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Multisystem inflammatory syndrome observed post-COVID-19: the role of natural products, medicinal plants and nutrients and the use of prediction tools supporting traditional forms of diagnosis

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Editorial: Multisystem inflammatory syndrome observed post-COVID-19: the role of natural products, medicinal plants and nutrients and the use of prediction tools supporting traditional forms of diagnosis

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Editorial on the Research Topic
Multisystem inflammatory syndrome observed post-COVID-19: the role of natural products, medicinal plants and nutrients and the use of prediction tools supporting traditional forms of diagnosis

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has had devastating global impacts since its emergence in late 2019. However, it also prompted an unprecedented scientific response (Cauchemez et al., 2024). While the acute phase of the pandemic has largely subsided due to quarantining, vaccination efforts and improved treatments, a new challenge has emerged in the form of post-COVID inflammatory syndromes. A hallmark of severe COVID-19 was excessive inflammation involving multiple organ systems, characterized by cytokine storms, coagulation dysfunction, and tissue damage. Even after recovery from acute infection, many patients continue to experience persistent inflammatory symptoms affecting various body systems, a condition now known as "long COVID" or "post-acute sequelae of SARS-CoV-2 infection" (PASC) (Barber et al., 2021; Davis et al., 2023; Liu et al., 2023; Porter et al., 2023).

The complex nature of post-COVID syndromes presents a significant challenge to healthcare systems worldwide. Symptoms can vary widely between patients and may affect

multiple organ systems, including the respiratory, cardiovascular, neurological, and gastrointestinal systems. Common complaints include fatigue, cognitive dysfunction ("brain fog"), shortness of breath, anxiety, depression, and sleep disturbances. The underlying mechanisms of these persistent symptoms are not fully understood but are thought to involve ongoing inflammation, autonomic dysfunction, and potential autoimmune processes triggered by the initial SARS-CoV-2 infection.

Conventional medical approaches to treating post-COVID syndromes have shown limited success, highlighting the need for novel and integrative strategies. Natural products and plant-based medicines have a long history of use in treating inflammatory conditions and modulating immune function. Many of these compounds have well-documented anti-inflammatory, antioxidant, and immunomodulatory properties that could potentially address the multifaceted nature of post-COVID syndromes.

The global impact of long COVID cannot be overstated. With millions of people worldwide experiencing persistent symptoms following SARS-CoV-2 infection, the social, economic, and healthcare burdens are immense. The development of effective treatments for post-COVID syndromes is therefore of paramount importance. As we continue to navigate the long-term consequences of the COVID-19 pandemic, interdisciplinary collaboration between conventional and complementary medicine will be crucial. By combining the strengths of various therapeutic approaches and diagnostic methods, we may be better equipped to tackle the complex challenges posed by post-COVID syndromes. The studies in this Research Topic demonstrate the potential of such integrative approaches and pave the way for future research in this critical area.

This Research Topic explored innovative approaches to address post-COVID inflammatory syndromes using natural products, medicinal plants, nutrients, and integrative diagnostic methods. The articles in this collection investigate a range of natural compounds and plant-based medicines for their anti-inflammatory and immunomodulatory properties that may help alleviate lingering post-COVID symptoms. Additionally, the potential of nutritional interventions and traditional diagnostic techniques to support patients with post-COVID syndromes is examined.

The research presented in this collection explores several promising avenues for natural interventions. These include the use of specific vitamins and nutrients to support immune function and reduce inflammation, herbal extracts with known anti-inflammatory properties, and comprehensive lifestyle interventions addressing diet and physical activity. By targeting multiple aspects of post-COVID syndromes simultaneously, these integrative approaches may offer advantages over single-target pharmaceutical interventions.

Moreover, this Research Topic delves into the potential of traditional diagnostic methods to complement conventional techniques in assessing and monitoring patients with post-COVID syndromes. These approaches may provide valuable insights into the complex interplay of symptoms and underlying physiological imbalances, allowing for more personalized and holistic treatment strategies. As our understanding of post-COVID syndromes continues to evolve, this research provides valuable insights into natural therapeutic options that may work in concert with conventional treatments to improve outcomes for affected patients.

1. Deng et al. examined the relationship between fat-soluble vitamin status and antibody responses to COVID-19 vaccination in a cohort of 141 healthy adults. They found that higher plasma vitamin D levels were associated with lower anti-SARS-CoV-2 antibody titers, both for wild-type and Omicron variants. This unexpected finding suggests that vitamin D may play a complex role in modulating vaccine-induced immunity, highlighting the need for further research on optimal vitamin D levels for vaccine efficacy.
2. Pourfarzi et al. conducted a randomized controlled trial testing a web-based lifestyle intervention focused on nutrition and physical activity for preventing COVID-19. The study involved 303 women aged 30–60 who had not previously contracted COVID-19. The intervention group received online educational sessions on healthy diet and physical activity. After 4 weeks, the intervention group showed significant improvements in weight, BMI, nutritional status, and physical activity levels compared to controls. Importantly, the intervention group also had a lower incidence of COVID-19 infection during the follow-up period, suggesting that lifestyle modifications may help reduce COVID-19 risk.
3. Gaylis et al. evaluated a nutraceutical supplement containing multiple compounds, including β-caryophyllene, pregnenolone, and various herbs and vitamins, for treating long COVID symptoms. In an open-label trial with 51 participants, the supplement significantly improved various persistent symptoms including fatigue, weakness, cognitive issues, and shortness of breath over 4 weeks of treatment. The study demonstrated the potential of this multi-component natural approach in addressing the complex symptomatology of long COVID.
4. Joung et al. investigated the effects of a herbal extract (Myelophil) on fatigue symptoms in long COVID patients. The study was a non-randomized, open-label observational study, without a control group. Myelophil was administered for 4 weeks to the 49 participants (18 males, 31 females) in this study. After 4 weeks of Myelophil administration, participants showed significant improvements in fatigue scores, physical weakness, and quality of life measures. This study provides evidence for the potential efficacy of traditional herbal medicine in managing long COVID symptoms.
5. Bioinformatics and systems biology approaches were employed by Qian et al. for the identification of hub genes, shared pathways, molecular biomarkers, and candidate therapeutics for the management of sepsis and sepsis-induced ARDS in the context of COVID-19 infection. 189 differentially expressed genes (DEGs) shared among COVID-19 and sepsis datasets were identified. Construction of protein-protein interaction networks revealed that six hub genes (CD247, CD2, CD40LG, KLRB1, LCN2, RETN) exhibited significant alterations across COVID-19, sepsis, and geriatric sepsis-induced ARDS. Functional analysis underscored the interconnection between sepsis/sepsis-ARDS and COVID-19, enabling the identification of

potential therapeutic targets, transcription factor-gene interactions, DEG-microRNA co-regulatory networks, and prospective drug and chemical compound interactions involving hub genes.

6. Qian and Zeng investigated the effects of Jinhua Qinggan Granules (JHQG), a Traditional Chinese Medicine formulation, on COVID-19 using mass spectrometry, network pharmacology, and single-cell RNA sequencing analysis. The researchers identified 73 chemical components in JHQG and constructed a network showing interactions between these compounds, target proteins, and immune cells. Results suggest JHQG may mitigate inflammation in COVID-19 by inhibiting the activity of activated neutrophils, monocytes, plasmoblasts, and effector T cells in peripheral blood. The findings provide insights into JHQG's mechanism of action and support its potential as a safe and effective treatment for viral infections like COVID-19.
7. Ahmad et al. published the first survey reviewing the application of AI methodologies on Long COVID. Twenty research papers that met the inclusion criteria (innovative AI approach with clear results published after 2020) employing AI techniques such as ML and NLP on Long COVID data are discussed. Thirteen papers were focused on using ML techniques and the other seven were on applying text mining to Long COVID data. The data, AI techniques implemented, accuracy, and precision of each of the 20 papers are reviewed in detail. The use of AI techniques to analyze temporal data such as a symptom or to physiologically monitor data over time, can assist in detecting early signs of worsening of Long COVID, facilitating both timely medical interventions and personalized adaptation of treatment protocols hence improving patient outcome.

This Research Topic has made significant strides in exploring natural and integrative approaches to address the complex challenge of post-COVID inflammatory syndromes. The collected studies investigated a diverse range of interventions, from specific nutrients and herbal formulations to comprehensive lifestyle modifications. Collectively, these studies offer valuable insights into natural therapeutic options that may complement conventional treatments, potentially improving outcomes for those affected by post-COVID syndromes. The research underscores the importance of integrative approaches in addressing the multifaceted nature of long COVID and opens new avenues for future investigations in this critical area of public health.

It is important to note that while the results presented in these studies are encouraging, further research is needed to fully establish the efficacy and safety of these natural interventions for post-COVID syndromes. Large-scale, randomized controlled trials will be crucial in validating these findings and determining optimal treatment protocols. Additionally, the potential interactions between natural products and conventional medications must be carefully considered to ensure patient safety.

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The results of a unique dietary supplement (nutraceutical formulation) used to treat the symptoms of long-haul COVID

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Long-COVID is a syndrome characterized by debilitating symptoms that persist over 3 months after infection with the SARS-CoV-2 virus. It affects 15 to 33% of COVID-19 recovered patients and has no dedicated treatment. First, we found that β-caryophyllene and pregnenolone have a significant synergistic effect in the resolution of LPS-induced sepsis and inflammation in mice. Then we combined these two compounds with seven others and designed a unique dietary supplement formulation to alleviate long COVID inflammatory and neurological disorders. We performed a one-arm open-labeled study at a single site with 51 eligible patients from 18 states. Each participant recorded the severity level of 12 symptoms (including fatigue, weakness, cardiac and neurological symptoms, shortness of breath, gastrointestinal disorders, ageusia or anosmia, anxiety, joint pain, rash, cough, and insomnia) at baseline, 2- and 4-week time points. On average, all the symptoms were significantly milder after 2 weeks, with further improvement after 4 weeks. Importantly, each symptom was significantly attenuated in 72 to 84% of the participants. There were no significant adverse effects. Our data indicate that the use of this nutraceutical product is a safe and significantly efficient option to reduce multiple symptoms of long COVID.

KEYWORDS

dietary (food) supplements, long-COVID-19, immunology and inflammation, cannabinoids, CB2 agonists

Introduction

Long-haul COVID is characterized by chronic and often debilitating symptoms following acute Coronavirus disease 2019 (COVID-19) caused by the SARS-CoV-2 virus. Long COVID is very challenging to diagnose, treat and categorize as it combines multiple and different symptomatic presentations in sufferers.

The Center for Disease Control (CDC) in June 2022 published a report that 40% of adults in the US reported having COVID-19 in the past, of which nearly 1 in 5 are still having symptoms of long COVID. The CDC defines Long COVID as symptoms that last three or more months after first contracting the virus and did not exist before the COVID-19 infection. Symptoms may last well over 1 year (1). Other studies reported a prevalence ranging from 1 in 8 to 1 in 3 (2, 3), significantly higher than after influenza (2). There appeared to be differences in the prevalence of long COVID between states (2).

Differences in the reported prevalence of long-COVID likely results from the disparity of symptoms and the lack of a unified definition for this condition. Importantly, this condition cannot be ignored as it presents a set of symptoms that often severely impact the quality of life and ability to carry out daily activities. For example, in one survey, 44% percent of patients with Long COVID reported not being able to work at all, compared to their pre-COVID-19 work capacity, and 51% had reduced their working hours (4). Overall, the economic burden may approximate $50 billion annually in lost salary only (4).

An international cohort of 3,762 participants from 56 countries identified 203 symptoms in 10 organ systems that persisted at least 4 weeks after a confirmed diagnosis of COVID-19 (5). The CDC listed the most common symptoms of COVID-19 in a survey they initiated in April 2022.[1] These included tiredness or fatigue, difficulty thinking, concentrating, forgetfulness, or memory problems (sometimes referred to as "brain fog"), difficulty breathing or shortness of breath, joint or muscle pain, fast-beating or pounding heart (also known as heart palpitations), chest pain, dizziness on standing, menstrual changes, changes to taste/smell, or inability to exercise.

The exact underlying cause of long-COVID remains uncertain, but most reports agree that this condition is associated with a persistent viral infection and long-lasting inflammation (6, 7). Specific neurological symptoms (fatigue, brain fog, anosmia, and ageusia/dysgeusia) in long COVID resemble "sickness behavior," a response of the autonomic nervous system to pro-inflammatory cytokines (8). The long-standing dysautonomia has been proposed to result from sympathetic/parasympathetic imbalance (7, 9).

To the best of our knowledge, no medicine is currently dedicated to treating long-haulers (patients suffering from long-COVID). In the absence of established protocols, each symptom is treated separately with different drugs for different symptoms, even though they all have a common cause. Even when combining the current therapeutic approaches with rehabilitation (10–14), success is minimal, and the treatments are associated with multiple adverse effects.

We thus designed a formula to address the underlying chronic inflammatory status and autonomic imbalance that characterize many long-haulers. The first challenge was to help restore the function of various organ systems; the second challenge was to use only US-recognized dietary supplements to be able to address the unmet need in a very short time.

Tel Aviv University, in collaboration with Arthritis & Rheumatic Disease Specialties (AARDS) based in Miami, FL, USA, formulated a unique oral nutraceutical supplement containing only approved dietary supplements and ingredients "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA). This combination would, by design, be natural, free of any known side effects, and with limited drug interactions. The final formulation relied on new experimental data (presented here), clinical experience in treating long-hauler patients at AARDS with multiple clinical symptoms over 2 years, and scientific literature (**Table 1**). We selected the ingredients for their unique immuno-modulation properties and activities, reduction in pain, anxiety and depression, potential effect on dysautonomia, anticoagulation activity, as well as the ability to inhibit viral replication. We report here the results of a clinical study aimed at testing this nutraceutical formula as a standalone treatment in a population of COVID long haulers.

We and others have reported that selective CB2 agonists are potent immunomodulators of the innate immune system, both *in vivo* and *in vitro* (15, 16). We showed that CB2 agonists inhibit cytokine expression in LPS-exposed macrophages in cultures and decrease ear edema in a skin inflammation model in mice (16). Following these results, we tested the hypothesis that β-caryophyllene (βCP), a dietary terpene that is also a selective CB2 agonist (17, 18), reduces the exaggerated inflammatory response induced by pathogens.

Another approach to attenuate inflammation and associated tissue damage is using steroids. In contrast to glucocorticosteroids such as dexamethasone, pregnenolone is a steroid hormone precursor with known anti-inflammatory properties in myeloid cells (19), does not induce lymphocyte apoptosis (20) and may even promote thymocyte survival and differentiation (21). Pregnenolone (Preg) and its metabolites suppress the secretion of tumor necrosis factor α and interleukin-6 mediated through TLR2 and TLR4 signaling in macrophages (22). Also relevant to long-haulers, Preg can help with cognitive and neurological issues, partly *via* binding to TRPM3, without causing bone loss (23–26).

In addition to βCP and Preg, we included Dehydroepiandrosterone (DHEA), Bromelain, St. John's Wort extract, Boswellia Serrata gum/resin extract (AKBA), Quercetin, zinc compound, and vitamin D. The rationale for including each compound is summarized in **Table 1**.

1 cdc.gov

TABLE 1 List of compounds included in our nutraceutical combination and their relevance for COVID long haulers.

Active ingredient	Properties and activities	References
β-caryophyllene (βCP)	Antioxidant, anti-inflammatory, analgesic	(18, 27–29)
Pregnenolone	Modulator of inflammation, regulator of neuroinflammation	(22, 30–32)
Dehydroepiandrosterone (DHEA)	Anti-inflammatory, improves dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, immune modulator	(33–35)
Bromelain	Anti-inflammatory, antiviral (Anti-SARS-CoV-2), fibrinolytic	(36–39)
St. John's Wort extract	SRI* for treating autonomic dysfunction and impaired balance between the sympathetic and parasympathetic nervous systems	(40, 41)
Boswellia Serrata gum/resin extract (AKBA)	Anti-inflammatory, COVID-19 therapeutic agent, respiratory support	(42–44)
Quercetin	Anti-inflammatory, antiviral, immune modulator, antioxidant	(45–47)
Zinc compound	Essential to preserve natural tissue barriers such as the respiratory epithelium, balanced function of the immune system and the redox system, antiviral	(48, 49)
Vitamin D	Reducing inflammation induced by SARS-CoV-2 infection	(50, 51)

*SRI, serotonin reuptake inhibitor.

Materials and methods

Mouse study

Male wild type 8–10 weeks old mice of inbred strain C57BL/6J-RCC were obtained from Envigo Ltd (Jerusalem, Israel). All experiments were in accordance and with the approval of the institutional animal care and use committee of Tel-Aviv University for these experiments (permit number 01-20-022). The mice were divided into treatment groups 24 h before LPS administration. All treatments were administered intraperitoneally (IP). β-Caryophyllene (βCP) and pregnenolone were injected every 12 h starting 24 h before the LPS injection. A single IP injection of LPS was administered at 25 μg/gr dose. This study had two control groups as indicated in the experiments below. One that received PBS instead of the LPS injection ("PBS"), and one that received PBS instead of the treatments (CB2 agonist or steroid) before the LPS injection ("LPS"). For humane reasons murine sepsis score (MSS) was used as a surrogate for survival (52). A low MSS score (0–2) served as a surrogate for survival while mice that reached the score 3 were defined as critically ill and were euthanized.

TABLE 2 Amount of each compound included in the nutraceutical formulation per serving. The participants were required to take one serving twice a day with food.

Active ingredient	Amount per serving
β-caryophyllene (βCP)	40 mg
Pregnenolone	40 mg
Dehydroepiandrosterone (DHEA)	30 mg
Bromelain (2400 GDU*/g)	416 mg
St. John's Wort extract	150 mg
Boswellia Serrata gum/resin extract (AKBA)	100 mg
Quercetin (*Sophora Japonica*)	40 mg
Zinc (as Zinc Picolinate)	12 mg
Vitamin D	25 μg (1000 IU)

*GDU, gelatin digesting unit.

Statistical analyses for the animal study

For survival experiments in mice, Gehan–Breslow–Wilcoxon test was used for multiple groups comparison. For disease progression we used Dunnett's multiple comparisons test. Differences between groups were considered significant when $p < 0.05$.

Clinical study performed at arthritis and rheumatic disease specialties

The new combination of nutraceuticals including the compounds listed in Table 1 was tested in a one-arm, open-label clinical trial. The amount of each compound in the formulation and the total amount per serving are detailed in Table 2. Participation was voluntary, recruitment was from local patient population as well as social media advertising. All subjects had to have documented evidence of prior COVID infection (PCR or Rapid test) and were having ongoing symptoms (1 or more) for a minimum of 3 months from the time of infection. The existence of any of these symptoms prior to contracting COVID was considered an exclusion. All subjects were required to complete a survey listing and rating their symptoms and all participants were interviewed by the research staff at baseline to verify inclusion criteria.

Following qualification, subjects were provided with the nutraceutical supplement for a 2-week period with dosing instructions (1 serving twice a day with food) after which they were required to repeat the process of documenting any change

in symptoms relative to baseline and their voluntary decision to continue with treatment for a further 2-week period. At the end of 4 weeks, their change in symptoms from baseline was documented as well as an overall global response.

The total number of patients completing the 4-week evaluation was 51, the age range was 21–73 years of age, female to male ratio was approximately 2 to 1 and the patients originated from 18 US states.

Statistical analysis of the clinical study

We compared the score of each symptom at 2 and 4 weeks to the baseline for each participant using multiple paired *t*-tests. Individual variance was assumed for each symptom and a Benjamini–Krieger–Yekutiel False Rate Discovery Rate (FDR) was used to account for the multiple comparisons. For all comparisons, differences between time points were considered significant when $p < 0.01$.

Results

Animal study

Testing of anti-inflammatory compounds in mice

Here we tested the therapeutic potential of β-caryophyllene and that of pregnenolone in a mouse model of LPS-induced sepsis. βCP (25 mg/kg) and Preg (10 mg/kg) were administered 24 and 12 h before LPS injection (25 mg/kg), and then every 12 h. All the injections were given i.p. A Murine Septic Score (MSS) was recorded every 12 h and survival was defined as MSS < 3. Over the 4-day follow-up, we found a slight beneficial effect of βCP in improving disease progression (improved "well-being"), while Preg alone had no significant effect (**Figure 1**). When measuring survival, both compounds induced a slight decrease in mortality, but none had a statistically significant effect (**Figure 1**). Next, we asked whether the combined treatment with both compounds can improve the outcome of the mice to LPS injection. Our results show that combining βCP and Preg had a significant positive effect on both disease progression and survival ($p = 0.026$, **Figure 2**). Indeed, the combined treatment with βCP and pregnenolone significantly improved the wellbeing by >twofold and the survival from a non-significant effect to a >90% increase (**Figure 2**). These data demonstrate that βCP and Preg have a synergistic effect in alleviating inflammation as well as disease severity and mortality from sepsis *in vivo*.

Following the demonstration of a synergistic effect of βCP and Preg in the resolution of inflammation, we combined these two compounds with seven additional supplements. We designed the resulting formulation to address the symptoms of long-COVID, related to persistent viral infection, long-lasting inflammation and neurological disorders.

Clinical trial

Clinical study on COVID long hauler patients

At baseline, 2- and 4-week time points, participants recorded the level of severity for each of the 12 symptoms in addition to their subjective assessment of wellbeing ("global reported"). The symptoms in the survey included fatigue, physical weakness, cardiac (e.g., palpitations) and neurological symptoms (e.g., “brain fog”), shortness of breath, gastrointestinal disorders, loss of smell or taste, anxiety, joint pain, rash or hives, cough and insomnia. All the participants took the recommended daily dose of the nutraceutical for 4 weeks. The surveys and scoring by the participants revealed that the severity score for all the symptoms improved already after 2 weeks and this beneficial effect tended to be even more pronounced after 4 weeks (**Figure 3**). Notably, the decrease in the severity level of all the symptoms was statistically significant at both the 2- and 4-week time points.

Next, we evaluated the number of symptoms that improved for each participant. The vast majority of the participants (46 out of 51) reported more symptoms that improved than symptoms that worsened. Among the remaining five participants, only one reported feeling a worsening in general wellbeing. We also calculated the percentage of participants who reported an improvement, worsening, or no change for each symptom over the 4-week treatment. For each symptom, we omitted any participants who reported a null severity score ("zero") for this symptom at all time points as these do not denote a lack of effect. Notably, 72 to 84% of the participants reported an improvement for each of the 12 symptoms (**Figure 4**). When asked about their general wellbeing ("global reported"), 59% reported a noticeable improvement. However, when we calculated the average score of all the symptoms for each participant ("global calculated"), we found that the general profile improved for 88% of the participants.

Safety data

Overall safety data was very good, and no major adverse events were noted. Minor adverse events included three patients with vertigo, one patient with increased anxiety, one patient with a gout attack, one patient with increased joint pain and one patient was reinfected with COVID.

Discussion

The results suggest this unique nutraceutical dietary supplement combination may afford significant symptomatic

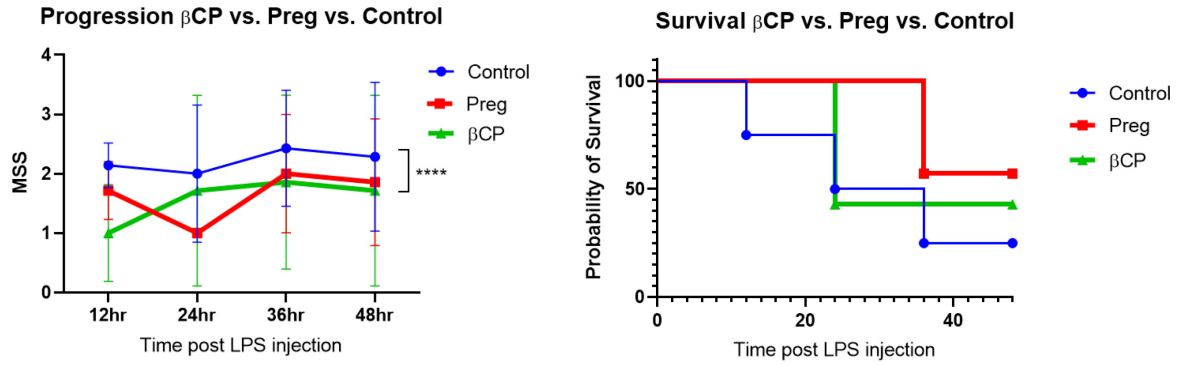

FIGURE 1
All groups were injected with LPS 25 μg/g. Mice were pre-treated with β-caryophyllene (BCP, 25 mg/kg) or Pregnenolone (Preg, 10 mg/kg) 24 and 12 h before LPS injection and then every 12 h. $N = 7$. ****$p < 0.0001$, 2-way ANOVA between βCP and Controls.

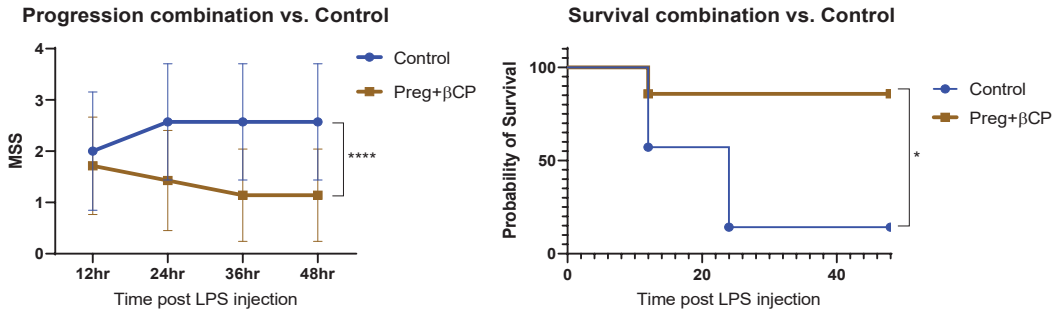

FIGURE 2
All groups were injected with LPS 25 μg/g. Mice were pre-treated with β-caryophyllene (BCP, 25 mg/kg) and Pregnenolone (Preg, 10 mg/kg) 24 and 12 h before LPS injection and then every 12 h. $N = 7$. ****$p < 0.0001$, 2-way ANOVA for disease progression; *$p = 0.0152$, Log-rank (Mantel-Cox) test for survival.

benefit to long COVID sufferers. The adverse events were minor and overall safety of the nutraceutical product was confirmed.

As the clinical study results indicate, there were statistically significant improvements in this study population in their overall symptoms after 2 and 4 weeks of treatment. It should be noted that different symptoms improved or worsened in different patients. There were some symptoms such as fatigue and brain fog that appeared to respond more than others, however, no baseline presentations were able to predict individual symptomatic responses.

It is unknown at this time if the duration of benefit will be complete, short term, or long term. Because the follow-up period ended after 4 weeks and treatment was taken until the last day, this study was specifically designed to demonstrate a beneficial effect on the long-COVID symptoms. Further studies are warranted to determine the optimal duration of the treatment and whether symptoms would recur upon cessation of the treatment.

We observed a discrepancy between the sum of improvements for each symptom and the reported global wellbeing. Indeed, calculating the average improvement of all the symptoms for each individual, 88% of the participants benefited from the treatment; however, only 59% of the participants reported an overall improvement of their wellbeing (**Figure 4**). A similar discrepancy has been reported by others in post-stroke patients where general wellbeing was poorly associated with changes in executive function and comorbidities (53). In our study, we may speculate that not all participants perceived all symptoms at the same level. For example, a person experiencing an improvement in 4 out of 5 symptoms may still consider no improvement in overall wellbeing if the one unchanged symptom has a severe impact on his or her quality of life.

Our *in vivo* experiment describes for the first time the synergistic effect of two different compounds in the attenuation of systemic inflammation. We could find no report on a putative interaction between βCP and Preg. However, a study showing that Preg may act as an allosteric modulator of CB1 (54), a receptor that shares a 44% homology with CB2 (55), may provide circumstantial evidence to a similar interaction between CB2 activation and Preg. In addition to

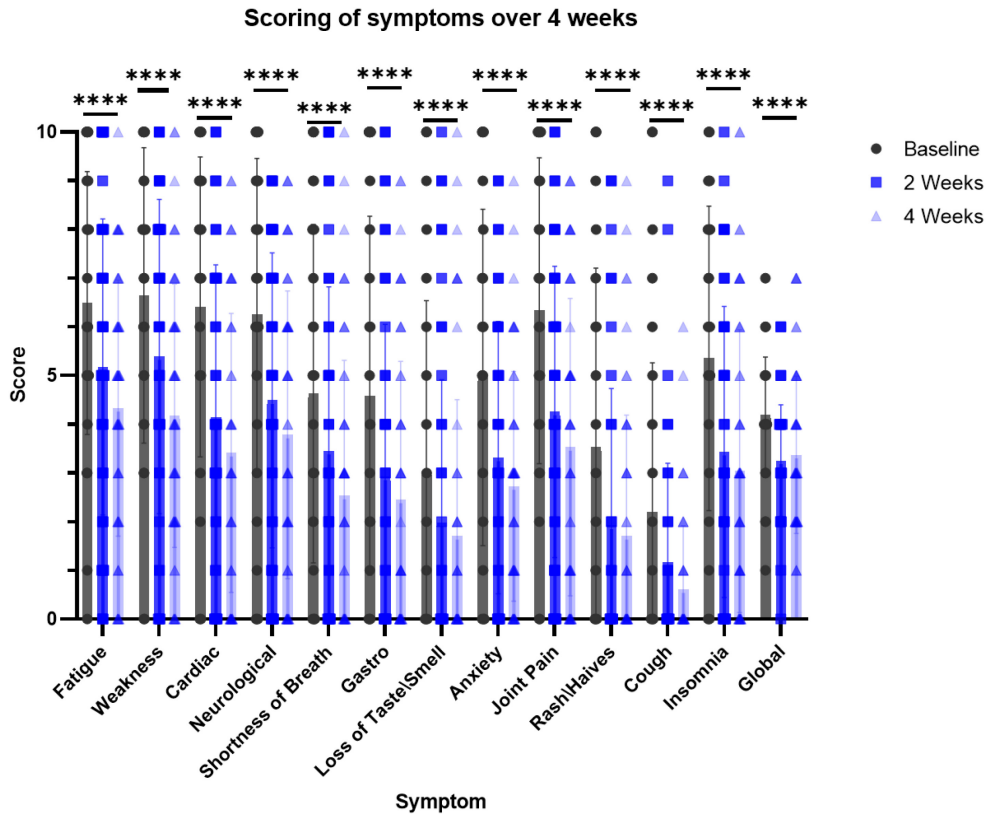

FIGURE 3
Survey-based scoring by participants at the intake to the trial (baseline), 2 and 4 weeks while taking the supplement. $N = 51$. Dunnett's multiple comparisons test, $p < 0.0001$ in 2 and 4 weeks for the effect of the supplement on all the symptoms (vs. baseline). ****$p < 0.006$ paired t-test, at 2 weeks vs. baseline and 4 weeks vs. baseline for each symptom.

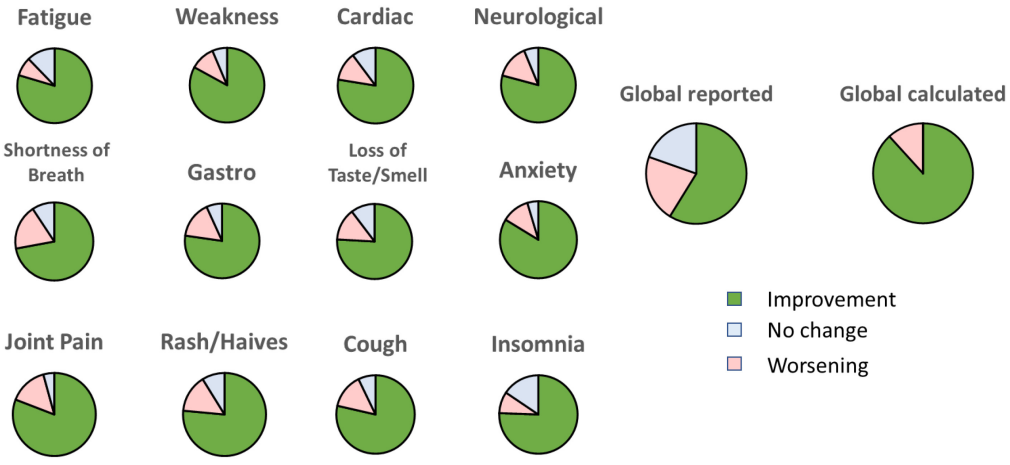

FIGURE 4
Percentage of participants reporting an improvement, no change or worsening for each symptom at week 4 relative to baseline. "Global reported" is the score given by each participant on general feeling. "Global calculated" is the average of all the scores for each symptom.

βCP and Preg that have potent anti-inflammatory role, we selected nutraceutical compounds that have been tested and proven effective as anti-viral and/or immunomodulatory agents (DHEA, Bromelain, AKBA, Quercetin, Zinc, and Vitamin D) or in managing neurological dysfunctions (DHEA, St John Wort, and Zinc, see Table 1). Further studies may be

warranted to elucidate the relative contribution of each of the nine compounds included in this nutraceutical to the various symptoms of Long-COVID.

This product is formulated for adults excluding pregnant and breastfeeding women. It is also contra-indicated in combination with serotonin-related antidepressants medications due to the St. John's Wort extract. When indicated, this new nutraceutical is a safe product to be used in combination with other standard of care therapies prescribed for long COVID. We emphasize that the product is not meant to replace standard recommendations of treatment, vaccinations or other suggested methods of prevention or treatment of COVID-19.

In this study, we compared the treatment outcome after 2 and 4 weeks to the same participant's baseline. While there was no placebo in this clinical study, every patient had tried and failed numerous nutraceutical and pharmaceutical products that were available to them along 3–20 months before the trial with no success. In addition, eligibility included persistence of the symptoms for at least 3 months, which reduces the likelihood that the positive effects are coincidental.

The use of nutraceuticals for specific long-COVID symptoms has been previously suggested (56). Here, we developed a nutraceutical for the treatment of a large array of long-COVID symptoms and we demonstrated statistically significant improvement in all tested endpoints in a relatively short time. Within the limitations of this study, our data indicate that the use of this nutraceutical product is a safe and significantly efficient option to reduce multiple symptoms of long COVID. To the best of our knowledge, our population study represents the largest group of patients to have shown statistically significant symptomatic improvement to a nutraceutical formulation.

Patents

Patent pending, International Patent Application No. PCT/IL2022/050676.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Tel Aviv University Animal Studies Ethics Committee.

Author contributions

NG, JM, and YG: conceptualization, writing – original draft preparation, and project administration. NG and YG: methodology, resources, supervision, and funding acquisition. NG, IK, JS, and YG: validation and investigation. IK, JS, and YG: formal analysis. JS and IK: data curation. IK and YG: writing – review and editing and visualization. All authors reviewed and approved the final version.

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Conflict of interest

The clinical study has been sponsored in part by NTN Enterprises LLC. Authors NG and JM own stock in NTN Enterprises LLC. Ramot, the technology transfer company of Tel Aviv University also owns stock in this company, and YG was an employee of Tel Aviv University. Authors JM and YG are the inventors of patent PCT/IL2022/050676, which exclusive rights has been acquired by NTN Enterprises LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Association of fat-soluble vitamins (A, D, and E) status with humoral immune response to COVID-19 inactivated vaccination

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Background: Fat-soluble vitamins (A, D, and E) are essential for the proper functioning of the immune system and are of central importance for infection risk in humans. Vitamins A, D, and E have been reported to be associated with the immune response following vaccination; however, their effects on the immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination remain unknown.

Methods: We measured the neutralizing antibody titers against wild type and omicron within 98 days after the third homologous boosting shot of inactivated SARS-CoV-2 vaccine (BBIBP-CorV or CoronaVac) in 141 healthy adults in a prospective, open-label study. High-performance liquid chromatography-tandem mass spectroscopy was used to determine the concentrations of plasma vitamins A, D, and E.

Results: We found that the anti-wide-type virus and anti-omicron variant antibody levels significantly increased compared with baseline antibody levels ($P < 0.001$) after the third vaccination. $25(OH)D_3$ was significantly negatively associated with the baseline anti-wide-type virus antibody concentrations [beta (95% CI) = −0.331 (−0.659 ∼ −0.003)] after adjusting for covariates. A potentially similar association was also observed on day 98 after the third vaccination [beta (95% CI) = −0.317 (−0.641 ∼ 0.007)]. After adjusting for covariates, we also found that $25(OH)D_3$ was significantly negatively associated with the seropositivity of the anti-omicron variant antibody at day 98 after the third vaccination [OR (95% CI) = 0.940 (0.883 ∼ 0.996)]. The association between plasma $25(OH)D_3$ with anti-wild-type virus antibody levels and seropositivity of anti-omicron variant antibodies were persistent in subgroup analyses. We observed no association between retinol/α-tocopherol and anti-wide-type virus antibody levels or anti-omicron variant antibody seropositive in our study.

Conclusion: The third inactivated SARS-CoV-2 vaccination significantly improved the ability of anti-SARS-CoV-2 infection in the human body. Higher vitamin D concentrations could significantly decrease the anti-wide-type virus-neutralizing antibody titers and anti-omicron variant antibody seropositive rate after the

inactivated SARS-CoV-2 vaccination in people with adequate levels of vitamin D, better immune status, and stronger immune response; further studies comprising large cohorts of patients with different nutritional status are warranted to verify our results.

KEYWORDS

SARS-CoV-2, Omicron, vaccine, neutralizing antibody, fat-soluble vitamins, cohort study

1. Introduction

Vaccination against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is undoubtedly an effective means of mitigating the coronavirus disease 2019 (COVID-19) pandemic. Different people obtain varying levels of protection from vaccines; therefore, the identification of sensitivity factors that affect vaccine protection is essential. The current research has focused on the association of unhealthy lifestyles and disease status with the immunogenicity of SARS-CoV-2 vaccines (1, 2). However, studies on the association between nutritional status and the immunogenicity of SARS-CoV-2 vaccines are relatively scarce and have yielded inconsistent results.

The optimal status of specific micronutrients is crucial for maintaining immune components within normal activity and improving host defenses against infections. The fat-soluble vitamins A, D, and E are essential for the proper functioning of the immune system (3) and play key roles at every stage of the innate and adaptive immune responses. Recent studies suggest that vitamin A may positively or negatively affect vaccine antibody response. Previous animal experiments have revealed that an adequate level of vitamin A is necessary to mount an efficient antibody response to many antigens (4); however, it has also been found that vitamin A-deficient animals can produce a strong antibody response to some antigens (4, 5). Furthermore, some studies have reported that vitamin A supplementation can stimulate an antibody response to vaccination, even in animals with normal vitamin A levels (6, 7). A human clinical trial also showed that vitamin A supplementation could improve immune responses to influenza virus vaccines in vitamin A-insufficient children at the baseline (8).

