Modified Proline Metabolism and Prolidase Enzyme in COVID-19

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ABSTRACT

Objective: The aim of the study was to evaluate proline metabolism in patients affected by COVID-19.

Materials and Methods: This case-control study consisted of 116 patients with COVID-19 and 46 healthy individuals. Tests related to proline metabolism (prolidase, proline, hydroxyproline, glutamic acid, manganese) and copper and zinc tests were analyzed.

Results: The levels of proline and hydroxyproline amino acids and the prolidase enzyme were found to be lower and glutamic acid was found to be higher in the COVID-19 group compared to the healthy group ($P = .012$, $P < .001$, $P < .001$, and $P < .001$, respectively). The copper/zinc ratio was higher in patients with COVID-19 than in healthy individuals ($P < .001$). Significant correlations were found between proline metabolism tests and inflammatory and hemostatic markers commonly used in COVID-19.

Conclusion: The proline metabolic pathway was affected in COVID-19. Relationships between proline pathway–related tests and inflammatory/hemostatic markers supported the roles of proline metabolism in proinflammatory and immune response processes.

The whole world continues to fight the SARS-CoV-2 infection, which causes COVID-19 according to the World Health Organization (WHO). Although many studies have been conducted on SARS-CoV-2 in a relatively short time, information about the pathogenesis of SARS-CoV-2 and the immune response against it in the host cell is still limited.[1,2]

COVID-19 displays a broad array of clinical features, from an asymptomatic infection to a severe lung illness and/or multiorgan failure.[3] The efficiency of the host's immune response has a considerable influence on the clinical presentation.[3] Throughout viral infection, the innate and adaptive immune systems are involved in the immune response.

In addition to their role as building blocks of proteins and polypeptides, some amino acids are substantial regulators of the fundamental metabolic pathways essential for maintenance, growth, reproduction, and immunity in organisms.[4] Proline and hydroxyproline are the most notable amino acids in the collagen structure. The proline metabolic pathway is strategically located in the metabolism. This pathway is connected to the Krebs cycle via glutamate and to the urea cycle via arginine.[5,6] Proline-containing peptides are involved in many biological processes such as proinflammatory response, immune response, and hemostasis.[7]

Prolidase (EC 3.4.13.9) is a dipeptidase that breaks down proline or hydroxyproline-containing aminopeptides and takes a crucial role in the remodeling of the collagen metabolism matrix and cell growth.[8] Prolidase is involved in numerous biological processes at the cellular level.[7] The catalytic function of prolidase provides for the delivery of proline or hydroxyproline, which modulates intracellular signaling. Prolidase is an epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) ligand that regulates the signaling pathways depending on these receptors. Under physiological conditions, prolidase stimulates these pathways and can act as an interface in regeneration processes involved with inflammation or tissue damage.[9] Prolidase also participates in the immune response by stimulating the expression and maturation of the interferon α/β receptor. It has also been observed that prolidase activity regulation modulates the biological effects of the nuclear factor κβ (NF-κβ) transcription factor, which has a crucial position in the activation of the inflammatory

response.[9,10] Lung inflammation may trigger transcription factors such as NF-κβ, which modulates the expression of proinflammatory genes.

Based on the role of proline-containing peptides in many biological processes such as proinflammatory and immune response and hemostasis, and the fact that prolidase enzyme activity is associated with inflammation and tissue damage, our study aimed to investigate proline metabolism in patients affected by COVID-19 and to evaluate molecules/tests related to this metabolism.

Materials and Methods

Study Design
The study was carried out with patients who had COVID-19 from March to May 2021 in Ankara City Hospital, which is one of the pandemic hospitals in Turkey. Clinical diagnoses were executed in accordance with WHO guidelines for COVID-19.[11] The study procedure was established in compliance with the Helsinki Declaration and confirmed by the local ethics board (number E1-20-1125). Patients with current clinical symptoms, signs of COVID-19 pneumonia on computed tomography, and/or positive real-time polymerase chain reaction (RT-PCR) test results of oro-nasopharyngeal swab specimens for SARS-CoV-2 were included in the study. All patients were hospitalized. Patients with negative RT-PCR results were not included in the study, nor were patients with an unverified diagnosis of SARS-CoV-2 infection. Healthy volunteers with a negative result from a RT-PCR test for SARS-CoV-2 infection constituted the control group. A detailed history was obtained from all patients. All participants underwent a comprehensive physical examination and routine clinical laboratory tests. In addition to the routine clinical examinations and blood tests, all participants had proline, hydroxyproline, and glutamic acid amino acid tests, along with prolidase enzyme, manganese, copper (Cu), and zinc (Zn) tests.

