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Research article

Ephrin-A1 and the sheddase ADAM12 are upregulated in COVID-19

Rachelle Mendoza [a], Nayanendu Saha [b], Amir Momeni [c], Elmer Gabutan [a], Mouyed Alawad [a], Amir Dehghani [a], John Diks [a], Bo Lin [a], Donghai Wang [a], Mohamed Alshal [a], William Fyke [a], Bingcheng Wang [d], Juha P. Himanen [b], Prem Premsrirut [e,f], Dimitar B. Nikolov [b,*]

[a] Department of Pathology, SUNY Downstate Health Sciences University, 450 Clarkson Avenue, Brooklyn, NY 11203, USA
[b] Structural Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
[c] Brigham and Women's Hospital, 75 Francis St, Boston, MA 02115, USA
[d] Rammelkamp Center for Research, Department of Medicine, MetroHealth Medical Center, 2500 MetroHealth Drive, Cleveland, Ohio 44109, USA
[e] Department of Cell Biology, SUNY Downstate Health Sciences University, 450 Clarkson Avenue, Brooklyn, NY 11203, USA
[f] Mirimus Inc., 760 Parkside Ave, Brooklyn, NY 11226, USA

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ABSTRACT

More than 3.5 million people have died globally from COVID-19, yet an effective therapy is not available. It is, therefore, important to understand the signaling pathways that mediate disease progression in order to identify new molecular targets for therapeutic development. Here, we report that the blood serum levels of ephrin-A1 and the sheddase ADAM12 were significantly elevated in COVID-19 patients treated at SUNY Downstate Hospital of Brooklyn, New York. Both ephrin-A1 and ADAM12 are known to be involved in inflammation and regulate endothelial cell permeability, thus providing a gateway to lung injury. The clinical outcome correlated with the ephrin-A1 and ADAM12 serum levels during the first week of hospitalization. In contrast, the serum levels of TNFα were elevated in only a small subset of the patients, and these same patients also had highly elevated levels of the sheddase ADAM17. These data indicate that ephrin-A1-mediated inflammatory signaling may contribute to COVID-19 disease progression more so than TNFα-mediated inflammatory signaling. They also support the notion that, in COVID-19 inflammation, ADAM12 sheds ephrin-A1, while ADAM17 sheds TNFα. Furthermore, the results suggest that elevated serum levels and activity of cytokines, such as TNFα, and other secreted inflammatory molecules, such as ephrin-A1, are not simply due to overexpression, but also to upregulation of sheddases that release them into the blood circulation. Our results identify ephrin-A1, ADAM12, and other molecules in the ephrin-A1 signaling pathway as potential pharmacological targets for treating COVID-19 inflammation.

1. Introduction

After being declared a pandemic by the World Health Organization (WHO) in March 11, 2020, COVID-19 has taken an unparalleled increase in the number of cases worldwide. According to the Johns Hopkins University Coronavirus Resource Center, by the end of April 2021 there were over 150 million global confirmed cases and 3.1 million deaths. The United States is leading in the cumulative number of cases, followed by India and Brazil. The causative agent, SARS-CoV-2, belongs to the subfamily of β-coronaviruses and shares 79.5% genetic sequence with the SARS-CoV virus that caused the epidemic in 2002–2004. The spike protein of coronaviruses facilitates viral entry into target cells that depends on binding of its S1 subunit to a cellular receptor, the Angiotensin 2 Converting Enzyme (ACE2) [1, 2, 3]. COVID-19 patients with severe presentation are reported to develop decreased lymphocyte count and interstitial pneumonia with high levels of pro-inflammatory cytokines, including IL-2, IL-2R, IL-6, IL-7, IL-8, IL-10, G-CSF, IP-10, MCP-1, MIP-1α and TNFα [4, 5, 6].

The Eph receptors are the largest family of receptor tyrosine kinases [7] and emerging evidence suggests that the Ephs and their ephrin ligands play important roles in injury [8], inflammation [9] and immunity [10, 11]. In particular, ephrin-A1 and EphA2 regulate endothelial cell permeability during inflammation [12] and vascular permeability in the lungs [13]. The EphA2/ephrin-A1-mediated upregulation of vascular inflammation was, for example, found to be crucial in atherosclerosis formation [14]. Ephrin-A1 was also reported to be important for both CD4+ and CD8+ T cells migration [15, 16]. Furthermore, ephrin-A1 was reduced in asthma patients and was shown to regulate IL2 and IL4

* Corresponding author.
E-mail address: nikolovd@mskcc.org (D.B. Nikolov).

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expression and Th2 cell activation [17]. Ephrin-A1 was initially identified as a TNFα–responsive gene in endothelial cells, further highlighting its importance in inflammatory responses [18].

ADAMs (A Disintegrin And Metalloproteases) are transmembrane metalloproteases that process and shed the ectodomains of membrane-anchored growth factors, cytokines and receptors [19]. Many ADAMs (or 'sheddases') are overexpressed in various tumors, as represented by ADAM12 overexpression in glioblastoma multiforme [20]. It was previously shown that ADAM12 cleaves the membrane attached ephrin-A1, releasing it in soluble, monomeric form in the blood circulation, which facilitates lung metastasis [21]. ADAM12 was also reported to be involved in tissue inflammation [22]. Another family member, ADAM17 (initially termed TNFα-converting enzyme or TACE), modulates the signaling activity of a variety of cytokines, including TNFα [23]. TNFα is a key proinflammatory cytokine contributing to various acute and chronic inflammatory pathologies, and several studies reported high levels of TNFα, among other pro-inflammatory cytokines, in the serum of critically ill COVID-19 patients [4]. The efficacy of TNFα inhibition in COVID-19 has been fully investigated but preliminary results did not show superiority over other anti-inflammatory treatments, such as steroids.

Although ephrin-A1 is known to mediate inflammation, particularly in the lungs [24], where it is shed by ADAM12 [21], the precise biological functions of both ephrin-A1 and ADAM12 in lung physiology and inflammation are still largely unknown. In addition, their possible roles in COVID-19 disease progression have not been investigated. We, therefore, measured the levels of ephrin-A1 and ADAM12 in the serum of the COVID-19 patients treated in The SUNY Downstate Hospital in Brooklyn, New York. We also measured the serum levels of TNFα, which was previously shown to be involved in COVID-19 inflammation, and of its sheddase ADAM17.

