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Co-Analysis of TLR7 and CCL2 as Predictive Biomarkers forH1N1 Influenza Infection Severity

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ABSTRACT

Background and Aim: Influenza A virus (H1N1) infection is still a high risk disease that involves inflammatory reactions. Chemokine (C-C motif) ligand 2 (CCL2) and Toll-like receptor 7 (TLR7) are important elements of immune system that regulate antiviral and inflammatory responses. This study aimed to assess the combined diagnostic and prognostic importance of TLR7 and CCL2, and investigate their relationship with clinical parameters and disease severity of H1N1.

Materials and Methods: In this case-control study, 60 influenza patients (divided into influenza-like illness [ILI] and severe acute respiratory infection [SARI] groups) were included along with 30 healthy people that served as control group. Blood samples were used to evaluate the expression of the TLR7 gene using RT-qPCR. The levels of CCL2 were measured using ELISA. ROC curve analysis, multivariate logistic regression, spearman correlation, and nonparametric tests were used to analyze the clinical data.

Results: H1N1 patients showed considerably higher levels of TLR7 expression than controls (median 8.6 vs 1.6; *P*<0.001). WBC and CRP levels were positively linked with elevated levels of TLR7. On the other hand, CCL2 was significantly lower in SARI group compared to ILI patients (37.5 vs 87.4 pg/mL; *P*<0.001). Predictive AUCs of 0.75 for TLR7 and 0.79 for CCL2 were found using ROC analysis; the combined model increased AUC to 0.85 (95% CI, 0.73–0.91). The presence of disease severity and diabetes was substantially correlated with both biomarkers.

Conclusion: In severe H1N1 infection, both CCL2 downregulation and TLR7 overexpression indicate increased immunological activity and inflammatory reactions dysregulation. The prognostic accuracy for identifying patients at risk of severe influenza is improved by their combined profiling.

Keywords: Biomarker, CCL2, Cytokine, H1N1, ILI, Influenza, Innate Immunity, TLR7, SARI

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1. Introduction

Severe acute respiratory infection (SARI) can develop from influenza A (H1N1) infection, which is still a leading source of respiratory morbidity globally, especially in susceptible groups. The clinical outcome of infection depends not only on viral load but also on the host's immunogenetic response. Chemokine (C-C motif) ligand 2 (CCL2) and Toll-like receptor 7 (TLR7) have become important regulators of immune regulation among the major molecular determinants **(1, 2)**.

TLR7 is found in endosomal compartments of dendritic cells and macrophages. Type I interferon and proinflammatory cytokines are induced when TLR7 detects single-stranded viral RNA and initiates MyD88-dependent signaling pathways. However, the "cytokine storm" feature of severe viruses may be exacerbated by increased activation of TLR7 **(1-3)**.

The powerful chemokine CCL2 (monocyte chemoattractant protein-1), which is encoded on chromosome 17q11.2, facilitates the recruitment of monocytes and T-cells **(4, 5)**.

In viral pneumonia, abnormal leukocyte trafficking and tissue damage have been associated with elevated or dysregulated levels of CCL2 expression **(6-8)**.

According to a recent study, TLR7 and CCL2 interact through common inflammatory and antiviral pathways **(9, 10)**. TLR7 stimulation increases cytokine cascades, but excessive CCL2 synthesis or depletion might upset immunological homeostasis and increase the risk of immunopathology or exhaustion **(11-13)**.

This integrated study evaluates the combined diagnostic and prognostic relevance of previously created single-biomarker datasets on CCL2 serum levels and TLR7 gene expression from the same Iraqi H1N1 patient group. Our hypothesis was that immunological dysregulation linked to influenza severity is concurrently reflected by the elevated TLR7 expression and downregulated CCL2, and that their combined profiling enhances the illness progression prediction.

2. Materials and Methods

2.1 Study Design and Participants

For this case-control study, sampling was conducted between November and December 2023 across multiple public hospitals in seventeen Iraqi provinces. The study included 90 participants, 60 of them were patients with laboratory-confirmed influenza A (H1N1) infection and 30 were control participants. To capture the acute phase responses, in ten days of symptom onset nasal and throat swabs and blood samples were taken. Patients were clinically classified according to the World Health Organization (WHO) criteria into two groups: influenza-like illness (ILI) and severe acute respiratory infection (SARI). ILI patients showed mild respiratory symptoms with fever and cough, whereas SARI patients exhibited dyspnea requiring supplemental oxygen therapy or hospitalization in addition to fever and cough.

Control subjects were hospital employees or blood donors who volunteered, and they had no clinical signs of respiratory infection, chronic inflammatory or autoimmune disease. Furthermore, demographic and clinical characteristics were registered in all participants, such as age, sex, diabetes mellitus, and hypertension. This study was approved by the Ethics Committee of the College of Science, University of Baghdad (approval No. CSE/0922/0084). All the participants signed a written informed consent form prior to the study.

2.2 Sample Collection and Handling

Nasal and throat swabs were collected using sterile, labeled, screw-capped tubes containing viral transport medium (VTM) to maintain viral integrity during transportation. Samples were promptly transferred to the central laboratory under cold-chain conditions and stored at −70°C until processing.

Five milliliter of venous blood samples were obtained from each participant and equally divided into ethylene diamine tetraacetic acid (EDTA) tubes and plain tubes. The plain tubes were left to clot for 30 min at room temperature (20-25°C) and then centrifuged for 15 min at 4ºC to separate serum for immunological and biochemical assays, while whole blood samples (EDTA-treated) were reserved for hematological analysis and RNA extraction.

2.3 Molecular Diagnosis of H1N1 Infection

Infection of H1N1 was confirmed molecularly using RT/Platinum™ Taq mix with fast 7500 Real-Time PCR system (Applied Biosystems). Positive samples for influenza A were subjected to subtyping with CDC Influenza Virus Real-Time RT-PCR Subtyping Influenza A (H3/H1pdm09) Panel kit (Atlanta, USA). Each 20 µL reaction contained: 0.5 µL of each primer and probe (40 µM primers and 10 µM probe concentrations), 0.5 µL Rox dye (1:10 dilution), 5.5 µL PCR grade water, and 5 µL of RNA template extracted from the swab sample. The thermal cycling conditions included reverse transcription at 50°C for 5 min, followed by initial denaturation at 95°C for 2 min, and 45 amplification

cycles consisting of denaturation at 95°C for 3 sec and combined annealing/extension at 55°C for 30 sec.

