JPET Fast Forward. Published on June 30, 2022 as DOI: 10.1124/jpet.122.001209
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Exosomal miR-145 and miR-885 regulate thrombosis in COVID-19

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Manuscript Pages: 37

Figures: 5

Abstract Word Count: 123

Introduction Word Count: 705

Discussion Word Count: 735

Manuscript Word Count: 4198

JPET Fast Forward. Published on June 30, 2022 as DOI: 10.1124/jpet.122.001209
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Running title: Exosomal miRNAs drive thrombosis in COVID-19

Abbreviations

ACE2: angiotensin-converting enzyme 2

COVID-19: coronavirus disease 2019

HUVECs: human umbilical vein endothelial cells

miRs: miRNAs (microRNAs)

TF: tissue factor

TMPRSS2: transmembrane protease serine 2

vWF: von Willebrand factor

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Abstract

We hypothesized that exosomal microRNAs (miRNAs) could be implied in the pathogenesis of thromboembolic complications in COVID-19. We isolated circulating exosomes from COVID-19 patients and then we divided our population in two arms based on the D-dimer level on hospital admission. We observed that exosomal miR-145 and miR-885 significantly correlate with D-Dimer levels. Moreover, we demonstrate that human endothelial cells express the main cofactors needed for SARS-CoV-2 internalization, including ACE2, TMPRSS2, and CD-147. Interestingly, human endothelial cells treated with serum from COVID-19 patients release significantly less miR-145 and miR-885, exhibit increased apoptosis, and display significantly impaired angiogenetic properties compared to cells treated with non-COVID-19 serum. Taken together, our data indicate that exosomal miR-145 and miR-885 are essential in modulating thromboembolic events in COVID-19.

Key words: COVID-19; SARS-CoV-2; coronavirus; long-COVID; non-coding RNA; microRNA; cardiovascular; thromboembolism.

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SIGNIFICANCE STATEMENT

In this work, we demonstrate for the first time that two specific microRNA (namely miR-145 and miR-885) contained in circulating exosomes are functionally involved in thromboembolic events in COVID-19. Our findings are especially relevant to the general audience when considering the emerging prominence of post-acute sequelae of COVID-19 systemic manifestations known as Long-COVID.

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Introduction

COVID-19 has caused an enormous number of deaths due to the poor information on SARS-CoV-2 and its exact mechanisms of action. Substantial progresses have been recently made in science and technology, improving the management of COVID-19 patients (Kaur and Gupta, 2020; Stasi et al., 2020; Tregoning et al., 2020; Ulinici et al., 2021; Gupta et al., 2022; Narhi et al., 2022; Vincent et al., 2022; Weerakkody et al., 2022). However, the war is not over yet, inasmuch as the COVID-19 pandemic leaves substantial aftermaths due to the long-term complications of the disease which are often disabling, reducing the quality of life (Crook et al., 2021; Iqbal et al., 2021; Michelen et al., 2021; Desai et al., 2022; Robineau et al., 2022; Whitaker et al., 2022). Several reports suggest that the risk of death for COVID-19 survivors is higher than the risk associated with other conditions, due at least in part to long-term complications (Davido et al., 2020; Basu et al., 2021; Moreno-Perez et al., 2021; Yang et al., 2021; Zhang et al., 2021b; Comelli et al., 2022; Smith, 2022). Of note, the number of deaths for long-term COVID-19 complications has not necessarily been recorded as deaths due to COVID-19, therefore the actual situation could be worse than what reported. In this context, the identification of useful biomarkers of fatal complications is sorely needed.

The symptoms of COVID-19 patients vary greatly, ranging from an asymptomatic state to debilitating respiratory failure due to bilateral pneumonia (Calica Utku et al., 2020; Iacobucci, 2022; Kaliszewski et al., 2022). Patients can also develop a systemic inflammatory state that favors multi-organ failure and increases susceptibility to systemic thromboembolic complications that may contribute to a rapid clinical deterioration (Mui et

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al., 2021). Arterial thrombotic events include end-organ ischemia to systemic organs, cerebrovascular accidents, and limb ischemia, and are associated with high D-dimer, prolonged PT, and elevated levels of fibrinogen, which indicate activation of coagulation pathways and thrombosis (Li et al., 2020; Gambardella et al., 2021). D-Dimer, in particular, is one of the most sensitive coagulation parameters in COVID-19 and indicates a greater risk for the development of thrombosis (Zhang et al., 2020; Conte et al., 2021; Ozen et al., 2021; Poudel et al., 2021). Thus, D-Dimer measurement is currently considered a critical approach in the clinical management of COVID-19 (Rostami and Mansouritorghabeh, 2020; Conte et al., 2021; Ghosh and Ghosh, 2022).

A finding that emerged from the intensive research on COVID-19 was that endothelium is a key target organ of COVID-19. We were among the first groups to describe the involvement of endothelial dysfunction in COVID-19 (Sardu et al., 2020) and successively both clinical and preclinical evidence supported our finding (Otifi and Adiga, 2022). The endothelium is instrumental in thrombosis, fibrinolysis, inflammation, and in maintaining a proper vasodilator/vasoconstrictor and antioxidant/pro-oxidant balance (Wu and Thiagarajan, 1996; Libby et al., 2006; Gambardella et al., 2020; Adebayo et al., 2021); therefore, an impaired endothelial function could be the mechanism underlying the systemic complications of COVID-19, especially but not exclusively thromboembolic events (Sardu et al., 2020; Adebayo et al., 2021).