2. Results

2.1. Ephrin-A1 serum levels are significantly elevated in COVID-19 patients

The characteristics of the patients (Table 1) are described in *Methods*. When compared to healthy controls, ephrin-A1 levels in the sera were significantly elevated in all of the patients across all disease severity groups (Kruskal-Wallis p-value: 0.02, Dunn's test Z values of 2.14, 2.95 and 2.65 in mild, severe and critically ill patients, respectively (Z-values >1.96 are significant); Figure 1A). The median ephrin-A1 level was highest in the severe patient group, and lowest in the mild patient group. For healthy individuals, the median overall measured serum level was 1185.3 pg/ml (Interquantile range (IQR): 1100.6–1556.8), as compared to average levels observed in critical (3599.9 pg/ml; IQR: 2742.6–4548.9), severe (3896.7 pg/ml; IQR: 3526.1–5300.6) and mild (3013.2 pg/ml; IQR: 2774.9–3333.5) patients. These levels are in good agreement with previous reports on ephrin-A1 levels in healthy and diseased individuals, including a study of diabetic retinopathy patients that reported plasma ephrin-A1 levels that were elevated to a similar extend (1520 pg/ml average in human plasma and 3630 pg/ml average in diabetic retinopathy patients) [25]. Interestingly, patients suffering from diabetic retinopathy present with blood vessel damage, which was also reported in critically ill COVID-19 patients [26]. Furthermore, diabetes mellitus was found to be a common co-morbidity among hospitalized COVID-19 patients [4].

In analyzing the time course progression, ephrin-A1 levels remained higher than normal throughout the disease course (Figure 1B and C). Mild patients generally displayed a downward trend from the beginning, while severe and critically ill patients had peak in ephrin-A1 serum levels in days 3–7 post-admission followed by a downward trend. Ephrin-A1 levels, particularly during the first week of hospitalization (Figure 2A), were higher in the patients who died of the disease compared to the patients who survived (Figure 1D), but this difference was short of statistical significance. However, when an adverse outcome of either death or prolonged hospitalization (>30 days) was considered, higher ephrin-A1 day 3 levels showed a strong correlation with adverse outcomes (Mann Whitney p value = 0.033) (Figure 3A).

2.2. ADAM12 serum levels are also significantly elevated in COVID-19 patients

Ephrin-A1 is initially expressed and predominantly functions as a cell-surface protein, but it has been reported that in the lungs, the metalloprotease ADAM12 cleaves ephrin-A1 off the cell membrane, promoting its entry into the blood circulation [21]. We, therefore, also measured the levels of soluble ADAM12 in the serum of the COVID-19 patients. Our results show that, when compared to healthy controls, ADAM12 levels were significantly elevated in all COVID-19 patients across all disease severity groups (Kruskal-Wallis p-value: 0.015, Dunn's test Z values of 3.22, 2.45 and 2.40 in mild, severe and critically ill patients, respectively (Z-values >1.96 are significant; Figure 4A). For healthy individuals, the median overall measured level was 146.7 pg/ml (IQR: 84–151.4), which was significantly lower as compared to the median levels observed in mild (13494.9 pg/ml; IQR: 5400.5–17462.2), severe (3017 pg/ml; IQR: 2866–8839.7) and critically ill (3166.1 pg/ml; IQR: 1394.5–8260.4) patients. Interestingly, in contrast to ephrin-A1, during the first week of hospitalization, ADAM12 levels were lower in critically ill patients as compared to patients with mild clinical course (Figure 2B, Mann Whitney p value = 0.018). This suggests that highly elevated ADAM12 levels in the early stages of infection might have some protective effect.

Table 1. Baseline characteristics of patients.

	All patients (N = 31)	Mild (N = 6)	Severe (N = 11)	Critical (N = 14)
Age ([median (IQR)]	62 y (52–76 y)	61 (55.25–72)	57 (53–62.5)	63.5 (61.25–70)
<50 [N (%)]	2 (6.5)	0 (0)	2 (100)	0 (0)
50–70 [N (%)]	22 (71.0)	4 (18.2)	7 (31.8)	11 (50)
>70 [N (%)]	7 (22.6)	2 (28.6)	2 (28.6)	3 (42.9)
Sex				
Male	16 (51.6)	1 (6.3)	8 (50.0)	7 (43.8)
Female	15 (48.4)	5 (33.3)	3 (20)	7 (46.7)
Race				
Black	24 (77.4)	6 (25.0)	9 (37.5)	9 (37.5)
White	5 (16.1)	0 (0)	2 (40)	3 (60)
Asian	1 (3.2)	0 (0)	0 (0)	1 (100)
Undisclosed	1 (3.2)	0 (0)	0 (0)	1 (100)

