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Original Article

CYR61 as a Potential Apoptosis Biomarker in Osteoarthritis with Comorbidities

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Abstract

Objective: Obesity and diabetes mellitus (DM) are major risk factors for osteoarthritis (OA), but it remains unclear how comorbidity affects apoptosis signaling in OA. This study investigated the effect of metabolic diseases on apoptosis and apoptosis-related intracellular and extracellular signaling in OA. **Methods:** Excision materials of human articular cartilage from total knee arthroplasties were collected. The samples were divided into four groups as, control OA, OA+DM, OA+Obese, and OA+DM+obesity. Protein activities were determined using Western blot and ELISA. **Results:** Caspase-3 levels were significantly increased in chondrocytes in which OA was associated with DM or obesity. However, an increase in Bcl-2 activity was also observed in these comorbidities. The increased levels of CaMKII in the same groups also indicate an increase in cellular activity in comorbidities. While IL-6 and TNF-α did not show significant changes, matrix regulatory protein CYR61 levels reflected the intracellular apoptotic activity. **Conclusion:** Metabolic diseases have a stimulatory effect on the etiopathology of osteoarthritis by enhancing cellular signaling towards apoptosis and that matrix signaling proteins may play a key role in regulating these effects. Examining OA with its accompanying diseases will lead to a better understanding of the cellular mechanisms that differ in OA.

Keywords: Apoptosis, CYR61, Diabetes Mellitus, Obesity, Osteoarthritis

Introduction

Osteoarthritis (OA) is a degenerative joint disease that occurs as a result of a disruption in the balance between the synthesis and breakdown of articular cartilage[1,2]. Chondrocytes, the sole cell type residing in cartilage tissue, maintain the synthesis of the extracellular matrix (ECM) and the degradation of ECM components at a notably slow pace in healthy adult cartilage[3,4]. Following disruptions in this balance due to various diseases such as OA or trauma, chondrocytes proliferate and increase ECM synthesis in an effort to repair the damage[4]. Despite anabolic activities aimed at repairing damage in OA, a shift towards catabolic activities such as matrix degradation and apoptosis leads to an imbalance, contributing to increased tissue damage[5]. In the late stages of OA, the cartilage becomes hypocellular, often accompanied by frequent lacunar emptying; This is widely accepted as evidence for the central role of chondrocyte death in OA[6]. However, the impact of chondrocyte apoptosis on the pathogenesis of OA is not yet fully elucidated.

Increased inflammation is a common feature of OA and it can trigger catabolic processes through pro-inflammatory cytokines[7]. These cytokines can stimulate the production of enzymes that contribute to cartilage degradation. In cartilage affected by OA accompanied by metabolic diseases such as Type 2 Diabetes Mellitus (DM), there is also a characteristic presence of chronic low-grade inflammation[8]. In particular in the context of OA, inflammatory cytokines such as IL-6 and TNF-α have been shown to play a key role in the insulin resistance and also in metabolic dysfunctions observed in Type 2 DM[9]. Obesity, another metabolic disease characterized by excessive fat accumulation and frequently associated with OA[10], is also linked to chronic low-grade

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inflammation throughout the body. In addition to systemic inflammation, the adipose tissue within and around the joints can locally produce inflammatory cytokines[11]. This local inflammation can further exacerbate joint inflammation, contributing to the development and progression of OA[12]. Obesity is often associated with insulin resistance, leading to elevated blood sugar levels. High blood sugar levels can contribute to inflammation and oxidative stress in the joints, potentially worsening OA symptoms[13].

