Comparative Study between Serum Collagen Type-II and Matrix Metalloproteinase III in Identifying Early Osteoarthritis among Atraumatic Knee Joint Pain Patients Attending Pain Clinics

Ahmed E Salem[a], Adel F Al-Kholy[b], Doaa M Ismail[c*]

[a]Department of Anesthesia, ICU, Pain, Faculty of Medicine, Tanta University, Tanta, Egypt.
[b]Department of Medical Biochemistry, Faculty of Medicine, Benha University, Benha, Egypt.
[c]Department of Physical Medicine, Rheumatology & Rehabilitation, Faculty of Medicine, Tanta University, Tanta, Egypt.

Abstract

Background: Degenerative changes due to osteoarthritis (OA) may cause atraumatic knee joint pain (KJP). The concept and clinical diagnosis of early KOA (EKOA) are ambiguous and must be investigated.

Objectives: This study tried to assess the possibility of using the estimated serum biomarkers' levels to discriminate EKOA patients among atraumatic KJP patients.

Patients and methods: Patients complaining of KJP were evaluated clinically for pain scoring and diagnosis of OA and radiologically for grading according to the Kellgren–Lawrence (KL) grading scale. Blood samples were obtained from patients and controls with a KL grade of zero for ELISA estimation of serum matrix metalloproteinase-3 (MMP-3), C-terminal cross-linked telopeptides of type II collagen (CTX-II), tumor necrosis factor-α (TNF-α), and interleukin-6.

Results: The median values of MMP-3, CTX-II, and TNF-α were significantly higher in OA than in control ($P < 0.001$) and EKOA samples ($P < 0.001$, 0.0003, and 0.0004, respectively) and in EKOA samples ($P = 0.034$, 0.011, and 0.0045, respectively) than in control samples. The estimated cytokine levels were positively related to the KL grading of KOA and pain score. Statistical analyses defined high levels of MMP-3 and CTX-II as the highly significant identifiers for the EKOA and high serum TNF-α and MMP-3 levels as the highly significant identifiers for patients with severe pain requiring intervention.

Conclusion: EKOA is a possible cause for atraumatic KJP, especially in older and obese subjects. No single cytokine can be used to discriminate EKOA patients. Estimating serum levels of MMP3, CTX-II, and TNF-α might be used to distinguish EKOA patients.

Keywords: Osteoarthritis; Knee joint pain; Early KOA; TNF-α.

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***Correspondence:** doaa.m.ismail2022@gmail.com

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Introduction

Osteoarthritis (OA) is characterized by the irreversible destruction of articular cartilage with bony overgrowth, inflammation of the synovial membranes, and subsequent fibrosis of the ligaments (**Olansen et al., 2024**). OA-associated degenerative processes may cause atraumatic knee joint pain (KJP) that was previously considered idiopathic **(Dippmann et al., 2024)**.

Pathologically, there is a failure of normal repair processes for OA-accompanied cartilage damage. OA is an inflammatory condition that may be preceded by molecular modifications **(Heffernan et al., 2024)**.

According to the Osteoarthrosis Committee of the Chinese Aging Well Association (2024) guidelines, the concept of early KOA (EKOA) is ambiguous, and its clinical diagnosis and treatment are often overlooked. Unfortunately, there is no current drug that can modify the progression of the inflammatory and destructive processes associated with OA, but intra-articular injections are effective for pain relief **(Wang et al., 2025)**. This illustrates the importance of identifying patients who had EKOA to effectively prevent the progress of OA and reduce the incidence of mid-to-late-stage KOA **(Chinese Association, 2024)**.

A recent review defined 17 biomarkers that have a statistically significant association with diagnostic hallmarks of OA. Interleukin-6 (IL-6), matrix metalloproteinase-3 (MMP-3), C-terminal telopeptide of type-II collagen (CTX-II), and collagen type-II cleavage product showed promise in predicting patients' outcomes **(Batty et al., 2024)**.

Mammalian MMP-3 comprises a highly conserved sequence of 475–478 amino acids. The structure of MMP-3 can be divided into three constituents: a propeptide domain, metalloproteinase catalytic domain, and a heme-protein domain that functions as a ligating peptide **(Adamcova and Simko, 2018)**. The catalytic domain of the MMP-3 is responsible for digesting the extracellular matrix components, especially the matrix proteins and adhesion molecules **(Ahmed et al., 2018)**.

