

1 **SARS-CoV-2 infection paralyzes cytotoxic and metabolic functions of immune cells**

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3 Yogesh Singh^{1,2,6*#}, Christoph Trautwein^{3*}, Rolf Fendel^{4*}, Naomi Krickeberg⁴, Jana Held⁴,
4 Andrea Kreidenweiss⁴, Georgy Berezhnoy³, Rosi Bissinger⁵, Stephan Ossowski^{1,2}, Madhuri S.
5 Salker⁶, Nicolas Casadei^{1,2}, Olaf Riess^{1,2#} and the Deutsche COVID-19 OMICS Initiative
6 (DeCOI)

7
8 ¹Institute of Medical Genetics and Applied Genomics, University of Tübingen, Calwerstrasse
9 7, 72076, Tübingen, Germany

10
11 ²NGS Competence Center Tübingen (NCCT), University of Tübingen, Calwerstrasse 7, 72076
12 Tübingen, Germany

13
14 ³Werner Siemens Imaging Center, University of Tübingen, Röntgenweg 13, 72076, Tübingen,
15 Germany

16
17 ⁴Institute of Tropical Medicine, University Hospital of Tübingen, Tübingen, Wilhelmstrasse 27,
18 72076, Tübingen, Germany

19
20 ⁵Department of Internal Medicine, Division of Endocrinology, Diabetology and Nephrology,
21 University Hospital of Tübingen, Germany

22
23 ⁶Research Institute of Women's Health, University of Tübingen, Calwerstrasse 7/6, 72076,
24 Tübingen, Germany

25
26 *Equal contributions

27
28
29 # Correspondence

30 Dr Yogesh Singh or Prof Olaf Riess

31 Institute of Medical Genetics and Applied Genomics, Tübingen University

32 Calwerstraße 7, 72076, Tübingen, Germany

33 Phone: 0049 7071 29 72257/78264 Fax: 0049 7071 29 25355

34 Email: yogesh.singh@med.uni-tuebingen.de or olaf.riess@med.uni-tuebingen.de

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40 **Short title:** Defective immune-metabolic functions in COVID-19 patient

49 **Abstract:**

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The SARS-CoV-2 virus is the causative agent of the global COVID-19 infectious disease outbreak, which can lead to acute respiratory distress syndrome (ARDS). However, it is still unclear how the virus interferes with immune cell and metabolic functions in the human body. In this study, we investigated the immune response in 10 acute or convalescent COVID-19 patients. We characterized the peripheral blood mononuclear cells (PBMCs) using flow cytometry and found that CD8⁺ T cells were significantly subsided in moderate COVID-19 and convalescent patients. Furthermore, characterization of CD8⁺ T cells suggested that patients with a mild and moderate course of the COVID-19 disease and convalescent patients have significantly diminished expression of both perforin and granzyme B in CD8⁺ T cells. Using ¹H-NMR spectroscopy, we characterized the metabolic status of their autologous PBMCs. We found that fructose, lactate and taurine levels were elevated in infected (mild and moderate) patients compared with control and convalescent patients. Glucose, glutamate, formate and acetate levels were attenuated in COVID-19 (mild and moderate) patients. Our findings reveal patients who suffer from an over activation of the immune system, a change of composition in infusion/intravenous fluids during infection with the aim to lower blood levels of glucose, glutamate, acetate and formate could avoid a life-threatening cytokine storm. In summary, our report suggests that SARS-CoV-2 infection leads to disrupted CD8⁺ T cytotoxic functions and changes the overall metabolic functions of immune cells.

97 **Introduction:**

98 The first cases of severe acute respiratory coronavirus-2 (SARS-CoV-2) infection appeared in
99 December 2019, in Wuhan, China¹. This zoonotic virus has infected by now more than 26.1
100 million people (03.09.2020) and killed more than 0.86 million^{2,3} worldwide. The containment of
101 the pandemic is challenging and is still continuing with roughly 200,000 or more new infections
102 being reported daily since July 2020^{2,3}. There is an urgent need for a better understanding of
103 the immunopathology, as SARS-CoV-2 has become the leading cause of morbidity and
104 mortality in many countries.

105
106 Coronaviruses (CoV) are a large family of viruses that can cause illnesses such as the
107 common cold and seasonal influenza⁴. Pathologically, SARS-CoV-2 infects angiotensin-
108 converting enzyme 2 (ACE2)-expressing nasal epithelial cells in the upper respiratory tract
109 and type II alveolar epithelial cells in patients exhibiting pneumonitis^{1,5}. The most severe
110 disease courses led to death frequently but not exclusively in older patients with and without
111 risk conditions. The primary symptoms of SARS-CoV-2 infections are fatigue, fever, sore
112 throat, dry cough, loss of smell and taste within 5-21 days of incubation of the virus⁶⁻⁹. COVID-
113 19 symptoms are heterogeneous and range from asymptomatic to mild, moderate, and severe
114 pathological symptoms, presenting with or without pneumonia^{10,11}, however, most infected
115 people develop mild to moderate illness and recover without hospitalization^{12,13}. Primarily the
116 older COVID-19 patients can develop acute severe respiratory distress syndrome (ARDS) due
117 to a cytokine storm which is a life-threatening situation, requiring ventilation and intensive care
118 support¹⁴⁻¹⁸. High serum levels of IL-6, IL-8, IL-10, TNF- α cytokines and an immune hyper-
119 responsiveness referred to as a 'cytokine storm' is connected with poor clinical outcome^{19,20}.

120
121 Several break-through discoveries have extended our understanding how the virus takes
122 advantage of the host and modulates immunity^{12,17,21-25}. Recovered COVID-19 patients have
123 an increased number of antibody-secreting cells, activated CD4⁺ and CD8⁺ T cells, and
124 immunoglobulin M (IgM) and SARS-CoV-2 reactive IgG antibodies were detected in blood
125 before full symptomatic recovery²⁶⁻²⁸. Most severely affected COVID-19 patients had a lower
126 T cell but elevated B cell counts^{13,19,29,30}. Interestingly, patients with mild symptoms were
127 shown to have increased T and B cells compared with severely affected patients^{26,29-31}. There
128 could be several reasons for different disease outcomes including over-activated innate or
129 hyper-activated adaptive immune responses leading to cytokine storms and resulting in
130 severe injury to the lungs^{10,13,25,32}. Despite of several ongoing efforts, the immunological
131 mechanisms of the host-pathogen interaction are not well understood³³.

132
133 There is an intricate balance between the metabolic state of immune cells and generation of
134 immune response^{17,34-37}. CD8⁺ T cells require energy to proliferate and accomplish their
135 effective functions³⁸. Most propagating cells such as lymphocytes utilize the most abundant
136 energy substrates including, glucose, lipids, and amino acids³⁹. In response to SARS-CoV-2
137 and other virus infections, CD8⁺ T cells play a pivotal role in profound growth and proliferation
138 to generate their effective functional cells which can produce copious amounts of effector
139 molecules such as cytokines and cytotoxic granules^{30,38-40}. An activated immune system is
140 coupled with a change in metabolic reprogramming to produce enough energy needed during
141 (viral) infection^{38,39}. Proliferating T cells ferment glucose to lactate even in the presence of
142 oxygen to meet high energy demands^{34,37-39}. Furthermore, glucose and glutamine are involved
143 in the hexosamine biosynthetic pathway, which regulates the production of uridine

144 diphosphate N-acetyl glucosamine necessary for T cell clonal expansion and function⁴¹. The
145 synthesis of lactate intracellularly is crucial for T cells to have an increased glycolytic flux³⁸.

146

147 Peripheral blood mononuclear cells (PBMCs) can be analyzed to measure the health status
148 of an individual and can serve as a health biomarkers⁴². Therefore, the metabolic status of
149 lymphocytes could help to predict disease severity or to select the optimal therapeutic
150 intervention to boost the immune function during infection. Generally, most of the metabolism-
151 related functions in PBMCs during SARS-CoV-2 infections were inferred based on
152 transcriptomics analysis^{34,43} and no functional data (biochemical level) have been presented.
153 Thereof, understanding the kinetics of adaptive immune response as well as the metabolic
154 functions during SARS-CoV-2 infections will help to elucidate the host immune response to
155 SARS-CoV-2 infection. In this study, using flowcytometry and proton nuclear magnetic
156 resonance (¹H-NMR) spectroscopy, we characterized the PBMCs from SARS-CoV-2 infected
157 and convalescent patients for the their immunophenotypic and metabolic functions.

158

159 **Results:**

160

161 *Characteristics of study participants*

162

163 PBMCs were isolated and cryopreserved from blood samples obtained from COVID-19
164 patients suffering from mild ('Mild (outpatient)') or moderate/severe ('Moderate (inpatient)')
165 disease or were already recovered ('Convalescent') and from healthy controls ('HC').
166 Classification of disease severity for this analysis was based on the requirement of
167 hospitalization. Patients with mild COVID-19 were recruited within three days after
168 confirmation of infection by RT-qPCR. From moderate to severe COVID-19 patient blood
169 samples were collected one week after their admittance. The moderate patients were admitted
170 to the hospital requiring medical care, however, they did not need ventilation or O₂ supply.
171 Recovered patients were included based on a positive SARS-CoV-2 antibody testing. Study
172 participant characteristics are described in Table 2.