While the profiles of cytokines in osteoarthritic cartilage associated with metabolic diseases have been demonstrated in the literature, the contribution of these diseases to OA and their correlation with apoptotic responses is not fully understood. Therefore, the main aim of this study is to investigate the effects of metabolic diseases on apoptotic signaling in OA chondrocytes. Moreover, we investigated whether apoptotic activity follows extracellular signaling by examining extracellular stimulatory molecules such as the pro-cytokines IL-6, TNF-alpha and CYR61 (cysteine-rich angiogenic inducer 61), a matricellular protein that regulates cartilage extracellular matrix components and molecular interactions between cells[14-16]. Non-surgical treatments for osteoarthritis (OA) are currently limited to symptomatic approaches, and the search for cellular signals is a crucial step in understanding the molecular mechanism of OA.

Material and Methods

Patients

Female OA patients undergoing total knee replacement (TKR) surgery at the University of Health Sciences, Adana City Hospital Orthopedics and Traumatology Clinic, Turkiye, were recruited to the study. The patients were divided into four groups (n = 8/group) according to their body mass index (BMI). These groups were; osteoarthritis (OA: BMI 25.9 - 26.5 kg/m^2), DM (OA+DM: BMI 25.5-29 kg/m^2), and obese (OA+Ob: BMI 32-42 kg/m^2), and DM with obesity (OA+DM+Ob: BMI 32- 42 kg/m^2).

Cartilage Homogenization and Western Blotting

The knee cartilage samples were stored at -80°C until homogenization. The samples were pulverized in liquid nitrogen and then homogenized in 500 µl of RIPA solution for 10 mg of tissue (containing 20 mM Tris–HCl, 150 mM NaCl, 2 mM KCl, 2 mM EDTA, 0.5 mM DTT, and 100 mM protease inhibitor cocktail at pH 7.4). Homogenized cartilage samples were centrifuged at 1000 × g (10 min, 4°C), and their supernatants were collected. The protein content of the tissues was measured using the Bradford assay (Pierce Biotechnology, USA). Lysates (10 µg) were exposed to SDS-polyacrylamide gels (10%) under a constant current of 20 – 40 mA for 2 h and then transferred to PVDF membranes and incubated overnight at 4°C with Bcl-2, Caspase-3, p-CaMKII or CaMKII antibodies. β-actin was used as a gel loading control.

Enzyme-linked immunosorbent assay (ELISA)

The protein levels of IL-6 (FineTest, China), TNF-a (FineTest, China) and CYR61 (SunRed, China) in the homogenates were measured by enzyme-linked immunosorbent assay (ELISA) in accordance with the kit protocols. Briefly, tissue samples were rapidly lysed with liquid nitrogen and weighed. Then RIPA buffer was added (100 mg/ml) and centrifuged at 4°C (1500 rcf, 10 min). Serial dilution of the standards was performed according to the kit procedure. 50 µl of standard was added to each designated standard well, and 40 µl of sample and 10 µl of the specific antibody were added to each designated sample well. They were incubated at 37°C for specified time intervals in accordance with the procedure. Finally, 50 ml of stop solution was added.

Protein concentration was measured as a change in absorbance at 450 nm using a microplate reader (Awareness Technology, USA).

Chemicals

All the chemicals used in the experiments were obtained from Sigma, except where stated otherwise.

Statistical Analysis

Data were presented as means ± SEM. Analysis of variance (ANOVA) was used to assess differences among multiple groups, followed by Tukey's post hoc analysis. p-values less than 0.05 were considered statistically significant. Data analyzes were conducted using GraphPad Prism 5.0 software (GraphPad Software, Inc, CA, USA).

Results

The expression of the apoptosis protein caspase-3 increases in metabolic diseases.

Caspase-3 plays a crucial role in apoptosis by executing the cell death process. The increase in apoptosis in osteoarthritis compared to healthy cells represents an important aspect of OA pathology. In this study, caspase-3 levels were examined using the Western blotting. Subsequently, the protein bands were quantified using image analysis software (ImageJ, USA).

