Enhanced Expression of RAGE AXIS is Associated with Severity of COVID-19 in Patients with Comorbidities

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Research Article

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Abstract

There is a limited understanding of molecular and cellular events that derive disease progression in patients with COVID-19. Receptor for Advanced Glycation End Products (RAGE) is hyperactive in development and complications of several diseases by mediating oxidative stress and inflammation in the body. The present study aims to explore activation of RAGE signaling in patients infected with SARS-CoV-2 with preexisting comorbidities. Enhanced levels of ligands of RAGE including AGEs, S100, and HMGB-1 were observed in Covid 19 patients with severe diseases, however, their level was significantly higher in COVID-19 patients with comorbidties as compared to COVID-19 patients without comorbidties. The Expression of RAGE in parallel to ligands accumulation, was significantly increased in patients with severe disease and comorbidities as compared to COVID-19 patients with sever disease without comorbidities. The expression of downstream effectors of RAGE including STAT-3 and NF-kB were also enahnced and their activity was increaed in COVID-19 patients with comorbisdities. Levels of inflammatory and oxidative stress biomarkers were markeldy in COVID-19 patients with comorbidties as compared to COVID-19 patients without comorbidties. We conclude that upregulated RAGE axis is favorable to worsen the severity of the SARS-CoV-2 infection in patients with preexisting comorbidities and partly explain inflammatory and oxidative stress strom in severe COVID-19 patietnts.

Introduction

Corona Virus disease 2019 (COVID-19) has effected nearly two third of the global population and resulted in more than six million death [1]. Clinical oucomes of COVID-19 infection depend upon preexisting comorbidities such as chronic lung disease, hypertension, obesity, diabtetes, cardiovascular diseases, kidney diseases. The presence of comorbidities is associated with worse outcome in COVID-19 infection [2-4]. Additionally, these comorbiditities are associated with aging, that is also strongly associated with severity of COVID-19. However, evidence is lacking to decipher molecular and cellular events of this association. RAGE is a multiligand receptor that is highly expressed in several tissues including brain, muscle, lungs and immune cells. Beside its physiological functions it has role in innate immune response. RAGE signaling is involved in development and complications of diabetes, cardiovascular diseases, neurological disorder and cancer [5]. Generation of inflammation and oxidative stress is one of the common mechanisms of RAGE induced cellular events. Cytokine storm due to inflammation and oxidative stress is hallmark of COVID-19 as well, that may promote severe illness, organ damage and death [6, 7]. Therefore, exploring underlying molecular mchanisms of SARCoV-2 infection with coexisting comorbidities is pivotal in undertsnading the pathology of the disease. Here we hypothesied that hyperreactive RAGE signaling may contribute in severity of COVID-19 disease and this contribution is significantly higher in patients with comorbidities. In the present study we investigated RAGE signaling in COVID-19 patients with comorbidities and COVID-19 patients without comorbidties.

Materials And Methods

Study subjects

A total of 442 subjects with COVID-19, from three reference hospitals in Karachi from November 2020 to July 2021, were recruited for the study (supplementary material) . Inclusion criteria was Covid 19 infection and one or more symptoms of COVID-19 including fever, headache, cough, sore throat, sputum production, fatigue, shortness of breath (SOB), nausea, vomiting, diarrhea, nasal congestion, conjunctival congestion, myalgia, and arthralgia. A set of 50 healthy volunteers were enrolled in the study. This group is referred as “control”. The patients were divided into two groups “Covid 19 positive patients with comorbidities” including diabetes and hypertension and “Covid 19 positive patients without comorbidities”. Each group was further sub-divided into two sub-groups, Without comorbidities as “Mild” and “Severe” and with comorbidities as “C-Mild” and “C-Severe”. The disease severity criteria found in supplementary material. Ethical approvals were taken from Ethical Review Board of Sohail University. The study was conducted in accordance with the Declaration of Helsinki. Written consent was taken from the patients.

