



Therapeutic application of estrogen for COVID-19: Attenuation of SARS-CoV-2 spike protein and IL-6 stimulated, ACE2-dependent NOX2 activation, ROS production and MCP-1 upregulation in endothelial cells

Ji Youn Youn¹, Yixuan Zhang¹, Yusi Wu, Maxime Cannesson, Hua Cai^{*}

Division of Molecular Medicine, Department of Anesthesiology and Perioperative Medicine, Division of Cardiology, Department of Medicine, David Geffen School of Medicine at University of California Los Angeles, USA

ARTICLE INFO

Keywords:

COVID-19
Estrogen
IL-6
SARS CoV-2 spike protein
Endothelial cells
Oxidative stress
NOX2
ACE2
MCP-1

ABSTRACT

The outbreak of COVID-19 has remained uncontained with urgent need for robust therapeutics. We have previously reported sex difference of COVID-19 for the first time indicating male predisposition. Males are more susceptible than females, and more often to develop into severe cases with higher mortality. This predisposition is potentially linked to higher prevalence of cigarette smoking. Nonetheless, we found for the first time that cigarette smoking extract (CSE) had no effect on angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) expression in endothelial cells. The otherwise observed worse outcomes in smokers is likely linked to baseline respiratory diseases associated with chronic smoking. Instead, we hypothesized that estrogen mediated protection might underlie lower morbidity, severity and mortality of COVID-19 in females. Of note, endothelial inflammation and barrier dysfunction are major mediators of disease progression, and development of acute respiratory distress syndrome (ARDS) and multi-organ failure in patients with COVID-19. Therefore, we investigated potential protective effects of estrogen on endothelial cells against oxidative stress induced by interleukin-6 (IL-6) and SARS-CoV-2 spike protein (S protein). Indeed, 17 β -estradiol completely reversed S protein-induced selective activation of NADPH oxidase isoform 2 (NOX2) and reactive oxygen species (ROS) production that are ACE2-dependent, as well as ACE2 upregulation and induction of pro-inflammatory gene monocyte chemoattractant protein-1 (MCP-1) in endothelial cells to effectively attenuate endothelial dysfunction. Effects of IL-6 on activating NOX2-dependent ROS production and upregulation of MCP-1 were also completely attenuated by 17 β -estradiol. Of note, co-treatment with CSE had no additional effects on S protein stimulated endothelial oxidative stress, confirming that current smoking status is likely unrelated to more severe disease in chronic smokers. These data indicate that estrogen can serve as a novel therapy for patients with COVID-19 via inhibition of initial viral responses and attenuation of cytokine storm induced endothelial dysfunction, to substantially alleviate morbidity, severity and mortality of the disease, especially in men and post-menopause women. Short-term administration of estrogen can therefore be readily applied to the clinical management of COVID-19 as a robust therapeutic option.

1. Introduction

The outbreak of the coronavirus disease 2019 (COVID-19) has turned into a global pandemic of more than 197 million cases and 4.2 million deaths (as of Aug 3rd, 2021) [1]. The uncontained outbreak remains challenging to manage with new strains/mutants of the virus emerging constantly. Currently, four variants of alpha (B.1.1.7) [2–4], beta (B.1.351) [4,5], gamma (P1, B.1.1.28.1) [5] and delta (B.1.617) [6],

first identified in United Kingdom, South Africa, Brazil, and India respectively, have been classified as variants of concern (VOC) due to their high transmissibility and potentially more challenging features for disease management [1,7]. Intensive research efforts have been devoted to understanding molecular mechanisms of viral infection and disease pathogenesis since the outbreak to result in many breakthroughs including very rapid identification of the viral strains and their RNA sequences, identification of viral receptor ACE2 and its partner of

^{*} Corresponding author. David Geffen School of Medicine, University of California, Los Angeles, USA.

E-mail address: hcai@mednet.ucla.edu (H. Cai).

¹ The authors contribute equally to this work.

<https://doi.org/10.1016/j.redox.2021.102099>

Received 24 May 2021; Received in revised form 10 August 2021; Accepted 11 August 2021

Available online 17 August 2021

2213-2317/© 2021 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

transmembrane protease serine 2 (TMPRSS2, primes S protein for host cell entry of SARS-CoV-2) for entry into host cells [8], development of multidimensional diagnostic methodologies and applications, and remarkable progress of vaccine development. Nonetheless, the molecular mechanisms underlying detailed pathological processes of COVID-19 have remained far from being fully understood.

We have previously reported sex difference of COVID-19 for the first time indicating male predisposition [9]. Epidemiological studies have shown that males are more susceptible to COVID-19 than females [9–12]. In a report of 1,099 COVID-19 cases from 552 hospitals in 30 provinces in China, 58.1% of the patients were men [11]. In another study, 62% (119 out of 191) of patients examined between December 29th, 2019 and January 31st, 2020 in China were men [12]. In addition, more males (60.6%, 238 out of 393) than females were admitted to hospital due to COVID-19 in New York City in March 2020 [10]. Of note, male gender was found to be independently associated with increased in-hospital mortality, oxygenation requirements, and outcome of intubation in a study of 200 COVID-19 patients admitted to the Montefiore Medical Center in Bronx, New York [13]. A systematic review of 32 studies enrolling 69,093 ICU patients indicates that 59% of the ICU patients were men, and that 76% of the patients with acute respiratory distress syndrome (ARDS) were men [14]. From a meta-analysis of 3, 111,714 patients, males have significantly higher risk than females for ICU admission (odds ratio 2.84, 95% CI 2.06–3.92) and higher mortality rate (odds ratio 1.39, 95% CI 1.31–1.47) [15]. In UK, the incidence of COVID-19 related death was 1.59-fold in males compared to that in females (males: 6,162 out of 8,630,403 patients; females: 4,764 out of 8, 647,989 patients; hazard ratio 1.59, 95% CI 1.53–1.65) [16]. These data clearly show that men are predisposing to be infected with SARS-CoV-2, and to develop into more severe cases with higher mortality.

The gender difference observed in COVID-19 patients is potentially linked to higher prevalence of cigarette smoking in men, particularly in China where 49.8% of men smoke (vs. 2.2% in women) [9,17]. Nonetheless, the difference in gender related prevalence in cigarette smoking is much smaller in other territories where COVID-19 is more prominent in men. In Italy, 26.0% of men vs. 17.2% women smoke [18]. In US, 15.6% of men are current smokers, vs. 12.0% in women, according to the 2018 National Health Interview Survey (NHIS) [19]. Therefore, the mechanistic impacts of cigarette smoking on COVID-19 remain to be fully understood. The potential regulation of ACE2 (angiotensin converting enzyme 2, the membrane receptor required for the entry into host cells by SARS-CoV-2) by cigarette smoking has remained controversial. Leung et al. reported that ACE2 is upregulated in lung tissues of smokers, especially in small airway epithelia [20]. However, a recent study of primarily cultured airway basal stem cells (ABSCs) isolated from human nonsmokers indicated no change in ACE2 expression after exposure to cigarette smoke [21]. No alteration of ACE2 expression was reported in large airway epithelia isolated from smokers, when compared to that from nonsmokers [22]. We therefore examined regulation of ACE2 and TMPRSS2 by cigarette smoking extract (CSE) in endothelial cells, in view of the central roles of endothelial infection and dysfunction in the pathogenesis of acute respiratory distress syndrome (ARDS) and multi-organ failure during COVID-19, and in mediating the responses to cytokine storm. We report for the first time that protein levels of ACE2 and TMPRSS2 were not altered in endothelial cells exposed to CSE, indicating that current smoking status might not contribute to more prevalent and severe disease of COVID-19 in males, who tend to smoke more than women in different ethnic groups and regional territories. On the other hand, it is most likely that chronic smokers develop into severe cases because of baseline respiratory diseases induced by chronic smoking, which make patients more prone to injuries following SARS-CoV-2 infection.

Endothelial cells are rich in the lung and endothelial barrier dysfunction is a hallmark of acute lung injury (ALI)/ARDS, which is a major pathological feature of COVID-19. Indeed, endotheliitis

(endothelial inflammation) has been observed in patients with COVID-19 [23,24]. Autopsy analyses of lung tissues from COVID-19 patients indicated severe endothelial injury and disrupted endothelial cell membranes [24]. Endothelial cell damage also contributes to other systematic organ failures such as that of heart and kidney [23]. Exposure of endothelial cells to inflammatory cytokines is one of the major mechanisms of endothelial cell injury. Of note, cytokine storm-induced multi-organ failure leads to mortality in COVID-19 patients [25]. Circulating levels of interleukin-6 (IL-6) are significantly elevated in patients with COVID-19 [12,26,27]. The levels of serum IL-6 were found increased in asymptomatic patients as well, although patients with moderate and severe symptoms had much higher elevation in IL-6 levels [28]. A recent meta-analysis indicates that elevated circulating levels of IL-6 correlate well with increased mortality of COVID-19 patients [29]. In a phase III clinical trial, a humanized monoclonal antibody against IL-6 receptor, tocilizumab (Actemra/RoActemra, Roche), has been shown to reduce the need for mechanical ventilation in COVID-19 patients with associated pneumonia [30]. We therefore hypothesized that perhaps less severe disease of COVID-19 in females is related to estrogen-dependent protection against endothelial cell injury induced by cytokine storm.

SARS-CoV-2 Spike protein (S protein) is a homotrimer, with each monomer composed of two functional subunits, S1 and S2. The receptor-binding domain (RBD) situated in S1 subunit binds to ACE2 on the cell membranes. S protein functions as an attachment to the host cell receptor to facilitate viral binding to the host cell receptor, as well as fusion of the virus with cellular membranes for host cell entry [31,32]. Recent studies have shown that incubation with recombination proteins of S1, S2, or RBD domain disrupted tight junctions in brain endothelial cells, thus inducing endothelial dysfunction [33]. Exposure of brain endothelial cells to the S protein resulted in regulation of 83 genes including C3, CD163, CCL8, CXCL8, and CCL24, all of which are known to play roles in inflammation and chemokine signaling [34]. In parallel to studies of IL-6 treatment of endothelial cells, we investigated whether and how exposure of endothelial cells to S protein also triggers reactive oxygen species (ROS) production through activation of NADPH oxidase, ACE2 upregulation, and induction of pro-inflammatory gene MCP-1, and whether these responses can be attenuated by estrogen.

NADPH oxidases (NOXs) represent major sources of ROS production during cardiovascular pathogenesis [35]. Activation of NOX family oxidases induces endothelial dysfunction, while augmenting inflammation to further deteriorate endothelial cell barrier dysfunction/injury. Therefore, we hypothesized that IL-6 or S protein induces oxidative stress via activation of selective isoform(s) of NOX in endothelial cells, which may be alleviated by estrogen. This might represent one of the most important mechanisms underlying the protection against pathogenesis and severe symptoms of COVID-19 in females. Indeed, we found that NOX2 mediates IL-6 induced ROS production in endothelial cells, which was completely abrogated by estrogen administration. Importantly, we found that exposure of endothelial cells to S protein resulted in marked upregulation in NOX2 dependent ROS production, indicating that NOX2-dependent ROS production triggered by S protein represents a direct effect on endothelial cells following SARS-CoV-2 infection. Furthermore, estrogen treatment reversed ACE2 mediated upregulation of NOX2 and ROS production in response to S protein, as well as MCP-1 induction, implicating therapeutic effects of estrogen on endothelial dysfunction and vascular inflammation. S protein induced upregulation of ACE2 was also attenuated by estrogen treatment. Taken together, NOX2 activation-mediated oxidative stress in endothelial cells in response to SARS-CoV-2 infection and cytokine storm might represent one of the most important mechanisms of endothelial cell injury during COVID-19, which in turn drives progression of the disease, ARDS/multi-organ failure, and mortality. Estrogen administration, via attenuation of NOX2-dependent oxidative stress in endothelial cells, can therefore serve as a robust therapeutic option for COVID-19 to effectively reduce disease severity and mortality, especially in men and

post-menopause women at both early stage of viral infection and later stage of cytokine storm mediated systematic injuries.