The CTX-II is produced secondary to the destruction of the articular cartilage, so it can be measured in synovial fluid, serum, and urine, but the urinary evaluation is advantageous for the easier accessibility of urine than synovial fluid for evaluation of the levels of CTX-II **(Corsetti et al., 2015)**. The preliminary animal studies detected significantly decreased levels of CTX II in serum during the initiation of OA and supposed that these levels were irrelevant to the severity of the pathological process **(Tonge et al., 2013)**. On the contrary, **Bai and Li (2016)** reported significantly higher serum concentrations of CTX-II at each time-point in the animal model of OA and concluded that early dynamic changes of serum CTX-II concentration may be used as an efficient biomarker to verify early cases of OA.

Objectives comprised the determination of the diagnostic performance of the estimation of serum biomarkers to identify patients with EKOA among those attending pain clinics with atraumatic KJP.

Patients and methods

Design: A multicenter prospective observational case-control study

Setting: Departments of Anesthesia, Surgical ICU and Pain Therapy, Physical Medicine, Rheumatology, and Rehabilitation, Faculty of Medicine, Tanta University, in conjunction with the Department of Medical Biochemistry, Faculty of Medicine, Benha University

Study population: Patients complaining of KJP and attending pain or physiotherapy clinics were evaluated for the inclusion criteria. The study also included a

similar number of age- and gender-cross-matched controls and accepted to undergo the same evaluation tools.

Inclusion criteria and grouping

The study participants were categorized according to the KL grading: participants with KL grade 0 with no diagnostic criteria of OA, especially pain, were grouped as the control group (C-Group). Participants with KL grade I with only KJP and were free of other diagnostic criteria of OA were categorized as the EKOA-Group. Other participants with the full diagnostic spectrum of OA were categorized as the OA group. The included participants had to sign the written, fully informed consent for study participation and give blood samples for estimation of the studied cytokines. No intervention for pain was provided to be included in the written consent only investigations and diagnosis.

Exclusion criteria: The presence of a history of rheumatic fever, positive rheumatoid factor, autoimmune diseases, inflammatory disorders, and diabetes mellitus were the exclusion criteria. Also, participants who refused to sign the study participation consent or missed during the application of the study protocol were excluded from the study.

Trial registration: The Research Ethics Committee, Faculty of Medicine, Tanta University, approved the protocol study with reference number 36264PR1008/12/24.

Evaluation items

1. **Pain scoring:** the study participants were asked to determine their pain score during rest and movement on a 10-point numeric rating scale (NRS) with 10 indicating the worst pain **(Williamson and Hoggart, 2005)**.
2. **Clinical and laboratory diagnosis of OA:** KOA was diagnosed on the presence of KJP and the detection of at least three criteria, of the diagnostic criteria according to the ACR Clinical Classification Criteria including age > 50 years, morning stiffness of < 30 min, crepitus on active motion, bony enlargement, and bony tenderness with no palpable warmth of synovium. Laboratory diagnostic data included negative qualitative detection of rheumatoid factor (RF) or quantitatively <1:40 and erythrocyte sedimentation rate (ESR) of <40 mm/h **(Altman et al., 1986)**.
3. **Radiological grading of OA according to the Kellgren–Lawrence (KL) grading scale**: all the study participants had bilateral weight-bearing anteroposterior knee radiographs and were graded as KL-grade 0 on detection of no radiologic changes of OA, KL-grade I was suggested on the detection of doubtful narrowing of joint space with or without osteophytic lipping, and KL-grade II was diagnosed on detecting possible narrowing of joint space with definite osteophytes. The KL-grade III was diagnosed by detecting definite narrowing of joint space, moderate multiple osteophytes, some sclerosis, and possible deformity of bone contour. The KL-grade–IV was identified on detecting marked narrowing of joint space, large osteophytes, severe sclerosis, and definite deformity of bone contour **(Kellgren and Lawrence, 1957)**.

The sample size: The sample size was calculated using G*Power (Version 3.1.9.2). The sample size was calculated to be 30 patients per group to ensure the certainty of objectives and to give the study a power of 80% with α-error of 0.05 and an effect size of 0.20 **(Faul et al., 2007)**.

