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Identification Of Serum Micro-Rnas Of Early Knee Osteoarthritis In A Cohort OfSaudi Patients

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Abstract:

Background: *Osteoarthritis (OA), is a prevalent low-grade inflammatory synovial joint disease characterized by progressive cartilage degradation and alteration of the entire joint. Knee OA (KOA) is the commonest form of OA. Osteoarthritis is the 4th leading cause of disabilityworldwide with no cure and therefore identifying persons with early OA of the knee (KOA), isimportant to retard progression of the disease process. Several proinflammatory cytokines and microRNAs (miRNAs) are involved in OA pathogenesis.* ***Objective:*** *The aim of the work was to investigate the serum miRNA-136 (miR-136) and miR- 155-5p expression profile, IL-8 and IL-17 levels in subjects with early KOA and to further evaluate and compare the expression profile of these miRNAs, and level of serum IL-8 and IL-17 in subjects with early and severe KOA.* ***Methods:*** *Serum miR-136 and miR-155-5p expression profile in 40 patients with early KOA were compared to 40 age and sex matched healthy controls and 10 patients with severe KOA. Clinical, laboratory, and disease parameters were assessed. Serum and synovial fluid miRNAs were assessed by real-time polymerase chain reaction. Levels of IL-17 and IL-8 levels were determined by the enzyme linked immunosorbent assay test.* ***Results:*** *miR-155-5p in serum was significantly higher in early KOA patients compared to healthy controls and to patients with severe OA. S[1]ynovial miRNA-155-5p expression levels were significantly higher than serum miRNA-155-5p in severe KOA patients compared to early OA patients. When we compared the expression levels of miRNA-136 between cases andhealthy controls, there was no statistical significant difference, p=0.413. Serum IL-8 levels were significantly higher in early OA patients compared to healthy controls.* ***Conclusions:*** *Serum miR-155-5p and IL-8 levels are potential useful biomarkers for the early detection of OA and in particular, early KOA.*

Introduction

Osteoarthritis (OA) is characterized by cartilagedegradation and alteration of the whole joint

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structure [1]. OA has been relabeled as a whole organ disease, because pathologic abnormalities such as periarticularmuscle weakness, lax ligaments, and meniscal degeneration are frequently present in these patients, with progressive synovial inflammation and changes on thesubchondral bone and osteophyte formation [2]. OAis a leading cause of chronic pain and physical dis- ability in older individuals [3].
OA is better thought of as a group of overlapping disorders, of various etiologies and arising froma combination of systemic factors (age, gender, body mass index (BMI), and genetics) and local factors suchas knee biomechanics (joint injury, malalignment, abnormal mechanical loading, overweight/obesity,and muscle weakness), which gradually converge to produce a condition with definable morphologic and clinical outcomes [4]. OA is one of the most costly anddisabling forms of joint disease, being far more common than rheumatoid arthritis and other formsof joint disease [5]. The knee is the most common form of OA joint disease and it the most prevalent form of both radiographically evident and symptomatic OA. Females have a higher prevalence thanmales [6]. The prevalence of OA is expected to increase worldwide due to the aging of the populationsand to the increase in the incidence of obesity becauseage and body mass index (BMI) are both two major risk factors for OA and in particular for KOA [7].

Knee OA is a multi-factorial disease. Knee OA is characterized by structural changes in and around the knee joint [4–6]. The predominant structural changes are the loss of cartilage and the formation of osteophytes [7,8]. These changes are easily demonstrated radiographically, and objective measures of disease severity are based on the amount of joint space loss(a reflection of cartilage loss) and the presence of osteophytes [9]. A common sign of knee OA is synovial inflammation, detected using ultrasonography. Furthermore, magnetic resonance imaging as well as arthroscopic inspection of the knee joint has also provided insights to the presence of inflammation in knee OA [10,11].

OA is a low-grade synovial joint inflammatory dis-order where all tissues of the entire joint are affected characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity [12– 14]. Synovial inflammation plays an important and increasingly recognized role in OA pathophysiology. Cartilage breakdown products increase synovial inflammation. In turn, the inflamed synovium pro- duces catabolic and proinflammatory mediators that lead to excess production of proteolytic enzymes responsible for cartilage breakdown, creatinga positive feedback loop [15,16]. The inflammatory response is amplified by activated synovial T cells,B cells, and infiltrating macrophages [17]. Inflammatory cells and their cytokines are present in both early and late OA [18].
Numerous studies have shown that inflammatory
cytokines provide essential biochemical signals that simulate chondrocytes to release cartilage-degrading enzymes [18]. Moreover, chondrocytes have the capa- city to produce these cytokines themselves andrespond to them by acting via autocrine/paracrine pathways [19]. Abnormal mechanical forces can also activate chondrocytes to produce several cytokines[19]. In addition, oxidative stress may also participate in the induction of cytokine release [20]. Among the many cytokines released, the most important involvedin OA pathophysiology include interleukin-1 (IL-1) β,tumor necrosis factor alpha (TNFα), IL-6, IL-15, IL- 17, IL-8, and IL-18 [17–23].

Understanding the cellular and molecular processesand mediators involved as well as the communication between cells and tissues should may help in the iden-tification of specific strategies to prevent, treat and cure OA.

