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Correlation of nasopharyngeal viral load and pro-inflammatory cytokines with COVID-19 disease severity

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Abstract

Introduction: Most individuals with SARS-CoV-2 experience mild symptoms, however, many can experience a severe form of the disease, which can also be fatal. Several studies have associated serum pro-inflammatory cytokines with disease severity. However, very few have associated early cytokine changes, following viral infection of the nasopharyngeal region, with disease severity. Therefore, our study aimed to associate changes in viral loads and the expression of various cytokines in nasopharyngeal samples with COVID-19 disease severity.

Material and Method: A total of 118 SARS-CoV-2 nasopharyngeal samples in the viral transfer medium were collected. The samples were characterized as mild and severe, based on the WHO criteria, and qPCR was performed to determine the viral loads and also evaluate the expression of eight cytokines (IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ, TGF-β1 and TNF-α) expression in the mild and severe group. Subsequently, appropriate statistical tests were applied to determine the differential expression of cytokines in the mild and severe groups and to correlate the viral loads and cytokine expression with disease severity.

Results: Out of 118 nasopharyngeal samples, 71 were characterized as mild, while 47 as severe. The mean viral load between the mild and severe groups was comparable (mild group: 27.07I 5.22; severe group: s 26.37 n7.89). Analysis of the cytokines showed the expression of IL-2, IFN- γ and TNF-α to be significantly higher in the severe groups as compared to the mild group. Further, we also observed a significant positive correlation between all the cytokines in the severe group.

Conclusion: We found IL-2, TNF-α, and IFN-γ expression to be significantly higher in severe cases as compared to mild. The increased expression of pro-inflammatory cytokines in the nasopharyngeal milieu may be considered as biomarkers for disease severity in COVID-19 positive patients.

Introduction

The novel coronavirus (SARS-CoV-2) infection is one of the most serious public health issues at the current time. This virus may result in severe pneumonia with the onset of multiple other illnesses [1]. The SAR-CoV-2 was found to be more prevalent in adult male patients, with a median age of 34-59 years, while the highest percentage of severe cases were observed at 60 years of age of the patient [2]. Patients with pre-existing conditions (co-morbidities), such as diabetes, cardiovascular disease, immunodeficiency, asthma, kidney disease, lung disease, cancer, chronic liver/ gastrointestinal disorders, etc. show the worst prognosis and high mortality rate regardless of their age and gender [3]. Clinical features of SARS-CoV-2 infection are similar to previous of SARS-CoV, which includes fever, dry cough, tiredness, dyspnea, myalgia, and chest discomfort [4]. Additional symptoms are nausea, vomiting, diarrhea, stomach pain, headache, dizziness, however, these are less common symptoms [5].

It has been suggested that increased production of various inflammatory cytokines in serum, such as Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, IL-6, IL-10, and IL-8 is the hallmark of viral infection, presumably by activation of the nuclear factor transcription factor (NF)-B, activator protein (AP)-1 and

activating factor (ATP-2). Recent studies have shown that overexpression of pro-inflammatory cytokines (e.g. IL-6, IL-12, IFNγ, IL1B, MCP1, and IP-10) in serum is strongly correlated with multiple organ failure in patients with COVID-19 disease [6]. Similarly, another study showed high levels of IP10, MCP1, GCSF, MIP1A, and TNF-α in patients who required ICU admittance, indicating that the severity of the infection was correlated with the cytokines [7].

While most of these studies have focused on cytokine changes in serum, few have associated early cytokine changes [8], following viral infection of the nasopharyngeal region, with disease severity. One study from China showed that pro-inflammatory cytokines and chemokine can be detected in the nasopharyngeal sample, where levels of cytokines can be valuable for the diagnosis and prognosis of systemic disease [9]. This suggests that estimating the cytokine battery in the nasopharyngeal samples may make it possible to predict the outcome of COVID-19 disease, and such cytokines can serve as early biomarkers predicting the severity of the disease. In this study, which is part of the larger study assessing immunological response against SARS-CoV-2 in HIV-positive and HIV-negative individuals, we aimed to associate changes in viral loads and the expression of various cytokines in nasopharyngeal samples with COVID-19 disease severity. We found differential expression of various cytokines in nasopharyngeal samples, which correlated with COVID-19 disease severity.

