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**“Study of Morpho-Molecular Mechanisms and Tissue  
Engineering involved in the Musculoskeletal Disorders”**

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## CHAPTER I

## 1. Summary

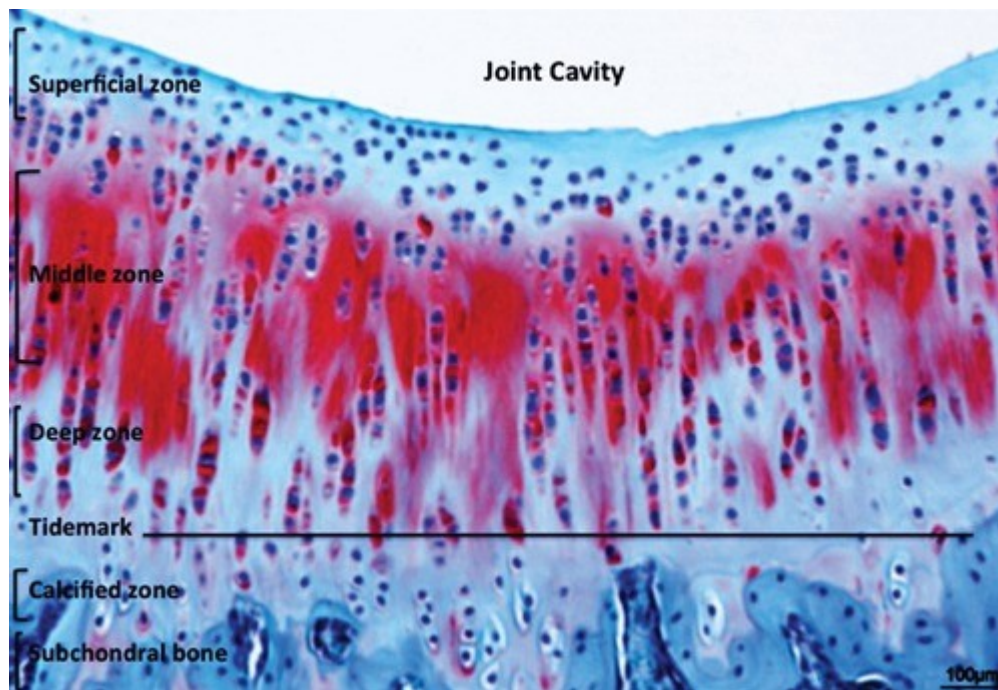
Osteoarthritis (OA), one of the most commonly occurring musculoskeletal conditions, is a nearly universal, slowly progressive degenerative condition of articular cartilage, affecting of men and women as they age. Articular cartilage is characterized by its extreme fragility, due to the fact that it is neither innervated nor vascularized, so once injured it is not able to repair and it undergoes the degenerative process. Although a lot is known about this complex and multifactorial disease, the only existing therapy consists in the attenuation of its symptoms but no resolutive therapy is available. Considering numerous aspects and multifactorial nature of the OA disease, the aim of this research was to improve the knowledge of the morpho-molecular mechanisms occurring in the osteoarthritic and normal cartilage and to find the possible therapeutic solutions for this complicated disease. The present study focused on the most important aspects of OA. Primarily, we took into account its principal risk factor and triggering element represented by aging process, to pass subsequently to investigate about its main cause given by the inflammatory process and altered lubricating ability of the cartilage tissue, to end with the investigation of the solution to the problem given by the tissue engineering approach based on adipose tissue-derived MSCs. In conclusion, the present research highlights some important aspects concerning molecular alterations of articular cartilage tissue occurring in the pathological conditions and provides new insights for the treatments aimed to prevent, attenuate or solve the osteoarthritic process, as well as to restore the lost cartilage tissue.

**Keywords:** Osteoarthritis, Articular cartilage, Aging, Inflammation, Lubricin, Chitinases, Tissue Engineering, Mesenchymal Stem Cells.

