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A urinary proteomic landscape of COVID-19 progression identifies signaling pathways and therapeutic options

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Signaling pathway alterations in COVID-19 of living humans as well as therapeutic targets of the host proteins are not clear. We analyzed 317 urine proteomes, including 86 COVID-19, 55 pneumonia and 176 healthy controls, and identified specific RNA virus detector protein DDX58/RIG-I only in COVID-19 samples. Comparison of the COVID-19 urinary proteomes with controls revealed major pathway alterations in immunity, metabolism and protein localization. Biomarkers that may stratify severe symptoms from moderate ones suggested that macrophage induced inflammation and thrombolysis may play a critical role in worsening the disease. Hyper activation of the TCA cycle is evident and a macrophage enriched enzyme CLYBL is up regulated in COVID-19 patients. As CLYBL converts the immune modulatory TCA cycle metabolite itaconate through the citramalyl-CoA intermediate to acetyl-CoA, an increase in CLYBL may lead to the depletion of itaconate, limiting its anti-inflammatory function. These observations suggest that supplementation of itaconate and inhibition of CLYBL are possible therapeutic options for treating COVID-19, opening an avenue of modulating host defense as a means of combating SARS-CoV-2 viruses.

COVID-19, urine, proteome, CLYBL, itaconate

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INTRODUCTION

The coronavirus disease 2019 (COVID-19) has emerged as a pandemic with massive loss of lives worldwide. As of July 30, 2021, the virus has infected over 196 million people and killed over 4.2 million. Treatment is based mainly on supportive and symptomatic care according to the diagnosis and treatment of pneumonia and coagulopathies caused by COVID-19 (Pascarella et al., 2020). Powerful antiviral neutralizing antibodies and vaccines are developed and used

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in patients with great success to combat the pandemic (Jiang et al., 2020; Lu et al., 2020). Notwithstanding the great progress, effective and affordable therapies for COVID-19, particularly for those who have missed the therapeutic window of antibody treatment are urgently needed (Hu et al., 2021). As emerging of the mutant strains such as the delta variant that may evade the protection of the vaccine against the WT virus, the pandemic continues. More options to combat the disease are urgently needed (Zhang et al., 2020b).

Much has been learned from the omics studies of blood from COVID-19 patients. One striking feature of the disease is the dysfunctional immune response. Critically ill COVID-19 patients showed higher amounts of cytokines and chemokines (Chen et al., 2020; Giamarellos-Bourboulis et al., 2020; Huang et al., 2020; Ong et al., 2020) with an impaired type I interferon (IFN) response (Blanco-Melo et al., 2020; Hadjadj et al., 2020) in the blood plasma. High frequencies of interleukin-6 (IL-6)-secreting $CD14^{hi}CD16^{hi}$ monocytes were often observed with the appearance of proliferating, type-I-IFN-activated $CD14^{+}$ HLA^{lo} suppressive monocytes (Bernardes et al., 2020). T cell lymphopenia and exhaustion were also suggested as hallmarks of severe COVID-19 (Diao et al., 2020; Giamarellos-Bourboulis et al., 2020; Guan et al., 2020; Huang et al., 2020; Zheng et al., 2020). Further analyses revealed that STAT1 and IRF3 signaling pathways were activated in COVID-19 (Zhu et al., 2020). The other important feature of the disease is pulmonary embolism and thrombosis (Deshpande, 2020). Patients exhibited elevated D-dimer levels and widespread thrombotic microvascular injuries (De Voeght et al., 2020; Rapkiewicz et al., 2020). Plasma proteomic data have also identified changes of coagulation and platelet degranulation (Shen et al., 2020; Shu et al., 2020).

At the early stage of the infection, identifying disease progression markers is important to predict which patients may develop into diseases with severe manifestations. Shen et al. (2020) characterized 99 plasma samples, including 46 COVID-19 and 53 healthy controls with proteomics. 29 potential serum proteins and metabolite biomarkers were identified for severity assessment, manifesting dysregulation of macrophage, complement system pathways, platelet degranulation, and metabolic suppression. Another plasma proteomic study identified disorders of the immune system, inflammatory response, platelet degranulation, coagulation, and metabolism. It also identified 11 plasma protein biomarkers that can accurately distinguish or predict outcomes in patients with COVID-19 (Shu et al., 2020).

Urine is a non-invasive sample and over 5,000 proteins have been detected in the urine proteome (Leng et al., 2017; Tian et al., 2020; Zhang et al., 2020a). We previously found that the urinary ACE2 protein level was correlated with the risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (Ni et al., 2020). The influence of sex, age, and disease types on the ACE2 abundance was consistent with the epidemiological data of COVID-19. This finding suggests that urine may serve as a source for biomarker discovery in COVID-19. Indeed, a urinary proteomic study of 37 samples, including 14 COVID-19, 13 non-COVID-19 pneumonia and 10 healthy controls, showed that immunosuppression and tight junction impairment occurred in the early stage of COVID-19, and an activated immune response emerged in severely affected patients (Tian et al., 2020). Not only alterations of the urinary proteome were observed (Chavan et al., 2021), biomarkers indicating adverse COVID-19 clinical outcomes were also generated (Wendt et al., 2021). These demonstrated the feasibility of urinary proteomics as a non-invasive method in investigating COVID-19.

In this study, we present the analyses of urine proteomes from 317 samples, including 176 healthy controls, 55 pneumonia patients, and 86 COVID-19 patients. By correlating the proteomic data with the clinical features and health records, we grouped samples from different clinical courses and categorized them into early, middle and recovery stage of the disease, allowing the identifications of signaling pathways during COVID-19 progression and the possible therapeutic options.

RESULTS

Clinical and proteomics characteristics of the samples

This retrospective analysis included 86 COVID-19 patients (Figure 1A; Table S1 in Supporting Information). 176 healthy individuals and 55 pneumonia patients were included as case controls with matching age and gender (non-significant difference). Baseline characteristics of the study groups are shown in Table S1 in Supporting Information. Urine samples of COVID-19 patients were collected at various disease stages during the hospitalization. The mean±SD age of COVID-19 patients was (49±15.3) years, with 34.1% men and 65.9% women. All COVID-19 patients were hospitalized when the diagnosis of COVID-19 was made. The most common symptoms were fever (75.6%), cough (67.4%), expectoration (40.7%), short of breath (22.1%), fatigue (11.6%), diarrhea (11.6%), and anorexia (9.3%). The median follow-up time was 27 d (interquartile range (IQR): 13–37) from the date of COVID-19 diagnosis. At the end of the study period, 3 patients were transferred to another hospital for treatment and survived, and the others were discharged after full recovery.

