



## Research article

# Phenylalanine metabolism and tetrahydrobiopterin bio-availability in COVID-19 and HIV

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## ABSTRACT

Various metabolomics studies have reported increased phenylalanine serum concentrations in SARS-CoV-2 positive cases and have correlated increased phenylalanine with COVID-19 severity. In this study, we report similar results based upon metabolomics analysis of serum collected from a South African cohort of adults with confirmed COVID-19. The novelty of this study is the inclusion of HIV positive cases in the African context. We found that pre-existing HIV co-infection exacerbates the disruption of phenylalanine metabolism in COVID-19. What is lacking in literature is biological context and deeper understanding of perturbed phenylalanine metabolism in COVID-19. We delve deep into the metabolism of phenylalanine in COVID-19 and posit new insights for COVID-19 cases co-infected with HIV; namely, HIV-COVID-19 co-infected individuals do not have sufficient bioavailability of tetrahydrobiopterin (BH<sub>4</sub>). Hence, we identify BH<sub>4</sub> as a potential supplement to alleviate/lessen COVID-19 symptoms.

## 1. Introduction

The COVID-19 pandemic has caused devastation around the world with over 630 million confirmed cases and an estimated 6 583 588 deaths reported by November 11, 2022 [1]. The most vulnerable are those with comorbidities such as advanced age, cardiovascular disease, diabetes, chronic respiratory disease, or cancer [2]. While it is well known that people living with primary or secondary immunodeficiencies are more likely to be infected with and die from respiratory pathogens [3], the data around HIV and COVID-19 are conflicting.

In a review of data on the Global Clinical Platform for the clinical characterization of COVID-19 among hospitalized individuals, Bertagnolio et al. (2022) [4] reported that the odds of severe COVID-19 in people living with HIV (PLWH) was increased by 15% (OR 1.15, 95% CI 1.10–1.20) and were 38% more likely to die in hospital (HR 1.38, 1.34–1.41) compare to COVID-19 cases without HIV. Importantly, 96% of the 16 955 PLWH included in the analysis were from Africa. In contrast, a systematic review of case reports and case series from the USA, China, and Italy found a mortality rate of 9% in PLWH admitted with COVID-19. They speculated that the low morbidity and mortality could be secondary to the compromised immunity of PLWH that prevented the development of a hyper immune response [5].

South Africa has the highest number of PLWH in the world, with an estimated adult (15–49 years old) prevalence of 18.3%. In 2021,

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approximately 74% of the roughly 7.5 million PLWH were on antiretroviral therapy (ART) [6]. South Africa has also been hard hit by the COVID-19 pandemic, with over 4 million reported cases and more than 100 000 deaths [7]. Although, the actual mortality is estimated to be much higher, with an increase of over 300 000 in total deaths reported since the start of the pandemic [8]. Local data show that HIV co-infection increases the odds of death during COVID-19 hospitalization by a fold of 1.34. PLWH not on ART and with a high HIV viral load (>1000 copies/mL) and low CD4 count (<200 cells/ $\mu$ L) have the worst outcomes [9].

Metabolomics is a relatively new field in systems biology compared to genomics, transcriptomics, and proteomics [10,11] and is a powerful tool with which to investigate the metabolic effects of infectious diseases like COVID-19. Therefore, it is not surprising that the COVID-19 pandemic triggered a lot of research using metabolomics-based approaches for the diagnosis and prognosis of COVID-19 [12]. Indeed, metabolomics has been utilized to examine and understand the SARS-CoV-2 virus in humans [13,14]. However, studies on the metabolic consequences for PLWH becoming infected with COVID-19 are lacking. Consequently, we aimed to use a holistic multi-platform metabolomics approach to investigate systemic metabolic alterations in patients with HIV and COVID-19 co-infection.

## 2. Methods

### 2.1. Sampling

Adults (>18 years of age) admitted to the Steve Biko Academic Hospital (Tshwane District Hospital complex) in South Africa with moderate to severe PCR-confirmed SARS-CoV-2 infection were recruited between May 2020 and March 2021. Blood was collected in EDTA tubes on the day of admission, prior to the administration of treatment, spun down and the serum collected. The ROX score (ratio of oxygen saturation index) was used as a marker of COVID-19 severity in this cohort, as it is based on clinically available measurements and has evidence linking it to clinical outcomes of interest [15].

### 2.2. $^1\text{H}$ NMR analysis

Due to limited volume of serum,  $^1\text{H}$  NMR analysis was done using an established miniaturized method [16] that requires only 100  $\mu$ L serum samples. The method used is described in detail by van Zyl et al. (2020) [17]. Briefly, all samples were filtered using Amicon Ultra-2 mL 10 000 MWCO centrifugal filters at 4500 g for 20 min – physical removal of all macromolecules, especially proteins. A digital syringe (eVol®) was used to pipette a 90%:10% ratio of sample:NMR buffer solution (pH 7.4) into a 2 mm glass NMR tube. The NMR buffer solution contained the internal standard (trimethylsilylpropionic acid [TSP]), with 1.5 M sodium phosphate and 1% sodium azide in deuterium oxide. The settings for the NMR (Bruker Avance III HD 500 MHz) analysis were identical to those of van Zyl et al. (2020) [17].

### 2.3. LC-MS/MS analysis

Sample preparation and LC-MS/MS analysis was done based on the methods described in detail by Moutloatse et al. (2017) [18], with slight deviation. Briefly, a 50  $\mu$ L serum sample was mixed with an ice cold 410  $\mu$ L isotope mixture in methanol and acetonitrile (1:5 ratio), centrifuged (protein crash) and supernatant dried under nitrogen gas. Deuterated isotopes of Tyr, Phe and Trp were included in the isotope mix at a concentration of 1  $\mu$ g/mL. Dried samples were derivatized with 100  $\mu$ L butanolic-HCl at 60 °C for 30 min. Samples were dried under nitrogen and then re-dissolved in 100  $\mu$ L acetonitrile:water (1:1) mixture. One  $\mu$ L sample was injected and analyzed with a 1200 series Agilent HPLC system linked to an Agilent 6410 MSMS system. The settings for the LC-MS/MS were identical to those of Moutloatse et al. (2017) [18].