IL-17, as a potent inflammatory factor, plays an important role in the development of a variety of inflammatory diseases, and it is also an importantregulator in the development of bone metabolism and bone diseases. IL-17A can increase the gene or protein expression of selected

inflammatory media- tors, including IL-6, IL-8, CXCL1, CCL2, COX2, andinducible nitric oxide (iNOS). In addition, IL-17A hasbeen shown to affect ECM by increasing matrix metalloproteinase (MMP) production. IL-17 also stimulatesIL-1 production in chondrocytes [24–26]. IL-8 is a keymediator in the pathogenesis of OA. It is responsible for certain pathogenic events such as: secretion ofMMP-13, accumulation and activation (the activated cells secrete enzymes like elastase which degrades typeII collagen crosslinks and proteoglycan in articular cartilage) of neutrophils, leukocyte stimulation and migration to synovium, induction of angiogenic changes associated with chronic inflammation within the joint, chondrocyte differentiation, and hypertro- phy induction [27–34]. In addition to cytokines, microRNAs (miRNAs) have received considerable attentionas biomarkers of disease activity due to their ease of detection and their stability in body fluids. Many miRNAs control pro-inflammatory factors like IL-1, TNF-α, IL-6, inducible cyclo-oxygenase − 2 (COX-2), NO, MMPs, and reactive oxygen species (ROS) that are elevated in OA [31–34]. MiRNA expression is so common and very stable in plasma and serum sam- ples, miRNAs may be appropriate blood-based diag- nostics for OA.

MicroRNAs (miRNAs) are short, non-coding RNAs that are 19 to 25 nucleotides in length that fine-tune the cell response to a changing environ- ment by modulating the cell transcriptome [35–39]. They regulate gene expression post-transcriptionally by pairing with complementary nucleotide sequences in the 3'-untranslated regions of specific mRNA tar- gets, and have an overarching regulatory role in both normal cellular function and in many diseases including OA [40–42]. MiRNAs have been shownto be involved in the pathology of OA. MicroRNA- 155 (miR-155) is a multifunctional miRNA in cells ofthe immune system whose regulation is highly linked to its role as an immune modulator [43–45]. MiR- 155-5p can control cell proliferation and apoptosis in many diseases [44]. However, when deregulated, miR-155 contributes to the development of chronic inflammation, autoimmunity, cancer, and fibrosis [43]. MiR-155-5p has been reported to be expressed and identified as a potent regulator of cytokine expression in inflammatory arthritis like rheumatoid arthritis [43–45]. MiR-136-5p has been shown to effectively enhance inflammatory factors and chemo- kines via activating nuclear factor kappa beta (NF- κB)/A20 signaling in the IL-17-mediated inflammatory response both in vitro and in vivo. Over- expression of miR-136-5p appears to inhibit cartilage degeneration [46,47].

The diagnosis of OA is largely based on clinical examination and radiology, both methods lacking sensitivity and specificity and do not detect early OA. There is now a growing interest in molecular markers in OA due to their potential usefulness in formulating early diagnosis, in assessing disease activity and severity and in evaluating drug effects. There is thus, an urgent need for new diagnostic biomarkers [48,49]. The numerous participants in OA (bone, synovitis, cartilage, and systemic inflammation) as well as the varied stages of these tissues can be represented by candidates for biochemical markers of joint tissues in OA [50–54]. Biomarkers are helpful in elucidating the disease physiopathology and supporting an impression of the OA disease state in cohort studies and clinical trials.

The aim of the present study was to investigate the expression profile of miR-136 and miR-155-5p, as well as the IL-8 and IL-17 levels in subjects with early KOAand further assess and compare the expression profile of these miRNAs, and level of serum IL-8 and IL-17 insubjects with early and severe KOA.

Subjects and methods

Study subjects

In this case–control study, 90 subjects were recruited from the Rheumatology and Clinical Immunology Unit of the Medicine Department, Makkah hospitals Hospital. An informed

consent was taken from all patients before the beginning ofthe study. The study was conducted on three groups. The first group (group A) consisted of 40 adult patients with early osteoarthritis defined by WOMAC score ≤ 60 [48] and Kellgren-Lawrence (KL) grade ≤1 [49]. The second group (group B) consisted of 40 adult persons (age and sex matched) and were the control group. The third group (groupC) consisted of 10 adult patients with severe osteoarthritis defined by WOMAC score ≥81 [48] and Kellgren-Lawrence (KL) grade 4 [49]. Patients with systemic infection, other rheumatic diseases, other autoimmune diseases and trauma to the joint were excluded from the study. This study was approved by the local ethics committee of our institution. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Methods

All patients were subjected to a detailed history takingincluding age, sex, body mass index, and duration of the disease. A complete clinical examination and assessment of OA severity by the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire and the Kellgren-Lawrence (KL) scale using radiographs. Radiologic evaluation ofthe knees consisted of bilateral posteroanterior weight-bearing knee radiographs. The severity of OA was graded according to Kellgren and Lawrence (KL)system according to the following criteria: grade 1 (doubtful narrowing of joint space and possible osteo- phytes), grade 2 (definite osteophytes and possible narrowing of joint space), grade 3 (moderate multiple osteophytes, definite narrowing of joint space and some sclerosis, and possible deformity of bone ends), and grade 4 (large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity ofbone ends). Grade: early stage (WOMAC score ≤ 60; KL Grade: I/II) and advanced stage (WOMAC score ≥81; KL Grade: III/IV).

Venous blood samples were collected from all sub-jects after proper disinfection. Synovial samples were aspirated from the knee joints of 10 patients with severe OA. Laboratory evaluation included: CBC, ESR, CRP, ANA, RF, Serum IL-17, IL-8 level by ELISA technique. Micro-RNA expression was deter- mined using real-time polymerase chain reaction. Total micro-RNA was extracted from peripheralblood mono-nuclear cells using the miRNeasy Mini kit (Qiagen, Germany cat no. 217004). The Protocol ofRT was according to instructions of the miRCURY LNA miRNA PCR assay kit (Qiagen, Germany).

Detection and principle of the test

Blood samples were collected into citrate tubes, with- out using a tourniquet. Immediately after collection of the samples, blood cells were removed by a 2-step centrifugation protocol performed at room tempera- ture (1,800 g for 10 minutes, then 3,000 g at 10 min- utes) to obtain platelet-poor, cell-free plasma. Aliquots(250 µL) were snap frozen in liquid nitrogen. Samples were drawn into EDTA-coated tubes and left for1 hour before 1-step centrifugation (1,600 g for 10 min-utes at room temperature). All samples were stored at
−80°C until analyzed.