## **2. General Introduction**

### **2.1. Structure, function and composition of articular cartilage**

Articular cartilage is a specialized connective tissue that covers the opposing ends of the bones of diarthrodial joints (1). It is also found in the growth plate of the metaphysis (2). Its principal function is to provide a continuous and almost frictionless surface for articulation and to allow the transmission of low friction loads. Articular cartilage is avascular and aneural so it has a limited capacity for intrinsic healing and repair. Therefore, since this tissue is continuously subjected to a severe biomechanical environment, its preservation and health are fundamental for the joint health. Articular cartilage is a hyaline cartilage and is composed of highly specialized cells called *chondrocytes*. Chondrocytes are metabolically active and play a fundamental role in the development, maintenance, and repair of the extracellular matrix (ECM). They originate from mesenchymal stem cells through the process of chondrogenesis and constitute about 2% of the total volume of articular cartilage. Depending on the anatomical zone, these cells vary in shape, number, and size. Articular cartilage thickness is about 2 to 4 mm. It is composed of a dense ECM with a sparse distribution of chondrocytes within it. The ECM is principally composed of water, collagen type II, and proteoglycans such as aggrecan, with other non-collagenous proteins and glycoproteins in smaller quantities (3,4). Type II collagen is responsible for the tensile strength of the cartilage, while aggrecan provides the osmotic resistance for cartilage, which is critical to maintain its unique viscoelastic and mechanical properties (1). In the hyaline cartilage, the chondrocytes are located in lacunae within the ECM and represent only 5% to 10% of the total cartilage volume, but they are crucial to the maintenance of a stable cartilage. Nutrients and cellular repair components are transported to the chondrocytes by diffusion from the synovial fluid, generated by joint motion. Thanks to histochemistry and microscopy, it is possible to highlight the characteristic organization of articular cartilage that shows a heterogeneous distribution of cell and matrix components through its width (5). Four different zones are identified in the articular cartilage 1) superficial zone; 2) intermediate (or middle zone); 3) radial zone (or deep zone); 4) calcified cartilage (or calcified zone) (Figure 1).



**Figure 1.** Safranin-O staining (cartilaginous proteoglycans detection) of the healthy hyaline cartilage layers. Scale bars: 100 µm. The figure taken from the paper by Musumeci et al., 2014 (5).

- 1) The thin superficial zone, where chondrocytes produce and lay mainly collagen type II and IX in a parallel direction to the surface of the tissue, protects deeper layers from shear, tensile, and compressive stresses imposed by articulation. It contains a relatively high number of flattened chondrocytes. It is in direct contact with synovial fluid and is responsible for most of the tensile properties of cartilage.
- 2) A middle zone is characterized by randomly oriented collagen fibres. It provides a functional and anatomic link between the superficial and deep zones. It represents the major part of the total cartilage volume and contains more proteoglycans, which maintain the fluid within the tissue and are responsible of the hyaline cartilage's distinctive compression resistance properties. In this layer, the collagen is organized obliquely and the chondrocytes are spherical and at low density.
- 3) A deep zone, located at the cartilage-bone interface where the collagen fibres are aligned perpendicular to the surface. Thanks to this collagen fibres distribution this zone is responsible of providing the greatest resistance to compressive forces. Here we can find the highest proteoglycan content, and the lowest water concentration. The chondrocytes are typically arranged in columnar orientation and parallel to the collagen fibres. The deep zone is separated from the calcified one by the tide mark as we can observe in the Figure 1.

4) The calcified zone plays a basic role in connecting the cartilage to the bone, by implanting the collagen fibrils of the deep zone to the subchondral bone. In this zone, we can notice a scarce number of chondrocytes, which become hypertrophic.

It is important to mention that only some minor defects in articular cartilage's ECM can be regenerated by chondrocytes. With the avascular and aneural features of articular cartilage, it should resist to the not indifferent mechanical load over years without degenerative changes. Due to its unique properties, cartilage shows little or no intrinsic repairing capacity for an effective healing response. When more extensive defects exceed the reparative capacity, the damage can become permanent and triggers the development of pathological conditions such as osteoarthritis.

## **2.2. Musculoskeletal disorders: Osteoarthritis**

Over the past century, with the world's population growth, increased average age and decreased death rates, people are becoming increasingly susceptible to the age-related diseases, including musculoskeletal disorders. The recent Global Burden of Disease (GBD) Study estimated that the burden disability from musculoskeletal conditions worldwide is exceptionally high. Osteoarthritis, in particular, as well as rheumatoid arthritis and gout are significant contributors to the global disability burden (6). The impact of this disability on the life quality, economic independence and the costs to society due to health and social care and work loss, are not indifferent.

