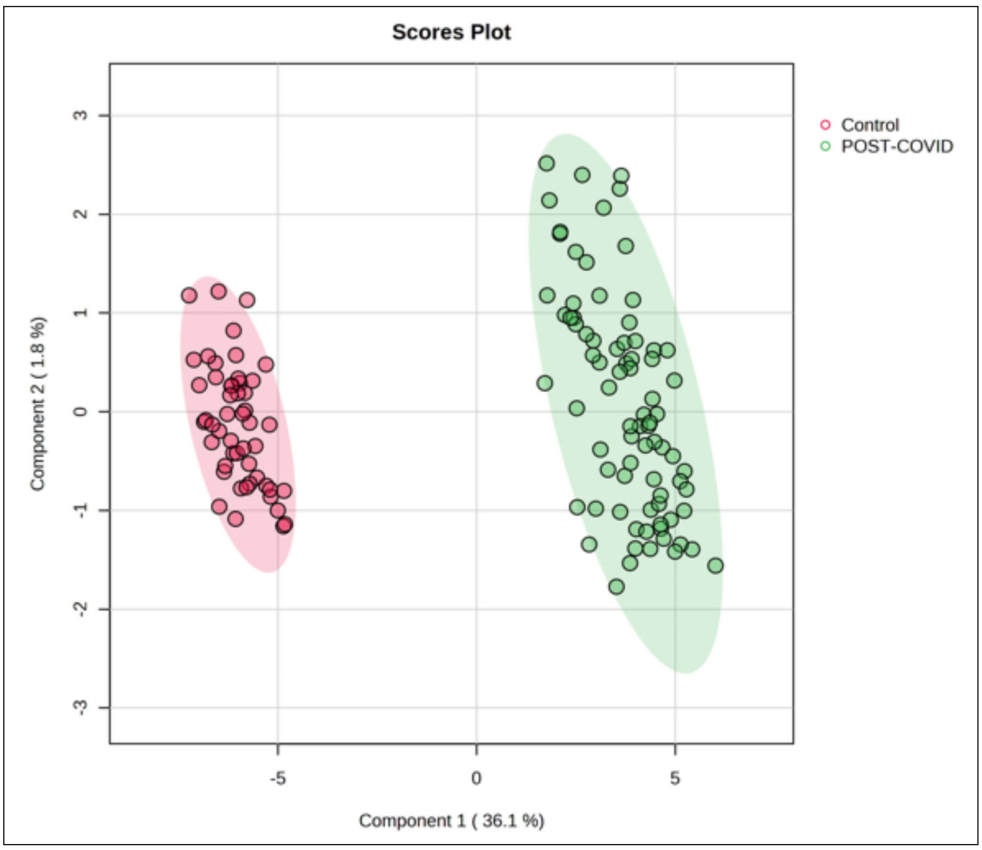

Figure 1a. Control and long-COVID patients have distinct proteomic profiles. By applying a multivariate analysis (PLS-DA) to the literature-selected proteins, it was possible to observe a clear distinction between the two groups (**a**).