Laboratory Analysis
Venous blood specimens were obtained from each participant by venipuncture immediately on admission to the hospital after being confirmed with SARS-CoV-2 infection. Afterward, serum was separated by centrifugation at 1500*g* for 10 minutes. Proline, hydroxyproline, and glutamic acid amino acid concentrations were analyzed using a liquid chromatography/mass spectrometry instrument (Sciex QTrap 4500, Foster City, CA). The specimens were studied using a ready-to-use commercial kit (Immuchrom, Heppenheim, Germany). Serum prolidase enzyme concentrations were determined with Chinard reagent according to the spectrophotometric assay defined by Myara et al.[12] Measurements were made using a Siemens Advia 1800 chemistry analyzer (Siemens Healthcare, Erlangen, Germany). Serum manganese levels were determined in a graphite furnace containing an atomic absorption spectrometer (AAS; Thermo Fisher Scientific ICE 3000 series, Waltham, MA) using the method proposed by Lisboa et al.[13] Serum Zn levels were measured using a Zn hollow cathode lamp (HCL) at a wavelength of 213.9 nm in an AAS-flame unit (Thermo Fisher Scientific ICE 3000 series).[14] Serum Cu levels were measured using a Cu HCL at a wavelength of 324.8 nm in an AAS-flame unit (Thermo Fisher Scientific ICE 3000 series).[14] Among the routine laboratory tests, albumin (ALB), total protein (TP), lactate dehydrogenase (LDH), iron, and C-reactive protein (CRP) levels were detected using Advia Chemistry-XPT systems (Siemens Healthcare Diagnostics Erlangen, Germany). Procalcitonin (PCT) and ferritin tests were analyzed using the Atellica IM analyzer (Siemens Healthcare Diagnostics). Complete blood cell counts were measured using the Siemens Advia 2120 Hematology Analyzer (Siemens Healthcare Diagnostics). D-dimer tests were analyzed using the Sysmex CS-5100 coagulation analyzer. Erythrocyte sedimentation rates (ESRs) were analyzed using Vision c (YHLO Biotech, Shenzen, China).

Statistical Analysis
Visual (histograms) and statistical methods (Shapiro-Wilk test) were used to determine whether the data were normally distributed. Descriptive analyses were conducted using the mean and standard deviation for normally distributed variables. Because of the normal distribution of the data, independent-sample *t*-tests were performed to determine the significance levels of the investigated tests between the 2 groups. Correlation analyses were performed using Pearson correlation. In all comparative statistical analyses performed, the level of significance was accepted as <5% ($P < .05$). The SPSS software program (version 26; IBM, Armonk, NY) was performed for statistical utilizations.

Results
A total of 116 patients with confirmed COVID-19 were included in the study. Of these, 45 were female and 71 were male. The control group consisted of 46 healthy individuals (31 female, 15 male). Although the mean age of the patient group was 60.8 years, the mean age of the control group was 37.5 years. The most common symptoms seen in the patients were fever (67.2%), cough (56%), fatigue (46.5%), and shortness of breath (28.4%), respectively. Hypertension (33.6%) and diabetes (26.7%) were the most common comorbidities. The demographic characteristics of the study group are summarized in **TABLE 1**.

The test results regarding the proline metabolism of the participants and other laboratory findings are shown in **TABLE 2**. The prolidase enzyme level was found to be significantly lower in patients with COVID-19 compared to the control group ($P < .001$). The levels of the proline and hydroxyproline amino acids were found to be lower in the COVID-19 group compared to the healthy group, whereas the glutamic acid amino acid was found to be higher in the COVID-19 group ($P = .012$, $P < .001$, and $P < .001$, respectively). There was no significant difference between the groups in terms of manganese level ($P = .299$). When the study groups were evaluated in terms of Cu and Zn, Cu was found to be significantly higher in patients with COVID-19 compared to the control group ($P < .001$); Zn was significantly lower in the patient group than in healthy individuals ($P < .001$). The Cu/Zn ratio was higher in patients with COVID-19 than in healthy individuals ($P < .001$).

