PRO-INFLAMMATORY BIOMARKERS AND CYTOKINES IN THE VARIOUS STAGES OF OSTEOARTHRITIS

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Abstract

Introduction: Studies have shown, the burden and impact of OA in India are substantial and it is increasing. The current pilot study compared the levels of biomarkers in cases to those in healthy controls and within grades.

Materials and method: 95 subjects between the ages of 40 and 75 were a part of the study. Out of 95, knee OA cases were 63, and normal healthy subjects were 32. It also assessed the relationship between proinflammatory cytokines and biomarkers (IL-6, TNF-a, CTX-I, and CTX-II) from patients with Kellegren-Lawrence grades 1-4. Chi-square, Mann-Whitney U test, correlation, and ROC were used for comparison, and association, to find sensitivity, specificity, and predictive values.

Result: 20% of the participants were in stage 3 and 4 categories of K-L grade. Cases showed significantly higher levels of IL-6, TNF-α and CTX-I compared to controls. A consecutive significant (p = .04) increase was observed with mean IL-6 within 4 grades of OA while mean TNF-α showed an increase in 3rd and 4th grade as compared to 1st and 2nd grade (P = .01). Conclusion: From the ROC curve, the biomarkers (IL-6, TNF-α, CTX-I and TNF-α, and IL-6 together) are good predictors of Severe OA. The highest predictive value was observed with IL-6 and TNF-α a together. There is a need for research, development, Quantification, and application of biomarkers with large sample sizes. A tool is required for the validation of biomarkers in OA.

Keywords: Osteoarthritis; obesity; Prevalence; biomarkers; IL6; TNF- α.

INTRODUCTION

Knee Osteoarthritis (OA) is a degenerative joint disease with a 28.7% prevalence in India (Pal et al.,2016). It is as high as 22% to 39% in India. OA is considered as an inability of chondrocytes to replace the cartilage matrix that has been lost by wear and tear by producing a new viable matrix. This results in pain and immobility of the affected patients by the loss of shielding joint cartilage consequential in bone rubbing over bone articulations. Recent studies on OA indicate there is an inflammatory ailment associated with molecular and biochemical changes. Aging, diabetes, obesity, and mechanical misalignment of joints are examples of associated comorbidities. There is no precise pathophysiology known about the progression of OA; though, several heterogeneous factors are known to cause it. Currently, physical exams and radiological studies are used to diagnose OA. The Kellgren-Lawrence (K-L) scale, which classifies OA severity from 0 to 4 based on radiological visualization, is the most commonly used scale for OA severity. The physical examination determines the severity of pain present and mobility compromised. These methods do not help in forecasting before OA sets in.

OA is an elderly age-related degenerative joint disease (1). Multi-articular OA incidence corresponds with an increase in age. Around 60% of elderly OA patients require treatment (2). To date there is no clear pathogenesis of OA has been established and clinically, early diagnosis of OA is often missed. OA is commonly diagnosed by X-ray and Magnetic resonance imaging (MRI) with certain limitations. Early diagnosis of OA with joint involvement might be difficult with X-ray Although MRI provides a superior resolution for early OA diagnosis, its use is constrained by its high cost (3,4). OA can be treated clinically in a variety of ways, but the process is lengthy and the results are frequently unsatisfactory. Joints steadily deteriorate as OA

progresses, and the interior structure of the joint experiences pathological alterations and abnormalities. In clinical practice, it frequently presents as poor joint stability, joint discomfort, edema, and morning stiffness. Joint dysfunction and deformity can also happen in extreme situations (5,6). Therefore, it is crucial to diagnose and treat OA as soon as possible. With the advancement of molecular biology, the use of biological markers in OA diagnosis has gained increased attention. Investigations demonstrated that the pathophysiology of OA is heavily influenced by low-grade, persistent inflammation (7). The clinical value of inflammatory mediators in the early diagnosis and prognosis of the disease is a common area of emphasis for researchers (8). The development of tests that are predictive rather than reactive is the aim of OA biomarker research. OA biomarkers, which show changes in blood, synovial fluid, or urine levels and show tissue development or degeneration, can be recognized earlier than radiographic abnormalities. Numerous novel biomarkers for OA should possess certain qualities in it to be used for early-stage prognostic and diagnostic purposes (9)