Preparations before starting the analysis

Template RNA and 5x miRCURY RT SYBR® GreenReaction Buffer was thawed on ice. RNase-free water was thawed at room temperature. Each solution was mixed by flicking the tubes, centrifuged briefly tocollect residual liquid from the sides of the tubes and then kept on ice. UniSp6 RNA spike-in was resuspended by adding 80 µl nuclease-free water to the tube, mixed by vortexing and briefly centrifuged. It was then left for 20–30 min on ice to fully dissolve theRNA spike-in. It was then stored in aliquots at −30 to−15°C. Immediately before use, the 10× miRCURY RT enzyme was removed from the freezer, mixed by flick-ing the tube

and placed on ice. It was then briefly centrifuged to collect residual liquid from the sidesof the tubes, and then kept on ice (Table 1).

miR base ID	NCBI accession number	miRCURY LNA miRNA PCR assay, Qiagen	Sequence of the mature miRNA ([3–5])
Has-miR-136-5p	406,927	YP00204779	ACUCCAUUUGUUUUGAUGAUGGA
Has-miR-155-5p	406,947	YP00204308	UUAAUGCUAAUCGUGAUAGGGGU

RNA isolation and miRNA profiling

(1) The volume of RNA was calculated corresponding to 16 µl of original serum/plasma sample for each 20 µl RT reaction (e.g. forRNA isolated from 200 µl plasma and elutedin 50 µl, use 4 µl eluate in each RT reaction or RNA isolated from 200 µl plasma and eluted in14 µl, use 1.12 µl eluate in each 20 µl RT reac- tion [14 µl/200 µl * 16 µl]).

Template RNA [µl] = Elution volume [µl]/Original sample volume [µl] * 16 [µl]

2. The reverse transcription reactions were prepared on ice according to (Table 1), mixed and then placed on ice.
3. It was incubated for 60 min at 42°C.
4. It was incubated for 5 min at 95°C to heat,inactivate the reverse transcriptase.
5. It was then immediately cooled to 4°C (Table 2).
6. The reverse transcription reactions were placed on ice and proceeded directly with real-time PCR. Therecommendations were followed for proper cDNA dilution provided in the protocol for the PCR Assayor Panel to be used. The quantity and purity of RNA were assayed using Nanodrop 2000c spectrophotometer (serial no 4354, Thermo Scientific, USA). The predesigned miRNA primers used were miRNA 155–5p and 136–5p and U6snRNA as housekeeping gene primer for normal- ization. (All were ready made, miRCURY LNA miRNA PCR assay, Qiagen, Germany.)

Parameter	Group A (n = 40)	Group B (n = 40) p C (n = 10)	p-value Grou
Sex			
Male	6(15)	16(40) 4 (40)	0.174
Female	34(85)	24(60) 6(60)	
Significance between groups		$p_1 = 0.580$, $p_2 < 0.001$*, $p_3 < 0.001$*	
Weight (kg)			<0.001*
Min. – Max	32.0–78.0	34.0–80.0 8.0–78.0	5

Mean (SD)	50.63(11.45)	48.25(10.44)	67
		.60(7.31)	
Significance between groups		$p_1 = 0.580$, $p_2 < 0.001$*, $p_3 <$ 0.001*	
Height (cm)			0.064
Min. – Max	159.0–182.0	156.0–185.0	15
		6.0–175.0	
Mean (SD)	168.2(5.52)	170.0(6.61)	16
		5.0(5.85)	
BMI (kg/m^2)			^{MC}p = 0.001*
Underweight, n(%)	0(0.0)	0(0.0)	<0.001*
		0(0.0)	
Normal, n(%)	12(30)	22(55.0)	
		0(0.0)	
Overweight, n(%)	14(35)	13(32.5)	2
		(20.0)	
Obese class I, n(%)	9(22.5)	2(5.0)	5
		(50.0)	
Obese class II, n(%)	3(7.5)	2(5.0)	1
		(10.00)	
Obese class III, n(%)	2(5.0)	1(2.5)	2
		(20.0)	
Min. – Max	22.50–44.10	20.80–41.50	27
		.50–43.60	
Mean (SD)	29.22(5.25)	26.09(4.82)	34
		.24(5.34)	
Significance between groups		$p_1 = 0.019$*,$p_2 = 0.017$*,$p_3 <$ 0.001*	

Data handling and statistical analysis

Average raw quantification cycle (Cq) values of >30 were removed from all data sets. Average Cq values ofthe duplicate analysis of each miRNA were then sub- tracted from the average Cq value of the three cell- miRNAs for that particular sample, yielding the Δ Cq values used in further analyses. All ΔCq values were then row-normalized to correct for variations in total input RNA. The average value of 26 miRNAs that were detected in all samples (miR-155-5p &miR-136) was subtracted from all miRNA _Cq values in each sample.These row normalized expression values were used forthe statistical analyses.

The data was inserted into Microsoft excel andanalyzed statistically using the statistical package for social sciences – SPSS 18 software package (SPSS Inc., Chicago, Illinois, USA). The normally distributed datawere expressed as mean ± SD. Multiple group comparisons were performed by one-way analysis of variance. Univariate correlations between study variables were calculated with Spearman's rank correlation coefficients (r). P-values less than 0.05 were considered significant. The diagnostic ability of miR-155-5p and miR-136 was identified using the ROC curve (receiveroperating characteristic curve). The data was tested fornormality using Kolmogorov–Smirnov test which was significant indicating the use of nonparametric tests. The statistical significance was analyzed using Spearman's correlation coefficient, Mann–Whitney test, and Monte-Carlo test. $P < 0.05$ was accepted as statistically significant. ROC curves with the area under the curve (AUC) were used to assess the diag- nostic performance of miR-155-5p and miR-136, an area = 1.00 (100%) denoting (a gold standard-like) performance, while an area = 0.5 (50%) denoting (a chance-like) performance. Significant areas ($p < 0.05$)indicate that the diagnostic performance is signifi-cantly better than chance. Cutoff values were chosen as the points that maximize the sensitivity and specificity.

