



Saliva metabolomic profile of COVID-19 patients associates with disease severity

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Abstract

Introduction Coronavirus disease 2019 (COVID-19) is strongly linked to dysregulation of various molecular, cellular, and physiological processes that change abundance of different biomolecules including metabolites that may be ultimately used as biomarkers for disease progression and severity. It is important at early stage to readily distinguish those patients that are likely to progress to moderate and severe stages.

Objectives This study aimed to investigate the utility of saliva and plasma metabolomic profiles as a potential parameter for risk stratifying COVID-19 patients.

Method LC–MS/MS-based untargeted metabolomics were used to profile the changes in saliva and plasma metabolomic profiles of COVID-19 patients with different severities.

Results Saliva and plasma metabolites were screened in 62 COVID-19 patients and 18 non-infected controls. The COVID-19 group included 16 severe, 15 moderate, 16 mild, and 15 asymptomatic cases. Thirty-six differential metabolites were detected in COVID-19 versus control comparisons. SARS-CoV-2 induced metabolic derangement differed with infection severity. The metabolic changes were identified in saliva and plasma, however, saliva showed higher intensity of metabolic changes. Levels of saliva metabolites such as sphingosine and kynurenine were significantly different between COVID-19 infected and non-infected individuals; while linoleic acid and Alpha-ketoisovaleric acid were specifically increased in severe compared to non-severe patients. As expected, the two prognostic biomarkers of C-reactive protein and D-dimer were negatively correlated with sphingosine and 5-Aminolevulinic acid, and positively correlated with L-Tryptophan and L-Kynurenine.

Conclusion Saliva disease-specific and severity-specific metabolite could be employed as potential COVID-19 diagnostic and prognostic biomarkers.

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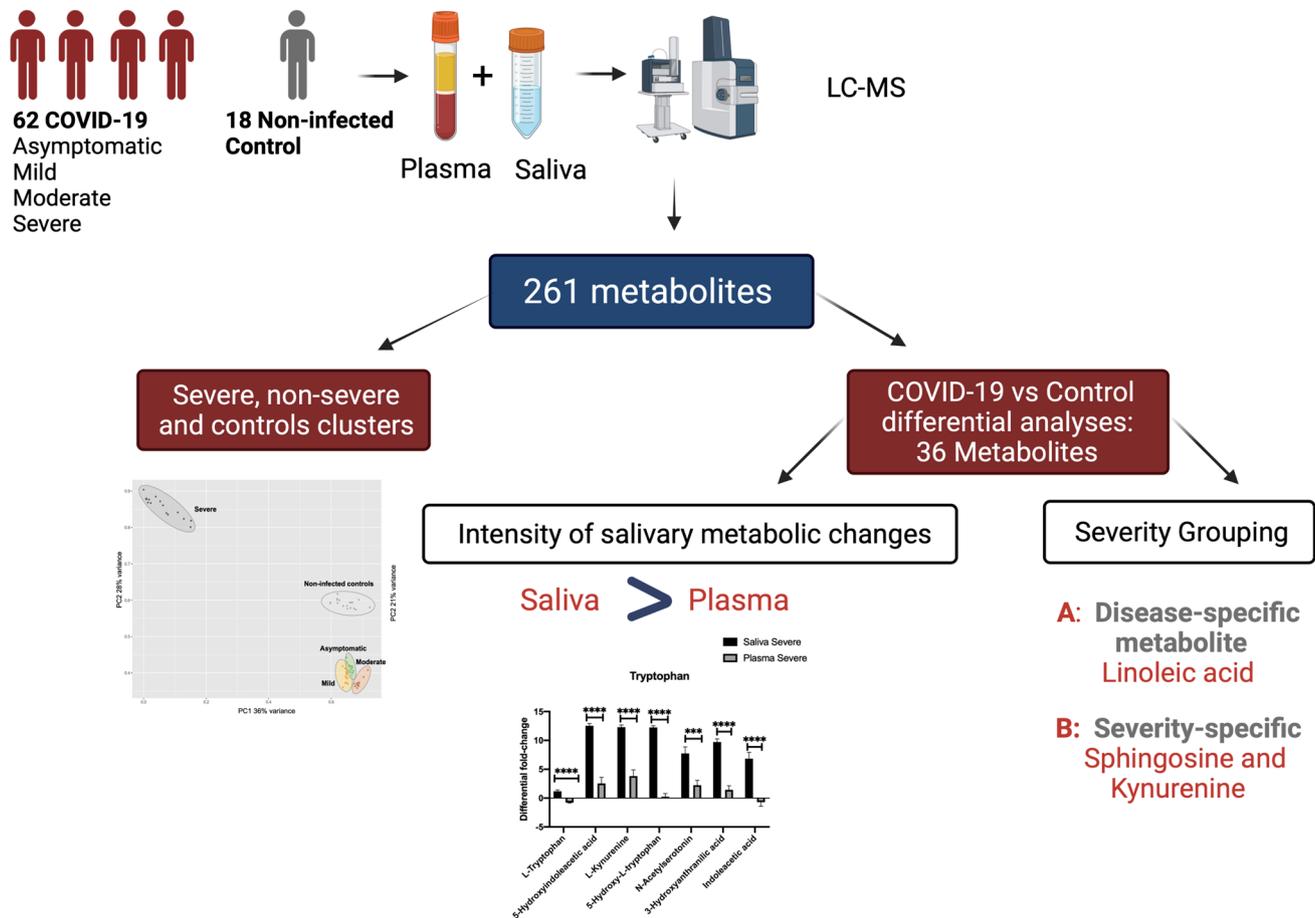
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Graphical abstract



Keywords SARS-CoV-2 · COVID-19 · Metabolomics · Liquid chromatography-mass spectrometry · Plasma · Saliva

1 Introduction

The global outbreak coronavirus disease 2019 (COVID-19) viral pneumonia caused by severe acute respiratory syndrome corona virus 2 (SARS-CoV-2), which originated in Wuhan, China, in December 2019 has become a worldwide pandemic claiming several thousands of lives worldwide. According to last update 4th of October 2021 of John Hopkins- Coronavirus Resource Center (<https://coronavirus.jhu.edu/map.html>), this virus has infected more than 234,987,662 people worldwide and 4,803,300 confirmed deaths. Despite the fact that more than 80% of COVID-19 patients have mild symptoms, such as anosmia (loss of smell) and ageusia (loss of taste) and in some cases accompanied with other more moderate symptoms including fever, exhaustion, cough, and shortness of breath (Hui et al., 2020; Yang et al., 2020). A small percentage (around 5%) of patients however may

develop life-threatening acute respiratory distress syndrome (ARDS) requiring invasive mechanical ventilation (Yang et al., 2020). Both of geriatrics and people with certain medical conditions including hypertension, obesity, or diabetes are most likely prone to get infected with COVID-19 manifesting severe symptoms (Richardson et al., 2020). However, there have been episodes of patients with COVID-19 symptoms who do not have any pre-existing medical conditions and still progress rapidly from mild or/moderate to severe conditions, particularly in the absence of adequate medical care (Yang et al., 2020). Thus, given the high mortality rate among COVID-19 patients, early diagnostic and prognostic of the disease and appropriate interventions are critical in reducing complication and mortality. It is critical to differentiate between patients who are likely to progress to moderate/severe stages and those who will remain mild or asymptomatic and eventually recover. There are currently no diagnostic or prognostic markers that may reliably predict

