**Running title:** Metabolomic profiling in severe COVID-19 patients.

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Abstract

The heterogeneity in severity and outcome of COVID-19 cases points out the urgent need for early molecular characterization of patients followed by risk-stratified care. The main objective of this study was to evaluate the fluctuations of serum metabolomic profiles of COVID-19 patients with severe illness during the different disease stages in a longitudinal manner. We demonstrate a distinct metabolomic signature in serum samples of 32 hospitalized patients at the acute phase compared to the recovery period, suggesting the tryptophan (tryptophan, kynurenine, and 3-hydroxy-DL-kynurenine) and arginine (citrulline and ornithine) metabolism as contributing pathways in the immune response to SARS-CoV-2 with a potential link to the clinical severity of the disease. In addition, we provide evidence for glutamine metabolism in M2 macrophages as a complementary process and contribution of phenylalanine and tyrosine in the molecular mechanisms underlying the severe course of the infection. In conclusion, our results provide several functional metabolic markers for disease progression and severe outcome with potential clinical application.

Importance

Although the host defense mechanisms against SARS-CoV-2 infection are still poorly described, they are of central importance in shaping the course of the disease and the possible outcome. Metabolomic profiling may complement the lacking knowledge of the molecular mechanisms underlying clinical manifestations and pathogenesis of COVID-19. Moreover, early identification of metabolomics-based biomarker signatures is proved to serve as an effective approach for the prediction of disease outcome. Here we provide the list of metabolites describing the severe, acute phase of the infection and bring the evidence of crucial metabolic
pathways linked to aggressive immune responses. Finally, we suggest metabolomic phenotyping as a promising method for developing personalized care strategies in COVID-19 patients.

**Main text**

More than a year has passed since the World Health Organization (WHO) announced the COVID-19 outbreak as a pandemic in March 2020, following the rapid spread of the SARS-CoV-2 virus (1). The clinical course of COVID-19 is versatile, the infection of the SARS-CoV-2 virus not only varies in its severity from asymptomatic or mild and moderate respiratory disease (80%) to clinically severe or critical life-threatening disease (20%) but also varies in a range of organs the disease can affect (2, 3). Diverse clinical trajectories seem to be the result of the immune response differences between individuals (4).

Multiple innate and adaptive immune system pathways that produce inflammatory molecules against the virus and virus-infected human cells are triggered after the SARS-CoV-2 entry in the cell, with characteristic overexpression of proinflammatory cytokines (e.g. IL-6, TNFα, IFN-γ) known as cytokine storm in the most severe cases (4–6). The host's immune responses typically involve changes in metabolic processes at the cellular level, reflecting the host-defense mediators and underlying mechanisms (7).

Detailed understanding of the molecular mechanisms behind COVID-19 pathogenesis and inflammatory response is needed to predict and reduce individual risks, develop therapeutic strategies, and reduce the overall ~2% mortality rate (mortality in hospitalized patients can be up to 30%) (8, 9). The human blood sera metabolome (defined as small molecules <1500–2000 Da) reflects the organism's metabolic state and is widely used to gain a deeper understanding of the pathogenesis of diseases. Recent reports of metabolomics studies highlight the pivotal role of cellular metabolites in programming immune response to SARS-CoV-2 infection (10–13).
Considering the extremely high heterogeneity of the COVID-19 disease and lack of promising predictive biomarkers, we believe that implications of longitudinal metabolite profiling may be beneficial in understanding the underlying mechanisms of the diverse course of the disease and promote the early identification of people at increased risk of severe illness from COVID-19 and related complications.

We performed quantitative targeted metabolome analysis with liquid chromatography-mass spectrometry (LC-MS) in blood sera of 32 hospitalized COVID-19 patients at the acute phase (time of admission at the hospital) and the recovery phase (40 ± 14.92 days) of the disease (see Text S1 in the supplemental material for a detailed description of methods). Written informed consent was obtained from every participant before their inclusion in the study, and the study protocol was approved by the Central Medical Ethics Committee of Latvia (No. 01-29.1.2/928).

