ANGIOTENSIN II AS A BIOMARKER OF COVID-19 SEVERITY AND PROGNOSIS IN CHILDREN

By

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ABSTRACT

Background: *Ang II inhibits fibrinolysis and promotes the production of thrombin. The elevation in Ang II levels is substantially linked to lung injury and viral load in cases with severe Corona Virus Infectious Disease 2019 (COVID-19).*

Aim of the work: *This study aimed to determine the level of serum Ang II as a diagnostic biomarker of COVID-19 severity and a prognostic biomarker for the outcome in confirmed COVID-19 children.*

Patients and Methods: *This case-control study was carried out on 45 positive COVID-19 Real-time polymerase chain reaction (RT-PCR) children as the cases and 45 healthy children as the controls. Clinical examination, laboratory assays, and imaging studies were done on the patients to determine COVID–19 infection severity. Serum Ang-II was evaluated utilizing Enzyme-Linked Immunosorbent Assay (ELISA) for both groups as well as linked to patients' radiological, laboratory, and clinical parameters.*

Results: *The levels of serum Ang II were substantially elevated in the cases compared to controls (p-value= 0.001) and in the non-survivor COVID-19 group than in the survivor group (p-value = 0.0001). The median serum Ang II level in COVID-19 cases was 100 interquartile range (IQR):88-137) ng/L and 20 (IQR:15-25) ng/L in the controls respectively. There were statistically significant positive correlations between serum Ang II as well as clinical parameters and different grades of clinical severity classifications, laboratory parameters and radiological parameters according to COVID-19 Reporting and Data System (Co-RAD) score of Computed tomography (CT) chest of the patient group.*

Conclusion: *We concluded that early measurement of Ang II serum levels in confirmed COVID-19 children might be a helpful diagnostic and prognostic biomarker to identify high-risk patients for highly severe disease progression.*

Keywords: *Angiotensin II, SARS-CoV-2, COVID-19, ACE2, biomarkers.*

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a newly discovered RNA betacoronavirus infection induced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) **(Pedersen and Ho, 2020)** Since the onset of the novel coronavirus pandemic, the scientific community urgently needs accurate biomarkers associated with the progression of the disease for the early identification of high-risk cases. The fast spread of the disease necessitates immediate categorization of patients upon diagnosis **(Ponti et al.,2020)**.

SARS-CoV-2 utilizes ACE-2 as a functional receptor to enter the target cells, activating the RAS. After (ACE-2) is inhibited, the vasoconstrictor Ang II is significantly secreted, which consequently results in reducing its counter-regulating molecules angiotensin 1–7 **(Miesbach, 2020)**.

Furthermore, the diminished ACE-2 is significantly correlated with severe pulmonary pathology, indicating that unopposed Ang II functions as endogenous toxins. Accumulating Ang II leads to upregulated reactive oxygen species (ROS) and interleukin-6 (IL-6) which impairs adaptive and innate immunity. These immunological deficiencies impede viral clearance, resulting in a vicious cycle and a dismal prognosis for COVID-19. Intracellular Ang II is an endogenous mitochondrial toxin that results in premature endothelial senescence, thereby damaging end organs as well as impairing the prognosis of COVID-19 **(Sfera et al., 2020)**.

Accumulating Ang II stimulates many severe and fatal conditions, including sepsis, acute respiratory distress syndrome (ARDS), coagulopathy, thrombosis, fulminant myocarditis, as well as multi-organ failure **(Zaimet al., 2020)**. Ang II elevates the formation of thrombin, in addition to impairing fibrinolysis. Ang II elevated levels have been substantially linked to lung injury and viral load in severe COVID-19 cases. Consequently, severe COVID-19 patients' complex clinical picture is due to the multiple impacts of highly expressed Ang II on inflammation, coagulopathy, and vasculopathy.

Future treatment alternatives in COVID-19 cases must concentrate on inhibiting the inflammatory and thrombogenic Ang II properties **(Miesbach, 2020)**. Critically ill cases with COVID-19 can benefit from Ang II blockers by the reversal of premature vascular senescence and maintaining

immunological homeostasis **(Meng et al., 2020)**.