Material And Methodology

Sample collection and characterization of the sample as mild and severe based on patient’s symptoms

A total of 118 nasopharyngeal swabs in viral transport medium (VTM) were collected from SARS-CoV-2 PCR positive patients. The samples were characterized as mild and severe based on their symptoms. The severe and samples were procured, respectively, from the Aga Khan University Hospital SARS-CoV-2/COVID-19 sample biorepository, and Cancer Foundation Hospital, Karachi-Pakistan. This study was approved by the Ethics Review Committee, Aga Khan University Hospital (ERC# 2021-5456-15382). Written consent forms were taken from each patient. To ensure the patient's confidentiality the sample were given unique IDs. All methods were performed in accordance with the relevant guidelines and regulations.

The patients with mild clinical symptoms (fever, cough, sore throat, etc) with no requirement of oxygen therapy or oxygen by mask or nasal cannula were categorized in the mild group; whereas samples from patients with at least one of the following symptoms were included in the severe group: 1) non-invasive ventilation or less than 93 percent oxygen saturation at rest, 2) invasive mechanical ventilator without other organ support, 3) invasive mechanical ventilation with other organ support (example: continuous renal replacement therapy, extracorporeal life support, vasopressors, etc.) [10].

RNA extraction and cDNA synthesis

From all the SARS-CoV-2 positive nasopharyngeal samples, viral RNA was extracted using QIAamp viral RNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was stored at -80C till further use.

RNA was reverse transcribed using ONE SCRIPT PLUS cDNA Synthesis Kit (CAT # G236) from Applied Biological Materials inc. (ABM) following the manufacturer's instructions. In the first step, 5ul of each RNA sample was mixed with 1ul of Oligo DT, 1 ul of 10uM dNTP mix, and 20ul of nuclease-free water. The reaction tubes were incubated at the preheated block at 65°C. Immediately after the incubation the reaction mixture was chilled on ice for 5 minutes and centrifuged briefly to bring the content to the bottom of the tube. Subsequently, 1ul of reverse transcriptase enzyme was added and the volume was made up to 20ul. The reaction mixture was incubated in a thermal cycler under the following conditions: initial incubation for 15 minutes at 55°C, followed by another incubation at 85°C for 5 minutes, and then hold at 4°C. Subsequently, the freshly synthesized cDNA mixture was incubated for 1 minute on ice.

Detection of viral load:

The viral load was determined using the COVID-19 genesig® Real-Time PCR assay (Primerdesign). Each of 20µL triplex reaction mix containing 8µL of eluted RNA, and 12µL of the master mix was subjected to the following thermocycling conditions: 55°C for 10 min, 95°C for 2 min followed by 45 cycles of 95°C for 10 s, 60°C for the 60s. Ct values of Internal control and Target (Orf1ab) genes were measured on Hex and FAM channels, respectively, on Rotor Gene™ 3000 (Qiagen, USA).

Quantitative Polymerase Chain Reaction (qPCR) for estimation of cytokines gene expression:

The cDNA samples were used in the qPCR assay to estimate the expression level of each of the cytokine's genes using gene-specific primers (Table 1). The β-actin gene was used as a housekeeping gene and also to normalize the gene expression.

Table 1
List of primers for cytokine and β-actin genes used in the qPCR array.

Genes	Forward Primer (5'—3')	Reverse Primer (3'—5')
IL- 1	5-ATGATGGCTTACSGTGGCAA-3	3-GTCGGAGATTCGTAGCTGGA-5
IL-2	5-GAAGATCGTCATGGGAAGGAAGC-3	3-CGGGTATTTATAGTGGCATGGG-5
IL- 4	5- CCAACTGCTTCCCCCTCTG-3	3-TCTGTTACGGTCAACTCGGTG-5
IL-6	5- ACTCACCTCTTCAGAACGAATTG-3	3-CCATCTTTGGAAGGTTCAGGTTG-5
Il-10	5-GACTTTAAGGGTTACCTGGGTTG-5	3-TCACATGCGCCTTGATGTCTG-5
IFN-γ	5-GAGGCCAAGCCCTGGTATG-3	3- CGGGCCGATTGATCTCAGC-5'
TNF- ∝	5- TCGGTAACTGACTTGAATGTCCA-3	3 - $TCGC$ T ℂ CTG T T $AGCTGC$ - 5
TGF-β1	5-CAATTCCTGGCGATACCTCAG-3	3-GCACAACTCCGGTGACATCAA-5
B-actin	5-CAACTTCATCCAGCTTCACC-3	3-TCGAGGACGCCCTATCATGG-5

In order to make the reaction mixture for qPCR analysis, 2ul of cDNA was mixed with 4ul of BlasTaq 2X qPCR Mastermix (Cat # G891; ABM), and 0.5ul of each reverse and forward primers were added. The qPCR was performed using the following thermal cycling conditions: 95°C for 3 minutes, 40 cycles of 95°C for 15 seconds, and 57.8°C to 64°C (depending on the primer) for 1 minute with a melt curve at 55-95C. All reactions were run in duplicate. The relative gene expression was calculated using the comparative Ct (threshold cycle) method [11, 12].

