Original Article

Discovery Phase of Serum Proteomic Analysis in Post-COVID-19 Syndrome Patients using Two-Dimensional Electrophoresis

Aisyah Badhrulhisham[1], Norbaiyah Mohamed Bakrim*[1], Wan Nurliyana Wan Ramli[2], Nor Zamzila Abdullah[3], Salmah Anim Abu Hassan[4], Hairul Aini Hamzah[1], Mohd Yusri Idorus[5], Alfi Khatib[6]

ABSTRACT

Objective

Post-COVID-19 Syndrome (PCS) significantly, adversely affects the quality of life of persons who continue to have symptoms following an initial COVID-19 infection. Hence, the identification of reliable biomarkers for PCS is crucial for understanding its pathophysiology, aiding in diagnosis, prognosis, and devising therapeutic strategies. This study aimed to compare the serum proteomic profiles between PCS and non-PCS (NPCS) patients to identify potential protein biomarkers that could distinguish these two groups.

Materials and methods

PCS patients were recruited at the post-COVID-19 clinic and confirmed diagnosis by a physician, with prolonged symptoms beyond three months post- infection. While non-PCS patients were individuals who had fully recovered from an acute COVID-19 infection. Proteins from pooled serum of six PCS patients and ten NPCS participants, matched for age, gender, and race were isolated and separated by two-dimensional electrophoresis (2-DE). PD Quest software was used for analysis, and protein expression with more than a twofold and significant difference were recognised. Subsequently, proteins of interest were identified using Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF) Mass Spectrometry.

Results and discussion

All subjects were Malay females with a mean age of 38 ± 9.7 for the PCS group and 40 ± 11.1 for the NPCS. The most common clinical symptoms were persistent cough, dysponea, and fatigue. There were 182 protein spots expressed in serum PCS patients in a range of pH 4 to 7. Two proteins, Haptoglobin and T-cell surface glycoprotein CD8 alpha chain, were found to be significantly overexpressed, while two proteins, namely Vitamin D-binding protein and Immunoglobulin heavy constant alpha 1, were under-expressed in PCS patients when compared to NPCS subjects.

Conclusion

This discovery phase of proteomic analysis revealed several candidate proteins that are predominantly involved in inflammation and immune response in PCS. These proteins require additional examination during the verification phase to assess their capability in identifying COVID-19 individuals at an elevated risk of developing PCS. (309 words)

Keywords

post COVID-19 syndrome; proteomic analysis; two-dimensional electrophoresis

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is an infectious ailment caused by a unique strain of coronavirus known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which was identified in December 2019 in Wuhan, China. The World Health Organization has reported approximately 775 million cases and recorded 7 million deaths since the disease's emergence nearly four and a half years ago.[1] Significant efforts have focused on understanding both the acute phase and the lasting effects of COVID-19, which continue to raise complex and persistent concerns. Growing

1. Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
2. Department of Internal Medicine, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
3. Department of Pathology and Laboratory Medicine, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
4. Department of Orthopaedics, Traumatology & Rehabilitation, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
5. Institute of Medical Molecular Biotechnology, Faculty of Medicine, UITM Sungai Buloh, 4700 Sg. Buloh, Selangor, Malaysia.
6. Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.

Correspondence

Norbaiyah Mohamed Bakrim, Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia. Email: drbaiyah@iium.edu.my , HP: 013 673 0407

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evidence shows that some individuals who recover from the initial infection still experience lingering symptoms for weeks or months, affecting multiple organ systems and reducing quality of life. This condition has been referred to by various terms, including post-COVID-19 syndrome,[2] post-COVID-19 condition,[3] long COVID,[4,5] chronic COVID, and post-acute COVID-19.[6]

Post-COVID-19 Syndrome (PCS) refers to "signs and symptoms that develop during or following an infection consistent with COVID-19 that continue for more than 12 weeks and are not explained by an alternative diagnosis."[7] Meta-analysis of estimated pooled prevalence of post-COVID-19 condition showed the prevalence of 43% to 45% globally.[3,8] Reports from Malaysia indicated that 3.4% of respondents to a questionnaire using the My Sejahtera application matched the post-COVID-19 condition.[9] Numerous studies indicated that fatigue is among the most prevalent symptoms reported, alongside forgetfulness and a persistent cough.[8,9] It is noteworthy that the introduction of COVID-19 vaccination programs led to a reduction in the prevalence of PCS. Receiving two vaccine doses has been shown to reduce the risk of developing PCS compared to one dose or no vaccination.[10,11] However, vaccines do not significantly alleviate symptoms if administered during active PCS.[12,13] Despite the vaccination rollout, PCS continues to impact many, highlighting the need for further research to better understand and address this condition.

