Morphological Study: Ultrastructural Aspects of Articular Cartilage and Subchondral Bone in Patients Affected by Post-Traumatic Shoulder Instability

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ABSTRACT

Post-traumatic shoulder instability is a frequent condition in active population, representing one of most disabling pathologies, due to altered balance involving joints. No data are so far available on early ultrastructural osteo-chondral damages, associated with the onset of invalidating pathologies, like osteoarthritis-OA. Biopsies of glenoid articular cartilage and subchondral bone were taken from 10 adult patients underwent arthroscopic stabilization. Observations were performed under Transmission Electron Microscopy-TEM in tangential, arcuate and radial layers of the articular cartilage and in the sub-chondral bone. In tangential and arcuate layers chondrocytes display normal and very well preserved ultrastructure, probably due to the synovial liquid supply; otherwise, throughout the radial layer (uncalcified and calcified) chondrocytes show various degrees of degeneration; occasionally, in the radial layer evidences of apoptosis/autophagy were also observed. Concerning sub-chondral bone, osteocytes next to the calcified cartilage also show signs of degeneration, while osteocytes farther from the osteo-chondral border display normal ultrastructure, probably due to the bone vascular supply. The ultrastructural features of the osteo-chondral complex are not age-dependent. This study represents the first complete ultrastructural investigation of the articular osteo-chondral complex in shoulder instability, evaluating the state of preservation/viability of both chondrocytes and osteocytes throughout the successive layers of articular cartilage and sub-chondral bone. Preliminary observations here collected represent the morphological basis for further deepening of pathogenesis related to shoulder instability, enhancing the relationship between cell shape and microenvironment; in particular, they could be useful in understanding if the early

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Key words: shoulder instability; osteoarthritis; chondrocytes; osteocytes; ultrastructure

Shoulder instability represents one of the most disabling post-traumatic pathologies affecting young and adult subjects, due to altered balance involving the complex formed by several anatomical structures (glenoid surface, capsule, gleno-humeral ligaments, and rotator cuff tendons). Patients with chronic shoulder instability may present recurrent dislocation, subluxation, or chronic shoulder pain. For this reason, joint instability creates a clinical and economic burden in the *Health Care System*. Traumatic shoulder dislocation can cause a variety of well-known pathologic lesions of gleno-humeral ligaments, glenoid and humeral bone (Bankart, 1923, 1938; Hill and Sachs, 1940); in the US the estimated incidence of shoulder dislocation is 23.9 per 100.000 person/year (Zacchilli and Owens, 2010) with risk factors for shoulder dislocation including contact sports, male gender, young age. The recurrence of shoulder dislocation is the first significant clinical problem; studies on young and adult patients have evaluated that the chance of recurrent shoulder instability after standard nonoperative treatments is 55–67%, but recurring dislocation rate rises at 87% during 5-year follow-up if the only young male population is consider (Robinson et al., 2006; Owens et al., 2007). Randomized clinical trials demonstrate that surgical stabilization of the shoulder is more effective in preventing recurrence of dislocation with respect to immobilization and rehabilitation alone (Kirkley et al., 1999, 2005; Jakobsen et al., 2007). A relevant question is to understand if traumatic dislocation and disorders, that directly damage shoulder joint structures or lead to joint instability, are associated with disabling pathologies like osteoarthritis (OA) that is one of the most common joint diseases. A significant relationship between previous joint injury and resultant joint instability, followed by the onset of OA, has been clearly established in the literature for lower limbs (Lohmander et al., 2007; Richmond et al., 2013; Blalock et al., 2015). It is estimated that more than 40% of ligament/meniscus lesions-associated joint instability and articular surface injury of the knee will develop a post-traumatic OA (Blalock et al., 2015). The evolution of OA in the unstable shoulder after surgical stabilization is mostly reported in surgical series (Hawkins and Angelo, 1990; Allain et al., 1998; Hovelius et al., 2001; Flury et al., 2007). Samilson and Prieto (1983) coined the term "dislocation artropathy," suggesting a classification of shoulder artropathy and showing that shoulders without surgical stabilization can also develop OA even after a single dislocation. Hovelius et al. (1996) showed 20% incidence of artropathy, 10 years after the primary single shoulder dislocation, without any references to morphological alterations. Later, Hovelius and Saeboe (2009) indicate that the surgical repair may decrease the artropathy evolution. Thus, the mechanism of joint instability and the identification of which component in the articular/skeletal complex is primarily affected in instability acquire relevant clinical significance, particularly in the light of deepening knowledge on the onset and development of OA. The present study represents the first complete ultrastructural investigation of the glenoid articular osteo-chondral complex in post-traumatic shoulder instability, evaluating in detail cell features throughout the articular cartilage and sub-chondral bone that is the morphological prerequisite to understand bio-molecular mechanisms of such disabling pathology.

MATERIAL AND METHODS

The study includes 10 patients with anterior post-traumatic shoulder instability underwent arthroscopic stabilization with at least two episodes of gleno-humeral dislocations, at the Orthopedic University Department of Modena. The analysis included withdrawals from

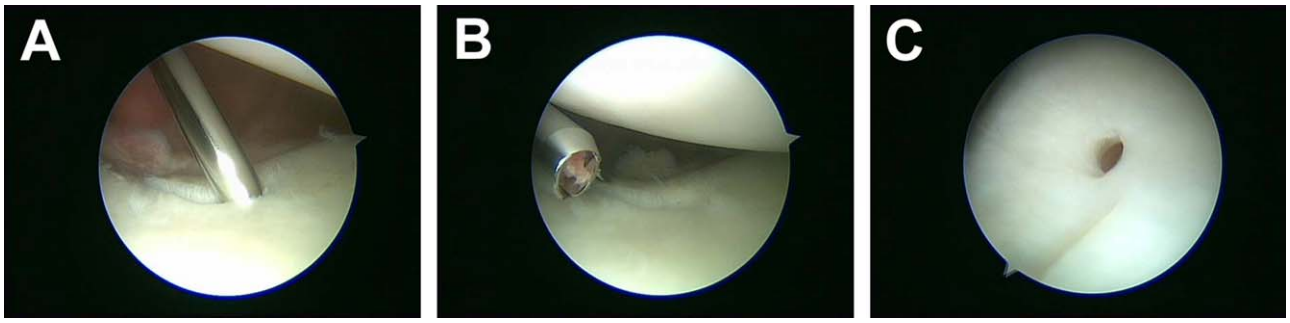

Fig. 1. Photographs showing a representative withdrawal (area = 9 mm^2; depth = 3 mm) by means of Arthrex curette (diameter = 3.4 mm) from a patient affected by shoulder instability. From A to C successive phases during arthroscopy.