Caspase-3 bands (Figure 1A) in all groups were first normalized to β-actin bands and then, to express the data as fold increase, the data were normalized with the caspase-3 data of the OA group. As shown in Figure 1B, caspase-3 expression was significantly higher in the OA+DM, OA+Ob, and OA+DM+Ob groups (Mean ± SEM); OA+DM: 2.25 ± 0.27, OA+Ob: 2.4 ± 0.3, OA+DM+Ob: 2.79 ± 0.35.

The significant increase in caspase-3 in the OA+DM, OA+Ob, and OA+DM+Ob groups compared to the OA group suggests more active apoptotic signaling according to the OA group, which has no comorbidities.

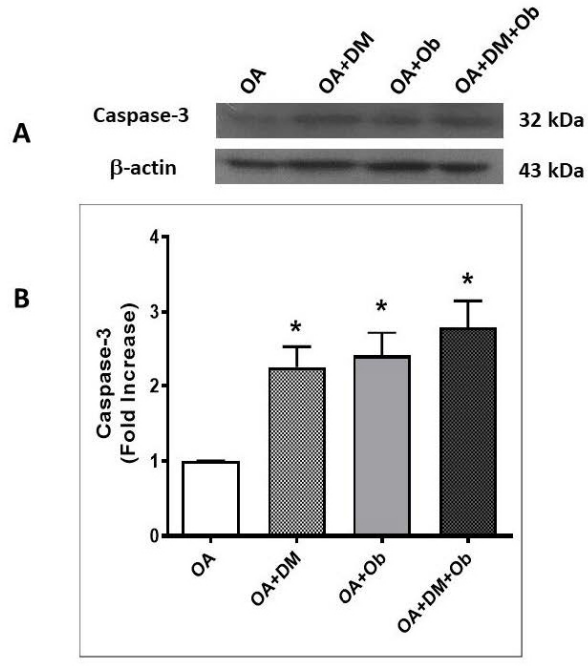

Figure 1. The effect of metabolic diseases associated with OA on caspase-3 levels in cartilage. A. Western blot data showing variation in caspase-3 between groups. B. The bar graph is presented as a fold increase compared to the OA group. Results in the graph are mean ± SEM, n = 9-10 samples/group. *p < 0.05 vs. OA group (by one-way ANOVA).

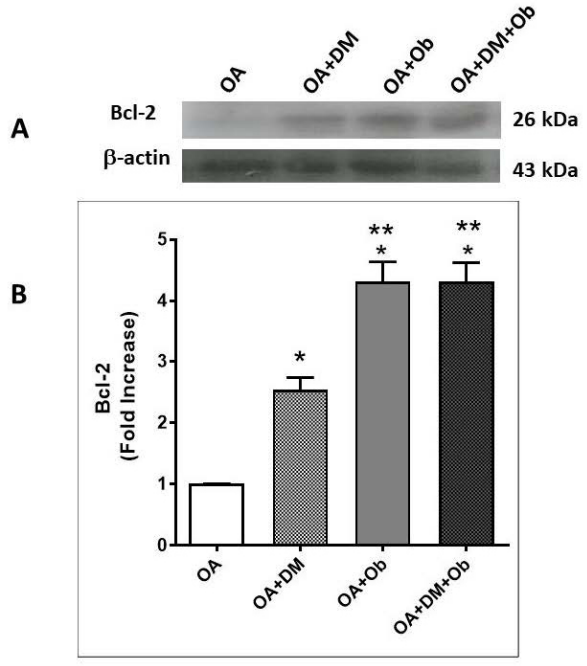

Figure 2. Bcl-2 protein levels in cartilage samples with OA and comorbidities. A. Western blot data for Bcl-2 protein levels between groups. B. The bar graph is presented as a fold increase compared to the OA group. Results in the graph are mean ± SEM, n = 9-10 samples/group. *p < 0.05 vs. OA group, **p < 0.05 vs. OA+DM group (by one-way ANOVA).

