

## Article

# Dynamic Neuroimmune–Endothelial Network Remodeling in Long COVID: A Longitudinal Multilayer Graph Analysis

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## Abstract

**Background:** Long COVID is a heterogeneous post-viral condition in which persistent neurological, autonomic, cognitive, and psychometric symptoms often occur without clear isolated biomarker abnormalities. This mismatch suggests that disease persistence may be driven not only by changes in individual markers, but by longitudinal reorganization of biological and clinical interactions. **Materials and Methods:** This observational longitudinal study evaluated patients with persistent symptoms after confirmed SARS-CoV-2 infection at 3 and 6 months. Clinical assessment included neurological examination, Hospital Anxiety and Depression Scale, Beck Depression Inventory, and COMPASS-31. Biomarkers representing hypoxia signaling, oxidative/redox stress, endothelial and renin–angiotensin system activity, glycation-related processes, and complement regulation were analyzed. Correlation analysis, association-level biomarker–clinical network modeling, and complementary Graphical LASSO-based sparse network estimation were used to compare network density, community organization, centrality, and edge rewiring between time points. **Results:** Conventional paired analysis identified HIF-1 $\alpha$  as the only continuous variable with a statistically significant longitudinal change (Wilcoxon statistic = 610.0,  $p = 0.000350$ ), whereas association-level network analysis revealed a broader systems-level signal. The association-level biomarker–clinical network preserved a similar global size at 3 and 6 months, with 16 nodes, 27 versus 26 edges, and densities of 0.225 versus 0.217. However, this apparent stability concealed substantial rewiring: 19 edges were shared, 8 were lost, and 7 emerged. Complementary Graphical LASSO analysis with 1000 bootstrap resamples supported this pattern by identifying a conservative sparse conditional-dependency core, including seven shared conditional-dependency edges across time points and selective weakening of four early conditional dependencies. The C3–C4 relationship reversed from negative to positive correlation ( $r = -0.618$  to  $r = 0.618$ ), indicating marked remodeling of complement-associated regulation. A psychometric–autonomic module involving Beck, HADS I, HADS II, and COMPASS-31 remained stable across both assessments. **Conclusions:** Long COVID progression was characterized by dynamic remodeling of immune, endothelial/RAS, oxidative-redox, hypoxia-related, autonomic, and psychometric interactions. Longitudinal network analysis identified a systems-level interaction structure that was not captured by isolated biomarker comparisons alone and that was further supported by complementary sparse conditional-dependency analysis.



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**Keywords:** long COVID; multilayer graph analysis; network remodeling; neuroimmune interactions; endothelial dysfunction; oxidative stress; autonomic dysfunction; biomarker networks; Graphical LASSO; longitudinal analysis

## 1. Introduction

Long COVID has emerged as a major chronic post-viral condition following SARS-CoV-2 infection, with persistent symptoms that may continue for months after the acute phase of disease [1]. Its clinical importance lies not only in its frequency, but also in its heterogeneity and long-term impact on daily functioning, quality of life, and work capacity [2]. Large cohort and global burden studies have shown that post-COVID manifestations commonly include persistent fatigue, respiratory complaints, cognitive impairment, mood-related symptoms, and other multisystem disturbances [3]. Neurological, autonomic, cognitive, and psychometric manifestations are particularly important because they frequently overlap, fluctuate over time, and may persist even when routine clinical or laboratory findings are not clearly abnormal [4,5]. This complexity highlights the need to move beyond descriptive symptom reporting and toward a deeper understanding of the biological mechanisms and system-level interactions that sustain long COVID.

Several biological mechanisms have been proposed to explain the persistent manifestations of long COVID. Early studies primarily focused on direct viral effects mediated through angiotensin-converting enzyme 2 (ACE2), which is expressed in endothelial, neuronal, and glial tissues and may contribute to neurological and vascular involvement during acute infection [6,7]. However, increasing evidence suggests that persistent post-COVID manifestations are more likely associated with chronic dysregulation of interconnected biological systems rather than ongoing direct viral injury alone [8]. Proposed mechanisms include sustained neuroinflammation, endothelial dysfunction, oxidative stress, mitochondrial impairment, altered hypoxia signaling, autonomic nervous system dysregulation, and persistent immune activation [9,10]. Consistent with this mechanistic framework, previous gene-expression analysis in acute COVID-19 and the recovery period reported altered expression profiles involving ACE2, Toll-like receptors, TMPRSS13, IFN-( $\gamma$ ), and IL-4, including changes observed six months after recovery [11]. Disturbances involving complement pathways, cytokine signaling, and microvascular dysfunction have also been implicated in the persistence of neurological, cognitive, and psychometric symptoms [12,13]. Importantly, these biological abnormalities appear to interact dynamically rather than independently, suggesting that long COVID may represent a complex systems-level disorder involving continuous remodeling of neuroimmune and vascular regulatory pathways [14,15].

Despite substantial progress in characterizing long COVID, important limitations remain in the current literature. Many studies primarily focus on isolated biomarkers or single biological pathways, often producing inconsistent findings across patient cohorts [16]. In addition, most investigations employ cross-sectional designs, which provide only static representations of a condition that is clinically dynamic and temporally heterogeneous [17]. Although neurological, autonomic, psychometric, immune, endothelial, and oxidative stress abnormalities have each been independently reported, integration between clinical manifestations and interconnected biological systems remains limited [18]. Consequently, longitudinal interactions between biomarkers and symptom domains, as well as their temporal remodeling during disease progression, remain insufficiently understood [19]. Furthermore, conventional statistical approaches are often optimized for detecting isolated group differences and may inadequately capture the complex, nonlinear, and network-

like interactions that characterize multisystem disorders such as long COVID [20]. These limitations highlight the need for longitudinal systems-oriented analytical frameworks capable of identifying hidden interaction structures and dynamic biological remodeling beyond individual biomarker abnormalities.

Biological systems are inherently organized as complex and interconnected networks rather than isolated linear pathways, and pathological conditions frequently emerge through alterations in interaction structure rather than single molecular abnormalities [21]. Network science and graph-based analytical approaches therefore provide an opportunity to identify hidden organizational patterns, interaction hubs, and system-level remodeling that may not be detectable using conventional statistical methods [22]. In recent years, approaches such as Graphical LASSO have been increasingly applied to high-dimensional biomedical data to infer conditional dependency structures and reduce spurious associations within complex biological systems [23]. Similarly, multilayer graph frameworks and patient similarity networks have enabled simultaneous investigation of relationships between biomarkers, clinical manifestations, and patient-specific phenotypes across multiple biological domains [24]. In the present study, this systems-level rationale was operationalized through longitudinal biomarker–clinical association networks, Graphical LASSO-based conditional-dependency networks, and edge-rewiring analysis, which directly correspond to the study objective and the analyses reported in the Results. This graph-based perspective is particularly relevant for long COVID because the disorder involves heterogeneous and temporally evolving interactions among immune, endothelial, autonomic, oxidative-stress, and psychometric domains [25]. In this context, longitudinal biomarker–clinical graph-based modeling may provide deeper insight into dynamic network remodeling and hidden biological interactions underlying persistent post-COVID manifestations, thereby supporting a more integrative and systems-oriented understanding of long COVID pathophysiology [26].

Long COVID is increasingly recognized as a dynamic condition in which symptoms and biological abnormalities may evolve over time rather than remain clinically or mechanistically static [4,14]. Importantly, longitudinal progression may involve changes in relationships among biological pathways even when individual biomarkers show only modest absolute changes [10]. Therefore, temporal network rewiring may provide information about disease evolution that is not captured by isolated biomarker comparisons alone [27]. This is particularly relevant because long COVID symptoms often follow heterogeneous trajectories, with different patterns of persistence, improvement, or fluctuation across patients [28]. Comparing early and later post-infection stages, such as 3 and 6 months, may therefore help identify whether neuroimmune, endothelial, autonomic, oxidative stress, and psychometric interactions remain stable or undergo systems-level remodeling. Accordingly, time-dependent graph-based analysis provides a suitable framework for detecting hidden interaction patterns and biological adaptation during the progression of persistent post-COVID manifestations.

To examine long COVID as a systems-level disorder, the present study investigated longitudinal interactions between biological and clinical domains using a multilayer graph-based framework. Patients with persistent post-COVID manifestations were evaluated at 3 and 6 months after SARS-CoV-2 infection through neurological examination, psychometric and autonomic assessment, and laboratory analysis of biomarkers related to oxidative stress, endothelial dysfunction, hypoxia signaling, and immune regulation. Rather than analyzing biomarkers as isolated variables, the analytical strategy reconstructed the evolving architecture of long COVID through biomarker interaction networks, Graphical LASSO-based biomarker–clinical conditional-dependency networks, and longitudinal network rewiring analysis. We hypothesized that the fundamental pathological signature of long

COVID may emerge from dynamic remodeling of interactions between neuroimmune, endothelial, autonomic, oxidative stress, and psychometric systems over time, thereby uncovering hidden organizational patterns, interaction hierarchies, and trajectories of disease evolution that remain inaccessible to conventional biomarker-centered analyses and revealing systems-level mechanisms underlying persistent post-COVID manifestations that cannot be identified through isolated statistical comparisons alone. Accordingly, the present analysis combined broad association-level networks with sparse conditional-dependency modeling to capture both overall biomarker–clinical relationship remodeling and more conservative network structures supported by regularized estimation.

## 2. Materials and Methods

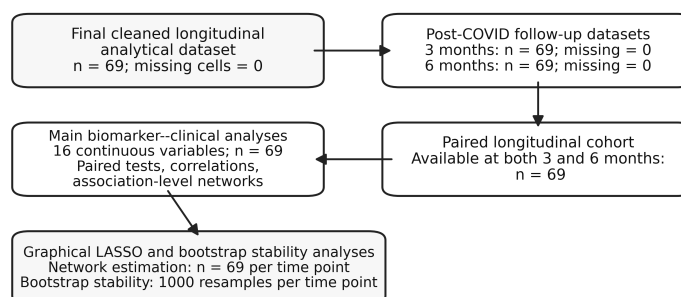
### 2.1. Study Design and Participants

The present study was designed as a prospective longitudinal observational study conducted in Karaganda, Republic of Kazakhstan. Patients were recruited from May to August 2021 after recovery from PCR-confirmed COVID-19 and discharge from infectious disease hospitals. Participants were followed during the post-COVID period according to each patient's post-infection timeline, with two predefined observation points: a short-term assessment at 3 months after infection and a longer-term assessment at 6 months after infection. No healthy control group was included because the study was designed to evaluate longitudinal within-cohort changes and interaction patterns during post-COVID follow-up.

Eligible participants were adults older than 18 years with mild, moderate, or severe COVID-19 confirmed by polymerase chain reaction testing. Acute disease severity was classified according to World Health Organization clinical management guidance [29]. Mild cases were defined as asymptomatic or mildly symptomatic infection without radiological lung changes; moderate cases were defined by clinical manifestations of infection with radiological or clinical evidence of pneumonia but without signs of severe disease; and severe cases were defined by clinical signs of pneumonia accompanied by at least one severity criterion, including respiratory rate >30 breaths/min, severe respiratory distress, or reduced oxygen saturation on room air. Patients were excluded if they were pregnant or lactating, immunocompromised, including if they had HIV, or had active malignancy at the time of examination. These exclusion criteria were applied to reduce potential confounding from conditions that could independently affect immune, endothelial, inflammatory, or oxidative stress-related biomarker profiles.