2.4 Blood Biomarkers Assessment

Biomarkers in serum samples were evaluated to measure inflammation and participants immune response. C reactive protein (CRP) was quantified using an electro-chemiluminescence immunoassay system (ECLIA), and ferritin was measured using the Enzyme-Linked Fluorescent Assay (ELFA) on a miniVIDAS analyzer. Concentrations of CCL2 were quantified using an enzyme-linked immunosorbent assay (ELISA) kit with minimum detectable limit of 5 pg/mL. An automated hematology analyzer was used to analyze white blood cell (WBC) counts. All biochemical and immunological assays were performed according to the manufacturers' instructions.

2.5 RNA Extraction and Quantitative Gene Expression Analysis

Following the manufacturers' protocol, RNA was extracted from EDTA-treated whole blood samples using Viral RNA Mini Kit (Qiagen, Germany). Afterward, the extracted RNA underwent complementary DNA (cDNA) synthesis by EasyScript One-Step gDNA Eraser and cDNA Synthesis SuperMix (TransGen, China). Reaction tubes contained 3-5 µL RNA, 1 µL Anchored Oligo (dT) Primer (0.5 µg/µL) with 1 µL Random Primer (0.1 µg/µL) and incubated for five min at 65°C and for 10 min at 4°C in a Thermocycler, following the incubation, 10 µL of EX reaction mix, 1 µL of gDNA remover, and 1 µL of EasyScript® RT/RI enzyme mix were added. The final reaction volume was adjusted to 20 µL by RNase free water. The thermocycling program was consisted of: 25°C for 10 min, 42°C for 30 min, and 85°C for 5 sec. Utilizing the TransStart® Green qPCR SuperMix Kit (TransGen, China), quantitative polymerase chain reaction (qPCR) was performed. The used primers were as follow:

TLR7 (forward 5'-AATGTCACAGCCGTCCCTAC-3' **(14)** and reverse -5'- GCGCATCAAAAGCATTTACA-3' **(14)**) and Beta-actin as housekeeping gene (forward 5'-GAAGGATTCCTATGTGGGCG-3' **(15)** and reverse -5'-TGGTGGTAAAGCTGTAC-3' **(15)**).

The reaction consisted of 3 µL cDNA, 1 µL of each primer, 10 µL SYBR Green master mix, and 5 µL nuclease free water. The initial denaturation of amplification was at 94°C for 30 sec, next was 40 cycles of 94°C for 5 sec, 58°C for 15 sec, and 72°C for 20 sec. Finally, to confirm amplicon specificity, the dissociation curve was generated at 55-95ºC for one cycle. Relative expression of TLR7 was calculated using the $2^{\Delta\Delta Ct}$ method, normalized to β-actin expression levels.

2.6 Statistical Analysis

Kolmogorov-Smirnov and Shapiro-Wilk test was used to test continuous variables for normality before hypothesis testing. Continuous variables that followed a normal distribution were expressed as mean ± standard deviation (SD) and compared using the Student's *t*-test. Non-normally distributed (skewed) variables were expressed as the median with interquartile range (IQR) and compared using Mann–Whitney *U* test for two groups or the Kruskal–Wallis test for multiple groups.

Data were summarized as frequencies and percentages then analyzed by the Pearson Chi-square test or the two-tailed Fisher's exact test as appropriate for the categorical variables. Meanwhile, to evaluate the relationships between quantitative variables and to assess the associations between TLR7 expression, CCL2 serum levels, and inflammatory markers such as CRP, ferritin, and WBC counts the Spearman's rank-order correlation coefficient (ρ) was used. Binary logistic regression analysis was applied to identify independent predictors of disease severity (SARI vs. ILI) for predictive modeling. With control group as the reference category, patients were categorized into "low" and "high" production groups for each biomarker based on the median value (≤median vs. >median) and results were reported as odds ratios (ORs) with 95% confidence intervals (CIs).

The diagnostic performance of the test was assessed using receiver operating characteristic (ROC) curve analysis. For TLR7, CCL2, and the combined model the area under the curve (AUC), 95% CI, optimal cutoff point, sensitivity, and specificity were calculated. The optimal threshold was determined using Youden's index (sensitivity + specificity − 1) to maximize discriminatory accuracy.

All statistical tests were two-tailed and as statistically significant a probability (*P*) value ≤0.05 was taken. GraphPad Prism version 10.0.0 (San Diego, California, USA) and IBM SPSS Statistics version 27.0 (Armonk, NY: IBM Corp.) were used to perform all statistical analyses. To determine the power of sample size the G*Power software (version 3.1.9.7) was used.

3. Results

3.1 Expression levels of TLR7 gene using RT-qPCR

The median of TLR7 gene expression increased in H1N1 patients (8.6) compared to controls (1.6) (Figure 1).

The median levels of TLR7 fold stratified according to ILI, SARI patients, and controls are listed in Table 1. There was an increase in high median of SARI vs. low median (*P*=0.008).

Figure 2 illustrated the ROC curve analysis of TLR7 Fold in H1N1 patients compared to controls. TLR7 Fold less than 3.85 can predict patients at lower risk (Area under the curve=0.751; 95% CI=0.619-0.881; *P*<0.001; Sensitivity=76.1%; Specificity=75.2%).

The elevation in the level of TLR7 was more obvious in SARI group compared to ILI, particularly in the age group 46-67, males more than females, and diabetes (Table 2).

As depicted in Figure 3, there was a positive correlation between TLR7 and WBC as well as CRP (*P*<0.001).

3.2 CCL2 protein levels in the sera of patients and controls

There was no significant difference in the level of CCL2 in the patients (80.8) (range 47.3 - 98.6) compared to the control group (86.8) (range 69.9-108.9); *P*=0.195 (Figure 4).

No significant difference was observed in the level of CCL2 between patients and controls in relation to age group and hypertension, except for sex factor that increased in males [90.7 (87.9–115.5)] in control group compared to females [81.1 (60.6–91.8)] (*P*=0.023). Also, there was significant difference in the level of CCL2 in patient with diabetes [positive 95.5 (71.6-123.8) vs. negative 73.6 (44.5-95.3); *P*=0.034] and control [positive 110.5 (87.8–117.8) vs. negative 83.5 (64.7–91.8); *P*=0.041] (Table 3).