PATIENTS AND METHODS

Ethical Considerations:

1. An approval by ethical guidelines of the Faculty of Medicine's Research Ethics Committee at Ain Shams University was done before the study (NO. FWA 000017585).
2. A written informed consent was obtained from patients or their legal guardians.
3. All the data of the patients and results of the study are confidential and the patients have the right to keep it or withdraw from the study at any time.
4. The researcher explains the aim of the study to the patient.

Funding:

This research has not received any funds regarding the study or publication.

Conflict of Interest:

The authors declared no potential conflicts of interest with respect to the research, authorship, and/ or publication of this article.

Calculation Of Sample Size: Estimation was done using the Epi info7 program for sample size calculation, with 0.05 alpha error, a confidence interval of 0.95, and the study's power of 0.80. The minimum sample size was calculated to evaluate serum Ang II level as a diagnostic biomarker in confirmed COVID-19 children.

Study Design: Our study was a case-control study. They were selected by a simple random method. It was conducted in the Pediatric Hospital, Ain Shams University, Cairo, Egypt, during the period of October 2020 to March 2021.

Inclusion criteria:

1. Both genders were included in the study.
2. All patients who presented to the hospital with aged 0-18 years.
3. The study included 45 cases with positive COVID-19 RT-PCR with clinical and radiological findings of COVID-19 infection.

Exclusion Criteria were any patients with:

1. Underlying chronic lung disease.
2. Renal disease.
3. Hypertensive patients.
4. Patients on medications like angiotensin-receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEIs).

Study procedure: all enrolled COVID-19 cases were subjected to:

- **Complete history taking** (age, sex, and history of close contact with confirmed COVID-19 patients within two weeks of the onset of symptoms) and clinical symptoms such as (fever, cough, dyspnea, sudden loss of smell or taste, diarrhea, nausea, vomiting, and hemoptysis).
- **Thoroughly clinical examination:** a general examination of vital signs, anthropometric measurement, chest examination, and systematic examination (GIT, CNS, and CVS).
- Classification of clinical severity of pediatric COVID-19 patients into mild, moderate, severe, and critically ill **(Mostafa et al., 2020)**.

Laboratory investigations were measured for all COVID-19 children in our study:

- **Complete blood count (CBC)** with differential white blood cells (WBC), lymphocytes, neutrophils, platelets, and haemoglobin (Hb) by Sysmex XN-1000 (Sysmex Corporation, Japan).
- **Liver function test** as AST, ALT, and albumin using automated BECKMAN COULTER AU480 analyzer (Beckman Coulter, USA).
- **Coagulation profile** (prothrombin time (PT), partial Prothrombin time (PTT), INR, D-dimer) using STA Compact Max (Stago, USA).
- **Renal function test** (creatinine and BUN) using automated BECKMAN COULTER AU480 analyzer (Beckman Coulter, USA), Inflammatory markers such as CRP, ESR (by Westergren method).
- **Serum ferritin and Lactate Dehydrogenase (LDH)** by using an automated BECKMAN COULTER AU480 analyzer (Beckman Coulter, USA).
- **Computerized tomography (CT) scan:** The radiological grading was evaluated utilizing the classification of coronavirus disease 2019 reporting and data system (CO-RAD), which included grades ranging from 1 to 6 **(Prokop et al., 2020)**.
- **Serum human Ang II assay:** The assay was done using a commercially available kit obtained from Bioassay Technology Laboratory (1008 Junjiang Inter. Bldg. 228 Ningguo, Shanghai, China). A

total volume of 3 mL of venous blood samples was collected from all subjects included in this study using the aseptic venepuncture technique. The serum was separated by centrifugation and kept frozen at -20°C. The test principle applied in the kit was the Sandwich enzyme-linked immune-sorbent assay (ELISA) technology. The microwells of the plate provided in the kit have been pre-coated with an Ang II antibody. Each standard and sample were dispensed into a specific well and incubated. A biotin-conjugated anti-human Ang II antibody was added. After incubation and washing steps, Streptavidin-HRP was pipetted into all wells to bind to the biotin-conjugated anti-human Ang II antibody. After incubation and washing steps, substrate solution was added and color developed in proportion to the amount of human Ang-II. A stop solution was applied to each well, and the absorbance was determined at 450 nm by a spectrophotometer. The concentration of Ang II in each sample was determined according to the standard curve (normal plasma Ang II in children 37.8 ±3.7) **(FIONA et al., 1981)**.

