Erciyes Med J 2022; 44(6): 594–602 • DOI: 10.14744/etd.2022.32955
ORIGINAL ARTICLE – OPEN ACCESS

The Effect of Sex-Specific Genetic Factors on the Host Immune Response to COVID-19: A Pilot Study

Dilara Fatma Akın Balı[1], Tuğba Arslan Gülen[2], Esma Özmen[3], Zeynep Türe Yüce[4], Orhan Yıldız[4], Tuba Turunç[5], Üner Kayabaş[2]

Cite this article as:
Akın Balı DF, Arslan Gülen T, Özmen E, Türe Yüce Z, Yıldız O, Turunç T, et al. The Effect of Sex-Specific Genetic Factors on the Host Immune Response to COVID-19: A Pilot Study. Erciyes Med J 2022; 44(6): 594-602.

[1]Department of Medical Biology, Niğde Ömer Halisdemir University Faculty of Medicine, Niğde, Türkiye
[2]Department of Infectious Diseases and Clinical Microbiology, Niğde Ömer Halisdemir University Faculty of Medicine, Niğde, Türkiye
[3]Deparment of Medical Biochemistry, Niğde Ömer Halisdemir University Faculty of Medicine, Niğde, Türkiye
[4]Department of Infectious Diseases and Clinical Microbiology, Erciyes University Faculty of Medicine, Kayseri, Türkiye
[5]Department of Infectious Diseases and Clinical Microbiology, Health Sciences University Adana Faculty of Medicine, Adana, Türkiye

Submitted
16.02.2022

Revised
04.04.2022

Accepted
22.05.2022

Available Online
28.06.2022

Correspondence
Üner Kayabaş,
Niğde Ömer Halisdemir University Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Niğde, Türkiye
Phone: +90 388 225 60 50
e-mail: ukayabas@gmail.com

ABSTRACT

Objective: The aim of this study was to investigate the impact of sex-specific genetic factors in the pathogenesis and prognosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2)-induced macrophage activation syndrome (MAS), independent of age and comorbidity presence.

Materials and Methods: Patients aged 18–50 years who had been diagnosed with coronavirus 2019 (COVID-19), the disease caused by the SARS-CoV2 virus, were enrolled in a prospective, case-control, multi-center study. Genetic alterations and messenger RNA (m-RNA) expression levels of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL genes were determined using DNA sequencing analysis, and gene expression was determined using quantitative reverse transcriptase polymerase chain reaction testing. PolyPhen-2 (Polymorphism Phenotyping v2; Adzhubei et al., 2010) and SNAP2 (Rostlab, Munich, Germany) genetic analysis tools were used to define the pathogenic effects of detected mutations by sequencing the selected genes in hotspot regions.

Results: The study group consisted of 80 patients diagnosed with COVID-19 and was divided into groups based on sex and MAS status. Twenty-nine mutations were detected in 6 genes. Among the alterations, 15 were identified in this study for the first time and 9 were pathogenic. Pathogenic missense mutations in the TLR7, TLR8, ACE2, and TASL genes were detected in the MAS (+) group. In males, decreased TLR7, TLR8, and CXCR3 expression was statistically significant in the MAS (+) group ($p<0.050$). CXCR3 expression was lower in the female and male MAS (+) groups compared with the MAS (-) groups ($p<0.050$).

Conclusion: In the absence of major risk factors for COVID-19, the TLR7/8, ACE2, and CXCR3 variants and decreased m-RNA expression levels associated with genetic susceptibility may be independent prognostic risk factors for COVID-19.

Keywords: ACE2, COVID-19, host genetics, mutation, sex difference, TLR7, X chromosome

INTRODUCTION

An outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) and the resulting disease, coronavirus 2019 (COVID-19) led to a pandemic. The epidemiological data indicate that the elderly and men have been severely affected in terms of the number of cases, disease severity, and mortality ratio (1–4). There may be a gender bias in susceptibility. Pre-existing comorbidities and lifestyle contribute to disease risk, however, biological factors underlying the host immune response are of critical importance (5–8). Generally, women develop stronger immune responses to infections and vaccines and live longer than men (4–10). Therefore, it may be that sex-based genetic/biological factors underlying the immune response are significant determinants of susceptibility to infection, disease outcomes, and mortality (9–11). It has been established that the sex chromosomes can play a crucial role in disease outcomes. Differences in the immune response formation of men and women are directly related to the number of X chromosomes and the effect of sex-related hormones on the functions of immune cells (10–12). Most of the genes associated with the immune system and the pathogenesis of COVID-19 (ACE2, TLR7, TLR8, CXCR3, NEMO, TASL) are localized on the X chromosome (9–13). Though males have 1 copy of each X-linked gene and females have 2 copies, the amount of gene product of a single allele in the male and a pair of alleles in the female is usually equivalent (14). The current mechanism for achieving dosage compensation is based on the principle of X inactivation (Xi), known as the Lyon hypothesis. X inactivation is fundamental to providing balanced gene expression between the genders; however, about 15% to 30% of genes, most localized on the short arm (Xp) of the X chromosome, escape inactivation. The genes in question are randomly distributed on the X chromosome. A larger percentage of genes on Xp escape inactivation compared with Xq. This causes biallelic gene expression with double dosage and distinctions in gene dosage between genders (13–15).

This study was designed to examine a gender-related genetic background to the pathogenesis of COVID-19. The study group comprised adults <50 years of age without a comorbidity. Macrophage activation syndrome (MAS) due to SARS-CoV2 infection was examined for a role in the pathogenesis. The aim was to analyze possible dif-

Table 1. The primer sequences and amplicon lengths for the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL genes

