Iraqi Journal of Biotechnology, 2024, Vol. 23, No. 3, 249-259

Detection of NLRP3 Gene Polymorphism and Evolution of Some Biomarkers in SARS-CoV-2 Patient

[1]Raghad KH. Maeh, [1]Hula Y. Fadhi

[1]Department of Biology, College of Science, University of Baghdad

Received: November 14, 2023 / Accepted: December 20, 2023 / Published: December 30, 2024

Abstract: The polymorphisms in the genes of NLR family pyrin domain containing protein 3 (NLRP3) and inflammatory markers are related to the severity and Susceptibility of coronavirus 2019 (COVID-19). The aim of the study to determine the role of variants in the genes of NLRP3 and their effects on severity and/ or susceptibility in COVID-19 disease we used allele-specific primer method to analyze NLRP3 variants rs35829419, rs10754558 also, The infection with COVID-19 in the first stage leads to Neutrophilia, while the increased severity of the disease leads to lymphopenia, and the first indicator for storms of cytokine is the neutrophil-to-lymphocyte ratio (NLR). Therefore, the current study is designed to offer data for local information about parameters of hematology among patients with COVID-19 and evaluate their relationship with further factors. Between December and October, 2022 blood specimens and nasopharyngeal swabs (99) were taken from patients with COVID-19. Samples were established by antibody IgM and IgG for COVID-19, real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay, as well as analysis of blood samples to test the hematological parameters. The results exhibited NLPR3 *rs*35829419 *A* allele and genotype CA are related to susceptibility and the presence of COVID-19. NLR, CRP, D-dimer, Ferritin, LDH were increased significantly and associated with the disease severity. It was concluded the NLR can be used as a suitable biomarker assistant to laboratory records in the progression of disease and evaluating the severity of the cases.

Keywords: COVID-19, polymorphism,biomarkers, rRT-PCR, NLRP3.

Corresponding author: Email: (raghadnoor79@gmail.com)

Introduction

There are many signs with SARS-CoV-2 such as cough, shortness of breath, pneumonia, formation of sputum, fever, and fatigue or myalgia (1). This indicates that SARS-CoV-2 affect on respiratory system . Infection with SARS-CoV-2, also, can lead to diseases of tissue, such as weak appetite, diarrhea, vomiting, nausea, headache, and confusion, as well as chest distress, heart injury systems, and palmus (2,3). Depending on the severity of the disease and clinical symptoms the World Health Organization (WHO), rules separate the patients into three groups, critical, severe, and mild. Severe COVID-19 can causes a lack in supply of oxygen by alveoli into the blood. Therefore, to deliver O2 to lungs, mechanical ventilators are needed for patients. Despite these concentrated efforts, 40% of patients do not live (4). To classify patients of COVID-19 into risk groups which is essential in the therapeutic management and clinical setting, the Laboratory registers, taking into account biochemical, hematological, immunological parameters, and

inflammatory (5,6). COVID-19 is a systemic disease with a significant effect on the hemostasis and hematopoietic system. Infection with SARS-CoV-2 causes Neutrophilia in an early stage of the disease, while lymphopenia is related with increased severity of the disease as well as the storms of cytokine are related to neutrophil-to-lymphocyte ratio (NLR). The essential problem of the outbreak of COVID-19 is the disease symptoms that are varied and may affect in diverse appearances between the patients (7,8,9). The polymorphisms in the genes of NLR family pyrin domain containing protein 3 (NLRP3) and inflammatory markers are related to the severity and Susceptibility of coronavirus2019 (COVID-19), Genetic variations in the gene of NLRP3 can change its activity ,There for , two variants of NLRP3 genes rs35829419, rs 10754558 were analyzed by allele-specific primer method to establishment their role in COVID-19,also The current study is designed to recognize the correlation between some risk factors and the alteration in the severity of disease among patients with COVID-19, as well as to offer local data about hematological parameters in moderate, and severe cases and evaluate their correlation with further factors.

Materials and methods

Subject

We focused on patients of Iraq in Baghdad Teaching Hospital, patients (42 – 55) age and control age (37 – 51). There are two groups of patients severe and moderate (31 and 68). WHO Interim Guidance recognized Criteria:

1- Moderate (patient infected with pneumonia and without severe pneumonia).
2- Severe (pulse saturation of oxygen (SpO2) ≤ 93% or the rate of respiration ≥ 30 breaths/min, severe respiratory distress) (10) . In a patient group of 99 individuals, some of them had hypertension and some diabetes (53.5 and 51.5%). A group of control (96) were without chronic diseases (diabetes and cardiovascular) and in the past 12 months, they were without respiratory infections, as well as, by use of COVID-19 IgM and IgG antibodies tests, their serum was negative.

Detection of SARS-CoV-2

After4–5 days of hospitalization, nasopharyngeal swabs were obtained from patients. The RNA of virus was isolated by Mini kit QIAamp Viral RNA. SARS was diagnosed by real-time polymerase chain reaction analysis using a commercial kit and followed by the manufacturer's instructions.

