Systemic perturbations in amine and kynurenine metabolism associated with acute SARS-CoV-2 infection and inflammatory cytokine responses

Supporting information

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1. Materials and Methods

1.1. Quantification of amino acids

1.1.1. Reagents

Unlabelled amino acid standards (physiological amino acids; acidics, basics and neutrals), ammonium formate, sodium hydroxide and formic acid were purchased from Sigma-Aldrich (North Ryde, NSW, Australia). Stable isotope labelled internal standard non-canonical and canonical amino acid mixes were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Water, acetonitrile, methanol and isopropanol (all Optima grade) were purchased from Thermo Fisher Scientific (Malaga Western Australia). AccQTag Ultra 3X derivatization kit was purchased from Waters Corporation (Milford, MA, USA).

1.1.2. Calibrators and quality controls

Calibrators and quality controls were prepared from a stock solution of physiological amino acids (acidics, basics and neutrals) at 500 µmol/L (Sigma-Aldrich, North Ryde, NSW, Australia). On each day, asparagine and glutamine were prepared at 0.5 mmol/L due to instability. A working stock solution containing all amino acids was prepared at 400 µmol/L. The stable isotope labelled internal standard solution (ISTD, 12.5 µmol/L in water) was prepared from stocks of canonical and non-canonical amino acids at 2.5 mmol/L in water and stored at -20°C until use.

1.1.3. Sample Preparation

Calibrators, quality control (QC) and biofluid samples (plasma, serum and urine) were treated using a Biomek i5 sample automation system. Calibrators and QCs were prepared from a working stock solution at 400 µmol/L in water, and diluted to 200, 100, 40, 20, 10, 4, 2 and 1 µmol/L for calibrators and 3, 15, 75, and 300 µmol/L for quality controls. Biofluids were vortex mixed and centrifuged at 13,000 g for 10 minutes and 10 µL was transferred to a PCR 96-well plate (Axygen, Fisher Biotec, Wembley, WA, Australia). 20 µL of each calibrator and quality control were transferred to the sample plate and the following process performed: dilution of biological samples with water (1:1 v/v); addition of internal standard mixture (20 µL of a 12.5 µmol/L solution in water); protein precipitation with 90 µL methanol. After mixing, centrifugation was performed at 200 g for 10 minutes, and 10 µL of the supernatant was transferred into a Waters 700 µL 96-well plate for derivatization. The derivatization processes consisted of the addition of 70 µL borate buffer and 20 µL AccQTag Ultra derivatization reagent to each well. After mixing the plate was incubated at 55 °C for 10 minutes. The subsequent derivatized samples were diluted with water 1:4 (v/v) for analysis.

1.1.4. Liquid chromatography-mass spectrometry

The chromatographic separation of analytes was performed on a Waters Acquity I-class UHPLC systems (comprising a Binary Solvent Manager, thermostatic Column Manager and FL Sample Manager, Waters Corp., Milford, MA, USA) using an Acquity UPLC HSS T3 1.8 µm 2.1
x 150 mm column. Eluent A consisted of 2 mM ammonium acetate in water and eluent B consisted of 2 mM ammonium acetate acetonitrile/water 95/5 (v/v). The flow rate was 0.6 mL/min and column temperature was maintained at 45 °C. The autosampler compartment was cooled to 4 °C and 2 µL injection volume was performed using full-loop injection mode. Gradient elution was performed starting with 5 % B for 0.2 min, increasing to 30 % B at 5 min, 100 % B at 5.1 min for 1 min before returning to 5 % B until 7.5 min. The weak and strong washes were water/acetonitrile 95/5 (v/v) and isopropanol respectively.

Positive electrospray ionization (ESI) was performed on a quadrupole time-of-flight (QToF) mass spectrometer (Bruker impact II (Bruker Daltonics, Billerica, MA)) operated in broadband collision-induced dissociation (bbCID) mode. This bbCID function offers MS and MS/MS spectra within the same injection. The ion source settings were: capillary voltage = 4.5 kV; end plate offset = 500 V; drying gas flow = 12.0 L/min; nebulizer gas = 5.0 bar; drying temperature = 250 °C. The data acquisition rate was set to 8 Hz over the mass range of m/z 30 – 1000. The collision energy for the MS scan was set to 6.0 eV and alternating low and high energy for MS/MS were set at 20 and 50 eV. An internal calibration was performed by injection of 5mM sodium formate solution in water:isopropanol (50:50 v/v) at the beginning of every run.

