Supplementary material

COVID-19 specific metabolic imprint yields insights into multi-system perturbations

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Supplementary material and methods

Characteristics of patients and controls
SARS-CoV-2 RNA+ patients with moderate (n=10) or severe (n=17) COVID-19 admitted to the Karolinska University Hospital, Stockholm, Sweden, were recruited to the study. COVID-19 patients were sampled 5-24 days after symptom debut and 0-8 days after being admitted to hospital. Healthy controls were SARS-CoV-2 IgG seronegative at time of inclusion, median age was 50-59 years, and 11 out of 17 were male (65%). The study was approved by the Swedish Ethical Review Authority and all patients gave informed consent. More details are available on www.covid19cellatlas.com.

SARS-CoV-2 serology
SARS-CoV-2 IgG titers were measured in serum in all the patients and controls using the clinical routine assay at the Clinical Microbiology Department, Karolinska University Hospital; and finally interpreted by a clinical microbiologist as positive or negative, according to clinical practice.

Virus titers
Virus load was assessed as serum SARS-CoV-2 PCR positivity, examined in serum from all the patients following the pipeline at the Clinical Microbiology Department, Karolinska University Hospital. Briefly, two independent tests were used and finally interpreted by a clinical microbiologist as positive or negative, according to the clinical routine.

Sample preparation for deep metabolomics
All serum samples from patients and controls were processed within 4 hours from them being taken, frozen, and maintained at -80 degree C until processed. From ICU-patients, peripheral arterial samples were obtained and from regular ward patients, peripheral venous samples were taken. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company). Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was stored as a backup. Samples were placed briefly on a TurboVap® (Zymark) until dried to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis. Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowing instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled
matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

**Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)**

Metabolomics was performed in collaboration with Metabolon. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS
analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. The library is based on authenticated standards that contain the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis. A variety of curation procedures were carried out. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Library matches for each compound were checked for each sample and corrected if necessary. Peaks were quantified using area-under-the-curve.
Defining a corrected metabolome

We aimed to distinguish the metabolome specifically associated with COVID-19 features rather than with its risk factors (either to develop the disease, or its severe form) and/or patent’s management. For this, we first identified all metabolites that varied according to (thus are dependent on) a range of risk factors (such as BMI and diabetes) and management criteria (such as time of hospitalization and administration of steroids). We then removed these metabolites from the global metabolome to define a “corrected metabolome” containing only metabolites that do not vary according to potential confounding factors.

Targeted proteomic

Serum proteomic was performed using the Proximity Extension Assay (PEA) technology. The full library (Olink Explore 1536) consists of 1472 proteins and 48 controls assays divided into four 384-plex panels. The analytical performance of PEA is rigorously validated for each protein assay included in the library and performance data is publicly available at www.olink.com. Technical criteria include assessing specificity, sensitivity, dynamic range, precision, scalability, endogenous interference and detectability in both healthy and pathological plasma and serum samples. In the immune reaction, 2.8 µl of the samples are incubated overnight with PEA probes at +4° C. Following the immune reaction, a combined extension and pre-amplification mix is added to the incubated samples at room temperature for PCR. The PCR products are thereafter pooled before a second PCR step is performed with additions of individual sample index sequences. After pooling of samples, bead purification and QC of the generated libraries are followed on a Bioanalyzer. Finally, sequencing takes place using Illumina’s NovaSeq 6000 instrument with two S1 flow cells with 2 x 50 base read lengths. Counts of known sequences are thereafter translated into normalized protein expression (NPX) units through a QC and normalization process. The PEA QC process consists of specifically
engineered controls, enabling full control over the technical performance of assays and samples. Three internal controls are spiked into every sample: an immune control (incubation step) using a non-human assay, an extension control (extension and pre-amplification step) composed of an antibody coupled to a unique DNA-pair always in proximity, and a detection control (amplification step) based on a double stranded DNA amplicon. In addition, each sample plate includes one column with sample controls used to estimate the precision (intra- and inter-CVs). Three negative controls (buffer) were utilized to set background levels and calculate limit of detection (LOD), three plate controls (plasma pool) to adjust levels between plates and two sample controls (reference plasma) are included in duplicate to estimate CV between runs. NPX is Olink’s relative protein quantification unit on a log2 scale and values are calculated from the number of matched counts on the NovaSeq run. Data generation of NPX consists of three main steps: normalization to the extension control (known standard), log2-transformation, and level adjustment using the plate control (plasma sample).

**Pathogenic biological events and tissue-specific damage**

We aimed to gain insights into the pathophysiology of COVID-19 by considering scales of severity that were both specifically and significantly associated to parameters such as organ damage or various biological processes. For organ damage, we defined signatures based on sets of proteins (Supplementary table 10) that associated specifically to unique human tissues (Supplementary fig 1, https://www.proteinatlas.org) and that were significantly altered in COVID-19 patients as compared to healthy donors (Supplementary fig 2, based on the above described targeted proteomic from serum). Since each set of proteins were expressed (RNA) specifically in a tissue (at steady state), we hypothesize that the variations of this set of proteins were likely to reflect a COVID-19-related pathogenic event occurring in a particular tissue. For the pathogenic biological events, pathway analysis based on several sets of proteins
significantly associated with specific biological events (such as the set of 86 proteins associated with the biological event “neuron death” with P-value of 10e-23, Supplementary table 10) that at the same time were significantly altered in COVID-19 patients as compared to healthy donors (Supplementary fig 2) were considered. In a similar manner, we hypothesize that the variation of these sets of proteins were likely to reflect a COVID-19-related pathogenic biological event involving the defined specific biological processes, cell component, signaling pathway, molecular functions, or cell components.