When the study groups were compared in terms of routine laboratory tests, we found that TP and ALB values were lower in the COVID-19 group than in the control group ($P = .010$ and $P = .034$, respectively). Although the serum iron level was lower in the patient group than in healthy individuals ($P < .001$), LDH enzyme activity was found to be higher in individuals with COVID-19 than in the healthy group ($P < .001$). Furthermore, PCT, ferritin, ESR, and CRP levels were all significantly higher in patients with COVID-19 than in healthy control individuals ($P < .001$ for all). When we considered the hemogram parameters white blood cell (WBC) and lymphocytes (LYM), we found that the WBC value

TABLE 1. Demographic Characteristics of Patients with COVID-19 and Control Group[a]

Characteristic	Patients with COVID-19 (n = 116)	Control Group (n = 46)	P Value[b]
Age (y, mean ± SD)	60.8 ± 10.8	37.5 ± 10.1	<.001
Sex (female/male)	45/71	31/15	.001
Signs and symptoms			
Fever	78 (67.2)	…	
Cough	65 (56)	…	
Dyspnea	33 (28.4)	…	
Fatigue	54 (46.5)	…	
Myalgia	17 (14.6)	…	
Nausea/vomiting	10 (8.6)	…	
Headache	6 (5.17)	…	
Diarrhea	5 (4.31)	…	
Comorbidities			
Diabetes	31 (26.7)	…	
Hypertension	39 (33.6)	…	
Coronary artery disease	18 (15.5)	…	
Chronic lung disease	16 (13.7)	…	
Malignancy	5 (4.31)	…	

SD, standard deviation.
[a]Data are expressed as numbers (%).
[b]P < .05, statistically significant.

was higher in patients with COVID-19 compared to those in the control group (P = .041), whereas the LYM value was significantly lower in patients with COVID-19 compared to healthy individuals (P < .001). D-dimer test results were more than 6 times higher in patients with COVID-19 than in patients in the control group (P < .001).

The relationship between the tests related to the proline pathway and other laboratory tests in patients, as seen in **TABLE 3**, was examined. A significant correlation was observed between prolidase enzyme level and LYM (r = 0.56, P = .001) and Zn (r = 0.30, P = .029). Statistically significant negative correlations were obtained between prolidase enzyme and ferritin (r = −0.24, P = .042,) D-dimer (r = −0.28, P = .022), CRP (r = −0.22, P = .038), Cu (r = −0.18, P = .025), and Cu/Zn ratio (r = −0.21, P = .043). Similarly, a significant correlation was observed between proline amino acid and LYM (r = 0.46, P = .009) and Zn (r = 0.28, P = .025). Negative correlations were found between proline amino acid and ferritin (r = −0.19, P = .009), D-dimer (r = −0.15, P = .162), CRP (r = −0.50, P = .006), Cu (r = −0.47, P = .009), and Cu/Zn ratio (r = −0.53, P = .003). While statistically significant negative correlations were observed between hydroxyproline amino acid and ferritin (r = −0.52, P = .004), D-dimer (r = −0.36, P = .040), CRP (r = −0.71, P < .001), Cu (r = −0.35, P = .039), and Cu/Zn ratio (r = −0.42, P = .014); a positive correlation was found between hydroxyproline amino acid and the LYM (r = 0.38, P = .041) and Zn (r = 0.28, P = .041). Unlike other amino acids, statistically significant negative correlations were observed between glutamic acid amino acid and LYM (r = −0.32, P = .041) and Zn (r = −0.36, P = .012); positive and significant correlations were found between glutamic acid and ferritin (r = 0.32, P = .037), D-dimer (r = 0.39, P = .038), CRP (r = 0.30, P = .03), Cu (r = 0.33, P = .017), and Cu/Zn ratio (r = .40, P = .009).

Discussion

The results of our study not only provided information about the underlying causes of infection and inflammation in patients with COVID-19 whose proline metabolism was investigated but also revealed the relationships between inflammatory and prognostic markers, the prolidase enzyme, and the proline pathway for COVID-19. Our study has the feature of being the first research in this area to examine proline metabolism together with the prolidase enzyme in COVID-19.

