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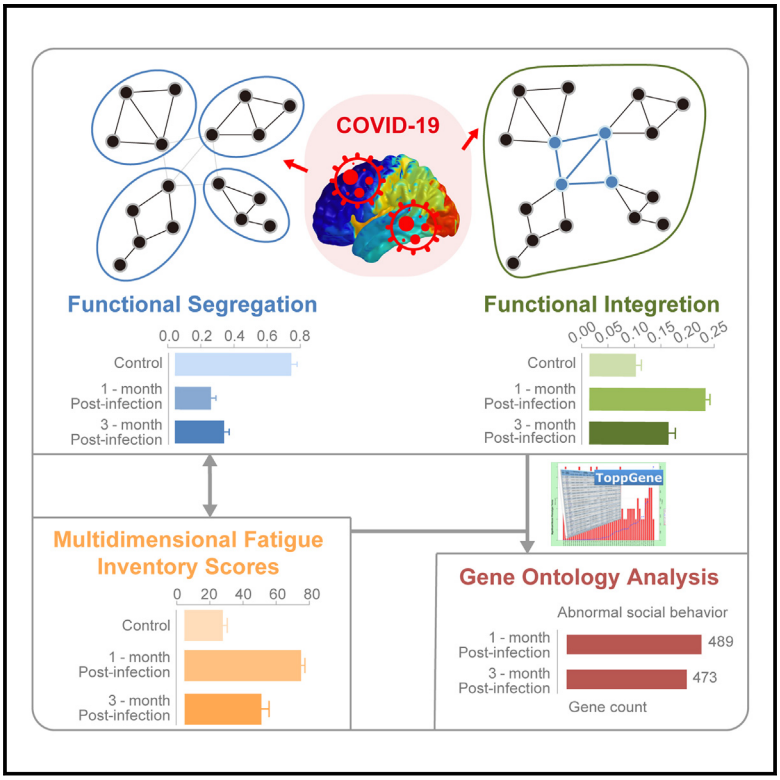
Segregation and integration of resting-state brain networks in a longitudinal long COVID cohort

Graphical abstract



Highlights

- Systematically explore multiscale neural mechanisms of post-viral fatigue progression
- Patients with long COVID show higher fatigue scores and less segregated brain states
- Visual system and attention network are important in shaping long COVID fatigue perception
- Genes linked to abnormal social behavior may relate to long COVID fatigue

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In brief

Health sciences; Medicine; Medical specialty; Immunology; Virology; Internal medicine; Neurology

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Segregation and integration of resting-state brain networks in a longitudinal long COVID cohort

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SUMMARY

Long COVID is characterized by debilitating fatigue, likely stemming from abnormal interactions among brain regions, but the neural mechanisms remain unclear. Here, we utilized a nested-spectral partition (NSP) approach to study the segregation and integration of resting-state brain functional networks in 34 patients with long COVID from acute to chronic phase post infection. Compared to healthy controls, patients with long COVID exhibited significantly higher fatigue scores and shifted the brain into a less segregated state at both 1 month and 3 months post infection. During the recovery of fatigue severity, there was no significant difference of segregation/integration. A positive correlation between network integration and fatigue was observed at 1 month, shifting to a negative correlation by 3 months. Gene Ontology analysis revealed that both acute and long-term effects of fatigue were associated with abnormal social behavior. Our findings reveal the brain network reconfiguration trajectories during post-viral fatigue progression that serve as functional biomarkers for tracking neurocognitive sequelae.

INTRODUCTION

The COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has a profound impact on patients' quality of life while imposing substantial economic burdens.[1] Following the acute phase, which typically lasts around 4 weeks after onset,[2,3] approximately 10%–25%[4,5] of patients develop long COVID—a chronic condition with persistent symptoms for over 12 weeks post infection.[6] Long COVID induced diverse symptoms,[7] wherein the fatigue was consistently reported as one of the most debilitating manifestations.[8,9] Understanding the progression of long COVID fatigue and its neurological basis is essential for the prevention and control of post major infectious diseases.[10,11]

Normal brain functions depend on both the relatively independent processing within specialized systems (segregation) and the global cooperation between different systems (integration).[12] This segregation and integration can reflect various cognitive abilities[13] and also be changed by task conditions and brain disorders.[14,15] Evidence has shown that COVID-19 infection can negatively impact brain health both acutely and for months following the initial infection.[16] Neuroimaging studies have identified a characteristic pattern of functional connectivity (FC) changes in patients with COVID-19, e.g., decreased connectivity within higher-order cognitive networks, including the default mode network (DMN), salience network, frontoparietal network (FPN), and somatomotor network (SMN).[17] These alternations are associated with a variety of cognitive and neuropsychiatric symptoms, e.g., cognitive deficits and depression.[18] It is thus expected that long COVID has an effect on the segregation and integration of brain FC networks. More importantly, fatigue is a prominent symptom of long COVID, and it is thought to be linked to the dynamics of integration and segregation.[17] However, it is still unclear how the long COVID alternates the segregation and integration and how this alteration associates with the fatigue symptom.

The current landscape of research was primarily composed of cross-sectional, retrospective studies conducted 6 months or

Table 1. Characteristics of samples (n = 58)

	Control (N = 24)	Long COVID (N = 34)	p value
Male sex, no. (%)	11 (45.83)	21 (61.76)	>0.05
Age, median (Q1–Q3) – year	43 (36, 49)	36 (31, 45)	>0.05
Educational level, median (Q1–Q3) – year	16 (12, 16)	17 (15, 17)	0.002
BMI, no. (%)			
Underweight	0 (0.00)	0 (0.00)	>0.05
Normal weight	15 (62.50)	14 (41.18)	
Overweight	9 (37.50)	16 (47.06)	
Obesity	0 (0.00)	4 (11.76)	
Presence of chronic diseases, no. (%)			
No	20 (83.33)	30 (88.24)	>0.05
Yes	4 (16.67)	4 (11.76)	
Vaccination status, no. (%)			
None	2 (8.33)	1 (2.94)	>0.05
Booster dose	18 (75.00)	27 (79.41)	
Partially vaccinated	0 (0.00)	2 (5.88)	
Two vaccine doses	4 (16.67)	4 (11.77)	
Mean FD, median (Q1–Q3)	0.05 (0.04, 0.09)	0.06 (0.04, 0.07)	>0.05
LC-SSS, median (Q1–Q3)	–	24 (4, 14)	
Total	24 (100.00)	34 (100.00)	

Mean FD, volume-level mean FD (Jenkinson) for accounting head motion at group-level analysis. $p < 0.05$ considered significant. LC-SSS, Long COVID Symptoms and Severity Score.