Statistical analysis: Data was collected, tabulated and analyzed using statistical package for social sciences (SPSS) version 23. Quantitative variables will be first subject to the normality test (Kolmogorov v Simonov). Significance of the obtained results was judged at the (0.05) level. Continuous variables were present mean ±SD, and their differences were assessed by the independent T-test. Categorical variables were described as numbers (percentage), and were compared by chi-squared test. Mann-Whitney U, Kruskal-Wallis test, Spearman's correlation and Operating Characteristic (ROC) curve analysis were conducted.

RESULTS

All results will be demonstrated in the following tables and figures:

Table (1): Statistical comparison between COVID-19 children and controls regarding gender and age

	Cases (n =45)		Controls (n =45)		Test of Sig.	p
	No.	%	No.	%		
Gender						
Male	17	37.8	17	37.8	x2= 0.001	1
Female	28	62.2	28	62.2		
Age (years)						
Range	0.17- 16		0.57 - 16		t=-0.75	0.45
(Mean + SD)	5.87 ± 4.77		6.61 ± 4.31			

There was no statistically significant difference between cases and control as regards gender and age **(Table 1)**.

Table (2): Statistical comparison between COVID-19 children and controls serum Ang II level

	COVID-19 cases (n = 45)	Control group (n = 45)	Test of sig.	P-value
Serum ANG II			U= 2	0.001**
Range	(75-275)	(10-45)		
Median (IQR)	100 (88-137)	20 (15-25)		

Serum Ang II level was substantially elevated in COVID-19 children compared to controls (p-value= 0.001). Additionally, the median serum Ang level in COVID-19 patients was 100 (IQR:88-137) ng/L, whereas it was 20 (IQR:15-25) ng/L in controls **(Table 2)**.

Table (3): Correlation of serum Ang II to clinical findings of the studied COVID-19 children

Variables \ Numbers		Serum Ang II		Test value (U)	P-value	Sig.
		Median (IQR)	Range			
Fever	42	100 (75 – 112.5)	75 – 162.5	1.214	0.225	NS
	93.3 %	100 (87.5 – 150)	75 – 275			
Cough	16	95 (87.5 – 112.5)	75 –212.5	3.952	**0.000**	**HS**
	35.5 %	137.5 (106.25 – 180)	90 – 275			
Dyspnea	7	95 (87.5 – 112.5)	75 – 212.5	3.842	**0.000**	**HS**
	15.6 %	180 (175 – 225)	150 – 275			
Loss of smell	4	100 (87.5 – 150)	75 – 275	1.587	0.113	NS
	8.9 %	87.5 (80 – 101.25)	75 – 112.5			
Loss Of Taste	4	100 (87.5 – 150)	75 – 275	1.587	0.113	NS
	8.9 %	87.5 (80 – 101.25)	75 – 112.5			
Diarrhea	9	100 (88.75 – 156.25)	75 – 275	1.338	0.181	NS
		100 (82 – 112.5)	75 – 115			
	20 %	97.5 (87.5 – 187.5)	85 – 225			
ICU admission	10	95 (87.5 – 112.5)	75 – 180	4.322	**0.000**	**HS**
	22.2 %	177.5 (150 – 212.5)	112.5 – 275			
Mechanical ventilation	8	97.5 (87.5 – 112.5)	75 – 275	3.006	**0.003**	**HS**
	17.8 %	162.5 (115 – 180)	112.5 – 212.5			

In line of the current study, there were highly significant positive correlations between serum Ang II, and cough, dyspnea, and COVID-19 children who underwent ICU admission and were mechanically ventilated **(Table 3)**.