Estimation of some biomarker

Venous blood (5ml) was collected and spread into EDTA and plain tubes (2 and 3 mL). CRP was tested in Serum by using an electro-chemiluminescence immunoassay system. White blood cells were counted using EDTA blood by an automated hematology analyzer. The Neutrophil/lymphocyte ratio was found by dividing the neutrophil absolute by the lymphocyte absolute.

Detection of NLRP3 genes polymorphism

Two SNPs of NLRP3 genes rs35829419 and rs10754558, were recognized. EDTA blood was used to isolate genomic DNA with the

gSYNC DNA extraction kit (Geneaid, Taiwan). To detect target SNPs, DNA isolation was exposed to PCR. SNP data and The DNA sequence of the NLRP3 gene were downloadedfirst (http://asia.ensembl.org), and then, Primers were designed in this study by using the Amplifx program as follows ;

rs35829419F C/A(CCGACACCTTGATATGGTGC)
rs35829419R (TGCTCCAAGTAGCTTACAAGAAA)
rs10754558F G/C(CAGCATCGGGTGTTGTTG)
rs10754558R (CCAGCTACAAAAAGCATGGA)

The PCR was performed in a total volume of 25 µL, with 5 µL AccuPower PCR PreMix (Bioneer, Korea), 3 µL DNA with 1 µL from each primer (forward and reverse), and N.F.W 15 µL, The tube was transported to a thermal cycler (Eppendorf, Germany) that was programmed as follow: Cycle of denaturation (94 ˚C for 3 min), then 35 denaturation cycles of (94 C for 30 s), annealing (51˚C for rs10754558; 53 ˚C for rs35829419) extension (72 ˚C for 30 s), and a final extension cycle (72 ˚C for 5 min). The PCR products were electrophoresed in agarose gel (1.5%; 5 V/cm^2 for 55 min), and a gel documentation system was used to visualize the migrating bands.

Statistical analysis

Percentage and Number were depended on to define significant differences and categorical variables were evaluated by the two-tailed Fisher exact and Chi-square test. For normality, continuous variables were tested (Kolmogorov-Smirnov and Shapiro-Wilk test). Usually spread variables were evaluated as, mean with standard deviation (SD), and the Student *t*-test was used to check significant differences. The median and interquartile range were used to express the nonparametric variables (skewed), to conclude significant differences the Mann-Whitney *U* test was used. GraphPad Prism version 8.0.0 (San Diego, California USA) and IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) were used for these graphs and analyses. To determine the power of the sample size the G*Power software (version 3.1.9.7) was used.

Results and discussion

Baseline characters of patients with COVID-19

Higher rates of platelets and WBC were exhibited in moderate cases compared with severe (241.7 *vs*. 144; 6.48 *vs*. 3.49; respectively $p < 0.001$). A higher rate of Neutrophil was exhibited in severe cases compared with moderate (2.4 vs 1.5 $p < 0.001$). Higher rates of Ferritin and CRP were exhibited in severe cases compared with moderate (981 vs 360; 29 vs 9.7; respectively $p < 0.001$). Higher the rate of D-dimer and LDH were exhibited in severe cases compared with moderate (995 vs 322; 289 vs 200 respectively; $p < 0.001$) , hypertension and diabetes were higher in severe cases compared to moderate (29 vs 24; 27 vs 24: respectively. $p < 0.001$) (Table 1).

Table (1): Baseline blood parameters and chronic disease in COVID-19 patients.

Parameter	COVID-19 patients; N = 99		Reference range†	*p* -Value
	Mild-moderate; N = 68	Severe; N = 31		
Platelets ($\times 10^9$/L)	241.7 (191.3 – 275.5)	144 (111 – 160)	135 - 317	< **0.001**
WBC ($\times 10^9$/L)	6.48 ± 1.4	3.49 ± 0.84	3.4 - 9.6	< **0.001**
Neutophile / lymphocyte ratio	1.5 (1.2 – 1.6)	2.4 (2.2 – 2.9)	1.8 - 2	< **0.001**
CRP (mg/L)	9.7 (5 – 16.2)	29 (27.3 – 61.1)	8 - 10	< **0.001**
Ferritin (ng/ml)	360 (260 – 521)	981 (780 – 1297)	20 - 250	< **0.001**
D. dimer (mg/L)	322 (230 – 507)	995 (810 – 2106)	≤ 500	< **0.001**
LDH (IU/L)	200 (165 – 215)	289 (265 – 395)	105 - 333	< **0.001**
Diabetes	24 (35.3)	27 (87.1)	----	< **0.001**
Hypertension	24 (35.3)	29 (93.5)	----	< **0.001**

Data are liable as mean ± standard deviation, median, and interquartile range (IQR) (continuous variables) or number and percentage (categorical variables as present). Means were compared with the Student t-test. Medians were compared with the Mann-Whitney U test. Significant p-value is indicated in bold. WBC: White blood cell count; CRP: C-reactive protein; LDH: L-dehydrogenase; SD: Standard deviation; †: Data source from https://www.mayoclinic.org.

