Increased kynurenine-to-tryptophan ratio in the serum of patients infected with SARS-CoV2: An observational cohort study.☆

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ABSTRACT

Immune dysregulation is a hallmark of patients infected by SARS-CoV2 and the balance between immune reactivity and tolerance is a key determinant of all stages of infection, including the excessive inflammatory state causing the acute respiratory distress syndrome. The kynurenine pathway (KP) of tryptophan (Trp) metabolism is activated by pro-inflammatory cytokines and drives mechanisms of immune tolerance. We examined the state of activation of the KP by measuring the Kyn:Trp ratio in the serum of healthy subjects ($n = 239$), and SARS-CoV2-negative ($n = 305$) and -positive patients ($n = 89$). Patients were recruited at the Emergency Room of St. Andrea Hospital (Rome, Italy). Kyn and Trp serum levels were assessed by HPLC/MS-MS. Compared to healthy controls, both SARS-CoV2-negative and -positive patients showed an increase in the Kyn:Trp ratio. The increase was larger in SARS-CoV2-positive patients, with a significant difference between SARS-CoV2-positive and -negative patients. In addition, the increase was more prominent in males, and positively correlated with age and severity of SARS-CoV2 infection, categorized as follows: $1 =$ no need for intensive care unit (ICU); $2 \leq 3$ weeks spent in ICU; $3 \geq 3$ weeks spent in ICU; and $4 =$ death. The highest Kyn:Tr*p* values were found in SARS-CoV2-positive patients with severe lymphopenia. These findings suggest that the Kyn:Trp ratio reflects the level of inflammation

associated with SARS-CoV2 infection, and, therefore, might represent a valuable biomarker for therapeutic intervention.

1. Introduction

One of the most challenging aspects of SARS-CoV2 infection is to unravel the complex metabolic interplay between the virus and the immune system, which drives all phases of infection. Immune responses observed in severely ill patients, such as lymphopenia/eosinopenia and the cytokine storm leading to acute respiratory distress, are unique to SARS-CoV2 and are rarely observed in other respiratory viral infections [1]. Of note, SARS-CoV2 may infect T lymphocytes by interacting with the surface protein, CD147 [2]. There are only a few studies exploring the profile of distinct subsets of CD4$^+$ and CD8$^+$ T lymphocytes in SARS-CoV2 infection. Flow cytometric analysis showed increases in naïve T cells and reductions in memory CD4$^+$ and cytotoxic CD8$^+$ T cells in severe SARS-CoV2 patients in Wuhan [3]. Absolute levels of T regulatory (T$_{reg}$) cells were also reduced as a result of lymphopenia, but the ratio between T$_{reg}$ and the whole number of CD4$^+$Th cells was greater in severely ill patients (calculated from the data presented in Table 3 of ref. [3]). Whether mechanisms of immune tolerance are triggered during SARS-CoV2 infection, and how they associate with the severity of infection is unclear. In respiratory virus infections, immune tolerance can limit lung immunopathology [4], but it might delay viral clearance. Understanding how SARS-CoV2 influences the immune system is a mandatory step for a correct use of immunomodulatory agents in the treatment of infected patients and may pave the way to novel therapeutic interventions.

A large body of evidence indicates that the kynurenine pathway of tryptophan (Trp) metabolism has a key role in the regulation of the immune system. The pathway is activated by three enzymes, tryptophan 2,3-dioxygenase (TDO), and types 1 and 2 indoleamine 2,3-dioxygenase (IDO-1 and -2), which convert Trp into N-formylkynurenine. N-Formylkynurenine is transformed into kynurenine (Kyn), which is transaminted into kynurenic acid or, alternatively, hydroxylated by kynurenine 3-monooxygenase into 3-hydroxykynurenine, the precursor of 3-hydroxyanthranilic acid, quinolinic acid, and nicotinic acid. Anthranilic, xanthurenic, and cinnabarinic acids are also generated by cleavage of the side chain of Kyn, transamination of 3-hydroxykynurenine, and non enzymatic oxidation of 3-hydroxyanthranilic acid, respectively [5]. Interferon gamma (IFN-γ) and other inflammatory cytokines potently induce IDO expression in macrophages, dendritic cells, and other cell types (see Discussion and References therein). Activation of the kynunenine pathway in IDO-competent cells, such as antigen-presenting cells and epithelial cells, restrains inflammation and induces long-term immune tolerance *via* the following mechanisms: (i) Trp depletion activates the *general control non-depressible 2* (GCN2) stress kinase in dendritic cells and macrophages enhancing the production of anti-inflammatory cytokines and causing differentiation and recruitment of T$_{reg}$ cells [6]; (ii) Kyn binds to, and activates, the aryl hydrocarbon receptor (AhR) in both dendritic cells (DCs) and T cells. This promotes the conversion of T effector cells into T$_{reg}$ cells, and also promotes IDO induction, thereby creating a Kyn/AhR/IDO loop that sustains immune suppression [7,8]; interestingly, AhR has been implicated in the pathogenesis of SARS-CoV2 infection [9,10]; (iii) other kynurenine metabolites, *e.g.*, kynurenic, 3-hydroxyanthranilic, and cinnabarinic acids, display antinflammatory action and promote immune tolerance [11]; and (iv) IDO-1 may lead to immune tolerance independently of its enzymatic activity by acting as a signaling molecule in dendritic cells [12,13]. The Kyn:Trp ratio, measured in blood, is a valuable clinical benchmark of IDO-1 activation, and reflects the state of immune activation or tolerance in cancer immunity, autoimmunity, infection and other disorders. Changes in Kyn:Trp ratio have been associated with viral infections, such as HIV and HIV-human herpes

virus co-infection [14], seasonal influenza [15], Dengue virus [16], and HBV [17].

Thomas et al. [18] performed a metabolomic analysis in the plasma of 33 SARS-CoV2-positive and 16 negative subjects, the latter group including both sieronegative subjects and convalescent plasma donors. They found reductions in blood serotonin, 3-hydroxykynurenine, and anthranilic acid levels and increases in Kyn and kynurenic acid levels in SARS-CoV2-positive patients, which positively correlated with IL-6 levels, *i.e.*, with the extent of inflammation. No sex-related analysis was performed [18].

Here, we measured the ratio between Kyn and Trp serum levels, which reflects the state of activation of the kynurenine pathway, in SARS-CoV2-positive and SARS-CoV2-negative patients recruited from the Emergency Room of the St. Andrea Hospital in Rome, and in healthy control subjects. We found a large increase in the Kyn:Trp ratio in SARS-CoV2-positive subjects, which correlated with the severity of the disease, and was more pronounced in male patients and in patients with severe lymphopenia.

