Sex, but not age and bone mass index positively impact on the development of osteochondral micro-defects and the accompanying cellular alterations during osteoarthritis progression

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Abstract

Background: Osteoarthritis (OA) is characterized by cartilage breakdown and subchondral sclerosis. Micro-fractures of the calcified tissues have been, also, detected, but their exact role has not been elucidated yet. This study was to examine the frequency of cracks during OA progression and to correlate them with the underlying cellular modifications and matrix metalloproteinase-2 (MMP-2) expression using histological/immunohistological methods.

Methods: Overall, 20 patients and 3 controls (9 specimens per patient), aged 60–89 years, diagnosed with hip/knee OA were included. The development of cracks was examined in 138 sections, whereas the expression of MMP-2 was examined in 69 additional sections.

Results: Based on Mankin score, three groups of OA severity were analyzed: Group I (mild) was constituted of sections with score 1–5 while Groups II (moderate) and III (severe) with score 6–7 and greater or equal to 8, respectively. Demographic characteristics did not reveal any association between the number of microdefects and age or body mass index (BMI). Cartilage micro-cracks were increased during moderate and severe OA, while bone cracks were increased during mild and severe OA. In knee OA, cartilage cracks were not correlated with Mankin score, whereas in hip OA they appeared association with severity score. Bone cracks were positively correlated with matrix apoptotic osteocytes and osteoblastic cells, but not with osteoclasts. MMP-2 immunostaining was increasing by OA severity in the osteochondral unit. Similarly, MMP-2 was expressed on the microcracks’ wall mainly in Group III.

Conclusion: Our data displayed that bone cracks during primary OA stages, represent an early adaptative mechanism aiming to maintain cartilage integrity. Accumulation of bone defects and concomitant increase of apoptotic osteocytes activated an abnormal remodeling due to osteoblastic activity, in which MMP-2 played a pivotal role, leading to subchondral sclerosis promoting further osteochondral deformities.
1 | INTRODUCTION

Osteoarthritis (OA) is a clinical syndrome of joint pain accompanied by varying degrees of functional limitation and reduced quality of life. Pathologically, OA is characterized by cartilage breakdown along with subchondral bone abnormal remodeling, associated synovial inflammation and degenerative changes in meniscus or articular ligaments.1 Predicated on the above evidence, OA is currently classified as a whole joint degenerative disease.

The chronological events of OA progression are still under debate, but recent studies emphasized in the pathophysiological role of the calcified tissues during the early stages of OA. The most predominant injury of the osteochondral plate in OA is presented in the form of micro-cracks. Cracks and micro-fractures of the calcified tissues, which are interstitial cracks, have been detected in human OA,2,3 in animal models following joint trauma3,4 and in equine joint disease.5–8 Cracks are mainly localized in the calcified cartilage, in the subchondral plate and in the metaphyseal trabecular network6,7 and they represent a physical separation of the matrix that grows longitudinally along the lamellar interfaces of the tissue.7 Micro-cracks size is about 50–100 μm in width and 200–400 μm in length8 occurring in most connective tissues along with the cartilage matrix but their role in the initiation and progression of OA is still under investigation. To explain the physiologic function of micro-cracks in OA, researchers proposed two possible mechanisms. First of all, it is supported that cracking of the calcified cartilage or subchondral bone stimulates an abnormal remodeling which results to the removal of the damaged tissue.7,10 In both layers, the remodeling process is achieved by periodic osteoclastic removal, replacement, and refreshment of the junction.2 Additionally, cracks may be repaired spontaneously by the formation of a highly density infill material11 via a dystrophic calcification process.12 Second, Burr and Radin developed the idea that micro-cracks of the subchondral bone promote the loss of osteochondral integrity due to channels or vessels invasion to the articular cartilage. Current studies also showed that these channels allow the diffusion of several inflammation cytokines into the cartilage, promoting its degradation and/or degeneration.¹ Microcracks were also observed that altered the biomechanical properties of cancellous bone, not only by reducing fatigue life during cyclic loading in human vertebra but by decreasing hardness and elastic modulus during cracks repair too, contributing to the accumulation of micro- and/or macroscopical fractures to these locations.13,14 Furthermore, cartilage cracks in OA animal models were accompanied by acellularity of the cracked tissue and by regions of necrotic/apoptotic chondrocytes leading to the notion that cracking is an important determinant in trauma-induced cell death due to apoptosis15,16 and a remarkable contributor in the pathophysiology of OA.17