Bcl-2 expression is elevated in chondrocytes in OA with metabolic diseases

To investigate the anabolic signaling in metabolic diseases along with the increased apoptotic activity in osteoarthritis, the expression of Bcl-2, an anti-apoptotic protein, was examined in chondrocytes. Figure 2.A shows Western blot images for Bcl-2. The bar graph in Figure 2.B was created by comparing the Bcl-2 bands with their own B-actin band (Fig. 2.A). The data were then normalized with the Bcl-2 data of the OA group. The graph is expressed as a fold increase.

In our experiments, Bcl-2 increased in OA+DM, OA+Ob, and OA+DM+Ob groups compared to the OA group. However, the expression of Bcl-2 in the articular cartilage in the OA+Ob and OA+DM+Ob groups was higher than in the OA+DM group: OA+DM: 2.5 ± 0.19, OA+Ob: .4.3 ± 0.3, OA+DM+Ob: 4.31 ± 0.31.

The anti-apoptotic activity in the OA+Ob and OA+DM+Ob groups was higher than in the OA+DM group. However, the presence of diabetes in the obese group (OA+DM+Ob) had no difference in Bcl-2 activity according to the obese group alone (OA+Ob).

pCaMKII and CaMKII expressions

Our study also examined CaMKII, which plays a key role in regulating intracellular signals in articular cartilage. Figure 3.A shows the bands of pCaMKII (the phosphorylated form of CaMKII), and CaMKII (the total form of CaMKII) and β-actin bands. In Figure 3.B, the pCaMKII bands of each group were compared to the CaMKII band and these values were divided with the OA data to create the bar graph in Figure 3.B. The graph is expressed as a fold increase compared to the OA data. In the bar graph in Figure 3.C, each group's CaMKII bands were first normalized to their own B-actin band and these values were divided by the OA group to express the data as a fold increase compared to the OA data.

The total amount of CAMKII did not change between groups, while pCaMKII expression showed similar activation patterns to the Bcl-2 data; Activation of this protein also increased more in the obese groups than in the DM group alone. pCaMKII increased in the OA+DM, OA+Ob, and OA+DM+Ob groups compared to the OA group. This increase was even greater in both obese groups than in the DM group. Furthermore, DM caused no additional difference compared to Obese group in the OA+DM+Ob samples (fold increase