Gene/exon	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon (bp)
TLR7-DNA	AAGCTGCTACAAGTATGGGC	GTATCTGTTATCACCTTCTCTCC	460
TLR8- DNA	ACCTCTCTAGCACTTCCCTC	CGTTTCCGGATATGACGTTGA	484
ACE2-1- DNA	TGGGTACTCAAGATTCACTGGT	ACAAGCCATGAGAAAATGTCCA	384
ACE2-2- DNA	ACTAGTTATGCCCACCTGCT	AGCTTGGTAGTTATGGGTGGA	307
ACE-3- DNA	GTATCTCTTTGGTTACTTGGGCT	TGAGGACATTTTGAGGCCATAAA	355
CXCR3-DNA	TCAGCCTGAACTTCGACCG	GCTCCTGCGTAGAAGTTGATG	277
CD40L-DNA	GGGAAACAGCTGACCGTTAA	CACTTATTACAGTTCTACATGCC	480
TASL-DNA	TGACTTACAGATTGCAGGGG	ACTCCACAACCTTCTGCTCC	287
TLR7 (m-RNA)	CACATACCAGACATCTCCCCA	CCCAGTGGAATAGGTACACAGTT	92
TLR8 (m-RNA)	ATGTTCCTTCAGTCGTCAATGC	TTGCTGCACTCTGCAATAACT	143
ACE2 (m-RNA	ACAGTCCACACTTGCCCAAAT	TGAGAGCACTGAAGACCCATT	103
CXCR3 (m-RNA)	CCACCTAGCTGTAGCAGACAC	AGGGCTCCTGCGTAGAAGTT	141
CD40L (m-RNA)	GAGCAACAACTTGGTAACCCT	GGCTGGCTATAAATGGAGCTTG	134
TASL (m-RNA)	GTGACAACAGATTTTCCCTCTGA	GTACCGCTGGATAGGTTTTTGG	135
RPLP0 (m-RNA)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	197

ferences in gender-related genetic factors on disease prognosis, not related to age or comorbidity, by examining the TLR7, TLR8, ACE2, CD40L, CXCR3, TASL genes localized on the X chromosome that have escaped inactivation and may be associated with COVID-19 susceptibility.

MATERIALS and METHODS

Ethics Statement

This study was approved by the local ethics committee of Niğde University Faculty of Medicine on April 08, 2021 (no: 2021/50) and performed in accordance with the principles of the Declaration of Helsinki. Written, informed consent was received from all of the participants.

Study Design and Collection of Blood Samples

This prospective, case-control, multi-center study was conducted with COVID-19 patients who presented at the Infectious Diseases and Clinical Microbiology department of the Niğde University Faculty of Medicine Training and Research Hospital, the Erciyes University Faculty of Medicine, and the Adana Health Sciences University Faculty of Medicine. The COVID-19-associated MAS diagnostic criteria used in patient selection for the study were ≤94% oxygen saturation/tachypnea (>30/min) at rest and the presence of at least 2 of the following: high C-reactive protein level (>100 mg/L), elevated serum ferritin level (>900 µg/L single measurement or 2-fold increase in 48 hours compared to admission), and a high D-dimer level (>1500 µg/L). For the DNA and RNA analysis, 2-mL samples of blood were collected into in tubes containing 2% ethylenediaminetetraacetic acid (EDTA) from the patients and controls.

Mutation Screening

DNA isolation was performed using Zymo Quick DNA kits (Zymo Research Corp., Irvine, CA, USA). Genomic DNA quality and concentration were determined using a spectrophotometer. The exon regions of the TLR7, TLR8, ACE2, CD40L, CXCR3, TASL genes to be studied were amplified using polymerase chain reaction (PCR) testing. The primers were designed using Primer3 software (https://primer3.org/) (Table 1). Approximately 200 ng of DNA was utilized as a template for PCR using an Applied Biosystems 96-well thermal cycler (Applied Biosystems Corp., Waltham, MA, USA). After PCR testing, the products were run on 2% agarose gel stained with Safe-Red (Applied Biological Materials Inc., Richmond, BC, Canada) in 1x Tris/boric acid/EDTA solution for 40 minutes at 90 volts. GeneRuler DNA Ladder (100 base pairs) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized as a marker. Gels were visualized using Syngene UV transilluminator (Syngene International Ltd., Bangalore, India). The PCR products were then sequenced using the Applied Biosytems 3500 XL DNA sequencer (Applied Biosystems Corp., Waltham, MA, USA). The raw data were analyzed using data collection software and Chromas 2.6.5 (Technelysium Pty. Ltd., South Brisbane, Queensland, Australia) to determine nucleotide changes in comparison with the reference TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL sequences in the Ensembl genome browser (www.ensembl.org).

Mutation Impact Analysis

The PolyPhen-2 tool (Polymorphism Phenotyping v2; Adzhubei et al., 2010) helps to determine effects on the stability and function of a protein using constructional and comparative analysis data of amino acids affected by mutations in genes. The program provides a qualitative prediction (benign, possibly damaging, or probably damaging) based on the data (16). The evolutionary conservation analysis of mutant codons identified during the study using the multiple sequence alignment section included in the software were compared between species. SNAP2 (Rostlab, Munich, Germany) is a tool that discriminates between impact and neutral variants/non-synonym single-nucleotide polymorphisms (SNPs) by assessing various sequence and variant properties. A SNAP2 score of -100–0 is categorized as neutral, and a score of 0–100 is considered non-neutral/effect on function (17).

Table 2. Demographic, clinical, and genetic data of COVID-19 patients with MAS and without MAS

Characteristic	MAS(+)/Male n=21 (%)	MAS(-)/Male n=23 (%)	MAS(+)/Female n=5 (%)	MAS(-)/Female n=31 (%)	p
Gender, male/female	21/M (26%)	23/M (29%)	5/F (6%)	31/F (39%)	–
Mean age, years (range)	41.5 (23–50)	39 (28–50)	39.4 (24–46)	35.5 (21–46)	0.190
Body mass index (kg/m^2)	24.2 (19–29)	26.8 (24–29)	27.4 (22–30)	25.7 (22–29)	0.610
HGB (g/dL) (mean±SD)	14.2 (13.1–16)	15.2 (13.2–16.4)	11.5 (9.6–13.0)	13.1 (11.7–15.0)	0.0001
D-dimer (mg/mL) (mean±SD)	2.2 (0.2–9.5)	0.4 (0.1–1.1)	4.5 (1.5–7.6)	1.3 (2.4–7.7)	0.004
Ferritin ng/mL	1178 (415–3400)	237.3 (119–387)	1590 (195–5397)	98.75 (12–375)	0.0001
WBC (X10^3/L)	11118 (5000–20000)	8630 (5200–15500)	11037 (3560–15200)	6372 (3400–12000)	0.001
PLT (X10^3/L)	213 (87–395)	224 (151–345)	183 (101–383)	248 (145–338)	0.080
CRP	136.6 (25–304)	21.3 (0.5–97)	142.6 (30.1–153.6)	45.9 (0.4–218)	0.00005
Neutrophils	9284.8 (2120–18280)	6340 (1820–14900)	8685.7 (2100–15500)	3687 (1590–7020)	0.0006
Lymphocytes	836 (350–2250)	1392 (800–3330)	423 (340–510)	1854 (700–4410)	0.0003
Fibrinogen	694 (254–1082)	285 (65–544)	521 (190–775)	NA	–
ALT	87.4 (18–286)	38.2 (23–65)	120.7 (33–201)	25.8 (11–51)	0.0006
AST	60.9 (11–154)	45 (16–78)	126.3 (26–236)	25.6 (13–45)	0.0005
CK	245 (40–1237)	84 (30–153)	105.4 (21–192)	97.6 (45–248)	0.0008
Creatinine	0.9 (0.53–1.26)	0.8 (0.5–1.4)	0.8 (0.5–1.1)	0.71 (0.6–0.9)	0.018
LDH	427 (180–788)	181 (65–491)	305 (89–563)	195 (137–350)	0.0003