COVID-19 PCR Testing: The patients were tested for COVID-19 in their nasopharyngeal sample, by a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) by using a COVID-19 Nucleic Acid Detection Kit according to the manufacturer’s protocol (Genesig, Primerdesign Ltd, Chandler’s Ford, UK) Patients with a confirmed diagnosis of SARS-CoV-2 infection were included in the study.

Sample collection: A total of 10 mL peripheral blood was collected for laboratory experiments. Nasopharyngeal swab was taken for SARS-CoV-2 testing. Sera and plasma were isolated from the blood accordingly and stored at −80 °C until further assay.

Cell isolation:

Peripheral blood was drawn in sodium heparinized tubes and allowed to ‘rest’ at room temp for at least 1 hour. Peripheral Blood Mononuclear Cells (PBMCs) were prepared from Buffy- Coats. PBMCs were isolated by gradient density centrifugation using histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). The cell pellets were used for RNA extraction and protein extraction. Following density gradient centrifugation, PBMCs were also harvested for culturing.

Cell stimulation

PBMCs were cultured at 20×10^6/mL in Iscove Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, Schnelldorf, Germany). PBMCs were stimulated in the presence or absence of TNF-alpha or IL-10 for various time points. PBMCs were incubated in a humidified atmosphere at 37°C with 5% CO_2.

Nuclear Extraction: After incubation, PBMCs were washed with PBS containing 10% endotoxin-free FBS (Gibco, New York, USA). Washed cells were then treated with a nuclear extract kit (Abcam, Shanghai, China) according to the manufacturer's instructions. A cytoplasmic extract and a nuclear extract for each culture condition was obtained and conserved at -80°C until further assays.

Immunological Assays

Serum HMGB1, S-100B and CML levels were measured by the quantitative sandwich enzyme immunoassay technique. using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (BioVision Inc., CA, USA). Following kits were used to analyze the inflammatory cytokines: Human IL-10 Quantikine ELISA kit Immunoassay, Human IL-6 Quantikine ELISA kit Immunoassay, Human TNF-alpha Quantikine ELISA kit Immunoassay (R&D System, Minneapolis, MN, USA),

Gene expression by real time PCR

The mRNA expression levels of RAGE, NF-kB and STAT-3 were quantified, using quantitative PCR (Startagene MX 3000P, Agilent technologies, Germany) as described, previously [8]. Using Trizol reagent (Invitrogen, CA, USA), RNA was extracted from the peripheral blood. cDNA synthesis was carried out using 1 microgram RNA by SuperScript III First-Strand Synthesis System (Thermoscientific Karlsruhe, Germany). Quantitative PCR was performed by SYBR Green Realtime PCR Master Mix (Thermoscientific Mannheim, Germany) in accordance with manufacturer's instruction. Each measurement was performed in triplicate. The mRNA expression levels were normalized to beta-actin. Primer sequences are available in supplementary material.

Western Blot

PBMCs were isolated and homogenized at 4 °C (Stuart Homogeniser, UK) in lysis buffer. The cells were then processed as described previously [9]. Briefly, homogenates were solubilized on ice for 30 min followed by centrifugation at 12,000 × g for 15 min. The supernatant was separated and protein content was determined by the Bradford method. 400 µg of protein was used for immunoprecipitation. Immunoprecipitated proteins were separated by SDS-PAGE, and western blot analysis was performed as described [10] using anti RAGE antibody (Abcam, Cambridge, UK).

Measurement of Activation of NF-kB and STAT-3

Phosphorylated STAT3 and phosphorylated NF-kB levels were measured, in nuclear extracts of PBMCs after stimulation, by transcription factor ELISA kit, Phospho-Stat3 (pTyr705) and pan-Stat3 ELISA Kit (Sigma-Aldrich, St. Louis, MO, USA), total and phosphorylated NFkB p65 by human InstantOne ELISA Kit (ThermoFisher, MA USA).