Direct effects of COVID-19 as well as indirect effects of the infection (inflammation, hypoxia) might predispose patients to thrombotic events (Bikdeli et al., 2020) which then play a decisive role in the clinical outcome (Cryer et al., 2022). Thus, an early biomarker

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of the development of thromboembolic events and predictor of associated clinical outcomes could be useful for a timely intervention with targeted therapies.

In this context, measuring the levels of microRNAs (miRNAs) within extracellular vesicles (EVs) represents a useful strategy as diagnostic and prognostic biomarker in numerous disease states (Guo et al., 2020; Cho et al., 2021; He et al., 2021; Lyu et al., 2021; Ning et al., 2021; Ueta et al., 2021; Ying et al., 2021; Zhang et al., 2021a; Lin et al., 2022; Mahmoudi et al., 2022; Wang et al., 2022). The microRNA cargo of extracellular vesicles could not only contribute to the pathogenesis of thrombotic and thromboembolic complications of COVID-19 but most likely also be a diagnostic/prognostic marker. We recently identified a significant association linking endothelial exosomal miR-24 and cerebrovascular disorders, indicating that this approach could be an extremely useful tool for diagnosis and prognosis (Gambardella et al., 2021). Herein, we aim at identifying specific circulating miRNAs associated with thromboembolic events in COVID-19 patients.

Materials and Methods

To test our hypothesis that exosomal miRNAs are a major determinant of thrombosis in COVID-19, we enrolled 26 patients positive for COVID-19 admitted to the Sant'Anna and San Sebastiano Hospital of Caserta and Naples University (Italy). The serum from 10 non-COVID-19 subjects was used as control. All subjects underwent a SARS-CoV-2 test by RT-qPCR to rule out or confirm the COVID-19 diagnosis. The study was conducted

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in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments; we obtained a written informed consent from all participants or their legal representatives.

We isolated circulating exosomes from equal amounts of serum, as previously reported by our research group (Gambardella et al., 2021). Purity and absence of contamination were assessed by immunoblot (Wang et al., 2020b), whereas morphology and size distribution were examined by dynamic light scattering and electron microscopy (not shown). Levels of miRNAs were quantified by RT-qPCR (Morelli et al., 2019; Wang et al., 2020b; Gambardella et al., 2021).

In vitro experiments

We performed in vitro assays in human umbilical vein endothelial cells (HUVECs), cultured in F12 medium enriched with specific growth factors for endothelial growth (Lonza). The cells were cultured at 37 °C in 95% air and 5% CO_2. All experiments were performed at least in triplicate using cells between passages 5 and 9. The experimental protocol on HUVECs consisted of 24h incubation with serum from COVID-19 patients or from SARS-COV-2 negative patients as control. The serum was used with a final dilution of 1:50 directly in the medium.

Angiogenesis assay

The formation of network-like structures by HUVECs on an extracellular matrix (ECM)-like 3D gel was performed as previously described (Santulli et al., 2011; Gambardella et

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al., 2018). HUVECs (5×10^4) were seeded on Matrigel Matrix and incubated at 37°C for 24 h, in presence of COVID-19 serum or control serum.

Lipid peroxidation assay

The level of malondialdehyde (MDA) was measured by using a lipid peroxidation assay Kit (#ab118970, Abcam, Cambridge, UK), as we previously described (Tang et al., 2021).

Immunoblot analysis

Total lysates were prepared as we described (Sorriento et al., 2009). Immunoblot analyses were performed as previously reported (Wang et al., 2020b). Briefly, lysates were electrophoresed by SDS/PAGE and transferred to nitrocellulose. Angiotensin converting enzyme 2 (ACE2), transmembrane protease serine 2 (TMPRSS2), C147, cleaved caspase 3, and GAPDH, were visualized by specific antibodies (Cell Signaling Technology, Danvers, MA), while fluorochrome-conjugated anti-rabbit and anti-mouse were used as secondary antibodies (LI-COR, Lincoln, NE). The nitrocellulose membrane with fluorescent signal was scanned using the LI-COR imaging system, as we described (Matarese et al., 2020a; Dridi et al., 2022).

Statistical Analysis

Data are expressed as means ± SE. All data were analyzed using GraphPad Prism version 9 (GraphPad by Dotmatics, Boston, MA) with a significant difference established at a p-value <0.05. The normal distribution of values was verified by the

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Shapiro-Wilk test; the Student's 2-tailed t-test was applied to compare values between groups; the correlation between miRNA and D-Dimer levels was determined by Pearson's correlation analysis.

Results

miR-145 and miR-885 downregulation was associated with thrombotic risk and mortality in COVID-19 patients

To verify our hypothesis that exosomal miRNAs play a crucial role in the pathogenesis of thrombosis in COVID-19, we divided our population in two groups based on the serum D-dimer level on hospital admission, using a cut-off of 3 μg/ml. We did not detect any significant differences in the main clinical characteristics when comparing patients with low versus high D-Dimer. Strikingly, we found that exosomal miR-145, and miR-885 were significantly downregulated in the subjects in the high D-dimer arm compared to subjects in the low D-Dimer arm (**Fig. 1A**-**B**).