173

174 *Immunophenotyping of COVID-19 mild, moderate and convalescent COVID-19 patients*

175

176 To compare the number of lymphocytes and monocytes amongst the four study groups,
177 PBMCs were stained and analysed by flow cytometry. Both, lymphocytes (p=0.005) and
178 monocytes (p=0.04), were significantly decreased in moderate COVID-19 patients compared
179 with HC (Suppl. Fig. 1a, b). However, mild and convalescent patients also had a reduced, but
180 not significantly reduced, count of lymphocytes/monocytes compared to HC.

181

182 *Increased inflammatory monocytes and reduced NK cells in moderate COVID-19 patients*

183

184 Monocytes were further classified into classical, non-classical and intermediate based on
185 expression of CD14 and/or CD16 and we used the same gating strategies as described
186 earlier⁴⁴ (Suppl. Fig. 1c). We found that CD16⁺⁺CD14⁺ patrolling (non-classical) monocytes
187 were significantly increased (p=0.0008) in numbers in moderate patients compared to HC,
188 whereas this number is decreased again significantly compared with convalescent patients
189 (p=0.01) (Fig. 1a). The percentage of CD16⁺⁺CD14⁺ monocytes was also significantly
190 increased (p=0.006) in mild patients (outpatients) compared with moderate patients (Fig. 1a
191 Panel I). Interestingly, CD16⁺⁺CD14⁺⁺ pro-inflammatory monocytes (intermediate) were again

192 significantly increased in moderate ($p=0.003$) compared with HC as well as between mild and
193 HC ($p=0.02$) (Fig. 1a panel II). Furthermore, we observed a significantly reduced percentage
194 of CD14⁺⁺CD16⁻ phagocytic monocytes (classical) in moderate compared with mild
195 ($p=0.0009$), HC ($p<0.0001$) and convalescent ($p=0.0003$) patients (Fig. 1a Panel III). Finally,
196 we explored the lymphoid cells compartment for NK cells (CD56⁺CD3⁻CD19⁻). We found that
197 both mild ($p=0.0003$) and moderate ($p=0.0002$) patients were significantly different from
198 convalescent and HC ($p<0.0001$; HC vs mild or moderate) patients (Fig. 1b).

199

200 *Dynamics of B and T cells in mild, moderate and convalescent patients*

201

202 Both T and B cells are indispensable for the immune response against viral infections such as
203 SARS-CoV-2. Firstly, we compared the number of B cells amongst the study groups, which
204 give rise to virus-specific antibodies (see gating strategy in Suppl. Fig. 1c). The CD19⁺CD3⁻
205 cells (B cells) were significantly increased in mild ($p=0.008$; 1.7x times) and moderate
206 ($p=0.0008$; 1.9x times) patients compared with HC (Fig. 2a). Whilst, B cells were significantly
207 decreased in moderate compared to convalescent ($p=0.03$) patients (Fig. 2a). Comparing
208 CD3⁺CD19⁻ lymphocytes among the different patient groups we observed no significant
209 difference. However, there was an increased trend of CD3⁺ cells in the outpatients, inpatients
210 and convalescent groups compared with HC.

211

212 CD3⁺ cells were analysed for the CD4⁺ and CD8⁺ T cell compartment. There was a tendency
213 of increased CD4⁺ T cells for outpatients, inpatients and convalescent patients compared to
214 HC, but no significant difference was observed among any of the groups. CD8⁺ T cells were
215 significantly different between HC compared to moderate ($p=0.04$) or convalescent ($p=0.04$)
216 patients (Fig. 2b). Finally, we characterized CD4⁺Foxp3⁺CD45R⁻ regulatory T cells (Tregs),
217 however, no significant difference was observed among the different groups (Suppl. Fig. 2).

218

219 *Impaired activation and defective cytotoxic functions of CD8⁺ T cells*

220

221 We found that the percentage of CD8⁺ T cells was decreased in mild and convalescent
222 patients compared to HC. Thus, we explored the activation status of CD8⁺ T cells based on
223 HLA-DR expression. We found that CD8⁺ T cell activation status in all three groups of infected
224 patients were significantly different from HC (mild $p=0.01$, moderate $p=0.009$, and
225 convalescent $p=0.008$, Fig. 3a). We characterized the cytotoxic potential of CD8⁺ T cells based
226 on granzyme B and perforin levels and found that there was a tendency of decreased
227 granzyme B expression in mild, moderate and convalescent patients compared with HC (Fig.
228 3b), however it did not reach significance. Perforin was significantly decreased in convalescent
229 ($p=0.03$) patients compared with HC (Fig. 3b), although mild patients also had borderline
230 significantly reduced levels ($p=0.06$). Furthermore, we studied the expression of CD38, a
231 marker of cell activation, which was significantly upregulated in convalescent patients
232 compared with HC ($p=0.01$), mild ($p=0.03$) and moderate ($p=0.02$) patients (Fig. 4a). Similarly,
233 convalescent patients had significantly increased numbers of CD38⁺PD-1⁺ cytotoxic CD8⁺ T
234 cells compared with HC ($p=0.005$), moderate ($p=0.002$) and mild ($p=0.002$), which reflects the
235 exhaustion and non-responsiveness (anergy) of CD8⁺ T cells (Fig. 4b). Overall, our data
236 suggested that CD8⁺ T cells have reduced activation, diminished expression of cytotoxic
237 molecules such as perforin and granzyme B and severely exhausted phenotype.

238

239 *Dynamics of metabolites production in mild, moderate and convalescent patient*

240 PBMCs from all patient groups were subjected to ¹H-NMR spectroscopy analysis. We
 241 identified and quantified a total of 18 metabolites (Fig. 5a). Hereby, unsupervised PCA showed
 242 that spectral data from mild and moderate patients formed overlapping clusters clearly distinct
 243 from a cluster formed by HC and convalescent patients (Fig. 5b), indicating a strong difference
 244 in metabolite levels between infectious state compared to healthy or recovered state.
 245 Statistical analysis of the four different groups, revealed that 15 metabolites showed p-values
 246 < 0.05, with highest significance for metabolites from the energy metabolism (Fig. 5c, Suppl.
 247 Fig. 3 & Table 1). The data indicate a strong consumption of glucose, acetate, formate during
 248 infection, while with lactate levels are increased. Furthermore, we also found very high levels
 249 of fructose in PBMCs from mild patients, medium concentrations in moderate and, low levels
 250 in HC and convalescent patients (Fig. 5c). Furthermore, glutamate was almost abolished in
 251 mild and moderate patients, potentially as a consequence of enhanced production of α-
 252 ketoglutarate in the TCA cycle in PBMCs *via* glutamate dehydrogenase (Fig. 5c).

253
 254 To find an association between different metabolites, we applied the variable importance of
 255 projection (VIP) score. We found that formate and glucose had the highest score compared to
 256 another other metabolites (Fig. 6A). In order to determine if additional metabolites are
 257 positively associated with changes in glucose, lactate and fructose, we performed a pattern
 258 hunter analysis for all metabolites. We found that high glucose levels correlated with high
 259 formate, acetate and glutamate and low lactate and fructose (Fig. 6b), indicating enhanced
 260 glycolysis and TCA cycle in PBMCs. Similarly, fructose, that is entered *via* fructose-1-
 261 phosphate and dihydroxy acetone phosphate (DAP) into the glycolysis, is correlated positively
 262 with lactate and citrate and a decrease in acetate and formate, respectively (Fig. 6b).
 263 Interestingly, levels of the ROS scavenger taurine are only positively correlated with lactate
 264 and fructose, but not glucose (Fig. 6).

265

266 **Table 1: Summary of metabolites dysregulated in PBMCs**

267

| No | Metabolites | HC | Mild | Moderate | Convalescent |
|----|-------------|----|------|----------|--------------|
| 1 | Glucose | ↑↑ | ↓↓ | ↓↓ | ↑↑ |
| 2 | Formate | ↑↑ | ↓↓↓ | ↓↓ | ↑↑ |
| 3 | Acetate | ↑ | ↓↓ | ↓ | ↑ |
| 4 | Lactate | ↓↓ | ↑↑ | ↑↑ | ↓↓ |
| 5 | Fructose | ↓ | ↑↑ | ↑ | ↓ |
| 6 | Glutamate | ↑ | ↓ | ↓ | ↑ |
| 7 | Citrate | ↓ | ↑↑ | ↑ | ↓ |
| 8 | Taurine | ↓↓ | - | ↑↑ | ↓ |
| 9 | Creatine | ↓ | ↑ | - | - |
| 10 | Alanine | ↓ | - | ↑↑ | ↓ |
| 11 | Glycine | ↑ | ↓ | ↓ | - |
| 12 | Isoleucine | - | ↓↓ | ↓ | ↑ |

268

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 270 projection (VIP) score. We found that formate and glucose had the highest score compared to
 271 other metabolites (Fig. 6A). In order to determine if additional metabolites are positively
 272 associated with changes in glucose, lactate and fructose, we performed a pattern hunter
 273 analysis for all metabolites. We found that high glucose levels correlated with high formate,

274 acetate and glutamate and low lactate and fructose (Fig. 6b), indicating enhanced glycolysis
275 and TCA cycle in PBMCs. Similarly, fructose, that is entered *via* fructose-1-phosphate and
276 dihydroxy acetone phosphate (DAP) into the glycolysis, is correlated positively with lactate
277 and citrate and a decrease in acetate and formate, respectively (Fig. 6b). Interestingly, levels
278 of the ROS scavenger taurine are only positively correlated with lactate and fructose, but not
279 glucose (Fig. 6).

