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Is there any relationship between serum zinc levels and angiotensin-converting enzyme 2 gene expression in patients with coronavirus disease 2019?

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ABSTRACT

Background: The level of angiotensin-converting enzyme 2 (ACE2) expression in different tissues is essential in the sensitivity, symptoms and consequences of COVID-19 infection. It seems that zinc is involved in the structure of the ACE2 enzyme has been identified; nonetheless, the relationship between *ACE2* expression and zinc serum levels in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected patients is still unclear. This study aimed to evaluate the expression of *ACE2* in peripheral blood-derived immune cells of COVID-19 patients and its relationship with serum zinc levels.

Methods: Thirty healthy subjects and thirty patients with COVID-19 were enrolled in this study. The COVID-19 infection was confirmed by positive real-time polymerase chain reaction (RT-PCR) and radiological data. Peripheral blood samples were taken from healthy subjects and COVID-19 patients. Whole blood samples were also used to measure *ACE2* gene expression by RT-PCR technique. The correlation matrix evaluated the relationship between *ACE2* expression, serum zinc levels, and other related variables.

Results: The outcomes showed no considerable alteration in serum zinc levels between patients and the control group. Likewise, the *ACE2* gene expression results showed a significant decrease in this receptor's expression in COVID-19 patients compared with the healthy subjects. A significant positive correlation was observed between serum zinc level and *ACE2* gene expression in patients with COVID-19.

Conclusion: The immune system seems to reduce the mRNA expression of the *ACE2* in the peripheral blood leukocytes following SARS-CoV-2 infection. Moreover, zinc deficiency can make patients more susceptible to SARS-CoV-2 infection.

1. Introduction

Coronaviruses (CoV) are considered a large family of viruses responsible for various disorders, from the common cold to the more severe respiratory disorders and even death (Perlman, 2020). The new coronavirus, SARS-CoV-2, is emerging not previously reported in humans (Novel, 2020). The virus is transmitted between people through droplets or direct contact, and the average incubation period is reported as 6.4 days (Lai et al., 2020). During a preliminary period in an infected patient, the virus is replicated in the upper respiratory tract. After this period, the lower respiratory tract cells' infection leads to infiltration of immune cells, the formation of an inflammatory milieu and lesions, as well as lung tissue injury (Abbasifard and Khorramdelazad, 2020). Evidence showed that the main symptoms of COVID-19 patients are fever, sore throat, dry cough, runny nose, headaches, body aches, fatigue, diarrhea and vomiting, as well as discoloration of fingers or toes and skin rash (Khorramdelazad et al., 2020). It has been reported that severe symptoms including pneumonia and shortness of breath were observed

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in 14% of patients; however, only in 5% of patients, the condition was severe, which is associated with acute respiratory distress syndrome (ARDS), infectious shock, multi-organ failure, and even death (Zaim et al., 2020). The *ACE2* gene encodes the ACE2, an enzyme located on the outer surface of epithelial cells in the lungs, blood vessels, heart, kidneys, and intestines (Hamming et al., 2004; Donoghue et al., 2000). ACE2 is a receptor for the SARS-CoV-2 to enter the target cells (Devaux et al., 2020). The spike (S) surface glycoprotein of SARS-CoV-2 can bind to the ACE2 receptor, allowing the virus to enter the target cells, particularly epithelial type1 and 2 in the respiratory tract tissue (Xu et al., 2020). Therefore, this receptor plays a crucial role in tissue infection susceptibility, clinical symptoms, and consequence of SARS-CoV-2 infection (Cao et al., 2020). Previous studies reported a positive and significant association between ACE2 expression and SARS-CoV-2 infection in vitro (Hofmann et al., 2004; Li et al., 2007).