2. Materials and methods

2.1. Cell treatment and siRNA transfection

Primary bovine aortic endothelial cells (BAECs, Genlantis, passages 4 to 6) were cultured in M199 supplemented with 10% fetal bovine serum (FBS), 1% vitamin, and 1% L-glutamine as previously described [36–39]. Primary human aortic endothelial cells (HAECs, Lonza, passages 4 to 6) were cultured in EGM-2 media supplemented with 10% fetal bovine serum and EGM-2 SingleQuots (#CC-4176, Lonza) as previously described [40]. Confluent BAECs were starved overnight in M199 containing 5% FBS, and treated with different concentrations of cigarette smoke extract (CSE, 0, 2.5%, 5%, 10%), recombinant human interleukin-6 (IL-6, 100 ng/mL, #206-IL-010, R&D), or recombinant SARS-CoV-2 Spike protein (S protein, 500 ng/mL, #10549-CV-100, R&D) for 24 h before harvested for analyses. In the experiment when estrogen was applied, cells were pre-treated with IL-6 (100 ng/mL) or S Protein (500 ng/mL) for 30 min prior to exposure to various doses of 17 β -estradiol (10 nmol/L to 10 μ mol/L, #E2758, MilliporeSigma) for 24 h.

In additional experiments, BAECs at 80% confluence were transfected with siRNA targeting bovine NOX2 (#sc-270683, Santa Cruz Biotechnology) (200 pmol) or scrambled control siRNA (#sc-37007, Santa Cruz Biotechnology) according to the manual of Lipofectamine RNAiMAX reagent (#13778150, ThermoFisher). Forty-eight hours post-transfection, cells were starved overnight and then treated with IL-6 (100 ng/mL) or S protein (500 ng/mL) for 24 h. To examine specificity of ACE2 antibody, two μ g of pcDNA3.1-hACE2 (#145033, Addgene) or 200 pmol of siRNA targeting ACE2 (customized #CTM-785249, Dharmacon) was used for overexpression of human ACE2 or knockdown of ACE2. For experiments of ACE2 neutralization, 1 μ g/mL of ACE2 antibody (#ab15348, Abcam) or control Rabbit IgG (#N101, Calbiochem) was used to pre-treat endothelial cells for 30 min prior to S protein stimulation.

2.2. Cigarette smoking extract (CSE) preparation

Fresh CSE was prepared by bubbling the smoke generated from one 3R4F reference cigarette (University of Kentucky) (350 mL smoke) into 10 mL M199 according to previous publications [41,42]. The resulted CSE was considered as 100% stock (350 mL smoke/10 mL medium = 35 mL smoke/mL medium) and diluted as required for experimentation after filtering through a 0.22 μ m filter. The concentration of the cigarette smoke in 10% dilution of the CSE stock (3.5 mL smoke/mL medium) is approximately equivalent to the concentration of cigarette smoke in one's circulation for human subjects who consume more than 2 packs of cigarettes per day (2 packs * 20 cigarettes/pack * 350 mL smoke/cigarette/5–7 L blood = 2–2.8 mL smoke/mL blood) [43,44].

2.3. Western blotting and RT-PCR

After indicated treatment protocols, BAECs and HAECs were harvested in lysis buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, 1 mmol/L sodium orthovanadate, 1% Triton X-100, supplemented with protease inhibitor cocktail). The cell lysates were incubated on ice for 20 min before supernatant was separated by centrifugation at 12,000 rpm for 10 min at 4°C. Then the protein concentration was determined by DC protein assay (#5000112, Bio-Rad). Twenty-five to forty μ g proteins were separated in 10% or 15% SDS-PAGE, followed by standard Western blotting protocol by probing with antibodies for ACE2 (1:1000, #ab15348, Abcam), TMPRSS2 (1:1000, #ab92323, Abcam), NOX1 (1:250, #5821, Santa Cruz

Biotechnology), NOX2 (1:250, #611414, BD Biosciences), NOX4 (1:1000, #NB110-58849, Novus), MCP-1 (1:500, #ab9669, Abcam), and β -actin (1:1000, #A2066, MilliporeSigma). The densities of protein bands were quantified by NIH Image J program.

For RT-PCR determination of ACE2, NOX2, and MCP-1 mRNA expression, total RNA was isolated from cultured BAECs using TRIzol (#15596018, Invitrogen) according to the protocol provided by manufacturer. Reverse transcription was performed using iScript reverse transcriptase supermix (#170–8840, Bio-Rad). For real-time PCR amplification, iQ SYBR green supermix (#64102502, Bio-Rad) was used and primer sequences for PCR were summarized in the [Supplementary Table 1](#). PCR amplifications were carried out on a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with the protocol of [(95°C/3 min), (95°C/10 s, 60°C/30 s) \times 39 cycles, melt curve (55°C–95°C, increment 0.5°C), (95°C/30 s)]. The PCR mix contained GAPDH primers to generate GAPDH amplicon that served as an internal control and $\Delta\Delta$ Cq expression was used to calculate the relative expression levels over control.

2.4. Determination of superoxide production by electron spin resonance (ESR)

Superoxide production in BAECs and HAECs was determined by ESR (eScan, Bruker) as we previously published [35–39,45–51]. After indicated treatment protocols, BAECs or HAECs were collected in cold modified Krebs/HEPES (KHB) buffer (99 mmol/L of NaCl, 4.69 mmol/L of KCl, 1.03 mmol/L of KH₂PO₄, 2.50 mmol/L of CaCl₂, 1.20 mmol/L of MgSO₄, 25.0 mmol/L of NaHCO₃, 5.6 mmol/L of glucose, and 20.0 mmol/L of Na-HEPES, pH 7.35). Cell suspension was mixed with superoxide-specific spin trap CMH (1 mmol/L, #ALX-430-117-M250, Enzo Life Sciences) in nitrogen gas bubbled KHB buffer containing diethyldithiocarbamic acid (5 μ mol/L, #D3506, MilliporeSigma) and deferoxamine (25 μ mol/L, #D9533, MilliporeSigma). Then the cell mixture was loaded in glass capillaries and analyzed immediately by ESR. Superoxide signal was measured in the presence or absence of polyethylene glycol-superoxide dismutase (PEG-SOD, 20 U/mL, #S9549, MilliporeSigma). The PEG-SOD inhabitable superoxide signal was calculated and normalized to protein concentrations. The ESR settings used were as follows: center field 3477 or 3480 G; sweep width 9.00 G; microwave frequency 9.79 GHz; microwave power 21.02 mW; modulation amplitude 2.47 G; 512 points of resolution; and receiver gain 1000.

2.5. Statistical analysis

All data are presented as Mean \pm SEM. One-way ANOVA was used to compare the mean among multiple groups with a Newman-Keuls post hoc test. $p < 0.05$ was considered statistically different.

3. Results

3.1. CSE has no effects on endothelial ACE2 and TMPRSS2 expression

We first examined whether exposure of endothelial cells to CSE upregulates expression ACE2 and TMPRSS2. To validate the specificity of ACE2 antibody, Western blot analysis was conducted from cell lysates prepared from ACE2 overexpressed cells using pcDNA3.1-hACE2 plasmid, and cell lysates prepared from ACE2 silenced cells using RNA interference. As a result, ACE2 antibody we used in the present study was able to detect ACE2 protein specifically by picking up bands around 150 kDa for overexpressed ACE2 with C9 tag in pcDNA3.1-hACE2 transfected cells but not in empty vector transfected cells ([Fig. 1A](#)), and showing completely diminished bands in ACE2 siRNA transfected cells but not in control siRNA transfected cells ([Fig. 1B](#)). As shown in [Fig. 1C–D](#), Western blotting results indicate that CSE had no effects on protein abundance of ACE2 and TMPRSS2 in endothelial cells. It has

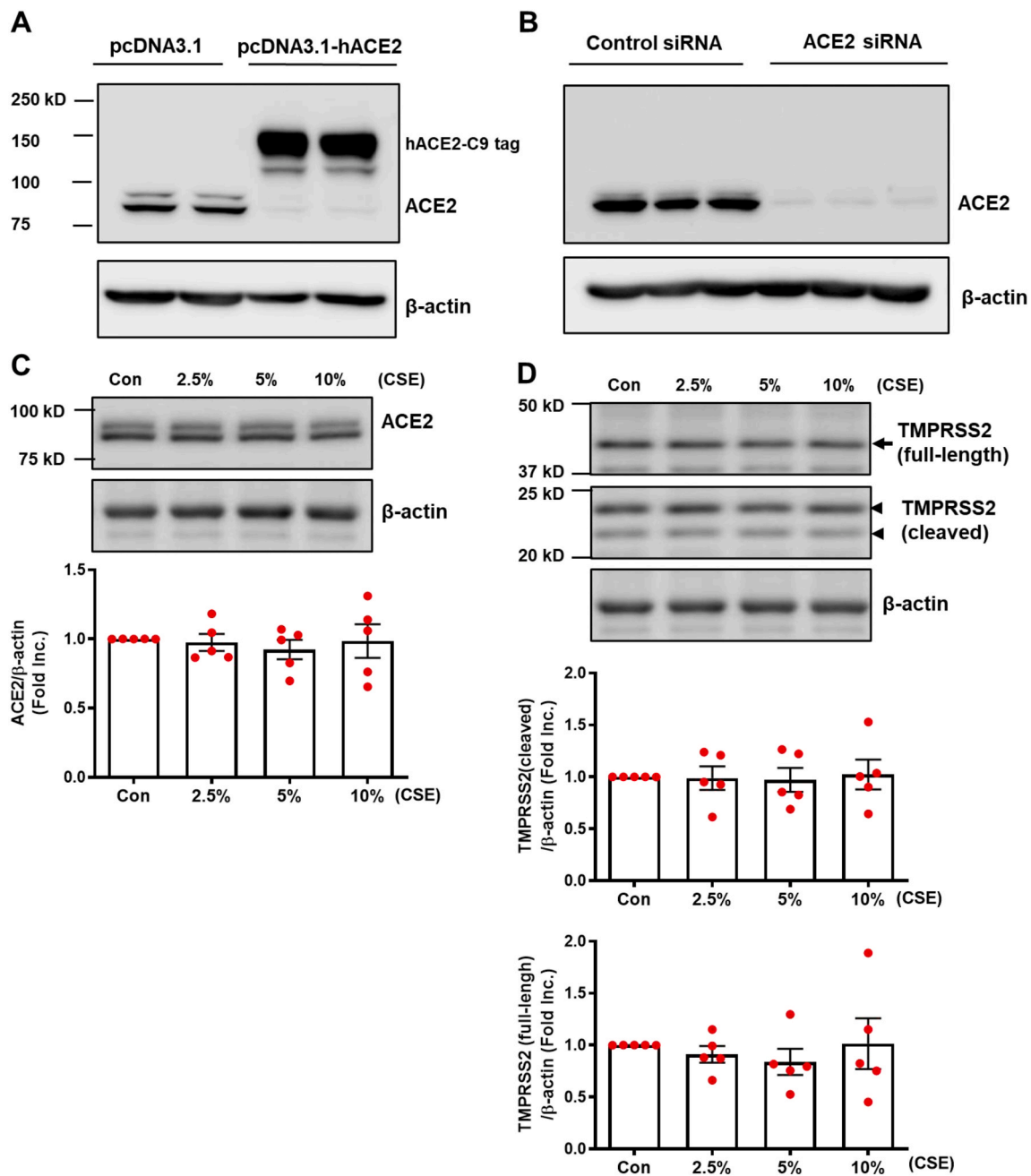


Fig. 1. CSE exposure had no effects on protein expression levels of ACE2 and TMPRSS2 in endothelial cells. Bovine aortic endothelial cells (BAECs) were transfected with empty plasmid or plasmid containing hACE2 for 48 h and cell lysates were subjected to Western blotting analysis of ACE2 protein expression **A**. Overexpression experiments confirmed specificity of ACE2 antibody in detecting ACE2 specifically. The hACE2 insert includes a C9 tag, which is 63 kDa, so bands were detected at around 150 kDa for C9-tagged ACE2 in pcDNA3.1-hACE2 transfected cells, but not in empty vector transfected cells, confirming the specificity of ACE2 antibody which was used subsequently for all experiments in the present study. BAECs were transfected with bovine specific ACE2 siRNA for 48 h and cell lysates were subjected to Western blotting analysis of ACE2. **B**, RNA interference (RNAi) experiments confirmed specificity of ACE2 antibody in detecting ACE2 specifically. In ACE2 siRNA transfected endothelial cells, ACE2 protein bands disappeared between 75 and 100 kDa at the target size of 97 kDa, confirming the specificity of ACE2 antibody which was used subsequently for all experiments in the present study. One day postconfluent BAECs were incubated with CSE at indicated concentrations for 24 h prior to Western blotting analysis of ACE2 protein expression. **C**, Representative Western blots and grouped data indicating that exposure of BAECs to CSE had no effects on ACE2 protein expression. Data are shown as Mean \pm SEM, n = 5. One day post-confluent BAECs were incubated with CSE at indicated concentrations for 24 h prior to Western blotting analysis of TMPRSS2 protein expression. **D**, Representative Western blots and grouped data of TMPRSS2 protein expression indicating that exposure of BAECs to CSE had no effects on TMPRSS2 protein expression. Arrow/arrow heads indicates full-length/cleaved (activated) TMPRSS2 respectively. Data are shown as Mean \pm SEM, n = 5.