280

281 **Discussion:**

282

283 SARS-CoV-2 infections are an intense and rapidly evolving area of research due to the
284 ongoing global pandemic^{19,25}. In this study, we used flow cytometry and ¹H-NMR to decipher
285 the cell proportions and functional state of immune cells (PBMCs) in mild, moderate and
286 convalescent COVID-19 patients compared to HC. Recent reports from COVID-19 patients
287 suggested that mild and severe patient had lymphopenia^{11,45-47}. Here, we found that mild
288 patients have reduced lymphocyte numbers whereas convalescent patients have recovered
289 the total lymphocyte counts. Similarly, monocytes were also reduced in mild patients, which is
290 in agreement with other recent studies^{29,48,49}. Importantly, our characterization of myeloid cell
291 compartment based on CD16 and CD14 markers suggested that non-classical and
292 intermediate monocytes were increased during an active mild or moderate SARS-CoV-2
293 infection, once infections are cleared the monocyte numbers return to normal. These results
294 are in accordance with some of the recent published studies^{48,50,51}, while another study
295 suggested the opposite⁵².

296

297 In our cohort, specifically CD56⁺NK cells were dramatically decreased during the course of
298 active SARS-CoV-2 viral infections (mild and moderate), while during recovery the numbers
299 were comparable to HC as report by others⁵³. Similarly another recent study suggested the
300 decrease in number of NK cell subsets in COVID-19 patients, with no change in CD56^{bright} or
301 CD56^{dim} cells⁵⁴. Thus, these data point to a crucial role of CD56⁺NK cells in eliminating SARS-
302 CoV-2 infections⁴⁷. CD19⁺ B lymphocytes were increased during the course of infection and
303 remain slightly higher than HC, thus reflecting the antibody response against the COVID-19
304 virus. Thus, this data implicated that these patients were able to generate the SARS-CoV-2
305 specific B cells.

306

307 A major difference was found in the T lymphocytes compartment. On the one hand, CD4⁺ T
308 cells were increased during infection, but not dramatically. On the other hand, CD8⁺ T cells
309 were significantly decreased in moderate and convalescent patients as reported earlier⁵³.
310 Thus, it appears that during viral infection non-virus specific CD8⁺ T cells are dead, while the
311 viral-specific surviving CD8⁺ T cells are clonally expanded but appeared to lost their effector
312 functions⁵⁵. To confirm this, we first measured the activation status of CD8⁺ T cells and found
313 that CD8⁺ T appeared to be less activated based on their HLA-DR activation marker²⁶. Further,
314 CD8⁺ T cells were examined for another activation marker CD38 which is involved in cell
315 adhesion, signal transduction and calcium signalling⁵⁶ and was found to be upregulated in
316 convalescent patients but not during active infection. These CD38⁺CD8⁺ T cells, were also
317 expressing higher levels of PD-1, which is an immune checkpoint and marker of
318 exhaustion^{24,30,49,57,58}. It guards against autoimmunity, promotes apoptosis of antigen-specific
319 T cells and promotes self-tolerance by suppressing T cell inflammatory activity. Thus, viral
320 infection leaves convalescent patients with exhausted phenotypes. We found that although
321 there was not a significant change in the numbers of Tregs in COVID-19 patients, there was

322 a trend towards elevated levels of Tregs in COVID-19 patients and rescued Tregs in
323 convalescent patients, in agreement with previous studies⁵⁷.

324

325 A key finding of our study was the surprising observation that granzyme B and perforin
326 secreting CD8⁺ T cells were significantly reduced in convalescent patients. The possible
327 implication of our finding is that convalescent patients, specifically including cancer patients
328 under treatment, could be susceptible to future opportunistic infections with other viruses
329 including different strains of SARS-CoV-2.

330

331 To date, the general metabolic physiology of PBMCs is not well defined in literature. However,
332 it is clear that PBMCs are dependent on circulating nutrients and hormones in the blood
333 system⁵⁹. The defective immune response in COVID-19 patients prompted us to investigate
334 the metabolic functions of these immune cells. Our metabolomics data indeed shows that
335 PBMCs from actively infected patients have a distinct metabolic profile from convalescent or
336 healthy individuals. The most notable difference we observed were for metabolites from the
337 glycolysis and oxidative phosphorylation (TCA cycle) pathway, which is in accordance with
338 recently published transcriptome data for PBMCs^{39,43}. Metabolites such as glucose, formate,
339 acetate and choline were also reduced in PBMCs in infected patients whereas, HC and
340 convalescent patients had a normal profile. Accordingly, the glycolytic pathway end products
341 such as lactate were higher in active mild and moderate COVID-19 patients compared with
342 HC and convalescent individuals. Therefore, our data suggests that PBMCs (which constitute
343 a major fraction of T lymphoid cells: 70- 80%) may have changed their metabolic functions,
344 particularly favouring the oxidative phosphorylation pathway over the glycolytic pathway, to
345 meet the high demands of energy needed to combat the ongoing viral infection.

346

347 A recent report suggested that elevated glucose levels enhance SARS-CoV-2 replication and
348 cytokine expression in monocytes and glycolysis sustains the viral-induced monocyte
349 response⁶⁰. Recently, it was emphasized that glucose consumption in PBMCs during COVID-
350 19 disease could be also a read-out of cytokine storms³⁴. Further, a higher abundance of
351 citrate in PBMCs suggested that perhaps T cells could use the oxidative phosphorylation
352 pathway for energy consumption to endure the infection, as recent transcriptomic data also
353 suggested that higher expression of genes related to oxidative phosphorylation both in
354 peripheral mononuclear leukocytes and bronchoalveolar lavage fluid (BALF) could play a
355 crucial role in increased mitochondrial activity during SARS-CoV-2 infection³⁴.

356

357 Another interesting finding of our study was the increase of fructose levels in PBMCs during
358 the course of infection. Previous findings suggested that fructose is involved in the
359 inflammatory pathways for the production of IL-1 β and IL-6 production⁶¹. Thus, it is possible
360 that the immune cells (most probably monocytes) could be triggered by higher fructose and
361 simultaneously induce inflammation and IFN- γ production by T cells⁶¹. These findings are
362 correlating with recent transcriptomic studies on the BALF from infected COVID-19 patients
363 and plasma of COVID-19 patients that also identified changes in fructose metabolism^{34,62}.

364

365 We finally observed a reduction of granzyme B and perforin in CD8⁺ T cells and detected the
366 antioxidant amino acid taurine, which could be involved in the cytotoxic functions of CD8⁺ T
367 cells. Both granzyme B and perforin are involved in ROS production and taurine serves as
368 ROS scavenger^{63,64}. Thus, decreased granzyme B and perforin could be implicated in reduced
369 ROS production for the impaired effectiveness of CD8⁺ T cells in convalescent or COVID-19

370 patients. This should be the case, as taurine levels that are generally increased during an
371 active infection in mild patients compared to healthy controls are not specifically decreasing
372 due to granzyme B and perforin lacking ROS activity in COVID-19 patients. However, this
373 finding needs further investigation to validate this hypothesis. In summary, the metabolomics
374 data generated in this study provides first and crucial insights into the complex metabolic
375 changes of PBMCs during SARS-CoV-2 infections, warranting further investigation.

376

377 **Conclusions:**

378 Using immunophenotyping and metabolomics approaches we detected significant changes in
379 PBMC samples of mildly and moderately affected COVID-19 as well as convalescent patients
380 compared to healthy controls. The significantly reduced amount of NK cells in both mild and
381 moderate patient groups corresponded with the clustering of PBMCs metabolite levels in the
382 principal component analysis distinct from the cluster formed by healthy and convalescent
383 individuals. The dramatic changed metabolic activity and pathways, such as glycolysis and
384 TCA cycle, might not only lead to a vulnerability of COVID-19 patients to subsequent
385 infections, but can also offer insights into how PBMCs could be manipulated towards a better
386 survival and personalized treatment of moderate and severe COVID-19 patients.

387

388 **Materials and Methods:**

389

390 **Ethics statement**

391 The study protocols were approved by the University of Tübingen, Germany Human Research
392 Ethics Committee (TÜCOV: 256/2020BO2 (convalescent study), COMIHY: (225/2020AMG1)
393 (outpatient study)-COMIHY, EUDRA-CT: 2020-001512-26, ClinicalTrials.gov
394 ID: NCT04340544, and COV-HCQ: (190//2020AMG1) (inpatient study)-COV-HCQ, EUDRA-
395 CT: 2020-001224-33, ClinicalTrials.gov ID: NCT04342221, 556/2018BO2) and all associated
396 procedures were carried out in accordance with approval guidelines. All participants provided
397 written informed consent in accordance with the Declaration of Helsinki.