Moreover, the ACE2 enzyme, as a metalloprotein, can chelate and retain zinc due to its reduced zinc levels favor the interaction of the angiotensin-converting motif (Vickers et al., 2002). Zinc is considered one of the essential trace elements in the human body. Since there is no zinc storage system in the body, zinc's daily consumption is essential to maintain a stable body condition (Tapiero and Tew, 2003). Studies have shown that zinc is essential for the optimum of several enzyme activities, cellular processes, including growth, DNA synthesis, and RNA transcription (Prasad, 1979). Fluctuations in the intracellular concentration of zinc are essential to inhibit virus transcription (Read et al., 2019). In this context, studies have shown that the binding and elongation pattern of RNA-dependent RNA polymerase (RdRP, RDR) or RNA replicase in SARS is inhibited by zinc (Te Velthuis et al., 2010). In vitro studies also showed that high concentrations of zinc and the addition of complexes that induce zinc entry into the cell could inhibit the proliferation of RNA-positive viruses (Te Velthuis et al., 2010). Protease activity was also inhibited by zinc in rhinovirus and poliovirus (Baum et al., 1991; Cordingley et al., 1989).

Further details on the effect of zinc on SARS-CoV-2 infection are currently mysterious. Based on the latest studies, zinc's role in the ACE2 structure has been identified; nonetheless, the relationship between *ACE2* expression and serum zinc level in COVID-19 patients is still unclear. Therefore, this study aimed to measure the serum level of zinc and gene expression of *ACE2* in peripheral blood-derived immune cells of COVID-19 patients compared to healthy subjects, as well as the association between gene expression of *ACE2* with zinc levels and other related variables.

2. Materials and methods

2.1. Subjects

In this investigation, thirty patients with COVID-19 and thirty healthy age and sex-matched subjects were enrolled. Inclusion criteria for COVID-19 patients were hospitalization in Ali Ibn Abitaleb Hospital (Rafsanjan, Iran), positive RT-PCR test and radiological data, and inflammation signs. Subjects with other viruses' infection, respiratory system-related disorders, immune-based diseases such as autoimmunity, allergies, cancer, and immunocompromised patients were excluded from the study. The subjects' demographic and clinical data, including age, sex, hospitalization duration, type of medication before and during the infection, disease outcome (discharge, death), underlying diseases, duration symptom onset, the severity of patients' symptoms (CT scan result, chest x-ray data), and vital signs of patients, were recorded. Additionally, laboratory parameters such as leukocyte count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), platelet (PLT) count were also collected. Informed consent has been received from each patient and control group based on the Declaration of Helsinki (DoH). Furthermore, this study has been approved by the Ethics Committee of Rafsanjan University of Medical Sciences, Rafsanjan, Iran (IR.RUMS.REC.1399.025).

Table 1
The sequences of primers used in the study.

Gene	Forward	Reverse
U6	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
ACE2	TCCATTGGTCTTCTGTCACCCG	AGACCATCCACCTCCACTTCTC

ACE2; Angiotensin-converting enzyme 2 (ACE2).

2.2. Blood analysis

Blood samples were collected from both patients and healthy subjects and divided into two fractions for serum separation, cell blood count, and RNA extraction. After collection, the sample was transferred to the laboratory, and isolated serums were kept at −20 °C until further experiments. The blood indexes, CRP, and ESR levels were measured using appropriate methods and laboratory instruments. In addition, serum zinc level was measured with a Hitachi 912 biochemistry auto-analyzer (Roche, Germany), and absorption was measured at 530–570 nm based on a calorimetric method.

2.3. Gene expression assay

Total RNA was extracted from fresh blood samples using an RNA extraction kit (Karmania pars gene, Iran). Extracted RNAs were evaluated for purity and integrity by spectrophotometry (260/280 nm) and agarose gel electrophoresis. Next, cDNAs were synthesized using a cDNA synthesis kit (Karmania pars gene, Iran) with a 15 μL cDNA master mix and a 5 μL RNA template (1 ng to 5 μg). The cDNA synthesize procedure was performed regarding the one-step protocol suggested by the mentioned manufacturer: 42–50 °C for 30 min, followed by 90 °C for 5 min for reverse transcriptase (RT) enzyme inactivation, and finally, the microtubes were cooled on the ice for 2 min, and 1/5 ratio (1 for cDNA and 5 for total volume) for PCR amplification was used for next step. Also, specific forward and reverse primers (concentration of the primers: 0.5 μM), 2× qPCRBIO SYGreen Mix Hi-ROX (PCRBiosystem, England), and nuclease-free water were prepared for RT-PCR and amplification using a Rotor-Gene Q 2plex System (Qiagen) regarding the recommended program: 1 cycle of 95 °C for 2 min; 40 cycles of 95 °C for 5 s, and 60–65 °C (annealing) for 20 to 30 s. Additionally, the melting curve step was used later by 10 s at 95 °C and then 10 s each at 0.2 °C enhancements between 62 and 95 °C. The designed sequence primers of the studied genes are shown in Table 1. The RT-PCR was completed in triplicate, and U6 was employed as the reference gene to standardize the acquired signals. Furthermore, the relative expression of the amplified products was correspondingly calculated by the $2^{-\Delta\Delta Ct}$ formula.

