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Vitamin B12 as an epidrug for regulating peripheral blood biomarkers in long COVID-associated visuoconstructive deficit

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Approximately four months after recovering from a mild COVID-19 infection, around 25% of individuals developed visuoconstructive deficit (VCD), which was found to be correlated with an increase in peripheral immune markers and alterations in structural and metabolic brain imaging. Recently, it has been demonstrated that supplemental vitamin B12 regulates hyperinflammation during moderate and severe COVID-19 through methyl-dependent epigenetic mechanisms. Herein, whole peripheral blood cultures were produced using samples obtained from patients with confirmed persistent VCD, and controls without impairment, between 10 and 16 months after mild COVID-19. This experimental model was used to assess the leukocyte expression patterns of 11 biomarkers previously associated with VCD in long COVID and explore the potential of pharmacological B12 in regulating these genes. The results showed that patients with persistent VCD displayed continued upregulation of *CCL11* and *LIF* compared to controls. It is worth noting that elevated serum levels of CCL11 have been previously linked to age-related neurodegenerative diseases. Notably, the addition of 1 nM of vitamin B12 to blood cultures from individuals with VCD normalized the mRNA levels of *CCL11*, upregulated the neuroprotective *HGF*, and, to a lesser extent, downregulated *CSF2* and *CXCL10*. There was an inverse correlation observed between *CCL11* mRNA levels and methylation levels of specific cytosines in its promoter region. These findings underscore the significance of systemic inflammation in persistent VCD associated with long COVID. Moreover, the study provides evidence suggesting that B12, acting as an epidrug, shows promise as a therapeutic approach for addressing this cognitive impairment.

Keywords Long COVID, Visuoconstructive deficit, *CCL11*, DNA methylation, Epigenetics.

Abbreviations

BBB	Blood brain barrier
BSP	Bisulfite sequencing PCR
CNS	Central nervous system
COVID-19	Coronavirus disease 2019
CpG	Cytosine-phosphate-guanine
DML	Differentially methylated locus
HCY	Homocysteine
MS	Multiple sclerosis
PET-CT	Positron emission tomography - computed tomography
ROCF	Rey-Osterrieth complex figure
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

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TF	Transcription factor
TFBS	Transcription factor binding site
VCD	Visuoconstructive deficiency
WHO	World Health Organization

Long COVID is a term used to describe the prolonged symptoms of COVID-19, including cognitive dysfunction, also known as "brain fog," which can harm memory, attention, and problem-solving abilities. These symptoms may persist for weeks or even months after the infection has resolved[1,2]. While the exact cause of long COVID is not fully understood, it is thought to be related to the immune system's response to the virus and the systemic inflammation that it causes (reviewed in:[3]).

In a previous prospective cohort study of individuals who had mild COVID-19, it was found that approximately 25% of young adults (with an average age of 38 years) exhibited significant cognitive impairment in the copy stage of the Rey-Osterrieth Complex Figure (ROCF) Test four months after their recovery from the infection[4]. The ROCF is a neuropsychological test that involves drawing a complex figure to assess visuospatial abilities, executive functions, and memory retention (reviewed in:[5]). Performance on visuoconstruction and memory tests is mainly associated with learning, problem-solving skills, and several activities of daily living, such as orientation and navigation[6]. These processes integrate the perception and interpretation of visual information with memory and executive systems by involving various brain areas, including the occipito-parietal regions, the dorsal and ventral streams, and connections with the cingulate, medial temporal, and frontal cortices[7]. Notably, negative correlations were found between performance on the ROCF test and white matter volume in both the left and right genu of the corpus callosum, extending to the cingulum bundle, as well as glucose metabolism in the right dorsal anterior cingulate gyrus of individuals diagnosed with visuoconstructive deficit (VCD). Moreover, a functional interaction network was observed among ten out of 11 plasma biomarkers that were upregulated in VCD patients. Four of these biomarkers are components of the *Neuroinflammation Signaling* pathway, and four are components of the *IL-17 Signaling* pathway. Indeed, evidence suggests that peripheral innate immune system molecules can trigger a secondary neuroinflammatory response in the central nervous system (CNS)[8–10].

Epigenetic mechanisms regulate the expression of genes crucial for immune response, neuronal function, and synaptic plasticity in neuroinflammatory processes. In this regard, it is possible that epigenetic changes may contribute to cognitive dysfunction in long COVID. For example, Lee and colleagues reported that individuals with long COVID had higher levels of DNA methylation in certain genes involved in immune response and inflammation[11]. DNA methylation is a process that involves the addition of a methyl group (-CH3) to a cytosine base in DNA. This modification can regulate gene expression by altering the interaction between the DNA and the proteins that bind to it[12,13]. It is worth noting that dysregulation of DNA methylation patterns has been linked to various brain disorders, including depression, anxiety, and cognitive decline (reviewed in:[14,15]).

Vitamin B12 is an essential cofactor for the enzyme methionine synthase, which catalyzes the conversion of homocysteine to methionine in the sulfur amino acid pathway. S-adenosylmethionine, the methyl donor utilized in all cellular methylation processes, is derived from methionine[16]. Accordingly, adjuvant vitamin B12 has been shown to increase methylation of key genes in inflammatory disorders, resulting in their downregulation. This effect has been previously demonstrated in an infant rat model of pneumococcal meningitis[17] and in whole blood cultures from human patients with acute COVID-19[18]. These findings highlight the potential therapeutic role of vitamin B12 as an epidrug in attenuating hyperinflammation, providing promising directions for further research in neuroinflammatory conditions associated with long COVID.