been shown that TMPRSS2 undergoes autoactivation to result in self-cleavage. Nonetheless, both full-length (~41 kD) and cleaved (activated) (~22/23 kD) TMPRSS2 were not altered in CSE stimulated endothelial cells (Fig. 1D). Our results seem consistent with recent findings in primary cultured human airway basal stem cells (ABSCs)

from nonsmokers, where CSE failed to change ACE2 expression level [21]. These data indicate that current status of cigarette smoking might not necessarily explain more prevalent and severe disease of COVID-19 in men (or women) who smoke, at least not via regulation of ACE2 and TMPRSS2. Development of more severe disease of COVID-19 in smokers

is however likely linked to baseline respiratory pathologies induced by chronic cigarette smoking.

3.2. IL-6 specifically upregulated endothelial expression of NOX2 and NOX2-dependent ROS production

To examine whether IL-6 induces NOX-dependent oxidative stress in endothelial cells, we treated BAECs with IL-6 and examined expression of NOX isoforms (NOX1, NOX2, and NOX4) by Western blotting analysis. Interestingly, exposure of cells to IL-6 (100 ng/mL) for 24 h resulted in upregulation of NOX2, but not NOX1 or NOX4 (Fig. 2A–C). We next measured superoxide production specifically and quantitatively using electron spin resonance (ESR), in IL-6 treated BAECs transfected of NOX2 siRNA or negative control siRNA. As shown in Fig. 3, we found that IL-6 treatment resulted in marked increase in superoxide production in endothelial cells, which was completely attenuated by NOX2 siRNA but not by scrambled control siRNA, indicating that IL-6

specifically increases endothelial ROS production via activation of NOX2.

3.3. Estrogen administration alleviated IL-6-induced upregulation of NOX2 and MCP-1

To test the hypothesis that estrogen is involved in the protection of endothelial cells from IL-6-induced oxidative stress, BAECs were treated with various doses of 17β-estradiol (10 nmol/L to 10 μmol/L, 24 h) following IL-6 exposure (100 ng/mL, 30 min). Of note, estrogen receptors, ERα and ERβ, have been previously described to be functional in BAECs [52,53]. At a minimal dose of 10 nmol/L, 17β-estradiol almost completely abrogated IL-6-induced upregulation of NOX2 (Fig. 2B). Treatment of 17β-estradiol did not change protein expression of NOX1 or NOX4 in the presence of IL-6 (Fig. 2A and C). As a downstream effector of IL-6, MCP-1 was also examined (Fig. 2D), and found upregulated by IL-6, while this response was abolished by estrogen

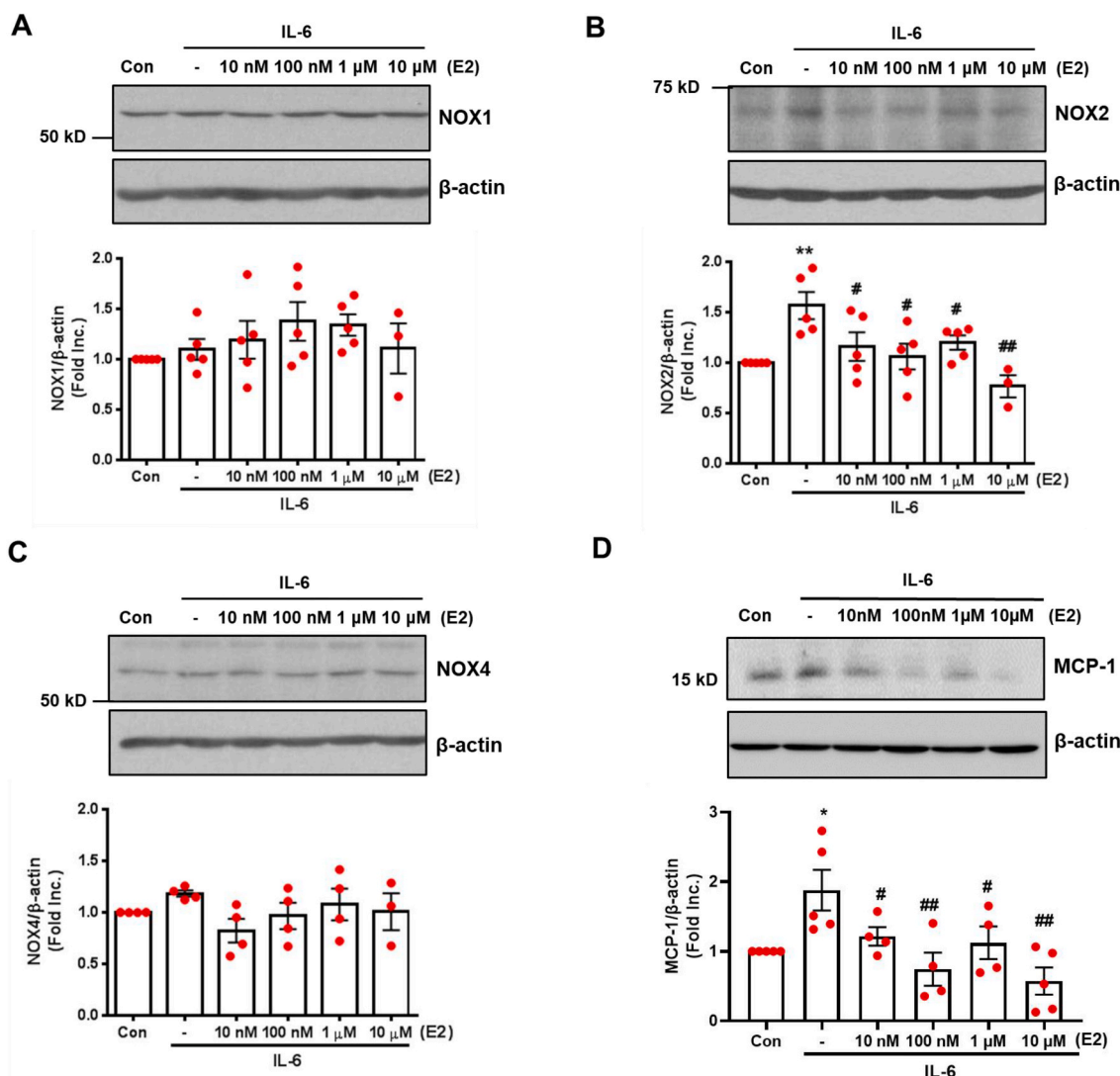


Fig. 2. IL-6 selectively upregulated protein expression levels of NOX2, and MCP-1, which were substantially attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were pre-treated with IL-6 (100 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. A, Representative Western blots and grouped data of NOX1 protein expression indicating no effects of IL-6 or 17β-estradiol. Data are shown as Mean ± SEM, n = 3–5. B, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 expression by IL-6, which was substantially attenuated by 17β-estradiol treatment at concentration as low as 10 nmol/L. Data are shown as Mean ± SEM, n = 3–5. C, Representative Western blots and grouped data of NOX4 protein expression indicating no significant effect by IL-6 or 17β-estradiol. Data are shown as Mean ± SEM, n = 3–4. D, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation by IL-6 and reversal by 17β-estradiol. Data are shown as Mean ± SEM, n = 4–5. *p < 0.05, **p < 0.01 vs. Control group; #p < 0.05, ##p < 0.01 vs. IL-6 group by One-Way ANOVA.

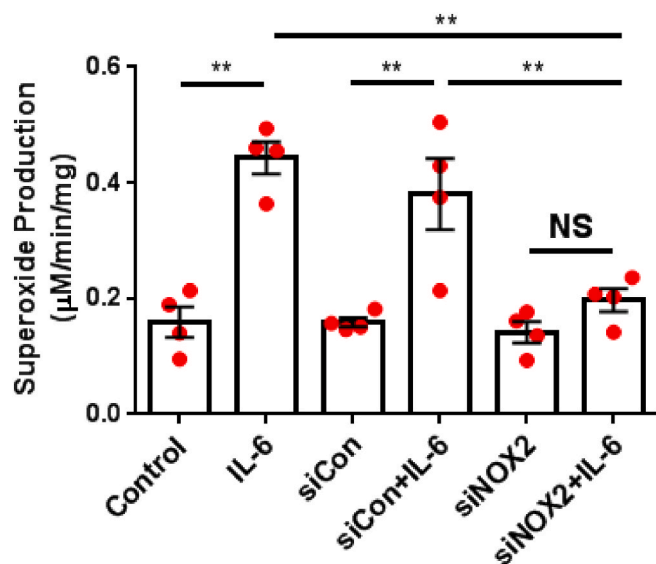


Fig. 3. IL-6 stimulated ROS production in endothelial cells is NOX2-dependent. Bovine aortic endothelial cells (BAECs) were transfected with siRNA (200 pmol) targeting NOX2 or scrambled negative control siRNA, and then treated with IL-6 (100 ng/mL) for 24 h. Superoxide production was selectively and quantitatively determined by electron spin resonance (ESR) as we previously published. IL-6 induced marked increase in endothelial superoxide production, which was completely abrogated by transfection with NOX2 siRNA (siNOX2). Of note, there was no effect by transfection of scrambled negative control siRNA (siCon). Shown are grouped data of superoxide production with indicated treatments. Data are shown as Mean \pm SEM, $n = 4$. ** $p < 0.01$ by One-Way ANOVA. NS, not significant.

treatment. These data indicate that estrogen administration protects endothelial cells from IL-6 induced oxidative stress via attenuation of NOX2 and MCP-1 activation, hence may be used as a potential therapy for cytokine storm mediated ARDS/multi-organ injury during the pathogenesis of COVID-19, to effectively reduce disease severity and mortality especially in men. Of note, we are proposing acute treatment of COVID-19 patients with estrogen, so the potential side effects of chronic treatment with estrogen are not relevant.

3.4. SARS-CoV-2 S protein dose-dependently stimulated endothelial ROS production

To examine whether S protein directly induces endothelial superoxide production, which might be the underlying mechanism of SARS-CoV-2 promoted endothelial dysfunction, BAECs were treated with S protein at doses of 0.5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, and 5 $\mu\text{g/mL}$ for 24 h. As is clear in Fig. 4, treatment with S protein at low dose of 0.5 $\mu\text{g/mL}$ induced a marked, 4.4 fold increase in endothelial superoxide production ($p < 0.001$). The superoxide production was aggravated in a dose dependent manner. These data for the first time demonstrate that S protein alone stimulates an excessive endothelial ROS production. These results not only validate our experimental model using S protein to examine ROS production implicated in endothelial dysfunction in vitro, but also suggest that S protein provoked endothelial superoxide production might be the underlying mechanism of endothelial dysfunction and inflammation following SARS-CoV-2 infection.