### 2.4. Statistics

Statistical results reported support interpretation in terms of generalizability (statistical significance indicated by a p-value), but primarily practical relevance (effect sizes), as advocated by Sullivan & Feinn (2012) [19]. SPSS (2020) [20] was used to perform univariate analyses on the available clinical and demographic data. Nonparametric analyses were performed as far possible, to avoid violating assumptions and data transformations, which hinders translation into clinical settings. Therefore, metabolites are summarized using median and inter quantile ranges (IQR). Where nonparametric analysis became cumbersome, statistical approaches robust to violating the assumption of normality and homogeneity of variances were used. Specifically, the Mann-Whitney (MW) test was performed when comparing two groups with the associate effect size calculated and interpreted as described in Field (2009) [21], with  $0.3 < \eta < 0.5$  indicating practical relevance and  $\eta \geq 0.5$  practical significance; the Brown-Forsythe (BF) test was used to compare more than two groups, using Games-Howell (GH) as a post-hoc test and  $\eta^2$  as an effect size (interpretation based on Field (2009) [21]:  $0.06 < \eta^2 < 0.14$  indicating practical relevance and  $\eta^2 \geq 0.14$  practical significance). Pearson's Chi-squared (PC) test was used to evaluate association between categorical variables, using odds ratios (OR) or Phi values ( $\omega$ ) as effect sizes (interpretation based on Field (2009) [21]:  $0.3 < \omega < 0.5$  indicating practical relevance and  $\omega \geq 0.5$  practical significance). Kendall tau b correlation coefficient ( $\tau$ ) was used to evaluate the strength of relationships between continuous variables (absolute  $r > 0.5$  is considered noteworthy).

Box plots and correlation networks were constructed using R [22]. Box plots were generated with the ggpubr package in with the following information overlaid: (i) Mann-Whitney (MW) p-values after adjusting for multiple testing (stats package in base R) indicated as an asterisks (\*) if below a 10% significance level; and (ii) Cohen's d effect sizes (d) to determine the practical relevance of differences (interpretation based on Field (2009) [21]:  $d \geq 0.8$  is noteworthy). To better understand the statistical and practical

significance of relationships between metabolites, Spearman's rho correlation networks were drawn using the corrr package.

### 3. Results

#### 3.1. Admissions clinical data

The experimental groups consisted of 15 control cases (COVID-19 and HIV negative) and 66 COVID-19 positive (COVID-19+) cases categorized by severity and HIV status. COVID-19 cases were considered severe if the patient's ROX score was less than 6 ( $N = 28$ , ignoring HIV status) or nonsevere given a ROX score greater than 6 ( $N = 38$ , ignoring HIV status). Of all COVID-19+ cases, 14 were HIV positive (HIV+,  $N = 14$ ), of which only three had severe COVID-19. The full stratification of experimental cases is presented in [Table 1](#).

The association between HIV status and ROX score proved to be statistically significant albeit not necessarily noteworthy (MW  $p = 0.03$ ,  $\eta = 0.27$ ). The influence of age on the stratification of samples by COVID-19 and HIV status was significant (BF  $p = 0.015$ ,  $\eta^2 = 1.37$ ), but significant pairwise differences were only found between controls (mean age of 43 years) and severe COVID-19 HIV- cases (mean age of 55 years) with GH  $p = 0.017$ . NOTE: the age of six cases were not available. There was no significant association between gender and HIV status or COVID-19 severity or ROX score. There was, however, a significant association between COVID-19 status and gender (PC  $p < 0.001$ ) with males being almost twice as likely to contract COVID-19 (OR = 1.8). However, we report this result with caution as five of the controls did not have information on gender, reducing this group to only ten. The mean CD4 count for PLWH was 338 cells/ $\mu\text{L}$  and the mean HIV viral load was 18 630 copies/mL. There was also a notable correlation between the ROX scores and viral load (Kendall's tau b  $p = 0.089$ ,  $r = 0.441$ ). Eleven of the 14 PLWH were on ART and had higher median CD4 counts (285 cells/ $\mu\text{L}$  [IQR 137–625] versus 76 cells/ $\mu\text{L}$  [IQR 44–108]) and lower HIV viral loads (lower than detectable versus a median of 25 449 copies/mL [IQR 1360–22 613]) than those not on ART. Since our focus was only on four targeted metabolites, we cannot speculate the holistic metabolic effects of ART on HIV patients; however, an assessment of untargeted metabolomics data may shed light on long-term pharmacologic treatment of HIV.

Regarding common comorbidities, hypertension was more common in individuals with severe COVID-19 and no HIV co-infection, but the association was not significant. Diabetes was present mostly in COVID-19 infected individuals with HIV co-infection, the relationship showed some significance with an effect size of  $\omega = 0.358$  (medium effect), however PC  $p = 0.172$ . There was no evidence that heart disease influenced either COVID-19 infection, severity, or HIV status. Limited information was available on the presence/absence of lung disease ( $N = 28$ ), kidney disease ( $N = 29$ ), cancer ( $N = 29$ ) and tuberculosis ( $N = 31$ ), but of the cases with a classification, no association to COVID-19 infection, severity, or HIV status was observed. Overweight individuals without HIV co-infection were more likely to present with COVID-19 severe/nonsevere (PC  $p = 0.034$  and  $\omega = 0.496$  indicating a notable effect).

Correlations were found between clinical measures for COVID-19 cases which seem to be dependent on HIV status. There was a weaker correlation between absolute lymphocyte count and neutrophil:lymphocyte ratio for PLWH ( $r = -0.382$  vs  $r = -0.534$ ). The associations with D-dimer became significant in PLWH for absolute neutrophil count ( $r = 0.714$  vs  $r = 0.276$ ), neutrophil:lymphocyte ratio ( $r = 0.810$  vs  $r = 0.198$ ), and white cell count ( $r = 0.714$  vs  $r = 0.262$ ).

#### 3.2. Phenylalanine and tyrosine

For this study we focused on the phenylalanine metabolism in the sera of COVID-19 and HIV cases. Our assessment involved the targeted analysis of phenylalanine, tyrosine and the phenylalanine:tyrosine (Phe:Tyr) ratio using  $^1\text{H}$  NMR analysis. The Phe:Tyr ratio provides additional information regarding flux; namely, the degree to which phenylalanine is converted to tyrosine. In addition, we assessed kynurenic acid, tryptophan and the kynurenic acid:tryptophan (KA:Trp) ratio using LC-MS/MS analysis. The reasoning for including these latter metabolites, as well as Fischer's ratio, is explained in more detail later. [Table 1](#) gives the median (IQR) concentrations, in micromolar ( $\mu\text{mol/L}$ ), of the targeted metabolites and ratios.

