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Reactivation of herpesvirus type 6 and IgA/IgM-mediated responses to activin-A underpin long COVID, including affective symptoms and chronic fatigue syndrome

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Abstract

Background: Persistent infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), reactivation of dormant viruses, and immune-oxidative responses are involved in long COVID. *Objectives:* To investigate whether long COVID and depressive, anxiety, and chronic fatigue syndrome (CFS) symptoms are associated with IgA/IgM/IgG to SARS-CoV-2, human herpesvirus type 6 (HHV-6), Epstein-Barr Virus (EBV), and immune-oxidative biomarkers. *Methods:* We examined 90 long COVID patients and ninety healthy controls. We measured serum IgA/IgM/IgG against HHV-6 and EBV and their deoxyuridine 5′-triphosphate nucleotidohydrolase (duTPase), SARS-CoV-2, and activin-A, C-reactive protein (CRP), advanced oxidation protein products (AOPP), and insulin resistance (HOMA2-IR). *Results:* Long COVID patients showed significant elevations in IgG/IgM-SARS-CoV-2, IgG/IgM-HHV-6, and HHV-6-duTPase, IgA/IgM-activin-A, CRP, AOPP, and HOMA2-IR. Neural network analysis yielded a highly significant predictive accuracy of 80.6% for the long COVID diagnosis (sensitivity: 78.9%, specificity: 81.8%, area under the ROC curve = 0.876); the topmost predictors were as follows: IGA-activin-A, IgG-HHV-6, IgM-HHV-6-duTPase, IgG-SARS-CoV-2, and IgM-HHV-6 (all positively) and a factor extracted from all IgA levels to all viral antigens (inversely). The top 5 predictors of affective symptoms due to long COVID were IgM-HHV-6-duTPase, IgG-HHV-6, CRP, education, IgA-activin-A (predictive accuracy of $r = 0.636$). The top 5 predictors of CFS due to long COVID were in descending order: CRP, IgG-HHV-6-duTPase, IgM-activin-A, IgM-SARS-CoV-2, and IgA-activin-A (predictive accuracy: $r = 0.709$). *Conclusion:* Reactivation of HHV-6, SARS-CoV-2 persistence, and autoimmune reactions to activin-A combined with activated immune-oxidative pathways play a major role in the pathophysiology of long COVID as well as the severity of its affective symptoms and CFS.

Significant outcomes

1. Long COVID prevalence is associated with the reactivation of latent viruses, in particular, human herpes type 6.
2. Immune reactions to SARS-CoV-2 and latent viruses (HHV-6) contribute to the activation of immune-oxidative pathways that result in autoimmunity.
3. The immunoregulatory protein Activin-A, which plays a pivotal role in both the innate and adaptive immune systems, is a major target of autoimmunity in long COVID and its overlap with ME/CFS.
4. Development of ME/CFS and affective symptoms are the results of SARS-CoV-2 viral persistence and reactivation of latent viruses.

Original Article

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Limitations

- The results of the study would be even more informative if a broader spectrum of latent viruses was included, such as cytomegalovirus (CMV) and HHV-7, and if we had assessed the gut microbiome.
- The results of the study would be more informative if we had measured more immune-inflammatory pathways, including the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and hypernitrosylation.

Introduction

Between three and six months following acute COVID-19 infection, many individuals may acquire long coronavirus (long COVID) or post-COVID illness (Groff *et al.*, 2021). Despite the wide range of symptomatology, chronic fatigue, symptoms of depression and anxiety, problems with neurocognition, shortness of breath, and gastrointestinal dysfunctions are the most common long COVID symptoms (Renaud-Charest *et al.*, 2021; Sandler *et al.*, 2021; Titze-de-Almeida *et al.*, 2022). We found that fatigue, physiosomatic, depressive, and anxiety symptoms are manifestations of a single core (latent vector) referred to as the physio-affective phenome of acute COVID-19 infection and long COVID (Al-Hadrawi *et al.*, 2022; Al-Jassas *et al.*, 2022; Almulla *et al.*, 2023). Based on the results of the Hamilton Depression (HAMD) and Anxiety (HAMA) Rating Scales, the Fibromyalgia and Fatigue (FF) Rating Scale scores, this physio-affective phenome was created using a precision nomothetic technique (Maes, 2022; Maes and Stoyanov, 2022).

We discovered that abnormalities in the immune-inflammatory (as indicated by higher C-reactive protein or CRP, and NOD-, LRR- and pyrin domain-containing protein 3, NLRP3 inflammasome biomarkers), oxidative stress (as indicated by increased advanced oxidation protein products (AOPP)), as well as increased insulin resistance (IR) partially explain the physio-affective phenome of acute and long COVID (Al-Jassas *et al.*, 2022; Almulla *et al.*, 2023). We demonstrated in our long COVID studies that decreased oxygen saturation (SpO2) and elevated peak body temperature (PBT) during the acute infection stage are related to persistent fatigue and depressive symptoms many months later (Al-Hadrawi *et al.*, 2022). Increased PBT and decreased SpO2, both of which are linked to higher morbidity, are indicators of the severity of the inflammatory response during the acute phase of disease (Al-Jassas *et al.*, 2022). Therefore, the severity of the acute infectious phase contributes to aberrations in immune and oxidative pathways, with low SpO2 linked to changes in oxidative stress pathways, and increased PBT linked to increased CRP and decreased antioxidant defences (Al-Hakeim *et al.*, 2022a).

Nevertheless, the precise pathophysiology of long COVID and the mechanisms underlying the activated immune pathways in long COVID have not yet been clarified. Regular detection of SARS-CoV-2 viral RNA or antigens in blood, faeces, and other tissue samples up to many months after the initial diagnosis of COVID-19 led to the assumption that persistent SARS-CoV-2 infection may be involved (Li *et al.*, 2021; Cheung *et al.*, 2022; Natarajan *et al.*, 2022; Rahmani *et al.*, 2022). As suggested by (Chen *et al.*, 2023; Vojdani *et al.*, 2023; Yang *et al.*, 2023), the continued presence of the virus might lead to a sustained activation of the immune system, potentially resulting in the persistent neuropsychiatric symptoms and chronic fatigue syndrome (CFS) observed in individuals with long COVID.

A growing body of research also demonstrates that acute and critical SARS-CoV-2 infection can trigger the reactivation of other viruses, such as the Epstein-Barr Virus (EBV), human herpesvirus type 6 (HHV-6), and other viruses (Balc'h *et al.*, 2020; Lehner *et al.*, 2020). A high proportion of patients with long COVID show IgG titres that are positive for the EBV reactivation markers early antigen-diffuse (EA-D) and small viral capsid antigen p23 (Gold *et al.*, 2021, Jon *et al.*, 2022). It is not yet known, however, if these viruses contribute to the development of neuropsychiatric disorders and signs of chronic fatigue syndrome (CFS) during long COVID (Proal and VanElzakker, 2021).