398

399 **Study participants**

400

401 SARS-CoV-2 positive patients were used for this study and no other virus species were
402 analysed in this study (COMIHY and COV-HCQ). Blood was collected from COVID-19 patients
403 enrolled into two different prospective randomized, placebo-controlled, double blind clinical
404 trials evaluating safety and efficacy of hydroxychloroquine in COVID-19 outpatients
405 (COMIHY) and hospitalized patients (COV-HCQ). We analysed subsets of these study cohort
406 and used outpatient (n=3; COMIHY) which came to a specified outpatient ward at in the
407 Institute of Tropical Medicine with mild symptoms and blood was taken and usually defined as
408 D1 outpatients. Inpatients (n=3; COV-HCQ), blood was taken after 7-9 days after study
409 inclusion defined as D7. These patients had moderate symptoms needing hospital care,
410 however not being transferred to the intensive care unit in the hospital. Furthermore,
411 convalescent COVID-19 patients (n=4) were defined as positive for serum antibody reactive
412 to SARS-CoV-2 and blood was taken when they visited the Institute of Tropical Medicine for
413 testing of antibody levels. Amongst this cohort, 3 persons reported mild fever for 10-11 days
414 and 1 individual reported no fever but found positive for SARS-CoV-2 antibodies. Blood from
415 healthy controls (n=5) was obtained from the hospital blood bank.

416

417

418

419 **Table 2: Overview of study participants**
 420

| No | COVID-19 status | Blood sampling | COVID-19 severity | Sex | Age |
|----|-------------------------|----------------|--------------------|-----|-----|
| 1 | Outpatient (mild) | Day1 | mild | F | 21 |
| 2 | Outpatient (mild) | Day1 | mild | M | 59 |
| 3 | Outpatient (mild) | Day1 | mild | F | 40 |
| 4 | Inpatient (moderate) | Day7 | Moderate | M | 57 |
| 5 | Inpatient (moderate) | Day7 | Moderate | M | 47 |
| 6 | Inpatient (moderate) | Day7 | Moderate | F | 78 |
| 7 | Convalescent (Sero +ve) | Convalescent | Recovered, healthy | F | 50 |
| 8 | Convalescent (Sero +ve) | Convalescent | Recovered, healthy | F | 24 |
| 9 | Convalescent (Sero +ve) | Convalescent | Recovered, healthy | M | 50 |
| 10 | Convalescent (Sero +ve) | Convalescent | Recovered, healthy | F | 51 |
| 11 | HC1 | - | None | F | 36 |
| 12 | HC2 | - | None | M | 60 |
| 13 | HC3 | - | None | M | 40 |
| 14 | HC4 | - | None | M | 37 |
| 15 | HC5 | - | None | M | 47 |

421
 422 **Flow cytometry**

423
 424 PBMCs were isolated by standard Ficoll method⁶⁵. A total of 1-2 x10⁶ PBMCs per participants
 425 were used for three FACS panels (Table 2). In brief, cells were stained with surface markers
 426 in DPBS (Sigma) with Super Bright stain Buffer (ThermoFisher) for 30 minutes at room
 427 temperature (RT). To distinguish between live from dead, the cells were also incubated with
 428 LIVE/DEAD Fixable Infra-Red Dead stain (ThermoFisher). After surface staining cells were
 429 also stained for intracellular (IC) markers. Before IC staining, cells were fixed for 30-45 minutes
 430 and permeabilized for 5 minutes followed by IC antibody incubation for additional 30 minutes
 431 at RT. Cells were washed and resuspended in DPBS containing 2%FBS. Fixing of cells was
 432 performed irrespective of whether panel was used for IC staining or not to prevent the possible
 433 contamination during acquisition of the samples. For each sample 200,000 cells were acquired
 434 using BD LSRFortessa (core facility) equipped with 4 lasers (violet, blue and yellow-green and
 435 Red). Data were analysed using Flow Jo (Tree Star) and fluorescence minus one controls
 436 (FMO) were used for setting up the arbitrary gates for the major cell markers.

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441 **Table 2: Antibodies and other reagents used for Flow cytometry**
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| No. | Product Name | Clone | Fluorochrome | Product ID | Company |
|-----|---------------|--|------------------|------------|--------------|
| | | NK cells and Monocytes (Panel 1) | | | |
| 1 | CD3 | UCHT1 | eFluor 450 | 48-0038-42 | Thermofisher |
| 2 | CD4 | SK3 | SuperBright 600 | 63-0047-42 | Thermofisher |
| 3 | CD8a | SK1 | PerCP-eFluor 710 | 46-0087-42 | Thermofisher |
| 4 | CD19 | HIB19 | eFluor 506 | 69-0199-42 | Thermofisher |
| 5 | CD45-RA | HI100 | PE-Cy7 | 25-0458-42 | Thermofisher |
| 6 | HLA-DR | L243 | Alexa Fluor647 | A51010 | Thermofisher |
| 7 | CD38 | HIT2 | PE-eFluor610 | 61-0389-42 | Thermofisher |
| 8 | CD56 | MEM188 | PE | MA119638 | Thermofisher |
| 9 | CD16 | 3G8 | Super Bright702 | 67-0166-42 | Thermofisher |
| 10 | CD14 | 61D3 | Alexa Fluor700 | 56-0149-42 | Thermofisher |
| 11 | Foxp3 (IC) | PCH101 | FITC | 11-4776-42 | Thermofisher |
| | | CD8 exhaustion, T helper follicular cells (Tfh) and antibody secreting cell (ASC) (Panel 2) | | | |
| 1 | CD3 | UCHT1 | eFluor 450 | 48-0038-42 | Thermofisher |
| 2 | CD19 | HIB19 | eFluor 506 | 69-0199-42 | Thermofisher |
| 3 | CD4 | SK3 | Super Bright 600 | 63-0047-42 | Thermofisher |
| 4 | CD8a | SK1 | PerCP-eFluor 710 | 46-0087-42 | Thermofisher |
| 5 | CD38 | HIT2 | PE-eFluor 610 | 61-0389-42 | Thermofisher |
| 6 | CD27 | O323 | Alexa Fluor700 | 56-0279-42 | Thermofisher |
| 7 | CXCR5 (CD185) | MU5UBE E | FITC | 11-9185-42 | Thermofisher |
| 8 | ICOS (CD278) | C398.4A | PE | 12-9949-81 | Thermofisher |
| 9 | PD-1 (CD279) | eBioJ105 (J105) | PE-Cy7 | 25-2799-42 | Thermofisher |
| 10 | HLA-DR | L243 | Alexa Fluor647 | A51010 | Thermofisher |
| | | Cytotoxic potential | | | |
| 1 | CD4 | SK3 | SuperBright600 | 63-0047-42 | Thermofisher |
| 2 | CD8 | SK1 | PerCP-eFluor710 | 46-0087-42 | Thermofisher |
| 3 | CD19 | HIB19 | eFluor 506 | 69-0199-42 | Thermofisher |
| 4 | CD38 | HIT2 | PE-eFluor 610 | 61-0389-42 | Thermofisher |
| 5 | HLA-DR | L243 | Alexa Fluor 647 | A51010 | Thermofisher |

| | | | | | |
|---|-----------------------------------|----------------------------|-----------------|------------|--------------|
| 6 | GZMA (IC) | CB9 | Alexa Fluor 488 | | Thermofisher |
| 7 | GZMB (IC) | GB11 | PE | MA523639 | Thermofisher |
| 8 | Perforin (IC) | dG9 | PE-Cy7 | 12-9177-42 | Thermofisher |
| | | Other Flow reagents | | | |
| 1 | Ultracompensation bead | | | 01-2222-42 | Thermofisher |
| 2 | FOXP3/TRN FACTOR STAIN BUFFER SET | | | 00-5523-00 | Thermofisher |
| 3 | FLOW STAIN BUFFER SOLN | | | 00-4222-57 | Thermofisher |
| 4 | SB COMPLETE STAINING BUFFER | | | SB-4401-42 | Thermofisher |
| 5 | DPBS | | | D8537 | Sigma |
| 6 | Pancoll human | | | P04-601000 | Pan Biotech |

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¹H-NMR metabolomics

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Statistical analysis

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Bar diagrams were prepared using GraphPad Prism 6.0. FACS data were analysed using one-way ANOVA for multiple group comparisons (mild, moderate, convalescent and HC) in GraphPad Prism software. No matching or pairing was used. Assumed Gaussian distribution with equal standard deviations (SDs) for experimental design. Mean of each group was compared with the mean of every other group and Tukey's post-hoc tests for multiple comparisons. P value considered significant less than 0.05. Metabolites data were analysed with MetaboAnalyst 4.0 software.

474 **Figure legends:**

475

476 **Fig. 1:** Comparison of monocytes and NK cell percentage amongst study groups.

477

478 A. The stained PBMCs were gated on the monocyte population and CD3+CD19+ cells
479 were excluded. Cell populations are displayed for CD16 and CD14 expression (upper
480 FACS panel). One exemplary dot plot is shown per study group. The bar diagrams
481 (lower panel) show the non-classical (CD16⁺⁺CD14⁺), intermediate (CD16⁺⁺CD14⁺⁺)
482 and classical (CD16⁻CD14⁺⁺) monocytes. *P value <0.05, **P value <0.01 and ***P
483 value <0.001.