3.3 CCL2 levels in the sera of SARI and ILI groups

There was increase in the level of CCL2 in ILI patients [87.4 (range 58.1 - 103)] compared to SARI patients [37.5 (26.8-56.7); *P*=0.00.1], while there was decrease in the level of CCL2 in SARI patients [(37.5 (range 26.8-56.7)] compared to control [86.8 (range 69.9-108.9); *P*=0.00.1]. Also, there was no significant difference between ILI patients [87.4 (range 58.1 - 103) and controls [86.8 (range 69.9-108.9); *P*=0.731] (Figure 5).

ROC curve analysis of CCL2 level less than 76.33 pg/mL can distinguish patients with influenza type A progressive (Area under the curve=0.794; 95% CI=0.703 – 0.886; *P*< 0.0001; Sensitivity=70.3%; Specificity=67.6%), as shown in Figure 6.

A positive correlation was shown between the levels of CCL2 and CRP, as well as ferritin level. Data are shown in Figure 7.

There were no significant differences between ILI and SARI patients in case of age group, except for the sex factor in ILI patients that was significant between male (17) and female (33) (*P*=0.024), while regarding diabetes and hypertension, there were significant differences with ILI. Also, significant differences were highlighted between ILI and SARI patients in the level of CRP [ILI, 13.7 (13-14.2); *P*<0.001], WBC in ILI 11 (10.7-11.8), in SARI 42 (40.1-49.3), SARI 12.5 (10-14.2); *P*<0.001, ferritin in ILI 387 (360-444), in SARI 780.5 (671.967.5); (*P*<0.001) (Table 4).

Table 1. Median TLR7 expression stratified by disease group and corresponding odds ratios.

Group	High (>median)	Low (≤median)	OR	95% CI	*P*-value
Control	7	23	Reference	—	—
ILI	12	33	1.19	0.41–3.44	0.793
SARI	10	5	6.57	1.74–24.83	0.008

OR: odds ratio; CI: confidence interval; *P*-value calculated by Fisher's exact test.

Table 2. Median levels of TLR7 fold stratified according to characteristics of H1N1 patients and severity of illness.

Characteristic	ILI (n=45)	SARI (n=15)	P-value
Age 25–35	0.2 (0.12–1.1)	0.22 (0.32–42)	0.770
Age 36–45	0.12 (0.03–3.6)	0.10 (0.01–0.2)	0.109
Age 46–55	1.2 (0.09–3.7)	7.3 (1.1–55.3)	0.105
Age 56–67	0.77 (0.15–1.7)	6.2 (0.01–6.3)	0.926
Sex (Male)	1.0 (0.06–3.7)	3.66 (0.01–36.8)	0.928
Sex (Female)	0.31 (0.03–3.25)	1.1 (0.01–6.3)	0.646
Diabetes (Yes)	0.1 (0.01–6.3)	0.77 (0.17–2.4)	0.305
Diabetes (No)	0.2 (0.06–3.6)	3.7 (0.12–31.3)	0.408

SARI: severe acute respiratory infection, ILI: influenza-like illness; Age values are given as median with IQR (continuous variables); *P*: probability of Kruskal-Wallis test and Mann-Whitney *U* test.

Table 3. CCL2 Median levels stratified according to characteristics of patients with influenza virus and controls.

Characteristic		Control (n=30)	Patient (n=60)	*P*-value
Age group (years)	25–35	75.5 (61.9–88.5)	70.1 (43.6–106.2)	0.167
	36–45	88.3 (78.8–101.6)	81.9 (44.3–99.1)	0.950
	46–55	109.5 (88.2–114.3)	75.4 (51.4–95.2)	—
	56–67	—	79.7 (55.6–106.1)	—
Sex	Male	90.7 (87.9–115.5)	66.4 (41.2–93.9)	0.023
	Female	81.1 (60.6–91.8)	86.2 (47.8–110.5)	0.118
Hypertension	Positive	91.8 (83.7–110.5)	57.7 (41.1–94.1)	0.134
	Negative	81.1 (64.7–90.7)	86.0 (53.5–101.6)	0.172
Diabetes mellitus	Positive	110.5 (87.8–117.8)	95.5 (71.6–123.8)	0.034
	Negative	83.5 (64.7–91.8)	73.6 (44.5–95.3)	0.041

IQR: Interquartile range; *P*: Mann-Whitney *U* test and Kruskal-Wallis test probability.

Table 4. Baseline characteristics of patients with influenza virus stratified by infection severity.

Variable	ILI (n=50)	SARI (n=10)	*P*-value
Age, years	44 (40–56)	56 (47–64)	0.074
Sex (Male/Female)	17/33	5/5	0.599
Diabetes mellitus, n (%)	9 (18)	3 (30)	0.278
Hypertension, n (%)	10 (20)	5 (50)	0.221
CRP (mg/L)	13.7 (13–14.2)	42.5 (40.1–49.3)	<0.001
WBC (×10^9/L)	11.0 (10.7–11.8)	12.5 (10–14.2)	<0.001
Ferritin (ng/mL)	387 (360–444)	780.5 (671.9–967.5)	0.029

SARI: severe acute respiratory infection, ILI: influenza-like illness; Age values are given as median with IQR (continuous variables) or number and percentage (categorical variables); *P*: probability of Mann-Whitney *U* test (to compare continuous variables), two-tailed Fisher's exact test, or Pearson Chi-square test (to compare categorical variables).

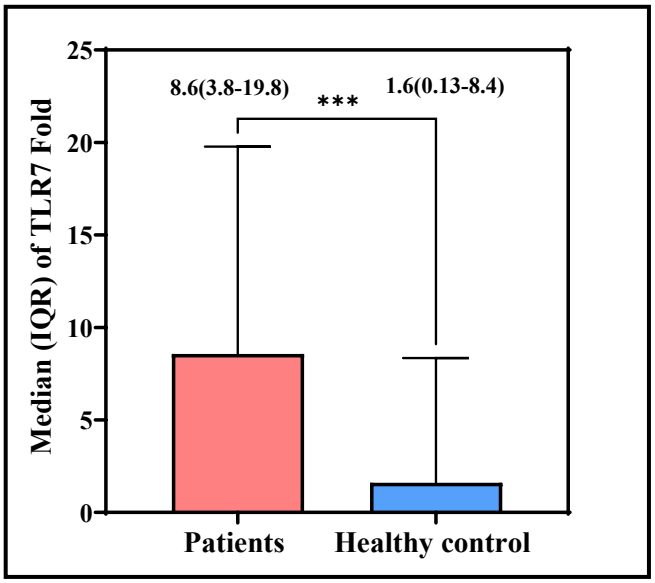

Figure 1. TLR7Gene expression in H1N1 patients and healthy controls. Bars represent the median and IQR. (***: *P*<0.001) **(Prepared by Authors, 2025)**.