COVID-19 disease development (Statsenko et al., 2021; Yan et al., 2020). Nonetheless, COVID-19 pathology is strongly linked to dysregulation of various molecular, cellular, and physiological processes, including a significant disturbance in the cytokines that might be generated by a subset of inflammatory monocytes (Zhou et al., 2020), lymphopenia (Guan et al., 2020; Qin et al., 2020) and T cells exhaustion (Zheng et al., 2020; Zhou et al., 2020). As a result, such disease-related processes result in alter abundance of different biomolecules not only cytokines but also peptides, and metabolites that ultimately may be used as prognostic markers for COVID-19 progression and severity. In this line, a number of proteomics studies in peripheral blood samples have shown that proteins such as IL6, CKAP4, Gal-9, IL-1ra, LILRB4 and PD-L1 (Alaiya et al., 2021; Patel et al., 2021) and more recently Geyer et al., 2021 (Geyer et al., 2021) reported that proteins of innate immune system (CRP, SAA1, CD14, LBP) and coagulation regulators (APOH, FN1, HRG) are associated with COVID-19 severity and may have a potential value as disease prognostic biomarkers. Although additional research is needed to confirm those protein biomarkers, nevertheless this alone opens new avenues in this field of study and suggests that multi-omics approaches may lead to the identification of small molecules that inform us about COVID-19 disease status and progression. In this regard, metabolomics is an appealing complementary analysis to proteomics because protein deregulation may result in an altered level of several metabolites. Furthermore, metabolite extraction is simpler and faster than protein extraction, allowing not only for protocol standardization in biomarker research, but also for the analysis of simpler body fluid samples such as saliva, sweat, tears, and so on (Blanco-Melo et al., 2020). Untargeted metabolomics is a powerful high-throughput tool for identifying disease diagnostic and/or prognostic biomarkers. We used LC-MS/MS-based untargeted metabolomics in this study to investigate the utility of saliva and/or plasma metabolome profiles as a potential parameter for risk stratifying COVID-19 patients into four groups: asymptomatic, mild, moderate, and severe.

2 Materials and methods

2.1 Patients and samples

Non-infected controls and COVID-19 patients were recruited from Rashid Hospital, Dubai, UAE. Diagnosis of COVID-19 was confirmed by a positive SARS-CoV-2 infection polymerase chain reaction (PCR) test while non-infected control was asserted by PCR-negative SARS-CoV-2 test. National Institutes of Health (NIH)

COVID-19 guidelines were used to grade the severity of patients into asymptomatic, mild, moderate, and severe (NIH). Asymptomatic cases were defined as those who did not present any symptoms at the time of SARS-CoV-2 testing or diagnosis, mild cases were defined as those who presented with minor respiratory presentation without need of hospitalization, moderate cases was defined by infection requiring hospitalization, while severe cases were defined by disease requiring hospitalization and respiratory support (Alamer et al., 2021; Al-Muhsen et al., 2022; Ma et al., 2021). Saliva and blood were collected from each participant. Plasma was isolated from blood via standard Ficoll-Paque density gradient centrifugation (Sigma, Histopaque-10771). Precautions recommended by CDC for safe collecting, handling and testing of biological fluids were followed (www.cdc.gov).

2.2 Sample collection and preparation

A total of 1 mL or 4 mL of saliva or blood was collected from each subject into a sterile container. An aliquot of sample and cold methanol was added in a micro centrifuge tube at 3:1 ratio, the tube was vortexed, and then allowed to sit for two hours at -20°C . Next the samples were centrifuged for 15 min at $20,817\times g$ and the supernatant transferred to a new micro centrifuge tube. Dried samples were either stored at -80°C until further use or dissolved in 200 μL of 0.1% formic acid in deionized water-LC-MS CHROMASOLV from Honeywell (Wunstorfer Strasse, Seelze, Germany) and vortexed for 2 min to be mixed totally. Finally, the samples were filtered using a hydrophilic nylon syringe filter of 0.45 μm pore size and returned to the glass insert within LC glass vials, after that the samples were placed in the autosampler at 8°C for LC-MS/MS analysis.

TIMS-Q-TOF Mass Spectrometer (BRUKER, Germany) along with Metaboscape software version 4 were employed for separation and detection of the cell metabolites. It was equipped with Trapped Ion Mobility Quadrupole Time-of-Flight mass spectrometer and composed of Solvent delivery systems pump (ELUTE UHPLC Pump HPG 1300), Autosampler (ELUTE UHPLC), Thermostat column compartment (ELUTE UHPLC), Computer System, Windows 10 Enterprise 2016 LTSC, Data Management Software, Bruker Compass HyStar 5.0 SR1 Patch1 (5.0.37.1), Compass 3.1 for otofSeries, otofControl Version 6.0. Metabolites were analyzed in auto MS/MS within the range of 20–1300 m/z utilizing electrospray ionization (ESI). The ESI source was 10 L/min (nitrogen) and the drying temperature equal to 220°C .

The capillary voltage of the ESI was 4500 V with 2.2 bar nebulizer pressure and end Plate Offset as 500 V.

The acquisition involved two segments; auto MS scan, which ranged from 0 to 0.3 min for the calibrant sodium formate, and auto MS/MS scan with CID acquisition, which included fragmentation and ranged from 0.3 to 30 min. The acquisition in both segments was performed using the positive mode at 12 Hz. The width of the precursor ion was ± 0.5 , the number of precursors was 3, the cycle time was 0.5 s., and the threshold was 400 cts. Active exclusion was excluded after 3 spectra and released after 0.2 min. For the auto MS scan, the collision energy was set at 7 eV and for MS2 acquisition the data dependent acquisition (DDA) was used, and the collision energy stepping fluctuated between 100 and 250% set at 20 eV. A HAMILTON® Intensity Solo 2 C18 column (100 $\mu\text{m} \times 2.1 \text{ mm} \times 1.8 \mu\text{m}$) was utilized for the separation of metabolites. Sodium Formate was used as a calibrant for the external calibration step. For metabolite analysis, solvent A (Water + 0.1% FA and solvent B (Acetonitrile + 0.1% FA) were used in gradient elution mode as the following: 0 to 2 min, 99% A; 2 to 17 min, 99–1% A; 17 to 20 min, 1% A; 20 to 20.1 min, 1–99% A; 20.1 to 30 min, 99% A. The flow rate was set as (0.250–0.350 mL/min) for 30 min in gradient mode with a maximum pressure of 14,993 psi. The elute autosampler temperature was set at 8°C and the column oven temperature was at 35°C. And a total volume of 10 μL was injected into the Q-TOF-MS. The quality control (QC) sample was prepared by pooling the same volume (10 μL) of each sample. The QC sample was injected five times at the beginning of the sequence, then one QC injection was performed every (9–10 samples). LC total ion chromatograms (TIC) and fragmentation patterns of the metabolites were identified using HMDB Mass Spectral Library (2 technical replicates).

The performance of the column and the mass spectrometer was tested using a test mixture of (TRX-2101/RT-28-calibrants for Bruker T-ReX LC-QTOF solution from Nova Medical Testing Inc.) to check the performance of reversed phase liquid chromatography (RPLC) separation and perform multipoint retention time calibration, and (TRX-3112-R/MS Certified Human serum for Bruker T-ReX LC-QTOF solution from Nova Medical Testing Inc.) to check the performance of sample preparation protocols as well as LC–MS instruments. This product is prepared from pooled human blood.

The Initial Processing were performed using MetaScape® 4.0 software (Bruker Daltonics). Bucketing in T-ReX 2D/3D workflow the parameters set for molecular features detection were as follows- intensity threshold equal to 1000 counts along with minimum peak length equal to 7 spectra were fixed for peak detection using peak area as feature signal. The mass recalibration was done within a retention time range between 0 and 0.3 min. On the other hand, the MS/MS import method was done using the average

spectrum out of all MS/MS spectra. Then, parameters set for data bucketing were assigned as follows: Retention time range started at 0.3 min and ended at 25 min, while mass range started at 50 m/z and ended at 1000 m/z. Identification of metabolites was based on mapping the MS/MS spectra and retention time in the HMDB 4.0, an annotated resource designed to satisfy the needs of the metabolomics community. The compounds with MS/MS were identified using library matching through the annotation process. Then, the selected metabolites were filtered by choosing the set with a higher annotation quality score (AQ score) representing the best retention time values, MS/MS score, m/z values, mSigma, and analyte list spectral library.