Osteoarthritis (OA), one of the most commonly occurring musculoskeletal conditions, is a nearly universal, slowly progressive degenerative condition affecting of men and women as they age. OA of the hip and knee represent two of the most significant causes of pain and physical disability in adults. In the United States, OA is the second most common form of disability. OA is a clinical condition of joints involved in the movement of limbs. OA is a degenerative and slowly progressing joint disease, regarding the entire joint and especially the articular cartilage tissue (7). The latter is characterized by its extreme fragility, due to the fact that it is neither innervated nor vascularized, so once injured it is not able to repair and it undergoes the degenerative process. The condition is characterized by degeneration of the cartilage with reactive formation of new bone at the articular margins. This degeneration and new bone formation result in pain and stiffness of the affected joints (8). All joints can be affected, but OA is seen most commonly in the hand, hip, and knee joints (9). Primary OA is the most common form and has no known cause, although it is often related to aging and heredity. Aging is the most prominent risk factor triggering the initiation of OA. A fundamental mechanism that mediates age-related dysfunctions is represented by cellular senescence, characterized by the phenomenon of telomere shortening, resulting in cell proliferation

arrest. The telomeres are replicated by a special reverse transcriptase called telomerase, which level in normal human somatic tissues is insufficient to prevent telomere shortening. Secondary OA may occur in any joint as a result of articular injury, such as fracture, repetitive joint use, obesity, or metabolic disease, and may occur at any age (10).

It has been widely demonstrated that inflammatory mediators participate in the onset and progression of OA after joint injury (11). Moreover, the evidences from literature indicate that the flow of pro-inflammatory cytokines following the acute injury seems to be directly associated with the altered lubricating ability occurring in the joint tissue. The latter is associated with the reduction of lubricin, which is one of the major joint lubricants (12).

Actually, although a lot is known about this complex and multifactorial disease, the only existing therapy consists in the attenuation of its symptoms but no resolutive cure is available.

### **2.3. Mesenchymal stem cells and tissue engineering in cartilage regeneration**

OA disease is represented by the poor regenerative properties of the articular cartilage once injured. For this reason the field of articular cartilage tissue engineering, which aims to repair, regenerate, and/or improve injured or diseased articular cartilage functionality, evokes intense interest and holds great potential for improving articular cartilage therapy. As a rapidly expanding field, tissue engineering may provide alternative solutions for articular cartilage repair and regeneration through developing biomimetic tissue substitutes. In the orthopedic field the bioinspired scaffolds are designed to mimic structural and biological cues of the native cartilage unit, supporting both cartilaginous repair and the integration of the newly formed matrix with the surrounding tissues. Successful articular cartilage tissue engineering strategy relies largely on several essential components including cellular component, supporting 3D carrier scaffolding matrix, bioactive agent and proper physical stimulants. Utilization of mesenchymal stem cells (MSCs) for articular cartilage tissue engineering is continuously increasing compared to use of autologous chondrocytes thanks to their high proliferative capacity and chondrogenic differentiating potency (13). Chondrogenesis of MSCs involves several processes such as cell recruitment, migration, expansion, differentiation and maturation, which are regulated by an amount of growth and transcriptional factors. Various sources of MSCs have been investigated including adipose tissue, amniotic fluid, blood, bone marrow, dermis, embryonic stem cells, infrapatellar fat pad, muscle, periosteum, placenta, synovium, trabecular bone, and umbilical cord (14). A wide range of matrices have been investigated to develop tissue engineering-based strategies including hydrophilic polymers, carbohydrate-based scaffolds, protein-based scaffolds, etc. TGF- $\beta$ 1, TGF- $\beta$ 3, BMP-2, and hypoxic environment are the recommended bioactive agents to induce optimum



chondrogenesis of MSCs and the combination of shear forces/dynamic compression is designed as the best maturation-promoting physical stimulant (15-17).

The perfect cartilage for articular repair should be characterized by the phenotypic stable chondrocytes that remain in their quiescent state without differentiating in hypertrophic chondrocytes with inferior biological and mechanical features. Unfortunately, the progression of MCS-derived chondrocytes to hypertrophic phenotype under in vitro chondrogenic culture conditions seems to be unavoidable. Maintaining MSC-derived chondrocytes in their quiescent healthy state represents then the biggest challenge for clinical application of MSCs in articular cartilage repair. The further studies in this field seem necessary for providing new insights in understanding the regulation of stem cell function and potentially open novel therapeutic avenues for OA treatments.

