ORIGNAL RESEARCH

# Oxidant-Antioxidant Status in Patients with Knee Osteoarthritis in Duhok City, Kurdistan Region of Iraq

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## ABSTRACT

The major famous degenerative joint disease is osteoarthritis (OA), which leads to disability, reduced motion, pain, swelling, and crepitus. Thus, this research aimed to determine the statuses of oxidative stress (OS) and antioxidants in blood patients with primary OA and to compare serum levels of some inflammatory markers between control and knee OA patients groups. In this case-control study, 126 knee OA patients and 49 control individuals were taken, who visited the Center of Rheumatoid in Duhok city, Kurdistan region of Iraq. For the participant's information, a study questionnaire was employed. Serum total bilirubin (TBIL), uric acid (UA), catalase, peroxynitrite (PN), malondialdehyde (MDA), and ceruloplasmin (CP) were evaluated. Some inflammatory markers were measured, including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Also, body mass index (BMI) was measured. In the present study, the % of the control group was 28% and patients groups were 29.71, 28, and 14.29% for mild, moderate, and severe cases, respectively. The mean values of MDA and CP showed significant differences between knee OA and control groups. On the other hand, the mean values of catalase, PN, UA, and TBIL showed no significant differences among control and knee OA groups. A significant difference was found among control and knee OA groups for ESR, but CRP was non-significant. In the current study, elevated levels of lipid-peroxidation and CP in patients with knee OA were observed. OS may be involved in OA.

**Keywords:** C-reactive protein, Ceruloplasmin, Erythrocyte sedimentation rate, Knee osteoarthritis, Malondialdehyde, Peroxynitrite.

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## INTRODUCTION

The OA causes cartilage and disc degeneration, and osteophyte formation at limbs and spine joints. It is a major public health problem that affects daily living activities and life quality in the elderly leading to increased morbidity and mortality.[1] One-third of all adults have signs of radiological OA. The most common type of OA is knee OA.[2] A recent WHO report mentioned that it is a global burden disease and is becoming the 4th cause of women's disability, and for men, it is the 8th most important cause.[3]

OA historically has been identified as "primary" since no discernible cause was proved, and "secondary" since the appearance of the triggering factor.[4] In metabolic and physiological processes, reactive oxygen species (ROS) are released, where a harmful oxidative reaction in organisms may occur, which is removed through anti-oxidative mechanisms (enzymatic and non-enzymatic). Under some circumstances, oxidative/anti-oxidative balance shifts toward the oxidative status due to oxidants' elevation and antioxidants' depletion. About 100 disorders had been developed as a result of OS, where OA is one of them.[5]

The study tried to investigate some serum OS parameters (like MDA and PN) and some antioxidant parameters (catalase, CP, UA, and TBIL) in knee OA patients and control groups. Also, some inflammatory markers were measured, such as, CRP and ESR. The objectives of this research were to determine the OS and antioxidant statuses in patients' blood with primary knee OA.

## PATIENTS AND METHODS

This case-control study, included 175 subjects (110 females and 65 males), was performed at the Duhok Specialized Centre of Rheumatic Diseases and Medical Rehabilitation. The study was conducted from October 2018 to April 2019. A questionnaire form was filled for every patient of knee OA and control. X-rays of the knee joints that suspected to have OA were examined. According to Kellgren Lawrence (KL) grading scale, radiographic knee OA was defined.[6] Patients' groups of

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both sexes (35–80 years old) suffering from primary knee OA were joined in the study. The excluded cases were participants with a history of corticosteroid medication, diabetes, cancer, other forms of arthritis, or other chronic inflammatory diseases, alcoholics, and those who were taking antioxidant drug treatments. Informed consent from all participants was obtained and approved by the Ethics Committee, Directorate of Health, Duhok. For each participant, BMI was measured. From control and patients groups, fasting blood samples were withdrawn. Two mL of whole blood was kept in an ethylenediamine tetraacetic acid (EDTA) tube, which was used for ESR. The other parts of the blood samples were drawn into tubes with anticoagulants, which ultimately are separated and portioned appropriately and kept at -20ºC, till the analyzing time of CP, catalase, PN, and MDA. UA, TBIL, and CRP were measured using commercial kits provided by (Biolabo SA 02160, Maizy, France).