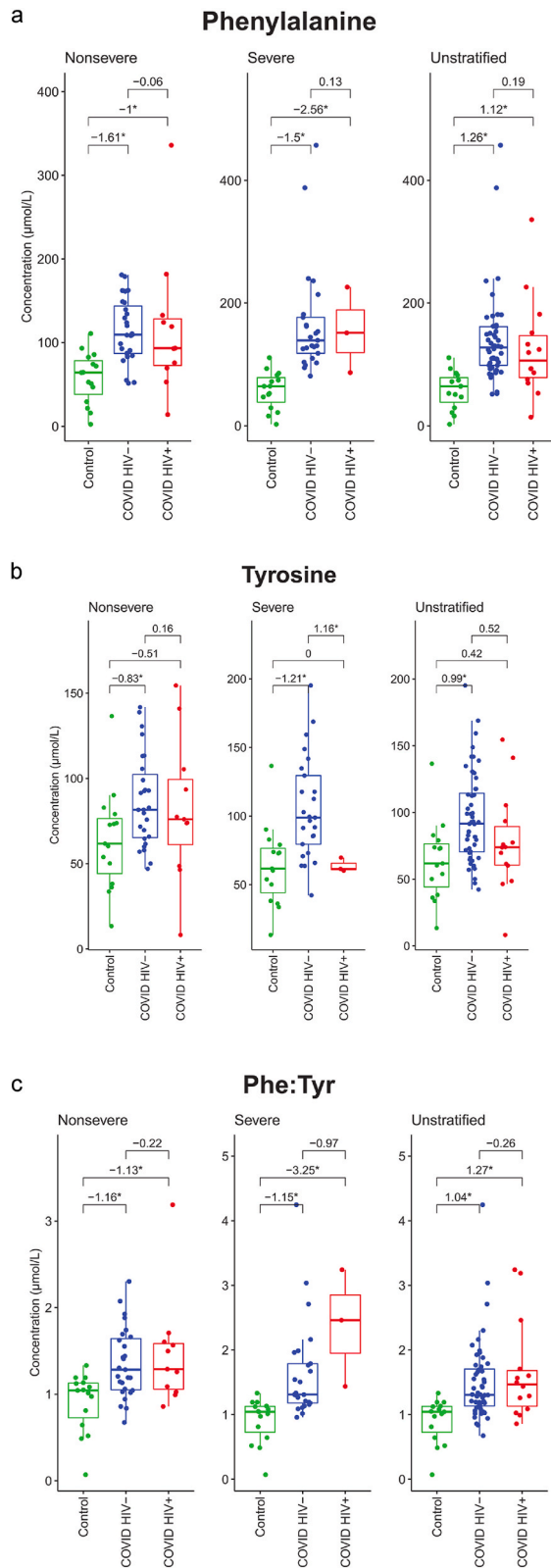
[Figs. 1 and 2](#) compares measurement across different groupings with significance concluded if MW p-values were below 0.1

**Table 1**  
Concentrations ( $\mu\text{mol/L}$ ) of targeted metabolites, and their ratios, given as medians and IQRs for analyzed serum.

Group		Phe <sup>A</sup>	Tyr <sup>A</sup>	Phe:Tyr	Trp <sup>B</sup>	KA <sup>B</sup>	KA:Trp	Fischer's ratio
Controls	(N = 15)	64.22 (38.11–78.4)	61.77 (44.19–76.48)	1.04 (0.73–1.13)	3.14 (2.24–5.49)	0.5 (0.36–0.53)	0.19 (0.07–0.22)	3.7 (3.04–4.66)
COVID-19+ & HIV-	Nonsevere (N = 27)	109.5 (86.88–143.65)	81.65 (65.29–102.34)	1.28 (1.05–1.64)	3.58 (2.91–6.72)	1.19 (0.64–1.58)	0.28 (0.11–0.45)	2.84 (2.02–3.65)
	Severe (N = 25)	138.85 (117.86–176.73)	98.87 (79.49–129.5)	1.31 (1.18–1.79)	3.51 (2.49–4.48)	0.74 (0.5–1.13)	0.19 (0.15–0.3)	2.56 (1.98–3.2)
COVID-19+ & HIV+	Nonsevere (N = 11)	93.24 (72.65–128.42)	76.05 (61.21–99.45)	1.29 (1.06–1.59)	4.66 (2.48–5.33)	0.73 (0.52–1.32)	0.16 (0.13–0.35)	2.47 (1.98–3.45)
	Severe (N = 3)	151.42 (118.97–188.75)	61.5 (60.85–65.61)	2.46 (1.95–2.85)	9.51 (6.34–10.23)	0.62 (0.53–0.7)	0.07 (0.07–0.11)	2.2 (1.9–2.44)

<sup>A</sup>  $^1\text{H}$  NMR analysis, <sup>B</sup> LC-MS/MS analysis.

Phe = phenylalanine; Tyr = tyrosine; Trp = tryptophan; KA = kynurenic acid.



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**Fig. 1. Box plots of phenylalanine (a), tyrosine (b) and phenylalanine:tyrosine (Phe:Tyr; c) for controls (green) and the COVID-19 cases stratified as nonsevere (ROX score >6), severe (ROX score <6) and unstratified COVID-19 cases, and HIV status (positive – red or negative – blue). Number by the brackets indicates effect size (d-value) and statistical significance (adjusted p-value <0.1) is presented by \*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)**

(indicated by a \*) and absolute d-values exceeded >0.8. NOTE: no emphasis should be placed on comparisons to severe COVID-19 cases with HIV coinfection due to the low sample size (N = 3). To address this, COVID-19 cases, stratified by HIV status, are compared to controls without taking severity into account (unstratified), displayed in the box plots, but also compared using BF tests.

Considering Fig. 1a, phenylalanine was significantly increased when comparing experimental groups to controls (BF p = 0.001,  $\eta^2 = 0.179$ ), but none of the comparisons between HIV+ and HIV- within the COVID-19 groups (split by severity or unstratified) showed any significant differences. Tyrosine (Fig. 1b) was significantly elevated in the HIV- cases, but not in the HIV + cases, in the COVID-19 groups when compared to the controls (BF p = 0.006,  $\eta^2 = 0.133$ ). All the COVID-19 groups showed significantly increased Phe:Tyr (BF p = 0.004,  $\eta^2 = 0.15$ ) when compared to the controls (Fig. 1c). These results will be the main talking points in our discussion.

### 3.2.1. Kynurenic acid and tryptophan

Additional results (Fig. 2a–c) show kynurenic acid (KA) as being significantly increased in all COVID-19 cases (except for the severe COVID-19 HIV + cases where N = 3), when compared to the controls (BF p = 0.003,  $\eta^2 = 0.14$ ). Tryptophan shows no significant differences, except for practical significance in comparisons to HIV + cases with severe COVID-19. However, differences are driven by only two HIV + cases with severe COVID-19 observation and, as such, cannot be considered. There were also no significant differences between HIV+ and HIV- within the COVID-19 groups for both KA and tryptophan. For the KA:Trp ratio, only statistically significant (d < 0.8) differences were found between HIV- COVID-19 nonsevere cases and controls. Significance was noted between HIV+ and HIV-cases with severe COVID-19, but given the sample size, this could be purely by chance.

