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Specific Immune Marker Associated With Coronavirus Disease 2019

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Abstract **Background:** The infection that caused the pneumonia, the virus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the source of coronavirus disease 2019 (COVID-19) is the source of the global pandemic. The pneumonia cases began in Wuhan, China in December 2019 and had no apparent reason. Essential inflammatory mediators Chemokines are necessary for an immune response to eliminate pathogens. However, the fundamental cause of hyper inflammation is their excessive release. Chemokines may be directly responsible for the acute respiratory illness syndrome in the current COVID-19 outbreak. **Objective:** The current study aimed to estimate hematological changes with immunogenic markers CD177, GNLY, and CXCR4 gene expression in COVID-19 patients, and compare these parameters among patients and healthy individuals. **Materials and Methods:** the study was conducted at Al- Yarmouk Teaching Hospital and Medical Teaching Laboratories. Finally, to detect the immune markers level in samples of patients, by RT-PCR. **Results:** the results revealed the gene expression results in patients with COVID-19 means Ct difference of CD177 (24.70) fold more than (10.5) elevated when compared with the mean of Ct control healthy group is (28.21) fold of gene expression is 1.00 of CD177 when our study showed increased gene expression the mean of GNLY Ct receptor was reached to (28.70 ng/L) with fold more than 15.0 in COVID-19 patients, while mean Ct in healthy control was (32.61 ng/L) with fold 1.00, the current study found mean CT of CXCR4 gene expression (31.2) with fold (1.1) compared with healthy control CT(31.3) fold of gene expression was (1.0). **Conclusion:** It was concluded that COVID-19 caused significant changes in many immunological parameters and found the titer of immune markers was higher in infection compared with control.

Key Words CXCR4, VTM, CD177, GNLY, COVID19, RT PCR

1. Introduction

Regarding COVID-19, the hyper immune response brought on by SARS-CoV-2 is most likely the reason for mortality in those cases. The virus SARS-CoV-2 interacts closely with an individual's immune system to produce a range of clinical indications of COVID-19 disease; the elimination of the virus is dependent on adaptive immune responses. When COVID-19 first appears, RT-qPCR should be the main technique used for diagnosis [1]. A severe acute respiratory syndrome coronavirus2 (SARS-CoV-2) is a new coronavirus, responsible for the current pandemic of the infectious disease COVID-19, which has already infected and caused the death of millions of people around the world. This highly contagious and dangerous coronavirus endangers public health and safety [2]. The total number of afflicted individuals and the geographic scope of the outbreak have significantly exceeded that of SARS and MERS. The ongoing COVID-19 outbreak has put public health around the world at extreme risk [3], [4].

Chemokines play a role in a number of human illnesses as well as various COVID-19 infection phases with in the pathogenesis related acute respiratory illness syndrome, a significant complication that kills COVID-19 patients, they are essential. A particular indicator of neutrophil activity is CD177. With a significant amount of CD177, a particular neutrophil activation marker, unsupervised analysis verified the important function of neutrophil activation. The majority significantly variable gene expression that contributed to the severe patient clustering was CD177, and the abundance of this gene was associated with the levels of CD177 protein by using serum. The patients which had COVID19 there were from French and "confirmatory" Swiss had greater CD177 levels, one of the most strongly expressed basophil receptors in COVID-19 patients is CXCR4, which may be related to basophil trans-endothelial migration [5]. Since the GNLY

Primer	Sequence (5'→3' direction)
(Gene Expression) huCD177	
Forward	TATTACTGCTGGCCCTCCTG
Reverse	TGTTCTTAGGGGTCCATTGC
hu GNLY	
Forward	TTCCTCGATCCAGAATCCAC
Reverse	CCCTCAAATCCACCAAAGAA
huCXCR4	
Forward	CCGTGGCAAACTGGTACTTT
Reverse	TTTCAGCCAACAGCTTCCTT
GADPH	
Forward	GAAATCCCATCACCATCTTCCAGG
Reverse	GAGCCCCAGCCTTCTCCATG

Table 1: The study's designed primers of Genes

receptor is well recognized to play a significant role in the pathogenicity of COVID-19 infection, the gene expression approach was used to measure the GNLY receptor level in whole blood for this portion of the investigation. When comparing the patient's group to the healthy control, the GNLY receptor level was higher [6], [7]. This study aims the levels of some immunological markers were determined and assessment the immune response of COVID-19 convalescent patients in comparison to healthy individuals .