2.3 Statistical analysis

Metabolite levels were normalized using the Bioconductor package limma-voom and presented as log₂ (Ritchie et al., 2015a). Metabolites normality was confirmed using Shapiro–Wilk test and Kolmogorov–Smirnov test. Log-transformed normalized intensities were used in LIMMA analyses to identify differential changes in metabolites between diseased and control groups. We used the default Benjamini–Hochberg correction for multiple testing (Dudoit et al., 2002; Smyth Gordon, 2004). One-way analysis of variance (ANOVA) and post hoc Tukey multiple comparison analyses were applied to compare between more than two independent variables. Correlation of sphingosine, 5-Aminolevulinic acid, L-Tryptophan, and L-Kynurenine saliva concentrations of COVID-19 patients and serum markers of COVID-19 severity such as D-dimer and C-reactive protein was evaluated using Pearson's coefficient test with two-tailed p-value < 0.05 considered significant.

Principal component analysis (PCA) was implemented using PCAGO R Shiny framework which is available at github.com/hoelzer-lab/pcagom. jHeatmap was used to create the heatmaps which is available at [http://jheatmap.github.io/jheatmap/](https://jheatmap.github.io/jheatmap/) (Deu-Pons et al., 2014). Metabolite Set Enrichment Analysis (MSEA) were carried using MetaboAnalystR3 package which is available at github.com/xia-lab/MetaboAnalystR (Pang et al., 2020). MSEA contains metabolic set libraries and it is considered to be the metabolic version of Gene Set Enrichment Analysis (GSEA) (Xia & Wishart, 2010). Statistical analyses were performed using R software (v 3.5.2), SPSS 27.00 (SPSS Inc., Chicago, IL, USA), and Prism (v8; GraphPad Software). p-value of < 0.05 considered statistically significant.

3 Results

3.1 Clustering of severe, non-severe and non-infected controls

A total of 62 COVID-19 patients and 18 non-infected controls were included. COVID-19 group comprised 15 asymptomatic, 16 mild, 15 moderate, and 16 severe patients. The participants were age and gender matched. Supplementary Table 1 presents the demographic and clinical profile of the patients.

Metabolites from plasma and saliva of all patients were analyzed. Total of 261 metabolites were detected and identified in saliva and plasma samples (Supplementary Table 2). Downstream analyses were performed to identify COVID-19 infection related metabolites and their correlation to the disease severity (Fig. 1). PCA scores for saliva metabolites revealed three distinct clusters representing the non-infected controls (tested PCR negative for COVID-19), severe, and non-severe COVID-19 patients (Fig. 2A), while the PCA scores for plasma showed an additional fourth cluster for the asymptomatic COVID-19 (Fig. 2B).

3.2 Comparison of saliva and plasma metabolite dysregulation during COVID-19 infection

Metabolite values were normalized with limma-voom method (Ritchie et al., 2015b). Differential metabolite analyses were run independently between the non-infected controls and three COVID-19 severities. The top 50 metabolites from each four comparison were selected, medication and repeated names were removed to remain with 36 metabolites that presented the COVID-19 differential metabolites (Figs. 1 and 3A, B). We next conducted Metabolite Set Enrichment Analysis to determine the metabolic pathways involving these metabolites. This revealed enhancement of top pathways including tryptophan metabolism, purine metabolism, phenylalanine, and citrate cycle (TCA) pathways (Supplementary Fig. 1).

The top 36 metabolites and their respective pathways were plotted in heatmaps separately for saliva (Fig. 3A) and plasma (Fig. 3B). Heatmap visualization presented more noticeable COVID-19 induced metabolism change in saliva versus plasma samples. To quantify this observation, the differential metabolite results of severe COVID-19 versus controls were compared between saliva and plasma for top three enriched pathways: phenylalanine, purine, and tryptophan displayed in Fig. 3A and B. Following heatmap pattern, differential metabolite analyses showed more significant change in saliva compared to plasma during severe SARS-CoV-2 infection (Fig. 4). Phenylalanine pathway comparison

