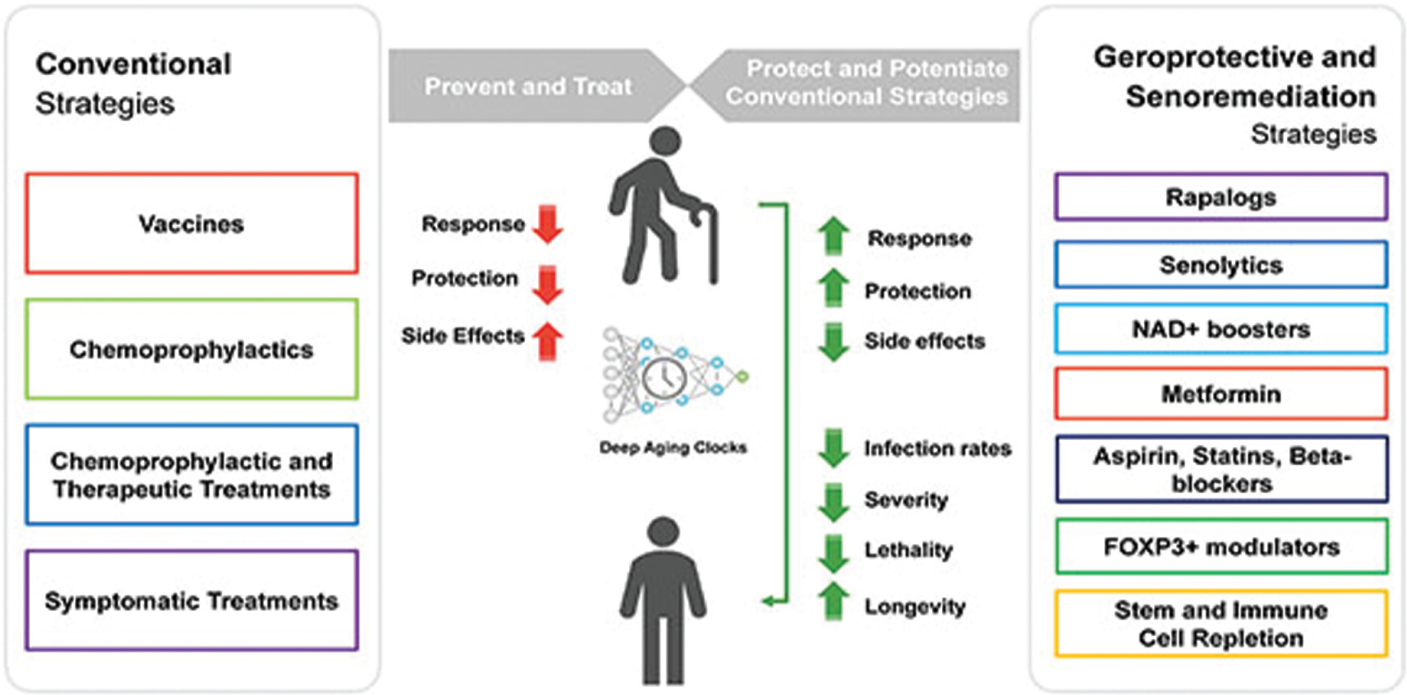

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COVID-19 | AGING

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Research Paper

Predictors for imaging progression on chest CT from coronavirus disease 2019 (COVID-19) patients

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ABSTRACT

Objective: This study aimed to investigate the potential parameters associated with imaging progression on chest CT from coronavirus disease 19 (COVID-19) patients.

Results: The average age of 273 COVID-19 patients enrolled with imaging progression were older than those without imaging progression (p = 0.006). The white blood cells, platelets, neutrophils and acid glycoprotein were all decreased in imaging progression patients (all p < 0.05), and monocytes were increased (p = 0.025). The parameters including homocysteine, urea, creatinine and serum cystatin C were significantly higher in imaging progression patients (all p < 0.05), while eGFR decreased (p < 0.001). Monocyte-lymphocyte ratio (MLR) was significantly higher in imaging progression patients compared to that in imaging progression-free ones (p < 0.001). Logistic models revealed that age, MLR, homocysteine and period from onset to admission were factors for predicting imaging progression on chest CT at first week from COVID-19 patients (all p < 0.05).

Conclusion: Age, MLR, homocysteine and period from onset to admission could predict imaging progression on chest CT from COVID-19 patients.

Methods: The primary outcome was imaging progression on chest CT. Baseline parameters were collected at the first day of admission. Imaging manifestations on chest CT were followed-up at (6±1) days.

INTRODUCTION

Since the end of 2019, a novel coronavirus with person-to-person transmission has spread to many other countries worldwide [1–5]. Previous epidemiology report uncovered that the epidemic of coronavirus disease 2019 (COVID-19) has doubled every 7.4 day in its early stage, with an average serial interval of 7.5 days [3]. Early information estimated that the basic reproductive number R_0 was estimated to be 1.4 – 2.5 reported by WHO [2]. The pandemic is accelerating at an exponential rate and at risk of escalating into a global health emergency [2]. The mortality of coronavirus disease 2019 (COVID-19) patients in China is approximately 2.3%, compared with 9.6% of severe acute respiratory syndrome (SARS) and 34.4% of middle east respiratory syndrome (MERS) reported by WHO [6]. Even this virus is not as fetal as people thought, the transmissibility is far exceeding that of SARS and MERS [7]. Although many clinical and epidemiological literatures have been published [3–6, 8–10], the spread in still ongoing and the early warning parameters for disease progression remain incomplete.

Compared to symptoms, chest CT findings were more rapid and frequent [11, 12]. The imaging performance on

chest CT scans from COVID-19 patients mainly manifested as bilateral ground-glass opacities (GGOs) in the lung periphery [13]. In a retrospective cohort, chest CTs of 121 symptomatic COVID-19 patients have been reviewed. Bilateral lung involvement was observed in 10/36 early patients (28%), 25/33 intermediate patients (76%), and 22/25 late patients (88%) [11]. Currently, chest CT is used to assess the severity of lung involvement in COVID-19 pneumonia [14]. In a cohort study, 85.7% (54/63) confirmed COVID-19 patients developed imaging progression including enlarged and increased extent of GGOs and consolidation at early follow-up chest CT scans [12]. That is, short-term imaging progression on chest CT from COVID-19 patients should be early predicted and intervened.

In this analysis, we summarized the baseline characteristics and investigated the potential predictive parameters for imaging progression on chest CT scans at first week after admission of COVID-19 patients, in the hope that the data may provide novel biomarker candidates as well as useful insights into the pathogenesis and progression of COVID-19 patients.

RESULTS

Imaging performance of progression and progression-free patients

As shown in Figure 1, most mild type COVID-19 patients had bilateral and peripheral GGOs, consolidation and linear opacities imaging involvements on chest CT at the first admission day. Some patients had no remarkable hallmarks. At the first six (±1) day, enlarged and increased GGOs, consolidation, solid nodules and fibrous stripes were observed for patients suffered from imaging progression on chest CT scans. On the contrary, the GGOs, consolidation and linear opacities were partly resolved and decreased for imaging progression-free patients.

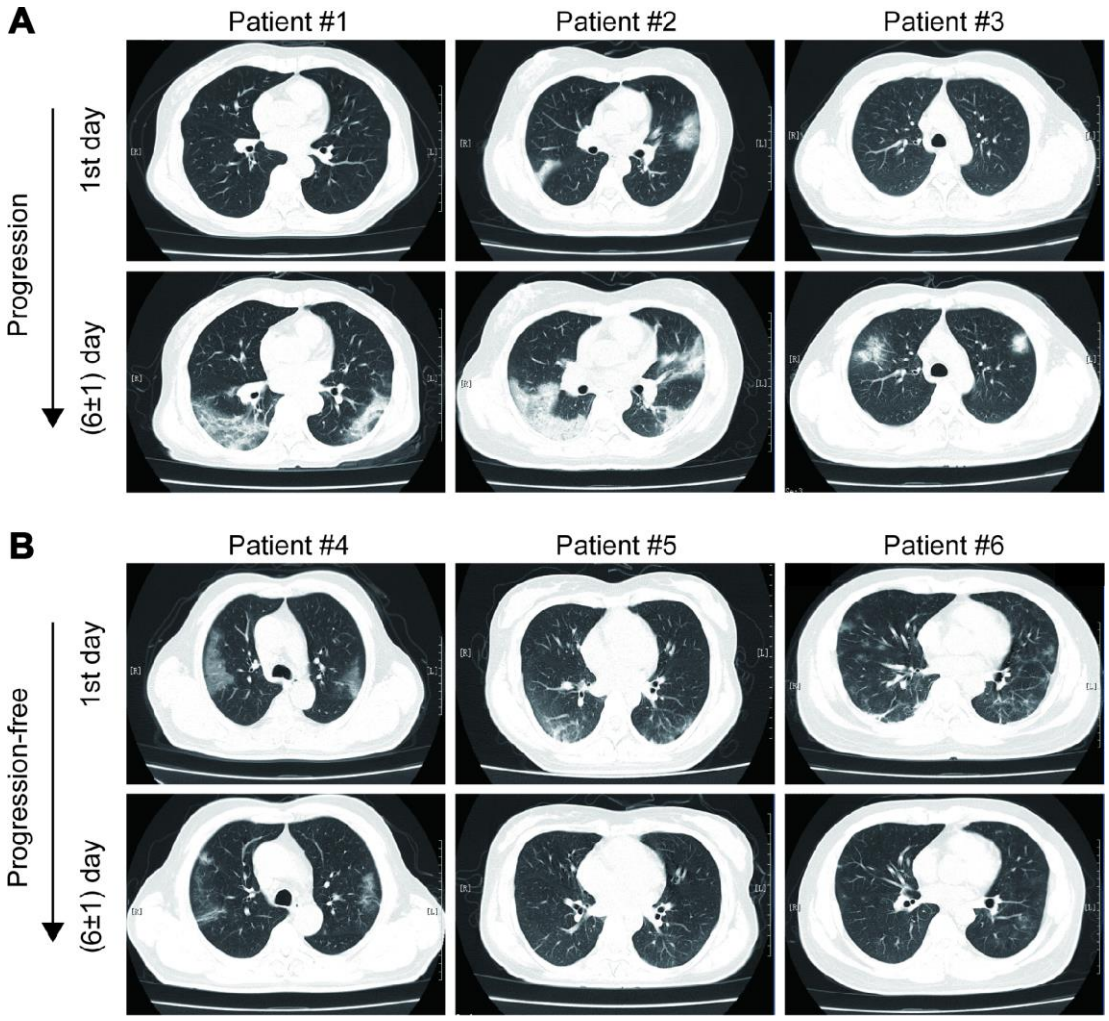

Figure 1. Examples of imaging progression (**A**) and progression-free (**B**) in chest CT from COVID-19 patients.

Baseline characteristics and inflammatory model comparisons between imaging progression and progression-free patients

In total, 71 COVID-19 patients suffered from imaging progression on chest CT at first week after admission, and the other 202 patients were imaging progression-free on chest CT. As summarized in Table 1, the patients in imaging progression group were significantly older than those in imaging progression-free group ($p = 0.006$, Table 1). More patients were treated with gamma globulin and thymosin in imaging progression group compared to those without imaging progression ($p = 0.022$ and $p = 0.001$, respectively, Table 1). In blood routine tests, the white blood cells (WBC), platelets and neutrophils were significantly lower in imaging progression patients than those in imaging progression-free ones ($p = 0.025$, $p = 0.044$ and $p = 0.014$, respectively, Table 1), while the monocytes were significantly higher in imaging progression patients ($p = 0.025$, Table 1). Additionally, acid glycoprotein was significantly lower in imaging progression patients ($p = 0.037$, Table 1). In liver function tests, gamma-glutamyl transferase (GGT) levels were significantly higher in imaging progression-free patients ($p = 0.045$, Table 1), while homocysteine levels were significantly higher in imaging progression patients ($p = 0.006$, Table1). In kidney function tests, urea, creatinine and serum cystatin C levels were significantly higher in imaging progression patients compared to those in imaging progression-free ones ($p = 0.011$, $p = 0.007$, respectively, Table 1). As we expected, the estimated glomerular filtration rate (eGFR) levels were significantly decreased in imaging progression patients ($p < 0.001$, Table 1). No differences were found in cardiac markers and coagulation function tests.

Six inflammatory models were compared between imaging progression and progression-free patients. As shown in Figure 2, monocyte-lymphocyte ratio (MLR) levels were significantly higher in imaging progression patients than those in imaging progression-free ones ($p < 0.001$, Figure 2C), while no differences were found among aspartate aminotransferase-lymphocyte ratio index (ALRI), aspartate aminotransferase-platelet ratio index (APRI), neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) between these two groups (Figure 2A, 2B, 2D–2F).

Co-manifestations on chest CT and outcomes

As summarized in Table 2, except for common manifestations on chest CT, chronic inflammatory manifestation, chronic bronchitis / emphysema, pericardial effusion, pleural effusion, bullae of lung and obsolete tuberculosis were the most frequent imaging co-manifestations in COVID-19 patients. COVID-19 patients with imaging progression had significantly higher frequency of chronic inflammatory manifestation than those without imaging progression (12.7% vs. 3.5%, $p = 0.005$, Table 2). No differences were found in distributions of chronic bronchitis / emphysema, pericardial effusion, pleural effusion, bullae of lung and obsolete tuberculosis between these two groups (Table 2).

Moreover, no acute bacterial or other viral co-infection performances on chest CT were found in these COVID-19 patients.

All these COVID-19 patients did not develop severe conditions, no one died during our follow up.

Parameters associated with imaging progression on chest CT

Variables including age, gender, disease history, epidemiology, chest CT imaging, therapeutic strategies, period from onset to admission, ALRI, APRI, MLR, NLR, PLR, SII, WBC, neutrophils, lymphocytes, monocytes, platelet, red blood cells (RBC), hemoglobin, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), GGT, lactate dehydrogenase (LDH), total bilirubin (TBiL), albumin, globulin, urea, creatinine, eGFR, lactic acid, haptoglobin, acid glycoprotein, cystatin C, homocysteine, retinol-binding protein, cardiac troponin (cTnI), myoglobin, brain natriuretic peptide prohormone (pro-BNP), prothrombin time, prothrombin activity (PTA), international normalized ratio (INR), D-dimer were included in the univariate analysis. As presented in Table 3, age, gamma globulin therapy, thymosin therapy, MLR, serum cystatin C, homocysteine, eGFR and period from onset to admission were potential parameters associated with imaging progression (all $p < 0.05$, Table 3). When these parameters were included in the multivariate model, age, MLR and homocysteine were significantly correlated with imaging progression on chest CT from COVID-19 patients (RR = 2.28, 95%CI = 1.12 – 4.34, $p = 0.012$; RR = 7.69, 95%CI = 1.67 – 35.55, $p = 0.009$ and RR = 3.17, 95%CI = 1.01 – 9.96, $p = 0.048$; respectively, Table 3). In addition, COVID-19 patients with period from onset to admission ≥ 4 days might have lower risk to develop imaging progression on chest CT at first week after admission (RR = 0.35, 95%CI = 0.19 – 0.67, $p = 0.001$, Table 3).

Table 1. Baseline characteristics of COVID-19 patients.

Variables	Chest CT		p value
	Progression group (n = 71)	Progression-free group (n = 202)	
Age, years, mean ± SD	53.5 ± 1.9	47.6 ± 1.1	0.006
Male, n (%)	33 (46.5)	101 (50)	0.61
Disease history, n (%)			0.614
None	48 (67.6)	143 (70.8)	
Hypertension	13 (18.3)	27 (13.4)	
Diabetes	7 (9.9)	11 (5.4)	
Fatty liver disease	12 (16.9)	27 (13.4)	
Others	3 (4.2)	21 (10.4)	
Epidemiology, n (%)			
Hubei sojourning history	43 (56.3)	108 (53.5)	0.301
Contact with COVID-19 patients	27 (38.0)	72 (35.6)	0.719
Therapeutic strategy, n (%)			
Antivirus drugs	58 (81.7)	141 (69.8)	0.053
Antibiotics	22 (31.0)	46 (22.8)	0.169
Gamma globulin	13 (18.3)	17 (8.4)	0.022
Thymosin	20 (28.2)	23 (11.4)	0.001
Glucocorticoid	10 (14.1)	17 (8.4)	0.169
TCM decoction	5 (7.0)	25 (12.4)	0.216
TCM patent	27 (38.0)	58 (28.7)	0.145
Chest CT imaging, n (%)			0.504
Bilateral lung lesion	60 (84.5)	177 (87.6)	
Single lung lesion	11 (15.5)	25 (12.4)	
Blood routine tests, mean ± SD			
WBC, $10^3/mm^3$	4.6 ± 0.1	5.2 ± 0.1	0.025
RBC, $10^4/mm^3$	4.4 ± 0.1	4.5 ± 0.04	0.334
Hemoglobin, g/L	135.1 ± 1.7	136.7 ± 1.1	0.465
Platelet, $10^3/mm^3$	176.0 ± 6.6	195.0 ± 5.1	0.044
Neutrophils, $10^3/mm^3$	2.9 ± 0.1	3.5 ± 0.1	0.014
Lymphocytes, $10^3/mm^3$	1.2 ± 0.1	1.3 ± 0.04	0.342
Monocytes, $10^3/mm^3$	0.5 ± 0.03	0.4 ± 0.01	0.025
Hypersensitive CRP, mg/L, mean ± SD	17.5 ± 2.4	18.7 ± 1.6	0.697
ESR, mm/Hour, mean ± SD	56.9 ± 4.3	64.5 ± 2.7	0.148
Procalcitonin, ng/ml, mean±SD	0.05 ± 0.01	0.09 ± 0.05	0.687
Acid glycoprotein, mg/dl, mean ± SD	140.9 ± 5.6	154.5 ± 3.3	0.037
Liver function tests, mean ± SD			
ALT, U/L	27.6 ± 2.3	27.6 ± 1.4	0.995
AST, U/L	29.4 ± 1.7	29.2 ± 1.6	0.958
GGT, U/L	29.5 ± 2.5	38.6 ± 2.5	0.045
LDH, U/L	244.4 ± 10.4	248.8 ± 5.8	0.703
TBiL, μmol/L	8.4 ± 0.4	9.2 ± 0.3	0.116
Albumin, g/L	40.8 ± 0.4	41.1 ± 0.3	0.537
Globulin, g/L	28.8 ± 0.5	29.0 ± 0.3	0.693
Homocysteine, μmol/L	10.7 ± 0.5	9.3 ± 0.2	0.006
Renal function test, mean ± SD			
Urea, mmol/L	5.1 ± 0.2	4.5 ± 0.1	0.011
Creatinine, μmol/L	70.7 ± 3.0	63.0 ± 1.3	0.007
Serum cystatin C, mg/L	1.0 ± 0.04	0.8 ± 0.01	< 0.001
eGFR, ml/(min×1.73m^2)	101.3 ± 3.1	116.3 ± 1.9	< 0.001
Lactic acid, mmol/L, mean ± SD	2.8 ± 0.1	2.8 ± 0.04	0.936
Haptoglobin, mg/dl, mean ± SD	209.2 ± 12.0	229.6 ± 7.0	0.142
Retinol-binding protein, mg/L, mean ± SD	27.8 ± 1.4	26.4 ± 0.7	0.327
Cardiac markers, mean ± SD			

cTnI, ng/ml	0.029 ± 0.004	0.033 ± 0.003	0.455
Myoglobin, ng/ml	17.5 ± 3.0	14.7 ± 2.9	0.59
Pro-BNP, pg/ml	73.5 ± 13.7	67.6 ± 7.2	0.692
Coagulation function tests, mean ± SD			
INR	1.01 ± 0.008	1.02 ± 0.008	0.424
PTA	99.9 ± 1.2	99.0 ± 0.8	0.579
Prothrombin time, second	13.4 ± 0.08	13.5 ± 0.08	0.402
D-Dimer, μg/ml	0.55 ± 0.06	0.77 ± 0.11	0.254

TCM, Traditional Chinese Medicine; WBC, white blood cells; RBC, red blood cells; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; TBiL, total bilirubin; eGFR, estimated glomerular filtration rate; cTnI, cardiac troponin; Pro-BNP, Brain natriuretic peptide prohormone; INR, international normalized ratio; PTA, prothrombin activity.

Predictive values of MLR and age for imaging progression on chest CT

Using OptimalCutpoints package in R program, we detected that the optimal cutoff of MLR was 0.51. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MLR for predicting imaging progression on chest CT were 0.44, 0.79, 0.42 and 0.80, respectively (Figure 3A and Table 4). And, the AUC of MLR for predicting imaging progression on chest CT was 0.63 (Figure 3A).

The optimal cutoff of age for predicting imaging progression on chest CT was 51 years. The sensitivity, specificity, PPV and NPV were 0.65, 0.58, 0.35 and 0.83 respectively (Figure 3B and Table 4). ROC curve

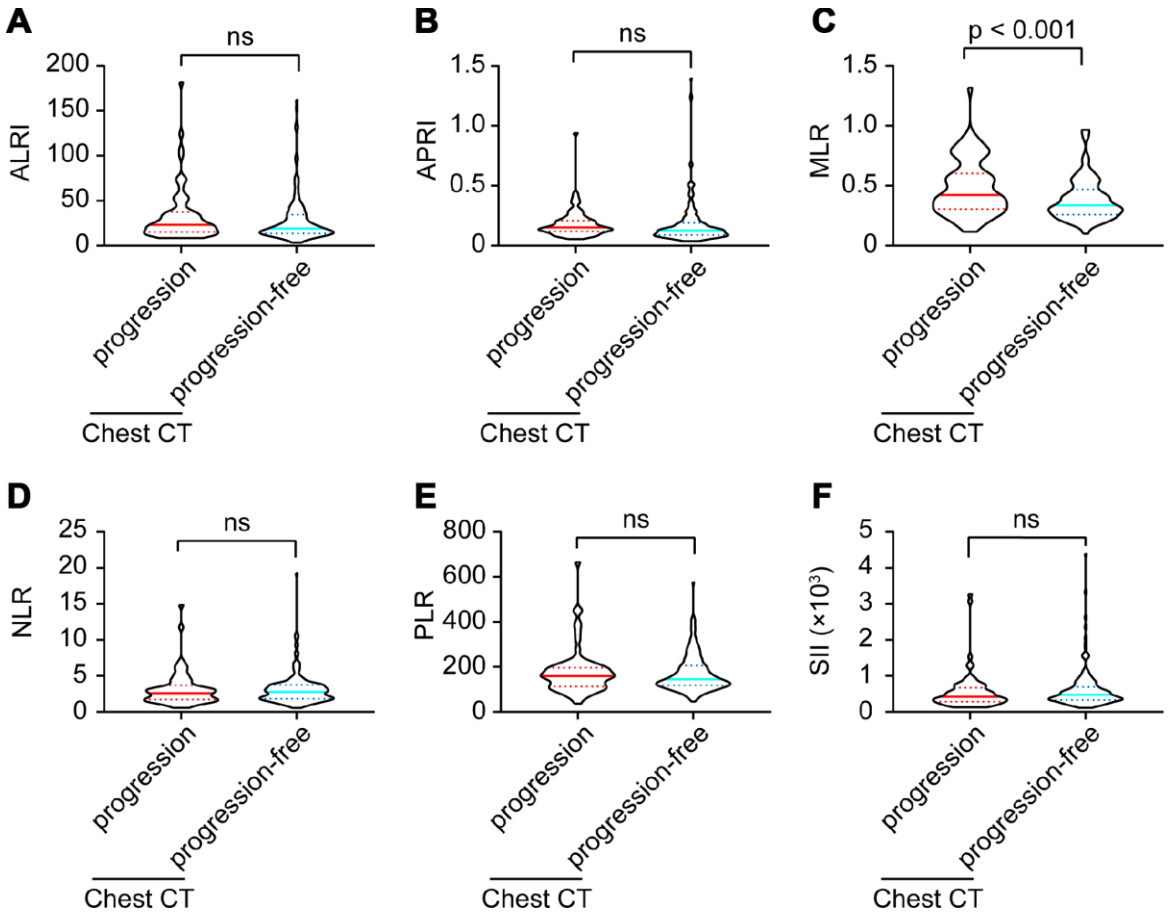

Figure 2. ALRI (**A**), APRI (**B**), MLR (**C**), NLR (**D**), PLR (**E**) and SII (**F**) model comparisons between imaging progression and progression-free COVID-19 patients.

Table 2. Co-manifestations on chest CT in COVID-19 patients.

Co-manifestations, n (%)	Chest CT		p value
	Progression group (n = 71)	Progression-free group (n = 202)	
Chronic inflammatory manifestations	9 (12.7)	7 (3.5)	0.005
Chronic bronchitis / emphysema	2 (2.8)	2 (1.0)	0.271
Pericardial effusion	1 (1.4)	1 (0.5)	0.438
Pleural effusion	1 (1.4)	0 (0)	0.091
Bullae of lung	1 (1.4)	2 (1.0)	0.771
Obsolete tuberculosis	2 (2.8)	1 (0.5)	0.107

Table 3. parameters associated with imaging progression in chest CT from COVID-19 patients[#].

Variables	Univariate		p value	Multivariate		p value
	RR	95%CI		RR	95%CI	
Age, years						
<60	reference	-	1.0	reference	-	1.0
≥60	2.72	1.55-4.78	< 0.001	2.28	1.12-4.34	0.012
Gamma globulin, yes vs. no	2.44	1.12-5.32	0.025	1.08	0.38-3.08	0.89
Thymosin, yes vs. no	3.05	1.55-6.0	0.001	2.32	0.94-5.73	0.069
MLR, per increase 1 unit	12.2	3.09-48.23	< 0.001	7.69	1.67-35.55	0.009
Serum cystatin C, mg/L						
< 1.03	reference	-	1.0	reference	-	1.0
> 1.03	2.8	1.35-5.82	0.006	0.79	0.28-2.2	0.65
Homocysteine, μmol/L						
< 15.4	reference	-	1.0	reference	-	1.0
> 15.4	3.54	1.23-10.14	0.019	3.17	1.01-9.96	0.048
eGFR, ml/(min×1.73m^2)						
> 90	reference	-	1.0	reference	-	1.0
< 90	2.97	1.54-5.75	0.001	1.63	0.67-4.0	0.281
Period from onset to admission, days						
< 4	reference	-	1.0	reference	-	1.0
≥ 4	0.36	0.20-0.64	0.001	0.35	0.19-0.67	0.001

Variables including age, gender, disease history, epidemiology, chronic inflammatory co-manifestation on chest CT, therapeutic strategies, period from onset to admission, ALRI, APRI, MLR, NLR, PLR, SII, WBC, neutrophils, lymphocytes, monocytes, platelet, RBC, hemoglobin, CRP, ESR, procalcitonin, ALT, AST, GGT, LDH, TBiL, albumin, globulin, urea, creatinine, eGFR, lactic acid, haptoglobin, acid glycoprotein, cystatin C, homocysteine, retinol-binding protein, cTnI, myoglobin, pro-BNP, prothrombin time, PTA, INR, D-dimer were included in the univariate analysis. Only variables with $p < 0.05$ in univariate model were included in the multivariate analysis.
[#] Only variables significantly associated with imaging progression in chest CT in univariate analysis were presented.

revealed that the AUC of age in the prediction model was 0.6 (Figure 3B).

In addition, the optimal cutoff of homocysteine for predicting imaging progression on chest CT from COVID-19 patients was 10.58 μmol/L. The sensitivity, specificity, PPV and NPV were 0.42, 0.79, 0.41 and 0.80, respectively (Figure 3C and Table 4).

We performed ROC comparison in MLR, age and homocysteine using ROC regression. As showed in Figure 3D, no difference among these three indexes was found ($p = 0.834$, Figure 3D).

DISCUSSION

According to the Chinese guidelines, imaging progression-free on chest CT scans was one of discharge criteria for COVID-19 patients. At present stage, the long-term imaging features of COVID-19 are not yet known [13, 15]. Follow-up imaging in COVID-19 patients often demonstrated the disease progression. Generally, imaging manifestations are in line with the severity of COVID-19 [16]. Hence, a short-term follow up with identification of imaging progression is of great importance for early warning of disease aggravation from COVID-19 patients, which could help clinicians to

manage quickly and accurately [12]. Considered that, we defined the imaging progression at first week on chest CT as the primary outcome.

In this outbreak, age was considered as one critical content during the disease occurrence and development. Our results also revealed that the average age of patients with imaging progression was older than those without. Logistic model confirmed that age should be a risk factor for predicting imaging progression. Previous reports suggested that COVID-19 is more susceptible to infect older adults [3, 8, 10]. Research with small samples of 2019-nCoV infected infants have been reported [17]. In a study included 34 COVID-19 children, the authors concluded that the clinical manifestations in children with 2019-nCoV infection are non-specific and are milder than that in adults [18]. In a nationwide retrospective study, 2143 pediatric patients were included. They found that more than 90% patients were asymptomatic, mild, or moderate, even though young children, particularly infants, were vulnerable to infection [19]. The first deaths of COVID-19 occurred frequently among elderly people, who may progress more faster [20]. In a multicenter cohort study with 137 patients enrolled, age was shown to be associated with high risk of death in COVID-19 patients. Middle-aged and elderly patients with underlying comorbidities are prone to respiratory failure

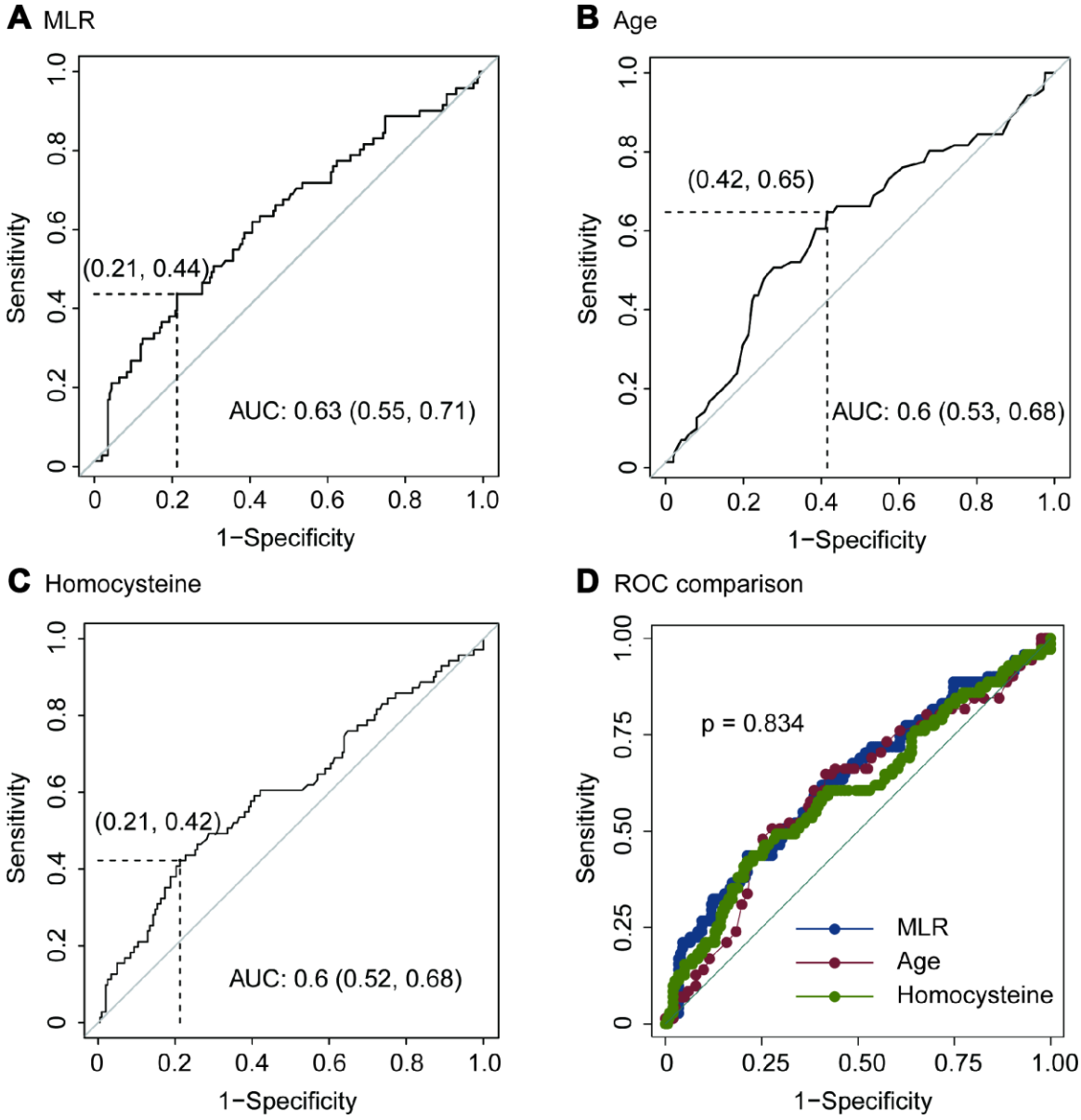

Figure 3. ROC of MLR (**A**), age (**B**), homocysteine (**C**) and ROC comparison (**D**) for imaging progression in chest CT from COVID-19 patients.

Table 4. Predictive values of MLR model, age and homocysteine for imaging progression on chest CT from COVID-19 patients.

	Estimate	95%CI
MLR		
Cutoff	0.51	-
Sensitivity	0.44	0.32 – 0.56
Specificity	0.79	0.72 – 0.84
Positive predictive value	0.42	0.34 – 0.54
Negative predictive value	0.80	0.71 – 0.85
Age, years		
Cutoff	51	-
Sensitivity	0.65	0.53 – 0.76
Specificity	0.58	0.51 – 0.65
Positive predictive value	0.35	0.29 – 0.48
Negative predictive value	0.83	0.74 – 0.86
Homocysteine, μmol/L		
Cut off	10.58	
Sensitivity	0.42	0.31 – 0.55
Specificity	0.79	0.72 – 0.84
Positive predictive value	0.41	0.33 – 0.53
Negative predictive value	0.80	0.70 – 0.85

and have a poorer prognosis [21, 22]. Combined the previous literatures and our results, we assumed that age also should be a risk factor for imaging progression at the early stage of COVID-19.

Among the six inflammatory models, MLR was significantly higher in COVID-19 patients with imaging progression on chest CT scans, and correlated with imaging aggravation. Previous evidence demonstrated that monocytes/macrophages were susceptible to human coronavirus (HCoV) 229E infection, but strongly restricted OC43 replication [23]. Differs from HCoV-229E, SARS-CoV poorly infects human purified monocytes/macrophages, and production of interferon-alpha by these cells further limits the infection [24]. Following infection of monocytes/macrophages by HCoV-OC43, viability remained high over 6 days and no apoptosis was observed [25]. These clues suggested that monocytes might be stable in function and quantity levels during HCoV infection like SARS and 2019-CoV. Conversely, SARS-CoV frequently targets for cytotoxic T lymphocytes [26, 27]. Lymphopenia is one of hematological abnormalities during SARS-CoV infection, and lymphocyte counts could predict the severity and clinical outcomes [28]. Previous study showed that lymphocytes and its subsets significantly decreased in SARS patients, while those with severe clinical illness or those who died had more remarkable CD4+ and CD8+ lymphopenia [28]. Also, MERS-CoV could efficiently infected T lymphocytes from the peripheral blood and from human lymphoid organs and induced apoptosis in T lymphocytes [29]. Similar with SARS-CoV and MERS-CoV, 2019-nCoV infection also related with loss of lymphocytes, which was supported by Chinese guidelines [30, 31]. Thus, the MLR increased especially in patients with disease progression.

Homocysteine is a potent toxic agent that involved in oxidative stress and neurotoxicity promotion, endothelial dysfunction, and acceleration of the atherosclerotic process [32–34]. Emerging evidences revealed that hyperhomocysteinemia contributed to a spectrum of disease development, including cardiovascular disease, diabetes, chronic kidney disease and fatty liver disease [35–37]. Previous reports uncovered that homocysteine concentrations were greater in many virus infections including human immunodeficiency virus, hepatitis virus and human papilloma virus [38–40]. However, the roles of homocysteine in coronavirus infection have not been well illustrated. Based on our results, homocysteine concentrated in imaging progression patients and showed predictive value for imaging progression.

Our results also demonstrated that COVID-19 patients with period from onset to admission ≥ 4 days had lower risk to develop imaging progression on chest CT at first week after admission. On the one hand, patients with

period over 4 days might have mild clinical symptoms, which in line with mild or slow progression of this disease. On the other hand, the period from onset to admission should be counted in the natural process of 2019-nCoV infection.

This study has some limitations. First, only mild type of COVID-19 patients was included, and severe type and life-threating types were excluded in this analysis. Second, MLR and age did not have powerful prognostic values for imaging progression on chest CT in our study. Therefore, we suggest that they be used in combination in clinical practice. Third, the follow-up period was short-term, more solid outcomes should be considered in future. And, subgroup analysis of category manifestation of imaging progression on chest CT should also be considered. Even though, age, MLR model, homocysteine and period from onset to admission might be useful for evaluating disease progression in COVID-19 patients.

MATERIALS AND METHODS

Ethic statement

All participants provided written informed consent during their admission. The study protocol and informed consent documents were reviewed and approved by the Ethics Committee of Shanghai Public Health Clinical Center, Fudan University.

Patients

In accordance to the 4th edition of "Diagnosis and management program of novel coronavirus-infected pneumonia" released by National Health Commission of The People's Republic of China [30], 273 diagnosed COVID-19 patients with mild category in Shanghai Public Health Clinical Center were included in this analysis. 2019 novel coronavirus (2019-nCoV) nucleic acid of sputum samples from all participants were positive detected by real-time polymerase chain reaction. The influenza A and B antigens of all participants were negative. All participants had no other lymphatic system disorders or malignant hematologic diseases, ensuring that the whole blood parameters were representative of normal baseline values. Patients with renal and/or hepatic failure, acute coronary syndromes, valvular heart diseases, autoimmune thyroid diseases, or systematic inflammatory diseases were excluded from our study.

Study design

This was a prospective single-center cohort study. The baseline characteristics, including demographics, treatment strategies, routine blood tests, liver-kidney function parameters, coagulation function tests, cardiac markers and chest CT imaging, were all collected at the first admission day. Chest CT imaging were also performed at the (7 ± 2) day during their admission. All the tests and examines were conducted in the Department of Medical Laboratory and the Department of Radiology in Shanghai Public Health Clinical Center, Fudan University.

Definition

The primary outcome was defined as imaging progression on chest CT at first week. Any one of the following criteria was considered as imaging progression on chest CT: 1) Increased ground-glass lesions in the underlying involvements; 2) Newly occurred lesions beyond underlying involvements. The chest CT imaging performance was diagnosed by two radiologists independently and inconsistency was discussed and determined by the director of Department of Radiology who acted as an arbiter.

Six inflammatory models, including ALRI, APRI, MLR, NLR, PLR and SII were included in this analysis. The definitions of these models are as follows: ALRI = AST / L; APRI = AST / P; MLR = M / L; NLR = N / L; PLR = P / L; and SII = P × N / L, where M, L, N and P are the peripheral monocyte, lymphocyte, neutrophil and platelet counts, respectively.

Statistical analysis

Differences of variables between the individual groups were analyzed using student t test and Chi-square test based on variables types. Parameters associated with the outcome were assessed by univariate and multivariate logistic regression. Only variables significantly associated with the outcome at univariate analysis (two-sided $p < 0.05$) included in the multivariate model. Results were reported as risk ratios (RR) with 95% confidence intervals (CI). OptimalCutpoints package [41] in R program was used to perform ROC analysis to evaluate predictive values of potential factors for the outcome. Stata software version 16.0 (Stata Corp LLC, Texas, USA) was used for other statistics. A two-tailed $p < 0.05$ were considered significant for all tests.

CONFLICTS OF INTEREST

The authors have declared that no Conflicts of interest exist.

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Research Paper

Does comorbidity increase the risk of patients with COVID-19: evidence from meta-analysis

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ABSTRACT

Currently, the number of patients with coronavirus disease 2019 (COVID-19) has increased rapidly, but relationship between comorbidity and patients with COVID-19 still not clear. The aim was to explore whether the presence of common comorbidities increases COVID-19 patients' risk. A literature search was performed using the electronic platforms (PubMed, Cochrane Library, Embase, and other databases) to obtain relevant research studies published up to March 1, 2020. Relevant data of research endpoints in each study were extracted and merged. All data analysis was performed using Stata12.0 software. A total of 1558 patients with COVID-19 in 6 studies were enrolled in our meta-analysis eventually. Hypertension (OR: 2.29, P<0.001), diabetes (OR: 2.47, P<0.001), chronic obstructive pulmonary disease (COPD) (OR: 5.97, P<0.001), cardiovascular disease (OR: 2.93, P<0.001), and cerebrovascular disease (OR:3.89,P=0.002) were independent risk factors associated with COVID-19 patients. The meta-analysis revealed no correlation between increased risk of COVID-19 and liver disease, malignancy, or renal disease. Hypertension, diabetes, COPD, cardiovascular disease, and cerebrovascular disease are major risk factors for patients with COVID-19. Knowledge of these risk factors can be a resource for clinicians in the early appropriate medical management of patients with COVID-19.

INTRODUCTION

Coronavirus Disease 2019(COVID-19) is a viral respiratory disease caused by the 2019 novel coronavirus (2019-nCoV), which has caused the pneumonia epidemic in the world [1–3]. As of March 5, 2020, a total of 96539 cases with laboratory-confirmed COVID-19 infection have been detected in the world reported by the World Health Organization (WHO). In China, there have been 80567 accumulated confirmed cases of COVID-19, and 5952 of them were existing severe patients. Given the rapid spread and high mortality rate of COVID-19, it is absolutely necessary to evaluate the possible risk factors affecting the progression of disease in COVID-19 patients.

Previous studies show that COVID-19 patients with comorbidity may lead to a poor prognosis [5]. Identifying the most important risk groups is essential when making decisions anti-2019-nCoV therapy. To date, there has been no systematic review that comprehensively explores whether the presence of common comorbidities increase COVID-19 patients' risk, to guide clinical practice better. Therefore, we performed a meta-analysis of the available studies to explore relationship between comorbidity and patients with COVID-19.

RESULTS

Literature search and screening

The database searches identified a total of 324 potentially relevant articles. After the exclusion of duplicate references,243 articles were considered for the meta-analysis. Of these,208 studies were excluded after screening the title and abstract. After careful review of the full texts, 29 articles were excluded because they were reviews, cases, and insufficient data. Six studies qualified for inclusion [1, 4–8]. The flow diagram (Figure 1) showed the detailed literature search steps.

Characteristics and quality of studies

A total of 1558 samples from 6 retrospective studies were enrolled in this meta-analysis [1, 4–8]. All six studies were performed in China. Six studies [1, 4–8] reported that hypertension, diabetes, and COPD, five covered liver disease [1, 4–7], four investigated malignancy [1, 4, 5, 8], renal disease [4–7], and cardiovascular disease [4–7], and three [4, 5, 7] researched cerebrovascular disease. Two studies [1, 5] used whether patients experienced ICU care to judge the severity of the disease, and the other four studies used clinical symptoms to judge the severity of the disease. All articles are of high quality because of NOS score no less than 6. Detailed descriptions of the studies included are shown in Table 1.

Hypertension, diabetes, and COPD

Six studies, including 324 severe group cases and 1234 non-severe group cases, provided the data in terms of hypertension, diabetes, and COPD [1, 4–8]. The

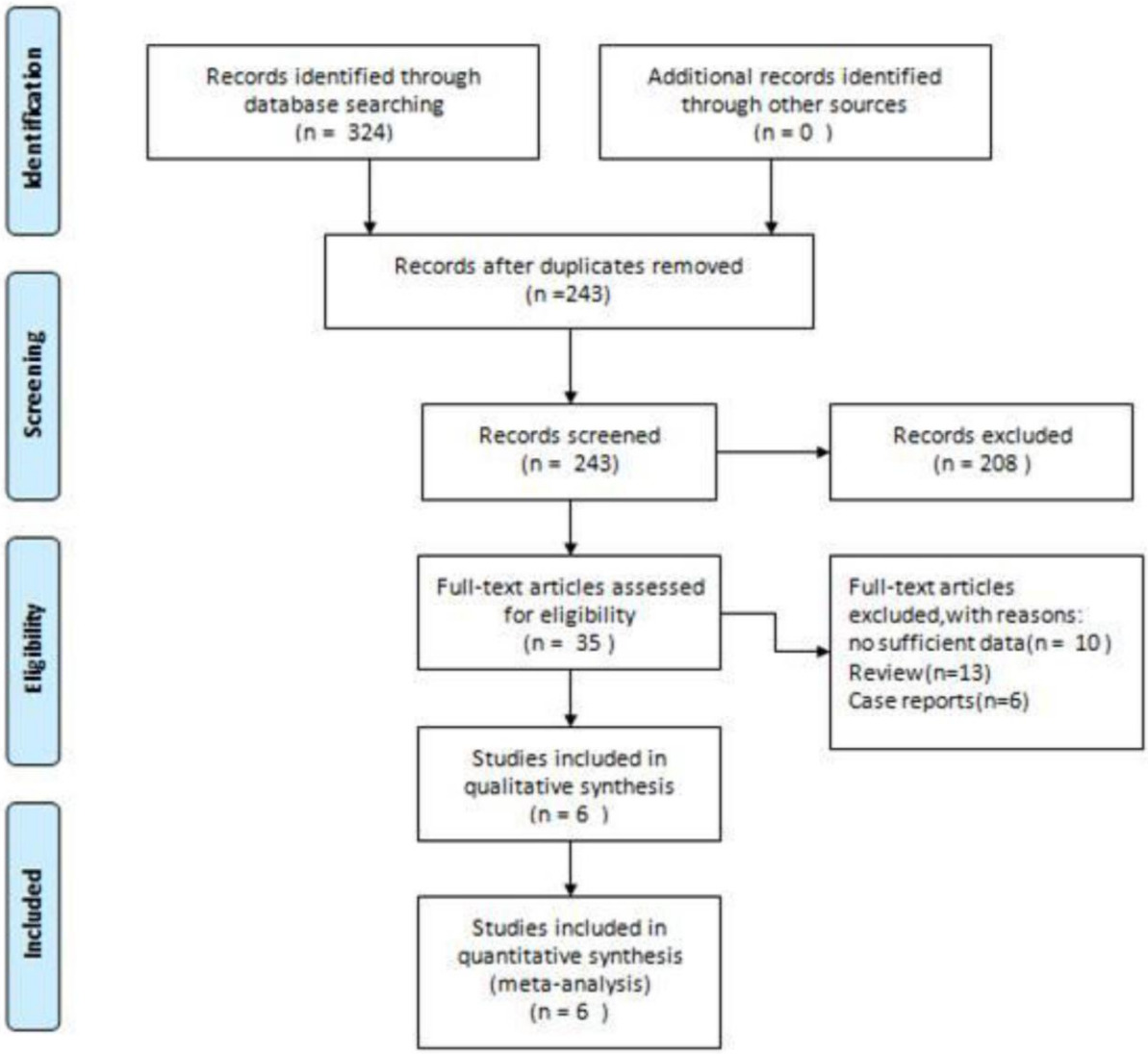


Figure 1. Flow diagram of the literature search and selection process in the meta-analysis.

Table 1. Main characteristics of the included studies in our-analysis.

Study	Year	Country	Sample	Median Age (years)	Sex		Diseases severity		Basis of disease severity	NOS
					Men	Women	Non-severe	Severe		
C.Huang	2020	China	41	49.0 (41.0–58.0)	30	11	28	13	ICU care	7
D.Wang	2020	China	138	56.0 (42.0-68.0)	75	63	102	36	ICU care	7
W.Guan	2020	China	1099	47.0 (35.0–58.0)	640	459	926	173	clinical symptoms	8
W.Liu	2020	China	78	38.0 (33.0-57.0)	39	39	67	11	clinical symptoms	7
X.Xu	2020	China	62	41.0 (32.0-52.0)	36	26	29	33	clinical symptoms	6
J.Zhang	2020	China	140	57.0 (25.0-87.0)	71	69	82	58	clinical symptoms	7

NOS, Newcastle-Ottawa Scale.

heterogeneity test showed low heterogeneity among these studies, and a fixed-effects model was used for the meta-analysis. The results find that COVID-19 patients with hypertension (OR: 2.29, 95% CI: 1.69-3.10,P<0.001) (Figure 2A), diabetes (OR: 2.47, 95% CI: 1.67-3.66, P<0.001) (Figure 2B), or COPD (OR: 5.97, 95% CI: 2.49-14.29, P<0.001) (Figure 2C) had a higher risk of exacerbation.

Cardiovascular disease and cerebrovascular disease

Four included studies reported the relationship between cardiovascular disease and patients with severe COVID-19 [1, 4–6]. No significant heterogeneity was found (I^2 =0, P=0.989) among these trials, so a fixed effect pattern was selected. The results showed that cardiovascular disease is a risk factor for patients with COVID-19 (OR:2.93, 95% CI: 1.73-4.96, P<0.001) (Figure 2G).

Three studies provided the data in terms of cerebrovascular disease [4, 5, 7]. A fixed-effects model was used since the heterogeneity test suggested that there was no significant heterogeneity (I^2=44.8%, P=0.163). The meta-analysis shows a significant relationship between patients with severe COVID-19 and cerebrovascular disease (OR:3.89, 95% CI: 1.64-9.22, P=0.002) (Figure 2H).

Liver disease, malignancy, and renal disease

Five studies comprising 313 severe group cases and 1167 non-severe group cases evaluated the role of liver disease in patients with COVID-19 [1, 4–7]. The meta-analysis showed that patients with the previous liver disease did not increase the risk of disease progression (OR:0.67, 95% CI: 0.30-1.49, P=0.326) (Figure 2D).

The relative risk assessments associated with malignancy and kidney disease are presented in Figure 2E and 2F, respectively. The meta-analysis suggested that there was no correlation between malignant tumor (the 95% confidence interval includes 1) or kidney disease (P=0.070) and COVID-19 patients' aggravation.

Subgroup analysis

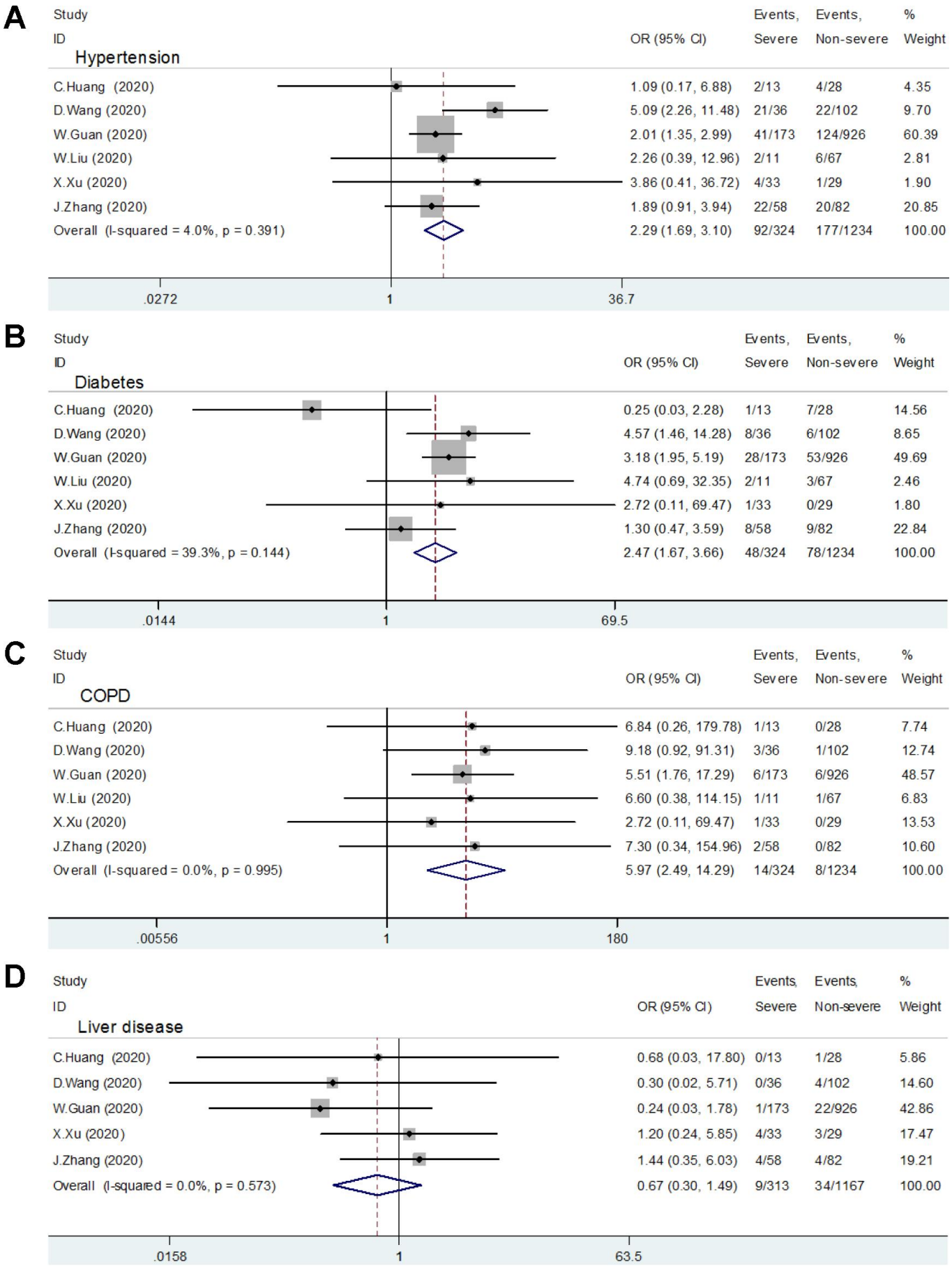
To further verify the correlation of comorbidity and COVID-19 patients' aggravation, subgroup analysis was conducted. The results of the subgroup analysis are presented in Table 2. The Subgroup analysis results further support the results of hypertension, COPD, liver disease, and renal disease. In the clinical symptom group, we further observed that hypertension, diabetes, COPD, malignancy, and cardiovascular disease were a risk factor in COVID-19 patients.

Publication bias

The risk of publication bias was analyzed in the following comorbidities: hypertension, diabetes, COPD, and liver disease. Figure 3 shows the results of publication bias, which were evaluated by funnel plots and Eggers test. Begg's test (All Pr>0.05) and Egger's regression test (All P >0.05) suggest no significant publication bias.

DISCUSSION

Currently, the increasing number of cases and extensive geographical expansion of the COVID-19 are causing widespread concern in the world [9]. Tian et al [10]. described that the proportion of severe versus common cases of the COVID-19 infection, which was approximately 1:4, the ratio of severe to mild were 18%


A
Study
ID
OR (95% CI)
Events, Severe
Events, Non-severe
% Weight
Hypertension
C.Huang (2020) 1.09 (0.17, 6.88) 2/13 4/28 4.35
D.Wang (2020) 5.09 (2.26, 11.48) 21/36 22/102 9.70
W.Guan (2020) 2.01 (1.35, 2.99) 41/173 124/926 60.39
W.Liu (2020) 2.26 (0.39, 12.96) 2/11 6/67 2.81
X.Xu (2020) 3.86 (0.41, 36.72) 4/33 1/29 1.90
J.Zhang (2020) 1.89 (0.91, 3.94) 22/58 20/82 20.85
Overall (I-squared = 4.0%, p = 0.391) 2.29 (1.69, 3.10) 92/324 177/1234 100.00
.0272
1
36.7
B
Study
ID
OR (95% CI)
Events, Severe
Events, Non-severe
% Weight
Diabetes
C.Huang (2020) 0.25 (0.03, 2.28) 1/13 7/28 14.56
D.Wang (2020) 4.57 (1.46, 14.28) 8/36 6/102 8.65
W.Guan (2020) 3.18 (1.95, 5.19) 28/173 53/926 49.69
W.Liu (2020) 4.74 (0.69, 32.35) 2/11 3/67 2.46
X.Xu (2020) 2.72 (0.11, 69.47) 1/33 0/29 1.80
J.Zhang (2020) 1.30 (0.47, 3.59) 8/58 9/82 22.84
Overall (I-squared = 39.3%, p = 0.144) 2.47 (1.67, 3.66) 48/324 78/1234 100.00
.0144
1
69.5
C
Study
ID
OR (95% CI)
Events, Severe
Events, Non-severe
% Weight
COPD
C.Huang (2020) 6.84 (0.26, 179.78) 1/13 0/28 7.74
D.Wang (2020) 9.18 (0.92, 91.31) 3/36 1/102 12.74
W.Guan (2020) 5.51 (1.76, 17.29) 6/173 6/926 48.57
W.Liu (2020) 6.60 (0.38, 114.15) 1/11 1/67 6.83
X.Xu (2020) 2.72 (0.11, 69.47) 1/33 0/29 13.53
J.Zhang (2020) 7.30 (0.34, 154.96) 2/58 0/82 10.60
Overall (I-squared = 0.0%, p = 0.995) 5.97 (2.49, 14.29) 14/324 8/1234 100.00
.00556
1
180
D
Study
ID
OR (95% CI)
Events, Severe
Events, Non-severe
% Weight
Liver disease
C.Huang (2020) 0.68 (0.03, 17.80) 0/13 1/28 5.86
D.Wang (2020) 0.30 (0.02, 5.71) 0/36 4/102 14.60
W.Guan (2020) 0.24 (0.03, 1.78) 1/173 22/926 42.86
X.Xu (2020) 1.20 (0.24, 5.85) 4/33 3/29 17.47
J.Zhang (2020) 1.44 (0.35, 6.03) 4/58 4/82 19.21
Overall (I-squared = 0.0%, p = 0.573) 0.67 (0.30, 1.49) 9/313 34/1167 100.00
.0158
1
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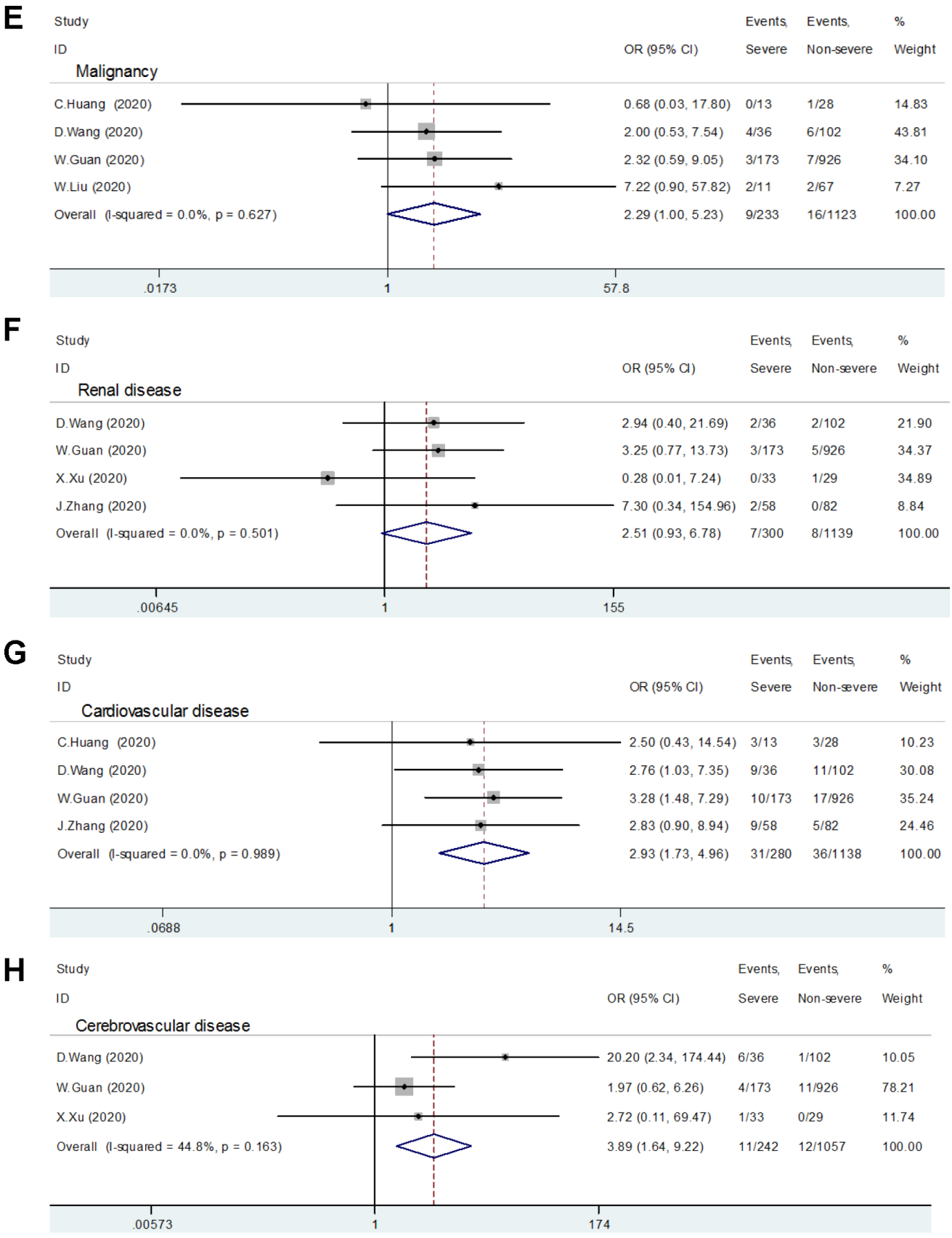

Figure 2. Relationship between comorbidity and patients with COVID-19. (**A**) Hypertension; (**B**) Diabetes; (**C**) COPD; (**D**) Liver Disease; (**E**) Malignancy; (**F**) Renal disease; (**G**) Cardiovascular disease; (**H**) Cerebrovascular disease.

Table 2. Results of meta-analysis and subgroup analysis.

	No. of studies	OR(95%CI)	P-Value	Heterogeneity		Model used
				I^2	P_h	
Hypertension	6	2.29(1.69-3.10)	<0.001	4.0%	0.391	Fixed
ICU care	2	2.97(0.70-12.55)	<0.001	55.7%	0.133	Romdon
Clinical symptoms	4	2.03(1.45-2.85)	<0.001	0	0.947	Fixed
Diabetes	6	2.47(1.67-3.66)	<0.001	39.3%	0.144	Fixed
ICU care	2	1.24(0.07-22.98)	0.883	82.0%	0.018	Romdon
Clinical symptoms	4	2.66(1.73-4.10)	<0.001	0	0.429	Fixed
COPD	6	5.97(2.49-14.29)	<0.001	0	0.995	Fixed
ICU care	2	8.30(1.26-54.43)	0.027	0	0.885	Fixed
Clinical symptoms	4	5.37(1.99-14.46)	0.001	0	0.973	Fixed
Liver disease	5	0.67(0.30-1.49)	0.326	0	0.573	Fixed
ICU care	2	0.41(0.05-3.53)	0.713	0	0.416	Fixed
Clinical symptoms	3	0.74(0.31-1.75)	0.492	16.9%	0.300	Fixed
Malignancy	4	2.29(1.00-5.23)	0.049	0	0.627	Fixed
ICU care	2	1.67(0.49-5.61)	0.410	0	0.547	Fixed
Clinical symptoms	2	3.18(1.05-9.64)	0.041	0	0.370	Fixed
Renal disease	4	2.51(0.93-6.78)	0.070	0	0.501	Fixed
ICU care	1	2.94(0.40-21.69)	0.290	-	-	-
Clinical symptoms	3	2.38(0.76-7.50)	0.237	15.0%	0.308	Fixed
Cardiovascular disease	4	2.93(1.73-4.96)	<0.001	0	0.989	Fixed
ICU care	2	2.69(1.14-6.34)	0.023	0	0.924	Fixed
Clinical symptoms	2	3.10(1.59-6.02)	0.001	0	0.834	Fixed
Cerebrovascular disease	3	3.89(1.64-9.22)	0.002	44.8%	0.163	Fixed
ICU care	1	20.20(2.34-174.44)	0.006	-	-	-
Clinical symptoms	2	2.07(0.70-6.12)	0.189	0	0.852	Fixed

and 73%. Until now, the source and pathogenesis of the COVID-19 remain unclear, and no specific treatment has been recommended for coronavirus infection except for meticulous supportive care [8, 11]. Unfortunately, in severe patients with COVID-19, the disease progresses rapidly, and respiratory failure can occur within a short time, even leading to death. Early data from Wuhan Jinyintan Hospital showed that 61.1% of patients in ICU had respiratory failure, 44.4% had arrhythmia, and 30.6% had a shock [11]. Therefore, early identification of severe patients is of great significance for improving the therapeutic effect of COVID-19 and reducing mortality.

Previous studies have described that the presence of common comorbidities increase COVID-19 patients' risk [5]. Besides, some scholars think that the presence of any coexisting illness was more common among patients with severe disease than among those with the non-severe disease [4]. However, the specific comorbidity by which can lead to disease progression remain unknown in COVID-19 patients.

A total of 1558 COVID-19 patients were included in the analyses, 324 (20.8%) of whom were severe. The meta-analysis of retrospective studies confirms that COPD is associated with a dramatically increased risk of aggravation in patients with COVID-19. COVID-19 patients with COPD had a 5.9-fold higher risk of progression than patients without COPD. Moreover, we identify an increased risk of aggravation in

individuals who have hypertension, diabetes, cardiovascular disease, or cerebrovascular disease. Our meta-analysis did not provide sufficient evidence that there was a correlation between liver disease, malignant tumor or kidney disease, and COVID-19 patients' aggravation.

However, this conclusion needs to be taken with caution, as this study has several limitations. Firstly, the small sample size may reduce the significance of the results. Secondly, the judgment criteria for severe and non-severe patients included in the study were not uniform. Thirdly, some included patients who had more

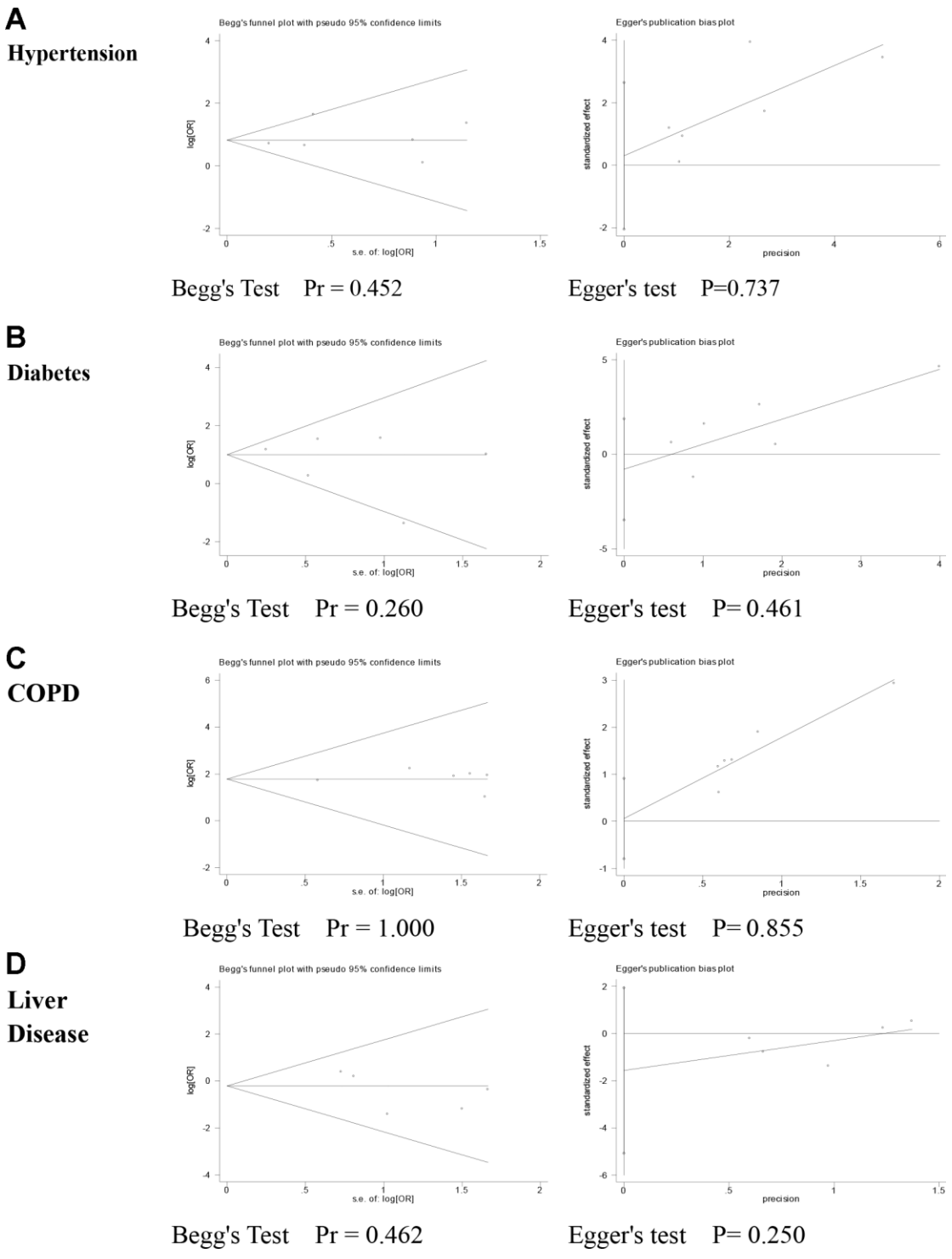

Figure 3. Publication bias assessment. (**A**) Hypertension; (**B**) Diabetes; (**C**) COPD; (**D**) Liver Disease.

than one coexisting illness. Fourth, the quality of different studies was different, which might lead to bias.

CONCLUSIONS

The meta-analysis identified hypertension, diabetes, COPD, cardiovascular disease, and cerebrovascular disease as significant risk factors for COVID-19 patients. The knowledge of these factors can better define those COVID-19 patients at higher risk, and thus allow a more targeted and specific approach to prevent those deaths. Given the limitations of this conclusion, well-designed trials of high quality are needed to explore the relationship between comorbidity and patients with COVID-19.

MATERIALS AND METHODS

Search strategy and study selection

The Meta-analysis was performed according to the Preferred Reporting Items for Systematic reviews and Meta-analysis (PRISMA) statement [12]. Relevant literature was extracted by systematic retrieval of PubMed (Medline), EMBASE, Springer, Web of Science, and Cochrane Library databases up to date to March 1, 2020. Our search strategy included terms for "2019-nCoV" or "Coronavirus" or "COVID-19" or "SARS-CoV-2" or "2019-nCoV" or "Wuhan Coronavirus." Besides, we manually screened out the relevant potential article in the references selected. The above process was performed independently by two participants.

Inclusion criteria are as follows: (1) Types of Studies: published studies reported the relationship between comorbidity and patients with COVID-19; (2) Subjects: diagnosed patients with COVID-19; (3) Exposure intervention: COVID-19 patients with comorbidity included: hypertension, diabetes, chronic obstructive pulmonary disease (COPD), liver disease, malignancy, renal disease, cardiovascular disease, cerebrovascular disease; (4) Outcome indicator: the odds ratios (OR) with 95% confidence intervals (CI) for each comorbidity.

The exclusion criteria: (1) Case reports, reviews, summaries of discussions, (2) Insufficient data information provided; (3) Patients were not stratified for the degree of severity.

Data extraction and quality assessment

Two participants separately conducted literature screening, data extraction, and literature quality evaluation, and any differences could be resolved through discussion or a third analyst. Information extracted from the included literature: first author surname, year of publication, country of the population, sample size, relevant data on comorbidity of severe and non-severe patients, etc.

The Newcastle-Ottawa scale (NOS) was adopted to evaluate the process in terms of queue selection, comparability of queues, and evaluation of results [13]. The quality of the included studies was assessed independently by two participants. NOS scores of at least six were considered high-quality literature. Higher NOS scores showed higher literature quality.

Statistical analysis

All data analysis was performed using Stata12.0 software (Stata Corp, College Station, Texas). The OR and relevant 95% CI were used to estimate pooled results from studies. After that, the heterogeneity test was conducted. When $P \geq 0.05$ or $I^2 < 50\%$ was performed, it indicated that there was no obvious heterogeneity, and the fixed-effect model should be applied for a merger. Otherwise, the random-effect model was applied. Results were considered significant statistically when the p-value less than 0.05.

Studies were grouped according to the type of disease severity judgment basis. One subgroup is based on the clinical symptoms of patients, and the other subgroup is based on whether patients experience ICU care or not. Subgroup sensitivity analyses were conducted to explore potential sources of heterogeneity.

Publication bias was assessed using Begg funnel plot and Egger test linear regression test (where at least five studies were available). If $P < 0.05$ indicates obvious publication bias.

AUTHOR CONTRIBUTIONS

Bolin Wang made the substantial contributions to the conception and design of the work; Zhong Lu, Ruobao Li, and Bolin Wang searched, selected materials and extracted data; Bolin Wang wrote this manuscript; Yan Huang and Bolin Wang revised the paper carefully and also contributed to the statistical analysis. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Editorial

Biomarkers of biological age as predictors of COVID-19 disease severity

Gordan Lauc and David Sinclair

Early epidemiological studies suggest the most important predictor of severity of COVID-19 disease course is age. Pre-existing conditions, including diabetes, CVD, hypertension, obesity and other consequences of an unhealthy lifestyle are also associated with increased mortality, indicating that the biological age is more relevant than the chronological age. Because a reliable COVID-19 vaccine is unlikely to available before the maximal infection of COVID-19 has occurred, it is essential to establish reliable tools for patient stratification and identification of individuals at high risk of severe disease.

A number of biomarkers aimed at objective estimation of biological age have been developed in the past several years, the most prominent ones being the epigenetic clock and the glycan clock. A key feature of a good biomarker of biological age is that the difference between chronological and biological age should correlate with known biomarkers of unhealthy lifestyle and that increased biological age should predict future disease development. The original epigenetic clock relied, in part, on chronological age, so several alternative epigenetic clocks, such as the GrimAge methylation clock, were developed. This has been demonstrated for both methylation and glycans. The difference between glycan age and chronological age associates with biomarkers of unhealthy lifestyle [1], while changes in glycans predict future diabetes and cardiovascular events [2]. Several different epigenetic clocks were recently also shown to predict prevalence and incidence of leading causes of death and disease [3].

Glycans, or polysaccharides, are carbohydrate-based polymers that regulate a variety of processes, including immunity [4]. In fact, glycan diversity represents one of the main defenses of all higher organisms against pathogens, and the repertoire of glycans changes with age, especially in the age ranges that are most susceptible to SARS-CoV2. Furthermore, both the SARS-Cov-2 virus and its principal cellular target ACE2 are known to be highly glycosylated [5], a pattern that likely changes with age. Recent study analysed site-specific N-linked glycosylation of MERS and SARS S glycoproteins, indicating that each of these glycosylation sites can be occupied by up to ten different glycans (called glycoforms), which greatly extends epitope diversity [6].

Glycans are the primary molecular basis inter-individual differences within the human population, including the ABO blood groups. Furthermore, glycans are one of the principal regulators of antibody effector functions and many other aspects of the immune system. Based on

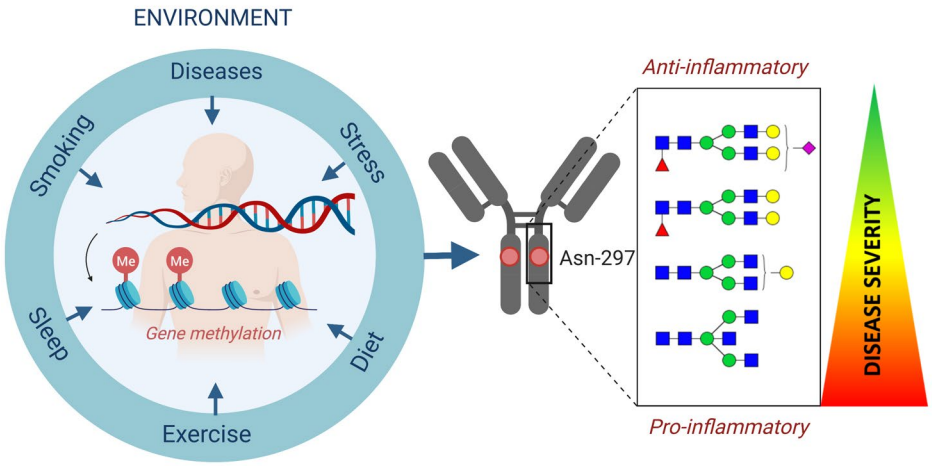

Figure 1. Information from genetic, epigenetic and direct environmental factors integrate at the level of protein glycosylation and result in inter-individual differences in both expression of surface antigens and regulation of the immune system.

these and other findings, we believe that glycans should be in the focus of biomarker discovery in COVID-19 cases. Since glycans are structurally complex and their analysis is technically challenging, until recently they were largely ignored by clinical researchers. However, the situation changed dramatically in the last few years and through the Human Glycome Project over 100,000 glycome profiling has been performed, resulting in many prominent discoveries of promising glycan biomarkers.

Glycans are inherited as complex traits and also affected by epigenetic memory of environmental factors [7]. Environmental factors such as smoking and diabetes could alter the glycan repertoire directly or by increasing biological (Figure 1), [2, 8].

Reports from Italy and US indicate that in case of insufficient ICU capacity triage of COVID-19 patients is based on subjectively defined criteria that are not based on strong data. At present, we still do not understand the molecular basis of severe COVID-19 symptoms, so research is urgently needed to identify biomarkers that could enable early identification of high-risk individuals. Therefore, it is of utmost importance to biobank large number of plasma samples of both severe and mild cases, so that modern profiling technologies can be used to identify molecular risk factors during this and for future outbreaks. We understand that our colleagues at the frontlines of this pandemics are overwhelmed with saving lives, but biobanking samples has a potential to save many more lives in the future.

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Research Perspective

Geroprotective and senoremediative strategies to reduce the comorbidity, infection rates, severity, and lethality in gerophilic and gerolavic infections

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ABSTRACT

The recently identified SARS-CoV-2 betacoronavirus responsible for the COVID-19 pandemic has uncovered the age-associated vulnerability in the burden of disease and put aging research in the spotlight. The limited data available indicates that COVID-19 should be referred to as a gerolavic (from Greek, géros "old man" and epilavís, "harmful") infection because the infection rates, severity, and lethality are substantially higher in the population aged 60 and older. This is primarily due to comorbidity but may be partially due to immunosenescence, decreased immune function in the elderly, and general loss of function, fitness, and increased frailty associated with aging. Immunosenescence is a major factor affecting vaccination response, as well as the severity and lethality of infectious diseases. While vaccination reduces infection rates, and therapeutic interventions reduce the severity and lethality of infections, these interventions have limitations. Previous studies showed that postulated geroprotectors, such as sirolimus (rapamycin) and its close derivative rapalog everolimus (RAD001), decreased infection rates in a small sample of elderly patients. This article presents a review of the limited literature available on geroprotective and senoremediative interventions that may be investigated to decrease the disease burden of gerolavic infections. This article also highlights a need for rigorous clinical validation of deep aging clocks as surrogate markers of biological age. These could be used to assess the need for, and efficacy of, geroprotective and senoremediative interventions and provide better protection for elderly populations from gerolavic infections. This article does not represent medical advice and the medications described are not yet licensed or recommended as immune system boosters, as they have not undergone clinical evaluation for this purpose.

INTRODUCTION

Aging is a complex, multifactorial process [1] that leads to loss of function and is the primary risk factor for major human pathologies including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases [1, 2]. Although there is still much debate in the scientific community, proposals have been made to classify aging as a disease in order to develop therapeutic strategies to prevent or delay the onset of age-related illnessess [3–5]. Increasing frailty with age leads to an increased risk of many diseases. These diseases are commonly referred to as age-related [6]. Many pathogens are more infectious and prevalent in the elderly, [7–10] and may be referred to as gerophilic (from Greek, *géros* "old man" and *philia*, "love"). Some infections, including COVID-19, are not exclusively gerophilic, as younger people may also become infected. However, these individuals have mild symptoms or remain asymptomatic, while the elderly experience substantially more severe symptoms and lethality. The term gerolavic (from Greek, *géros* "old man", and *epilavís*, "harmful") may more appropriately

describe infections that cause the most harm in the elderly.

COVID-19 is a gerophilic and gerolavic infection

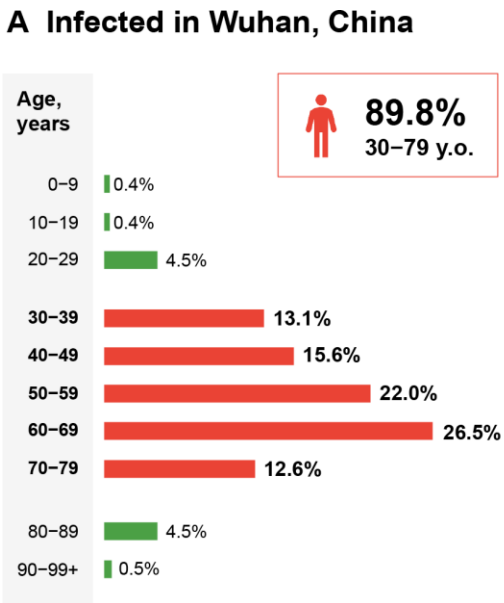
Statistics from the COVID-19 pandemic indicate that COVID-19 is a gerolavic infection, one that disproportionately affects the elderly (Figure 1). The majority of the infected population are 50 and older, while the majority of the deceased are 60 and older [11]. At the same time, fewer than 10% of the infected were 30 or younger, while mortality rates are 2.8% in males versus 1.7% in females [12]. However, these values for the young are skewed upwards because of higher mortality among the elderly. According to Worldometers [13], an online resource aggregating data on COVID-19, of the 139,580 people infected worldwide as of March 13, 2020, 70,733 patients had recovered and 5,120 had died. Based on these data, the mortality rates (number of deaths/number of cases), or the probability of dying if infected by the virus, were determined to be 3.6% for individuals aged 60-69, 8% for individuals aged 70-79, and 14.8% for patients aged 80 years or older. An open coronavirus analysis project by the Nobel Laureate Michael Levitt [14] and a recent study by Mizumoto et al. [15] provide further insight into the mortality rates of COVID-19, specifically using data from the Diamond Princess Cruise, where all passengers were exposed to SARS-CoV-2 for an extended period. Of the approximately1,690 passengers over 65 years of age, 7 passengers died, suggesting a death rate of 0.41%. This death rate is approximately 4.3 times higher than that of influenza. As more countries start reporting statistics, these death rates are likely to be adjusted. These statistics indicate that the infectivity of SARS-CoV-2, and the severity and lethality of COVID-19, are age-related.

The challenges of assessing the burden of gerolavic epidemics

The online resources Our World in Data [16] and The Lancet's Global Burden of Disease [17] provide deep visual insight into the global burden of disease by cause and demographics. In 2017, there were 56 million deaths globally; over two-thirds of these (76%) were in people over 50 years of age. According to the online period life tables put out by the US Social Security Administration [18], the annual chance of death in 2015 (the probability of dying within one year) for a person over 80 was 5.2%, increasing to 14.8% by the age 89.

According to estimates by the US Centers for Disease Control [19], approximately 5,945,690 individuals older than 65 had symptomatic influenza during the 2017-2018 season, resulting in 3,329,586 medical visits, 540,517 hospitalizations, and 50,903 deaths. Hence, the death rate for those hospitalized with influenza was 9.4% for patients over 65.

However, many of the COVID-19 patients over 65 years have one or more comorbities [20], and it is often difficult



B Infected and Diseased in China

Age	Infected, N	Infected, %	Deaths, N	Deaths, %
0–9	416	0.9%	0	0.0%
10–19	549	1.2%	1	0.1%
20–29	3,619	8.1%	7	0.7%
30–39	7,600	17.0%	18	1.8%
40–49	8,571	19.2%	38	3.7%
50–59	10,008	22.4%	130	12.7%
60–69	8,583	19.2%	309	30.2%
70–79	3,918	8.8%	312	30.5%
≥80	1,408	3.2%	208	20.3%
Total:	44,672	100.0%	1,023	100.0%

89.7%

99.2%

Figure 1. COVID-19 as a gerophilic and gerolavic infection. (**A**) Distribution of patients diagnosed in the city of Wuhan only through February 11, 2020. (**B**) Age distribution of the infected and diseased patients in Mainland China through February 11, 2020. The figures are adopted and generated from [12] (http://weekly.chinacdc.cn/en/article/id/e53946e2-c6c4-41e9-9a9b-fea8db1a8f51).

to attribute the cause of death exclusively to the gerolavic coronavirus. Currently, there are no accurate statistics linking smoking status, lifestyle, and behavior to the severity and lethality of COVID-19. Despite this, it is possible that these factors, as well as frailty and comorbidities, play a substantial role. Gerolavic diseases such as COVID-19 may not significantly increase the yearly death rates for each individual age group; however, these diseases substantially accelerate death from multiple conditions, and compress the process to less than two weeks. Comorbidity is also the likely cause of the substantial differences in death rates among different countries due to differences in patient demographics, levels of preparedness, when the epidemic began locally, and reporting [13]. Only the data available from China, where the epidemic has subsided, and the Diamond Princess cruise ship were used for this study.

Aging and immunosenescence

One of the possible causes of the age-associated increases in COVID-19 infection rate, severity, and lethality is immunosenescence. Immunosenescence is a well-known age-related process contributing to the global burden of disease [21]. It is among the major factors underlying the difference between younger and older populations in the response rate to vaccinations and the virulence of infectious diseases [22–24]. Among the factors contributing to immunosenescence is the chronic involution of the thymus gland with increased age. Indeed, the infection rates of COVID-19, separated by age, are correlated with involution of the thymus [12]. The thymus gland is most active early in life, reaching maximum size within the first year. Its activity then declines with age until an individual reaches 40 to 50, after which there are negligible traces of the thymus remaining, replaced by fibrotic tissue [25]. As a result of thymic involution, the number of naïve T cells exiting the thymus decreases significantly, with substantial declines in older age [26].

Besides thymic involution, there are many other factors driving immunosenescence and the increase in multimorbidity that occurs during aging [22, 27, 28]. Figure 2 illustrates the hypothesised reciprocal relationship between immunosenescence and infectious disease acquisition. In this model, age-associated immunosenescence leads to a reduced ability to resist infection, while infection produces biological damage and loss of homeostasis. This ultimately contributes to accelerated aging and the development of age-related diseases, and further accelerates immunosenescence. In support of this model, infections and other age-related diseases are among the main causes of death in the developed world and in developing countries.

Addressing a gerolavic virus: classical versus geroprotective and senoremediative strategies

Due to the gerolavic nature of COVID-19, the classical preventative measures and treatment strategies used for targeting infectious diseases may not be as effective, and there is a need for alternative geroprotective and senoremediative strategies. There are multiple clinical trials in progress using established medical interventions to treat COVID-19, with the number of studies rapidly increasing [23]. For a list of promising SARS-CoV-2/COVID-19 targets and treatment approaches, please refer to the Global Health Drug Discovery Institute's portal dedicated to COVID-19 [29].

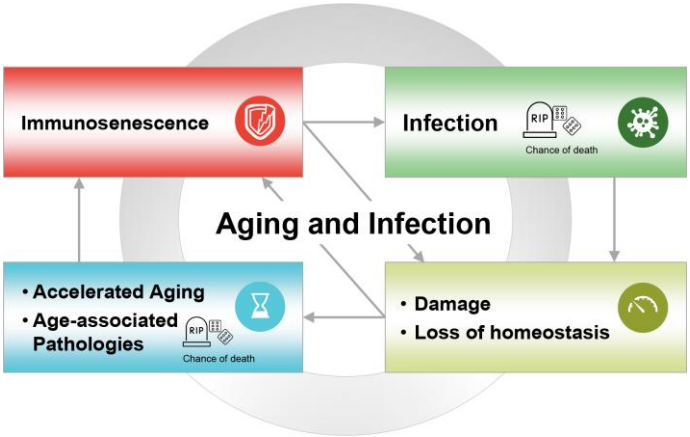


Figure 2. The vicious circle of aging and infection, where age-associated immunosenescence leads to reduced ability to resist infection; infection leads to increased damage, loss of homeostasis, and accelerated aging; which in turn leads to age-related diseases, further accelerating immunosenescence. Infections and other age-related diseases are among the main causes of death in the developed world and developing countries.

Here we compare the expected benefit of treatments for elderly populations (60 years and older) that are currently in development, including standard preventative strategies such as vaccines and antivirals targeting SARS-CoV-2, and the potential added benefit of speculative geroprotective strategies such as rapalogs, NAD+ boosters, senolytics, and stem cell treatment. These additional measures may be used in isolation or as adjuvant therapies to reduce infection risk, symptom severity, or improve vaccine efficacy.

Vaccines

Vaccine development is one of the most successful approaches for combating viral diseases globally, and is often regarded as one of the greatest advances in biomedical science and integrated healthcare. Currently, there are around 60 active clinical trials related to a SARS-CoV-2 vaccine, most of them taking place in China [30]. Broadly speaking, the success of a vaccine partly depends on the similarity of the vaccine strain with the viral pathogenic strain in question. In addition, an individual's immune response must be sufficiently strong to mount a reaction to the vaccine that can later confer protection against the pathogen, should exposure occur. Our current strategy for targeting annual influenza viral outbreaks focuses on effective vaccination based on predictions of strain variants. People >60 years of age with chronic medical conditions, such as type 2 diabetes or cardiovascular disease, direct immunosuppression from HIV, post-transplant or biologic treatment, pregnant individuals, or those with BMI>40, are believed to be at higher risk for influenza infection due to a weakened immune response [31]. Similarly, vaccines do not provide complete protection in older populations due to age-related declines in immune function and accumulation of multi-morbidities. Outbreaks can occur in elderly nursing homes even when vaccination rates reach 80-98% uptake [32]. Thus, even when a successful vaccine for SARS-CoV-2 becomes available, a geroprotective agent might be used in combination with the vaccine to boost the immune response. Currently in most countries, the influenza vaccine formulation is determined 6-9 months before the expected outbreak season and the strains are based on the precedent season's viruses. As a result, vaccine efficacy is expected to differ from season to season. Thus, an ongoing additive geroprotective therapy is of high importance [33, 34] and is applicable beyond the current pandemic. While vaccines may be the best preventative strategy for reducing the infection rates, severity, and lethality of COVID-19, the rates to vaccines in the elderly will likely be lower [35] and vaccine potentiation strategies [36] may be explored and evaluated in clinical trials.

Chemoprophylactic and therapeutic therapies

While chemoprophylaxis is not routinely indicated and is not considered a replacement for vaccination, using influenza as an example, prophylactic treatment prior to symptom onset in high-risk groups or after close contact exposure to the virus is an alternative preventative strategy against viral disease [31]. For influenza, the neuraminidase inhibitors oseltamivir and zanamivir are occasionally given prophylactically to high-risk individuals in long-term care facilities during outbreaks [37]. Nevertheless, there is currently no definitive benefit proven for antiviral treatment outside of these specific circumstances, as it comes at a cost and may be associated with side effects; for example, zanamivir can induce bronchospasms in patients with chronic respiratory disease and asthma. Pharmacotherapy for individuals with infection remains the cornerstone of clinical practice. The success of antiviral treatment is condition-specific, ranging from new, direct-acting antiviral drugs that offer a potential cure for hepatitis C [38]; to the highly active antiretroviral drugs that enable HIV positive individuals the prospect of a healthy life expectancy while on treatment; to antiviral drugs for herpes simplex types 1 and 2 that lead to symptom alleviation but do not eradicate the latent infection; to antivirals for seasonal influenza that are believed to reduce symptom duration, and reduce complications and transmission risk.

Other anti-influenza medications licensed for treatment, aside from oseltamivir and zanamivir, consist of an intravenous neuraminidase inhibitor, peravamir, and a novel oral inhibitor of cap-dependent endonuclease, baloxavir. Neuraminidase inhibitors are effective against both influenza A and B, while an additional class of antivirals that are no longer recommended for treatment of influenza due to reduced efficacy, neurological side effects, and widespread resistance, adamantanes (M2 inhibitors, amantadine and rimantadine), are only active against influenza A [31]. Although many patients with influenza exhibit minimal clinical improvement upon treatment with these medications, they are currently recommended for treatment of all hospitalised patients, even prior to laboratory confirmation of influenza infection. Evidence shows that the greatest benefit is seen when these drugs are administered 24-30 hours prior to symptom onset, in which case they reduce symptom duration by 0.5-3 days and reduce transmission risk [39–42].

Symptomatic treatments

According to the recent COVID-19 treatment guidelines in China [43], symptomatic treatment for COVID-19 patients is recommended for mild cases and consists of

rest, isolation, adequate hydration, analgesia, and antipyretic medication. Moderate and severe cases (mostly hospitalized) require additional measures, such as careful fluid balance, intravenous antibiotics for superinfections, oxygen supplementation, non-invasive ventilation with or without positive pulmonary pressure, and in some cases intubation and mechanical ventilation. Although the projected global infection rates are variable, we share a common concern that outside of China there may be an insufficient number of beds for hospitalization and ventilation units if the disease spread does not slow down.

COVID-19 rehabilitation

Even asymptomatic COVID-19 infections can induce lung fibrosis, which may lead to reduced function of the respiratory system. Further, severe cases are often complicated by bacterial infections and pneumonia, leading to fibrosis. Therefore, COVID-19 rehabilitation may include antifibrotic compounds, anti-COPD, and regenerative medicine therapies.

Geroprotective and senoremediative strategies for COVID-19 gerolavic infection

There are multiple interventions proposed in the academic literature to remedy age-associated increases in infection rates, severity, and lethality for a variety of infections. For example, regular increased physical activity has been proposed to reduce immunosenescence [44]. Fahy et al. [45] and Horvath [46] have suggested that a combination of the potentially geroprotective compound metformin, recombinant human Growth Hormone (rhGH), and dehydroepiandrosterone (DHEA) may reverse biological age, as measured using the methylation aging clock, and immunosenescence [45]. Geroprotectors were previously proposed to enhance human radioresistance in extreme conditions [47]. While there is no clinical evidence yet suggesting age reversal or improved immune function in the elderly, efforts are being made to identify new geroprotectors using human data and artificial intelligence [48–50]. Further, the use of natural compounds that mimic the effects of known geroprotectors is generally recognized as safe [51]. However, attempts have been made to develop criteria for the evaluation of geroprotectors for clinical validation.

There are multiple strategies proposed to restore immune function in the elderly [52], and multiple databases of geroprotectors exist [53, 54]. However, to date the only known geroprotectors backed by promising clinical evidence of improved immune response to viral infection in the elderly, although still limited by a lack of large clinical trials, are sirolimus (rapamycin) and everolimus. These may be used as single agents in combination with other treatments (Figure 3).

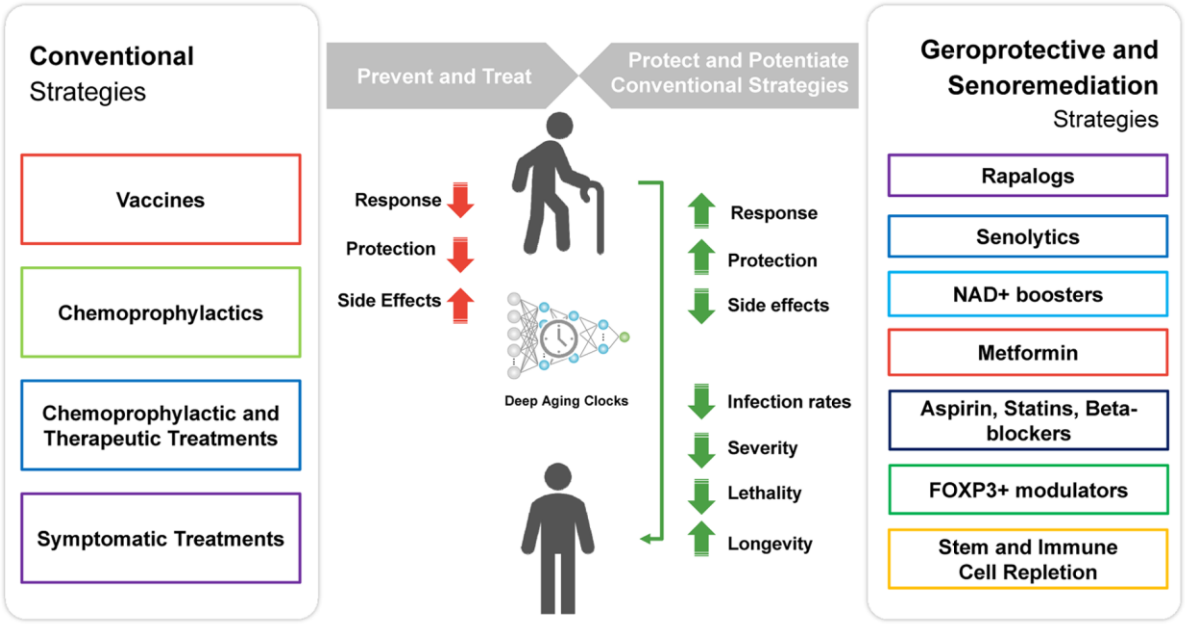


Figure 3. Geroprotective and senoremediative strategies, such as a course of low-dose rapamycin, may potentiate the response to conventional prevention and treatment strategies, prevent infection, reduce disease severity and lethality, and may also increase longevity.

Rapamycin and rapalogs

Sirolimus (rapamycin) is a well-known geroprotector, known to effectively increase lifespan and slow aging in many species, including yeast [55, 56], *Drosophila* [57, 58], *C. elegans* [59], and mice [60–64]. It also delays age-related diseases in humans [65–68], and Blagosklonny proposed rapamycin for the prevention of multiple age-related diseases in humans [69–72].

Sirolimus and rapalogs are commonly used as immunosuppressants. Rapalogs, the derivatives and mimetics of rapamycin, target critical factors in the rapamycin (TOR) pathway. Everolimus (RAD001), another close structural derivative of sirolimus developed by Novartis, acts as an immunosuppressant; but like sirolimus, it has many other properties beyond immunosuppression [73]. Paradoxically, these compounds also exert immunostimulatory effects, such as boosting T cell responses in reaction to pathogen infection and vaccination [74]. Nevertheless, this would not be the first case of a physiological paradox in clinical medicine. The administration of beta-blockers to heart failure patients at first seemed contradictory, as these compounds slow down an already failing heart, but proved to provide the most benefit for the treatment of heart failure patients. Likewise, hormonal treatment of hormone-dependent cancers, such as testosterone-dependent prostate cancer, seems incongruous. However, administration of a synthetic version of gonadotropin-releasing hormone (GnRH) in a different dosing regime from the cyclical secretion that occurs physiologically, which normally indirectly increases testosterone levels, actually reduces hormone levels. Therefore, it might be possible that a drug that is known to be an immunosuppressant might in a different dosing regimen prove to be an immunostimulant. However, extremely cautious clinical validation is required as this treatment might carry significant risks; indeed, there is some indication that morbidity from coronavirus infections occurs from secondary overactive immune responses [75, 76]. In addition to rapamycin, other agents that inhibit mTOR, such as Torin1, Torin2, AZD8055, PP242, KU-006379 and GSK1059615, may act similarly to rapamycin in low-doses and may have a geroprotective effect [77–79]. Substantial pre-clinical validation would be required to apply these compounds to specific age-associated diseases and to explore clinical applications of these compounds in human clinical trials.

Multiple clinical observations suggested that patients with cytomegalovirus (CMV) disease who were treated with rapamycin demonstrated better outcomes and were better able to control CMV viremia than patients treated with standard calcineurin inhibitor-based immunosuppression following transplantation [74, 80]. In 2009, two seminal studies of sirolimus demonstrated the immunostimulatory effects of rapamycin on the CD8+ memory T cell response following pathogen infection [74, 80]. Later studies also showed that monkeys treated with sirolimus exhibited increased recall responses and enhanced differentiation of memory T cells following vaccination with Modified Vaccinia Ankara [81].

Additional clinical studies by Mannick et al. [82, 83] demonstrated the immunostimulatory role of rapalogs in the elderly using the Novartis rapalog everolimus (RAD001), a close structural analog of sirolimus (rapamycin). Administration of everolimus ameliorated immunosenescence in healthy elderly volunteers and enhanced the response to the influenza vaccine by around 20% at doses that were well tolerated [82]. Further studies demonstrated enhanced immune function and reduced infection in elderly patients receiving tolerable doses of everolimus. Mannick et al. also conducted a phase 2a randomized, placebo-controlled clinical trial which demonstrated that a low-dose combination of dactolisib (BEZ235) and everolimus in an elderly population was safe and associated with a significant (P=0.001) decrease in the rate of reported infections [83].

Mannick and colleagues further conducted a phase 2a randomized, placebo-controlled clinical trial that demonstrated that a low-dose combination of dactolisib (BEZ235), a PI3K inhibitor [84] and catalytic mTOR inhibitor, and everolimus in an elderly population was safe and associated with a significant (P=0.001) decrease in the rate of reported infections [83]. A follow-up trial of dactolisib alone (BEZ235 rebranded as RTB101) for prevention of respiratory tract infections in the elderly did not meet the primary endpoint and further trials were withdrawn [85]. In prior studies, everolimus (RAD001) was used as a standalone agent or in combination with dactolisib, which may explain the phase 3 failure of BEZ235/RTB101. There are over 95 phase 3 and phase 4 studies for these agents [86], and they are generally well tolerated even in high doses. Even though it may not be commercially viable due to the patent expirations, clinical trials should be conducted to evaluate the effectiveness of these agents for protection against SARS-CoV-2 (COVID-19) and other gerophilic and gerolavic infections.

Metformin

Metformin is a drug approved to treat type 2 diabetes but appears to target a number of aging-related mechanisms, including decreasing IGF-1 levels, inhibiting mTOR, and inhibiting mitochondrial complex 1. Metformin is currently in the first large-scale human

clinical trial of aging, the Targeting Aging with Metformin (TAME) study, which is investigating its effect on time to a new occurrence of a composite outcome that includes cardiovascular events, cancer, dementia, and mortality [87]. Metformin would likely still be contraindicated in elderly patients with advanced chronic kidney disease and eGFR<15. A reduced dose would potentially be required for eGFR<30 due to a risk of lactic acidosis. The effects on gerophilic and gerolavic infections should be carefully examined in the context of the TAME study, and other clinical trials involving metformin.

NAD boosters

Nicotine adenine dinucleotide (NAD) is a cofactor of multiple fundamental enzymes. It is involved in metabolic regulation through the Krebs (citric acid) cycle, oxidative phosphorylation, and cellular signaling, as well as cellular senescence and DNA repair through the poly-ADP-ribose polymerases (PARPs), sirtuins, and CD38. NAD levels decrease with aging, and benefits of NAD supplementation have been reported in multiple animal studies. Although no proof of a similar effect in humans has been shown, several clinical trials are in progress [88–91]. Supplementation with nicotinamide riboside (NR) in one human study produced an improvement in exercise capacity in a population with a mean age of 71 [92]. This compound was also shown to reduce blood pressure in hypertensive patients [93].