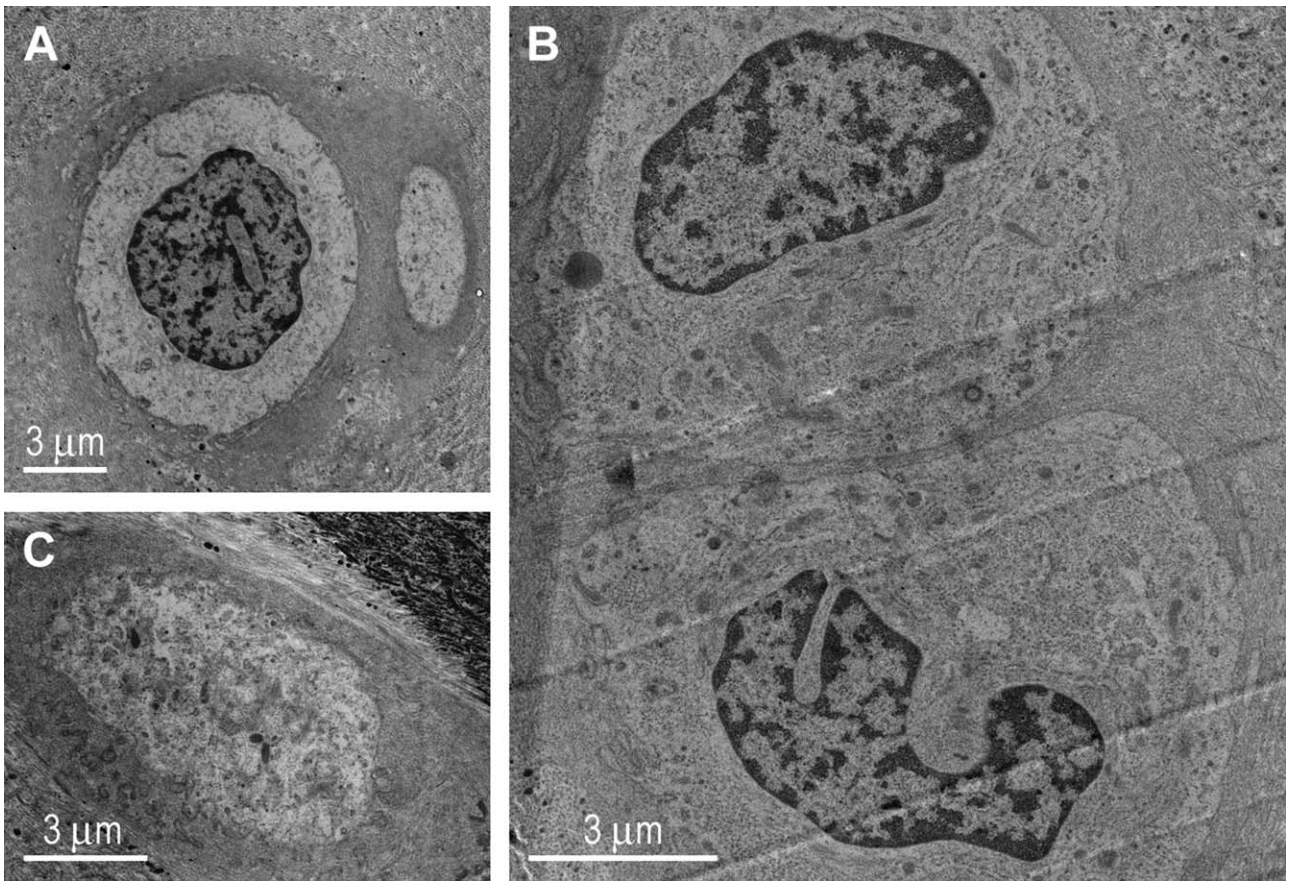

Fig. 2. TEM micrographs from a representative control subject, showing the ultrastructure of chondrocytes inside arcuate (**A**), un-calcified radial (**B**) and calcified radial (**C**) layers. Note, in B the very well preserved ultrastructure of chondrocytes (to be compared with chondrocytes from patients with shoulder instability showed in Figs. 5C and 9B–G).

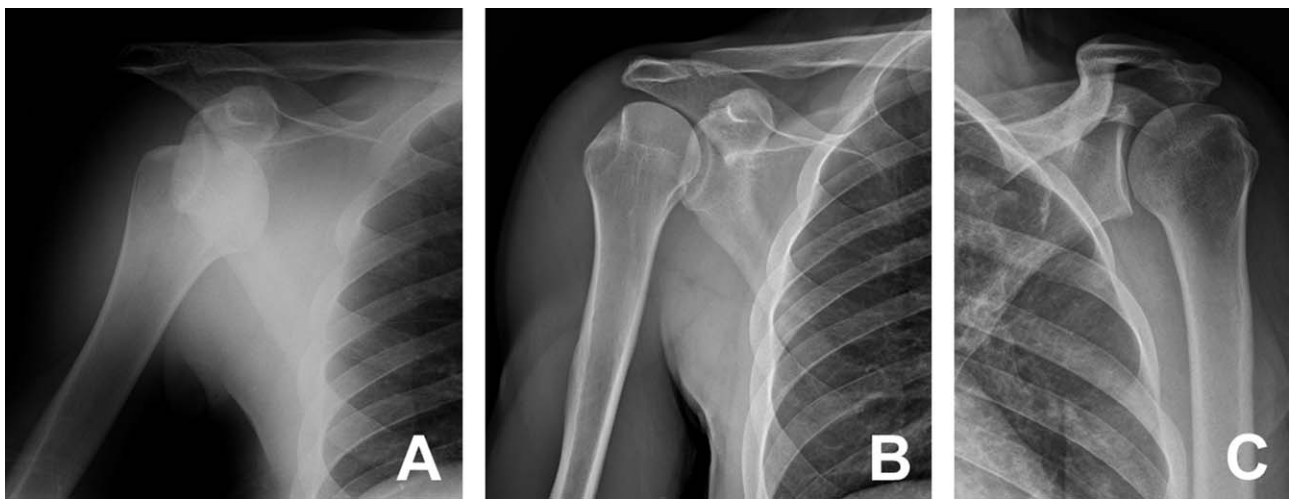

Fig. 3. X-ray taken from a recruited patient with shoulder lower anterior dislocation (**A**) and after reduction with external technique (**B**); control subject (**C**).

