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Association of Toll-like receptor-4 polymorphism with SARS CoV-2 infection in Kurdish Population

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ABSTRACT

Genetic variations are critical for understanding clinical outcomes of infections including server acute respiratory syndrome coronavirus 2 (SARS CoV-2). The immunological reactions of human immune genes with SARS CoV-2 have been under investigation. Toll-like receptors (TLRs), a group of proteins, are important for microbial detections including bacteria and viruses. TLR4 can sense both bacterial lipopolysaccharides (LPS) and endogenous oxidized phospholipids triggered by Covid-19 infection. Two TLR4 single nucleotide polymorphisms (SNPs), Asp299Gly and Thr399Ile have been linked to infectious diseases. No studies have focused on these SNPs in association with Covid-19. This study aims to reveal the association between Covid-19 infection with these SNPs by comparing a group of patients and a general population. Restriction fragment length polymorphisms (RFLP) were used to identify the TLR4 SNPs in both the general population ($n = 114$) and Covid-19 patient groups ($n = 125$). The results found no association between the TLR4 polymorphisms and Covid-19 infections as the data showed no statistically significant difference between the compared groups. This suggested that these TLR4 SNPs may not be associated with Covid-19 infections.

1. Introduction

The patho-immunology of Coronavirus disease 2019 (Covid-19), has been under investigation. Nevertheless, based on previous studies of other viruses and current knowledge on Severe Acute Respiratory Syndrome Coronavirus 2 (SARS CoV-2), it has been known that the immune reactions occur between SARS COV-2 viral particles (e.g., spike protein) and human cellular receptors (e.g., human angiotensin converting enzyme 2 (ACE2)) (Sironi et al., 2020). Simultaneously, human innate immune receptors are also activated by the components of the virus or secondary bacteria that consequently trigger cytokine secretions to attract phagocytic cells which in turn can elicit adaptive immunity to limit viral replication (Forbester and Humphreys, 2021). Ultimately, the immune response is usually able to control the infection leading to recovery. Nonetheless, the disease often results in inflammations of the lungs leading to acute respiratory distress syndrome (ARDS) and death, especially in old or comorbid people with cardiovascular and metabolic diseases (Brandão et al., 2021; Forbester and Humphreys, 2021).

One of the most important innate immune receptors is toll-like receptors (TLRs) which can sense several microbial pathogens associated molecular patterns (PAMPs) including components of either viruses or bacteria. Interestingly, TLR4 can recognise both bacterial lipopolysaccharide (LPS) and endogenous oxidized phospholipids induced by lung tissue injury due to viral infections such as SARS CoV-2 (Akpınar et al., 2021; Birra et al., 2020; Brandão et al., 2021; Imai et al., 2008; Khanmohammadi and Rezaei, 2021). Therefore, TLR4 may play dual roles in fighting against both SARS CoV-2 and the secondary Gram-negative bacterial infections that may lead to septic shock in some Covid-19 patients. Additionally, TLR4 is unique among the human TLRs that can be activated via two adapter protein pathways including myeloid differentiation factor (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) leading to express proinflammatory cytokines and

Abbreviations: **ARSD**, Acute respiratory distress syndrome; **ACE2**, Angiotensin converting enzyme 2; **Covid-19**, Coronavirus disease 2019; **IL**, Interleukin; **LPS**, Lipopolysaccharides (LPS); **PCR-RFLP**, Polymerase chain reaction-restriction fragment polymorphism; **MyD88**, Myeloid differentiation factor; **SARS CoV-2**, Server acute respiratory syndrome coronavirus 2; **SNPs**, Single nucleotide polymorphisms; **TLRs**, Toll-like receptors; **TNF-α**, Tumour necrosis factor alpha;; **TRIF**, TIR-domain-containing adapter-inducing interferon-β..

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interferon, respectively. In other words, induction of the MyD88 pathway enhances pro-inflammatory cytokines (e.g., IL-1, IL-6 and TNF-α) that may lead to hyper inflammations, cytokine storm and organ damage (Forbester and Humphreys, 2021), while activation of the TRIF pathway results in secretion of interferons (type 1 interferons) to control the virus (Aboudounya and Heads, 2021; Beck and Aksentijevich, 2020; Brandão et al., 2021).