2. Material and methods

2.1. Patients

During the period from 10 March 2020 to 5 May 2020 we recruited 394 patients (181 males, 213 females) admitted to the Emergency Room of St. Andrea Hospital in Rome; 305 patients were negative (138 males, 167 females) and 89 positive (43 males, 46 females) for SARS-CoV2 testing performed at admission to the Emergency Room. SARS-CoV2-negative subjects were transferred from the Emergency Room to intensive care unit (ICU) (8.20%), pulmonology ward (2.95%), infectious disease ward (0.98%), internal medicine ward (7.54%), stroke unit (1.97%), surgery wards (7.87%), gastroenterology ward (5.90%), cardiology ward (3.93%), home management (39.02%) or other wards (21.64%). SARS-CoV2-positive patients were transferred from the Emergency Room to ICU (36.11%), pulmonology ward (16.66%), infectious disease ward (19.44%), internal medicine ward (9.72%) or low-intensity Covid-19 ward (18.07%). The clinical severity of the SARS-CoV2 infection was evaluated according to the following score system: 1 = no need for ICU; 2 ≤3 weeks spent in ICU; 3 ≥ 3 weeks spent in ICU; 4 = death. SARS-CoV2-negative healthy subjects (*n* = 239, 87 males, 152 females) were recruited among Sant'Andrea Hospital healthcare workers from October 10, 2020 to November 20, 2020.

There were no inclusion or exclusion criteria for patients recruited at the Emergency Room. None of the patients had cancer. Inclusion criteria for healthy control subjects were the absence of chronic inflammatory, autoimmune, neurological, psychiatric, and endocrine disorders (even in the asymptomatic stage), cardiovascular or chronic kidney disorders, and cancer. Subjects with mild hypertension were included in the control group. All patients and control subjects were > 18 years old. This study was approved by the local ethics committee and followed the Declaration of Helsinki.

2.2. Detection of SARS-CoV2 in nasopharyngeal and oropharyngeal swabs

Nasopharyngeal and oropharyngeal specimens were collected using specific COPAN UTM-RM virus transport medium (Copan Diagnostics Inc., Murrieta, CA, USA). To increase the SARS-CoV2 viral load into COPAN transport medium, nose and throat swabs were combined in a single collection tube for each patient. A volume of 200 μl from nasopharyngeal and oropharyngeal samples was subjected to viral RNA

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Table 1

SARS-Cov2-negative and -positive patients' comparison for sex, age and serum levels of Kyn, Trp, and the Kyn:Trp ratio. Data are reported as median (interquartile range).

	Healthy subjects (*N*= 239)	Overall (*N* = 394)	Negative (*N* = 305)	Positive (*N* = 89)	*p* Value
Sex (M, %)	36.4	45.94	45.24	48.3	**0.044**[†,1]
					0.057[†,2]
					0.63[†,3]
Age	46.00 (38.00–53.00)	62.00 (46.75–78.00)	60.00 (45.00–78.00)	66.00 (55.50–77.00)	**<0.0001**[‡,1 2]
					0.096[‡,3]
Kynurenine (µg/ml)					
Overall	0.30 (0.26–0.37)	0.42 (0.30–0.59)	0.40 (0.28–0.55)	0.55 (0.38–0.94)	**<0.0001**[‡,1,2,3]
Males	0.33 (0.27–0.39)	0.46 (0.34–0.68)	0.43 (0.32–0.61)	0.66 (0.44–1.09)	**<0.0001**[‡,1,2,3]
Females	0.30 (0.24–0.36)	0.39 (0.28–0.54)	0.35 (0.27–0.49)	0.48 (0.34–0.62)	**<0.0001**[‡,1,2,3]
Tryptophan (µg/ml)					
Overall	11.9 (10.1–13.9)	8.04 (5.96–10.90)	9.05 (6.35–11.20)	6.63 (4.91–8.04)	**<0.0001**[‡,1,2,3]
Males	12.6 (10.5–14.1)	8.49 (6.23–10.95)	9.02 (6.78–11.12)	6.62 (4.88–9.58)	**<0.0001**[‡,1,2,3]
Females	11.45 (9.8–13.5)	7.85 (5.80–10.60)	9.18 (6.08–11.50)	6.64 (4.97–7.80)	**<0.0001**[‡,1,2,3]
Kyn:Trp ratio					
Overall	0.03 (0.02–0.03)	0.05 (0.03–0.10)	0.040 (0.03–0.08)	0.10 (0.06–0.15)	**<0.0001**[‡,1,2,3]
Males	0.03 (0.02–0.03)	0.06 (0.04–0.11)	0.05 (0.03–0.09)	0.11 (0.06–0.20)	**<0.0001**[‡,1,2,3]
Females	0.03 (0.02–0.03)	0.05 (0.03–0.09)	0.04 (0.03–0.08)	0.08 (0.05–0.14)	**<0.0001**[‡,1,2,3]

Statistically significant p Values are highlighted in bold.

[†] Fisher's exact test
[‡] Mann Whitney U test
[1] Healthy subject *Vs* Negative
[2] Healthy subject *Vs* Positive
[3] Negative *Vs* Positive

extraction using QIAamp Viral RNA mini kit following manufacturer instructions. Specifically, elution was performed in a volume of 60 µl of Qiagen elution buffer. Real-time RT-PCR was performed by using All-plex™ 2019-nCoV Assay of Seegene (CE-marked assay; Seul, Republic of Korea). An exogenous internal control (IC; 10 µl) provided with All-plex™ 2019-nCoV kit was added to each aliquot of 200 µl nasopharyngeal and oropharyngeal samples before viral RNA extraction to monitor the quality of the entire molecular workflow. Afterward, 8 out of 60 µl eluted RNA samples was RT-PCR amplified as suggested by the Seegene manufacturer on Biorad CFX96 real-time system. The Allplex™ 2019-nCoV Assay is based on the simultaneous detection of E gene (Sabercovirus, β-coronavirus), RdRP and N genes (specific for Sars-CoV2) and exogenous IC. Results were analysed by the aim of Seegene viewer software settled for the analysis of fluorescent curves produced by Allplex™ 2019-nCoV Assay. The interpretation criteria were the following: a positive signal detected in IC and at least two in E, RdRP and N genes with a cycle threshold (CT) values ≤40 were considered positive for Sars-CoV2 RNA whereas positive signals in IC and only one in E, RdRP and N genes with a CT values ≤40 were considered as inconclusive and addressed to a second nasopharyngeal and oropharyngeal sampling. No fluorescent signals or over the 40th CT in E, RdRP and N genes were considered nonspecific and reported as negative for Sars-CoV2 RNA (in these cases CT values of IC must be <40). The declared LoD is 100 RNA copies/reaction.

