1	Title						
2		Vitamin B12 attenuates leukocyte inflammatory signature in COVID-19 via methyl-					
3		dependent changes in epigenetic marks					
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5	Short	Title					
6		Vitamin B12 attenuates inflammation in COVID-19					
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26	Abstr	act					
27		been shown that vitamin P12 downrogulates some inflammatory genes via methyl					
20 20		dependent enigenetic mechanisms. In this work, whole blood cultures from moderate or					
30		severe COVID-19 patients were used to assess the potential of B12 as adjuvant drug. The					
31		vitamin normalized the expression of a panel of inflammatory genes still dysregulated in					
32		the leukocytes despite glucocorticoid therapy during hospitalization. B12 also increased					
33		the flux of the sulfur amino acid pathway, raising the bioavailability of methyl.					
34		Accordingly, B12-induced downregulation of CCL3 strongly and negatively correlated					
35		with the hypermethylation of CpGs in its regulatory regions. Transcriptome analysis					
36		revealed that B12 attenuates the effects of COVID-19 on most inflammation-related					
51		painways affected by the disease. As far as we are aware, this is the first study to					
30 30		regulates central components of COVID-19 physionathology					
39 40		regulates central components of CO v ID-17 physiopathology.					
41	Tease	r					
42		B12 has great potential as an adjuvant drug for alleviating inflammation in COVID-19.					
43							

44 Introduction

- Coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, has been reported, as of 45 August 2022, in more than 583 million confirmed cases and has led to more than 6.4 46 million deaths worldwide (1), although recent studies estimate a much higher death toll 47 (2). Since the end of 2020, large-scale population vaccination has drastically reduced the 48 mortality rate and the burden on health systems, but factors such as inequality in access to 49 immunizations, rapid waning neutralizing antibody titers induced either by vaccines or by 50 51 exposure to SARS -CoV-2 and the emergence of new genotypic variants of the virus have contributed to successive new waves of the pandemic with more dramatic impacts on 52 unvaccinated individuals or those with poor response to vaccines. In this context, efforts 53 have been made in the search for treatments for COVID-19, such as the repositioning of 54 chemicals and monoclonal antibodies that act as anti-inflammatory agents, and the 55 development of new antiviral small molecules that target SARS-CoV-2 replication and 56 57 antibodies that bind to the viral spike protein blocking virus entry into cells (3). Unfortunately, most of these drugs have limited effectiveness or are very expensive, 58 making them unsuitable to be used on a global scale. Therefore, there is still an urgent 59 need for new efficient treatments with safe, inexpensive, and widely available drugs (4). 60
- While most COVID-19 patients will experience mild symptoms, some will develop severe 61 acute respiratory distress syndrome, systemic inflammation, multiple organ failure, and 62 other more serious complications that can lead to death. Several components of the host 63 immune system are dramatically altered during SARS-CoV-2 infection and the extent of 64 immune dysregulation is related to the worsening of COVID-19 and progression to death. 65 (5). Patients with COVID-19 exhibit inflammatory signatures defined by low levels of 66 type I and III interferons (IFNs) and elevated levels of some cytokines and chemokines. 67 These two branches of the innate immune response contribute to the clinical evolution of 68 the patients (6). As the disease worsens, increased levels of inflammatory cytokines and 69 chemokines are observed, which can lead to depletion and exhaustion of T cell 70 populations resulting in a significant elevation of the neutrophil/lymphocyte ratio (NLR) 71 (7-9). The increase in neutrophil population (7, 8) with transcriptional signatures related to 72 its activated state (10) also contributes to the elevation of NLR in COVID-19. 73
- DNA methylation is an epigenetic mechanism that, in mammals, occurs mainly in 74 dinucleotides of a cytosine followed by a guanine (CpG) and can affect the accessibility of 75 transcription factors (TF) and RNA polymerases to DNA, thus modulating gene 76 77 expression. (11). In general, DNA hypomethylation in gene promoters is associated with the activated state of their expression (12). It is already known that promoters of genes 78 encoding cytokines are rapidly demethylated (~ 6 hours) after T cell activation (13) and 79 that several genes encoding key cytokines and chemokines have increased systemic 80 expression in patients with COVID-19 (14) are regulated by the methylation of CpGs in 81 their promoters or enhancers (15-18). The ability of RNA viruses to hijack the epigenome 82 of host immune cells in order to evade antiviral defense is widely acknowledged (19, 20). 83 In early 2021, Corley and colleagues reported that the DNA methylation signature of 84 peripheral blood mononuclear cells from critically ill COVID-19 patients is characterized 85 by hypermethylation of genes related to the IFN-mediated antiviral response and 86 hypomethylation of inflammatory genes (17). This finding, in light of previous knowledge 87 about the epigenetic regulation of genes involved in the inflammatory storm of COVID-88 19, points to the therapeutic potential of drugs capable of modulating the epigenetic 89 90 landscape as a strategy for the control of exacerbated inflammation resulting from SARS-CoV-2 infection. 91