A Pearson's correlation analysis confirmed these findings (**Fig. 1C-D**). Furthermore, when we dichotomized our COVID-19 population in survivors and non-survivors, we noted that the levels of both miR-145 and miR-885 were significantly lower in the patients who did not survive (**Fig. 1E-F**). These data indicate that the downregulation of miR-145 and miR-885 is associated to worst prognosis, correlating with a higher

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thrombotic risk and mortality in COVID-19. It is noteworthy that miRs were assessed on hospital admission, hence their alterations already at time 0 denote miR-145 and miR-885 as powerful prognostic predictors of adverse outcome.

Endothelial dysfunction as responsible for miR-145 and miR-885 downregulation

Endothelial cells are leading actors in producing and releasing Tissue Factor (TF) and von Willebrand Factor (vWF), thus regulating the thrombotic cascade. We hypothesized that the endothelial dysfunction occurring during SARS-CoV-2 infection, determines a dysregulation of this mechanism, reducing the capability of endothelial cells to release miR-145 and miR-885 and resulting in an uncontrolled coagulation. To test this hypothesis we employed human endothelial cells (HUVECs) as an in vitro model, and exposed them to serum from COVID-19 patients, compared to HUVECs exposed to serum from patients negative for SARS-CoV-2.

Importantly, HUVECs expressed all the major SARS-CoV-2 receptors, including ACE2, TMPRSS2, and CD-147 (Matarese et al., 2020b; Wang et al., 2020a; Zipeto et al., 2020; Evans and Liu, 2021), providing a further evidence of endothelial cells susceptibility to SARS-CoV-2 infection (**Fig. 2A**). This expression pattern of viral receptors also confirms that our in vitro setting is a valuable model to study endothelial response and damage in COVID-19 context.

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The exposure to COVID-19 serum induces apoptosis of endothelial cells, as indicated by caspase 3 activation (**Fig. 2B-D**), supporting the hypothesis of a significant endothelial damage.

We further explored the endothelial dysfunction evaluating a key phenotype of viable endothelial cells, the angiogenic competence. Consistently, we observed an impaired capacity of forming network-like structures on Matrigel, indicating that COVID-19 serum exposure compromises the angiogenic capacity of endothelial cells (**Fig. 3A-B**).

In order to assess the effects of COVID-19 on lipid peroxidation, an established hallmark of severity in COVID-19 patients (Lage et al., 2021; Martin-Fernandez et al., 2021; Zarkovic et al., 2021; Soto et al., 2022), we measured MDA level in HUVEC lysate and we observed a significantly increased peroxidation in the lysate for endothelial cells incubated for 24 hours with COVID-19 serum compared to cells incubated with non-COVID-19 serum (**Fig. 4**).

Then, we verified whether human endothelial cells were able to produce miR-145 and miR-885 and if such production was regulated by COVID-19. We detected both miRNAs in the HUVEC lysate; interestingly, the levels of both miRNAs decreased in response to COVID-19 serum exposure (**Fig. 5A-B**). This phenomenon confirms that endothelial cells are able to actively release these miRNAs and that stress condition can affect miR-145 and miR-885 production by endothelial cells.

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Discussion

One of the main findings of the present study is the identification of two miRNAs, miR-145 and miR-885, as potential predictors of thrombotic risk in COVID-19 patients. Indeed, miR-145 and miR-885 are significantly downregulated in COVID-19 patients with high D-Dimer, and the correlation analysis confirms that miR-145 and miR-885 inversely correlate with D-Dimer. Moreover, the downregulation of these two miRNAs seems to predispose or at least to predict mortality in COVID-19 patients. Indeed, the group of patients that died for COVID-19 showed lower levels of both miR-145 and miR-885 at baseline. These data unveil the clinically relevant predictor value of these miRNAs, also suggesting a causal implication of these molecules in determining higher thrombotic risk and mortality in COVID-19 patients. Consistently, these two miRNAs are implicated in the coagulation pathway; indeed, Tissue Factor (TF) has been identified as a direct target of miR-145 (Sahu et al., 2017), while miR-885 targets the von Willebrand Factor (vWF) (Zhang et al., 2019). Consistent with our findings, the downregulation of these two miRs should promote higher levels of TF and vWF, thus determining a prothrombotic state. By modulating these two factors endothelial cells can regulate the activation of the coagulation cascade. One limitation of our study is that we did not determine the exact source of exosomes in our population; nevertheless, since endothelial dysfunction is a prominent feature of COVID-19, functionally contributing to the pro-inflammatory and pro-thrombotic state of the vasculature (Bikdeli et al., 2020), we speculate that at least one of the main sources of exosomes could be represented by endothelial cells, which indeed do express these miRNAs in normal conditions (Santulli, 2016). To test this hypothesis we performed in vitro experiments on human

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endothelial cells. Specifically, we set an in vitro model where we verified the expression of cofactors needed for the internalization of SARS-CoV-2 in host cells, and we explored endothelial stress responses to a “COVID-like environment” represented by serum from COVID-19 patients. We found that COVID-19 serum (collected on hospital admission) induces endothelial damage including cell apoptosis and alterations of a specialized endothelial feature like angiogenic capacity. This is in line with numerous reports from we and others supporting endothelial involvement in COVID-19 clinical manifestations (Gu et al., 2020; Libby and Luscher, 2020; Perea Polak et al., 2020; Teuwen et al., 2020; Fiorentino et al., 2021; Gambardella and Santulli, 2021; Mesquida et al., 2021; Mone et al., 2021; Qin et al., 2021; Schmaier et al., 2021; Yin et al., 2021; Kelliher et al., 2022; Mone et al., 2022; Otifi and Adiga, 2022; Robles et al., 2022).