The aim of our study was to examine the frequency of cartilage and subchondral bone crack lesions during different OA stages and to correlate their presence with the osteoblastic and osteoclastic activity, the degree of apoptotic/necrotic chondrocytes and osteocytes, and the degenerative changes of the osteochondral unit by using histological and immunohistological methods. Matrix metalloproteinase–2 (MMP-2), which was found to be upregulated in the articular cartilage and synovial fluid of patients suffering from OA18 playing significant role in the pathogenesis of OA,19 was used as molecular index of disease severity.

2 | METHODS

2.1 | Ethical approval

The study design was approved by the scientific committee for Postgraduate studies of Patras University (Registration No: 45700/18-06-2008). Tissue samples were obtained with informed consent from the patients undergoing arthroplasty and the research complies with the 1964 Declaration of Helsinki and its later amendments.

2.2 | Patient samples

Overall, 20 patients aged 60–89 years diagnosed with idiopathic OA of the hip and knee in accordance with the criteria of the American College of Rheumatology,20,21 were included and compared with 3 controls aged 68–83 years, as previously described.22,23 Inclusion criteria were the presence of idiopathic OA, while exclusion criteria were: (a) the presence of secondary arthritis; (b) hemochromatosis; (c) hip fractures; (d) posttraumatic arthritis; (e) inflammatory, autoimmune, or bone metabolic diseases; (f) malignancies; (g) suspected osteonecrosis; (h) treatment with bone anabolic medication like bisphosphonates; and (i) intra-articular administration of steroids.

OA clinical severity was estimated based on pain, functionality, and clinical data using the Harris score for hip OA and the index of severity (ISK) for knee OA, as
Radiographing imaging of the knees and hips were evaluated blindly by two senior orthopedic surgeons and were graded according to Ahlbach classification for the knee OA and the Kellgren & Lawrence for hip OA, as previously described. The same orthopedic surgeons evaluated all X-rays.

Eleven patients suffered from hip OA and nine from knee OA. Controls consisted of two femoral heads, which were received after hip hemiarthroplasty due to fracture of the femoral neck, and a tibial plateau after amputation above the knee due to leg dismemberment. All control patients had no known history of bone or joint disease and their cartilage was macroscopically free of OA lesions (Grade 0), according to the Collins scale modified by Muehleman et al. For each patient, clinical data of sex, age, weight, height, and body mass index (BMI) were documented.

2.3 Histological sample collection and grading

Nine specimens per patient and per control were assessed. In total, the development of cartilage and subchondral bone defects was examined in 138 sections, while the expression of MMP-2 was assessed in 69 additional sections.

Lateral and medial condyles of the tibial and the femur were collected during total knee arthroplasty (TKA), while femoral heads were collected during total hip arthroplasty (THA). Osteochondral specimens of the TKA (10 × 10 × 6 mm) and THA (10 × 10 × 10 mm) were removed starting from the weight-bearing center portion of each anatomic location. The distance between the centers of the samples was more than 15 mm. The above methodology provided sufficient number of histological specimens, variety of OA Mankin grading and included the characteristic features of OA tissue severity.