2.3. Serum kynurenine and tryptophan analysis by LC-MS/MS method

Blood samples were collected within 24 h after admission to the Emergency Room, centrifuged to obtain serum samples, and stored at −80 °C until analysis. Sixty microliters of serum sample were treated using 60 µl of deproteinizing solution. The mixture was mixed for 30 s and centrifuged at 14.000 rpm for 15 min. Ten microliters of clean supernatant were injected into the chromatographic system. Compounds were detected using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) analytical method [19]. Chromatographic separation of analytes was performed using an Agilent Liquid Chromatography System series 1100 (Agilent Technologies, USA), on a F5 column (100 × 2.1 mm, Kinetex 2.6 µm F5, 100 Å, Phenomenex, CA, USA) equipped with a security guard pre-column (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of a solution of 0.1% aqueous formic acid (A) and 100% acetonitrile (B); elution was performed at flow rate of 300 µl/min, using an elution gradient. The mass spectrometry method was performed on a 3200 triple quadrupole system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray source. The detector was set in the positive ion mode. The instrument was set in the Multiple Reaction Monitoring (MRM) mode. Data were acquired and processed by the Analyst 1.5.1 Software.

2.4. Statistics

Statistical analysis was performed using GraphPad PRISM version 8.2.0. Normality of the data was assessed by the Kolmogorov-Smirnov test. Continuous variables were expressed as median and interquartile range (IQR). Fisher's exact test was used to evaluate sex distribution between SARS-CoV2-positive and -negative patients. Kruskall-Wallis test was executed to analyse the relationships among Kyn, Trp and Kyn:Trp ratio and positivity to SARS-CoV2, Mann-Whitney *U* test was used to analyse sex, lymphocytes counts of patients and COVID-19 severity score. Spearman's correlation was used to assess the relation between Kyn:Trp ratio and age in the overall population and in the subgroups of male and female patients. Statistical significance was set at $p < 0.05$.

3. Results

Serum Kyn and Trp levels and the Kyn:Trp ratio were determined in 239 healthy subjects, 305 SARS-CoV2-negative, and 89 SARS-CoV2-positive patients. In SARS-CoV2-positive and –negative patients, blood was collected within 24 h after admission to the Emegency Room. There were no inclusion or exclusion criteria in the recruitment of SARS-CoV2-positive or -negative patients. Thus, both groups were formed by patients with any disorder who required medical support in the Emergency Room. Median age was 46 years (range, 38–53) in the healthy control group; 60 years (range, 45–78) in SARS-CoV2-negative patients; and, 66 years (range, 55.7–77) in SARS-CoV2-positive patients. There was no difference in sex and age distribution between SARS-CoV2-positive and –negative patients, whereas sex and age distributions in control group were significantly different compared to patients groups (Table 1).
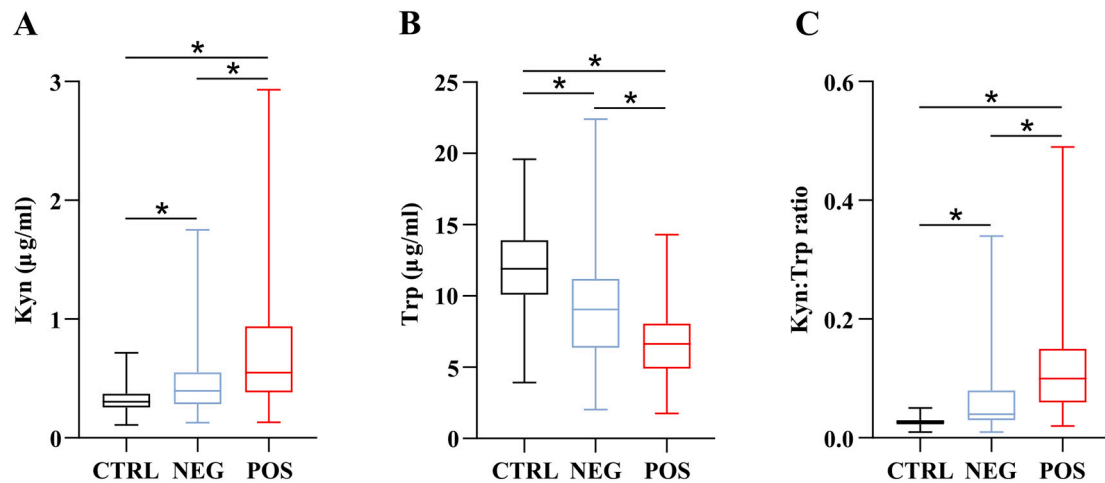
Fig. 1. Changes in serum Kynurenine and Tryptophan levels in healthy subjects, SARS-CoV2-positive and -negative patients.
Box plots of Kynurenine and Tryptophan levels are shown in (A) and (B), respectively. The Kynurenine:Tryptophan (Kyn:Trp) ratio is shown in (C). Kruskal-Wallis one-way ANOVA; A: H = 109.8; B: H = 169.8; C: H = 244.5. CTRL = healthy subjects, NEG and POS=SARS-CoV2-negative and positive patients. * $p < 0.0001$.
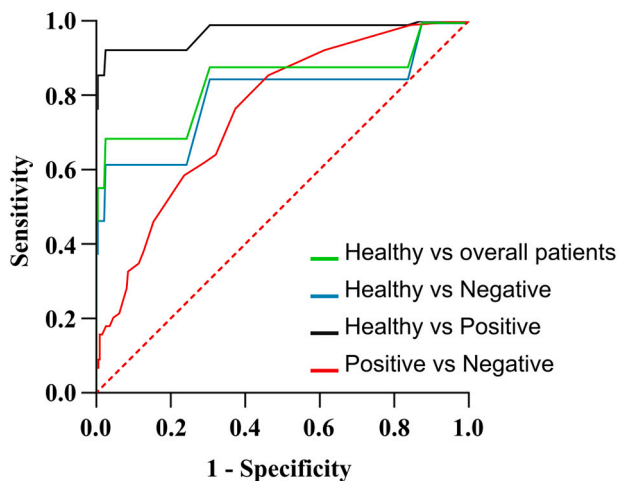

Fig. 2. Receiver operating characteristic (ROC) curve analysis of the Kynurenine:Tryptophan (Kyn:Trp) ratio for differentiating healthy subjects, SARS-CoV2-positive and SARS-CoV2–negative patients.

Kyn levels were significantly increased and Trp levels significantly reduced in both SARS-CoV2-negative and -positive patients as compared to healthy controls, and in SARS-CoV2-positive patients, as compared to SARS-CoV2-negative patients ($p < 0.0001$) (Fig. 1A, B). The Kyn:Trp ratio was 1.5-fold greater in SARS-CoV2-negative patients and 3.7-fold greater in SARS-CoV2-positive patients as compared to healthy controls ($p < 0.0001$; Fig. 1C, Table 1). The receiver operating characteristic (ROC) curves for the Kyn:Trp ratio for differentiating healthy subjects from i) overall patients, ii) negative patients, iii) positive patients, and differentiating SARS-CoV2-negative from -positive patients, showed a 0.8366, 0.7977, 0.9700 and 0.7552 AUC (area under curve) respectively (i) 95%CI:0.8053–0.8679; p < 0.0001; ii) 95%CI:0.7595–0.8359, p < 0.0001; iii) 95%CI:0.9463–0.9937, p < 0.0001; iv) 95% CI:0.7012–0.8091; p < 0.0001) (Fig. 2). To differentiate SARS-CoV2-positive from SARS-CoV2-negative patients, the Kyn:Trp ratio cut-off maximizing the Youden index (difference between true positive rate and false positive rate) was 0.045, while the highest sensitivity *plus* specificity value was 0.055.
Sex-related differences were detected for Kyn levels in healthy controls, SARS-CoV2-negative and -positive patients, with SARS-CoV2-positive male patients showing a 37% increase in the median value