Nicotinic acid is another NAD precursor that is converted in the body to NAD by the enzymes NAPRT, NMNAT, and NADS. Large-scale trials of nicotinic acid for cardiovascular disease [94, 95] showed some efficacy, but produced adverse side effects, such as headache, skin flushing, and dizziness [96].

NAD acts at a cellular level and it is still unclear whether oral or intravenous supplementation with NAD donors, such as NR and nicotinic acid, will increase NAD levels and exert a clinical benefit in humans. However, COVID-19 patients may benefit tremendously from these compounds, as SARS-CoV-2-infected patients have increased levels of CD38+, and NAD has been shown to enhance DNA repair via PARP pathways [97].

Caution should be exercised when conducting any clinical trials for NAD boosters against gerophilic and gerolavic infections, as the underlying biology of NAS metabolism and viral infections is still poorly understood. Recent studies in humans demonstrate that NR supplementation reduces the levels of circulating inflammatory cytokines [98], while Nicotinamide Mononucleotide (NMN) may reduce the expression of these cytokines [99]. Other studies implicate NAD in increased cytokine production [100] and the NAD+-consuming enzyme CD38 in increased inflammation [101]. Additional immunological studies of NAD boosters must be performed before clinical trials may be conducted. However, considering the large consumer base of NR and NMN supplements, it may be possible to conduct metastudies on influenza and SARS-CoV-2 infectivity, severity, and lethality.

Senolytics

Senolytics are drugs that are postulated to selectively destroy senescent cells, which accumulate with aging and exhibit senescence-associated secretory phenotype (SASP), through senolysis, apoptosis, immunosurveillance, or other mechanisms of action [102]. SASP is now hypothesised to lead to NAD depletion and thus initiate or perpetuate an increase in sterile chronic inflammation. Many drug classes, ranging from fibrates to cardiac glycosides, have been reported to have senolytic properties in animal models [103]. However, recent promising human data have been reported with the tyrosine kinase inhibitor dasatinib in combination with the plant flavonol quercetin in a trial by the Mayo Clinic [104]; flavonoid polyphenols have also proven beneficial. In addition, pre-clinical and clinical data suggest that flavonoids may be used for prophylaxis in upper respiratory tract infections [105].

Although senolytic drugs would have a scientifically plausible role in biological age reversal and thus reduction of mortality from gerolavic viruses like SARS-CoV-2, it has not been shown that these classes of drugs would protect against infection or could be used as adjuncts to vaccination. In addition, there remains the risk that senolytics would not be sufficiently specific to discriminate between deleterious senescent cells and quiescent (dormant) cells, which might still differentiate into the mature cell types of a given tissue, and could thus deplete beneficial protective stem cell reserves.

Intermittent caloric restriction

It has been shown in multiple studies that calorie restriction leads to increased lifespan and improved cardiometabolic markers, even when initiated in middle age [106]. Caloric restriction should be considered as a preventive measure on a long-term basis and is indicated for younger individuals. Some elderly patients already have frailty syndromes and evident sarcopenia/ osteopenia, which limits the suitability of intermittent caloric restriction. Nevertheless, the

benefits of time-restricted feeding and intermittent fasting go beyond simple caloric restriction due to the production of ketones. Ketones are active signaling molecules that play a major role in the PPAR, sirtuin, NAD and CD38 pathways, encourage autophagy (the removal of damaged cellular materials), modulate the immune response, and have been explored in clinical trials as an adjuvant therapy for cancer treatments [107]. Within 8-12 hours of food restriction, ketones are believed to rise to 0.2 to 0.5mM and continue to increase within the first 48 hours to 1 to 2mM [108]. Under fasting conditions the major body ketone in the plasma, beta-hydroxybutyrate (BHB), increases. BHB is believed to confer the major metabolic benefit of fasting and is in development as an independent therapeutic supplement.

T cell activation

An age-related decrease of thymic function consequently reduces the levels of specific T cell subsets [109]. FOXP3+ regulatory T (Treg) cells are critical in homeostasis of the immune system and are believed to start declining in numbers at around 50-60 years of age; this remains one of the fundamental drivers of immunosenescence. There are two known origins for Treg cells: thymus-derived Treg cells and peripherally-derived Treg (pTreg) cells. Thus, inducing a peripheral Treg response in older individuals might be a feasible strategy for increasing Treg cell levels until we have more plausible options for thymic rejuvenation. FOXP3 transcription factor (TF) is the most important regulator of Tregs and age-associated immunosenescence. FOXP3 TF expression is regulated by chemical modification by sirtuin (sirt) and histone deacetylases, in particular Sirt1 and HDAC9 [110, 111]. Interestingly, NAD is essential for sirtuin action. Therefore, it is plausible that NAD and NAD-related compounds such as NR and NMN, which are under investigation as therapeutic interventions that increase serum and cellular NAD levels, also act via Sirt1 along the FOXP3 and Treg axis, and play a role in immunosenescence and "inflammaging".

A brief summary of the conventional and geroprotective and senoremediative strategies for patients 60 or older is provided in Table 1.

On the timing of geroprotective interventions

While there are decades of clinical evidence supporting the use of rapalogs, such as sirolimus, everolimus, and metformin, substantial meta-analysis and additional clinical trials must be conducted to understand the population-level and individual effects of these drugs taken as single agents and in combination in the context of gerolavic diseases. In this paper I propose conducting clinical trials on these known geroprotectors as a preventative measure before patients are exposed to disease (Figure 4). In the case of COVID-19 as the number of cases worldwide increases, meta-analysis of infection rates, severity, and lethality should be performed rapidly to evaluate the effects of geroprotectors, with particular focus on rapamycin. Since COVID-19 engages the immune system to damage the lungs, it may be entirely plausible that the immunomodulatory properties of rapamycin may go beyond prevention and may provide an effective treatment option. However, this hypothesis must be validated using meta-analysis before being proposed for a clinical trial.

As COVID-19 causes substantial lung damage, antifibrotics, senolytics and other geroprotectors may be explored in clinical trials to assist in patient recovery to prevent a reduction in respiratory function.

Using biological aging clocks as markers of immunosenescence

Since senescence varies among individuals, a person's chronological age is not as important as their biological age. For several years, scientists have sought accurate aging biomarkers that may predict an individuals' biological age and, independently of immunosenescence, their risk of morbidity and mortality. These biomarkers, or "clocks", could then be used to test for the effectiveness of proposed geroprotective treatments and as surrogate markers in anti-aging clinical trials. While there are no reliable aging clocks to evaluate immunosenescence and inflamaging [112], these biomarkers may be rapidly developed using historical data. At present, age clocks trained on clinical blood tests [113], transcriptomic [114] and proteomic data [115], methylation clocks [46, 116], microbiomic clocks [117] and other clocks have been described. Recent advances in artificial intelligence have enabled the development of multi-modal multi-omics age-predictors, able to learn complex non-linear patterns and extract the most important features [113, 118]. None of these currently have robust clinical validation and cannot yet serve as companion biomarkers for geroprotective and anti-aging interventions intended to ameliorate the population-level effects of infectious diseases during flu seasons and pandemics. We call for rigorous clinical validation and further development of biological aging clocks that could, in the future, allow us to measure the effectiveness of the numerous speculative geroprotective and senoremediative interventions described herein.

Table 1. The benefits and risks of conventional and geroprotective strategies for patients 60 or older.

Intervention	Mechanism	Validation	Expected Benefit	Side Effects and Risks
Conventional Antiviral Strategies				
Vaccines	Contains antigenic material that mounts an immune response which is then augmented on exposure to the virus and offers protection against the disease.	Major global antiviral strategy to prevent new infective cases and fight epidemics. Viral illnesses eradicated/controlled due to previous successful vaccination programs.	Vaccinated people do not develop an infection or develop a milder infection. Reduces the number of new cases and the progression of the epidemic. Lower effectiveness in the elderly.	COVID-19 is a gerolavic infection. Efficacy of the vaccine will likely be significantly reduced due to immunosenescence and multimorbidity. Possible immunogenicity and mild viral prodrome symptoms as a result of vaccination.
Targeted Antibodies for COVID-19	Antibodies of serum of recovered individuals. Antibodies targeting specific SARS-CoV-2 proteins.	Successfully trialed in other viral diseases including Ebola.	Reduction in disease severity and lethality in exposed individuals.	Risk of systemic immune reactions and certain blood borne infections.
Targeted Small Molecule Drugs for SARS-CoV-2	Selective small molecule inhibitors targeting SARS-CoV-2 proteins such as 3C-like protease.	Multiple examples from Influenza. Neuraminidase and endonuclease inhibitors.	Reduction in disease duration, severity, and lethality in exposed individuals.	Mild side effects such as nausea, vomiting, diarrhea, etc.
Symptomatic Treatments	Non-steroidal anti-inflammatory drugs (NSAIDs), antibacterials, pain management.	Multiple clinical trials, common use.	Reduction in severity of disease.	Mild side effects such as nausea, vomiting, diarrhea, etc.
Geroprotective and Senoremediation Strategies				
Rapalogs	mTOR inhibition.	Mouse, monkey, and human phase 2a studies in aging, over 95 phase 3 and phase 4 studies in humans for multiple diseases.	Infectivity: reduced; significant reduction when administered with vaccines. Severity: reduced. Lethality: reduced. Other benefits: Improved immune function that might increase the probability of increased longevity.	Risk of pancytopenia and potentially fatal infection risk. Risk of nephrotoxicity although milder side effect profile reported in preliminary studies.
Metformin	mTOR inhibition. Mitochondrial complex 1 inhibition. Reduction of hepatic gluconeogenesis.	Long-term use in humans. 1st line treatment for type 2 diabetes, leading to weight loss and improved glucose	Reduction in cardiovascular events, cancer, dementia, and mortality.	Diarrhea in 20% of patients. Contraindicated in patients with eGFR <15 and reduced dose required if eGFR <30

		metabolism. Improved fertility in polycystic ovarian syndrome.		due to risk of lactic acidosis.
NAD Boosters	Sirtuin Activation. PARP efficacy augmentation. Improvement in cellular metabolism and repair mechanisms.	Reduction in blood pressure. Improvement in exercise capacity.	Reduction in immunosenescence, thus reduced infection risk and improved vaccine response. Improved endothelial function and reduced cardiovascular disease risk, potential increased lifespan.	No increase in cellular NAD levels with supplementation and no improvement in immune function. Side effects of nicotinic acid include headache, skin flushing and dizziness. Uncertain effects on circulating inflammatory cytokines.
Senolytics	Reduction in anti-apoptotic senescent inactive cells. Multiple drug classes mentioned with varying disease mechanisms.	Reduced adipose tissue senescent cell burden in a short human trial. Improvement in pulmonary fibrosis treatment.	Reduction in senescent cell burden and thus chronic sterile inflammation; reduction of chronic disease burden.	Removal of quiescent stem cells and depletion of stem cell reserve. Drug-specific side effects. Possible elimination of pre-senescent cells leading to cardiovascular, kidney, liver, and CNS damage.
Caloric Restriction and Intermittent Fasting	Promotion of autophagy, metabolic flexibility. Increase in beta hydroxybutyrate.	Human studies showing improvement in weight, glucose levels, fasting insulin and plasma lipids that go beyond weight loss.	Activated self-defenses, reduction in body weight, improvement of insulin resistance, thus reduced cardiovascular risks and certain cancer type risks.	Sarcopenia, osteoporosis. Risk of nutritional deficiencies and worsening of eating-disorders.
Growth Hormone (GH)	Action via GH receptor and IGF-1 axis.	Used in humans with GH deficiency. Improvement in anabolic function and increase in muscle mass.	Prevention of thymus degeneration and increase in immune function. Improved mood and body composition.	Increased cancer risk. Type 2 diabetes. Connective tissue proliferation.
FOXP3+ activation	One of the key mediators of immune regulation, Tregs express high levels of FOXP3. FOXP3 expression is essential for Treg development and function.	Tregs were unable to develop in a mouse receiving FOXP3-deficient progenitor cells from another animal and retroviral expression of FOXP3 in human T-cells enabled the conversion of non-regulatory naïve T-cells into a Treg-like phenotype.	FOXP3 is a Treg - specific transcription factor essential for Treg functions. Rapamycin facilitates the expansion of functional CD4+CD25+FOXP3+ Treg cells.	Treg cells are a heterogeneous population and their stability and plasticity under inflammatory conditions may pose serious problems for their clinical usage. No therapy yet available to modulate this cell subgroup safely in humans.

DISCUSSION

SARS-CoV-2 is a betacoronavirus that causes respiratory illness, and is genetically most similar to SARS-CoV, the betacoronavirus that caused the SARS epidemic of 2003. The Middle East respiratory syndrome (MERS) epidemic was also caused by a betacoronavirus that induces severe respiratory illness. Both SARS and MERS were contained before they became pandemics, and much of what we predict about the trajectory of COVID-19 comes from what we learned about SARS and MERS [24]. Theoretically, treatments found to be effective against SARS and MERS are the most promising starting points for treatments likely to be effective against SARS-CoV-2.

The SARS outbreak of 2002 was rapidly contained, and no new cases have been reported since 2004 [119]. Since the scale of the outbreak did not provide any commercial benefit for the pharmaceutical industry to develop effective drugs for SARS, much of the discovery efforts stopped after the epidemic. When the news of SARS-CoV-2/COVID-19 emerged in early January 2020, it was difficult to justify a business case for small biotechnology companies to allocate resources to the effort. By January 28th, however, Insilico Medicine allocated resources to generate and test small molecules against the SARS-CoV-2 3C-like protease [120, 121]. As the scale of the current COVID-19 pandemic remains uncertain, it is still difficult to justify allocating scarce company resources to full-scale drug discovery and drug development programs, which may cost tens or even hundreds of millions of dollars [122]. Multiple biotechnology companies are in the same situation and will not be able to proceed without substantial backing from government agencies, non-profit organizations, or bigger pharmaceutical companies. However, given the gerophilic and gerolavic nature of COVID-19, strategies targeting age-associated pathologies and immunosenescence, which could decrease the comorbidity, infection rates, severity, and lethality of the disease, will remain commercially-viable even when the pandemic subsides. In addition, respiratory infections are now the third leading cause of death in the world, following cardiac disease and stroke [123], further justifying the need for these interventions.

Considering the gerolavic nature of COVID-19, where the majority of the seriously affected population is older than 60, classical prevention and treatment strategies may not be effective. Given the severity and lethality of the pandemic, even healthcare systems in developed countries will find it challenging to cope with the increased disease burden and hospital needs. Conventional approaches to prevention such as vaccines are much needed, but even these do not offer complete protection in the elderly due to multi-morbidity and age-related immune declines. Therefore, interventions that enable immunocompromised elderly to mount an immune response to newly developed vaccines are necessary to help eradicate the disease and reduce the associated mortality.

To avoid substantial loss of life and quality of life, primarily among the elderly and vulnerable populations, governments and healthcare systems should investigate preventative and intervention strategies stemming from recent advances in aging research. As discussed in this paper, small clinical studies have shown that several geroprotective and senoremediative interventions, such as treatment with

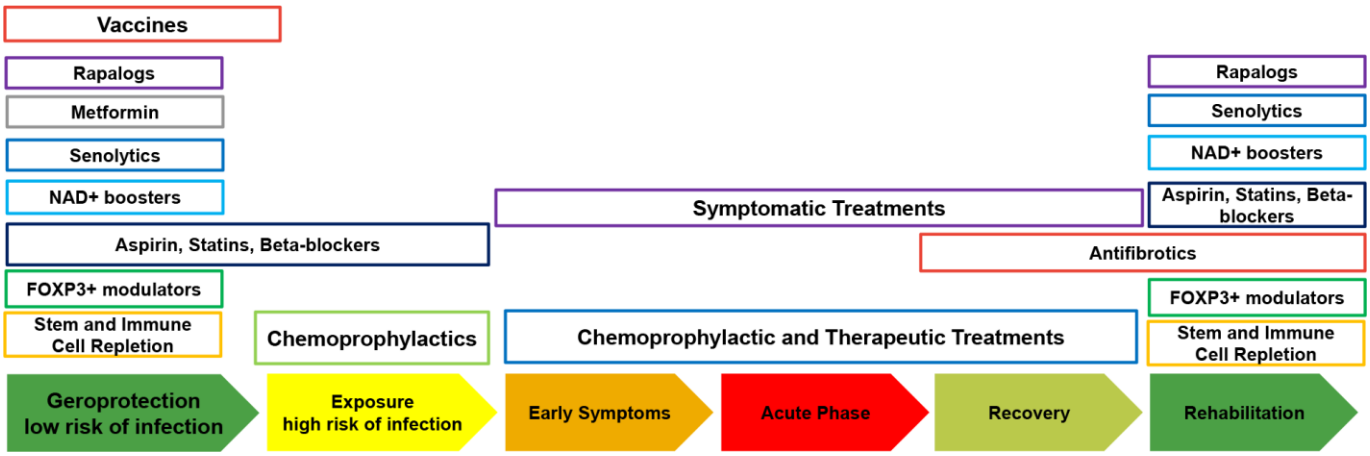


Figure 4. The timing of the administration of geroprotectors for prevention, treatment and rehabilitation of gerolavic respiratory diseases.

sirolimus and rapalogs, can induce immunopotentiation, increase resistance to infection, and reduce disease severity in the elderly, without severe side effects. Serendipitously, during the revision of this article, another group utilizing computational approaches proposed using melatonin and sirolimus (rapamycin) in combination to treat the COVID-19 infection outside the context of geroprotection [124].

Many of these predicted geroprotectors are available as supplements; however, no meta-analysis or metaclinical trials have been performed at scale to evaluate their effectiveness. The COVID-19 pandemic highlights the paucity of clinical trials on the effects of dietary supplements and drugs on aging and immunosenescence. The existence of pseudoscience and anecdotal promotion in the supplement industry does not mean that protective compounds do not exist. Dietary supplement vendors and pharmaceutical companies need to actively engage in preclinical and clinical research to evaluate the effectiveness of the currently available products on immunosenescence and aging.

CONCLUSIONS

This paper is not intended to encourage the use of rapalogs or other potential geroprotectors during the COVID-19 pandemic. It may be possible that some of the potential geroprotectors described in this paper are harmful to the elderly after infection, and may actually increase disease severity and lethality. However, it may be possible to conduct clinical trials on the efficacy of geroprotectors previously tested in human clinical trials in treating COVID-19 and other gerophilic and gerolavic infections.

To combat the growing COVID-19 pandemic, researchers have united globally to tackle a disease that is impacting lives and healthcare systems around the world. After carefully analysing preliminary data, we suggest that COVID-19 has a gerophilic and gerolavic profile, being more infectious and more severe in the elderly. In this paper, we review the current literature on speculative aging reversal treatments, such as experimental geroprotective strategies using everolimus (RAD001) and sirolimus (rapamycin). We summarize the current possible interventions and identify the lack of clinical evidence to support their immediate use with the aim of encouraging further, more rigorous reviews of geroprotective compounds such as rapalogs, metformin, senolytics, and conventional and investigational NAD+ boosters. We also suggest that further clinical studies should be carefully designed and adequately powered to determine if these interventions might provide clinical benefit as adjuncts to vaccines and antiviral treatments by acting as immune response potentiators. Lastly, as with many other diseases, COVID-19 is more common and severe in elderly populations, and we thus invite further research and clinical validation in the field of biological aging clocks. These markers could potentially be used in the future to measure and analyze immunosenescence and the efficacy of interventions claimed to slow down or reverse age-related immune decline.

Disclaimer and limitations

This perspective is of a highly speculative nature presented during the time of a global COVID-19 pandemic. It is intended for a professional audience to stimulate ideas and aid the global efforts of the scientific community to develop effective new treatments for this disease. This article does not represent medical advice or recommendations to patients. There is no clinical evidence to support the use of the treatments described in this article for this indication and the authors do not advise anyone to self-administer these drugs as COVID-19 prevention or treatment. Furthermore, this perspective is based on the limited data from the first weeks of the COVID-19 outbreak. The demographic distribution of the infected and diseased may change and differ in different countries with different social customs and different ethnicities. The media should exercise caution and seek expert medical advice for interpretation when referring to this article to avoid misinterpretation or unsafe messages being delivered to the community amidst exceptional coverage of this disease in the media at present.

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CONFLICTS OF INTEREST

No funding has been provided for this work. Alex Zhavoronkov is a co-founder of Insilico Medicine, a leading artificial intelligence company specializing in target discovery and small molecule generation, and Deep Longevity, a company specializing in deep aging clocks, multimodal age predictors built using deep learning. He has multiple granted patents and patent applications on deep aging clocks, geroprotective

interventions, generative chemistry, generative biology, and artificial intelligence techniques.

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Research Perspective

COVID-19 and chronological aging: senolytics and other anti-aging drugs for the treatment or prevention of corona virus infection?

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Abbreviations: SASP: senescence-associated secretory phenotype

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ABSTRACT

COVID-19, also known as SARS-CoV-2, is a new emerging zoonotic corona virus of the SARS (Severe Acute Respiratory Syndrome) and the MERS (Middle East Respiratory Syndrome) family. COVID-19 originated in China and spread world-wide, resulting in the pandemic of 2020. For some reason, COVID-19 shows a considerably higher mortality rate in patients with advanced chronological age. This begs the question as to whether there is a functional association between COVID-19 infection and the process of chronological aging. Two host receptors have been proposed for COVID-19. One is CD26 and the other is ACE-2 (angiotensin-converting enzyme 2). Interestingly, both CD26 and the angiotensin system show associations with senescence. Similarly, two proposed therapeutics for the treatment of COVID-19 infection are Azithromycin and Quercetin, both drugs with significant senolytic activity. Also, Chloroquine-related compounds inhibit the induction of the well-known senescence marker, Beta-galactosidase. Other anti-aging drugs should also be considered, such as Rapamycin and Doxycycline, as they behave as inhibitors of protein synthesis, blocking both SASP and viral replication. Therefore, we wish to speculate that the fight against COVID-19 disease should involve testing the hypothesis that senolytics and other anti-aging drugs may have a prominent role in preventing the transmission of the virus, as well as aid in its treatment. Thus, we propose that new clinical trials may be warranted, as several senolytic and anti-aging therapeutics are existing FDA-approved drugs, with excellent safety profiles, and would be readily available for drug repurposing efforts. As Azithromycin and Doxycycline are both commonly used antibiotics that inhibit viral replication and IL-6 production, we may want to consider this general class of antibiotics that functionally inhibits cellular protein synthesis as a side-effect, for the treatment and prevention of COVID-19 disease.

The earliest retrospective study of the COVID-19 outbreak in Wuhan, China, published in the Lancet, was among one of the first clinical studies to identify older age as a significant risk factor for in-hospital mortality, suggesting that advanced chronological age may play an epidemiological role in patient clinical outcomes [1].

Mortality was also associated with other co-morbidities, normally considered to be aging-associated diseases, such as diabetes or coronary heart disease, as well as a critical inflammatory mediator of the senescence-associated secretory phenotype (SASP), namely IL-6 [1].

This specific association of COVID-19 fatality with advanced chronological age was directly validated by the CDC in the US population [2] and published in the Morbidity and Mortality Weekly Report (MMWR) on the 18th of March, as follows: "This first preliminary description of outcomes among patients with COVID-19 in the United States indicates that fatality was highest in persons aged ≥85, ranging from 10% to 27%, followed by 3% to 11% among persons aged 65–84 years, 1% to 3% among persons aged 55-64 years, <1% among persons aged 20–54 years, and no fatalities among persons aged ≤19 years".

What could be the biological mechanism(s) by which the COVID-19 virus preferentially targets patients with advanced chronological age?

Two host receptors have been proposed for COVID-19. One is CD26 [3] and the other is ACE-2 (angiotensin-converting enzyme 2) [4]. Interestingly, both CD26 and the angiotensin system show associations with senescence. For example, ACE-2 is a known inhibitor of cell proliferation and the angiotensin system is upregulated in both premature and replicative senescence [5,6]. Remarkably, CD26 is known to be a *bonafide* cell surface marker of senescent cells [7]. Similarly, myofibroblasts (which are considered to be senescent and pro-fibrotic cells) also over-express CD26 and ACE-2 [8,9]. Senescent cells produce large amounts of inflammatory cytokines, as a result of the senescence-associated secretory phenotype (SASP), including IL-6.

Interestingly, the host receptor for MERS-CoV, a highly-related corona virus, is CD26, also known as dipeptidyl-peptidase IV (DPP4) [10-12]. Genetic evidence, including functional studies of existing CD26 human polymorphisms and humanized CD26 transgenic mouse animal models, has directly shown that CD26 is the functional host receptor for MERS-CoV, which is specifically required for host cell attachment, entry and, therefore, productive host cell infections, as well as species restrictions [10-12] Moreover, recent structural studies predict that the COVID-19 spike glycoproteins also directly interact with host cell CD26 [3].

Thus, one hypothesis is that the COVID-19 virus significantly increases mortality in patients with advanced chronological age, because these patients have an increased number of senescent lung cells, which are the host target for COVID-19 viral infection. Interestingly, senescent cells also show an increased propensity for enhanced protein synthesis, which is required to produce SASP inflammatory mediators, which would make senescent cells an ideal host target for efficient viral replication.

Therefore, it would be predicted that senolytic drugs could have a beneficial effect for the treatment and/or prevention of COVID-19 disease. Is there any evidence to support this attractive hypothesis?

Recently, a clinical trial was conducted using COVID-19 positive hospitalized patients, which assessed COVID-19 virus production in response to treatment with two FDA-approved drugs, namely Hydroxy-chloroquine (Plaquenil) and Azithromycin (Z-PAC) [13]. Hydroxy-chloroquine alone, at the standard dosages, was surprisingly effective in reducing COVID-19 viral production. However, the combination of Hydroxy-chloroquine and Azithromycin appeared to be even more effective. The mechanism(s) by which this drug combination halts COVID-19 virus production remains unknown.

What is the known relationship between Hydroxy-chloroquine, Azithromycin and senescence?

Chloroquine and its derivatives, such as Hydroxy-chloroquine, alkalinize the pH in lysosomes, which accumulate in large numbers in senescent cells. This Chloroquine-induced alkalinization functionally prevents the induction and accumulation of one of the most widely-recognized markers of senescence, known as beta-galactosidase (Beta-Gal), a lysosomal enzyme [14]. Hydroxy-chloroquine is also used clinically for the treatment of chronic inflammatory diseases, such as Sjögren's syndrome, and it effectively reduces the salivary and serum levels of IL-6, a key component of the SASP [15].

Azithromycin also has a key relationship with senescence [16]. Recent studies have shown that Azithromycin, and the closely related drug Roxithromycin, both act as senolytic drugs that can target and selectively remove senescent cells, with an efficiency of nearly 97% [16]. Interestingly, in patients with Cystic Fibrosis, Azithromycin is known to have an anti-fibrotic effect, which significantly extends their lifespan, by targeting myofibroblast cells (Discussed in Ref [16]). Cystic Fibrosis patients normally die from lung inflammation and fibrosis, resulting in lung stiffening and an inability to respire. Fibrosis is also known to be an age-related phenomenon, associated with increased numbers of myofibroblasts (senescent cells), which increases with chronological age. Azithromycin functionally acts as an anti-inflammatory drug and reduces SASP mediators, such as IL-1beta and IL-6 [17,18]. This may be due to Azithromycin's high senolytic activity and/or inhibition of protein synthesis.

Interestingly, Azithromycin also inhibits the replication of other viruses, such as Zika and Ebola [19-21]. If this

inhibitory activity reflects Azithromycin's ability to inhibit protein synthesis, then other inhibitors of protein synthesis, such as Rapamycin, should be considered as well (see Supplementary Figure 1).

Consistent with this hypothesis, Rapamycin has been shown to potently inhibit HIV-1 replication [22]. Moreover, Rapamycin shows key anti-aging properties and prevents the onset of senescence [23-25].

Similarly, Doxycycline inhibits mammalian cell protein synthesis as an off-target side effect [26], effectively blocks replication of Dengue virus [27], reduces IL-6 serum levels during viral infection [28] and behaves as an anti-aging drug [29]. Therefore, Doxycycline could provide another inexpensive, but very attractive, option for the treatment or prevention of COVID-19 infection.

Finally, a recent study, using supercomputer-based *in silico* drug-docking to the COVID-19 viral spike protein identified Quercetin as a potential binding partner, to reduce virus-host interactions, with ACE-2 [30]. Quercetin has also been identified as a dietary supplement with senolytic properties [31].

Therefore, we propose that the clinical relationship between advanced chronological age and COVID-19 mortality may suggest the use of senolytic or anti-aging drugs in COVID-19 disease prevention (Figure 1). Of course, clinical trials will be necessary to test this attractive, but speculative, hypothesis experimentally.

Fortunately, several promising senolytic and other anti-aging drugs are already FDA-approved for other disease indications. This approach can significantly accelerate their clinical evaluation through drug repurposing, as they have already been evaluated for their clinical safety, in Phase I trials. As such, these FDA-approved drugs can directly enter into Phase II clinical trials, to test their potential efficacy against COVID-19. Alternatively, in the United States, FDA-approved drugs can be medically-prescribed for an "off-label" use, at the discretion of the practicing physician.

Interestingly, SARS-CoV, a close relative of COVID-19 (SARS-CoV-2), also shows increased susceptibility in patients with advanced chronological age, which has been recapitulated in a mouse animal model of disease pathogenesis [32,33]. Briefly, in young mice (4-8 weeks-old), the SARS-CoV infection is cleared very rapidly, which is accompanied by mild pneumonitis, without the activation of cytokine production. In contrast, in older mice (12-14-months-old), productive infection with SARS-CoV led to a more severe interstitial pneumonitis, with alveolar damage, significant fibrosis and scarring, as well as severe activation of cytokine production, including TNF-α, IL-6, CCL-2, CCL-3, CXCL-10, and IFN-γ [32,33]. This latter mouse model more closely resembles the SARS-CoV disease phenotype, observed in patients with advanced chronological age. Therefore, such a mouse model would also be useful for testing the efficacy of new therapies, specifically targeting the senescent cell

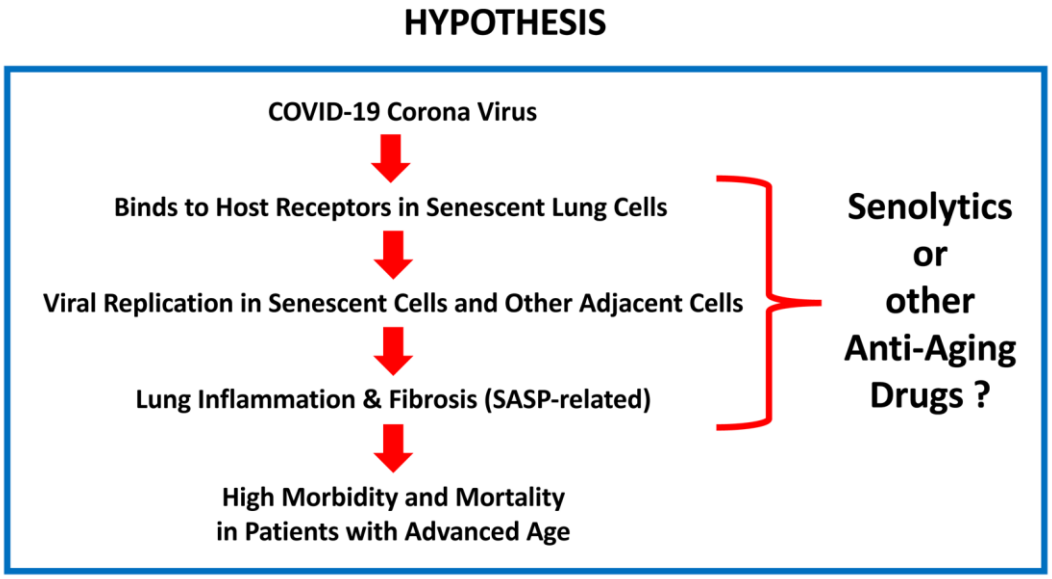

Figure 1. What is the relationship between COVID-19 and advanced chronological age? Here, we suggest that the COVID-19 corona virus preferentially targets senescent lung cells, resulting in increased morbidity and mortality in the aging population. One possible solution for prevention/treatment would be the use of senolytics or other anti-aging drugs. Testing this hypothesis will require the necessary clinical trials, with a focus on drug repurposing.

population and SASP, for the repurposing of these FDA-approved anti-aging drugs.

Disclaimer

This perspective is intended for a professional audience, to stimulate new ideas and to aid the global efforts to develop effective treatments for COVID-19 disease. This article does not represent medical advice or recommendations to patients. The media should exercise caution and seek expert medical advice for interpretation, when referring to this article.

Note Added in Proof

While this Perspective was being reviewed, another clinical trial was published online, confirming the efficacy of Hydroxy-chloroquine and Azithromycin, for treating COVID-19 patients, in a larger cohort of 80 patients. See the following article:

Philippe Gautret, Jean-Christophe Lagier, Philippe Parola, Van Thuan Hoang, Line Meddeb, Jacques Sevestre, Morgane Mailhe, Barbara Doudier, Camille Aubry, Sophie Amrane, Piseth Seng, Marie Hocquart, Julie Finance, Vera Esteves Vieira, Hervé Tissot Dupont, Stéphane Honoré, Andreas Stein, Matthieu Million, Philippe Colson, Bernard La Scola, Véronique Veit, Alexis Jacquier, Jean-Claude Deharo, Michel Drancourt and Didier Raoult. Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: an observational study. https://www.mediterranee-infection.com/wp-content/uploads/2020/03/COVID-IHU-2-1.pdf

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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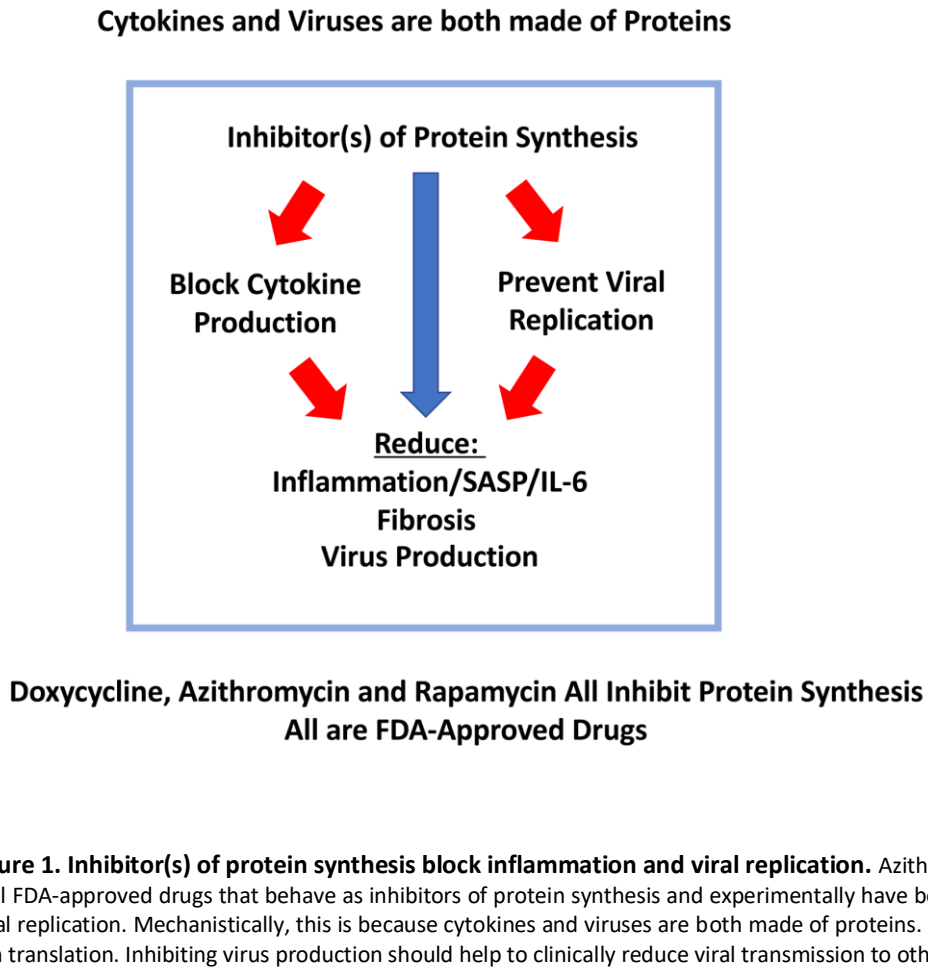
SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Inhibitor(s) of protein synthesis block inflammation and viral replication. Azithromycin, Doxycycline and Rapamycin are all FDA-approved drugs that behave as inhibitors of protein synthesis and experimentally have been shown to reduce inflammation and viral replication. Mechanistically, this is because cytokines and viruses are both made of proteins. Both use the cellular ribosomes for protein translation. Inhibiting virus production should help to clinically reduce viral transmission to other patients.

Research Paper

ACE2 correlated with immune infiltration serves as a prognostic biomarker in endometrial carcinoma and renal papillary cell carcinoma: implication for COVID-19

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ABSTRACT

Angiotensin-converting enzyme 2 (ACE2) is a member of the renin-angiotension system, however, the correlation between ACE2 and prognosis in UCEC (Uterine Corpus Endometrial Carcinoma) and KIRP (Kidney Renal Papillary Cell Carcinoma) is not clear. We analyzed the expression levels of ACE2 in the Oncomine and TIMER databases, the correlation between ACE2 and overall survival in the PrognoScan, GEPIA and Kaplan-Meier plotter databases. The correlation between ACE2 and immune infiltration level and the type markers of immune cells was investigated in TIMER database. A prognosis analysis based on the expression levels of ACE2 was further performed in related immune cells subgroup. The ACE2 promoter methylation profile was tested in the UALCAN database. In addition, we used GSE30589 and GSE52920 databases to elucidate the changes of ACE2 expression in vivo and in vitro after SARS-CoV infection. ACE2 was elevated in UCEC and KIRP, and high ACE2 had a favorable prognosis. The expression of ACE2 was positively correlated with the level of immune infiltration of macrophage in KIRP, B cell, CD4+T cell, neutrophil and dendritic cell immune infiltration levels in UCEC. ACE2 was significantly positively correlated with the type markers of B cells and neutrophils, macrophages in UCEC, while ACE2 in KIRP was positively correlated with the type markers of macrophages. High ACE2 expression level had a favorable prognosis in different enriched immune cells subgroups in UCEC and KIRP. And the promoter methylation levels of ACE2 in UCEC and KIRP were significantly reduced. What's more, we found that the expression of ACE2 decreased in vivo and in vitro after SARS-CoV infection. In conclusion, ACE2 expression increased significantly in UCEC and KIRP, elevated ACE2 was positively correlated with immune infiltration and prognosis. Moreover, tumor tissues may be more susceptible to SARS-CoV-2 infection in COVID-19 patients with UCEC and KIRP, which may worsen the prognosis.

INTRODUCTION

UCEC (Uterine Corpus Endometrial Carcinoma) is a common gynecological cancer in the world [1–3]. It is an epithelial malignant tumor of endometrium, which has a high mortality rate and seriously threatens the health of women [4, 5]. It can be divided into two types: estrogen dependent and non estrogen dependent [6]. The incidence of non estrogen dependent tumors is low, but the malignancy is high and the prognosis is poor [4, 7]. The prognoses of endometrial cancer patients with metastasis are poor regardless of grade or stage, and the overall survival rate of patients is significantly reduced [8]. There is evidence that microsatellite unstable endometrial cancer has infiltration of granzyme B + cells, activated cytoxic T-lymphocytes, and PD-L1 + cells [9], which suggests that endometrial cancer can be treated with immunotherapy to improve prognosis.

KIRP (Kidney Renal Papillary Cell Carcinoma) accounts for 15%-20% of renal cancer [10]. KIRP is a malignant parenchymal tumor of the kidney, which is

characterized by a papillary or tubular papillary structure [11]. It can be divided into type 1 and type 2 according to the histologic features, and type 2 KIRP has a high grade, a late stage and a poor prognosis [12, 13]. Moreover, the immune cell response is closely connected with the clinical prognosis of KIRP, and tumor related macrophages can represent the indicator of good prognosis of KIRP [14, 15]. Thus, it is necessary to clarify the relationship between UCEC and KIRP and immune invasion, and find an immune related biomarker to indicate the prognosis of UCEC and KIRP.

Angiotensin-converting enzyme 2 (ACE2) is a member of the renin-angiotension system. It's open reading frame encodes a polypeptide containing 805 amino acids [16]. The extracellular surface of ACE2 enzyme contains a catalytic metal peptidase domain, which has 42% sequence homology with the N-terminal catalytic domain of ACE [17]. ACE2 mainly splits angiotensin II (ANG II) into angiotensin-(1-7) and acts as a vasodilator in the renin-angiotension system [18]. A recent study has shown that it can block the angiogenesis, tumor cell growth and metastasis of pancreatic cancer, breast cancer and colon cancer [19–21]. But the related prognosis and possible immune mechanisms of ACE2 in UCEC and KIRP are still ambiguous.

Since December 2019, coronavirus disease 2019 (COVID-19) was found in Wuhan City, Hubei Province, China [22]. It is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously tentatively named 2019-nCoV), which belongs to the beta coronaviruses (β-CoV) genus [23]. SARS-CoV-2 and severe acute respiratory syndrome coronavirus (SARS-CoV) have 79.5% homologous sequences, and it uses ACE2 as receptor to enter the cell like SARS-CoV [24]. As of March 4, 2020, there were 80409 laboratory confirmed cases and 3012 dead cases of COVID-19 in China [25]. It has been widely spread all over the world, and has been recognized as a public health emergency of international concern by the World Health Organization [26]. However, the prognosis of COVID-19 patients with UCEC and KIRP are still unclear.

In this study, we first analyzed the expression of ACE2 in different tumors in the oncomine and Tumor Immune Estimation Resource (TIMER) databases, and then used the PrognoScan, GEPIA and Kaplan-Meier plotter databases to study the prognostic relationship between ACE2 and various tumors. After screening tumors prognosis related to ACE2, the relationship between ACE2 and immune infiltration levels in different tumors was investigated in the Timer database. The ACE2 promoter methylation profile was also tested in the UALCAN database. Besides, we made use of the GSE30589 and GSE52920 databases to clarify the changes of ACE2 in cells and animals following SARS-CoV infection. Our findings shed light on the important role of ACE2 in UCEC and KIRP and also provided a potential mechanism related to immune infiltration in these tumors. It also illustrated the possible susceptibility of tumors to SARS-CoV-2 and prognosis of COVID-19 patients with UCEC and KIRP.

RESULTS

The mRNA expression levels in human cancers

In order to study the changes of ACE2 expression levels in different tumor tissues compared with normal tissues. We first analyzed Oncomine database, 11 databases including 739 samples were selected. The analysis showed that the expression levels of ACE2 in Invasive Breast Carcinoma, Esophageal Cancer, Head and Neck cancer, Liver cancer, Lung cancer and other cancer (Testicular Intratubular Germ Cell Neoplasi) increased significantly, while in breast cancer (Intraductal Cribriform Breast Adenocarcinoma and Invasive Breast Carcinoma), colorectal cancer, Esophageal Cancer, kidney cancer, Lymphoma, other cancer (Yolk Sac Tumor, Seminoma, Mixed Germ Cell Tumor, Embryonal Carcinoma, Testicular Embryonal Carcinoma, Testicular Yolk Sac Tumor, Testicular Seminoma, Uterine Corpus Leiomyoma, Malignant Fibrous Histiocytoma), pancreatic cancer and sarcoma decreased significantly (Figure 1A). The detailed results were summarized in Supplementary Table 1.

Then, we further studied the expression levels of ACE2 between different tumors and normal tissues based on the RNA-seq data of malignant tumors in TCGA database. The expression levels were higher in KIRP (Kidney Renal Papillary Cell Carcinoma) and UCEC (Uterine Corpus Endometrial Carcinoma) (Figure 1B). In addition, the expression level was also higher in LUAD (Lung Adenocarcinoma) (Figure 1B). Nevertheless, the expression levels of ACE2 were lower in BRCA (Breast Invasive Carcinoma), KICH (Kidney Chromophobe), LIHC (Liver Hepatocellular Carcinoma), PRAD (Prostate Adenocarcinoma), STAD (Stomach Adenocarcinoma) and THCA (Thyroid Carcinoma) (Figure 1B). We analyzed the above databases and found that the expression levels of ACE2 in breast cancer, eophagal cancer, kidney cancer, liver cancer and sarcoma were different due to different subtypes, most of which were lower than that in normal tissues except for liver cancer. What's more, ACE2 acts as a receptor for SARS-CoV-2 to enter cells, which means that tumor tissues that highly express ACE2 may be susceptible to SARS-CoV-2 infection.

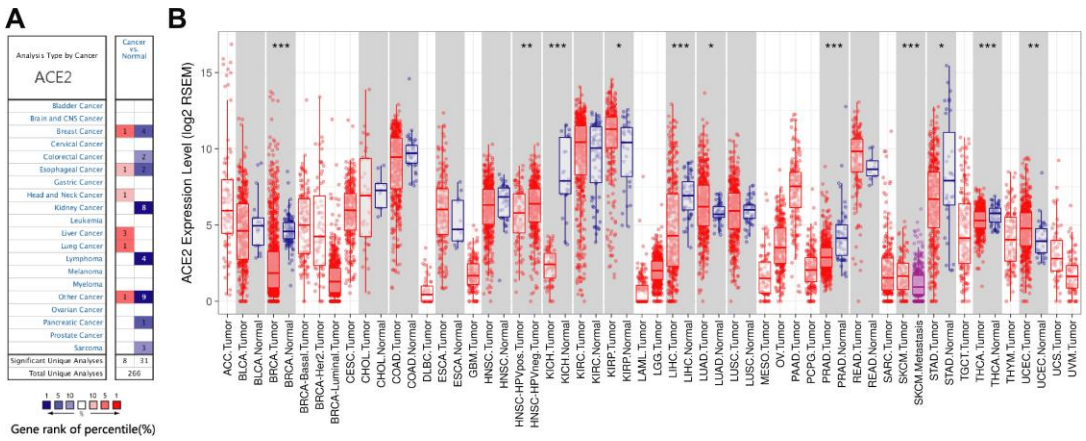

Figure 1. The expression levels of ACE2 in different cancers. (**A**) ACE2 in different cancers compared to normal tissues in the Oncomine database. (**B**) ACE2 expression levels of different tumor types in the TCGA database were detected by TIMER (*P<0.05, **P<0.01, ***P<0.001).

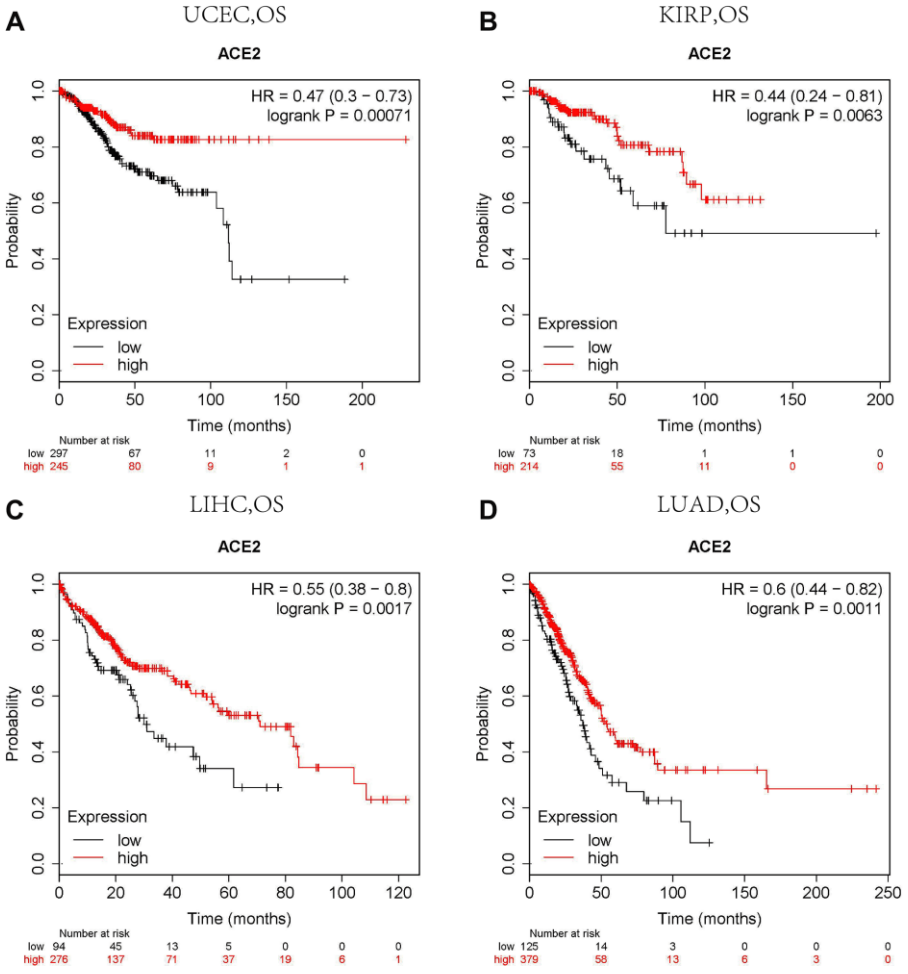

Figure 2. Comparison of Kaplan-Meier survival curves of ACE2 overexpression and underexpression in different cancers. (**A**) High ACE2 expression in the Kaplan Meier plotter database had favorable OS in UCEC (n=543), (**B**) KIRP (n=288), (**C**) LIHC (n=371) and (**D**) LUAD (n=513). OS, overall survival; UCEC, Uterine Corpus Endometrial Carcinoma; KIRP, Kidney Renal Papillary Cell Carcinoma; LIHC, Liver Hepatocellular Carcinoma; LUAD. Lung Adenocarcinoma.

ACE2 predicted prognosis in different cancers

According to the difference of ACE2 expressions in some tumors, we further analyzed the relationship between ACE2 expression and prognosis in these tumors, so it is necessary to clarify whether ACE2 is the promoter or suppressor of tumors. PrognoScan was first used to study the relationship between the expression of ACE2 and the overall survival rate of different tumors. The analysis results showed that the high expression of ACE2 in breast cancer was related to the poor prognosis. However, the high expression of ACE2 in renal cell carcinoma had a favorable prognosis (Supplementary Table 2).

Then we analyzed TCGA database by GEPIA and explored the potential prognostic relationship between ACE2 expressions and human tumors. Interestingly, there was no significant relationship between the expressions of ACE2 and the prognosis of breast invasive carcinoma, kidney chromophobe, prostate adenocarcinoma, stomach adenocarcinoma, thyroid carcinoma, colon adenocarcinoma and head and neck squamous cell carcinoma (Supplementary Figure 1A–1G).

The Kaplan Meier plotter is a large database containing GEO, EGA and TCGA. It can be used as a tool to evaluate genes on survival in 21 cancer types. Therefore, we used the Kaplan Meier plotter to further check the relationships between ACE2 and prognoses of different tumors. ACE2 expressions have no significant correlations with the prognoses of breast cancer, head neck squamous cell carcinoma, stomach adenocarcinoma and thyroid carcinoma (Supplementary Figure 1H–1K). However, high ACE2 expression levels in uterine corpus endometrial carcinoma and kidney renal papillary cell carcinoma showed significant favorable prognoses (OS HR0.47, 95%CI=0.30 to 0.73, OS HR0.44, 95%CI=0.24 to 0.81, respectively) (Figure 2A, 2B). Similar prognoses were also observed in liver hepatocellular carcinoma and lung adenocarcinoma (Figure 2C, 2D).

The transcription levels of ACE2 were correlated with tumor immune infiltration

Previous studies had shown that tumor infiltration was related to the prognoses of renal cancer and endometrial cancer [9, 14, 15]. So, we tested whether the transcription levels of ACE2 in different tumors were correlated with immune infiltration. TIMER database was used to analyze the correlations between ACE2 level and uterine corpus endometrial carcinoma, kidney real papillary cell carcinoma, Liver hepatocellular carcinoma and lung adenocarcinoma. The results showed that ACE2 was very weakly negatively correlated with B cell, CD4 + T cell, dendritic cell and neutrophil of Liver hepatocellular carcinoma and lung adenocarcinoma (Supplementary Figure 2).

However, the expression of ACE2 was positively correlated with the level of immune infiltration of macrophage (r=0.322, p<0.001) in kidney renal papillary cell carcinoma. Similarly, ACE2 has a positive correlation with B cell (r=0.166, p<0.01), CD4 + T cell (r=0.154, p<0.01), neutrophil (r=0.223, p<0.001) and dendritic cell (r=0.271, p<0.001) immune infiltration levels of uterine corpus endometrial carcinoma (Figure 3).

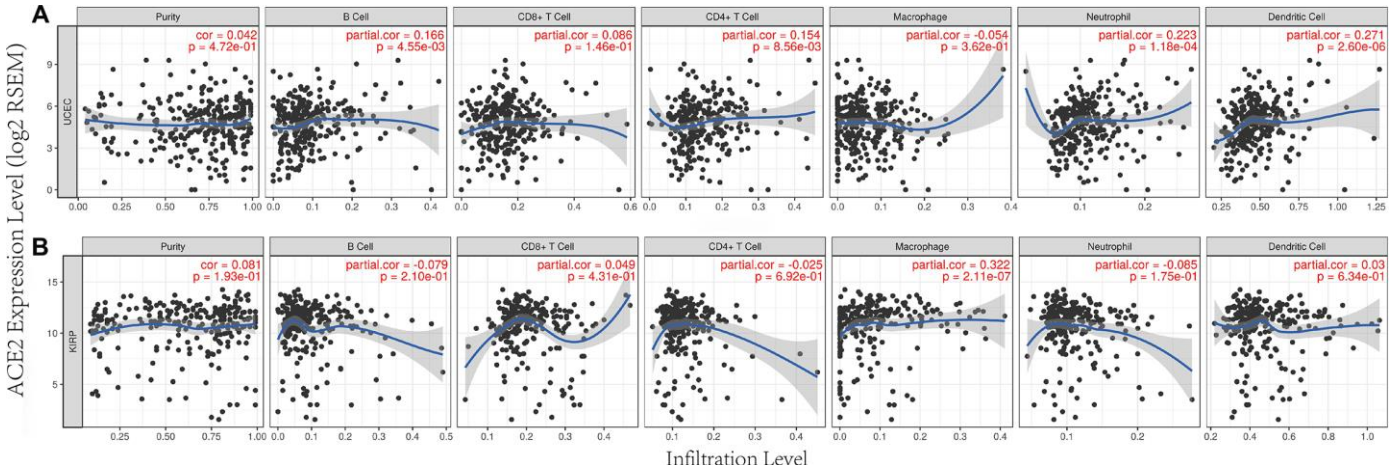

Figure 3. Correlation between ACE2 expression and immune infiltration in UCEC and KIRP in TIMER database. ACE2 expressions were positively correlated with (**A**) B cel, CD4 + T cell, neutrophil and dendritic cell immune infiltration levels of uterine corpus endofamilial carcinoma (UCEC), (**B**) the level of immune infiltration of macrophage in kidney renal papillary cell carcinoma (KIRP).

ACE2 expressions were correlated with immune cell type markers

We further explored the relationships between the expressions of ACE2 and the type markers of different immune cells in endometrial and renal carcinoma. The type markers of B-cells, CD8 + T cells, neutrophils, macrophages, dendritic cells NK cells, Th1 cells, Treg cells and monocyte were analyzed by TIMER database.

The results showed that ACE2 in UCEC was positively correlated with FCRL2 and MS4A1 in B cells. ACE2 in UCEC was also positively correlated with FCGR3B, CEACAM3, SIGLEC5, CSF3R, S100A12 in neutrophils and CD84 in macrophages (Table 1). Similarly, ACE2 in KIRP was positively correlated with CD68 and CD84 in macrophages and C3AR1 in monocyte (Table 1). These correlations remained unchanged after tumor purity and age correction (Table 1). This further confirmed that ACE2 expressions in uterine corpus endofamilial carcinoma and kidney renal papillary cell carcinoma were correlated to immune infiltration.

Prognostic analysis of ACE2 expressions in different tumors based on immune cells

We have confirmed that the expressions of ACE2 were correlated with the immune infiltration in uterine corpus endometrial carcinoma and kidney renal papillary cell carcinoma, and the expressions of ACE2 were also related to the favorable prognoses of these tumors. So we speculated that the expressions of ACE2 in these tumors affected the prognosis partly because of immune infiltration.

We did a prognosis analysis based on the expression levels of ACE2 of different tumors in ralated immune cells subgroup via the Kaplan Meier plotter. The results showed that the high expression of ACE2 of uterine corpus endometrial carcinoma in enriched B cells (HR = 0.24), enriched CD4+ memory T cells (HR = 0.28), enriched CD8+ T cells (HR = 0) and enriched macrophages (HR = 0.09) cohort had better prognosis respectively (Figure 4A, 4C, 4E, 4G). Similarly, the high expression of ACE2 of Kidney Renal Papillary Cell Carcinoma had better prognosis in enriched regulatory T cells (HR = 0.27) and enriched type 1 T helper cells (HR = 0.23) cohort respectively (Figure 4I, 4K). But there was no significant correlation between the high ACE2 and the prognosis of Kidney Renal Papillary Cell Carcinoma in the enriched macrophages cohort (OS HR0.63, 95%CI=0.3 to 1.3, logrank P=0.21), and the high expressions of ACE2 of UCEC and KIRP had no significant correlation in decreased immune cells subgroup (Figure 4B, 4D, 4F, 4H, 4J, 4L). The above analysis suggested that high ACE2 expressions in UCEC and KIRP may affect prognoses in part due to immune infiltration.

Promoter methylation levels of ACE2 decreased in UCEC and KIRP

The significant increases of ACE2 expressions in UCEC and KIRP were observed. Therefore, we further studied the reason for the elevated ACE2. DNA methylation is an important event in the epigenetic modification of the genome and is closely related to the process of the disease [33]. In particular, hypomethylation can lead to genome instability [34, 35], and may activate related genes. So we used UALCAN database to verify the methylation levels of ACE2 promoter in UCEC and KIRP. Interestingly, the methylation levels of ACE2 promoter in UCEC and KIRP were significantly lower than that in normal tissue (Figure 5A, 5F). Also, we stratified UCEC and KIRP according to patients' age, individual cancer stages, tumor grade, tumor histology and nodal metastasis status.

The results showed that ACE2 promoter methylation levels of the older people, higher grade tumors and serous tumors groups were lower than control in UCEC (Figure 5B, 5D, 5E). Moreover, the ACE2 promoter methylation levels of tumors with lymph node metastasis group in KIRP were lower than that in normal tissue (Figure 5I). And the ACE2 promoter methylation levels of different individual cancer stage groups decreased significantly compared with normal tissues groups in UCEC and KIRP (Figure 5C, 5H). However, the ACE2 promoter methylation levels of ACE2 did not change significantly in different age subgroups of KIRP and individual cancer stages subgroups of UCEC and KIRP (Figure 5C, 5G, 5H). It is suggested that ACE2 promoter hypomethylation in UCEC and KIRP may activate itself and increase its level respectively.

SARS-CoV-2 infection may reduce the expression of ACE2

ACE2 can be used as a receptor for SARS-CoV-2 to enter the cell [24]. It is necessary to study the changes of ACE2 in tumors after SARS-CoV-2 infection. Because SARS-CoV-2 and SARS-CoV have high homology [24], the change of ACE2 expression after cells or animals infected with SARS-CoV can be used as a reference for SARS-CoV-2. GSE30589 and GSE52920 databases were used to analyze the changes of ACE2 expression after SARS-CoV infected Vero E6 cells and mice lung. The results showed that the expressions of ACE2 in Vero E6 cells and mouse lung decreased significantly compared with control group (Figure 6). This finding suggested that ACE2 expression may decrease after SARS-CoV-2 infection.

Table 1. Correlation analysis between ACE2 and immune cell type markers in TIMER database.

Cell type	Gene markers	UCEC						KIRP					
		None		Purity		Age		None		Purity		Age	
		COR	P	COR	P	COR	P	COR	P	COR	P	COR	P
B cells	FCRL2	0.325	6.7E-15	0.290	4.4E-07	0.331	2.9E-15	-0.046	4.4E-01	-0.072	2.5E-01	-0.044	4.6E-01
	CD19	0.151	4.2E-04	0.153	8.9E-03	0.159	2.1E-04	-0.040	5.0E-01	-0.026	6.8E-01	-0.040	5.1E-01
	MS4A1	0.210	7.9E-07	0.231	6.6E-05	0.217	3.6E-07	0.043	4.7E-01	0.059	3.4E-01	0.050	4.1E-01
CD8+ T cells	CD8A	0.150	4.6E-04	0.152	9.4E-03	0.149	5.0E-04	0.059	3.2E-01	0.038	5.5E-01	0.064	2.9E-01
	CD8B	0.161	1.7E-04	0.159	6.5E-03	0.158	2.3E-04	0.021	7.2E-01	0.007	9.1E-01	0.023	7.0E-01
Neutrophils	FCGR3B	0.279	3.2E-11	0.239	3.6E-05	0.274	9.0E-11	0.045	4.5E-01	0.037	5.5E-01	0.048	4.2E-01
	CEACAM3	0.307	2.6E-13	0.283	8.4E-07	0.308	2.5E-13	-0.015	8.0E-01	-0.052	4.1E-01	-0.024	6.8E-01
	SIGLEC5	0.210	7.7E-07	0.204	4.5E-04	0.215	4.6E-07	0.171	3.5E-03	0.146	1.9E-02	0.172	3.6E-03
	FPR1	0.195	4.7E-06	0.187	1.3E-03	0.199	3.1E-06	0.081	1.7E-01	0.037	5.6E-01	0.071	2.3E-01
	CSF3R	0.234	3.2E-08	0.184	1.5E-03	0.248	5.4E-09	0.085	1.5E-01	0.051	4.2E-01	0.091	1.2E-01
	S100A12	0.247	5.4E-09	0.244	2.4E-05	0.249	4.7E-09	-0.087	1.4E-01	-0.113	7.0E-02	-0.079	1.8E-01
Macrophages	CD68	0.189	9.1E-06	0.173	3.0E-03	0.196	4.3E-06	0.390	7.8E-12	0.372	7.1E-10	0.388	1.3E-11
	CD84	0.210	7.9E-07	0.194	8.3E-04	0.216	3.9E-07	0.259	8.2E-06	0.269	1.2E-05	0.266	5.6E-06
	CD163	0.094	2.8E-02	0.092	1.2E-01	0.102	1.7E-02	0.180	2.1E-03	0.173	5.2E-03	0.173	3.5E-03
	MS4A4A	0.094	2.8E-02	0.079	1.8E-01	0.098	2.3E-02	0.162	5.8E-03	0.157	1.1E-02	0.157	7.9E-03
Dendritic cells	CD209	0.077	7.4E-02	0.113	5.3E-02	0.081	5.9E-02	-0.045	4.5E-01	-0.033	6.0E-01	-0.044	4.6E-01
NK cells	KIR3DL3	0.145	7.0E-04	0.121	3.8E-02	0.140	1.1E-03	-0.048	4.1E-01	-0.052	4.0E-01	-0.043	4.7E-01
	NCR1	0.186	1.2E-05	0.137	1.9E-02	0.181	2.3E-05	0.039	5.1E-01	0.032	6.0E-01	0.035	5.6E-01
Th1 cells	TBX21	0.132	2.1E-03	0.123	3.6E-02	0.135	1.7E-03	0.069	2.4E-01	0.056	3.7E-01	0.060	3.2E-01
Treg	FOXP3	0.160	1.7E-04	0.146	1.3E-02	0.155	3.0E-04	-0.095	1.1E-01	-0.110	7.8E-02	-0.085	1.5E-01
	CCR8	0.165	1.1E-04	0.149	1.1E-02	0.158	2.2E-04	-0.014	8.2E-01	-0.046	4.7E-01	-0.007	9.0E-01
Monocyte	C3AR1	0.138	1.2E-03	0.116	4.6E-02	0.143	8.8E-04	0.231	7.6E-05	0.231	1.8E-04	0.231	8.3E-05
	CD86	0.164	1.1E-04	0.161	5.7E-03	0.171	6.6E-05	0.166	4.7E-03	0.153	1.4E-02	0.166	5.0E-03
	CSF1R	0.147	5.6E-04	0.150	1.0E-02	0.150	4.6E-04	0.134	2.3E-02	0.110	7.7E-02	0.132	2.6E-02

UCEC, Uterine Corpus Endometrial Carcinoma; KIRP, Kidney Renal Papillary Cell Carcinoma; NK cells, Natural killer cells; Th 1 cells, type I helper T cells; Treg, regulatory T cells; COR, r value of Spearman's correlation; Purity, correlation adjusted by purity; Age correlation adjusted by age.

DISCUSSION

In this study, the changes of ACE2 mRNA in UCEC and KIRP were analyzed in Oncomine and TIMER databases. And we analyzed the correlations between ACE2 expression levels and immune infiltration and the prognoses of these tumors. Moreover, we predicted the susceptibility of different tumor tissues to SARS-CoV-2 and the potential prognoses of patients after SARS-CoV-2 infection in UCEC and KIRP.

We analyzed the TCGA database using TIMER database and found that ACE2 was elevated in both UCEC and KIRP (Figure 1B), which suggested that tumor tissues were more likely to be infected with SARS-CoV-2 in UCEC and KIRP. The Kaplan Meier plotter was used to investigate the effect of ACE2 on tumor prognosis, the results showed that high ACE2 had a favorable prognosis in UCEC and KIRP (Figure 2A, 2B). In addition, TIMER database was also used to analyze the correlation between ACE2 and immune infiltration in UCEC and KIRP. The results showed that ACE2 and B cell, CD4 + T cell, neutrophil and dendritic cell infiltration levels were positively correlated in UCEC (Figure 3A). There was also a positive correlation between macrophage infiltration level and ACE2 in KIRP (Figure 3B). The immune cell type markers in UCEC and KIRP were further studied, after correction of tumor purity, ACE2 in UCEC was significantly positively correlated with FCRL2 and MS4A1 in B cells, it was also positively correlated with FCGR3B, CEACAM3, SIGLEC5, CSF3R and S100A12 in neutrophils and CD84 in macrophages, while ACE2 in KIRP was positively correlated with CD68 and CD84 in macrophages

(Table 1). These strongly confirmed the positive correlation between ACE2 and immune infiltration in UCEC and KIRP. Prognostic analysis of ACE2 expression levels in different tumor based on immune cells was performed, high ACE2 expression level in UCEC had a favorable prognosis in the enriched B cells, CD4 + memory T cells, CD8 + T cells and macrophages subgroups (Figure 4A–4H), and high ACE2 expression in KIRP had a favorable prognosis in the enriched regulatory T cells and type 1 T helper cells subgroups (Figure 4I, 4K). The analysis suggests that the high expressions of ACE2 in UCEC and KIRP may affect the prognoses of cancer patients in part due to immune infiltration.

In order to explore the causes of elevated ACE2 in UCEC and KIRP, we investigated the level of methylation in UCEC and KIRP. Surprisingly, the promoter methylation levels of ACE2 in UCEC and KIRP were significantly reduced (Figure 5A, 5F). ACE2 may be activated and up-regulated due to its hypomethylation, which to some extent explained the elevated ACE2 in UCEC and KIRP. SARS-CoV-2 can use ACE2 as a receptor to enter cells, and it has high homology with SARS-CoV [24]. Therefore, we used the GSE30589 and GSE52920 databases to study the changes of ACE2 of Vero E6 cells and mouse that were infected with SARS-CoV. The results showed that ACE2 expression levels in both of them were reduced after SARS-CoV infection (Figure 6). This finding suggested that tumor tissues may also have decreased ACE2 levels after SARS-CoV-2 infection in UCEC and KIRP.

In the above analysis, ACE2 was confirmed to be elevated in UCEC and KIRP, Thus, after patients with UCEC and KIRP are infected with SARS-CoV-2, their tumor tissues are more susceptible to virus interference in addition to the respiratory system. Afterwards, tumor tissues infected with SARS-CoV-2 in turn underwent a decrease in ACE2, and reduced ACE2 brought about tumor microenvironment disorders because of reduced

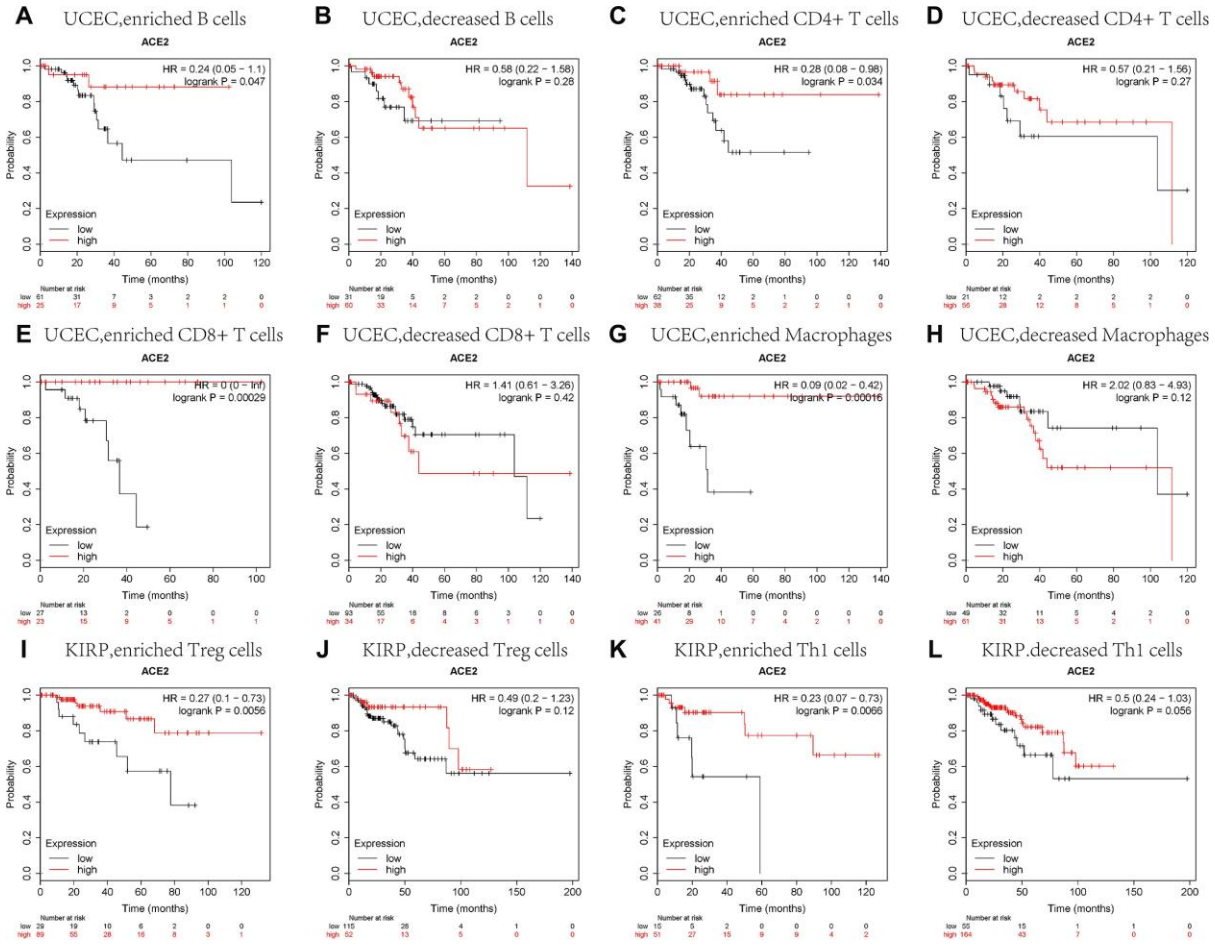

Figure 4. Comparison of Kaplan-Meier survival curves of the high and low expression of ACE2 in UCEC and KIRP based on immune cells subgroups. Relationships between ACE2 of different immune cells subgroup and prognoses in (**A**–**H**) Uterine Corpus Endometrial Carcinoma (UCEC), and (**I**–**L**) Kidney Renal Papillary Cell Carcinoma (KIRP).

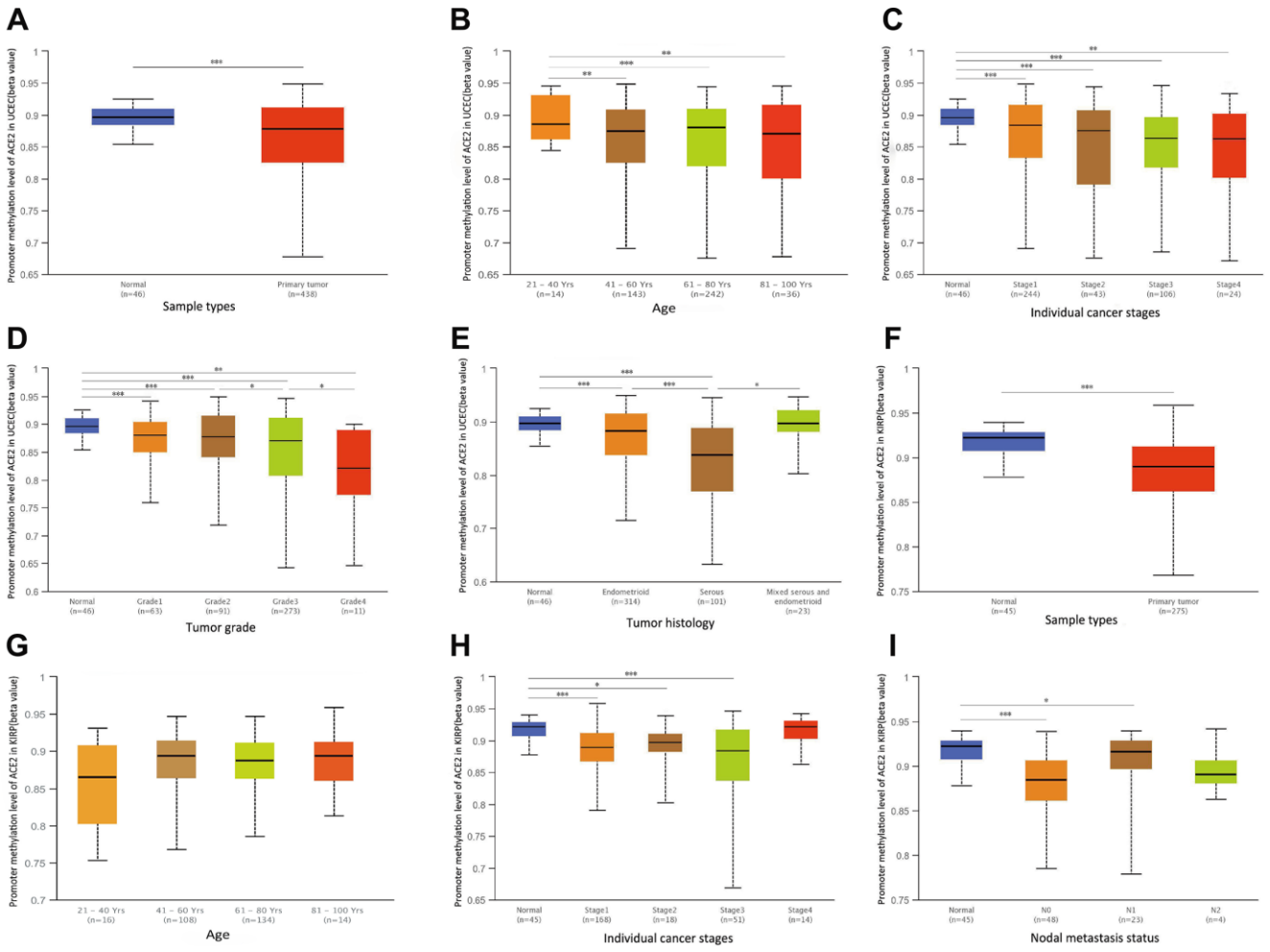

Figure 5. The promoter methylation levels of ACE2 in UCEC and KIRP. Promoter methylation levels of ACE2 were low in (**A**–**E**) Uterine Corpus Endometrial Carcinoma (UCEC) and (**F**–**I**) Kidney Renal Papillary Cell Carcinoma (KIRP) (*P<0.05, **P<0.01, ***P<0.001).

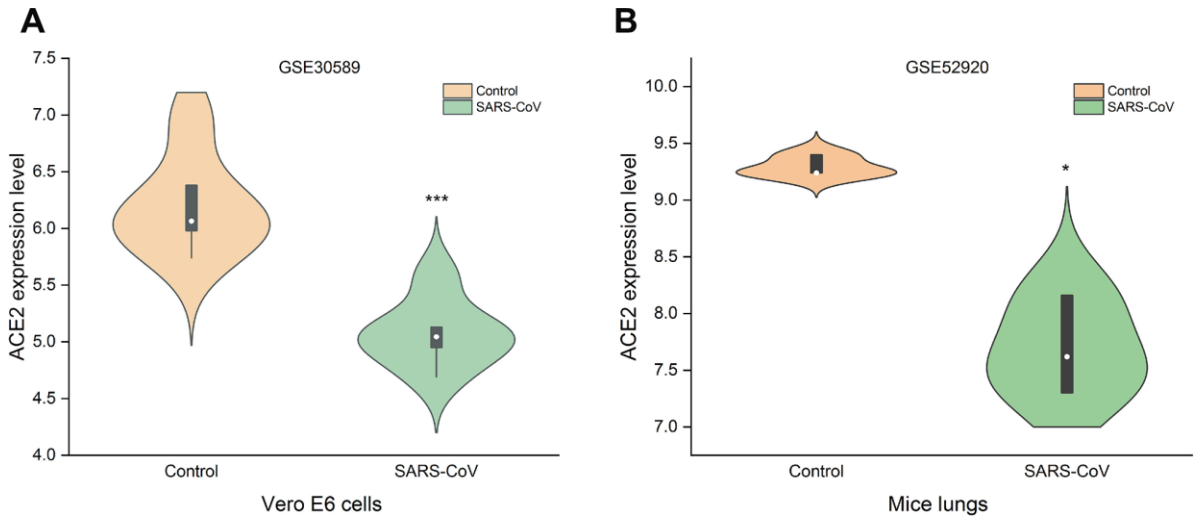

Figure 6. Changes of ACE2 after SARS-CoV infection. SARS-CoV reduced the expression levels of ACE2 in (**A**) Vero E6 cells and (**B**) mice lungs (***P<0.001, *P<0.05 vs. Control).

immune infiltration, which may worsen the prognoses of UCEC and KIRP patients after SARS-CoV-2 infection.

As an important member of the renin-angiotensin system (RAS), ACE2 has shown different roles in some pathological processes. One study showed that overexpression of ACE2 can protect endothelial cells by inhibiting the inflammatory response, which is beneficial to early prevention of atherosclerosis [36]. Walters et al. [37] found a direct relationship between atrial structural remodeling and plasma ACE2 activity in patients with atrial fibrillation. While ACE2 not only affects the progress of cardiovascular diseases, but also plays a new role in tumor pathology. Yu C et al. [20] found that down-regulating the ACE2/Ang-(1-7)/Mas axis caused breast cancer metastasis by activating store-operated calcium entry (SOCE) and PAK1/NF-κB/Snail1 pathways. ACE2 also down-regulates VEGFa expression in breast cancer cells and inactivates phosphorylation of VEGFR2, MEK1/2 and ERK1/2 in human umbilical endothelial cells, furthermore, ACE2 can prevent breast cancer cell metastasis in zebrafish models [38]. A study carried out by Yu X et al. [39] showed that ACE2 can block the inflammatory response of pancreatic acinar cells by blocking the p38 MAPK/NF-κB signaling pathway. Li J et al. [40] examined the role of ACE2 and FZD1 in squamous cell/adenosquamous carcinoma (SC / ASC) of the gallbladder, the results showed that negative ACE2 expression in SC/ASC was associated with high TNM stage and lymph node metastasis, and survival analysis showed ACE2 and SC/ASC overall survival were positively correlated. ACE2 was also decreased in non-small cell lung cancer (NSCLC), but overexpression of ACE2 in vitro exerted protective effects by inhibiting cell growth and VEGFa production [41]. In addition, ACE2 overexpression in non-small cell lung cancer can inhibit tumor angiogenesis induced by acquired platinum resistance [42]. In general, ACE2 mainly affected tumor metastasis by intervening signaling pathways, but the mechanism by which ACE2 affected the prognosis of UCEC and KIRP is unclear. Here, we found that ACE2 may affect the prognosis of UCEC and KIRP through a new mechanism, that was, immune infiltration, which can provide a direction for future in-depth research. But this research also has some limitations, due to the limitation of the database, we did not continue to analyze the deep relationship between ACE2 and immune infiltration. Also, experiments are urgently needed to verify the analysis results in our research.

CONCLUSIONS

To sum up, ACE2 expression increased significantly in UCEC and KIRP. Elevated ACE2 was positively correlated with immune infiltration and prognoses of UCEC and KIRP. Moreover, tumor tissues were more susceptible to SARS-CoV-2 infection in COVID-19 patients with UCEC and KIRP. In the end, tumor tissues infected with SARS-CoV-2 may undergo a decrease in ACE2, and reduced ACE2 can bring about reduced immune infiltration in the tumor microenvironment, which may worsen the prognosis of COVID-19 patients with UCEC and KIRP.

MATERIALS AND METHODS

Oncomine database analysis

ACE2 expression in different tumors was identified in the Oncomine database (https://www.oncomine.org/resource/main.html) [27]. The threshold was a P-value of 0.01, a 1.5-fold change, and a top 10% of gene ranking. The data must come from mRNA.

Survival analysis in PrognoScan, GEPIA and Kaplan-Meier plotter databases

To analyze the prognosis of ACE2 expression in various tumors, PrognoScan (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html) [28], GEPIA (http://gepia.cancer-pku.cn/) [29] and Kaplan-Meier plotter (http://kmplot.com/) [30] databases were used separately. This threshold was cox p-value<0.05 in PrognoScan database, logrank p value <0.05 in GEPIA and Kaplan-Meier plotter database.

TIMER database analysis

TIMER is a comprehensive database that can analyze the levels of immune invasion in different tumors and the differences in gene expression of different tumors (https://cistrome.shinyapps.io/timer/) [31]. We confirmed the expression of ACE2 in various tumors using the TIMER database. Then the correlation of ACE2 with immune infiltration (B cells, CD4 + T cells, CD8 + T cells, Neutrophils, Macrophages, and Dendritic cells) in the tumor was estimated using the TIMER algorithm. Finally, the correlation of ACE2 with the type markers of B-cells, CD8 + T cells, neutrophils, macrophages, dendritic cells NK cells, Th1 cells, Treg cells and monocytes in UCEC and KIRP were verified. In addition, we used tumor purity and patient's age for p-value correction.

UALCAN database analysis

UALCAN is a comprehensive interactive web resource for analyzing cancer OMICS data (http://ualcan.path.uab.edu/index.html) [32]. It is built on PERL-CGI and can be used to assess the methylation levels of

different genes. So the ACE2 promoter methylation profile was tested in the UALCAN database. Moreover, we performed a stratified analysis based on patients' age, individual cancer stages, tumor grade, tumor histology and nodal metastasis status.

Microarray data collection

GEO (https://www.ncbi.nlm.nih.gov/geo/) is a public repository that can archive microarrays and other forms of high-throughput functional genomics data, and the expression profiles of GSE30589 and GSE52920 were obtained in the GEO database. The GSE30589 database which contained 12 SARS-CoV infected samples and 9 control samples was based on the GPL570 platform ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array). While the GSE52920 database which included 3 lung tissue samples of mice infected with SARS-CoV and 3 normal mice lung tissue samples was based on the GPL13912 platform (Agilent-028005 SurePrint G3 Mouse GE 8x60K Microarray).

Statistical analysis

The statistical results of the survival analysis were obtained from a log-rank test, and the correlations of ACE2 with immune infiltration and type markers of immune cells were evaluated using Spearman's correlation. Student's t test was used to compare two independent samples. p-values less than 0.05 were considered statistically significant.

Abbreviations

ACC: Adrenocortical Carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast Invasive Carcinoma; CESC: Cervical and Endocervical Cancer; CHOL: Cholangiocarcinoma; COAD: Colon Adenocarcinoma; DLBC: Diffuse Large B-cell Lymphoma; ESCA: Esophageal Carcinoma; GBM: Glioblastoma Multiforme; HNSC: Head and Neck Cancer; KICH: Kidney Chromophobe; KIRC: Kidney Renal Clear Cell Carcinoma; KIRP: Kidney Renal Papillary Cell Carcinoma; LGG: Lower Grade Glioma; LIHC: Liver Hepatocellular Carcinoma; LUAD: Lung Adenocarcinoma; LUSC: Lung Squamous Cell Carcinoma; MESO: Mesothelioma; OV: Ovarian Serous Cystadenocarcinoma; PAAD: Pancreatic Adenocarcinoma; PCPG: Pheochromocytoma and Paraganglioma; PRAD: Prostate Adenocarcinoma; READ: Rectum Adenocarcinoma; SARC: Sarcoma; SKCM: Skin Cutaneous Melanoma; STAD: Stomach Adenocarcinoma; TGCT: Testicular Germ Cell Tumors; THCA: Thyroid Carcinoma; THYM: Thymoma; UCEC: Uterine Corpus Endometrial Carcinoma; UCS: Uterine Carcinosarcoma; UVM: Uveal Melanoma; NK cells: Natural killer cells; Th 1 cells: typeIhelper T cells; Treg: regulatory T cells.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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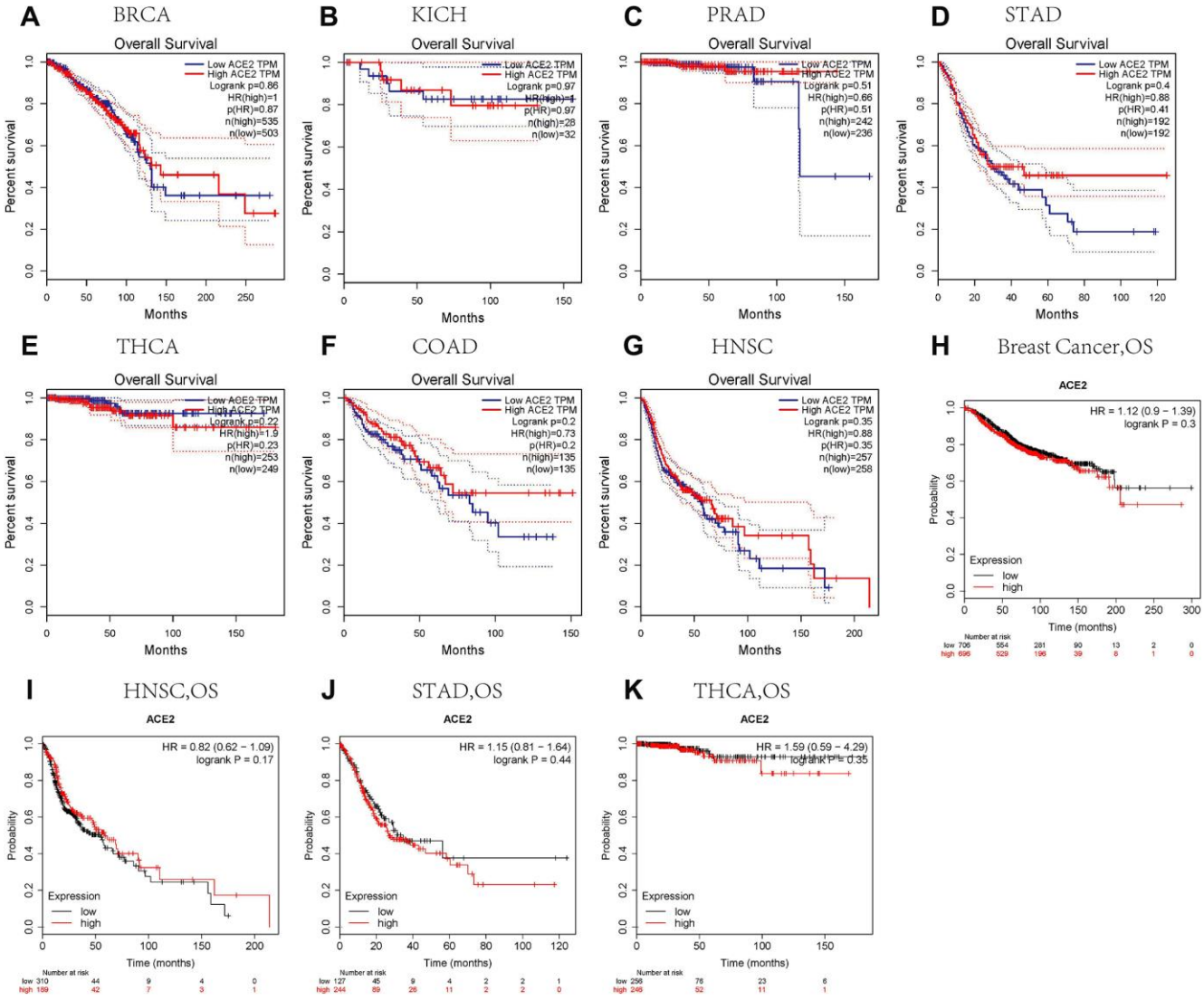
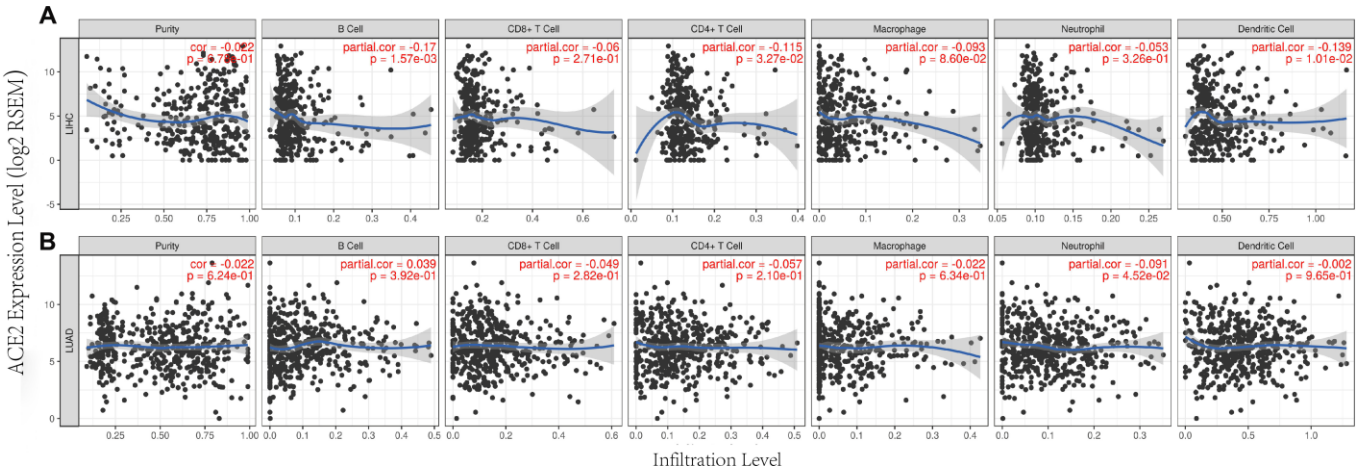
SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Correlations between ACE2 expression and immune infiltration in different cancers. Comparison of overall survival of ACE2 over-expression and under-expression in (**A**–**G**) Breast Invasive Carcinoma (BRCA), Kidney Chromophobe (KICH), Prostate Adenocarcinoma (PRAD), Stomach Adenocarcinoma (STAD), Thyroid Carcinoma (THCA), Colon Adenocarcinoma (COAD) and Head and Neck Cancer (HNSC) in GEPIA database, (**H**–**K**) Breast Cancer, Head and Neck Cancer (HNSC), Stomach Adenocarcinoma (STAD) and Thyroid Carcinoma (THCA) in Kaplan Meier plotter database.



Supplementary Figure 2. Correlation between ACE2 expression and immune infiltration in LIHC and LUAD in TIMER database. ACE2 was very weakly negatively correlated with (**A**) B cell, CD4 + T cell, dendritic cell of Liver hepatocellular carcinoma (LIHC), and (**B**) neurophil of lung adenocarcinoma (LUAD).

Supplementary Tables

Supplementary Table 1. The expression levels of ACE2 in Oncome database compared with normal tissues.

Cancer	Cancer subtype	P-value	Fold change	Rank (%)	Sample	Reference (PMID)
	Invasive Breast Carcinoma	2.11E-16	2.279	4%	725	18438415
	Invasive Ductal Breast Carcinoma	1.11E-4	-1.751	2%	324	19187537
	Invasive Lobular Breast Carcinoma	1.93E-11	-3.134	5%	931	TCGA
	Invasive Ductal Breast Carcinoma	9.75E-26	-2.684	6%	1124	TCGA
	Intraductal Cribriform Breast Adenocarcinoma	0.008	-5.450	10%	2016	TCGA
	Colon Mucinous Adenocarcinoma	9.11E-5	-2.690	8%	1413	17615082
	Colon Adenoma	8.55E-5	-3.739	9%	1650	20957034
	Barrett's Esophagus	1.21E-4	3.893	10%	1820	21152079
	Esophageal Adenocarcinoma	0.002	-5.143	3%	445	16952561
	Barrett's Esophagus	0.006	-2.947	4%	560	16952561
Head and Neck cancer	Tongue Squamous Cell Carcinoma	0.002	1.537	10%	1763	18254958
	Renal Wilms Tumor	4.08E-4	-64.062	1%	156	20440404
	Chromophobe Renal Cell Carcinoma	0.003	-21.769	4%	779	20440404
	Renal Oncocytoma	0.003	-13.376	5%	958	20440404
	Chromophobe Renal Cell Carcinoma	1.50E-10	-5.131	2%	212	16115910
	Renal Pelvis Urothelial Carcinoma	1.12E-6	-3.856	7%	841	16115910
	Renal Oncocytoma	1.17E-7	-4.528	9%	1026	16115910
	Renal Wilms Tumor	0.005	-1.959	7%	843	16299227
	Clear Cell Sarcoma of the Kidney	0.010	-2.843	10%	1255	16299227
	Cirrhosis	5.12E-4	2.842	5%	874	17393520
	Liver Cell Dysplasia	0.005	1.956	5%	969	17393520
	Cirrhosis	6.51E-9	1.755	9%	1054	19098997
Lung cancer	Lung Adenocarcinoma	1.36E-11	2.039	5%	866	23028479
	Germinal Center B-Cell-Like Diffuse Large B-Cell Lymphoma	2.94E-11	-1.872	1%	118	
	Activated B-Cell-Like Diffuse Large B-Cell Lymphoma	1.06E-10	-1.841	3%	556	
	Follicular Lymphoma	1.81E-9	-1.649	9%	1568	
	Diffuse Large B-Cell Lymphoma	2.15E-10	-1.809	10%	1800	
	Testicular Intratubular Germ Cell Neoplasi	0.009	4.922	2%	170	15994931
	Yolk Sac Tumor	1.61E-12	-17.251	1%	25	
	Seminoma	3.01E-12	-14.554	1%	46	
	Mixed Germ Cell Tumor	1.48E-19	-12.891	1%	50	
	Embryonal Carcinoma	2.54E-10	-13.188	2%	282	
	Testicular Embryonal Carcinoma	3.39E-4	-23.719	4%	477	
	Testicular Yolk Sac Tumor	6.09E-4	-20.922	4%	554	
	Testicular Seminoma	0.001	-30.784	6%	809	
	Uterine Corpus Leiomyoma	3.01E-4	-1.641	5%	799	19622772
	Malignant Fibrous Histiocytoma	0.003	-3.649	9%	1058	15994966, 16603191
pancreatic cancer	Pancreatic Ductal Adenocarcinoma	0.006	-2.373	2%	333	16053509

sarcoma	Malignant Fibrous Histiocytoma	0.003	-3.649	9%	1058	15994966, 16603191
	Clear Cell Sarcoma of the Kidney	0.010	2.843	10%	1255	16299227
	Gastrointestinal Stromal Tumor	8.51E-5	-3.067	7%	1335	21447720, 29725014, 23112551

Supplementary Table 2. Relationships between ACE2 expressions and prognoses in different cancers in PrognoScan database.

DATASET	CANCER TYPE	SUBTYPE	ENDPOINT	N	COX P-VALUE	HR [95% CI-low CI-upp]
GSE9893	Breast cancer	-	Overall Survival	155	0.207469	0.83 [0.61 - 1.11]
GSE1456-GPL96	Breast cancer	-	Overall Survival	159	0.749893	1.05 [0.78 - 1.40]
GSE1456-GPL96	Breast cancer	-	Overall Survival	159	0.24964	1.28 [0.84 - 1.93]
E-TABM-158	Breast cancer	-	Overall Survival	117	0.263366	1.39 [0.78 - 2.47]
E-TABM-158	Breast cancer	-	Overall Survival	117	0.531078	1.19 [0.69 - 2.07]
GSE7390	Breast cancer	-	Overall Survival	198	0.002524	1.23 [1.08 - 1.41]
GSE7390	Breast cancer	-	Overall Survival	198	0.006852	1.18 [1.05 - 1.33]
GSE12945	Colorectal cancer	-	Overall Survival	62	0.081404	0.68 [0.44 - 1.05]
GSE12945	Colorectal cancer	-	Overall Survival	62	0.112689	0.68 [0.43 - 1.09]
GSE17536	Colorectal cancer	-	Overall Survival	177	0.841356	0.98 [0.82 - 1.18]
GSE17536	Colorectal cancer	-	Overall Survival	177	0.929774	0.99 [0.85 - 1.16]
GSE17537	Colorectal cancer	-	Overall Survival	55	0.592087	1.11 [0.75 - 1.64]
GSE17537	Colorectal cancer	-	Overall Survival	55	0.494492	1.11 [0.82 - 1.49]
GSE11595	Esophagus cancer	Adenocarcinoma	Overall Survival	34	0.974499	0.98 [0.38 - 2.53]
GSE2837	Head and neck cancer	Squamous cell carcinoma	Relapse Free Survival	28	0.308794	1.75 [0.60 - 5.12]
GSE11117	Lung cancer	NSCLC	Overall Survival	41	0.118476	1.37 [0.92 - 2.03]
GSE3141	Lung cancer	NSCLC	Overall Survival	111	0.362302	0.87 [0.63 - 1.18]
GSE3141	Lung cancer	NSCLC	Overall Survival	111	0.542736	0.91 [0.68 - 1.22]
GSE14814	Lung cancer	NSCLC	Overall Survival	90	0.973271	1.02 [0.35 - 2.93]
GSE14814	Lung cancer	NSCLC	Overall Survival	90	0.442885	1.52 [0.52 - 4.42]
GSE4573	Lung cancer	Squamous cell carcinoma	Overall Survival	129	0.809129	0.94 [0.56 - 1.57]
GSE17710	Lung cancer	Squamous cell carcinoma	Overall Survival	56	0.149829	0.76 [0.52 - 1.10]

GSE17710	Lung cancer	Squamous cell carcinoma	Overall Survival	56	0.104661	0.73 [0.50 - 1.07]
E-DKFZ-1	Renal cell carcinoma	-	Overall Survival	59	0.021041	0.17 [0.04 - 0.77]

Research Paper

Anti-SARS-CoV-2 virus antibody levels in convalescent plasma of six donors who have recovered from COVID-19

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ABSTRACT

Background: Anti-SARS-CoV-2 virus antibody levels in convalescent plasma (CP), which may be useful in severe Anti-SARS-CoV-2 virus infections, have been rarely reported.

Results: A total of eight donors were considered for enrollment; two of them were excluded because of ineligible routine check. Of the six remaining participants, five samples were tested weakly positive by the IgM ELISA. Meanwhile, high titers of IgG were observed in five samples. The patient treated with CP did not require mechanical ventilation 11 days after plasma transfusion, and was then transferred to a general ward.

Conclusions: Our serological findings in convalescent plasma from recovered patients may help facilitate understanding of the SARS-CoV-2 infection and establish CP donor screening protocol in COVID-19 outbreak.

Methods: Anti-SARS-CoV-2 antibodies including IgM and IgG were measured by two enzyme-linked immunosorbent assays (ELISA) in convalescent plasma from six donors who have recovered from coronavirus disease 2019 (COVID-19) in Nanjing, China. CP was also utilized for the treatment of one severe COVID-19 patient.

INTRODUCTION

By late 2019 the outbreak of coronavirus disease 2019 (COVID-19) was unchecked in China [1, 2]. Apart from supportive care, specific drugs for this disease are still being researched [3, 4]. The absence of efficacy-proven antiviral treatment has led to attempts to treat severe SARS-CoV-2 infection with convalescent plasma containing SARS-CoV-2 specific antibodies from recovery patients-a precedent established with pathogen-specific immunoglobulin therapy for Ebola virus disease, influenza, severe acute respiratory syndrome, and severe fever and thrombocytopenia syndrome [5–8].

Previous reports on other viral infections have suggested that convalescent plasma with higher antibody levels may have great effect on virus load [9, 10], and our study was designed to test anti-SARS-CoV-2 virus antibody levels to select those with high titers, desiring a meaningful serologic response after CP infusion.

In accordance with CP infusion therapeutics guidelines approved by the National Health Commission of People's Republic of China, we used ELISA to screen for anti-SARS-CoV-2 IgM and IgG. In this report, we present our preliminary findings of anti-SARS-CoV-2 antibody levels in convalescent plasma obtained from six donors and clinical effects of one case treated with CP in Nanjing, China.

RESULTS

Characteristics of the six CP donors

We recruited a total of six donors including four males and two females, aged from 30 to 50 years old, with laboratory confirmed SARS-CoV-2 infection during the COVID-19 outbreak and the subsequent recovery certificated by two consecutively negative SARS-CoV-2 PCR assays and resolution of clinical symptoms. All the donors had fever and cough during the course of COVID-19. None of the donors were currently smoking. Donor D had a history of brain surgery due to a benign tumor. The other five donors did not have any underlying comorbidities. The baseline blood examinations of the donors, when they were admitted to the hospital due to COVID-19, were summarized in Table 1. At the time of admission, two donors had lymphocytopenia (lymphocyte counts$<0.8\times10^9$/L), one donor had increased alanine aminotransferase level (144 IU/L), one donor had elevated creatine kinase level (490 U/L), three donors had abnormal lactate dehydrogenase (ranged from 261 to 286 IU/L) and four donors had a C-reactive protein level of more than 10 mg/L (Table 1). Chest CT scans demonstrated bilateral pneumonia in all six donors.

During hospitalization, all donors were routinely given antiviral therapy with interferon-α (500 WU, twice a day, aerosol inhalation) and lopinavir/ritonavir (400/100mg, twice a day). Donor B, C, D, and E also received intravenous immunoglobulin. A 3-day course of corticosteroids (methylprednisolone 40 mg per day) was administered to donor B, D and F. None of donor needed mechanical ventilation or required to be transferred to the intensive care unit. The time from onset of symptoms to clearance of virus, defined as two consecutive negative nucleic acid tests from throat swab samples, were varied from 8 to 18 days. The donors were discharged after virus clearance and substantially improvement of their pneumonia.

