Serum metabolomic profile of symptomatic and asymptomatic SARS-CoV-2 infected patients

Jacopo Troisi (✉ troisi@theoreosrl.com)

Theoreo srl – Spin-off company of the University of Salerno, Italy <https://orcid.org/0000-0003-2962-7379>

Pierpaolo Cavallo

Department of Physics, University of Salerno, Italy <https://orcid.org/0000-0003-3111-5241>

Mario Masarone

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy <https://orcid.org/0000-0003-0550-8201>

Immacolata Sepe

Diagnostica Cavallo srl, Via C. Calò, 2, 84123 Salerno, Italy

Giovanni Scala

Hosmotic srl Via R. Bosco, 178, 80069 Vico Equense, Italy

Pietro Campiglia

Department of Pharmacy, University of Salerno, 84084 Fisciano, SA, Italy <https://orcid.org/0000-0002-1069-2181>

Francesco De Caro

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy

Giovanni Boccia

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy

Carolina Ciacci

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy <https://orcid.org/0000-0002-7426-1145>

Sergio Poto

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy

Pasquale Pagliano

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy

Marcello Persico

Department of Medicine and Surgery and Dentistry, “Scuola Medica Salernitana”, University of Salerno, Italy <https://orcid.org/0000-0002-1399-6498>

Research Article

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Abstract

Background: One of the most challenging aspects related to Covid-19 is to establish which are the characteristics that may explain clinical variability among patients. To achieve this objective, we compared serum metabolomic profiles of asymptomatic SARS-CoV-2 carriers to patients affected by mild and severe symptoms.

Methods: All subjects underwent quantitative assays for anti-SARS-CoV-2 antibody detection. Serum samples for metabolomic analysis were obtained from 109 healthy controls; 15 seroconverted, asymptomatic subjects (AS); 16 Covid-19 patients with mild symptomatology (MI) and 12 patients with severe symptomatology (SE). Metabolites were identified using Mass Spectrometry coupled to Gas Chromatography. Uni- and multivariate approaches were used to select the most relevant metabolites.

Results: Anti SARS-CoV-2 IgG showed an increasing trend from controls to asymptomatic and mild severity patients. Tyrosine, phenylalanine, acetoacetate and fumarate showed a decreased concentration in MI compared to both CTRL and AS subjects; on the contrary, mandelic acid showed an increased concentration. The shortest route among these metabolites resulted the Tyrosine metabolism. Aspartic acid, alanine, isoleucine, valine and proline were decreased in MI patients, while methionine and oxo-leucine resulted increased. An increased concentration of fatty acids lauric and myristic acid, phospholipids (phosphatidyl myo-inositol and lyso-phosphatidyl inositol) and histamine were recorded in MI and SE.

Conclusion: The reported metabolite levels could be explained by an increase production of L-DOPA, resulting in a following increased production of noradrenaline and adrenaline that could, at least partially, explain the different clinical presentation of the infection.

Introduction

In December 2019, the city of Wuhan, the capital of Hubei province, in China, became the center of a pneumonia epidemic of unknown cause. On January 7th, 2020, Chinese scientists isolated a novel Coronavirus from these patients, which was responsible of the acute respiratory syndrome called SARS-CoV-2 (formerly known as 2019-nCoV) [1,2]. The disease caused by this virus was subsequently designated by the World Health Organization (WHO) Coronavirus Disease 2019 (Covid-19) [3].

Although the outbreak is likely to have started from a zoonotic transmission event associated with a fish market that also traded with live wildlife, it soon became clear that this virus was also efficient in person-to-person transmission [4]. The clinical spectrum following SARS-CoV-2 infection appears to be broad, ranging from asymptomatic infection, to mild upper respiratory tract disease up to severe viral pneumonia with major respiratory distress and even death. A significant number of affected subjects need hospitalization [5,6]. Although some case series were published, many patients in these series

remain hospitalized at the time of publication [7,8]. The estimates of risk factors for serious illness and death in these, are currently not yet robust.

To date, this infection has taken on the characteristics of the pandemic, affecting over five million people worldwide with a death toll close to 350,000 [9]. Although many nations have undertaken drastic containment actions, including lockdowns, and important social distancing policies, the infection is still exponentially growing in many countries, including the United States of America, which is today the most heavily affected country.

Asymptomatic or poorly symptomatic subjects represent an important viral basin that will potentially widen the temporal expansion of the infection. Contagion monitoring policies are being studied around the world. To date, some progresses have already been made in the study of seroconversion following the infection [10,11]. These results will be the basis on which to monitor and map contagions, in order to establish policies for the recovery of activities and the gradual exit from the lockdown state.

Currently, it is strategic to investigate the biochemical characteristics of infected subjects that present widely different clinical features, with a particular interest in the conditions that allow to overcome the infection asymptotically.

The respiratory symptoms caused in the Covid-19 by the Sars-CoV-2 infections are accompanied, in the most severe cases, by other major events such as high blood pressure, arterial and venous thromboembolism, kidney disease, neurologic disorders, and diabetes mellitus. The multiorgan involvement indicates that the virus is targeting the endothelium [12] causing a systemic disease. The pathologic mechanisms underlying the broad spectrum of the clinical courses of the Covid-19, from asymptomatic to multiorgan failure cases, is going to be the object of study and research for the next future. Metabolomics is an emerging field of research that allows the simultaneous evaluation of many compounds in a single biological sample, in a precise moment of the disease and of the treatment. Metabolomic may contribute to the understanding of different onset and course of the same diseases. In the field of infection disease, the utility of metabolomics is wide [13–17].

The aim of the present study was to characterize serum metabolomic profiles of asymptomatic SARS-CoV-2 carriers as well as patients affected by mild and severe symptoms, in order to understand the mechanisms underlying the predisposition towards the different disease forms.

