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DEPARTMENT OF BIOMOLECULAR SCIENCES

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MECHANICALLY STRESSED JOINT MODIFICATIONS IN DIFFERENT PHYSIOPATHOLOGICAL CONDITIONS: A MORPHOFUNCTIONAL ANALYSIS

SSD: BIO/16 HUMAN ANATOMY

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INTRODUCTION

Joint degeneration and articular lesions represent a common cause of temporary or permanent disability, both in elderly and in young people, and their onset could be associated to different physiological and pathological conditions.

Mechanical overloading is one of the most relevant risk factor for joint diseases and traumas promoting. As already demonstrated in the literature, conditions such as obesity (often due to sedentariness and wrong diet), working physical overuse and excessive or incorrect physical activity can induce articular disorders or injuries.[1,2] These last, may also depend, for instance, on repetitive microtrauma, acute sport trauma or aging. Joint injuries, especially if recidivist, together with mechanical overloading may induce articular structure degeneration, which can contribute to the development and the progression of articular pathologies.[3,4,5] Two of these most common joint diseases are osteoarthritis (OA) and calcium pyrophosphate dihydrate crystal deposition disease (CPPD).

OA is today the leading cause of pain and disability in the elderly population.[6,7] Aging is the most relevant risk factor, together with mechanical overuse, obesity, joint trauma and impact or contact sports.[8,9,10] OA is characterized by joint pain, stiffness, swelling and limited functions, with a consequent quality of life reduction.[10,11,12] In radiographic images osteophytes and joint space impairment mainly appear.[13] This chronic-degenerative disease may affects all synovial joints, but in particular interest knee, hip, vertebral column and hands.[8,9]

CPPD is a common rheumatological disorder in elderly population, characterized by the presence of radiographic articular cartilage calcifications.[14,15] This condition is also called chondrocalcinosis.[16] It is strictly correlated to aging and/or previous joint trauma,[16,17] and the most affected joint is knee, followed by wrist, hip, pubis symphysis and shoulder.[18] Symptoms are difficult to interpret, in fact CPPD can be characterized by acute joint pain attacks, swelling and stiffness, but it may also be asymptomatic, or comparable with a chronic inflammatory arthritis.[19] Moreover, CPPD and OA often coexist in the same joint, probably inducing a symptom worsening,[20,21,22] but their interaction is still unclear.

An appropriate, constant over time and balanced physical activity can be the most powerful preventive and rehabilitative treatment for articular degeneration or joint traumas. In the preventive strategy, a proper nutrition and the maintenance of the BMI (Body Mass Index) in the normal-weight threshold are also added.[8] Concerning the rehabilitation, we could have different approaches in terms of conservative or surgical treatment. *American College of Sports Medicine's guidelines* represent a valid international reference for safe physical exercise prescription in subjects affected by articular pathologies such as OA and CPPD or post-articular trauma.[23] This activity is important to maintain muscular trophism, to improve proprioception and balance, to limit the progression of the disease, to avoid further joint traumas and also to avoid an eventual surgery.[24] If this latter becomes necessary, it is very important to use specific pre-operative exercise protocols, to reduce as much as possible recovery time after surgery.[12] Finally, also the post-operatory rehabilitation has a crucial role in maximizing the surgery results, in articular and muscular recovery and in returning to sport activity as soon as possible, in case of athletes.[12]

As previously reported, given that overload is one of the main risk factors for articular pathology onset or joint trauma, during my doctoral course I focused my attention on two of the most mechanically stressed joints, which are the knee and the shoulder. These are always subjected to stress, wear and mechanical loading, and they consequently have a high probability to incur in injuries or joint degeneration.

In particular, I analysed knee and shoulder joints modifications in different physiopathological conditions: OA, CPPD and meniscal trauma.

AIM OF THE WORK

In this thesis, I analysed:

- knee joint alterations in patients affected by CPPD;
- shoulder joint modifications in subjects affected by CPPD;
- traumatic knee joint changes in median-age patients, compared with knee joint alteration of subjects affected by OA.

Each condition is explained in detail in the three following publications.

Briefly, the main aims of the first study were to analyse the differences between knee loaded and non-loaded area, and to investigate morphological modifications of femoral condyle articular cartilage in patients affected by CPPD, in particular analysing chondrocyte behavior.

The main goal of the second work was to investigate calcium crystal distribution and its interaction with cell behavior in glenohumeral joint, in particular in humeral articular cartilage, long head of biceps brachii tendon sheath, articular capsule and loose bodies, in patients affected by CPPD.

The aim of the third study was to investigate extracellular matrix (ECM) composition and cell morphology of normal, injured, and OA menisci from human knee. In addition, the age importance to determine the pre- and post-operative outcome was examined.

As reported in the results, samples were fixed and processed to be analysed by means of light microscopy, transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM) with microanalysis capability.

RESULTS

Curzi D, Fardetti F, Beccarini A, Salucci S, **Burini D**, Gesi M, Calvisi V, Falcieri E, Gobbi P. Chondroptotic chondrocytes in the loaded area of chondrocalcinotic cartilage: a clinical proposal? *Clinical Anatomy*, 2017. 31(8):1188-92. DOI: 10.1002/ca.22988.

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Letter to the Editor

Chondroptotic Chondrocytes in the Loaded Area of Chondrocalcinotic Cartilage: A Clinical Proposal?

To the Editor, *Clinical Anatomy*:

Chondrocalcinosis is a crystal-induced arthropathy characterized by calcium pyrophosphate dihydrate (CPPD) crystal deposition in the connective tissues, and it is strictly correlated to aging, one of the most important risk factors. The articular cartilage of the knee, where the crystal deposition is associated to inflammation and tissue degeneration, is one of the most affected tissue (Abhishek et al., 2013). The chondrocyte behavior is strictly correlated to the integrity of articular cartilage, given its ability in maintaining tissue homeostasis. Cell death, which characterizes chondrocytes of control tissues during natural cell turnover, appears quantitatively increased in the cartilage of patients. For this reason, chondrocyte cell death has been largely investigated in cartilage to deeply understand the pathogenesis of chronic joint diseases, such as osteoarthritis, rheumatoid arthritis, and chondrocalcinosis. Although different kinds of cell death (e.g., apoptosis, necrosis, autophagy) have been revealed in the most common articular pathologies, their role in the pathophysiology of these diseases is still unknown (Lee et al., 2011). In particular, there are contradictory researches and it is not clear whether chondrocyte death is an inducer of cartilage degeneration or an effect of its degeneration (Zamli and Sharif, 2011). Mechanical stress, induced by loading, seems to have a key role in chondrocyte death. In fact, in literature a cause–effect relationship has been demonstrated between loading compression and cell death. A significant increase of chondrocyte necrosis and apoptosis has been revealed following single-loading compression or prolonged cyclic compression (Chen et al., 2001; Lucchinetti et al., 2002).

Chondroptosis, a different kind of chondrocyte apoptosis, has been observed in patients affected by osteoarthritis (Pérez et al., 2005). This type of cell death has some features in common with classical apoptosis such as cell shrinkage, chromatin condensation, and the probable involvement of caspases. In addition, chondroptosis shows some peculiar characteristics, including cytoplasmic vacuolization, autophagic vacuoles, extrusion of cellular material into the extracellular space, and a final disruption of cell remnants (Battistelli et al., 2014). To date, no evidence has revealed chondroptosis in patients affected by chondrocalcinosis. For that, a morphological study of loading effects in the femoral condyle cartilage of a patient affected by chondrocalcinosis has been performed, to identify the possible chondroptotic death presence in the chondrocytes of the cartilage middle layer.

Human cartilage samples were obtained from femoral condyle of the left knee of the patient (female; age 61) undergoing total knee replacement surgery. Pre-surgery X-ray images of the weight bearing knee displayed the typical degenerative joint features, such as joint space narrowing, osteophytes, and bone sclerosis (Figs. 1A and 1B). At high magnification (Fig. 1C), the presence of the articular cartilage and/or meniscal fibrocartilage calcification is a macroscopic and common feature of chondrocalcinosis. Histological analysis, microanalysis, transmission, and environmental scanning electron microscopies were performed in cartilage specimens.

Compared to unloaded area (Fig. 2A), the loaded sites of cartilage showed a general breakdown, characterized by the occurrence of empty lacunae in the underlying tissue layers. In the upper layers of the cartilage, the accumulation of CCPD crystals was visible (red staining in Fig. 2B) and some of them were observable within the tissue (Fig. 2C). While specimens from the unloaded area revealed an apparently undamaged articular surface (Fig. 2D), samples from tissue sites subjected to repeated mechanical stress displayed a first cartilage layer wear. This superficial zone appeared somewhere absent, revealing the underlying layers directly facing the articular cavity (Fig. 2E). CCPD crystals appeared on cartilage surface (Fig. 2F) and their nature has been confirmed by energy dispersive X-ray spectroscopy (EDS) spectrum (Fig. 2G). Indeed, the relationship between calcium and phosphorous peaks was in line with the stoichiometric ratio of CCPD.

Analyzing the chondrocyte behavior in the middle layer of cartilage specimens from area subjected to mechanical loading, the presence of a diffuse cell death has been observed. Some chondrocytes displayed necrotic features, the other ones showed morphological patters suggestive of apoptotic cell death. Among these, a group of chondrocytes revealed the typical features of chondroptosis. In particular, these cells showed chromatin margination and condensation followed by

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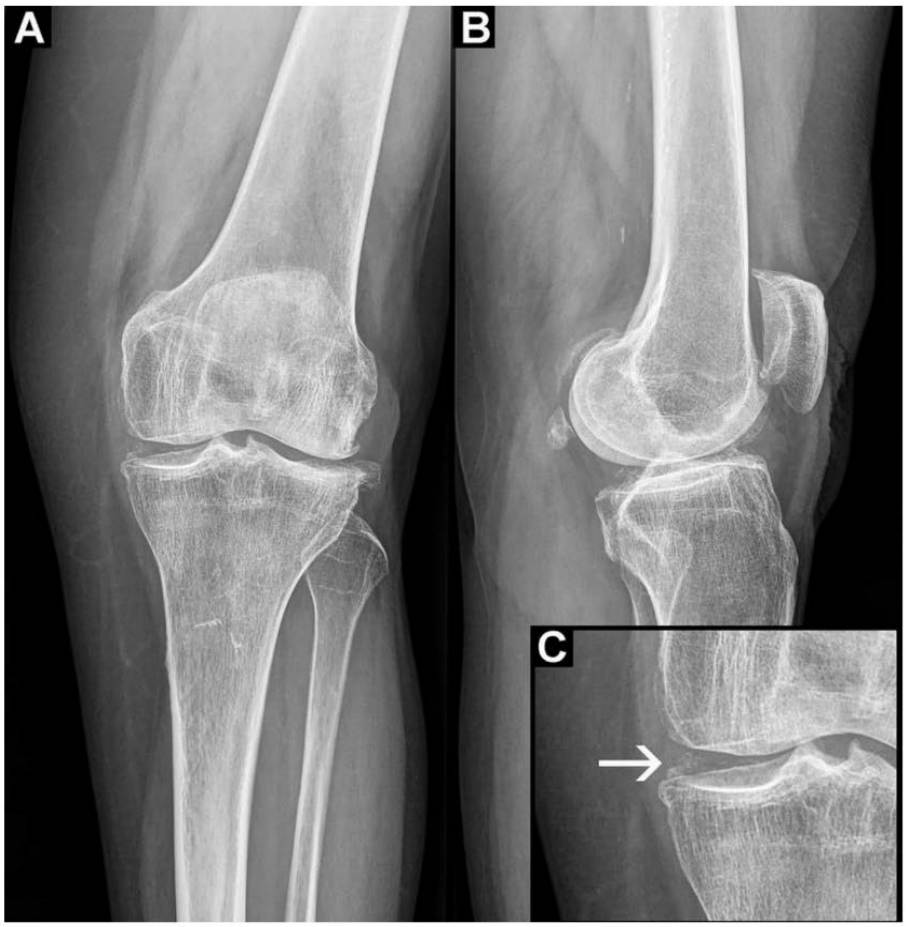

Fig. 1. Standard pre-surgery radiographs of weight bearing left knee: anteroposterior (**A**) and lateral view (**B**). High magnification of anteroposterior view (**C**), where the presence of articular cartilage and/or meniscal fibrocartilage calcification is observable (arrow).

multilobed nuclear morphology in the presence of an outer nuclear membrane detachment. In addition, cytoplasm vacuolization could be observed (Fig. 3A). A translocation of nuclear pores, with the formation of clusters, was evident (Fig. 3B). Sometimes, small patches of condensed chromatin, diffusing throughout the nucleus, appeared in the degenerated chondrocytes (Fig. 3C). Round and swollen mitochondria and a diffuse presence of autophagic vacuoles occurred (Figs. 3C–3F).