Measurement of oxidative stress parameters

For measurement of AOPP, plasma samples were diluted in PBS and levels of AOPP were measured by colorimetry using OxiSelect™ AOPP Assay Kit, (Cell Biolabs Inc San Diego, USA) as per manufacturer instructions by comparison with Chloramine standard curve**.** The optical density was read at 340 nm using a Multimode Microplate Reader (Varioskan™ LUX multimode microplate reader, USA). The levels of Thiobarbituric Acid Reactive Substances (TBARS) were measured by fluorometry using TBARS assay kit (Cayman Chemical MI, USA).

Statistical Analysis

Statistical analysis was performed using SPSS version 26 software (SPSS Inc., Chicago, IL). The results were adjusted for age through propensity score matching. Groups of data were compared, after calculating mean +/- SEMs, either using two way ANOVA, followed by post hoc analysis (using Bonferroni test) or one-way ANOVA, followed by post hoc analysis (using Dunnett's multiple comparison tests). The Student's *t* tests and chi-squared test was used to compare the distribution of a categorical variables, when appropriate. A p-value ≤ 0.05 was taken to indicate statistical significance.

Results

Determination of RAGE ligand levels in COVID-19 Patients

Glucose modified proteins; Advance Glycation End Products (AGEs) activate RAGE in several pathological states. However, RAGE is multiligand receptor and its activation by members of the high mobility group box (HMGB)-1 and S-100 is well documented [11, 12]. All three ligands of RAGE were measured in serum of COVID-19 patients (Fig. 1 A, B, C). It was found that the levels of all RAGE ligands were significantly higher in patients with severe COVID-19 and comorbidities as compared to COVID-19 patients without comorbidities.

Determination of expression of receptor for AGE in COVID-19 Patients

RAGE expression in PBMC may reflect RAGE axis activity in diseases where RAGE ligands are accumulated [13, 14]. Next, we sought to determine expression of RAGE in peripheral blood mononuclear cells (PBMC) in COVID-19 positive and control subjects using Real Time-Polymerase Gene Expression (Fig. 2 A). We found significantly increased expression of RAGE gene in PBMC of COVID-19 patients with severity of disease and comorbidities as compared to all the subgroups of control group (Fig.2 B). Interestingly, the expression of RAGE gene was also found significantly higher in COVID-19 patients with severe disease as compared to mild cases. Expression of RAGE protein was measured by western blot and found to be significantly higher in sever COVID-19 patients with comorbidities as compared to COVID-19 patients without comorbidities.

Investigation of downstream signal transduction in COVID-19 Patients.

To further investigate downstream RAGE signaling, we addressed the expression of transcriptional factors that ultimately manifest inflammation and oxidative stress in the patients. NF-kB (nuclear factor kappa B) and STAT-3 (signal transducer and activator of transcription) expression was enhanced in severely ill COVID-19 patients with comorbidities as compared to patients without comorbidities (Fig.3 A, B). Additionally, activation of these transcriptional factors was analyzed in nuclear extracts of PBMC of COVID-19 patients, using ELISA (Fig.3 C, D). We found that after in vitro stimulation with TNF-alpha and IL-10, higher levels of phosphorylated NF-kB and STAT3 were translocated in nucleus, in monocytes of patients with severe COVID-19 and comorbidities as compared to monocytes of severe COVID-19 patients without comorbidities.

Evaluation of level of inflammatory markers in COVID-19 Patients

Inflammatory markers including TNF-alpha, IL-6, and IL-10 were analyzed in COVID-19 positive patients and control group. The level of inflammatory markers in serum was significantly higher in severely ill COVID-19 patients with comorbidities as compared to COVID-19 patients without comorbidities (Fig. 4 A, B, C).

Measurement of systematic oxidative stress in COVID-19 Patients

As indicator of oxidative stress, we measured endogenous lipid peroxidation by measuring levels of Thiobarbituric Acid Reactive Substances (TBARS) in serum and endogenous protein oxidation by measuring Advanced Oxidation Protein Products (AOPP) in plasma. We found that the oxidative stress was elevated in severe COVID-19 patients, however, the amount of oxidative stress was significantly increased in severe COVID-19 patients with Comorbidities as compared to without comorbidities (Fig.4 D, E).