Methods

Study population and study design

The prospective study described herein was conducted at the A.U.O. S. Giovanni di Dio e Ruggi D'aragona, University of Salerno, Campania Region, Southern Italy, during the month of April 2020. One hundred fifty-two subjects were enrolled and stratified in 4 classes: subjects without symptoms referable to viral infection (e.g. temperature higher than 37 °C, cough, muscle pain, tiredness, breathing difficulties) and

with a negative serological evaluation against SARS-CoV-2 Spike protein (see below), defined as controls (CTRL); asymptomatic subjects carrying a higher than diagnostic cut-off immunoglobulin IgM or IgG level, defined as asymptomatic (AS); patients with a mild symptomatology, requiring ordinary hospitalization, defined as mild symptomatic patients (MI) and patients with a severe symptomatology, requiring ICU hospitalization (SE).

The study was approved by the ethics committee CE Campania Sud (IRB n.9/2020, prot. 0061907/20) and a written consent form was signed by each participant or their legal representative. The recruited patients (or a legal representative) completed a questionnaire addressing anamnestic and demographic characteristics. A complete clinical evaluation was obtained for all patients.

Statistical analysis of demographic and clinical data

Study data were collected and managed using the REDCap electronic data capture tools [18] hosted at the INFN (Istituto Nazionale di Fisica Nucleare), University of Salerno (Italy). Statistical analysis was performed using R-Studio ver. 1.2.5042 [19]. Data are presented as mean $\pm$ standard deviation for continuous variables and number (percentage) for categorical variables. Demographic and clinical data were tested for normality via the Kolmogorov-Smirnov test. Since data resulted to be normally distributed, the Student-t-test and analysis of variance (ANOVA) with Tukey post-hoc test were employed to compare the results among the several classes.

Sample collection

Human tissue collection strictly adhered to the guidelines outlined in the Declaration of Helsinki IV edition [20]. All patients were asked to respect a 12-h fast before blood collection.

Blood samples were collected using a BD vacutainer (Becton Dickinson, Oxfordshire, UK) blood collection tube (red top with no additives). After centrifugation, serum samples were immediately frozen to -80 °C until analysis.

Serum antibody anti SARS-CoV-2 quantification

The quantitative assays for antibody detection were performed using the MAGLUMI™ 2000 Plus 2019-nCov IgM and IgG assays (Snibe, Shenzhen, China).

The test was considered positive for an IgG or IgM level higher than 1.1 AU/mL. Test's precision around the threshold level, expressed as CV%, was 5.05% at 0.61 UA/mL and 3.31% at 1.96 UA/mL.

Metabolite extraction, derivatization and analysis

The metabolome extraction, purification, and derivatization were conducted by the MetaboPrep GC kit (Theoreo, Montecorvino Pugliano, Italy) according to manufacturer instructions. Instrumental analyses were performed with a GC-MS system (GC-2010 Plus gas chromatograph and QP2010SE mass spectrometer; Shimadzu Corp., Kyoto, Japan). The analytical details are reported in Troisi et al. [21–26].

Metabolite identification was performed according to Troisi et al. [23], briefly, the linear index difference maximum tolerance was set to 10, while the minimum matching for NIST library search was set to 85% (Level 2 identification according to Metabolomics Standards Initiative [MSI]) [27]. Metabolites that emerged as the most relevant in separating cases from controls (see below) were further confirmed using external standards (MSI level = 1).

Dataset preparation

Within each total ion count (TIC) chromatogram, > 290 signal peaks were detected in each specimen. Chromatograms were first aligned by means of parametric time warping (PTW) using the PTW package [28]. Some of the peaks were not investigated further as they were not consistently found in at least 80% samples, too low in concentration, or of poor spectral quality to be confirmed as metabolites. A total of 229 endogenous metabolites were detected consistently. The aligned chromatograms were tabulated with one sample per row and one metabolite area ratio (with respect to the internal standard area) per column. Each value was transformed by taking the natural log and then scaled by mean-centering and dividing by the standard deviation of that column (i.e., autoscaled) [29].

Features selection

To reduce the dataset dimension and focus the analysis on the most relevant metabolites, a process referred to as feature selection was performed using a genetic algorithm that is a heuristic search that mimics the process of natural evolution such as inheritance, mutation, selection, and crossover [30]. In genetic algorithms for feature selection, “mutation” means switching features on and off and “crossover” means interchanging used features. Feature selection was performed by means of the “Optimize Selection (Evolutionary)” algorithm implemented in Rapid Miner Studio ver. 9.6.0 (RapidMiner GmbH, Boston, MA, USA) [31]. These features were used to train the classification models.

Partial Least Square Discriminant Analysis (PLS-DA)

PLS-DA was performed in order to find the combination of metabolites that best separated the different classes on the basis of a specific metabolomic profile.

PLS-DA is a supervised method that uses multivariate regression techniques to extract, by means of linear combinations of original variables, the information able to predict class membership. PLS regression was performed by means of the MetaboanalystR [32] package that uses the `pls` function from

the R pls package [33]. Classification and cross-validation were performed using the wrapper function from the caret package [34]. Permutation test was performed to verify the significance of class discrimination. For each permutation, a PLS-DA model was built between the data and the permuted class labels, using the optimal number of components determined by cross validation for the model based on the original class assignment. Two types of test statistics were used to measure class discrimination. The first was based on prediction accuracy during training. The second made use of separation distance based on the Between/Within distance ratio (B/W). If the observed test statistics was part of the distribution based on the permuted class assignments, class discrimination could not be considered significant from a statistical point of view [35].

The “Metacost” algorithm [36] was used to correct the imbalance effect for each class, which was expected to be minimal but higher in comparison with CTRL. A cost matrix was built based on the number of samples in each class.