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; COVID-19: Coronavirus 2019; CRP: C-reactive protein; F: Female; HGB: Hemoglobin; LDH: Lactate dehydrogenase; M: Male; MAS: Macrophage activation syndrome; PLT: Platelet; WBC: White blood cell

Gene Expression Analysis

RNA isolation was performed using TRIzol solution (Life Technologies Corp., Carlsbad CA, USA) according to the manufacturer's recommended protocol. The concentration and purity of the samples of RNA obtained were measured with a spectrophotometer and then stored at -80 °C until use. The primer sequences are given in Table 1. The quantitative reverse transcriptase (q-RT) PCR technique was employed to calculate the messenger RNA (m-RNA) expression rate of the human acidic ribosomal protein (RPLP0) housekeeping gene of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL genes. Complementary DNA synthesis was performed with a synthesis kit according to the manufacturer's recommended protocol. Messenger RNA levels were assessed using SYBR Green I Master (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the protocol proposed by the manufacturer and a Rotor-Gene device (Qiagen N.V., Hilden, Germany). Each sample was studied as 3 replicates. The quantification level was determined using the 2−ΔΔCT method based on the manufacturer's recommendations. Fold-change values >2.00 were accepted as statistically significant. The specificity of the RT-PCR was confirmed using melting curve analysis.

Statistical Analysis The Kolmogorov-Smirnov test indicated that the data demonstrated a normal distribution. All of the variables were compared between all groups using one-way analysis of variance. The Tukey test was applied to determine means that were significantly different between groups. All of the data were expressed as the mean±SD. A p value of <0.050 was considered statistically significant. The calculation of amplification efficiency was an important step in the RNA analysis. This method was used to calculate the comparative expression of the samples in the measurement of relative quantitation. A p value of <0.050 was considered statistically significant.

RESULTS

Demographic Data and Clinical Characteristics of Patient Groups

The study group consisted of 80 male and female patients aged 18–50 years who had been diagnosed with COVID-19. The study sample was composed of 4 groups: Group 1, Female/diagnosed with COVID-19-associated MAS, aged 18–50 years (premenopausal), no comorbidity, (5 patients); Group 2, Female/not diagnosed with COVID-19-associated MAS, aged 18–50 years (premenopausal), no comorbidity, (31 patients); Group 3, Male/ diagnosed with COVID-19-associated MAS, aged 18–50 years, no comorbidity, (21 patients); and Group 4, Male/not diagnosed with COVID-1-associated MAS, aged 18–50 years, no comorbidity, (23 patients). All of the demographic and clinical data of the study samples evaluated are presented in detail in Table 2. The blood value parameters reveal a statistically significant difference between groups.

Results of Genotyping Analysis

Mutation detection from DNA isolated from the blood samples of the 80 patients in our study group was performed using the Sanger DNA sequencing method. The mutation analysis detected 29 variants (20 missense mutations, 5 synonymous nucleotide changes, 2 SNPs and 2 non-coding untranslated regions (3' UTR) in 6 genes; 14 had previously been reported in the Human Gene Mutation Database (HGMD; Cardiff University, Cardiff, Wales), and 15 were