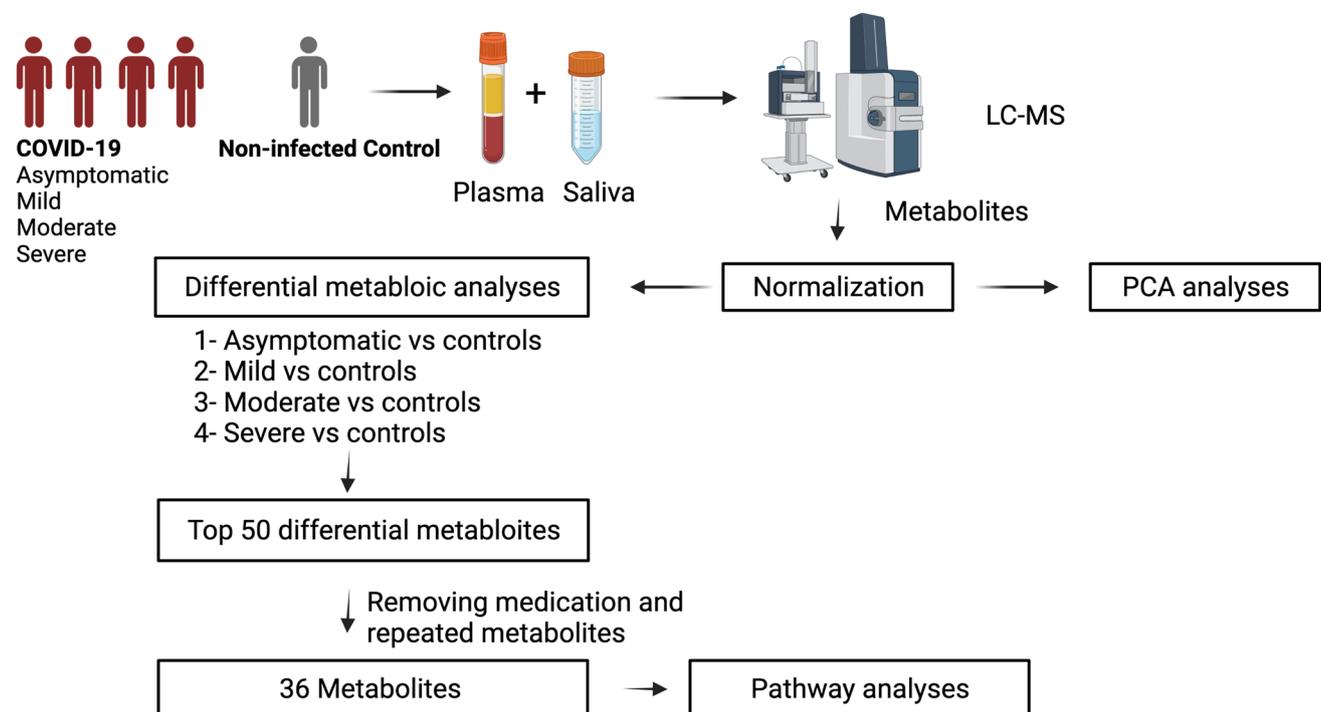


Fig. 1 Metabolomics analyses flowchart of patients with COVID-19

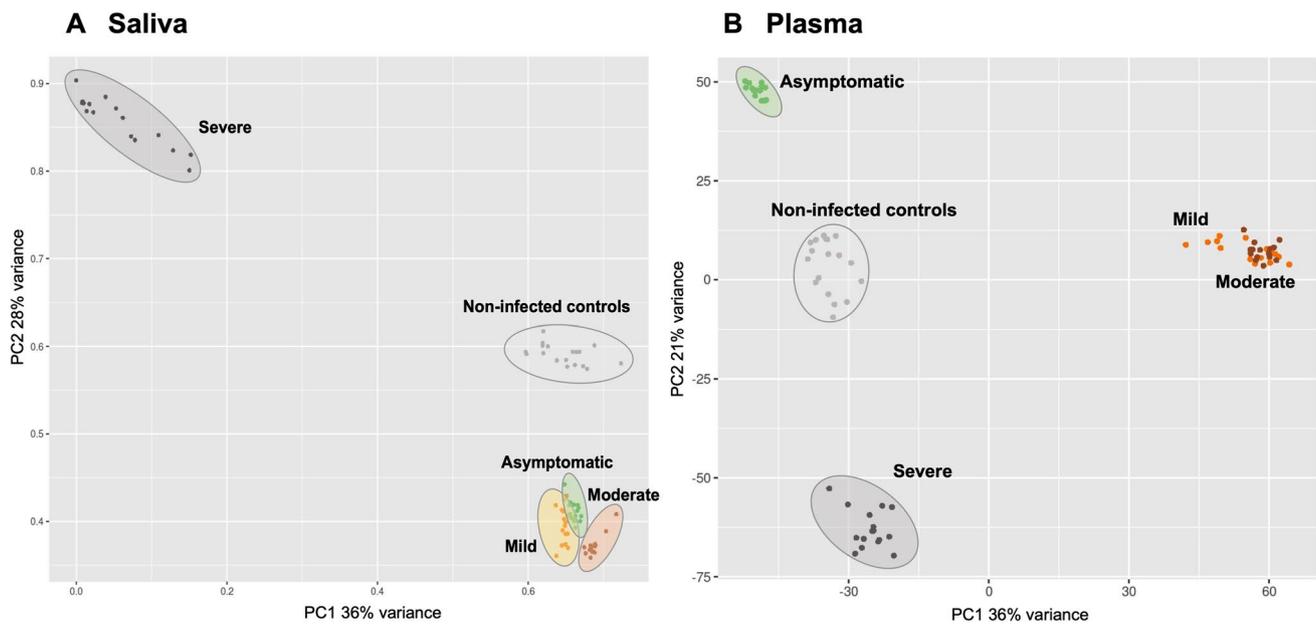


Fig. 2 Principal component analysis (PCA) clustering showing separation of severe, non-severe and control cases in both saliva (A) and plasma (B) samples. Samples included 18 non-infected controls and

62 COVID-19 patients that comprised 15 asymptomatic, 16 mild, 15 moderate, and 16 severe

showed similar increase in L-Phenylalanine part in both saliva and plasma of severe COVID-19 (1.2 ± 0.2 log-fold vs 2.3 ± 1.2 log-fold; p -value = 0.39), hippuric metabolite was significantly higher in saliva samples (8.7 ± 1.2 log-fold vs 0.4 ± 0.15 log-fold; p -value < 0.0001) while the levels of phenylacetaldehyde was not changed in saliva but increased in severe plasma (4.7 ± 0.66 log-fold) samples (Fig. 4). During severe COVID-19, overall metabolites change along purine and tryptophan pathway was at least twofold higher in saliva compared to plasma samples. Metabolism of hypoxanthine from purine pathway was similar in both severe saliva and plasma (6.1 ± 1.6 log-fold vs 5.2 ± 1.3 log-fold; p -value = 0.67), while metabolism of guanosine was not changed in severe plasma but increased in saliva samples (10.8 ± 1.3 log-fold).

Among the top enriched pathways, tryptophan metabolism that included L-Kynurenine, 3-Hydroxyanthranilic acid, L-Tryptophan were distinctly enriched in severe COVID-19 group (Figs. 3A, B and 5). L-Kynurenine overall metabolite level was higher in plasma than saliva, but in both sample types, it was elevated noticeably in severe COVID-19 (Fig. 5A). Salivary L-Tryptophan levels followed the pattern of L-Kynurenine specific elevation in severe COVID-19; however, the plasma pattern was different where the levels compared to controls were not changed in severe and decreased in non-severe groups (Fig. 5B).

Next, we wanted to identify the metabolites and their respective pathways that were enriched in non-severe COVID-19 groups. Sphingolipid metabolism,

glycerophospholipid metabolism, and glycine, serine and threonine metabolism were increased in less severe groups of asymptomatic, mild, and moderate (Fig. 3A, B). We have displayed the sphingosine levels within sphingolipid metabolism pathway (Fig. 6A), and 5-Aminolevulinic acid within glycine, serine, and threonine metabolism (Fig. 6B). 5-Aminolevulinic acid showed an increase in non-severe groups but were not detected in non-infected controls and severe patients (Fig. 6B). In contrast, sphingosine metabolite levels showed a decreasing pattern moving from asymptomatic to mild to moderate with diminished levels in severe COVID-19 (Fig. 6A).

We then correlated C-reactive and D-dimer protein prognostic levels with four salivary metabolites of sphingosine, 5-Aminolevulinic acid, L-Tryptophan, and L-Kynurenine. The two prognostic markers were negatively correlated with sphingosine and 5-Aminolevulinic acid (Fig. 7A, B, E, F), and positively correlated with L-Tryptophan and L-Kynurenine (Fig. 7C–D and G–H).

4 Discussion

Metabolic derangement in COVID-19 is triggered directly by SARS-CoV-2 and indirectly by the host response (Sid-diqi & Mehra, 2020). Herein we have profiled salivary and plasma metabolites in different COVID-19 severities. Our results revealed the top metabolic pathways deranged during COVID-19 and identified the metabolites that

