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REVIEW ARTICLE

A Review on Biochemical and Immunological Biomarkers used for Laboratory Diagnosis of SARS-CoV-2 (COVID -19)

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Abstract:

COVID-19 caused by SARS-CoV-2 is considered an emerging disease that results in severe acute respiratory syndrome. Coronaviruses, as single-stranded RNA viruses, have envelope and positive-sense genome. Virion's nucleocapsid contains genomic RNA and phosphorylated nucleocapsid protein, which is located within the phospholipid layers and is covered with spike proteins. Although considerable research improvements have occurred, the virus origin is yet obscure. There are several factors that contribute to the development of COVID-19, such as mutations, viral loads, and the survival of the virus in the laboratory, or related factors such as age, sex, and immune status. Proper diagnosis of changes in biochemical and immunological factors affecting COVID-19 leads to the rapid detection of this disease. Finding suitable biochemical and immunological biomarkers could help us in early diagnosis and reducing the casualties of this disease. However, further studies for finding a good solution to early diagnosis require a better understanding of the mechanism of action of this virus. In this review, we review the biochemical and immunological biomarkers used for laboratory diagnosis of SARS-CoV-2 (COVID -19).

Keywords: Biochemical, Immunological, Biomarkers , COVID-19 , Laboratory diagnosis , Hepatic enzyme , SARS-CoV-2.

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1. INTRODUCTION

The pandemic of COVID-19 began in December 2019 in Wuhan, China. More than 24.8 million cases of COVID-19 and more than 838,000 deaths have been reported until 29 August 2020 [1].

Coronaviruses (CoVs), which belong to the Nidovirales order, Coronaviridae family, Coronavirinae subfamily, are single-stranded RNA viruses that are also enveloped with positive-sense [2]. Due to the crown-like appearance of the CoVs, this name has been given to this virus [2]. Alphacoronavirus (α CoV), Betacoronavirus (β CoV), Deltacoronavirus (δ CoV) and Gammacoronavirus (γ CoV) are the four different types of coronavirus [3]. Human Coronaviruses (HCoVs) are recognized as respiratory pathogens related to respiratory and intestinal infections [4]. SARS-COV-2, which includes CoVs, is found in humans, bats, and other wildlife species (SARS-CoV, in bats and others) and is a beta coronavirus [5, 6].

A study of 12 sequences of viral genomes and their sampl-

ing dates (24 December 2019 and 13 January 2020) as well as mapping and phylogenetic analysis of the virus genome along with the geographic location (first time in Wuhan, Hubei Province, China) indicated the first generation of potential human-to-human virus transmission. In a report, it has been estimated that SARS-CoV-2 probably emanated on 9 November 2019 (95% valid: 25 September 2019 and 19 December 2019) [7]. Based on another report, the outbreak of the virus was from 15 October to 10 November 2019, or 16 November to 22 December 2019 (contingent on the calculation method) [8].

Patino Galindo presented a double scenario for the emergence of the SARS-CoV-2 virus, according to which the ancestors of the virus in bats initially obtained the genetic traits of SARS by recombining SARS-like Receptor-binding Domain (RBD) in 2009, which later evolved under convergent evolution [9]. According to this report, the consumption pattern of amino acids in SARS-CoV-2 is generally identical to that of the bat and human severe acute respiratory syndrome-related coronavirus (SARSr-CoVs) [10].

Compared to SARS, bat SARS and MERS CoV, Pangolin β -CoV had little difference with SARS-CoV-2 in terms of the Effective Number of codon (ENc) values [11]. Tang provided

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new data about the evolution and origin of SARS-CoV. However, the researchers found merely a 4% variation in genomic nucleotides between SARS-CoV bat CoV RaTG13 and SARS-CoV-2; the 17% difference in neutral sites indicates a greater variance between two viruses, unlike the previous estimation. Based on this report, new variants in functioning sites of spike RBD have been seen in SARS-CoV-2, and viruses from pangolin SARS-CoVs have probably resulted from natural selection and mutations as well as recombination. After analyzing 103 SARS-CoV-2 genomes, it was indicated that these viruses underwent evolution into two main types. These two types include L and S, which were found outside China. Considering that type L is more prevalent (70%) than type S (30%), it can be concluded that type S is the ancestral version [12]. Although considerable research improvements have occurred, the virus origin is yet obscure [13].