The histological and immunohistochemical processing was previously described. The specimens were fixed in 10% buffered formalin for 24–36 h, decalcified in neutral Ethylenediaminetetraacetic acid (EDTA) for 6–8 weeks at room temperature, and embedded in paraffin blocks. Three micrometer thick histological sections were obtained and stained with Hematoxylin/Eosin. The quantitative histological evaluation of the specimens was based on a modified form of the Mankin scale. Three groups of OA were formed: Group I (Mild OA) constituted of sections with Mankin score 1–5, Groups II (Moderate OA) and III (Severe OA) with Mankin score 6–7, and ≥8, respectively.

Necrotic and apoptotic cells were defined by their histological features. Apoptotic cells were characterized by cell shrinkage, nuclear condensation, and cellular fragmentation. The morphological changes of necrotic cells include cell blebbing and leaking. The number of cells of interest per microscope field (objective ×20) was counted by two observers in a double-blind fashion. The number of cracks or cells per microscope field was calculated in all tissue sections of each group.

2.4 Immunohistochemistry (IHC) assay

For immunohistochemical analysis, the slices were deparaffinized in xylene and graded alcohols and immersed in distilled water. The endogenous peroxidase was blocked with 3% H₂O₂ for 30 min in a dark chamber at room temperature. The sections were then washed once in distilled water and three times with Tris-buffered saline pH 7.4 (TBS) and incubated for 1 h at room temperature with anti-MMP-2 (8B4, Santa Cruz, sc-13595, is a mouse monoclonal IgG1, with high specificity for the detection of both active and inactive forms of MMP-2) diluted 1:50 in antibody diluents (DAKO REAL S2022), washed and incubated for 45 min at room temperature with peroxidase-labeled anti-mouse/rabbit IgG (Envision Kit, DAKO Detection System, Peroxidase/DAB + , Rabbit/Mouse K5007). Then the slices were washed three times with TBS, stained for 10 min in a dark chamber at room temperature with 3-amino-9-ethylcarbazole/H₂O₂, washed in distilled water and counterstained with hematoxylin.

2.5 Statistical analysis

Statistical analysis was carried out using the SPSS software v21.0 (SPSS Inc.). All data were expressed as mean ± standard deviation (SD). The comparison was performed with Mann–Whitney U test. The correlations between microcracks measurements and demographic parameters were analyzed by the Pearson correlation coefficient test. A value of \( p \leq 0.05 \) was considered significant.

3 RESULTS

3.1 Population characteristics

A total of 207 immunohistological samples were included in the analysis. Eleven patients suffered from hip OA and nine from knee OA. No significant differences between groups were observed in the patient age, gender, height, weight, and BMI (Table 1). Demographic characteristics of the participants were summarized in Table 1.

3.2 Osteochondral cracks and Mankin score in OA

Histological findings were identical for all the osteochondral cracks and were based on the criteria of Burr and Stafford that were used to define microcracks and to distinguish them from vessels. In specific,
The definition of cracks was defined by the following criteria: (a) Characteristic intermediate size appearance that was larger than canaliculi and smaller than vessels, (b) their tissue borders were sharp, and (c) they were stained through the depth of the section. Cracks were measured using light microscopy and the number of cracks per section was calculated. Microcracks were identified in the calcified cartilage and in the subchondral bone plate tissue in all specimens of OA patients (Figure 1).

Overall, the frequency of cartilage micro-cracks was increased in the groups of moderate and severe OA stages, while the prevalence of subchondral bone cracks was higher in the groups of mild and severe OA (Table 2 and Figure 2). Moreover, the greatest frequency of cartilage cracks was observed in the Group II while the maximal composition of subchondral bone cracks was detected in Group III and its immunostaining was increasing by the severity of OA in the cartilage and subchondral bone of patients with knee and hip OA (p = 0.0042 and p = 0.0019, respectively). Similarly, MMP-2 was expressed on the wall of subchondral (Figure 5A-D) and calcified cartilage (Figure 5A,C) microcracks mainly in Group III, whereas MMP-2 expression was undetectable in Groups I and II (Figure 1A-D).