We isolated the high-speed sediments from the urine, which were digested in solution, and applied a 30 min MS runs to measure the proteins (Zhang et al., 2018a) (Figure 1B). A total of 4,255 high-confidence proteins (≥1 unique and strict peptides and ≥2 strict peptides (ion score>20), or ≥3 strict peptides) were identified (Table S2 in Supporting

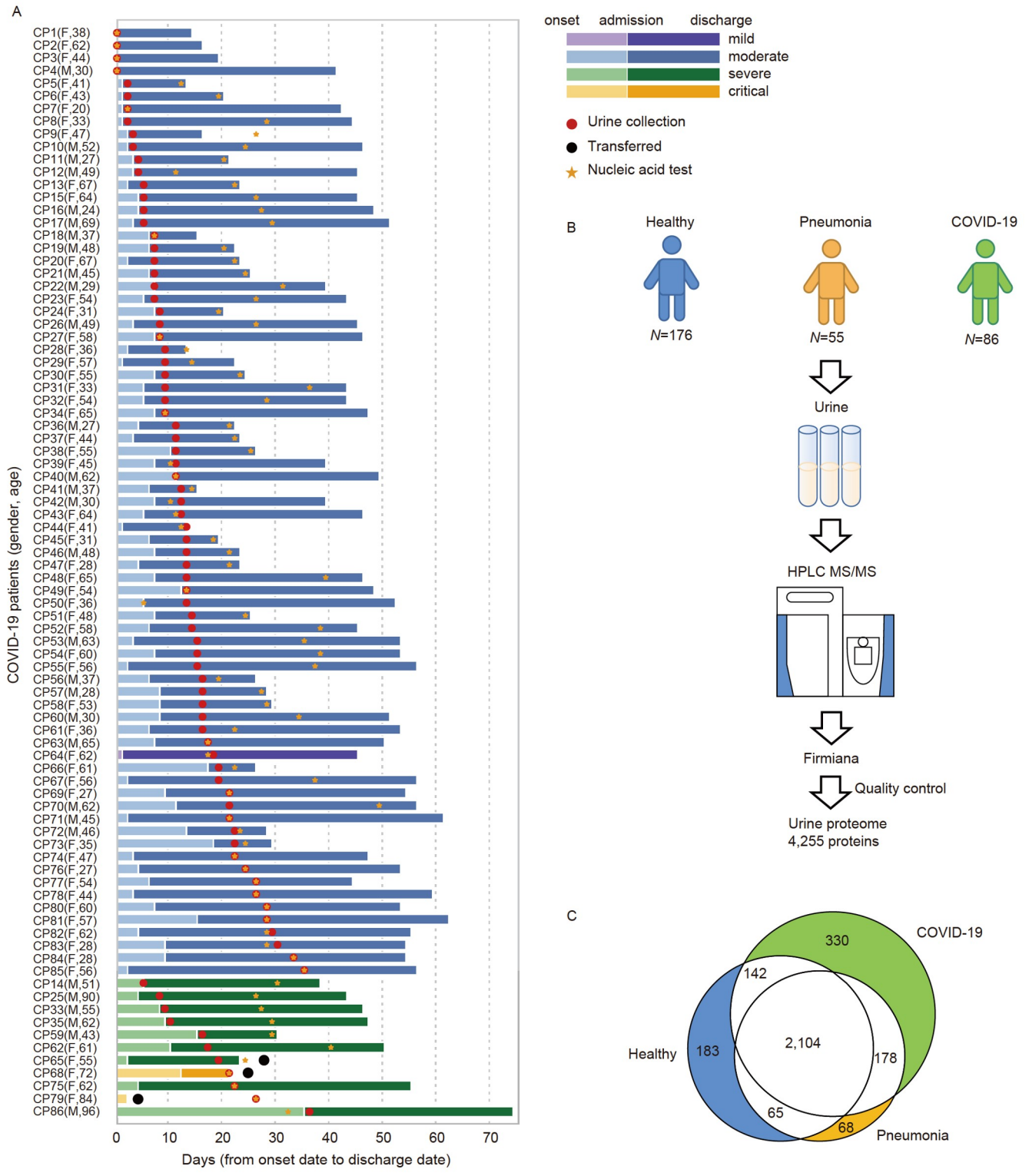

Figure 1 A summary of clinical and proteomic analysis of healthy, pneumonia, COVID-19 samples. A, A summary of the clinical information of the COVID-19 patients in the study, including mild (n=1), moderate (n=74), severe (n=9), and critical (n=2) patients. Gender and age are indicated in the parenthesis. For more details, see Table S1 in Supporting Information. B, A workflow for urine proteome measurement. A total of 4,255 urine proteins from 176 healthy controls, 55 pneumonia patients, and 86 COVID-19 patients were identified. C, A venn diagram showing the number of high confident proteins identified in each group.

Information) with a median of 952 proteins identified in one urine sample. The performance of the MS system was monitored daily by running quality control (QC) 293T lysate, and the reproducibility was confirmed by Pearson correlation

coefficients of the QC samples (min.: 0.84, avg.: 0.91; Figure S1A in Supporting Information). The protein accumulation curve suggested that the number of urine proteins started to saturate, indicating that the sample size met a basic requirement (Figure S1B in Supporting Information).

In the subsequent data analyses, we included proteins detected in at least 2.5% samples in one group of the healthy, pneumonia, or COVID-19 cases. Consequently, 2,494, 2,415, and 2,754 proteins were detected in the healthy, pneumonia, and COVID-19 group, respectively. The Venn diagram (Figure 1C) showed that although the number of COVID-19 patients was ~50% of that of the healthy controls, the COVID-19 proteomes contained the largest number of proteins among the 3 groups, suggesting its diversity.

A urinary proteomic landscape of COVID-19 during disease progression

The disease progression time between symptom appearance and discharge from the hospital is an important parameter for disease evaluation. As the urgency of the pandemic prevented careful clinical experimental design, the urine samples used in this study were collected at the most convenient times for patients and healthcare workers, thus were distributed at seemingly random time points during disease progression. We classified these samples as early stage, middle stage, and recovery stage based on the status between hospital admission and discharge (See METHODS for details).

We identified COVID-19 specific DEPs (differentially expressed proteins) during the three stages in comparison with the pneumonia and healthy controls. As a result, 396, 115, and 156 COVID-19 specific proteins were found at each of the three stages, respectively (Mann-Whitney *U* test *P*-value<0.05), it was increased >2-fold than both healthy or pneumonia controls (Figure S2A and Table S3 in Supporting Information). The COVID-19 DEPs from each stage were combined with those found from the entire period for pathway analysis. Reactome pathway enrichment analysis using the 519 DEPs showed that 3 major categories (Tier 1) of pathways, including immune, metabolism, and protein localization, were significantly enriched (Figure 2A). The enrichment in the immune system pathway can be further assigned to Tier 2 pathways of cytokine signaling and adaptive immune systems, which were further detailed to the Tier 3 pathways of interferon signaling, class I MHC mediated antigen processing & presentation and the immunoregulatory interactions between a lymphoid and a non-lymphoid cell. Similarly, the citric acid (TCA) cycle and respiratory electron transport were the Tier 3 metabolism pathways that were specifically enriched (Figure 2A; Table S3 in Supporting Information).

To examine pathway activation in a more specific and semi-quantitative manner, we defined a pathway index based on the statistical representation of the DEPs in the aforementioned Tier 3 pathways at each stage (See METHODS for details). As shown in Figure 2B, the immune system and metabolism pathways showed the highest degree of activation in the early stage, but protein localization did not show enrichment in Tier 3 pathways at any specific stages, due to the insufficient number of proteins identified.

To investigate the detailed pathways in COVID-19 progression, we selected the most up-regulated proteins (fold change>4) in the above pathways plus ACE2 to illustrate a urinary proteomic landscape of COVID-19 progression. The results were summarized in the bubble plot (Figure 2C; Figure S2B and Table S3 in Supporting Information). Protein expression patterns were depicted for healthy control, pneumonia case control, COVID-19, moderate case, severe case, and patients with viral nuclear acid detected or not-detected.

Five pathways can be summarized: SARS-CoV2 infection, including proteins for viral Spike protein processing and maturation, interferon signaling, TCA and respiratory electron transport, fatty acid metabolism, and class I MHC mediated antigen processing and presentation.

