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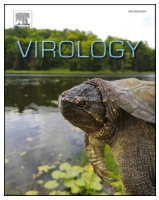

Humoral anti-SARS-CoV-2 response in patients with different long COVID phenotypes

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ABSTRACT

Long COVID (LC) is characterized by persistent symptoms following SARS-CoV-2 infection, with various mechanisms offered to explain its pathogenesis. This study explored whether adaptive humoral anti-SARS-CoV-2 responses differ in LC. Unvaccinated COVID-19 convalescents (n = 200) were enrolled, with 21.5% (n = 43) presenting LC three months post-infection. LC diagnosis was based on persistent symptom(s) and alterations in biochemical/clinical markers; three phenotypes were distinguished: cardiological, pulmonary, and psychiatric LC. All three phenotypes were characterized by significantly decreased seroprevalence of IgG antibodies against nucleocapsid (anti-NP). LC was associated with decreased odds of testing positive for anti-NP (OR = 0.35, 95%CI: 0.16–0.78, p = 0.001). Seropositive LC patients had lower anti-S1 and anti-S2 levels than individuals without LC, and those with pulmonary and psychological phenotypes also revealed decreased anti-RBD concentrations. The results indicate that LC can be characterized by diminished humoral response to SARS-CoV-2. The potential implication of this phenomenon in post-acute viral sequelae is discussed.

1. Introduction

Long COVID (LC), also known as post-COVID-19 syndrome or post-acute sequelae of SARS-CoV-2, is a multifaceted condition manifested by a range of symptoms that persist or onset following the SARS-CoV-2 infection. Although chronic fatigue is the most commonly reported one, LC can have broad manifestations, including neurological, cardiological, respiratory, cognitive, and mental (Davis et al., 2023). According to the definition established by the World Health Organization (WHO), these symptoms must be present at least three months post-infection and cannot be attributed to any other cause (WHO, 2022). The global prevalence of LC is substantial, with a recent meta-analysis estimating it at 14% (95%CI: 12–18) (Natarajan et al., 2023), which would translate into 107 million cases by assuming 765 million confirmed SARS-CoV-2 infections by the time WHO declared COVID-19 as no longer Public Health Emergency of International Concerns (May 5, 2023). Therefore, LC is also generating a significant economic burden at an average individual rate projected at 9000 USD per year (Cutler, 2022).

Various hypotheses to explain LC pathogenesis have been offered, including immune dysregulation and reactivation of dormant viruses (e.g., EBV or HHV-6), microbiota disruption, abnormalities of clotting and endothelial function, altered neurological signaling, induction of autoimmune processes and generation of autoantibodies (Davis et al., 2023; Dobrowolska et al., 2023; Hallmann et al., 2023). Some studies also suggest the role of persistence of SARS-CoV-2 and its antigens in selected locations, triggering a dysregulated immune system response, leading to the release of proinflammatory cytokines and chronic low-grade inflammation, ultimately resulting in multifaceted symptomatology (Buonsenso et al., 2022). If so, it is plausible that such prolonged exposure to viral proteins could also affect adaptive immune responses, including the production of virus-specific antibodies in individuals suffering from LC. However, this issue has been subject scarcely

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explored. One study involving healthcare workers found no difference in peak titers of anti-SARS-CoV-2 antibodies between individuals with and without persistent symptoms post-infection (Altmann et al., 2023). However, this investigation included a small group of patients, encompassed only individuals with asymptomatic or mild SARS-CoV-2 infection, did not account for confounding variables, and, similarly to most of the research on LC, was based on symptom self-reporting (Altmann et al., 2023). In another study, longer persistence of post-infection symptoms was found in patients with higher initial levels of antibodies against viral nucleocapsid, but this was not explored in relation to particular LC phenotype (Jia et al., 2022). Therefore, further studies are required to better understand the relationship between adaptive immune responses in LC.