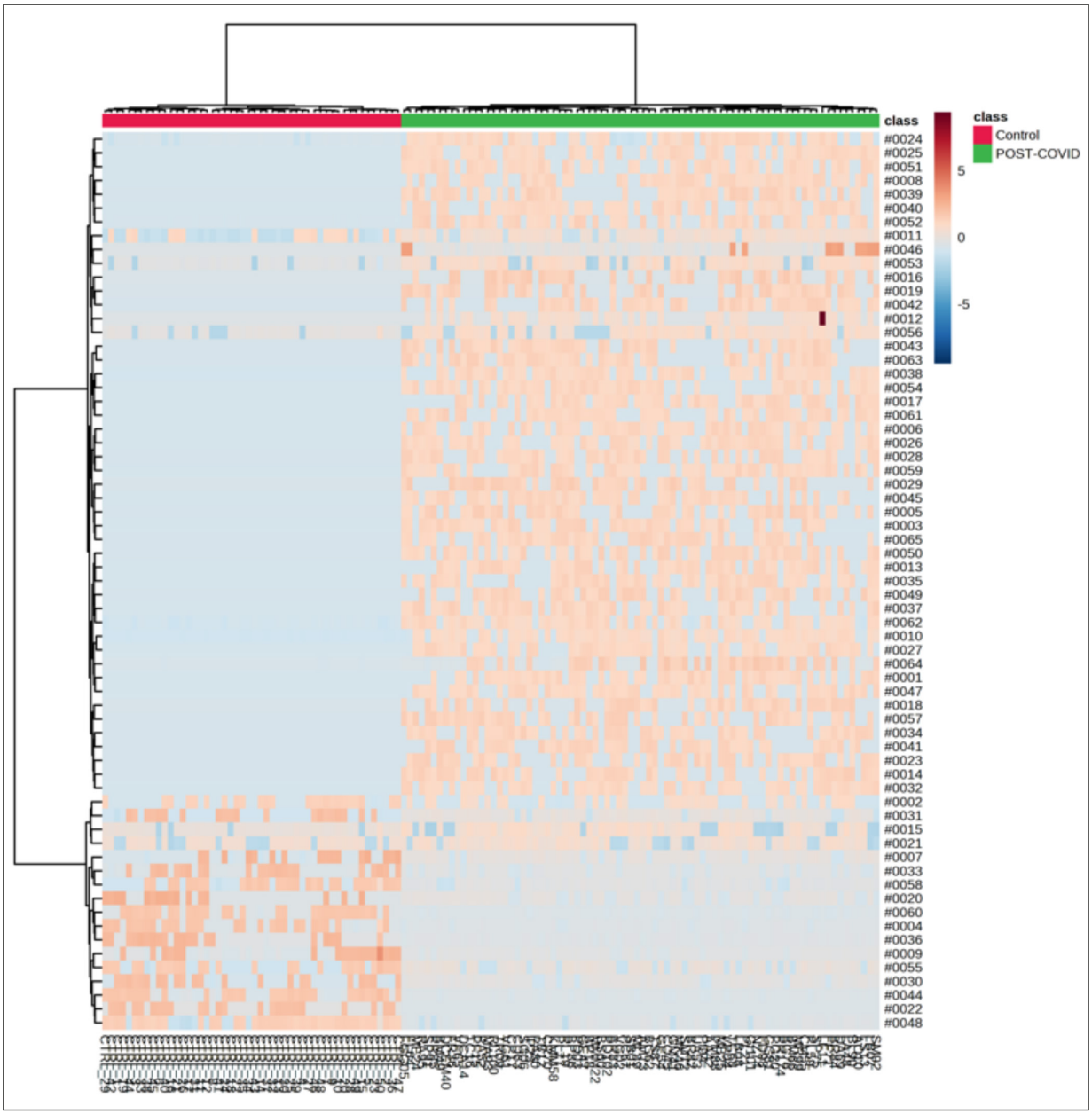

Figure 1b. From this heatmap, we can observe how clustering analysis segregated the two groups, Patients *vs.* Control, and the differences in the proteomic profile that we found in it (**b**).

was found between dyspnea and glucocorticoid receptors (Figure 3b) and between memory deficit and antithrombin (Figure 3e). Moreover, Arachidonate 5-Lipoxygenase was negatively correlated (below -0.5) with tremors and hair loss (Figure 3c, d). Also, although with a weaker correlation, Mannose Binding Lectin 2 was found to be positively linked to asthenia (**Supplementary Figure 4b**), Antithrombin with concentration deficit (**Supplementary Figure 4c**), and Endothelin-2 with muscle aches (**Supplementary Figure 4d**).

