Assessment of the relation between IL-6 expression and the severity of COVID-19 infection

Alyaa Ezz AbdelKader¹, Mahmoud Shoukry¹, Noha Anwar Hassouna¹, Mohammed Zain-Alabdeen Abdel Kareem², Shaimaa Hassouna Zaki¹
¹ Department of Medical Microbiology and Immunology, Faculty of Medicine, Minia University, Minia, Egypt
² Department of Medical Microbiology and Immunology, Faculty of Medicine, Al-Azhar University, Assiut, Egypt.

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Abstract:
Background: The 2019 coronavirus pandemic (COVID-19) was accused by the SARS-CoV-2 virus, which causes severe acute respiratory syndrome. The cytokine storm may influence the severity and course of illness when levels of proinflammatory cytokines, such interleukin-6 (IL-6), are elevated. The purpose of this research is to determine if there is a correlation between COVID-19 infection severity and IL-6 expression levels. Methods: One hundred people were enlisted for this investigation; fifty patients and fifty were healthy controls. To measure the amount of IL-6 expression, RNA was isolated from patients' peripheral blood leukocytes and subjected to Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). Results: There was a significant difference in the level of IL-6 expression in severe cases than in mild and moderate COVID-19 cases. Conclusion: Based on the results, IL-6 is a key factor in the disease's development and progression. Therefore, it has potential as a diagnostic indicator.

Keywords: COVID-19; IL-6; qRT-PCR.

Introduction
Wuhan, China, was the site of the first detection of a new coronavirus in December 2019. individuals were admitted with acute pneumonia that had no apparent cause (1). After going by names like novel coronavirus 2019 (nCoV-2019) and SARS-CoV-2, the sickness caused by this virus was finally referred to as COVID-19 in February 2020 by the World Health Organization (WHO)(2), Middle East respiratory syndrome coronavirus (MERS-CoV) originated in the Arabian Peninsula in 2012, responsible for respiratory distress syndromes that proved lethal, after the emergence of SARS-CoV-1 in China in 2002. The second coronavirus-related worldwide health concern in the last 20 years is SARS-CoV-2(3).

The primary receptor for SARS-CoV-2 attachment and subsequent host cell entry is angiotensin-converting enzyme 2 (ACE2). A cough, fever, and dyspnea are symptoms that 70% of patients experience after an incubation period of around five days(4). Some patients have an immunological response characterized by a worsening of respiratory symptoms and inflammatory syndrome, often within eight to ten days after the initial clinical symptoms, after this stage of viral invasion (5).

Cytokine storms describe this ineffective immunological stage. Coagulopathy and viral sepsis are possible complications(6). Endothelial damage, brought on by the cytokine storm, makes acute respiratory distress syndrome worse(7).
In the aftermath of tissue injury or infections, several cells release IL-6. These include fibroblasts, macrophages, mast cells, dendritic cells, monocytes, T and B lymphocytes, and vascular endothelial cells (ECs). In order to increase the risk of thrombosis, it stimulates the inflammatory response, which in turn increases levels of acute phase proteins including ferritin and C-reactive protein (CRP). It may also cause leukocytosis, lymphopenia, and thrombocytopenia, as well as induce the tissue factor gene and its procoagulant profile.

Tissue damage may result from cytokine storms, which IL-6 and other inflammatory cytokines can trigger. Cytokine storm and lymphopenia are two immunological pathologic characteristics seen in individuals with viral infections like SARS-CoV-2. To further emphasize the impact of interleukin 6 expressions on the course of COVID-19, this research aims to examine the correlation between higher levels of IL-6 in patients with severe COVID-19 and damage to lung tissue as well as infection.

Materials and methods:

Study design:

This is a case-control study that was conducted in the Medical Microbiology and Immunology Department laboratories at the Faculty of Medicine, Minia University, Egypt. From April 2022 to March 2023, one hundred people participated in the research. They included two groups, a group of healthy controls (N=50) and a group of COVID-19 patients (N=50). The patients included in the study were diagnosed clinically by their manifestations (fever, cough, dyspnea, chest pain, fatigue, GIT manifestations, anosmia, and loss of taste sensation) and confirmed by real-time PCR. Patients excluded from the study include those who were below 16 years old, with a History of COVID-19 vaccination, Pregnant females, and individuals diagnosed with bronchial asthma, malignancies, immunological disorders, cardiac conditions, or other forms of chronic obstructive pulmonary disease (COPD).