Discussion

Deciphering pathobiological mechanisms underlying the severity and risk of infection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is escalating. Recent reports provided putative links of association of RAGE ligands with severity of COVID-19 in patients [15]. A study found the role of RAGE gene polymorphisms in predisposing patients to severe COVID-19 and another recent study explored function of RAGE in mice infected with SARS-CoV-2 [16]. However, expression of RAGE axis has not been explored in COVID-19 patients yet. We propose that RAGE axis is hyperactive in severity of COVID-19 and

that this contribution is significantly higher in COVID-19 patients with comorbidities. We found higher RAGE ligand expression in serum of severely ill COVID-19 patients with comorbidities as compared to COVID-19 patients without comorbidities. Interestingly, CML levels did not changed between COVID-19 patients with mild and severe disease without comorbidities. Indicating Function of HMGB-1 and S100B in COVID severity while AGEs have limited role in enhancing severity of the COVID-19. HMGB-1 and S100 B may be involved in tissue injury in severely ill COVID-19 patients [17] our study, support this hypothesis. In parallel, we found significantly higher gene and protein expression of RAGE in severely ill COVID-19 patient as compared to all other groups of the study. A recent study corroborates higher HMGB-1 and RAGE expression in plasma of severely ill COVID-19 patients [18]. The downstream effectors of RAGE including NF-kB and STAT-3 are transcriptional factors that upon activation translocate into nucleus and stimulate transcription of cytokines and oxidative biomarkers to contribute to the severity of several disease. Such hypothesis has been proposed for the severity of COVID-19 as well [19, 20]. We found enhanced activation and nuclear translocation of NF-kB and STAT-3 in the nucleus of monocytes from COVID-19 patients with severe disease. Since interaction of RAGE with its ligands culminate in inflammation and oxidative stress, we found higher levels of inflammatory markers including IL-6, IL-10, TNF-alpha and oxidative stress markers including lipid peroxidation and Advanced Oxidation Protein Products in severe COVID-19 patients with comorbidities. The outcome of this study provides for the first time the evidence that the active RAGE signaling in monocytes of COVID-19 patients partly explain the severity of COVID-19 infection in patients with comorbidities.

Declarations

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Consent for publication:

Ethical approvals were taken from Ethical Review Board of Sohail University, Protocol Number: 000173/20. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was taken from the patients to participate in the study. The participant has consented for publication of their information, data, and all images in the manuscript.

Availability of data and materials:

Authors accept journal's type-1 research data policy. Authors agree for data sharing and data citation.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Gulnaz Khan], [Fadielhe A Soahil] [Bushra Khan] [Sidra Rafi] [Shumaila Nasir] and [Rizwana Sanaullah Waraich]. The first draft of the manuscript was written by [Rizwana Sanaullah Waraich] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