Identification of metabolites related to the SARS-CoV-2 infection symptomatology

Two separate selection strategies were used to find the most relevant metabolites. First, the importance of each metabolite in class separation was evaluated using the variable importance in projection (VIP) scores [37] calculated for each metabolite used in the PLS-DA classification model. Second, metabolites were selected based on their fold change (FC) and t-test-based p-values. Metabolites which showed both $FC > 2$ or $FC < -2$ and p-value lower than the false discovery rate (FDR) adjusted cut-off were selected. The molecular identity of the metabolites of interest (i.e. metabolites with a VIP-score > 2.0 [38], or in the interest areas in FC, p-value diagram) was determined comparing the corresponding mass spectrum with a mass spectrum library [39]. These identified metabolites were further confirmed using external standards, according to level 1 Metabolomics Standards Initiative (MSI) [27]. The selected metabolites' ontology was reported in Supplementary S1.

Metabolites occurrences in the several features selection strategies were summarized in a UpSet diagram [40].

Next, the metabolites were investigated by metabolomic pathway analysis using the interactive pathways explorer iPath ver. 3.0 [41]. This application allows the visualization and interpretation of metabolomic data in the context of human metabolism, analyzing networks of genes and compounds, identifying enriched pathways, and visualizing changes in metabolite data. iPath uses data from the KEGG (Kyoto Encyclopedia of Genes and Genomes) [42].

Results

Enrolled subjects

The 152 subjects enrolled in the study were grouped according to both the presence and severity of Covid-19 related symptoms and the evidence of SARS-CoV-2 infection in 4 classes: healthy controls (CTRL, n=109); asymptomatic patients (AS, n=15); patients with a mild symptomatology (MI, n=16) and patients with a severe symptomatology (SE, n=12) (see “Study population and study design” section for further details).

The demographic and clinical characteristics of all enrolled subjects were reported in Table 1. Age, transaminase (both aspartate transaminase [AST] and alanine transaminase [ALT]) levels and C-reactive protein (CRP) levels resulted significantly higher in MI patients compared to controls. MI mean age resulted higher also compared with AS and SE. Moreover, the anti SARS-CoV-2 IgG showed an increasing trend from controls to asymptomatic and mild severity patients. Unfortunately, it was not possible to quantify seroconversion in patients with a severe Covid-19, due to a poor serum sample availability. However, diagnosis in these patients was confirmed by means of a PCR-based viral genome detection using a rhino-pharyngeal swab. IgM levels were not statistically different in the CTRL class compared to AS, while the IgM serum concentration in MI was higher than both CTRL and AS. AS subjects showed higher title of IgG compared to CTRL.

Table 1. Demographic and clinical characteristics of the enrolled subjects: Controls (CTRL), Asymptomatic (AS), patients with mild (MI) and severe symptomatology (SE). *=p-value<0.05 Vs CTRL; §=p-value<0.05 Vs Asymptomatic; ¥=p-value<0.05 Vs Mild.

Abbreviations: IgM= anti SARS-CoV-2 type M immunoglobulin; IgG=anti SARS-CoV-2 type G immunoglobulin; WBC=With Blood cells; ALT=alanine transaminase; AST= aspartate transaminase; CK=creatinine kinase; LDH=lactic dehydrogenase; CRP=C-reactive protein; ESR=erythrocyte sedimentation rate

	CTRL	AS	MI	SE
Sample size	109	15	16	12
Age [years]	50±13	53±8	79±28*§¥	60±12
Sex [M/F] (%)	40/69 (36.7/63.3)	8/7 (53.3/46.6)	9/7 (56.3/43.7)	5/7 (41.7/58.3)
IgM [AU/mL]	0.11±0.15	0.16±0.26	2.07±1.89*§	ND
IgG [AU/mL]	0.50±0.20	1.36±0.45*	41.86±22.68*§¥	ND
WBC [cells/μL]	6544±1586	6865±1558	ND	ND
Lymphocytes [cells/μL]	2352±754	2587±698	ND	ND
Lymphocytes [%]	36.4±8.8	38.6±10.5	ND	ND
ALT [U/L]	26.2±6.6	24.6±4.6	33.4±16.8*	35.0±26.9
AST [U/L]	27.4±15.9	25.1±7.1	67.8±70.7*§	39.5±30.4
CK [U/L]	107.8±72.9	80.5±23.9	60.0±105.2	89.5±53.0
LDH [U/L]	185.2±51.8	170.9±21.9	185.9±73.4	327.5±137.9*§
CRP [mg/dL]	0.24±0.54	0.18±0.13	2.20±2.72*	10.89±8.86*§
ESR [mm]	12.5±8.5	13.0±8.8	ND	ND

Altered serum metabolome

To the best of our knowledge, no investigation is currently available about the metabolic perturbations of the several clinical syndromes related to SARS-CoV-2 affected patients. For this reason, we firstly focalized our attention to understand if a symptom-related metabolomic signature does exists. Although the PLS-DA model, built using all the analyzed serum samples, showed a robust statistical significance without overfitting, ($R^2=0.79$, $Q^2=0.68$, $p<0.001$), CTRL and AS were full overlapped (Figure 1A). This was further confirmed by the model built using only CTRL and AS (Figure 1B) that, despite showing a graphical separation, lacked statistical significance as confirmed by the negative Q^2 value ($=-0.23$) and the high p-value (0.64). On the contrary, both MI vs SE (Figure 1C) and AS vs MI (Figure 1D) models resulted statistically robust, showing $R^2=0.92$, $Q^2=0.80$, $p<0.001$ and $R^2=0.87$, $Q^2=0.83$, $p<0.001$, respectively. The ANOVA, based on all classes, highlighted 45 metabolites showing an FDR corrected p-value (q-value) below the cut-off value. These metabolites were clustered by means of the Ward algorithm [43] based on the Minkowski distance, resulting in 4 groups (Figure 2A). One showed low abundance in MI and SE and higher in CTRL and AS (2-hydroxy-3-methyl butyrate, phenylalanine, homocysteine, acetoacetate, palmitic acid, alanine, linoleic acid, glucose, 2-pyrrolidone, creatinine, proline, valine, isoleucine, tyrosine and oxalic acid). The other 3 groups showed higher abundances in MI and SE compared with AS and CTRL. Ten metabolites were selected by VIP-score, namely creatinine, isoleucine, isopropyl alcohol, 2-hydroxy-3-methyl butyrate, proline, erythrose, tyramine, propanoic acid, glucose and stearic acid (Figure 2B).