with respect to female patients ($p = 0.004$; Fig. 3A, Table 1). Interestingly, sex-related differences were found in Trp levels in the control group ($p = 0.0167$), but not in both SARS-CoV2-positive ($p = 0.56$) and -negative patients ($p = 0.523$) (Fig. 3B). The Kyn:Trp ratio was significantly greater in male than in female in both SARS-Cov2-negative and -positive patients ($p = 0.013$ and $p = 0.027$, respectively; Fig. 3C, Table 1).
Spearman correlation analysis showed that the Kyn:Trp ratio increased with age in healthy subjects, (Spearman's $\rho = 0.349$, 95% CI 0.229–0.459; $p < 0.0001$), SARS-CoV2-negative (Spearman's $\rho = 0.567$, 95% CI 0.483–0.641, p < 0.0001) and SARS-CoV2-positive patients (Spearman's $\rho = 0.376$, 95% CI 0.176–0.547; $p = 0.0003$) (Fig. 4A), and the increase was significant in both males and females in SARS-CoV2-negative (Spearman's $\rho = 0.5418$, 95% CI 0.3762 to 0.6309, p < 0.0001) (Spearman's $\rho = 0.5980$, 95% CI 0.4873 to 0.6898, p < 0.0001) and -positive patients (Spearman's $\rho = 0.4312$, 95% CI 0.1413 to 0.6529, $p = 0.0039$) (Spearman's $\rho = 0.4047$, 95% CI 0.1209 to 0.6273, $p = 0.0053$) (Fig. 4B-C).
Searching for a correlation between the Kyn:Trp ratio and the severity of SARS-CoV2 infection, we used an arbitrary scale in which the following scores were assigned to infected patients: 1 = no need for ICU; 2 ≤3 weeks spent in ICU; 3 ≥ 3 weeks spent in ICU; 4 = death. All subgroups of SARS-CoV2-positive patients showed a significant increase in the Kyn:Trp ratio as compared to the control group and to the whole population of SARS-CoV2-negative patients (score = 0) (p(1 *vs* 0) = 0.0196, p(2 *vs* 0) = 0.039, p(3 *vs* 0) < 0.0001, p(4 *vs* 0) < 0.0001; Fig. 5). Patients with a more severe score (3–4) showed a significant increase of the Kyn:Trp ratio compared to patients who did not need intensive care (p(3 *vs* 1) = 0.0181, p(4 *vs* 1) < 0.0001; Fig. 5).
We also examined whether the presence of severe lymphopenia could affect the Kyn:Trp ratio in SARS-CoV2-positive patients. Lymphocyte counts were available for 80 SARS-CoV2-negative (38 males, 42 females) and 44 SARS-CoV2-positive (25 males, 19 females) patients. Lymphocytes were significantly decreased in SARS-CoV2-positive patients ($p = 0.0237$; Fig. 6A), and were lower in male patients ($p = 0.018$; Fig. 6B). Moreover, the Kyn:Trp ratio was significantly greater in SARS-CoV2-positive patients with severe lymphopenia (defined by an arbitrary cut-off value of $<0.6 \times 10^3$ lymphocytes/µl) with respect to SARS-CoV2-positive patients with lymphocyte counts $>0.6 \times 10^3$ lymphocytes/µl ($p = 0.0016$; Fig. 6C).

4. Discussion

We evaluated the involvement of KP activation in COVID-19

A

B

C
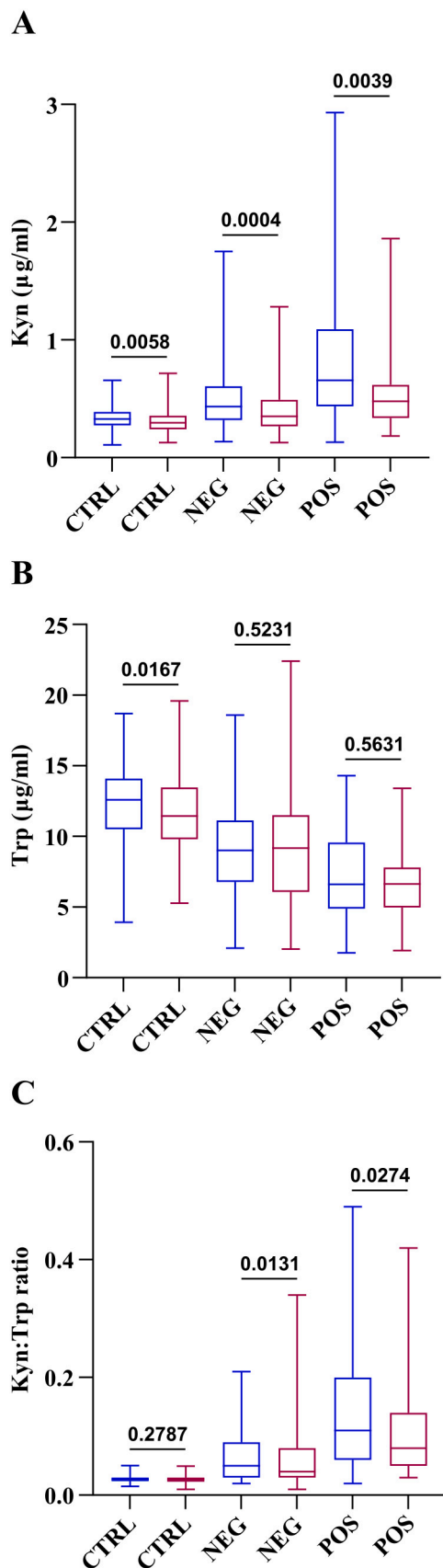
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Fig. 3. Sex-related analysis of serum Kynurenine and Tryptophan levels, and the Kynurenine:Tryptophan (Kyn:Trp) ratio in healthy control subjects and in SARS-CoV2-negative and -positive patients.