#### **2.4. Physical activity**

Nowadays the concept of health is closely related with proper nutrition and adequate physical activity. Several lines of evidence have confirmed the beneficial role of both, but especially their synergy in gets well-being. The information about the best way to feed and about the proper physical activity is not always based on scientific evidence and often the general population, more and more interested in their own well-being, falls victim of misunderstandings or wrong informations. Physical activity increases energy metabolism, contributes to a healthy energy balance and can be used to increase lean mass, bone, strengthen joints, can be useful in controlling pain and improves cardio-respiratory efficiency by promoting psychological well-being. Physical activity is also useful to mitigate or improve the condition of chronic diseases, for example people with diabetes and with osteoarthritis (18). OA Research Society International (OARSI) recommends physical activity programs after surgical interventions in cases of severe and advanced cases of OA, to maintain joint health and keep the patient mobile. Indeed, physical therapy is crucial for the success of any surgical procedure and can promote recovery of muscle strength, range of motion, coordinated walking, proprioception and mitigate joint pain (19). The beneficial effects of the conjugation of physical activity and Mediterrean Diet, based on the consumption of extra virgin olive oil, has been also investigated and confirmed. It was shown that this conjugation was able to improve the articular cartilage condition by increasing the expression of lubricin, the major lubricating molecule present in synovial fluid and cartilage, and by decreasing the expression of pro-inflammatory mediators such as interleukin 1, responsible of the degenerative responses that follow the acute injury (20).

## **2.5. The purpose and design of the present research**

Having regard of what said above and considering a numerous aspects and multifactorial nature of the OA disease, the aim of this research was to improve the knowledge of the morpho-molecular mechanisms occurring in the osteoarthritic and normal cartilage and to find the possible therapeutic solutions for this complicated and multifactorial disease.

### **2.5.1. Aims of the single researches:**

- ***Age-related degeneration of articular cartilage in the pathogenesis of osteoarthritis: molecular markers of senescent chondrocytes.***

Aging is the most prominent risk factor triggering the initiation and progression of OA. Many hypothesis link aging and OA and the most accredited one is that chondrocytes undergo premature aging due to several stressors, such as excessive mechanical loading or oxidative stress, inducing the so called "stress-induced senescent state". The aim of the review was to focus on molecular markers and mechanisms implicated in chondrocyte aging and the pathogenesis of OA. We discussed the most important age-related morphological and biological changes that affect articular cartilage and chondrocytes with aging and identified their main senescence markers. Published in Musumeci G, Szychlinska MA, Mobasher A. Age-related degeneration of articular cartilage in the pathogenesis of osteoarthritis: molecular markers of senescent chondrocytes. *Histol Histopathol.* 2015;30:1-12.

- ***Physical activity ameliorates cartilage degeneration in a rat model of aging: a study on lubricin expression.***

Since aging is primarily implicated in the OA onset, we wondered whether OA might be prevented by undertaking regular physical activity, since its beneficial effects have been widely reported in literature. The aim of the research was to investigate if the moderate physical activity would improve the articular cartilage condition of aged rats by increasing the lubricin expression. Published in Musumeci G, Castrogiovanni P, Trovato FM, Imbesi R, Giunta S, Szychlinska MA, Loreto C, Castorina S, Mobasher A. Physical activity ameliorates cartilage degeneration in a rat model of aging: a study on lubricin expression. *Scand J Med Sci Sports.* 2015;25:e222-30.

- ***Altered joint tribology in osteoarthritis: Reduced lubricin synthesis due to the inflammatory process. New horizons for therapeutic approaches.***

It is evident that inflammatory process is strongly implicated in the progression of articular cartilage degeneration and OA onset. Moreover, the flow of pro-inflammatory cytokines following an acute injury seems to be associated also with the altered lubricating capacity of the tissue. Thus, the aim of this review was to consolidate the evidence that implicates the inflammation in the attenuation of the joint tribology and synovial lubrication leading to the decreased joint function and pain. Published in Szychlinska MA, Leonardi R, Al-Qahtani M, Mobasheri A, Musumeci G. Altered joint tribology in osteoarthritis: Reduced lubricin synthesis due to the inflammatory process. New horizons for therapeutic approaches. *Ann Phys Rehabil Med.* 2016;59:149-56.