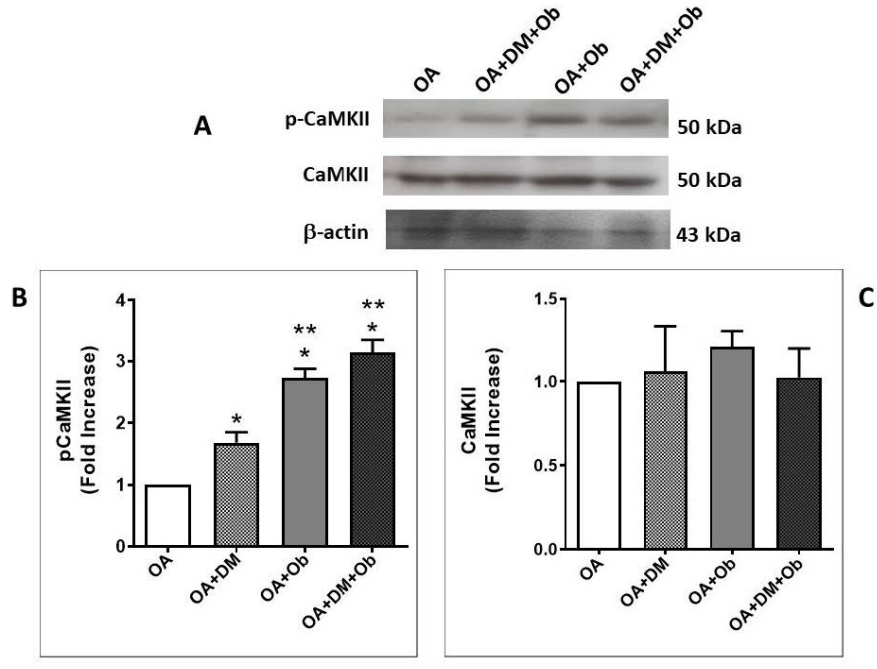

Figure 3. pCaMKII and CaMKII protein levels in cartilage samples with OA and comorbidities. A. Representative Western blot data for pCaMKII and CaMKII. B and C. The bar graph is presented as a fold increase compared to the OA group. Results in the graph are mean ± SEM, n = 9-10 samples/group. *p < 0.05 vs. OA group, **p < 0.05 vs. OA+DM group (by one-way ANOVA).

compared to the control OA group: DM: 1.68 ± 0.16, Obese: .2.73 ± 0.14, DM+ Ob: 3.14 ± 0.19).

Cytokine Levels in OA Tissues with Metabolic Diseases

To investigate whether the extracellular signals reflect the observed apoptotic signaling differences in metabolically diseased tissues, two proinflammatory cytokines interleukin-6 (IL-6) and TNF-α, as well as matricellular protein CYR61 were examined. Bar graphs of optical density values obtained from IL-6, TNF-α and CYR61 assays using the ELISA method. Data are presented as mean and standard error of the mean (SEM).

IL-6 unchanged in tissues with comorbidities

The study investigated whether IL-6, one of the pro-inflammatory cytokines, mirrors the mechanism that generates the response differences in the cellular destructive response caspase-3 and the anabolic response Bcl-2. Surprisingly, the presence of metabolic diseases accompanying OA had no difference in IL-6 levels on cartilage tissue (Figure 4).

No difference in TNF-α levels was observed in OA samples with metabolic diseases

Protein levels of TNF-α, another procytokine that plays an active role in OA metabolism in cartilage tissue, were also

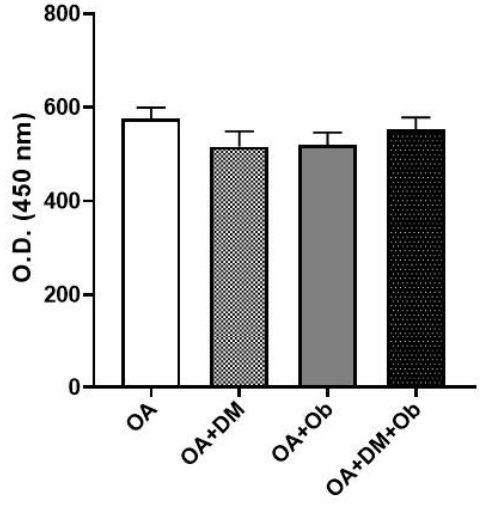

Figure 4. IL-6 expression levels in cartilage samples. Bar graph for changes of IL-6 in cartilage using ELISA. Results in the graph are mean ± SEM, n = 9-10 samples/group.

measured by ELISA (Figure 5). The presence of metabolic diseases in the samples did not result in a difference in TNF-α levels. The DM and Obese groups showed no difference between each other and also in comparision to the OA+DM+Ob group.

CYR-61 increased in all metabolic disease groups

Cyr-61, an extracellular signaling protein with targets other than inflammatory cytokines was investigated in the comorbidity groups. CYR 61 increased in all metabolic disease groups compared to the OA group (OA: 256 ± 54, OA+DM: 722 ± 72.2, OA+Ob: 796 ± 59.4, OA+DM+Ob: 654 ± 80) in Figure 6.