Mann-Whitney *U* test; A: $U_{CTRL} = 5199$, n1 = 87, n2 = 152; A: $U_{NEG} = 8797$, n1 = 138, n2 = 167; A: $U_{POS} = 640$, n1 = 43, n2 = 46; B: $U_{CTRL} = 5384$, n1 = 87, n2 = 152; B: $U_{NEG} = 11,033$, n1 = 138, n2 = 167; B: $U_{POS} = 918$, n1 = 43, n2 = 46; C: $U_{CTRL} = 6055$, n1 = 87, n2 = 152; C: $U_{NEG} = 9641$, n1 = 138, n2 = 167; C: $U_{POS} = 721.5$, n1 = 43, n2 = 46; CTRL = healthy subjects, NEG and POS-=SARS-CoV2-negative and positive patients. Blue: males; red: females.

comparing the Kyn:Trp ratio between SARS-CoV2-positive and SARS-CoV2-negative subjects. SARS-CoV2-negative subjects included a group of healthy controls, and a group of patients recruited at the Emergency Room. The latter was a spurious group formed by patients admitted to the Emergency Room for any possible acute disorder. However, in our opinion, this was a valuable reference group because SARS-CoV2-positive patients were also recruited from the Emergency Room. In SARS-CoV2-positive patients the Kyn:Trp ratio was much greater than in healthy controls, and also significantly greater than in SARS-CoV2-negative patients. These findings are in line with data shown in the elegant article by Thomas et al. [18], who performed targeted and untargeted metabolomic analyses in SARS-CoV2-positive and negative patients (the latter group included patients who had been infected before). They found reductions in Trp, serotonin, indole-pyruvate, 3-hydroxykynurenine, and anthranilic acid, and increases in Kyn, kynurenic acid, nicotinic acid, and picolinic acid levels, which, in most of the cases, correlated with IL-6 levels [18]. In this article, calculation of ROC curves suggested that Trp levels <105 μM and Kyn levels >5.3 μM could distinguish between SARS-CoV2-positive and -negative patients [18]. Our results support the hypothesis [18] that activation of the kynurenine pathway is a potential marker of clinical severity of SARS-CoV2 infection, and might be targeted by therapeutic intervention.

SARS-CoV2-positive patients who had the highest Kyn:Trp ratio were those with the most severe outcome of infection. This suggests two possibilities: (i) activation of the kynurenine pathway develops in response to inflammation and represents a sterile attempt to restrain an excessive immune reactivity; or, (ii) immune tolerance resulting from activation of the kynurenine pathway weakens the immune response to the virus and delays its clearance, thus paving the way to the development of acute respiratory distress syndrome and multiorgan failure. These two hypotheses are not mutually exclusive.

The extent of activation of the kynurenine pathway in SARS-CoV2-positive patients was different in the two sexes, and the increase in the Kyn:Trp ratio was larger in males, who are more susceptible to infection. Interestingly, male and female patients showed the same drop in Trp levels, but the increase in Kyn levels was more pronounced in males. This suggests that either other pathways of Trp metabolism are activated, or, alternatively, Kyn has a more rapid turnover rate in female patients.

We also found that the Kyn:Trp ratio increased to a greater extent in SARS-CoV2 patients with severe lymphopenia. Lymphopenia is a specific hallmark of severe SARS-CoV2 infection with respect to other viral infections, and occurs as a result of multiple mechanisms, including infiltration and trapping of T lymphocytes in the lower respiratory tract, poly-ADP ribose polymerase (PARP) activation and NAD^+ depletion in immune cells, or expression of protein 7a [20]. In SARS-CoV2 patients, the kynurenine pathway might be induced in antigen-presenting cells and mucosal epithelial cells of the respiratory tract in response to proinflammatory cytokines produced by resident cells of the innate immunity or by infiltrating T lymphocytes [21]. IDO is induced by type I and II interferons (IFNs) [22,23], TNF-α [24], IL-1β [25], CTL-A4 [26], poly(I:C) and CpG through IFNs [23,27], corticosteroids [28], CD200 activation [29], and AhR ligands, including Kyn and its metabolites [13]. A key role for interferons in the induction of IDO is supported by the evidence that the kynurenine pathway is constitutively active in
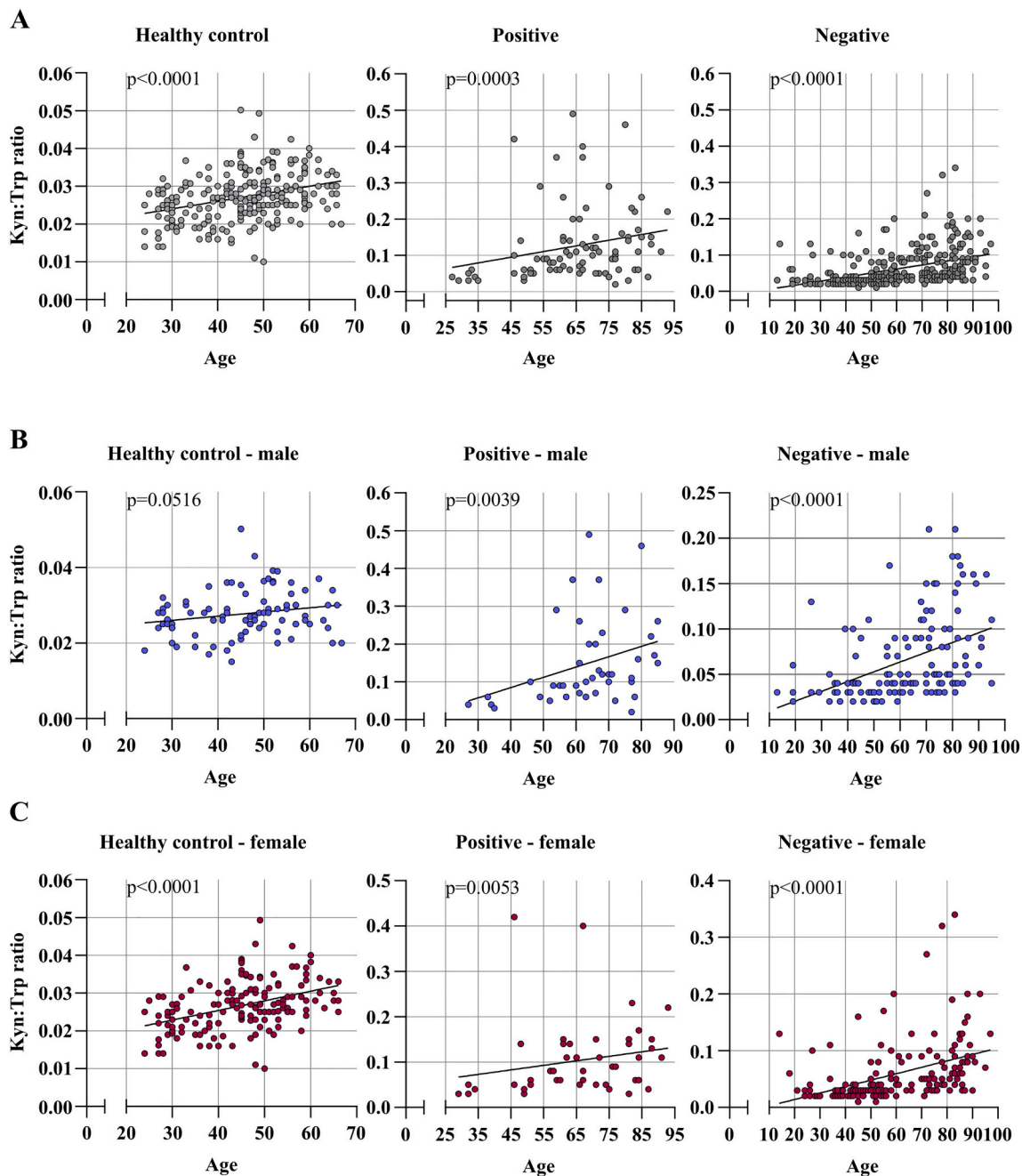
Fig. 4. Spearman correlation analysis between Kynurenine:Tryptophan ratio and age.
Correlation between the Kyn:Trp ratio and age in healthy subjects, SARS-CoV2-positive and -negative patients is shown in (A). The same correlation grouping healthy subjects, SARS-CoV2-positive and -negative patients in relation to sex is shown in (B) and (C).

individuals with Down syndrome, owing to the mapping on chromosome 21 of 4 of the 6 genes coding for interferon-α and -γ receptors [30].