Finding suitable biochemistry and immunological biomarkers could help us in early diagnosis and reducing the casualties of this disease. In this review, we aimed to review the previous investigations of biochemical and immunological biomarkers to recommend suitable diagnostic biomarkers for laboratory diagnosis of SARS-CoV-2 (COVID-19).

2. IMMUNOLOGY ANALYSES

2.1. Antibody Response

The host's humoral response, including IgA, IgM, and IgG, to SARS-CoV-2, has been investigated using recombinant viral nucleocapsids and an ELISA based assay. Two hundred- eight plasma samples, including 82 confirmed cases and 58 suspected cases, were obtained from the patients. By these samples, the IgM diagnostic value was assessed. IgM with 85.4% and IgA with 92.7% were detected after 5 days (IQR 3-6), and IgG with 77.9% was detected after 14 days (IQR 10-18) of the onset of symptoms. The positive IgM antibody level was estimated to be 75.6% in confirmed cases and 93.1% in suspected cases. IgM ELISA had higher diagnostic efficiency than qPCR in 5 days from the onset of symptoms. Compared to the single qPCR test, the use of IgM ELISA with PCR for each patient significantly increased the rate of positive diagnosis so that this value for qPCR and IgM ELISA with PCR was (51.9%) and (98.6%), respectively [14].

To *et al.* conducted a cohort study at two hospitals in Hong Kong and assessed the serum antibody value against two essential proteins including surface spike RBD and internal nucleoprotein for 14 days or more after the onset of symptoms using the EIA method. They observed that 15 (94%), 14 (88%), 16 (100%), and 15 (94%) out of 16 patients were positive for anti- internal nucleoprotein IgG, anti- internal nucleoprotein IgM, anti-RBD IgG, and anti-RBD IgM, respectively. They believed that IgG anti-SARS-CoV-2- internal nucleoprotein or anti-SARS-CoV-2-RBD levels are associated with virus neutralization ($R_2 > 0.9$) [15].

In another study, Wu and co-workers measured the antibody level against RBD, S1, and S2 proteins in the 175 recovered patients using ELISA. All patients manifested mild symptoms. 10 to 15 days after the onset of disease, specific neutralizing antibodies against SARS-CoV-2 were detected in

patients' samples without cross-reactivity with SARS-CoV. Plasma neutralizing antibody titers and S-antibodies targeting S1, RBD, and S2 were in correlation and were significantly lower in young people than in middle-aged people ($P < 0.0001$ and $P = 0.0003$, respectively).

The neutralizing antibody titers were positively correlated with plasma C-reactive Protein (CRP) levels but negatively correlated with the lymphocyte counts of patients at the time of admission, indicating an association between humoral response and cellular immune response [16]. The neutralizing antibody titers were negatively associated with the number of lymphocytes in patients at the time of hospitalization and were positively correlated with the levels of the plasma CRP, indicating a link between cellular immune and humoral response. According to some observations in patients, there are some memory B cells specified for viruses and capable of detecting RBD on the surface of SARS-CoV-2. They produced 206 specific SARS-CoV-2 RBD monoclonal antibodies using B-cell Receptor (BCR) sequencing and single-cell sorting. Antibodies were derived from different genes of immunoglobulins families with no evident enrichment for any particular family. Two clones of 98-99% showed blocking activity against virus entry, which correlated with high competition capacity against ACE2 receptors (which is considered a vector for COVID-19) [17, 18].