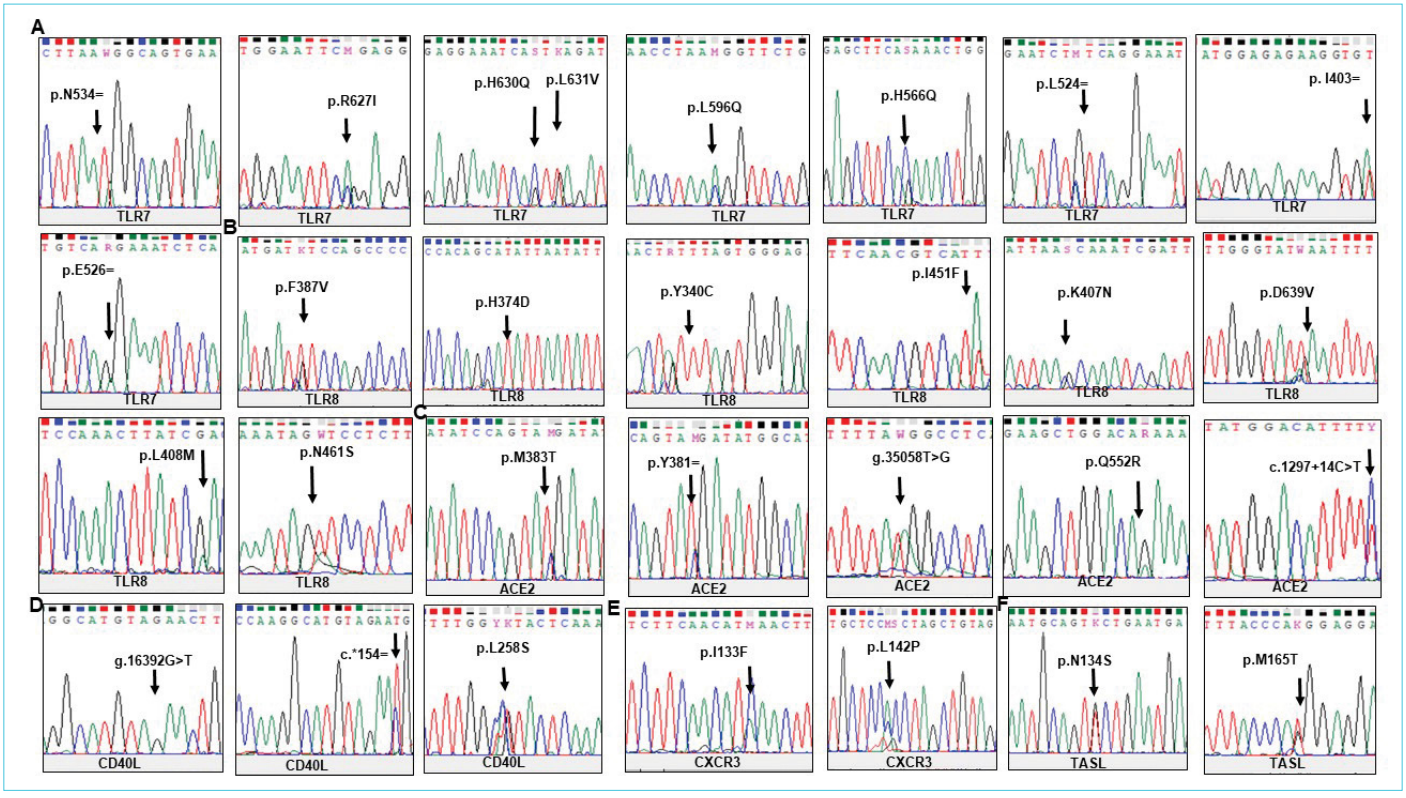

Figure 1. The sequencing electropherograms of TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL gene mutations and variants. The arrow indicates the localization of the mutation and variant

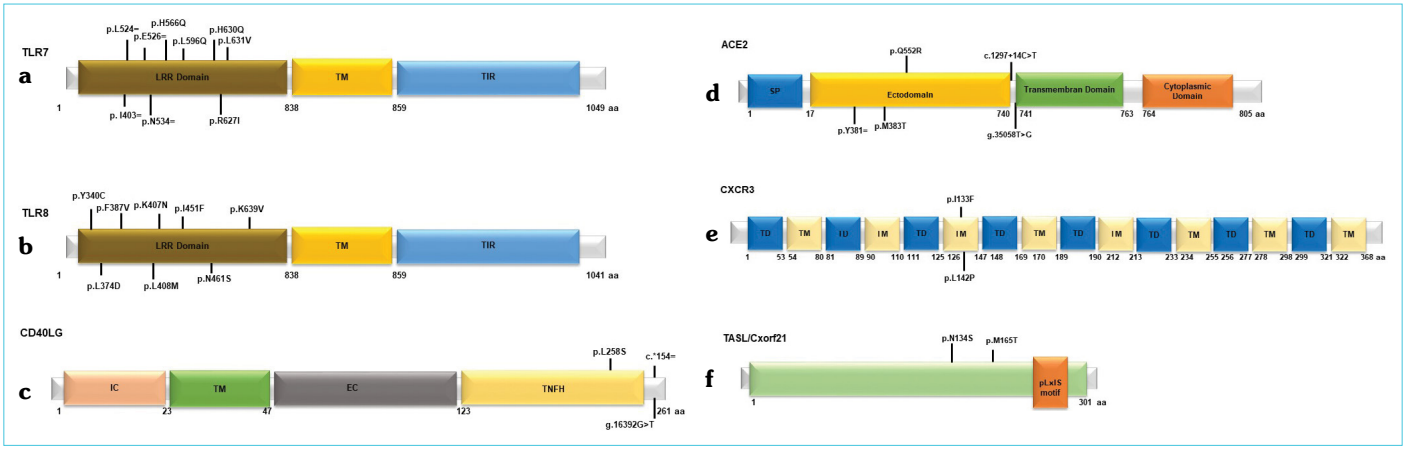

Figure 2. Schematic representation of domain structure of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL proteins and mutations detected in the study groups. (a) TLR7 is a polypeptide of 1049 amino acids (b) TLR8 is a polypeptide of 1041 amino acids (c) CD40L is a polypeptide of 261 amino acids (d) ACE2 is a polypeptide of 805 amino acids (e) CXCR3 is a polypeptide of 368 amino acids (f) TASL is a polypeptide of 301 amino acids

defined for the first time in the present study. The identified mutations were heterozygous and the variants detected in the DNA sequence analysis electropherograms are shown in Figure 1. Variants were detected in 39 (48.75%) of 80 individuals. The gene with the greatest number of variants was TLR7 (9 alterations), while the fewest alterations were detected in the CXCR3 and TASL genes (2 changes). An illustration of the domain structure of the variants found in the TLR7, TLR8, ACE2, CD40L, CXCR3, TASL genes of the study groups is provided in Figure 2 and Figure 3. The variants determined are summarized in detail in Table 3.

TLR8 Analysis

TLR8 is a member of the family of Toll-like receptors in the immune system and functions as a pattern recognition receptor (18). In TLR8, 7 missenses and 1 synonym variants were determined, all in the ligand recognition region (LRR) encoded by exon 2. Six of these variants were defined for the first time in our study. The frequency of carrying a TLR8 variant was 6.25% (5 individuals) in this group of patients. Missense mutations p.Y340C, p.L407Asn, and p.F461S were detected in a MAS (+) male patient. All of the TLR8 changes were observed in males.

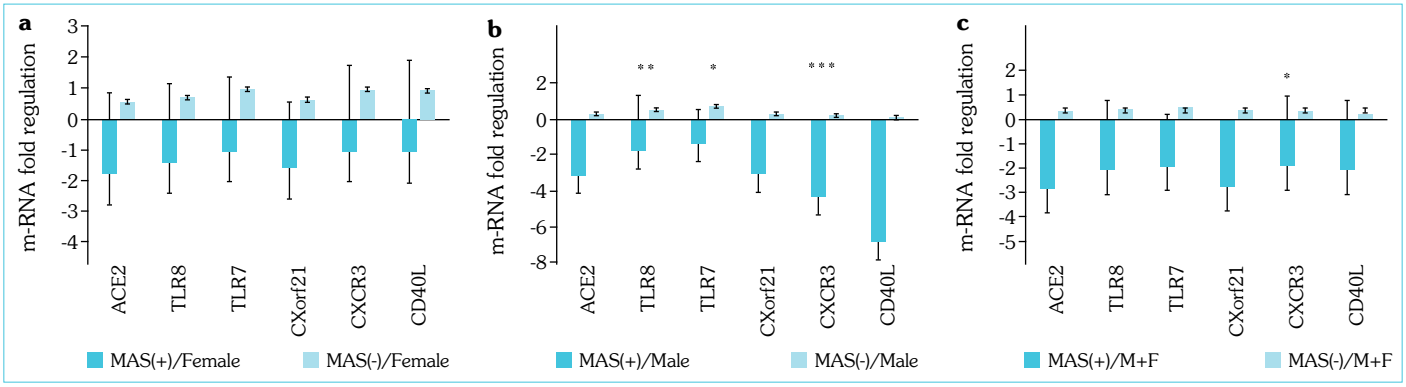

Figure 3. Comparative representation of fold regulation of m-RNA expression of ACE2, TLR7, TLR8, CD40L, CXCR3, and TASL genes. (a) Comparison of expression level of genes in female MAS(+)/MAS(-) groups. (b) Comparison of expression level of genes in male MAS(+)/MAS(-) groups. (c) Comparison of expression level of genes in female/male MAS(+)/MAS(-) groups