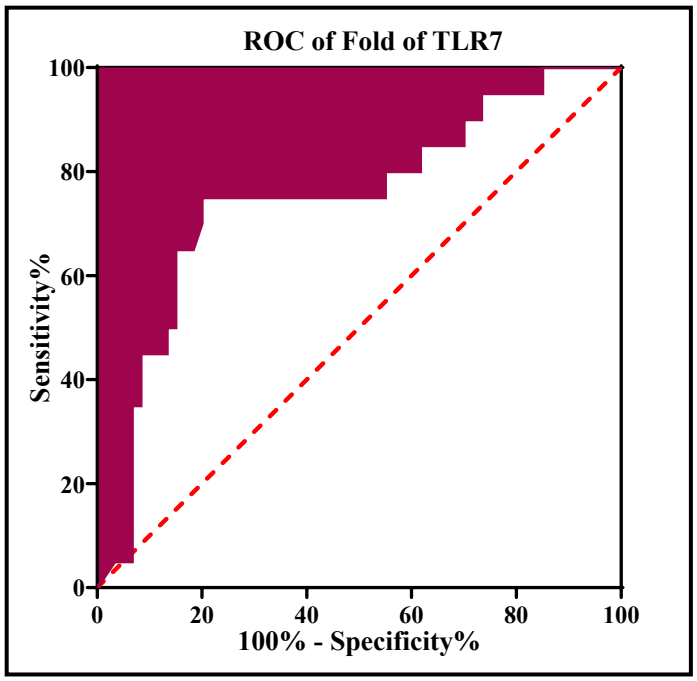

Figure 2. ROC curve analysis of TLR7 Fold in H1N1 patients vs. controls. TLR7 Fold less than 3.85 can predict patients at lower risk **(Prepared by Authors, 2025)**.

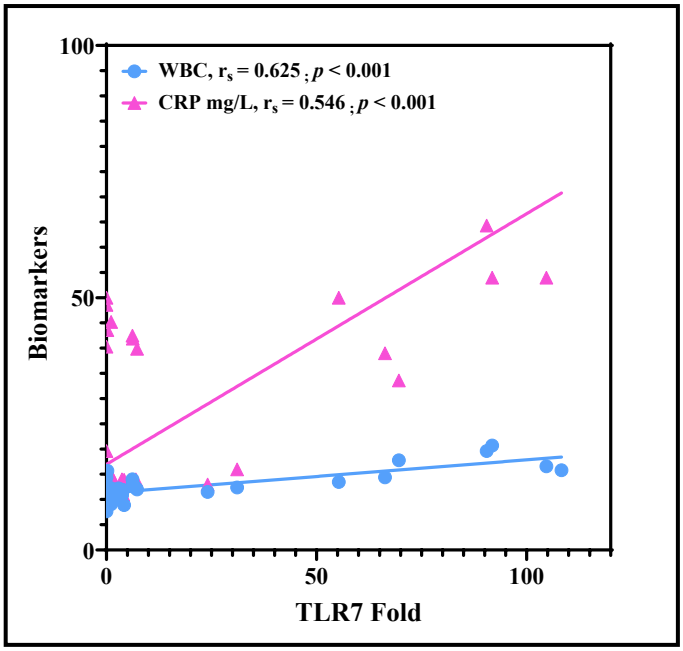

Figure 3. Scatter plot Spearman rank-order correlation coefficient (rs). The image shows the relationship of TLR7 fold analysis with CRP and WBC among influenza patients **(Prepared by Authors, 2025)**.

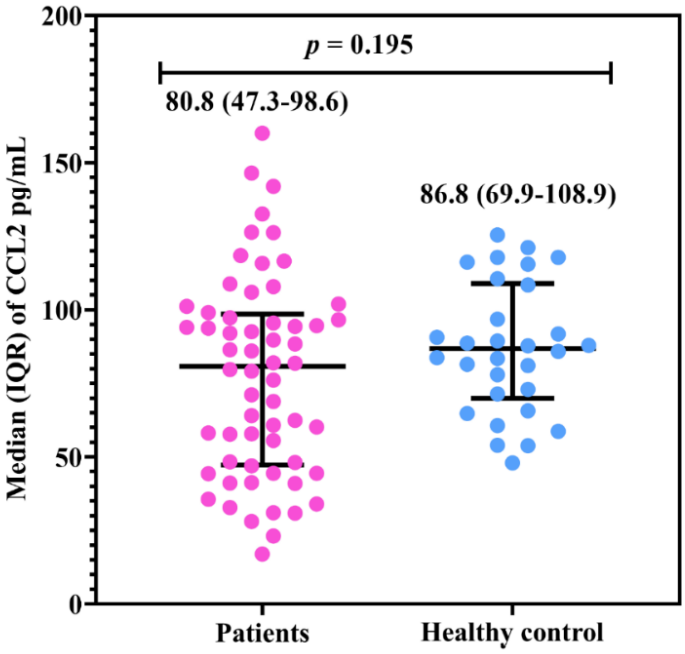

Figure 4. Scatter dot plot of CCL2 levels in patients with influenza virus and controls. The median and IQR are indicated by horizontal and vertical lines, respectively. *P*: probability of Mann-Whitney *U* test between continuous variables **(Prepared by Authors, 2025)**.

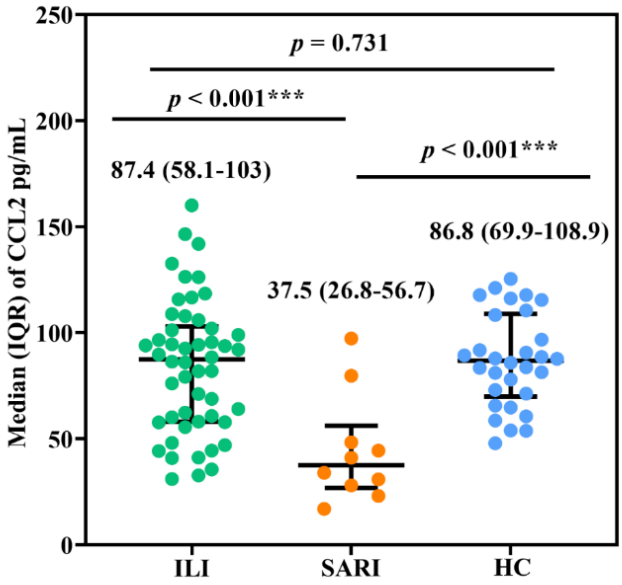

Figure 5. Scatter dot plot of CCL2 levels in SARI and ILI patients compared to controls (HC). *P*: the probability of Mann-Whitney *U* test between continuous variables **(Prepared by Authors, 2025)**.