As the probability of TLR4 involvement in SARS-COV-2 induced inflammatory responses have been strongly suggested (Choudhury et al., 2021b). Thus, the investigation of TLR4 is feasibly important for understanding inflammatory conditions, viral entrance, therapeutics and immunizations against SARS CoV-2 in Covid-19 patients. For instance, spike protein of SARS CoV-2 has an affinity to bind TLR4, which is proposed to increase ACE2 expression in type 2 pneumocytes and cardiomyocytes that lead to induction of the pro-inflammatory cytokines causing hyper inflammation through the MyD88 dependent pathway; in this manner, TLR4 potentially helps SARS CoV-2 enter directly into host cells (Aboudounya and Heads, 2021; Brandão et al., 2021). Understanding the association between TLR4 and infection with Covid-19 is essential in the development of novel treatments. Likewise, researchers have candidate TLR4 and COVID-19 spike protein interaction for targeting as an anti-SARS COV-2 therapy (Choudhury et al., 2021b; Patra et al., 2021a) and recent studies have shown that TLR4 antagonists can inhibit acute respiratory distress syndrome (ARDS) and thrombosis caused by activation of TLR4 on lung cells and platelets (Aboudounya and Heads, 2021; Brandão et al., 2021). This association is also important for designing vaccines. For instance, an immune-informatic study has revealed that multi-epitopes of designed vaccines have an affinity to bind TLR4 enhancing immune response potentially during vaccinations (Naz et al., 2020). Moreover, *in silico* peptide vaccines have been designed for prophylaxis against all coronaviruses (Choudhury et al., 2022). Recent *in silico* studies have also used phytochemicals (Das et al., 2022) and ivermectin (Choudhury et al., 2021a) to block SARS CoV-2 proteins that may reduce the proinflammatory effects of the TLR4 pathway. Additionally, both preventive and therapeutic strategies have been recently under clinical trials for combating Covid-19 pandemic by using both TLR agonists (e.g., Imiquimod) and antagonists (e.g., Hydroxychloroquine) (Patra et al., 2021a). Applications of both immunization and therapeutic strategies against Covid-19 pandemics can be facilitated by using artificial intelligence along with experimental, socioeconomic, *in silico* and clinical studies (Patra et al., 2021b).

There are two *co*-segregating single nucleotide polymorphisms (SNPs) in the coding region of TLR4 protein that is known to impact on function and structure of the receptor and can be hypothetically linked to Covid-19 infections. One SNP that changes an amino acid, aspartate 299 to glycine is called TLR4 Asp299Gly (A896G) and the other SNP is caused by a substitution of threonine 399 with isoleucine, TLR4 Thr399Ile (C1196T). These SNPs may impact the binding ability of the TLR4 receptor with the spike protein of SARS CoV-2 since crystallography data showed that TLR4 SNPs particularly Asp299Gly causes focal structural modifications in LPS binding compared to the wildtype type (Hold et al., 2014). These SNPs may not only impact the virus entry into the human cells but it is also possible to affect the TLR4 pathways (Richard et al., 2021), in particular MyD88 which is the pathway for pro-inflammatory cytokines. A study also shows that both TLR4 Asp299Gly and Thr399Ile SNPs impact on the expression of genes of the TRIF pathway and pro-inflammatory cytokines (Hold et al., 2014) that may lead to immune imbalances. These suggest that the TLR4 SNPs can have effects on the pathways in response to microbial infections and may cause dysregulations of the inflammatory signals produced in each of the TLR4 pathways. Thus, differential responses to stimulate the TLR4 pathways due to those SNPs may result in failure of Covid-19 patients to control the SARS CoV-2 replications that may eventually develop severe symptoms.