The levels of platelets decreased in severe cases .COVID-19 display proplatelet formation and an abnormal megakaryocyte distribution (11, 12, 13). Platelets can be stimulated by the complexes of viral antigen-antibody or the responses of host inflammation, stimulated platelets are more readily cleared from the circulation by the reticuloendothelial system. Platelet interacts directly with the virus by a variety of receptors. The released products of platelets can destroy the virus (14). The synthesis of platelets can be reduced by the interaction of viruses with megakaryocyte(15). Also, the levels of WBC were decreased in severe cases because the virus infected those cells, which caused lysis of these cells. Moreover, apoptosis may be promoted by the storm of cytokine and increased levels of tumor necrosis factor TNFα and interleukins (16) . The neutrophil to lymphocyte ratio (NLR) was increased as shown in Figure 1, the poor clinical outcome with higher disease severity led to an increase in NLR and neutrophil count(17,18). The results refer to CRP, LDH, D.dimer, Ferritin, hypertension, and diabetes were used as danger factors to recognize severe sickness in patients and recognize the risk classification to the probability of severe illness in patients. CRP was produced by hepatocytes, it's a nonspecific protein that plays a role during inflammation and infection. 68% of severe cases established increased levels of CRP (19,20). Oxygen saturation was related to the levels of CRP, and there is an increase in the levels of CRP in patients with SpO2 ≤ 90% compared to patients with SpO2 > 90%, Moreover, the patients of COVID-19, increased levels of CRP, IL-10, and IL-6, were related with decreased SpO2 (21,22,23).

In this study, the severe cases had hypertension and diabetes. some study establishes a bad result in COVID-19 was related with hypertension and T2DM, NLRP3 inflammasome is stimulated by T2DM as showed by the activity of NLRP3 and increase the levels of caspase-1, monocytes, IL-18, and IL-1β behind activation of PBMCs (24, 25). Moreover, NLRP3 plays an important role in atherosclerosis and endothelial

inflammation in diabetes (26) In patients with T2DM in Chinese, insulin resistance is related to the rs10754558 NLRP3 GG genotype (27) The NLRP3 controls on beta cells in the pancreas and obesity is related to the resistance of insulin therefore, NLRP3 contributes to T2DM and insulin resistance (28). NLRP3 inflammasome contributes to hypertension by the role of low-grade inflammation , The variants of NLRP3 rs7512998 are related to n hypertension (29). Therefore, COVID-19 is associated with the presence of hypertension and diabetes due to those two conditions displaying stimulation of NLRP3 inflammasome that is stimulated with the infection.

In this study, ferritin was associated with severe cases, Ferritin is the key to deregulation of the immune system, mainly with hyperferritinemia. Ferritin plays an essential role in the storm of cytokine by its pro-inflammatory effects and suppressive of the immune system. SARS CoV-2 causes Inflammation and this led to an increased production of ferritin to reduce the iron effect. cytokines and macrophages cause hyperferritinemia by the Production of active ferritin, which leads to the increased creation of IL- 10 (immune suppression) and IL-1β (proinflammatory cytokines). Extra ferritin causes fibrosis or tissue damage by the creation of reactive oxygen species (ROS). Therefore, ferritin may be a factor that effects COVID-19 severity(30,31,32). The LDH levels are related with severe cases , LDH is an enzyme created in all cells in the body and responsible for the production of energy. LDH was used as indicater for damage in tissues also its related to interstitial lung disease and diseases of the liver. The increased level of LDH is considered a symbol of cell/tissue damage, suggestive of lung damage, e.g. pneumonia produced by SARS (33). The D-dimers increase in severe cases, It is used for the prediction and diagnosis of thrombosis recurrence and intravascular coagulation (34, 35). The value of D-dimers, in patients was related to mortality and severe disease progression (36, 37). Several studies detected mild lobular, portal inflammatory, and microvesicular steatosis by postmortem liver biopsy(38), Further studies showed vascular walls with fibrous thickening, luminal ectasia, and thrombosis, suggesting in COVID-19 liver failure includes coagulation, dysfunction and endothelial lesion (39). Therefore, an increased level of D-dimers can refer to damage to the liver Figure 1: refer to a significant difference ($p<0.001$) between patients 47 (42-55) age and control age (HC) 42 (37 – 51); and in the concentration of CRP stratified by age group/year (≤ 45 and > 45 years), while there is no significant differences between the two group of sex (male and female) in COVID-19 patients; $p = 0.758$. Moreover, a significant difference ($p < 0.001$) in the Concentration of Ferritin (ng/ml), D-dimer (mg/L), and LDH (IU/L) stratified by age group/year (≤ 45 and > 45 years), While the differences are not significant between the two groups of sex (female and male) in patients.