In response to this need, the present study aimed to evaluate the seroprevalence and titers of various anti-SARS-CoV-2 antibodies in a well-defined group of COVID-19 convalescents, which included individuals reporting persistent, post-infection symptoms three months after COVID-19, who additionally revealed altered diagnostic markers of cardiological, pulmonary or psychological disturbances. Although SARS-CoV-2 infection is associated with the production of different immunoglobulin classes, we have chosen to explore the serum IgG due to their longer half-life and persistence than IgM and IgA (Post et al., 2020). Therefore, it can be speculated that if humoral response plays a role in LC, it may more likely relate to immunoglobulins of prolonged circulation, i.e., IgG.

2. Methods

2.1. Patients

This research was a continuation of SILCOV-19, a prospective observational registry-based cohort study in Poland (Niedziela et al., 2022). The study was approved by the Bioethics Committee of the Medical University of Silesia (approval number 17/2020) and the Bioethics Committee of the Poznan University of Medical Sciences (approval no. 429/22). Adult patients (n = 200) with confirmed, symptomatic SARS-CoV-2 infection (with RT-PCR or antigen test) were enrolled in June 2020–March 2021 for testing the presence of post-acute sequelae of COVID-19. The enrolled patients were infected during the period (March–October 2020) dominated in Poland by infections with Nextstrain clades 20A, 20B, and 20C (GISAID, 2024), which did not reveal major differences in clinical outcomes (Flisiak et al., 2021; Hoang et al., 2021). None of these patients received COVID-19 vaccination and none had a documented history of reinfection. The patients underwent detailed clinical and biochemical examinations three months after SARS-CoV-2 infection as described elsewhere (Niedziela et al., 2022). Three phenotypes of LC were distinguished: cardiological (card-LC), pulmonary (pulm-LC), and psychological (psych-LC). Their diagnosis required a patient reporting a persistence of at least one LC symptom (chronic fatigue, chest pain, dyspnea, muscle pain, or any bothersome symptom reported by the patient that could not be attributed to any other cause than previous SARS-CoV-2 infection) and the following values of biochemical/clinical markers: (i) card-LC: N-terminal pro–B-type natriuretic peptide >125 pg/mL and/or Left Ventricular Global Longitudinal Strain > −16, (ii) pulm-LC: transfer factor of the lung for carbon monoxide <80% and/or Borg score >2, (iii) psych-LC: ASB score ≥6 and/or depression subscale of the Hospital Anxiety and Depression Scale score >10 and/or State-Trait Anxiety Inventory X1 (state anxiety) score ≥4.

2.2. Assessment of anti-SARS-CoV-2 humoral immunity

A diagnostic using the CE-IVD certified immunoblot microarray (TestLine Clinical Diagnostics, Brno, Czech Republic) was used to measure serum titers of IgG antibodies against SARS-Co-2 nucleocapsid (anti-NP), receptor binding domain (anti-RBD), epitopes of subunit 1 of the spike protein other than RBD (anti-S1), and epitopes of subunit 2 of the spike protein (anti-S2) (Poniedziałek et al., 2022). The employed diagnostic assay is based on recombinant and purified native antigens that are immobilized on specific spots of nitrocellulose membrane fixed at the bottom of the microplate well (Montesinos et al., 2021). According to the information provided by the manufacturer, the assay demonstrates a diagnostic sensitivity and specificity of 98.7% and 99.3%, respectively. In this assay, recombinant and purified native antigens are immobilized on specific spots of nitrocellulose membrane fixed at the bottom of the microplate well. The concentrations for all four antibodies were given as U/mL and interpreted as positive if above 210 U/mL according to the manufacturer's instruction; the concentrations below this level were not considered seropositive and were excluded from calculating mean concentrations of antibodies.

2.3. Statistical analyses

Data were analyzed with Statistica v.13.3 (StatSoft Inc., USA). A comparison of antibody titers between non-LC and patients with different LC phenotypes was conducted using the Student's T-test. The prevalence of antibodies in LC and non-LC groups was compared with Fisher's exact test. Stepwise multiple logistic regression models were used to evaluate the association between the presence of particular anti-SARS-CoV-2 antibodies and patient's characteristics, including age (≥60 years), sex, obesity (≥30 kg/m^2), cigarette smoking, hospitalization due to COVID-19, and LC onset. When $p < 0.05$, differences were deemed statistically significant.