Several factors may play roles in the appearance of clinical manifestations of Covid-19 including ages, gender, comorbidities, and genetic background of susceptible individuals to the virus (Casanova et al., 2020). It has been known for decades that genetic variations among individuals in populations are associated with infectious, metabolic and autoimmune diseases. Recent reviews have highlighted several genetic variants of human genes including angiotensin converting enzyme 2 (ACE2), Toll-like receptor 3 (TLR3), TLR7, Transmembrane serine protease 2 (TMPRSS2), Furin, interferon receptor alpha (IFNRA), Apolipoprotein Epsilon (Apo E), Interferon Induced Transmembrane Protein 3 (IFITM3), Interferon regulatory factors (IRF), human leukocyte antigen (HLA), blood group (ABO), CD26 or Dipeptidyl peptidase-4 (DPP4), Sodium-Dependent Imino Acid Transporter 1 (SLC6A20), Glutathione S-transferases (GSTT1), Vitamin D binding protein (DBP) and Interleukin 6 (IL-6) that are known to have correlations with Covid-19 infections (Kaltoum, 2021; Yildirim et al., 2021). Investigations of SNPs are important not only for understanding the disease severity but also for identifying risk groups who are vulnerable to Covid-19 infections. Therefore; such groups should be given priority for vaccinations and intensive therapies as Norin and colleagues suggest for black minority ethnic groups who have HLA B53 variant (Norin et al., 2021).

Covid-19 linked candidate genes can be nominated according to the literature on genetics correlated with the disease, microbial life, patients' clinical manifestations concerning biological pathways, and in particular the receptors through which the virus interacts with the human cells (Murray et al., 2020). Review studies recommended investigations of human genetic variations in response to Covid-19 infections (Forbester and Humphreys, 2021; Ghafouri-Fard et al., 2020). These studies suggested that the severity of Covid-19 may be related to SNPs in innate immune genes including cytokines and toll-like receptors. Furthermore, the COVID Human Genetic Effort studies have been searching for candidate genes which might be associated with resistance or susceptibility to Covid-19 infections and this would help the development of therapeutics (Casanova et al., 2020). The authors argued that the causality of the disease severity might be due to a single or multiple defective genes (e.g. interferon) that contribute to immune responses against the viral infection. It is worth remembering that interferon is one of the products of the TLR4 pathways and thus it is interesting to discover the TLR4 SNPs and other defective genes in association with Covid-19 patients.

Despite the important roles of TLR4 SNPs in several infectious and inflammatory diseases (Medvedev, 2013), up to our best knowledge, during the writing of this manuscript, there is only one study investigating the association between TLR4 SNPs and Covid-19 infection (Taha et al., 2021). Within the last two years, our group has been studying the genotypic frequencies of the two TLR4 SNPs (Asp299Gly and Thr399Ile) (Niranji and Al-Jaf, 2022) and Apolipoprotein E alleles (Al-Jaf, 2021) of a general population in Iraqi Kurds. We have also found that the Apo E4 allele is associated with Covid-19 infection (Al-Jaf et al., 2021). The current study aims to investigate the association between the TLR4 SNPs and susceptibility to Covid-19 infection by comparing the general Kurdish population as a reference control group and Covid-19 patients who visited clinics seeking treatments.

2. Materials and methods

2.1. Sampling information

We conducted a retro-perspective case control study. Only Kurdish ethnic backgrounds and unvaccinated individuals were included in both general population control and patient groups. Three (3) ml EDTA-conserved blood samples were collected from Covid-19 real-time PCR-positive patients ($n = 125$) who visited polyclinics in Kalar town, Sulaymaniyah province, Kurdistan Region of Iraq. Patient information included in a questionnaire form: age, sex, comorbidities (Asthma, obesity, diabetes, hypertension, stroke, and ischemic heart disease), SpO2, CT scan, and laboratory parameters (ESR, CRP, Ferritin, D-dimer). General population samples ($n = 114$) were used from the previous

retro-perspective study (Niranji and Al-Jaf, 2022). Informed consent forms were taken from patients prior to sampling. Ethical approval was performed by an ethical committee in the Department of Biology, the University of Garmian that adhered to the Declaration of Helsinki.

2.2. Patients information

Details of clinical features of the COVID-19 patients are summarised in Table 1. The demographic data showed that the percentage of male and female patients was 46.4% and 53.6%, respectively. The patient's ages below 45 years were 43.2% while those above 45 years were 56.8%. Among the comorbidities, both hypertension (28.8%) and obesity (18.4%) were the most prevalent medical conditions in the Covid-19 patients. However, other conditions such as diabetes (8.8%), ischemic heart diseases (8%), asthma (3.2%) and stroke (0.8%) were less common. The parameter data showed that most patients had higher acute phase reactants such as CRP (83.4%) and ESR (71.2%) while other parameters including ferritin (34.4%) and D-dimer (31.2%) were also found. Concerning oxygen saturations, 47.2% had low SpO_2 while 52.8% had normal SpO_2. Among the patients, only 24.8% had signs of pneumonia that were required to be screened with CT scan which showed that 13.6% and 11.2 were moderate and severe, respectively.