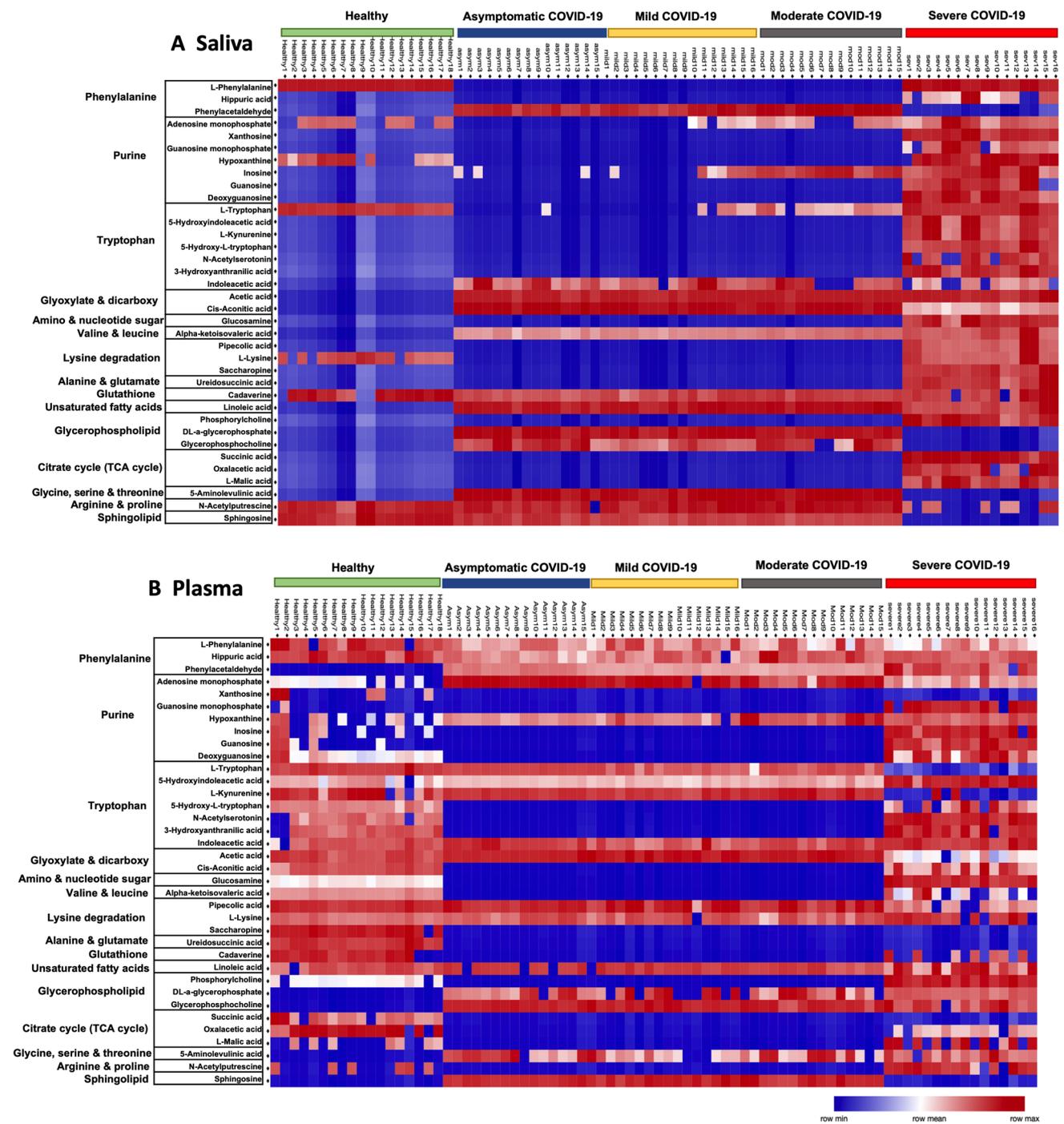


Fig. 3 Heatmaps of top 36 differential metabolites and their respective pathway during COVID-19 infection. **A** Salivary metabolites and **B** plasma metabolites were displayed for 62 COVID-19 patients and

18 non-infected controls. COVID-19 comprised 15 asymptomatic, 16 mild, 15 moderate, and 16 severe. Red color indicated higher metabolite values and blue color indicated lower metabolite values

differentiated the SARS-CoV-2 infected from non-infected controls, and those that segregated the severe infection from non-severe subtype.

Several previous reports have identified plasma metabolites to be used for diagnosis and prognosis of COVID-19 (Masuda et al., 2021; Thomas et al., 2020), however salivary COVID-19 metabolic dysregulation has not been

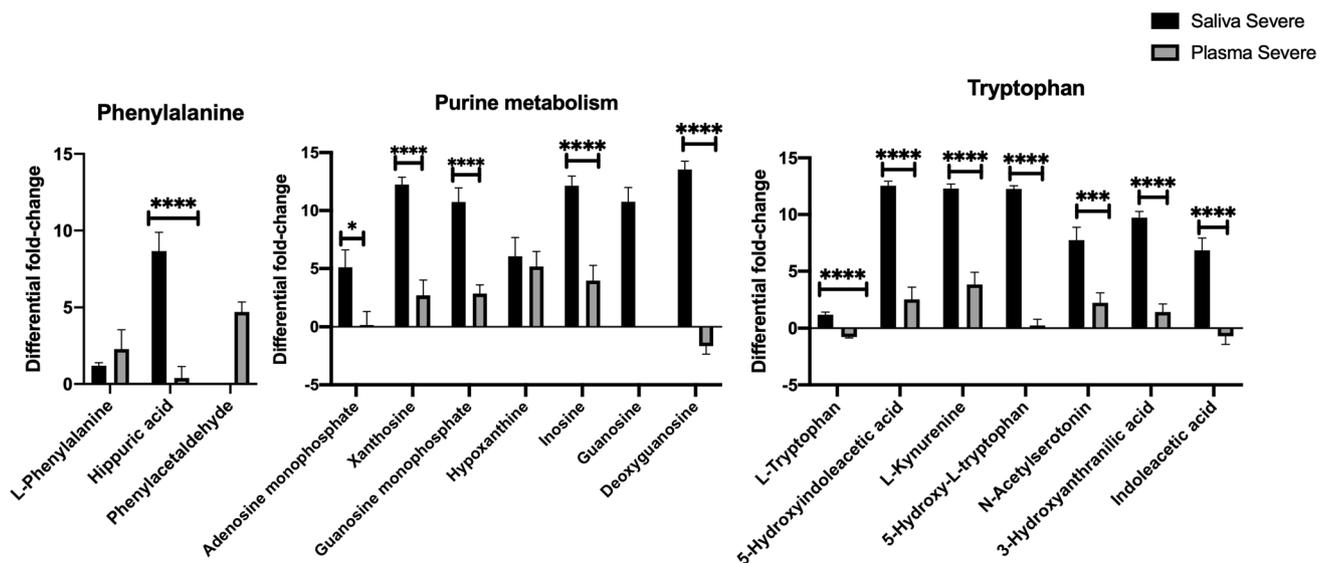


Fig. 4 Saliva and plasma differential metabolites of top enriched pathway derived from comparison between severe and non-infected controls. Higher fold-change were observed in saliva versus plasma samples. One-way analysis of variance (ANOVA) and post hoc Tukey

multiple comparison analyses were applied to compare between more than two independent variables. Samples included 18 non-infected controls 16 severe COVID-19. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results are presented as mean (\pm SEM)

well defined and mainly proposed in a number of published review papers (Costa dos Santos Junior et al., 2020; Hyvärinen et al., 2021; Sapkota et al., 2021). A preprint by Baily and colleague showed that salivary metabolic changes could be used to distinguish severe and non-severe COVID-19 but could not differentiate COVID-19 positive and negative patients (Frampas et al., 2021). The previous study used different settings than ours that could have affected their findings. They used hospitalized patients as controls which could have similar inflammatory levels to COVID-19 patients, and they did not include critical COVID-19 patients in severe group (Frampas et al., 2021). To our knowledge this is the first study to profile and compare salivary and plasma metabolites during COVID-19 infection. When compared to plasma, salivary metabolites were superior in differentiating both COVID-19 positive and negative samples as well as distinguishing the severe from non-severe COVID-19.

Saliva fluid is a good reservoir for different respiratory viruses (To et al., 2019; Wyllie et al., 2020; Yoon et al., 2017). Salivary fluid transcriptome has been highlighted as a potential non-invasive sample for detection of SARS-CoV-2 (Wyllie et al., 2020). SARS-CoV-2 infects host cells mainly using angiotensin-converting enzyme 2 (ACE2) receptor that is abundantly expressed in salivary gland and oral epithelial cells (Hoffmann et al., 2020; Xu et al., 2020). In addition to oral cavity shedding and salivary gland secretion, saliva fluid could be infected further through viral particles transmitted from upper and lower respiratory tract (Sapkota et al., 2020). Beside microbes and viral particles, other

important diagnostic, and prognostic contents such as hormones, enzymes and antibodies were also detected within saliva fluid (Lima et al., 2010; Sapkota et al., 2020). Salivary glands are surrounded by rich blood circulation which facilitate exchange of contents between blood and salivary fluid (Zhang et al., 2016). Therefore, the noticeable metabolic derangements observed in saliva could be directly induced by active SARS-CoV-2 infection within the oral cavity and salivary gland, besides the defused metabolites from blood circulation.