### 3.2.2. Fischer's ratio

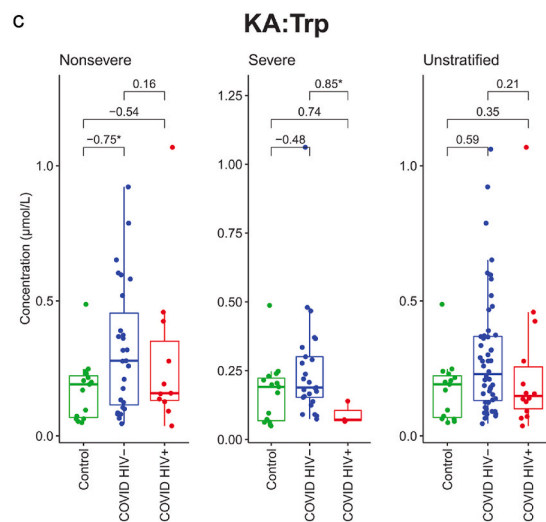
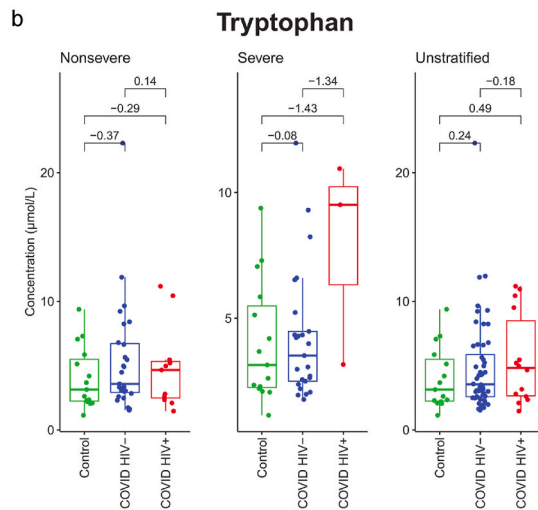
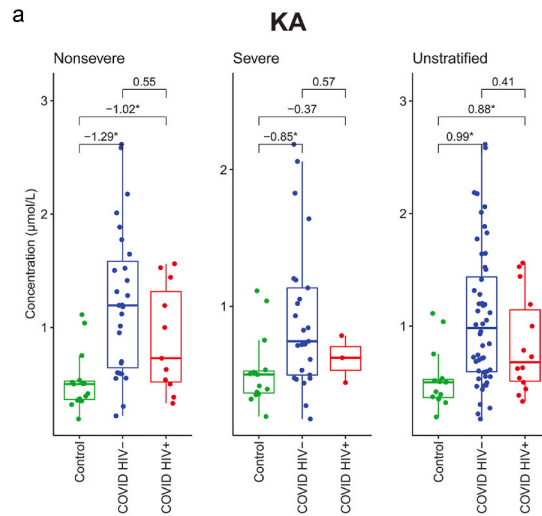
Lastly, we also looked at Fischer's ratio. Fischer's ratio is the sum of the branched chain amino acids (valine, leucine and isoleucine) divided by the sum of the aromatic amino acids (phenylalanine, tyrosine and tryptophan). Fischer's ratio, first described in 1976 [23], is a clinical indicator of hepatic function. In principle, branched chain amino acids can be metabolized in various compartments in the human body; however, catabolism of aromatic amino acids occurs primarily in the liver. Hence, an increase in aromatic amino acids, relative to branched chain amino acids, is indicative of decreased liver function. A Fischer's ratio between 3.0 and 3.5 is considered normal. Table 1 shows that the Fischer's ratio for the controls gave a median of 3.7, with only 1 case having a value below 3.0. For many of the experimental cases the Fischer's ratio dropped below 3.0, indicating moderate to severe hepatic stress, but did not drop low enough (e.g., <1.0) to be indicative of severe hepatic damage and indicate liver damage. Fig. 3 illustrates that the Fischer's ratio is significantly lower in COVID-19 cases without HIV coinfection compared to the controls, regardless of severity (GW p = 0.061).

### 3.2.3. Correlation networks

The data from this study showed a significant positive correlation (r = 0.37) between COVID-19 severity and phenylalanine within the unstratified COVID-19 HIV- group (i.e., for the HIV- group, phenylalanine increases with COVID-19 severity). Phenylalanine also showed significant positive correlation with Phe:Tyr in the unstratified COVID-19 HIV- (r = 0.63) and unstratified COVID-19 HIV+ (r = 0.74) groups, and significant negative correlation with Fischer's ratio in the control (r = -0.61) and unstratified COVID-19 HIV- (r = -0.5) groups. Lastly, tyrosine showed significant positive correlation with phenylalanine in the unstratified COVID-19 HIV- (r = 0.58) group. All data for correlations is given in the supplementary information (SI). Fig. 4 is a compilation of graphical representations of the correlations between compounds given various data subsets. Each set of correlations is represented as a network displaying the strength (i.e.,  $-1 \leq r \leq 1$ ) and direction (i.e., positive or negative) of the correlation, as indicated by the color scale, after removing weak correlations ( $|r| < 0.3$ ). Compounds are then positioned together to highlight strong associations as well as a lack of covariation between compounds. Fig. 4 highlight changes in correlations between metabolites associated with stratification by COVID-19 and HIV infection. A stronger correlation between Fischer's ratio and tryptophan was observed in co-infected cases, while Fischer's ratio is less dependent on leucine, isoleucine and valine in controls. KA:Trp, KA and tryptophan have negligible correlations to other compounds in HIV- COVID-19 infected cases.

## 4. Discussion

In this study we examined the targeted  $^1\text{H}$  NMR and LC-MS/MS metabolomics profile of the sera from COVID-19 cases, with and without HIV co-infection (HIV+ and HIV-), in a South African adult cohort. We stratified the COVID-19 cases based upon severity (nonsevere and severe) and compared the experimental cases to healthy controls from the same population. For the purpose of this paper, we focused on four targeted metabolites – phenylalanine, tyrosine, tryptophan and kynurenic acid. We also looked at the ratios of phenylalanine:tyrosine (Phe:Tyr) and kynurenic acid:tryptophan (KA:Trp), and Fischer's ratio. The primary results of this study are that phenylalanine is significantly increased in serum of COVID-19 cases. The pre-existing presence of HIV co-infection does not significantly increase the amount of phenylalanine in COVID-19 cases. Tyrosine was only significantly elevated in COVID-19 cases without HIV coinfection. Our focus was more upon the Phe:Tyr ratio and the trend observed – an increase in Phe:Tyr in relation to COVID-19 and HIV + status, with limited evidence pointing to a potential further increase due to severity. This finding suggests that



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**Fig. 2. Box plots of kynurenic acid (KA; a), tryptophan (b) and kynurenic acid:tryptophan (KA:Trp; c) for controls (green) and the COVID-19 cases stratified as nonsevere (ROX score >6), severe (ROX score <6) and unstratified COVID-19 cases, and HIV status (positive – red or negative – blue). Number by the brackets indicates effect size (d-value) and statistical significance (adjusted p-value <0.1) is presented by \*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)**

the flux of phenylalanine to tyrosine is impaired, as described in more detail later.

Our metabolomics findings about phenylalanine and tyrosine are supported by other metabolomics studies on the sera/plasma from stratified and unstratified COVID-19 cases (Table 2) that report similar metabolite measurements (Table 3). Table 2 shows that each experimental group in literature-reported metabolomics studies of COVID-19 had approximately 20–30 cases, on average. In this study we had similar numbers with 28 severe and 38 nonsevere COVID-19 cases, but after stratifying further by HIV status (14 HIV + cases), sample sizes dropped to 11 and 3 for severe COVID-19 cases, highlighting an important limitation.