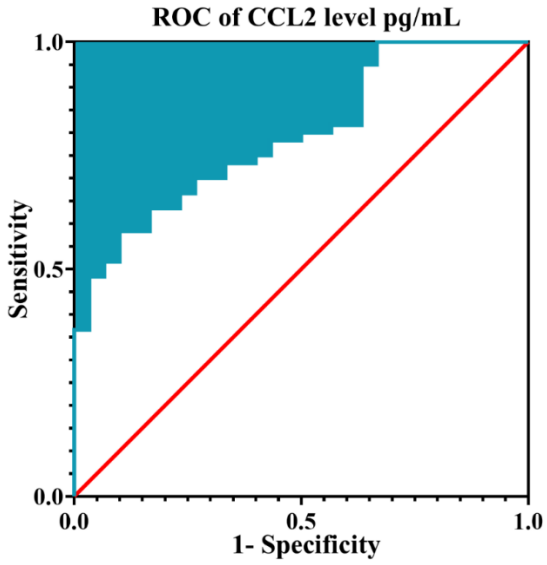

Figure 6. ROC curve analysis of CCL2 in patients with influenza type A vs. controls. A CCL2 level of less than 76.33 pg/mL can distinguish patients with influenza type A progressive **(Prepared by Authors, 2025)**.

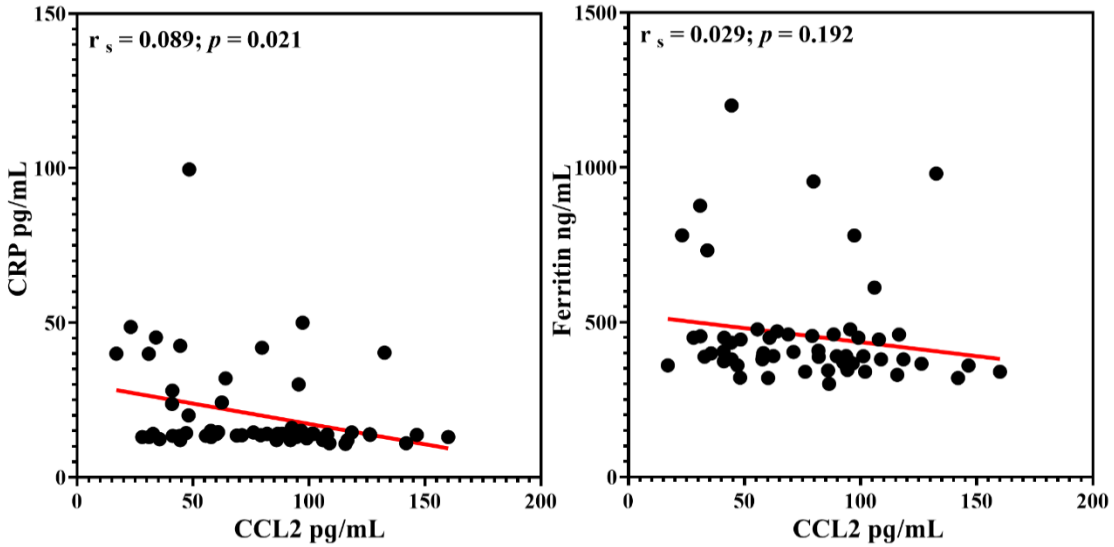

Figure 7. Scatter plot Spearman rank-order correlation coefficient (rs). The image shows the relationship of CCL2 level with CRP and ferritin levels in patients with influenza virus **(Prepared by Authors, 2025)**.

4. Discussion

The present study demonstrates that patients with influenza A (H1N1) exhibited significant upregulation of TLR7 gene expression, while CCL2 levels showed a biphasic pattern, remaining relatively stable in mild cases (ILI) but markedly decreased in severe cases (SARI). An increase in TLR7 gene expression in H1N1 patients was observed in comparison with controls in this study.

TLR7, an endosome-resident molecule, is a critical pattern recognition receptor for antiviral immunity. It is pro-activated by interaction with virus single stranded RNA, like influenza A. This activation is important in innate and adaptive immunity, being the responsibles for the initiation of signaling pathways which mediate an augmentation of proinflammatory cytokines release and dendritic cell maturation.

Improved antiviral response is achieved while TLR7 is stimulated. For instance, TLR7 agonists administration like imiquimod increased type I interferons (IFNs) remarkably. Intranasal administration of imiquimod in murine models showed a rise in levels of type I interferons in lung tissues for five-fold within three days that correlated with reduced replication of influenza infection. Such

results suggest TLR7 as potential target for the viral infection treatment **(4, 16)**.

Besides TLR7 direct antiviral effects, it also enhances wider immune response through increasing inflammatory cytokines production leading to recruitment and activation of immune cells, which in return strengthen the antiviral defense. Furthermore, influenza-infected mice lacking functional TLR7 showed signs of impaired inflammatory response and diminished adaptive immunity, most likely due to insufficient maturation of dendritic cells as studies revealed **(17, 18)**.

Results listed in Table 1 displayed an increase in TLR7 levels in SARI patients compared to ILI patients. In individuals with H1N1, the variations in TLR7 expression levels have the ability to impact the clinical outcomes of SARI significantly. An excessive inflammatory response is a consequence of overactive TLR7 signaling pathway commonly referred to as cytokine storm; therefore, patients with elevated or altered TLR7 expression levels could manifest more severe SARI symptoms for intensified inflammatory response **(19, 20)**. This correlation between severity or SARI and expression levels of TLR7 imply that monitoring these levels might be a beneficial biomarker for predicting H1N1 clinical outcome. Additionally, and as shown in Figure 2, TLR7 predictive value area under the curve conducted by ROC curve analysis is a valuable technique for assessing a statistical model precision and evaluating the efficacy of diagnostic procedures. Data revealed that TLR7 levels during H1N1 infection was an auspicious biomarker for the disease progression.