- ***Expression of CHI3L1 and CHIT1 in osteoarthritic rat cartilage model. A morphological study.***

Recently, the biological role of chitinases such as human cartilage glycoprotein 39 (CHI3L1) and chitotriosidase (CHIT1) has been studied in relation to several inflammatory and degenerative disorders. Hence, the aim of this study was to evaluate for the first time the expression of these two chitinases in the osteoarthritic and normal rat articular cartilage, to assess their potential involvement in the OA disease. Published in Di Rosa M, Szychlinska MA, Tibullo D, Malaguarnera L, Musumeci G. Expression of CHI3L1 and CHIT1 in osteoarthritic rat cartilage model. A morphological study. *Eur J Histochem.* 2014;58:2423.

- ***Co-Expression and Co-Localization of Cartilage Glycoproteins CHI3L1 and Lubricin in Osteoarthritic Cartilage: Morphological, Immunohistochemical and Gene Expression Profiles.***

Since CHI3L1, studied in the previous research, was shown to be implicated in the OA disease, and is also known as a potential marker for the activation of chondrocytes, while lubricin was shown to be chondroprotective, the aim of this study was to investigate the co-expression and co-localization of CHI3L1 and lubricin in normal and osteoarthritic rat articular cartilage and to correlate their altered expression to the specific grade of OA. Published in Szychlinska MA, Trovato FM, Di Rosa M, Malaguarnera L, Puzzo L, Leonardi R, Castrogiovanni P, Musumeci G. Co-Expression and Co-Localization of Cartilage Glycoproteins

CHI3L1 and Lubricin in Osteoarthritic Cartilage: Morphological, Immunohistochemical and Gene Expression Profiles. *Int J Mol Sci.* 2016;17:359.

- ***Biosynthesis of collagen I, II, RUNX2 and lubricin at different time points of chondrogenic differentiation in a 3D in vitro model of human mesenchymal stem cells derived from adipose tissue.***

Since there is no resolutive therapy for the OA disease and the MSC-based approaches and tissue engineering for the cartilage regeneration seem to be very promising, we wanted to further investigate the potential use of adipose tissue-derived MSCs for the cartilage regeneration. The first aim of this study was to assess the best time for differentiation of adipose tissue derived MSCs to chondrocytes, through the self-assembly process. The second aim was to demonstrate that the expression of lubricin, such as the expression of collagen type II, could be a possible biomarker for the detection of chondrocytes well-being and viability in the natural self-assembling constructs, called 'cell pellets'. Published in Musumeci G, Mobasher A, Trovato FM, Szychlinska MA, Graziano AC, Lo Furno D, Avola R, Mangano S, Giuffrida R, Cardile V. Biosynthesis of collagen I, II, RUNX2 and lubricin at different time points of chondrogenic differentiation in a 3D in vitro model of human mesenchymal stem cells derived from adipose tissue. *Acta Histochem.* 2014;116:1407-17.

- ***Assessment of osteoblast and chondrocyte biomarkers in adipose tissue derived-mesenchymal stem cells and relative study of Lubricin and Caspase-3 expression.***

As the continuation of the previous study, the aim of the present research was to demonstrate the morphological and biochemical structure of a healthy hyaline cartilage constituted by chondrocytes differentiated from adipose tissue derived-MSCs growing on a Collagen Cell Carrier (CCC) scaffold, through the evaluation of the expression of some osteoblasts (RUNX2 and osteocalcin), chondrocytes (collagen I, II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated MSCs, chondrocytes cultured in 2D and chondrocytes grown on the CCC scaffolds. In submission (Szychlinska MA, Castrogiovanni P, Nsir H, Di Rosa M, Guglielmino C, Parenti R, Calabrese G, Pricoco E, Salvatorelli L, Magro G, Mobasher A, Musumeci G. Assessment of osteoblast and chondrocyte biomarkers in adipose tissue derived-mesenchymal stem cells and relative study of Lubricin and Caspase-3 expression. A bioengineering model for cartilage regeneration. *International Journal of Molecular Sciences*).