Tryptophan showed no significant changes in this cohort; however, kynurenic acid was significantly increased in all COVID-19 cases, and KA:Trp was only significantly increased somewhat notably in nonsevere COVID-19 cases without HIV co-infection. No clear trend in KA:Trp was observed. Anson et al. (2021) [24], similarly, did not find any significant changes in tryptophan, but Caterino et al. (2021) [25] and Herrera-van Oostdam et al. (2021) [26] reported decreasing tryptophan levels with increasing COVID-19 severity. Thomas et al. (2020) [27] reported hyperactivation of the kynurenine pathway in COVID-19.

Based upon the measurements of Fischer's ratio in this study, the COVID-19 cases with HIV co-infection showed increased hepatic stress (2.2–2.5) than the COVID-19 cases without HIV co-infection (2.6–2.9), but no hepatic damage. This additional hepatic stress in the HIV co-infected cases is likely due to the concurrent anti-retroviral therapy that is required for the management of HIV. Looking back at the clinical data available in this cohort, PLWH had significantly higher levels of the canalicular enzymes – alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), but similar levels of the transaminases – alanine transaminase (ALT) and aspartate transaminase (AST). Hence, the clinical data supports the hypothesis that PLWH have additional hepatic stress. Schmelter et al. (2021) [28] also reported a decreased Fischer's ratio in COVID-19 cases. Based upon the metabolite values reported by Anson et al. (2021) [24], we calculated that the COVID-19 cases had a Fischer's ratio of  $2.09 \pm 0.36$ . Similarly, for Bruzzone et al. (2020) [29] we calculated the Fischer's ratio to be an average of 1.90 in the COVID-19 cases. Thus, the Latvian cohort analyzed by Anson et al. (2021) [24] and the Spanish cohort analyzed by Bruzzone et al. (2020) [29] had COVID-19 cases that had more severely impaired hepatic amino acid metabolism, suggesting possible liver problems associated with COVID-19.

Some of the metabolomics studies on COVID-19 in Table 2 [28,30,31,34,35] did not report metabolite concentration values, nor provide supplementary data from which metabolite concentrations could be calculated, hence could not be incorporated into Table 3. Atila et al. (2021) [30] reported that phenylalanine increased with disease severity and had high diagnostic power for COVID-19 under non-parametric assumption when compared to controls (N = 30). Atila et al. (2021) [30] also found tyrosine to be increased when comparing mild to moderate COVID-19 and tryptophan to decrease when comparing moderate to severe COVID-19. Dierckx et al. (2020) [31] examined three large cohorts of COVID-19 cases (N = 581) and found phenylalanine to be increased and associated with COVID-19 severity. Páez-Franco et al. (2021) [34] found phenylalanine to be increased in both mild (fold change = 1.5) and severe (fold change = 1.6) COVID-19 cases. Schmelter et al. (2021) [28] reported increased levels of phenylalanine in COVID-19. Shi et al. (2021) [35] reported increased phenylalanine and decreased tyrosine in COVID-19. The resounding conclusion from all these emerging metabolomics studies on COVID-19, supporting the findings of this study, is that increased phenylalanine occurs in serum of COVID-19 patients, and phenylalanine increases with more severe symptoms of SARS-CoV-2 infection, and even more so with a pre-existing chronic co-infection (e.g., HIV).

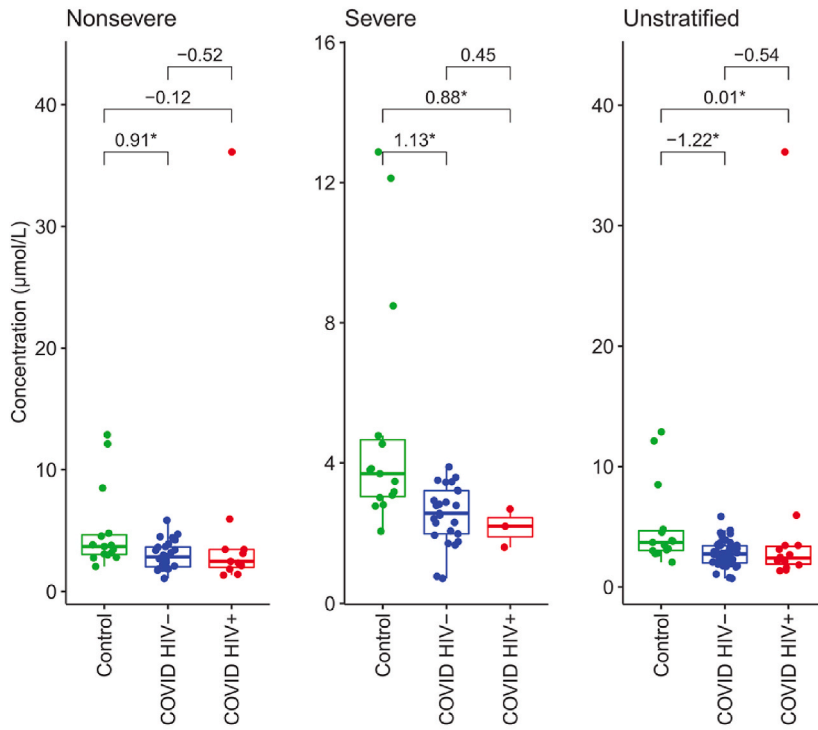
In this study we also found that phenylalanine had a significant positive correlation with COVID-19 severity in the unstratified COVID-19 HIV- group (MW p = 0.009). Some of the other metabolomics studies on COVID-19 have found similar correlations between phenylalanine and co-variables in COVID-19 – Table 4.

The observation that phenylalanine plays a significant role within severe infections is not new. In cases with severe general infections (with a sequential organ failure assessment (qSOFA) score  $\geq 2$ , excluding HIV), Huang et al. (2019) [37] showed that increased phenylalanine in sera correlates with renal failure and low albumin levels and is an independent predictor of death. Murr et al. (2014) [38] found a significant positive correlation between an increased phenylalanine:tyrosine ratio and high C-reactive protein levels in cardiovascular disease. Increased phenylalanine has also been linked to immune activation in cases of sepsis [39] and HIV infection [40,41]. Even in chronic low-inflammatory neuropsychiatric states [42,43], increased phenylalanine is present. Hence, it is quite clear that accumulated inflammation and persistent immune activation leads to an accumulation of phenylalanine in the serum. The big question is – why? It is our belief that the bioavailability of the cofactor tetrahydrobiopterin (BH<sub>4</sub>) – an essential cofactor needed for conversion of phenylalanine to tyrosine, is a major limiting factor, along with other intertwined metabolic pathways – described below.