Results:

This study included 6 males (15%) and 34 females (85%), with a mean age of (50.63 ± 11.45 year) in group A, 16 males (40%) and 24 females (60%) witha mean age of (48.25 ± 10.44) in group B and 4 males(40%) and 6 females (60%) with a mean age of (67.60 ± 7.31) in group C. There was no statistically significant difference regarding age and sex between group A and group B. There was a statistically significant increase in age in group C compared to group A ($p2 < 0.001$) and compared to group B ($p1 < 0.001$)[Table 3]. There was a statistically significant increasein BMI in group A compared to group B ($p1 < 0.002$),increase in group C compared to group A ($p2 < 0.017$) and compared to group B (Table 3). The mean value of microRNA-155-5p expression levels in cases (groups A and C) was significantly higher than the mean value of healthy controls (group B) ($p < 0.001$) [Figure 1, Table 4]. The expression levels of miRNA- 155-5p in the three groups are shown in Figure 2. TheROC curve of microRNA-155-5p expression levels (Table 5, Figure 3) shows that of microRNA-155-5p expression levels can significantly discriminate between patients with OA and persons without OAat a cutoff level >1.88 as diagnostic of OA. The mean value of microRNA-155-5p expression levels in groupA was significantly higher than the mean value of group B ($p1 < 0.001$), in comparison between groupA and group C, the mean value of microRNA-155-5p expression levels in group A was significantly higher than the mean value of group C ($p2 = 0.002$) and in comparison between group B and group C, the mean value of microRNA-155 expression levels in group B,there was no a statistical significant difference betweenthe 2 groups, $p3 = 0.942$ (Table 6, Figure 4).

Table 3:

Parameter	No.	%	No.	%	No.	%	p-value
Sex							
Male	6	15.0	16	40.0	4	40.0	0.174
Female	34	85.0	24	60.0	6	60.0	
Significance between groups			$p_1 = 0.172$, $p_2 = 0.097$, $p_3 = 0.462$				
Age (years)							
Min. – Max.	32.0–78.0		34.0–80.0		58.0–78.0		<0.001*
Mean ± SD	50.63 ±		48.25 ± 10.44		67.60 ± 7.31		

	11.45					
Significance between groups		$p_1 = 0.580$, $p_2 < 0.001$*, $p_3 < 0.001$*				
Weight (kg)						
Min. – Max.	60.0–115.0	54.0–120.0			75.0–106.0	0.002*
Mean ± SD	82.50 ± 13.90	75.48 ± 14.83			92.60 ± 9.99	
Significance between groups		$p_1 = 0.069$, $p_2 = 0.108$, $p_3 = 0.002$*				
Height (cm)						
Min. – Max.	159.0–182.0	156.0–185.0			156.0–175.0	0.064
Mean ± SD	168.2 ± 5.52	170.0 ± 6.61			165.0 ± 5.85	
BMI (kg/m^2)						
Underweight						$^{MC}p = 0.001$*
Normal	12	30.0	22	55.0	0.0	
Overweight	14	35.0	13	32.5	20.0	
Obese class I	9	22.5	2	5.0	50.0	
Obese class II	3	7.5	2	5.0	10.0	
Obese class III	2	5.0	1	2.5	20.0	
Min. – Max.	22.50–44.10	20.80–41.50			27.50–43.60	<0.001*
Mean ± SD	29.22 ± 5.25	26.09 ± 4.82			34.24 ± 5.34	
Significance between groups		$p_1 = 0.019$*, $p_2 = 0.017$*, $p_3 < 0.001$*				

Figure 1:

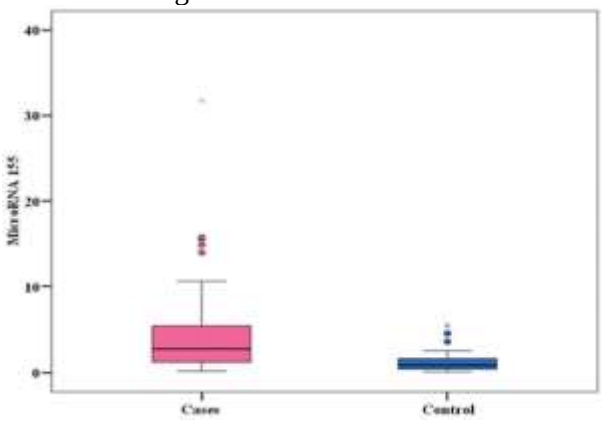


Figure 2

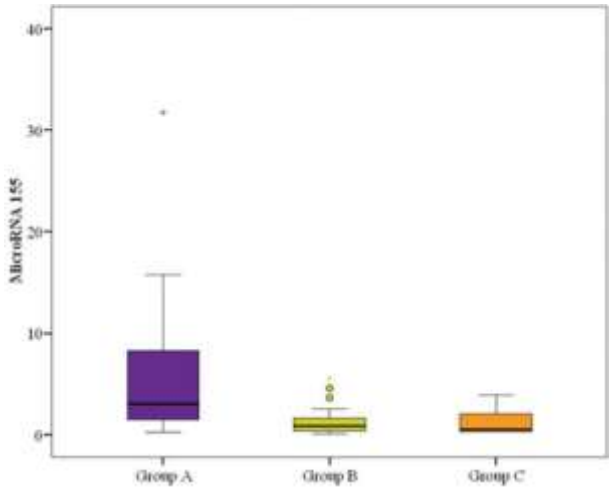


Figure 3:

