

## Title

Vitamin B12 attenuates leukocyte inflammatory signature in COVID-19 via methyl-dependent changes in epigenetic marks

## Short Title

Vitamin B12 attenuates inflammation in COVID-19

## Authors

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## Abstract

COVID-19 induces chromatin remodeling in host immune cells, and it had previously been shown that vitamin B12 downregulates some inflammatory genes via methyl-dependent epigenetic mechanisms. In this work, whole blood cultures from moderate or severe COVID-19 patients were used to assess the potential of B12 as adjuvant drug. The vitamin normalized the expression of a panel of inflammatory genes still dysregulated in the leukocytes despite glucocorticoid therapy during hospitalization. B12 also increased the flux of the sulfur amino acid pathway, raising the bioavailability of methyl. Accordingly, B12-induced downregulation of *CCL3* strongly and negatively correlated with the hypermethylation of CpGs in its regulatory regions. Transcriptome analysis revealed that B12 attenuates the effects of COVID-19 on most inflammation-related pathways affected by the disease. As far as we are aware, this is the first study to demonstrate that pharmacological modulation of epigenetic marks in leukocytes favorably regulates central components of COVID-19 physiopathology.

## Teaser

B12 has great potential as an adjuvant drug for alleviating inflammation in COVID-19.

## 44 Introduction

45 Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has been reported, as of  
46 August 2022, in more than 583 million confirmed cases and has led to more than 6.4  
47 million deaths worldwide (1), although recent studies estimate a much higher death toll  
48 (2). Since the end of 2020, large-scale population vaccination has drastically reduced the  
49 mortality rate and the burden on health systems, but factors such as inequality in access to  
50 immunizations, rapid waning neutralizing antibody titers induced either by vaccines or by  
51 exposure to SARS -CoV-2 and the emergence of new genotypic variants of the virus have  
52 contributed to successive new waves of the pandemic with more dramatic impacts on  
53 unvaccinated individuals or those with poor response to vaccines. In this context, efforts  
54 have been made in the search for treatments for COVID-19, such as the repositioning of  
55 chemicals and monoclonal antibodies that act as anti-inflammatory agents, and the  
56 development of new antiviral small molecules that target SARS-CoV-2 replication and  
57 antibodies that bind to the viral spike protein blocking virus entry into cells (3).  
58 Unfortunately, most of these drugs have limited effectiveness or are very expensive,  
59 making them unsuitable to be used on a global scale. Therefore, there is still an urgent  
60 need for new efficient treatments with safe, inexpensive, and widely available drugs (4).

61 While most COVID-19 patients will experience mild symptoms, some will develop severe  
62 acute respiratory distress syndrome, systemic inflammation, multiple organ failure, and  
63 other more serious complications that can lead to death. Several components of the host  
64 immune system are dramatically altered during SARS-CoV-2 infection and the extent of  
65 immune dysregulation is related to the worsening of COVID-19 and progression to death.  
66 (5). Patients with COVID-19 exhibit inflammatory signatures defined by low levels of  
67 type I and III interferons (IFNs) and elevated levels of some cytokines and chemokines.  
68 These two branches of the innate immune response contribute to the clinical evolution of  
69 the patients (6). As the disease worsens, increased levels of inflammatory cytokines and  
70 chemokines are observed, which can lead to depletion and exhaustion of T cell  
71 populations resulting in a significant elevation of the neutrophil/lymphocyte ratio (NLR)  
72 (7-9). The increase in neutrophil population (7, 8) with transcriptional signatures related to  
73 its activated state (10) also contributes to the elevation of NLR in COVID-19.

74 DNA methylation is an epigenetic mechanism that, in mammals, occurs mainly in  
75 dinucleotides of a cytosine followed by a guanine (CpG) and can affect the accessibility of  
76 transcription factors (TF) and RNA polymerases to DNA, thus modulating gene  
77 expression. (11). In general, DNA hypomethylation in gene promoters is associated with  
78 the activated state of their expression (12). It is already known that promoters of genes  
79 encoding cytokines are rapidly demethylated (~6 hours) after T cell activation (13) and  
80 that several genes encoding key cytokines and chemokines have increased systemic  
81 expression in patients with COVID-19 (14) are regulated by the methylation of CpGs in  
82 their promoters or enhancers (15-18). The ability of RNA viruses to hijack the epigenome  
83 of host immune cells in order to evade antiviral defense is widely acknowledged (19, 20).  
84 In early 2021, Corley and colleagues reported that the DNA methylation signature of  
85 peripheral blood mononuclear cells from critically ill COVID-19 patients is characterized  
86 by hypermethylation of genes related to the IFN-mediated antiviral response and  
87 hypomethylation of inflammatory genes (17). This finding, in light of previous knowledge  
88 about the epigenetic regulation of genes involved in the inflammatory storm of COVID-  
89 19, points to the therapeutic potential of drugs capable of modulating the epigenetic  
90 landscape as a strategy for the control of exacerbated inflammation resulting from SARS-  
91 CoV-2 infection.

92 The bioavailability of methyl is a determinant of the DNA methylation state and can be  
93 regulated by vitamin B12, a cofactor of the enzyme methionine synthase (MS), which  
94 transfers methyl groups from 5-methyltetrahydrofolate to homocysteine (HCY) forming  
95 methionine. This is then converted to S-adenosylmethionine (SAM), the universal methyl  
96 donor. In this same pathway of sulfur amino acids, HCY can be metabolized producing  
97 glutathione (GSH) (Fig. 1) (21). It has already been demonstrated that adjuvant therapy  
98 with B12 increases DNA methylation and reduces the expression of inflammatory  
99 mediators in the central nervous system of infant rats with pneumococcal meningitis (18).  
100 Among these genes, *IL1B* and *CCL3* also play a central role in the pathophysiology of  
101 COVID-19. Vitamin B12, also known as cobalamin (Cbl), is an essential micronutrient,  
102 which, once absorbed, binds to transcobalamin II (TC-II) and is transported into the cells  
103 through specific receptors (23). Although the liver is the main source of TC-II, its  
104 unsaturated form (not bound to cobalamin) is abundant in the blood (24).

105 The hypothesis of this study is that, during the advanced phase of COVID-19,  
106 characterized by hyperinflammation (cytokine storm), supplemental vitamin B12 would  
107 increase the flow of the sulfur amino acid pathway, favoring the production of SAM and  
108 the antioxidant GSH. The increased methylation capacity of cells, provided by higher  
109 concentrations of SAM, would lead to the hypermethylation of regulatory regions of pro-  
110 inflammatory genes attenuating inflammation. This hypothesis was tested using the *ex*  
111 *vivo* model of whole blood culture collected from patients with moderate or severe forms  
112 of COVID-19 and healthy controls.

## 113 Results

### 114 Patients and healthy volunteers

115 The median age of patients and non-infected healthy volunteers included in the study was  
116 64 years (minimum = 45; maximum = 86), 55% of which were women, with no  
117 statistically significant differences between the groups in these aspects. All patients had a  
118 confirmatory clinical diagnosis of COVID-19 and confirmation of SARS-CoV-2 virus  
119 infection by RT-qPCR carried out, on average, 5 days before the collection of blood  
120 samples for this project. The time elapsed between the onset of symptoms and admission  
121 to the hospital was 6.3 days, and the length of stay before sample collection was 11.8  
122 days, for patients in the MOD and SEV groups, with no statistically significant difference  
123 between the two groups. Cardiovascular diseases (73.08%) and diabetes mellitus (38.46%)  
124 were the most frequent comorbidities among patients.

125 All patients were on glucocorticoid treatment (dexamethasone: 25 patients; prednisone: 1  
126 patient) but those with severe COVID-19 most often received combinations of two or  
127 three drugs of this category, namely dexamethasone, hydrocortisone and beclomethasone  
128 for an average of 11 days before their blood samples were taken, with no differences  
129 between MOD and SEV regarding the duration of this treatment. Among the cytochemical  
130 parameters evaluated, differences were found between MOD and SEV groups for blood  
131 glucose (mg/dL; means MOD = 131.7 and SEV = 196.7;  $P = 0.0111$ ), total leukocytes  
132 (cells per  $\text{mm}^3$ ; means MOD = 9,650 and SEV = 16,355;  $P = 0.0041$ ), percentage of  
133 lymphocytes (%; medians: MOD = 14.85 and SEV = 6.95;  $P = 0.0048$ ), percentage of  
134 neutrophils (%; medians: MOD = 73.85 and SEV = 86.1;  $P = 0.0139$ ) and for the NLR  
135 (means MOD = 5.2 and SEV = 13.1;  $P = 0.0038$ ). The increase in the percentage of  
136 neutrophils in SEV patients coincides with the occurrence of more frequent bacterial  
137 coinfections in this group (20% for MOD and 75% for SEV;  $P = 0.0138$ ). Arterial blood

138 gas analysis of MOD and SEV patients revealed significant differences in pH (medians  
139 MOD = 7.435 and SEV = 7.375;  $P = 0.0223$ ), O<sub>2</sub> pressure (mmHg; medians MOD = 58.15  
140 and SEV = 80.10;  $P = 0.0028$ ), CO<sub>2</sub> tension (mmol/L; medians MOD = 20.30 and SEV =  
141 23.25;  $P = 0.0309$ ) and O<sub>2</sub> saturation (%; means MOD = 90.25 and SEV = 94.94;  $P =$   
142 0.0123), compatible with the fact that SEV patients were intubated with mechanical  
143 ventilation. Potassium concentrations were also higher in patients in the SEV group  
144 (mmol/L; medians MOD = 3.820 and SEV = 4.365;  $P = 0.0022$ ). The combination of  
145 Clavulanate and Amoxicillin was more frequent among MOD patients than SEV (%;  
146 MOD = 70 and SEV = 25;  $P = 0.0426$ ). Although there were no statistically significant  
147 differences between MOD and SEV groups regarding the occurrence of clinical  
148 complications, the outcomes were significantly worse for patients in the SEV group, with  
149 longer total hospitalization time (days; means MOD = 16.2 and SEV = 27.94;  $P = 0.0132$ )  
150 and more frequent deaths (%; MOD = 10% and SEV = 75%;  $P = 0.0036$ ). All information  
151 compiled from medical records is presented in Table S1.

