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A Case-Control Study

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Published in:
International Journal of Infectious Diseases

DOI (link to publication from Publisher):
10.1016/j.ijid.2025.108032

Publication date:
2025

Document Version
Publisher's PDF, also known as Version of record

International Journal of Infectious Diseases 159 (2025) 108032

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Classic pathway persistent complement activation is associated with specific symptoms in individuals with post–COVID-19 condition: A case-control study

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ARTICLE INFO

Article history:
Received 5 August 2025
Revised 24 August 2025
Accepted 26 August 2025

Keywords:
Long-COVID
Complement
post–COVID-19
Plasma
Fatigue
Dyspnea

ABSTRACT

Objectives: The complement system is a crucial part of the immune system. The role of complement activation in post–COVID-19 condition is still not conclusive. We present a case-control study investigating long-lasting complement hyperactivation in COVID-19 survivors with/without post–COVID-19 condition.
Methods: A case-control (2:1 design) study was performed. Concentration levels of four proteins from classic complement pathway (C3, C4, C5, C7) and total hemolytic complement (CH50) activity were obtained from blood samples in group of survivors with post–COVID-19 condition and in a comparison group of survivors without post–COVID-19 condition, matched by age, sex, and vaccination status. Post–COVID-19 condition was defined when individuals self-reported at least one of these post–COVID-19 symptoms: fatigue, arthralgia, myalgia, memory problems, new-onset headache, or palpitations.
Results: A total of 57 (56.1% women, age: 46.5 years, SD: 9.0 years) survivors with post–COVID-19 condition and 27 (55.5% women, age: 46.5 years, SD: 11.5 years) survivors without post–COVID-19 condition were evaluated 1.7 (SD 1.2) and 2.0 (SD 1.7) years after SARS-CoV-2 infection, respectively. Overall, the results did not reveal differences in complement protein levels and CH50 activity between survivors with or without post–COVID-19 condition. Patients reporting post-COVID fatigue exhibited lower C3 levels ($P = 0.025$) than those without post-COVID fatigue, whereas survivors with post-COVID dyspnea reported lower levels of C3 ($P = 0.001$), C5 ($P = 0.015$), and C7 ($P = 0.030$) proteins than those without post-COVID dyspnea.
Conclusions: This explorative case-control study did not observe overall complement activation from classic pathway in survivors with post–COVID-19 condition up to 2 years after. Some complement proteins were elevated in individuals with specific post-COVID symptoms, such as fatigue or dyspnea.

Introduction

The world has experienced one of the worst health crises of this century in the last years due to the rapid spread of COVID-19, the infection caused by the SARS-CoV-2. The COVID-19 outbreak caused up to 778 million confirmed cases and 7.1 million deaths

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https://doi.org/10.1016/j.ijid.2025.108032

worldwide [1]. To date, the spread and virulence of SARS-CoV-2 has been controlled due to the advances in their pathogenesis and the development of preventive strategies such as vaccination [2].

Several molecular mechanisms have been identified to be involved during the acute COVID-19 phase. One of the mechanisms suggested in the pathogenesis of COVID-19 is potential hyperactivation of the complement system [3] because an increased complement activation, as seen in autoimmune diseases, is associated with COVID-19 severity due to compensatory immune regulatory mechanisms [4]. Thus, a meta-analysis found that C3 and C4 complement protein levels were significantly lower (indicative of complement hyperactivation causing inflammation and damage to healthy cells and tissues) in people with severe COVID-19 or in non-survivors compared with patients with less severe COVID-19 or survivors, indicating that these complement proteins are more readily consumed in patients with a severe or fatal course of COVID-19 disease [5]. Because control of complement activation observed at the acute COVID-19 phase has been used as a therapeutic target for managing these patients [6], identification of long-lasting complement activation in COVID-19 survivors experiencing post-COVID symptoms could open therapeutic strategies for these patients. As an example, the US Food and Drug Administration provided emergency use authorization for anti-C5 antibodies drugs for treating severe COVID-19 in the acute phase [7], a situation which provoked speculation about its potential applicability in patients with post–COVID-19 condition.

Evidence suggests that up to 25-30% of COVID-19 survivors exhibit long-lasting symptoms 2 [8] or 3 [9] years after the infection. Nevertheless, the diagnosis of post–COVID-19 condition is not properly reported because only 0.5% of participants with at least one post-COVID symptom 1 year after infection receive an appropriate diagnostic code [10]. This can be related to the fact that the manifestations of post–COVID-19 condition is heterogeneous and different clusters of patients, grouped by predominant symptoms, are described [11]. This heterogeneity in post-COVID symptoms led to the hypothesis that different pathophysiological mechanisms can operate [12]. Thus, a better understanding of operating immune regulatory mechanisms behind post–COVID-19 condition is crucial for a personalized-medicine approach. Among those underlying mechanisms proposed to be involved in post–COVID-19 condition, persistent complement hyperactivation has been raised as a factor potentially contributing to long-lasting post-COVID symptomatology.