2.3. Genotyping of TLR4 SNPs

The common TLR4 SNPs, Asp299Gly (A896G) and Thr399Ile (C1196T) were genotyped using a polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP) method as described previously (Ajdary et al., 2011; Niranji and Al-Jaf, 2022). In this method, Genomic DNA extraction was performed using a kit from (GENET BIO CO., Daejeon, KR), then the manufacturer's instructions were followed. This was followed by PCR amplification of the TLR4 gene using primers described by Ajdary et al., 2011 for each SNP (Ajdary et al., 2011). The TLR4 Asp299Gly PCR products were cleaved by *Nco1* enzyme. While TLR4 Thr399Ile PCR products were cleaved by *Hinf1* enzyme (NEB, USA).

2.4. Statistical analysis

Data were analysed using $\chi 2$ (Fisher's exact test) employing Graphpad prism 9.1.1 software (GraphPad Software, San Diego, California USA) to compare Covid-19 and general population groups. *P*-value (<0.05) was considered statistically significant. Both odd ratios and confident intervals (95%) were also presented.

3. Results

3.1. Genotyping of TLR4 SNPs

The homozygous wildtype: Asp299/Asp299 (AA), produced a single PCR product of 249 bp DNA bands which was seen in 3% agarose gel; the heterozygous SNP: Asp299/Gly299 (AG) has two PCR products of 249 bp and 223 bp; and the homozygous SNP: Gly299/Gly299 (GG) shows a PCR product of 223 bp. The homozygous wildtype: Thr399/Thr399 (CC) has a single PCR product of 406 bp. The heterozygous SNP: Thr399/Ile399 (CT) has two PCR products of 406 bp and 379 bp. The homozygous SNP: Ile399/Ile399 (TT) has a single PCR product of 379 bp.

3.2. Association of TLR4 SNPs genotypes with Covid-19

The genotypic study showed that both TLR4 SNPs are not associated with Covid-19 infections compared to the general population as shown in Table 2. The results revealed that the wildtype TLR4 Asp299 (AA) genotype is more frequent among both the general population and COVID-19 patients comprising around 92 to 89% of all genotypes, respectively. While the heterozygous TLR4 Asp299Gly (AG) was less common in both patients and general population groups with around 8 to 10% of all genotypes, respectively. Similarly, the wildtype TLR4 Thr399 (CC) is more prevalent among both studied groups compared to the heterozygous TLR4 Thr399Ile (CT) genotype. The wildtype TLR4 Thr399 (CC) genotype was around 90 to 93% in the general population and COVID-19 patients, respectively. However, the heterozygous TLR4 Thr399Ile (CT) was less common among both the general population and patients with around 10 to 6% of all genotypes, respectively. Surprisingly, we found only one individual which carried the homozygous mutant genotype of both TLR4 Gly299 (GG) and TLR4 Ile399 (TT) which was in the patient group.

3.3. Association of TLR4 haplotypes with Covid-19

Likewise, when the co-segregated TLR4 SNPs were analyzed, there was no association between the general population and the Covid-19 patient groups. The majority of studied groups carried wildtype genotypes for both investigated SNPs TLR4 Asp/Asp and Thr/Thr (AACC) as shown in Table 3. Noticeably, TLR4 Asp/Gly Thr/Thr (AGCC) is present in ten Covid-19 patients while 5 individuals carried this haplotype in the general population, however, this difference was not statistically significant. Probably due to the low frequency of the mutant alleles in the studied population and the relatively low number of samples, the co-segregation of these genotypes (AATT, AGTT, GGCC, GGCT) was not found in the current study.

4. Discussion

In our previous study, we showed that the genotypic frequencies of the wildtype TLR4 were around 90%, and the heterozygous TLR4 Asp299Gly (7.9%) and TLR4 Thr399Ile (9.7%) were also common in this population. While homozygous mutant genotypes of either SNPs were absent in this population (Niranji, 2020; Niranji and Al-Jaf, 2022) The current study hypothesized that TLR4 SNPs (Asp299Gly and Thr399Ile) are associated with Covid-19 infection. Nonetheless, no association was found between the frequency of TLR4 genotypes and alleles among both Covid-19 patients and the general population in Kurdish people. A recent study concluded that the TLR4 SNPs are associated with the

Table 1
Demographic and clinical information of Covid-19 patients.