2. Methods

The COVID-19 sample collection and the practical work of this study extended through 2023. The Ethics Committee, the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies committee, the Research Committee in the Health Departments of the Iraqi Ministry of Health in Baghdad, the Ministry of Health and Environment, and others all approved this study. Before they were included in the study tests.

A. Subjects

Patient cohorts: In accordance with the World Health Organization's standards, the samples were split into 2 clinical groups based on clinical evaluation, as follows: Group 1: Contains 50 whole blood COVID-19 for gene expression; Group 2: Contains 50 COVID-19 swabs. The Control sample: consists of fifty groups of healthy individuals

3. Materials

A. Primers

The University Code of Student Conduct (UCSC) programs double-checked the primers, which were designed with Primer 3 plus, V4, and had their reference sequences verified by the National Center for Biotechnology Information (NCBI) database. Alpha DNA Ltd. (Canada) produced and lyophilized them. All primer sequences used in the tests for this study are shown in Table 1.

B. Preparing the Primers

For each assay in this study, the required primers as shown in Figure 2- 9 were prepared as follows: Following the manufacturer's instructions and dissolving the lyophilized material in nuclease-free water, a stock solution containing 100μM was created and kept at -20°C. A working solution was prepared with a concentration of 10μM and diluting 10μL of each primer stock solution in 90μL of nuclease-free water. This solution was then stored at (-20°C) until it was needed.

	Step 1	Step 2	Step 3
Temperature	25 ℃	42°C	85 ℃
Time	10 min	15 min	5 Sec.

Table 2: Thermal cycler steps for cDNA reverse transcription conditions

C. Nasal Swap Collection

Nasopharyngeal or oropharyngeal swabs that freshly collected from each patient and put quickly in a sampling collector tube that contains viral transport medium (VTM) for (transport, storage, and prevention of viral nucleic acid from degradation) for RNA extraction and purification steps, diagnosis, and confirmatory of SARS-CoV-2 by using (RT)PCR TRANSGEN Biotech company.

D. Gene Expression for Immune Markers

1- RNA extraction from Blood sample

All samples were extracted to yield total RNA. use the TransGen, biotech. ER501-01 TransZol Up Plus RNA Kit.

2. Evaluation of RNA purity and concentration

To determine if samples were suitable for RT-qPCR analysis, the Once Nanodrop spectrophotometer system (Thermo Fisher Scientific, USA) was used to measure the concentration and purity of extracted RNA. The samples ranged in RNA concentration from 85-96 ng/μl, while the absorbance of the samples was measured at two distinct wavelengths to determine RNA purity (260 and 280nm). The presence of an A260/A280 ratio of around 2.0 suggested that the RNA sample was pure.

E. Synthesis of the cDNA form mRNA

1. First strand cDNA synthesis, reaction component

Using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit, whole RNA was reverse transcribed to complementary DNA (cDNA). Reverse transcription of 4 μl of total RNA was required, and Following the manufacturer's directions, a volume of reaction of 20 μl was used for the operation.

2. Incubation

For ten minutes, a random primer was incubated at 25°C. An attached oligo (dT) 18 primer and GSP were incubated for 15 minutes at 42°C for qPCR. The enzymes were incubated for 5 seconds at 85°C to render them inactive. as displayed in Table 2.

F. Quantitative Real-Time PCR (qRT–PCR) Runs

The Quantitative Real-Time PCR (qRT–PCR) process was carried out using the QIAGEN Rotor gene Q Real-time PCR

Step	Temperature (ºC)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	58,56,56,54	15	
Extension	72	20	
Dissociation	55 ºC-95 ºC		1

Table 3: The thermal profile of GADPH and CD177, CXCR4, and GNLY gene expression respectively

Step	Temperature (ºC)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing	58	15	
Extension	72	20	
Dissociation	55 ºC-95 ºC		1

Table 4: The thermal profile of GDPH and CD177, CXCR4, and GNLY gene expressions

System (Germany). By measuring the threshold cycle (Ct) and utilizing the TransStart® Top Green qPCR Super Mix kit, the expression levels and fold changes of the genes were evaluated. Each response was run through twice.

The cycling routine was set up in accordance with the thermal profile displayed in Table 3 and optimized for the subsequent cycles.

To determine the fold changes and gene expression levels, the threshold cycle (Ct) was determined using the components of the TransStart® Top Green qPCR Super Mix kit. Each response was given twice. The cycling regimen was set up for the following optimum cycles based on the thermal profile, as shown in Table 4.