484 B. The stained PBMCs were gated on lymphocyte population and further excluded the
485 CD3+CD19+ cells and examined for the CD56 and CD16 expression in HC, mild,
486 moderate and convalescent (upper FACS panel). One exemplary dot plot is shown per
487 study group. The bar diagram shows the CD56⁺CD3⁻CD19⁻ NK cells. **P value <0.01
488 and ****P value <0.0001.

489

490 **Fig. 2:** Increased B cells in mild and moderate patients and reduced CD8⁺ cytotoxic T cells in
491 mild and convalescent patients

492

493 A. The stained PBMCs were gated on lymphocyte population and examined for the CD19
494 and CD3 expression in HC, mild, moderate and convalescent (upper FACS panel).
495 One exemplary dot plot is shown per study group. The bar diagram shows CD3⁻CD19⁺
496 B cells. *P value <0.05, **P value <0.01 and ***P value <0.001.

497 B. The CD19⁻CD3⁺ lymphocytes were examined for CD4⁺ and CD8⁺ T marker expression.
498 One exemplary dot plot is shown per study group. There was statistically significant
499 difference among HC, mild, moderate and convalescent (upper FACS panel).
500 However, CD8⁺ T cells were significantly reduced in outpatient and convalescent
501 patients. *P value <0.05.

502

503 **Fig. 3:** Decreased activation and cytotoxic functional protein expression of CD8⁺ T cells in
504 convalescent patients

505

506 A. CD8⁺ T cells were examined for the expression of activation marker HLA-DR (upper
507 FACS panel). One exemplary dot plot is shown per study group. The bar diagram
508 (lower panel) shows that HLA-DR was significantly lower on CD8⁺ T cells in mild,
509 moderate and convalescent COVID-19+ patients compared with HC.

510 B. CD8⁺ T cells were examined for the expression of their cytotoxic potential using
511 granzyme B and perforin expression using IC staining (upper FACS panel). One
512 exemplary dot plot is shown per study group. There was statistically significant
513 difference among HC, mild, moderate and convalescent (upper FACS panel) for
514 granzyme B. The bar diagram (lower panel) shows that perforin expression was
515 significantly lower on CD8⁺ T cells in convalescent COVID-19+ patients compared with
516 HC, though mild and moderate represent lower expression of perforin, but it did not to
517 a significant level. *P value <0.05.

518

519 **Fig. 4:** Increased exhausted CD8⁺ T cells in convalescent patients

520

521 A. Expression of activation marker CD38 on CD8⁺ T cells (upper FACS panel). One
522 exemplary dot plot is shown per study group. The bar diagram (lower panel) shows
523 that CD38 expression was significantly higher on CD8⁺ T cells in convalescent COVID-
524 19+ patients compared with HC. *P value <0.05.

525

526 B. Expression of activation marker CD38 and PD-1 on CD8⁺ T cells (upper FACS panel).
527 One exemplary dot plot is shown per study group. The bar diagram (lower panel)
528 shows that PD-1⁺CD38⁺ expression on was significantly higher on CD8⁺ T cells in
529 convalescent COVID-19+ patients compared with HC. *P value <0.05, **P value <0.01.

530

531 **Fig. 5:** ¹H-NMR spectroscopy of PBMC extracts

532 A. Heatmap of featured metabolites' concentrations plotted with SARS-CoV-2
533 progression group clustering.

534 B. Principle component analysis (PCA) was performed to identify the clustering of two
535 different groups. HC and convalescent COVID-19 patient samples cluster together
536 while SARS-Co-2 infected mild and moderate patients cluster in a separate cluster with
537 PC1: 90.7% and PC2: 2.6%.

538 C. Box plots for differentially abundantly present metabolites in different group including
539 HC, mild, moderate, and convalescent COVID-19 patient. *P value <0.05, **P value
540 <0.01 and ***P value <0.001.

541

542 **Fig. 6:** Pattern hunter plots provide an insight of close correlations with other metabolites
543 during COVID-19 infection.

544 A. Variable Importance in Projection (VIP) scores for all metabolites in the four studied
545 groups.

546 B. Pattern hunter plot for glucose.

547 C. Pattern hunter plot for lactate and fructose.

548

549 **Suppl. Fig. 1:** Total % counts of monocytes and lymphocytes from PBMCs of COVID-19
550 patients.

551 A. Fixed PBMCs samples were acquired on flow cytometry on 2-3 different days for the
552 entire experiments. Total 200,000 cells were acquired by flow cytometry and gating
553 was performed based on FSC and SSC parameters for lymphocytes, monocytes and
554 dead cells as described earlier⁶⁷⁻⁶⁹.

555 B. The bar graphs represent the % of lymphocytes and monocytes.

556 C. Gating strategy for T lymphocytes (CD3, CD4 and CD8) monocytes (CD14 and
557 CD16)⁴⁴, NK cells (CD56) using FMO controls.

558

559 **Suppl. Fig. 2:** Kinetics of regulatory T cells is not affected significantly in mild, moderate and
560 convalescent patients.

561

562 Foxp3⁺ expression on CD19⁻CD3⁺CD4⁺CD45RA⁻ T cells to identify the regulatory T cells in
563 HC, outpatient, outpatient and convalescent (upper FACS panel). There was statistically
564 significant difference among HC, mild, moderate and convalescent (upper FACS panel).

565

566 **Suppl. Fig. 3:** Metabolite analysis in COVID-19 patients

567 A. Analysis of Variance (ANOVA) for multi-group comparisons

568 B. Partial Least Squares Discriminant Analysis (PLSDA) scores plot

569 C. Hierarchical clustering of metabolites (distance measured with Pearson r correlation
570 coefficient)

571 D. Boxplots for branched chain amino acids valine and leucine

572

573 **Authors contributions:**

574

575 YS: Overall study design and project coordination, flow cytometry experiments, data analysis
576 and interpretation and initial metabolic sample preparation, funding generation, and writing the
577 manuscript.

578 CT: Metabolites sample preparation, Processing of the sample on ¹H-NMR, data analysis,
579 data interpretation and writing

580 MO: Metabolites sample preparation, Processing of the sample on ¹H-NMR

581 RF, NK: Provided patient materials, Isolation of PBMCs, Performed the experiments for
582 flowcytometry in BSL-2 facility, flow data interpretations.

583 JH, AK: Coordination of the clinical trials, revision of the manuscript.

584 RB: Isolation of PBMCs from HC and sample preparation for flow cytometry.

585 SO, MS, NC, OR: Study design, providing research tools, funding generation, data
586 interpretation and writing and amending the manuscript.

587

588 All the authors have seen the manuscript, substantially contributed and agreed to be co-
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605

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607

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609

610 **Consortia**

611 The members of the Deutsche COVID-19 Omics Initiative (DeCOI) are Angel Angelov, Robert
612 Bals, Alexander Bartholomäus, Anke Becker, Daniela Bezdán, Ezio Bonifacio, Peer Bork,
613 Nicolas Casadei, Thomas Clavel, Maria Colome-Tatche, Andreas Diefenbach, Alexander
614 Dilthey, Nicole Fischer, Konrad Förstner, Julia-Stefanie Frick, Julien Gagneur, Alexander
615 Goemann, Torsten Hain, Michael Hummel, Stefan Janssen, Jörn Kalinowski, René Kallies,
616 Birte Kehr, Andreas Keller, Sarah Kim-Hellmuth, Christoph Klein, Oliver Kohlbacher, Jan O.