**Determination of Serum Catalase Levels**

Serum catalase determination was performed by Hadwan and Abed method (2016).[7] Catalase estimated by incubating the sample of the enzyme in 1 mL substrate (65 µmol/mL $H_2O_2$ in 60 mmol/L sodium-potassium phosphate buffer, pH 7.4) at 37ºC for 3 minutes. Ammonium molybdate will act to stop the reaction. Molybdate and $H_2O_2$ yellow complex formation was measured at an absorbance of 374 nm against the blank.[7]

**Determination of Serum CP Levels**

Serum CP was estimated by Menden *et al*., a method which is the p-phenylenediamine oxidase method.[8] Molar extinction coefficient of CP $\varepsilon = 0.68\ M^{-1}.cm^{-1}$ was used for the determination of sample concentration. In an ice-water bath, test tubes were placed, and a mixture of 1 mL of refrigerated substrate solution and 0.l-mL of serum was added. The substrate solution was prepared by dissolving 50 mg of crystalline p-phenylenediamine + 1 mL of glacial acetic acid + 5 mL of double distilled water (DDW). In another container, 8.15 grams of sodium acetate trihydrate was dissolved in 30 mL of DDW, and then this solution was added to the p-phenylenediamine solution, and completed to the final volume of 50 mL in a volumetric flask with DDW, and incubated at 37ºC for 15 minutes. Blanks were prepared for each sample by combining the substrate, the inactivating solution, and 0.1-mL of the sample. After incubation, the tubes were placed in an ice-water bath and shacked for 30 seconds. Five mL of refrigerated inactivating solution (100 mg of sodium azide + 500 mL of DDW) were added, and the test tubes contents were mixed by inversion and brought to 25ºC in a 27ºC water bath. The absorbance of the test and blank was read at 525 nm.

**Determination of Serum MDA Levels**

MDA levels were measured according to Tüközkan and Erdamar (2006).[9] The molar extinction coefficient of MDA is $1.65 \times 10^5$ L/mol.cm. At room temperature, trichloroacetic acid was added to the mixture of 50 µL of serum + 50 µL of thiobarbituric acid (TBA) reagent. Mixing and heating the solution at about 100ºC in a water bath was done for 20 to 30 minutes. Next, the cooling process was preformed; centrifugation was done for 3 minutes at 3,000 rpm to remove the supernatant. Absorbance for the colored supernatant solution was determined against blank at 532 nm.[9]

**Determination of Serum PN Levels**

The molar extinction coefficient of PN 4,400 $M^{-1}.cm^{-1}$ was used for determination of sample concentration by the method of Vanuffelen *et al*. (1998), which is spectrophotometrically detected; 0.6-mL of serum was added to a mixture of 1.4 mL of phenol (5 mm) in sodium phosphate buffer (50 mm) with pH 7.4. After incubation for 2 hours at 37ºC, 15 µL of 0.1-M NaOH was added, and the absorbance of the samples at 412 nm immediately was recorded.[10]

**Statistical Analysis**

SPSS 20.0 packaged program was used to conduct the obtained data. Results were expressed as mean values X ± SD. The difference among mean values of groups was determined by using the unpaired t test, chi-square test, and one way (ANOVA) test for continuous and categorical variables, respectively. In the results, $p < 0.05$ was accepted as significant at a confidence interval of 95%. The Pearson correlation was used to examine the relationship between variables.

## RESULTS

In the current study, general characteristics and inflammatory markers of the study population of knee OA patients and control groups are depicted in Table 1. According to the chi-square test for n (%), there were significant differences observed in control and patients' groups for number, % of gender, and hypertension ($p < 0.05$). Conversely, no significant differences were observed for smoking habits ($p > 0.05$). A significant difference was observed in age. Similarly, BMI and WC illustrated significant differences ($p < 0.001$) between knee OA patients and control groups.

Table 2 shows the OS parameters; significant differences between knee OA and control groups ($p < 0.001$) of serum CP level was observed in mean ± SD (24.05 ± 6.98, 31.22 ± 9.84, 28.58 ± 9.08, and 26.17 ± 8.72) for control, mild, moderate, and severe knee OA groups, respectively. Similarly, serum MDA showed significant differences in mean ± SD (0.64 ± 0.24, 0.83 ± 0.47, 0.86 ± 0.56, and 0.97 ± 0.57) for control, mild, moderate, and severe knee OA groups, respectively. On the other hand, there were no significant differences among groups ($p > 0.05$) in terms of serum UA, TBIL, serum PN, and antioxidant catalase.

According to the one-way ANOVA test, Table 3 reveals that there were significant differences in ESR ($p < 0.05$) among control and knee OA patients groups in mean ± SD. On the other hand, there was no significant variation in CRP across the groups ($p > 0.05$).