Evidence on complement hyperactivation in post-COVID is still on its infancy if compared with data at the acute COVID-19 phase. Thus, data on complement activation at the post-COVID phase are heterogeneous because some studies suggested an increased complement activation in survivors with post-COVID symptoms [13,14], whereas other did not [15]. However, previous studies in patients with long-COVID have investigated one or two complement protein from different pathways. In fact, complement activation is complex because it involves classic complement pathway, alternative complement pathway, and mannan-binding lectin (MB-lectin) pathway. Classic complement pathway includes the native components of the complement. Previous studies on individuals with post–COVID-19 condition have investigated single proteins associated with either classic or alternative pathways [13–15].

We present here a case-control, explorative study investigating the presence of long-lasting complement activation in the classic pathway in COVID-19 survivors with and without post–COVID-19 condition. We hypothesized that individuals who recover from a SARS-CoV-2 infection and develop post–COVID-19 condition will exhibit long-lasting complement hyperactivation (expressed by lower C3, C4, C5, and C7 protein levels, as well as lower by lower CH50 activity) compared with participants who recovered from acute SARS-CoV-2 infection but did not develop post–COVID-19 condition. If immune system hyperactivation in the classic component pathway is identified in people with post–COVID-19 condition, it may be a target for management.

Methods

Study population

A cross-sectional case-control (2:1) study including a group of participants who had recovered from an acute SARS-CoV-2 infection with the presence of post-COVID symptoms and a comparative control group of participants who had recovered from a SARS-CoV-2 infection without the presence of post-COVID symptoms was conducted. The study design was approved by the institutional ethics committees of the involved hospitals (HUIL/092-20; H12OCT23/418). All participants provided their informed consent before collecting data.

Potential eligible participants should report that they have passed a SARS-CoV-2 infection confirmed by reverse transcription-polymerase chain reaction and/or antigen test against SARS-CoV-2 using nasopharyngeal swabs, and the diagnosis should have been confirmed by a medical doctor. Volunteer participants were recruited from the Madrid Long-COVID Society (Spain). Eligible participants were scheduled for a face-to-face appointment at the long-COVID unit of two urban hospitals of Madrid (Spain). An exhaustive clinical examination was done to confirm the diagnosis of post–COVID-19 condition. Demographic (age, sex, height, weight), medical comorbidities, vaccine status, COVID-19–associated onset symptoms, days at the hospital, and intensive care unit admission were collected from medical records.

For defining the case group, we used the definition of post–COVID-19 condition proposed by Soriano et al. [16]: “post–COVID-19 condition occurs in individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of infection, with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis. Common symptoms include but are not limited to fatigue, shortness of breath (dyspnea), and cognitive dysfunction and generally have an impact on everyday functioning. Symptoms might be new onset after initial recovery from an acute COVID-19 episode or persist from the initial illness. Symptoms might also fluctuate or relapse over time.” To be included, survivors report the presence of at least one of the following post-COVID symptoms starting the first 3 months after the infection and persisting at the time of the study: fatigue, dyspnea, myalgias, memory problems, concentration problems, new-onset headache, or palpitations.

Participants in both groups were excluded if they (1) had confirmed or suspected current SARS-CoV-2 infection or (2) had confirmed or suspected SARS-CoV-2 infection within the last 3 months.

Post-COVID data collection

Individuals who fulfilled the inclusion criteria and agreed to participate were scheduled for a telephone interview by experienced health care researchers. Participants were asked to self-report any symptom that appeared 3 consecutive months after the acute SARS-CoV-2 infection and that persisted at the time of the study. A predefined list of long-lasting symptoms, including fatigue, dyspnea, anosmia, ageusia, pain, brain fog, hair loss, pain, or concentration loss, was systematically used, but individuals were free to report any symptom that they suffered from.

Anxiety and depressive symptoms were evaluated using the hospital anxiety and depression scale (HADS) [17], whereas sleep quality was evaluated using the Pittsburgh sleep quality index [18] because both self-rated questionnaires can be properly evaluated

by telephone [19]. Anxiety (HADS-A, seven items, 0-21 points) and depressive (HADS-D, seven items, 0-21 points) subscales of the HADS were included because they have shown good validity in people with post–COVID-19 condition [20].

The functional impairment checklist (FIC) is an eight-item disease-specific questionnaire evaluating the symptoms of severity and functionality in post–COVID-19 condition [21]. Four items (e.g. breathlessness at rest, breathlessness exertion, general fatigue, and muscle weakness) evaluate symptoms severity (FIC symptoms, 0-12 points), whereas another four items evaluate function, such as limitations in occupational daily living activities, leisure/social activities, basic daily living activities, and instrumental activities of daily living (FIC disability, 0-12 points) [21].