Higher expression of ACE2 in early stage and severe COVID-19 patients

ACE2 is the COVID-19 virus receptor (Hoffmann et al., 2020; Zhu et al., 2021). We found that ACE2 in urine was higher in patients with COVID-19 than in patients with other diseases (Figure 3A; Figure S3A in Supporting Information). Our previous study examined the correlation between ACE2 levels in our urine proteome database containing 7,191 subjects, including healthy controls and 22 types of diseases, and the epidemiology has led to the hypothesis that urinary ACE2 level may serve as a biomarker for the risk of SARS-CoV-2 infection (Ni et al., 2020). Our data showed that the ACE2 level was higher in severe patients and the early stage (Figure 3B). The fold change from the moderate and severe COVID-19 group to healthy control was 2.0 and 3.7, respectively (Mann-Whitney *U* test *P*-value: 0.013 and 0.247, respectively, Figure 3B). The fold change from the early, middle, and recovery stage in COVID-19 patients to healthy control was 2.46, 2.09, and 1.87, respectively (Mann-Whitney *U* test *P*-value: 0.001, 0.134, and 0.881, respectively, Figure 3B).

Activation of the DDX58/RIG-I and STAT pathway

Several proteins involved in the anti-virus response were detected in urine. For example, the viral dsRNA sensor DDX58/RIG-I (retinoic-acid inducible gene I) was up-regulated, particularly in the early time. In contrast, the viral DNA sensor cGAS was not detected, consistent with the fact

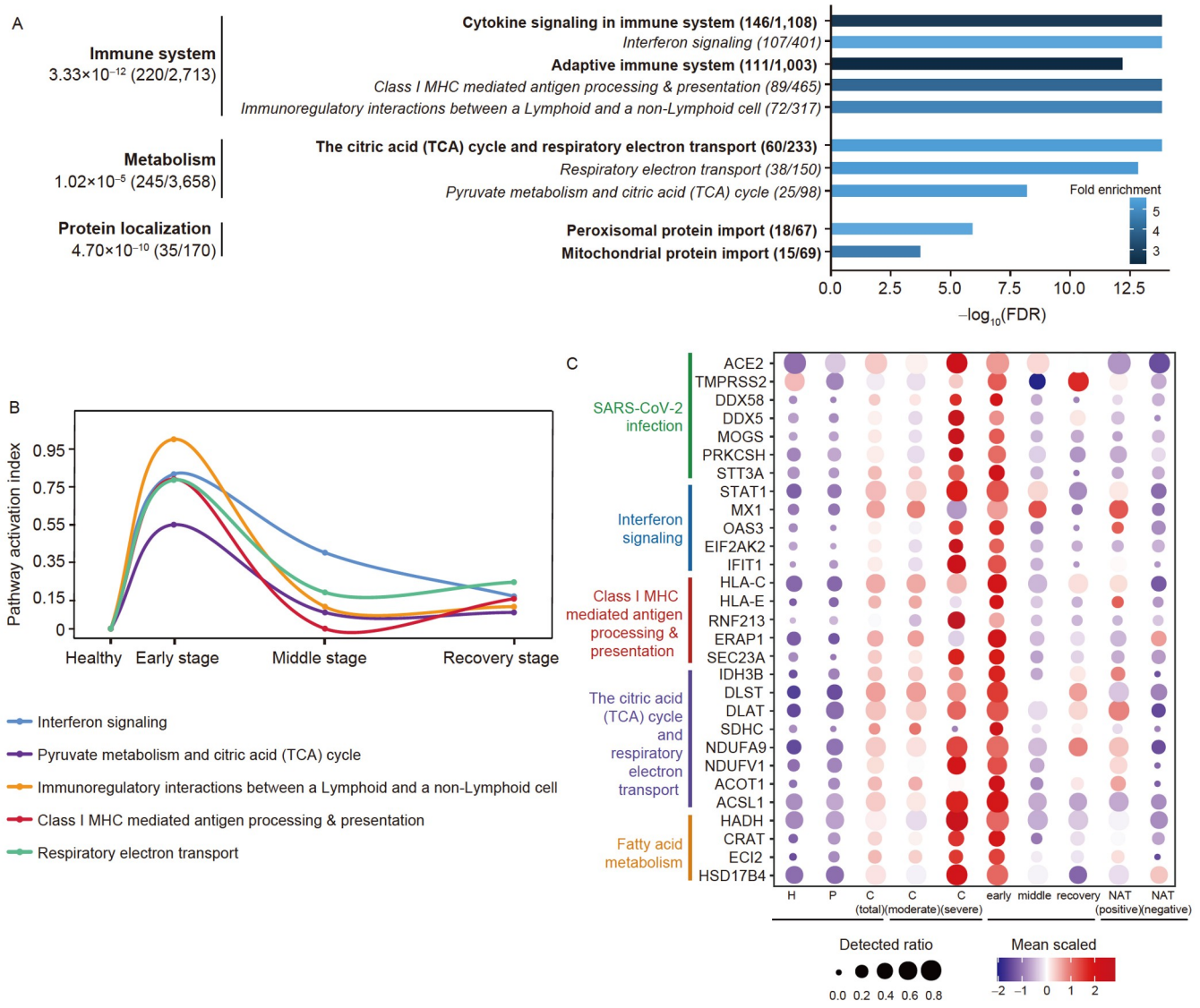

Figure 2 A urinary proteomic landscape and pathway activation during COVID-19 progression/recovery. A, Reactome pathway enrichment analysis using the 519 DEPs. Three significantly enriched major categories of pathways (Tier 1), including immunity, metabolism and protein localization, and the more specific categories in the pathway hierarchy were shown. The color represents fold enrichment. B, Pathway activation indices at each disease progression/recovery stages calculated by the DEPs in the Tier 3 pathways according to Reactome annotations. C, Bubble plot showing representative urinary proteins changed during COVID-19 progression in the 5 pathways. H: healthy; P: pneumonia; C(total): total COVID-19; C(moderate): moderate COVID-19; C (severe): severe COVID-19; early: early stage COVID-19; middle: middle stage COVID-19; recovery: recovery stage COVID-19; NAT(postive): nucleic acid test was positive; NAT(negative): nucleic acid test was negative.

that SARS-CoV-2 is an RNA virus. The detection of STAT1 as one of the most up-regulated proteins suggested that activation of the JAK/STAT pathway was in agreement with the specificity of STAT activation by IFN (Figure 3C). Consequently, IFN-stimulated genes (ISGs), including ISG15, MX1, MX2, IFIT1, IFIT3, IFITM1, OAS2, OAS3, and EIF2AK2, were among the most up-regulated compared with the healthy controls, with a fold-change ranging from 7 to 55. It is worth mentioning that 8 up-regulated proteins, including ISG15, MX1, IFIT1, IFIT3, IFITM1, OAS2, OAS3, and EIF2AK2, were in the interferon alpha/beta signaling pathway, with a high elevation in intensity (Fold change>16, or Mann-Whitney *U* test *P*-value<1×10^{-5}, Table S3 in Supporting Information).