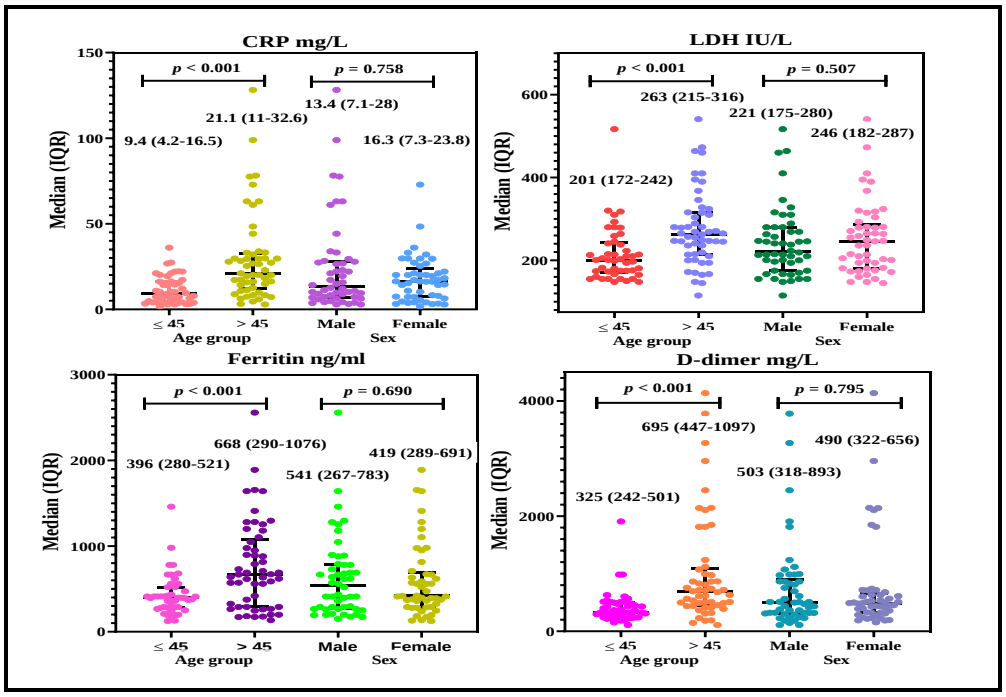

Figure (1): Concentration of CRP, LDH, ferritin, and D-dimer as a biomarker stratified by age group/year (≤ 45 and > 45 years) and gender (female and male) in patients of COVID-19.

The study exhibited that CRP, LDH, D.dimer, and Ferritin increased in older people >45 (severe cases) and they more susceptible to being infected with the virus. Older people have been described with difficulty breathing and don't have signs of COVID-19, for example: smallness of breath and fever while they have dementia, delirium, and confusion, which lead to further death(40). The immune system of older people is responsible for an increased susceptibility to COVID-19 (41, 42). A mark of elderly is the change of the adaptive and innate immunity to forming states of inflammation in the body (43). Moreover, aging is related to downregulated in the functions of cells with the adaptive immunity; such as T-cytotoxic, antigen-presenting, and B cells, and this may respond to the adaptive immunity to control inflammation and infection (44). Then, age was related to reduced regulation and functions of the immune system, described as inflammation and immunosenescence, that responsible for increased infection with COVID-19 moreover, increased stimulation of the NLRP3 is accompanied by increasing in age by an explanation of death because of COVID-19(45). In terms of sex, patients showed males 50 (51.5%) and females 49 (49.5%), and there is no significant difference was showed between groups (p =0.944).

Table (2): Distribution of gender among COVID-19 patients and controls groups

COVID-19	Patients (N = 99)		control (N = 96)	
	N	%	N	%
Male	50	51.5	48	50
Female	49	49.5	48	50
Statistical analysis	D.F. = 1; Pearson X2 = 0.005; p = 0.944 (NS)			

P: Probability ; NS: Not Significant (p > 0.05); D.F; Degree of freedom.

The current study showed that sex affected on appears COVID-19. Much research revealed potential causes of sex differences such as sex hormones (46), X chromosome, the effect of COVID-19 infection, vitamin D, and obesity (47). Sex hormones have a number of distinct effects on the immune system that are crucial to the pathophysiology of COVID-19 (48,49,50)**.**

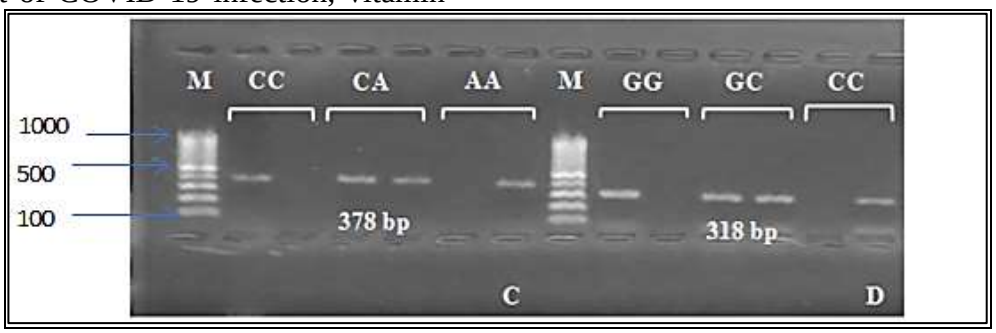

Figure (2): Refer to agarose gel electrophoresis (1.5%; 5 V/cm2 for 55Min.) of DNA–PCR products for SNPs of *NLRP3* gene; C: 378 bp for rs35829419 (C/A), D: 318 pb for rs10754558 (G/C),. M: DNA ladder (100bp).

