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BRIEF REPORT

Impact of Innate Immunity, Endothelial Damage, and Metabolic Biomarkers on COVID-19 Severity and Mortality

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In this study, abnormal levels of myeloid activation, endothelial damage, and innate immune markers were associated with severe coronavirus disease 2019 (COVID-19), while higher levels of metabolic biomarkers (irisin, leptin) demonstrated a protective effect. These data support a model for COVID-19 immunopathogenesis linking robust inflammation and endothelial damage in metabolically predisposed individuals.

Keywords. COVID-19; endothelial damage; inflammation; metabolic biomarkers.

Coronavirus disease 2019 (COVID-19) is a heterogeneous clinical disease ranging from asymptomatic infection to life-threatening acute respiratory distress syndrome that continues to cause significant morbidity and mortality worldwide [1–5]. Despite significant progress in understanding the role of innate immune signaling and robust inflammation in the pathogenesis of COVID-19, disease heterogeneity and variability in host factors hamper identification of predictors with translational clinical implications. We sought to expand the understanding of COVID-19 pathophysiology by integrating clinical data and soluble biomarkers of innate immunity, myeloid cell activation, vascular injury, control of energy metabolism, and coagulation from a cohort covering a broad range of disease severity.

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METHODS

De-identified clinical blood samples from 128 patients with molecularly confirmed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection were collected near the time of hospital admission and before any medical treatment including corticosteroids or tocilizumab at 2 Southern Italian medical centers (Bari and Foggia) between February and September 2020. At the time of this study, antivirals such as remdesivir and monoclonal antibodies were unavailable. Clinical characteristics and outcomes including disease severity and in-hospital mortality were recorded. Disease severity was categorized as mild–moderate or severe–critical based on the greatest disease severity experienced throughout admission. Briefly, cases were designated: mild (n = 24)—minimal symptoms, no imaging findings; moderate (n = 36)—fever, respiratory symptoms, and radiographic pneumonia; severe (n = 49)—respiratory distress (≥30 breaths/min) OR oxygen saturation ≤93% at rest; critical (n = 19)—shock, respiratory failure, or other organ failure requiring intensive care [6]. This study was approved by local internal review boards and performed in accordance with the Declaration of Helsinki.

Plasma levels of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-27, interferon (IFN)-γ, tumor necrosis factor (TNF)–α, C-reactive protein (CRP), serum amyloid A (SAA), thrombopoietin (TPO), soluble thrombomodulin (sTM), intercellular adhesion molecule–1 (sICAM-1), sICAM-3, sVCAM-1, E-selectin, P-selectin, pentraxin-3 (PTX3), tissue factor pathway inhibitor (TFPI), antithrombin III (AT3), elastase, peptidylarginine deaminase–4 (PAD-4), serum levels of IL-7, C3a, and C5a, circulating immune complexes–C1q (CIC-C1q), CIC-C3d, C1q, irisin, and leptin were quantified using electrochemiluminescence from Meso-Scale Discovery (MSD; Gaithersburg, MD, USA), while sCD14 and sIL-6Ra were quantified by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

To investigate the impact of clinical characteristics and biomarkers on COVID-19 severity and in-hospital mortality, logistic regression modeling was performed while controlling for time from symptom onset to sample collection for all variables. Significant biomarkers were fed into a multivariate stepwise selection process (Supplementary Methods). Spearman`s correlation was used to assess associations between biomarkers, and a decision tree model was performed to identify which markers were most predictive of severe–critical COVID-19.

RESULTS

In this retrospective cohort study, we collected data from 128 consecutive patients with COVID-19 admitted to

2 Southern Italian hospitals (60 patients with mild–moderate and 68 with severe–critical disease) (Table 1). The median age (interquartile range) was 59 (50–79) years, and 66 patients (51.6%) were women. Ten total deaths were recorded in the cohort; all other patients survived to hospital discharge. As the time from symptom onset to sample collection differed between patients with mild–moderate and severe–critical disease, this variable was adjusted for in all our regression analyses. Clinical characteristics associated with severe–critical disease by adjusted logistic regression included age (OR, 1.06 per year; 95% CI, 1.04–1.10), hypertension (OR, 3.91; 95% CI, 1.67–9.7), and chronic obstructive pulmonary disease (COPD; OR, 17.51; 95% CI, 3.17–329.05). COPD was the only clinical factor that remained significant in the multivariate regression analysis (OR, 12.9; 95% CI, 1.53–294.1). Among clinical laboratory values, D-dimer (OR, 4.48; 95% CI, 1.77–12.76) was associated with severe–critical disease, whereas a higher absolute lymphocyte count was protective (OR, 0.11; 95% CI, 0.02–0.54).