2.4. Statistical analyses

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was employed to perform statistical analysis. The one-sample Kolmogorov–Smirnov (KS) and Shapiro–Wilk tests were used for the variable's normality evaluation. Differences between the groups in the study were also assessed using independent sample *t*-test, Mann–Whitney U, and Chi-square statistic tests. Furthermore, the correlation matrix test was used to estimate the association between targeted variables. All data are presented as mean ± SEM. A p-value<0.05 was considered statistically significant.

3. Results

Thirty patients with COVID-19, including 17 women and 13 men with a mean age of 60 ± 3.39 years and thirty healthy individuals, including 19 women and 11 men with a mean age of 57 ± 1.98 years, enrolled in the study. The statistical analysis results showed no significant difference between the patient and control groups regarding age and sex ($P > 0.05$). The demographic and clinical data of the study

Table 2
Clinical and laboratory data of studied patients with COVID-19.

COVID-19 patient (n = 30)	WBC/ (μL)	PLT/ (μL)	Neutrophil count /(μL)	Lymphocyte count /(μL)	CRP (mg/ mL)	O2 (mm Hg)	Fever (C°)	Zinc (mg/ mL)	Medication used	Age (Years)	Duration of hospitalization (Days)
1	3900	176	65	33	55	80	38	55	Hydroxychloroquine sulfate, interferon beta, Tamiflu-oseltamivir, methylprednisolone	91	15
2	5900	166	69	19	9	84	38.9	49	Hydroxychloroquine sulfate, interferon beta, Tamiflu-oseltamivir, methylprednisolone	65	31
3	6500	229	71	34	50	86	38.5	45	Lopinavir & ritonavir (Kaletra ®)	61	9
4	7300	342	77	20	8	94	38	78	Hydroxychloroquine sulfate	65	6
5	7000	211	65	33	6	93	37	45	Hydroxychloroquine sulfate	38	8
6	4100	165	60	37	53	92	37.5	62	Lopinavir & ritonavir (Kaletra ®), Tamiflu-oseltamivir, hydroxychloroquine sulfate	79	11
7	4100	152	52	39	32	96	36.6	40	Lopinavir & ritonavir (Kaletra ®), Tamiflu-oseltamivir, hydroxychloroquine sulfate	70	6
8	5300	140	72	21	30	92	37	61	Interferon beta, hydroxychloroquine sulfate, methylprednisolone	50	8
9	3800	342	46	50	55	95	37	45	Hydroxychloroquine sulfate	56	3
10	`4500	157	63	25	10	93	37.2	47	Hydroxychloroquine sulfate	60	5
11	4500	221	70	28	6	98	37	40	Hydroxychloroquine sulfate	88	3
12	2300	210	61	31	50	97	36.4	52	Interferon beta, hydroxychloroquine sulfate, methylprednisolone	58	8
13	5100	205	65	29	24	89	38	80	Hydroxychloroquine sulfate, methylprednisolone	56	3
14	2300	195	67	31	6	85	37.2	39	Interferon beta, hydroxychloroquine sulfate, methylprednisolone	70	15
15	5400	95	75	19	16	92	37	84	interferon beta, lopinavir & ritonavir (Kaletra ®), selenium, methylprednisolone, dexamethasone	56	26
16	4200	215	70	30	35	95	38	68	Interferon beta, selenium, methylprednisolone	61	15
17	3100	242	64	30	8	87	36.2	40	Lopinavir & ritonavir (Kaletra ®)	63	6
18	5000	181	55	40	9	88	37.5	62	Lopinavir & ritonavir (Kaletra ®), Tamiflu-oseltamivir, hydroxychloroquine sulfate	63	6
19	7300	225	79	20	24	90	37	68	Interferon beta, hydroxychloroquine sulfate, methylprednisolone	59	8
20	8300	190	70	25	25	90	38	35	Hydroxychloroquine sulfate Tamiflu-oseltamivir,	52	5
21	1380	259	87	11	16	89	40	95	hydroxychloroquine sulfate, lopinavir & ritonavir (Kaletra ®)	65	7
22	1900	67	78	22	20	94	37	71	Interferon beta, methylprednisolone	88	11
23	1180	288	65	25	127	92	37	64	Interferon beta, dexamethasone	77	4
24	4600	220	75	25	15	80	38.9	55	Interferon beta	70	8
25	6100	150	85	13	42	95	37.5	70	Dexamethasone, interferon beta	61	7
26	7000	174	86	12	21	92	37.9	68	Interferon beta, methylprednisolone	67	8
27	3500	183	70	30	14	75	37.2	54	Interferon beta, dexamethasone	96	9
28	5300	215	60	37	51	99	37	21	Interferon beta, methylprednisolone	45	9
29	6600	222	79	16	20	87	37.5	57	Interferon beta, hydroxychloroquine sulfate, methylprednisolone	75	15
30	5300	105	82	16	20	66	37	66	Interferon beta, lopinavir & ritonavir (Kaletra ®), hydroxychloroquine sulfate, dexamethasone	73	11
All (mean ± SEM)	4768 ± 347.8	198.1 ± 11.2	69.43 ± 1.8	26.70 ± 1.67	28.57 ± 4.53	89.5 ± 1.3	37.5 ± 0.14	57.2 ± 2.97		65.93 ± 2.44	9.533 ± 1.14