The pathophysiology of post-COVID syndrome is unclear, with suggested mechanisms comprising immune dysregulation, persistent inflammatory responses, autoimmunity, endothelial dysfunction, viral persistence, coagulation activation, ongoing circulation of SARS-CoV-2 spike proteins, and the emergence of mast cell activation syndrome (MCAS).[5,14–16] However, researchers are currently investigating the precise molecular signatures and biomarkers associated with this syndrome, which remain unclear. Clinical diagnosis of PCS still relies on the clinical symptoms and the exclusion of other causes.[17]This approach is time- consuming, involves various tests and procedures, and leads to delayed initiation of interventions and management. Identifying reliable biomarkers for post-COVID syndrome is paramount for understanding its aetiology, improving diagnosis and predicting outcomes, and developing targeted therapeutic interventions.[18]

Proteomics, the extensive examination of proteins and their activities, provide a robust method for elucidating the molecular framework of illnesses and disorders.[19] Serum, an easily accessible biofluid, contains a wealth of protein biomarkers that reflect physiological and pathological states within the body. By employing proteomic techniques, researchers can comprehensively analyse the serum proteome to identify alterations specific to post-COVID syndrome.[20] A plasma proteomic study utilising proximity extension assay identified 119 protein biomarkers in long-COVID patients that are distinct from those in acutely well COVID-19 inpatients and healthy control participants.[21] A meta-analysis encompassing 28 research identified 113 proteins substantially associated with prolonged COVID, highlighting Interleukin 6, C-reactive protein and tumor necrosis factor alpha as promising biomarkers.[22] Despite the widespread use of mass spectrometry in shotgun proteomics analysis, two-dimensional electrophoresis (2DE) which is often considered as outdated tool, continues to provide valuable information and plays a significant role in the field. This is due to its robustness, its ability to separate protein variants which is closer to cellular physiology compared to peptides in mass spectrometry, and due to its cost effectiveness.[23] While most of proteomic analysis of PCS have employed mass spectrometry modalities, there have been minimal studies using two-dimensional electrophoresis to complement these discoveries and offer insights from an alternative viewpoint.

In this study, we aimed to analyse serum proteomics profiling associated with post-COVID syndrome using two-dimensional electrophoresis. By comparing the serum proteome of individuals with persistent post-COVID symptoms to those who recovered, we sought to describe molecular signatures suggestive of disease persistence and PCS development. Through this approach, we hope to shed light on the underlying mechanisms driving post-COVID syndrome and facilitate the development of targeted diagnostic and therapeutic strategies.

MATERIALS AND METHODS

Subject recruitment and sample collection

This study was a comparative cross-sectional study, which included two groups of adults−with PCS and NPCS. The subjects were recruited among female adults. Patients with PCS were recruited in the PCS Clinic of a tertiary hospital, meeting inclusion criteria

that included the presentation of protracted symptoms such as cough, dyspnoea, chest discomfort, and exhaustion, a confirmed diagnosis by physicians, and the exclusion of any potential differential diagnoses. Patients with underlying neurological disease, chronic fatigue syndrome, sepsis of other causes, inflammatory diseases, trauma, myocardial infarction, and respiratory failure of other causes, as well as pregnant or breastfeeding mothers, were excluded. The NPCS group comprised individuals who had been infected with COVID-19 at least three months before enrolment and shown no lingering symptoms.

Following informed consent from all subjects, 5 ml of blood was collected via venepuncture at the cubital fossa. For proteomic analysis, 2.5 ml of blood was collected in a gel separator tube and left for 30 minutes and not more than 60 minutes for the blood to clot. The blood was subsequently centrifuged at 2,500 revolutions per min (rpm) for 10 minutes to isolate the serum. Serum samples were aliquoted in small aliquots and stored in -80°C freezer until further analysis.