152 Control subjects had no RT-qPCR detectable SARS-CoV2 in their oropharynx and  
153 nasopharynx and no antibodies against the virus in their blood. No statistically significant  
154 differences were found between baseline plasma B12 levels of patients in the SEV, MOD  
155 and CTRL groups (Fig. S1). No SARS-CoV-2 RNA was detected by RT-qPCR in raw  
156 blood or blood cultures of any participant in this study.

### 157 **Validation of the experimental model**

158 The RT-qPCR analyses revealed distinctive transcriptional signatures for MOD and SEV  
159 forms of COVID-19 and the CTRL group in the aliquots of endpoint Z, which remained  
160 mostly preserved after 24h of incubation (endpoint A) (Fig.S2 and Fig. 2). Importantly,  
161 patients were already on glucocorticoid treatment before blood collection for this study,  
162 which may explain why higher levels of mRNA were not found for some genes, such as  
163 *CCL2*, *CXCL9*, *IL6*, *IL17A*, *CCL1* and *TNF* in MOD and/or SEV patients compared to  
164 CTRL at endpoints Z and A (Fig.S2 and Fig. 2). High levels of *CCL3* and *IL1B* mRNA  
165 were observed in the SEV and MOD groups compared to the CTRL, indicating that these  
166 mediators of COVID-19 inflammation are not sufficiently responsive to glucocorticoid  
167 treatment. Reduced levels of mRNA of marker genes of CD4 and CD8a lymphocyte  
168 lineages were observed in MOD and SEV patients compared to CTRL at endpoints Z and  
169 A. At endpoint Z, these two genes showed a positive correlation (MOD:  $r = 0.5553$ ,  $P =$   
170 0.004 and SEV:  $r = 0.5665$ ,  $P = 0.0032$ ) with the percentage of lymphocytes in the  
171 patients' blood counts (Table S1). On the other hand, mRNA levels of *HAVCR2*  
172 lymphocyte exhaustion marker did not differ between patients and CTRL.

### 173 **Vitamin B12 favorably modulated critical inflammatory mediators**

174 The biomarker genes *CCL3* and *IL1B*, which were upregulated in patients despite previous  
175 glucocorticoid therapy, responded very well to treatment with B12, which reduced *CCL3*  
176 mRNA levels of MOD and SEV, and *IL1B* of SEV, matching them to the CTRL at  
177 endpoint A (Fig. 2, A to D). Importantly, B12 did not affect *CCL3* or *IL1B* expression in  
178 the CTRL group at endpoint B. Other genes had lower mRNA levels in MOD (*IL17A*, *IL6*  
179 and *CCL1*) and SEV (*IL17A* and *IL6*) compared to CTRL at endpoint A. B12 raised  
180 mRNA levels of *IL6* and *IL17A* in both MOD and SEV, matching them to CTRL at  
181 endpoint A. The vitamin also raised *CCL1* mRNA levels in cultures of MOD and SEV  
182 patients. In this case, *CCL1* mRNA levels of MOD group equaled those of the CTRL at

183 endpoint A and the levels of the SEV exceeded this reference. *CXCL9* mRNA levels,  
184 which did not differ between patients and CTRL at endpoint A, increased in SEV cultures  
185 treated with B12 so that its mRNA levels exceeded those of the CTRLs at endpoint A.

186 *TNF*, whose baseline mRNA levels were low only in the SEV group compared to CTRL at  
187 endpoint A, did not have their mRNA levels significantly altered by B12 in either of the  
188 two patient groups. B12 also did not affect *CD4* and *CD8A* mRNA levels in MOD or  
189 SEV, but slightly increased those of *HAVCR2* in SEV. However, this increase was not  
190 enough to achieve a statistically significant difference compared to CTRL at endpoint A.

191 Finally, the treatment of whole blood cultures of individuals in the CTRL group with B12  
192 did not change the expression levels of any gene assessed. Overall, these results reinforce  
193 the security of the 1 nM dose of B12 and prove its efficiency in regulating the mRNA  
194 levels of several critical inflammatory mediators in whole blood cultures of patients with  
195 moderate and severe COVID-19. This regulation of mRNA levels of inflammatory  
196 mediators by B12 was done by down- or upregulation depending on the gene.

### 197 **Vitamin B12 reduced intracellular protein levels of critical inflammatory mediators**

198 MOD cultures treated with B12 had reduced intracellular protein levels of CCL3 and IL-  
199 1B ( $P < 0.0001$ ), both of which were elevated at endpoint A compared to CTRL ( $P =$   
200  $0.0011$  and  $P < 0.0001$ , respectively) (Fig. 2, E and F). Interestingly, B12 did not affect  
201 the intracellular protein levels of IL-1B in the cultures of the SEV group, and, regardless  
202 of the treatment, the intracellular protein levels of IL-1B were very low, close to or below  
203 the detection limit of the method (Fig. 2F).

### 204 **Vitamin B12 increased the flow of the sulfur amino acid pathway**

205 B12 caused an increase in HCY concentrations of all groups (MOD,  $P = 0.0037$ ; SEV,  $P <$   
206  $0.0001$ ; CTRL  $P = 0.0313$ ) and in CYS and GSH levels of MOD and SEV (MOD:  $P =$   
207  $0.0029$  and  $P = 0.0002$ ; SEV:  $P = 0.0007$  and  $P < 0.0001$ ) compared to their respective  
208 levels at endpoint A, which indicates an increased flow of the sulfur amino acid pathway  
209 (Table 1). However, the expected increase in the SAM/SAH ratio in response to B12 was  
210 not observed. In fact, at endpoint B, decreased SAM levels were observed in MOD group  
211 ( $P = 0.0010$ ), without, however, any change in SAM/SAH ratio. In the SEV group, B12  
212 increased SAH ( $P = 0.0091$ ) and reduced SAM/SAH ( $P = 0.0021$ ).

### 213 **Vitamin B12 increased methylation levels of CpGs in regulatory regions of *CCL3***

214 The effects of COVID-19 and B12 on the methylation levels of the 21 CpGs located in the  
215 promoter region and proximal portion of the first exon of the *CCL3* gene (GRCh38/hg38  
216 chr17: 36,090,276-36,090,005) were evaluated by BSP in an NGS platform from DNA  
217 libraries produced with aliquots of endpoints A and B from individuals representing  
218 MOD, SEV and CTRL groups. At endpoint A, when compared with the CTRL group, no  
219 changes were found in the percentage of methylation of any of the evaluated positions in  
220 the cultures of MOD patients, while SEV patients had an hypomethylated CpG at  
221 chr17:36,090,102 (Fig. 3). Treatment of cultures with B12 increased methylation levels of  
222 CpGs at chr17:36,090,097 in MOD subjects and at chr17:36,090,097, 36,090,102 and  
223 36,090,246 in SEV subjects compared to their respective cultures at endpoint A. Note that  
224 for methylation of the chr17:36,090,102 position in SEV group, COVID-19 and B12 had



inverse effects. The methylation levels of all the aforementioned CpGs had negative and statistically significant correlations with the gene expression levels.

## **Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients with COVID-19**

The effects of COVID-19 and B12 on the global gene expression in leukocytes in whole blood cultures from representative individuals of the MOD, SEV and CTRL groups were assessed by RNA-Seq. Aiming at identifying differentially expressed genes (DEG), the following contrasts were analyzed: 1) MOD vs. CTRL at endpoint A; 2) SEV vs. CTRL at endpoint A; 3) MOD (endpoint B) vs. CTRL (endpoint A); and 4) SEV (endpoint B) vs. CTRL (endpoint A); 5) CTRL at endpoint A vs. CTRL at endpoint B.

A large number of genes had their expression affected by COVID-19 (Fig. 4). In the MOD group, 3,034 DEGs (2,041 upregulated and 993 downregulated) were found in contrast 1 and 3,636 DEGs (2,361 upregulated and 1,275 downregulated) in contrast 3. In cultures of the SEV group, 8,565 DEGs (4,464 upregulated, 4,101 downregulated) were found in contrast 2 and 8,894 DEGs (4,520 upregulated and 4,374 downregulated) in contrast 4. Among the DEGs identified, 2,699 had decreased (1,364) or increased (1,335) expression after treatment with B12 (Fig. 4). In the leukocytes of patients in MOD group, B12-upregulated genes had GO annotations related to *phagocytosis*, *regulation of miRNA transcription*, *response to virus* and *monocyte extravasation*. In the same group, B12-downregulated genes had GO annotations related to *NF-kappaB signaling*, *T-cell receptor signaling pathway* and *negative regulation of histone H3-K9 trimethylation* (Fig. S3). Regarding the SEV group, B12-upregulated genes had GO annotations related to *cytoplasmic translation* (tRNA and rRNA processing), *adaptive immune response*, *ncRNA processing*, *miRNA-mediated gene silencing*, *proteasome-mediated ubiquitin-dependent protein catabolic process*, *cobalamin transport*, and *negative regulation of transcription by RNA polymerase II* (Fig. S3). B12-downregulated genes in this group were related to *positive regulation of transcription by RNA polymerase II*, *processing and transport of mRNA*, *mRNA splicing*, *cellular response to DNA damage stimulus*, *activation of innate immune response*, *positive regulation of IL-6 and IL-2 production*, *T-cell differentiation in thymus*, *histone modification*, *viral transcription* and *DNA conformational change*. Finally, no DEGs were found in contrast 5, which reinforces the safety profile of B12.