**Cell preparation and flow cytometry**

Peripheral blood samples were collected in heparin tubes and peripheral blood mononuclear cells (PBMC) isolated using Ficoll gradient centrifugation. PBMC were thereafter stained fresh with the antibody mix. Live/Dead cell discrimination was performed using fixable viability dyes (Invitrogen). After staining, cells were fixed with 1% paraformaldehyde (PFA) for two hours before being acquired on a BD LSR Symphony with 355 nm, 405 nm, 488 nm, 561 nm, and 640 nm lasers. More details on flow cytometry panels used are available on https://covid19cellatlas.com.

**Trucount**

Absolute counts from the different samples were obtained using BD Multitest™ 6-color TBNK reagents with bead-containing BD Trucount™ tubes (337166) according to manufacturer’s instructions. Samples were fixed with 2% PFA for two hours prior to acquiring.

**Statistics, analysis principles, and composite scores**

If not specified, Mann-Whitney U-test and Spearman’ rank coefficient was used to analyze differences between groups and correlations respectively. False discovery rate was applied to
correct for multiple comparisons when mentioned. Clustering was based on Euclidian distances and Wards’ method. Concerning the categorical parameters, BMI above 30 was considered as cut off (lower group ranging from 23 to 30 with median of 26.9, higher group ranging from 31 to 55 with median of 35.5), smoking status was based on reported ongoing or previous smoking, any corticosteroid treatment started at hospital prior to sampling was used as readout. Age below 57 years old was taken as cut off (lower group ranging from 18 to 56 with median of 46.5 years old, older group ranging from 57 to 78 with median of 63 years old), time spent in the ICU below 5 days before sampling was taken as cut off (shorter group ranging from 1 to 5, with median of 4 days, longer group ranging from 6 to 20, with median of 7 days) and time spent in the infectious clinic below 5 days before sampling was considered as cut off (shorter group ranging from 2 to 3, with median of 2 days, longer group ranging from 5 to 7, with median of 7 days). Clinical gradings were defined at the time of sampling. For composite continuous scales related to immune activations (flow cytometry-based), parameters used to build the scale were normalized to the median value of COVID-19 patients and the medians of those were taken for each individual. For the other composite continuous scales (proteomic-based), the cumulative NPX value of selected proteins normalized to the median value of COVID-19 patients was considered. For organ damage-related scales, the selection of these proteins was based on tissue-specific expression derived from the human protein atlas (https://www.proteinatlas.org). For other scales (biological processes, cell components, molecular functions, and signaling pathways (KEGG)), the selection of proteins was based on gene ontology signatures. The combined clustering of severity scales and metabolites was based on the three highest and three lowest Pearson correlation coefficients for each scale. Online tools and resources used include the human protein atlas (https://www.proteinatlas.org), MetaboAnalyst 5.0 (https://www.metaboanalyst.ca), ClustVis (https://biit.cs.ut.ee/clustvis), and the Karolinska KI/K COVID-19 immune ATLAS (https://covid19cellatlas.com).
Version 1.3.1056 with R version 4.0.2 (packages ggplot2, pheatmap, UpSetR, AC-PCA, StepReg) and GraphPad Prism v9 were used for generation of figures.
Supplementary figure legends

Supplementary fig. 1
Specificity of organ damages signatures based on normalized transcriptional data from the human protein ATLAS. Cumulative expression (normalized consensus expression, NX, from the human protein ATLAS) of the proteins/genes included in the signatures, normalized to the highest compartment is displayed.

Supplementary fig. 2
Comparison of the composite severity scales between healthy donors and hospitalized COVID-19 patients. Bar plots depict medians of each group and p-values from Mann-Whitney tests are indicated.

Supplementary fig. 3
Metabolic trajectories across COVID-19 severities. Correlation-based metabolic trajectories across the 69 composite severity scales of the Munsell chart. R, Pearson’s coefficient of correlation. Combined clustering (using Ward’s method) of severity scales and metabolites based on the three highest and three lowest Pearson correlation coefficients identified for each scale.
Supplementary table legends

Supplementary table 1. Metabolite ID and info.

Supplementary table 2. Normalized metabolite concentrations and comparison between COVID-19 patients and controls.

Supplementary table 3. Correlations between xenobiotic metabolites and other metabolites.

Supplementary table 4. Interdependence of all metabolites among each other as well as specifically for xenobiotics compared to other metabolites.

Supplementary table 5. Metabolite presence in relation to clinical confounding factors.

Supplementary table 6. Most variables or most significant effects of metabolites in relation to confounders.

Supplementary table 7. Metabolites present in the corrected metabolome.

Supplementary table 8. Metabolites significantly different before and after SARS-CoV-2 seroconversion (switch 1 and 2) for the total and corrected metabolome.

Supplementary table 9. Metabolites significantly associated with fatal outcome of COVID-19.

Supplementary table 10. Definition of the 69 composite severity scales.
Supplementary table 11. Overlaps between proteomic data and the defined severity scales.

Supplementary table 12. SNPs associated with the studied metabolites.

Supplementary table 13. Raw data for the PCA analysis of subjects and severity scales.
Supplementary fig. 1
Supplementary fig. 2

COVID-19 severity composite scales (arbitrary unit)

Natural Killer cells
Immune activations
Molecular functions
Biological processes

Cellular components

Signaling pathways

Immune activations

Organ damage

Healthy donors COVID-19 patients