This study was executed in compliance with the Declaration of Helsinki and received approval from the Ministry of Health Medical Research and Ethics Committee (MREC) under National Medical Research Registry (NMRR) ID-22-02261-PTG.

Two-Dimensional Electrophoresis

Protein extraction

Proteomic profiling was done using 2-Dimensional Electrophoresis method. Some 50µL of each sample were pooled according to the groups. Proteins of high abundance such as albumin, together with ionic pollutants including detergents, lipids, and phenolic compounds, were eliminated from protein samples with the Bio-Rad ReadyPrep™ 2-D Cleanup Kit (USA). The samples from each group were pooled in triplicates and were cleaned up. A total of 500 µg of protein was initially precipitated using 300 µL of precipitating agent 1 and 300 µL of precipitating agent 2. Subsequently, the samples were centrifuged at 15,000 rpm, followed by washing and air-drying in accordance with the protocol. The protein pellet was subsequently resuspended in 125 µL of rehydration buffer with 7 mol/L, 4% CHAPS, 0.2% Bio-Lyte, 3/10 ampholyte (pH 4–7), and 0.002% bromophenol blue (w/v).

First dimension separation

First dimension separation was done using isoelectric focusing. The protein samples were passively rehydrated on 7 cm-IPG strips (Bio-Rad, ReadyStrip; pH 4-7) for 12 hours. Isoelectric focusing was conducted at a peak current of 50µA per strip at 20°C, adhering to the protocol of the PROTEAN IEF Cell System (Bio-Rad, USA). Linear and fast voltage-ramping were performed at 250 V for Step 1 and 4000 V for Steps 2 and 3.

Second dimension separation

Before the second-dimensional electrophoresis, the IPG strips were equilibrated. The initial 10 minutes involved an equilibrium solution I [6 M urea, 0.375 M Tris-HCl, 2% sodium dodecyl sulphate (SDS), 20% glycerol, 2% dithiothreitol (DDT); pH [8.8], succeeded by equilibrium solution II (Equilibrium solution I, in which 2% DTT was replaced by 2.5% iodoacetamide) for the subsequent 10 minutes.

The strips were positioned above the two-dimensional gels (12% SDS-polyacrylamide) containing 0.5% agarose. A steady voltage of 120 V was applied for vertical separation until the blue dye line reached the gel's bottom. The gels were stained over the night with Coomassie Brilliant Blue R-250 stain and then destained with mili-Q water until the background was acceptably clear. The stained gels were scanned with a Bio-Rad GS-900 Calibrated Densitometer (USA), and the pictures were processed using PD Quest 8.0.1 2-D image analysis software (Bio-Rad, USA). The notable protein spots that varied between the case and control gels were manually removed with a biopsy punch and forwarded for identification purposes.

Protein Identification

The protein samples underwent trypsin digestion, followed by peptide extraction utilising conventional methodologies, and were subsequently analysed by Matrix-assisted laser desorption/ionisation – Time-of-flight mass spectrometry (MALDI-TOF MS). The peptide sample was combined with an equivalent volume of matrix solution of 10 mg/ mL R-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% ACN and deposited onto a 384-well stainless steel MALDI target plate (Applied Biosystems, Foster City, CA). A MALDI TOF/TOF mass spectrometer (Applied Biosystems), namely the ABI 4800 Proteomics Analyser, was employed to examine the spotted samples. Both MS and MS/MS spectra were recorded in combination mode and subsequently searched in databases. Protein ID was conducted by submitting combined MS and

MS/MS data using GPS Explorer (version 3.6, Applied Biosystems) to the Mascot server (version 2.0, Matrix Science) for database searching.

Statistical Analysis

The participants' baseline characteristics were presented as means (standard deviations) for normally distributed data and medians (interquartile ranges) for non-normally distributed data. The difference between proteins expression of case and control gels were analysed using Student t-test. The intersection of minimum two-fold difference and significant t-test with 90% confidence interval was considered as significant protein spots difference. All data were analysed by using analytical software RStudio version 2024.04.1.