Laboratory Investigations

At the time of enrolment, blood samples were aseptically obtained from the antecubital vein from all the enrolled

participants. Blood samples were put in plain numbered tubes, allowed to clot, and centrifuged at 3000 rpm for 5 minutes to separate serum. The resultant serum was collected in clean, dry Eppendorf tubes and frozen at -20ºC till ELISA was assayed for the estimation of

1. Serum matrix metalloproteinase-3 (MMP-3; MyBiosource.com, Cat. No. MBS764270, San Diego, CA, USA).
2. Serum human C-terminal cross-linked telopeptides of type II collagen (CTX-II; MyBiosource.com, Cat. No. MBS039244, San Diego, CA, USA).
3. Serum human tumor necrosis factor-α (TNF-α) was measured with an ELISA kit (Abcam Inc., Cat. No. ab181421, San Francisco, USA).
4. Serum interleukin-6 (IL-6) was estimated with an ELISA kit (Abcam Inc., Cat. No. ab46027, San Francisco, USA).

Study outcome

1. The ability of the estimated serum cytokine levels for differentiation of the study participants to identify EKOA patients.
2. The differences in the estimated serum cytokines levels according to patients' enrollment data and diagnostic tools.

Statistical analysis

The data normality was assessed using the Kolmogorov- Smirnov test of normality and the normal Q-Q plots. The significance of the intergroup differences was assessed using the unpaired t-test and the chi-square test (X^2 test) to evaluate the differences regarding the data presented as percentages. Pearson's correlation analysis was applied to evaluate correlations between studied variables. Receiver characteristic curve (ROC) analysis was used to determine the identifiers of EKOA patients among the studied population and to identify patients with pain necessitating interference. Multivariate regression analysis was used to ensure the ability of the defined variates by the ROC curve as identifiers. IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) was used for these analyses with a P value < 0.05 indicating a significant difference.

Results

The preliminary evaluation of patients with atraumatic KJP included 42 patients; 12 were excluded: 5 patients had previous knee trauma since variable durations, 4 patients were older than 50 years, and 3 patients had previous arthroscopic knee surgeries. Also, 7 patients with full diagnostic criteria of OA were excluded; 3 had inflammatory disorders, 2 patients were maintained on intra-articular steroid therapy, and 2 were preparing for total knee replacement surgery (**Fig. 1**).

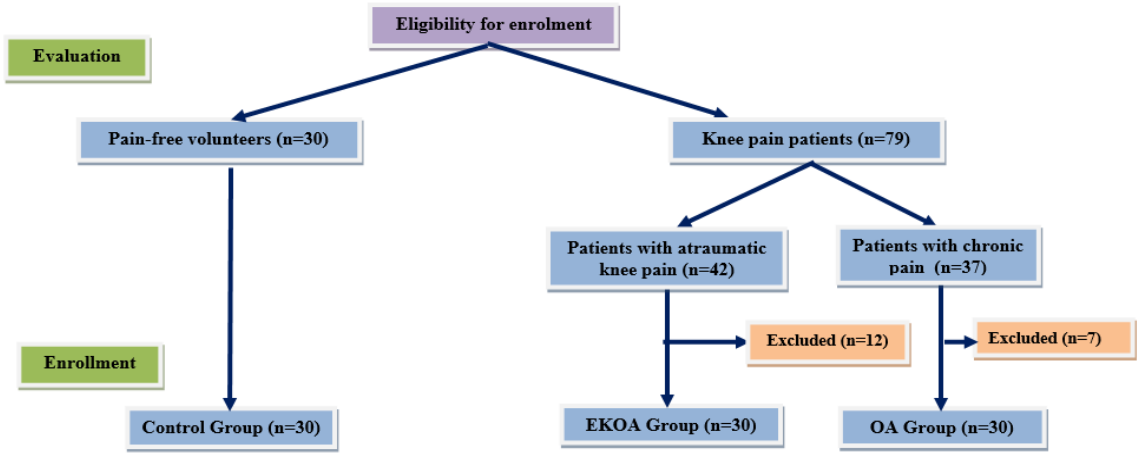

Fig.1. Study participants' flowchart

OA patients were significantly older and more obese than other participants (**Table.1**). EKOA patients were more obese than controls. Control and EKOA groups had similar other characteristics.

