Increased levels of circulating neurotoxic metabolites in patients with mild Covid19

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Abstract

SARS-CoV-2 corona virus causes a multi-faceted and poorly defined clinical and pathological phenotype involving hyperinflammation, cytokine release, and long-term cognitive deficits, with an undefined neuropathological mechanism. Inflammation increases the activity of the kynurenine pathway, which is linked to neurodegenerative and psychiatric disorders. We sought to determine whether the kynurenine pathway is impacted in patients with mild COVID-19, leading to elevated neurotoxic metabolites in blood, and whether such changes are associated with pro-inflammatory cytokines. Serum samples were taken from 150 patients and analyzed by ELISA and ultra-high performance liquid chromatography (UHPLC). The data were analyzed using multiple linear regression models adjusted for age and sex. We found increased levels of kynurenine, quinolinic acid and 3-hydroxykynurenine in serum from patients with mild COVID-19, together with increased levels of IL-6, ICAM-1, VCAM-1 and neopterin. The levels of neurotoxic metabolites were significantly associated with key inflammatory cytokines including IL-6 and TNFα. The COVID-19 risk-factor hypertension was associated with the highest levels of neurotoxic metabolites in plasma. These neuroactive metabolites could be part of the pathological mechanisms underlying cognitive impairment during and post-COVID and should be explored as potential biomarkers for long-COVID symptoms.

Keywords: Mild COVID-19, Tryptophan metabolism, 3-HK, quinolinic acid

Introduction

COVID-19, caused by SARS-CoV-2, can lead to systemic disease, including pneumonia, acute respiratory distress syndrome, and impaired consciousness (1). COVID-19 also leads to an activation of innate and adaptive immune responses which result in a substantial inflammatory response (2). In a certain percentage of patients, symptoms persist over time with the potential of symptoms lasting months and up to years after initial illness. In these patients, there is a predominance for those effected by the illness to present neuropsychiatric symptoms (3). Recent studies have shown an increased risk for acute and long-term sequalae after COVID-19 in both vaccinated and unvaccinated patients (4). However, there is currently a lack of understanding for the factors that drive the neuropsychiatric symptoms present during acute and long-term COVID-19. Thus, there is an urgent need to identify biomarkers that can indicate the disease progression during long-COVID, as well as contribute to the understanding of its underlying pathology.

The kynurenine pathway is the major route for tryptophan (TRP) metabolism, and it contributes to several fundamental biological processes, all converging in energy metabolism. Infections and inflammatory conditions can alter the activity of the enzymes in this pathway (5). Several neuroactive metabolites of the kynurenine pathway that bind neuronal receptors have an underlying role in both neurological and psychiatric symptoms (5). As such, quinolinic acid (QUIN) is an agonist of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor and kynurenic acid (KYNA) is an antagonist of the same receptor (6). In high concentrations, QUIN induces excitotoxic neuronal death by allowing excessive amounts of calcium to enter the cell (6). While KYNA blocks the cholinergic *α*7

83 nicotinic receptor, it also antagonizes the glycine site of the NMDA-receptor, thus
84 preventing calcium influx (7, 8).
85
86 Another kynurenine pathway metabolite, 3-hydroxykynurenine (3-HK) is also neurotoxic
87 and pro-inflammatory (9). 3-HK promotes reactive oxygen species (ROS) generation
88 through several oxidative conversions (10), and 3-HK can accelerate endothelial cell
89 apoptosis (11). The neurotoxic effects of QUIN and 3-HK are additive (5). Entry of QUIN
90 and KYNA into the central nervous system (CNS) is likely partially prohibited by an intact
91 blood brain barrier (BBB). KYN (the metabolite produced from TRP metabolism) and 3-
92 HK, can freely pass the BBB (12). Within the brain, KYN is metabolized to KYNA by
93 astrocytes (5, 13), or to 3-HK and then further to QUIN by microglia and macrophages (5,
94 14). SARS-CoV-2 infection is known to affect the KYN levels by inducing the production
95 of the pro-inflammatory cytokine, interferon-gamma (IFN-ɣ) that stimulates the rate-
96 limiting enzyme in the kynurenine pathway, indoleamine-2,3-dioxygenase (IDO), thus
97 affecting KYN levels (15).

98 As previously mentioned, SARS-CoV-2 can lead to a substantial inflammatory response,
99 which affects the levels of different metabolites from the kynurenine pathway. However,
100 there are also other proteins involved in the inflammatory response, such as the
101 intercellular cell adhesion molecule-1 (ICAM-1) and the vascular cell adhesion protein-1
102 (VCAM-1), in which we were interested. Both ICAM-1 and VCAM-1 are cell surface
103 glycoproteins that govern immune cell migration to sites of inflammation and T-cell-
104 mediated immunity in tissues (16-18). They are expressed in vascular endothelial cells
105 and in response to inflammation, their expression is induced in epithelial and immune

106 cells (19, 20). ICAM-1 and VCAM-1 play a central role in leukocyte trafficking, lymphocyte
107 activation and several immune responses, and the upregulation of ICAM-1 is a signature
108 event during inflammation (21). A recent study showed that monocyte-derived
109 macrophages drive the inflammatory response to SARS-CoV-2 and long-term changes
110 in inflammatory response of monocytes can be detected in convalescent SARS-CoV-2
111 patients following mild infection (22).Therefore, it is of interest to determine whether the
112 levels of ICAM-1 and VCAM-1 are affected in patients with mild COVID-19, and whether
113 they are linked to kynurenine pathway activation.