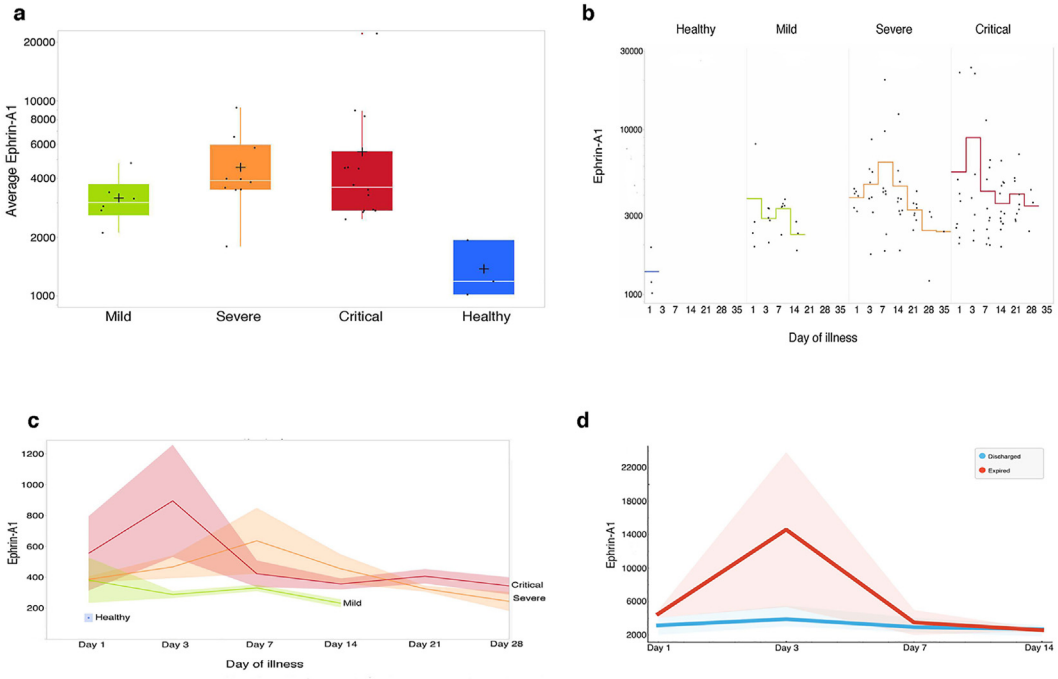

Figure 1. Ephrin-A1 serum levels were elevated across all COVID-19 patients compared to healthy controls. A) Ephrin-A1 serum levels in COVID-19 patients and healthy controls. Green - mild patients, orange - severe patients, red - critical patients, blue - healthy controls. Each dot represents the average ephrin-A1 level for one patient. In the box-and-whisker plot, the white horizontal lines indicate the median, the top and bottom of the boxes - interquartile range, the whiskers - the range, and the black crosses represent the mean (average) values. B) Time progression of ephrin-A1 levels in the serum of COVID-19 patients. The dots represent the individual patient values for ephrin-A1 for each time point and the lines represent the average values for all patients in each time point. Colors are as in Panel A. C) Another representation of the time progression of ephrin-A1 serum levels in COVID-19 patients. The solid lines represent the average value for each patient group in each time point and the shaded areas indicate the standard deviation (SD). Colors are as in Panel A. D) Higher ephrin-A1 levels during the first week of illness were observed among those who died from COVID-19 than among those who recovered. Blue line - average levels in discharged patients, red line - average levels in expired patients. The solid lines represent the average values in each time point and the shaded areas indicates the SD.

In analyzing the time course progression, ADAM12 levels remained higher than normal throughout the disease course (Figure 4B and C). The patients generally had a peak in ADAM12 levels in days 3–7 post-admission. That was followed by a downward trend in the mild and severe patients. Interestingly, in the critical patients there was an upward trend after 3 weeks of hospitalization. Average ADAM12 serum levels were not a significant predictor of death or survival (Figure 4D, Mann Whitney p value = 0.168), however, day 3 ADAM12 levels correlated with outcome, with patients who had higher ADAM12 levels being less likely to have adverse outcomes (death or prolonged hospitalization (>30 days)) (Mann Whitney p-value = 0.023) (Figure 3B). We next evaluated whether the ADAM12 levels in patient sera correlated with the ephrin-A1 levels. Figure 5A shows that ADAM12 and ephrin-A1 levels showed no correlation independently of disease severity (p-value = 0.652).

2.3. Elevated ephrin-A1 serum levels are not a result of elevated TNFα levels. TNFα is elevated in only a small subset of COVID-19 patients

It has been previously reported that elevated TNFα can induce ephrin-A1 expression [27] and, therefore, we measured the TNFα serum levels in the COVID-19 patients. We observed that only 2 of the 31 patients (patients 7 and 12, Table 2) had significantly elevated TNFα levels (>300 pg/ml) throughout their hospital stay. This was in clear contrast to the ephrin-A1 levels, which were elevated in all patients. The median overall TNFα level for severe patients was found to be 39.1 pg/ml (IQR:

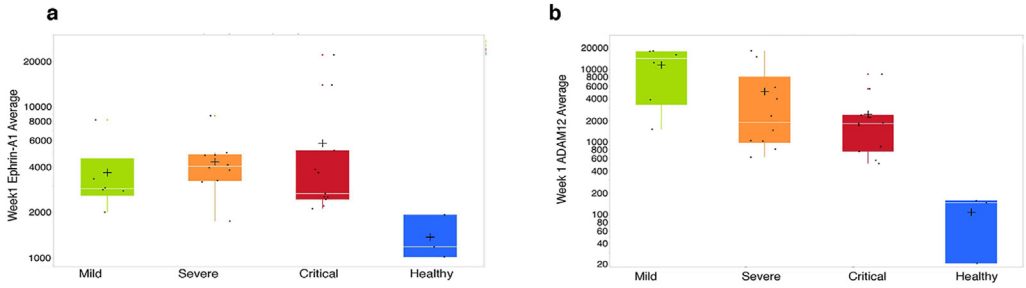

Figure 2. Week 1 levels of ephrin-A1 and ADAM12 were elevated in COVID-19 patients. Green - mild patients, orange - severe patients, red - critical patients, blue - healthy controls. Each dot represents the average week 1 level for one patient. A) Ephrin-A1 week 1 levels in COVID-19 patients and healthy controls. B) ADAM12 week 1 levels in and COVID-19 patients and healthy controls. In the box-and-whisker plot, the white horizontal lines indicate the median, the top and bottom of the boxes - interquartile range, the whiskers - the range, and the black crosses represent the mean (average) values.