Complement fractions

Blood serum was collected from all participating and refrigerated at 4°C until the main analysis. Levels of four different complement fractions from classic pathway were calculated using commercial kits and following the manufacturer's instructions: C3 (Complement 3 reagent) and C4 (complement 4 reagent) were analyzed with the Beckman (Coulter, USA) kit, C5 (complement 5 reagent) with N C5 kit (Trimero diagnostics), and C7 (complement 7 reagent) with Quidel Ortho C7 antibody polyclonal kit (Cat N° A308). The study of C3-C4 components is based on immunoturbidimetric tests, the study of C5 component is based on nephelometric immunoassays, and the analysis of C7 component is based on radial immunodiffusion. According to the information provided in the manufacturer kit, the reference intervals of these fractions are 90-180 mg/dl for C3, 10-40 mg/dl for C4, 9.02-16.7 mg/dl for C5, and 80-120 for C7 component.

Total hemolytic complement activity (CH50)

Blood serum was frozen at –20°C for this analysis. The study of CH50 activity was performed following the manufacturer's instructions (Autokit CH50/Autokit CH50 small, Fujifilm Wako Chemicals, Japan). A minimum of 10 μl of blood serum was required for each analysis.

When the sample is added to the liposome and the substrate, the antibodies in the reagent combines with dinitrophenyl on the liposomes and activates the complement. Consequently, the liposome membrane is broken by the activated complement. The enzyme glucose-6-phosphate dehydrogenase, contained in the liposome, reacts with Nicotinamide Adenine Dinucleotide (NAD) and glucose 6-phosphate, and, as a result, the NAD is reduced to NADH, thus increasing the absorbance at 340 nm. The absorbance increase is proportional to the complement activity.

The manufacturer's reference interval (RI) for CH50 activity ranges from 31.6 to 57.6 U/ml, based on data from a Japanese population [22]. However, it is known that geographical differences can influence the complement system [23] and because of this, RIs too. To establish an accurate RI for the population included in our study, we analyzed CH50 activity in a cohort of 17,084 individuals who underwent testing in our laboratory in 2024. Hence, an estimation of the biological RI (reference values or normal ranges) was conducted using indirect sampling techniques, following the guidelines of the Clinical and Laboratory Standards Institute EP28-A3C for percentiles and confidence intervals. These indirect sampling techniques assume—confirmed by observation—that most results, even in patients in the hospital and clinic, appear and are "normal." Thus, to estimate RIs, laboratory values from an existing database established for other purposes (e.g. reference laboratory information system) can be used. To apply this indirect approach, an outlier analysis was first conducted to exclude values from non-healthy individuals that might be present in the database. Outlier detection was performed using the method proposed by Tukey. After outlier detection, we obtained a sample size of 15,707 individuals.

The RI estimation was carried out following the Clinical and Laboratory Standards Institute EP28-A3C protocol through an indirect method based on bootstrap techniques. These techniques fall within the scope of non-parametric statistics because they do not require any assumption regarding the distribution of the studied populations. The bootstrapping method generates multiple pseudo-samples through resampling, with replacement from the available data. For the outlier analysis and the production of reference values, MedCalc Statistical Software version 23.1.1 was used. Based on these results, we calculated a double-sided 95% RI of 36.5-83.7 U/ml. The 90% confidence interval (CI) for lower limit was 36.2-36.7 and the upper limit was 83.3-84.0. The median of our population was estimated to be 57.9 U/ml. In addition, we also estimated the left-sided 95% RI that should be more appropriate for clinical use. This 95% RI was <39.8 U/ml (95% RI 39.6-40.0). These values closely align with data from a similar study on CH50 activity in a Caucasian population [24].

Statistical analysis

Data were analyzed with SPSS Statistics, version 25.0. Mean and SD are presented for quantitative data, and number of cases (percentages) are presented for categorical data. The Shapiro–Wilk test was used to assess normality. Differences in demographic, clinical data, and complement levels between COVID-19 survivors with and without post–COVID-19 condition were compared with chi-square or analysis of variance tests, as appropriate. Because this was an explorative study, a secondary analysis investigating differences in clinical data and complement levels according to the presence/absence of post-COVID fatigue or post-COVID dyspnea were conducted using analysis of variance tests. The level of significance was set at a priori 0.05, with *P*-values being corrected with Holm–Bonferroni correction.

Results

A sample of 100 individuals with a positive history of SARS-CoV-2 infection was invited to participate in the study. A total of 16 (16%) patients refused to participate. Accordingly, a total of 88 COVID-19 survivors were included. A total of 57 patients had a diagnosis of post–COVID-19 condition, whereas the remaining 27 did not exhibit any post-COVID symptom (comparative group). Both groups were similar in clinical data, preexisting medical conditions and COVID-19–associated onset symptoms (Table 1). Thus, the participants reported infection with the Omicron variant (based on time of infection) and had received at least two vaccine doses before infection. Thus, SARS-CoV-2 reinfections were reported by 21% of the participants (n = 12 post–COVID-19 condition group, n = 6 comparative group, $P = 0.922$).