Functional enrichment analysis of DEGs identified in the contrasts above mentioned revealed 90 metabolic and signaling pathways differentially regulated in the groups MOD or SEV at endpoint A directly or indirectly related to the inflammatory response in blood, although patients had received glucocorticoid treatment for approximately 11 days prior to collection of samples to the study (Fig. 5A and Fig. S4). In the cultures treated with B12, 45 and 74 out of these 90 pathways were still differentially regulated in MOD and SEV groups, respectively, although with a minimum 20% difference in Z-scores relatively to their untreated condition. An overall favorable effect on inflammation control was predicted for B12 treated cultures of MOD (Fig. 5B and Fig. S5) and SEV (Fig. 5C and Fig. S6) groups.

## **Discussion**

Epigenetic changes in host cells during COVID-19 have already been described and are associated with the regulation of the SARS-CoV-2 cycle (25, 26), the disease severity (17, 26) or the prognosis for critically ill patients (16, 26). However, to the best of our

270 knowledge, this is the first work to demonstrate that pharmacological modulation of the  
271 leukocyte epigenetic marks favorably regulates central components of hyperinflammation  
272 in moderate and severe forms of the disease. Initially, the whole blood culture model was  
273 validated for evaluation of anti-inflammatory drugs for COVID-19 by the identification of  
274 stable transcriptional signatures distinguishing between moderate and severe forms of the  
275 disease and between these and controls without infection, despite the fact that patients  
276 were on glucocorticoids before sample collection. Then, using this *ex vivo* model, it was  
277 demonstrated that vitamin B12 favorably modulates, by methyl-dependent epigenetic  
278 mechanisms, the expression of inflammatory genes and the activity of metabolic and  
279 signaling pathways related to the hyperinflammation associated with COVID-19 forms  
280 that require hospitalization.

281 Expression analysis of a panel of COVID-19 related genes at endpoint Z (blood aliquots  
282 added with culture medium and B12 excipient but not incubated) (Fig. S2) in addition to  
283 revealing distinctive transcriptional signatures of moderate and severe forms of the  
284 disease, have also shed light on the effects of glucocorticoid therapy on circulating  
285 leukocytes. Exacerbated activation of inflammatory cytokines is associated with COVID-  
286 19 severity and poor prognosis (27). However, at endpoint Z, mRNA levels of *CCL2*,  
287 *CXCL9*, *IL6*, *IL17A*, *CCL1* and *TNF* were lower in the MOD and/or SEV groups  
288 compared to the controls, probably due to previous glucocorticoid treatment that patients  
289 received during hospitalization. Although these genes are components of the  
290 hyperinflammation caused by SARS-CoV-2 (27, 28), it is debatable whether their early  
291 inhibition and drastic reduction are beneficial for patients. For instance, dexamethasone is  
292 known to have a strong inhibitory effect on *IL6* and *IL17A* in patients with COVID-19  
293 (28), but it is still unclear to what extent the beneficial effects of IL-6 blockers depend on  
294 dose, time of administration, clinical condition, among other factors (29, 30). It is also  
295 worth mentioning that IL-17A play a role in induce protective inflammatory responses,  
296 hindering viral infection (31, 32). In contrast, some biomarkers remained overexpressed in  
297 MOD and SEV cultures, despite the patients having been previously treated with  
298 glucocorticoids. Among the genes most refractory to glucocorticoid treatment, *CCL3* and  
299 *IL1B* stand out, both with a central role in the pathophysiology of COVID-19 and highly  
300 expressed in peripheral blood mononuclear cells (PBMC) during the disease (14, 33).  
301 *CCL3* is a critical pyrogenic cytokine that is involved in leukocyte recruitment and  
302 activation in acute inflammation. It has been reported that *CCL3* expression is higher in  
303 COVID-19 patients with an unfavorable outcome (34). IL-1B has a broad spectrum of  
304 biological functions and participates in innate and adaptive immunity. In infections, IL-1B  
305 induces gene expression and synthesis of various cytokines and chemokines in  
306 macrophages and mast cells. SARS-CoV-2 activated IL-1B stimulates the secretion of  
307 TNF, IL-6 and other cytokines, a pro-inflammatory complex that can lead to cytokine  
308 storm and be deleterious both in the lung and systemically (35).

309 The transcriptional signatures observed at endpoint Z remained for the most part  
310 conserved after incubation of cultures for 24h (endpoint A), thus validating the *ex vivo*  
311 model of whole blood cultures for researching drugs with the potential to fight  
312 hyperinflammation in COVID-19.

313 The whole blood culture model was used to test whether vitamin B12 can regulate, via  
314 methyl-dependent epigenetic mechanisms, the expression of inflammatory genes in the  
315 leukocytes of patients with moderate and severe forms of the disease and who are already  
316 being treated with glucocorticoids. Indeed, in the cultures of the MOD group, B12 equaled

317 the mRNA levels of almost all the cytokine or chemokine genes tested to those of the  
318 CTRL group that did not receive the vitamin. These genes had not been regularized by  
319 glucocorticoid therapy previously received by the patients before sample collection to this  
320 study. An exception was *IL1B*, whose mRNA levels were not significantly affected by  
321 B12. Interestingly, B12 decreased intracellular protein levels of IL-1B in the MOD group  
322 (Fig. 2F). It is worth noting the magnitude of reduction of *CCL3* mRNA levels by B12 in  
323 this group, which was accompanied by the reduction of their intracellular protein levels.  
324 Likewise, the treatment of SEV group cultures with B12 brought the mRNA levels of  
325 most of the evaluated biomarkers closer to those of untreated CTRLs (Fig. 2, A, B and D).  
326 In this group, *CCL3* and *IL1B* were the most responsive genes, at the transcriptional level,  
327 to B12, but no differences were observed in the intracellular levels of the respective  
328 proteins (Fig. 2, E and F). The choice to analyze the intracellular protein concentrations of  
329 IL-1B and *CCL3* was based on the premise that the inhibition of gene transcription in  
330 response to B12 added to the turnover of these proteins by the proteasome machinery  
331 present inside the cells would result in detectable reductions in their concentrations after  
332 24 hours of culture incubation. As opposite, in the extracellular medium, a detectable  
333 decrease in the concentrations of these proteins would be less likely due to the absence of  
334 the cellular machinery for protein degradation. This strategy was suitable to detect the  
335 reduction of *CCL3* and IL-1B proteins in cultures of the MOD group. However, in  
336 cultures of the SEV group, the reduction of *CCL3* by B12 did not reach statistical  
337 significance and intracellular IL-1B levels were very low, close to the detection limit of  
338 the technique, suggesting that, in severe COVID-19, leukocytes are more prone to  
339 promptly export IL-1B. Still in the SEV group, B12 increased the mRNA levels of *CCL1*  
340 and *CXCL9* beyond those observed in the untreated CTRL group. *CCL1* is produced by  
341 activated T lymphocytes and monocytes/macrophages, and is the only known chemokine  
342 capable of interacting with CCR8, a receptor expressed on Th2 cells and regulatory T cells  
343 (Treg). This chemokine mediates the inhibition of dexamethasone-induced thymocyte  
344 apoptosis (36) and its stimulation is related to autocrine anti-apoptosis (37), suggesting  
345 that *CCL1*-CCR8 interactions may provide survival signals for T cells at sites of  
346 inflammation. *CXCL9*, which is inducible by IFN- $\gamma$ , is related to the activation of the Th1  
347 antiviral immune response, as well as the trafficking of Th1, CD8 and natural killer (NK)  
348 cells (38). B12 treatment also brought *IL17A* levels of SEV closer to those of untreated  
349 CTRL. Therefore, these results indicate that B12 can stimulate T cell survival in patients  
350 with severe COVID-19. As expected, since lymphocytes expressing *CD4* and *CD8A*  
351 multiply and differentiate by clonal expansion in the thymus (39), treatment of whole  
352 blood cultures with B12 did not affect *CD4* and *CD8A* mRNA levels in either MOD or  
353 SEV groups (Fig. 2, A to D).

354 Quantification of sulfur amino acid pathway metabolites revealed an increased flux as  
355 evidenced by higher concentrations of HCY, CYS and GSH in the cultures of the MOD  
356 and SEV groups treated with B12 compared to their untreated cultures. The increased  
357 concentration of GSH favors the attenuation of exacerbated inflammation and prevents  
358 cell damage caused by COVID-19 due to its antioxidant effect (40). However, a reduction  
359 in SAM and SAM/SAH was also observed in cultures from MOD and SEV groups,  
360 respectively, in response to B12 (Table 1). When HCY and B12 levels are high, the  
361 enzyme MS converts HCY to methionine, which is then converted to SAM. Then,  
362 methyltransferases transfer the methyl group of SAM to cytosine residues in the DNA or  
363 to other acceptors, such as amino acid residues in histone tails, resulting in the formation  
364 of SAH (41). In fact, an increase in SAH was observed in cultures of the SEV group  
365 treated with vitamin B12. This set of results suggests that SAM, whose concentration was



366 probably increased in cultures treated with B12, was readily consumed in methylation  
367 reactions of either DNA or other acceptors of methyl groups during the incubation period  
368 of 24 h. This increase in the methylation capacity of leukocytes induced by B12 was  
369 confirmed by analyzing the methylation profile of CpGs of the *CCL3* gene.