The primary objective of this study was to identify peripheral blood biomarkers that are linked to persistent VCD in long COVID patients who have previously experienced a mild acute COVID-19 episode. Additionally, the study aimed to explore the methyl-dependent epigenetic mechanisms that play a role in regulating these biomarkers as well as investigate the potential of vitamin B12 in modulating the gene expression of these genes in leukocytes.

Methods

Patients

This study is part of a longitudinal research project involving a cohort of individuals who experienced mild COVID-19 before completing their vaccination schedule and prior to the emergence of the Omicron variants. This prospective observational cohort study received approval from the Institutional Review Board (IRB) of the Federal University of Minas Gerais (UFMG) (CAAE3768820.1.0000.5149). All participants provided written informed consent. The study has been performed in accordance with the Declaration of Helsinki. Volunteers were recruited approximately four months after having mild COVID-19 confirmed through RT-qPCR testing. Disease severity was categorized as 1 or 2 according to the WHO clinical ordinal scale[19]. During the recruitment appointment and subsequent follow-up appointments at six and twelve months, the clinical status and mental health of all volunteers were evaluated, peripheral blood samples were collected, 45 plasma biomarkers (cytokines, chemokines, and growth factors) were assessed, and a comprehensive investigation was conducted using neuropsychological and ophthalmological tests, PET-CT, and magnetic resonance neuroimaging. These procedures are thoroughly detailed in[4]. Exclusion criteria were self-reported history of autoimmune diseases, chronic mental or neurological disorders, recurrent infections, substance abuse, previous brain surgery, and endotracheal or orotracheal intubation during COVID-19 treatment. Detailed information on neuropsychological assessment and exclusion criteria have been thoroughly documented in previous publications based on this cohort study[4,20]. The research timeline can be found in Fig. 1. For the present study, seven individuals diagnosed with VCD during the recruitment appointment were enrolled. Additionally, five individuals who had mild COVID-19 but did not exhibit any signs of VCD or other neuropsychological sequelae that could be related to the infection at the recruitment and at six- and 12-month follow-ups were

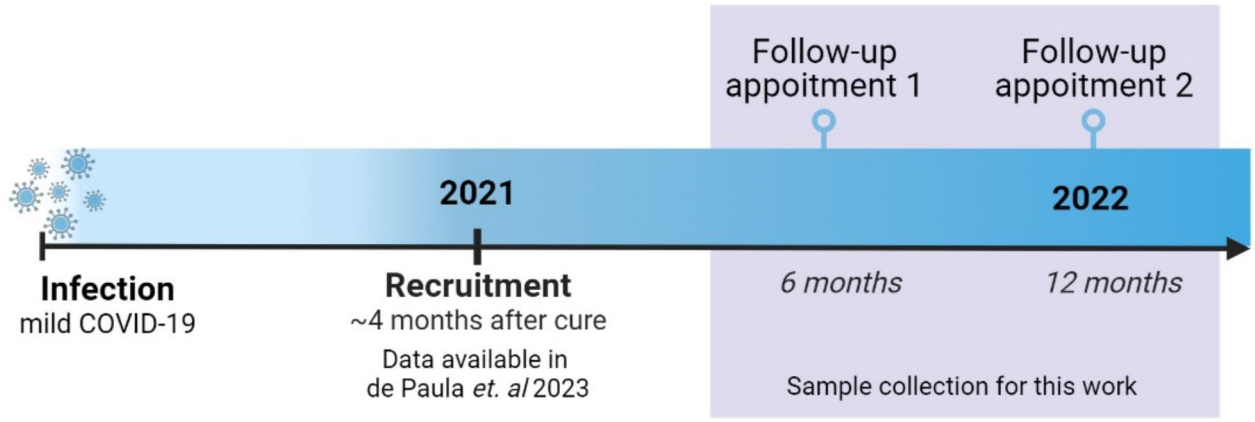

Fig. 1. Research timeline (Created with BioRender.com).

included as controls. Approximately 30% of participants discontinued their involvement between recruitment and the 6-month follow-up[20]. Women were more consistent in attending follow-ups, while men participation significantly declined after six months. Consequently, men were excluded from the present study. Due to the unique characteristics of the studied population - individuals infected before completing vaccination and prior to the Omicron variants - it is not feasible to increase the sample size.

Assessment of folate, homocysteine and vitamin B12 basal levels

The standard chemiluminescence method was used by a commercial clinical laboratory (Geraldo Lustosa, Belo Horizonte, Brazil) to quantify the basal levels of folate (vitamin B9), homocysteine (HCY), and vitamin B12 in plasma samples from all volunteers.

Whole blood cultures

Whole peripheral blood cultures were generated using samples collected in sodium heparin at 8 a.m., following the protocol outlined by Cassiano et al.[18]. In brief, the blood samples were mixed with 50% (v/v) RPMI 1640 medium (Sigma-Aldrich, Saint Louis, Missouri). Two different conditions were employed: endpoint A, where the medium was supplemented with an excipient (citrate-phosphate buffer, pH 5, Merck, Darmstadt, Germany), and endpoint B, where the medium was supplemented with cyanocobalamin (Merck) at a final concentration of 1 nM. The cultures were then incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2.