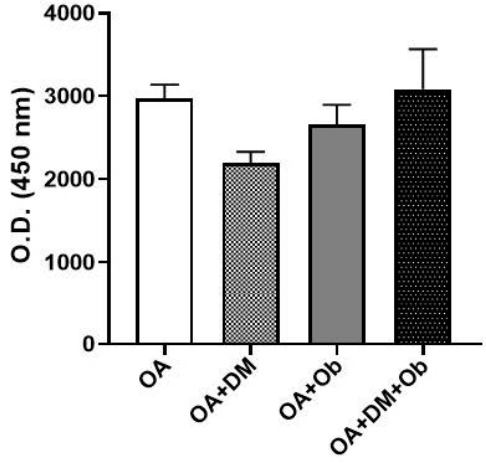

Figure 5. Expression level of the proinflammatory cytokine TNF-α in cartilage samples. Bar graph for changes of IL-6 in cartilage detected using ELISA. Results in the graph are mean ± SEM, n = 9-10 samples/group.

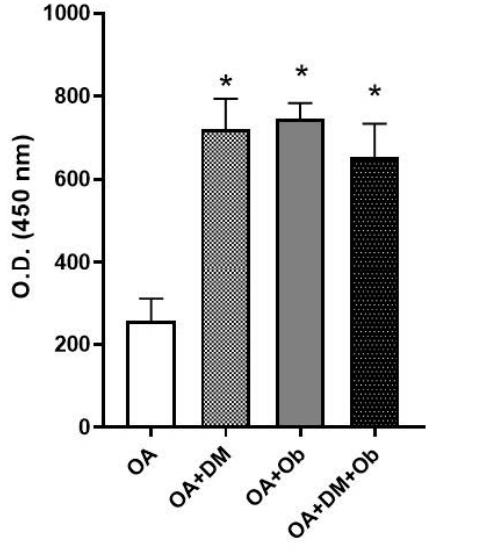

Figure 6. Expression level of the TNF-α in cartilage samples. Bar graph for changes of IL-6 in cartilage detected using ELISA. Results in the graph are mean ± SEM, n = 9-10 samples/group.

Discussion

While knee OA has a significant impact on the quality of life of the elderly population, the molecular mechanisms leading to cartilage loss in osteoarthritis are not well understood. Chondrocyte apoptosis has been demonstrated to play a significant role in developing osteoarthritis in human knee cartilage[17-19]. The aim of this study is to understand the apoptosis that develops with knee osteoarthritis in the presence of metabolic diseases such as obesity and DM and to identify a possible link between intracellular signaling and extracellular cytokines to assign a biomarker for apoptosis in OA with metabolic diseases. According to the hypothesis of this study, metabolic diseases will increase apoptosis in the cell, and this increase will also occur in parallel with cytokine expressions outside the cell that play a role in apoptosis.

Our results provided initial evidence that apoptotic and anti-apoptotic molecules such as caspase-3 and Bcl-2 as well as the apoptosis regulatory molecule CaMKII[20] exhibit increased expression in the presence of metabolic diseases compared to osteoarthritis alone. This observation suggests increased cellular signaling activity, particularly in the context of obesity. Interestingly, the pro-inflammatory cytokines IL-6 and TNF-α[21] did not emerge as predominant factors in shaping intracellular apoptotic signals in metabolic diseases. However, increased expression levels of CYR61, a matricellular protein with diverse regulatory roles outside the cell[22], showed a correlation with increased caspase-3 activity in the context of metabolic diseases.

When chondrocytes are exposed to cellular stresses such as mechanical loading and diabetes mellitus, they initiate matrix destruction through the activation of various cytokines and mediator molecules[23,24]. It was shown in chondrocytes from patients with osteoarthritis that matrix degradation leads to an increase in apoptotic activity[25]. Upregulation of caspase-3 in osteoarthritis is considered a crucial step in cartilage loss induced by increased catabolic processes[6,25,26]. In our study, carried out on knee cartilages from patients with osteoarthritis (OA) and comorbidities such as DM and/or obesity, the increased expression of caspase-3 indicates a significantly increased apoptotic activity in the presence of systemic pathologies accompanied by OA.