Candidate biomarkers to stratify high-risk patients

The proteomic landscape of COVID-19 progression offered opportunities to ask additional questions about proteins or pathways that may determine the transition from moderate to severe disease. To identify putative biomarkers to stratify high-risk patients, we identified proteins whose abundance was increased by >10-times and detected in >50% of the severe patients at the early stage. This yielded CD14, RBP4,

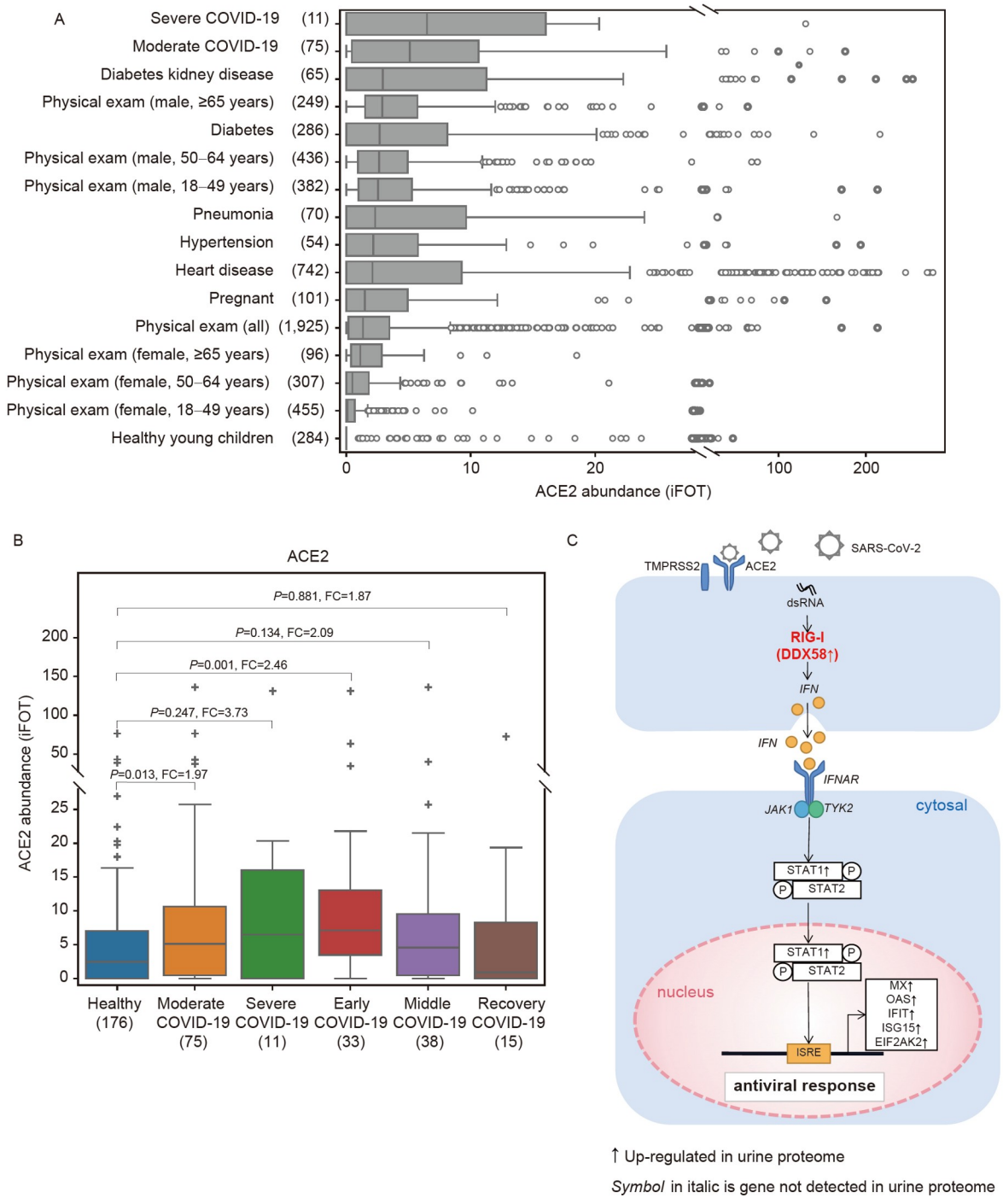

Figure 3 Urinary ACE2 levels and the pathway ACE2 activates during SARS-CoV-2 infection. A, ACE2 abundance in patients with COVID-19, pneumonia, or other underlying health problems and in healthy controls. Boxplot depicts the protein abundance distribution in each group (center line: median; bounds of box: 25th and 75th percentiles; and whiskers: from Q1−1.5*IQR to Q3+1.5*IQR). All data except COVID-19 patients were obtained from (Ni et al., 2020). B, ACE2 abundance across healthy, moderate COVID-19, severe COVID-19, and early, middle, and recovery stage COVID-19. C, The viral response pathway activated by SARS-CoV-2 infection. Using ACE2 as an entry receptor and TMPRSS2 as an activator, SARS-CoV-2 induces double-stranded RNA-mediated innate immune responses. dsRNA sensing by DDX58 can lead to IFN production. Its release triggers the activation of JAK/STAT pathway to induce ISGs, including ISG15, MX1, MX2, IFIT1, IFIT3, IFITM1, OAS2, OAS3, and EIF2AK2.

SPON2, GMFG, SERPINA1, SERPINB6, SERPINC1, TTR, RAB3A, and SAA2 (Figure 4; Figure S3B and Table S4 in Supporting Information).

CD14 is a surface antigen preferentially expressed on monocytes/macrophages. Along with other proteins, it mediates innate immune responses to viral and bacterial infections. CD14 has been identified as a potential therapeutic target in patients with COVID-19, with the potential to