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

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

113 **Results**

114 **Patients and healthy volunteers**

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

All patients were on glucocorticoid treatment (dexamethasone: 25 patients; prednisone: 1 125 patient) but those with severe COVID-19 most often received combinations of two or 126 three drugs of this category, namely dexamethasone, hydrocortisone and beclomethasone 127 for an average of 11 days before their blood samples were taken, with no differences 128 between MOD and SEV regarding the duration of this treatment. Among the cytochemical 129 130 parameters evaluated, differences were found between MOD and SEV groups for blood glucose (mg/dL; means MOD = 131.7 and SEV = 196.7; P = 0.0111), total leukocytes 131 (cells per mm³; means MOD = 9,650 and SEV = 16,355; P = 0.0041), percentage of 132 lymphocytes (%; medians: MOD = 14.85 and SEV = 6.95; P = 0.0048), percentage of 133 neutrophils (%; medians: MOD = 73.85 and SEV = 86.1; P = 0.0139) and for the NLR 134 (means MOD = 5.2 and SEV = 13.1; P = 0.0038). The increase in the percentage of 135 neutrophils in SEV patients coincides with the occurrence of more frequent bacterial 136 coinfections in this group (20% for MOD and 75% for SEV; P = 0.0138). Arterial blood 137

138	gas analysis of MOD and SEV patients revealed significant differences in pH (medians
139	MOD = 7.435 and SEV = 7.375; <i>P</i> = 0.0223), O ₂ pressure (mmHg; medians MOD = 58.15
140	and SEV = 80.10 ; $P = 0.0028$), CO ₂ tension (mmol/L; medians MOD = 20.30 and SEV =
141	23.25; $P = 0.0309$) and O ₂ 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

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

173 Vitamin B12 favorably modulated critical inflammatory mediators

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

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 at187endpoint A, did not have their mRNA levels significantly altered by B12 in either of the188two patient groups. B12 also did not affect *CD4* and *CD8A* mRNA levels in MOD or189SEV, but slightly increased those of *HAVCR2* in SEV. However, this increase was not190enough to achieve a statistically significant difference compared to CTRL at endpoint A.

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

197 Vitamin B12 reduced intracellular protein levels of critical inflammatory mediators

198MOD cultures treated with B12 had reduced intracellular protein levels of CCL3 and IL-1991B (P < 0.0001), both of which were elevated at endpoint A compared to CTRL (P =2000.0011 and P < 0.0001, respectively) (Fig. 2, E and F). Interestingly, B12 did not affect201the intracellular protein levels of IL-1B in the cultures of the SEV group, and, regardless202of the treatment, the intracellular protein levels of IL-1B were very low, close to or below203the detection limit of the method (Fig. 2F).