*: P<0.050; **: P<0.001; ***: P<0.0001; MAS: Macrophage activation syndrome

TLR7 Analysis

The binding of the S glycoprotein on the SARS-CoV2 envelope to ACE2 is recognized by TLR7 (18). In the TLR7 gene, 9 (5 missense and 4 synonym) variants were detected, all on the LRR domain. Four of these variants were reported for the first time in this study. Coexistence of p.L631V and p.H630Q mutations was seen in MAS (+) male individuals, and p.H566Q and p.R627I changes were also present only in MAS (+) male patients. TLR7 variants were detected in 9 (11.25%) individuals.

ACE2 Analysis

ACE2, a transmembrane protein that functions as a protease through its catalytic activity, consists of 805 amino acids, is located on the Xp22 chromosome, and includes 18 exons (19). In the ACE2 gene, 2 missense (p.Q552R, and p.M383T), 1 synonym (p.Y381=) and 2 SNPs (c.1297+14C>T and g.35058T>G) were identified. p.M383T and p.Y381= mutations were determined in a MAS (+) male patient. A p.Q552R missense mutation was detected in a MAS (+) female patient.

CD40L Analysis

In the CD40L gene, 1 missense (p.L258S) and 1 non-coding region variant were detected. CD40L variants were identified in 4 MAS (-) individuals (5%), and a p.L258S mutation was found on the sequences encoding the TNFH domain.

TASL Analysis

Missense mutations p.N134S and p.M165T were detected. The p.N134S variant was present in 20 (25%) individuals, while p.M165T was present in 3 (3.75%) individuals. Coexisting p.N134S and p.M165T mutations were noted in 1 MAS (+) male patient.

CXCR3 Analysis

In the CXCR3 gene, 2 mutations (p.I133P, p.L142P) were detected, both on the transmembrane domain coding sequences. These 2 variants were present in 2 MAS (-) females (2.5%) in the study group.

Results of Impact Analysis of Detected Mutations

Analysis using PolyPhen-2 and SNAP2 revealed that the of 29 mutations detected in our study, the pathogenic score of 9 mutations (ACE2 p.M383T; TASL p.N134S; CXCR3 p.I133P, p.K142P; CD40LG p.L258S; TLR7 p.L631V, p.R627I; TLR8, p.L374D, p.Y340C) was close to 1.0 (damaging) and 0–100 (non-neutral), respectively. "Effect" variants are predicted to be pathogenic and capable of causing disease susceptibility. The amino acid sequences of the missense mutations were compared between species utilizing the multiple sequence alignment capability of PolyPhen-2 and it was determined that 13 of 20 missense mutations identified changed critically important amino acids that were highly protected among different species during the evolutionary process.

Results of Gene Expression Analysis

The expression rate of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL target genes and the RPLP0 reference gene in the study groups was determined using q-RT PCR. As shown in Figure 3a, there was no statistically significant difference in the expression level of the MAS (+) and MAS (-) female groups. Figure 3b illustrates that in the MAS(+)/(-) male groups, the m-RNA expression level was low in the MAS (+) group, and the expression of CXCR3, TLR7, and TLR8 were statistically significant (p value: 0.001, 0.030, and 0.010, respectively). The CXCR3 expression was statistically significant between the male and female groups (p=0.020) (Fig. 3c).

DISCUSSION

Numerous studies have linked the different course of COVID-19 in women than men to genes encoded by the X chromosome, escaping X inactivation, and sex hormones (1, 3, 8, 9, 14, 19). These and similar genetic and biological factors support the reports that the rate of hospitalization and death due to COVID-19 is higher among men than women. Genotyping analysis of DNA fragments was performed in this study using Sanger sequencing analysis to detect mutations/SNPs of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL genes. In addition to a genetic profile study, expression analysis of RNA fragments was performed using the q-RT PCR technique to determine the effect of the identified mutations at the gene expression level. Among 80 individuals diagnosed with COVID-19, 29 variants (20 missense mutations, 5 synonymous nucleotide changes, 2 SNPs and 2 non-coding untranslated regions (3' UTR) were detected in 6 genes which escape X chromosome inactivation. Of these 29 variants, 14 had been previously registered in the HGMD e-database and 15 were identified for the first time in the current study. The TLR7 gene displayed the greatest number of variants, and CXCR3 and TASL revealed the fewest. Genetic alterations were detected in 39 (48.75%) of the 80 individuals in our study group.

Table 3. Characteristics and clinical features of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL gene mutations in the study groups