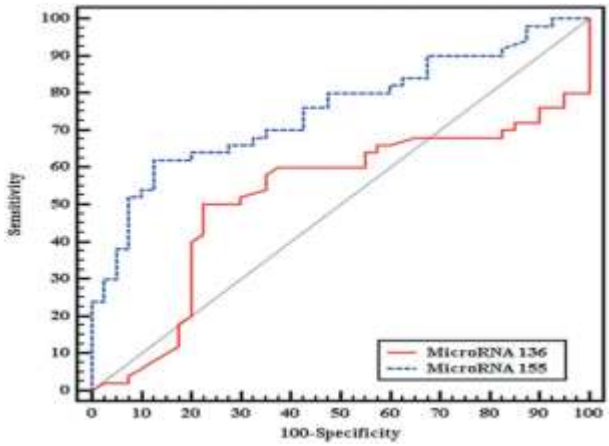


Table 4:

	Cases(n = 50)	Control (n = 40)	U	p
MicroRNA-136				
Min. – Max.	0.01–9.01	0.01–2.81	793.50	0.950
Mean ± SD.	1.41 ± 1.96	0.96 ± 0.81		
Median (IQR)	0.35 (0.13–2.24)	0.68 (0.30–1.51)		
MicroRNA-155				
Min. – Max.	0.20–31.72	0.10–5.46	495.0*	<0.001*
Mean ± SD.	4.72 ± 5.82	1.24 ± 1.16		
Median (IQR)	2.70 (1.25–5.42)	0.87 (0.39–1.62)		

Table 5:

AUC		p		95% C.I	Cutoff#	Sensitivity	Specificity	PPV
MicroRNA-155-5p	0.753*	<0.001*	0.653–0.852	>1.88#	62.0	87.5	86.1	64.8
AUC		p		95% C.I	Cutoff#	Sensitivity	Specificity	PPV

ROC curve of miRNA-155-5p expression levels (Table 7, Figure 5 shows that of miRNA-155-5p expression levels can significantly discriminate between patients with early OA and patients with severe OA at a cutofflevel > 0.61 diagnostic of early OA. Values of micro RNA-136 in slightly less than half of the studied patients without OA (45.0%) were below the 3rd quar-tile as compared to only one-tenth of patients with OA(10.0%), with an odds ratio of 0.131 denoting that the probability of having microRNA-136 values below the 3rd quartile in patients without OA is 0.131 times greater than the probability in patients with OA. This difference was statistically significant (p = 0.002). The value of microRNA-155-5p in nearlyone-third of the studied patients with OA (34.0%) wasbelow the 4th quartile compared to only minority of patients without OA (5.0%), the probability of having values of microRNA-155-5p below 4th quartile in patients with OA was 15.93 times greater than that in patients without OA as denoted by odds ratio (OR = 15.93), this difference was statistically signifi- cant (p = 0.001) [Table 8]. Univariate linear regression for the parameters affecting MicroRNA-136 and MicroRNA-155-5p for cases showed that WOMAC score was the only significant predictor of MicroRNA-136, with each one unit increase of WOMAC score decreasing MicroRNA-136 by 0.024 units (Figure 6). The KL score was the only significantpredictor of MicroRNA-155-5p; where each unit increase of KL score was associated with a decrease inmicroRNA-155-5p by 1.470 units (Table 9a). The mean microRNA-155-5p in patients with early OA was (5.58 ± 6.20) as compared to (1.27 ± 1.24) in patients with severe OA, the probability of having microRNA-155-5p in patients with early OA was 1.977 times greater than patients with severe OA, with statistically significant difference between thetwo (Table 9b). Subjects without OA (57.5%) and patients with OA (64.0%) had microRNA-136 values below the median (0.51) with no statistically significant difference between the two groups. While nearly two-thirds of patients with OA (66.0%) had microRNA-155-5 p values above the median (≥1.56) as compared to less than one-third of subjects without OA (30.0%) with OR = 2.750 denoting that the probability of having microRNA-155-5p values above the median in patients with OA is 2.750 times greater thanthe probability in subjects without OA with statistically significant difference between the two groups(p = 0.003) (Table 10).

On analyzing the distribution of microRNA-136and microRNA-155-5p in patients with severe OA as compared to patients with early OA, it was found that comparable proportions of patients with severe OA 40.0%) and patients with early OA (52.6%) havemicroRNA-136 values above the median (≥0.35) withno statistical significant difference between the two groups, whereas three-fifths of patients with early OA (60.0%) had microRNA-155-5p values above the median as compared to one-tenth of patients with severe OA (10.0%), the probability of having microRNA-155-5p values above the median in patients with early OA was 13.5 times greater thanthe probability in patients with late OA (OR = 13.5), with statistical significant difference between the two groups (p = 0.018) [Table 11]. In comparison between the mean value of microRNA-136 expression levels inserum of group C (0.69 ± 1.01) and the mean value of microRNA-136 expression levels in synovial fluid of group C (0.70 ± 1.0), there was no statistical significantdifference, p = 0.385. The mean value of microRNA- 155-5p expression levels in synovial fluid of groupC (1.29 ± 1.23) was significantly higher than the mean value of microRNA-155-5p expression levels inserum of group C (1.27 ± 1.24), p = 0.024 (Table 12, Figure 7). The mean values of IL-8 and IL-17 levels ingroup A were significantly higher than the mean values of group B (p1 = 0.027) [Table 13 and 14, Figure 8].