Real-time quantitative PCR (RT-qPCR)

The total RNA was extracted from leukocytes using the QIAamp RNA Blood Kit (Qiagen, Hilden, Germany), and cDNA was produced with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA) as previously described[18]. The mRNA levels of *CCL2* (C-C Motif Chemokine Ligand 2), *CCL11* (C-C Motif Chemokine Ligand 11), *CSF2* (Colony Stimulating Factor 2), *CXCL10* (C-X-C Motif Chemokine Ligand 10), *HGF* (Hepatocyte Growth Factor), *IL1RA* (Interleukin 1 Receptor Antagonist), *IL6* (Interleukin 6), *IL10* (Interleukin 10), *IL31* (Interleukin 31), *LIF* (LIF Interleukin 6 Family Cytokine), and *NGF* (Nerve Growth Factor) were quantified using specific primers in SYBR Green (Thermo Fisher) or TaqMan (Thermo Fisher) platforms following the manufacturer's instructions. These genes encode for the plasma inflammatory and growth factors previously associated with VCD in long COVID in this cohort[4]. Target gene expression levels were normalized by 18 S ribosomal RNA (18 S ribosomal N1), and relative gene expression was calculated using the 2e(-ΔCt) method[21]. The primer sequences and TaqMan assay IDs are listed in Additional file 1. Each sample was tested in two technical replicates.

Bisulfite sequencing PCR

To assess the impact of VCD and supplemental vitamin B12 on the methylation levels of 17 CpG sites located in the promoter region and proximal portion of the first exon of *CCL11*, DNA libraries obtained from cultures of endpoints A and B were analyzed using Bisulfite Sequencing PCR (BSP). The individuals included in the analysis represented both the impaired and non-impaired groups.

The production of BSP libraries, sequencing, and bioinformatics analysis were performed as described in[18] with minor modifications. Following DNA isolation, unmethylated cytosine nucleotides were converted into uracil with the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, California). The DNA region spanning from −2 569 pb to +310 pb of *CCL11* (GRCh38/hg38 chr17: 34283173–34286052) was amplified by PCR from bisulfite-converted DNA using a primer set designed to account for cytosine conversion to uracil (Bisulfite Primer Design Tool, Zymo Research) (Additional file 2) and GoTaq DNA Polymerase (Promega, Madison, Wisconsin). The mapping of filtered reads, as well as the calculation of methylation percentages at CG loci, were performed using the Bismark software[22]. Only reads that uniquely mapped to the reference sequence chr17:

34,283,173–34,286,052 of the *Homo sapiens* genome (Gencode, release 38) were considered for the methylation analysis. The functional annotations for *CCL11* were obtained from the UCSC Genome Browser[23].

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0.2) from GraphPad Software Inc., Irvine, CA. The data distribution was assessed using Anderson-Darling, D'Agostino & Person, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Data were considered parametric if they passed (alpha = 0.05) at least one of the normality tests. Based on the distribution, a two-tailed Student's t-test, paired Student's t-test, Mann-Whitney test, or Wilcoxon test were employed to compare the groups. Statistical significance was defined as a *P*-value less than 0.05. The data were presented as median ± interquartile range.

The fold change methylation levels of each CG locus were compared between groups using an unpaired t-test. Only the loci that exhibited statistically significant differences between the compared groups were chosen for further analysis. To evaluate the correlation between the fold change methylation levels of differentially methylated loci (DML) and *CCL11* gene expression levels quantified by RT-qPCR in the same cultures, Pearson or Spearman tests were employed based on data distributions.

Search for transcription factor biding sites at the DML

Transcription factor binding sites (TFBS) at the DML were searched in the JASPAR CORE 2022 database (https://jaspar.genereg.net), an open-access and regularly updated resource featuring manually curated transcription factor (TF) binding profiles[24,25].

Results

Sample characterization

The study included female participants with an average age of 40.7 ± 8.5 years. There were no statistically significant differences in age between the groups initially diagnosed with or without VCD, as indicated in Table 1. It is worth mentioning that all participants had received at least two doses of SARS-CoV-2 vaccine (Sinovac-CoronaVac, Pfizer or AstraZeneca) at the time of the follow-up appointments.

Among the seven individuals initially diagnosed with VCD, four (57%) still exhibited the condition during the follow-up appointments, while three showed no further signs of impairment. The five non-impaired control individuals maintained their initial diagnosis, as shown in Table 1. No significant statistical differences were observed in the basal levels of serum folate, HCY, and B12 among individuals with VCD, those who had recovered from the impairment, and non-impaired individuals, as detailed in Additional file 3.

Supplemental B12 favorably modulates persistently dysregulated inflammatory genes in the leukocytes of patients with VCD

After 6 to 12 months from the initial diagnosis, *CCL11* ($P = 0.0127$) and *LIF* ($P = 0.0485$) continued to show upregulation in leukocytes from individuals with persistent VCD compared to those from non-impaired subjects (Fig. 2). Importantly, when the whole peripheral blood cultures were incubated with 1 nM of B12 for 24 h, the expression of *CCL11* in the impaired group was normalized to the levels observed in the cultures from the non-impaired group incubated with the excipient ($P > 0.05$). B12 also downregulated *CSF2* ($P = 0.0071$) and *CXCL10* ($P = 0.0205$) in leukocytes from impaired individuals, although these genes were not differentially regulated when compared to the non-impaired group. Additionally, B12 increased the mRNA levels of *HGF* in the impaired group compared to the non-impaired group incubated with the excipient ($P = 0.0081$). *CCL2*, *IL10*, *IL1RA*, and *IL6* were not differentially expressed when impaired and non-impaired individuals were compared, and these genes did not respond to incubation with B12 (Additional file 4). *IL31* and *NGF* mRNA were undetectable in leukocytes from all volunteers.