ESR was classified into three groups.[11] The present study showed that 18.3% of the study population has ESR

**Table 1:** Principal and demographic characteristics of knee OA patients and control groups

| | *Mean ± SD* | | | | |
|---|---|---|---|---|---|
| *Particulars* | *Control group (n = 49)* | *Group I Mild (n = 52)* | *Group II Moderate (n = 49)* | *Group III Severe (n = 25)* | *p value* |
| Age (years) | 7.673 ± 42.489 | 8.854 ± 50.153 | 9.134 ± 52.6326 | 7.0237 ± 58 | < 0.001 |
| BMI (kg/m$^2$) | 3.789 ± 29.0789 | 5.789 ± 31.4076 | 4.3539 ± 33.265 | 7.110 ± 33.296 | < 0.001 |
| WC (cm) | 11.647 ± 96.775 | 10.86 ± 103.173 | 9.035 ± 108.43 | 108.44 ± 12.416 | < 0.001 |
| Gender | | | | | |
| Males | 28 (16%) | 19 (10.9%) | 10 (5.7%) | 8 (4.6%) | |
| Females | 21 (12%) | 33 (18.9%) | 39 (22.3%) | 17 (9.7%) | 0.002 |
| Hypertension | | | | | |
| Yes | 0 (0%) | 5 (2.9%) | 7 (4.0%) | 5 (2.9%) | |
| No | 49 (28%) | 47 (26.9%) | 42 (24.0%) | 20 (11.4%) | 0.024 |
| Smoking habits | | | | | |
| No | 49 (28%) | 49 (28%) | 47 (26.9%) | 22 (12.6%) | |
| Yes | 0 (0%) | 3 (1.7%) | 2 (1.1%) | 3 (1.7%) | 0.127 |

N = patients number; p value is significant when p < 0.05

**Table 2:** OS between knee OA and control groups

| *Parameters* | *Control group (n = 49)* | *Group I Mild (n = 52)* | *Group II Moderate (n = 49)* | *Group III Severe (n = 25)* | *p value* |
|---|---|---|---|---|---|
| UA (mg/dL) | 1.83 ± 5.57 | 1.56 ± 5.98 | 1.56 ± 5.71 | 1.45 ± 5.66 | 0.634 |
| TBIL (mg/dL) | 0.53 ± 0.31 | 0.29 ± 0.56 | 0.36 ± 0.51 | 0.22 ± 0.52 | 0.892 |
| CP (mg/mL) | 24.05 ± 6.98 | 31.22 ± 9.84 | 28.58 ± 9.08 | 26.17 ± 8.72 | < 0.001 |
| MDA (nmol/mL) | 0.64 ± 0.24 | 0.83 ± 0.47 | 0.86 ± 0.56 | 0.97 ± 0.57 | 0.024 |
| PN (mmol/L) | 0.26 ± 0.1 | 0.27 ± 0.107 | 0.281 ± 0.12 | 0.298 ± 0.13 | 0.673 |
| Cat. (KU/L) | 5.05 ± 4.39 | 7.26 ± 7.91 | 7.81 ± 7.5 | 6.18 ± 3.81 | 0.174 |

One way ANOVA; p value is significant when p < 0.05; n = patients' number

**Table 3:** Inflammatory markers in control and knee OA patients

| | *Mean ± SD* | | | | |
|---|---|---|---|---|---|
| *Parameters* | *Control group (n = 49)* | *Group I Mild (n = 52)* | *Group II Moderate (n = 49)* | *Group III Severe (n = 25)* | *p value* |
| ESR (mm/hr) | 15.637 ± 15.687 | 17.044 ± 23.153 | 19.52 ± 24.97 | 16.199 ± 26.5 | 0.024 |
| CRP (mg/L) | 1.9592 ± 0.199 | 1.8654 ± 0.3446 | 1.8571 ± 0.3535 | 1.9200 ± 0.2768 | 0.308 |

p value is significant when p < 0.05; n = patients' number

**Table 4:** Classification of ESR in knee OA

| | *ESR (mm/hr) levels* | | |
|---|---|---|---|
| *ESR levels* | *< 20 mm/hr* | *20–40 mm/hr* | *> 40 mm/hr* |
| N (%) | 65 (51.6%) | 38 (30.2%) | 23 (18.3%) |
| Mean ± SD | 11.48 ± 5.94 | 28.94 ± 4.79 | 53.43 ± 15.69 |