Table 6:

	Group A(n = 40)	Group B(n = 40)	Group C(n = 10)	H	p
MicroRNA-136					
Min. – Max.	0.01–9.01	0.01–2.81	0.05–3.27	1.767	0.413

Mean ± SD.	1.59 ± 2.11	0.96 ± 0.81	0.69 ± 1.01		
Median (IQR)	0.42 (0.14–2.62)	0.68 (0.30–1.51)	0.18 (0.08–1.11)		
MicroRNA -155-5p					
Min. – Max.	0.20–31.72	0.10–5.46	0.30–3.83	26.847*	<0.001*
Mean ± SD.	5.58 ± 6.20	1.24 ± 1.16	1.27 ± 1.24		
Median (IQR)	3.01 (1.46–8.25)	0.87 (0.39–1.62)	0.54 (0.32–2.02)		
Significance between groups		$p_1 < 0.001$*,$p_2 = 0.002$*, $p_3 = 0.942$			

Figure 4:

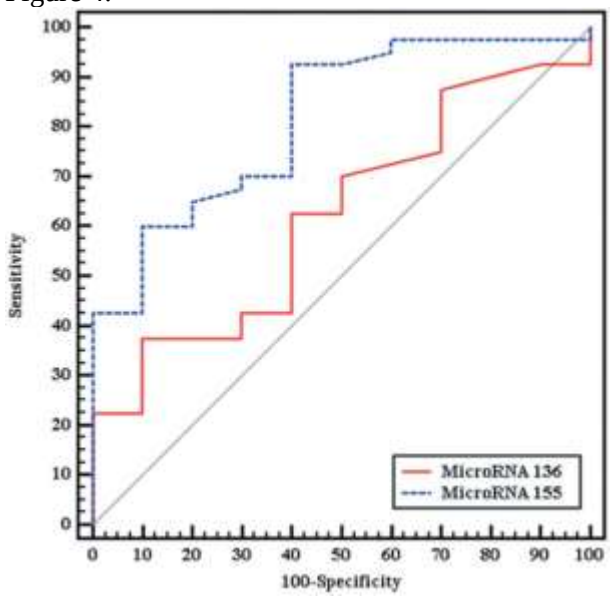


Figure 5:

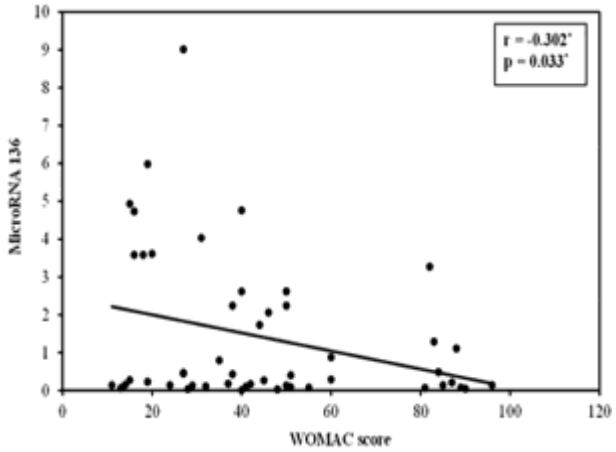


Table 7:

	AUC	p	95% C.I	Cutoff#	Sensitivity	Specificity	PPV	NPV
MicroRNA-136	0.620	0.244	0.439–0.801					
MicroRNA-155-5p	0.815*	0.002*	0.675–0.955	>0.61	92.50	60.0	90.2	66.7

Table 8:

	Without OA(n = 40)	With OA(n = 50)	OR (95% C.I)	p
MicroRNA 136				
1st quartile ®	8 (20%)	17 (34%)	1.000	—
2nd quartile	7 (17.5%)	13 (26%)	0.874 (0.252–3.036)	0.832
3rd quartile	18 (45%)	5 (10%)	0.131* (0.036–0.479)	0.002*
4th quartile	7 (17.5%)	15 (30%)	1.008 (0.295–3.447)	0.989
MicroRNA 155				
1st quartile ®	15 (37.5%)	8 (16%)	1.000	—
2nd quartile	13 (32.5%)	9 (18%)	1.298 (0.388–4.343)	0.672
3rd quartile	10 (25%)	16 (32%)	3.0 (0.934–9.631)	0.065
4th quartile	2 (5%)	17 (34%)	15.93*(2.91–87.06)	**0.001***

Figure 6:

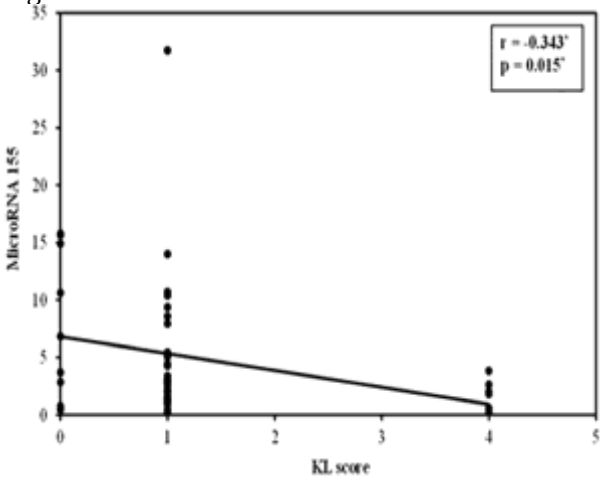


Table 9:

	MicroRNA 136		MicroRNA 155	
Cases (n = 50)	B (95% C.I)	p	B (95% C.I)	p
Age (years)	–0.020 (–0.065–0.024)	**0.363**	–0.107 (–0.236–0.023)	**0.103**
BMI (kg/m^2)	–0.092 (–0.191–0.006)	**0.065**	–0.184 (–0.481–0.113)	**0.220**
KL score WOMAC	−0.312 (–0.723–0.098)	**0.132**	−1.470*(–2.640 – –0.30)	**0.015***
score	–0.024*(–0.046 – –0.002)	**0.033***	–0.054 (–0.120–0.012)	**0.109**

Table 10:

	Group C Severe (n = 10)	Group A Early (n = 40)	AOR#(95% C.I)	p
MicroRNA 155	1.27 ± 1.24	5.58 ± 6.20	1.977*(1.034–3.781)	**0.039***

Table 11:

Total sample	Without OA (n = 40)	With OA (n = 50)	OR (95% C.I)	p
MicroRNA 136				

Below median (<0.51) ®	23 (57.5%)	32 (64%)	1.000	–
Above median (≥0.51)	17 (42.5%)	18 (36%)	0.761 (0.325–1.785)	**0.530**
MicroRNA 155				
Below median (<1.56) ®	28 (70%)	17 (34%)	1.000	–
Above median (≥1.56)	12 (30%)	33 (66%)	2.750*(0.090–0.540)	**0.003***