The effect of vitamin D on the antibody response to different vaccines is inconsistent. Vitamin D enhances the vaccine antibody response to tetanus and hepatitis B (9, 10); however, it is negatively correlated with the antibody response to the human papillomavirus (HPV) vaccine (11). Some studies have suggested that vitamin D does not influence the antibody response to the influenza vaccine (12–14). Similarly, animal studies have observed that vitamin E supplementation increases the antibody response in poultry (15, 16). However, randomized clinical trials have revealed that vitamin E supplementation has no effect on the antibody response to tetanus toxoid and pneumococcal polysaccharide vaccines in humans (17, 18), and a population study also showed no association between serum vitamin E levels and influenza vaccine response (19). The nutritional status of vitamins has different effects on antibody responses to different antigens or vaccines. However, it is unknown whether vitamins A, D, and E affect the immune response to SARS-CoV-2 vaccines.

An ecological study demonstrated that the intake levels of relevant micronutrients, especially vitamin D, were inversely associated with COVID-19 incidence and mortality (20). Moreover, a nutrigenetic study has shown that micronutrients, including vitamins A and D, and relevant genetic factors can help strengthen the immune system of individuals and prepare populations to fight against COVID-19 (20). A recent study showed that serum-neutralizing antibody levels gradually decreased after the second dose of inactivated vaccines within half a year; therefore, a third booster dose is necessary to maintain the effectiveness of inactivated vaccines (21). With the repeated outbreaks of the COVID-19 epidemic, a third booster SARS-CoV-2 vaccine shot has been administered in many Chinese cities. This study aimed to explore the association of vitamins A, D, and E with dynamic changes in neutralizing antibody titers (wild-type and omicron) after the third booster shot in a prospective cohort and provided evidence and clues for nutrition education during the COVID-19 pandemic.

2. Materials and methods

2.1. Study population

We conducted a prospective, open-label study (chictr.org.cn identifier: ChiCTR2200059259) at Zhuhai People's Hospital in Zhuhai, China, to explore the relationship of vitamins A, D, and E with dynamic changes of neutralizing antibody titers within 98 days after the third homologous boosting shot of inactivated SARS-CoV-2 vaccine (CoronaVac; Sinovac or BBIBP-CorV; Sinopharm) in healthy adults aged 18–59 years from December 2021 to April 2022. The subjects entered the study according to the inclusion criteria: no past or current SARS-CoV-2 infection had received two doses of inactivated whole-virion vaccines more than 6 months, and women were not pregnant or puerperal. A total of 183 subjects met the inclusion criteria and were invited to participate in the study, and 42 subjects were excluded according to established criteria: receipt of COVID-19 vaccine other than CoronaVac or BBIBP-CorV; allergy to any ingredient of vaccines; acute diseases attack or chronic diseases with/without acute exacerbation (including uncontrolled hypertension, diabetes complications, malignant tumor, renal diseases, and known autoimmune disease); the appearance of 10 symptoms of COVID-19 such as fever, cough, runny nose, and sore throat within 7 days before the third boost with the vaccine; using immunosuppressive medications and vitamin supplements for 15 days before and after the vaccine; a shot of other vaccines 14 days before the third vaccine or other vaccines planned within

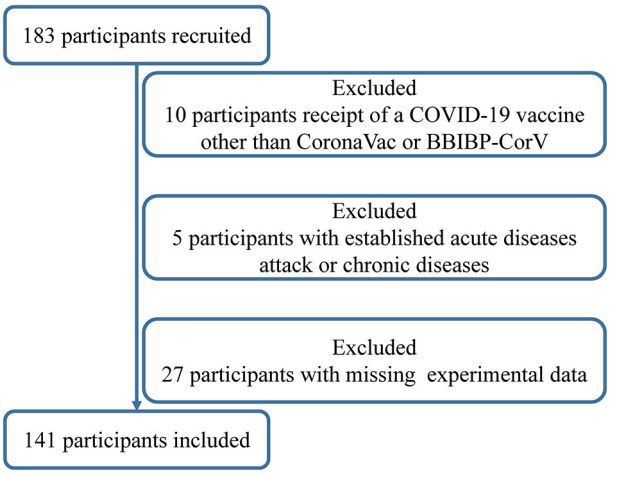

FIGURE 1
Flowchart of the participants' enrollment process.

28 days; having participated in other clinical studies; missing experimental data at any time point; and any condition that could interfere with the primary objectives. Written informed consent had been obtained from all participants before the enrolment (Figure 1). The study's informed consent and protocol were reviewed and approved by the Ethics Committee of Zhuhai People's Hospital.

2.2. Vaccination procedure and blood collection

All subjects received the third booster shot inactivated SARS-CoV-2 vaccines (CoronaVac or BBIBP-CorV, according to the previous vaccination program) more than 6 months after the second shot. All subjects underwent three blood draws that were handled according to the standards of practice before the third inoculation, 14 days, and 98 days after inoculation, respectively. The procedure is shown in Figure 2. Next, plasma was separated from blood cells immediately, and then aliquots (20 μL) were pipetted onto the imprinted circles of the dried plasma spots (DPS) card for vitamin analysis. The spots were dried for 2 h in the dark and then stored at −20°C with desiccant in resealable aluminum foil bags until analysis. The remaining plasma was stored at −80°C for further analysis.

2.3. Detection of vitamins A, D, and E

Plasma vitamin concentrations were measured using high-performance liquid chromatography-tandem mass spectroscopy (LC-MS/MS, Nexera UHPLC LC-30A, and SCIEX Triple Quad^{TM} 6500+), online coupled with fully automated dried blood spot extraction (CAMAG DBS-MS 500). DPS cards were moved by a robotic arm from the racks toward the different workstations, including an optical recognition system (locating the position of the spots on the cards), an IS module (spraying of an IS solution onto the cards before extraction), and an extraction module containing a 4 mm clamp head for clamping of a DPS card. A total of 10 μL internal standard composed of retinol-d5, 25(OH)D_3-13C5, and α-tocopherol-d6 in acetonitrile was sprayed onto the cards. Subsequently, 70% methanol aqueous solution as the extraction solvent is horizontally guided through the clamped area of the DPS, and the resulting extract is then sent into a sample loop. The autosampler is equipped with a wash station to avoid cross-contamination between subsequent samples.

For LC-MS/MS analysis, the optimized mass transition ion pairs (m/z) for quantitation were 269.2/93 for retinol, 274.2/93 for retinol-d5, 383.3/257 for 25(OH)D_3, 388.5/262.3 for 25(OH)D_3-13C5, 431.3/165 for α-tocopherol, and 437.3/171 for α-tocopherol-d6. The chromatographic separation was achieved on a Phenomenex Kinetex PFP (4.6 mm × 100 mm, 2.6 μm) column with a flow rate of 0.6 mL/min, using gradient elution with acetonitrile and 0.1% formic acid 0.05% heptafluorobutyric acid in water as the mobile phase. For quality check, each batch contained 48 samples with four quality control samples inserted, and intra- and inter-batch coefficients of all vitamins were below 15%. All participants had plasma vitamins above the quantitation limit (4.0 ng/L for retinol, 1.0 ng/L for 25(OH)D_3, and 10.0 μg/L α-tocopherol).

2.4. Detection of the anti-SARS-CoV-2 receptor-binding domain neutralizing antibody

At the same time, we evaluated the anti-RBD responses in fasting blood samples at three-time points above by the serum surrogate virus neutralization test (sVNT) to assess the dynamic changes of the neutralizing antibody. Circulating NAb against SARS-CoV-2 was detected which blocked the interaction between the RBD of the viral spike glycoprotein with the angiotensin-converting enzyme 2 cell surface receptor in the experiment. Recombinant S-RBD from the wild type (Wuhan-Hu-1) and omicron (B.1.1.529) strains were used in this study. All experimental operations and the use of the SARS-CoV-2 sVNT kit (GenScript) were performed according to the manufacturer's instructions. Tests were performed on Varioskan Lux (ThermoFisher).

For all assays, the limit of quantitation (LOQ) was 9.38 U/mL, and levels < LOQ were substituted with LOQ/Sqr(2).

2.5. Statistical analysis

Demographic characteristics of the study are summarized as mean ± standard deviation (SD) or median and interquartile range (IQR) for continuous variables, as no. (%) for categorical variables. Neutralizing antibody titers are presented as geometric mean titers (GMTs) with 95% confidence intervals (CIs). The

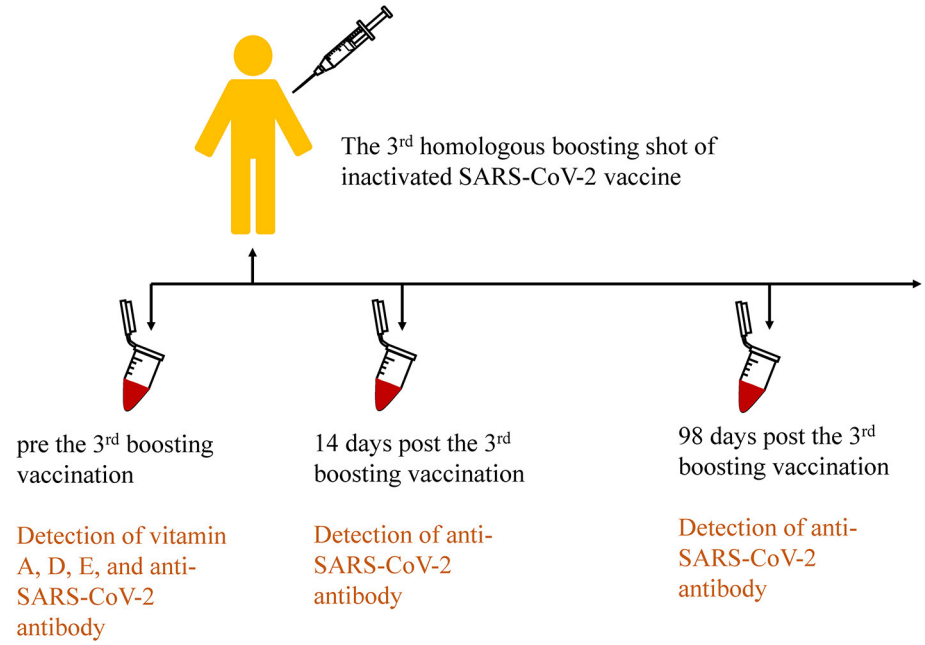

FIGURE 2
Procedures of the third homologous boosting shot of inactivated SARS-CoV-2 vaccination and sample collection in the cohort study.

seropositivity rate was defined as the serum anti-SARS-CoV-2 antibody concentrations exceeding 4 × LOQ. The Kolmogorov–Smirnov test was used to assess the normality of variables and those not normally distributed were log-transformed. The Mann–Whitney *U*-test was used to compare the difference in anti-SARS-CoV-2 neutralizing antibody growth and decay between subgroups. The Spearman correlation test was used to assess the association of antibody growth and decay with the target vitamin concentrations. We performed multivariate linear regression (anti-wide-type antibody as the dependent variable) and logistic regression (anti-omicron antibody seropositivity or not as the dependent variable) analyses to assess the relationships between antibody levels and the concentrations of plasma retinol, 25(OH)D_3, and α-tocopherol. Statistical analyses were performed using R software (version 4.1.3), with the two-sided significant level at 0.05.

3. Results

3.1. Study participants characteristics

The distribution of the participants' characteristics is presented in Table 1. After excluding participants with missing experimental data, 141 were included in the study. The mean age of patients was 30.16 years, and the mean body mass index (BMI) was 22.01 kg/m^2; 54.61% of patients had regular physical activity, and 71.63% were injected with the BBIBP-CorV vaccine. Over 90% of participants were of Han ethnicity, did not smoke or drink, and had a college education or higher.

3.2. Distribution of anti-wild-type virus and anti-omicron variant antibody titers at the baseline and 14 and 98 days after the third booster dose

We detected anti-wild-type virus and anti-omicron variant antibodies at the baseline and on days 14 and 98 after the third vaccination (Table 2). We found that the third vaccination induced a significantly higher degree of humoral immunogenicity for either the wild-type or omicron variant (days 14 and 98 after the third vaccination vs. baseline, $P < 0.001$, respectively). The third vaccination induced not only a significantly high humoral immunogenicity of the wild-type virus but also of the omicron variant. The seropositivity rates were 98.58%, 100.00%, and 100% for the anti-wild-type virus antibodies and 11.34%, 50.35%, and 35.46% for the seropositivity rates of anti-omicron variant antibodies at the baseline and on days 14 and 98, respectively ($P < 0.001$, compared with the baseline).

3.3. Associations of retinol, 25(OH)D_3, and α-tocopherol with anti-wild-type virus and the anti-omicron variant neutralizing antibodies

We performed multivariate regression analyses to assess the effect of the target vitamin levels at the baseline on anti-wild-type virus-neutralizing antibodies before and after the third booster dose. The results are presented in Figure 3. After adjusting for

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TABLE 1 Characteristics of the study participants by sex group.

Variable	Total ($N = 141$)	Male ($N = 49$)	Female ($N = 92$)
Age, years, mean ± SD	30.16 ± 7.84	29.35 ± 7.24	30.59 ± 8.14
Race, Han, no. (%)	136 (96.45)	48 (34.04)	88 (62.41)
BMI, kg/m^2, mean ± SD	22.01 ± 4.15	23.73 ± 3.12	21.10 ± 4.34
Smoking no. (%)			
Never	133 (94.33)	42 (29.79)	91 (64.54)
Former	1 (0.71)	1 (0.71)	0
Current	7 (4.96)	6 (4.26)	1 (0.71)
Drinking no. (%)			
Never	136 (96.45)	46 (32.62)	90 (63.83)
Former	1 (0.71)	1 (0.71)	0
Current	4 (2.84)	2 (1.42)	2 (1.42)
Education levels no. (%)			
Middle school	3 (2.13)	0	3 (2.13)
High school	4 (2.84)	1 (0.71)	3 (2.13)
College or above	134 (95.04)	48 (34.04)	86 (61.00)
Physical activity, yes, No. (%)	77 (54.61)	34 (24.11)	43 (30.50)
First-to-second dose interval, days, median (IQR)	31.00 (22.00–41.00)	29.00 (18.00–41.00)	31.00 (23.00, 40.25)
Second-to-third dose interval, days, median (IQR)	252.00 (200.00–293.00)	257.00 (207.00–296.00)	245.50 (197.00–291.25)
Manufacturer of vaccine no. (%)			
BBIBP-CorV	101 (71.63)	37 (26.24)	64 (45.39)
CoronaVac	40 (28.37)	12 (8.51)	28 (19.86)

SD, standard deviation; IQR, interquartile range.

age, sex, BMI, physical activity status, the intervals between the first and second vaccination and between the second and third vaccination, and the manufacturer of the vaccine, we found that 25(OH)D_3 was significantly negatively associated with baseline anti-wild-type virus antibody concentrations (beta [95% CI] = −0.331 [−0.659–−0.003]). After adjusting for covariates, there was a potentially negative association between 25(OH)D_3 and anti-wild-type virus antibody concentrations on day 98 after the third vaccination (beta [95% CI] = −0.317 [−0.641–0.007]). No statistically significant associations were observed between retinol and anti-wild-type virus antibody concentrations after adjusting for covariates (all $P > 0.050$).

Logistic regression analyses were used to assess the effect of target vitamin levels at the baseline on the seropositivity of anti-omicron variant neutralizing antibodies before and after the third booster dose (Figure 4). After adjusting for covariates, we also found that 25(OH)D_3 was significantly negatively associated with the seropositivity of the anti-omicron variant antibody upon day 98 after the third vaccination (OR [95% CI] = 0.940 [0.883–0.996]). Similarly, no statistically significant associations were observed between the retinol and α-tocopherol and the seropositivity of the anti-omicron variant antibodies after adjusting for covariates (all, $P > 0.050$).

Subgroup analyses showed an association between 25(OH)D_3 and the baseline anti-wild-type virus antibody concentrations, which seemed to be more pronounced among subjects with the female, with the intervals between the first and second vaccination ≤30 days (Supplementary Figure 1A). The association between 25(OH)D_3 and the anti-wild-type virus antibody concentrations at day 98 after the third vaccination seemed to be more pronounced among subjects with the intervals between the first and second vaccination being >30 days (Supplementary Figure 1B). We also found the association of 25(OH)D_3 with the seropositivity of anti-omicron variant antibody at day 98 which after the third vaccination seemed to be more pronounced among subjects with male, CoronaVac-vaccinated, with the intervals between the first and second vaccination being >30 days and the intervals between 2nd and 3rd vaccination being >240 days (Supplementary Figure 1D).

To further explore the relationship between target vitamins and changes in anti-wild-type virus-neutralizing antibodies on days 14 and 98 after the third vaccination, we assessed the

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TABLE 2 Distribution of antibody titers against wild-type and Omicron at three-points time.

	Baseline	14 days after 3rd vaccination	98 days after 3rd vaccination
Prototype, U/mL			
GMT (95% CI)	142.43 (127.74–159.17)	831.34 (742.48–925.19)	436.27 (391.51–482.99)
Seropositivity rate	98.58%	100.00%	100.00%
textitP[a] value		< 0.001	< 0.001
Omicron, U/mL			
GMT (95% CI)	11.93 (10.18–14.01)	31.03 (25.03–38.47)	20.19 (16.78–24.29)
Seropositivity rate	11.34%	50.35%	35.46%
P[a] value		< 0.001	< 0.001

GMT, geometric mean titer; IQR, interquartile range; LOQ, limit of quantitation.
The seropositivity rate was defined as at least a 4-fold higher than the limit of quantitation of the assays.
[a]The P-value of the t-test.

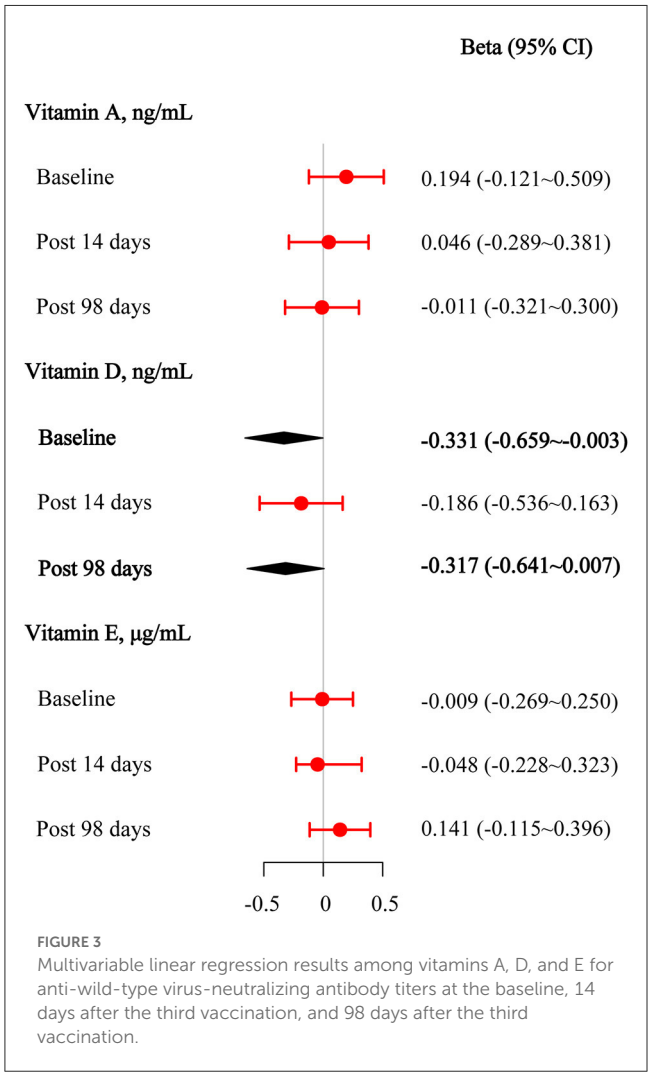

FIGURE 3
Multivariable linear regression results among vitamins A, D, and E for anti-wild-type virus-neutralizing antibody titers at the baseline, 14 days after the third vaccination, and 98 days after the third vaccination.

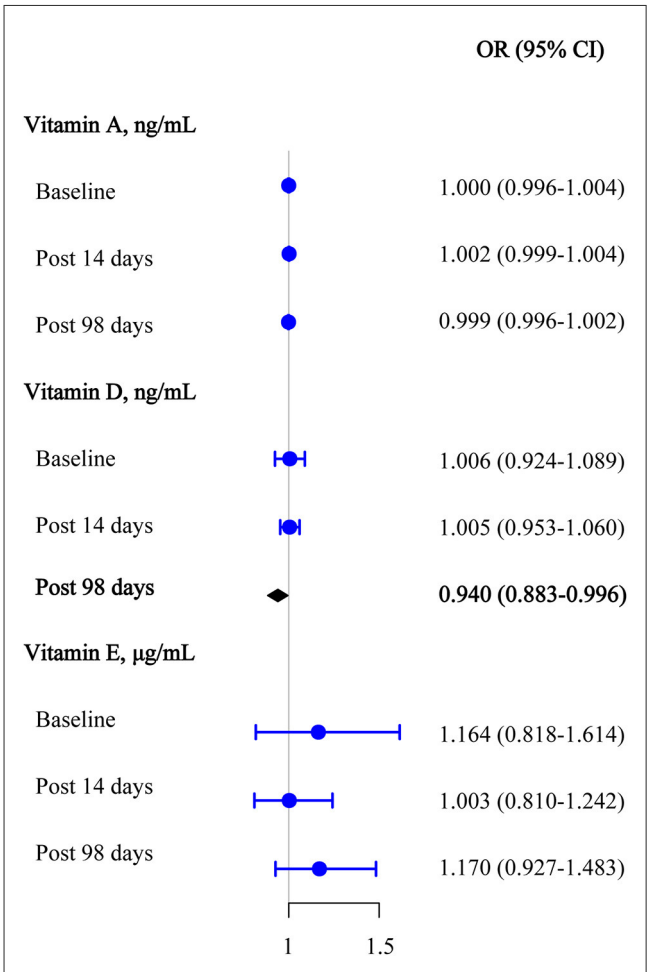

FIGURE 4
Multivariable logistic regression results among vitamins A, D, and E for anti-omicron variant neutralizing antibody seropositive at the baseline, 14 days after the third vaccination, and 98 days after the third vaccination.

correlations between target vitamins, fold increases in antibody titers (comparison between the baseline and 14 days after the third vaccination), and fold decreases in antibody titers (comparison between 14 days after the third vaccination and 98 days after the third vaccination). However, we observed no correlation between the target vitamins and fold increase/decrease in antibody titers (all $P > 0.050$; Supplementary Table 2).

4. Discussion

In this study, we used a prospective, open-label design to assess the relationships between vitamins A, D, and E nutritional status and neutralizing antibodies (anti-wild-type and anti-omicron) before and after the third inactivated SARS-CoV-2 vaccination. The third vaccination induced a significantly high degree of humoral immunogenicity for both the wild-type virus and the omicron variant. We found that plasma vitamin D levels were significantly negatively associated with the baseline anti-wild-type virus antibody levels, potentially negatively associated with anti-wild-type virus antibody concentrations on day 98 after the third vaccination, and significantly negatively associated with the seropositivity of the anti-omicron variant antibody on day 98 after the third vaccination. The inverse associations between plasma vitamin D and anti-wild-type virus antibody levels and seropositivity of the anti-omicron variant antibodies persisted in the subgroup analyses. We observed no significant association between vitamin A/E and anti-wild-type virus antibody levels or seropositivity of the anti-omicron variant antibodies.

The inactivated SARS-CoV-2 vaccine is widely used to prevent infection and severe COVID-19, and neutralizing antibody levels are vital predictors of vaccine efficiency (22). Similar to the results of Ai et al., the third homologous booster vaccination enhanced participants' immune responses against SARS-CoV-2 (23). However, the neutralizing capacity against the omicron variant was significantly lower than that against the wild-type virus, confirming the results of another study (24).

Numerous previous studies have found that low vitamin D levels were associated with significantly higher antibody titers after antiviral vaccination in people with a relatively high vitamin D nutritional status, which is consistent with our study. Linder et al. found significantly higher mean geometric rubella antibody titers in winter-inoculated children than in summer-inoculated children; vitamin D was stimulated by ultraviolet radiation (25). A study on HPV vaccines in college-aged men suggested that antibody titers for all HPV strains were significantly higher in individuals with lower vitamin D levels than in those with higher vitamin D levels (11). A cross-sectional study found that low serum vitamin D levels were associated with higher antibody titers against partial influenza virus vaccines in children (26). Another cross-sectional analysis of the National Health and Nutrition Examination Survey (NHANES) highlighted a negative association between serum 25(OH)D levels and measles antibody titers (27). Many studies have demonstrated the expression of the vitamin D receptor (VDR) in almost all immune cells (28). Therefore, vitamin D has potent direct effects on active B lymphocytes (i.e., VDR expression is upregulated) (29). Vitamin D inhibits immunoglobulin production through various mechanisms, including the inhibition of cytokine-mediated B-cell activation by acting on T-helper cells, suppressing the differentiation of mature B cells into plasma cells and class-switched memory B-cells, and inducing apoptosis of both activated B- and plasma cells (30).

However, the effect of vitamin D on the immunogenicity of vaccines is complex, and the association between vitamin D and antibody response to COVID-19 vaccines is inconsistent in the current research. Several studies have reported no clear correlation between vitamin D levels and antibody responses to anti-SARS-CoV-2 mRNA vaccination (31, 32). A sub-study nested within the CORONAVIT randomized controlled trial also reported that vitamin D supplementation did not influence the protective efficacy or immunogenicity of SARS-CoV-2 mRNA or adenovirus vaccinations in old adults (33). Moreover, a few studies reported results contrary to ours, suggesting that adequate levels of vitamin D may improve the antibody response to SARS-CoV-2 mRNA vaccines (34, 35). A differential immune response has been observed for different SARS-CoV-2 vaccine types (36); therefore, vitamin D potentially has different effects on different vaccine types. Rubella, HPV, influenza, and measles vaccines, as well as the anti-SARS-CoV-2 vaccine used in our study, are all inactivated antiviral vaccines. Vitamin D deficiency can cause dysregulation of the immune response (37), and correcting this deficiency can effectively improve the immune response. Positive associations between vitamin D and antibody response to the SARS-CoV-2 vaccine were obtained by comparing the adequate status of vitamin D and insufficient/deficiency status (34, 35). However, positive associations were not observed in another study of older adults (33), which might have been caused by different population backgrounds; the former included middle-aged people with better immune status and stronger immune responses. In our study, the subjects were mainly middle-aged people with better immune status and stronger immune response and had a relatively high vitamin D nutritional status; <30% of patients had vitamin D insufficiency [i.e., a 25(OH)D level of 20–30 ng/mL], and none had vitamin D deficiency [i.e., a 25(OH)D level <20 ng/mL] (38). Additionally, the genetic and ethnic backgrounds of different populations should be considered (39, 40).

No significant associations were observed between vitamins A and E and the antibody response of both wild-type and omicron variant disease following inactive anti-SARS-CoV-2 vaccination in our study; this result was consistent with the results of previous studies. Gardner et al. found that vitamins A and E were not associated with antibody responses to influenza vaccines in healthy elderly individuals (41). An observational prospective cohort study reported that vitamins A and E levels were not related to the odds of seroprotection or seroconversion to the influenza vaccine in older adults (19). A prospective randomized controlled clinical trial suggested that vitamins A and E supplementation did not affect the IgG response to tetanus toxoid in healthy children (17). However, a clinical trial suggested that weekly maternal vitamin A supplementation during pregnancy and postpartum could enhance prenatal H1N1-vaccine responses in mothers with low vitamin A status (42). In our study, only approximately 2% of subjects had vitamin A deficiency [retinol level <200 ng/mL as vitamin A deficiency (43)], approximately 95% of subjects had vitamin E deficiency [α-tocopherol level <5 μg/mL as vitamin E deficiency (44)], and none had vitamin A or E supplementation. Therefore, the association of vitamins A and E with the antibody response to inactive anti-SARS-CoV-2 vaccination needs to be verified in individuals with different vitamin A and E statuses.

Our study has several advantages. Our study is a prospective cohort study to estimate the anti-wild-type virus and anti-omicron variant antibody response in the medium-to-long-term following

the third inactive anti-SARS-CoV-2 vaccination. It is also, to the best of our knowledge, the first to explore the associations between vitamins A, D, and E with anti-wild-type virus/anti-omicron variant antibody response to inactive anti-SARS-CoV-2 vaccination. This study reveals the relationship between vitamin D and antibody response, which provides new clues for precise nutrition during the COVID-19 pandemic, especially for the present omicron pandemic. However, this study had several limitations. First, most subjects had a relatively high vitamin D and vitamin A nutritional status, and most subjects had a relatively poor vitamin E nutritional status; therefore, we were unable to assess the effects of different vitamin A, D, and E nutritional statuses on the antibody response. Second, the study did not include people who used vitamin supplements to further explore the effects of vitamin supplementation on the antibody response. Third, our subjects were mainly aged 24–42 years, which meant that they had a better immune status and stronger immune response; therefore, younger and older people were ignored. Finally, different vaccine types and populations with different genetic backgrounds should be considered because of the complex effects of vitamins on immune responses. In the future, the relationship between vitamins A, D, and E and antibody response to inactive anti-SARS-CoV-2 vaccination should be comprehensively described in different populations.

5. Conclusion

Our study suggested that the third homologous boosting vaccination enhanced subjects' immunity response against SARS-CoV-2, and low vitamin D levels were associated with significantly higher antibody titers for the anti-wild-type virus and higher antibody seropositivity for the anti-omicron variant after the third inactive anti-SARS-CoV-2 vaccination in people with adequate levels of vitamin D, better immune status, and stronger immune response; further studies comprising large cohorts of patients with different nutritional status are warranted to verify our results.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Zhuhai People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YD, TS, LH, JL, and PL conceived the study, performed manuscript revision, and took accountability for all aspects of the work. YD, LH, and PL designed the methodology and did the software analysis. TS and JL were in charge of supervision and administration. All authors performed the data interpretation, drafted and revised the manuscript, and read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1167920/full#supplementary-material

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A hub gene signature as a therapeutic target and biomarker for sepsis and geriatric sepsis-induced ARDS concomitant with COVID-19 infection

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Background: COVID-19 and sepsis represent formidable public health challenges, characterized by incompletely elucidated molecular mechanisms. Elucidating the interplay between COVID-19 and sepsis, particularly in geriatric patients suffering from sepsis-induced acute respiratory distress syndrome (ARDS), is of paramount importance for identifying potential therapeutic interventions to mitigate hospitalization and mortality risks.

Methods: We employed bioinformatics and systems biology approaches to identify hub genes, shared pathways, molecular biomarkers, and candidate therapeutics for managing sepsis and sepsis-induced ARDS in the context of COVID-19 infection, as well as co-existing or sequentially occurring infections. We corroborated these hub genes utilizing murine sepsis-ARDS models and blood samples derived from geriatric patients afflicted by sepsis-induced ARDS.

Results: Our investigation revealed 189 differentially expressed genes (DEGs) shared among COVID-19 and sepsis datasets. We constructed a protein-protein interaction network, unearthing pivotal hub genes and modules. Notably, nine hub genes displayed significant alterations and correlations with critical inflammatory mediators of pulmonary injury in murine septic lungs. Simultaneously, 12 displayed significant changes and correlations with a neutrophil-recruiting chemokine in geriatric patients with sepsis-induced ARDS. Of these, six hub genes (CD247, CD2, CD40LG, KLRB1, LCN2, RETN) showed significant alterations across COVID-19, sepsis, and geriatric sepsis-induced ARDS. Our single-cell RNA sequencing analysis of hub genes across diverse immune cell types furnished insights into disease pathogenesis. Functional analysis underscored the interconnection between sepsis/sepsis-ARDS and COVID-19, enabling us to pinpoint potential therapeutic targets, transcription factor-gene interactions, DEG-microRNA co-regulatory

networks, and prospective drug and chemical compound interactions involving hub genes.

Conclusion: Our investigation offers potential therapeutic targets/biomarkers, sheds light on the immune response in geriatric patients with sepsis-induced ARDS, emphasizes the association between sepsis/sepsis-ARDS and COVID-19, and proposes prospective alternative pathways for targeted therapeutic interventions.

KEYWORDS

COVID-19, sepsis, sepsis-ARDS, hub gene, disease biomarker, bioinformatics

Introduction

Sepsis, a life-threatening condition resulting from an uncontrolled immune response to infection, can be instigated by various pathogens, including SARS-CoV-2, the virus responsible for COVID-19 (1, 2). With respiratory and gastrointestinal bacterial and viral infections being the most prevalent, sepsis accounts for nearly 20% of global deaths (3). Furthermore, severe COVID-19 presents similarities to sepsis-induced acute respiratory distress syndrome (ARDS), such as pulmonary inflammation, dense mucus secretion, microthrombosis, and systemic proinflammatory cytokine elevation (4, 5). Given the immense global impact of both conditions, it is imperative to understand the pathophysiology of sepsis/sepsis-ARDS in the context of COVID-19 and refine intensive care therapies for critically ill patients.

Geriatric patients with sepsis-induced ARDS are at a higher risk of poor outcomes when infected with COVID-19 (5–7). The interplay between COVID-19 and sepsis makes this vulnerable group particularly susceptible to respiratory failure and mortality (7, 8). Therefore, early identification of key diagnostic targets is crucial for potentially mitigating COVID-19 and sepsis-induced ARDS effects. Moreover, despite the severity of this issue, the cellular and molecular events that contribute to the effects of COVID-19 on geriatric sepsis-induced ARDS have not been clearly defined.

In this study, we employ bioinformatics and systems biology approaches to examine the effects of COVID-19 on sepsis and geriatric sepsis-induced ARDS. Our investigation aims to uncover shared cellular signaling pathways, gene networks, potential biomarkers, and therapeutic targets. Furthermore, we explore candidate drugs and their underlying molecular mechanisms for the treatment of sepsis and geriatric sepsis-ARDS patients co-infected with COVID-19. To corroborate our findings, we validate hub genes in murine sepsis-ARDS models and geriatric patients with sepsis-induced ARDS, utilizing single-cell RNA sequencing (scRNA-seq) to assess their expression patterns across various immune cell populations. This comprehensive analysis furnishes therapeutic targets/biomarkers and imparts invaluable insights into the pathogenesis of these intricate conditions.

Materials and methods

Study population

In this study, patient samples were collected from critically ill individuals aged 60 years or older who were undergoing treatment in the emergency intensive care units (ICUs) at Zhongshan Hospital and Minhang Hospital, both affiliated with Fudan University, China. We prospectively enrolled 17 patients diagnosed with sepsis-induced ARDS, following the diagnostic criteria established in the 2016 international Sepsis 3.0 consensus proposed by sepsis experts and the Berlin definition and diagnostic criteria for ARDS (9, 10). These patients did not have SARS-CoV-2 infection. Table S1 presents an overview of the demographic and clinical features of the study population. For three of these patients, blood samples were collected on Day 1 and Day 7 and subsequently subjected to scRNA-seq. Based on their clinical manifestations, Day 1 and Day 7 were designated as the onset and recovery phases, respectively. For additional information, please refer to Table S2.

Gene expression datasets

To investigate the shared genetic correlations between COVID-19 and sepsis, we utilized RNA-seq and microarray datasets from the Gene Expression Omnibus (GEO) database. Specifically, we analyzed the GSE171110 dataset, which comprises whole-blood RNA-seq profiles from COVID-19 patients and healthy donors, and the GSE137342 dataset, which includes whole blood cells from sepsis patients and healthy volunteers and was sequenced using microarrays (11). Further information regarding the datasets is outlined in Table S3.

Identification of differentially expressed genes

DEGs were determined from expression values utilizing the "limma" package in R software (version 4.2.0), applying Benjamini-Hochberg correction to regulate the false discovery rate. The DEGs

were considered significant if they met the cutoff criteria (adjusted *P-value* < 0.05 and |logFC| ≥ 1.0). The common DEGs between COVID-19 and sepsis were identified using an online VENN analysis tool called Jvenn (12).

Gene ontology and pathway enrichment analysis

Utilizing the "clusterProfiler" package in R, potential functions and pathways associated with DEGs were identified. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed, and a standardized metric (*P*-value < 0.05, *Q*-value < 0.25) was used to prioritize the top functional items and pathways.

Protein-protein interaction network analysis

The PPI network was generated based on the proteins encoded by the common DEGs between COVID-19 and sepsis, as determined through the STRING database (13). The PPI network was further processed and analyzed using Cytoscape software, and gene clusters were identified using the Markov cluster method. The prominent nodes in the PPI network modules were predicted using the CytoHubba plugin to identify hub genes.

Gene regulatory networks analysis

Hub gene-microRNA (Hub-miRNA) interaction networks and hub gene-transcription factor (Hub-TF) interaction networks were analyzed using the NetworkAnalyst tool (14). TarBase (15) and miRTarBase (16) databases were used to identify Hub-miRNA interactions, while the JASPAR database (17) was used to analyze Hub-TF interactions. The hub genes common to COVID-19 and sepsis were used in the gene regulatory networks analysis to identify the transcriptional elements and miRNA that regulate hub genes at the post-transcriptional level.

Immune infiltration analysis

The composition of immune cells in blood samples was analyzed utilizing the CIBERSORT (18), a deconvolution algorithm designed to quantify the representation of 22 distinct subpopulations of infiltrating lymphocytes. GraphPad Prism 8.0.2 software was utilized to contrast immune cell proportions between the COVID-19 and healthy control samples, as well as between the sepsis and healthy control samples.

Evaluation of applicant drugs

The Enrichr web server (19) and the DSigDB database (20) were used to identify pharmacological compounds connected to the common hub genes of COVID-19 and sepsis, based on a statistical threshold of adjusted *P-value* < 0.05.

Gene-disease association analysis

The DisGeNET database (21) was utilized to investigate gene-disease associations and identify diseases and chronic issues linked to the common hub genes of COVID-19 and sepsis. The NetworkAnalyst tool (22) was also used in this analysis.

Blood sample collection, processing, and analysis

Peripheral blood samples were collected from each patient in a 5 mL EDTA tube on the day of enrollment and processed within two hours to isolate all peripheral blood mononuclear cells (PBMCs). The PBMCs were either subjected to scRNA-seq or cryopreserved and stored at -80°C for subsequent analysis.

scRNA-seq

Blood-derived PBMCs were subjected to single-cell gel bead-in-emulsion generation using the Chromium Controller Instrument (10×Genomics) and the Single Cell 3' Library and Gel Bead Kit V3.1 (10×Genomics) according to the manufacturer's recommended protocol. The resulting GEM libraries were then sequenced on an Illumina Novaseq 6000 using a custom paired-end sequencing mode of 150 bp for the first read and 150 bp for the second read. The raw data was processed using the Cell Ranger Single-Cell Software Suite (v5.0.0) with default parameters and aligned to the genomic reference. To maintain data quality, cells were discarded if they had gene counts below 200 or over 5000, or mitochondrial gene expression greater than 15%. The most variable genes among the single cells were identified, and their log-transformed gene-barcode matrices were subjected to principal component analysis to reduce their dimensionality. The resulting data was visualized in a t-SNE plot constructed with Seurat (v3.1.1) to differentiate among the PBMC cell types.