1.1.5. Data and statistical analyses

QToF mass spectrometric data were collected with Compass HyStar 5.1 and O-TOF Control version 5.2. Data were processed using TASQ 2.2 (Bruker Daltonics, Bremen, Germany). The molecular formula or exact mass of the derivatized amino acid was used to extract the precursor ions with a mass error of < 3 mDa. Calibration curves were linearly fitted with a weighting factor of 1/x.
1.2. Quantification of tryptophan pathway metabolites

1.2.1. Reagents

Analytical standards were purchased from Sigma-Aldrich (North Ryde, NSW, Australia), Novachem (Victoria, Australia). Stable isotope labelled (SIL) standards were purchased from Novachem (Victoria, Australia). Water, acetonitrile, methanol and isopropanol (all Optima grade) were purchased from Thermo Fisher Scientific (Malaga Western Australia). Phenomenex PHREE solid-phase extraction plates were purchased directly from Phenomenex (Phenomenex, NSW, AUS).

1.2.2. Calibrators and quality controls

Two diluents were prepared for use in the preparation of calibration and quality control (QC) samples. Diluent 1 (D1) consisted of water with 1 mg/mL (0.1%) citric acid, and diluent 2 (D2) consisted of water with 0.1 mg/mL (0.01%) citric acid.

1 mg/mL stock solutions were made using D1 for 5-hydroxyindole-3-acetic acid (5-HIAA), nicotinamide adenine dinucleotide (NAD+), citrulline, dopamine, picolinic acid, serotonin, quinolinic acid, 3-hydroxykynurenine (3-HK), nicotinic acid, kynurenine, β-nicotinamide mononucleotide (βNM), tryptophan, and nicotinamide riboside.

1 mg/mL stock solutions were made using 40:60 D1 to 0.1 M sodium hydroxide (NaOH) for xanthurenic acid, 3-hydroxyanthranilic acid (3-HAA), indole-3-acetic acid (I-3-AA), and kynurenic acid.

A 1 mg/mL stock solution of neopterin was prepared using 100% dimethyl sulfoxide (DMSO).

Parent stock solutions were diluted using D2 to produce suitable calibration and QC ranges for each target analyte.

1.2.3. Sample Preparation

Sample extraction was completed using a Biomek i5 sample automation system. Stable isotope labelled internal standards (20 μL) were added to all samples prior to protein precipitation via the addition of 250 μL of methanol containing 2 mM ammonium formate. After mixing, samples were transferred to a Phenomenex PHREE™ phospholipid removal solid phase extraction plate (Phenomenex, NSW, AUS). PHREE™ plates were then washed with an additional 150 μL of methanol containing 2 mM ammonium formate. Eluent collection plates were dried using a SpeedVac vacuum concentrator (Thermo Fisher, Massachusetts, USA). Dried extracts were re-suspended in 100 μL of water with 0.1% formic acid prior to LC-MS analysis.

1.2.4. Liquid chromatography-mass spectrometry

LC-MS analysis was performed using a Waters Acquity UPLC® (Waters Corp., Milford, MA, USA) coupled to a Waters Xevo TQ-XS MS (Waters Corp., Wilmslow, UK). The LC column used
was a Waters HSS T3 2.1 × 150 mm, 1.8 μm column maintained at 45 °C. Linear gradient elution was performed at 0.6 mL/min. The mobile phase was composed of 0.1% formic acid in 2 mM ammonium formate (v/v) (A) and 0.1% formic acid in acetonitrile (v/v) (B), starting at 1% B increasing to 10% B over 3 min, then increasing to 90% B at 4 min, and finally returning to 1% B at 4.1 min for column re-equilibration, which was completed at 5 min. The weak and the strong washes were 95:5 water/acetonitrile (0.2% formic acid) (v/v) and 100% isopropanol (0.5% formic acid), respectively.
### Table S1. Patient demographics for each of the study groups.

|                  | SARS-CoV-2 Positive (+) (n=10) | SARS-CoV-2 Negative (-) Non-Hospitalised (N = 23) | Healthy Controls (n=16) | SARS-COV-2 Negative (-) Hospitalised (N = 10) |
|------------------|---------------------------------|--------------------------------------------------|-------------------------|-----------------------------------------------|
| M:F              | 1.5:1                           | 1:1.18                                          | 1.67:1                  | 1.5:1                                         |
| Age (SD)         | 68.4 [±12.65]                   | 48.63 (± 15.85)                                 | 50.31 (± 16.70)         | 67.45 (± 21.03)                              |
| BMI (SD)         | 34.43 [±9.98]                   | 29.6 (± 7.82)                                   | 26.13 (± 3.27)          | 25.73 (± 5.01)                               |
| Pre-Diabetic/ Diabetic | 1 (10%)                    | 3 (12.5%)                                      | 1 (6.25%)               | 1 (4.54%)                                    |
| Hypertension     | 5 [50%]                         | 5 (20.83%)                                     | 0                       | 0                                             |
| Asthma           | 2 [20%]                         | 6 (25%)                                        | 0                       | 0                                             |
| COPD             | 0                               | 1 (4.17%)                                      | 0                       | 2 (9.09%)                                    |
| Arthritis        | 2 [20%]                         | 0                                               | 0                       | 0                                             |
| Glaucoma         | 1 [10%]                         | 0                                               | 0                       | 0                                             |
| Dyslipidaemia    | 1 [10%]                         | 0                                               | 0                       | 0                                             |
| Chronic Renal Disease | 1 [10%]                | 0                                               | 0                       | 3 (13.64%)                                   |
| Chronic Heart Disease | 1 [10%]                | 0                                               | 0                       | 2 (9.09%)                                    |
Table S2. Cytokine univariate analysis. Analysis was completed between Healthy Control (n=10) and SARS-CoV-2 (+) (n=7) groups. Statistical comparisons were performed with Mann-Whitney rank sum test. To control the false discovery rate (FDR), q values were generated from the Mann-Whitney p values using the method proposed by Benjamini and Hochberg. Boxplots of the data are presented in Figure S2.