n = patients' number; results are expressed as mean ± SD

**Table 5:** Pearson correlation (r) of ESR and OS in mild, moderate, and severe cases

| | *Mild grade* | | *Moderate grade* | | *Severe grade* | |
|---|---|---|---|---|---|---|
| *Variable* | *r value* | *p value* | *r value* | *p value* | *r value* | *p value* |
| TBLU (mg/dL) | -0.327 | 0.018 | -0.125 | 0.393 | -0.396 | 0.055 |
| UA (mg/dL) | -0.302 | 0.03 | 0.039 | 0.791 | -0.2 | 0.35 |
| Cat. (KU/L) | 0.054 | 0.704 | -0.145 | 0.319 | -0.085 | 0.695 |
| MDA (nmol/mL) | 0.025 | 0.859 | 0.033 | 0.828 | 0.149 | 0.531 |
| PN (mmol/L) | -0.16 | 0.255 | -0.066 | 0.654 | -0.04 | 0.852 |
| Cer. (mg/mL) | 0.303 | 0.029 | 0.048 | 0.743 | 0.467 | 0.021 |

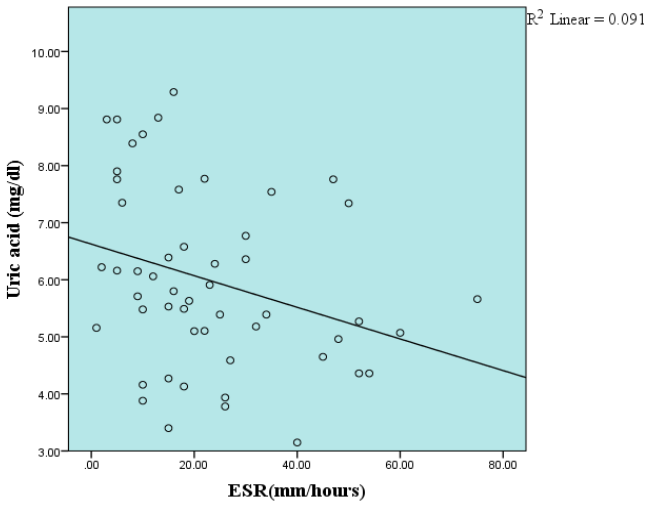


**Figure 1:** Negative correlation between serum (ESR) and serum TBIL in mild case (r = -0.327, p = 0.018)

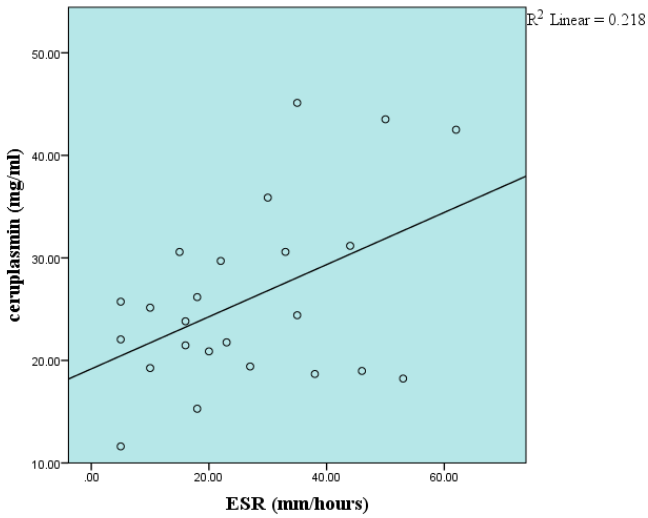


**Figure 2:** Negative correlation between serum ESR and serum UA in mild case (r = -0.302, p = 0.03)

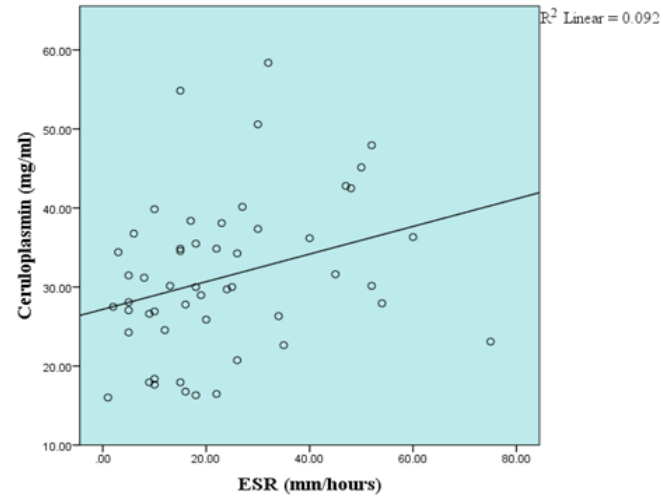


**Figure 3:** Positive correlation between serum ESR and CP in mild case (r = 0.303, p = 0.029)

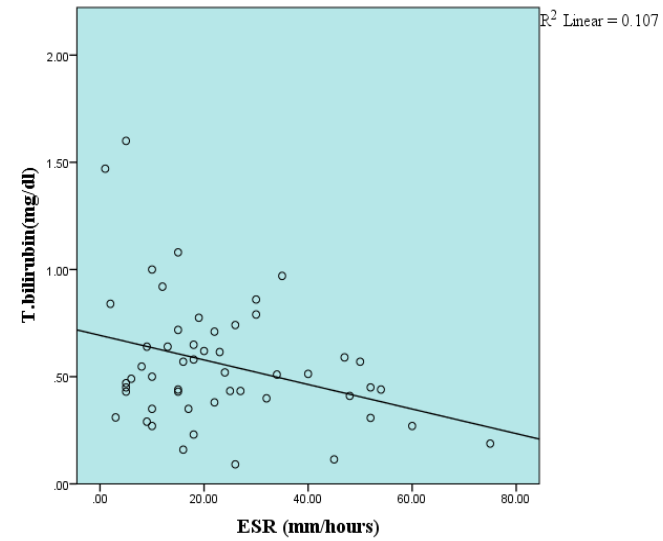


**Figure 4:** Positive correlation between serum ESR and CP in severe case (r = 0.467, p = 0)

levels more than 40 mm/hr with mean ± SD (53.43 ± 15.69). Conversely, 51.6% of the study groups were with high ESR levels < 20 mm/hr with mean ± SD (11.48 ± 5.94). Finally, among the study groups, 30.2% with mean ± SD (28.94 ± 4.79) belonged to the levels of 20–40 mm/hr (Table 4).

According to Pearson correlation coefficient, Table 5 reveals that there were no significant correlation of the ESR with serum catalase, MDA, and PN in mild cases of knee OA.

Figures 1 and 2 show a negative significant correlation of TBIL and UA with ESR, while a positive significant correlation effect in serum CP level was observed in Figure 3. No significant correlation of the ESR with serum catalase, PN, UA, TBIL, MDA, and CP in moderate and severe case of knee OA was noticed, except CP in severe case has a positive significant correlation (Figure 4).