While IFN-α and -β production is suppressed in SARS-CoV2 infection [31], the kynurenine pathway might be activated by proinflammatory cytokines, such as IFN-γ, TNF-α, and IL-1β, which are extensively produced by lymphocytes sequestered in the lower respiratory tract and are ultimately responsible for SARS-CoV2-associated respiratory distress syndrome. Thus, a high Kyn:Trp ratio likely reflects the extent of ongoing inflammation in the lung and other organs, and might be considered as a candidate biomarker for predicting the severity of SARS-CoV2 infection. Accordingly, an increase in Kyn:Trp ratio has been reported as a marker of inflammation and pathological signature of SARS-CoV2 infection in a recent article [32].

5. Conclusions

We have found an increase in the Kyn:Trp ratio in a relatively large cohort of SARS-CoV2 infected patients. This is indicative of activation of the kynurenine pathway of Trp metabolism, and fully support recent findings [18,32]. The increase correlated with the severity of infection and the extent of lymphopenia. A weakness of the study is that we did not investigate the individual metabolites of the kynurenine pathway downstream of Kyn formation.

In spite of this limitation, we suggest that an early determination of the Kyn:Trp ratio in SARS-CoV2-positive patients might drive the decision to start immunosuppressive treatments, such as glucocorticoids, anti-IL6 drugs, or JAK inhibitors, in order to improve the outcome of the
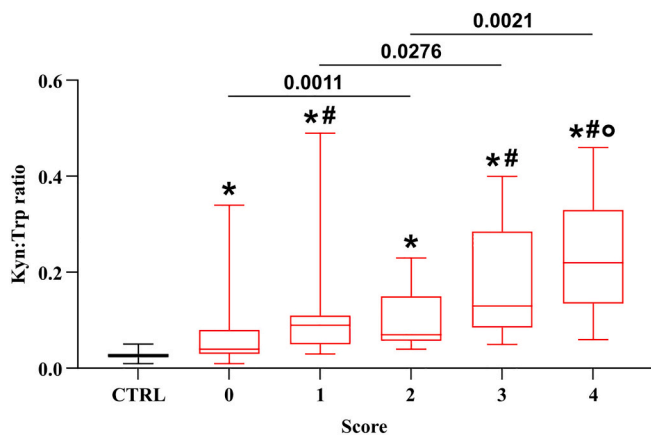
Fig. 5. The Kynurenine:Tryptophan ratio in healthy subjects, SARS-CoV2-negative patients and SARS-CoV2-positive patients, grouped by severity of infection.

Healthy subject: $n = 239$; SARS-CoV2-negative: $n = 296$; SARS-CoV2-positive, score 1: $n = 42$; score 2: $n = 14$; score 3: $n = 9$; score 4: $n = 13$. Mann-Whitney U test; (CTRLvs0): U = 14,480; (CTRLvs1): U = 353.5; (CTRLvs2): U = 7.5; (CTRLvs3); U = 1; (CTRLvs4): U = 0; (1vs0): U = 3721; (2vs0): U = 1041; (3vs0): U = 360; (4vs0): U = 282; (2vs1): U = 263.5; (3vs1): U = 101; (4vs1): U = 77.50; (3vs2): U = 38; (4vs2): U = 30. Severity score: 1 = no need for ICU; 2 ≤3 weeks spent in ICU; 3 ≥3 weeks spent in ICU; 4 = death. * p < 0.0001 (*Vs* CTRL), # p < 0.0001 (*Vs* score 0), ° p < 0.0001 (*Vs* score 1).

infection in spite of a possible delay of viral clearance. Activation of the kynurenine pathway in severe SARS-CoV2-positive patients might have additional implications in the complex scenario of infection. For example, some kynurenine metabolites, such as kynurenic, quinolinic, xanthurenic, and cinnabarinic acids, are neuroactive and have a strong impact on excitatory synaptic transmission by activating or inhibiting several classes of glutamate receptors [5,33,34]. At least some of these metabolites are able to cross the blood-brain barrier and affect animal behavior in response to peripheral administration [19,34]. Neurological and psychiatric manifestations are frequently associated with SARS-CoV2 infection, and our understanding of their pathophysiology is still largely unknown. An attractive possibility is that neuroactive kynurenine metabolites produced in response to SARS-CoV2-associaed inflammation contribute to functional alterations and neurodegenerative processes occurring in the CNS, as already hypothesized for HIV-associated neurocognitive disorders. This hypothesis warrants further investigation.

CRediT authorship contribution statement

Luana Lionetto: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Martina Ulivieri:** Formal analysis, Visualization, Writing – review & editing. **Matilde Capi:** Data curation, Investigation, Writing – review & editing. **Donatella De Bernardini:** Data curation, Investigation, Writing – review & editing. **Francesco Fazio:** Conceptualization, Formal analysis, Visualization, Writing – review & editing. **Andrea Petrucca:** Investigation, Writing – review & editing. **Leda Marina Pomes:** Conceptualization, Writing – review & editing. **Ottavia De Luca:** Investigation, Writing – review & editing. **Giovanna Gentile:** Investigation, Writing – review & editing. **Barbara Casolla:** Conceptualization, Writing – review & editing. **Martina Curto:** Conceptualization, Writing – review & editing. **Gerardo Salerno:** Investigation, Writing – review & editing. **Serena Schillizzi:** Data curation, Writing – review & editing. **Maria Simona Torre:** Resources, Writing – review & editing. **Iolanda Santino:** Resources, Writing – review & editing. **Monica Rocco:** Resources, Writing – review & editing. **Paolo Marchetti:** Resources, Writing – review & editing. **Antonio Aceti:** Resources, Writing – review & editing. **Alberto Ricci:** Resources, Writing – review & editing. **Rita Bonfini:** Resources, Writing – review & editing. **Ferdinando Nicoletti:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Maurizio Simmaco:** Conceptualization, Resources, Supervision, Writing – review & editing. **Marina Borro:** Conceptualization, Data curation, Formal analysis, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
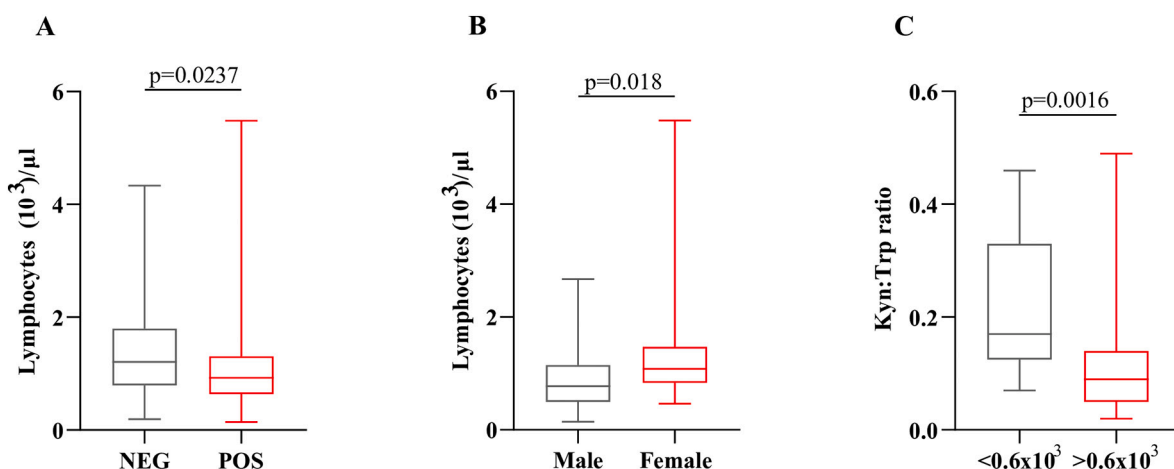