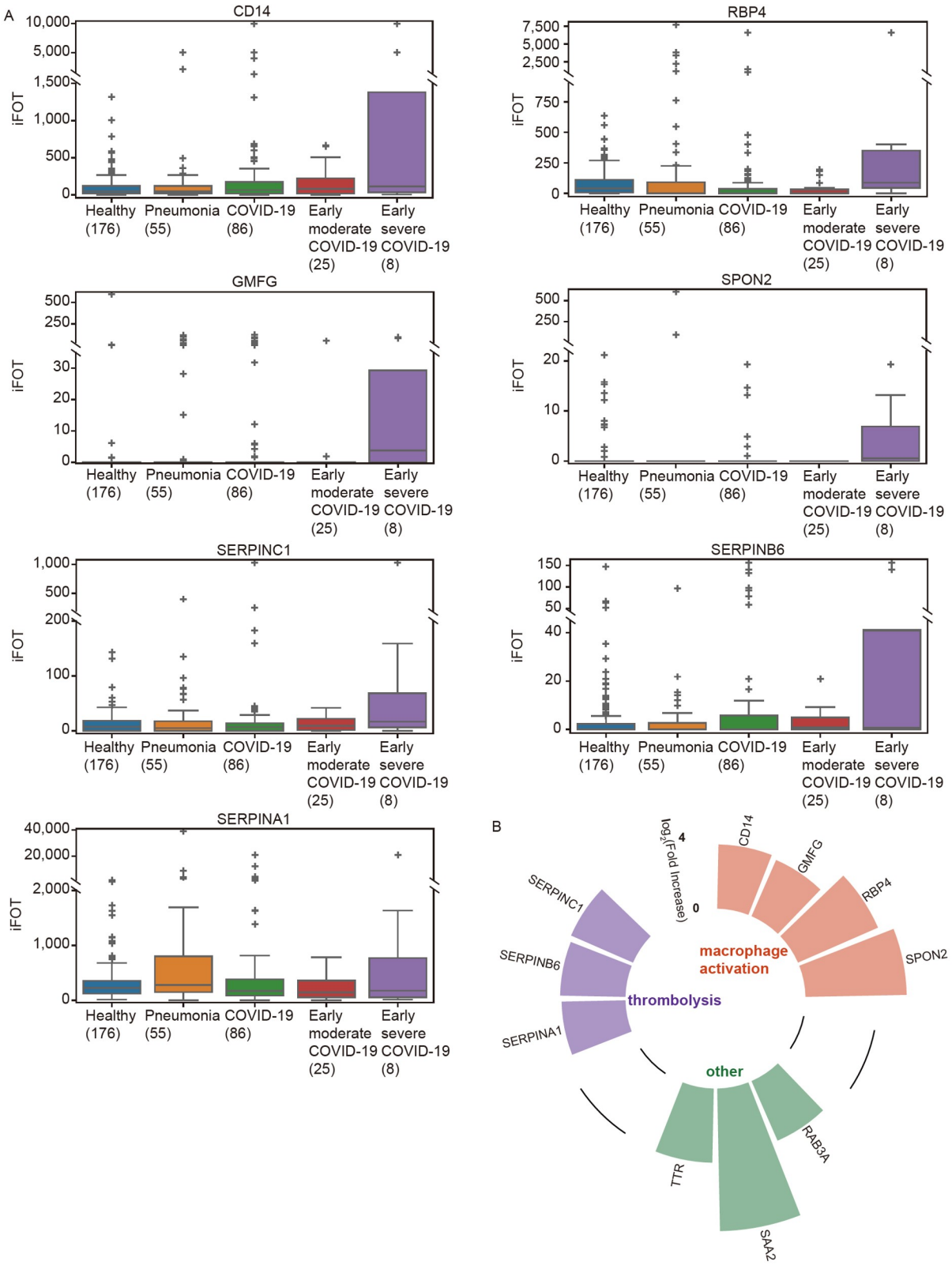

Figure 4 Candidate biomarkers to stratify high-risk patients. A, Boxplot displaying the abundance of proteins that are associated with macrophage activation and thrombolysis across 5 groups (Healthy: n=176; pneumonia: n=55; COVID-19: n=86; early moderate COVID-19: n=25; early severe COVID-19: n=8). Boxplot center line: median; bounds of box: 25th and 75th percentiles; and whiskers: from Q1−1.5*IQR to Q3+1.5*IQR. B, Circular histogram displaying increased levels of proteins involved in macrophage activation pathway and thrombolysis for patients with severe COVID-19 compared to those with moderate COVID-19 during early stage of the disease progression.

lessen or suppress severe inflammatory responses (Martin et al., 2020). RBP4 elevation induces pro-inflammatory cytokine secretion from macrophages. RBP4 primes the NLRP3 inflammasome by signaling through Toll-like receptors 2 and 4 (Moraes-Vieira et al., 2020). Matricellular protein SPON2 is essential for recruiting lymphocytes and initiating immune responses and promotes M1-like Macrophage Recruitment (Zhang et al., 2018b). GMFG also has functions in macrophage recruitment (Aerbajinai et al., 2013). SERPINA1, SERPINC1, and SERPINB6 are serine protease inhibitors belonging to the serpin superfamily, whose target includes thrombin (Coughlin et al., 1993; Rau et al., 2007; Strik et al., 2004). SERPINC1, in particular, also inhibits other activated serine proteases of the coagulation system, regulates the blood coagulation cascade. A reduction in the serum level of this protein is known to be associated with severe cases of COVID-19 (Mir et al., 2021).

These biomarkers for the stratification of severe COVID-19 patients all pointed to macrophage activation and thrombolysis (Figure 4B), suggesting that macrophage-induced inflammation and thrombolysis may play a critical role in worsening the COVID-19 disease.

Potential drug target CLYBL and immune modulatory metabolite itaconate for COVID-19 treatment

Since databases such as Reactome rely on the published studies to calculate pathway enrichment, under studied proteins can be underrepresented in these analyses. CLYBL is such an example. CLYBL encodes an expressed mitochondrial protein, and its function was unknown until 2017, when it was reported as a citramalyl-CoA lyase in mammalian cells (Shen et al., 2017). CLYBL is an intermediate of C5-dicarboxylate metabolic pathway that includes the transition from itaconate to acetyl-CoA. It can convert citramalyl-CoA feeding into the TCA cycle (Figure 5B; Figure S3C in Supporting Information). As shown in Figure 5A, CLYBL was specifically up-regulated in COVID-19 patients (Figure 5A), especially in those with severe manifestations.

Itaconate is an immunomodulatory and anti-microbial metabolite derived from *cis*-aconitate, a TCA cycle intermediate, in activated macrophages (Jha et al., 2015; Michelucci et al., 2013; Strelko et al., 2011). Itaconate is first converted to itaconyl-CoA, then to citramalyl-CoA, pyruvate and acetyl-CoA (Shen et al., 2017). As CLYBL functions as a citramalyl-CoA lyase, its accumulation would lead to a loss of upstream metabolites, thus result in the down-regulation of itaconate. A recent study in a cohort of 76 subjects confirmed the down-regulation of itaconate in the plasma of COVID-19 patients (Song et al., 2020).

Itaconate was shown to play an important role in anti-oxidation, cellular protection, and anti-inflammation (Hooftman et al., 2020; Li et al., 2020; Mills et al., 2018) (Figure 5B). It was thought to inhibit succinate dehydrogenase (SDH) and reduce reactive oxygen species (ROS) generation (Li et al., 2020). It may also activate NRF2/NFE2L2 via alkylation and inactivation of KEAP1as a cellular protective mechanism (Figarska et al., 2014; Mills et al., 2018). In addition, itaconate may inhibit the activation of inflammasome via alkylation of NLRP3 (Hooftman et al., 2020), which leads to the down regulation of caspase-1 (CASP1)-mediated cleavage of IL-1β, IL-18, and gasdermin D to avoid excessive inflammation. Consistent with these knowledges, CASP1 and IL18 were up regulated in our dataset. Together, up regulation of CLYBL may serve as a key factor causing the consumption of anti-inflammatory metabolite itaconate. These observations led us to speculate that supplement of itaconate might be a means for treating COVID-19 patients.

Itaconate modulates the expression of proteins that provide antiviral and protective functions in macrophage RAW264.7 cells

To investigate whether itaconate modulates the expression of proteins that may provide antiviral and immune functions in macrophages, we treated RAW264.7 cells with itaconate for 8 and 16 h, and measured their dynamic proteome changes. Immune and metabolism pathways were found to be the most significantly up-regulated (Figure 6A and B; Table S5 in Supporting Information). Specifically, among significantly up-regulated proteins, they function in inhibiting virus entry into cells, regulating immunity, and providing cellular protections (Figure 6C).

For example, CD74 has duo functions in combatting virus. CD74 is a major histocompatibility complex class II (MHC-II) invariant chain and has an important function in antigen presentation. Recently a systematic CRISPR screen for inhibition of SAR-CoV-2 replication in human cells identified CD74 as a gene. Functionally, up-regulation of CD74 blocked cathepsin-mediated cleavage of viral glycoproteins (GPs), thereby preventing viral fusion (Bruchez et al., 2020). Similarly, cystatin 3 (Cst3) was also reported to inhibit SARS-CoV-2 entry into cells. Cst3 is ubiquitously expressed and acts as a potent inhibitor of cathepsins B, H, L, and S (Jakoš et al., 2019). Both Cathepsin B and Cathepsin L participated in the cleavage of the spike protein from SARS-CoV-2 upon its entry to the human host cell (Zhao et al., 2021). Clinically, CD74 was also found at lower levels in severe COVID-19 patients compared with healthy controls and moderate COVID-19 patients (Kvedaraite et al., 2021).