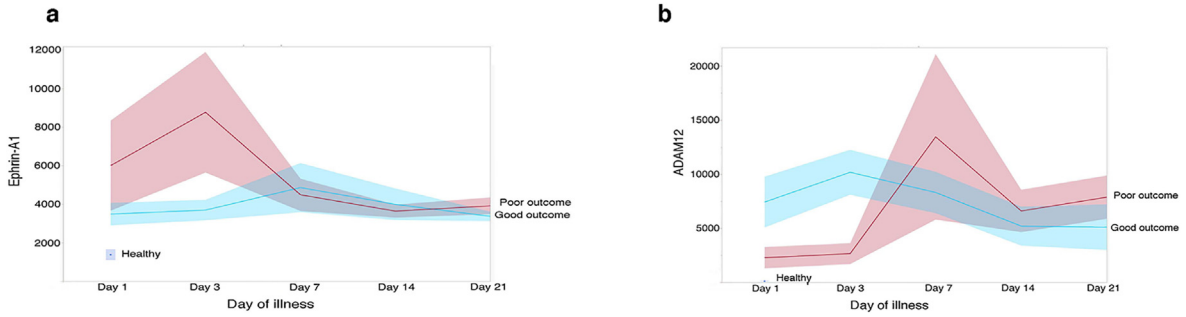

Figure 3. Time progression of the plasma levels of ephrin-A1 (Panel A) and ADAM12 (Panel B) in patients who had favorable clinical outcome (early discharge (<30 days), colored in blue), vs adverse outcome (late discharge (≥30 days) or death, colored in red). High day 3 ephrin-A1 levels displayed a strong correlation with adverse outcomes (p value = 0.033), while high day 3 ADAM12 showed a strong correlation with favorable outcomes (p value = 0.023).

18.3–109.7), compared to median healthy levels of 139.2 pg/ml (IQR: 127–189.6), revealing only a marginal difference in TNFα (Figure 6A). The median overall TNFα levels for mild and critical patients were actually lower than the ones for healthy controls, 3 pg/ml (IQR: 2.4–4.1) and 19.2 pg/ml (IQR: 10.9–61.2), respectively. Since the TNFα serum levels were not elevated in the large majority of the COVID-19 patients (and there was no correlation between TNFα and ephrin-A1 or ADAM12 levels), we conclude that the increased ephrin-A1 levels in the patients are not due to TNFα-mediated upregulation of ephrin-A1 expression.

2.4. ADAM17 serum levels are highly elevated in only a small subset of COVID-19 patients: exactly those with elevated TNFα levels

Similar to ephrin-A1, TNFα is initially expressed as a cell-surface protein and metalloproteases, most notably ADAM17, cleave TNFα off the cell membrane so that it can enter the blood circulation. Interestingly, ADAM17 is known to shed a variety of cell surface proteins, in addition to TNFα, including the ephrins [28]. We, therefore, measured the levels of soluble ADAM17 in the COVID-19 patients. Only 2 of the 31 patients had highly elevated ADAM17 levels (>10,000 pg/ml). The median overall

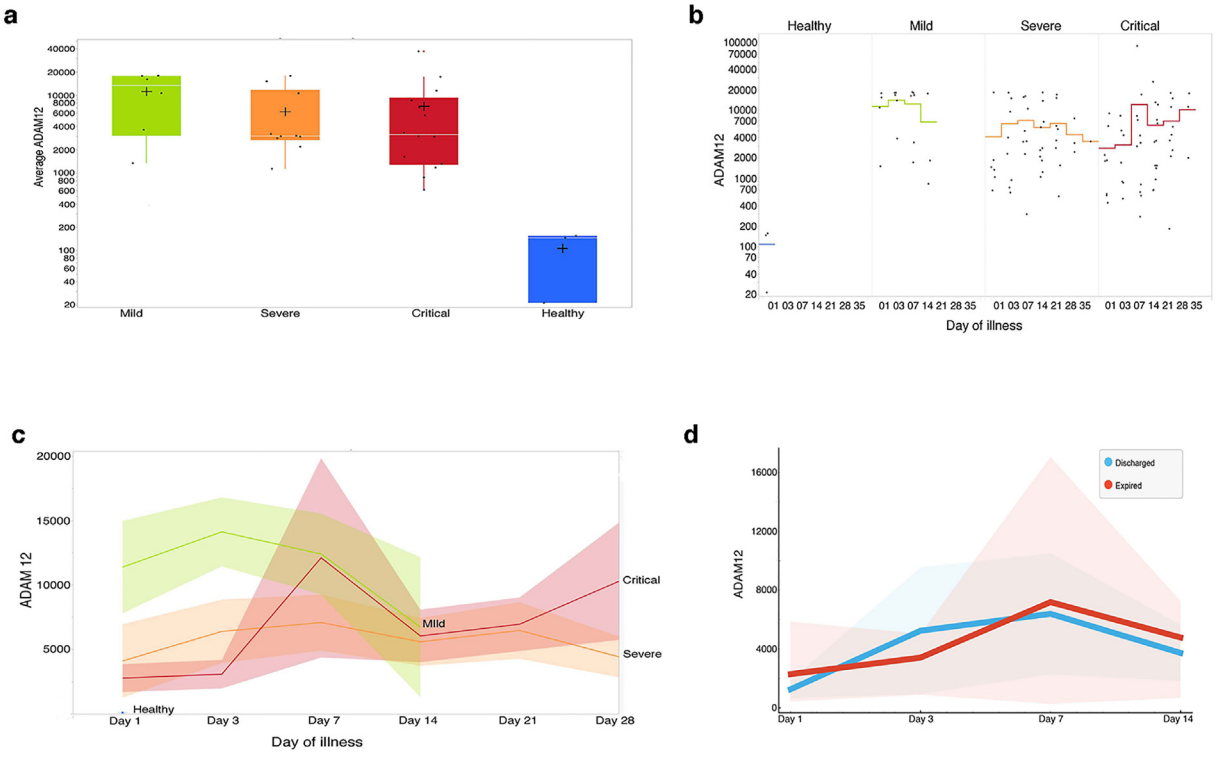

Figure 4. ADAM12 serum levels were elevated across all COVID-19 patients compared to healthy controls. A) ADAM12 levels in healthy controls and COVID-19 patients. Green - mild patients, orange - severe patients, red - critical patients, blue - healthy controls. Each dot represents the average ADAM12 level for one patient. In the box-and-whisker plot, the white horizontal lines indicate the median, the top and bottom of the boxes - interquartile range, the whiskers - the range, and the black crosses represent the mean (average) values. B) Time progression of ADAM12 levels in the serum of COVID-19 patients. The dots represent the individual patient values for epherin-A1 for each time point, and the lines represent the average values for all patients in each time point. Colors are as in Panel A. C) Another representation of the time progression of ADAM12 serum levels in COVID-19 patients. The solid lines represent the average value for each patient group in each time point, and the shaded areas indicate the SD. Colors are as in Panel A. D) ADAM12 levels did not significantly correlate with clinical outcome (death vs. survival). Blue line - average levels in discharged patients, red line - average levels in expired patients. The solid lines represent the average values in each time point and the shaded areas indicate the SD.