3. Results

The characteristics of the studied group are given in Table 1. Among 200 enrolled unvaccinated COVID-19 convalescents, 43 (21.5%) presented with LC three months post-infection. Among them, 12 had card-LC (27.9%), 39 – pulm-LC (90.1%), and 42 – psych-LC (97.6%). LC patients were more frequently women, had obesity, and required hospitalization due to COVID-19 (Table 1). The seropositivity rate for anti-RBD, anti-S1, anit-S2, and any anti-SARS-CoV-2 antibodies in LC groups was comparable to that in non-LC individuals. However, there was a significantly lower prevalence of anti-NP antibodies in LC and particular LC phenotypes (Fig. 1A).

This was further confirmed with stepwise multiple logistic regression, indicating that LC was associated with significantly lower odds of testing positive for anti-NP (OR: 0.35, 95%CI: 0.16–0.78, Fig. 1), or conversely, lack of anti-NP antibodies was related to increased odds of LC (OR: 2.86, 95%CI: 1.28–6.25). Age ≥60 years, male sex, obesity, and cigarette smoking were not related to seropositivity of anti-SARS-CoV-2 antibodies, whereas being hospitalized due to COVID-19 increased the

Table 1
Characteristics of the studied group and comparison between groups with and without long COVID.

Parameter	All (n = 200)	Long COVID (n = 43)	No long COVID (n = 157)	*p*-value
Age (years), mean ± SD	52.1 ± 11.5	52.1 ± 11.8	51.9 ± 11.5	>0.05
≥60 years, n (%)	54 (27.0)	14 (32.5)	40 (25.5)	>0.05
Women/men, n (%)	98/102 (49.2/50.8)	26/17 (62.0/38.8)	72/85 (45.9/54.1)	0.04
BMI (kg/m^2), mean ± SD	28.6 ± 5.1	30.9 ± 6.1	28.0 ± 4.6	0.0009
obesity (≥30 kg/m^2), n (%)	69 (34.7)	22 (52.4)	47 (29.9)	0.006
Smokers, n (%)	11 (5.8)	1 (2.6)	10 (6.6)	>0.05
Hospitalized due to COVID-19, n (%)	86 (43.2)	26 (60.5)	60 (38.2)	0.008

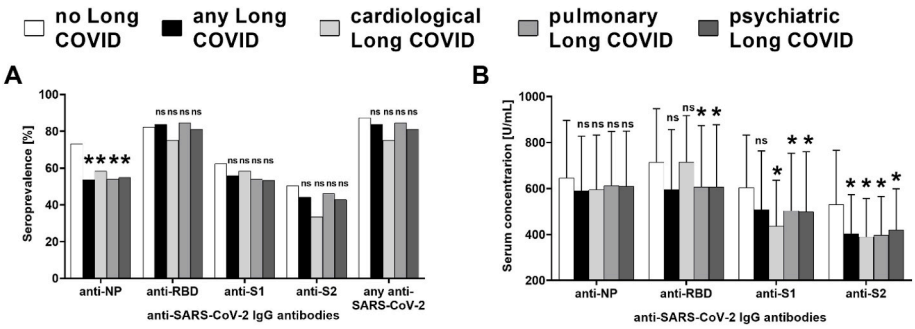

Fig. 1. **(A)** Seropositivity (%) of anti-SARS-CoV-2 IgG antibodies in patients with different long COVID (LC) phenotypes compared to patients not presenting any LC phenotype**. (B)** Anti-SARS-CoV-2 IgG antibody titers in seropositive patients with different LC phenotypes compared to a group without LC. Seronegative patients (defined as those with titers below <210 U/mL) were excluded from this analysis. The columns indicate mean, the whiskers indicate standard deviation, the asterisk indicates $p < 0.05$ compared to no LC; ns – statistically non-significant compared to no LC ($p > 0.05$); anti-S1 antibodies represent those recognizing S1 epitopes other than RBD.

odds of testing positive for all of them (Fig. 2).