3.5. Estrogen administration alleviated SARS-CoV-2 S protein upregulation of NOX2, ACE2 and MCP-1

To examine whether infection by SARS-CoV-2 can directly induce NOX-dependent oxidative stress in endothelial cells, we treated endothelial cells with S protein. As shown in Fig. 5B, expression of NOX2 was

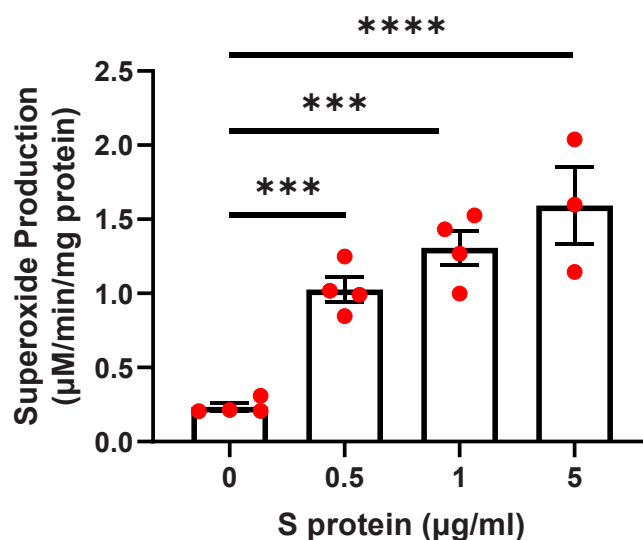


Fig. 4. SARS-CoV-2 Spike protein (S protein) induced an excessive ROS production in a dose dependent manner in endothelial cells. Bovine aortic endothelial cells (BAECs) were treated with different concentrations of SARS-CoV-2 S protein as indicated concentrations for 24 h. Superoxide production was determined selectively and quantitatively by electron spin resonance (ESR) as we previously published. S protein induced a marked increase in endothelial cell superoxide production in a dose dependent manner. Shown are grouped data of superoxide production in endothelial cells treated with indicated concentrations of S protein. Data are shown as Mean \pm SEM, $n = 3-4$. *** $p < 0.001$, **** $p < 0.0001$ by One-Way ANOVA.

robustly upregulated by S protein (500 ng/mL) after 24 h incubation, which was however significantly attenuated by 17 β -estradiol treatment at concentration as low as 100 nmol/L. Similar to observations in IL-6 treated endothelial cells, neither NOX1 nor NOX4 was upregulated by S protein treatment (Fig. 5A and C). Of note, ACE2 was abundantly expressed in endothelial cells as shown in Fig. 6A. The protein abundance of ACE2 was significantly upregulated by S protein treatment, which was substantially attenuated by 17 β -estradiol (at 10 $\mu\text{mol/L}$). As shown in Fig. 6B, upregulation of MCP-1 by S protein treatment was evident, which was abolished by 17 β -estradiol at all doses used (10 nmol/L to 10 $\mu\text{mol/L}$). Of note, S protein significantly upregulated protein expression of NOX2, ACE2, and MCP-1 in cultured human aortic endothelial cells (HAECs) as well (Fig. 7A–C), which was completely attenuated by 100 nmol/L of 17 β -estradiol to control levels.

3.6. Estrogen administration alleviated ACE2-dependent upregulation of NOX2, MCP-1 and ROS production

Consistent with changes in protein levels, mRNA levels of *ace2*, *nox2*, and *mcp-1* were upregulated in S protein treated endothelial cells, all of which were significantly reversed by 17 β -estradiol (Fig. 8A–C). Of note, expression of *nox2* and *mcp-1* at mRNA levels were completely suppressed by ACE2 antibody neutralization, indicating an intermediate role of ACE2 in these responses (Fig. 8D–E). Moreover, S protein treatment induced marked increase in superoxide production in endothelial cells, which was completely alleviated by NOX2 siRNA transfection (Fig. 9A). Also, S protein induced superoxide production was abolished by treatment with 17 β -estradiol in both BAECs and HAECs (Fig. 9B and C) at concentration as low as 100 nmol/L, which was effective to inhibit NOX2 activation, implicating a protective role of estrogen in attenuating S protein induced endothelial dysfunction via inhibition of NOX2-dependent superoxide production. Of interest, ACE2 antibody neutralization significantly blocked S protein induced superoxide production as shown in Fig. 9D, suggesting that S protein activation of NOX2 and ROS production is ACE2 dependent. However, acute exposure of cigarette

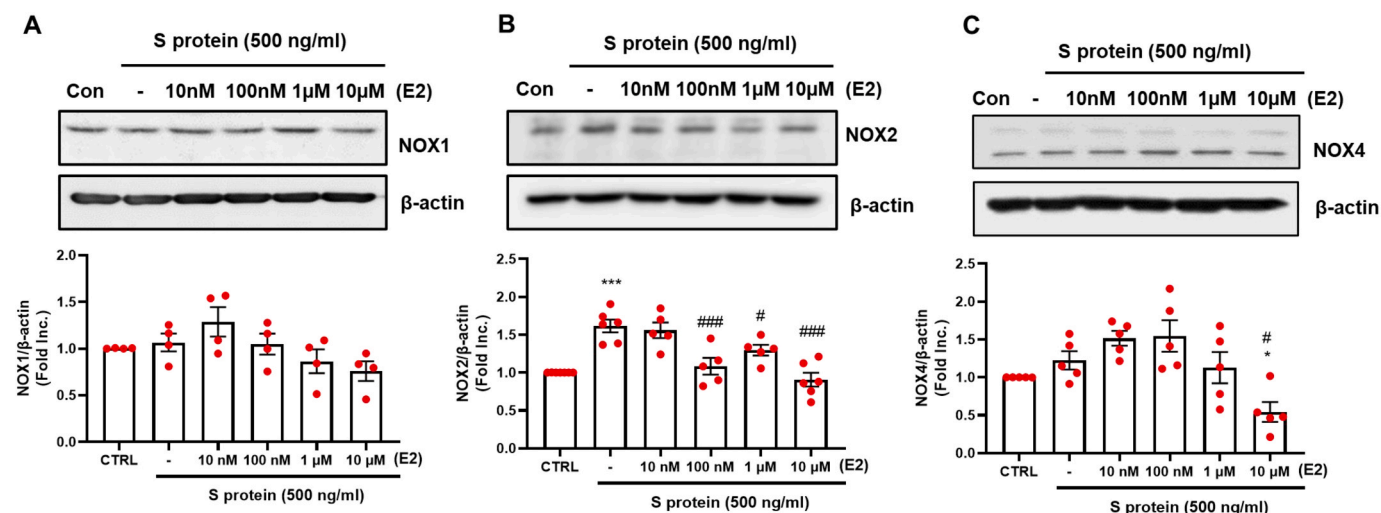


Fig. 5. SARS-CoV-2 Spike protein (S protein) selectively upregulated protein expression of NOX2, but not NOX1 or NOX4, in endothelial cells, which was substantially attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. **A**, Representative Western blots and grouped data of NOX1 protein expression indicating no effects of S protein or 17β-estradiol. Data are shown as Mean ± SEM, n = 6. **B**, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 by S protein, which was markedly attenuated by 17β-estradiol treatment at concentration as low as 100 nmol/L (effective range: 100 nmol/L to 10 μmol/L). Data are shown as Mean ± SEM, n = 5–6. **C**, Representative Western blots and grouped data of NOX4 protein expression indicating no significant effect by S protein. Data are shown as Mean ± SEM, n = 5. *p < 0.05, ***p < 0.001 vs. Control group; #p < 0.05, ###p < 0.001 vs. S protein group by One-Way ANOVA.

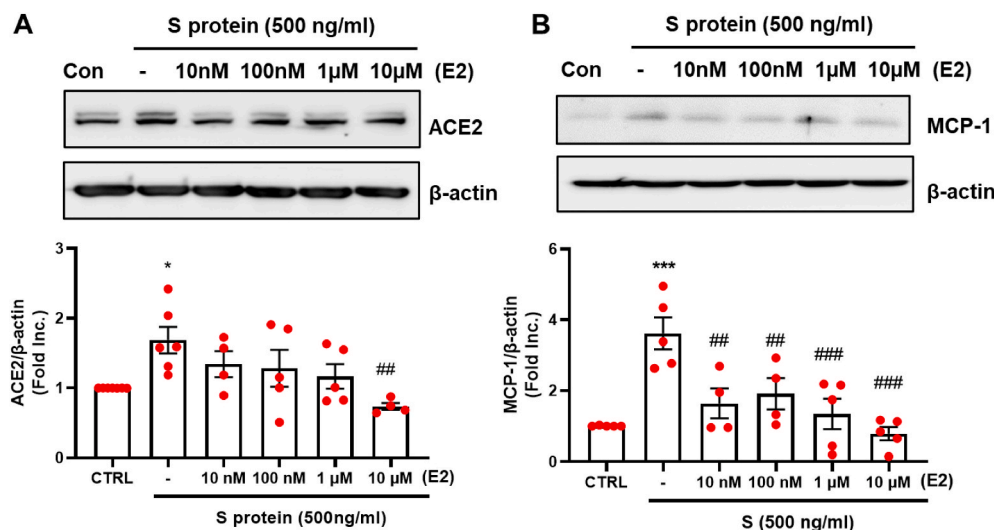


Fig. 6. SARS-CoV-2 Spike protein (S protein) upregulated protein expression levels of ACE2 and MCP-1 in endothelial cells, which was substantially attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. **A**, Representative Western blots and grouped data of ACE2 protein expression indicating upregulation of ACE2 by S protein, which was completely attenuated by 10 μM of 17β-estradiol. Data are shown as Mean ± SEM, n = 4–6. **B**, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation of MCP-1 by S protein, which was substantially attenuated by 17β-estradiol at concentration as low as 10 nmol/L (effective range: 10 nmol/L to 10 μmol/L). Data are shown as Mean ± SEM, n = 4–5. *p < 0.05, ***p < 0.001 vs. Control group; ##p < 0.01, ###p < 0.001 vs. S protein group by One-Way ANOVA.

smoke extract (CSE) prior to S protein treatment did not affect S protein activation of superoxide production (Fig. 10), again indicating that current smoking status might not be related to more severe disease in chronic smokers. Taken together, as summarized in Graphical abstract, these data indicate that viral infection itself and consequent cytokine storm (IL-6) can both trigger NOX2-dependent ROS production, ACE2 upregulation, and inflammatory cytokine expression in endothelial cells, resulting in endothelial dysfunction and inflammation to mediate ARDS/multi-organ failure and mortality during pathogenesis of COVID-19. These responses however can be completely alleviated by estrogen treatment, indicating a robust therapeutic effect of estrogen on COVID-19, especially in men and post-menopause women.