2.2. Virus' Cellular Responses

Liu *et al.* investigated the variation of the subsets of lymphocyte and cytokines in 40 patients using flow cytometry and immunoassay. 13 out of 40 patients with severe symptoms showed considerable lymphocyte reduction, especially of CD8⁺ T cells, but exhibited a neutrophil increase in comparison with the 27 mild patients. In addition, IL-6, IL-10, IL-2, and IFN- γ levels in the peripheral blood were significantly higher in patients with severe symptoms. In surviving patients with acute symptoms, the levels of T cells and cytokines gradually decreased and reached levels similar to those patients with mild symptoms. The authors believed that the ratio of neutrophil to lymphocyte and neutrophil to CD8⁺ T cells could be a valuable prognostic indicator to screen the severe diseases in the early stage [19]. In another study on the 60 patients who suffered from COVID-19, the peripheral blood lymphocyte subscales were investigated using flow cytometry both before and after treatment. The levels of all lymphocytes, including CD4⁺ and CD8⁺ T cells, B cells, and NK cells, were reduced, and it was directly related to the severity of the disease. There has also been a correlation between inflammation and subsets, including CD8⁺ T cells and the CD4 / CD8 ratio. In addition, in 67% of treated patients, increased rates of CD8⁺ T cells and B cells were observed [20].

According to another study, the level of CD8⁺ T and NK cells in SARS-CoV-2 infection patients has been significantly reduced. In addition, NKG2A expression was upregulated on NK cells and CTLs in patients with a reduced ability to produce CD107a, IFN- γ , IL-2, granzyme B, and TNF- α . Also, in the patients, NK and CTLs increased the expression of NKG2A, although the ability to produce CD107a, IFN- γ , IL-2, granzyme B, and TNF- α was decreased. The level of NKG2A

+ cytotoxic lymphocytes has also reduced after the patients' recovery. It can be due to the functional exhaustion of cytotoxic lymphocytes [21]. Zheng *et al.* provided a detailed analysis of the immunological characteristics of peripheral blood leukocytes from 16 patients, including 10 mild cases and 6 severe cases. The levels of IFN- γ and TNF- α in CD4⁺ T cells were lower in the severe group than in the mild group, whereas the levels of granzyme B and perforin in CD8⁺ T cells were higher in the severe group than in the mild group. Another study was conducted on 16 patients infected with COVID-19 (10 mild and 6 severe cases) for evaluating peripheral blood leukocytes and their immunological characteristics. The study showed that the more group of acute patients had lower levels of IFN- γ and TNF- α in their CD4⁺ T cells compared to the mild patients group, and on the other hand, they had a higher level of granzyme B and perforin in CD8⁺ T cells. Although the activation molecules displayed no differentiation in CD4⁺ T cells, the mild group had lower levels of HLA-DR and TIGIT in CD8⁺ T cells. According to this report, COVID-19 behaves in a similar manner to some chronic infections, so that it disrupts the function of CD4⁺ T cells and makes CD8⁺ T cells more active and possibly exhausts them. The number of CD4⁺ T multifunctional cells (with at least two positive cytokines) in the severe group was reported to be less than the healthy control group and the mild group. The amount of the non-exhausted (PD-1-CTLA-4-TIGIT-) subsets of CD8⁺ T cells in the severe group was significantly lower compared to the healthy control group and the mild group [22].

3. LABORATORY FINDINGS & BIOMARKERS

Various laboratory researches are ongoing to search for signs for early diagnosis and understanding the pathological mechanism of the virus.

3.1. Virus Load

The virus load in patients' respiratory secretions using RT-PCR is one of the ways for diagnosing COVID-19. The following are some of the findings regarding it:

3.1.1. Virus Load And Disease Severity

One of the early diagnosis methods for the severity of the disease is to evaluate viral load from the patient's respiratory tract. According to a report from 76 patients with COVID-19, the virus load can be used to assess disease severity and prognosis, so in acute cases, the viral load was found to be at least 60 times higher than that of the mild cases [23, 24]. However, it has been reported that the rate of viral load in the respiratory tract of two patients with mild symptoms was 5.2 and 7.4 log₁₀ copies per 1000 cells 24 hours after the onset of the disease [25]. There are conflicting reports on the relationship between viral load and age, which sometimes links older age to higher viral load, and some reports do not associate the two [15, 26].