Statistical Analysis:

An unpaired T-test was applied to compare the statistical difference in mean expression of each cytokine between the mild and severe groups. Similarly, an unpaired T-test was also used to measure the difference in viral load between the two groups. In all tests, a $p<0.05$ was considered significant. We also applied the Pearson correlation test to establish a correlation between disease severity, viral load, and cytokines expression. In these analyses, a $p<0.05$ was considered significant. Social sciences (SPSS) software, version 20 used for statistical analysis.

Results

- **Association of viral loads with disease severity**

For this study, a total of 118 nasopharyngeal swabs samples in viral transfer medium were collected, out of which, 71 were mild and 47 were severe patients. The mean viral loads for the mild and severe groups were 27.07 ± 5.22 and 26.37 ± 7.89, respectively. Statistically, no significant difference was found between the two groups, and no association was found between viral loads and disease severity.

- **Differential expression of cytokines between the mild and severe group**

Analysis of differential expression of cytokines in the mild and severe group showed the expression of IL-2, TNF-α, and IFN-γ to be significantly ($p<0.05$) higher in the severe group as compared to the mild group (Figure 1). The mean relative expression of IL-2 in the severe group was 3.41 ± 5.33, while 1.11 ± 1.96 in the mild group (Figure 1). Similarly, the mean relative expression of TNF-α in the severe group was 0.72 ± 4.96, while -1.01 ± 3.40 in the mild group (Figure 1). Comparably, the mean relative expression of IFN-γ in the severe group was 1.57 ± 5.18, while -0.85 ± 4.76 in the mild group (Figure 1). Interestingly, the expression of IL-6 was downregulated in both groups, where the mild group exhibited significant downregulation of IL-6 expression as compared to the severe group ($p<0.05$; Figure 1).

- **Correlation between cytokine expression in mild and severe groups**

Pearson correlation test was applied to investigate the relationship between cytokine expression in mild and severe groups (Table 2). In the mild group, a statistically significant weak positive correlation was found between IL-2 and IL-4 ($r =0.403$, $p= 0.00$), IL-2 and TGF- β ($r=0.334$, $P=0.004$), IL-1 and IL-6 ($r=0.31$, $p=0.01$), and IL-10 and IL-4 ($r=0.403$, $p= 0.00$). The detailed correlations between cytokines in the mild group are shown in Table 2.

Table 2
Correlation between cytokine expressions in the mild group. Each column shows the coefficient of correlation (r). Correlations with significant p-values ($p<0.05$) are shown in bold.

Cytokines	IL- 1	IL-2	IL-4	IL-6	IL-10	IFN- γ	TNF–α	TGF-β
IL- 1	-	0.011	0.116	**0.311**	0.191	0.014	0.198	-0.027
IL-2	0.011	-	0.403	-0.11	0.225	0.13	0.22	0.334
IL- 4	0.116	**0.403**	-	-0.149	**0.46**	0.78	-0.005	-0.171
IL-6	**0.31**	-0.11	-0.149	-	0.144	-0.148	0.127	0.180
IL-10	0.191	0.225	0.462	0.144	-	0.031	0.316	-0.140
IFN-γ	0.014	0.13	0.78	-0.148	0.031	-	-0.03	0.091
TNF- α	0.198	0.22	0.127	-0.148	0.031	-0.03	-	0.12
TGF-β	-0.027	**0.334**	- 0.171	0.180	-0.140	0.091	0.12	-

In the severe group, a significant moderate to strong positive correlation was observed between all the cytokines, except IL-6 (Table 3), where the cytokines IL2 and IL4, IL2 and IL10, IL2 and TNF-α, IL-2 and IFN-γ, IL-2 and TGF-β, IL-4 and IL-10, IL-4 and IFN-γ, IL-4 and TNF- α, IL-10 and IFN- γ, IL-10 and TNF-α, IFN-γ and TNF- α and IFN- γ and TGF-β exhibited the strongest positive correlation ($r>0.5$, $p<0.05$; Table 3).