There may be several mechanisms explaining why reactivation of these viruses increases susceptibility to neuropsychiatric symptoms and CFS. First, the viruses listed above might make it easier for SARS-CoV-2 to enter cells (Vojdani *et al.*, 2023). Second, according to several studies (Gold *et al.*, 2021; Zubchenko *et al.*, 2022; Vojdani *et al.*, 2023), these viruses probably lead to mitochondrial breakdown and dramatically change energy metabolism. Since mitochondrial dysfunctions have been linked to the pathobiology of immune-mediated illnesses such as CFS and affective disorders, a same mechanism could lead to long COVID (Morris and Maes, 2014; Anderson *et al.*, 2023). Third, while a small number of studies have investigated the presence of immunoglobulins (IgA, IgM, and IgG) against persistent SARS-CoV-2 and reactivated viruses, no studies have investigated its deoxyuridine 5′-triphosphate nucleotidohydrolase (duTPase), a nucleotide metabolic enzyme that is an important regulator of the innate and adaptive immune responses (Vojdani *et al.*, 2023). The release of the latter in exosomes may contribute to CFS by increasing the synthesis of cytokines, activin-A, plasma cells, and antibodies, and consequently abnormal immunological responses (Cox *et al.*, 2022). The immune responses against human growth proteins like activin-A, interferon (IFN)-α^2, a master cytokine in type 1 IFN signalling, heat shock protein (HSP)60, and HSP90, which support proteostasis and regulate apoptosis and the cell cycle, are not studied in relation to neuropsychiatric symptoms and CFS caused by long COVID (Hu *et al.*, 2022). The aetiology of depressive symptoms and CFS is associated with changes in activin-A, HSP60 and HSP90 (Dow *et al.*, 2005; Ageta *et al.*, 2008; Zheng *et al.*, 2017; Sagulkoo *et al.*, 2022). However, there is little or no information on the relationship between altered IgA/IgG/IgM responses to activin-A, IFN-α^2, HSP60, and HSP90 and affective symptoms and CFS caused by long COVID.

Hence, the aim of the current study is to examine the levels of IgA/IgG/IgM antibodies against SARS-CoV-2, HHV-6, and HHV-6-dutPase, EBV and EBV-duTPase, activin-A, HSP60, HSP90, and IFN-α^2 in long COVID patients versus healthy persons. We also want to see if the severity of the affective symptoms and CFS due to long COVID is predicted by these IgA/IgG/IgM levels.

Participants and methods

Participants

The participants in this study included 90 long COVID patients and ninety healthy controls. Senior clinicians diagnosed the patients, using criteria specified by the World Health Organization (WHO, 2021). According to these guidelines, a long COVID patient must satisfy the following criteria: a confirmed COVID-19 infection; at least two symptoms, such as fatigue, memory or concentration issues, muscle aches, loss of smell or taste, emotional distress, and cognitive impairment that interfere with daily life;

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symptoms persisting after the acute phase of the illness or appearing 2–3 months later; and the continuity of these symptoms for at least two months, even 3–4 months after the acute phase of the illness. The research strategy employed two study designs. The first part ($n = 72$) was a case-control and retrospective cohort study to determine the influence of PBT, SpO2, and duration of the acute infection on the biomarkers and the effects of the biomarkers on the symptoms of long COVID. The second study ($n = 180$) was a case-control study of the associations between biomarkers and long COVID versus healthy controls. Group 1 consisted of Iraqi participants. Acute COVID-19 infection was diagnosed by virologists and specialist physicians. When determining the prognosis of long COVID, the following factors were taken into account: (a) presence of symptoms of long COVID (see above) that lasted for 12–16 weeks or longer; (b) the acute infectious phase is characterised by severe infection symptoms, including fever, cough, shortness of breath, and the loss of senses of smell and taste; (c) during the acute phase of illness, a positive test result for reverse transcription real-time polymerase chain reaction (rRT-PCR) and immunoglobulin M (IgM) antibodies against SARS-CoV-2. Patients in subgroup 1 were treated at a variety of hospitals, including the Imam Sajjad Hospital, the Hassan Halos Al-Hatmy Hospital for Transmitted Diseases, the Middle Euphrates Center for Cancer, the Al-Najaf Teaching Hospital, and the Al-Sader Medical City of Najaf, Iraq. In Najaf, controls were recruited from the same catchment area. Controls were excluded if they exhibited symptoms suggestive of COVID-19 or another infectious disease, a positive rRT-PCR test, or elevated IgM antibodies against SARS-CoV-2. Participants were excluded if they had a lifetime history of a major depressive episode, bipolar disorder, dysthymia, generalised anxiety disorder, panic disorder, schizoaffective disorder, schizophrenia, psycho-organic syndrome, or substance use disorder (except for nicotine dependence). This investigation excluded patients with neuroimmune, autoimmune, and immune disorders including Parkinson's disease, CFS, Alzheimer's disease, multiple sclerosis, stroke, psoriasis, chronic kidney disease, COPD, and scleroderma. Women who were pregnant or nursing were excluded. The mean age of the subjects was 28.1 ($SD = 5.9$) years old, and the female / male ratio was 20/52. Subgroup 2 consisted of 32 long COVID patients who were examined at the Cyrex Laboratory in California, United States. These patients exhibited symptoms of fatigue, brain fog, neurocognitive decline, shortness of breath, headaches or vertigo, difficulty sleeping, cough, chest pain, joint or muscle pain, gastrointestinal disorders, and irregular menstruation that persisted for at least 12 weeks following the initial infection with COVID-19. All individuals were screened for elevated IgG antibodies against the spike protein and nucleoprotein of SARS-CoV-2. In addition, seventy-six pre-COVID control sera were acquired from Innovative Research (Novi, MI). The sera were extracted from healthy volunteers and tested for the presence of HIV and hepatitis C. Using the SARS-CoV-2 IgG antibody assay by Zeus Scientific, all seventy-six sera were tested for the presence of SARS-CoV-2 antibody and were found to be negative. The mean age of the subjects was 42.1 ($SD = 14.8$) years old, and the female / male ratio was 72/36. All sera were stored at -20°C until their use in the antibody assays.

The College of Medical Technology Ethics Committee of the Islamic University of Najaf in Iraq (Document No. 34/2023) granted ethical approval for this investigation. Before participating in our study, all participants or their legal guardians gave their written consent. The International Conference on Harmonization of Good Clinical Practice, the Belmont Report, the Council of International Organizations of Medicine Guideline, as well as Iraqi and international ethical and privacy statutes, were all adhered to in the planning and execution of this study. Our institutional review board follows the International Guidelines for the Conduct of Safe Human Research (ICH-GCP).

Clinical measurements

Three to four months after recovering from acute COVID-19, all participants in study 1 were interviewed by an experienced psychiatrist. The purpose of this interview was to collect information regarding the sociodemographic and clinical characteristics of the participants. The psychiatrist evaluated the presence and severity of CFS symptoms using the Fibro-fatigue (FF) scale (Zachrisson *et al.*, 2002). The Hamilton Depression Rating Scale (HAMD) (Hamilton, 1960) and the Beck Depression Inventory-II (BDI-II) (Hautzinger, 2009) were used to measure the severity of depression. To evaluate the severity of anxiety symptoms, the Hamilton Anxiety Rating Scale (HAMA) (Hamilton, 1959) was utilised. The severity of the long COVID phenome was estimated by computing a z unit-based composite score calculated as z HAMD + z HAMA + z BDI (labelled as comp_AFFECT). The diagnostic criteria for tobacco use disorder were derived from the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). The body mass index (BMI) was calculated by dividing the individual's weight in kilograms by the square of their height in meters. We extracted the SpO2 and PBT values from the patients' hospitalisation records during the acute infection phase. A highly competent paramedical professional took these measurements using an electronic oximeter manufactured by Shenzhen Jumper Medical Equipment Co. Ltd and a digital sublingual thermometer with an audible alert, respectively. The duration of the patient's illness was deduced from their medical records.