3.1.2. Virus Load in Different Types of Specimens

In a research study, the samples of 82 patients were examined. In the daily examination of the samples of throat swab and sputum of 2 patients, it was observed that the viral

load reached its peak in 5-6 days from the onset of symptoms (10^4 to 10^7 copies per mL). At different severity levels of infection, the viral load ranged from 641 to 1.34×10^{11} copies per mL. The average viral loads for throat samples and the sputum samples were 7.99×10^4 and 7.52×10^5 , respectively. In deceased patients, the sputum samples collected in 8 days after the onset of the disease had a high amount of 1.34×10^{11} copies per mL. Positive results of RT-PCR in 2 people who were exposed to infection before the onset of symptoms indicate the possibility of people getting infectious before the onset of symptoms. Stool samples from 11 of the 17 cases, 0-11 days after the onset of the disease, were positive with a lower viral load using RT-PCR analysis [27]. In another report, the authors found RNA of SARS-CoV-2 in the blood of 6 of 57 patients. Since all of these 6 patients showed severe symptoms, they concluded that there is a strong association between the disease severity and the serum viral RNA (p-value = 0.0001) [28].

3.2. Cell Counts

Lagunas-Rangel investigated the neutrophil to lymphocyte and Lymphocyte-to-CRP ratio in COVID-19 patients to understand if these values can predict the severity of the disease. He conducted a meta-analysis on six studies with 828 patients and reported in severe COVID-19 patients, the neutrophil to lymphocyte ratio is significantly increased (SMD=2.404, 95% CI=0.98 to 3.82), whereas the lymphocyte-to-CRP ratio is decreased (SMD= -0.912, 95% CI= -1.275 to -0.550) [29].

According to the study findings presented by Liu *et al.*, in which 61 patients were monitored, it was concluded that neutrophil to lymphocyte ratio could be a prognostic marker for the identification of severe diseases. This marker has overridden the MuLBSTA score known for monitoring COVID-19 patients [30]. The following report from 40 patients' data verified the conclusion above [19]. The number of lymphocytes in severe cases is considerably lower (0.7×10^9 /L) than in moderate cases (1.1×10^9 /L), based on Chen's report. The absolute number of T lymphocytes, CD4⁺ T, and CD8⁺ T cells experienced a decline in almost all the patients and was significantly lower in severe cases (294.0, 177.5, and 89.0×10^6 /L) than that in the moderate cases (640.5, 381.5 and 254.0×10^6 /L). The expressions of IFN- γ by CD4⁺ T cells were lower in severe cases (14.1%) than in moderate cases (22.8%) [31]. Moreover, Zheng *et al.* assessed the differences between 103 COVID-19 and 22 non-COVID-19 pneumonia cases through examining the laboratory parameters. The lymphocyte subsets number had a remarkable negative relationship with biochemical indices that are related to organ injury in the patients infected by COVID-19 [32]. In a similar way, the phenomenon of lymphocyte depletion (PLD) was explained by Zeng *et al.* This phenomenon was reported in a 100% proportion of severe or critical cases [ICU]. With the advancement of the disease, the lymphocyte amount fell drastically [33]. An investigation by Tan *et al.* affirmed the observation of Lymphopenia. In the beginning, the number of lymphocytes declined in severe patients, after which it rose by above 10% till discharge. However, there was a brief

fluctuation in the number of lymphocytes after initiation of disease, followed by an increase above 20% during discharge in moderate patients [34]. Since eosinopenia is regularly noticed in COVID-19 patients (79% in SARS-CoV-2 positive patients compared to 36% in SARS-CoV-2 negative patients), a straight forward alternate method has been offered to assist triage of patients. This method resulted in a diagnosis specificity and sensitivity of 64% and 79%, respectively [35]. As reported in previous studies, severe cases had the tendency to have higher leukocytes amount, lower number of lymphocytes, a lower proportion of eosinophil, monocytes, basophils, and a higher neutrophil to lymphocyte ratio. The majority of severe cases showed an increased amount of inflammatory cytokines and infection-related biomarkers. Lymphocyte subgroups were examined in 44 patients with COVID-19 on admission. There was a dramatic decrease in total number of T cells, B cells, and NK cells in patients with COVID-19, especially in severe cases. On the other hand, the proportion of naïve helper T cells ($CD3^+$, $CD4^+$, $CD45RA^+$) rose while that of memory helper T cells ($CD3^+CD4^+CD45RO^+$) fell in severe cases [36].