370 Leukocytes from cultures of patients with severe COVID-19 at endpoint A showed  
371 hypomethylation of the chr17:36,090,102 locus, in the 5' UTR region of *CCL3*, when  
372 compared to the CTRL group (Fig. 3). Contrarily, no DML was identified in leukocyte  
373 cultures from patients in the MOD group at endpoint A. The hypothesis that B12  
374 downregulates inflammatory genes of COVID-19 via methyl-dependent epigenetic  
375 mechanisms was proved by the hypermethylation of three CpGs located in the 5' UTR  
376 region and in the proximal portion of the first exon of *CCL3* (Chr17: 36,090,276 -  
377 36,090,005) of leukocytes from cultures of MOD and SEV groups treated with the  
378 vitamin. The B12-induced increment in methylation levels of each of these DMLs  
379 negatively correlated with the *CCL3* expression level assessed by RT-qPCR (Fig. 3). Two  
380 of the three DMLs in response to B12 are located at transcription factor binding sites  
381 (TFBS). TFs FOS (JASPAR ID: MA1800.1) and SMARCA4 (OREgAnno ID:  
382 OREG1238466) bind to the region encompassing the DML chr17:36,090,246 (a), while  
383 NFIC (JASPAR ID: MA0161.2) binds to that containing the DML chr17:36,090,102 (b).  
384 NFIC is a component of the CTF/NF-I family and a SARS-CoV2-upregulated  
385 inflammation-associated TF (42). At endpoint A, the gene encoding NFIC is upregulated  
386 in the SEV group compared to controls (contrast 2), suggesting an important role for this  
387 TF in the transcription of inflammatory genes in severe COVID-19. AP-1, a complex  
388 formed by FOS and other TFs, is crucial for transcription of *CCL3*. The AP-1 signaling  
389 pathway can be activated by cytokines, growth factors, stress, and bacterial or viral  
390 infections, including SARS-CoV proteins (43). Interestingly, the gene encoding FOS was  
391 downregulated in the SEV group at endpoint B compared to controls at endpoint A  
392 (contrast 4), indicating that B12, in addition to blocking the binding of this TF to the  
393 regulatory region of *CCL3*, also reduces its expression in severe COVID-19. SMARCA4  
394 is a protein present in ATP-dependent chromatin remodeling complexes of the SWI/SNF  
395 type. Members of this family have helicase and ATPase activities and regulate the  
396 transcription of certain genes by altering the chromatin structure in their surroundings.  
397 Recruitment of this complex is associated with increased expression of *CCL3* (44).

398 Therefore, the severe form of COVID-19 induces hypomethylation of a CpG locus in the  
399 regulatory region of *CCL3*, which is coherent with its upregulation. This result  
400 corroborates the findings of Corley et al (17), who had already demonstrated the  
401 hypomethylation of upregulated inflammatory genes in PBMC of patients with severe  
402 COVID-19. More importantly, the results presented herein prove that vitamin B12  
403 downregulates *CCL3* in leukocytes of patients with moderate or severe COVID-19 via the  
404 hypermethylation of CpGs and subsequent inhibition of TF binding to the analyzed  
405 regulatory region.

406 As expected, functional enrichment analysis of RNA-Seq data revealed different immune  
407 response profiles depending on the severity of the disease (Fig. 5A and Fig. S4). However,  
408 although it has already been shown that greater activation of hyperinflammation-related  
409 pathways is associated with disease severity (45), in the present study, cultures from  
410 patients with severe COVID-19 showed less activation or greater inhibition of pathways  
411 directly or indirectly related to inflammation compared to patients with moderate disease.  
412 It is very likely that the less inflamed profile of SEV group is due to the administration of

413 more potent glucocorticoids to these patients during hospitalization, prior to the collection  
414 of blood samples for the study (Table S1). These differences in the inflammatory profile  
415 of leukocytes of patients with severe and moderate COVID-19 previously treated with  
416 glucocorticoids are evident in the activation patterns of the *Coronavirus Pathogenesis*  
417 *Pathway* (Fig. S7).

418 Gene ontology (GO) functional annotation analysis of the 2,699 genes differentially  
419 expressed in response to B12 (Fig. S3) indicate that, in leukocytes of patients with  
420 moderate COVID-19, the vitamin stimulates the antiviral response induced by IFNs,  
421 regulates the exacerbated immune response induced by NF-kappaB and favors the  
422 inhibition of gene expression. Regarding the SEV group, the results indicate that in  
423 addition to suppressing viral infection, B12 induces inflammatory attenuation during  
424 severe COVID-19 and promotes activation of the adaptive immune response. These  
425 processes seem to have been regulated by epigenetic mechanisms, such as changes in  
426 chromatin status, gene silencing by miRNAs and alternative splicing reduction, which  
427 favored the reduction of gene transcription and translation.

428 Functional enrichment analysis also disclosed B12-modulated pathways in the MOD (Fig.  
429 5B and Fig. S5) and/or SEV (Fig. 5C and Fig. S6) groups. It should be noted that the  
430 effect of B12 was pleiotropic, that is, it regulates several metabolic or intracellular  
431 signaling pathways maladjusted by COVID-19 despite the glucocorticoid treatment that  
432 patients received during hospitalization. Most of the differentially regulated pathways in  
433 contrasts 1 and 2 (MOD vs. CTRL and SEV vs. CTRL, all at endpoint A) had their  
434 activation or inhibition attenuated by B12 (contrasts 3 and 4 - MOD at endpoint B vs.  
435 CTRL at endpoint A; and SEV at endpoint B vs. CTRL at endpoint A). In the cultures of  
436 patients with moderate COVID-19, B12 favored the overall reduction of inflammation and  
437 activation of the Th1-type antiviral response (Fig. 5B and Fig. S5). The few pathways  
438 predicted to be more activated in response to B12, such as *iNOS signaling*, regulate  
439 desirable mechanisms in the context of COVID-19. Through S-nitrosylation of cysteine  
440 residues of viral and host proteins, NO reduces the activity of viral proteases, inhibiting  
441 the fusion and replication of SARS-CoV-2 in host cells (46). Another example is the *NK*  
442 *cell signaling pathway*. During infection, the coronavirus depletes NK cells and impairs  
443 their antiviral effects while activating macrophages and other immune cells leading to the  
444 cytokine storm (47). Furthermore, the activation of *TREM1 signaling* and *Acute phase*  
445 *response signaling* pathways favors the production of IFN and activation of Th1 and Th17  
446 cells, which are fundamental components of the antiviral response (32, 48, 49). Activation  
447 of the *Th1 pathway* was also observed, which further reinforces the idea that B12  
448 stimulates the antiviral response. Regarding the few pathways where the result seems  
449 incongruous, when analyzing their topology, it is clear that their activation by B12 is  
450 potentially beneficial to the patient. For example, in the *Inflammasome pathway*, NLRP1  
451 was inhibited in the MOD group and B12 abrogates this inhibition (Fig. S8). This  
452 inflammasome interacts with double-stranded RNA (ds), being an important detector of  
453 dsRNA viruses such as SARS-CoV-2 (50). NLRP1 activity is regulated by anti-apoptotic  
454 proteins, BCL-2 and BCL-XL, which associate with the inflammasome and inhibit its  
455 activity (51). In this study, the genes encoding these two proteins were predicted to be  
456 upregulated in the leukocytes of patients in the MOD group, however B12 did not affect  
457 their expression. Thus, the regulatory effect of B12 on NLRP1 likely depends on other  
458 mediators. In the *Coronavirus Pathogenesis Pathway*, angiotensin 1-7 was predicted to be  
459 the activated in cultures treated with B12 (Fig. S7). It binds to MAS1 receptors leading to  
460 the activation of the anti-inflammatory branch of the renin-angiotensin pathway (52).

461 Moreover, in this same pathway, *Accumulation of lungs edema fluids* and *SARS CoV*  
462 *Replication* are predicted to be inhibited and *Type I interferon response* and *Adaptive*  
463 *Immunity* are predicted to be activated by B12. The vitamin caused even more evident  
464 attenuation of most of the inflammatory pathways activated by the disease in the cultures  
465 of patients with severe COVID-19 (Fig. 5C). In summary, the pro-inflammatory  
466 transcriptional profile observed in leukocytes from patients with moderate and severe  
467 COVID-19 was clearly attenuated by vitamin B12, with no adverse effects predicted from  
468 these results.

469 Besides regulating DNA methylation acting as a cofactor of the enzyme MS in the sulfur  
470 amino acid pathway, B12 also modulates different chromatin remodeling mechanisms that  
471 can affect gene expression. In the Krebs cycle, AdoCbl can induce the accumulation of  
472 succinate, an antagonist of histone and DNA demethylation reactions catalyzed by  
473 enzymes of the 2-oxoglutarate-dependent dioxygenase family, which may favor the  
474 activation or inhibition of gene expression (53). The antioxidant activity of B12 (54) can  
475 also indirectly influence the epigenetic landscape by regulating oxidative stress, which,  
476 depending on the context, can determine an accessible or non-accessible state of  
477 chromatin. Oxidative stress inactivates the histone-deacetylating enzyme HDAC2,  
478 contributing to the greater accessibility of transcriptional machinery to DNA. However,  
479 oxidative stress also activates signaling pathways that increase the expression of the  
480 enzyme DNMT1 that methylates DNA (53, 55). All these mechanisms may contribute to  
481 the down- (1,364) or upregulation (1,335) of the 2,699 genes in the leukocytes of patients  
482 in the MOD and/or SEV groups in response to B12 (Fig. 4).

483 It is noteworthy that when patients were recruited to this study, in July 2020, vaccines  
484 were not yet available and dexamethasone and other glucocorticoids had recently been  
485 integrated to the COVID-19 therapeutic protocol. At that time, there was no record of  
486 SARS-CoV-2 variants of interest/concern circulating in Brazil. Therefore, caution is  
487 needed when extrapolating the findings of this work to COVID-19 patients previously  
488 vaccinated or infected with SARS-CoV-2 variants.

489 In conclusion, vitamin B12 has a great potential as an adjuvant drug for alleviating  
490 inflammation in patients with moderate or severe COVID-19 in addition with the other  
491 established treatments. Beyond favorably regulating the expression of several  
492 inflammatory genes via methyl-dependent epigenetic mechanisms, B12 also acts as an  
493 antioxidant, has an excellent safety profile and is widely available at low cost. Therefore,  
494 phase II/III clinical trials are enthusiastically recommended. Furthermore, the results  
495 presented herein neither endorse the prophylactic use of B12 to prevent SARS-CoV-2  
496 infection, nor its therapeutic use in mild COVID-19.