Assays

Blood samples were collected from fasting participants early in the morning, between 7:30 and 9:00 a.m. Venous blood (five millilitres) was drawn and placed into sterilised, clear serum tubes. Samples that showed signs of haemolysis were not used. After allowing the blood to clot for ten minutes, the samples were centrifuged for five minutes at a speed of 3000 rpm. The resulting serum was then carefully transferred into several new Eppendorf tubes. Measurements for CRP in human serum were performed using the CRP latex slide test, a product of Spinreact® (Barcelona, Spain). Serum AOPP levels were determined using enzyme-linked immunosorbent assay (ELISA) kits sourced from Nanjing Pars Biochem Co., Ltd (Nanjing, China). The Homeostatic Model Assessment for Insulin Resistance (HOMA2-IR), a measure of insulin resistance, was computed from fasting insulin and serum glucose levels using a HOMA2 calculator available at https://www.dtu.ox.ac.uk/homacalculator/.

Antigens and sera

SARS-CoV-2 spike protein superantigen (amino acid sequence of 2 671-700 (NH2) CASYQTQTNSPRRRARSVASQSIIAYTMSLGA (COOH)) which has some sequence and structure similarity to Staphylococcus enterotoxin B (Cheng *et al.*, 2020); EBV lytic protein that is produced during reactivation of the virus (Williams *et al.*, 2016), amino acid sequence [NH2]WAT[C(Cam)]AFEEVPGLA [M(OX)]GDSGLSEALEGR[COOH] of duTPase; human herpesvirus 6 A&B (HHV-6A&B); HHV-6-duTPase that function as pathogen-associated molecular pattern proteins for TLR2 (Cox

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et al., 2022) amino acid sequence of (NH2) CHGLLIETYIWNKD TIPSIKIFNST (COOH); human molecular chaperones, HSP60 (amino acid sequence (NH2) AEIPKEEVKPFITESKPSVEQR KQDDKK (COOH)) and HSP90 (amino acid sequence (NH2) KTFPPTEPKKDKKKKADETQALPQRQKKQQ(COOH)) which share antigenic epitopes with SARS-CoV-2, were synthesised with purity of greater than 85% by Bio-Synthesis (Lewisville, TX, USA). Citrullinated EBV EBNA2 peptide (sequence CQGRCGRWRG-cit-GRSKGRGCRMH) was synthesised by Bio-Synthesis (Lewisville, TX, USA) (Trier *et al.*, 2018). Recombinant human interferon alpha-2 (IFN-α2) protein was purchased from Novus Biologicals LLC (CO, USA). Human activin-A was purchased from ProSpec (Rehovot, Israel). Recombinant human activin-A Ab151687 was purchased from Abcam (Cambridge, MA, USA).

Antibody measurements

We used an ELISA to measure the presence of antibodies (IgA, IgG, IgM) against the SARS superantigen, HHV-6, HHV-6-duTPase, Citrullinated EBV EBNA2 peptide, EBV-duTPase, HSP60 and HSP90, IFN-α2, and activin-A. SARS superantigen and HHV-6 were synthesised and provided with purity greater than 85% by Bio-Synthesis (Lewisville, TX, USA). Additionally, we obtained activin-A from ProSpec (Rehovot, Israel). The procedure was as follows: (a) A Tris buffer with a pH of 7.2 was used to dissolve the proteins and peptides. Then, in a 0.1 M carbonate buffer at pH 9.6, we put 100 ml of each at concentrations ranging from 0.5 to 1 microgram to a series of microwell plates, (b) After 16 hours in room temperature (25°C) incubation, the plates were subjected to refrigeration for another 8 hours, (c) After removing the contents, the plates were washed three times with 250 ml of a 0.01 M phosphate buffer saline solution with a pH of 7.4, containing 0.05% Tween 20, (d) We added 250 ml of a solution containing 2% bovine serum albumin (BSA) and 2% dry milk in the same PBS buffer to each well, and then incubated the plates again to prevent nonspecific binding of serum immunoglobulins (e) After four washes in PBS buffer, we applied serum dilutions of 1:100 for IgG and IgM and 1:50 for IgA antibodies to duplicate wells of the microtitre plates, (f) After incubating for 1 hour at 25°C, we washed the ELISA plates five times with the PBS buffer. We then added 100 ml of alkaline phosphatase-labelled anti-human IgG (dilution at 1:800), anti-human IgM (dilution at 1:600), and anti-human IgA (dilution at 1:200) antibodies to the appropriate sets of plates, (g) After additional washing, we added 100 ml of substrate and stopped the colour development by adding 50 ml of 1 N NaOH. The intensity of the colour was measured using an ELISA reader at a wavelength of 405 nm. We also used several wells coated with BSA, human serum albumin (HAS), and foetal bovine serum as negative controls or blanks, and used sera from patients with ME/CSF and long COVID with known antibody titres as calibrators and positive controls. The ELISA optical densities (ODs) were converted to indices by dividing each sample OD by the calibrator OD, after subtracting the background OD from both, using the flowing formula:

$$\text{Antibody ELIS Aindex} = \frac{\text{OD of sample} - \text{OD of negative control}}{\text{OD of calibrator} - \text{OD of negative control}}$$

Statistical analysis

All statistical analyses were performed using the 29th version of IBM's SPSS Windows software. We employed analysis of variance to assess differences in continuous variables across study groups. Additionally, we used contingency table analysis to investigate the relationships between categorical variables. In the total study group ($n = 180$) we performed binary logistic regression analysis with long COVID as dependent variable (and healthy controls as reference group) using the IgA/IgG/IgM responses as explanatory variables while adjusting for the effects of age, sex, and research site. The putative effects of these possible confounding variables were accounted for by forced entry of those three variables in hierarchical logistic regression analyses (first set comprised the immune data, and the next set contained age, sex, and site, forced entry). We computed B (SE), and Wald statistics with exact p values, adjusted Odds ratios with 95 confidence intervals (CI), the classification table, and Nagelkerke pseudo-R square (used to estimate the effect size). A neural network analysis was conducted to predict the differentiation between individuals with long COVID and control subjects, as well as the clinical continuous data. In our multilayer perceptron neural network models, we used the most significant IgG, IgM, IgA responses to the antigens, age, gender, level of education, with or without AOPP, HOMA2-IR, and CRP. An automatic feedforward network model with one or two hidden layers was constructed using up to eight nodes. The models were trained using the batch type, with 250 epochs. The stopping criterion was set to a single successive step that did not further decrease the error term. We calculated the error and the relative error, the percentage of misclassifications, and the area under the receiver operating characteristic curve (AUC ROC). The predictive accuracy of the models was estimated using the confusion matrix (for binary outputs), or the predicted versus observed coefficient of determination (R^2). The input factors' significance and relative importance were also assessed and displayed in an importance chart. We initially divided the participants into three categories: (a) a training group, which constituted 46.7 % of all participants and was used to determine the model's parameters; (b) a testing group, which made up 20% of all participants and was used to avoid overfitting; and (c) a holdout group, which accounted for 33.3% of all participants and was used to assess the model's predictive validity. We used a data level oversampling correction (adding instance observations to the minority sample) to compensate for a disbalanced data set when performing binary regressions and neural networks with a binary output variable. Principal component analysis was employed for feature reduction purposes. The PC was accepted as a validated concept when the explained variance (EV) was at least 50%, the anti-image correlation matrix was adequate, the factoriability metrics were adequate with the Kaiser-Meyer-Olkin (KMO) metric > 0.7, and Bartlett's sphericity test were significant, and PC loadings > 0.7. The significance level for all tests was set at a p-value of 0.05, and all tests were two-tailed. A priori power analysis (G*Power 3.1.9.7.) shows that the estimated sample size should be at least 126 to detect differences in a chi-square test with an effect size of 0.25 given $p = 0.05$, power = 0.8 and df = 1.