At the time of study (mean: 1.7, SD: 1.2 years after acute infection), participants in the post–COVID-19 condition group reported a mean of 2.0 (SD 1.7) symptoms (Table 2). Fatigue (49%) and dyspnea (35%) were the most prevalent post-COVID symptoms (Table 1). Survivors with post–COVID-19 condition reported higher related disability, anxiety and depressive levels, and poor sleep quality than those without post–COVID-19 condition (P <0.001, Table 2).

Overall, the analysis did not reveal significant differences in complement fraction levels as well as CH50 activity between individuals with and without post–COVID-19 condition (Table 2). Five (8.8%) patients in the post–COVID-19 condition group had C4 levels higher than the reference value of 40 mg/dl. In addition, 11 (19.3%)

Table 1
Clinical data according to the presence or absence of post–COVID-19 condition.

	Post–COVID-19 condition (n = 57)	No post–COVID-19 condition (n = 27)	*P*-value
Female n (%)	32 (56.1%)	15 (55.5%)	0.958
Age (years)	46.5 ± 9.0	46.5 ± 11.5	0.955
Weight (kg)	70.0 ± 9.0	72.0 ± 9.5	0.589
Height (cm)	166.5 ± 5.0	168.5 ± 5.5	0.533
Number of pre-existing medical conditions	0.7 ± 0.5	0.6 ± 0.3	0.665
Obesity (preexisting)	5 (8.8%)	2 (7.4%)	0.845
Hypertension (preexisting)	5 (8.8%)	3 (11.1%)	0.757
Diabetes mellitus (preexisting)	3 (5.2%)	1 (3.7%)	0.764
Asthma (preexisting)	1 (2.0%)	0 (0.0%)	0.492
Chronic obstructive pulmonary disease (preexisting)	0 (0.0%)	1 (3.7%)	0.151
Cardiac diseases (preexisting)	1 (2.0%)	0 (0.0%)	0.492
Musculoskeletal Pain (preexisting)	11 (19.3%)	4 (14.8%)	0.673
Other diseases (preexisting)	5 (8.8%)	3 (11.1%)	0.757
Number of COVID-19 onset symptoms	2.2 ± 0.8	2.0 ± 1.1	0.543
Fever (COVID-19 onset)	21 (36.8%)	10 (37.1%)	0.909
Dyspnea (COVID-19 onset)	7 (12.3%)	3 (11.1%)	0.890
Myalgia (COVID-19 onset)	14 (24.6%)	6 (22.2%)	0.853
Cough (COVID-19 onset)	4 (7.0%)	2 (7.4%)	0.951
Headache (COVID-19 onset)	12 (21.1%)	5 (18.5%)	0.825
Diarrhea (COVID-19 onset)	1 (2.0%)	0 (0.0%)	0.492
Anosmia (COVID-19 onset)	3 (5.2%)	1 (3.7%)	0.764
Ageusia (COVID-19 onset)	2 (3.5%)	1 (3.7%)	0.965
Throat pain (COVID-19 onset)	2 (3.5%)	1 (3.7%)	0.965

Table 2
Post-COVID symptomatology and functionality and complement levels according to the presence or absence of post–COVID-19 condition.

	Post–COVID-19 condition (n = 57)	No post–COVID-19 condition (n = 27)	*P*-value
Fatigue	28 (49.1%)	–-	–-
Dyspnea	20 (35.1%)	–-	–-
Cognitive blurring–brain fog	15 (26.3%)	–-	–-
Pain	15 (26.3%)	–-	–-
Memory loss	12 (21.0%)	–-	–-
Gastrointestinal problems	12 (21.0%)	–-	–-
Anosmia	11 (19.3%)	–-	–-
Ageusia	10 (17.5%)	–-	–-
Concentration loss	10 (17.5%)	–-	–-
Ocular problems	7 (12.3%)	–-	–-
Hair loss	5 (9.0%)	–-	–-
Palpitations-tachycardia	5 (9.0%)	–-	–-
diarrhea	5 (9.0%)	–-	–-
Skin rashes	3 (5.2%)	–-	–-
FIC symptoms (0-12)[a]	2.3 (2.1)	0.6 (0.2)	0.001
FIC disability (0-12)[a]	1.4 (1.3)	0.0 (0.0)	0.001
HADS-A (0-21)[a]	1.6 (0.8)	0.8 (0.2)	0.001
HADS-D (0-21)[a]	2.8 (1.7)	0.7 (0.4)	0.001
PSQI (0-21)[a]	7.7 (3.8)	5.4 (2.5)	0.015
C3 component (mg/dl)	129.4 (24.8)	122.1 (24.7)	0.211
C4 component (mg/dl)	31.3 (9.6)	27.8 (6.7)	0.196
C5 component (mg/dl)	16.9 (3.7)	18.0 (5.2)	0.278
C7 component (mg/dl)	90.9 (13.0)	94.3 (13.8)	0.300
CH50 level	60.3 (10.6)	59.0 (10.2)	0.772

CH50: total hemolytic complement activity; FIC: functional impairment checklist; HADS: hospital anxiety and depression scale (A: anxiety; D: depression); PSQI: Pittsburgh sleep quality index.
[a] Statistically significant differences ($P < 0.05$).

patients in the post–COVID-19 condition group and five (18.5%) individuals in the comparative group showed C5 levels higher than the reference value (16.7 mg/dl, $P = 0.944$). Values of C3 and C7 fractions were within the normal range values in both groups.