G. Genes Expression Calculation

The approach of estimating fold changes in the observed expression of mature RNAs was first published by [8] and involved the use of the relative cycle threshold (2-ΔΔCt). It is the proportion of the test group's relative gene expression to that of the control group. Gene expression is downregulated or decreased when the number is between 0 and 1, upregulated or enhanced when the number is larger than 1.and a fold change of 1 signifies no change. The target genes' expressions were normalized by setting appropriate thresholds so that the qRT-PCR instrument would provide accurate Ct values.

H. Statistical Analysis

The Examination of Statistics the IBM SPSS Statistics 26 program was utilized to determine how various factors affected the study's parameters. The T-test and one-way ANOVA were utilized to compare means statistically. A meaningful comparison between percentages (0.05 and 0.01 probability) was made using the chi-square test. estimated values for the study's CI and odd ratio. The study using the GraphPad Prism 9 application to create the figures. The SPSS software and WINPEPI were utilized to find genotyping.

4. Results

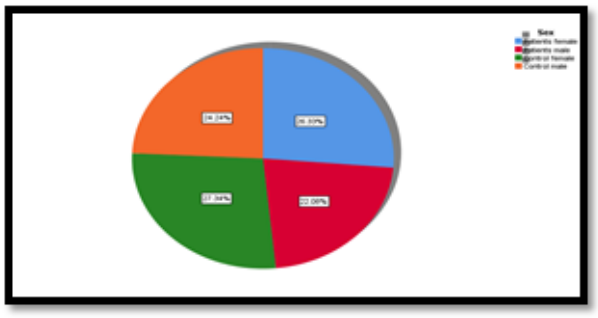

Figure 1: Age and gender comparison between the COVID -19 infection and control groups

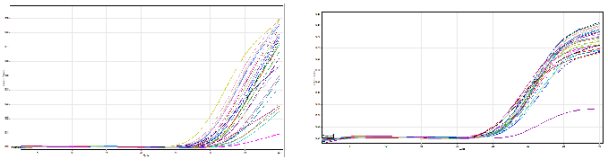

Figure 2: a-b Curve Amplification of CD177

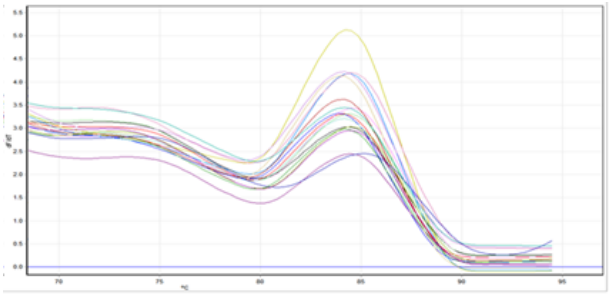

Figure 3: Amplification of Comparative between GADPH and CD177

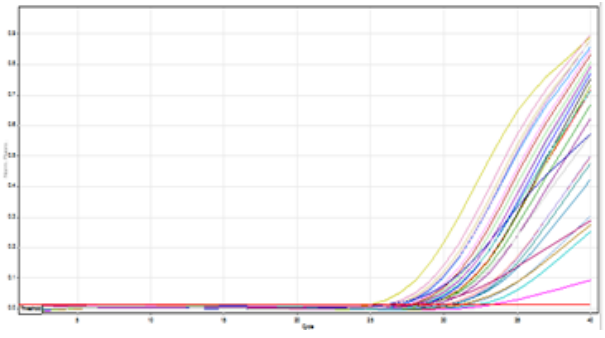

Figure 4: Amplification GNLY gene

Groups	Means Ct of CD177	Means Ct of GAPDH	ΔCt (Means Ct of CD177)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Patients	24.703	15.055	9.647	0.001246	0.001246/0.000114	10.9
Control	28.211	15.113	13.098	0.000114	0.000114/0.000114	1.00

Table 5: Comparing the CD177 patient and control groups

groups	Means Ct of GNY	Means Ct of GAPDH	ΔCt (Means Ct of GNY)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Patients	28.70621	15.05586	13.65034	0.000078	0.000078/0.000005	15.6
Control	32.61828	15.11345	17.50483	0.000005	0.000005/0.000005	1.00