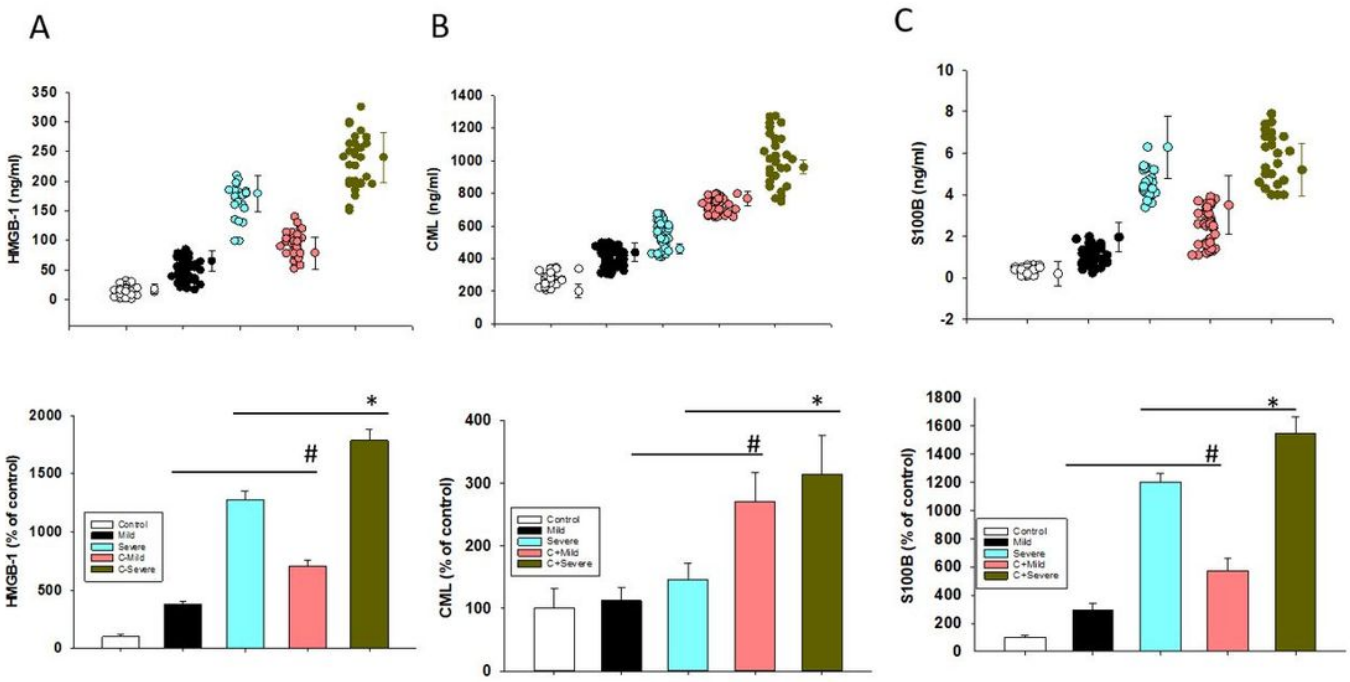

Figure 1

Determination of RAGE ligand levels in COVID-19 Patients (A) Levels of HMGB-1 (B) Levels of CML (C) Levels of S100B, in serum of COVID-19 patients and controls. Results represent mean ± SEMs. n=20-30 in each group, *$P < 0.05$, Severe COVID-19 patients with comorbidities (C-Severe) vs Severe COVID-19 patients without comorbidities. # $P < 0.05$, Severe COVID-19 patients with comorbidities (C-Severe) vs Severe COVID-19 patients without comorbidities.