One of the main new findings unveiled here is that the dysfunctional endothelium could participate to thrombotic manifestations by changes of the miRNA production profile. As proposed here, endothelial cells physiologically produce miR-145 and miR-885 to target vWF and TF blocking their release and controlling coagulation. Indeed, we observed that endothelial cells are able to produce these miRNAs and under stress condition (*i.e.* when exposed to COVID-19 serum), their levels significantly decrease. Consistently, in patients in which these adaptive mechanisms could be compromised, the resultant lower availability of miR-145 and miR-885 predispose to a detrimental prothrombotic status, denoted by high D-Dimer, and eventually high mortality. We reckon that we did not completely prove this mechanism, however our data in humans and in vitro are highly suggestive of this view. Certainly, this report further exposes the endothelium as

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a central player in orchestrating the adaptative and maladaptive response to SARS-CoV-2 infection, acting as a main trigger of thrombotic manifestations.

Consistent with our findings, other investigators have emphasized the importance of miRs and other non-coding RNAs in the management of COVID-19 patients (Amini-Farsani et al., 2021; Battaglia et al., 2021; Dash et al., 2021; Farr et al., 2021; Lukiw, 2021; Narozna and Rubis, 2021; Plowman and Lagos, 2021; Saha et al., 2021).

Our data can be useful also for the management and treatment of long COVID; in fact, the so-called “Long COVID-19 Syndrome”, indicating the set of disorders and clinical manifestations that remain long after COVID-19 infection, is emerging as the next challenge in biomedical research (Yan et al., 2021; Antoniou et al., 2022; Cattadori et al., 2022; Desai et al., 2022; Martinez-Salazar et al., 2022; Murray et al., 2022; Oikonomou et al., 2022; Thye et al., 2022). Henceforward, the deep understanding of the molecular mechanisms underlying the major complications of COVID-19 will allow us to intervene and tackle Long-COVID.

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Acknowledgments

We thank Dr. Varzideh for insightful discussion.

Author Contributions

Participated in research design: Gambardella, and Santulli.

Conducted experiments and *contributed new reagents or analytic tools:* Gambardella, Kansakar, Messina, Jankauskas, Marfella, Maggi, Mone, Paolisso, and Sorriento.

Performed data analysis: Gambardella, Kansakar, and Santulli.

Wrote or contributed to the writing of the manuscript: Gambardella, Kansakar, Sorriento, and Santulli.

All authors contributed to the article and approved the submitted version.

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JPET Fast Forward. Published on June 30, 2022 as DOI: 10.1124/jpet.122.001209
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Footnotes

Funding

The Santulli's Lab is supported in part by the National Institutes of Health (NIH): National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK: R01-DK123259, R01-DK033823), National Heart, Lung, and Blood Institute (NHLBI: R01-HL159062, R01-HL146691, T32-HL144456) to G.S., by the Diabetes Action Research and Education Foundation (to G.S.), and by the Monique Weill-Caulier and Irma T. Hirschl Trusts (to G.S.). J.G. and S.S.J. are supported by a postdoctoral fellowship of the American Heart Association (AHA-20POST35211151 and AHA-21POST836407, respectively).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

JPET Fast Forward. Published on June 30, 2022 as DOI: 10.1124/jpet.122.001209
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Figure legends

Figure 1. **miR-145 and miR-188 are associated with a higher risk of thrombosis and mortality in COVID-19 patients**.

Expression level of exosomal miRNAs in COVID-19 patients. Different levels of miRNAs (miR-145, miR-885) in patients with high D-Dimer and low D-Dimer (**A-B**); in the violin plots, median (solid line) and quartiles (dotted lines) are indicated. The trend was confirmed by linear regression analysis (**C-D**). In panels E and F, miRNAs levels are quantified as mean±SE among survivors and non survivors (**E-F**); *: $p<0.001$.

Figure 2. Endothelial cell damage induced by COVID-19 environment.

Representative immunoblots showing the expression of the main viral receptors on human endothelial cells (**A**). The exposure of HUVECs to COVID-19 serum induces the cleavage of caspase 3, indicating apoptosis activation (**B**), quantified in panels **C** and **D**. Data are from triplicate experiments; *: $p<0.001$.

Figure 3. Endothelial cell angiogenic capacity is impaired by COVID-19 environment.

Angiogenesis assay on Matrigel. COVID-19 serum significantly affects the angiogenic capacity of endothelial cells. Representative pictures of network-like formation (dimensional bar: 100 μm) from independent triplicate experiments (**A**) and relative quantification (**B**); *: $p<0.001$.