Participant ID	Age (years)	Baseline diagnosis	Follow-up appointment (months)	Diagnosis at follow-up appointment
BRA-106	34	Impaired	12	Impaired
BRA-108	51	Impaired	12	Normal
BRA-111	32	Normal	12	Normal
BRA-112	56	Normal	12	Normal
BRA-126	31	Impaired	6	Normal
BRA-138	35	Normal	6	Normal
BRA-140	33	Normal	6	Normal
BRA-143	36	Impaired	6	Impaired
BRA-187	49	Impaired	6	Impaired
BRA-196	44	Impaired	6	Impaired
BRA-200	51	Normal	6	Normal
BRA-202	37	Impaired	6	Normal

Table 1. Participant's data.

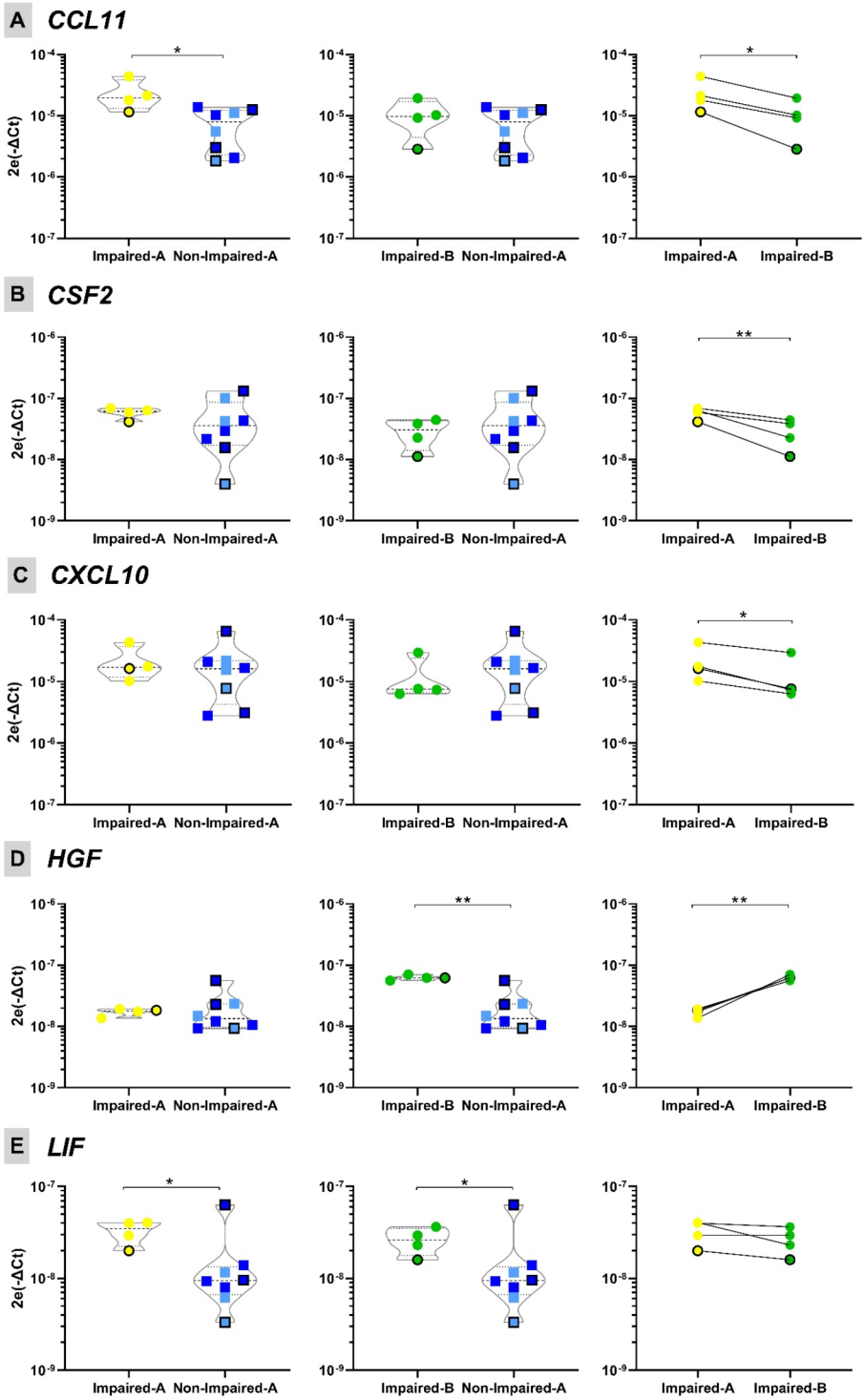

Fig. 2. Supplemental B12 favorably modulates persistently dysregulated inflammatory genes in the leukocytes of patients with VCD. Violin charts depicting the expression levels of a panel of inflammatory and growth factor genes in leukocytes from whole peripheral blood cultures (WPBC). Endpoint A: WPBC incubated with excipient. Endpoint B: WPBC incubated with 1 nM vitamin B12. Light blue squares represent individuals diagnosed with VCD at the time of recruitment who subsequently recovered before the follow-up appointment. Black-outlined circles or squares denote individuals who attended the 12-month follow-up appointment. In all other cases, both circles and squares represent individuals who attended the 6-month follow-up appointment. The pairwise comparison of the gene expression values (2e(-ΔCt)) between groups was conducted using two-tailed Student's t, paired Student's t, Mann-Whitney, or Wilcoxon tests according to the experimental design and data distribution. Values were expressed as medians. * $P < 0.05$; ** $P < 0.01$.