The FC and VIP-scores of the metabolites selected in the AS vs MI and MI vs SE comparison are reported in figure 3. No metabolite showed a significative q-value in CTRL vs AS comparison. All the selected metabolites were further grouped based on their occurrence in the several models using the UpSet graph (Figure 3C). According to this evaluation, glucose emerged as the most involved metabolite, selected in 5 comparison, followed by mandelic and gluconic acid with 4 involvements. All the other metabolites were selected 3 or less times.

Metabolomics pathways

AS vs MI relevant metabolites were analyzed in the contest of the cellular metabolism using the “m01100” KEGG metabolic pathway (Figure 4). Tyrosine, phenylalanine, acetoacetate and fumarate showed a decreased concentration in MI compared to both CTRL and AS subjects; on the contrary, mandelic acid showed an increased concertation. The shortest route among these metabolites resulted the Tyrosine metabolism (KEGG id mp0350). Further perturbations were related to a decreased concentration of several amino acids (aspartic acid, alanine, isoleucine, valine and proline) in MI patients, while methionine and oxo-leucine (2-ketoisocaproic acid) resulted increased. An increased concertation of

fatty acids lauric and myristic acid, phospholipids (phosphatidyl myo-inositol and Lyso-phosphatidyl inositol) and sugar and sugar-related metabolites (fructose, glyceric and glyoxylic acid, lactic acid), glycerol and urocanate were also recorded. Moreover, histamine showed a higher concentration in MI compared to CTRL and AS.

Discussion

Pandemic influenza have a quadratic ("U"-shaped) lethality curve (being more lethal for infants and elderly than for adults), perhaps because adults have an higher chance to be, at least partially, immune against the infection [44]. On the contrary, Covid-19 lethality increases with age. The severity and the clinical picture could even be related to the massive activation of the immune response.

Nevertheless, Covid-19 mortality, although rare, is not absent in young people. The Italian "Istituto Superiore di Sanità" on April 30th, reported 5% of the 25,450 Italian COVID-19 related death occurred in patients younger than 60 years old, as well as 4% in subjects with no comorbidity [45]. Moreover, an extensive population-based SARS-CoV-2 screening in the Italian village Vo' Euganeo [46], showed a significative number of people older than 60 years faced the infection being asymptomatic. These evidences further showed the need to better understand the physiological mechanism under the different clinical presentations of the disease.

Here we reported a metabolomics-based approach to describe the difference among infected people reporting different disease severity. According to our results, asymptomatic and healthy subjects shared the same metabolomics aspects, showing no significant differences. On the contrary, both mild and severe patients have a specific metabolomics fingerprint. Nevertheless, the most important difference seems to be related to the hospitalization more than to the disease *per se*. Indeed, both glucose and gluconic acid reported differences among the analyzed samples, emerged as relevant in 5 and 4 features selection strategies, respectively. This finding could be due to the parenteral nutrition to which ICU hospitalized patients are subjected. ICU hospitalized patients showed a deep metabolic impairment due to their specific clinical conditions and treatments. For this reason, we focalized our attention on mild symptomatic patients compared to asymptomatic. The combined perturbations recorded in the tyrosine, phenylalanine, acetoacetate and fumarate (decreased in MI compared to both CTRL and AS subjects), and mandelic acid (increased), suggest a relevant Tyrosine metabolism perturbation.

According to this pathway, the reported metabolite levels could be explained by an increase production of L-DOPA, resulting in a following increased production of noradrenaline and adrenaline, finally metabolized in vanillylmandelic acid, a mandelic acid metabolite. Although adrenaline, noradrenaline and dopamine levels resulted, in all the analyzed samples, under the detection limits of the used GCMS approach, we can make inference about their hyperproduction by looking at the molecular pathways involved. The increased adrenaline production could be in line with the severity of the immune response. Indeed, as early as 1948, a Lancet annotation reported that "*When a bacterial suspension is inoculated into guinea-pig skin and muscle, the resulting infection is much more severe if a small quantity of*

adrenaline is injected at the same time" [47]. Moreover, since 1971 [48,49] several cases of adrenaline-induced pulmonary edema were reported. Pulmonary edema is one of the most relevant Covid-19 clinical feature. On the other hand, adrenaline has an important effect in lung dynamics, opening up the bronchioles and facilitating the air income [50].

Although a rapid and well-coordinated innate immune response is the first line of defense against viral infections, its dysregulated activation may cause immune damage to the human body. Covid-19 lung injury seems to be sustained prevalently by a cytokine storm-syndrome rather than the virus itself. [51]. Indeed, the evidence of Interleukin-6 (IL-6) involvement was provided by the effectiveness of the treatment of several patients with tocilizumab, a humanized monoclonal antibody against IL-6 [52]. Circulating adrenaline induces a dose-dependent increase in plasma IL-6 concentrations [53]. This evidence further supports the potential adrenaline role in Covid-19 severity.