No.	Gene	Nt alteration	Rs number	Alteration type	Localization	AA position	Clinical significance		MAS status	Frequency of variant n (%)
							PolyPhen-2 (score)	SNAP (score)		
C-1	ACE-2	c.1297+14C>T	rs776241279	SNP	Non-coding region	–	NA	NA	MAS (-)	10 (12.5)
C-2	ACE-2	c.1655A>G	rs1124935570	Missense variant	Exon-12/peptidase domain	p.Q552R	Benign score 0.023	Neutral score-79	MAS (-)	1 (1.25)
C-3	ACE2	c.1143T>C	Novel	Synonymous variant	Peptidase domain/exon-9	p.Y381=	NA	NA	MAS (+)	1 (1.25)
C-4	ACE2	c.1148T>C	rs1396769231	Missense variant	Peptidase domain/exon-9	p.M383T	Probably damaging score 1.00	Effect score 17	MAS (+)	1 (1.25)
C-5	ACE2	g.35058T>G	rs895807533	SNP	Intron variant	–	–	–	MAS (-)	13 (16.25)
C-6	CXCR3	c.397A>T	rs1166679147	Missense variant	Exon-2/transmembrane receptor	p.I133F	Probably damaging score 0.99	Effect score 61	MAS (-)	1 (1.25)
C-7	CXCR3	c.422A>C	Novel	Missense variant	Exon-2/transmembrane receptor	p.L142P	Probably damaging score 1.00	Effect score 83	MAS (-)	1 (1.25)
C-8	Cxorf21/ TASL	c.401A>G	rs1467341267	Missense variant	Exon-3	p.N134S	Probably damaging score 0.99	Effect score 75	MAS (+) MAS (-)	18 (22.5)
C-9	Cxorf21/ TASL	c.627T>G	rs887369	Synonymous variant	Exon-3	p.M165T	Probably damaging score 0.49	NA	MAS (+)	3 (3.75)
C-10	TLR8	c.1404A>T	Novel	Missense variant	Exon-2	p.I451F	Probably damaging score 0.78	Neutral score-61	MAS (-)	1 (1.25)
C-11	TLR8	c.1275C>G	Novel	Missense variant	Exon-2	p.K407N	Neutral score 0.01	Neutral score-14	MAS (+)	1 (1.25)
C-12	TLR8	c.1212A>C	Novel	Missense variant	Exon-2	p.F387V	Probably damaging score 0.84	Neutral score-30	MAS (-)	1 (1.25)
C-13	TLR8	c.1119C>G	Novel	Missense variant	Exon-2	p.H374D	Probably damaging score 1.00	Effect score 81	MAS (-)	1 (1.25)
C-14	TLR8	c.1019T>C	Novel	Missense variant	Exon-2	p.Y340C	Probably damaging score 0.99	Effect score 49	MAS (+)	1 (1.25)
C-15	TLR8	c.1222C>A	COSV54320805	Missense variant	Exon-2	p.L408M	Probably damaging score 0.83	Neutral score-30	MAS (-)	1 (1.25)
C-16	TLR8	c.1382A>G	rs1337623910	Missense variant	Exon-2	p.N461S	Benign score 0.001	Neutral score 5	MAS (+)	1 (1.25)
C-17	TLR8	c.1916T>A	Novel	Missense variant	LRR domain/exon-3	p.K639V	Probably damaging score 0.95	Neutral score-17	MAS (-)	1 (1.25)
C-18	TLR7	c.1786A>C	rs779750615	Missense variant	LRR domain/Exon-3	p.L596Q	Benign score 0.001	Effect score 34	MAS (+)	1 (1.25)
C-19	TLR7	c.1570C>T	rs774525175	Synonymous variant	LRR domain/Exon-3	p.L524=	Benign	NA	MAS (+)	1 (1.25)

Table 3 (cont.). Characteristics and clinical features of the TLR7, TLR8, ACE2, CD40L, CXCR3, and TASL gene mutations in the study groups

No.	Gene	Nt alteration	Rs number	Alteration type	Localization	AA position	Clinical significance		MAS status	Frequency of variant n (%)
							PolyPhen-2 (score)	SNAP (score)		
C-20	TLR7	c.1697G>C	Novel	Missense variant	LRR domain/Exon-3	p.H566Q	Benign score 0.001	Neutral score-16	MAS (+)	1 (1.25)
C-21	TLR7	c.1892 A >C	Novel	Missense variant	LRR domain/Exon-3	p.L631V	Probably damaging score 1.00	Effect	MAS (+)	1 (1.25)
C-22	TLR7	c.1890 G>C	Novel	Missense variant	LRR domain/Exon-3	p.H630Q	Benign score 0.014	Effect score 78	MAS (+)	1 (1.25)
C-23	TLR7	c.1576G>A	Novel	Synonymous variant	LRR domain/Exon-3	p.E526=	Benign	NA	MAS (+)	1 (1.25)
C-24	TLR7	c.1602T>C	rs1259652414	Synonymous variant	LRR domain/Exon-3	p.N534=	Benign	NA	MAS (-)	1 (1.25)
C-25	TLR7	c.1880G>T	rs34014664	Missense variant	LRR domain/Exon-3	p.R627I	Probably damaging score 0.98	Effect score 61	MAS (+)	1 (1.25)
C-26	TLR7	c.1263 A>T	Novel	Synonymous variant	Exon-2	p. I403=	NA	NA	MAS (+) /MAS (-)	5 (6.25)
C-27	CD40L	c.*154=	Novel	3 prime UTR variant	Non-coding region	–	NA	NA	MAS (-)	6 (7.5)
C-28	CD40L	c.773T>C	rs1569377884	Missense variant	–	p.L258S	Probably damaging score 1.00	Effect score 19	MAS (-)	2 (2.5)
C-29	CD40L	g.16392G>T	Novel	3 prime UTR variant	3'UTR	–	NA	NA	MAS (-)	2 (2.5)

C: Change; LRR: Ligand recognition region; MAS: Macrophage activation syndrome; NA: Not applicable; SNP: Single nucleotide polymorphism; UNK: Unknown; UTR: Untranslated region

The SARS-CoV-2 spike (S) protein activity appears to be similar to that of other members of the coronavirus family, severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Following the 2019 outbreak of the COVID-19 pandemic, several studies have focused on the role of ACE2 mutations in disease susceptibility and seriousness (13, 15, 19, 20). The viral S protein receptor-binding domain binds to ACE2 to enter the host cell. (19–21). ACE2 is a protein that consists of 805 amino acids and has 2 domains: the N-terminal peptidase M2 domain and the C-terminal collectrin domain (20, 21). Experimental and *in-silico* studies have reported that a pathogenic variant, including R219C, R219H, M383T, P389H, D427Y, R514G, R708W, R710H, R710C, R716C, L731F, and R768W, affects susceptibility to COVID-19 (20–22). Regarding the M383T mutation detected in a MAS (+) male patient in our study, previous studies reported that the M383 amino acid residue inhibits SARS-CoV2 binding (22). We think that the methionine>threonine change in this amino acid codon may change the binding affinity of SARS-CoV2. Moreover, our pathogenicity estimation analysis determined that a M383T missense mutation was pathogenic. ACE2 expression at cellular levels differs among individuals. It is well established that both ACE2 variants and expression levels can affect individual sensitivity and disease severity to SARS-CoV2 infection (13, 15, 20–22). The localization of ACE2 on Xp.22.2 has been considered a disadvantage for male carriers of alleles due to a lower expression of ACE2 than in females; this may clarify the higher prevalence of severe COVID-19 among men (13, 15, 19–22).