## 497 **Materials and Methods**

### 498 **Patients**

499 This study was approved by the National Research Ethics Committee of Brazil (CONEP:  
500 32242620.6.0000.5091). All patients or their legal representatives were informed about  
501 the study and signed an informed consent form. Patients with moderate (MOD; n = 10)  
502 and severe (SEV; n = 16) forms of COVID-19, classified according to the World Health  
503 Organization's COVID-19 clinical severity scale (56), admitted to the Metropolitan  
504 Hospital Doctor Célio de Castro (HMDCC) and uninfected volunteers (CTRL; n = 6) with  
505 biological sex and age parity with the patients were recruited from 20th to 26th July 2020.

506 Participants had peripheral venous blood samples (10 mL) collected in sodium heparin, at  
507 8 a.m., and data from medical records of COVID-19 patients were analyzed. Uninfected  
508 volunteers included as controls in the study were tested for the presence of SARS-CoV2  
509 in the oropharynx and nasopharynx by RT-qPCR and for the presence of IgM and IgG blood  
510 antibodies against the virus by lateral flow immunochromatography (Wondfo, Guangzhou,  
511 China).

## 512 **Sample processing and production of whole blood cultures**

513 Each peripheral venous blood sample was divided into 4 aliquots of 1 mL and processed  
514 as follows: endpoint U) unprocessed blood; endpoints A and Z) added with 498  $\mu$ l of  
515 RPMI 1640 culture medium (Sigma-Aldrich, Saint Louis, Missouri) and 2  $\mu$ l of pH 5  
516 citrate-phosphate buffer excipient (Merck, Darmstadt, Germany); and endpoint B) added  
517 with 500  $\mu$ l of RPMI 1640 culture medium with cyanocobalamin (Merck) to a final  
518 concentration of 1 nM. Aliquots Z were immediately processed with no incubation.  
519 Aliquots A and B were incubated in 6-well plates for 24 hours at 37°C in a humidified  
520 atmosphere with 5% CO<sub>2</sub> (Fig. 6).

## 521 **Quantification of basal B12**

522 Basal levels of vitamin B12 were quantified of plasma aliquots from endpoint U using the  
523 standard chemiluminescence method by a commercial clinical laboratory (Hermes Pardini,  
524 Belo Horizonte, Brazil).

## 525 **Real time quantitative PCR (RT-qPCR)**

526 Total RNA was obtained from 1.5 mL of aliquots from endpoints Z, A and B, using the  
527 QIAamp RNA Blood kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed  
528 from 2  $\mu$ g of total RNA using the High-Capacity cDNA Reverse Transcription Kit  
529 (ThermoFisher, Waltham, Massachusetts), both according to manufacturers' protocols. All  
530 samples had their RNA quantified by fluorometry using the Qubit RNA HS Assay Kit  
531 (#Q32852) and the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA).

532 Specific primers (Table S2) were used to detect the human mRNAs of *CCL1* (C-C Motif  
533 Chemokine Ligand 1) (NM\_002981.2), *CCL2* (C-C Motif Chemokine Ligand 2)  
534 (NM\_002982.4), *CCL3* (C-C Motif Chemokine Ligand 3 ) (NM\_002983.3), *CXCL9* (C-  
535 X-C motif chemokine ligand 9) (NM\_002416.3), *IL1B* (Interleukin 1 beta)  
536 (NM\_000576.3), *IL6* (Interleukin 6) (NM\_000600.5), *IL17A* (Interleukin 17A)  
537 (NM\_002190.3), *TNF* (Tumor Necrosis Factor) (NM\_000594.4), *HAVCR2* (Hepatitis A  
538 Virus Cellular Receptor 2) (NM\_032782.5), *CD4* (CD4) (NM\_000616.5) and *CD8A*  
539 (CD8a) (NM\_001768.7). For all RT-qPCR assays, target gene expression levels were  
540 normalized by 18S ribosomal RNA (18S ribosomal N1) (NR\_145820.1) levels. qPCR  
541 reactions were performed with Fast SYBR Green Master Mix (ThermoFisher) plus 10 ng  
542 of cDNA in a final volume of 10  $\mu$ L. Thermal cycling and fluorescence detection were  
543 performed using the ViiA 7 real-time PCR system (ThermoFisher) according to the  
544 manufacturer's recommendations. The relative expression of target genes was calculated  
545 using the  $2e^{-\Delta Ct}$  method (57).

## 546 **ELISA**



547 To access the intracellular protein levels of CCL3 and IL-1B, cell precipitates of aliquots  
548 from endpoints A and B were homogenized in 1X PBS, diluted in Milli-Q H<sub>2</sub>O 1:2 and the  
549 cells disrupted by mechanical lysis in the TissueLyser (Qiagen) for 2 minutes at 30 Hz and  
550 three freeze/thaw cycles (20° to -20°C).

551 ELISA kits (R&D Systems, Abingdon, UK) for CCL3 (#DY270) and IL-1B (#DY201)  
552 were used and assays performed as per the manufacturer's instructions. The absorbance  
553 was read at 450 nm with the Multiskan GO spectrophotometer (ThermoFisher). Standard  
554 curves were constructed from 0.1 to 100 pg/mL and cytokine concentrations were  
555 calculated with SkanIt Software 4.1 for Microplate Readers RE, version 4.1.0.43  
556 (ThermoFisher).

### 557 **High performance liquid chromatography (HPLC)**

558 For measurements of HCY, cysteine (CYS), GSH, SAM and S-adenosyl-L-homocysteine  
559 (SAH), cell precipitates from aliquots A and B were diluted in Milli-q H<sub>2</sub>O 1:2 and the  
560 cells disrupted by three freeze/thaw cycles (20° to -20°C). Metabolites were quantified by  
561 high performance liquid chromatography (HPLC) on the Shimadzu Prominence-i LC-  
562 2030C 3D Plus (GMI, Ramsey, Minnesota) according to Pfeiffer *et al.* (58) with some  
563 modifications. HCY, CYS and GSH were quantified using a C18 Luna column (5 mm x  
564 150 mm x 4.6 mm) and mobile phase composed of 0.06 M sodium acetate, 0.5% acetic  
565 acid and 2% methanol (pH 4.7 adjusted with acetic acid). The flow rate was 1.1 mL ×  
566 min<sup>-1</sup> and the retention time was 4.1 min for CYS, 5.9 min for HCY and 10.3 min for  
567 GSH. SAM and SAH were quantified using a method adapted from Blaise *et al.* (59).  
568 HClO<sub>4</sub> was added to the homogenized cell precipitate for protein precipitation. The  
569 supernatant was injected into a C18 LiChroCart column (5 mm x 250 mm x 4 mm) and the  
570 mobile phase applied at a flow rate of 1 mL × min<sup>-1</sup>, consisting of 50 mM sodium  
571 phosphate (pH 2.8), 10 mM heptane sulfonate and 10% acetonitrile. Retention time was  
572 8.7 min for SAH and 13.6 min for SAM. All metabolites were detected by UV absorption  
573 at a wavelength of 254 nm.

574 The intracellular concentrations of the metabolites were normalized by hemoglobin  
575 concentrations, obtained by colorimetry (Agabe hemoglobinometer) adding 10 µL of the  
576 sample in an ampoule (Hemoglobin AP; Labtest).

### 577 **RNA-Seq**

578 Total RNA from three samples from each group (MOD, SEV and CTRL) at endpoints A  
579 and B (total 18 samples) was used to construct cDNA libraries with the TruSeq Stranded  
580 mRNA kit (Illumina, San Diego, CA) and the indexed fragments were sequenced on the  
581 NGS NextSeq 500 (Illumina) with the NextSeq 500/550 High Output 2x75 cycles kit  
582 (Illumina). All sequenced samples were quality assessed by capillary electrophoresis with  
583 the Agilent RNA 2100 Nano kit (Bioanalyzer, Santa Clara, CA).

### 584 **Bisulfite Sequencing PCR (BSP)**

585 Genomic DNA from five samples from each group (MOD, SEV and CTRL) at endpoints  
586 A and B (total of 30 samples) was extracted with QIAamp DNA Blood Mini kit (Qiagen)  
587 according to the manufacturer's instructions. DNA was subjected to bisulfite conversion  
588 using EpiTect Bisulfite kit (Qiagen). The region -107pb to +164pb of *CCL3*

589 (GRCh38/hg38 Chr17: 36,090,276-36,090,005) that cover the promoter region and  
590 proximal portion of the first exon of the gene was PCR amplified with GoTaq DNA  
591 Polymerase (Promega, Madison, Wisconsin) from bisulfite-converted DNA using the  
592 primer listed in Table S3. DNA libraries were purified with AMPure XP beads (Beckman  
593 Coulter, Indianapolis, IN), indexed with Nextera XT DNA Library Preparation Kit  
594 (Illumina) and sequenced on the NGS MiSeq (Illumina) with MiSeq Reagent kit v2 (300  
595 cycles) (Illumina). All sequenced samples had their quality evaluated by capillary  
596 electrophoresis with Agilent High Sensitivity DNA Kit (Bioanalyzer).

## 597 **Bioinformatics analysis**

598 Hierarchical clustering analyzes were performed using the GenePattern software (60)  
599 using Pearson correlation as a comparison method and average linkage as a linkage  
600 method.