Fig. 6. Association of lymphocytes counts with SARS-CoV2 infection, sex, and the Kynurenine:Tryptophan ratio.

Lymphocyte counts in SARS-CoV2-positive and -negative patients are shown in (A); lymphocyte counts in male and female SARS-CoV2-positive patients are shown in (B). (Mann-Whitney U test; A: U = 1328, $n_{NEG} = 80$, $n_{POS} = 44$; B: U = 139, $n_{MALE} = 25$, $n_{FEMALE} = 19$). The Kynurenine:Tryptophan ratio in SARS-CoV2-positive patients with severe lymphopenia (lymphocytes counts <0.6 × 10^3/μl) *vs* SARS-CoV2-positive patients with counts >0.6 × 10^3/μl is shown in (C). Mann-Whitney U test; A: U = 138.5; $n < 0.6 × 10^3$/μl = 9; $n > 0.6$ x 10^{3}μl = 35.

References

[1] Y. Wang, Y. Chen, Q. Qin, Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID-19) implicate special control measures, J. Med. Virol. 92 (6) (2020) 568–576, https://doi.org/10.1002/jmv.25748.

[2] H. Ulrich, M.M. Pillat, CD147 as a target for COVID-19 treatment: suggested effects of azithromycin and stem cell engagement, Stem Cell Rev. Rep. 16 (2020) 434–440, https://doi.org/10.1007/s12015-020-09976-7.

[3] C. Qin, L. Zhou, Z. Hu, et al, Dysregulation of immune response in patients with COVID-19 in Wuhan, China, Clin. Infect. Dis. (2020) ciaa248. doi:https://doi.org/10.1093/cid/ciaa248.

[4] J. Loebbermann, L. Durant, H. Thornton, C. Johansson, P.J. Openshaw, Defective immunoregulation in RSV vaccine-augmented viral lung disease restored by selective chemoattraction of regulatory T cells, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 2987–2992, https://doi.org/10.1073/pnas.1217580110.

[5] R. Schwarcz, Kynurenines and glutamate: multiple links and therapeutic implications, AdvPharmacol 76 (2016) 13–37, https://doi.org/10.1016/bs.apha.2016.01.005.

[6] T. Eleftheriadis, G. Pissas, G. Antoniadi, et al., Indoleamine 2,3 dioxygenase downregulates T cell receptor complex ζ chain and c Myc, and reduces proliferation, lactate dehydrogenase levels and mitochondrial glutaminase in human T cells, Mol. Med. Rep. 13 (2016) 925–932, https://doi.org/10.3892/mmr.2015.4595.

[7] U. Grohmann, P. Puccetti, The coevolution of IDO1 and AhR in the emergence of regulatory T-cells in mammals, Front. Immunol. 6 (2015) 58, https://doi.org/10.3389/fimmu.2015.00058.

[8] G. Mondanelli, A. Coletti, F.A. Greco, et al., Positive allosteric modulation of indoleamine 2,3-dioxygenase 1 restrains neuroinflammation, Proc. Natl. Acad. Sci. U. S. A. 117 (2020) 3848–3857, https://doi.org/10.1073/pnas.1918215117.

[9] G. Anderson, A. Carbone, G. Mazzoccoli, Aryl hydrocarbon receptor role in co-ordinating SARS-CoV-2 entry and symptomatology: linking cytotoxicity changes in COVID-19 and cancers; modulation by racial discrimination stress, Biology (Basel) 9(9) (2020) E249. doi: https://doi.org/10.3390/biology9090249.

[10] W.A. Turski, A. Wnorowski, G.N. Turski, C.A. Turski, L. Turski, AhR and IDO1 in pathogenesis of Covid-19 and the "systemic AhR activation syndrome:" translational review and therapeutic perspectives, Restor. Neurol. Neurosci. (2020), https://doi.org/10.3233/RNN-201042.

[11] F. Fazio, C. Zappulla, S. Notartomaso, et al., Cinnabarinic acid, an endogenous agonist of type-4 metabotropic glutamate receptor, suppresses experimental autoimmune encephalomyelitis in mice, Neuropharmacology 81 (2014) 237–243, https://doi.org/10.1016/j.neuropharm.2014.02.011.

[12] A.L. Mellor, D.H. Munn, IDO expression by dendritic cells: tolerance and tryptophan catabolism, Nat Rev Immunol. 4 (2004) 762–774, https://doi.org/10.1038/nri1457.

[13] G. Mondanelli, R. Bianchi, M.T. Pallotta, et al., A relay pathway between arginine and tryptophan metabolism confers immunosuppressive properties on dendritic cells, Immunity 46 (2017) 233–244, https://doi.org/10.1016/j.immuni.2017.01.005.

[14] S.H. Yap, N.K. Abdullah, M. McStea, et al., HIV/human herpesvirus co-infections: impact on tryptophan-kynurenine pathway and immune reconstitution, PLoSOne 12 (2017), e0186000, https://doi.org/10.1371/journal.pone.0186000.

[15] A. Pizzini, K. Kurz, J. Santifaller, et al., Assessment of neopterin and indoleamine 2,3-dioxygenase activity in patients with seasonal influenza: a pilot study, Influenza Other Respir.Viruses. 13 (2019) 603–609, https://doi.org/10.1111/irv.12677.

[16] S. Geisler, S.D. Lytton, N.L. Toan, et al., Neopterin levels and Kyn/Trp ratios were significantly increased in dengue virus patients and subsequently decreased after recovery, Int. J. Infect. Dis. 91 (2020) 162–168, https://doi.org/10.1016/j.ijid.2019.12.005.