Furthermore, studies have established that the binding of the virus to its cellular receptor, ACE2, and subsequent invasion of the host cell, results in downregulation of the ACE2 receptor. Similar to reports in the literature, our study determined that the ACE2 expression level was lower in the male MAS (+) group than in the female group. Expression levels were significantly lower in all of the MAS (+) groups. TLRs are type 1 transmembrane proteins expressed by macrophages and dendritic cells, which are components of innate immunity. During virus infection, TLRs are capable of recognizing different pathogen-associated molecular patterns and triggering the innate immune response (12, 23, 24). TLR7/TLR8 recognizes single-stranded viral RNA. Deleterious germline mutations (p.Gln710Argfs*18 and p.V795P) of TLR7 on the X chromosome have been demonstrated in young men with a severe clinical course of COVID-19 and have been shown to affect the folding of the LRR domain (24, 25).

The genes that revealed the most variants in our study were TLR7 and TLR8. The 9 variants we detected are on the LRR domain. The LRR domain is important for ligand binding and related signaling and acts as a pattern recognition receptor (12, 23–25). Two of the mutations (p.L631V, p.R627I) that we detected on this domain and only in the male MAS (+)patient group were pathogenic. These variants are on conserved amino acid codons in the evolutionary process; studies have shown that this domain is highly conserved among mammals. The variants detected on the LRR domain have previously been associated with severe COVID-19 (25). The literature indicates that greater expression of TLR7 can cause a higher immune response, leading to a better prognosis in single-stranded RNA (ssRNA) viral infections. Consistent with the literature, our m-RNA expression analysis demonstrated statistically significantly lower TLR7 gene expression in the male MAS(+) group compared with the MAS(-) group. We think that this may adversely affect the prognosis of the disease by altering the m-RNA level of pathogenic missense variants in the MAS (+) group (decreased viral RNA recognition feature). TLR8, located on the X chromosome, resides in endosomes where it recognizes nucleic viral ssRNA, then initiates signaling to induce inflammatory cytokines and type I interferons (24, 25). p.I451P, p.P387V, p.L374D, p.Y340C, p.L408M, p.N461S, p.K639V mutations detected in the TLR8 in our study were classified as pathogenic, and all were on the LRR domain. p.Y340C, p.N461S, p.K639V were detected only in 1 male MAS(+) patient. Furthermore, m-RNA expression analysis comparing the TLR8 expression level of male MAS(+)/MAS(-) groups revealed a statistically significantly lower expression level in the MAS(+) group. As with TLR7, function-impairing pathogenic variants may affect TLR8 expression, and we think that this may be a common cause of progression to severe stages of COVID-19, especially in males.

The gene-encoding CD40L, localized on the q arm of the X chromosome, acts as an important co-stimulatory molecule that can modulate the adaptive immune response by regulating B cell activation/differentiation and T cell survival (26). It encodes a 33kDa type II transmembrane protein belonging to the tumor necrosis factor (TNF) family (26, 27). The p.L258S we detected on the TNF homology domain, known as the extracellular domain, is pathogenic. The expression analysis did not detect a significant difference in the CD40L m-RNA expression level between the groups. TASL is expressed in both adaptive and innate immune cells, particularly monocytes, primary B cells, and neutrophils. TASL is known to be more highly expressed in females than males and elicits a sex-dimorphic response to TLR7 activation (28). The coexistence of p.N134S and p.M165T missense mutations was detected in 1 male MAS (+)patient, and both missense variants were pathogenic. However, we observed no difference in m-RNA expression between the male and female groups. CXCR3 is an important X-linked immune-associated gene, an inducible chemokine receptor, and a significant regulator of Th1 immune responses. CXCR3 is located on the Xq13.1 and contains 3 exons (29). The receptor is mainly expressed on activated T cells. The 4 NH2-terminal residues are encoded by exon 1 of CXCR3, and all remaining amino acids are encoded by exon 3 (30). A variant was detected in female MAS(+) individuals. The m-RNA expression analysis yielded a statistically significant difference between the male and female MAS(+)/(-) groups. Similarly, other studies have shown that CXCR3 m-RNA was overexpressed in T cells in women compared with men. CXCR3 and its ligands appear to play a crucial role in resistance to infectious agents, and despite similar pathogenic exposure, men are more susceptible than women (29–31).

CONCLUSION

Gender is increasingly recognized as an important factor in COVID-19 prognosis. A common feature of epidemiological data and observational reports from both the original SARS epidemic and the current COVID-19 pandemic is that men are more likely than women to experience both higher disease severity and a higher mortality rate. In the present study, we performed comprehensive molecular profile analyses of the TLR7, TLR8, ACE2, CD40L, CXCR3, TASL genes encoded on the X chromosome involved in the pathogenesis of the virus and the formation of the immune response, and which may account for increased susceptibility in men. Especially in the absence of COVID-19-related clinical risk factors, the initiation of early preventive and therapeutic interventions may be possible by determining the ACE2 and TLR pathogenic mutations for COVID-19 genetic susceptibility and the expression of TLR7/8 and chemokine receptors involved in immunopathogenesis. However, we are aware of the limited sample size of this study and believe that further research and larger samples are needed to identify variants and expression differences in gender-biased genes responsible for COVID-19 pathogenesis to further clarify the epidemiology of COVID-19.

Ethics Committee Approval: The Niğde University Clinical Research Ethics Committee granted approval for this study (date: 08.04.2021, number: 2021/50).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – DFAB, ÜK; Design – ÜK, DFAB; Supervision – DFAB., ÜK, OY, TT; Resource – DFAB, ÜK, TAG, EÖ; Materials – TAG, ZTY, OY, TT, ÜK; Data Collection and/or Processing – DFAB, EÖ, TAG, ZTY; Analysis and/or Interpretation – DFAB, EÖ; Literature Search – DFAB; Writing – DFAB; Critical Reviews – DFAB, TAG, ZTY, OY, TT, ÜK.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: This work was supported by grants from the Scientific Research Projects of Niğde Ömer Halisdemir University (BAP; Project no SAT 2021/2-ÖNAP).