114 In the current study, we analyzed serum samples from 150 patients, 44 of whom tested
115 positive for COVID-19 but exhibited mild disease and were non-hospitalized. We sought
116 to determine whether the production of neuroactive and neurotoxic metabolites along the
117 kynurenine pathway, as well as several proteins involved in inflammation, is altered in
118 patients with mild COVID-19, as this could be associated to the underlying
119 neuropsychiatry symptoms. Kynurenine pathway metabolites are correlated with the
120 severity and predicted negative outcomes of symptoms in COVID-19 patients; and could
121 potentially serve as biomarkers or predictors of neuropsychiatric long-covid symptoms.
122

Results

Demographics of study participants

Serum samples were taken from 150 individuals, 44 were diagnosed with mild COVID-19 (defined for this purpose as positive, but not requiring hospitalization or treatment) and 106 were controls who tested negative for COVID-19. Demographics of study participants are shown in **Table 1**. The average age of SARS-CoV-2 positive individuals was 44.2 ± 13.1 years and controls were 45.6 ± 13.4 years. 47 females (44.3%) were included as controls and 25 females (56.8%) were enrolled with mild COVID-19. Of all participants, 8 controls (7.5%) and 7 COVID +ve (15.9%) were Asian, 5 controls (4.7%) and 0 COVID +ve (0%) were Black/African, 84 controls (79.2%) and 35 COVID +ve (79.5%) were White/Caucasian, 4 controls (3.8%) and 2 COVID +ve (4.5%) were Other, and 5 control (4.7%) and 0 COVID +ve (0%) opted to not answer.

Higher levels of IL-6 present in patients with mild COVID-19

Individuals with mild COVID-19 demonstrated significantly higher levels of IL-6 when compared to negative controls (ANOVA F: 5.260, p= 0.0028**) (**Figure 1a**). TNF-α levels were not altered when both groups were compared (ANOVA F: 2.347, p= 0.075 ns) (**Figure 1b**). No significant differences were observed for the other inflammatory markers including IL-13 (F: 1.847, p= 0.141 ns), IL-8 (F: 1.223, p= 0.304 ns), IFN-ɣ (F: 2.054, p= 0.109 ns, data not shown). All data was corrected for age and sex.

144 ***Higher levels of several proteins related to inflammation present in patients with***
145 ***mild COVID-19***

146 The level of ICAM-1 was increased in patients who tested positive for COVID-19 when
147 compared to the ones that did not (data corrected for sex and age, ANOVA test F: 5.823,
148 p<0.001 ***) (**Figure 2a**). Similarly, patients positive for COVID-19 also showed increased
149 levels of VCAM-1 when compared to the PCR-negative patients (data adjusted for sex
150 and age, ANOVA F: 3.307, p= 0.022 *). The inflammatory related protein, neopterin was
151 also increased in COVID-19 positive patients (data adjusted for age and sex, ANOVA test
152 F: 3.309, p= 0.022 *) (**Figure 2b and 2c)**

153 ***Metabolites of the kynurenine pathway are increased in patients with mild COVID-***
154 ***19***

155 Previous research demonstrated alterations in kynurenine pathway activity following
156 infection (23-25). Therefore, we investigated alterations in kynurenine pathway
157 metabolite levels. Kynurenine (KYN), the first metabolite of the pathway, was significantly
158 increased in patients with mild COVID-19 when compared to patients who tested negative
159 for SARS-CoV-2 (ANOVA test F: 11.195, p= 0<0.001 ***, **Figure 3**). Additionally, 3-HK
160 and QUIN, were increased in patients who tested positive for COVID-19 when compared
161 to patients who did not (ANOVA test F: 3.990, p= 0.009 **; F: 8.492, p<0.001 ***,
162 respectively, **Figure 3**). Further, picolinic acid (PIC) levels in COVID-19 positive patients
163 were significantly decreased when compared to COVID negative patients (ANOVA test
164 F: 4.399, p= 0.005 **). Finally, anthranilic acid (AA) was also significantly increased in
165 patients with mild COVID-19 when compared to the patients who tested negative for the

166 virus (ANOVA test F: 4.024, p= 0.009 **, **Figure 2d**). All data was corrected for age and
167 sex.

168 The ratio of several metabolites was used to further investigate the induction of the
169 kynurenine pathway. The KYN/TRP ratio was quantified to determine the induction of the
170 kynurenine pathway. When both groups were compared, COVID-19 patients showed
171 significantly increased levels of the induction of the kynurenine pathway (ANOVA test F:
172 6.377, p< 0.001 ***, **Figure 3**). The QUIN/TRP ratio, which has been previously identified
173 as a biomarker for neurological diseases (26), was also analyzed. COVID-19 positive
174 cases showed a statistically significant increase in this ratio when compared to the non-
175 COVID-19 patients (ANOVA test F: 5.837, p<0.001 ***, **Figure 3**). Another neurotoxic
176 ratio that was measured was QUIN/KYNA. In this study, COVID-19 positive patients
177 presented significantly higher ratios when compared to the control group (ANOVA test F:
178 2.847, p= 0.040 *, **Figure 3**). All data was corrected for age and sex.

179 ***Correlation between neurotoxic metabolites of the kynurenine pathway and pro-***
180 ***inflammatory cytokines***

181 Previous research has shown the relationship between inflammation and the kynurenine
182 pathway activity. Therefore, we subsequently investigated whether the levels of
183 kynurenine metabolites and inflammatory cytokines were correlated. As shown in **Figure**
184 **4**, there was a positive correlation between the levels of TNF-α and KYN (Pearson
185 correlation: 0.453; p=0.002**), 3-HK (Pearson correlation: 0.527; p<0.001***) and QUIN
186 (Pearson correlation: 0.482; p<0.001***). Furthermore, a positive correlation between IL-
187 6 and both 3-HK (Pearson correlation: 0.328; p=0.03*) and QUIN (data adjusted for age

188 and sex, Pearson correlation: 0.418; p=0.005**) was also found. All data was corrected
189 for age and sex.