articular cartilage and sub-chondral bone in unloaded glenoid anterior rim (2 mm from the glenoid rim), without soft tissues damage, performed by a curette of 3.4 mm diameter (by Arthrex Inc.) that corresponds to an area of 9 mm^2 and about 3 mm of depth (Fig. 1). The enrolment of patients was performed among the subjects with shoulder instability waiting for an arthroscopic stabilization, meeting the inclusion/exclusion criteria. Features of instability group were the following: average age 27.4 years (min 21 to max 40); 9 males and 1 female; 9 caucasians and 1 african; 8 right side (5 dominant) and 2 left side (no dominant). In 6 patients the first shoulder dislocation was caused by contact sport, in the others by accidental fall. The dislocation mean number was 4.8 (range 2–10), the average time from the first episode and the surgical stabilization was 6.5 years (range 4.8–8.4). Biopsies were also performed in three subjects undergoing arthroscopic rotator cuff tear repair (considered as controls). The inclusion criteria for controls were: 18–60 years, no previous interventions or humeral/glenoid fractures, informed consensus; the exclusion criteria were: radiographic OA evidence or arthroscopic cartilage damage, rotator cuff tear in the instability group. The study was approved by the *ethics Committee* of the Modena Policlinico Hospital (Ref. n° 46/13).

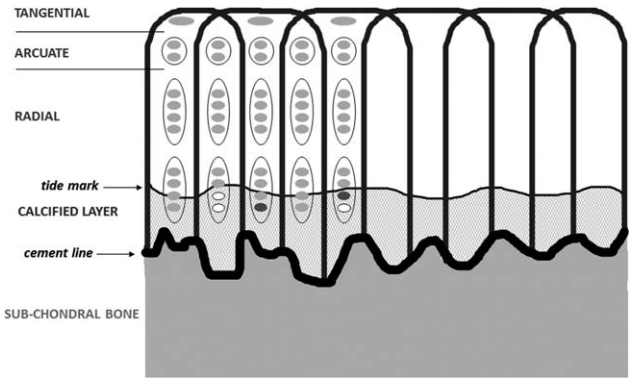

Fig. 4. Simplified schematic drawing showing articular cartilage stratification. From top to bottom: tangential layer (with collagen fibrils parallel to the joint surface); arcuate layer (with collagen fibrils forming arched systems); radial layer divided in un-calcified and calcified portions by means of the tide mark (with collagen fibrils orthogonal to the joint surface). The cement line represents the border between calcified cartilage and sub-chondral bone.

All withdrawals were immediately fixed for 2 h with 4% paraformaldehyde in 0.13 M phosphate buffer pH 7.4, postfixed for 1 h with 1% osmium tetroxide in 0.13 M phosphate buffer pH 7.4, dehydrated in graded ethanol and embedded in epoxy resin (Durcupan ACM); samples were then sectioned with a diamond knife mounted in an Ultracut-Reichert Microtome. Semi-thin sections (1 μm) were stained with toluidine blue and examined by Axiophot-Zeiss light microscope (LM). Ultrathin sections (70–80 nm) were mounted on Formvar- and carbon-coated copper grids, stained with 1% uranyl acetate and lead citrate, and examined by Zeiss EM109 transmission electron microscope (TEM). All specimens were nondecalcified before osmium postfixation; for this reason, some ultrathin sections show few damage tracks, whose presence, however, doesn't prevent data interpretation.

RESULTS

In control subjects, all chondrocytes display the typical morphology of a healthy articular cartilage (Fig. 2).

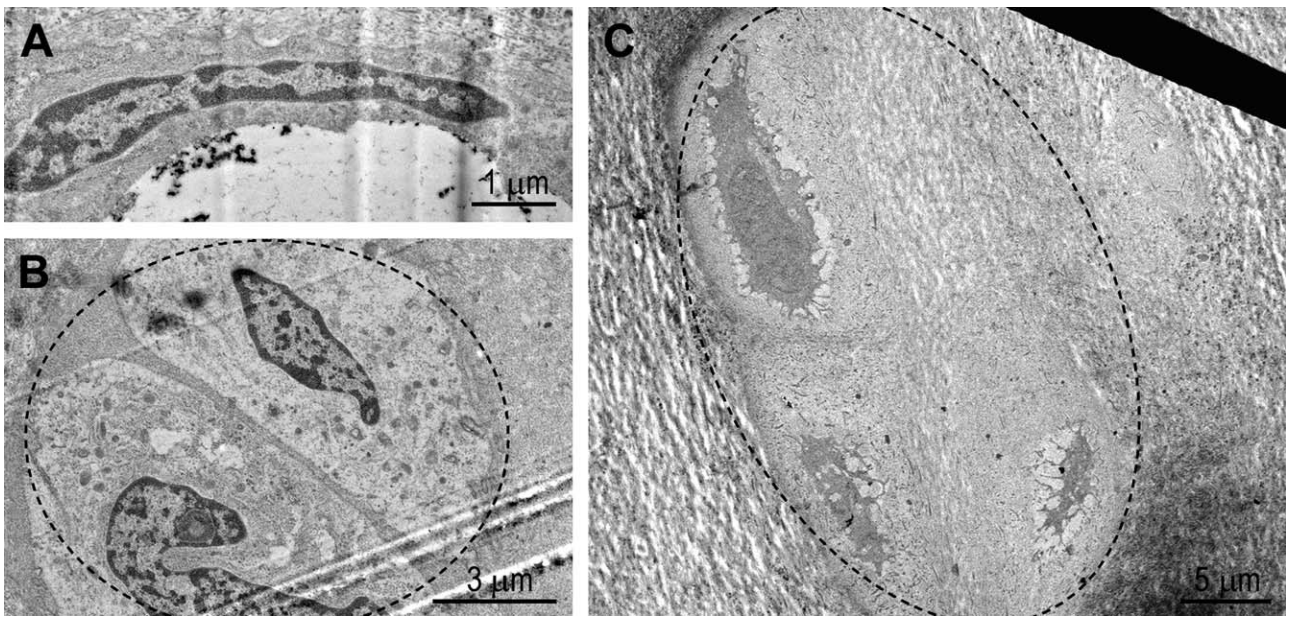

Fig. 5. TEM micrographs from a patient with shoulder instability showing differently-shaped chondrocytes in the various layers: (**A**) fibroblast-like flattened chondrocytes inside tangential layer; (**B**) globular-shaped chondrocytes arranged in a spheroid isogenic group (dashed circle) inside the arcuate layer; (**C**) less-globular chondrocytes organized in an elongated isogenic group (dashed oval) inside the radial layer.