Results

Sociodemographic data, psychological measurements, and immunological parameters

The current study presents sociodemographic data, PBT, SpO2 and duration of the acute phase in Table 1. This table also encompasses scores from several psychological and biomarker assessments, including the FF, HAMA, HAMD, BDI, CRP, AOPP and HOMA2-IR levels. Sociodemographic data (except residency) such as age, gender, BMI, marital state, smoking, and education did

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Table 1. Sociodemographic and clinical data, body temperature, oxygen saturation (SpO2), and psychological rating scales in healthy controls (HC) and long COVID patients

Variables	HC ($n = 90$)	Long COVID ($n = 90$)	F/X^2	df	p-value
Age (Years)	38.24(13.02)	34.67(14.53)	3.005	1/178	0.085
Gender (M/F)	52/38	40/50	3.20	1	0.074
BMI (m^2/Kg)	25.36(2.41)	25.54(3.40)	0.035	1/70	0.852
Marital state (Y/N)	8/6	25/33	0.895	1	0.344
Smoking (N/Y)	9/5	40/18	0.114	1	0.483
Residency (R/U)	0/14	15/43	4.57	1	0.032
Education (years)	15.6(1.6)	15.7(1.5)	0.089	1/70	0.767
Peak body temperature (°C)	36.87(0.11)	38.05(0.82)	28.67	1/70	<0.001
Oxygen saturation, SpO2 (%)	96.28(1.20)	91.05(3.35)	32.68	1/70	<0.001
FF-Total	8.9(3.1)	26.1(11.9)	28.22	1/70	<0.001
comp_AFFECT (z score)	−1.083(0.241)	0.262(0.935)	28.23	1/70	<0.001
HAMA-Total	6.9(3.5)	15.0(8.0)	13.90	1/70	<0.001
BDI-Total	11.1(4.1)	24.7(7.3)	45.10	1/70	<0.001
HAMD-Total	7.9(3.2)	17.2(5.0)	43.86	1/70	<0.001
CRP (mg/L)	5.07(0.27)	7.25(3.83)	4.50	1/70	0.037
AOPP (uM)	0.82(0.22)	1.64(1.16)	6.81	1/70	0.011
HOMA2-IR	1.28(0.23)	1.67(0.57)	6.34	1/70	0.014

R, Rural; U, Urban; SpO2, Oxygen saturation; FF, Fibro-Fatigue scale; comp_AFFECT, composite reflecting severity of affective symptoms due to long COVID; HAMA, Hamilton anxiety rating scale; BDI, Beck Depression Inventory; HAMD, Hamilton Depression rating scale; CRP, C-reactive protein; AOPP, advanced oxidation protein products; HOMA2-IR, Homeostatic Model Assessment for Insulin Resistance. Results are shown as mean (SD).

not significantly differ among study groups. We found that PBT was significantly increased and SpO2 significantly decreased during the acute infectious phase in long COVID patients versus controls. Our results also revealed that long COVID patients show significantly elevated scores of FF, HAMA, BDI and HAMD. Additionally, CRP, AOPP, and HOMA-2IR levels were significantly higher in long COVID patients as compared to controls. In the long COVID patients, the mean (±SD) duration of acute infectious illness was 14.1 (±5.6) days.

PBT was significantly and positively correlated with the HAMD ($r = 0.698$, $p < 0.001$), HAMA ($r = 0.586$, $p < 0.001$), BDI ($r = 0.675$, $p < 0.001$), and FF ($r = 0.619$, $p < 0.001$) scores, CRP ($r = 0.723$, $p < 0.001$), and HOMA2-R ($r = 0.241$, $p = 0.041$). SpO2 was significantly and inversely correlated with the HAMD ($r = -0.501$, $p < 0.001$), HAMA ($r = -0.310$, $p = 0.008$), BDI ($r = -0.401$, $p < 0.001$), and FF ($r = -0.597$, $p < 0.001$) scores, CRP ($r = -0.400$, $p < 0.001$), AOPP ($r = -0.255$, $p = 0.031$), and HOMA2-R ($r = -0.245$, $p = 0.038$).

Associations between long COVID and activated immune responses

To identify the most robust predictors of long COVID disease, we have executed binary logistic regression analyses in the total study group ($n = 180$), designating long COVID as the dependent variable (with the control group serving as the reference group) and employing the IgA/IgG/IgM responses to the antigens as independent variables, while adjusting for age, sex, and study site (Table 2). Regression #1-3 revealed that IgG and IgM-SARS-CoV-2 were significantly and positively associated with long COVID, while IgA against SARS-CoV-2 was inversely associated. The effect sizes were (without age, sex, and research site) 0.018, 0.085 and 0.028 for IgA, IgG, and IgM, respectively. There were no significant effects of age, sex, and research site (and this for all variables). Regressions #4-6 reveal positive associations between IgG and IgM-HHV-6 and long COVID, with effect sizes of 0.211 and 0.023, respectively. Regressions #7-9 indicate that both IgG and IgM-HHV-6-duTPase are highly significantly associated with long COVID, with Nagelkerke effect sizes of 0.073 and 0.086, respectively. We found that IgA and IgM directed to activin-A were significantly and positively associated with long COVID (effect sizes were 0.146 and 0.048, respectively), while IgG-activin-A showed an inverse association (effect size was 0.196). Figure 1 shows that patients with long COVID have significantly elevated levels of IgA and IgM towards activin-A as compared to controls.

There were no significant associations between the Ig responses to citrullinated EBV EBNA2 peptide and EBV-duTPase and long COVID, except EBV-duTPase which was significantly and negatively associated with long COVID (Wald = 17.29, df = 1, $p = 0.010$, Odds ratio = 0.232, 95% CI interval: 0.117 – 0.462) with an effect size of 0.080. Interestingly, one PC could be extracted from the IgA responses to IgA responses to SARS-CoV-2, HHV-6, HHV-6-duTPase, citrullinated EBV EBNA2 peptide, and EBV-duTPase (labelled PC_IgA, EV = 73.6%, KMO = 0.845, $X^2 = 1203.72$, df = 10, $p < 0.001$). Table 2 shows that this PC was significantly and inversely associated with long COVID with an effect size of 0.022. There were no significant associations between long COVID and IgA/IgG/IgM responses to IFN-α2, HSP60, and HSP90.