Table 2. Results of Multidimensional Fatigue Inventory (MFI-20), median (Q1–Q3)

	Control (N = 24)	1-month post-infection (N = 34)	3-month post-infection (N = 34)
MFI-20			
Total score	37.5 (31.5, 46)	56.5 (47.5, 62)	48.5 (36.25, 57)
General fatigue	8 (6, 11.25)	12.5 (10, 15.75)	10 (8, 12)
Physical fatigue	7.5 (5.75, 11)	11.5 (9.25, 13)	10 (6.25, 12)
Mental fatigue	6 (4.75, 10.25)	11 (7.5, 12.75)	9.5 (6.25, 11.75)
Reduced activity	8 (5.75, 11)	11 (9.25, 12.75)	10.5 (7.25, 12)
Reduced motivation	7 (5, 9.25)	10.5 (9, 12)	9 (5.25, 10.75)

MFI-20, Multidimensional Fatigue Inventory.

more after the initial infection.[19] Such studies were prone to recall bias and lack standardized criteria for selecting patients with long COVID. Additionally, the scarcity of prospective cohort studies leaves significant gaps in understanding the mechanisms driving symptom onset and progression over time. This underscores the need for longitudinal prospective studies[20] to elucidate the temporal and biological dynamics of the neurological effects associated with long COVID fatigue. Furthermore, inconsistencies remain regarding the regions and extent of FC alterations in patients with long COVID,[21,22] highlighting the critical need for robust findings to elucidate the neural underlying mechanisms. Therefore, we anticipate observing dynamic changes of segregation and integration in the brain's resting state in a longitudinal long COVID cohort from acute to chronic phase, corresponding with fluctuations in fatigue severity over time.

In this study, 34 patients with long COVID recovered from an initial mild SARS-CoV-2 infection underwent neuropsychological measurements and MRI scans at 1 month and 3 months post infection. 24 healthy controls followed the same protocol as the 1-month post-infection group simultaneously. The selection of participants with long COVID was based on the Long COVID Symptoms and Severity Score, a validated tool addressing a significant gap in the standardized and quantifiable monitoring of long COVID.[23] We aimed to investigate the characteristics and neurological basis of long COVID fatigue through a prospective longitudinal study. First, we reported the fatigue symptom in patients with long COVID tracking its recovery over time. Second, we studied the segregation and integration of brain networks in patients with long COVID at different phases as well as their relationship with fatigue measurement. Finally, we performed Gene Ontology (GO) enrichment analysis to identify human disease phenotypes associated with the fatigue.

RESULTS

Characteristics of samples

In this longitudinal study, the healthy controls (HCs) included 24 non-infected individuals, and the long COVID group consisted of 36 individuals who had previously been diagnosed with mild SARS-CoV-2 infections and were experiencing symptoms of long COVID, but 2 patients with long COVID were excluded because of poor image quality. Assessments were conducted at two time points: 1 month post infection, with a time since symptom onset of 24 days (interquartile range: 21–27 days), and at 3 months post infection, with the time since symptom onset at 101 days (interquartile range: 92–106 days). The temporal distribution was aligning with the initial wave of COVID-19 in China. We analyzed categorical variables using the chi-squared test and Fisher's exact test. For continuous variables, the Mann-Whitney U test was employed to assess differences between groups. The educational level was the only variable exhibiting significant differences between the two groups, which was used as a covariate in subsequent analyses. The participant characteristics are summarized in Table 1.

Higher MFI-20 score in long COVID infection

Table 2 shows the Multidimensional Fatigue Inventory (MFI-20) assessment results. Compared to HCs, patients with long COVID at 1 month post infection exhibited significantly higher scores on the MFI-20 total score ($p < 0.001$, Figure 1A) and all subscales (general fatigue, physical fatigue, mental fatigue,