SARS-CoV-2 is the cause of the ongoing COVID-19 pandemic. Unlike the majority of coronaviruses, SARS-CoV-2 multiplies in the lower respiratory tract and in severe cases causes the development of acute respiratory distress syndrome (ARDS) and progressive pneumonia, with fatal destruction of the human organism. The general step in the development of ARDS is the elevation of plasma proinflammatory cytokines and rapid lung infiltration by immune cells. Pulmonary fibrosis and cytokine release syndrome (CRS) are often present in the advanced stages of COVID-19. The widespread release of proinflammatory cytokines, combined with sepsis and major multiorgan damage, is responsible for at least 30% of fatal COVID-19 cases.[15] The molecular pathways responsible for the development of SARS-CoV-2-induced fibrosis, ARDS, and CRS are not yet understood.[15,16]

Proline and hydroxyproline are the most important amino acids in the collagen structure. The proline metabolic pathway is strategically located in the metabolism.[17] Proline-containing peptides are involved in many biological processes such as the proinflammatory response, immune response, and hemostasis.[7] Prolidase is a dipeptidase that breaks down proline or hydroxyproline-containing aminopeptides and plays an important role in the remodeling of the collagen metabolism matrix and cell growth.[8] It plays a regulatory role in the function of other biological molecules.[8,9] Prolidase is an EGFR and HER2 ligand that regulates signaling pathways dependent on these receptors such as PI3K/Akt/mTOR, ERK1/2, and JAK/STAT3. Under physiological conditions, prolidase stimulates these pathways and can act as an interface in regeneration processes involved with inflammation or tissue damage.[9] It has also been observed that the regulation of prolidase activity modulates the biological effects of the nuclear factor κβ (NF-κβ) transcription factor.[9] The NF-κβ is a transcription factor that plays an important role in the activation of the inflammatory response. Lung inflammation can stimulate transcription factors such as NF-κβ, which regulate the expression of proinflammatory and antioxidant genes.[18]

The results of our study showed that the prolidase enzyme activity in patients with COVID-19 was statistically significantly lower than in healthy individuals. The role of prolidase in the modulation of NF-κβ and its involvement in the signaling pathways in inflammation and tissue damage explain its low level in patients with COVID-19. When the amino acids in the proline pathway were examined, proline and hydroxyproline were found to be lower in patients with COVID-19 compared to the control group, whereas the glutamic acid level was found to be higher. Prolidase deficiency reduces circulating proline levels. The hydroxylation of proline is an important factor in regulating the stability of collagen. In addition, the hydroxylation of proline can prepare functional sites to interact with proteins and receptors. The low level of proline in the circulation may also have led to the decrease in hydroxyproline formed by the hydroxylation of proline.

The regulation of proline is critical to ensure tissue integrity. Mammals can synthesize proline from arginine, glutamine, and glutamate. It is known that glutamine plays a key role in protein metabolism.

TABLE 2. Proline Metabolism Profile and Other Laboratory Results[a]

Results	Patients with COVID-19 (n = 116)	Control Group (n = 46)	P Value[b]
Prolidase, U/L	788 ± 86.5	855 ± 52.3	<.001
Proline, μmol/L	196 ± 44.1	228 ± 46.2	.012
Hydroxyproline, μmol/L	4.93 ± 1.14	7.92 ± 1.43	<.001
Glutamic acid, μmol/L	219 ± 63.4	65 ± 13.7	<.001
Mn, mcg/L	2.52 ± 0.73	2.33 ± 1.01	.299
Cu, mcg/dL	138 ± 24.6	113 ± 21.3	<.001
Zn, mcg/dL	136 ± 19.4	159 ± 18.1	<.001
Cu/Zn ratio	1.01 ± 0.22	0.71 ± 0.17	<.001
TP, g/L	61 ± 5.81	68.3 ± 4.59	.010
ALB, g/L	36.4 ± 4.03	40.1 ± 3.6	.034
Iron, μg/dL	27.93 ± 7.89	69.2 ± 10.7	<.001
LDH, U/L	301.7 ± 42.63	198 ± 20.6	<.001
PCT, μg/L	0.066 ± 0.022	0.03 ± 0.01	<.001
Ferritin, μg/L	389 ± 41.55	48 ± 9.6	<.001
D-dimer, mg/L	1.38 ± 0.3	0.21 ± 0.07	<.001
WBC, ×10^9/L	7.01 ± 1.8	5.99 ± 1.23	.041
LYM, ×10^9/L	1.18 ± 0.25	1.93 ± 0.46	<.001
ESR, mm/h	43.09 ± 9.9	9.2 ± 2.8	<.001
CRP, g/L	0.042 ± 0.015	0.002 ± 0.0006	<.001