Regression #14 shows the results of an automatic multivariable logistic regression analysis with long COVID as outcome variable (and no long COVID as reference group). Thus, the best prediction of long COVID ($X^2 = 138.46$, df = 5, $p < 0.001$) was obtained when

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Table 2. Results of binary logistic regression analysis with the diagnosis long COVID as dependent variable (healthy controls as reference group)

Regression number	Explanatory variables	*B*	*SE*	Wald	*p*	*OR*	95% CI
1	IgA-SARS-CoV-2	− 0.609	0.297	4.197	0.040	0.544	0.304–0.974
2	IgG-SARS-CoV-2	1.448	0.399	13.153	<0.001	4.255	1.945–9.306
3	IgM-SARS-CoV-2	0.506	0.253	4.012	0.045	1.659	1.011–2.723
4	IgA-HHV-6	0.186	0.299	0.39	0.533	1.205	0.670-2.164
5	IgG-HHV-6	2.295	0.417	30.326	<0.001	9.924	4.385–22.459
6	IgM-HHV-6	0.475	0.23	4.271	0.039	1.608	1.025–2.523
7	IgA-HHV-6-duTPase	− 0.114	0.287	0.158	0.691	0.892	0.509–1.565
8	IgG-HHV-6-duTPase	1.580	0.424	13.863	<0.001	4.855	2.113–11.153
9	IgM-HHV-6-duTPase	0.956	0.260	13.501	<0.001	2.601	1.562–4.331
10	IgA-activin-A	1.267	0.239	28.038	<0.001	3.551	2.221–5.676
11	IgG-activin-A	− 2.497	0.446	31.316	<0.001	0.082	0.034–0.197
12	IgM-activin-A	0.796	0.298	7.123	0.008	2.216	1.235–3.975
13	PC_IgA	− 0.415	0.154	7.321	0.007	0.660	0.489-0.892
14	IgG-SARS-CoV-2	0.726	0.199	13.32	<0.001	2.067	1.400; 3.052
	IgG-HHV-6	0.818	0.226	13.10	<0.001	2.265	1.455; 3.527
	IgM-HHV-6-duTPase	0.617	0.205	9.06	0.003	1.853	1.240; 2.769
	IgA-activin-A	1.681	0.272	38.12	<0.001	5.369	3.149; 9.153
	PC_IgA	− 1.786	0.276	42.01	<0.001	0.168	0.098; 0.288

OR, Odds ratio; 95 CI, 95% confidence intervals. All results are adjusted for age; sex; and research centre. Ig, Immunoglobulin; HHV, Human herpes virus; duTPase, deoxyuridine 5′-triphosphate nucleotidohydrolase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PC_IgA, principal component extracted from all IgA values against viral antigens.

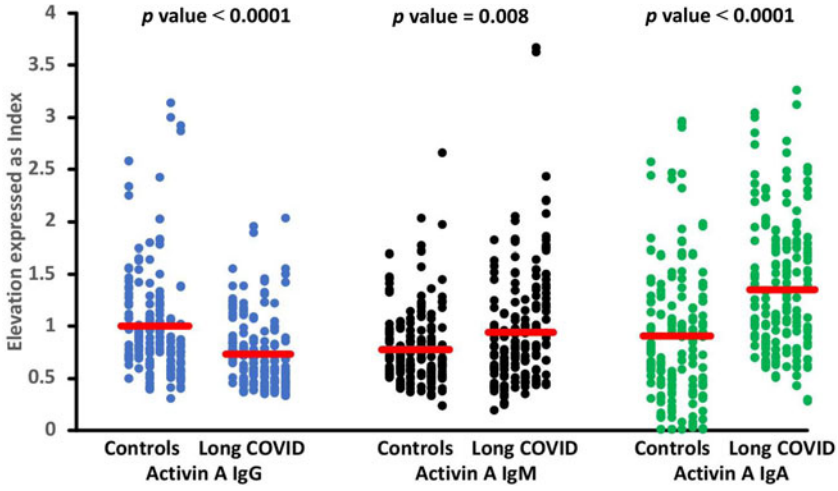

Figure 1. Differences in immunoglobulins igM, igA and igG levels against activin-A between long COVID patients and controls.

PC_IgA (inversely associated) was combined with IgG-SARS-CoV-2, IgG-HHV-6, IgM-HHV-6-duTPase, and IgA-activin-A (all positively associated). The Nagelkerke effect size was 0.533 and 79.8% of all subjects were correctly classified with a sensitivity of 74.6% and specificity of 84.2%.

Table 3, neural network model #1 (NN#1) shows the characteristics of a first neural network model discriminating long COVID from controls. This model was constructed using two hidden layers with seven nodes in hidden layer 1, 5 in hidden layer 2, and 2 units in the output layer. The error term was significantly lower in the testing than in the training sample, whilst the percentage of incorrect classifications was fairly constant between the three samples, indicating that the model was not overtrained. This solution was better than the logistic regression, with a predictive accuracy (computed in the holdout sample) of 80.6% (sensitivity = 78.9% and specificity = 81.8%) and an area ROC curve of 0.876. The topmost important biomarkers (Fig. 2) were in descending order of importance: IgA-activin-A, IgG-HHV-6, PC_IgA, IgM-HHV-6-duTPase, and IgG-SARS-CoV-2

There were no significant correlations between the biomarkers, PBT or SpO2, and the IgA/IgG/IgM responses listed in Table 2, except between PBT and IgA-activin-A ($r = 0.323$, $p = 0.006$), but after false discovery p-correction, the latter was no longer significant.

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Table 3. Results of neural networks (NN) with the diagnosis long COVID or the severity of the long COVID phenome as output variables and immune variables as input data

	Models	NN#1	NN#2	NN#3
Input Layer	Number of units	9	15	15
Hidden layers	Number of hidden layers	2	2	2
	Activation function	Sigmoid	Sigmoid	Hyperbolic tangent
	Number of units in hidden layer 1	7	1	2
	Number of units in hidden layer 2	5	1	21
Output layer	Dependent variable	Long COVID vs controls	Comp_AFFECT (HAMD + HAMA + BDI)	Total FF
	Number of units	2	1	1
	Activation function	Softmax	Sigmoid	Identity
Training	Error term	49.549 (cross-entropy)	0.679 (sum of squares)	9.877 (sum of squares)
	%incorrect or relative error	21.2%	0.821	0.664
	Prediction (sens, spec)	74.5%, 82.5%	–	–
Testing	Sum of squares error	30.259	0.366	0.803
	% incorrect or relative error	26.2%	0.847	0.589
	Prediction: sens, spec	75.8%, 71.4%	–	–
	AUC ROC	0.876	–	–
Holdout	%incorrect or relative error	19.4%	0.950	0.764
	Predictive accuracy: Sens, spec, or r value (predicted vs observed)	78.9%, 81.8%	$r = 0.632$	0.709

Sens, Sensitivity; Spec, Specificity; AUC, Area under curve; ROC, Receiver operating characteristic; comp_AFFECT, severity of affective symptoms due to long COVID (computed as z HAMD + z HAMA + z BDI); HAMA, Hamilton anxiety rating scale; BDI, Beck Depression Inventory; HAMD, Hamilton Depression rating scale; FF, Fibro-Fatigue scale score.