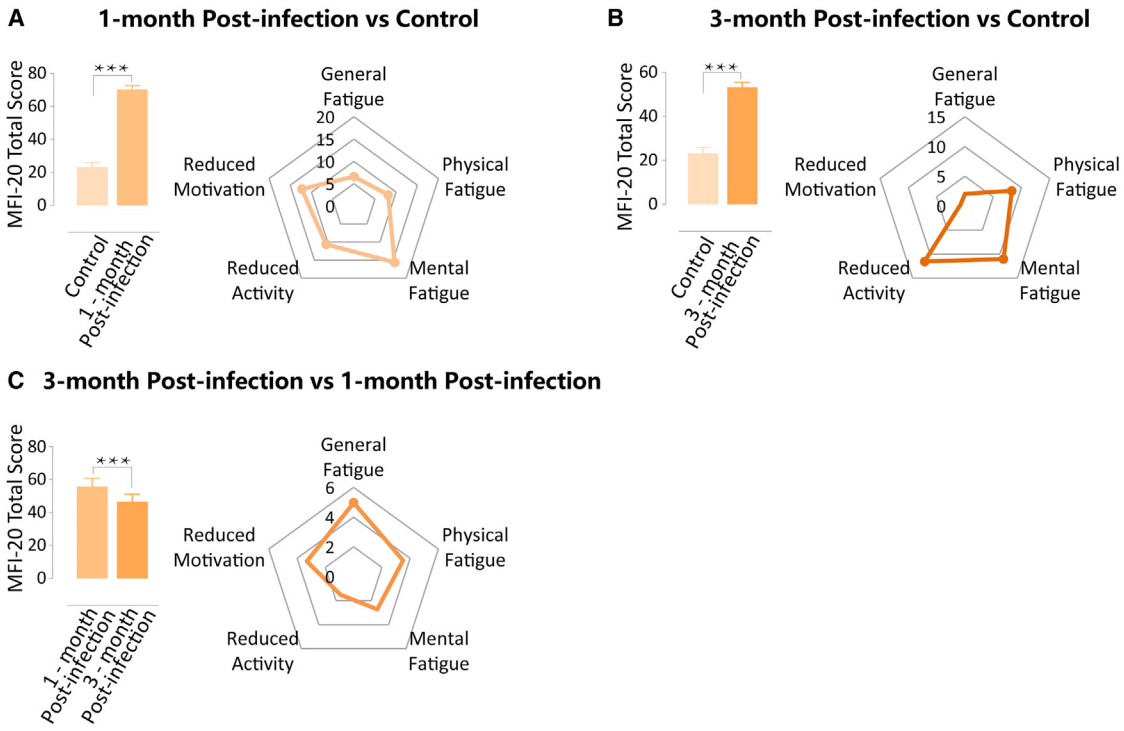

Figure 1. Comparative analysis of fatigue in patients with long COVID and healthy controls

(A) Comparisons of Multidimensional Fatigue Inventory (MFI-20) scores between the 1-month post-infection and healthy control (HC) groups, (B) the 3-month post-infection and HC groups, and (C) the 3-month and 1-month post-infection groups. Radar plots represent the t value in different dimensions of MFI-20 (dot: Bonferroni-corrected, $p < 0.05$).

Data are represented as mean ± SEM.

reduced activity, and reduced motivation, all $p < 0.001$, Bonferroni-corrected, Figure 1A). Between 1 month and 3 months post infection, the reductions in the MFI-20 total score and all subscales were observed, but only the decrease in MFI-20 total score and general fatigue was statistically significant ($p = 0.032$, $p = 0.014$, Bonferroni-corrected, Figure 1C), reflecting a potential recovery of MFI at 3 months. But when directly comparing the control and 3-month post-infection, the MFI-20 total score and most subscales in 3-month post-infection are still significantly higher than those for HC (MFI-20 total score: $p < 0.001$; physical fatigue, mental fatigue, and reduced activity: all $p < 0.001$, Bonferroni-corrected, Figure 1B), except for the reduced motivation (Bonferroni-corrected, $p = 1$) and general fatigue (Bonferroni-corrected, $p = 1$), indicating the long-lasting effect of COVID on MFI in individuals. Specific results are detailed in Table S3.

Increased network integration and decreased segregation in long COVID infection

We next investigated how the long-lasting effect of COVID and the recovery of MFI specifically affect the brain network configuration. We here employed the nested-spectral partition (NSP) method to measure the functional integration and the segregation across both the whole-brain and local scales. Compared to HCs, 1-month post-infection patients showed a significant increase in network integration (0.132 ± 0.014, $p < 0.001$, Figure 2A) and a decrease in segregation (−0.485 ± 0.046, $p < 0.001$, Figure 2B). These alterations were consistently observed in network integration and segregation across nearly all networks, including FPN, ventral attention network (VAN), DMN, dorsal attention network (DAN), SMN, limbic network, visual network (VN), and subcortical network (SCN) (all of them: Bonferroni-corrected, $p < 0.05$, Figure 2). At the regional level, most of regions (Bonferroni-corrected, $p < 0.05$, Figures 3A and 3B) has the increased integration and decreased segregation at 1 month post infection relative to HC. To improve the statistical power, we here focused on the regions of interest (ROIs) that have significant differences (Bonferroni-corrected, $p < 0.05$, Figures 3A and 3B) in both integration and segregation, generating 134 ROIs.

There was no significant difference of segregation/integration between the 1-month and 3-month post-infection groups in the whole-brain, system and regional scales (Figure S1), even the recovery of MFI (Figure 1C). But compared to the HCs, 3-month post-infection patients had insignificant change of integration (−0.485 ± 0.046, $p = 0.918$, Figure 2C) and significantly decreased segregation (−0.405 ± 0.048, $p < 0.001$, Figure 2D). This decrease is also significant in seven systems (Bonferroni-corrected, $p < 0.05$, Figure 2D) except for the SCN (Bonferroni-corrected, $p = 1$, Figure 2D). At the regional scale (Figures 3C and 3D), individuals with 3-month post-infection exhibited significant differences in both integration and segregation in 14 ROIs,