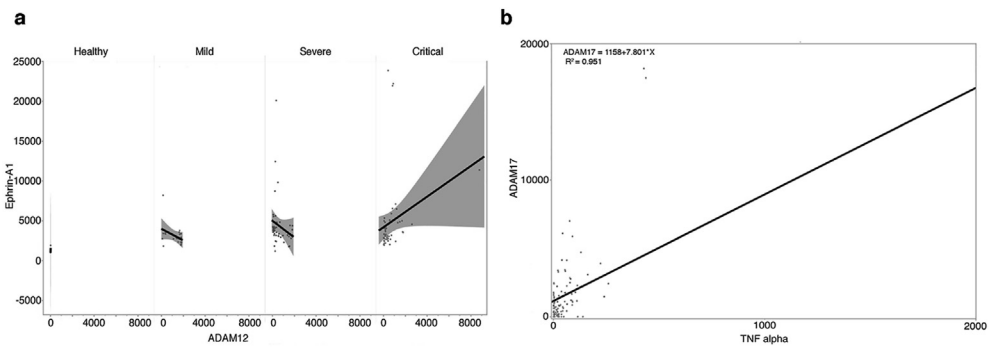

Figure 5. Correlation between the serum levels of ephrin-A1 and ADAM12, as well as between the serum levels of TNFα and ADAM17, in COVID-19 patients. A) Ephrin-A1 and ADAM12 serum levels did not have significant statistical correlation (p value = 0.652). The dots represent the individual values for each patient, the solid line indicates the best-fit linear regression line of all values, and the shaded areas represent the SD. B) TNFα and ADAM17 serum levels were highly correlated (p value < 0.001).

ADAM17 levels were 587 pg/ml (IQR: 284.3–1468.8), 1483.2 pg/ml (IQR: 774.1–4991.2), and 779.9 pg/ml (IQR: 295.2–1396.9) in mild, severe and critically ill COVID-19 patients, respectively (Figure 6B). The median ADAM17 level was 0.0 pg/ml (IQR: 0–733.7) in healthy controls. These levels are consistent with previous reports that the range for circulating (soluble) ADAM17 levels in healthy individuals is 56–1114 pg/ml with a mean of 350 pg/ml [29]. The fact that the patients' ADAM17 serum levels were highly correlated with the TNFα levels (p value < 0.001; Figure 5B), but not with the ephrin-A1 levels, suggests that ADAM17 is the sheddase for TNFα in COVID-19, but is not the sheddase for ephrin-A1.

3. Discussion

Despite intensive global efforts to quell the COVID-19 pandemic, no effective antiviral therapy for SARS-CoV-2 is available to date. It is, therefore, imperative to understand the signaling pathways that mediate disease progression and to identify new therapeutic targets for anti-COVID-19 drug development. It is especially important to understand and find ways to impede the inflammatory response, as it seems to be the primary cause for COVID-19 morbidity and mortality, in order to reduce the risks of associated complications and death. The ephrin-A1/ADAM12 and TNFα/ADAM17 signaling pathways are known to be involved in inflammatory responses associated with various diseases, such as cancer, rheumatoid arthritis and fibrosis. We, therefore, measured the levels of these four proteins in the serum of COVID-19 patients treated in the SUNY Downstate University Hospital of Brooklyn, New York.

We observed that all patients had elevated levels of both ephrin-A1 and ADAM12, with most patients having very high levels, as compared to healthy controls. This shows that the ephrin-A1/ADAM12-mediated signaling plays an important role in COVID-19 disease. Furthermore, while the serum levels of ephrin-A1 were highly elevated in the large majority of our COVID-19 patients, the serum levels of TNFα were only elevated in a small subset of the cases, indicating that the ephrin-A1-mediated inflammatory signaling is more relevant to COVID-19 disease progression than the TNFα-mediated inflammatory signaling. This is in contrast to previous reports that TNFα is elevated in a large fraction of COVID-19 patients [1], but is consistent with other reports, including Wan et al (2020) [30] and Chen et al (2020) [31], which find no elevation of TNFα levels or no differences between mild and critical COVID-19 patients. While it has been previously shown that TNFα can induce ephrin-A1 expression, the ephrin-A1 serum levels did not correlate with the TNFα levels in the COVID-19 patients. This indicates that in COVID-19, the increase of ephrin-A1 in the blood circulation is not due to TNFα-induced upregulation. This is consistent with similar observations in other diseases, such as metastatic lung malignancies [21] and lung injury due to viral infection and hypoxia [24], where the increased ephrin-A1 levels are also not caused by TNFα.

The COVID-19 patients with significantly elevated ADAM17 levels also had significantly elevated TNFα, indicating that ADAM17 upregulation results in increased shedding of TNFα and consequent increase in TNFα serum levels. This is consistent with previous reports postulating that ADAM17 is the primary and nonredundant sheddase for TNFα during inflammation [32]. In addition, the fact that ADAM12 levels were elevated in all patients with increased ephrin-A1 levels suggests that ADAM12 is the ephrin-A1 sheddase in COVID-19. Thus, our results show that elevated serum levels and activity of cytokines (e.g. TNFα) and other secreted inflammatory molecules (e.g. ephrins) in diseases that manifest with inflammation (e.g. COVID-19) are not simply due to overexpression but also to an increase in the levels and/or activity of the sheddases that release them into the blood circulation.