A subgroup analysis in the post–COVID-19 condition group revealed that participants specifically reporting post-COVID fatigue or dyspnea reported higher related disability and symptom severity and anxiety or depressive symptoms than those without post-COVID fatigue or dyspnea (Table 3). Participants with post-COVID fatigue exhibited lower C3 levels ($P = 0.025$) than those without fatigue, whereas survivors with post-COVID dyspnea had lower C3 ($P = 0.001$), C5 ($P = 0.015$), and C7 ($P = 0.030$) levels than those without dyspnea (Table 3).

Discussion

Long-lasting complement activation in post–COVID-19 condition

Persistent inflammation has been implicated in post–COVID-19 condition [12], which can be caused by immune hyperactivation. Thus, it is hypothesized that viral reactivation, complement dysregulation, thrombin-mediated pathway, and inflammatory responses can interplay in the pathogenesis of post–COVID-19 condition [12]. However, it is unclear whether complement hyperactivation persists after the acute COVID-19 phase and can be long-lasting and sustained in survivors developing post–COVID-19 condition. Because SARS-CoV-2 activates the complement system di-

Table 3
Post-COVID functionality and complement levels according to the presence or absence of post-COVID fatigue or dyspnea.

	Post-COVID fatigue (n = 28)	No post-COVID fatigue (n = 29)	*P*-value
FIC symptoms (0-12)[a]	4.25 (1.6)	0.6 (0.6)	0.001
FIC disability (0-12)[a]	2.9 (1.8)	0.6 (0.2)	0.001
HADS-A (0-21)[a]	2.0 (1.4)	0.75 (0.3)	0.001
HADS-D (0-21)[a]	3.5 (3.1)	1.0 (0.6)	0.001
PSQI (0-21)	8.1 (4.9)	7.2 (2.4)	0.386
C3 component[a]	119.6 (19.3)	132.7 (23.8)	0.025
C4 component	32.5 (13.8)	29.3 (8.4)	0.298
C5 component	15.5 (1.5)	17.3 (4.5)	0.065
C7 component	89.0 (14.6)	90.3 (10.8)	0.703
CH50 level	56.3 (7.7)	61.0 (11.8)	0.085
	Post-COVID dyspnea (n = 20)	**No post-COVID dyspnea (n = 37)**	***P*-value**
FIC symptoms (0-12)[a]	4.7 (1.9)	1.6 (1.5)	0.001
FIC disability (0-12)[a]	3.3 (2.7)	1.9 (0.8)	0.001
HADS-A (0-21)[a]	2.5 (2.2)	0.6 (0.25)	0.001
HADS-D (0-21)[a]	4.3 (4.2)	1.2 (0.9)	0.001
PSQI (0-21)	7.7 (2.6)	7.6 (4.3)	0.915
C3 component[a]	114.6 (16.5)	130.8 (23.2)	0.001
C4 component	30.2 (8.6)	30.9 (11.8)	0.827
C5 component[a]	15.0 (1.4)	17.0 (4.0)	0.015
C7 component[a]	84.5 (10.4)	91.3 (12.7)	0.030
CH50 level	55.7 (4.3)	60.0 (11.5)	0.070

CH50: total hemolytic complement activity; FIC: functional impairment checklist; HADS: hospital anxiety and depression scale (A: anxiety; D: depression); PSQI: Pittsburgh sleep quality index.
[a] Statistically significant differences ($P < 0.05$).

rectly (alternative complement and MB-lectin pathways) and indirectly (classic complement pathway), particularly, via amplification of loop and drive endothelial damage and inflammation, it would be possible that long-lasting inflammation observed in individuals with post–COVID-19 condition can be related to persistent complement hyperactivation [25].