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

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

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

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

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 235 group, 3,034 DEGs (2,041 upregulated and 993 downregulated) were found in contrast 1 236 and 3,636 DEGs (2,361 upregulated and 1,275 downregulated) in contrast 3. In cultures of 237 the SEV group, 8,565 DEGs (4,464 upregulated, 4,101 downregulated) were found in 238 contrast 2 and 8,894 DEGs (4,520 upregulated and 4,374 downregulated) in contrast 4. 239 Among the DEGs identified, 2,699 had decreased (1,364) or increased (1,335) expression 240 after treatment with B12 (Fig. 4). In the leukocytes of patients in MOD group, B12-241 upregulated genes had GO annotations related to phagocytosis, regulation of miRNA 242 transcription, response to virus and monocyte extravasation. In the same group, B12-243 downregulated genes had GO annotations related to NF-kappaB signaling, T-cell receptor 244 signaling pathway and negative regulation of histone H3-K9 trimethylation (Fig. S3). 245 Regarding the SEV group, B12-upregulated genes had GO annotations related to 246 cytoplasmic translation (tRNA and rRNA processing), adaptive immune response, ncRNA 247 processing, miRNA-mediated gene silencing, proteasome-mediated ubiquitin-dependent 248 protein catabolic process, cobalamin transport, and negative regulation of transcription 249 by RNA polymerase II (Fig. S3). B12-downregulated genes in this group were related to 250 positive regulation of transcription by RNA polymerase II, processing and transport of 251 mRNA, mRNA splicing, cellular response to DNA damage stimulus, activation of innate 252 immune response, positive regulation of IL-6 and IL-2 production, T-cell differentiation in 253 thymus, histone modification, viral transcription and DNA conformational change. 254 Finally, no DEGs were found in contrast 5, which reinforces the safety profile of B12. 255

Functional enrichment analysis of DEGs identified in the contrasts above mentioned 256 revealed 90 metabolic and signaling pathways differentially regulated in the groups MOD 257 or SEV at endpoint A directly or indirectly related to the inflammatory response in blood, 258 although patients had received glucocorticoid treatment for approximately 11 days prior to 259 collection of samples to the study (Fig. 5A and Fig. S4). In the cultures treated with B12, 260 45 and 74 out of these 90 pathways were still differentially regulated in MOD and SEV 261 groups, respectively, although with a minimum 20% difference in Z-scores relatively to 262 263 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 264 Fig. S6) groups. 265

266 **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

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

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

309The transcriptional signatures observed at endpoint Z remained for the most part310conserved after incubation of cultures for 24h (endpoint A), thus validating the *ex vivo*311model of whole blood cultures for researching drugs with the potential to fight312hyperinflammation in COVID-19.

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

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

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

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

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

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

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

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

Gene ontology (GO) functional annotation analysis of the 2.699 genes differentially 418 expressed in response to B12 (Fig. S3) indicate that, in leukocytes of patients with 419 moderate COVID-19, the vitamin stimulates the antiviral response induced by IFNs, 420 regulates the exacerbated immune response induced by NF-kappaB and favors the 421 inhibition of gene expression. Regarding the SEV group, the results indicate that in 422 addition to suppressing viral infection, B12 induces inflammatory attenuation during 423 severe COVID-19 and promotes activation of the adaptive immune response. These 424 processes seem to have been regulated by epigenetic mechanisms, such as changes in 425 chromatin status, gene silencing by miRNAs and alternative splicing reduction, which 426 favored the reduction of gene transcription and translation. 427