190 ***Increased levels of neurotoxic metabolites in COVID-19 patients with hypertension***

191

192 We then analyzed patients who presented with hypertension, a well-known risk factor for
193 severe COVID-19, and compared them with the patients that were not hypertensive in
194 both COVID-19 positive and negative patients. Regression analysis adjusted for age and
195 sex revealed that COVID-19 hypertensive patients presented evidence of higher levels
196 when compared to non-hypertensive COVID-19 patients in the following proteins: IL-2
197 (fold change 2.66, p=0.003**), IL-6 (fold change 1.45, p-value=0.014*), TNF-α (fold
198 change 1.38, p=0.012*), 3-HK (fold change 1.26, p=0.08), and the QUIN/TRP ratio (fold
199 change 1.14, p = 0.089) (**Figure 5**).

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Discussion

In the current study, we investigated the inflammatory and kynurenine pathway metabolite signatures between mild COVID-19 cases and controls. We found increases in several kynurenine pathway metabolites, such as KYN, QUIN, 3-HK and PIC, together with higher levels of IL-6. Additionally, the increased levels in QUIN/TRP, QUIN/KYN, QUIN/KYNA and KYN/TRP, further support a recent published study by Cihan and colleagues (23). Pro-inflammatory pathway proteins, along with metabolites from the kynurenine pathway were significantly increased in patients that presented with both COVID-19 and hypertension when compared to COVID-19 patients that were not hypertensive. Those proteins included IL-2, IL-6, TNFα, ICAM-1, VCAM-1, 3-HK, QUIN, QUIN:TRP and KYN:TRP.

Kynurenine pathway metabolites are correlated with severity and predicted negative outcomes of symptoms in COVID-19 patients; therefore, it is important to understand the role of kynurenine metabolites in mild COVID-19 patients and long-haulers, in particular those with neuropsychiatric symptoms (15). In this study, we found the levels of ICAM-1 and VCAM-1 to be significantly increased in patients with mild SARS-CoV-2, in particular those with hypertension. Increased levels of ICAM-1 and VCAM-1 in mild and severe cases of COVID-19 infection has already been reported in a small study (27), where it was observed that the severity of COVID-19 disease was associated with increased levels of ICAM-1 and VCAM-1. The main caveat with that study was the small number of patients analyzed. Our new findings support the prior observations and link increases in endothelial cell adhesion molecules to kynurenine pathway metabolites, such as IDO.

228 IDO is expressed in endothelial cells of vessel walls, and under pathological states, a
229 decrease in IDO leads to an increase in VCAM-1 (28). Therefore, understanding how the
230 kynurenine pathway is involved in inflammatory diseases and how it can alter the levels
231 of ICAM-1 and VCAM-1 will be important to understand, especially in the context of
232 COVID-19.
233
234 Lionetto et al., measured the levels of KYN and TRP in the serum of healthy patients,
235 SARS-CoV-2 negative patients, and SARS-CoV-2 positive patients (29). In SARS-CoV-2
236 positive patients, the KYN/TRP ratio was higher when compared to the negative and the
237 healthy controls (29). We also found an increase in the KYN/TRP ratio in SARS-CoV-2
238 positive patients when compared to those who tested negative. The KYN/TRP ratio is
239 usually used as an indirect measure of the activity of the IDO enzyme (30); which
240 catalyzes tryptophan. Previous research has demonstrated that IDO is regulated by IFN-
241 ɣ (31, 32). We observed no differences in the levels of serum IFN-ɣ in mild COVID-19
242 samples. However, IDO activation and gene expression has also been shown to be
243 altered by noncanonical pathways in addition to IFN-ɣ (33). This potentially explains the
244 increased kynurenine pathway activation that we observed in our cohort. The activation
245 of the kynurenine pathway may cause long-lasting inflammation rather than the acute
246 inflammation observed early in COVID-19. Therefore, it will be of interest to follow the
247 kynurenine metabolites as markers in patients with symptoms of long-COVID.
248
249 Together with the KYN/TRP ratio, the QUIN/TRP ratio was increased in patients with
250 COVID-19 when compared to controls. In a previous study, Drewes and colleagues

251 suggest that cerebrospinal fluid (CSF) QUIN/TRP ratio could be an early, predictive
252 marker of CNS disease (26). In simian-immunodeficient virus (SIV)-infected macaques,
253 the ratio of QUIN/TRP was significantly increased in cases that led to severe encephalitis
254 (26). Encephalitis is one of the many potential consequences of COVID-19 patients (34);
255 and the QUIN/TRP ratio could be used to determine the outcome of patients. Our data
256 show a significant increase in this ratio in COVID-19 patients, when compared to those
257 that tested negative for the virus. We also found that the ratio between QUIN/TRP was
258 also increased in hypertensive COVID-19 patients, suggesting that patients with
259 hypertension might be prone to a worse outcome.
260
261 Cihan and colleagues analyzed kynurenine pathway metabolites and inflammatory
262 cytokines and found a positive correlation between IL-6 and various metabolites from the
263 kynurenine pathway (23). Our study supports a positive correlation between IL-6 and
264 QUIN as well as between IL-6 and 3-HK. Since Cihan's study analyzed severe and ICU
265 cases of COVID-19 patients, it is possible that the lack of correlation between IL-6 and
266 other kynurenine metabolites in our study is because only mild COVID-19 cases were
267 analyzed. The correlation of IL-6 and kynurenine metabolites could also potentially be
268 used as a biomarker of disease severity.
269
270 The correlation between IL-6, IFN-ɣ, and TNF-α and kynurenine metabolites supports the
271 link between inflammation, SARS-CoV-2 and the kynurenine pathway. We found a
272 positive correlation between TNF-α and three metabolites: 3-HK, KYN, and QUIN. TNF-
273 α affects the kynurenine pathway in patients with schizophrenia, as a positive correlation