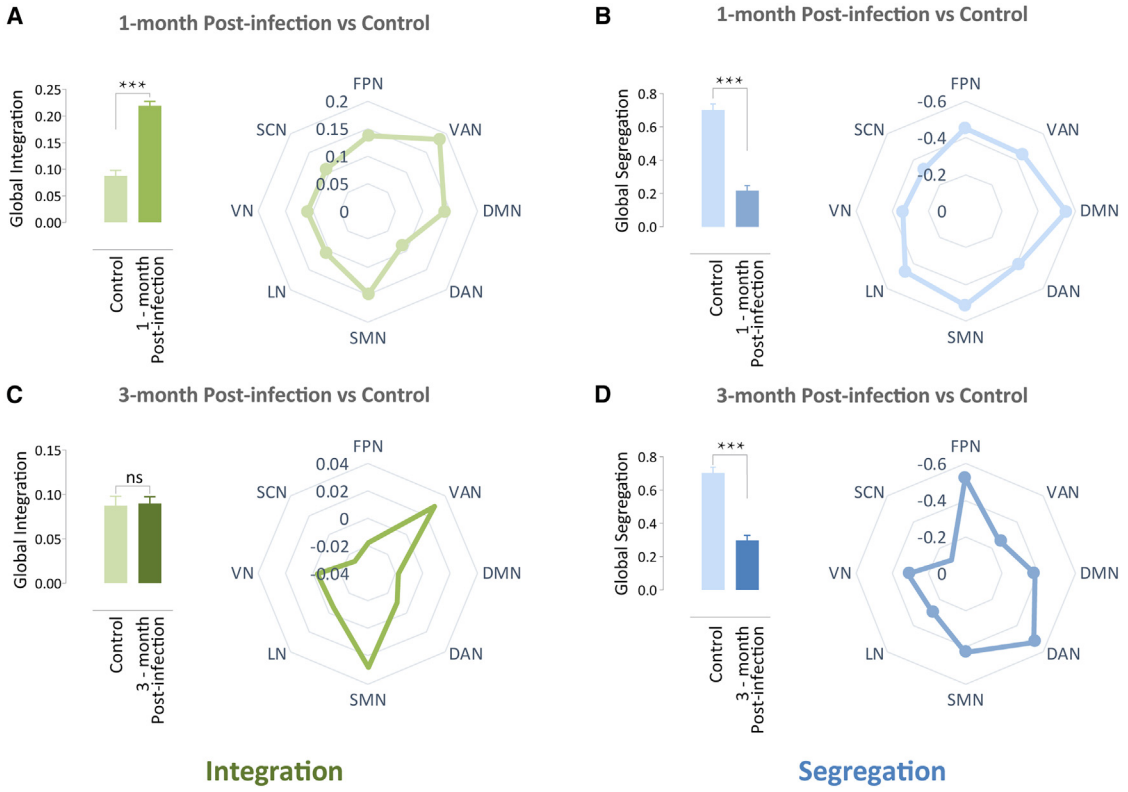

Figure 2. Comparative analysis of integration and segregation

The changes in network integration (A) between the 1-month post-infection and healthy control (HC) groups, and (C) between the 3-month post-infection and HC groups. The changes in network segregation were provided on (B) and (D). Radar plots indicate the differences in eight networks (dot: $p < 0.05$, Bonferroni-corrected). SCN, subcortical network; VN, visual network; SMN, somatosensory-motor network; DAN, dorsal attention network; VAN, ventral attention network; LN, limbic network; FPN, frontoparietal network; DMN, default mode network.

Data are represented as mean ± SEM.

including the right precentral gyrus, superior temporal gyrus, left parahippocampal gyrus, insular gyrus, left superior frontal gyrus, lateral occipital cortex (LOcC), left hippocampus, left basal ganglia, right inferior frontal gyrus, and left thalamus (Bonferroni-corrected, $p < 0.05$). Specific results are detailed in Table S3.

Shift in correlations between brain networks and MFI scores in 1-month and 3-month infection

While the MFI had a recovery across MFI-20 (total score and all subdimensions [general fatigue, physical fatigue, mental fatigue, reduced activity, and reduced motivation]) from 1 month to 3 months, the brain network did not show a significant change of integration and segregation, such that the correlation between brain networks and MFI-20 may be alternated from 1 month to 3 months. In 1-month post-infection group, the MFI-20 total score was insignificantly correlated with integration ($r = 0.123$, $p = 0.325$, Figure 4) and segregation ($r = -0.238$, $p = 0.080$, Figure 4) at the global level, but the physical fatigue was significantly correlated with segregation ($r = -0.328$, $p = 0.006$, Figure 4). At the network level, the correlations between the MFI-20 total score and its subdimensions with integration and segregation followed a similar trend, though almost none were statistically significant. Only the physical fatigue is significantly negatively correlated with the segregation of VN ($r = -0.374$, $p = 0.011$, Bonferroni-corrected, Figure 4). In regional scale, left medioventral occipital cortex (MVOcC) and LOcC showed significant negative correlations with physical fatigue (MVOcC: $r = -0.487$, $p < 0.001$; LOcC, $r = -0.539$, $p = 0.015$, Bonferroni-corrected, see Table S4). These correlations were similar in the HC group (Figure S2).

But for the 3-month post-infection group, the correlation trends were opposite. MFI-20 total score and nearly all the subdimensions of MFI-20 were significantly negatively correlated with network integration and positively correlated with segregation (all $p < 0.05$, Figure 4). The only exception was the correlation between mental fatigue and integration ($r = -0.254$, $p = 0.162$, Figure 4). At the system scale, the MFI-20 total score was positively correlated with segregation and negatively correlated with integration in DAN (with integration: $r = -0.458$, $p < 0.001$; with segregation: $r = 0.541$, $p < 0.001$), DMN (with integration: $r = -0.381$, $p = 0.004$; with segregation: $r = 0.434$, $p < 0.001$), FPN (with integration: $r = -0.430$, $p = 0.002$; with segregation: $r = 0.524$, $p < 0.001$), and VN (with integration: $r = -0.358$, $p = 0.004$; with segregation: $r = 0.450$, $p < 0.001$, Bonferroni-corrected) networks (Figure 4). More specifically, reduced