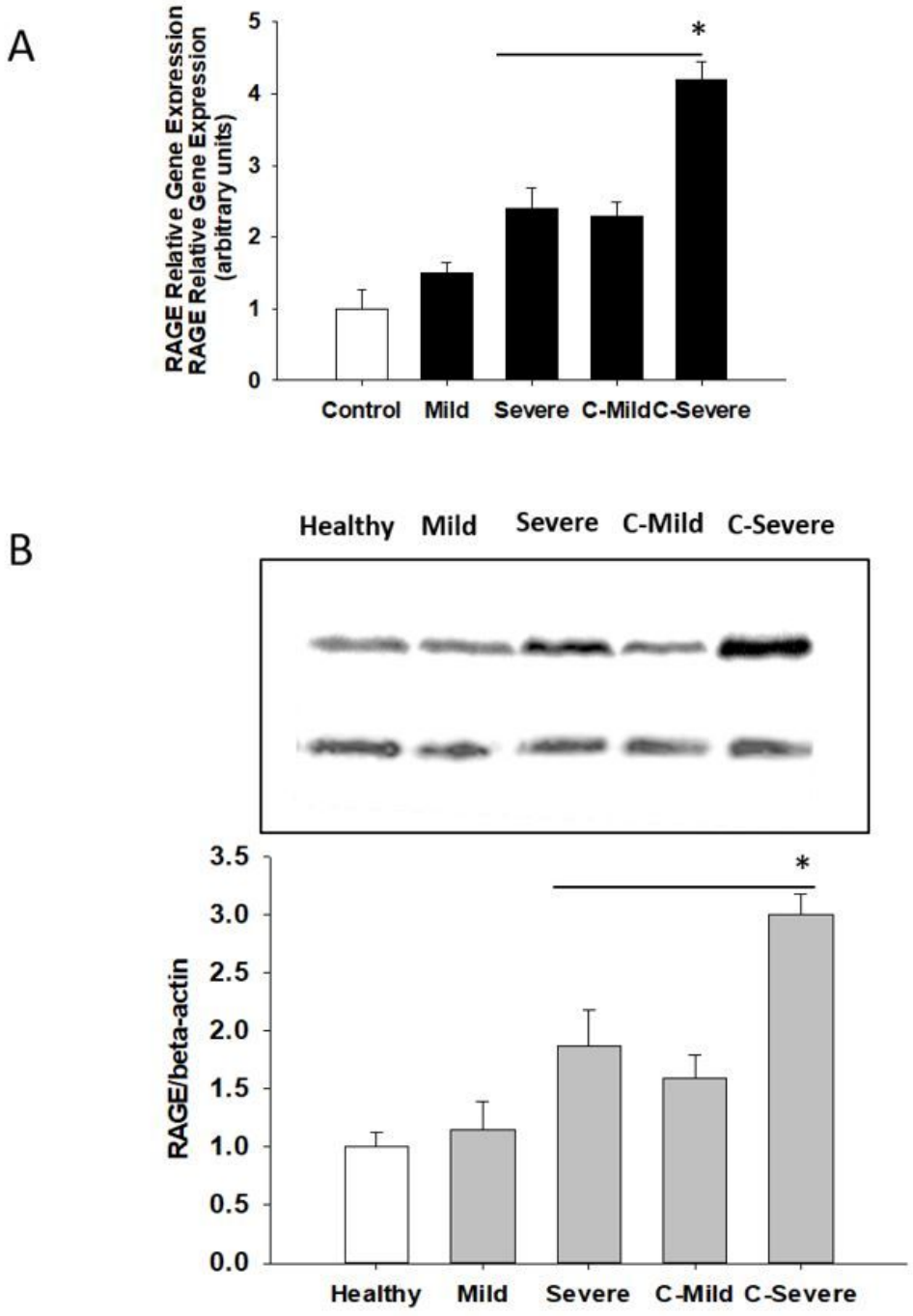

Figure 2

Determination of expression of receptor for AGE in COVID-19 Patients (A) RAGE gene expression in PBMC (B) RAGE protein expression in PBMC. Total protein from skeletal muscle of all mice groups was separated on 7.5% SDS-PAGE gels, and immunoblotted with anti-RAGE antibody. The levels of protein in the immunoblots were quantified using densitometry and normalized to beta-actin protein expression.

The data are presented as mean ± SEMs, n = 20-30, *P < 0.05, Severe COVID-19 patients with comorbidities (C-Severe) vs Severe COVID-19 patients without comorbidities.