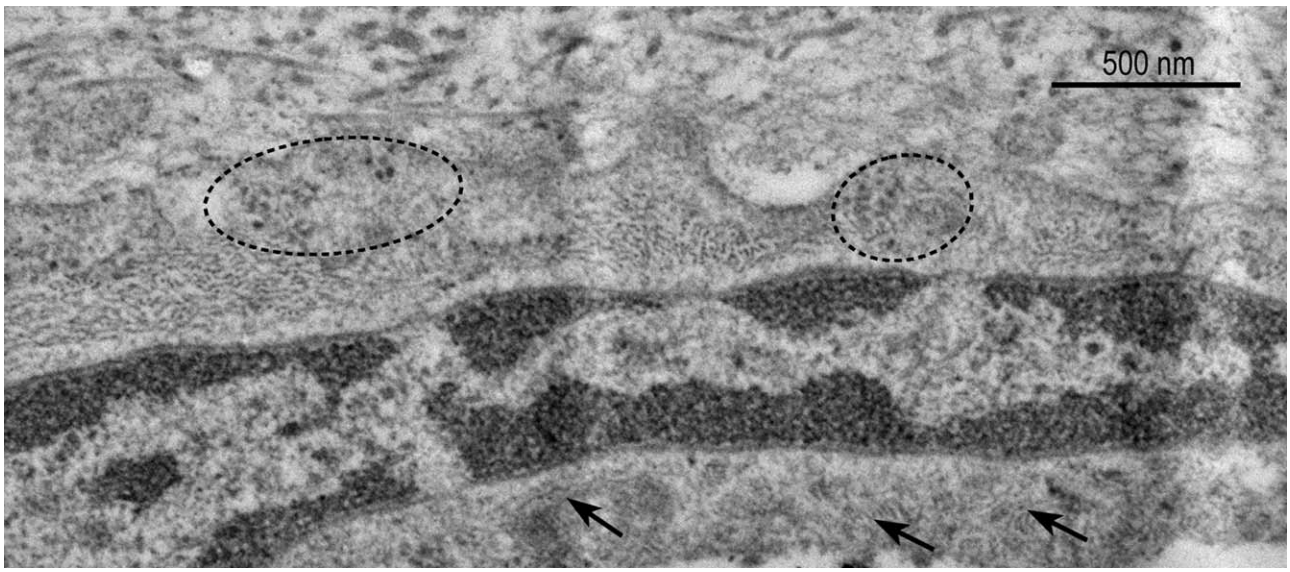

Fig. 6. TEM micrographs from a patient with shoulder instability showing, in the tangential layer, a flattened chondrocyte displaying a well preserved heterochromatic nucleus and developed cytoplasmic organelle machinery. Note the abundance of cytoskeleton (arrows) and ribosomes (dashed ovals).

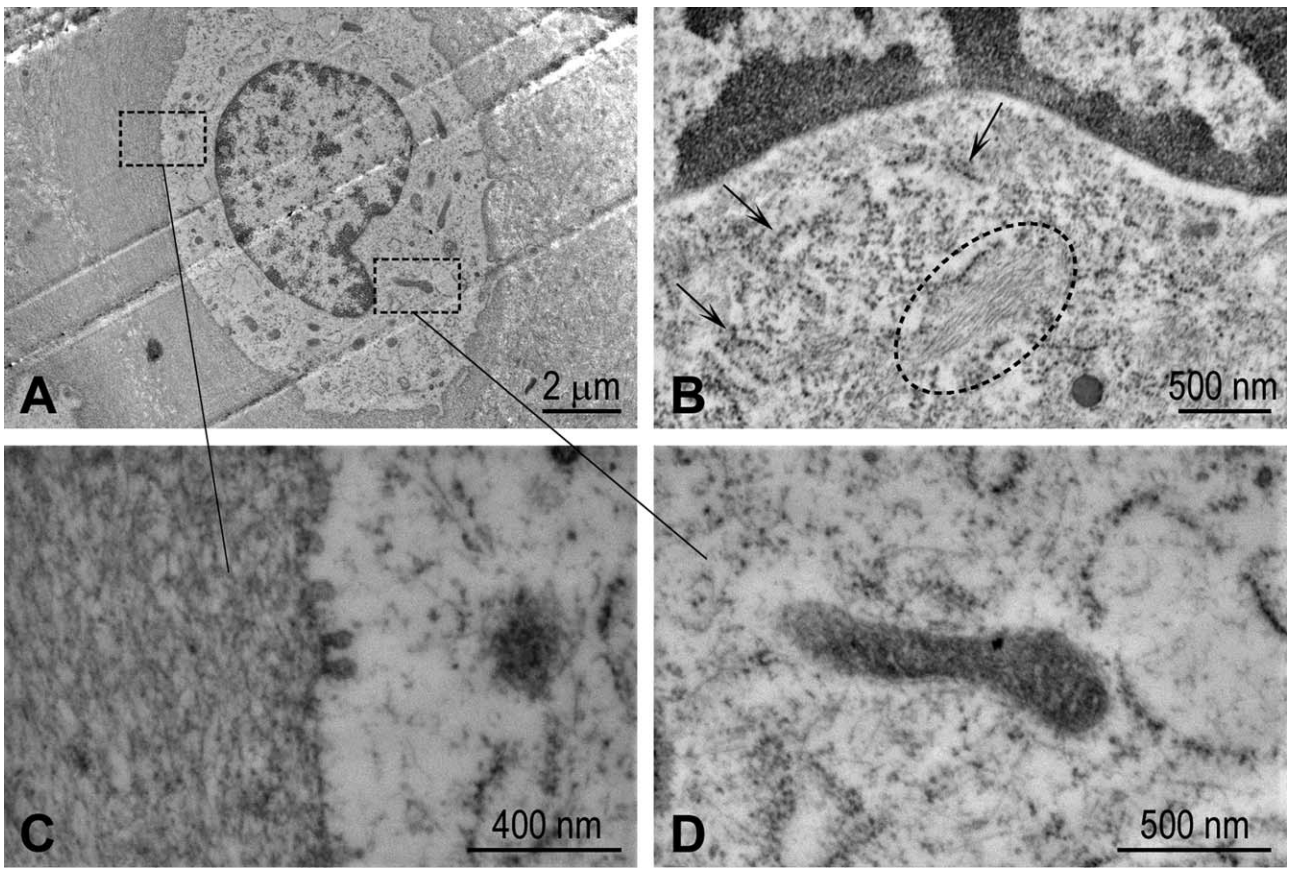

Fig. 7. TEM micrographs from a patient with shoulder instability showing, in the arcuate layer, two chondrocytes (**A** and **B**) with well-preserved cytoplasmic organellar machinery. Note in B the abundant cytoskeleton (dashed oval) and abundant polysomes (arrows). Note also in **C** and **D** (enlargements of the squared areas in A) various micro-pinocytosis vesicles and one mitochondrium with clearly defined tubular crests, respectively.