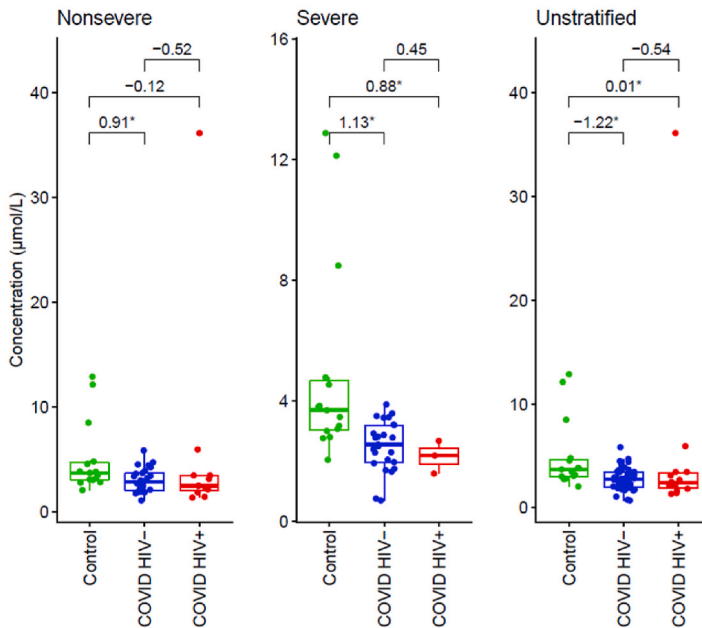
We posit that a sufficiently acute and/or persistent state of inflammation and/or immune activation decreases the bioavailability of BH<sub>4</sub>, impairing the functioning of important enzymes, such as phenylalanine hydroxylase. To understand how fragile the BH<sub>4</sub> pool is in the human body, it is important to understand where and how BH<sub>4</sub> is used. Fig. 5 illustrates five enzymatic reactions that require the cofactor BH<sub>4</sub>. If the bioavailability of BH<sub>4</sub> becomes limited, then the functioning of these five enzymatic reactions becomes impaired. Hence, the *de novo* biosynthesis of BH<sub>4</sub> and the salvaging/recycling pathways of BH<sub>4</sub> in the human body becomes very important.

Fig. 6 illustrates the *de novo* biosynthesis pathway of BH<sub>4</sub>. Also shown in Fig. 6 is the expected effect of SARS-CoV-2 infection. We explain Fig. 6 in more detail here. The SARS-CoV-2 infection elicits a Th1-type immune response which is the initial pro-inflammatory response aimed at attacking and killing the invading pathogen. The main cytokine in this pro-inflammatory storm is interferon gamma

### Fischer's Ratio



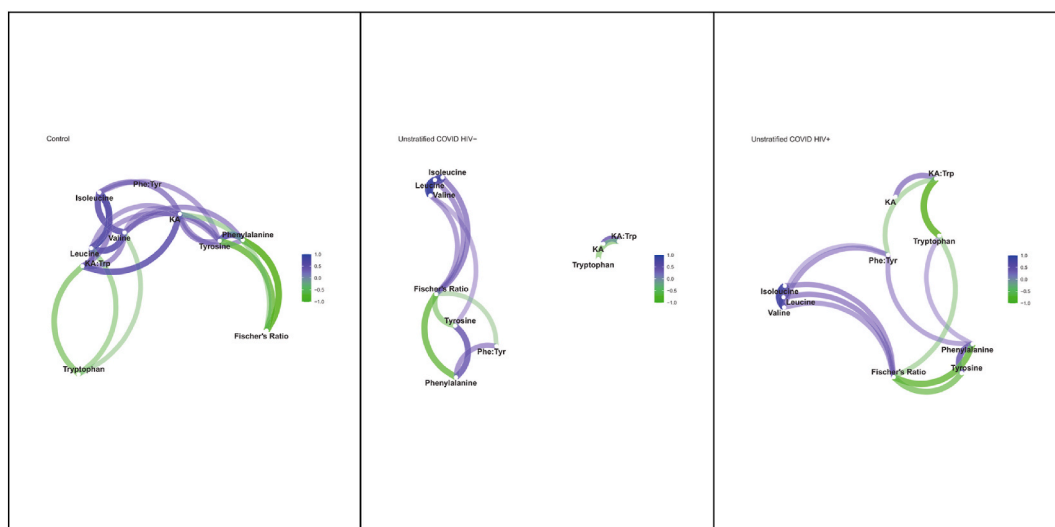
### Fischer's Ratio



(caption on next page)



**Fig. 3. Box plot of Fischer's ratio** for controls (green) and the COVID-19 cases stratified as nonsevere (ROX score >6), severe (ROX score <6) and unstratified COVID-19 cases, and HIV status (positive – red or negative – blue). Number by the brackets indicates effect size (d-value) and statistical significance (adjusted p-value <0.1) is presented by \*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4. Correlation networks** for controls (left) and COVID-19 cases split by HIV status (middle: HIV-; right HIV+). Correlations of note ( $|r| \geq 0.3$ ) are represented by the lines. The strength and direction of correlations are indicated by the color scale. Groups of compounds that do not have a noteworthy correlation are distanced from groups of compounds that do. The positioning itself is arbitrary. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**

Metabolomics studies from literature that report increased phenylalanine in COVID-19.

Reference	Region	Biological Matrix	Metabolomics platform	Stratified COVID-19 (N)			Unstratified COVID-19 (N)
				Mild	Moderate	Severe	
Ansone et al. (2021) [24]	Latvia	Serum	LC-MS				32
Caterino et al. (2021) [25]	Italy	Serum	LC-MS/MS	20	16	16	
Herrera-van Oostdam et al. (2021) [26]	Mexico	Plasma	LC-MS/MS	41	35	45	
Schmelter et al. (2021) [28]	Germany	Serum	$^1\text{H}$ NMR				5
Bruzzone et al. (2020) [29]	Spain	Serum	$^1\text{H}$ NMR				263
Atila et al. (2021) [30]	Turkey	Serum	LC-MS/MS	29	31	28	
Dierckx et al. (2020) [31]	Belgium	Serum & plasma	$^1\text{H}$ NMR				581, across 3 cohorts
Luporini et al., 2021 [32]	Brazil	Serum	$^1\text{H}$ NMR	21	84	61	
Meoni et al., 2021 [33]	Italy	Serum	$^1\text{H}$ NMR				30
Páez-Franco et al. (2021) [34]	Mexico	Serum	GC-MS		19	46	
Shi et al., 2021 [35]	China	Serum	GC-MS				79

(IFN- $\gamma$ ), as well as others such as tumor necrosis factor alpha (TNF- $\alpha$ ) and various interleukins (e.g. interleukin-2). These pro-inflammatory cytokines upregulate the initial reaction of the pathway of *de novo* biosynthesis, namely guanosine-5'-triphosphate cyclohydrolase I (GTP cyclohydrolase, EC:3.5.4.16). GTP is a purine nucleoside triphosphate that undergoes a 4-step reaction to produce 7,8-dihydroneopterin-3-triphosphate (DHNTTP). Under primary metabolism, DHNTTP is converted to 6-pyruvoyltetrahydropterin (PTP) by 6-pyruvoyltetrahydropterin synthase (EC:4.2.3.12). Increased flux in this pathway opens a secondary pathway whereby alkaline phosphatase (EC:3.1.3.1) produces increased levels of 7,8-dihydroneopterin (DHN). DHN is then enzymatically converted to 6-hydroxymethyl-7,8-dihydropterin via dihydroneopterin aldolase (EC:4.1.2.25). However, in a less understood pathway, DHN can also be acted upon by superoxide hyperchlorite – originating from oxidative stress, which is responsible for the nonenzymatic synthesis of neopterin – an established laboratory diagnostic marker of immune activation [44]. Neopterin is a low abundance metabolite that is only measurable via LC-MS based analytical methods. We were not able to measure neopterin in this study, but we predict that neopterin should be elevated in COVID-19 patients.