Tryptophan metabolism was one of the most enriched pathways in the severe COVID-19 group (Fig. 3A, B and Supplementary Fig. 1). Significant increase of Kynurenine pathway, part of tryptophan metabolism, was observed in both plasma and saliva samples of severe COVID-19 patients compared to controls as presented in Fig. 5A. Several studies have associated kynurenine serum metabolite upregulation with worse COVID-19 prognosis (Cai et al., 2021; Kimhofer et al., 2020; López-Hernández et al., 2021; Thomas et al., 2020; Xiao et al., 2021), however, this was not previously analyzed in saliva samples. Severe COVID-19 disease is associated with innate immune dysregulation, lymphocytopenia, and cytokine storm (Blanco-Melo et al., 2020; Lee et al., 2020; Liao et al., 2020; Vardhana & Wolchok, 2020). Thereby it is expected that metabolite reprogramming observed in severe COVID-19 disease to correlate to this immune dysregulation. Following this assumption, Xiao and colleague studied the plasma metabolite programming during COVID-19 infection (Xiao et al., 2021). They revealed

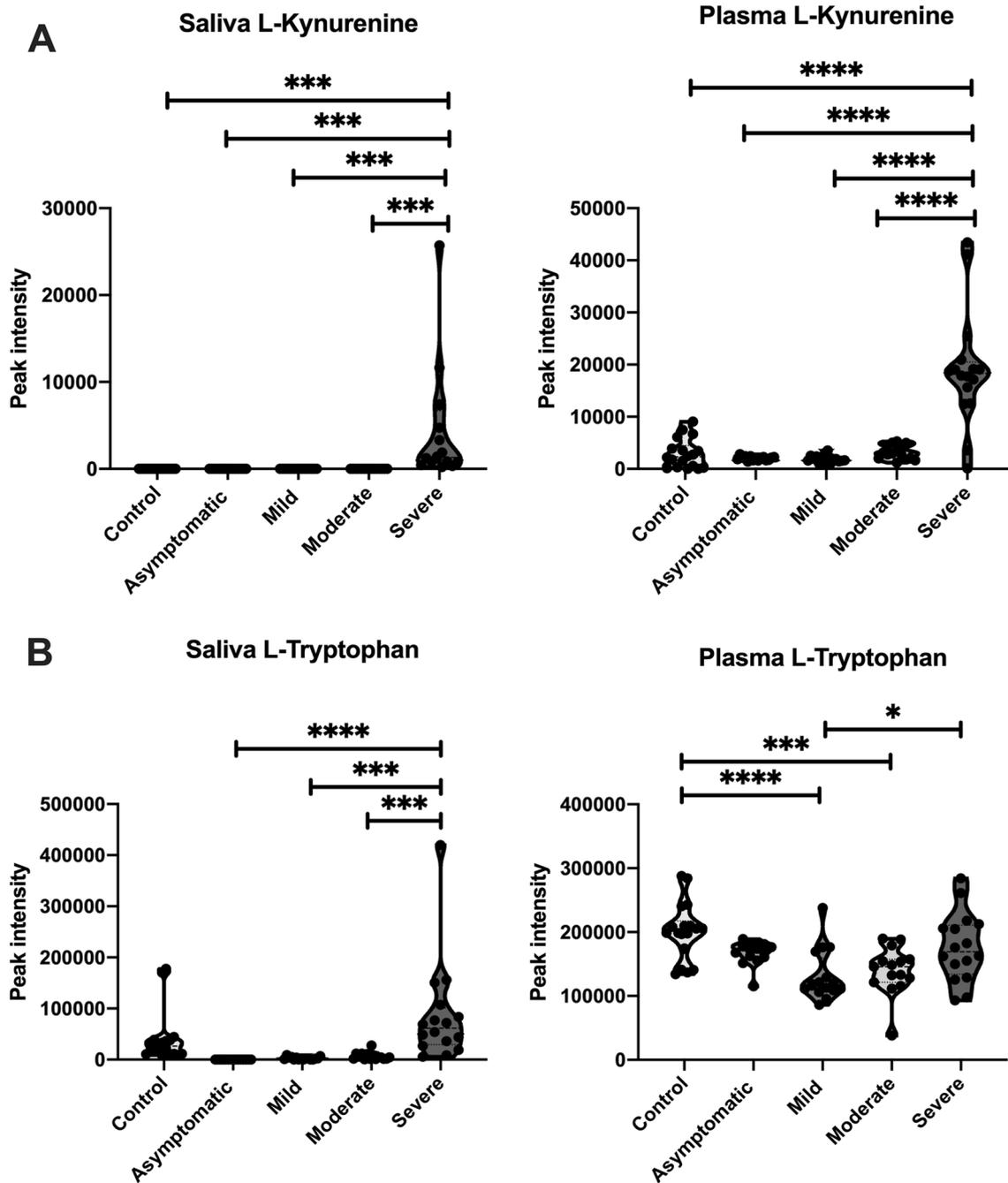


Fig. 5 Kynurenine and Tryptophan levels in different COVID-19 severities. **A** Kynurenine was noticeably increased in severe saliva and plasma of severe group, while **B** tryptophan was increased in saliva samples, it is overall level was decreased in COVID-19 plasma samples. Data presented as truncated violin plot with median and interquartile range. One-way analysis of variance (ANOVA) and

post hoc Tukey multiple comparison analyses were applied to compare between more than two independent variables. Samples included 18 non-infected controls and 62 COVID-19 patients that comprised 15 asymptomatic, 16 mild, 15 moderate, and 16 severe. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results are presented as mean (\pm SEM)

strong correlation between metabolite dysregulation and hyperinflammation found in severe COVID-19 (Xiao et al., 2021). Further, they confirmed this association by modulating viral induced inflammation using metabolite supplements (Xiao et al., 2021). In another study, kynurenic

acid to Kynurenine ratio, specifically in males was positively associated with age, inflammatory marker levels and negatively associated with T cell immune response during COVID-19 (Thomas et al., 2020).