Demographic data No. (%)		Parameters No. (%)			Comorbidities No. (%)
Age groups	**No. (%)**	Positive CRP	103 (82.4)	Hypertension	36 (28.8)
below 45 years	54 (43.2)	High ESR	89 (71.2)		
above 45 years	71 (56.8)	High Ferritin	43 (34.4)	Obesity	23 (18.4)
		High D-Dimer	39 (31.2)		
Gender	**No. (%)**	SpO_2		Diabetes	11 (8.8)
		Higher than 93	66 (52.8)		
Male	58 (46.4)	Lower than 93	59 (47.2)	Ischemic heart disease	10 (8.0)
Female	67 (53.6)	CT scan	31 (24.8)	Asthma	4 (3.2)
Total	125 (100)	Moderate	17 (13.6)		
		Severe	14 (11.2)	Stroke	1 (0.8)

Table 2
Association between genotypes of TLR4 SNPs with Covid-19 infection.

SNPs	Genotype/allele distribution	General pop. $n = 114$ (%)	COVID-19 Patients n = 125 (%)	OR (95% CI.)	*p*-value
	AA	105 (92.1)	111 (88.8)	1.000 (Ref.)	
	AG	9 (7.9)	13 (10.4)	1.366 (0.545 to 3.27)	0.512
	GG	0 (0.0)	1 (0.8)	Inf. (0.104 to Inf.)	>0.999
TLR4 Asp299Gly	A	219 (96)	235 (94)	1.000 (Ref.)	
	G	9 (7.2)	15 (6)	1.553 (0.695 to 3.774)	0.402
	GG versus AG + AA	0 (0.0) vs. 114 (100)	1 (0.8) vs. 124 (99.2)	0.00 (0.00 to 9.87)	>0.999
	GG + AG versus AA	9 (7.9) vs. 105 (92.1)	14 (11.2) vs. 111 (88.8)	1.126 (0.459 to 2.70)	0.820
	CC	103 (90.4)	116 (92.8)	1.000 (Ref.)	
	CT	11 (9.6)	8 (6.4)	0.646 (0.255 to 1.647)	0.474
	TT	0 (0.0)	1 (0.8)	Inf. (0.098 to Inf.)	>0.999
TLR4 Thr399Ile	C	217 (95.18)	240 (96)	1.0000 (Ref.)	
	T	11 (4.82)	10 (4)	0.8220 (0.3424 to 1.9735)	0.6609
	TT + CT versus CC	11 (9.6) vs. 103 (90.4)	9 (7.2) vs. 116 (92.8)	1.376 (0.561 to 3.294)	0.641
	TT versus CT + CC	0 (0.0) vs.114 (100)	1 (0.8) vs.124 (99.2)	0.000 (0.000 to 9.868)	>0.999

OR = Odds ratio, CI = Confidence interval, Ref. = Reference, Inf. = Infinity.

Table 3
Co-segregation of TLR4 SNPs haplotypes in both Covid-19 patients and the general population.

Genotypes	COVID-19 patients n = 125 (%)	General popul. n = 114 (%)	OR (95% CI.)	*P*-value
AACC	106 (84.8)	98 (85.96)	1.014 (0.703 to 1.468)	0.999
AACT	5 (4.0)	7 (6.14)	1.535 (0.519 to 4.357)	0.560
AATT	0 (0.0)	0 (0.0)	N/A	N/A
AGCC	10 (8.0)	5 (4.4)	0.548 (0.204 to 1.522)	0.302
AGCT	3 (2.4)	4 (3.5)	1.462 (0.380 to 5.893)	0.731
AGTT	0 (0.0)	0 (0.0)	N/A	N/A
GGCC	0 (0.0)	0 (0.0)	N/A	N/A
GGCT	0 (0.0)	0 (0.0)	N/A	N/A
GGTT	1 (0.8)	0 (0.0)	0 (0.000 to 9.947)	>0.999

OR = Odds ratio, CI = Confidence interval, N/A = Not applicable.

severity of Covid-19 patients (Taha et al., 2021). The authors have compared severe and mild/moderate patients without focusing on the frequency of the alleles in Egyptian general populations. It is reasonable that the alleles or genotypes of TLR4 SNPs should be compared to a healthy control group or a general population of the ethnic group study.