| Cytokine      | SARS-CoV-2 (-) Median Concentration (µM) | SARS-CoV-2 (+) Median Concentration (µM) | Mann-Whitney p value | BH q value | Lower 95% confidence interval | Upper 95% confidence interval | Mann-Whitney Effect Size (r) |
|---------------|------------------------------------------|------------------------------------------|----------------------|------------|------------------------------|------------------------------|-----------------------------|
| IL-1 RA       | 3.57E+02                                 | 1.73E+03                                 | 7.38E-04             | 1.03E-02   | -1.24E+04                    | -9.09E+02                    | 8.30E-01                    |
| IP-10 (CXCL10)| 1.05E+01                                 | 5.14E+01                                 | 1.95E-03             | 1.36E-02   | -1.01E+02                    | -1.48E+01                    | 7.10E-01                    |
| IL-8 (CXCL8)  | 5.46E-01                                 | 1.70E+01                                 | 7.37E-03             | 2.58E-02   | -3.03E+01                    | -2.55E+00                    | 6.62E-01                    |
| SDF-1α        | 3.33E+02                                 | 5.46E+02                                 | 6.79E-03             | 2.58E-02   | -5.22E+02                    | -8.00E+01                    | 6.39E-01                    |
| TNF-α         | 1.59E+00                                 | 1.26E+01                                 | 1.09E-02             | 3.05E-02   | -1.81E+01                    | -7.26E-01                    | 6.30E-01                    |
| MIP-β (CCL4)  | 3.89E+01                                 | 9.61E+01                                 | 1.36E-02             | 3.17E-02   | -8.57E+01                    | -1.45E+01                    | 5.92E-01                    |
| MCP-1 (CCL2)  | 8.25E+01                                 | 2.52E+02                                 | 1.85E-02             | 3.70E-02   | -2.33E+02                    | -3.03E+01                    | 5.68E-01                    |
| IL-18         | 1.72E+01                                 | 5.16E+01                                 | 5.08E-02             | 8.88E-02   | -6.22E+01                    | 4.25E-05                     | 4.86E-01                    |
| IFN-γ         | 3.04E+00                                 | 5.35E+00                                 | 5.79E-02             | 8.88E-02   | -1.08E+01                    | 1.25E-05                     | 4.72E-01                    |
| IL-7          | 2.80E+00                                 | 9.53E+00                                 | 6.34E-02             | 8.88E-02   | -1.29E+01                    | 3.92E-01                     | 4.62E-01                    |
| RANTES (CCL5) | 9.98E+00                                 | 1.26E+01                                 | 1.33E-01             | 1.69E-01   | -1.42E+01                    | 7.99E-01                     | 3.79E-01                    |
| Eotaxin (CCL11)| 2.53E+01                                | 3.69E+01                                 | 1.61E-01             | 1.88E-01   | -3.06E+01                    | 7.02E+00                     | 3.55E-01                    |
| IL-17A (CTLA8)| 4.67E-01                                 | 2.63E+00                                 | 1.82E-01             | 1.96E-01   | -3.41E+00                    | 3.22E+00                     | 3.36E-01                    |
| MIP-1α (CCL3) | 4.00E+01                                 | 1.12E+01                                 | 6.01E-01             | 6.01E-01   | -8.63E+00                    | 5.62E+01                     | 1.42E-01                    |
Figure S1. Two group boxplot comparison of metabolites between HC/SARS-CoV-2 (-) vs SARS-CoV-2 (+) groups for those metabolites that reported no significant differences in the univariate Mann-Whitney U analysis.
Figure S2. Three group boxplot comparison of plasma metabolites between HC vs SARS-CoV-2 (-) vs SARS-CoV-2 (+) groups for those metabolites that reported significant differences in the univariate Mann-Whitney U analysis.
Figure S3. Three group boxplot comparison of plasma metabolites between HC vs SARS-CoV-2 (-) vs SARS-CoV-2 (+) groups for those metabolites that reported no significant differences in the univariate Mann-Whitney U analysis.
**Figure S4.** Two group boxplot comparison of serum cytokines in SARS-CoV-2 (-)/HC vs SARS-CoV-2 (+).