Severe–critical disease was strongly associated with innate inflammatory biomarkers including IL-6 (OR, 3.70; 95% CI, 1.79–8.53), TNF-α (OR, 9.38; 95% CI, 1.57–69.97), pentraxin-3 (PTX3; OR, 9.78, 95% CI, 3.48–32.78), C1q (OR, 18.43; 95% CI, 2.68–151.11), and soluble CD14 (sCD14; OR, 24.21; 95% CI, 1.47–497.9). Multiple markers of endothelial activation were also associated with severe–critical disease, specifically soluble thrombomodulin (sTM; OR, 15.76; 95% CI, 2.12–145.5), sICAM-1 (OR, 42.1; 95% CI, 3.22–828.0), and sVCAM-1 (OR, 92.0; 95% CI, 7.62–1696.7) (Figure 1*A*). Stepwise multivariate logistic regression identified PTX3 (OR, 10.5; 95% CI, 1.84–79.1), IL-6 (OR, 7.37; 95% CI, 1.82–40.3), and C1q (OR, 48.3; 95% CI, 2.29–1663) as the most important biomarkers associated with severe–critical disease (Supplementary Table 1). Consistent with these findings, a decision tree analysis identified PTX3 >8.68 ng/mL as the most robust predictor of severe disease, with a second node at age >62 years (Supplementary Figure 1).

Interestingly, irisin, a myokine produced during exercise and with anti-inflammatory properties in different clinical settings [7–9], was strongly correlated with protection against severe–critical disease (OR, 0.03; 95% CI, 0.0–0.68). Logistic regression analysis for in-hospital mortality also demonstrated that leptin, an adipokine, was associated with lower mortality (OR, 0.23; 95% CI, 0.06–0.76), highlighting the importance of metabolism in innate and adaptive immunological functions [10] impacting the severity of COVID-19.

Logistic regression for in-hospital mortality also identified a greater risk of death with increased D-dimer (OR, 3.82; 95% CI, 1.11–13.95) and CRP (OR, 7.12; 95% CI, 1.52–50.34), with a similar cytokine profile as found in those with severe–critical disease (TNF-α, PTX3, IL-6, sCD14, sTM, and sVCAM-1) (Figure 1*B*). Unique biomarkers associated with increased mortality included IL-10 and IL-27. Because of the small total number of deaths in our cohort, multivariate analysis for mortality was not performed due to low statistical power. Overall, the biomarker patterns in both analyses were overlapping and consisted primarily of markers involved in innate immune signaling, monocyte activation, and endothelial injury. Due to the inherent subjectivity of reported symptom onset, a sensitivity analysis was performed by excluding outlier individuals with sample collection >30 days from reported symptom onset. No notable differences in the logistic regression for severe–critical COVID-19 and in-hospital mortality were identified in this subset analysis (Supplementary Tables 2 and 3), further corroborating the lack of confounders due to the variability in time from symptom onset to sample collection in the observed differences in COVID-19 clinical severity.

Heat maps comparing biomarker correlations in the mild–moderate vs severe–critical cohorts revealed a network of significant links between markers of innate immunity, endothelial activation, and complement in the mild–moderate cohort (Figure 1*C*). However, in severe disease a unique pattern emerged with a reduced number of overall correlations and stronger associations between innate immune and endothelial markers consistent with a more rigid and dysregulated cytokine network (Figure 1*D*).