Table 1. Demographic data of the study participants

Data Groups		Control	EKOA	OA	Significance of difference		
					Control vs. EKOA	Control vs. OA	EKOA vs. OA
Age (years)*		43.3±7.1	43.8±7.9	48.4±6.1	0.758	0.003	0.005
Gender	**Males**	13 (43.3%)	16 (53.3%)	10 (33.3%)	0.438	0.425	0.118
	Females	17 (56.7%)	14 (46.7%)	20 (66.7%)			
Body mass index (kg/m^2)	**Mean (±SD)***	27.65±3.55	30.89±3	31.25±2.15	0.0008	< 0.001	0.418
	Average	13 (43.3%)	3 (10%)	1 (3-3%)	0.001	< 0.001	0.131
	Overweight	12 (40%)	9 (30%)	4 (13.3%)			
	Obese	5 (16.7%)	18 (60%)	25 (83.4%)			

Chi-square test; Unpaired t-test*;

EKOA patients had significantly lower pain scores (p=0.0002) than OA patients, and a higher, though not significant (p=0.091), frequency of NRS scores < 4 (**Table.2**). Most EKOA patients (93.3%) met one or no ACR criteria for OA, while most OA patients (86.6%) met three or more. This difference in ACR criteria frequency was significant (p<0.001), with EKOA patients having a significantly lower mean number of criteria. All EKOA patients were KL grade I, as were 33.3% of OA patients. The remaining OA patients were grade II (53.3%) or III (13.4%).

Table 2. OA-diagnostic criteria and grading of patients of the EKOA and OA groups

Clinical variables			EKOA	OA	Significance
Pain score	**Mean (±SD)***		3.3±1	4.4±1.1	0.0002
	Frequency of NRS score	**<4**	12 (40%)	6 (20%)	0.091
		>4	18 (60%)	24 (80%)	
ACR criteria	**Mean (±SD)***		0.63±0.6	3.7±0.7	< 0.001
	Frequency of criteria	**0**	13 (43.3%)	0	< 0.001
		1	15 (50%)	0	
		2	2 (6.7%)	0	
		3	0	13 (43.3%)	
		4	0	13 (43.3%)	
		5	0	4 (13.4%)	
The mean number of OA diagnostic criteria *			1.63±0.6	4.7±0.7	< 0.001
Kellgren–Lawrence (KL) grading scale	**I**		30 (100%)	10 (33.3%)	<0.001
	II		0	16 (53.3%)	
	III		0	4 (13.4%)	

Chi-square test; Unpaired t-test*

The median value of the estimated serum MMP-3 level was significantly ($P < 0.001$) higher in OA-group than the other groups with significantly higher median levels of MMP-3 in KOA than control group (**Fig. 2**). Similarly, the median value of serum CTX-II level was higher in OA-group than control ($P < 0.001$) and EKOA ($P = 0.0003$) groups and was significantly ($P = 0.011$) higher in EKOA than in control samples (**Fig. 3**). Additionally, the median value of the levels of serum TNF-α was significantly higher in OA samples than in control ($P < 0.001$) and EKOA samples ($P = 0.0004$) and was significantly ($P = 0.0045$) higher in EKOA than in control samples (**Fig. 4**). In contrast, the median level of IL-6 was significantly lower in control samples in comparison to samples of EKOA ($P = 0.0015$) and OA samples ($P < 0.001$) with insignificantly lower ($P = 0.109$) in EKOA than OA samples (**Table.3, Fig. 5**).

Table 3. Median levels and the IQRs of the estimated serum levels of the studied cytokines in samples of the study participants

Cytokines	Control	EKOA	OA	Significance of difference		
				Control vs. EKOA	Control vs. OA	EKOA vs. OA
MMP-3 (ng/ml)	18.95 [15.3-22.18]	28.85 [12.8-32.4]	57.75 [33.8-67.5]	0.034	< 0.001	< 0.001
CTX-II (ng/ml)	364.5 [239.75-390]	459 [275-517]	660 [506-756]	0.011	< 0.001	0.0003
TNF-α (pg/ml)	2.15 [1.68-3.13]	3.15 [2.2-4.33]	4.95 [3.75-6.13]	0.0045	< 0.001	0.0004
IL-6 (pg/ml)	0.325 [0.21-0.58]	0.545 [0.38-0.84]	0.68 [0.49-0.89]	0.0015	< 0.001	0.109