Complement hyperactivation is reflected by a decrease in serum concentration of several complement proteins due to increased product consumption [26]. We hypothesized there are lower protein levels of the classic complement pathway in patients with post–COVID-19 condition; however, we did not find significant differences in C3, C4, C5, or C7 complement proteins nor in complement total activity (CH50). In fact, previous studies had found contradictory results. For instance, Cervia-Hasler et al. [13] observed a decrease in C7 protein levels in a sample of COVID-19 survivors with post-COVID symptoms compared with survivors without post-COVID symptoms 6 months after the infection. On the contrary, Baillie et al. found higher levels of the C5a fragment protein in participants with long-COVID, with a heterogeneous follow-up ranging from 2 years to 3 months after the infection [15]. Our study did not find long-lasting complement hyperactivation in survivors with post–COVID-19 condition up to 2 years after infection in four proteins of the classic complement pathway. Importantly, most COVID-19 survivors with and without post–COVID-19 condition showed complement fraction protein levels within normal values, with some individual exceptions. It is possible that complement hyperactivity would decrease with time, and long-term follow-up lead to a normalization of fraction protein levels, at least in classic pathway. Thus, the fact that no differences in total complement activity (CH50) were seen between both groups would support this hypothesis. Longitudinal studies investigating this hypothesis are needed.

Complement activation associated with specific post-COVID symptoms

Some data suggest that complement dysregulation could be related to specific post-COVID symptoms. We observed that some proteins from the classic complement pathway had lower levels in survivors specifically reporting post-COVID fatigue (C3 protein level) or dyspnea (C3, C5, and C7 protein levels). Decreased C3 and C4 protein levels in serum indicate activation of the classic complement pathway. Vlaicu et al. proposed that decreased C3-C5 protein levels are associated with neuroinflammatory and immune responses, suggesting that terminal complement complex (i.e. membrane attack complex, MAC) explains neurological post-COVID symptomatology [27]. A higher neuroinflammatory and immune response may also explain the development of post-COVID fatigue or dyspnea symptoms. We also found lower C5 and C7 protein levels in survivors with post-COVID dyspnea. Interestingly, C5 inhibitors appear to be the most promising for suppressing the cytokine storm and for reducing COVID-19 severity [28]. Based on current data, we inferred that inhibitors of specific complement proteins may possibly mitigate/alleviate specific post-COVID symptomatology. Future clinical trials investigating this hypothesis are needed.

In agreement with the results of the current study, Hagiya et al. [14] found higher CH50 activity in COVID-19 survivors reporting post-COVID neurologic symptoms (e.g. poor concentration, brain fog) 3 months after the acute infection but no differences in CH50 activity in individuals with post-COVID fatigue and/or dyspnea. Because elevated CH50 levels are related to increased total complement activity, current and previous results did not support an overall hyperactivity (response) of the immune system in survivors with post–COVID-19 condition in response to infection.

Strengths and limitations

The results of this explorative study should be considered according to its potential strengths and limitations. The use of a case-control design with a group of COVID-19 survivors fulfilling an accepted definition of post–COVID-19 condition and with the inclusion of a comparative/control group of survivors without post-COVID symptoms matched to the case group by age, sex, and vaccination status is a significant strength of the study. Thus, both groups exhibited similar preexisting medical comorbidities, which could not explain the results. In fact, this is an important topic because some pre-infection medical comorbidities (e.g. type II diabetes mellitus) can exacerbate complement system dysregulation (particularly, C3 protein) in those with post-COVID symptoms [29]. The number of individuals with diabetes mellitus in this ex-

ploratory study was small, and no association with the presence of post–COVID-19 condition was found. Second, to the best of our knowledge, our study has included the longest follow-up period after a SARS-CoV-2 infection of studies published to date in relation to complement hyperactivity in participants with post–COVID-19 condition, almost 2 years after the acute infection.

Nevertheless, some potential limitations should be also analyzed. First, due to the heterogeneity in the published literature related to complement proteins analyzed and the different follow-up periods, we were unable to conduct an "a priori" sample size calculation, accordingly, our sample size could be considered relatively small. It should also be considered that we included non-hospitalized COVID-19 survivors, so we do not know if the same results would be observed in hospitalized survivors. Second, the cross-sectional nature of the study design did not permit us to longitudinally investigate a potential complement hyperactivity from the time of infection. Because post–COVID-19 has a fluctuating nature, it is possible that complement hyperactivity could also exhibit a potential fluctuating nature, explaining the heterogeneity results found in the literature. Third, we only evaluated the serum levels of some proteins from the native components of the complement. In fact, the assessment of complement activation products requires stringent preanalytical conditions (immediate processing of plasma with addition of ethylenediaminetetraacetic acid (EDTA plasma), addition of inhibitors, and careful handling to avoid *ex vivo* activation). Future studies evaluating alternative complement and MB-lectin pathways using EDTA plasma would support or refute the current findings.

Conclusion

This case-control, explorative study did not find overall long-lasting complement hyperactivation evaluating the classic pathway in individuals who had survived an acute SARS-CoV-2 infection and developed post–COVID-19 condition up to 2 years after infection. Some proteins of the classic complement pathway were elevated in patients with specific symptoms, such as post-COVID fatigue (C3 protein level) or post-COVID dyspnea (C3, C5, C7 protein levels). These findings deserve future research and may lead to new therapeutic strategies.

Declaration of competing interest

The authors have no competing interests to declare.