Mouse models of sepsis-induced ARDS

To induce sepsis-induced ARDS in mice, we used the cecal ligation and puncture (CLP) method (23, 24). In brief, mice underwent an 8-hour fast and 4-hour water deprivation before surgery. Under 2% isoflurane anesthesia, a sterile abdominal incision provided access to the cecum. The cecum was ligated 1 cm from its end, punctured twice with a 22-gauge needle, repositioned, and the incision closed in two layers. Control mice experienced a similar procedure without CLP. At 24 hours post-CLP, lungs were collected, and RNA was isolated from the tissue using a Tiangen kit. RNA was reverse transcribed with the Tiangen kit, and relative gene expression was determined via the ΔΔCt method, using actin as an internal control.

Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM). Statistical analyses were executed using GraphPad Prism and R software. For normally distributed data, a two-tailed, unpaired Student's t-test was applied to assess differences between groups. Non-normally distributed data were evaluated using the Mann-Whitney test. Correlation analyses were performed employing the Pearson method. Statistical significance was established for P-values below 0.05 (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

Results

Investigation of the genetic overlap between COVID-19 and sepsis

To reveal shared genetic pathways between COVID-19 and sepsis, we comprehensively analyzed human transcriptomic data from the GEO database (Figure S1). Our results showed that COVID-19 and sepsis patients had 4082 and 551 DEGs, respectively, compared to healthy controls (Table S3). The DEGs of the highest significance for COVID-19 and sepsis are depicted in heatmaps and volcano plots in Figures 1A–D. We then utilized Jvenn to uncover 189 DEGs that were shared between the sepsis and COVID-19 datasets (Figure 1E). The complete list of these 189 DEGs can be found in Table S4.

Functional enrichment analysis of GO terms and significant signaling pathways

To understand the functional significance of the shared DEGs between COVID-19 and sepsis, we conducted a functional enrichment analysis using the "clusterProfiler" package. The analysis consisted of GO enrichment, linking genes to GO terms, and KEGG pathway enrichment, which revealed gene-pathway associations. The GO analysis was categorized into biological process, cellular component, and molecular function (25). Our results showed that the DEGs were significantly enriched in defense against fungus in the biological process category, specific granule lumen in the cellular component category, and MHC class II receptor activity in the molecular function category (Figures 2A, B). The KEGG pathway analysis revealed the top 10 pathways, including asthma, inflammatory bowel disease, hematopoietic cell lineage, intestinal immune network for IgA production, legionellosis, staphylococcus aureus infection, leishmaniasis, Th1 and Th2 cell differentiation, systemic lupus erythematosus, and cytokine-cytokine receptor interaction (Figures 2C, D).

Functional networks and hub genes identified by PPI analysis

The shared DEGs between COVID-19 and sepsis were subjected to a PPI analysis using the STRING database, aiming to reveal functional networks and associated pathways. The PPI network is illustrated in Figure 3A, where highly interconnected nodes represent hub genes. The maximal clique centrality method of cytoHubba in Cytoscape was employed to identify the top 15 most influential DEGs, which include CD4, CD3E, IL7R, CD5, CD247, CD2, CCR7, CD40LG, ITK, KLRB1, MPO, MMP9, TLR2, LCN2, and RETN. These hub DEGs hold potential as biomarkers and therapeutic targets for COVID-19 and sepsis, presenting novel opportunities for therapeutic intervention. A submodule network was constructed to facilitate a better understanding of the relationships and positions of these genes, as shown in Figure 3B.

Validation of the identified hub genes

To assess the potential of hub genes as biomarkers for predicting COVID-19 and sepsis-induced ARDS, we employed a murine model of sepsis-induced ARDS as a proxy for both COVID-19 and sepsis-related lung injuries. This choice was guided by the shared pathophysiological characteristics of ARDS in COVID-19 and sepsis, and their substantial mechanistic overlap. The use of the murine model was also influenced by the absence of a biosafety level 3 facility in our laboratory, which precluded the development of a specific SARS-CoV-2 infection model. Our results showed that genes such as IFN-γ, TNF-α, IL-6, MIP-2, IL-10, KC (CXCL1), CCL-2, MPO, MMP9, TLR2, and LCN2 were significantly upregulated in the lungs of sepsis-induced ARDS mice compared to control mice. In contrast, genes such as CD247, CD2, CD40LG, KLRB1, and RETN were significantly downregulated (Figures 4A, B).

TNF-α, IFN-γ, and IL-6 represent vital inflammatory mediators in the progression of lung injury in both sepsis and COVID-19 scenarios (1, 26). Positive correlations were observed between MPO, TLR2, LCN2 and TNF-α, and between MPO, MMP9, LCN2 and IL-6. Conversely, negative correlations were observed between CD2 and IFN-γ, and between CD40LG and IL-6 (Figures 4C, D, S2A–C).

KC and CCL-2 serve as significant chemokines that attract neutrophils and monocytes to infection sites, thereby playing a pivotal role in the pathophysiology of COVID-19 and sepsis-induced lung injury (1, 27, 28). The results showed that MMP9 and LCN2 were positively correlated with KC, while CD40LG and KLRB1 were negatively correlated with KC. Furthermore, MPO, MMP9, and LCN2 were positively correlated with CCL-2, while CD40LG was negatively correlated with CCL-2 (Figures 4E, F, S2D, E).

Finally, we assessed the potential of the discovered hub genes as targeted biomarkers in elderly sepsis-induced ARDS. We investigated the expression levels of these hub genes in PBMCs collected from elderly patients with sepsis-induced ARDS. Our findings revealed significant decreases in CD4, CD3e, IL-7R, CD5, CD247, CD2, CD40LG, ITK, and KLRB1, and significant elevations in MMP9, LCN2, and RETN in sepsis-induced ARDS compared to controls (Figure 5A). Similar results were also observed in COVID-19 and sepsis (Figure S3). Moreover, we found that CD3, CD247, CD2, and CD40LG were negatively correlated with KC, whereas

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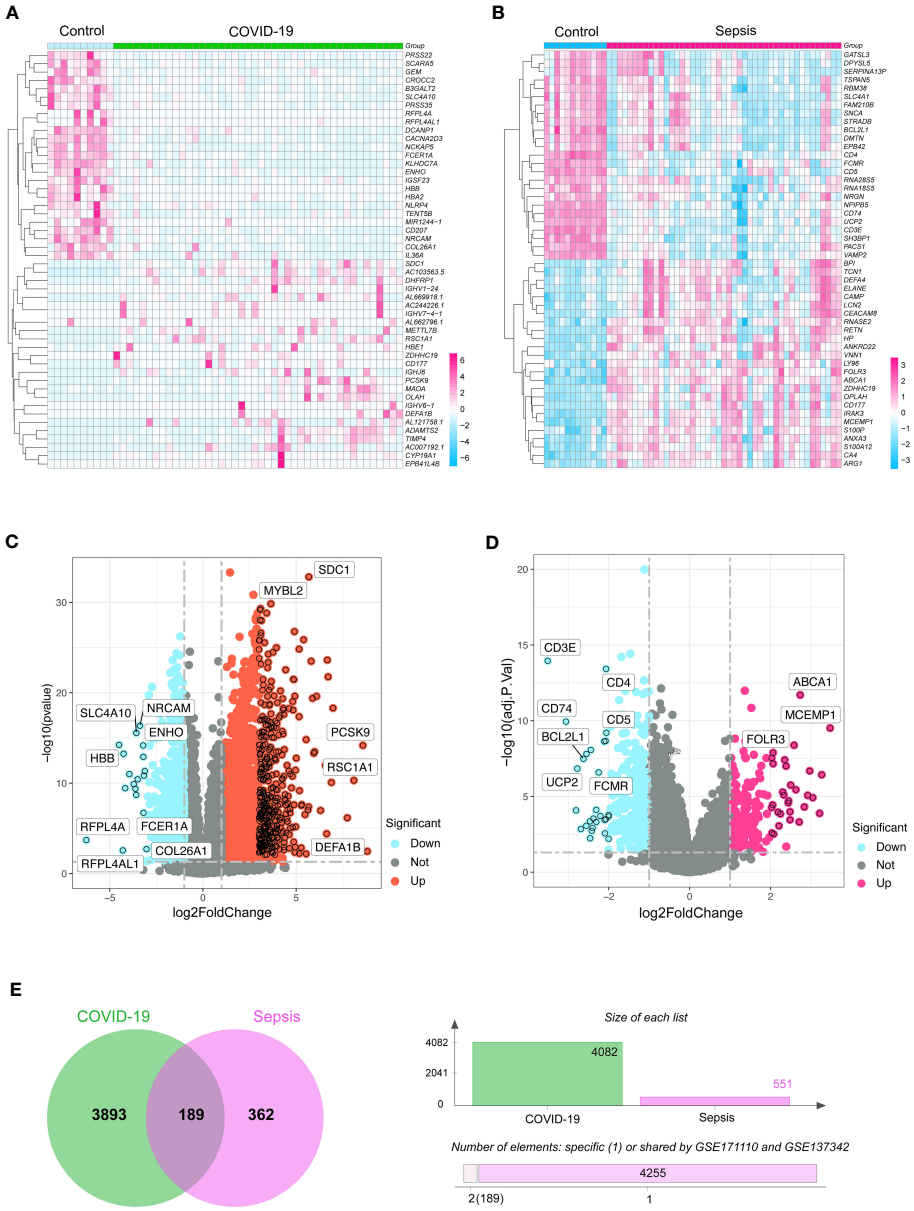

FIGURE 1
The heatmaps exhibit differentially expressed genes (DGEs) for **(A)** COVID-19 (GSE171110) and **(B)** sepsis (GSE137342) cases. Volcano diagrams represent the DGEs for **(C)** COVID-19 and **(D)** sepsis. The DEGs were considered significant if they met the cutoff criteria (adjusted *P-value* < 0.05 and |logFC| ≥ 1.0). **(E)** A Venn chart highlights the overlapping DGEs in both COVID-19 and sepsis situations.

MMP-9, LCN2, and RETN were positively correlated with KC (Figure 5B), suggesting that these candidate hub genes may regulate geriatric sepsis-induced ARDS by modulating the recruitment of neutrophils to the lung.

scRNA-seq of PBMCs from elderly patients with sepsis-induced ARDS

In order to provide additional support for the involvement of hub genes in the development of sepsis-induced ARDS among elderly individuals, we executed scRNA-seq on PBMCs procured from geriatric patients diagnosed with sepsis-induced ARDS. Drawing upon the insights gleaned from our murine sepsis-induced ARDS model and human subjects afflicted with sepsis-induced ARDS, we selected CD247, CD2, CD40LG, KLRB1, LCN2, and RETN as the foci of our subsequent investigation. Our findings revealed that CD247, CD2, and KLRB1 were predominantly expressed in natural killer (NK) cells, CD4+ T cells, and CD8+ T cells, respectively; CD40LG was chiefly expressed in CD4+ T cells; LCN2 was primarily expressed in monocytes; and RETN was principally expressed in monocytes and DCs (Figures 6A, S4). Additionally, we detected elevated expression of CD247 in NK cells, CD4+ T cells, and CD8+ T cells on Day 7 (recovery phase) in comparison to Day 1 (onset phase). CD2 expression was heightened in CD8+ T cells on Day 7 relative to Day 1. CD40LG expression demonstrated a decline in CD8+ T cells on Day 7 compared to Day 1.

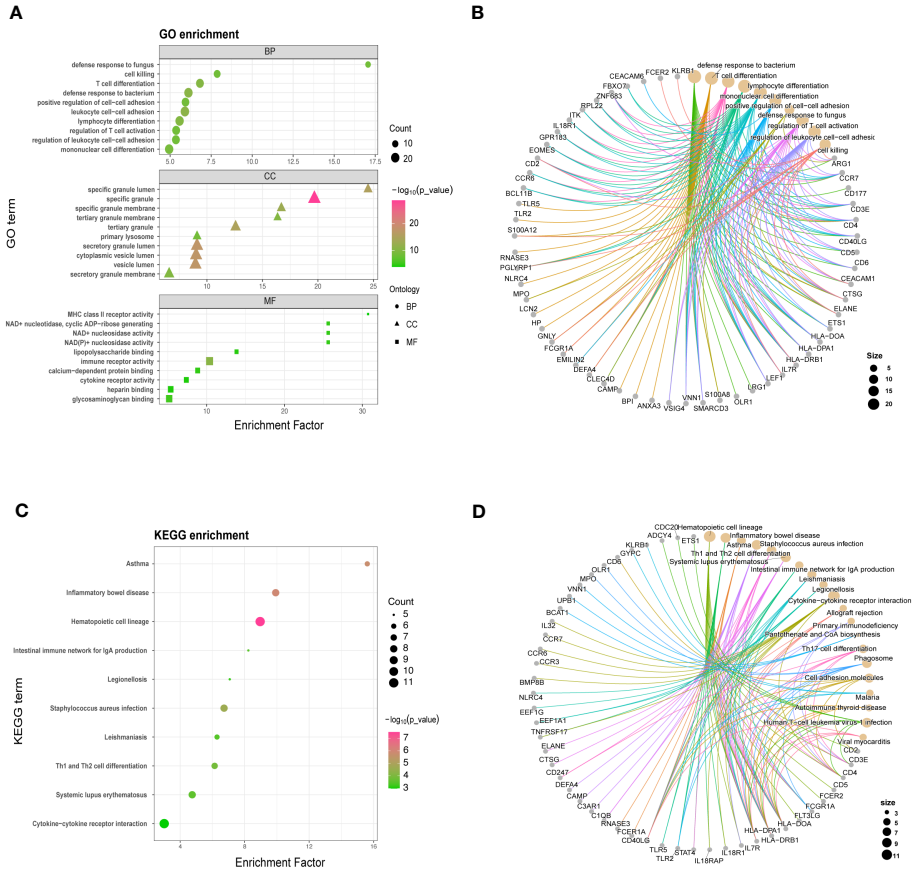

FIGURE 2
GO **(A, B)** and KEGG analysis **(C, D)** for shared differentially expressed genes in COVID-19 and sepsis. GO and KEGG pathway analyses were conducted, prioritizing significant functional items and pathways based on a standardized metric ($P < 0.05$, $Q < 0.25$).

KLRB1 expression exhibited an increase in NK cells and CD8+ T cells and a decrease in CD4+ T cells on Day 7 relative to Day 1. LCN2 expression witnessed an augmentation in monocytes on Day 7 in comparison to Day 1. Lastly, RETN expression manifested a reduction in monocytes and DCs on Day 7 as opposed to Day 1 (Figure 6B). These findings indicate that the six identified hub genes could be closely related to the development of sepsis-induced ARDS in older individuals, with their expression patterns and alterations potentially having a crucial impact on disease progression and recovery.

Immune infiltration analysis

To delve deeper into the potential roles of the discovered hub genes in developing COVID-19 and sepsis, we examined the proportions of immune cells and their associations with these hub genes in patients affected by COVID-19 and sepsis. Our results revealed that memory B cells, CD8+ T cells, and CD4+ memory resting T cells were reduced in COVID-19, while plasma cells, CD4+ memory activated T cells, and neutrophils were increased when compared to healthy controls (Figures 7A, B). Similarly, memory B cells, CD8+ T cells, CD4+ memory resting T cells, and resting NK cells were decreased in sepsis, while gamma delta T cells, monocytes, M0 macrophages, activated DCs, and resting mast cells were increased when compared to healthy controls (Figures 7C, D). Neutrophil levels were also elevated in sepsis, although not to a statistically significant extent. Furthermore, our analysis showed strong associations between multiple immune cell types, including memory B cells, CD8+ T cells, CD4+ memory resting T cells, and neutrophils, in both COVID-19 and sepsis (Figures 7E, F). Specifically, in COVID-19, memory B cells, CD8+ T cells, and CD4+ memory resting T cells were negatively correlated with neutrophils (Figure 7E). In sepsis, CD8+ T cells and gamma delta T cells were negatively correlated with neutrophils (Figure 7F).

Subsequently, we employed the Pearson correlation coefficient to assess the association between immune cell abundance and hub gene expression in both COVID-19 and sepsis cases. Results revealed a negative correlation between neutrophils and CD247, CD2, and KLRB1, and a positive correlation with RETN in both COVID-19 and sepsis (Figures 8A, B). LCN2 was positively correlated with neutrophils in COVID-19 but not in sepsis cases (Figures 8A, B). In sepsis, monocytes were negatively correlated with CD247, CD2, and CD40LG, while positively correlated with LCN2 and RETN (Figure 8B). This correlation was weaker in COVID-19. CD247, CD2, CD40LG, and KLRB1 displayed a positive correlation with CD8+ T cells and CD4+ T cells in both COVID-19 and sepsis cases, while LCN2 and RETN showed a negative correlation (Figures 8A, B). These results demonstrate the key relationships between immune cells and hub gene expressions,

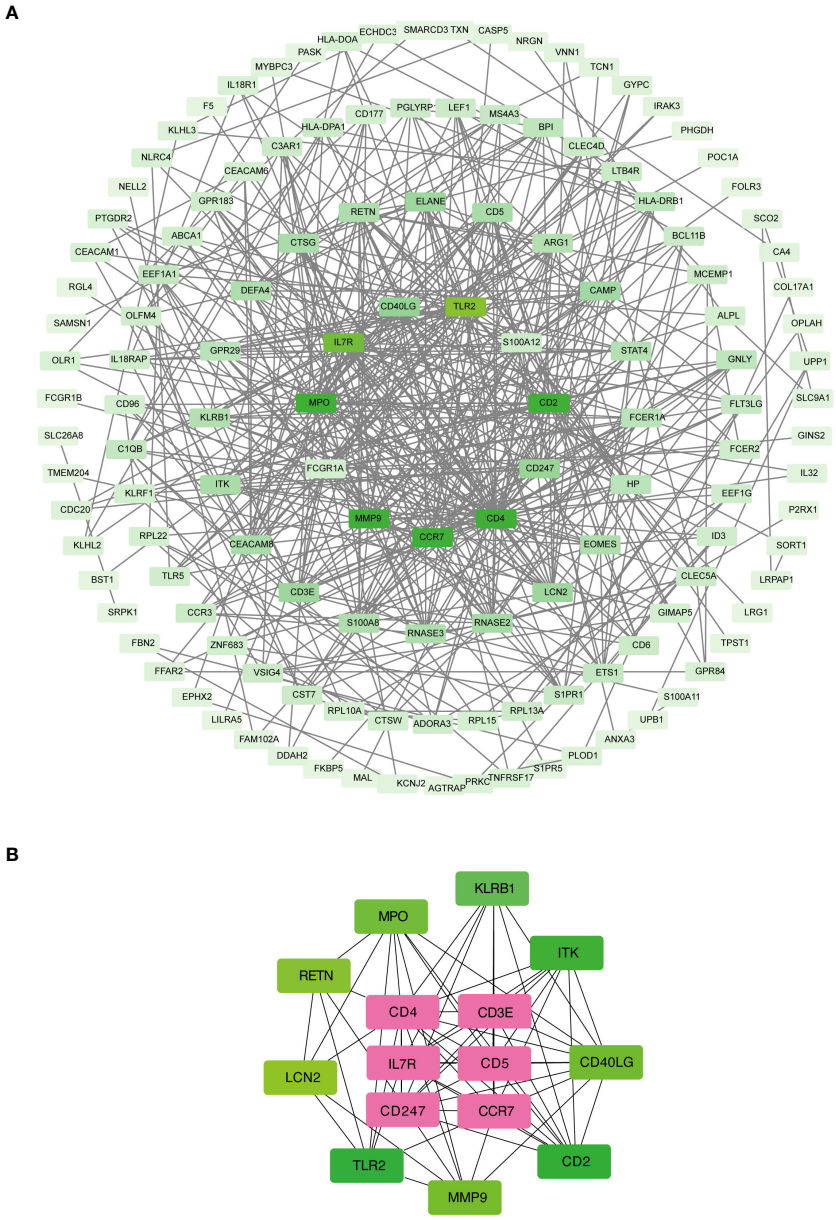

FIGURE 3
Protein-protein interaction network **(A)** and hub genes **(B)** of shared differentially expressed genes in both COVID-19 and sepsis cases. This network underwent processing and analysis in Cytoscape. Prominent nodes within the network modules were predicted using the CytoHubba plugin to identify hub genes.

highlighting differences and similarities in immune responses across COVID-19 and sepsis-induced ARDS cases.

Network-based analysis of transcriptional and post-transcriptional regulators

To elucidate the regulatory molecules controlling the identified hub genes (including CD4, CD3E, IL7R, CD5, CD247, CD2, CCR7, CD40LG, ITK, KLRB1, MPO, MMP9, TLR2, LCN2, and RETN) at the transcriptional level, we utilized a network-based approach to identify the key TFs and miRNAs involved. The interaction between TF regulators and hub genes is depicted in Figure 9, while Figure 10 shows the interactions of miRNA regulators with the hub genes. Our analysis revealed a total of 62 TFs and 125 miRNAs that are potentially involved in regulating the hug genes, indicating a significant interplay between them. For more detailed information, Tables S5, S6 provide the regulatory network of target TF-genes and target miRNA-genes, as well as the topology table.

Identification of potential therapeutic drug molecules

Protein-drug interaction analysis is critical for understanding the structural features of receptor sensitivity, which can aid in discovering new drugs (29). To identify potential therapeutic drugs for COVID-19, sepsis, and geriatric sepsis-ARDS, we focused on the hub genes

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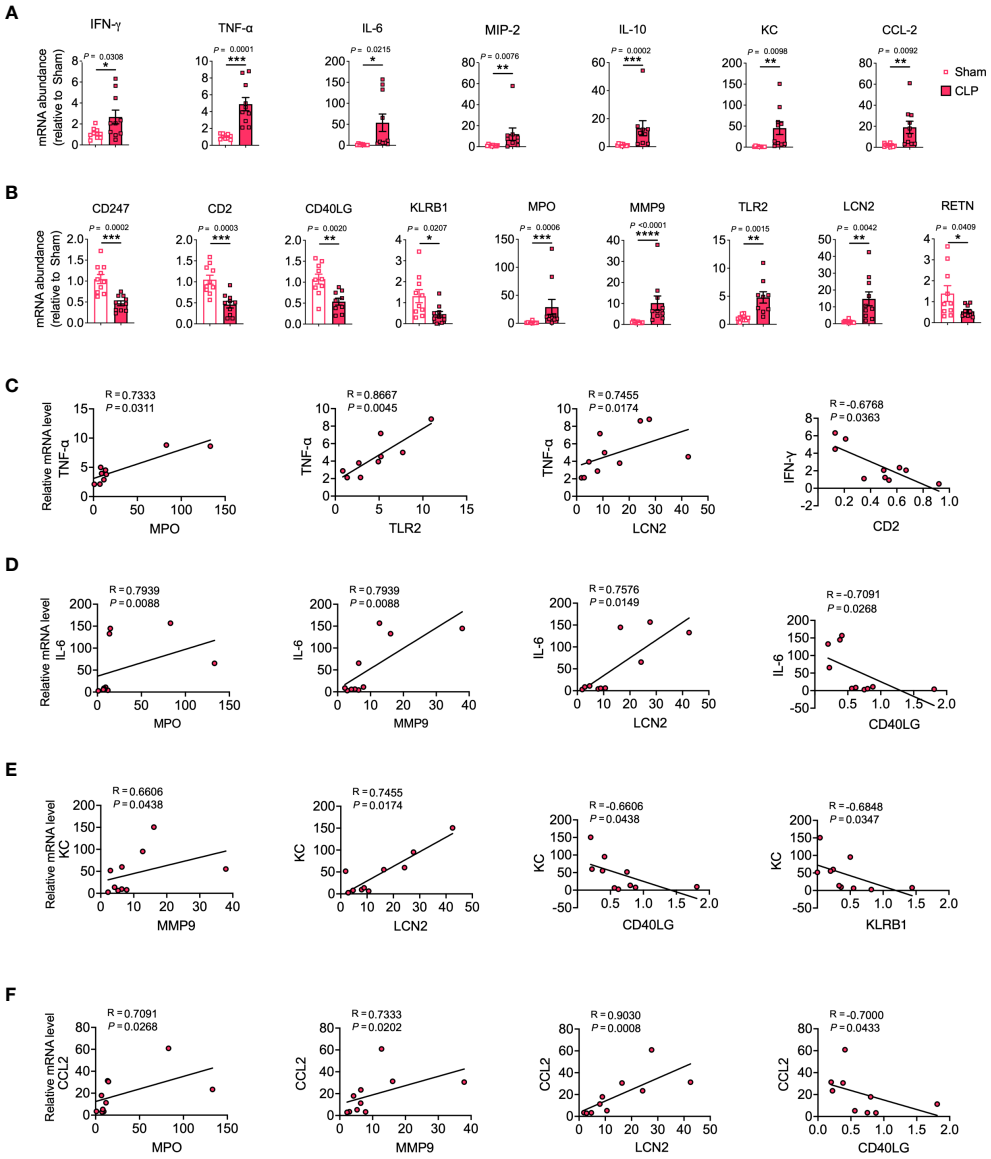

FIGURE 4
Validation of hub genes through a mouse model of sepsis-induced ARDS. mRNA expression levels of cytokines, chemokines **(A)**, and hub genes **(B)** were assessed in lung tissue homogenates. **(C–F)** Relevant scatterplots were created to study the relationships between mRNA expression levels of hub genes and TNF-α, IFN-γ, IL-6, KC, and CCL2 in the lungs using Spearman's rank correlation. **(A–F)** Data from two experiments were combined, with a total of n = 10 mice. **(A, B)** Student's t-test.

common to these diseases. By utilizing the Enrichr tool and analyzing transcriptional characteristics from the DSigDB database, we identified ten candidate compounds. These drugs were selected based on their P-values and are listed in Table 1. Our findings suggest that these compounds may have promising therapeutic effects and could serve as the basis for developing new treatment options for these diseases.

Identification of disease association

The relationship between diseases can be established based on shared genetic factors, which is a crucial step toward developing effective treatments for various disorders (30). Using NetworkAnalyst, we thoroughly examined the associations between our identified hub genes and various disease states. Our analysis revealed that autosomal recessive predisposition, rheumatoid arthritis, ulcerative colitis, severe combined immunodeficiency, hepatomegaly, eosinophilia, COVID-19, and sepsis have the strongest connections with our reported hub genes. Figure 11 illustrates the gene-disease relationships, highlighting the potential correlations between these diseases. This emphasizes the potential impact of these diseases on each other and provides valuable insight into their complex relationships.

Discussion

The COVID-19 pandemic and sepsis represent significant public health challenges. The close association between COVID-

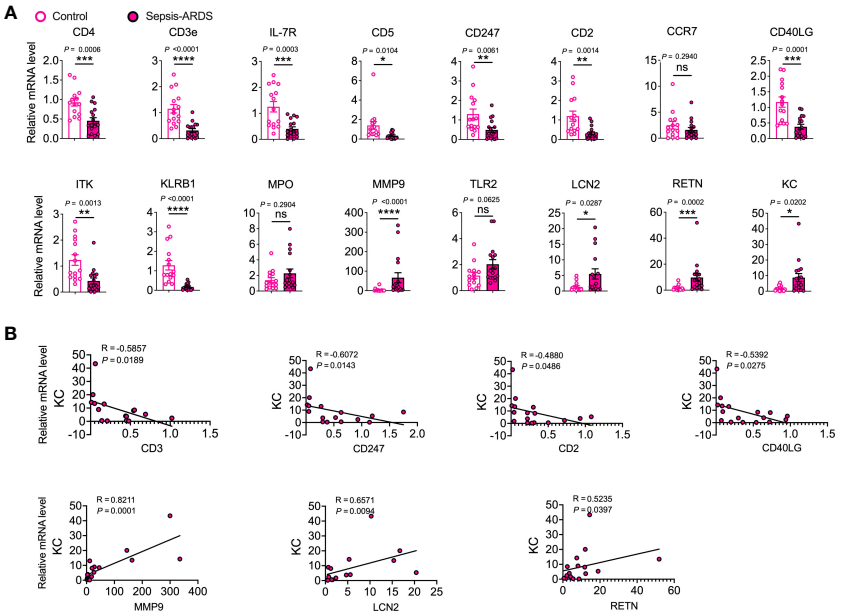

FIGURE 5
Validation of hub genes in elderly sepsis-induced ARDS. **(A)** mRNA expression levels of the identified hub genes and chemokine (KC) were evaluated in PBMCs. *n* = 15–17. Student's *t*-test and Mann-Whitney test. **(B)** Appropriate scatterplots were generated to investigate the association between mRNA expression levels of hub genes and KC in PBMCs utilizing Spearman's rank correlation coefficient (R).

19 and sepsis is well-established, despite a limited understanding of the underlying molecular pathways (5, 27). Studies indicate that the severity of both COVID-19 and sepsis are mutually influenced, underscoring the need to understand the association between these conditions (2, 27, 31). Both diseases can cause severe symptoms, including ARDS, organ damage, immune system dysregulation, and long-term complications. Recent research indicates a genetic correlation between COVID-19 and sepsis, supporting that they share a common underlying biological mechanism (31, 32). Older adults are particularly vulnerable to COVID-19 and sepsis, experiencing higher rates of complications and case fatality (3, 6). Therefore, understanding the association between these conditions is crucial for improving treatment strategies, especially for geriatric patients with sepsis-induced ARDS.

Identifying common genetic pathways between COVID-19 and sepsis provides valuable insights into the underlying mechanisms of both illnesses. Our study identified 189 common DEGs in COVID-19 and sepsis, significantly enriched in defense response to fungus, specific granule lumen, MHC class II receptor activity, and various signaling pathways. Fungal infections are a risk factor for sepsis and COVID-19, and gene expression differences in sepsis are involved in the defense response to fungus (33). The lumen of specific granules, predominantly present in mature neutrophils, may hold considerable importance in modulating COVID-19 and sepsis (32), while MHC class II receptors instigate the immune response and serve as a vaccine target (34). Manipulation of MHC class II expression or signaling presents a potential therapeutic strategy for ameliorating outcomes in COVID-19 and sepsis. Further inquiry is merited to explicate the direct association between specific granule lumen/MHC class II receptor activity and these afflictions, as well as to corroborate the prospective therapeutic approaches intimated by our findings.

KEGG pathway analysis revealed several shared pathways between sepsis and COVID-19, including asthma, inflammatory bowel disease (IBD), hematopoietic cell lineage, intestinal immune network for IgA production, legionellosis, staphylococcus aureus infection, leishmaniasis, Th1 and Th2 cell differentiation, systemic lupus erythematosus, and cytokine-cytokine receptor interactions. We and others have shown a strong relationship between COVID-19 and asthma, and further connections have been established between COVID-19, asthma, and legionellosis (35, 36). IBD, COVID-19, and sepsis share a link to the gut microbiota, which is a critical factor in regulating the host's susceptibility to SARS-CoV-2 infection and clearance (37, 38). The gut microbiota also impacts the host's response and tolerance to treatment drugs for IBD and sepsis (38, 39). Identifying and understanding these common pathways can provide valuable insights into potential therapeutic targets and strategies for COVID-19 and sepsis management.

We have identified top hub genes (CD4, CD3E, IL7R, CD5, CD247, CD2, CCR7, CD40LG, ITK, KLRB1, MPO, MMP9, TLR2, LCN2, and RETN) associated with both sepsis and COVID-19. These hub genes may serve as important therapeutic targets or biomarkers for both diseases. In particular, the upregulation of MPO, MMP9, TLR2, and LCN2 in the lungs of sepsis-induced ARDS mice suggests their crucial role in developing lung injury in COVID-19 and sepsis. Positive correlations between MPO, TLR2, LCN2, TNF-α, and IL-6 also suggest their potential as targets for reducing inflammation and lung injury in these diseases. TLR2 has been shown to sense the SARS-CoV-2 envelope protein, leading to

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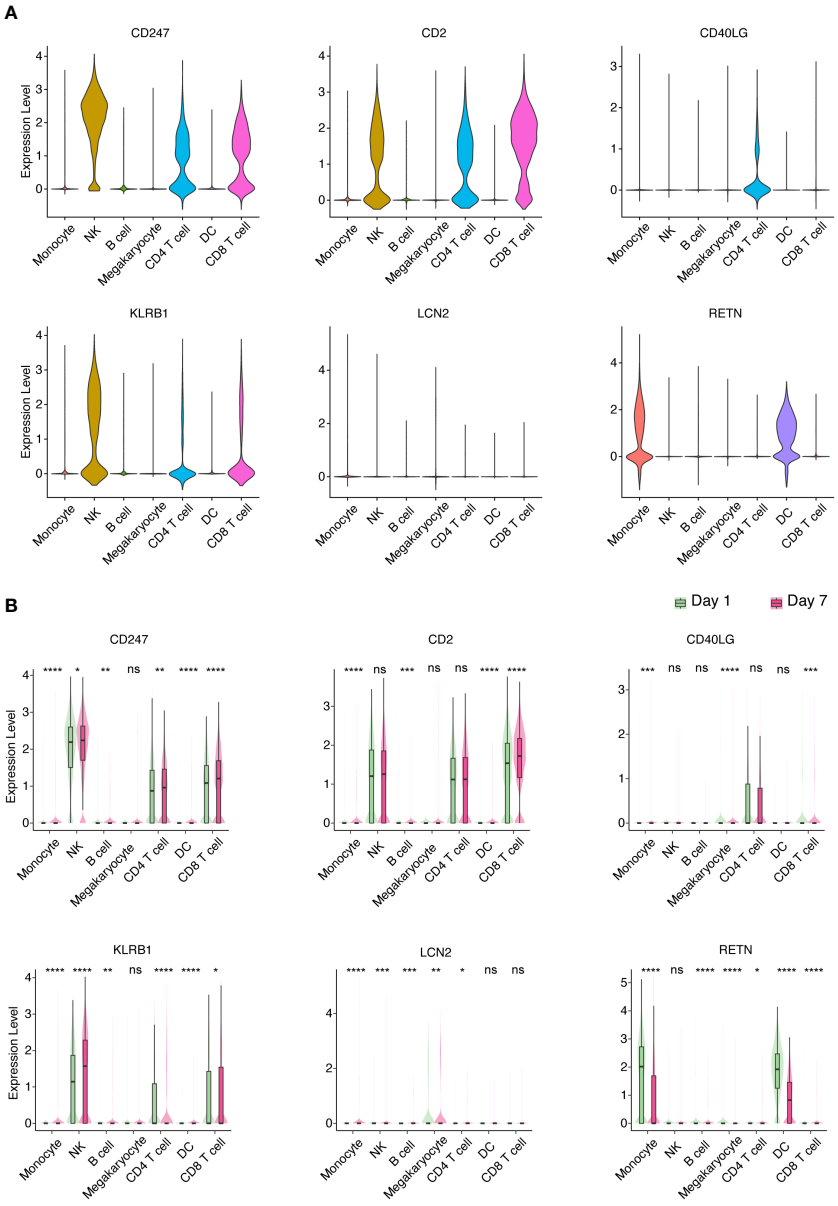

FIGURE 6
Identification of key PBMC populations expressing hub genes in aged sepsis-induced ARDS. **(A)** Detection of the primary PBMC cell population expressing hub genes in patients with elderly ARDS using scRNA-seq. **(B)** Differential expression of selected hub genes in various PBMC cell populations throughout disease progression. **(A, B)** Student's t-test and Mann-Whitney test.

the production of inflammatory cytokines (40). In addition, MPO and LCN2 have been identified as critical genes in sepsis and sepsis-related ARDS, with LCN2 showing diagnostic value in sepsis-related ARDS (41).

In contrast, negative correlations observed between CD2 and IFN-γ, and between CD40LG and IL-6, as well as the downregulation of CD247, CD2, CD40LG, KLRB1, and RETN in the lungs of sepsis-induced ARDS mice, suggest their potential role in modulating the immune response and reducing inflammation in COVID-19 and sepsis. Bioinformatics and meta-analysis have identified CD247 as a critical gene for septic shock, making it a promising candidate for becoming a new biomarker for this condition (42).

Moreover, in elderly sepsis-induced ARDS patients compared to controls, we observed a significant decrease in CD4, CD3e, IL-7R, CD5, CD247, CD2, CD40LG, ITK, and KLRB1 gene expression levels, and a substantial elevation of MMP9, LCN2, and RETN, indicating their potential as targeted biomarkers for predicting COVID-19 and sepsis-induced ARDS in elderly patients. The negative correlations between CD3, CD247, CD2, CD40LG, and the positive correlations between MMP-9 and LCN2, and RETN with KC suggest their potential role in regulating neutrophil recruitment to the lung and modulating lung injury in these diseases. LCN2 and RETN are proteins produced by neutrophils and stored in their secondary granules, which are released upon neutrophil activation (43).

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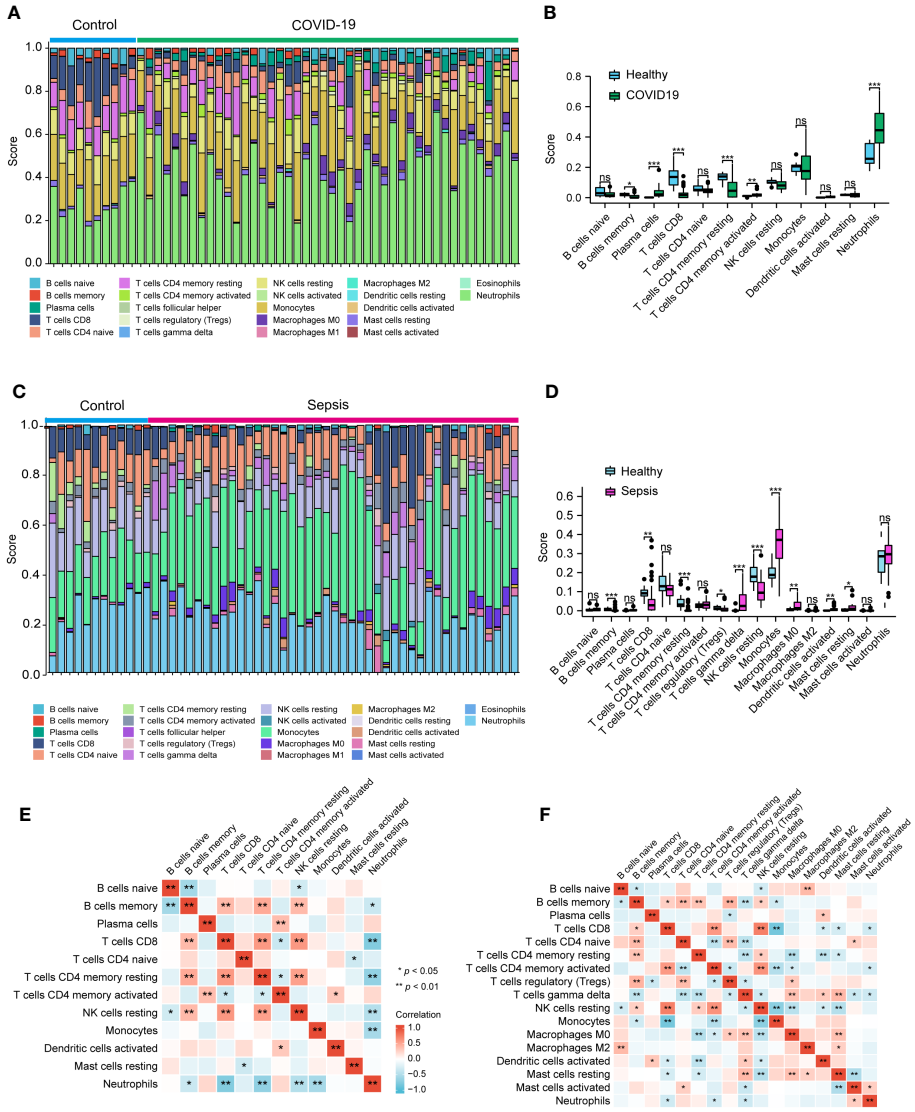

FIGURE 7
Analysis of the immune infiltration levels in the COVID-19 and sepsis. The ratio of immune cells in COVID-19 **(A, B)** and sepsis **(C, D)** were analyzed using CIBERSORT. The relationship between each immune cell for COVID-19 **(E)** and Sepsis **(F)**. **(B, D)** Student's t-test. **(E, F)** Spearman.