274 between the levels of TNF-α and KYN has been reported (35). TNF-α may accelerate the
275 formation of KYN from the catabolism of TRP (35). We did not observe differences in
276 TNF-α levels between negative and positive SARS-CoV-2 patients, however, we did find
277 a correlation between TNF-α and KYN levels, similar to what has been reported for
278 schizophrenia (35). In this study, QUIN, 3-HK and KYN were significantly increased in
279 patients with mild COVID-19. Both QUIN and 3-HK are neurotoxic metabolites and were
280 positively correlated with TNF-α and IL-6 in the case of QUIN, and TNF-α in the case of
281 3-HK, it is possible that the inflammation observed in COVID-19 patients further
282 contributes to the neuronal damage caused by the neurotoxic metabolites.
283 To summarize, we found an increase in neurotoxic metabolites of the kynurenine pathway
284 in patients with mild COVID-19. Furthermore, the neurotoxic metabolites were correlated
285 with inflammatory markers and vascular injury markers, such as TNF-α, IL-6, VCAM-1
286 and ICAM-1. We hypothesize that the activation of the neurotoxic branch of the
287 kynurenine pathway might contribute to neurological, cognitive, and psychiatric
288 symptoms experienced in COVID-19 and its aftermath. We suggest that these
289 metabolites should be studied further for their potential as biomarkers of long COVID and
290 as potential contributors to the disease mechanisms underlying long COVID.
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296

297 **Materials and Methods**

298

299 *Blood samples*

300 Blood was drawn by venipuncture of the right or left antecubital vein. Blood was allowed
301 to clot at room temperature prior to centrifugation. Serum was aliquoted into cryovials and
302 immediately transferred to -80°C for storage until biological assays.

303

304 *Detection of tryptophan, serotonin, and kynurenine metabolites*

305 Serum samples were mixed with extraction solution, briefly vortexed and centrifuged. The
306 supernatant, which contains the metabolites of interest was removed and dried under
307 reduced pressure conditions for ninety minutes in a GeneVac EZ-2 Plus speedvac (SP
308 Scientific, Warminster,PA). Dried down extracts were then resuspended in 0.1% formic
309 acid in Milli-Q water, once resuspended samples were centrifuged through a COSTAR
310 Spin-X 0.22-um filter tube and transferred to an amber vial containing a glass insert.

311

312 *Quantification of kynurenine metabolites*

313 Kynurenine pathway metabolites (KYN, KYNA, 3-HK, QUIN, PIC, NTA, NIC, AA), TRP
314 and serotonin were quantified using reverse phase ultra-high-performance liquid
315 chromatography (UHPLC) coupled to a triple quadrupole mass spectrometer (1290
316 Infinity II LC System, 6470 Triple quadrupole, Agilent Technologies, Santa Clara, CA).
317 Five-microliters of samples were injected onto a Vanguard HSS T3 Pre-column that was
318 connected to an Acquity HSS T3 analytical column. Elution conditions used a combination
319 of Solvent A (0.1% formic acid in LC/MS grade Water) and Solvent B (0.1% formic acid

320 in 90% LC/MS grade acetonitrile) at a flow rate of 0.4mL/min. Agilent Masshunter
321 Quantitative Analysis Software (v9.0, Agilent) was used to analyze and export data.
322 Intra-assay coefficients of variability (CV) for plasma analytes: TRP 4.1%, KYN 2.5%,
323 KYNA 1.6%, 3-HK 5.0%, QUIN 2.8%, PIC 3.3%, NTA 1.6%, NIC 5.4%, AA 8.4% and 5-
324 HT 2.2%.
325 Inter-assay CVs: TRP 4.8%, KYN 1.8%, KYNA 1.5%, 3-HK 4.9%, QUIN 1.8%, PIC 1.9%,
326 NTA 1.8%, NIC 6.5%, AA 7.2% and 5-HT 4.9%.
327 Lower limits of detection (LLOD) were found to be as follows: TRP 36.6 nM, KYN 2.2 nM,
328 KYNA 0.16 nM, 3-HK 0.29 nM, QUIN 4.15 nM, PIC 0.63 nM, NTA 0.98 nM, NIC 0.07 nM,
329 AA 0.98 nM and 5-HT 0.73 nM.
330
331 *Quantification of cytokines and alpha-synuclein*
332 Three Meso Scale Discovery (MSD) multiplex kits (Meso Scale Diagnostics LLC,
333 Rockville, MD) were read using a MESO QuickPlex SQ 120 plate reader. Samples were
334 run in duplicate according to the manufacturer's instructions and sample concentrations
335 were generated through MSD Discovery Workbench 4.0 software. Samples below the
336 average LLOD were denoted as the average LLOD across plates. For α-synuclein, the
337 U-PLEX human α-synuclein kit was used. Samples were diluted 1:8 with sample diluent
338 and generated an inter-plate CV of 3%, an average intra-assay CV of 5.2%, and the LLOD
339 of 0.876 pg/mL.
340
341 Using the MSD V-PLEX human vascular injury II kit, we quantified C-reactive protein
342 (CRP), serum amyloid A (SAA), ICAM-1, and VCAM-1. Samples were diluted 1:1000 with