Adrenaline also improves renin production by the kidney [54], by stimulating the adenylate cyclase-5. Hoffmann et al. recently reported that the angiotensin converting enzymes 2 (ACE2), a homologue of ACE that negatively regulates angiotensin II, is a cellular receptor for SARS-CoV-2 [55]. This has led to hypothesize that decreasing ACE2 levels in cells might help in fighting the infection. Conversely, ACE2 has shown to have a protective effect against virus-induced lung injury, by increasing the production of the vasodilator angiotensin 1–7 [56]. Moreover, according to studies conducted on mice, the interaction of the SARS-CoV-2 spike protein with ACE2 induces a decrease in the levels of ACE2 on the cell surface, since it induces the internalization and degradation of the protein, contributing to lung damage [56,57]. Thus, adrenaline synthesis increase could be read as a compensatory response to the ACE2 decreased levels.

Strengths And Limitations

The novelty concerning our study is that the metabolomic approach was not yet used to describe the metabolic signatures of patients affected by the several clinical presentations of SARS-CoV-2 infection. The most severe clinical presentation has shown to be particularly disabling and, even in case of survival, recovery is very long. This was also shown in SARS patients, which showed metabolic perturbations as late as 12 years after the infection [16]. Untargeted metabolomics allows to provide hypotheses that can be further studied with other approaches [58,59]. Here we reported a tyrosine metabolism impairment hypothesis that could be in line with the severity of the Covid-19 and is supported by several concomitant observations.

On the other hand, our findings should be considered in the context of several study limitations, including the lack of adrenaline and noradrenaline direct quantitation, due to a very low concentration, and a limited samples size.

Moreover, our study design, lacking pneumonia patients with a different etiology, did not allow to exclude the observed signature is an acute lung infection non-specific reaction. Anyway, both a plasma metabolomics study of community-acquired pneumonia patients [60] and plasma metabolomics study

of H1N1 influenza pneumonia [61] did not reported similar results supporting the SARS-CoV-2 pneumonia specific metabolomics signature.

Conclusions

Concluding, although a metabolic signature of symptomatic SARS-CoV-2 patients is here reported and supported an increased tyrosine metabolism in the L-DOPA-Noradrenaline-Adrenaline production, our experimental design does not allow us to understand if this is an effect of the established symptomatology or a predisposing condition to develop a severe clinical picture. Further studies are needed to understand the exact role of this imbalance in Covid-19 patients.

Declarations

Conflict of interest: All the authors have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local ethics committee “Comitato Etico Campania SUD” n.9/2020, prot. 0061907/20) and a written consent form was signed by each participant.

Author contribution: JT, PC and MP conceived and designed the experimental study. MM, FDC, GB, CC, PP and MP enrolled subjects and performed the clinical evaluations and the samples’ collection. PC, and IS performed the serological evaluation. JT and GS performed metabolome extraction, purification, derivatization and analysis. JT performed statistical analysis and machine learning training and test. JT, PC and MM wrote the manuscript. MP, PA and CC edited the manuscript. All the authors approved the final version of the manuscript.

References

1. Gorbalenya, A.E.; Baker, S.C.; Baric, R.S.; de Groot, R.J.; Drosten, C.; Gulyaeva, A.A.; Haagmans, B.L.; Lauber, C.; Leontovich, A.M.; Neuman, B.W.; et al. Severe acute respiratory syndrome-related coronavirus: The species and its viruses – a statement of the Coronavirus Study Group. *bioRxiv* 2020, 2020.02.07.937862, doi:10.1101/2020.02.07.937862.
2. Phelan, A.L.; Katz, R.; Gostin, L.O. The Novel Coronavirus Originating in Wuhan, China: Challenges for Global Health Governance. *JAMA* 2020, 323, 709–710, doi:10.1001/jama.2020.1097.
3. WHO Naming the coronavirus disease (COVID-19) and the virus that causes it Available online: [https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/naming-the-coronavirus-disease-\(covid-2019\)-and-the-virus-that-causes-it](https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/naming-the-coronavirus-disease-(covid-2019)-and-the-virus-that-causes-it).
4. Li, Q.; Guan, X.; Wu, P.; Wang, X.; Zhou, L.; Tong, Y.; Ren, R.; Leung, K.S.M.; Lau, E.H.Y.; Wong, J.Y.; et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus–Infected Pneumonia. *N. Engl.*