Funding

The project was supported by a grant from the Novo Nordisk Foundation NNF21OC0067235 (Denmark) and by a grant associated with the Fondo Europeo De Desarrollo Regional - Recursos REACT-UE del Programa Operativo de Madrid 2014-2020, en la línea de actuación de proyectos de I+D+i en materia de respuesta a COVID 19 (LONG-COVID-EXP-CM). Both sponsors had no role in the design; collection; management; analysis; or interpretation of the data, draft, review, or approval of the manuscript or its content. The authors were responsible for the decision to submit the manuscript for publication, and the sponsor did not participate in this decision.

Author contributions

All the authors cited in the manuscript had substantial contributions to the concept and design, execution of the work, or analysis and interpretation of data; and drafting or revising the manuscript. All authors have read and approved the final version of the paper. C. Fernández-de-las-Peñas: conceptualization, visualization, methodology, validation, data curation, writing-original, writing-review, editing. M. Ruiz-Ruigómez: methodology, validation, data curation, writing-original draft, writing-review and editing. L. Esparcia-Pinedo: conceptualization, methodology, validation, data curation, writing-original draft, writing-review, editing. B. Colom-Fernández: conceptualization, methodology, data curation validation, writing-original draft, writing-review, editing. F Cava-Valenciano: conceptualization, methodology, data curation validation, writing-original draft, writing-review, editing. J. Torres-Macho: methodology, validation, data curation, writing-original draft, writing-review, editing. E. Arrieta-Ortubay: methodology, validation, data curation, writing-original draft, writing-review and editing. M. Akasbi-Moltalvo: validation, writing-original draft, writing-review and editing. C. Lumbreras-Bermejo: validation, writing-original draft, writing-review, editing. L. Arendt-Nielsen: validation, writing-original draft writing-review, and editing. A. Franco-Moreno: methodology, validation, data curation, writing-original draft, writing-review and editing.

References

[1] World Health Organization. Coronavirus (COVID-19) Dashboard, https://covid19.who.int/; n.d. [accessed 01 July 2025].
[2] Park MB, Sim B. Vaccine effectiveness of COVID-19 and rebound in the real world. *Clin Exp Med* 2023;**23**:4975–83. doi:10.1007/s10238-023-01204-z.
[3] Li Q, Chen Z. An update: the emerging evidence of complement involvement in COVID19. *Med Microbiol Immunol* 2021;**210**:101–9. doi:10.1007/s00430-021-00704-7.
[4] Jarlhelt I, Nielsen SK, Jahn CXH, Hansen CB, Pérez-Alós L, Rosbjerg A, et al. SARS-CoV-2 antibodies mediate complement and cellular driven inflammation. *Front Immunol* 2021;**12**:767981. doi:10.3389/fimmu.2021.767981.
[5] Zinellu A, Mangoni AA. Serum complement C3 and C4 and COVID-19 severity and mortality: a systematic review and meta-analysis with meta-regression. *Front Immunol* 2021;**12**:696085. doi:10.3389/fimmu.2021.696085.
[6] Bosmann M. Complement control for COVID-19. *Sci Immunol* 2021;**6**:eabj1014. doi:10.1126/sciimmunol.abj1014.
[7] West EE, Woodruff T, Fremeaux-Bacchi V, Kemper C. Complement in human disease: approved and up-and-coming therapeutics. *Lancet* 2024;**403**:392–405. doi:10.1016/S0140-6736(23)01524-6.
[8] Fernandez-de-las-Peñas C, Notarte KI, Macasaet R, Velasco JV, Catahay JA, Ver AT, et al. Persistence of post-COVID symptoms in the general population two years after SARS-CoV-2 infection: a systematic review and meta-analysis. *J Infect* 2024;**88**:77–88. doi:10.1016/j.jinf.2023.12.004.
[9] Rahmati M, Udeh R, Kang J, Dolja-Gore X, McEvoy M, Kazemi A, et al. Long-term sequelae of COVID-19: A systematic review and meta-analysis of symptoms 3 years post-SARS-CoV-2 infection. *J Med Virol* 2025;**97**:e70429. doi:10.1002/jmv.70429.
[10] Wang HI, Doran T, Crooks MG, Khunti K, Heightman M, Gonzalez-Izquierdo A, et al. Prevalence, risk factors and characterisation of individuals with long COVID using Electronic Health Records in over 1.5 million COVID cases in England. *J Infect* 2024;**89**:106235.
[11] Kuodi P, Gorelik Y, Gausi B, Bernstine T, Edelstein M. Characterization of post-COVID syndromes by symptom cluster and time period up to 12 months post-infection: a systematic review and meta-analysis. *Int J Infect Dis* 2023;**134**:1–7. doi:10.1016/j.ijid.2023.05.003.
[12] Fernández-de-las-Peñas C, Raveendran AV, Giordano R, Long Arendt-Nielsen L. COVID or post-COVID-19 condition: past, present and future research directions. *Microorganisms* 2023;**11**:2959. doi:10.3390/microorganisms11122959.
[13] Cervia-Hasler C, Brüningk SC, Hoch T, Fan B, Muzio G, Thompson RC, et al. Persistent complement dysregulation with signs of thromboinflammation in active Long COVID. *Science* 2024;**383**:eadg7942. doi:10.1126/science.adg7942.
[14] Hagiya H, Tokumasu K, Otsuka Y, Sunada N, Nakano Y, Honda H, et al. Relevance of complement immunity with brain fog in patients with long COVID. *J Infect Chemother* 2024;**30**:236–41. doi:10.1016/j.jiac.2023.10.016.
[15] Baillie K, Davies HE, Keat SBK, Ladell K, Miners KL, Jones SA, et al. Complement dysregulation is a prevalent and therapeutically amenable feature of long COVID. *Med* 2024;**5**:239–53 .e5. doi:10.1016/j.medj.2024.01.011.
[16] Soriano JB, Murthy S, Marshall JC, Relan P, Diaz JV. WHO Clinical Case Definition Working Group on Post-COVID-19 Condition. A clinical case definition of post-COVID-19 condition by a Delphi consensus. *Lancet Infect Dis* 2022;**22**:e102–7. doi:10.1016/S1473-3099(21)00703-9.
[17] Herrmann-Lingen C, Buss U, Snaith RP. *Hospital anxiety and depression scale – deutsche version (HADS-D).* Bern: Verlag Hans Huber; 2011.
[18] Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989;**28**:193–213. doi:10.1016/0165-1781(89)90047-4.
[19] Hedman E, Ljótsson B, Blom K, El Alaoui S, Kraepelien M, Rück C, et al. Telephone versus internet administration of self-report measures of social anxiety, depressive symptoms, and insomnia: psychometric evaluation of a method to reduce the impact of missing data. *J Med Internet Res* 2013;**15**:e229. doi:10.2196/jmir.2818.