In parallel with the increase in caspase-3, Bcl-2 expression also increased in groups with metabolic diseases. In particular, the expression of Bcl-2 was higher in both the OA+Ob and OA+DM+Ob groups than in the OA+DM group, indicating the activity of cellular balance mechanisms

which amplified even more with obesity. Kim et al. (2000) demonstrated that apoptotic chondrocytes were less prevalent in normal cartilage compared to osteoarthritis[27]. Furthermore, their data showed more apoptotic cells in the OA lesional area than in the non-lesional area on the same cartilage. Additionally, their study showed higher expression of Bcl-2 and Fas (an apoptosis-inducing protein) in cartilage with OA lesions than in the non-lesional area. A different study on human osteoarthritic cartilage also found increased expression of Bcl-2 and p53 (an apoptosis regulator protein) and suggested that the increased Bcl-2/p53 ratio keeps chondrocytes metabolically active[28]. The concurrent increase in Bcl-2 expression alongside apoptotic protein expression was also observed in patients with Kashin-Beck syndrome (KBD), a type of osteochondropathy that leads to secondary osteoarthritis[29]. In that study, increased Bcl-2 expression coupled with increased expression of apoptotic proteins such as Bax and Fas in KBD articular cartilage was interpreted as a shift in tissue balance towards apoptosis.

In our study, metabolic diseases, which serve as an additional stressor in osteoarthritis tissues, led to an increased cumulative apoptotic response, implying a possible worsening in the extent of damage compared to the OA group (without comorbidity). Hence, the observed elevation in Bcl-2 expression consistent with literature findings, may suggest a robust cellular activity within osteoarthritic cartilage concurrent with increased apoptosis.

Ca^{2+} is a crucial intracellular messenger and plays an indispensable role in maintaining cellular homeostasis and various metabolic processes[30,31]. CaMKII, a protein that is central to the activation of numerous signaling pathways, is involved in the synthesis and regulation of the extracellular matrix, as well as in inflammatory and apoptotic processes in cartilage tissue and also responds to changes in the cytoplasmic Ca^{2+} concentration in chondrocytes[32]. The increased phosphorylation of CaMKII in the cell is significant for indicating the activation of Ca^{2+}-dependent pathways in chondrocytes, concurrently with the rise in intracellular Ca^{2+} concentration. Studies have demonstrated that CaMKII phosphorylation is involved in chondrocyte proliferation and extracellular matrix (ECM) synthesis[33]. Moreover, existing literature provides evidence for an increase in p-CaMKII in OA[34]. In our data, the presence of metabolic diseases in osteoarthritic chondrocytes enhances apoptotic signaling. Despite the prevailing tissue destruction, increased Bcl-2 and CaMKII phosphorylation may indicate increased cellular activation to maintain homeostatic balance in OA.

The onset and progression of OA are closely linked to immune activation and subsequent inflammatory responses[35]. Consequently, osteoarthritis is considered a degenerative joint disease that is associated with low-grade chronic systemic inflammation. The development of the disease is mediated by an imbalance in cytokine and enzyme expression, which compromises the integrity of articular cartilage tissue[36]. The catabolic events that lead to cartilage matrix degradation in osteoarthritis involve the release of catabolic cytokines[37]. However, it remains unclear whether the expression levels of these cytokines in cartilage change when osteoarthritis is accompanied with a metabolic disease and whether such changes are reflected in intracellular apoptotic signaling.

Obesity is a disease characterized by changes in the levels of various hormones and cytokines. These changes in metabolic concentrations are known to induce insulin resistance and thus the development of diabetes mellitus[38]. The influence of obesity on the development of OA is therefore thought to involve many systemic regulators[39,40]. Furthermore, in DM alone, increased production of several inflammatory cytokines has been documented to contribute particularly to the development of osteoarthritis[8]. The involvement of the pro-inflammatory cytokines IL-6 and TNF-α in human osteoarthritic chondrocytes and their roles in apoptosis induction and cartilage defects have been discussed previously[41]. Exposure of chondrocytes to IL-6 and TNF-α leads to increased expression of genes encoding inducible nitric oxide synthase (iNOS) and stimulates nitric oxide release. This contributes to joint inflammation and destruction by enhancing the activation and production of NO and MMPs, inhibiting the synthesis of anabolic macromolecules such as collagen and proteoglycans, and inducing chondrocyte apoptosis[42-45].