Functional enrichment analysis also disclosed B12-modulated pathways in the MOD (Fig. 428 5B and Fig. S5) and/or SEV (Fig. 5C and Fig. S6) groups. It should be noted that the 429 effect of B12 was pleiotropic, that is, it regulates several metabolic or intracellular 430 signaling pathways maladjusted by COVID-19 despite the glucocorticoid treatment that 431 patients received during hospitalization. Most of the differentially regulated pathways in 432 contrasts 1 and 2 (MOD vs. CTRL and SEV vs. CTRL, all at endpoint A) had their 433 activation or inhibition attenuated by B12 (contrasts 3 and 4 - MOD at endpoint B vs. 434 CTRL at endpoint A; and SEV at endpoint B vs. CTRL at endpoint A). In the cultures of 435 patients with moderate COVID-19, B12 favored the overall reduction of inflammation and 436 activation of the Th1-type antiviral response (Fig. 5B and Fig. S5). The few pathways 437 predicted to be more activated in response to B12, such as *iNOS signaling*, regulate 438 desirable mechanisms in the context of COVID-19. Through S-nitrosylation of cysteine 439 residues of viral and host proteins, NO reduces the activity of viral proteases, inhibiting 440 the fusion and replication of SARS-CoV-2 in host cells (46). Another example is the NK441 cell signaling pathway. During infection, the coronavirus depletes NK cells and impairs 442 their antiviral effects while activating macrophages and other immune cells leading to the 443 cvtokine storm (47). Furthermore, the activation of *TREM1 signaling* and *Acute phase* 444 response signaling pathways favors the production of IFN and activation of Th1 and Th17 445 cells, which are fundamental components of the antiviral response (32, 48, 49). Activation 446 of the *Th1 pathway* was also observed, which further reinforces the idea that B12 447 stimulates the antiviral response. Regarding the few pathways where the result seems 448 incongruous, when analyzing their topology, it is clear that their activation by B12 is 449 potentially beneficial to the patient. For example, in the Inflammasome pathway, NLRP1 450 was inhibited in the MOD group and B12 abrogates this inhibition (Fig. S8). This 451 inflammasome interacts with double-stranded RNA (ds), being an important detector of 452 453 dsRNA viruses such as SARS-CoV-2 (50). NLRP1 activity is regulated by anti-apoptotic proteins, BCL-2 and BCL-XL, which associate with the inflammasome and inhibit its 454 activity (51). In this study, the genes encoding these two proteins were predicted to be 455 upregulated in the leukocytes of patients in the MOD group, however B12 did not affect 456 their expression. Thus, the regulatory effect of B12 on NLRP1 likely depends on other 457 mediators. In the Coronavirus Pathogenesis Pathway, angiotensin 1-7 was predicted to be 458 the activated in cultures treated with B12 (Fig. S7). It binds to MAS1 receptors leading to 459 the activation of the anti-inflammatory branch of the renin-angiotensin pathway (52). 460

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

- Besides regulating DNA methylation acting as a cofactor of the enzyme MS in the sulfur 469 amino acid pathway, B12 also modulates different chromatin remodeling mechanisms that 470 can affect gene expression. In the Krebs cycle, AdoCbl can induce the accumulation of 471 succinate, an antagonist of histone and DNA demethylation reactions catalyzed by 472 enzymes of the 2-oxoglutarate-dependent dioxygenase family, which may favor the 473 activation or inhibition of gene expression (53). The antioxidant activity of B12 (54) can 474 also indirectly influence the epigenetic landscape by regulating oxidative stress, which, 475 depending on the context, can determine an accessible or non-accessible state of 476 chromatin. Oxidative stress inactivates the histone-deacetylating enzyme HDAC2, 477 contributing to the greater accessibility of transcriptional machinery to DNA. However, 478 oxidative stress also activates signaling pathways that increase the expression of the 479 enzyme DNMT1 that methylates DNA (53, 55). All these mechanisms may contribute to 480 the down- (1,364) or upregulation (1,335) of the 2,699 genes in the leukocytes of patients 481 in the MOD and/or SEV groups in response to B12 (Fig. 4). 482
- It is noteworthy that when patients were recruited to this study, in July 2020, vaccines 483 were not vet available and dexamethasone and other glucocorticoids had recently been 484 integrated to the COVID-19 therapeutic protocol. At that time, there was no record of 485 SARS-CoV-2 variants of interest/concern circulating in Brazil. Therefore, caution is 486 needed when extrapolating the findings of this work to COVID-19 patients previously 487 vaccinated or infected with SARS-CoV-2 variants. 488
- In conclusion, vitamin B12 has a great potential as an adjuvant drug for alleviating 489 inflammation in patients with moderate or severe COVID-19 in addition with the other 490 established treatments. Beyond favorably regulating the expression of several 491 492 inflammatory genes via methyl-dependent epigenetic mechanisms, B12 also acts as an antioxidant, has an excellent safety profile and is widely available at low cost. Therefore, 493 phase II/III clinical trials are enthusiastically recommended. Furthermore, the results 494 presented herein neither endorse the prophylactic use of B12 to prevent SARS-CoV-2 495 infection, nor its therapeutic use in mild COVID-19. 496

Materials and Methods 497

Patients 498

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

506Participants had peripheral venous blood samples (10 mL) collected in sodium heparin, at5078 a.m., and data from medical records of COVID-19 patients were analyzed. Uninfected508volunteers included as controls in the study were tested for the presence of SARS-CoV2 in509the oropharynx and nasopharynx by RT-qPCR and for the presence of IgM and IgG blood510antibodies against the virus by lateral flow immunochromatography (Wondfo, Guangzhou,511China).