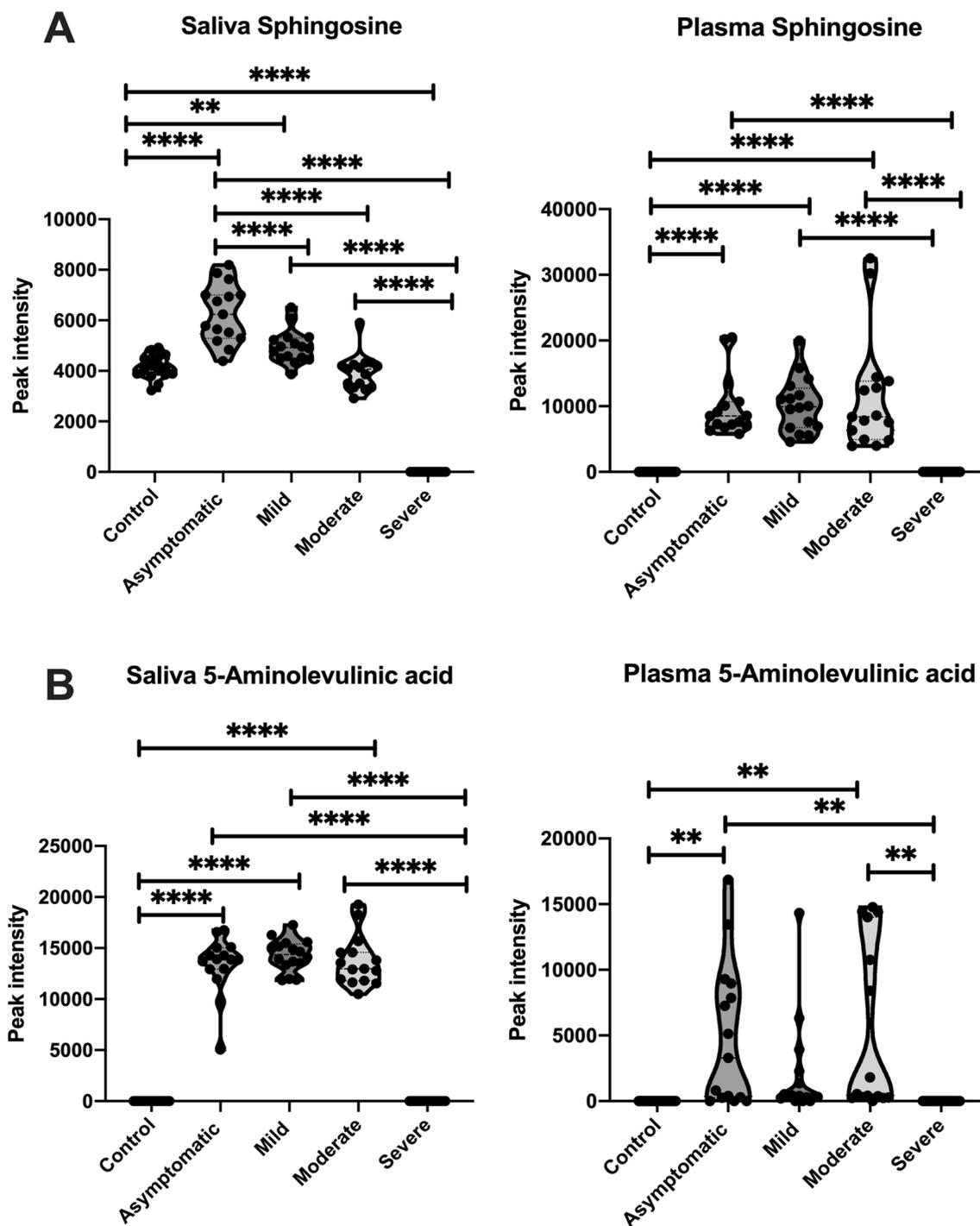


Fig. 6 Sphingosine and 5-Aminolevulinic acid levels in different COVID-19 severities. The two metabolites (**A**) Sphingosine (**B**) 5-Aminolevulinic acid levels were higher in non-severe groups of asymptomatic, moderate, and severe compared to severe and non-infected controls. Sphingosine was detected in salivary controls but not in plasma controls while 5-Aminolevulinic acid was not detected in non-infected controls for both plasma and saliva. Data presented

as truncated violin plot with median and interquartile range. One-way analysis of variance (ANOVA) and post hoc Tukey multiple comparison analyses were applied to compare between more than two independent variables. Samples included 18 non-infected controls and 62 COVID-19 patients that comprised 15 asymptomatic, 16 mild, 15 moderate, and 16 severe. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results are presented as mean (\pm SEM)

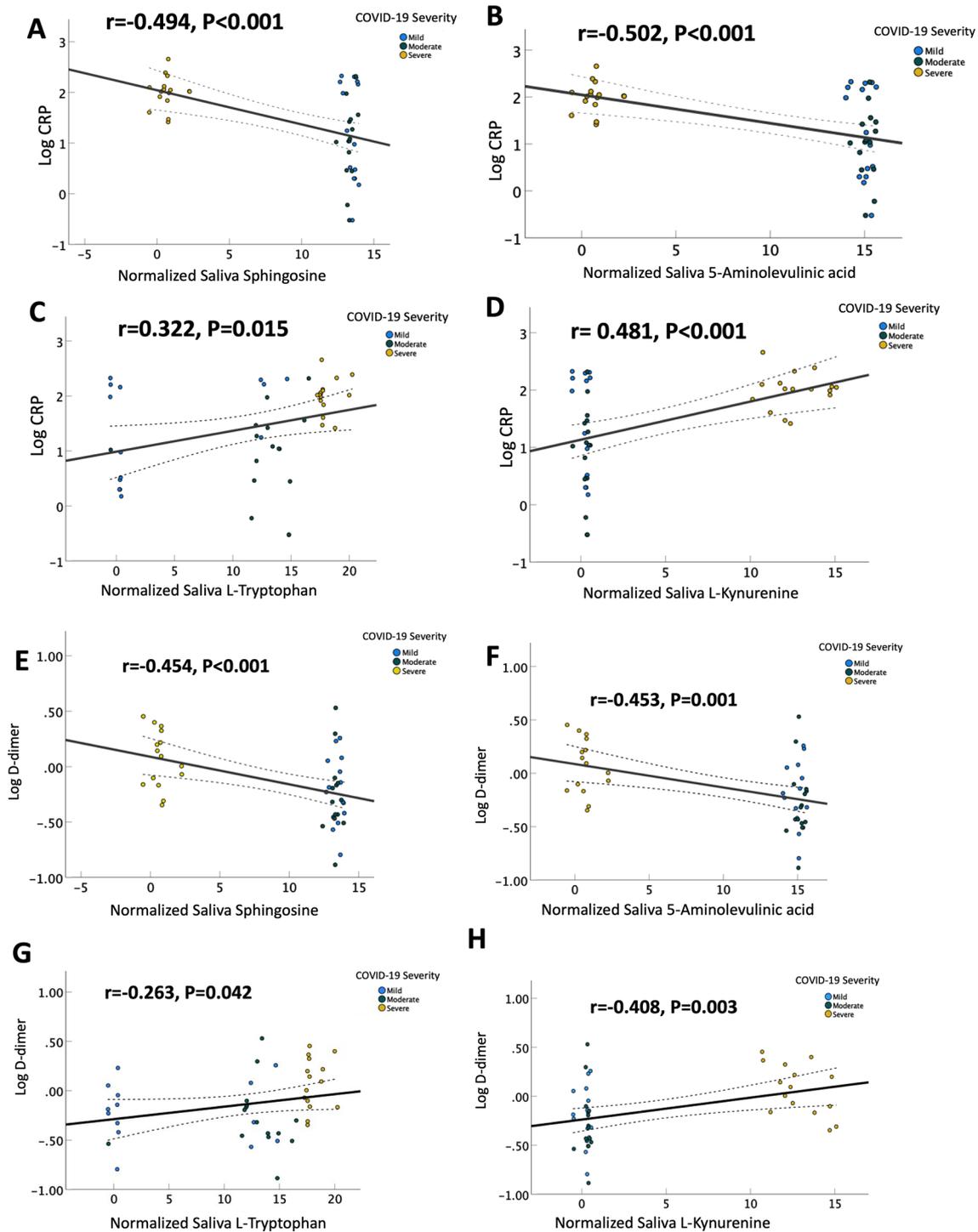


Fig. 7 Correlation of four salivary metabolites of sphingosine, 5-Aminolevulinic acid, L-Tryptophan, and L-Kynurenine with serum markers of COVID-19 severity. The two serum markers C-reactive protein and D-dimer were negatively correlated with saliva levels of

sphingosine and 5-Aminolevulinic acid (**A**, **B** and **E**, **F**), and positively correlated with L-Tryptophan and L-Kynurenine (**C**, **D** and **G**, **H**) of COVID-19 patients. Statistical test: Pearson's coefficient test with two-tailed p-value < 0.05 considered significant

Kynurenine has a central role in modulating inflammation and immune response (Chen & Guillemin, 2009).

Kynurenine shunt that moves the tryptophan degradation toward kynurenine and away from serotonin has been

associated independently with depression, fatigue, muscle weakness, and other neurologic diseases (Mangoni, 1974; Németh et al., 2005). COVID-19 disease causes long-lasting symptoms months after recovery from acute infection that resembles kynurenine shunt presentations (Eroğlu et al., 2021; Huang et al., 2021). It is therefore possible for the metabolic programming to play a role in manifestation of both acute and long-term symptoms observed in COVID-19 (Huang et al., 2021; Ramakrishnan et al., 2021; Saheb Sharif-Askari et al., 2021). More studies are needed to profile the long-term metabolic change in those suffering from lingering long COVID symptoms.