3.4 | Osteochondral cracks and cellular alterations

Even though cartilage and bony cracking were both present during mild OA stages, our analysis displayed that increased bone erosions were correlated with reduced number of cartilage defects during OA progression (Figure 2 and Table 2). Further analysis of the immunochemical results identified the association between the number of osteochondral defects in OA and cellular degenerative changes either in the cartilage and in the subchondral plate tissue (Table 2). In specific, increased number of subchondral bone cracks were positively correlated with high concentration of apoptotic osteocytes and osteoblastic cells (p < 0.005 and p < 0.0001, respectively) in bone matrix. Interestingly, the number of osteoclasts was not correlated with the severity of bone cracks as it appeared gradual reduction with OA histological severity (Table 2). Furthermore, cartilage cracking was not associated with cartilage apoptotic cells (Table 2).

### Table 1: Demographic characteristics of the study population

| Characteristics          | Patients with hip OA (n = 11) | Patients with knee OA (n = 9) | Control (n = 3) |
|-------------------------|-------------------------------|-------------------------------|-----------------|
| Sex                     | 7 females, 4 males            | 8 females, 1 male             | 2 females, 1 male |
| Mean age (years)        | 73.3 ± 8.01                  | 72.75 ± 7.76                 | 72.3 ± 6.66     |
| (Min:60, Max:89)        | (Min:60, Max:89)             | (Min:65, Max:78)             |
| Mean height (cm)        | 165.45 ± 5.3                 | 163.93 ± 5.8                 | 172.67 ± 2.52   |
| (Min:156, Max:175)      | (Min:154, Max:171)           | (Min:170, Max:175)           |
| Mean weight (kg)        | 83.5 ± 13.1                  | 83.18 ± 12.5                 | 77.10 ± 7.94    |
| (Min:64, Max:110)       | (Min:62, Max:106)            | (Min:68, Max:83)             |
| Body mass index (BMI)   | 30.1 ± 5.28                  | 29.875 ± 5.03                | 25.98 ± 1.61    |
| (kg/m²)                 | (Min:23, Max:41)             | (Min:22, Max:40)             | (Min:23.5, Max:27.7) |

Note: Data are expressed as number of individuals or mean ± standard deviation. Abbreviation: OA, osteoarthritis.
Defects of the osteochondral unit were firstly described by Frost in 1960 being considered to induce bone remodeling and degenerative changes during OA progression. Among those defects, osteochondral cracks constituted the most prominent findings and their presence was associated with abnormal bone remodeling during OA progression. However, the relevance of cracking to demographic characteristics of the population and the degenerative tissue and cellular changes during OA progression remains unclear. To the best of our knowledge, this is the first research that examines substantially the impact of demographic characteristics on cracks development but also the association of microcracking with cellular changes into the calcified tissues during OA progression.

Our results displayed that demographic characteristic, such as age and BMI, were not correlated with the frequency of cartilage or bone microdefects in OA. This is in agreement with the analysis of Zarka et al. in which microcracks number in calcified cartilage or subchondral bone was not affected by age or BMI. Similarly, the age was not a determinant factor for the development of microcracks during OA in the study of Rabelo et al. Although increased BMI has been implicated in the pathophysiology of OA, either by inducing the secretion of catabolic factors or by promoting bone adaptation process and remodeling, our results supported the notion that crack development was not directly linked to BMI. A possible explanation could be that repetitive complex mechanical loading, such as the combination of impact energy and cyclic compression, has been reported to be necessary to promote microtraumas in the collagen network, to initiate the