Pro-inflammatory cytokines, in particular, IL-1 and TNF-, are essential for OA onset and progression. The cartilage breakdown is brought on by IL-1, whilst inflammation is sparked by TNF-alpha. Both of these mediators can cause synovial cells and chondrocytes to release additional pro-inflammatory cytokines like IL-6 (10,11). Another pro-inflammatory cytokine, IL-6, stimulates the growth of chondrocytes and amplifies the effects of IL-1 by increasing the number of inflammatory cells in synovial tissue (11). According to Streich NA, an increase in CTX-1 levels is associated with an increase in the local production of inflammatory cytokines, which causes the uncoupling of bone turnover and ultimately the degeneration of cartilage (12). According to Davis CR, there is a link between CTX-I levels and K-L grades as well as joint space width, which indicates general pathological alterations in the knee (13). According to Garnero P, bone degeneration follows cartilage disintegration just as CTX-1 release followed CTX-II. The biomarker C- terminal telopeptides of collagen type II (CTX-II) can show degenerative alterations and joint tissue metabolism (14). It can be found in the synovial fluid, blood, and urine. When OA develops, the articular cartilage repair and rebuilding process is expedited, and the level of CTX-II in bodily fluids also rises (15). In the form of nano-collagen, CTX-II travels through joint blood, synovial fluid, and urine after being generated by the cleavage of mature collagen type II (16). According to studies, the OA in the population in our society is substantial, hence this group needs to be targeted. Therefore, it is essential to create new serological markers that are non-invasive and economical to reduce the global burden of disease in the future. The lack of research conducted on the Indian population based on the examination of blood biomarkers in the various stages of osteoarthritis are lacunae in the literature. The current pilot study assessed the relationship between proinflammatory cytokines and biomarkers from patients with early-stage (Kellegren-Lawrence grade1-2) and late-stage (grade 3-4) to those in healthy donors under the hypothesis that relationships between these chemokines and clinical evaluations in OA patients are possible. The objectives of this study were

1) To estimate the levels of pro-inflammatory cytokines and biomarkers in the various stages of osteoarthritis

2) To compare the pro-inflammatory cytokines and biomarkers levels in healthy controls with cases.

3) To compare the levels of pro-inflammatory cytokines and biomarkers in an early stage with the late stage of osteoarthritis i.e., within grades.

MATERIALS AND METHODOLOGY:

This case-control series was conducted in the Orthopaedics and Biochemistry departments at a Medical College in central Maharastra from January 2020 to July 2021. The study was approved by the institutional research and ethics committee. Sample size estimation is done as the Fisher's formula Sample Size 18.

(n) = Z2 pq/d2

For a 95 % ca confidence interval the value of Z=1.96 Calculation of Prevalence/Incidence (p): The global age-standardized prevalence of knee OA was estimated to be 3.8% by a study published in 2014.19 Thus p= 3.8% q= (100-p) =96.2% d=Error percentage. We considered an error of 5%.

Sample Size= 1.96x1.96x3.8x96.2/5x5; Sample Size=56.17 rounded off to 56.

To make provision the incomplete or inaccurate data, withdrawal of consent, drop, out, etc. an estimated 10 % was added to the calculated sample. Therefore, the minimum final sample size came to 56+ 5.6=61.6 rounded off to 62 For comparison of pro-inflammatory cytokines and biomarkers in normal healthy individuals, age-gender-matched controls were also enrolled. The number of subjects enrolled as controls were taken roughly in the ratio of 2:1 with the cases. Thus, 32 healthy individuals between the ages of 40 and 75 and a total of 63 cases of knee osteoarthritis were enrolled.