RESULTS

Baseline characteristics

Table 1 presents the basic demographic and clinical characteristics of PCS and NPCS subjects. The NPCS group recruited were matched by age and sex with the PCS group. The mean ages of PCS and NPCS were 37.67 (9.69) and 39.50 (11.08), respectively, and all study subjects were Malay females. There was no severe COVID-19 infection, as most subjects had clinical Category 2 and one subject had clinical Category 3 in both groups. The majority of the subjects completed their vaccination consisting of first, second, and at least one booster dose. The mean duration from initial infection to recruitment visit was not significantly different: 129.3 (14.95) days for PCS and 141 (52.13) days for NPCS. The most common symptoms complained about during the acute phase of infection were fever, cough, sore throat, and runny nose for both groups, while persistent cough, fatigue, and exertional dyspnoea were the most common presentations in PCS patients.

Table 1: Basic demographic and clinical characteristics of PCS and NPCS subjects

Variables	PCS (n=6)	NPCS (n=10)	p-value
Age (yrs), mean (sd)	37.67 (9.69)	39.50 (11.08)	0.7427
BMI (kg/m^2), mean (sd)	31.36 (5.68)	25.40 (5.68)	0.0520
Vaccination status, no (%)			
Completed	5 (83.3%)	10 (100%)	
Not completed	1 (16.7%)	0 (0%)	
Hospitalisation, no (%)	1 (16.7%)	0 (0%)	
Clinical category			
Category 2	5 (83.3%)	9 (90%)	
Category 3	1 (16.7%)	1 (10%)	
Presenting symptoms at acute infection, no (%)			
Fever	6 (100%)	10 (100%)	
Cough	6 (100%)	8 (80%)	
Sore throat	5 (83.3%)	9 (90%)	
Runny nose	4 (66.7%)	8 (80%)	
Shortness of breath	3 (50%)	1 (10%)	
Nausea/vomiting/diarrhea	2 (33.3%)	0 (0%)	
Anosmia	4 (66.7%)	2 (20%)	
Ageusia	4 (66.7%)	2 (20%)	
Days from infection to recruitment, mean (sd)	129.3 (14.95)	141 (52.13)	0.6054
Persistent symptoms, no (%)			
Cough	5 (83.3%)	2 (20%)	
Exertional dyspnea	4 (66.7%)	0 (0%)	
Chest pain	0 (0%)	0 (0%)	
Fatigue	5 (83.3%)	0 (0%)	
Musculoskeletal pain	2 (33.3%)	0 (0%)	
Headache	3 (50%)	0 (0%)	
Poor appetite	2 (33.3%)	0 (0%)	
Nausea/vomiting/diarrhoea	0 (0%)	0 (0%)	
Depression	1 (16.7%)	0 (0%)	
Anxiety	1 (16.7%)	0 (0%)	
Insomnia	0 (0%)	0 (0%)	
Brain fog	1 (16.7%)	0 (0%)	
Cognitive impairment	4 (66.7%)	0 (0%)	

Categorical variables were summarised as counts and percentages (%); continuous variables are expressed as means and standard deviation (sd). Student's t-test, was used and p-value<0.05 was considered significant. BMI = body mass index.

Two-dimensional electrophoresis and protein identification

The experiment was performed in triplicates for each group and the representative image for the gels are shown in Figure 1. A total of 182 unique protein spots were identified in the serum of PCS patients, with molecular weights between 15 kDa and 200 kDa. Following comparison with the protein profiles of NPCS subjects, four protein spots were recognised to be significantly differently expressed between PCS and NPCS, whereby SSP 1505 and SSP 4302 were overexpressed while SSP 4603 and SSP 4404 were underexpressed in PCS group.