According to Table 2 and in comparison, with ILI, a rise in the levels of TLR7 in SARI patients appeared in particular between age group, sex (males>females), and diabetes indicating an active immune response in patients with SARI. Older adults, especially over sixty ages, have compromised immune reaction due to immnunosenescence making them more vulnerable to complications of severe influenza compared to younger individuals as research showed.

Taking into consideration, the variation in immune response between sexes are essential for deciding therapy strategies. These differences are due to hormonal influence in general and estrogens specifically, that modulate immune response as studies indicated **(21)**.

People with diabetes have an altered immune response notably regarding TLR7 signaling that leads to a modified immune response and cause complications in effective management of influenza and other viral infections. This may attribute to differences in expression levels of TLR7 **(22)**.

Results from the current study as depicted in Figure 3 indicated a positive correlation between TLR7 and WBC and CRP. The WBC counts and CRP levels increase during inflammations and infections as part of the H1N1 immune response **(23)**. Due to migration to the infection site the WBC count rises, meanwhile, in response to inflammatory signals the liver produces CRP that leads to recording elevated levels **(24)**.

A dual biomarker profile could be established from this interplay of these biomarkers that is useful for assessing the inflammation severity. Understanding this interaction may enhance the accuracy of diagnosis and support clinical strategies through shedding light on inflammatory processes underlying metabolic disorders.

In the present study, as shown in Figure 4, there were no important differences in the levels of CCL2 between control and H1N1 patients. CCL2 plays an important role in inflammatory response in respiratory illnesses like influenza. Several studies demonstrated a rise in the level of CCL2 in severe cases. CCL2 is essential for controlling inflammation during the acute stage of H1N1 infection because it makes easier for different immune cells to enter the infected tissues **(18)**. Elevated CCL2 levels in H1N1 infections promote the immune cells migration into the lungs, which is essential for pathogen clearance. Nevertheless, if left unchecked it can be potentially harmful leading to tissue damage. The receptor of CCL2 is CCR2 that recruits various cells, for instance, dendritic cells, monocytes, fibroblasts, basophils, and so forth. The fibroblasts and monocytes recruitment may lead to progression of fibrosis through several mechanisms like collagen production, TGF-β upregulation, and αSMA expression **(25)**.

Vangeti and Falck-Jones **(26)** documented that increased inflammation and severe clinical outcomes correlate with higher concentrations of CCL2 in blood. Heightened levels of CCL2 together with proinflammatory cytokines such as IL-6 and other related markers showed hyperactivation in H1N1 infection cases where patients developed severe pneumonia **(27)**. Moreover, concerning respiratory infections, this observation emphasises a possible dual role for CCL2 as an indicator of active infection as well as a measure of the disease severity.

Furthermore, valuable insights could be provided for the patients' care and treatment strategies by tracking changes in CCL2 levels throughout the illness. This evaluation may confer an advantage in differentiating between mild and severe cases by providing support for the clinical decisions regarding interventions and hospitalisation for H1N1 patients who are at increased risk of serious complications **(28)**. Sample size and the participants' demographic

characteristics are among various factors influenced the findings of this research. The validity of this statistical analysis can be weakened by a small sample size, which identifies significant differences in CCL2 levels challenging among different patient groups. Regarding sample collection, timing of the collection is important relative to the onset and progression of the disease.

In the present study, as demonstrated in Figure 5, the examination of CCL2 levels among individuals with SARI, ILI, and non-affected participants revealed clear trends that point to the potential of CCL2 as a biomarker in respiratory infections. Compared to SARI patients, ILI patients had noticeably greater CCL2 concentrations. This demonstrates an intensified immune response aiming to boost recruitment of monocytes to the infection site. Such increase may reflect early stage of the disease, where massive virus replication is controlled by a vigorous immune response. In contrast, CCL2 levels in SARI subjects were dysregulated and could not be closer to the degree of illness and may be attributed to variations in immune modulation or prior physical conditions. In addition, pathogen burdens or immunological responses could be reflected by the differences in CCL2 levels between ILI and SARI. Briefly, these results emphasize the role of CCL2 as a disease severity marker and potential therapeutic target for treating respiratory infections elicited by influenza.

As depicted in Figure 6, ROC analysis is a valuable technique for evaluating the efficacy of diagnostic procedures and assessing the precision of a statistical model (e.g., logistic regression). The predictive value of CCL2 area under the curve was demonstrated by ROC curve analysis. Results showed that the amount of CCL2 during influenza type A infection was a promising biomarker for disease progression.

There were significant differences in CCL2 levels between males and females (Table 3) likely due to hormonal influences, particularly estrogens, which modulate immune responses. Studies indicated that females generally have higher levels of CCL2 than males. Macrophages produce more inflammatory cytokines in females, among them CCL2 exacerbates inflammation in chronic health issues **(29)**.

Compared to controls, data in Table 3 indicated an increase in the level of CCL2 in patients with diabetes. By mediating inflammation and insulin resistance, CCL2 plays a significant role in the pathophysiology of diabetes. In obesity cases, the adipose tissue inflammatory condition leads to an increase in production of CCL2, attracting inflammatory cells to the area. This influx aggravates local inflammation, which is closely linked to insulin resistance and eventually contributes to type 2 diabetes development. Additionally, insulin resistance has been linked to genetic variants like single-nucleotide polymorphisms (SNPs) in the CCL2 genes in a few populations. Increased CCL2 levels are repeatedly observed in diabetes patients and correlate with markers that indicate metabolic dysfunction. Addressing the underlying inflammatory processes that affect insulin sensitivity suggests that targeting CCL2 could be a promising therapeutic strategy for managing diabetes **(30)**. Especially regarding CCL2 levels, ferritin is recognized as an important biomarker for inflammation and a positive correlation found between CCL2 and ferritin, along with CRP. Higher levels of ferritin regularly imply iron overload and have been linked to various inflammatory conditions **(30)**.

Highlighting the link between systemic inflammation and metabolic imbalance, ferritin is an acute phase reactant and its levels can increase significantly during episodes of inflammation. Elevated ferritin levels are often associated with increased expression of CCL2 and regardless of other known risk factors research concludes that higher serum ferritin levels serves as a predictor for the onset of type 2 diabetes as well as reflecting ongoing inflammatory activity. The possible significance of monitoring ferritin in healthcare facilities to assess inflammatory status and its implications for metabolic disorders is emphasized by this relationship **(31)**.