Discussion

Both supervised and unsupervised multivariate analysis allowed a clear separation between the control and long-COVID groups (Figure 1a and **Supplementary Figure 1a**). However, it is possible to observe that the control group is more homogeneous than the long-COVID group. This idea is reinforced after the selection of the proteins of interest, where the control group shows a smaller dispersion within the group. Interestingly, it is

Table I. Enrichment (Fold change) of proteins in long-COVID *vs.* normal control groups.

Protein name	Fold change
Coenzyme Q2	0.21
Translocase Inner Mitochondrial Membrane 50	0.36
Adenosine Monophosphate Deaminase 1	0.61
* Interferon Induced Transmembrane Protein 1	0.65
Angiotensin I Converting Enzyme	1.67
Organic Cation Transporter 1	2.40
* Sirtuin 1	2.60
* Adiponectin	2.88
Contactin 2	2.93
* Prostaglandin-Endoperoxide Synthase 2	2.94
* Anterior Gradient 2, Protein	3.08
Translocase Inner Mitochondrial Membrane 23	3.34
* Pirin	3.39
DNA Topoisomerase III Beta	3.71
Endothelin-2	3.72
Endothelin Converting Enzyme 1	3.81
Carnitine Palmitoyltransferase 2	3.91
Tumor Necrosis Factor-Alpha	4.14
Transmembrane Serine Protease 2	4.34
Dipeptidyl Peptidase 4	5.10
VWF-Cleaving Protease	5.24
Alpha-1 Antitrypsin	5.71
Von Willebrand Factor	5.90
* Cystatin C	6.19
* Prostaglandin Reductase 1	6.45
Growth Differentiation Factor 15	6.52
PPARgamma Coactivator	8.55
* Mannose Binding Lectin 2	9.41
* Hemopexin	10.93
Glucocorticoid Receptor	16.84
* Natriuretic Peptide B	19.65
* Arachidonate 5-Lipoxygenase	27.22
* Antithrombin	31.08

possible to cluster the groups of long-COVID patients and normal controls based on the PCA model (Figure 2a). This heterogeneity might be related to sex, medication, or other pathologies (for which no information was provided by the clinicians).

Antithrombin was the stand-alone biomarker with the highest score. It is a plasma protease involved in the coagulation cascade that is responsible for inactivating thrombin[16]. However, the literature indicates that antithrombin III levels were decreased in non-survivors of COVID-19[17]. In the present study, the levels of antithrombin were also positively correlated with concentration and memory deficits that manifested after COVID-19 infection.

Other unique biomarkers were identified as potential biomarkers. Sirtuin-1 is a protein deacetylase, involved in response to oxidative stress and inflammatory processes[18]. In fact, this protein has already been described[19] to be involved in the inflammatory processes triggered by COVID-19 infection. Another potential biomarker was Arachidonate 5-Lipoxygenase, which is involved in processes that modify arachidonic acid and consequently modulate chemokine production and inflammation[20]. This enzyme has already been described[21] to mediate COVID-19 infection. Here, our team identified that the levels of this protein were negatively correlated with tremors and hair loss as long-COVID sequelae.

Moreover, Natriuretic Peptide B is a hormone released by heart ventricles, and its levels are proportional to heart failure[22]. This molecule has already been proposed[23] as a COVID-19 biomarker since it is known for being elevated in patients with severe symptomatology. Finally, Hemopexin, a glycoprotein that is responsible for protecting the organism from heme toxicity, was also identified as a candidate biomarker[24]. The literature describes a tendency of increased levels of this protein in infected patients, compared to healthy volunteers, although without statistical significance[25].