Table (4): Correlation of serum Ang II to the morbidity and mortality of the studied COVID-19 children

Variables \ Numbers		Serum Ang II		Test value (U)	P-value	Sig.
		Median (IQR)	Range			
Morbidity	No	88.75 (85 – 106.25)	75 – 187.5	20.984	**0.002**	**HS**
	Pneumonia	100 (95 – 112.5)	87.5 – 175			
	ARDS	162.5 (112.5 – 212.5)	112.5 – 212.5			
	MIS-C	125 (125 – 125)	125 – 125			
	Septic shock	175 (162.5 – 180)	150 – 275			
	Acute kidney injury	180 (180 – 180)	180 – 180			
	Multi-organ failure	225 (225 – 225)	225 – 225			
Mortality: Survivor	39(86.7%)	97.5 (87.5 – 112.5)	75 – 187.5	3.753	**0.000**	**HS**
non-survivor	6(13.3%)	196.25 (175 – 225)	175 – 275			

Serum Ang II was statistically elevated in COVID-19 children associated with comorbid conditions (P-value=0.0002). and the non-survivor group (P-value=0.0001) **(Table 4)**.

Table (5): Correlation of serum Ang II to clinical severity in studied COVID-19 children

Clinical severity	Angiotensin II (ng/L)	N (%)	Test of Sig. H	P
Mild Range Median (IQR)	 75-82 75 (75-78.5)	 5 (11.1)	36.9	0.001**
Moderate Range Median (IQR)	 85-100 92 (88-98)	 20 (44.5)		
Severe Range Median (IQR)	 100-150 112 (112-117)	 10 (22.2)		
Critically ill Range Median (IQR)	 150-275 180 (171.75-215.25)	 10 (22.2)		

There were substantial differences between various grades of clinical severity classifications of the studied COVID-19 cases group regarding serum Ang II levels (the median serum Ang II was 75 (IQR:75-78.5) for mild cases, 92 (IQR:88-98) for moderate cases, 112 (112-117) for severe cases and 180 (171.75-215.25) for critically ill cases) (p-value=0.001) **(Table 5)**.

Table (6): Correlation of serum Ang II to radiological severity in studied COVID-19 children

Co-RAD classification	Angiotensin II (ng/L)	N (%)	Test of Sig. H	P
1 Range Median (IQR)	75-88 83.5 (75-88)	10 (22.2)	33.03	0.001**
2 Range Median (IQR)	85-100 95 (90-100)	5 (11.1)		
3 Range Median (IQR)	88-150 95 (89-122)	5 (11.1)		
4 Range Median (IQR)	90-100 100 (92.5-100)	5 (11.1)		
5 Range . Median (IQR)	88-188 112 (112-162)	7 (15.6)		
6 Range . Median (IQR)	100-275 175 (112-196)	13 28.9		

There were substantial differences between different classifications of Co-RAD score of CT chest and serum Ang II level (p-value=0.001). Overall, there were statistically significant positive correlations between serum Ang II and clinical severity and radiological parameters (Co-RAD score of CT chest) of the patient group **(Table 6)**.

Table (7): Correlation of serum Ang II to clinical, laboratory parameters of the studied COVID-19 children

Variables	Serum Level of Ang II	
	Spearman's Correlation (rs)	p-value
Age	-0.209	0.163
Temperature	0.189	0.208
Pulse	**0.417***	**0.004***
RR	**0.724***	**0.000***
Spo2 (Room air)	**-0.507***	**0.000***
SBP	**-0.454***	**0.002***
DBP	-0.244	0.103
TLC	0.223	0.137
Lymphocytes	-0.017	0.909
Neutrophils	0.250	0.094
HB	-0.102	0.501
Platelets	0.038	0.800
ESR	**0.361***	**0.014***
CRP	**0.331***	**0.025***
LDH	0.289	0.051
D-dimer	**0.382***	**0.009***
Ferritin	**0.443***	**0.002***
PT	0.149	0.322
PTT	0.221	0.141
INR	**0.297***	**0.045***
Clinical severity classification	**0.92***	**0.001***

There were statistically significant positive correlations between serum Ang II and clinical parameters (O2 saturation, respiratory rate, systolic blood pressure, heart rate, and different grades of clinical severity classifications), also with laboratory parameters (CRP, ESR, D- dimer, serum ferritin, AST, ALT, and INR) of the patient group. On the contrary, there were no statistically significant correlations between serum Ang II and leucocytes, lymphocytes, neutrophils, haemoglobin, platelets, LDH, PT, PTT, blood urea nitrogen, and creatinine **(Table 7)**.