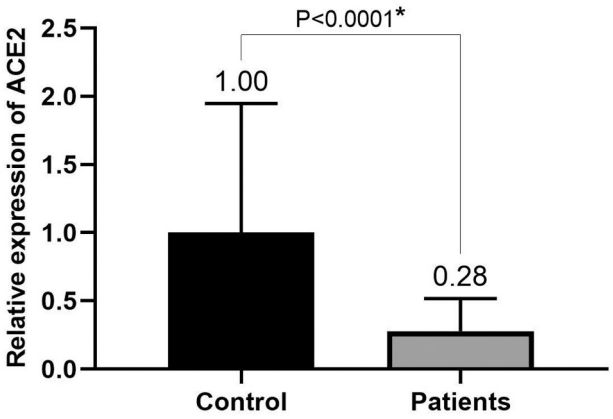

Fig. 1. Relative expression of ACE2 in control group and patient with COVID-19. All the tests were performed in triplicate. Data are presented as mean ± SEM, * significant ($P < 0.05$).

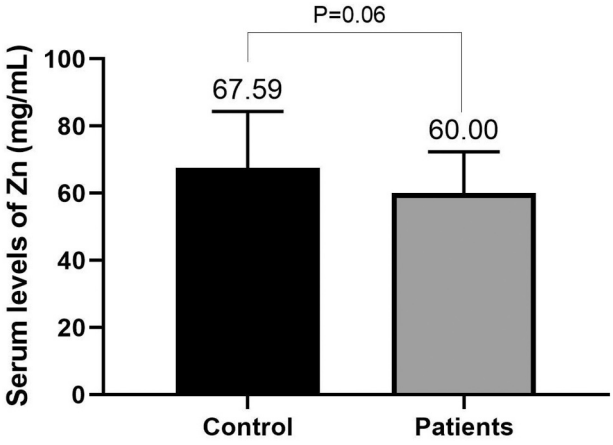

Fig. 2. The differences between serum levels of zinc in healthy subjects (control) and patients with COVID-19. Data are presented as mean ± SEM; A *p*-value<0.05 was considered statistically significant.