NLRP3 polymorphism

(Figure 2) shows conventional PCR was used to detection of SNPs in the gene of NLRP3 the outcomes of gel electrophoresis exhibited genotypes for each SNP rs35829419, . The products of DNA PCR for the gene of NLRP3 SNPs;,. **M**: DNA ladder (100bp) as shown in Figure (2). (CC, CA, AA) for rs35829419, (GG, GC, CC) for rs10754558 ,**A**: 378 bp for rs35829419 (C/A), **B**: 318 pb for rs10754558 (G/C) In COVID-19 controls and patients the frequencies of genotype rs35829419, rs10754558, SNPs were in good agreement with Hardy-Weinberg (HWE). The genotype and allele frequencies did not display significant variation with the exception the rs35829419 A allele and genotype CA in patients more than controls (28.8 *vs*. 18.8 %; OR= 1.75; 95% CI = 1.09-2.81; p<0.013 for allele), (45.5 vs 30.5 %; OR=1.82; 95% CI =1.01-3.26; p <0.032 for genotype), respectively (Table 3).

Table (3): Hardy-Weinberg and Logistic regression analyses of *NLRP3* gene SNPs inCOVID-19 patients and healthy controls.

SNP	Allele/ genotype	COVID-19		HC		OR	95% CI	*p*-value (*pc*)
		N	%	N	%			
rs35829419	C	141	71.2	156	**81.3**	Reference		
C/A	A	57	28.8	36	18.8	1.75	1.09 – 2.81	**0.013**
	CC	48	48.5	62	**66**	Reference		
	CA	45	45.5	32	30.5	1.82	1.01 – 3.26	**0.032**
	AA	6	6.1	2	3.5	3.88	0.83 – 18.13	0.088
HWE-*p*-value		0.280		0.357				
rs10754558	G	149	75.3	130	67.7	Reference		
G/C	C	49	24.7	62	32.3	0.69	0.44 – 1.07	0.062
	GG	59	56.6	47	45.9	Reference		
	GC	31	37.3	36	43.7	0.69	0.37 – 1.26	0.147
	CC	9	6.1	13	10.4	0.55	0.22 – 1.38	0.152
HWE-*p*-value		0.113		0.163				

SNP (Single nucleotide polymorphism); HC(Healthy controls) , HWE (Hardy-Weinberg equilibrium); CI (Confidence interval) ; OR(Odds ratio); *p*: Two-tailed Fisher's exact probability;(The bold line is refer to significant p-value).

This study finding the NLPR3 rs 35829419 *A* allele and genotype CA are related to CoV-19 susceptibility. The gene of NLRP3 is found in 1q44, with a length of ~30 kbp, involving 8 introns and 9 exons(51). NLRP3 is fellow to the family of NLR and plays an important role in increasing the production of inflammatory cytokine (51) Alteration in the gene of NLRP3 could change its activity. NLRP3 gene with SNPs was related to inflammatory disorders with genetic origin and increased secretion of IL-1β (52). Mutations in the genes of inflammasome causes inflammatory disorders such as increased secretion of IL-1β lead to chronic inflammation, and involved infection with virus (53). The polymorphism of NLRP3 rs35829419 is related to susceptibility to many diseases such as colorectal cancer, leprosy, rheumatoid arthritis, HIV-1 infection, inflammatory bowel disease, abdominal aortic aneurysms, ulcerative atopic dermatitis and colitis. The polymorphism of NLRP3 rs35829419 C>A is found in 3'-UTR of the gene and may affect the expression of NLRP3 mRNA and stability (54). This variant is an increased of function mutation, it causes high secretion of IL-18 and IL-1β. Therefore, NLRP3 rs35829419 affects a range of inflammatory diseases.

The polymorphism of NLRP3 rs10754558 C >G is found in the 3 –UTR. alteration in the 3 -UTR leads to alter in the function of the inflammasome through the affecting on the release of IL-1β, IL-18, and stability of mRNA(55). Hitomi et al. (2009) showed The NLRP3 rs10754558 affected to mRNA stability, and increase gene expression(56). Moreover, a study in Brazilian establish a relationship between protection against HIV-1 and rs10754558 G allele(57).Also Brazilian group displayed the NLRP3 rs10754558 C/C genotype affect on the levels of IL-1β(58,59). Numerous studies displayed the relationship of this polymorphism with many diseases (60,61).