As expected, the clinical blood tests revealed abnormal hematological parameters for the majority of study participants at the time of hospitalization, with a high variation in platelet (202.94 ± 65.26 µL) levels and low lymphocyte measurements (0.64 ± 0.56 µL), which coincides with previously reported lymphopenia as the hallmark of severe COVID-19 cases. We also observed a high variation of several markers (e.g. alanine aminotransferase, bilirubin, lactate C-reactive protein) indicating renal and hepatic dysfunction, myocarditis, inflammation, and coagulation, which confirms the systemic response to the infection in our study cohort(2, 14) (see Table S1 in the supplemental material).

Out of 51 metabolites analyzed by LC-MS, 22 metabolites showed significantly altered levels (paired t-test, FDR<0.05) in the serum samples during the acute phase in comparison to the recovery phase (Table 1), where concentrations for 16 compounds were significantly
elevated, whereas 8 metabolites were decreased. The hierarchical clustering and principal component analysis of the obtained metabolomic profiles showed clear metabolomics-based discrimination of samples collected in different phases of the disease (Figure 1A and B), indicating an altered metabolic activity during infection. Pathway analysis revealed 13 significantly enriched pathways (FDR<0.05), including phenylalanine, tyrosine and tryptophan biosynthesis, D-glutamine and D-glutamate metabolism and arginine biosynthesis (Figure 1C, Table S3). Statistical analysis was done with Metaboanalyst version 5.0 (15).

We found L-glutamine (Figure 1D) as the most significantly changed amino acid between the paired samples with reduced acute phase concentrations. It is known that glutamine deprivation and decreased glutaminolysis inhibits M2 macrophage polarization, which may partly explain the hyperinflammatory state in severe COVID-19 cases (16, 17). Moreover, the beneficial effect of glutamine has been proposed in multiple studies, where adding enteral L-glutamine to the regular nutrition shortened the duration of hospitalization and improved the outcome in moderate to severe COVID-19 cases (18, 19).

Two other amino acids involved in arginine catabolism through the Urea cycle: citrulline (Figure 1D) and ornithine (Table 1) were found to significantly change between the acute and recovery phases. However, alterations in the levels of L-arginine itself were not detected. Low blood plasma citrulline levels have already been reported in COVID-19 patients, whereas in patients with severe sepsis, the decreased citrulline levels are associated with acute respiratory distress syndrome (11, 13, 20). Since arginine can be metabolized to creatine and then to creatinine, both varying highly in our study cohort according to regular blood tests performed during hospitalization, arginine catabolism may be implicated in disturbed kidney function of COVID-19 patients (21).
Three out of 22 metabolites showing significantly changed levels between acute infection and recovery phase are involved in the tryptophan-kynurenine pathway: L-tryptophan, kynurenine, and 3-hydroxy-DL-kynurenine (Figure 1D, Table 1). The reduction of tryptophan levels and increase of kynurenine, 3-hydroxy-DL-kynurenine in the acute phase supports the conclusions of previous reports and confirms this pathway's key role in severe COVID-19 cases (10, 11). In vitro experiments have shown that tryptophan deprivation sensitizes T cells to apoptosis, inhibits proliferation of T cells, and plays a role in CD8 T-cell suppression in cancer (22, 23). Notably, the tryptophan-kynurenine pathway shows a modulatory effect on the macrophage-mediated responses by targeting the synthesis of the metabolic coenzyme NAD+(24, 25). According to Thomas et al. dysregulated tryptophan metabolism, an essential regulator of inflammation and immunity may be a potential explanation for severity in older COVID-19 patients (Thomas et al., 2020).

Finally, we observed a significant difference in L-phenylalanine (Figure 1D) and tyrosine levels in our cohort between the analyzed disease phases, both already suggested as metabolic hot spots of COVID-19 before (26). In sepsis and HIV-1 infection, the increased phenylalanine sera concentrations are linked to immune activation and increased cardiovascular event risk (27–29). Although the mechanisms behind this association are not well studied, it is in line with microvascular endothelial damage and higher coagulation risk characteristic to both: coronary heart disease and severe COVID-19 (29). Phenylalanine and tyrosine are catabolized to dopamine and epinephrine, and the latter has been employed in cardiac arrest as a result of cytokine storm, characteristic to severe COVID-19 patients(30).