While IL-6, a key determinant of structural changes in osteoarthritis, plays a critical role in the development of cartilage damage associated with OA[45,46], it has previously been identified as a target molecule for the treatment of OA[47]. The elevated IL-6 levels in the synovial fluid of osteoarthritis and obese patients, compared to normal-weight OA patients, underscore the crucial role of this molecule in OA[48]. However, it is recognized that IL-6 plays a dual role in OA and has a protective effect on the extracellular matrix (ECM) in cartilage by affecting the production of tissue inhibitors of metalloproteinases (TIMPs)[49]. In our study, encompassing patient samples with OA across all groups, IL-6 levels did not change in DM and/or obesity groups, despite its dual catabolic and anabolic effects in OA.

Another cytokine that plays a critical role in regulating ECM destruction in osteoarthritis (OA) is TNFα. TNFα downregulates collagen II and proteoglycan synthesis, key components of the extracellular matrix (ECM), by inhibiting the anabolic activities of chondrocytes[50,51]. However, in this study, metabolic diseases did not result in differences in TNFα levels in OA tissues. Neither IL-6 nor TNF-α showed any signal difference that may indicate the divergence of apoptotic responses observed in metabolically diseased chondrocytes. This could be suggested as IL-6 and TNF-α may not have any effect on the increase in cellular activity caused by comorbidity.

Since pro-inflammatory cytokines did not play a prominent role in the differentiation of intracellular signaling in the presence of metabolic diseases, this study subsequently asked whether a matricellular molecule involved in the extracellular matrix and apoptosis regulation would mirror the intracellular dynamics of apoptosis. To understand this, matricellular Cyr61 was examined, which plays a regulatory

role in apoptosis and signaling in the extracellular matrix (ECM) by regulating biological processes such as fibrogenesis through various integrins and proteoglycans[52,53]. Cyr61, which also plays an important role in various cellular processes including cell adhesion, migration, proliferation, and differentiation, was also shown to induce ER stress when overexpressed in the cell, leading to activation of caspase-3 and subsequent apoptosis[54].

In our study, CYR61 exhibited increased signaling in cartilage, reflecting the enhanced apoptosis responses in metabolic diseases. The altered response pattern of CYR61 suggested that intracellular and extracellular communication in metabolic diseases in osteoarthritis could be mediated through matricellular proteins and Cyr61 could be considered as a biomarker for these intracellular signals.

In conclusion, these results suggest that the metabolic diseases in OA lead to altered cellular activities and also chondrocyte apoptosis depending on the type of disease and that matricellular proteins such as CYR61 may play a key role in regulating apoptosis signals. Nevertheless, understanding how tissue cells maintain homeostasis in the face of increasing chondrocyte death and activate their signaling mechanisms that contribute to disease is a crucial prerequisite for the development of effective therapeutic approaches.

Ethics approval

Ethical approval was granted by the Ethics Committee for Non-invasive Clinical Research of Cukurova University (2019-91/52), and the Helsinki Declaration was considered. In addition, permission for the study was obtained from the Department of Orthopedics and Traumatology of Adana City Hospital.

Consent to participate

Informed consent was obtained from all patients before sample collection.

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Authors' Contributions

F. Cicek and H. Cicek conceived the study concept and design and were committed to the development of the manuscript. F. Cicek performed data analyses. M. Tokus, H. Ates, C. Coskun, and Y. Dogus carried out data acquisition. M. Tokus was the primary author. All authors approved the final version of this manuscript.

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