In patients with shoulder instability, post-traumatic damage (like dislocation) can be easily recognizable by X-ray imaging (Fig. 3). Concerning the histological evaluation, patients with shoulder instability showed the classical stratification of the articular cartilage, a peculiar type of hyaline cartilage, characterized by ordered texture of matrix fibrillar components: (i) superficial or tangential layer, in which collagen fibrils lie parallel to the joint surface; (ii) transitional or arcuate layer, in which the collagen fibrils are arranged in forming arched systems; (iii) radial layer, distinguished in middle and deep (depending on the consistence of the matrix, uncalcified or calcified, respectively), in which collagen fibrils run orthogonal to the articular surface. In Figure 4 is depicted (only for schematic purpose) the articular cartilage stratification (for better detail concerning fibril arrangement see Nickien et al., 2013). In the various layers, the cell organization adapts to the typical orientation of matrix fibril components; thus, the shape and arrangement of chondrocytes showed, as in control subjects, different morphology in the various layers: fibroblast-like flattened chondrocytes in the tangential layer, never organized in isogenic groups; globular-shaped cells arranged in spheroid isogenic groups inside the arcuate layer; less-globular chondrocytes organized in elongated isogenic groups in the radial layer (Fig. 5).

All observations obtained from all patients with shoulder instability included in the study showed similar ultrastructural features, regardless of age. In the tangential layer, the flattened chondrocytes display a well preserved nucleus and developed cytoplasmic organelle machinery. In particular, chondrocytes show well-represented rough endoplasmic reticulum (RER) and heterochromatic nuclei (Fig. 6). In the arcuate layer, chondrocytes appear very well structured with developed RER, evident juxtanuclear Golgi apparatus, abundant polysomes, numerous mitochondria and various micro-pinocytosis vesicles (Fig. 7) as well as a well-developed cytoskeleton (Fig. 8). Otherwise, differently from control subjects, the middle uncalcified radial layer contains chondrocytes at various degrees of degeneration, also well recognizable under LM (Fig. 9A), separated inside the isogenic group by enlarged intercellular spaces; nuclei often appear pyknotic and the cytoplasm appears partially shrunk with variously-sized vacuoles abundant throughout the cytosol (Fig. 9B–G, to be compared with Fig. 2B). Occasionally, in uncalcified radial layer, chondrocytes with morphological signs of apoptosis or autophagy were also observed (Fig. 10). In the deep calcified radial layer (Fig. 11A,B), some chondrocytes display small size and very poor organellar machinery; however, mostly of chondrocytes clearly show signs of degeneration. As far as subchondral bone is concerned, also osteocytes next to the deep calcified cartilage, within 80–100 μm from the cement line, show cell shrinkage and not well defined cytoplasmic structure, suggestive of degeneration (Fig. 11C), while osteocytes more distant from the osteo-chondral border (farther than 100 μm from the cement line) display normal ultrastructure (Fig. 11D).

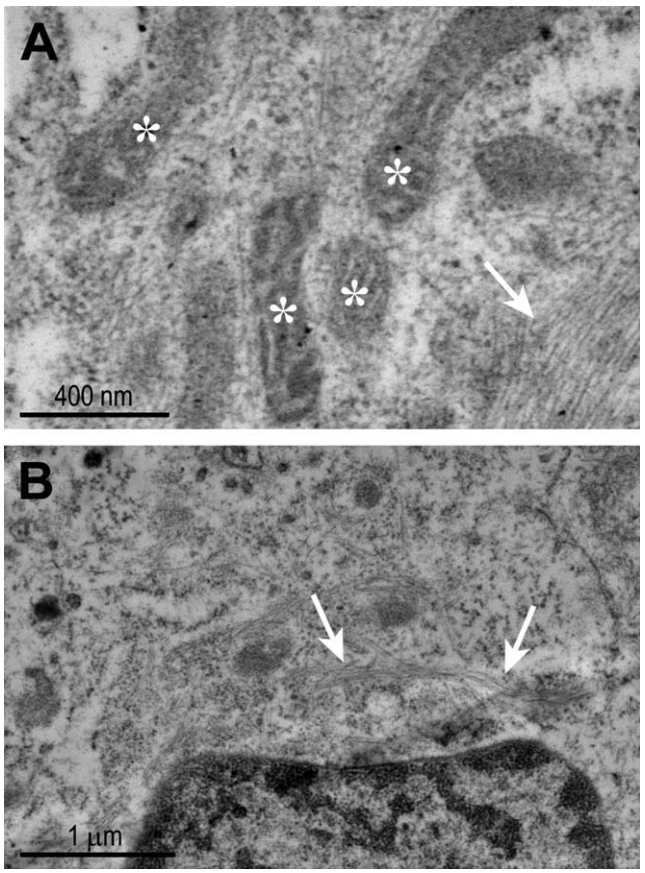

Fig. 8. TEM micrographs from a patient with shoulder instability. Particular of chondrocytes in the arcuate layer, showing the well-developed cytoskeleton (arrows) and well-structured mitochondria (asterisks).

DISCUSSION

This article describes in detail, for the first time, cell ultrastructural modifications inside the complex formed by glenoid articular cartilage and sub-chondral bone in shoulder instability, particularly evident inside the uncalcified radial layer.