In conclusion the blood biomarkers, hypertension, diabetes, age and gender group were played an essential role in exposure to COVID-19, as well as the polymorphism of NLRP3 is related to the presence of COVID-19 and severity of disease this led to a many studies in the future about the correlation between polymorphism of NLRP3, hematological Parameters and the disease severity.

References

1. Huang, C.; Wang, Y.; Li, X. and Ren, L. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. The Lancet, 395(20): 497-506.
2. Chen, N.; Zhou, M.; Dong, X.; Qu, J. and Gong, F. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. The Lancet, 395(20): 507-513.
3. Guan, W.; Guan, W.; Ni, Z. and Hu, Y. (2020). Clinical Characteristics of Coronavirus Disease 2019 in China, New England Journal of Medicine,382(1) :1708-1720.
4. Sun, X.; Wang, T.; Cai, D.; Hu, Z. and Chen, J. (2020).Cytokine storm intervention in the early stages of COVID19pneumonia,Cytokine&GrowthFActor,53(18):38-42.
5. Ponti, G.; Maccaferri, M.; Ruini, C.; Tomasi, A. and Ozben, T. (2020). Biomarkers associated with COVID-19 disease progression, Critical Reviews in Clinical Laboratory Sciences, (57) 6: 389-399.
6. Angioni, R.; Sánchez-Rodríguez, R.; Munari, F.; Bertoldi, N.; Arcidiacono, D.; Cavinato, S., *et al*. (2020). Age-severity matched cytokine profiling reveals specific signatures in Covid-19 patients. Cell death and Disease, 11(11): 957.
7. Zainab, S.; Hula, Y. and Ali, H. (2021). Estimation of Hematological Parameters

of Disease Severity in Iraqi Patients with COVID-19. Iraqi Journal of Science, 62 (10): 3487-3496

8. Alaa, M.; Ali, H. and Hula, Y. (2022). Potential Role of TLR3and RIG-IGenes Expression in Surviving COVID-19 Patients with Different Severity of Infection . Iraqi Journal of Science, (63)7: 2873-2883.
9. Abdul Hussein, T. and Fadhil, H. (2023). Impact of inflammatory markers, dread diseases and cycle threshold (Ct) Values in COVID-19 progression. Revis Bionatura, 8(1): 33-38.
10. World Health Organization. (2020). Clinical Management of Severe Acute Respiratory Infection When Novel Coronavirus (2019-nCoV) Infection is Suspected: Interim Guidance.
11. Zhang, S.; Liu, Y. and Wang, X.(2020).SARS-CoV-2 binds platelet ACE2 to enhance thrombosis in COVID-19. J Hematol Oncol, 13(01): 120-129.
12. Bikdeli, B.; Madhavan, M. and Jimenez, D.(2020).Global COVID-19 Thrombosis Collaborative Group, Endorsed by the ISTH, NATF, ESVM, and the IUA, Supported by the ESC Working Group on Pulmonary Circulation and Right Ventricular Function. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up: JACC state- of-the-art review. Journal of the American College of Cardiology, 75(23): 2950–2973.
13. Bi, X.; Su, Z. and Yan, H. (2020).Prediction of severe illness due to COVID-19 based on an analysis of initial fibrinogen to albumin ratio and platelet count. Platelets, 31(5): 674–679
14. Seyoum, M.; Enawgaw, B.and Melku, M. (2018) Human blood platelets and viruses defense mechanism and role in the removal of viral pathogens. Thrombosis Journal, 16(1):1-6.
15. Assinger, A. (2014). Platelets and infection–an emerging role of platelets in viral infection. Frontiers in Immunology, 5(1): 649-653.
16. Singh, S.; Sharma, A. and Arora, S. (2014). High producer haplotype (CAG) of-863C/A,-308G/A and-238G/A polymorphisms in the promoter region of TNF-α gene associate with enhanced apoptosis of lymphocytes in HIV-1 subtype C infected individuals from North India. PLoS One, 9(5): 98020.
17. Zheng, M.; Gao, Y.; Wang, G.; Song, G.; Liu, S.; Sun, D., *et al*. (2020).Functional exhaustion of antiviral lymphocytes in COVID-19 patients. Cellular and Molecular Immunology, 17(5): 533-535.
18. Zainab, S.; Hula, Y.; Thaer, A. and Ali, H.(2022) .Severity of coronavirus disease 19: Profile of inflammatory markers and ACE (rs4646994) and ACE2 (rs2285666) gene polymorphisms in Iraqi patients. Meta Gene, 31(6): 101014.
19. Ali, N. (2020). Elevated level of C-reactive protein may be an early marker to predict risk for severity of COVID-19. Journal of Medical Virology, 92(8): 2409–2411.
20. Xie, J.; Covassin, N.; Fan, Z.; Singh, P.; Gao, W.; Li, G., *et al*. (2020) Association between hypoxemia and mortality in patients With COVID-19. Mayo Clinic Proceedings, 95(5): 1138–1147.
21. Jinan, J. and Hula, Y. (2022). Relationship between Caspase-8 and SARS-CoV-2 Variants in a Sample of Iraqi Population. The Egyptian Journal of Hospital Medicine , 89(6): 5392- 5399.
22. Khadija, T. and Hula, Y. (2022). Evaluation Role of CXCL-8 levels and SARS-CoV-2 Variants in Progression of Infection. The Egyptian Journal of Hospital Medicine ,89(7):5743- 5751
23. Lee, H.; Kim, J.; Kim, H.; Shong, M.; Ku, B. and Jo, E. (2013). Upregulated NLRP3 inflamma- some activation in patients with type 2 diabetes. Diabetes, 62(8): 194–204.
24. Dixit, V.(2013).Nlrp3 inflammasome activation in type 2diabetes: is it clinically rele- vant? Diabetes, 62(8):22–4.
25. Wan, Z.; Fan, Y.; Liu, X.; Xue, J.; Han, Z. and Zhu, C. (2019). NLRP3 inflammasome promotes diabetes-induced endothelial inflammation and atherosclerosis. Diabetes Metab Syndr Obes, 12(9): 1931–42.
26. Zheng, Y.; Zhang, D.; Zhang, L.; Fu, M.; Zeng, Y. and Russell, R. (2013). Variants of NLRP3 gene are associated with insulin resistance in Chinese Han population with type-2 diabetes. Gene, 530(4): 151–154.
27. Grant, R. and Dixit, V. (2013). Mechanisms of disease: inflammasome activation and the development of type2 diabetes. Front Immunology, 4(9): 50.
28. Rheinheimer, J.; de Souza, B.; Cardoso, N.; Bauer, A. and Crispim, D. (2017). Current role of the NLRP3 inflammasome