Table 6: Comparison between patients and control groups in GNY

groups	Means Ct of CXCR4	Means Ct of GAPDH	ΔCt (Means Ct of CXCR4)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Patients	31.23241	15.05586	16.17655	0.000014	0.000014/0.000013	1.1
Control	31.35689655	15.11344828	16.24344828	0.000013	0.000013/0.000013	1.00

Table 7: Comparison between patients and control groups in CXCR4

groups	Means Ct of CXCR4	Means Ct of GAPDH	ΔCt (Means Ct of CXCR4)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Patients	31.23241	15.05586	16.17655	0.000014	0.000014/0.000013	1.1
Control	31.35689655	15.11344828	16.24344828	0.000013	0.000013/0.000013	1.00

Table 8: Person correlations between patients and control groups

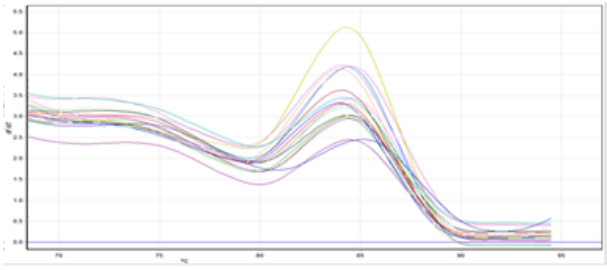

Figure 5: Comparative Quantitation Analysis of GNLY gene and GADPH

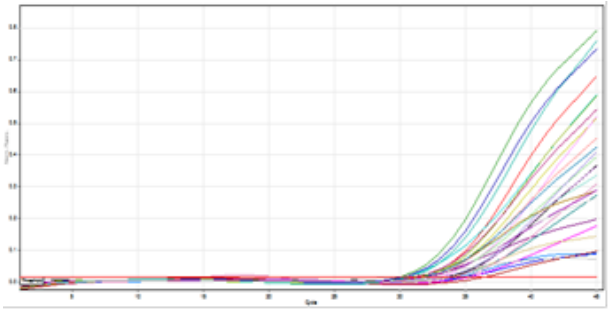

Figure 6: The GNLY Gene expression of COVID-19 patients

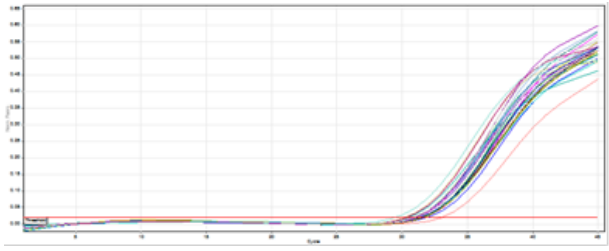

Figure 7: Amplification curve of CXCR4 Gene

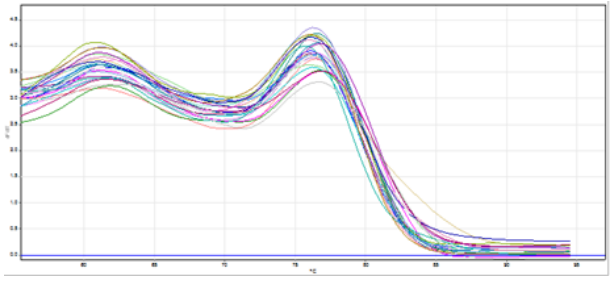

Figure 8: Comparative Quantitation Analysis of the CXC4 gene and GADPH gene

5. Disscussion

A. Distribution of COVID-19 Patients according to Gender

The patients' ages ranged from 13 to 50 years old, indicating that the condition impacted people of all ages. Our results did not show any major differences in the disease between genders Figure 1. The current study's findings are consistent with other local investigations carried out by Iraqi researchers in a prior study [9]. The distribution of genders did not substantially correlate with the level of disease illness (P>0.05).

These results were at odds with those of research conducted by [10], [11] which demonstrated that the prevalence of COVID-19 rose with age and that males experienced more