The predominant presence of CPPD crystal accumulations in the area subjected to the compressive stress, suggests a possible indirect role of mechanical loading in promoting the deposition through cartilage degeneration. In fact, the numerous hollows or clefts in the cartilage could be the sites for the deposition of crystals that are contained in the synovial fluid or the ways for their passage to the deepest layers of cartilage. Many studies suggest the noxious role of CPPD crystal accumulations in cartilage degradation and synovial inflammation (McCarthy and Cheung, 2009), assuming that crystal deposition induces tissue injury and cell death (Ea et al., 2011). However, the presence of crystal accumulations in the cartilage middle layers lets also hypothesize a possible different origin of these accumulations. Thus, as well as for other diseases, the calcification process may develop within the same tissue and in this case the crystal presence on the surface could be related to tissue wear and the role of mechanical loading might be that of inductor of this process.

Analyzing at morphological level the chondrocyte behavior in a patient affected by chondrocalcinosis, our findings reveal the presence of chondroptosis associated to other cell death types. Chondrocytes, which showed chondroptotic features, have been found in the cartilage middle layer from the area particularly subjected to mechanical loading, suggesting a strong relationship between compressive stress and chondroptosis, as well as for the other cell death types and osteoarthritis (OA) (Levin et al., 2001). Chondroptotic chondrocytes, already showed in osteoarthritis and alkaptonuria, are characterized by a specific morphology, in the absence of apoptotic patterns, like chromatin clumping into large solid masses at nuclear periphery (known as cup-shaped masses) and presence of micronuclei, allowing to clearly distinguish between them (Battistelli et al., 2014; Hwang and Kim, 2015; Millucci et al., 2015). One of the

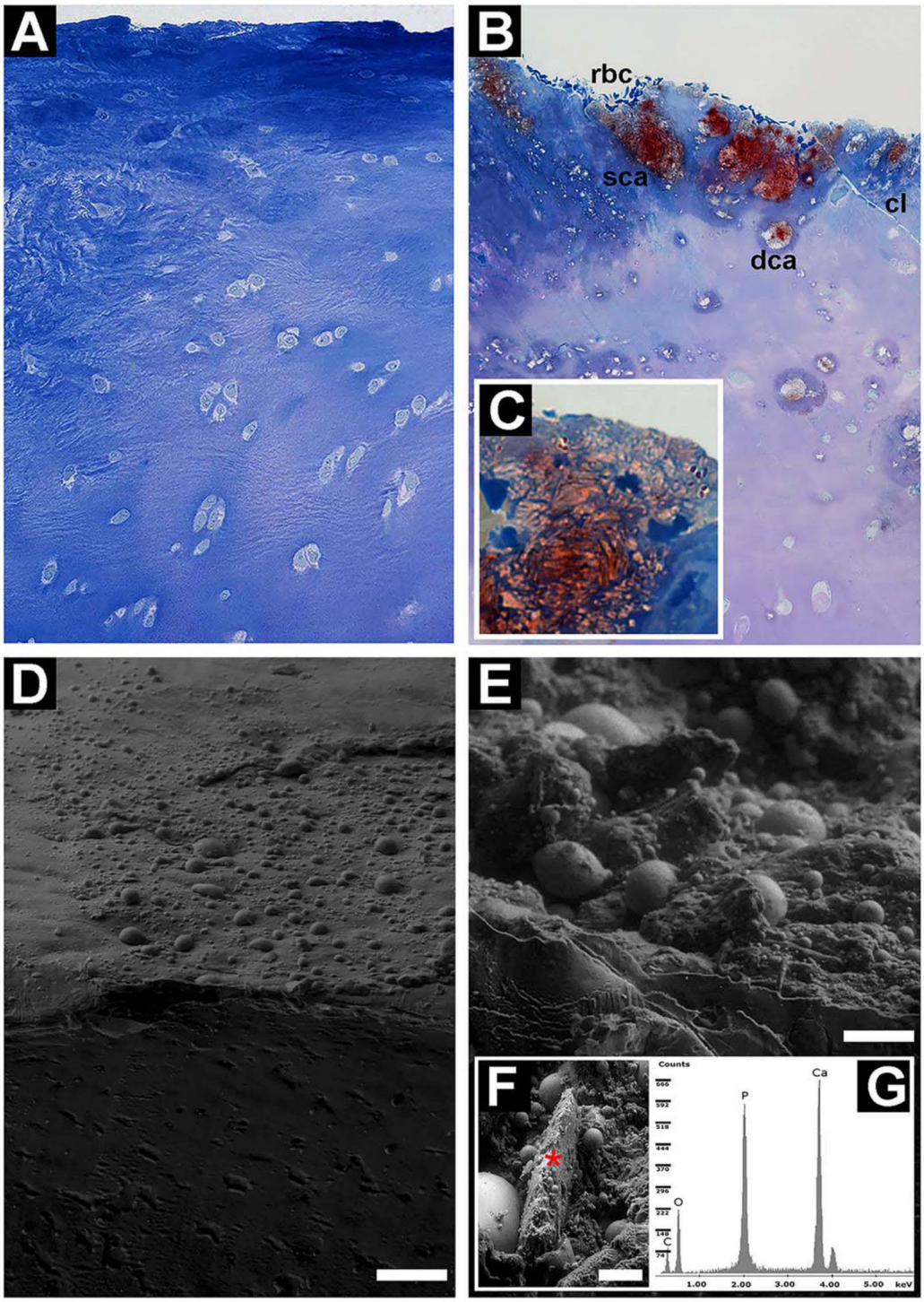

Fig. 2. Light microscopy (**A**–**C**) and ESEM (**D**–**F**) images of chondrocalcinotic cartilage cross-sections in unloaded (A, D) and loaded area (**B**, C, **E**, F). In B, C, the accumulations of CCPD crystals appear red stained and the nature of one (*) of these crystals (F) is confirmed by its EDS spectrum (**G**) (sca: superficial CCPD crystal accumulations; dca: deep CCPD crystal accumulations; rbc: red blood cells; cl: cartilage cleft). Magnification: A, B: 20×; C: 100×. Bars: D: 100 μm; E: 40 μm; F: 20 μm. [Color figure can be viewed at wileyonlinelibrary.com]

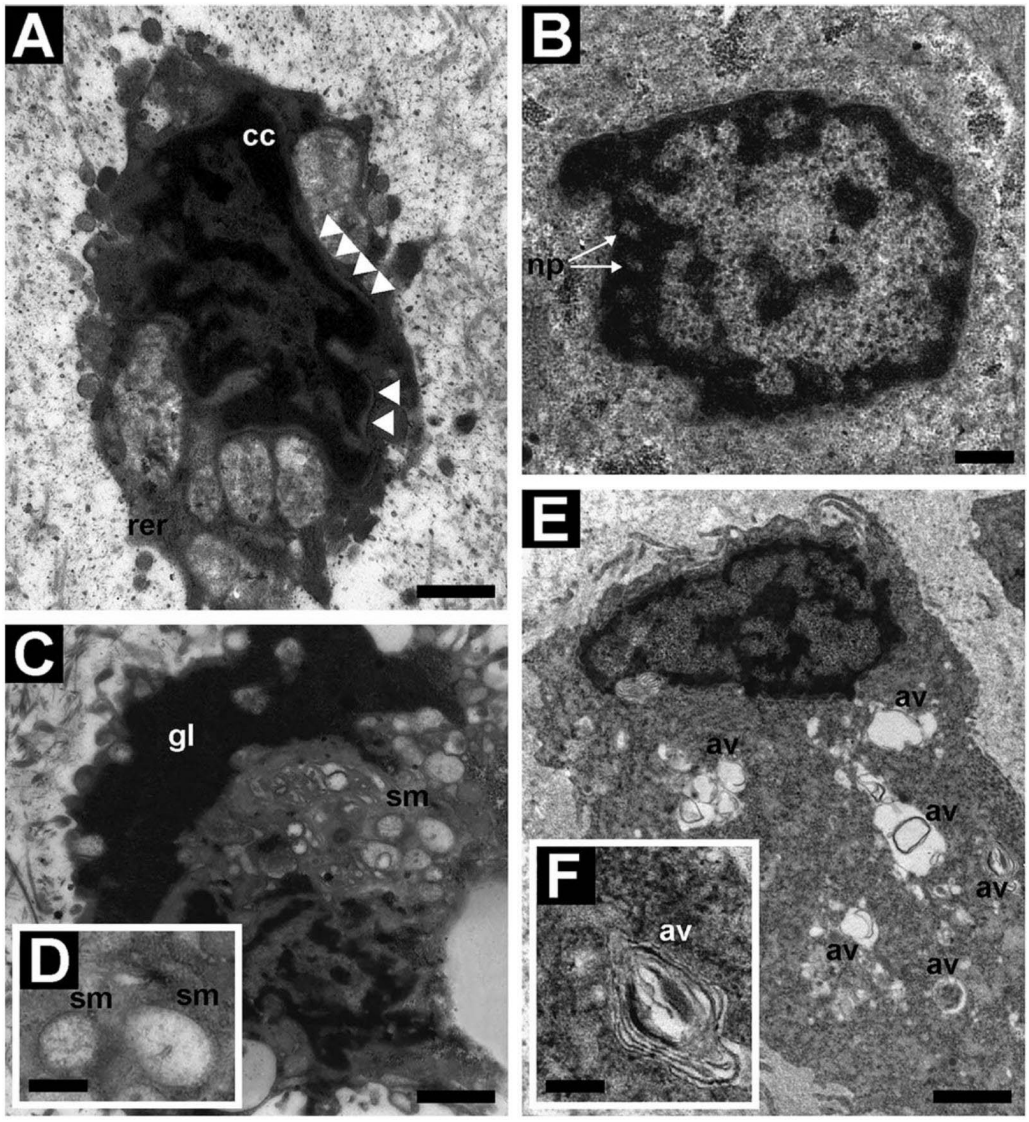

Fig. 3. TEM images of chondroptotic chondrocytes and their morphological features (**A**–**F**), such as: chromatin condensation (cc) and margination close to the nuclear membrane and detachment of the outer membrane (arrow-head), RER (rer) (**A**), nuclear pores translocation and clustering (np) (**B**), glycogen (gl), swollen mitochondria (sm) (**C**, **D**), and autophagic vacuoles (av) (**E**, **F**). Bars: A, B: 0.8 μm; C, E: 2 μm; D, F: 0.25 μm.

most interesting feature of chondroptosis is its ability to reduce the cellular components without producing residual material, which could induce an inflammation process. The end of chondroptotic event is not yet completely clarified but seems to involve the autophagic pathway and to be independent of the macrophage activation (Roach et al., 2004). For these reasons chondroptosis could be the priority programmed cell death for the chondrocytes, given their particular position in the lacunae enclosed by a narrow extracellular matrix.

Considered the role of cell death as a potential target for pharmacological treatments against cartilage degeneration, the identification, for the first time, of chondroptosis in chondrocalcinosis could open a new research field in the study of this disease.

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Original article

Chondrocalcinosis: a morphofunctional study of crystal deposition in mechanically stressed shoulder soft tissues

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Summary

***Introduction*: Chondrocalcinosis is a pathological condition characterized by the presence of calcium pyrophosphate dihydrate (CPPD) crystal deposition in the soft tissues. Even if knee articular cartilage is the most involved anatomical area, different kind of tissue and joint can be affected by this disorder.**
***Methods*: The aim of this manuscript is to analyze at histological and ultrastructural level the crystal deposition in shoulder soft tissue subjected to mechanical stress of patients affected by CPPD disease. Moreover, the cellular behavior in the same specimens has been investigated by means of transmission electron microscopy at variable distances from crystal deposits.**
***Results*: An interesting relationship between CPPD and cellular impairment appearis in humeral articular cartilage, joint capsule and long head of biceps brachii tendon sheath, where respectively chondrocytes and fibroblasts, close to crystal deposits, reveal numerous cell damages, such as chromatin condensation, dilation of organelles or cell membrane rupture.**
***Conclusion*: Considering that cells far to the crystals are healthy, their behavior appears to be different from that of neighboring cells, then our preliminary results suggest a possible cause-effect relationship between events.**
Level of evidence: basic science study.

KEY WORDS: shoulder, chondrocalcinosis, articular cartilage, long head of biceps brachii, joint capsule, calcium crystals.