All patients who presented to the Orthopaedics OPD with primary knee osteoarthritis symptoms and signs underwent screening. The study's goal and relevance were conveyed to the volunteered recruited subjects (cases and controls) were added to the study after giving informed, written consent. There was no dropout and all the data could be collected from all of the 95 enrolled cases.

Inclusion criteria:

Cases:

Those who meet the American College of Rheumatology's criteria for primary knee osteoarthritis

Controls:

Healthy subjects with age between 40 and 75 years

No symptoms or signs of osteoarthritis

preferably, the cases' first-degree relatives

Exclusion criteria:

1. Intraarticular Steroids or any other Injections

2. Secondary OA – Fracture, Rheumatoid arthritis, seronegative spondyloarthropathies,

3. Pregnancy and lactating mothers

Methodology: -

Clinically and radiographically diagnosed patients were considered.

- Demographic data like age, gender, weight, height, body mass index (BMI), and occupational details were taken.
- Blood samples: - blood samples of recruited cases and controls were collected, centrifuged and serum stored at -80℃ until processing
- Blood volume collected- 5ml/patient
- Samples were measured by ELISA (Enzyme-Linked Immuno-sorbent Assay) (Elabscience; catalog no: E-EL-H0835) according to the manufacturer's protocols
- Primary data was collected in paper-based proforma and the data was then entered in Microsoft Excel spreadsheets 2016.

IBM SPSS STATISTICS VERSION 20 was used for the statistical analysis. Cross tabulations were performed for the selected parameters, and column proportions were compared using the Chi-square test. Categorical variables were taken in the form of frequencies and proportions. Since the data distribution was not normal, continuous variables were expressed in the descriptive statistics tables as means and standard deviation, and they were compared using the Mann-Whitney test (for two independent samples) and the Kruskal-Wallis test (for more than two independent samples).

Sensitivity and 1-sensitivity were used to plot the receiver operating characteristic curve (ROC). Biomarkers for predicting the existence of knee osteoarthritis were discovered by their area under the curve (with a 95% confidence range). Sensitivity, specificity, positive predictive value, and negative predictive value were determined using the cut-off values of the relevant markers. P value <0.05 and p value<0.01 were regarded as significant and very significant, respectively.

We used the ROC curve to understand how likely the individual will have the disease. The more specific the test, the less likely an individual with a positive test will be free from disease and will show a greater positive predictive value. Based on how many persons have the disease, sensitivity is determined. Sensitivity was previously calculated as follows: sensitivity = number of true positives / (number of true positives + number of false negatives). The highest true positive rate and lowest false positive rate are found in the best cut-off.

RESULTS:

Table 1: Differences in cases and controls demographic and clinical characteristics

Variables	**Control N (%) 32 (33.65)**	**Cases N (%) 63 (66.31)**	**Total N (%) 95 (100)**	**P-value**
Gender Male Female	 17 (53.13) 15 (46.88)	 25 (39.68) 38 (60.32)	 42 (44.21) 53 (56.79)	 0.275
History of Strenuous Activities Squatting or kneeling	 4 (12.05)	 24 (38.10)	 28 (29.47)	 0.01
Continuous standing for more than 1 hour a day	10 (31.25)	25 (39.68)	35 (36.84)	.503
Frequent Lifting of heavy weights (> 10 Kg)	2 (6.23) 16 (50)	12 (19.05) 2 (03.17)	24 (25.26) 8 (8.42)	0.13
Kellegren & Lawrence grading of OA Knee Stage 1		 19 (30.2)		

Stage 2 Stage 3 Stage 4		30 (47.6) 8 (12.7) 6 (9.5)		
	Mean ±std			
Age (years)	57.22 ±9.99	60.13 ±11.22	58.67 ± 10.01	0.236
Height (meters) Weight (KG) BMI (kg/m 2)	1.66 ±0.08 66.77 ±4.92 22.63 ± 6.06	1.65± 0.07 68.6± 5.56 25.2±1.52	1.65 ±0.75 67.68±5.24 23.92±3.79	0.532

Table 1: showed significantly (P =0.01) more participants who suffered from OA showed a history of strenuous activities like squatting or kneeling compared to participants in the control group. Prevalence was more in women than in men but the result was not significant. One-fifth (20%) of the participants were in stage 3 and 4 categories of OA.