[20] Fernández-de-las-Peñas C, Rodríguez-Jiménez J, Palacios-Ceña M, de-la-Llave-Rincón AI, Fuensalida-Novo S, Florencio LL, et al. Psychometric properties of the hospital anxiety and depression scale (HADS) in previously hospitalized COVID-19 patients. *Int J Environ Res Public Health* 2022;**19**:9273.
[21] Fernández-de-las-Peñas C, Palacios-Ceña M, Rodríguez-Jiménez J, de-la-Llave-Rincón AI, Fuensalida-Novo S, Cigarán-Méndez M, Florencio LL, et al. Psychometric properties of the functional impairment checklist (FIC) as a disease-specific patient-reported outcome measure (PROM) in previously hospitalized COVID-19 survivors with long-COVID. *Int J Environ Res Public Health* 2022;**19**:11460.
[22] Akiyama M, Sasaki T, Kaneko Y, Yasuoka H, Suzuki K, Yamaoka K, et al. Serum soluble interleukin-2 receptor is a useful biomarker for disease activity but not for differential diagnosis in IgG4-related disease and primary Sjögren's syndrome adults from a defined population. *Clin Exp Rheumatol* 2018;**36**:S157–64.
[23] Delanghe JR, Speeckaert R, Speeckaert MM. Complement C3 and its polymorphism: biological and clinical consequences. *Pathology* 2014;**46**:1–10. doi:10.1097/PAT.0000000000000042.
[24] Capiau S, Delanghe JR, De Kesel PM. Evaluation of reference intervals for classical and alternative pathway functional complement assays. *Clin Chem Lab Med* 2022;**60**:e7–9. doi:10.1515/cclm-2021-0902.
[25] Van Damme KFA, Hoste L, Declercq J, De Leeuw E, Maes B, Martens L, et al. A complement atlas identifies interleukin-6-dependent alternative pathway dysregulation as a key druggable feature of COVID-19. *Sci Transl Med* 2023;**15**:eadi0252. doi:10.1126/scitranslmed.adi0252.
[26] Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement system Part II: Role in immunity. *Front Immunol* 2015;**6**:257. doi:10.3389/fimmu.2015.00257.
[27] Vlaicu SI, Tatomir A, Cuevas J, Rus V, Rus H. COVID, complement, and the brain. *Front Immunol* 2023;**14**:1216457. doi:10.3389/fimmu.2023.1216457.
[28] Lim EHT, van Amstel RBE, de Boer VV, van Vught LA, de Bruin S, Brouwer MC, et al. Complement activation in COVID-19 and targeted therapeutic options: a scoping review. *Blood Rev* 2023;**57**:100995. doi:10.1016/j.blre.2022.100995.
[29] Ji W, Xie X, Bai G, Fan Y, He Y, Zhang L, et al. Type 2 diabetes mellitus aggravates complement dysregulation and affects cortisol response in patients with post-COVID-19. *Diabetes Metab Syndr Obes* 2024;**17**:3849–61. doi:10.2147/DMSO.S480457.