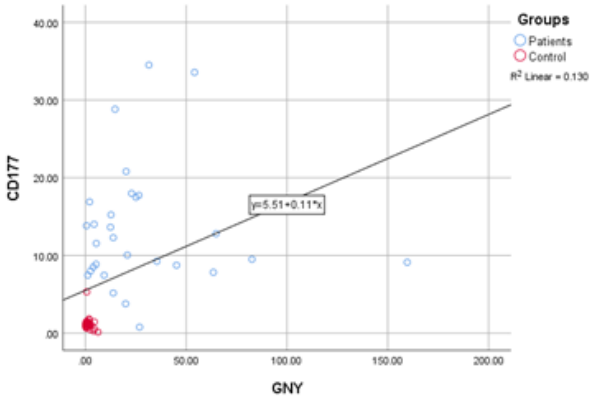

Figure 9: Person correlations between patients and control groups

cases than females. The difference in COVID-19 prevalence between males and females may be explained by social gender behavior. For example, an American study found that women wash their hands approximately four times more frequently than men do, and that women are less likely than men to go out during curfew, which may have reduced their exposure to the virus [12] . Given that men are more likely than women to smoke, different outcomes for men and women may result from different health behaviors [13] .In addition, men are more likely than women to have hypertension and cardiovascular disease, two additional risk factors for the severity of COVID-19.

B. Molecular Study

1) Results of quantitative Real-time PCR

1. RT PCR quantification of GAPDH Expression:

The Ct value of GAPDH, the housekeeping gene used in the present study is shown in Table 5. The range of Ct value for GAPDH in the COVID-19 patients' group was 15.055 with the control healthy 15.113 group.

2. b- Gene expression of CD177 in COVID-19:

In our investigation, we noted the gene expression results in patients with COVID-19 Means Ct of CD177 (24.70) fold more than 10.5 and the mean of Ct control healthy group is (28.21) fold of gene expression is 1.00 Figure 2 Table 5.

This is consistent with the research. Significantly more CD177 was upregulated in COVID-19 patient neutrophils than in healthy controls, according to Lourda et al.'s observation in 2021. This finding is consistent with a prior study by(6) that discovered a strong correlation between the RNA-seq-measured CD177 gene expression and the ELISA-measured CD177 protein levels.

In the previous work [6], [14], the gene that most significantly contributed to the grouping of seriously ill individuals was CD177, whose abundance was correlated with serum levels of the CD177 protein. The levels of CD177 were higher in French and "confirmatory" Swiss COVID-19 patients. These findings highlight the significance of neutrophil activation as an indicator of a serious illness and the validity of CD177 measurement as a predictive factor for traditional treatment. Investigating the neutrophil direction revealed a higher abundance of genes mostly involved in movement, endothelial cell contact, and neutrophil activation, despite the fact many of these paths encompassed numerous cell types. This signature contained CD177, a particular marker of neutrophil adherence to the endothelium and transmigration, among its most highly expressed genes [15]. We looked for neutrophil-activation characteristics that would serve as potential trustworthy indicators of the progression of the disease, given the role that the neutrophil activation pathway played in the COVID-19 patient cluster. Since CD177 is a marker exclusive to neutrophils and is the gene that exhibits the greatest variation in expression in patients, concentrated on it as a representation of neutrophil activation. the previous A study [6] comprehensive phenotypic study of immune cells, amalgamated measurements of a broad panel of serum analytes, and Utilizing whole-blood RNA-seq studies in a multicentric French cohort to investigate variables influencing the clinical outcomes of individuals with severe COVID-19. A comprehensive and worldwide analysis of host indicators identified multiple pathways linked to the development of COVID-19 infection, one of which is neutrophil activation. This profile included the neutrophil marker CD177, which is specific to activation, endothelium adherence, and transmigration. The correlation between serum protein levels and CD177 gene abundance in COVID-19 patient blood emphasizes the marker's importance.. Measuring CD177 protein levels is a dependable method that is generally available in regular treatment [16], COVID-19 [17], and severe influenza [18]. Clinical observations in lung autopsies of deceased individuals have included the observation of neutrophil chemotaxis, endothelial cell infiltration, and extravasation into alveolar spaces. Patients with acute Kawasaki disease have also been reported to have elevated CD177 mRNA expression. [19] A syndrome that has been identified as identified as a potential side effect of SARS-CoV-2 infection in children [20], [21] and resistant to IV Ig treatment [22], [23] .Previous findings, which indicated a significant neutrophil activation's significance in the severity of respiratory virus infection through their migration toward the lungs of infected individuals and during influenza infections in humans, were also in line with those obtained using animal models [24]–[26].

3. C- GNLY gene expression:

This portion of the study measured the GNLY receptor level in whole blood using a gene expression approach since the GNLY receptor is known to play a significant role in the pathogenicity of COVID-19 infection. According to [7], the patients' group had higher levels of GNLY receptor than the healthy control group, with a highly significant difference ($P<0.01$). which is consistent with a recent study that found that COVID-19 patients' mean GNLY Ct receptor level was reached at (28.70 ng/L) with a fold higher than 15.0. while mean Ct in healthy control was (32.61 ng/L) with fold 1.00 as demonstrated in Table 6.