As it is well known, articular cartilage is a highly specialized and structured tissue that is devoid of blood vessels, lymphatics and nerves; it is a viscoelastic tissue due to the presence of various macro-molecules inside chondromucoid matrix (Khalsa and Eisenberg, 1997; Setton et al., 1999), like decorin, biglycan, fibromodulin, and aggrecan. Negatively charged carboxyl and sulfate groups found on these glycosaminoglycan chains (namely, keratin sulfate and chondroitin sulfate) have a high affinity for water that interferes with the integrity of the cellular components, that is, chondrocytes. These cells are anaerobic, have limited cell-to-cell contacts and low self-renewal potential. The maintenance over time of the constant composition and arrangement of both fibrillar and non-fibrillar elements of chondromucoid results in the normal ultrastructure of the cellular component; thus, the first point to discuss is the importance of cell ultrastructural observation in the articular cartilage from subjects with shoulder instability that, reflecting the physiologic composition of the chondromucoid, acquires clinical relevance in the orthopedic practice. Both fibril-level organization and chondrocyte ultrastructure are crucial in articular cartilage physiology: recently Nickien et al. (2013) studied in detail the former, in the

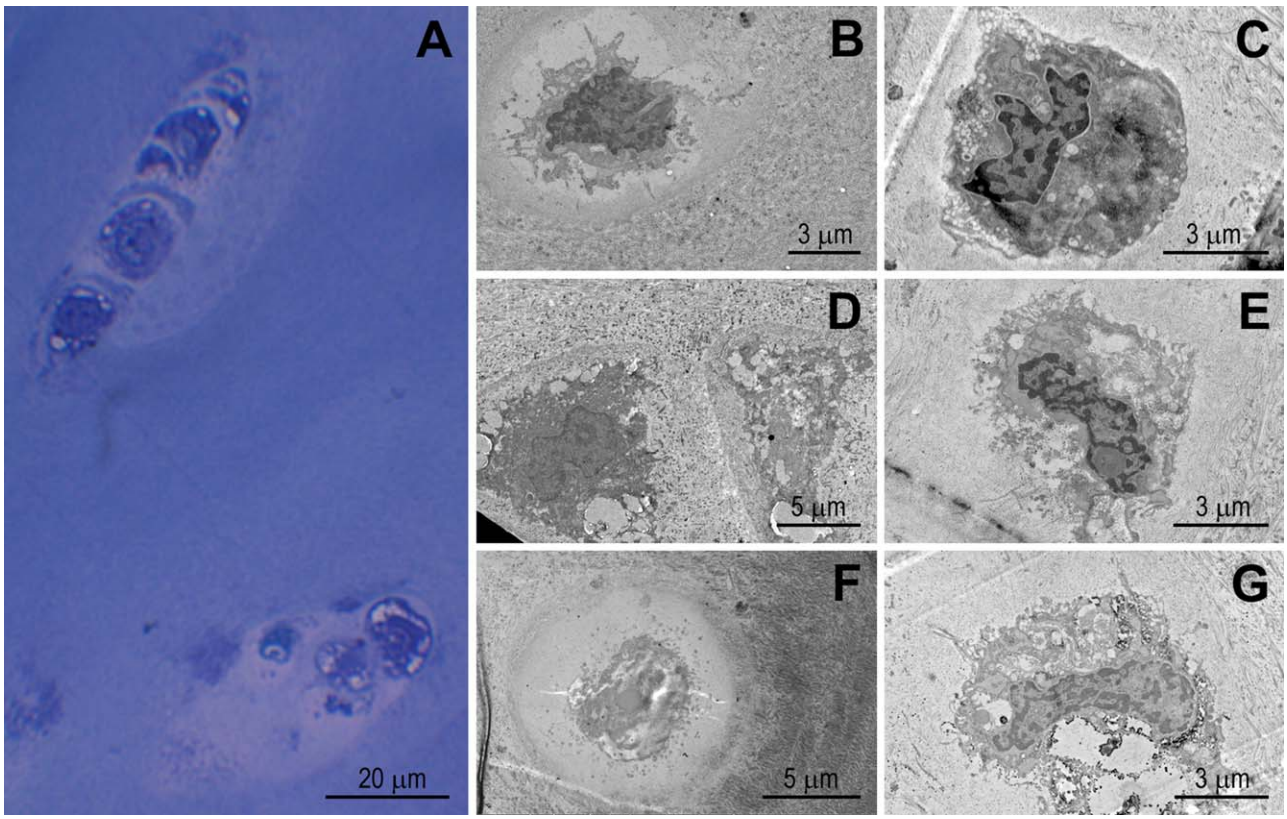

Fig. 9. LM (**A**) and TEM (**B**–**G**) micrographs from a patient with shoulder instability, showing chondrocytes at various degrees of degeneration, inside isogenic groups, in the middle un-calcified radial layer. Note partially coerced chondrocytes with pyknotic nuclei and variously-sized vacuoles abundant throughout the cytosol.

present article we report in detail the latter. Therefore, it is important recognize the peculiar ultrasturctural features of chondrocytes arranged in the various zones of the articular cartilage that determine its unique properties (Poole, 1997). (1) The superficial tangential zone contains flattened chondrocytes within tightly packed collagen fibers (primarily types I, and IX) oriented mostly parallel to the articular surface. This zone serves to protect the underlying zones and provides resistance to shear forces (Guo and Torzilli, 2016). (2) The arcuate zone contains spherical-like chondrocytes (Fig. 2A) in a transitional layer composed of proteoglycans and thick, obliquely oriented collagen fibrils. The oblique orientation of the collagen fibrils marks a transition from a resistance-to-shear-forces to resistance-to-compressive-forces (Nickien et al., 2013; Quinn et al., 2013). (3) The un-calcified radial zone contains chondrocytes and collagen fibrils aligned roughly perpendicular to the joint (Fig. 2B), whose arrangement allows for the greatest resistance to compressive forces; the highest proteoglycan content and the lowest water concentration are found just in this zone. (4) The calcified radial zone, containing chondrocytes partially surrounded by calcified matrix (Fig. 2C), is separated from the deep zone by the wavy tidemark. The primary role of the calcified radial zone is to secure firmly the articular cartilage to the sub-chondral bone, between which the irregular cement line is present. The essential function of articular cartilage is based on the ability to modulate its metabolic response in relation to mechanical stimuli (Sah et al., 1989; Guilak et al., 1994; Smith et al., 1995; Torzilli et al., 1997; Grodzinsky et al., 2000), in turn influenced by both the local profile of interstitial fluid flow and the matrix deformation (Wong et al., 1997; Quinn et al., 1998; Buschmann et al., 1999) as well as the chondrocyte location within the different zones (Lee et al., 1998). Moreover, the articular cartilage provides a lubricated surface for low-friction movements and an equilibrated transmission of loads to the sub-chondral bone (immediately below the articular cartilage) with the primary function to attenuate the forces acting on the joint. Subchondral bone is composed of a superficial layer of compact cortical bone and an underlying layer of cancellous bone. The complex formed by articular cartilage and sub-chondral bone allows the transformation of shear forces into tensile and compressive ones (Khalsa and Eisenberg, 1997; Setton et al., 1999; Hoemann et al., 2012). The different ultrastructural features in shoulder instability (with respect to the normal conditions, above described) are extremely crucial to define strategies aimed to avoid OA onset. Moreover, the morphological evidences from arthroscopic analysis, preliminary to surgery in the recovery of shoulder instability, are important to understand if the early surgical treatment in shoulder instability could retard or prevent the beginning of OA.