3.3. Biochemistry Tests

High CRP is a significant trait of COVID-19 [37]. A study of 12 patients demonstrated that blood biochemistry indexes such as lactate dehydrogenase (LDH), CRP and albumin might be a hallmark of disease severity [23]. Also, based on the report by Liu *et al.*, CRP was found to be high in a group of patients with progressive disease compared with improved/stabilized group (38.9 [14.3, 64.8] vs. 10.6 [1.9, 33.1] mg/L, $U = 1.315$, $P = 0.024$). The level of albumin was considerably higher in the improvement/stabilization group than that of the progression group (41.27 ± 4.55 g/L vs. 36.62 ± 6.60 , $U = 2.843$, $P = 0.006$) [38]. Based on the observations by Li *et al.*, there was a dramatic rise in the levels of CRP and Serum Amyloid A (SAA). Over the disease progression, the levels of SAA and CRP increased, while the number of lymphocytes declined. By analyzing the ROC curve, it could be concluded that the levels of CRP, SAA, lymphocyte numbers, and SAA/lymphocyte ratio are useful data for assessing the severity of COVID-19 and separation of severe cases from mild ones. Also, it is more likely that the CT images of patients who have initially high levels of SAA are poor [39]. As explained in the report by Fan *et al.*, 50.7% of 148 patients had an aberrant liver function on admission with increased Gamma-glutamyl Transferase (GGT), AKP, Aspartate Aminotransferase (AST), and Alanine Aminotransferase (ALT) levels [40]. Levels of LDH, high-sensitivity CRP, ALT, and ferritin were considerably higher in severe cases (41.4 U/L, 567.2 U/L, 135.2 mg/L and 1734.4 ug/L) than that in moderate cases (17.6 U/L, 234.4 U/L, 51.4 mg/L and 880.2 ug /L). Moreover, the concentrations of TNF- α , IL-2R and IL-10 were higher in severe cases (1202.4 pg/mL, 10.9 pg/mL and 10.9 pg/mL) compared to mild cases (441.7 pg/mL, 7.5 pg/mL and 6.6 pg/mL) on admission [31].

Also, the level of the plasma angiotensin II from COVID-19 patients was raised and had a linear correlation with lung injury and viral load [23]. The enhanced amount of procalcitonin is related to an almost 5-fold higher risk of severe

SARS-CoV-2 infection (OR, 4.76; 95% CI, 2.74-8.29) based on a meta-analysis by Lippi *et al.* Among various studies, the heterogeneousness was moderate (*i.e.*, 34%). Since this biomarker synthesis is prevented by INF- γ , its concentration is assumed to rise over the period of viral infections; the authors think that an elevated amount of procalcitonin could illustrate bacterial superinfection in severe cases. However, more researches are required to be carried out to determine the biomarker's origin [41]. Lippi *et al.* evaluated the level of cardiac troponin I (cTnI) in patients with COVID-19 by conducting a meta-analysis. Despite high heterogeneity, the amount of cTnI rose in the patients with severe illness compared to those without severity (SMD, 25.6 ng/L; 95% CI, 6.8-44.5 ng/L) [42].

Serum ferritin, IL-10, and IL-6 levels were considered an essential determinant for severe diseases. According to the findings by Terpos *et al.*, different criteria are capable of determining the severity. For instance, thrombocytopenia, lymphopenia, and neutrophilia could anticipate acute respiratory distress syndrome (ARDS), as well as cardiovascular complications. Increased levels of ferritin, CRP, LDH, IL-6, procalcitonin, and coagulation disorders (PTT, D-dimer, aPT, and increased fibrin degradation) have also been emphasized [43].

In another study on 47 patients by Han *et al.*, the author reported that LDH had the most positive relationship with the severity of disease; there is also a strong correlation of LDH with lung damage. They also reported that LDH had a positive correlation with CRP, AST, Blood Urea Nitrogen (BUN) and cTnI, and a negative correlation with lymphocytes. In severe cases, during the 14-days monitoring period, they observed an increase in CRP and a decrease in lymphocytes, especially $CD3^+$, $CD4^+$, and $CD8^+$ T cells [44].