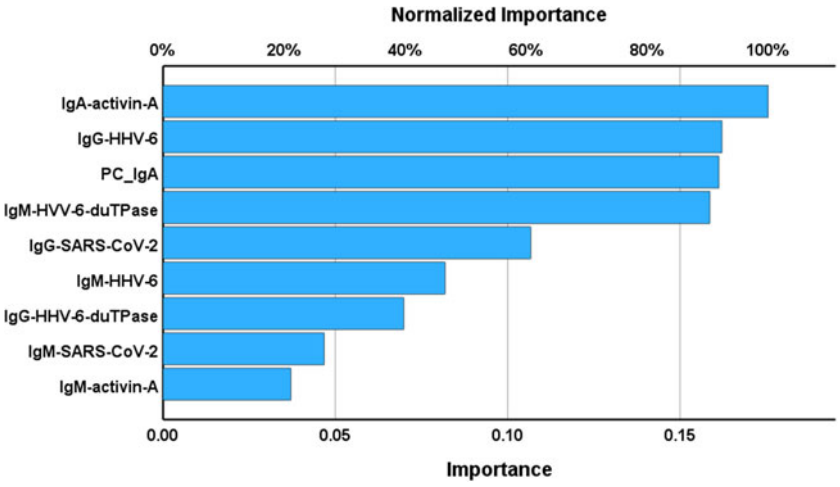

Figure 2. Importance chart of a neural network analysis with long COVID patients versus controls as output variables. Ig: immunoglobulin. HHV: human herpes virus, duTPase: deoxyuridine 5′-triphosphate nucleotidohydrolase, SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, PC_IgA: principal component extracted from all igA values against viral antigens.

Results of neural networks with phenome rating scores as output variables

We also analysed the effects of the abovementioned biomarkers on affective and FF scores. Figure 3 and Table 3 depict the features of NN#2, which used affective symptoms (comp_AFFECT) as the output variable. This model was trained with two hidden layers, each with one unit, and with sigmoid as the activation function for the hidden and output layers. During training, the neural network model improved its ability to generalise from the trend, as measured by a decrease in the error term. In addition, the model is not overfitted because the relative error terms were quite similar in the training, testing, and holdout samples. The predicted versus observed r value was 0.632. The relative (normalised) importance of all input variables is depicted in Figure 3's relevance chart. The top predictors that showed the highest predictive value of the model were in descending order: IgM-HHV-6-duTPase, IgG-HHV-6, CRP, education, and IgA-activin-A, followed at a distance by IgG-SARS-CoV-2, IgM-HHV-6, and IgG-HHV-6-duTPase.

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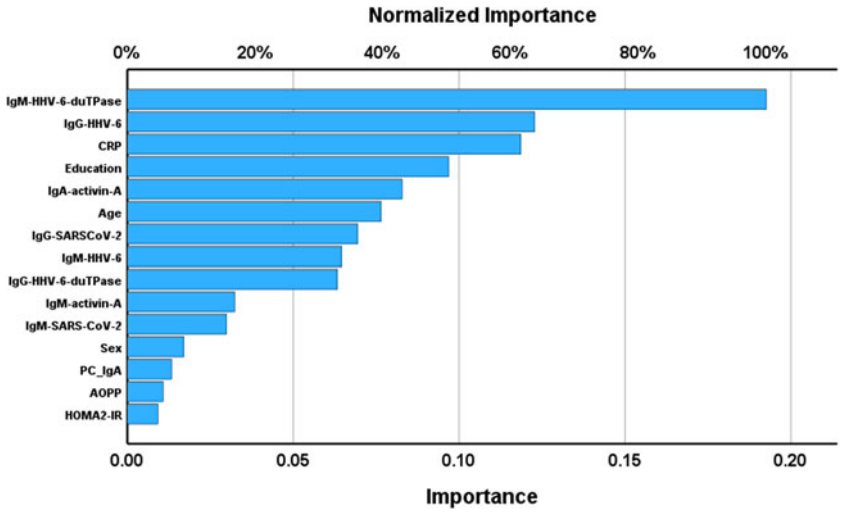

Figure 3. Importance chart of neural network analysis with an affective composite score as dependent variable. Ig: immunoglobulin. HHV: human herpes virus, duTPase: deoxyuridine 5′-triphosphate nucleotidohydrolase, SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, PC_IgA: principal component extracted from all igA values against viral antigens, AOPP: advanced oxidation protein products, CRP: C-reactive protein, HOMA2-IR: homeostatic model assessment for insulin resistance.

NN#3 used the total FF score as an output variable and was trained using a two-layer architecture. The hyperbolic tangent was used as the activation function during training on this layer and identity in the output layer. After training, the neural network model could more accurately predict future values by reducing the error term (sum of squares) from 9.877 to 1.213. The training (0.746), testing (0.589), and holdout (0.764) samples had quite similar relative error terms, showing that the model is not overfitted. The predicted versus observed r value was 0.709. The relative (normalised) importance of the input variables is depicted in Figure 4's relevance chart. The top 5 predictors were in descending order of importance: CRP, IgG-HHV-6-duTPase, IgM-activin-A, IgM-SARS-CoV-2, and IgA-activin-A, followed at a distance by IgM-HHV-6, AOPP, sex, IgG-SARS-CoV-2, and education. Figure 5 shows the predicted versus observed value of the NN#3 model.

Discussion

Long COVID is associated with an immune response

The primary outcome of this research indicates that long COVID disease is characterised by an enhanced immune-inflammatory response compared to controls. This is evidenced by a marked elevation in specific immunoglobulins reactive to viral and self-antigens, along with increased levels of CRP and AOPP. Prior studies have provided evidence indicating that the activation of immune-inflammatory pathways, characterised by heightened cytokine production, increased CRP levels, the involvement of the NLRP3 inflammasome, oxidative stress pathways, and lowered antioxidant levels play a crucial role in the pathophysiology and persistence of long COVID disease (Al-Hakeim *et al.*, 2022a; Al-Hakeim *et al.*, 2022b; Almulla *et al.*, 2023; Low *et al.*, 2023; Al-Hakeim *et al.*, 2023b). The current investigation additionally demonstrates that long COVID is characterised by elevated levels of IgA, IgG and/or IgM antibodies targeting HHV-6, HHV-6-duTPase, SARS-CoV-2, and activin-A. These findings extend those of a recent review showing that persistence of inflammation, and immune dysregulation are common patterns leading to or maintaining long COVID (Vojdani *et al.*, 2023).

Therefore, the results of our study may indicate a potential link between the activated immune and oxidative pathways in long COVID and the elevated levels of immunoglobulins in response to SARS-CoV-2, HHV-6, HHV-6-duTPase, and activin-A. The latter immune responses to viruses and activin-A may, in theory, play a role in initiating or maintaining the immune-inflammatory response that is characteristic of long COVID (Vojdani *et al.*, 2023). However, the current study did not find any statistically significant correlations between CRP or AOPP and any of the IgA/IgG/IgM responses. Furthermore, our study did not observe any discernible impacts of PBT or SpO2 on the IgA/IgG/IgM responses during the acute infectious phase. In contrast, we found significant correlations between PBT (positively) and SpO2 (inversely) and CRP, AOPP, and the HOMA2-IR index. As previously mentioned in the introduction, it can be inferred that the intensity of the acute infectious stage is indicative of the subsequent immune and oxidative responses, but not the IgA/IgM/IgG responses to viral antigens and self-antigens measured here.

In agreement with our previous studies (Al-Hakeim *et al.*, 2023a), the current study found that the HOMA-2IR index was higher in long COVID patients than in controls. Nevertheless, no associations were detected between the increased HOMA2-IR index and the IgA/IgM/IgG responses examined in the present study.