512 Sample processing and production of whole blood cultures

Each peripheral venous blood sample was divided into 4 aliquots of 1 mL and processed 513 as follows: endpoint U) unprocessed blood; endpoints A and Z) added with 498 ul of 514 RPMI 1640 culture medium (Sigma-Aldrich, Saint Louis, Missouri) and 2 µl of pH 5 515 citrate-phosphate buffer excipient (Merck, Darmstadt, Germany); and endpoint B) added 516 with 500 µl of RPMI 1640 culture medium with cyanocobalamin (Merck) to a final 517 concentration of 1 nM. Aliquots Z were immediately processed with no incubation. 518 Aliquots A and B were incubated in 6-well plates for 24 hours at 37°C in a humidified 519 atmosphere with 5% CO₂ (Fig. 6). 520

521 Quantification of basal B12

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

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

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

- Specific primers (Table S2) were used to detect the human mRNAs of CCL1 (C-C Motif 532 Chemokine Ligand 1) (NM 002981.2), CCL2 (C-C Motif Chemokine Ligand 2) 533 (NM 002982.4), CCL3 (C-C Motif Chemokine Ligand 3) (NM 002983.3), CXCL9 (C-534 X-C motif chemokine ligand 9) (NM 002416.3), *IL1B* (Interleukin 1 beta) 535 (NM 000576.3), IL6 (Interleukin 6) (NM 000600.5), IL17A (Interleukin 17A) 536 (NM 002190.3), TNF (Tumor Necrosis Factor) (NM 000594.4), HAVCR2 (Hepatitis A 537 Virus Cellular Receptor 2) (NM 032782.5), CD4 (CD4) (NM 000616.5) and CD8A 538 (CD8a) (NM 001768.7). For all RT-qPCR assays, target gene expression levels were 539 normalized by 18S ribosomal RNA (18S ribosomal N1) (NR 145820.1) levels. qPCR 540 reactions were performed with Fast SYBR Green Master Mix (ThermoFisher) plus 10 ng 541 of cDNA in a final volume of 10 μ L. Thermal cycling and fluorescence detection were 542 performed using the ViiA 7 real-time PCR system (ThermoFisher) according to the 543 manufacturer's recommendations. The relative expression of target genes was calculated 544 using the 2e(- Δ Ct) method (57). 545
- 546 ELISA

547To access the intracellular protein levels of CCL3 and IL-1B, cell precipitates of aliquots548from endpoints A and B were homogenized in 1X PBS, diluted in Milli-Q H2O 1:2 and the549cells disrupted by mechanical lysis in the TissueLyser (Qiagen) for 2 minutes at 30 Hz and550three 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)

- For measurements of HCY, cysteine (CYS), GSH, SAM and S-adenosyl-L-homocysteine 558 (SAH), cell precipitates from aliquots A and B were diluted in Milli-q H₂O 1:2 and the 559 cells disrupted by three freeze/thaw cycles (20° to -20° C). Metabolites were quantified by 560 high performance liquid chromatography (HPLC) on the Shimadzu Prominence-i LC-561 2030C 3D Plus (GMI, Ramsey, Minnesota) according to Pfeiffer et al. (58) with some 562 modifications. HCY, CYS and GSH were quantified using a C18 Luna column (5 mm x 563 150 mm x 4.6 mm) and mobile phase composed of 0.06 M sodium acetate, 0.5% acetic 564 acid and 2% methanol (pH 4.7 adjusted with acetic acid). The flow rate was 1.1 mL \times 565 min⁻¹ and the retention time was 4.1 min for CYS, 5.9 min for HCY and 10.3 min for 566 GSH. SAM and SAH were quantified using a method adapted from Blaise et al. (59). 567 HClO₄ was added to the homogenized cell precipitate for protein precipitation. The 568 supernatant was injected into a C18 LiChroCart column (5 mm x 250 mm x 4 mm) and the 569 mobile phase applied at a flow rate of 1 mL \times min⁻¹, consisting of 50 mM sodium 570 phosphate (pH 2.8), 10 mM heptane sulfonate and 10% acetonitrile. Retention time was 571 8.7 min for SAH and 13.6 min for SAM. All metabolites were detected by UV absorption 572 at a wavelength of 254 nm. 573
- 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