Table (8): ROC curve analysis for serum Angiotensin II in COVID-19 children

Lab parameter	AUC	Significance	Sensitivity (%)	Specificity (%)	95%CI	Cutoff
Angiotensin II	0.99	0.001**	91.1	100	0.99-1	>78.5

AUC: Area under the curve, 95%CI: 95% Confidence Interval, **: statistically significant.

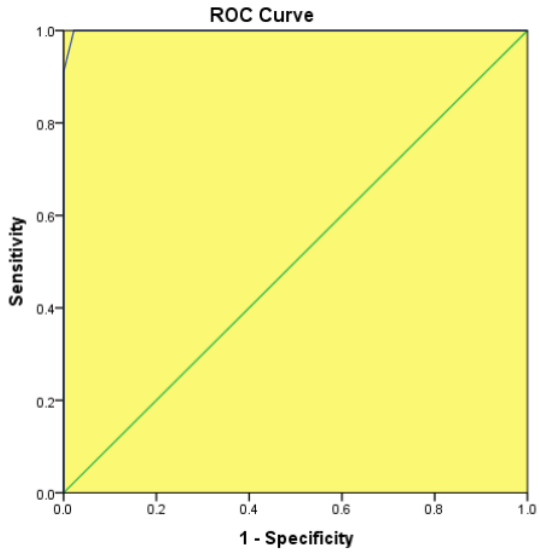

Figure (1): ROC curve analysis for serum Angiotensin II in COVID-19 children.

The receiver Operator Characteristics (ROC) curve was constructed to estimate serum Ang II's validity to predict COVID-19 infection. It was found at the cut-off point ≥78.5 ng/L with an Area under the ROC curve (AUC) of 0.99 **(Table 8) (Figure 1)**.

DISCUSSION

Most studies had been conducted on adults as the reported number of pediatric patients with COVID-19 was relatively smaller **(Ding et al., 2020)**. Notably, 62.2% of the cohort had female predominance. In concordance with our results, females represented 66% of the **Tagarro et al., (2020)** study that was conducted in Madrid-Spain, Additionally, this cohort study revealed that 8 (17.8%) of the case group had close contact with infected patients. In contrast, **Qiu et al. (2020)** found that 32 (89 %) of their 36 studied children had a

history of close contact with COVID-19 patients.

Fever was presented in 42 (93.3%) of our studied cases. This finding was consistent with the findings of **de Munain et al., (2021)** who performed a study on 44 hospitalized pediatric in Navarre, Spain, and fever was presented in 15 (88.24 %) of 17 infected infants <12 months, 18 (78.26 %) of 23 infected children aged 1-12 years old and 4 (100%) of infected Adolescents >12-year-old.In adults, fever was found in 78% of patients in Grant et al., (2020) study Contrary to our findings, fever was found only in 71 (41.5 %) of 171 children in a study by **Lu et al., (2020)** in China.

In the current study, lymphopenia was presented in 34 (75.6%) of our cases, compared with another study conducted by **Zheng et al., (2020)** in China on 25 confirmed pediatric cases of COVID-19 infection. They found that 10 (40%) of their 25 studied patients presented with lymphopenia.

Moreover, leucopenia and thrombocytopenia were observed in 15.5% and 13.3% of our studied cases, respectively. Compared to another study conducted by Guan et al. in China, they found that 33.7% and 36.2% of their patients had leucopenia and thrombocytopenia **(Guan et al., 2020)**. As regards inflammatory markers, the current study revealed statistically significant positive correlations between serum Ang II, clinical severity classifications, and laboratory parameters as inflammatory markers (CRP, ESR, and ferritin), coagulation function (D-dimer), liver dysfunction (ALT, and AST).