4. Discussion

In the present study, we have demonstrated that CSE stimulation of endothelial cells has no effects on the expression levels of ACE2 and TMPRSS2, indicating that the prevalence and severity predisposition to COVID-19 in men may not result from higher rate of current smoking status in men to induce more efficient viral entry via ACE2 and TMPRSS2. We therefore hypothesized that lower incidence and less severe disease in women might be related to estrogen mediated protection. We found that the major mediator of cytokine storm, IL-6, specifically induced upregulation of NOX2, but not that of NOX1 or NOX4, in endothelial cells. Superoxide production, determined selectively and quantitatively by ESR, was markedly increased by IL-6 but completely alleviated by transfection of endothelial cells with NOX2 siRNA. In

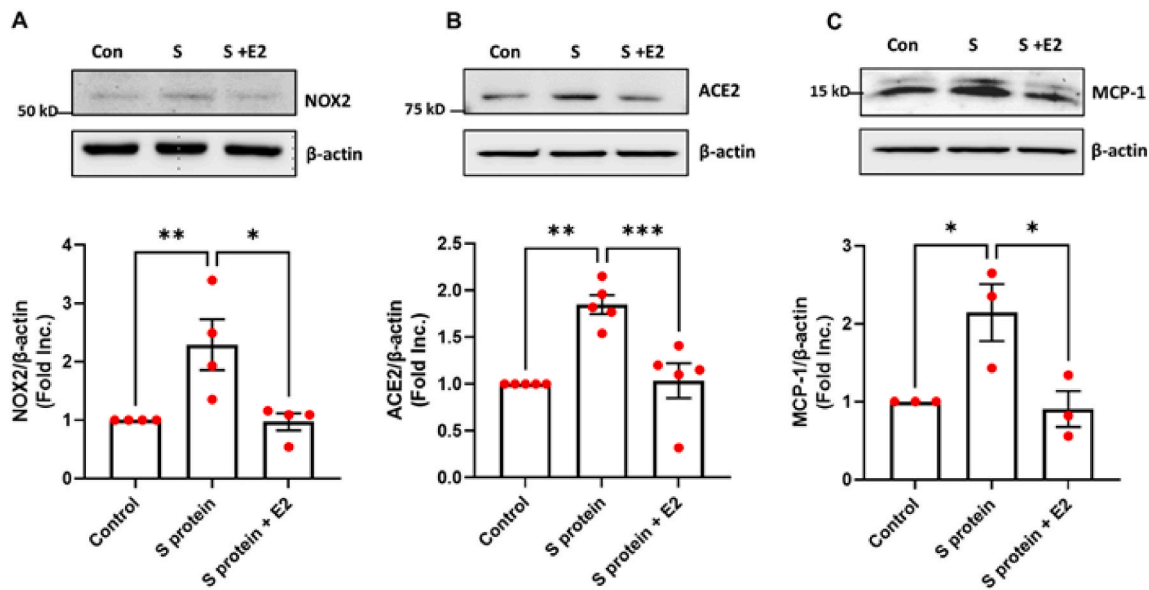


Fig. 7. SARS-CoV-2 Spike protein (S protein) upregulated protein expression levels of NOX2, ACE2, and MCP-1 in human aortic endothelial cells, which was completely attenuated by 17 β -estradiol. Human aortic endothelial cells (HAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 100 nmol/L of 17 β -estradiol (E2) for 24 h. **A**, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 by S protein, which was completely attenuated by 100 nmol/L of 17 β -estradiol. Data are shown as Mean \pm SEM, n = 4. **B**, Representative Western blots and grouped data of ACE2 protein expression indicating upregulation of ACE2 by S protein, which was completely attenuated by 100 nmol/L of 17 β -estradiol. Data are shown as Mean \pm SEM, n = 5. **C**, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation of MCP-1 by S protein, which was completely attenuated by 100 nmol/L of 17 β -estradiol. Data are shown as Mean \pm SEM, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 by One-Way ANOVA.

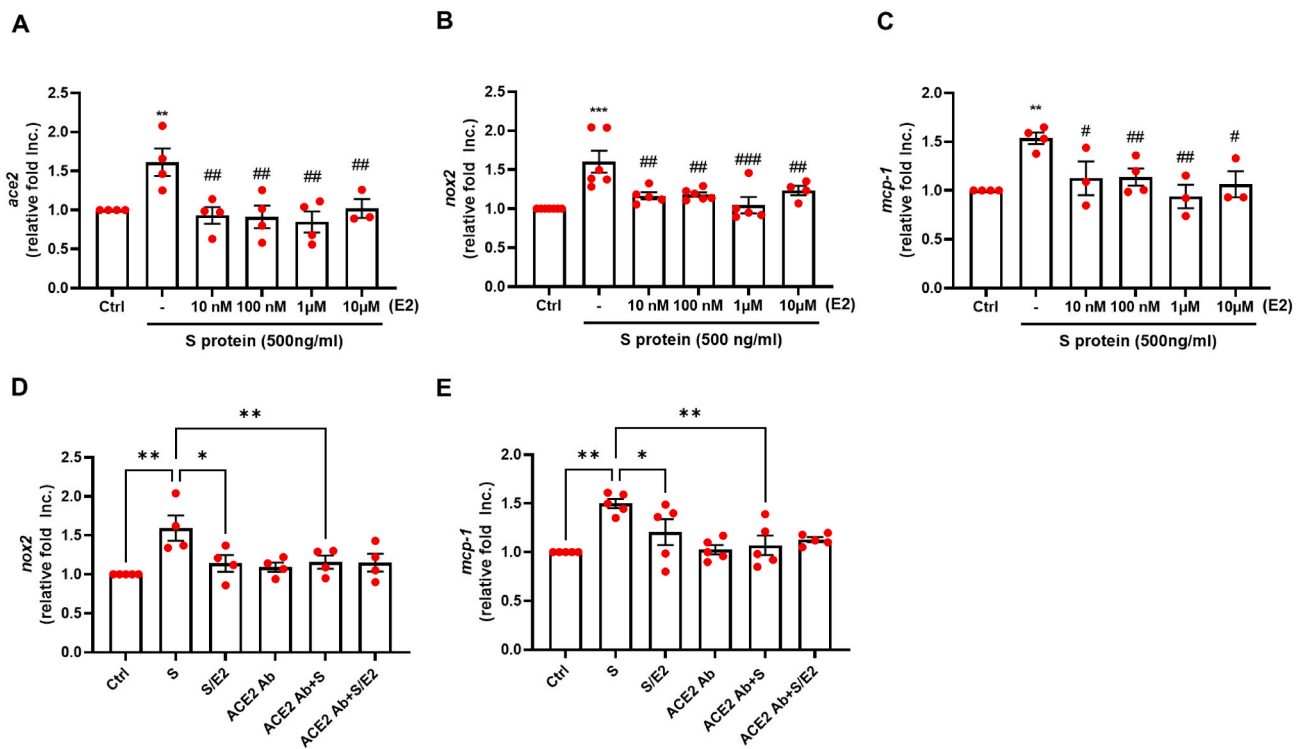


Fig. 8. SARS-CoV-2 Spike protein (S protein) upregulated *ace2* mRNA expression, and ACE2 dependent upregulation of *nox2* and *mcp-1* mRNA in endothelial cells, which were completely attenuated by 17 β -estradiol. Bovine aortic endothelial cells (BAECs) were pre-treated with S protein (500 ng/mL) for 30 min prior to exposure to 17 β -estradiol (E2) for 24 h at indicated concentrations. **A**, Grouped data of *ace2* mRNA expression indicating upregulation of *ace2* by S protein, which was completely attenuated by 17 β -estradiol. Data are shown as Mean \pm SEM, n = 3–4. **B**, Grouped data of *nox2* mRNA expression indicating upregulation of *nox2* by S protein, which was substantially attenuated by 17 β -estradiol. Data are shown as Mean \pm SEM, n = 5–6. **C**, Grouped data of *mcp-1* mRNA expression indicating upregulation of *mcp-1* by S protein, which was substantially attenuated by 17 β -estradiol. Data are shown as Mean \pm SEM, n = 3–4. BAECs were pretreated with 1 μ g/mL of ACE2 antibody to neutralize membrane receptor of ACE2 for S protein binding prior to exposure of endothelial cells to S protein. This was followed by treatment with 17 β -estradiol (E2) for 30 min at 100 nmol/L. **D**, Grouped data of *nox2* mRNA expression, indicating ACE2 dependent upregulation of *nox2* mRNA by S protein and its reversal by estrogen treatment. Data are shown as Mean \pm SEM, n = 4. **E**, Grouped data of *mcp-1* mRNA expression, indicating ACE2 dependent upregulation of *mcp-1* mRNA expression by S protein and its reversal by estrogen treatment. Data are shown as Mean \pm SEM, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control group; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. S protein group by One-Way ANOVA.

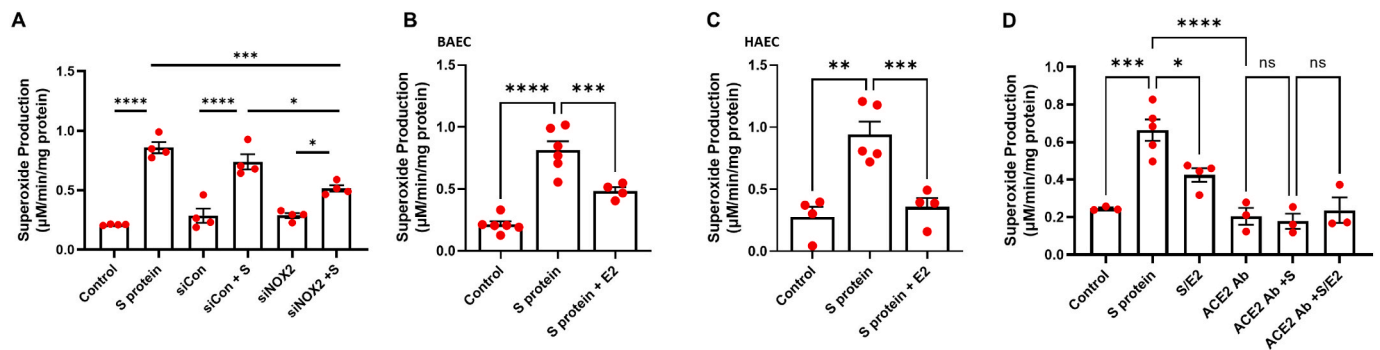


Fig. 9. SARS-CoV-2 Spike protein (S protein) stimulated ACE2 and NOX2-dependent ROS production, which was attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were transfected with siRNA (200 pmol) targeting NOX2 or scrambled negative control siRNA, and then treated with S protein (500 ng/mL) for 24 h. Superoxide production was selectively and quantitatively determined by electron spin resonance (ESR) as we previously published. **A**, Grouped data of superoxide production indicating NOX2 dependent endothelial cell superoxide production in response to S protein exposure. Of note, S protein induced a marked increase in endothelial superoxide production, which was significantly abrogated by transfection with NOX2 siRNA (siNOX2). There was no effect by transfection of scrambled negative control siRNA (siCon). Data are shown as Mean ± SEM, n = 4. BAECs were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 100 nmol/L of 17β-estradiol (E2) for 24 h. **B**, Grouped data of superoxide production indicating that S protein induced endothelial cell superoxide production was substantially abrogated by estrogen treatment in BAECs. Data are shown as Mean ± SEM, n = 4–6. Human aortic endothelial cells (HAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 100 nmol/L of 17β-estradiol (E2) for 24 h. **C**, Grouped data of superoxide production indicating that S protein induced endothelial cell superoxide production was substantially abrogated by estrogen treatment in HAECs. Data are shown as Mean ± SEM, n = 4–5. BAECs were pretreated with 1 μg/mL of ACE2 antibody (E2) for 30 min at 100 nmol/L. **D**, Grouped data of superoxide production indicating S protein induced superoxide production is mediated by ACE2. The markedly increased superoxide production by S protein was substantially attenuated by ACE2 antibody neutralization, indicating an intermediate role of ACE2 in S protein induced ROS production. Of note, in the presence of ACE2 neutralization antibody, estrogen had no additional effects on superoxide production. Data are shown as Mean ± SEM, n = 3–5, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by One-Way ANOVA.