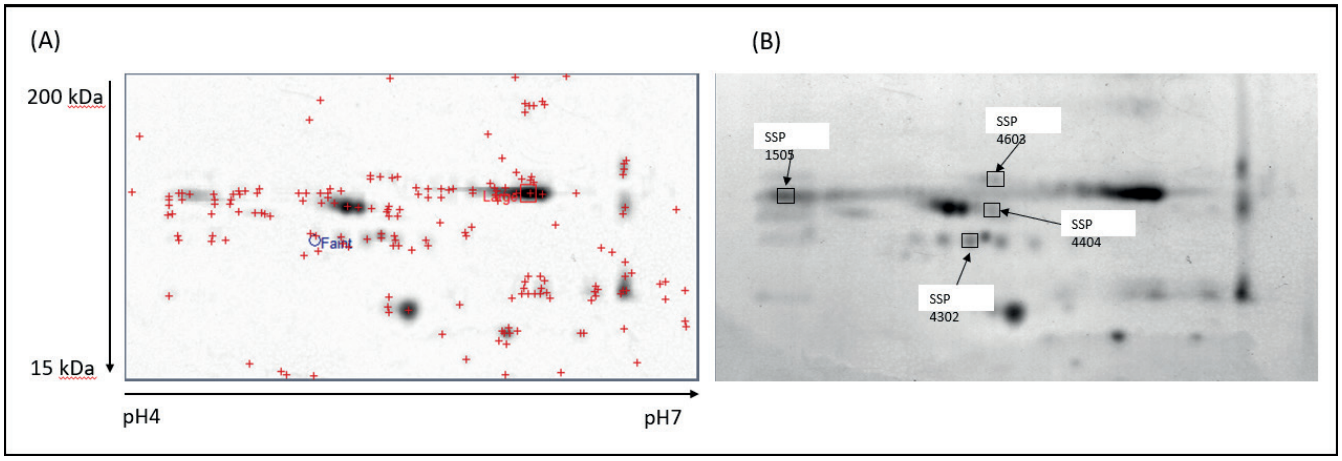
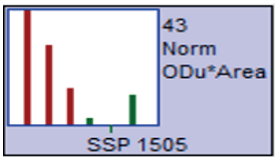
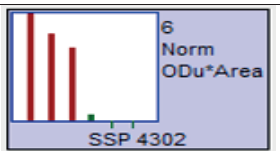
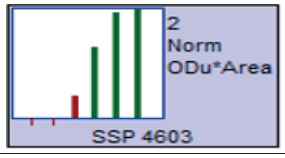
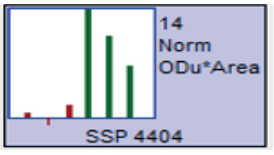


Figure 1: A. Representative (2-DE) gel image of serum from PCS patients using a pH range of 4 to 7. **B.** 2-DE gel image showing the location of protein spots that were expressed differently (p<0.1) in PCS patients compared to NPCS using Independent Student's T-test done by PD Quest software.

Table 2 demonstrates the results of further analysis on the comparison of the spots protein intensity, measured in optical density units for these four proteins of interest.

Table 2: Protein spots intensity of SSP 1505, SSP 4302, SSP 4603, and SSP 4404

Protein spot ID	Protein density graph	PCS (OD) (Mean)	NPCS (OD) (Mean)	p-value
SSP 1505		29.0677	4.5119	0.09032
SSP 4302		4.681833	0.178767	0.01137
SSP 4603		0.199067	1.531133	0.01214
SSP 4404		0.7357	10.57447	0.04081

Data were analysed using Student's T-test and p-value of <0.1 was considered as significantly different. The height of each bar corresponded with the intensity of protein as expressed in PCS patients (red bar) and NPCS (green bar) measured in optical density unit.

These protein spots of interest were manually excised and identified using MALDI-TOF mass spectrometer analysis. The two overexpressed proteins were identified as T-cell surface glycoprotein CD8 alpha chain and Haptoglobin, while the underexpressed proteins were Immunoglobulin heavy constant alpha 1 and Vitamin D-binding protein. Table 3 summarises the basic characteristics of the proteins based on the Uniprot database, a comprehensive and freely accessible online database for sequence and functional information of proteins.

Table 3: Protein spots basic characteristics based on the Uniprot database

Protein spot	Protein name	Uniprot ID	Molecular weight	Peptide sequence	Main molecular function
SSP 1505	T-cell surface glycoprotein CD8 alpha chain	P01732	26.225	CPRPVVK	Immune system response
SSP 4302	Haptoglobin	P00738	45.860	DYAEVGR	Inflammatory response
SSP 4603	Immunoglobulin heavy constant alpha 1	P01876	38.486	YLTWASR	Immune system response
SSP 4404	Vitamin D-binding protein	P02774	54.479	THLPEVFLSK	Immune system response