Table No.2: Comparison of biomarkers between controls and cases

	Controls	Cases	P value
	Mean ± Std	Mean ± std	
IL-1β	10.78 ±6.09	15.67± 13.24	0.302
IL-6	1.27 ± 1.15	3.9±3.53	*<0.001
CTX-I	0.027 ± 0.01	0.05±0.083	0.016*
CTX-lI	0.22 ± 0.3	0.23±0.2	0.106
TNF-α	51.95 ± 97.8	69.24±42.69	<0.001*

Table 2 showed Levels of IL-6, TNF-α and CTX-I are significantly higher in cases as compared to controls.

Table No.3: Bio markers by OA grades

GRADE		IL-1β	IL-6	CTX-I	CTX-lI	TNF-α
Grade 1	Mean	12.04	**2.47**	0.04	0.22	**65.31**
	N	19	19	19	18	19
	Std. Deviation	11.31	2.48	0.03	0.25	48.75
	Median	5.86	1.47	0.02	0.09	44.70
Grade 2	Mean	16.53	**4.03**	0.07	0.27	**57.62**
	N	30	30	30	30	30
	Std. Deviation	12.05	3.22	0.12	0.19	38.70
	Median	15.10	3.34	0.04	0.21	49.06
Grade 3	Mean	10.41	**4.59**	0.04	0.17	**113.20**
	N	8	8	8	8	8
	Std. Deviation	9.29	4.85	0.04	0.13	13.67
	Median	6.99	1.98	0.03	0.11	118.94
Grade 4	Mean	29.92	**6.95**	0.05	0.21	**81.27**
	N	6	6	6	6	6
	Std. Deviation	20.23	4.49	0.02	0.18	31.14
	Median	39.48	7.67	0.05	0.13	72.06
P		0.08	***0.04**	0.37	0.08	***0.01**

Within OA grades, mean IL-6 values increased significantly (P = .04) with an increase in grades. TNF -α did not show a significant consecutive increase with all four grades but mean TNF-α showed an increase in 3rd and 4th grade as compared to

1st and 2nd grade (P = .01). No significant trend was observed with OA grades and mean IL - 1, CTX1, and CTXII.

Correlations:

We looked at the correlation of biomarkers with age, BMI, and OA grade. The correlation between IL6 and BMI is -0.012, TNF-α and BMI are – 0.04, CTX-I and BMI are 0.002, and CTX-II and BMI is 0.177. No parameters were significant with BMI or with age.