## DISCUSSION

Most of the people over 65 years of age are suffering from OA as a common reason for long-term disability. The manifestation of OA, including erosion and loss of articular cartilage, subchondral sclerosis, and osteophytes formation are the most serious indicators.[11] Regarding epidemiology, OA is the most common disease affecting humans and a common cause of disability. As the population ages, dramatic increase of OA is expected during the next two decades.[12]

In the current study, serum MDA was investigated to know the relation between knee OA and the OS marker MDA. The results indicated that concentrations of MDA elevated in patients of knee OA compared with control group (p-value < 0.05), as listed in Table 2. In patients of knee OA, lipid peroxidation was elevated due to the diminishing of antioxidant defense mechanisms, therefore, MDA was increased due to this

damage as an end-product in the patient's plasma and synovial fluid.[13] Our findings agreed with Katti *et al*. (2015), who demonstrated that MDA levels were significantly high in study group subjects, which clarified the role of lipid peroxidation in knee OA progression. The increased OS in OA patients may be attributed to the elevated levels of lipid peroxidation or due to decreased levels of antioxidants.[14] The elevated MDA level in our knee OA patients coincided with the results of Dwivedi *et al*. (2016), who reported that lipid peroxidation levels were significantly elevated.[15]

PN ($OONO^-$) is thought as the main tissue damaging species that cause various changes in proteins via oxidation of sulfhydryl groups of cysteine and methionine, as well as, selectively nitrating tryptophan and tyrosine residues.[16] $ONOO^-$ is decomposed rapidly to $OH^-$ and $NO_2$, where collectively reacts with other radicals and molecules.[17] The obtained results (Table 2) showed that there were no significant differences among the participant groups ($p < 0.05$) with respect to serum PN concentration. However, recent studies suggested that NO redox derivatives could have a role as anti-inflammatory in chondrocytes. There is no straight forward role of NO and its derivatives in knee OA progression, and probably that PN and NO may have opposing role in the development of inflammation and arthritis.[18] However, there are limited studies focusing on PN ($ONOO^-$) role in bone metabolism and pathologies.[19]