Impaired individuals exhibit hypomethylation of CpG sites in the *CCL11* promoter region, which are then hypermethylated upon supplementation with B12

In impaired individuals, three CpG sites exhibited hypomethylation when compared to the non-impaired group. Notably, treatment of cultures with B12 significantly increased the methylation level of two of these CpG sites. Furthermore, a strong and negative correlation was observed between the methylation status of these DML and the mRNA levels of *CCL11*. These DML are located at predicted transcription factor binding sites (TFBS) according to the JASPAR CORE 2022 database (Fig. 3).

Discussion

VCD, characterized by an impaired ability to visually perceive and construct complex visual-spatial designs or tasks, is a well-recognized feature of aging-related neurodegenerative diseases, including Alzheimer's disease[26]. The occurrence of this symptom in the young adults enrolled in this study is noteworthy. It suggests the possibility that individuals who experience mild COVID-19 may be at a higher risk of developing severe conditions associated with neurodegeneration. Furthermore, the high prevalence (57%) of persistent VCD six to 12 months after the initial diagnosis is a cause for concern.

Of the 11 plasma inflammatory and growth factors previously associated with VCD in long COVID at the recruitment, four months after mild COVID-19[4], only *CCL11* and *LIF* displayed a transcriptional upregulation in leukocytes from individuals with persistent impairment. Accordingly, elevated CCL11 protein levels were identified in the raw plasma from individuals who developed VCD, compared with individuals who recovered from the impairment between the recruitment and the six-month appointment[20]. CCL11, also known as eotaxin-1, is a chemotactic agent for eosinophils into inflammatory sites. In the CNS, CCL11 has been implicated in age-associated cognitive decline, psychiatric disorders, multiple sclerosis (MS), and Alzheimer's disease (AD) (reviewed in:[27]). Under stimuli, CCL11 can be transported from blood to the brain across the blood-brain barrier