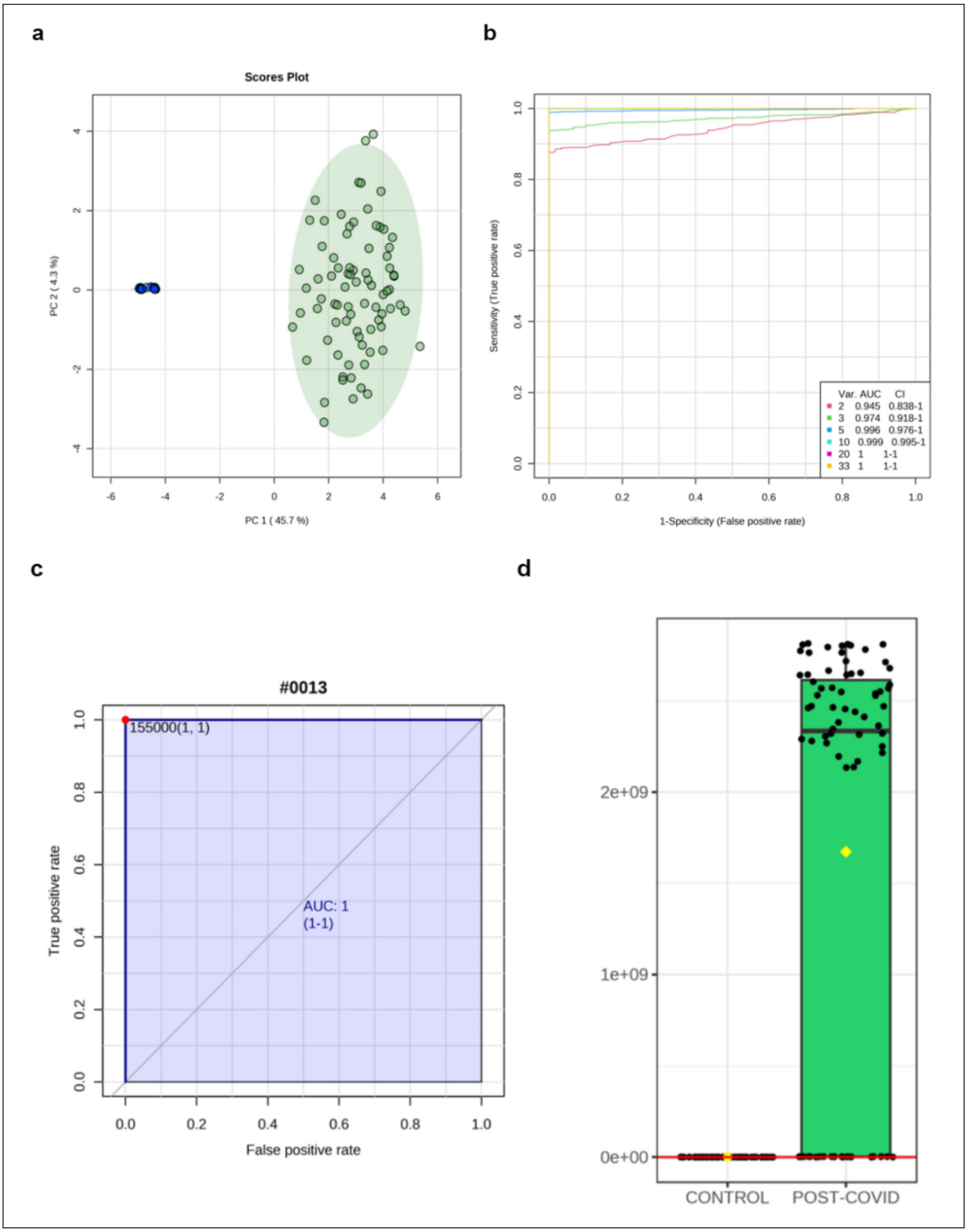

Figure 2. Panel of biomarkers distinguishing control and long-COVID groups. A PCA model was built using the filtered proteins (**a**). Then, selected proteins were analyzed *via* ROC curve analysis, using both a multivariate approach (**b**) and a univariate one (**c**). Antithrombin was the stand-alone protein with the highest score (**d**).