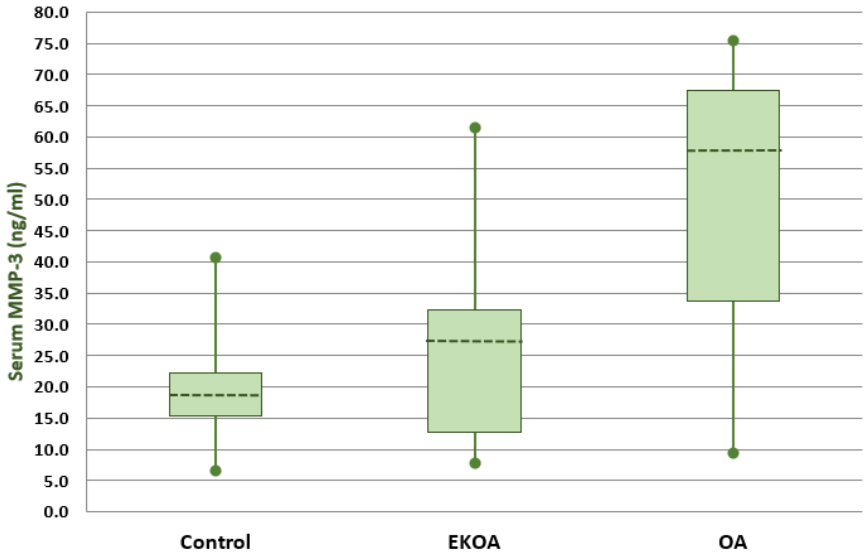

Fig.2. Box-blot for the median value (the dotted line) with the IQR of serum MMP-3 levels

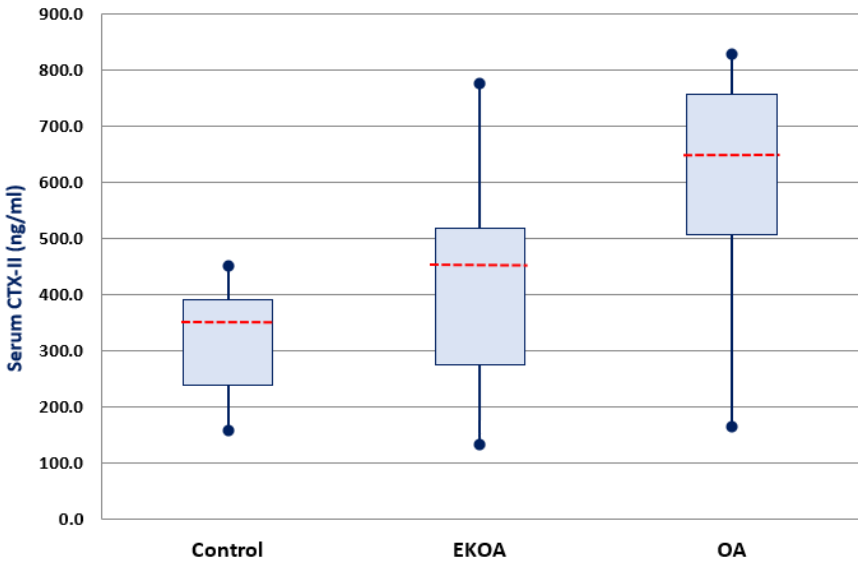

Fig.3. Box-blot for the median value (the dotted line) with the IQR of serum CTX-II levels

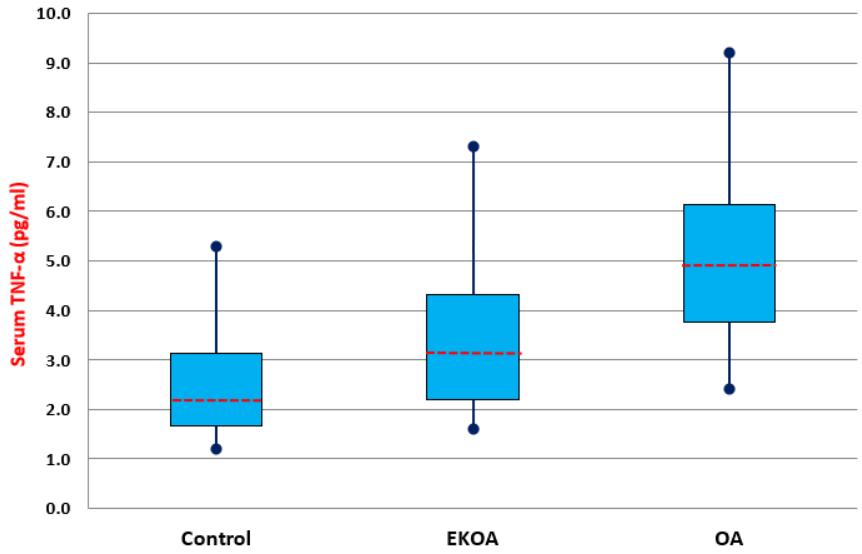

Fig. 4. Box-blot for the median value (the dotted line) with the IQR of serum TNF-α level