Clinical information related to the acute phase of COVID-19 was obtained from electronic medical records, whereas post-COVID follow-up assessments included clinical evaluation and blood sampling for biomarker analysis. For the main longitudinal analyses, the analytical cohort was defined as participants who completed both scheduled post-COVID follow-up assessments at 3 and 6 months and had complete paired records for the variables used in the principal statistical and network analyses. This complete paired longitudinal cohort consisted of 69 participants and provided a consistent participant denominator for paired tests, correlation matrices, association-level biomarker–clinical networks, Graphical LASSO network estimation, and bootstrap stability analysis. The study was conducted in accordance with the Declaration of Helsinki [30] and was approved by the Bioethics Committee of Karaganda Medical University, Kazakhstan, Protocol No. 18, dated 14 April 2021. Written informed consent was obtained from all participants. The participant flow for the longitudinal analytical cohort is summarized in Figure 1.

### STROBE-style participant flow diagram for the longitudinal analytical cohort



The diagram summarizes the final analytical cohort used for paired statistical, correlation-based, association-level network, Graphical LASSO, and bootstrap stability analyses.

**Figure 1.** STROBE-style participant flow diagram for the longitudinal analytical cohort. The diagram summarizes the final cleaned dataset, completion of the scheduled 3-month and 6-month post-COVID follow-up assessments, the complete paired longitudinal cohort denominator, and the fixed analytical denominators used for paired statistical tests, correlation matrices, association-level biomarker-clinical network analysis, Graphical LASSO network estimation, and bootstrap stability analysis.

## 2.2. Clinical and Neurological Assessment

Clinical and neurological assessments were performed at both post-COVID observation points, corresponding to 3 and 6 months after SARS-CoV-2 infection. At each visit, participants underwent a standard clinical neurological examination performed by a neurologist. The neurological assessment included evaluation of the main components of neurological status, including sensory function, coordination, balance, tremor, Romberg test performance, signs of hypesthesia, local pain on palpation, and other clinically relevant neurological findings. Neurological status was first recorded descriptively for clinical characterization of post-COVID neurological manifestations and was subsequently encoded into binary symptom variables for inclusion in the network-based analyses. Dedicated objective cognitive screening tests or formal neuropsychological batteries were not part of the present protocol. Accordingly, cognitive manifestations are discussed as part of the broader neurological and neuropsychiatric context of long COVID, whereas the cohort-level analyses were based on the neurological examination findings, psychometric scores, autonomic symptom scores, and biomarker measurements available in the dataset.

Psychometric assessment was performed using the Beck Depression Inventory [31] and the Hospital Anxiety and Depression Scale [32]. Both instruments were administered at the 3-month and 6-month follow-up assessments. Beck scores were recorded as quantitative indicators of depressive symptom burden, while HADS I and HADS II were recorded as separate psychometric variables. HADS I corresponded to the HADS anxiety subscale (HADS-A), whereas HADS II corresponded to the HADS depression subscale (HADS-D). For consistency with the original variable names used in the analytical dataset, statistical outputs, network figures, and supplementary files, these variables are reported as HADS I and HADS II throughout the manuscript, tables, and figures. Numerical questionnaire scores were used in subsequent statistical and network-based analyses where applicable.

Autonomic symptoms were assessed using the Composite Autonomic Symptom Score 31 [33] at both follow-up time points. COMPASS-31 was used to characterize autonomic symptom burden during the post-COVID period, and the recorded scores represented summed questionnaire responses. Together with Beck and HADS scores, COMPASS-31 values were incorporated as clinical variables in the analysis of interactions between psychometric, autonomic, neurological, and biological domains.

### 2.3. Laboratory Biomarker Analysis

Blood samples were collected during the post-COVID follow-up period for laboratory assessment of biological pathways potentially involved in long COVID. For the present study, the final analytical dataset included biomarker data from 69 patients. Venous blood was collected into EDTA tubes and centrifuged at  $1500\text{--}2000\times g$  for 10–15 min at  $+4\text{ }^{\circ}\text{C}$ . Plasma was aliquoted and stored at  $-80\text{ }^{\circ}\text{C}$  until laboratory analysis.

The biomarker panel was selected to represent several biological domains relevant to post-COVID pathophysiology. Hypoxia-signaling markers included hypoxia-inducible factor 1-alpha and hypoxia-inducible factor 2-alpha. Oxidative stress and redox-related markers included reduced glutathione, oxidized glutathione, malondialdehyde, and methylglyoxal. Endothelial, vascular, and renin-angiotensin system-associated markers included angiotensin-converting enzyme 2, angiotensin I, angiotensin II, and advanced glycation end products. Immune and complement-related markers included complement components C3 and C4. These biomarkers were incorporated into subsequent statistical, longitudinal, and graph-based analyses to evaluate both individual biomarker variation and interaction-level remodeling across biological domains.

Concentrations of angiotensin-converting enzyme 2, complement components C3 and C4, and hypoxia-inducible factor 1-alpha were determined using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Optical density was measured at 450 nm with reference wavelength correction, and concentrations were calculated using a four-parameter logistic standard curve. Additional biomarkers, including angiotensin I, angiotensin II, and advanced glycation end products, were measured using plate-based photometric assay procedures, with absorbance readings recorded at 450/620 nm. For biomarkers measured across more than one analytical plate, plate 1 and plate 2 used the same calibration standard series for the corresponding biomarker; therefore, measurements from both plates were expressed in the same units. Plate identifiers were retained for laboratory tracking and did not represent distinct biological analytes or different measurement units.

Quality control was performed using standard calibrators and control samples. Detection limits, intra-assay coefficients of variation, and inter-assay coefficients of variation were within the ranges recommended by the manufacturers and did not exceed 10% for the available assays. Final biomarker values were exported for statistical, longitudinal, and network-based analyses.

### 2.4. Data Preprocessing and Normalization

The clinical and laboratory data were organized into structured analytical matrices corresponding to the two post-COVID observation points: 3 months and 6 months after SARS-CoV-2 infection. Each matrix included the final set of variables used for analysis, including biomarker concentrations, psychometric and autonomic questionnaire scores, and encoded neurological examination findings. Continuous clinical and laboratory variables were retained as quantitative features, whereas neurological signs derived from the clinical examination were transformed into binary variables indicating the presence or absence of specific findings.

The main longitudinal biomarker-clinical analyses were conducted using the complete paired longitudinal analytical cohort defined in Section 2.1. For the 16 continuous variables included in the principal paired statistical tests, correlation matrices, association-level networks, Graphical LASSO network estimation, and bootstrap stability analyses, the final cleaned 3-month and 6-month analytical matrices contained complete paired records for all 69 participants. Therefore, no additional participant exclusion was applied for these main continuous-variable analyses. Before z-score standardization for correlation and network

modeling, biomarker variables were retained in their original laboratory reporting units and clinical variables were retained in their questionnaire score scales. Table 1 summarizes the analysis-specific denominators, variable-level missing-data accounting, original laboratory reporting units or clinical score scales, and assay/QC information for the main longitudinal biomarker–clinical analyses.

Variable names and biological annotations were harmonized before analysis to ensure consistency across datasets and time points. Biomarkers were grouped according to their main biological relevance, including hypoxia signaling, oxidative stress and redox regulation, endothelial and renin–angiotensin system activity, complement-associated immune regulation, and glycation-related pathways. Clinical variables included Beck scores, HADS I and HADS II scores, COMPASS-31 scores, and neurological symptom indicators. This organization allowed the dataset to be used for both conventional longitudinal comparisons and graph-based modeling of interactions between biological and clinical domains.

Before network construction, continuous variables were standardized to reduce the influence of differences in measurement units and numerical ranges across biomarkers and clinical scales. Standardization was performed using z-score scaling, in which each continuous variable was centered by subtracting its mean and scaled by its standard deviation within the corresponding analytical dataset. Standardized values were used for correlation-based analysis, association-level biomarker–clinical network construction and Graphical LASSO-based network estimation. Binary neurological variables were retained in their encoded form for analyses requiring symptom-level representation. The resulting preprocessed datasets provided consistent input matrices for evaluating longitudinal changes, interaction structures, and network remodeling between the 3-month and 6-month post-COVID assessments.

## 2.5. Correlation and Statistical Analysis

Classical statistical analyses were performed to evaluate longitudinal changes in clinical and laboratory variables between the 3-month and 6-month post-COVID assessments. Continuous variables were summarized using descriptive statistics, including measures of central tendency and dispersion. Longitudinal comparisons were performed to identify variables showing significant changes over time, while recognizing that the primary focus of the study was the interaction structure among variables rather than isolated biomarker differences.

Associations between biomarkers, psychometric scores, autonomic scores, and encoded neurological variables were assessed using correlation analysis. Pearson correlation was used for the biomarker–clinical correlation matrices, and association-level network edges were retained when the absolute correlation coefficient met the predefined threshold of  $|r| \geq 0.155$ . Correlation matrices were generated separately for the 3-month and 6-month datasets to evaluate time-specific relationships among biological and clinical features. These matrices were subsequently used to support the construction and interpretation of interaction networks.

For inferential statistical tests, a two-sided  $p$ -value  $< 0.05$  was considered statistically significant. Paired longitudinal comparisons were reported with the Wilcoxon signed-rank statistic, paired median change, bootstrap percentile 95% confidence interval for the paired change, standardized effect size, nominal  $p$ -value, and Benjamini–Hochberg false-discovery-rate-adjusted  $p$ -value. Multiple-testing adjustment was applied across the family of 16 paired longitudinal biomarker–clinical comparisons. Statistical analyses were performed as an initial step before graph-based modeling, allowing conventional longitudinal findings to be compared with network-level results.

**Table 1.** Analytical denominators, original laboratory reporting units, and variable-level missing-data accounting for the main longitudinal biomarker–clinical analyses.