578Total RNA from three samples from each group (MOD, SEV and CTRL) at endpoints A579and B (total 18 samples) was used to construct cDNA libraries with the TruSeq Stranded580mRNA kit (Illumina, San Diego, CA) and the indexed fragments were sequenced on the581NGS NextSeq 500 (Illumina) with the NextSeq 500/550 High Output 2x75 cycles kit582(Illumina). All sequenced samples were quality assessed by capillary electrophoresis with583the Agilent RNA 2100 Nano kit (Bioanalyzer, Santa Clara, CA).

584Bisulfite Sequencing PCR (BSP)

585Genomic DNA from five samples from each group (MOD, SEV and CTRL) at endpoints586A and B (total of 30 samples) was extracted with QIAamp DNA Blood Mini kit (Qiagen)587according to the manufacturer's instructions. DNA was subjected to bisulfite conversion588using EpiTect Bisulfite kit (Qiagen). The region -107pb to +164pb of CCL3

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

597 **Bioinformatics analysis**

598Hierarchical clustering analyzes were performed using the GenePattern software (60)599using Pearson correlation as a comparison method and average linkage as a linkage600method.

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

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

633 Statistical analysis

- Statistical analyses were performed using GraphPad Prism software (version 8.0.2) 634 (GraphPad Software Inc., Irvine, CA). Data distribution was analyzed using Anderson-635 Darling, D'Agostino & Person, Shapiro-Wilk and Kolmogorov-Smirnov tests. For 636 comparisons between three or more groups, one-way analysis of variance (ANOVA) or 637 Kruskall-Wallis tests were used, followed by the multiple comparison tests of Tukey or 638 Dunn, for parametric or non-parametric data, respectively. Two-way ANOVA followed by 639 Tukey's multiple comparison test was used for comparisons involving the effect of two 640 641 factors on a dependent variable in three or more groups. Two-tailed Student's t, paired Student's t, Mann-Whitney or Wilcoxon tests were used for comparisons between two 642 groups, according to the experimental design and data distribution. Correlations were 643 tested using Pearson or Spearman tests according to data distribution. Outliers identified 644 by ROUT test (Q = 1%) were excluded. Data were expressed as median \pm interquartile 645 (nonparametric) or mean \pm standard deviation (parametric). Differences were considered 646 statistically significant when P < 0.05. 647
- For BSB analysis, the means of methylation percentages of each CG locus of MOD, SEV
 and CTRL groups at endpoints A and B were compared using two-tailed Wilcoxon test
 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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865 Acknowledgments

- This work received financial support from FIOCRUZ, CAPES, INCT-Vacinas and from a
 gentle personal money donation by Claudia Garcia Martins. Technical support was
 provided by FIOCRUZ Technological Platforms: Real Time PCR and Digital PCR (P04006), NGS Sequencing (P01-007), and Bioinformatics (P08-002). We thank Dr. Ludmila
 R.P. Ferreira for valuable discussions about the functional enrichment analysis, and Dr.
 Diana Bahia and Prof. Dulciene M.M. Queiroz for critically reviewing this manuscript.
- Author contributions: LC performed the experiments, contributed to experimental 872 design, analyzed data and wrote the manuscript; VCS and VD'A - performed the HPLC 873 assays, analyzed data and reviewed the manuscript; CC, BP and SF – recruited patients, 874 collected blood samples and clinical and sociodemographic data from patients records; 875 MO - contributed to data analysis and reviewed the manuscript; AS - made substantial 876 contributions to the NGS experiments; GF - contributed to the experimental design and 877 data analysis and reviewed the manuscript; RC - designed and oversaw the study, 878 analyzed data and wrote the manuscript. All authors read and approved the final 879 manuscript. 880
- 881 **Competing interests:** The authors declare that they have no competing interests.
- 882Data and materials availability: The dataset supporting the conclusions of this article is883available in the SRA repository, [https://www.ncbi.nlm.nih.gov/sra/PRJNA862565]. Other884data needed to evaluate the conclusions in the paper are present in the Supplementary885Materials.
- 886 Figures and Tables