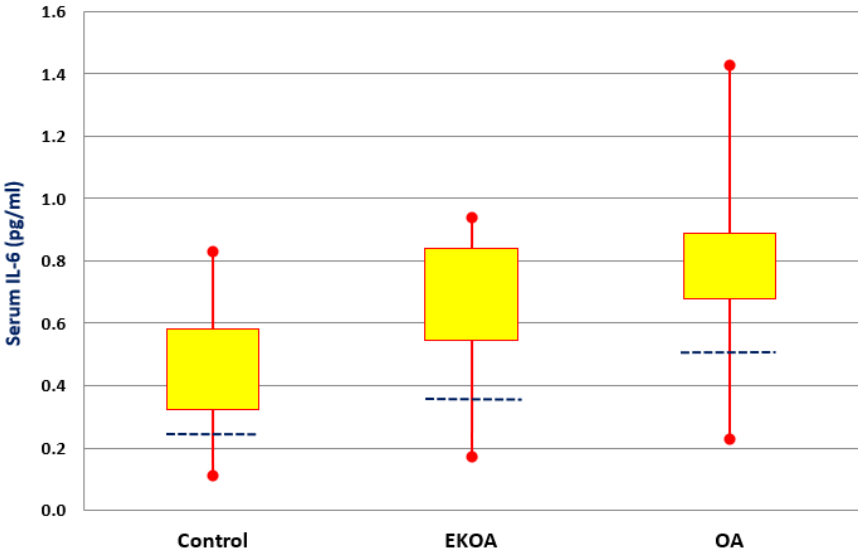

Fig. 5. Box-blot for the median value (the dotted line) with the IQR of serum IL-6 levels

KL grade significantly correlated ($p<0.001$) with serum cytokine levels and positively correlated with BMI ($p=0.002$) and age ($p=0.013$), but negatively with male gender ($p<0.001$). Pain scores highly correlated ($p<0.001$) with BMI and cytokine levels, and significantly with age ($p=0.029$). MMP-3 levels correlated with age ($p<0.001$) and BMI ($p=0.001$). CTX-II ($p=0.014$) and TNF-α ($p=0.002$) levels correlated with BMI (**Table. 4**).

Table 4. Pearson's correlation between the studied variables

Variables	KL grading		Pain score		Age		BMI	
	"r"	P	"r"	P	"r"	P	"r"	P
Age	0.261	0.013	0.230	0.029				
BMI	0.320	0.002	0.520	< 0.001	0.268	0.006		
Male	-0.354	< 0.001	-0.034	0.751	0.140	0.189	0.009	0.935
MMP-3	0.659	< 0.001	0.595	< 0.001	0.414	< 0.001	0.336	0.001
CTX-II	0.570	< 0.001	0.505	< 0.001	0.129	0.225	0.257	0.014
IL-6	0.454	< 0.001	0.390	< 0.001	0.111	0.299	0.189	0.075
TNF-α	0.521	< 0.001	0.657	< 0.001	0.135	0.206	0.320	0.002

The ROC curve analysis defined the high serum level of MMP-3 and TNF-α as the highly significant ($P < 0.001$) identifier of patients with EKOA among the study participants. Also, obesity and high serum levels of CTX-II and IL-6 are identifiers for EKOA with variable significance values; $P = 0.003$, 0.005, and 0.006, respectively. However, old age and male gender were excluded by the ROC curve analysis as an identifier for EKOA patients (**Table. 5, Fig. 6a**). The ROC curve analysis for predictors of pain necessitating intervention defined high serum TNF-α and MMP-3 as the biomarkers that might predict pain severity with significant AUC; $P < 0.001$ and $= 0.006$, respectively (**Table.5, Fig. 6b**).