| <b>Panel A. Analysis-Specific Participant Denominators</b>                                     |                            |                          |                          |                         |                                                                                                                                           |
|------------------------------------------------------------------------------------------------|----------------------------|--------------------------|--------------------------|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Analysis Layer</b>                                                                          | <b>3-Month n</b>           | <b>6-Month n</b>         | <b>Paired n</b>          | <b>Input Variables</b>  | <b>Analytical Use</b>                                                                                                                     |
| Paired longitudinal tests                                                                      | 69                         | 69                       | 69                       | 16 continuous variables | Within-participant comparison of 3-month and 6-month biomarker–clinical values.                                                           |
| 3-month correlation matrix                                                                     | 69                         | –                        | –                        | 16 continuous variables | Time-point-specific Pearson correlation matrix.                                                                                           |
| 6-month correlation matrix                                                                     | –                          | 69                       | –                        | 16 continuous variables | Time-point-specific Pearson correlation matrix.                                                                                           |
| 3-month association-level network                                                              | 69                         | –                        | –                        | 16 continuous variables | Network constructed from retained pairwise biomarker–clinical associations.                                                               |
| 6-month association-level network                                                              | –                          | 69                       | –                        | 16 continuous variables | Network constructed from retained pairwise biomarker–clinical associations.                                                               |
| 3-month Graphical LASSO network                                                                | 69                         | –                        | –                        | 16 continuous variables | Sparse conditional-dependency network estimated from the 3-month analytical matrix.                                                       |
| 6-month Graphical LASSO network                                                                | –                          | 69                       | –                        | 16 continuous variables | Sparse conditional-dependency network estimated from the 6-month analytical matrix.                                                       |
| Bootstrap stability analysis                                                                   | 69                         | 69                       | –                        | 16 continuous variables | Bootstrap resampling was performed separately at each time point using the corresponding 69-participant analytical matrix.                |
| <b>Panel B. Variable-level missing-data accounting, original units, and assay/QC reporting</b> |                            |                          |                          |                         |                                                                                                                                           |
| <b>Variable</b>                                                                                | <b>Original unit/scale</b> | <b>3-month missing n</b> | <b>6-month missing n</b> | <b>Paired n</b>         | <b>Domain and assay/QC reporting</b>                                                                                                      |
| ACE2                                                                                           | U/L                        | 0                        | 0                        | 69                      | Endothelial/RAS marker; plate-based ELISA/immunoassay; 450/620 nm reading; standard-curve quantification; plate-level QC; CV ≤ 10%.       |
| Advanced glycation end products                                                                | ng/mL                      | 0                        | 0                        | 69                      | Glycation-related marker; plate-based immunoassay; standard-curve quantification; shared calibration standards; plate-level QC; CV ≤ 10%. |
| Angiotensin I                                                                                  | pg/mL                      | 0                        | 0                        | 69                      | Endothelial/RAS marker; plate-based immunoassay; standard-curve quantification; shared calibration standards; plate-level QC; CV ≤ 10%.   |
| Angiotensin II                                                                                 | pg/mL                      | 0                        | 0                        | 69                      | Endothelial/RAS marker; plate-based immunoassay; standard-curve quantification; shared calibration standards; plate-level QC; CV ≤ 10%.   |
| C3                                                                                             | mg/dL                      | 0                        | 0                        | 69                      | Complement/immune marker; plate-based ELISA/immunoassay; 450/620 nm reading; standard-curve quantification; plate-level QC; CV ≤ 10%.     |
| C4                                                                                             | mg/dL                      | 0                        | 0                        | 69                      | Complement/immune marker; plate-based ELISA/immunoassay; 450/620 nm reading; standard-curve quantification; plate-level QC; CV ≤ 10%.     |
| HIF-1α                                                                                         | pg/mL                      | 0                        | 0                        | 69                      | Hypoxia-signaling marker; plate-based ELISA/immunoassay; 450/620 nm reading; standard-curve quantification; plate-level QC; CV ≤ 10%.     |
| HIF-2α                                                                                         | pg/mL                      | 0                        | 0                        | 69                      | Hypoxia-signaling marker; plate-based ELISA/immunoassay; standard-curve quantification; plate-level QC; CV ≤ 10%.                         |
| MDA                                                                                            | μmol/L                     | 0                        | 0                        | 69                      | Oxidative stress marker; plate-based biochemical assay; calibrator-based quantification; plate-level QC; CV ≤ 10%.                        |
| GSSG                                                                                           | nmol/L                     | 0                        | 0                        | 69                      | Oxidized glutathione/redox marker; plate-based biochemical assay; calibrator-based quantification; plate-level QC; CV ≤ 10%.              |
| GSH                                                                                            | nmol/L                     | 0                        | 0                        | 69                      | Reduced glutathione/redox marker; plate-based biochemical assay; calibrator-based quantification; plate-level QC; CV ≤ 10%.               |
| Methylglyoxal                                                                                  | nmol/L                     | 0                        | 0                        | 69                      | Dicarbonyl/glycation-stress marker; plate-based biochemical assay; calibrator-based quantification; plate-level QC; CV ≤ 10%.             |
| Beck Depression Inventory                                                                      | Score                      | 0                        | 0                        | 69                      | Psychometric measure; continuous clinical score included in paired tests, correlation matrices, and network analyses.                     |
| HADS I                                                                                         | Score                      | 0                        | 0                        | 69                      | Psychometric measure; continuous clinical score included in paired tests, correlation matrices, and network analyses.                     |
| HADS II                                                                                        | Score                      | 0                        | 0                        | 69                      | Psychometric measure; continuous clinical score included in paired tests, correlation matrices, and network analyses.                     |
| COMPASS-31                                                                                     | Score                      | 0                        | 0                        | 69                      | Autonomic symptom score; continuous clinical score included in paired tests, correlation matrices, and network analyses.                  |

Note: The final longitudinal analytical cohort consisted of 69 participants who completed both scheduled post-COVID follow-up assessments. Original units/scales indicate the laboratory reporting units or clinical score scales used before z-score standardization for network modeling. For the 16 continuous biomarker–clinical variables used in the main paired statistical tests, correlation matrices, association-level networks, Graphical LASSO network estimation, and bootstrap stability analyses, no missing values were present in the final cleaned 3-month or 6-month analytical matrices. Therefore, paired tests used 69 paired observations, whereas each time-point-specific correlation matrix, association-level network, and Graphical LASSO network used the corresponding 69-participant analytical matrix. Biomarkers were quantified using calibrated plate-based assays with standard-curve or calibrator-based quantification, plate-level quality control, and coefficients of variation not exceeding 10%.

## 2.6. Graph-Based and Network Analysis

Graph-based analysis was used to examine long COVID as an interconnected system of biological and clinical features rather than as a collection of isolated variables. In this framework, biomarkers, psychometric scores, autonomic scores, and encoded neurological findings were represented as network nodes, while statistical or model-derived relationships between these features were represented as edges. Separate networks were constructed for the 3-month and 6-month post-COVID assessments, allowing time-specific interaction patterns to be evaluated and compared. This multilayer approach was designed to identify hidden structures of association, connectivity, and systems-level organization across biological and clinical domains that may not be evident from conventional variable-by-variable analysis alone.

The network analysis included two complementary levels. Association-level biomarker-clinical networks were first constructed separately for the 3-month and 6-month datasets using the same node set to describe broad pairwise relationships among biological and clinical variables. In these networks, edges represented retained pairwise Pearson associations with an absolute correlation coefficient of  $|r| \geq 0.155$ . Graphical LASSO was then applied as a complementary sparse conditional-dependency analysis to characterize a more conservative sparse network structure among the same variables.

Biomarker-clinical interaction networks were constructed to evaluate pairwise relationships among laboratory markers and clinical variables at each follow-up time point. In these networks, nodes represented individual biomarkers, psychometric scores, autonomic scores, and encoded neurological features, while edges represented statistical associations between pairs of variables. Separate interaction networks were generated for the 3-month and 6-month datasets using the same analytical procedure, allowing direct comparison of network organization across time. This approach enabled identification of relationships that remained stable, disappeared, or emerged during follow-up, thereby providing a basis for evaluating longitudinal remodeling of biological and clinical interaction structures in long COVID.

To complement the pairwise association networks with a more conservative conditional-dependency layer, Graphical LASSO-based network modeling was applied to estimate sparse conditional dependency structures among the analyzed biological and clinical variables [34]. This method estimates the inverse covariance matrix with regularization, allowing the construction of a sparse network in which edges represent conditional relationships between variables after accounting for the influence of other variables in the dataset. Compared with simple pairwise correlation networks, Graphical LASSO can reduce indirect or potentially spurious associations and highlight more specific patterns of connectivity. In the present study, this approach was used to model conditional interactions among biomarkers, psychometric scores, autonomic scores, and encoded neurological features at the 3-month and 6-month post-COVID assessments.

Network topology was evaluated using graph-level and node-level measures to characterize the organization of biological and clinical interaction networks at each time point. Network density was used to describe the overall degree of connectivity within each network, while centrality measures were used to identify biomarkers or clinical features occupying more influential positions in the interaction structure. Community detection was performed to identify clusters of closely connected variables, reflecting potential functional grouping across biological and clinical domains. Longitudinal network rewiring was assessed by comparing the 3-month and 6-month networks, with edges classified as shared, lost, or gained according to their presence across time points. This edge-level comparison allowed identification of stable interactions as well as newly emerging or disappearing re-

lationships, providing a direct measure of dynamic remodeling in the network architecture of long COVID.

2.7. Graphical LASSO and Bootstrap Stability Analysis

Graphical LASSO was applied as a complementary sparse conditional-dependency analysis using the 16 continuous biological and clinical variables included in the main biomarker–clinical network. For each time point, the standardized 3-month and 6-month data matrices were analyzed separately. Regularization was selected using GraphicalLassoCV, resulting in selected  $\alpha$  values of approximately 0.171733 for the 3-month dataset and 0.180488 for the 6-month dataset. The estimated precision matrices were converted into partial-correlation matrices, and sparse conditional-dependency edges were retained when the absolute partial correlation was at least 0.05. The main model settings and stability parameters are summarized in Table 2.

Table 2. Graphical LASSO parameters and bootstrap stability settings.

| Parameter                     | Specification                                                                                  |
|-------------------------------|------------------------------------------------------------------------------------------------|
| Input matrix                  | Standardized biomarker–clinical matrix                                                         |
| Variables included            | 16 continuous biological and clinical variables                                                |
| Time-point analysis           | 3-month and 6-month datasets analyzed separately                                               |
| Estimator                     | Graphical LASSO                                                                                |
| Regularization method         | GraphicalLassoCV                                                                               |
| Selected $\alpha$ at 3 months | 0.171733                                                                                       |
| Selected $\alpha$ at 6 months | 0.180488                                                                                       |
| Network matrix                | Partial-correlation matrix derived from the precision matrix                                   |
| Edge-retention rule           | Absolute partial correlation $\geq 0.05$                                                       |
| Bootstrap iterations          | 1000 resamples per time point                                                                  |
| Bootstrap procedure           | Resampling with replacement, model refitting, edge re-extraction, and centrality recalculation |
| Edge stability                | Edge-selection frequency across bootstrap resamples                                            |
| Centrality stability          | Top-N frequency and bootstrap summaries                                                        |
| Primary centrality measures   | Degree and strength                                                                            |

Bootstrap stability was evaluated using 1000 resamples per time point. In each bootstrap iteration, patients were resampled with replacement, the Graphical LASSO model was refitted, sparse edges were re-extracted, and node centrality measures were recalculated. Edge stability was reported as the frequency with which each sparse conditional-dependency edge was selected across bootstrap resamples. Centrality stability was summarized using top-N frequency and bootstrap summaries, with degree and strength emphasized as the primary centrality measures.

2.8. Longitudinal Network Remodeling Analysis

Longitudinal network remodeling was evaluated by directly comparing the biological and clinical interaction networks constructed for the 3-month and 6-month post-COVID assessments. Networks generated at both time points were compared using the same node set and analytical framework to ensure that observed differences reflected changes in interaction structure rather than differences in variable inclusion. This comparison focused on changes in edge presence, edge direction or sign where applicable, edge strength, node connectivity, centrality patterns, and community organization.

Dynamic network changes were characterized by identifying shared edges that persisted across both time points, lost edges that were present at 3 months but absent at 6 months, and gained edges that emerged at 6 months. Changes in network topology

were interpreted as evidence of longitudinal remodeling in the relationships among neuroimmune, endothelial, renin–angiotensin, oxidative stress, autonomic, psychometric, and neurological domains. Particular attention was given to interactions showing reversal or marked change over time, as these patterns may indicate evolving regulatory relationships that are not captured by isolated biomarker comparisons. This analysis provided the main framework for evaluating whether long COVID progression was associated with dynamic reorganization of biological and clinical interaction networks.

### 2.9. Visualization and Software Environment

All data preprocessing, statistical analysis, graph construction, network comparison, and visualization procedures were implemented in Python 3.10.12. Data handling and matrix preparation were performed using pandas and NumPy, while statistical analyses, including correlation analysis and paired longitudinal comparisons, were performed using SciPy. Standardization procedures were implemented using scikit-learn preprocessing tools. Graphical LASSO-based conditional dependency networks were estimated using the covariance module of scikit-learn.