OR: Odd's ratio
CI: Confidence interval
LL: Lower limit
UL: Upper Limit
*: Statistically significant at $p \leq 0.05$

Table 12:

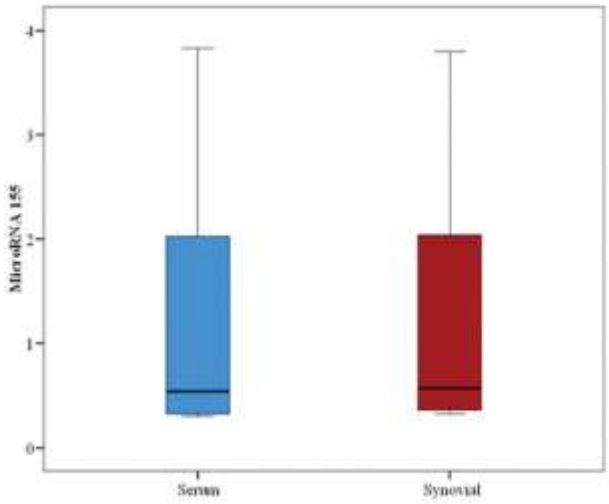
Cases Severe (n = 10)	Group	CGroup A Early (n = 40)	OR (95% CI)	p
MicroRNA 136				
Below median (<0.35) ® (60%)	6	19 (47.5%)	1.000	—
Above median (≥0.35) (40%)	4	21 (52.5%)	1.658 (0.405–6.785)	0.482
MicroRNA 155				
Below median (<2.70) ® (90%)	9	16 (40%)	1.000	—
Above median (≥2.70) (10%)	1	24 (60%)	13.50*(1.556–117.14)	0.018*

OR: Odd's ratio
CI: Confidence interval
LL: Lower limit
UL: Upper limit
*: Statistically significant at $p \leq 0.05$

Table 13:

	Serum	Synovial	Z	p
MicroRNA-136				
Min. – Max.	0.05–3.27	0.01–3.20	0.868	0.385
Mean ± SD.	0.69 ± 1.01	0.70 ± 1.0		
Median (IQR)	0.18 (0.08–1.11)	0.25 (0.09–1.15)		
MicroRNA-155-5P				
Min.– Max.	0.30–3.83	0.32–3.80	2.256*	0.024*
Mean ± SD.	1.27 ± 1.24	1.29 ± 1.23		
Median (IQR)	0.54 (0.32–2.02)	0.57 (0.36–2.04)		

Figure 7:



Discussion:

One of the hallmarks of OA is inflammation in the joints[2,3]. TNF-α, IL-6, IL-1, inducible cyclooxygenase-2 (COX-2), MMPs, NO, and ROS are among the pro- inflammatory cytokines that are greatly elevated in OA. Chondrocytes, synoviocytes, osteoblasts, osteoclasts, and macrophages are among the cell types affected by theseproinflammatory mediators in the OA damaged joints. OA affects the expression of many miRNAs that regulate pro-inflammatory factors, at least in vitro [35–37].

There is increasing evidence that abnormal expression of miRNAs play an important role in the pathogenesis of OA and that circulating miRNAs are potentially derived from tissue injury-cartilage degradation, synovial inflammation, and apoptosis. Biomarkers or markers of OA disease activity that can differentiate between early- and late-stage KOA are not known yet.

Our findings provide evidence for the involvementof miRNAs in OA pathology, and emphasize their possible application as a diagnostic tool or biomarker. The present study attempted to investigate poten- tial OA biomarkers. In the present work, we report the upregulation of miR-155-5p in serum and synovialfluid of early OA patients as we studied the serummiRNA miR-136 and miR-155-5p expression profilein KOA (early and advanced-stage/severe). We reporta significant association, independent of age, sex and BMI, of serum miR-155-5p and KOA.

We evaluated and compared the circulating serum miR-136, miR-155-5p, IL-8, and IL-17 levels in early and late-stage KOA. In the present study, there was nostatistical significant difference between early OA patients and healthy controls regarding age ($P = 0.172$); however, there was a statistically significant difference in age in late-stage OA compared to early OA patients ($p2 < 0.001$) and compared to con- trols ($p3 < 0.001$). Also, in this study, there were morefemales (60%) in the early OA and in the late-stage OA. In agreement with our results, Fernandez-Cuadros et al. found that OA increases exponentially with age, with a clear predominance in women [55]. Inour study group, there was a statistically significant increase in BMI in cases compared to controls and there was a statistically significant increase in BMI in the severe OA group when compared to early OA (p1 0.002) (p2 0.017). Blagojevic et al. [56], after reviewing 36 publications on BMI, found that being obese and overweight were major risk factors for KOA in all studies.

In this study, microRNA-155-5p expression levels were significantly higher in OA patients comparedto healthy controls and microRNA-155-5p expres- sion levels were significantly higher in early OA patients compared to severe OA patients. Ata cutoff level of >0.61 as diagnostic of early OA, our study found that microRNA-155-5p expressionlevels can

significantly differentiate between indivi- duals with early OA and those with advanced OA. Furthermore, at a cutoff level of >1.88, microRNA- 155-5p expression levels can significantly distinguish between patients with OA and those without OA asa diagnostic of OA.

Our findings showed that the probability of having values of micro RNA-155-5p below 4th quartile in patients with OA is 15.93 times greater than that in patients without OA (OR = 15.93), this difference wasstatistically significant (p = 0.001).

In this study, KL score was the only significant predictor of microRNA-155-5p; where with each unit increase of KL score was associated with a decrease ofmicroRNA-155-5p expression by 1.470 units. In addi-tion, the probability of having microRNA- 155-5pexpression in patients with early OA was 1.977 times greater than patients with severe OA.