601 Raw RNA-Seq reads were pre-processed with Trimmomatic software (61) to remove  
602 adapters, poor quality bases or very short reads (less than 36nt). The filtered reads were  
603 mapped to the *Homo sapiens* reference transcriptome (Gencode, release 36) and the total  
604 number of reads mapped per transcript and the number of transcripts per million (TPM)  
605 were calculated with Salmon software (62). Transcript counts and abundances were  
606 summarized at the gene level using the *summarizeToGene* function of the R Tximeta  
607 package (63). Contrast analyses between MOD vs. CTRL at endpoint A (contrast 1), SEV  
608 vs. CTRL at endpoint A (contrast 2), MOD at endpoint B vs. CTRL at endpoint A  
609 (contrast 3), SEV at endpoint B vs. CTRL at endpoint A (contrast 4), and CTRL at  
610 endpoint A vs. CTRL at endpoint B (contrast 5) were performed with the R DESeq2  
611 package (64). Genes with Fold Change greater than 1.5 and *P* value less than 0.05  
612 obtained in the False Discovery Rate (FDR) analysis were considered differentially  
613 expressed (DEG) and submitted to functional enrichment analysis with Ingenuity  
614 Pathways Analysis software (IPA, Qiagen) using default parameters. Pathways with  $-\log P$   
615 value greater than 2 were considered to be differentially activated or inhibited. To assess  
616 the differences between moderate and severe COVID-19 (contrast 1 vs. contrast 2) and the  
617 effect of B12 (contrast 3 vs. contrast 1, and contrast 4 vs. contrast 2), only differentially  
618 regulated pathways that had more than 20% difference in Z-scores between the two  
619 contrasts were selected. Genes differentially expressed in response to B12 were submitted  
620 to gene ontology (GO) functional annotation analysis using *DAVID Bioinformatics*  
621 software (65) with default parameters. The GO terms for Biological Processes with  $P <$   
622 0.05 were summarized according to their ontology with REVIGO (66).

623 For BSP analysis, raw reads were preprocessed with Trimmomatic (61) as described  
624 above, except that the minimum read size threshold was 50nt. Since no single nucleotide  
625 polymorphisms (SNPs) that influence CpGs dinucleotides, which can generate or abolish a  
626 CpG site, were identified in the analyzed region, the reference sequence chr17:  
627 36,090,276-36,090,005 of the genome of *Homo sapiens* (Gencode, release 38), was used  
628 to map the filtered reads. The mapping, as well as the calculation of the percentages of  
629 methylation of cytosines in CG context were performed with the Bismark software (67).  
630 *CCL3* functional annotations, such as transcription start site (TSS), SNPs and predicted  
631 transcription factor binding sites (TFBS) were identified with the UCSC Genome Browser  
632 (68).

## 633 **Statistical analysis**

634 Statistical analyses were performed using GraphPad Prism software (version 8.0.2)  
635 (GraphPad Software Inc., Irvine, CA). Data distribution was analyzed using Anderson-  
636 Darling, D'Agostino & Person, Shapiro-Wilk and Kolmogorov-Smirnov tests. For  
637 comparisons between three or more groups, one-way analysis of variance (ANOVA) or  
638 Kruskal-Wallis tests were used, followed by the multiple comparison tests of Tukey or  
639 Dunn, for parametric or non-parametric data, respectively. Two-way ANOVA followed by  
640 Tukey's multiple comparison test was used for comparisons involving the effect of two  
641 factors on a dependent variable in three or more groups. Two-tailed Student's t, paired  
642 Student's t, Mann-Whitney or Wilcoxon tests were used for comparisons between two  
643 groups, according to the experimental design and data distribution. Correlations were  
644 tested using Pearson or Spearman tests according to data distribution. Outliers identified  
645 by ROUT test ( $Q = 1\%$ ) were excluded. Data were expressed as median  $\pm$  interquartile  
646 (nonparametric) or mean  $\pm$  standard deviation (parametric). Differences were considered  
647 statistically significant when  $P < 0,05$ .

648 For BSB analysis, the means of methylation percentages of each CG locus of MOD, SEV  
649 and CTRL groups at endpoints A and B were compared using two-tailed Wilcoxon test  
650 and only those that presented a statistically significant difference were considered.  
651 Correlation between methylation levels of differentially methylated loci (DML) at  
652 endpoint B compared to endpoint A and *CCL3* gene expression levels from the same  
653 cultures were tested with Pearson or Spearman correlation for parametric or nonparametric  
654 distributions, respectively.

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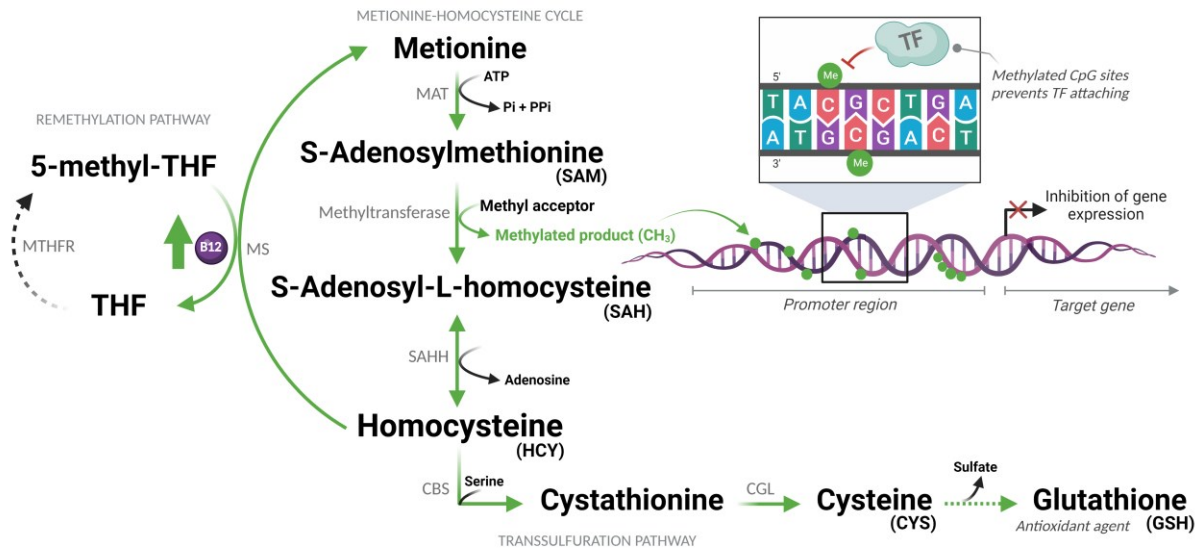
872 **Author contributions:** LC - performed the experiments, contributed to experimental  
873 design, analyzed data and wrote the manuscript; VCS and VD'A – performed the HPLC  
874 assays, analyzed data and reviewed the manuscript; CC, BP and SF – recruited patients,  
875 collected blood samples and clinical and sociodemographic data from patients records;  
876 MO - contributed to data analysis and reviewed the manuscript; AS - made substantial  
877 contributions to the NGS experiments; GF - contributed to the experimental design and  
878 data analysis and reviewed the manuscript; RC - designed and oversaw the study,  
879 analyzed data and wrote the manuscript. All authors read and approved the final  
880 manuscript.

881 **Competing interests:** The authors declare that they have no competing interests.

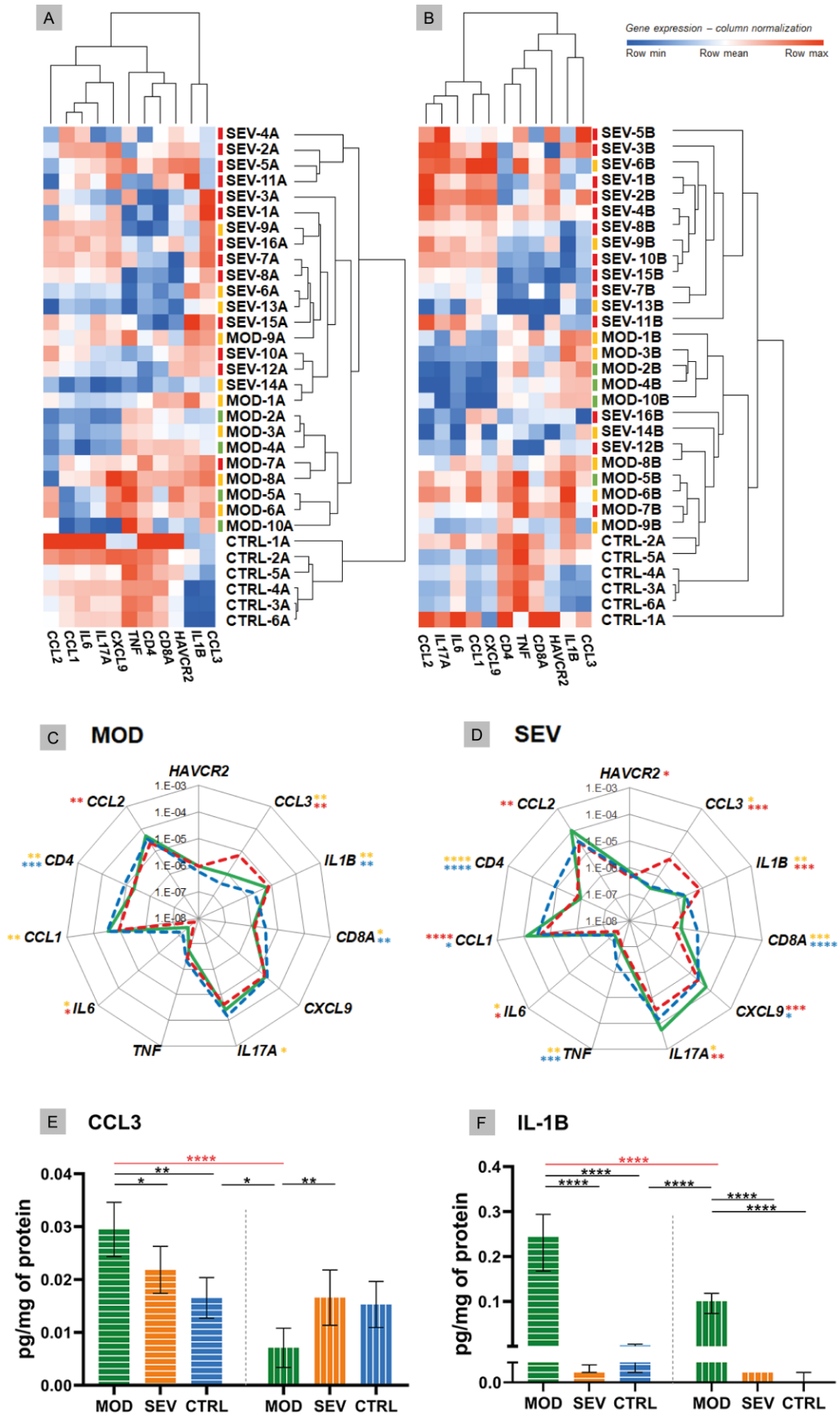
882 **Data and materials availability:** The dataset supporting the conclusions of this article is  
883 available in the SRA repository, [<https://www.ncbi.nlm.nih.gov/sra/PRJNA862565>]. Other  
884 data needed to evaluate the conclusions in the paper are present in the Supplementary  
885 Materials.