on obesity and insulin resistance: a systematic review. Metabolism,74(4):1-9.
29. Rodrigues, J.; Lima, D.; Leal, V.; Bosco, A.; Sandrim, V. and Pontillo, A. (2018). Gain-of-function SNPs in NLRP3 and IL1B genes confer protection against obesity and T2D: undiscovered role of inflammasome genetics in metabolic homeostasis. Endocrine, 60(6): 368–371.
30. Perricone, C. (2020). COVID-19 as part of the hyper ferritinemic syndromes: the role of iron depletion therapy. Immunologic Research, 68 (7): 213–224.
31. Vargas, M. (2020). Ferritin levels and COVID-19. Revista Panamericana de Salud Pública, 45: 1-21.
32. Kishaba, T.; Tamaki, H.; Shimaoka, Y.; Fukuyama, H. and Yamashiro, S. (2014). Staging of acute exac- erbation in patients with idiopathic pulmonary fibrosis. Lung. 192: 141–9.
33. Chen, N.; Zhou, M. and Dong, X. (2020).Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 395(10223): 507–513.
34. Guan, W.; Ni, Z. and Hu, Y. (2020). Clinical characteristics of coronavirus disease 2019 in China. The New England Journal of Medicine, 382(18): 1708–1720.
35. Nugroho, J.; Wardhana, A. and Maghfirah, I. (2021). Relationship of D-dimer with severity and mortality in SARS-CoV-2 patients: a meta-analysis. International Journal of Laboratory Hematology, 43(1):110–115.
36. StefanopolI, A.; Miulescu, M.; Baroiu, L.; Anghele, A.; Danila, D. and Tiron, Z. (2021). An unusual case of Meckel diverticulitis misdiagnosed as an infected urachal cyst. Medicina-Lithuania,57(5): 495.
37. Xu, Z.; Shi, L. and Wang, Y. (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. The Lancet Respiratory Medicine Home Page, 8(4): 420–422.
38. Baroiu, L.; Dumitru, C. and Iancu, A. (2021). COVID-19 impact on the liver. World Journal of Clinical Cases. 9(16): 3814–3825.
39. Alaa, M.; Hussein, A. and Hula, Y. (2022).Exploring the significant role of IFIH gene polymorphism with severity infection of SARS-CoV-2 Iraqi patient. International Journal of Special Education, 37(3): 56-66.
40. Liu, K.; Chen, Y.; Lin, R. and Han, K. (2020) Clinical features of COVID-19 in elderly patients: a comparison with young and middle-aged patients. International Journal of Information Security, 34(8): 67-77.
41. Chen, Y.; Klein, S.; Garibaldi, B.; Li, H.; Wu, C.; Osevala, N., *et al*. (2020).Aging in COVID-19: Vulnerability, immunity and intervention. Ageing Research Reviews, 65(8): 45-55.
42. Jinan, J.; Hula, Y. and Iman, M. (2023). Impact of SARS-COV-2 Variants on the Infection Severity among Iraqi Patients. Iraqi Journal of Science, 64(7): 4163-4172 .
43. Bajaj, V. Gadi, N.; Spihlman, A.; Wu, S. and Choi, C. (2020).Aging, immunity, and COVID-19: how age influences the host immune response to coronavirus infections. Front. Physiology, 67(9): 23-30.
44. Zhu, X.; Chen, Z.; Shen, W.; Huang, G.; Sedivy, J.; Wang, H., *et al*. (2021). Inflammation, epigenetics, and metabolism converge to cell senescence and ageing: the regulation and intervention. Signal Transduct. Target. Ther, 6: 1–29.
45. Salah, H.; Fadhil, H. and Alhamdani, G. (2020). Multiplex syber green assay for Coronavirus detection using fast real time RT-PCR. Iraqi Journal of Agricultural Sciences, 51(2): 556-564
46. Thaer, A .and Hula, Y. (2023). Analysis of Mutations in Conserved and Susceptible Regions Across the Whole Genome Sequencing Analysis for SARS-CoV-2 in Iraqi Patients. Iraqi Journal of Science, 64(1): 56-64.
47. Schurz, H.; Salie, M.; Tromp, G.; Hoal, E. G.; Kinnear, C. J. and Möller, M. (2019). The X chromosome and sex-specific effects in infectious disease susceptibility. Human genomics, 13(1) :1-12.
48. Maggio, M.; Basaria, S.; Ceda, G. P.; Ble, A.; Ling, S. M.; Bandinelli, S., *et al*. (2005).The relationship between testosterone and molecular markers of inflammation in older men. Journal of Endocrinological Investigation, 28(11): 116-119.
49. Chen, L.; Li, X.; Chen, M.; Feng, Y. and Xiong, C. (2020). The ACE2 expressionin human heart indicates new potential mechanism of heart injury among patients infected with SARS-CoV-2.