Furthermore, OA patients had a 2.750 timesincreased chance of having microRNA-155-5p values above the median compared to subjects without OA, while the probability of having microRNA- 155-5p values above the median in patients with early OAwas 13.5 times greater than the probability in patients with late OA (OR = 13.5).

MiR-155-5p has recently been discovered as a key regulator of cytokine expression in inflammatory arthritis, with high levels of expression. MiRNA-155 is activated by proinflammatory mediators such as IL-1 and TNF [57]. MiR-155-5p upregulates pro-inflammatory cytokines like IL-1B, IL-6, IL-8, and TNF-α [57].

In line with our findings, Okuhara et al. showed that in OA patients, miRNA-155 expression was sig- nificantly greater than healthy subjects [58].

In contrast to our results, Soyocak et al. observed that the expression of miRNA-155 increased in the successive stages of OA in patients classified usingthe K-L grading technique (grades 3 and 4) [59]. In vivo and in vitro research revealed that miRNA-155 has a role in immune cell formation and innate immunity control, as well as playing a role in acquired immunity. The modulation of miRNA-155 levels is aided by mitogen-activated protein kinase (MAPK) signaling [60]. c-Jun N-terminal kinase (JNK), a key regulator of many cellular events, including apoptosis,has also been demonstrated to influence the production of miRNA-155, indicating that the pro inflammatory transcription factor NF-B plays a key role in the response to inflammatory stimulation [57–61]

In the current study, microRNA-155-5p expressionlevels in synovial fluid of the severe OA group (1.29 ± 1.23) was significantly higher than microRNA-155-5p expression levels in their serum (1.27 ± 1.24), p = 0.024. In accordance to and as a possible explanation for these findings, Li et al. discovered that miR 1555p influences macrophage apoptosis and polarization inKOA synovial fluid [62]. When macrophages isolatedfrom the synovial fluid of KOA patients were comparedto macrophages isolated from healthy subjects, miR- 155-5p expression was shown to be increased [62].

The current study showed that microRNA-155-5p expression levels (4.72 ± 5.82) was significantly higherthan microRNA-136 expression levels in (1.41 ± 1.96),p < 0.001 in OA patients. When we compared microRNA-136 expression levels among all three groups, there was no statistical significant difference between 3 groups, p = 0.413.

Wan et al. studied miRNA-136 expression in plasmasamples from 74 patients and 79 healthy volunteers [63]. Plasma levels of miRNA-136 were found to be significantly lower in individuals with OA as comparedto healthy controls. MiRNA-136 levels were also foundto be inversely linked to illness severity and IL-17 [62].This discrepancy between our results and the results ofWan et al. may be attributed first of all to the selectionof the OA patients, we investigated patients with earlyOA. In addition, geographical and ethnic differences between our study and the Wan et al. study may also have contributed to the discrepancy in the results between the two studies.

In the present study, we found no statistical significantdifference in IL-17 levels between three groups,p = 0.983. There was also no statistical significant difference in IL-17 levels between

cases and controls, p = 0.929. In accordance to our findings, Chabaud et al.showed that IL-17 was present in higher concentrations in RA synovium supernatants than in synovium supernatants from individuals with OA [64]. Chen et al. alsoreported no significant variations in blood IL-17 levelsbetween cases with grade 2 or grade 3 OA and healthyparticipants, which is in agreement with our findings [27].

In disagreement with our results, de Boer et al. showed that IL-17 in the serum of patients with knee OA was considerably higher than in controls [65].

In yet another study, serum IL-17 concentrations were considerably greater in patients with knee OA than in control subjects [66]. Snelling et al. showedthe existence of IL-17 in the synovial fluid of a subsetof individuals with end-stage primary OA, with the hypothesis that the occurrence of IL-17 in the synovialfluid constitutes a distinct OA phenotype that may be targeted for therapeutic intervention [66]. The existenceof IL-17 in synovial fluid may thereby identify a patientsubset that would otherwise be classified as atrophic orquickly degenerative, with a lack of bone involvementbut a higher rate of development and synovitis.

Previous results linking IL-17 to OA synovitis corroborate the inflammatory nature of this patient population. However, the presence of synovitis ina significant proportion of OA patients who do not have detectable IL-17 indicates that OA has an independent inflammatory component in these persons,and more research is needed to uncover the cytokine networks involved [67–69]. The existence of IL-17 in the synovial fluid and serum of a subgroup of end-stageOA patients implies that inflammation is still present and unresolved, which is a hallmark of chronic inflammatory illness [67–70]. The discrepancy between our findings and those of other authors may be explained on the basis of racial/ethnic differences. The incidence of lateral knee OA is substantially greater in North African populations [71]. In addition, genetic connections that underpin OA differ between Caucasian and Asian populations [69]. As a result, discrepancies in OA susceptibility and different features between individuals of different ethnic background could explain the differential expression of IL-17 in serum and synovial fluid as found in our study.

The mean value of IL-8 levels in the early OA was
substantially greater than the mean value of healthy controls (p1 = 0.027). The mean value of IL-8 levels inall OA cases was considerably greater than the mean value of controls. Studies have shown that synovium expresses increased quantities of IL-8 messenger RNAin early OA cultures [69,70]. Bi-compartmental OA appears to be driven by both CD14+ macrophages and CD4 + T cells, according to Moradi et al, and hasa greater inflammatory profile in synovial fluid samples, with increased IL-8 levels [71]

conclusion:

In conclusion, our findings revealed a profile of dysrgulated miRNA signatures in KOA patients, suggestingthat these miRNAs may have a role in the etiology of KOA. Micro-RNA-155-5p expression is a sensitive andspecific diagnostic marker of early OA; hence, itcould be used as a biomarker for early disease identification. The level of IL-8 in the blood could also be used as a biomarker to identify patients with early OA.Early identification of OA is critical for determining the course of the disease and evaluating the disease's
response to treatment.

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