Often reflecting the underlying pathological mechanisms found in various diseases such as diabetes and hypertension makes CCL2 and CRP as significant indicators of inflammation. CCL2 primarily facilitates the monocytes recruitment to the areas of inflammation; meanwhile, the liver produces CRP in response to inflammatory signals. A dual biomarker profile could be established by this interaction, which is beneficial for assessing the severity of inflammation and enlightening the inflammatory processes underlying metabolic disorders. Understanding the interplay between these biomarkers can enhance diagnostic accuracy and shape the therapeutic approaches **(32)**.

Patients with SARI had higher levels of WBC, CRP, and ferritin than patients with ILI, as seen in Table 4. The increase in these factors due to inflammation refers to an active immune response from SARI patients compared to ILI patients. When compared to ILI patients, SARI patients have an active immune response due to the rise in these variables brought on by inflammation. When used as integrated biomarkers for prognosis and illness monitoring, their combined evaluation (TLR7 and CCL2) produced a higher prediction accuracy for disease severity than either marker alone, supporting their potential use.

Limitation

There are contributing factors affecting the latest research, specifically such as participants' demographic characteristics and sample size. The validity of analysis affects a small sample weakens the statistical constitutes from small sample. In addition, timing is a crucial element to biomarkers detection in sample collection as it correlates to the disease's onset and progression. Furthermore, as observed in various groups of patients, ranges to environmental factors and viral strains may act in varying immune responses.

5. Conclusion

The TLR7 expression levels can serve as a biological marker to foresee a severity of disease in H1N1 patients. By understanding the intricate association between TLR7 expression and blood markers provides a tool for potential therapeutic interventions and diagnostic approaches. Moreover, it assists to enhance the management and prevention of patients who are affected by severe type of influenza infection. Furthermore, in the latest study, CCL2 demonstrated an essential role in immunity response to ILI. Another result showed a positive relationship between Diabetes and CCL2. A positive correlation was also found between CCL2, CRP, WBC, and ferritin. The findings suggested that CCL2 may serve both as factor influencing and biomarker in severe influenza infections. The findings recommend TLR7 and CCL2 may serve as a complementary biomarkers for predicting H1N1 infection progression and severity.

6. Declarations

6.1 Acknowledgment

Not applicable.

6.2 Ethical Considerations

Approved by the University of Baghdad, College of Science, Ethics Committee (Approval No. CSE/0922/0084).

6.3 Authors' Contributions

RKH Maeh and AI AL-Tameemi designed the study and prepared the manuscript; ZS Mahmood and HY Fadhil contributed to data collection, analysis, and critical revision; MS Jabir performed validation and statistical review. All authors have read and approved the final submitted version.

6.4 Conflict of Interests

The authors declare no competing interests.

6.5 Financial Support and Sponsorship

No funding.

6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this study, including the writing of this manuscript.

References

1. Li S, Pan M, Zhao H, Li Y. Role of CCL2/CCR2 axis in pulmonary fibrosis induced by respiratory viruses. J Microbiol Immunol Infect. 2025;58(4): 397-405. [DOI:10.1016/j.jmii.2025.02.003] [PMID]
2. Singh S, Anshita D, Ravichandiran V. MCP-1: Function, regulation, and involvement in disease. Int Immunopharmacol. 2021;101: 107598. [DOI:10.1016/j.intimp.2021.107598] [PMID] [PMCID]
3. Gschwandtner M, Derler R, Midwood KS. More than just attractive: how CCL2 influences myeloid cell behavior beyond chemotaxis. Front Immunol. 2019;10:2759. [PMCID] [DOI:10.3389/fimmu.2019.02759] [PMID]
4. Maschalidi S, Hässler S, Blanc F, Sepulveda FE, Tohme M, Chignard M, et al. Asparagine endopeptidase controls anti-influenza virus immune responses through TLR7 activation. PLoS Pathog. 2012;8(3):e1002841. [PMCID] [DOI:10.1371/journal.ppat.1002841] [PMID]
5. Ebli FM, Heshmatipour Z, Daneshjou K, Siadat SD. Simultaneous Genetically Detection of Streptococcus pyogenes, Streptococcus pneumoniae and Haemophilus influenzae in Patients with Treatment-Resistant Respiratory Infection. Iran J Pathol. 2023;18(3):356. [DOI:10.30699/ijp.2023.1991067.3075] [PMID] [PMCID]
6. Jeisy-Scott V, Kim JH, Davis WG, Cao W, Katz JM, Sambhara S. TLR7 recognition is dispensable for influenza virus A infection but important for the

induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. J Virol. 2012;86(20):10988-98. [DOI:10.1128/JVI.01064-12] [PMID] [PMCID]