Introduction

Calcium pyrophosphate dihydrate crystal deposition disease (CPPD) is an articular disorder, characterized by the presence of radiographic articular fibro- o hyaline cartilage calcifications. This condition is also called chondrocalcinosis. CPPD is the third most common form of arthritis in elderly and epidemiological studies highlight that 4 to 7% of European and USA populations are affected by chondrocalcinosis[1,2]. The prevalence shows no gender difference and CPPD onset is strictly correlated to aging, to date identified as the most relevant risk factor together with previous joint trauma[3]. In fact, Neame et al. have demonstrated an increased prevalence of knee CPPD in patients aged 80-84 (17.5%), respect to those aged 55-59 (3.7%), without difference between man and woman[4]. Moreover, CPPD is frequently associated to mechanical stress induced by repetitive trauma, as confirmed by its relationship with sport practice or injuries[5].
CPPD and osteoarthritis (OA) often coexist in the same joint, probably inducing a symptom worsening. The presence of calcium crystals has been observed in cartilage specimens in 25-30% of patients affected by OA, following knee arthroplasty[6,7].
The most frequent anatomical site of CPPD is knee, followed by wrist, hip, pubis symphysis and shoulder[8]. From a radiological point of view, crystal deposition has been observed in the connective tissues (mainly fibrocartilage and hyaline cartilage) of different joint anatomical structures, such as menisci, articular capsule, tendon, ligament, synovial bursae, synovial fluid, synovial membrane, articular cartilage[9]. Meniscus fibrocartilage is the most affected tissue (from 86.3 to 95% of patients affected by CPPD in two different studies), followed by articular cartilage (from 45 to 56.8%) and articular capsule fibrous con-

nective tissue (about 30%)[4,8]. From a histological point of view, crystal depositions in human connective tissue were found close to degenerating collagen fibers in the matrix surrounded hypertrophic chondrocytes. This kind of matrix was characterized by electron-dense amorphous material, including proteoglycans and debris of cellular components[10]. Moreover, crystal deposition induced fibrosis, angiogenesis and neutrophils accumulation in the ligament flavum[11].
Unlike knee, epidemiological data on glenohumeral joint crystals distribution are unknown and a limited literature describes this pathological condition on shoulder tissue. Frequently it is difficult to discern CPPD and OA is this anatomical area, due to their similar radiographic features, such as subchondral bone sclerosis and osteophytes[12]. The presence of calcifications has been observed, by means of magnetic resonance imaging and X-rays technique, in humeral articular cartilage, tendons, capsule, synovial fluid, synovial membrane and bursae[13,14].
Although the mechanisms of connective tissue calcification are not completely understood even for the most common and studied crystal deposition diseases, an impaired chondrocyte behavior seems to be involved in articular cartilage calcification of patients affected by OA[15]. In particular, similarly to other kinds of crystals, calcium pyrophosphate dihydrate precipitates may induce cell death, involving the necroptosis pathway[16].
The aim of this work was to investigate at microscopic and histological level the crystal deposition on shoulder soft tissues of patients affected by CPPD. Electron microscopy analysis has been performed in the same specimens to investigate the cellular behavior close to crystal and far from them. This manuscript allows correlate the crystal microscopic location and cell behavior, suggesting an interesting relationship.

Materials and methods

Patients and specimen handling

The study meets the ethical standards of the journal. In particular, all experimental procedures were carried out according to the journal guidelines[17].
Specimens from humeral articular cartilage, joint capsule, long head of biceps brachii and loose bodies were withdrawn from 6 patients with CPPD, aged 67±5, during shoulder arthroplasty. For each patient, pre-surgery X-ray images have been performed to confirm the typical degenerative features of CPPD. In particular, shoulders revealed a diffuse calcium crystal deposit in the soft tissue (Figs. 1A, B). Fragments

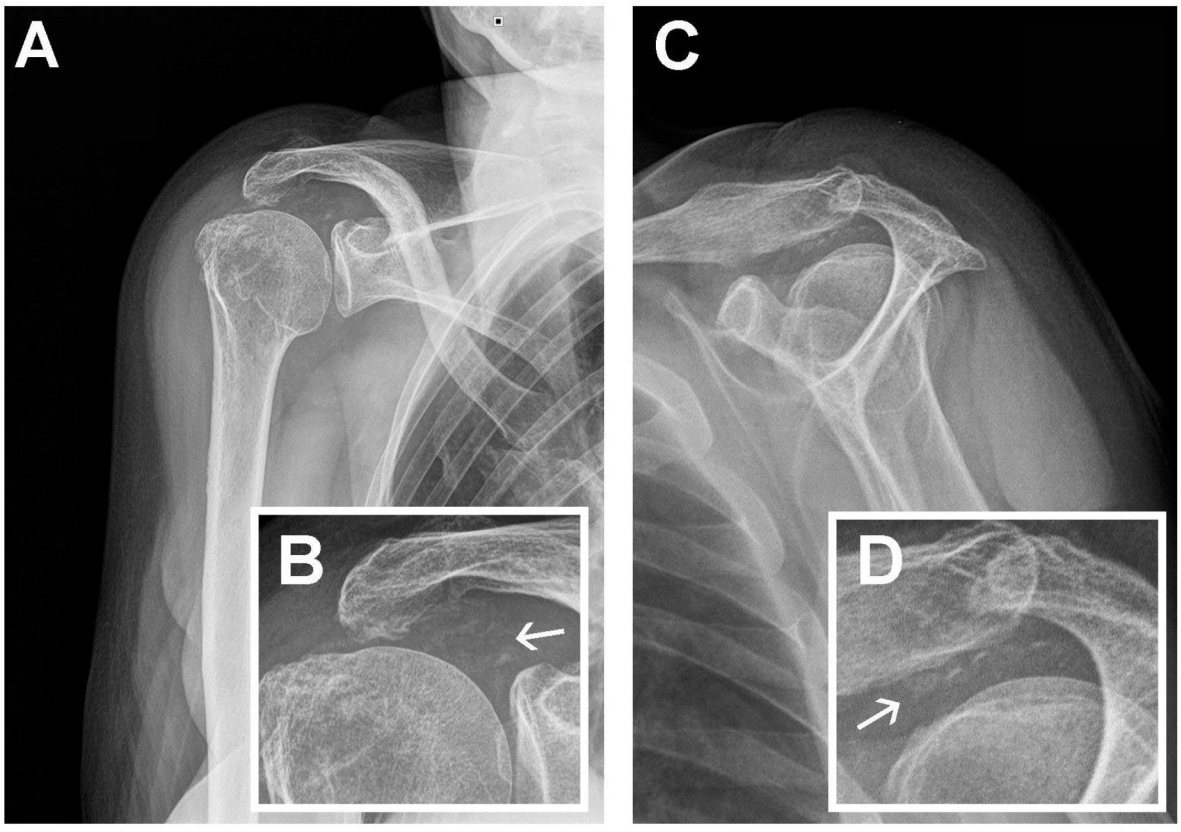

Figure 1. Radiographs of right and left shoulders of patients affected by CPPD: anteroposterior (A) and outlet-view (C). At high magnification (B, D), the presence of widespread calcium crystal accumulations in soft tissues is observable (arrow).

from each tissue were fixed with 2.5% glutaraldehyde solution in a 0.1 M phosphate buffer for 3 h and then minced into smaller specimens (3 mm^3), which were fixed in the same solution for a supplementary hour. After washing, samples were post-fixed with 1% osmium tetroxide solution in the same buffer for 1 h, rinsed and dehydrated in a graded series of ethanol[18].

Environmental scanning electron microscopy (ESEM) and microanalysis

Fixed samples from each tissue were dried and mounted on conventional SEM stubs, and then observed with a FEI QUANTA 200 environmental scanning electron microscope (mode: low vacuum; detector: secondary electrons; high voltage: 30 kV). To confirm the diagnosis of chondrocalcinosis, an energy dispersive X-ray spectroscopic microanalysis (EDS) has been performed in the same conditions of observation[19].

Histological analysis

Other fixed fragments were embedded in araldite and sectioned by an LKB 2088 ultramicrotome. Semithin sections were stained with 1% toluidine blue in distilled water for 2 minutes. After washing, they were stained with 2% alizarin red in distilled water for 2 minutes. To better fix alizarin red staining, specimen sections were immersed for 20 seconds in acetone, acetone-xylene (1:1) solution and xylene, respectively. Then, samples were washed and observed by means of Olympus optical microscope at 10x, 20x, 40x or 100x (oil)[20].

Transmission electron microscopy (TEM)

The stained semithin sections were used to identify areas where to perform TEM analysis. Thin sections were then obtained from these chosen regions, stained with uranyl acetate and lead citrate solutions and then observed with Philips CM10 transmission electron microscope (voltage: 80 kV)[21].

Results

Humeral articular cartilage

Chondrocalcinotic humeral articular cartilage displayed a general degradation. In transverse sections (Fig. 2A), numerous clefts occurred from the articular surface to the deeper tissue layers, and some areas of cartilage surface layer appeared completely destroyed. Calcium crystal depositions (red stained) were visible on the superficial zones, as well as in the middle layers of tissue (Fig. 2B). These crystals appear to be generally collected in deposits and rarely can be found alone. When this happens, it affects the deeper levels of the tissue and not the superficial ones.

The cellular distribution was deeply modified, as confirmed by the almost complete absence of cells on the superficial layers. In the middle zone, TEM images revealed the presence of rare healthy chondrocytes, which showed a regular organization of nucleus, nuclear pores and chromatin, as well a diffuse endoplasmic reticulum and numerous glycogen granules (Fig. 2C). However, several chondrocytes displayed necrotic features, such as vacuolized cytoplasm, degraded organelles, plasma and nuclear membrane discontinuities, with lack of chromatin condensation (Fig. 2D).

Long head of biceps brachii tendon sheath

Optical images of the long head of biceps brachii tendon revealed a high tissue mineralization. In particular, the outer fibrous sheath, that, together with the inner synovial ones, forms the tendon sheath, appeared characterized by crystal accumulations, as well as numerous single crystals scattered in the tissue. Analyzing at optical microscopy the tissue areas close to these sediments, fibroblasts showed an evident vacuolization, suggesting a compromised cell vitality (Fig. 3A). TEM micrographs confirmed the impaired cell morphology. Fibroblasts showed an evident cell shrinkage with visible cytoplasmic alterations characterized by numerous vacuoles, many of which containing cellular material inside. In addition, chromatin condensation appeared (Fig. 3C). On the other hand, tissue areas far from crystal deposit, showed preserved healthy cells (Fig. 3B). In most of these cells, the cytoplasmic vacuoles were completely absent and, when present, they had a clear reduced dimension if compared to those close to crystal sediments.

Joint capsule

Optical transverse sections of articular capsule displayed wide crystal accumulations on the synovial membrane, and the presence of single crystals scattered within the tissue (Figs. 4A, B). The capsule integrity appeared compromised by tissue clefts, extending from the joint cavity to the tissue deeper layers. From optical images, a relationship between single crystal and clefts locations was observable. In particular, the sediments in the depth layers of tissue appeared neighboring to the cleft wall.

At ultrastructural level, the fibroblast-like synoviocyte, if compared to few preserved cells (Fig. 4C), revealed the presence of autophagic vacuoles and swollen organelles (Fig. 4D).