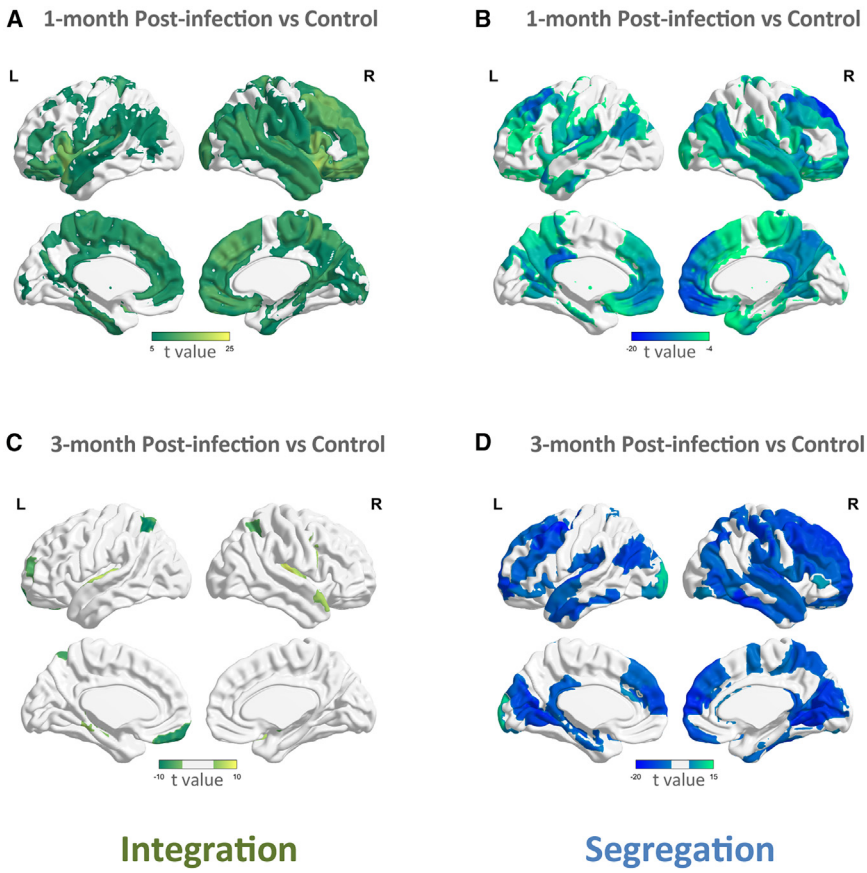

Figure 3. Comparative analysis of integration and segregation in regions

The changes in regional integration (A) between the 1-month post-infection and healthy control (HC) groups, and (C) between the 3-month post-infection and HC groups. The changes in regional segregation were provided on (B) and (D). Brain maps represent the t values of statistics in significant regions ($p < 0.05$, Bonferroni-corrected).

motivation showed a significantly positive correlation with segregation and negative correlation with integration in DAN (with integration: $r = -0.418$, $p < 0.001$; with segregation: $r = 0.493$, $p < 0.001$) and VN (with integration: $r = -0.399$, $p < 0.001$; with segregation: $r = 0.532$, $p < 0.001$, Bonferroni-corrected) networks (Figure 4). At the regional scale, the integration of two ROIs (left posterior superior temporal sulcus [pSTS] and LOcC) was both significantly negatively correlated with reduced motivation (left pSTS: $r = -0.440$, $p < 0.05$; left LOcC, $r = -0.527$, $p < 0.05$, Bonferroni-corrected). Meanwhile, the segregation of these ROIs showed significant positive correlations with reduced motivation (left pSTS, $r = 0.592$, $p < 0.05$; left LOcC, $r = 0.544$, $p < 0.05$, Bonferroni-corrected). All the results are detailed in Table S4.

Linking effects of fatigue to gene transcriptional profiles

We collected whole-brain gene expression profiles of 246 parcellation regions from the Allen Human Brain Atlas and linked the components dominantly affected by fatigue to gene expression. First, we calculated the effect (estimated by regression coefficient) of MFI-20 total score on integration/segregation in each region at 1 month and 3 months post infection separately. This helped identify key genes that were statistically significant (Bonferroni-corrected, $p < 0.05$). Then, we calculated the correlation between the effect and gene transcriptional profiles in all regions. At 1 month post infection, 4,604 significant genes were identified, with 1,561 genes related to integration and 3,043 genes related to segregation (Bonferroni-corrected, $p < 0.05$, Figure S3). At 3 months post infection, 4,133 significant genes were identified, with 4,111 genes related to integration and 22 genes related to segregation (Bonferroni-corrected, $p < 0.05$, Figure S3). We then used these significant genes to perform GO annotation analysis using the ToppGene Suite. We observed that the human disease phenotypes included abnormal social behavior, abnormal communication in both 1-month and 3-month infection, indicating the same association between COVID-indicated social problem and fatigue Figure 5).

DISCUSSION

This work studied functional segregation and integration in brain FC networks with long COVID fatigue from acute to chronic phases. We first found that patients with long COVID exhibited significantly higher fatigue scores than healthy controls, with self-recovery from 1 month to 3 months. Second, the brain networks of patients with long COVID tend to be more integrated and less segregated, with more pronounced changes at 1 month. Third, the correlation between brain network segregation and fatigue severity shifted from negative at 1 month to positive at 3 months post infection. Finally, GO analysis revealed that the effects of fatigue in patients with long COVID were associated with abnormal social behavior. Our findings systematically delineate multiscale neural mechanisms underlying post-viral fatigue progression, revealing dynamic brain network reconfiguration trajectories from acute to chronic phases that serve as functional biomarkers for tracking neurocognitive sequelae.

It is increasingly recognized that higher-order network functions, which are important in the context of long COVID fatigue, mainly depend on distributed large-scale brain networks rather than isolated regions.[24] Our results further confirm that during the acute phase, patients with long COVID suffered a highly integrated and less segregated brain, underscoring the widespread impact of long COVID on overall brain connectivity. Specifically, the reduced segregation of DMN and the increased integration of VAN showed the most pronounced alterations. VAN has a crucial function of regulating and providing input into networks involved in cognition such as the DMN, DAN, and FPN,[25] even functions