Fig. 1. Sulfur amino acid pathway. Methionine is converted to S-adenosylmethionine 888 (SAM), which is the methyl donor for numerous reactions. Upon losing its methyl 889 group, SAM becomes S-adenosyl-L-homocysteine (SAH), which is then converted 890 to homocysteine (HCY). This is then converted back to methionine or enters the 891 transsulfuration pathway to form other sulfur-containing amino acids. 892 Abbreviations: MAT = Methionine adenosyltransferase; ATP = Adenosine 893 triphosphate; Pi = Phosphate (inorganic); PPi = Pyrophosphate; THF = 894 Tetrahydrofolate; MTHFR = Methylenetetrahydrofolate reductase; MS = 895 Methionine synthase; B12 = Vitamin B12; CH₃/Me = Methyl group; TF = 896 Transcription factors; SAHH = Adenosylhomocysteinase; CBS = Cystathionine 897 beta-synthase; CGL = Cystathionine gamma-lyase. (Created with BioRender.com) 898

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



Fig. 3. Vitamin B12 increased methylation levels of CpGs in regulatory regions of

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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).



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.



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-Lhomocysteine; PCR = Polymerase Chain Reaction; RT-gPCR = Real-time quantitative PCR. (Created with BioRender.com).

	Intracellular concentrations (μmol/g Hb) (mean [SD] or median [IQ])					P value (AxB)		
Metabolites	Group	MOD	SEV	CTRL	MOD	SEV	CTRL	
НСУ	Α	0.04096 (0.007656)	0.04312 (0.01524)	0.054 (0.05133- 0.0638)	_ 0.0037 (**)	<0.0001 (****)	0.0313 (*)	
IIC1	В	0.0561 (0.01653)	0.05762 (0.01784)	0.067 (0.06225- 0.0985)				
CVS	Α	0.5869 (0.2176)	0.6795 (0.2867)	0.5635 (0.04167)	_ 0.0029 (**)	0.0007 (***)	0.1253	
	В	0.7326 (0.2542)	0.9097 (0.3812)	0.8457 (0.2764)				
CSH	Α	12.52 (2.123)	10.6 (4.292)	15.35 (2.735)	0.0002 (***)	<0.0001 (****)	0.0612	
USH	В	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	
	В	0.001789 (0.0003617)	0.002673 (0.002365- 0.004239)	0.002081 (0.001825- 0.00227)				
SAH	Α	0.0002819 (0.00009389)	0.0001972 (0.00008667)	0.0001817 (0.00003608)	- 0.0722	0.0015 (**)	0.0087 (**)	
	В	0.0002293 (0.00004081)	0.0003267 (0.0001541)	0.0002277 (0.00004052)				
	Α	9.093 (2.571)	16.44 (12.09)	9.632 (3.123)	0.1511	0.0021 (**)	0.7341	
SAM:SAH	В	8.041 (2.28)	8.058 (4.557)	9.243 (2.087)				

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

983The parametric data were compared using two-tailed paired Student's T test and represented as \pm standard deviation. The nonparametric data984were compared using two-tailed Wilcoxon paired test and represented as \pm interquartile range. * P < 0.05; ** P < 0.01; *** P < 0.001 ****; P < 0.001 ****; P < 0.001. 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).

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988 Supplementary Materials

- 989 Fig. S1. Vitamin B12 basal levels
- Fig. S2. Samples added to culture medium with excipient and incubated for 24h have their
- 991 transcriptional signatures preserved
- Fig. S3. Biological Processes affected by B12
- Fig. S4. Patients with moderate and severe COVID-19 previously treated with
- 994 glucocorticoids had distinct global gene expression patterns
- Fig. S5. Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patients
 with moderate COVID-19
- Fig. S6. Vitamin B12 attenuated the pro-inflammatory profile of leukocytes from patientswith moderate COVID-19
- 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