Table 3

Correlation between cytokine expressions in the severe group. Each column shows the coefficient of correlation (r). Correlations with significant p-values (p<0.05) are shown in bold.

Cytokines	IL- 1	IL-2	IL-4	IL-6	IL-10	IFN- γ	TNF-α	TGF-β
IL- 1	-	**0.449**	**0.373**	-0.105	**0.455**	**0.544**	**0 .504**	**0.378**
IL-2	**0.449**	-	**0.646**	0.300	**0 .818**	**0.915**	**0 .916**	**0.809**
IL- 4	**0.373**	**0.646**	-	0.162	**0.653**	**0.736**	**0.689**	**0.583**
IL-6	-0.105	0.300	0.162	-	0.052	0.206	0.193	0.227
IL-10	**0.455**	**0.818**	**0.653**	0.052	-	**0.887**	**0.905**	**0.728**
IFN-γ	**0.544**	**0.915**	**0.736**	0.206	**0.887**	-	**0.952**	**0.803**
TNF- α	**0.50**	**0.916**	**0.689**	0.193	**0.905**	**0.952**	-	**0.803**
TGF-β	**0.378**	**0.809**	**0.583**	0.227	**0.728**	**0.803**	**0.803**	-

- **Correlation of cytokine expression with the viral loads of the mild and severe group**

The relationship between cytokine expression and the viral load of mild and severe cases was examined. No statistically significant correlation was found between viral loads and cytokine expression in both mild and severe groups (Table 4).

Table 4

Correlation of cytokines with the viral loads of mild and severe groups. Each column shows the coefficient of correlation (r) and p-value.

Cytokines	Mild patients Total N=71 r (p-value)	Severe patient Total N= 47 r (p-value)
IL- 1	-0.116 (0.336)	-0.015 (0.918)
IL-2	0.013 (0.914)	0.080 (0.594)
Il- 4	0.087 (0.470)	0.176 (0.236)
Il-6	0.053, (0.682)	-0.026 (0.861)
Il-10	-0.200, (0.094)	0.163 (0.274)
IFN-γ	0.145, (0.228)	0.199 (0.180)
TNF-α	-0.203, (0.089)	0 .196 (0.187)
TGF-β	-0.079, (0.514)	0.218 (0.141)

Discussion

In this study, nasopharyngeal samples from mild and severe patients were used and associated changes in viral loads and the expression of various cytokines in nasopharyngeal samples with COVID-19 disease severity were explored [13]. It is important to mention that this study is part of the larger study assessing immunological response against SARS-CoV-2 in HIV-positive and HIV-negative individuals. We found a significant difference in cytokine expression in nasopharyngeal samples from (HIV-negative) mild and severe groups.

According to clinical observations, SARS-CoV-2 infection can vary from asymptomatic infection to a mild respiratory disease with rising fever and dry cough to a severe form of disease accompanied by the development of acute respiratory distress syndrome or/and unusual upper respiratory tract pneumonia [14]. In severely sick patients with pneumonia, aberrant and uncontrolled cytokine production has been seen [15]. On January 30, 2020, the first guidelines for the diagnosis and treatment of SARS-CoV-2-infected pneumonia were released, which for the first time recommended monitoring of cytokine expression, to increase the curative rate and minimize mortality [15]. Following the evidence of the "cytokine storm's" pathogenic significance, numerous investigations were performed to see if different chemokines may contribute to promoting COVID-19 development. The results of these investigations suggest the role of increasing or decreasing levels of one or more specific chemokines in COVID-19 patients [16]. Such studies have also highlighted the need for biomarkers that can be used to predict the likelihood of developing a mild or severe form of the disease. However, most current approaches rely on invasive blood sampling, which may overlook early viral-host processes in infection that aid in viral illness progression. Estimation in cytokines in the nasopharyngeal region can be particularly beneficial, as nasopharyngeal swabs/samples provide a non-invasive way of studying early changes in viral titers and immunological biomarkers that can be linked to illness severity.