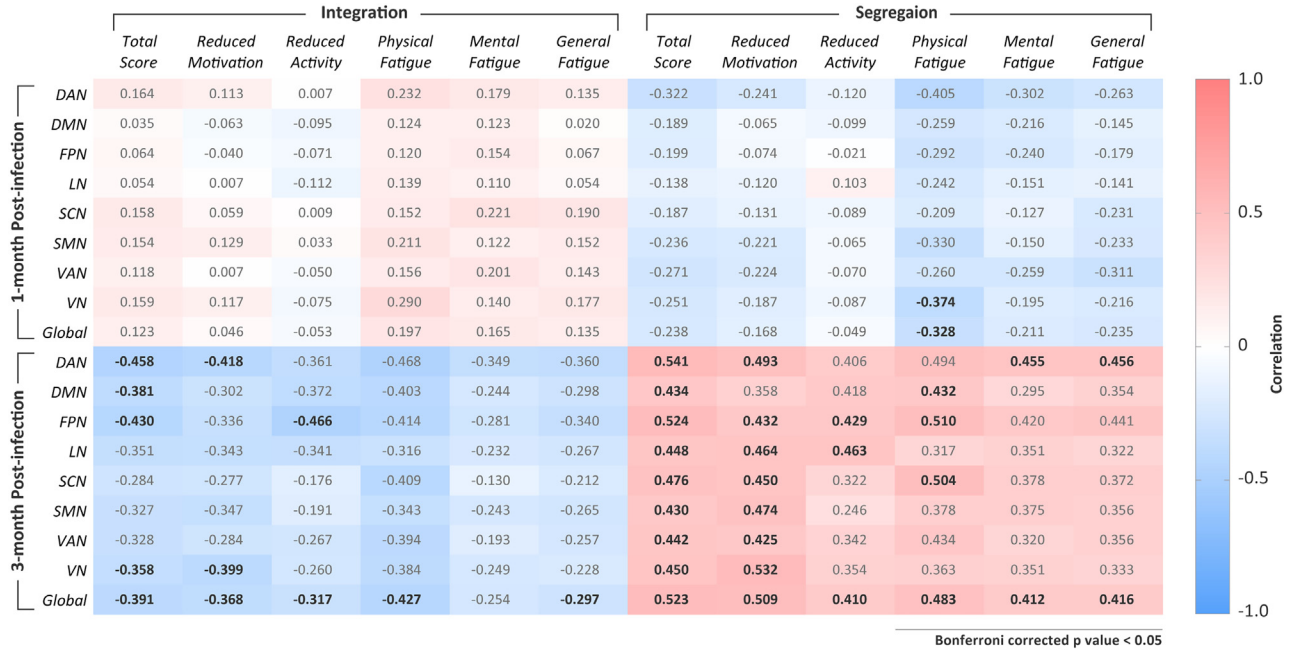

Figure 4. Correlation of NSP metrics with fatigue scores across different groups in global and network levels

Depicting the correlation of network segregation and integration with various dimensions of fatigue (general fatigue, mental fatigue, physical fatigue, reduced activity, and reduced motivation) as measured by the Multidimensional Fatigue Inventory (MFI-20) for patients with long COVID at 1-month and 3-month post infection.

as a switch between these networks.[26,27] This regulatory function arises from the sensitivity of the VAN for detecting salient environmental stimuli.[28,29] Therefore, the changes observed in VAN and DMN may represent a rapid neural mechanism in response to COVID-19 in acute phase of patients with long COVID. This aligns with the theory that COVID-19 stress induces a dynamic reallocation of resources between brain networks to maintain a hypervigilant state, promoting threat detection and survival.[30] At 3 months post infection, global- and network-level integration was no longer significantly different from that of healthy controls, and the reduction in segregation had partially diminished, indicating a partial recovery of brain network function in tandem with improvements in fatigue symptoms over time. Nevertheless, a decrease in segregation remained evident, particularly in FPN. Prior research highlighted that the reduced segmentation of top-down (FPN) system may act as a compensatory mechanism for cognitive decline associated with persistent fatigue following infection, thereby supporting the maintenance of normal cognitive function.[31,32] Therefore, the persistent alterations in brain network connectivity, especially within the attention network, may serve as neural correlates of fatigue in patients with COVID.

Our results further support the hypothesis that segregation and integration correlated with long COVID fatigue. In the acute phase, though not statistically significant, we observed a positive relationship between integration and fatigue, and a negative relationship between segregation and fatigue. A reversal in the correlations observed was noted in the chronic phase: fatigue was positively associated with segregation and negatively associated with integration. And these relationships were stronger

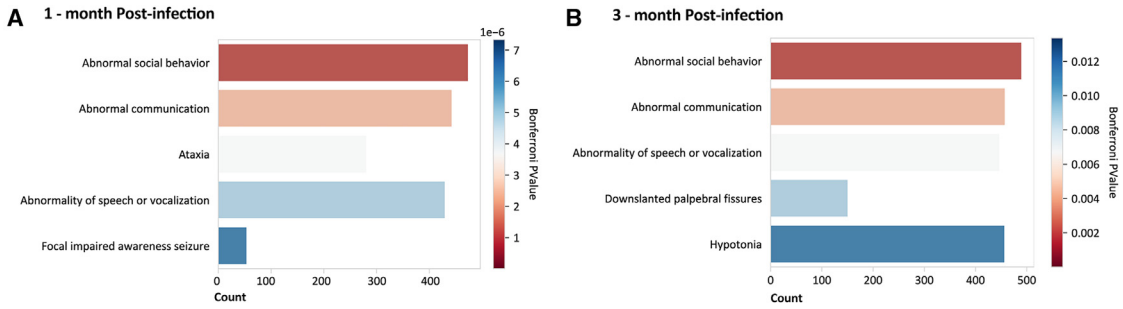

Figure 5. Effects of fatigue and gene profiles

Gene Ontology (GO) enrichment results for human disease phenotypes using ToppGene for (A) 1-month post-infection group and (B) 3-month post-infection group.

than those observed in the acute phase or in healthy individuals, highlighting fatigue as a key neurological symptom in long COVID. This shift from patterns seen at 1 month may indicate a reduction in the acute illness response. In patients with chronic fatigue syndrome, fatigue is negatively correlated with integration,[33,34] which is consistent with our results, implying long COVID fatigue aligning closely with typical pathological changes associated with chronic fatigue conditions. More specifically, our results indicate that long COVID fatigue is linked to increased segregation and decreased integration, particularly in DAN and VN, and is most evident in reduced motivation. In line with this, reduced motivation was positively correlated with integration and negatively correlated with segregation in the left LOcC (in VN) and left pSTS (in DMN). This shift from physical fatigue to reduced motivation may be driven by altered brain segregation in VN and DAN. Since visual processing plays a critical role in selective attention[35–37] and the VN can influence mental health by facilitating communication between higher-order cognitive networks, the FPN and the DMN,[4] our findings emphasize the importance of the visual system and attention network in shaping long COVID fatigue perception.