Plasma samples were collected at times ranging from 29 to 46 days after symptom onset, and 13 to 27 days after their discharge, respectively (Table 2). At the time of blood donation, the donors were free of any symptom. The complete blood count, liver and renal function, lactate dehydrogenase, and C-reactive protein were within the normal range. The lymphocyte subsets counts were summarized in Table 3. All ABO types were involved in the study except AB type. Additionally, as part of the routine check, the donated plasma was confirmed free of hepatitis B and C virus, human immunodeficiency virus (HIV) and residual SARS-CoV-2 by RT-PCR and serologic negative for hepatitis B and C virus, HIV, and syphilis.

Serological findings of anti-SARS-CoV-2 antibodies detected by ELISA

The anti-SARS-CoV-2 IgM antibody was weakly reactive (OD ratio from 1.22 to 2.01) for all donors except donor F, with a slightly higher OD ratio of 5.63, and IgG ELISA assay were also positive (OD ratio from 3.92 to 8.36) for all six donors who had IgM reactive plasma samples (Table 2).

All donors but one had high IgG titers (≥1:320) (Figure 1), meeting the criteria (≥1:160) sponsored by the National Health Commission. However, donor D had a low IgG titer (1:40) (Figure 1), therefore this donor was not considered as an eligible donor. This donor, a 42-year-old man had the longest duration (46 days) from symptom onset to plasma collection and he had the longest duration (19 days) of hospital stay. Also, this donor had the lowest CD19+ B-cell count as well as percentage in the lymphocyte subsets analysis (Table 3).

Clinical utility of CP in a critically ill patient

The recipient for CP was a 64-year-old female. The patient was admitted to the hospital because of fever, fatigue, nausea and vomiting for 3 days, and was then confirmed of COVID-19. The underlying commodities included hypertension and diabetes. There was a fast progression of the clinical condition. On day 4 of hospitalization, the patient was transferred to Intensive Care Unit (ICU) and 1 week later received invasive mechanical ventilation. SARS-CoV-2 was undetectable from throat swab sample by nucleic acid test at the time of intubation. On day 17 of hospitalization, while the patient was still receiving invasive mechanical ventilation with a PaO2/FiO2 of 166 mmHg, she was given 200 mL CP from donor B. At the time of plasma transfusion, the lymphocyte count was 0.44×10^9/L.

Table 1. Baseline blood examinations of the six donors when they were admitted to the hospital due to COVID-19.

Donor No.	Age, y/sex	WBC, $\times 10^9$/L	Lymphocyte counts,$\times 10^9$/L	ALT, IU/L	Creatinine, μmol/L	CK, U/L	LDH, IU/L	Troponin I, ng/mL	D-dimer, μg/L	PT, s	Procalcitonin, ng/mL	IL-6	CRP, mg/L
A	30/M	5.52	1.67	22.7	84	140	261	0.05	0.18	12	0.024	0.014	< 10.00
B	37/M	4.7	0.63	22.1	47	490	265	0.01	NA	12.4	0.039	0.055	63.77
C	45/F	3.42	1.41	28.1	43	34	141	0.05	0.53	11.9	0.013	0.006	16.09
D	42/M	5.65	0.71	12.5	64.5	39	223	0.009	0.19	13.0	0.076	0.084	21
E	32/M	4.32	1.46	16	57	60	188	0.25	0.26	12	0.410	0.031	< 10.00
F	50/F	4.06	0.99	144	38	47	286	0.06	0.19	10.1	0.013	0.031	12.4

WBC, white blood cell counts; ALT, alanine aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase; PT, prothrombin time; IL-6, interleukin 6; CRP, C-reactive protein; NA, not available.

Table 2. Antibody levels of six donors recovered from COVID-19.

Donor No.	Blood group	Days from symptom onset to plasma collecting	Days from discharge to plasma collecting	Anti-SARS-CoV-2 IgM levels (OD ratio)[a]	Anti-SARS-CoV-2 IgG levels (OD ratio)[a,b]
A	A	29	13	1.47	7.58
B	O	36	17	1.22	6.59
C	B	37	23	1.55	7.84
D	A	46	27	2.01	3.92
E	O	40	22	1.95	7.52
F	A	39	27	5.63	8.36

[a] Negative controls and positive controls were included in every run.
[b] Serial tests were performed (Figure 1).

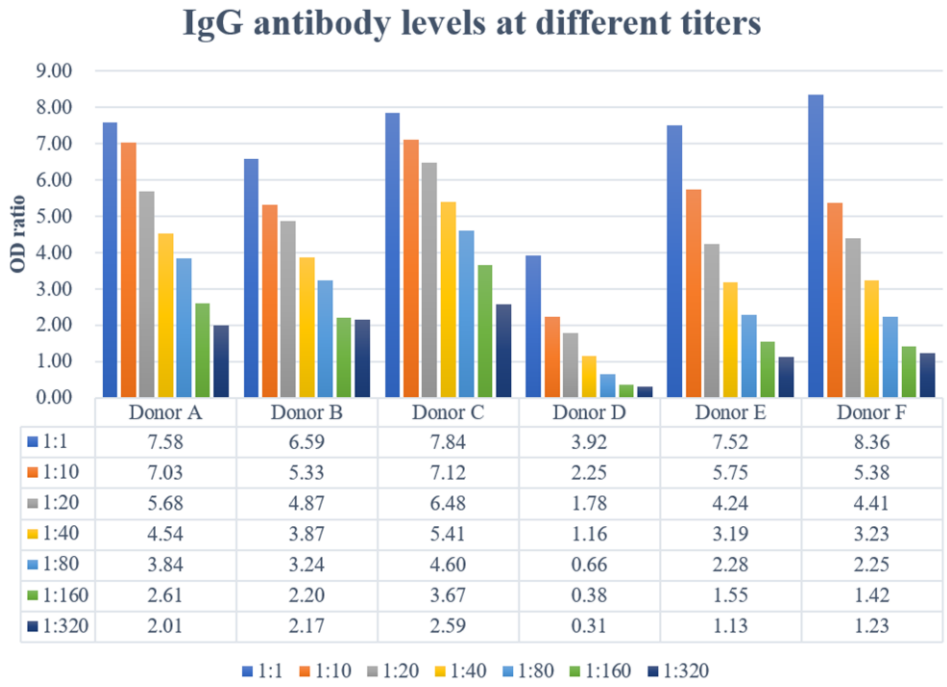

Figure 1. Specific immunoglobulin IgG were titrated by semiquantitative ELISA. Plasma IgG antibody titers ranged from 1:40 to >320.

Table 3. Lymphocyte subsets counts of the six donors at the time of blood donation.

Donor No.	Lymphocyte counts (cells/ul)	CD3+ (%)	CD3+ (cells/ul)	CD3+ CD4+(%)	CD3+CD4+ (cells/ul)	CD3+ CD8+(%)	CD3+CD8+ (cells/ul)	CD4/CD8 ratio	CD16+CD56+ NK cells (%)	CD16+CD56+NK cells (cells/ul)	CD19+ (%)	CD19 (cells/ul)
A	2619	63.23	1656	20	512	30	792	0.65	20.43	535	13.21	346
B	1970	67.36	1327	30	585	33	643	0.91	19.39	382	8.07	159
C	1690	67.81	1146	28	465	30	503	0.92	20.41	345	9.7	164
D	1796	70.99	1275	36	653	30	542	1.2	21.55	387	5.57	100
E	1841	57.47	1058	34	625	17	318	1.97	17.82	328	20.64	380
F	1645	80.3	1321	52	850	25	416	2.04	9.3	153	9.18	151

Other blood examinations, including renal and liver function, prothrombin time, creatine kinase, lactate dehydrogenase and myocardial enzymes, did not significantly changed, although the D-dimer was increased (2.31mg/L). There was no transfusion related adverse event. Lymphocyte count remained below 0.5×10^9/L for 1 week. The patient did not require mechanical ventilation 11 days after plasma transfusion, and was then transferred to a general ward.

DISCUSSION

We reported the serological findings of SARS-CoV-2 infection in a CP donor population. Our preliminary findings suggest that recently recovered COVID-19 patients may be suitable potential donors, provided they meet other blood donation criteria.

Although our experience is limited in a few cases, a possibility could be suggested that, different from other viruses like MERS-CoV infection [11], antibody to SARS-CoV-2 in serum or plasma was frequently reactive by ELISA. All of the six donors showed positive IgM results, indicating that a negative result for IgM, a serologic marker which usually represents a recent or current infection [12–14], may not be suitable to be taken as a mandatory requirement for CP donor selection of limited availability of eligible potential donors in a COVID-19 outbreak. Of the six donors, only one donor had IgG titers of 1:40, which did not meet the criteria 1:160 recommended by the National Health Commission. Of note, compared with other donors, he experienced a severe disease, and had the longest duration from symptom onset to plasma collection, we suspected whether this phenomenon was related to his low CD19+ B-cell count or he had experienced a viral reactivation-an observation that requires further investigation.

However, due to limitations imposed by sample size, reactivity of ELISA tests may also be affected by the timing of plasma collection, severity of illness or corticosteroids administration. In addition, although this life-threatening disease appear to be under control following nationwide efforts and implementation of quarantine policy in China, but it is still developing in the other parts of the world. As yet no reference materials of anti-SARS-CoV-2 antibodies has been made available to evaluate the performance of the kits. Our study highlights the need for prospective serology studies and good laboratory quality assurance to better understand the humoral response to SARS-CoV-2 infection.

The weakness of the study should be noted that the clinical relevance of antibody titers in protecting against subsequent SARS-CoV-2 infection is uncertain. Compared to ELISAs, neutralization assays require virus culture, are much more labor-intensive, and need to be conducted in laboratories with higher biosafety levels [15, 16]. We are currently conducting neutralization studies to further investigate whether ELISA results were correlated with neutralization results so far as to substitute for the neutralization test in resource-limited situations.

Although a favorable outcome was achieved in one patient after CP transfusion, the efficacy of CP remains inconclusive due to the very small sample size and other concomitant treatments, which might confound the result.

In summary, we presented serologic findings from six CP donors recovered from COVID-19 and one case treated with CP. This report may help facilitate understanding of the SARS-CoV-2 infection and establish donor screening protocol for CP infusion therapeutics in the COVID-19 outbreak.

MATERIALS AND METHODS

Study design

Under the first and second edition of CP infusion therapeutics guidelines approved by the National

Health Commission, we developed a protocol for donor screening, plasma collection and specimen analysis to screen potential donors and collect high-titer plasma. Donor screening, specimen collecting and convalescent plasma collecting were conducted at the Second Hospital of Nanjing, a designated medical institution for COVID-19. The antibody testing was conducted in Nanjing Red Cross Blood Center, and its Department of Laboratory Medicine is accredited by China National Accreditation Service for Conformity Assessment. This study was approved by the ethics committee of the Second Hospital of Nanjing (reference number: 2020-LS-ky003). Written informed consent was obtained from all the donors and the recipient.

Donor population

We screened potential convalescent plasma donors from patients who were confirmed SARS-CoV-2 infection by PCR and had recovered at least four weeks from symptom onset. A total of eight volunteers were recruited as potential plasma donors for assessment. Two were excluded because of elevated alanine transaminase for one case and unexpected hemoglobin levels for the other case. The remaining six provided written, informed consent to become qualified donors.

Collection of specimens for antibody levels

Convalescent plasma was collected by apheresis from COVID-19 recovered donors, and specimen for antibody testing were collected from an integrated bypass collection reserved sample bag. Plasma for determination of anti-SARS-CoV-2 IgG antibody levels was collected in EDTA tubes and serum for anti-SARS-CoV-2 IgM antibody levels was collected in tubes with coagulation accelerators. Samples were delivered to Nanjing Red Cross Blood Center immediately after collecting, followed by sample centrifuging and antibody testing.

Serology tests

Two solid-phase microplate ELISAs were employed, based on the nucleocapsid (N) protein of SARS-CoV-2 (Livzon, Diagnostics Inc., Zhuhai, China).

The first kit was a capture enzyme-linked immunosorbent assay for IgM antibody using horseradish peroxidase (HRP)-labeled SARS-CoV-2 antigens. To reveal IgM, serum samples were diluted 1:100 in dilution buffer and allowed to incubate for 60 min with plates coated by anti-human IgM μ chain. Plates were washed and HRP-labeled antigens were added. After 30 min incubation, unbound components were washed away, following adding of TMB substrate with its buffer. For a further 15 min incubation, stop buffer was added and absorbance values were measured at 450nm and 630nm dual-wavelength using a microplate reader.

The second kit was an indirect enzyme-linked immunosorbent assay designed for IgG antibody. After a formulated 1:20 predilution according to the ELISA manufacturer's instructions, plasma specimens were serially titrated 1:1, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 in microplates by plasma from unexposed donors and added to plates coated with SARS-CoV-2 antigens. Following 60 min incubation at 37°C, plates were washed and incubated with horseradish peroxidase-labeled anti-human IgG secondary antibody. Again, plates were washed following 30 min incubation at 37°C and TMB substrate was added with its buffer. 15 min later, stop buffer was added and absorbance values were measured at 450nm and 630nm dual-wavelength using a microplate reader.

Results were reported as the optical density (OD) ratio, which was calculated as the OD value of the donor's sample divided by the cutoff OD value. We used cutoff values recommended by the ELISA kit manufacturer: a ratio of <1 was considered negative, and ≥1 was considered positive.

Statistical methods

All data from measurements were displayed as tables and a histogram.

Abbreviations

CP: convalescent plasma; COVID-19: coronavirus disease 2019; WBC: white blood cell counts; ALT: alanine aminotransferase; CK: creatine kinase; LDH: lactate dehydrogenase; PT: prothrombin time; IL-6: interleukin 6; CRP: C-reactive protein; NA: not available; OD: optical density; ICU: Intensive Care Unit.

AUTHOR CONTRIBUTIONS

L.B.Z. and R.R.P. performed the experiments and wrote the paper. X.X. participated in the design of the study and contributed with comments during the writing. J.J.B. and S.Y. performed the experiments and conducted data analysis. Y.D.D. reviewed the manuscript. Y.S.Z participated in the clinical utility of CP. Q.F. and Z.L.H. conceived the study and collected the data. Y.X.Y. participated in the design of the study. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

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Letter to the Editor

Modified management mode for colorectal cancer during COVID-19 outbreak – a single-center experience

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ABSTRACT

During the epidemic of COVID-19, the management model of colorectal cancer has to be changed at our center due to relatively limited medical resources. Outpatient visits are reduced under well protected after appointment, and rigorous investigation of epidemiological history and clinical symptoms are needed. We prefer a simple and convenient treatment regimen, which may also be postponed appropriately. Minimally invasive CRC surgery combined with a perioperative program of enhanced recovery after surgery should be recommended. We also focus on mental health treatments and healthy lifestyle education. In addition, routine follow-up can be moderately delayed. In total, adequate doctor-patient communication is also recommended throughout the treatment.

INTRODUCTION

From the end of 2019, a war without gunpowder has begun in China. Novel coronavirus pneumonia (COVID-19) from Wuhan city has now spread to the whole country and even the world [1]. The major routes of the coronavirus infection are the respiratory droplets, close contact transmission, and also when exposed to high concentrations of aerosol in a relatively closed environment for a long time [2]. The incubation period of COVID-19 is up to 24 days. The most common symptoms were fever and cough, and some severe cases can quickly progress to acute respiratory distress syndrome [3]. The Chinese government has initiated a first-level response to major public health emergencies, mobilized the whole country to fight against the epidemic, made comprehensive deployments, and implemented the strongest and strictest prevention and control measures. By the end of March, the epidemic situation has been under control across China. However, the pandemic of COVID-19 is global now.

Colorectal cancer (CRC) is the malignancy with the fourth highest prevalence among females and fifth among males in China [4]. CRC patients were generally in poor immunity and physical fitness, which are susceptible to COVID-19. A prospective cohort study has found that cancer patients were at higher risk of COVID-19 infection and had a worse prognosis than those without tumors [5].

During this particular period, most hospitals have suspended or postponed outpatient and elective surgery. Therefore, how to deal with CRC patients is challenging and essential. As follows, we introduce our single center's experience in the management of CRC patients during COVID-19 outbreak and present a series of issues of our clinical work (Table 1).

Outpatient

If CRC patients have obvious symptoms of bleeding, perforation, obstruction, or extreme discomfort, we recommend them to go to the emergency as soon as possible. Regular outpatient visits can be postponed appropriately. With the joint efforts of the whole country, the epidemic situation has changed positively, and outpatient in various places has gradually restored.

Table 1. Changes in clinical practice during COVID-19 outbreak.

Terms		Details
Outpatient	Appointment	Online
	Escort	One at most
	Protection	A medical surgical mask or a general medical mask at least
	Screening	Check body temperature; Check for signs and symptoms; Epidemiological investigation
	Treatment	Reduce infusion time; Long prescription policy
Non-surgical treatment	Adjuvant chemotherapy	Prefer simple and convenient regimen in principle; Continue the original regimen in principle
	Neoadjuvant therapy	Prefer neoadjuvant chemotherapy alone; Expand neoadjuvant therapy indications for low to moderate locally advanced rectal cancer
	mCRC patient	Develop, improve or change the regimen by online MDT clinics or Wechat group
	Traditional Chinese Medicine	Follow the physicians' advice
Surgery	Before surgery	A comprehensive examination to exclude COVID-19
	Surgery procedures	Prefer minimally invasive surgery plus ERAS program; Not recommend colorectal and liver resection simultaneously; Postpone surgery where condition permits
Daily life		Mental health; Healthy lifestyle
Follow up		Postpone review time appropriately; The principle of proximity hospital; Online follow-up

Appointment

Not as the previous, all outpatient clinics request appointments now. CRC patients should make an appointment in advance, and only one family member is allowed to accompany to prevent cross-infection.

Protection

The medical workers, patients and families need to strengthen their own protection, such as wearing surgical masks, goggles, and so on. We also pay attention to the disinfection of outpatient equipment and the environment.

Epidemiological history

At the clinic, a rigorous investigation of epidemiological history and clinical symptoms is needed. Patients with a history of living or traveling in the affected area, close contacts with a confirmed or probable case, or having fever and respiratory symptoms, should be checked during pre-diagnosis. And then, if suspected COVID-19 manifestations, the patient should be sent to the fever clinic. If a suspected or confirmed case is diagnosed, the patient shall be immediately quarantined and reported.

Outpatient treatment

At the outpatient, we choose simple and convenient regimen, and also establish a long prescription policy to facilitate patients to receive drugs for 2 to 3 months at a time, to reduce the times of visiting.

Non-surgical treatment

The Chinese Society of Clinical Oncology guidelines recommend that adjuvant chemotherapy should be started as soon as possible after recovery, generally about 3 weeks after CRC operation, and no later than 2 months [6]. A meta-analysis of 15 410 CRC patients showed that the start of postoperative adjuvant chemotherapy was delayed every 4 weeks, patients' overall survival time and disease-free survival time will be significantly reduced [7]. Therefore, we recommend postponing adjuvant chemotherapy appropriately at the local hospital as the first choice. Moreover, we prefer the three-week CapeOX regimen to biweekly FOLFOX regimen, so that we can minimize the chance of cross-infection. Oral capecitabine monotherapy also could be used as much as possible. In addition, we recommend that the patients can contact the physicians to reduce the treatment intensity and switch to oral therapy.

During the epidemic, many hospitals suspended radiotherapy. The Chinese FOWARC Trial showed that no significant difference in outcomes was found between mFOLFOX6 without radiotherapy and fluorouracil with radiotherapy for locally advanced rectal cancer [8]. Therefore, neoadjuvant chemotherapy alone with the mFOLFOX6 regimen is also an option.

For patients with metastatic CRC, Multi-Disciplinary Treatment (MDT) is the best choice. If the condition is stable, the original chemotherapy regimen can be maintained for another 1-2 cycles until the MDT outpatient restore. If there is obvious progression, we recommend online MDT clinics or communicating with physicians via WeChat or telephone to change the regimen.

Undoubtedly, traditional Chinese medicine has certain effects on improving the physical condition of CRC patients, which can reduce the side effects of chemotherapy and improve the quality of life [9]. Considering the patients' resistance during chemotherapy is relatively low, we also recommend regular thymosin to improve immunity as prescribed [10].

Surgery

CRC surgeons should control the routine operation to reduce the patient's exposure time in the hospital [11]. For CRC patients with mass bleeding, perforation, or obstruction, emergency surgery should be considered, and COVID-19 infection needs to be ruled out before.

Endoscopic surgery is recommended for early-stage CRC when it is completely removed clearly with good histological features, and no additional surgical treatment is required. Whether surgery delay affects survival remains controversial for advanced CRC. We prefer to expand neoadjuvant therapy indications for low to moderate locally advanced rectal cancer. And we try to conduct surgery for advanced colon cancer as early as possible. A detailed investigation and a comprehensive examination (chest CT or viral nucleic acid test) should be performed to exclude COVID-19 before elective surgery. In addition, we also do not recommend to perform colorectal and liver resections simultaneously at the current situation, so as to avoid a prolonged hospital stay and increased risk of infection.

At the surgical ward, we prefer minimally invasive surgery plus a perioperative program of enhanced recovery after surgery (ERAS) as the best treatment strategy, which could accelerate patient recovery and shorten hospital stay [12].

The hospital should strictly implement the National Health Commission's requirements for infection control in medical institutions [13]. Ordinary patients who underwent CRC resection can be transferred to the general ward after surgery. It is necessary to reduce the movement of accompanying staff and personnel. Patients with postoperative fever should be carefully identified and isolated according to the suspected COVID-19 criteria [2]. And then suspected or confirmed patients should be transferred to a designated negative pressure isolation monitoring room for single room isolation.

Daily life

CRC and COVID-19 are double blows to patients. Many patients have mental health problems of anxiety and depression, so we should give positive psychological support during the epidemic. We inform that the prognosis of CRC is not so bad, and even with recurrence, a considerable part of the patients will be cured when metastases are detected and resected early [14]. Stoma patients are also encouraged to communicate with families and friends, learn to self-regulate bad moods, and actively integrate into society. At the same time, we also inform the patients that they will not be infected if actively protect, and the epidemic situation is getting better now, which will return to normal soon. Even if the treatment is appropriately delayed, it will not affect the treatment effect. At present, several public institutions and domestic hospitals have launched psychological hotline services, and we recommend patients could contact when needed.

A healthy lifestyle is especially important for CRC patients. Studies have shown that smokers have a significantly increased risk of developing and dying from CRC compared with never-smokers, and heavy drinking also increases the risk of developing CRC [15]. We recommend CRC patients a healthy and balanced lifestyle diet, avoiding high fat and low fiber diet, reducing the intake of red meat and processed meat. The American Gastroenterological Association has recommended calcium supplements for the primary or secondary prevention of colon cancer, so we also recommend appropriate intake of calcium-rich food such as dairy products [16]. CRC patients also need to appropriately increase the intake of cellulose and decrease irritating and too much oily food. Stoma patients can properly consume dairy products and vegetables to reduce the odor at the stoma. In addition, we also suggest that during the epidemic, patients can arrange indoor physical exercise under the guidance of the physicians, avoiding prolonged bed rest, which can promote the recovery of intestinal function and prevent deep vein thrombosis.

Recently, many health organizers have opened public-interest online lectures and free mobile applications for different patient groups, to provide disease education and answer questions online. Furthermore, several university hospitals have also opened up various online clinics, including online fever clinics, psychological clinics, specialist clinics and online MDT clinics. In addition, we also have established several follow-up

WeChat groups for CRC patients. When CRC patients have questions, they can get medical advice quickly from multiple experts at home.

Follow up

For patients who need to be reviewed after CRC surgery during the epidemic, we recommend that the review time can be appropriately postponed. We encourage patients to complete routine review projects at the nearest medical institution. After they obtain the review results, it is recommended to adopt an online network method for consultation.

In summary, our current clinical work model has to be changed due to COVID-19 outbreak, which includes the above outpatient, inpatient, psychological treatment and health education.

CONFLICTS OF INTEREST

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Letter to the Editor

Preventive strategy for the clinical treatment of hip fractures in the elderly during the COVID-19 outbreak: Wuhan's experience

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ABSTRACT

Hip fractures in the elderly account for more than half of osteoporotic fractures and represent a substantial economic and social burden. Novel coronavirus pneumonia (COVID-19), which began to spread in December 2019, has created challenges in the management of elderly hip fracture patients, not only by influencing the choice of operation and postoperative rehabilitation methods, but also by generating new risks for the medical staff. During this period, our infection and orthopedic treatment unit in the center of the epidemic area effectively treated 82 elderly patients with hip fracture, and no cross-infection occurred. Therefore, our experience in prevention and treatment is worth recommending to frontline anti-epidemic personnel.

INTRODUCTION

In December 2019, novel coronavirus pneumonia cases were first reported in Wuhan, Hubei province, and the infection spread rapidly throughout Hubei province and eventually to the whole country [1, 2]. At present, most countries in the world have a large number of people infected with the virus, which has become a serious threat to human safety. Novel coronavirus pneumonia is caused by the 2019 novel coronavirus (SARS-CoV-2) [3]. On February 11, 2020, the World Health Organization announced an official name for the disease: COVID-19. SARS-CoV-2 has been classified as a lineage of β-coronavirus, and has characteristics typical of the coronavirus family. A recent study indicated that SARS-CoV-2 is very similar to another coronavirus carried by bats, leading to the speculation that bats may have hosted the novel virus [4].

The population is generally susceptible to SARS-CoV-2, with an incubation period of 1-14 days. COVID-19 patients are already infectious in the incubation period when they have no specific symptoms, creating great obstacles to the early detection of SARS-CoV-2 carriers and the early implementation of strict isolation measures [5]. The transmission routes include droplet transmission, contact transmission, and possibly fecal-oral transmission and aerosol transmission, so both orthopedic patients and medical staff may become infected with SARS-CoV-2.

Hip fractures in the elderly (aged > 65 years) account for more than half of osteoporotic fractures [6], causing a huge economic and social burden. The death rate and disability rate due to hip fractures in the elderly are very high, and the 30-day mortality rate is greater than 5% [7]. In principle, active surgical treatment should be performed unless the patient's health condition is very poor, the patient cannot tolerate the operation, the risk of death during the operation is very high or postoperative nursing would be very difficult. In the perioperative period, multidisciplinary comprehensive treatment is needed to improve patients' exercise abilities and quality of life.

The outbreak of COVID-19 has presented new challenges in the management of fractures and the

protection of medical staff. During this period, our infection and orthopedic treatment unit in the center of the epidemic area effectively treated 82 elderly patients with hip fracture, and no-cross infection occurred. Therefore, we would like to share our treatment experience for the reference of frontline medical personnel at present.

Why should we pay more attention to the treatment of hip fractures in elderly patients with COVID-19?

The prognosis of COVID-19 is relatively poor for elderly patients, especially those who are already at much higher risk for mortality than younger patients due to basic diseases such as heart disease, hypertension, diabetes, etc. [8]. Hip fractures in the elderly should generally be treated surgically at an early stage to prevent complications related to staying in bed, except in patients who cannot tolerate or are unwilling to undergo surgery [6]. Elderly patients with hip fractures often need to stay in bed for half a year or more, which makes it difficult for them to discharge lower respiratory tract secretions, and thus alters the treatment process for diseases such as COVID-19. In addition, hypostatic pneumonia due to staying in bed may exhibit similar symptoms to COVID-19, increasing the difficulty of clinical diagnosis and treatment. Thus, the surgical treatment of hip fractures in elderly COVID-19 patients should enhance both the fracture recovery and the COVID-19 treatment. On the other hand, weakness, fevers, immune responses and other systemic manifestations associated with COVID-19 may interfere with incision healing and postoperative rehabilitation training. Furthermore, a recent study indicated that surgical stress may activate or aggravate the progression and mortality of COVID-19 [9]. Therefore, orthopedic doctors should comprehensively analyze each patient's situation in order to create the most favorable treatment plan for the individual.

Typical clinical features of COVID-19 and hip fractures in the elderly

A fever, cough and fatigue are the main symptoms of COVID-19, and are sometimes accompanied by nasal obstruction, a runny nose, sore throat and diarrhea [10]. Severe patients usually experience dyspnea within one week, and gradually develop refractory hypoxemia, acute respiratory distress syndrome, septic shock, acid-base metabolic imbalance and other manifestations [11]. The diagnosis of suspected cases should be based on both their distinct epidemiological histories and their clinical symptoms, especially for those who have been exposed to infected persons or relevant environments within 14 days. Confirmed cases should have additional pathogenic or serologic evidence of SARS-CoV-2 obtained using quantitative real-time PCR, gene sequencing or antibody analysis [12].

With increasing age, age-related factors such as poor visual sensitivity, nervous system disease, altered drug sensitivity, myasthenia, abnormal gait and poor balance will increase, thus increasing the risk of fractures due to falling. Clinically, hip fracture often manifests as a history of hip injury, hip pain or a shortening/rotating deformity of the affected limb [13].

Emergency treatment of hip fractures in elderly patients

During the COVID-19 epidemic, patients who come to the emergency department should first be questioned about their etiology and epidemiological history. Doctors should also measure each patient's body temperature to determine whether further treatment is needed. Any patient with a fever or respiratory symptoms should immediately be transferred to the designated medical institution and isolated. Based on clinical experience, mild cases of COVID-19 can be discharged with simply symptomatic treatment; otherwise, computed tomography (CT) examination is needed to ascertain the diagnosis.

Patients who need to be hospitalized for elective surgery should undergo pulmonary CT and SARS-CoV-2 nucleic acid and antibody tests immediately. Patients requiring immediate orthopedic surgery should also have their overall condition assessed by an expert panel. Pulmonary CT and SARS-CoV-2 antibody analyses are also necessary for accompanying family members. Any detection of positive results of SARS-CoV-2 should be reported immediately, and patients should be transferred to the designated hospital for further treatment before surgery. If the results of SARS-CoV-2 analysis suggest a negative diagnosis for a patient who has typical characteristics on pulmonary CT, the patient should immediately be admitted to the infection department for isolation, and an expert panel should be consulted to exclude the differential diagnosis; then, the necessity of elective surgery should be assessed again.

At the same time, if hip fracture is suspected in a received patient, the patient should be questioned about his/her medical history, basic diseases and medications, and should undergo an orthopedic physical examination to determine the injury mechanism (e.g., a fall; then the cause of the fall, the stress on the hip during the fall, etc.). Patients should be asked to lie on their back, straighten their legs, avoid sitting and prevent hip stress [14]. Elderly hip fracture patients who are willing and able to meet the requirements of operation should have hospitalization treatment arranged as soon as possible, and should undergo elective surgery [15].

Therapeutic principles for hip fractures in elderly patients during the COVID-19 epidemic

Conservative treatment is only applicable to patients who are unable (due to severe underlying diseases) or unwilling to undergo surgery [16]. Conservative treatment cannot effectively reduce and repair the broken ends of the fracture, which may lead to delayed union or even non-union of the fracture. Staying in bed long-term increases the risk of respiratory system infections, deep vein thrombosis of the lower extremities, bedsores and so on [17]. During the outbreak of COVID-19, confirmed patients may be forced to choose conservative treatment because their poor lung condition would prevent them from tolerating surgery. Uninfected patients may be less willing to undergo surgical treatment because they are afraid of cross-infection during their hospitalization.

For patients who are not infected with COVID-19, surgery should be performed as soon as possible, because the 30-day mortality after surgery decreases from 6.5% to 5.8% when the wait time for surgery is less than 24 hours [7]. The risk of cross-infection with SARS-CoV-2 in the perioperative period should be strictly controlled during the operation, and safety precautions such as isolation treatment and the use of an independent regional operating room should be taken to accelerate the process of entering and leaving the hospital and to reduce the length of stay.

For patients with suspected or confirmed COVID-19 infections, full preparation should be made before the operation, the operation should be performed with close monitoring of vital signs, limb function should be recovered as soon as possible, discharge should occur as soon as possible after the SARS-CoV-2 has been eliminated, and rehabilitation should be performed outside the hospital.

Choice of operation methods

In terms of operation methods, the first choice is hollow screw fixation, which can maintain the stability of the fracture. Dynamic hip screw (DHS) fixation can be used for patients in better physical condition. For displaced femoral neck fractures (Garden type III, IV), hip arthroplasty is the first choice. Hemi or total hip replacement should be chosen according to the patient's age, physical condition, activity before injury, acetabulum wear and mental health [18]. For stable intertrochanteric fractures, a DHS or intramedullary nail is the best choice. For unstable intertrochanteric fractures, an intramedullary nail is the first choice. In the treatment of subtrochanteric fractures, intramedullary nail fixation is the first choice, and long nail fixation can be used if necessary [19, 20]. Regardless of which operation method is adopted, to improve the prognosis of the elderly, it is very important to shorten the operation time and minimize soft tissue injury, blood loss and complications.

Pre-operation discussion

At our treatment unit, senior doctors from the orthopedic department presided over the pre-operation discussions, and experts from the respiratory, infection, anesthesia and other relevant departments participated in the consultations. Pre-operation discussions must clarify key issues such as the initial diagnosis, COVID-19 infection status, pulmonary function classification and infectivity, proposed operation mode, personnel needed for the operation, required surgical instruments and consumables, blood product infusion demand, antibiotic demand, etc. For severe COVID-19 patients, the first task should be to save the patients' lives, and surgical treatment should be carefully implemented. Suspected and confirmed patients must wear surgical masks. Hospital transport requires medical staff to travel together to ensure that the shortest distance is followed in the fastest amount of time without stopping on the way.

Anesthesia management during operation

In principle, general anesthesia should be used to anesthetize COVID-19 patients or suspected patients. A disposable filter should be placed between the tracheal tube and the respiratory circuit to reduce the pollution of the respiratory circuit. At our treatment unit, before induction, two pieces of wet gauze were used to cover the nose and mouth, oxygen was given through a mask, and 100% pure oxygen was recommended for all patients. General anesthesia patients were advised to undergo rapid anesthesia induction and tracheal intubation after total muscle relaxation. The anesthesiologist completed the endotracheal intubation at a long distance with the help of an assistant. After the intubation, the disposable appliance was discarded into a designated garbage can, which was not to be taken out of the operating room.

Routine electrocardiogram results, blood oxygen saturation, end-tidal carbon dioxide partial pressure, invasive arterial blood pressure, body temperature, urine volume, arterial blood gas levels and coagulation function were monitored. After the operation, we recommend sending patients to the intensive care unit isolation ward, and then removing the endotracheal tube after their general condition has stabilized. We used a closed endotracheal suction system. The tracheal tube should be removed under analgesia to reduce choking.

Intraoperative management

All medical staff should follow the principles of standard prevention and three-level prevention (Table 1). The participants in the anesthesia and operation procedures should be as few as possible, and should avoid entering other operating rooms. During the operation, surgeons should be under level III protection, and anesthesiologists may adopt level II protection, but their heads and faces should be equipped with a screen to prevent infection during tracheal intubation. Indoor personnel should not leave the room during the operation, and outdoor personnel should not enter the infected room.

After the operation, the medical personnel leaving the operating room must first replace their gloves, remove their protective clothing and foot covers and discard them in the designated garbage can. After removing their gloves, personnel should wash their hands thoroughly with a disinfectant, remove their mask, protective eyepiece or screen, and then wash their hands under running water for two minutes after leaving the operating room. After the operation, the goggles and masks should be sterilized with disinfectant paper towels and wiped with clean gauze for reuse. All the operation personnel should leave the operating room after bathing and changing their clothes.

In this process, strict requirements should be followed for putting on and taking off protective equipment. Protective equipment should be put on in the following order: hand disinfection → putting on working cap → putting on medical protective mask → putting on goggles or face shield / eye protection medical mask → putting on isolation clothing or protective clothing → putting on shoe covers → putting on gloves. Protective equipment should be taken off in the following order: taking off shoe covers → taking off gloves → hand disinfection → taking off isolation clothing or protective clothing → hand disinfection → taking off goggles or face shield → hand disinfection → taking off medical protective mask → hand disinfection → taking off working cap → hand disinfection and hand washing → replacing medical protective mask and disposable working cap.

Medical personnel can apply for exemption from isolation if there was no accidental exposure during the whole process. Otherwise, the medical staff involved in the operation should be observed for 14 days, and if there are any suspected symptoms during the observation period, they should be isolated and treated without delay.

Postoperative patient management

The management of personnel in the ward should be strengthened, and personnel should have their temperature checked before entering the ward. Daily protection should be emphasized during the epidemic period, and coveralls and medical surgical masks should be worn. Once suspected patients are identified, they should be isolated in a single room immediately, and the COVID-19 diagnosis procedure should be started [21]. If contact with patients' blood, body fluids, secretions, excreta, vomitus or pollutants may occur, personnel should wear latex gloves and wash their hands after removing the gloves. Personnel who may be splashed with patients' blood or body fluids should wear goggles or a protective face shield and impermeable protective clothing.

If a patient develops a fever after the operation, it is important to determine whether the fever is due to COVID-19 infection, trauma or the operation itself by comprehensively examining various inflammatory indexes (white blood cell, neutrophil, lymphocyte, C-reactive protein and procalcitonin levels), the drainage tube and the wound exudate, in addition to markers of COVID-19. If postoperative dyspnea and reduced blood oxygen saturation occur, serious complications such as pulmonary embolism should be excluded [22]. At the same time, stress ulcers, gastrointestinal bleeding, venous thrombosis and other complications should be prevented and treated [23].

Suspected or confirmed COVID-19 patients should continue to be treated in isolation with their body temperature continuously monitored. If a patient's body temperature returns to normal for more than three days, the lung CT images reveal obvious absorption of inflammation, and negative results have been obtained for respiratory pathogenic nucleic acids two consecutive times (with an interval of at least one day between samplings), the patient can be regarded as clinically cured of COVID-19. Patients can be discharged normally after being clinically cured; however, to avoid the risk of virus transmission as much as possible, it is still recommended that patients continue to practice centralized isolation under medical observation for two weeks [24, 25].

Rehabilitation of hip fractures in elderly patients

The overall goal of postoperative rehabilitation for elderly hip fracture patients is to restore motor function to the lower limbs as soon as possible. In patients who are able to bear exercise activities, rehabilitation exercise can be started within six hours after the operation [26], and the help of a multidisciplinary rehabilitation team can be provided. Early rehabilitation exercise can reduce complications such as pressure sores or deep vein thrombosis while also accelerating postoperative recovery and shortening the hospital

Table 1. Classification of protection levels.

Classification	Content
Protection level I	Suitable for pre-examination triage, fever and infection clinics. Involves wearing a disposable working cap, disposable surgical mask (N95 protective mask when contacting patients with an epidemiological history), working clothes, medical protective clothing (disposable protective clothing when necessary for pre-examination and triage) and disposable latex gloves when necessary, and washing hands thoroughly.
Protection level II	Suitable for medical staff engaging in diagnosis and treatment activities in close contact with suspected or confirmed patients. Involves wearing a disposable working cap, protective goggles/mask, medical protective mask, protective clothing, disposable latex gloves and disposable shoe covers, and washing hands thoroughly.
Protection level III	Applicable to medical personnel who may be exposed to aerosol from suspected or confirmed patients due to sputum aspiration, respiratory sampling, tracheal intubation, tracheotomy, etc. When such personnel are working under the possibility of being sprayed or splashed with respiratory secretions or other substances, they should wear a disposable working cap, protective mask (or comprehensive respiratory protective device or positive pressure type head cover), medical protective mask, preventive clothing, disposable latex gloves and disposable shoe covers, and should wash their hands thoroughly.

stay [27]. Rehabilitation plans including aerobic training of the upper limbs can increase patients' adaptation and utilization of oxygen. Based on the results of postoperative rehabilitation, the patient should increase his/her weight-bearing exercise and seek to enhance balance. Rehabilitation exercise outside the hospital under the guidance of doctors can improve physical function and quality of life.

Summary

Elderly patients with hip fractures often have a variety of underlying diseases, and are prone to related complications. The outbreak of COVID-19 has created great challenges for orthopedic doctors managing such patients. According to our experience, standardized and effective diagnosis and treatment is the key to ensuring that elderly hip fracture patients recover as soon as possible in the context of the COVID-19 epidemic. We recommend these orthopedic surgical practices for global medical workers fighting against the COVID-19 epidemic, especially among elderly hip fracture patients.

AUTHOR CONTRIBUTIONS

Study design: Hui Li, Guohui Liu. Funding: Guohui Liu. Data collection: Jing Liu, Bobin Mi, Liangcong Hu, Yuan Xiong. Data interpretation: Hang Xue, Wu Zhou, Faqi Cao, Mengfei Liu. Manuscript preparation: Jing Liu, Bobin Mi, Lang Chen, Yan Chenchen. Critical revision of the manuscript: all authors.

CONFLICTS OF INTEREST

All authors have declared no conflicts of interest.

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Research Paper

Immune environment modulation in pneumonia patients caused by coronavirus: SARS-CoV, MERS-CoV and SARS-CoV-2

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ABSTRACT

Currently, we are on a global pandemic of Coronavirus disease-2019 (COVID-19) which causes fever, dry cough, fatigue and acute respiratory distress syndrome (ARDS) that may ultimately lead to the death of the infected. Current researches on COVID-19 continue to highlight the necessity for further understanding the virus-host synergies. In this study, we have highlighted the key cytokines induced by coronavirus infections. We have demonstrated that genes coding interleukins (Il-1α, Il-1β, Il-6, Il-10), chemokine (Ccl2, Ccl3, Ccl5, Ccl10), and interferon (Ifn-α2, Ifn-β1, Ifn2) upsurge significantly which in line with the elevated infiltration of T cells, NK cells and monocytes in SARS-Cov treated group at 24 hours. Also, interleukins (IL-6, IL-23α, IL-10, IL-7, IL-1α, IL-1β) and interferon (IFN-α2, IFN2, IFN-γ) have increased dramatically in MERS-Cov at 24 hours. A similar cytokine profile showed the cytokine storm served a critical role in the infection process. Subsequent investigation of 463 patients with COVID-19 disease revealed the decreased amount of total lymphocytes, CD3+, CD4+, and CD8+ T lymphocytes in the severe type patients which indicated COVID-19 can impose hard blows on human lymphocyte resulting in lethal pneumonia. Thus, taking control of changes in immune factors could be critical in the treatment of COVID-19.

INTRODUCTION

The family of coronaviruses (CoV) are enveloped RNA viruses which can be highly pathogenic to human beings [1]. Before long, the epidemics of the two highly infectious coronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV) [2] and Middle East respiratory syndrome coronavirus (MERS-CoV) [3] had resulted disastrous effects to human beings globally. The outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and Coronavirus disease-2019 (COVID-19) originated from Wuhan, China in the end of 2019 has caused thousands of deaths [4]. Phylogenetic analysis of SARS-CoV-2 indicated that it is closely related to SARS-CoV (~79%) and a little more distant to MERS-CoV(~50%) [5]. The pathological changes of COVID-19 dead puncture suggest that its pathological characteristics are very similar to SARS-CoV and MERS-CoV-induced viral pneumonia [6]. Thus, it is critical to identify common patterns between these lethal pathogens and immune response.

Coronavirus has specific immune response and immune escape characteristics, and then causes severe pathogenic mechanisms through inflammation, which leaded to severe pneumonia, pulmonary oedema, ARDS, or multiple organ failure and even death [7]. Cytokine storm, also known as cytokine cascade, or hypercytokinemia, is caused by infection, drugs or autoimmune diseases of the body's excessive immunity response [8]. Pioneering investigations have confirmed that increased volumes of pro-inflammatory cytokines in serum (e.g., IL-1B, IL-6, IL-12, IFN-γ) correlated with pulmonary inflammation and severe lung impairment in SARS patients [9]. MERS-CoV infection was also described to provoke increased concentrations of cytokines (IL-15,IL-17,

TNF-α, and IFN-γ) [10]. It is reported that victims infected with SARS-CoV-2 also demonstrate high amounts of IL-1B, IFN-γ, IP10, and MCP1, which may attribute to activated Th1 (T helper) cell responses [11]. Although these virus invaded human bodies through various proteins(SARS-CoV: angiotensin-converting enzyme 2, Angiotensin-Converting Enzyme 2 (ACE-2), MERS-CoV: Dipeptidyl Peptidase-4 (DDP-4), SARS-CoV-2: ACE-2 possibly), the similar cytokine cascade from immune response which caused severe damage has been widely covered [12].

Hence, identifying the key cytokines induced by coronavirus infection and the cells involved in the regulation of cytokine storms, blocking their signal transduction, will greatly reduce the inflammatory response and damage to the lung tissue and multiple organs of patients.

RESULTS

Invasion process and immune response of SARS-CoV, MERS-CoV and SARS-CoV-2

SARS-CoV-2 shows 88% identity to the sequence of SARS-like coronaviruses and about 50% to the sequence of MERS-CoV. Due to the similar structure, their pathogenesis is similar. SARS-CoV-2, just like SARS-CoV, requires the ACE-2. MERS-CoV enters target cells not via ACE-2, but via binding to DPP-4. Both ACE-2 and DPP-4 are expressed in several human tissues. While the virus enters the cells, antigen presentation subsequently stimulates the body's humoral and cellular immunity, which are mediated by virus-specific immune cells. Immune response causes a lot of symptoms and the main death cause of coronavirus is cytokine storm, which is the deadly uncontrolled systemic inflammatory response. COVID-19 induced strong immune response is resulting from the release of large amounts of pro-inflammatory cytokines and chemokines, which are similar to the symptoms of SARS-CoV and MERS-CoV infections. Hence, although the pathogenesis of COVID-19 is poorly understood, the similar mechanisms of SARS-CoV and MERS-CoV still can give us a lot of information on the pathogenesis of SARS-CoV-2 infection to facilitate our recognition of COVID-19 (Figure 1).

SARS-CoV-induced immune responses

To explore SARS-CoV induced immune responses, infected mice group was analyzed. Lungs from mice were harvested at 12, 24, and 48 hours post-infection and at least 3 biological replicates were collected. As pneumonia in the elderly is more susceptible to infection and the symptoms are heavier, the changes in inflammatory factors at 12, 24, and 48 hours after the infection of the SARS virus in elderly rats were analyzed, and multiple factors were found to occur. IL-1α, IL-1 β, IL-6 and IL-10 presented a significant higher level and was more obvious at 24 hours while the level of IL-7 showed moderate fluctuation and IL-23α a decreased trend (Figure 2). The results showed that SARS-CoV infection induced a cytokine storm.

As for interferon system which protects mammals against virus infections, we analyzed the changes of interferon at 12h, 24h and 48h after infection with SARS virus in elderly rats. We found IFN-α2, IFN-β1 and IFN2 all demonstrated higher expression volumes especially in 24h (Figure 3) which suggest the onset such as plasmacytoid dendritic cells (pDCs) and proinflammatory monocytes. In terms of changes in chemokines which synergistically induce a proinflammatory recruitment, the level of CCL2, CCL3, CCL5 and CCL10 are all drastically elevated in 24h and remained high level in 48h. In the meantime, CXCL3 expression increased in 24h but decreased in 48h. And CXCL5 expression showed a decreased trend in 24h and 48h compared to 12h (Figure 4). Taken together, these rising molecules reflected anti-viral response from the host in the early phase.

MERS-CoV-induced immune responses

In order to explore the common pattern of immune response after coronavirus contagion, we analyzed the situation in MERS-CoV infected human microvascular endothelial cells. So we analyzed the expression genes of interleukins and interferons after 24h. And we found interleukins (IL-6, IL-23α, IL-10, IL-7, IL-1α, IL-1β) and interferons (IFN-α2, IFN2, IFN-γ) have increased dramatically (Figure 5) which indicated an elevated anti-virus immune response.

Differences in immune responses in young and aged mice

To explore the immune differences between young and aged mice, we analyzed the cytokine variation after SARS-CoV infected for 12 and 24 hours. The results showed that several cytokines increase more significantly in aged mice than young mice (Figure 6). It indicated that coronavirus may cause more severe cytokine storms in elderly patients. To quantify the immune response on cell level, we applied ssGSEA method to compare the variation of different immune cells of aged and young mice after SARS-CoV infection. The level of T cells, NK cells and monocytes increased significantly both in aged and young mice. Lymphoid cells show an elevated level in young mice but remained stable comparatively in aged mice. And

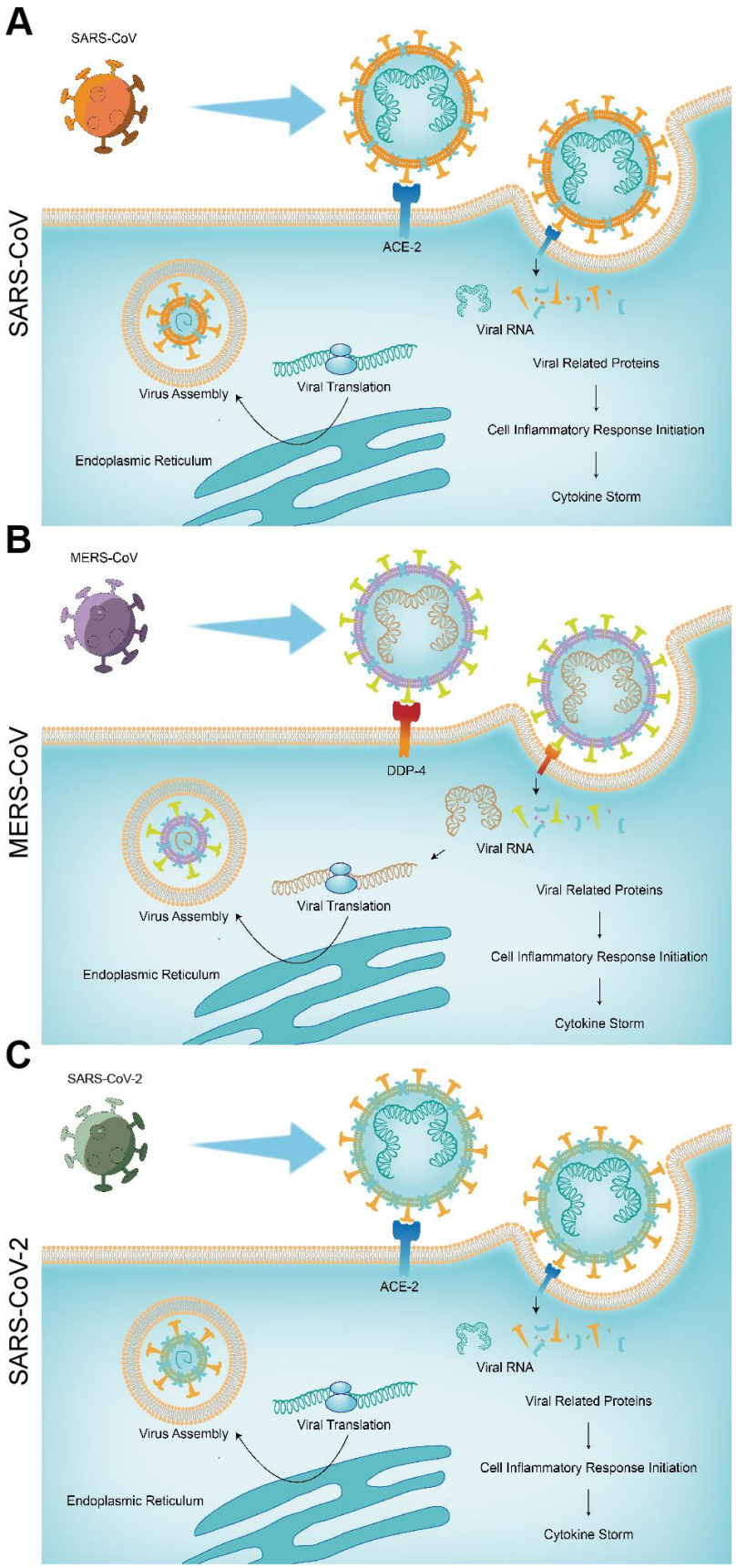

Figure 1. The pathogenic mechanisms of the three pneumonias. (**A**) SARS-CoV; (**B**) MERS-CoV; (**C**) SARS-CoV-2.

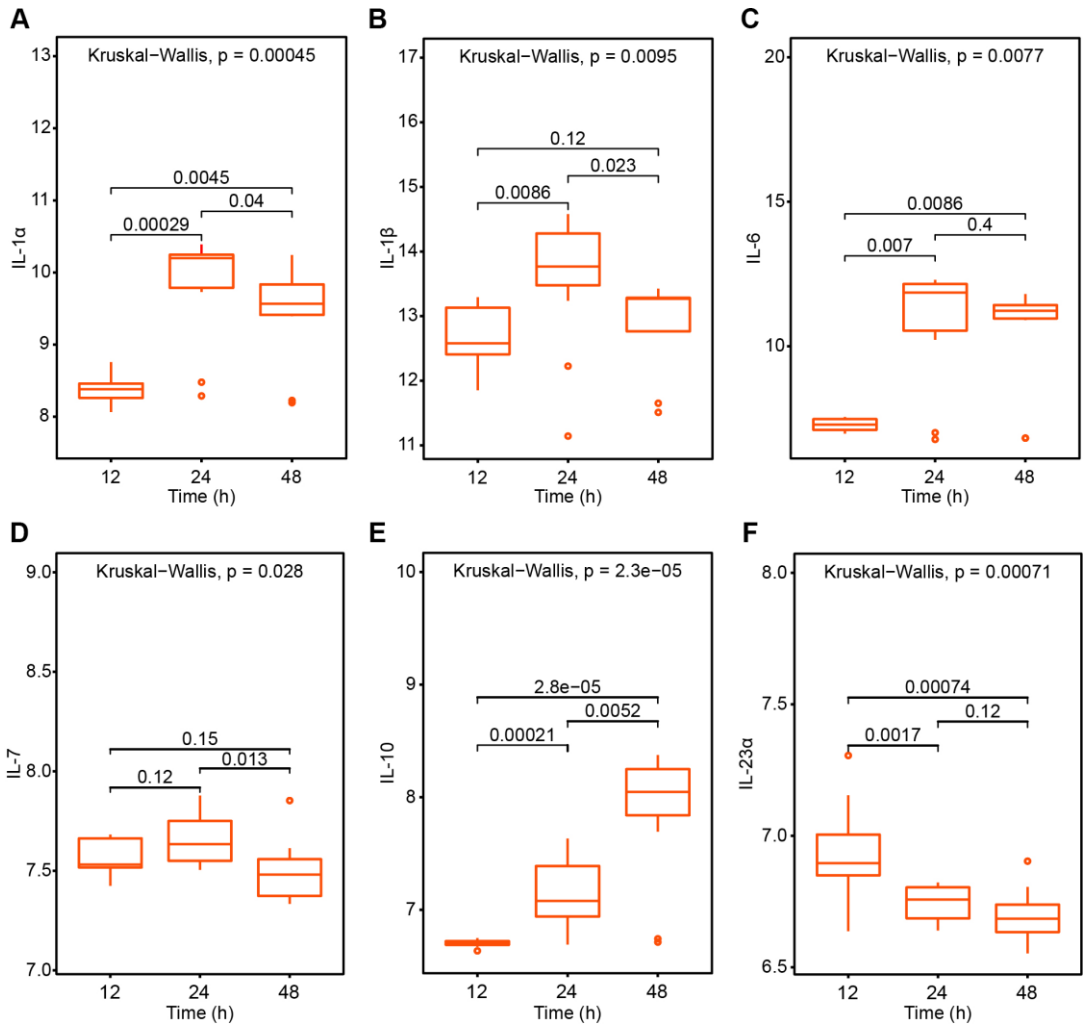

Figure 2. The pneumonia related interleukin cytokines variation trend after SARS-CoV treatment 12h, 24h and 48h respectively. (**A**) IL-1α; (**B**) IL-1β; (**C**) IL-6; (**D**) IL-7; (**E**) IL-10; (**F**) IL-23α.

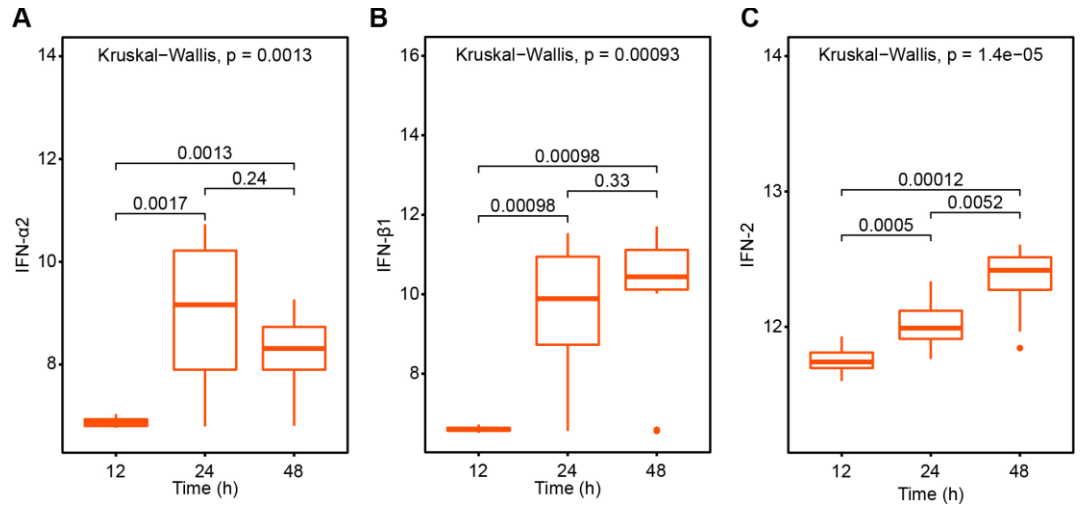

Figure 3. The interferon variation trend after SARS-CoV treatment 12h, 24h and 48h respectively. (**A**) IFN-α2; (**B**) IFN-β1; (**C**) IFN-2.

granulocytes tend to decrease both in aged and young mice after the infection. Interestingly, monocytes aged mice increased more quickly (24h) than in the young mice (48h) (Figure 7). The results showed that coronavirus infection can cause strong immune response in both young and old mice. Lymphocyte-mediated immune responses are more severe in young mice, but monocyte-mediated immune responses are more rapid in older mice.

Clinical immunoassay of COVID-19 patients

For further study, we analyzed immune cells in peripheral blood of 463 patients with COVID-19 disease (Table 1). We found that total lymphocytes, CD3+, CD4+ and CD8+ T lymphocytes significantly went down in the severe type patients compared to the common type (Figure 8) which indicated SARS-CoV-2 can impose hard blows on human lymphocyte resulting in lethal pneumonia. Moreover, total lymphocytes, and CD8+ T lymphocyte counts decreased more severely in patients >= 50 years old than those below 50 which suggest that young patients are more likely to bounce back. And CD3+ or CD4+ lymphocyte counts showed no significant difference between different age groups.

DISCUSSION

Pathological manifestations of COVID-19 greatly resemble what has been seen in SARS and MERS

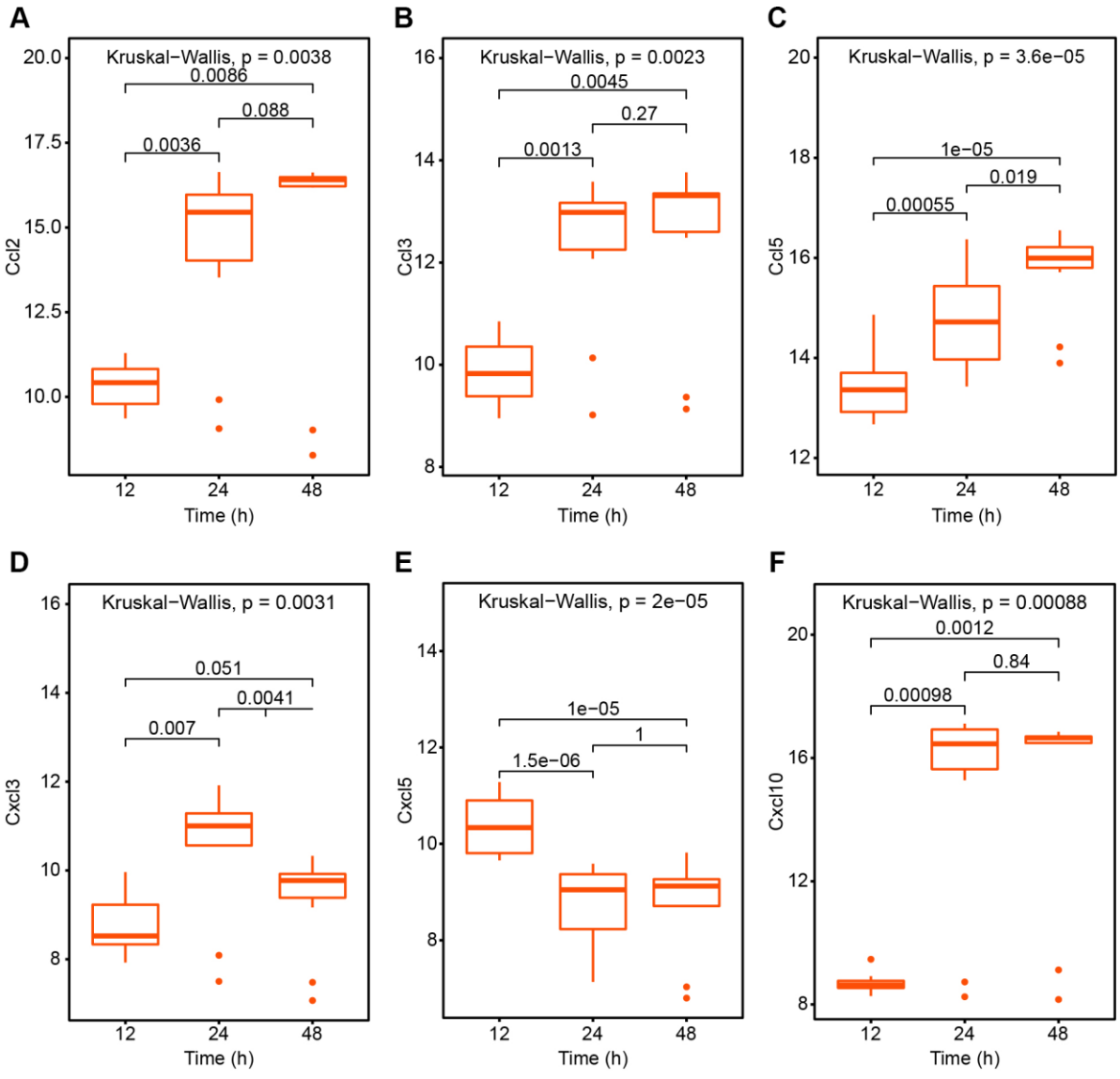

Figure 4. The variation trend of chemokines after SARS-CoV treatment 12h, 24h and 48h respectively. (**A**) Ccl2; (**B**) Ccl3; (**C**) Ccl5; (**D**) Cxcl3; (**E**) Cxcl5; (**F**) Cxcl10.

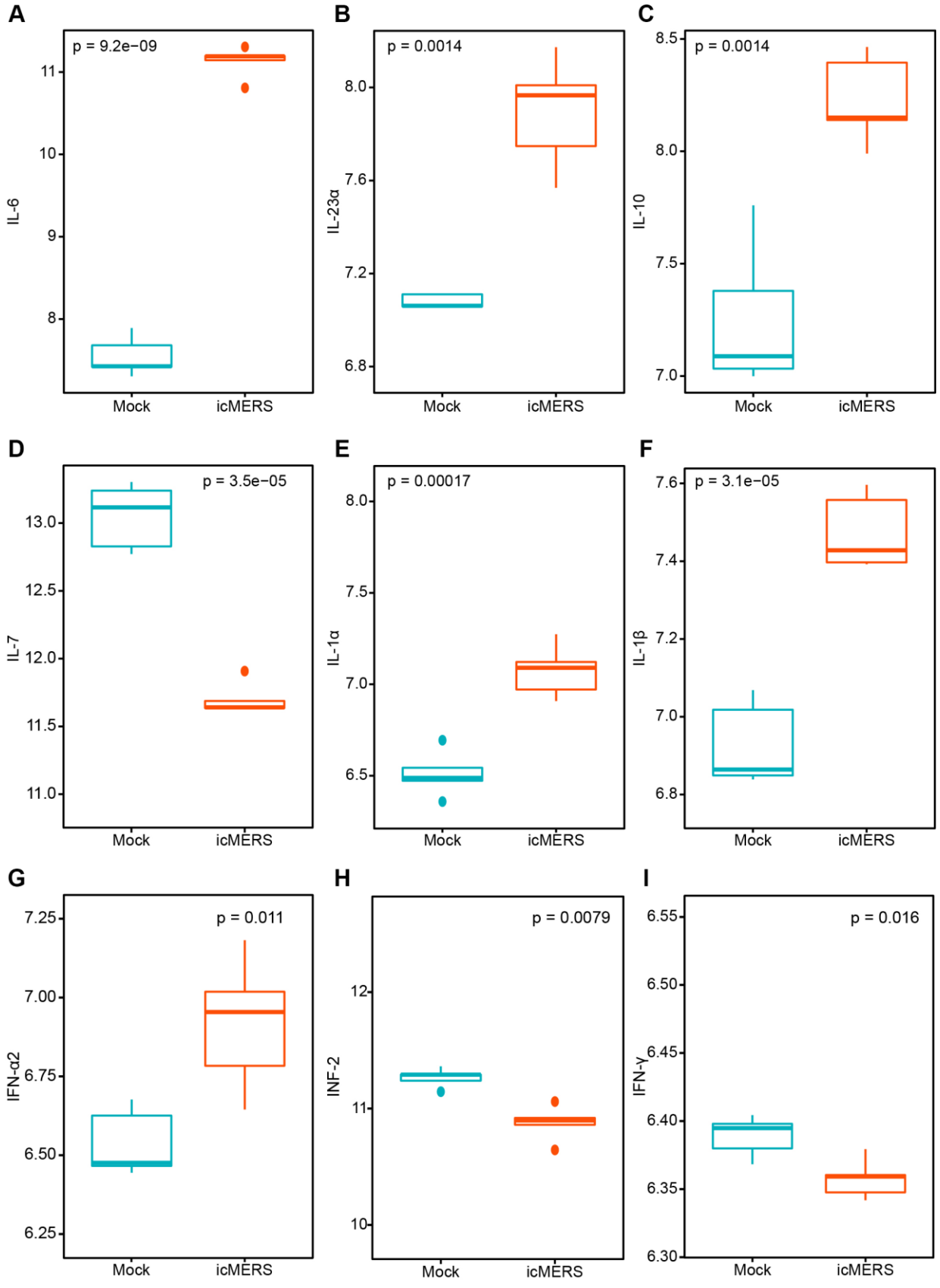

Figure 5. The interleukin cytokines and interferon variation trend after MERS-CoV treatment in 24 hours. (**A**) IL-6; (**B**) IL-23α; (**C**) IL-10; (**D**) IL-7; (**E**) IL-1α; (**F**) IL-1β; (**G**) IFN-α2; (**H**) IFN-2; (**I**) IFN-γ. (Mock: Control group; icMERS: MERS-CoV treated group).

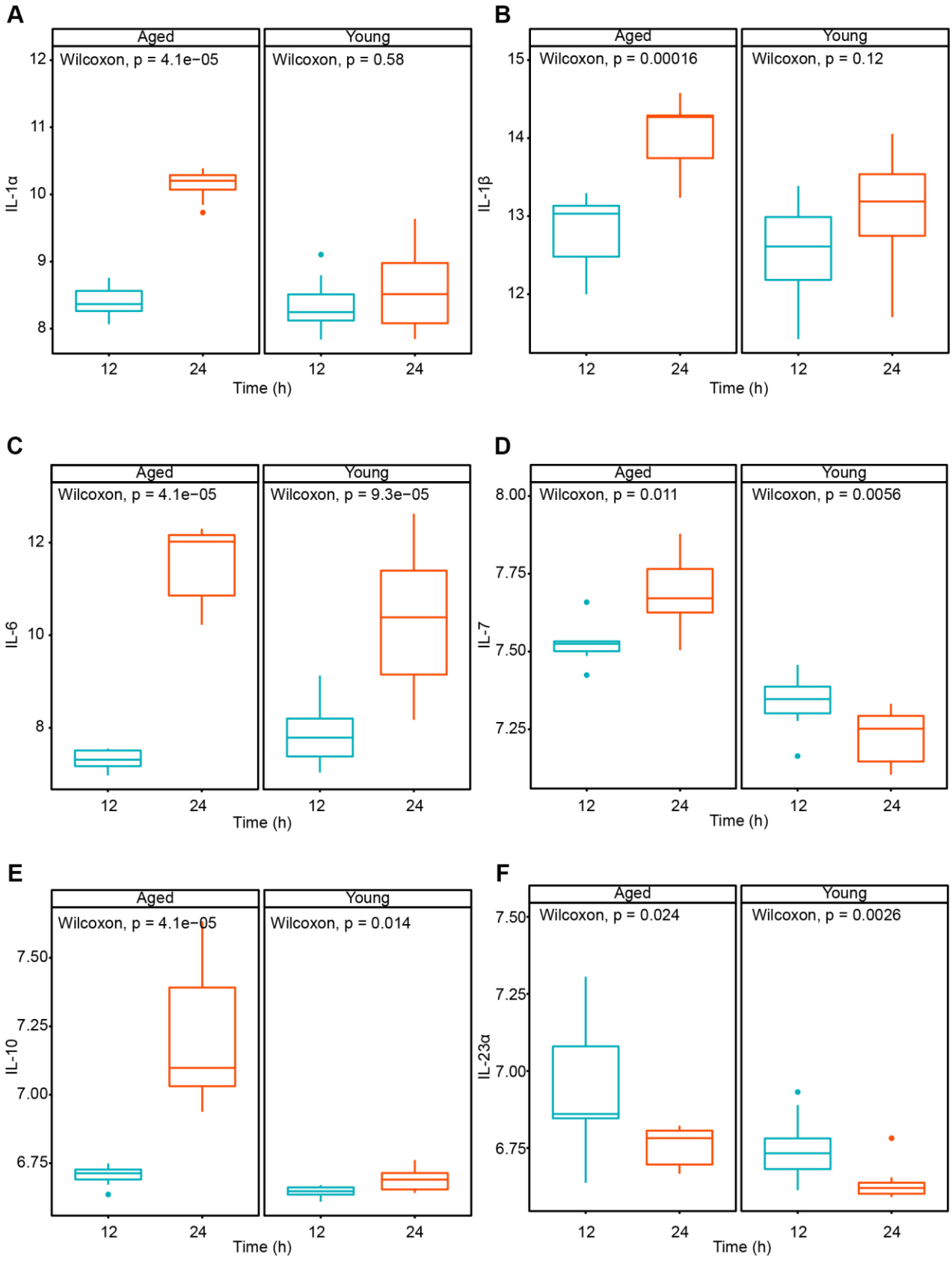

Figure 6. Cytokine variation in young and aged mice after MERS-CoV treated for 12 and 24 hours. (**A**) IL-1α; (**B**) IL-1β; (**C**) IL-6; (**D**) IL-7; (**E**) IL-10; (**F**) IL-23α.

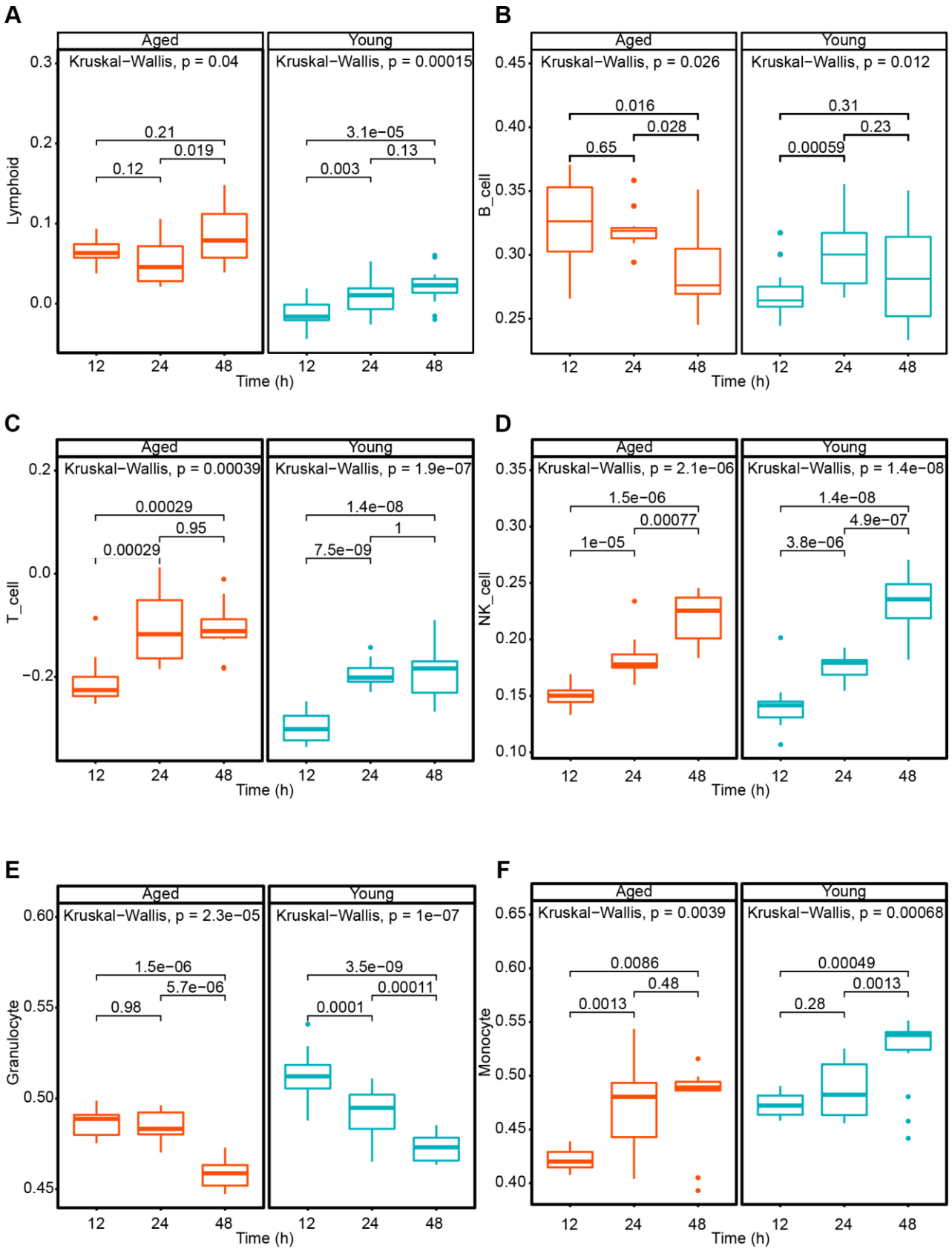

Figure 7. The quantification of immune cell in SARS-CoV infected different age groups mice for 12 and 24 hours based on ssGSEA method. (**A**) IL-1α; (**B**) IL-1β; (**C**) IL-6; (**D**) IL-7; (**E**) IL-10; (**F**) IL-23α.

Table 1. Characteristics of 463 COVID-19 patients.

	Overall (%)	Severe (%)	Common (%)	*P* value
Age (year)	51(43-60)	54(46-64)	49(42-58)	<0.001
15~49	208/463(44.92)	60/181(33.15)	148/282(51.06)	<0.001
50~64	177/463(38.23)	79/181(43.65)	98/282(32.62)	0.054
≥ 65	78/463(16.85)	42/181(23.20)	36/282(12.77)	0.034
Gender				
Male	244/463(52.70)	99/181(54.70)	145/282(51.42)	0.492
Female	219/463(47.30)	82/181(45.30)	137/282(48.58)	
Symptoms				
LOS (day)	12(9-14)	13(9-16)	11(9-14)	0.006
Fever (°C)	38.5(38-39)	38.9(38.2-39)	38.4(37.8-39)	＜0.001
Cough	356/463(77.06)	145/181(80.11)	211/282(74.82)	0.188
Difficulty breathing	194/463(41.90)	82/181(45.30)	112/282(39.72)	0.234
Expectorant	146/463(31.53)	64/181(35.36)	82/282(29.08)	0.156
Fatigue	130/463(28.08)	49/181(27.07)	81/282(28.72)	0.699
Muscle ache	61/463(13.17)	21/181(11.60)	40/282(14.18)	0.423
headache	25/463(5.40)	10/181(5.52)	15/282(5.32)	0.924
Diarrhea	13/463(2.81)	4/181(2.21)	9/282(3.19)	0.533
Sore throat	13/463(2.81)	3/181(1.66)	10/282(3.55)	0.230
Runny	11/463(2.38)	3/181(1.66)	8/282(2.84)	0.618
Hemoptysis	9/463(1.94)	4/181(2.21)	5/282(1.77)	0.990

*LOS: Length of stay.

infection which massive interstitial inflammatory infiltrates diffused in the lung [6]. The cellular fibro-myxoid exudate which caused severe alveolar impairment from postmortem autopsy indicates the cytokine storm may play a critical role in patient rapid death. In this study, we found that genes coding interleukins(Il-1α, Il-1β, Il-6, Il-10), chemokines (Ccl2, Ccl3, Ccl5, Ccl10), and interferons (Ifn-α2, Ifn-β1 and Ifn2) raised significantly in SARS-CoV treated mice within 24h which in line with the elevated infiltration of T cells, NK cells and monocytes. And similar pattern of cytokine projection were found in the MERS-CoV infected group.

Investigating the inflammatory profile in SARS and MERS may advance our knowledge of the immune-pathological process in COVID-19 treatment. In this study, we reviewed SARS-infected mice and MERS-treated human micro vascular endothelial cells to clarify the association between temporal changes in cytokine/ chemokine profiles and the six immune cell infiltration patterns. We retrospectively reviewed the clinical data of 463 cases with common and severe type COVID-19, who discharged before February 6, 2020. We found that severe type of patients suffered more serious symptoms like higher fever and took more time to recover which may suggest the fluctuation of immune indices is of predictive value.

To explore the specific mechanism of immune environment changes, we analyzed potential influencing factors. Cytokines, not merely aid in the process of antimicrobial immunity but are liable for immune-pathological damage to owner cells, causing significant morbidity or even fatality in multiple respiratory disorders as well [17, 18]. Chemokines like CXCL10 (IP10) and CCL2 (MCP-1) proved to be up-regulated in monocytes/macrophages by SARS-CoV which is consistent with our results [19]. The clinical progression of MERS cases proves that secretion of monocyte chemo-attractant protein-1 (MCP-1), CXCL10 is out of control [20]. Pro-inflammatory cytokines (IL-6, CCL5), and interferon-stimulated genes (CXCL10) are involved in Toll-like receptors (TLR) signaling [21]. These molecules are effectors on the process of respiratory virus infections towards the context of Acute Respiratory Distress Syndrome (ARDS) which is lethal to the COVID-19 patients [22]. IL-12 is the main cytokine secreted by DCs that manages the differentiation of CD4+ T cells into Th1 cells and serves essential duty in cell-mediated immunity. And IL-23 which includes in the IL-12 Family are

predominantly pro-inflammatory cytokines which contribute critical roles in the growth of Th17 cells [23, 24]. Increased expression of IL-12 and IL-23 after SARS-infected lung tissue in mice may indicate the activated response of Th1 and Th17 cells which is observed in MERS victims as well [10]. Interesting, in the SARS-CoV infected cells, the ACE-2 was significantly correlated with neutrophils, NK cells, Th17 cells, Th2 cells, Th1 cells, DC which may call for further investigations [25].

IFN-α/β is regarded as one of the body's primary antiviral defenses. IFN-β exerts its effects through intercellular communication resulting the induction of IFN-α/β and interferon-stimulated genes (ISGs), which make up an important aspect of host antiviral defense [26]. Notwithstanding, particular cell types, such as pDCs and monocytes, have been confirmed to produce more IFN than other cell types when viral infection committed [27]. And elevated level of IFN and monocyte infiltration in our analysis validates this. The

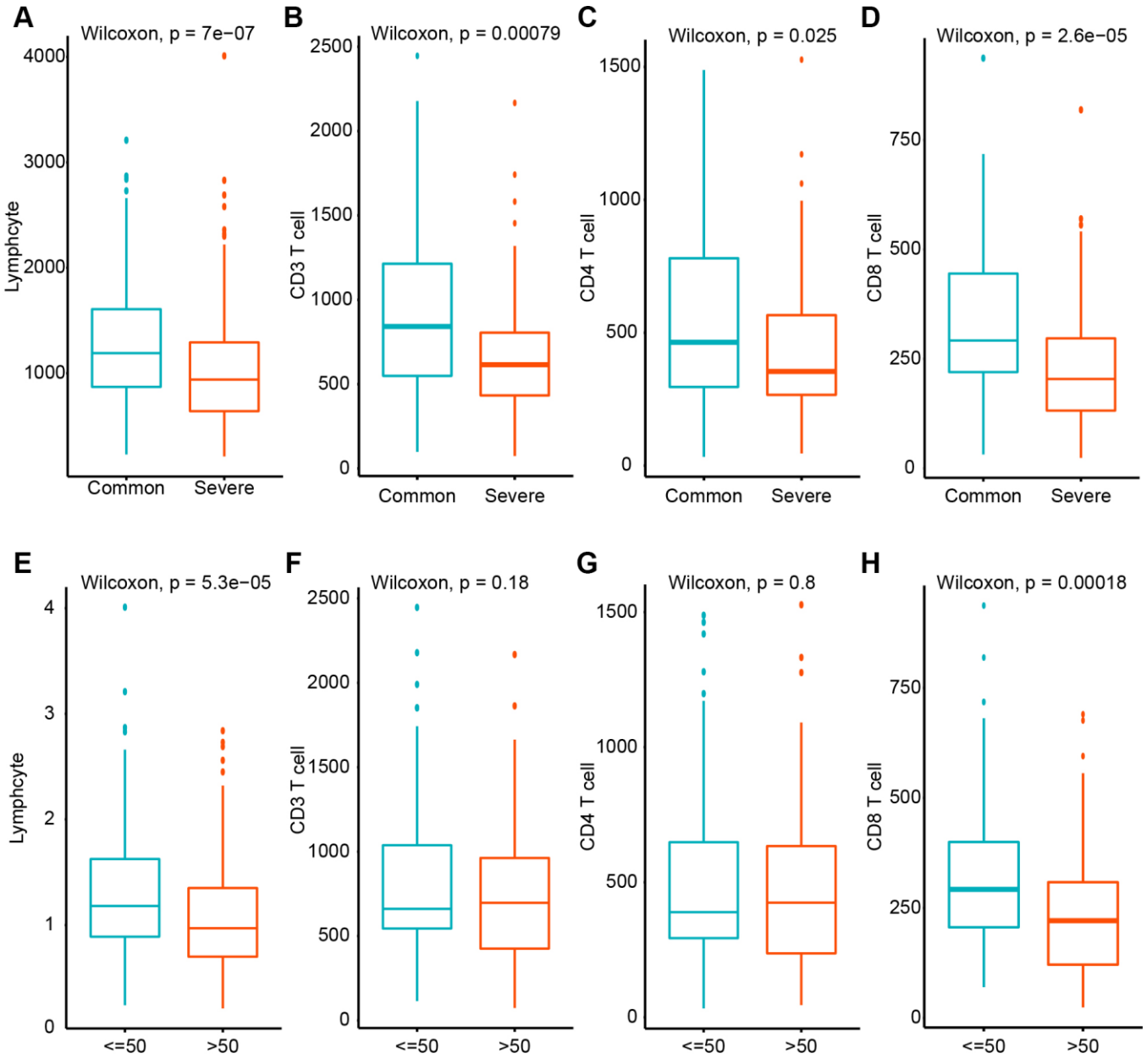

Figure 8. The quantification of total lymphocytes, CD3+, CD4+ and CD8+ T lymphocytes from peripheral blood from COVID-19 patients by flow cytometry. (**A**–**D**) Count variation between common and severe type disease. (**E**–**H**) Count variation between different age groups.

innate immune response on the basis of pDCs and monocytes may play substantial role in the formation of the cytokine storm which damages the lung severely.

Lymphopenia is common in COVID-19 patients. Severe lymphocyte reduction occurred in about 10% of patients, especially in the heavy group, which is consistent with the latest reported results [28]. Flow cytometry showed that CD3+, CD4+ and CD8+ T lymphocytes had decreased to varying degrees. And aged patients suffered a more severe decrease in total lymphocytes and CD8+ T lymphocytes. About 40% of patients had a decrease in CD4 + T lymphocytes, and the incidence was higher in the heavy group than in the common group. This shows that SARS-CoV-2 may mainly attack lymphocytes in the body, which can cause the reduction of CD4 + T lymphocytes, resulting in decreased immune function and infection, and severe cases of severe pneumonia.

CONCLUSIONS

In a word, we analyze the cytokine profiles in SARS-CoV infected mice and MERS-CoV infected human micro vascular cells. Interleukin (Il-1α, Il-1 β, Il-6, Il-10), chemokine (Ccl2, Ccl3, Ccl5, Ccl10), and interferon (Ifn-α2, Ifn-β1 and Ifn2) increased dramatically in SARS-CoV treated mice within 24h. As for MERS-CoV treated cells, interleukins (IL-6, IL-23α, IL-10, IL-7, IL-1α, IL-1β) and interferon (IFN-α2, IFN2, IFN-γ) showed a significant ascending trend in 24h. Subsequent analysis revealed elevated abundance of T cells, NK cells and monocytes in both young and aged mice group treated by SARS-CoV. And impaired lymphocyte system in severe and aged COVID-19 patients indicates the disease is more likely to progress when cytokines exhausted and functional lymphocytes suppressed. Thus, catching the window of treatment for COVID-19 according to these immune molecules may be critical.

MATERIALS AND METHODS

Microarray analysis

Microarray datasets related to gene expression were obtained from the GEO database For SARS-CoV dataset (GSE36969), young (8 weeks old) and aged (1 year old) female BALB/c mice were intranasally infected with 10^5 PFU of MA15 epsilon (SARS-CoV pathogenic virus). For MERS-CoV dataset (GSE79218), human microvascular endothelial cells were infected with MERSCOV002 (MERS-CoV pathogenic virus) or mocks and the 24h post-infection time point was picked for analysis. All gene expression datasets above were independently log2 transformed and quantile normalized in the linear models for microarray data (LIMMA) package in the R language environment.

Clinical data

Patients who were diagnosed with COVID-19 and collected from Wuhan Jinyintan Hospital from January 1 to February 6, 2020 were collected. This study was approved by the Ethics Review Committee of Wuhan Jinyintan Hospital. Diagnostic criteria are according to the "Diagnosis and Treatment of New Coronavirus Pneumonia " issued by the General Office of the National Health and Health Commission as the diagnostic standard [13]. We classified patients into 2 types: (1) Common: fever, respiratory tract and other symptoms, with or without pneumonia manifestations on imaging; (2) Severe: meet any of the following: ① Respiratory distress, RR ≥ 30 beats / min; ② In resting state, refers to oxygen saturation ≤ 93%; ③ partial pressure of arterial oxygen (PaO2) / oxygen concentration (FiO2) ≤ 300mmHg.

Statistical analysis

The Wilcoxon t-test were used to determine differences between two groups for continuous variables and the Kruskal – Wallis rank sum test for more than two groups, respectively. And we applied Single Cell Gene Set Enrichment Analysis (ssGSEA) to estimate the infiltration of immune cells [14] using the GSVA R package [15]. Fingerprint genes of granulocytes, monocytes, NK cells, activated and naive T cells, B cells and lymphoid cells are extracted from the previous study [16]. Statistical analyses were performed in the R (version 3.6.1) language environment and P-value <0.05 (two-sided) is considered to be significant.

AUTHOR CONTRIBUTIONS

JHZ conceived the initial concept and designed the study, KW, ZZ and ZXY participated to design the study and in the data extraction. ZZ and ZXY wrote the manuscript. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Research Paper

Typical radiological progression and clinical features of patients with coronavirus disease 2019

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ABSTRACT

We aimed to describe typical radiological features and progression of Coronavirus disease 2019 (COVID-19) patients. We reviewed the chest CT scans, laboratory findings, and clinical records of 66 COVID-19 patients who were admitted to affiliated hospitals of Nanchang university, Nanchang, China, from Jan 21 to Feb 2, 2020. CT was used to evaluate the radiological characteristics of COVID-19 patients. Only 4 patients (4/66, 6%) claimed their exposure to COVID-19 pneumonia patients. The major symptoms were fever (60/66, 91%) and cough (37/66, 56%). The predominant features of lesion were scattered (43/66, 65%), bilateral (50/66, 76%), ground-glass opacity (64/66, 97%), and air bronchogram sign (47/66, 71%). Forty-eight patients (48/66, 73%) had more than two lobes involved. Right lower lobe (58/66, 88%) and left lower lobe (49/66, 74%) were most likely invaded. Twelve patients (12/66, 18%) had at least one comorbid condition. Pleural traction (29/66, 44%), crazy paving (15/66, 23%), interlobular septal thickening (11/66, 17%), and consolidation (7/66, 11%) were also observed. The typical radiology features of COVID-19 patients are scattered ground-glass opacity in the bilateral lobes. Fever and cough are the major symptoms. Evaluating chest CT, clinical symptoms, and laboratory results could facilitate the early diagnosis of COVID-19, and judge disease progression.

INTRODUCTION

Since December 2019, a series of unknown pneumonia caused by a novel coronavirus broke out in Wuhan, Hubei, China. This new coronavirus was named as severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) or 2019 novel coronavirus (2019-nCoV) [1]. The disease caused by 2019-nCoV is coronavirus disease 2019 (COVID-19), which had been confirmed to be a global pandemic by the World health organization (WHO). By April 8 2020, more than 1, 350, 000 infected cases and 79, 000 deaths have been caused by COVID-19 [2]. COVID-19 has been effectively prevented and controlled in China, Singapore, South Korea, and Japan right now, but 2019-nCoV is spreading fast in Europe and the United State. Obviously, the threat to the global health and economy by 2019-nCoV will last for a long time [3, 4].

2019-nCoV, a betacoronavirus, is a member of family Coronaviridae [5]. In total, six types of coronavirus have been identified including middle east respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV), NL-63, OC-43, and 229E, among which MERS-CoV and SARS-CoV could cause severe respiratory diseases [6]. 2019-nCoV, a novel coronavirus, could interact with the human angiotensin converting enzyme 2 receptor through its spike protein [7, 8]. 2019-nCoV spread among population mainly through respiratory droplets and direct contact, and could cause several different symptoms including fever, cough, and fatigue [9].

Table 1. Clinical characteristics and laboratory results of patient with COVID-19 pneumonia (n=66).

Characteristic	Number (%)
Male	43 (65%)
Female	23 (35%)
> 50 years old	21 (32%)
≤ 50 years old	45 (68%)
Exposure to COVID-19 pneumonia patients	4 (6%)
Lived in or visited Wuhan during the epidemic	21 (32%)
Unknown exposure	41 (62%)
Symptoms	
Fever	60 (91%)
Cough	37 (56%)
Sore throat	17 (26%)
Sputum	16 (24%)
Fatigue	15 (23%)
Dyspnoea	14 (21%)
Dizziness	9 (14%)
Myalgia	7 (11%)
Headache	3 (5%)
Diarrhoea	3 (5%)
Nausea	3 (5%)
Rhinorrhea	2 (3%)
C-reactive protein (mg/L; normal range 0-10)	
Increased	38 (58%)
Decreased	0
Normal	28 (42%)
Leucocytes ($\times 10^9$, normal range 3.5-9.5)	
Increased	1 (2%)
Decreased	14 (21%)
Normal	51 (77%)
Lymphocyte ($\times 10^9$, normal range 1.1-3.2)	
Increased	0
Decreased	29 (44%)
Normal	37 (56%)
Comorbid conditions	
Any	12 (18%)
Hepatitis or liver cirrhosis	8 (12%)
Hypertension	4 (6%)
Diabetes	2 (3%)
Chronic pulmonary disease	1 (2%)
Cardiovascular disease	1 (2%)

Early diagnose of 2019-nCoV is important for the next isolation and treatment. However, shortage of nucleic acid detection reagent has been reported in some countries. CT characterized by convenience and accuracy plays a key role in the diagnose of respiratory diseases. CT provides a simple, direct, and convenient auxiliary diagnosis method for the patients, who cannot be tested by RT-PCR. However, there are very few studies focusing on the lung CT features of COVID-19 patients so far.

In this study, we summarized the radiological characteristics and clinical features of 66 COVID-19 patients. We aimed to unfold the typical radiology characteristics and progression of COVID-19 patients. This study may provide helpful images for early diagnose and treatment.

RESULTS

Total 66 COVID-19 pneumonia patients were admitted to three affiliated hospitals of Nanchang university between Jan 21 to Feb 2, 2020 (Table 1). Epidemiological investigation indicated that 4 (4/66, 6%) patients had direct exposure to COVID-19

pneumonia patients, 21 (21/66, 32%) patients lived in or visited Wuhan during epidemic, and 41 (41/66, 62%) patients did not have obvious exposure. In this cohort, the average age of all patients was 44 years (SD 14; range 18-75), and there were 43 male patients (43/66, 65%) and 23 female patients (23/66, 35%). 45 (45/66, 68%) patients were younger than 50 years, and 21 (21/66, 32%) patients were elder than 50 years.

The most common symptoms were fever (60/66, 91%) and cough (37/66, 56%). Some other symptoms, such as sore throat (17/66, 26%), sputum (17/66, 24%), fatigue (15/66, 23%), and dyspnoea (14/66, 21%), were also observed frequently. Other non-specific symptoms included myalgia (7/66, 11%), headache (3/66, 5%), diarrhea (3/66, 5%), and nausea (3/66, 5%). 38 (38/66, 58%) patients had a higher level of C-reactive protein, and the rest of patients were normal. The leucocytes level of 51 (51/66, 77%) patients was normal. 29 (29/66, 44%) patients had decreased lymphocyte level, and 37 (37/66, 56%) patients' lymphocyte count was normal. Comorbid conditions were not common in these patients, and only 12 (12/66, 18%) patients had at least one complication.

Scattered lesions found in 43 (43/66, 65%) patients were most common, and 23 (23/66, 35%) patients shown subpleural distribution. Lesion involved bilateral lungs was observed in 50 (50/66, 76%) patients. Lesion invaded more than two lobes was found in 48 (48/66, 71%) patients. Right lower lobe (58/66, 88%) and left lower lobe (49/66, 74%) were most likely to be involved.

In this study, we presented some common and typical radiology changes (Figures 1 and 2). The most common radiology characteristic seen on the CT was ground-glass opacity (64/66, 97%). Most ground-glass opacities were characterized by scattered and bilateral lesions (Figure 1A and 1B). The CT scans of 15 (15/66, 23%) patients shown crazy paving (Figure 1C), and consolidation was observed in 7 (7/66, 11%) patients (Figure 2A). In addition, air bronchogram sign (47/66, 71%, Figure 1D), pleural traction (29/66, 44%), interlobular septal thickening (11/66, 17%), and halo sign (3/66, 5%, Figure 2B) were also observed (Table 2). Bronchiectasia was observed in the right lower lobe of one patient with bilateral ground-glass opacity (Figure 2C).

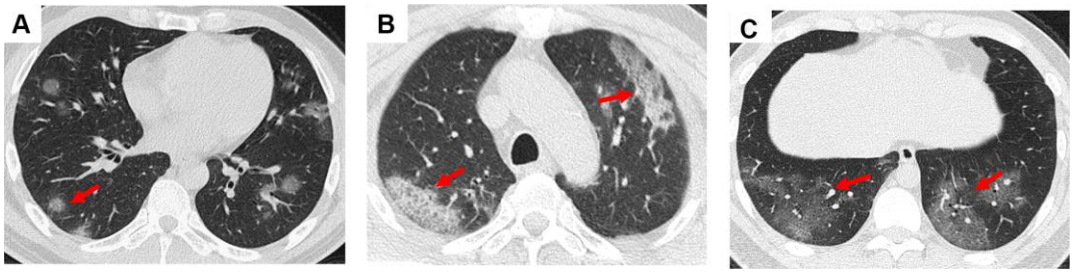

Figure 1. Ground-glass opacity and crazy paving in the CT scans of COVID-19 pneumonia patients. (**A**) Multiple nodular ground-glass opacity scattered in both lungs of a 44-year-old male patient; (**B**) Mixed ground-glass opacity along the long axis of subpleural in both lungs of a 67-year-old male patient; (**C**) Crazy paving was observed in the bilateral lower lungs of a 67-year-old male patient at the fourth day since admission. Typical lesions were marked with red arrows.

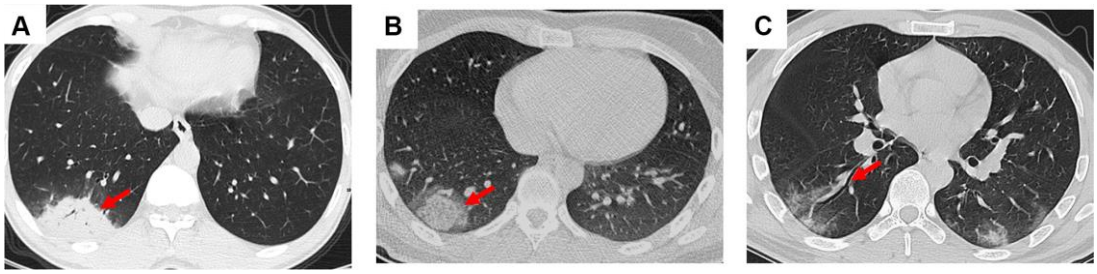

Figure 2. Consolidation, halo sign, and bronchiectasia in the CT scans of COVID-19 pneumonia patients. (**A**) Consolidation accompanying air bronchogram sign was found in the right lower lobe of a 46-year-old male patient; (**B**) Halo sign was observed in the right lower lobe of a 18-year-old male patient; (**C**) Bronchiectasia was observed in the right lower lobe of a 30-year-old male patient with bilateral ground-glass opacity. Typical lesions were marked with red arrows.

Table 2. CT findings of patient with COVID-19 pneumonia (n=66)

CT features	Number (%)
Distribution	
Subpleural	23 (35%)
Central	0
Scattered	43 (65%)
Number of lobes involved	
1	13 (20%)
2	5 (8%)
3	10 (15%)
4	11 (17%)
5	27 (41%)
More than two lobes involved	48 (73%)
Lobe of lesion distribution	
Right upper lobe	42 (64%)
Right middle lobe	37 (56%)
Right lower lobe	58 (88%)
Left upper lobe	44 (67%)
Left lower lobe	49 (74%)
Lesion involved bilateral lungs	50 (76%)
Lesion involved unilateral lung	16 (24%)
Lesion characteristics	
Ground-glass opacity	64 (97%)
Crazy paving	15 (23%)
Consolidation	7 (11%)
Lesion shape	
Patch	66 (100%)
Circular	13 (20%)
Reticular spline	12 (18%)
Other signs in the lesion	
Air bronchogram sign	47 (71%)
Pleural traction	29 (44%)
Interlobular septal thickening	11 (17%)
Vacuole Halo sign	3 (5%)
Other findings	
Pulmonary emphysema	4 (6%)
Pulmonary fibrosis	2 (3%)
Pleural effusion	1 (2%)
Bronchiectasis	1 (2%)
Tuberculosis	1 (2%)

By Mar 23, 2020, 60 (60/66, 91%) patients had been discharged. 6 (6/66, 9%) patients were still in hospital, and two patients had died because of ARDS. Patient 1, 78-year-old man with hypertension, who died on day 15 after admission (Figure 1B). Patient 2, 47-year-old man with type 2 diabetes, whose CT scan presented rapid radiology progression (Figure 3A, 3B). The radiological change of COVID-19 pneumonia develops fast during the first seven days (Figure 3C, 3D). Some of patch lesion could be absorbed and change into reticular spline lesion (Figure 4A, 4B). Meanwhile, some patients achieved rapid recovery with significant improvement of CT sign (Figure 4C, 4D) and clinical symptoms. We also did some CT follow-up scans for few patients, which showed the aggravated progression of disease since admission and rapid recovery after treatment (Figures 5 and 6). Disappearance of lesions and significant improvement of clinical symptoms were observed in two patients (Figure 5: a 54-year-old male patient; Figure 6: a 54-year-old female patient).

DISCUSSION

2019-nCoV, an enveloped positive-sense RNA virus, is the seventh member of the coronaviridae family [10]. It is estimated that 2019-nCoV could cause 1%-6% mortality rate depending on different regions, which is lower than MERS-CoV (10%) and SARS-CoV (37%)

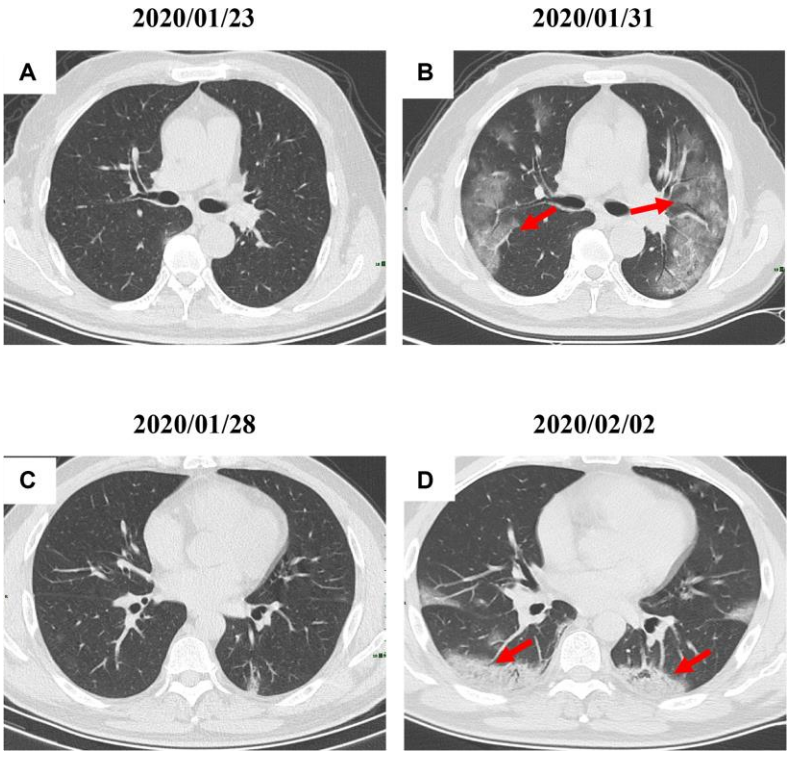

Figure 3. Radiological worsen progression of two COVID-19 pneumonia patients. (**A**, **B**): Bilateral, large, and multiple ground-glass opacity was observed in a 47-year-old male patient with type 2 diabetes after 8 days since admission; (**C**, **D**) Consolidation accompanying air bronchogram were found in the bilateral lower lungs of a 29-year-old male patient after 5 days since admission. Typical lesions were marked with red arrows.