Table 1. Patient Characteristics of the Mild–Moderate and Severe–Critical COVID-19 Cohorts

Characteristic	Mild–Moderate (n = 60)	Severe–Critical (n = 68)
Age, y	53 (43–62)	67 (56.8–84.2)
Female, No. (%)	32 (53.3)	34 (50)
Race, No. (%)		
Caucasian	57 (100)	65 (97)
Other	0 (0)	2 (3)
BMI, kg/m^2	24.3 (22.2–26.0)	25.6 (23.1–27.3)
Symptoms to specimen collection, d	9.0 (7.0–13.5)	15.0 (10.0–30.2)
Total neutrophil count, cells/μL	3.48 (2.71–4.57)	3.64 (2.36–5.55)
Total lymphocyte count, cells/μL	1.39 (0.86–1.89)	1.10 (0.66–1.61)
Comorbidities, No. (%)		
Hypertension	13 (21.7)	35 (51.5)
Chronic kidney disease	2 (3.3)	9 (13.2)
Diabetes mellitus	10 (16.7)	17 (25)
COPD/asthma	2 (3.3)	15 (22.1)
Cancer/autoimmunity	7 (11.7)	12 (17.6)
Treatment, No. (%)		
Antibiotics	2 (3.3)	17 (25)
Tocilizumab	2 (3.3)	11 (16.2)
Steroids	0 (0)	5 (7.5)

Continuous variables are represented as median with interquartile range, and categorical variables as number with percentage. All samples included in this cohort were collected before the administration of corticosteroids or tocilizumab. No patients received monoclonal antibodies or remdesivir, as these treatments were unavailable at the time of this study.

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019.