In addition to severe metabolic pathways, we have also profiled those that were specific to non-severe group which included sphingosine. Our differential metabolite analyses in both saliva and plasma samples have shown higher sphingosine levels in non-severe than severe COVID-19 (Fig. 3A, B and Fig. 6A). Interestingly, salivary sphingosine pattern revealed an inverse relationship between sphingosine levels and disease severity with significant increase of sphingosine levels in asymptomatic and its complete absence in severe disease. We detected sphingosine in salivary controls but not in plasma controls. Similar to our result, Janneh et al. (Janneh et al., 2021) and Shen et al. (Shen et al., 2020) associated the reduced plasma sphingosine levels to COVID-19 severity. In their findings plasma sphingosine levels differentiated symptomatic from non-symptomatic infection but it could not differentiate severe from non-severe disease (Janneh et al., 2021; Shen et al., 2020).

Sphingosine present abundantly in healthy nasal, tracheal, and bronchial epithelial cells protecting the respiratory tracts from both bacterial and viral respiratory infection (Verhaegh et al., 2020). It prevent SARS-CoV-2 viral entry into host cells; sphingosine binds to ACE2 receptor and block the interaction between SARS-CoV-2 viral spike protein and host ACE2 receptor (Edwards et al., 2020). It is involved in synthesis and regulation of multivesicular bodies which modulate diverse cellular processes (Lang et al., 2020). Additional antiviral effect of sphingosine was shown against herpes simplex virus in macrophages where sphingosine binds, traps and degrades the virus within the multivesicular bodies (Lang et al., 2020). The bactericidal effect of this sphingolipid have been shown against different bacterial strains (Grassmé et al., 2017; Seitz et al., 2019). Verhegh and colleagues demonstrated that protonated amino group (NH_3^+) group of sphingosine binds to the—negatively charged lipid cardiolipin in *Pseudomonas aeruginosa* and *Staphylococcus aureus* plasma membranes which then induce rapid permeabilization and loss of bacterial metabolic activity (Verhaegh et al., 2020).

Sphingosine is phosphorylated by sphingosine kinases to generate Sphingosine-1-phosphate (Liu et al., 2002).

In our study we did not detect sphingosine-1-phosphate, however recently Marfia group linked the decreased serum level of sphingosine-1-phosphate with severe COVID-19 disease (Marfia et al., 2021). Sphingosine-1-phosphate is a pleotropic sphingolipid that acts as a major modulator of vascular and immune system (Törnquist et al., 2021). It displays dose-dependent effect on inflammation; at normal nanomolar physiologic concentration it acts as anti-inflammatory while at higher micromolar concentration it shows pro-inflammatory activity (Fettel et al., 2019; Whetzel et al., 2006). Airway smooth muscles treated with micromolar concentration of sphingosine-1-phosphate induced their proinflammatory effect which was inhibited upon treatment with corticosteroid, dexamethasone (Che et al., 2013). In small dose, Sphingosine-1-phosphate analogue, Fingolimod, showed reduction of inflammatory response and entered a clinical trial for treatment of cytokine storm during COVID-19; however the trial was stopped due to occurrence of lymphopenia adverse reaction (www.clinicaltrials.gov NCT04280588)(Marfia et al., 2021). Further studies are required to assess the potential of sphingolipid modulation towards control of SARS-CoV-2 viral infection as well as cytokine storm.

Resembling sphingosine findings, 5-Aminolevulinic acid metabolite levels were detected in non-severe COVID-19, while this metabolite was not present in control and severe COVID-19 in both saliva and plasma results. This metabolite has antiviral effect and thereby their detection in the less severe infection suggest that it could have played a role in the control of infection severity. Ben-Hur and colleague showed that 5-Aminolevulinic Acid supplementation followed by photodynamic therapy was effective both in-vivo and in-vitro to inactivate intracellular herpes simplex virus (HSV) and human immunodeficiency virus (HIV) (Smetana et al., 1997). More recently, Japanese researchers have shown that pretreatment with this amino acid blocked the SARS-CoV-2 infection in both VeroE6 cells and human colon-derived Caco-2 cells in a time-dependent manner (Sakurai et al., 2021). Mechanistically, 5-ALA supplementation induced accumulation of protoporphyrin IX and heme inside host cells, that interfered with viral replication through targeting the G-quadruplex (G4) structure within the virus nonstructural protein 3 (Nsp3) (Sakurai et al., 2021). 5-Aminolevulinic Acid is bioavailable orally and could potentially be used for SARS-CoV-2 infection prevention (Sakurai et al., 2021).

Beside sphingosine, other lipid mediators such as eicosanoids were previously shown to be upregulated during COVID-19 (Regidor et al., 2021). Eicosanoids were associated with lipid mediator storm during critical SARS-CoV-2 infection as well as post COVID-19 syndrome following mild disease (Archambault et al., 2021; Bohnacker et al., 2022). SARS-CoV-2 infection induced a long-term immune

abnormalities in macrophage effector functions and eicosanoid metabolism which persisted up to 3–5 month following mild COVID-19 (Bohnacker et al., 2022). Future investigations are needed to study the role of other bioactive lipids during and after severe viral infections. LC–MS/MS analysis could be used for simultaneous detection of bioactive lipids such as eicosanoids in different patients' samples of serum, bronchial alveolar lavage fluid (BALF), and sputum (Thakare et al., 2018).

The current study identified the metabolites that are specific to severe and non-severe groups. Like sphingosine, metabolites presented in Fig. 3A, B such as *N*-Acetylputrescine could play a key role in development or resistance to severe viral infection and thereby further research is needed to understand the mechanism that regulate their abundance. Moreover, many of the dysregulated metabolites are expected to be directly or indirectly associated with clinical manifestation observed in acute phase and long-term sequel of COVID-19 and deserve further investigation. As expected, the two prognostic biomarkers of C-reactive protein and D-dimer were negatively correlated with sphingosine and 5-Aminolevulinic acid (Fig. 7A, B and E, F), and positively correlated with L-Tryptophan and L-Kynurenine (Fig. 7C, D and G, H).

This study was conducted to profile the global metabolic dysregulation during acute COVID-19 disease, and to focus on those metabolites that are specific to non-severe and severe groups. Our study only provides a snapshot of SARS-CoV-2 induced metabolic reprogramming. Future longitudinal studies are required to better characterize the early, acute, and long-term change in COVID-19. Our study was limited by the sample size and larger cohort could be required to confirm the current findings. Although we have used age and gender matched cohort (Supplementary Table 1), metabolite change observed could be cofounded or not by other factors such as diet, disease, and medication. To control for the potential cofounder's larger sample size will be needed. Another limitation was that deuterium-labeled internal standards were not used prior to sample extraction.

5 Conclusion

In conclusion, SARS-CoV-2 infection induce distinctive metabolic derangement in severe and non-severe COVID-19. Metabolic change could be detected in both salivary and plasma samples, however saliva collected from active side of infection presented with higher intensity of metabolic dysregulation. General COVID-19 metabolites (such as Linoleic acid and Alpha-ketoisovaleric in Fig. 3A, B) could be used for infection detection while metabolites distinguishing severe from non-severe stages (such as sphingosine and kynurenine) could be used as a prognostic marker. Further,

metabolites such as L-Kynurenine, sphingosine and 5-aminolevulinic could play active role in modulating immune system inflammatory and antiviral response and could represent an effective therapeutic approach for the treatment of COVID-19 early and late immune dysregulations.

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Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was approved by Dubai Scientific Research Ethics Committee (DSREC). Written, informed consents were obtained from all study participants prior to inclusion.

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