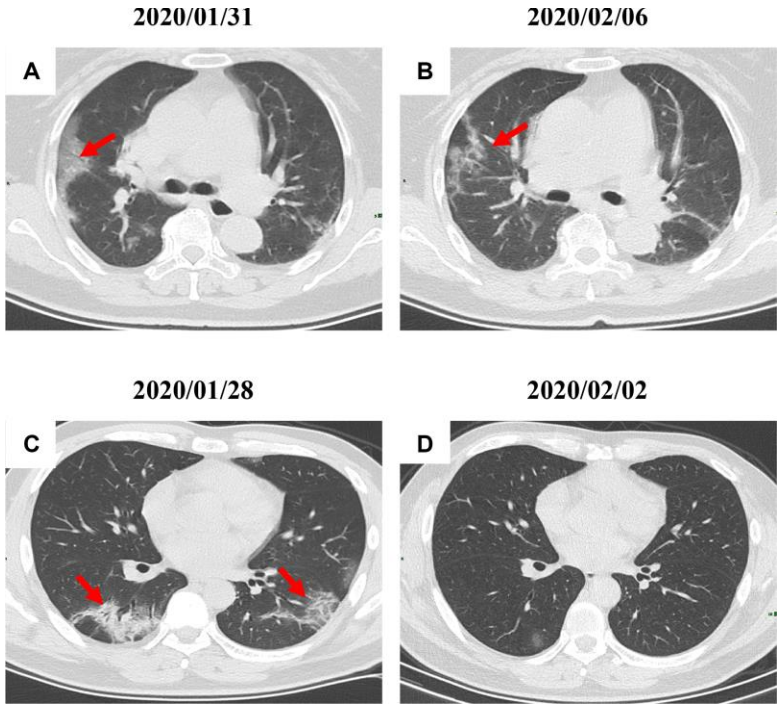

Figure 4. Radiological improvement of two COVID-19 pneumonia patients. (**A**, **B**) Patch lesions were absorbed and changed into reticular spline ones (a 31-year-old female patient); (**C**–**D**) Significant improvement of CT sign was achieved in a 22-year-old male patient. Typical lesions were marked with red arrows.

[5, 11], but the high infectivity of the pathogen has caused a global pandemic. 2019-nCoV has became a huge threat for the global health, economic development, and social stability.

Previous study indicated that old age population with comorbidities were susceptible to infection of 2019-nCoV [12]. In our cohort, there were 45 (45/66, 68%) patients under 50 years-old, and only 12 (12/66, 8%) patients had at least one comorbid condition. Small cohorts and differences in demographic characteristics might account for this discrepancy. Previous study suggested that 73% (30/41) patients were male [1], which is inconsistent with another study [13]. In our study, male infected patients account for 65% (43/66). The difference of gender distribution might also due to small cohorts. By Mar 23, 2020, two patients (78 and 47 years old, respectively) in this study had died, and both had comorbid conditions.

It is worth mentioning that 41 patients (41/66, 62%) patients had no obvious exposure history indicating that they might be infected by latent infection patients. Latent infection should attract the attention of people, because the clinical appearance of latent infection patients is not consistent with real disease progression. Meanwhile, the latent infection patients indeed have infectivity. When participating in group activities or gathering, wearing mask should be an effective method to prevent infectivity by asymptomatic patients. Some countries such as India and Indonesia have a large population and the medical condition of them is not optimistic. For the people who lack sufficient medical protection, it is effective to prevent and control virus spreading by avoiding gathering, wearing mask in the crowd, regular ventilation at home.

Fever and cough were the most common symptoms in the COVID-19 patients. Self-isolation and wearing mask are still effective and economic method for fever people who have mild symptoms, but if symptoms aggravate, professional and medical measures should be taken because of the high mortality rate.

Due to special structure, right lower lobe and left lower lobe were most commonly involved, which is in line

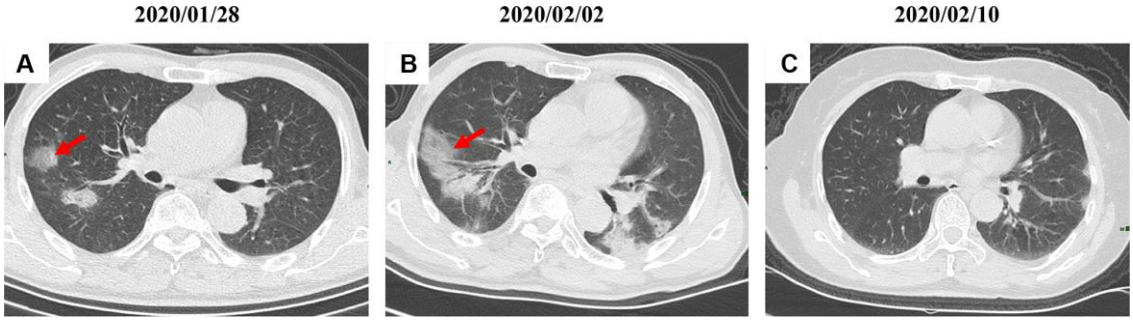

Figure 5. A serial CT images after admission of a 54-year-old male patient. (**A**) Patch ground-glass opacity was observed in the middle right lobe. (**B**) 5 days later, significant larger patch ground-glass opacities were observed in bilateral lungs. (**C**) Follow-up CT scans on day 13 after admission show a remarkable improvement. Typical lesions were marked with red arrows.

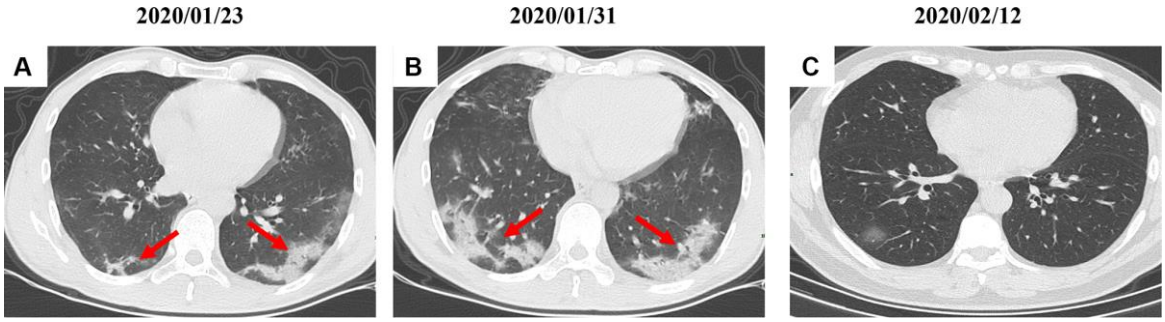

Figure 6. A serial CT images after admission of a 54-year-old female patient. (**A**) Patch ground-glass opacity mainly located in the left lower lobe. (**B**) Significant larger patch ground-glass opacities were observed in both lower lobes after 8 days. (**C**) Follow-up CT scans on day 20 after admission show a remarkable improvement. Typical lesions were marked with red arrows.

with previous study [13]. Most COVID-19 patients presented bilateral lungs lesion with scattered distribution. However, unilateral lesion is more common in the early infection stage of MERS-CoV and SARS-CoV [14, 15]. The most common image feature was ground-glass opacity, which was found in 64 (64/66, 97%) patients. Other features such as crazy paving, consolidation, air bronchogram sign, and pleural traction were also observed. However, these radiological characteristics could be found in other viral pneumonia caused by MERS-CoV, SARS-CoV, and adenovirus.

RT-PCR has been viewed as the gold standard for COVID-19 pneumonia diagnosis. While, many countries are facing the shortage of nucleic acid test reagent. Meanwhile, the it nucleic acid test costs at least 4-5 hours including throat swab collection, RNA extraction, and RT-PCR. Chest CT could provide effective and fast evidence for the clinical diagnosis of COVID-19 pneumonia. Imaging findings could also indicate the prognosis. The radiological features of some patients might worsen fast indicating a poor prognosis (Figure 3A, 3B).

Our study had some limitations. Due to short time for data collection, we did not conduct long-term follow-up CT, which is necessary to evaluate the prognosis of patients. In addition, we did not systematically investigate the radiology progression of patients, which could help to judge disease course of COVID-19 pneumonia.

In summary, the typical radiology features of COVID-19 pneumonia were characterized by bilateral and scattered ground-glass opacity accompanying with air bronchogram sign, and predominant lesion location in the left lower lobe and right lobe. Sometimes, the clinical symptoms were not consistent with imaging features indicating that asymptomatic patients may account for a certain proportion. Therefore, CT should be an effective, fast, and simple method for the screening, diagnose, and treatment of COVID-19 pneumonia.

MATERIALS AND METHODS

Patients

The retrospective study was approved by the ethical committee of affiliated hospitals of Nanchang university. The written informed consent of this research has been waived by the ethics committee of our hospital for the reason that there is no potential risk and this is a retrospective study. The COVID-19 patients identified by RT-PCR or nest-generation sequencing were admitted from Jan 21 to Feb 2, 2020. A total of 66 patients were enrolled (43 men and 23 women, 18-75 years old, average age: 44 years). Throat swab samples were collected by experienced nurses, and total RNA extraction was conducted using TRIzol reagent (Thermo scientific, CA, USA). According to previous study [13], related primers (forward primer: 5′-TCAGAATGCCAATCTCCCCAAC-3′; reverse primer: 5′-AAAGGTCCACCCGATACATTGA-3′) were used to detect SARS-CoV-2.

CT data acquisition

All patients were examined by CT for 2-6 times at different time points. The patients in the supine position were scanned using Siemens Emotion 16 (Siemens Healthineers, Forchheim, Germany), Phillips iCT 256 (Phillips Healthcare, Andover, MA, USA), or GE revolution frontier (GE Healthcare, Issaquah, WA, USA). Scans were conducted from the apex of lung to the base of lung on the condition that patients were instructed to hold breath during examination. The following scan parameters were used: tube voltage 120 kV, tube current 70-168mAs, pitch 08-1.2 mm, slice thickness 5 mm, matrix 512×512, FOV 55*35cm, axial reconstruction image layer thickness 1-1.5mm. Three experienced radiologists blinded to nucleic acid results of patients, reviewed all CT scans.

Data analysis

Data analysis was performed on SPSS 22.0 (IBM, Armonk, NY, USA). Categorical variables were presented as number (%), and continuous variables were shown as a range.

Abbreviations

COVID-19: Coronavirus disease 2019; WHO: World health organization; SARS-Cov-2: severe acute respiratory syndrome coronavirus 2; 2019-nCoV: 2019 novel coronavirus; MERS-CoV: Middle east respiratory syndrome coronavirus; SARS-CoV: Severe acute respiratory syndrome coronavirus.

CONFLICTS OF INTEREST

These authors declare no conflicts of interest.

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Research Perspective

Metformin and SARS-CoV-2: mechanistic lessons on air pollution to weather the cytokine/thrombotic storm in COVID-19

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ABSTRACT

Pathological signaling in the lung induced by particulate matter (PM) air pollution partially overlaps with that provoked by COVID-19, the pandemic disease caused by infection with the novel coronavirus SARS-CoV-2. Metformin is capable of suppressing one of the molecular triggers of the proinflammatory and prothrombotic processes of urban PM air pollution, namely the mitochondrial ROS/Ca^{2+} release-activated Ca^{2+} channels (CRAC)/IL-6 cascade. Given the linkage between mitochondrial functionality, ion channels, and inflamm-aging, the ability of metformin to target mitochondrial electron transport and prevent ROS/CRAC-mediated IL-6 release might illuminate new therapeutic avenues to quell the raging of the cytokine and thrombotic-like storms that are the leading causes of COVID-19 morbidity and mortality in older people. The incorporation of infection rates, severity and lethality of SARS-CoV-2 infections as new outcomes of metformin usage in elderly populations at risk of developing severe COVID-19, together with the assessment of bronchial/serological titers of inflammatory cytokines and D-dimers, could provide a novel mechanistic basis for the consideration of metformin as a therapeutic strategy against the inflammatory and thrombotic states underlying the gerolavic traits of SARS-CoV-2 infection.

Particulate matter air pollution and SARS-CoV-2/COVID-19: A mechanistically linked pathway illuminating a therapeutic opportunity for metformin

Particulate matter (PM) air pollution concentrations frequently encountered in major cities can trigger the release of proinflammatory interleukins (e.g., IL-6) from alveolar macrophages, promoting an acceleration of arterial thrombosis [1]. Analogously, infection with the novel SARS-CoV-2 coronavirus can stimulate a *too-little-too-late* type-I interferon-mediated innate immune response, which is inherently accompanied by dysregulated secretion of IL-6 from alveolar macrophages [2, 3]. The so-called *cytokine storm* – involving overproduction of proinflammatory cytokines and over-activation of immune cells (hyperinflammation) – ultimately drives an acute respiratory distress syndrome (ARDS), one of the leading causes of mortality in patients with severe COVID-19 disease [4, 5]. Intriguingly, patients with severe COVID-19 admitted to the intensive care unit are at highest thrombotic risk, with acute pulmonary embolism being the most common thrombotic complication [6]. The ability of COVID-19 to predispose to thromboembolism, which can fuel futile cycles of hyperinflammatory responses that aggravate SARS-CoV-2 pathogenesis [7, 8], is increasingly viewed as a major factor in disease severity and mortality. It is thus not surprising that long-term exposure to PM has been recently proposed as a key contributor to COVID-19 mortality in the United States [9]. Likewise, the elevated levels of PM air pollution in Northern Italy and central Spain have been postulated as a putative risk factor underlying the extremely high COVID-19 fatality rates observed in these European regions [10–12].

The link between air pollution and COVID-19 severity can be viewed merely as the passive result of a *carrier* action of virus particles by PM; yet, one should acknowledge that PM air pollution is also a principal cause of chronic systemic and airway inflammation, ultimately leading to innate immune system hyper-activation, elevated production of proinflammatory cytokines, and thrombosis [1, 10, 13–16]. The physio-pathological overlap between PM-driven inflammatory cytokine production and the cytokine/thrombotic storm in patients with COVID-19 might also suggest a *boosting* action of the former on the SARS-CoV-2 mechanism of disease (Figure 1). Therapeutically, if the chronic pulmonary effects of PM impact the prognosis of COVID-19, it then follows that small molecules with acceptable risk profiles that can block the molecular trigger(s) of IL-6 release from alveolar macrophages in response to PM might also mitigate the aggressive proinflammatory/prothrombotic nature of COVID-19. Using sophisticated cell and mouse models, a groundbreaking study by the Budinger group established that the anti-diabetic drug metformin – through its capacity to inhibit mitochondrial complex I – suppressed the mitochondrial reactive oxygen species (ROS) signaling necessary for the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the generation of IL-6 from alveolar macrophages upon exposure to PM (Figure 2) [1]. Because the use a respiratory filter in people residing in areas with high levels of PM air pollution validated the causal link between PM

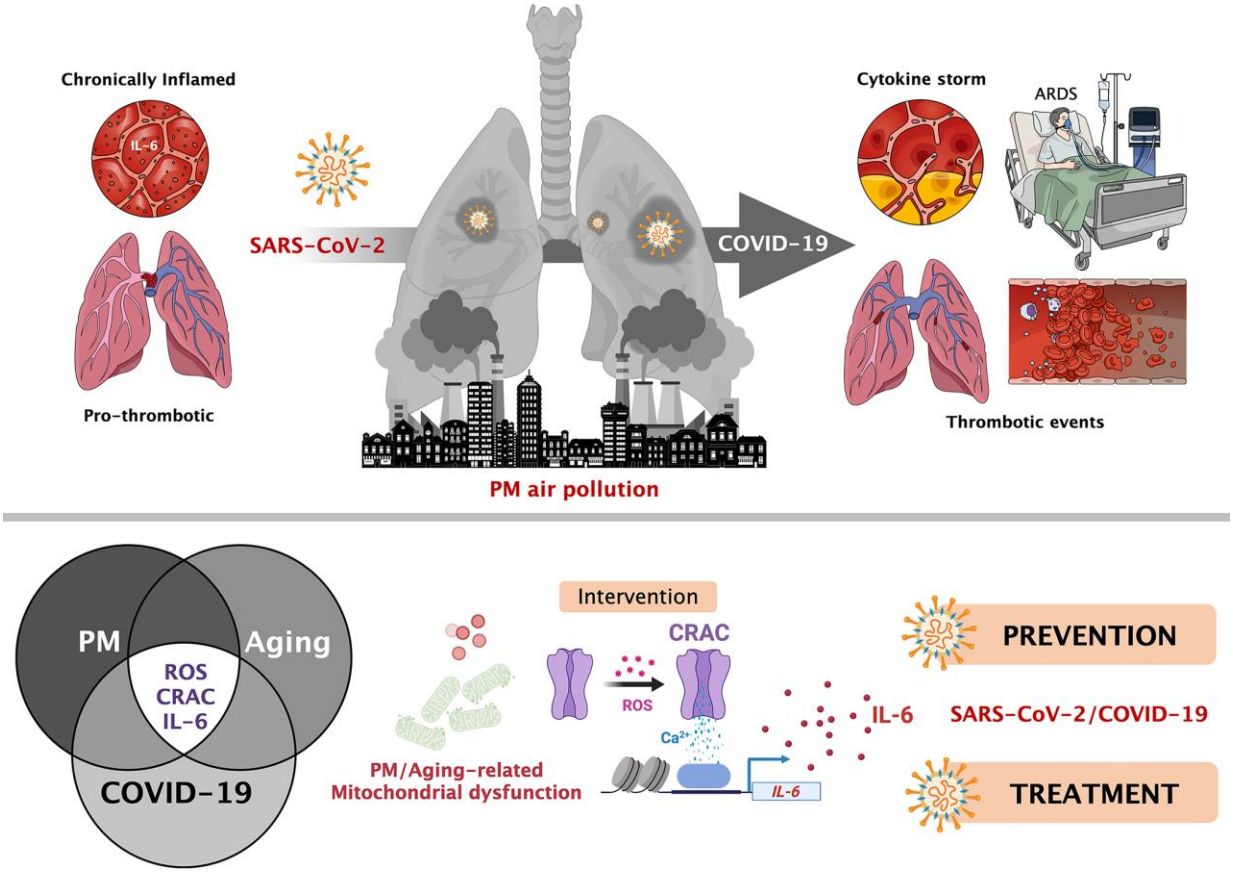

Figure 1. Particulate matter air pollution and SARS-CoV-2/COVID-19: A mechanistically linked pathway illuminating a therapeutic opportunity for metformin. *Top*. Pathological signaling in the lung induced by particulate matter (PM) air pollution partially overlaps with that caused by severe SARS-CoV-2/COVID-19, namely the release of proinflammatory interleukins (e.g., IL-6) from alveolar macrophages via mitochondrial reactive oxygen species (ROS)-driven activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels, lastly promoting an acceleration of thrombotic events. Patients already experiencing a chronic cytokine response might be at higher risk of COVID-19 lethal complications after SARS-CoV-2 infection. *Bottom*. Given the linkage between mitochondrial functionality, ion channels, and inflammation in human aging, therapeutic interventions capable of targeting mitochondrial electron transport and prevent mitochondrial ROS/CRAC-mediated IL-6 release (e.g., metformin) might illuminate a preventive/prophylactic mechanism of action to quell the raging of the cytokine and thrombotic-like storms that are the leading causes of COVID-19 morbidity and mortality in older people. In an acute scenario of SARS-CoV-2-driven hyperinflammation, small molecule CRAC channel inhibitors may also be contemplated as a means of treating patients with severe COVID-19 at risk for progressing to typical/atypical ARDS.

exposure and levels of IL-6-related systemic markers [17], these findings altogether support metformin use as a preventive strategy for the mortality attributable to PM air pollution worldwide [1]. In the same line, it would be relevant to test whether metformin could suppress the cytokine and thrombotic-like storms in COVID-19 before they begin, thereby lowering the risk of severe disease in high-risk individuals.

ROS/CRAC/IL-6-targeted activity of metformin: From preventive therapy of the premature death attributable to PM air pollution to geroprotector against the gerophilic and gerolavic traits of SARS-CoV-2 infection

Severe COVID-19 illness and death is more common in people aged 60 and older with underlying conditions, which can include chronic respiratory system disease not only due to chronic exposure to PM air pollution but also to immuno-senescence and inflamm-aging phenomena [18–22]. Given the linkage between mitochondria functionality, ion channels including CRAC, and inflamm-aging [23], the ability of metformin to target mitochondrial electron transport and prevent ROS/CRAC-mediated IL-6 release might illuminate a preventive (and prophylactic) measure to quell the raging of the cytokine and thrombotic-like storms that are the leading causes of COVID-19 morbidity and mortality in older people. Such CRAC-related mechanism of action [24] capable of preventing systemically IL-6-driven thrombotic events [1], together with the multi-faceted capacity of metformin to ameliorate immunometabolism-related inflammation and alleviate ARDS [25–27] -which are believed to be the main risk factors for a worse outcome in the elderly with COVID-19 [28, 29]- could provide a novel mechanistic basis for the recently proposed geroprotective role of metformin against the *gerophilic* and *gerolavic* traits of SARS-CoV-2 infection [30].

Previous randomized clinical trials and numerous retrospective observational studies have consistently associated metformin administration with significant improvement in risk factors of aging-related diseases (cardiovascular, neurodegenerative, and cancer) beyond type 2 diabetes [31–33]. Research is now urgently needed to test whether metformin might additionally

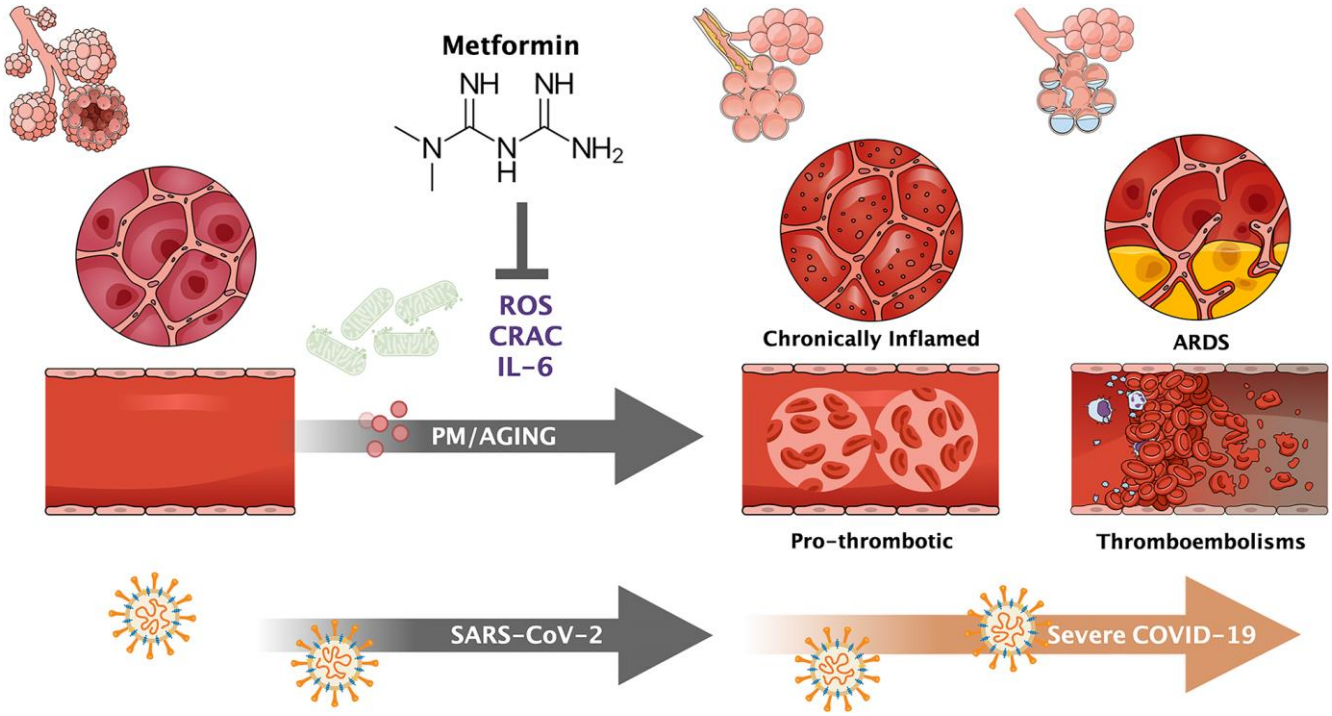

Figure 2. CRAC-targeted activity of metformin: From preventive therapy of the premature death attributable to PM air pollution to geroprotector against the gerophilic and gerolavic traits of SARS-CoV-2 infection. The ability of metformin to suppress the signaling by mitochondrial reactive oxygen species (ROS) that are necessary for the opening of Ca^{2+} release-activated Ca^{2+} channels in the generation of IL-6 from alveolar macrophages upon exposure to PM air pollution might mechanistically extend to the immune dysregulation/inflammation and thrombotic events driven by the systemic release of IL-6 from lung macrophages in response to SARS-CoV-2 infection. By restraining the raging of cytokine and thrombotic-like storms, two of the leading causes of morbidity and mortality in SARS-CoV-2 infection, metformin might be considered a putative geroprotector against the gerophilic and gerolavic traits of COVID-19 disease.

reduce the comorbidity, infection rate, severity, and lethality of SARS-CoV-2 infection, with special emphasis on the elderly risk groups accounting for the majority of severe COVID-19 disease and fatalities to date. Clinical trial strategies such as the TAME (Targeting Aging with Metformin) study, which plans to enroll 3,000 older subjects (ages 65–79) without type 2 diabetes who will be randomly assigned to 1,500 mg metformin daily or placebo for 4 years to measure time to a new occurrence of a composite outcome that includes cardiovascular events, cancer, dementia, and mortality [31, 33], provides an ideal opportunity to explore the recently proposed strategy of metformin as a low-cost geroprotector for prevention of SARS-CoV-2 [30]. In the meanwhile, observational studies in residential care nursing homes and day-care centers where older adults at significant risk of COVID-19 outbreaks are receiving metformin for treatment of type 2 diabetes (along with the assessment of bronchial/serological levels of inflammatory cytokines and markers of pro-thrombotic/hypercoagulable states such as D-dimers) might provide a deeper comprehension of how metformin can protect and potentiate common preventive strategies such as distancing measures, mask-wearing, and hand-washing.

CRAC channels and treatment of typical/atypical ARDS in severe COVID-19

Targeting hyperinflammation in severe COVID-19 patients may be critical for reducing mortality. One might therefore wonder whether treatment with indirect (e.g., metformin [1]) or direct (e.g., CM4620 [34]) small-molecule inhibitors of CRAC channels could improve clinical outcomes in hospitalized patients with moderate/severe COVID-19. CM4620-IE, a potent and selective small molecule CRAC channel inhibitor that prevents channel overactivation and has demonstrated efficacy in patients with hypoxemia secondary to systemic inflammatory response syndrome in acute pancreatitis [34], will be trialed in patients with severe COVID-19 pneumonia at risk for progressing to ARDS (ClinicalTrials.gov identifier: NCT04345614). Because a subset of severe COVID-19 infections have a delayed onset of respiratory distress despite the severity of hypoxemia that clearly differs from classic ARDS but principally involves a catastrophic microvascular injury and thrombosis [35–37], it might be relevant to carefully evaluate the impact of targeting the mitochondrial ROS/CRAC/IL-6 signaling cascade in the respiratory, inflammatory, and survival outcomes during the “typical” and “atypical” presentation of ARDS in COVID-19 patients. Nonetheless, the findings from the clinical testing of CRAC-targeting drugs might link, at a mechanistic level, the metformin lessons on air pollution to ride (out) the cytokine/thrombotic storm in severe SARS-CoV-2/COVID-19.

DISCLAIMER AND LIMITATIONS

This perspective merely aims to stimulate new ideas as part of the global efforts aimed to develop new preventive/treatment strategies against the SARS-CoV-2/COVID-19 outbreak. Accordingly, this perspective does not represent medical advice or therapeutic recommendations to either COVID-19 patients or people at risk of SARS-CoV-2 infection.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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Review

Why does COVID-19 disproportionately affect older people?

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ABSTRACT

The severity and outcome of coronavirus disease 2019 (COVID-19) largely depends on a patient's age. Adults over 65 years of age represent 80% of hospitalizations and have a 23-fold greater risk of death than those under 65. In the clinic, COVID-19 patients most commonly present with fever, cough and dyspnea, and from there the disease can progress to acute respiratory distress syndrome, lung consolidation, cytokine release syndrome, endotheliitis, coagulopathy, multiple organ failure and death. Comorbidities such as cardiovascular disease, diabetes and obesity increase the chances of fatal disease, but they alone do not explain why age is an independent risk factor. Here, we present the molecular differences between young, middle-aged and older people that may explain why COVID-19 is a mild illness in some but life-threatening in others. We also discuss several biological age clocks that could be used in conjunction with genetic tests to identify both the mechanisms of the disease and individuals most at risk. Finally, based on these mechanisms, we discuss treatments that could increase the survival of older people, not simply by inhibiting the virus, but by restoring patients' ability to clear the infection and effectively regulate immune responses.

INTRODUCTION

Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), which is responsible for the worldwide pandemic of coronavirus disease (COVID-19) originated in Wuhan, China, in late 2019 [1]. COVID-19 has so far killed more than 350,000 people, with the majority of deaths (74%) occurring in people over the age of 65 [2, 3]. Why the disease is particularly dangerous in older people is not yet known and poorly understood at the molecular level. It is clear, however, that age alone is by far the most significant risk factor for death due to COVID-19 [4, 5]. Even prior to SARS-CoV-2, human coronaviruses and influenza viruses have been known to impact older people disproportionately [6], yet therapeutic strategies to protect this fraction of the population, with the exception of vaccines, have largely failed. The severity of COVID-19 is, of course, strongly associated with comorbidities such as hypertension, diabetes, obesity, cardiovascular disease, and respiratory system diseases [2]. Whether these comorbidities contribute specifically to SARS-CoV-2 pathogenesis or whether they are primarily indicators of biological age remains an open question. For example, simple explanations for the impact of age that are based solely on co-morbidities or on a general lack of resilience in aging, for example, fail to explain why the immune system often reacts uncontrollably.

SARS-CoV-2 is transmitted through respiratory droplets or by direct contact. Entering the nose, mouth or eyes, the virus spreads to the back of the nasal passages, where it binds to and enters via the dimerized angiotensin-converting enzyme 2 (ACE2) [7] on the surface of airway epithelial cells [8]. From there, it spreads to the mucous membranes of the throat and bronchial tubes, eventually entering the lungs where it infects type 2 alveolar epithelial cells called pneumocytes. This can lead to acute respiratory distress syndrome (ARDS), characterized by a loss of beneficial lung surfactant and an increase in oxidative stress and inflammation [9, 10] (Figure 1).

Particularly the older people, severe cases of the disease are characterized by acute lung injury and ARDS, the latter of which is typically treated by positive airway pressure with oxygen and pronation or invasive ventilation. This stage is characterized by neutrophilia, lymphocytopenia, lung consolidation, and bilateral nodular and peripheral ground glass opacities on chest X-rays. The ACE2 protein is widely expressed on the surface of both epithelial and microvascular pericytes, that traverse multiple organs, allowing both cell types to be infected by the virus [11, 12]. The recruitment of immune cells to sites of infection results in widespread inflammation and endothelial dysfunction in the lung, heart, kidney, and liver and brain, with prominent endotheliitis of the submucosal vessels and apoptotic bodies [11].

Even if viral loads decline in the patient,, a type of cytokine release syndrome can rapidly develop, characterized by disseminated intravascular coagulation (DIC), causing liver damage, renal dysfunction, cardiovascular inflammation, coagulopathy and death [13, 14]. There are very few studies that definitely connect the known mechanisms of aging to the pathogenesis of viruses. In this perspective, we offer potential mechanistic explanations as to why COVID-19 advances in some people and not others, and especially in older patients, including differences in the immune system, glycation, the epigenome, inflammasome activity, and biological age. We also discuss therapies that may improve immunity against viral infection while enhancing the ability of older people to recover from severe COVID-19.

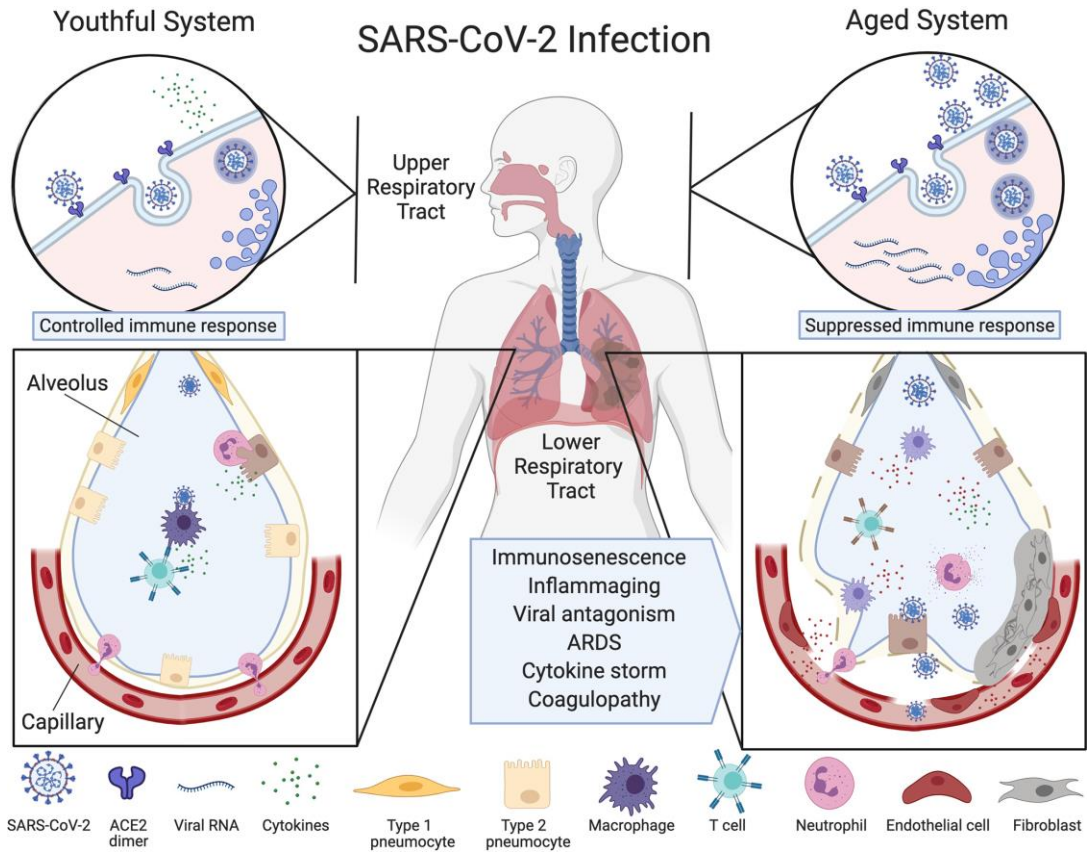

Figure 1. Ineffective clearance of SARS-CoV-2 infection in the aged respiratory system. The SARS-CoV-2 virus binds to ACE2 enzymes on airway epithelial cells in the upper respiratory tract where they are endocytosed and replicated (top left), alerting the immune system. Viruses then travel to the alveoli and infect type 2 pneumocytes which, in the youthful system (lower left), are recognized by alveolar macrophages (AMs) or dendritic cells (not pictured) that release cytokines and present antigens to T cells and other adaptive immune cells. T cells with the appropriate receptors activate other lymphocytes or directly kill infected cells, preventing the spread of the virus. Neutrophils migrate to the sites of infection to clear infected cell debris. In the aged system (top right), viral alert signals are initially slow, resulting in greater viral replication. Defective macrophages and T cells with a limited repertoire of receptors are less effective (lower right). More cells are infected, inducing high levels of inflammatory cytokine signaling. The endothelial cell lining of the capillary becomes inflamed, fibroblasts are activated, and SARS-CoV-2 viral components and cytokines enter the bloodstream. Fluid fills the alveolus, reducing lung capacity and the virus infects microvascular pericytes in other organs. A cytokine storm initiates microvasculature clotting, causing severe hypoxia, coagulopathy and organ failure. Created with BioRender.

The aging immune system

The ability to control viral load is one of the best prognostics of whether a patient will have mild or severe COVID-19 symptoms [15]. For the immune system to effectively suppress then eliminate SARS-CoV-2, it must perform four main tasks: (1) recognize, (2) alert, (3) destroy and (4) clear. Each of these mechanisms are known to be dysfunctional and increasingly heterogeneous in older people [16, 17]. But which tasks are most relevant to COVID-19 progression in older people is not yet clear [18].

During aging, the immune system changes in two major ways. One is a gradual decline in immune function called immunosenescence, which hampers pathogen recognition, alert signaling and clearance. This is not to be confused with cellular senescence, an aging-related phenomenon whereby old or dysfunctional cells arrest their cell cycle and can become epigenetically locked into a pro-inflammatory state in which they secrete cytokines and chemokines. The other classic immune system change during aging is a chronic increase in systemic inflammation called inflammaging, which arises from an overactive, yet ineffective alert system [19].

An abundance of recent data describing the pathology and molecular changes in COVID-19 patients points to both immunosenescence and inflammaging as major drivers of the high mortality rates in older patients. Within immunosenescence, there are defects in both the innate and adaptive immune systems. Innate immuno-senescence is characterized by ineffective pathogen recognition and macrophage activation, and a reduction in natural killer (NK) cell cytotoxicity, whereas adaptive immunosenescence is characterized by thymic atrophy and accumulation of anergic memory lymphocytes. In both cases, these age-related changes are thought to be due to pathogenic, genetic, and lifestyle factors that affect the cells' epigenetic status and the diversity of immune cells.

The aging innate immune system

The innate immune system is the body's first line of defense against coronaviruses. Sentinel cells, such as macrophages and dendritic cells, recognize structurally conserved viral proteins via single-pass membrane-spanning receptors called Toll-like receptors (TLRs) expressed on their cell surfaces. Defects in TLR function in innate immune cells are known to increase the severity of pneumonia in mice, especially in the context of aging and chronic inflammaging [20]. Alveolar macrophages (AMs) are mononuclear phagocytes that surveil the lungs for dust, allergens and the remnants of pathogens. When their TLRs detect an invader, AMs respond by producing type I interferons, which attract immune cells to the site of infection and present antigens to lymphocytes [21, 22]. Although AMs increase in number during aging, their plasticity to convert between pro- and anti-inflammatory states is greatly reduced [23], exemplified by a weak cytokine response after TLR activation [24] (Figure 1).

The inability of AMs in older individuals to recognize viral particles and convert to a pro-inflammatory state likely accelerates COVID-19 in its early stages, whereas in its advanced stages, AMs are likely to be responsible for the excessive lung damage. A recent study comparing immune cell composition of bronchoalveolar lavage fluid from moderate and severe COVID-19 patients showed in severe cases, macrophages were phenotypically more pro-inflammatory, expressing higher levels of CCR1 and CXCR2 that recruit other innate immune cells, compared to macrophages from moderate COVID-19 cases that expressed more T-cell attracting chemokines [25]. Prolonged monocyte activation is a well-known cause of severe lung injury in rhesus monkeys [26] and in cases of SARS (caused by SARS-CoV-1), higher numbers of pulmonary neutrophils and macrophages correlated with the development of ARDS and greater lung damage [27]. A decline in neutrophil activity might also be partly responsible because, during aging, these cells progressively lose their ability to migrate to sites of infection and kill infected cells [28, 29]. NK cells, a major component in innate immunity with potent cytotoxic activity, are an unlikely cause of COVID-19 severity. Their numbers are relatively stable during aging [30] and in a mouse model of SARS, they were not necessary for normal viral clearance [31]. To discern which of these cell types play the most destructive roles, more detailed analyses of COVID-19 patient autopsy tissue will be needed.

Additionally, the production and diversity of mucins, protective glycoproteins found in mucosal barriers throughout the body, also change in aging [32, 33], although their role in immunity against coronaviruses in humans is understudied.

The aging adaptive immune system

Immunosenescence of the adaptive immune system is also a likely factor that determines whether a patient progresses to severe COVID-19 (Figure 2). Situated just above the heart, the thymus – a primary lymphoid organ and the site of T cell development and maturation of early thymic progenitors from the bone marrow – is one

of the first tissues to experience aging. By age 65, the thymus is on average ~40% its original size [34], coincident with activation of the inflammasome component NLRP3 and Caspase-1, a pro-apoptotic protease [35, 36]. A build-up of intrathymic adipocytes further reduces thymic cellularity and deteriorates the thymic microenvironment. Thymic atrophy also contributes to a reduction of naïve T cells and an accumulation of memory lymphocytes, resulting in defective immunosurveillance and an exhaustion of B cells, cytotoxic T cells, and helper T cells [37]. Other common effects of aging on the adaptive immune system include a decline in the production of fresh naïve T cells, a less expansive T cell receptor (TCR) repertoire, T cell metabolic dysfunction, and weaker activation of T cells [38, 39]. Clonal populations of $CD8^+$ T cells expand during aging, limiting their diversity, whereas $CD4^+$ T cells retain fairly diverse TCRs [40] and, instead, suffer activation deficits [39].

Interestingly, one study found that supercentenarians – defined as adults over 110 years old – tend to have an unusual population of cytotoxic $CD4^+$ T cells whose activation doesn't decline with age and can take on the effector functions usually performed by $CD8^+$ T cells [41]. This T cell behavior may explain why some older people, even some people over 100, are able to survive COVID-19. Measuring the repertoire and frequency of TCRs in patients from a spectrum of ages and disease severity should be performed to determine if a loss of T cell diversity is a reason why SARS-CoV-2 viral loads tend to spike in older people but not the young.

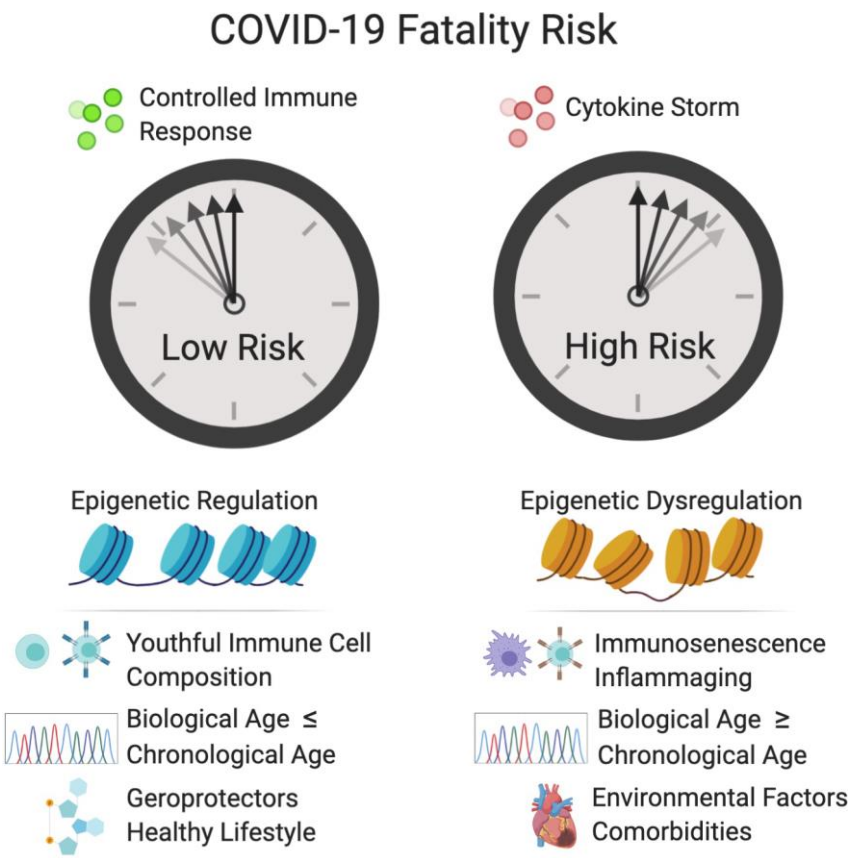

Figure 2. Factors that increase the fatality risk of COVID-19. Epigenetic dysregulation, immune defects, advanced biological age, and other factors increase the risk of cytokine storm and COVID-19 fatality. Tightly controlled activation of the innate immune system is essential for viral recognition and clearance. Cytokine storm is the result of sustained activation of the inflammatory signaling cascade and can result in hypercoagulation in small blood vessels, which leads to tissue damage, DIC and multi-organ failure. Inflammaging and immunosenescence contribute to the development of cytokine storm. D-dimer, a fibrin degradation product and prognostic of disseminated intravascular coagulation (DIC), and elevated levels of the cytokine, IL-6, are associated in the clinic with increased fatality. Epigenetic dysregulation of the immune system and of the renin-angiotensin system (RAS) may increase fatality risk. A variety of biological clocks have been shown to predict human health and longevity more accurately that chronological age. An individual with a biological age greater than their chronological age is thought to be undergoing accelerated aging, which may increase the risk of COVID-19 fatality. Individuals with comorbidities such as cardiovascular disease, diabetes, obesity and COPD, are at greater risk for COVID-19 fatality. Conversely, individuals who live healthy lifestyles and consume geroprotectors such as metformin, resveratrol and NAD^+ boosters may have a decreased risk of fatality. Created with BioRender.

Not only does the repertoire of T cells decline in aging, so do their numbers. Those over 60 years old increasingly have low T cell numbers, a condition known as lymphopenia [42]. Because T cells express very low levels of ACE2, the lymphopenia in COVID-19 patients is unlikely to be caused by direct SARS-CoV-2 infection [43], as in the case for HIV. One proposed cause of the T cell paucity is an exhaustion of the immune system driven by repeated exposures to viruses over one's lifetime [42, 44, 45]. This hypothesis is based on several studies that tracked the morbidity and mortality of people over 60 who had been chronically infected with human cytomegalovirus (CMV) [46, 47]. Cycles of CMV reemergence were associated with vast immune system remodeling, including a pronounced exhaustion of $CD8^+$ T cells that was more predictive of all-cause mortality than chronological age. Other studies indicate that T cell depletion is due to the cumulative exposure to many different pathogens and lifestyle factors, not CMV alone [46, 48]. At the chromosomal level, a major cause of immune exhaustion is telomere shortening in viral-specific memory $CD8^+$ T cells, which induces cellular senescence, a state of cell cycle arrest and hyper-inflammation that prevents expansion upon re-infection [49]. The fact that in the most severe COVID-19 cases bronchoalveolar $CD8^+$ T cells appear to have reduced expansion capability [25] and peripheral blood T cells express high levels of the immune-exhaustion marker PD-1 [42] make this theory plausible.

B cells – adaptive immune cells which produce antibodies in response to coronavirus antigens [21] – are also less diverse and less responsive in aging [50, 51]. While total B cells numbers do not decrease in aging, memory B cells accumulate and naïve B cells are depleted, which may lead to loss of diversity of the B cell repertoire, although this has not yet been definitively demonstrated in humans [51]. Changes in IgG glycosylation patterns, however, have been shown to strongly associate with age and inflammation, and predict age-associated disease development [52]. In particular, IgG N-glycans appear to be the most predictive of biological aging, however B-cell intrinsic and extrinsic regulation of glycosylation in aging require further study.

Due to a lack of sun exposure and decreased production of vitamin D, about half of all older people have a deficiency in this vitamin [53], which reduces the efficacy of both adaptive and innate immune responses and increases the risk of infection [54]. Vitamin D levels in older people are correlated with preserved features of immunity such as the $CD4^+/CD8^+$ ratio and lower levels of pro-inflammatory cytokines after stimulus [54, 55]. Although not all studies see a benefit of vitamin D supplementation on the risk or duration of lower respiratory infections [56], the majority have, especially in those with an antibody deficiency or increased susceptibility to respiratory tract infections [57, 58]. A recent meta-analysis of 25 randomized, placebo-controlled trials concluded that vitamin D supplementation prevented about 20% of acute respiratory infections [59]. As such, some health professionals have recommended vitamin D supplementation for older people in general and especially for aged-care residents and critically ill patients as a strategy for improving chances of COVID-19 survival.

Increased inflammation and cytokine storms in the aged

During the course of COVID-19, older patients can reduce their viral titers, only to rapidly descend into a state of shock involving hyperactivation of the immune system and hypercoagulation in small blood vessels [42, 60]. This rapid and uncontrolled inflammatory signaling cascade typically occurs in the later stages of infection. Known as a "cytokine storm," it exacerbates the dyspnea and hypoxemia, and triggers inflammation in major tissues such as the lungs, kidneys, heart, liver and brain. Cytokine storm syndrome is defined as life-threatening organ dysfunction caused by a maladaptive host response to an infectious trigger [61]. The resulting vascular inflammation is emerging as the cause of complement-associated microvascular injury and thrombosis in severe COVID-19 cases [62]. The initial trigger for cytokine storm is not yet known but it likely involves the immune system's detection of a large quantity of viral antigens released by dying cells. Why older people are particularly prone to cytokine storms is also unclear.

The cytokine profiles of late-stage COVID-19 patients are similar to patients with secondary haemo-phagocytic lymphohistocytosis, a type of cytokine storm that can be triggered by systemic viral infection, including increased levels of interleukin (IL)-2, IL-6, IL-7, C-reactive protein (CRP), granulocyte-colony stimulating factor (GCSF), interferon-γ inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-α (MIP1-α) and tumor necrosis factor-α (TNF-α) [45, 63, 64].

Even more predictive of death than serum cytokine profiles is an increase in the fibrin degradation product D-dimer, released from blood clots in the micro-vasculature, and a prognostic for DIC [9]. As such, D-dimer is now widely regarded as a key indicator of the severity of late-stage COVID-19. D-dimer levels naturally increase with age, most likely reflecting a

higher basal level of vascular inflammation [65], which could predispose patients to severe COVID-19. It would, therefore, be informative to know if pre-cytokine storm levels of D-dimer levels could predict who is likely to develop a cytokine storm.

In cytokine storms, high levels of IL-6 cause vascular endothelial cells to secrete fibrin, which causes DIC. In the lung, this may underlie the hypoxemia seen in patients with seemingly functional lungs. If left untreated, clots leach additional clotting factors from the bloodstream, increasing the risk of bleeding (coagulopathy) and multi-organ failure. Drugs such as tocilizumab (Actemra), which block IL-6 receptor activity, are currently being used in patients in advanced stages [66].

One in two fatal cases of COVID-19 experience a cytokine storm, 82% of whom are over the age of 60 [67]. Though there may be many simultaneous triggers of the storm, abundant evidence indicates that inflammaging is a major driver, exacerbated by obesity, poor diets and oral health, microbial dysbiosis, and sedentary lifestyles [68, 69]. For example, in rodents, inflammaging increases the risk of cytokine storm syndrome [70] and, in humans, age correlates with higher basal circulating levels of pro-inflammatory cytokines including IL-6, TNF-α, IL-1α and CRP [71, 72].

A central player that could help explain the predisposition to cytokine storms is NLRP3, the major protein component of the inflammasome. During aging, there is a steady increase in the abundance and activity of NLRP3 in immune cells, including AMs of the lung which, upon chronic stimulation, contribute to pulmonary fibrosis [73]. NLRP3 inflammasome activation requires two steps, the first of which is the priming step, induced by TLRs or tumor necrosis factor receptor activation. This leads to the activation of NF-κB and promotes the expression of NLRP3, pro-IL-1β, and pro-IL-18. The second step, also called the activation step, is triggered by a range of stimuli that emerge during infections, such as tissue damage, nucleic acids, and invading pathogen proteins [74].

In older individuals, NLRP3 may be poised for hyperactivation by SARS-CoV-2 antigens. NLRP3 activity is under the direct control of sirtuin 2 (SIRT2), a member of the NAD^+-dependent sirtuin family of deacetylases (SIRT1-7) [75]. During aging, NAD^+ levels decline, reducing the activity of the sirtuins [76]. Old mice, especially those deficient in SIRT2, have decreased glucose tolerance and increased insulin resistance [77]. This decline, exacerbated by COVID-19, might promote hyperactivation of NLRP3 and the trigger cytokine storms in COVID-19 patients [14]. Maintaining NAD^+ levels may therefore alleviate COVID-19 symptoms, a possibility supported by recent data showing that SARS-CoV-2 proteins hyperactivate poly-ADP-ribose polymerases PARP9, -10, -12, and -14 and deplete cellular NAD^+ [78]. Additionally, NAD^+ precursors lower inflammation in human subjects [79, 80].

Mechanisms of infection in other coronaviruses support the hypothesis that NLRP3 activation is a trigger of cytokine storms in the aged. The SARS-CoV-1 ORF3a protein, for example, is a potent activator of pro-IL-1β gene transcription and protein maturation, the two main signals required for activation of NLRP3 [81]. In macrophages, SARS-CoV-1 ORF8b robustly activates the NLRP3 inflammasome by interacting directly with the Leucine Rich Repeat domain of NLRP3 in cytosolic dot-like structures [82], suggesting another two-step model, in which inflammaging and the NLRP3 basal overactivation is the first step and SARS-CoV-2 antigen-mediated hyperactivation is the second step that triggers a cytokine storm.

In chronic diseases, hyperactivity of the inflammasome plays a dominant role in the development of type 2 diabetes and other age-related diseases [83]. Indeed, in older adults, the upregulation of two inflammasome-related gene sets correlate with increased risk of hypertension, metabolic dysfunction, oxidative stress and mortality [84]. Individuals over the age of 85 that expressed lower levels of these inflammasome modules were less likely to die within seven years [84]. Taking together, the known effects of coronavirus proteins on NAD^+, NLRP3, and the two stages of inflammasome activation, these data provide a plausible explanation as to why co-morbidities positively correlate with cytokine storms and fatality in COVID-19 patients.

After age and hematological cancers, obesity is the next major risk factor for COVID-19 fatality, similar to type 2 diabetes [85]. Obesity is well known to increase the activity of NLRP3 and stimulate low grade inflammation in mice, including higher levels of serum chemokines, and lower neutralizing antibodies and effector memory T cells during a viral infection [86]. Accordingly, this may help explain why obesity is associated with lower survival in COVID-19, SARS-CoV-1 and MERS-CoV infections, and why obesity-related human diseases such as cardiovascular disease, chronic kidney disease, and diabetes, predispose patients to cytokine storms (Table 1) [87–89]. In addition, by causing the endothelium of the microvasculature to become leaky, obesity and type 2 diabetes, may increase the ability of SARS-CoV-2 to infect surrounding pericytes that appear to express ACE2 at levels far greater than surrounding cells [12].

Table 1. Risk factors for adverse outcomes in human coronavirus infections.

Risk factor	Virus	References
Advanced age	SARS-CoV-2, SARS-CoV-1, MERS	[4, 175–181]
Cardiovascular disease, hypertension and coronary artery disease	SARS-CoV-2, SARS-CoV-1, MERS	[4, 176, 179, 181–184]
Diabetes	SARS-CoV-2, SARS-CoV-1, MERS	[4, 176, 182, 183, 185–188]
Obesity	SARS-CoV-2, SARS-CoV-1, MERS	[4, 182, 183, 189]
Male Sex	SARS-CoV-2, MERS	[4, 176, 178]
Respiratory diseases	SARS-CoV-2, MERS	[4, 176, 181]
Kidney disease	SARS-CoV-2, MERS	[4, 176, 187, 190]
Immunological disorders	SARS-CoV-2	[4, 175]
Cancer	SARS-CoV-2, SARS-CoV-1	[4, 179]
Other factors	SARS-CoV-2, SARS-CoV-1	[4, 179, 180, 186, 191]

Epigenetic changes with age

The dysregulation of the epigenome and resulting changes in gene expression during aging are strongly implicated as biomarkers, and potentially underlying causes, of chronic disease states and of aging itself. The "relocalization of chromatin modifiers" theory of aging postulates that symptoms of aging and the loss of resilience are a result of a lifetime accumulation of epigenetic changes [90, 91]. These changes may be caused, in part, by the redistribution of chromatin factors, such as the nuclear proteins SIRT1/6/7, HDAC1 and PARP1 away from regular loci to sites of dsDNA break repair, then back again, causing epigenetic "noise" to accumulate, which may iteratively erase cellular identity [90–94]. This process is thought to manifest as DNA methylation changes that set the pace of the biological clock in tissues and in hematopoietic cells [95, 96].

There is an abundance of evidence indicating that age-related changes to the host's epigenome compromise immune cell composition and function [97] and negatively impact viral defenses [98, 99], including adaptive immune memory [100, 101]. Coronaviruses are known to mediate epigenetic alterations, potentially accelerating the rate that the immune system ages. MERS-CoV, for example, antagonizes host antigen presentation by altering DNA methylation, a mark that silences genes encoding major histocompatibility complexes [102]. Similarly, SARS-CoV-1 changes histone methylation and long non-coding RNAs, which is accompanied by the activation of interferon-response genes [103]. Measuring the DNA methylation age of immune cells and other blood cell types before, during, and after infection could help elucidate both how the aged epigenome impacts disease severity and how the virus alters the aged epigenome.

The vulnerability of the aged to SARS-CoV-2 may also have to do with the effects of the epigenome on viral entry, which is initiated by physical interaction between the viral spike glycoprotein receptor and the ACE2 cell surface protein [104]. While genetic differences in ACE2 are being pursued as a cause of COVID-19 severity [105], there is little attention being paid to epigenetic differences. In humans, ACE2 is ubiquitously expressed in epithelial tissues of the body, most highly in alveolar epithelial cells and enterocytes of the small intestine [106]. ACE2 is regulated in the body transcriptionally, post-transcriptionally, and post-translationally [107], although its role and regulation in COVID-19 is still poorly understood.

In both mice and rats, ACE2 expression decreases with age and is associated with an increase in aortic fibrosis and inflammation [108, 109]. In healthy human lungs, ACE2 expression does not appear to change with age, [110]. Even though ACE2 is more highly expressed in the lungs of cigarette smokers [111]. A meta-analysis of COVID-19 deaths, however, did not identify smoking as a significant risk factor [4]. ACE2 promoter hypo-methylation in lymphocytes correlates with trans-criptional activation in patients with lupus [112], implying that transcription of ACE2 is controlled by methylation, although this mechanism has not been systematically investigated. It is known, however, that methylation at one of seven CpGs in the ACE2 promoter decreases with age and these CpGs are bordered by long-range promoter-enhancer contacts that may change over time [113]. Bisulfite sequencing of the ACE2 gene paired with transcriptomic and four-dimensional chromatin analyses will be necessary to understand if there is a causal relationship between promoter methylation, ACE2 expression, and disease outcome.

The elucidation of SARS pathogenesis is complicated by the fact that ACE2 is also part of the renin-angiotensin system (RAS) that regulates immunity, fibrosis, blood pressure, and metabolism. ACE2 counteracts vasoconstriction caused by angiotensin

converting enzyme (ACE) by cleaving its product, angiotensin II. Most likely due to its role in vasodilation and reducing inflammation, ACE2 partially protects against sepsis-induced- and SARS-induced severe acute lung injury in mice [114, 115] and asthma-induced airway inflammation in rats [116]. Changes in DNA methylation during aging are known to affect the RAS [14, 117, 118]. Analysis of ACE2 gene expression in the lungs of COVID-19 patients with pulmonary arterial hypertension and chronic obstructive pulmonary disease found a correlation between ACE2 expression and COVID-19 severity [111]. Thus, age-related dysregulation of ACE2 could explain why age is such a risk factor for COVID-19 complications and why cardiovascular disease and hypertension predispose patients to develop a more aggressive form of COVID-19.

The effects of ACE inhibitors, used commonly beyond middle age to control blood pressure, are generally believed to be neutral in COVID-19 [119, 120]. Due to their opposing roles in the RAS, ACE2 expression appears to increase when ACE is inhibited, likely providing a yet unknown protective function [121]. Inhibiting ACE2 expression or blocking ACE2 accessibility could prevent viral entry but may lead to vasoconstriction and hypertension. Instead, the most promising ACE2-targeted therapeutic strategy is to infuse human recombinant soluble ACE2 into the airway or bloodstream to bind the SARS-CoV-2 spike glycoprotein receptor, preventing it from binding ACE2 on host cell surfaces [122] and slowing cell infection rates.

Sirtuins and NAD^+

The sirtuins are a family of NAD^+-dependent lysine deacylases that control numerous aspects of stress resistance and pathogen defenses. SIRT1 is a nuclear histone deacetylase that suppresses viral replication and chronic inflammation [123]. By binding to the promoter region of ACE2, SIRT1 upregulates transcription under conditions of cell stress [124]. During aging, and perhaps particularly during the course of COVID-19, levels of NAD^+ decline. This is likely due to increased NAD^+ consumption by the $CD38^+$ glycohydrolase [125] and increased transcription of the poly-ADP-ribosyl transferases, PARP9, PARP10, PARP 12 and PARP14 in mice and humans infected with SARS-CoV-2 [78]. Corona-viruses also possess an ADP-ribosylhydrolase that further depletes NAD^+, apparently to disrupt cell signaling, DNA repair, gene regulation and apoptosis [14, 126, 127].

By negatively regulating activity of NLRP3, SIRT1 and the related protein SIRT2, seem to play key roles in suppressing acute lung inflammation during sepsis [75]. Mice lacking SIRT1, for example, display aggravated inflammasome activation, with increased production of lung proinflammatory mediators, including intercellular adhesion molecule 1 (ICAM-1) and high-mobility group box 1 (HMGB1), and a dramatic reduction of lung claudin-1 and vascular endothelial-cadherin expression [128]. Further, as a result of NAD^+ depletion in mouse models of uncontrolled diabetes, DNA repair is blunted leading to pulmonary inflammation, senescence and fibrosis [129], which could explain why diabetics are more susceptible to COVID-19. SIRT1 also attenuates the acute inflammatory response through deacetylation of H4K16 in the TNF-α promoter [130]. Another nuclear sirtuin, SIRT6 attenuates NF-kB signaling by deacetylating H3K9 [131]. Thus, a decline in NAD^+ and the known mis-localization of SIRT1 and SIRT6 across the genome during aging [90, 132], could be major contributors to the age-dependency of COVID-19 symptoms. As such, NAD^+ precursors, such as NMN and NR [133], have been suggested as possible treatments for COVID-19, especially in older people [78]. Clinical studies are needed to determine if NAD^+ supplementation would benefit in the early stages of SARS-CoV-2 to reduce replication or if NAD^+ treatment during acute COVID-19 can hasten recovery.

Biological clocks

Over the past decade, a variety of biological clocks have been developed to predict human health and longevity more accurately than chronological age, including those based on DNA methylation patterns [95, 134–136], inflammaging [137], gene expression patterns [138], frailty [139, 140], serum proteins [141], and IgG glycosylation [142–144]. Given that these clocks provide a quantitative measure of the rate of aging of an individual and their overall resilience, biological clocks may be useful for identifying at-risk populations and for predicting, within those populations, who will most likely progress to severe COVID-19.

Epigenetic clocks

Estimates based on twin studies place the contribution of non-genetic factors on predicted COVID-19 phenotype at 50% [145] and on total disease burden in old age at approximately 80% [146]. Indeed, lifestyle factors that affect the epigenome such as calorie intake may increase the susceptibility to COVID-19. Epigenetic age is greater than chronological age in various disease contexts and lower in long-lived humans, providing strong evidence that epigenetic age reflects biological age [134, 147]. Age-associated changes to the epigenome have profound effects on

the immune system, including T cell function, cytokine production and macrophage pattern recognition. DNA methylation is believed to set the pace of the aging clock in several mammalian tissues, including hematopoietic cells of the immune system [95, 96]. Epigenetic clocks that measure DNA methylation at specific CpG sites are the most widely used measure of biological age and disease susceptibility [134, 147]. Restoration of the thymus using a drug cocktail of metformin, growth hormone and dehydroepiandrosterone led to the reversal of features of immunosenescence, specifically increasing naïve T cells and a decreasing senescent PD-1^+ T cells, along with the reversal of the epigenetic clock by about 1.5 years [96]. Epigenetic age may be a better biomarker than chronological age in predicting how variation in lifestyle factors and age-associated comorbidities increase susceptibility to COVID-19 and may also help determine if COVID-19 infection accelerates epigenetic age. We hope to test both by measuring the DNA methylation ages of peripheral blood samples from thousands of COVID-19 patients and correlating methylation age measurements with clinical outcomes.

Glycosylation clocks

Changes in glycosylation during aging may also predispose older individuals to severe COVID-19 [148]. Glycosylation is the enzymatic process by which carbohydrates called glycans, such as sialic acid, mannose and fucose, are covalently attached to proteins or lipids, typically on the cell surface or in the bloodstream. An individual's repertoire of glycans – a notable example being the type of N-glycans attached to immunoglobulins [149] – changes with age and environmental factors, such as smoking and poor diet [148]. The type of glycans attached to IgGs affects their pro- and anti-inflammatory properties [150]. Decreased galactosylation of IgGs is associated with central adiposity [151] and inflammaging in the context of diabetes [152]. Biological clocks based on IgG glycosylation are able to predict chronological age within 10 years, and can be improved by inclusion of clinical parameters [144]. Thus, changes to the glycome with age could serve both as an indicator of biological age and could potentially predict COVID-19 severity.

Aging also changes the glycome via non-enzymatic glycation, by which reducing sugars circulating in extracellular compartments covalently bind to proteins and lipids to form advanced glycation end products (AGEs). AGEs are present in large quantities in the Western diet, and greater consumption of dietary AGEs increases serum TNF-α [153]. AGEs tend to accumulate under hyperglycemic conditions and contribute to the pathology of many age-related disease such as type 2 diabetes and obesity [154]. AGEs may increase COVID-19 severity in the aged by inhibiting the NLRP3 inflammasome during the early stages of viral infection [155] when the inflammatory program is activated by the SARS-CoV-1 3a protein [156]. AGEs also play a role in activating pro-coagulation pathways [154], potentially contributing to the DIC observed in COVID-19 patients.

Glycosylation patterns specific to older people may also impact viral entry. The SARS-CoV-2 spike protein is heavily glycosylated [157], modifications that are highly conserved between coronaviruses. SARS-CoV-2 shares 20 out of 22 of glycosylated N-linkages with SARS-CoV-1 [157]. In the case of the human influenza virus, variation in sialic acid structures on the surface of cells lining the upper and lower respiratory tracts dictates tropism and age-dependent binding efficiency of the virus [158] but how changes in the coronavirus spike protein during aging might affect viral transmission and pathogenesis is not yet known. If we are to use glycation as a prognostic marker for COVID-19, it will be necessary to map the glycome in hundreds of patient samples with varying degrees of COVID-19 severity, including asymptomatic individuals.

Immune clocks

Between individuals, heterogeneity of the immune system increases during aging [18] and may explain differences in susceptibility to infectious diseases. A biological clock based on the immune system called IMM-AGE was recently developed that predicts all-cause mortality in older adults more accurately than even DNA methylation clocks [137]. IMM-AGE overcomes the limitation of inter-human immune heterogeneity by tracking immune cell frequencies and gene expression changes longitudinally within individuals and then computationally predicting how an individual's homeostatic immune state changes over time. Though individuals exhibit variation in immune cell-type composition, these changes fall into three stages that converge on a common "attractor point" that correlates with age and is indicative of overall physiological resilience [137]. In this way, IMM-AGE measures the entropic relationship between age and immune system remodeling, the rate of which can predict survival. Because IMM-AGE is even able to capture and predict the effect of inflammaging on the cardiovascular system, and because COVID-19 fatality is so closely tied to cardiovascular disease and inflamm-aging, this clock may prove to be the most accurate at identifying COVID-19-susceptible individuals. More

studies are still needed to determine if and how viral infections alter these and other biological clocks, and whether variation in biological age predicts COVID-19 severity.

Geroprotectors to improve immunity

Advanced age is by far the greatest risk factor for COVID-19 fatality independent of underlying co-morbidities [4]. This striking fact has led many researchers to speculate whether molecules that target aging itself, called geroprotectors, could be used to combat infections in older people [5, 159]. Primarily via its regulation of cellular metabolism, the mammalian target of rapamycin (mTOR) signaling pathway controls several immune functions such as antigen presentation, immune activation, differentiation, and cytokine production [160, 161]. Low dose mTOR inhibitors exhibit a hormetic effect in older people, seemingly improving immunity and reducing rates of infection [162, 163]. People over 65 years old who took mTOR inhibitors for six weeks responded more robustly when challenged with an influenza vaccine and showed reduced levels of the T-cell exhaustion marker PD-1 [162]. In a similar clinical trial, protection from infection and an increase in anti-viral gene expression was observed even a year after the 6-week course of mTOR complex 1 (mTORC1) inhibitors [163], though the result was not reproduced in a Phase 3 trial.

Metformin, a blood glucose lowering geroprotector that activates 5'AMP-activated protein kinase (AMPK) and inhibits the mTOR pathway, has also been suggested as a possible drug to combat severe SARS-CoV-2 infection in older people. In addition to its potential insulin-sensitizing antiviral effect [164], metformin confers a myriad of anti-aging benefits including improving mitochondrial metabolism, decreasing inflammatory cytokines, protecting against genomic instability and decreasing cellular senescence [165], which may bolster the aging body's resistance to COVID-19. Results from the ongoing Targeting Aging with MEtformin (TAME) clinical trials and others should reveal whether these anti-aging drugs are protective against SARS-CoV-2 infection [165, 166].

Where do we go from here?

Why SARS-CoV-2 infections are more severe and fatal in the aged is not known, but viable hypotheses are emerging that include changes to the immune cell repertoire, the epigenome, NAD^+ levels, inflammasome activity, biological clocks, and covalent modifications of human and viral proteins (Figure 3). Much

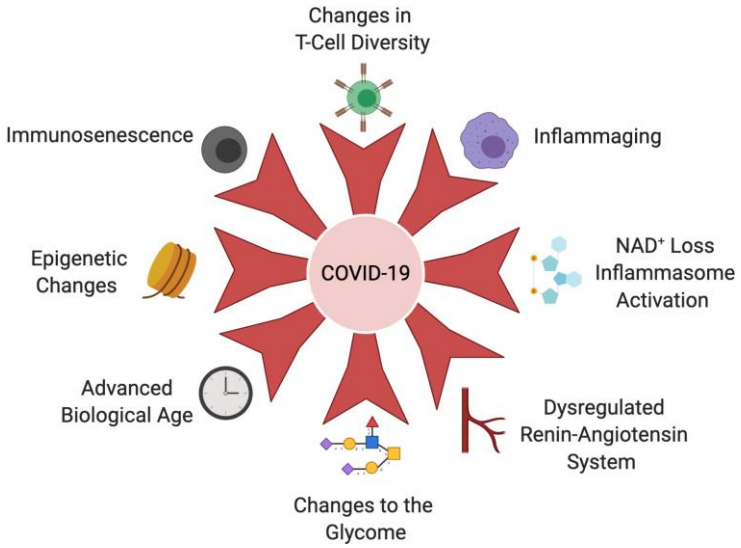

Figure 3. Age-related changes that increase COVID-19 susceptibility. The aging immune system undergoes immunosenescence, T-cell diversity alterations and chronic activation of the innate immune system known as inflammaging. These hallmarks of the aging immune system cripple the body's ability to clear the SARS-CoV-2 virus, initiate and sustain cytokine storms that cause acute organ injury, DIC and multi-organ failure. An age-associated decline in NAD^+ results in derepression of NLRP3 and inflammasome in older people, further exacerbating the cytokine storm. Coronaviruses also possess an ADP-ribosylhydrolase that further depletes already-low NAD^+ levels in older people. Leveling of the epigenetic landscape during aging results in changes in immune cell composition and function that decrease the immune system's ability to mount a response to infection. Epigenetic dysregulation of ACE2 may also impact increased viral loads in older people. Dysregulation of the RAS during aging and in the context of age-associated disease, such as cardiovascular disease, hypertension, COPD and obesity, contributes to severity of COVID-19 infection. The glycome which controls a variety of immune signaling pathways changes during aging and in the context of metabolic diseases. For example, decreases in IgG galactosylation contribute to chronic inflammation. Biological clocks that measure different biomarkers of biological age may explain increased COVID-19 susceptibility more accurately than advanced chronological age. Created with BioRender.

remains to be elucidated, still. Besides understanding the basis of the cytokine storms and coagulopathy, it is not known why SARS-CoV-2 so easily damages such a broad array of tissues in older people but rarely in the young. Nor is it clear whether older people develop stronger or weaker functional immunity during seroconversion, or how long their protection will last compared to younger people. In the aged, immune responses to vaccination are also often weak or defective [18, 167, 168], whereas autoimmunity increases [169]. Therefore, in designing vaccines against SARS-CoV-2, it will be important to consider that older people may not respond as well to vaccines as young people. Studies that follow the long-term consequences of SARS-CoV-2 infection in older people will also be critical to understand the long-term health consequences of COVID-19 pathology, such as fibrosis and scaring of the lungs, micro-ischemic events, cardiopulmonary dysfunction, and neuropsychological disability [170]. These could significantly reduce viral resistance and lifespan in older and middle-aged people who recover from severe cases of COVID-19. The most exciting and potentially impactful technologies to combat COVID-19 and other viral pandemics are those that activate the body's defenses against aging [5, 166]. Eventually, with advances in the field, it may even be possible to reverse the age of cells and tissues [171–174] so that high-risk older individuals can respond to viral infections as though they were young.

Abbreviations

SARS-CoV-1: severe acute respiratory syndrome coronavirus identified in 2003; SARS-CoV-2: severe acute respiratory syndrome coronavirus identified in 2019; MERS-CoV: middle east respiratory syndrome coronavirus; COVID-19: coronavirus disease 2019; ARDS: acute respiratory distress syndrome; TLR: Toll-like receptor; TCR: T cell receptor; DIC: disseminated intervascular coagulation; IL: interleukin; ACE: angiotensin-converting enzyme; ACE2: angiotensin-converting enzyme 2; RAS: renin-angiotensin system; SIRT1-7: sirtuin 1-7; AGE: advanced glycan end product; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; IMM-AGE: immune age; PARP: poly (ADP-ribose) polymerase; NAD^+: nicotinamide adenine dinucleotide; NMN: nicotinamide mono-nucleotide; NR: nicotinamide riboside;, SARS-CoV-1 ORF3a: SARS-CoV-1 open reading frame 3a; NK cell: natural killer cell; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PD-1: programmed cell death protein 1; IgG: immunoglobulin G; IgE: immunoglobulin E; AM: alveolar macrophages; CRP: C-reactive protein; CMV: cytomegalovirus; GCSF: granulocyte-colony stimulating factor; IP-10: interferon gamma-induced protein 10; MCP-1: monocyte chemoattractant protein-1; MIP-1α: macrophage inflammatory protein 1 alpha; TNF-α: tumor necrosis factor alpha; mTOR: mammalian target of rapamycin; mTORC1: mammalian target of rapamycin 1; AMPK: 5' AMP-activated protein kinase; TAME: targeting aging with metformin; HDAC: histone deacetylases; CCR1: C-C Motif Chemokine Receptor 1; CXCR2: CXC chemokine receptor 2; ICAM-1: intercellular adhesion molecule 1; HMGB1: high mobility group box 1.

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CONFLICTS OF INTEREST

AM and MM declare no conflicts. DAS is a board member, equity owner and inventor on patents licensed to MetroBiotech, Liberty Biosecurity, and Jumpstart Fertility, both developing molecules for the treatment of diseases by raising NAD^+ levels. Other affiliations are listed here https://genetics.med.harvard.edu/sinclair-test/people/sinclair-other.php.

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Review

From causes of aging to death from COVID-19

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ABSTRACT

COVID-19 is not deadly early in life, but mortality increases exponentially with age, which is the strongest predictor of mortality. Mortality is higher in men than in women, because men age faster, and it is especially high in patients with age-related diseases, such as diabetes and hypertension, because these diseases are manifestations of aging and a measure of biological age. At its deepest level, aging (a program-like continuation of developmental growth) is driven by inappropriately high cellular functioning. The hyperfunction theory of quasi-programmed aging explains why COVID-19 vulnerability (lethality) is an age-dependent syndrome, linking it to other age-related diseases. It also explains inflammaging and immunosenescence, hyperinflammation, hyperthrombosis, and cytokine storms, all of which are associated with COVID-19 vulnerability. Anti-aging interventions, such as rapamycin, may slow aging and age-related diseases, potentially decreasing COVID-19 vulnerability.

COVID-19 vulnerability: age, diseases, gender

COVID-19 is caused by coronavirus SARS-CoV-2. Most cases of COVID-19 are asymptomatic, but some are severe and lethal. Mortality is the simplest marker of COVID-19 vulnerability. COVID-19 vulnerability can be defined as a chance of death from COVID-19, once infected.

Age:
In all studies conducted in all countries, the mortality rate from COVID-19 increases exponentially with age [1–11]. Exact mortality rates varied in hundreds of studies because they depend on testing and therapeutic interventions. But the rule is clear: the mortality rate is increasing exponentially with age.

Age-related diseases:
Mortality is especially high in patients with pre-existing conditions [6, 9, 10, 12–23].

In Italy, 99% of patients, who died, had at least one illness.

https://www.bloomberg.com/news/articles/2020-03-18/99-of-those-who-died-from-virus-had-other-illness-italy-says.

In other words, infected people without pre-existing diseases do not die. This may seem paradoxical because we just discussed that age is sufficient to increase mortality exponentially. This is because pre-existing conditions are manifestations of biological age, whereas aging and diseases are two sides of the same coin [24–26]. These conditions are typical age-related diseases: hypertension, diabetes, obesity, ischemic heart disease (IHD) and chronic obstructive pulmonary disease (COPD) and other diseases [9, 12–23].

Of course, not all (only some) patients with age-related diseases die from COVID-19. In other words, age-related diseases are necessary but not sufficient for mortality from COVID-19.

Age and pre-existing (age-related) diseases are interdependent. A number and severity of diseases correlate with age. An average 60 year old person has

more age-related diseases than an average 50 your old person. Yet, a particular 60 year old person may have no age-related diseases, whereas a particular 50 year old person may have multiple diseases including hypertension, diabetes, obesity and cancer. In this case, it is a chronologically younger person who is biologically older. And it is the biological age that determines the likelihood of death from COVID-19.

Male Gender:
At the same age, the mortality rate is twice higher in men than in women [9, 27, 28], in part, because men age faster than women and, at any chronological age, men are biologically older than women [29].

So, three rules can be combined in one: COVID-19 vulnerability is determined by biological age. Biological age combines chronological age, age-related diseases and gender. A combination of all age-related diseases (and pre-diseases) is a biomarker of biological age. Figuratively, SARS-Cov-2 can "measure" biological age, which is thus the best predictor of mortality from both COVID-19 and other diseases.

Mortality from aging compared with COVID-19 mortality

Aging can be measured as an increase in the probability of death with age. Mortality increases exponentially, starting from age 8-9. Men have a higher "normal" age-related death rate than women because men age faster than women [29].

COVID-19 mortality rate parallels the "expected" aging-related death rate (Supplementary Figure 1) and see second graph in:
https://medium.com/wintoncentre/how-much-normal-risk-does-covid-represent-4539118e1196.

Chances to die from COVID-19 are proportional to chances to die from aging itself at any age. The only discrepancy between natural and COVID-19 mortality is observed below the age of 8 years old. Whereas natural death rate is relatively high, COVID-19 mortality is low (no mortality [11]). This discrepancy will be discussed later. But first how do animals, including humans, die from aging?

Age-related diseases

Humans and other animals (including the worm [30] and the fly [31]) do not die from aging itself but from age-related diseases such as ischemic heart disease (IHD), hypertension, diabetes, cancer, Alzheimer's and Parkinson's diseases, age-related macular degeneration, osteoporosis and sarcopenia (As we will discuss, even seemingly non-deadly diseases such as osteoporosis can lead to deadly complications). The incidence of these diseases increases exponentially with age. Some diseases such as obesity, hypertension and diabetes develop earlier in the course of aging. Other diseases, such as Alzheimer's disease and macular degeneration, are usually diagnosed later [32, 33]. Age-related diseases may also occur in younger people with genetic predisposition and environmental exposure hazards. But even without these factors, diseases develop because they are quasi-programmed (see "Quasi-programmed aging section"). These diseases are not diseases of civilization, as it may seem. Humans simply now live long enough to develop them. Of course, "hazards of civilization" can accelerate them at a younger age.

Aging and its diseases cannot be separated. Healthy aging, or aging without diseases, is merely a slow aging, when biological age is less than chronological age. During a period of seemingly healthy aging, pre-pre-diseases and pre-diseases are progressing until they eventually reach clinical manifestations. Thus, healthy aging progress to unhealthy and pre-diseases become diseases [34].

Age-related diseases and COVID-19 vulnerability are highly intertwined. Patients, who die from COVID-19, otherwise would die from age-related diseases such as heart disease, cancer, diabetes, hypertension, just a year later. COVID-19 approximately doubles a patient's aging-dependent risk of dying during one year. For example, (numbers are very approximate), a sixty year old woman has 1% chance to die from aging before her 61st birthday. At that age, if infected, the death rate from COVID-19 is around 1% for females. If infected, a patient has approximately doubled chances to die compared with usual age-related mortality during one year. As David Spiegelhalter put it: "getting COVID-19 is like packing a year's worth of risk into a week or two". https://medium.com/wintoncentre/how-much-normal-risk-does-covid-represent-4539118e1196.

Children and young adults have a very low risk of death from aging-related diseases, so that risk remains extremely low even when doubled.

Although natural mortality is relatively high in the youngest age group, especially in infants, they do not die from age-related diseases of course. Instead, infants are vulnerable to bacterial infections and candida infections due to underdeveloped immune system [35]. Low COVID-19 mortality in the pediatric age group [11] is consistent with the notion that COVID-19 vulnerability is not due to a "weak" immune system. In contrast, as we will discuss in the next section, it is hyper-functional immune response that leads to death from COVID-19 in the elderly by causing cytokine storm.

Cytokine storm as a hyperfunction

Severe COVID-19 is characterized by hyper-inflammation, cytokine storm, acute respiratory distress syndrome (ARDS), damage to the lung, heart and kidneys [36–39].

In response to viral replication, hyperfunctional monocytes and macrophages infiltrate the lung, causing hyper-inflammation and hyper-secretion of cytokines such as interleukin (IL)-6, IL-2, IL-7, IL-1ra, interferon-γ inducible protein (IP)-10, tumor necrosis factor (TNF)-α, ferritin, monocyte chemo-attractant protein (MCP)-1, macrophage inflammatory protein (MIP) 1-α, granulocyte-colony stimulating factor (G-CSF), C-reactive protein (CRP) and procalcitonin. [22, 36–42].

This leads to leukocyte recruitment, vascular permeability, edema and further pulmonary damage in vicious cycle [37, 38, 41, 43, 44]. Hyper-inflammation becomes systemic, in turn causing hyper-coagulation and thrombosis, disseminated intravascular coagulation [45]. This causes injury of distant organs such as the kidneys. Pre-existing organ damage (late stages of age-related diseases) exacerbates organ damage caused by cytokine storm [42, 43, 46]. In addition, cellular hyper-functions and systemic hyper-inflammation may lead to cellular exhaustion, such as exhaustion of lymphocytes (lymphopenia) [47–49]. Hypercoagulation is associated with hyperactive fibrinolysis and increased D-dimer blood levels [23]. Cytokine storm is a systemic hyperfunctional response (Figure 1).

Of course, age-related hyperfunctional response, such as cytokine storm, is not caused by lifelong accumulation of molecular damage. Aging is not caused by molecular damage after all. Instead it's a continuation of developmental/growth programs that lead to hyper-functions and in turn eventually to dysfunctions.

Hyperfunction theory of quasi-programmed aging

"Quasi" means "resembling" or "seemingly, but not really." Quasi-program of aging is not a program but a continuation of developmental programs that were not switched off upon their completion [24, 50]. They purposelessly unfold, leading to age-related diseases, secondary organ failure and death. Quasi-programmed (program-like) aging is associated with higher than optimal cellular and systemic functions, which eventually, via cellular exhaustion and organ damage, lead to functional decline (Figure 2). For example, starting from birth, blood pressure increases and continues to increase after organismal growth is completed. Therefore, hypertension is the most prevalent age-related disease. In turn, hypertension can cause organ damage: stroke, infarction and renal failure. Similarly, obesity develops in post-development as a continuation of growth (yet, it can be prevented by low caloric diets, illustrating that quasi-program of aging can be decelerated).

Hyperfunction is an excessive normal cellular function: contraction by smooth muscle cells (SMC), adhesion and aggregation by blood platelets, insulin secretion by

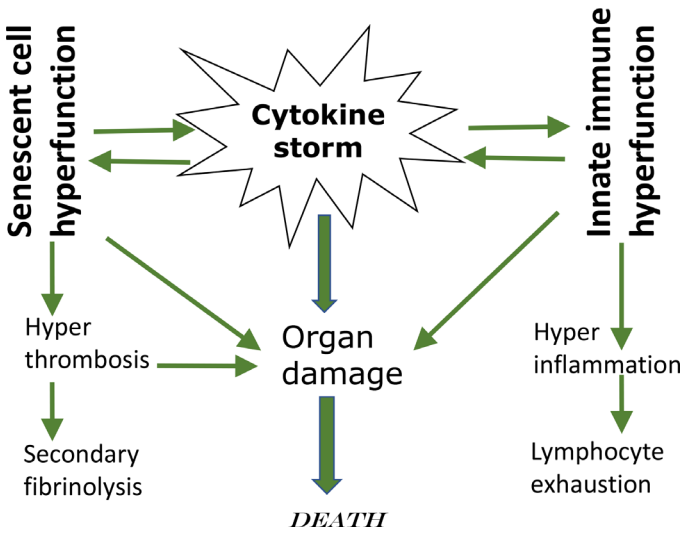

Figure 1. Cytokine storm as a systemic hyperfunction.

beta-cells, lipid accumulation by adipocytes, secretion by stromal and immune cells, oxidative burst by leukocytes, just to name a few. When higher than optimal, they cause vasoconstriction and hypertension, thrombosis, hyperinsulinemia, hypertrophy, hyperplasia, obesity, hyper-secretory phenotype or Senescence-associated secretory phenotype (SASP), hyper-inflammation and so on.

Hyper-function is not necessarily an absolutely increased function. It may be also insufficiently decreased function (relative hyperfunction). Levels of IGF-1 and growth hormone decrease during lifespan. Despite this decrease, IGF-1 levels are still higher than optimal (relative hyper-function) because further genetic decrease in IGF-1 levels (by genetic means) extends health span and lifespan in mammals [51–53].

Cellular hyperfunctions may eventually switch to cellular exhaustion and loss of functions at late stages. During the course of type II diabetes, mTOR overactivation and hyperinsulinemia eventually lead to beta-cell exhaustion and insulin insufficiency, from pre-diabetes to diabetes [54, 55]. As another example, after puberty, hyperstimulation of the ovary eventually leads to oocyte exhaustion and menopause (see Figure 3 in ref. [29]). Depletion of naïve lymphocytes is another example, as reviewed here later. Age-related alterations are mostly noticed when they switch to functional decline, which is a late event.

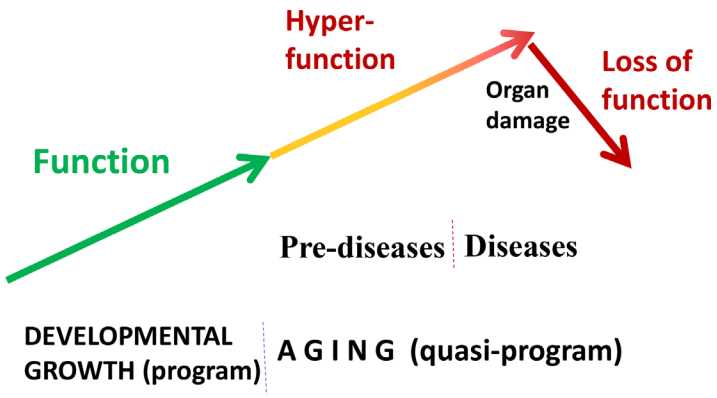

Figure 2. Quasi-programmed hyperfunctional aging. Aging is a continuation of developmental programs that were not switched off upon their completion. An increase in cellular and systemic functions (manifested as pre-diseases and then as diseases) leads to eventual organ damage and secondary loss of function.

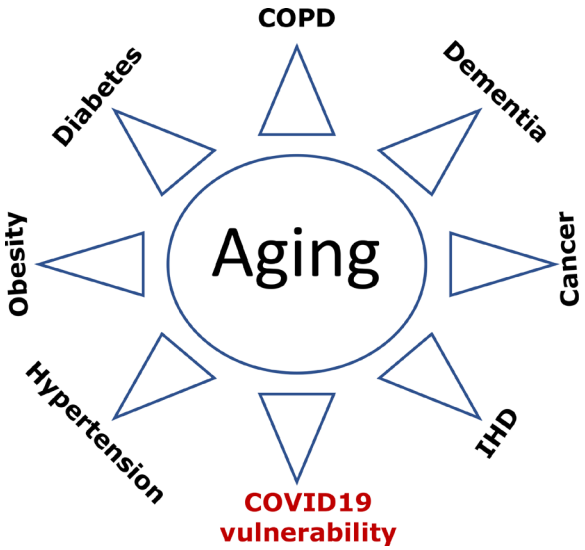

Figure 3. COVID-19 vulnerability as an age-related disease. Age-related diseases, including COVID-19 vulnerability, are manifestations of aging. Abbreviations: Ischemic heart disease (IHD); Chronic obstructive pulmonary disease (COPD).

In some cases, functional decline can be primary and programmed. For example, thymus involution (replacement of T cells by adipocytes) starts early in life, accelerates at puberty and continues later. Still loss of thymocytes and their niches may be in part due to adipocyte hyperplasia and hypertrophy [56]. In fact, obesity accelerates involution, whereas calorie restriction decelerates it [57, 58]. Furthermore, the oblation of sex hormones decelerates or even reverses thymus involution [59]. Thus, involution is triggered by adipocyte hyperplasia and increased production of sex hormones during puberty [56].

Quasi-programmed aging is not driven by molecular damage. It is driven by nutrient/hormone/cytokine-sensing and growth-promoting signaling pathways such as Target of Rapamycin (TOR; mTOR), which are involved in developmental growth and later cause hyperfunctional aging and its diseases [24, 26].

Covid-19 vulnerability as an age-related syndrome

What is the cause-effect relationship between age-related diseases and COVID-19 lethality? Do patients die from age-related diseases, complicated by COVID-19? Or, in contrast, do these various diseases make COVID-19 infection lethal? Both scenarios take place to some extent. However, the relationship is mostly indirect. Both age-related diseases and COVID-vulnerability result from the same underlying cause (Figure 3). This is why they are highly correlated. The cause is aging itself. Aging is manifested by a sum of deadly - and not so deadly - diseases and conditions ranging from cancer to grey hair. Although not all diseases seem to be deadly, they can cause complications such as stroke, ventricular fibrillation, renal failure, lung edema. Even sarcopenia and osteoporosis lead to falls and broken bones culminating in a deadly sequence of events. Cosmetic manifestations such as aging spots and wrinkles, while not deadly by themselves, can be manifestations of other diseases. For example, baldness correlates with prostate enlargement [60], and the later can lead to urinary obstruction and renal failure.

Diseases occur together. For example, chronic obstructive pulmonary disease (COPD) is associated with diabetes, cardiovascular disease and hypertension [61]. If a person has one disease (e.g., diabetes), this patient has higher chances of having other diseases (e.g., hypertension, IHD, cancer) or conditions, including COVID-19 vulnerability, which is revealed only during infection but can be predicted by pre-existing diseases.

Aging is initially driven by an increase in cellular and systemic functions (hyperfunction), leading to age-related conditions. For example, hypertension is a systemic hyperfunction due to hyperfunction of multiple cell types such as arterial smooth muscle cells (aSMC). Similarly, COVID-19-vulnerability is associated with hyperfunction of inflammatory cells that, in response to COVID-19 infection, causes cytokine storm, hyper-coagulation and damage of the lung and distant organs.

The COVID-19 vulnerability syndrome is an aging-related disease, strictly dependent on biological age, associated with other age-related diseases, and exemplified by hyper-functional response to infection**.**

Inflamm-aging and immunosenescence

With hundreds of cell types acting in concert, the immune system is so complex that we cannot discuss age-related alterations without oversimplification. The most noticeable alteration is that memory T and B cells replace naive T and B cells [62]. (This seems natural since life-long exposure to pathogens replaces naïve cells by memory cells). Replacement of naïve immune cells decreases adaptive responses to novel antigens such as SARS-CoV-2. In contrast, immune protection by memory T cells from viral re-infection with known pathogens is usually increased with age [62].

Immune responses are roughly divided into (a) innate responses, carried mostly by neutrophils, macrophages and NK cells, which react to pathogen rapidly and nonspecifically, and (b) adaptive responses, carried by T and B lymphocytes, which are delayed, slower and specific (e.g., antigen-specific clonal expansion of T and B lymphocytes and antibody production by B lymphocytes) [63–65]. In the elderly, immune responses to SARS-CoV-1/2 are "stuck in innate immunity," with insufficient progression to adaptive immunity [37]. However, decline in adaptive response, such as antibody production, plays little role in COVID-19 mortality. It is hyper-functional innate immunity, hyper-inflammation, cytokine storm and hyper-coagulation that lead to organ failure and death. In agreement, hyper inflammatory response rather than high virus numbers leads to death of SARS-CoV-infected old nonhuman primates [66].

Aging is associated with diseases of immune hyper-function such as autoimmune disorders with paradoxical increase in certain signaling pathways and cytokine levels [67–69].

In the elderly, innate immune cells are in a state of sustained activation, producing pro-inflammatory cyto-kines [67, 70–72]. Increased pro-inflammatory activity by the innate immune system, especially by monocytes/ macrophages, is a state of alertness and hyper-reactivity on the cost of potential age-related inflammatory diseases

[67, 70–72]. Whereas some functions are decreased, others are increased. According to the inflamm-aging concept, innate immune system overtakes adaptive immune system in aging. Cause-effect relationships are bi-directional: immunosenescence (namely, a decrease in adaptive response) is a cause and consequence of inflamm-aging [67, 70–72].

We can consider inflamm-aging as an example of hyper-function. While some functions are decreased, others are increased. Hyper-function is damaging. (In analogy, increased electric power, without an adaptor, would damage a laptop). Damaging hyper-functions can lead to loss of function and cellular exhaustion. And vice versa, loss of function may cause compensatory hyper-functions of another components.

Cellular senescence as a continuation of growth

Cellular senescence is a continuation of cellular growth, when actual growth is completed [73, 74]. In proliferating cells, cellular mass growth is balanced by cell division. Cells grow in size and then divide. When the cell cycle is blocked (e.g., p21 and p16), then growth-promoting pathways such as mTOR and MAPK drive conversion to senescence (geroconversion) [24, 74, 75]. During geroconversion, cells become hypertrophic and "fat". Cellular functions increase: hyper-secretion and lysosomal hyper-function are manifested by SASP and beta-Gal staining. Hyper-activated growth-promoting pathways cause compensatory resistance to growth factors/insulin, permanent loss of re-proliferative potential [74]. Rapamycin, everolimus, pan-mTOR and MAPK inhibitors slows down geroconversion, maintaining reversible quiescence instead of senescence [73, 76–88].

Geroconversion is a continuation of cellular growth [73, 74]. Similarly, aging is a continuation of developmental growth (see Figure 1 in ref. [89]). When the developmental program is completed, it becomes a quasi-program of aging. As discussed in detail, chronically activated nutrient-sensing and growth-promoting pathways drive age-related diseases, culminating in organismal death [24, 26].

Age-related diseases are quasi-programmed. Aging is a common cause of age-related diseases, a sum of all age-related diseases. They are diseases of hyper-function, secondary hypo-function and compensation reactions [25]; they are deadly manifestations of aging.

From activation of cellular functions to systemic hyperfunctions, from diseases to organ damage and death, hyperfunction theory of quasi-programmed aging describes the sequence of events [26]. And as discussed in 2006, suppression of aging by gero-suppressants, such as rapamycin, will prevent and treat all age-related diseases [24]. This point of view is becoming widely accepted and, in recent literature, quasi-programmed model of diseases (2006) is called "geroscience hypothesis" [2, 90].

Figuratively, rapamycin rejuvenates immunity [91]

If aging were functional decline due to accumulation of molecular damage, then it would be near to impossible to restore functions and rejuvenate the immune system. In contrast, if functional decline is secondary to hyperfunctions (see Figure 2 in ref. [89]), these hyperfunctions can be suppressed pharmacologically to restore lost functions. Typical drugs are inhibitors of their targets, rather than activators, so they decrease functions of their targets. By decreasing hyper-functions, which otherwise lead to secondary loss of functions, rapamycin may restore "lost" functions (Figure 4).

Rapamycin improves vaccination against viruses such as influenza in old mice, monkeys and humans [92–100]. Importantly, rapamycin increases pathogen-specific but not graft-reactive CD8+ T cell responses [95, 101]. Therefore, rapamycin and everolimus can both be used to prevent donor organ rejection and improve adaptive immunity against new pathogens [96].

Differentiation is an increase of tissue-specific cellular functions. Terminally differentiated B, T, and NK cells can rapidly react to already known pathogens [102]. Decrease in naïve T and B lymphocytes (and thus diminished response to novel antigens) results in part from cellular hyper-differentiation in the immune system [64, 103]. Hyper-functional differentiation can be counteracted by rapamycin [98].

As another example, age-related exhaustion of stem cells is partially due to loss of quiescence caused by growth over-stimulation [92, 104–106]**.** In general, senescent cells characterized by hyper-proliferative drive coupled with cell cycle arrest [77]. In young mice, mTOR hyper-activation causes senescence of hematopoietic stem cells (HSC) and decreases lymphopoiesis [92]. In old mice, rapamycin rejuvenates hematopoiesis, and improves vaccination against influenza virus [92].

Third, production of lymphoid cells may be decreased because of disruption of hypoxic niches due to adipocytes hyperplasia [56, 107]. Hypoxic niches can preserve HSC [108, 109] probably because hypoxia inhibits mTOR and cellular senescence [110]. In agreement, rapamycin preserves HSCs [92, 98, 111, 112] reduces the proportion of memory cells and maintains a pool of naïve T cells [92, 98].

Fourth, growth factor (GF)- and insulin-resistance is loss of function because cells cannot respond to GF/insulin. But it may be caused by over-activated mTOR, which via S6K/IRS feedback loop blocks insulin and GF signaling. Rapamycin abrogates the loop restoring signaling [113–118].

Anti-aging medicine

A high prevalence of age-related diseases, often called "diseases of civilization," is a success story of modern medicine. In the past, most people did not live long enough to develop age-related diseases and those who developed them died soon after. Due to medical advances, people survive to 85 on average, despite suffering from age-related diseases. Standard medicine preferentially extends life span, without necessarily affecting health span (see Figure 3 in ref. [119]). For example, defibrillation and coronary stenting can save life but not cure heart disease. It is anti-aging interventions that extend health span, delaying diseases, thus extending lifespan. Aging is a common cause of all age-related diseases. By suppressing aging, anti-aging interventions may delay all age-related diseases [119].

As a well-known example, low calorie diets such as calorie restriction, intermittent fasting, and low carbohydrate diets extend both health and lifespan. Figuratively, low calorie diets prolong life by improving health. Nutrients and obesity activate growth-promoting pathways (e.g., mTOR), thus accelerating development of quasi-programmed (age-related) diseases. Obesity is associated with all age-related diseases from cancer to Alzheimer's and from diabetes to sarcopenia. COVID-19 vulnerability is also associated with obesity [9, 19, 20, 22]. According to hyperfunction theory, obesity accelerates aging and all age-related conditions including COVID-19 vulnerability.

Diabetes is one of main risk factors of death in COVID-19 [5, 6, 12, 13, 15, 21]. Can type 2 diabetes, an age-related disease, be reversed? In remarkable studies, it was shown that a brief course (6-8 weeks) of very low calorie diets (VLCDs) can reverse type II diabetes. In one study, VLCD reversed diabetes in 46% of patients with up to a 6-year history of diabetes [120]. VLCD is most effective for its prevention and at early stages of diabetes [121]. This anti-aging modality is so simple that remission can be achieved at home by health-motivated individuals [122]. Simultaneously, it treats other age-related diseases such hypertension [123]. Obesity is associated with other diseases of hyper-function from diabetes and sarcopenia to cancer and Alzheimer's' disease. Since age-related diseases are predictors of COVID-19 mortality, VLCD in theory may decrease COVID-19 vulnerability.

Rapamycin and everolimus as anti-aging drugs

In the soil of Easter Island, a complex bacteria produces anti-fungal antibiotic rapamycin to suppress yeast growth but, as a by-product, it also suppresses yeast aging (quasi-programed aging is a continuation of growth). Approved for human use in 1999, Rapamycin

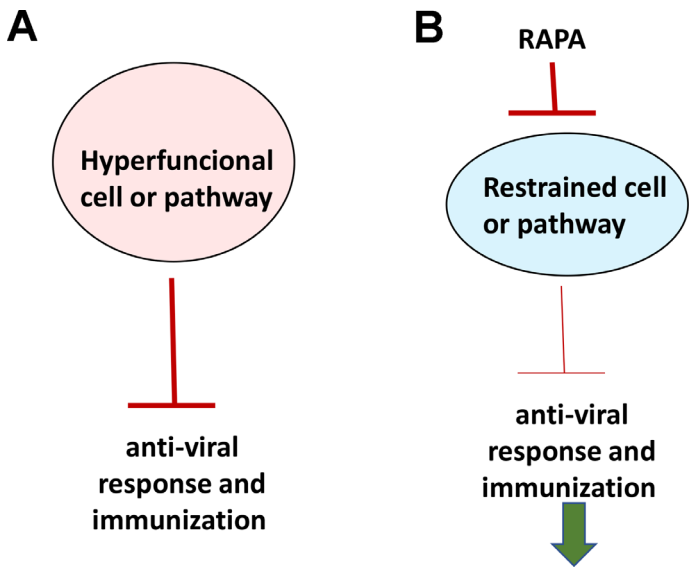

Figure 4. Rejuvenating immunity by inhibiting hyperfunction. (**A**) Specific hyper-functional cells (or signaling pathways) can inhibit some other cell types (or pathways) that are needed for proper anti-viral response and immunization. (**B**) By inhibiting hyper-functional cells or pathways, rapamycin can reactivate "loss-of-function" otherwise suppressed by hyper-functional cells or pathways.

(Sirolimus) and its close analog Everolimus are widely used in several diseases including cancer and organ transplantation. Hundreds of clinical trials (and twenty years of clinical practice) have ensured their safety and good tolerability especially in healthy older adults [119].

Currently, several anti-aging clinics prescribe rapamycin out of label to prevent age-related diseases and slow aging. Hundreds of recent reviews discussed rapamycin and everolimus in detail, so I will just emphasize a few points:

1. Crucial prediction of hyper-function theory of quasi-programmed aging in 2006 was that rapamycin will slow aging, extend healthspan and lifespan and decrease all age-related [124]. It has been confirmed: it extends lifespan in animals from worm to mammals. In some strains of short-lived mutant mice, it extends life span two fold [98, 125].

2. Rapamycin slows geroconversion to cellular senescence in cell culture [74].

3. mTOR is a potential therapeutic target in chronic obstructive pulmonary disease COPD [126], [127]. Rapamycin (sirolimus) is already approved and successfully used in lymphangioleiomyomatosis (LAM), a progressive, cystic lung disease, associated with inappropriate activation of mTOR [128]. Long-term daily use of rapamycin improves lung function without causing serious side effects (and of course no even minor side effects in the lung, given that rapamycin improves lung function) [128].