617 Korbel, Ingo Kurth, Markus Landthaler, Yang Li, Kerstin Ludwig, Oliwia Makarewicz, Manja
618 Marz, Alice McHardy, Christian Mertes, Markus Nöthen, Peter Nürnberg, Uwe Ohler, Stephan
619 Ossowski, Jörg Overmann, Silke Peter, Klaus Pfeffer, Anna R. Poetsch, Alfred Pühler,
620 Nikolaus Rajewsky, Markus Ralser, Olaf Rieß, Stephan Ripke, Ulisses Nunes da Rocha, Philip
621 Rosenstiel, Antoine-Emmanuel Saliba, Leif Erik Sander, Birgit Sawitzki, Philipp Schiffer, Eva-
622 Christina Schulte, Joachim L. Schultze, Alexander Sczyrba, Yogesh Singh, Oliver Stegle, Jens
623 Stoye, Fabian Theis, Janne Vehreschild, Jörg Vogel, Max von Kleist, Andreas Walker, Jörn
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658 NIKOLAOS dRAKOULIS, JOSEF M. dUMANOV, VIcTOR A. TUTELYAN, GENNAdII
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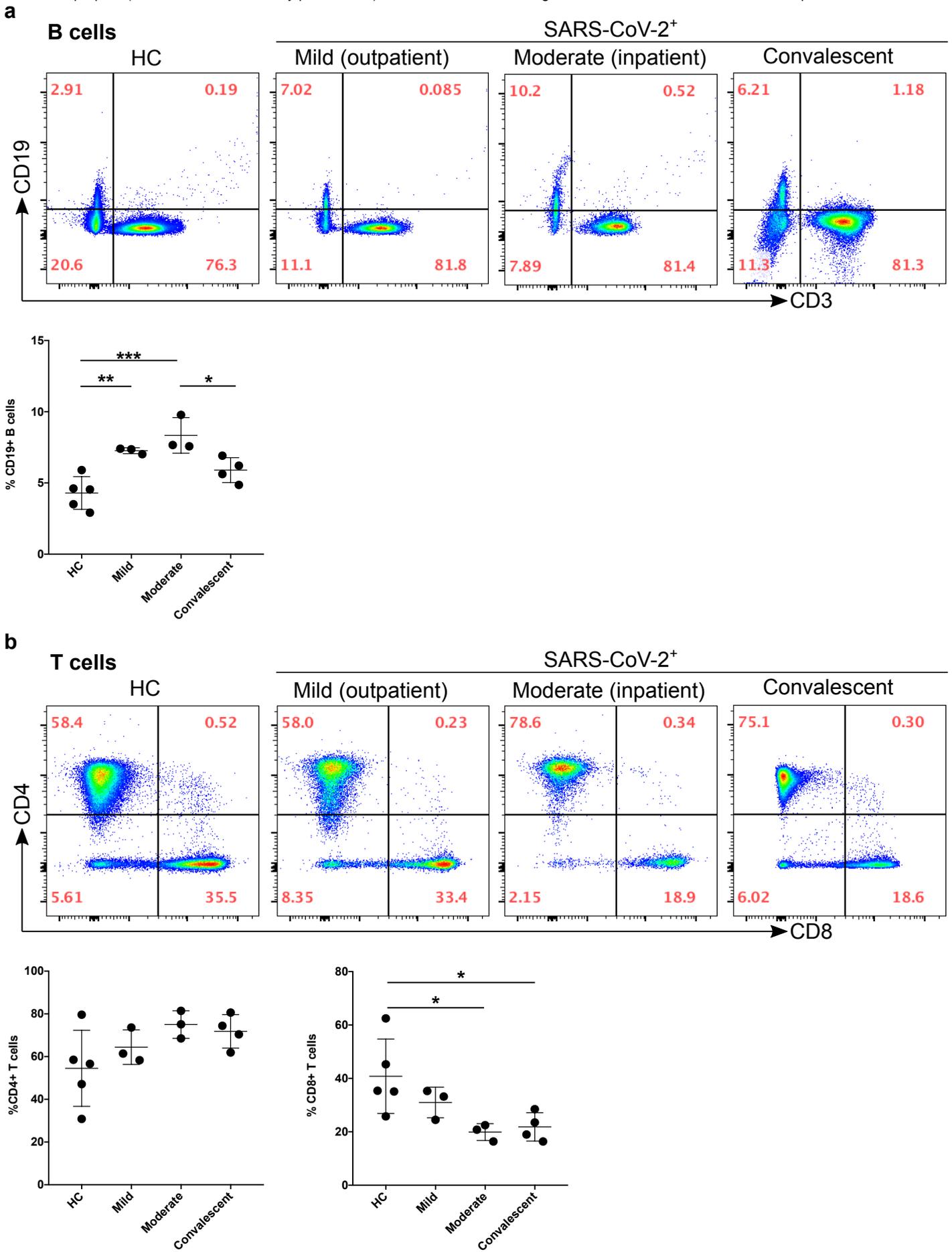