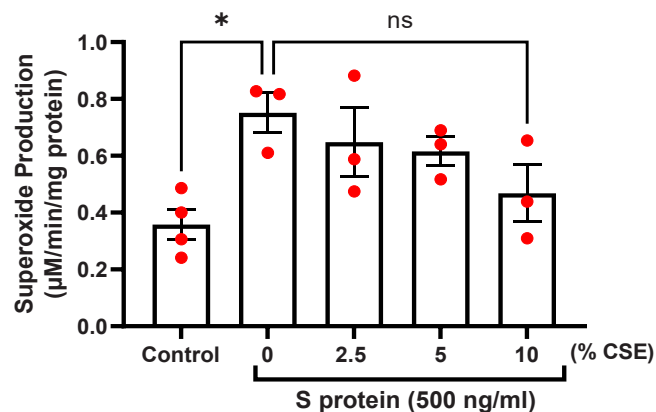


Fig. 10. Acute exposure of endothelial cells to CSE had no additional effects on SARS-CoV-2 Spike protein (S protein) induced ROS production. Bovine aortic endothelial cells (BAECs) were incubated with CSE at indicated concentrations for 30 min prior to S protein (500 ng/mL) treatment for 24 h. Superoxide production was selectively and quantitatively determined by electron spin resonance (ESR) as we previously published. The marked increase in S protein stimulated endothelial cell superoxide production was not affected by the acute pretreatment of the cells with CSE. Shown are grouped data of superoxide production with indicated treatments. Data are shown as Mean ± SEM. n = 3–4. *p < 0.05 by One-Way ANOVA. NS, not significant.

addition, estrogen administration substantially attenuated IL-6-induced NOX2 activation and hence NOX2-derived ROS production, as well as upregulation of pro-inflammatory gene MCP-1. NOX1 and NOX4 expression were not changed by IL-6 or estrogen. Similar results were found in endothelial cells treated with SARS-CoV-2 Spike protein (S protein). We found that endothelial cell NOX2 expression, ACE2 expression, and ROS production were all markedly upregulated by exposure to S protein. The S protein stimulated ROS production is attributed to ACE2 dependent NOX2 activation since it was completely

attenuated by transfection with NOX2 siRNA and neutralization of ACE2 with anti-ACE2 antibody. ACE2 antibody neutralization also attenuated NOX2 and MCP-1 mRNA expression. These data indicate that activation of NOX2 and induction of MCP-1 mediate oxidative stress and endothelial dysfunction triggered by SARS-CoV-2 infection upon its S protein binding to ACE2, and by infection related cytokine storm, which can be substantially alleviated by estrogen treatment. Therefore, estrogen may be used as a robust therapy for COVID-19, especially in men, both at the early stage of viral infection and the later stage of cytokine storm and ARDS/multi-organ failures. It is anticipated to effectively reduce disease severity and mortality.

Our current study investigated the underlying molecular mechanisms of sex difference in the morbidity and severity of COVID-19 patients, indicating that estrogen mediates the protection against COVID-19 in females. In addition, our data support the hypothesis that estrogen may be used to alleviate viral infection and cytokine storm-induced endothelial dysfunction, resulting in therapeutic effects to attenuate disease progression, severity and mortality. In an earlier report we described sex difference in patients with COVID-19 for the first time [9]. Indeed, studies of additional patient cohorts worldwide confirmed the observation of male predisposition to higher morbidity and mortality [10–16,54,55]. In addition, male gender predisposition was also seen in subgroups of COVID-19 patients with different conditions. For example, in 928 patients with cancer who were infected with SARS-CoV-2, male gender was significantly associated with increased 30-day all-cause mortality after adjustment for age, smoking status, and obesity (odds ratio 1.63; 95% CI 1.07–2.48) [56]. Of note, male gender-related higher infection incidence for SARS-CoV-2 has also been reported in pre-symptomatic patients with COVID-19 [57,58]. In the study of 196,738 COVID-19 patients from Mexico, 25,520 cases were presymptomatic [57]. In a study that analyzed 194,349,591 males and 201,715,364 females from the onset of the pandemic until June 21st by Bhopal et al., information of gender, age, and mortality in COVID-19 patients was collected by the National Institute for Demographic Studies from national statistical agencies of different countries, including England and Wales, France, Germany, Italy, Netherlands, Portugal, Korea, and Spain. The gender ratios (males vs. females) for mortality from COVID-10

ranged from 1.46 (10–19 years old age group) to 2.56 (60–69 years old age group) [59], indicating constantly higher mortality in males. Furthermore, sex difference was demonstrated in COVID-19 patients with different ethnic backgrounds, according to a study conducted in 848,166 patients with COVID-19, for which data were collected from daily COVID-19 report from the California Department of Public Health (CDPH) [60]. Males had higher rates of mortality resulted from COVID-19 than females across all age groups (0–34, 35–49, 50–59, 60–64, 65–69, 70–74, 75–79, 80+) and different ethnic backgrounds [60]. Therefore, sex difference is a real and very important phenomenon in COVID-19 patients that exists cross different age groups, disease severity, territories, and ethnic backgrounds. Hence, our present study aimed to investigate molecular mechanisms underlying sex difference in COVID-19, targeting of which may lead to novel therapeutics for the devastating disease of COVID-19.

ACE2 and TMPRSS2 are critically required for SARS-CoV-2 entry into host cells. Mixed results have been reported for cigarette smoking regulation of ACE2 expression. Smith et al. reported that cigarette smoke and inflammatory signaling upregulates ACE2 expression in respiratory tract [61]. Whereas others found ACE2 expression was not regulated in large airway epithelia isolated from smokers, or in primary cultured airway basal stem cells (ABSCs) isolated from human nonsmokers exposed to CSE [20,21]. In patients with chronic obstructive pulmonary disease (COPD) in which smoking prevalence is high, ACE2 expression was also not increased in the patient group [62]. Therefore, it is unclear as to whether cigarette smoking is consistently regulatory of ACE2 expression, and whether such possible regulation plays a role in the pathogenesis of COVID-19. In particular, whether or not cigarette smoking regulates ACE2 expression in endothelial cells has remained completely unknown. Our study represents the first evidence that CSE stimulation has no effects on protein expression levels of ACE2 and TMPRSS2 in endothelial cells, indicating that current smoking status may not play a role in SARS-CoV-2 induced endothelial inflammation and dysfunction, at least not via increased viral entry into host cells by enriched ACE2 and TMPRSS2. Also, CSE stimulation prior to SARS-CoV-2 S protein exposure had no additional effects on S protein activation of ROS production, indicating that current smoking status/acute cigarette smoking exposure itself might not explain morbidity and mortality predisposition in males who tend to smoke more. Rather, the more severe disease in smokers might be related to baseline respiratory diseases derived from chronic smoking.

Oxidative stress has been implicated in acute lung injury/ARDS. We have shown that S protein treatment of endothelial cells induces NOX2 activation and ROS production. Of note, cytokine storm developed following initial viral infection can function as a major trigger of oxidative stress, forming a vicious cycle (Graphical Abstract). The central mediator of cytokine storm during the development of COVID-19, IL-6, has been extensively studied as a treatment target [30,63]. Meanwhile, endothelial cell inflammation and dysfunction have been shown to play an important role in acute lung injury/ARDS, as well as systematic failures of other organs of kidney and heart during COVID-19 [23,24]. Therefore, the regulatory effects of IL-6 on endothelial cells to generate oxidative stress to result in endothelial dysfunction, need to be fully understood to reveal potential treatment targets for COVID-19. Our results indicate that IL-6 induces selective upregulation and activation of NOX2 isoform, but not that of NOX1 or NOX4, to result in increased superoxide production. This response was completely alleviated by siRNA transfection of endothelial cells with NOX2 siRNA, but not by scrambled negative control siRNA. The IL-6 upregulation of NOX2 was substantially attenuated by estrogen administration, at levels as low as 10 nmol/L. In addition, IL-6 exposure resulted in an upregulation of pro-inflammatory protein of MCP-1, which was abolished by estrogen treatment. These data indicate that IL-6-dependent NOX2 activation and ROS production may underlie endothelial cell injury in response to cytokine storm during the pathogenesis of COVID-19. In addition, estrogen treatment may be considered as an effective strategy to alleviate

cytokine storm-induced endothelial ROS production and endothelial dysfunction/injury in patients with COVID-19.

It is noteworthy that active form of S protein was used in this study to mimic the infection of SARS-CoV-2 in endothelial cells. Recombinant S protein treatment alone was found capable of activating MEK pathway, which is required for viral replication in host cells, indicating that S protein itself can trigger similar signalling mechanisms of viral infection [64]. Likewise, another study has demonstrated that S protein treatment elicits upregulation of genes related to cell growth signaling such as MAK2K and JAK2 in human umbilical vein endothelial cell (HUVEC), and upregulated genes related to signaling of IL-6, NF- κ B, and chemokines in brain endothelial cells [34]. Given that S protein has been shown to bind to neuropilin-1 (NRP-1) to inhibit VEGF-A signaling in neurons, S protein binding to NRP-1 in endothelial cells might result in impaired angiogenesis [65]. Moreover, prolonged treatment with recombinant S1 subunit for 48 h resulted in endothelial cell degeneration, indicating the deleterious effect of recombinant S1 subunit on vascular cells [66]. Collectively, these studies seem to implicate that S protein treatment of ACE2 expressing endothelial cells is a highly valuable tool for studying underlying mechanisms of SARS-CoV-2 infection mediated endothelial dysfunction. In a recent study that conducted functional assessment of ACE2 using ACE2 orthologs, the internalization of SARS-CoV-2 nucleocapsid (N) protein was confirmed in bovine ACE2 expressing cell, indicating the successful infectivity of SARS-CoV-2 via interaction with bovine ACE2 [67]. In addition, specificity of the antibody used to detect ACE2 was confirmed by either overexpression of ACE2 with pcDNA3.1-hACE2 plasmid or knockdown of ACE2 using RNA interference, indicating clearly that endothelial cells used in the present study expresses abundant ACE2 and that antibody neutralization experiments using ACE2 antibody is a valid approach to examine ACE2 dependency of NOX2 activation and ROS production in response to S protein exposure.

S protein treatment resulted in upregulation of ACE2, which was completely attenuated by estrogen administration. By exposure of endothelial cells to S protein, we demonstrated for the first time that S protein itself can cause an excessive ACE2 dependent endothelial oxidative stress, a crucial mediator of COVID-19 related endothelial dysfunction and inflammation. Moreover, estrogen treatment following S protein exposure abolished excessive superoxide production and upregulated expression of NOX2 and MCP-1 protein and mRNA, indicating that estrogen can be used as a therapeutic intervention to reduce subsequent ARDS and multi-organ injury resulted from endothelial dysfunction and vascular inflammation. Given that ACE2 upregulation by renin-angiotensin system (RAS) antagonist paradoxically favors SARS-CoV-2 binding to endothelial cells [68,69], downregulation of ACE2 expression by estrogen is beneficial in alleviating viral infection induced endothelial dysfunction especially in patients who need to take RAS antagonists for co-existing hypertension. Furthermore, considering that S protein increased endothelial ROS production is stronger and more robust than that of the response from IL-6 treatment, as soon as S protein binds to ACE2 on endothelial cells, it will prime excessive ROS production to contribute to endotheliitis. Of note, estrogen treatment showed reversal effects on NOX2 activation, ROS production, ACE2 upregulation and MCP-1 induction following 30 min of S protein pre-stimulation, further validating therapeutic applicability of estrogen treatment. Therefore, the protective effects of estrogen on both S protein and IL-6 induced NOX2 activation and oxidative stress in endothelial cells warrant a remarkable potential of estrogen to serve as a robust therapy for endothelial dysfunction/inflammation that is a critical mediator of ARDS/multi-organ failure and mortality in patients with COVID-19.

In conclusion, our data demonstrate for the first time that estrogen-mediated attenuation of NOX2 activation, ROS production and MCP-1 upregulation in response to S protein/IL-6 exposure of endothelial cells underlie protection against COVID-19 in females. These data therefore indicate that estrogen administration can be used as a robust

treatment option for COVID-19 to effectively reduce disease severity and improve survival, which is readily translatable into clinical practice to treat patients with COVID-19.

Declaration of competing interest

The authors declare no conflicts of interests for this work.

Acknowledgement

This work was supported by National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (NHLBI) grants HL077440 (H. Cai), HL088975 (H. Cai), HL142951 (H. Cai), and HL154754 (H. Cai). The authors thank Margaret Matern and Juyun Hwang for their technical contribution.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2021.102099>.