4. Despite widespread misunderstanding, rapamycin and everolimus do not cause diabetes. In contrast, they prevent diabetic complications in animals with diabetes (see for references [129]). In rodents, in some conditions they may cause symptoms of starvation pseudo-diabetes similar to prolong fasting and ketogenic diet [129]. Although, the *Johnson* study found a slight but significant correlation between Medicare billing for insulin and the use of rapamycin in renal transplant patients, this correlation was mechanistically explained by interaction of rapamycin with two other drugs used in the same patients [130, 131]. In cancer patients, everolimus may cause reversible hyperglycemia as a mild, infrequent and reversible side effect after several weeks of daily high doses of everolimus and rapamycin [132]. Mechanistically, everolimus decrease insulin production, not causing insulin resistance [132]. If anything, everolimus and rapamycin can be considered to treat complications of type II diabetes and prevent hyperinsulinemia and obesity ([129] and references within). What actually contributes to type 2 diabetes is excess of nutrients (and especially carbohydrates), which activate mTOR and cause hyperinsulinemia and insulin resistance.

Potential applications of rapamycin/everolimus to COVID-19

As soon as COVID-19 epidemic started, it become clear that COVID-19 vulnerability is an aging-dependent condition and the use of rapamycin (Sirolimus) was immediately suggested by independent researchers [1, 3, 133–137]. These proposals were based on a mixture of several rationales, which need to be clearly distinguished. In theory, there are at least three independent applications of rapamycin and everolimus for COVID-19. Currently, they all are still hypothetical.

1. Anti-aging effect (Figure 5). By decreasing biological age and preventing age-related diseases, a long-term rapamycin therapy may in theory decrease COVID-19 mortality rate in the elderly. Anti-aging application is especially important because it is beneficial regardless of COVID-19. After all, mortality rate from aging and its diseases is 100%, causing more than 2 million deaths in the USA annually. Continuous use of rapamycin is expected to improve health, decrease age-related diseases and extend healthy lifespan, rendering individuals less vulnerable, when infected with the virus.

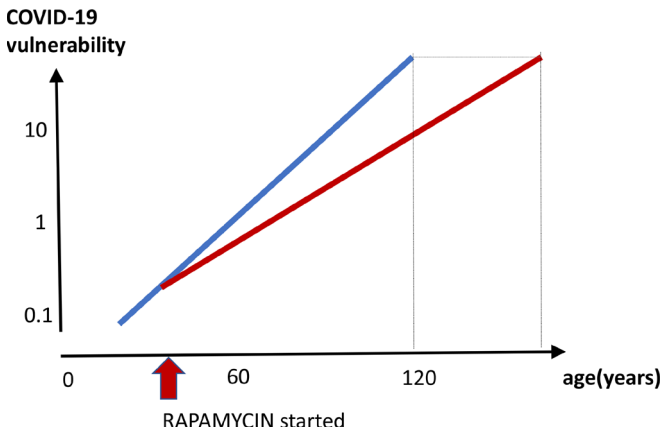

Figure 5. Prevention of COVID-19 vulnerability by staying young. Hypothetical graph in the absence of COVID-19. COVID-19 vulnerability (log scale) increases exponentially with age (blue line). The line ends at age 120, a maximum recorded age for humans. In theory, a continuous rapamycin treatment would slow down an increase of the vulnerability with age (red line). The increase is still logarithmic but at a different slope, because rapamycin slows the aging process. The maximum lifespan, in the absence of COVID-19, is extended because the 100% natural death threshold is achieved later.

2. Rejuvenating immunity. As we discussed in section "Figuratively, rapamycin rejuvenates immunity" [91], mTOR inhibitors can improve immunity to viral infections, improve immunization and vaccination to some viruses such as flu [92–100, 111, 112, 138]. In addition, viruses such as flu [139] and coronavirus (MERS-CoV) [140] depend on mTOR activity for replication. Currently, however, there are no data regarding COVID-19. Although aimed to evaluate safety, Phase 1 clinical trial "Sirolimus in COVID-19 Phase 1 (SirCO-1)" may reveal anti-viral effects too https://clinicaltrials.gov/ct2/show/NCT04371640.

3. Potential suppression of cytokine storm and hyper-inflammation (Figure 1). As we discussed in the section "Cytokine storm is a hyperfunction", cytokine storm and hyper-inflammation is a main cause of death in COVID-19 pneumonia [36–40, 42, 45, 135, 141–143] Rapamycin, an anti-inflammatory agent, inhibits hyper-functions, cellular senescence and decrease secretion of cytokines ([74, 81, 144]. Rapamycin inhibits the Jak2/Stat4 signaling pathway [145] and reduces IF-γ and TNF-α levels [112]. Rapamycin (Sirolimus) treatment improves outcomes in patients with severe H1N1 pneumonia and acute respiratory failure and was associated with improvement in virus clearance, and shortened ventilator days [146]. Clinical trial "Sirolimus Treatment in Hospitalized Patients With COVID-19 Pneumonia (SCOPE)" has been started https://clinicaltrials.gov/ct2/show/NCT04341675.

Disclaimer

This review is intended for a professional audience, to stimulate new ideas and to aid the global efforts to develop effective treatments for COVID-19 disease. This article does not represent medical advice or recommendations to patients. The media should exercise caution and seek expert medical advice for interpretation, when referring to this article.

CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Figure

Supplementary Figure 1. The mortality risk with COVID-19 superimposed on background annual risk. Annual risk of death (hazard) for England and Wales, 2016–2018, from Office for National Statistics. https://medium.com/wintoncentre/how-much-normal-risk-does-covid-represent-4539118e1196.

Review

Potential mechanisms of hemorrhagic stroke in elderly COVID-19 patients

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ABSTRACT

The novel severe acute respiratory syndrome coronavirus 2 is the causative agent of coronavirus disease 2019, a new human infectious disease. While fever, cough, and respiratory distress are typical first symptoms, a fraction of those affected present instead with neurological symptoms suggestive of central nervous system compromise. This review summarizes the potential contribution of coronavirus disease 2019 to hemorrhagic stroke in the elderly and proposes possible mechanisms. Reports show that the most affected patients have underlying chronic diseases such as hypertension and diabetes, which are two key risk factors for hemorrhagic stroke. Angiotensin-converting enzyme 2 is the main host cell surface receptor interacting with the severe acute respiratory syndrome coronavirus 2 spike glycoprotein to allow viral entry and infection. We speculate that ensuing downregulation of angiotensin-converting enzyme 2 expression may compound the risk conferred by pre-existing comorbidities and critically influence the pathogenesis of hemorrhagic stroke by elevating blood pressure and impairing cerebrovascular endothelial function. Additionally, both age- and/or disease-related immune dysfunction and enhanced catecholamine release secondary to anxiety and stress may also aggravate central nervous system symptoms of severe acute respiratory syndrome coronavirus 2 infection. Thus, assessment of systemic inflammatory biomarkers and tight control of hemodynamic parameters upon admission are crucial to minimize mortality and morbidity in coronavirus disease 2019 patients with central nervous system symptoms suggestive of incipient stroke.

INTRODUCTION

Since the initial report of cases in Wuhan, Hubei Province, China, in December 2019 and January 2020, coronavirus disease 2019 (COVID-19) has been recognized as a new human disease [1]. The causative agent was identified as a novel coronavirus strain, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the Coronavirus Study Group (CSG) [2]. The mortality rate of SARS-CoV-2 is lower than those of Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) [3]. However, SARS-CoV-2 spreads more rapidly than MERS-CoV and SARS-CoV because viral load and infectiousness peak before or around the time of symptom onset, i.e. much earlier than for both MERS-CoV and SARS-CoV [3]. The high transmissibility of SARS-CoV-2 is denoted by a basic reproduction number (R_0) of 3.39 over the whole epidemic period [4]. Moreover, COVID-19 can be transmitted by asymptomatic carriers during the incubation period [4–7], probably because they carry viral loads similar to those of symptomatic patients [8]. Although further studies are warranted to ascertain the epidemiological significance of

the asymptomatic cases, this suggests that asymptomatic transmission may be playing a substantial role in the outbreak [6, 9]. Notably, it is increasingly apparent that in many patients, neurological signs and symptoms are the first manifestations of COVID-19 infection [10, 11]. Although clinical data is not enough, there is still much concern that COVID-19 may increase the risk or trigger the onset of hemorrhagic stroke, especially in older patients. This review summarizes common risk factors for both stroke and COVID-19 severity, and potential mechanisms influencing the onset of hemorrhagic stroke in the elderly.

Identification of SARS-CoV-2 as the causative agent of COVID-19

Zhou et al. provided the first evidence that COVID-19 is associated with a novel coronavirus strain [12]. They used next-generation sequencing and pan-CoV Polymerase Chain Reaction (PCR) primers to determine the cause of the disease in 7 patients with COVID-19 in Hubei, most of whom were seafood market sellers or deliverers [12]. Their findings significantly strengthened the etiological association reported by investigators from India [13], Switzerland [14] and other places in China [15], who had also isolated the novel coronavirus from patients with COVID-19. These efforts, corroborated by statements from Chinese authorities, conclusively led to identification of SARS-CoV-2 as the causative agent of the COVID-19 outbreak [14].

Since its discovery, the sequence of the complete genome of SARS-CoV-2 has been determined [13, 16, 17]. It has ~29,000 nucleotides in length and like other CoVs, it contains at least six open reading frames (ORFs) and several accessory genes [13]. According to Chen et al. [15], the genome sequence of SARS-CoV-2 is 89% identical to the bat SARS-like-CoVZXC21 and 82% identical to the human SARS-CoV [15]. In addition, phylogenetic analysis indicated that two bat SARS-Like CoVs were the nearest homologs of SARS-CoV-2 [13]. Based on genomic structure and phylogenetic analysis, the subfamily Coronavirinae are divided into four genera, namely Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus [13, 18, 19]. Currently, seven human CoVs have been reported: 229E (HCoV-229E), OC43 (HCoV-OC43), NL63 (HCoV-NL63), HKU1 (HCoV-HKU1), SARS-CoV, MERS-CoV, and SARS-CoV-2. HCoV-229E and HCoV-NL63 belong to the Alphacoronavirus genus, while HCoV-HKU1, SARS-CoV, MERS-CoV, and HCoV-OC43 are Betacoronavirus members [18]. SARS-CoV-2 is also classified as a novel Betacoronavirus belonging to the subgenus Sarbecovirus of the Coronaviridae family [13, 15].

The 3' terminal one-third of SARS-CoV-2 genome sequence encodes four structural proteins, namely spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N). Among these, the S gene is particularly important for receptor binding and host specificity [13]. Infection by CoV begins with the binding of the S protein, a surface antigen determining viral tropism, to cell-surface molecules expressed in host cells [20]. As shown in Table 1, host receptors for the seven human CoVs include human aminopeptidase N (CD13) for HCoV-229E [21]; 9-O-acetylated sialic acid for HCoV-OC43 [22]; angiotensin-converting enzyme 2 (ACE2) for SARS-CoV [22]; ACE2 for HCoV-NL63 [23, 24]; 9-O-acetylated sialic acid for HCoV-HKU1 [25, 26]; dipeptidyl peptidase 4 (DPP4) for MERS-CoV [27]; and ACE2 by SARS-CoV-2 [18].

Potential impact of COVID-19 on hemorrhagic stroke in the elderly

At presentation, the most common symptoms in COVID-19 patients are fever, dry cough, and shortness of breath, whereas headache, diarrhea, and vomiting are more rare [3, 28–30]. However, early neurological symptoms (e.g. headache, epilepsy, and unconsciousness), without obvious respiratory symptoms, have been reported for numerous COVID-19 patients [10, 31]. A 2005 case report by Xu et al. provided the first direct evidence that SARS-CoV has the ability to infect the central nervous system (CNS) [32]. A predicted cDNA fragment specific for SARS-CoV was amplified by nested RT-PCR from Vero-E6 cell cultures inoculated with a brain tissue extract from a symptomatic patient, and presence of enveloped virus particles, 80–90 nm in diameter, was found by transmission electronic microscopy [32]. Shortly before this finding, another study had reported the case of a 32-year-old woman with SARS whose cerebrospinal fluid tested positive for SARS-CoV [33]. These findings were further supported by experiments in mice that demonstrated the ability of various CoVs to cause CNS infections [34–36]. Indeed, SARS-CoV-2 shares similar characteristics with SARS-CoV, and both anecdotal and statistical data indicate that neurologic symptoms are not common in COVID-19 patients [10]. Since it is well known that cerebral hemorrhage may result from viral infection of the CNS compromising the neurovascular unit [37–40], available evidence strongly suggest that SARS-CoV-2 infection may greatly increase the incidence of hemorrhagic stroke, especially in at-risk patients.

Shared risk factors

Hypertension is the most important risk factor for cerebral hemorrhage [41, 42]. Of note, for the 138 COVID-19

Table 1. Human coronavirus species and their receptors.

Coronavirus species	Discovery year	Cellular receptor
HCoV-229E	1966	Human aminopeptidase N (CD13)
HCoV-OC43	1967	9-O-acetylated sialic acid
SARS-CoV	2003	ACE2
HCoV-NL63	2004	ACE2
HCoV-HKU1	2005	9-O-acetylated sialic acid
MERS-CoV	2012	DPP4
SARS-CoV-2	2019	ACE2

confirmed cases analyzed by Wang et al. [30], 43 patients (31.2%) were hypertensive, a proportion that reflects, relative to other diseases, the higher susceptibility to SARS-CoV-2 infection conferred by hypertension. Similar results were recently reported by both Guan et al. [26] and the Novel Coronavirus Pneumonia Emergency Response Epidemiology Team [50]. SARS-CoV-2 infection in humans is mediated by binding of the receptor-binding domain (RBD) of the viral S glycoprotein to ACE2 receptors in host cells, and this in turn may lead to downregulation of ACE2 expression [20, 43]. Since reduced ACE2 expression implies increased Ang II availability, COVID-19 patients with pre-existing hypertension may experience large blood pressure (BP) fluctuations, making them especially susceptible to hemorrhagic stroke episodes.

There is a close relationship between systolic BP variability (SBPV) and poor prognosis of cerebral hemorrhage. Divani et al. reported that elevated SBPV in the first 24 h of admission was related to unfavorable in-hospital prognosis in patients with intracerebral hemorrhage (ICH) [44]. Since BP elevations resulting from downregulation of ACE2 expression may occur after SARS-CoV-2 infection, higher SBPV may be present on admission in hemorrhagic stroke patients affected by COVID-19. Therefore, the management of BP might require additional attention during the hyper-acute and acute hemorrhagic stroke phases in COVID-19 patients, as both high absolute BP levels and high BP fluctuations are main determinants of cerebral hemorrhage prognosis.

Diabetes is also an independent risk factor for hemorrhagic stroke [42]. Huang et al. reported that among 41 patients with laboratory- confirmed SARS-CoV-2 infection, 8 (20%) cases had diabetes; this again represents a higher proportion of comorbidity cases compared with other diseases [45]. Indeed, available data suggest that among COVID-19-confirmed cases with underlying chronic diseases, diabetes ranks second after hypertension [29, 45].

Elevated plasma D-dimer levels were associated with increased risk of hemorrhagic stroke [41]. Recently, Chen et al. conducted a retrospective, single-center study including 99 patients with COVID-19 and found elevated D-dimer levels in 36 patients (36%) [28]; however, mortality rate for this subgroup was not reported. Meanwhile, in a similar study assessing 191 COVID-19-confirmed patients, D-dimer greater than 1 mg/L on admission was associated with significantly increased odds ($p = 0.0033$) of in-hospital death [46]. Of note, a recently posted pre-print article reporting on 248 consecutive COVID-19 cases in Wuhan found D-dimer elevation ($\geq$ 0.50 mg/L) in 74.6% (185/248) of the patients. D-dimer levels correlated with disease severity, and values >2.14 mg/L predicted in-hospital mortality with a sensitivity of 88.2% and specificity of 71.3% [47].

Surprisingly, two recent studies have reported an association between SARS-CoV-2 infection and the incidence of stroke [31, 48]. A single center, retrospective, observational study by Li et al reported a 5% risk of ischemic stroke and a 0.5% risk of cerebral hemorrhage in 221 patients with SARS-CoV-2 infection from Wuhan, China [48]. In this cohort, patients with new onset stroke are obviously older, more likely to present with severe COVID-19 and have the above risk factors including hypertension, diabetes and elevated plasma D-dimer levels [48]. Another study of 214 patients reported 5 (5.7%) developed acute cerebrovascular diseases including 4 (4.6%) patients with ischemic stroke and 1 (1.1%) with cerebral hemorrhage in severe patients with COVID-19 [31]. Nevertheless, further studies including larger sample sizes, more exhaustive assessment of patients' clinical histories, and additional molecular analysis are clearly needed to determine in which cases stroke is directly triggered by SARS-CoV-2 infection, or it occurs coincidentally [49].

Convergence of inflammatory mediators

Inflammatory monocyte-macrophages (IMMs) and neutrophils are major sources of cytokines and

chemokines involved in the pathogenicity of SARS-CoV-2 [50]. Some of these factors represent classical inflammatory biomarkers associated with secondary brain injury following cerebral hemorrhage and may have prognostic value in hemorrhagic stroke patients [51–55]. Lattanzi et al. recently reviewed available evidence pointing to the relevance of assessing the neutrophil-to-lymphocyte ratio (NLR) to determine inflammatory status in ICH patients [54]. In turn, newer studies confirmed NLR's predictive value for prognosis of ICH [56, 57]. Neutrophil-derived matrix metalloproteinases (MMPs) are upregulated after acute ICH, contributing significantly to tissue destruction and activation of neuro-inflammatory cascades [54]. Accordingly, research suggests that it may be possible to mitigate brain damage by early, short-term inhibition of MMPs [53]. Napoli et al. reported that increased concentrations of serum C-reactive protein (CRP), a marker of inflammation, may be an independent predictor of ICH outcome [52]. Nevertheless, it should be considered that interethnic genomic differences may influence CRP status and its predictive values on different stroke phenotypes. Another marker, namely serum neutrophil gelatinase-associated lipocalin (NGAL), a member of the lipocalin family of proteins associated with transport of small hydrophobic molecules, plays an important role in the innate immune response and has also been identified as an independent predictor for outcome following hemorrhagic stroke [51]. Given that these inflammatory biomarkers have been associated with both SARS-CoV-2-related cytopathic effects and hemorrhagic stroke outcome, it would be worthwhile to explore which changes in inflammatory biomarkers occur after hemorrhagic stroke and their predictive value in patients with and without COVID-19. This would allow to better define reliable indices of hemorrhagic stroke severity and functional recovery.

Substantially reduced peripheral lymphocyte counts were evident in severe COVID-19 cases [28–30, 45, 58]. Xu et al. reported pathological findings of lung, liver, and heart biopsies, as well as blood cell analysis, from a patient who died of COVID-19 [59]. The findings showed infiltration of IMMs in the lung, whereas peripheral CD4 and CD8 T cells were reduced in number but overactivated. The authors suggested that severe immune injury in this patient was due to overactivation of T cells, manifested by increased representation of highly pro-inflammatory CCR6+ Th17 CD4 T cell subsets and enhanced cytotoxic capacity of CD8 T cells. These data suggest that although lymphopenia is a common feature in patients with COVID-19, it may be paralleled by a pro-inflammatory phenotypic switching in T cell subsets that could be critically associated with disease severity and mortality [9, 59].

In addition, it was suggested that like SARS-CoV, SARS-CoV-2 also acts on lymphocytes in the respiratory mucosa, leading to a systemic "cytokine storm" concomitant with reduced peripheral blood lymphocytes which impairs cellular immune function [28]. This effect will be clearly potentiated by immune senescence, a well-described phenomenon in many middle-aged and elderly people [60], and aggravated by underlying conditions such as hypertension, diabetes, and cerebrovascular disease. This evidence points to worsened outcomes for patients with COVID-19 and cerebral hemorrhage comorbidity.

Possible mechanisms underlying COVID-19 effects on hemorrhagic stroke in the elderly

ACE2 expression

Soon after the COVID-19 outbreak, investigations confirmed that the ACE2 receptor, abundantly expressed in lung alveolar epithelial cells, enables SARS-CoV-2 entry into host cells through the RBD of the virus' S glycoprotein [12, 61, 62]. The RBD that confers ACE2 binding specificity is part of the S1 subunit of the large ectodomain of the S protein. The ectodomain contains also an S2 subunit, which mediates fusion between the viral and host cell membranes [61]. A ternary structure of the RBD of SARS-CoV-2 was obtained by molecular simulation, revealing that the structure is essentially superimposable (72% identity) to that of SARS-CoV, except for a flexible loop with CNGVEGFNC that replaces the rigid loop with CTPPALNC present in SARS-CoV [61]. Further analysis indicated that the unique F486 residue in the flexible loop can penetrate deep into a hydrophobic pocket in ACE2 formed by F28, L79, Y83, and L97 [61].

ACE2 was identified in 2000 as a homolog of the angiotensin-converting enzyme (ACE), although with different substrate specificity [63]. ACE2 primarily acts on angiotensin II (Ang-II), a major bioactive peptide [43], to generate the vasodilatory heptapeptide Ang-(1-7), while ACE acts on angiotensin I (Ang-I) to generate Ang-II [43]. ACE2 counterbalances the vasopressor effect of the ACE/Ang-II/AT1 axis by stimulating vasodilation through the ACE2/Ang-(1-7)/MasR axis [64, 65]. Demonstrating the adversarial relationship between ACE and ACE2, Crackower et al. reported that heart function is impaired in *ace2*-deficient mice, and this effect can be rescued by ablation of ACE expression [66]. ACE2 expression is widely distributed across different cells and tissues. To date, it was identified in epithelial cells of the oral mucosa [62], pulmonary alveolar type II cells [67–69], esophagus upper and stratified epithelial cells, absorptive enterocytes from ileum and colon [69],

cholangiocytes [70], myocardial cells, kidney proximal tubule cells, and bladder urothelial cells [46]. In addition, ACE2 expression has also been detected in vascular endothelial and smooth muscle cells [71] and in some neurons [43, 64, 71–73], including those in the cardio-respiratory center of the brainstem [43]. The widespread expression of ACE2 is thus consistent with the reported effects of SARS-CoV-2 on multiple tissues and organs. Binding of SARS-CoV-2 to ACE2 receptors in brain blood vessels may trigger the release of proinflammatory cytokines and chemokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF), leading to activation and extravasation of lymphocyte subsets, neutrophils, and macrophages with subsequent neurological manifestations [74]. On the other hand, neuronal ACE2 expression could also be a significant factor in COVID-19 cases associated with cerebral hemorrhage. Research on the 2003 SARS outbreak concluded that downregulation of ACE2 expression occurred in infected organs, including lungs [75], kidney [43], heart [76], liver [43], and brain [43]. Similarly, a study by Chen et al. reported decreased ACE2 expression in the lungs of COVID-19 patients [61].

Downregulation of ACE2 expression may increase risk of hemorrhagic stroke in several ways: i) ACE2 deficiency in the brain may impair endothelial function in cerebral arteries, leading to a 4-fold elevation in the risk of cerebrovascular events, including hemorrhagic stroke [77]; ii) Downregulation of ACE2 expression may increase local Ang-II levels, which acting on AT1 receptors may rise BP and facilitate hypertrophy and fibrosis [64]; iii) Decreased ACE2 expression would also lead to reduced generation of Ang (1-7) and depression of Ang (1-7)/MasR signaling, thus preventing its vasodilatory, growth inhibiting, and antifibrotic actions [64, 78] (Figure 1).

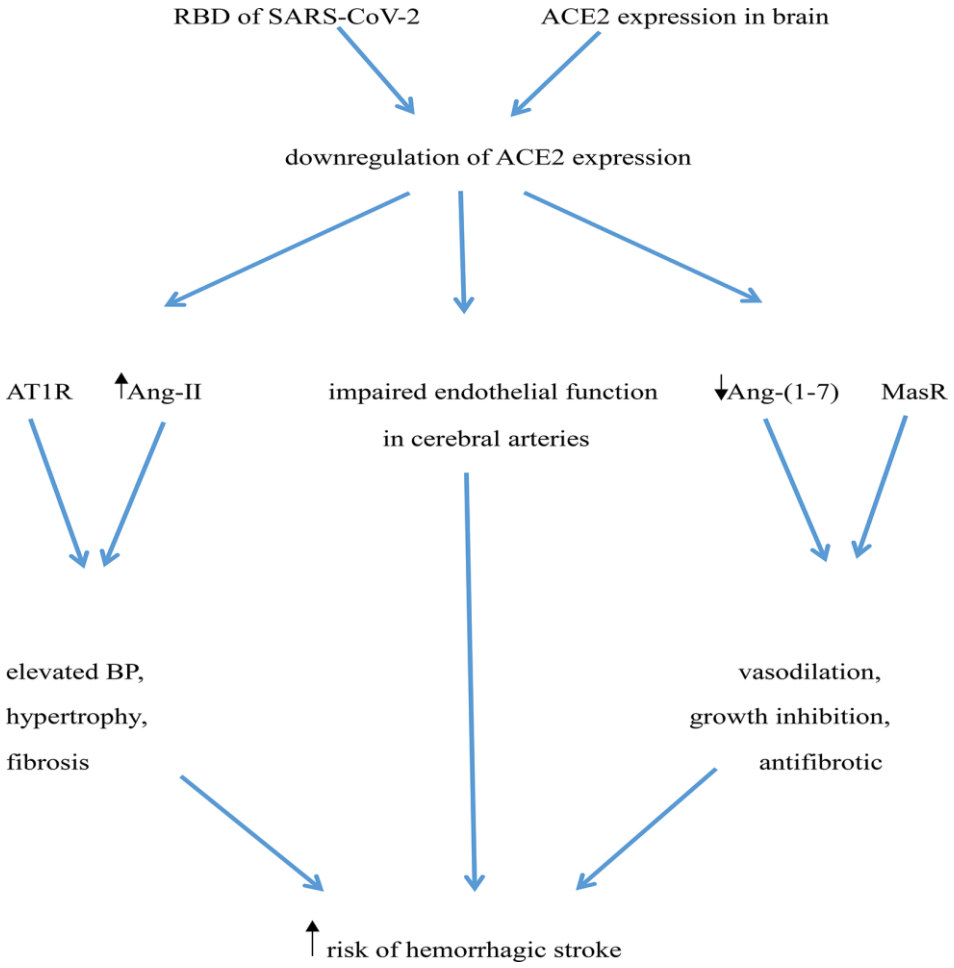

Figure 1. Potential mechanisms mediating increased risk of hemorrhagic stroke in COVID-19 patients. The RBD of SARS-CoV-2' spike protein interacts with ACE2, leading to ACE2 downregulation. ACE2 deficiency impairs endothelial function in cerebral arteries and determines an increase in Ang-II levels, which elevates BP through activation of AT1 receptors (AT1R). Simultaneously, reduced ACE2 leads to a decrease in Ang (1-7) levels, weakening its vasculo-protective effects mediated by Mas receptor (MasR) activation.

It calls for special attention the fact that COVID-19 may exacerbate any underlying hypertension and put patients at higher risk for hemorrhagic stroke. Several mechanisms may contribute to hemorrhagic stroke in hypertensive patients infected with SARS-CoV-2. These include fibrinoid necrosis, promoted by increased vascular pressure [79], and extensive structural and functional alterations in endothelium and smooth muscle in intracerebral arteries, often aggravated by atherosclerosis, especially in the elderly [80].

Endothelial dysfunction at the blood-brain barrier

The BBB is a semi-permeable structure consisting of a well-defined basement membrane and endothelial cells bound by tight junctions that limit the passage of macromolecules into the brain parenchyma. The BBB lies in close apposition to brain cell types, including pericytes, astrocytes, microglia, and neurons, and is especially susceptible to damage by both hypertension and diabetes [81, 82]. Xu et al. reported that a chemokine, i.e. the monokine/Mig/CXCL9, induced by IFN-g mostly in glial cells, might be involved in the brain immunopathology triggered by SARS [32]. Elevated Mig levels in the blood are correlated with brain infiltration of $CD68^+$ monocytes/macrophages and $CD3^+$ T lymphocytes in the brain [32]. Given the similarities between SARS-CoV-2 and SARS-CoV, this mechanism deserves further exploration as it may lead to therapeutic strategies to prevent or attenuate brain pathology in COVID-19 patients.

The BBB is a dynamic and complex structure that helps maintain brain homeostasis and compensates fluctuations in the systemic circulation [83]. Expression of ACE2 in endothelial cells of the BBB may be a gateway for SARS-CoV-2 entry into the brain [83]. Moreover, the ensuing ACE2 down-regulation, compounded by age-related ACE2 deficiency in older patients, might further increase endothelial dysfunction and risk of ICH [77]. More studies are needed to ascertain the impact of ACE2 expression at the BBB and its effect on SARS-CoV-2-mediated CNS symptoms, particularly ICH.

Immunity and inflammation

There is accruing evidence that viral CNS infections may cause hemorrhage stroke [37, 39, 84]. The pathogenesis may involve cytokine, chemokine, and protease actions increasing BBB permeability, and damage and/or demise of the neurovascular unit during the necrotizing process [37]. Although the specific mechanisms remain unclear, it is obvious that the type and extent of the immune response triggered by the SARS-CoV-2 determine symptoms severity. A recent study from Anderson et al. revealed that bats, the most likely source of the novel SARS-CoV-2, have evolved a highly specific innate immune response characterized by a large expansion of the type I interferon gene family [85]. While this may clarify the basis of bats' immune resistance to SARS-CoV-2, there are still many open questions about the mechanism(s) mediating immune defense against CoV-2 in humans. In this regard, it will be very valuable to ascertain and compare immunological (i.e. T cell status, cytokine expression) and genetic (i.e. HLA haplotypes) profiles between symptomatic and asymptomatic COVID-19 patients, which have shown to influence responses to recent viral outbreaks [86]. This should allow predicting why high viral replication early in the course of infection would lead to the "cytokine storm" characteristic of severe COVID-19 cases [50].

Anxiety and stress

The current COVID-19 outbreak has undoubtedly increased anxiety, fear, and stress in many people around the world. Social stress, anxiety, and depression are potential risk factors for hemorrhagic stroke, therefore adequate management of these conditions is a key aspect in primary prevention of cerebrovascular disease [87, 88]. The locus coeruleus, a structure in the brainstem, consists mainly of adrenergic neurons that play a crucial role in the genesis of anxiety by releasing catecholamines that critically influence the stress response [89]. Indeed, research has shown that excessive adrenergic stimulation by catecholamines could lead to severe vasospasm and microcirculation disturbances, thus increasing the risk of hemorrhagic stroke [90].

Aging

Although people of all ages can be infected, middle-aged and elderly people are most severely affected by COVID-19, suggesting that aging is a prominent risk factor. Accordingly, it seems logical that the risk of hemorrhagic stroke in COVID-19 patients would increase significantly with age, although a recent article by Oxley et al reported COVID-19-related stroke episodes occurred in five young patients [91]. Based on available evidence, Camacho et al. concluded that age is a strong risk factor for hemorrhagic stroke, the deadliest stroke type [92]. Their study highlights several age-related processes and pathologies, including cerebral microembolism, white matter lesions, vascular basement membrane thickening, and increased BBB permeability, which determine endothelial damage, changes in vessel elasticity, and ensuing fluctuations in blood flow and pressure that cause loss of autoregulation and increase the risk of ICH [92].

Research on both animal models and humans indicated that aging is closely associated with endothelial dysfunction and oxidative stress in cerebral arteries [93–97]. Moreover, studies in rodents suggested that these deleterious effects can be promoted by alterations in the RAS system in aged brains. Specifically, works by Pena-Silva et al. [77] and Labandeira-Garcia et al. [98] suggested that age-related downregulation of ACE2 and AT2 expression may promote vascular dysfunction because the anti-inflammatory/anti-oxidant effects of AngII/AT2 and Ang1-7/MasR signaling are overridden by pro-inflammatory/pro-oxidant signaling through the AngII/AT1 axis. Although confirmatory data in humans is still needed, these studies provide strong support for the overall concept that brain RAS activity has a critical effect on cerebrovascular function during aging and may contribute to endothelial dysfunction, oxidative stress, and risk of hemorrhagic stroke.

CONCLUSIONS

COVID-19 emerged as a new human infectious disease caused by SARS-CoV-2, a novel coronavirus. A significant proportion of COVID-19 cases, especially older patients, manifest neurological, rather than respiratory, symptoms on admission and may be at higher risk of developing cerebral hemorrhage. The mechanisms by which COVID-19 may promote hemorrhagic stroke in the elderly are not yet clear, but may involve downregulation of ACE2 expression secondary to SARS-CoV-2 binding to neurovascular ACE2 receptors. This might increase Ang-II expression and decrease Ang (1-7) expression, leading to severe BP elevation. increased BBB permeability, and extensive alterations in endothelium and smooth muscle function in intracerebral arteries. The patients most gravelly affected by COVID-19 have underlying hypertension disease, which greatly increases the risk of hemorrhagic stroke. Since SBPV in the first 24 h of admission predicts cerebral hemorrhage outcome, special attention should be paid to management of BP in at-risk COVID-19 patients. Predisposing factors may be compounded in COVID-19 patients by the inability of their immune system to efficiently prevent or counteract the pernicious effects of the pro-inflammatory cytokines released upon infection. In addition, anxiety and stress may lead to enhancement of adrenergic tone and trigger vasospasm and microcirculation disturbances, further contributing to cerebrovascular symptoms. In light of this, exploring the changes in inflammatory biomarkers occurring in COVID-19 patients with CNS symptoms suggestive of incipient stroke would aid diagnosis and treatment to avoid irreversible outcomes.

CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

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Research Perspective

Microdose lithium reduces cellular senescence in human astrocytes - a potential pharmacotherapy for COVID-19?

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ABSTRACT

Cell senescence is a process that causes growth arrest and the release of a senescence associated secretory phenotype (SASP), characterized by secretion of chemokines, cytokines, cell growth factors and metalloproteases, leading to a tissue condition that may precipitate cancers and neurodegenerative processes. With the recent pandemic of coronavirus, senolytic drugs are being considered as possible therapeutic tools to reduce the virulence of SARS-CoV-2. In the last few years, our research group showed that lithium carbonate at microdose levels was able to stabilize memory and change neuropathological characteristics of Alzheimer's disease (AD). In the present work, we present evidence that low-dose lithium can reduce the SASP of human iPSCs-derived astrocytes following acute treatment, suggesting that microdose lithium could protect cells from senescence and development of aging-related conditions. With the present findings, a perspective of the potential use of low-dose lithium in old patients from the "high risk group" for COVID-19 (with hypertension, diabetes and chronic obstructive pulmonary disease) is presented.

INTRODUCTION

Since the beginning of the coronaviral burst in December 2019, SARS-CoV-2 has widely spread in more than 50 countries around the world. The increased fatality of COVID-19 amongst older versus younger individuals has become more evident [1].

The aging process is characterized by increased levels of oxidative stress and chronic inflammation contributing to many age-related pathologies [2, 3]. It has been suggested that increases in inflammation may be promoted by the release of pro-inflammatory and other factors from senescent cells as part of what is known as the senescence-associated secreted phenotypes (SASP) [4, 5], which includes increase in senescence-associated β-galactosidase (SA-β-gal) activity, increased levels of the cyclin-dependent kinase (CDK) inhibitors p16 and p21, and pro-inflammatory cytokines including IL-6, IL-8 and IL-1α [6]. The SASP promotes the development of an inflammatory environment leading to tissue frailty contributing to many diseases including cancer, chronic obstructive pulmonary disease, diabetes and neurodegenerative diseases [5, 7–9].

In a recent research perspective, the use of senolytic drugs was suggested for the treatment and prevention of COVID-19 [10]. These drugs induce the apoptosis of senescent cells and reduce production of the SASP, reducing vulnerability to chronic diseases [11]. The authors described how many FDA-approved drugs including azithromycin, doxycycline and chloroquine have been shown to act as senolytics.

Recently our research group and others have identified additional compounds that may also inhibit the inflammation associated with aging and neuro-degenerative diseases. Lithium in microdose [12, 13], for example, was shown to enhance the maintenance of memory, decrease the density of senile plaques, and reduce neuronal cell loss both clinically and pre-clinically. A recent review highlighted the potential use of lithium as candidate for therapy of COVID-19 along with chloroquine or other drugs [14]. It is possible that one of the mechanisms by which microdose lithium may be eliciting its protective effects is via preventing inflammatory SASP induction.

In order to test this, human iPSCs-derived astrocytes were seeded in cell culture plates pre-coated with matrigel (Corning Matrigel Matrix, Tewksbury, MA, USA) and treated with different concentrations of Li_2CO_3 for 24 h and 48h. Concentrations up to 100 μM showed no toxicity in the astrocytes as determined by the MTT assay (Figure 1A, 1B). Based on this analysis and our previous pre-clinical studies [13], three

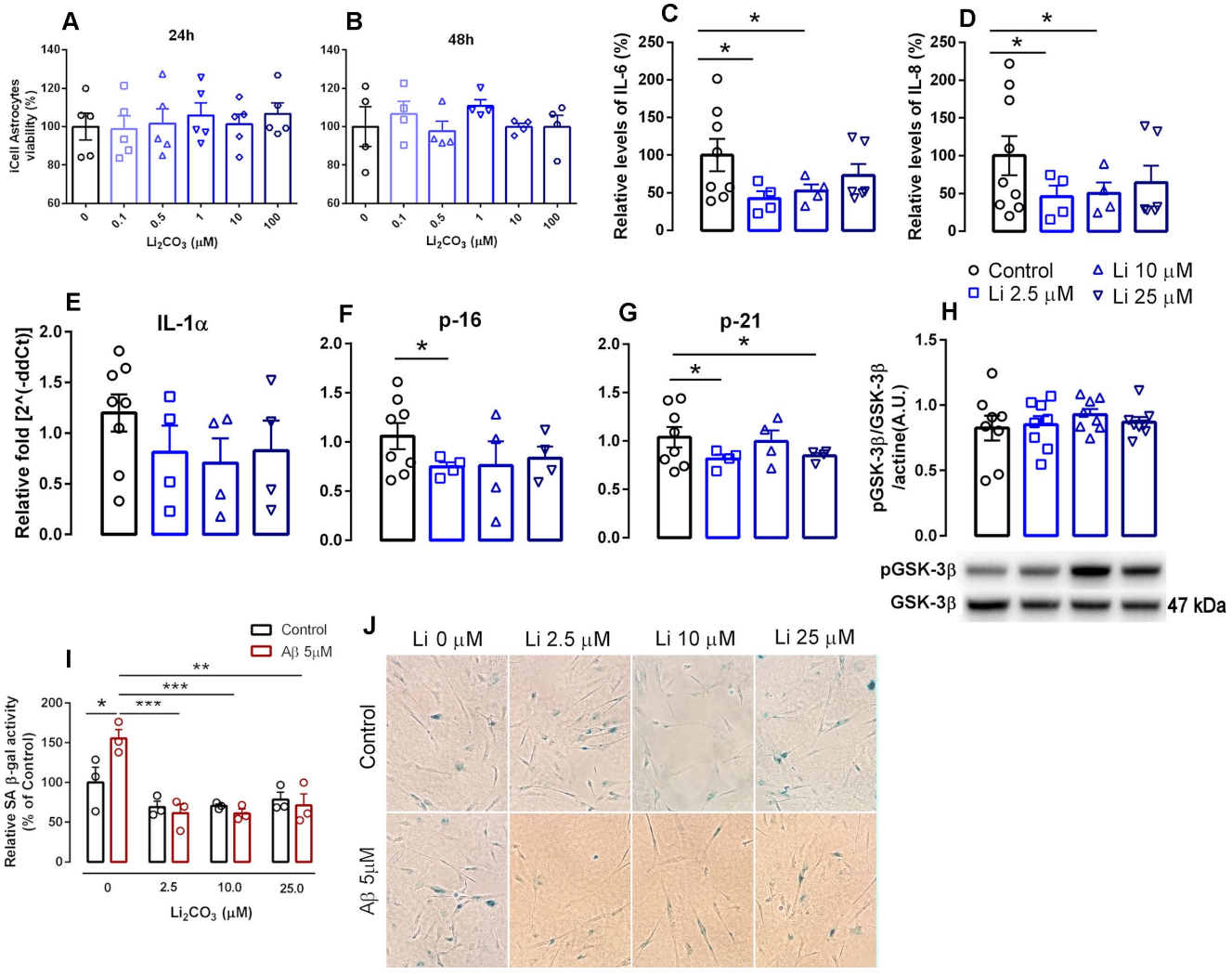

Figure 1. Effects of increasing lithium concentrations on cell viability and induction of senescence and the SASP in human iPSC-derived astrocytes. (**A**, **B**) cell viability measured by the MTT assay. Data are expressed as individual points, mean and SEM; performed in triplicate. (**C**, **D**) Relative levels of secreted IL-6 and IL-8. Conditioned media was collected 24h following induction of senescence with 1% FBS and data was normalized to cell number. (**E**–**G**) RNA isolated from human iPSCs-derived astrocytes was analyzed for IL-1α, $p16^{INK4a}$ and p21 mRNA levels by qPCR. Transcripts were normalized to actin and are shown as fold change over control levels. (**H**) GSK-3β activation measured as the proportion of phosphorylated and total GSK-3β. Data are expressed as individual points, mean and SEM. (**I**) SA β-gal in iPSC-derived astrocytes in the absence and presence of Aβ with increasing concentrations of lithium. Values show relative amounts of SA β-gal positive cells in three independent experiments. (**J**) Representative panels of SA-β gal staining under various treatment conditions. *p<0.05; **: p<0.01; ***:p < 0.001. For (**C**–**H**), data are expressed as individual points, mean and SEM of 4-5 independent experiments.

concentrations (2.5 μM, 10 μM and 25 μM) were selected for subsequent experiments; treatments were maintained for 24 h.

Concentrations of the hallmark SASP factors such as IL-6 and IL-8 were measured in the conditioned culture media using ELISA kits. Treatment with 2.5 μM and 10 μM Li_2CO_3 promoted a 57.6% ($P<0.05$) and 47.5% decrease ($P<0.05$), respectively, in the release of IL-6 and a 54.2% ($P<0.05$) and 49.6% ($P<0.05$) decrease in the release of IL-8 compared to untreated controls. Incubation of 25 μM Li_2CO_3 however did not alter the release of either cytokine (Figure 1C, 1D). These data are in agreement with recent studies from our lab and others showing anti-inflammatory properties of low-dose lithium as evidenced by reductions in pro-inflammatory cytokine density [15], Toricelli et al. (Toricelli M, Evangelista SR, Buck HS, Viel TA. Microdose lithium treatment reduced inflammatory factors and neuro-degeneration in organotypic hippocampal culture of old SAMP-8 mice. Submitted to Cellular and Molecular Neurobiology, March 2020). These results are of particular interest as a very recent report shows strong association of elevated IL-6 levels with respiratory failure in COVID-19 infected patients [16].

Similar expression profiles for the senescence markers p16 and p21 and the SASP factor IL-1α were also observed following treatment with Li_2CO_3 compared with untreated controls. 2.5 μM Li_2CO_3 significantly reduced expression of p16 and p21 and 25 μM Li_2CO_3 also reduced p21 expression. For IL-1α, however, the decrease in expression with Li_2CO_3 did not reach statistical significance (Figure 1E–1G).

Interestingly, positive effects of acute treatment with low dose lithium seems not to act via known mechanism of lithium (inhibition of GSK-3β activation) [17], as no differences in phosphorylation of Ser9-GSK-3β were observed following acute treatment with low concentrations of lithium (Figure 1H). In a previous study, treatment of WI-38 fibroblasts with 20 mM lithium chloride reduced GSK3-dependent increases in p53 and p21 nuclear levels [18], indicating that microdose lithium used in the present work has different cell effects than lithium in higher concentrations.

We further confirmed the antisenescence properties of lithium using an established amyloid β-induced senescence model [19]. We observed that low dose of Li_2CO_3 including 2.5 μM, 10 μM and 25 μM significantly suppressed amyloid-β (Aβ) increased SA β-gal staining in astrocytes, a hallmark of cellular senescence (Figure 1I, 1J). Overall our results highlight the potential of microdose lithium (a safe FDA approved drug) in suppressing cellular senescence.

Lithium carbonate is still widely used as a therapeutic for bipolar depression [20]. Recently, low-dose lithium has begun to be considered as a disease-modifying strategy for some neurodegenerative diseases [13, 15, 21–24]. Its neuroprotective effects in pre-clinical models may be due to its anti-inflammatory properties [15, 25], Toricelli et al.

This work was originally initiated by the authors to explore the beneficial effects of low-dose lithium in brain aging and age-related neurodegenerative diseases. However, in face of the recent COVID-19 pandemic and the urgency to identify anti-viral drugs, including the potential use of FDA-approved drugs displaying senolytic properties, we believe that these findings will be important to broaden the research community therapy possibilities. The fact that microdose lithium suppresses IL-6 and recent finding correlating IL-6 level with severity of the diseases in COVID-19 patients provides a strong rationale for why lithium treatment should be tested as treatment. In this way, low-dose lithium may constitute a novel potential therapeutic to reduce the virulence of SARS-CoV-2. It is important to highlight that no side effects were verified in old people with the use of low-dose lithium [12, 26].

MATERIALS AND METHODS

Culture of human iPSCs-derived astrocytes

Commercially available human iPSC-derived astrocytes (iCell, # 01434) were used for our studies. Cells were seeded at 1 x 10^4 cells/cm^2 in cell culture plates pre-coated with matrigel (Corning Matrigel Matrix, Tewksbury, MA, USA) and cultured to 70-80% confluence. Cells were then cultured at 37°C and 5% CO_2 in complete DMEM media (supplemented with N2 supplement and 2% penicillin/streptomycin) containing 10% fetal bovine serum (FBS). Cells were grown in physiological (3%) oxygen concentrations as previously described [27, 28]. Cells were incubated with concentrations of up to 1 mM Li_2CO_3 for 24-48 hrs and toxicity verified by the MTT assay.

Determination of IL-6 and IL-8 levels

Following treatment with 2.5 μM, 10 μM and 25 μM Li_2CO_3 for 24h, culture medium was prepared by washing cells once in PBS followed by incubation in DMEM with 1% FBS for 24 hr. The medium was collected and stored at -80 °C. Cell numbers were determined with an automated cell counter (Thermo Scientific). ELISA assays were performed using an alphaLISA IL-6 or IL-8 Immunoassay Research Kit (Perkin Elmer) following the manufacturer's

instructions. Data was normalized to cell number and expressed as picograms per 1,000 cells.

RT-qPCR analysis

Total RNA was prepared from human astrocytes using a Direct-zol RNA MiniPrep Kit (Genesee Scientific). Integrity of RNA was verified using a nanodrop system. RT-qPCR was performed using the Universal Probe Library System (Roche, South San Francisco, CA) with the following primers and probes:

IL-1a: forward (FW) 5′-ggttgagtttaagccaatcca-3′; reverse (RV) 5′-tgctgacctaggcttgatga-3′

$p16^{INK4a}$: FW 5′-cggaaggtccctcagacatc-3′; RV 5′-aaactacgaaagcggggtgg-3′

p21: FW 5′-ccagcatgacagatttctaccac-3′; RV 5′-cttcctgtgggcggattagg-3′

actin: 5′-ACCGAGCGCGGCTACAG-3′; 5′-CTTAATGTCACGCACGATTTCC-3′

Determination of GSK-3β activation

For protein extractions, astrocytes were collected and homogenized in lysis buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 10,000g for 10 min at 4 °C and supernatants collected. Total protein concentration was determined using the Bradford assay [29]. Proteins (10 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Membranes were blocked with TBST containing 5% non-fat milk for 1 hour and then incubated with the primary antibody GSK-3β (Cell Signaling Technology, 9315, 1:1000) and phospho-GSK-3β (Cell Signaling Technology, 5558, 1:1000). Bands were detected using an ECL system (EMD Millipore) and quantified densitometrically. Actin (1:2000) was used as a loading control.

Senescence-associated-β-galactosidase (SA-β-gal) assay

SA-β-gal staining was performed according to the method described by Bhat and co-workers [19]. Cells were plated at 1 x 10^4 cells/cm^2 in chamber slides and treated or not with 5 µM amyloid-β for 2 h. The medium was then replaced with fresh medium containing 0 µM, 2.5 µM, 10 µM or 25 µM Li_2CO_3. This treatment was maintained for three days after which cells were assessed for SA-β-gal activity. Positive (blue) cells were expressed as a percentage of total cell number.

Statistical analysis

Data were expressed as means ± SEM and analyzed with the Graph Pad Prism program (GraphPad Software, San Diego, CA, version 6). Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. In all analyses, only probability values (*P*) less than 0.05 were considered statistically significant.

Data availability statement

All data generated or analyzed during this study are included in this published article.

AUTHOR CONTRIBUTIONS

TAV, SC and JKA conceived and designed the experiments; TAV and AR performed the experiments; TAV, SC, MC and HSB performed data acquisition and analysis; TAV, MC, HSB and JKA contributed intellectually to the paper. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that there are no conflicts of interest in the present work.

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Research Paper

Infection with SARS-CoV-2 causes abnormal laboratory results of multiple organs in patients

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ABSTRACT

Aim: To evaluate the clinical value of abnormal laboratory results of multiple organs in patients with coronavirus disease 2019 (COVID-2019) and to help clinicians perform correct treatment.

Results: Elevated neutrophil-to-LYM ratio (NLR), D-dimer(D-D), interleukin (IL)-6, IL-10, IL-2, interferon-Y, and age were significantly associated with the severity of illness. However, significant and sustained decreases were observed in the LYM subset ($p<0.05$). D-D, T cell counts, and cytokine levels in severe COVID-19 patients who survived the disease gradually recovered at later time points to levels that were comparable to those of mild cases. Second, D-D increased from 0.5 to 8, and the risk ratio increased from 2.75 to 55, eventually leading to disseminated intravascular coagulation. Moreover, the acute renal function damage occurred earlier than abnormal heart and liver functions ($p<0.05$).

Conclusions: The degrees of lymphopenia and proinflammatory cytokine storm were higher in severe COVID-19 patients than in mild cases. The degree was associated with the disease severity. Advanced age, NLR, D-D, and cytokine levels may serve as useful prognostic factors for the early identification of severe COVID-19 cases.

Methods: Peripheral blood samples were collected from 93 confirmed COVID-19 patients. The samples were examined for lymphocyte (LYM) subsets by flow cytometry and cytokine profiles by specific immunoassays. The receiver operating characteristic curve was applied to determine the best diagnostic thresholds for laboratory results, and principal component analysis was used to screen the major risk factors. The prognostic values were assessed using the Kaplan–Meier curve and univariate and multivariate COX regression models.

INTRODUCTION

Coronavirus is a large virus family known to cause multiple system infections in various animals and mainly respiratory tract infections, such as severe acute respiratory syndrome (SARS) [1–3] and the Middle East respiratory syndrome (MERS) [4], in humans. Although the clinical characteristics of coronavirus disease 2019 (COVID-19) have been broadly defined [5], an outline of the most representative laboratory abnormalities observed in patients with COVID-2019 is still incomplete [6–7].

Laboratory medicine plays an essential role in the early detection, diagnosis, and management of numerous diseases [8]. COVID-2019 is no exception to this rule. Nevertheless, the role of laboratory diagnostics extends beyond etiological diagnosis and epidemiologic surveillance, whereby in vitro diagnostic tests are commonly used for assessing disease severity, defining the prognosis, patient follow-ups, treatment guide, and therapeutic monitoring [9]. Diagnostics identify the defining laboratory results and clinical characteristics with high precision and unravel the risk factors associated with mortality.

Lymphopenia and inflammatory cytokine storm are typical laboratory abnormalities observed during highly pathogenic coronavirus infections, such as SARS coronavirus (SARS-CoV) and MERS coronavirus (MERS-CoV) infections; these abnormalities are believed to be associated with disease severities [10]. Severe inflammatory responses contribute to the weakening of the adaptive immune response, which results in an imbalanced immune response and COVID-19. Therefore, circulating biomarkers that can represent the status of inflammation and immunity are recognized as potential predictors for the prognosis of COVID-19 patients [11]. Recent studies have also reported decreases in the lymphocyte (LYM) counts in the peripheral blood and increases in serum inflammatory cytokine levels in COVID-19 patients [12]. However, how different LYM subsets and the kinetics of inflammatory cytokines change in the peripheral blood in COVID-19 remain unclear. In this study, the changes in LYM subsets and cytokines profiles in the peripheral blood of COVID-19 patients with distinct disease severities were longitudinally characterized.

RESULTS

Results of white blood cell (WBC) count, LYM subset, and demographics of the study subjects

Table 1 showed the demographics and clinical characteristics of the study subjects. The proportion of randomly selected severe cases, including critical illness, was 25.8%. The average age of those was 58 years old, as well as 42 years old of non-severe patients. The age and WBC count, NLR, LYM–monocyte (MON) ratio, platelet-to-LYM ratio, CRP, d-NLR, and D-dimer (D-D) of severe ill patients were significantly higher than those of non-severe patients ($p<0.01$). By contrast, the results of CD3+, CD3+CD4+, CD3+CD8+, CD56+CD16+, and CD3-CD19+ were notably low ($p<0.01$). However, no significant difference was observed in terms of gender, Fib, albumin-to-fibrin ratio, and CD4+/CD8+ ($p>0.05$).

Results of clinical characteristics of the study subjects

All patients had no contact with wild animals. However, 27.8% (26/93) of the patients recently traveled to Wuhan, and 73.1% (68/93) of those had contact with people from Wuhan. Fever and cough were the first and most common symptoms before admission. A total of 50 (53.7%) patients in both groups had co-morbidities, including diabetes (22.5%; 21/93), hypertension (24.7%; 23/93), hepatitis B (11.8%; 11/93), abnormal liver function (13.9%; 13/93), heart disease (13.9%; 13/93), and renal dysfunction (10.7%; 10/93) (Table 2). A total of 70.8% of severe case patients and 79.7% of mild case patients had fever. Meanwhile, no significant difference was observed in the degrees of temperature ($p=0.37$), fatigue ($p=0.213$), cough ($p=0.496$), pharyngalgia ($p=0.748$), dizziness ($p=0.109$), headache ($p=0.831$), chest pain ($p=0.456$), vomiting ($p=0.762$), diarrhea ($p=0.999$), heart disease ($p=0.663$), and abnormal liver function ($p=0.659$) between the two groups (Table 2). The severe case patients showed significantly high frequencies in the occurrence of diabetes ($p<0.01$), hypertension ($p<0.01$), renal dysfunction ($p<0.05$), chill ($p<0.05$), shivering ($p<0.05$), sputum production ($p<0.01$), and nausea ($p<0.01$) (Table 2).

Analysis of inflammatory cytokine levels in the serum of COVID-19 patients

A previous study demonstrated the changes in the levels of inflammatory cytokines, such as IL-2, IL-7, IL-10, and tumor necrosis factor (TNF)-α, in the serum of COVID-19 patients [4]. Therefore, the changes in inflammatory cytokine levels, including IL-2 and IL-12P70, were further characterized in the serum of our patient cohort. The severe case patients showed significantly high levels of IL-2 ($p<0.05$), IL-6 ($p<0.01$), IL-8 ($p<0.05$), and IL-10 ($p<0.01$) (Table 3). No significant difference was observed in the degrees of IL-5, IFN-α, IL-1β, IFN-γ, IL-17, IL-4, and IL-12P70 between the two study groups (Table 3). The laboratory reference values for each cytokine are as follows: IL5 ≤ 3.1 pg/mL, IFN-α≤ 8.5 pg/mL, IL-2 ≤ 7.5 pg/mL, IL-6 ≤ 5.4 pg/mL, IL-1β≤ 12.4 pg/mL, IL-10 ≤ 12.9 pg/mL, IFN-γ≤ 23.1 pg/mL, IL-8 ≤ 5.6 pg/mL, IL-17 ≤ 21.4 pg/mL, IL-4 ≤ 8.56 pg/mL, and IL-12P70 ≤ 3.4 pg/mL.

Acute heart, liver, and kidney function damage in severe COVID-19 patients

Among the 24 severe COVID-19 patients, only 16 cases were selected because of their complete electronic

Table 1. Results of WBC count, lymphocyte subset and demographic in the study subjects.

Laboratory results	Total	non-sever (n=69)	Severe (including critical illness) (n=24)	P-value
age(M±SD)	46.4±17.6	42.1±18.6	57.9±11.8	<0.05
sex±M/F±	56/37	38/31	18/6	0.135
WBC(M±SD)	6.9±3.9	6.4±2.4	9.1±5.6	<0.01
LYM	1.04±0.64	1.17±0.63	0.65±0.54	<0.01
NEU	5.38±3.6	4.55±0.21	7.73±5.4	<0.01
MON	0.43±0.46	0.41±0.2	0.5±0.84	<0.05
NLR(M±SD)	10.8±15.6	4.8±3.5	20.7±24.1	<0.01
d-NLR(M±SD)	5.07±5.5	3.3±1.9	9.8±7.8	<0.01
LMR(M±SD)	3.42±4.6	4.1±6.0	2.1±1.6	<0.01
PLR(M±SD)	255.8±226.1	176.7±84.2	436.5±329.2	<0.01
CRP(M±SD)	33.8±48.4	20.1±24.5	53.9±60.1	<0.01
CD3+	629.4±489.4	763.8±483.3	222.2±195.2	<0.01
CD3+CD4+	370.6±264.3	448.7±254.9	132.6±98.5	<0.01
CD3+CD8+	219.8±209.3	264.6±217.4	83.9±97.2	<0.01
CD4+/CD8+	2.06±0.97	2.01±0.98	2.0±0.97	0.754
CD56+CD16+	148.7±132.3	169.3±141.3	85.9±76.7	<0.01
CD3-CD19+	124.8±103.9	141.3±111.2	75.2±53.7	<0.01
D-dimer	3.2±8.1	0.54±0.42	16.6±23.1	<0.01
Alb	38.6±6.9	41.4±5.8	31.9±4.4	<0.05
Fib	3.6±1.3	3.8±1.2	3.2±1.4	0.179
AFR	12.2±5.7	12.2±5.1	12.5±7.4	0.585

Albumin(Alb), Fibrin(Fib), Albumin-to-Fibrin (AFR), white blood count cell(WBC), neutrophil-to-lymphocyte ratio (NLR), derived neutrophil-to-lymphocyte ratio (d-NLR) (neutrophil count divided by the result of white cell count minus neutrophil count), platelet-to-lymphocyte ratio (PLR) and lymphocyte-to-monocyte ratio (LMR), C-reactive protein(CRP), lymphocyte (LYM), Neutrophils (NEU), Monocyte (MON).

medical records, subsequently, the abnormal laboratory results of multiple organs, such as kidney (creatinine and urea), liver (alanine aminotransferase and aspartate aminotransferase), and heart (High sensitivity troponin T and creatine kinase-MB), were further analyzed. The laboratory results of acute renal function injury (4.94±1.69) occurred earlier than those of liver (7.81±1.86) and heart functions (6.19±1.83). One-way analysis of variance showed a statistically significant difference in laboratory results ($p<0.01$) between kidney and heart ($p=0.056$), kidney and liver ($P<0.01$), and heart and liver functions ($P=0.014$). However, several patients presented opposite trends. Further checking of the electronic medical record revealed that these patients were infected with hepatitis B and other common diseases, such as liver fibrosis, cyst, fatty liver, and cirrhosis (Figure 1).

Prognostic factors for the identification of severe COVID-19 cases

PCA was performed by SPSS package "factor analysis" to identify correlated variables for distinguishing severe patients from mild case patients (Figure 2). The seven most contributing variables, namely, D-D, IL-6, IL-8, and NLR with a score of more than 2. CD3+, IL-10 and age with a score of more than 1, which were selected as potential prognostic factors for further detailed statistical analysis. To assess the diagnostic value of the seven selected parameters, we calculated the ROC curve and area under the ROC curve (AUC) by SPSS package (Figure 3). The results of this analysis identified D-D with a higher AUC (0.958) than IL-6 (0.795), NLR (0.789), IL-8 (0.774), age (0.728), and IL-10 (0.717). With values of 49.5 for age, 3.3 for NLR, and 2.1 for D-D dimer, CD3+could not be used as a potential

Table 2. Baseline characteristics of patients infected with COVID-2019.

Baseline variables	Total(n=93)	non-sever (n=69)	Severe (including critical illness)(n=24)	P-Value
Wuhan exposure(%)	29 (31.1)	21 (30.4)	9 (37.5)	0.524
co morbidities (%)	50 (53.7)	29 (42.1)	21 (87.5)	<0.01
Diabetes	21 (22.5)	8 (11.6)	13 (54.2)	<0.01
Hypertension	23 (24.7)	7 (10.1)	16 (66.8)	<0.01
hepatitis B	11 (11.8)	7 (10.1)	4 (16.7)	0.409
Heart disease	13 (13.9)	4 (5.8)	9 (37.5)	<0.01
Renal dysfunction	10(10.7)	2 (2.9)	8 (33.3)	<0.05
Abnormal liver function	13 (13.9)	9 (13.0)	4 (16.7)	0.659
others	5 5.4)	3 (4.3)	2 (8.3)	0.456
Signs and symptoms				
Fever	72 (77.4)	55 (79.7)	17 (70.8)	0.37
Chill	22 (23.6)	12 (17.4)	10 (41.6)	<0.05
Shivering	11 (11.8)	5 (7.2)	6 (25)	<0.05
Fatigue	60 (64.5)	42 (60.8)	18 (75)	0.213
Cough	67 (72.1)	51 (73.9)	16 (66.7)	0.496
Sputum production	44 (47.3)	25 (36.2)	19 (79.1)	<0.01
Pharyngalgia	10 (10.7)	7 (10.1)	3 (12.5)	0.748
Dizziness	17 (18.3)	10 (14.5)	7 (29.1)	0.109
Headache	18 (19.3)	13 (18.8)	5 (20.8)	0.831
Chest tightness	28 (30.1)	17 (24.6)	11 (45.8)	0.051
Chest pain	6 (6.5)	3 (4.4)	2 (8.3)	0.456
Shortness of breath	5 (5.4)	2 (2.9)	3 (12.5)	0.072
Nausea	10 (10.7)	4 (5.8)	6 (25)	<0.01
Diarrhoea	1 (1.1)	1 (1.5)	0	0.999
Vomiting	3 (4.3)	2 (2.9)	1 (4.2)	0.762

Table 3. Results of inflammatory cytokine levels in the serum of COVID-19 patients.

Baseline Variables	Total (n=93) Mean (Min-Max)	non-sever (n=69)	Severe (including Critical illness) (n=24)	P-Value
IL-5	2.39(0.2-40.3)	1.99(0.2-10.4)	2.95(0.24-40.3)	0.438
IFN-a	2.31(0.84-22.5)	2.03(0.96-4.83)	2.69(0.84-22.5)	0.617
IL-2	2.21(0.74-25.4)	1.74(0.74-4.76)	2.88(1.23-25.36)	<0.05
IL-6	26.5(0-1197.7)	6.91(0-109.5)	54.1(0-1197.7)	<0.01
IL-1B	14.6(0-121.5)	14.1(0-49.7)	15.5(0-121.5)	0.398
IL-10	3.27(0.96-39.5)	2.81(0.08-10.2)	4.81(1.15-39.5)	<0.01
INF-Y	6.32(0-150.9)	4.46(0.08-67.1)	8.96(0-150.9)	0.218
IL-8	108.3(0-3979.2)	36.1(0-454.3)	210.4(0.57-3979.2)	<0.05
IL-17	1.67(0.17-10.6)	1.56(0.17-10.3)	1.84(0.79-10.6)	0.399
IL-4	1.47(0.53-7.14)	1.51(0.53-7.14)	1.42(0.61-3.56)	0.804
IL-12P70	0.93(0-4.87)	0.85(0-4.5)	1.04(0-4.87)	0.44
TNF-a	19.8(0-1065)	8.29(0-54.4)	35.9(0-1065)	0.577

diagnostic biomarker for subsequent analysis with its AUC < 0.50. Meanwhile, the results for IL-10 in severe case patients were statistically higher than those in non-severe patients. However, the average results of both groups were within the reference range (IL-10≤12.9 pg/mL). The further univariate analysis including five factors, such as IL-6, IL-8, D-D, age, and NLR, was used to calculate the odds ratios (ORs) between the severe and non-severe case groups. The results were obtained for NLR (OR: 4.6, 95% Cl: 1.242–17.80), IL-6 (OR: 6.625, 95% Cl: 2.398–18.304), IL-8 (OR: 6.881, 95% Cl: 2.453–19.298), and age (OR: 4, 95% Cl: 1.493–10.714) with our patient cohort as predictive factors for severe COVID-19 (Table 4). D-D increased from 0.5 to 8. The risk ratio between severe and non-severe group increased from 2.75 to 55 and eventually leading to disseminated intravascular coagulation (DIC) ($y = 7.0651x - 0.4251$, $R^2 = 0.9893$).

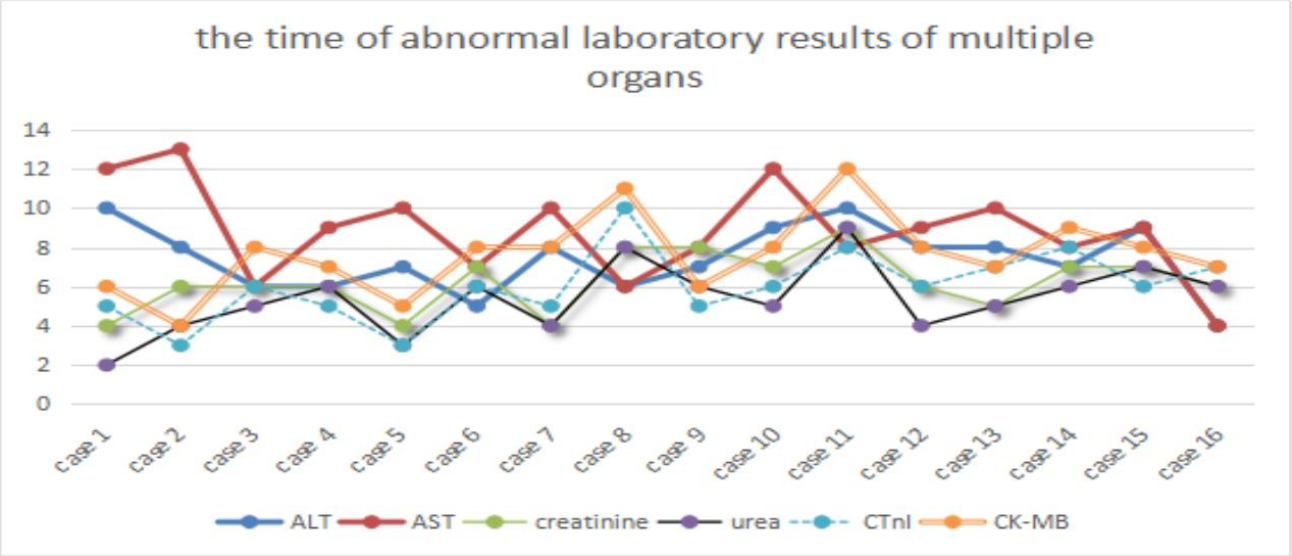

Figure 1. Time of abnormal laboratory results of multiple organs. ALT and AST, urea and creatinine, CTnI and CK-MB represent acute liver, renal and heart dysfunctions, respectively. X-axis represents the case number, whereas Y-axis denotes the time of abnormal laboratory results.

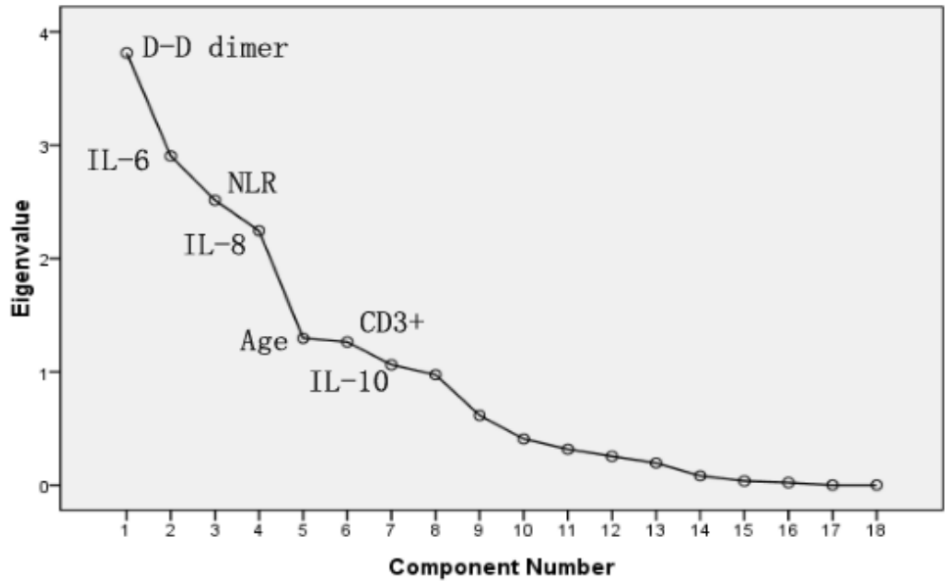

Figure 2. PCA Sreen point.

Kinetic analysis of WBC, NEU, LYM, MON, D-D dimer, and CRP in COVID-19 patients

The absolute numbers of total WBCs (A), NEU (B), LYM (C), MON (D), CRP (E), and D-D (F) in the peripheral blood of mild (blue line) and severe (red line) COVID-19 patients were analyzed at different time points after hospital admission. Error bars represent mean ± SD. From non-severe to severe cases, the time for D-D to change from lower normal limit to upper normal limit was significantly earlier than that for other biomarkers, and the change was more evident (Figure 4).

DISCUSSION

Since the outbreak of 2019-nCoV pneumonia in December 2019, the median incubation period was 4–7 days, and the fatality rate was relatively low [2]. By the end date of data collection (2020-02-29), less than 80,000 cases of COVID-19 were confirmed, and 2,835 patients died (CDC, China). The number of severe cases and deaths also increased every day [13]. In this study, severe case patients were older, and the proportion of underlying diseases was higher compared with mild case patients. Fever and cough were the first and most common symptoms before admission, whereas gastrointestinal symptoms, headache, shivering, pharyngalgia, and shortness of breath were rarely observed. This paper concluded the difference in viral tropism compared with SARS-CoV, MERS-CoV, and influenza [14, 15]. Fever occurred in 43.8% of patients upon initial presentation and developed in 83.4% after hospitalization. Among COVID-19, SARS–CoV (1%), and MERS–CoV (2%) infections, the absence of fever is most frequent in COVID-19 cases [14, 16], and the patients may be missed if the surveillance case definition focuses heavily on fever detection [3]. Significantly high frequencies of severe cases were observed in elderly patients with diabetes or hypertension (Tables 1 and 2). The clinical characteristics of these patients were similar to those reported in previous studies. [2, 3, 17]. From non-severe to severe, the time for D-D to change from lower normal limit to upper normal limit was significantly earlier than that for other biomarkers (Figure 4). Therefore, the D-D should be monitored every other day, which should also be beneficial for patients.

After the discussion of the clinical features of COVID-19, we analyzed the immunological characteristics of peripheral blood in patients with COVID-19. Firstly, the LYM counts were normal in COVID-19 patients with mild diseases [18]. By contrast, almost all patients with severe diseases had lymphopenia, and the LYM counts in patients with a mortal outcome remained at a low level [19]. This study also confirmed the higher rates of developing lymphopenia in severe case patients than in mild case patients (99.6% vs 67.2%). The development of lymphopenia in severe case patients was mainly related to the significantly decreased absolute counts of T cells, including CD4+ and CD8+T cells, similar to the absolute counts of B cells and natural killer cells but not

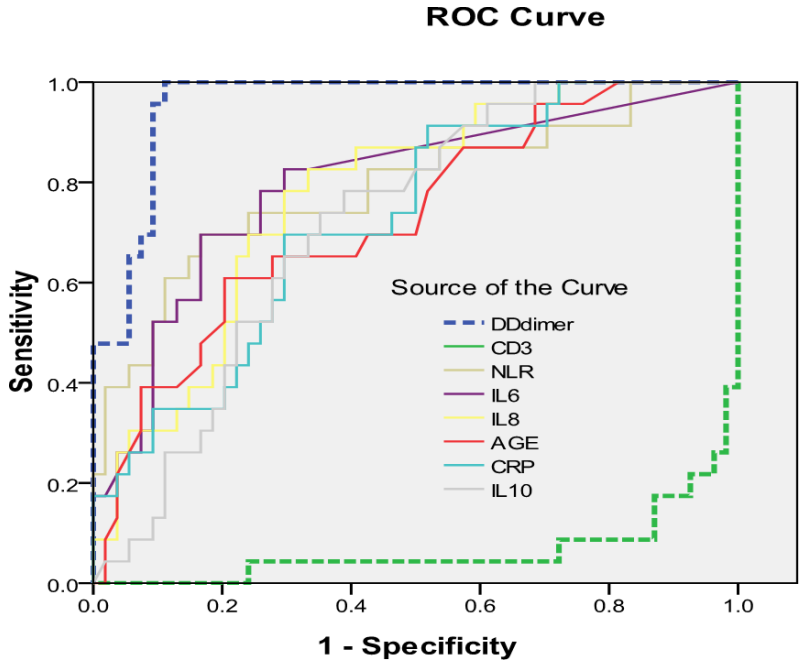

Figure 3. Receiver operating curve analysis used to identify patients with severe or non-severe cases of COVID-2019.

Table 4. The OR in each of the NLR, IL-6, IL-8, age, D-D dimer and double D-D dimer.

	OR	95%CI		OR	95%CI
IL-6	6.625	2.398-18.304	D-D >0.5	2.75	1.999-3.784
IL-8	6.881	2.453-19.298	D-D >1	3.733	2.241-5.756
AGE	4	1.493-10.714	D-D >2	16.5	6.832-42.656
D-D	16.5	6.382-42.656	D-D >4	29.4	7.148-120.9
NLR	4.6	1.242-17.08	D-D >8	55	6.523-463.7

to the proportion of LYMs. Interestingly, the ratio of CD4+ to CD8+ was mostly normal. This result is opposite that for acquired immunodeficiency syndrome. In addition, the immunoglobulin levels were normal, which may be explained by the acute infection caused by COVID-19 and half-life of immunoglobulin [20]. The number of T cells increased three to five days earlier than the relief of clinical symptoms. Thus, this course is associated with favorable outcomes among severe COVID-19 patients.

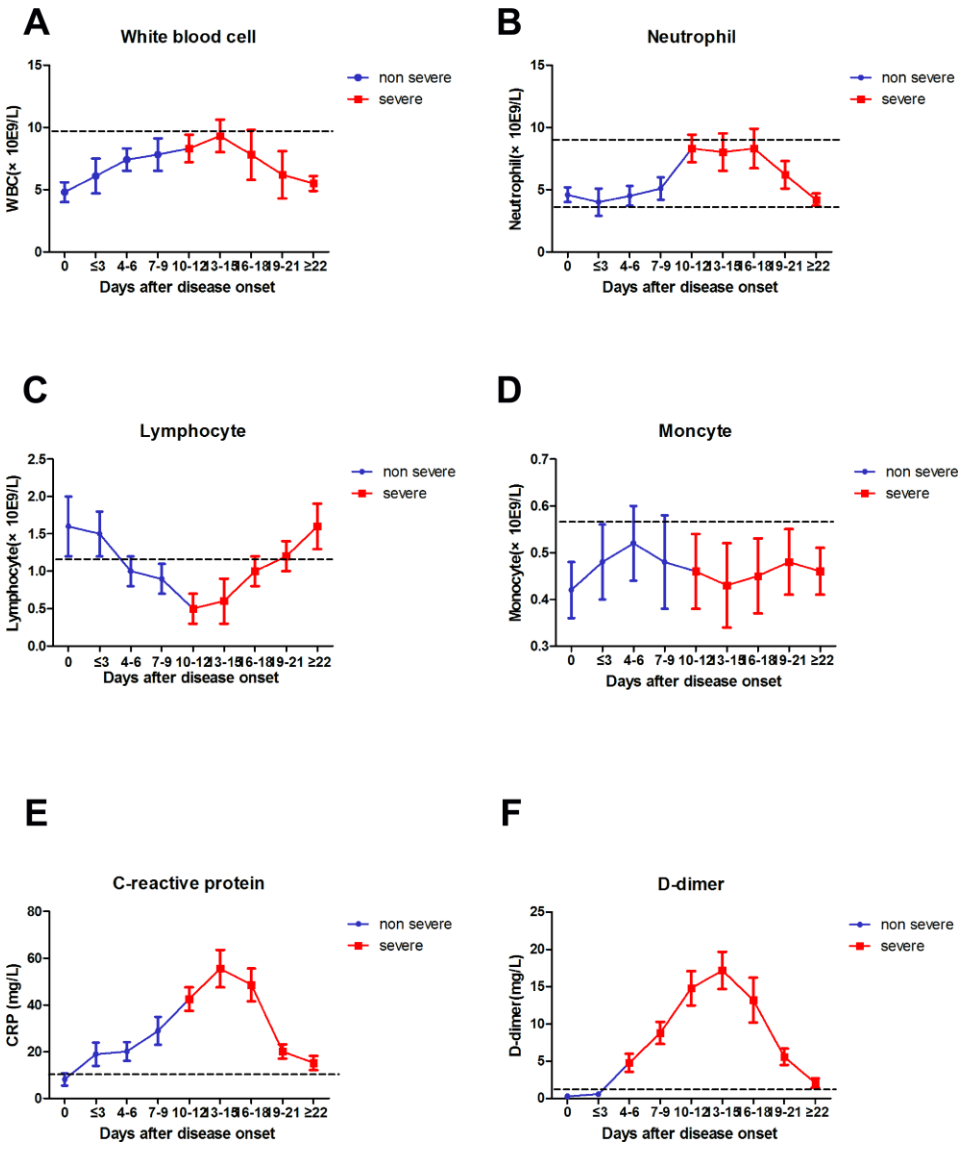

Figure 4. Dynamic results of WBC, NEU, LYM, MON, CRP, D-D dimer from non-severe to severe.

Elevated levels of proinflammatory cytokines, such as IFN, TNF-α, IL-6, and IL-8, are associated with severe lung injury and adverse outcome of SARS-CoV or MERS-CoV infection [20, 21]. Our results also demonstrated that severe COVID-19 patients had higher concentrations of IL-6, IL-8, IL-2,TNF-α, and IFN-γ in the serum than mild case patients, which suggested that the magnitude of cytokine storm was associated with the disease severity. The results indicated that COVID-19 may act on immune cells, especially T LYMs. T LYM damage is an important factor that causes patient deterioration. Additionally, T cells are important for dampening overactive innate immune responses during viral infection [22, 23]. Thus, the loss of T cells during COVID-19 may result in aggravated inflammatory responses, whereas restoration of T cell numbers may alleviate them. The courses of restoration of T cell numbers are associated with the decreases in serum IL-6, IL-10, IL-2, IL-4, TNF-α, and IFN-levels [24]. Therefore, the steady raise in the number of immune cells and the sustained decline in the levels of inflammatory factors are important laboratory manifestations for the clinical improvement of severe patients with COVID-19.

The decreased levels of immune cells and the increased number of inflammatory cells are important manifestations of COVID-19 infection. In the present study, the results supported the hypothesis which indicated that D-D dimer and elevated NLR are independent prognostic biomarkers affecting pneumonia progression in COVID-19 patients [25]. In addition, the integration of elevated NLR to prognostic nomograms may lead to improved prediction. The findings were consistent with those of previous studies on the relationship between NLR and prognosis of other infectious diseases [26]. The following reasons may account for the findings. On the one hand, NEU is a major component of leukocyte population that activates and migrates from the venous system to the immune organ or system. In addition, NEUs interact with distinct cell populations and produce numerous cytokines and effector molecules, such as circulating vascular endothelial growth factor. Furthermore, NEUs can be triggered by virus-related inflammatory factors, such as IL-6, IL-8, TNF-α, and granulocyte colony stimulating factor, and interferon gamma factors, which are produced by LYM and endothelial cells [27]. On the other hand, the increase in D-D is common in secondary hyperfibrinolysis conditions, such as hypercoagulable state, DIC, sepsis, and kidney disease [23, 28]. Hypercoagulable state blocks immune cell migration to infected organs and is incompatible with the novel coronavirus. Findings showed that the blocking factor is important given the doubled increase of D-D and the seven-fold increased risk ratio. Thus, infection-triggered inflammation increases NLR. Elevated NLR promotes COVID-19. The clinical symptoms become increasingly severe, and the progress from admission to intensive care unit, cure and discharge, or mechanical ventilation occurs rapidly. Thus, early identification of risk factors for severe COVID-19 patients may facilitate appropriate supportive care and prompt access to the intensive care unit if necessary.

In this study, the laboratory findings of acute renal function injury (4.94±1.69) appeared earlier than abnormal liver (7.81±1.86) and heart functions (6.19±1.83). However, several patients presented the opposite results. Further checking of electronic medical records revealed that these patients had hepatitis B infection and other common diseases, such as liver fibrosis, cyst, fatty liver, and cirrhosis. The causes are still unclear. Except for acute respiratory distress syndrome in patients caused by COVID-19, acute renal dysfunction may occur earlier than other organ dysfunctions, such as those of the liver and heart; these organs need to be monitored by clinicians. Hepatitis B infection is common in China [3, 29]; excluding this factor, whether we can arrive at the same conclusion remains to be further studied. Finally, PCA was performed to identify correlated variables for distinguishing severe and mild case patients (Figure 2). Five of the most contributing variables, namely, D-D dimer, IL-6, IL-8, age, and NLR, were selected as potential prognostic factors for further detailed statistical analysis. The optimal threshold of 3.3 for NLR indicated the superior prognostic possibility of clinical symptoms to change from light to heavy. D-D dimer had the highest sensitivity and specificity and the largest AUC.

Several notable limitations have been observed in this paper. First, the data were obtained from a single clinical research center. Second, the experimental data were limited to Han population of China. Furthermore, the conclusions of this study may differ from those of other scholars at home and abroad and must be further improved in clinical cases. Finally, accurate clinical data were lacking for the small number of patients with mild illness because of time constraints.

In conclusion, the study revealed that LYM subsets and cytokine profiles in the peripheral blood of COVID-19 patients were longitudinally characterized. D-D dimer increased from 0.5 to 8, and the risk ratio increased from 2.75 to 55, eventually leading to DIC. Acute renal function damage occurred earlier than the abnormal heart and liver functions. Finally, the kinetics features of immune parameters associated with the disease severity were determined, and D-D dimer and NLR were identified as the most useful prognostic factors for predicting severe COVID-19 cases.

MATERIALS AND METHODS

Patients

We performed a retrospective study on the clinical characteristics, epidemiological, demographic, laboratory data, and outcome data of laboratory-confirmed cases with 2019-nCoV. Cases were diagnosed based on the WHO interim guidance [5]; non-severe patients met all the following conditions: (1) epidemiological history, (2) fever or other respiratory symptoms, (3) typical computed tomography image of abnormities of viral pneumonia, and (4) positive result in reverse transcription polymerase chain reaction (RT-PCR) for SARS-CoV-2 RNA. Severe patients additionally met at least one of the following conditions: (1) shortness of breath, RR $\geq$ 30 times/min, (2) oxygen saturation (resting state) $\leq$ 93%, (3) $PaO_2/FiO_2 \leq 300$ mmHg. Infections with other respiratory viruses, including influenza A virus, influenza B virus, respiratory syncytial virus and parainfluenza virus were excluded by serological test. Informed consent was waived in light of the anonymous, retrospective, and observational character of this study

Clinical characteristics and laboratory data

The epidemiological characteristics (including recent exposure history), clinical symptoms and signs, and laboratory findings were extracted from electronic medical records. Laboratory assessments consisted of complete blood count, blood chemistry, coagulation test, liver and renal function, C-reactive protein (CRP), LYM subsets, and cytokines. The severity of COVID-19 was defined in accordance with the international guidelines for community-acquired pneumonia. The LYM test kit (FC 500 MCL, BECKMAN, USA) was used for LYM subset analysis (CD3+, CD3+CD4+, CD3+CD8+, CD16+CD56+, and CD3-CD19+). Plasma cytokines (interleukin (IL)-5, interferon (IFN)-α, IL-2, IL-6, IL-1β, IL-10, IFN-γ, IL-8, IL-17, IL-4, and IL-12P70) were detected with human Th1/2 cytokine kit II (ACEA NovoCyte, Guangzhou, China). All tests were performed in accordance with the product manual.

Statistical analysis

Continuous variables were expressed as means and standard deviations or medians and interquartile ranges as appropriate. Categorical variables were summarized as the counts and percentages in each category. Wilcoxon rank-sum tests were applied to continuous variables. Chi-square tests and Fisher's exact tests were used for categorical variables as appropriate. Optimal cutoff values of the continuous neutrophil (NEU)-to-LYM ratio (NLR), Age, D-D, IL-2, IL-6, IL-8, and CRP were calculated by applying the receiver operating curve analysis (ROC). Hazard risk and 95 % confidence interval were used as common measures to assess relative risk. Principal component analysis (PCA) was performed to identify the major contributing factors among clinical parameters to distinguish mild and severe cases of COVID-19 patients. $P<0.05$ was recognized as statistically significant. All statistical calculations were performed using SPSS 17.0 software (SPSS Inc, Chicago, USA).

AUTHOR CONTRIBUTIONS

Jian-ping Liu: Designed the study. Min Wang: Data curation. Ai-ping Yang: Methodology, software, writing - original draft preparation. Hui-ming Li: Collected the samples, detected serum marker levels. Wen-qiang Tao: Writing-reviewing. Xue-jing Yang and Wen-juan Yang: Editing. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

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Research Paper

Comparison of clinical characteristics and outcomes of patients with coronavirus disease 2019 at different ages

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ABSTRACT

Background: Information about the clinical characteristics and mortality of patients with coronavirus disease 2019 at different ages is limited.

Results: The older group had more patients with dyspnea and fewer patients with fever and muscle pain. Older patients had more underlying diseases, secondary infection, myocardial injury, renal dysfunction, coagulation dysfunction, and immune dysfunction on admission. More older patients received immunoglobulin therapy and mechanical ventilation. The proportions of patients with multiple organ injuries, critically ill patients and death increased significantly with age. The older groups had higher cumulative death risk than the younger group. Hypertension, cerebrovascular disease, comorbidities, acute cardiac injury, shock and complications are independent predictors of death.

Conclusions: The symptoms of the elderly patients were more atypical, with more comorbidities, secondary infection, organ injuries, immune dysfunction and a higher risk of critical illness. Older age was an important risk factor for mortality.

Methods: 1000 patients diagnosed with coronavirus disease 2019 from January 1, 2020 to February 14, 2020 were enrolled. According to age, patients were divided into group 1 (<60 years old), group 2 (60-74 years old) and group 3 (≥75 years old). The clinical symptoms, first laboratory results, CT findings, organ injuries, disease severity and mortality were analyzed.

INTRODUCTION

Since the outbreak of coronavirus disease 2019 (COVID-19) in Wuhan, it has spread globally [1]. According to data from the World Health Organization (WHO), as of April 22, 2020, at least 2,471,136 patients have been confirmed to have COVID-19, of whom 169,006 have died [2]. At present, the number of patients with severe

acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is still increasing. The numbers of infected patients and deaths both exceeded the respective figures associated with the outbreaks of severe acute respiratory syndrome (SARS) in 2003 [3] and Middle East respiratory syndrome (MERS) in 2015 [4]. Compared to the mortality of SARS (10%) and MERS (35%), COVID-19 has a lower fatality rate of 2.3% [5–7]. However, the rapidly increasing number of cases and increasing evidence of human-to-human transmission suggest that SARS-CoV-2 is more contagious than SARS-CoV and MERS-CoV [8, 9].

According to a report from the Chinese Centers for Disease Control and Prevention, older patients over 60 years old in Wuhan accounted for 44.1% of COVID-19 patients [9]. A recent study [10] from the Lancet indicated that the proportion of patients over 60 years old in Wuhan was significantly higher than that in the rest of China, which reflected a more severe illness in Wuhan. Older patients had a substantially higher case fatality ratio of 6.4% than younger patients. Increasing evidence has shown that elderly patients are more likely to develop severe illness and have higher risks of in-hospital death [11–14]. However, there were no reports about the clinical findings of patients diagnosed with SARS-CoV-2 infection at different ages. The mechanisms of the poor prognosis in elderly patients remain unclear.

In this study, we analyzed and compared the clinical characteristics and outcomes of COVID-19 patients at different ages.

RESULTS

Comparison of basic clinical characteristics among the three groups

A total of 1000 COVID-19 patients were analyzed during the study period, including 473 young patients (47.3%) (age group 1), 359 elderly patients (35.9%) (age group 2) and 168 super-aged patients (16.8%) (age group 3). A total of 466 (46.6%) were male, and 534 (53.4%) were female. The median (interquartile ranges) interval from disease onset to admission of the three groups was 10 (7-14), 11 (8-15) and 10 (6-13) days, respectively. The most common symptoms before admission of all the patients were fever (75.4%), followed by cough (59.7%), fatigue (33.5%) and dyspnea (25.5%). The percentage of patients with fever in age group 3 was lower than that in age group 2 (79.1% vs. 69.6%, P <0.05). With increasing age, the incidence of dyspnea increased (22% vs. 28.1% vs. 29.8%), and the rate of muscle pain decreased (3.6% vs. 5% vs. 8.9%). No significant difference was found for the other symptoms, such as cough, chest pain, catarrhal symptoms, fatigue, dizziness, headache and digestive symptoms (Table 1). The finger oxygen saturation on admission increased with age (median, 96% vs. 97% vs. 98%). During hospitalization, 623 patients (66.3%) had fever, and the proportion of patients with fever (59.7% vs. 71.9% vs. 73.2%) and shortness of breath (respiratory rate > 30 times per minute) (9.5% vs. 17.5% vs. 19.6%) increased with age (Table 2).

In addition, 405 patients (40.5%) had at least one comorbidity. The most common comorbidity was hypertension (28.2%), followed by diabetes (11.8%), coronary artery disease (CAD) (6%) and cerebrovascular disease (CVD) (3.2%). The proportion of patients with any comorbidity, more than one comorbidity, diabetes, hypertension, CAD, CVD and chronic renal disease all increased with advancement of age (Table 2).

Results of laboratory tests and CT scans

We collected and analyzed the initial laboratory tests after admission. According to the routine blood test results, the median white blood cell counts and neutrophil counts increased with age, while the lymphocyte counts decreased with age. The proportion of patients with lymphopenia in the older groups was higher than that in the younger group (43.1% vs. 62% vs. 70.7%). The probability of thrombocytopenia increased with age (5.3% vs. 11.3% vs. 21%) (Table 3).

Aspartate aminotransferase, direct bilirubin, globulin creatinine, blood urea nitrogen, uric acid, creatinine kinases MB isoenzyme (CKMB), hypersensitive troponin I (hs-TnI) and lactate dehydrogenase (LDH) all increased with advancement of age. The proportion of patients with increased aspartate aminotransferase (>120 U/L), CKMB (>5 ng/mL), hs-TnI (>0.0796 ng/mL), LDH (>250 U/L) and decreased estimated glomerular filtration (≤90 mL/min) all increased with age. The older group had more patients with myocardial injury, renal dysfunction, and liver dysfunction on admission (Table 3).

The results of arterial blood gas analysis showed that older patients presented with lower arterial oxygen saturation and arterial partial pressure of oxygen. No difference was discovered in blood pH among the three age groups. The coagulation function results showed that the activated partial thromboplastin time and D-dimer (median, 0.51 vs. 0.93 vs. 1.77 μmol/L) increased with age, but prothrombin time activity decreased with age (Table 3).

Regarding the biomarkers of nonspecific inflammation, the results showed that the levels of biomarkers including C-reactive protein (CRP) (median, 11.4 vs. 40 vs. 54.3 mg/L), high-sensitivity C-reactive protein (hs-CRP)

Table 1. Baseline characteristics of COVID-19 patients.

	all (n=1000)	age<60(n=473)	60-74(n=359)	age≥75(n=168)
Age, median (IQR), y	61 (46,70)	46 (34,53)	67 (64,70)*	80 (77,84)*#
Male (n, %)	466 (46.6%)	216 (45.7%)	164 (45.7%)	86 (51.2%)
Female (n, %)	534 (53.4%)	257 (54.3%)	195 (54.3%)	82 (48.8%)
Onset of symptom to admission, median (IQR), d	10 (7,14)	10 (7,14)	11 (8,15)*	10 (6,13)#
Initial symptoms, No. (%)				
Fever	754 (75.4%)	353 (74.6%)	284 (79.1%)	117 (69.6%)#
Symptoms of respiratory system				
Sore throat	43 (4.3%)	31 (6.6%)	7 (1.9%)*	5 (3%)
Cough	597 (59.7%)	290 (61.3%)	202 (56.3%)	105 (62.5%)
Expectoration	190 (19%)	81 (17.1%)	65 (18.1%)	44 (26.2%)*#
Chest tightness	209 (20.9%)	78 (16.5%)	97 (27%)*	34 (20.2%)
Chest pain	19 (1.9%)	9 (1.9%)	7 (1.9%)	3 (1.8%)
Dyspnea	255 (25.5%)	104 (22%)	101 (28.1%)*	50 (29.8%)*
Catarrhal symptoms	20 (2%)	12 (2.5%)	5 (1.4%)	3 (1.8%)
Neuromuscular symptoms	388 (38.8%)	199 (42.1%)	127 (35.4%)	62 (36.9%)
Fatigue	335 (33.5%)	172 (36.4%)	111 (30.9%)	52 (31%)
Dizziness	33 (3.3%)	15 (3.2%)	10 (2.8%)	8 (4.8%)
Headache	32 (3.2%)	19 (4%)	7 (1.9%)	6 (3.6%)
Lethargy	10 (1%)	6 (1.3%)	0 (0%)	4 (2.4%)#
Muscle ache	66 (6.6%)	42 (8.9%)	18 (5%)*	6 (3.6%)*
Digestive symptoms				
Anorexia	134 (13.4%)	61 (12.9%)	49 (13.6%)	24 (14.3%)
Nausea	21 (2.1%)	8 (1.7%)	8 (2.2%)	5 (3%)
Vomiting	26 (2.6%)	11 (2.3%)	8 (2.2%)	7 (4.2%)
Abdominal pain	7 (0.7%)	3 (0.6%)	3 (0.8%)	1 (0.6%)
Diarrhea	100 (10%)	48 (10.1%)	35 (9.7%)	17 (10.1%)

The total number of patients with available data: n(<60)=473, n(60-74)=359, n(≥75)=168. * p<0.05 vs. <60 group. # p<0.05 vs. 60-74 group.

(median, 5 vs. 5 vs. 5 mg/L) and procalcitonin (PCT) (median, 0.05 vs. 0.06 vs. 0.12 ng/mL) increased with age. The proportion of patients with elevated CRP, elevated serum amyloid protein (SAA), and elevated PCT all significantly increased with age, suggesting that the percentage of elderly patients with secondary infection was significantly increased. Interleukin 6 (IL-6) expression increased (median, 5.71 vs. 7.19 vs. 16.66 pg/mL) with age, although there were no significant differences in interferon-γ, IL-2, IL-5, IL-10 or tumor necrosis factor among the three groups. Older patients had higher levels of complement C3, and C4 and immunoglobulin A, G, and M than younger patients. In addition, the counts of CD19 cells, CD3 cells, CD4 cells (median, 412 vs. 328 vs. 266/μL) and CD8 cells (median, 263 vs. 177 vs. 120.5/μL) decreased with age (Table 4).

A total of 545 CT results were collected within 3 days before or after admission. A total of 509 patients (93.4%) had pneumonia. Compared with younger patients, older patients were more likely to have bilateral involvement and paving stone/reticulation/linear findings (Table 5).

Complications after admission and treatment

Of the 1000 patients, 191 patients (19.1%) experienced complications. The most common complication was acute cardiac injury (11.6%), followed by shock (8.1%) and acute liver injury (6.4%). The rates of patients with any complication, more than one complication, acute cardiac injury, shock and liver injury were higher in the older group than in the younger group. The proportion of patients with acute cardiac injury increased with age (4%

Table 2. Vital signs and comorbidity of COVID-19 patients.

	all (n=1000)	age<60(n=473)	60-74(n=359)	age≥75(n=168)
Characteristics on admission				
Fever, No. (%)[a]	200 (21.3%)	98 (22.1%)	71 (20.8%)	31 (20.3%)
Temperature	36.7 (36.4,37.1)	36.7 (36.4,37.1)	36.7 (36.5,37.1)	36.7 (36.5,37)
Temperature, No. (%)[a]				
< 37.3°C	739 (78.7%)	346 (77.9%)	271 (79.2%)	122 (79.7%)
37.3-38.0°C	117 (12.5%)	55 (12.4%)	46 (13.5%)	16 (10.5%)
38.1-39.0°C	72 (7.7%)	35 (7.9%)	23 (6.7%)	14 (9.2%)
≥ 39.1°C	11 (1.2%)	8 (1.8%)	2 (0.6%)	1 (0.7%)
Heart rate, median (IQR), bpm[b]	82 (76,92)	82(76,92)	83(76,92)	80(76,89)
Systolic pressure, median (IQR), mmHg[c]	126 (116,139)	123(112,132)	130(120,142)*	131(117,148)*
Diastolic pressure, median (IQR), mmHg[d]	76 (69,83)	75(68,80)	76(70,84)*	76(67,84)
Respiratory rate, median (IQR), bpm[e]	20 (18,20)	19(18,20)	20(18,21)*	19(18,21)
Finger oxygen saturation, median (IQR), %[f]	97 (95,99)	98(96,99)	97(95,99)*	96(92,98)*#
Characteristics during hospital admission, No. (%)				
Fever	623 (66.3%)	265 (59.7%)	246 (71.9%)*	112 (73.2%)*
Highest temperature[g]				
< 37.3°C	316 (33.7%)	179 (40.3%)	96 (28.1%)*	41 (26.8%)*
37.3-38.0°C	352 (37.5%)	150 (33.8%)	145 (42.4%)*	57 (37.3%)
38.1-39.0°C	203 (21.6%)	76 (17.1%)	85 (24.9%)*	42 (27.5%)*
≥ 39.1°C	67 (7.1%)	38 (8.6%)	16 (4.7%)*	13 (8.5%)
>41.0°C	1 (0.1%)	1 (0.2%)	0 (0%)	0 (0%)
Respiratory rate≥30 bpm	141 (14.1%)	45 (9.5%)	63 (17.5%)*	33 (19.6%)*
Comorbidity, No. (%)				
Diabetes	118 (11.8%)	34 (7.2%)	55 (15.3%)*	29 (17.3%)*
Hypertension	282 (28.2%)	62 (13.1%)	131 (36.5%)*	89 (53%)*#
Coronary heart disease	60 (6%)	6 (1.3%)	29 (8.1%)*	25 (14.9%)*#
COPD	23 (2.3%)	0 (0%)	17 (4.7%)*	6 (3.6%)*
Asthma	12 (1.2%)	3 (0.6%)	5 (1.4%)	4 (2.4%)
Cerebrovascular disease	32 (3.2%)	3 (0.6%)	8 (2.2%)*	21 (12.5%)*#
Chronic renal disease	24 (2.4%)	7 (1.5%)	7 (1.9%)	10 (6%)*#
Chronic liver disease	29 (2.9%)	12 (2.5%)	12 (3.3%)	5 (3%)
Malignancy	28 (2.8%)	7 (1.5%)	11 (3.1%)	10 (6%)*
Autoimmune disease	13 (1.3%)	3 (0.6%)	6 (1.7%)	4 (2.4%)
Organ transplantation	2 (0.2%)	2 (0.4%)	0 (0%)	0 (0%)
Only one comorbidity	237 (23.7%)	67 (14.2%)	115 (32%)*	55 (32.7%)*
≥2 comorbidities	168 (16.8%)	34 (7.2%)	75 (20.9%)*	59 (35.1%)*#
With comorbidity	405 (40.5%)	101 (21.4%)	190 (52.9%)*	114 (67.8%)*#

The total number of patients with available data: a: n(<60)=444, n(60-74)=342, n(≥75)=153; b: n(<60)=443, n(60-74)=352, n(≥75)=153; c: n(<60)=351, n(60-74)=314, n(≥75)=143; d: n(<60)=354, n(60-74)=314, n(≥75)=143; e: n(<60)=443, n(60-74)=342, n(≥75)=152; f: n(<60)=319, n(60-74)=302, n(≥75)=136; g: n(<60)=444, n(60-74)=342, n(≥75)=153. * p<0.05 vs. <60 group. # p<0.05 vs. 60-74 group.

vs. 12.5% vs. 31%). Shock and acute liver injury were most likely to occur in age group 3 (Table 6).

Regarding the treatment measures, 63 (6.3%) patients were sent to the intensive care unit (ICU). Older patients were more likely to be admitted to the ICU than younger patients. Patients in age groups 2 and 3 were more likely to receive nasal catheter oxygen inhalation, mask oxygen inhalation and noninvasive mechanical ventilation than patients in age group 1. Regarding medical treatment, the major treatments were antiviral treatment (92.7%), antibiotic treatment (78.3%),

Table 3. General laboratory findings of COVID-19 patients on admission to hospital.