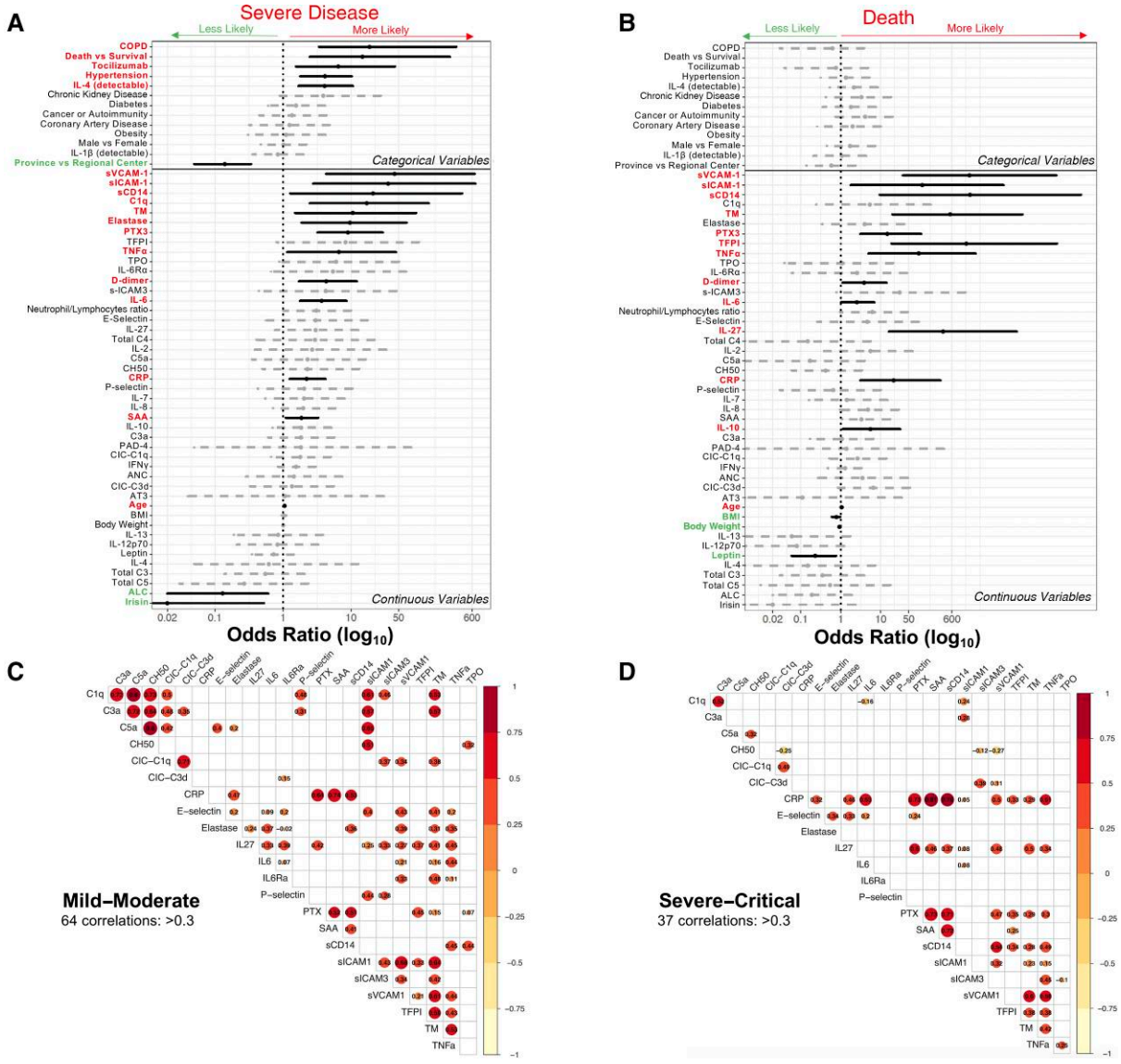

Figure 1. Logistic regression of clinical characteristics and biomarkers associated with severe–critical disease (*A*) and death (*B*) from COVID-19, adjusted for time from symptom onset to specimen collection. Multiparameter Spearman's correlation analysis was used to generate heat maps demonstrating correlations between biomarkers in those with mild–moderate COVID-19 (*C*) and severe–critical disease (*D*) (statistically significant [$P < .05$] correlations are highlighted by colored circles, with red indicating positive and yellow highlighting negative associations). The total number of positive correlations ($r > 0.30$) was notably different between those with mild–moderate disease compared with severe–critical. No negative correlations with $r < -0.30$ were identified. Abbreviations: ALC, absolute lymphocyte count; ANC, absolute neutrophil count; AT3, antithrombin III; BMI, body mass index; CIC-C1q, circulating immune complexes–C1q; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; IFNγ, interferon-γ; IL-6Rα, interleukin-6 receptor-α; PAD-4, peptidylarginine deaminase–4; PTX3, pentraxin-3; SAA, serum amyloid A; sICAM-1, soluble intercellular adhesion molecule–1; sVCAM-1, soluble vascular cell adhesion molecule-1; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; TNFα, tumor necrosis factor-α; TPO, thrombopoietin.

DISCUSSION

The pathogenesis of hyperinflammation in COVID-19 continues to be refined. As type I interferon signaling is involved in early sensing of SARS-CoV-2, as well as other respiratory viruses [11], the downstream inflammatory cascade converges on innate immune cytokines including IL-6, IL-10, and TNF-α, which are consistently increased in those progressing to severe COVID-19 [12–15]. Innate immune cytokines correlate with SARS-CoV-2 viral load when assessed longitudinally, and persistent stimulation through Toll-like receptors (TLRs) has been identified as a potential mechanism to explain this innate hypercytokinemia [12, 14].

We found a similar pattern with increases in IL-6 and TNF-α associating with severe–critical disease and death by regression analyses, with IL-6 remaining associated with severe disease in the multivariate stepwise model. In addition, a high level of

PTX3, a key component of innate immune signaling, was also significantly increased in severe COVID-19 by multivariate regression. These findings suggest that PTX3 may be more robustly associated with severe disease than CRP, as previously proposed [16, 17]. This was validated in our decision tree model, which identified elevated PTX3 as the strongest predictor of severe COVID-19.

Multiple studies have highlighted the role of inflammatory monocytes in severe COVID-19, and pathologic cytokine production is implicated in driving monocyte expansion [18–20]. Consistent with these reports, we found increased sCD14 and IL-27 levels in patients progressing to severe–critical disease. Increased IL-27 was also associated with greater in-hospital mortality in our analysis. Interestingly, IL-27 plays a crucial regulatory role in limiting inflammatory responses [21]. Therefore, increased IL-27 may represent failure of a negative feedback mechanism to suppress the pathologic inflammation driven by SARS-CoV-2. As a prior study found increased IL-27 in those requiring prolonged hospitalizations in an age-dependent manner [22], pathogenic signaling through IL-27 pathways may represent an intriguing area for future studies [21].