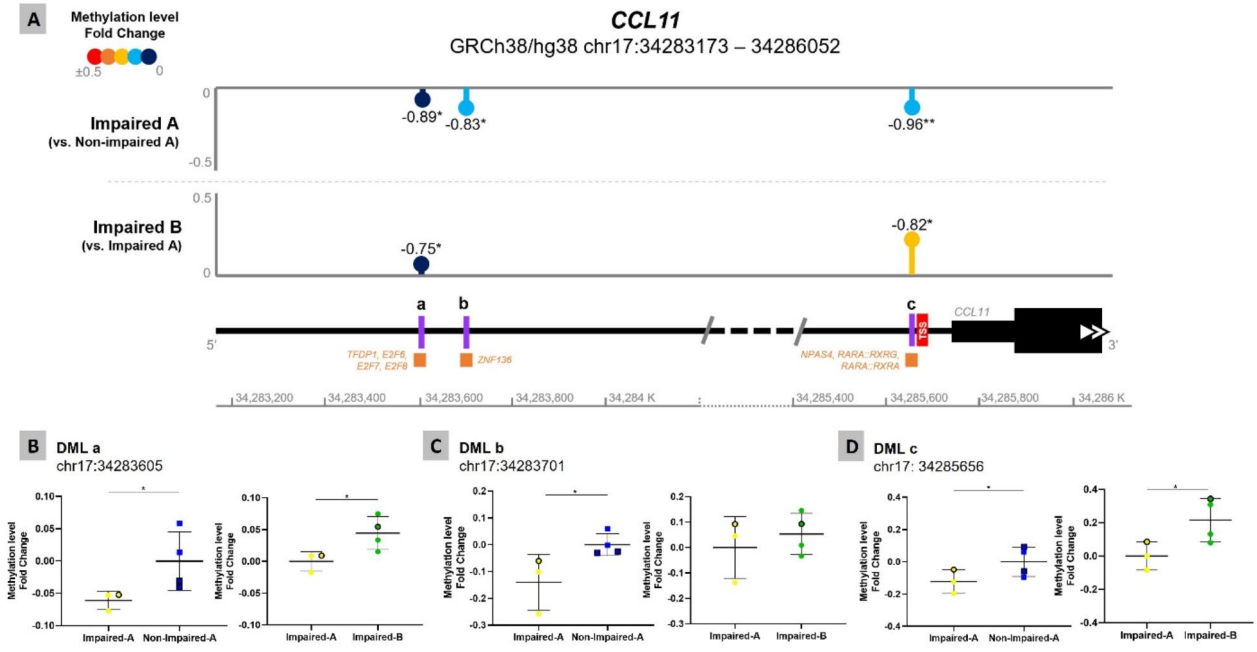

Fig. 3. VCD patients exhibit hypomethylation of CpG in *CCL11* promoter region, which are hypermethylated upon B12 supplementation. **A** = a lollipop plot depicting the methylation level fold change of DML in the promoter region and proximal portion of the first exon of *CCL11*. **B**, **C**, and **D** = The fold change methylation levels of CpG positions were compared between groups using an unpaired t-test. Values next to lollipops represent correlation coefficients (Pearson or Spearman) with statistical significance (* $P < 0.05$ or ** $P < 0.01$) between methylation level fold change in each DML and gene expression values. In the schematic representation of the analyzed gene region, the black line represents the *CCL11* promoter region, and the black blocks delimit the 5' UTR region (thin block) and proximal portion of the first exon of the gene (thick block). The red bold dash indicates the transcription start site of *CCL11*, and the orange boxes indicate predicted transcription factor binding sites. Purple lines correspond to the DML at coordinates (GRCh38/hg38): a = chr17: 34,283,605; b = chr17: 34,283,701; c = chr17: 34,285,656. In the graphs, black-outlined circles or squares denote individuals who attended the 12-month follow-up appointment. In all other cases, both circles and squares represent individuals who attended the 6-month follow-up appointment. Line at mean ± standard deviation. *$P < 0.05$. DML = differentially methylated locus; TSS = transcription start site. Suffixes: A = endpoint A (whole peripheral blood cultures (WPBC) incubated with excipient); B = endpoint B (WPBC incubated with 1 nM B12).

(BBB) or be primarily secreted by activated astrocytes. CCL11 predominantly acts via the CCR3 receptor, which is expressed by microglia, astrocytes, and neural progenitor cells (reviewed in:[28]). High levels of this chemokine can upregulate nicotinamide adenine dinucleotide phosphate-oxidase 1 (NOX1) in glial cells, triggering the production of reactive oxygen species (ROS) and potentiating glutamate-induced neuronal death[29]. CCL11 also inhibits neural progenitor cell proliferation, impairing neurogenesis and hippocampal-related learning and memory[30–32]. Recent findings have revealed CCL11 to be a critical biomarker of long-term brain damage in a mouse model of mild COVID-19[33]. One of the primary mechanisms postulated to underlie the neurodegenerative effects of CCL11 in long COVID involves the induction of hippocampal microgliosis, leading to diminished neurogenesis and the deterioration of myelinated subcortical axons[27]. Altogether, it is reasonable to conclude that CCL11 is a strong biomarker of persistent cognitive sequelae following mild COVID-19 and could be a key target for VCD treatment. LIF is a multifunctional neurotrophic cytokine from the interleukin-6 family. Despite its intricate and context-specific function within the CNS, exhibiting both protective and detrimental impacts on neuronal and glial survival and function, LIF signaling is crucial for the maintenance of the nervous system (reviewed in:[34]). LIF has been identified as a regulator of neuronal phenotype and a coordinator of glial and inflammatory cell responses in the CNS (reviewed in:[34,35]). Moreover, the LIF protein has been shown to increase the transcription of *HGF*, which encodes an important neuroprotective molecule[36]. Nonetheless, the specific role of LIF in the CNS during long COVID remains to be further elucidated.

No significant alteration in the mRNA levels of *CCL2*, *CSF2*, *CXCL10*, *HGF*, *IL10*, *IL1RA*, or *IL6* was observed in leukocytes from individuals with persistent VCD during the follow-up. However, it is unclear whether the reduction of some of these molecules before the resolution of the impairment is beneficial. HGF is a potent neurotrophic factor that promotes neurogenesis and has been shown to have neuroprotective effects in neurodegenerative diseases (reviewed in:[37]). IL-10 is an anti-inflammatory cytokine that negatively regulates the immune response, reduces inflammation, and protects against neuroinflammatory processes associated with neurodegenerative diseases (reviewed in:[38]). IL-1RA dampens the pro-inflammatory effects of IL-1, preventing excessive inflammation and mitigating neuroinflammatory processes associated with neurological disorders (reviewed in:[39]). These findings indicate an orchestrated decline in the neuroprotective and anti-inflammatory response in individuals with persistent VCD. It is important to mention that elevated CXCL10 and HGF levels, along with decreased IL-10 protein levels, have been associated with worsening cognitive function from recruitment to the six-month follow-up in the previous study by Souza-Silva et al.. 2024 with this prospective cohort[20]. These authors analyzed protein levels in raw plasma from patients whose cognitive status changed from VCD to healed, and vice versa, between recruitment and the six-month follow-up, while the present study evaluated the mRNA levels of these biomarkers in whole blood cultures from volunteers who maintained the same diagnosis between recruitment and the six or 12-month appointment. However, a direct comparison between both experiments is not recommended and further research is needed to elucidate the transcription-translation dynamics of the investigated biomarkers in long COVID-associated persistent VCD.

The absence of *IL31* and *NGF* mRNA in leukocytes from the volunteers suggests that the proteins they encode, which were previously found to be elevated in the plasma samples of patients with VCD during the recruitment appointment four months after mild acute COVID-19[4], might be primarily produced by other cell types[40–42].