## CHAPTER II

## REVIEW

# **Age-related degeneration of articular cartilage in the pathogenesis of osteoarthritis: molecular markers of senescent chondrocytes**

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## 1. Summary

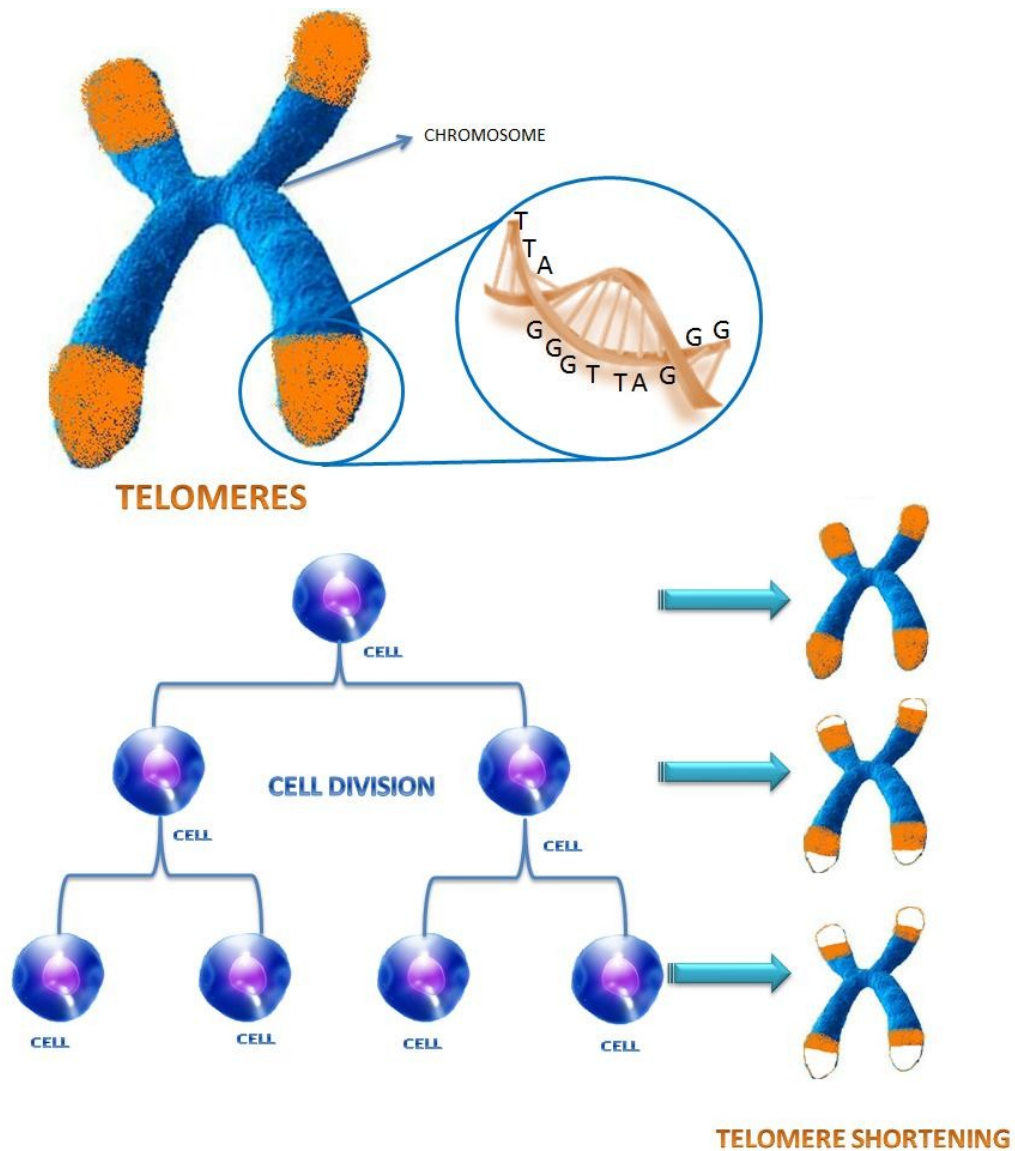
Aging is a natural process by which every single living organism approaches its twilight of existence in a natural way. However, aging is also linked to the pathogenesis of a number of complex diseases. This is the case for osteoarthritis (OA), where age is considered to be a major risk factor of this important and increasingly common joint disorder. Half of the world's population, aged 65 and older, suffers from OA. Although the relationship between the development of OA and aging has not yet been completely understood, it is thought that age-related changes correlate with other risk factors. The most prominent hypothesis linking aging and OA is that chondrocytes undergo premature aging due to several factors, such as excessive mechanical load or oxidative stress, which induce the so called “stress-induced senescent state”, which is ultimately responsible for the onset of OA. This review focuses on molecular markers and mechanisms implicated in chondrocyte aging and the pathogenesis of OA. We discuss the most important age-related morphological and biological changes that affect articular cartilage and chondrocytes. We also identify the main senescence markers that may be used to recognize molecular alterations in the extracellular matrix of cartilage as related to senescence. Since the aging process is strongly associated with the onset of osteoarthritis, we believe that strategies aimed at preventing chondrocyte senescence, as well as the identification of new increasingly sensitive senescent markers, could have a positive impact on the development of new therapies for this severe disease.