JPET Fast Forward. Published on June 30, 2022 as DOI: 10.1124/jpet.122.001209
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Figure 4. COVID-19 environment induces lipid peroxidation.

We evaluated lipid peroxidation by measuring the level of malondialdehyde (MDA) in HUVEC lysate. Data are from triplicate experiments; *: $p<0.001$.

Figure 5. Endothelial cells produce miR-145 and miR-885, and COVID-19 serum affects their levels.

Quantification of miR-145 and miR-855 in HUVEC lysate, after incubation with the indicated sera for 24 h. Data are from triplicate experiments; *: $p<0.001$.

Figure 1

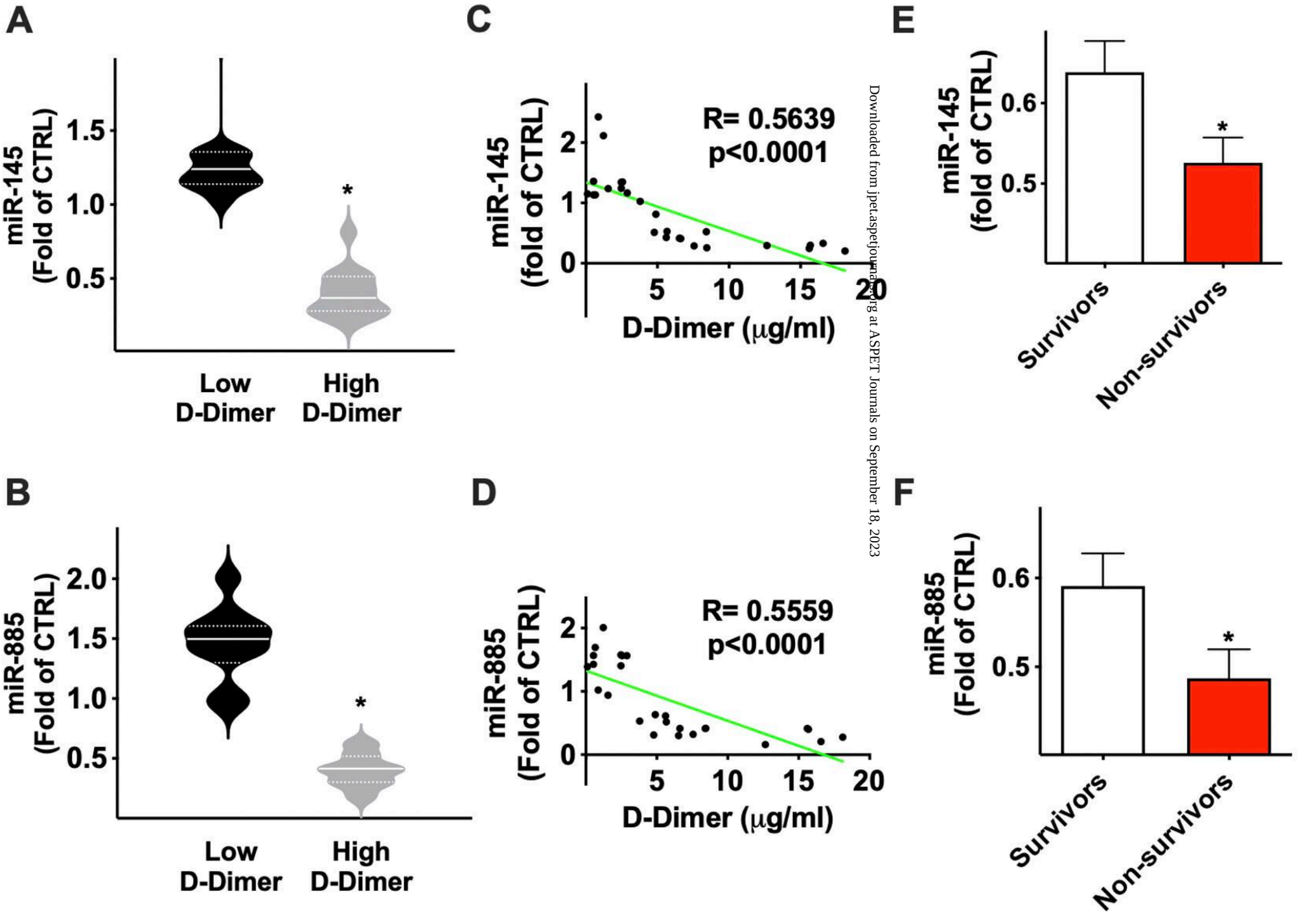


Figure 2

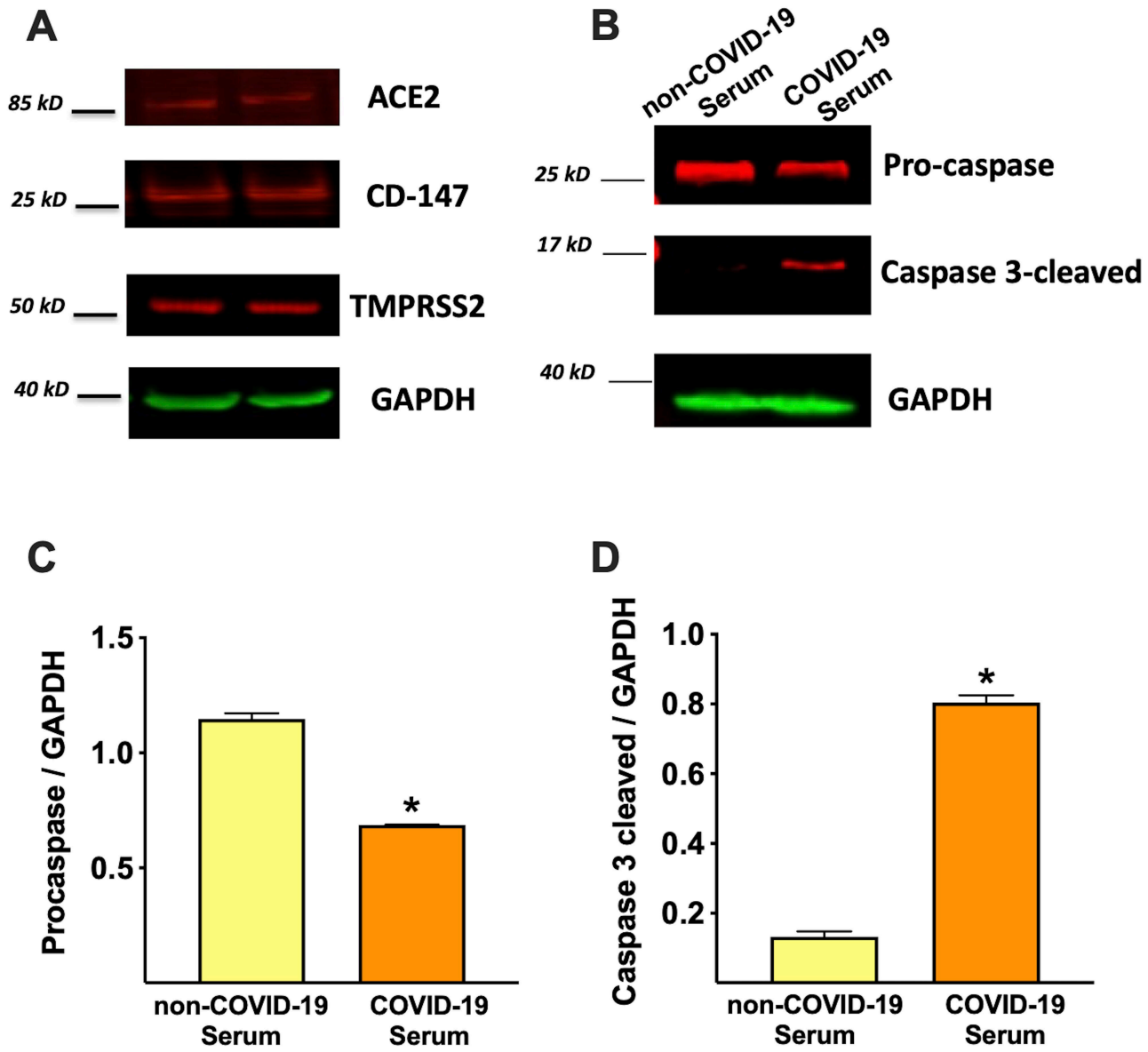

A
85 kD
ACE2
25 kD
CD-147
50 kD
TMPRSS2
40 kD
GAPDH
B
non-COVID-19 Serum
COVID-19 Serum
25 kD
Pro-caspase
17 kD
Caspase 3-cleaved
40 kD
GAPDH
C
Procaspase / GAPDH
1.5
1.0
0.5
*
non-COVID-19 Serum
COVID-19 Serum
D
Caspase 3 cleaved / GAPDH
1.0
0.8
0.6
0.4
0.2
*
non-COVID-19 Serum
COVID-19 Serum