Back to the primary metabolic pathway of *de novo* BH<sub>4</sub> biosynthesis, PTP is used to generate the end-product BH<sub>4</sub> via sepiapterin reductase (EC:1.1.1.153). However, this final enzymatic reaction step is inhibited by xanthurenic acid (XA) [45]. XA is a by-product

**Table 3**

Measurements of phenylalanine, tyrosine and tryptophan ( $\mu\text{mol/L}$ ), as reported by other metabolomics studies of stratified (mild, moderate and severe) and unstratified COVID-19 cases.

Metabolite	Reference	Stratified COVID-19			Unstratified COVID-19	Normal serum reference values
		Mild	Moderate	Severe		
Phenylalanine	Psychogios et al. (2011) [36]					78.1 $\pm$ 20.5
	Ansone et al. (2021) [24]				117.35 $\pm$ 38.07	
	Caterino et al. (2021) [25]	84.25 $\pm$ 50.58	172.5 $\pm$ 60.12	125.5 $\pm$ 47.53		
	Herrera-van Oostdam et al. (2021) [26]	76.1	83.9	94.15 $\pm$ 27.37		
	Bruzzone et al. (2020) [29]	[61.45–91.70]	[70.23–102]			
Tyrosine	Luporini et al., 2021 [32]	60.54	90.8	103.52	116 $\pm$ 43	
	Meoni et al., 2021 [33]	[51.46–81.72]	[69.01–135.0]	[86.57–131.36]	167 $\pm$ 48.9	
	Psychogios et al. (2011) [36]					54.5 $\pm$ 9.7
	Ansone et al. (2021) [24]				68.93 $\pm$ 21.50	
	Caterino et al. (2021) [25]	75.75 $\pm$ 20.17	91.9 $\pm$ 22.06	73.8 $\pm$ 28.82		
Tryptophan	Bruzzone et al. (2020) [29]				56 $\pm$ 21	
	Luporini et al., 2021 [32]	55.74	71.2	75.06		
	Meoni et al., 2021 [33]	[49.67–71.75]	[55.19–94.38]	[55.19–88.86]	173.3 $\pm$ 35.2	
	Psychogios et al. (2011) [36]					54.5 $\pm$ 9.7
	Ansone et al. (2021) [24]				59.57 $\pm$ 20.67	
	Caterino et al. (2021) [25]	78.55 $\pm$ 24.17	54.9 $\pm$ 18.81	41.3 $\pm$ 25.68		
	Herrera-van Oostdam et al. (2021) [26]	39.63 $\pm$ 13.58	36.98 $\pm$ 13.57	34.46 $\pm$ 15.45		

**Table 4**

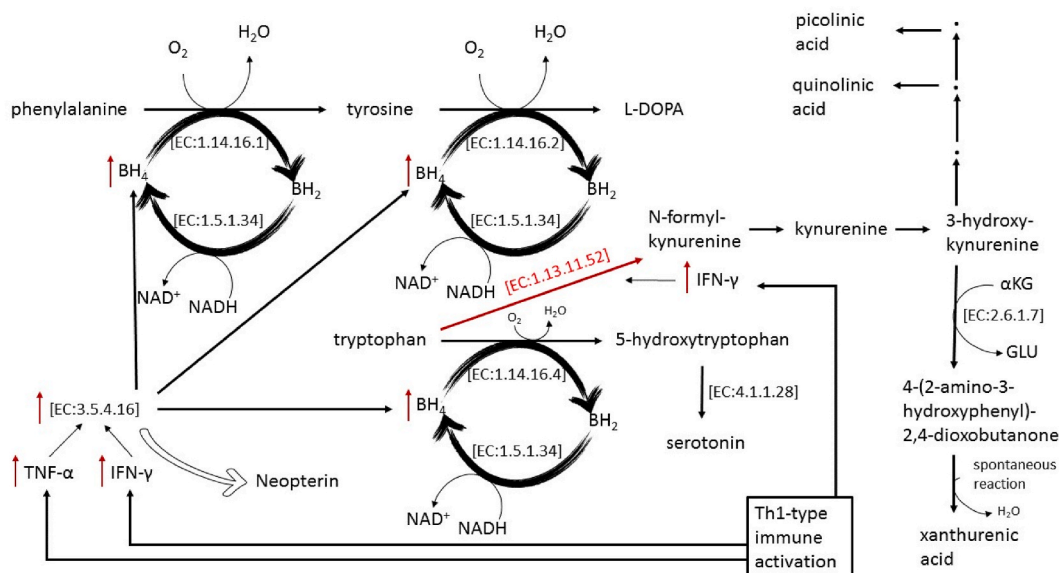
Significant correlations and/or associations between phenylalanine and other variables in COVID-19, as reported by literature studies.

Reference	Significant correlations between phenylalanine and other variables in COVID-19
Ansone et al. (2021) [24]	None
Caterino et al. (2021) [25]	None
Herrera-van Oostdam et al. (2021) [26]	Positive correlation between phenylalanine and IP-10 (cytokine produced in response to IFN- $\gamma$ activation)
Schmelter et al. (2021) [28]	None
Bruzzone et al. (2020) [29]	None
Atila et al. (2021) [30]	Phenylalanine showed significant association with COVID-19 severity
Dierckx et al. (2020) [31]	Phenylalanine showed significant association with COVID-19 severity
Luporini et al., 2021 [32]	Phenylalanine showed significant association with COVID-19 severity
Meoni et al., 2021 [33]	Phenylalanine exhibited a significant positive correlation with C-reactive protein and interleukin-6
Franco et al. (2021) [34]	Phenylalanine positively correlated with fraction of inspired oxygen
Shi et al., 2021 [35]	None

from increased tryptophan catabolism. As shown in Fig. 7, increased amounts of BH<sub>4</sub> are generated via *de novo* biosynthesis. BH<sub>4</sub> is used in the initial breakdown of all three aromatic amino acids – phenylalanine, tyrosine and tryptophan. Within the tryptophan metabolic pathway, tryptophan can either be used for the biosynthesis of serotonin via 5-hydroxytryptophan or tryptophan can be catabolized via the kynurenine pathway. The initial enzymatic reaction for the catabolism of tryptophan – indoleamine monooxygenase (EC:1.13.11.52), is upregulated via pro-inflammatory cytokines, such as IFN- $\gamma$  [46]. Thus, sufficiently acute and/or persistent inflammation and/or immune activation leads to increased tryptophan catabolism. The two main downstream end-products from the primary catabolism of tryptophan are quinolinic acid and picolinic acid (Fig. 7). Increased flux within the catabolism of tryptophan also results in the secondary pathway whereby 3-hydroxykynurenine is converted to 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanone via kynurenine aminotransferase (EC:2.6.1.7), which undergoes a spontaneous reaction to form XA. Activation of kynurenine aminotransferase also results in increased conversion of kynurenine to kynurenic acid. Increased kynurenic acid in COVID-19 has been found to be correlated with interleukin-6 [27]. In this study kynurenic acid significantly increased in COVID-19.