These findings aligned with an Egyptian study conducted on 180 COVID-19 subjects (age ≥ 18 years old) admitted to the Quarantine department of Ain-Shams University, Cairo, Egypt. The study revealed that severe disease and non-survivors showed substantially elevated ($P \leq 0.05$) inflammatory markers (CRP, ESR, and ferritin), tissue damage (LDH), coagulation function (D-dimer), liver dysfunction (ALT and AST) than the corresponding groups **(Taha et al., 2021)**.

In the context of radiological scoring, we found that 55.5 % of our cases were categorized as Co-RAD (1-4), 15.6 % as Co-RAD 5, and 28.9 % as Co-RAD 6. These findings are compatible with those of **Lu et al., (2020)** who observed that ground-glass opacity was presented in 56 (32.7 %), bilateral patchy shadowing in 21 (12.3 %), local patchy shadowing in 32

(18.7%), and interstitial abnormalities in 2 patients (1.2 %) of their 171 studied COVID-19 children.

In the line of this study, serum Ang II was substantially elevated in patients than in controls and in severe and critically ill cases than in mild and moderate cases. Therefore, these findings suggested that serum Ang II may contribute to determining COVID-19 progression. This finding agreed with **Wu et al., (2020)** who carried out a study on 82 COVID-19 children at the Department of Cardiovascular, Wuhan University. They also reported a positive correlation between plasma Ang II levels and COVID-19 severity, as plasma Ang II was found to be higher in 90.2% of their studied COVID-19 cases than in controls. In addition, they observed that plasma Ang II levels in critically ill COVID-19 cases were substantially elevated than in mild COVID-19 cases.

This finding also aligned with **Liu et al., (2020)** who conducted a study on 55 COVID-19 cases admitted to Wuhan University. They found that 34 (61.8%) of their 55 studied patients had elevated levels of serum Ang-II. They observed that COVID-19 severity was positively linked to the level of Ang II, and Ang II level was linearly correlated with viral loads and lung injury.

Henry and his colleagues conducted a study at the College of Medicine, USA, including 30 patients after the exclusion of those on renin-angiotensin-aldosterone system (RAAS) medications. Fourteen (14) healthy controls were enrolled as a comparison group. In contrast to our findings, the median plasma Ang II levels in their study were nearly identical between both groups (p-value = 0.990). COVID-19 patients had median plasma Ang II levels of 71.4 (IQR: 49.7–97.8) pg/mL, while healthy controls had median plasma Ang II levels of 71.5 (IQR: 65.1–95.8) pg/mL. They found no substantial differences between the two groups. In addition, they did not detect substantial differences between patients who needed ICU admission and those who did not (p-value = 0.440) **(Henry et al., 2020)**.

In contrast to our findings, **Ozkan et al., (2021)** conducted a study on 112 patients. They found that serum AngII was markedly diminished in COVID-19 patients compared to healthy controls (p-value < 0.001). They found that serum Ang II of the ARDS patients were substantially declined than subjects without ARDS (p-value < 0.05). Serum

Ang II of the patients who needed ICU admission was found to be substantially decreased compared to subjects who did not require admission to the ICU (p-value < 0.05). They reported low serum Ang II in dead patients.

A prospective single-centre study was conducted by **Rieder et al., (2021)** who measured ACE2, Ang II, and aldosterone serum levels in COVID-19 patients and compared them with control patients presenting with similar symptoms in the emergency unit. No evidence for altered RAAS activity, such as blood pressure, aldosterone, Ang II, and potassium levels, was detected in COVID-19 patients, as the mean serum concentrations of Ang II, ACE 2, and aldosterone did not vary between the SARS-CoV-2 positive cases and controls.

LIMITATION OF THE STUDY

- There were a lot of obstacles in the collection of the data and samples because of isolation.
- Risk of infection transmission inspite of the protective measures.

CONCLUSION

We concluded that early measurement of Ang II serum levels in confirmed COVID-19 children might be a reliable diagnostic and prognostic biomarker to identify cases at higher risk for extremely severe disease progression.

RECOMMENDATION

Targeting ACE2/Ang 1-7 axis and antagonizing ACE2 interaction are becoming very attractive therapeutic potential for the treatment and prevention of COVID-19 infection.

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