In the multifaceted pathogenesis of COVID-19 and sepsis-induced ARDS, the uncontrolled activation and subsequent degranulation of neutrophils are central (44, 45). These key cells in the innate immune response can, when dysregulated, release excessive granules containing proteolytic enzymes and reactive oxygen species, leading to substantial cytotoxicity (45, 46). This process damages the lung's alveolar epithelial cells and disrupts the extracellular matrix, contributing to endothelial barrier dysfunction (46). Simultaneously, the release of inflammatory mediators like cytokines and chemokines can amplify the inflammatory response into a "cytokine storm," potentially causing systemic inflammation that affects multiple organs (28, 44, 45, 47). Hence, our findings offer new insights into the molecular mechanisms underlying COVID-19 and sepsis-induced ARDS and suggest CD3, CD247, CD2, CD40LG MMP-9, LCN2, and RETN as the potential targets for neutrophils in developing novel therapies and diagnostic tools for these diseases.

By conducting scRNA-seq of PBMCs from elderly patients with sepsis-induced ARDS, we obtained further evidence for the role of hub genes in the pathogenesis of these conditions. The expression patterns and changes of these genes may play a crucial role in the disease's progression and recovery. Moreover, the analysis of immune infiltration indicated changes in immune cell proportions in COVID-19 and sepsis compared to healthy controls, with strong associations found between multiple immune cell types and hub genes. These findings provide valuable insights into the relationships between immune cells and hub gene expressions, highlighting similarities and differences in immune responses across COVID-19 and sepsis-induced ARDS cases. However, further research is necessary to validate the potential of these identified hub genes as targeted biomarkers for these conditions.

At the molecular level, we observed significant connections between COVID-19 and sepsis, particularly in the context of TFs

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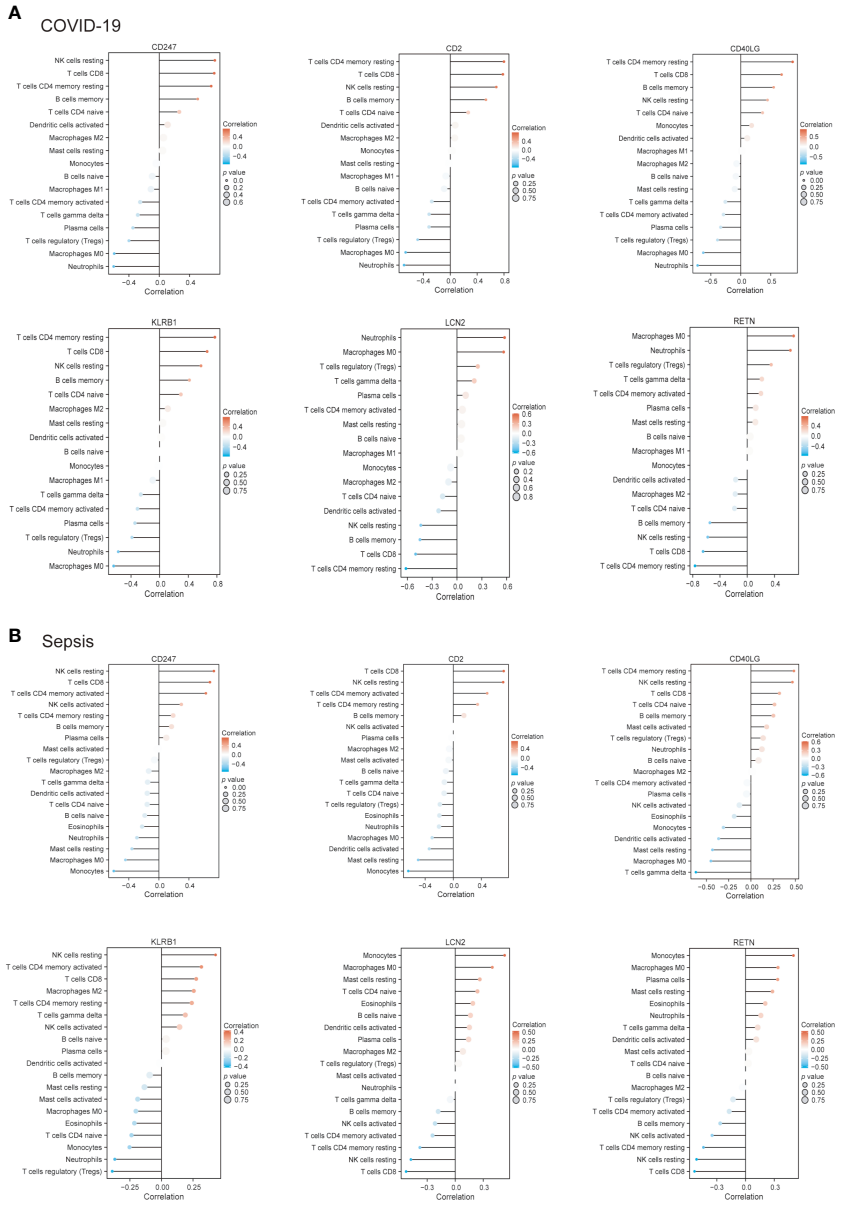

FIGURE 8
The connection between immune cells and hub genes in COVID-19 **(A)** and sepsis **(B)**. The association between the two factors was assessed employing Spearman's rank correlation coefficient.

and miRNAs. Both TFs and miRNAs play crucial roles in regulating gene expression, with TFs modulating mRNA expression and miRNAs acting post-transcriptionally via RNA silencing (30, 35). The identified TFs, including GATA3, STAT1, IRF2, NFKB1, RELA, and FOXC1, and miRNAs, such as hsa-mir-335-5p, hsa-mir-4505, hsa-mir-143-3p, hsa-miR-26b-5p, and hsa-miR-146a-5p, are associated with a range of respiratory diseases, including asthma, ARDS, and pulmonary fibrosis, as well as the pathogenesis and exacerbation of sepsis and COVID-19 (32, 35, 48–50). Intriguingly, many of these miRNAs have also been implicated in various types of cancer, including lung and gastric cancer (32, 35, 51).

Our gene-disease analysis further revealed relationships between identified hub genes and various disorders, including COVID-19 and sepsis. A notable finding is the identification of several genes related to severe combined immunodeficiency (SCID). Patients with SCID may be more susceptible to COVID-19, highlighting the need for additional preventive and therapeutic measures for this vulnerable population (52). Due to limited data, the safety and efficacy of COVID-19 vaccines for SCID patients remain uncertain. Moreover, our results suggest that individuals with COVID-19 and sepsis may also be affected by other disorders such as autosomal recessive predisposition, rheumatoid arthritis, ulcerative colitis, hepatomegaly, and eosinophilia.

We have also identified potential drug molecules with therapeutic value for COVID-19, sepsis, and geriatric sepsis-induced ARDS. Among them are several compounds with immunomodulatory effects, such as Etynodiol, Glycoprotein, and

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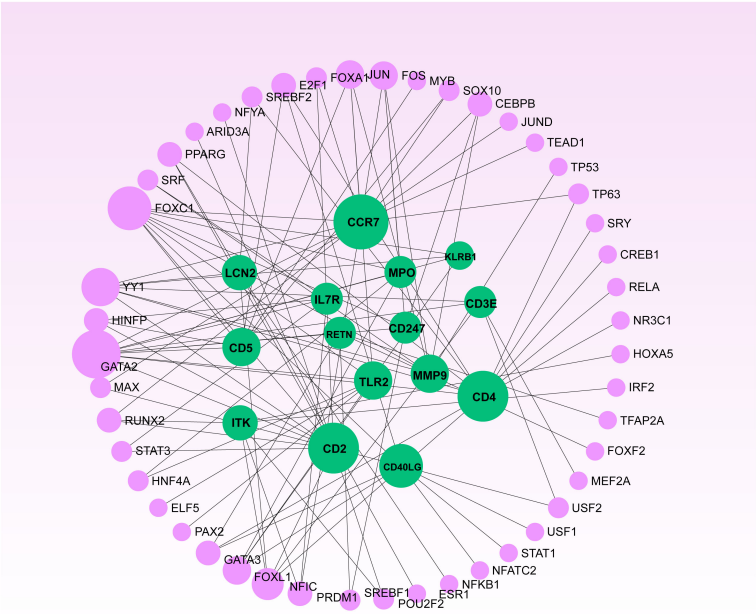

FIGURE 9
The Network Analyst produced a regulatory interaction network linking DEGs and Transcription Factors (TFs). In this network, purple nodes represent TFs, while green nodes illustrate the connections between gene symbols and TFs.

Vitamin D3, which may represent promising treatment options by modulating the immune response in affected patients. Diphenylpyraline, an antihistamine drug, may offer a therapeutic effect on COVID-19 and sepsis by affecting cytokine transport and release. Corticosteroids, including Alclometasone, Isoflupredone, and Fludroxycortide, commonly used for skin inflammation (53), can potentially suppress an overactive immune system in COVID-19 or sepsis, thereby reducing inflammation and mortality risk. It is important to note, however, that not all corticosteroids are suitable for these patients, and the World Health Organization currently recommends dexamethasone or hydrocortisone for severe or critical patients with COVID-19. While Alclometasone, Isoflupredone, and Fludroxycortide may hold promise as treatments for COVID-19 and sepsis, there is insufficient

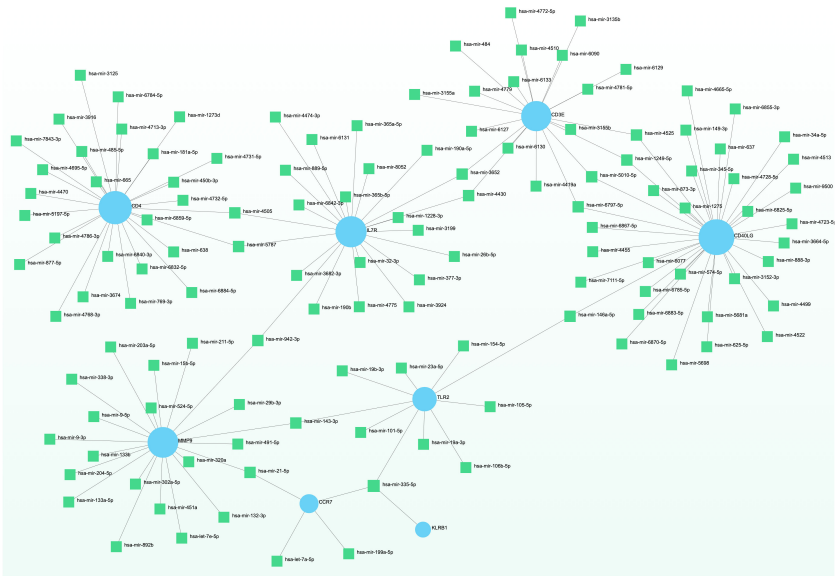

FIGURE 10
The displayed regulatory interaction network emphasizes the interconnectivity of DEGs and microRNAs. Within this network, blue circular nodes symbolize genes that interact with microRNAs.

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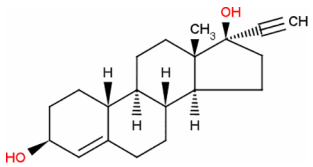
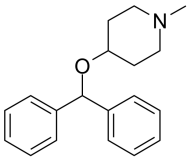
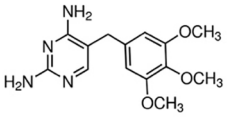
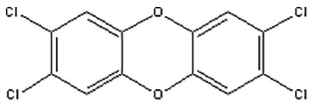
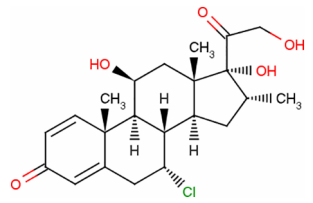
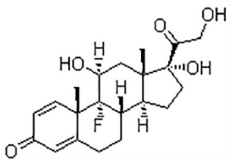
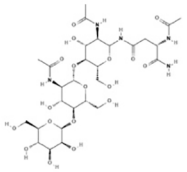
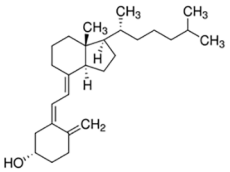
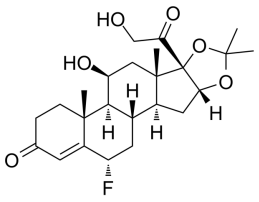
TABLE 1 The recommended medications.

Name	*P*-value	Chemical Formula	Structure
Etynodiol	3.70E-10	$C_{20}H_{28}O_2$	
Diphenylpyraline	6.99E-08	$C_{19}H_{23}NO$	
Trimethoprim	2.83E-07	$C_{14}H_{18}N_4O_3$	
Tetradioxin	6.23E-07	$C_{12}H_4Cl_4O_2$	
Alclometasone	7.09E-7	$C_{22}H_{29}ClO_5$	
Isoflupredone	1.41E-6	$C_{21}H_{27}FO_5$	
Glycoprotein	1.91E-06	$C_{28}H_{47}N_5O_{18}$	
Vitamin D3	3.81E-06	$C_{27}H_{44}O$	
Fludroxycortide	5.88E-06	$C_{24}H_{33}FO_6$	

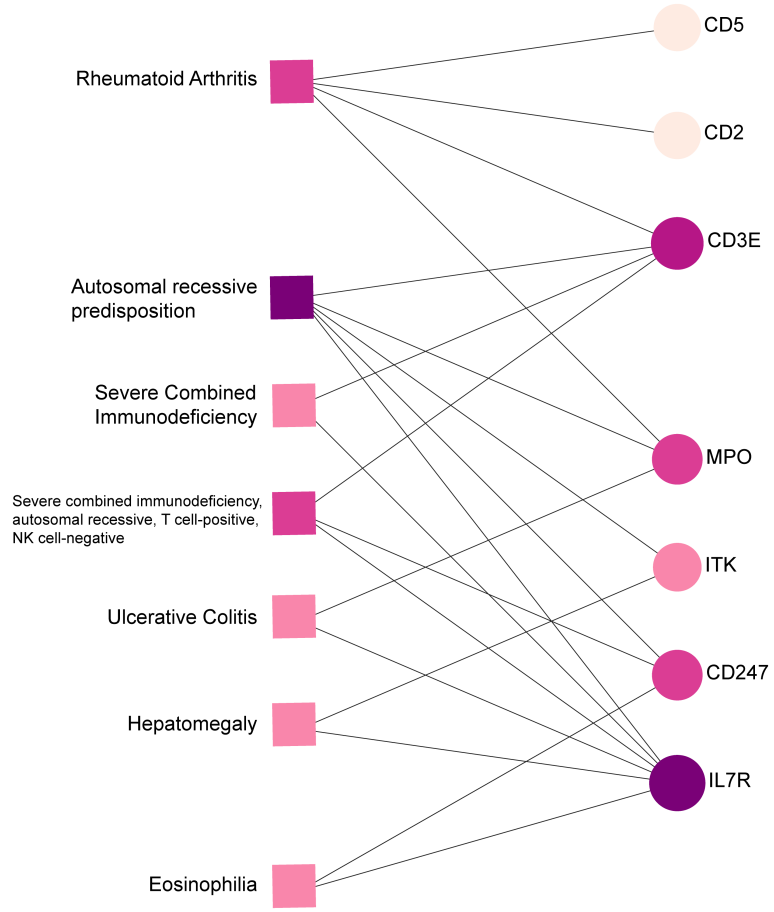


FIGURE 11
Gene-disease connection network. Square nodes symbolize diseases, and circular nodes indicate gene symbols interacting with the associated disease.

evidence to support their efficacy in these conditions. Further research is necessary to determine their effectiveness and safety in treating these diseases.

While revealing preliminary insights, this study has key limitations. Our bioinformatic findings require rigorous validation through conditional knockout and pharmacological testing to move from correlation to causation and assess clinical viability. The speculative therapeutic targets need extensive experimental characterization for safety and efficacy before consideration for treatments. Additionally, our focus on expression overlooks determinants like post-translational modifications, necessitating a comprehensive systems perspective. Critically, we lacked access to facilities for SARS-CoV-2 animal models and clinical samples collection, preventing experimental validation and highlighting the need for expanded biosafety infrastructure to enable COVID-19 research. Overall, these results should be interpreted as early findings that point to future research directions rather than definitive conclusions. Our study elucidates pathways and biomarkers but requires meticulous *in vitro* and *in vivo* follow-up to transition these leads into viable diagnostic and therapeutic approaches for managing sepsis and ARDS amidst the COVID-19 pandemic.

Conclusion

In conclusion, this investigation provides preliminary insights into the possible genetic links between COVID-19, sepsis, and geriatric sepsis-induced ARDS, suggesting potential biomarkers and therapeutic targets for these complex conditions. While the study offers a foundation for exploring innovative immunomodulatory therapies and pharmaceutical compounds, it is important to recognize that these findings are initial and require extensive further research. The targeting of the identified biomarkers and the utilization of suggested drug candidates could represent a promising direction in the efforts to improve patient outcomes for COVID-19 and sepsis/sepsis-ARDS, particularly in the elderly population. However, this must be approached with caution, as further exploration and rigorous validation are necessary to confirm the safety and efficacy of these potential therapeutic interventions. Ultimately, this study contributes to our evolving understanding of the intricate interplay between the immune system, genetics, and the pathogenesis of COVID-19 and sepsis/sepsis-ARDS. It is a stepping stone rather than a definitive solution, and it highlights the need for continued, comprehensive research to pave the way for more effective and validated treatment strategies in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GSE242127 (GEO- https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE242127).

Ethics statement

The studies involving humans were approved by Ethics Committees of Zhongshan Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Committee of Animal Experiments at Guangzhou Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GQ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. HWF: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. AC: Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. ZS: Data curation, Investigation, Methodology, Writing – original draft. MH: Investigation, Methodology, Writing – original draft. ML: Investigation, Methodology, Writing – original draft. EC: Investigation, Methodology, Writing – original draft. SZ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. XW: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. HF: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1257834/full#supplementary-material

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A survey on the role of artificial intelligence in managing Long COVID

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In the last years, several techniques of artificial intelligence have been applied to data from COVID-19. In addition to the symptoms related to COVID-19, many individuals with SARS-CoV-2 infection have described various long-lasting symptoms, now termed Long COVID. In this context, artificial intelligence techniques have been utilized to analyze data from Long COVID patients in order to assist doctors and alleviate the considerable strain on care and rehabilitation facilities. In this paper, we explore the impact of the machine learning methodologies that have been applied to analyze the many aspects of Long COVID syndrome, from clinical presentation through diagnosis. We also include the text mining techniques used to extract insights and trends from large amounts of text data related to Long COVID. Finally, we critically compare the various approaches and outline the work that has to be done to create a robust artificial intelligence approach for efficient diagnosis and treatment of Long COVID.

KEYWORDS

artificial intelligence, deep learning, machine learning, Long COVID, post-acute sequelae of SARS CoV-2 infection, PASC

1 Introduction

Patients that have been infected with the SARS-CoV-2 virus can experience persistent and long-term effects known as Long COVID (Callard and Perego, 2021; Cau et al., 2022). Long COVID is known by several terms, such as post-COVID conditions, long-haul COVID, post-acute COVID-19, and the prolonged effects of COVID (Fernández-de Las-Peñas et al., 2021). Moreover, post-acute sequelae of SARS CoV-2 infection (PASC) (Pfaff et al., 2022) is also adopted as an alternative term for Long COVID.

Patients experiencing Long COVID reported multiple post-COVID symptoms affecting different organs/systems (Davis et al., 2023). Figure 1 illustrates the multiple organs on which the Long COVID has effects. The virus can also have adverse effects causing sections of the immune system to become overactive and causing damaging inflammation throughout the body (Marshall, 2020).

The variety of potential symptoms and problems encountered by patients with Long COVID highlights the need for a deeper knowledge of the condition's clinical course. The most frequently reported symptoms of Long COVID that affect different organs are described in Table 1.

The study of the prevalence, duration, and clinical outcomes of Long COVID is still under investigation (Walia et al., 2021). The scope and complexity of healthcare data require advanced analytics to derive meaningful insights from longitudinal data

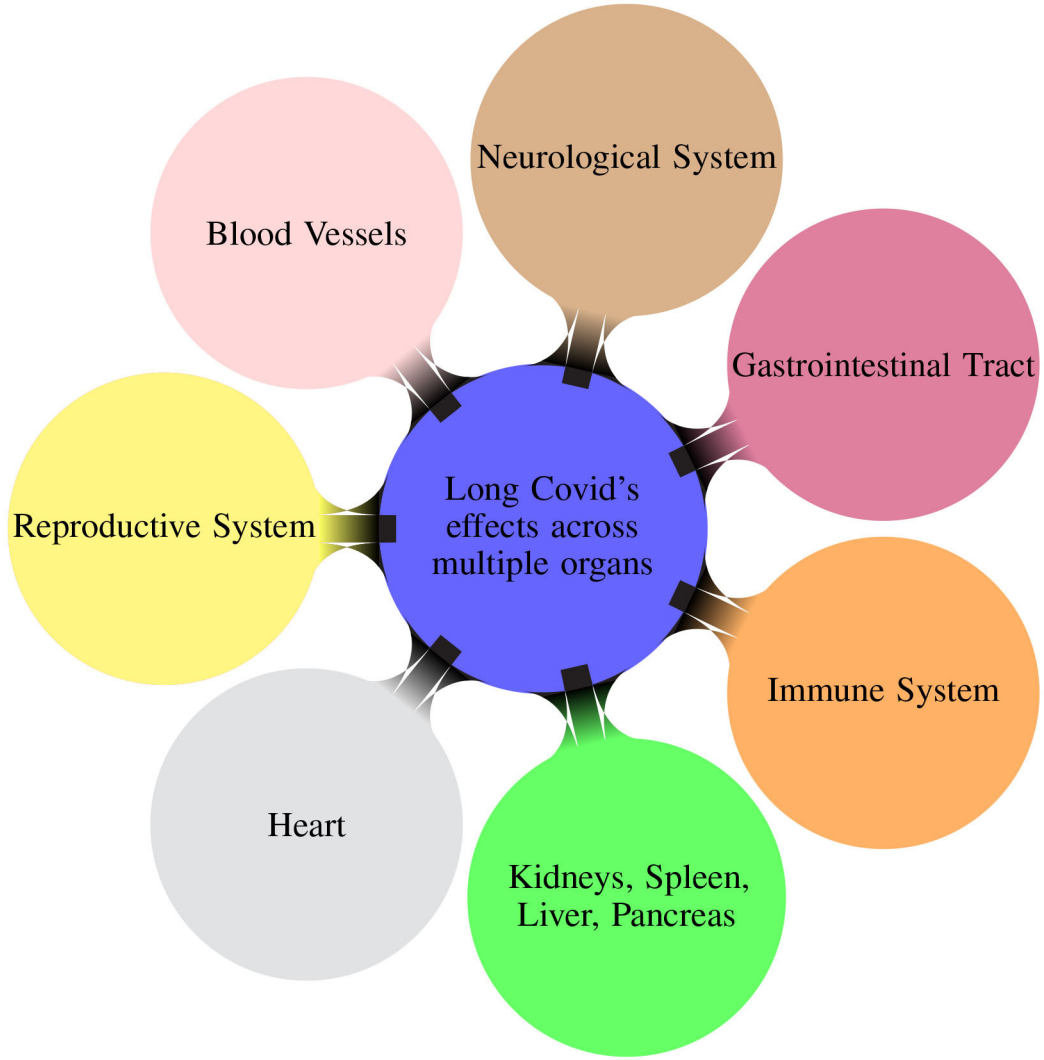

FIGURE 1
Mindmap illustrating the different organ systems on which the Long COVID has effects (Davis et al., 2023).

TABLE 1 **Overview of Long COVID's effects and diverse pathologies across multiple organs (Davis et al., 2023).**

Organ/system	Associated symptoms and pathologies
Lungs	Cough, dyspnoea, abnormal gas exchange
Heart	Chest pain, palpitations, cardiac impairment, myocardial inflammation, POTS
Kidneys, spleen, liver, pancreas	Organ injury
Immune system	Autoimmunity, MCAS
Gastrointestinal tract	Abdominal pain, nausea, gut dysbiosis, viral persistence and viral reservoir
Neurological system	Cognitive impairment, fatigue, disordered sleep, memory loss, tinnitus, dysautonomia, ME/CFS, neuroinflammation, reduced cerebral blood flow, small fiber neuropathy
Blood vessels	Fatigue, coagulopathy, deep vein thrombosis, endothelial dysfunction, microangiopathy, microclots, pulmonary embolism, stroke
Reproductive system	Erectile dysfunction, more severe and frequent premenstrual symptoms, irregular menstruation, reduced sperm count

encompassing symptoms, laboratory results, imaging, functional assessments, genomic information, data from wearable sensors, mobile health applications, clinicians' notes, and electronic health records (EHR). Artificial intelligence (AI) and machine learning (ML) techniques increasingly show the potential to bring insight into patient-level data from massive amounts of data to comprehend the effect of SARS-CoV-2 on patients. Techniques of AI have been largely exploited for analyzing COVID-19 data [see

for instance (Nayak et al., 2021)] but only a few works explore the trend patterns for Long COVID.

There is also an immediate need for enhanced care techniques that are more integrated to improve patient's clinical outcomes. These approaches would support and treat patients who have Long COVID establishing resilient healthcare systems to deliver efficient and effective responses to upcoming health challenges (Aiyegbusi et al., 2021). The deployment of AI can significantly improve everyday clinical practice (Recht et al., 2020) by answering physician inquiries concerning risk classification and the clinical outcome of COVID-19 patients. In fact, clinicians are faced with limited options as the existing diagnostic tools and therapeutics for Long COVID are still in the experimental stage, while early diagnosis and treatment would be crucial for improving patient outcomes. In this context, AI approaches would be helpful to automatize complex tasks that could hardly be produced manually.

In this survey, we consider data coming from symptoms, laboratory data and data from EHR on Long COVID. We specifically collect and analyze papers using AI techniques applied to Long COVID.

In the first part of the survey, we analyze ML techniques, such as Extreme Gradient Boosting (XGBoost), random forest, and Convolutional Neural Network (CNN), for predicting the prevalence of Long COVID and identifying the associated risk factors. Most of the papers in the literature perform a binary classification, and only a few works deal with predicting risk factors using regression methods, identifying blood proteins for Long COVID detection, and deriving Long COVID subphenotypes. The datasets employed comprise COVID-19 datasets, such as the National COVID Cohort Collaborative's (N3C) repository (Haendel et al., 2021), collections of surveys and health administrative data, laboratory data and patients' demographics comorbidities.

In the second part of the survey, we analyze natural language processing (NLP) approaches on textual data discussing Long COVID. In most cases, data are collected from Twitter, blogs, and clinical notes. Adopted approaches are mainly based on BERT models, topic modeling techniques, and association rule mining. The aim in these cases is to identify Long COVID symptoms and their co-occurrences.

In both cases, we focus only on papers that utilized AI techniques, including ML and NLP, to elaborate on the Long COVID data, discharging the considerable amount of work where basic statistics or other different models are exploited.

From the task point of view, we report and analyze different systems with the aim to: (i) identify and predict Long COVID from patients diagnosed as COVID-19 positive, (ii) predict the risk of developing different pathologies for patients who manifested COVID-19, and the potential long-term consequences of the emergence of the coronavirus, (iii) determine the associations between risk factors and Long COVID, (iv) distinguish between short and long COVID-19, (v) explore the characteristics, patterns and behavior of Long COVID symptoms, (vi) study the Long COVID course of the disease and evolution over time, and (vii) identify Long COVID symptom co-occurrences, topics of discussion about Long COVID, patient profiles and the challenges faced during treatment.

We gathered a total of 20 papers from the literature, with 13 of them focusing on ML techniques and the remaining seven on text mining applied to data related to Long COVID. We describe the individual contribution of each paper, the data and techniques adopted, and the results obtained, mainly in terms of accuracy, precision, recall, F1-score and AUC (Area Under the ROC Curve).

We critically analyze the different approaches and the results obtained, both in the ML and NLP categories, and also compare the two categories in terms of used datasets, methodologies, and obtained results. We show the current limitations of the approaches in the literature and outline future work directions in terms of AI methodologies and Long COVID target.

To the extent of our understanding, this is the first survey reviewing AI methodologies applied to Long COVID data.

2 The complexity of the Long COVID condition

Managing Long COVID is a complex issue, and the lack of effective pharmacological therapies and data to advise healthcare practitioners reflects this task's difficulty.

Long COVID has many different complications concerning manifestations, duration, and treatment. Diagnosing Long COVID can be difficult because of its wide variety of symptoms and its comorbidity with other illnesses. The development of precise diagnostic criteria is still ongoing. Establishing a worldwide standard for defining post-COVID-19 conditions is poised to enhance advocacy and research efforts significantly. However, this definition will likely undergo modifications in response to emerging evidence and the evolution of our comprehension of COVID-19's long-term effects. The Long COVID clinical definition was painstakingly crafted utilizing the exhaustive Delphi consensus approach. This approach relied on selecting relevant domains and variables for inclusion, as reflected in the WHO's ICD-10 diagnosis code U09. The process ensured the involvement and input of diverse stakeholders to ensure a well-rounded and inclusive understanding (Soriano et al., 2022). Long COVID solidified through patient-led surveys, self-appellation, case studies, and hashtag circulation. After patients, several new players and some typical scientific actors appear (Callard and Perego, 2021). Between December 2019 and May 2020, Davis et al. (2021) surveyed patients via an online questionnaire about their experiences with Long COVID symptoms, focusing on recovery and return to baseline from neurological and neuropsychiatric symptoms, including work impact.

Long COVID complexity and ongoing efforts to gather and prepare data make it an essential ground for multimodal ML techniques. Combining clinical and EHR data in Long COVID such as the National Institutes of Health (NIH) research initiative, MIDRC-N3C interoperability, pathology, wearable sensor data, imaging, and ML can help understand underlying physiology, explain heterogeneity, and identify therapeutic targets (Chen et al., 2023). The multimodal ML approaches' clinical usefulness depends on targeting the right clinical question, particularly Long COVID development, shared pathways, and response to treatment approaches.

3 Literature review

To discover relevant publications, we collected studies and data from different sources (PubMed, Scopus, WoS, MedRxiv, ArXiv). Identifying relevant papers was not a trivial task (Lever and Altman, 2021; Langnickel et al., 2022; Leaman et al., 2023).

In particular, we have considered, besides the basic term "Long COVID", also multiple synonyms: "Post-COVID conditions", "long-haul COVID", "post-acute COVID", "long-term effects of COVID", "chronic COVID", and also "post-acute sequelae of SARS CoV-2 infection" and "PASC" which refer to a subset of Long COVID cases. These terms have been used in combination with "artificial intelligence", "machine learning", "deep learning", "natural language processing", "NLP", and "text mining".

The search yielded substantial literature (121 papers selected from PubMed, Scopus, WoS, including only two papers from MedRxiv), including research articles, review articles, case studies, and reports. Among these articles, we selected all the contents pertaining to the application of AI methods to Long COVID data, and ended up with the 20 papers. The criteria used to select the papers were:

- relevance of the topic: we selected only papers with an innovative approach in the realm of AI. Accordingly, we discharged papers with basic statistic analysis;
- completeness and significance of the results: we selected the papers where AI is used to achieve some important result, removing those papers where AI was only discussed and not a clear result was obtained;
- publication date: we discharged all the papers published before 2020.

Figure 2 illustrates the process of selection of the relevant papers.

In the rest of the paper, we first analyze in Section 3.1 the literature where machine learning and deep learning models are developed, and then in Section 3.2 the studies where NLP techniques are applied. It is worth noting that medical images, electronic health records and other laboratory data can be the input to ML models for predicting a possible diagnosis of Long COVID, while clinical notes and tweets can be the input to NLP models to perform a risk factor or symptom co-occurrence analysis.

3.1 Machine learning and deep learning approaches to Long COVID data

This section presents recent research studies utilizing traditional and novel AI methods to detect Long COVID. We start with the authors applying ensemble learning techniques and then explore other approaches.

3.1.1 Ensemble learning

In the ensemble learning context, a strategy known as Optimized XGBoost was suggested by Jha et al. (2023). This supervised learning strategy used an ensemble approach based on the gradient boosting method, and its customized hyper-parameters were used to increase the performance of Long COVID prediction. The researchers looked at COVID-19 patients who had lung fibrosis 90 days after being discharged from the hospital. Analyses were conducted on a dataset of 1175 EHRs and associated High-Resolution Computed Tomography (HRCT) chest images from COVID-19 patients. The dataset included 725 cases of pulmonary fibrosis and 450 cases of standard lung. The dataset was divided into distinct groups for training and testing purposes, with 881 samples allocated for training and 294 for testing. The findings of the experiments had an accuracy of 99.37% on the EHR dataset and 98.48% on the HRCT scan dataset, respectively. In order to reduce the dependence of the performance results on the size of the considered dataset, the authors divided the dataset into distinct sets of train-test data from which they derived the performance metrics. The suggested ML model optimized XGBoost, compared to other ML approaches, such as decision tree, Support Vector Machine (SVM), random forest, logistic regression, Naive Bayes, and the traditional XGBoost approach. The suggested system's precision, recall, and accuracy were higher than those of other approaches in the literature.

XGBoost machine learning models were also exploited by Pfaff et al. (2022) to identify patients affected by Long COVID using the N3C EHR database (Haendel et al., 2021). The dataset comprises information from more than 8 million patients with diverse demographics and geographic locations, obtained from their EHRs. The population (n=1,793,604) was selected among a set of alive adult patients over the age of 18 who had either an International Classification of Diseases-10 Clinical Modification or a positive SARS-CoV-2 PCR or antigen test or a COVID-19 diagnostic code (U07.1) from an inpatient or emergency visit, whose COVID-19 index date has passed at least 90 days. The authors investigated 97,995 persons diagnosed with COVID-19 regarding their demographics, healthcare usage, diagnosis, and medicines. In the study, researchers collected 924 features from 597 patients diagnosed with Long COVID. These features were used to train three ML models to determine the possible cases of Long COVID among COVID-19-diagnosed patients, COVID-19-hospitalized patients, and COVID-19-positive patients who were not hospitalized. Essential characteristics include the healthcare usage, the patient's age, dyspnea, and other information on diagnoses and medications that are available inside the EHR. The dataset was split into different sets for training (80% of hospitalized and 75% of not hospitalized patients) and testing (20% of hospitalized and 25% of not hospitalized patients). After additional validation of the models using data from a fourth location, the authors achieved an AUC value of 0.92 for all patients, 0.90 for hospitalized patients, and 0.85 for outpatients.

A different approach was presented by Gupta et al. (2022) for the early diagnosis of cardiac problems in COVID-19 survivors to predict Long COVID. In this work, an ensemble was performed using a stacked approach. The proposed model was trained on heart-related data acquired from 180 COVID-19 patients with a questionnaire. The data of the 180 patients were first bootstrapped

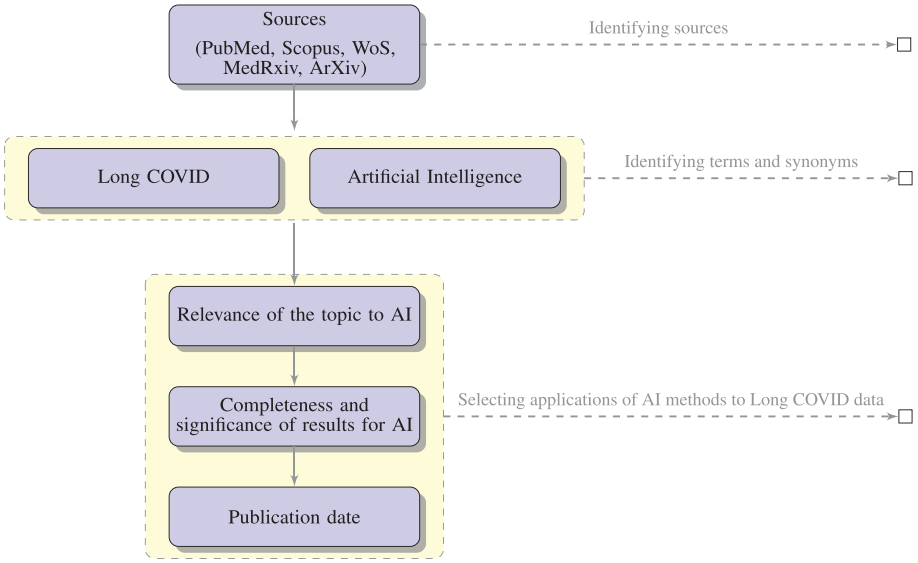

FIGURE 2
The literature selection process.

to 4700 records, using a tenfold cross-validation approach. Data were divided into a training set (70%), a validation set (20%) and a test set (10%). The performance of the suggested model was compared to that of standard ML techniques. Performance measurements included accuracy, specificity, precision, and recall with two other statistical measures: Mean Absolute Error (MAE), and Root Mean Square Error (RMSE). Accuracy in predicting heart disease using the stacking ensemble method was 93.23%. The suggested method outperforms conventional learning algorithms, including decision trees, random forests, SVM, and artificial neural networks. The minimal RMSE (0.32) and MAE (0.23) values further support the suggested model's robustness.