Seropositive LC patients had lower anti-S1 (by 17–29% on average, depending on LC phenotype) and anti-S2 levels (by 21–27%) compared to individuals without LC. In addition, patients with pulm-LC and psych-LC also revealed decreased anti-RBD concentrations (by 15% on average) compared to the non-LC group (Fig. 1B).

4. Discussion

The present study provides an overview of anti-SARS-CoV-2 humoral responses in patients with different LC phenotypes. Contrary to numerous other research on LC, based on patients' symptom self-reporting, which often does not correspond to the results of diagnostic tests (Holm et al., 2023), the strength of our investigation was considering clinical and biochemical markers to objectivize LC identification and decrease over-diagnosis bias. Moreover, the present study enrolled patients after primary SARS-CoV-2 infection, without a history of reinfection and not vaccinated with the COVID-19 vaccine. Therefore, the obtained results are not confounded by repeated antigen exposure. We documented an altered seroprevalence of anti-NP IgG antibodies, also when controlling for confounding variables known to influence adaptive immunity. Further, lower titers of antibodies against both subunits of spike protein (S1 and S2) were observed in all three LC phenotypes, and individuals with pulm-LC and psych-LC also revealed decreased levels of anti-RBDs. These findings clearly show that LC was associated with diminished humoral response to SARS-CoV-2 infection.

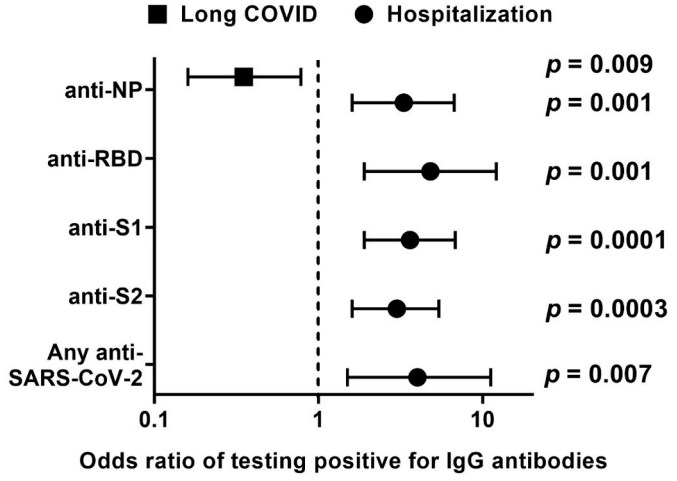

Fig. 2. The patient's characteristics found to influence the odds of testing positive for particular IgG anti-SARS-CoV-2 antibodies in the stepwise multiple logistic regression analyses when controlling for age, sex, BMI, and cigarette smoking. The dot represents the odds ratio, and the whiskers represent the 95% confidence interval.

According to one hypothesis, LC results from the prolonged existence of SARS-CoV-2 or its antigens in particular locations, e.g., intestines. Such a phenomenon could potentially lead to enhanced adaptive immune responses due to chronic exposure to viral proteins. However, the present study's findings do not support this since decreased prevalence and concentration of anti-SARS-CoV-2 antibodies were found in LC individuals. However, one could suggest, on the contrary, that a lack or diminished adaptive response would promote viral persistence due to insufficient pathogen recognition and subsequent neutralization. Although most LC individuals were seropositive for any SARS-CoV-2 IgG antibodies and did not differ in this regard from non-LC individuals and the general Polish population studied previously (Poniedziałek et al., 2022), they were characterized by a 20% lower frequency of detectable anti-NP antibodies. SARS'CoV-2's nucleocapsid, which exhibits multifunctional RNA binding properties, has also been shown to counteract the host antiviral immune response, e.g., by antagonizing type I interferon signaling (Mu et al., 2020). Considering that some studies have shown the prolonged persistence of nucleocapsid protein in some individuals infected with SARS-CoV-2 and that some research has associated this persistence with LC symptoms (Arostegui et al., 2022; El-Baky et al., 2024; Zollner et al., 2022), our results suggest that diminished immune response to nucleocapsid may be the sole reason for its prolonged detection in infected individuals, ultimately increasing odds for LC.