343 manufacturer's diluent reagent. The inter-plate CV was 10.3% (CRP: 8.3%, SAA: 9.7%,
344 ICAM-1: 15.2%, VCAM-1: 8.1 %), average intra-assay CV 5.6% (CRP 4.2%, SAA 6.5%,
345 ICAM-1 6.6%, and VCAM-1 5.2%), the LLOD were 1.077pg/mL CRP, 12.839 pg/mL SAA,
346 1.037 pg/mL ICAM-1, and 6.618 pg/mL VCAM-1.
347
348 The MSD V-PLEX human proinflammatory I kit was used to determine the level of IFN-ɣ,
349 interleukin (IL)-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, and tumor necrosis factor
350 alpha (TNF-α). Samples were diluted 1:1 with sample diluent. Inter-plate CV were
351 calculated (**see Supplementary Table 1**), and the LLOD was IFN-ɣ: 0.087 pg/mL, IL-10:
352 0.025 pg/mL, IL-12p70: 0.0385 pg/mL, IL-13: 0.209 pg/mL, IL-1β: 0.032 pg/mL, IL-2:
353 0.024 pg/mL, IL-4: 0.004 pg/mL, IL-6: 0.045 pg/mL, IL-8: 0.017 pg/mL, and TNF-α: 0.049
354 pg/mL.
355
356 *Quantification of Neopterin and S100B*
357 Neopterin ELISA kits were purchased from IBL America (Immuno-Biological Laboratories
358 Inc., Minneapolis, MN). Undiluted serum samples were used following the manufacturer's
359 protocol. S100B ELISA kits were purchased from Millipore (EMD Millipore, St. Louis, MO)
360 and samples were diluted 1:1. Plates were read using a Tecan Infinite M200 Pro plate
361 reader (Tecan Group Ltd, Männedorf, Switzerland). Sample concentrations were
362 generated using a 4 Parameter Logistic Curve Calculator (AAT Bioquest, "Quest Graph
363 Four Parameter Logistic (4PL) Curve Calculator" 15 Jul. 2021,
364 https://www.aatbio.com/tools/four-parameter-logistic-4pl-curve-regression-online-
365 calculator). The average inter-plate CV for Neopterin was 12.1%, the average intra-assay

366 CV was 2.2%, and the LLOD given by the manufacturer is 0.177 ng/mL. For S100B the
367 average inter-plate CV was 5.0%, the average intra-assay CV was 1.9%, and the LLOD
368 was 2.7 pg/mL as specified by the manufacturer.
369
370 *Statistical analysis*
371 Models were adjusted for age and sex in all instances. Robust linear regressions to
372 assess differences between PCR+ individuals with and without hypertension were
373 analyzed via R v 4.1.0 (https://cran.r-project.org/). Correlation analyses and ANOVAs
374 were done with SPSS Statistics (version 28.0.1.0). Graphs were generated using
375 GraphPad Prism version 9.0374 (GraphPad Software, La Jolla, CA). For all tests,
376 statistical significance was considered as $p<0.05$ and weak evidence as $0.05 < p < 0.1$.
377
378 *Study approval*
379 This study utilized a subset of samples from the Beaumont Health Large-Scale
380 Automated Serologic Testing for COVID-19 study (36) and was approved by the
381 Institutional Review Board (IRB) at the Beaumont Research Institute Detroit, Michigan,
382 USA (2021-110). The final cohort consisted of 150 individuals (44 Mild COVID-19 cases
383 and 106 controls) randomly selected from the registry. Individuals testing positive for
384 SARS-CoV-2 in a qPCR test at the time of sampling were assigned “Covid19 positive”
385 whereas individuals testing negative in a qPCR test for C SARS-CoV-2 at the time of
386 blood sample were assigned “Covid19 negative” for the purpose of this study.
387
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Author Contributions

Conceptualization, LB SFG, PB; data curation, ESM, ARB, CDC, CF, KH; formal analysis, ESM, LB, SFG, PB, regression analysis with R, ZM; methodology, LB; visualization, ESM, ARB; writing—original draft, ESM, ARB; writing—review and editing, SG, MXH, JAP, PB, SFG, LB.

Acknowledgements

We would like to thank the Farmer Family Foundation for their generous contribution, which allowed for this research to be conducted. Additionally, we would like to thank the Van Andel Institute Metabolomics and Bioenergetics Core for providing technical assistance. This project was also supported with Beaumont institutional funds as well as through the Beaumont Health Foundation with philanthropic gifts provided by the following: Sidney & Madeline Forbes, Nathan & Catherine Forbes, Edward C. Levy & the Linda Dresner Foundation, Stephen & Bobbi Polk, Warren & Carol Ann Rose & Family, Elizabeth Rose, Mickey Shapiro & Family, S. Evan & Gwen Weiner and The Hearst Foundations.

410 **List of abbreviations**

411 3-HK: 3-HydroxyKynurenine

412 AA: Anthranilic Acid

413 BBB: Blood Brain Barrier

414 COVID-19: Coronavirus Disease (2019)

415 CI: Confidence Interval

416 CSF: Cerebrospinal Fluid

417 Glu: Glutamate

418 ICAM-1: Intercellular Adhesion Molecule 1

419 ICU: Intensive Care Unit

420 IDO: Indoleamine 2,3-Dioxygenase

421 IFN-γ: Interferon Gamma

422 IL: Interleukins

423 KYN: Kynurenine

424 KYNA: Kynurenic Acid

425 PA: Picolinic Acid

426 PCR: Polymerase Chain Reaction

427 ROS: Reactive Oxygen Species

428 S100B: S100- Calcium-Binding Protein B

429 SAA: Serum Amyloid A

430 SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

431 SIV: Simian Immunodeficient Virus

432 TNF-α: Tumor Necrosis Alpha

433 TRP: Tryptophan

434 QUIN: Quinolinic acid

435

436 **Data availability**

437 Data generated is available upon request.

438

439 **Competing interests**

440

441 P.B. has received support as a consultant from Calico Life Sciences, CureSen,
442 Enterin Inc, Idorsia Pharmaceuticals, Lundbeck A/S, AbbVie, Fujifilm-Cellular Dynamics
443 International, and Axial Therapeutics. He has received commercial support for research
444 from Lundbeck A/S and F. Hoffman-La Roche. He has ownership interests in Acousort
445 AB, Axial Therapeutics, Enterin Inc and RYNE Biotechnology. During the time that this
446 paper was written he became an employee of F. Hoffman-La Roche, although none of
447 the data were generated by this company.