A recent study by Jiang et al. (2022) focused on the relationship between vital signs (oxygen levels, heartbeat, systolic/diastolic blood pressure) and Long COVID. Since no vital measurement data are available for all the patients in the N3C cohort, two subcohorts with abundant vital measurement data for the first week after hospitalization were selected. Various features (139) were designed from vital measurement readings, including daily averages and daily variability features. Using data from the first subcohort, an XGBoost model predicted a Long COVID outcome, while CNN and LSTM were used to process a multidimensional time series of vital measures in the second subcohort. The authors evaluated the performance of the models using the standard AUC metric with 5-fold cross-validation.

A retrospective case-control research was designed by Hill et al. (2022) to determine risk factors linked to PASC and Long COVID from thirty-one health systems in the United States (N3C). COVID-19 risk factors included patient age, gender, comorbidities, medications, and acute symptoms. 8,325 persons were diagnosed with PASC compared to 41,625 healthy individuals from the same health system. Using multivariate logistic regression, random forest, and XGBoost, the correlations between potential risks and PASC were examined. This study identified a number of significant risk variables for PASC, including middle age, severe COVID-19 illness, and particular comorbidities. Results from the XGBoost and logistic regression models were comparable, with an AUC of 0.73. The random forest model, which has an AUC of 0.69, comes next.

A supervised ML algorithm based on random forest with five folds and ten iterations of stratified repeated cross-validation techniques was developed by Sudre et al. (2021) to determine who is susceptible to Long COVID and organize therapy and rehabilitation. This study used data from mobile health apps, allowing users to self-report their symptoms, with a sample size of 2,149. A simple model to differentiate short (duration of symptoms less than ten days) and Long COVID at seven days has an AUC of 75.9%.

Patel et al. (2023) also used a random forest classifier to classify the most pertinent blood proteins for the identification of Long COVID cases. The study compared the expression of 2,925 different blood proteins in Long COVID outpatients to COVID-19 inpatients and healthy individuals. The data were stratified by subject group and divided using a dimensionality reduction with 70% designated for training and 30% reserved for testing. The Boruta method was used for the feature reduction dataset to select the most important characteristics. A 3-fold cross-validation with a random forest of 10 trees and a maximum depth of 3 was adopted to limit the overfitting. Experts explicitly obtained unstructured text on mRNA or protein expression at the cell or tissue level, which NLP then processed to produce protein expression tissue specificity. The results revealed 119 essential proteins for classifying Long COVID outpatients, with classification accuracy of 100%, AUC 100% and F1-score 100%. Also, NLP expression analysis confirmed widespread organ system involvement and identified key cell types as crucial elements related to Long COVID.

Finally, Patterson et al. (2021) used the random forest for the classification of healthy, mild-moderate, severe, and Long COVID patients from their immunological profile. Data from 224

individuals were compiled, including 29 healthy individuals, 26 with mild to moderate COVID-19, 48 with severe COVID-19, and 121 with Long COVID. The dataset comprised 16 columns, with 14 dedicated to cytokine/chemokine levels, one for patient IDs, and one for classification (healthy, mild-moderate, severe, or Long COVID). Training, validation, and testing used 60%, 20%, and 20% of the data. The Synthetic Minority Oversampling Technique (SMOTE) was employed to balance class representation. Three random forest classifiers were then developed: a multi-class predictor, a binary classifier for severe COVID-19, and another binary classifier for Long COVID. These models were evaluated to identify critical cytokines significant in disease assessment. The multi-class model achieved an 80% accuracy and a 63% F1-score, while the Long COVID model reached a 96% accuracy with a 95% F1-score, and lastly, the severe model secured a 95% accuracy and a 94% F1-score.

3.1.2 Deep learning

Using deep learning BiLSTM with a 1D CNN model, Sengupta et al. (2022) analyzed historical diagnosis code data from the N3C repository to identify possible risk factors of Long COVID. The study assessed patients for Long COVID infection using a chronological list of diagnosis codes up to 45 days following the initial positive test. The authors used Gradient-weighted Class Activation Mapping (Grad-CAM) to rate each input diagnosis. The diagnostic with the highest score was regarded as the most significant for making the proper diagnosis for a patient. The article proposed a method for collecting these leading diagnoses for each patient in the dataset and analysing their temporal trends to identify which codes are connected with a Long COVID positive diagnosis. Data were divided into training (75%), validation (15%), and testing (10%) sets. The study offered the mean AUC value of 3-fold stratified cross-validation for all models, achieving an accuracy of 70.48% despite the unbalanced dataset. Differently from the previous work where an LSTM was used, Subramanian et al. (2022) carried out diagnostic work for classification utilizing two CNN models, specifically VGG16 (Liu and Deng, 2015) and ResNet-50 (He et al., 2016), trained on 925 HRCT images, each with two different learning rates. The dataset was split into training (585 images), validation (65 images) and testing (275 images) sets. The best model produces an accuracy of 97.132%. An additional model was developed using a revised loss function that combines dice loss and binary cross-entropy, achieving an accuracy of 98.2%. The authors finally proposed a diagnostic model using the U-Net, which segmented and predicted the precise lung area infected with COVID-19 with an accuracy of 99.40%.

3.1.3 Regression models

Binka et al. (2022) proposed a machine learning technique which uses the elastic net regression model to identify Long COVID cases in a population-based cohort of COVID-19 that have been reported in British Columbia, Canada. The suggested model was trained using the known Long COVID cohort patients' characteristics, including their demographics, existing medical problems, and other unique symptoms and complaints from health administrative data recorded after the index date for COVID-19 with 10-fold cross-validation. The optimal model exhibited a high sensitivity and specificity rate of 86% and AUC of 93%, classifying 25,220 individuals out of 141,381 COVID-19 patients as Long COVID cases.

By contrast, Moreno-Pérez et al. (2021) used a traditional multiple logistic regression model to assess the acute infection phase risk variables linked to Long COVID. Data were collected from electronic medical records, and a follow-up assessment was conducted 10-14 weeks after either recovery from COVID-19 in an ambulatory setting or hospital discharge. This assessment comprised a clinical examination, blood tests, chest X-ray, pulmonary function tests, and a quality of life questionnaire. The study results showed that Long COVID was detected in half of COVID-19 survivors. Mild radiological and spirometric alterations were detected in less than 25% of the patients. Independent predictors were not found among the baseline clinical characteristics for the Long COVID development. The predictors of the outcome were examined using multiple logistic regression with a 95% cumulative incidence value.

Table 2 summarizes all the approaches presented in the previous sections. Note that several papers use the same datasets, looking at different features and using different techniques. Moreover, given the class imbalance of many of the datasets related to Long COVID, it is important to note that the accuracy measure can provide an inaccurate impression of the quality of a model and in general, of the overall analysis results.

3.1.4 Other approaches

Zhang et al. (2023) proposed a machine learning-based approach on topic modeling to derive Long COVID subcategories based on newly acquired medical conditions during the post-acute phase of a COVID-19 infection. The study focused on 30-180 days after confirmed COVID-19 infection. Development and validation cohorts were formed using EHRs from two large cohorts, INSIGHT and OneFlorida+, part of the National Patient-Centered Clinical Research Network, including 20,881 and 13,724 patients infected by COVID-19. The ML method analyzed more than 137 symptoms and conditions in the cohort of patients with newly incident conditions within 30-180 days after COVID-19 infection. After computing a 137-dimensional binary vector encoding of each patient with Long COVID diagnoses, it learned Long COVID topics from these vectors. Specifically, Long COVID subjects are sets of circumstances that occur together according to their respective event probabilities. Next, a topic modeling technique is used to infer patient representations in the low-dimensional Long COVID topic space. Based on how extensively each topic was covered in the patients' post-acute phase data, these themes are used to further characterize the patients. Finally, a clustering method was employed from the patient representations to detect the subphenotypes. The analysis detected four Long COVID subphenotypes: (i) cardiac and renal sequelae affected 33.75% of patients in the development cohort and 25.43% in the validation cohort; (ii) respiratory, sleep, and anxiety issues were observed in 32.75% and 38.48% of these cohorts, respectively; (iii) musculoskeletal and nervous system complications occurred in

TABLE 2 ML techniques for Long COVID diagnosis.

Study	Input data	AI method	Task	Output (%)
Jha et al. (2023)	1,175 EHR & HRCT	Optimized XGBoost	Binary classification of pulmonary fibrosis	Accuracy 99.37 precision 99.54
Pfaff et al. (2022)	N3C repository	XGBoost	Binary classification of Long COVID	AUC all patients 92 hospitalized 90 non-hospitalized 85
Jiang et al. (2022)	N3C repository	XGBoost CNN LSTM	Binary classification of Long COVID	AUC XGBoost 82.2 CNN 61.64 LSTM 59.94
Hill et al. (2022)	N3C repository	XGBoost Random forest	Risk factors associated with Long COVID	AUC XGBoost 73 Random forest 69
Gupta et al. (2022)	180 questionnaires	Stacking ensemble technique	Binary classification of heart diseases	Accuracy 93.23 precision 95.248
Sudre et al. (2021)	2,149 self-reported health status and symptoms	Random forest	Binary classification of short and Long COVID	AUC 75.9
Patel et al. (2023)	Expression of 2,925 unique blood proteins	Random forest NLP	Identification of blood proteins for Long COVID detection	AUC 100 accuracy 100 F1-score 100
Patterson et al. (2021)	Immunologic profiles from 224 individuals	Random forest	Classification of healthy, mild-moderate, severe and Long COVID patients	Multi-class: accuracy 80 F1-score 63 Long COVID: accuracy 96 F1-score 95 Severe: accuracy 95 F1-score 94
Sengupta et al. (2022)	N3C repository	BiLSTM with 1D CNN model	Binary classification of Long COVID	Accuracy 70.48
Subramanian et al. (2022)	925 HRCT	VGG-16 ResNet-50 U-Net	Binary classification of Long COVID	Accuracy from 97.132 to 99.4
Binka et al. (2022)	26,730 health administrative data	Elastic Net regression	Binary classification of Long COVID	AUC 93 sensitivity 86 specificity 86
Moreno-Pérez et al. (2021)	277 patients' demographics and comorbidities	Multiple logistic regression	Risk factors associated with Long COVID	Cumulative Incidence Value 95
Zhang et al. (2023)	34,605 EHR	Topic modeling clustering	Derive Long COVID subphenotypes	Four Long COVID subphenotypes

The first part of the table (6 rows) refers to ensemble learning, the second part (2 rows) to deep learning, and the last parts (2 rows and 1 row) refer to regression models and other approaches, respectively (all reported measures have the same number of decimal digits as the original paper).

23.37% and 23.35%; and (iv) digestive and respiratory system issues were seen in 10.14% and 12.74% of patients, each linked to specific patient demographics.

3.2 Text mining's role in Long COVID diagnosis and therapy

The latest developments in NLP offer the possibility of improving healthcare and public health. Massive amounts of unstructured data are continuously generated from various sources, including EHRs, social media, and recent literature. One of the goals of this investigation is to look ahead to potential uses of NLP-based technologies which can help enhance pandemic response preparedness, extracting textual patterns which can represent Long COVID symptoms and relationships between symptoms, and the discussion topics about COVID-19. *Pandemic response preparedness* means not just handling immediate issues but also planning for long-term effects, like Long COVID.

The aim is to improve public awareness of Long COVID, provide important insights to public health authorities, and learn more about the health effects of Long COVID.

In the following, we distinguish among the approaches based on BERT (Devlin et al., 2018) and other techniques.

3.2.1 BERT approaches

Miao et al. (2022) analyzed 30,327 user-generated conversations on Twitter about Long COVID symptoms. NLP was utilized to investigate how Twitter users described the nature of Long COVID symptoms in terms of demographic features such as patient's gender, age, and geographical location, as well as temporal parameters such as symptom severity and duration. Moreover, to address the Long COVID evolution over time, the study compared the results of datasets collected in different periods. The authors constructed two sets of tweets related to Long COVID; the first set spanned from 1st May to 31st December 2020, and the second was from October 2021. They randomly divided the annotated data into 80% for training and 20% for testing purposes. To ensure the accuracy of the automated labeling, they manually checked a subset of the labeled samples. On the demographic categories, the BERT classifier reached an accuracy of 89%, while on the symptom categories, it reached an accuracy of 95%.

BERT was also used by Zhu et al. (2022) on free-text clinical notes to identify patients with persistent symptoms following acute COVID-19 infection. Data from clinical notes of 719 patients seen by physicians were analyzed to look for patient similarities. The authors employed 5-fold cross-validation and divided the training, validation, and testing data by a ratio of 60%:20%:20%. The study applied three different pre-trained BERT models to automatically identify patients with Long COVID effects. The `ClinicalBERT` model achieved a sensitivity score of 0.88 for note-level prediction. The study identified potential phenotypes from the classification results.

To gain insight into the Italian perspective of the COVID-19 pandemic, Scarpino et al. (2022) discussed and compared two topic modeling techniques: Latent Dirichlet Allocation (LDA) and a BERT transformer (BERTopic). The authors analyzed texts written by patients with Long COVID, healthcare professionals, and citizens (without Long COVID), with the aim of characterizing patients affected by Long COVID based on the textual narration. BERTopic is a topic modeling technique that adopts transformers and c-TF-IDF to create clusters to represent topics and identify important words in the topic descriptions. The BERTopic-based method surpassed the LDA-based method, with 97.26% of documents correctly clustered and an overall accuracy of 91.97%.

3.2.2 Other approaches

Differently from the previous approaches where classification is adopted, Matharaarachchi et al. (2022) explored the trends and characteristics associated with Long COVID using the Apriori algorithm-based Association Rule Mining Technique. The focus of the study was to examine the common symptoms of patients with Long COVID and determine any correlations between them, using Twitter social media conversations as a reference. The authors set a minimum support threshold value of 0.001, a lift greater than 1, and a confidence level of 10% for positively correlated rules. According to the results, the three indications and symptoms that occurred most frequently were brain fog, fatigue, and breathing or lung issues.

Using EHR data, a comprehensive Long COVID symptom lexicon was developed by Wang et al. (2022). The authors evaluated PASCLex, a lexicon-based NLP approach that uses data-driven approaches based on medical ontologies to extract Long COVID symptoms from clinical notes. The primary dataset consisted of 23,505 patients, accounting for 90%, with 299,140 related clinical notes. In contrast, the validation subset comprised 2,612 patients, which is 10% of the total, and included 29,739 respective clinical notes. The developed method took advantage of the Unified Medical Language System (UMLS) and achieved precision and recall values of 94% and 84%, respectively.

Investigating the progression of the disease in its post-acute phase by analyzing 296,154 tweets, Banda et al. (2021) employed a blend of machine learning and NLP techniques, supplemented by clinician evaluations, to construct comprehensive symptom and condition timelines spanning 150 days. This process involved expert annotation of tweets, machine learning for filtering relevant content, and NLP for standardizing the data. The primary outcome of this approach was the evaluation of temporal symptoms, timeline visualization, and cluster identification.

Similarly, Déguilhem et al. (2022) collected and analyzed data from France on Long COVID most frequently reported symptoms, symptom combinations, challenges, and patient profiles. Data were gathered from the social media Twitter and the health-related online forum Doctissimo (https://www.doctissimo.fr). Symptoms were indexed using the MedDRA dictionary, ranked according to the times they were mentioned in posts, and summarized on a per-user basis. The study proposed to compute co-occurrences of terms in users' posts. The posted content was analyzed to identify common terms, and users were then grouped using hierarchical clustering based on these terms. The study looked at 289 users who used at least two distinct symptom phrases in their messages. A heat map was produced to illustrate the major co-occurrences. NLP-based text mining approach Biterm Topic Modeling (BTM) was used to analyze the conversations, and difficulties and unfilled needs were discovered through in-depth interviews. The analyses identified three major symptom clusters: asthenia-dyspnea (102/289, 35.3%), asthenia-anxiety (65/289, 22.5%), and asthenia-headaches (50/289, 17.3%).

Table 3 summarizes the aforementioned NLP approaches.

Figure 3 shows the expected outcomes of applying NLP-based text mining algorithms like BERT, BTM and LDA on data from clinical settings and social media data with the expected outcome of classification and clustering.

3.3 Task description

In this section, we recall the main tasks of the different papers. For the technical description of the input data and obtained results, the reader can refer to Tables 2, 3.

- Jha et al. (2023) identify the development risk of pulmonary fibrosis after 90 days of hospital discharge from clinical

TABLE 3 Long COVID diagnosis: recently applied data mining and NLP techniques.

Study	Input data	AI method	Task	Output (%)
Miao et al. (2022)	Tweets	NLP	Analysis of reported Long COVID symptoms in terms of demographics, geographical and temporal parameters	Accuracy demographic categories 89 symptom categories 95
Zhu et al. (2022)	Clinical notes	Pretrained BERT	Identification of Long COVID and potential computational phenotypes	Sensitivity score 88.1
Scarpino et al. (2022)	Blogs	LDA and BERT	Extract discussion topics in the Italian narration of COVID-19 pandemic	Accuracy of BERT 91.97
Matharaarachchi et al. (2022)	Tweets	Association rule mining	Relationships between symptoms	Confidence 77 for lung/breathing problems and loss of taste vs. loss of smell
Wang et al. (2022)	Clinical notes	PASCLex (NLP) model	Identification of symptoms	Precision 94 recall 84
Banda et al. (2021)	Tweets	NLP and SVM	Identification of symptoms	Accuracy 75 on a 20% random held-out test set
Déguilhem et al. (2022)	Tweets	Biterm Topic Modeling	Identification and co-occurrence of symptoms	Three major symptom co-occurrences: asthenia-dyspnea 35.3, asthenia-anxiety 22.5, asthenia-headaches 17.3

The division is between BERT (the first 3 rows) and other approaches (all reported measures have the same number of decimal digits as the original paper).

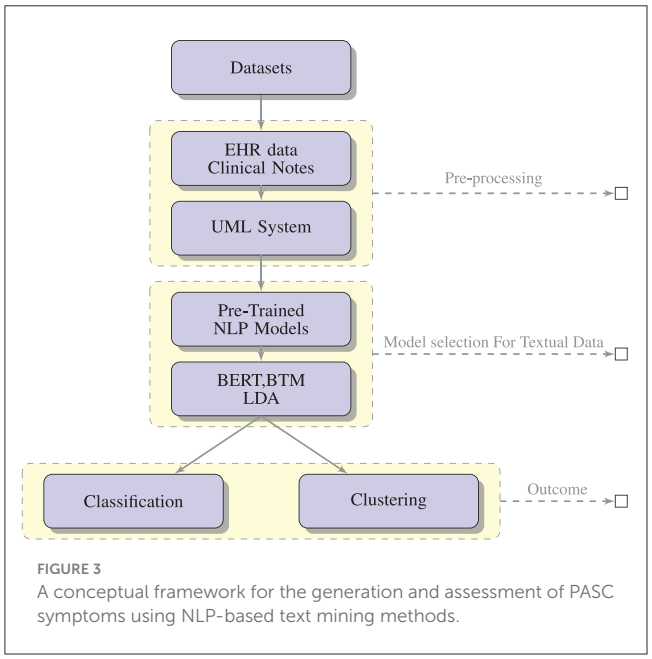

FIGURE 3
A conceptual framework for the generation and assessment of PASC symptoms using NLP-based text mining methods.

features retrieved at the time of follow-ups of COVID-19 patients.

- Pfaff et al. (2022) detect patients with Long COVID using EHR with diagnosis and medication characteristics from patients and for whom at least 90 days have passed since COVID-19 index date.
- Jiang et al. (2022) detect Long COVID from the features of vital measurements of patients with a diagnosis of COVID-19 and hospitalized. Measurements derived from the first seven days since the hospitalization started.
- Hill et al. (2022)'s objective was to identify links between risk factors such as demographics, comorbidities, and treatment, as well as acute characteristics associated with COVID-19, and Long COVID. Patients with Long COVID were included based on a prior diagnosis of SARS-CoV-2 infection or a positive polymerase chain reaction (PCR) or antigen (AG) lab test for SARS-CoV-2, with the initial acute infection date ranging from March 1, 2020, to December 1, 2020.
- Gupta et al. (2022) detect the risk of heart disease, as well as the long-term negative consequences of the coronavirus outbreak on recovered patients from data of patients who were diagnosed with COVID-19, in particular personal details, severity of disease, recovery days, hospital admission, symptoms during disease and Long COVID symptoms.
- Sudre et al. (2021) differentiate between short and long COVID-19 at seven days from fatigue, headache, dyspnea and anosmia symptoms. A connection was shown between more than five symptoms during the first week of sickness and the presence of Long COVID.
- Patel et al. (2023) find novel blood biomarkers for Long COVID by comparing protein expression in COVID-19 inpatients, healthy control individuals, and Long COVID outpatients. The discovered proteins represented a wide variety of cell types and organs.
- Patterson et al. (2021) identify and characterize the immunologic steps of COVID-19 (healthy, mild-moderate, severe and Long COVID) from the immunological profile in order to detect and monitor effective treatment plans. After the first symptoms appeared, the duration of Long COVID continued for more than 12 weeks.
- Sengupta et al. (2022) determine whether a patient is impacted by Long COVID by analysing a chronologically ordered set of diagnostic codes up to 45 days after the initial positive test or diagnosis. Looking at the overall temporal evolution for all

patients allows to identify which codes lead to a Long COVID positive diagnosis.

- Subramanian et al. (2022) make a binary classification of COVID-19 images, detect lungs region on HRCT images and identify COVID-19 region on HRCT images from patients with various recovery periods from COVID-19 infection.
- Binka et al. (2022) detect Long COVID cases from health administrative data, including demographic features, pre-existing conditions, COVID-19-related data and all symptoms recorded 28 days after the COVID-19 symptom index date and lasted up to 183 days afterwards.
- Moreno-Pérez et al. (2021) study the incidence of Long COVID and its features and assess the risk factors connected to the acute infection step from data of adult patients who had recovered from COVID-19 (from 27th February to 29th April 2020), with a systematic evaluation 10-14 weeks after disease occurrence.
- Zhang et al. (2023) find Long COVID subphenotypes based on newly incident conditions in the post-acute COVID-19 infection period, defined as 30-180 days after the confirmed infection, of patients with COVID-19.
- Miao et al. (2022) analyze the features of Long COVID symptoms included in Long COVID-related tweets from May to December 2020 in terms of the patient's gender, age, location and duration of symptoms, and also analyze the Long COVID evolution over time, making a comparison of the results between May-December 2020 and October 2021.
- Zhu et al. (2022) detect Long COVID from clinical notes of outpatient encounters of patients with constant symptoms after their positive COVID-19 tests between 30 days after the positive COVID-19 diagnosis and 365 days after diagnosis and characterize potential phenotypes.
- Scarpino et al. (2022) characterize textual narration of Long COVID patients by discussed topics from textual testimonies written about COVID-19 illness, which are parts of texts written by subjects affected by Long COVID, and texts of healthcare professional and general reflections by citizens.
- Matharaarachchi et al. (2022) analyze the patterns and behavior of Long COVID symptoms reported by patients from Twitter data retrieved from May 2020 to December 2021. Obtained results proved that patients with lung/breathing problems and loss of taste are likely to lose smell with 77% confidence.
- Wang et al. (2022) generate a comprehensive Long COVID symptom lexicon (PASCLex) from clinical notes (day 51–110 from first positive COVID-19 test) to assist the identification of symptoms. Among the symptoms with the highest frequency, there are pain, anxiety, depression, fatigue, joint pain, shortness of breath, headache, nausea and/or vomiting, myalgia, and gastroesophageal reflux.
- Banda et al. (2021) employ social media data derived from Twitter to define the Long COVID course of the disease, generating detailed timelines of symptoms and conditions and studying their symptomatology for a period of over 150 days. They rebuild a timeline for each Twitter user with the main phases (testing, symptoms, therapy, etc.).
- Déguilhem et al. (2022) detect and study Long COVID symptoms, symptom co-occurrences, topics of discussion, difficulties encountered, and patient profiles. Data were extracted based on a collection of pertinent keywords from public sites (e.g., Twitter) and health-related forums (e.g., Doctissimo) between January 2020 and August 2021. The analyses found three major symptom co-occurrences: asthenia-dyspnea, asthenia-anxiety, and asthenia-headaches.

4 Discussion

It is worth noting that all the papers reported in Table 2 discuss ML systems, and all the papers reported in Table 3 discuss NLP systems.

The *Input data* column in Table 2 summarizes the different datasets used in the papers. The variety of the datasets allowed us to understand Long COVID from various perspectives. The N3C dataset (Haendel et al., 2021) has been the most used one. It is a large-scale collection of EHRs collected with a collaborative effort from different healthcare systems and research institutions in the USA. The network consists of a collaborative partnership involving over 600 individuals and 100 organizations. This coalition focuses on national collaboration and governance, formulating regulatory strategies, defining COVID-19 cohorts through community-developed phenotypes, and standardizing data. The N3C facilitates community-led, replicable, and clear analysis of COVID-19 data, promoting the swift sharing of findings and precise attribution. EHR data derived from 14,026,265 patients who: (i) have tested positive for COVID-19 infection (5,409,269 patients), (ii) have symptoms that are compliant with a COVID-19 diagnosis, or (iii) have tested negative for COVID-19 infection (and have never tested positive) to support comparative analysis. EHR data have many features, including demographics, geographical locations of patients, healthcare visits, medical conditions, vital measurements of patients, and prescriptions. The N3C repository also includes specific COVID-19 diagnoses and service utilization dates. Additionally, it contains records of patients identified with the newly implemented ICD-103 U09.9 code, which is used to mark patients diagnosed with Long COVID. We believe that the strength of this dataset is its large sample size, which comprises millions of patients. When used in machine learning and deep learning approaches, this allows for a more robust analysis across diverse patient populations, even from different geographic regions. The number of works exploiting this dataset confirms that it is one of the most valuable resources for researchers studying Long COVID.

Table 2 shows that most papers use ensemble techniques, which are able to produce more accurate results, compared to the approaches using CNN. We believe that the reason could be the robustness of this approach, which can better handle noise and outliers in the data. In fact, note that the datasets include collections from surveys and self-reported status data (using an app), where these phenomena may easily happen. Another reason could be that Long COVID is a complex and multifaceted condition with different manifestations and risk factors. Ensemble techniques can handle this complexity by combining models, each of which

specializes in different aspects of the data so that the overall result enables a more comprehensive analysis and enhances the model's ability to capture the data complexities. In fact, the approach with the best performance is the random forest by Patel et al. (2023) followed by the Optimized XGBoost by Jha et al. (2023).

Table 2 also shows that most approaches focus on simple targets, such as binary classifications, whose primary goal is to diagnose and identify cases of Long COVID versus non-cases. This also includes the approach of Binka et al. (2022), which adopts an Elastic Net regression model but then produces a binary classification. Only Hill et al. (2022) (with XGBoost and random forest) and Moreno-Pérez et al. (2021) (with multiple logistic regression) examine the relationship between risk variables and the development of Long COVID. Finally, Patterson et al. (2021), Patel et al. (2023), and Zhang et al. (2023) adopted multiclass classification of Long COVID data.

Table 3 shows that the BERT model is the most used approach for pattern extraction from text data discussing Long COVID. In particular, it is adopted in Miao et al. (2022) on Twitter data for characterizing the nature of Long COVID symptoms in terms of demographic features, in Zhu et al. (2022) on free-text clinical notes to identify patients with persistent symptoms following acute COVID-19 infection, and in Scarpino et al. (2022) for characterizing patients affected by Long COVID based on the textual narration. Also, we can observe that BERT models are adopted for most of the different types of textual data, i.e., tweets (Miao et al., 2022), clinical notes (Zhu et al., 2022) and blogs (Scarpino et al., 2022). In terms of topic modeling, the approach introduced by Scarpino et al. (2022) using LDA and BERT models, and the approach introduced by Déguilhem et al. (2022) using Biterm Topic Modeling, adopted textual data based on patients' opinions, i.e., blogs and tweets.

Regarding the approaches using models different from BERT, they are mostly employed for identifying Long COVID symptoms [see Matharaarachchi et al. (2022) using Association Rule Mining, Wang et al. (2022) using PASCLex (NLP) model, and Banda et al. (2021) using NLP and SVM model] and for capturing symptom co-occurrences [see Déguilhem et al. (2022) using Biterm Topic Modeling].

In terms of data, it is worth noting that most of the techniques are employed on textual data from Twitter. Among these techniques, three out of four which are based on association rule mining (Matharaarachchi et al., 2022), NLP and SVM (Banda et al., 2021), and Biterm Topic Modeling (Déguilhem et al., 2022), are adopted for Long COVID symptom identification and co-occurrence, while one of them (Miao et al., 2022) aims to describe the nature of Long COVID symptoms in terms of demographic features.

From a comparison of Tables 2, 3, we can observe that most of the approaches in Table 2 are related to a binary classification of Long COVID, i.e., if the patient is affected or not by Long COVID. Also, only two papers in Table 2 (Moreno-Pérez et al., 2021; Hill et al., 2022) aim to predict the risk factors associated with Long COVID. Finally, Patterson et al. (2021), Patel et al. (2023), and Zhang et al. (2023) adopted multiclass classification of laboratory data for Long COVID identification. By contrast, the approaches based on NLP in Table 3 are mostly related to Long COVID symptoms identification [see Banda et al. (2021), Déguilhem et al. (2022), Matharaarachchi et al. (2022), Miao et al. (2022), Wang et al. (2022), and Zhu et al. (2022)].

Regarding the complexities of Long COVID, we can observe that Jiang et al. (2022), Gupta et al. (2022), and Miao et al. (2022) used input data characterized by a temporal duration of COVID-19, in particular measurements of patients from the first 7 days since the hospitalization start day, recovery days and symptom duration. It is worth noting that both Gupta et al. (2022) and Jiang et al. (2022) adopted ensemble learning (i.e. XGBoost and stacking ensemble technique), which is a more robust and reliable approach than traditional classifiers. Jiang et al. (2022) also used CNN and LSTM, which are deep learning methods specifically adopted for prediction tasks on temporal data. Still, Sengupta et al. (2022) used temporally ordered input data and looked at the temporal trends for all the patients, and Miao et al. (2022) studied the Long COVID evolution over time. For capturing the complexity of the task, Sengupta et al. (2022) used BiLSTM with a 1D CNN model, which is a powerful network for managing temporal data. Finally, Banda et al. (2021) analyzed the symptomatology of Long COVID conditions and symptoms over a period of more than 150 days using detailed timelines. From this analysis, we can observe that, although the length of symptoms is very important for Long COVID, many ML and NLP methods do not address this aspect. Only Miao et al. (2022) takes into account the symptom duration.

5 Recommendation and future work

In this survey, we have presented the applications of artificial intelligence in the Long COVID diagnostics, classification, risk factor prediction, and symptom occurrences. The ML approaches include (Optimized) XGboost, CNN, LSTM, random forest, stacking ensemble technique, Elastic Net regression, SVM, multiple logistic regression, topic modeling and clustering, BERT, and LDA.

Moreover, NLP approaches such as LDA and topic modeling based on the BERT transformer and Biterm Topic Modeling play a vibrant role in investigating how data from Twitter users and inpatients describe the nature of Long COVID symptoms in terms of demographic features (such as patient's gender, age, and geographical location), as well as temporal parameters such as symptom severity and duration. This will help to address the Long COVID evolution over time.

ML algorithms require massive datasets and high-quality information to construct effective models or discover meaningful patterns. The types of data used in the papers in Tables 2, 3 are EHR, health administrative data, patients' demographics and comorbidities, HRCT, other laboratory data, surveys, (free-text) clinical notes, tweets and blogs. These papers mainly focused on the following tasks: (i) identification and prediction of Long COVID from patients diagnosed as COVID-19 positive, (ii) prediction of the risk to develop different pathologies for patients who manifested COVID-19, along with the prolonged adverse impacts of the coronavirus pandemic, (iii) determining the associations between risk factors and Long COVID, (iv) distinction between short and long COVID-19, (v) exploring the characteristics,

patterns and behavior of Long COVID symptoms, (vi) study of the Long COVID course of the disease and evolution over time, and (vii) identification of Long COVID symptom co-occurrences, topics of discussion about Long COVID, difficulties encountered, and patient profiles.

However, the methods described in this survey have some limitations from the point of view of input data and adopted AI models. The first problem is that data on Long COVID are still relatively scarce, and existing datasets may be biased or incomplete (Pfaff et al., 2022). For instance, the N3C repository data is limited and may include healthcare access limitations. As a result, it may be challenging to construct accurate and generalized models over a wide range of patient data. Also, identifying the many factors, among age, sex, or infection severity, predicting the likelihood of developing Long COVID symptoms and their co-occurrence is still an open problem. Another source of data that, to the best of our knowledge, has not been explored comes from wearable devices that can remotely acquire data and share it with medical teams. Advances in digital technology have made it easier to collect electronic patient-reported data like temperature, oxygen saturation, and blood pressure. We believe that utilizing such data with machine learning and artificial intelligence (Lassau et al., 2021) could improve identifying and monitoring individuals at risk to enable early clinical intervention and rehabilitation. Moreover, considering the current availability of data on the phenomenon, most approaches in Table 2 focus on binary classification to detect cases of Long COVID. We believe that multiclass approaches or regression analyses are also possible and could bring more insightful results.

In conclusion, the approaches in Table 3, aiming to detect symptoms from textual data describing Long COVID, are quite different from the approaches in Table 2, aiming to identify the presence of Long COVID from symptoms, laboratory data and demographic features. In some sense, the former could be used for feeding the latter. More specifically, symptoms detected from textual data by an NLP approach can be inputted into a model for identifying the presence of Long COVID from the given symptoms. Also, note that most of the works in Table 2 deal with the diagnosis of Long COVID, namely with identifying and confirming that an individual is experiencing persistent symptoms following a COVID-19 infection. We believe that a prediction of prognosis in Long COVID could be interesting for the expected course of the condition, for instance, the duration of the persistent symptoms and the likelihood of symptom resolution. For example, using temporal data such as a symptom or physiological monitoring data over time, deep learning analyses could detect early signs of worsening of Long COVID, allowing for timely interventions and enabling personalized adaptations of therapies to improve patient outcomes.

Author contributions

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The effect of a web-based lifestyle intervention on nutritional status and physical activity on prevention of COVID-19: a randomized controlled trial in women's empowerment

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Background: Healthy dietary intake and physical activity affect the immune systems. The present study aimed to investigate the effects of a web-based lifestyle intervention on nutritional status, physical activity, and prevention of COVID-19.

Methods: Three hundred-three women (30–60 years old), who did not have COVID-19 in the City of Ardabil, participated in this study. Participants were randomized into an intervention (n = 152) or control group (n = 151). The intervention group received eight online educational sessions focusing on a healthy diet and physical activity via the website. There was no educational session for the control group during the intervention, but they were placed on the waiting list to receive the intervention and given access to the website and educational content after the follow-up. Outcomes were nutritional status, physical activity, immunoglobulin G (IgG), and immunoglobulin M (Ig M) antibody titers against the virus. They were evaluated at the baseline, after 4 and 12 weeks.

Results: Significant improvements in weight ($P < 0.001$), BMI ($P < 0.001$), total energy ($P = 0.006$), carbohydrate ($P = 0.001$), protein ($P = 0.001$), and fat ($P < 0.001$) were found for the intervention group compared to the control group during the study. MET-min/week for moderate physical activity increased during the time for the intervention and control groups ($P < 0.001$ and $P = 0.007$, respectively). MET-min/week for walking activity rose in the post-intervention and follow-up compared to that in the baseline in the groups ($P < 0.001$ for both groups). Total physical activity was increased during the study ($P < 0.001$) for both groups. The mean of serum IgG and IgM titers against the virus were increased during the study in both groups in time effect ($P < 0.001$). There was a significant time x group interaction for carbohydrate and fat intakes ($P = 0.005$ and $P = 0.004$, respectively).

Conclusion: The web-based lifestyle intervention may improve nutritional status and physical activity, and have the potential to reduce the risk of contracting a COVID-19 infection.

KEYWORDS

lifestyle, healthy diet, physical activity, COVID-19, web-based

Introduction

Coronavirus 2019 disease (COVID-19) was diagnosed as a pandemic by the World Health Organization (WHO) in March 2020, which has led to economic, public health, and social crisis (1). At the time of writing this article, there are more than 676 million coronavirus cases and the total deaths are more than six million worldwide. There have been more than 7 million COVID-19 cases and more than 144,000 deaths in Iran.[1] The clinical symptoms vary from fever, headache, sore throat, dry cough, and fatigue to progressed symptoms, including pneumonia and death (2). The progress of COVID-19 disease is associated with a rise in inflammatory cytokines and IgG and IgM. Therefore, detecting IgG and IgM antibodies has been more consistent than the nucleic acid detection assay. This method is cheap and simple. This method may play an important role in the diagnosis and epidemic control (3, 4). COVID-19 can be transmitted from person to person. It threatens human health, especially in vulnerable populations, such as women, children, and the elderly; moreover, vulnerable people are at a higher risk of infection (5, 6). Women include the majority of health-related roles. The development of gender-equitable disaster response and reconstruction results from the empowerment of women. Thus, gender remains a basic consideration in infectious disease and during pandemic planning and response. A group at risk during natural disasters and social crises are women. In the majority of societies, women play an important role as health liaisons in changing behaviors and controlling pandemics (7). Governments have put into practice certain strategies to prevent the spread of COVID-19 (8). Unfortunately, the COVID-19 lockdown and social distancing have affected people's lifestyles, especially nutrition patterns, and physical activity in the whole world (9). A sedentary lifestyle and poor eating behavior have increased, which is associated with numerous disorders (10). In accordance with individuals' lifestyles, women are the least active and spend more time watching TV than men (6, 7).

Physical activity training was proven to be one of the most effective lifestyle interventions capable of preventing metabolic disturbances and improving the inflammatory state (11). Physical activity affects the immune system, so its moderate practice boosts the body's immune response, reducing the incidence and severity of infectious processes, especially respiratory diseases (12). COVID-19 lockdown has caused a reduction in physical activity by 36.4% in adults. House confinement increases the consumption of unhealthy food (13). Studies have indicated that the diet during the lockdown includes further energy intake compared to that before COVID-19 (9, 14, 15). Healthy dietary intake affects the immune system and health outcomes during the COVID-19 pandemic (16). A balanced diet strengthens the immune system in response to infection and reduces the severity and complication of COVID-19 disease (17). Nowadays, unhealthy dietary habits have increased in most countries, and poor eating behavior is associated with a higher risk of diseases. There is an urgent need to improve the quality and eating behavior of humans (18). An active lifestyle with an increased level of physical activity affects the immune system (19). Meanwhile, physical activity enhances immune surveillance (20). Based on guidelines, adults are recommended to do 150 min/week of moderate to vigorous physical activity to prevent diseases (21). Therefore, lifestyle interventions increasing physical activity and improving nutritional status are of great necessity during the period of social distancing caused by the disease's pandemic; these strategies are effective in the management of chronic diseases (22). In addition, it seems that due to the widespread complications and high mortality rate of COVID-19 disease, nutrition and physical activity education is essential in strengthening immunity. Nowadays, the number of internet and smartphone users has increased, as a result of which electronic, virtual, and mobile health intervention programs are growing worldwide (23). Under this circumstance, for the prevention of COVID-19 spread, web-based lifestyle intervention would be beneficial and cost-effective. There is a lack of evidence of the effects of a web-based lifestyle intervention to prevent COVID-19.