	Median (IQR)			
	all (n=1000)	**age<60(n=473)**	**60-74(n=359)**	**age≥75(n=168)**
Blood routine [a]				
White blood cell count, × 10^9/L	5.48 (4.12,7.23)	5.16 (3.97,6.65)	5.72 (4.26,7.47)*	5.89 (4.53,8.45)*
<3.5, No. (%)	136 (13.9%)	77 (16.8%)	39 (11%)*	20 (12%)
>9.5, No. (%)	106 (10.8%)	37 (8.1%)	37 (10.4%)	32 (19.2%)*#
Neutrophil count, × 10^9/L	3.67 (2.48,5.44)	3.16 (2.22,4.6)	4.06 (2.74,5.97)*	4.51 (3.11,7.29)*#
Neutrophil %	68.9 (57.9,81.85)	64.6 (53.6,75.4)	72 (62.7,82.75)*	80 (66,86.8)*#
Lymphocyte count, × 10^9/L	1.02 (0.72,1.46)	1.2 (0.88,1.6)	0.93 (0.65,1.39)*	0.81 (0.53,1.13)*#
<1.1, No. (%)	535 (54.6%)	197 (43.1%)	220 (62%)*	118 (70.7%)*
Lymphocyte %	20.6 (11.6,30.15)	24.9 (16.9,34.7)	18.5 (10.55,26.35)*	13 (7.5,22)*#
Platelet count, × 10^9/L	208 (160,267.5)	209 (166,264)	215 (163.5,283.5)*	186 (137,233)*
<125, No. (%)	99 (10.1%)	24 (5.3%)	40 (11.3%)*	35 (21%)*#
>350, No. (%)	78 (8%)	32 (7%)	42 (11.8%)*	4 (2.4%)*#
Red blood cell count, × 10^{10}/L	4.07 (3.71,4.45)	4.28 (3.94,4.65)	3.99 (3.64,4.25)*	3.81 (3.38,4.22)*#
Liver function [b]				
Alanine aminotransferase, U/L	24 (16,42)	25 (16,47)	24 (17,39)	23 (15,38)
>150, No. (%)	17 (1.7%)	10 (2.2%)	5 (1.4%)	2 (1.2%)
Aspartate aminotransferase, U/L	28 (20,40)	25 (20,37)	29 (21,40)*	31.5 (22,46)*#
>120, No. (%)	15 (1.5%)	7 (1.5%)	4 (1.1%)	4 (2.4%)
Total bilirubin, μmol/L [c]	10.5 (7.9,14.13)	10.1 (7.48,13.4)	10.45 (8.03,14.1)	11.95 (8.65,16.27)*#
Direct bilirubin, μmol/L	3.8 (2.7,5.1)	3.5 (2.5,4.8)	3.9 (2.8,5.1)*	4.6 (3.4,6.6)*#
Total protein, g/L	60.9 (57.3,64.63)	62.2 (58.48,65.9)	59.65 (56.43,63.78)*	59.85 (55.7,63.08)*
Albumin, g/L	36.8 (33.4,39.83)	38.65 (35.8,41.4)	35.2 (32.6,37.98)*	34.4 (31.9,37.2)*#
Globulin, g/L	23.73 (21.6,26.8)	23.2 (21.3,25.7)	24.15 (21.7,27.2)*	24.85 (22.03,27.7)*
Kidney function [d]				
Creatinine, μmol/L	60 (49,73)	56 (47.75,70.25)	60 (50,70)*	69.5 (54.25,98.75)*#
Increase, No. (%)	116 (11.9%)	29 (6.4%)	34 (9.6%)	53 (31.9%)*#
Blood urea nitrogen, nmol/L	4.6 (3.59,6.2)	4.06 (3.15,5.04)	4.91 (3.81,6.5)*	6.95 (4.81,11.4)*#
Uric acid, μmol/L	249 (199,327)	252.5 (201,322.25)	233 (194,300.5)*	282.5 (217.25,393.25)*#
Increase, No. (%)	168 (17.2%)	72 (15.8%)	42 (11.8%)	54 (32.5%)*#
Estimated glomerular filtration rate, mL/min	98.86 (88.36,111.87)	112.98 (102.88,121.29)	94.87 (88.55,99.75)*	79.85 (53.68,88.94)*#
≤90, No. (%)	273 (27.9%)	43 (9.4%)	100 (28.2%)*	130 (78.3%)*#
Injury of cardiac and skeletal muscle				
Creatine kinase, U/L [e]	61 (38,104.25)	58 (37,101)	60.5 (38,100)	67 (42,138.25)*#
>310, No. (%)	60 (6.3%)	25 (5.7%)	15 (4.2%)	20 (12.2%)*#

Creatine kinase-myocardial band isoenzyme, ng/mL [f]	0.97 (0.64,1.79)	0.68 (0.48,0.97)	1.11 (0.81,1.88)*	1.85 (1.17,3.24)*#
>5, No. (%)	35 (4.6%)	4 (2%)	11 (3.5%)	20 (13.3%)*#
Lactate dehydrogenase, U/L [g]	264 (203.75,361.25)	236 (187,312)	284 (221.25,368.75)*	298 (222,439)*
>250, No. (%)	523 (54.5%)	197 (44.7%)	217 (61.3%)*	109 (66.1%)*
Myoglobin, μg/L [h]	44.54 (28.5,85.05)	33.27 (21.95,54.42)	44 (30.76,75.99)*	88.56 (55.55,207.42)*#
>110, No. (%)	132 (17.5%)	28 (9.6%)	43 (13.8%)	61 (40.7%)*#
Hypersensitive troponin I, ng/mL [i]	0.006 (0.006,0.018)	0.006 (0.006,0.006)	0.006 (0.006,0.017)*	0.028 (0.01,0.077)*#
>0.0796, No. (%)	66 (8.7%)	7 (2.4%)	22 (7.1%)*	37 (24.3%)*#
Cholinesterase, U/L [j]	8127 (6469.5,9827)	9091 (7521.75,10688.5)	7666.5 (6124.25,9072.25)*	6060 (4775.5,7030.5)*#
>11900	49 (8.2%)	39 (13.1%)	9 (4.5%)*	1 (1%)*
Arterial Blood Gas Analysis [k]				
Blood PH	7.42 (7.38,7.45)	7.42 (7.38,7.45)	7.43 (7.38,7.46)	7.42 (7.37,7.46)
<7.35, No. (%)	77 (13.6%)	23 (11%)	35 (15.2%)	19 (15.1%)
>7.45, No. (%)	176 (31.1%)	56 (26.8%)	81 (35.1%)	39 (31%)
Arterial oxygen saturation, %	96 (91,98)	97 (92,98)	96 (92,98)	95 (89,98)*
<95%, No. (%)	222 (39.2%)	64 (30.6%)	96 (41.6%)*	62 (49.2%)*
Arterial partial pressure of oxygen, mmHg	80 (60,105)	87 (62,107)	78 (62,102)	73 (55.25,99)*
<60 mmHg, No. (%)	140 (24.7%)	49 (23.4%)	52 (22.5%)	39 (31%)
Arterial partial pressure of carbon dioxide, mmHg l	40 (36,45)	42 (38,45)	40 (35.5,44)*	37 (34,42)*#
<35 mmHg, No. (%)	111 (20.1%)	23 (11.3%)	49 (21.6%)*	39 (32%)*#
>45 mmHg, No. (%)	153 (27.7%)	71 (35%)	55 (24.2%)*	27 (22.1%)*
Lactic acid, mmol/L m	2.1 (1.6,2.8)	2.1 (1.6,2.8)	2.1 (1.6,2.8)	2.2 (1.5,2.88)
Electrolytes [n]				
K+, mmol/L	3.84 (3.43,4.25)	3.88 (3.54,4.3)	3.8 (3.38,4.2)*	3.81 (3.4,4.21)
Na+, mmol/L	139.2 (136.1,142)	139.6 (137,142)	139 (136,142)	139 (135,142)
Cl -, mmol/L [o]	105.6 (102.8,107.8)	105.7 (103.28,107.5)	105.4 (102.7,107.8)	105.2 (102.35,108.38)
Coagulation function [p]				
Prothrombin time activity, % q	84 (74.9,93.3)	84.7 (76,96.75)	84.7 (76,92.8)*	78.85 (71.3,89.7)*#
Activated partial thromboplastin time, s	28.4 (26.2,31.2)	28.5 (26.35,31)	27.9 (25.6,31)*	29.05 (27.1,32)*#
D-dimer, μmol/L	0.81 (0.41,2.35)	0.51 (0.29,1.41)	0.93 (0.48,2.41)*	1.77 (0.7,5.49)*#

The total number of patients with available data: a: n(<60)=457, n(60-74)=355, n(≥75)=167; b: n(<60)=457, n(60-74)=355, n(≥75)=166; c: n(<60)=456, n(60-74)=354, n(≥75)=166; d: n(<60)=456, n(60-74)=355, n(≥75)=166; e: n(<60)=442, n(60-74)=354, n(≥75)=164; f: n(<60)=200, n(60-74)=312, n(≥75)=150; g: n(<60)=442, n(60-74)=354, n(≥75)=165; h: n(<60)=291, n(60-74)=312, n(≥75)=150; i: n(<60)=297, n(60-74)=312, n(≥75)=152; j: n(<60)=298, n(60-74)=202, n(≥75)=99; k: n(<60)=209, n(60-74)=231, n(≥75)=126; l: n(<60)=203, n(60-74)=227, n(≥75)=122; m: n(<60)=209, n(60-74)=230, n(≥75)=126; n: n(<60)=458, n(60-74)=357, n(≥75)=167; o: n(<60)=456, n(60-74)=355, n(≥75)=166; p:n(<60)=351, n(60-74)=337, n(≥75)=160; q: n(<60)=352, n(60-74)=338, n(≥75)=160. * p<0.05 vs. <60 group. # p<0.05 vs. 60-74 group.

Table 4. Inflammatory response and immunoreaction of COVID-19 patients on admission to hospital.

	Median (IQR)			
	all (n=1000)	**age<60(n=473)**	**60-74(n=359)**	**age≥75(n=168)**
Nonspecific inflammation index				
C-reactive protein, mg/L [a]	29.1 (5,70.9)	11.4 (5,46.98)	40 (7.35,83.85)*	54.3 (18.55,87.6)*#
>10, No. (%)	615 (64.8%)	226 (51.6%)	250 (72%)*	139 (84.8%)*#
High-sensitivity C-reactive protein, mg/L [b]	5 (5,5)	5 (1.75,5)	5 (5,5)*	5 (5,5)*#
>5, N0. (%)	138 (14.7%)	64 (14.8%)	46 (13.3%)	28 (17.2%)
Serum amyloid protein, mg/L [c]	59.42 (5,202.77)	9.94 (5,127.66)	200 (66.43,300)*	200 (61.48,300)*
>10, No. (%)	148 (61.2%)	78 (49.1%)	44 (83%)*	26 (86.7%)#
Erythrocyte sedimentation rate, mm/h [d]	54 (31,71)	40.5 (28,62.5)	62 (41,86)*	57.5 (39.75,83.5)*
Procalcitonin, ng/mL [e]	0.06 (0.04,0.13)	0.05 (0.03,0.1)	0.06 (0.04,0.13)*	0.12 (0.06,0.3)*#
>0.5, No. (%)	62 (7.4%)	13 (3.8%)	23 (7%)	26 (16.3%)*#
Cytokines [f]				
Interferon-γ, pg/mL	3.82 (2.72,5.53)	3.78 (3.01,6.02)	4 (2.81,5.4)	3.76 (2.16,4.58)
interleukin 2, pg/mL	3.49 (3.08,4.06)	3.48 (3.08,4.06)	3.55 (3.08,4.06)	3.2 (2.87,3.84)
interleukin 4, pg/mL	3.61 (3.01,4.16)	3.75 (3.16,4.29)	3.55 (3.01,4.1)	3.17 (2.81,3.85)*
Interleukin 5, pg/mL [g]	2.25 (2.13,2.35)	2.23 (2.13,2.34)	2.22 (2.11,2.42)	2.31 (2.31,2.33)
Interleukin 6, pg/mL [h]	7.04 (2.89,18.15)	5.71 (2.11,10.74)	7.19 (2.61,18.02)	16.66 (7.03,39.16)*#
interleukin 10, pg/mL	5.72 (4.75,7.38)	5.72 (4.74,7)	5.66 (4.59,7.4)	6.25 (4.93,7.54)
Tumor necrosis factor, pg/mL	3.11 (2.73,4.32)	3.06 (2.64,4.03)	3.26 (2.91,4.26)	3.04 (2.75,4.57)
Humoral immunity [i]				
Complement 3, g/L	1 (0.86,1.15)	1.02 (0.86,1.17)	1.01 (0.88,1.14)	0.95 (0.81,1.06)*#
Complement 4, g/L	0.25 (0.19,0.33)	0.26 (0.19,0.34)	0.24 (0.18,0.32)*	0.25 (0.2,0.32)
Immunoglobulin A, g/L	2.34 (1.76,3.01)	2.17 (1.64,2.61)	2.44 (1.85,3.21)*	2.68 (2.03,3.4)*
Immunoglobulin E, IU/mL	43.65 (18.3,122)	45.95 (18.3,131)	42.3 (18.3,115)	42.3 (18.3,120.5)
Immunoglobulin G, g/L	11.85 (10.03,14.2)	11.5 (9.9,13.58)	12.2 (10.2,14.6)*	12.5 (10.3,14.85)*
Immunoglobulin M, g/L	0.94 (0.68,1.23)	1.01 (0.74,1.29)	0.9 (0.66,1.2)*	0.84 (0.58,1.15)*
Cellular immunity [j]				
CD16+56, %	13.24 (8.52,20.58)	11.64 (7.54,18.08)	14.1 (9.26,20.29)*	16.82 (11.24,29.9)*#
CD16+56 counts, No./μL	118 (73,183)	117 (75.25,175)	118 (72.5,188)	118.5 (70,190.75)
CD19, %	15.29 (11.23,20.81)	14.92 (10.83,20.04)	16.32 (11.93,22.04)*	14.6 (10.15,21.76)#
CD19 counts, No./μL	136 (88,200)	142.5 (103,212.25)	137 (81.5,203.5)*	107 (60.25,151.75)*#
CD3, %	66.95 (57.01,74.25)	69.66 (61.85,75.67)	64.84 (56.05,72.72)*	61.24 (48.06,70.32)*#
CD3 counts No./μL	597 (378.5,904.5)	740 (508.25,1038.5)	552 (345.5,819)*	416 (243,639)*#
CD4, %	39.56 (31.64,46.06)	39.55 (32.52,45.65)	40.64 (32.76,47.79)	35.82 (29.07,45.89)*#
CD4 counts, No./μL	359 (217,548)	412 (273.5,613.75)	328 (209.5,531.5)*	266 (138,392.5)*#
CD8, %	22.17 (15.9,29.25)	25.21 (19.49,31.01)	20.19 (14.35,26.08)*	18.6 (12.12,24.99)*
CD8 counts, No./μL	211 (114,332)	263 (163.25,403.75)	177 (95,285)*	120.5 (64.75,230.5)*#
CD4/CD8	1.74 (1.24,2.65)	1.56 (1.16,2.12)	2.04 (1.4,3.07)*	1.93 (1.28,3.17)*

The total number of patients with available data: a: n(<60)=438, n(60-74)=347, n(≥75)=164; b: n(<60)=431, n(60-74)=347, n(≥75)=163; c: n(<60)=159, n(60-74)=53, n(≥75)=30; d: n(<60)=50, n(60-74)=39, n(≥75)=20; e: n(<60)=345, n(60-74)=329, n(≥75)=160; f: n(<60)=87, n(60-74)=69, n(≥75)=26; g: n(<60)=32, n(60-74)=15, n(≥75)=3; h: n(<60)=167, n(60-74)=165, n(≥75)=66; i: n(<60)=374, n(60-74)=309, n(≥75)=139; j: n(<60)=390, n(60-74)=319, n(≥75)=142. * p<0.05 vs. <60 group. # p<0.05 vs. 60-74 group.

Table 5. Initial pulmonary CT findings of COVID-19 patients.

Characteristics of lung CT, No. (%)	all (n=545)	age<60 (n=304)	60-74 (n=179)	age≥75 (n=62)
Pneumonia	509 (93.4%)	279 (91.8%)	174 (97.2%)*	56 (90.3%)
Unilateral lung	73 (13.4%)	60 (19.7%)	9 (5%)*	4 (6.5%)*
Bilateral lung	454 (83.3%)	227 (74.7%)	169 (94.4%)*	58 (93.5%)*
Ground-glass opacity	405 (74.3%)	227 (74.7%)	141 (78.8%)	37 (59.7%)*#
Paving stone/reticular/linear	141 (25.9%)	62 (20.4%)	58 (32.4%)*	27 (43.5%)*
Consolidation shadow	74 (13.6%)	46 (15.1%)	25 (14%)	3 (4.8%)*
Air bronchogram	59 (10.8%)	27 (8.9%)	27 (15.1%)*	5 (8.1%)

Abbreviation: CT: computerized tomography. * p<0.05 vs. <60 group. # p<0.05 vs. 60-74 group.

glucocorticoids (50%) and immunoglobulin therapy (51.3%). The proportion of patients receiving immunoglobulin therapy increased significantly with increasing age. No difference was found in patients receiving special supporting treatments, including continuous renal replacement therapy (CRRT), extracorporeal membrane oxygenation (ECMO), or artificial liver support system (ALSS) (Table 6).

Clinical classification and prognosis of COVID-19 patients

The proportion of patients with critical conditions increased (18% vs. 27.3% vs. 50.6%) with advancement of age. A total of 119 patients (11.9%) died, and 197 patients (19.7%) were discharged or transferred to isolation points during hospitalization. The percentage of deaths increased with age (5.1% vs. 11.7% vs. 31.5%). However, there was no significant difference in the duration from admission to death or duration from disease onset to death among the three groups (Table 7).

Age group 3 had a higher cumulative death risk than group 2 (p<0.05), which had a higher risk than group 1 (p<0.05). After adjusting for sex and comorbidity status, patients in group 2 (HR, 1.944, 95% CI, 1.156-3.271) and group 3 (HR, 4.777, 95% CI, 2.850- 8.008) were more likely to die than patients in group 1 according to observation from admission. Patients in group 2 (HR, 1.849, 95%CI 1.1-3.108) and group 3 (HR, 4.770, 95%CI, 2.841-8.008) were more likely to die according to observation from disease onset (Figure 1A and 1B).

Age and comorbidities were incorporated into the proportional hazards model, and the analysis showed that patients with hypertension (HR, 1.974, 95% CI, 1.297-3.003), cerebrovascular disease (HR, 2.1, 95% CI, 1.157-3.809) were more likely to die than those without. As compared with patients without comorbidity, the HR (95% CI) was 1.71 (1.063-2.75) among patients with one comorbidity and 2.348 (1.464-3.766) among patients with two or more comorbidities after adjusting for age groups (Figure 2A). Another proportional hazards model incorporating age and complications showed that patients with acute cardiac injury (HR, 4.876, 95% CI, 2.993-7.945), shock (HR, 3.855, 95% CI, 2.436-6.101) were more likely to die than those without. As compared with patients without complications, the HR (95% CI) was 6.793 (4.295-10.746) among patients with only one complication and 13.125 (8.249-20.884) among patients with two or more complications after adjusting for age groups (Figure 2B). (all P <0.05)

DISCUSSION

In this study, we found that age had a significant impact on the clinical characteristics and outcomes of COVID-19 patients. The symptoms of the elderly patients were more atypical than those of the young patients and were characterized by more comorbidities. More older patients had organ damage, immune dysfunction, and more severe inflammation on admission. During hospitalization, more older patients received oxygen therapy and experienced more complications. Most importantly, older patients were more likely to develop critical illness with a significantly higher mortality rate. The previous literature [10] discussed the mortality rate of COVID-19 patients at different ages by constructing models. It was speculated that the disease in Wuhan was more serious and that the mortality rate in the elderly population was higher. Our research further supports these views, and we found that the case fatality rate was significantly higher than that reported in this literature, which may be related to the fact that we did not include other patients, such as patients in mobile cabin hospitals. However, we described the clinical characteristics of patients at different ages and analyzed the relevant mechanisms that led to poor prognosis, which are complementary to the findings of the previous study [10].

According to the results of the study, we found that older patients, especially super-aged patients, may have

Table 6. Complications and treatments of COVID-19 patients.

	all (n=1000)	age<60(n=473)	60-74(n=359)	age≥75(n=168)
Complications, No. (%)				
Shock	81 (8.1%)	26 (5.5%)	29 (8.1%)	26 (15.5%)*#
Acute cardiac injury	116 (11.6%)	19 (4%)	45 (12.5%)*	52 (31%)*#
Acute renal injury	29 (2.9%)	6 (1.3%)	12 (3.3%)*	11 (6.5%)*
Acute liver injury	64 (6.4%)	36 (7.6%)	23 (6.4%)	5 (3%)*
≥1 complication	191 (19.1%)	62 (13.1%)	68 (18.9%)*	61 (36.3%)*#
Only one complication	125 (12.5%)	49 (10.4%)	40 (11.1%)	36 (21.4%)*#
≥2 complications	66 (6.6%)	13 (2.7%)	28 (7.8%)*	25 (14.9%)*#
Admission to ICU, No. (%)	63 (6.3%)	20 (4.2%)	26 (7.2%)	17 (10.1%)*
ICU treatment duration, day, Median (IQR)	7 (3,11)	7 (4,11)	10 (5,12)	4 (2,7)#
Oxygen therapy, No. (%)				
Nasal catheter oxygen inhalation	661 (66.1%)	270 (57.1%)	261 (72.7%)*	130 (77.4%)*
Mask oxygen inhalation	314 (31.4%)	120 (25.4%)	120 (33.4%)*	74 (44%)*#
HFBHTI	91 (9.1%)	29 (6.1%)	35 (9.7%)	27 (16.1%)*#
Non-invasive mechanical ventilation	147 (14.7%)	46 (9.7%)	57 (15.9%)*	44 (26.2%)*#
Invasive mechanical ventilation	43 (4.3%)	15 (3.2%)	18 (5%)	10 (6%)
Medical treatment, No. (%)				
Antiviral treatment	927 (92.7%)	435 (92%)	340 (94.7%)	152 (90.5%)
Antibiotic treatment	783 (78.3%)	362 (76.5%)	288 (80.2%)	133 (79.2%)
Antifungal treatment	32 (3.2%)	11 (2.3%)	15 (4.2%)	6 (3.6%)
Glucocorticoids	500 (50%)	220 (46.5%)	197 (54.9%)*	83 (49.4%)
Immunoglobulin therapy	513 (51.3%)	219 (46.3%)	200 (55.7%)*	94 (56%)*
Special treatment, No. (%)				
CRRT	15 (1.5%)	6 (1.3%)	6 (1.7%)	3 (1.8%)
ECMO	2 (0.2%)	2 (0.4%)	0 (0%)	0 (0%)
ALSS	10 (1%)	6 (1.3%)	4 (1.1%)	0 (0%)

Abbreviation: ALSS: artificial liver support system; CRRT: continuous renal replacement therapy; ECMO: extracorporeal membrane oxygenation; ICU: intensive care unit; IQR: interquartile range. P values indicate differences between male and female patients. * p<0.05 vs. <60 group. # p<0.05vs. 60-74 group.

atypical symptoms, and it may be harder to accurately identify older patients with COVID-19. Similar to previous reports [7, 15], the most common symptom was fever. However, the rate of fever in super-aged patients was significantly lower than that in younger patients. More elderly people showed symptoms of expectoration, dyspnea and chest tightness, which may be considered symptoms of underlying diseases such as COPD or coronary heart disease. The proportion of older patients with muscle pain decreased as they aged. Therefore, the symptoms of older patients seem to be more atypical than those of younger patients, making it harder to identify SARS-CoV-2 infection early. The results showed that the older patients presented with a slightly longer duration from the onset of symptoms to admission. A previous study showed that the main CT findings were ground-glass opacities and nodules [16]. However, our study found that super-aged patients showed a lower proportion of ground-glass opacities but more paving stone/reticular/linear findings than younger patients. It was suggested that CT images of super-aged patients may not be as typical as those of younger patients, and CT diagnosis alone was prone to misdiagnosis. It should be noted that the elderly with

Table 7. Clinical classification and prognosis of COVID-19 patients.

	all (n=1000)	age<60(n=473)	60-74(n=359)	age≥75(n=168)
Clinical classification, No. (%)				
Mild-Moderate	385 (38.5%)	250 (52.9%)	105 (29.2%)*	30 (17.9%)*#
Severe	347 (34.7%)	138 (29.2%)	156 (43.5%)	53 (31.5%)*#
Critical	268 (26.8%)	85 (18%)	98 (27.3%)*	85 (50.6%)*#
Prognosis, No. (%) or Median (IQR)				
Death	119 (11.9%)	24 (5.1%)	42 (11.7%)*	53 (31.5%)*#
Onset of disease to death, d	17 (13,21)	17 (13.8,20)	17 (13,21)	16 (12,22)
From hospitalization to death, d	6 (3,10)	7 (4,10)	6 (3,9)	7 (3,11)
Discharge or Transfer to the isolation point	197 (19.7%)	134 (28.3%)	52 (14.5%)*	11 (6.5%)*#
Staying in hospital	673 (67.3%)	310 (65.5%)	259 (72.1%)*	104 (61.9%)#

Abbreviation: d: day; IQR: interquartile range. * p<0.05 vs. <60 group. # p<0.05vs. 60-74 group.

atypical symptoms may be missed, leading to the spread of infection. Therefore, the diagnosis of the elderly should be more careful.

Deaths and serious consequences in older patients have also been reported for other human coronaviruses, such as HCoV-OC43 [17], SARS-CoV [3], and MERS-CoV [18]. Older age has been reported as an important independent risk factor for mortality in SARS [19] and MERS [20]. A recent study confirmed that increased age was associated with death in patients with COVID-19 [14]. However, the mechanisms between poor prognosis and older age remain unclear.

After comparing the characteristics of COVID-19 patients of different ages, we found that the poor prognosis may be related to the higher proportion of comorbidities in older patients. In this study, the major comorbidities were hypertension, diabetes, and CHD. The proportion of old patients with comorbidities was much higher than that of young patients. The super-aged patients were more likely to have more than one comorbidity. Old patients with chronic comorbidities were more sensitive to SARS-CoV-2 because metabolic diseases were reported to lead to weaker immune functions [21]. A series of studies showed that the combined comorbidities were one of the independent risk factors of the poor prognosis of COVID-19 patients [14, 22–24]. Besides, our study reported that the comorbidities were an independent risk factor of death. The poor outcome of the older COVID-19 patients may be associated with the higher rate of comorbidities.

In addition, comorbidities such as hypertension, CHD and COPD lead to cardiac and pulmonary dysfunction. The deteriorated lung and cardiac function in the elderly may be associated with poor prognosis. With increasing age, changes in the anatomy of the lungs and muscle atrophy in the elderly lead to changes in the physiological functions of the respiratory system, reduced airway clearance, reduced lung reserve, and reduced barrier function. As shown in the results, the proportion of patients with dyspnea in the older age group was significantly higher than that in the young age group. The arterial oxygen saturation and arterial partial pressure of oxygen decreased with age. The proportion of patients receiving oxygen therapy regardless of nasal catheter, mask, or noninvasive mechanical ventilation in the older group was significantly higher than that in the younger group. These results indicate that older patients were more likely to develop worse conditions. Although we did not determine the number of patients who died of respiratory failure or heart failure, there was a report indicating that heart failure was observed in addition to respiratory failure in the patients who died [23].

In addition, we found that the proportion of patients with complications increased with age. Older patients are more likely to have more than one complication, especially super-aged patients. Complications such as cardiac injury were reported to be related to the poor prognosis of COVID-19 patients [12, 25–27]. And our result showed that the complications were an independent predictor of fatality. Therefore, the high mortality of older patients may be associated with a higher rate of complications.

This study showed that increases in nonspecific inflammation biomarkers, including CRP, hs-CRP,

serum amyloid protein and procalcitonin, were more likely to occur in older patients. The proportion of patients with increased white blood cells and neutrophils in the older age groups was significantly higher than that in the younger age group. These findings suggest that older patients may be more likely to have a secondary infection with other bacteria, which may lead to poor prognosis [28]. Kim et al. reported that viral-bacterial coinfection was an independent predictor of mortality from viral pneumonia [29]. Although there was no difference in antibiotic treatment between the age groups, we believe that older patients need antibiotics more to prevent coinfection. Coinfection may be a risk factor for poor prognosis in older patients [30], but more research is needed to confirm this hypothesis.

Moreover, we speculate that the poor prognosis of older patients was related to the aging of the immune system. Immune system aging is an important process in the human aging process and is mainly manifested by the progressive degeneration of innate and adaptive

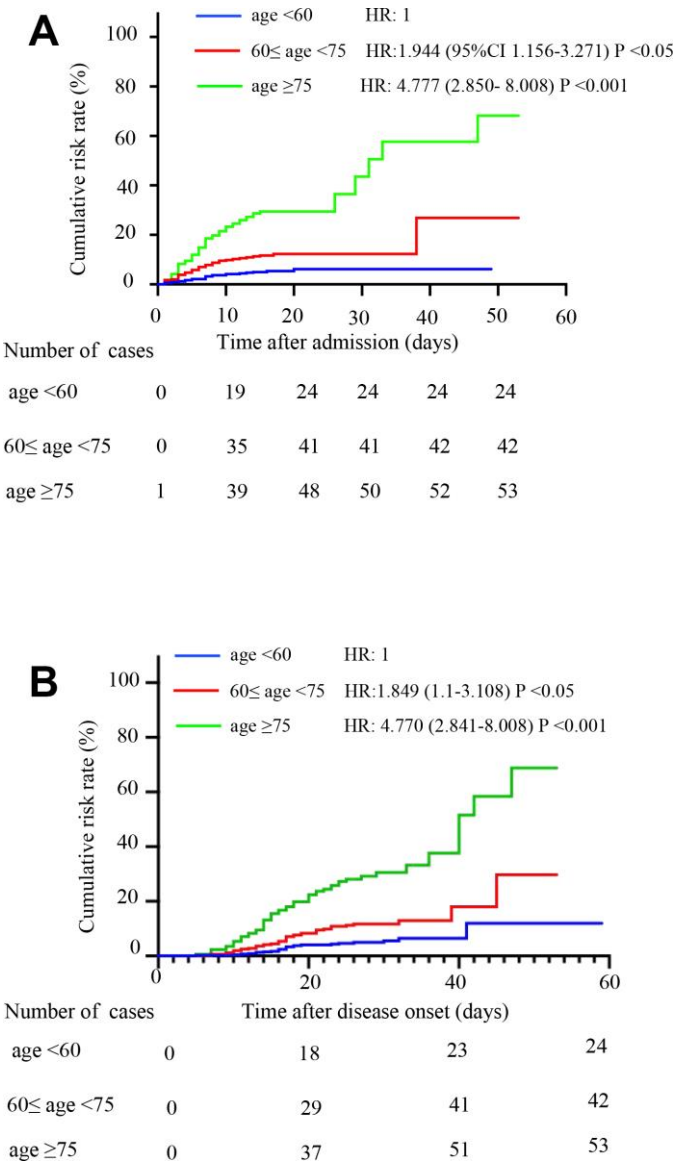

Figure 1. Comparison of the time-dependent risk of death. (**A**) The cumulative death risk after admission in age group 1 (blue curve), age group 2 (red curve) and age group 3 (green curve). Compared to age group 1, the hazard ratios (HRs) and 95% confidence intervals (95% CIs) of age groups 2 and 3 were HR: 1.944 (1.156-3.271; P <0.05) and HR: 4.777 (2.850- 8.008; P <0.001), respectively. The model was adjusted for sex and comorbidities. (**B**) The cumulative death risk after disease onset in age group 1 (blue curve), age group 2 (red curve) and age group 3 (green curve). Compared to age group 1, the HRs (95% CIs) of age groups 2 and 3 were HR: 1.849 (1.1-3.108; P <0.05) and HR: 4.77 (2.841-8.008; P <0.001). The model was adjusted for sex and comorbidities.

immunity [31]. Because of immune aging, older patients are more susceptible to and impacted by bacteria and viruses, especially with comorbidities such as COPD. Previous research on SARS-CoV-inoculated macaques has found that older macaques had a stronger innate response to virus-infected hosts and increased expression of genes related to inflammation [32]. Defects in age-dependent T and B cell function and overproduction of type 2 cytokines may lead to inadequate viral replication control and longer pro-inflammatory responses, which may lead to adverse results [33]. In this study, we observed that the rate of lymphopenia was higher in the older age groups than in the younger age group. The function of humoral and cellular immunity was significantly downregulated in older patients. The proportion of patients receiving immunoglobulin therapy increased significantly with age. The expression level of IL-6 in super-aged patients was significantly increased. These results suggest that the immune system and inflammatory reaction of COVID-19 patients were disturbed. It was reported that SARS-CoV-2 may mainly affect T lymphocytes, especially CD4+ T cells, resulting in a significant decrease in lymphocyte numbers [34]. The extent of lymphopenia and increase in inflammatory

A

Features	Hazard Ratio (95% CI)	P Value
Types of comorbidities		
Hypertension	1.974 (1.297-3.003)	0.001*
Diabetes	0.962 (0.576-1.608)	0.884
Coronary heart disease	0.972 (0.547-1.726)	0.922
COPD	1.477 (0.627-3.481)	0.373
Cerebrovascular disease	2.1 (1.157-3.809)	0.015*
Chronic renal disease	1.365 (0.634-2.942)	0.427
Chronic liver disease	1.218 (0.462-3.207)	0.69
Malignancy	0.737 (0.295-1.842)	0.514
Number of comorbidities		
1	1.71 (1.063-2.75)	0.027*
2 or more	2.348 (1.464-3.766)	<0.001*

B

Features	Hazard Ratio (95% CI)	P Value
Types of complications		
Shock	3.855 (2.436-6.101)	<0.001*
Acute cardiac injury	4.876 (2.993-7.945)	<0.001*
Acute renal injury	0.84 (0.484-1.457)	0.534
Acute liver injury	1.026 (0.534-1.973)	0.938
Number of complications		
1	6.793 (4.295-10.746)	<0.001*
2 or more	13.125 (8.249-20.884)	<0.001*

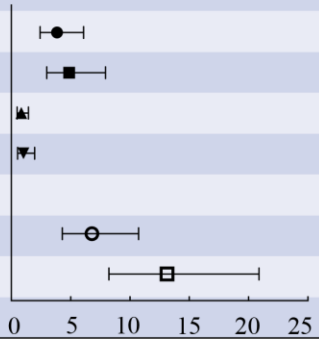

Figure 2. Predictors of the death in the proportional hazards model. (**A**) Shown in the figure are the hazards ratio (HR) and the 95% confidence interval (95%CI) for the risk factors of death after disease onset. The comorbidities were classified according to the organ systems as well as the number. (**B**), Shown in the figure are the hazards ratio (HR) and the 95% confidence interval (95%CI) for the risk factors of death after disease onset. The complications were classified according to the organ systems as well as the number. * means the P value <0.05. The scale bar indicates the HR. The model has been adjusted with age groups.

cytokines were related to the severity of the disease [35]. Therefore, we speculate that the poor prognosis of the older patients was associated with the disturbed immune system and inflammation.

In summary, the high case fatality rate of elderly patients was related to comorbidities, reduced heart and lung function, complications, secondary infections and disturbed immune system and inflammation.

There are several limitations to our study. First, the follow-up period was short, and data on the outcomes of many patients were not collected. An extended follow-up period may help us better understand the prognosis of older patients. Second, this study is a retrospective study. We analyzed only the initial laboratory results. The dynamic changes in different markers during hospitalization should be further analyzed. Third, this study is only a descriptive study, and the mechanisms underlying the relationship between poor prognosis and age require further research. Fourth, this study is a single-center study. Patients included in the study were all from Renmin Hospital. And they were mainly severe and critically ill patients.

MATERIALS AND METHODS

Study design and participants

This is a retrospective study and was approved by the Ethics Commission of Renmin Hospital of Wuhan University. Data from a total of 1000 confirmed COVID-19 patients admitted to both the Shouyi and East districts of Renmin Hospital of Wuhan University from January 1, 2020 to February 14, 2020 were collected. The patients' age ranges from 21 to 101 years; According to age, patients were divided into three groups: age group 1 (<60 years old), age group 2 (60-74 years old) and age group 3 (≥75 years old).

Diagnostic criteria

The diagnosis of COVID-19 were performed according to The Diagnosis and Treatment Guidelines of Pneumonia Caused by Novel Coronavirus (6th trial edition) published by the General Office of the National Health Commission and the General Office of the National Administration of Traditional Chinese Medicine [36].

Confirmed cases should be suspected cases with one of the following etiological evidences: 1) positive nucleic acid test; 2) sequencing of viral genes, highly homologous to known SARS-CoV-2.

The diagnosis of suspected cases needs to be combined with the following comprehensive analysis of epidemiological history and clinical manifestations. Epidemiology history: 1) travel history or residence history of Wuhan city and surrounding areas, or other communities with case reports within 14 days before onset; 2) had a history of contact with SARS-CoV-2 infection (positive nucleic acid test) within 14 days before onset; 3) had a history of contact with patients with fever or respiratory symptoms from Wuhan and surrounding areas, or from communities with case reports within 14 days before onset; 4) clustering onset of COVID-19 infection. Clinical manifestations: 1) fever or respiratory symptoms; 2) with the imaging features of COVID-19; 3) with normal or decreased number of white blood cells and reduced lymphocyte count in the early stage. Patients who met one of the following conditions were defined as suspected cases: 1) meet any one in the history of epidemiology and any two in the clinical manifestations; 2) no definite epidemiological history, but with all the three items in the clinical manifestations.

Evaluation of clinical results

The onset of a disease was defined as the time when the associated symptoms first appeared. The outcome information of these patients was collected until February 24, 2020, including whether they were still in the hospital, improved and were discharge or were transferred to the isolation area for continued isolation and death.

According to the sixth edition of the Novel Coronavirus Pneumonia Diagnosis and Treatment Plan, the disease is generally classified into four types: mild, moderate, severe, and critical. Patients who met one of the following conditions were defined as severe: 1) dyspnea, breathing frequency >30 times per minute or 2) finger oxygen saturation ≤ 93%. Patients who met any of the following conditions were defined as critical: 1) respiratory failure requiring mechanical ventilation; 2) vibration; and 3) any concomitant organ failure other than respiratory failure, requiring monitoring and treatment in the intensive care unit (ICU). Other patients were classified as mild-moderate.

Criteria for target organ injury

Plasma hypersensitivity troponin I (hs-TnI) levels above the 99% reference line were considered to indicate acute heart injury. Alanine aminotransferase (ALT≥150 U/L) increasing to three-fold higher than normal was considered to indicate acute liver injury (ALI). Patients with one of the following conditions could be diagnosed with acute kidney injury (AKI): 1) the highest serum creatinine (Scr) level increased by more than 26.5 μmol/L (0.3 mg/dL) within 48 hours; 2) Scr exceeded the baseline value by 1.5-fold

(confirmed or estimated to occur within 7 days); and 3) urine output <0.5 ml/kg * h), lasting more than 6 hours. Patients with septic shock were clinically identified by vasopressor requirement to maintain a mean arterial pressure of 65 mmHg or greater and a serum lactate level greater than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia [37].

Data collection

Information including the patient's hospitalization, medical history, clinical symptoms, signs, laboratory tests, chest computed tomography (CT) scan, treatment, and outcome or prognosis was obtained through the hospital's medical record system. We analyzed the image of the first CT scan in our hospital within three days after admission. The first results of laboratory tests after admission were analyzed.

Statistical analysis

Continuous variables are represented by medians and interquartile ranges (IQRs), and categorical variables are represented by numbers (percentages). The Mann-Whitney U test was used to analyze continuous variables. The χ^2 test or Fisher's exact test was used to analyze the classified variables. The Kaplan-Meier test was used to investigate the cumulative death rate among the three groups. Cox proportional hazard regression models were applied to determine the potential risk factors associated with the mortality, with the hazards ratio (HR) and 95% confidence interval (95%CI) being reported. The statistical software package of Social Sciences (SPSS 26.0) was used for analysis, and a P value < 0.05 was considered statistically significant.

Abbreviations

COVID-19: coronavirus disease 2019; WHO: World Health Organization; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SARS: severe acute respiratory syndrome; MERS: Middle East respiratory syndrome; CAD: coronary artery disease; CVD: cerebrovascular disease; hs-TnI: hypersensitive troponin I; CKMB: creatinine kinases MB isoenzyme; LDH: lactate dehydrogenase; CRP: C-reactive protein; hs-CRP: high-sensitivity C-reactive protein; PCT: procalcitonin; SAA: serum amyloid protein; IL: interleukin; ICU: intensive care unit; CRRT: continuous renal replacement therapy; ECMO: extracorporeal membrane oxygenation; ALSS: artificial liver support system; ALT: alanine aminotransferase; ALI: acute liver injury; AKI: acute kidney injury; Scr: serum creatinine; CT: computed tomography; IQR: interquartile ranges; HR: hazard ratio; CI: confidence interval.

AUTHOR CONTRIBUTIONS

Dr. Jun Wan and Dr. Menglong Wang had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Mengmeng Zhao, Menglong Wang, Jishou Zhang, Pingan Zhang and Jian Gu contributed equally to this work and are co–first authors.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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Research Paper

ACE2 and *TMPRSS2* variants and expression as candidates to sex and country differences in COVID-19 severity in Italy

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ABSTRACT

As the outbreak of coronavirus disease 2019 (COVID-19) progresses, prognostic markers for early identification of high-risk individuals are an urgent medical need. Italy has one of the highest numbers of SARS-CoV-2-related deaths and one of the highest mortality rates. Worldwide, a more severe course of COVID-19 is associated with older age, comorbidities, and male sex. Hence, we searched for possible genetic components of COVID-19 severity among Italians by looking at expression levels and variants in *ACE2* and *TMPRSS2* genes, crucial for viral infection. Exome and SNP-array data from a large Italian cohort were used to compare the rare-variants burden and polymorphisms frequency with Europeans and East Asians. Moreover, we looked into gene expression databases to check for sex-unbalanced expression.
While we found no significant evidence that *ACE2* is associated with disease severity/sex bias, *TMPRSS2* levels and genetic variants proved to be possible candidate disease modulators, prompting for rapid experimental validations on large patient cohorts.

INTRODUCTION

As we write, Italy, Europe, and the entire world are facing one of the worst medical emergencies spanning centuries, the coronavirus disease 2019 (COVID-19) pandemia due to infection by SARS-CoV-2 virus. The early identification of risk factors for COVID-19 is an urgent medical need to provide the appropriate support to patients, including access to intensive care units.

Presently, Italy has one of the highest rate of SARS-CoV-2 infection in the world among large countries, with 371 cases per 100,000 people, one of the highest number of deaths and apparently also one of the highest mortality rates, 14.1% vs. an average value of 6.6% (as of May 16th, 2020, data from https://coronavirus.jhu.edu/map.html). These data may have different explanations, including: 1) the number of tests performed, 2) the structure of the population (Italy has the oldest population in Europe) [https://ec.europa.eu/eurostat/data/database], 3) the percentage of smokers, even though no significant association was found between smoking and severity of COVID-19 in a very recent study on the Chinese population [1], 4) the possible existence of a different virus strain [2], 5) a high population density in some hot spot areas of the infection, 6) the concentration of severe cases in a limited region of the country, potentially overwhelming the available intensive care units, 7) differences in environmental factors (e.g. air pollution), as well as 8) social factors, such as trust in the institutions and tendency to socialize [3]. However, there could also be some peculiar genetic characteristics of the Italian population that may have an impact on the susceptibility to viral infection, the

disease severity, and the number of patients shedding huge amounts of virus.

What is unquestionable is a more severe course of the disease associated with older age and high number of comorbidities and with the male sex (male:female ratio in case fatality rate among Italians 1.75, data from the Italian National Institute of Health: https://www.epicentro.iss.it/coronavirus/), a feature shared with the 2003 SARS epidemic and MERS [4–6]. Indeed, while males and females have similar susceptibility to both SARS-CoV-2 and SARS-CoV, males are more prone to have higher severity and mortality, independently of age [4]. Among the many possible factors impacting on sex-related differences in disease manifestations, including the fact that females are known to mount a stronger immune response to viral infections compared to males due to more robust humoral and cellular immune responses [7], we decided to center our attention on possible genetic components, with a particular focus on the Italian population.

It was recently demonstrated that both angiotensin I converting enzyme 2 (ACE2) and the transmembrane protease, serine 2 (TMPRSS2) are crucial for SARS-CoV-2 entry into host cells [8, 9]. As previously described for SARS-CoV, ACE2 is the main receptor also for the spike (S) protein of SARS-CoV-2, mediating viral attachment to target cells. Moreover, both coronaviruses use TMPRSS2 for protein S priming, i.e. the cleavage of protein S at the S1/S2 and the S2' sites, allowing fusion of viral and cellular membranes [9]. Both genes have been proposed to modulate susceptibility to SARS-CoV [10, 11], and are good candidates to mediate sex-related effects: *ACE2* is located on the X chromosome, while *TMPRSS2* expression is responsive to androgen/estrogen stimulation [12]. Controversial data have been reported on the level of expression of *ACE2* in the lung of males and females [13–15], however, it must also be taken into account the effect of estrogen drop in postmenopausal life and the possible compensating effect of hormone replacement therapy in some females.

With this background, we searched for possible genetic components of COVID-19 severity among Italians by looking at expression levels and genetic variants in *ACE2* and *TMPRSS2*, two crucial genes for viral infection.

RESULTS AND DISCUSSION

ACE2

For most X-chromosome genes, the double allelic dosage in females is balanced by the epigenetic silencing of one of the X chromosomes in early development [16]. However, the X-chromosome inactivation (XCI) is incomplete in humans and up to one third of genes are expressed from both alleles, with the degree of XCI escape varying between genes and individuals [17]. *ACE2* is one of the genes escaping X inactivation, but it belongs to a subgroup of X-chromosome genes escaping XCI showing an uncharacteristically heterogeneous pattern of male-female expression, with higher expression in males in several tissues [13]. Specifically concerning the lung, a recent analysis on published expression data, reported a substantial similar level of *ACE2* transcript in males and females [14], however, another study, using single-cell sequencing, found a higher expression of *ACE2* in Asian males [15]. Figure 1 reports data on *ACE2* mRNA expression levels in the lung as retrieved from the largest datasets available in the literature; no substantial differences were found between males and females, nor between younger and older females, thus confirming what already observed by Cai and colleagues [14].

Another possible sex-related effect might be due to the fact that males are hemizygous for the gene, therefore, in the presence of an *ACE2* allelic variant increasing disease susceptibility or severity, males will have all cells expressing the risk variant. Based on this hypothesis, we looked into the genetic variation in *ACE2*. A recent manuscript explored this same topic in different populations using data from public databases [18]. However, a specific analysis of the Italian population is lacking.

We have therefore exploited the available data on 3,984 exomes obtained from an Italian cohort representative of the whole country [19, 20] to extract the variants in exons and splice junctions of *ACE2*. Variants were filtered for quality and classified according to their predicted effect at protein level and on splicing. Concerning rare variants (i.e. those with a minor allele frequency, MAF, <1%; to be used in burden tests), we considered only null variants, abolishing or significantly impairing protein production (nonsense, out-of-frame ins/dels, and splicing variants), and missense variants predicted to be deleterious or possibly deleterious by all the 5 prediction algorithms used (see Supplementary Methods, paragraph "Definition of disrupting variants and statistical analysis"). Concerning common variants (i.e., MAF>5%), all were retained for comparing their frequency with those of the European (non Finnish) and East Asian populations, retrieved from the GnomAD repository.

No significant differences in the burden of rare deleterious variants were observed comparing the Italian population with Europeans and East Asians (Table 1A). Concerning common exonic variants, the

only striking difference, as also noticed by Cao and colleagues [18], was observed for the single nucleotide polymorphism (SNP) rs2285666 (also called G8790A), with the frequency of the rare A allele being 0.2 in Italians and Europeans, and 0.55 in East Asians (uncorrected P=2.2*10-16 for difference in Italians vs East Asians; corrected P=7.9*10-15; Table 1B). This variant was extensively studied as a potential risk factor

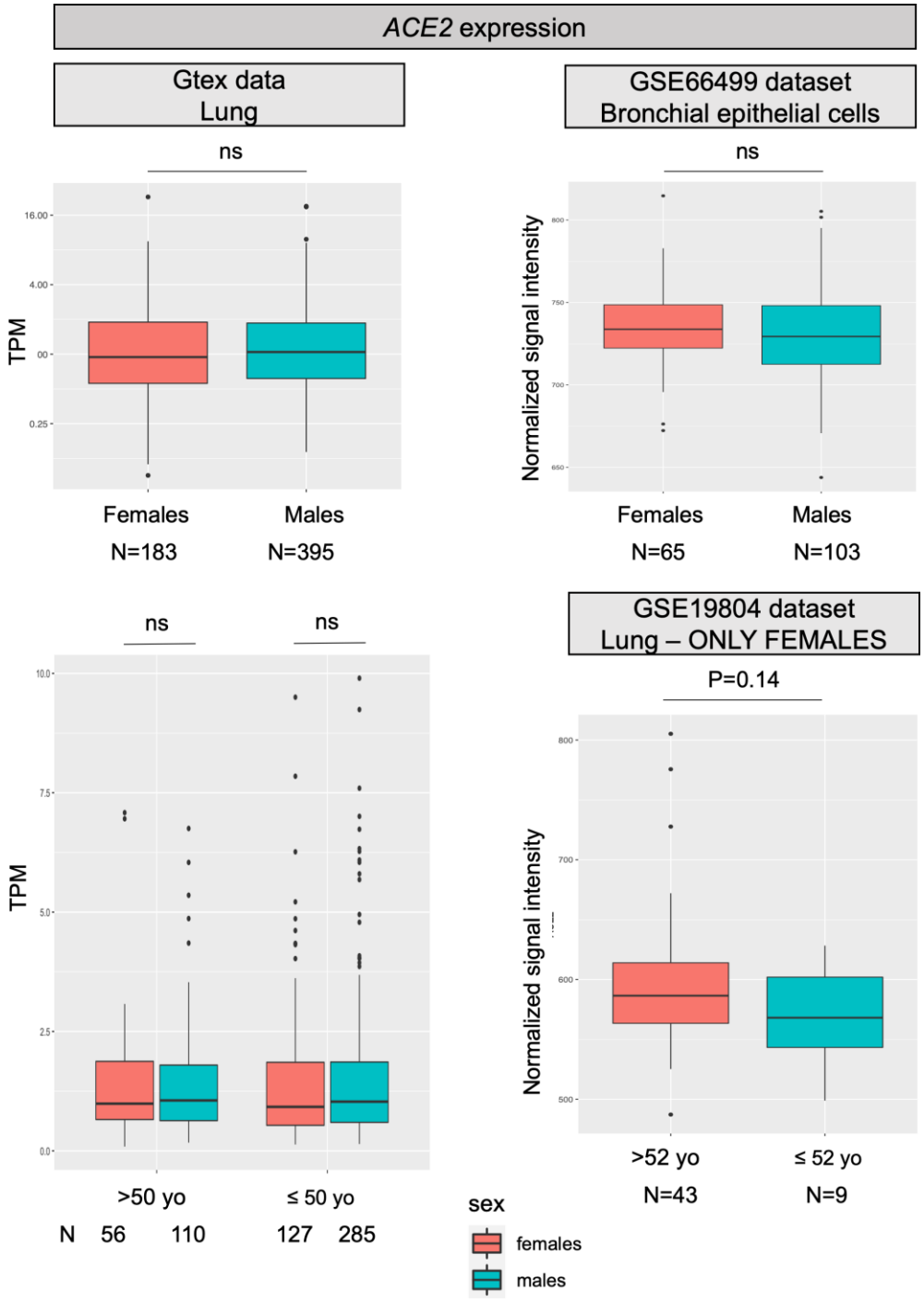

Figure 1. *ACE2* expression levels. All panels show *ACE2* mRNA expression levels in human normal lung samples stratified according to sex (or on sex and age). On left panels, data were retrieved for a total of 578 RNAseq experiments from the GTex repository. Expression levels are reported as transcripts per kilobase million (TPM). On the right, data were collected from two different datasets (GSE66499 and GSE19804) from the GEO database. Expression levels are reported as normalized signal intensities. P values were calculated by using either the Kruskal-Wallis or the student t test, using the R software (https://www.r-project.org/).

Table 1A. Burden of rare mutations in the *ACE2* gene in different populations.

Population	N alleles	T1	Freq T1	ITA	EUR	EAS
ITA	4422	7	0.0016	-	P=0.518	P=0.974
EUR	92545	200	0.0022	P=0.518	-	P=0.077
EAS	14840	21	0.0014	P=0.974	P=0.077	-

Total allele counts, carrier allele counts, and carrier frequencies are shown; only deleterious variants with MAF less than 1% were considered in the burden analysis. The 'deleterious' set is defined by missense variations predicted to be possibly damaging by all the 5 algorithms used (LRT score, MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT), and loss-of-function variants (nonsense, frameshift, and splicing variants affecting the donor/acceptor sites).
P values are presented as non-corrected; the number of statistical comparisons performed in Tables 1A, 1B, 2A, and 2B is collectively of 24, thus lowering the threshold for significance at P=0.0021 (Bonferroni threshold).
T1: alleles carrying damaging variants; Freq T1: frequency of T1 allele; ITA: Italian population; EUR: European population; EAS: East Asian population.

Table 1B. Common exon variants in the *ACE2* gene in different populations.

Variant ID	Consequence	A1/N alleles ITA	Freq ITA	A1/N alleles EUR	Freq EUR	A1/N alleles EAS	Freq EAS	ITA Vs EUR	ITA Vs EAS	EUR Vs EAS
rs2285666	c.439+4G>A	909/4408	0.206	17240/86164	0.200	7336/13387	0.548	0.331	**P<2.2e-16**	**P<2.2e-16**
rs35803318	p.Val749Val	235/4422	0.053	3935/88946	0.044	0/13918	0.0	P=0.0058	**P<2.2e-16**	**P<2.2e-16**

Total allele counts, carrier allele counts, and carrier frequencies are shown; only variants with MAF more than 5% were considered.
P values are presented as non-corrected; the number of statistical comparisons performed in Tables 1A, 1B, 2A, and 2B is collectively of 24, thus lowering the threshold for significance at P=0.0021 (Bonferroni threshold). Significant P values are indicated in bold.
A1: alleles carrying variants; Freq A1: frequency of A1 allele; ITA: Italian population; EUR: European population; EAS: East Asian population.

for hypertension, type 2 diabetes, and coronary artery disease [21, 22], hence possibly constituting a predisposing factor also for the comorbidities observed in COVID-19 patients. A single paper reports the association of the three rs2285666 genotypes with ACE2 protein level measured in serum by ELISA, with the A/A genotype having an expression level almost 50% higher than the G/G genotype, while heterozygous G/A individuals had intermediate levels [23]. Given the position of the variant, at nucleotide +4 in the donor splice site of intron 3 (c.439+4G>A), we calculated the predicted effect on splicing and indeed the substitution of G with an A is predicted to increase the strength of the splice site of about 9.2% (calculation made through the Human Splicing Finder v.3.1 webtool, http://www.umd.be/HSF/), consistently with the higher level of ACE2 protein in serum. It would be crucial to compare the frequency of this variant with *ACE2* expression in the lung and with susceptibility to viral infection and severity of COVID-19 manifestations. Of note, no eQTL for *ACE2* in the lung has been described so far in the GTEx database, and investigations on this topic are recommended.

TMPRSS2

TMPRSS2 is a gene well known to oncologists as genetic rearrangements producing a fusion between *TMPRSS2* and *ERG* (or, more rarely, other members of the ETS family) are the most frequent genetic lesions in prostate cancer patients [24]. As *TMPRSS2* is an androgen responsive gene, the fusion results in androgen dependent transcription of ERG in prostate tumor cells. Therefore, we can hypothesize that males might have higher *TMPRSS2* expression also in the lung, which might improve the ability of SARS-CoV-2 to enter cells by promoting membrane fusion. Looking into GTEx and GEO data, the overall expression of *TMPRSS2* in the lung is only slightly increased in males (P=0.029; Figure 2A). However, *TMPRSS2* expression is also promoted by estrogens [12], and therefore the situation might be different when considering individuals above 60 years, who are at higher risk of fatal events due to COVID-19, as in this group females will all be postmenopausal. According to this hypothesis, we checked the expression of the gene in lungs of males and females at different ages, but no

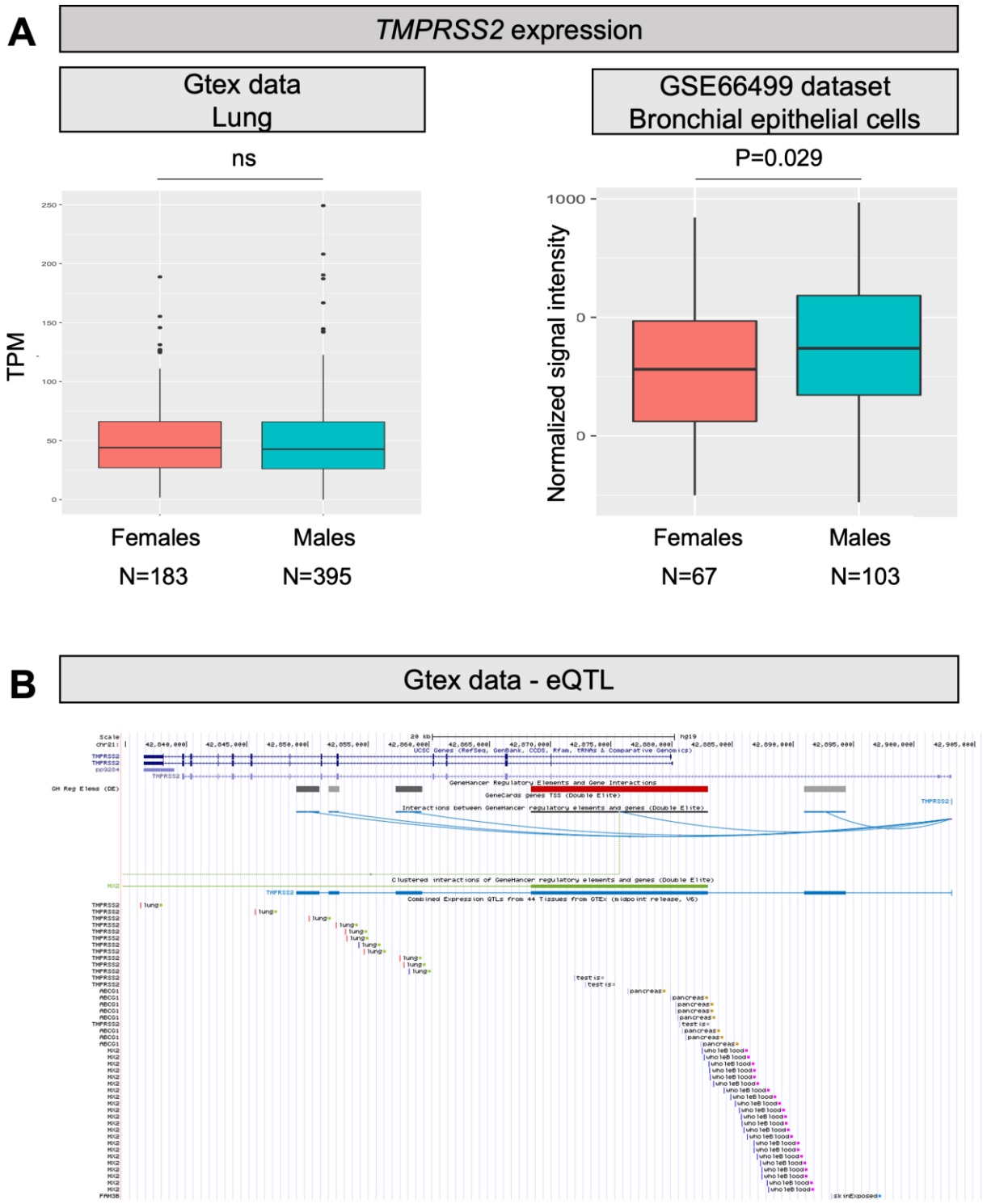

Figure 2. *TMPRSS2* expression levels and eQTLs. (**A**) Both panels show *TMPRSS2* mRNA expression levels in human normal lung samples stratified according to sex. On the left, data were retrieved for a total of 578 RNAseq experiments from the GTex repository. Expression levels are reported as transcripts per kilobase million (TPM). On the right, data were collected for a total of 170 microarray experiments from the GEO database. Expression levels are reported as normalized signal intensities. P values were calculated by using either the Kruskal-Wallis or the student t test. (**B**) Screenshot from the UCSC Genome browser (http://genome.ucsc.edu/; GRCh37/hg19) highlighting the *TMPRSS2* region (coordinates chr21: 42,835,000-42,905,000). The panel shows the following tracks: i) the ruler with the scale at the genomic level; ii) chromosome 21 nucleotide numbering; iii) the UCSC RefSeq track; iv) enhancers (grey and red bars) from GeneHancer database; v) interactions (curved lines) connecting GeneHancer regulatory elements and genes: all curved lines converge towards the androgen-responsive enhancer for the *TMPRSS2* gene described by Clinckemalie and colleagues [29].

substantial differences emerged between males and females (neither below, nor above 60 years of age; data not shown).

Finally, we explored genetic variation in *TMPRSS2* in search of variants, possibly already annotated as eQTL in the lung, which might have an impact on the serine protease expression as well as on its catalytic activity. Again, we used the available Italian exome data, as well as data deposited in GnomAD [25].

Firstly, we looked at the overall burden of deleterious rare variants, using the variant classification described above. Italians had a nominally significant decrease in the burden of deleterious variants compared to Europeans (uncorrected P=0.039, not significant after correction for multiple testing; Table 2A). This decrease was even more evident for the East Asian population (corrected P=8.6*10-4); however, in this case, we must consider that the number of individuals over 65 years of age in Italy is more than double the one in the Hubei province (22.7 vs. 10%, respectively) and this is a major determinant of disease lethality.

Focusing specifically on common exonic variants, 4 SNPs showed significantly (P<2.2*10-16) different frequencies when comparing the Italian population with East Asians (and with Europeans) (Table 2B); 3 of them are synonymous variants, whereas one is the missense substitution p.Val160Met, which impacts on a residue far from the serine protease catalytic triad. This variant was previously found significantly associated with genomic rearrangements involving *TMPRSS2*, with the risk of prostate cancer [26] and with shorter time to prostate cancer diagnosis for high-risk patients [27].

Concerning eQTLs, a number of variants significantly impacting on *TMPRSS2* expression in the lung (GTex data) are reported in the 3' region of the gene (Figure 2B). In Table 2C, a list of the most significant (P<1*10-8), together with their GnomAD frequencies in the East Asian and European populations, are reported. As for the Italian frequencies, we took advantage of the genome-wide association study (GWAS) performed on the above-described cohort (for a total of 3,284 individuals) [28]; in this case, we had to infer genotype frequencies by an imputation approach (for details, see Supplementary Methods). Interestingly, all these eQTLs appear to have extremely different frequencies among populations. In particular, 2 different haplotypes can be inferred from frequency data:

1) A frequent "European" haplotype (composed at least of SNPs rs463727, rs34624090, rs55964536, rs734056, rs4290734, rs34783969, rs11702475, rs35899679, and rs35041537), which is totally absent in the Asian population. Interestingly, this haplotype has been functionally linked to another eQTL (rs8134378), located at a known androgen-responsive enhancer for *TMPRSS2*, 13 kb upstream of the *TMPRSS2* transcription start site [29] (Figure 2B). Hence, this haplotype is expected to up regulate *TMPRSS2* gene expression in an androgen-specific way.

2) A second haplotype, predicted to be associated with higher *TMPRSS2* expression, is characterized by 3 SNPs (rs2070788, rs9974589, rs7364083), whose MAF is significantly increased in Europeans (9% increase in Italians respect to East Asians, corrected $P<6.8*10^{-9}$). Importantly, a small-scale GWAS, comparing the distribution of genetic variants in severe and mild cases of patients with A(H1N1)pdm09 influenza, identified rs2070788 as being associated with increased risk to both human A(H7N9) and severe A(H1N1)pdm09 influenza [11]. Of note, also in A(H7N9) influenza, the proportion of male patients was more than double that of female patients [30].

LIMITATIONS AND CONCLUSIONS

We are aware of the limitations of our study: first of all we focused our attention only to two candidate genes identified on the basis of their crucial role in viral infection and on the a priori probability that they might mediate sex-specific effects. A number of other X-linked genes (such as *IL13*, *IL4*, *IL10*, *XIST*, *TLR7*, *FOXP3)* and Y-linked genes (*SRY*, *SOX9*) may underlie sexually dimorphic immune responses [31]. Moreover, the number of non-genetic determinants of sex-biased severity and case fatality rates is huge and probably has to do not only with sex differences in both innate and adaptive immune responses [7], but also with gender and cultural habits in different countries. In particular, important gender-related factors might concern the social role of women (job, maternal and childcare role), the propensity to smoke, the hand hygiene compliance, as well as differences in the impact of the social role of women in the different countries.

In conclusion, we have explored possible genetic components impacting on COVID-19 severity, focusing on effects mediated by *ACE2* and *TMPRSS2* genes in the Italian population. From available data, it seems unlikely that sex-differences in *ACE2* levels can explain sex differences in disease severity. However, it remains to be evaluated if changes in *ACE2* levels in the lung correlate with susceptibility and severity of SARS-CoV-2 infection. Experimental data from patients with different disease manifestations are urgently needed. Among the analyzed hypotheses, the most interesting signals refer to sex-related differences in *TMPRSS2* expression and in genetic variation in *TMPRSS2*. In particular, we

Table 2A. Burden of rare mutations in the *TMPRSS2* gene in different populations.

Population	N alleles	T1	Freq T1	ITA	EUR	EAS
ITA	7968	30	0.0038	-	P=0.039	**P=3.6e-05**
EUR	129920	726	0.0056	P=0.039	-	**P=9.8e-16**
EAS	19979	25	0.0013	**P=3.6e-05**	**P=9.8e-16**	-

Total allele counts, carrier allele counts, and carrier frequencies are shown; only deleterious variants with MAF less than 1% were considered in the burden analysis. The 'deleterious' set is defined by missense variations predicted to be possibly damaging by all the 5 algorithms used (LRT score, MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and SIFT), and loss-of-function variants (nonsense, frameshift, and splicing variants affecting the donor/acceptor sites).
P values are presented as non-corrected; the number of statistical comparisons performed in Tables 1A, 1B, 2A, and 2B is collectively of 24, thus lowering the threshold for significance at P=0.0021 (Bonferroni threshold). Significant P values are indicated in bold.
T1: alleles carrying damaging variants; Freq T1: frequency of T1 allele; ITA: Italian population; EUR: European population; EAS: East Asian population.

Table 2B. Common exon variants in the *TMPRSS2* gene in different populations.

Variant ID	Consequence	A1/N alleles ITA	Freq ITA	A1/N alleles EUR	Freq EUR	A1/N alleles EAS	Freq EAS	ITA Vs EUR	ITA Vs EAS	EUR Vs EAS
rs2298659	p.Gly259Gly	1388/7968	0.174	28744/122880	0.234	5179/19478	0.266	**P<2.2e-16**	**P<2.2e-16**	**P<2.2e-16**
rs17854725	p.Ile256Ile	4131/7968	0.518	67712/122814	0.551	2544/19604	0.130	**P=1.16e-08**	**P<2.2e-16**	**P<2.2e-16**
rs12329760	p.Val160Met	1387/7968	0.174	29831/128604	0.232	7651/19934	0.384	**P<2.2e-16**	**P<2.2e-16**	**P<2.2e-16**
rs3787950	p.Thr75Thr	889/7968	0.112	9864/127666	0.077	2905/19600	0.148	**P<2.2e-16**	**P=1.39e-15**	**P<2.2e-16**

Total allele counts, carrier allele counts, and carrier frequencies are shown; only variants with MAF more than 5% were considered.
P values are presented as non-corrected; the number of statistical comparisons performed in Tables 1A, 1B, 2A, and 2B is collectively of 24, thus lowering the threshold for significance at P=0.0021 (Bonferroni threshold). Significant P values are indicated in bold.
A1: alleles carrying variants; Freq A1: frequency of A1 allele; ITA: Italian population; EUR: European population; EAS: East Asian population.

Table 2C. eQTL variants in the *TMPRSS2* gene in different populations.

Variant ID	P GTEx	NES GTEx	Freq ITA	Freq EUR	Freq EAS	ITA vs EUR	ITA vs EAS	EUR vs EAS
rs463727	5.0e-10	0.12	0.44	0.46	0.0051	P=0.038	**P<2.2e-16**	**P<2.2e-16**
rs2070788	8.9e-9	-0.11	0.55	0.53	0.66	P=0.003	**P=4.7e-15**	**P<2.2e-16**
rs9974589	7.4e-9	-0.12	0.55	0.53	0.66	P=0.002	**P=3.3e-15**	**P<2.2e-16**
rs34624090	9.2e-9	0.12	0.43	0.45	0.0051	P=0.005	**P<2.2e-16**	**P<2.2e-16**
rs7364083	3.3e-9	-0.12	0.56	0.53	0.65	**P=8.7e-05**	**P=1.9e-10**	**P<2.2e-16**
rs55964536	1.9e-9	0.12	0.46	0.49	0.0045	**P=4.6e-04**	**P<2.2e-16**	**P<2.2e-16**
rs734056	1.3e-9	0.12	0.47	0.49	0.0051	P=0.030	**P<2.2e-16**	**P<2.2e-16**
rs4290734	8.3e-10	0.12	0.47	0.49	0.0051	P=0.019	**P<2.2e-16**	**P<2.2e-16**
rs34783969	3.9e-10	0.12	0.47	0.49	0.0051	P=0.027	**P<2.2e-16**	**P<2.2e-16**
rs11702475	8.4e-10	0.12	0.47	0.49	0.0046	P=0.015	**P<2.2e-16**	**P<2.2e-16**
rs35899679	7.8e-9	0.11	0.44	0.46	0.0051	P=0.004	**P<2.2e-16**	**P<2.2e-16**
rs35041537	3.6e-9	0.12	0.44	0.47	0.0051	**P=8.6e-04**	**P<2.2e-16**	**P<2.2e-16**

P values are presented as non-corrected; the number of statistical comparisons performed in Table 2C is collectively of 36, thus lowering the threshold for significance at P=0.0013 (Bonferroni threshold). Significant P values are indicated in bold.
NES: normalized effect size; Freq: frequency of the minor allele; ITA: Italian population; EUR: European population; EAS: East Asian population.

identified an exonic variant (p.Val160Met) and 2 distinct haplotypes showing profound frequency differences between East Asians and Italians. The rare alleles of these haplotypes, all predicted to induce higher levels of *TMPRSS2*, are more frequent in the Italian than in the East Asian population; in one case, the haplotype could be regulated through androgens, thus possibly explaining the sex bias in COVID-19 severity, in the other case, a SNP belonging to the haplotype has been associated with increased susceptibility to influenza, possibly related to a higher susceptibility in Italians and Europeans.

Our data, beside suggesting possible explanations for the unusually high, relative to known data, lethality rates among Italians, provide reference frequencies in the general Italian population for candidate variants that can be compared to genetic data from patients infected by SARS-CoV-2 with different disease manifestations, as soon as they will be available on large numbers of patients. These studies will hopefully be of help in predicting the individual risk of infection and susceptibility to CoV-2 and in recognizing in advance infected individuals being at higher risk of poor prognosis.

MATERIALS AND METHODS

Gene expression data

Expression data for *ACE* and *TMPRSS2* genes were obtained through the: 1) genotype-tissue expression (GTEx) database (https://gtexportal.org/home/), which was also used to extract quantitative trait loci (eQTLs) for the two genes (all data based on RNAseq experiments); and 2) Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/). In particular, two GEO datasets were extracted and analyzed: 1) GSE66499, reporting microarray data on 152 normal lung samples from Caucasian individuals; 2) GSE19804, reporting microarray data on 60 normal lung samples from Taiwanese females (see also Supplementary Methods, paragraph "Datasets and statistical power estimations").

Genetic data

Genetic data for general European and East Asian populations were retrieved through the GnomAD repository, which contains data on a total of 125,748 exomes and 71,702 genomes (https://gnomad.broadinstitute.org/).

As for Italians, details on whole-exome sequencing (on 3,984 individuals) and genome-wide microarray genotyping (on 3,284 individuals) of the analyzed cohort are specified elsewhere [19, 20, 28], as well as in Supplementary Methods (paragraphs "Sequencing" and "Datasets and statistical power estimations"). Imputation procedures are detailed in Supplementary materials (paragraph "Dataset imputation").

Statistical analysis

Expression levels were compared by using either the Kruskal-Wallis test (RNAseq data) or the student t test (microarray data). Allele frequencies were compared using the chi square test. All calculations were performed using the R software (https://www.r-project.org/). P values are presented as non-corrected for multiple testing, but the Bonferroni-corrected threshold of significance is indicated below each set of comparisons presented in Tables. Power calculations have been described in Supplementary Methods (paragraph "Datasets and statistical power estimations").

AUTHOR CONTRIBUTIONS

All authors contributed to the study design. EMP did the genetic analysis, RA performed the statistical analysis, SD drafted the manuscript and supervised the entire study. All authors critically reviewed the manuscript and approved the final draft.

CONFLICTS OF INTEREST

No conflicts of interest to disclose

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Sequencing

Whole-exome sequencing (WES) was performed at the Broad Institute (Boston, MA). Demographic characteristics, as well as exome capture methods, sequencing, variant annotation, and data processing of the samples were described previously [1].

Definition of disrupting variants and statistical analysis

Using WES data, we searched the *ACE2* and *TMPRSS2* genes for loss-of-function variants (nonsense, frameshift, splicing, or disrupting missense mutations). Missense variants were considered damaging if they were predicted to be deleterious or possibly deleterious by all the 5 prediction algorithms used: LRT (likelihood ratio test) [2], MutationTaster [3], PolyPhen-2 HumDiv, PolyPhen-2 HumVar [4], and SIFT [5].

The positions of mutations were based on the cDNA reference sequence for *ACE2* and *TMPRSS2* (NM_021804 and NM_005656) with the ATG initiation codon numbered as residue 1 (p.Met1).

Burden test analyses were performed considering only those variants having a minor allele frequency (MAF) <1%. Significance in the differences of MAFs between different populations were calculated using chi-square tests, with the R software (https://www.r-project.org/). A $P<0.05$ was considered to indicate statistical significance.

Dataset imputation

When missing from exome data, intronic variant frequencies in *TMPRSS2* were retrieved from SNP-array data obtained from the same Italian cohort. Genome-wide genotyping was performed at the Broad Institute. Genotyping details and data processing of the samples have been already described [6].

Imputation was performed remotely using the Michigan Imputation Server (https://imputationserver.sph.umich.edu) [7], using the 1000G Phase 3 v5 as reference panel, ShapeIT v2.r790 for the phasing step [8], and Minimac3 [7] as imputation software. The imputed dataset was then filtered to retain only those variants with $r^2>0.3$.

Datasets and statistical power estimations

For expression data analyses, we took advantage of microarray data reported in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/). We specifically searched for the wider datasets reporting expression data on normal lung tissues derived from individuals whose sex and geographical origin were specified (search done by keywords, filters based on the number of available samples in the dataset, and by a final manual inspection of the retrieved data). This search allowed the identification of two datasets: GSE66499 and GSE19804, for a total of 115 samples from male individuals, and 135 samples from female subjects. Indeed, it is difficult to provide an accurate power estimate for a microarray study. Among others, [9] suggested that a sample size of 20 is necessary, at a P value of 0.01 and 90% power, to detect a two-fold change in the 75% least variable genes in a microarray study. Based on this observation, the data available through the GSE66499 and GSE19804 datasets were considered reasonably powered to identify possible altered levels in the ACE2 and TMPRSS2 genes.

As for genotype data, from one side we took advantage of exome and SNP-array in-house data on ~3,500 individuals; [1, 6], from the other of exome and genome data on the largest dataset freely accessible online, i.e. the GnomAD repository (https://gnomad.broadinstitute.org/). For GnomAD data, we extracted allele/genotype frequencies available for East Asian and European individuals, for a total of at least 9,967 and 64,302 subjects, respectively. The use of such large cohorts ensured us to be sufficiently powered to detect significant differences in allele frequencies between the analyzed populations. As an example, a sample size of 2,000 pairs has an approximately 80% power of detecting a significant allele difference at $P<0.05$ if the frequency of the rare allele is 2%. For higher frequencies of 10% or more, the power of detection increases to more than 90%.

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Editorial

Reversing immunosenescence for prevention of COVID-19

Robert T. Brooke and Gregory M. Fahy

Age is the strongest predictor of the severity and lethality of COVID-19 [1]. But is age - or at least biological age - a modifiable risk factor? Our recently published TRIIM trial [2] demonstrated that in healthy older adults it is possible to not only regenerate the thymus and reverse age-related immunological changes but also to reverse epigenetic aging - the most robust indicator of biological age available today [3]. These observations introduce new possibilities for preventive medicine in the elderly, and the nature of SARS-CoV-2 infection suggests new possibilities may be necessary.

The primary reason advanced age increases susceptibility to COVID-19, and all other infectious diseases, is an age-related decline of immune competence, or immunosenescence [1, 2]. Two hallmarks of this process are the well-known loss with age of naïve T cell generation and T cell diversity [1, 2], which provide the source of the resilience and versatility of the cellular component of the adaptive immune system and are also important for humoral immunity - the mounting of robust antibody responses. This problem, and potentially a good deal of aging more generally, is made inevitable by the involution of the thymus in early life [2]. The thymus produces naïve T cells that can help recognize and clear infectious agents, including viruses like SARS-CoV-2, from the body, through both helper CD4 T cells and cytotoxic CD8 T lymphocytes. Age-related loss of thymic T cell output leads eventually to a reduced capacity to mount robust adaptive immune responses to novel antigens in later life [2].

Virus-specific T cell responses have been shown to be present in 100% of individuals who recover from COVID-19 [4]. In the elderly, generation of a similar response is presumably more difficult due to the tendency of COVID-19 to induce a profound depression of circulating total T cells, $CD8^+$ T cells, and also NK cells and an increase in functionally exhausted T cells [5] in the presence of pre-existing deficits in T cell receptor repertoire. In principle, these deficits may be corrected by thymus regeneration, as our recent trial demonstrated that regeneration of the thymus was accompanied by increases in naïve T cells and recent thymic emigrants and by decreases in the number of exhausted CD8 T cells in normal aging men [2]. Interestingly, the first demonstrations of thymus regeneration in humans were inspired by the T cell depleting effects of the HIV virus, rather reminiscent of the effect of SARS-CoV-2, and those demonstrations were successful in improving T cell levels despite ongoing viral infection [6]. T cell repletion by thymus regeneration could also be long-lasting. In the case of SARS-CoV-1 infection, which is closely related, virus-specific CD8 T cells have been shown to be able to persist for at least 11 years post-infection [7].

Thymic involution also brings with it reduced production of thymic hormones such as thymosin alpha-1. Recently, thymosin alpha-1, by itself, was found to reduce COVID-19-associated mortality [8], providing additional evidence that thymus regeneration may help to prevent or moderate this disease. The potential for thymus regeneration to help protect older individuals from COVID-19 and immune system aging more generally will soon be further investigated through our expanded TRIIM-X clinical trial.

As a preventive medicine measure, the TRIIM treatment is unique in that it addresses the reversal of both epigenetic aging and immunosenescence, and does this using a combination of FDA-approved drugs that are already available today [2]. It involves use of growth hormone as a thymotrophic agent, which has been well established in both preclinical and clinical studies to induce the production of new naïve T cells and to enhance immune system function, and complementary agents that block side effects and may have independent benefits of their own.

Today's medicine, dramatic public health measures, new vaccines and treatments, and perhaps natural attenuation will presumably beat back COVID-19 later this year, but a more permanent and fundamental solution is needed. Immunosenescence, more than anything else, is what makes us susceptible to COVID-19, influenza, pneumonia, countless other infectious diseases, and very likely also many of the risks of cancer and even cardiovascular disease through increased inflammation. Unless we address immunosenescence in a powerful way, it will continue to plague us long after we've contained this particular pandemic. Fortunately, humanity has more treatments and diagnostics in its arsenal than ever before. We also have new digital health technologies that can enable more efficient, larger, and more definitive clinical trials - better science - than ever before. Combining these

new technologies with treatments to reverse immunosenescence could enable us to protect our elderly and provide health and economic benefit to the broader community for decades to come.

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Research Paper

Prognosis analysis of patients with mental disorders with COVID-19: a single-center retrospective study

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ABSTRACT

Our study aimed to investigate the factors affecting the prognosis of patients with mental disorders with COVID-19. All patients with mental disorders who were diagnosed with COVID-19 at the intensive care unit of Wuhan Mental Health Center during the period January 3 to March 1, 2020 were selected. The influence of the baseline characteristics, clinical symptoms, laboratory parameters and the types of mental disorders on prognosis were analyzed. According to their final prognosis, the patients were divided into the deceased group (5 patients) and the cured group (25 patients). The mortality rate of patients with dementia was significantly higher than that of patients with other mental disorders (P = 0.001). The levels of certain laboratory parameters in the serum of dementia patients were significantly increased compared with levels in nondementia patients (WBC count: 10.100±6.147 vs. 5.694±3.383, p = 0.029; neutrophil count: 8.504± 5.993 vs. 3.764 ± 2.733, P = 0.008; BUN: 8.300± 4.072 vs. 4.364 ± 1.196, P = 0.001). Our research indicated that the mortality rate of dementia patients with COVID-19 was higher than that of patients with other mental disorders. A focus on the inflammatory response of dementia patients may provide novel ideas for reducing mortality.

INTRODUCTION

An aggressive, acute respiratory disease caused by SARS-CoV-2, a novel coronavirus of zoonotic origin, called COVID-19, has become a new public health crisis threatening the world [1]. Public health and healthcare professionals are at the frontline and work hard to control and mitigate the spread of the pandemic. With the deepening of the understanding of the disease, patients with COVID-19 in various special groups have gradually attracted attention, such as cancer patients [2, 3], end-stage kidney disease patients [4], and pregnant women [5]. Patients with mental disorders need long-term treatment and specialized care, and their health and psychological status are different from those of the general population. The double hit of

Table 1. Baseline characteristics of mental disorders patients with COVID-19.

Clinical parameters	Nonsevere symptoms group (n=19)	Severe symptoms group (n=11)	ES	P
Age(years)	61.47±14.74	66.73±7.30	-1.01	0.28
Gender (n, Male/Female)	6/13	4/7	0.07	1.00
Maximum body temperature(°C)	37.74±1.05	38.09±1.30	-0.82	0.42
Heart rate (bpm)	81.42±9.78	88.55±15.48	-1.55	0.13
Systolic pressure (mmHg)	128.53±16.76	122.09±11.16	1.13	0.27
Diastolic pressure (mmHg)	79.63±8.45	77.91±11.18	0.47	0.64
Blood oxygen saturation (%)	95.58±1.02	88.45±3.56	8.25	**< 0.01**
History of basic diseases (n)				
Hypertension	11	3	2.63	0.14
Chronic bronchitis	1	0	0.60	0.22
Atherosclerosis	4	4	0.84	0.36
Symptom (n)				
Fever	11	8	0.66	0.42
Cough	9	9	3.45	0.06
Muscle soreness	0	1	1.79	0.18
Expectoration	3	2	0.03	0.87
Hemoptysis	0	0	-	-
Dizzy	1	2	1.29	0.26
Headache	0	0	-	-
Diarrhea	4	3	0.15	0.70
Fatigue	8	8	2.63	0.11
Pharyngalgia	0	0	-	-
Stuffy nose/Runny nose	0	0	-	-
Anorexia/Nausea	1	6	9.46	**0.02**
Dyspnea	1	10	22.00	**< 0.01**
Lumbago	0	2	3.70	0.05

ES=effect size

mental disorder and COVID-19 in the pandemic has raised great concerns. However, little information about this special group has been reported.

It has been acknowledged that mental disorders are a diverse group of conditions that primarily impair cognition, emotion, and behavioral control. Mental disorders can occur early in life and have a high aggregate prevalence in all countries, especially in low- and middle-income countries. People living with mental disorders have limited access to or difficulties understanding public health information, which predisposes them to an increased chance of infection compared with the general population. Obviously, the double hit of mental disorder and COVID-19 in the pandemic leads to increased danger. Therefore, this study focused on the factors affecting the prognosis of patients with mental disorders (especially dementia) with COVID-19.

RESULTS

The clinical and laboratory parameters of this study are given in Table 1. The cases of a total of 30 patients were reviewed, and patients were divided into the nonsevere symptoms group (n=19) and the severe symptoms group (n=11). There were significant differences in blood oxygen saturation (95.58±1.02% vs. 88.45±3.56%, $P < 0.01$) and the incidence of certain clinical symptoms (anorexia/nausea, $P = 0.02$, and dyspnea, $P < 0.01$) between the nonsevere symptoms group and the severe symptoms group. However, there was no significant difference in baseline data such as age, sex, the type of mental disorder, and heart rate ($P > 0.05$).

We regrouped the patients according to their final prognosis and divided the patients into the deceased group (n = 5) and the cured group (n = 25). There were significant differences in some clinical symptoms (fatigue, anorexia/nausea and dyspnea) and laboratory parameters (AST and BUN) between the two groups ($P < 0.05$, Table 2). Surprisingly, the mortality rate of patients with dementia was significantly higher than that of patients with other mental disorders ($P = 0.001$, Table 2).

To further explain this phenomenon, we compared the blood indexes of dementia patients and nondementia

Table 2. Univariate analysis based on the prognosis of patients.

Clinical parameters	Cured(n=25)	Deceased(n=5)	ES	P
Age (years)	62.72±13.640	66.80±5.070	-0.652	0.520
Basic mental illness (n)				
Dementia	2	3	8.112	**0.004**
Nondementia	23	2		
Gender (n)			1.920	0.166
Male/Female	7/18	3/2		
Symptom severity (n)				
Severe cases	6	5	10.364	**0.001**
Non Severe cases	19	0		
Symptom (n)				
Fever	14	5	3.474	0.062
Cough	15	3	0.000	1.000
Muscle soreness	1	0	0.207	0.649
Expectoration	5	0	1.200	0.273
Dizziness	2	1	0.667	0.414
Diarrhea	6	1	0.037	0.847
Fatigue	11	5	5.250	**0.022**
Anorexia/Nausea	4	3	4.509	**0.034**
Dyspnea	6	5	10.364	**0.001**
Blood routine				
WBC (10^9/L)	5.877±3.419	9.184±6.720	-1.633	0.107
Neutrophil count (10^9/L)	3.978±2.776	7.434±6.743	-1.949	0.061
Lymphocyte count (10^9/L)	1.393±0.680	1.180±0.481	0.666	0.511
Monocyte count (10^9/L)	0.464±0.301	0.454±0.106	0.075	0.941
Hemoglobin (g/L)	125.640±15.196	131.200±7.050	-0.793	0.435
Platelet count (10^9/L)	180.720±49.526	143.800±34.172	1.582	0.125
Albumin(g/L)	37.550±5.534	34.180±0.838	1.199	0.241
Blood biochemistry				
AST (U/L)	24.360±12.086	49.250±27.585	-3.157	**0.004**
ALT (U/L)	22.760±13.758	31.250±29.296	-0.971	0.340
TBil (umol/L)	7.396±3.517	7.525±1.611	-0.071	0.944
SCr(umol/L)	76.816±43.071	111.180±56.265	-1.552	0.132
BUN (mmol/L)	4.212±1.613	9.060±3.464	-4.984	**<0.001**
UA (umol/L)	324.720±97.053	351.800±97.513	-0.569	0.574

ES=effect size; TBil=total bilirubin; SCr=serum creatinine; BUN=blood urea nitrogen; UA=uric acid

patients. The results showed that certain laboratory parameters in the serum of dementia patients were significantly increased (WBC count: 10.100 ± 6.147 vs. 5.694 ± 3.383, P = 0.029; neutrophil count: 8.504 ± 5.993 vs. 3.764 ± 2.733, P = 0.008; BUN: 8.300 ± 4.072 vs. 4.364 ± 1.196, P = 0.001; Figure 1).

Is there a correlation between the upregulation of inflammation indicators (WBC and neutrophil counts) and the impairment of renal function (BUN)? Our results suggest that WBC and neutrophil counts and BUN levels in nondementia patients were significantly positively correlated (WBC count: r^2=0.376, P < 0.05, Figure 2A; neutrophil count: r^2=0.325, P < 0.05, Figure 2B). However, we did not find such a significant correlation in patients with dementia (Figure 2C and 2D).

DISCUSSION

In this study, we collected data on baseline characteristics, clinical symptoms, laboratory parameters and mental disease types in patients with mental disorders with COVID-19. The above information was used to find internal associations or differences as much as possible. Differences between

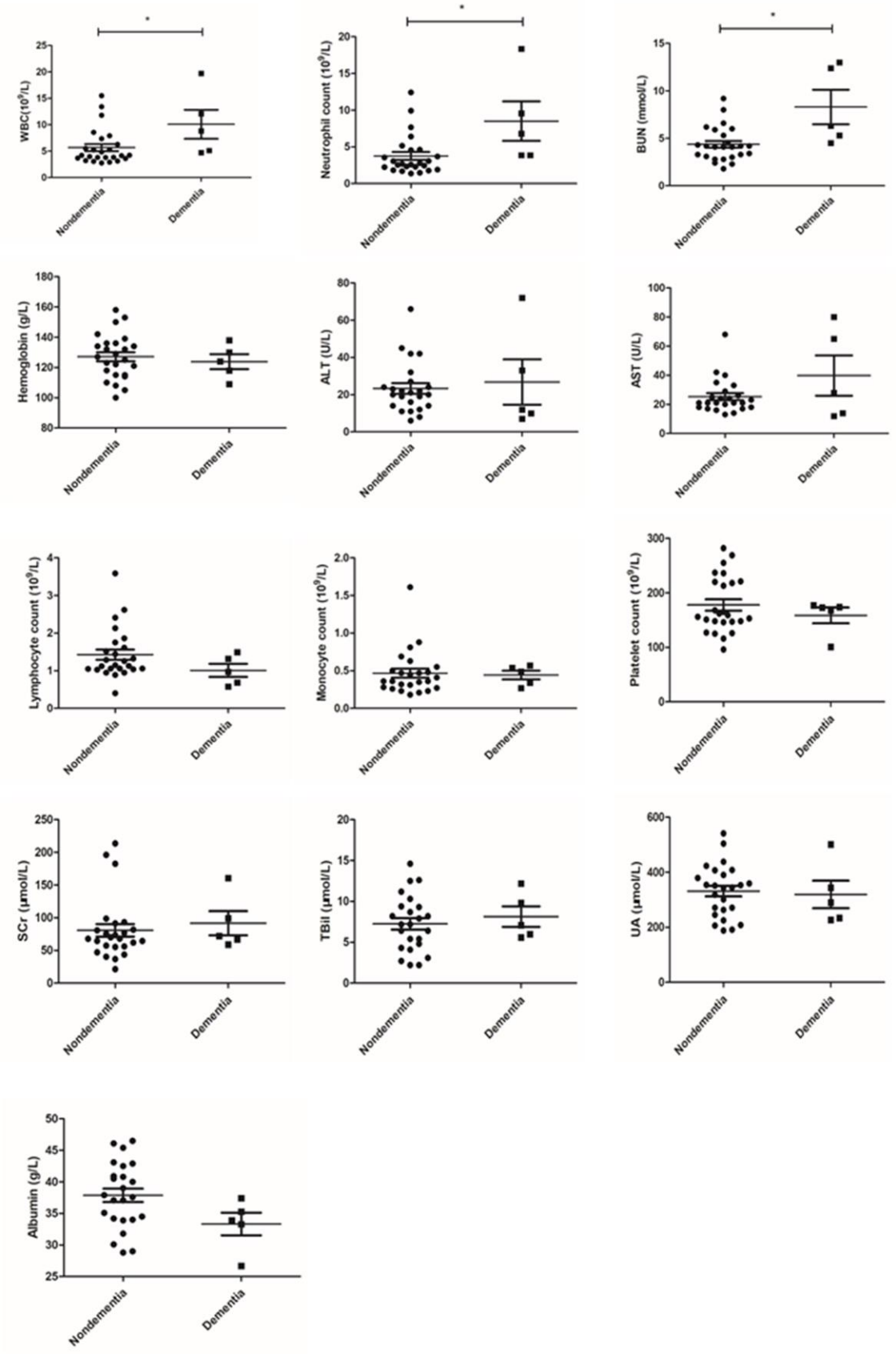

Figure 1. Comparison of laboratory parameters between dementia and nondementia patients.

patients with moderate/mild symptoms and patients with severe symptoms were mainly reflected in blood oxygen saturation, anorexia/nausea, and dyspnea. We analyzed the prognosis of patients and found that the mortality rate of dementia patients was significantly higher than that of patients with other mental disorders. In addition, the WBC count, neutrophil count and BUN level of patients with dementia were significantly higher than those of patients with other mental disorders.

Facing such a very interesting result, we needed to explore why the mortality rate of dementia patients was so high. First, dementia tends to have severe mental and behavioral symptoms. Second, due to less activity and long-term bed rest, the incidence of serious complications, such as pressure ulcers, lung infections, and cardiopulmonary insufficiency, is higher in dementia patients than in nondementia patients. Last but not least, inflammation might play an important role in the pathogenesis of dementia [6–8]. Inflammation is a characteristic of Alzheimer's disease (AD; the most important cause of dementia). AD dementia patients also have different degrees of inflammation [9]. Chronic inflammation is a common feature of various types of vascular dementia (the second most important cause of dementia) [10]. In addition to amyloid protein, inflammatory molecules, including acute inflammatory reactants and inflammatory cytokines, have been found in the cerebrospinal fluid of dementia patients [11]. Peripheral infection will also aggravate the onset and development of AD [12]. However, in another study [13] of general patients with COVID-19, patients with severe cases tended to have higher leukocyte counts and neutrophil-lymphocyte ratios. Therefore, the persistent inflammatory state of dementia patients may be the cause of the increased peripheral blood WBC and neutrophil counts in dementia patients with COVID-19.

At present, there is not enough evidence to support that the renal function damage of dementia patients with COVID-19 is worse than that of patients with other mental disorders. In our study, the inflammatory

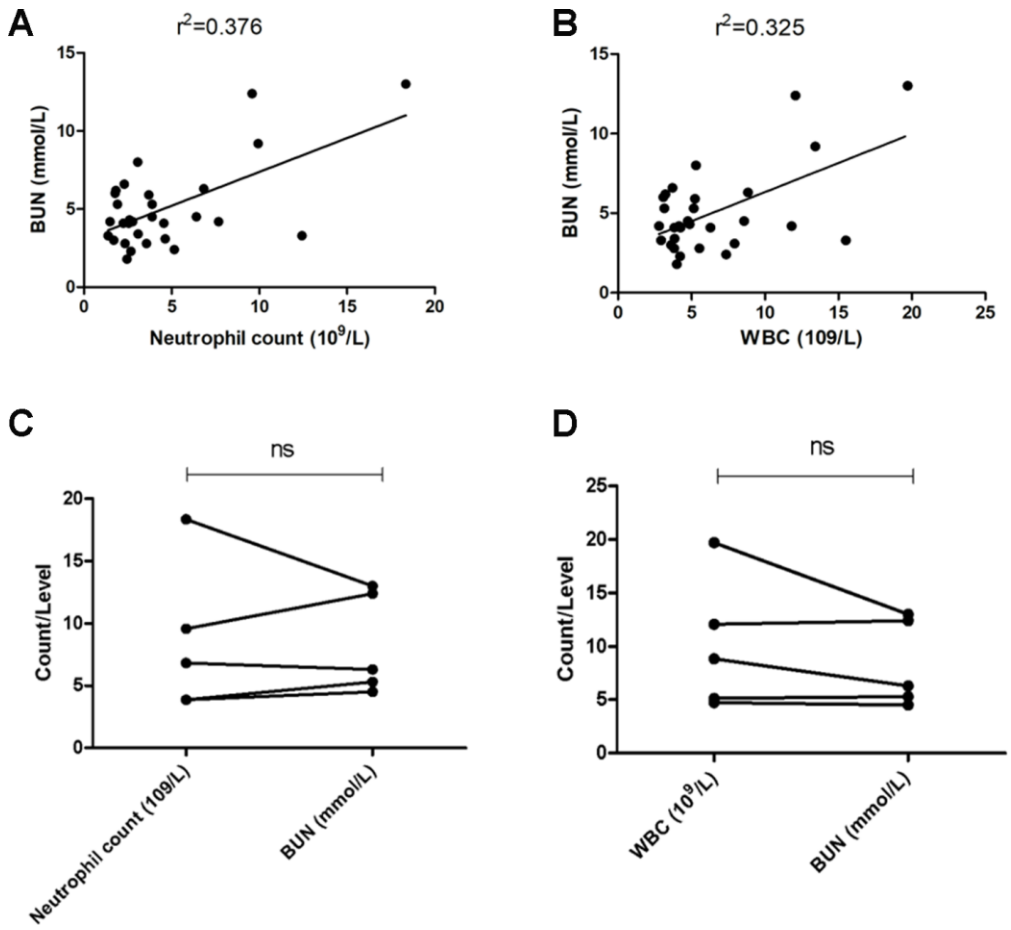

Figure 2. Correlation between inflammation and renal function. (**A**) Correlation analysis of BUN and neutrophil count in all patients with mental disorders; (**B**) correlation analysis of BUN and WBC count in all patients with mental disorders; (**C**) matched test of BUN and neutrophil count in dementia patients; (**D**) matched test of BUN and WBC count in dementia patients. ns: no significance.

response of patients with mental disorders was associated with renal impairment, but this phenomenon was not statistically significant in patients with dementia. Therefore, the increase in BUN levels may not be unique to patients with dementia with COVID-19. It may be that these patients have severe symptoms, which lead to damage to renal function. Of course, due to the limitation of the sample size of dementia patients, the trend of the correlation analysis may be masked. Therefore, the correlation between the inflammatory response and renal function impairment in dementia patients needs to be treated with caution. At least, based on the current results and previous evidence, it is not enough to deny this conclusion.

It is worth noting that the following limitations of this study cannot be ignored. Since our research is limited to patients with mental disorders, which makes the sample size insufficient, the probability of false-positive errors seen in small-sample clinical studies is difficult to avoid. In addition, dementia may also be accompanied by other mental disorders, and the heterogeneity between patients may result in an overestimation of the statistical results. Nonetheless, our research indicated that the mortality rate of dementia patients with COVID-19 was higher than that of patients with other mental disorders. A focus on the inflammatory response of dementia may provide novel ideas for reducing mortality.

MATERIALS AND METHODS

Participants and materials

All patients who were diagnosed with mental disorders and with COVID-19 at the intensive care unit of Wuhan Mental Health Center during the period January 2 to March 1, 2020 were selected. The definition of the mental disorders was based on the International Classification of Diseases-10 (ICD-10). The diagnosis of COVID-19 was made according to the standards for the "Diagnosis and Treatment Scheme of New Coronavirus Infected Pneumonia" (trial version 6 [14]). Finally, a total of 30 newly diagnosed COVID-19 patients with mental disorders were considered candidates in our study. Exclusion criteria included other infectious diseases, hepatic or renal insufficiency, and malignancies. No patient received COVID-19-related antiviral or symptomatic treatment before entering this study.

Data collection

We reviewed electronic patient records retrospectively; clinical and laboratory parameters were extracted, including sex, age, the types of mental disorders, COVID-19 nucleic acid detection, routine blood test and laboratory biochemical examination results, such as total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), blood urea nitrogen (BUN), and uric acid (UA), and so on. These laboratory parameters were evaluated when patients first underwent laboratory tests in the hospital. The study was approved by the Ethics Committee of Wuhan Mental Health Center, and all patients provided informed consent.

Statistical analysis

All data were analyzed by using SPSS 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 5. Continuous variables are shown as the mean ± the standard deviation (SD), and categorical variables are shown as percentages. Before analysis, the Kolmogorov–Smirnov test was conducted to identify variable normality. Continuous variables with normal distribution were analyzed by an independent-sample t test, and non-normally distributed data were compared by a rank-sum test. $P < 0.05$ was considered statistically significant.

Ethical statement

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 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration among all authors. YDX, JW and QX defined the research theme and discussed the analyses, interpretation, and presentation. CY and LHN drafted the manuscript, analyzed the data, developed the algorithm and interpreted the results. YW, QQL, CJZ, FF and HL worked together on associated data collection and helped to draft the manuscript. CY and CJZ helped to perform the statistical analysis and reference collection. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Research Paper

Lactate dehydrogenase, an independent risk factor of severe COVID-19 patients: a retrospective and observational study

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ABSTRACT

Background: The World Health Organization has declared coronavirus disease 2019 (COVID-19) a public health emergency of global concern. Updated analysis of cases might help identify the risk factors of illness severity.

Results: The median age was 63 years, and 44.9% were severe cases. Severe patients had higher APACHE II (8.5 vs. 4.0) and SOFA (2 vs. 1) scores on admission. Among all univariable parameters, lymphocytes, CRP, and LDH were significantly independent risk factors of COVID-19 severity. LDH was positively related both with APACHE II and SOFA scores, as well as P/F ratio and CT scores. LDH (AUC = 0.878) also had a maximum specificity (96.9%), with the cutoff value of 344.5. In addition, LDH was positively correlated with CRP, AST, BNP and cTnI, while negatively correlated with lymphocytes and its subsets.

Conclusions: This study showed that LDH could be identified as a powerful predictive factor for early recognition of lung injury and severe COVID-19 cases.

Methods: We extracted data regarding 107 patients with confirmed COVID-19 from Renmin Hospital of Wuhan University. The degree of severity of COVID-19 patients (severe vs. non-severe) was defined at the time of admission according to American Thoracic Society guidelines for community acquired pneumonia.

INTRODUCTION

First reported in Wuhan, Hubei province, China, on December 2019, outbreak of a viral pneumonia has attracted extensive attention of international community [1]. The pathogen, a novel β-coronavirus, has currently been named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses [2]. The World Health Organization (WHO) has recently declared coronavirus disease 2019 (COVID-19) a public health emergency of global concern [3]. As of March 1, 2020, more than 500,000 confirmed cases have been documented all over the world, with more than 50,000 severe cases, and a mortality rate of 4-15% [2, 4].

Among recent studies, the presence of any coexisting illness was more common among patients with severe disease [2]. Most of the patients had elevated levels of C-reactive protein, and lymphocytopenia was common, especially in severe cases [1, 2, 5], which was thought

to be a result of reduction of $CD3^+$, $CD4^+$ and $CD8^+$ T cells [6]. Meanwhile, prolonged prothrombin time (PT) and elevated lactate dehydrogenase (LDH) was found in more than 40% cases during the whole disease period [5, 7], with elevated ALT and AST less common in COVID-19 patients [2].

Given the rapid spread of COVID-19, we considered that an updated analysis of risk factors may help early recognition of the severity of the disease. In this study, we analyzed the clinical and laboratory parameters of severe and non-severe COVID-19 patients in order to evaluate disease severity.

RESULTS

Demographics and characteristics of COVID-19 patients

Diagnosis of COVID-19 was made according to World Health Organization interim guidance [8]. A total of 107 diagnosed cases were enrolled in this study, with 48 severe and 59 non-severe cases (Table 1). 60 cases (56.1%) were male, of which 64.4% were severe ($P = 0.11$). The median age was 63 years (IQR, 49-71 years), and severe patients were significantly older than the non-severe ones (67 vs. 61, $P = 0.005$). A total of 49 (45.8%) patients had underlying conditions, including hypertension (28 [26.2%]), diabetes (13 [12.1%]), coronary heart disease (5 [4.7%]), and autoimmune disease (3 [6.3%]). Although there was no significant difference in the underlying conditions between the two groups, there were more patients with coronary heart disease (3 [6.3%]) and autoimmune disease (3 [6.3%]) in the severe group than the non-severe group. All patients enrolled had fever, with maximum temperatures of approximately 39ºC of which 30 (28%) had fever with dyspnea. Severe patients had higher Acute Physiology and Chronic Health Evaluation (APACHE) II (8.5 vs. 4.0, $P < 0.001$) and Sequential Organ Failure Assessment (SOFA) (2 vs. 1, $P < 0.001$) scores on admission, as well as the higher Pneumonia Severity Index (PSI) (83.35 vs. 55.76, $P < 0.001$), CURB (Confusion/Urea/Respiratory rate/Blood pressure)-65 (1 vs. 0, $P < 0.001$) and computed tomography (CT) semiquantitative rating scores (4 vs. 1, $P < 0.001$).