Fig. 2

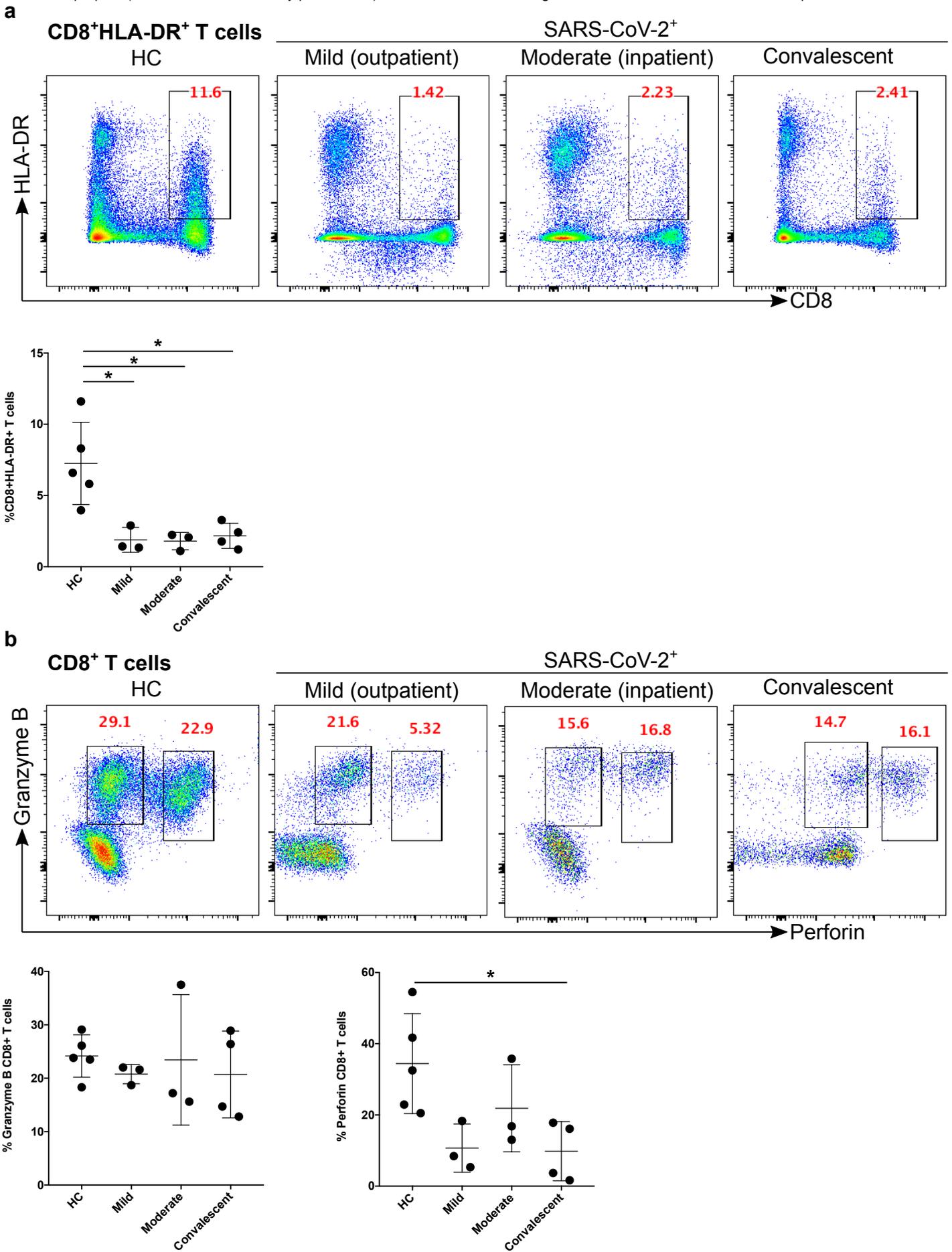


Fig. 3

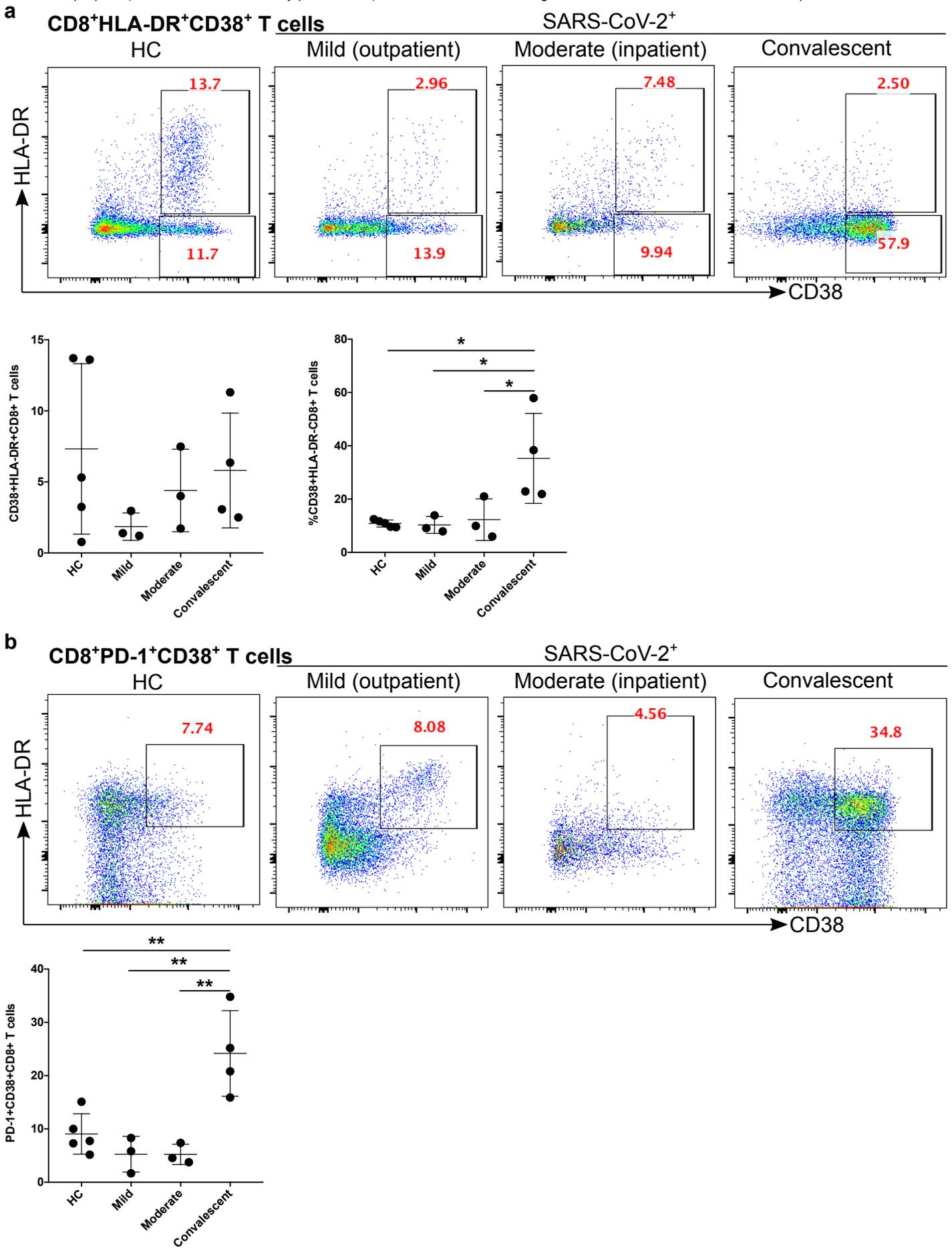


Fig. 4

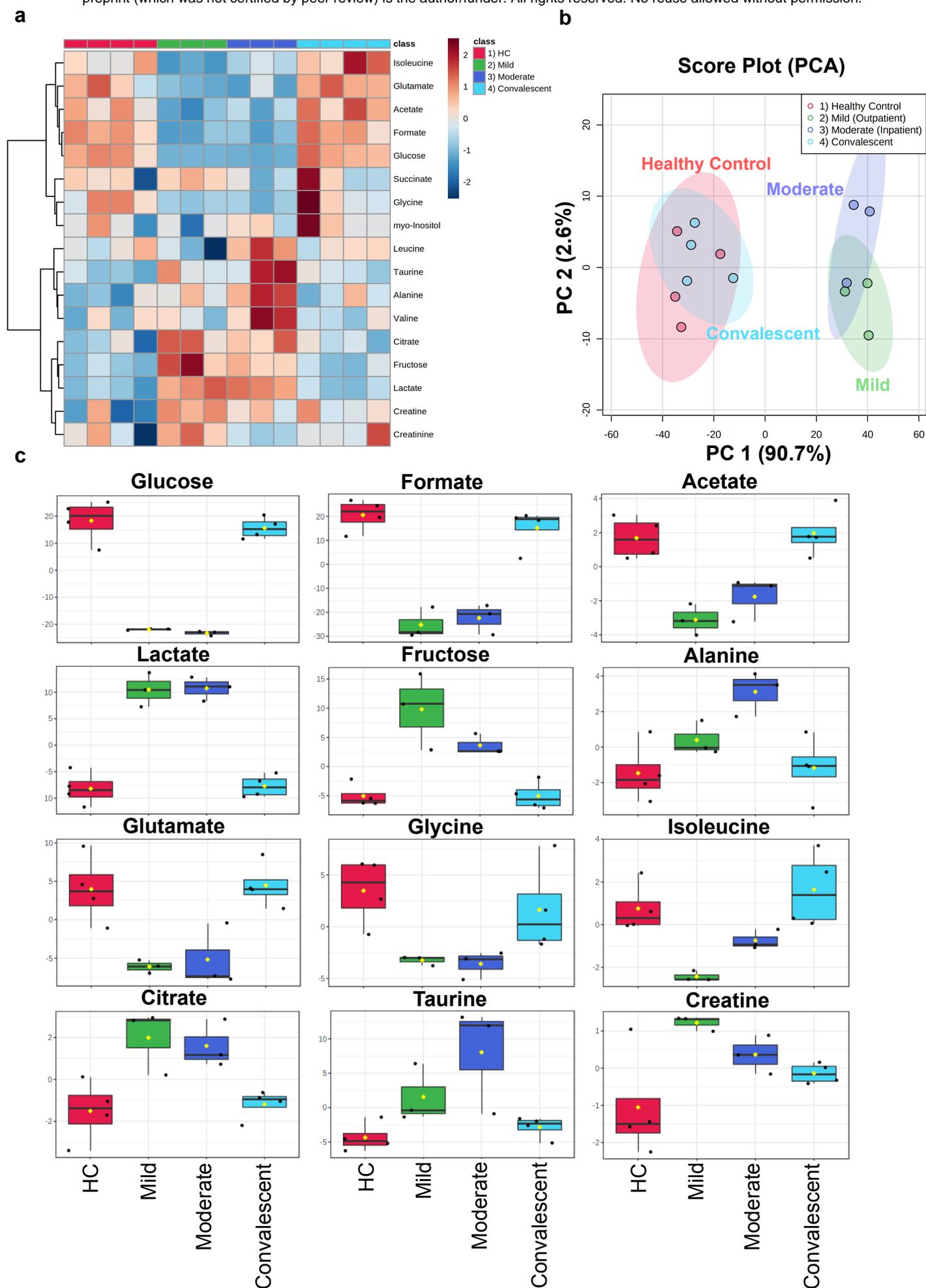
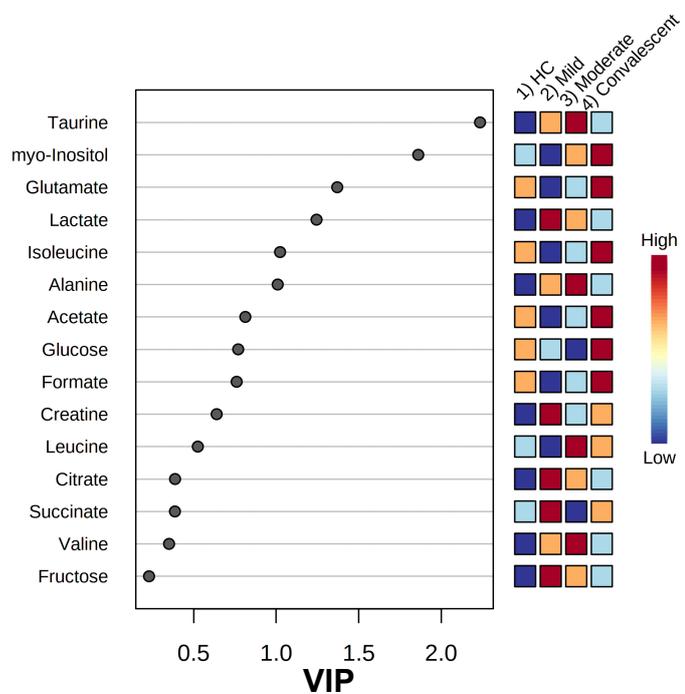
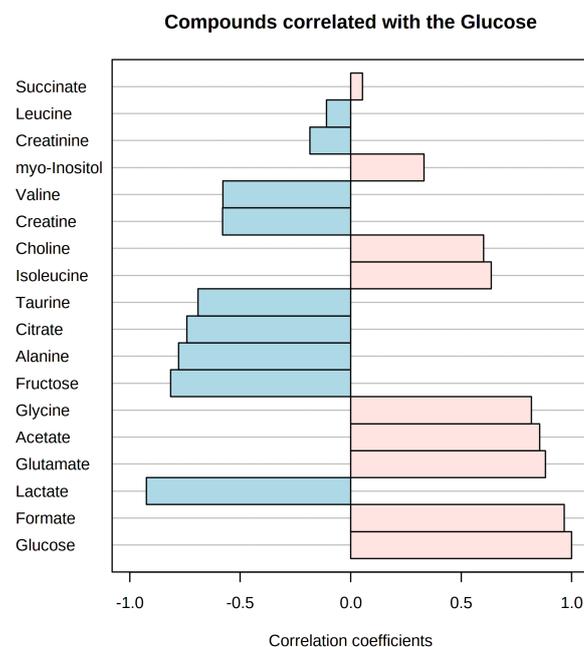