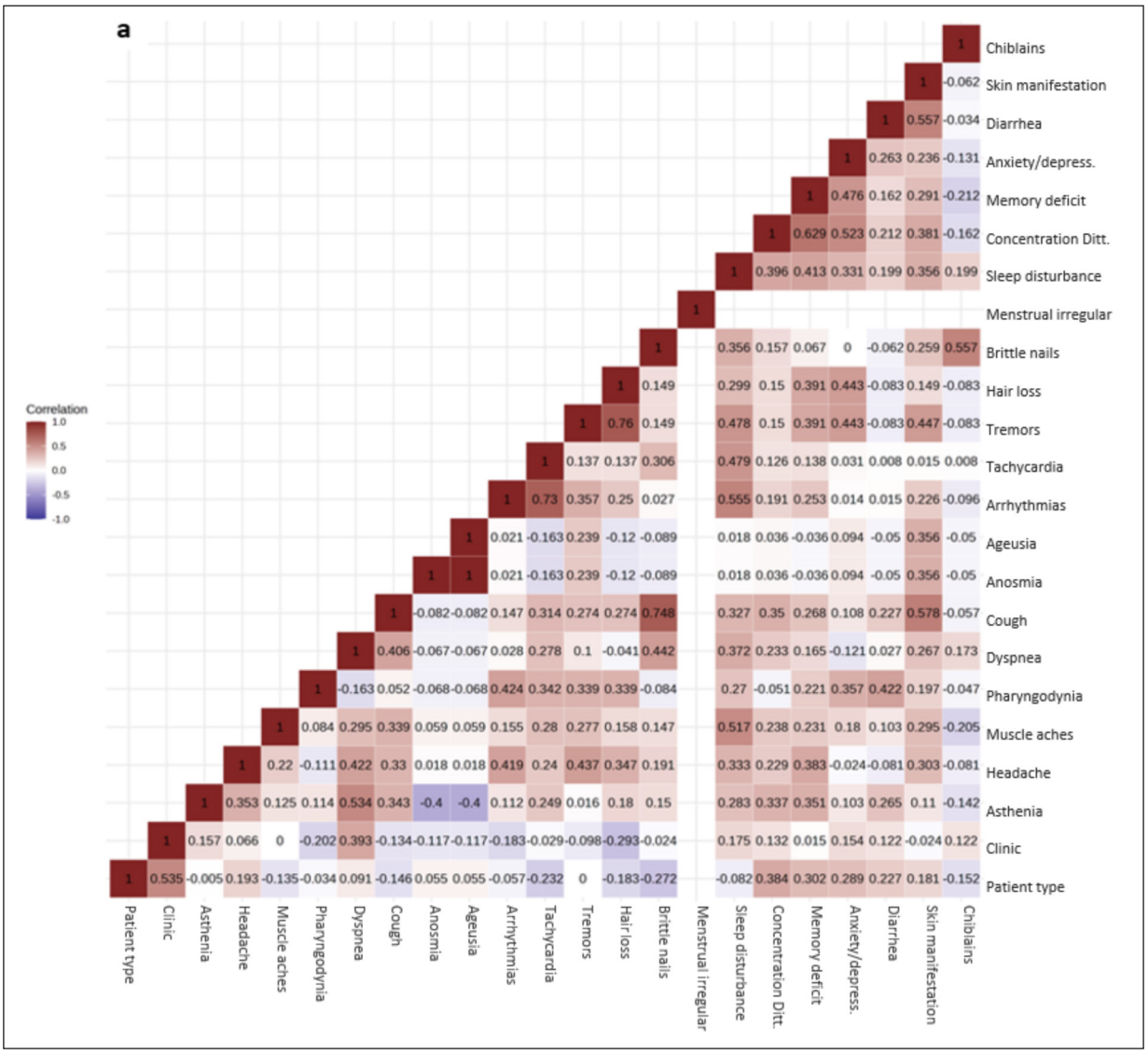

Figure 3a. long-COVID clinical manifestations correlations. The patient type was positively correlated with concentration and memory deficits, as well as anxiety or depression.