## 886 **Figures and Tables**



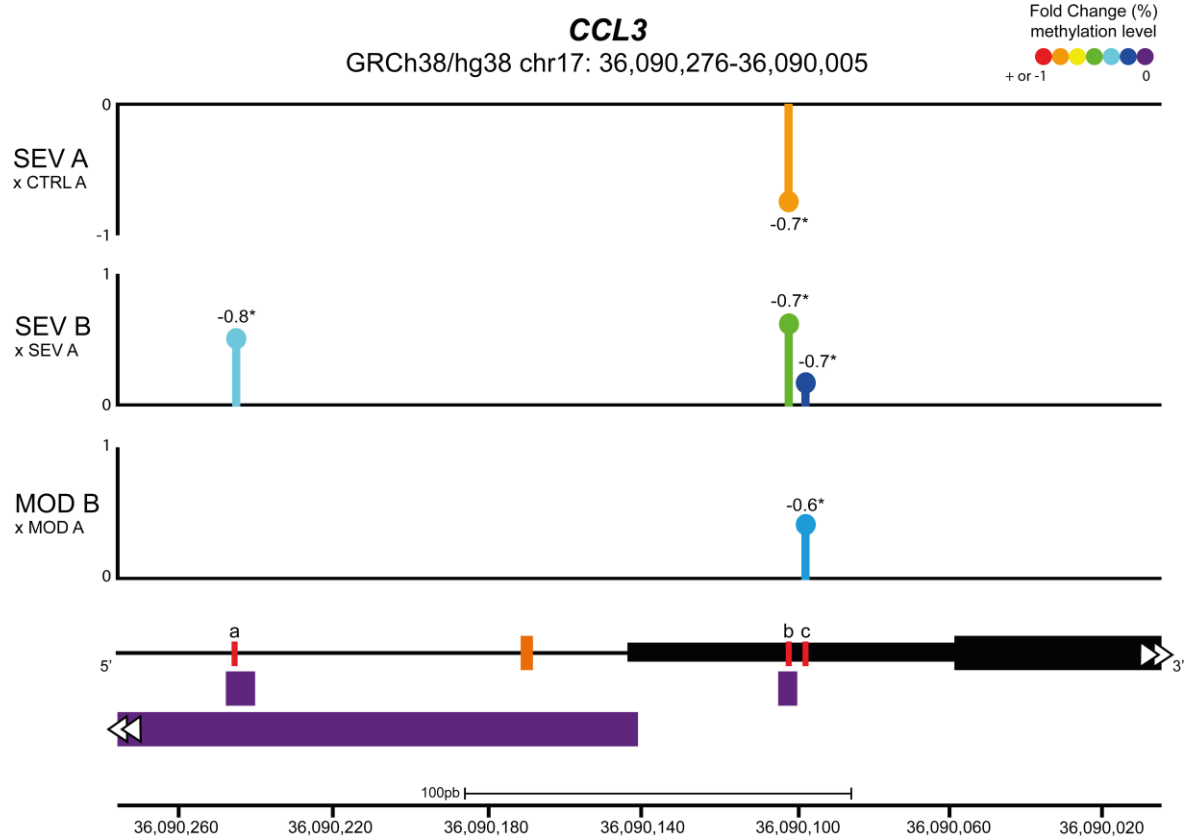


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 888 **Fig. 1. Sulfur amino acid pathway.** Methionine is converted to S-adenosylmethionine  
 889 (SAM), which is the methyl donor for numerous reactions. Upon losing its methyl  
 890 group, SAM becomes S-adenosyl-L-homocysteine (SAH), which is then converted  
 891 to homocysteine (HCY). This is then converted back to methionine or enters the  
 892 transsulfuration pathway to form other sulfur-containing amino acids.  
 893 Abbreviations: MAT = Methionine adenosyltransferase; ATP = Adenosine  
 894 triphosphate; Pi = Phosphate (inorganic); PPi = Pyrophosphate; THF =  
 895 Tetrahydrofolate; MTHFR = Methylene tetrahydrofolate reductase; MS =  
 896 Methionine synthase; B12 = Vitamin B12; CH<sub>3</sub>/Me = Methyl group; TF =  
 897 Transcription factors; SAHH = Adenosylhomocysteinase; CBS = Cystathionine  
 898 beta-synthase; CGL = Cystathionine gamma-lyase. (Created with BioRender.com)



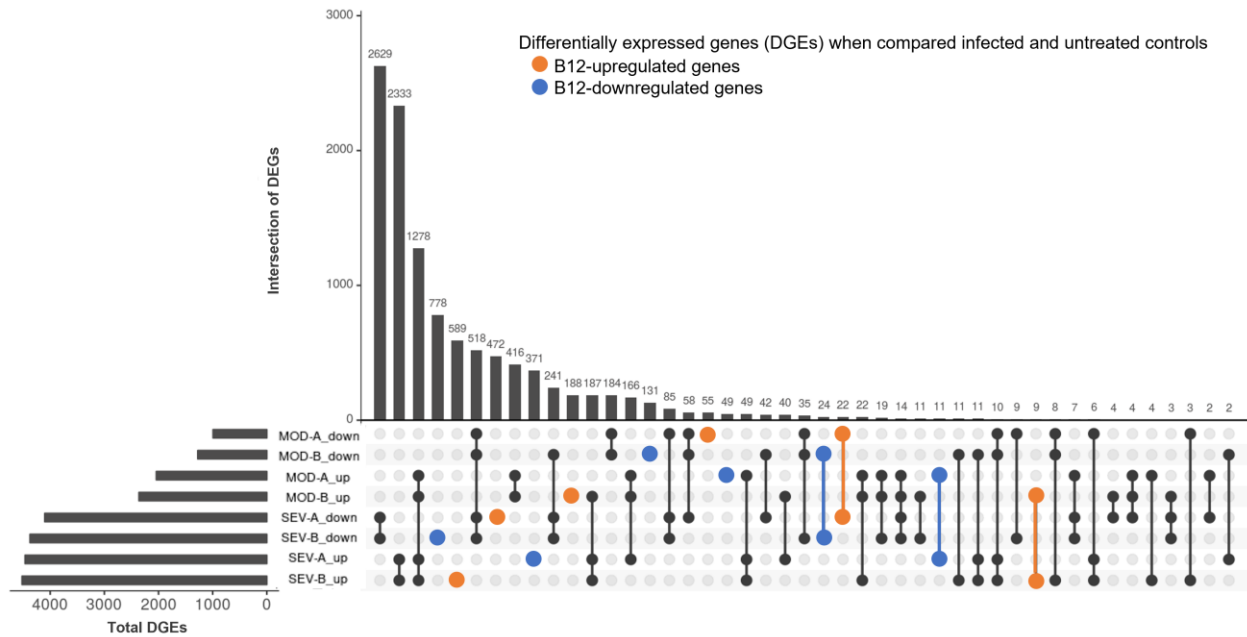
**Fig. 2. Vitamin B12 favorably modulated critical inflammatory mediators. A and B:**

Dendrogram and heatmap depicting the hierarchical clustering and expression levels of COVID-19 hyperinflammation-related genes panel in whole blood cultures of untreated MOD and SEV patients (endpoint A) (Panel A) or treated with B12 (endpoint B) (Panel B) and untreated non-infected controls (endpoint A). Colored dashes next to the sample identification correspond to the patient's outcome. Green dashes: patients discharged from hospital one day after sample collection; Yellow lines: patients discharged from hospital two or more days after sample collection; Red lines: patients died. **C and D:** Radar charts depicting the expression levels of COVID-19 hyperinflammation-related genes panel in whole blood cultures. The filled green lines correspond to gene expression values of infected groups (MOD, Panel C or SEV, Panel D) at endpoint B. Dashed red lines correspond to gene expression values of infected groups at endpoint A. Dashed blue lines correspond to gene expression values control group at endpoint A. The gene expression values ( $2e^{-\Delta Ct}$ ) of the groups were compared pairwise using two-tailed Student's t, paired Student's t, Mann-Whitney and Wilcoxon tests according to experimental design and data distribution. Values were expressed as medians. N sample = MOD (10) and SEV (16) for each endpoint (A or B), CTRL (6). Yellow asterisks = Infected A versus controls A; Red asterisks = Infected B versus Infected A; Blue asterisks = Infected B versus controls A. **E and F:** Bar charts depicting intracellular protein levels of CCL3 (Panel E) and IL-1B (Panel F). Filled bars with horizontal lines = endpoint A. Filled bars with vertical lines = endpoint B. Data were compared using two-way Analysis of Variance (ANOVA) test followed by Tukey's multiple comparison test. Values were represented as mean  $\pm$  standard deviation. N sample = MOD (7), SEV (8), CTRL (4) for each endpoint (A or B). Red asterisks and red lines denote the effect of B12 on cultures of patients with moderate COVID-19. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ . Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19; CTRL = non-infected controls. Suffixes: A = endpoint A (samples added to culture medium with excipient and incubated for 24h); B = endpoint B (samples added to culture medium with B12 and incubated for 24h).