Therefore, we found that early damage responses in the respiratory tract, converging on the IL-6 axis, facilitate the recruitment of inflammatory monocytes, and such early innate events may result in a concomitant endothelial injury. We identified a strong connection between increased levels of D-dimer and markers of endothelial activation such as sICAM-1, sVCAM-1, sTM, and TFPI in both severe disease and mortality. Increases in endothelial markers are found in severe COVID-19 [23–25]. SARS-CoV-2 can infect endothelial cells and causes substantial endotheliitis leading to diffuse microthrombi [26, 27], contributing to our understanding of the relationship between endothelial activation, coagulation, and severe infection. Future studies should evaluate the utility of endothelial activation markers as clinical predictors of severe disease and cardiovascular complications.

The protective effect of metabolic biomarkers, such as the adipo-myokines irisin and leptin, implicates these pathways in modulating inflammation at the systemic or local level [7–10, 28]. In our Southern Italian cohort, with a low prevalence of obesity, the relation of metabolic biomarkers to protection from severe disease may have been revealed due to increased muscle mass or nutritional patterns [29]. This association could be different in obese populations, where irisin production is driven more by adipocytes.

Our study has limitations. It was retrospective, with a variable time interval between symptom onset and sample collection. All logistic regression models adjusted for this important variable, controlling its impact on our findings, as documented also by a sensitivity analysis. Our cohort only included patients with COVID-19, and this unique immunometabolic profile may not be exclusive to those with SARS-Cov-2 infections. Although this study includes a population with a broad range of disease severity, which facilitated the identification of predictors of severe COVID-19 in a specific Mediterranean population, there were only 10 total deaths, which limits the precision of our mortality analysis. This study was conducted early in the pandemic, offering a perspective at the first appearance of SARS-CoV-2 in such a population. This provided valuable data for disease pathogenesis in a naïve host with all samples collected before any immunosuppressive treatments such as steroids or tocilizumab. However, in the current COVID-19 epidemiological environment, it is possible that newer variants and underlying active and passive adaptive immune responses could affect the relative contribution of the pathogenic mechanisms identified herein. Future prospective studies should evaluate the role of this unique immunometabolic signature in individuals who progress to severe–critical disease in the current epidemiological context and vaccine- and/or prior infection-associated SARS-CoV-2 immunity.

Despite these limitations, we provide an articulate analysis of a broad range of soluble biomarkers and their prognostic value for COVID-19 severity. We identified increased levels of myeloid activation markers supporting the role of inflammatory monocytes in COVID-19 hyperinflammation. The association of IL-27 with mortality and the potential protective effects of novel immunometabolic markers in severe disease could aid in identifying new mechanistic pathways. Finally, markers of coagulopathy and endothelial activation were significantly associated with severe disease and death. These markers and their link with immunometabolic characteristics of patients may have clinical utility in risk-stratifying hospitalized patients early in their disease course. The COVID-19 pandemic remains a worldwide challenge, and continued study of its pathogenesis is essential to improve treatment options and decrease adverse outcomes.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Patient consent. This study was approved by local internal review boards and performed in accordance with the Declaration of Helsinki. All blood samples and clinical data were de-identified, and direct patient consent was not required.