Thus, we hypothesized that the women who receive lifestyle intervention strategy web training strategies will be more likely to develop a healthy diet and physical activity, and will be less increasing IgM or IgG more than 1.1, will be less likely to develop COVID-19 than the control group. Herein, we conducted a web-based lifestyle intervention strategy in order to evaluate the effectiveness of women's empowerment in terms of a healthy diet and physical activity to prevent COVID-19.

Methods

Study protocol

This study was designed as a parallel randomized controlled trial and single-blind and was conducted over 6 months. The last participants were recruited on 20 January 2021. This study had a 3-month follow-up until 21 May 2021. This research was conducted according to the guidelines laid down in the Declaration of Helsinki, and received the approval of the research ethics committee of Ardabil University of Medical Sciences and

1 https://www.worldometers.info/coronavirus

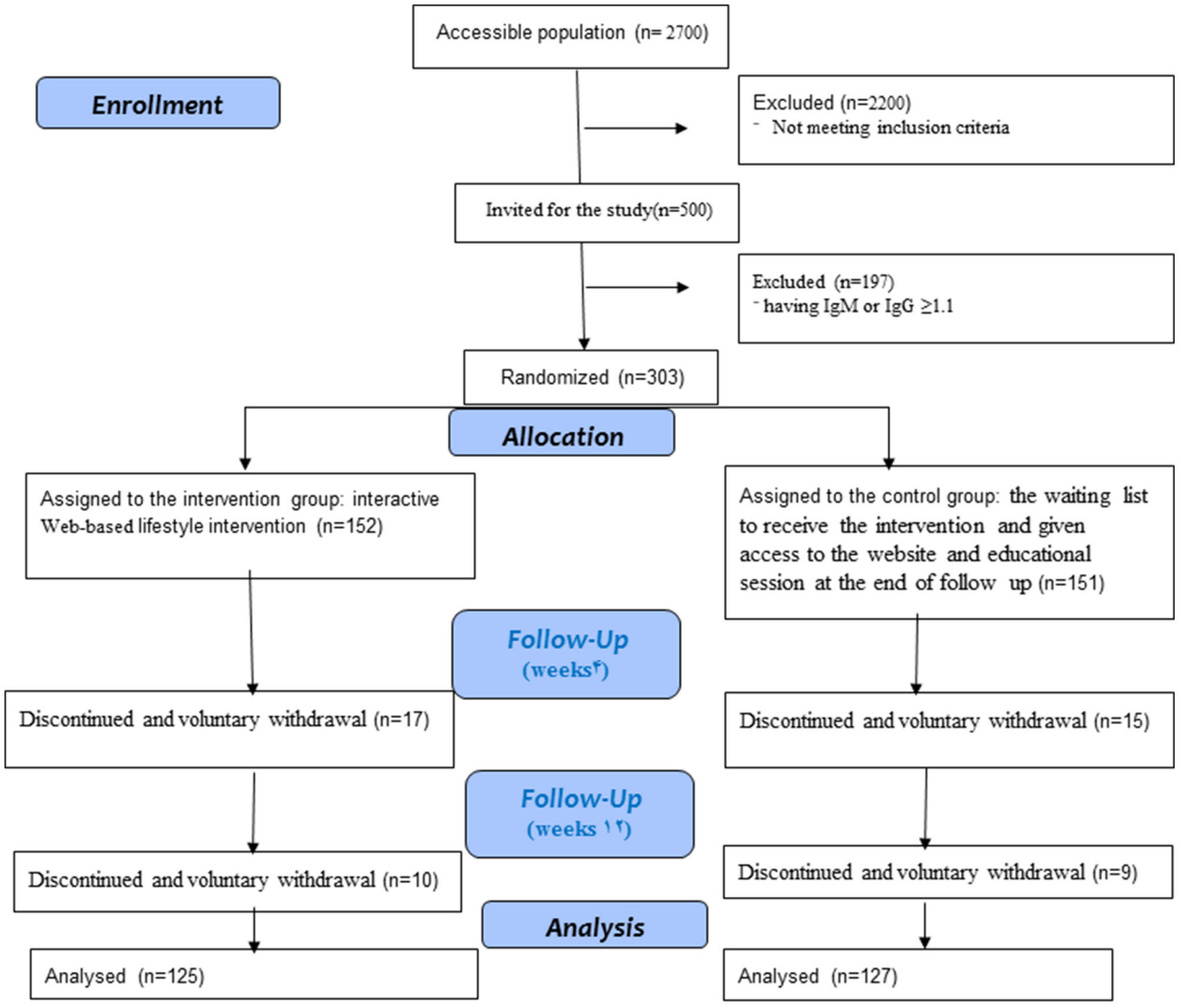

FIGURE 1
CONSORT flow diagram of lifestyle intervention during COVID-19 pandemic.

the clinical trial, IR.ARUMS.REC.1399.284, Approval code Irct.ir: IRCT20221228056969N.

Participants who completed and signed a written informed consent form participated in this study.

Study population

Admission began among the healthcare centers in Ardabil, Iran. Ardabil province is located in the northwest of Iran, with a population of over 1 million and 300 thousand people who speak Azeri, and it is divided into five regions. Ardabil, the capital of Ardabil province, was chosen for this study (24). Participants were contacted via a telephone call and screened for the inclusion criteria. Finally, the eligible interested individuals were invited to a free assessment performed by the staff of the Digestive Disease Research Center (DDRC). The women were screened in the baseline by an examination test. Randomization was performed after the baseline with Random Allocation Software (RAS). The population that had inclusion criteria was 500 participants, 197 of whom had IgM or IgG $\geq$1.1. According to the kit manufacturer guidelines, the cut-off index was calculated, where IgG titers and Ig M titers $\leq$1.1 were negative and IgG titers and Ig M titers $\geq$1.1 were positive. Therefore, 303 participants were randomized into two groups, the intervention group (n = 152) and the control group ($n = 151$), by a researcher. Figure 1 shows the study process. Because of the nature of this study, only the analysts were masked in group allocation.

The eligible participants were women (30–60 years of age); literate; having IgM or IgG $\leq$1.1 at baseline of the study; having access to the Internet, a computer, or smartphone; having the necessary skills to work with the Internet; and having consented to participate in this investigation. The exclusion criteria were having a history of chronic disease, being pregnant or breastfeeding, having

IgM or IgG ≥1.1 (which indicates that the person had COVID-19) prior to the intervention, and being vaccinated individuals.

Interventions

All participants, the intervention and control groups, received information about how to work Big Blue Button and a website designed by a researcher.

The intervention group was given access to a WhatsApp mobile group and website by code access. The mobile app was created to coordinate virtual classes. It allows the users to review their weekly class plans. Web developers and graphic designers created a professional, attractive, and user-friendly website with the address https://edusarscov.com. The website includes a home page, instructions, online and offline classes, and contacts us. Before the beginning of the intervention, the researcher explained the intervention objectives and sessions in the mobile app group. Four sessions of training courses on healthy diet were held once a week. Physical activity sessions were held for four sessions, once a week. This intervention program was conducted via virtual courses through Big Blue Button in the online class section on the website for the intervention group. The details of the class of the session on a healthy diet and physical activity are provided in Supplementary material (23, 25, 26). A number of researchers specializing in healthy diet and physical activity held online classes. All the educational content of the course was uploaded in the offline class section on the website. At the end of the intervention, an online test was conducted to evaluate the participants.

The participants in the control group had a meeting with DDRC employees in the baseline assessments. After the assessments in the baseline, 4 weeks (post-intervention), and 12 weeks (follow-up), the control subjects were offered the intervention and given access to the website and educational content. Actually, there was no educational session for the control group during the intervention, but they were placed on the waiting list to receive the intervention.

Measures and outcomes

Socio-demographic

The General Information Questionnaire was administered at the baseline to collect the following information: age (year), monthly income (million Iranian Rials, IRR), family history of COVID-19, and having a particular diet.

Outcome measures

Blood pressure (BP) was measured with a digital sphygmomanometer after the subjects were seated at rest for 15 min. The height and body weight of the participants were measured without shoes, and they were dressed in light clothing with a stadiometer (RASA, Manufactured in Iran) to the nearest of 0.1. The body mass index (BMI) was calculated using the body weight in kilograms divided by the square of height in meters (BMI, in kg/m^2). Blood samples were collected via venepuncture (Ayrik, Iran) 10 ml. Immunoglobulin G (IgG) and immunoglobulin M (Ig M) antibody titers for COVID-19 were detected via enzyme-linked immunosorbent assay, using kits (ELISA; Pishtaz, Iran) according to the manufacturer instructions.

Physical activity

Physical activity was measured with the short form of the International Physical Activity Questionnaire (IPAQ): a seven-item

TABLE 1 Demographic characteristics of participants' intervention and control groups.

		Study group		*P*-value
		Intervention	Control	
		Frequency (%)	Frequency (%)	
Age—y	30≤	51 (33.6)	48 (32%)	0.163[a]
	40–50	63 (41.4)	59 (39.3%)	
	≥50	38 (25.0)	44 (29.1%)	
Monthly Income—million Iranian Rials—IRR	10–20	62 (41.3)	78 (52.3)	0.019[a]
	20–30	42 (28.0)	39 (26.2)	
	30–40	10 (6.7)	14 (9.4)	
	40–50	15 (10.0)	9 (6.0)	
	≥50	21 (14.0)	9 (6)	
Family history of COVID-19 infection	Yes	52 (34.2)	35 (23.2)	0.034[b]
	No	100 (65.8)	116 (76.8)	
Having a special diet	Yes	2 (1.3)	1 (0.7)	0.623[b]
	No	151 (99.3)	149 (98.7)	

Data are *n* (%). Analyses are [a]Mann–Whitney, and [b]Fisher's exact test. There is no significant difference between the study groups in demographic characteristics, except monthly income and family history of COVID-19 infection ($p < 0.05$).

questionnaire validated in Iran. Finally, the Metabolic Equivalent of Task (MET) was calculated for minutes in the week for each physical activity level (PAL). PAL is classified into three categories, namely the low category (the lowest level of physical activity with MET<600), moderate category (five or more days of moderate-intensity activity and/or walking of at least 30 min per day with 600 < MET < 1,500), and high category (1,500 MET-min per week of vigorous-intensity physical activity spread over at least 3 days per week, or 3,000 MET-min per week of moderate to vigorous-intensity physical activity spread over the seven days of the week) (27).

A physical activity behavior questionnaire was also utilized. This questionnaire consists of five items answered as never, sometimes, usually, and always, where responses range from "1"= never to "4" =always. Based on the Likert scale, the minimum score is 5 and the maximum is 20. A score of 5–10 is considered a low score, 10–15 is moderate, and 15–20 is a good score. This questionnaire reliability was established and the Cronbach's alpha of this instrument was 0.821.

Nutritional status

To assess nutritional status, a 24-h dietary recall was used (28). The subjects were answered to complete a 24-h dietary recall about the food items that they consumed during the 24 h preceding the interview. Household handy measures were taken to aid the subjects in the estimation of the portion size of their food intake and beverage and the portions were converted into grams. Data on a 24-h dietary recall as grams were entered in Nutrition 4 (N4), a computer program, and the levels of total energy (kcal), carbohydrate, protein, and total fat intake were calculated.

A 14-item healthy eating behavior questionnaire was developed by the authors to assess healthy eating. The Cronbach's alpha of this tool was.617, which shows it is a reliable tool.

The subjects were asked to rate their responses on a four-point scale as never, sometimes, usually, and always, with scores ranging from "1"= never to "4" =always. The scores on Likert scoring ranged from 14 to 56 points. A score of 14–28 was low, 28–42 was moderate, and 42–56 was good eating behavior.

TABLE 2 Changes in outcome variables from baseline to follow-up.

	Group	Time			*p*-value		
		Baseline	Post-intervention	Follow up	Time effect	Group effect	Interaction effect
		Mean (SD)	Mean (SD)	Mean (SD)			
Blood pressure—mm Hg							
Systolic	Intervention	121 (17)	121 (17)	122 (18)	0.310	0.292	0.310
	Control	118 (13)	119 (13)	119 (13)			
Diastolic	Intervention	85 (21)	85 (20)	85 (20)	0.310	<0.001	0.310
	Control	76 (11)	76 (12)	77 (11)			
Weight—kg	Intervention	76.10 (12.77)	75.67 (12.81)	75.46 (12.98)	<0.001	0.893	<0.001
	Control	74.40 (11.19)	75.93 (11.00)	76.20 (10.89)			
BMI—kg/m^2	Intervention	28.96 (4.80)	28.84 (4.87)	28.80 (4.80)	<0.001	0.838	<0.001
	Control	28.51 (4.12)	29.09 (4.09)	29.20 (4.08)			
Nutrition intake							
Total energy-Kcal/day	Intervention	1,995.38 (735.80)	1,779.73 (480.78)	1,938.61 (531.86)	0.006	0.395	0.081
	Control	1,904.54 (727.10)	1,890.38 (623.36)	2,040.40 (716.72)			
Carbohydrate-g/day	Intervention	249.62 (173.01)	188.35 (76.62)	199.77 (80.16)	0.001	0.211	0.005
	Control	233.54 (137.80)	221.81 (107.37)	233.57 (91.80)			
Protein-g/day	Intervention	72.67 (25.63)	71.30 (21.37)	79.76 (23.25)	0.001	0.393	0.190
	Control	74.97 (30.53)	67.85 (19.16)	75.31 (24.59)			
Fat-g/day	Intervention	77.49 (97.86)	48.21 (17.71)	47.20 (23.17)	<0.001	0.929	0.004
	Control	61.70 (34.99)	55.77 (26.87)	57.82 (29.88)			
Healthy dietary behavior	Intervention	43.8 (5.4)	43.8 (5.3)	44.3 (5.7)	0.316	0.205	0.942
	Control	42.9 (5.2)	43.2 (5.3)	43.8 (5.1)			

Data are means ± SD. Analysis is repeated measure ANOVA. BMI, body mass index is the weight in kilograms divided by the square of the height in meters.

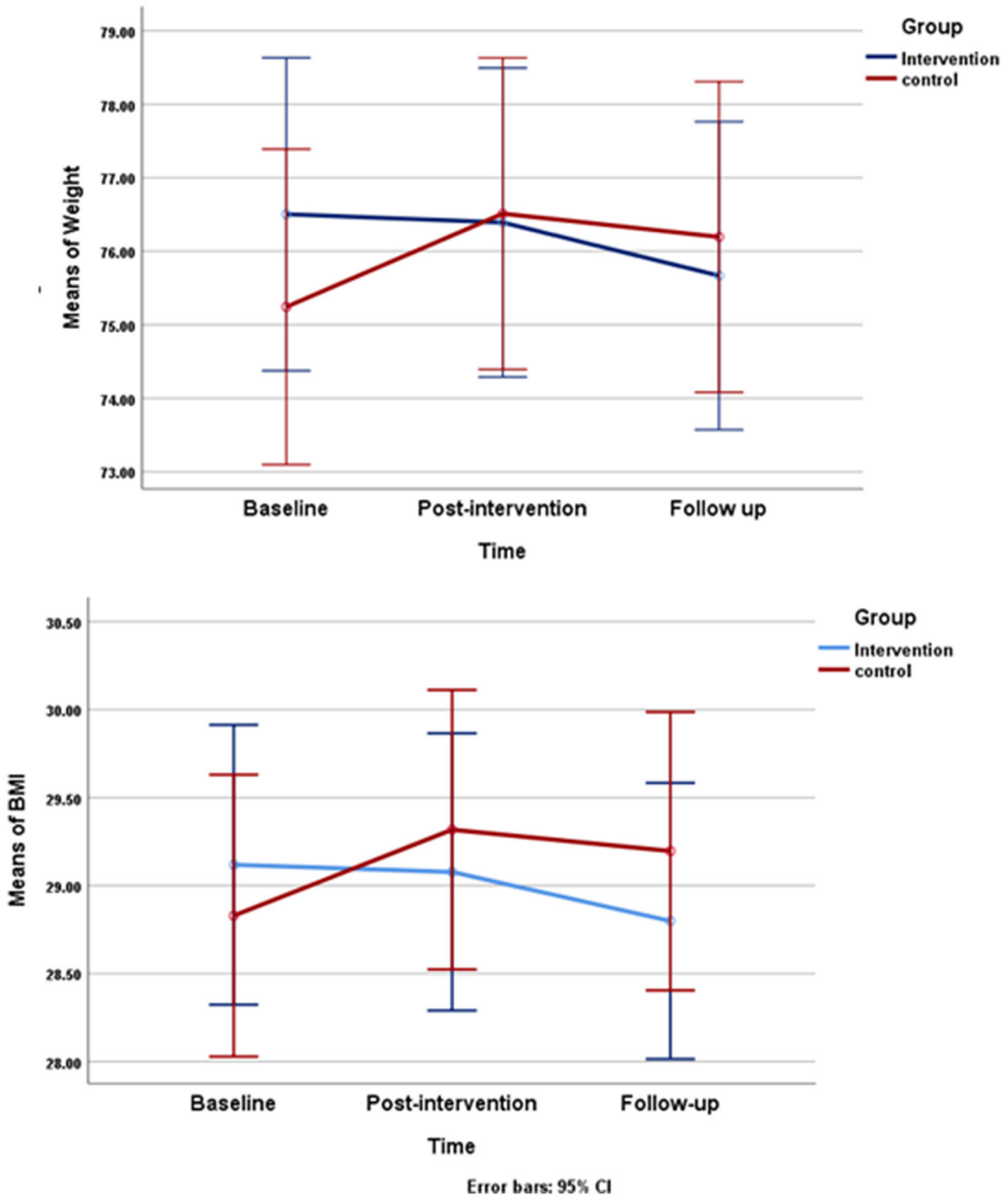

FIGURE 2
Changes in mean of weight and body mass index (BMI) during study changes in mean of body weight (kg) and changes mean of BMI (kg/m^2) from baseline through follow up are shown in the control and intervention group. Repeated measures ANOVA indicated that there is a statically significant difference among the three data collection between both of group.

Statistical analysis

The sample size was set considering a type 1 error of 0.05, type 2 error of 0.20, and success rate of p1 = 0.25 and p2 = 0.40; the minimum required sample size was 101 in each group. Considering the sample loss rate of about 50%, the minimum final sample volume in each group will be about 151 people in each group (29).

All the statistical analyses were performed using SPSS software version 25.0 (IBM, Chicago, Illinois, USA). The obtained data are shown as (mean standard deviation) and frequency (percentage) for quantitative and qualitative variables, respectively. The normality of data distribution was assessed using the Kolmogorov–Smirnov test. The Chi-square test (or Fisher exact test) was employed to compare qualitative factors between the two groups. An independent sample t-test was used to compare quantitative variables among the groups. Cochran's Q test was utilized to determine if there are differences concerning the dichotomous dependent variables between the groups across time. Through the use of the repeated measures ANOVA and Friedman test, continuous data in groups were evaluated. Mann-Whitney test compared the mean outcome quantities between the two groups in each time studied. To eliminate the effects of the confounding factors, a general linear model (GLM) with generalized estimating equations (GEE) approach was performed to assess the response variables changes by adjusting the confounding variables including age,

TABLE 3 Physical activity level and physical activity behavior among participants within three data collection times.

	Group	Time						*p*-value†
		Baseline		Post-intervention		Follow up		
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Physical activity level MET-min/week								
Vigorous	Intervention	23.7 (209.9)	0 (0, 0)	26.7 (22.7)	0 (0, 0)	13.4 (97.8)	0 (0, 0)	0.368
	Control	22.5 (145.9)	0 (0, 0)	23.2 (152.5)	0 (0, 0)	46.9 (219.4)	0 (0, 0)	0.819
p-value‡		0.312		0.487		0.082		
Moderate	Intervention	644.3 (933.2)[ab]	240 (120, 720)	989.5 (1,179.9)[b]	480 (240, 1,440)	972.9 (1,114.8)[a]	480 (240, 1,440)	<0.001
	Control	574.9 (873.4)	240 (120, 720)	681.7 (922.8)	390 (160, 960)	661.8 (930.1)	240 (160,720)	0.007
p-value‡		0.427		0.024		0.019		
Walking	Intervention	149.1 (246.9)[ab]	33 (0, 198)	215.38 (365.3)[a]	99 (0, 297)	310.2 (493.8)[b]	132 (33, 396)	<0.001
	Control	159.6 (295.4)[a]	33 (0, 198)	238.40 (534.4)	82.5 (0, 198)	299.8 (555.5)[a]	132 (16.5, 396)	<0.001
p-value‡		0.785		0.635		0.642		
Total physical activity level	Intervention	817.1 (1,020.5)[ab]	537 (201.2, 939)	1231.5 (1,296.1)[a]	753 (278, 1,836)	1,296.6 (1,282.5)[b]	876 (339, 1,986)	<0.001
	Control	757.1 (949.0)[a]	480 (198, 852)	943.3 (1,099.7)	568.5 (249.5, 1,212)	1,008.6 (1,086.9)[a]	678 (273, 1,356)	<0.001
p-value‡		0.614		0.058		0.105		
Physical activity behavior	Intervention	8.8 (3.2)	10.0 (6.0, 10.0)	8.8 (3.2)	10.0 (6.0, 10.0)	8.8 (3.1)	10.0 (6.0, 10.0)	0.834
	Control	8.4 (2.5)	8.0 (6.0, 10.0)	8.4 (2.5)	8.0 (6.0, 10.0)	8.5 (2.6)	8.0 (6.0, 10.0)	0.459
p-value‡		0.568		0.429		0.746		

Data are means ± SD and median ± IQR.
† Analyses are Freidman test.
‡ Mann–Whitney test.
[a,b] Similar letters show statistically significant $P < 0.05$.
MET, denotes metabolic equivalents task; IQR, interquartile range.

salary, and family history. A $P < 0.05$ was considered to be statistically significant.

Results

Baseline data

Most women in both groups were between the ages of 40–50 years. In the intervention group, 41.3% of the subjects, and in the control group, 52.3% of them had a monthly income range of 10–20 million Iranian Rials (IRR). COVID-19 infection was observed in the family history of 52% (34.2%) and 35(23.2%) of the participants in the intervention and control groups, respectively. The majority of them in both groups did not follow a special diet. The monthly income and family history of COVID-19 were significantly different between the two groups ($P = 0.019$ and $P = 0.034$, respectively) (Table 1).

Outcomes

Table 2 depicts the changes in outcome variables from the baseline to follow-up. However, there were no significant differences concerning systolic blood pressure between the two groups. However, the diastolic blood pressure was statistically significant between them ($P < 0.001$). Significant within-group and interaction differences were found regarding weight ($P < 0.001$) and BMI ($P < 0.001$). As shown in the plots, the weight and BMI in the control group were lower at the start of the experiment, but higher in this group in the follow-up compared to those in the intervention (Figure 2). Repeated measure ANOVA showed a significant difference during the time in terms of dietary intake: total energy ($P = 0.006$), carbohydrate ($P = 0.001$), protein ($P = 0.001$), and fat ($P < 0.001$). A significant time x group interaction effect was observed for carbohydrate and fat intakes ($P = 0.005$ and $P = 0.004$, respectively). Therefore, the effect of the treatment on carbohydrates and fat would depend on time (Table 2). We did not find any significant differences in healthy dietary behavior between the intervention and control groups and also during the time.

There are no significant differences among the three data collection times relating to vigorous physical activity in both groups. In the intervention group, MET-min/week for moderate physical activity increased during the time ($P < 0.001$). In the control group, MET for moderate activity rose among the three data collection times ($P = 0.007$). In MET-min/week for moderate physical activity, there were significant differences between the

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TABLE 4 Characteristics of plasma antibodies in participants within three data collection times.

Group			Time			*p*-value†
			Baseline	Post-intervention	Follow up	
			Frequency (%)	Frequency (%)	Frequency (%)	
Intervention	IgG (Binned)	<1.1	152 (100%)[a]	135 (100%)[b]	119 (95.2%)[ab]	0.002
		≥1.1	0 (0%)	0 (0%)	6 (4.8%)	
Control	IgG (Binned)	<1.1	151 (100%)[ab]	136 (98.6%)[b]	116 (91.3%)[a]	<0.001
		≥1.1	0 (0%)	2 (1.4%)	11 (8.7%)	
p-value‡			–	0.498	0.222	
Intervention	IgM (Binned)	<1.1	148 (97.4%)	134 (99.3%)	118 (94.4%)	0.078
		≥1.1	4 (2.6%)	1 (0.7%)	7 (5.6%)	
Control	IgM (Binned)	<1.1	149 (98.7%)[a]	136 (98.6%)[b]	118 (92.9%)[ab]	0.023
		≥1.1	2 (1.3%)	2 (1.4%)	9 (7.1%)	
p-value‡			0.684	1.00	0.797	

Data are *n* (%). Analyses are † Cochran's *Q*-test and ‡ chi-square (or Fisher's exact test).
[a,b]Similar letters show statistically significant $P < 0.05$.
IgG, immunoglobulin G; IgM, immunoglobulin M, ELISA, enzyme-linked immune sorbent assay antibodies to SARS-CoV-2, determined by ELISA of plasma samples obtained from subjects.

intervention and control groups in the post-intervention ($P = 0.024$) and follow-up ($P = 0.019$). The level of walking activity rose in the post-intervention and follow-up compared to that in the baseline in the groups ($P < 0.001$ for both groups). There were no significant differences between the intervention and control groups in terms of walking activity at each time. Total PAL in minutes per week indicated an increasingly significant difference between the three data collections during the study in both groups ($P < 0.001$ for both groups). In total PAL, we detected no significant differences between the intervention and control groups at each of the three data collection times. Finally, the result of the Friedman test and Mann–Whitney test in the intervention and control groups did not indicate any significant differences during the study, neither in the groups nor between them, in terms of physical activity behavior (Table 3).

There was no significant difference between the intervention and control groups in terms of characteristics of plasma antibodies and the means of plasma antibody titers against COVID-19. The result of the Cochran's Q test revealed a statistically significant difference in the intervention group concerning IgG against the virus ($P = 0.002$). Moreover, it indicated a statistically significant difference in the control group in terms of Ig G and Ig M against the virus ($P < 0.001$ and $P = 0.023$, respectively) (Table 4).

The result of repeated measures ANOVA demonstrated an increased mean of serum Ig G and Ig M titers against the virus among the three data collection times in both groups in time effect ($P < 0.001$). However, according to the group and interaction effect, no significant trend was observed in either of the groups in terms of Ig G and Ig M titers against the virus (Figure 3).

Table 5 shows weight, BMI, physical activity behavior, total energy, carbohydrate, protein, fat, and healthy dietary behavior, along with Ig G and Ig M titers changes in the groups by adjusting the effect of confounding variables. After adjusting the confounding variables, age, salary, and family history, there were no significant group differences in terms of weight, BMI, physical activity behavior, total energy, carbohydrates, protein, fat, healthy dietary behavior, and IgG and Ig M titers.

Discussion

The study results revealed a significant difference in terms of diastolic blood pressure between the two groups during the study. The result indicated that the intervention group had a decreasing body weight in the post-intervention (0.271 kg) and the follow-up (0.661 kg) compared with the control group. This study indicated that a lifestyle intervention program could lead to decreased total energy in the post-intervention and the follow-up in the intervention group in comparison with the control group. In the former, carbohydrates decreased in the post-intervention and the follow-up compared to the baseline. Total fat consumption decreased in the post-intervention and the follow-up in the intervention group. Daily protein intake rose during the follow-up in the intervention group. In both groups from baseline through follow-up, the healthy dietary behavior score increased. However, healthy dietary behavior did not indicate significant differences during the study. From the beginning study, in the intervention group, a healthy dietary behavior score was a good score; however, in the control group, the healthy dietary behavior score changed from medium to good scores. The healthy dietary behavior score did not indicate significant differences. Physical activity education did not vary according to the baseline concerning vigorous level. Although the analyses indicated a significant improvement in the post-intervention and follow-up in the majority of physical activity levels in both groups, web-based intervention showed greater improvements (moderate, walking, and total physical activity) in the intervention group than in the control group. The obtained findings did not show a significant modification in the mean score of physical activity behavior. In both groups, the mean score for

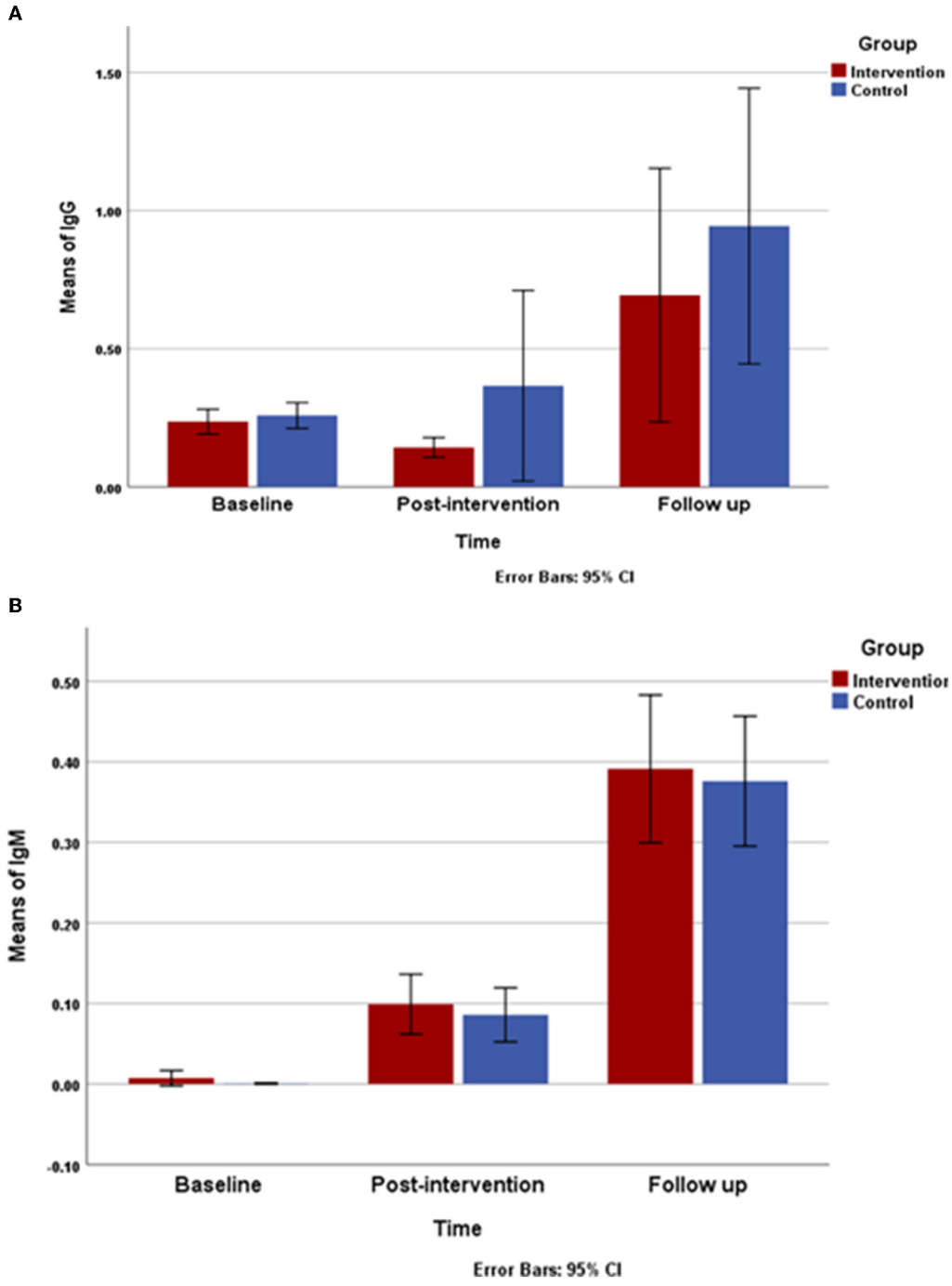

FIGURE 3
Changes in mean of immunoglobulin G (IgG) and immunoglobulin M (IgM) titers during study **(A)**, changes mean of IgG titers and **(B)**, changes mean of IgM titer from baseline through follow-up are shown in the intervention and control group. Repeated measures ANOVA indicated that there is a statically significant difference among the three data collection between both of group.

physical activity behavior was low. In the present study, we found that lifestyle intervention programs in women without COVID-19 infection resulted in a lower risk of getting infected in the intervention group, and the mean of Ig M and IgG against the coronavirus titers increased in the follow-up in both groups.

A previous paper indicated that a web-based intervention on nutritional status, physical activity, and health-related quality significantly decreased systolic and diastolic blood pressure within groups in a patient with metabolic syndrome (22). Our results in terms of blood pressure were contrary to these findings. In 2021, a systematic literature review and meta-analysis yielded a significant decrease in body weight and BMI (30). Our findings are consistent with a recent systematic literature review and meta-analysis. The meta-analysis showed that web-based digital

TABLE 5 Evaluation of main outcome changes using a general linear model (GLM) with generalized estimating equations (GEE) approach.

	Group effect[a]		Time effect				Interaction (group) * (Time)			
	B (CI)	*p*-value	Post-intervention[b]		Follow up[b]		(Group = intervention) * (time = post-intervention)		(Group = intervention) * (time = follow up)	
			B (CI)	*p*-value	B (CI)	*p*-value	B (CI)	*p*-value	B (CI)	*p*-value
Weight—kg	1.1 (−1.5 to 3.7)	0.392	1.2 (1.02 to 1.4)	<0.001	0.9 (0.6 to 1.3)	<0.001	−1.4 (−1.7 to 1.2)	<0.001	−1.8 (−2.3 to −1.2)	<0.001
BMI—kg/m^2	0.42 (−0.56 to 1.4)	0.402	0.45 (0.40 to 0.57)	<0.001	0.36 (0.22 to 0.50)	<0.001	−0.54 (−0.67 to −0.41)	<0.001	−0.69 (−0.90 to −0.48)	<0.001
Physical activity behavior	0.24 (−0.38 to 0.85)	0.442	0.01 (−0.008 to 0.02)	0.286	0.04 (−0.10 to 0.2)	0.615	–	–	–	–
Total energy-Kcal/day	91.8 (−68.9 to 252.6)	0.263	−11.3 (−130.1 to 107.4)	0.852	127.7 (−33.9 to 289.4)	0.122	−206.5 (−377 to −35.9)	0.018	−183.6 (−395.7 to 28.5)	0.090
Carbohydrate-g/day	12 (−23.2 to 47.3)	0.50	−10.8 (−38.9 to 17.3)	0.452	1.1 (−24.3 to 26.6)	0.930	−50.7 (−91.9 to −9.4)	0.016	−51.3 (−90.2 to −12.4)	0.010
Protein-g/day	1.6 (−2.2 to 5.4)	0.409	−4.3 (−8.1 to −0.49)	0.027	3.60 (−0.40 to 7.6)	0.078	–	–	–	–
Fat-g/day	16.1 (−1.4 to 33.6)	0.071	−6.1 (−12.7 to 0.44)	0.068	−4 (−11.2 to 3)	0.260	−23.2 (−40.1 to −6.3)	0.007	−26.2 (−43.1 to −9.3)	0.002
Healthy dietary behavior	0.58 (−0.32 to 1.5)	0.205	0.13 (−0.75 to 1)	0.771	0.66 (−0.23 to 1.5)	0.147	–	–	–	–
IgG Titers	−0.14 (−0.39 to 0.10)	0.248	0.01 (−0.16 to 0.18)	0.913	0.57 (0.24 to 0.90)	0.001	–	–	–	–
IgM Titers	0.01 (−0.03 to 0.06)	0.483	0.09 (0.06 to 0.11)	<0.001	0.38 (0.32 to 0.44)	<0.001	–	–	–	–

OR, odds ratio; CI, confidence interval.

[a]Intervention group is compared with the control group (reference).

[b]Post-intervention and follow-up time are compared with baseline.

Analyses are based on general linear model (GLM) with generalized estimating equation (GEE).

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intervention led to greater weight loss in the short term (31). A lifestyle intervention showed that the mean bodyweight of the subjects in the intervention group decreased compared with that in the control group (32). Sevilla et al. conducted lifestyle exercise and nutrition intervention, and body weight did not change after the intervention. Our results in terms of body weight were different from the findings reported by Sevilla et al. (33). We observed a reduction in body weight and BMI. Our findings are consistent with a recent systematic literature review and meta-analysis, showing that multi-component worksite wellness programs improve diet and body weight (30). The present analysis supported previous research implying that web-based intervention, decreased total calorie, fat, and carbohydrate could be of benefit (22). Nevertheless, the dietary behavior included in this study indicated an increased mean score that was not statistically significant. In line with the current study, Sevilla et al. reported that nutrition and adherence to the Mediterranean diet were effective (33). In a cross-sectional study conducted on undergraduate nursing and medical students, a 10-index scale increment of digital healthy diet literacy was associated with increased healthy eating behavior in students (34). However, social distancing during the COVID-19 pandemic has had an impact on individuals' behaviors, reducing the level of physical activity and worsening dietary habits (35); therefore, lifestyle intervention is necessary during the pandemic. A systematic review and meta-analysis concluded that the intervention increased the physical activity of the participants in vigorous and moderate physical activities (36). According to a systematic review, when moderate- to vigorous-intensity physical activity is >150 min/week, it could prevent weight gain (37). Poppe's study reported an increase in moderate and moderate to vigorous intensity levels of physical activity (38). A randomized controlled trial indicated that an educational intervention increased physical activity during the COVID-19 pandemic (33). The results herein are in accordance with those of previous investigations.

Several possible explanations could be considered for the decreased weight and BMI in the intervention group. The main reason is the decrease in carbohydrate and total fat consumption in this group in comparison with the control group. Further increase in physical activity level in the intervention group compared to that in the control group is another reason behind weight loss and BMI. The growth in the level of physical activity and reduction in certain macronutrients in the intervention group compared to those in the control group was using proper educational curriculums in the intervention group. Despite social distancing and mobility restrictions, intensive physical activity had an increasingly significant effect on both groups and the intervention group had a MET-min/week closer to 1,500 MET-min; this score is categorized as a moderate level of physical activity (27). Greater improvement in the total physical activity in the intervention group may be owing to the effectiveness of the web-based intervention. Of course, vaccination started worldwide at the time of the follow-up of this study, and the decrease in home quarantine caused all levels of physical activity to increase in the control group in the follow-up compared to the baseline.