Network construction and graph-theoretical analyses were performed using NetworkX. The analysis pipeline generated biomarker–clinical interaction networks, Graphical LASSO-based conditional-dependency networks, and longitudinal edge-rewiring outputs. Network-level and node-level metrics included density, degree measures, weighted degree, degree centrality, betweenness centrality, closeness centrality, eigenvector centrality, connected-component structure, clustering measures, and community structure. Community detection was performed using greedy modularity optimization implemented in NetworkX.

Visualizations were generated in Python using Matplotlib 3.9.0 and NetworkX 3.4.2 layout functions. Network graphs were visualized using force-directed layouts, and additional plots were generated for correlation heatmaps, centrality rankings, metric changes, community sizes, and edge-rewiring summaries. Output files were saved as tabular reports, figures, and GraphML files to support reproducibility and downstream inspection of network structures.

## 3. Results

### 3.1. Analytical Dataset and Variable Domains

The study dataset comprised 69 patients evaluated at two post-COVID follow-up time points, corresponding to 3 and 6 months after SARS-CoV-2 infection. For each time point, a structured analytical matrix was generated using the same set of clinical and biological variables, enabling matched longitudinal comparison between the short-term and longer-term post-COVID periods. This organization allowed subsequent analyses to evaluate both variable-level changes and changes in the interaction structure among biological and clinical domains.

Cohort context and targeted analytical characteristics of the complete paired longitudinal cohort are summarized in Table 3. The present analysis was derived from a larger post-COVID research project and was designed to evaluate longitudinal biochemical–clinical interaction patterns. Accordingly, the table reports the cohort-level clinical context, paired follow-up structure, and biological and clinical variable framework used for the main statistical and network analyses.

The analytical framework included 12 biological markers, four questionnaire-based clinical scores, and encoded neurological examination findings. The biological variables covered hypoxia signaling, oxidative/redox stress, endothelial and renin–angiotensin system activity, glycation-related processes, and complement-associated immune regulation.

The clinical component included psychometric and autonomic symptom scores, while neurological examination findings were first recorded descriptively and then encoded as binary variables indicating the presence or absence of specific neurological signs. Thus, the dataset was structured to support a multilayer network analysis in which laboratory biomarkers, questionnaire scores, and neurological findings could be evaluated within a unified longitudinal framework. The clinical and biological variable domains included in the analysis are summarized in Table 4.

**Table 3.** Cohort context and targeted analytical characteristics of the complete paired longitudinal cohort.

| Characteristic                                                                          | Complete Paired Cohort ( <i>n</i> = 69)                                                                                                                        |
|-----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Participants included in the complete paired longitudinal analytical cohort             | 69                                                                                                                                                             |
| Participants completing both 3-month and 6-month post-COVID follow-up assessments       | 69 (100%)                                                                                                                                                      |
| Acute COVID-19 severity at admission                                                    | Mild, moderate, or severe                                                                                                                                      |
| Mild acute COVID-19, <i>n</i> (%)                                                       | 47 (68.1%)                                                                                                                                                     |
| Moderate acute COVID-19, <i>n</i> (%)                                                   | 19 (27.5%)                                                                                                                                                     |
| Severe acute COVID-19, <i>n</i> (%)                                                     | 3 (4.3%)                                                                                                                                                       |
| Continuous biomarker–clinical variables used in the main longitudinal analyses          | 16                                                                                                                                                             |
| Biochemical variables included in the main continuous-variable analyses                 | 12                                                                                                                                                             |
| Questionnaire-based clinical scores included in the main continuous-variable analyses   | 4                                                                                                                                                              |
| Neurological examination indicators encoded for symptom-level clinical characterization | 14 binary indicators at each follow-up time point                                                                                                              |
| Primary analytical focus of the cohort                                                  | Longitudinal biochemical–clinical association patterns, Graphical LASSO conditional-dependency structure, network remodeling, and bootstrap stability analysis |

Note: The table reports cohort-level characteristics relevant to the specific objective of the present biochemical–clinical network analysis. Acute COVID-19 severity at admission is provided as clinical context for the paired longitudinal cohort. The main analytical objective was to characterize longitudinal relationships among biochemical markers, questionnaire-based clinical scores, and encoded neurological examination findings.

**Table 4.** Clinical and biological variable domains included in the longitudinal network analysis.

| Domain                                    | Variables Included                                                   | Data Type                 |
|-------------------------------------------|----------------------------------------------------------------------|---------------------------|
| Hypoxia signaling                         | HIF-1 $\alpha$ , HIF-2 $\alpha$                                      | Continuous biomarkers     |
| Oxidative/redox and dicarbonyl stress     | GSH, GSSG, MDA, methylglyoxal                                        | Continuous biomarkers     |
| Endothelial/RAS/glycation-related markers | ACE2, angiotensin I, angiotensin II, advanced glycation end products | Continuous biomarkers     |
| Complement/immune regulation              | C3, C4                                                               | Continuous biomarkers     |
| Psychometric assessment                   | Beck Depression Inventory, HADS I, HADS II                           | Questionnaire scores      |
| Autonomic assessment                      | COMPASS-31                                                           | Questionnaire score       |
| Neurological examination                  | Encoded neurological signs                                           | Binary clinical variables |

Abbreviations: RAS, renin–angiotensin system; HIF, hypoxia-inducible factor; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; ACE2, angiotensin-converting enzyme 2; HADS, Hospital Anxiety and Depression Scale; COMPASS-31, Composite Autonomic Symptom Score 31.

### 3.2. Longitudinal Changes in Individual Biomarkers and Clinical Scores

Longitudinal paired comparisons were performed to determine whether individual biological and clinical variables changed between the 3-month and 6-month post-COVID assessments. Across the 16 continuous variables included in the main biomarker–clinical network, most markers demonstrated limited isolated longitudinal change. The only variable

showing a statistically significant longitudinal difference after multiple-testing adjustment was HIF-1 $\alpha$ , with supporting effect-size and adjusted-significance estimates (Wilcoxon statistic = 610.0, nominal  $p$  = 0.000350, false-discovery-rate-adjusted  $p$  = 0.00560, standardized effect size  $r$  = 0.43). No other biomarker, psychometric score, or autonomic score remained statistically significant after false-discovery-rate adjustment.

Although several variables showed visible median shifts, including ACE2, C4, angiotensin II, HADS I, and COMPASS-31, these changes were not statistically significant. The absence of broad variable-level significance is important because it indicates that the main longitudinal signal was not dominated by large isolated biomarker changes. Instead, the findings suggest that the progression of long COVID in this cohort may be more strongly reflected by changes in the relationships among biological and clinical variables than by changes in single markers alone. Raw-unit biomarker summaries are presented in Table 5 to support clinical interpretability. The full longitudinal comparison of standardized continuous variables used for the statistical and network analyses is presented in Table 6.

**Table 5.** Raw-unit biomarker summaries at 3 and 6 months after SARS-CoV-2 infection.

| Domain                       | Biomarker      | Original Unit | 3 Months, Median [IQR]  | 6 Months, Median [IQR]  |
|------------------------------|----------------|---------------|-------------------------|-------------------------|
| Hypoxia signaling            | HIF-1 $\alpha$ | pg/mL         | 0.0140 [0.0110, 0.0170] | 0.0534 [0.0432, 0.0617] |
| Hypoxia signaling            | HIF-2 $\alpha$ | pg/mL         | 0.0160 [0.0130, 0.0220] | 0.0391 [0.0293, 0.0541] |
| Oxidative/redox stress       | GSH            | nmol/L        | 44.5 [16.9, 71.1]       | 45.2 [16.3, 72.3]       |
| Oxidative/redox stress       | GSSG           | nmol/L        | 2973.2 [1898.3, 4921.0] | 3054.3 [2023.6, 4771.2] |
| Oxidative/redox stress       | MDA            | $\mu$ mol/L   | 0.0400 [0.0304, 0.0474] | 0.0410 [0.0330, 0.0460] |
| Oxidative/glycation stress   | Methylglyoxal  | nmol/L        | 438.0 [273.7, 777.9]    | 438.3 [288.0, 838.2]    |
| Endothelial/RAS              | ACE2           | U/L           | 0.629 [0.463, 0.824]    | 9.675 [8.323, 22.579]   |
| Endothelial/RAS              | Angiotensin I  | pg/mL         | 0.3 [0.2, 0.4]          | 52.8 [34.8, 79.7]       |
| Endothelial/RAS              | Angiotensin II | pg/mL         | 2.714 [2.111, 2.901]    | 4.230 [2.405, 15.391]   |
| Glycation-related            | AGEs           | ng/mL         | 0.5 [0.5, 0.6]          | 226.5 [157.6, 278.3]    |
| Complement/immune regulation | C3             | mg/dL         | 1.3 [1.0, 1.8]          | 45.5 [32.2, 68.3]       |
| Complement/immune regulation | C4             | mg/dL         | 1.318 [1.124, 1.807]    | 0.632 [0.263, 0.812]    |

Note: Values are presented in original laboratory reporting units as median [interquartile range] for the complete paired longitudinal analytical cohort ( $n$  = 69). These raw-unit summaries are provided for clinical interpretability. Standardized values were used for correlation analysis, association-level network construction, Graphical LASSO network estimation, and bootstrap stability analysis to place biomarkers and clinical scores on a comparable scale. Abbreviations: ACE2, angiotensin-converting enzyme 2; AGEs, advanced glycation end products; GSH, reduced glutathione; GSSG, oxidized glutathione; HIF, hypoxia-inducible factor; MDA, malondialdehyde; RAS, renin–angiotensin system.

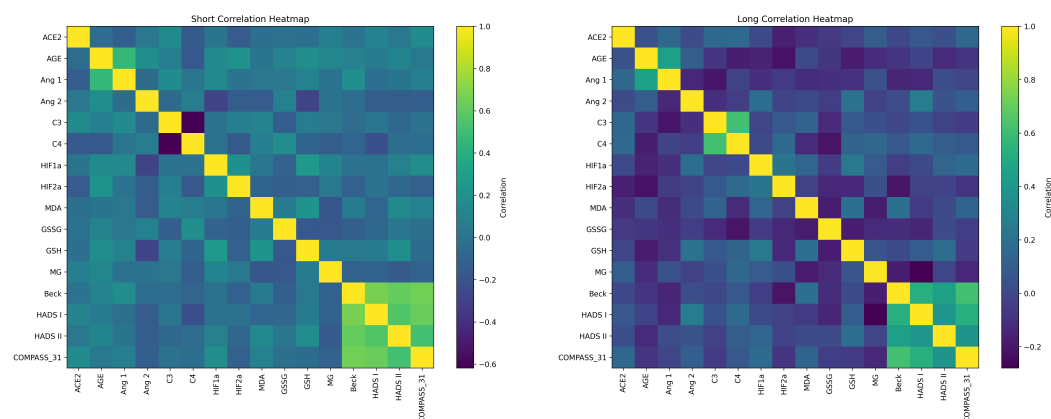
### 3.3. Time-Dependent Correlation Structure

Correlation analysis was performed separately for the 3-month and 6-month datasets to evaluate whether relationships among biological and clinical variables changed over time. This correlation-based analysis captured the broader association-level structure and provided the pairwise biomarker–clinical context for the subsequent sparse conditional-dependency modeling with Graphical LASSO. Figure 2 shows that the overall association structure was not static between the two post-COVID time points. While several relationships persisted across both assessments, other associations weakened, disappeared, emerged, or changed direction, indicating time-dependent remodeling of the biomarker–clinical correlation structure.