Laboratory indices of COVID-19 patients

Compared to the non-severe patients, neutrophil levels ($P < 0.001$), alanine transaminase (ALT) ($P = 0.001$), aspartate transaminase (AST) ($P < 0.001$), LDH ($P < 0.001$), Urea ($P = 0.006$), C-reactive protein (CRP) ($P < 0.001$), troponin I (cTnI) ($P < 0.001$), creatine kinase-MB (CKMB) ($P < 0.001$), B-type natriuretic peptide (BNP) ($P < 0.001$), prothrombin time (PT) ($P < 0.001$), activated partial thromboplastin time (APTT) ($P = 0.022$), and D-dimer ($P < 0.001$) in severe patients were significantly higher at admission. Conversely, lymphocyte ($P < 0.001$), monocyte ($P < 0.001$), $CD3^+$ ($P < 0.001$), $CD4^+$ ($P < 0.001$), $CD8^+$ ($P < 0.001$), $CD19^+$ ($P = 0.015$) and $CD16^+56^+$ ($P = 0.010$) T cells in severe patients were significantly lower, as well as PaO_2/FiO_2 (P/F) ratio ($P < 0.001$) (Table 2). No significant differences in the serum levels of immunoglobulins (IgA, IgE, IgG and IgM) or complement C3 and C4 were observed between the two groups (Table 2).

Independent risk factors of severe COVID-19 patients

To assess the risk factors of the demographics, characteristics, and laboratory indicators on the severity of COVID-19 patients, logistic regression analysis was performed on the parameters of significant difference using *t* test. In univariable analysis, odds ratio of serum CKMB concentration and PT level were the highest in severe patients. Male patients infected with SARS-CoV-2 showed as an independent risk factor for being in a more severe condition as 1.89 (0.86-4.12). Apart from the risk factors above, patient age, white blood cell count, neutrophil count, serum AST, ALT, LDH, Urea, CRP, and D-dimer level were all associated with the severity of COVID-19 patients. Meanwhile, we found that the lymphocytes, monocytes, $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ T cells and P/F ratio were protective factors (OR < 1) for COVID-19 patients. Based on the condition we mentioned, age, gender, dyspnea, and laboratory indicators of lymphocytes, CRP, cTnI and LDH were chosen for a multivariable logistic regression model. As a result, serum lymphocytes (OR:0.2, 95% CI:0.04-0.96, $P < 0.05$), CRP (OR:1.026, 95% CI:1.006-1.046, $P < 0.05$), and LDH (OR:1.009, 95% CI:1.002-1.016, $P < 0.05$) were found to be independent risk factors for the severity of COVID-19 patients (Table 3).

The predictive factors correlated with severity of COVID-19

We used clinical severity scores (APACHE II and SOFA) to assess the disease severity in COVID-19 patients. The average APACHE II score was 8.5 in severe cases versus 4.0 in non-severe cases, with SOFA 2.0 versus 1.0 (Table 1, $P < 0.001$). We used speculated factors such as lymphocytes, AST, CRP and LDH performing Pearson and Kendall's tau_b correlation analysis with APACHE II and SOFA score. The results showed that lymphocytes had a negative correlation with APACHE II ($R = -0.437$, $P < 0.001$) and SOFA

Table 1. Demographic and characteristics of COVID-19 patients.

	All patients (N = 107)	Nonsevere patients (N = 59)	Severe patients (N = 48)	*P* value
Age(median, IQR)years	63, (49-71)	61, (43-69)	67, (56-76)	0.005
Gender	-	-	-	0.110
Male	60, 56.1%	29, 49.2%	31, 64.4%	
Female	47, 43.9%	30, 50.8%	17, 35.4%	
History	-	-	-	
Hypertension	28, 26.2%	14, 23.7%	14, 29.2%	0.524
DM	13, 12.1%	9, 15.3%	4, 8.3%	0.276
CHD	5, 4.7%	2, 3.4%	3, 6.3%	0.655
AD	3, 2.8%	0, 0%	3, 6.3%	0.087
Symptoms	-	-	-	
Fever(Highest) (median, IQR)°C	38.3, (38-38.7)	38, (38-38.5)	38.4, (37.9-39)	0.478
Dyspnea	30, 28%	12, 20.3%	18, 37.5%	0.049
APACHE II	6.0, (3-8)	4.0, (2-6)	8.5, (6-11)	<0.001
SOFA	2, (1-2)	1, (0-1)	2, (2-3)	<0.001
PSI	68.1±30.7	55.76±24.53	83.35±30.95	<0.001
CURB65	1, (0-1)	0, (0-1)	1, (1-2)	<0.001
CT score	2,(1-4)	1, (1-2)	4, (3-5)	<0.001

(CHD: Coronary Heart Disease; AD: Autoimmune Disease)

(R = -0.486, $P < 0.001$), while other indicators CRP (R = 0.484 $P < 0.001$, R = 0.580 $P < 0.001$), LDH (R = 0.352 $P < 0.001$, R = 0.560 $P < 0.001$) and AST (R = 0.287 $P < 0.001$, R = 0.425 $P < 0.001$) were positively associated with both APACHE II and SOFA scores (Figure 1, Table 4).

The predictive factors correlated with the severity of lung damage

We used P/F ratio to assess the severity of pneumonia induced lung injury in COVID-19 patients. The average P/F ratio was 214 mmHg in severe cases versus 413 mmHg in non-severe cases (Table 2, $P < 0.001$). We also evaluated the extent of inflammation on chest CT using a semiquantitative rating system (Supplementary Table 1); the median score was 4.0 in severe cases while only 1.0 in non-severe cases (Table 1, $P < 0.001$). Indicators above were further performed Pearson and Kendall's tau_b correlation analysis with P/F ratio and CT rating score to determine the potential biomarkers for the lung injury. As a result, the serum LDH level showed the highest R value, positively with CT score (R = 0.556, $P < 0.001$) and negatively with P/F ratio (R = -0.249, $P = 0.017$) in all the indicators (Figure 2D, Table 4). Nevertheless, although the serum CRP (R = 0.507, $P < 0.001$), AST (R = 0.519, $P < 0.001$) and lymphocytes (R = -0.411, $P < 0.001$) were significantly related to CT scores, they showed no correlation with P/F ratio (Figure 2A–2C).

The predictive factors for identification of severe COVID-19 cases

To assess the diagnostic value of these selected parameters, receiver operating characteristic (ROC) curve and area under ROC curve (AUC) were calculated using R package "pROC". As indicated in Figure 3, the area under curve (AUC = 0.878) implied a perfect accuracy of the serum LDH level more than 344.5 U/L in COVID-19 patients as a predictive factor for identification of severe condition, with the high specificity (96.9%) and sensitivity (68.8%) (Figure 3D). The serum AST level over 28 U/L and CRP over 88.85 mg/L showed relative moderate accuracy with AUC = 0.827 and AUC = 0.859 (Figure 3B, 3C). As a protective factor, the lymphocytes less than 0.985 x 10^9 /L showed a good accuracy for identification of severe patients with AUC = 0.868, the maximum specificity (84.1%) and sensitivity (80.0%) (Figure 3A). Furthermore, AUC of P/F ratio was 0.889, CT score was 0.881, and APACHE II was 0.852. The other indicators were relatively poor accuracy factors in ROC curve analysis (Figure 3, Table 5).

Table 2. Laboratory Indices of COVID-19 patients.

	All patients (N = 107)	Nonsevere patients (N = 59)	Severe patients (N = 48)	*P* value
White blood cell (×10^9/L)	5.81, (4.23-8.11)	5.38, (4.23-7.26)	7.11, (4.22-10.4)	0.032
Neutrophil (×10^9/L)	3.88, (2.52-6.08)	3.41, (2.14-4.64)	5.67, (3.22-9.57)	<0.001
Lymphocyte (×10^9/L)	1.0, (0.68-1.5)	1.3, (1.01-1.71)	0.72, (0.58-0.92)	<0.001
Monocyte (×10^9/L)	0.46±0.21	0.54±0.21	0.35±0.17	<0.001
CD3(/uL)	550, (343-846.5)	819.5, (572-1083.5)	365, (289-527.5)	<0.001
CD4(/uL)	336, (226.5-539.5)	509, (350-697.75)	240, (172-317)	<0.001
CD8(/uL)	191, (104.5-311.5)	259.5, (191.25-366.5)	118, (64-187)	<0.001
CD19(/uL)	123, (81-197)	175.5, (101.25-226.25)	102, (76-167)	0.015
CD16+56(/uL)	115, (70.5-186.5)	135.5, (97.75-227.5)	95, (61.5-161.5)	0.010
ALT(U/L)	24, (17-39)	19.5, (16-29.5)	29, (21-51)	0.001
AST(U/L)	27, (19-39)	21, (17.25-27)	39, (29-56)	<0.001
ALB(g/L)	37.91±5.95	40.18±5.84	34.95±4.68	<0.001
TB(umol/L)	12, (8.5-15.7)	10.8, (8.2-14.4)	13.6, (9.4-17.3)	0.037
LDH(U/L)	273, (195-414)	206, (174-272)	426, (298.25-516.25)	<0.001
Urea (mmol/L)	4.76, (3.98-6.51)	4.39, (3.82-5.84)	6.3, (4.2-8.4)	0.006
Crea (umol/L)	64, (53-74)	64.5, (53-73.5)	63, (54-74)	0.846
BG (mmol/L)	5.46, (4.78-6.93)	5.12, (4.61-6.0)	6.12, (5.26-7.54)	0.003
CRP (mg/L)	35.8, (5.0-85.1)	6.35, (5.0-38.18)	93.4, (37.73-158.38)	<0.001
cTnI (ng/mL)	0.006, (0.006-0.011)	0.006, (0-0.006)	0.01, (0.006-0.073)	<0.001
CKMB (ng/mL)	1.16, (0.64-1.85)	0.96, (0.62-1.33)	1.48, (0.91-2.96)	<0.001
BNP (pg/mL)	147, (50.43-437.7)	84.33, (22.64-170.68)	289, (135-911.2)	<0.001
PT (s)	12, (11.28-13)	11.8, (11.2-12.3)	12.4, (11.85-13.45)	<0.001
APTT (s)	28.05, (26.1-30.6)	26.9, (25.85-29.65)	28.8, (26.3-32.1)	0.022
D-Dimer (mg/L)	0.76, (0.36-2.14)	0.45, (0.26-1.04)	2.06, (0.76-8.37)	<0.001
Fib (g/L)	4.54±1.57	4.05±1.53	5.21±1.37	<0.001
IgA (g/L)	2.28, (1.76-3.03)	2.07, (1.65-2.63)	2.65, (1.83-3.97)	0.053
IgE (IU/mL)	89.4, (37.8-157.5)	73.8, (31.7-128)	97.65, (38.42-272)	0.374
IgM (g/L)	0.96±0.40	1.0±0.44	0.91±0.36	0.279
IgG (g/L)	11.7, (9.97-13.2)	11.65, (9.95-12.7)	11.9, (10.01-15.2)	0.252
C3 (g/L)	1.04±0.25	1.03±0.23	1.07±0.28	0.467
C4 (g/L)	0.27±0.12	0.26±0.15	0.28±0.08	0.546
PaO2/FiO2 (mmHg)	250.26±142.87	413.22±141.31	214.01±116.89	<0.001

Table 3. Univariable OR and multivariable OR of severe COVID-19 patients.

	Univariable OR (95% CI)	P value	Multivariable OR (95% CI)	P value
Demographics and clinical characteristics				
Age(years)	1.04 (1.01-1.07)	0.005	0.99 (0.94-1.05)	0.786
Gender(Male)	1.89 (0.86-4.12)	0.111	0.40 (0.09-1.87)	0.244
Dyspnea	2.35 (0.99-5.57)	0.052	2.51 (0.59-10.62)	0.212
Labaray paremeters				
White blood cell (×10^9/L)	1.20 (1.04-1.38)	0.011		
Neutrophil (×10^9/L)	1.34 (1.14-1.57)	<0.001		
Lymphocyte (×10^9/L)	0.019 (0.009-0.11)	<0.001	0.20 (0.04-0.96)	0.044
Monocyte (×10^9/L)	0.005 (0.00-0.067)	<0.001		
CD3(/uL)	0.995 (0.992-0.997)	<0.001		
CD4(/uL)	0.992 (0.988-0.995)	<0.001		
CD8(/uL)	0.989 (0.984-0.994)	<0.001		
CD19(/uL)	0.994 (0.989-0.999)	0.017		
CD16+56(/uL)	0.996 (0.991-1.00)	0.076		
ALT(U/L)	1.03 (1.01-1.05)	0.005		
AST(U/L)	1.10 (1.05-1.14)	<0.001		
LDH(U/L)	1.01 (1.01-1.02)	<0.001	1.009 (1.002-1.016)	0.016
Urea(mmol/L)	1.08 (0.97-1.20)	0.167		
BG (mmol/L)	1.18 (0.99-1.41)	0.060		
CRP (mg/L)	1.03 (1.02-1.05)	<0.001	1.026 (1.006-1.046)	0.012
cTnI (ng/mL)	7832.09 (0.29-2.1x10^9)	0.085	1.803 (0-132491.35)	0.918
CKMB (ng/mL)	2.18 (1.28-3.73)	0.004		
BNP (pg/mL)	1.00 (1.00-1.00)	0.638		
PT (s)	2.50 (1.49-4.19)	0.001		
APTT (s)	1.17 (1.02-1.33)	0.022		
D-Dimer (mg/L)	1.61 (1.14-2.27)	0.007		
Fib (g/L)	1.70 (1.24-2.31)	0.001		
PaO2/FiO2 (mmHg)	0.988 (0.980-0.997)	0.005		

Relationship between LDH and inflammation, cardiac and liver injury biomarkers

As the serum LDH level showed the highest Correlation Coefficient in the correlation with APACHE II, SOFA, CT score, and P/F ratio, we evaluated the relationship between LDH and lymphocytes (including subsets), serum CRP, AST, BNP, and cTnI level. We found that LDH was positively correlated with CRP, AST, BNP, and cTnI, while negatively correlated with lymphocytes and its subsets, including $CD3^+$, $CD4^+$ and $CD8^+$ T cells ($P < 0.01$) (Figure 4).

DISCUSSION

In this study, we analyzed the clinical features in 107 patients with COVID-19 who were admitted to Renmin Hospital of Wuhan University between February 1 and March 1, 2020. Although the clinical characteristics of patients enrolled were somewhat akin to those reported in previous studies [1, 5, 7], there were no differences in gender and proportion with underlying diseases between severe and non-severe patients. Patients with advanced age were more likely to progress into severe pneumonia, which was not unexpected, in concert with recent studies [9]. We also found that the clinical characteristics of COVID-19 mimic those of SARS-CoV [1, 5, 10]. Fever and cough were the dominant symptoms, both groups had similar maximum temperature and number of patients who had dyspnea. APACHE II and SOFA scores were calculated based on admission data. PSI, CURB-65, and the CT semiquantitative rating score were used to assess the severity of lung damage. Significantly higher scores were found in severe cases.

Among the risk factors we investigated in this study, we surprisingly discovered that LDH had the most positive relationship between both P/F ratio and CT score. In addition, it was also most positively relevant to APACHE II and SOFA scores, which reflected a strong correlation between LDH with lung damage as well as disease severity. LDH is a major player in glucose metabolism which is present in tissues throughout the body and catalyzes pyruvate to lactate. It is released from cells upon damage of their cytoplasmic membrane [11]. Previous studies also had noted the importance of LDH as an indicator of lung diseases. In a study on Epstein-Barr virus (EBV), researchers found that EBV infected B cells had more LDH transcripts than the uninfected B cells [12]. In addition, the serum levels of LDH increased in pneumocystis pneumonia (PcP) patients, probably was due to lung injury [13, 14]. Among patients who were infected during the 2009 influenza A (H1N1) pandemic, 77.8% whose laboratory test showed LDH > 225U/L had lung involvement, without differences between adult and children [15], which indicated that LDH elevation was associated with various pathogens including viruses, and was relevant to lung injury. Furthermore, there was a case reported in 2017 that a patient with human Zika virus infection had markedly elevated LDH, which was associated with 70% mortality in further a Zika-infected animal study. They considered LDH as an indicator of multiorgan injury, not only affecting liver or cardiac function [16].

LDH is found in all human cells, especially in myocardial and liver cells. In our study, LDH elevation was positively associated with AST, cTnI and BNP, which verified it as an isozyme of heart and liver. However, it was somewhat surprising that cTnI and BNP were not associated with P/F ratio, while was relevant with disease severity (data not shown). Exactly similar with which, AST, that was associated with the APACHE II and SOFA score, was not related to P/F ratio, either. This rather intriguing finding might be explained by the fact that the myocardial and liver injury caused by SARS-CoV-2 might be due to the direct damage of the virus to targeted organs, not because of hypoxia induced by lung injury. Since the outbreak of COVID-19, structural analysis of the virus has suggested that SARS-CoV-2 might be able to bind to the angiotensin-converting enzyme 2 (ACE2) receptor in humans [17, 18]. The ACE2 receptor is abundantly present in the epithelia of lung and small intestine [19], which might provide possible routes of entry for SARS-CoV-2. This epithelial expression, together with its presence in vascular endothelium [19], also provides a step in understanding the pathogenesis of ARDS, cardiac injury, liver injury, and even MODS.

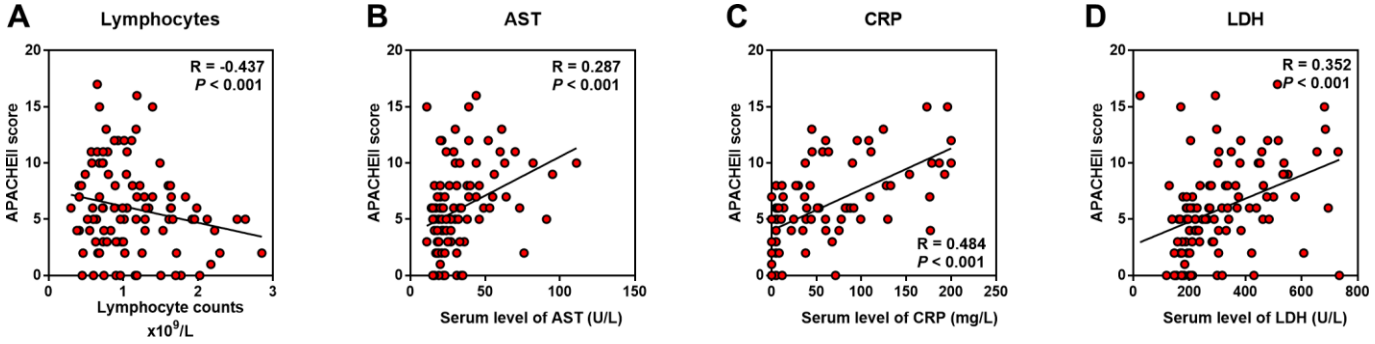

Figure 1. Predictive factors correlated with severity of COVID-19 patients. Correlation analysis was performed between candidate indicators with APACHE II score. (**A**) Lymphocyte counts was negatively correlated with APACHE II; (**B**–**D**) AST, CRP and LDH were positively correlated with APACHE II.

Table 4. Correlation with SOFA and CT semiquantitative rating score.

Predictive factors	SOFA score		CT score	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
Lymphocyte (×10^9/L)	-0.486	<0.001	-0.411	<0.001
AST (U/L)	0.425	<0.001	0.517	<0.001
LDH (U/L)	0.560	<0.001	0.556	<0.001
CRP (mg/L)	0.580	<0.001	0.507	<0.001

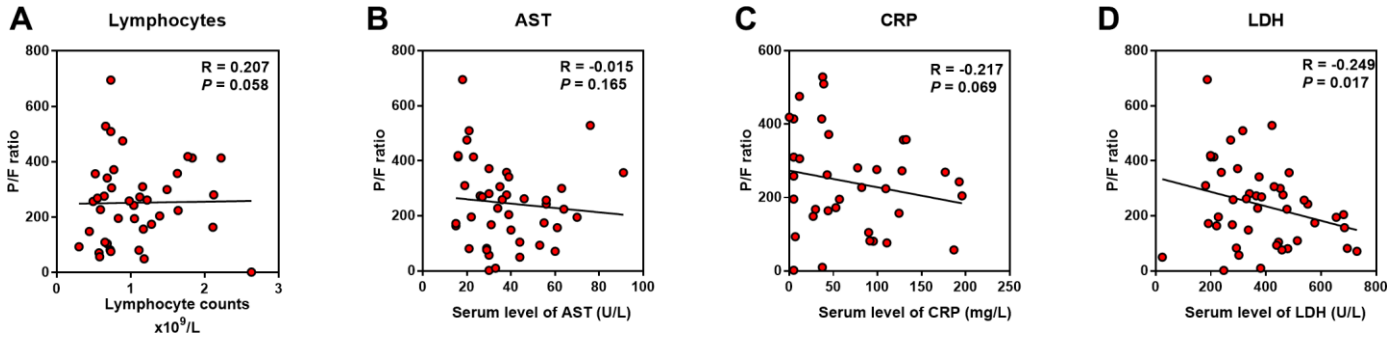

Figure 2. Predictive factors correlated with lung injury of COVID-19 patients. Correlation analysis was performed between the indicators with P/F ratio. (**A**–**C**) Lymphocyte counts, AST and CRP were not correlated with P/F ratio; (**D**), LDH was negatively correlated with P/F ratio.

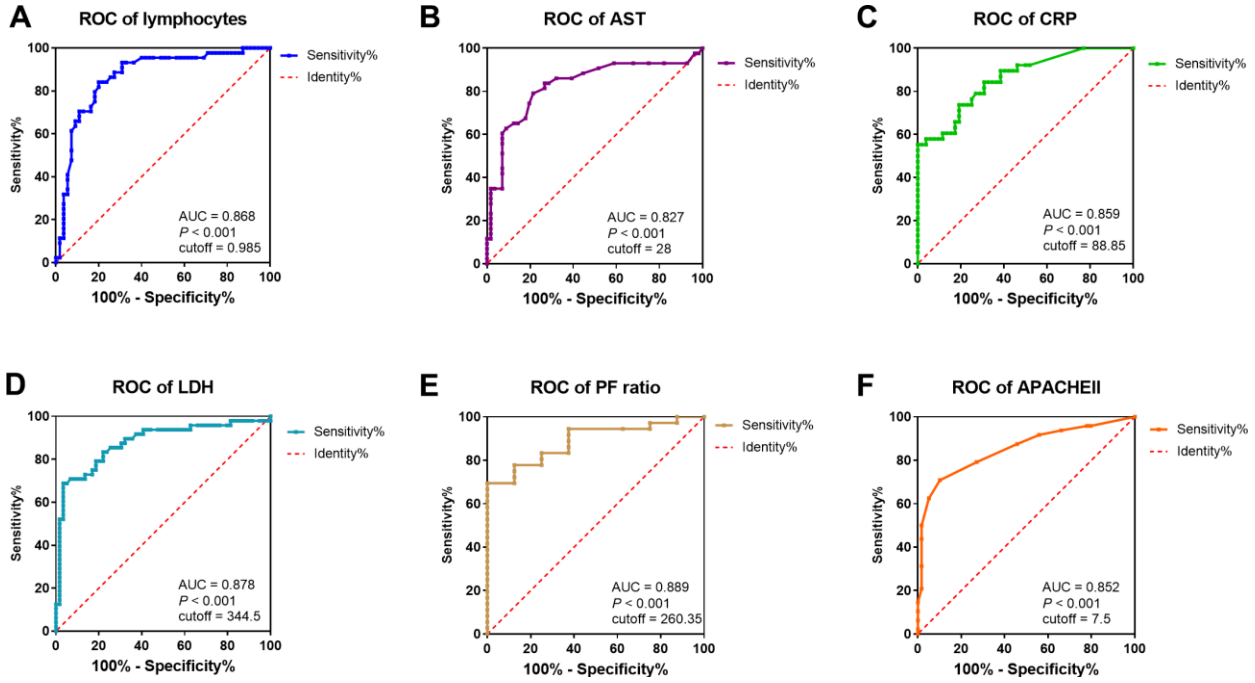

Figure 3. ROC curve and cutoff value of predictive factors. The factors for the prediction of COVID-19 patients getting severe condition. (**A**–**F**) ROC curve of lymphocytes, AST, CRP, LDH, P/F ratio, and APACHE II. AUC, area under curve.

Table 5. Cutoff value of predictive factors.

	Sensitivity	Specificity	Cutoff value	AUC	P value
Lymphocytes (×10^9/L)	0.80	0.841	0.985	0.868	<0.001
CRP (mg/L)	0.553	1.0	88.85	0.859	<0.001
LDH (U/L)	0.688	0.966	344.5	0.878	<0.001
AST (U/L)	0.791	0.786	28	0.827	<0.001
P/F Ratio (mmHg)	1.0	0.694	260.35	0.889	<0.001
CT score	0.818	0.857	2.5	0.881	<0.001
APACHE II	0.708	0.898	6.5	0.852	<0.001
PSI	0.604	0.814	76.5	0.757	<0.001

Furthermore, LDH was found to be positively associated with CRP and negatively with lymphocytes. An increase in CRP and decrease in lymphocytes were observed in severe cases during the 14-day observation period, which was consistent with findings of recent reports [1, 5, 6]. In our study, the development of lymphopenia in severe patients was mainly related to the significantly decreased absolute counts of T cells, especially $CD3^+$, $CD4^+$, and $CD8^+$T cells, but not to B cells or NK cells. The decrease of T cells in severe cases reached its trough within three days, and then slightly increased from the first week while still maintaining low levels and not recovering to the level of non-severe patients over two weeks (Supplementary Figure 1).

LDH is not only a metabolic but also an immune surveillance prognostic biomarker, its elevation is a harbinger of poor outcomes in immunosuppressed patients [20]. LDH increases production of lactate, leads to enhancement of immune-suppressive cells, including macrophages and dendritic cells (DCs), and inhibition of cytolytic cells, such as natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs) [11]. LDH is often induced upon T cell activation and proliferation [21, 22]. In a retrospective analysis of a CTLs antigen-4 antibody which could enhance T-cell activity and proliferation, the results showed that an increase in LDH level was indicative of a poor outcome [23], which confirms the inhibition

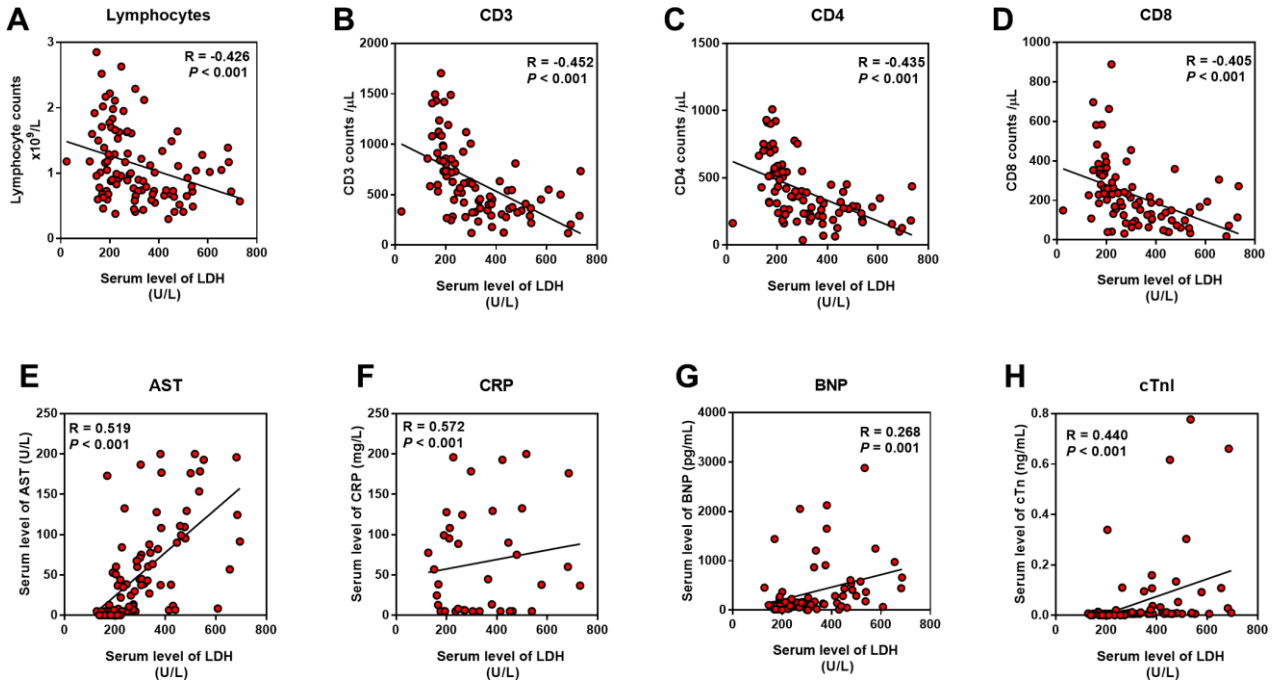

Figure 4. Relationship between LDH and inflammation, cardiac and liver injury. Pearson correlation analysis was performed between the indicators with the serum LDH level. (**A**–**D**) LDH was negatively correlated with lymphocyte and its subsets; (**E**–**H**) LDH was positively correlated with AST, CRP, BNP and cTnI.

effect of LDH on CTLs. Furthermore, $CD4^+$ T cells produce less IFN-γ in the absence of LDH, demonstrating a critical role for LDH in promoting T cell responses [22].

It was also hypothesized that change in lactate modulated the inflammatory response in macro-phages [24]. Suppression of LDH has anti-inflammatory effects due to the downregulation of several inflammatory mediators including cytokines and NO [24]. Also, significant correlations were found between LDH and cytokines/chemokines, therefore suggesting that LDH may be a useful biomarker to assist the clinician in the decision to hospitalize a child with bronchiolitis [25]. In our study, lymphocytes, especially $CD3^+$, $CD4^+$, and $CD8^+$ T cells were significantly decreased and relevant with LDH elevation. The decrease in T cell counts was strongly correlated with the severity of disease, which was in keeping with previous studies on SARS [26, 27]. On the other hand, elevation of LDH, the immune-related factor, could be considered as a predictive factor, that reflected a poor prognosis in severe COVID-19 patients.

Our study had some limitations. This study was conducted at a single-center with limited sample size. Furthermore, because many patients remained in hospital and outcomes were unknown at the time of writing, we only collected clinical data within two weeks for our analysis. COVID-19 has spread rapidly and has a wide spectrum of severity. A larger cohort study of patients with COVID-19 globally would help to further define the clinical characteristics and risk factors of the disease.

CONCLUSION

In summary, this study showed that LDH could be identified as a powerful predictive factor for early recognition of lung injury and severe COVID-19 cases. And importantly, lymphocytes, especially $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the peripheral blood of COVID-19 patients, which was relevant with serum LDH, were also dynamically correlated with the severity of the disease.

MATERIALS AND METHODS

Data collection

107 confirmed COVID-19 patients at Renmin Hospital of Wuhan University between February 1 to March 1, 2020 were enrolled into this retrospective observational study. A confirmed case of COVID-19 was defined as a positive result on real-time reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay of nasal-pharyngeal swab specimens.

A trained team of physicians and medical students reviewed and collected demographic, epidemiological, clinical, physical examination findings, and laboratory data from electronic medical records. Laboratory assessments consisted of complete blood count, liver and renal function, markers of cardiac injury, measures of electrolytes, C-reactive protein and procalcitonin, and assessment of coagulation and lactate dehydrogenase, among other parameters. We defined the degree of severity of COVID-19 patients (severe vs. non-severe) at the time of admission, according to American Thoracic Society (ATS) guidelines for CAP [28]. If imaging scans were available, the radiologic assessments of chest computed tomography (CT) were reviewed and scored by an experienced senior radiologist who extracted the data. The APACHE II and SOFA score were calculated based on clinical and experimental data on admission, and CT score was calculated based on a semiquantitative rating system (Supplementary Table 1).

Patients were followed up for 14 days after admission. Patient information was confidentially protected by assigning a deidentified ID to each patient. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University.

Statistical analysis

A sample size of at least 17 patients per group is needed to achieve 91% power to detect a difference of 0.3 between the area under the ROC curve (AUC) under the null hypothesis of 0.5 and an AUC under the alternative hypothesis of 0.8 using a two-sided test at a significance level of 0.05 (PASS 15, NCSS, LCC).

Because the patients enrolled in our study were not randomly assigned, all statistical findings should be interpreted as descriptive only. Quantized variables were presented as means ± standard deviation, and significance was tested by t-test. Nonparametric variables were expressed as medians and interquartile ranges or simple ranges as appropriate, and we used the Mann Whitney U or Kruskal Wallis tests to compare differences. Continuous and categorical variables were summarized as counts and percentages, and significance was detected by chi square or Fisher's exact test. To explore the risk factors associated with severity of COVID-19, univariable and multivariate logistic regression models were used. Correlation analysis was performed by using Pearson and Kendall's tau_b Correlation Coefficient. The sensitivity and specificity of the risk factors for the patient diagnosis were represented and analyzed by receiver operating characteristic curve (ROC curve).

All the analyses and figures were performed with SPSS software (Version 26) and Graphpad Prism (Version 7.0). $P < 0.05$ was considered statistically significant in all analyses.

Abbreviations

ACE2: angiotensin-converting enzyme 2; ALT: alanine transaminase; APACHEII: Acute Physiology and Chronic Health Evaluation II; APTT: activated partial thromboplastin time; AST: aspartate transaminase; ATS: American Thoracic Society; AUC: area under the ROC curve; BNP: B-type natriuretic peptide; CAP: community-acquired pneumonia; CKMB: creatine kinase-MB; COVID-19: coronavirus disease 2019; CRP: C-reactive protein; cTnI: troponin I; CT: computed tomography; CURB-65: Confusion/Urea/Respirotory rate/Blood pressure 65; EBV: Epstein-Barr virus; LDH: lactate dehydrogenase; OR: odds ratio; PcP: pneumocystis pneumonia; PSI: Pneumonia Severity Index; PT: prothrombin time; ROC curve: receiver operating characteristic curve; RT-PCR: real-time reverse-transcriptase-polymerase-chain-reaction; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SOFA: Sequential Organ Failure Assessment

AUTHOR CONTRIBUTIONS

Y.H. collected, analyzed data and drafted the manuscript; H.Z. acquired CT image and evaluated lung infiltration by a semiquantitative scoring system; S.M. organized, analyzed and interpreted the data. Y.H., H.Z. and S.M. are co-first authors, the order of the authorship was based on their contributions to this study. G.G. designed the study, and took responsibility for the integrity of data and the accuracy of data analysis; Y.X. provided the administrative, technical and material support; Y.Z. helped with technical support of radiology. G.G., Y.X., and Y.Z. are co-corresponding authors. W.W. and C.J. helped organize data and perform literature search; Z.S. helped revise the manuscript; C.T. was the supervisor of this study, who provided essential theoretical guidance of this study.

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CONFLICTS OF INTEREST

All authors declared that they had no conflict of interest.

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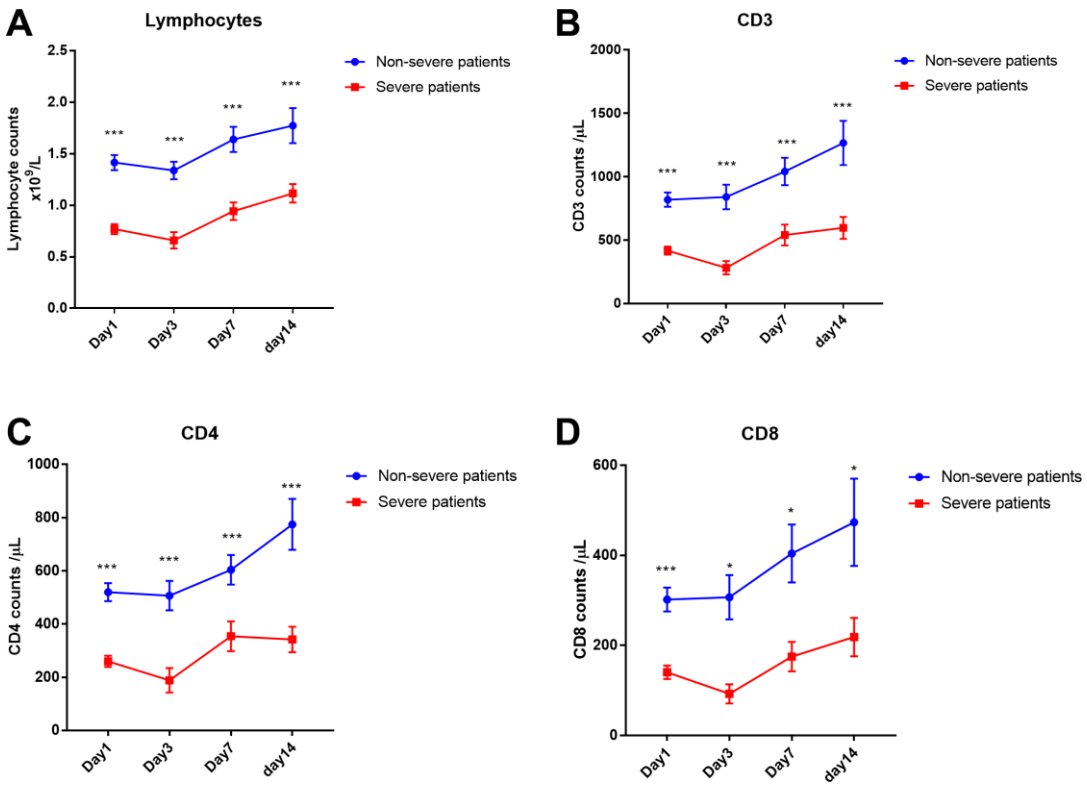
SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Change of lymphocyte and its subsets during 14-day observation period. (In severe cases, the decrease of cells reached its trough within three days, and then slightly increased from the first week while still maintaining low levels and not recovering to the level of non-severe patients over two weeks. (**A**) change of lymphocyte; (**B**–**D**) change of lymphocyte subsets.).

Supplementary Table

Supplementary Table 1. Semiquantitative rating system based on CT image.

Methods	Based on Lobes		Based on Whole Lung
	6-part	5-part	
Evaluation Range	0, 1, 2, 3, 4	0, 1, 2, 3, 4, 5	Disease dimension/whole lung dimension (%)
Evaluation Standard	Based on the lobes involved		Based on whole lung
	0, % lesion 1, <25% 2, 26-49% 3, 50-75% 4, >75%	0, 0% lesion 1, <5% 2, <25% 3, 26-49% 4, 50-75% 5, >75%	
Advantage	Easy	Standard	More quantitative

Research Paper

Lessons from a lumbar burst fracture patient infected with SARS-CoV-2

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ABSTRACT

In December 2019, the 2019 novel coronavirus (SARS-CoV-2) began spreading in China. At present, there are no special protocols for treating lumbar burst fracture (LBF) patients infected with SARS-CoV-2. Here, we present our lessons and experiences with a patient presenting with a severe LBF complicated by an occult SARS-CoV-2 infection. The clinical data for a 52-year-old male LBF patient were collected during the incubation period of COVID-19. The patient exhibited no obvious COVID-19-related symptoms prior to his surgery, and his vital signs were stable on the first day after the operation. By postoperative day 3, however, the patient was exhibiting chills and high fever. A chest CT showed a patchy high-density shadow surrounded by ground-glass opacity in the lower portion of his right lung. A nucleic acid test for SARS-CoV-2 was positive, and the patient was then transferred to the Department of Infectious Disease for further special treatment. This case taught that when treating patients with severe trauma within an epicenter of this pandemic, it is crucial for healthcare workers to be vigilant so as to avoid potential widespread outbreaks of COVID-19 within hospitals.

INTRODUCTION

SARS-CoV-2 refers to a novel coronavirus that firstly reported in Wuhan, Hubei province of China in December 2019 and quickly spread to the world [1]. SARS-CoV-2 belongs to the genus coronavirus, which is able to infect mammals, including humans [2]. The genetic characteristics of SARS-CoV-2 are significantly different from the acute respiratory syndrome coronavirus (SARS-CoV) and the middle east respiratory syndrome coronavirus (MERS-CoV) [3]. A recent study reported that SARS-CoV-2 is very similar to another virus in its family, which is carried by bats, leading some investigators to speculate that bats may host this new virus [4]. Currently, around 3.27 million cases have been confirmed, with 234,000 deaths worldwide [5].

Lumbar burst fracture (LBF) is very common in the elderly, and it is well known that elderly fracture patients are susceptible to pulmonary infection. This is especially true for patients for spinal fractures, which make it difficult for them to walk. This raises the question, what do we do when a fracture patient is infected with SARS-CoV-2? There is currently no protocol for this situation. Here, we summarize our experience and lessons learned when diagnosing and surgically treating a patient with a severe LBF who is also infected with SARS-CoV-2.

Patient and treatment

A 52-year-old male suffering with a L3 LBF presented with lower back pain and serious weakness in both lower limbs. The patient had history of shopping while

wearing an ordinary mask. Physical examination indicated that his bilateral hallux dorsal extensor muscle strength level was 2, bilateral tibialis anterior muscle strength was level 2, bilateral quadriceps muscle strength was level 4, and bilateral iliopsoas muscle strength was level. X-ray, magnetic resonance imaging (MRI), and computed tomography (CT) examinations revealed an L3 LBF as well as severe spinal cord compression (Figure 1). The patient denied experiencing fever, cough, sputum production, dyspnea, nausea or vomiting. There was no significant lung abnormality on preoperative CT examination (Figure 2), and tests for viruses indicated the patient to be influenza a virus RNA (-), influenza b virus RNA (-), and respiratory syncytial virus RNA (-). Routine blood counts showed leucocytes 11.43 g/L, erythrocytes 4.87 g/L, hemoglobin 139 g/L, platelets 208 g/L, neutrophils 88.5%, lymphocytes 5.9%, monocytes 5.5%, eosinophils 0%, and basophils 0.1%.

Lumbar posterior decompression and fixation was performed in a laminar flow, negative pressure operating room. All procedures were performed while strictly adhering to biosafety level 3 standards. In addition to routine personal protection, goggles were worn and postoperative disinfection was performed. The surgery took around 1 hour and 30 minutes, and there were 200 ml of blood loss without blood transfusion. After the surgery, the patient was transferred to the intensive care unit for medical observation and treatment. On the second postoperative day, the patient was safely returned back to the ward with a lumbar brace. Postoperative radiography and CT revealed that the internal repair was well positioned (Figure 3).

On the first postoperative day, the patient had no obvious cough or sputum production, and his vital signs were stable. By the third postoperative day, however, the patient had developed chills and a high fever, which reached 39.5°C. Routine examination showed that his leukocyte count was 7.7 g/L, hemoglobin 126 g/L, neutrophils 81.3%, lymphocytes 8.9%, monocytes 9.5%, eosinophils 0% and basophils 0.3%. Levels of procalcitonin and c-reactive protein were 0.146 ng/mL and 144.9 mg/L, respectively. A chest CT examination showed a patchy high-density shadow surrounded by a ground-glass opacity in the lower portion of the right lung (Figure 4). After consultation with specialists from the Department of Infectious Disease, the patient was immediately isolated in a single room to prevent potential widespread infection. By postoperative day 5, the patient's temperature had decreased to 37.2°C, and his cough had significantly diminished. A nucleic acid test for SARS-CoV-2 was positive, and the patient was transferred to the Department of Infectious Disease for further treatment, which consisted of cefoperazone sulbactam 1.5 g every 12 hours, abidole hydrochloride 0.2 g orally three times a day, and oseltamivir 75 mg orally twice a day. The patient's vital signs remained stable, and his cough and fever eventually resolved.

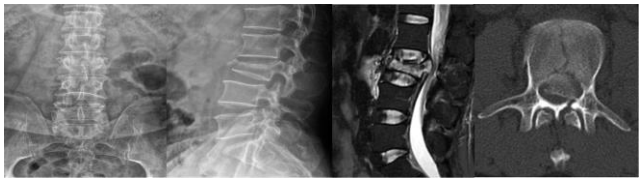

Figure 1. Lumbar X-rays, MRI, and CT examinations of the patient.

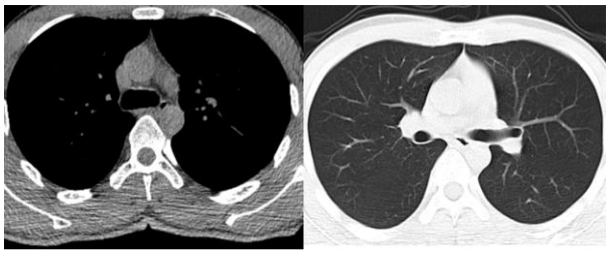

Figure 2. Preoperative chest CT examination of the patient.

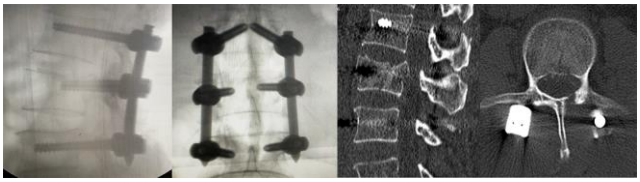

Figure 3. Postoperative X-rays and CT examination of the patient.

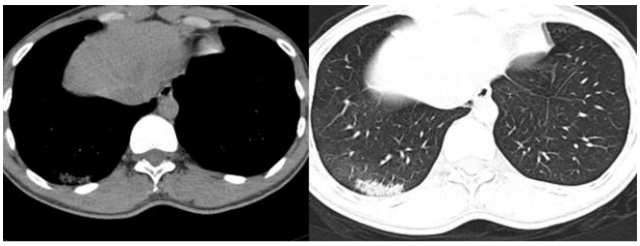

Figure 4. Chest CT examination of the patient on postoperative day 3.

DISCUSSION

A previous study reported that the incubation period for COVID-19 ranges from 1 to 14 days, with a average period from 3 to 7 days [6]. However, a recent retrospective study by the team of Nanshan Zhong, who is credited with detecting SARS in 2003, indicated that the incubation period for COVID-19 could be as long as 24 days, or 10 days longer than previously understood. That study, which has yet to be peer-reviewed, also suggested that patients can infect other people during the incubation period, echoing the findings of other reports about this virus. The most common symptoms at onset of COVID-19 are fever, fatigue, dry cough, myalgia, and dyspnea. Less common symptoms are headache, dizziness, abdominal pain, diarrhea, nausea, and vomiting [7]. The data from a recent study showed that up to 29% of COVID-19 patients are healthcare workers. Indeed, updated data indicate that in China, a total of 1716 healthcare workers were infected by SARS-CoV-2, and 16 of them have died. The adverse effect of infection of healthcare workers on the prevention and treatment of COVID-19 is enormous. On the one hand, loss of workers due to hospital-related transmission and infection exacerbates the existing shortage of healthcare workers. On the other hand, widespread infection of healthcare workers increases the public's fear of COVID-19, which is harmful to social stability.

Fever occasionally develops after surgery as a stress response to the procedure [8–9]. In the present case, the elevated neutrophils and the reduction in lymphocytes indicated the possibility of infection, though the chest CT revealed no obvious abnormity. However, by the time the patient developed fever with an obvious dry cough on the day postoperative day 5, the chest CT definitely showed signs of pneumonia, and a nucleic acid test confirmed the SARS-CoV-2 infection on postoperative day 7. The symptoms were initially occult in this case. This patient had no significant symptoms of pulmonary infection at admission, and the chest CT results also did not support a diagnosis of pulmonary infection. Nonetheless, because of the ongoing COVID-19 epidemic, we decided to perform the spinal surgery in a laminar flow, negative pressure operating room. All procedures were performed strictly according biosafety level 3 standards, which are vital for avoiding widespread outbreak of COVID-19. Moreover, to avoid potential cross-infection, all the healthcare workers involved in this case self-isolated for 14 days. This including 3 surgeons, 1 anesthesiologist, 2 operating room nurses, and 2 ward nurses. Fortunately, no one was infected by this patient.

Several lessons can be learned from this case. First, for emergency surgery, comprehensive preoperative examinations should be performed to exclude the possibility of SARS-CoV-2 infection, and use of laminar flow, negative pressure operating rooms is strongly recommended during this COVID-19 pandemic. Second, the incubation period of COVID-19 should be given full attention, even for patients with negative results on the routine tests, perioperative isolation measures should be strong enough to prevent cross-infection among healthcare workers. Finally, for patients with confirmed SARS-CoV-2 infections, consultation with infectious disease specialists should be completed in a timely manner, and the necessary reports should be produced and submitted. If available, patients should be transferred to designated or specialized departments or hospitals for further diagnosis and treatment when necessary.

CONCLUSION

To reduce the possibility of infection of healthcare workers and to avoid potential widespread infection in hospitals, healthcare workers must take the incubation period of COVID-19 into consideration and be highly vigilant when treating emergency patients during this pandemic.

Ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Puren Hospital, Wuhan University of Science and Technology, reference number 3-2020-0131) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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PMID:32379114

Research Paper

De novo design of protein peptides to block association of the SARS-CoV-2 spike protein with human ACE2

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ABSTRACT

The outbreak of COVID-19 has now become a global pandemic that has severely impacted lives and economic stability. There is, however, no effective antiviral drug that can be used to treat COVID-19 to date. Built on the fact that SARS-CoV-2 initiates its entry into human cells by the receptor binding domain (RBD) of its spike protein binding to the angiotensin-converting enzyme 2 (hACE2), we extended a recently developed approach, EvoDesign, to design multiple peptide sequences that can competitively bind to the SARS-CoV-2 RBD to inhibit the virus from entering human cells. The protocol starts with the construction of a hybrid peptidic scaffold by linking two fragments grafted from the interface of the hACE2 protein (a.a. 22-44 and 351-357) with a linker glycine, which is followed by the redesign and refinement simulations of the peptide sequence to optimize its binding affinity to the interface of the SARS-CoV-2 RBD. The binding experiment analyses showed that the designed peptides exhibited a significantly stronger binding potency to hACE2 than the wild-type hACE2 receptor (with -53.35 vs. -46.46 EvoEF2 energy unit scores for the top designed and wild-type peptides, respectively). This study demonstrates a new avenue to utilize computationally designed peptide motifs to treat the COVID-19 disease by blocking the critical spike-RBD and hACE2 interactions.

INTRODUCTION

The continuing pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, previously known as 2019-nCoV) has now become an international public health threat, causing inconceivable loss of lives and economic instability [1]. As of May 17, 2020, there have been more than 4500000 confirmed cases and over 300000 deaths caused by COVID-19 worldwide [2]. Exacerbating the problem, there is no specific antiviral medication toward COVID-19, though development efforts are underway [3–6]. Although vaccines are thought to be the most powerful weapon to fight against virus invasion, it may take quite a long time to develop and clinically test the safety of a vaccine. Moreover, vaccines are usually limited as preventative measures given to uninfected individuals. Thus, as an emergency measure, it is desirable to develop effective antiviral therapeutics that can take effect rapidly not only to treat COVID-19, but also to prevent its further transmission.

It has been confirmed that SARS-CoV-2 initiates its entry into host cells by binding to the angiotensin-converting enzyme 2 (ACE2) via the receptor binding domain (RBD) of its spike protein [7, 8]. Therefore, it is possible to develop new therapeutics to block SARS-CoV-2 from binding to ACE2. Although small molecule compounds are commonly preferred as therapeutics, they are not effective at blocking protein-protein interactions (PPIs) where a deep binding pocket may be missing at the interface [9]. On the contrary, peptide binders are more suitable for disrupting PPIs by specifically binding to the interface binding region [10]. Also of importance, small

peptides have reduced immunogenicity [11]. These positive features make peptides great candidates to serve as therapeutics [12, 13]. Recently, Zhang et al. [14] reported that the natural 23-mer peptide (a.a. 21-43) cut from the human ACE2 (hACE2) α1 helix can strongly bind to SARS-CoV-2 RBD with a disassociation constant (K_d) of 47 nM, which was comparable to that of the full-length hACE2 binding to SARS-CoV-2 RBD [15]; they also showed that a shorter 12-mer peptide (a.a. 27-38) from the same helix was not able to bind the virus RBD. In an earlier report, Han et al. [16] performed a study to identify the critical determinants on hACE2 for SARS-CoV entry, and they found that two natural peptides from hACE2 (a.a. 22-44 and 22-57) exhibited a modest antiviral activity and inhibited the binding of SARS-CoV RBD to hACE2 with IC50 values of about 50 μM and 6 μM, respectively, implying that the peptide composed of residues 22-57 had a stronger binding affinity for SARS-CoV RBD. They also generated a peptide by linking two discontinuous fragments from hACE2 (a.a. 22-44 and 351-357) with a glycine, and this 31-mer exhibited a potent antiviral activity with an IC50 of about 0.1 μM, indicating that this artificial peptide had a much stronger binding affinity for SARS-CoV RBD than the peptides composed of residues 22-44 or 22-57. Due to the high similarity of the binding interfaces between SARS-CoV RBD/hACE2 and SARS-CoV-2 RBD/hACE2, we hypothesize that this artificial peptide may also bind to SARS-CoV-2 more strongly than the peptide 21-43 tested by Zhang et al. [14], which is similar to the peptide 22-44 from Han et al. [16]. Although the natural peptides are promising, it has been argued that the sequence of hACE2 is suboptimal for binding the S protein of SARS-CoV-2 [17]. Therefore, further redesign of the natural peptides may significantly enhance its binding affinity to the virus RBD and the improved peptide binders may have the potential to inhibit SARS-CoV-2 from entering human cells and hinder its rapid transmission.

In this work, we computationally designed thousands of peptide binders that exhibited a stronger binding affinity for SARS-CoV-2 than the natural peptides through computational experiments. Based on the crystal structure of the SARS-CoV-2 RBD/hACE2 complex, we constructed a hybrid peptide by linking two peptidic fragments from hACE2 (a.a. 22-44 and 351-357) with a glycine. Starting from the peptide-protein complex, we used our protein design approaches, EvoEF2 [18] and EvoDesign [19], to completely redesign the amino acid sequences that match the peptide scaffold while enhancing its binding affinity for SARS-CoV-2. Detailed analyses support the strong binding potency of the designed binders, which not only recapitulated the critical native binding interactions but also introduced new favorable interactions to enhance binding. Due to the urgency caused by COVID-19, we share these computational peptides to the community, which may be helpful for further developing antiviral peptide therapeutics to combat this pandemic.

RESULTS

Initial peptide scaffold construction

Several experimental SARS-CoV-2 RBD/hACE2 complex structures have been reported [20–22] and deposited in the Protein Data Bank (PDB) [23]. Specifically, PDB ID 6m17 is a 2.9 Å structure of the SARS-CoV-2 RBD/ACE2-B0AT1 complex determined using cryogenic electron microscopy (Cryo-EM) [22]. Furthermore, PDB ID 6m0j is a 2.45 Å X-ray crystal structure of SARS-CoV-2 RBD/hACE2 [20], while 6vw1 is a 2.68 Å X-ray structure of SARS-CoV-2 chimeric RBD/hACE2 [21], where the chimeric RBD is comprised of the receptor binding motif (RBM) from SARS-CoV-2 S and the core from SARS-CoV, with the mutation N439R. The three experimental complex structures are quite similar to each other in terms of global folds (Figure 1A). Since 6vw1 does not contain the wild-type SARS-CoV-2 RBD, we did not use it as a template. Based on a preliminary examination, we found that the structure quality of 6m0j was better than 6m17 (see below), and therefore we only considered 6m0j as the template complex.

Two peptide fragments (a.a. 22-44 and 351-357) from hACE2 (6m0j, chain A) were extracted because they formed extensive contacts with the SARS-CoV-2 RBD (6m0j, chain E). The positions 44 and 351 were chosen because the distance between their Cα atoms was only 5.5 Å (see Supplementary Figure 1), and therefore only one residue was required to link them. To reduce the interference to the surrounding amino acids, the linker residue was initially chosen as glycine. The small loop, 44S-glycine-351L, was then reconstructed using MODELLER [24], while the other parts of the whole peptide were kept constant. Five similar loop conformations were produced and the one with the best DOPE [25] score was selected, where DOPE is a built-in scoring function in the MODELLER package for model assessment and loop modeling. For the sake of simplifying the discussion, the initial hybrid peptide constructed in this manner was denoted as the wild-type (note that it was not a truly native peptide), and the complex structure of SARS-CoV-2 RBD/hACE2 hybrid peptide was used as the template for computational peptide design (Figure 1B).

Evaluation of EvoEF2 score on experimental complexes

At the very beginning of the outbreak of SARS-CoV-2, to determine its relative infectivity, many computational

studies were performed to compare the binding affinity of SARS-CoV-2 RBD for hACE2 with that of SARS-CoV RBD for hACE2 based on homology modeling structures; all these studies came up with the conclusion that SARS-CoV-2 showed much weaker binding affinity to hACE2 than SARS-CoV and SARS-CoV-2 might not be as infectious as SARS-CoV [26–28]. However, recent biochemical studies demonstrated that SARS-CoV-2 exhibits much stronger binding affinity to hACE2 than SARS-CoV [3, 15, 21], implying that the homology models may not have been sufficiently accurate for binding affinity assessment based on atomic-level scoring functions, although the global folds of these models were correct.

Here, we used the EvoEF2 energy function to evaluate the binding affinity of SARS-CoV and/or SARS-CoV-2 (chimeric) RBD for hACE2 based on the experimental structures described above. As shown in Table 1, SARS-CoV-2 RBD showed stronger binding potency (lower EvoEF2 scores indicate stronger binding affinity) to hACE2 than SARS-CoV based on the calculations performed on two X-ray crystal structures (PDB IDs: 2ajf and 6m0j), regardless of whether or not the residues at the protein-protein interfaces were repacked; the computational estimations were consistent with the experimental results (Table 1). However, the EvoEF2 binding scores calculated using the Cryo-EM structure (i.e. 6m17) were much higher than those obtained from the X-ray structure 6m0j, suggesting that the Cryo-EM structure might not be as high quality as its X-ray counterparts. We examined the possible steric clashes in these experimental structures using a criterion of $d_{ij} < 0.7(R_i+R_j)$, where d_{ij} is the distance between non-hydrogen atoms i and j, R_i and R_j are the van der Waals radii for i and j, respectively. A clash was counted if the formula holds. The d_{ij} values were calculated from the atom coordinates in the experimental structures and the van der Waals radii were adapted from the EvoEF2 force field [18]. Five clashes were detected in 6m17 but none in 6m0j or 2ajf according to this criterion. Moreover, Shang et al. [21] demonstrated that the artificial SARS-CoV-2 chimeric RBD showed improved binding affinity to hACE2, compared to the wild-type SARS-CoV-2, and this improvement was also somewhat captured by EvoEF2 (Table 1). Thus, out of the two wild-type SARS-CoV-2 RBD/hACE2 structures (6m0j and 6m17), only 6m0j was used as a template structure for the peptide design study because it was better refined.

Peptide design based on the physical score

Eight out of the 1000 low-energy sequences that were designed using the EvoEF2 energy function were duplicates, resulting in 992 non-redundant designs. The EvoEF2 total energy values of the designed protein complex structures ranged from -829 to -816 EvoEF2 energy units (EEU), the majority of which varied from -827 to -822 EEU (Figure 2A). The EvoEF2 binding energies of the 992 designed peptides to SARS-CoV-2 RBD ranged from -53 to -40 EEU, centering around -50 to -47 EEU (Figure 2B). The sequence identities between the designed peptides and the wild-type peptide was diversely distributed, varying from 15% to 50% and centering around 37% (Figure 2C), which was much higher than the sequence recapitulation rate obtained for the protein surface residues during the benchmarking of EvoEF2 [18]. Although the peptide residues were considered to be highly exposed, the high sequence identity revealed that a large number of critical binding residues should be correctly predicted, indicating that the designed peptides are reasonable.

The wild-type peptide showed an EvoEF2 binding energy of -46.46 EEU, whereas the total energy of the

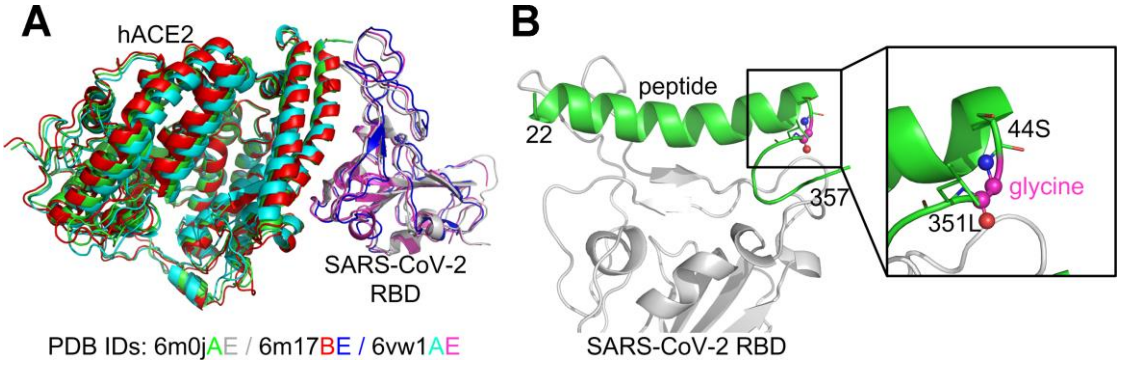

Figure 1. Comparison of the SARS-CoV-2 RBD/hACE2 complex structures (**A**) and the constructed SARS-CoV-2 RBD/hACE2 peptide complex (**B**). The superposition of the three complex structures was performed using MM-align [45]; the TM-score [46] between each complex pair was >0.98.

Table 1. Comparison of binding affinities for different PPIs.

PPI	Experiment K_d (nM)	EvoEF2 score (EEU)	
		Interface not repacked	Interface repacked
SARS-CoV RBD/hACE2	325.8 [15] 185 [21]	-40.73 (2ajfAE)	-51.12 (2ajfAE)
SARS-CoV-2 RBD/hACE2	14.7 [15] 44.2 [21]	-49.95 (6m0jAE) -19.84 (6m17BE) -19.84 (6m17DF)	-55.67 (6m0jAE) -30.50 (6m17BE) -30.50 (6m17DF)
SARS-CoV-2 chimeric RBD/hACE2	23.2 [21]	-53.15 (6vw1AE)	-58.81 (6vw1AE)

EEU stands for EvoEF2 energy unit.

wild-type peptide/SARS-CoV-2 RBD complex was -802 EEU (Figure 2D). 757 out of the 992 designs exhibited better binding affinities to SARS-CoV-2 RBD and showed lower total energies than the wild-type, and some designs showed good binding and stability simultaneously (Figure 2D), indicating that the wild-type peptide can be improved through design. Figure 2E illustrates the binding energy as a function of sequence identity for the designed peptides; it illustrates that a majority of the designs showed weaker binding affinity to SARS-CoV-2 than the wild-type peptide when the sequence identity was <25%, whereas most of the designs with sequence identities >35% exhibited stronger binding to SARS-CoV-2. These results suggest that, in general, low sequence identity designs may not be as good as high sequence identity designs. However, we can also see from Figure 2E that it does not necessarily mean that higher sequence identity always ensures better designs, since the two designs with the highest sequence identity (15/31=48.4%) did not always show stronger binding than those with sequence identities around 35%. Thus, the results suggest that good binders showed a high similarity to the wild-type, but the similarity should not be too high in order to leave room for the designs to be improved. This is in line with the common thinking that the critical binding residues (i.e. hot spot residues) should be conserved while some other residues can be mutated to enhance binding. Note that the wild-type peptide was comprised of a helix (a.a. 22-44) and a short loop (a.a. 351-357) with a glycine linker. To ensure good binding to SARS-CoV-2 RBD, the designed peptides should be able to preserve the secondary structure of this motif. To check this point, we used an artificial neural network-based secondary structure predictor [29] implemented in EvoDesign to predict the secondary structure of the designed peptides; the predictor that we used here was much faster than some other state-of-the-art predictors, e.g. PSIPRED [30] and PSSpred [31], but showed similar performance [29]. To quantify the similarity between the secondary structure of a designed peptide and that of the wild-type, we calculated the secondary structure match rate, which was defined as the ratio of the number of residues with correctly assigned secondary structure elements (i.e. helix, strand, and coil) to the total number of residues (i.e. 31). As shown in Figure 2F, 892 out of the 992 designed peptides had >90% secondary structure elements predicted to be identical to that of the wild-type peptide, indicating the high accuracy of the designs, although the EvoEF2 scoring function does not include any explicit secondary structure-related energy terms [18].

We used WebLogo [32] to perform a sequence logo analysis for the 992 designed sequences to investigate the residue substitutions and the results are shown in Figure 3A. 16 residues from the initial peptide scaffold were at the protein-peptide surface in contact with residues from SARS-CoV-2 RBD; these residues were Q24, T27, F28, D30, K31, H34, E35, E37, D38, F40, Y41, Q42, K353, G354, D355, and R357. Of these residues, Q24, D30, E35, E37, D38, Y41, Q42, and K353 formed hydrogen bonds or ion bridges with the binding partner (i.e. SARS-CoV-2 RBD) and the designed residues at these positions maintained favorable binding interactions. As shown in Figure 3A, the native residue types at these positions were top ranked out of all 20 canonical amino acids, suggesting that these residues may play critical roles in binding. For the nonpolar residues that were originally buried in the hACE2 structure (e.g. A25, L29, F32, L39, L351, and F356), they were likely to be mutated into polar or charged amino acids (Figure 3A), because they were largely exposed to the bulk solvent. The three glycine residues, including the one that was artificially introduced, were conserved, probably due to the narrow space at these positions.

To further examine what interactions improved the binding affinity of most designs, we carried out a detailed examination of some designed structures. We found that favorable hydrogen bonds or hydrophobic interactions were introduced in the binder that had the lowest EvoEF2 binding score (Figure 3B, 3C); the amino acid sequence of this binder was "EQEERI QQDKRKNEQEDKRYQRYGRGKGHQP". For this design, T27 was mutated to isoleucine (Figure 3B). In the wild-type structure, the threonine was enveloped by

four hydrophobic residues on SARS-CoV-2 RBD (i.e. Y489, F456, Y473 and A475), but its hydroxyl group did not form any hydrogen bonds with the hydroxyl group of either Y489 or Y473, and the mutation enhanced the favorable burial of nonpolar groups. The interface residue H34 was substituted for asparagine (Figure 3B), introducing a hydrogen bond to Y453 on SARS-CoV-2 RBD. Additionally, two mutations, F28Q and Q24E, simultaneously formed hydrogen bonds with the amide group of N487 from SARS-CoV-2 RBD (Figure 3C). Although the mutation D355H did not form hydrogen bonds with any residues from SARS-CoV-2, it simultaneously formed two hydrogen bonds with the hydroxyl group of Y41 and the main-chain carbonyl group of G45 on the peptide, which may help stabilize the loop region (a.a. 351-357).

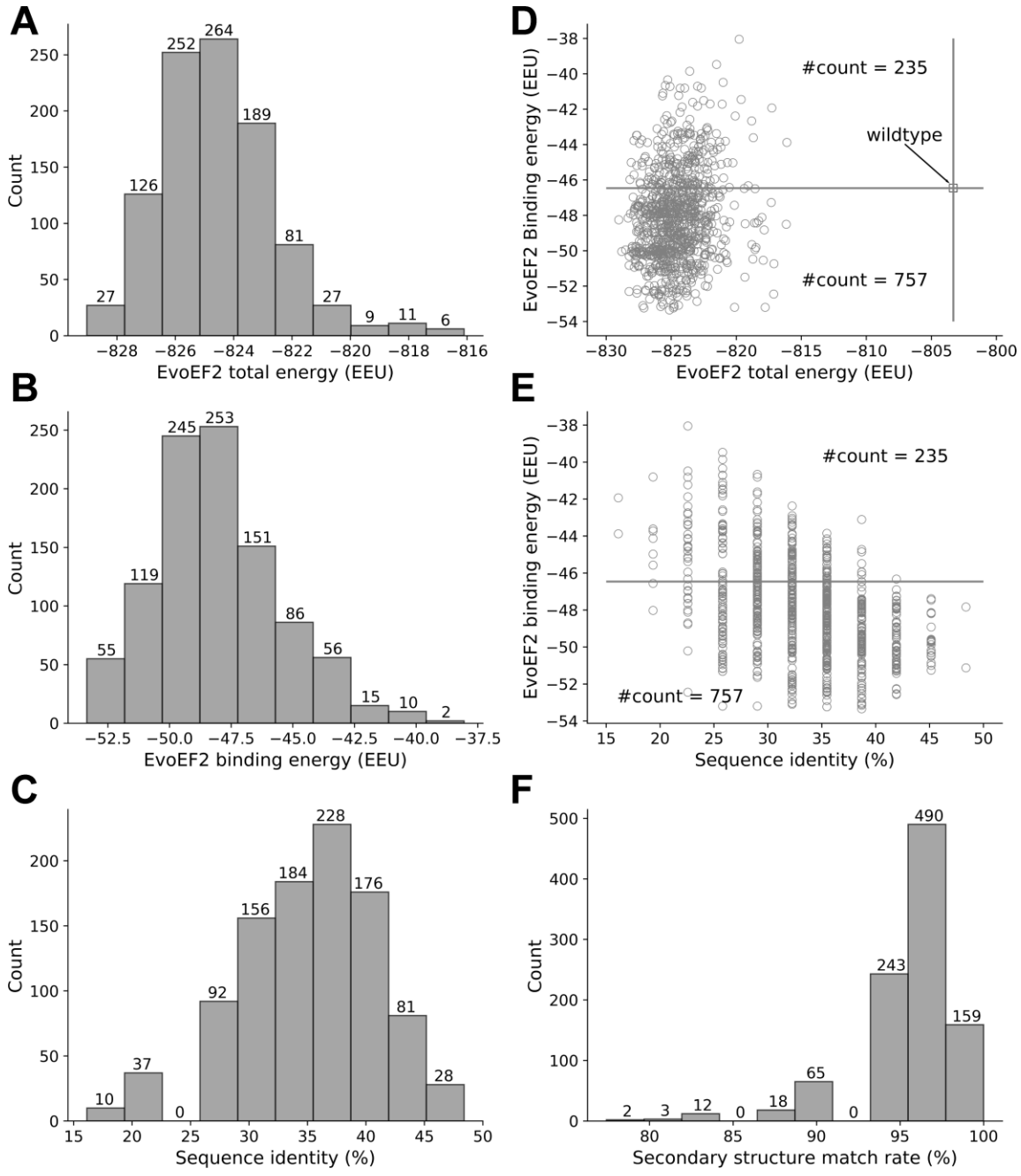

Figure 2. Overview of the characteristics of the EvoEF2 designs. (**A**) Distribution of total energy, (**B**) distribution of binding energy, (**C**) distribution of sequence identity, (**D**) binding energy as a function of total energy, (**E**) binding energy as a function of sequence identity, and (**F**) distribution of secondary structure match rate.

Peptide design based on the physical and evolutionary score

In previous studies, we found that evolutionary information can facilitate the design of proteins, improving their ability to fold into desired structures [29, 33]. To examine whether the evolutionary profile is important for peptide design here, we also performed four sets of designs with different weight settings for the evolution energy; for each design set, 1000 independent design simulation trajectories were carried out and the unique sequences out of the 1000 lowest energy designs were analyzed (Table 2). In general, giving a higher weight to the evolutionary energy facilitated the convergence of the design simulations, as indicated by the reduced number of unique designed sequences. It also helped identify sequences that were closer to the wild-type peptide as demonstrated by the higher sequence identities and the lower average evolutionary energy, which were both much more similar to those of the wild-type than the designs created using the physical score alone. We also found that incorporation of the profile energy moderately increased the ability of the designed sequences to

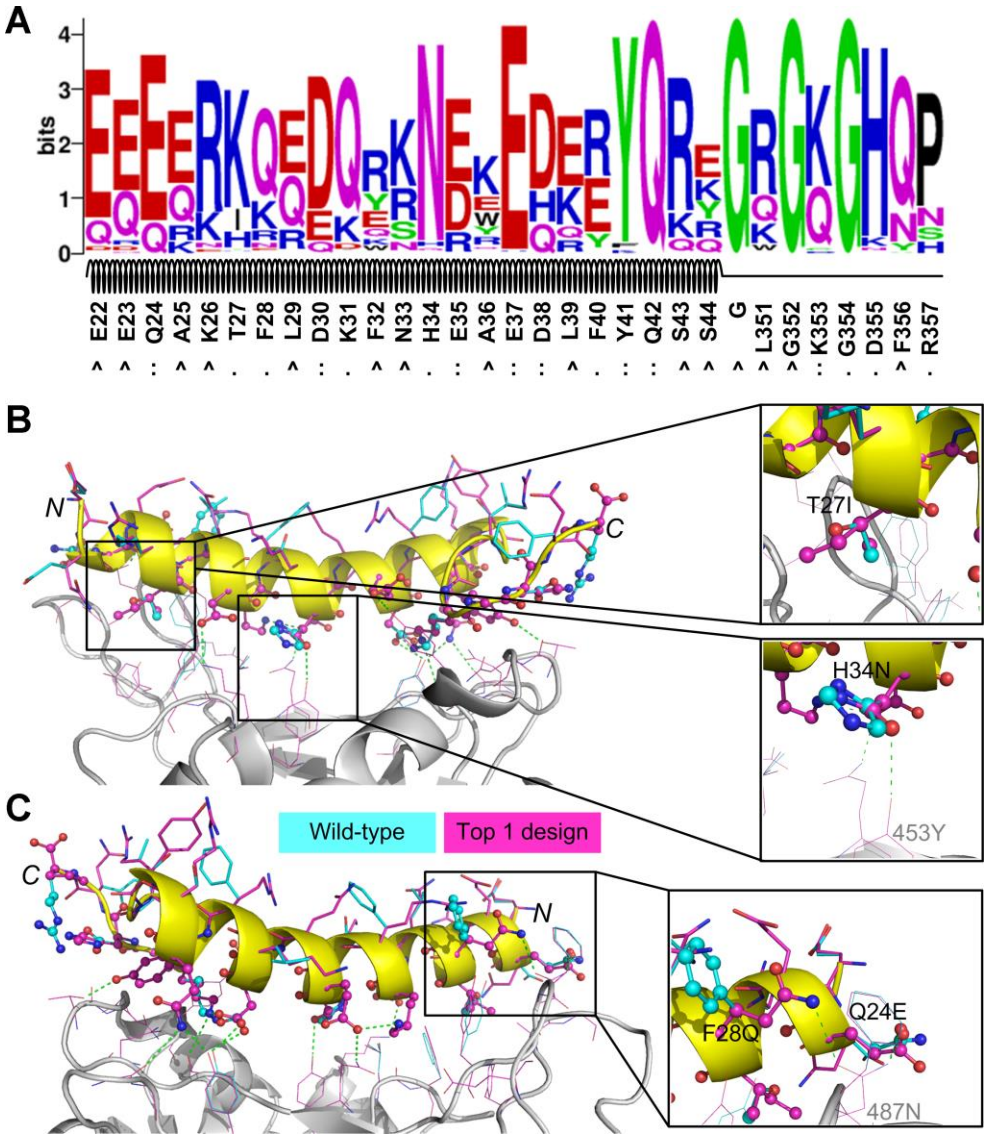

Figure 3. Sequence logo analysis of 992 unique peptide binders designed by EvoEF2 (**A**) and favorable interactions introduced in the top binder (B and C). In figure (A), the interface residues on the wild-type peptide are marked with ':' if hydrogen bonds or ion bridges exist, or '.' otherwise; non-interface residues are marked with '^'. In figures (**B**) and (**C**), the residues on the wild-type and designed structures are colored in cyan and magenta, respectively; interface and non-interface residues on the peptide are shown in ball-and-stick and stick models, respectively, while residues on SARS-CoV-2 RBD are shown in lines. Hydrogen bonds and/or ion bridges are shown using green-dashed lines.

Table 2. Summary of evolution-based peptide design results.

Comparison items [a]	Weight of evolutionary profile energy				
	0.00	**0.25**	**0.50**	**0.75**	**1.00**
Number of unique designs	992	991	966	877	695
Number of better binders [b]	757	636	392	340	226
EvoEF2 binding energy	-48.1±2.5	-47.2±2.2	-46.1±1.7	-45.8±1.6	-45.5±1.6
EvoEF2 total energy	-824.6±2.0	-823.4±2.2	-818.4±2.3	-813.3±1.9	-809.7±2.3
Profile energy [c]	6.7±2.7	-0.8±3.6	-13.3±3.3	-21.6±2.0	-25.6±1.6
EvoEF2+profile energy	-824.6±2.0	-823.6±1.8	-825.0±1.4	-829.5±1.2	-835.3±1.4
Sequence identity (%)	33.7±5.6	39.1±5.5	44.2±5.1	46.2±5.6	48.3±6.0
Sec. Str. match rate (%) [d]	95.7±3.3	96.2±3.0	97.5±2.7	97.5±2.5	97.7±2.4

[a] The units for the EvoEF2 and profile energies are EEU. [b] The EvoEF2 binding energy of the wild-type peptide binder was -46.46 EEU; this row shows the number of designed peptide binders with EvoEF2 binding energies lower than -46.46 EEU. [c] The profile energy of wild-type peptide binder was -22.2 EEU. [d] Secondary structure match rate.

maintain the original secondary structure. However, despite these improvements, giving a higher value to the profile weight hindered the identification of binders that exhibited better binding energy than the wild-type.

We performed sequence logo analyses of the four sets of designs obtained from the evolution-based method and the results are illustrated in Figure 4. Overall, the evolutionary profile did not have a dramatic effect on most interface residues (e.g. Q24, K31, H34, E35, E37, D38, Y41, Q42 and K353), because the dominating residue types identified in the EvoEF2-based designs were also top ranked (Figure 3A and Figure 4). However, some interface residues were indeed influenced. For instance, T27 could be substituted for either lysine or isoleucine without evolution (Figure 3A), but it was only mutated to lysine when the evolutionary weight was ≥0.75 (Figure 4C–4D). Additionally, without evolutionary profiles, F28 preferred glutamine over all other residues (Figure 3A), but it was conserved as phenylalanine when the evolutionary weight was ≥0.5 (Figure 4B–4D). The naturally occurring residues, glutamic acid, and arginine never appeared at positions 335 and 337, respectively, without evolutionary profile-guided design (Figure 3A); however, both of them were ranked second when a weight of 1.0 was given to the profiles. The residues that were most affected by evolution were those nonpolar residues that were not at the interface (e.g. A25, L29, F32, A36, L39, L351, and F356); without the evolutionary profile, polar or charged residue types were preferred at these positions (Figure 3A), while nonpolar residues were more frequently chosen for most of them when the weight of the profile energy was high (Figure 4B–4D). As discussed above, most of these residues were buried in the original hACE2 structure, but they were solvent exposed in the peptide, and therefore it might not be necessary to maintain the hydrophobic nature at these positions.

DISCUSSION

Although many different strategies are being employed to develop therapeutics or vaccines to treat COVID-19, there are, however, no effective antiviral drugs to combat the pandemic at present. Built on the fact that SARS-CoV-2 initiates its entry into human cells by the RBD of its spike protein binding to hACE2 [7, 8], we believe that molecules that can effectively block association of the SARS-CoV-2 spike protein with hACE2 may have the potential to treat COVID-19. In this regard, we extended a recently developed protein design approach, EvoDesign [19], to design novel peptides that can competitively bind to the SARS-CoV-2 RBD to inhibit the virus from entering human cells.

We constructed a novel hybrid peptide by linking two discontinuous peptide fragments from hACE2 with a linker glycine (denoted as 22-44G351-357), and utilized it as a template for designing new sequences with enhanced binding affinities for SARS-CoV-2 RBD. Based on the previous work by Han et al. [16], a peptide constructed using a similar approach exhibited a potent antiviral activity with an IC50 of about 0.1 μM when inhibiting the binding of SARS-CoV to hACE2, which was much higher that of two other peptides (a.a. 22-44 and 22-57). Since both SARS-CoV-2 and SARS-CoV use hACE2 as the receptor for entry into human cells and SARS-CoV-2 has much stronger binding toward hACE2 than SARS-CoV [15, 21], we believe that the wild-type hybrid peptide may also possess a high antiviral activity for inhibiting SARS-CoV-2 from binding to hACE2. Recently, Zhang et al. [14] reported that a natural hACE2 peptide (a.a. 21-43) can strongly bind to SARS-CoV-2 RBD with a K_d of 47 nM. We believe that the binding affinity of this peptide to SARS-CoV-2 may be weaker than peptide 22-44G351-357, because essentially it is almost identical to the natural hACE2 peptide 22-44 with only one residue

shifted, and Han et al. [16] demonstrated that peptide 22-44 showed much weaker binding to SARS-CoV than peptide 22-44G351-357. Therefore, it may be more promising to perform *de novo* sequence design starting with 22-44G351-357.

Computational design experiments showed that the binding energy of the peptide for SARS-CoV-2 RBD could be significantly enhanced, though the wild-type peptide already attained a good binding affinity. For instance, the wild-type peptide had an EvoEF2 binding score of -46.46 EEU, while the top designed binder achieved a score of -53.35 EEU. In contrast, the peptide used by Zhang et al. [14] had a binding score of only -37.37 EEU in our computational experiment. In the EvoDesign procedure, new peptides were designed starting from randomly generated sequences, where no wild-type sequence information was used [19]. However, sequence logo analysis suggested that the wild-type amino acid types were quite conserved for a large number of positions at the protein-peptide interface (Figures 3 and 4), indicating that some residues were critical for binding and they were correctly recapitulated by our design approach. Detailed inspection confirmed this point and also revealed that some extra favorable interactions were introduced to enhance binding in the top designed binders. Most of the *de novo* designed peptide binders shared a sequence identity of >30% to the wild-type peptide. This, on the one hand, indicates that our protein design potential was of high accuracy, and on the other hand, implies that good binders should not be random, and interestingly

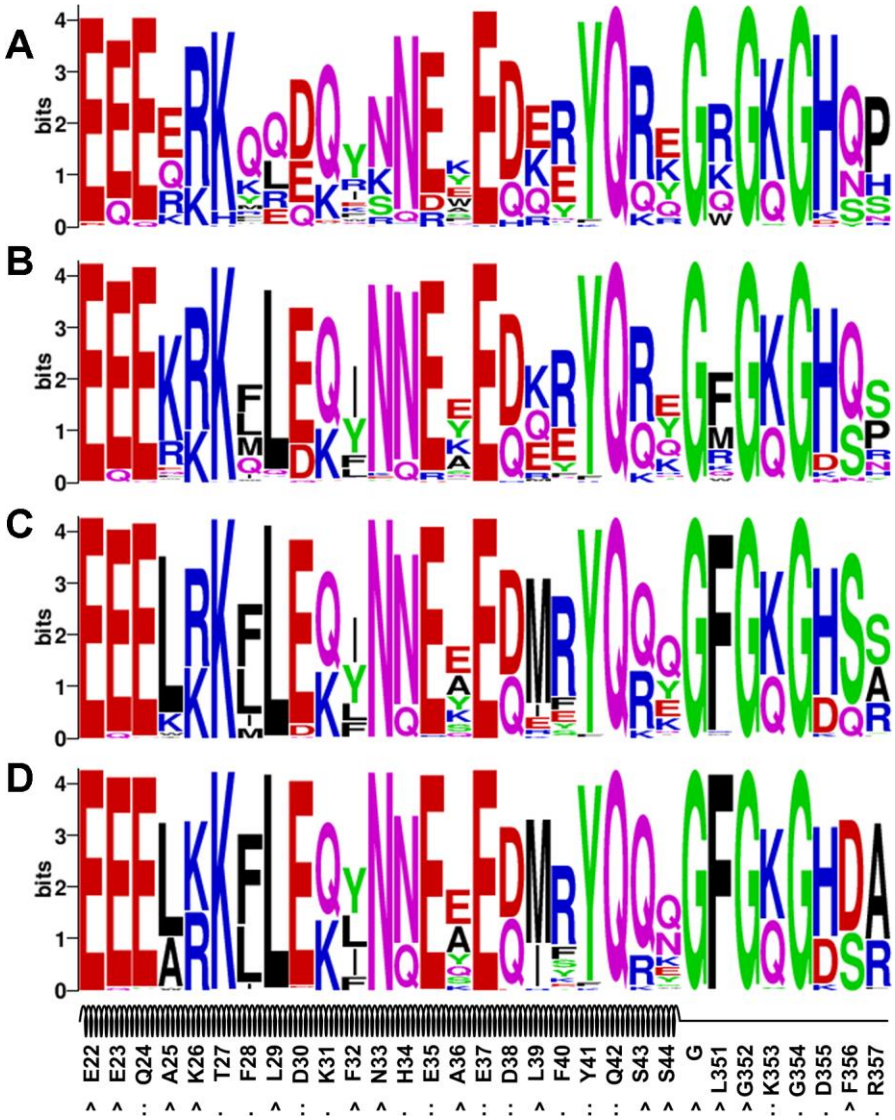

Figure 4. Sequence logo analysis of the evolution-based design results. Four sets of profile energy weight were used: 0.25 (**A**), 0.50 (**B**), 0.75 (**C**) and 1.00 (**D**).

they were somewhat similar to the wild-type peptide. Additionally, the machine-learning-based secondary structure prediction results showed that the *de novo* designed sequences should preserve the initial secondary structure topology of the peptide motif, which is important for facilitating the protein-peptide binding interaction.

In summary, we constructed a novel hybrid peptide from the interface of the natural hACE2 protein, and based on this peptide scaffold, we designed multiple novel peptide sequences with enhanced affinity toward SARS-CoV-2 RBD in computational binding experiments. Detailed analyses showed that the designed peptides were reasonable, as indicated by the recapitulation of critical binding interactions at the protein-peptide interface and the introduction of new favorable binding interactions, as well as the preservation of secondary structure to maintain the interactions. This work demonstrates the possibility of designing novel peptide therapeutics using computational algorithms. Other approaches can also be employed to engineer the hybrid peptide constructed based on the hACE2 protein, such as directed evolution [34, 35], which is widely used in the field of enzyme engineering [36–38]. Moreover, structure-based computational protein design can be combined with experiment-based approaches like directed evolution [39]. It is noteworthy that the experimental investigation of these designed peptides is of great importance for both methodology validation and drug design. We are working with our collaborators on the related experiments, which are still being conducted given that significantly more time is required for wet-lab experimental validation than a computational study. Due to the urgent situation caused by COVID-19 worldwide, we share our computational data with the community, which may help favorably combat the COVID-19 pandemic.

MATERIALS AND METHODS

Peptide design procedure

Based on the constructed protein-peptide complex structure (SARS-CoV-2 RBD/hACE2-22-44G351-357), we performed 1000 independent design trajectories individually, using (1) EvoEF2 [18], a physics- and knowledge-based energy function specifically designed for protein design and (2) a new version of EvoDesign [19], which combines EvoEF2 and evolutionary profiles for design scoring. A simulated annealing Monte Carlo (SAMC) [40] protocol was used to search for low total energy sequences as previously described [18]. For each trajectory, only the single lowest energy in that design simulation was selected, and therefore 1000 sequences each were collected from the EvoEF2 and EvoDesign designs. The EvoEF2 and EvoDesign designs were separately analyzed to determine the impact of the physics- and profile-based scores. Since SAMC is a stochastic searching method, some of the 1000 sequences were duplicates and thus excluded from the analysis. The backbone conformations of the hACE2 peptide and SARS-CoV-2 RBD were held constant during the protein design simulations, all the residues on the peptide were redesigned, and the side-chains of the interface residues on the virus RBD were repacked without design. The non-redundant designed peptides are listed in Supplementary Tables 1–5, and the raw data and computational protein design tools are freely available at https://zhanglab.ccmb.med.umich.edu/EvoEF/.

Evolutionary profile construction

To construct reliable structural evolutionary profiles, we used the hACE2 protein structure instead of the hybrid peptide to search structural analogs against a non-redundant PDB library. Only structures with a TM-score ≥0.7 to the hACE2 scaffold were collected to build a pairwise multiple sequence alignment (MSA). A total of nine structural analogs were identified. The corresponding alignment for residues 22-44 and 351-357 were directly extracted from the full-length MSA and combined to build an MSA for the hybrid peptide. Since an arbitrary glycine was used to link positions 44 and 351, a gap '-' was inserted in the peptide MSA for the glycine position. The peptide MSA constructed in this manner is described in Supplementary Figure 2. The peptide MSA was used to construct the evolutionary profile position-specific scoring matrix (PSSM) as previously described [29].

In previous studies, we also proposed incorporating protein-protein interface evolutionary profiles to model PPIs [19, 41, 42]. However, no interface structural analogs were identified from the non-redundant interface library (NIL) [42], and no interface sequence analogs were found from the STRING [43] database with a PPI link score ≥0.8. Therefore, the interface evolutionary profile scoring was excluded from the design.

Abbreviations

ACE2: angiotensin-converting enzyme 2; COVID-19: coronavirus disease 2019; Cryo-EM: cryogenic electron microscopy; EEU: EvoEF2 energy unit; hACE2: human angiotensin-converting enzyme 2; MSA: multiple sequence alignment; NIL: non-redundant interface library; PDB: protein data bank; PPI: protein-protein interaction; PSSM: position-specific scoring matrix; RBD: receptor binding domain; RBM: receptor binding

motif; SAMC: simulated annealing Monte Carlo; SARS-CoV: severe acute respiratory syndrome coronavirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

AUTHOR CONTRIBUTIONS

X.H performed computational experiments, analyzed data, and drafted the manuscript; R.P advised and edited the manuscript; Y.Z. conceived the project, acquired funding, designed computational experiments, assumed supervision, and edited the manuscript.

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CONFLICTS OF INTEREST

The authors declare that no conflict interest exists.

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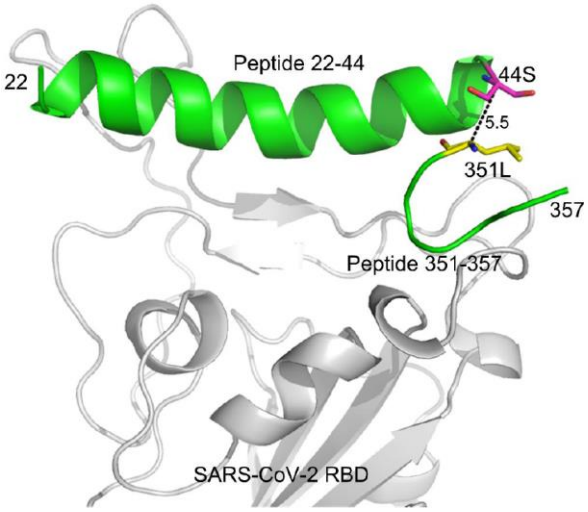
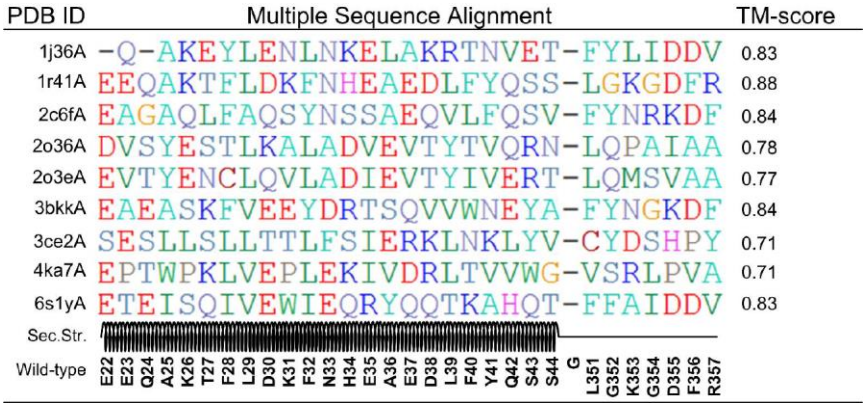
SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Illustration of the scaffold fragments used for design. Two discontinuous peptide fragments (a.a. 22-44 and a.a. 351-357) were used for peptide scaffold construction. The hACE2 peptides and SARS-CoV-2 RBD are shown in green and gray, respectively. The residues Ser44 and Leu351 are shown in magenta and yellow sticks, respectively. The distance between the Cα atoms of Ser44 and Leu351 is 5.5 Å.



Supplementary Figure 2. Peptide multiple sequence alignment for evolutionary profile construction.

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1 to 5.

Supplementary Table 1. Summary of 992 peptide sequences designed using EvoEF2 only.

Supplementary Table 2. Summary of 991 peptide sequences designed using EvoEF2 and the evolutionary profile (weight = 0.25).

Supplementary Table 3. Summary of 966 peptide sequences designed using EvoEF2 and the evolutionary profile (weight = 0.50).

Supplementary Table 4. Summary of 877 peptide sequences designed using EvoEF2 and the evolutionary profile (weight = 0.75).

Supplementary Table 5. Summary of 695 peptide sequences designed using EvoEF2 and the evolutionary profile (weight = 1.00).

Research Paper

Re-analysis of SARS-CoV-2-infected host cell proteomics time-course data by impact pathway analysis and network analysis: a potential link with inflammatory response

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ABSTRACT

Coronavirus disease 2019 (COVID-19), caused by an outbreak of the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) in Wuhan, China, has led to an unprecedented health and economic crisis worldwide. To develop treatments that can stop or lessen the symptoms and severity of SARS-CoV-2 infection, it is critical to understand how the virus behaves inside human cells, and so far studies in this area remain scarce. A recent study investigated translatome and proteome host cell changes induced *in vitro* by SARS-CoV-2. Here, we use the publicly available proteomics data from this study to re-analyze the *in vitro* cellular consequences of SARS-CoV-2 infection by impact pathways analysis and network analysis. Notably, proteins linked to the inflammatory response, but also proteins related to chromosome segregation during mitosis, were found to be altered in response to viral infection. Upregulation of inflammatory response proteins is in line with the propagation of inflammatory reaction and lung injury that is observed in advanced stages of COVID-19 patients and which worsens with age.

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by an outbreak of the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) in Wuhan, China, has led to an unprecedented health and economic crisis worldwide [1]. Initially reported in December 2019 in the Chinese city of Wuhan, and potentially linked to a zoonosis related to a wild animal market, COVID-19 has rapidly spread globally, and the World Health Organization (WHO) declared a pandemic on March 11th 2020. As of May 25th 2020, there are 5,304,772 confirmed cases and 342,029 confirmed deaths, with 216 countries affected (WHO, https://www.who.int, data accessed on May 25th 2020). These figures make COVID-19 the biggest health emergency of the 21st century. With neither an effective treatment nor vaccine available, the main controlling measure taken by nations has been social distancing followed by partial or total preventative lockdown. These control measures alone have led to the biggest global economic crisis of the 21st century.

COVID-19 typically manifests as an acute respiratory distress syndrome with fever, dry cough and breathing difficulties. Some patients, especially those with specific comorbidities, can rapidly deteriorate and die [2, 3]. A large number of undocumented cases is expected, as a result of asymptomatic carriers and patients with mild symptoms who are not tested for SARS-CoV-2 [4, 5]. The crude global mortality rate is estimated to be 6.5% in WHO reports [6]. However, given the likely high number of undocumented cases it is difficult to calculate the true mortality rate globally and on a nation-wide basis. In any case, COVID-19 spreads at an alarming rate, and both mortality rate and severity increase with age and depend on pre-existing comorbidity, such as hypertension and diabetes, which are age-related diseases. This defines COVID-19 as an

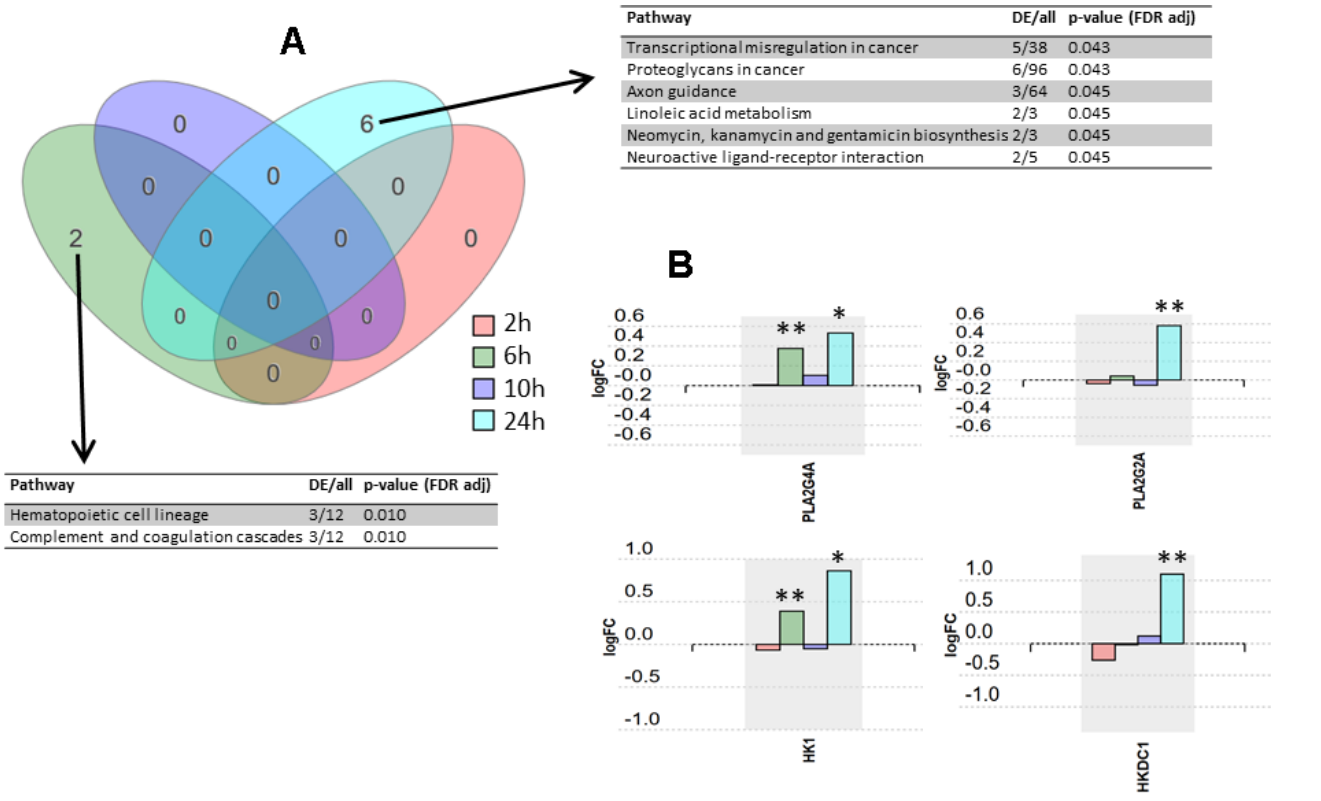
aging-dependent disease with outcomes determined by biological age.

To develop treatments that can stop or ameliorate the effects of SARS-CoV-2, we need to understand the biology of the virus and how it behaves inside human cells. This creates an urgent need to decipher the host cell molecular mechanisms that are triggered by viral infection. Cellular factors exploited by SARS-CoV-2 to gain entry into cells have recently been studied, revealing that the virus uses the angiotensin-converting enzyme 2 (ACE2) host cell receptor, together with the serine protease TMPRSS2. On this basis, a TMPRSS2 inhibitor has been proposed as a treatment option [7]. Elsewhere, it has been reported that ACE2 expression is protective against lung injury and that this is downregulated by SARS-CoV-1 [8, 9], which might promote lung injury, therefore worsening the prognosis of the disease, but it has not been demonstrated yet whether SARS-CoV-2 also interferes with ACE2 expression [7].

However, knowledge about what goes on inside human cells after the entrance of SARS-CoV-2 remains scarce. Host cell proteomics studies that measure changes in protein abundance following viral entry and subsequent global pathway and network analysis can shed some light on the mechanisms that are used and/or altered by the virus and may reveal novel drug targets. To the best of our knowledge, the first available study describing translatome and proteome host cell changes induced by SARS-CoV-2 was conducted by Bojkova et al. [10]. Here, the authors used Cytoscape and ReactomeFI to propose overrepresented pathways that could be targeted by potential treatment compounds. In our study, we use the publicly available proteomics data from Bojkova et al. [10] to re-analyze the cellular mechanisms altered upon viral infection by impact pathways analysis [11] and network analysis.

RESULTS

The input dataset for the analysis, formatted from Bojkova et al. [10], is compiled in Supplementary Table 1. The implicated pathways were analyzed using iPathwayGuide software. Significantly impacted pathways according to our analysis are shown in Figure 1 (see Supplementary Tables 2, 3 for the results



Pathway	DE/all	p-value (FDR adj)
Transcriptional misregulation in cancer	5/38	0.043
Proteoglycans in cancer	6/96	0.043
Axon guidance	3/64	0.045
Linoleic acid metabolism	2/3	0.045
Neomycin, kanamycin and gentamicin biosynthesis	2/3	0.045
Neuroactive ligand-receptor interaction	2/5	0.045

Pathway	DE/all	p-value (FDR adj)
Hematopoietic cell lineage	3/12	0.010
Complement and coagulation cascades	3/12	0.010

Figure 1. Pathway analysis results. (**A**) Venn diagram representing the intersections of pathway sets associated with the four post-infection time points. Pathways were considered significant according to a p-value calculated by iPathway Guide software using a hypergeometric distribution and adjusted using false discovery rate. DE, differentially expressed proteins. (**B**) Expression changes over four post-infection time points for proteins PLA2G4A, PLA2G2A, HK1, and HKDC1. * p-value < 0.05, ** p-value < 0.001.

from the pathway analysis for the metanalysis and for the 24 h time point, respectively). After false discovery rate (FDR) correction, six pathways were found to be significantly impacted at 24 h post-infection, two pathways were found to be significantly impacted at 6 h post-infection, and no pathways were found to be significantly impacted at 2 h and 10 h post-infection (Figure 1A). Expression changes for selected proteins over post-infection time points are shown in Figure 1B.

The differentially expressed proteins at the time point that revealed the most pronounced changes (24 h post-infection), were subjected to network analysis using iPathwayGuide. The interactions included were activation, binding, catalysis, expression, and inhibition. Confidence score for protein-protein interaction was set at 900 (high). The resulting network is shown in Figure 2A. One of the subnetworks with the highest number of interactions, comprised of six proteins, is shown in Figure 2B, together with the expression change profile over post-infection time for these six proteins.