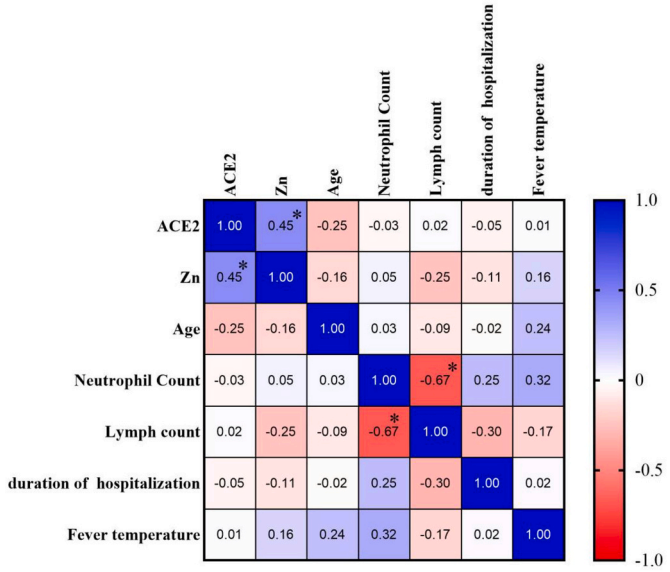

Fig. 3. The correlations between the expression of ACE2, zinc serum level, and other related variables in patients with COVID-19. The confidence interval of r is shown in the right rectangle (between −1 and 1). * Significant ($P < 0.05$).

groups are shown in Table 2. The results showed that in the patient group, the expression of the *ACE2* gene had a significant decrease compared to the control group ($P < 0.0001$) (Fig. 1). Furthermore, serum zinc levels were not significantly different in patients and controls ($P = 0.06$) (Fig. 2). Statistical analysis and correlation matrix test also showed a positive and significant correlation between serum zinc level and *ACE2* gene expression ($r = 0.44$, $p = 0.02$). Also, a significant negative correlation was observed between the number of neutrophils and lymphocytes, which shows that in patients with COVID-19, the severity of the disease increases, and the progression of the infection increases active neutrophils and decreases lymphocytes ($r = -0.66$, $p < 0.0001$) (Fig. 3).

4. Discussion

This study investigated the expression of the *ACE2* gene and its relationship with serum zinc levels. The results of this study showed that there was no significant difference in serum zinc levels between patients with COVID-19 and healthy individuals. However, *ACE2* gene expression was significantly reduced in the patient group compared to the control. The correlation results showed a positive and significant relationship between serum zinc levels and *ACE2* gene expression.

Recent studies reported that SARS-CoV-2 might infect $ACE2^{+}$ dendritic cells (DCs), macrophages, and monocytes. However, infection of these immune system cells can also occur in an ACE2-independent pathway through ligation with CD147 and CD209/CD209L (Abbasifard and Khorramdelazad, 2020; Jafarzadeh et al., 2020). Furthermore, SARS-CoV-2 can directly infect B cells and T cells, impairing their specific function in immune responses. This could elucidate the effect of SARS-CoV-2 infection on lymphoid tissues such as lymph nodes and the spleen (Feng et al., 2020). Following the infection of macrophages, DCs, and monocytes, the viruses can repress interferon-mediated antiviral responses by these cells. Infected monocytes can also infect resident macrophages after migrating to various tissues in the body, such as the lungs, spreading the viruses along with infection progression. However, there are still several controversies about whether ACE2 restrains or activates the immune system.

Additionally, pro-inflammatory mediators could be produced by infected macrophages and monocytes, resulting in local and systemic inflammation and cytokine storm, which play a pivotal role in expanding COVID-19-associated disorders, such as ARDS in infected patients with infected patients SARS-CoV-2 (Jafarzadeh et al., 2020). Evidence proposes that ACE2 is regulated by several pathways (Gallagher et al., 2008; Clarke et al., 2014). In line with this study's results, it has been shown that ACE2 is significantly down-regulated in COVID-19 (Du et al., 2020). Although few studies have been done in this area, there is evidence that zinc can affect virus-induced alterations in B and T cells (Wessels et al., 2020). It has also been argued that zinc can reduce *ACE2* expression (Skalny et al., 2020). This study showed that despite the lack of significant differences between the patient and control groups in the serum level of zinc, the amount of this trace element in both groups was less than normal, and both groups were zinc deficient. Due to this zinc deficiency, reduced *ACE2* expression in patients with COVID-19 may have other unknown mechanisms. Consistent with our data, another study also reported that a large number of patients with COVID-19 had zinc deficiency, developing more complications, and the deficiency was associated with an extended hospital stay and enlarged mortality rates (Jothimani et al., 2020). An experimental investigation reported that zinc with a concentration of 100 μM could decrease recombinant human ACE2 activity in rat lungs (Speth et al., 2014). However, the modulation of SARS-CoV-2 infection by administration of high doses of zinc is still hypothetical and needs further study (Chilvers et al., 2001).