Loose body

Optical microscope images revealed wide calcified tissue area in the intra-articular loose body. Given the nature of this anatomical structure, which is usually composed by cartilage or cartilage and bone, the calcification presence is apparently not noteworthy[22]. However, given the pathology of the patient, understanding the nature of these crystals was necessary and the crystal analysis confirmed results comparable to bone tissue (data not shown). Morphological observations were not able to identify single or accumulated calcium pyrophosphate dihydrate crystals in this

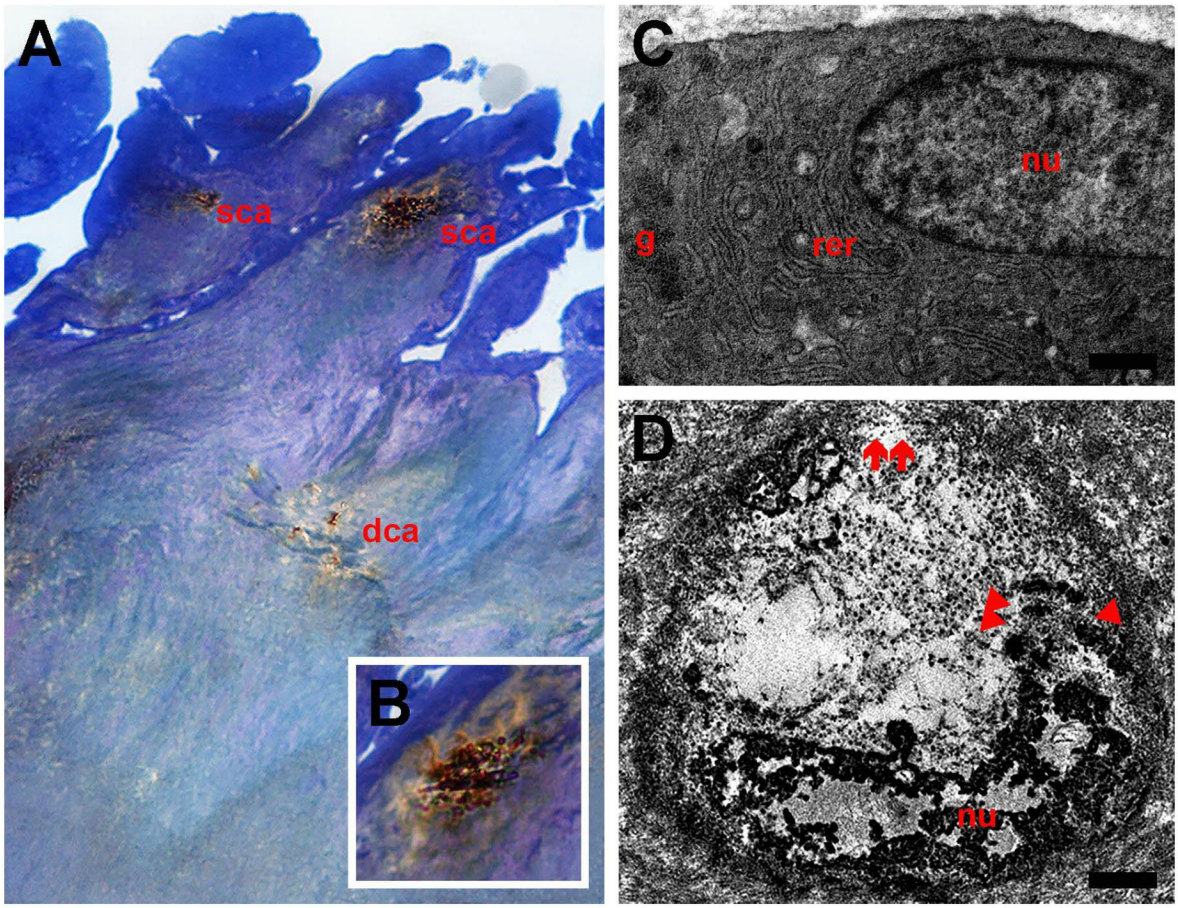

Figure 2. Light microscopy (A, B) and TEM (C, D) images of chondrocalcinotic cartilage cross-sections. In A and B the accumulations of CPPD crystals appear red stained. In A, red stained superficial (sca) and deep crystal accumulations (dca) are observable. TEM micrographs of cartilage middle layers reveal preserved chondrocytes far to crystal deposits, which show an undamaged nucleus (nu), an abundant rough endoplasmic reticulum (rer) and several glycogen granules (g). Close to crystal deposits, necrotic chondrocytes with discontinuous nuclear (arrow heads) and plasma membrane (arrows), appear. Magnification: A: 20x; B: 100x. Bars: C, D: 1 µm.

tissue (Fig. 5A), however in the high calcified zones the identification of specific kinds of crystals appeared really hard. Even though the loose body is a free fragment in the articular space, its cells showed a high vitality if compared to those of other tissues. TEM micrographs confirmed the chondrocyte morphofunctional integrity. In particular, cells showed a regular arrangement of nuclear and cytoplasmic components (Fig. 5B).

Calcium pyrophosphate dihydrate crystal deposition

Single crystal ultrastructure and accumulation morphology were respectively evaluated by ESEM and TEM in each tissue (Figs. 6A, B). The crystal chemical nature was investigated by EDS spectrum (Fig. 6C), showing the relationship between calcium and phosphorous peaks, in line with the stoichiometric ratio of CPPD crystals.

Discussion

Although the diagnoses of CPPD of shoulder soft tissues are increasing, many questions regarding this pathological condition, such as its origin or its development, still have no answers. Numerous scientific works have analyzed this condition at macroscopic level through radiological investigation, but, to the best of our knowledge, this is the first study which examined, by means of various microscopy techniques (previous described), CPPD crystal deposition in different human shoulder anatomical structures, evaluating a possible relationship between cell behavior and crystal deposits location.

If cell death is the origin or the consequence of crystal deposition is still under discussion. The molecular mechanisms of CPPD crystal formation have not yet been fully understood, but the most quoted hypothesis in human cartilage argues that chondrocyte dys-

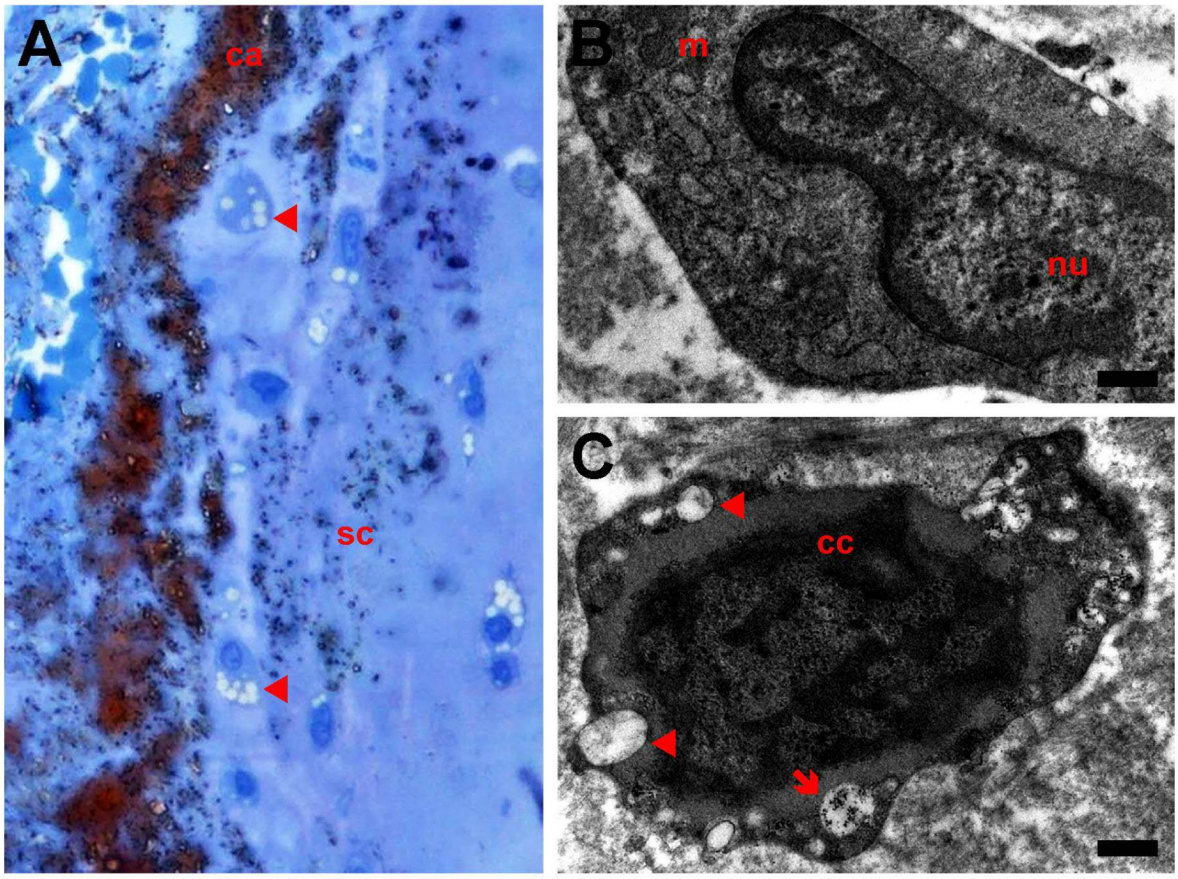

Figure 3. Light microscopy (A) and TEM (B, C) images of the outer fibrous layer of long head of biceps brachii tendon sheath. In A, red stained crystal accumulations (ca) and single crystal (sc) are observable near to high vacuolated cells (arrow head). At high magnification, fibroblasts far to crystal deposits display a healthy morphology (B), characterized by a regular disposition of chromatin in the nucleus (nu) and undamaged mitochondria (m). On the other hand, cells close to sediments show a compromised ultrastructure (C), with a starting chromatin condensation and numerous vacuoles in cytoplasm (arrow head), some of which seems containing cellular material inside (arrow). Magnification: A: 40x. Bars: B, C: 0,5 µm.

function in maintaining extracellular matrix turnover would be the starting point. In particular, these cells seem to produce excessive extracellular inorganic pyrophosphate in the tissue. These molecules are stored in synovial fluid through the direct contact between tissues and the weeping lubrication mechanism of cartilage. In the synovia, a significant increase of inorganic pyrophosphate induces CPPD crystal deposits[2,3,22], which in turn are allocated, as shown by our results, in the different soft tissues in direct or indirect contact with the synovial fluid, such as humeral articular cartilage, inner synovial membrane of joint capsule and long head of biceps brachii tendon sheath. Our preliminary results suggest a key role of tissue wear, related to mechanical stress and to crystal deposits themselves. In fact, even if the calcification process may develop within the same tissue, as well as for other pathological conditions, these results reveal a clear relationship between crystal location and tissue clefts, suggesting the ability of this crystals to insert themselves into the clefts through the synovial fluid, and then their capacity to pass from that space to the close tissue areas. Compared to other shoulder soft tissue, morphological and chemical techniques were not able to reveal CPPD crystals in the loose body. Nevertheless, the osteochondral nature of this structure could have affected our analysis. In particular, the identification of CPPD crystals in the large calcified area appeared particularly complex. According to the literature[23], despite the loose body is a free fragment in the joint cavity, its cartilaginous area was characterized by numerous healthy chondrocytes, confirming the synovial fluid ability to regularly make contact with it. Although our observations have not confirmed it, being the synovial fluid the carrier of crystals, this contact suggests a probable crystals deposit in this tissue.

The crystal deposition effects on each tissues are not yet clear. Some studies suggest a relationship between them and acute inflammation, which probably depend on the crystals interaction with neutrophils in the synovial fluid[24,25]. CPPD crystal accumulation in articular cartilage could also alter its mechanical properties, contributing to joint damage[2]. There are