- J. Med.* 2020, *382*, 1199–1207, doi:10.1056/NEJMoa2001316.
5. Chen, N.; Zhou, M.; Dong, X.; Qu, J.; Gong, F.; Han, Y.; Qiu, Y.; Wang, J.; Liu, Y.; Wei, Y.; et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *The Lancet* 2020, *395*, 507–513, doi:10.1016/S0140-6736(20)30211-7.
 6. Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *The Lancet* 2020, *395*, 497–506, doi:10.1016/S0140-6736(20)30183-5.
 7. Bhatraju, P.K.; Ghassemieh, B.J.; Nichols, M.; Kim, R.; Jerome, K.R.; Nalla, A.K.; Greninger, A.L.; Pipavath, S.; Wurfel, M.M.; Evans, L.; et al. Covid-19 in Critically Ill Patients in the Seattle Region – Case Series. *N. Engl. J. Med.* 2020, doi:10.1056/NEJMoa2004500.
 8. Lescure, F.-X.; Bouadma, L.; Nguyen, D.; Parisey, M.; Wicky, P.-H.; Behillil, S.; Gaymard, A.; Bouscambert-Duchamp, M.; Donati, F.; Le Hingrat, Q.; et al. Clinical and virological data of the first cases of COVID-19 in Europe: a case series. *Lancet Infect. Dis.*, doi:10.1016/S1473-3099(20)30200-0.
 9. Green, M.S. Did the hesitancy in declaring COVID-19 a pandemic reflect a need to redefine the term? *The Lancet* 2020, *395*, 1034–1035, doi:10.1016/S0140-6736(20)30630-9.
 10. Guo, L.; Ren, L.; Yang, S.; Xiao, M.; Chang, D.; Yang, F.; Dela Cruz, C.S.; Wang, Y.; Wu, C.; Xiao, Y.; et al. Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19). *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 2020, doi:10.1093/cid/ciaa310.
 11. Long, Q.-X.; Liu, B.-Z.; Deng, H.-J.; Wu, G.-C.; Deng, K.; Chen, Y.-K.; Liao, P.; Qiu, J.-F.; Lin, Y.; Cai, X.-F.; et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat. Med.* 2020, doi:10.1038/s41591-020-0897-1.
 12. Sardu, C.; Gambardella, J.; Morelli, M.B.; Wang, X.; Marfella, R.; Santulli, G. Hypertension, Thrombosis, Kidney Failure, and Diabetes: Is COVID-19 an Endothelial Disease? A Comprehensive Evaluation of Clinical and Basic Evidence. *J. Clin. Med.* 2020, *9*, 1417.
 13. Decuypere, S.; Pyke, J.; Tull, D.; Buddhisa, S.; McConville, M.; Blackwell, J.; Lertmemongkolchai, G. Metabolomics based biomarker discovery for infectious diseases, the case of melioidosis. *Int. J. Infect. Dis.* 2012, *16*, e216, doi:10.1016/j.ijid.2012.05.810.
 14. Kim, D.-H.; Creek, D.J. What role can metabolomics play in the discovery and development of new medicines for infectious diseases? *Bioanalysis* 2015, *7*, 629–631, doi:10.4155/bio.15.5.
 15. Pacchiarotta, T.; Deelder, A.M.; Mayboroda, O.A. Metabolomic investigations of human infections. *Bioanalysis* 2012, *4*, 919–925, doi:10.4155/bio.12.61.
 16. Wu, Q.; Zhou, L.; Sun, X.; Yan, Z.; Hu, C.; Wu, J.; Xu, L.; Li, X.; Liu, H.; Yin, P.; et al. Altered Lipid Metabolism in Recovered SARS Patients Twelve Years after Infection. *Sci. Rep.* 2017, *7*, 9110, doi:10.1038/s41598-017-09536-z.
 17. Zurfluh, S.; Baumgartner, T.; Meier, M.A.; Ottiger, M.; Voegeli, A.; Bernasconi, L.; Neyer, P.; Mueller, B.; Schuetz, P. The role of metabolomic markers for patients with infectious diseases: implications for

- risk stratification and therapeutic modulation. *Expert Rev. Anti Infect. Ther.* 2018, *16*, 133–142, doi:10.1080/14787210.2018.1426460.
18. Harris, P.A.; Taylor, R.; Thielke, R.; Payne, J.; Gonzalez, N.; Conde, J.G. Research Electronic Data Capture (REDCap) - A metadata-driven methodology and workflow process for providing translational research informatics support. *J. Biomed. Inform.* 2009, *42*, 377–381, doi:10.1016/j.jbi.2008.08.010.
 19. RDevelopment CORE TEAM, R. *R: A language and environment for statistical computing*, R foundation for statistical computing Vienna, Austria, 2008;
 20. Wolinsky, H. The battle of Helsinki: Two troublesome paragraphs in the Declaration of Helsinki are causing a furore over medical research ethics. *EMBO Rep.* 2006, *7*, 670–672, doi:10.1038/sj.embor.7400743.
 21. Troisi, J.; Masarone, M.; Caruso, R.; Persico, M. Metabolomics in the progression of fatty liver disease. *J. Hepatol.* 2018, *68*, S568–S569.
 22. Troisi, J.; Cinque, C.; Giugliano, L.; Symes, S.; Richards, S.; Adair, D.; Cavallo, P.; Sarno, L.; Scala, G.; Caiazza, M.; et al. Metabolomic change due to combined treatment with myo-inositol, D-chiro-inositol and glucomannan in polycystic ovarian syndrome patients: a pilot study. *J. Ovarian Res.* 2019, *12*, 25–25, doi:10.1186/s13048-019-0500-x.
 23. Troisi, J.; Sarno, L.; Martinelli, P.; Di Carlo, C.; Landolfi, A.; Scala, G.; Rinaldi, M.; D'Alessandro, P.; Ciccone, C.; Guida, M. A metabolomics-based approach for non-invasive diagnosis of chromosomal anomalies. *Metabolomics* 2017, *13*, 140, doi:10.1007/s11306-017-1274-z.
 24. Troisi, J.; Landolfi, A.; Sarno, L.; Richards, S.; Symes, S.; Adair, D.; Ciccone, C.; Scala, G.; Martinelli, P.; Guida, M. A metabolomics-based approach for non-invasive screening of fetal central nervous system anomalies. *Metabolomics* 2018, *14*, 77, doi:10.1007/s11306-018-1370-8.
 25. Troisi, J.; Landolfi, A.; Vitale, C.; Longo, K.; Cozzolino, A.; Squillante, M.; Savanelli, M.C.; Barone, P.; Amboni, M. A metabolomic signature of treated and drug-naïve patients with Parkinson's disease: a pilot study. *Metabolomics* 2019, *15*, 90, doi:10.1007/s11306-019-1554-x.
 26. Troisi, J.; Sarno, L.; Landolfi, A.; Scala, G.; Martinelli, P.; Venturella, R.; Di Cello, A.; Zullo, F.; Guida, M. Metabolomic Signature of Endometrial Cancer. *J. Proteome Res.* 2018, *17*, 804–812, doi:10.1021/acs.jproteome.7b00503.
 27. Sumner, L.W.; Amberg, A.; Barrett, D.; Beale, M.H.; Beger, R.; Daykin, C.A.; Fan, T.W.-M.; Fiehn, O.; Goodacre, R.; Griffin, J.L.; et al. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics Off. J. Metabolomic Soc.* 2007, *3*, 211–221, doi:10.1007/s11306-007-0082-2.
 28. Wehrens, R.; Bloemberg, T.G.; Eilers, P.H.C. Fast parametric time warping of peak lists. *Bioinforma. Oxf. Engl.* 2015, *31*, 3063–3065, doi:10.1093/bioinformatics/btv299.
 29. van den Berg, R.A.; Hoefsloot, H.C.; Westerhuis, J.A.; Smilde, A.K.; van der Werf, M.J. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics* 2006, *7*, 142, doi:10.1186/1471-2164-7-142.