In combination with Natriuretic Peptide B, a panel of biomarkers was identified using a multivariate ROC approach, composed of Anterior Gradient 2 Protein, Adiponectin, Endothelin Converting Enzyme 1, Interferon Induced Transmembrane Protein 1, Mannose Binding Lectin 2, Prostaglandin-Endoperoxide Synthase 2, Pirin, Prostaglandin Reductase 1, and Cystatin C. Anterior Gradient 2 is involved in protein folding[26], and it has been previously associated with in-ICU mortality[27]. Adiponectin plays a role in carbohydrate and lipid metabolism[28], and its lower levels were associated with COVID-19 hospitalization[29]. In the present study, levels of Adiponectin are significantly elevated in long-COVID patients. Endothelin Converting Enzyme 1 produces active endothelin 1, a vasoconstrictor peptide[30,31] that was correlated with admission to ICU and severe symptoms[32].

Furthermore, Interferon-Induced Transmembrane Protein 1 was already described[33] to restrict RNA viruses. Of all the identified potential biomarkers, this was the only one with a statistically significant decreased level. Particularly, the isoform Interferon-Induced Transmembrane Protein 3 has been associated[34] with severe SARS-COVID-19 and hospitalization. Prostaglandin-endoperoxide Synthase 2 and Prostaglandin Reductase 1 are involved in arachidonic metabolization and inflammatory pathways[35,36]. Taken together, several