Interestingly, zinc as an immune regulator can participate in both the inflammatory and anti-inflammatory responses in viral infections. Studies have shown that zinc can increase IFN-α production on the one hand and reduce the pathological inflammation leading to cytokine

storm by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway. Zinc can also activate regulatory T cells (Tregs), supporting the homeostasis process (Zhang et al., 2020). However, the association between zinc levels and *ACE2* gene expression in patients' peripheral blood cells with COVID-19 has not yet been determined.

In contrast with this study's findings, a flow cytometric investigation demonstrated slight to no ACE2 expression by peripheral blood-derived immune cells and platelets while alveolar macrophages can significantly express ACE2 (Song et al., 2020). Furthermore, in vitro investigations have revealed that decreased zinc levels support the interaction of ACE2 with SARS-CoV-2S protein and elevated zinc levels hinder ACE2 expression, reducing viral interaction, while the statistical analysis in this study showed that there was a positive and significant association between serum level of zinc and *ACE2* expression by peripheral blood-derived immune cells in patients with COVID-19 (Devaux et al., 2020; Li et al., 2020). These discrepancies in outcomes may be due to patients' conditions, treatment protocol, and other unknowns. The finding of an investigation demonstrated that zinc supplementation increased the suppressive impacts of emetine and triclabendazole on the expression of ACE2 in Calu-3 and H322M cells (lung cancer cell lines) (Lee et al., 2021). These findings indicated that the ACE2 expression could be modulated by the reactive oxygen species (ROS) and NF-κB signaling in human lung cells, and the combination of triclabendazole or emetine with zinc may have acceptable clinical outcomes for the treatment of patients with COVID-19 by regulating of ACE2 expression (Lee et al., 2021).

Regarding the findings of our study conducted on leukocytes of patients with COVID-19, there was a positive and significant association between serum levels of zinc and the expression of *ACE2*. In this context, studies showed that targeted cell subsets by COVID-19 in host tissues and the regulators of *ACE2* expression remain unidentified. It has been revealed that ACE2 might regulate immune response through activation of immunological signaling pathways such as the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) in the SARS-Cov-2 infection, resulting in the activation of helper T cells 1 (Th1 cells), B cells, macrophages and the inhibition of $CD8^+$ T cells and $Foxp3^+$ Tregs (Luo et al., 2021). Therefore, in patients under study, factors other than zinc may have played a role in increasing *ACE2* expression, as *ACE2* expression has been shown to increase significantly 72 h after SARS-CoV-2 infection, which can occur in both zinc-deficient patients and patients with sufficient zinc levels (Luo et al., 2021). Regrettably, due to the invasiveness of the biopsy method from the damaged lung tissue and the dissatisfaction of patients and their families, the measurement of ACE2 tissue expression was limited.

Collectively, this study's results showed that zinc plays an essential role in modulating immune responses in people with COVID-19 and can also play a protective role against viral infections such as SARS-CoV-2 because serum levels in most studied patients are lower than normal. This zinc deficiency can make patients more susceptible to the infection. On the other hand, gene expression of *ACE2* was reduced, although this decrease in mRNA expression was detected in peripheral blood cells and required further studies on the ACE2 protein level in various tissues such as the lung epithelium.

Credit author statement

Zahra Bagheri-Hosseinabadi: data collection, writing original draft preparation, Mitra Abbasifard: writing, reviewing and editing, supervision, Ali Pirsadeghi: data analysis and molecular tests, Fatemeh Bahrehmand: sample collection, laboratory and molecular tests, Amir Rahnama; data collection and troubleshooting.

Declaration of Competing Interest

None.

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