On average, the ephrin-A1 serum levels were highest in the critical patients and lowest in the mild patients. Furthermore, the clinical outcome (death/prolonged hospitalization vs. early discharge) depended on the ephrin-A1 levels during the initial days of hospitalization. These observations indicate that ephrin-A1-mediated inflammation may contribute to COVID-19 morbidity and mortality. They also suggest that ephrin-A1, as well as other molecules within the ephrin-A1 signaling pathway, including ADAM12 and EphA2, could be new pharmacological targets for anti-COVID-19 therapeutics. ADAM12 levels were shown to be elevated in several other diseases and ADAM12 inhibitors are already being developed to prevent inflammation-induced fibrosis (European patent, EP2470909A2). Ephrin-A1 and its Eph receptors are also emerging targets for cancer therapy [33] and in injury and inflammation [34], specifically for pulmonary inflammation in acute lung injury.

Our results suggest that targeting the ADAM proteinases that process cytokines and other inflammatory molecules could be a potentially effective and general approach to treat inflammation. In the case of COVID-19, several anti-TNFα therapeutics are being evaluated in early clinical trials but only sparse or insufficient data have been reported [35]. TNFα blockers have also been investigated for the treatment of Kawasaki disease in children with inconclusive results [36]. As TNFα seems elevated only in a subset of COVID-19 patients, it might be beneficial to screen patients for TNFα/ADAM12 levels in order to select suitable candidates for anti-TNFα therapy. In addition, since the currently evaluated anti-TNF therapies do not look particularly promising for COVID-19, ADAM17 could be targeted instead. Several ADAM17 inhibitors are currently being evaluated in the clinic for various pharmacological purposes, including tumor immunosurveillance and overcoming drug and radiation resistance in cancer, as well as treatments for cardiac hypertrophy and inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis [37].

In summary, the results reported here reveal that ephrin-A1 signaling is important in COVID-19 disease progression and suggest new therapeutic targets, namely ADAM12, ephrin-A1, and possibly ADAM17 in select patients, for the development of urgently needed anti-COVID-19 treatment strategies.

Table 2. Serum levels of ephrin-A1, ADAM12, TNFα and ADAM17 in the COVID-19 patients. All measurements are in pg/ml. NA denotes "not applicable" due to missing data.

Patient No.	Severity	Day of Illness	ephrin-A1	ADAM12	TNFα	ADAM17
1	Mild	Day 3	3337.7	3861.8	2.7	965.4
		Day 7	3453.5	3374.9	4.0	594.7
2	Mild	Day 1	2750.2	10991.4	1.5	2419.2
		Day 3	2807.0	13943.5	1.8	2471.0
		Day 7	3553.5	16227.0	2.7	2414.8
3	Mild	Day 1	1950.7	17773.0	0.0	1784.5
		Day 3	2065.3	17980.9	0.0	1605.9
		Day 7	2300.7	18521.5	0.7	1704.8
4	Mild	Day 1	NA	NA	4.6	641.9
		Day 3	2912.1	18115.5	2.8	107.0
		Day 7	3382.3	17935.3	3.8	242.1
		Day 14	2343.2	17554.9	6.1	0.0
5	Mild	Day 1	8197.8	1505.0	0.0	800.6
		Day 7	3421.9	1714.8	36.0	381.4
		Day 14	2761.1	832.9	20.9	0.0
6	Mild	Day 1	2357.2	15309.4	1.6	0.0
		Day 3	3312.7	16738.4	5.4	0.0
		Day 7	3772.0	16681.2	0.0	0.0
7	Severe	Day 3	1754.2	15014.5	437.3	17484.3
		Day 7	1833.3	15587.7	427.9	18182.4
8	Severe	Day 7	9806.1	4988.6	162.2	3092.8
		Day 14	3841.1	1057.0	110.8	1152.8
		Day 21	3569.4	547.0	103.3	1794.0
9	Severe	Day 3	8735.1	2302.7	89.5	5873.0
		Day 7	20088.1	3446.1	76.6	7004.2
		Day 14	5627.9	2785.4	39.9	4153.3
		Day 21	2450.9	2794.7	43.2	6106.5
10	Severe	Day 3	3817.6	622.2	55.6	3342.7
		Day 14	12431.5	2601.3	20.8	3393.7
		Day 21	3348.5	5879.4	19.0	1100.2
11	Severe	Day 1	4209.6	1335.8	17.0	2228.0
		Day 3	5770.5	732.9	25.2	1544.1
		Day 14	4827.3	6766.4	64.2	922.6
		Day 21	3529.2	3608.6	24.8	833.8
		Day 28	3172.0	3293.9	24.8	191.0
		Day 35	2405.3	3484.1	48.3	0.0
12	Severe	Day 1	4383.8	18318.2	7282.0	56802.6
		Day 3	3879.3	18141.2	7416.3	59914.9
		Day 14	3231.2	18288.6	6928.8	50380.7
		Day 21	2820.1	17031.4	6051.5	52266.5
13	Severe	Day 1	4086.7	668.7	37.4	1843.3
		Day 3	5531.6	943.9	28.4	802.6
		Day 7	4059.0	2284.7	42.8	1532.6
		Day 14	3058.6	5645.7	50.1	NA
		Day 21	3109.4	5293.4	62.3	416.2
14	Severe	Day 1	3191.5	1463.9	4.5	1084.6
		Day 7	4231.3	298.8	3.1	1389.0
		Day 14	3646.2	1236.3	1.2	143.9
		Day 21	4228.0	1557.0	2.2	286.0
15	Severe	Day 1	3954.7	1050.0	19.2	356.4
		Day 3	NA	NA	NA	1518.0
		Day 7	4414.3	16109.8	0.0	1590.0
		Day 14	3203.5	13707.3	16.5	74.7
		Day 21	3022.2	15150.2	26.1	0.0
		Day 28	2948.2	7575.0	7.5	173.1

(*continued on next page*)

Table 2 (*continued*)