This study also delineates the evolving gene transcriptional profiles associated with long COVID fatigue. The COVID-19 pandemic, as a massive social stressor, may have triggered changes in social behavior, which could manifest as part of the broader symptomatology of long COVID.[38] The possible linkage between the genes associated with abnormal social behavior and long COVID fatigue may reflect the broader biopsychosocial impact of long COVID, influencing both the brain's functional connectivity and the patient's emotional and social response[39] to the condition.

Limitations of the study

A limitation of this study is the limited sample cohort and the lack of a contemporaneous healthy control group at the 3-month post-infection. Larger sample size may contribute to identifying more significant relationships between brain networks and fatigue symptoms. Besides, due to the rapid spread of the first wave of infections in China, it was challenging to find enough uninfected individuals as controls. Another limitation is the reliance on self-reported measures of fatigue, which may be subject to reporting bias. Furthermore, this study did not account for the categorization of symptom severity of patients with long COVID. It may limit the understanding of how the condition impacts patients with long COVID differently.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xuan Niu (niuxuan@xjtu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All the data were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. The data are available from the corresponding authors upon acceptance of this manuscript and a reasonable request.
- The code for analyzing segregation and integration at the ROI, network, and global levels using the NSP method is available at https://github.com/TobousRong/ADHD. Additional data analysis code can be accessed at https://github.com/LouiseRyan95/Segregation-and-Integration-of-Resting-state-Brain-Networks-in-a-Longitudinal-Long-COVID-Cohort.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

X.N. and R.W. supervised the project and acquired funding. Y. Zhang, G.Y., X.N., and R.W. conceived the project and designed the experiments. X.N., R.W., C.L., Y. Zhu, D.L., J.L., W.W., P.L., and L.F. organized sample collection. Y. Zhang, G.Y., W.Z., R.Z., C.L., Y. Zhu, D.L., and J.L. performed and analyzed all experiments. Y. Zhang, G.Y., X.N., and R.W. interpreted the results and wrote the paper. J.L., W.W., P.L., L.F., R.W., and X.N. revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Healthy adults	First Affiliated Hospital of Xi'an Jiaotong University	
Long COVID patients	First Affiliated Hospital of Xi'an Jiaotong University	
Software and algorithms		
MATLAB R2023a	MathWorks	https://github.com/TobousRong/ADHD
RStudio Desktop 2024.12.1	Posit Software	https://github.com/LouiseRyan95/Segregation-and-Integration-of-Resting-state-Brain-Networks-in-a-Longitudinal-Long-COVID-Cohort

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The Ethics Committee of First Affiliated Hospital of Xi 'an Jiaotong University, has approved this study, which conducted from January to April 2022. All participants provided informed consent after receiving a comprehensive explanation of the study's aims and procedures.

We enrolled 58 participants across two groups for this cohort study. The first group consisted of 34 participants (age: 31–45, median age = 36 years, 13 females) diagnosed with long COVID, assessed using the LC-SSS[23] within 48 h of the MRI examination. The LC-SSS was specifically designed for individuals experiencing post-COVID-19 conditions lasting at least three months and evaluates a wide range of potential symptoms along with their severity levels. In this study, inclusion criteria aligned with the World Health Organization definition requiring persistence of at least one symptom for $\geq$12 weeks following SARS-CoV-2 infection.[40] The criteria were applied based solely on the presence of any symptom, without considering severity. Consequently, a total LC-SSS score greater than 0 was used to identify long COVID patients. These participants were evaluated at both 1-month and 3-month post-initial mild SARS-CoV-2 infection. The control group included 24 healthy, non-infected individuals (age: 36–49, median age = 43 years, 13 females). Eligible participants had no history of neurological or psychiatric diseases, brain tumors, and met the criteria for MRI safety. Additionally, all the participants completed the Multidimensional Fatigue Inventory (MFI-20)[41] within 48 h before their MRI evaluations to assess their fatigue levels. SARS-CoV-2 infection in the first group was confirmed via RT-PCR or positive antibody tests. Healthy controls were required to undergo repeated negative RT-PCR tests, and have no clinical signs or symptoms associated with COVID-19. All participants underwent blood tests, including IgG and IgM analyses, which helps to determine whether there was a history of SARS-CoV-2 infection.

METHOD DETAILS

MRI acquisition and preprocessing

MRI examinations were acquired using a 3 Tesla scanner. Imaging parameters are described in Table S1. DPABI v8.1[42,43] was used to preprocess resting-state fMRI data. The preprocessing steps were as follows: first 10 time points deletion; slice timing correction; The realignment on functional volumes used a six-parameter (rigid body) linear transformation with a two-pass procedure (registered to the first image and then registered to the mean of the images after the first realignment). After the realignment, individual T1-weighted MPRAGE images were co-registered to the mean functional image using a 6-degree-of-freedom linear transformation without re-sampling to ensure that the T1 images and functional images were spatially aligned before segmentation. Then T1-weighted MPRAGE images were segmented into gray matter, white matter (WM), and cerebrospinal fluid (CSF). Finally, transforming images from individual native space to MNI space with the Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra (DARTEL) tool[44]; nuisance covariance regression, including head motion (Friston 24-parameter model[45]); white matter signals and cerebral spinal fluid signals (A principal component analysis was used to extract the first five principal components to explain 90% of signal variance from the white matter and the cerebral spinal fluid); functional images normalization (T1 images were used); smoothing (4 mm full-width at half-maximum (FWHM) Gaussian kernel); and band-pass filtering (0.01–0.1 Hz). The time point with an FD power (Jenkinson) of <0.2 mm[46] was regarded as good.[32] The global signal regression would introduce more negative functional connectivity, and its use remains controversial in resting-state fMRI analyses.[47,48] Therefore, we did not remove the whole-brain global signal.