Proteins up-regulated by Itaconate, including Ifitm2, Ifitm3, and Nos2 restrict infections of many viruses. SARS-CoV-2 Spike-pseudo type virus and genuine SARS-CoV-2 infections are generally restricted by human and mouse IFITM2, and IFITM3 (Shi et al., 2021). Nitric Oxide

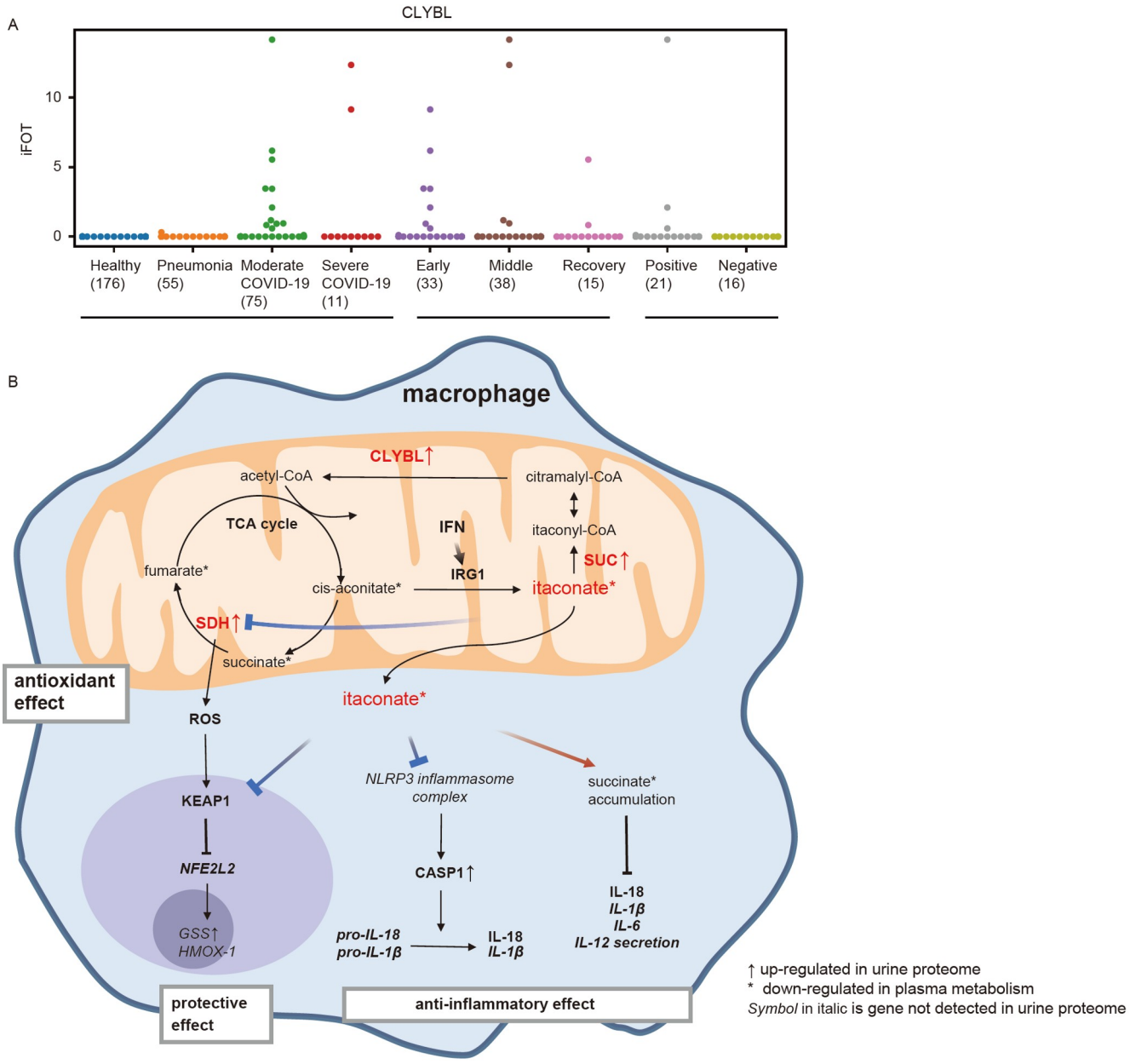

Figure 5 A potential COVID-19 drug target CLYBL and its mechanism of action. A, Dot plot displaying the abundance of CLYBL across patient groups, disease stages, and status of nuclear acid test results. B, Increase in CLYBL could lead to the loss of upstream metabolites, thus resulting in the reduction of itaconate. Itaconate can inhibit SDH, activate NFE2L2 via alkylation of KEAP1, and inhibit caspase-1-mediated cleavage of IL-1β, IL-18 to avoid excessive inflammation via blockage of NLRP3 inflammasome activation.

Synthase 2 (Nos2) produces nitric oxide (NO). During viral infection, intracellular NO production increases, aiding in the control of infections (Lisi et al., 2021). Along the line of antigen presentation, Tapbp and Psmb3 played roles in antigen presentation of MHC class I. Tapasin is involved in the stable assembly of major histocompatibility complex class I (MHC-I) molecules with peptide (Li et al., 2000; van Hateren and Elliott, 2021). Psmb3 is a component of the immune-proteasome that functions in processing of class I MHC peptides (Yano et al., 2004).

Hmox1, Sqstm1, and Ftl1 can provide antioxidant and anti-inflammatory protection. HMOX1 can protect against oxidative damages, regulate apoptosis and inflammation, and promote angiogenesis (Loboda et al., 2016). SQSTM1/p62 can sense the saturation of ROS buffer systems, and maintain redox-sensitivity to increase autophagy, therefore it ensures the survival of cells under oxidative stress (Carroll et al., 2018). In RAW264.7 cells, the overexpression of FTL inhibits lPS-induced MAPKs and nuclear factor-κB (NF-κB) activation. FTL plays an anti-inflammatory role in LPS-stimulated murine macrophages and has therapeutic potential in inflammatory diseases (Fan et al., 2014).