Table 5. ROC curve analysis for the identifiers for EKOA and pain severity

Variables	KL grade 1				Pain score ≥ 4			
	AUC	SE	P	95% CI	AUC	SE	P	95% CI
MMP-3	0.829	0.051	< 0.001	0.729-0.929	0.730	0.072	0.006	0.589-0.875
CTX-II	0.722	0.079	0.005	0.596-0.848	0.639	0.085	0.099	0.472-0.806
IL-6	0.716	0.067	0.006	0.585-0.847				
TNF-α	0.824	0.056	< 0.001	0.719-0.929	0.845	0.049	<0.001	0.749-0.942
Age	0.591	0.054	0.242	0.437-0.746				
BMI	0.733	0.064	0.003	0.623-0.843	0.629	0.072	0.126	0.488-0.770
Male	0.450	0.077	0.526	0.300-0.601				

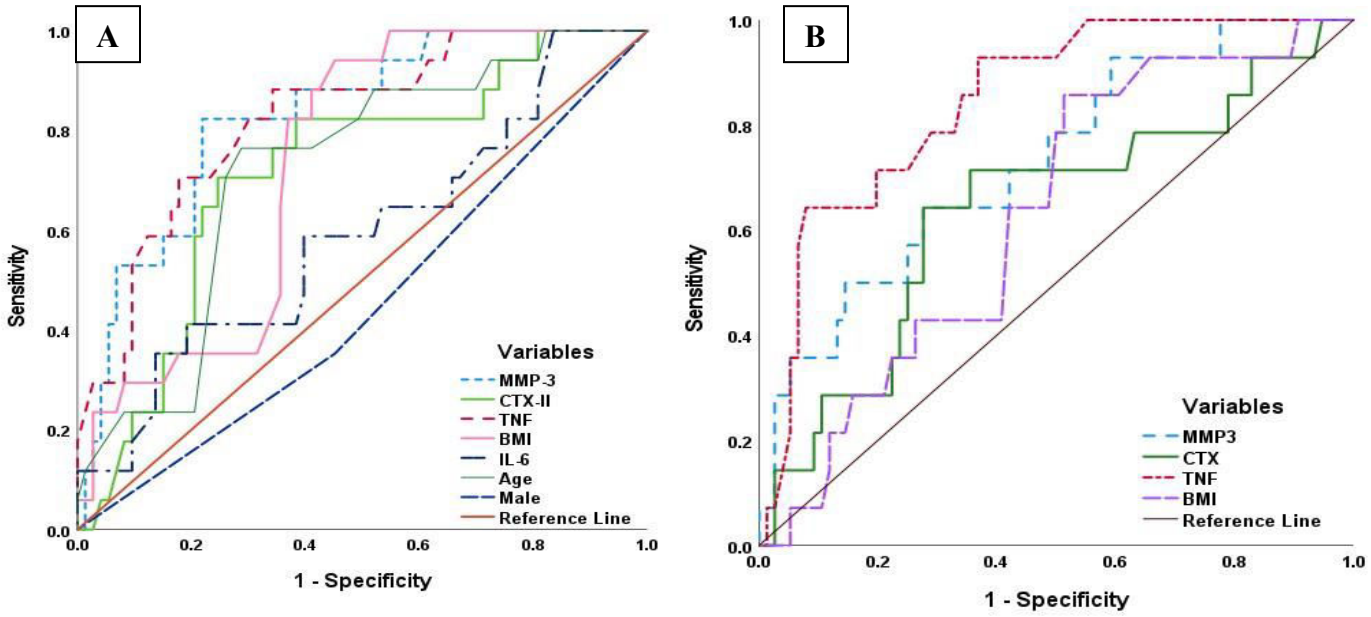

Fig. 6. A) ROC curve for the identifiers for patients who had EKOA. B) ROC curve for the identifiers for patients who had pain necessitating interference

Multivariate Regression analysis of the demographic data and serum levels of the studied cytokines defined high serum levels of MMP-3 and CTX-II as the highly significant (β = 0.512 and 0.363, respectively; P <0.001) identifiers for the presence of EKOA and defined high serum TNF-α and MMP-3 as the highly significant (P < 0.001) identifiers for patients with severe pain that necessitates intervention with β = 0.498 and 0.397, respectively.

Discussion

The reported interrelations between serum cytokine levels and the radiological grading of OA and the severity of OA-induced pain assured the study hypothesis that inflammatory cytokines play a critical role in the diagnosis of OA and its severity radiological grading **(Kellgren and Lawrence, 1957)**.