**Table 6.** Longitudinal comparison of biomarkers and clinical scores between 3 and 6 months after SARS-CoV-2 infection, including paired change estimates, effect sizes, bootstrap confidence intervals, and multiple-testing adjustment.

| Domain                       | Variable       | 3 Months, Median [IQR]  | 6 Months, Median [IQR]  | Paired Median Change [Bootstrap 95% CI] | Wilcoxon Statistic | Effect Size <i>r</i> | Nominal <i>p</i> -Value | FDR-Adjusted <i>p</i> -Value |
|------------------------------|----------------|-------------------------|-------------------------|-----------------------------------------|--------------------|----------------------|-------------------------|------------------------------|
| Hypoxia signaling            | HIF-1 $\alpha$ | −0.230 [−0.270, −0.189] | −0.179 [−0.273, −0.103] | +0.035 [+0.017, +0.050]                 | 610.0              | 0.430                | <b>0.000350</b>         | <b>0.00560</b>               |
| Hypoxia signaling            | HIF-2 $\alpha$ | −0.260 [−0.292, −0.196] | −0.256 [−0.380, −0.068] | −0.015 [−0.039, +0.028]                 | 1192.0             | −0.011               | 0.926                   | 0.993                        |
| Oxidative/redox stress       | GSH            | 0.156 [−0.928, 0.689]   | 0.165 [−0.963, 0.697]   | −0.019 [−0.067, +0.027]                 | 1196.0             | −0.008               | 0.945                   | 0.993                        |
| Oxidative/redox stress       | GSSG           | −0.002 [−0.736, 0.822]  | 0.013 [−0.725, 0.814]   | −0.032 [−0.119, +0.113]                 | 1206.0             | −0.001               | 0.993                   | 0.993                        |
| Oxidative/redox stress       | MDA            | −0.078 [−0.648, 0.363]  | −0.038 [−0.619, 0.322]  | +0.009 [−0.257, +0.126]                 | 1171.0             | 0.026                | 0.827                   | 0.993                        |
| Oxidative/glycation stress   | Methylglyoxal  | −0.268 [−0.664, 0.549]  | −0.284 [−0.647, 0.684]  | −0.019 [−0.046, +0.004]                 | 1055.0             | −0.110               | 0.362                   | 0.993                        |
| Endothelial/RAS              | ACE2           | −0.038 [−0.716, 0.757]  | −0.569 [−0.735, 1.017]  | +0.079 [−0.057, +0.189]                 | 1156.0             | 0.037                | 0.758                   | 0.993                        |
| Endothelial/RAS              | Angiotensin I  | −0.271 [−0.561, 0.027]  | −0.170 [−0.724, 0.659]  | +0.101 [−0.261, +0.748]                 | 1100.0             | 0.077                | 0.520                   | 0.993                        |
| Endothelial/RAS              | Angiotensin II | −0.278 [−0.520, −0.203] | −0.446 [−0.524, 0.027]  | −0.215 [−0.298, −0.068]                 | 1058.0             | −0.108               | 0.371                   | 0.993                        |
| Glycation-related            | AGEs           | −0.121 [−0.678, 0.738]  | −0.137 [−0.655, 0.254]  | −0.016 [−0.808, +0.641]                 | 1115.0             | −0.067               | 0.580                   | 0.993                        |
| Complement/immune regulation | C3             | −0.141 [−0.778, 0.816]  | −0.221 [−0.799, 0.775]  | −0.028 [−0.056, −0.009]                 | 1024.0             | −0.132               | 0.273                   | 0.993                        |
| Complement/immune regulation | C4             | −0.222 [−0.673, 0.913]  | 0.063 [−0.861, 0.514]   | +0.286 [−0.791, +0.704]                 | 1156.0             | 0.037                | 0.758                   | 0.993                        |
| Psychometric assessment      | Beck           | −0.166 [−0.814, 0.320]  | −0.137 [−0.845, 0.394]  | +0.044 [−0.031, +0.074]                 | 1135.0             | 0.052                | 0.664                   | 0.993                        |
| Psychometric assessment      | HADS I         | 0.146 [−0.727, 0.582]   | −0.096 [−1.045, 0.378]  | +0.118 [−0.204, +0.232]                 | 1193.0             | 0.010                | 0.931                   | 0.993                        |
| Psychometric assessment      | HADS II        | 0.230 [−0.755, 0.559]   | 0.088 [−1.156, 0.918]   | −0.056 [−0.211, +0.186]                 | 1109.0             | −0.071               | 0.556                   | 0.993                        |
| Autonomic assessment         | COMPASS-31     | −0.389 [−0.666, 0.439]  | −0.316 [−0.695, 0.347]  | −0.021 [−0.037, +0.065]                 | 1205.0             | −0.002               | 0.988                   | 0.993                        |

Note: Values are presented as standardized median [interquartile range]. Paired median change was calculated from within-participant 6-month minus 3-month differences and is reported with a bootstrap percentile 95% confidence interval. Paired comparisons were performed using the Wilcoxon signed-rank test. Standardized effect size was reported as *r*. Adjusted *p*-values were calculated using the Benjamini–Hochberg false-discovery-rate procedure across the 16 paired longitudinal biomarker–clinical comparisons. Formal interpretation of statistical significance was based on the FDR-adjusted *p*-values.



**Figure 2.** Time-dependent correlation structure of biological and clinical variables at 3 and 6 months after SARS-CoV-2 infection. The (**left heatmap**) represents the 3-month post-COVID assessment, and the (**right heatmap**) represents the 6-month post-COVID assessment. Heatmaps show pairwise correlation patterns among biomarkers, psychometric scores, autonomic scores, and clinical variables included in the longitudinal analysis.

A particularly notable finding was the reversal of the relationship between complement components C3 and C4. At 3 months, C3 and C4 showed a strong negative correlation, whereas at 6 months the association shifted to a strong positive correlation. This represented the largest observed correlation shift in the analysis and suggests a marked change in complement-associated immune regulation over the post-COVID follow-up period. Table 7 summarizes this reversal together with other major direction-changing relationships, including associations involving angiotensin II, GSH, HIF-1 $\alpha$ , HADS I, C4, HIF-2 $\alpha$ , ACE2, and methylglyoxal.

**Table 7.** Key longitudinal correlation changes between 3 and 6 months after SARS-CoV-2 infection.

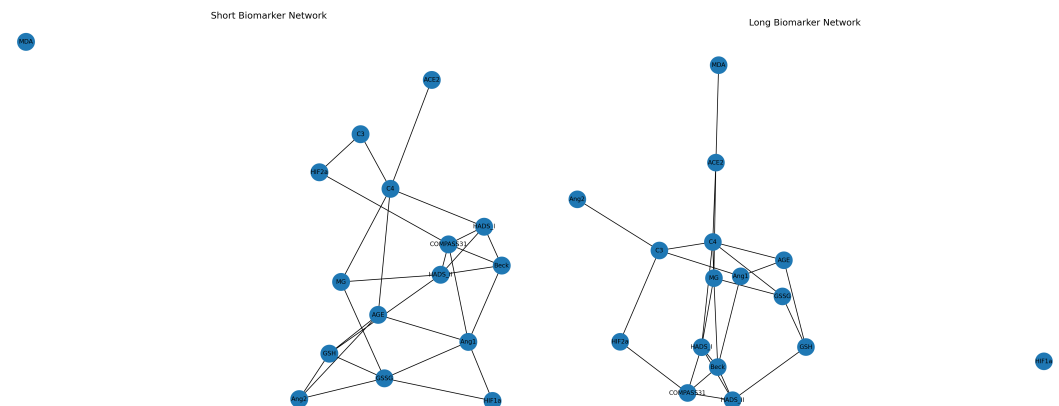
| Variable Pair                 | 3 Months, $r$ | 6 Months, $r$ | $\Delta r$ | Correlation Pattern                                           |
|-------------------------------|---------------|---------------|------------|---------------------------------------------------------------|
| C3–C4                         | −0.618        | 0.618         | +1.236     | Strong reversal from negative to positive association         |
| Angiotensin II–GSH            | −0.289        | 0.196         | +0.485     | Directional shift from negative to positive association       |
| Angiotensin II–HIF-1 $\alpha$ | −0.288        | 0.184         | +0.472     | Directional shift from negative to positive association       |
| Angiotensin II–HADS I         | −0.139        | 0.263         | +0.403     | Directional shift from negative to positive association       |
| C4–HADS I                     | −0.225        | 0.157         | +0.382     | Directional shift from negative to positive association       |
| C4–HIF-2 $\alpha$             | −0.169        | 0.156         | +0.325     | Directional shift from negative to positive association       |
| ACE2–C4                       | −0.158        | 0.159         | +0.317     | Directional shift from negative to positive association       |
| C4–methylglyoxal              | −0.143        | 0.149         | +0.292     | Directional shift from negative to positive association       |
| Beck–COMPASS-31               | 0.655         | 0.619         | −0.035     | Stable positive psychometric–autonomic association            |
| Beck–HADS I                   | 0.667         | 0.526         | −0.142     | Stable positive psychometric association                      |
| HADS I–COMPASS-31             | 0.633         | 0.527         | −0.107     | Stable positive psychometric–autonomic association            |
| HADS II–COMPASS-31            | 0.517         | 0.381         | −0.136     | Stable positive psychometric–autonomic association            |
| AGEs–angiotensin I            | 0.462         | 0.449         | −0.013     | Stable positive endothelial/RAS–glycation-related association |

Note: Correlation coefficients are shown for selected variable pairs demonstrating either major longitudinal shifts or stable clinically relevant associations.  $\Delta r$  was calculated as the 6-month correlation coefficient minus the 3-month correlation coefficient.

In contrast, the psychometric–autonomic variables showed a more stable pattern across time points. Beck, HADS I, HADS II, and COMPASS-31 remained positively associated at both 3 and 6 months, supporting the presence of a persistent psychometric–autonomic correlation structure during long COVID follow-up. Together, Figure 2 and Table 7 demonstrate two complementary patterns: dynamic remodeling among immune, endothelial/RAS, oxidative, and hypoxia-related variables, alongside relative stability of the psychometric–autonomic cluster.

### 3.4. Association-Level Biomarker–Clinical Interaction Networks

Association-level biomarker–clinical interaction networks were constructed separately for the 3-month and 6-month post-COVID assessments to visualize the organization of retained pairwise relationships among the 16 continuous biological and clinical variables. In these networks, nodes represented biomarkers and questionnaire-based clinical scores, including Beck, HADS I, HADS II, and COMPASS-31, while edges represented retained Pearson associations meeting the predefined threshold of  $|r| \geq 0.155$ . Figure 3 shows the resulting association-level networks at the two time points. Because association-level networks may include indirect relationships, these findings were further evaluated using Graphical LASSO-based conditional-dependency modeling and bootstrap stability analysis.



**Figure 3.** Association-level biomarker–clinical interaction networks at 3 and 6 months after SARS-CoV-2 infection. The (**left network**) represents the 3-month post-COVID assessment, and the (**right network**) represents the 6-month post-COVID assessment. Nodes represent biomarkers and questionnaire-based clinical scores, while edges represent retained Pearson associations meeting the predefined threshold of  $|r| \geq 0.155$ . The comparison illustrates longitudinal remodeling of biological interaction structure together with persistence of the psychometric–autonomic cluster.