Reactivation of HHV-6 and autoimmune responses to activin-A predict long COVID

The second important finding from this study is that the clinical diagnosis of long COVID disease is predicted by elevated levels of IgM/IgG against HHV-6 and its duTPase, and IgG/IgM directed to SARS-CoV-2, as well as IgA/IgM against activin-A, while there are no increased immunoglobulin responses to EBV, IFN-α2 or HSP60/90. Our findings indicate that the pathophysiology of long COVID is associated with reactivation of HHV-6, SARS-CoV-2 persistence, and autoimmunity to activin-A. A previous review (Vojdani *et al.*, 2023) summarised that persistent SARS-CoV-2 infection, HHV-6 reactivation, and associated processes during long COVID contribute to the pathophysiology of long COVID. Nevertheless, it is unclear whether latent viruses amplify the immunological response that is important in initiating long COVID or whether they directly contribute to the disease.

The findings presented in this study extend those of prior research which documented reactivation of HHV-6 by detecting DNA of the virus in 25% of the individuals with long COVID (Zubchenko *et al.*, 2022). The underlying mechanism may be

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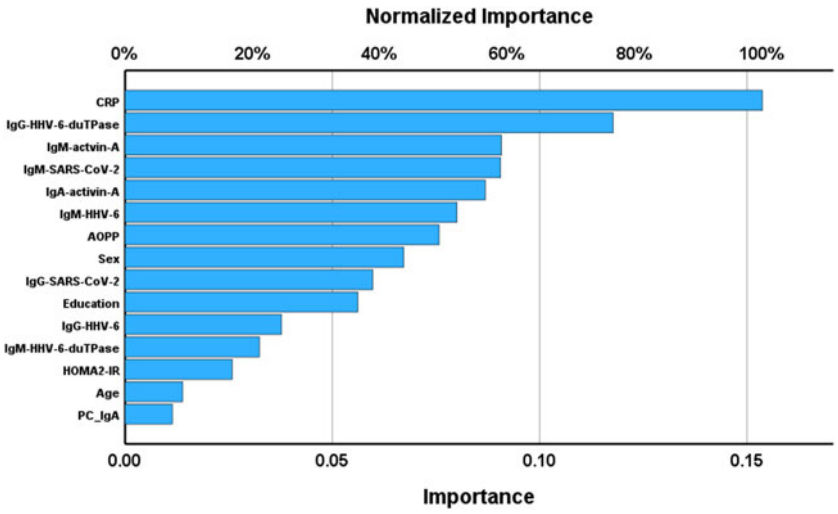

Figure 4. Importance chart of neural network analysis with to total fatigue-fibromyalgia (FF) score as dependent variable. Ig: immunoglobulin. HHV: human herpes virus, duTPase: deoxyuridine 5′-triphosphate nucleotidohydrolase, SARS-CoV-2: severe acute respiratory syndrome coronavirus 2, PC_IgA: principal component extracted from all igA values against viral antigens, AOPP: advanced oxidation protein products, CRP: C-reactive protein, HOMA2-IR: homeostatic model assessment for insulin resistance.

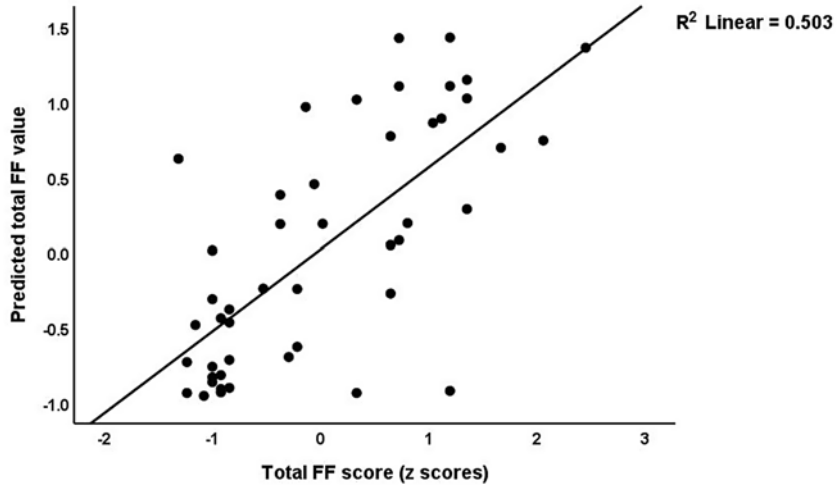

Figure 5. Predictive accuracy of the neuronal network (NN#3) shown in Figure 4 and Table 3; the predicted versus observed value of the fibro-fatigue score.

explained by the interference with the host's type I interferon response, which may be disrupted by autoantibodies (Acharya *et al.*, 2020), resulting in a compromised ability to control latent pathogens (Rojas *et al.*, 2022; Vojdani *et al.*, 2023). However, the current study did not find any significant alterations in the IgA/IgG/IgM-mediated responses to IFN-α2.

Furthermore, SARS-COV-2 persistence may be a factor in some patients since IgM to SARS-CoV-2 is connected to the long COVID diagnosis, albeit with a smaller impact size (see neural network data). Previously, Su et al., (Su *et al.*, 2022) reported that many individuals with long COVID had antibodies against the receptor-binding domain of the SARS-CoV-2 spike protein. This indicates the persistence of SARS-CoV-2 virus even after months of full recovery. As a result, the ongoing presence of SARS-CoV-2 and its secreted superantigens, which are known to trigger polyclonal T-cell activation, may cause immune activation, including dendritic cell activation, and apoptotic processes in the hosts' cells. Consequently, this process may lead to auto-immune responses seen in long COVID patients (Jacobs, 2021; Vojdani *et al.*, 2023).

Previous reports found EBV reactivation in long COVID, as indicated by an increase in VCA IgM and EA-D IgG levels (Gold *et al.*, 2021). Only a few patients showed positive EBV viraemia from nasal swabs 2-3 months after recovery (Su *et al.*, 2022). Likewise, in the current investigation, we were unable to demonstrate elevated immunoglobulin responses to EBV antigens in long COVID.

Intriguingly, our analysis found that the long COVID diagnosis was inversely correlated with the first component extracted from all IgA levels (labelled: PC_IgA) to viral antigens (SARS-CoV-2, HHV-6, EBV, and duTPase from HHV-6 and EBV). IgA is effective against certain viruses, including SARS-CoV-2 (Quinti *et al.*, 2021; Sterlin *et al.*, 2021). The severity of COVID-19 and prolonged viral shedding may be aggravated by decreased anti-SARS-Cov-2 (and other viruses) IgA and secretory IgA (sIgA) (Quinti *et al.*, 2021). As a result, we may posit that long COVID may develop, especially in subjects with decreased IgA against viral antigens or abnormalities in IgA class switching.

Importantly, our neural network analysis shows that the diagnosis of long COVID is well classified with a predictive accuracy of 80.6% (sensitivity of 78.9% and specificity of 81.8%); the top discriminatory variables are IgA-activin-A, IgG-HHV-6, IgM-HVV-6-duTPase, IgG-SARS-CoV-2, and IgM-HHV-6 (all positively) and PC_IgA (inversely). These results of our univariate and multivariate analyses show that long COVID is largely the consequence of reactivation of HVV-6, autoimmune responses to activin-A, activated immune and oxidative stress pathways, and to a lesser extent SARS-CoV-2 persistence.