Studies on associations of the TLR4 SNPs with other viral diseases have been conducted. For instance, genetic variations in TLR4 and its pathways (TRIF and MyD88) have been studied in association with susceptibility or severity of viral diseases (e.g., respiratory syncytial and swine influenza viruses and vaccinia virus) (Bourdon et al., 2020). TICAM2 (Toll-Like Receptor 4 Adaptor Molecule 2) is also found to be associated with the susceptibility of SARS CoV-1 in mice models that lack the gene of this adaptor protein (Gralinski et al., 2017).

Several studies have envisaged that TLR4 could be associated with Covid-19 infection. For example, studies showed that stimulations of TLR4, associated with diseases (such as pulmonary diseases, atherosclerosis, diabetes, obesity, hypertension and aged people), make COVID patients have a poor prognosis, due to high pro-inflammatory cytokines (e.g., IL-6, IL-1β, TNF-α) in lungs and circulations leading to pulmonary and systemic hyper inflammations (Brandão et al., 2021; Forbester and Humphreys, 2021). Another study showed that the expression of genes of TRIF pathway is reduced in unstimulated phagocytic cells which carried TLR4 Asp299Gly/ Thr399Ile SNPs compared to the wildtype TLR4. The same study found that interferon B and NF-κB are down-regulated in response to LPS challenge in the SNP-carried cells indicating that the SNPs have influences on the TLR4 signaling pathways (Hold et al., 2014). Additionally, the NF-κB-MyD88 pathway plays role in the severity of the infections (Brandão et al., 2021). It is not known whether SARS CoV-2 triggers which pathways. It would be interesting if the viral particles can induce the pro-inflammatory pathway leading to cytokine storm in some patients.

Studies on other TLRs and their pathways found an association with Covid-19 infection. For example, a systematic review has found 40 genes associated with viral infections and 21 genes with the severity of both SARS CoV-1 and SARS CoV-2 infections, including genes that involve in both TLR and inflammasome pathways, such as TLR3 and Nod-like 1 (NLRP1) receptor (Elhabyan et al., 2020). An in silico molecular docking analysis revealed that both wildtype TLR3 and SNP rs73873710 can recognize SARS CoV-2 RNA better than TLR3 SNPs, rs3775290 and rs3775291 (Teimouri and Maali, 2020). Studies have found an association between Covid-19 infections and human genetic defects, for instance, 'inborn errors' in TLR3 and interferon receptors or regulatory factors (Casanova et al., 2020; Smieszek et al., 2021; Yildirim et al., 2021; Zhang et al., 2020), low expression of interferon stimulating genes and high expression of pro-inflammatory cytokines (Blanco-Melo et al., 2020). Defects in the interferon receptor and the presence of auto-antibodies in patients' sera against interferons have also been linked with Covid-19 severity (Bastard et al., 2020; Casanova et al., 2020; Zhang et al., 2020). Other studies have revealed the association of a TLR3 variant (L412F, rs3775291, c.1234C > T) with the severity of Covid-19 patients (Croci et al., 2021; Dhangadamajhi and Rout, 2021). A nested case control study has found 2.1% of TLR7 defective SNPs in male Italian Covid-19 patients (Fallerini et al., 2021). A whole-genome study of 80 severe Covid-19 patients showed IFNAR2 Tyr322Ter (stop of function), particularly in South Asian people (Smieszek et al., 2021). These studies suggest that TLRs and their pathways should be taken into consideration for genetic association with Covid-19. However, we have not found a such association. The limitations of our study were the small sample size and the inability to follow additional SNPs in all TLRs and their pathway genes.

5. Conclusions

The current study found no association in TLR4 SNPs (Asp299Gly and Thr399Ile) between a general population and Covid-19 patients in the Kurdistan region of Iraq. Future studies should investigate polymorphisms of genes which participate in the TLR4 pathways including IL-1, IL-6, TNF-a, and INF, particularly in different ethnic backgrounds.

CRediT author statement

All authors are contributed equally in developing of this study.

Declaration of Competing Interest

None.

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