The 3-month network consisted of 16 nodes and 27 edges, with a network density of 0.225. The 6-month network contained the same 16 nodes and 26 edges, with a density of 0.217. Although the overall density and number of edges were similar between time points, the arrangement of connections changed substantially. At 3 months, HIF-1 $\alpha$  remained integrated into the biological network through connections with angiotensin I and GSSG, whereas MDA appeared as an isolated node. By 6 months, HIF-1 $\alpha$  became isolated, while MDA was incorporated into the network through its connection with ACE2. This shift indicates that the network changed not only in edge number, but also in the positioning of specific biological markers.

The biological interaction structure also showed clear redistribution of complement, renin–angiotensin, oxidative/redox, and glycation-related relationships. In the 3-month network, C3, C4, HIF-2 $\alpha$ , ACE2, methylglyoxal, AGE, angiotensin I, angiotensin II, GSH, and GSSG were connected through several partially overlapping paths. At 6 months, C3 and C4 became more visibly embedded within the central network structure, with C4 connected to several biological and clinical nodes, including ACE2, methylglyoxal, AGE, angiotensin I, GSSG, and HADS I. This pattern supports longitudinal remodeling of immune, endothelial/RAS, oxidative, and glycation-related interactions.

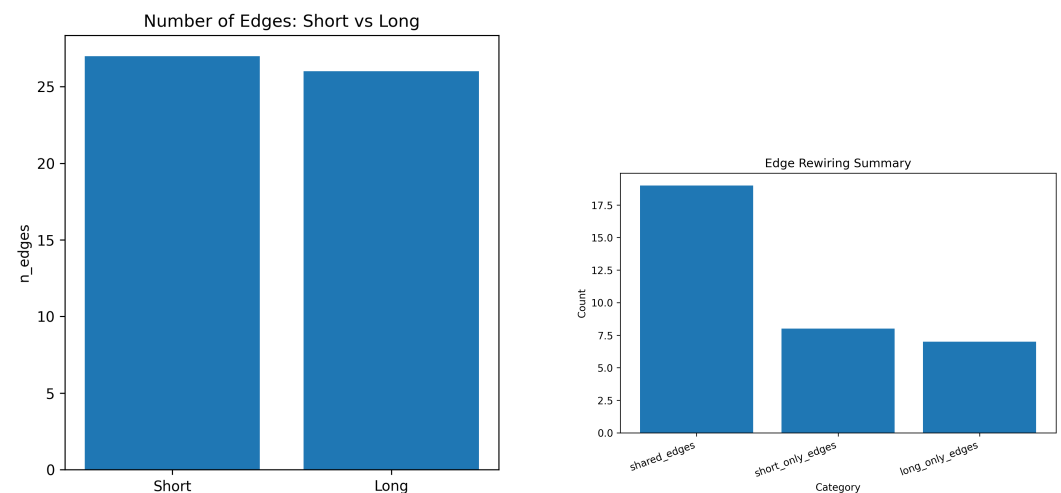
In contrast, the psychometric–autonomic component remained stable across both time points. Beck, HADS I, HADS II, and COMPASS-31 formed a consistent connected cluster at both 3 and 6 months, indicating persistent coupling between psychometric burden and autonomic symptom severity. Thus, Figure 3 demonstrates two parallel features of long

COVID network organization: dynamic remodeling among biological markers and relative stability of the psychometric–autonomic cluster.

### 3.5. Longitudinal Network Rewiring and Topological Remodeling

The central finding of the network analysis was that the global size of the biomarker–clinical network remained nearly stable, whereas the identity and organization of its connections changed over time. The 3-month network contained 27 edges and the 6-month network contained 26 edges, indicating only a minimal difference in total connectivity. However, direct edge-level comparison showed that this apparent stability masked substantial rewiring of the interaction structure.

Figure 4 illustrates this contrast between preserved global connectivity and changing edge identity. Of the observed network connections, 19 edges were shared between the 3-month and 6-month networks, while 8 edges were present only at 3 months and 7 edges emerged only at 6 months. Thus, almost half of the interaction pattern was either lost or newly formed across the follow-up interval, despite the similar total number of edges. This finding indicates that long COVID progression was not characterized by simple network weakening or strengthening, but by reorganization of the relationships among biological and clinical variables.



**Figure 4.** Association-level longitudinal network rewiring and edge turnover between the 3-month and 6-month post-COVID assessments. The **(left panel)** shows the total number of edges in the association-level biomarker–clinical interaction networks at each time point, demonstrating comparable global connectivity. The **(right panel)** summarizes edge-level remodeling, including shared edges retained across both time points, edges present only at 3 months, and edges emerging at 6 months. Together, these plots show that the overall association-level network size remained relatively stable while the identity of network connections changed over time.

The rewiring pattern was consistent with structured association-level remodeling within the biomarker–clinical network framework. At 3 months, HIF-1 $\alpha$  was connected within a community containing AGE, angiotensin I, angiotensin II, GSH, and GSSG, whereas by 6 months, HIF-1 $\alpha$  became isolated. In contrast, C3 and C4 became incorporated into a larger 6-month community with AGE, angiotensin I, angiotensin II, and HIF-2 $\alpha$ . MDA showed the opposite behavior to HIF-1 $\alpha$ : it was isolated at 3 months but became connected to ACE2 at 6 months. These transitions suggest redistribution of hypoxia-related, complement-associated, renin–angiotensin, oxidative/redox, and glycation-related interactions over time.

Table 8 summarizes the main topological and rewiring metrics. The number of communities remained unchanged at four, but modularity decreased from 0.438 at 3 months to

0.374 at 6 months, suggesting a less sharply separated community structure at the later time point. The maximum node degree increased from five to six, indicating that some variables became more connected even though the overall edge count remained similar. Together, Figure 4 and Table 8 show that the dominant longitudinal signal was not a widespread change in individual biomarkers but a remodeling of network architecture, with stable psychometric–autonomic coupling occurring alongside dynamic biological rewiring.

**Table 8.** Network topology and longitudinal rewiring metrics for the biomarker–clinical interaction networks.

| Category            | Metric                    | 3 Months                          | 6 Months                          | Longitudinal Pattern                                   |
|---------------------|---------------------------|-----------------------------------|-----------------------------------|--------------------------------------------------------|
| Global topology     | Number of nodes           | 16                                | 16                                | Same node set used at both time points                 |
| Global topology     | Number of edges           | 27                                | 26                                | Similar global connectivity                            |
| Global topology     | Network density           | 0.225                             | 0.217                             | Slight decrease in overall density                     |
| Global topology     | Connected components      | 2                                 | 2                                 | Stable component structure                             |
| Global topology     | Mean degree               | 3.375                             | 3.250                             | Slight decrease in average connectivity                |
| Global topology     | Median degree             | 4                                 | 3                                 | Reduced typical node connectivity                      |
| Global topology     | Maximum degree            | 5                                 | 6                                 | Increased maximum local connectivity                   |
| Edge rewiring       | Shared edges              | 19                                | 19                                | Persistent interactions across both time points        |
| Edge rewiring       | 3-month-only edges        | 8                                 | –                                 | Edges lost by 6 months                                 |
| Edge rewiring       | 6-month-only edges        | –                                 | 7                                 | Edges emerging by 6 months                             |
| Edge rewiring       | Total rewired edges       |                                   | 15                                | 8 lost and 7 gained edges                              |
| Community structure | Number of communities     | 4                                 | 4                                 | Same number of detected communities                    |
| Community structure | Modularity                | 0.438                             | 0.374                             | Less sharply separated community structure at 6 months |
| Community structure | Isolated node             | MDA                               | HIF-1 $\alpha$                    | Shift in isolated biological feature                   |
| Community structure | Stable clinical community | Beck, HADS I, HADS II, COMPASS-31 | Beck, HADS I, HADS II, COMPASS-31 | Preserved psychometric–autonomic cluster               |

Note: Network metrics were calculated from the association-level biomarker–clinical interaction networks constructed separately for the 3-month and 6-month post-COVID assessments. Total rewired edges represent the sum of edges present only at 3 months and edges emerging only at 6 months.

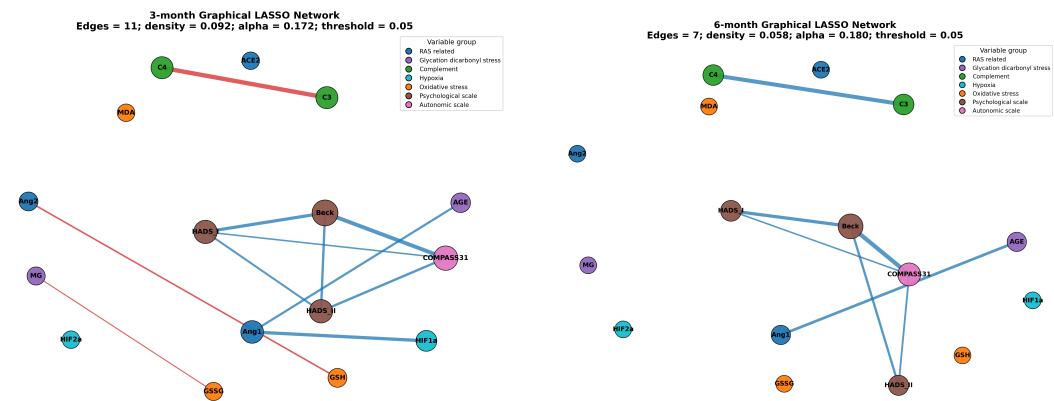
### 3.6. Graphical LASSO Conditional-Dependency and Bootstrap Stability Analysis

Complementary Graphical LASSO analysis produced a stricter sparse conditional-dependency network than the association-level biomarker–clinical network. Both time points retained the same 16-node structure, but the number of conditional-dependency edges decreased from 11 edges at 3 months to 7 edges at 6 months, corresponding to a reduction in network density from 0.092 to 0.058 (Table 9; Figure 5). This indicates that, under regularized conditional-dependency estimation, the network retained a smaller and more conservative set of conditional relationships, which is also visually reflected by the Graphical LASSO conditional-dependency networks shown in Figure 5.

**Table 9.** Summary metrics for Graphical LASSO conditional-dependency networks.

| Metric       | 3 Months | 6 Months |
|--------------|----------|----------|
| Nodes        | 16       | 16       |
| Edges        | 11       | 7        |
| Density      | 0.092    | 0.058    |
| Shared edges | 7        | 7        |
| Lost edges   | 4        | –        |
| Gained edges | –        | 0        |

Note: Metrics refer to Graphical LASSO sparse conditional-dependency networks constructed from the 16 continuous biomarker–clinical variables using the predefined absolute partial-correlation threshold of 0.05.



**Figure 5.** Graphical LASSO conditional-dependency networks at 3 and 6 months after SARS-CoV-2 infection. The left panel shows the 3-month sparse conditional-dependency network, and the right panel shows the 6-month sparse conditional-dependency network. Nodes represent the 16 continuous biomarker–clinical variables, while edges represent retained partial correlations after Graphical LASSO regularization using the predefined absolute partial-correlation threshold of 0.05. The figure illustrates the reduction from 11 conditional-dependency edges at 3 months to 7 edges at 6 months, together with preservation of a smaller psychometric–autonomic and complement-related sparse network core.

Direct comparison of the Graphical LASSO networks identified seven shared conditional-dependency edges, four lost edges, and no gained edges from 3 to 6 months. The retained and lost Graphical LASSO edges, together with their partial-correlation values and bootstrap selection frequencies, are summarized in Table 10.