Segregation and integration in brain FC networks

The brain was parcellated into $N = 246$ regions according to the Human Brainnetome Atlas,[49] accompanied by their corresponding Yeo seven-network parcellations[50] (Table S2).[51] The Pearson correlation coefficient between the time series of two regions was calculated to measure FC. The static FC network for each individual was constructed using whole fMRI time series, and the stable FC network was obtained by concatenating all-time series across all participants in each group. The negative correlations in the FC matrices were set to zero, following the previous studies.[13,52,53]

To analyze segregation and integration at the region of interests(ROI), network, and global levels, the nested-spectral partition (NSP) method[13,54] was employed to detect hierarchical modules of brain FC networks.[55] We began by performing an eigen-decomposition of FC matrix C, obtaining its eigenvectors U and eigenvalues Λ. These eigenvalues were arranged in descending order, and the NSP method was then applied to detect hierarchical modules as follows:

1. In the first mode, the eigenvector entries of all regions shared the same sign. This mode was considered the first level, consisting of a single module (i.e., the entire brain network).
2. In the second mode, regions with positive eigenvector values were grouped into one module, and those with negative values formed a second module. This mode represented the second level, containing two modules.
3. At the third mode, each of the two modules from the previous level was further divided based on the sign of the corresponding eigenvector entries, resulting in the third level. This process continued, producing progressively more modules at higher levels. When a level was reached where each module contained only a single region, the subdivision process stopped. Additionally, if all regions within a module maintained the same sign in the next mode, that module remained indivisible and had no further effect on subsequent iterations.

During the partitioning process, the number of modules M_i $(i = 1, \cdots, N)$ and the size of each module m_j $(j = 1, \cdots, M_i)$ were documented at each level. Unlike classical approaches that focus on segregation and integration at a single level, the NSP method defines these concepts across multiple levels.[13] The first level, with a single large module, captures global network integration and corresponds to the largest eigenvalue Λ. The second level, which splits the network into two modules, captures local integration within each module and segregation between modules. As more modes are considered, additional modules emerge, each representing more profound levels of segregation and associated with progressively smaller eigenvalues Λ. The segregation and integration components at each level can be defined as[56]

$$H_i = \frac{\Lambda_i^2 M_i (1 - p_i)}{N} \quad \text{(Equation 1)}$$

With

$$p_i = \frac{\sum_j |m_j - N/M_i|}{N} \quad \text{(Equation 2)}$$

Here, N is the number of regions, and p_i is a correction factor for the heterogeneous modular size and reflects the deviation from the optimized modular size $m_j - N/M_i$ in the i-th level. The global integration component is thus taken from the first level:

$$H_{In} = \frac{H_1}{N} \quad \text{(Equation 3)}$$

and the segregation component is summed from the 2^{nd} - N^{th} levels:

$$H_{Se} = \sum_{i=2}^{N} \frac{H_i}{N} \quad \text{(Equation 4)}$$

Based on the orthogonal and standard eigenvectors, the network integration and segregation components in each level could be mapped to each region j:

$$H_{In}^j = H_1 U_{1j}^2 \text{ and } H_{Se}^j = \left| \sum_{i=2}^{N} H_1 U_{ij}^2 \right| \quad \text{(Equation 5)}$$

where U_{ij} is the eigenvector value for the j-th region at the i-th level, such that regional features can be compared and linked to fatigue symptom. All computational analyses were conducted using MATLAB R2021b.

Gene expression analysis and enrichment

The gene expression data for this study were sourced from the Allen Human Brain Atlas,[56] an open-access resource offering 3,700 tissue samples from six individuals, complete with the Montreal Neurological Institute coordinates. Notably, the samples from four of these donors are exclusively from the left hemisphere, while those from the remaining two cover the entire brain. We employed the abagen toolbox to align the microarray gene expression data with 246 defined regions in the Human Brainnetome Atlas,[57] utilizing its standardized process to generate a regional gene expression matrix based on the atlas specifications, adhering to the recommended

default settings.[58] Despite the availability of right hemisphere gene expression data from only two donors, we opted to analyze whole-brain gene expression to address potential asymmetries between hemispheres. We performed Pearson correlation analyses to link gene expression with network components impacted by the MFI-20 total scores. Statistically significant genes were then analyzed using the ToppGene Suite[59] for functional enrichment to pinpoint associated human disease phenotype, applying a significance threshold of 0.05 with Bonferroni correction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Metrics of integration and segregation were computed using the NSP method across different hierarchical levels (ROI, network and global) of brain functional connectivity networks. We applied Analysis of Covariance (ANCOVA) to compare the NSP metrics along with MFI-20 (including its various dimension and total score) between the long COVID group at each post-infection time point (1-month and 3-month) and the healthy control group. These comparisons were conducted separately for each time point, while controlling multiple confounders to enhance the robustness of our findings. In a longitudinal analysis within the long COVID group, paired t-tests were utilized to detect significant changes in NSP metrics and MFI-20 (including its various dimension and total score) from 1-month to 3-month post-infection. To further explore the association between NSP metrics and fatigue, partial correlation analyses were conducted between these metrics and the dimensions of fatigue as measured by the MFI-20. These analyses were performed for each group separately, controlling for potential confounding variables.

Additionally, Bonferroni correction (BF) was applied to the corresponding p-values (ratio of 0.05 to the number of effective comparisons in the current analysis).[60] 50,000 permutations were performed to achieve the necessary p-value precision for BF.

ADDITIONAL RESOURCES

Ethics approval was granted by Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (XJTU1AF2023LSK-013). Trial registration number NCT05745805. All the participants gave written informed consent for themselves. All ethical regulations relevant to human research participants were followed.