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618 **Table 1. Demographics of the patients included in the current study**

Mild SARS-CoV-2 Demographics		
	SARS-CoV-2 Negative (n = 106)	**SARS-CoV-2 Positive** (n = 44)
Age (Mean ± SD)	45.65 ± 13.39	44.18 ± 13.12
Sex F (%)	47 (44.3%)	25 (56.8%)
Race n (%)		
Asian	8 (7.5%)	7 (15.9%)
Black/African	5 (4.7%)	0 (0%)
White/Caucasian	84 (79.2%)	35 (79.5%)
Other	4 (3.8%)	2 (4.5%)
No Answer	5 (4.7%)	0 (0%)

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620 SD: Standard Deviation
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Figures:

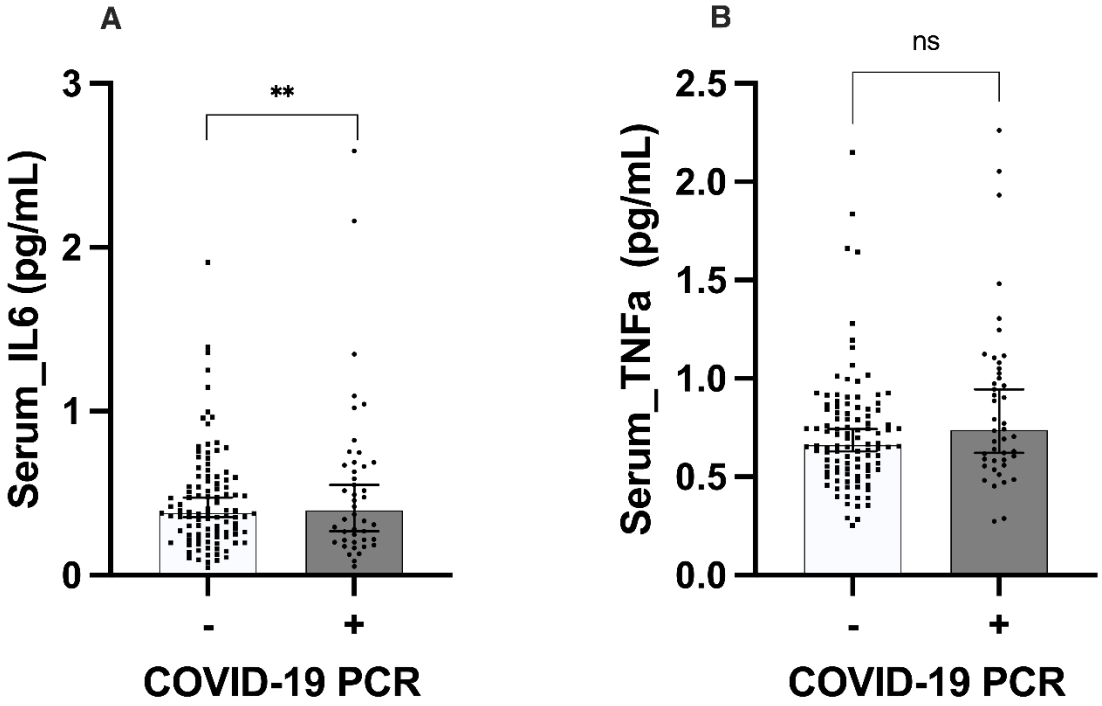

Figure 1. Significantly higher levels IL-6 in patients with mild COVID-19 when compared to controls. a) Significantly higher levels of IL-6 were found in patients with COVID-19 when compared to the negative controls (data adjusted for age and sex, ANOVA test F: 5.260, p=0.002 **). **b)** No differences were found in the level of TNF-α between the two groups (data adjusted for age and sex, ANOVA test F: 2.347, p= 0.075 ns). Graphs are represented by median with 95% of confidence interval (CI).

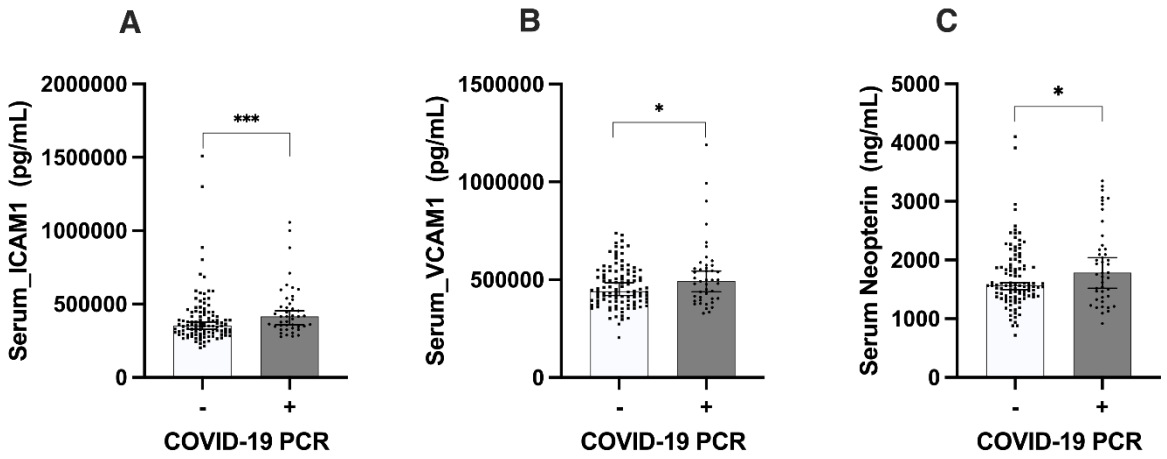

Figure 2. Significantly higher levels of serum ICAM-1, VCAM-1, and neopterin in patients with COVID-19. When the levels of all three proteins were analyzed, patients with COVID-19 presented significantly higher levels of **a)** ICAM-1 (data adjusted for age and sex, ANOVA test F: 5.823, p<0.001 ***), **b)** VCAM-1 (data adjusted for age and sex, ANOVA test F: 3.307, p= 0.022 *), and **c)** neopterin (data adjusted for age and sex, ANOVA test F:3.309, p= 0.022*). Graphs are represented by median with 95% of confidence interval (CI).