There was a tendency to increased expression of the cytotoxic proteins (GZMB and GNLY) and the cytokine IFN-γ, but it was not statistically significant. Similar findings were obtained by several other studies [27]. Using intracellular cytokine labeling, the study examined 14 COVID-19 patients and discovered elevated Expression of GZMB and PRF1 in CD8+ T cells from COVID-19 patients. They also observed a decrease in CD4+ T cells. When comparing GZMA expression in CD8+ T cells between COVID-19 patients and healthy controls, no differences were found [28]. the two groups. This also applied to B cells. Additionally, they discovered higher PRF1 expression, however it was not statistically significant. Increases in PRF1, GNLY, soluble Fas, and GZMB were also noted in COVID-19 patients by other investigations. Consistent with our findings, [29] when the severe cases and regular controls are contrasted, the lungs' GNLY performs a variety of physiological tasks, the majority of which are known to directly protect alveolar epithelial cells from lung injury. In 2020, Jiang Y. et al. [30].

4. D-Gene expression of CXCR4:

The current study found mean CT of CXCR4 gene expression (31.2) with a fold of 1.1 compared with healthy control CT(31.3) fold of gene expression was(1.0) shown Table 7, Reduced responsiveness of the signaling pathway in COVID-derived CD4+ naïve T cells due to decreased expression of CXCR4 and class-A1 Rhodopsin-like receptors may lead to immunodeficiency in patients [31], the previous result by [32] found markers and a reduction in CXCR4, markers in COVID-derived CD4+ naïve T cells concerning control cells interest, our study disagrees with study [33] reported a noteworthy elevation of CXCR4, whereas [34] demonstrated the involvement of chemokines in various diseases affecting humans and COVID-19 infection stages. In the pathogenesis of the associated acute respiratory illness syndrome, a significant complication that kills COVID-19 patients, they are essential. Particularly, it was discovered that COVID-19 patients had high expression levels of CXC chemokine receptor 4 (CXCR4), which is inconsistent with our findings.

[32] discovered that several immunological subsets from COVID-19 patients had lower expression of the chemokine receptor CXCR4, which is known to stimulate hematopoiesis, cell migration/homing, and bone marrow retention [35]. Interesting findings on COVID-19 include the expression of certain receptors involved in migration, adhesion, and activation being altered. These findings highlight particular immunophenotypes that may be predictive of clinical outcomes [33].

5. Correlation person:

Negatively Correlation person between CD177 and CXCR4 gene -0.078 NO significant P 0.555, when correlation person Positively 0.361 with highly significant P 0.005 between CD177 and GNLY, Figure 9 Table 8. RNA-seq information from COVID-19 patients' nasal swabs and entire blood compared to healthy donors. Immune response-related genes (such as interferon signaling, T- and B-cell response, neutrophil and myeloid activation), according to the earlier work by [36]–[38] MAITα cells were classified as immunologically active by the authors due to their enhanced expression of cytotoxic T cell-associated genes (GNLY, migration/adhesion (CXCR4)

6. Conclusion

1- When compared to a healthy control, the whole blood samples of COVID-19 patients with respect to gene expression of CD177 and GNLY showed a highly significant difference.

2-The study confirmed the significant differences of GNLY receptor distribution between COVID 19 patients and healthy individuals and clarified the role of this receptor in the pathogenesis of SARS-COV2.

3-In our study we found markers and a reduction in CXCR4, markers in COVID- lower expression.

Acknowledgments

My profound thanks and appreciation to all patients which there were help us to get all samples and qualification about their status during infection stage so for my mother and father to support funding to my research .

Conflict of interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

Authors Contribution

1. Zainab Fayadh Shubrem, Conceived and designed the analysis; Collected the data; and Contributed data or analysis tools, writing the paper.

2. Prof Dr. Wathiq Abbass, Performed the analysis and helped in writing the paper.

Ethics Approval

This study was performed in line with the principles of the Research Committee in Health Departments of Iraqi Ministry of Health (Form number 01/2021). Approval was granted by the Ethics Committee of University Baghdad "This study was approved by Institute of Genetic Engineering and Biotechnology for Post Graduate Studies committee at Baghdad University. (Date28-5-2023/No2446)."

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