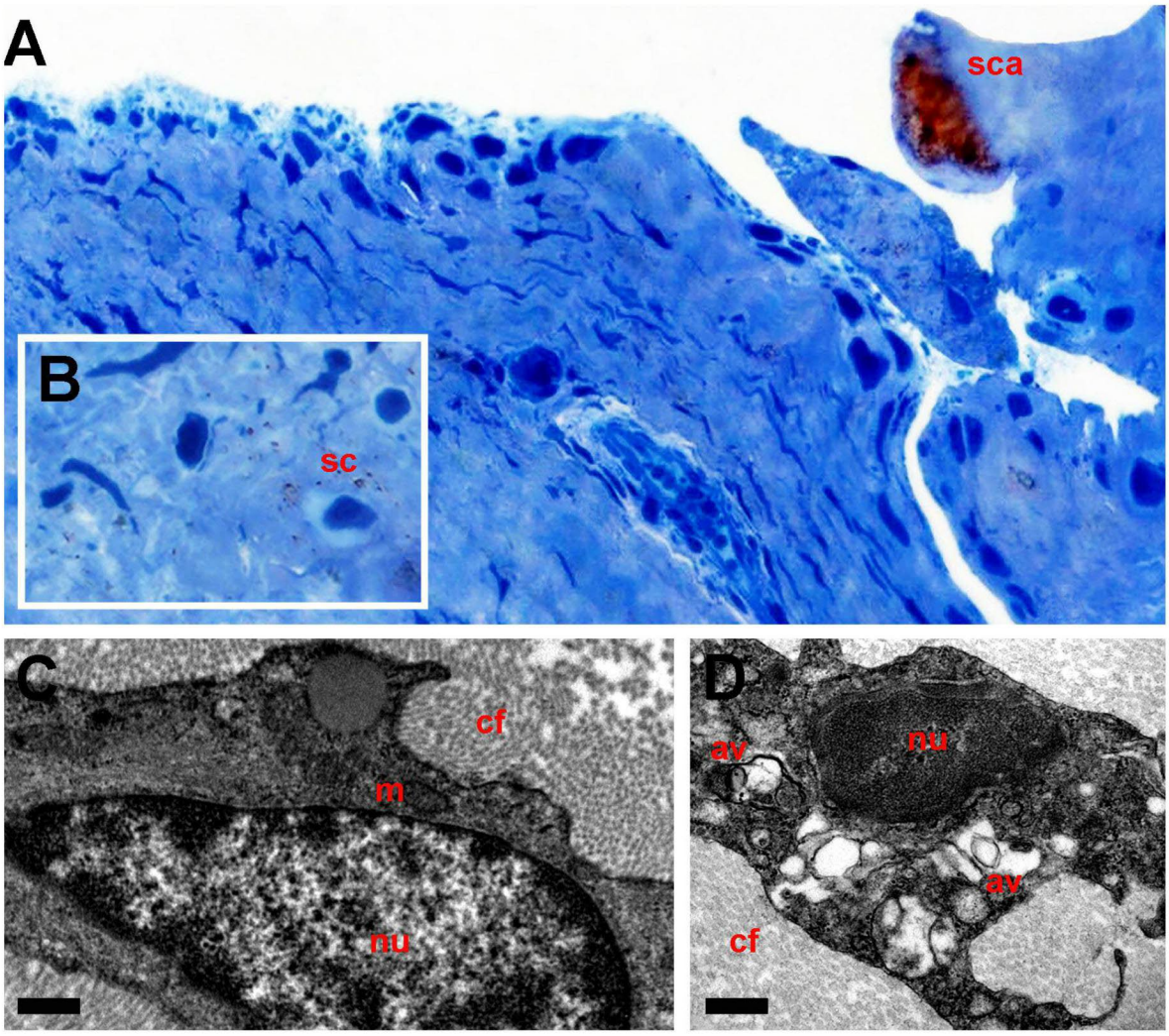

Figure 4. Light microscopy (A, B) and TEM (C, D) images of synovial membrane and joint capsule. In A, red stained superficial crystal accumulation (sca) appears at the synovial membrane, while in B, the presence of single crystals (sc) in the deeper layers of joint capsule are observable. Synoviocytes, supported by collagen fibers (cf), show a regular chromatin disposition in the nucleus (nu), far from sediments, as well as undamaged mitochondria in the cytoplasm. Contrary, synoviocytes close to crystal deposits display an electron dense nucleus and numerous autophagic vacuoles (av) in the cytoplasm, suggesting a compromised cell vitality. Magnification: A: 20X; B: 40X. Bars: C, D: 0,5 µm.

no evidences, instead, about a possible cell death associated to CPPD. There are only rare studies referred to other pathologies, for instance calcium oxalate stones seem to induce death in renal epithelial cells, but the mechanism is unclear[25].

In this study, a relationship between crystal sediments location and cell death has been observed. In humeral cartilage superficial zone, where calcium deposits are more significant, an almost total absence of chondrocyte is observable. Even if their death can be easy related to tissue wear, near single CPPD crystals, in cartilage middle layer numerous necrotic cells have been revealed. Similarly, in the long head of biceps brachii tendon sheath, impaired and healthy fibroblasts are major concentrated, respectively, close and far away from the calcium crystals. The synovial membrane of joint capsule displayed a comparable condition, where several impaired cells are specifically located close to CPPD crystal accumulations. Taken together, these preliminary findings suggest a connection between cell death and CPPD crystals. However, in order to understand their cause and effect relationship, future studies will be necessary and the possible existence of a positive feedback system between the two events should be considered as a hypothesis worth examining.

Compliance with ethical standards

Funding

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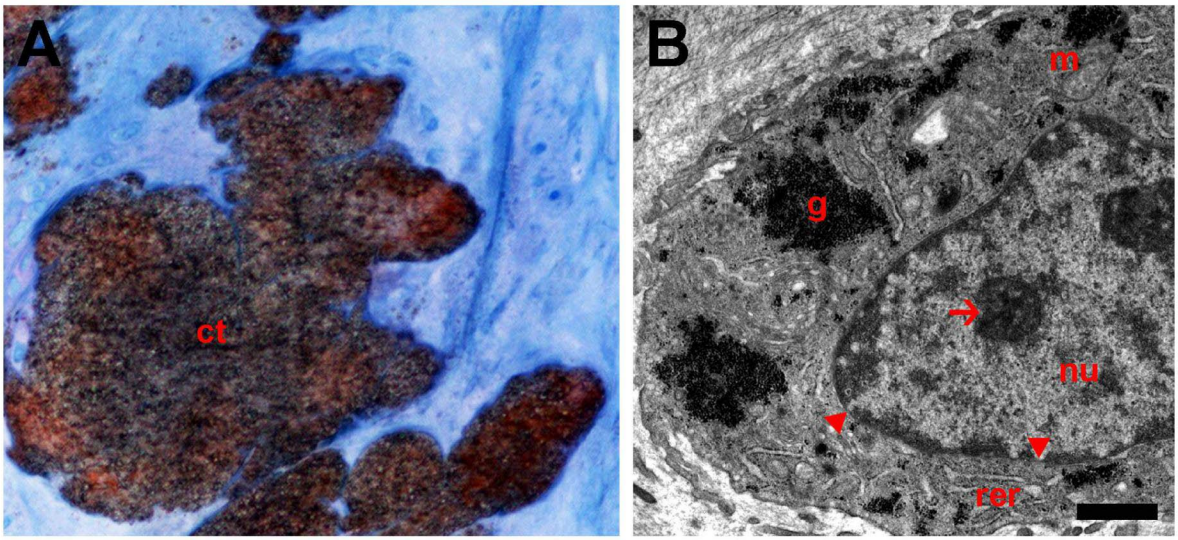

Figure 5. Light microscopy (A) and TEM (B) images of loose body. Numerous and wide calcified area (ca) from bone origin (red stained) surrounded from cartilaginous tissue are observable (A). A preserved chondrocyte appears, displaying an undamaged nucleus (nu) and nucleolus (n) and, in the cytoplasm, a high concentration of glycogen granules (g) and rough endoplasmic reticulum (rer) is observable. Magnification: A: 20X. Bars: B: 1 µm.

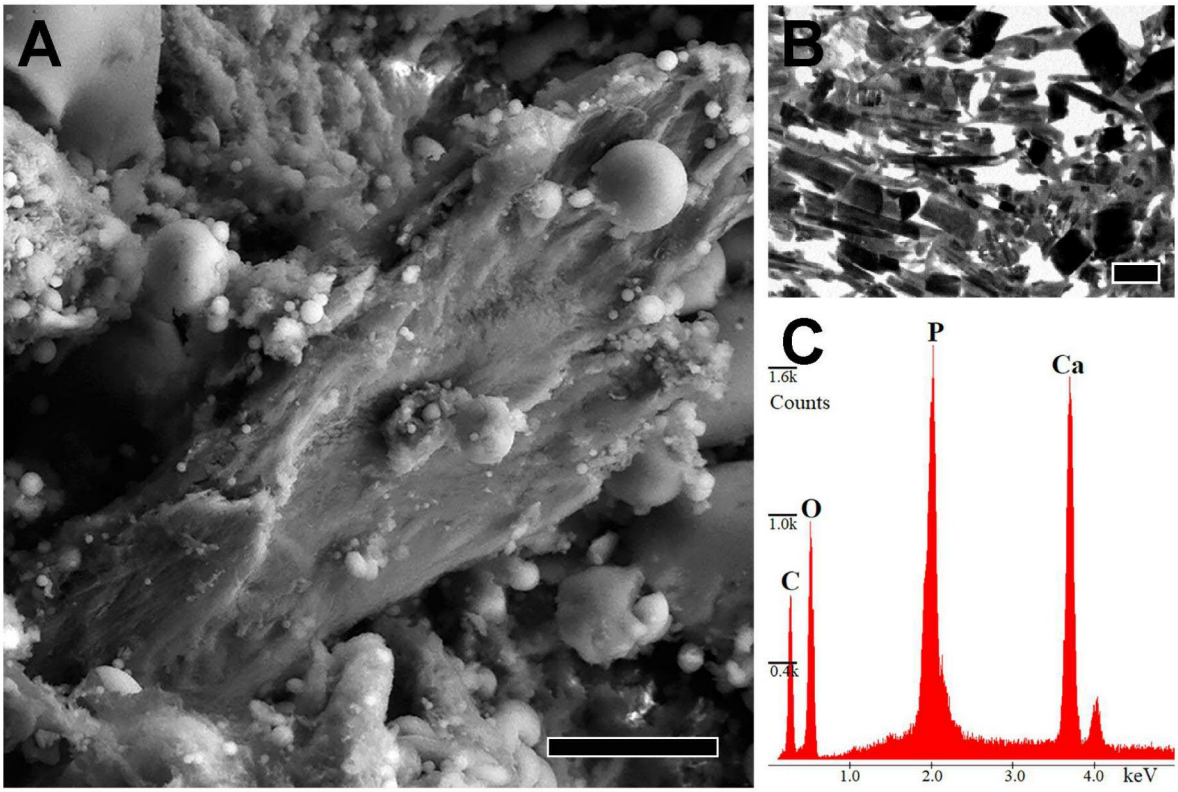

Figure 6. ESEM (A) and TEM (B) images of a single calcium pyrophosphate dihydrate crystal and an accumulation of numerous crystals in cartilaginous tissue respectively. In C, EDS spectrum confirms the nature of crystals. Bars: A: 2 µm; B: 5 µm.

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Conflict of interest

The Authors declare that they have no conflict of interest.

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Morphological and ultrastructural analysis of normal, injured and osteoarthritic human knee menisci

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Abstract

The human meniscus plays a crucial role for transmission and distribution of load across the knee, as well as shock absorption, joint stability, lubrication, and congruity. The aim of this study was to compare the complex geometry, and unique ultrastructure and tissue composition of the meniscus in healthy (control) and pathological conditions to provide understanding of structural changes that could be helpful in the future design of targetted therapies and improvement of treatment indications. We analyzed meniscus samples collected from 3 healthy multi-organ donors (median age, 66 years), 5 patients with traumatic meniscal tear (median age, 41 years) and 3 patients undergoing total knee replacement (TKR) for end-stage osteoarthritis (OA) (median age, 72 years). We evaluated the extracellular matrix (ECM) organization, the appearance and distribution of areas of calcification, and modifications of cellular organization and structure by electron microscopy and histology. The ECM structure was similar in specimens from traumatic meniscus tears compared to those from patients with late-stage OA, showing disorganization of collagen fibers and increased proteoglycan content. Cells of healthy menisci showed mainly diffuse chromatin and well preserved organelles. Both in traumatic and in OA menisci, we observed increased chromatin condensation, organelle degeneration, and cytoplasmic vacuolization, a portion of which contained markers of autophagic vacuoles. Areas of calcification were also observed in both traumatic and OA menisci, as well as apoptotic-like features that were particularly prominent in traumatic meniscal tear samples. We conclude that meniscal tissue from patients with traumatic meniscal injury demonstrate pathological alterations characteristic of tissue from older patients undergoing TKR, suggesting that they have high susceptibility to develop OA.

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Key words: Meniscus; meniscal tear; osteoarthritis; transmission electron microscopy; extracellular matrix degeneration.

Contributions: EO, MF, MB, study concept and design; GT, arthroscopic and THR surgeries; EO, histology and immunohistochemistry experiments; MB, TEM analysis; EO, MB, data acquisition; LDF, multiorgan donor samples collection; EO, MF, MB, GT, substantial contribution to data analysis and interpretation; GF, contribution to patient selection, sample collection; EO, MF, GT, MB, GF, EF, BG, MG, SG, contribution to manuscript editing. All the authors participated in drafting the article or in providing critical revisions for important intellectual content and gave the final approval of the version to be published.