30. Whitley, D. A genetic algorithm tutorial. *Stat. Comput.* 1994, 4, 65–85, doi:10.1007/BF00175354.
31. Jungermann, F. Information extraction with rapidminer.; Citeseer, 2009; pp. 50–61.
32. Chong, J.; Yamamoto, M.; Xia, J. MetaboAnalystR 2.0: From raw spectra to biological insights. *Metabolites* 2019, 9, 57.
33. Mevik, B.-H.; Wehrens, R. The pls Package: Principal Component and Partial Least Squares Regression in R. *J. Stat. Softw.* 2007, 1.
34. Kuhn, M. Building Predictive Models in R Using the caret Package. *J. Stat. Softw.* 2008, 1.
35. Bijlsma, S.; Bobeldijk, I.; Verheij, E.R.; Ramaker, R.; Kochhar, S.; Macdonald, I.A.; van Ommen, B.; Smilde, A.K. Large-scale human metabolomics studies: a strategy for data (pre-) processing and validation. *Anal. Chem.* 2006, 78, 567–574, doi:10.1021/ac051495j.
36. Domingos, P. MetaCost: a general method for making classifiers cost-sensitive. In Proceedings of the Proceedings of the fifth ACM SIGKDD international conference on Knowledge discovery and data mining; ACM: San Diego, California, USA, 1999; pp. 155–164.
37. Wold, S.; Sjöström, M.; Eriksson, L. Partial least squares projections to latent structures (PLS) in chemistry. *Encycl. Comput. Chem.* 1998.
38. Akarachantachote, N.; Chadcham, S.; Saithanu, K. Cutoff threshold of variable importance in projection for variable selection. *Int. J. Pure Appl. Math.* 2014, 94, 307–322.
39. Stein, S. Mass spectral reference libraries: an ever-expanding resource for chemical identification. 2012.
40. Lex, A.; Gehlenborg, N.; Strobel, H.; Vuilleumot, R.; Pfister, H. UpSet: Visualization of Intersecting Sets. *IEEE Trans. Vis. Comput. Graph.* 2014, 20, 1983–1992, doi:10.1109/TVCG.2014.2346248.
41. Darzi, Y.; Letunic, I.; Bork, P.; Yamada, T. iPath3.0: interactive pathways explorer v3. *Nucleic Acids Res.* 2018, 46, W510–W513, doi:10.1093/nar/gky299.
42. Kanehisa, M.; Goto, S.; Sato, Y.; Kawashima, M.; Furumichi, M.; Tanabe, M. Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res.* 2014, 42, D199-205, doi:10.1093/nar/gkt1076.
43. Ward Jr, J.H. Hierarchical grouping to optimize an objective function. *J. Am. Stat. Assoc.* 1963, 58, 236–244.
44. Ma, J.; Dushoff, J.; Earn, D.J.D. Age-specific mortality risk from pandemic influenza. *J. Theor. Biol.* 2011, 288, 29–34, doi:10.1016/j.jtbi.2011.08.003.
45. Istituto Superiore di Sanità Characteristics of COVID-19 patients dying in Italy Available online: <https://www.epicentro.iss.it/en/coronavirus/sars-cov-2-analysis-of-deaths>.
46. Day, M. Covid-19: identifying and isolating asymptomatic people helped eliminate virus in Italian village. *Bmj* 2020, 368, m1165.
47. Adrenaline and Infection. *Orig. Publ. Vol. 2 Issue 6514* 1948, 252, 21, doi:10.1016/S0140-6736(48)91799-1.