Zhou *et al.* performed blood and urine analysis in 178 patients. The serum creatinine (Scr) did not increase in patients; also, BUN increased in 2.8% of the patients, indicating "kidney dysfunction" in a few cases. In 54.2% of 83 patients without any kidney disease history, 45 (54.2%) patients showed proteinuria, hematuria, and leukocyturia, in their urinalysis, whereas no Acute Kidney Injury (AKI) parameter was observed. It is noteworthy that patients who show such abnormalities in their urinalysis, usually have higher liver injury inflammation and coagulation parameters, and they are more severe cases compared to the others. It has been suggested to use urinalysis which could be an excellent method to evaluate disease severity.

Tian *et al.* studied the patterns of the longitudinal changes in immunological and biochemical parameters in 59 patients with COVID-19. At the onset of the disease, the value of eosinophils reduced by 52.6%, and it increased significantly after that. Also, in 40.4% of cases, the number of lymphocytes reduced at the onset of the disease, and then after 5 days, it started to increase gradually. The platelet count in 12.3% cases reduced at the onset of the disease, and after 7 days, the average value of mean platelet volume reduced significantly. After day 7, in 30.6% of patients, the values of AST, LDH, creatine kinase, creatinine kinase-muscle/brain activity, and cTnI, serum cardiac markers, were more than the upper limit of

RI. Also the abnormality of liver function tests, kidney function tests, electrolytes was 2.0%~59.2%, 2.0%~4.1%, 6.0%~30.0%, respectively [45].

3.4. Coagulation Parameters

The coagulation data of 183 successive patients with verified COVID-19 pneumonia was explained by Tang *et al.* The levels of Fibrin Degradation Product (FDP) and D-dimer were higher in non-survivors. Also, longer activated partial thromboplastin time and prothrombin time were revealed in non-survivors compared to survivors on admission ($P < 0.05$) [46].

Zhou *et al.* realized that increased likelihood of in-hospital death is related to the concentration of D-dimer higher than 1.0 $\mu\text{g/L}$ (18-42, 2-64-128-55; $p = 0.0033$) on admission [47]. In addition, according to the findings of Gao *et al.*, levels of IL-6 and D-dimer were associated with the occurrence of severe COVID-19 infection in adult patients, and their combination provides the most sensitive and specific detection for predicting the severity of disease in the early stage. In this study, the specificity of prediction was almost 93.3%, while the sensitivity stood at 96.4% [48].

CONCLUSION

The development of COVID-19 infection is dependent on various parameters, all of which contribute to a person's illness, severity, and duration of illness, as well as the likelihood of death. Some of the biochemical and immunological factors have been different in various studies and groups.

General results from different researches on COVID-19 are similar to that of other infectious diseases, which contribute to an increase in lung damage factors and liver damage factors, as well as an increase in inflammatory factors. The reduction of lymphocytes is also confirmed in most of the articles. Proper diagnosis of changes in biochemical and immunological factors caused by COVID-19 infection leads to early detection of this disease and the reduction in mortality rate. However, further studies are needed for a better understanding of this virus and finding an effective strategy for early diagnosis.

LIST OF ABBREVIATIONS

RBD	=	Receptor-Binding Domain
PCR	=	Polymerase Chain Reaction
EIA	=	Enzyme-Linked Immunosorbent Assay
CRP	=	C-Reactive Protein
BCR	=	B Cell Receptor
NK	=	Natural Killer Cells
CTLs	=	Cytotoxic T Cell
TNF	=	Tumor Necrosis Factor
HLA-DR	=	Human Leukocyte Antigen – DR Isotype
TIGIT	=	T Cell Immunoreceptor
PD-1	=	Programmed Cell Death Protein 1
CTLA-4	=	Cytotoxic T-Lymphocyte-Associated Protein 4

PLD	=	Phenomenon Of Lymphocyte Depletion
SAA	=	Serum Amyloid A
ROC	=	Receiver Operating Characteristic
GGT	=	Gamma Glutamyl Transferase
Akp	=	Alkaline Phosphatase
Ast	=	Aspartate Aminotransferase
Alt	=	Alanine Aminotransferase
Ldh	=	Lactate Dehydrogenase
Inf-Γ	=	Interferon Gamma
BUN	=	Blood Urea Nitrogen
cTni	=	Cardiac Troponin I
Scr	=	Serum Creatinine
AKI	=	Acute Kidney Injury
FDP	=	Fibrin Degradation Product

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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