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Introduction

In the recent decades extensive studies have demonstrated the importance of meniscus integrity in preserving knee function and stability,[1] as well as the association of meniscal damage with development and progression of osteoarthritis (OA).[2] Both traumatic and degenerative meniscal changes are associated with elevated risk of OA,[3,4] but the risk is greater after degenerative-type tears.[5] Meniscal degeneration, which can also occur in both young and older people, is defined as structural and functional failure of the tissue and it is caused by various factors such as repetitive trauma and joint malalignment.[6] Aside from age, gender, body mass index (BMI), and sports activities, multiple studies have indicated that a longer interval between anterior cruciate ligament injury and surgical reconstruction increases the risk of meniscal degeneration and tears.[7]

Pauli *et al.*[8] found several differences that distinguish meniscus degeneration caused by aging versus progressive OA. The major changes in aged menisci are increased Safranin O staining intensity, decreased cell density, and mucoid degeneration associated with loss of collagen fiber organization.[8] These age-related meniscal tissue degeneration changes, even in the absence of overt tears is associated with molecular and cellular alterations indicative of processes characteristic of cellular senescence.[9] Additional alterations are observed in advanced OA, including severe fibrocartilaginous disruption, fine fibrillations, and loss of structure. Of interest, biomechanical and functional properties and, consequently, the resistance to injuries are dependent on the meniscal integrity with no reported difference observed between degenerative and traumatic meniscal tear.[10]

The meniscus sustains many different forces such as shear, tension, and compression. It also plays a crucial role in load bearing and transmission, shock absorption, as well as lubrication and nutrition of articular cartilage. These multiple functions are supported by the extracellular matrix (ECM) components and the activities of the resident cell populations.[1]

The aim of this study was to investigate ECM composition and cell morphology of normal, injured, and OA[11-13] menisci from

human knees employing transmission electron microscopy (TEM). Possible correlations between meniscal integrity and duration of symptoms after meniscal injury were investigated to understand structural changes, which could be helpful to design future targetted therapies and improve treatment indications.

Materials and Methods

Patient recruitment, clinical data, and sample collection

This work was designed as an observational study, in accordance with the approval of the Local Ethical Committees, within the framework of a multicenter prospective cohort study, and funded by the Italian Ministry of Health (GR-2010-2317593). Traumatic meniscal tissue samples from the inner superficial zone were harvested from 5 patients with symptomatic meniscal tears undergoing arthroscopic partial meniscectomy (males, median age 41 years with interquartile range (IQR) of 41-42; median of the body mass index (BMI) of 27.6 Kg/m^2 with IQR of 24.5-27.8). End-stage OA (Kellgren-Lawrence grades 3-4) tissue samples of meniscus were harvested from 3 patients undergoing total knee replacement (TKR) (2 males and 1 female, median age 72 years with IQR of 72-73.5). All the 5 patients selected for the study sustained a knee sprain during recreational sports activities (2 soccer; 2 running; 1 sailing). Specimens were collected after informed patient consent was obtained from each subject. As control, fragments from 3 multi-organ donors without a history of joint disorders (two males and one female, median age 66 years with IQR of 63-69) were collected from the Musculoskeletal Tissue Bank (IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy). The meniscal injuries were evaluated by MRI before surgery. The meniscal injury was also confirmed by the surgeon during arthroscopy and described according to Trisolino *et al.*[14] For patients undergoing arthroscopic partial meniscectomy pre- and post-operative symptoms were evaluated using the Knee Injury and Osteoarthritis Outcome Score (KOOS) total.

The meniscal samples used in the study were collected from multi-organ donors. Routinely, the fresh menisci are prepared by the Musculoskeletal Tissue Bank of our Institution for allograft and used for meniscal transplantation. The IRCCS Istituto Ortopedico Rizzoli (Bologna, Italy) is one of the leading institutions all around the world for this kind of surgery. Correct sizing is one of the most important factors in the success of the transplant. A screening process is done before selecting a possible donor and once selected the donor tissue undergoes many tests. The safety of the tissue is monitored and it is tested for viruses like those that cause HIV/AIDS, West Nile virus, hepatitis B and C, as well as for bacteria. The rate of discarded joint tissues is very low and only in this case those precious tissues are used for research. Moreover, the Italian law and the IOR ethical committee do not allow for tissue sampling from donors for research purposes; so we can obtain fresh samples from donors

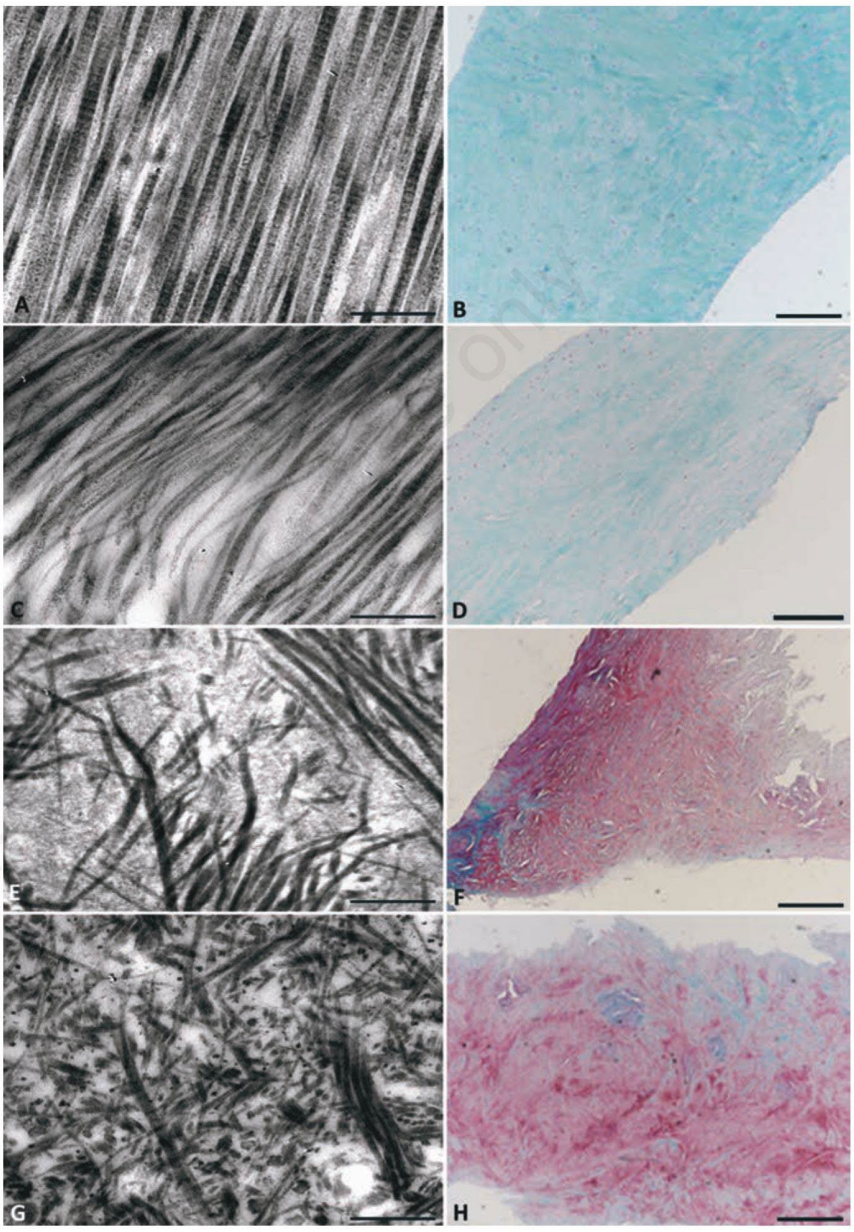

Figure 1. Meniscal tissue morphology. Meniscal samples collected from one representative multi-organ donor showed collagen fibers with homogenous distribution and orientation, as observed by TEM (A) and Safranin O/Fast green staining (B). Meniscal samples collected from a 25-year-old patient during the arthroscopic procedure for meniscal tear showed collagen fibers with regular size and distribution (C) and virtually no staining for proteoglycans (D). In samples from one representative patient with meniscal tear (53 years of age) (E and F), and one representative patient with end-stage OA (G and H), TEM revealed collagen fibers with a structural disorganization of the ECM and strong staining for proteoglycans. Scale bars: A,C,E,G) 1 µm; B,F,H) 500 µm; D) 200 µm.

only in case they were not used during the operation after assignment.

Histological analyses

Samples of meniscus from all subjects were fixed in 4% formaldehyde (Kaltek, Padua, Italy) for histological examination. After fixation, tissues were embedded in paraffin, and sections of 5 µm were prepared. The sections were stained with Gill III hematoxylin-eosin (Bioptica, Milan, Italy) and 0.25% Safranin O/0.3% Fast Green (Sigma Aldrich, St Louis, MO, USA) to evaluate the general morphology and proteoglycan and observed at 10x magnification. The histological meniscal specimens' degeneration grade was assessed by a Pauli's microscopic grading system, which is validated to evaluate changes in three separate areas (femoral and tibial side and inner border) of aging and OA menisci.[8] In our study, we modified Pauli's score because the meniscal biopsies were taken only from the inner border. The range of possible total scores was 0-12, was converted into 4 grades: G1=0-2, G2=3-5, G3=6-9, and G4=10-12. Grade 1 represents normal tissue, Grade 2 is mild degeneration, Grade 3 is moderate degeneration, and Grade 4 is severe degeneration. To evaluate calcification in meniscal samples, the sections were stained with 1.4% Alizarin Red at pH 4.2 (Sigma A5533). All images were captured with a Nikon Eclipse 90i microscope equipped with Nikon Imaging Software elements.

Transmission electron microscopy (TEM)

Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h, incubated with 1% OsO_4 in the same buffer for 1 h, alcohol dehydrated, and embedded in araldite, as reported previously.[15] Thin sections were collected on 400 mesh nickel grids and stained with uranyl acetate and lead citrate.

The observations were carried out with a Philips CM 10 electron microscope at 80 kV.[16] A qualitative analysis of the ECM features was performed on meniscal tissues, as described in Olivotto *et al.*[17] To measure the collagen fiber diameter, we carried out a careful analysis of 20 sections for each sample, recording observations in 10 different fields for each section.[18]

A qualitative analysis was performed to identify viable cells and cells undergoing different types of cell death, known to occur in fibrochondrocytes. As described in Olivotto *et al.*,[17] cells were considered "viable" when both the nuclear and cytoplasmic membranes appeared intact and euchromatin was present. A cell was "non-viable" when the plasma membrane or nuclear membrane appeared fragmented

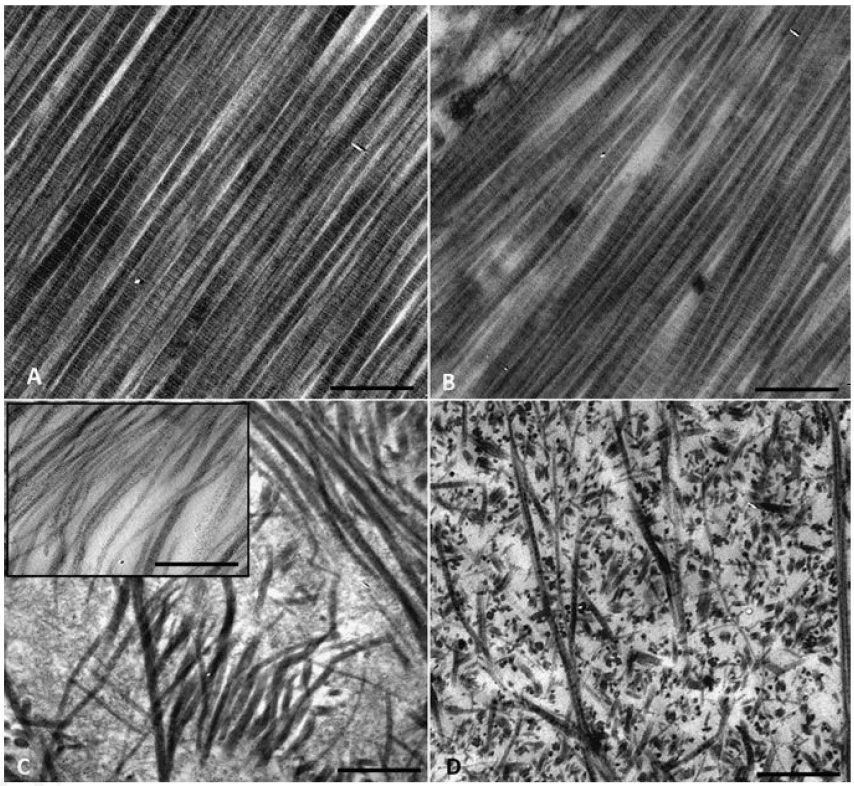

Figure 2. Collagen fiber organization and diameter. In samples from one representative multi-organ donor, the collagen fibers of the inner zone varied from 70 to 80 nm and showed the characteristic periodicity of collagen fibrillar organization (A). A meniscus biopsy collected from the 25-year-old patient undergoing arthroscopy for meniscal tear showed regular distribution of collagen fibers, appearing similar to that in the multi-organ donor (B). The sample from the 53-year-old patient showed meniscal degeneration with disorganized collagen fibers of between 35 to 45 nm in diameter (C and insert). The representative OA patient showed disorganized collagen fibers with diameters varying from 35 to 45 nm (D). Scale bars: 1 µm.