References

- [1] WHO, Coronavirus disease 2019 (COVID-19) weekly Epi update 51. <https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19-3-august-2021>, 2021. (Accessed 3 August 2021).
- [2] J. Wise, Covid-19: new coronavirus variant is identified in UK, *Bmj* 371 (2020) m4857.
- [3] E. Mahase, Covid-19: what have we learnt about the new variant in the UK? *Bmj* 371 (2020) m4944.
- [4] E. Callaway, A bloody mess': confusion reigns over naming of new COVID variants, *Nature* (2021).
- [5] E. Mahase, Covid-19: what new variants are emerging and how are they being investigated? *Bmj* 372 (2021) n158.
- [6] V.V. Edara, L. Lai, M.K. Sahoo, K. Floyd, M. Sibai, D. Solis, M.W. Flowers, L. Hussaini, C.R. Ciric, S. Bechnack, K. Stephens, E.B. Mokhtari, P. Mudvari, A. Creanga, A. Pegu, A. Derrien-Coleman, A.R. Henry, M. Gagne, B.S. Graham, J. Wrammert, D.C. Douek, E. Boritz, B.A. Pinsky, M.S. Suthar, *Infection and Vaccine-Induced Neutralizing Antibody Responses to the SARS-CoV-2 B.1.617.1 Variant*, *bioRxiv*, 2021.
- [7] WHO, WHO announces simple, easy-to-say labels for SARS-CoV-2 Variants of Interest and Concern. <https://www.who.int/news/item/31-05-2021-who-announces-simple-easy-to-say-labels-for-sars-cov-2-variants-of-interest-and-concern>, 2021. (Accessed 31 May 2021).
- [8] M. Hoffmann, H. Kleine-Weber, S. Schroeder, N. Kruger, T. Herrler, S. Erichsen, T. S. Schiergens, G. Herrler, N.H. Wu, A. Nitsche, M.A. Muller, C. Drosten, S. Pohlmann, SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor, *Cell* 181 (2020) 271–280 e8.
- [9] H. Cai, Sex difference and smoking predisposition in patients with COVID-19, *Lancet Respir Med* 8 (2020), e20.
- [10] P. Goyal, J.J. Choi, L.C. Pinheiro, E.J. Schenck, R. Chen, A. Jabri, M.J. Satlin, T. R. Campion Jr., M. Nahid, J.B. Ringel, K.L. Hoffman, M.N. Alshak, H.A. Li, G. T. Wehmeyer, M. Rajan, E. Reshetnyak, N. Hupert, E.M. Horn, F.J. Martinez, R. M. Gulick, M.M. Safford, Clinical characteristics of covid-19 in New York city, *N. Engl. J. Med.* 382 (2020) 2372–2374.
- [11] W.J. Guan, Z.Y. Ni, Y. Hu, W.H. Liang, C.Q. Ou, J.X. He, L. Liu, H. Shan, C.L. Lei, D. S.C. Hui, B. Du, L.J. Li, G. Zeng, K.Y. Yuen, R.C. Chen, C.L. Tang, T. Wang, P. Y. Chen, J. Xiang, S.Y. Li, J.L. Wang, Z.J. Liang, Y.X. Peng, L. Wei, Y. Liu, Y.H. Hu, P. Peng, J.M. Wang, J.Y. Liu, Z. Chen, G. Li, Z.J. Zheng, S.Q. Qiu, J. Luo, C.J. Ye, S. Y. Zhu, N.S. Zhong, C. China Medical Treatment Expert Group for, Clinical characteristics of coronavirus disease 2019 in China, *N. Engl. J. Med.* 382 (2020) 1708–1720.
- [12] F. Zhou, T. Yu, R. Du, G. Fan, Y. Liu, Z. Liu, J. Xiang, Y. Wang, B. Song, X. Gu, L. Guan, Y. Wei, H. Li, X. Wu, J. Xu, S. Tu, Y. Zhang, H. Chen, B. Cao, Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study, *Lancet* 395 (2020) 1054–1062.
- [13] L. Palaioodimos, D.G. Kokkinidis, W. Li, D. Karamanis, J. Ognibene, S. Arora, W. N. Southern, C.S. Mantzoros, Severe obesity, increasing age and male sex are independently associated with worse in-hospital outcomes, and higher in-hospital mortality, in a cohort of patients with COVID-19 in the Bronx, New York, *Metab. Clin. Exp.* 108 (2020) 154262.
- [14] R.B. Serafim, P. Povoia, V. Souza-Dantas, A.C. Kalil, J.I.F. Salluh, Clinical course and outcomes of critically ill patients with COVID-19 infection: a systematic review, *Clin. Microbiol. Infect.* (2020).
- [15] H. Peckham, N.M. de Grujter, C. Raine, A. Radziszewska, C. Ciurtin, L. R. Wedderburn, E.C. Rosser, K. Webb, C.T. Deakin, Male sex identified by global COVID-19 meta-analysis as a risk factor for death and ICU admission, *Nat. Commun.* 11 (2020) 6317.
- [16] E.J. Williamson, A.J. Walker, K. Bhaskaran, S. Bacon, C. Bates, C.E. Morton, H. J. Curtis, A. Mehrkar, D. Evans, P. Inglesby, J. Cockburn, H.I. McDonald, B. MacKenna, L. Tomlinson, L.J. Douglas, C.T. Rentsch, R. Mathur, A.Y.S. Wong, R. Grieve, D. Harrison, H. Forbes, A. Schultze, R. Croker, J. Parry, F. Hester, S. Harper, R. Perera, S.J.W. Evans, L. Smeeth, B. Goldacre, Factors associated with COVID-19-related death using OpenSAFELY, *Nature* 584 (2020) 430–436.
- [17] C. Wang, J. Xu, L. Yang, Y. Xu, X. Zhang, C. Bai, J. Kang, P. Ran, H. Shen, F. Wen, K. Huang, W. Yao, T. Sun, G. Shan, T. Yang, Y. Lin, S. Wu, J. Zhu, R. Wang, Z. Shi, J. Zhao, X. Ye, Y. Song, Q. Wang, Y. Zhou, L. Ding, T. Yang, Y. Chen, Y. Guo, F. Xiao, Y. Lu, X. Peng, B. Zhang, D. Xiao, C.S. Chen, Z. Wang, H. Zhang, X. Bu, X. Zhang, L. An, S. Zhang, Z. Cao, Q. Zhan, Y. Yang, B. Cao, H. Dai, L. Liang, J. He, G. China Pulmonary Health Study, Prevalence and risk factors of chronic obstructive pulmonary disease in China (the China Pulmonary Health [CPH] study): a national cross-sectional study, *Lancet* 391 (2018) 1706–1717.
- [18] A. Lugo, P. Zuccaro, R. Pacifici, G. Gorini, P. Colombo, C. La Vecchia, S. Gallus, Smoking in Italy in 2015–2016: prevalence, trends, roll-your-own cigarettes, and attitudes towards incoming regulations, *Tumori* 103 (2017) 353–359.
- [19] M.R. Creamer, T.W. Wang, S. Babb, K.A. Cullen, H. Day, G. Willis, A. Jamal, L. Neff, Tobacco product use and cessation indicators among adults - United States, 2018, in: *Mmwr-Morbidity and Mortality Weekly Report* 68, 2019, pp. 1013–1019.
- [20] J.M. Leung, C.X. Yang, A. Tam, T. Shaipanich, T.L. Hackett, G.K. Singhera, D. R. Dorscheid, D.D. Sin, ACE-2 expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19, *Eur. Respir. J.* 55 (2020).
- [21] A. Purkayastha, C. Sen, G. Garcia Jr., J. Langerman, D.W. Shia, L.K. Meneses, P. Vijayaraj, A. Durra, C.R. Koloff, D.R. Freund, J. Chi, T.M. Rickabaugh, A. Mulay, B. Konda, M.S. Sim, B.R. Stripp, K. Plath, V. Arumugaswami, B.N. Gomperts, Direct exposure to SARS-CoV-2 and cigarette smoke increases infection severity and alters the stem cell-derived airway repair response, *Cell Stem Cell* 27 (2020) 869–875 e4.
- [22] H. Zhang, M.R. Rostami, P.L. Leopold, J.G. Mezey, S.L. O'Beirne, Y. Strulovici-Barel, R.G. Crystal, Expression of the SARS-CoV-2 ACE2 receptor in the human airway epithelium, *Am. J. Respir. Crit. Care Med.* 202 (2020) 219–229.
- [23] Z. Varga, A.J. Flammer, P. Steiger, M. Haberecker, R. Andermatt, A.S. Zinkernagel, M.R. Mehra, R.A. Schuepbach, F. Ruschitzka, H. Moch, Endothelial cell infection and endotheliitis in COVID-19, *Lancet* 395 (2020) 1417–1418.
- [24] M. Ackermann, S.E. Verleden, M. Kuehnel, A. Haverich, T. Welte, F. Laenger, A. Vanstapel, C. Werlein, H. Stark, A. Tzankov, W.W. Li, V.W. Li, S.J. Mentzer, D. Jonigk, Pulmonary vascular endotheliitis, thrombosis, and angiogenesis in covid-19, *N. Engl. J. Med.* 383 (2020) 120–128.
- [25] M.Z. Tay, C.M. Poh, L. Renia, P.A. MacAry, L.F.P. Ng, The trinity of COVID-19: immunity, inflammation and intervention, *Nat. Rev. Immunol.* 20 (2020) 363–374.
- [26] D. Branco-Melo, B.E. Nilsson-Payant, W.C. Liu, S. Uhl, D. Hoagland, R. Moller, T. X. Jordan, K. Oishi, M. Panis, D. Sachs, T.T. Wang, R.E. Schwartz, J.K. Lim, R. A. Albrecht, B.R. tenOever, Imbalanced host response to SARS-CoV-2 drives development of COVID-19, *Cell* 181 (2020) 1036–1045 e9.
- [27] A.G. Laing, A. Lorenc, I. Del Molino Del Barrio, A. Das, M. Fish, L. Monin, M. Munoz-Ruiz, D.R. McKenzie, T.S. Hayday, I. Francos-Quijorna, S. Kamdar, M. Joseph, D. Davies, R. Davis, A. Jennings, I. Zlatareva, P. Vantourout, Y. Wu, V. Sofra, F. Cano, M. Greco, E. Theodoridis, J.D. Freedman, S. Gee, J.N.E. Chan, S. Ryan, E. Bugallo-Blanco, P. Peterson, K. Kisand, L. Haljasmagi, L. Chadli, P. Moingeon, L. Martinez, B. Merrick, K. Bisnauthsing, K. Brooks, M.A.A. Ibrahim, J. Mason, F. Lopez Gomez, K. Babalola, S. Abdul-Jawad, J. Cason, C. Mant, J. Seow, C. Graham, K.J. Doores, F. Di Rosa, J. Edgeworth, M. Shankar-Hari, A.C. Hayday, A dynamic COVID-19 immune signature includes associations with poor prognosis, *Nat. Med.* 26 (2020) 1623–1635.
- [28] L.H. Tjan, K. Furukawa, T. Nagano, T. Kiriu, M. Nishimura, J. Arai, Y. Hino, S. Iwata, Y. Nishimura, Y. Mori, Early differences in cytokine production distinguish severity of COVID-19, *J. Infect. Dis.* (2021).
- [29] M. Aziz, R. Fatima, R. Assaly, Elevated interleukin-6 and severe COVID-19: a meta-analysis, *J. Med. Virol.* (2020).
- [30] C. Salama, J. Han, L. Yau, W.G. Reiss, B. Kramer, J.D. Neidhart, G.J. Criner, E. Kaplan-Lewis, R. Baden, L. Pandit, M.L. Cameron, J. Garcia-Diaz, V. Chavez, M. Mekebe-Reuter, F. Lima de Menezes, R. Shah, M.F. Gonzalez-Lara, B. Assman, J. Freedman, S.V. Mohan, Tocilizumab in patients hospitalized with covid-19 pneumonia, *N. Engl. J. Med.* 384 (2021) 20–30.
- [31] A.C. Walls, Y.J. Park, M.A. Tortorici, A. Wall, A.T. McGuire, D. Velesler, Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein, *Cell* 183 (2020) 1735.
- [32] M.A. Tortorici, D. Velesler, Structural insights into coronavirus entry, *Adv. Virus Res.* 105 (2019) 93–116.
- [33] T.P. Buzhdygan, B.J. DeOre, A. Baldwin-Leclair, T.A. Bullock, H.M. McGary, J. A. Khan, R. Razmpour, J.F. Hale, P.A. Galie, R. Potluta, A.M. Andrews, S. H. Ramirez, The SARS-CoV-2 spike protein alters barrier function in 2D static and 3D microfluidic in-vitro models of the human blood-brain barrier, *Neurobiol. Dis.* 146 (2020) 105131.
- [34] N. Kaneko, S. Satta, Y. Komuro, S.D. Muthukrishnan, V. Kakarla, L. Guo, J. An, F. Elahi, H.I. Kornblum, D.S. Liebeskind, T. Hsiai, J.D. Hinman, Flow-mediated susceptibility and molecular response of cerebral endothelia to SARS-CoV-2 infection, *Stroke* 52 (2021) 260–270.
- [35] Y. Zhang, P. Murugesan, K. Huang, H. Cai, NADPH oxidases and oxidase crosstalk in cardiovascular diseases: novel therapeutic targets, *Nat. Rev. Cardiol.* 17 (2020) 170–194.
- [36] K. Chalupsky, H. Cai, Endothelial dihydrofolate reductase: critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 9056–9061.