REFERENCES

1. Pradhan A, Olsson PE. Sex differences in severity and mortality from COVID-19: are males more vulnerable? Biol Sex Differ 2020; 11(1): 53. [CrossRef]
2. Gebhard C, Regitz-Zagrosek V, Neuhauser HK, Morgan R, Klein SL. Impact of sex and gender on COVID-19 outcomes in Europe. Biol Sex Differ 2020; 11(1): 29. [CrossRef]
3. Scully EP, Haverfield J, Ursin RL, Tannenbaum C, Sabra L. Klein. Considering how biological sex impacts immune responses and COVID-19 outcomes. Nat Rev Immunol 2020; 20(7): 442–7. [CrossRef]
4. Haitao T, Vermunt JV, Abeykoon J, Ghamrawi R, Gunaratne M, Jayachandran M, et al. COVID-19 and sex differences: Mechanisms and biomarkers. Mayo Clin Proc 2020; 95(10): 2189–203. [CrossRef]
5. Callender LA, Curran M, Bates SM, Mairesse M, Weigandt J, Betts CJ. The impact of pre-existing comorbidities and therapeutic interventions on COVID-19. Front Immunol 2020; 11: 1991. [CrossRef]

6. Ejaz H, Alsrhani A, Zafar A, Javed H, Junaid K, Abdalla AE, et al. COVID-19 and comorbidities: Deleterious impact on infected patients. J Infect Public Health 2020; 13(12): 1833–9. [CrossRef]
7. Totura AL, Baric RS. SARS coronavirus pathogenesis: host innate immune responses and viral antagonism of interferon. Curr Opin Virol 2012; 2(3): 264–75. [CrossRef]
8. Gadi N, Wu SC, Spihlman AP, Moulton VR. What's sex got to do with COVID-19? Gender-based differences in the host immune response to Coronaviruses. Front Immunol 2020; 11: 2147. [CrossRef]
9. Foresta C, Rocca MS, Di Nisio A. Gender susceptibility to COVID-19: a review of the putative role of sex hormones and X chromosome. J Endocrinol Invest 2021; 44(5): 951–6. [CrossRef]
10. Penna C, Mercurio V, Tocchetti CG, Pagliaro P. Sex-related differences in COVID-19 lethality. Br J Pharmacol 2020; 177(19): 4375–85.
11. Chanana N, Palmo T, Sharma K, Kumar R, Graham BB, Pasha Q. Sex-derived attributes contributing to SARS-CoV-2 mortality. Am J Physiol Endocrinol Metab 2020; 319(3): E562–7. [CrossRef]
12. Sarmiento L, Svensson J, Barchetta I, Giwercman A, Cilio CM. Copy number of the X-linked genes TLR7 and CD40L influences innate and adaptive immune responses. Scand J Immunol 2019; 90(2): e12776.
13. Viveiros A, Gheblawi M, Aujla PK, Sosnowski DK, Seubert JM, Kassiri Z, et al. Sex- and age-specific regulation of ACE2: Insights into severe COVID-19 susceptibility. J Mol Cell Cardiol 2022; 164: 13–6. [CrossRef]
14. Migeon BR. X-linked diseases: susceptible females. Genet Med 2020; 22(7): 1156–74. [CrossRef]
15. Gemmati D, Bramanti B, Serino ML, Secchiero P, Zauli G, Tisato V. COVID-19 and Individual Genetic Susceptibility/Receptivity: Role of ACE1/ACE2 genes, immunity, inflammation and coagulation. Might the double x-chromosome in females be protective against SARS-CoV-2 Compared to the single x-chromosome in males? Int J Mol Sci 2020; 21(10): 3474. [CrossRef]
16. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013; Chapter 7: Unit7.20. [CrossRef]
17. Bromberg Y, Rost B. SNAP: predict effect of non-synonymous polymorphisms on function. Nucleic Acids Res 2007; 35(11): 3823–35.
18. Ovsyannikova IG, Haralambieva IH, Crooke SN, Poland GA, Kennedy RB. The role of host genetics in the immune response to SARS-CoV-2 and COVID-19 susceptibility and severity. Immunol Rev 2020; 296(1): 205–19. [CrossRef]
19. Ilikci Sagkan R, Akin-Bali DF. Structural variations and expression profiles of the SARS-CoV-2 host invasion genes in lung cancer. J Med Virol 2020; 92(11): 2637–47. [CrossRef]
20. Saih A, Baba H, Bouqdayr M, Ghazal H, Hamdi S, Kettani A, et al. In silico analysis of high-risk missense variants in human ACE2 gene and susceptibility to SARS-CoV-2 infection. Biomed Res Int 2021; 2021: 6685840. [CrossRef]
21. Suryamohan K, Diwanji D, Stawiski EW, Gupta R, Miersch S, Liu J, et al. Human ACE2 receptor polymorphisms and altered susceptibility to SARS-CoV-2. Commun Biol 2021; 4(1): 475. [CrossRef]
22. Devaux CA, Rolain JM, Raoult D. ACE2 receptor polymorphism: Susceptibility to SARS-CoV-2, hypertension, multi-organ failure, and COVID-19 disease outcome. J Microbiol Immunol Infect 2020; 53(3): 425–35. [CrossRef]
23. Khanmohammadi S, Rezaei N. Role of Toll-like receptors in the pathogenesis of COVID-19. J Med Virol 2021; 93(5): 2735–9. [CrossRef]
24. Moreno-Eutimio MA, López-Macías C, Pastelin-Palacios R. Bioinformatic analysis and identification of single-stranded RNA sequences recognized by TLR7/8 in the SARS-CoV-2, SARS-CoV, and MERS-CoV genomes. Microbes Infect 2020; 22(4-5): 226–9. [CrossRef]
25. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol 2014; 5: 461. [CrossRef]
26. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol Rev 2009; 229(1): 152–72. [CrossRef]
27. Aloui C, Prigent A, Sut C, Tariket S, Hamzeh-Cognasse H, Pozzetto B, et al. The signaling role of CD40 ligand in platelet biology and in platelet component transfusion. Int J Mol Sci 2014; 15(12): 22342–64. [CrossRef]
28. Odhams CA, Roberts AL, Vester SK, Duarte CST, Beales CT, Clarke AJ, et al. Interferon inducible X-linked gene CXorf21 may contribute to sexual dimorphism in Systemic Lupus Erythematosus. Nat Commun 2019; 10(1): 2164. [CrossRef]
29. Oghumu S, Varikuti S, Stock JC, Volpedo G, Saljoughian N, Terrazas CA, et al. Cutting edge: CXCR3 escapes X chromosome inactivation in T cells during infection: Potential implications for sex differences in immune responses. J Immunol 2019; 203(4): 789–94. [CrossRef]
30. Fathi A, Addo MM, Dahlke C. Sex differences in immunity: Implications for the development of novel vaccines against emerging pathogens. Front Immunol 2021; 11: 601170. [CrossRef]
31. Metzemaekers M, Vanheule V, Janssens R, Struyf S, Proost P. Overview of the mechanisms that may contribute to the non-redundant activities of interferon-inducible CXC Chemokine Receptor 3 Ligands. Front Immunol 2018; 8: 1970. [CrossRef]