The total energy intake restriction, increased protein intake, and physical activity might play a role in the changes in adipose cells' size and affect anti-inflammatory (11). Obesity affects immunity and there is a relationship between obesity and various infectious diseases. Obesity causes mild chronic inflammation violating innate immunity and adaptation (39). Physical activity affects the immune system and its anti-viral defenses. Several mechanisms interfere with the effect of exercise on cytokines, the increase in physical activity is related to the reduction in fat mass and subsequently a decrease in adipokine secretion and induction of an anti-inflammatory effect through releasing cytokines from contracting skeletal muscle (40). There is the concept of the inverted J theory, where moderate exercise, such as walking, reduces susceptibility to infection, and prolonged, high-intensity exercise increases it (41). In our study, moderate physical activity and walking indicated a further increase in the intervention group than in the control group. In general, the level of physical activity was moderate in the present study, which contributed to boosting the immune response (42). Owing to the importance of the spread of worldwide pandemics, especially COVID-19, lifestyle interventions must be effective and available to vulnerable populations. The Internet, smartphone, and technology programs prepare opportunities for implementing lifestyle interventions (22).

In the current study, we used modern technologies, such as web-based lifestyle intervention strategies for women who are among the high-risk populations. The participants had similar social and cultural (socio-cultural) characteristics because they lived in a province with the same socio-cultural characteristics. Herein, we observed favorable results. These findings indicated that web-based lifestyle intervention could effectively improve body weight, BMI, total energy and carbohydrate intakes, total fat, and protein consumption (total and moderate physical activity, and walking) levels, and healthy dietary behavior scores strengthen the immune response led to a lower prevalence of COVID-19 in the intervention group.

The strengths of the present study include a randomized control trial, large sample size, three months of follow-up, the use of educational approaches, such as PowerPoint, and the use of web-based lifestyle intervention. However, this investigation has certain limitations. Using 24-hour recall dietary in data collection, the error measurement was unavoidable. Accordingly, the intervention programs were relatively short so that they would not overlap with the vaccination process.

Conclusion

Our results give support to the effectiveness of interactive web-based lifestyle programs in improving weight, BMI, nutritional status, and physical activity which can be effective in boosting immunity and could help prevention of COVID-19. The integration of interactive web-based programs into primary health care practices such as prevention of the pandemics, especially COVID-19, offers possibilities for on-time interaction in a high-risk population with several advantages for administrators of the preventive strategies. Furthermore, the findings of the current study show that web-based lifestyle interventions could be considered beneficial for

decreasing the risk of chronic diseases, particularly in vulnerable populations. However, further research is required to corroborate these findings and apply newer technology in the prevention of pandemics.

Data availability statement

The datasets presented in this article are not readily available because restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided. Requests to access the datasets should be directed to MZ, zaremaryam119@gmail.com.

Ethics statement

The studies involving humans were approved by AIR.ARUMS.REC.1399.284, Approval code Irct.ir: IRCT20221228056969N. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MZ designed the study, secured the funding, designed the curriculum training strategy of trial and site education coordinated the study, analyzed data, and drafted the manuscript. AK designed the study, designed the training strategy content of the trial and held training for the class, and drafted the manuscript. FP coordinated the study and drafted the manuscript. JM contributed to the design of the trial and drafted the manuscript. All the authors have made an essential contribution to the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1172014/full#supplementary-material

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Evaluating myelophil, a 30% ethanol extract of *Astragalus membranaceus* and *Salvia miltiorrhiza*, for alleviating fatigue in long COVID: a real-world observational study

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Background: Persistent post-infectious symptoms, predominantly fatigue, characterize Long COVID. This study investigated the efficacy of Myelophil (MYP), which contains metabolites extracted from *Astragalus membranaceus* and *Salvia miltiorrhiza* using 30% ethanol, in alleviating fatigue among subjects with Long COVID.

Methods: In this prospective observational study, we enrolled subjects with significant fatigue related to Long COVID, using criteria of scores of 60 or higher on the modified Korean Chalder Fatigue scale (mKCFQ11), or five or higher on the Visual Analog Scale (VAS) for brain fog. Utilizing a single-arm design, participants were orally administered MYP (2,000 mg daily) for 4 weeks. Changes in fatigue severity were assessed using mKCFQ11, Multidimensional Fatigue Inventory (MFI-20), and VAS for fatigue and brain fog. In addition, changes in quality of life using the short form 12 (SF-12) were also assessed along with plasma cortisol levels.

Results: A total of 50 participants (18 males, 32 females) were enrolled; 49 were included in the intention-to-treat analysis with scores of 66.9 ± 11.7 on mKCFQ11 and 6.3 ± 1.5 on the brain fog VAS. After 4 weeks of MYP administration, there were statistically significant improvements in fatigue levels: mKCFQ11 was measured at 34.8 ± 17.1 and brain fog VAS at 3.0 ± 1.9. Additionally, MFI-20 decreased from 64.8 ± 9.8 to 49.3 ± 10.8, fatigue VAS dropped from 7.4 ± 1.0 to 3.4 ± 1.7, SF-12 scores rose from 53.3 ± 14.9 to 78.6 ± 14.3, and plasma cortisol levels also elevated from 138.8 ± 50.1 to 176.9 ± 62.0 / mL. No safety concerns emerged during the trial.

Conclusion: Current findings underline MYP's potential in managing Long COVID-induced fatigue. However, comprehensive studies remain imperative.

Clinical Trial Registration: https://cris.nih.go.kr, identifier KCT0008948.

KEYWORDS

Long COVID, fatigue, Myelophil, *Astragalus membranaceus*, *Salvia miltiorrhiza*

1 Introduction

Long COVID, often referred to as post-acute sequelae of COVID-19, is a multifaceted condition marked by persistent and frequently severe symptoms that emerge 2–3 months after an infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Common manifestations include fatigue, body pain, mood disturbances, cognitive issues, and respiratory complications (Chuang et al., 2023). Fatigue, often accompanied by cognitive complaints like "brain fog", is one of the most challenging symptoms of Long COVID (Chasco et al., 2022). The prevalence of post-COVID-19 fatigue ranges from 9% to 58%, influenced by follow-up duration, study population characteristics, recruitment methods, and evaluation depth (Verveen et al., 2022). Given the profound medical and socio-economic implications of Long COVID's fatigue, particularly its effects on work productivity and quality of life (Lunt et al., 2022), it's imperative that affected individuals receive specialized care and support.

While treatments for Long COVID fatigue are still emerging, behavioral interventions have shown potential efficacy in addressing post-infection fatigue conditions (Kuut et al., 2023). Currently, there's no established drug treatment targeting Long COVID fatigue. However, strategies initially designed for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), a condition that shares pathophysiological similarities with Long COVID, are under investigation (Qanneta, 2022). Recent studies have particularly highlighted their shared hallmarks in immune dysregulation, energy metabolism, and the pivotal role of the hypothalamic-pituitary-adrenal (HPA) axis (Komaroff and Lipkin, 2023).

One such potential therapeutic agent is Myelophil (MYP), a 1:1 mixture of the 30% ethanol extracts of *Astragalus membranaceus* and *Salvia miltiorrhiza*. Traditionally used to treat chronic fatigue-related disorders, MYP exhibited moderate benefits in a recent phase 2 RCT with 98 ME/CFS patients, showing pronounced effectiveness for those with severe symptoms (Joung et al., 2019a). Its potential benefits for ME/CFS highlight the need to investigate its effectiveness specifically against Long COVID-related fatigue, which may involve unique pathophysiological pathways influenced by SARS-CoV-2.

In this prospective observational study, our objective is to evaluate the effectiveness of MYP in alleviating fatigue symptoms in Long COVID patients. We aim to observe and analyze real-world data from patients who have opted to include MYP in their treatment regime, providing valuable insights into its utility in a practical healthcare setting.

2 Materials and methods

2.1 Participants

This study aims to analyze the outcomes --in individuals who had recovered from COVID-19 and were experiencing severe fatigue or brain fog symptoms and have opted to use MYP as part of their treatment. The data collection is conducted at Daejeon Korean Medicine Hospital of Daejeon University.

Eligible participants were those aged between 13 and 70 who, following a 4-week recovery period, reported these persistent symptoms. The diagnosis of COVID-19 for these participants was verified using the South Korean government's public health system, which provided online access to medical records of individuals diagnosed with COVID-19 until 30 May 2023. Polymerase Chain Reaction (PCR) testing to monitor viral loads was not employed as the study focused on individuals beyond the acute phase of infection, confirmed using rapid antigen tests to verify recovery status and align with the study's aim of assessing MYP's effects on Long COVID symptoms.

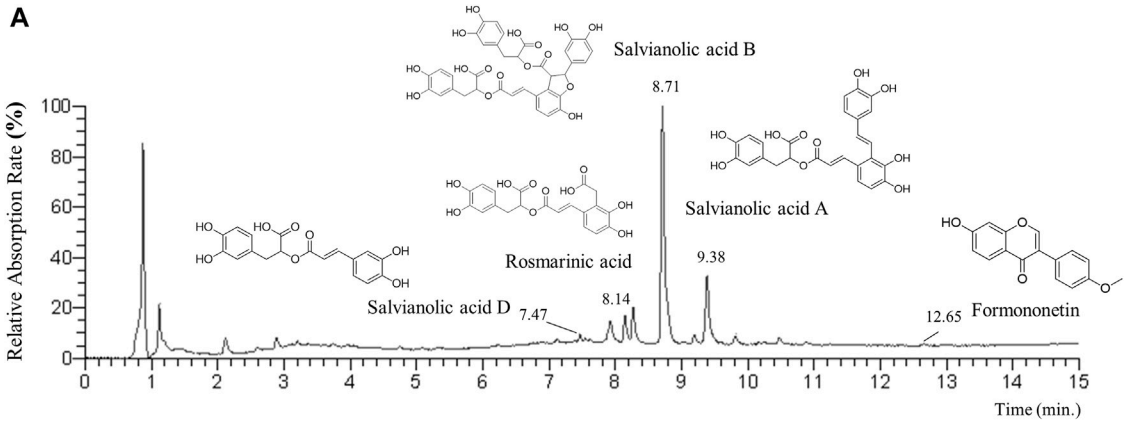
Eligibility for data inclusion requires a score of 60 or higher on the Modified Korean version of the Chalder Fatigue Scale (mKCFQ11), or a score of five or higher on the Visual Analog Scale (VAS) for brain fog. We exclude data from individuals with potential alternative causes for fatigue such as chronic hepatic, cardiovascular, neurological diseases, hypothyroidism, or clinically significant anemia, and those taking other supplements for fatigue/brain fog, with major physical or mental health issues, or recently involved in other clinical trials. For detailed inclusion and exclusion criteria, refer to Supplementary Table S1.

All participants provided informed consent prior to participation. The study was based on the principles of the Declaration of Helsinki and has received ethical clearance from the Institutional Review Board (IRB) of Daejeon University's Korean Medicine Hospital, with the reference number DJDSKH-21-BM-19. Additionally, the study was registered in the Clinical Research Information Service of the Republic of Korea (KCT0008948).

2.2 Study design and treatment

In this prospective observational study, we reference prior clinical trials in ME/CFS where MYP was administered for 4 weeks, providing a basis for our focus on a similar treatment duration for Long COVID symptoms (Joung et al., 2019a). We observed the effects of MYP over this 4-week period in individuals recovering from COVID-19 who report experiencing fatigue and brain fog. During this time, participants are typically advised to consume two MYP capsules orally, twice daily, leading to a total daily dosage of 2,000 mg. According to the Consensus statement on the Phytochemical Characterisation of Medicinal Plant extracts (ConPhyMP) guidlines (Heinrich et al., 2022), MYP, not listed in any country's pharmacopoeia, is classified as a Type B extract due to its commercial utilization.

The MYP capsules were manufactured by Hankook BioPharm Pharmacy, adhering to Korean Good Manufacturing Practice guidelines. Each capsule contained 500 mg of a dried extract prepared with 30% ethanol. This extract was derived in equal proportions from two botanical sources: 1.389 g each of *A. membranaceus* Fisch. ex Bunge (Fabaceae; *A. membranaceus* radix et rhizoma) and *S. miltiorrhiza* Bunge (Lamiaceae; *Salviae miltiorrhizae* radix et rhizoma). The *A. membranaceus* was sourced from Jecheon, South Korea (Batch No. 20191104-JC-HG), and the *S. miltiorrhiza* came from Hebei, China (Batch No. 20200228-CHN-DS), both purchased from Jeong-Seong Drugstore in Daejeon, Korea. The extraction of MYP involved a 20-h process at 80°C with 30% ethanol, yielding a final product concentration of 20.52%



B

Compounds	Quantitative Analysis (mg/g of Myelophil)		
	Astragali Radix	Salviae Radix	Myelophil
Astragaloside IV	1.81 ± 0.01	Not detected	0.87 ± 0.01
Formononetin	0.77 ± 0.01	Not detected	0.21 ± 0.01
Salvianolic acid B	Not detected	86.89 ± 0.19	36.21 ± 0.04
Rosmarinic acid	Not detected	2.53 ± 0.01	1.03 ± 0.01

C

FIGURE 1
(A) UHPLC-MS chromatogram of MYP. **(B)** Quantitative LC-MS analysis of MYP with four reference compounds. **(C)** Image of MYP capsules.

(w/w), which was then stored for subsequent use. The extraction of MYP was performed over a 20-h period at 80°C using 30% ethanol. This process resulted in a final product concentration of 20.52% (w/w), corresponding to a drug-extract ratio of 4.87:1, indicating that approximately 4.87 g of raw material were used to obtain 1 g of extract. Subsequently, the extract was stored for future use. The detailed specifications of MYP are shown in Supplementary Table S2.

To ensure the consistency of MYP's components, we performed fingerprint analysis as previously outlined (Kim et al., 2014), using four reference compounds: astragaloside IV and formononetin from *A. membranaceus*, and salvianolic acid B and rosmarinic acid from *S. miltiorrhiza*. For this analysis, 20 mg of MYP and 10 μg of each reference compound were dissolved in 1 mL of 90% methanol and filtered through a 0.45 μm filter. The samples were analyzed with ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) and liquid chromatography-mass spectrometry (LC-MS) using an LTQ Orbitrap XL system equipped with an electrospray ionization source. Chromatographic separation was carried out on an Acquity BEH C18 column using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 0.3 mL/min. The elution gradient was programmed to maintain 10% B isocratically for 0–1 min, linearly increase from 10% to 90% B over 1–10 min, and hold at 100% B from 10–12 min, ensuring thorough and consistent analysis of the metabolites. The representative sample chromatogram and the corresponding quantitative analysis are presented in Figures 1A, B. The capsule image is shown in Figure 1C.

2.3 Assessment of fatigue and safety

The primary outcome was the change in mKCFQ11 scores after 4 weeks of MYP administration, a tool specifically designed to assess fatigue severity with established reliability and validity (Ahn et al., 2020). The mKCFQ11 comprises 11 questions: seven on physical fatigue (up to 63 points) and four on mental fatigue (up to 36 points), with a combined maximum of 99 points. For secondary measures, the study employed the Multidimensional Fatigue Inventory (MFI-20), the VAS for general fatigue, a specific VAS for brain fog, and the Quality of Life (SF-12) scale to gauge the participants' overall wellbeing.

To explore MYP's pharmacological effects, we measured plasma cortisol with an R&D Systems assay kit (cat. No. KGE008B, Minneapolis, United States) and recorded absorbance at 450 and 570 nm using a Molecular Devices spectrophotometer (Sunnyvale, CA, United States) during fasting hours pre- and post-treatment. Furthermore, a complete blood count (CBC), chemistry profile, and urinalysis were performed to ensure the safety of MYP.

2.4 Estimation of sample size

Using G*Power software (version 3.1.9.7) (Faul et al., 2007), we estimated the necessary sample size for our study. Given that our study design involves a single-arm pre-post comparison, we used the standardized mean difference

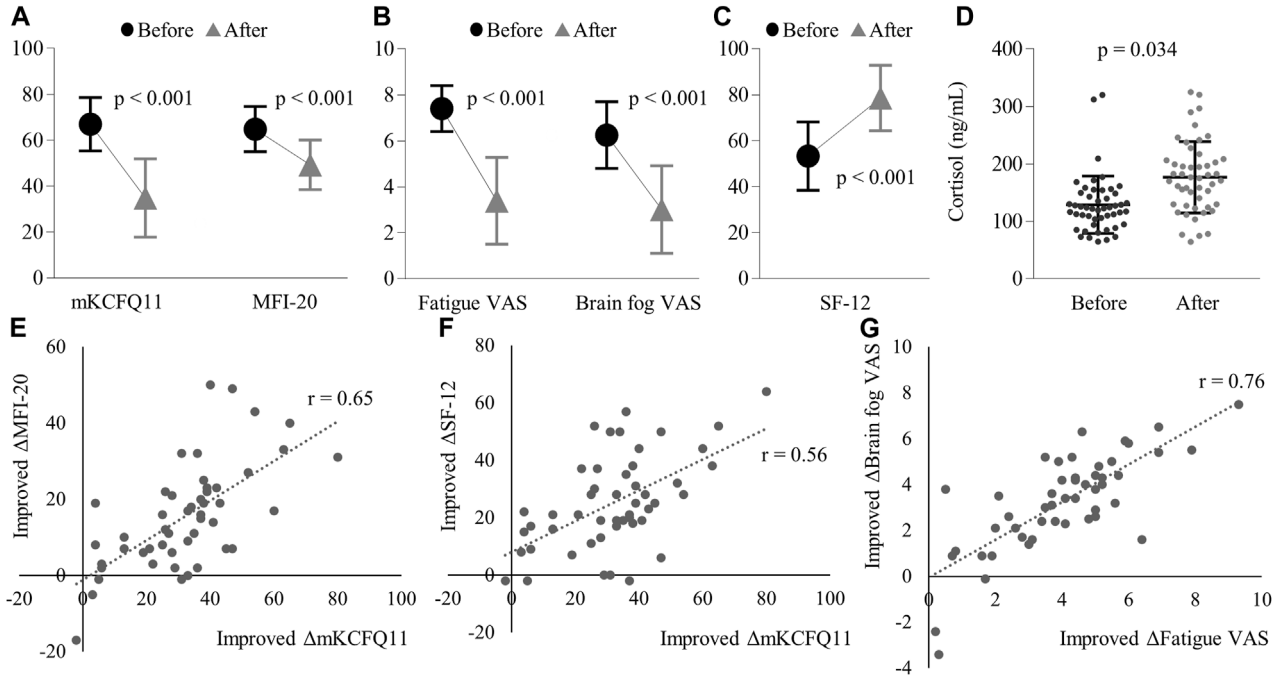


FIGURE 2
Changes before and after treatment in: **(A)** mKCFQ11 and MFI-20 scores, **(B)** Fatigue VAS and Brain fog VAS scores, **(C)** SF-12 scores, and **(D)** Plasma cortisol levels (ng/mL). The figure also demonstrates the correlations of improved changes between **(E)** mKCFQ11 and MFI-20, **(F)** mKCFQ11 and SF-12, and **(G)** Fatigue VAS and Brain fog VAS. Note: 'r' represents the correlation coefficient. Error bars represent standard deviations. Significant reductions were observed in mKCFQ11, MFI-20, Fatigue VAS, Brain Fog VAS, and SF-12 (all $p < 0.001$, data followed Gaussian distribution). Plasma cortisol levels increased significantly post-treatment ($p = 0.034$, non-Gaussian distribution).

(SMD) as our effect size measure. We set the significance level at 0.05, effect size at 0.5, and statistical power at 0.90. An SMD of 0.5, indicating a medium effect size, was chosen based on clinical experience and a recent phase 2 RCT involving MYP, which demonstrated benefits in reducing fatigue among patients with chronic fatigue syndrome (Joung et al., 2019a). This resulted in a minimum required sample size of 44 participants. Anticipating a dropout rate of approximately 10%, we decided to enroll 50 participants to ensure the robustness of our findings. Detailed calculations are provided in the Supplementary Table S3.

2.5 Statistical analysis

Analyses were conducted on the intention-to-treat (ITT) population using the baseline observation carried forward (BOCF) method, which included all participants who completed the baseline assessment and received at least one dose of MYP. A safety analysis encompassed all participants who received at least one dose of the trial medication. Continuous measures, including primary and secondary outcomes from mKCFQ11, MFI-20, VAS, SF-12, and cortisol levels, were compared pre- and post-intervention. The normality of the data was assessed using the Shapiro-Wilk test. For data following a Gaussian distribution, paired *t*-tests were employed. For non-Gaussian data, the Wilcoxon Signed-Rank Test was utilized. Additionally, correlation analyses were performed for these indices. A *p*-value of less than 0.05 was considered statistically significant.

3 Results

3.1 Study population

From December 2021 to April 2023, a total of 50 participants (18 males and 32 females) were enrolled. However, one female participant withdrew due to personal circumstances before the drug administration began. Of the remaining 49 participants (18 males and 31 females) who successfully finished the 4-week treatment and maintained an adherence rate above 75%, all were considered for the ITT analysis.

The mean age of the 49 participants was 42.0 ± 12.2 years (males: 43.3 ± 11.8; females: 41.9 ± 12.2), with a mean BMI of 23.6 ± 3.4 (males: 24.9 ± 2.6; females: 22.9 ± 3.6). On average, participants commenced the trial 139.3 ± 81.4 days after their COVID-19 diagnosis. At baseline, participants displayed pronounced fatigue, with an average mKCFQ11 score of 66.9 ± 11.9 (physical fatigue: 45.1 ± 6.6 and mental fatigue: 21.9 ± 6.6). Their average brain fog VAS score was 6.3 ± 1.5.

3.2 Changes in primary assessment: mKCFQ11 score

After 4 weeks of treatment, the mKCFQ11, our primary assessment, exhibited a significant shift from 66.9 ± 11.7 to

34.8 ± 17.1 ($p < 0.001$). Both components, physical fatigue (from 45.1 ± 6.6 to 23.8 ± 11.3) and mental fatigue (from 21.9 ± 6.6 to 11.1 ± 7.1), showed marked reductions ($p < 0.001$ for both) (Figure 2A). The changes in mKCFQ11 scores were tested for normality using the Shapiro-Wilk test, and the results indicated that the data followed a Gaussian distribution (Shapiro-Wilk statistic = 0.969, $p = 0.218$).

3.3 Changes in secondary assessment

Secondary assessments showed statistically significant improvements with p-values less than 0.001. MFI-20 shifted from 64.8 ± 9.8 to 49.3 ± 10.8, Fatigue VAS changed from 7.4 ± 1.0 to 3.4 ± 1.9, Brain Fog VAS decreased from 6.3 ± 1.5 to 3.0 ± 1.9, and SF-12 increased from 53.3 ± 14.9 to 78.6 ± 14.3 (Figures 2A–C).

The normality of these changes was also tested using the Shapiro-Wilk test. The results indicated that MFI-20 (Shapiro-Wilk statistic = 0.967, $p = 0.192$), Fatigue VAS (Shapiro-Wilk statistic = 0.981, $p = 0.592$), Brain Fog VAS (Shapiro-Wilk statistic = 0.959, $p = 0.089$), and SF-12 (Shapiro-Wilk statistic = 0.969, $p = 0.216$) followed a Gaussian distribution.

In the correlation analysis, the mKCFQ11 showed strong positive correlations with MFI-20 (correlation coefficient $r = 0.65$), Fatigue VAS ($r = 0.70$), and Brain Fog VAS ($r = 0.54$), while demonstrating a strong negative correlation with SF-12 ($r = -0.59$). (Figures 2E–G).

Additionally, there was a marked rise in cortisol levels post-treatment. The cortisol levels increased from 138.8 ± 50.1 ng/mL pre-treatment to 176.9 ± 62.0 ng/mL post-treatment ($p < 0.001$). (Figure 2D). The changes in cortisol levels did not follow a Gaussian distribution (Shapiro-Wilk statistic = 0.952, $p = 0.046$), so the Wilcoxon Signed-Rank Test was used to analyze these changes. This elevation in cortisol showed no significant correlation with mKCFQ11, with r being 0.15.

3.3.1 Safety

One participant (2.04%) experienced mild indigestion but recovered without any specific treatment. No other adverse reactions, including liver and kidney function in blood tests, were observed (data not shown).

4 Discussion

In traditional Korean and Chinese medicine, *A. membranaceus and S. miltiorrhiza* are respectively regarded as fundamental botanical drugs for enhancing two essential components of the human body, Qi and blood, respectively. Qi, understood as the vital energy, sustains bodily operations, including metabolism and growth, whereas blood serves as the crucial nourishing agent. Deficiencies in Qi or blood are linked to symptoms of physical and mental exhaustion (Kim et al., 2016). MYP, as a mixture of these two botanicals, has shown potential in treating ME/CFS.

Preclinical studies have demonstrated that MYP not only protects central neurons from stress-induced damage but also relieves fatigue and cognitive impairment by modulating the HPA axis, inhibiting neuroinflammation, and regulating cholinergic activity (Lee et al., 2015; Song et al., 2021). The optimal dosage for MYP, informed by these studies and further animal toxicity investigations, achieved peak effectiveness in mice at dosages exceeding 200 mg/kg/day (Kim et al., 2014). Additionally, the safe dosage for humans, or the no-observed-adverse-effect level (NOAEL), was established at 694 mg/kg, following toxicity evaluations with both rodents and non-rodents (beagle dog) (Joung et al., 2019b). A phase 2 clinical trial highlighted MYP's potential, showing notable benefits in treating ME/CFS, especially for individuals with severe symptoms (Joung et al., 2019a).

Recent research into Long COVID has revealed significant disruptions such as T-cell dysregulation, systemic inflammation, and a disjointed immune response to SARS-CoV-2 (Yin et al., 2024). This condition is marked by increased migration of $CD4^+$ T-cell to inflamed tissues, exhaustion of SARS-CoV-2-specific $CD8^+$ T-cell, and heightened antibody levels, creating a mismatch between cellular and humoral responses. MYP might counteract these issues through its actions on both the central nervous system and systemic inflammation. It modulates neurotransmitter pathways, notably serotonin and dopamine, which are known to alleviate neuroinflammatory processes and neurotransmitter imbalances, issues prevalent in Long COVID (Song et al., 2021; Reiss et al., 2023). Additionally, MYP's regulation of key mediators like transforming growth factor β (TGF-β) and its influence on the HPA axis provide anti-inflammatory benefits across multiple organ systems, potentially reducing the widespread inflammation characteristic of Long COVID (Kim et al., 2013; Lee et al., 2019). Given these properties, we initiated this real-world observational study to investigate the potential of MYP in alleviating symptoms of Long COVID fatigue.

The radical reduction of fatigue symptoms post-MYP treatment, as reflected in the mKCFQ11 scores (approximately 50% of baseline severity), offers a promising insight into potential interventions for Long COVID-induced fatigue. Additionally, this anti-fatigue efficacy of MYP is strongly supported by other measurements using fatigue-related tools: 24% in MFI-20, 54% in fatigue VAS, and 52% in brain fog VAS (Figures 2A, B). As expected, the QOL level also improved notably, showing a 47% increase from the baseline score of SF-12 (Figure 2C). Furthermore, there are strong and consistent correlations among the changed scores of these measurements (Figures 2E–G). Such a transition underscores a significant overall improvement in fatigue in Long COVID patients following MYP administration. Based on our prior research, the mKCF11 scale scores can be interpreted as follows: 0–25 points suggest no/mild fatigue; 25–40 indicate general fatigue; 40–60 represent idiopathic chronic fatigue levels; and scores exceeding 60 are indicative of ME/CFS levels (Lim and Son, 2022). At the baseline, 40 participants exhibited intense fatigue comparable to ME/CFS levels. However, after 4 weeks of MYP treatment, only four participants still had scores above 60. Remarkably, post-treatment, 35 participants had scores of 40 or below, of which 14 achieved scores of 25 or less (data not shown).

In our real-world observational study, we documented significant effects of MYP on Long COVID-related fatigue, highlighting the importance of patient-centered, value-based outcomes in contemporary medical practice. While our study's design, a non-randomized, open-label observational study

without a control group, calls for a careful interpretation of these results, the observed improvements are nonetheless compelling. When compared to placebo effects reported in prior RCTs, our findings suggest a potentially greater efficacy of MYP. For example, previous RCTs on Long COVID fatigue using the Chalder Fatigue Questionnaire (CFQ-11, with a maximum possible score of 33) or the Visual Analog Fatigue Scale (VAFS, with a maximum possible score of 10) demonstrated varying placebo responses: one exhibited a 5.7% decrease over 4 weeks, reducing from 28.1 to 26.5 on the CFQ-11 (Finnigan et al., 2023), while another reported a 22.5% decline in just 14 days, moving from 25.7 to 20.0 on the CFQ-11 (Rathi et al., 2021), and a further study noted an 18.7% reduction in VAFS scores over 2 weeks, from 7.34 to 5.97 (Harandi et al., 2024, p. 19). In their respective treatments, the metabolic modulator group experienced a 19.9% decrease in CFQ-11 (from 26.2 to 21.0), whereas the enzyme complex and probiotic group achieved a 67.1% improvement in CFQ-11 (from 25.8 to 8.5). Similarly, the Amantadine group demonstrated a 57.3% reduction in VAFS (from 7.90 to 3.37). These comparisons suggest that MYP may offer benefits beyond those attributable to placebo, highlighting the need for further controlled research to validate these promising results.

Given that 55.9% of patients with Long COVID-attributed fatigue reported enduring symptoms for six to 12 months and 17.6% for over a year (Oliveira et al., 2023), there's a clear persistence of these fatigue symptoms. Strikingly, this enduring fatigue closely resembles the core symptoms of ME/CFS, especially evident in shared manifestations like Brain Fog such as memory and concentration decline (Komaroff and Lipkin, 2023). Such parallels have rekindled interest in the traditional hypothesis associating viral infections with the etiology of ME/CFS (Wong and Weitzer, 2021). The 52% reduction in brain fog VAS in our study suggests that the alleviation of fatigue may be intricately linked to the pharmaceutical activities of MYP on brain pathology. In our previous animal studies, MYP demonstrated notable brain focused effects by modulating neurotransmitter pathways, regulating TGF-β expression, and protecting against chronic cold-stress-induced brain damage in mice (Kim et al., 2013; Song et al., 2021). Based on these findings, we cautiously suggest that improvements related to MYP could offer a potential therapeutic advantage, though not conclusively establishing its dominance.

A noteworthy aspect of our findings was the marked elevation in cortisol levels post-treatment. Cortisol, often termed the "stress hormone", plays intricate roles in metabolism, immune responses, and the maintenance of circadian rhythms (Russell and Lightman, 2019). Its post-treatment rise, in tandem with the observed reduction in fatigue symptoms, raises an intriguing hypothesis. This pattern could suggest a potential recalibration of the HPA axis, which is frequently dysregulated in chronic fatigue conditions (Tomas et al., 2013). However, the correlation between cortisol levels and fatigue scores was not statistically significant, necessitating cautious interpretation of these initial results. To further understand this relationship and explore MYP's therapeutic potential for Long COVID fatigue, larger-scale research using double-blind, placebo-controlled studies is essential.

This study has several limitations that are important to consider. Firstly, the absence of a control group in this observational study significantly limits our ability to definitively attribute observed effects to the intervention alone, without potential placebo influences. Secondly, the open-label nature of the study could introduce bias, as participants' awareness of the treatment might affect their symptom reporting. Thirdly, with a limited participant pool, our results might not be universally applicable, as the demographic may not reflect the diverse spectrum of Long COVID patients. Fourthly, the 4-week timeframe may not sufficiently capture the long-term efficacy or potential side effects of MYP.

Additionally, our study did not control for variables such as participants' diets or exercise routines, which could influence the effects observed and introduce bias. Nutritional supplements and a balanced diet, recommended for alleviating symptoms of post-COVID-19 fatigue syndrome, may include essential fatty acids, antioxidants, and nutrients like vitamin C, B vitamins, sodium, magnesium, and zinc, which are known to mitigate symptom severity (Barrea et al., 2022). Additionally, physical activity is considered a potential method to alleviate Long-COVID fatigue, although definitive data supporting its effectiveness is currently insufficient (Coscia et al., 2023). Lastly, the absence of assessments for oxygen saturation and detailed pulmonary evaluations is a notable limitation. Pulmonary impairments, often identified through tests like pulmonary function tests, 6-min walk tests, and quality of life assessments, are frequently reported in Long COVID cases (Christopher et al., 2024). Such declines in pulmonary function are crucial contributors to the fatigue, malaise, and decreased quality of life experienced by patients with Long COVID.

Given the prevalent challenges of Long COVID-induced fatigue and the absence of effective treatments, our findings hint at the potential therapeutic role of MYP. However, rigorous and extensive studies are needed to validate its efficacy and mechanism in treating Long COVID fatigue.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Institutional Review Board (IRB) at Daejeon Korean Medicine Hospital of Daejeon University, with the reference number DJDSKH-21-BM-19. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

J-YJ: Formal Analysis, Writing–original draft. J-SL: Methodology, Validation, Writing–review and editing. YC: Investigation, Validation, Writing–review and editing. YK: Data

curation, Methodology, Writing–review and editing. H-MO: Investigation, Writing–review and editing. H-SS: Investigation, Writing–review and editing. C-GS: Conceptualization, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1394810/full#supplementary-material

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Network-based pharmacology and UHPLC-Q-Exactive-Orbitrap-MS reveal *Jinhua Qinggan* granule's mechanism in reducing cellular inflammation in COVID-19

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Introduction: The outbreak of SARS-CoV-2, leading to COVID-19, poses a major global health threat. While specific treatments and vaccines are under development, Traditional Chinese Medicine (TCM) has historically been effective against pandemics, including viral pneumonias. Our study explores the efficacy and mechanisms of Jinhua Qinggan Granules (JHQG) in treating COVID-19.

Methods: We analyzed JHQG's components using UHPLC-Q-Exactive-Orbitrap-MS, identifying 73 compounds. Network pharmacology and single-cell RNA sequencing (scRNA-seq) were used to assess JHQG's effects on immune cells from peripheral blood mononuclear cells (PBMCs). Literature review supported the antiviral and anti-inflammatory effects of JHQG.

Results: JHQG targets were found to interact with immune cells, including neutrophils, monocytes, plasmablasts, and effector T cells, reducing their overactivation in severe COVID-19. JHQG's modulation of these cells' activity likely contributes to reduced inflammation and improved clinical outcomes.

Discussion: Our findings provide insights into JHQG's mechanism of action, highlighting its potential in controlling the inflammatory response in COVID-19 patients. The study supports the use of JHQG as a safe and effective treatment for COVID-19 and similar viral infections, leveraging its ability to modulate immune cell activity and reduce inflammation.

KEYWORDS

***Jinhua Qinggan* granules, traditional Chinese medicine, COVID-19, single-cell RNA sequencing, cellular inflammation**

Main

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared official in 2020. Four years on, the global crisis remains unabated (1–3). Infection with SARS-CoV-2 triggers a myriad of immune reactions in the peripheral blood, including heightened pro-inflammatory cytokine levels (4–6), the emergence of inflammatory monocyte subsets (7), lymphopenia (8, 9), T-cell exhaustion (10, 11), and plasma-cell overreactivity (12). Such amplified immune responses can precipitate a cytokine storm, which worsens patient prognoses (13).

After a thorough evaluation, the World Health Organization (WHO) has endorsed Traditional Chinese Medicine (TCM) as a valuable complementary approach for treating mild to moderate cases of COVID-19. TCM has demonstrated efficacy in accelerating viral clearance, alleviating clinical symptoms, and reducing hospitalization durations (14). Similarly, China's health authorities have sanctioned various TCM treatments for COVID-19 (15). One such therapy, Jinhua Qinggan Granules (JHQG), is advocated for fatigue and fever symptoms in affected individuals (16).

Studies confirm that JHQG not only addresses viral infections but also modulates immune responses, thereby slowing the disease progression (17). In this context, recent clinical investigations have provided evidence supporting JHQG's properties in reducing cellular inflammation. For instance, a clinical study observed a significant decrease in C-reactive protein (CRP) - a marker of inflammation - following the administration of JHQG in COVID-19 patients, indicating a substantial anti-inflammatory response ($P<0.05$) (17). Another pivotal aspect of JHQG's mechanism of action involves the mitigation of the cytokine storm, a severe hyperinflammatory condition associated with COVID-19. A honeysuckle extract component of JHQG was found to significantly reduce cytokine levels (18). Moreover, IL-6, a cytokine critically involved in immune dysregulation leading to cytokine storm, was observed to be significantly reduced in COVID-19 patients treated with JHQG (19). These findings are incrementally establishing JHQG's role not just in treating viral infection, but importantly, in regulating the immune system response that is critical to patient recovery.

Nevertheless, JHQG's intricate makeup, comprising 12 distinct medicinal components, including Lonicera japonica Thunb. (Jinyinhua, 金銀花), Gypsum Fibrosum (Shigao, 石膏), Ephedra sinica Stapf (Mahuang, 麻黄), Prunus armeniaca L. (Kuxingren, 苦杏仁), Scutellaria baicalensis Georgi (Huangqin, 黄芩), Forsythia suspensa (Thunb.) Vahl (Lianqiao, 連翹), Fritillaria thunbergii Miq. (Zhebeimu, 浙貝母), Anemarrhena asphodeloides Bunge (Zhimu, 知母), Arctium lappa L. (Niubangzi, 牛蒡子), Artemisia annua L. (Qinghao, 青蒿), Mentha canadensis L. (Bohe, 薄荷), and Glycyrrhiza inflate Batalin (Gancao, 甘草) (20). Although Traditional Network Pharmacology attempts to extract compounds from HERB databases (21), discrepancies between these databases and JHQG's actual constituents complicate the identification of its anti-inflammatory ingredients.

Addressing this complexity, our investigation involved the analysis of 73 authentic compound components identified in JHQG via the HPLC-Q-Exactive-Orbitrap-MS technique (20). We pinpointed the target proteins associated with these compounds within a comprehensive Network Pharmacological database (21). By leveraging single-cell sequencing data from a COVID-19 patient cohort and healthy individuals (22), we constructed an innovative interaction network connecting herbal compounds, target proteins, and peripheral blood cells. This enabled us to identify monocytes, plasma cells, granulocytes, and effector T cells as pivotal in JHQG's mitigation of inflammation.

Results

Mass spectrometry-based target protein network of the active ingredients in Jinhua Qinggan Granules against COVID-19

Mass spectrometry has revealed the complex network of target proteins interacting with the active constituents in Jinhua Qinggan Granules (JHQG), offering insights into their therapeutic effects against COVID-19. Recent study identified 73 components within JHQG through mass spectrometry analysis (Supplementary Table 1) (20). We standardized the names and classification details of these ingredients using the HERB database. Predominantly, flavonoid analogs represented 41% of these components. Notably, caffeoylquinic acid, phenolic acid, and alkaloids also comprised significant proportions, exceeding 5%, 9%, 7%, and 6%, respectively (Figure 1A). We determined the distribution of these compounds across various herbs; for instance, Lianqiao contributed 17%, Huangqin 14%, Jinyinhua 13%, Mahuang 14%, and Niubangzi 8% of the constituents (Figure 1B).