ALB, albumin; CRP, C-reactive protein; Cu, copper; ESR, erythrocyte sedimentation rate; LDH, lactate dehydrogenase; LYM, lymphocytes; Mn, manganese; PCT, procalcitonin; TP, total protein; WBC, white blood cells; Zn, zinc.
[a]Values are given as mean ± standard deviation.
[b]P < .05, statistically significant.

TABLE 3. Relationship Between Proline Metabolism Tests and Other Laboratory Tests

	LYM	Ferritin	D-dimer	CRP	Cu	Zn	Cu/Zn
Prolidase	r = 0.56 P = .001	r = −0.24 P = .042	r = −0.28 P = .022	r = −0.22 P = .038	r = −0.18 P = .25	r = 0.30 P = .029	r = −0.21 P = .043
Proline	r = 0.46 P = .009	r = −0.19 P = .009	r = −0.15 P = .162	r = −0.50 P = .006	r = −0.47 P = .009	r = 0.28 P = .025	r = −0.53 P = .003
Hydroxyproline	r = 0.38 P = .041	r = −0.52 P = .004	r = −0.36 P = .040	r = −0.71 P < .001	r = −0.35 P = .039	r = 0.28 P = .042	r = −0.42 P = .014
Glutamic acid	r = −0.32 P = .041	r = 0.32 P = .037	r = 0.39 P = .038	r = 0.30 P = .03	r = 0.33 P = .017	r = −0.36 P = .012	r = 0.40 P = .009

CRP, C-reactive protein; Cu, copper; LYM, lymphocyte; Zn, Zinc.

Therefore, glutamine is considered a regulatory amino acid of proline availability for collagen biosynthesis. Glutamic acid levels in patients with COVID-19 in our study may have been elevated to compensate for low proline levels and to provide a proline source. There was no difference between the patients with COVID-19 and the control group in terms of manganese levels. Manganese is located in the active site of prolidase. However, there are other divalent cations in the active site of prolidase. In this study, it was determined that manganese is not associated with low levels of the prolidase enzyme.

In our study, increased CRP, PCT, ESR, and ferritin, markers of inflammation, were found in patients with COVID-19 compared with healthy control individuals. Significant correlations were found between proline, hydroxyproline, glutamic acid amino acids, and the prolidase enzyme, which play a role in proline metabolism, with LYM, ferritin, D-dimer, and CRP parameters used in the diagnosis, treatment, and follow-up of the prognosis of COVID-19. The important role of prolidase in the modulation of NF-κβ and signaling pathways in inflammation and tissue damage explains the negative correlation between prolidase levels and inflammatory markers in patients with COVID-19. These data support the relationship of the proline pathway with inflammation, hemostasis, and immune response in COVID-19.

There are reports of the clinical relevance of prolidase in disorders of collagen metabolism,[19-21] metabolic disorders,[22,23] and oncological disorders.[24,25] In a study on the influenza A virus, it was shown that prolidase is a cellular factor required by the influenza virus for successful entry into target cells.[26] In addition, it has been shown that prolidase is required by the influenza virus in the early period of infection, and in the absence of prolidase, early viral events change, which leads to a decrease in the amount of virus in the early and late endosomes and fewer fusion events.[26]

In a metabolomics study in patients with COVID-19, in contrast to non–COVID-19 specimens, COVID-19 specimens showed reduced

proline in serum.[27] In this study, the researchers suggested that lactate and L-proline metabolites may help reduce the risk of SARS-CoV-2 infection because proteomic analysis of host cells infected with SARS-CoV-2 revealed that the inhibition of central carbon metabolism prevents viral replication.[27] Although the low proline levels in the earlier study are in line with the results of our study, the analysis of other metabolites in the proline pathway is an advantage of our study. In another metabolomics study, patients with COVID-19 were reported to exhibit low amino acid levels.[28] In addition, the study emphasized that arginine/proline/citrulline metabolism is an important pathway affected by COVID-19.[28] In the study, the low proline level in patients with COVID-19 was parallel to that reported in our study, whereas glutamine levels were found to be lower in patients with COVID-19 compared to the control group, unlike in our study.