**Keywords:** Osteoarthritis, Aging, Cartilage, Chondrocyte, Lubricin, Senescence, Senescence markers

## 2. Introduction

Our body is made up of an incredibly large number of cells, around 100 billion, some of them have a rather short life, others such as chondrocytes remain for a lifetime, but at a certain point, the mechanism slows down, cell duplication starts to fail and the cells are no longer replaced by other ones. The cells arrest their cell cycle progression. Each cell has a limited number of possible divisions, which is fixed between 50 and 70. The reason lies in the structure of chromosomes that are duplicated at each division in order to obtain one copy for itself and another for the daughter cell that will play the same role in the body. The division process, however, is not perfect, the cellular mechanism fails to copy the ends of chromosomes, the so-called telomeres (Fig. 1). After each cell division, the telomeres become shorter and shorter and are eventually completely worn out and parts of chromosomes that contain essential genetic information begin to erode. At

this point the cell is at the end of its life and it approaches death by activating specific mechanisms. In order to avoid the aging process, a “magic” molecule able to stretch the ends of chromosomes would be necessary.



**Figure 1.** Telomere shortening. A telomere is a region of repetitive DNA (TTAGGG repeats) at the end of a chromosome, which protects the end of the chromosome from deterioration. Each time cells divide, telomeres shorten, and there is a limit to the number of times a given cell can go on dividing, the so-called “Hayflick limit”. When the length of the telomeres is too short cell division stops.

This normally happens in cells and it is due to telomerase. However, telomerase is active only in embryonic cells and in a few adult cells such as cells of the immune system, in the other ones its activity runs out over time. It is for this reason that we talk about “cell senescence”. Cellular senescence is strongly associated with the development of several serious diseases such as



cancer, diabetes, cardiovascular and neurodegenerative diseases, and osteoarthritis (OA) (Burnet and Berger, 2014). OA is a degenerative joint disease, which affects especially the articular cartilage, leading to pain and stiffness of the affected joint. OA is one of the most disabling musculoskeletal disorders in the world and it affects mostly the elderly population (Musumeci et al., 2014a). In this review we discuss how cellular senescence can influence the onset of a complex joint disease such as OA. We will discuss the most important features of cellular senescence and how the age-related changes, which arise at cellular and tissue level, influence the development and progression of OA. Lastly, we will list some of the most important senescence markers used to evidence the senescence of chondrocytes.