FIGURE 1 Representative photomicrographs of immunohistological staining in the presence of anti-MMP-2 antibody. (A) Presence of linear microcracks (black arrows) in the calcified cartilage and subchondral bone in a section of a patient with Mankin score 3 (original magnification ×10). (B) Presence of microcracks (black arrows) and subchondral bone cysts (green arrows) in a section of a patient with Mankin score 7 (original magnification ×4). (C) Crack (black arrows) and subchondral cyst (SBC) (green arrows) in a patient with Mankin score 3. Notice that the wall of the crack and SBC was covered by osteoblastic cells without the expression of MMP-2 (original magnification ×10). (D) Osteoblastic cells on the wall of the cracks (black arrows). Adjacent, we observed an aggregation of multinuclear cells (osteoclasts, red arrows) from a slice with a Mankin score of 8 (original magnification ×10). MMP-2, matrix metalloproteinase-2
degradation of calcified tissues and affect the propagation of microcracks. Contrariwise, female sex was positively associated with crack development. The combination of different hormonal factors and low bone density in female participants may be responsible for the accumulation of microdefects.

Interestingly, our analysis showed that cartilage and subchondral bone development followed different chronological order. Indeed, the number of cartilage micro-cracks was increased in the groups of moderate and severe OA stages, while the density of subchondral bone cracks was raised in the groups of mild and severe OA. This finding may reflect an early cartilage adaptative mechanism by which the energy of mechanical loading was spread through the subchondral bone cracks. This was also supported by the observation that subchondral bone development followed different chronological order. Indeed, the number of cartilage micro-cracks was increased in the groups of moderate and severe OA stages, while the density of subchondral bone cracks was raised in the groups of mild and severe OA. This finding may reflect an early cartilage adaptative mechanism by which the energy of mechanical loading was spread through the subchondral bone cracks.
bone microcracks were associated with less cartilage defects and might be required for maintaining cartilage homeostasis.29

An important finding is that in the patients suffering from knee OA the number of cracks in the cartilage and subchondral bone was not statistically correlated with Mankin score, whereas in hip OA cracks appeared to have significant statistical association with histological severity score. In the study of Zarka et al., it was reported that crack density was not linked to the severity of Pritzker histological score in knee OA specimens.29 However, our analysis showed that, while crack numbers were not affected by the severity of Mankin score in knee OA, they were indeed influenced in hip OA. We must, also, highlight the fact that in our study the above association between cracks and knee and hip OA severity, was also verified by immunohistochemical data, where MMP-2 was applied as biomarker. Despite the fact that the small sample size may affect the validity of these data, the above findings may reflect differences in

**FIGURE 3** Representative photomicrographs of immunohistological staining in the presence of anti-MMP-2 antibody. Cartilage and subchondral bone without OA changes in a control immune-negative for MMP-2 (original magnification ×10). MMP-2, matrix metalloproteinase-2; OA, osteoarthritis

**FIGURE 4** Representative photomicrographs of immunohistological staining in the presence of anti-MMP-2 antibody. (A) MMP-2 expression in cartilage matrix and in chondrocytes (yellow arrows) in a patient with Mankin score of 7 (original magnification ×10). (B) MMP-2 expression was observed in the matrix and in enlarged chondrocytes and in clusters of aggregating chondrocytes (yellow arrows) in a section from a patient with Mankin score 8 (original magnification ×40). (C) Intense immunostaining for MMP-2 of the disorganized cartilage in a patient with Mankin score 9 (original magnification ×10). (D) MMP-2 expression in the osteoblastic cells (black arrows) and osteoclasts (red arrows) in a slice from a patient with Mankin score 9 (original magnification ×20). MMP-2, matrix metalloproteinase-2
early physiological modification process, biomechanical changes and/or genes expression between knee and hip OA pathophysiological mechanisms.