Patient No.	Severity	Day of Illness	ephrin-A1	ADAM12	TNFα	ADAM17
16	Severe	Day 1	NA	NA	0.1	830.8
		Day 3	4783.0	3965.9	10.4	1241.6
		Day 7	4178.4	3509.1	1.6	796.6
		Day 14	3790.0	2083.3	0.5	323.2
		Day 28	1208.4	2440.5	0.0	0.0
17	Severe	Day 1	3356.8	1861.1	259.9	2425.0
		Day 3	3145.3	9548.1	80.8	2777.4
		Day 7	2317.8	10504.8	78.2	2498.0
		Day 14	1849.6	1825.7	4.2	1016.6
18	Severe	Day 1	2033.6	5848.9	1.7	0.0
		Day 3	3042.6	5045.9	25.5	816.1
		Day 7	2482.5	4254.3	23.2	108.6
		Day 14	3186.0	7253.7	16.3	197.2
19	Severe	Day 7	11393.7	87928.5	53.8	1751.2
		Day 14	6473.5	12092.8	84.0	1764.4
		Day 21	7100.6	11132.0	43.8	712.1
20	Severe	Day 1	3674.7	1843.2	222.7	3906.7
		Day 14	4808.3	1404.7	129.8	4718.8
		Day 21	5083.8	5735.7	56.5	1759.4
21	Severe	Day 1	NA	NA	62.7	4136.8
		Day 3	3849.7	1701.9	54.3	3429.9
		Day 14	2897.0	3575.4	131.7	2228.0
		Day 21	3091.3	3607.2	112.9	2285.6
22	Severe	Day 1	2529.2	563.5	86.5	1670.7
		Day 7	3313.6	557.9	25.6	1517.8
		Day 14	4091.4	1546.8	20.9	588.8
		Day 21	4047.4	2603.5	15.9	488.5
23	Severe	Day 3	NA	NA	22.5	3281.9
		Day 7	5897.5	7197.1	77.5	2839.6
		Day 14	4756.2	13086.0	46.1	620.1
		Day 21	4881.5	14985.0	43.5	421.1
		Day 28	2416.7	11201.3	90.1	1080.4
		Day 35	NA	NA	88.6	1147.8
24	Severe	Day 1	2207.8	1796.6	2.7	1469.5
		Day 7	2921.9	2970.5	1.7	991.3
		Day 14	3094.9	1547.7	3.0	274.2
		Day 21	2850.2	182.9	4.3	43.0
25	Severe	Day 1	4084.8	438.8	18.7	1254.6
		Day 3	23867.2	4306.5	10.1	1203.3
		Day 7	4992.4	17046.2	0.5	382.5
		Day 14	2683.9	6422.1	0.5	620.9
26	Severe	Day 1	NA	NA	6.7	691.9
		Day 3	2440.8	866.8	4.9	0.0
		Day 7	2193.6	817.1	10.7	313.2
		Day 14	2771.5	966.2	19.9	107.4
27	Severe	Day 1	4980.3	593.6	14.9	494.1
		Day 3	5345.9	897.3	0.0	0.0
		Day 7	2020.2	274.1	33.3	864.3
		Day 14	2471.1	679.7	0.0	0.0
28	Severe	Day 3	2118.2	506.6	11.6	714.9
		Day 7	1990.6	631.2	0.0	345.0
		Day 14	1945.9	672.2	0.0	305.3
		Day 21	3281.5	2109.3	2.4	401.6
		Day 28	4314.1	2008.0	13.2	519.6
29	Severe	Day 1	22213.3	9013.8	37.5	167.2
		Day 3	21958.8	8311.5	5.8	196.2
30	Severe	Day 1	2673.7	2201.1	0.0	0.0
		Day 7	2799.3	3337.9	26.2	0.0
		Day 14	2677.3	3428.9	37.5	0.0
		Day 21	2785.2	4382.6	3.3	0.0

(*continued on next page*)

Table 2 (*continued*)

Patient No.	Severity	Day of Illness	ephrin-A1	ADAM12	TNFα	ADAM17
31	Severe	Day 7	6546.5	8254.6	62.8	2256.0
		Day 14	4564.0	26079.4	82.8	1208.7
		Day 21	3513.8	17947.4	88.6	858.5
		Day 28	3607.1	17668.3	53.3	1116.6
Healthy-1	NA	NA	1015.2	21.3	139.2	0.0
Healthy-2	NA	NA	1185.1	156.1	239.9	1467.5
Healthy-3	NA	NA	1928.5	146.7	114.7	0.0

4. Methods

4.1. Patient samples

De-identified serum samples from thirty-one (31) hospitalized COVID-19 patients in the SUNY Downstate University Hospital of Brooklyn, New York were included in this study (IRB protocol number: 1587443, date of approval: April 10, 2020). Limited demographics and clinical information were also collected. The clinical status is categorized into three levels of severity: mild - patients who were hospitalized for 7 days or less, and did not require any invasive oxygen support (i.e. intubation) until discharge; severe - patients who were hospitalized for more than 7 days and did not require any invasive oxygen support until discharge; critical - patients who required invasive oxygen support and/or died. Serum samples from three, COVID-19 negative, healthy donors were utilized for baseline comparison.

Ten to 15 ml of whole blood samples were collected from antecubital veins of patients directly into serum separator vacutainer tubes. The blood was allowed to clot at room temperature for 15–30 min before centrifuging at 10,000 rpm for 10 min. Serum was aspirated into 1.5-ml cryotubes and frozen at -80 °C until further analysis.

4.2. Characteristics of the patients

Table 1 shows the baseline characteristics of the 31 patients included in this study. Twenty-four patients were African American (77.4%), five were white (16.1%), one was Asian (3.2%) and one did not reveal this information. The median age was 62.0 y (interquartile range [IQR], 52–76 y). The majority of patients were between the age of 50 y and 70 y (22/31, 71.0%). There were 16 males (51.6%) and 15 females (48.4%) in the study. In patients with mild clinical course, the majority were females (5/6, 83.3%), while those with severe presentation had more males than females (7/10, 70.0%). The critically ill patients had equal number of males and females. All of the patients had at least one co-morbidity (data not shown). The most common co-existing illness was hypertension (25/30, 83.3%), followed by diabetes mellitus (20/30, 66.7%).