To establish a compound-target protein interaction network that could influence serum inflammation in COVID-19 patients, we first analyzed peripheral blood mononuclear cells (PBMCs) from seven hospitalized COVID-19 patients, including four with acute respiratory distress syndrome, and compared them to six healthy controls. We identified 145 up-regulated and 475 down-regulated differentially expressed genes (DEGs) (Figure 1C) and linked these to the 73 target proteins associated with the JHQG compounds from the HERB database (Supplementary Table 2). The analysis revealed significant DEG representation in proteins associated with herbs such as Zhimu, Zhebeimu, and Qinghao, each exceeding 5% (Figure 1D). The top ten compounds implicated in the network—peimisine, sophoricoside, scutellarin, irigenin, rosmarinic acid, pectolinarigenin, diosmin, daidzein, liquiritin, and diosgenin—were also identified based on the percentage of DEGs (Figure 1E). Intriguingly, the compounds with the most considerable DEG correspondence—daidzein and pectolinarigenin—were not those with the highest percentage representation alone (Figure 1F). a Leveraging the connections between these compounds, target proteins, and DEGs in COVID-19 patients, we constructed a detailed interaction network for JHQG, visualized using plotpy,

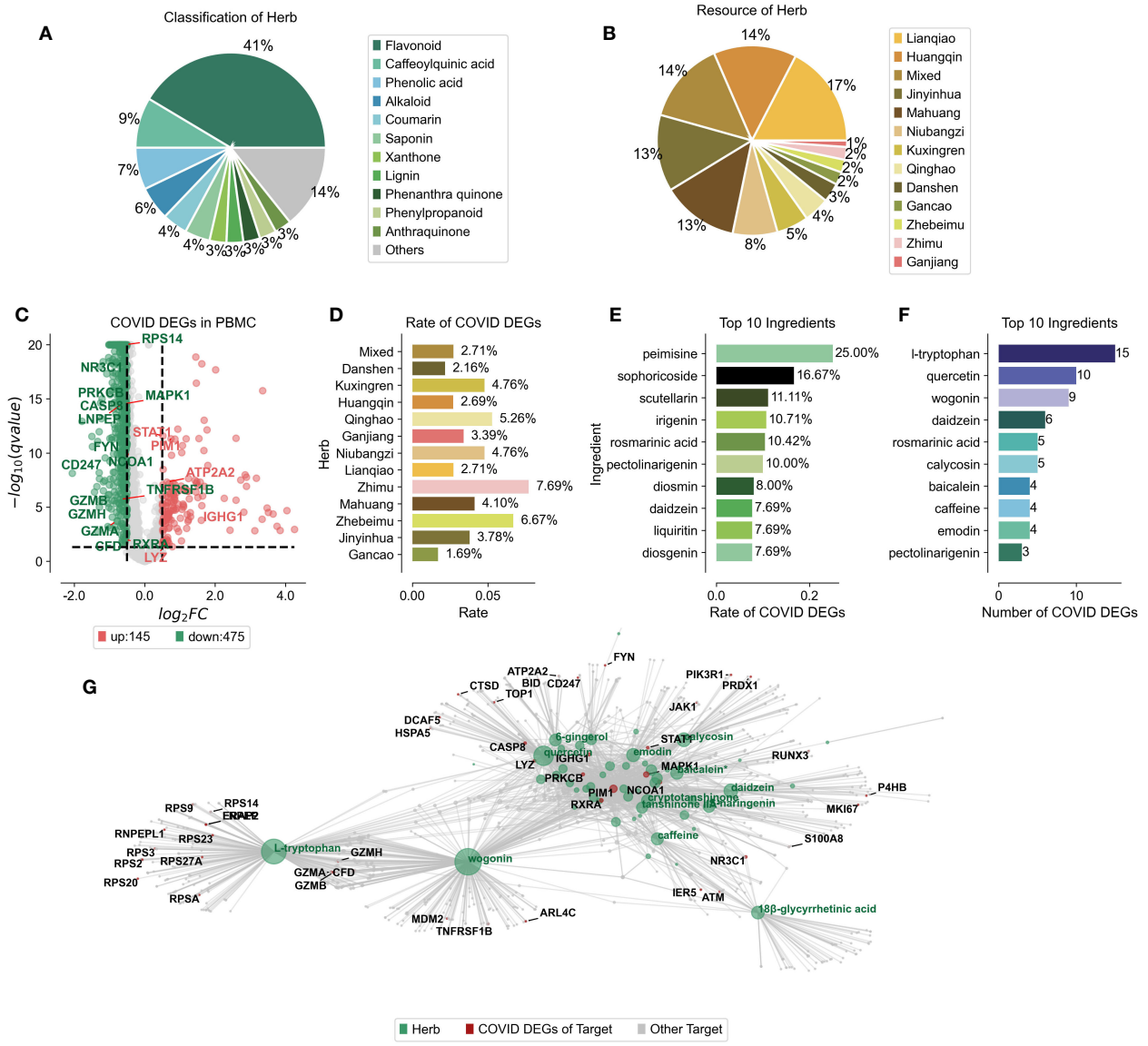

FIGURE 1
Ingredient Composition and Compound-Target Protein Network of Jinhua Qinggan Granules. **(A)** Percentage of compounds classification in the Chinese herbs of Jinhua Qinggan Granules. **(B)** Percentage of compounds resource in the Jinhua Qinggan Granules. **(C)** Differentially expressed genes (DEGs) in PBMC from COVID-19 patients. Horizontal coordinates represent fold changes of differential expression and vertical coordinates represent two-tailed ttest significance test pvalue. **(D)** Proportion of COVID-19 DEGs in the target proteins of different Chinese herbs in Jinhua Qinggan Granules. **(E)** Proportion of top 10 COVID-19 DEGs in the target proteins of compounds in Jinhua Qinggan Granules. **(F)** Number of top 10 COVID-19 DEGs in the target proteins of compound in Jinhua Qinggan Granules. **(G)** Compound-target protein interactions network of Jinhua Qinggan Granules, where green represents compounds, red represents target proteins differentially expressed at COVID-19, and grey represents other target proteins.

with an extended view accessible on GitHub (https://starlitnightly.github.io/Analysis_JHQG_COVID/) (Figure 1G).

An Atlas illustrating the impact of Jinhua Qinggan granules on peripheral blood mononuclear cells in COVID-19 patients

To investigate the immunomodulatory effects of JHQG on peripheral blood mononuclear cells (PBMCs) in COVID-19, we conducted an analysis of scRNA-seq data obtained from eight peripheral blood samples collected from seven hospitalized patients and six healthy individuals, as sampled by Wilk et al's study (22). The seven patients profiled were male, aged 20 to >80 years, healthy controls were asymptomatic, four male and two female, and aged 30–50 years (Supplementary Table 3). Following quality control, a total of 44,721 cells were categorized, including activated granulocytes, B cells, various T cell subsets (CD4 naïve, CD4+, CD4 memory, CD8 effector, CD8 memory), monocytes (CD14+ and CD16+), class-switched B cells, dendritic cells (DCs), plasma cells (IgA+ and IgG+), natural killer (NK) cells, neutrophils, platelets, red blood cells (RBCs), stem cells and eosinophils, and gamma delta (γδ) T cells. Of

these, 28,094 cells were derived from COVID-19 patients, while 16,627 cells were from healthy controls (Figures 2A, B). To enhance the precision and robustness of our analysis, we applied SEACells to calculate 447 metacells from the PBMC data (Figure 2C), resulting in metacells with high purity (over 0.9), low separation (below 0.25), and compactness close to zero, indicating successful metacell extraction (Figures 2D, E).

Analysis of metacells derived from PBMCs of COVID-19 patients showed that the target proteins associated with JHQG were primarily expressed in monocytes, dendritic cells, and plasma cells (Figures 2F, G). Interestingly, upon examining the 45 proteins differentially expressed in COVID-19 against the granules' target proteins, it became apparent that nearly all cell types were affected by Jinhua Qinggan Granules (Figures 2H, I).

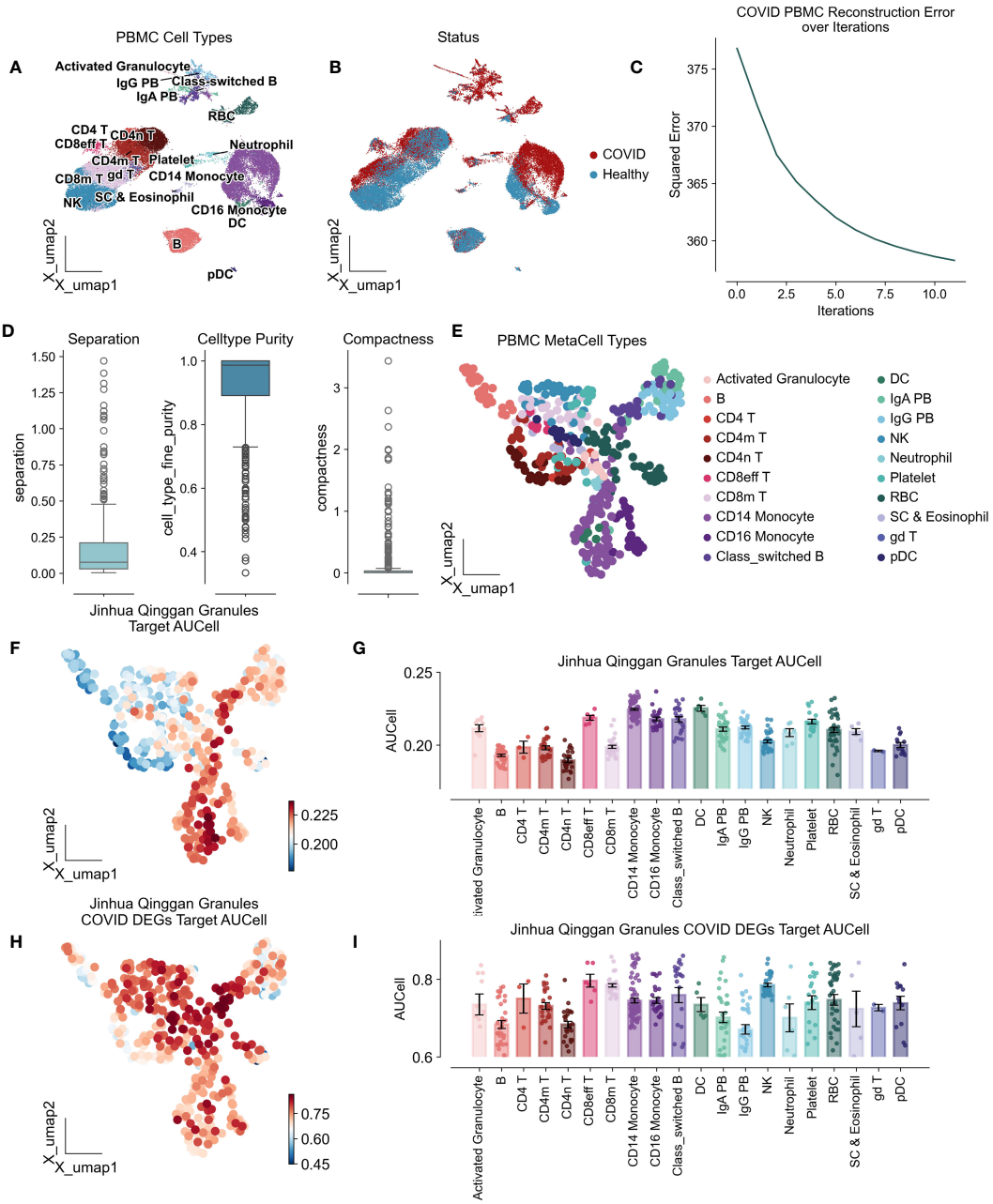

FIGURE 2
Atlas of peripheral mononuclear cells of COVID-19 by the action of Jinhua Qinggan granules. UMAP plot visualizes scRNA-seq from PBMCs in COVID-19 patients and healthy individuals, colored by cell types **(A)** and COVID status **(B)**. **(C)** Metacellular model iteration loss curves with horizontal coordinates representing the number of iterations and vertical coordinates representing the standard deviation. **(D)** Metacellular quality assessment indicators: metacell separation (distance between nearest metacell neighbor in diffusion space; Methods). Greater separation indicates better performance. metacell compactness (average diffusion component standard deviation; Methods). A lower score indicates more compact metacells. **(E)** UMAPs highlighting metacells of the PBMC in COVID-19 patients and healthy individuals. **(F)** UMAPs plot showing the AUCells score of Jinhua Qinggan Granules' Target proteins. **(G)** The AUCells score of JHQG' Target proteins in different cells. **(H)** UMAPs plot showing the AUCells score of JHQG' Target proteins in COVID-19 DEGs. **(I)** The AUCells score of JHQG' Target proteins in COVID-19 DEGs in different cells.

Jinhua Qinggan granules alleviate cellular immune inflammatory networks

In our investigation into the immunomodulatory potential of Jinhua Qinggan Granules in reducing inflammation within peripheral blood, we analyzed the top 20 highly variable genes for each cell type. These findings were cross-referenced with the differentially expressed genes observed in COVID-19 patients and the target genes affected by JHQG. Notably, S100A8, a surface antigen on activated granulocytes, is regulated by rutin, while the proliferation marker MKI67 in CD8+ effector T cells is targeted by daidzein. Additional key genes, such as GZMH in CD8+ memory T cells, CTSD in CD14+ monocytes, RXRA and CFD in CD16+ monocytes, IGHG1 and HSPA5 in class-switched B cells, and CD247 in NK cells, are regulated by various compounds including wogonin, L-tryptophan, quercetin, tanshinone IIA*, formononetin, irigenin, emodin, pectolinarigenin, and calycosin (Figures 3A, B).

Increased levels of S100A8, MKI67, HSPA5, LYZ, CTSD, and IGHG1 were observed in COVID-19 patients. However, the interaction with Jinhua Qinggan Granules components led to a downregulation of these genes. Reducing the expression levels of S100A8, LYZ, and CTSD can mitigate the hyperactive immune response from monocytes, diminishing inflammation. Moreover, lower expression of MKI67 can curtail the overactivity of CD8 effector T cells, while reduced HSPA5 and IGHG1 can decrease the inflammatory antibody release from plasma cells (Figures 3C, D). Collectively, we constructed a network depicting the interactions

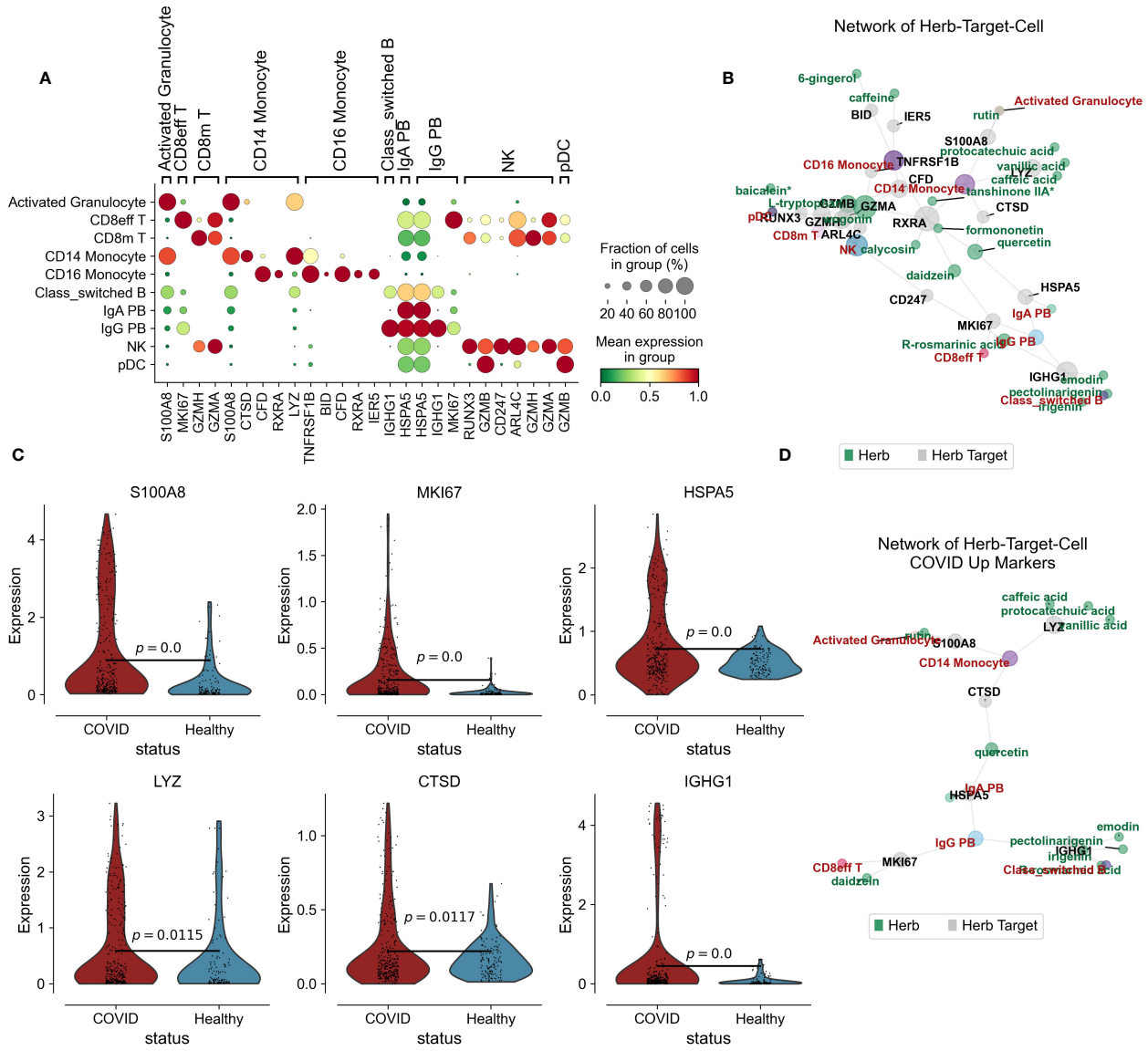

FIGURE 3
Herb-Target-Cell Network of Jinhua Qinggan Granules. **(A)** Dotplot showing the target marker genes' mean expression and fraction of cells in group (%). **(B)** Network of Jinhua Qinggan Granule Herb-Target (COVID-19 DEGs)-Cell (PBMC). Grey represent the Herb Target protein in COVID-19 DEGs, Green represent the compound of Jinhua Qinggan Granule, Red represent the target PBMC. **(C)** Violin plot showing the expression of S100A8, MKI67, HSPA5, LYZ, CTSD and IGHG1 in different states of PBMC. **(D)** Network of Herb-Target (COVID-19 Upregulated DEGs)-Cell (PBMC).

among the components of Jinhua Qinggan Granules, COVID-19-specific target proteins, and cellular responses, aiming to reduce excessive inflammatory and immune reactions.

Additionally, molecular docking simulations were performed to validate the interactions within the network. Among these, the binding affinity of CTSD with quercetin was notable at -5.588 kcal/mol, as was the affinity between HSPA5 and quercetin at -7.975 kcal/mol. Similarly, the binding energies for IGHG1 with different compounds such as emodin, irigenin, pectolinarigenin, and rosmarinic acid showed promising values (-6.64, -7.819, -7.453, and -6.561 kcal/mol, respectively), as did the interaction of LYZ with caffeic acid (-5.688 kcal/mol), MKI67 with daidzein (-5.688 kcal/mol), and S100A8 with rutin (-6.313 kcal/mol) (Figure 4).

Discussion

Traditional Chinese Medicine (TCM) offers a gentler therapeutic approach for COVID-19 patients with varying disease severity, mitigating clinical deterioration (23). This study suggests that early administration of JHQG alleviates viral infection symptoms. Individuals who survived Omicron infection have subjectively reported alleviation of symptoms associated with upper respiratory tract infections, including cough and sore throat, following treatment with JHQG (24). Furthermore, cohort studies demonstrated that the time until viral nucleic acid clearance (test negative) and recovery from pneumonia were significantly shorter in the JHQG group compared to the control group, averaging 10 ± 4 days versus 10 ± 5 days, and 8 ± 4 days versus 10 ± 5 days, respectively (P = 0.010 and 0.021). Moreover, the JHQG group exhibited a significantly higher 7-day viral clearance rate of 56.82% compared to 27.78% in the control group (P = 0.009), with no reported adverse effects of the treatment (25). Overall, these results collectively demonstrate the effectiveness of JHQG in treating COVID-19.

Beyond promoting viral clearance, JHQG also appears to mitigate immune inflammation and reduce the duration of such inflammation in patients (26). However, the mechanisms underlying JHQG's anti-inflammatory effects and its role in reducing the duration of inflammation remain to be fully elucidated (17). Our study introduces a novel network pharmacological framework that leverages mass spectrometry data on authentic JHQG components, a compound-target database, and single-cell patient data to pinpoint the immune cells that JHQG modulates in peripheral blood. Specifically, we discovered that rutin inhibits activated granulocytes, cells frequently associated with severe COVID-19 progression (27), while monocytes and plasmablasts—which can elicit a strong

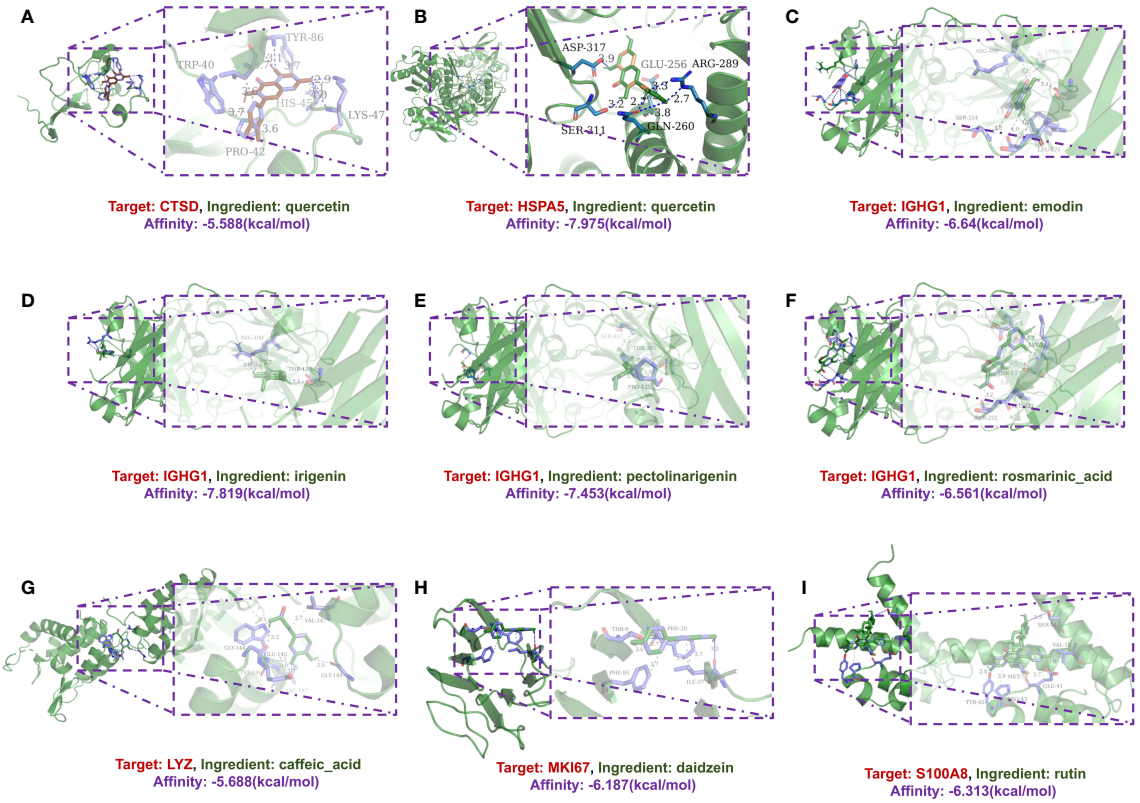

FIGURE 4
A schematic 3D representation of the molecular docking model. Active sites, binding distances, and ray tracing of compound and target proteins were predicted. **(A)** Quercetin in the protein CTSD (PDB ID: 4OBZ). **(B)** Quercetin in the protein HSPA5 (PDB ID: 3IUC). **(C–F)** Emodin, Irigenin, Pectolinarigenin and Rosmarinic acid in protein IGHG1 (PDB ID: 6HYG). **(G)** Caffeic acid in protein LYZ (PDB ID: 1XJU). **(H)** Daidzein in protein MKI67 (PDB ID: 2AFF). **(I)** Rutin in protein S100A8 (PDB ID: 5HLV).

peripheral humoral response—are also dampened following JHQG administration (27, 28).

Nonetheless, certain limitations in our research warrant mention. The study did not incorporate scRNA-seq from COVID-19 patients treated with authentic JHQG formulations. Instead, potential effector cells were inferred through target protein analysis. As such, we could only deduce cells affected by inhibitory actions of JHQG, not those possibly stimulated by the treatment. Moreover, while the study drew on known compound-protein interactions from the database and utilized Autodock-vina for docking simulations to confirm these relationships, considering protein isoforms and complex cellular contexts, the authenticity of JHQG target proteins demands further experimental validation. These analyses greatly narrowed the scope and cost required for subsequent experimental verification.

In summary, the innovative network we devised, integrating herbs, compounds, target proteins, and cells, offers insights into the network pharmacology of TCM and its therapeutic implications. Notably, our findings demonstrate that JHQG suppresses the activity of activated granulocytes, monocytes, plasmablasts, and effector T cells in COVID-19 peripheral blood, potentially limiting disease progression and diminishing humoral responses and inflammation duration.

Materials and methods

Component detection of Jinhua Qinggan granules

The UHPLC-Q-extractive-Orbitrap-MS provided all compound of Jinhua Qinggan Granule (20). Astragalin (kaempferol 3-O-glucoside, Cas. 480–10-4, C21H20O11, 448.38, 97%), oroxin A (baicalein 7-O-glucoside, Cas. 57396–78-8, C21H20O10, 432.38, 97%), mangiferin (Cas. 4773–96-0, C19H18O11, 422.34, 97%), luteolin 7-O-glucuronide (29741–10-4, C21H18O12, 462.36, 97%), ethyl caffeate (Cas. 102–37-4, C11H12O4, 208.12, 97%), scutellarin (Cas. 27740–01-8, C21H18O12, 462.37, 97%), 6-gingerol (Cas. 23513–14-6, C17H26O3, 293.39, 97%), methyl benzoate (Cas. 93–58-3, C8H8O2, 136.148, 97%), rutin (Cas. 153–18-4, C27H30O16, M.W. 610.518, 98%), baicalein (Cas. 491–67-8, C15H10O5, 270.24, 97%), wogonin (Cas. 632–85-9, C16H12O5, 284.26, 97%), chlorogenic acid (Cas. 327–97-9, C16H18O9, M.W. 354.31, 98%), isoquercitrin (Cas. 482–35-9, C21H20O12, M.W. 464.38, 98%), isochlorogenic acid C (4,5-O-dicaffeoylquinic acid, Cas. 57378–72-0, C25H24O12, M.W. 516.45, 98%), vanillic acid (Cas. 121–34-6, C8H8O4, M.W. 168.15, 98%), luteoloside (Cas. 5373–11-5, C21H20O11, M.W. 448.38, 98%), and salidroside (Cas. 10338–51-9, C14H20O7, 300.304, 97%) were obtained from Chengdu Alfa Biotechnology Co., Ltd (Chengdu, China).

18β-Glycyrrhetinic acid (Cas. 471–53-4, C30H46O4, M.W. 4470.7, 98%), cosmosiin (apigenin 7-O-glucoside, Cas. 578–74-5, C21H20O10, 432.4, 98%), licoricesaponin H2 (Cas. 135815–61-1, C42H62O16, 822.9, 98%), liquiritin (Cas. 551–15-5, C21H22O9, 418.4, 98%), quinic acid (Cas. 77–95-2, C7H12O6, M.W. 192.2, 98%), rhein (Cas. 478–43-3, C15H8O6, 284.2, 98%), cryptotanshinone (35825–57-1, C19H20O3, 296.4, 98%), scoparone (Cas. 120–08-1, C11H10O4, 206.2, 98%), and tanshinone IIA (Cas. 568–72-9, C19H18O3, 294.4, 98%) were from Shaanxi Herbest Co. Ltd. (Boji, China).

Acteoside (verbascoside, Cas. 61276–17-3, C29H36O15, M.W. 624.59, 98%), isoviolanthin (Cas. 40788–84-9, C27H30O14, M.W. 578.519, 98%), isoliquiritigenin (Cas. 961–29-5, C15H12O4, M.W. 256.253, 98%), formononetin (Cas. 485–72-3, C16H12O4, M.W. 268.264, 98%), 1,3-O-dicaffeoylquinic acid (Cas. 19870–46-3, C25H24O12, 516.455, 97%), 3,4-dicaffeoylquinic acid (3,4-O-dicaffeoylquinic acid, Cas. 14534–61-3, C25H24O12, 516.455, 97%), pectolinarigenin (Cas. 520–12-7, C17H14O6, 314.29, 97%), neomangiferin (Cas. 64809–67-2, C25H28O16, 584.48, 97%), diosmin (Cas. 520–27-4, C28H32O15, 608.54, 97%), peimisine (ebeiensine, Cas. 19773–24-1, C27H41NO3, 427.629, 98%), solancarpidine (Cas. 126–17-0, C27H43NO2, 413.62, 98%), sophocarpine (13,14-Didehydromatridin-15- one, Cas. 145572–44-7, C15H22N2O, 246.35, 98%), daidzein (Cas. 486–66-8, C15H10O4, 254.24, 97%); calycosin (Cas. 20575–57-9, C16H12O5, 284.27, 97%), scutellarein (Cas. 529–53-3, C15H10O6, 286.24, 97%), 5-O-caffeoylquinic acid (neochlorogenic acid, Cas. 906–33-2, C16H18O9, 354.311, 97%), 4-O-caffeoylquinic acid (cryptochlorogenic acid, Cas. 905–99-7, C16H18O9, 354.311, 97%), and irigenin (548–76-5, C18H16O8, 360.31, 97%) were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China).

Chrysin (Cas. 480–40-0, C15H10O4, M.W. 254.24, 98%), viscidulin I (Cas. 92519–95-4, C15H10O7, M.W. 302.24, 98%), 2′,6′-dihydroxypinobanksin (Cas. 80366–15-0, C15H12O7, 304.24, 98%), sophoricoside (Cas. 152–95-4, C21H20O10, 432.38, 98%), isorhamnetin-3-O-β-D-glucoside (Cas. 5041–82-7, C22H22O12, 478.4, 98%), 6-prenylapigenin (Cas. 68097–13-2, C20H18O5, 338.36, 98%), forsythoside B (Cas. 81525–13-5, C34H44O19, 756.7, 98%), dalbergioidin (Cas. 30368–42-4, C15H12O6, 288.65, 98%), (−)-epipinoresinol (Cas. 10061–38-8, C20H22O6, 358.39, 96%), and (+)-epipinoresinol (Cas. 24404–50-0, C20H22O6, 358.39, 96%) were purchased from BioBioPha Co., Ltd. (Kunming, China).

Esculetin (Cas. 305–01-1, C9H6O4, M.W. 178. 41, 98%), scopoletin (Cas. 92–61-5, C10H8O4, M.W. 192.17, 98%), vitexin (Cas. 3681–93-4, C21H20O10, M.W. 432.10, 98%), and isoschaftoside (apigenin-6-arabinoside-8-glucoside, Cas. 52012–29-0, C26H28O14, M.W. 564.49, 98%), quercetin (Cas. 117–39-5, C15H10O7, M.W. 302.23, 98%), S-naringenin (Cas. 480–41-1, C15H12O5, M.W. 272.25, 98%), vicenin-2 (Cas. 23666–13-9, C27H30O15, M.W. 594.518, 98%), and schaftoside (apigenin-6-glucoside-8-arabinoside, Cas. 51938–32-0, C26H28O14, M.W. 564.49, 98%) were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (Chengdu, China).

Chloesteryl acetate (Cas. 604–35-3, C29H48O2, 428.69, 97%) and protocatechuic acid (Cas. 99–50-3, C7H6O4, 154.12, 97%) were form Sigma–Aldrich (Shanghai, China); Caffeic acid (Cas. 331–39-5, C9H8O4, 97%) and emodin (Cas. 518–82-1, C15H10O5, M.W. 270.24, 97%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

D-Gluconic acid (Cas. 526–95-4, C6H11O7, M.W. 195.15, 98%) was from TCI Chemical Co. (Shanghai, China). R-rosmarinic acid (Cas. 20283–92-5, C18H16O8, M.W. 360.3, 98%) and ferulic acid (Cas. 1135–24-6, C10H10O4, M.W. 194.19, 98%) were purchased from Aladdin Chemistry Co. (Shanghai, China). L-Tryptophan (Cas. 73–22-3, C11H12N2O2, M.W. 204.23, 98%) was from J&K Scientific Co., Ltd. (Beijing, China). Danshensu (Cas. 76822–21-4, C9H10O5, M.W. 198.17, 97%) was from Shanghai Acmec Biochemical Co., Ltd (Shanghai, China). Caffeine (Cas. 58–08-2, C8H10N4O2, M.W. 194.191, 98%) was prepared using sublimation method from Green tea [9]. Methanol and water were of mass spectra purity grade.

Intregient-target protein network construction of Jinhua Qinggan Granules's compound

We retrieved target proteins from the HERB database (21) (http://herb.ac.cn/) based on the Ingredient Name of the compounds in 73, and a list of database-supported as well as literature-supported target proteins is included.

We then used the plot_network function in omicverse (29) to visualize the 73 components and for that matter the target proteins.

Analysis of differentially expressed genes in peripheral blood of patients with COVID-19

We first selected annotated single-cell sequencing data from the public database COVID-19 Cell Atlas (https://www.covid19cellatlas.org/#wilk20) for eight peripheral blood samples from seven COVID-19 hospitalized patients and six peripheral blood samples from six healthy individuals. The cohort encompassed seven male patients ranging in age from 20 to over 80 years. Samples were obtained between 2 and 16 days post symptom onset. In contrast, healthy controls, comprising four males and two females, were asymptomatic individuals aged between 30 and 50 years. Half of the eight COVID-19 specimens came from mechanically ventilated patients diagnosed with acute respiratory distress syndrome (ARDS). Distinctively, patient C1 provided two samples: the first at nine days after exhibiting symptoms, at which time he required supplemental oxygen, and a subsequent sample was taken two days later post-intubation. Remdesivir treatment in the hospital setting was given to five patients, with four receiving it before their samples were collected (Supplementary Table 3).

Raw sequencing data are available at NCBI Gene Expression Omnibus (accession number GSE150728). Cells with less than 1,000 UMI or more than 15,000 UMI, as well as cells containing more than 20% of reads for mitochondrial genes or rRNA genes (RNA18S5 or RNA28S5), were considered low quality and excluded from further analysis. To remove putative multiplex states (where there may be multiple cells loaded into a given well on the array), cells expressing more than 75 genes per 100 UMI were also filtered out. Genes expressed in fewer than 10 cells were removed from the final count matrix. There were 44,721 cells after quality control.

We then extracted 447 high-quality metacells using the SEACells (30) module in omicverse and performed compaction and segregation assessments. Given that metacells represent distinct cell states of the biological system under consideration, inferred metacells should (1) be compact, meaning that they exhibit low variability among aggregated cells and that most of this variability is a result of measurement noise, and (2) be well separated from neighboring metacells.

We then used the pyDEG module in omicverse to analyse the differential expression of two different status metacells, Contrl and COVID-19, and the significance was calculated using the ttest model. We finally selected differential genes with a differential expression multiplicity of 0.5 in the threshold and an ADJUST p-value of less than 0.05.

Intregient-target-cell network construction of Jinhua Qinggan Granules's compound

To construct the Intregient-Target-Cell network, we first used omicverse's get_celltype_marker function to obtain the marker genes for each cell type for scRNA-seq of COVID-19 peripheral blood samples. Then we took the intersection of COVID-19 differentially expressed genes, cell-specific marker genes and target proteins of JHQG to obtain the cell-specific target genes of COVID-19 differences of JHQG. Subsequently still the plot network function of omicverse was used to draw the Intregient-Target-Cell network for visualization.

Molecular docking

2D structures of HE obtained from the PubChem database were downloaded as SDF files and then imported into chem3D software to generate their respective free energy-minimized 3D conformations. Additionally, crystal structures of hub genes' proteins were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). The ADFR Suite was utilized to eliminate water molecules and ligands from protein structures, followed by addition of non-polar hydrogens and conversion to PDBQT format (31). Additionally, small molecule ligands (HE) were converted to PDBQT format for docking using the Meeko python package (https://github.com/forlilab/Meeko.git). The protein receptor structure was displayed in secondary structure representation without lines. The active pocket location was determined using AutoGrid4 (32). Subsequently, protein-ligand docking was conducted using Autodock Vina 4.0 software, with lower binding energy indicating greater stability. Ligand-receptor interactions, such as π stacking (parallel and perpendicular), π-cation interactions, hydrogen bonding, water bridges, and salt bridges, were visualized using the Protein-Ligand Interaction Profiler (PLIP) website (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) (33).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material. The original codes presented in the study are publicly available. This data can be found here: https://github.com/Starlitnightly/Analysis_JHQG_COVID.

Author contributions

LQ: Conceptualization, Formal analysis, Investigation, Software, Writing – original draft. ZZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1382524/full#supplementary-material

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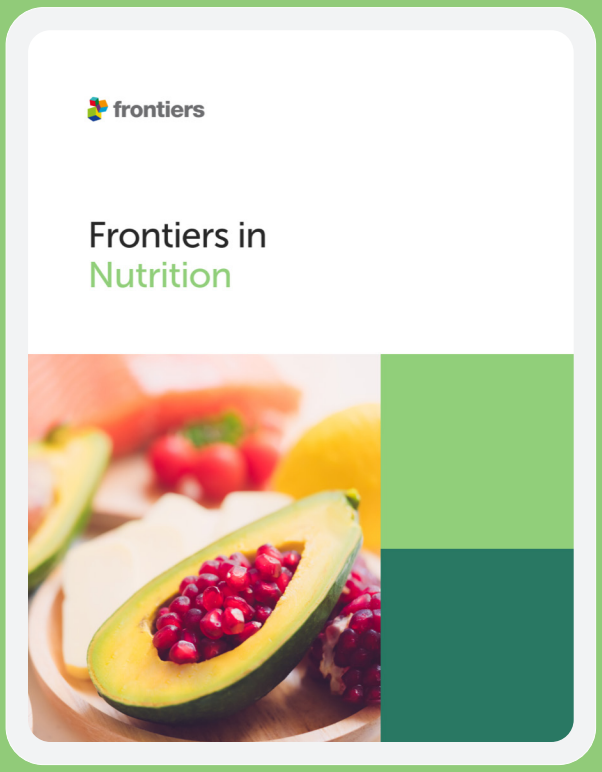

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