Table 1. Components of Pauli's meniscal degeneration score (surface, cellularity, collagen organization and Safranin O/Fast green staining) and total degeneration score.

Pathology	Patient #	Surface	Cellularity	Collagen	Saf-O/FG	Total score (grade)
Meniscal tear	#1	1	0	3	3	7 (moderate)
	#2	1	0	2	2	5 (mild)
	#3	1	0	3	3	7 (moderate)
	#4	1	0	2	2	5 (mild)
	#5	1	0	2	2	5 (mild)
Osteoarthritis	#1	3	0	2	2	7 (moderate)
	#2	3	0	3	3	9 (moderate)
	#3	2	0	3	3	8 (moderate)

Total score: G1, 0-2 normal; G2, 3-5 mild; G3, 6-9 moderate; G4, 10-12 severe; Saf-O/FG, Safranin O/Fast green staining.

(which is indicative of necrosis or apoptosis, respectively).

Results

Extracellular matrix changes

Careful visual inspection of more than 10 TEM fields for the presence of organized ECM (including collagen fibers, fibrils, and proteoglycans) in all meniscal samples collected from multi-organ donors showed homogenous distribution and orientation of collagen fibers. The inner zone showed a high prevalence of collagen fibers with diffuse proteoglycans (Figure 1A), which was confirmed by light microscopy showing low staining intensity for Safranin O (Figure 1B). In general, after trauma, collagen fibers were reduced and proteoglycans increased (Figure 1C-F). Meniscal samples collected during the arthroscopic procedure for meniscal tear showed mild (3 patients) to moderate (2 patients) degeneration, assessed by the total of components of Pauli's meniscal degeneration score: surface, cellularity, collagen organization, and Safranin O/Fast green staining, as listed in Table 1. In the 25-year-old patient examined after trauma, the collagen fibers, which showed more regular size and distribution, were more abundant than proteoglycans (Figure 1 C,D). In the other 4 patients, median age 45 years (Figure 1 E,F), and in subjects with OA (Figure 1 G, H), TEM observations, supported by histology (Figure 1H), revealed the strong prevalence of proteoglycans compared to collagen fibers and structural disorganization of the ECM. Interestingly, among those patients with meniscal tear, the 53-year-old patient showed the worst meniscal degeneration with ECM loss. The collagen fiber diameter was also analyzed in all samples. In the inner zone of the multi-organ donor samples, the collagen fibers were aligned with a regular pattern for both distribution and diameter, which varied from 70 to 80 nm (Figure 2A), as described in the literature for the standard collagen fibers.[19] Periodic collagen organization, mainly collagen Type II, which is the major fibrillar component of the inner-zone, was observed. Interestingly the meniscal biopsy collected from the 25-year-old patient, who had undergone arthroscopic surgery 2 years after the injury, showed the most regular distribution of collagen fibers, appearing similar to that in tissue from the multi-organ donor. Moreover, the fiber diameter appeared similar to those in the control samples from the multi-organ donors (Figure 2B). TEM observations showed that the other 4 patients with meniscal tears, in which the surgery was performed within 8 months from the injury, had structural disorganization of the ECM, including loss of collagen fiber organization. The collagen fibers were disorganized and the fiber diameter was inhomogeneous within the same specimen. The fiber diameter was between 45 and 60 nm (Figure 2C). Among those patients, the 53-year-old patient showed the worst meniscal degeneration with a collagen fiber diameter between 35 and 45 nm (Figure 2C, inset). As expected, OA patients showed disorganized collagen fibers and the fiber diameter varied from 35 to 45 nm (Figure 2D).

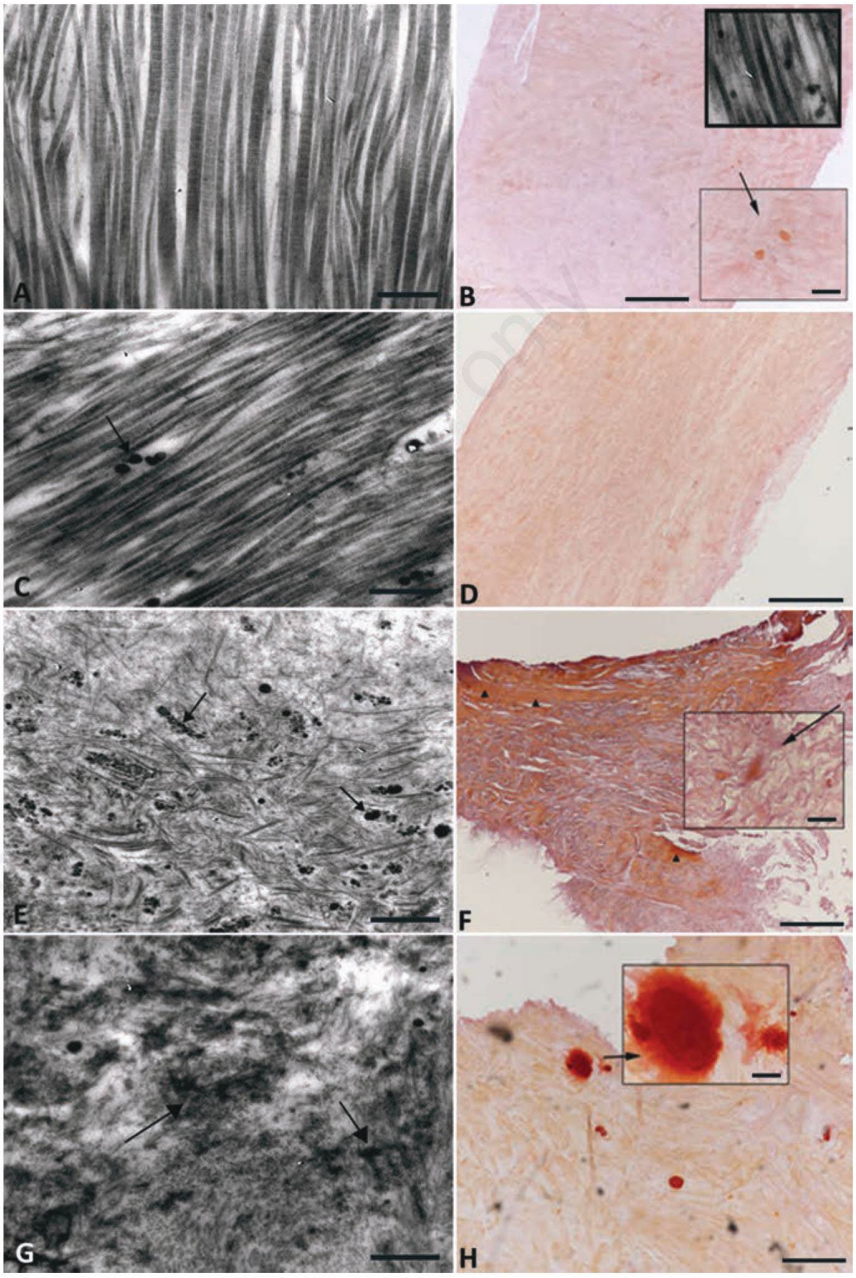

Figure 3. Calcium deposition. Meniscal samples collected from one representative multi-organ donor showed small calcium deposits, evaluated by Alizarin red staining (A) and rarely observed with TEM (B and insert). In meniscus samples collected from the 25-year-old patient, calcium deposits were not visible with Alizarin red (C) after trauma, but a few calcifications were detected by TEM (D). Large calcium deposits were observed in the 53-year-old patient (E and F). Extensive calcified areas were observed in one representative OA patient (G and H). Scale bars: A,C,E,G) 500 nm; B,F,H) 500 µm; D) 200 µm; insert) 50 µm.

Calcium deposits

Meniscal samples were evaluated for calcium deposits by TEM and Alizarin red staining. Among those collected from multi-organ donors, we observed by TEM rare calcium deposits (Figure 3A) and small calcium deposits (Figure 3B, inset) revealed by histology only in one patient. In general, after trauma we observed the presence of calcium deposits in all patients (Figure 3 C-F). Interestingly, in the 25-year-old patient with traumatic injury, few calcifications were detected by TEM and histology (Figure 3 C,D), contrary to the other patients with meniscal tear (Figure 3 E,F). In addition, we observed large calcium deposits only in the 53-year-old trauma patient. As expected, we observed extensive calcified areas in meniscal samples from all subjects with OA (Figure 3 G,H).

Cell morphology

In the menisci of multi-organ donors, the cells embedded in the ECM showed rounded healthy morphology, characteristic of viable and metabolically active cells. The nuclei exhibited diffuse chromatin with small condensed areas near the nuclear membrane, both in the chondroblast-like cells (Figure 4A) and in those more similar to fibrochondrocytes (Figure 4B). The cytoplasm contained a high amount of glycogen, mitochondria were round and swollen, and the rough endoplasmic reticulum was well preserved. We observed small autophagic vacuoles (Figure 4 A,B).

In the young patient with traumatic meniscal tear, the chromatin showed small condensed areas (Figure 4 C,D), similar to the chromatin in the multi-organ donor, whereas small rare vacuoles and autophagic vacuoles could be observed in the cytoplasm (Figure 4 C,D). In the older patients with traumatic meniscal tears, the nuclear chromatin appeared to be more condensed (Figure 4 E,F) compared to that in the healthy menisci of the multi-organ donors. Chromatin margination and condensation with a specific pattern, termed by Roach *et al.*[20] as "chondroptosis", was present in all samples from patients with traumatic injury (Figure 4). In the cytoplasm we observed abundant vacuoles but sparse cytoplasmic organelles, swollen and emptied mitochondria embedded in a relatively empty matrix (Figure 4 E,F). Autophagic vacuoles, presumably due to oxidative stress, were also present in the cytoplasm (Figure 4 E,F). In subjects with OA we observed large areas of condensed chromatin (Figure 4G) and large vacuoles within the cytoplasm (Figure 4H).

Clinical data

In all patients with meniscal tear (examples of the macroscopic findings are shown