Interestingly, the potential decreased production of anti-NP in LC was observed earlier in a study involving members of a social media group who suspected they may have sequelae from COVID-19 (Fogh et al., 2022). Over 30% of the participants were not reported to have detectable anti-NP antibodies. However, the authors concluded that this indicates that in a large number of cases, symptoms initially attributed to LC may have another origin (Fogh et al., 2022). Our findings point to the contrary, implying that LC individuals are characterized by increased odds of testing negative exclusively for anti-NP but not other anti-SARS-CoV-2 antibodies. In addition, another study reported that lower concentrations of anti-NP antibodies (but not other anti-SARS-CoV-2 antibodies) were associated with a longer duration of LC symptoms (Jia et al., 2022). Although the levels of anti-NP antibodies did not differ between LC and non-LC individuals in our study, we have taken into account only the concentrations considered to indicate seropositivity, odds for which were significantly lower in LC patients. According to a recent hypothesis, SARS-CoV-2 infection can decrease serotonin concentration, and its reduced peripheral levels may be responsible for neurocognitive manifestations of LC (Wong et al., 2023). Although we did not measure serotonin in our patients, it should be noted that *in vivo* experiments show that its peripheral levels blunt the

production of IgG antibodies, while pharmacological reduction of its concentration leads to the contrary (Devoino and Idova, 1973). Therefore, it is unlikely that lowered serotonin levels were responsible for decreased antibody generation by B cells in LC individuals, though further studies are necessary to assess it.

These findings raise some provocative questions regarding anti-NP antibodies' role in decreasing the risk of LC. Further research may validate the lack of anti-NP IgG antibodies as the potential biomarker of LC. The advantage of such a biomarker is the possibility of assessment with certified, non-expensive, and widely available diagnostic tests. On the other hand, one should note that in the present study, over half of LC subjects were seropositive for anti-NP IgG antibodies, implying that lack of these immunoglobulins following the SARS-CoV-2 infection may only be a contributing factor rather than a hallmark of LC. The other avenue to explore in the context of our findings is the role different COVID-19 vaccines may play in decreasing the risk of LC. Considering that the majority of authorized COVID-19 vaccines use spike protein as an antigen and that their use is consistently revealed to decrease odds for LC, though the effects are rather not highly substantial (Marra et al., 2023), it would be of interest to understand whether the vaccines that also employ NP as an antigen could provide further benefits in this regard. The NP is one of the antigens of COVID-19 vaccines based on inactivated SARS-CoV-2 (Wang et al., 2022), but comparative research on how they perform regarding the LC risk on par with other vaccines, e.g., mRNA, adenoviral, or subunit, are lacking. It would also be interesting to understand whether mRNA vaccines encoding both spike protein and NP would be superior in preventing LC compared to their counterparts expressing only a spike protein. Such vaccines are not yet authorized, but preclinical studies have shown that they may offer robust adaptive responses (Hajnik et al., 2022; McCafferty et al., 2022).

Moreover, the present study demonstrated significantly lower titers of anti-spike antibodies in LC patients, including anti-RBD, anti-S1, and anti-S2, all of which play a role in viral neutralization. However, one should note that they were 15–29% lower than non-LC individuals, and it remains unknown if such a difference would translate into diminished viral control. In addition, compared to the pulm/psych LC and non-LC individuals, card-LC patients were less frequently positive for anti-RBD (by 6–10% and 9%, respectively), anti-S2 (by 10–13% and 17%, respectively) and any anti-SARS-CoV-2 antibodies (by 6–10% and 12%, respectively), although these differences were not statistically significant, likely due to small size of card-LC group. Nevertheless, they are worth exploring in a larger cohort of COVID-19 convalescents presenting with altered cardiovascular function since there is evidence that spike protein may be implied in cardiovascular complications during SARS-CoV-2 infection by prompting cardiac cell dysfunction and triggering inflammatory responses (Avolio et al., 2021; Clemens et al., 2023). Diminished anti-NP and anti-S responses in LC subjects, coupled with antigen persistence, could ultimately lead to prolonged circulation and adverse effects exerted on the cardiovascular system (Yonker et al., 2023). Interestingly, similar observations of lower anti-S1/S2 titers in LC individuals, accompanied by low-degree chronic inflammation, were reported in a prospective cohort of hospitalized Spanish patients one year after primary SARS-CoV-2 infection (García-Abellán et al., 2022). Authors suggested that this phenomenon could arise from the greater and persistent damaging effect of viral infection on germinal centers in severely ill patients (García-Abellán et al., 2022).