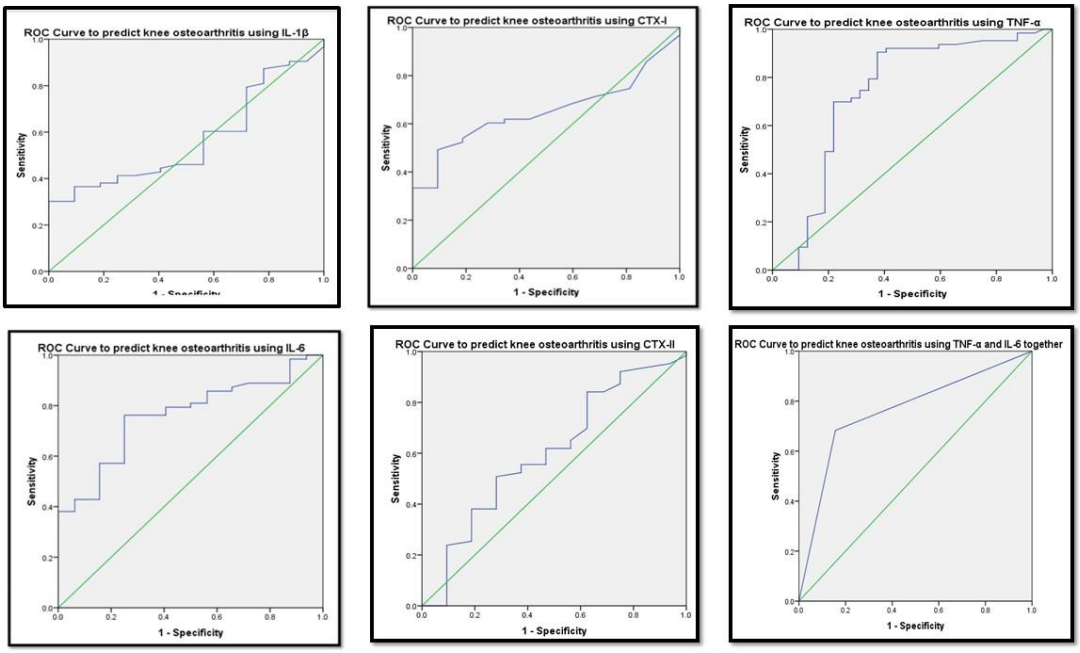

Figure 1: ROC to predict knee osteoarthritis using IL - 1□, CTX-I, CTX -II, TNF-□, IL-6, using TNF-A and IL-6 together.

Table 4: Statistical analysis of Prediction of OA using IL - 1□, CTX-1, CTX -II, TNF-□, and IL-6.

	IL-1β		IL6		CTX -1		CTX-II		TNF-α	
Statistic	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	38.10%	26.15% to 51.20%	76.19%	63.79% to 86.02%	53.97%	40.94% to 66.61%	56.45%	43.26% to 69.01%	90.48%	80.41% to 96.42%
Specificity	81.25%	63.56% to 92.79%	75.00%	56.60% to 88.54%	81.25%	63.56% to 92.79%	62.50%	43.69% to 78.90%	59.38%	40.64% to 76.30%
Positive Predictive Value	80.00%	64.55% to 89.78%	85.71%	76.42% to 91.74%	85.00%	72.67% to 92.35%	74.47%	63.94% to 82.75%	81.43%	74.11% to 87.04%
Negative Predictive Value	40.00%	34.05% to 46.26%	61.54%	49.63% to 72.21%	47.27%	39.55% to 55.13%	42.55%	33.39% to 52.25%	76.00%	58.40% to 87.72%

Table 5: Statistical analysis of Prediction of OA using TNF-A and IL-6 together

Statistic	Value	95% CI
Sensitivity	68.25%	55.31% to 79.42%
Specificity	84.38%	67.21% to 94.72%
Positive Predictive Value	89.58%	79.07% to 95.14%
Negative Predictive Value	57.45%	47.71% to 66.63%

ROC with TNF □ and IL-6 together showed the ability to detect 68% of patients with OA disease. TNF □ and IL-6 together have shown 90% of test positives are true positives. With the ROC curve, it can be concluded that the biomarkers (IL-6, TNF-α, CTX-I and TNF □ and IL-6 together) are good predictors of OA, and we get optimum sensitivity, specificity, and cut-off value. The highest predictive value was observed with IL-6 and TNF □ together.

DISCUSSION:

The elderly population touched 13.8 crores in 2021(22). Elderly adults are prone to developing Osteoarthritis (OA) and it is an important genetic component. MRI and traumatic arthroscopy are very expensive treatments while molecular biology markers are available at an affordable price and early detection is possible (23). Many biomarkers are developed recently for the analysis, detection, and follow-up of OA. Realizing, the vast and diverse population with a growing number of elderlies in India, the paucity of research on this topic, and the importance of early diagnosis, the present case-control study of OA patients was implemented to check levels of pro-inflammatory cytokines plus biomarkers in various stages of osteoarthritis as compared to healthy controls.

This case-control study showed cases and controls were similar in age and gender-wise. A significantly higher mean was observed for IL-6, CTX1, and TNF-α in cases as compared to controls.