Figure 3

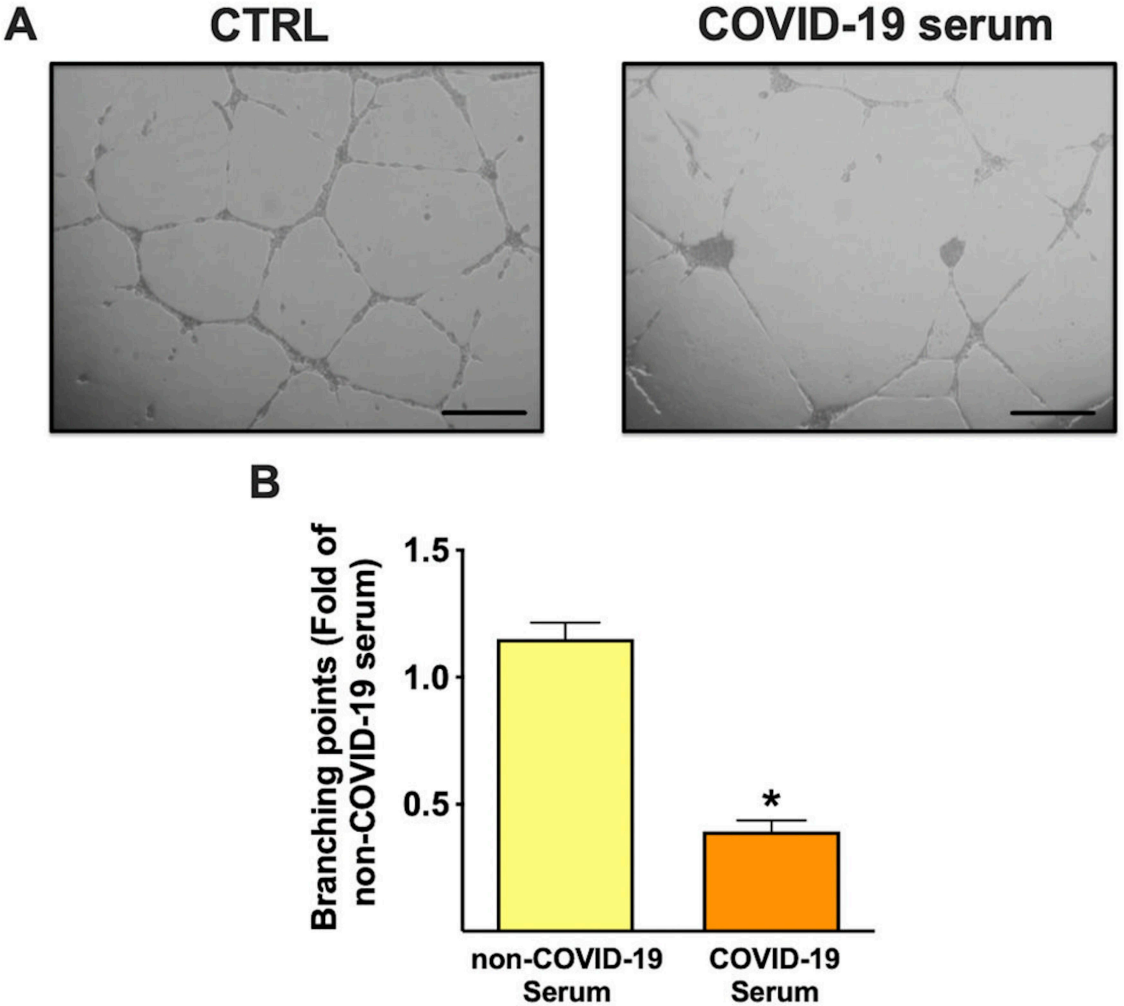

A
CTRL
COVID-19 serum
B
Branching points (Fold of non-COVID-19 serum)
1.5
1.0
0.5
*
non-COVID-19 Serum
COVID-19 Serum