[17] L. Ma, B. Xu, W. Wang, W. Deng, M. Ding, Analysis of tryptophan catabolism in HBV patients by HPLC with programmed wavelength ultraviolet detection, Clin. Chim. Acta 405 (2009) 94–96, https://doi.org/10.1016/j.cca.2009.04.009.

[18] T. Thomas, D. Stefanoni, J.A. Reisz, et al., COVID-19 infection results in alterations of the kynurenine pathway and fatty acid metabolism that correlate with IL-6 levels and renal status, JCI Insight. 5 (2020), e140327, https://doi.org/10.1172/jci.insight.140327.

[19] F. Fazio, L. Lionetto, M. Curto, et al., Xanthurenic acid activates mGlu2/3 metabotropic glutamate receptors and is a potential trait marker for schizophrenia, Sci. Rep. 5 (2015) 17799, https://doi.org/10.1038/srep17799.

[20] I. Rahimmanesh, R. Fatehi, Systems biology approaches toward autosomal dominant polycystic kidney disease (ADPKD), Clin. Transl. Med. 9 (2020) 1, https://doi.org/10.1186/s40169-019-0254-5.

[21] W.A. Aldajani, F. Salazar, H.F. Sewell, A. Knox, A.M. Ghaemmaghami, Expression and regulation of immune-modulatory enzyme indoleamine 2,3-dioxygenase (IDO) by human airway epithelial cells and its effect on T cell activation, Oncotarget 7 (2016) 57606–57617. doi:10.18632/oncotarget.11586.

[22] M.W. Taylor, G.S. Feng, Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism, FASEB J. 5 (1991) 2516–2522.

[23] A.L. Mellor, B. Baban, P.R. Chandler, A. Manlapat, D.J. Kahler, D.H. Munn, Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN type 1 signaling, J. Immunol. 175 (2005) 5601–5605, https://doi.org/10.4049/jimmunol.175.9.5601.

[24] C.M. Robinson, P.T. Hale, J.M. Carlin, The role of IFN-gamma and TNF-alpha-responsive regulatory elements in the synergistic induction of indoleamine dioxygenase, J. Interf. Cytokine Res. 25 (2005) 20–30, https://doi.org/10.1089/jir.2005.25.20.

[25] P.A. Zunszain, C. Anacker, A. Cattaneo, S. Choudhury, K. Musaelyan, A.M. Myint, S. Thuret, J. Price, C.M. Pariante, Interleukin-1β: a new regulator of the kynurenine pathway affecting human hippocampal neurogenesis, Neuropsychopharmacology. 37 (2012) 939–949, https://doi.org/10.1038/npp.2011.277.

[26] U. Grohmann, C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M.L. Belladonna, R. Bianchi, M.C. Fioretti, P. Puccetti, CTLA-4-Ig regulates tryptophan catabolism in vivo, Nat. Immunol. 3 (2002) 1097–1101, https://doi.org/10.1038/ni846.

[27] B. Wang, K. Koga, Y. Osuga, I. Cardenas, G. Izumi, M. Takamura, T. Hirata, O. Yoshino, Y. Hirota, M. Harada, G. Mor, Y. Taketani, Toll-like receptor-3 ligation-induced indoleamine 2, 3-dioxygenase expression in human trophoblasts, Endocrinology. 152 (2011) 4984–4992, https://doi.org/10.1210/en.2011-0278.

[28] U. Grohmann, C. Volpi, F. Fallarino, S. Bozza, R. Bianchi, C. Vacca, C. Orabona, M.L. Belladonna, E. Ayroldi, G. Nocentini, L. Boon, F. Bistoni, M.C. Fioretti, L. Romani, C. Riccardi, P. Puccetti, Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy, Nat. Med. 13 (2007) 579–586, https://doi.org/10.1038/nm1563.

[29] F. Fallarino, C. Asselin-Paturel, C. Vacca, R. Bianchi, S. Gizzi, M.C. Fioretti, G. Trinchieri, U. Grohmann, P. Puccetti, Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement, J. Immunol. 173 (2004) 3748–3754, https://doi.org/10.4049/jimmunol.173.6.3748.

[30] R.K. Powers, R. Culp-Hill, M.P. Ludwig, K.P. Smith, K.A. Waugh, R. Minter, K.D. Tuttle, H.C. Lewis, A.L. Rachubinski, R.E. Granrath, M. Carmona-Iragui, R.B. Wilkerson, D.E. Kahn, M. Joshi, A. Lleó, R. Blesa, J. Fortea, A. D'Alessandro, J.C. Costello, K.D. Sullivan, J.M. Espinosa, Trisomy 21 activates the kynurenine pathway via increased dosage of interferon receptors, Nat. Commun. 10 (2019) 4766, https://doi.org/10.1038/s41467-019-12739-9.

[31] D. Acharya, G. Liu, M.U. Gack, Dysregulation of type I interferon responses in COVID-19, Nat Rev Immunol. 20 (2020) 397–398, https://doi.org/10.1038/s41577-020-0346-x.

[32] T. Kimhofer, S. Lodge, L. Whiley, N. Gray, R.L. Loo, N.G. Lawler, P. Nitschke, S.H. Bong, D.L. Morrison, S. Begum, T. Richards, B.B. Yeap, C. Smith, K.C.G. Smith, E. Holmes, J.K. Nicholson, Integrative Modelling of quantitative plasma lipoprotein, metabolic and amino acid data reveals a multi-organ pathological signature of SARS-CoV-2 infection, J. Proteome Res. (2020), https://doi.org/10.1021/acs.jproteome.0c00519.

[33] F. Fazio, L. Lionetto, G. Molinaro, H.O. Bertrand, F. Acher, R.T. Ngomba, S. Notartomaso, M. Curini, O. Rosati, P. Scarselli, R. Di Marco, G. Battaglia, V. Bruno, M. Simmaco, J.P. Pin, F. Nicoletti, C. Goudet, Cinnabarinic acid, an endogenous metabolite of the kynurenine pathway, activates type 4 metabotropic glutamate receptors, Mol. Pharmacol. 81 (2012) 643–656, https://doi.org/10.1124/mol.111.074765.

[34] M. Ulivieri, J.M. Wierońska, L. Lionetto, K. Martinello, P. Cieslik, A. Chocyk, M. Curto, L. Di Menna, L. Iacovelli, A. Traficante, F. Liberatore, G. Mascio, N. Antenucci, G. Giannino, M. Vergassola, A. Pittaluga, V. Bruno, G. Battaglia, S. Fucile, M. Simmaco, F. Nicoletti, A. Pilc, F. Fazio, The trace kynurenine, cinnabarinic acid, displays potent antipsychotic-like activity in mice and its levels are reduced in the prefrontal cortex of individuals affected by schizophrenia. Schizophr Bull. 7 (2020) sbaa074. doi: https://doi.org/10.1093/schbul/sbaa074.