In conclusion, our study shows that metabolomic profiling provides novel insights into the pathogenesis of host-defense mechanisms and may be further applied for rapid biomarker
discovery in infectious disease. These discoveries could show novel therapeutic strategies as indirect targets to fasten the recovery process after severe COVID-19. To the best of our knowledge, this is the first longitudinal study covering metabolomic profiling of severe COVID-19 patients.

**Data availability**

The raw data supporting the current study are available from the corresponding author on request.

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**Author contributions**

LA, KK, IP, LB performed the sample analysis; MU, VR, LA, BR, LV, OK were involved in patient recruitment process and sample collection; AT ensured the clinical data acquisition; MU, LA drafted the manuscript; JK provided critical revision of the manuscript; LA, KK, MU performed the data analysis and interpretation; MU, JK collaborated in funding acquisition, JK
performed supervision and conceptualization of the study. All authors read and approved the final manuscript.

Declaration of Interests

The authors declare no competing interests.

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**Figures**

**Figure 1. Targeted metabolomic analysis of longitudinal serum samples of hospitalized COVID-19 patients.** (A) Heatmap and hierarchical clustering of top 22 significantly altered metabolites. Each column represents one sample: (0, green) - samples collected in the acute phase, (1, red) - samples collected during the recovery phase, each row conforms to a specific metabolite expressed in normalized, log transformed concentration value. (B) Principal component analysis showing clear discrimination of samples collected in the acute and recovery phases of infection based on the obtained metabolite profiles. (C) Scatter plot representing the most relevant metabolic pathways from KEGG library arranged by adjusted p-values (obtained by Global Test pathway enrichment analysis) on Y-axis, and pathway impact values (from pathway topology analysis) on X-axis. The node color is based on its p-value and the node radius is determined based on their pathway impact values. (D) Boxplots showing the normalized levels of the most functionally relevant metabolites during the acute phase (0) and the recovery phase (1) of the infection, described as the minimum value, the first quartile, the median, the third quartile, and the maximum value with the black dots representing each sample.
## Tables

Table 1. Serum metabolites showing significantly altered levels comparing measures obtained during the acute phase and recovery phase of the disease.

| Compound                        | Fold change | False discovery rate |
|---------------------------------|-------------|-----------------------|
| L-Glutamine                     | 0.72        | 5.71E-08              |
| Citrulline                      | 0.48        | 9.16E-08              |
| 3-Hydroxy-DL-Kynurenine         | 10.51       | 6.79E-07              |
| L-Phenylalanine                 | 1.33        | 6.79E-07              |
| L-Methionine                    | 1.47        | 1.62E-06              |
| Isovalerylcarnitine             | 2.01        | 1.78E-06              |
| 4-Hydroxyproline                | 0.42        | 1.99E-06              |
| Kynurenine                      | 1.71        | 2.67E-06              |
| L-Asparagine                    | 1.51        | 3.88E-05              |
| L-Glutamic acid                 | 1.65        | 4.61E-05              |
| L-Valine                        | 1.26        | 7.73E-05              |
| Carnitine                       | 1.25        | 8.90E-05              |
| L-Tryptophan                    | 0.83        | 2.87E-04              |
| L-Tyrosine                      | 1.14        | 2.87E-04              |
| L-Threonine                     | 1.45        | 2.15E-03              |
| L-Proline                       | 0.86        | 3.23E-03              |
| Taurine                         | 1.36        | 6.01E-03              |
| Metabolite                | Ratio | p-Value |
|--------------------------|-------|---------|
| Ornithine                | 1.37  | 9.45E-03|
| L-Lysine                 | 1.08  | 1.15E-02|
| L-Isoleucine             | 1.21  | 1.25E-02|
| L-Acetylcarnitine        | 1.21  | 2.34E-02|
| L-Octanoylcarnitine      | 0.75  | 3.85E-02|

Appendixes

Text S1. Detailed Methods Description.

Table S1. Characteristics of the study participants.

Table S2. Data matrix with quantified metabolite concentrations for targeted compounds, determined by ultra-performance liquid chromatography-mass spectrometry.

Table S3. Significant pathways based on enrichment procedures.