48. ERSOZ, N.; FINESTONE, S.C. ADRENALINE-INDUCED PULMONARY OEDEMA AND ITS TREATMENT: A Report of Two Cases. *Br. J. Anaesth.* 1971, *43*, 709–712, doi:10.1093/bja/43.7.709.
49. Syal, K.; Ohri, A.; Thakur, J.R. Adrenaline induced pulmonary oedema. *J. Anaesthesiol. Clin. Pharmacol.* 2011, *27*, 132–133.
50. Vardanyan, R.; Hruby, V. Chapter 11 - Adrenergic (Sympathomimetic) Drugs. In *Synthesis of Best-Seller Drugs*; Vardanyan, R., Hruby, V., Eds.; Academic Press: Boston, 2016; pp. 189–199 ISBN 978-0-12-411492-0.
51. Ye, Q.; Wang, B.; Mao, J. The pathogenesis and treatment of the 'Cytokine Storm' in COVID-19. *J. Infect.* 2020, doi:10.1016/j.jinf.2020.03.037.
52. Xu, X.; Han, M.; Li, T.; Sun, W.; Wang, D.; Fu, B.; Zhou, Y.; Zheng, X.; Yang, Y.; Li, X.; et al. Effective treatment of severe COVID-19 patients with tocilizumab. *Proc. Natl. Acad. Sci.* 2020, 202005615, doi:10.1073/pnas.2005615117.
53. DeRijk, R.H.; Boelen, A.; Tilders, F.J.; Berkenbosch, F. Induction of plasma interleukin-6 by circulating adrenaline in the rat. *Psychoneuroendocrinology* 1994, *19*, 155–163, doi:10.1016/0306-4530(94)90005-1.
54. Vandongen, R.; Greenwood, D.M. The stimulation of renin secretion by non-vasoconstrictor infusions of adrenaline and noradrenaline in the isolated rat kidney. *Clin. Sci. Mol. Med.* 1975, *49*, 609–612, doi:10.1042/cs0490609.
55. Hoffmann, M.; Kleine-Weber, H.; Schroeder, S.; Krüger, N.; Herrler, T.; Erichsen, S.; Schiergens, T.S.; Herrler, G.; Wu, N.-H.; Nitsche, A.; et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020, *181*, 271-280.e8, doi:10.1016/j.cell.2020.02.052.
56. Imai, Y.; Kuba, K.; Penninger, J.M. The discovery of angiotensin-converting enzyme 2 and its role in acute lung injury in mice. *Exp. Physiol.* 2008, *93*, 543–548, doi:10.1113/expphysiol.2007.040048.
57. Jia, H. Pulmonary Angiotensin-Converting Enzyme 2 (ACE2) and Inflammatory Lung Disease. *Shock Augusta Ga* 2016, *46*, 239–248, doi:10.1097/SHK.0000000000000633.
58. Schrimpe-Rutledge, A.C.; Codreanu, S.G.; Sherrod, S.D.; McLean, J.A. Untargeted Metabolomics Strategies-Challenges and Emerging Directions. *J. Am. Soc. Mass Spectrom.* 2016, *27*, 1897–1905, doi:10.1007/s13361-016-1469-y.
59. Troisi, J.; Cavallo, P.; Colucci, A.; Pierri, L.; Scala, G.; Symes, S.; Jones, C.; Richards, S. Metabolomics in genetic testing. 2019.
60. Seymour, C.W.; Yende, S.; Scott, M.J.; Pribis, J.; Mohny, R.P.; Bell, L.N.; Chen, Y.-F.; Zuckerbraun, B.S.; Bigbee, W.L.; Yealy, D.M.; et al. Metabolomics in pneumonia and sepsis: an analysis of the GenIMS cohort study. *Intensive Care Med.* 2013, *39*, 1423–1434, doi:10.1007/s00134-013-2935-7.
61. Banoei, M.M.; Vogel, H.J.; Weljie, A.M.; Kumar, A.; Yende, S.; Angus, D.C.; Winston, B.W.; Canadian Critical Care Translational Biology Group (CCCTBG) Plasma metabolomics for the diagnosis and prognosis of H1N1 influenza pneumonia. *Crit. Care Lond. Engl.* 2017, *21*, 97–97, doi:10.1186/s13054-017-1672-7.

Figures

Figure 1

Relevant metabolites selected in all classes' comparison. (A) Heatmap reporting the ANOVA selected metabolites. Cluster analysis allowed to recognize 4 grupes (A, B, C and D) of metabolites based on their concentration mean levels in the 4 classes. (B) Metabolites showing a VIP-score >2.0 in the PLS-DA

Analysis. Abbreviations: AS= asymptomatic subjects, CTRL= healthy controls, MI=patients showing mild symptomatology, SE= patients with severe symptoms, PLS-DA=Partial Least Square Discriminant Analysis, VIP=Variable Important in Projection, ANOVA=Analysis of Variance, 2-OH-3-MBA=2-hydroxy-3methylbutanoic acid, 2-KICA=2-keto isocaproic acid, MeSA= Methyl Succinic acid, TEG= tetraethylene glycol, LysPI= Lysophosphatidylinositol, P-myo-I= Phosphatidyl myo-inositol

Figure 2

Partial least squares-discriminant analysis (PLS-DA) based on serum metabolites determined by GC-MS. (A) Two-dimensional score plot showing clustering and separation between all subjects' classes: healthy controls (CTRL, green), asymptomatic (AS, orange), patients with a non-severe symptomatology (MI, yellow) and patients with a severe symptomatology (SE, purple). (B) PLS-DA model discriminating CTRL and MI. (C) PLS-DA model discriminating MI and SE. (D) PLS-DA model discriminating AS and MI For each latent variable (LV), percentage of explained variance was reported in brackets. Ellipses represent 95% confidence intervals.

Figure 3

Relevant metabolites selected. (A-B) Bar-diagrams reporting the base 2 logarithm of the FC of the metabolites selected by means of the combined evaluation of p-value and FC in (A) AS Vs MI comparison and (B) MI Vs SE comparison. (C) Upset diagram reporting the metabolites occurrence in the several selection strategies. Set size indicates the number of metabolites involved by each metabolites' selection strategy, while Intersection Size indicates the number of metabolites selected from each strategies' combination. * indicates the following metabolites: 2-Ketoisocaproic acid, Acetoacetate, Alanine, Boric acid, Fructose, Fumarate, Galactose, Glyceric acid, Glycerol, Glyoxylic acid, Histamine, Hydroxylamine, Lactic acid, Lysophosphatidylinositol, Methoxyacetic acid, Methylsuccinic acid, Myristic acid, Phenylalanine, Pyrogallol, Tetraethylene glycol, Threonic acid, Threose, Tyrosine Valine. (D-F) VIP-score selected metabolites in AS Vs CTRL PLS-DA model (D), AS Vs MI model (E) and MI Vs SE model (F). Abbreviations: AS= asymptomatic subjects, CTRL=healthy controls, MI=patients showing mild

symptomatology, SE=patients with severe symptoms, PLS-DA=Partial Least Square Discriminant Analysis, VIP=Variable Important in Projection, FC= Fold Change.

Figure 4

Metabolic systems map summarizing the shortest biochemical route that may explain the interactions among the most relevant metabolites selected in AS-MI comparison. Red dots indicate the metabolites showing an increased concentration in MI samples. Red dots indicate the metabolites with a lower concentration. Blue dots indicate metabolites involved in tyrosine pathway but not emerged in our analysis. Blue lines show the involved reactions.

Supplementary Files

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