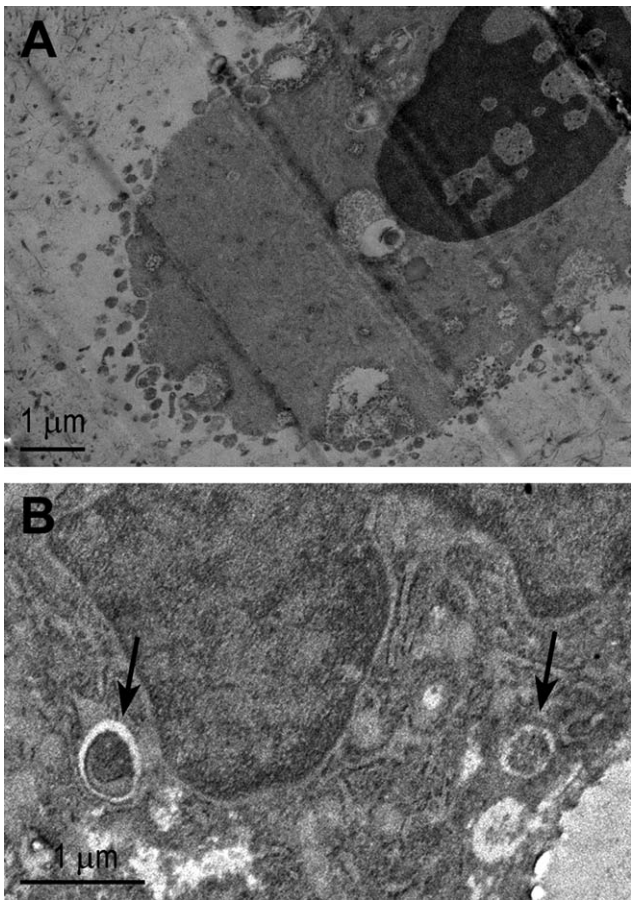

Fig. 10. TEM micrographs from a patient with shoulder instability. Note, inside the un-calcified radial layer, an apoptotic chondrocyte with the condensed nucleus (in **A**) and a chondrocyte containing autophagic vacuoles (in **B**, arrows).

OA is the most common form of joint disease characterized by loss of articular cartilage, remodelling of subchondral bone, osteophyte formation, pain and limitation of the range of motion. A significant association between previous joint injuries and joint instability, inducing the development of secondary osteoarthritis (i.e., post traumatic OA), has been clearly established in the literature (Lozito et al., 2013; Sena et al., 2014; Adams et al., 2015; Carter et al., 2015; Genemaras et al., 2015; Lieberthal et al., 2015). Injuries that directly damage the articular cartilage represent the main causes of the post-traumatic OA. The features that underlie the progression of post-dislocation OA are not fully understood. It has been demonstrated that joint instability significantly increased contact stress directional gradients in conjunction with dynamic articular surface incongruity (McKinley et al., 2004; Hovelius and Saeboe, 2009). Joint instability may result from abnormal mechanical forces and subsequent disruption of the extracellular matrix; this damage may lead to release of glycosylated aminoglycans and collagen molecules, which are sensed by surrounding chondrocytes through mechano-receptors and surface receptors (Villemure et al., 2002; Zignego et al., 2015; Chen et al., 2016). This leads to changes of gene expression and cartilage metabolism, including increased expression of catabolic factors and decreased expression of cartilage structural proteins (type II collagen, aggrecan); in addition, abnormal loading can lead to chondrocyte death mediated by oxidative stress or integrin-cytoskeleton inter-reactions (Chang et al., 2015; Yu et al., 2015). Thus, chondrocyte death decreases cartilage ability in producing and maintaining its extracellular matrix. Most of these considerations are in line with our results, showing that the main feature in patients affected by shoulder instability is the degeneration of chondrocytes inside the un-calcified radial layer (some of them also displaying autophagic or apoptotic evidences) as well as the osteocytes of sub-chondral bone next to the calcified cartilage. Some authors, indeed, suggest that tissue structural adaptations are performed primarily by the superficial and transitional zones (Pritzker et al., 2006); Quinn et al. (2013) explain such evidences on the basis of variations observed in both matrix (zone thickness) and chondrocyte morphology (cellular density) under changing joint and biomechanical environments. Otherwise, Sulzbacher (2013) observed that changes of the subchondral bone were found to precede cartilage damage, suggesting a primary alteration of the subchondral region: this observation agrees with our results, but the precise sequence of events leading to these changes is still not clear. Other studies were focused on the metabolic activity of chondrocytes in healthy cartilage of patients with OA. Interestingly, in shoulder instability, we observed a relationship between cell ultrastructure and metabolic context. The normal or altered ultrastructure of chondrocytes and osteocytes, observed in different sites, is likely due to their metabolic conditions: (i) in tangential and arcuate layers of the articular cartilage, the well preserved ultrastructure of chondrocytes is probably due to the synovial liquid supply (unlike chondrocytes throughout the radial layer, in both the un-calcified and calcified portions, showing various degrees of degeneration); (ii) in sub-chondral bone, the normal ultrastructure of osteocytes farther from the osteo-chondral border is probably due to the greater proximity to the bone vascular supply (unlike osteocytes next to the calcified cartilage showing signs of degeneration). Therefore, it should not be a coincidence that precisely those chondrocytes/osteocytes further away from synovial liquid and bone vascular supply, respectively, are the first cells to show signs of distress; consequently, they are undoubtedly elements more susceptible to the onset of OA.

It is also to be underlined that some authors reported that the formation of chondrocyte cluster may occur in addition to degeneration of some cells (Pritzker et al., 2006; Lotz et al., 2010); in our shoulder instability samples, instead, we never observed chondrocyte clusters, but only simple isogenic groups containing degenerated chondrocytes in the radial layer of articular cartilage.