The findings revealed IL-6, TNF-α and CTX-I are good predictors of OA. In this study, age and gender-wise significant difference were not observed in Pro-inflammatory cytokines and biomarkers. A significant difference was observed in mean Pro-inflammatory cytokines and biomarkers in various stages of OA with IL-6, and TNF-α.

Researchers frequently look at BMI to measure the effect of additional weight on Knee. Elizabeth Weiss confirmed knee pain increases with OA severity. Her study further stated individuals with higher BMIs experience more pain than individuals having lower BMI (24). The result of Thomas Mabey's study showed levels of IL-6 did not correlate with body mass index (BMI), age, or OA severity (KL) (25). Our analysis proved IL-6 did not correlate with BMI, and age. BMI was higher in cases of OA as compared to controls (25.2 vs 22.6) but the result was not significant.

The review article by Y. Henrotin mentioned the association of IL-6 and TNF-α with osteophytes (26). Other researchers also have noticed elevated levels of IL-6, and TNF-α associated with OA progression. This indicates IL-6, and TNF-α may serve as a biomarker for OA pathogenesis (27). Our study also showed similar results.

The present study showed Levels of IL-6, were significantly higher in cases compared to controls. Similar results are mentioned in the review article by Thomas Mabey and Hikmat Our study indicated a significant ($p<.05$) +ve relationship between IL-6 and OA grading and TNF-α and OA low and high grading (25, 28).

The result of our study that IL-6 was significantly positively correlated with OA grades did not match with the study mentioned above [Thomas Mabey]. A study conducted in Japan with OA patients detected measurable levels of TNF □ and IL-6 (29). The same study showed IL-6 had a significantly negative correlation with KL grade and TNFα was not correlated with OA grade. Our study showed a negative correlation of IL-6 with OA grade and the result was not significant. TNFα was not correlated with OA grade.

Our study results were similar to the findings of the study carried out by Sudhir Singh [30] that showed CTX-I levels in the case groups were significantly higher than in the control Group. However, our results did not get a significant association between K-L grading and urinary CTX-I as reported by Singh.

Using IL-6, TNF-α, and IL6 + TNFα our study could detect >75% of the cases with OA [fair AUC values are noticed]. These results conclude that the biomarkers (IL-6, TNF-α, and IL6 + TNFα) indicated optimum sensitivity, specificity, and cut-off value and hence are good predictors of OA. Using the combination of two biomarkers (IL-6 and TNF-α), we can say that it can decrease the false positive outcomes of disease as their levels are positively increasing. The strength of the present lies in the ability to predict OA with the help of IL-6, TNF-α, and (IL-6 and TNF-α) together.

Limitation of this study: 1. Small sample size. This may prevent extrapolating the results. 2. The number of Controls should have been more (at least double) than the cases. Since matching controls to cases lessen the effects of confounders. This was a single-center study; hence sample is not representative. Lastly, due to the small sample size, we could not look at age-category-wise and gender-wise associations of biomarkers.

CONCLUSIONS:

The goal of the current study is to determine the function of biomarkers in an early diagnosis and assessment of osteoarthritis severity in the Indian population. It is one of the few studies of its kind.

Our study also demonstrated that different biomarkers from tissues that experience metabolic alterations may be useful for diagnosing conditions or creating new treatments. Future consideration of a tailored medicine plan for the treatment of OA will involve the combined assessment of the biomarkers associated with various joint tissue types, such as cartilage, bone, and

synovium, products of pathological pathways, and even hereditary variables. Research, development, quantification, application, and the use of biomarkers are required. A procedure for the validation and quantification of OA biomarkers is required.

Future research should examine their function in OA pharmacodynamics. It would offer a reliable response about whether these biomarkers might be used in therapeutic settings. The use of biomarkers for early OA diagnosis would assist doctors in formulating a plan for treating OA at its earliest stages and might even prove to be cost-effective for patient care.

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