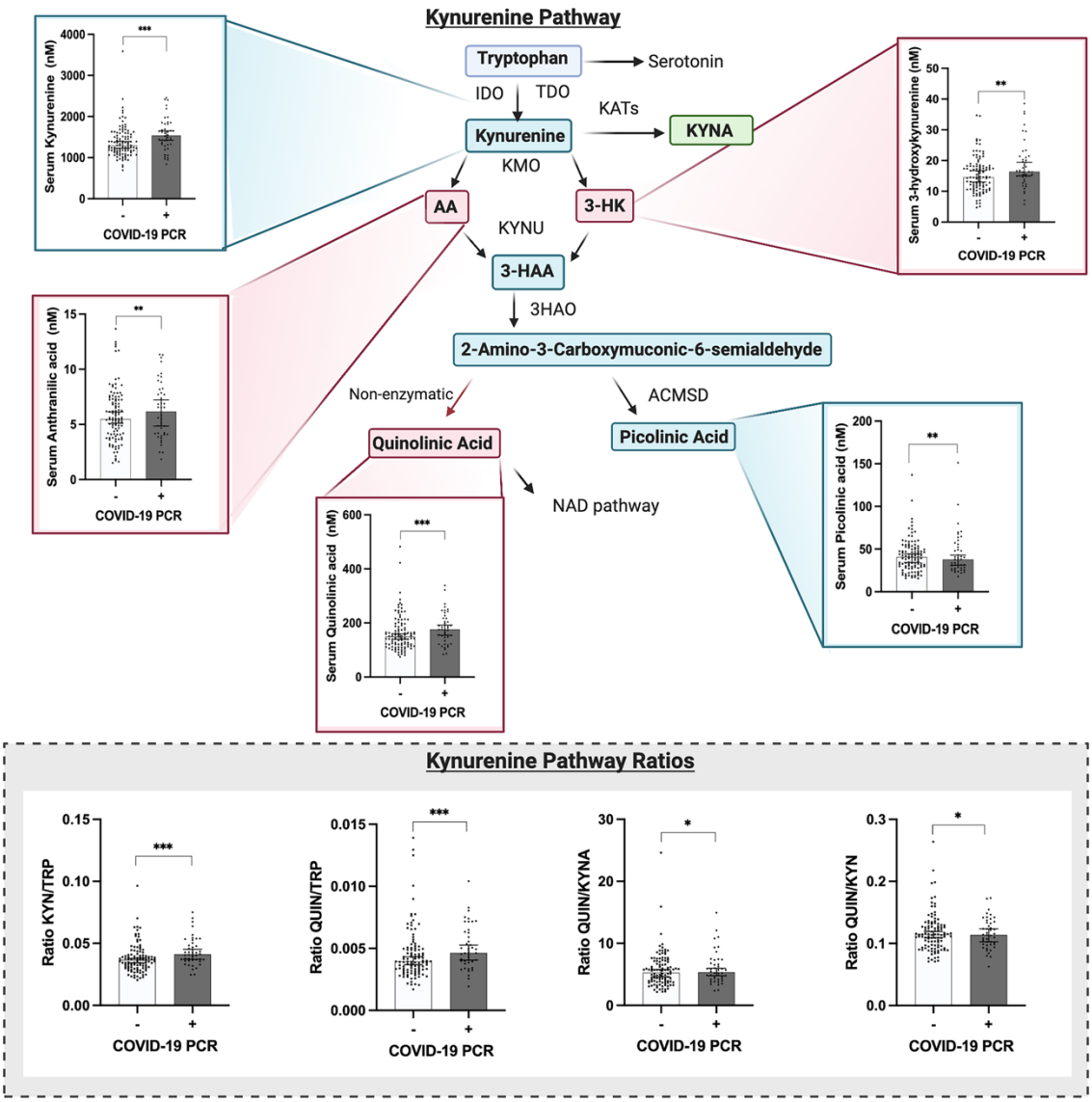

Figure 3. The kynurenine pathway is altered in patients with mild COVID-19, who present increased levels of neurotoxic metabolites. Significantly increased levels of kynurenine (data adjusted for age and sex, ANOVA test F: 11.195, p<0.001 ***), 3-hydroxykynurenine (data adjusted for age and sex, ANOVA test F: 3.390, p=0.009 **), anthranilic acid (data adjusted for age and sex, ANOVA test F: 4.024, p=0.009 **), and quinolinic acid (data adjusted for age and sex, ANOVA test F: 8.492, p<0.001 ***) were found in patients with mild COVID-19 when compared to controls. When the ratio of the metabolites was analyzed, significantly increased levels of KYN/TRP (data adjusted for age and sex, ANOVA test F: 6.377, p<0.001 ***) and QUIN/TRP (data adjusted for age and sex, ANOVA test F: 5.837, p<0.001 ***), as well as QUIN/KYNA (data adjusted for age and sex, ANOVA test F: 2.847, p= 0.040 *) were found in patients with COVID-19. Graphs show the median with 95% of CI. Abbreviations: IDO, Indoleamine 2,3-dioxygenase; TDO, Tryptophan 2,3-dioxygenase; KATs, Kynurenine aminotransferase; KYNA, kynurenic acid; KMO, Kynurenine 3-monooxygenase; AA, anthranilic acid; 3-HK, 3-hydroxykynurenine; KYNU, Kynureninase; 3-HAA, 3-hydroxyanthranilic acid; 3HAO, 3-hydroxyanthranilate oxidase; ACMSD, Aminocarboxymuconate-semialdehyde decarboxylase, and NAD, Nicotinamide adenine dinucleotide.