Methylation is a fundamental biochemical mechanism that plays a critical role in modulating gene expression during neuroinflammation and neurodegenerative disorders[43–45]. Folate and vitamin B12 are important cofactors of this mechanism, acting in the one-carbon metabolism. Insufficient levels of these vitamins hinder the efficient conversion of HCY to methionine, thereby disrupting the methylation cycle and diminishing the cells' methylation capacity. At the same time, HCY accumulates in the blood, leading to hyperhomocysteinemia. The association between the severity of acute COVID-19 and low concentrations of folate and B12 has been reported[46,47]. Conversely, a few studies reported high levels of vitamin B12 in the blood of patients with a worse COVID-19 outcome[48,49] potentially due to cytolysis leading to the release of intracellular B12 into the plasma and disrupted B12 clearance by the liver, which is often affected in these patients[50]. Nevertheless, the impact of B12 levels on cognitive sequelae following mild COVID-19 still needs to be investigated. In the present study, no change was observed in the basal serum levels of folate, vitamin B12 and HCY among patients with VCD, those who recovered from the impairment, and those without impairment. Notably, incubating peripheral whole blood cultures with vitamin B12 resulted in the normalization of *CCL11* expression, the increase of the neuroprotective *HGF* and the decrease *CSF2* and *CXCL10* mRNA levels in leukocytes from individuals with persistent VCD (Fig. 2). It is important to highlight that *CSF2* and *CXCL10* encode pro-inflammatory proteins linked to neurodegenerative diseases[51–53], and, as mentioned above, elevated levels of CXCL10 protein are associated with persistent VCD in individuals from the same cohort of the present study[20]. In addition to its role in regulating gene expression through methyl-dependent epigenetic mechanisms, vitamin B12 is recognized for its neuroprotective, antioxidant, and anti-inflammatory properties[54]. In the acute COVID-19 context, previous studies have demonstrated that vitamin B12 increases the flow of the sulfur amino acid pathway, favoring production of glutathione, an important antioxidant agent, in leukocytes of patients with moderate and severe COVID-19[18]. Moreover, daily B12 supplementation has improved the clinical outcome of hospitalized patients with COVID-19[55] and was also effective in alleviating long COVID symptoms from a patient recovered from mild COVID-19[56]. Altogether, these findings suggest a potential beneficial effect of vitamin B12 in reducing neuroinflammation and promoting neuroprotection in patients with persistent VCD long after experiencing mild acute COVID-19.

The observed differently methylated CpG sites in the promoter region of *CCL11* are located at predicted TFBS[25]. The transcription factors (TF) E2F7 (JASPAR ID: MA0758.1) and E2F8 (JASPAR ID: MA0865.2) can bind to the region encompassing the DML a (chr17: 34283605), while E2F6 (JASPAR ID: MA0471.2) and TFDP1 (JASPAR ID: MA1122.1) can bind to the complementary region of this DML in the minus strand. E2F6, E2F7, and E2F8 are members of the E2F family of TF, which play important roles in cell cycle regulation,

cell proliferation and survival, and gene expression[57]. TFDP1 heterodimerizes with E2F proteins to enhance their DNA-binding activity and promote transcription from E2F target genes[57]. Unfortunately, there is limited research specifically investigating the relationship between E2F6-8 and *CCL11* transcription in the CNS context. Regarding DML b (chr17: 34283701), ZNF136 (JASPAR ID: MA1588.1) can bind to the complementary region in the minus strand, but there is no evidence of its action on *CCL11* expression. NPAS4 (JASPAR ID: MA2042.1) can bind to the region containing the DML c (chr17: 34285656), and RARA::RXRA (JASPAR ID: MA0159.1) and RARA::RXRG (JASPAR ID: MA1149.1) to the complementary region of this locus. NPAS4 is a neuroprotective protein and acts as a TF, regulating the transcription of a diverse set of genes involved in synaptic plasticity, neurotransmission, and neuronal survival[58]. However, there is limited research on its specific interaction with *CCL11*. The RARA::RXRA and RARA::RXRG complexes are heterodimeric protein complexes formed by the retinoic acid receptor alpha (RARA) in conjunction with either retinoid X receptor alpha (RXRA) or retinoid X receptor gamma (RXRG)[59]. These complexes are involved in mediating the effects of retinoic acid signaling, which plays crucial roles in development, cell differentiation, and immune response[59]. Previous studies have demonstrated that the use of RXR partial agonists can reduce eosinophilic airway inflammation, a condition mediated, in part, by the chemoattractant activity of CCL11[60]. RXR agonists are also effective in the treatment of some neuropathies, such as Alzheimer's disease[61] and Parkinson's disease in animal models[62]. While direct evidence linking the RARA::RXRA and RARA::RXRG complexes to *CCL11* expression in the CNS is currently not well established, it is plausible to speculate that retinoic acid signaling through these complexes could inhibit *CCL11* expression in specific brain conditions. Furthermore, the DML c is situated within regulatory regions that contain binding sites for EZH2, CTCF, GPS2, and TCF21 across various cell types[63]. These TF act as epigenetic regulators of chromatin structure through interactions with histone modifiers, chromatin remodeling complexes, transcriptional regulators, and/or DNA methylation machinery that modulate gene expression. It is reasonable to assume that changes in CpG methylation levels in this region may reflect on the epigenetic landscape, possibly favoring the expression of *CCL11*. Overall, this significant discovery highlights the potential of using CpG methylation patterns as diagnostic biomarkers for neurodegenerative conditions characterized by *CCL11* upregulation.