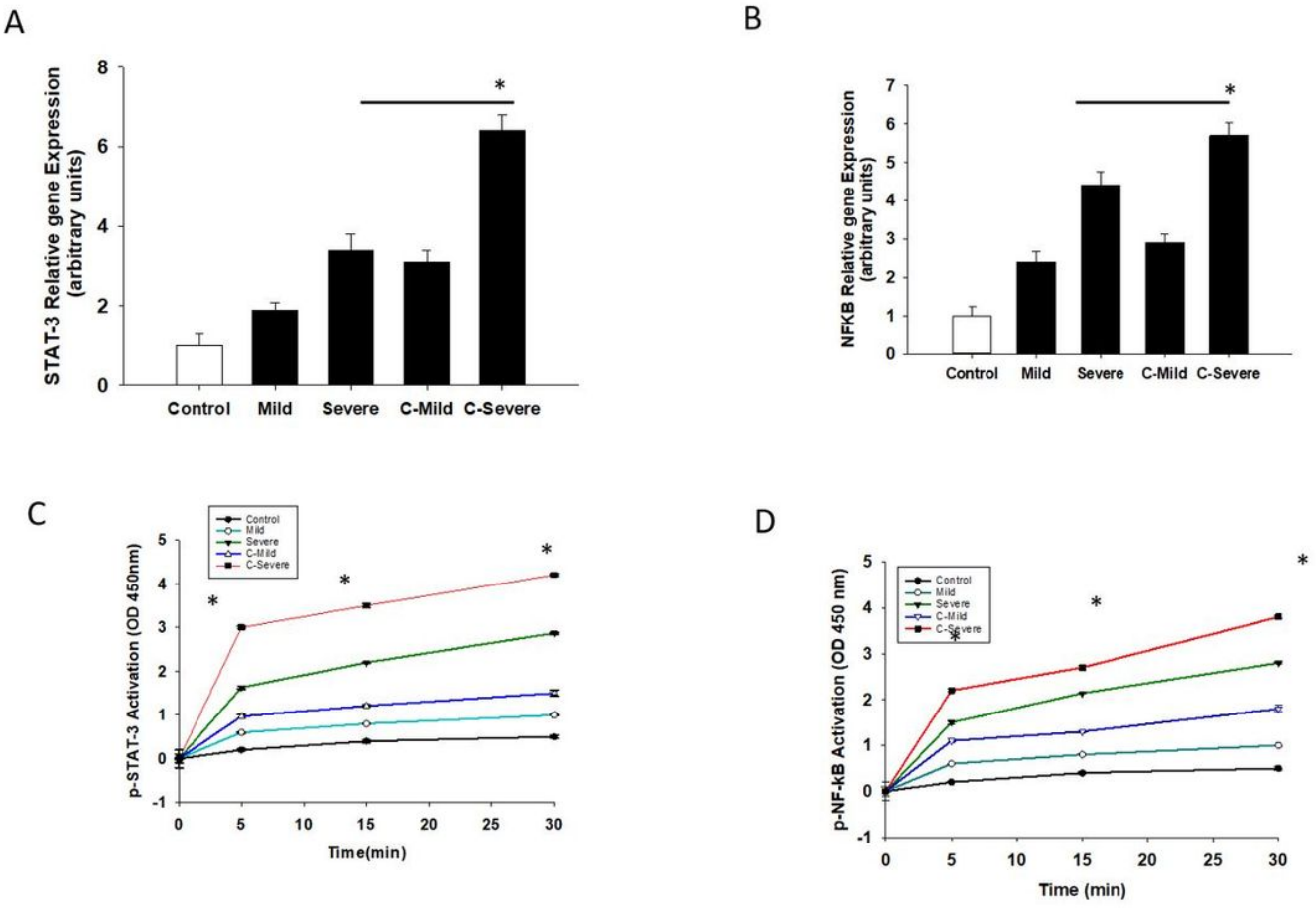

Figure 3

Investigation of downstream signal transduction in COVID-19 Patients. (A) STAT-3 expression in PBMC. (B) NF-kB expression in PBMC (C) Measurement of phosphorylation of STAT-3 after stimulation with IL-10 (100 ng/mL) in nuclear extracts of PBMC (D) Measurement of phosphorylation of NF-kB after stimulation with TNF-alpha (10 ng/mL) in nuclear extracts of PBMC. The data are presented as mean ± SEMs, n = 20-30, *P < 0.05, Severe COVID-19 patients with comorbidities (C-Severe) vs Severe COVID-19 patients without comorbidities.