4.3. Assays

4.3.1. Ephrin-A1

The level of soluble ephrin-A1 in each serum specimen was determined using a commercially available sandwich enzyme-linked immunesorbent assay (ELISA) kit (MyBioSource, Inc., San Diego, California, USA) following manufacturer's protocol. Fifty microliters of serum sample was diluted with 50 μl of the kit's sample diluent and added to pre-coated 96-well plate wells. One hundred microliters of serially diluted standards were also added to designated wells. Samples and standards were incubated at 37 °C for 90 min. After washing the plate two times with the kit wash buffer, 100 μl of biotin-labeled anti-ephrin-A1 antibody were added to sample and standard wells and incubated at 37 °C for 60 min. The wells were washed three times, then 100μl of HRP-conjugated reagent were added to each well followed by incubation at 37 °C for 30 min. After washing the plate 5 times, 90μl of TMB substrate were added into each well, followed by incubation at 37 °C in the dark for 10–20 min until development of blue color. Fifty microliters of the kit's stop solution were then added facilitating color change from blue to yellow. The absorbance in each well was immediately measured at 450 nm. The optical density was proportional to the amount of ephrin-A1 in the sample and the concentration was determined by interpolating the optical density in the standard curve using CurveExpert version 2.5.6.

4.3.2. ADAM12, TNFα and ADAM17

The levels of soluble ADAM12, TNFα and ADAM17 were determined using a different commercially available sandwich ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) following manufacturer's protocol. Fifty microliters of serum sample were diluted with 50 μl of the kit's sample diluent and added to pre-coated 96-well plate wells. One hundred microliters of serially diluted standards were also added to designated wells. Samples and standards were incubated at room temperature for 2 h. After washing three times with phosphate-buffered saline (PBS) with 0.05% tween solution, 100 μl of the kit's Detection Antibody were added to each well, followed by incubation for 2 h at room temperature. After washing again with the same wash solution three times, 100 μl of streptavidin-horse radish peroxidase (HRP) were added to each well,

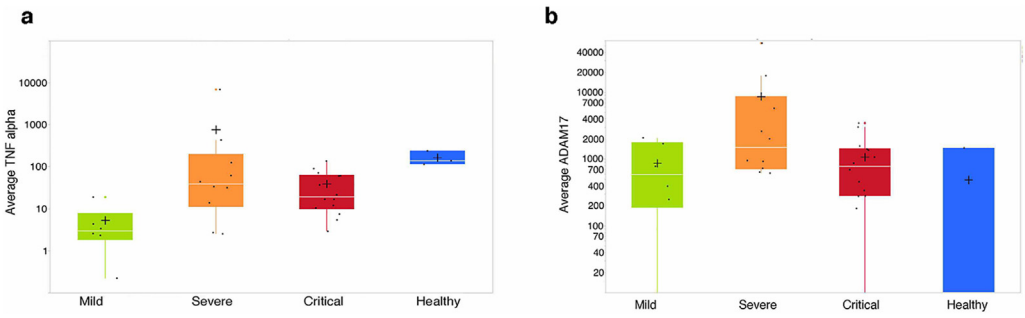

Figure 6. TNFα and ADAM17 serum levels were not elevated in the majority of COVID-19 patients as compared to healthy controls. Green - mild patients, orange - severe patients, red - critical patients, blue - healthy controls In the box-and-whisker plot, the white horizontal lines indicate the median, the top and bottom of the boxes - interquartile range, the whiskers - the range, and the black crosses represent the mean (average) values. A) TNFα levels in healthy controls and COVID-19 patients. B) ADAM17 levels in healthy controls and COVID-19 patients.

followed by incubation for 20 min at room temperature. The wells were again washed three times. One hundred microliters of a color substrate were added to each well and incubated for another 20 min at room temperature until development of yellow color. Fifty microliters of 2N H_2SO_4 was applied to each well as a stop solution, changing the color from blue to yellow. The absorbance in each well was immediately measured at 450 nm. The optical density was proportional to the amount of ADAM-12, TNFα or ADAM-17 in the sample and the concentration was determined by interpolating the optical density in the standard curve using CurveExpert version 2.5.6.

4.4. Statistical analysis

Statistical analyses were carried out with the NCSS 2020 Statistical Software (NCSS, LLC. Kaysville, Utah, USA) and R. The laboratory values measured were ephrin-A1, ADAM12, ADAM17 and TNFα. The outcome variables included disease severity (as determined by need for ICU admission, need for intubation and/or need for supplemental oxygen), and outcome (early discharge, late discharge (>30 days of hospitalization) and death). Averages were determined for patients across multiple measurements performed at predetermined intervals with overall average, and weekly averages used in comparisons in addition to moving averages to determine relevant time trends. Kolmogorov-Smirnov normality test was performed on all the main parameters, which showed that they do not follow normal distribution. As such non-parameteric Mann-Whitney U test was used for two group comparisons of quantitative measures. Comparisons of more than two groups was performed using Kruskal-Wallis test with post-hoc analysis using the Dunn's test. Associations of categorial variables were evaluated using the chi-square test, Fisher's exact test, or Spearman's r. Linear regression analysis was used to investigate correlations between quantitative variables. Alpha was set at 0.05 with two-sided p-values less than the alpha considered as significant for single comparisons, with Bonferroni adjustments made when multiple comparisons were made.

4.5. Study approval

The study protocol and other related documents were submitted for review to the SUNY Downstate Medical Center Institutional Review Board prior to implementation. Approval was secured on April 10, 2020 (protocol number: 1587443).

Declarations

Author contribution statement

Rachelle Mendoza: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Dimitar B. Nikolov: Conceived and designed the experiments; Wrote the paper.

Prem Premsrirut: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Elmer Gabutan, Mouyed Alawad, Amir Dehghani, John Diks, Bo Lin, Donghai Wang, Mohamed Alshal and William Fyke: Performed the experiments.

Amir Momeni: Analyzed and interpreted the data.

Bingcheng Wang and Juha P. Himanen: Contributed reagents, materials, analysis tools or data.

Nayanendu Saha: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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