Blood samples were collected for determination of IL-6 expression using Real-Time qRT-PCR.

Subjects:

A total of one hundred individuals (50 healthy controls, 15 patients with mild COVID-19, 15 patients with moderate disease, and 20 patients with severe disease) were enrolled in this study. Patients were presented to Minia University Hospital, Minia, Egypt. They were diagnosed as positive by nasopharyngeal swab sample examined by qRT-PCR test. Those in the mild illness group had anorexia, arthralgia, sore throat, and generalized malaise. Fever, cough, headache, and lethargy were seen in the moderate group. Due to respiratory distress and low arterial blood oxygen concentration, the severe group was admitted to the Minia University Hospital critical care unit (ICU).

Methods:

RNA extraction from blood samples:

We centrifuged the blood samples that we took at 800×g for 8 minutes. A 1.5 ml RNase-free microcentrifuge tube was used to transfer 200 µ of each sample. Next, a 3:1 ratio of GENEzol Reagent to sample was established, and a thorough mixing was accomplished by vortexing the mixture. The mixture was incubated for 5 minutes at room temperature. Samples were centrifuged at 14000×g for 1 minute to remove cell debris then the clear supernatant was transferred to a new 1.5 ml microcentrifuge tube.

* 1 volume of absolute ethanol was added directly to 1 volume of sample mixture (1:1) in GENEzol Reagent. Before placing the RB column in a 2 ml collection tube, they were well mixed using a vortex. After transferring 700 µ of the sample mixture to the RB column, it was centrifuged at 14000×g for 1 minute, and the byproduct was removed. Centrifugation was performed at 14000×g for 30 seconds after 400 µ of wash buffer was added. The flow through was discarded and the RB column was placed back in a 2 ml collection tube DNase I solution was prepared as follows:
DNase 1 5 µ, DNase 1 reaction buffer 45 µ (total 50 µ), and added for each sample. Then incubation for 15 minutes at room temperature was done.

* The RNA wash was performed by adding 400 µ of prewash buffer to the RB column and then centrifuging it at 14000×g for 30 seconds. After discarding the flow-through, the RB column was returned to the 2 ml collecting tube. The RB column was centrifuged at 14,000×g for 30 seconds after 600 µ of the wash buffer was poured to it. Following this, the flow-through was removed. The column matrix was dried by centrifugation at 14,000×g for three minutes.

To extract RNA, the RB column was dried in an RNase-free microcentrifuge tube. To make sure the column matrix absorbed all of the RNase-free water, 50 µ of it was put to the middle and let to stand for 3 minutes. The pure RNA was eluted by centrifugation at 14,000×g for 1 minute.

**Real-Time Quantitative reverse-transcription PCR (qRT-PCR):**

**Reaction tube of real-time PCR:**

A real-time qRT-PCR master mix was prepared with a final volume of 20 µ that consists of 10 µ Lo-Rox SYBR one-step mix, 1 µ Forward primer, 1 µ Reverse primer, up to 6 µ Water, and finally 2 µ of RNA template.

RT-PCR cycles were as follows; 42°C for 20 minutes of Reverse transcription, 95 °C for 3 minutes of Polymerase activation, 45 cycles; 95 °C for 15 seconds, 50°C for 30 seconds, and 72 °C 30 seconds. The primer sequence for amplification of IL-6 gene is as follows; forward primer: 5’-3’ TGCAATAACCCCTGACC and reverse primer: 5’-3’ ATTTGCGGAAGGCCCCTCAG. The primer sequence for amplification of the GUS-B gene (house-keeping gene) is as follows; forward primer: 5’-3’ CAGTCCCTCCAGCTTCAATG and reverse primer: 5’-3’ ACCCAGCCGACAAAATGC (13,14).

**Gene expression** was measured using real-time RT-PCR (Applied Biosyst 7500 fast, Techne (Cambridge) LTD., UK). It was normalized to the expression of the housekeeping gene GUS-B. CT (Curve Threshold) was measured for the target case and subtracted from the CT of the housekeeping gene, to get delta CT (ΔCT), and then we subtracted ΔCT of the case from the mean ΔCT of the control to calculate ΔΔCT. From these equations, we calculate relative quantity (RQ), RQ= 2^ {-ΔΔCT} which roughly indicates the gene expression (Livak & Schmittgen, 2001). Melting curve was done to ensure the specificity of amplification.