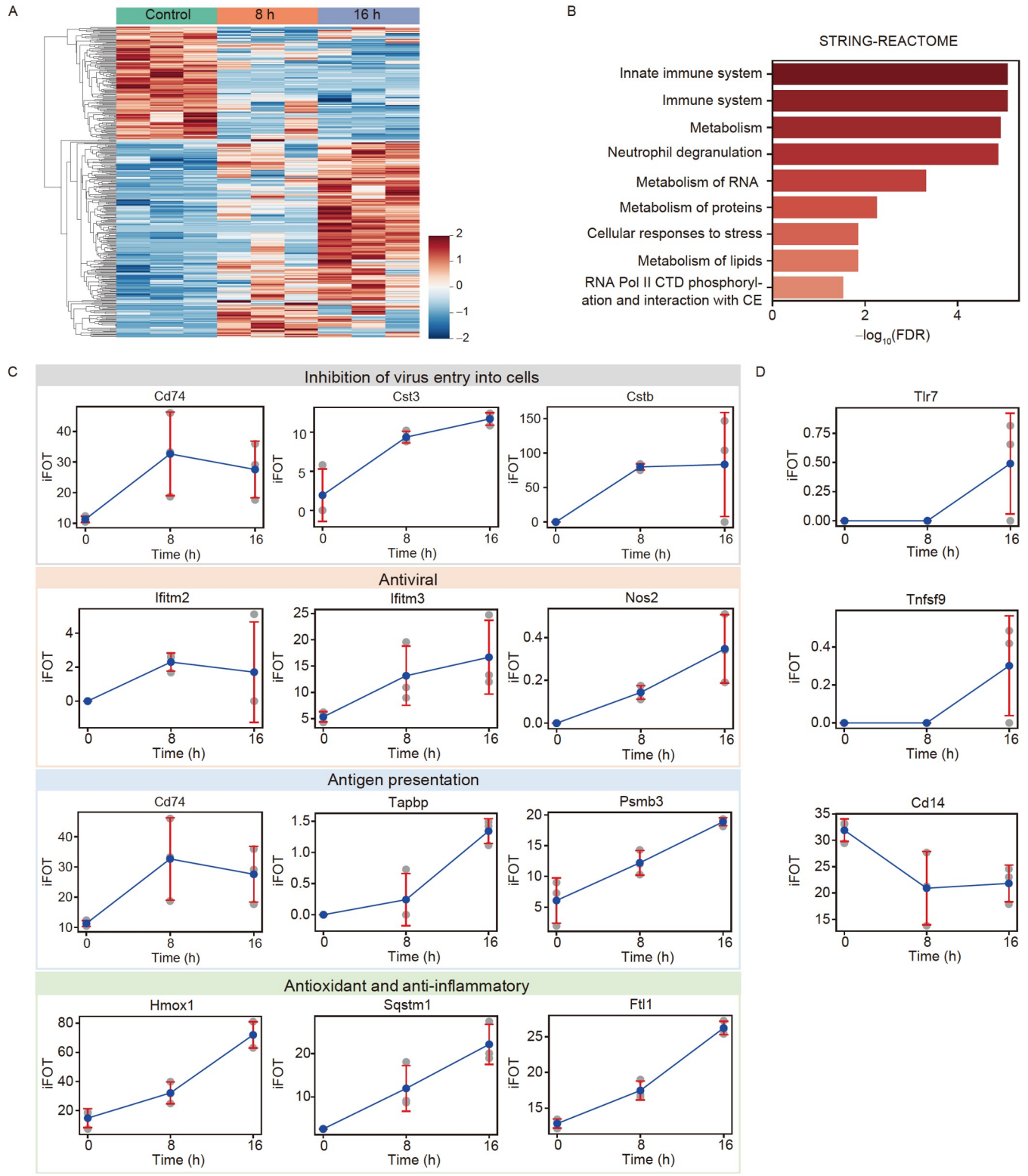

Figure 6 Itaconate modulates the expression of proteins that provide antiviral and protective functions in macrophage RAW264.7 cells. A, Heatmap showing the expression of 240 DEPs after the RAW264.7 cells were treated with itaconate for 8 and 16 h. B, STRING Reactome pathway enrichment analysis using the 240 DEPs. C, Itaconate can inhibit the entry of viruses into cells, and has antiviral, antioxidant, anti-inflammatory functions. Data are shown as mean±SD. D, Tlr7, Tnfsf9, and Cd14 play important roles in immunity. Data are shown as mean±SD.

Three other proteins that play important roles in immunity included Tlr7, Tnfsf9/4-1BBL, and Cd14 (Figure 6D). A human genetic study has identified young male patients of severe COVID-19 with a rare loss-of-function variant of

TLR7 (van der Made et al., 2020), supporting the idea that TLR7 is a positive factor for suppressing COVID-19. TNFSF9 is a cytokine, which is the ligand of a costimulatory receptor molecule TNFRSF9 in T lymphocytes. It is involved in cytotoxic T cell generation and antigen presentation. TNFSF9 can also promote T lymphocyte proliferation and reactivate anergic T lymphocyte (Li et al., 2008). More importantly, CD14, a candidate biomarker for severe COVID-19 that we previously identified, was down-regulated in cells stimulated by itaconate.

In summary, proteins up-regulated by itaconate play roles in anti-virus entry into cells, anti-inflammatory and cell-protections. Thus, supplement of itaconate may be a potential therapeutic option for COVID-19 patients.

DISCUSSION

We presented urine proteomic data and bioinformatics analysis from a cohort of 86 COVID-19 patients during different stages of the disease, revealing a urinary proteomic landscape of COVID-19 disease progression. This proteomic landscape allowed us to deduce significantly changed signaling pathways, including host viral response, STAT1 activation, and TCA cycles. We also identified potential drug targets and the immune-modulating metabolite itaconate as a potential therapy for treating the disease.

Although the origins of the proteins detected from urine are not clear, the detection of proteins involved in the host-viral response pathway, particularly DDX58/RIG-I, the specific RNA virus detector, is reassuring that the urinary protein may reflect what is happening inside the human body and reveal disease progressions. Since the samples come from high-speed urine sediment, which contains substantial proportion of macrovesicles and exosomes, the proteins detected in the urine may come from the exosomal pathways in disposing the dead cells from various tissues and organs. In this context, like serum and blood, urine could provide materials to inquire overall health and disease status in an indirect and non-invasive way, and complement serum and blood analyses with deeper protein coverage and more in-depth biological insights.

Our data pointed to monocytes/macrophage activation as one mechanism for COVID-19 disease. A number of up-regulated proteins between the severe and moderate cases, including CD14, RBP4, SPON2, and GMFG, all have important functions in activated macrophages (Aerbajinai et al., 2013; Martin et al., 2020; Yang et al., 2005; Zhang et al., 2018b). These proteins are also in a high concentration in the pneumonia case controls, indicating that lung inflammation is an import early sign of severe cases. Furthermore, the detection of CLYBL, an enzyme enriched in the macrophage and degrades the immune modulatory metabolite itaconate in the early stage of the disease progression, provides additional support for this hypothesis. Importantly, a metabolomic study showed that itaconate was indeed significantly lower in the serum of COVID-19 patients (Song et al., 2020). As an endogenous metabolite, itaconate modulates immune function mainly by playing a role in anti-inflammation. Lack of itaconate together with the increase in other inflammation inducing cytokines may lead to cytokine release, hyper inflammation, and eventually morbidity. Proteome changes in the treatment of RAW264.7 cells with itaconate suggested that itaconate may function in inhibiting SARS-CoV-2 entry into cells, regulating immunity and metabolism, and providing cellular protections. We suggest that itaconate can be used as a supplement for potential COVID-19 therapy. Itaconate and its cell membrane permeable derivatives are safe, and have shown efficacy in treating several mouse models of sepsis or auto-immune diseases (Bambouskova et al., 2018; Zhang et al., 2021). Itaconate was also validated to be a key negative regulator of monocyte function in a model of *in vivo* human endotoxemia (Domínguez-Andrés et al., 2019). Itaconate and its derivatives are inexpensive, readily available as an anti-inflammation reagent.

Similarly, CLYBL inhibitors may also be candidates for COVID-19 treatment. Nearly 3% of the human population carries loss-of-function variants in CLYBL gene. These individuals are largely healthy despite having lower levels of circulating vitamin B_{12} (Reid et al., 2017). It will be informative to determine the CLYBL genetic status in the COVID-19 morbidity group. If the death rate is significantly lower in the CLYBL knockout population, it would lend more support to our hypothesis and further justification in developing of CLYBL inhibitors to boost the itaconate level in combating COVID-19.

One limitation of our study was the small sample size and heterogeneity in disease stages. Because of the COVID-19 outbreak, our samples were real world samples and were not well designed clinical cohort. We thus assigned patients into the early, middle and recovery stages during the disease progression with a normalized scale, which simplified the progression and clinical manifestation of this complex disease. The other limitation was that this was a single center study with all patients including those severe cases were alive. This may limit the generality of our findings and conclusions. A multi-center study would be more accurate and informative.

MATERIALS AND METHODS

Urine samples

Urine samples from 86 COVID-19 patients who visited Guangzhou Eighth People's Hospital from January 26, 2020 to February 15, 2020 were collected. They were diagnosed as

COVID-19 according to the Chinese Government Diagnosis and Treatment Guideline (NHCPRC, 2020). Nucleic acid extracted from sputum or throat swab was used for real-time fluorescent RT-PCR. COVID-19 patients were classified into four subgroups: (i) Mild: mild symptoms without pneumonia; (ii) Moderate: fever or respiratory tract symptoms with pneumonia; (iii) Severe: respiratory distress, and/or low oxygen saturation, and/or $PaO_2/FiO_2 \leq 300$ mmHg (1 mmHg =0.133 kPa); (iv) Critical: respiratory failure, and/or septic shock, and/or admission to ICU with multiple organ dysfunction. Among the 86 patients, 1 (1.2%) had mild manifestations, 74 (86.0%) had moderate manifestations, 9 (10.5%) had severe manifestations, and 2 (2.3%) had critical manifestations. Because few patients were in the mild and critical categories, they were combined into moderate (n=75) and severe (n=11), respectively.