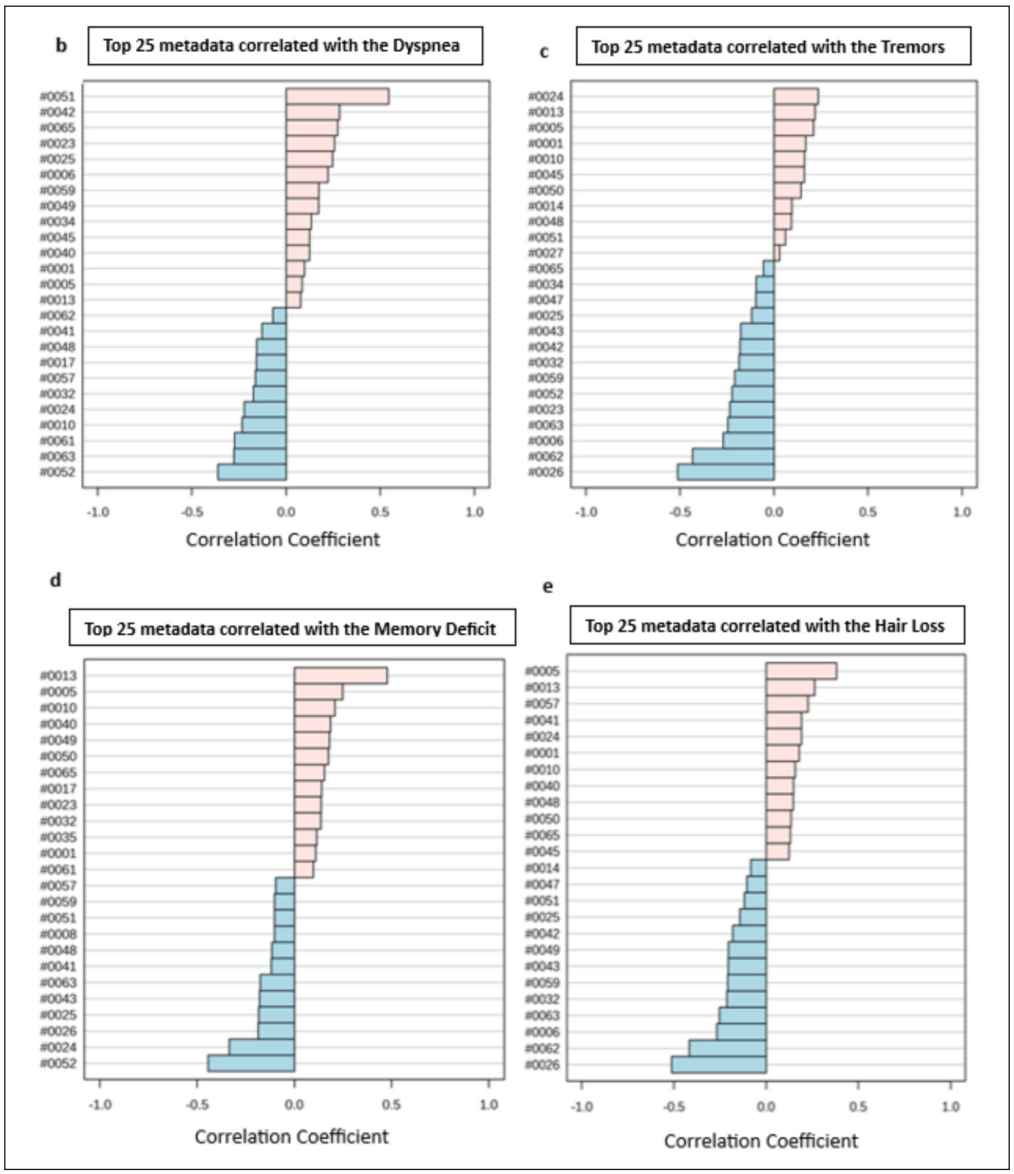

Figure 3b-e. A positive correlation was found between dyspnea and Glucocorticoid Receptor (#0051) (**b**) and between memory deficit and Antithrombin (#0013) (**d**). Moreover, Arachidonate 5-Lipoxygenase (#0026) was negatively correlated (below -0.5) with tremors and hair loss (**c, e**).

proteins identified as potential biomarkers are involved in inflammation and arachidonic pathway. Since the samples were collected after recovery, this might indicate that individuals still exhibit a chronic inflammatory state over time. Also, due to the increased levels of Mannose Binding Lectin 2, complement activation appears to be altered[37]. Alternative complement activation is associated with mortality due to COVID-19[38,39].

Comparable to the role of Sirtuin-1, pirin is also an oxidative stress sensor[40]. Polymorphisms and ethnic differences were associated with different SARS-CoV-19 severity, suggesting that it plays a crucial role in the pathophysiology of the disease[41].

Finally, Cystatin C is a marker of acute renal failure[42], and its levels are associated with a worse prognosis[43]. Taken together, these data suggest that, apart from chronic inflammation, other organs (such as the kidneys and the heart) are deeply affected, which could be harmful in the long term.

Despite not being associated as a biomarker, Glucocorticoid receptor levels were associated with dyspnea. This receptor has already been used[44] as a therapeutic target for many inflammatory diseases requiring an anti-inflammatory respon-

se. In fact, this therapeutic approach was already recommended even for COVID-19 treatment[45,46].

Limitations

One of the limitations of this study is its reduced cohort size. To validate these data, a higher number of samples must be collected. Also, individuals from different centers should be included, to increase heterogeneity among the participants. This is particularly important when analyzing follow-up clinical manifestations, and in this study, the sample size was deeply affected by the availability of such data. Moreover, additional time points would enrich the study and the assessment of its outcomes.

In summary, the present study identified potential biomarkers that allowed to distinguish between healthy controls and long-COVID individuals. A panel of multiple proteins mainly involved in the inflammatory response has also been identified, suggesting that a chronic inflammation state persists in a number of patients even after recovery from COVID-19 infection. Moreover, proteins associated with specific organs (like the heart or the kidneys) should be used to monitor possible sequelae in peripheric organs.

Conclusions

The identified biomarkers are associated with inflammatory processes, corroborating literature evidence that long-COVID patients develop an inflammatory state that damages many tissues. Nevertheless, our results should be validated in a larger cohort.

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Availability of Data and Materials

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

Ethics Approval

The study was conducted in accordance with the 1975 Declaration of Helsinki. 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

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

Conflicts of Interest

The authors declare no conflict of interest.

Informed Consent

A written informed consensus was obtained from all patients at the time of enrollment, and each of them was assigned a unique alphanumeric code to protect their anonymity.

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