**Statistical analysis:**

Excel spreadsheets were used to record all data, and SPSS for Windows version 19.0 (IBM, USA) was used for statistical analysis. Statistical significance was determined by a p-value less than 0.05. We used the Eta coefficient test to look for associations between the qualitative variables. The Pearson correlation was used to determine the correlation between the quantitative variables. Correlation coefficient values (Eta or r) between 0.00 and 0.24 indicate little or no link; values between 0.25 and 0.49 indicate reasonable association; values between 0.50 and 0.74 indicate moderate association; and values between 0.75 and 0.75+ indicate high association.

**Ethical Approval:**

This research protocol was approved by the Scientific Ethical Committee of the Faculty of Medicine, Minia University. Patient informed consent was fulfilled before sample collection.

**Results**

In this study, 50 positive cases and 50 healthy controls were included. The included cases were 15 mild, 15 moderate, and 20 severe cases. The mild group included nine males and six females with a mean age of 40 years, the moderate group included eleven males and four females...
with a mean age of 40 years while the severe group included twelve males and eight females with a mean age of 70 years.

The expression levels of IL-6 were higher in COVID-19 cases than in healthy control with a mean expression level in cases about 901-fold higher than that of control ($P=0.22$); Table 1. There was a weak association (Coefficient value Eta =0.16) between the incidence of covid19 and the level of expression of IL6.

**Table 1: Expression levels of IL-6 in cases and control groups**

<table>
<thead>
<tr>
<th>Study group</th>
<th>Expression levels of IL-6 (Mean±SD)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COVID-19 cases</td>
<td>1658.22 (4215.47)</td>
<td>0.22</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>1.87 (1.97)</td>
<td></td>
</tr>
</tbody>
</table>

The expression of IL6 was higher in severe cases versus mild to moderate group.

<table>
<thead>
<tr>
<th>COVID-19 case groups</th>
<th>Expression of IL-6 expression (Mean±SD)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>25.22 (36.21)</td>
<td>0.007</td>
</tr>
<tr>
<td>Moderate</td>
<td>499.8 (699.94)</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>3751.78 (6139.06)</td>
<td></td>
</tr>
</tbody>
</table>

There was a medium association between severity of covid19 and level of expression of IL6 (Coefficient value Eta= 0.41), and a positive correlation between expression of IL6 and severity of covid 19 ($r =0.383$, $P<0.006$) This indicates the role of IL-6 as an important prognostic factor for the course of illness in COVID-19 patients.

**Discussion**

The active signaling pathway explains why IL-6, a pleiotropic cytokine, may both promote and inhibit inflammation\(^{(15,16)}\). Important cellular processes including leukocyte survival, proliferation, differentiation, and chemotaxis are mediated by this cytokine \(^{(15)}\). This research used 100 samples (50 cases and 50 controls) to look for a correlation between IL-6 expression and COVID-19 severity.

Compared to healthy controls, COVID-19 patients had higher levels of IL-6 expression, according to this research. Also, compared to mild and moderate instances, severe patients had higher IL-6 expression.

Intensive care unit admission and illness recovery are both significantly correlated with elevated IL-6 levels, according to Pedersen's research\(^{(17)}\). Patients in the intensive care unit had 52% higher IL-6 levels than those in the general population, according to research by Prompetchara et al.,\(^{(18)}\). According to Chen et al., IL-6 levels in patients are inversely related to illness severity \(^{(19)}\). Additional research has shown that individuals with elevated IL-6
expression levels may have a poorer prognosis, accelerated inflammatory response, and cytokine release syndrome (CRS). The circulating amount of this substance is strongly correlated with both the severity of the illness and the dismal prognosis. Patients who succumbed to COVID-19 had significantly elevated IL-6 levels compared to those who made a full recovery.\(^{(20)}\)

**Conclusion**

Levels of IL-6 are correlated to the infection with COVID-19 and can be used as a predictor of the transition from mild to severe infection.

**Author contributions**

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by [Alyaa Ezz AbdelKader], [Shaimaa Hassouna Zaki], and [Noha Anwar Hassouna]. The first draft of the manuscript was written by [Alyaa Ezz AbdelKader] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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