176 healthy individuals and 55 pneumonia patients from Tianjin Baodi Hospital and The Second Affiliated Hospital of Guangzhou University of Chinese Medicine were included as case controls with matching age and gender (non-significant difference).

Urine sample preparation for MS analysis

To ensure biosafety, urine samples were heated at 56°C for 30 min to inactivate the virus and stored at −80°C. Sample preparation was conducted in a Bio-safety level II lab. One milliliter of urine sample was centrifuged at 176,000×g for 1 h and the pellet was collected. The pellet was resuspended with 40 μL of resuspension buffer (50 mmol L^{-1} Tris, 250 mmol L^{-1} sucrose, pH 8.5) with 50 mmol L^{-1} dithiotheitol (DTT). The suspension was then heated at 65°C for 30 min. Then 160 μL wash buffer (100 mmol L^{-1} NaCl, pH 7.4) was added and a second ultracentrifugation was carried out at 176,000×g for 30 min. The pellet was resuspended with 30 μL NH_4HCO_3, heated at 95°C for 3 min and cooled to room temperature, then digested by trypsin for 12 h. The digested peptides were vacuum dried and re-dissolved in 0.1% formic acid and resolved on an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific, USA) operating on a 30-min linear gradient (5%–35% acetonitrile in 0.1% formic acid) at a flow rate of 800 nL min^{-1}.

Cell culture and treatment

The RAW264.7 cells were grown in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO_2. The RAW264.7 cells were stimulated with 10 mmol L^{-1} itaconate for 8 and 16 h.

Cell sample preparation for MS analysis

The cell precipitates were collected by washing with pre-cooled PBS for 3 times. They were lysed in 1% sodium deoxycholate, 10 mmol L^{-1} Tris(2-carboxyethyl) phosphine, 40 mmol L^{-1} 2-chloroacetamide and 100 mmol L^{-1} Tris-HCl at pH 8.5, 95°C for 5 min, then sonicated for 5 min (3 s on and 3 s off, amplitude 25%). After 16,000×g centrifugation at 4°C for 10 min, the supernatant was retained, 100 μg protein was taken and digested overnight with 1:50 trypsin (Promega, USA). After that, the final 1% formic acid was added to stop digestion, and the precipitated sodium deoxycholate was removed by centrifugation at 16,000×g, 4°C for 10 min. The peptides in the supernatant were desalted at C18 StageTips. The desalted peptides were vacuum-dried and stored at −80°C until subsequent LC-MS/MS analysis.

LC-MS/MS analysis

Peptide samples were redissolved (0.1% FA-H_2O), dried in a vacuum concentrator, and then analyzed by LC-MS/MS. Peptide samples were loaded onto a trap column (100 μm× 2 cm, homemade; particle size, 3 μm; pore size, 120 Å; SunChrom, USA), separated by a homemade silica microcolumn (150 μm×10 cm, particle size, 1.9 μm; pore size, 120 Å; SunChrom) with a gradient of 5%–35% mobile phase B (20% H_2O/80% ACN, 0.08% FA) at a flow rate of 800 nL min^{-1} for 30 min. LC-MS/MS was performed on an Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). The instrument was operated in the data-dependent acquisition mode. The full scan was processed in the Orbitrap from m/z 300–1,400 at a resolution of 60,000, the automatic gain control (AGC) target was $3e^6$ and maximum injection time was 20 ms. The top 40 most intense ions in each scan cycle were selected for HCD fragmentation with normalized collision energy of 27%. For MS/MS scan, the fragment ions were detected in the Orbitrap with a resolution of 7,500, the AGC target was $5e^4$ and maximum injection time was 12 ms, and dynamic exclusion was 15 s. Quality control samples were prepared from trypsin digests of 293T cells and routinely analyzed to assess the LC-MS/MS sensitivity and reproducibility.

Protein identification and label-free quantification

The mass spectrometry data were processed on the Firmiana platform (Feng et al., 2017). Protein identification was performed using the MASCOT search engine (Matrix Science, version 2.3.01) in the NCBI human RefSeq protein database (released on 04/07/2013, 32,015 entries). The mass tolerance of precursor ion was set as 20 ppm, and the mass tolerance of product ion was set to 0.05 Da. Trypsin digestion may miss at most one cleavage. Methionine oxidation and N-terminal acetylation were considered as dynamic modifications. Only ≥1 unique and strict peptides and ≥2 strict peptides (ion score>20), or ≥3 strict peptides, with protein levels

equivalent to 1% FDR, were used for subsequent analyses. Intensity based absolute quantification (iBAQ) algorithm was used for protein quantification (Schwanhäusser et al., 2011). To normalize the differences in sample amounts, we converted the iBAQ to iFOT (fraction of total) (Leng et al., 2017), which was the iBAQ value of each protein divided by the total iBAQ of the sample, multiplied by 10^5. All missing values were replaced with zeros.

Bioinformatics and statistical analysis

Pathway enrichment analysis was performed using Reactome (Fabregat et al., 2017) (https://reactome.org/). The correlation analysis of quality control was calculated by scipy (version 1.4.1) package with python. In the urine proteome, Protein differential expression was assessed with the Mann-Whitney *U* test. The up-regulated DEPs were defined as follows: COVID-19 specific: *P*-value<0.05 and up-regulated >2 times; most up-regulated proteins: compared with healthy controls, *P*-value<0.05, up-regulated >4 times in early stage but not up-regulated >4 times in recovery stage. In experiments with RAW264.7 cells, Protein differential expression was assessed with Student's *t*-test. The up-regulated DEPs were defined as follows: *P*-value<0.05 and up-regulated >2 times. Statistical analyses were calculated by scipy (version 1.4.1) package with python.

To facilitate quantitative correlation between clinical and proteomic data, we devised an algorithm to normalize the disease progression and recovery from 0 to 1 (0 as the time of symptom appearance and 1 as the time of hospital discharge). The scale was then divided into three stages: early stage (from 0 to 0.25), middle stage (from 0.25 to 0.55), and recovery stage (from 0.55 to 1). Three patients that were transferred without recovery were classified as early stage. Two patients whose samples were collected at progression/recovery scale>0.55 but had positive nucleic acid test were classified as middle stage. One patient whose sample was collected at scale 0.48 (on the 2nd day of hospital admission) and had a prolonged hospital stay (75 d) was classified as early stage.

To examine pathway activation in a more quantitative manner, we defined an index based on the number of DEPs identified in the Tier 3 pathway and their *P* values. We defined a pathway activation index $S_{pi}(i, j)$, which is

$$S_{pi}(i,j) = -\log_{10}(\text{Pvalue}_i) * \frac{n_{i,j}}{\sum n_i},$$

where Pvalue_i is the *P*-value of the pathway, $n_{i,j}$ is the number of matched proteins within the pathway *i* in stage *j*, and $\sum n_i$ is the sum of the number of matched proteins within the pathway *i* in the early, middle and recovery stages.

Compliance and ethics *J.Q. is a cofounder and co-owner of the Beijing Pineal Health Management Co., Ltd. X.N., H.W., and X.Y. are employees of Beijing Pineal Health Management Co., Ltd. All other authors declare that they have no conflict of interest. All work performed in this study was approved by The Second Affiliated Hospital of Guangzhou University of Chinese Medicine Ethics Committee and written informed consents were obtained from patients.*

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SUPPORTING INFORMATION

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