DISCUSSION

All sixteen subjects in this study were Malay females whose difference in mean age and BMI were insignificant. The participants were matched by gender, age, and race to mitigate confounding variables in the proteomic profiling. Studies have shown that female and working age (30 to 60 years old) are the risk factors of developing PCS.[24,25] Since the recruitment was done after the initiation of the COVID-19 vaccination program, most of the participants were completely vaccinated. The patients initially presented mostly with fever, cough, and sore throat during acute infection, which is similar to the previous report.[26] In cases with chronic symptoms, cough, tiredness, and exertional dyspnoea were the most prevalent manifestations, consistent with earlier studies.[24] The mean duration of recruitment also corresponded to the definition of PCS which states that the persistent symptoms occurred for more than 120 days from initial infection.[27] This study's primary discovery was the over-expression of the T-cell surface glycoprotein CD8 alpha chain and Haptoglobin, with the under expression of Immunoglobulin heavy constant alpha 1 and Vitamin D-binding protein in PCS patients relative to NPCS patients.

The T-cell surface glycoprotein CD8 alpha chain is an essential element of the CD8 glycoprotein complex located on cytotoxic T cells, especially cytotoxic T lymphocytes (CTLs), commonly referred to CD8+ T cells. The main role of this protein is to enhance the T cells sensitivity to antigen.[28] This study suggests that overexpression of this glycoprotein in PCS patients, could lead to an increase in CD8+ T cells, and in PCS patients, this protein might be chronically activated in response to the ongoing immune response mechanism/process. When compared to healthy controls, a study by Santa Cruz et al. revealed higher levels of peripheral blood CD8+ T cells in convalescent COVID patients six months after infection, indicating higher expression levels of PD-1 (programmed cell death-1) as a sign of activation rather than exhaustion.[29] Another research corroborates this finding, demonstrating markedly elevated levels of proliferating effector T-cells (CD8+) in patients experiencing chronic dyspnoea, amnesia, disorientation, and chest discomfort.[30] Another study suggests that the production of CD8+ T cell increased in PCS patients, but these cells were dysfunctional.[31] It was proposed that there is an overactivation of CD8 T-cells leading to malfunction, resulting in a reduced ability to g Interferon-gamma (IFN-γ) or tumour necrosis factor-alpha (TNF-α) in long-COVID patients in comparison to healthy controls.[31] Therefore, dysfunctional CD8+

T cells may prevent PCS patients from achieving the appropriate immune system responses, despite the increased expression of the T-cell surface glycoprotein CD8 alpha chain protein.

In this current study, haptoglobin is another protein that was significantly overexpressed in PCS patients compared to non-PCS subjects. Haptoglobin (Hp) is a glycoprotein that binds to free haemoglobin (Hb) in the bloodstream, playing a crucial role in protecting tissues from oxidative damage and carrying out various regulatory functions.[32] An elevation of haptoglobin levels in PCS patients may be associated with persistent inflammation and immune system dysregulation, which are key features of the condition. This inflammation triggers the acute phase response, prompting the liver to produce more haptoglobin to help the body cope with stress. Other investigations have demonstrated significantly elevated levels of haptoglobin in acute COVID-19 patients, irrespective of disease severity, and a strong positive association exists between haptoglobin plasma levels and the acute phase of COVID-19, attributable to its established function as a positive acute phase reactant.[33] Meanwhile, the prolonged elevation of haptoglobin expression in current PCS patients may indicate ongoing inflammatory responses, which underlie the progression of PCS. This agrees with what Gulhar et al. claimed that the rise in haptoglobin is linked to other inflammatory markers like IL-6, CRP, and D-dimer, which are often high in PCS.[34]