Figure 4

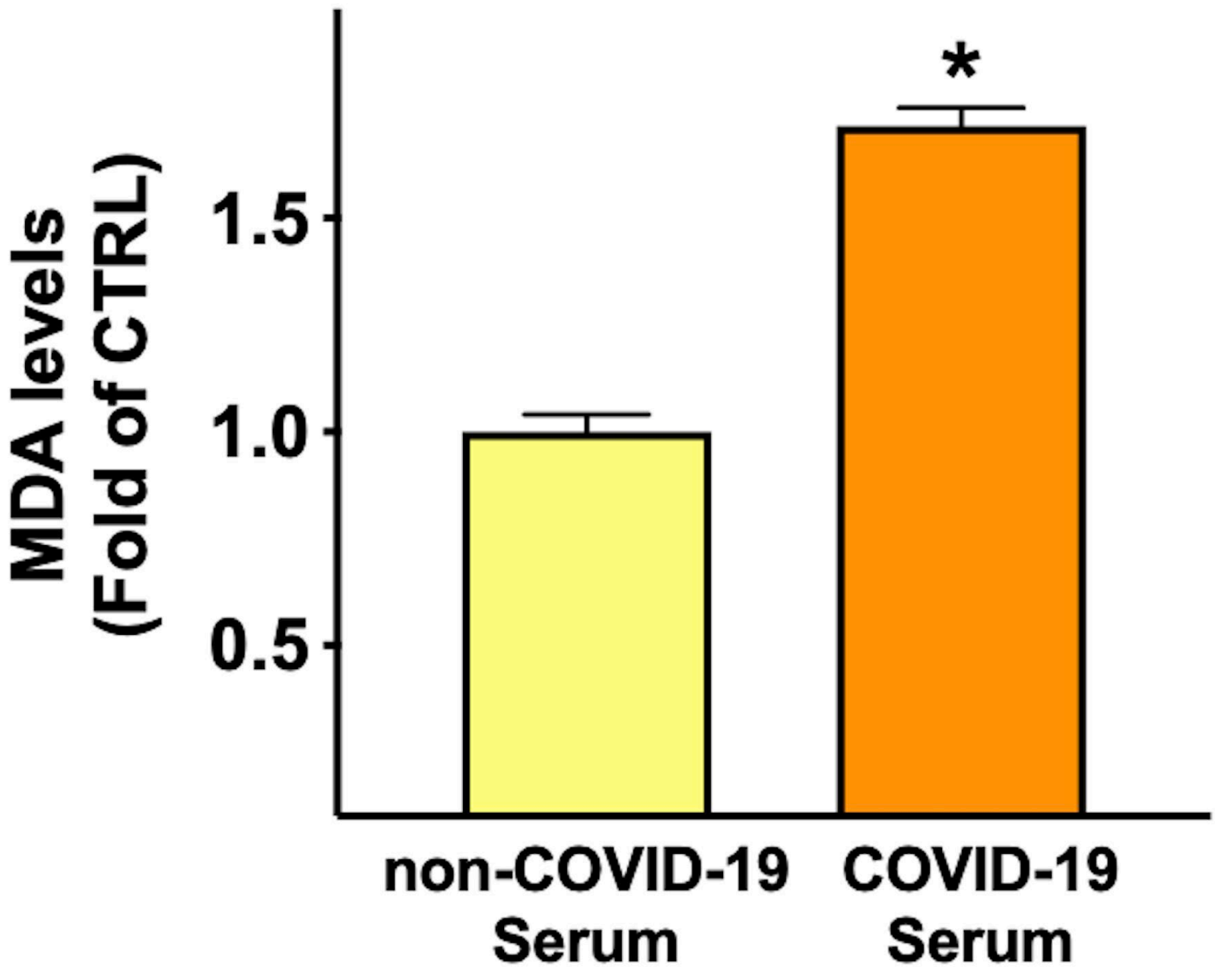
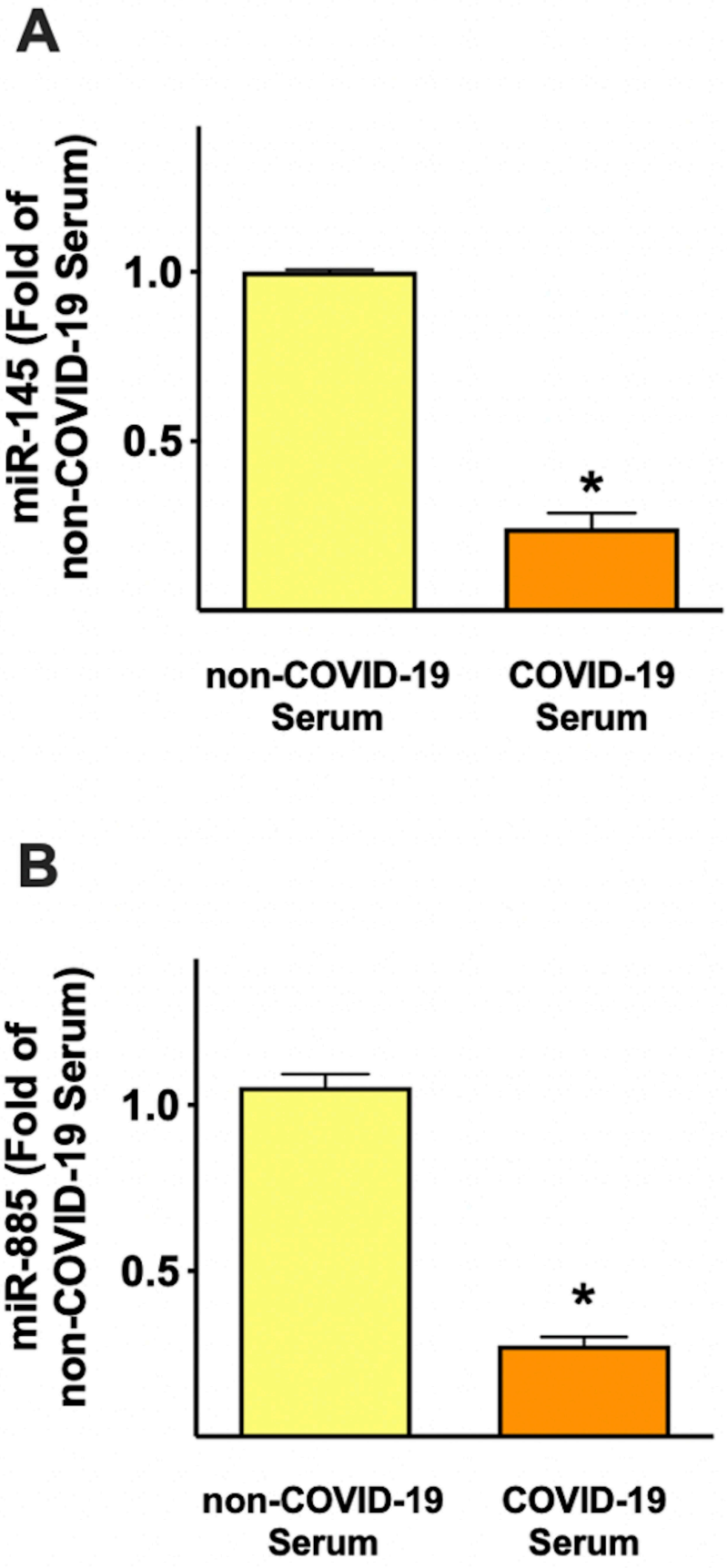

MDA levels
(Fold of CTRL)
1.5
1.0
0.5
*
non-COVID-19
Serum
COVID-19
Serum

Figure 5


A
miR-145 (Fold of non-COVID-19 Serum)
1.0
0.5
*
non-COVID-19 Serum
COVID-19 Serum
B
miR-885 (Fold of non-COVID-19 Serum)
1.0
0.5
*
non-COVID-19 Serum
COVID-19 Serum