The hypothesis that B12 could modulate *CCL11* expression during persistent VCD via methyl-dependent epigenetic mechanisms was confirmed by the hypermethylation of DML a and c in leukocytes from cultures of impaired individuals incubated with the vitamin. The B12-induced increment in methylation levels of each of these DML negatively correlated with the *CCL11* expression level (Fig. 3). These findings provide compelling evidence for the role of epigenetic regulation of *CCL11* and suggest that supplemental B12 has potential as an epidrug to treat neurodegenerative conditions associated with aberrant *CCL11* expression.

This study offers valuable insights into the mechanisms underlying persistent VCD associated with long COVID and highlights the therapeutic potential of vitamin B12 as an epidrug to prevent or mitigate this impairment. However, several limitations should be considered when interpreting the findings. The small sample size, restricted to women, limits the generalizability of the results.

It is noteworthy that the volunteers included in this study had mild COVID-19 in 2021, prior to the emergence of the SARS-CoV-2 Omicron variant and when the Brazilian population had not completed the full COVID-19 vaccination schedule. COVID-19 vaccination has been associated with a lower risk of several, but not all, post-COVID-19 symptoms[64]. The present study was not designed to assess the protective effect of vaccination against VCD as a post COVID-19 sequelae. Therefore, caution should be taken when extrapolating findings from this work to individuals who were infected after completing their vaccination or were infected with more recent SARS-CoV-2 variants.

The whole blood culture model serves as a valuable tool for studying immune responses, inflammation, and molecular processes in a physiologically relevant context, offering a more realistic representation of blood tissue complexity compared to in vitro studies with single type cells. However, further research using animal models is needed to evaluate the potential of B12 as an epidrug for in vivo modulation of peripheral biomarkers associated with persistent VCD in long COVID and to determine whether this contributes to neuroprotection.

Conclusion

This study provides compelling evidence highlighting the importance of systemic inflammation in persistent VCD associated with long COVID (Fig. 4). Notably, the study reports a persistent upregulation of *CCL11* in peripheral leukocytes in patients with VCD. This upregulation has been found to be strongly and negatively correlated with the methylation state of three cytosines in the promoter region of the *CCL11* gene. Collectively, these findings support the potential use of *CCL11* mRNA levels or promoter methylation patterns as biomarkers for VCD in long COVID. Moreover, the results suggest that supplementation with vitamin B12 holds promise as a therapeutic approach for patients with VCD in the context of long COVID.

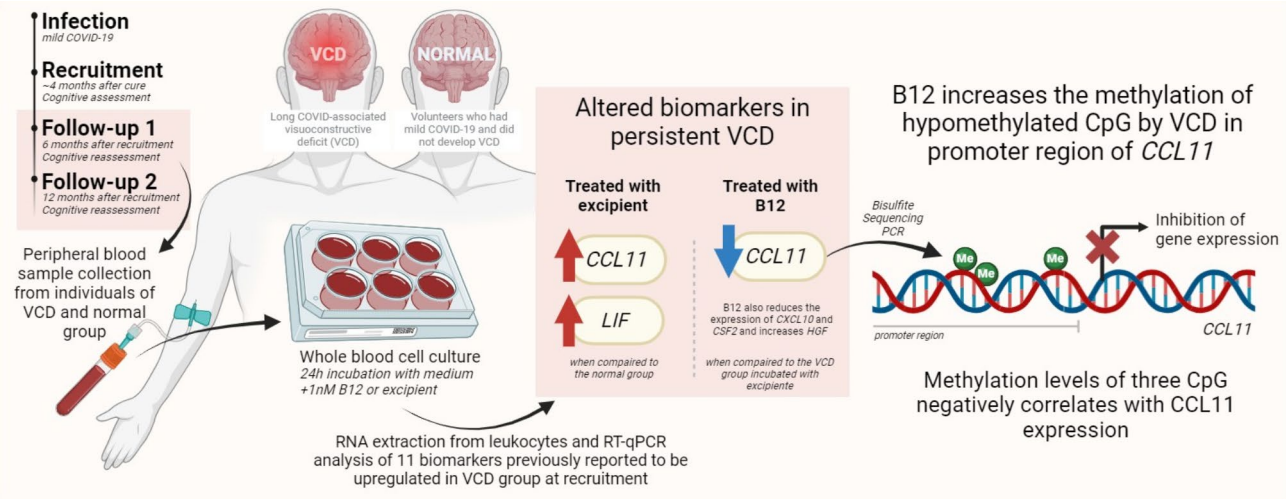

Fig. 4. Graphical representation of the study (created with BioRender.com).

Data availability

The dataset supporting the conclusions of this article is available in the SRA repository [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA992778].Supplementary information is available in the Additional files 1 to 4.

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Author contributions

Study conception and/or design: JJP, DMM, MAR-S, and RSC. Clinical assessment: JJP. Data acquisition: LMGC, JJP, DVR. Data analysis: LMGC, JJP, DVR, DMM, MAR-S, and RSC. Manuscript writing and/or revision: LMGC, JJP, DVR, DMM, MAR-S, and RSC.

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Declarations

Ethics approval and consent to participate
This study received approval from the Institutional Review Board (IRB) of the Federal University of Minas Gerais (UFMG) (CAAE3768820.1.0000.5149).

Consent for publication
All participants included in this study provided written informed consent.

Competing interests
The authors declare no competing interests.

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