It would be crucial to correlate ultrastructural alterations inside the glenoid articular osteo-chondral complex, showed for the first time in this article, with the different gene expression observed by many authors in shoulder (Marino et al., 2004; Mayan et al., 2013; Casagrande et al., 2015). In particular, Casagrande et al. (2015) showed that certain genes are markedly up-regulated in osteoarthritic shoulders compared with non-osteoarthritic ones; in particular, Cx43, Cox-2, versican, collagen type I, ADAMTS5, MMP-3, and TNF-α expression resulted

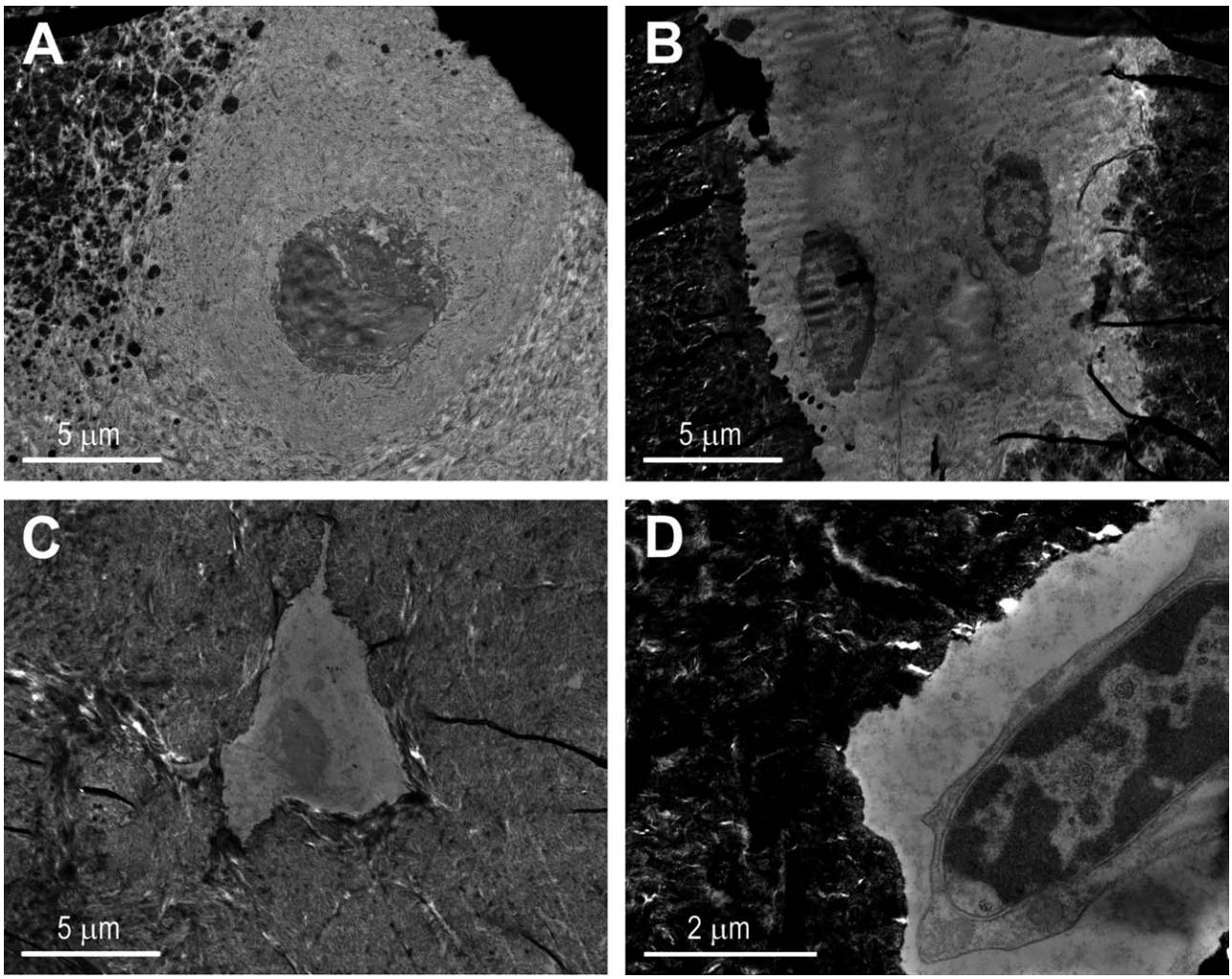

Fig. 11. TEM micrographs from a patient with shoulder instability. In **A** and **B**: small-sized chondrocytes with very poor organellar machinery and signs of degeneration in deep calcified radial layer. In C: an osteocyte showing evidences of degeneration inside sub-chondral bone, next to the deep calcified cartilage. In **D**: an osteocytes displaying normal ultrastructure inside sub-chondral bone, farther from the osteochondral border.

significantly increased. The same authors also suggest that these genes might be useful biomarkers for examining shoulder OA. Moreover, histologic degrees of cartilage degeneration in humeral articular cartilage were recently deduced by MR imaging and tomography (Bittersohl et al., 2015; Pawson et al., 2015), so that these authors suggest that bone and cartilage changes can be valuable by means of a sophisticated targeted diagnostic imaging approach to provide reconstructed 3D datasets of the cartilage and cortical/trabecular bone tissue, that allow a better classification of the disease, leading to improved therapies. Furthermore, these imaging techniques that reflect the ultrastructural features, can make a positive contribution to the currently evolving knowledge and the practice of cartilage prognostic profiles.

In conclusion, notwithstanding the zone-specific metabolic activity and the gene expression of chondrocytes are strongly related to the chondrocyte shape (Benya et al., 1988), the mechanisms by which chondrocyte shape and function are related to the mechanical response of the extracellular matrix (when subjected to external joint loading or in the onset of OA) are yet to be precisely elucidated. The present work represents the essential morphological basis (that must necessarily precede the biomolecular insights) for further deepening of the pathogenesis related to shoulder instability, since the relationship between cell ultrastructure and microenvironment is important for understanding both cell biomechanics and changes in chondrocyte function after post-traumatic injury as well as in the initiation and progression of OA.

AUTHOR CONTRIBUTIONS

Paolo Baudi, Fabio Catani, Marzia Ferretti and Carla Palumbo: conception and design. Manuela Rebuzzi, Gabriele Campochiaro, Fabio Serafini and Alberto Smargiassi: collection and assembly of data. Paolo Baudi,

Manuela Rebuzzi, Marzia Ferretti and Carla Palumbo: data analysis, interpretation and drafting of the manuscript. All authors: critical revision of the manuscript and approval of the article.

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