Putting everything together, we posit that for normal, healthy individuals (no pre-existing chronic inflammatory conditions) that the bioavailability of the BH<sub>4</sub> pool is sufficient to handle any immediate stress, such as a sudden, acute inflammatory infection (e.g. nonsevere form of COVID-19). Thus, phenylalanine should increase slightly, but tyrosine and phenylalanine:tyrosine measurements should not increase significantly. However, if the inflammatory conditions become sufficiently more severe (severe form of COVID-19), then both phenylalanine and tyrosine should spike in concentrations due to increased BH<sub>4</sub> production, but if the bioavailability of BH<sub>4</sub> is sufficient then the phenylalanine:tyrosine measurement should not significantly increase. For individuals that have a pre-existing chronic inflammatory condition – in this study it was HIV co-infection, but this can be extended to other co-morbidities (e.g. diabetes, obesity and hypertension), then the bioavailability of BH<sub>4</sub> becomes an issue. We posit that when individuals with pre-existing





**Fig. 7.** Initial catabolism of aromatic amino acids – phenylalanine, tyrosine and tryptophan, is upregulated by pro-inflammatory cytokines due to increased tetrahydrobiopterin (BH<sub>4</sub>) production. Pro-inflammatory cytokines also upregulate the formation of kynurenine, and the by-product xanthurenic acid. Neopterin is also indicated to be increased due to upregulated BH<sub>4</sub> production. αKG = α-ketoglutaric acid; GLU = glutamic acid; BH<sub>2</sub> = dihydrobiopterin; EC:1.14.16.1 = phenylalanine hydroxylase; EC:1.14.16.2 = tyrosine hydroxylase; EC:1.14.16.4 = tryptophan hydroxylase; EC:1.5.1.34 = dihydropteridine reductase; EC:3.5.4.16 = guanosine triphosphate cyclohydrolase; EC:1.13.11.52 = indoleamine 2,3-dioxygenase; EC:2.6.1.7 = kynurenine aminotransferase; EC:4.1.1.28 = 5-hydroxytryptophan decarboxylase.

phenylalanine to tyrosine is impaired, but not completely inhibited. This pathology is like what is seen in patients with inborn errors of metabolism (IEMs) that result due to BH<sub>4</sub> deficiency. These IEMs present with a more subtle form of primary phenylketonuria (PKU) – the complete absence of activity of the enzyme phenylalanine hydroxylase. Our hypothesis is supported by the fact that it is known that tryptophan catabolism is highly upregulated via pro-inflammatory cytokines, along with increased amounts of the by-product XA. XA has been shown to inhibit the final enzymatic step in the *de novo* biosynthesis of BH<sub>4</sub>. Hence, pre-existing inflammatory conditions result in limited bioavailability of BH<sub>4</sub>, and subsequent acute inflammation inhibits BH<sub>4</sub> biosynthesis.

Lastly, based upon the model described in Fig. 7, if the flux of tryptophan is toward the kynurenine pathway (catabolism), then the biosynthesis of serotonin should theoretically be decreased in COVID-19 cases. Moreover, based upon our hypothesis, if BH<sub>4</sub> bioavailability is limited in HIV + cases then the serotonin biosynthesis pathway, which needs the cofactor BH<sub>4</sub>, should be further impaired. We were unable to obtain measurements for serotonin in this study but the LC-MS/MS metabolomics study by Caterino et al. (2021) [25] reported a decrease of serotonin from mild ( $0.682 \pm 0.19 \mu\text{mol/L}$ ) to moderate ( $0.57 \pm 0.15 \mu\text{mol/L}$ ) and severe ( $0.486 \pm 0.17 \mu\text{mol/L}$ ) COVID-19. These serotonin data from Caterino et al. (2021) [25] support the model that we describe here.

## 5. Conclusion

In this study, we used a targeted metabolomics approach to analyze serum from COVID-19 patients, with and without HIV co-infection. We utilized two analytical platforms – <sup>1</sup>H NMR and LC-MS/MS, to obtain metabolite data. In this paper we focused upon four metabolites from the metabolomics data pool – phenylalanine, tyrosine, tryptophan and kynurenic acid, and their ratios, and include the Fischer's ratio. Based upon these targeted metabolomics data we formulated a hypothesis – when individuals with pre-existing chronic inflammatory conditions (e.g. HIV) are hit with an acute inflammatory condition (e.g. COVID-19) that they do not have sufficient bioavailability of BH<sub>4</sub>. This hypothesis is intended to give insights into the biochemistry behind acute inflammatory infections, such as SARS-CoV-2 infection, and persistent/chronic inflammatory diseases, such as HIV.

We acknowledge that a major limitation of this study is that the sample sizes are small (even more so for the HIV + co-infected cases) and further testing, which we encourage, is needed to test our hypothesis, particularly in other geographical cohorts. While our focus was on HIV, we believe other co-morbidities (e.g., diabetes, hypertension, cardiovascular disease) would exhibit a similar insufficient bioavailability of BH<sub>4</sub> in COVID-19 patients and our hypothesis should also be tested in these cases. Other limitations of the study include: only examined COVID-19 cases infected with an earlier variant of the SARS-CoV-2 virus, no children were included in the study, the effects of gender [47] and diets on the metabolome were not assessed, only serum samples (no other sample types) were collected/examined, and other 'omics analyses (e.g., proteomics, transcriptomic, genomics) were not incorporated into this study.

We propose that more targeted metabolomics analyses be performed to assess: neopterin, xanthurenic acid, the flux through BH<sub>4</sub> biosynthesis, and a closer examination of tryptophan catabolism, to either confirm or refute our hypothesis. Furthermore, we suggest a follow-up clinical study testing the effects of BH<sub>4</sub> supplementation in severe COVID-19 cases with pre-existing chronic inflammatory

conditions, because BH<sub>4</sub> supplementation may help this overstressed pathway, and potentially alleviate/lessen COVID-19 symptoms.

### Author contribution statement

Shayne Mason: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mari van Reenen: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Theresa Roussouw: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Zander Lindeque: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Roan Louw: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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### Data availability statement

Data will be made available on request.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e15010>.

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