DISCUSSION

The significantly affected pathways were analyzed using iPathwayGuide software, which implements an 'impact analysis' approach, taking into consideration not only the over-representation of differentially expressed genes in a given pathway (i.e. enrichment analysis), but also topological information such as the direction and type of all signals in a pathway, and the position, role, and type of each protein [11]. Although

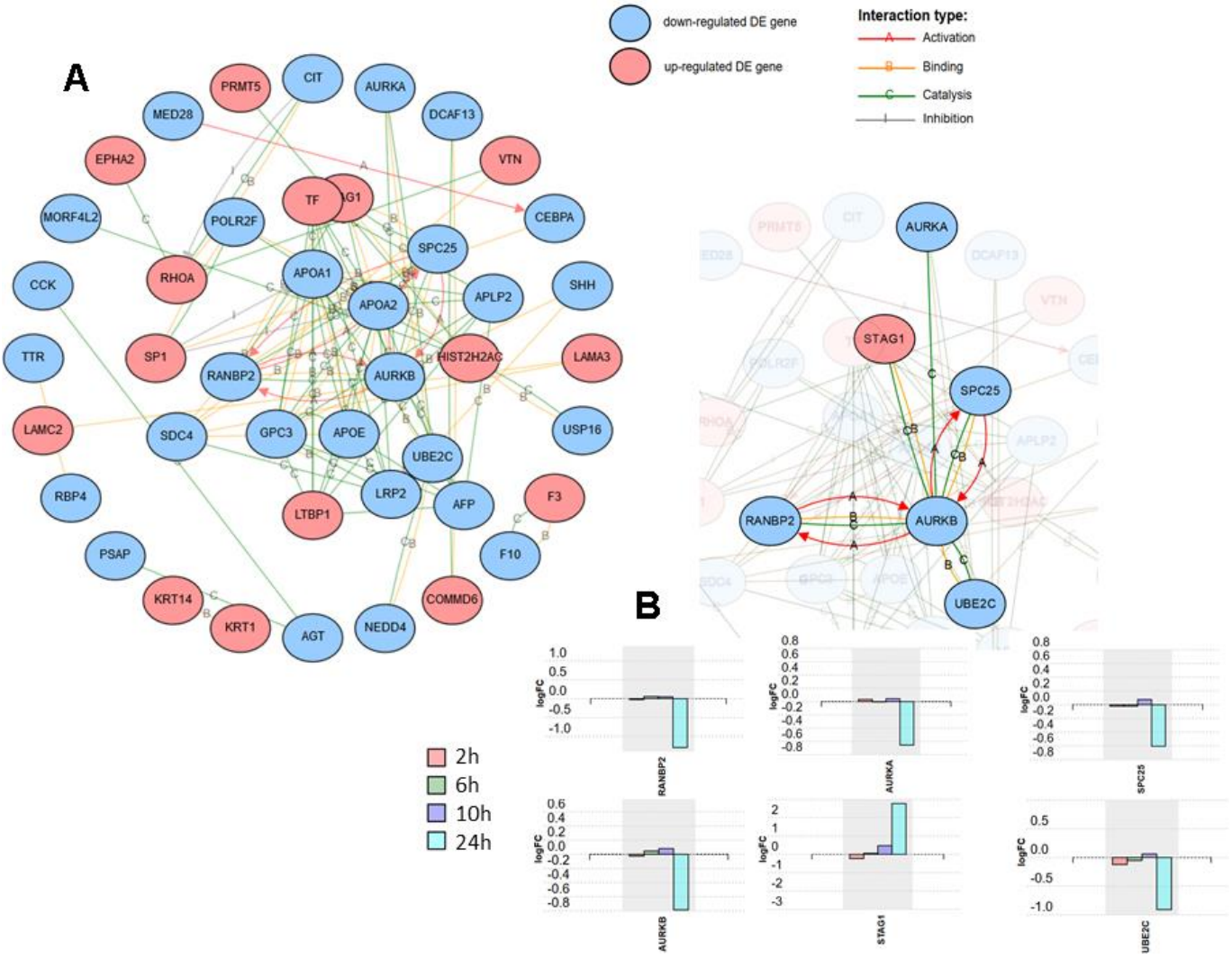


Figure 2. Network analysis including the 125 differentially expressed proteins at 24 h after SARS-CoV-2 in Caco-2 cells. Activation, binding, catalysis, and inhibition regulatory interactions are included. (**A**) Network with the isolated nodes hidden. (**B**) Six-protein subnetwork with the interactions for RANBP2, showing the expression changes for each time point for the six proteins.

six pathways were found to be significantly impacted at 24 h post-infection, and two at 6 h post-infection, the number of differentially expressed (DE) proteins in these pathways was low (ranging from 2 to 6 proteins). For instance, the pathway 'transcriptional misregulation in cancer' had 5 DE proteins out of the 38 proteins included in the pathway, the 'proteoglycans in cancer' pathway had 6 DE proteins out of 96 in total in that pathway, and the 'axon guidance' pathway had 3 DE proteins out of a total of 64 proteins in that pathway. Thus, we consider the experimental evidence for SARS-CoV-2 having an impact on these mechanisms to be relatively weak. However, while the overall number of DE proteins was low, the ratio of DE proteins to total proteins in three other significant pathways was higher, and these warranted further attention. These three pathways are 'linoleic acid metabolism' pathway, 'neomycin, kanamycin and gentamicin biosynthesis' pathway, and 'neuroactive ligand-receptor interaction' pathway. The linoleic acid metabolism pathway is linked to arachidonic acid metabolism and eicosanoids pathway, and it could therefore play a role in the inflammatory response observed in disease stages II and III in COVID-19 patients [12]. In fact, the two proteins found to be differentially expressed in this pathway at 24 h post-infection, PLA2G4A (cytosolic phospholipase A2) and PLA2G2A (phospholipase A2, membrane associated), are key components of the phospholipase A2 group, which has previously been suggested to participate in a key mechanism of the inflammatory reaction [13]. Additionally, the contribution of the phospholipase A2 group to inflammation and eicosanoid profile in arthritis [14] and in cardiovascular diseases has been demonstrated [15]. When looking at the overall trend in protein expression over the whole time-course, PLA2G4A and PLA2G2A appear to share the same expression profile with a clear increase at 24 h post-infection (Figure 1B). This observation suggests that these two proteins may serve as early systemic biomarkers for COVID-19 infection.

Two proteins from the neomycin, kanamycin and gentamicin biosynthesis pathway were significantly up-regulated at 24 h post-infection (Figure 1B). These are HK1 (hexokinase 1) and HKDC1 (hexokinase domain containing 1), which are proteins related to glucose use and homeostasis [16, 17]. These proteins also belong to the glycolysis/gluconeogenesis pathway, since they participate in the first step of glycolysis where the glucose ring is phosphorylated. The 'glycolysis/ gluconeogenesis' pathway also appeared in our pathway analysis for the 24 h post-infection time point (although not statistically significant) (Supplementary Table 3), and, according to the nature of the cells used in the assay, it should be a better match than the aminoglycoside antibiotics biosynthesis pathway. Interestingly, HK has previously been associated with the inflammatory response in autoimmune disorders, and deoxy-D-glucose (2-DG), an inhibitor of HK, has been proposed to ameliorate autoimmune inflammation [18]. Recently, 2-DG has been shown to inhibit SARS-CoV-2 replication in Caco-2 cells [10], as well as inhibiting rhinovirus infection and inflammation in a murine model [19]. Given these findings, we believe that a potential link between hexokinase and SARS-CoV-2 infection and the related inflammation response deserves further investigation.

Figure 2A shows the network formed by the DE proteins, excluding isolated nodes. One of the subnetworks with a higher number of connections is the one formed by RANBP2 (E3 SUMO-protein ligase RanBP2) (Figure 2B). RANBP2 forms a complex at the nuclear pore with TRIM5α, a cytoplasmic restriction factor that blocks post-entry retroviral infection and is regulated by SUMO. It has been demonstrated that loss of RANBP2 blocked SUMOylation of TRIM5α, suppressing its anti-retroviral activity [20]. Here, RANBP2 exhibited a statistically significant fold-change (log) of -1.295 at 24 h post-infection, thus the role of RAMBP2-TRIM5α in coronavirus infection deserves further consideration. In the same subnetwork as RANBP2, four other proteins that are interestingly related to cell cycle progression, AURKA, AURKB, SPC25 and STAG1, also deserve further attention (Figure 2B). They all participate in the regulation of chromosome segregation during mitosis [21–24]. Three of these four proteins were found to be down-regulated at 24 h post-infection except STAG1, which was strongly up-regulated. In this subnetwork, closely related to AURKB, and also down-regulated, is UBE2C (Ubiquitin-conjugating enzyme E2 C), which is an essential factor of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated ubiquitin ligase that controls progression through mitosis [25].

In parallel to re-analyzing the data with alternative tools, we also noticed a trend towards down-regulation of ACE2 over time post-infection (Figure 3). This had not been highlighted by the original authors [10]. In fact, at 24 h post-infection ACE2 presented a fold-change in expression (log) of -0.168 (p-value = 0.01). Coronavirus entry into target cells depends on binding of its spike (S) proteins to a cellular receptor, which facilitates viral attachment to the surface of target cells. ACE2 was reported as the entry receptor for SARS-CoV [26], another coronavirus closely related to SARS-CoV-2, therefore playing a key role in SARS-CoV transmissibility [27]. Similar findings were recently made for ACE2 and SARS-CoV-2 [7]. ACE2 is also a peptidase in the renin-angiotensin system, that converts

angiotensin I to angiotensin (1-9) and angiotensin II to angiotensin (1-7), which is a vasodilator. ACE2's protective role in lung injury is therefore related to its ability to cleave angiotensin II [28, 29]. It was also reported that ACE2 expression protects from lung injury and is downregulated by SARS-CoV [8, 9], which might promote lung injury, therefore worsening the prognosis of the disease. Here, we highlight that SARS-CoV2 also seems to interfere with ACE2 expression, and this may be related to a higher level of lung injury as was demonstrated for SARS-CoV. When inspecting the quantitative data for other proteins in the renin-angiotensin system, two other proteins were found to be down-regulated 24 h post-infection, namely cathepsin A (CTSA) and angiotensinogen (AGT) (Figure 3). We hypothesize that dysregulation of some of the key components of the renin-angiotensin system could be related to the lung injury and worsening observed in COVID-19.

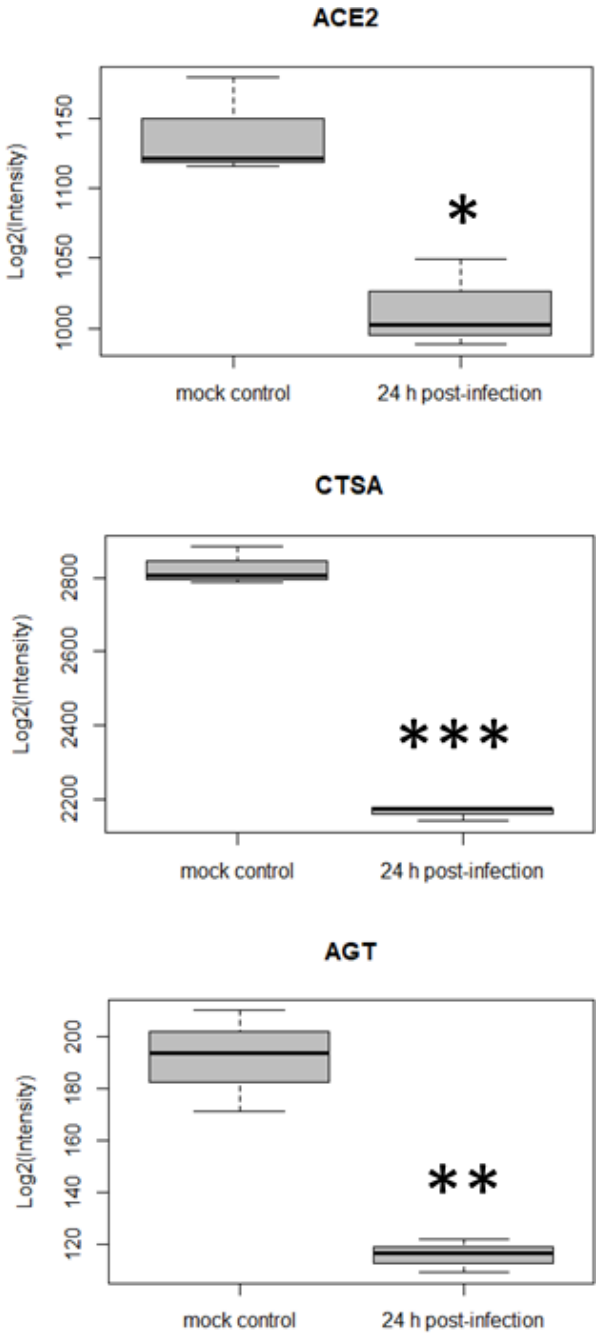


Figure 3. Differential expression for three host cell proteins in the renin-angiotensin system at 24 h post SARS-CoV-2 infection (*p-value <0.05, **p-value <0.01, ***p-value <0.0001, comparison to mock control).

It has also been suggested that differential levels of ACE2 in the cardiac and pulmonary tissues of younger versus older adults may be at least partially responsible for the worse outcomes seen in elderly COVID-19 patients [30]. Elderly people, especially those with hypertension and diabetes, have reduced ACE2 expression and increased levels of angiotensin II proinflammatory signaling. This cohort is therefore potentially more vulnerable to the ACE2 down-regulation that is exasperated by SARS-CoV-2, and we hypothesize that this is one explanation for the exaggerated inflammation and worse outcomes observed in elder populations. The impact of reduced ACE2 expression, together with the poorer clinical outcomes observed when comorbidities are present [31], which is also generally associated to age, makes the link between COVID-19 and aging strong.

ACE inhibitors (ACE-Is) and angiotensin II receptor blockers (ARBs), two common therapies for hypertension, increase ACE2 levels in some tissues such as the myocardium, contributing to protection against cardiovascular disease (CVD) [32]. The observed down-regulation of ACE2 by SARS-CoV-2 could suppress that increase in ACE2 levels mediated by ACE-Is and ARBs, thus counteracting their beneficial effects on hypertension and CVD [32], and explaining the poorer clinical outcome observed when these comorbidities are present. In any case, discontinuation of ACE-Is/ARBs in hypertension patients with COVID-19 might further decrease ACE2 levels, therefore worsening disease prognosis. In support of this notion, a recent study found that COVID-19 patients with untreated hypertension presented higher mortality than those treated with ACE-Is/ARBs [33]. On the other hand, since ACE2 is the receptor for SARS-CoV-2 entry, some authors have speculated about a greater susceptibility to viral infection and disease severity upon the use of ACE-Is and ARBs [34]. However, as of today there is no scientific evidence pointing in that direction, and several scientific societies recommend that hypertension and CVD patients continue their treatments [32].

It is important to note that the use of a colon cell line could be seen as a potential limitation of the study. Superior airways and lungs are the primary targets for SARS-CoV-2, and therefore a primary airway epithelial cell type, such as human bronchial or tracheal epithelial cells (HBEpC/HTEpC) is likely to be a more appropriate model to predict the cell infection profile than the colon cell line used by Bojkova et al. [10]. However, although it was initially thought that SARS-CoV-2 could only infect airway cells, it has since been found to affect numerous tissues and organs, including the intestinal tract [35, 36]. ACE2, the host cell receptor used by SARS-CoV-2 to enter cells, is distributed broadly across human tissues, with similar expression levels in the colon and lung [37, 38]. SARS-CoV-2 has also been found to replicate in gastrointestinal cells *in vivo* and it has been frequently detected in stool, with many patients developing gastrointestinal symptoms [39, 40]. These findings qualify colon cells as an appropriate model for studying SARS-CoV-2 infection and the primary human cell response. On the other hand, Caco-2 cells have been extensively used to study SARS-CoV and are highly permissible for SARS-CoV-2, allowing an analysis of a human model response to viral infection [10, 41, 42].

In summary, this work, through a re-analysis of previous data on the protein expression changes caused by SARS-CoV-2 infection in a cellular model, we point out several proteins related to the inflammatory response and chromosomal segregation that might be modulated by SARS-CoV-2 infection. In the case of proteins related to inflammation, the up-regulation observed could be linked to the propagation of the inflammatory reaction and lung injury that is observed during advanced stages of COVID-19.

MATERIALS AND METHODS

Publicly available proteomics data

Proteome measurements from Bojkova et al. [10] were downloaded and used for subsequent analysis. This data consisted of the quantification of 6,381 proteins in human Caco-2 cell secretomes at four time points after infection with SARS-CoV-2 virus. According to Bojkova et al. [10], a TMT-labeling bottom-up quantitative proteomics approach was used to obtain the data, with high pH reverse phase peptide fractionation and mass spectrometry measurement of the peptides using a Thermo QExactive and a nano-liquid chromatography configuration.

Impact pathway analysis and network analysis

iPathwayGuide (Advaita Corporation, Plymouth, MI, USA) v1910, within the PIPPR pathways analysis framework (COBO Technologies Aps, Maaloev, Denmark), was used to identify significantly impacted pathways and for GO analysis. All quantified proteins were included in the analysis, and the threshold for considering a protein as differentially expressed (DE) was fold-change (log2) higher than 0.5 and p-value below 0.05. Data was analyzed in the context of pathways obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 90.0+/05-29, May 2019). iPathwayGuide was also used for network analysis, using String v11.0 Jan 2019 and BioGRID v3.5.171 Mar 2019 as data sources. The interactions included were activation, binding, catalysis, expression, and inhibition. The confidence score for protein-protein interaction was set at 900 (high).

Statistical analysis

For impact pathway analysis, iPathwayGuide software calculated a p-value using a hypergeometric distribution. P-values were adjusted using false discovery rate (FDR).

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1 to 3.

Supplementary Table 1. Input dataset formatted from Bojkova et al. [10]. P-values and fold-changes are included for each of the quantified proteins at four different time points following SARS-CoV-2 infection of Caco-2 cells.

Supplementary Table 2. Pathway analysis meta-analysis results for the four time points investigated. Proteins were considered as differentially expressed (DE) when statistical analysis led to a p-value <0.05 and a fold change(log)>0.5 or <-0.5.

Supplementary Table 3. Expanded pathway analysis result for the 24 h time point. Proteins were considered as differentially expressed (DE) when statistical analysis led to a p-value <0.05 and a fold change(log)>0.5 or <-0.5.

Research Paper

Serum calcium as a biomarker of clinical severity and prognosis in patients with coronavirus disease 2019

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ABSTRACT

The aim of this study was to investigate the correlations between serum calcium and clinical outcomes in patients with coronavirus disease 2019 (COVID-19). In this retrospective study, serum calcium levels, hormone levels and clinical laboratory parameters on admission were recorded. The clinical outcome variables were also recorded. From February 10 to February 28, 2020, 241 patients were enrolled. Of these patients, 180 (74.7%) had hypocalcemia on admission. The median serum calcium levels were 2.12 (IQR, 2.04-2.20) mmol/L, median parathyroid hormone (PTH) levels were 55.27 (IQR, 42.73-73.15) pg/mL, and median 25-hydroxy-vitamin D (VD) levels were 10.20 (IQR, 8.20-12.65) ng/mL. The serum calcium levels were significantly positively correlated with VD levels (*P* =0.004) but negatively correlated with PTH levels (*P* =0.048). Patients with lower serum calcium levels (especially ≤2.0 mmol/L) had worse clinical parameters, higher incidences of organ injury and septic shock, and higher 28-day mortality. The areas under the receiver operating characteristic curves of multiple organ dysfunction syndrome, septic shock, and 28-day mortality were 0.923 (*P* <0.001), 0.905 (*P* =0.001), and 0.929 (*P* <0.001), respectively. In conclusion, serum calcium was associated with the clinical severity and prognosis of patients with COVID-19. Hypocalcemia may be associated with imbalanced VD and PTH levels.

INTRODUCTION

In December 2019, clusters of acute pneumonia cases of unclear etiology were identified in Wuhan City, the capital of Hubei Province in China [1–3]. The pathogen has been reported as a novel coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The World Health Organization (WHO) has made the assessment that coronavirus disease 2019 (COVID-19) can be characterized as a pandemic because the disease is still spreading rapidly around the world, especially in the United States, Spain, and Russia [4].

As of May 26, a total of 82993 cases (4634 deaths) were confirmed in China, including 50340 cases (3869 deaths) in Wuhan city [5].

The National Health Commission of China has issued a series of diagnosis and treatment recommendations and suggested classifying the disease into four grades: mild, moderate, severe and critical [5]. Recent studies have reported the clinical characteristics and prognosis of the varied severity grades of COVID-19 [1, 2, 6–8]. The underlying mechanisms of the novel coronavirus leading to disease exacerbation and organ dysfunction remain to be further explored. Due to the high mortality and the lack of effective treatments in critically ill patients [7, 9], early identification and prediction of these patients are crucial. What are the risk factors for severe illness or death [10]? How can we identify groups that are most likely to have poor outcomes so that we can focus prevention and treatment efforts [10]? These studies are needed. Huang et al [8] reported that patients admitted to the intensive care unit (ICU) had more severe clinical symptoms and more abnormal serum parameters. However, fewer studies have been published that confirm an early and sensitive biomarker to estimate the disease severity and prognosis of COVID-19. During our clinical work against the COVID-19 epidemic in Wuhan, we observed a high incidence of hypocalcemia in critically ill patients. Therefore, we hypothesized that serum calcium levels were associated with the disease severity and prognosis of patients with COVID-19. This study was performed to test this hypothesis and explore the causes of hypocalcemia.

RESULTS

A total of 241 patients with confirmed COVID-19 were enrolled in this clinical retrospective study. The median age was 65 (IQR, 55-72) years, and 129 (53.5%) were women. Of the patients, 192 (79.7%) were classified as severe (167/214, 69.3%) or critical (25/214, 10.5%). Fever (108/214, 44.8%) and cough (65/214, 27.0%) were the main initial symptoms. One hundred and eighty (180/214, 74.7%) patients had hypocalcemia on admission. The detailed clinical data of the patients are presented in Table 1. A total of 231 patients were discharged from the hospital, and the median hospital stay was 25 (IQR, 17-32) days. MODS developed in 17 (7.1%) patients, and septic shock developed in 6 (2.5%) patients. Ten (10/241, 4.1%) patients died within 28 days of admission, and all of the decedents were critically ill. In other words, the 28-day mortality of critically ill patients was 40.0% (10/25).

Serum calcium and clinical variables

The median serum calcium levels were 2.12 (IQR, 2.04-2.20) mmol/L on admission. We divided the patients into three groups based on the serum calcium values: ≤2.0 mmol/L (defined as group A, n = 43), 2.0-2.2 mmol/L (defined as group B, n = 137), and >2.2 mmol/L (defined as group C, n = 61). As shown in Table 2, significant differences in the clinical variables except for serum creatinine were found among the three groups, and the same differences were found between groups A and B (P <0.05). There were also differences in the clinical variables except for WBC count (P =0.07) and serum creatinine (P =0.244) between groups A and C, whereas differences in the variables except for WBC count (P =0.60), ALT (P =0.839), the lowest SpO2 (P =0.328), and serum creatinine (P =0.635) were found between groups B and C. These results indicated that patients with lower serum calcium levels had worse clinical variables.

Of the 241 patients, 26 were tested to determine levels of parathyroid hormone (PTH) and 25-hydroxy-vitamin D (VD) according to clinical needs. The median serum calcium level of the 26 patients was 2.13 (IQR, 2.03-2.16) mmol/L. The median PTH level was 55.27 (IQR, 42.73-73.15) pg/mL, and the median VD level was 10.20 (IQR, 8.20-12.65) ng/mL. All of these patients had low levels of VD (VD deficiency).

The SPSS scatterplots and correlation analyses of serum calcium and the blood biomarkers are shown in Figures 1 and 2. The serum calcium levels were significantly positively correlated with lymphocyte count (Figure 1A, P <0.001), albumin levels (Figure 1B, P <0.001), VD levels (Figure 1C, P =0.004), and lowest SpO2 (Figure 1D, P< 0.001), whereas they are significantly negatively correlated with CRP (Figure 2A, P< 0.001), D-dimer (Figure 2B, P< 0.001) and PTH (Figure 2C, P =0.048) levels. These results indicated that hypocalcemia may be associated with imbalanced VD and PTH in the acute phase of COVID-19.

Serum calcium and clinical severity

ARDS developed in 19 of 241 (7.9%) patients, and liver injury developed in 16 (6.6%), AKI developed in 14 (5.8%), and cardiac injury developed in 12 (5.0%) patients during the research period. Twelve patients received MV, and 7 patients received CRRT. As shown in Table 3, significant differences in the clinical severity and outcome variables were found among the abovementioned three groups (A, B, and C) (P <0.001) and between groups A and C (P <0.001). There were also differences in all these variables except for liver injury incidence (P =0.201). No differences were found between groups B and C (P >0.05). These results indicated that patients with serum calcium values ≤2.0 mmol/L had higher 28-day mortality, and a higher incidence of organ injury. Moreover, the serum calcium values were significantly lower in patients who died and in patients with MODS, septic shock, and organ injury, requiring MV or CRRT (P <0.001) (Table 4).

Table 1. Demographic data and clinical parameters.

Variables	
Categorical variables	***N (%)***
Sex (Male: Female)	112:129
Initial symptoms or signs	
Fever	108 (44.8%)
Cough	65 (27.0%)
Chest tightness or pain	22 (9.1%)
Fatigue	10 (4.1%)
Dyspnea	9 (3.7%)
Diarrhea	7 (2.9%)
Pharyngalgia	5 (2.1%)
Myalgia	5 (2.1%)
Nausea or vomiting	4 (1.7%)
Abdominal pain	3 (1.2%)
Other	3 (1.2%)
Classifications	
Mild	0 (0%)
Moderate	49 (20.3%)
Severe	167 (69.3%)
Critical	25 (10.4%)
Organs injury	
ARDS	19 (7.9%)
Liver injury	16 (6.6%)
AKI	14 (5.8%)
Cardiac injury	12 (5.0%)
Septic shock	6 (2.5%)
Need for NIV /HFNC	7 (2.9%)
Need for MV	12 (5.0%)
Need for CRRT	7 (2.9%)
Discharged	94 (39.0%)
Death	10 (4.1%)
Continuous variables	***Median (IQR)***
Age (years)	65 (55-72)
Days from onset to admission	13 (10-16)
Blood parameters	
Calcium (mmol/L)	2.12 (2.04-2.20)
CRP (mg/L)	6.30 (1.70-34.85)
WBC (10^9/L)	5.48 (4.55-7.15)
Lymphocyte (10^9/L)	1.26 (0.93-1.63)
ALT (U/L)	22.0 (14.0-36.0)
Albumin (g/L)	35.6 (31.6-38.8)
Creatinine (umol/L)	66.0 (56.0-80.0)
TNI (pg/mL)	3.80 (1.95-7.45)
D-dimer (ug/mL)	0.73 (0.34-1.42)
Worst SpO2 (%)	97.0 (96.0-98.0)

ARDS, acute respiratory distress syndrome; AKI, acute kidney injury; NIV, noninvasive ventilation; HFNC, high-flow nasal cannula; MV, mechanical ventilation; CRRT, continuous renal replacement therapy; IQR: interquartile ranges; CRP, C-reactive protein; WBC, white blood cells; ALT, alanine aminotransferase; TNI, troponin I; SpO2, pulse oxygen saturation.

Table 2. Serum calcium and clinical parameters.

	Group A (*n*= 43)	Group B (*n*= 137)	Group C (*n*= 61)	*P* value
Calcium (mmol/L)	1.96 (1.91-2.00)	2.11 (2.06-2.13)	2.22 (2.21-2.26)	<0.001
CRP (mg/L)	47.4 (20.5-105.7)	6.3 (1.9-25.8)	2.0 (0.8-6.2)	<0.001
WBC (10^9/L)	6.58 (4.57-9.01)	5.36 (4.38-6.79)	5.29 (4.67-6.96)	0.042
Lymphocyte (10^9/L)	0.75 (0.50-1.19)	1.27 (1.01-1.63)	1.53 (1.17-1.75)	<0.001
ALT (U/L)	32.0 (18.0-51.0)	20.0 (14.0-34.5)	20.0 (13.0-35.5)	0.027
Albumin (g/L)	30.6 (28.2-32.6)	35.6 (31.7-38.4)	40.0 (36.1-43.2)	<0.001
Creatinine (umol/L)	66.0 (61.0-79.0)	67.0 (55.5-80.0)	64.0 (57.0-80.0)	0.565
TNI (pg/mL)	8.80 (3.90-16.70)	3.40 (1.95-6.20)	2.50 (1.90-4.55)	<0.001
D-dimer (ug/mL)	1.30 (0.74-8.29)	0.68 (0.34-1.37)	0.43 (0.27-0.80)	<0.001
Worst SpO2 (%)	96.0 (90.0-97.0)	97.0 (96.0-98.0)	97.0 (96.0-98.0)	<0.001

Group A, the serum calcium values: ≤2.0 mmol/L; Group B, the serum calcium values: 2.0-2.2 mmol/L; Group C, the serum calcium values: >2.2 mmol/L; CRP, C-reactive protein; WBC, white blood cells; ALT, alanine aminotransferase; TNI, troponin I; SpO2, pulse oxygen saturation.

ROC curves were also performed to assess the associations between serum calcium and MODS, septic shock, and 28-day mortality. As shown in Figure 3, the area under the curves (AUCs) of MODS (Figure 3A), septic shock (Figure 3B), and 28-day mortality (Figure 3C) were 0.923 (*P* <0.001), 0.905 (*P* =0.001), and 0.929 (*P* <0.001), respectively. Optimal cut-off points of serum calcium values were derived from the ROC curves. The optimal cut-off point for MODS was 2.035 mmol/L, the sensitivity was 88.2%, and the specificity was 82.6%. The optimal cut-off point for septic shock was 2.01 mmol/L, the sensitivity was 100.0%, and the specificity was 84.3%. The optimal cut-off point for 28-day mortality was 2.01 mmol/L, the sensitivity was 100.0%, and the specificity was 85.7%. Figure 4 shows the significant differences in the 28-day mortality among groups A, B, and C. The 28-day mortality of group A was significantly higher than that of groups B or C (*P* <0.001). No difference was found between groups B and C (*P* >0.05).

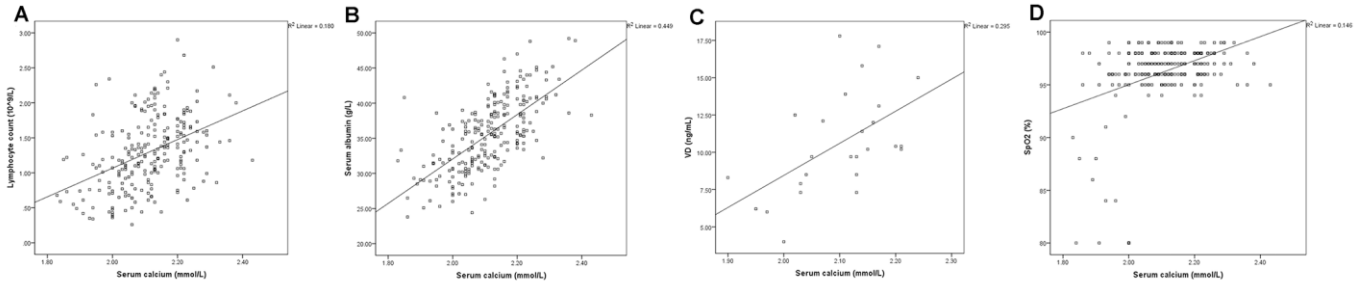

Figure 1. The serum calcium levels were positively correlated with lymphocyte count (**A**, *P* <0.001), and albumin (**B**, *P* <0.001), 25-hydroxy-vitamin D (VD) (**C**, *P* =0.004), and lowest SpO2 (**D**, *P*< 0.001) levels.

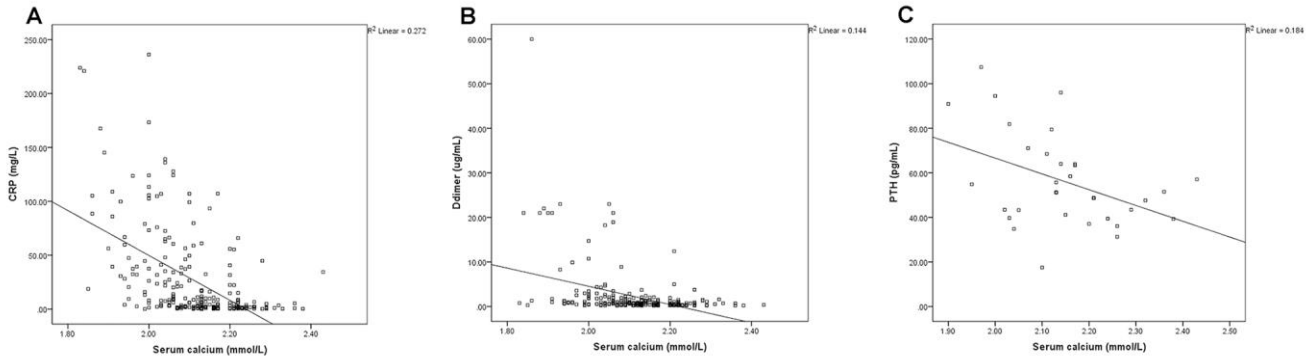

Figure 2. The serum calcium levels were negatively correlated with C-reactive protein (CRP) (**A**, *P*< 0.001), D-dimer (**B**, *P*< 0.001) and parathyroid hormone (PTH) (**C**, *P* =0.048) levels.

Table 3. Serum calcium and clinical variables of severity and outcomes.

	Group A (*n*= 43)	Group B (*n*= 137)	Group C (*n*= 61)	*P* value
Death	10 (23.3%)	0 (0.0%)	0 (0.0%)	<0.001
MODS	14 (32.6%)	3 (2.2%)	0 (0.0%)	<0.001
Septic shock	6 (14.0%)	0 (0.0%)	0 (0.0%)	<0.001
ARDS	15 (34.9%)	3 (2.2%)	1 (1.6%)	<0.001
Liver injury	6 (14.0%)	9 (6.6%)	1 (1.6%)	<0.001
AKI	8 (18.6%)	5 (3.6%)	1 (1.6%)	<0.001
Cardiac injury	7 (16.3%)	5 (3.6%)	0 (0.0%)	<0.001
Need for MV	12 (27.9%)	0 (0.0%)	0 (0.0%)	<0.001
Need for CRRT	7 (16.3%)	0 (0.0%)	0 (0.0%)	<0.001

Group A, the serum calcium values: ≤2.0 mmol/L; Group B, the serum calcium values: 2.0-2.2 mmol/L; Group C, the serum calcium values: >2.2 mmol/L; MODS, multiple organ dysfunction syndrome; ARDS, acute respiratory distress syndrome; AKI, acute kidney injury; MV, mechanical ventilation; CRRT, continuous renal replacement therapy.

Table 4. Values of serum calcium in relation to the presence or absence of clinical variables of severity and outcomes.

Clinical variables	Presence		Absence		*P* value
	serum calcium	*n*	serum calcium	*n*	
Death	1.96 (1.90-2.00)	10	2.12(2.05-2.20)	231	< 0.001
MODS	1.95 (1.88-2.00)	17	2.13 (2.06-2.20)	224	< 0.001
Septic shock	1.98(1.92-2.00)	6	2.12(2.05-2.20)	235	< 0.001
ARDS	1.95 (1.90-2.00)	19	2.13(2.06-2.20)	222	< 0.001
Liver injury	2.04 (1.96-2.06)	16	2.12(2.05-2.20)	225	< 0.001
AKI	1.97(1.87-2.07)	14	2.12(2.05-2.20)	227	< 0.001
Cardiac injury	2.00 (1.90-2.07)	12	2.12(2.05-2.20)	229	< 0.001
Need for MV	1.94(1.89-2.00)	12	2.12(2.05-2.20)	229	< 0.001
Need for CRRT	1.91(1.84-1.94)	7	2.12(2.05-2.20)	234	< 0.001

MODS, multiple organ dysfunction syndrome; ARDS, acute respiratory distress syndrome; AKI, acute kidney injury; MV, mechanical ventilation; CRRT, continuous renal replacement therapy.

DISCUSSION

This clinical retrospective study investigated the correlations between serum calcium and clinical severity and outcomes in patients with COVID-19. The incidence of hypocalcemia was 74.7%. We found that patients with lower serum calcium levels (especially ≤2.0 mmol/L) had worse clinical variables, higher incidences of MODS and septic shock, and higher 28-day mortality. Hypocalcemia may be associated with imbalanced VD and PTH in the acute phase of COVID-19. The overall mortality was 4.1% (10/241), whereas the mortality of critically ill patients was increased to 40.0% (10/25).

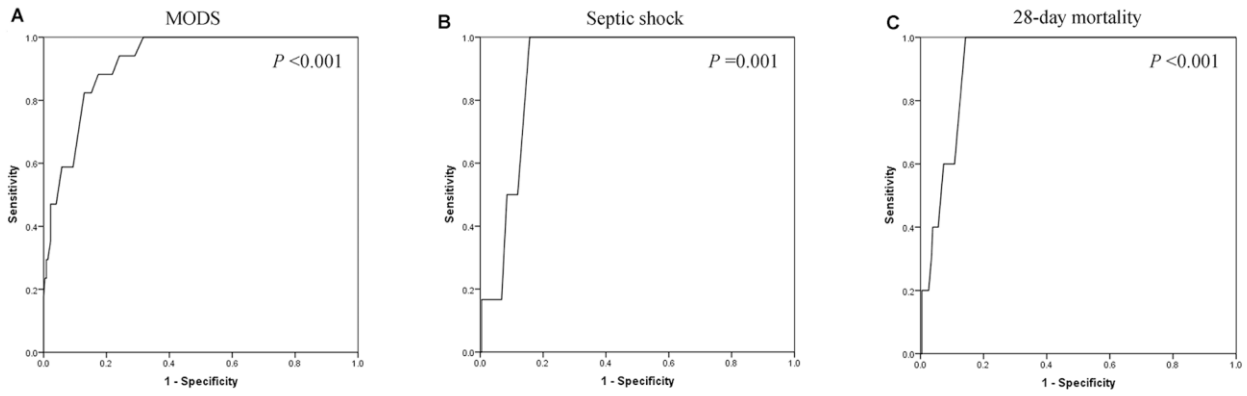

Figure 3. The areas under the receiver operating characteristic curves of multiple organ dysfunction syndrome (MODS) (**A**), septic shock (**B**), and 28-day mortality (**C**) were 0.923 (*P* <0.001), 0.905 (*P* =0.001), and 0.929 (*P* <0.001), respectively.

The WHO declared that COVID-19 was a pandemic because the disease is still spreading rapidly around the world [4]. More than 5700000 patients have been diagnosed with COVID-19 worldwide, and nearly 360000 have died [4]. Although the National Health Commission of China and the WHO have issued a series of diagnosis and treatment recommendations, the mortality of critically ill patients is still extremely high. The underlying mechanisms of the novel coronavirus causing organ dysfunction are yet unknown. It is crucial to identify the risk factors for severe illness or death [10]. However, few reports have been published to establish an early and sensitive biomarker to predict the disease severity and prognosis of COVID-19. In this study, we found that serum calcium levels were associated with the disease severity and prognosis of patients with COVID-19.

Hypocalcemia is common in critically ill patients. The causes of hypocalcemia include oversecretion of PTH, VD deficiency, decreased dietary intake, hypoproteinemia, hypomagnesemia drug interactions, and so on [15]. Hypocalcemia was defined as a serum calcium level less than 2.2 mmol/L in our clinical laboratory. At present, there is no specific severity grading system for hypocalcemia. Previous studies reported that serum calcium levels less than approximately 2.0 mmol/L were associated with worse clinical outcomes in critically ill patients [15–17]. Therefore, we divided patients into three groups based on serum calcium values of ≤2.0 mmol/L, 2.0-2.2 mmol/L, and >2.2 mmol/L. We found that patients with serum calcium values ≤2.0 mmol/L had higher 28-day mortality, and a higher incidence of organ injury. The findings of this study were consistent with previous reports. However, this was the first study to investigate the correlations between serum calcium and clinical outcomes in patients with COVID-19. These results suggested that correcting hypocalcemia could be an important strategy to improve the prognosis of patients with COVID-19, especially for patients with serum calcium values less than 2 mmol/L. In addition, our study also revealed that hypocalcemia was associated with hypoproteinemia and imbalanced VD and PTH in the acute phase of COVID-19. Hypoproteinemia and VD deficiency were also common and correlated with increased mortality in critically ill patients [18, 19]. Therefore, improvement of hypoproteinemia and imbalanced hormone levels may also be useful in the treatment of COVID-19.

Increased CRP, ALT, TNI, and D-dimer levels and lymphocytopenia were present in most critical COVID-19 patients [2, 7, 8]. Our results also showed that patients with lower serum calcium values had higher levels of CRP, ALT, TNI, and D-dimer and lower lymphocyte counts. The serum calcium values were significantly correlated with lymphocyte count and CRP and D-dimer levels. Moreover, CRP and D-dimer were also indicators to predict the prognosis of critically ill patients [19]. The findings of this study were consistent with previous reports and confirmed that serum calcium levels were associated with the disease severity and

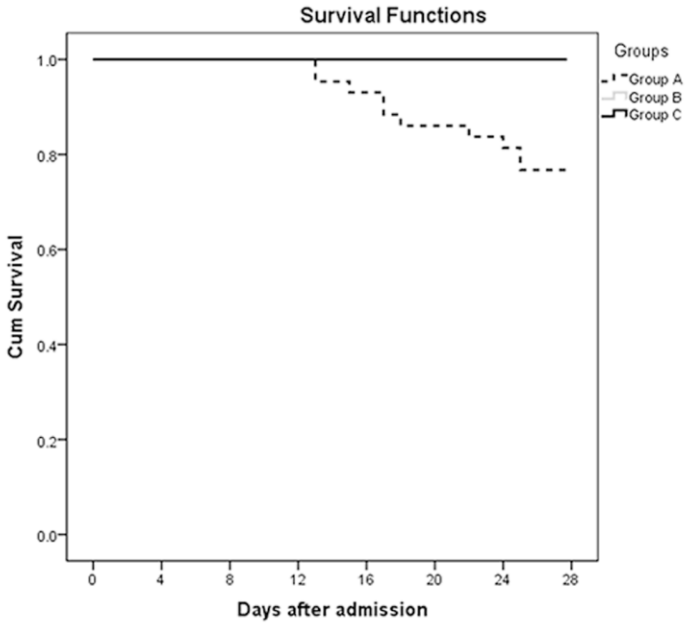

Figure 4. The survival curves at 28 days after admission of the three groups: Group A (serum calcium values ≤2.0 mmol/L, *n* =43), Group B (serum calcium values 2.0-2.2 mmol/L, *n* =137), and Group C (serum calcium values >2.2 mmol/L, *n* =61) (*P* <0.001).

prognosis of patients with COVID-19. The lung is the main organ affected by this disease. In this study, the median lowest SpO2 was 97.0% (IQR, 96.0%-98.0%; range, 80%-99%), and the SpO2 values were significantly positively correlated with serum calcium levels. Patients with serum calcium values ≤2.0 mmol/L had higher ARDS incidence, while patients with ARDS also had lower serum calcium values. These phenomena indicated that hypocalcemia might be crucial in the development of ARDS. Early diagnosis and treatment of hypocalcemia may alleviate organ injury in the acute phase of COVID-19.

Some limitations of the study should be discussed. Because of our single-center retrospective design and small sample size, the results might be inconclusive, and the accuracy should be confirmed by large-scale prospective clinical studies. Moreover, because the study was not based on pathophysiological models, the results were hypothesis generating, and the exact mechanisms of hypocalcemia and VD deficiency should be tested by more fundamental experiments. In addition, the values of serum calcium were of total calcium rather than ionized calcium in this study, which may not precisely reflect the extent of decreased calcium.

CONCLUSIONS

In conclusion, this retrospective clinical study found that the incidence of hypocalcemia and VD deficiency was very high in patients with COVID-19. Hypocalcemia may be associated with imbalanced VD and PTH levels. Patients with lower serum calcium levels (especially ≤2.0 mmol/L) had worse clinical variables, higher incidences of MODS and septic shock, and higher 28-day mortality. The overall mortality of COVID-19 was 4.1%, whereas the mortality of critically ill patients was 40.0%.

MATERIALS AND METHODS

Patients

From February 10 to February 28, 2020, adult patients (age ≥18 years) with confirmed COVID-19 admitted to our specialized isolation units, Tongji Hospital of Huazhong University of Science and Technology in Wuhan, were enrolled in this clinical retrospective study. Patients with chronic organ dysfunction (e.g., hepatic or renal dysfunction), terminal cancer, immunodeficiency, and a history of long-term use of hormones were excluded. Written informed consent was waived by our institutional review board because this was a retrospective study that assessed deidentified data and included no potential risk to patients. The diagnosis of COVID-19 was made according to the WHO interim guidance and the recommendations of the National Health Commission of China [4, 5], and confirmed by RNA detection of SARS-CoV-2 in the clinical laboratory of Tongji Hospital.

Definitions

An identified case of COVID-19 was defined as a positive finding by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) assay of nasal and pharyngeal swab specimens [4, 5, 7]. Only laboratory-confirmed cases were enrolled in the analysis. The clinical classifications of COVID-19 were in accordance with the Chinese recommendations [5]: Mild, with minor clinical symptoms (e.g., fever, cough) without imaging manifestations. Moderate, with fever or respiratory tract infection symptoms with imaging indicating pneumonia. Severe, met any of the following, I—respiratory distress and respiratory rate ≥30 breaths/min; II—pulse oxygen saturation (SpO2) ≤93% at rest; or III—arterial partial pressure of oxygen (PaO2)/ fraction of inspired oxygen (FiO2) ≤300 mmHg (1 mmHg =0.133 kPa). Critical, met any of the following, I—respiratory failure with mechanical ventilation (MV); II—shock; or III—multiple organ failure requiring ICU treatment. Hypocalcemia was defined as a serum calcium level less than 2.2 mmol/L in our clinical laboratory. Sepsis was defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, and septic shock was defined as a subset of sepsis with circulatory and cellular/metabolic dysfunction that is associated with a higher risk of mortality [11]. The diagnostic criteria of acute respiratory distress syndrome (ARDS) were in accordance with the Berlin definitions [12]. The definitions of acute kidney injury (AKI) were based on the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines [13]. Cardiac injury was defined if serum levels of cardiac biomarkers (e.g., troponin I) were more than twice the reference upper limit or new abnormalities were found in electrocardiography and echocardiography [2]. Liver injury was defined if serum levels of hepatic biomarkers (e.g., alanine aminotransferase) were more than twice the reference upper limit or if there was disproportionate elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared with alkaline phosphatase levels [14]. Multiple organ dysfunction syndrome (MODS) was defined as the combined dysfunction of two or more organs.

Data collection

The baseline clinical characteristics, including age, sex, days from onset to admission, initial symptoms or signs, and clinical classifications were collected from electronic medical records, and all laboratory tests were performed according to the clinical needs of patients. The levels of serum calcium, C-reactive protein (CRP),

ALT, albumin, creatinine, troponin I (TNI), and plasma D-dimer and white blood cell (WBC) count, lymphocyte count, and the lowest SpO2 within 24 hours of admission were recorded. The hormone levels associated with blood calcium (e.g., parathyroid hormone, 25-hydroxy-vitamin D) were also recorded. All blood parameters were detected by the clinical laboratory of Tongji Hospital. Moreover, the numbers of patients with ARDS, AKI, cardiac injury, liver injury, septic shock and MODS and patients receiving noninvasive ventilation (NIV), high-flow nasal cannula (HFNC), MV, and continuous renal replacement therapy (CRRT) were also recorded. The primary endpoints were the development of septic shock, MODS, and 28-day mortality. The secondary endpoints were the other disease severity parameters (e.g., organ injury or not).

Statistical analysis

The Kolmogorov-Smirnov test was first performed to test the normal distribution of the data. Normally distributed data were expressed as the means ± standard deviation and were compared by t tests. Abnormally distributed data were expressed as the medians (interquartile ranges, IQR) and were compared by the Mann-Whitney U test or the Kruskal-Wallis test. Categorical variables were presented as absolute numbers or percentages and were analyzed using the χ^2 test or Fisher's exact test. To take into account the repeated nature of the variables, analysis of variance (ANOVA) for repeated measurements of the general linear model was implemented. Receiver operating characteristic (ROC) curves were used to evaluate the associations between serum calcium and septic shock, MODS, and 28-day mortality. IBM Statistical Package for the Social Sciences (SPSS, version 22.0, NY, USA) software was used for statistical analysis, and P <0.05 was considered statistically significant. SPSS scatterplots and a correlation analysis were performed to evaluate the relevance between serum calcium and blood biomarkers. The statistical methods of this study were reviewed by Qiao Liu, a biostatistician from the Center for Disease Control and Prevention of Jiangsu Province in China.

AUTHOR CONTRIBUTIONS

Sun JK, Zhang WH, Shi QK, Yuan ST, Gu W, and Qi JW designed the research; Sun JK, Zhang WH, Zou L, Liu Y, Li JJ, Kan XH, Dai L performed the research; Sun JK, Zhang WH, Yu WK, and Xu HY analyzed the data; Sun JK and Zhang WH wrote the paper.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Research Paper

Clinical characteristics of older and younger patients infected with SARS-CoV-2

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ABSTRACT

Background: SARS-CoV-2 causes high mortality risk in older patients. This study aims to characterize the clinical features of older and younger SARS-CoV-2 infected patients.

Results: A total of 239 patients were divided into the younger group (<60 years; n=181) and the older group (≥60 years; n=58). In both groups, fever and cough were common symptoms. However, dyspnea was more frequent in older patients than younger patients (20.7% versus 9.9%, p=0.032). Compared with younger patients, older patients harbored more severe cases (37.9% versus 17.1%, p=0.001) and comorbidities (58.6% versus 21.0%, p<0.001) such as hypertension and diabetes. The baseline values of eosinophils and C-reactive protein were abnormal in older and younger groups. From baseline to day 14, significant decreases of three biomarkers (C-reactive protein, hemoglobin, albumin) and dramatic increases of three biomarkers (lymphocytes, platelets, blood urea nitrogen) were observed in older patients.

Conclusion: Older and younger patients exhibited differences in dyspnea, comorbidities, and proportions of severe cases. Moreover, the disease progression of SARS-CoV-2 in older patients is observed with the dynamics of laboratory biomarkers, supporting their potential use in disease monitoring.

Methods: We retrieved clinical symptoms, laboratory findings, comorbidities, and hospitalization information of SARS-CoV-2 cases in Changsha.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious coronavirus that causes pneumonia-like deaths and spreads fast through human-to-human contact [1–3]. Since the first suspected cases were documented in early December 2019, the increasing number of SARS-CoV-2 cases has reached more than six millions, including >350,000 deaths worldwide by June 1th, 2020.

It has been hypothesized that older people are more vulnerable to SARS-CoV-2. An early study reported a high prevalence of SARS-CoV-2 in older males with comorbidities [4]. Subsequent studies confirmed that SARS-CoV-2 was often observed in older patients with comorbidities [5] and severe disease progression [6, 7]. A study of 138 hospitalized patients reported a higher rate of ICU admission in older patients compared with younger patients [8]. Another study revealed a high risk of mortality in older patients with comorbidities and acute respiratory distress syndrome [9]. The overall case-fatality rate was 1.38% in China, but this rate increased to 3.99% in older patients between 60 and 69 years, 8.61% in older patients between 70 and 79 years, and 13.4% in ≥80 patients [10]. However, few studies have revealed clinical differences between older and younger groups.

Our study aims to characterize the clinical features of SARS-CoV-2 in younger and older groups based on a large cohort of 239 patients in Changsha - a neighboring city of Wuhan. Moreover, we assessed clinical symptoms, laboratory findings, comorbidities, and hospitalization information to monitor the disease progression of SARS-CoV-2.

RESULTS

Demographic profiles and clinical characteristics

A total of 239 patients confirmed with SARS-CoV-2 infections were hospitalized in Changsha. Table 1 summarizes their demographic and clinical characteristics. The median age of 239 patients was 45 years (interquartile range: 34 to 59 years) and 58 (24.3%) patients had at least 60 years of age (Figure 1A). Nearly half of the 239 patients were males. The youngest patient was a one-year-old girl discharged on February 18 after a 15-day hospitalization, while the eldest patient was an 84-year-old woman who had a 19-day hospitalization (from February 6 to February 25). Fifty-three (22.2%) patients were categorized into the severe group and severe cases were often observed in the elderly patients (Figure 1B).

At hospital admission, fever (67.4%) was the most common symptom, followed by cough (58.2%), fatigue (33.9%), dyspnea (12.6%), sore throat (11.3%), myalgia (9.6%), diarrhea (8.4%), and others (Table 1). In addition, 72 (30.1%) patients had at least one comorbidity such as hypertension (13.4%), diabetes (6.3%), cardiovascular disease (4.2%), hepatitis (2.9%), chronic obstructive pulmonary disease (2.1%), cerebral infarction (2.1%), peptic ulcer (1.7%), cardiac arrhythmia (1.3%), and abnormal lipid metabolism (1.3%). Furthermore, the increased risk of comorbidities was associated with the patient age that elderly patients were more likely to develop comorbidities (Figure 1B). HIV infection was absent in all patients.

Older patients (≥60 years) and younger patients (<60 years)

Clinical features of 58 older patients and 181 younger patients were summarized in Table 1. The median ages of older and younger patients were 66 and 40 years, respectively ($p<0.001$). The percentage of females was higher in the older group than the younger group (62.1% versus 47.0%, $p=0.045$). Compared with younger patients, older patients had pronounced key features such as: (i) older patients were more likely to be severe (37.9% versus 17.1%, $p=0.001$); (ii) older patients harbored more comorbidities such as hypertension (36.2% versus 6.1%, $p<0.001$), diabetes (15.5% versus 3.3%, $p=0.001$), and cardiovascular disease (10.3% versus 2.2%, $p=0.007$); and (iii) older patients had more cases of dyspnea (20.7% versus 9.9%, $p=0.032$).

Comparisons of CT diagnostics in younger and older patients revealed no difference in the risk of abnormal lungs ($p=0.972$). Two males were severely ill during hospitalization and died thereafter. CT images showed the accumulation of ground-glass opacities and pulmonary consolidation during the disease progression (Figure 2).

Biomarker dynamics during the disease progression of SARS-CoV-2

We compared laboratory biomarkers in older and younger patients at baseline (Table 2). The baseline values of eosinophils, C-reactive protein, lactic acid were abnormal in older and younger groups, while six biomarkers (white blood cells, neutrophils, alanine aminotransferase, total bilirubin, creatinine, lactic acids) were similarly expressed in both groups (Table 2). Of interest, the decrease of lymphocytes (Figure 1C) and the increase of C-reactive protein (Figure 1D) were observed along with the increasing age when patients were categorized based on their ages in decades (1 to 19, 20 to 29, 30 to 39, 40 to 49, 50 to 59, 60 to 69, ≥70 years).

Compared with younger patients, older patients had many abnormal biomarkers at baseline, including (i) higher levels of C-reactive protein (30.1 mg/L versus 12.1 mg/L, $p<0.001$); (ii) high levels of aspartate aminotransferase (27.90 U/L versus 23.38 U/L, $p<0.001$) and blood urea nitrogen (4.7 mmol/L versus 4.1 mmol/L, $p=0.003$); (iii) lower levels of hemoglobin (123 g/L versus 132 g/L, $p=0.001$) and albumin (35.56 g/L versus 38.98 g/L, $p<0.001$); and (iv) lower levels of

Table 1. Clinical features of 239 patients infected with SARS-CoV-2.

	Total (n=239)	Age<60 (n=181)	Age≥60 (n=58)	p-value
Age	45.0(34.0-58.5)	40(31.0-47.0)	66.0(64.0-70.8)	<0.001
Male	118 (49.4%)	96 (53.0%)	22 (37.9%)	0.045
Severe cases	53 (22.2%)	31 (17.1%)	22 (37.9%)	0.001
Comorbidity				
Any	72 (30.1%)	38 (21.0%)	34 (58.6%)	<0.001
Hypertension	32 (13.4%)	11 (6.1%)	21 (36.2%)	<0.001
Diabetes	15 (6.3%)	6 (3.3%)	9 (15.5%)	0.001
Cardiovascular disease	10 (4.2%)	4 (2.2%)	6 (10.3%)	0.007
Hepatitis	7 (2.9%)	4 (2.2%)	3 (5.2%)	0.244
Chronic obstructive pulmonary disease	5 (2.1%)	3 (1.7%)	2 (3.4%)	0.407
Cerebral infarction	5 (2.1%)	2 (1.1%)	3 (5.2%)	0.060
Peptic ulcer	4 (1.7%)	4 (2.2%)	0 (0.0%)	0.254
Abnormal lipid metabolism	3 (1.3%)	1 (0.6%)	2 (3.4%)	0.085
Cardiac arrhythmia	3 (1.3%)	2 (1.1%)	1 (1.7%)	0.712
Chronic kidney disease	1 (0.4%)	1 (0.6%)	0 (0.0%)	0.571
Symptoms				
Any	222 (92.9%)	167 (92.3%)	55 (94.8%)	0.509
Fever	161 (67.4%)	124 (68.5%)	37 (63.8%)	0.505
Cough	139 (58.2%)	107 (59.1%)	32 (55.2%)	0.596
Fatigue	81 (33.9%)	56 (30.9%)	25 (43.1%)	0.089
Dyspnea	30 (12.6%)	18 (9.9%)	12 (20.7%)	0.032
Sore throat	27 (11.3%)	22 (12.2%)	5 (8.6%)	0.459
Myalgia	23 (9.6%)	17 (9.4%)	6 (10.3%)	0.830
Diarrhea	20 (8.4%)	14 (7.7%)	6 (10.3%)	0.532
Headache	18 (7.5%)	12 (6.6%)	6 (10.3%)	0.351
Dizziness	10 (4.2%)	7 (3.9%)	3 (5.2%)	0.666
Nausea or vomiting	8 (3.3%)	5 (2.8%)	3 (5.2%)	0.375
Runny nose	5 (2.1%)	3 (1.7%)	2 (3.4%)	0.407

lymphocytes (0.98 versus 1.25 $\times 10^9$ cells/L, p<0.001). The percentage of patients with lymphocytopenia was higher in older patients than younger patients (32.8% versus 17.7%, p=0.015), while normal leukocytes (range: 0.8 to 4×10^9 cells/L) were observed in most patients (185, 77.4%).

We next evaluated laboratory biomarkers of C-reactive protein, albumin, lymphocytes, and blood urea nitrogen on days 0, 7, and 14. (Figure 3). First, serum levels of C-reactive protein were higher (30.11 mg/L) in older patients at hospital admission, but it dropped sharply after treatment and returned to the normal status (5.2 mg/L) on day 14. In contrast, the lower level of C-reactive protein was observed in younger patients (12.1 mg/L) at baseline and it decreased slowly compared with that in older patients. Second, lymphocytes increased from 1.14×10^9 cells/L at baseline to 1.34×10^9 cells/L on day 7 and 1.46 $\times 10^9$ cells/L on day 14. Similar increasing patterns were observed for blood urea nitrogen from baseline (4.22 mmol/L) to day 14 (5.64 mmol/L). Third, serum levels of albumin in older patients continuously decreased from 35.56 g/L at baseline, to 34.6 g/L on day 7 and 33.6 g/L on day 14.

Clinical outcome

The median duration from symptom onset to virus clearance was 19 days (interquartile range: 15 to 28 days). This duration was much longer in older patients than younger patients (24 versus 19 days, p=0.014) (Figure 4A). Compared with younger patients, older patients had longer hospital stays (18 versus 15 days, p=0.047) (Figure 4B). We further analyzed the associations of baseline biomarkers with the short (<3 weeks) or long (≥3 weeks) hospital stay in older and younger patients (Supplementary Table 1). Blood urea

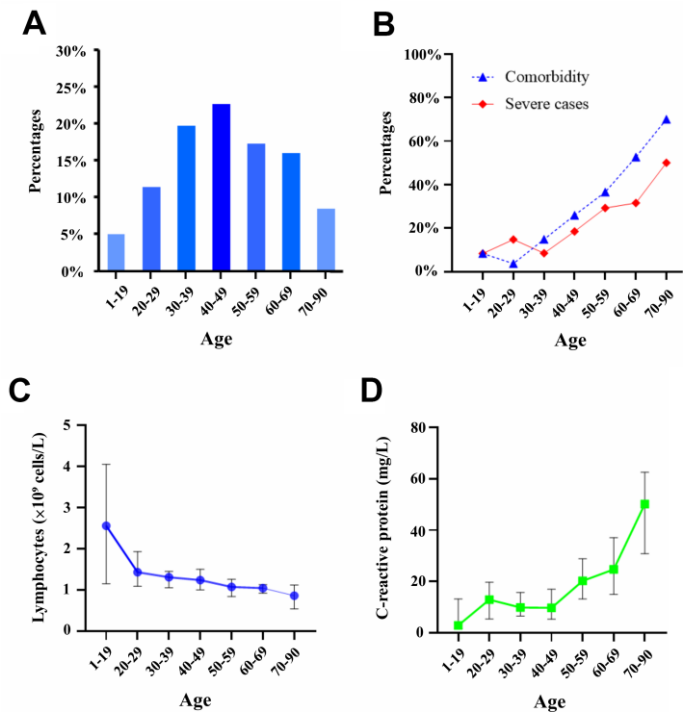

Figure 1. Distribution of patient age and age-related biomarkers. (**A**) Distribution of patients within decades of age. (**B**) Percentages of severe cases and patients with at least one comorbidity. (**C**) Serum levels of lymphocytes in seven age classes. (**D**) Serum levels of C-reactive protein in seven age classes.

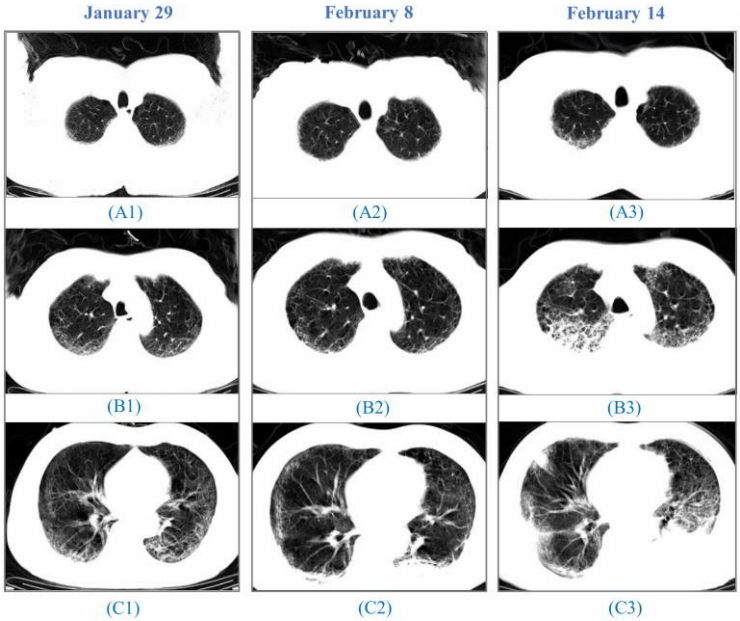

Figure 2. CT images from a 64-year-old man. A 64-year-old man, who had a fever and pneumonia, was suspected as the SARS-CoV-2 carrier on January 28 and confirmed on January 30. (**A1**), (**B1**) to (**C1**): On January 29, initial CT scans at the hospital admission showed multifocal ground-glass opacity (GGO) and reticulation, predominantly in the subpleural areas of both lungs. (**A2**), (**B2**) to (**C2**): On February 8, CT images indicated progressing GGOs. Newly-appeared patchy and core-like consolidation were visible in lower lobes of both lungs. The patient showed high fever, cough, blood in the sputum, reduced SpO2, and a sign of heart failure. (**A3**), (**B3**) to (**C3**): On February 14, CT images showed progressing lesion with multiple newly-appeared GGO and consolidation. Irregular interlobular septal thickening was observed in the upper lobe of the right lung. The patient passed away on February 15.

Table 2. Baseline characteristics of biomarkers in 239 patients with SARS-CoV-2.

	Total (n=239)	Age<60 (n=181)	Age≥60 (n=58)	p-value
White blood cells ($\times10^9$ cells/L)	4.58(3.48-5.69)	4.59(3.47-5.73)	4.55(3.63-5.41)	0.523
Lymphocytes ($\times10^9$ cells/L)	1.14(0.85-1.60)	1.25(0.90-1.70)	0.98(0.64-1.19)	<0.001
Neutrophils ($\times10^9$ cells/L)	2.89(2.12-3.64)	2.86(2.03-3.61)	3.02(2.39-3.66)	0.261
Eosinophils ($\times10^9$ cells/L)	0.01(0-0.04)	0.01(0-0.05)	0.01(0-0.03)	0.010
Hemoglobin (g/L)	130(120-141)	132(122-143)	123(115.50-134.75)	0.001
Platelets ($\times10^9$/L)	171(138-227)	179(146-228)	147.50(117.25-206.50)	0.005
D-dimer (mg/L)	0.27(0.14-0.54)	0.22(0.13-0.48)	0.38(0.17-0.71)	0.013
C-reactive protein (mg/L)	15.60(4.36-30.85)	12.10(3.55-24.07)	30.11(15.80-55.19)	<0.001
Alanine aminotransferase (U/L)	19.45(14.21-27.48)	19.69(14.20-27.79)	18.22(14.22-26.36)	0.486
Aspartate aminotransferase (U/L)	24.40(19.80-31.37)	23.38(18.85-28.75)	27.90(23.46-35.87)	<0.001
Total bilirubin (μmol/L)	10.87(8.19-15.80)	10.92(8.02-15.60)	10.80(9.02-16.07)	0.665
Albumin (g/L)	38.23(35.36-40.97)	38.98(36.21-41.75)	35.56(32.17-38.32)	<0.001
Albumin/globulin	1.50(1.31-1.72)	1.53(1.39-1.78)	1.33(1.23-1.48)	<0.001
Blood urea nitrogen(mmol/L)	4.22(3.19-5.11)	4.12(3.14-4.88)	4.70(3.88-6.45)	0.003
Creatinine (μmol/L)	50.21(39.99-63.34)	49.85(39.8-62.19)	52.44(43.41-64.90)	0.227
Lactic acid	768.35(392.65-824.70)	761.40(391.60-808.10)	773.40(742.50-837.30)	0.482

nitrogen was significantly lower in older patients with a short hospital stay than older patients with a long hospital stay (p-value=0.037). Compared to older patients, younger patients with a short hospital stay usually had lower baseline levels of C-reactive protein and aspartate aminotransferase, but higher levels of lymphocytes, albumin, and albumin/globulin at baseline (p-values<0.05, Supplementary Table 1).

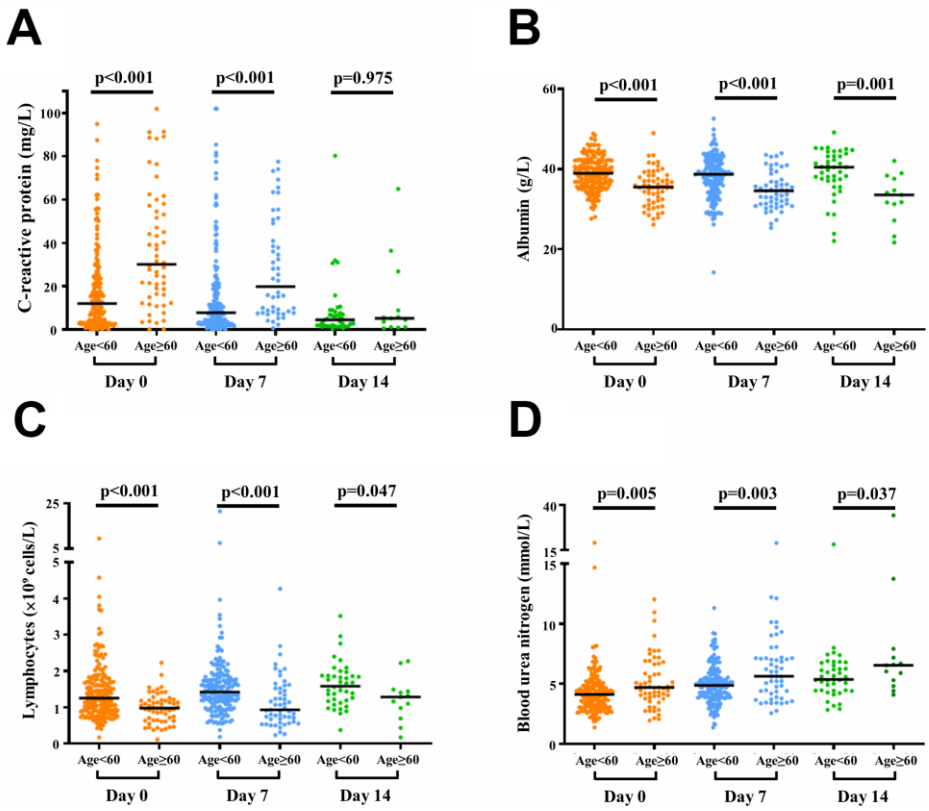

Figure 3. Dynamics of laboratory biomarkers of 239 SARS-CoV-2 cases. Scatter plots of C-reactive protein (**A**), albumin (**B**), lymphocytes (**C**), and blood urea nitrogen (**D**) in older and younger patients are illustrated on days 0, 7, and 14. Blood tests on days 0, 7, and 14 were conducted for 239, 229, and 54 patients, respectively. Laboratory biomarkers on day 14 were assessed for 54 patients who had positive SARS-CoV-2 and remained in hospital on day 14.

By March, 15th, 2020, 237 (99.2%) patients fulfilled the discharge criteria, and a 58-year-old male and a 64-year-old male had died. After the 14-day hospitalization, 129 patients were diagnosed with virus clearance, and 101 of them were discharged for 14-day home isolation. After hospital discharge, the presence of SARS-CoV-2 was not reported in any discharged patient over a follow-up period of two months.

DISCUSSION

Based on a cohort of 239 patients, our study revealed three major findings: (i) older and younger patients exhibited differences in dyspnea, comorbidities, and proportions of severe cases; (ii) compared with younger patients, older patients exhibited higher levels of C-reactive protein, D-dimer, aspartate aminotransferase, blood urea nitrogen and lower levels of lymphocytes, hemoglobin, platelet, albumin at baseline; and (iii) the disease progression of SARS-CoV-2 was associated with the dynamics of laboratory biomarkers such as C-reactive protein and lymphocytes, supporting their clinical use in disease monitoring.

SARS-CoV-2 is a highly pathogenic coronavirus of bat origin [11] that causes upper respiratory tract diseases and pneumonia-like diseases [3, 9]. Increased mortality risk was previously reported in critically ill patients with chronic comorbidities and acute respiratory distress syndrome [9]. Similar to the prevalence of older patients in Wuhan [4], 58 (24.3%) of 239 patients were ≥60 years in our study. Although the cutoff of 60 years was used to categorize older and younger patients, key factors such as comorbidities, severe cases, lymphocytes, and C-reactive protein showed increasing or decreasing patterns over seven age groups (Figure 1). In agreement with previous studies [5], severe cases were often observed in older patients in our study (Table 1). Older patients often have many comorbidities such as diabetes, hypertension, and cardiovascular disease, which potentially cause the difficulty of clinical treatment. Fever, cough, and fatigue were common symptoms but there were no differences in older and younger patients, indicating that symptoms and signs were not unique features to distinguish the impact of SARS-CoV-2 in both groups. However, dyspnea was more common in older patients, implying the potential risk of lung lesions after SARS-CoV-2 infection.

In agreement with previous studies [6, 12, 13], our study revealed key laboratory markers such as white blood cells, lymphocytes, eosinophils, C-reactive protein, albumin, blood urea nitrogen, aspartate aminotransferase, and lactic acid. These biomarkers are commonly used to monitor disease progression, inflammatory/immune responses, and/or physiological changes associated with viral infections. For instance, lymphocytes are a type of white blood cell that could play protection roles in defending viral infections [14]. Lymphocytopenia might be a biomarker to reveal disease severity or antiviral immunity [15]. Lymphocytopenia was observed in 51 (21.3%) patients in our study, while this percentage was lower than the national study (83.2%) [3]. This discordance may be due to the condition of mildly ill patients in our cohort and timely antiviral treatment. Moreover, C-reactive protein was much higher in older patients (30.11 mg/L versus 12.11 mg/L), indicating that severe inflammatory reactions could be observed in older patients. Whether these biomarkers could be effective predictors of treatment responses requires further investigations.

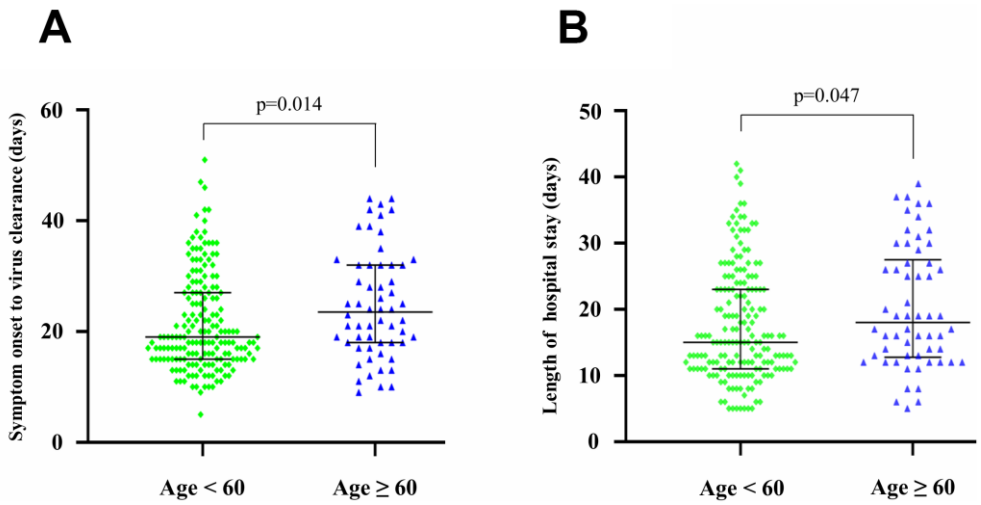

Figure 4. Clinical features of 239 SARS-CoV-2 infected patients. (**A**) The duration from symptom onset to virus clearance in younger and older patients. (**B**) The length of hospital stay in younger and older patients.

This study has several limitations. First, our study characterized older and younger patients in Changsha, but the clinical features of older patients should be analyzed from a global perspective, including those from different countries. Second, we evaluated the dynamics of laboratory biomarkers in a 14-day period because of data availability, but future studies should report the full course of disease progression. Third, only two deaths were observed in our study and future studies should characterize the mortality risk of older patients in larger cohorts.

CONCLUSION

Overall, our study characterized the clinical features of younger and older patients infected with SARS-CoV-2. Older and younger patients exhibited differences in dyspnea, comorbidities, and proportions of severe cases. Higher levels of C-reactive protein, aspartate amino-transferase, blood urea nitrogen, and lower levels of lymphocytes and albumin were observed in older patients. Furthermore, the dynamics of laboratory biomarkers such as lymphocytes and C-reactive protein can be used for monitoring the disease progression in older patients.

MATERIALS AND METHODS

Study design and participants

This study was conducted at The First Hospital of Changsha, designated as the single hospital to treat all SARS-CoV-2 cases in Changsha. Patients who were infected with laboratory-confirmed SARS-CoV-2 according to the WHO interim guidance [16] were transferred from local hospitals to The First Hospital of Changsha between January 23 and March 15, 2020. Clinical outcomes were monitored up to the hospital discharge of all patients. This study was performed following the Helsinki Declaration and was approved by the Ethics Committee of The First Hospital of Changsha. In light of the rapid emergence of SARS-CoV-2, written informed consent was waived for this observational study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Data collection

We retrieved electronic medical records, clinical symptoms or signs, laboratory findings, comorbidities, and hospitalization information of hospitalized patients infected with SARS-CoV-2. Clinical information was retrieved using a customized collection form. Any missing or uncertain record was clarified by direct communications with doctors and patients. To verify data accuracy, two study investigators (HYX and CCL) reviewed the clinical data independently.

Diagnostics of SARS-CoV-2

To identify the presence of SARS-CoV-2, throat swab specimens were collected for real-time RT-PCR analyses using the SLAN-96P real-time PCR system (Hongshitech, Shanghai, China) and SARS-CoV-2 nucleic acid diagnostic kits (PCR-Fluorescent Probe) from Sansure Biotech, Changsha, China. The latter was approved by the China National Medical Products Administration (registration number: 20203400064) and the European CE approval (ID: CMB 8764-2020). The detection limit of this nucleic acid kit was 200 copies/mL. SARS-CoV-2 tests were independently conducted at two medical centers: the First Hospital of Changsha and the Changsha Municipal Center for Disease Prevention and Control. A positive case was reported if SARS-CoV-2 was identified by two medical centers above, while a negative case was reported if two medical centers consistently reported an undetectable viral load. Negative cases were considered from their discharge if they fulfilled three requirements: (i) no respiratory symptoms of fever or cough were observed for three consecutive days; (ii) two consecutive nucleic acid tests were negative (three days apart from each test); and (iii) computed tomography images became normal. All discharged cases remained on home isolation for another 14 days.

Laboratory assessments

Computed tomography (CT) diagnostics were performed using the 128-slice SOMATOM go. Top CT systems from Siemens Healthineers. Hematologic assessments of white blood cells, hemoglobin, lymphocytes, neutrophils, eosinophils, and platelets were proceeded using the Mindray BC-6800 automated hematology analyzer. Biochemical data of albumin, alanine aminotransferase, albumin/globulin, aspartate aminotransferase, blood urea nitrogen, creatinine, C-reactive protein, D-dimer, and total bilirubin were quantified using the ARCHITECT c16000 clinical chemistry analyzer.

Classification of severe and non-severe cases

Based on the New Coronavirus Diagnosis and Treatment Guideline (version 7) in China, a severe case was classified if a patient had any of the following conditions: (i) respiratory distress with the respiration rate ≥30 times per minute; (ii) oxygen saturation ≤93% in the resting state; (iii) the ratio of the arterial partial pressure of oxygen to fraction of inspired oxygen ≤300 mmHg (1mmHg = 0.133 kPa); and (iv) the area of the

lung affected with pneumonia increased >50% within 24 to 48 hours. Non-severe cases included mild or moderate patients who had the conditions of fever (≥37.5°C) and/or the respiratory tract.

Statistical analyses

We measured median (interquartile range) of continuous variables as well as counts and percentages of categorical variables. Normal distribution was examined by Shapiro-Wilks normality tests. To explore differences between patient groups, the chi-square and Fisher's exact tests were conducted for categorical variables; two-tailed t-tests were performed for continuous variables following normal distributions; the Wilcoxon rank-sum tests were used for non-normal continuous variables in paired groups; Mann-Whitney U tests were applied for non-normal continuous variables in unpaired groups. A common approach called pairwise deletion was applied to handle missing data. Statistical analyses were conducted using SPSS 16.0. Differences were considered significant at $p<0.05$.

AUTHOR CONTRIBUTIONS

ZZ, MZ, and YW collected data, performed statistical analyses and drafted the manuscript; YH and CC offered technical and clinical support; KH and FZ performed data acquisition; XX, JL, and YT performed statistical analyses and data interpretation; QY and DC did the biomarker measurements; EDC contributed to the critical revision of the manuscript; GL, GG, and YX supervised the study, obtained funding, and revised the manuscript. All authors contributed to the final article.

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CONFLICTS OF INTEREST

The authors disclose no conflicts.

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SUPPLEMENTARY MATERIALS

Supplementary Table

Supplementary Table 1. Baseline features of laboratory biomarkers in older and younger patients.

Biomarkers	Group 1 (G1) Younger patients Hospital stay < 21 d (N=122)	Group 2 (G2) Younger patients Hospital stay ≥21 d (N=59)	G1 vs G2 p-value	Group 3 (G3) Older patients Hospital stay < 21d (N=35)	Group 4 (G4) Older patients Hospital stay ≥21d (N=23)	G3 vs G4 p-value	G1 vs G3 p-value	G1 vs G4 p-value	G2 vs G3 p-value	G2 vs G4 p-value
White blood cells	4.67(3.49-5.90)	4.53(3.41-5.61)	0.299	4.94(3.81-5.53)	4.10(3.12-5.03)	0.129	0.871	0.123	0.356	0.366
Lymphocytes	1.35(0.93-1.79)	1.15(0.84-1.61)	0.094	1.05(0.86-1.21)	0.74(0.63-1.14)	0.133	**0.002**	**<0.001**	**0.045**	**0.003**
Neutrophils	2.85(2.05-3.66)	2.90(2.05-3.50)	0.675	3.08(2.52-3.85)	2.74(2.00-3.26)	0.215	0.131	0.764	0.081	0.918
Eosinophils	0.02(0.01-0.07)	0.01(0.00-0.04)	0.053	0.01(0.00-0.03)	0.01(0.00-0.03)	0.150	0.058	0.002	0.981	0.097
Hemoglobin	132(120-142)	134 (124-145)	0.291	120 (115 -132)	125 (118-143)	0.224	0.001	0.428	<0.001	0.141
Platelet	176(144-227)	179 (152-234)	0.507	150 (134-213)	138(112-187)	0.192	0.172	0.011	0.075	0.006
D-dimer	0.21(0.12-0.52)	0.28(0.16-0.45)	0.393	0.38(0.18-0.85)	0.36(0.16-0.57)	0.338	0.009	0.257	0.053	0.535
C-reactive protein	10.30(4.14-20.29)	13.05(3.29-25.14)	0.443	24.90(14.97-44.20)	43.30(20.23-62.12)	0.141	**0.001**	**<0.001**	**<0.001**	**<0.001**
Alanine aminotransferase	19.45(13.75-27.21)	19.91(15.12-28.31)	0.666	19.70(14.86-28.00)	16.29(13.76-20.82)	0.117	0.669	0.148	0.941	0.093
Aspartate aminotransferase	23.23(18.87-30.13)	23.74(19.11-28.53)	0.712	27.50(22.87-34.71)	28.29(24.78-38.67)	0.431	**0.005**	**0.005**	**0.042**	**0.022**
Total bilirubin	10.92(8.05-15.32)	10.78(7.99-16.86)	0.605	10.72(8.89-17.37)	10.87(9.42-14.20)	0.691	0.483	0.963	0.639	0.84
Albumin	38.86(36.26-41.40)	39.41(36.17-42.17)	0.637	35.46(32.69-38.22)	36.0(31.22-38.3)	0.886	**<0.001**	**0.003**	**<0.001**	**0.003**
Albumin/globulin	1.52(1.39-1.79)	1.55(1.39-1.74)	0.951	1.34(1.21-1.57)	1.30(1.24-1.41)	0.499	**0.001**	**<0.001**	**0.002**	**<0.001**
Blood urea nitrogen	4.01(3.12-4.83)	4.29(3.20-5.05)	0.273	4.47(3.50-5.12)	5.23(4.14-7.07)	**0.037**	0.133	<0.001	0.647	0.007
Creatinine	48.81(38.43-58.91)	53.13(41.93-64.33)	0.092	51.45(43.11-63.08)	57.0(44.04-66.07)	0.413	0.320	0.105	0.719	0.512
Lactic acid	768.35(397-845)	742(387-789)	0.373	798(636-830)	769(742-841)	1.000	0.887	0.541	0.516	0.416

Research Paper

ADL-dependency, D-Dimers, LDH and absence of anticoagulation are independently associated with one-month mortality in older inpatients with Covid-19

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ABSTRACT

Background: To assess factors associated with one-month mortality among older inpatients with Covid-19.

Results: The mean age was 78 ± 7.8 years, 55.5% were men, CT scan lung damage was observed in 76% of the patients (mild 23%, moderate 38%, extensive 22%, and severe 7%). The mortality rate was 26%. Dependency/Activities of Daily Living (ADL) score ≤ 5/6, D-Dimers, LDH, and no anticoagulation by reference for curative were independently associated with one-month mortality. A score derived from the multivariate model showed good calibration and very good discrimination (Harrell's C index [95%CI] = 0.83 [0.79-0.87]).

Conclusion: ADL-dependency, high serum levels of D-Dimers and LDH and the absence of anticoagulation were independently associated with one-month mortality among older inpatients with Covid-19.

Methods: 108 consecutive older inpatients aged 65 and over with Covid-19 confirmed by RT-PCR and/or typical CT chest scan were prospectively included in a French single-centre cohort study from March to April 2020. A systematic geriatric assessment was performed. Covariates were lymphocyte count, serum levels of albumin, C-Reactive Protein, D-Dimers and Lactate Dehydrogenase (LDH), anticoagulation level, and exposure to the hydroxychloroquine and azithromycin combined therapy. Cox uni- and multivariate proportional-hazard regressions were performed to identify predictors of one-month mortality.

INTRODUCTION

Since the end of 2019, the SARS-Cov-2 pandemic (named Covid-19) exposes older patients to the risk of early death [1–3]. As with other diseases, chronological age should not be the only element in the therapeutic decision.

To date, factors associated with short-term mortality among older inpatients with Covid-19 have not been characterized. Given the heterogeneity of the older inpatient population, these factors are needed to avoid under- and over-treatment, particularly intensive care.

We used the Geriatric Assessment (GA) to try to identify predictive factors associated with one-month mortality among older inpatients with Covid-19 [4].

RESULTS

Patients

The Covid-19 outbreak has been particularly severe in Paris and its suburbs since February 2020. The university hospital of Paris-Seine Saint Denis (Avicenne hospital) set aside nearly 200 hospital beds for Covid-19 patients. In this health emergency, a Geriatric Assessment (GA) was systematically performed for older inpatients with Covid-19 to help clinical teams in their therapeutic strategy. This prospective observational cohort study consecutively included all older (65 and over) inpatients with a Covid-19 diagnosis. The diagnosis of Covid-19 was based on a positive SARS-Cov-2 RT-PCR test on a nasopharyngeal sample [5] and/or on a typical CT chest scan [6]. Informed consent was obtained from the patients before inclusion in accordance to national ethical rules.

Three hundred and twenty-five new consecutively admitted patients for a confirmed Covid-19 infection were recorded between 03/28/2020 and 04/13/2020, of whom 120 (37%) concerned individuals 65 years of age or older. We assessed 108 (90%) of them.

Baseline characteristics of the patients

Among the 108 Covid-19 patients studied, RT-PCR testing for SARS-Cov-2 was positive for 85% of the patients (n=92/108), and CT scan was available for 84% (n=91/108). On CT scans, there was no lung disease for 10% (n=9/91), mild damage for 23% (n=21/91), moderate damage for 38% (n=35/91), extensive damage for 22% (n=20/91), and severe damage for 7% (n=6/91).

The mean age was 78.4 ± 7.8 years (min-max: 66-95), and 55.5% were men. The geriatric domains impaired concerned ranged from 16% (BMI < 21 kg/m^2) to 87% (muscle weakness). Median serum levels of D-Dimers and LDH were 1308.5 ng/mL and 341.5 UI/L respectively. 93/108 patients had an anticoagulation either curative (30%) or preventive (56%). 27/108 of the patients received the combination of hydroxychloroquine and azithromycin for 1 to 9 days (Table 1).

Univariate and multivariate factors associated with one-month inpatient mortality

All patients were followed up without loss until discharge from acute care unit. The median follow-up time was 10 days (IQR = 15) (min-max: 0-37). 7 patients (6.5%) were admitted to intensive care and three died. On 05/02/2020, the inpatient mortality rate was 26% (n=28/108).

In univariate analyses, age (per one IQR of more), comorbidities (total CIRSG ≥ 11), dependency (ADL ≤ 5/6 and IADL ≤ 3/4), D-Dimers (per one IQR of more) were significantly associated with one-month inpatient mortality. None of the following were associated with one-month inpatient mortality: gender, CT chest scan damage, malnutrition (BMI < 21 kg/m^2 or weight loss ≥ 5%), muscle weakness, depressed mood (mini GDS ≥ 1/4), serum levels of albumin, age-adjusted D-Dimers, CRP and LDH, absolute lymphocyte cell count, anticoagulant therapy, and hydroxychloroquine and azithromycin combined therapy (Table 1).

In multivariate analyses, comorbidities (total CIRSG ≥ 11) were not anymore associated with mortality. Only ADL-dependency (aHR = 4.33 [1.39-13.5], P = 0.01), D-Dimers per one IQR of more (aHR = 1.00 [1.00-1.00], P = 0.0008), LDH per one IQR of more (aHR = 1.00 [1.00-1.00], P = 0.03), and no anticoagulation by reference for curative (aHR = 4.20 [1.36-12.9], P = 0.02) were significantly associated with one-month inpatient mortality (Table 1). There was no significant interaction between predictors (P for interaction ≥ 0.05).

Derivation score for one-month inpatient mortality

The derivation score ranged from 3 to 63 with a median score of 10 (IQR = 5). Two groups were identified: 58 patients (54%) were at low risk (3 to 10), and 50 (46%) at high risk (score > 10). Overall, the score was well calibrated (P = 0.24), and discrimination was very good with a Harrell's C index of 0.83 (0.79-0.87). The Kaplan-Meier plot showed significant discrimination (P = 0.0004) across the two risk groups. In particular, the one-month inpatient risk of mortality was 9.1% (low risk), and 85.5% (high risk) respectively (Figure 1). For internal validation, using a bootstrapping method with 1000 resamples, the Harrell's C index was 0.81

Table 1. Baseline characteristics of 108 older inpatients with Covid-19, uni- and multivariate factors associated with one-month mortality.

Variables	Whole cohort N = 108 (%)	Univariate analysis		Multivariate analysis		
		HR [95%CI]	*P**	aHR [95%CI]	*P**	Scoring
Age (y), median (IQR)	78 (13)	1.05 [1.00-1.10]	**0.03**	-		
Gender (male)	60 (55.5)	1.45 [0.67-3.11]	0.34			
Comorbidities						
Total CIRSG ≥ 11	57 (53)	2.38 [1.01-5.62]	**0.04**	-		
Hypertension	77 (71)	1.34 [0.54-3.32]	0.52			
Diabetes	30 (28)	1.57 [0.72-3.44]	0.25	-		
Dependency						
ADL ≤ 5/6	54 (50)	6.65 [2.30-19.2]	**0.0004**	4.33 [1.39-13.5]	**0.01**	4
IADL ≤ 3/4	68 (63)	7.93 [1.88-33.4]	**0.004**	-		
Nutrition						
BMI < 21 kg/m2	17 (16)	0.89 [0.34-2.35]	0.81			
Weight loss ≥ 5% (yes)	49 (45)	1.38 [0.65-2.93]	0.40			
Mobility						
Muscle weakness (yes)	94 (87)	4.92 [0.66-36.6]	0.12	4.44 [0.57-34.5]	0.15	4
Depressed mood						
Mini GDS ≥ 1/4	65 (60)	2.27 [0.95-5.41]	0.06	2.30 [0.81-6.49]	0.11	2
Covariates (median, IQR)						
Albumin level (g/L)	27 (7.0)	0.94 [0.87-1.02]	0.12	-		
CRP level (mg/L)	85.5 (110.5)	1.00 [0.99-1.01]	0.23	-		
Lymphocyte count	955 (650.0)	1.00 [0.99-1.00]	0.89			
D-dimers (ng/mL)	1308.5 (1405.0)	1.00 [1.00-1.01]	**0.02**	1.00 [1.00-1.00]	**0.0008**	1
LDH (IU/L)	341.5 (195.5)	1.00 [0.99-1.00]	0.08	1.00 [1.00-1.00]	**0.03**	1
Intensive cares (yes)	7 (6.5)	1.02 [0.30-3.47]	0.97			
Converting enzyme inhibitors (yes)	42 (39)	1.18 [0.55-2.52]	0.67			
Anticoagulation			0.12		**0.02**	
Curative	32 (30)	1 (reference)		1 (reference)		0
Preventive	61 (56)	1.45 [0.55-3.78]		1.20 [0.43-3.31]		1
None	15 (14)	2.91 [1.00-8.47]		4.20 [1.36-12.9]		4
Hydroxychloroquine + azithromycin (yes)	27 (25)	0.49 [0.19-1.29]	0.15	-		

* Log rank test; Bold: significant *P* value at the threshold of 5%; IQR: Inter Quartile Range; HR: Hazard Ratio; aHR: adjusted HR; Continuous variables are expressed by one IQR of more.
CIRSG: Cumulative Illness Rating Scale Geriatric; ADL: Activity of Daily Living; IADL: Instrumental-ADL; BMI: Body Mass Index; Mini-GDS: Mini Geriatric Depression Scale; CRP: C-Reactive Protein; LDH: Lactate Dehydrogenase.

[0.75-0.88], close to the original C index. Overall, we showed that our prognostic score is reliable to predict short-term mortality in older inpatients with Covid-19.

DISCUSSION

This is the first report on a prospective observational cohort study of older inpatients with Covid-19 that specifically assessed geriatric conditions and factors associated with one-month mortality. We found that ADL-dependency before hospitalization, serum levels of D-Dimers and LDH, and the absence of anticoagulation were the factors independently associated with one-month mortality in older inpatients with Covid-19.

To overcome the heterogeneity of older inpatients with Covid-19 in terms of comorbidities, dependency, nutrition, mobility and mood, we used the Geriatric Assessment to detect vulnerabilities. In this frail population where half of the patients had significant

comorbidities and two-thirds had pre-admission dependency, the mortality rate of 26% is closed to the 34.5% mortality rate reported for 55 Chinese patients from Wuhan over 65 years [7]. In our study, we identified one clinical factor independently associated with one-month mortality: ADL-dependency (≤ 5/6) which is a typical complication of frailty among older adults [8]. We also identified two biological factors independently associated with one-month mortality, high serum levels of D-Dimers and LDH, previously reported as risk factors in younger patients [9, 10].

Strikingly, curative anticoagulation was strongly and independently associated with decreased risk of one-month mortality. Over-incidence of thromboembolism events in Covid-19 patients has been reported [11], and this protective effect of anticoagulation with high serum levels of D-Dimers suggest associated vascular impairment and possible direct effect of SARS-Cov-2 on normal endothelial cells [12].

From these four variables combined with depressed mood (i.e. mini-GDS) and muscle weakness, we derived a score to predict inpatient mortality with good calibration and very good discrimination. Other scores have been proposed to predict the risk of progression, but not for older inpatients [13]. This is a major strength of our study. Thus, for a patient over 65 years with pre-admission ADL-dependency, muscle weakness and depressed mood, and high serum levels of D-Dimers, the risk of short-term mortality is very high (85%), and should lead to cautious routing to intensive care. In contrast, a patient with no ADL-dependency and no depressed mood, and thus a very low risk of one-month mortality, should be actively transferred to intensive care unit if his/her respiratory condition requires it, regardless of his/her chronological age. Among older patients with Covid-19, as with most diseases [14], chronological age should not be the only factor considered for therapeutic decision, to avoid under- or over-treatment.

In addition, our original score includes thromboembolic-related risk of death and could help to choose the appropriate level of anticoagulation. Let us consider the real case of a 74-year-old woman hospitalized after 8 days of symptoms, with a muscle weakness, D-Dimers at 2950 ng/mL and LDH at 290 UI/L. Thus, the score is 8 (low risk). However, in the absence of anticoagulation,

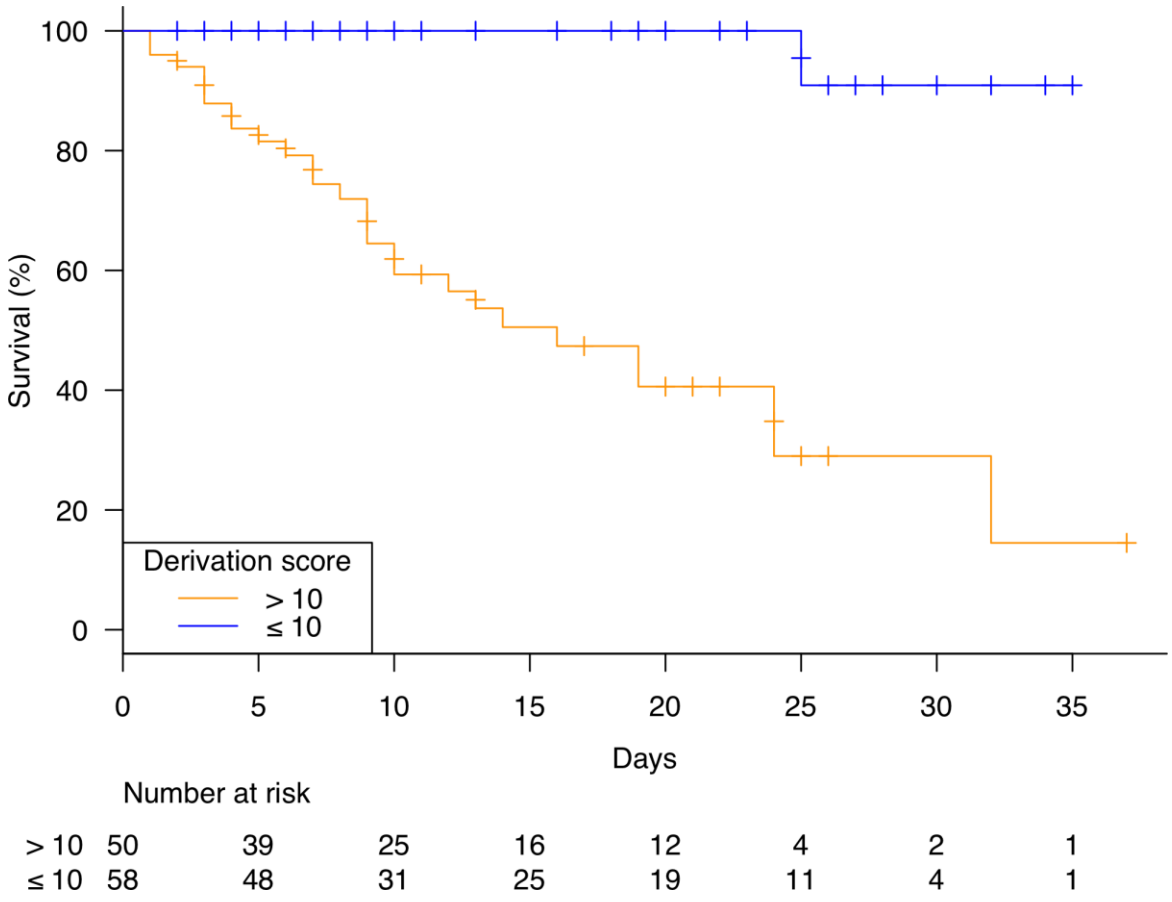

Figure 1. Kaplan-Meier survival curves for short-term inpatients mortality according to derivation score.

the score is 12 with a high risk of mortality. For this reason, she should be offered at least preventive anticoagulation.

The limitations of our study are the one-single center recruitment with the limited number of patients, and the absence of external validation. This is counterbalanced by a rigorous methodology and high prognostic performances of our scoring system to predict short-term mortality in older inpatients with Covid-19. Our results are also of particular importance in identifying the most at-risk older patients and protecting them as well as possible from the second wave, once confinement measures are lifted. In the latter case, a further validation of our study results will be required.

CONCLUSIONS

ADL-dependency, high serum levels of D-Dimers and LDH and the absence of anticoagulation were independently associated with one-month mortality among older inpatients with Covid-19. A simple derivation score was developed to help clinicians in their daily therapeutic strategy.

MATERIALS AND METHODS

Demographic and disease characteristics

Demographic data (age, gender), and severity of the Covid-19 based on CT chest scan for lung damage extent (none 0%, mild < 10%, moderate 10-25%, extensive 25-50%, or severe > 50%) were collected at the first GA [6].

The geriatric assessment (GA)

The GA was performed by two clinicians (GB and FP) and included five domains. The GA is easily performed even in this context of acute care and only takes a few additional ten minutes. Comorbidities were assessed using the Cumulative Illness Rating Scale for Geriatrics (CIRS(G)) which covers all diseases including hypertension, cardiovascular diseases, diabetes, chronic bronchitis, and their long-term complications (Supplementary Table 1) [15]. Impairment was defined as a total CIRS(G) score above the median of 11. Dependency before hospitalization was defined from a six-item activities of daily living (ADL) score of 5 out of 6 or less, and from a four-item simplified instrumental ADL score (IADL, using the telephone, transport, medications, and money management) of under 4 [16, 17] (Supplementary Table 2). Malnutrition was defined as a body mass index (BMI) under 21 kg/m^2 or unintentional weight loss in the previous year ≥ 5% [18, 19]. Depressed mood was defined from a Mini-Geriatric Depression Scale score of 1 or more out of 4 (Supplementary Table 3) [20]. Impaired mobility was defined by the presence of muscle weakness (MW) assessed from hand-grip strength. Maximum handgrip strength (in kg) was measured twice for each hand using a hand-held dynamometer (model EH101; Zhongshan Camry Electronic Co., Ltd, Guangdong, China). MW was defined by thresholds adjusted for gender and BMI derived from the frailty phenotype established by Fried et al. [19].

Covariates

At the time of diagnosis, we collected total lymphocyte count, serum levels of albumin (g/L), C-reactive protein (mg/L), D-Dimers (ng/mL), and Lactate Dehydrogenase (LDH, UI/L). These covariates were expressed as continuous variables. We also tested D-Dimers serum level as an age-adjusted categorical variable according to National consensus (i.e. abnormal D-Dimers ≥ age x 10) [21]. Anticoagulation was classified as follows: curative, preventive or none. Exposure to converting enzyme inhibitors was noted. Exposure to the hydroxychloroquine and azithromycin combined therapy was noted to assess the predictive value for the risk of death with this treatment [22].

Outcome

Data was collected from 03/28/2020 to 04/13/2020. On 05/02/2020, inpatient mortality following the diagnosis of Covid-19 until discharge from acute care unit was determined. Vital status was obtained from medical records.

Statistical analyses

Categorical data were expressed as numbers and proportions, and continuous data as means and standard deviation (SD) or medians and interquartile range (IQR).

Comparisons of baseline characteristics between survivors and non-survivors were performed using the log-rank test. A Cox uni- and multivariate proportional-hazard regression model was run to assess factors associated with one-month mortality. Model assumptions were verified. Variables yielding P values ≤ 0.25 in the univariate analysis were considered for inclusion in the multivariate analysis using a backward procedure according to the lowest Akaïke Information Criteria. Continuous variables were expressed per one IQR of more. We then assessed interaction terms between predictors. A derivation score for each predictor was created using Hazard Ratio point-based scoring system [23]. We categorized this score by the median. The calibration of the derivation score was assessed by using the Grönnesby and Borgan test. A P value ≥ 0.05 was

considered to indicate good calibration. Discrimination by the derivation score was assessed using Harrell's C index with 95%CI. Survival curves were plotted according to the Kaplan-Meier method with the derivation score divided by median. Internal validation was performed with the bootstrap-adjusted Harrell's C index with 1000 resamples as recommended by the TRIPOD guidelines [24].

All tests were two-sided, and the threshold for statistical significance was set at P <0.05. The data was analysed using R statistical software (version 4.0.0, R Foundation for Statistical Computing, Vienna, Austria; http://www.rproject.org).

AUTHOR CONTRIBUTIONS

Conception and design: GB, GF, OB, FP Acquisition, analysis, or interpretation of data: All authors Drafting the work: GB, GF, FP Final approval: All authors Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: All authors

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Supplementary Table 1. The Cumulative Illness Rating Scale for Geriatrics (CIRS(G)) [15].

Disease	Severity				
	0	1	2	3	4
Heart					
Vascular (including hypertension)					
Hematopoietic					
Respiratory					
Eyes, ears, nose, throat, and larynx					
Upper Gastrointestinal					
Lower Gastrointestinal					
Liver, pancreas, and biliary					
Renal					
Genitourinary					
Musculoskeletal and skin					
Neurologic					
Endocrine and breast					
Psychiatric illness					
Total (0-56)					

Supplementary Table 2. Score for Activities of Daily Living (ADL) [16].

Questions	Points		
	Does alone 1	Does with help 0.5	Cannot do alone 0
Washing			
Getting dressed			
Moving about indoors			
Going to the toilet			
Eating			
Continence			

Scoring:
Scores range from 0 to 6.
Total score ≤ 5/6 indicates ADL-dependency.

Supplementary Table 3. The mini Geriatric Depression Scale (mini-GDS) [20].

Questions	Points	
	Yes	No
Do you feel discouraged and sad?	1	0
Do you feel your life is empty?	1	0
Are you happy most of the time?	0	1
Do you feel hopeless?	1	0

Scoring:
Score ranges from 0 to 4.
Total score ≥ 1/4 indicates depressed mood.