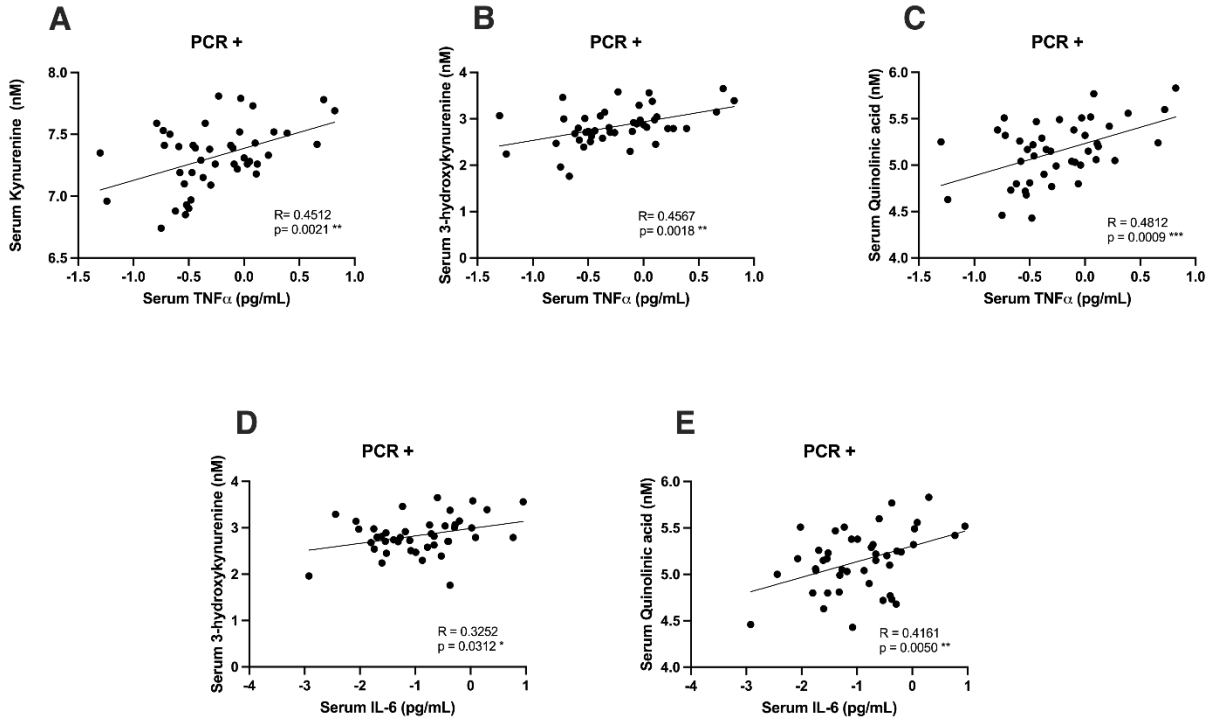

Figure 4. There is correlation between inflammatory cytokines and metabolites of the kynurenine pathway in patients with mild COVID-19. Patients with mild COVID-19 present a positive correlation between TNF-α and kynurenine (data adjusted for age and sex, Pearson R: 0.4512; p=0.0021 **), TNF-α and 3-hydroxykynurenine (data adjusted for age and sex, Pearson R: 0.4567; p=0.0018 **), TNF-α and quinolinic acid (data adjusted for age and sex, Pearson R: 0.4812; p=0.0009 ***), IL-6 and 3-hydroxykynurenine (data adjusted for age and sex, Pearson R: 0.3252, p=0.0312 *) and between IL-6 and quinolinic acid (data adjusted for age and sex, Pearson R: 0.4161, p=0.0050 **).

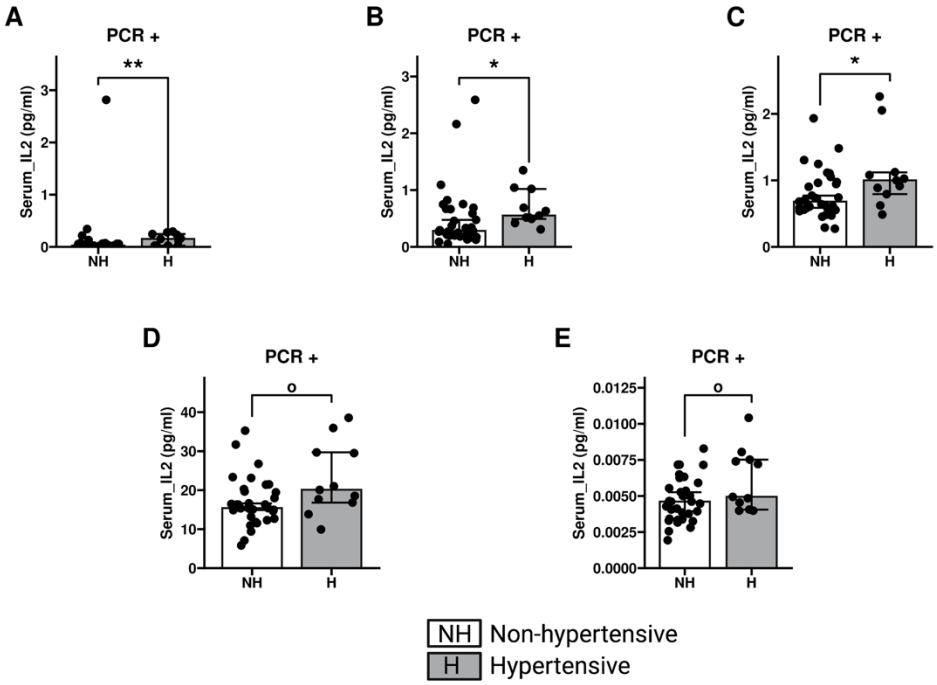

Figure 5. Increased metabolites and ratio in COVID-19 positive hypertensive patients when compared to non-hypertensive COVID-19 patients. Higher levels of IL-2 (fold change 2.66, p=0.003**), IL-6 (fold change 1.45, p-value=0.014*), and TNF-a (fold change 1.38, p=0.012*) in COVID-19 hypertensive patients when compared to COVID-19 non-hypertensive patients. Weak evidence of increased levels in 3-HK (fold change 1.26, p=0.08º), and the QUIN/TRP ratio (fold change 1.14, p = 0.089º). Graphs are represented by median with 95% of confidence interval (CI).