**Table 10.** Graphical LASSO conditional-dependency edge status and bootstrap stability.

| Edge                 | Status | 3-Month Partial Correlation | 6-Month Partial Correlation | Bootstrap Selection Frequency |
|----------------------|--------|-----------------------------|-----------------------------|-------------------------------|
| C3–C4                | Shared | −0.451                      | 0.401                       | 1.000/1.000                   |
| Beck–COMPASS-31      | Shared | 0.397                       | 0.416                       | 0.999/1.000                   |
| Beck–HADS I          | Shared | 0.309                       | 0.308                       | 0.999/0.977                   |
| Beck–HADS II         | Shared | 0.214                       | 0.202                       | 0.971/0.971                   |
| AGEs–angiotensin I   | Shared | 0.194                       | 0.250                       | 0.953/0.986                   |
| COMPASS-31–HADS I    | Shared | 0.114                       | 0.113                       | 0.837/0.826                   |
| COMPASS-31–HADS II   | Shared | 0.218                       | 0.145                       | 0.980/0.883                   |
| HADS I–HADS II       | Lost   | 0.169                       | –                           | 0.948/0.467                   |
| Angiotensin II–GSH   | Lost   | −0.120                      | –                           | 0.875/0.530                   |
| Angiotensin I–HIF-1α | Lost   | 0.322                       | –                           | 0.795/0.340                   |
| GSSG–methylglyoxal   | Lost   | −0.055                      | –                           | 0.716/0.510                   |

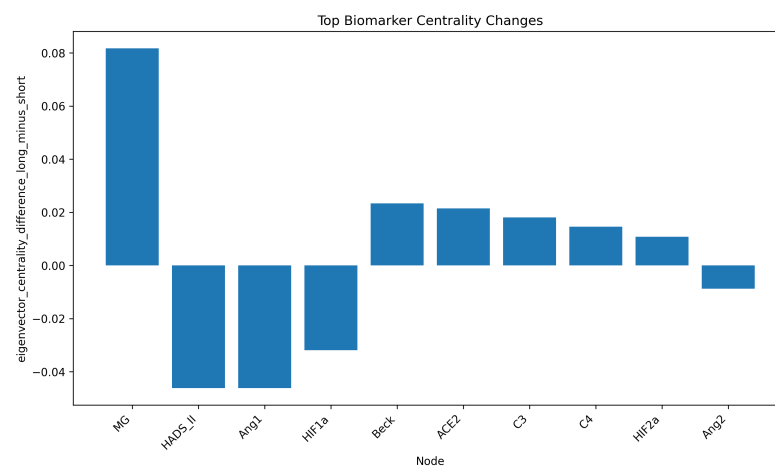
Note: Bootstrap selection frequencies are shown as 3-month/6-month values. Dashes indicate that the edge was not retained in the final 6-month Graphical LASSO network using the predefined absolute partial-correlation threshold of 0.05.

The stable shared Graphical LASSO core included complement-related, endothelial/RAS–glycation-related, and psychometric–autonomic conditional dependencies. In contrast, the lost conditional-dependency edges involved HADS I–HADS II, angiotensin II–GSH, angiotensin I–HIF-1α, and GSSG–methylglyoxal. Thus, while the broader association-level network showed extensive remodeling, the Graphical LASSO analysis revealed a conservative conditional-dependency core with selective weakening or loss of early conditional dependencies over time.

3.7. Centrality and Bootstrap Centrality Stability

Centrality analysis was performed to identify which variables changed their relative influence within the biomarker–clinical interaction network between the 3-month and 6-month assessments. Unlike the previous rewiring analysis, which focused on the presence or absence of edges, centrality analysis evaluated how the network position of individual variables changed over time. Centrality was evaluated using two complementary network

perspectives. In the broader association-level biomarker–clinical network, eigenvector centrality was used to identify variables whose relative influence changed over time. In the Graphical LASSO bootstrap analysis, degree and strength centrality were emphasized to identify variables that remained consistently central within the stricter sparse conditional-dependency network. Together, these two centrality perspectives allowed both dynamic redistribution of network influence and stability of central conditional-dependency nodes to be evaluated. Figure 6 shows the variables with the most prominent changes in eigenvector centrality between the two follow-up time points.



**Figure 6.** Centrality changes in the biomarker–clinical interaction network between the 3-month and 6-month post-COVID assessments. The plot shows variables with the most prominent changes in association-level eigenvector centrality, highlighting features that increased or decreased in relative network influence over time. Positive values indicate increased centrality at 6 months compared with 3 months, whereas negative values indicate decreased centrality.

The strongest increase in centrality was observed for methylglyoxal, indicating that this dicarbonyl stress-related marker became more embedded within the 6-month interaction structure. Additional increases were observed for Beck score, ACE2, C3, C4, and HIF-2 $\alpha$ , suggesting greater network involvement of psychometric, endothelial/RAS, complement-associated, and hypoxia-related features at the later follow-up point. These changes indicate that the 6-month network was not simply a weakened or strengthened version of the 3-month network, but a reorganized system in which different variables acquired greater relative importance.

In contrast, HADS II, angiotensin I, HIF-1 $\alpha$ , and angiotensin II showed decreases in eigenvector centrality. The reduction in HIF-1 $\alpha$  centrality was especially consistent with its transition from an integrated node at 3 months to an isolated node at 6 months. Similarly, the decreased centrality of angiotensin I and angiotensin II suggests a shift in the organization of renin–angiotensin-related interactions over time. Together, these centrality changes support the presence of systems-level feature remodeling, in which the most influential variables in the network changed across the post-COVID follow-up period.

Bootstrap centrality stability from the Graphical LASSO analysis complemented the association-level centrality results by identifying variables that remained consistently central under sparse conditional-dependency modeling. The two centrality perspectives were not expected to be identical because they reflect different aspects of network architecture: association-level eigenvector centrality captured broader redistribution of relative influence across the biomarker–clinical interaction network, whereas Graphical LASSO bootstrap centrality identified the more conservative stability core of the sparse conditional-dependency network. Across the full variable set, Beck, C4, COMPASS-31, and HADS I

showed stable centrality across time points, while C3 showed increased centrality stability at 6 months. The overlap of Beck, C4, and C3 across the two centrality perspectives supports the importance of psychometric–autonomic and complement-related components, whereas differences involving methylglyoxal, ACE2, and HIF-2 $\alpha$  indicate broader association-level remodeling that was not necessarily preserved as stable conditional-dependency centrality. The bootstrap centrality stability results for all variables are summarized in Table 11.

**Table 11.** Bootstrap centrality stability across all variables in the Graphical LASSO network.

| Node           | 3-Month Mean Strength | 6-Month Mean Strength | 3-Month Top-N Frequency | 6-Month Top-N Frequency |
|----------------|-----------------------|-----------------------|-------------------------|-------------------------|
| Beck           | 1.163                 | 1.172                 | 0.857                   | 0.865                   |
| C4             | 1.050                 | 1.038                 | 0.710                   | 0.734                   |
| COMPASS-31     | 1.024                 | 1.000                 | 0.679                   | 0.672                   |
| HADS I         | 1.010                 | 0.995                 | 0.659                   | 0.698                   |
| HADS II        | 0.929                 | 0.738                 | 0.461                   | 0.184                   |
| Angiotensin I  | 0.879                 | 0.825                 | 0.408                   | 0.319                   |
| C3             | 0.875                 | 0.946                 | 0.299                   | 0.581                   |
| AGEs           | 0.766                 | 0.716                 | 0.163                   | 0.208                   |
| GSH            | 0.759                 | 0.711                 | 0.152                   | 0.170                   |
| GSSG           | 0.673                 | 0.551                 | 0.115                   | 0.069                   |
| Angiotensin II | 0.651                 | 0.413                 | 0.132                   | 0.014                   |
| HIF-1 $\alpha$ | 0.614                 | 0.242                 | 0.132                   | 0.015                   |
| Methylglyoxal  | 0.604                 | 0.646                 | 0.055                   | 0.104                   |
| MDA            | 0.498                 | 0.583                 | 0.078                   | 0.129                   |
| ACE2           | 0.497                 | 0.610                 | 0.024                   | 0.099                   |
| HIF-2 $\alpha$ | 0.403                 | 0.483                 | 0.075                   | 0.138                   |

Note: Mean strength represents the average bootstrap strength centrality across 1000 resamples. Top-N frequency represents the proportion of bootstrap resamples in which a node appeared among the five most central nodes.

Taken together with the edge-rewiring, Graphical LASSO bootstrap stability, and community findings, the centrality results indicate that long COVID progression was associated with redistribution of network influence across biological and clinical domains. This reinforces the central observation of the study: longitudinal changes in long COVID were expressed not only through isolated biomarker differences, but through reorganization of the positions and relationships of variables within the broader biomarker–clinical network.

## 4. Discussion

### 4.1. Main Interpretation of the Findings

The main finding of this study is that longitudinal changes in long COVID were expressed more strongly through remodeling of relationships among biological and clinical variables than through broad isolated changes in individual markers. Classical paired comparisons showed that most biomarkers and clinical scores demonstrated only modest changes between the 3-month and 6-month assessments, with HIF-1 $\alpha$  being the only variable reaching statistical significance in the individual longitudinal analysis, as shown in Table 6. However, the relative stability of most isolated variables did not indicate biological stability at the systems level. Instead, correlation analysis, interaction networks, edge-rewiring patterns, community structure, and centrality changes, together with complementary Graphical LASSO/bootstrap stability analysis, collectively showed that the organization of the biomarker–clinical system changed over time.

This distinction is central to the interpretation of the study. Figure 2 and Table 7 show that several variable relationships changed direction or strength between the two follow-up points, most notably the reversal of the C3–C4 association. Figures 3 and 4 further demonstrate that the overall number of network connections remained relatively stable, while the identity of these connections changed, with shared, lost, and newly emerging edges across time. Table 8 supports this interpretation by showing that global network density changed only slightly, whereas edge composition, community structure, isolated nodes, and network organization were remodeled. Figure 6 extends this observation by showing that the relative network influence of individual variables also shifted over time.

Taken together, these findings suggest that long COVID may not be adequately characterized by isolated biomarker abnormalities alone. Rather, persistent post-COVID manifestations may reflect altered coordination among immune, endothelial/RAS, oxidative/redox, hypoxia-related, autonomic, and psychometric systems. This interpretation is consistent with the growing view that long COVID is a multisystem disorder involving interacting biological pathways rather than a single dominant pathological mechanism [4,14]. In this context, the graph-based framework used in the present study provided a way to detect hidden longitudinal organization within the data, revealing a pattern of systems-level remodeling that would be difficult to identify from conventional variable-by-variable comparisons alone.

An important implication of the present multilayer analysis is that the longitudinal remodeling pattern was supported by two complementary levels of network evidence rather than by a single network construction strategy. The association-level biomarker–clinical networks captured broad reorganization of pairwise relationships, including changes in edge composition, community structure, and node influence. In parallel, the Graphical LASSO analysis provided a more conservative sparse conditional-dependency perspective, identifying a stable core of retained relationships together with selective loss of early conditional dependencies. The convergence between these broader association-level findings and the stricter Graphical LASSO/bootstrap stability results strengthens the interpretation that the observed remodeling reflects a reproducible systems-level signal rather than an artifact of one network method. Thus, the findings support the view of long COVID as a dynamic multisystem disorder in which disease persistence may be expressed through altered coordination among biological and clinical domains, despite the absence of widespread statistically significant longitudinal changes in individual biomarkers.