**Fig. 3. Vitamin B12 increased methylation levels of CpGs in regulatory regions of *CCL3*.** Lollipop plot depicting the percent fold change of methylation levels of CpG sites in the promoter region and proximal portion of the first exon of the *CCL3* gene. Values next to lollipops represent correlation coefficients (Pearson or Spearman) with statistical significance (\*  $P < 0.05$ ) between methylation levels in each CpG position and gene expression values. The % fold change of methylation levels of CpG positions were compared pairwise using two-tailed Mann-Whitney. N sample = MOD (5) and SEV (5) for each endpoint (A or B), CTRL (5). In the schematic representation of the analyzed gene region, the black line represents the *CCL3* promoter region and the black blocks delimit the 5' UTR region (thin block) and proximal portion of the first exon of *CCL3* (thick block). The orange dash indicates the transcription start site (TSS) of *CCL3* and the purple blocks indicate transcription factor binding sites (TFBS). Red lines correspond to differentially methylated CpG sites, coordinates (GRCh38/hg38): a = chr17:36,090,246; b = chr17:36,090,102; c = chr17:36,090,097. Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19; CTRL = untreated non-infected controls. Suffixes: A = endpoint A (samples added to culture medium with excipient and incubated for 24h); B = endpoint B (samples added to culture medium with B12 and incubated for 24h).





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**Fig. 4. Differently expressed genes (DEGs).** DEGs in infected patients (at endpoint A or B) when compared to untreated non-infected controls (endpoint A). Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19. Suffixes: A = endpoint A (samples added to culture medium with excipient and incubated for 24h); B = endpoint B (samples added to culture medium with B12 and incubated for 24h); up = upregulated; down = downregulated.



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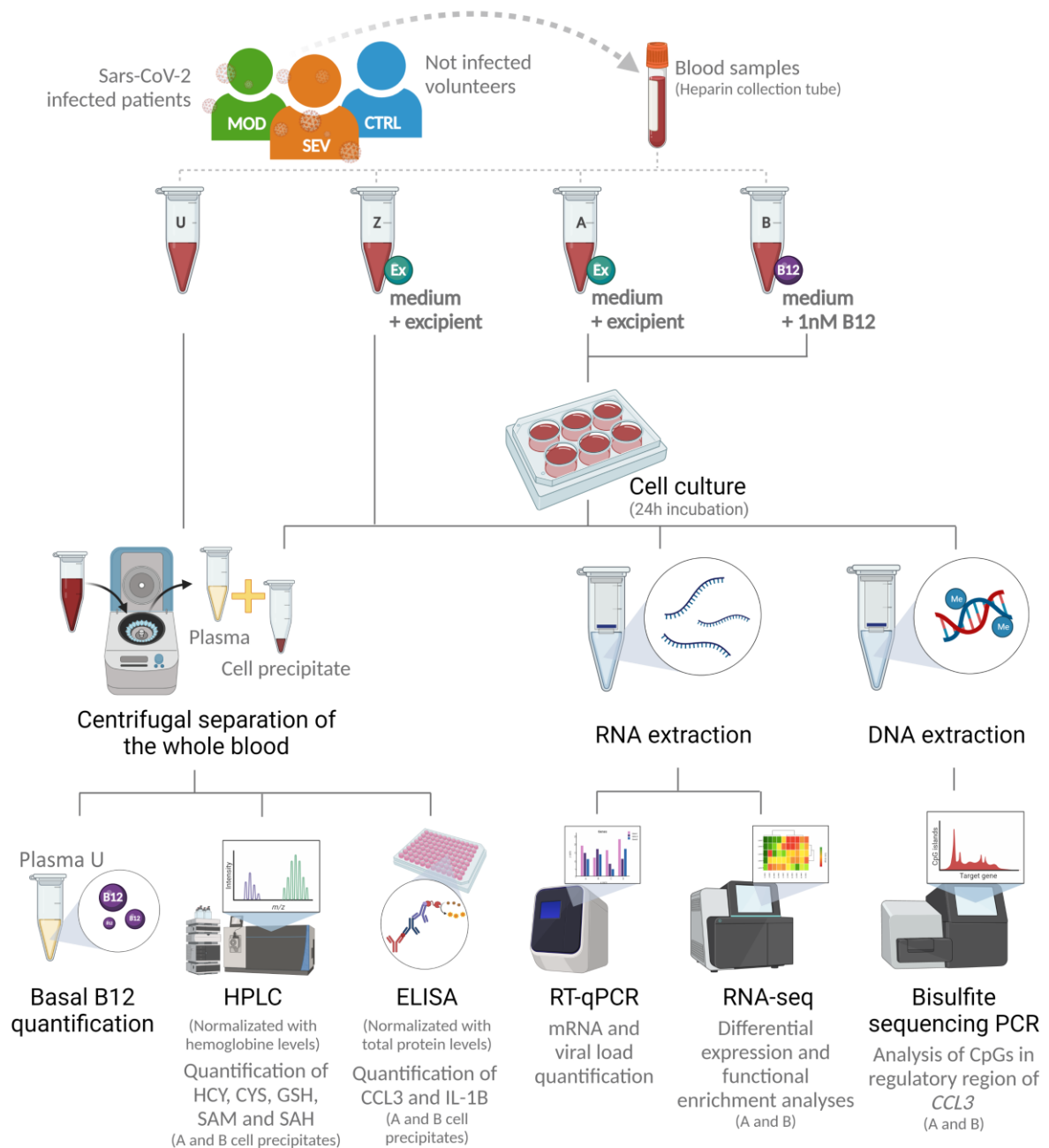
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**Fig. 5. Canonical Pathways affected by COVID-19 and B12.** Patients with moderate and severe COVID-19 previously treated with glucocorticoids had distinct global gene expression patterns (A) and vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients with moderate (B) or severe (C) forms of the disease. Green bars = contrast 1 (MOD vs. CTRL at endpoint A); Orange bars = contrast 2 (SEV vs. CTRL at endpoint A); Pink bars = contrast 3 (MOD at endpoint B vs. CTRL at endpoint A); Purple bars = contrast 4 (SEV at endpoint B vs. CTRL at endpoint A); Faded bars indicate pathways with  $P$  value less than 0.05 (statistically non-significant). Abbreviations; SEV = severe COVID-19; MOD = moderate COVID-19. Suffixes: A = endpoint A (Samples added to culture medium

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with excipient and incubated for 24h); B = endpoint B (Samples added to culture medium with B12 and incubated for 24h).



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**Fig. 6. Research design.** Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19; CTRL = non-infected controls; U = endpoint U (unprocessed blood); Z = endpoint Z (samples immediately processed after addition of culture medium with excipient); A = endpoint A (Samples added to culture medium with excipient and incubated for 24h); B = endpoint B (Samples added to culture medium with B12 and incubated for 24h); Ex = Excipient; B12 = Vitamin B12; HPLC = High Performance Liquid Chromatography; HCY = Homocysteine; CYS = Cysteine; GSH = Glutathione; SAM = S-adenosylmethionine; SAH = S-adenosyl-L-homocysteine; PCR = Polymerase Chain Reaction; RT-qPCR = Real-time quantitative PCR. (Created with BioRender.com).

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**Table 1. Vitamin B12 increases the flow of the sulfur amino acid pathway.**

Metabolites	Group	Intracellular concentrations (μmol/g Hb) (mean [SD] or median [IQ])			P value (AxB)		
		MOD	SEV	CTRL	MOD	SEV	CTRL
HCY	A	0.04096 (0.007656)	0.04312 (0.01524)	0.054 (0.05133- 0.0638)	0.0037 (**)	<0.0001 (****)	0.0313 (*)
	B	0.0561 (0.01653)	0.05762 (0.01784)	0.067 (0.06225- 0.0985)			
CYS	A	0.5869 (0.2176)	0.6795 (0.2867)	0.5635 (0.04167)	0.0029 (**)	0.0007 (***)	0.1253
	B	0.7326 (0.2542)	0.9097 (0.3812)	0.8457 (0.2764)			
GSH	A	12.52 (2.123)	10.6 (4.292)	15.35 (2.735)	0.0002 (***)	<0.0001 (****)	0.0612
	B	15.1 (2.877)	14.94 (6.213)	16.24 (2.878)			
SAM	A	0.002436 (0.000673)	0.003405 (0.00225- 0.00466)	0.0018 (0.001418- 0.001815)	0.0010 (**)	0.4954	0.0938
	B	0.001789 (0.0003617)	0.002673 (0.002365- 0.004239)	0.002081 (0.001825- 0.00227)			
SAH	A	0.0002819 (0.00009389)	0.0001972 (0.00008667)	0.0001817 (0.00003608)	0.0722	0.0015 (**)	0.0087 (**)
	B	0.0002293 (0.00004081)	0.0003267 (0.0001541)	0.0002277 (0.00004052)			
SAM:SAH	A	9.093 (2.571)	16.44 (12.09)	9.632 (3.123)	0.1511	0.0021 (**)	0.7341
	B	8.041 (2.28)	8.058 (4.557)	9.243 (2.087)			

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The parametric data were compared using two-tailed paired Student's T test and represented as ± standard deviation. The nonparametric data were compared using two-tailed Wilcoxon paired test and represented as ± interquartile range. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*;  $P < 0.0001$ . Abbreviations: SEV = severe COVID-19; MOD = moderate COVID-19; CTRL = non-infected controls; A = endpoint A (samples added to culture medium with excipient and incubated for 24h); B = endpoint B (samples added to culture medium with B12 and incubated for 24h).

## Supplementary Materials

989 Fig. S1. Vitamin B12 basal levels  
990 Fig. S2. Samples added to culture medium with excipient and incubated for 24h have their  
991 transcriptional signatures preserved  
992 Fig. S3. Biological Processes affected by B12  
993 Fig. S4. Patients with moderate and severe COVID-19 previously treated with  
994 glucocorticoids had distinct global gene expression patterns  
995 Fig. S5. Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients  
996 with moderate COVID-19  
997 Fig. S6. Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients  
998 with moderate COVID-19  
999 Fig. S7. Coronavirus Pathogenesis Pathway (Contrast 1, 2, 3 and 4)  
1000 Fig. S8. Inflammasome Pathway (Contrast 1 and 3)  
1001 Table S1. Patients' data (separate file)  
1002 Table S2. COVID-19 hyperinflammation-related genes panel primers  
1003 Table S3. BSP primers