References

1. Gustine JN, Jones D. Immunopathology of hyperinflammation in COVID-19. Am J Pathol **2021**; 191:4–17.
2. Wolff D, Nee S, Hickey NS, Marschollek M. Risk factors for COVID-19 severity and fatality: a structured literature review. Infection **2021**; 49:15–28.
3. Szabo PA, Dogra P, Gray JI, et al. Longitudinal profiling of respiratory and systemic immune responses reveals myeloid cell-driven lung inflammation in severe COVID-19. Immunity **2021**; 54:797–814.e6.
4. Abers MS, Delmonte OM, Ricotta EE, et al. An immune-based biomarker signature is associated with mortality in COVID-19 patients. JCI Insight **2021**; 6: e144455.
5. Marik PE, Iglesias J, Varon J, Kory P. A scoping review of the pathophysiology of COVID-19. Int J Immunopathol Pharmacol **2021**; 35:20587384211048026.
6. . Wei PF, ed. Diagnosis and treatment protocol for novel coronavirus pneumonia (trial version 7). Chin Med J **2020**; 133:1087–95.
7. Arhire LI, Mihalache L, Covasa M. Irisin: a hope in understanding and managing obesity and metabolic syndrome. Front Endocrinol **2019**; 10:524.
8. Li Q, Zhang M, Zhao Y, Dong M. Irisin protects against LPS-stressed cardiac damage through inhibiting inflammation, apoptosis, and pyroptosis. Shock **2021**; 56:1009–18.
9. Pinkowska A, Podhorska-Okolow M, Dziegiel P, Nowinska K. The role of irisin in cancer disease. Cells **2021**; 10:1479.
10. La Cava A, Matarese G. The weight of leptin in immunity. Nat Rev Immunol **2004**; 4:371–9.
11. Ziegler CGK, Miao VN, Owings AH, et al. Impaired local intrinsic immunity to SARS-CoV-2 infection in severe COVID-19. Cell **2021**; 184:4713–33.
12. Lucas C, Wong P, Klein J, et al. Longitudinal analyses reveal immunological misfiring in severe COVID-19. Nature **2020**; 584:463–9.
13. Laing AG, Lorenc A, Del Molino Del Barrio I, et al. A dynamic COVID-19 immune signature includes associations with poor prognosis. Nat Med **2020**; 26: 1623–35.
14. Dorgham K, Quentric P, Gokkaya M, et al. Distinct cytokine profiles associated with COVID-19 severity and mortality. J Allergy Clin Immunol **2021**; 147: 2098–107.
15. Ahmad R, Haque M. Surviving the storm: cytokine biosignature in SARS-CoV-2 severity prediction. Vaccines **2022**; 10:614.
16. Brunetta E, Folci M, Bottazzi B, et al. Macrophage expression and prognostic significance of the long pentraxin PTX3 in COVID-19. Nat Immunol **2021**; 22: 19–24.
17. Assandri R, Accordino S, Canetta C, et al. Long pentraxin 3 as a marker of COVID-19 severity: evidences and perspectives. Biochem Med **2022**; 32:020901.
18. Vanderbeke L, Van Mol P, Van Herck Y, et al. Monocyte-driven atypical cytokine storm and aberrant neutrophil activation as key mediators of COVID-19 disease severity. Nat Commun **2021**; 12:4117.
19. Knoll R, Schultze JL, Schulte-Schrepping J. Monocytes and macrophages in COVID-19. Front Immunol **2021**; 12:720109.
20. Bonnet B, Cosme J, Dupuis C, et al. Severe COVID-19 is characterized by the co-occurrence of moderate cytokine inflammation and severe monocyte dysregulation. EBioMedicine **2021**; 73:103622.
21. Yoshida H, Hunter CA. The immunobiology of interleukin-27. Annu Rev Immunol **2015**; 33:417–43.
22. Angioni R, Sanchez-Rodriguez R, Munari F, et al. Age-severity matched cytokine profiling reveals specific signatures in COVID-19 patients. Cell Death Dis **2020**; 11:957.
23. Goshua G, Pine AB, Meizlish ML, et al. Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. Lancet Haematol **2020**; 7:e575–82.
24. Vieceli Dalla Sega F, Fortini F, Spadaro S, et al. Time course of endothelial dysfunction markers and mortality in COVID-19 patients: a pilot study. Clin Transl Med **2021**; 11:e283.
25. Flaumenhaft R, Enjyoji K, Schmaier AA. Vasculopathy in COVID-19. Blood **2022**; 140:222–35.
26. Liu F, Han K, Blair R, et al. SARS-CoV-2 infects endothelial cells in vivo and in vitro. Front Cell Infect Microbiol **2021**; 11:701278.
27. Ma Z, Yang KY, Huang Y, Lui KO. Endothelial contribution to COVID-19: an update on mechanisms and therapeutic implications. J Mol Cell Cardiol **2022**; 164:69–82.
28. Alves HR, Lomba GSB, Goncalves-de-Albuquerque CF, Burth P. Irisin, exercise, and COVID-19. Front Endocrinol **2022**; 13:879066.
29. Anastasilakis AD, Polyzos SA, Saridakis ZG, et al. Circulating irisin in healthy, young individuals: day-night rhythm, effects of food intake and exercise, and associations with gender, physical activity, diet, and body composition. J Clin Endocrinol Metab **2014**; 99:3247–55.