Fig. 5

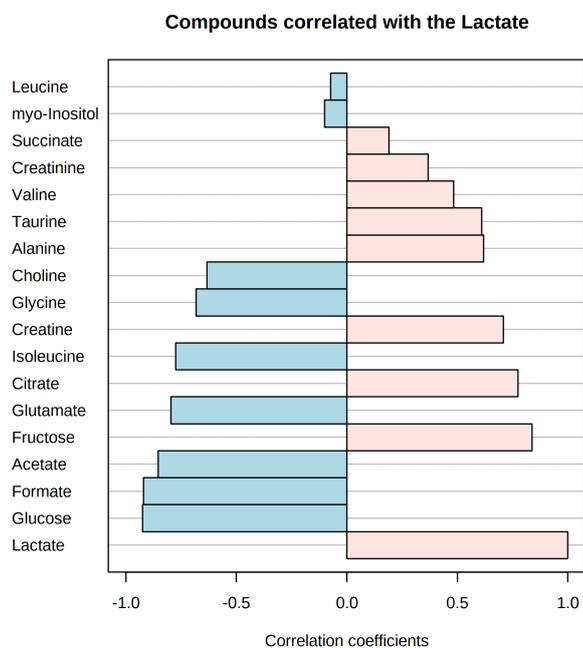
a



b



c



Compounds correlated with the Fructose

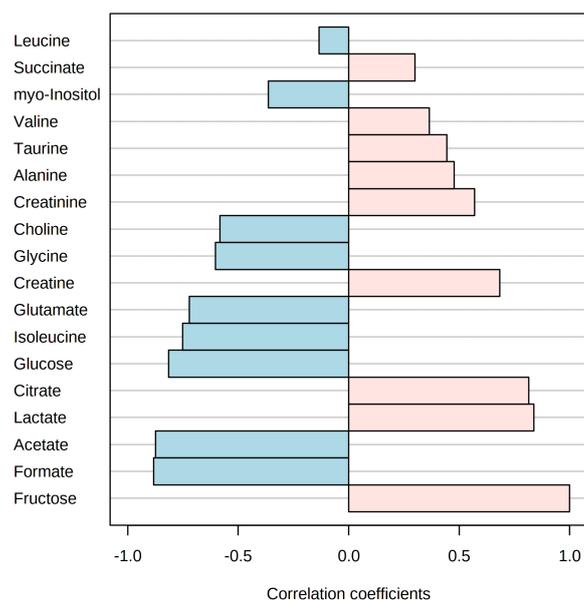
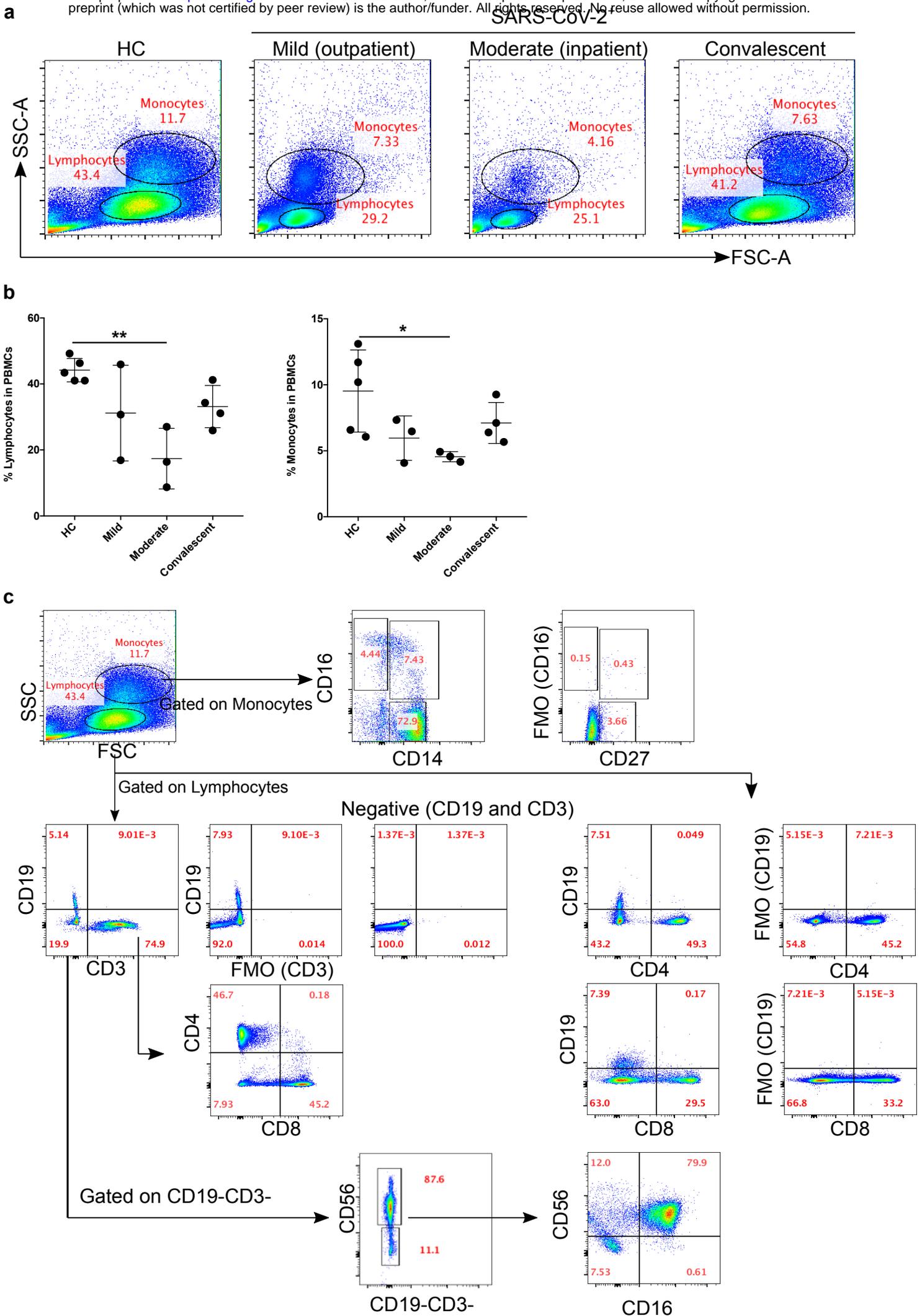
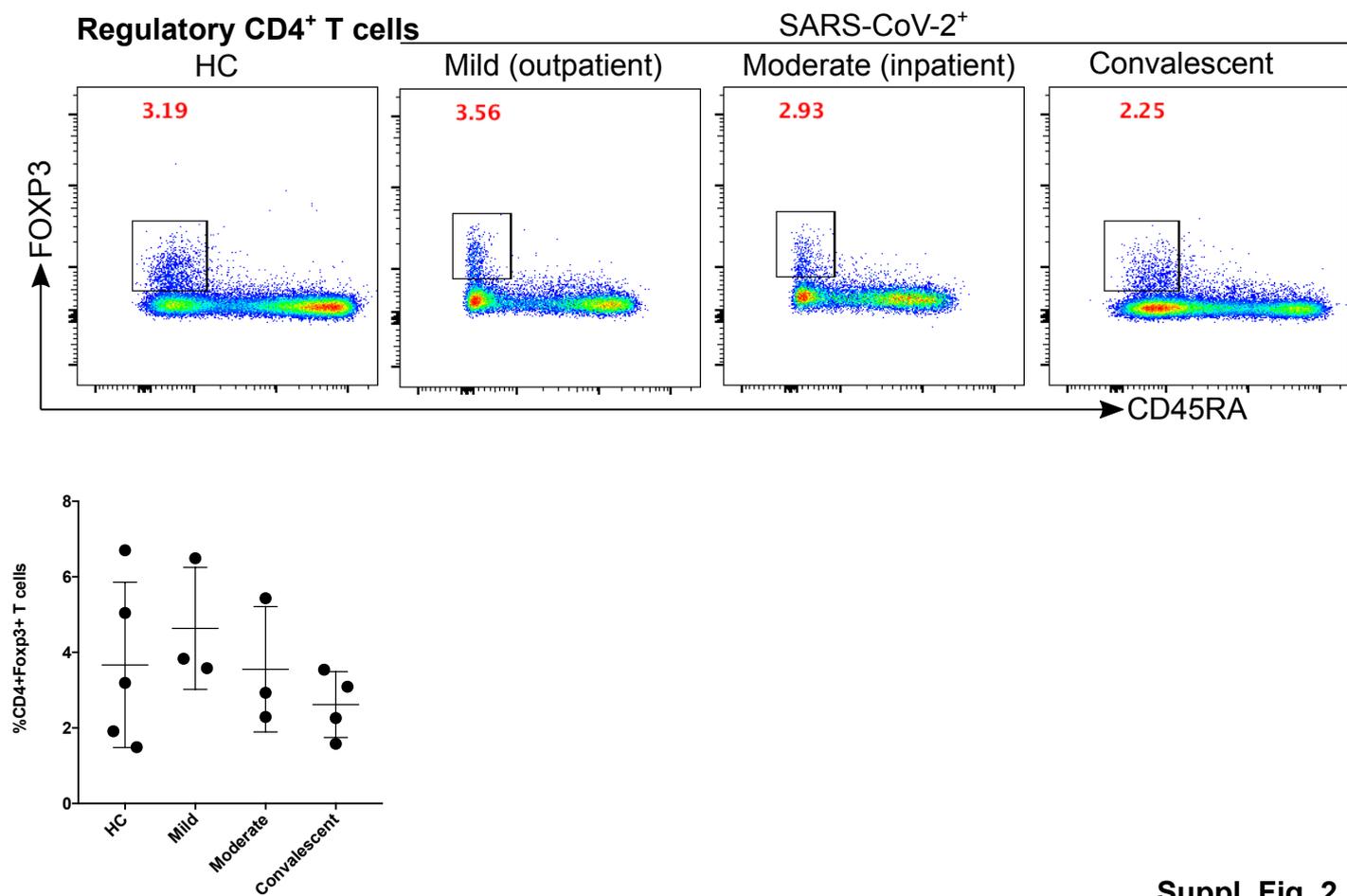


Fig. 6

SARS-CoV-2





Suppl. Fig. 2