7. To EE, Erlich J, Liong F, Luong R, Liong S, Bozinovski S, et al. Intranasal and epicutaneous administration of Toll-like receptor 7 (TLR7) agonists provides protection against influenza A virus-induced morbidity in mice. Sci Rep. 2019; 9(1):2366. [DOI:10.1038/s41598-019-38864-5] [PMID] [PMCID]
8. Flerlage T, Boyd DF, Meliopoulos V, Thomas PG, Schultz-Cherry S. Influenza virus and SARS-CoV-2: pathogenesis and host responses in the respiratory tract. Nat Rev Microbiol. 2021; 19(7):425-41. [PMID] [PMCID] [DOI:10.1038/s41579-021-00542-7]
9. Xue J, Wang X, Wang H, Qiao B, Gao P, Ren B, et al. Unraveled role of TLR7-mediated interferon signaling activation in COVID-19. Front Cell Infect Microbiol. 2025;15:1658249.[PMCID] [DOI:10.3389/fcimb.2025.1658249] [PMID]
10. Ghimire R, Shrestha R, Amaradhi R, Liu L, More S, Ganesh T, et al. Toll-like receptor 7 (TLR7)-mediated antiviral response protects mice from lethal SARS-CoV-2 infection. J Virol. 2025;99(5): e01668-24. [DOI:10.1128/jvi.01668-24] [PMID] [PMCID]
11. Zhou J, Wang D, Gao R, Zhao B, Song J, Qi X, et al. Biological features of novel avian influenza A (H7N9) virus. Nature. 2013;499(7459):500-3. [DOI:10.1038/nature12379] [PMID]
12. Davey Jr RT, Lynfield R, Dwyer DE, Losso MH, Cozzi-Lepri A, Wentworth D, et al. The association between serum biomarkers and disease outcome in influenza A (H1N1) pdm09 virus infection: results of two international observational cohort studies. PLoS One. 2013; 8(2):e57121. [PMID] [PMCID] [DOI:10.1371/journal.pone.0057121]
13. Vangeti S, Falck-Jones S, Yu M, Österberg B, Liu S, Asghar M, et al. Human influenza virus infection elicits distinct patterns of monocyte and dendritic cell mobilization in blood and the nasopharynx. Elife. 2023;12:e77345. [DOI:10.7554/eLife.77345] [PMID] [PMCID]
14. Nian H, Geng WQ, Cui HL, Bao MJ, Zhang ZN, Zhang M, et al. R-848 triggers the expression of TLR7/8 and suppresses HIV replication in monocytes. BMC Infect Dis. 2012;12(1):5. [DOI:10.1186/1471-2334-12-5] [PMID] [PMCID]
15. Sikand K,Singh J, Ebron JS, Shukla GC. Housekeeping gene selection advisory: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are targets of miR-644a. PLoS One. 2012;7(10):e47510. [PMCID] [DOI:10.1371/journal.pone.0047510] [PMID]
16. Lee N, Wong CK, Chan PK, Chan MC, Wong RY, Lun SW, et al. Cytokine response patterns in severe pandemic 2009 H1N1 and seasonal influenza among hospitalized adults. PLoS One. 2011;6(10):e26050. [PMID] [PMCID] [DOI:10.1371/journal.pone.0026050]
17. Jiang Q, Zhang Y, Duan D, Retout S, Upmanyu R, Glavini K, et al. Using exploratory pharmacokinetic and pharmacodynamic analyses to predict the probability of flu-like symptoms in healthy volunteers and patients with chronic hepatitis B treated with the toll-like receptor 7 agonist ruzotolimod. Clin Transl Sci. 2024;17(8):e13896. [DOI:10.1111/cts.13896] [PMID] [PMCID]
18. Rappe JC, Finsterbusch K, Crotta S, Mack M, Priestnall SL, Wack A. A TLR7 antagonist restricts interferon-dependent and-independent immunopathology in a mouse model of severe influenza. J Exp Med. 2021; 218(11):e20201631. [DOI:10.1084/jem.20201631] [PMID] [PMCID]
19. Dong Y, Dong Y, Zhu C, Yang L, Wang H, Li J, et al. Targeting CCL2-CCR2 signaling pathway alleviates macrophage dysfunction in COPD via PI3K-AKT axis. Cell Commun Signal. 2024;22(1): 364. [DOI:10.1186/s12964-024-01746-z] [PMID] [PMCID]
20. Guo S, Zhang Q, Guo Y, Yin X, Zhang P, Mao T, et al. The role and therapeutic targeting of the CCL2/CCR2 signaling axis in inflammatory and fibrotic diseases. Front Immunol. 2025;15: 1497026. [DOI:10.3389/fimmu.2024.1497026] [PMID] [PMCID]
21. Maeh RK, Fadhil HY. Downregulation of CCL20 in Severe COVID-19 Infections and its Association with NLRP3 Gene Polymorphisms. Iraqi J Sci. 2025;66(5):9. [DOI:10.24996/ijs.2025.66.5.9]
22. Forouhi NG, Harding AH, Allison M, Sandhu MS, Welch A, Luben R, et al. Elevated serum ferritin levels predict new-onset type 2 diabetes: results from the EPIC-Norfolk prospective study. Diabetologia. 2007;50(5):949-56. [DOI:10.1007/s00125-007-0604-5] [PMID]
23. Sun L, Franco OH, Hu FB, Cai L, Yu Z, Li H, et al. Ferritin concentrations, metabolic syndrome, and type 2 diabetes in middle-aged and elderly

Chinese. J Clin Endocrinol Metab. 2008;93(12): 4690-6. [DOI:10.1210/jc.2008-1159] [PMID]

24. Files JK, Boppana S, Perez MD, Sarkar S, Lowman KE, Qin K, et al. Sustained cellular immune dysregulation in individuals recovering from SARS-CoV-2 infection. J Clin Invest. 2021; 131(1):e140491. [DOI:10.1172/JCI140491] [PMID] [PMCID]
25. Zou Y, Ding M, Zheng Y, Dai G, Wang T, Zhu C, et al. Peripheral blood cytokine expression levels and their clinical significance in children with influenza. Transl Pediatr. 2025;14(2):286. [DOI:10.21037/tp-2024-534] [PMID] [PMCID]
26. Bober A, Mika J, Piotrowska A. A Missing Puzzle in Preclinical Studies-Are CCR2, CCR5, and Their Ligands' Roles Similar in Obesity-Induced Hypersensitivity and Diabetic Neuropathy?-Evidence from Rodent Models and Clinical Studies. Int J Mol Sci. 2024;25(20):11323. [DOI:10.3390/ijms252011323] [PMID] [PMCID]
27. Wang YL, Koh WP, Yuan JM, Pan A. Plasma ferritin, C-reactive protein, and risk of incident type 2 diabetes in Singapore Chinese men and women. Diabetes Res Clin Pract. 2017;128:109-18. [DOI:10.1016/j.diabres.2017.04.012] [PMID]
28. Ali N. Elevated level of C-reactive protein may be an early marker to predict risk for severity of COVID-19. J Med Virol. 2020;92(11):2409. [DOI:10.1002/jmv.26097] [PMID] [PMCID]
29. Aufi IM, Fadhil HY, Ali AW, Alhamdani FG, Owaid FM. Epidemiology of seasonal influenza outbreak among Iraqi population: 2018. Iraqi J Agric Sci. 2020;51(1):454-61. [DOI:10.36103/ijas.v51i1.944]
30. Klein SL, Passaretti C, Anker M, Olukoya P, Pekosz A. The impact of sex, gender and pregnancy on 2009 H1N1 disease. Biol Sex Differ. 2010;1(1):5. [PMID] [PMCID] [DOI:10.1186/2042-6410-1-5]
31. Varghese M, Clemente J, Lerner A, Abrishami S, Islam M, Subbaiah P, et al. Monocyte trafficking and polarization contribute to sex differences in meta-inflammation. Front Endocrinol. 2022;13: 826320. [DOI:10.3389/fendo.2022.826320] [PMID] [PMCID]
32. Wang M, Chen X, Shang Y, Chen B, Chen H, Zhou L, et al. Oligopeptide-strategy of targeting at adipose tissue macrophages using ATS-9R/siCcl2 complex for ameliorating insulin resistance in GDM. Biomed Pharmacother. 2024;175:116775. [DOI:10.1016/j.biopha.2024.116775] [PMID]