In this study, we analyzed the differential viral loads and expression of eight cytokines (IL-1, IL-2, IL-4, IL-6, IL-10 IFN-γ, TNF-α, and TGF-β-) in a total of 118 laboratory-confirmed cases, both mild and severe, of COVID-19. We found no significant difference in viral loads of the mild and severe groups, suggesting a lack of association between viral titers and disease severity. A similar result was reported by Julia R. et al, who found the total quantity of respiratory viral titer is indifferent to the severity of the COVID-19 disease [17].

We estimated the differential expression of cytokines in mild and severe groups. We found the expression of IL-2, TNF-α, IFN-gamma to be significantly higher in severe cases as compared to mild cases. Similar studies have been done which reported that IL-2 and TNF-alpha levels are higher in serum of patients with severe COVID-19 disease as compared to the mild patients and healthy group [13, 18–21]. Another study by Bastard *et al*, identified high titers of neutralizing autoantibody against IFN-γ in roughly 10% of patients with severe COVID-19 pneumonia. These antibodies were not present in infected patients who were asymptomatic or had a milder disease or healthy people.[22]. Similarly, another study also reported high expression of pro-inflammatory serum cytokines (IFN-alpha and IFN-γ) and interferon-stimulated

genes CXCL10 and CCL-2 in patients with severe COVID-19 disease as compared to healthy people or those with mild-moderate sickness [23, 24]. *Boan Li et al*, investigating the differential serum cytokine expression in individuals with severe and mild COVID-19 disease, reported IL-6 and IL-10 to be significantly increased in COVID-19 of the severe group as compared to the mild group [25]. This observation is in contrast to our results where we found the expression of IL-6 and IL-10 to be significantly decreased in both groups. This difference may be due to the type of samples used in the study (serum vs nasal swabs) and may suggest that downregulation of IL-6 and IL-10 in the nasopharyngeal milieu and not serum may be linked to disease severity. Our results are, however, supported by the study by *Suxin Wan et al*, who reported dysregulation of IL-6 levels in more than 50% of their mild patients [26]. Our results also showed decreased levels of IL-10 in both groups. One of the previous studies reported that patients with the severe illness show extremely low levels of anti-inflammatory cytokines IL-10. [27].

In the correlation analysis, we found a positive correlation between IL-1 and TNF-α, as well as a positive correlation between-6 and TNF-α. A previous study on acute-phase cytokines in the nasopharynx also reported cytokines IL-1, IL-6, and TNF-α to be positively correlated with each other [28]. A study on COVID-19 infected pregnant women indicated that IFN- and IL-6 have statistically significant positive relationships with disease severity [29]. Another study was conducted by Meira *et al.*, showed the positive correlation between IL-2 and IFN- which is the Th1 subset's definer. In contrast to the study by Meira *et al.*, in COVID-19 nasopharyngeal swab samples, we observed a strong positive correlation of IL-2 with IFN-γ, IL-4, IL-10, TNF-α, and TGF-β1 [30]. Another study indicated a positive correlation between IL-4 and TNF-α, but a negative correlation between IL-4 and IL-6 after ERCP (Endoscopic Retrograde Cholangio Pancreatography) [31]. We also observed a positive correlation between IL-4 and TNF-α but no correlation was observed between IL-4 and IL-6.

In conclusion, we found the nasopharyngeal expression of certain pro-inflammatory cytokines to be significantly higher in the severe group as compared to the mild group. These findings were demonstrated that high expression of pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α in the nasopharyngeal milieu may initiate the development of cytokine storm, which may later be observed in serum and may correlate with the severe form of the disease. Early detection of high expression of these cytokines may, therefore, be useful in predicting the development of the severe form of the disease in patients with COVID-19, and such patients can be provided with appropriate therapy (for example, cytokine inhibitors or anti-inflammatory agents), which may stop the disease from taking a severe form and can provide patients with a chance of early recovery.

Declarations

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Conflict of Interest: None

Author's contribution:

Conceptualization: SHA; Methodology and sample collection: UG, FN, NF, MFA, AJ, AH; Paper writing and initial review: UG, HAK, NM, AH; Final review and overall supervision: SHA. All authors read and approved the study.

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Figures

Figure 1

Cytokines mean expression of interleukin-1, -2, -4, -6, -10, TFN-α, TGF-β, and INF-γ in mild vs severe group: The Y-axis show mean relative expression of genes and X-axis show cytokines tested in the mild vs severe groups. The line above bars with asterisk sign indicates a significant difference (**p < 0.001, * p<0.05) in the expression of the tested genes between the mild and severe groups.