Other viral infections also reported the association between decreased virus-specific antibodies and long-term outcomes and complications. For example, lower levels of serum IgG antibodies against tick-borne encephalitis virus were a significant predictor of post-encephalitic syndrome 2–7 years after infection (Bogovič et al., 2021). Similarly, an increase in titers of neutralizing IgG/IgM antibodies results in the clearance of the Chikungunya virus, while the lack of it might influence the progression to the chronic phase and manifestation of long-term clinical complications (Srivastava et al., 2020).

In addition, our research also found that hospitalization due to COVID-19 was a main predictor of being seropositive to various anti-SARS-CoV-2 antibodies in the considered cohort. This finding is consistent with other studies showing significantly higher seroprevalence and titers of different anti-SARS-CoV-2 antibodies following severe COVID-19 (including when accompanied by pneumonia) compared to mild or asymptomatic cases (Chansaenroj et al., 2021, 2022; Yan et al., 2022). It is likely linked to higher viral loads and excessive immune responses in severely ill individuals, ultimately leading to enhanced and prolonged B-cell receptor stimulation (Chen et al., 2020).

The limitations of the present study should be stressed. Although lower seroprevalence and titers of selected antibodies were evidenced in LC, it does not necessarily imply the causation but rather advocates further research to understand the exact nature of this relation. Moreover, the possibility of reverse causation, i.e., conditions in LC altering the adaptive humoral immunity, cannot be excluded. One should also note that the biochemical and clinical examinations employed to identify LC individuals were assessed only post-infection as their baseline level, prior to the acquisition of SARS-CoV-2, was not available. Importantly, this study explores the association between LC phenotypes and anti-SARS-CoV-2 antibodies within three months from infection, while LC symptoms are known to persist longer. On the other hand, follow-up studies, taking into account extended periods of observation, would be challenging due to the impact of COVID-19 vaccinations and reinfections on antibody titers. Moreover, exploring other immunoglobulins, especially IgA, in the context of LC, could provide further information on the role of humoral responses in the condition's onset and its symptomatology. Last but not least, further studies are advocated to understand whether altered humoral responses in LC are differentiated by the SARS-CoV-2 variant or COVID-19 vaccination status. However, one should note that exploring the association between LC and seroprevalence of antibodies against S protein may be challenging in the future since a large number of patients may have pre-existing immunity due to a previous history of SARS-CoV-2 infections and COVID-19 vaccination.

5. Conclusions

LC was associated with lower seroprevalence of anti-NP antibodies, and particular LC phenotypes were associated with lower concentrations of selected anti-SARS-CoV-2 antibodies, excluding the involvement of enhanced humoral immunity in the studied cohort. Further research is required to establish whether diminished adaptive response to SARS-CoV-2 may play a role in post-acute viral sequelae and whether vaccines that employ NP as an antigen may be superior in decreasing the odds of LC..

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CRediT authorship contribution statement

Piotr Rzymski: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Jacek Niedziela:** Writing – review & editing, Methodology. **Barbara Poniedziałek:** Writing – review & editing, Investigation. **Joanna Rosińska:** Writing – review & editing, Investigation. **Dorota Zarębska-Michaluk:** Writing – review & editing. **Barbara Sobala-Szczygieł:** Writing – review & editing. **Robert Flisiak:** Writing – review & editing, Supervision, Methodology. **Mariusz Gąsior:** Writing – review & editing, Supervision, Conceptualization. **Jerzy Jaroszewicz:** Writing – review & editing, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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