This study revealed the under-expression of two additional proteins: Vitamin D binding protein (VDBP) and immunoglobulin heavy constant alpha 1 protein (IgA1). VDBP serves the primary carrier protein for 25-hydroxy Vitamin D (25(OH)D) in plasma, with the fraction bound to VDBP regarded as inactive, while those associated with albumin or in free from (25(OH)D) are deemed actively bioavailable.[35] VDBP, synthesised in the liver, is essential for Vitamin D regulation; however, its synthesis is influenced by oestrogen, inflammatory cytokines, and glucocorticoids, rather than by Vitamin D levels.[36–38] In addition to its primary role as a Vitamin D carrier, it also performs other crucial biological functions such as an actin scavenger, transporting fatty acid, activating macrophages, and enhancing the chemotactic effect.[39] While there have been few studies of VDBP in long-term COVID, several studies on VDBP in COVID-19 have yielded contradicting results. Jiang et al. investigated the serum level of VDBP in COVID-19 patients with and without acute lung injury (ALI). The study indicated an elevation in VDBP levels in ALI, suggesting a notion of heightened inflammatory response and death bronchial epithelial cells.[37] Another study demonstrated an association between reduced VDBP level and in-hospital mortality, but another study showed no association.[40] Apart from VDBP, the insight into the level of Vitamin D itself in long COVID-19 might give valuable information. Some studies presented no correlation between Vitamin D levels and long COVID-19 or recurrent symptoms, [41,42] whereas another study documented those low levels of (25(OH)D) are associated with long COVID-19 and the persistence of neurocognitive symptoms.[43] This disparity might be due to the different method and component of Vitamin D that were measured. The under-expression of VDBP in the current study can be hypothesised to be manifested as a low level of Vitamin D, due to the ongoing inflammation underlying the establishment of PCS.

The current study has demonstrated an under-expression of the immunoglobulin- heavy constant alpha-1 protein (IgA1). IgA1 is one of the subclasses of Immunoglobulin A (IgA), which is present in both serum and mucosal secretion. IgA1 encompasses 90% of the subclass distribution of the main IgA form, which is monomeric.[44] This protein plays a significant role in protective mechanisms by neutralising and blocking pathogen activity, activating the complement system via an alternative pathway, and mediating various effector functions in order to kill and clear pathogens.[44] Yu et al. conducted a study on the humoral response to COVID-19 infection, revealing that during the acute phase, severe patients had considerably elevated levels of IgA and IgG in comparison to non-severe patients.[45] Considering the functions of mucosal and systemic IgA in COVID-19, the induction of IgA production has been suggested as an innovative treatment for severe COVD-19. Consequently, the under-expression of IgA1 proteins in this study suggests as inadequate immune response, which may contribute to the unresolved initial infection and subsequently manifest as persistent, debilitating symptoms in PCS patients. This aligns with the study suggesting ongoing dysregulation of the immune system's complement cascade, marked by changes in diverse immune cell types and chronic inflammation, including immunoglobulins such as IgA, which underpins PCS.[46]

This work not only identifies possible protein indicators, but also has some limitations that should be acknowledged. The limited sample size of post-COVID-19 patients is due to the difficulty in recruiting individuals who satisfy the stringent diagnostic criteria. Additionally, to reduce confounding variables, the study required samples to be homogeneous, limiting them to a specific gender, race, and age group. The study's results are also limited to low molecular weight proteins that can be seen on SDS gels. Also, because of limited funds, only a few spots could be analysed for identification, which limited the level of protein profiling that could be done.

CONCLUSION

This study found that there are significant proteomic alterations in people with PCS. These changes include over-expression of the T-cell surface glycoprotein CD8 alpha chain and haptoglobin and under-expression of the immunoglobulin heavy constant alpha 1 and Vitamin D-binding protein. These findings suggest ongoing immune activation, chronic inflammation, and potential immune dysregulation as part of the pathophysiology of PCS. Additional research, particularly concentrating on the function of these specific proteins in larger cohorts during the verification phase, is essential to elucidate the underlying mechanisms of this syndrome, assess their potential in predicting COVID-19 patients at elevated risk of developing PCS, and subsequently help the design of targeted therapeutic interventions for PCS.

Source of fund

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Conflict of Interest

The authors confirm that they have no conflict of interests to disclose.

Ethical Clearence

The study was approved by IIUM Research Ethics Committee (IREC) (approval number: IREC 2021-300) and by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (MOH) (approval number: NMRR ID-22-02261-PTG).

Author's contribution

All authors were involved in generating ideas for this study. The study design of this study was contributed by AB, NMB, and WNWR. Subjects' recruitment, sample collection, and data analysis were done by AB, SAAH, NZA, DM, MYI, and AK. Meanwhile, AB and NMB prepared the initial draft, and then it was reviewed by all other authors for editing and approval of the final draft.

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