However, elevated serum MMP-3 levels showed higher diagnostic ability and

might be used as identifiers of cases that had EKOA with a stepwise rise with increasing severity of the disease and its induced manifestations. In line with these findings, **Noorwali et al. (2024)** experimentally documented that cartilage damage leads to increased levels of inflammatory cytokine and levels of biomarkers related to cartilage degradation with an inverse relation between these levels and treatment of OA. Additionally, using a rabbit model of knee OA, **Fu et al. (2024)** detected significantly increased synovial fluid TNF-α and IL-1β levels with up-regulation of the expression levels of MMPs and down-regulation of the expression levels of COL-II in the knee cartilage than in the healthy animals.

Clinically, **Ishibashi et al. (2020)** investigated the relationship between knee synovitis and EOKA and detected higher levels of serum MMP-3 and effusion-synovitis volume in EKOA patients than controls with a positive relation between MMP-3 concentration and pain scores. Also, **Singh et al. (2023)** documented that estimated serum levels of MMP-3 have good discriminatory power for subjects with KL grade I among healthy subjects and patients with KL grade II of KOA. Additionally, **Hutcherson et al. (2024)** found serum concentrations of MMP are increased, especially after tilted walking with direct relation to levels of TNF-α and cartilage oligomeric protein, and these increased levels of pro-inflammatory mediators seriously affect the remodeling and repair of the cartilage tissues.

There was a stepwise increase of serum CTX-II with increased KL grade, especially in OA patients, than in EKOA patients and controls, with a direct, meaningful relation to the radiologic grading and pain severity. These findings support the results obtained by **Liem et al. (2020),** who found urinary CTXII had a strong and consistent association with clinical and/or radiological features of OA and can diagnose symptomatic patients who were radiographically free. Also, **Hu et al. (2022)** found serum COMP and urine CTX-II levels were positively related to the presence of EKOA in premenopausal women. Moreover, **Puts et al. (2024)** detected relationships between high levels of intra-articular inflammatory biomarkers and biomarkers of cartilage degeneration and peripheral and central pain sensitization, respectively.

Statistical analyses defined high serum levels of MMP-3 and CTX-II as the highly significant identifiers for the presence of EKOA with minimal differences between both biomarkers. These findings support the previously documented work by **Singh et al. (2023)** that CTX-II is superior to MMP-3 for the discrimination between KL Grade 0 (normal) and Grade I (EKOA) subjects, while MMP-3 is superior to CTX-II for differentiation between KL Grade I and Grade II (mild KOA).

Also, high serum levels of TNF-α and IL-6 showed positive significant relations with the KL grade with the ability to differentiate between patients with KL Grade I among patients presenting with KJP. However, as a predictor for pain necessitating intervention, TNF-α was superior to IL-6, which showed no ability to predict pain. Per these data, **Coleman et al. (2024)** detected significant differences in MPO and TNF-α level between OA patients and control subjects but not in levels of IL-6. In contrast, **Heffernan et al. (2024)** evaluated inflammatory markers among subjects with EKOA and asymptomatic controls and documented a possibility for IL-6 to be diagnostic for EKOA.

Statistical analyses defined high serum levels of TNF-α as being found to predict extensive pain severity that requires intervention. In line with this finding, **Yu et al. (2024)** experimentally demonstrated that eggshell membrane oral administration

efficiently decreases pain of the joint and articular cartilage destruction in parallel with significant down-expression of mRNA of pro-inflammatory mediators. Additionally, a direct relation was found between TNF-α levels, KL grades pain scores, and BMI as a measure of obesity. In support of the relation between obesity and TNF-α level, **Vasileva et al. (2024)** detected significantly higher levels of adipocytokines in knee OA patients with a positive relation with TNF-α level.

Limitations: Wider-scale studies are required to establish the obtained results. Also, the assessment of the effect of various therapies on both pain and cytokines levels was required.

Recommendations: Estimation of the studied cytokines' levels after provision of pain therapy trials is recommended to assess the effect of therapy on these biomarkers.

Conclusion

Early OA is a possible cause for atraumatic KJP, especially in older and more obese subjects. Regrettably, no single cytokine can be used to discriminate EKOA patients. Estimation of serum levels of MMP3 and CTX-II in conjunction with TNF-α might be used to distinguish EKOA patients from patients complaining of KOA.

Authors' contributions: Case collection was the duty of the pain therapist; Salem AE. Assessment and diagnosis of OA and case differentiation to assure enrolment criteria was the duty of the physiotherapist; Ismail DM. Laboratory investigations were performed by the biochemist; Al-Kholy AF.

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