- [37] A. Nguyen, H. Cai, Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 6530–6535.
- [38] J.Y. Youn, T. Wang, H. Cai, An ezrin/calpain/PI3K/AMPK/eNOSs1179 signaling cascade mediating VEGF-dependent endothelial nitric oxide production, *Circ. Res.* 104 (2009) 50–59.
- [39] H. Li, Q. Li, Y. Zhang, W. Liu, B. Gu, T. Narumi, K.L. Siu, J.Y. Youn, P. Liu, X. Yang, H. Cai, Novel treatment of hypertension by specifically targeting E2F for restoration of endothelial dihydrofolate reductase and eNOS function under oxidative stress, *Hypertension* 73 (2019) 179–189.
- [40] K. Huang, T. Narumi, Y. Zhang, Q. Li, P. Murugesan, Y. Wu, N.M. Liu, H. Cai, Targeting MicroRNA-192-5p, a downstream effector of NOXs (NADPH oxidases), reverses endothelial DHFR (dihydrofolate reductase) deficiency to attenuate abdominal aortic aneurysm formation, *Hypertension* 78 (2021) 282–293.
- [41] S.E. Michaud, S. Dussault, J. Groleau, P. Haddad, A. Rivard, Cigarette smoke exposure impairs VEGF-induced endothelial cell migration: role of NO and reactive oxygen species, *J. Mol. Cell. Cardiol.* 41 (2006) 275–284.
- [42] A. Janoff, H. Carp, Possible mechanisms of emphysema in smokers: cigarette smoke condensate suppresses protease inhibition in vitro, *Am. Rev. Respir. Dis.* 116 (1977) 65–72.
- [43] Y. Su, W. Han, C. Giraldo, Y. De Li, E.R. Block, Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells, *Am. J. Respir. Cell Mol. Biol.* 19 (1998) 819–825.
- [44] D. Bernhard, C.W. Huck, T. Jakschitz, G. Pfister, B. Henderson, G.K. Bonn, G. Wick, Development and evaluation of an in vitro model for the analysis of cigarette smoke effects on cultured cells and tissues, *J. Pharmacol. Toxicol. Methods* 50 (2004) 45–51.
- [45] Q. Li, P. Wang, K. Ye, H. Cai, Central role of SIAH inhibition in DCC-dependent cardioprotection provoked by netrin-1/NO, *Proc. Natl. Acad. Sci. U. S. A.* 112 (2015) 899–904.
- [46] Z. Guo, Y. Zhang, C. Liu, J.Y. Youn, H.L. Cai, Toll-like receptor 2 (TLR2) deficiency abrogates diabetic and obese phenotypes while restoring endothelial function via inhibition of NOX1, *Diabetes* (2021).
- [47] K. Huang, Y. Wang, K.L. Siu, Y. Zhang, H. Cai, Targeting feed-forward signaling of TGFbeta/NOX4/DHFR/eNOS uncoupling/TGFbeta axis with anti-TGFbeta and folic acid attenuates formation of aortic aneurysms: novel mechanisms and therapeutics, *Redox Biol.* 38 (2021) 101757.
- [48] J.H. Oak, H. Cai, Attenuation of angiotensin II signaling recouples eNOS and inhibits nonendothelial NOX activity in diabetic mice, *Diabetes* 56 (2007) 118–126.
- [49] K.L. Siu, Q. Li, Y. Zhang, J. Guo, J.Y. Youn, J. Du, H. Cai, NOX isoforms in the development of abdominal aortic aneurysm, *Redox Biol.* 11 (2017) 118–125.
- [50] J.Y. Youn, L. Gao, H. Cai, The p47phox- and NADPH oxidase organizer 1 (NOXO1)-dependent activation of NADPH oxidase 1 (NOX1) mediates endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction in a streptozotocin-induced murine model of diabetes, *Diabetologia* 55 (2012) 2069–2079.
- [51] J.Y. Youn, K.L. Siu, H.E. Lob, H. Itani, D.G. Harrison, H. Cai, Role of vascular oxidative stress in obesity and metabolic syndrome, *Diabetes* 63 (2014) 2344–2355.
- [52] L.H. Smith, S.R. Coats, H. Qin, M.S. Petrie, J.W. Covington, M. Su, M. Eren, D. E. Vaughan, Differential and opposing regulation of PAI-1 promoter activity by estrogen receptor alpha and estrogen receptor beta in endothelial cells, *Circ. Res.* 95 (2004) 269–275.
- [53] F. Bayard, S. Clamens, G. Delsol, N. Blaes, A. Maret, J.C. Faye, Oestrogen synthesis, oestrogen metabolism and functional oestrogen receptors in bovine aortic endothelial cells, *Ciba Found. Symp.* 191 (1995) 122–132, discussion 132–8.
- [54] G. Grasselli, A. Zangrillo, A. Zanella, M. Antonelli, L. Cabrini, A. Castelli, D. Cereda, A. Coluccello, G. Foti, R. Fumagalli, G. Iotti, N. Latronico, L. Lorini, S. Merler, G. Natalini, A. Piatti, M.V. Ranieri, A.M. Scandroglio, E. Storti, M. Ceconi, A. Pesenti, C.-L.I. Network, Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the lombardy region, Italy, *JAMA* 323 (2020) 1574–1581.
- [55] O.T. Ranzani, L.S.L. Bastos, J.G.M. Gelli, J.F. Marchesi, F. Baiao, S. Hamacher, F. A. Bozza, Characterisation of the first 250 000 hospital admissions for COVID-19 in Brazil: a retrospective analysis of nationwide data, *Lancet Respirat. Med.* (2021).
- [56] N.M. Kuderer, T.K. Choueiri, D.P. Shah, Y. Shyr, S.M. Rubinstein, D.R. Rivera, S. Shete, C.Y. Hsu, A. Desai, G. de Lima Lopes Jr., P. Grivas, C.A. Painter, S. Peters, M.A. Thompson, Z. Bakouny, G. Batist, T. Bekaii-Saab, M.A. Bilen, N. Bouganim, M. B. Larroya, D. Castellano, S.A. Del Prete, D.B. Doroshov, P.C. Egan, A. Elkrief, D. Farmakiotis, D. Flora, M.D. Galsky, M.J. Glover, E.A. Griffiths, A.P. Gulati, S. Gupta, N. Hafez, T.R. Halfdanarson, J.E. Hawley, E. Hsu, A. Kasi, A.R. Khaki, C. A. Lemmon, C. Lewis, B. Logan, T. Masters, R.R. McKay, R.A. Mesa, A.K. Morgans, M.F. Mulcahy, O.A. Panagiotou, P. Peddi, N.A. Pennell, K. Reynolds, L.R. Rosen, R. Rosovsky, M. Salazar, A. Schmidt, S.A. Shah, J.A. Shaya, J. Steinharter, K. E. Stockerl-Goldstein, S. Subbiah, D.C. Vinh, F.H. Wehbe, L.B. Weissmann, J.T. Wu, E. Wulff-Burchfield, Z. Xie, A. Yeh, P.P. Yu, A.Y. Zhou, L. Zubiri, S. Mishra, G. H. Lyman, B.I. Rini, J.L. Warner, Covid, C. Cancer, Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study, *Lancet* 395 (2020) 1907–1918.
- [57] M.A. Fernandez-Rojas, M.A.L. Esparza, A.C. Romero, D.Y. Calva-Espinosa, J. L. Moreno-Camacho, A.P. Langle-Martinez, A. Garcia-Gil, C.J. Solis-Gonzalez, A. Canizalez-Roman, N. Leon-Sicairos, J. Alcantar-Fernandez, Epidemiology of COVID-19 in Mexico, symptomatic profiles and presymptomatic people, *Int. J. Infect. Dis.* (2021) official publication of the International Society for Infectious Diseases.
- [58] J. He, Y. Guo, R. Mao, J. Zhang, Proportion of asymptomatic coronavirus disease 2019: a systematic review and meta-analysis, *J. Med. Virol.* 93 (2021) 820–830.
- [59] S.S. Bhopal, R. Bhopal, Sex differential in COVID-19 mortality varies markedly by age, *Lancet* 396 (2020) 532–533.
- [60] D. Xiong, L. Zhang, G.L. Watson, P. Sundin, T. Bufford, J.A. Zoller, J. Shamshoian, M.A. Suchard, C.M. Ramirez, Pseudo-likelihood based logistic regression for estimating COVID-19 infection and case fatality rates by gender, race, and age in California, *Epidemics* 33 (2020) 100418.
- [61] J.C. Smith, E.L. Sausville, V. Girish, M.L. Yuan, A. Vasudevan, K.M. John, J. M. Sheltzer, Cigarette smoke exposure and inflammatory signaling increase the expression of the SARS-CoV-2 receptor ACE2 in the respiratory tract, *Dev. Cell* 53 (2020) 514–529 e3.
- [62] G. Li, X. He, L. Zhang, Q. Ran, J. Wang, A. Xiong, D. Wu, F. Chen, J. Sun, C. Chang, Assessing ACE2 expression patterns in lung tissues in the pathogenesis of COVID-19, *J. Autoimmun.* 112 (2020) 102463.
- [63] E. Huang, S.C. Jordan, Tocilizumab for covid-19 - the ongoing search for effective therapies, *N. Engl. J. Med.* 383 (2020) 2387–2388.
- [64] Y.J. Suzuki, S.I. Nikolaienko, V.A. Dibrova, Y.V. Dibrova, V.M. Vasylyk, M. Y. Novikov, N.V. Shults, S.G. Gychka, SARS-CoV-2 spike protein-mediated cell signaling in lung vascular cells, *Vasc. Pharmacol.* 137 (2021) 106823.
- [65] A. Moutal, L.F. Martin, L. Boinon, K. Gomez, D. Ran, Y. Zhou, H.J. Stratton, S. Cai, S. Luo, K.B. Gonzalez, S. Perez-Miller, A. Patwardhan, M.M. Ibrahim, R. Khanna, SARS-CoV-2 spike protein co-opts VEGF-A/neuropilin-1 receptor signaling to induce analgesia, *Pain* 162 (2021) 243–252.
- [66] G.J. Nuovo, C. Magro, T. Shaffer, H. Awad, D. Suster, S. Mikhail, B. He, J. J. Michaille, B. Liechty, E. Tili, Endothelial cell damage is the central part of COVID-19 and a mouse model induced by injection of the S1 subunit of the spike protein, *Ann. Diagn. Pathol.* 51 (2020) 151682.
- [67] Y. Liu, G. Hu, Y. Wang, W. Ren, X. Zhao, F. Ji, Y. Zhu, F. Feng, M. Gong, X. Ju, Y. Zhu, X. Cai, J. Lan, J. Guo, M. Xie, L. Dong, Z. Zhu, J. Na, J. Wu, X. Lan, Y. Xie, X. Wang, Z. Yuan, R. Zhang, Q. Ding, Functional and genetic analysis of viral receptor ACE2 orthologs reveals a broad potential host range of SARS-CoV-2, *Proc. Natl. Acad. Sci. U. S. A.* (2021) 118.
- [68] L. Fang, G. Karakioulakis, M. Roth, Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Respir Med* 8 (2020) e21.
- [69] M. Esler, D. Esler, Can angiotensin receptor-blocking drugs perhaps be harmful in the COVID-19 pandemic? *J. Hypertens.* 38 (2020) 781–782.