#### *4.2. Biological Meaning of Network Remodeling*

The biological remodeling observed in the network analysis suggests that long COVID progression may involve changing coordination among complement activity, endothelial and renin–angiotensin system signaling, oxidative–redox balance, dicarbonyl/glycation stress, and hypoxia-related pathways. One of the most striking findings was the reversal of the C3–C4 relationship from a negative association at 3 months to a positive association at 6 months, as shown in Table 7. Because C3 and C4 are central components of complement-associated immune regulation, this reversal may reflect a transition in immune coordination during the post-COVID period rather than a simple increase or decrease in complement activity. Previous studies have implicated persistent immune activation and inflammatory dysregulation in long COVID, but the present analysis adds a longitudinal interaction-based perspective by showing that complement markers may also change their relational structure over time [13,18].

This finding is biologically relevant because complement dysregulation has increasingly been proposed as one mechanism contributing to long COVID pathogenesis, particularly through persistent innate immune activation and inflammatory pathway remodeling [35]. In addition, recent clinical evidence has linked classical-pathway complement proteins to symptom-specific post-COVID manifestations; in a case–control study of individuals with post-COVID-19 condition, classical-pathway proteins including C3 and C4 were evaluated, and lower C3 levels were reported among participants with post-COVID fatigue [36]. In this context, the reversal of the C3–C4 relationship in the present longitudinal cohort suggests that complement involvement in long COVID may be reflected not only by isolated concentration differences, but also by changes in the coordination between complement components over time.

The network results further suggest that complement-associated remodeling was linked to vascular and renin–angiotensin system-related pathways. At 6 months, C3 and C4 became more embedded within the biological network, particularly in relation to ACE2, angiotensin I, angiotensin II, AGEs, and HIF-2 $\alpha$ , as shown in Figure 3 and Table 8. This pattern may indicate a shift toward more integrated immune–vascular regulation during the later post-COVID phase. Although endothelial dysfunction, microvascular injury, and multisystem biological dysregulation have been widely discussed in long COVID, our findings suggest that their relevance may depend not only on isolated marker levels, but also on how these variables become connected with complement and hypoxia-related pathways over time [12,14,26].

Oxidative, redox, and dicarbonyl/glycation stress markers also appeared to participate in this remodeling process. Methylglyoxal, AGEs, GSH, GSSG, and MDA represent interconnected aspects of oxidative stress, redox balance, lipid peroxidation, and glycation-related cellular injury. In the present network, methylglyoxal showed one of the strongest increases in centrality, while MDA shifted from an isolated node at 3 months to a connected feature at 6 months, as shown in Figure 6 and Table 8. These findings suggest that oxidative and glycation-related pathways may become more integrated into the biological interaction structure over time, even when their isolated longitudinal changes are not statistically significant.

The hypoxia-related findings further illustrate the difference between isolated biomarker change and network-level interpretation. HIF-1 $\alpha$  was the only variable showing a significant individual longitudinal difference in Table 6, but its network role decreased over time, becoming isolated in the 6-month network. In contrast, HIF-2 $\alpha$  became more embedded within the 6-month biological community involving C3, C4, AGEs, angiotensin I, and angiotensin II, as shown in Figures 3 and 6. This divergence suggests that HIF-1 $\alpha$  may reflect a measurable longitudinal biomarker shift, whereas HIF-2 $\alpha$  may better reflect integration into the evolving network architecture at the later stage.

This HIF-1 $\alpha$  pattern is biologically meaningful because HIF-1 $\alpha$  is a central hypoxia-responsive transcriptional regulator that also participates in metabolic adaptation and inflammatory activation. Experimental and clinical COVID-19 studies have shown that SARS-CoV-2 infection can induce HIF-1 $\alpha$ -related signaling and that HIF-1 $\alpha$  may promote pro-inflammatory responses during infection [37]. In addition, single-cell analysis of convalescent COVID-19 patients identified altered expression of *HIF1A*, the gene encoding HIF-1 $\alpha$ , as a severity-sensitive long-term immunological scar in circulating monocytes, supporting the relevance of this pathway beyond the acute phase of infection [38]. In the present cohort, the significant paired change in HIF-1 $\alpha$  therefore supports an evolving hypoxia-inflammatory signal, whereas its reduced network integration at 6 months suggests that isolated biomarker change and systems-level connectivity capture complementary aspects of post-COVID remodeling.

Overall, the biological network remodeling observed in this study does not point to a single dominant pathway. Instead, it suggests a dynamic redistribution of interactions among complement regulation, endothelial/RAS activity, oxidative-redox and dicarbonyl stress, glycation-related processes, and hypoxia signaling. This interpretation is consistent with current mechanistic models of long COVID as a multisystem condition, but the distinctive contribution of the present study is the demonstration that these mechanisms may be organized through time-dependent network remodeling rather than through isolated pathway abnormalities alone [4,14,25].

#### 4.3. Clinical Relevance of the Psychometric-Autonomic Module

A clinically important feature of the network analysis was the persistence of a psychometric–autonomic module across both follow-up time points. Beck, HADS I, HADS II, and COMPASS-31 remained positively connected at 3 and 6 months, as shown in Figure 3 and Table 7. This pattern suggests that psychometric burden and autonomic symptom severity were organized as a coherent clinical axis rather than as isolated or secondary manifestations. In contrast to the biological network, which underwent clear longitudinal rewiring, the psychometric–autonomic module remained comparatively preserved.

This stability is clinically meaningful because it suggests that persistent symptom burden in long COVID may be maintained by coupling between affective, psychometric, and autonomic domains, with potential relevance to the broader neuropsychological and cognitive symptom spectrum described in long COVID. Even when most individual biomarkers showed modest longitudinal changes, the continued association between Beck, HADS, and COMPASS-31 scores indicates that psychometric and autonomic manifestations may form a stable clinical layer of the syndrome. This interpretation is consistent with clinical descriptions of long COVID as a condition involving overlapping neuropsychological, autonomic, and multisystem symptoms [2,4].

The contrast between a stable psychometric–autonomic module and a dynamically remodeling biological network has important implications for clinical evaluation. It suggests that persistent symptoms should not be interpreted solely as direct reflections of single biomarker abnormalities. Instead, long COVID may involve a relatively stable clinical burden interacting with biological systems that continue to reorganize over time. Therefore, assessment and management may benefit from an integrated approach that combines laboratory biomarkers with structured neurological, psychometric, and autonomic evaluation, addressing measured neurological, psychometric, and autonomic domains as interconnected components of the post-COVID condition, while recognizing their potential relevance to broader cognitive and neuropsychiatric manifestations described in long COVID [39].

#### 4.4. Methodological Implications and Analytical Scope

The present study demonstrates the value of applying a longitudinal graph-based framework to long COVID data. Conventional paired comparisons identified limited isolated variable-level changes, whereas network-based analyses revealed changes in correlation structure, edge composition, community organization, and centrality. This distinction indicates that graph-based modeling can complement standard statistical testing by identifying interaction-level patterns that may remain hidden when biomarkers and clinical scores are analyzed separately.

A strength of this approach is that it integrates multiple dimensions of post-COVID assessment within a unified analytical framework. Biological markers, psychometric scores, autonomic symptom burden, and encoded neurological findings were evaluated not only as individual variables, but also as components of an evolving interaction system. The use of correlation-based biomarker–clinical networks, Graphical LASSO-based conditional dependency modeling, edge-rewiring analysis, community detection, and centrality profiling allowed the dataset to be examined at both association and topology levels. This is consistent with network medicine approaches that interpret complex disorders through patterns of interaction rather than isolated abnormalities alone [21,22].

The analytical scope of the study should be considered when interpreting the findings. The longitudinal within-cohort design reduces between-participant heterogeneity; however, potential cohort-specific clinical context and residual confounding should be considered when interpreting network patterns, and evaluation in independent populations will

help establish their broader generalizability. Network edges represent statistical or model-derived associations and should not be interpreted as direct causal mechanisms. In addition, because the analysis was performed in a moderate-sized longitudinal cohort without external validation, the network findings should be interpreted as exploratory rather than confirmatory. Nevertheless, the bootstrap stability analysis provided internal support for several shared and lost conditional-dependency edges, strengthening their value as biologically informative hypotheses for future validation. This framing does not weaken the main finding; rather, it defines the appropriate interpretation of the network results and supports their use as a systems-level exploratory framework for studying long COVID.

The analytical scope of the present study was defined by its focus on longitudinal biochemical–clinical network remodeling in a complete paired post-COVID cohort. Cohort-level clinical information was used to describe the study population, whereas the main analytical structure was centered on biochemical markers and questionnaire-based clinical scores. This design was appropriate for evaluating temporal changes in interaction patterns among biological and clinical domains, rather than for performing individual-level demographic risk stratification. The repeated-measures within-cohort structure further supported this objective by allowing the same participants to be evaluated across the 3-month and 6-month post-COVID assessments. Accordingly, the findings should be interpreted as longitudinal biochemical–clinical network patterns within this analytical framework.

## 5. Conclusions

This study demonstrates that the longitudinal biology of long COVID may be more clearly reflected by changes in the organization of biomarker–clinical interactions than by isolated biomarker abnormalities alone. Although conventional paired comparisons identified limited variable-level changes, multilayer graph analysis revealed a distinct pattern of network remodeling across the 3-month and 6-month post-COVID assessments. The reversal of the C3–C4 association, redistribution of complement, endothelial/RAS, oxidative-redox, dicarbonyl/glycation, and hypoxia-related interactions, and shifts in node centrality collectively indicate that persistent post-COVID manifestations are accompanied by time-dependent reorganization of interconnected biological systems.

The findings also highlight the clinical relevance of a stable psychometric–autonomic module. The persistent coupling of Beck, HADS I, HADS II, and COMPASS-31 suggests that psychometric burden and autonomic symptom severity form a durable clinical axis of long COVID, even while biological interaction networks continue to remodel. This distinction between stable clinical clustering and dynamic biological rewiring supports the need to evaluate long COVID through integrated biological and clinical frameworks.

The main novelty of this study is the demonstration that longitudinal multilayer graph analysis can uncover hidden patterns of systems-level remodeling that remain largely inaccessible to conventional biomarker-centered approaches. By shifting attention from isolated marker changes to evolving interaction architecture, this framework provides a more informative view of long COVID as a dynamic post-viral systems disorder. Such an approach may support future patient stratification, mechanism-oriented biomarker discovery, and the development of multimodal assessment strategies for chronic post-COVID and other post-infectious syndromes.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/covid6070120/s1>: Supplementary Analytical Outputs: S1 Longitudinal Statistics, including short- and long-term correlation matrices, paired test results, and top correlation tables; S2 Graphical LASSO Networks, including edge and node tables for short- and long-term networks; S3 Network Comparison and Rewiring, including basic network metrics, edge rewiring, edge-weight changes, and node centrality comparisons; S4 Bootstrap Stability, including

bootstrap settings, centrality stability results, and stability summary tables. The Supplementary Materials include supporting analytical outputs for the results reported in this manuscript, including longitudinal statistical outputs, correlation matrices, Graphical LASSO edge and node files, network comparison and rewiring outputs, and bootstrap centrality stability summaries. These files are provided to support transparency and verification of the reported statistical and network findings. Participant-level source records are not publicly shared because of privacy and ethical restrictions. Full analysis code is not publicly released because it forms part of an ongoing research pipeline.

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