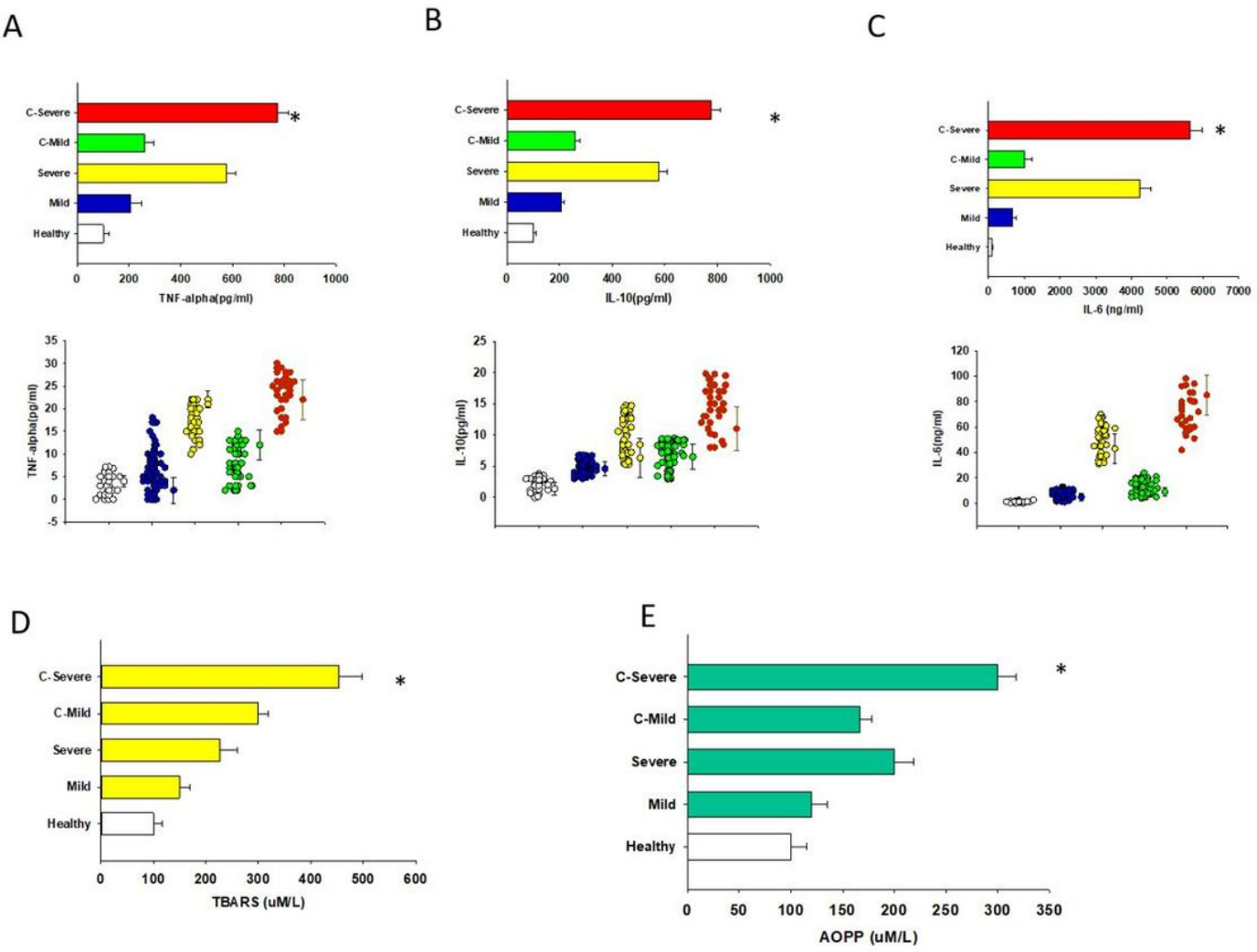

Figure 4

Evaluation of level of inflammatory markers and oxidative stress in COVID-19 Patients. (A) Measurement of blood TNF-alpha levels (B) Measurement of blood IL-10 levels (C) Measurement of blood IL-6 levels (D) Measurement of serum, Thiobarbituric Acid Reactive Substances (TBARS) (E) Measurement of plasma, Advanced Oxidation Protein Products (AOPP). The data are presented as mean ± SEMs, n = 20-30, *$P <$ 0.05, Severe COVID-19 patients with comorbidities (C-Severe) vs Severe COVID-19 patients without comorbidities.

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