In the present study, no significant difference was observed between control and knee OA groups in term of catalase ($p > 0.05$). The study of Pinto *et al*. (2008) proved that ROS toxicity can decline by chondrocytes antioxidant enzyme system, as they constitutively express catalase.[20] On the other hand, a study of Altindag *et al*. (2007) showed that catalase activity significantly increased in patients with OA compared to the control group.[5]

During inflammatory status, CP used as a measure of acute phase reactivity, behaves as an antioxidant via scavenging free radical species and hence, protects surrounding cells against oxidative damage.[21] In the current study, no significant differences were found between knee OA and control groups in serum CP. Similar results had been reported by Al-Gebori (2012) who detected that the levels of serum CP in patients with OA were significantly higher as compared to the control group.[21]

Our study showed that ESR values for participants were 51.6% less than 20 mm/hr, 30.2% with the range of 20 to 40 mm/hr, and 18.3% more than 40 mm/hr (Table 4). Similar findings were demonstrated by other studies.[13-15] Those findings support that inflammation plays a part in the pathogenesis of OA.[15] Generally, ESR, according to Kellgren Lawrence grade, II, III and IV groups were significantly higher than control group (Table 3). The obtained results were in line with those reported by Hanada *et al*. (2016).[22]

Another inflammatory marker studied was CRP; it is a pentameric ring-shaped protein, whose levels rose in response to inflammation, and was thereby classified as an acute-phase reactant and biomarker for inflammation.[17] In the current study, no significant differences were observed in serum CRP ($p > 0.05$) among knee OA and control groups as shown in Table 3. The obtained results were in accordance with those obtained by John *et al*. (2008), which proved no significant association between the presence of radiographic knee OA and any inflammatory marker.[18] On the contrary, a recent study by Kozijn *et al*. (2019) revealed the absence of the correlation between CRP and knee OA development. Even though the action mechanism for CRP association in OA is not yet understood, it was suggested that amelioration of metabolic OA progression could be a result of interventions selectively directed against CRP activity.[20] On the other hand, a positive association between circulating CRP has been clarified in patients with OA, obesity, and metabolic syndrome, as reported by Farnaghi *et al*.[21]

The consequence of this study indicated a positive correlation between ESR and CP in mild and severe grades of knee OA (Figures 3 and 4; Table 5). In contrast with this finding, El-Barbary *et al*. (2011) proved that serum CP in OA patients was insignificantly higher than in the control group.[23]

Bilirubin in multiple clinical applications has a vital function and a promising biological parameter in predicting prognosis. It has anti-inflammatory, anti-oxidative, and immunosuppressive characteristics. Actually, these properties have been considered to behave as a central link in the pathogenesis of various diseases. Therefore, serum bilirubin should be considered as closely associated with its exact mechanism not fully explained yet.[24] In the present study, there were no significant differences between the control and knee OA groups in terms of serum TBIL ($p > 0.05$). Mean values of serum TBIL were $0.53 \pm 0.31$, $0.29 \pm 0.56$, $0.36 \pm 0.51$, and $0.22 \pm 0.52$ in control, mild, moderate, and severe grades of knee OA, respectively, as shown in Table 2. The negative correlation between serum TBIL and ESR in mild grade ($r = -0.327$, $p = 0.018$), as shown in Figure 1 and Table 5. Juping *et al*. (2017) supported the obtained results.[25]

Our findings indicated that there were no significant differences between control and knee OA patients groups in terms of serum UA. No correlation between serum UA and knee OA had been reported,[26] which was confirmed, where there was no significant rise in UA levels in all cases. Also, the study found the level of serum UA in increasing order, parallel to the disease cases.[27] Jos *et al*. (2018) reported a significant positive correlation between the development of knee joint OA serum and uric acid.[28] Our study showed a negative correlation between ESR and serum UA in mild cases ($r = -0.302$, $p = 0.03$), as shown in Figure 2 and Table 5.

## CONCLUSION

In conclusion, this case-control study showed a significant relationship between ages, genders, and BMI with knee OA. ESR concentration was higher in patients with knee OA. There was no significant association between OS parameters and duration of knee OA disease. Also, there were no significant

differences between the groups ($p > 0.05$) in terms of UA, TBIL, serum PN, and antioxidant catalase. Conversely, there were significant differences between the knee OA and the control group ($p < 0.001$) of serum CP and serum MDA levels.

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