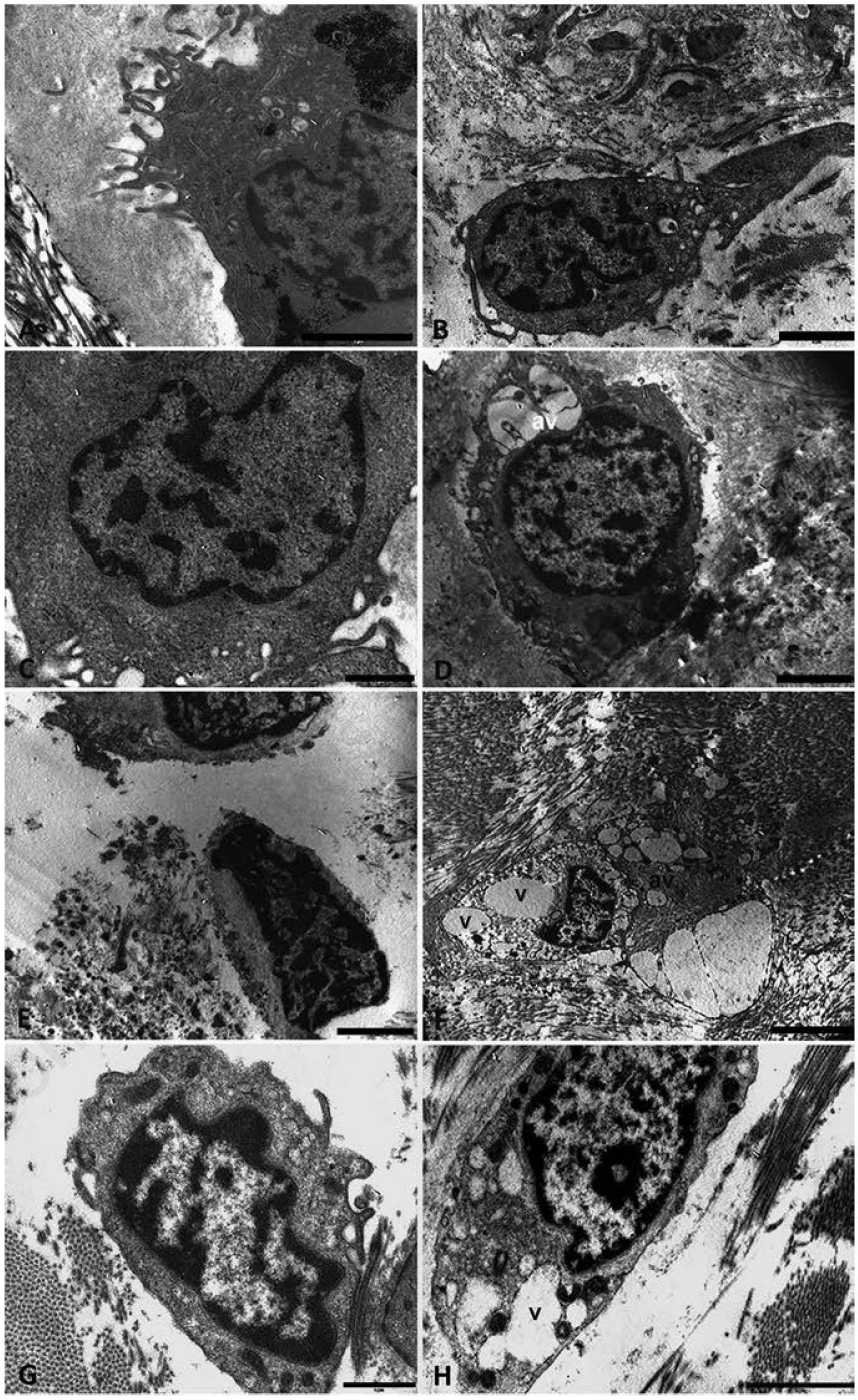

Figure 4. Cell morphology. In the menisci from multi-organ donors, the cells embedded in the ECM were round and showed a healthy morphology, consistent with viable and metabolically active cells. In the nucleus, the chromatin appeared diffuse with small condensed areas near the nuclear membrane (A and B). In the young patient the chromatin showed small condensed areas (C and D), that were similar to those in the multi-organ donors with the cytoplasm containing small rare vacuoles (v) and autophagic vacuoles (av). In the older patients with traumatic meniscal tear meniscal cells contained nuclear chromatin that was more condensed than in the donor cells, with a specific pattern, called "chondroptosis". In the cytoplasm abundant vacuoles were present with scarcity of cytoplasmic organelles and swollen and emptied mitochondria (E, F). Autophagic vacuoles, consistent with oxidative stress, were also present in the cytoplasm. In the representative OA patients, large areas of condensed chromatin (G) and large vacuoles within the cytoplasm were observed (H). Scale bars: A) 2 µm; B-H) 1 µm.

in Supplementary Figures 1 and 2) we observed a post-operative improvement of the KOOS at 1 and 2 years after arthroscopy (*data not shown*).

Interestingly, the 25-year-old patient also showed post-operative improvement despite having surgery 2 years after the injury.

Discussion

The analyses performed in our study indicate that different histological and morphological changes occur in the menisci from traumatic and end-stage OA patients that can be attributed to the different pathological conditions compared to the healthy donors. Meniscus samples collected from multi-organ donors showed low staining intensity for Safranin O, consistent with the median age of the donors. As described in Pauli *et al*.,[8] the major changes attributed to age included increased Safranin-O staining intensity. Despite early signs of aging, the morphological analysis revealed highly organized ECM with intact collagen fibers having periodic collagen organization and homogenous distribution and orientation. Contrary to Pauli's observation,[8] we did not note any decrease in cell density in the donor samples and the morphological analysis showed healthy and active cells. Consistent with reports that calcifications occur in the menisci of 20% of elderly people without a history of joint disorders,[21,22] we observed small calcium deposits in only one donor.

Meniscal biopsies collected from patients with meniscal tear showed similar features to those in OA patients, as described by Pauli *et al*.,[8] even though they were collected from injured patients of median age of 41 years, who were about 30 years younger than the patients with end-stage OA. Of importance, the features of both groups were very different from those of the menisci from the healthy organ donor controls. The histological analysis of meniscal samples showed fibrocartilage disruption in patients with both meniscal tear and end-stage OA. These samples were characterized by proteoglycans increase and loss of structure with bands of degenerated ECM and with mostly disorganized collagen fibers. Evidence of increased proteoglycan deposition was shown by a moderate to strong staining intensity for Safranin-O. Interestingly, in contrast to meniscal samples from OA patients, the surface of those collected from patients with meniscal tear was often intact and there were distinct changes in ECM organization. This pattern is a typical age-related meniscal change in knees without history of major trauma.[8]

It is well established that meniscal tear is a risk factor for the development of OA.[23] Among the mechanisms proposed, the release of inflammatory mediators in the space joint may trigger or accelerate the degenerative processes in the joint tissues, including the meniscus,[2,24] as we observed in our study. Another factor is the duration of symptoms before the arthroscopic procedure is performed. Although a short duration of symptoms is one of the clinical variables that orthopedic surgeons consider, the evidence of the impact on clinical outcome is scarce.[25] For example, Eijgenraam *et al.* found moderate evidence that duration of symptoms of longer than 3-12 months is associated with worse clinical outcome following meniscectomy.[25]

Our histological and morphological analysis showed that the meniscal degeneration is more related to the age of the individual rather than the duration of symptoms. In the 25-year-old patient, in spite of the 2 years of symptom duration before the arthroscopic procedure for partial meniscectomy, the features of the meniscal samples were similar to those from the multi-organ donors; furthermore, post-operative improvement was comparable to that observed in patients treated within 12 months following the injury. Therefore, age appeared to be the strongest incident factor determining the pre- and post-operative outcome.

In conclusion the limitation of our study is the low sample number of patients; thus, further research involving larger numbers of patients per group would enhance the significance of data. However, our observations at microscopic and ultrastructural levels confirm that meniscal injury activates ECM degeneration in meniscal tissue, and that meniscal tears in middle-aged patients present features similar to the OA degenerated meniscus. This potentially could account for the poorer clinical outcomes observed in treating older patients, thus suggesting the need for regenerative therapies to improve the results of treating traumatic lesions in middle-aged patients, as well as providing more information to support clinical treatment indications.

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DISCUSSION

Knee and shoulder joint modifications in different physiopathological conditions have been widely analysed in these three studies.

In the first work, according to the literature, we showed the predominant presence of cartilage degeneration in knee loaded zone, suggesting a possible role of mechanical loading to promote articular diseases. In addition, a particular kind of chondrocyte apoptosis called chondroptosis, until now detected only in patients affected by OA,[25,26] for the first time has been observed in chondrocalcinotic cartilage. But further studies are necessary to better characterize this death mechanism, which is still uncertain. Therefore, considered the role of cell death as a potential target for pharmacological treatments, the identification of chondroptosis in CPPD could open a new research field in the study of this disease.

In the second paper, we have reported that until now shoulder CPPD has been analysed only through radiological investigation,[27] and, as far as we know, this is the first study which examined crystal deposition in human shoulder by means of various microscopy techniques (previous described). In addition, we demonstrated that in each joint structure analysed, impaired and healthy cells are mostly concentrated close and far away from the calcium crystal deposits, respectively. Thus, taken together, these preliminary findings suggest a connection between cell death and crystal accumulation. However, if cell death is the origin or the consequence of crystal deposition is still under discussion,[28,29,30] and future studies will be necessary to better understand the probable cause-effect relationship.

Finally, in the last study we displayed that meniscal injury increases meniscal tissue degeneration, which becomes similar to the OA damage. Moreover, we found that in the 25-year-old patient, in spite of the 2 years of symptom duration before the partial meniscectomy, the meniscal features were similar to those from the multiorgan donors, and post-operative improvement was comparable to those in patients treated within 12 months following the injury. Therefore, age seems to significantly affect the pre- e post-operative joint structures stability.

The limits of our studies are the low number of patients and the absence of molecular analysis. Thus, to enhance the data significance, in our future studies we would like to increase the patient number and to better characterize molecular pathways of the cellular and tissue alterations by means of molecular biology techniques as reported in literature.[11,22,31,32,33,34,35,36,37]

In conclusion, our results have highlighted many innovative and interesting aspects concerning the incidence of mechanical overload in the onset of joint pathologies, the microscopical articular modifications in OA and CPPD, the first identification of chondroptosis in CPPD, the probable cause-effect relationship between cell death and calcium crystals, the similarity between meniscal degeneration from OA and meniscal trauma, and the young age importance in pre- and post-operative joint stability.

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APPENDIX

LIST OF SCIENTIFIC PUBLICATIONS

Full papers (5):

- Salucci S, Battistelli M, Baldassarri V, **Burini D**, Falcieri E, Burattini S. Melatonin prevents mitochondrial dysfunctions and death in differentiated skeletal muscle cells. *Microscopy Research and Technique*, 2017. 80:1174-81. DOI: 10.1002/jemt.22914.

- Curzi D, Fardetti F, Beccarini A, Salucci S, **Burini D**, Gesi M, Calvisi V, Falcieri E, Gobbi P. Chondroptotic chondrocytes in the loaded area of chondrocalcinotic cartilage: a clinical proposal? *Clinical Anatomy*, 2017. DOI: 10.1002/ca.22988.

- **Burini D**, Salucci S, Fardetti F, Beccarini A, Calvisi V, Gobbi P, Falcieri E, Curzi D. Chondrocalcinosis: a morphofunctional study of crystal deposition in mechanically stressed shoulder soft tissues. *Muscle, Ligaments and Tendons Journal*, 2018. 8(4):465-72. DOI: 10.11138/mltj/2018.8.4.465.

- Battistelli M, Favero M, **Burini D**, Trisolino G, Dallari D, De Franceschi L, Goldring SR, Goldring MB, Belluzzi E, Filardo G, Grigolo B, Falcieri E, Olivotto E. Morphological and ultrastructural analysis of normal, injured and osteoarthritic human knee menisci. *European Journal of Histochemistry*, 2019. 63(1):2998. DOI: 10.4081/ejh.2019.2998.

- Ciacci C, Grimmelpont Margot, Corsi Ilaria, Bergami E, Curzi D, **Burini D**, Bouchet V, Ambrogini P, Gobbi P, Ujiié Y, Ishitani Y, Coccioni R, Bernhard J, Frontalini F. Nanoparticle-biological interactions in a marine microbial eukaryote. *Scientific Reports*, 2019. In press.

Abstracts (30):

- Curzi D, Fardetti F, Beccarini A, Salucci S, **Burini D**, Falcieri E, Gobbi P. Morphological study of cartilage cell death in patients affected by osteoarthritis and chondrocalcinosis. 70° congresso SIAI (Società Italiana di Anatomia e Istologia), Roma 15-17/09/2016. *Italian Journal of Anatomy and Embryology*, 121(supplement 1):104.

- Curzi D, Fardetti F, Beccarini A, Salucci S, **Burini D**, Gobbi P, Falcieri E. Load-dependent articular cartilage damage in patients affected by osteoarthritis and chondrocalcinosis. 8°

Congresso Nazionale SISMES (Società Italiana delle Scienze Motorie e Sportive), Roma 7-9/10/2016. *Sport Sciences for Health*, 12(supplement 1):64.

- Curzi D, Salucci S, **Burini D**, Fardetti F, Beccarini A, Falcieri E, Gobbi P. Morphological study of chondrocyte cell death in patients affected by chondrocalcinosis. 15th congress ICHC (International Congress of Histochermistry and Cytochemistry), Antalya (Turkey) 18-21/05/2017. *Abstract Book*, 178. (ORAL PRESENTATION)

- Giordano FM, **Burini D**, Burattini S, Canonico B, Falcieri E, Papa S, Battistelli M, Salucci S. Autophagy modulation in preserving skeletal muscle integrity. 15th congress ICHC (International Congress of Histochermistry and Cytochemistry), Antalya (Turkey) 18-21/05/2017. *Abstract Book*, 179.

- Salucci S, Giordano FM, Curzi D, Battistelli M, **Burini D**, Falcieri E, Burattini S. Natural anti-oxidant for skeletal muscle death prevention. 15th congress ICHC (International Congress of Histochermistry and Cytochemistry), Antalya (Turkey) 18-21/05/2017. *Abstract Book*, 458.

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AWARDS WON DURING PhD COURSE

- Young Histochemist Award (of the Italian Society of Histochemistry), for attendance to the 15th International Congress of Histochemistry and Cytochemistry (ICHC 2017), Antalya (Turkey) 18-21/05/2017.

- EMS Scholarship (of the European Microscopy Society), for attendance to the 13th Multinational Congress on Microscopy (MCM 2017), Rovinj (Croatia) 24-29/09/2017.

- SISM Scholarship (of the Italian Society for Microscopical Sciences), for attendance to the 14th Multinational Congress on Microscopy (MCM 2019), Belgrade (Serbia) 15-20/09/2019.

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