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IgA/IgG/IgM responses to antigens predict the severity of the long COVID phenome

The third major finding of this study is that a combination of different immunoglobulins to viral and self-antigens and inflammatory biomarkers predicts the severity of the affective symptoms and CFS due to long COVID; the top predictors were (in descending order): CRP, IgA-Activin-A, IgG-HHV-6-duTPase, IgG-HHV-6, IgM-HHV-6-duTPase, and IgM-HHV-6. These findings indicate that reactivation of HHV-6, IgA-mediated responses to activin-A, and activated immune-inflammatory pathways predict the severity of the long COVID phenome.

Long-term COVID patients display symptoms comparable to those observed in myalgic encephalomyelitis (ME)/CFS, a condition characterised by severe fatigue, musculoskeletal pain, and post-exercise malaise, as well as affective symptoms and neurocognitive deficits (Morris and Maes, 2013a). A previous longitudinal study demonstrated that, in long COVID, fatigue was associated with EBV viraemia and that in acute COVID-19 memory impairment was associated with EBV viraemia and SARS-CoV-2 RNAemia (Su *et al*., 2022). There is evidence that ME/CFS and affective disorders are associated with activated immune-inflammatory and oxidative pathways, while ME/CFS also has strong associations with a variety of autoimmune processes (Twisk and Maes, 2009; Morris and Maes, 2013b; Gerwyn and Maes, 2017; Komaroff and Bateman, 2021; Kedor *et al*., 2022). In addition, ME/CFS is frequently precipitated by viral infections, such as HHV-6, or repeated exposure to pathogens, suggesting that infectious agents such as HHV-6 play a role in both ME/CFS and long COVID (Maes *et al*., 2012; Morris and Maes, 2013b; Rasa *et al*., 2018). Importantly, the substantial increase in virus-specific HHV-6 antibodies in saliva is especially pronounced in patients with ME/CFS (Apostolou *et al*., 2022). These findings emphasise similarities in antiviral profiles against latent viruses in ME/CFS and long COVID (Apostolou *et al*., 2022).

Our findings suggest that activin-A abnormalities play a significant role in the severity of affective symptoms and CFS due to long COVID. Indeed, elevated IgA-mediated autoimmunity against activin-A is the most significant predictor of the diagnosis long COVID, while IgA/IgM responses to activin-A are major predictors of the severity of the affective phenome and CFS symptoms. As described in the Introduction, activin-A, as a member of the TGF-β family, is a crucial component of immune function. Inflammatory cytokines, Toll-like receptor ligands, and oxidative stress stimulate the production and release of activins (Gravelsina *et al*., 2021). Activin-A regulates the growth and differentiation of various immune cell types and immune responses and plays a role in the activin/follistatin axis, which is significantly disrupted in COVID-19 cases and is linked to higher mortality rates (Bilezikjian *et al*., 2006; Megan *et al*., 2021; Synolaki *et al*., 2021). Activin-A has been shown to inhibit virus replication in multiple infected cell lines (Lidbury *et al*., 2017) and inhibits virus replication in the A549 lung epithelial cell line following Zika virus infection, either alone or in combination with IFN-α (Eddowes *et al*., 2019). Activin-A plays a dual function in autoimmune diseases, exhibiting both pro-inflammatory and anti-inflammatory properties. Specifically, it regulates the inflammatory responses associated with pathogenic T helper 1 and T helper 17 cells in the central nervous system (Morianos *et al*., 2019).

Importantly, aberrations in activin are known to influence depressive and anxiety-related behaviours (Dow *et al*., 2005; Ageta *et al*., 2008), and play a role in the pathophysiology of ME/CFS (Lidbury *et al*., 2017). Additionally, activin is involved in cognitive functioning (Zheng *et al*., 2017). The expression of activin-A increases in response to neuronal activity (Andreasson and Worley, 1995; Inokuchi *et al*., 1996) and may protect neurons from ischaemic injury (Tretter *et al*., 2000). It influences dendritic spine morphology, a critical factor for synaptic plasticity in the hippocampus (Fukazawa *et al*., 2003; Shoji-Kasai *et al*., 2007). Recent research by Dow and colleagues (Dow *et al*., 2005) demonstrated that the administration of activin into the hippocampus produced similar effects to antidepressants. In addition, the concentration of activin in the hippocampus appears to influence behaviours associated with depression and anxiety. Therefore, activin may be a novel modulator of affective symptoms (Ageta *et al*., 2008). Activin B, which shares 65% sequence homology with Activin-A (Massagué, 1987), has also been identified as a novel biomarker for CFS. Lidbury et al. (2017) suggest that analysing levels of activin and its binding protein, follistatin, could be a valuable tool for distinguishing CFS from other fatigue-related disorders (Lidbury *et al*., 2017). Activin may therefore play an important role in the pathophysiology of affective and CFS symptoms due to long COVID.

Limitations

While interpreting the current data, it is imperative to acknowledge certain limitations. Specifically, an examination of immunoglobulins against a broader spectrum of latent viruses – including CMV, HHV-7, Herpes Simplex Virus types 1 and 2, Human Papillomavirus, and Adenovirus – will enrich future studies. Second, in the case-control part of this study we combined Iraqi and American participants to estimate the adjusted Odds ratios of the immune data predicting long COVID. All these results were adjusted for age, sex, and research site. Moreover, all IgA/IgM/IgG biomarker analyses were performed in the same centre (LA, USA) by the same operator, thereby minimising analytical error. The cohort part of this study, which examined the associations between the biomarkers and the phenome of long COVID, was performed on the Iraqi sample only. Likewise, the CRP and AOPP data were analysed on the Iraqi samples by the same operator at the Medical Laboratory Technology Department, The Islamic University of Najaf, Iraq. More studies are required to investigate the associations between immune-inflammatory pathways, including the NLRP3 inflammasome, and oxidative and nitrosative stress, including hypernitrosylation, and SARS-CoV-2 persistence, latent virus reactivation, and autoimmune reactivity in long COVID disease.

Conclusions

Long COVID patients demonstrate elevated levels of CRP, AOPP, HOMA2-IR, IgG/IgM-SARS-CoV-2, IgG/IgM-HVV-6 and HVV-6-duTPase, and IgA/IgM-activin-A. Using the combination of these data in neural networks, we obtained adequate predictive accuracy, predicting long COVID. The severity of the long COVID phenome, which includes depression, anxiety, and CFS symptoms, was predicted by immune and oxidative biomarkers, with CRP, IgM/IgG-HHV-6, IgM/IgG-HHV-6-duTPase, and IgM/IgA-activin-A as top predictors. These data show that interactions among these different biomarkers are associated with the development or maintenance of long COVID. The results show that reactivation of HHV-6, persistence of SARS-CoV-2, and autoimmune reactions to activin-A, in conjunction with activated immune-oxidative

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pathways and increased insulin resistance, play a significant role in the pathophysiology of long COVID.

Data availability. The corresponding author (MM) will make the SPSS file used in the current study available upon receipt of an appropriate request and once the author has fully exploited the data.

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Author contributions. AA and AV oversaw taking blood samples and managing other patient-related duties. AV quantified the biomarkers in the blood serum. MM conducted the study's statistical analysis. The work is written and edited by AA, MM, AV, and HAH. All authors have read and approved the final manuscript.

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Ethical approval. The College of Medical Technology Ethics Committee approved this study at the Islamic University of Najaf, Iraq (Document No. 34/2023). The research was conducted strictly per local, international, and Iraqi ethical and privacy laws. Written informed consent was obtained from patients and controls.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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