Our results, also, displayed that either cartilage or bone cracks in OA were correlated with cellular modifications in the osteochondral unit. However, these changes were more conspicuous in the subchondral bone, where statistical significance between calcified tissue cracks and osteoblastic and apoptotic activity was detected. Apoptosis of osteocytes seemed to have a key role in osteochondral remodeling process. Our analysis was consistent with the results of several studies that reported a close connection between cracks development and osteocyte apoptosis. Our results did not reveal any association between cracks and increased osteoclastic activity as osteoclastic activity was not remarkably altered during all stages of OA progression. On the contrary, significant increase in the matrix osteoblastic activity linked to crack density was observed. Additionally, MMP-2 expression on the wall of subchondral cracks and in the surrounding osteoblastic cells was induced during Groups II and III. In vivo studies showed that clinical features of MMP-2 knockout mice were characterized by severe craniofacial defects, osteopenia, decreased bone mineralization, joint erosions consistent with an underlying arthritis and defective bone remodeling procedure. In the same study, in vitro results of osteoblastic cell lines isolated by calvaria of MMP-2 knockout mice presented decreased ability of growth, proliferation, and differentiation that was not recovered by the administration of extraneous MMP-2, indicating the importance of its intrinsic

FIGURE 5 Representative photomicrographs of immunohistological staining in the presence of anti-MMP-2 antibody. (A) Presence of large calcified cartilage horizontal crack without expression of MMP-2 (black arrow) associated with vertical crack in the subchondral bone tissue with osteoblastic and osteoclastic concentration (red arrows) on its wall being immunopositive for MMP-2 from a patient with Mankin score 7 (original magnification x10). (B) Linear cracks of the subchondral bone from a section with Mankin score 8. Please notice the large number of apoptotic osteocytes around bony defects (yellow arrows) (original magnification x10). (C) Subchondral bone cracks from a patient with Mankin score 9 accompanied by large number of apoptotic osteocytes (yellow arrows) and osteoblastic cells (black arrows) (original magnification x10). (D) Section from a patient with Mankin score 8 that displayed linear crack of the subchondral bone with increased number of osteoblastic (black arrows) and osteoclastic cells (red arrows) on its wall that expressed MMP-2 indicating an advanced stage of remodeling process (original magnification x20). MMP-2, matrix metalloproteinase-2.
expression in remodeling process during trabecular and cortical bone formation. Therefore, our data suggested that crack-related osteocyte apoptosis and the concomitant reduction in bone strength triggered, not osteoclastic but, abnormal osteoblastic activity resulting in subchondral sclerosis and osteophyte formation. This study has several limitations. First of all, the small sample size of participated individuals was a significant drawback. Nevertheless, the analysis of remarkable large number of histological sections, the examination of both sexes, as well as the investigation of cracks development during all stages of OA progression increased the integrity of our results. Furthermore, the sample of the patients and the controls was representative of the whole area population for the age, sex, BMI, and OA radiological severity, as it was based on statistical preanalysis of the hospital admissions. Second, another weak point could be that the molecular severity of the study was established only on the tissue expression of MMP-2 without examining other biomarkers like MMP-3, MMP-13, or interleukin-1β (IL-1β). However, the examination of Mankin score, which consisted an objective grading system of OA degeneration, and the analysis of the accompanied cellular modifications along with MMP-2 expression, may offer an accurate approach to the pathophysiological role of osteochondral defects and provide an alternative therapeutic target especially during the initiation of degenerative changes.

In conclusion, our data displayed that subchondral bone cracks were established during primary stages of OA and they were positively correlated with female sex, whereas BMI and age were not determinant factors for their development. Moreover, cracks were associated with reduced cartilage loss supporting the notion that represent an early physiological mechanism which aimed to maintain cartilage integrity. Although, the exact mechanism was not clarified yet, it has been proposed that the prevention of cartilage breakdown was due to the downregulation of osteocytes activity and the reduction of the abnormal remodeling process in the subchondral bone. However, our data showed that the accumulation of bone defects and the concomitant increase of apoptotic osteocytes triggered an abnormal remodeling due to osteoblastic activity, where MMP-2 played a pivotal role, resulting in subchondral sclerotic alterations and further osteochondral defects resulting to cartilage degradation.

CONFLICT OF INTERESTS
The authors declare no conflict of interest.

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