Our results showed that iron levels were lower in patients with SARS-CoV-2 infection than in the control group. Because iron is a crucial factor in various processes involving DNA synthesis and adenosine triphosphate production, viruses principally count on iron to replicate in host cells.[29] The cytokines participating in the "cytokine storm" in COVID-19 are potent modulators of iron metabolism. For instance, interleukin (IL)-6 plays a role in many processes ranging from B-cell proliferation to hepcidin synthesis in the liver.[30,31] Hepcidin is the chief regulator of iron homeostasis. During infection or inflammatory conditions, hepcidin levels rise and limit the availability of iron in the plasma.[32] The emerging hypoferremia is an indivisible part of the host defense system.[32] Moreover, certain cytokines such as IL-1 and tumor necrosis factor enhance the generation of ferritin, which is the iron storage protein. As a result, more iron is kept mostly in the reticuloendothelial system, which processes most of the iron recycled from deformed red blood cells. This hypoferremia results in the impairment of iron uptake in many organs.[30-33]

Trace elements have an important place in maintaining a healthy body. For example, Zn is an essential trace element in the growth and maintenance of immune cells. It inhibits the RNA polymerase required to replicate RNA viruses such as those containing coronavirus.[34] Furthermore, Cu is an important micronutrient for viral infections for both pathogens and hosts. It has been shown to play an important role in immunity through its involvement in the production and differentiation of immune cells such as T-cell proliferation and natural killer activity in the host.[34] The Cu/Zn ratio is clinically more important than the concentration of these metals separately.[35] Previous studies have shown that Cu and Zn deficiencies predispose to infections, whereas systemic inflammation and infections result in a decreased serum Zn concentration during the acute-phase response because of the redistribution of serum Zn to the liver and other tissues.[35-37] In addition, acute infections cause an increased serum Cu concentration. Both responses end in an elevated serum Cu/Zn ratio.

The Cu/Zn ratio has been found to be high as an acute-phase response in several infectious diseases.[35,37] Researchers have extensively reviewed the relationship between the Cu/Zn ratio and health status.[36] Oxidative stress and inflammation impact the Cu/Zn ratio, and the Cu/Zn ratio modulates immune defense and stress response.[36,37] In our study, Cu levels and the Cu/Zn ratio were higher in patients with COVID-19 compared to the control group, and Zn levels were lower. In addition, we found significant correlations between tests of proline metabolism and Cu, Zn, and the Cu/Zn ratio. These results provide information that strengthens the relationship between proline metabolism and inflammation and immune response in COVID-19.

As in our study, Fromonot et al[38] found Zn levels lower in patients with COVID-19 than in patients without COVID-19. They also showed the association of low Zn with lymphopenia and inflammation in the early phase of COVID-19.[38] In another study, Skalny et al[39] found a high Cu level, low Zn level, and higher Cu/Zn ratio in patients with COVID-19 compared to healthy control individuals. In addition, as the severity of the disease increased, the Zn level gradually decreased and the Cu and Cu/Zn ratio gradually increased.[39] These data support the Cu, Zn, and Cu/Zn ratios we found in our study.

This study has some limitations. One of these is the age difference between the patient and control groups. The mean age of the patient group was higher than the mean age of the control group. When conducting a scientific study, it is often a challenge to create a control group of healthy individuals older than age 60 years. Another limitation is the sex distribution in the patient and control groups. We do not have any information on the effect of sex on amino acid levels.

Conclusion

In conclusion, changes in proline metabolism have been observed in patients with COVID-19. Low levels of the prolidase enzyme and the proline and hydroxyproline amino acids and high glutamic acid amino acid levels suggest that they are associated with inflammation, the release of proinflammatory cytokines, and immune response in COVID-19. The relationship between proline metabolism tests and commonly used inflammatory and hemostatic markers in COVID-19 supports this hypothesis. In addition, proline pathway metabolites have associations with the Cu/Zn ratio, which is recognized to modulate immune response.

Funding

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