Cardiovascular research, 116(6): 1097-1100.
50. Breithaupt-Faloppa, A. C.; Correia, C. D. J.; Prado, C. M.; Stilhano, R. S.; Ureshino, R. P. and Moreira, L. F. P. (2020). 17β-Estradiol, a potential ally to alleviate SARS-CoV-2 Infection. Clinics, 75(9): 56-66.
51. Zaki, M.H.; Lamkanfi, M. and Kanneganti, TD. (2011). The Nlrp3 inflammasome: contributions to intestinal homeostasis. Trends in Immunology, 32(4):171-179.
52. Van Reeth, K. (2000). Cytokines in the pathogenesis of influenza. In: Veterinary Microbiology. Elsevier, 67(12): 109–16.
53. Lasigliè, D.; Traggiai, E.;Federici, S.; Alessio, M.; Buoncompagni, A. and Accogli, A. (2011).Role of IL-1 beta in the development of human TH17 cells: Lesson from NLPR3 mutated patients. PLoS One, 6(5): 45-51.
54. Theodoropoulou, K.; Wittkowski, H.; Busso, N.; Von Scheven-Gete, A.; Moix, I. and Vanoni, F. (2020). Increased prevalence of NLRP3 Q703K variant among patients with autoinflammatory diseases: an international multicentric study. Frontiers in Immunology, 11(877): 123-133.
55. Hitomi, Y.; Ebisawa, M.; Tomikawa, M.; Imai, T.; Komata, T. and Hirota, T. (2009). Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. Journal of Allergy and Clinical Immunology, 124(4): 144-145.
56. Pontillo, A.; Oshiro, T.M.; Girardelli, M.; Kamada, A.J.; Crovella, S. and Duarte, AJS. (2012). Polymorphisms in Inflammasome ' Genes and Susceptibility to HIV-1 Infection. Basic to Translational Science, 59(2): 121–5.
57. Toro, D.M.; Ramasawmy, R.; Silva, Neto P.V.; Pereira, G.L.; Sarmento, P.S. and Dray, H.L.S.N. (2021). Inflammasome genes polymorphisms may influence the development of hepatitis C in the Amazonas, Brazil. Ciccacci C, editor. PLoS One, 16(6).
58. Ravimohan, S.; Nfanyana, K.; Tamuhla, N.; Tiemessen, C.T.; Weissman, D. and Bisson, GP. (2018).Common Variation in NLRP3 Is Associated With Early Death and Elevated Inflammasome Biomarkers Among Advanced HIV/TB Co-infected Patients in Botswana. Open Forum Infectious Diseases, 20(12): 1–8.
59. Alaa, M.; Ali, H. and Hula, Y. (2022). Exploring the significant role of IFIH gene polymorphism with severity infection of SARS-CoV-2 Iraqi patient. International journal of special aducation, 37(3): 16-26
60. Amer, M.; Abdul Hussein, M. and Ahmed, M. (2022). Alteration of M and N Genes of Corona Virus 2 (SARS-CoV-2) Iraqi Journal of Biotechnology. 21(2): 39-45.
61. Mahdi, B. S. and Abd Almohsen, W. S. (2023). Role of IFIH1 Gene Polymorphism rs3747517 and IL17A with its Receptor in Detection and Therapeutic Approach for Type 1 Diabetes Patients. Iraqi Journal of Biotechnology, 22(1): 134-139.