### **3. Osteoarthritis**

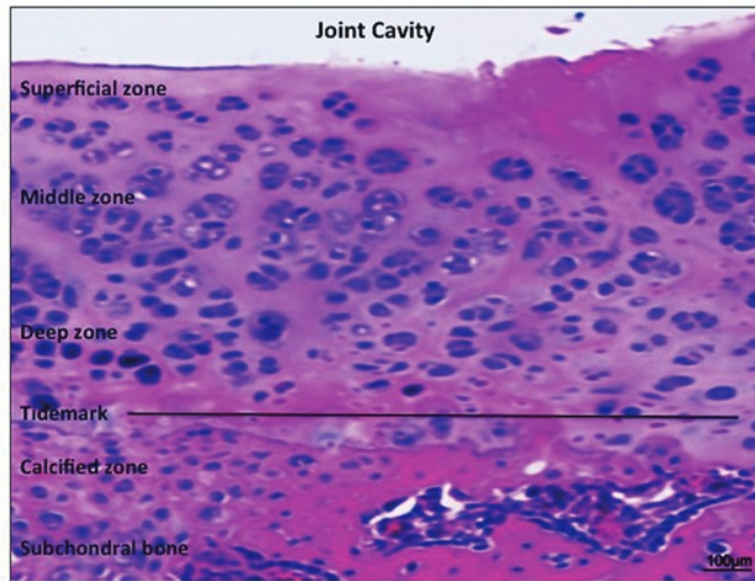
OA is the most common form of joint disease and affects mainly hips, knees, hands, and feet, leading to severe disability and loss of quality of life, particularly in the elderly population (Musumeci et al., 2013a). Indeed, half of the world's population, aged 65 and older, suffers from OA. It has been estimated that 9.6% of men and 18% of women in that age group, have symptomatic OA. Moreover, OA is considered the most important cause of impaired mobility and contributes to 50% of all the musculoskeletal diseases worldwide (Wolf and Pfleger, 2003). The disease is characterized by joint dysfunction due to gradual changes in several structures of the joint such as synovium, subchondral bone and especially articular cartilage (Fig. 2) (Buckwalter and Mankin, 1998a). Progressive wear and tear on articular cartilage can lead to a progressive cartilage tissue loss, further exposing the bony ends, leaving them without protection (Buckwalter and Mankin, 1998b). This finally deteriorates into the most common form of arthritis, or rather moderate OA at early stage (Fig. 3) and severe OA at advanced stage (Fig. 4) (Musumeci et al., 2014a).

The degradation, consequent loss of articular cartilage and formation of osteophytes lead to chronic pain and functional restrictions in the affected joint. Unfortunately, articular cartilage has a limited regenerative capacity (Musumeci et al., 2013b; Iwamoto et al., 2013;). Consequently, once injured, cartilage is much more difficult to self-heal and the only way to improve the patient's condition is therapeutic intervention. Different factors can be involved in the development of OA, such as joint injury, genetic predisposition, defective position of joints, obesity, malnutrition and excessive mechanical load, which all lead to similar alterations of the articular cartilage (Musumeci et al., 2014b,c; Goldring and Goldring, 2007; Lee et al., 2013).

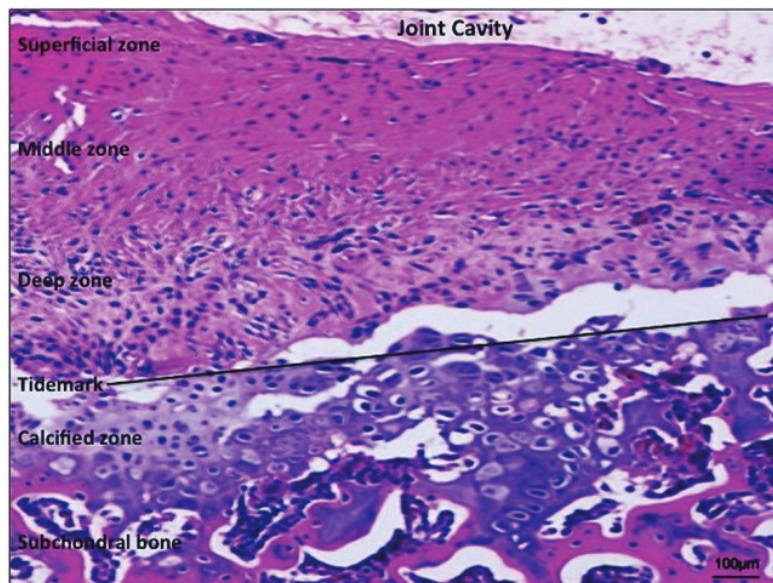


**Figure 2.** Healthy articular knee cartilage from rat. Hematoxylin and Eosin staining. Normal articular knee hyaline cartilage layers: superficial zone, intermediate (or middle zone), radial zone (or deep zone) and calcified cartilage (or calcified zone). Tidemark, the border between non-calcified and calcified cartilage. In the superficial zone, cells are flat and small; in the middle and deep zone, cells are organized in columns; the tidemark is evident. Magnification x20. Scale bars: 100 μm.

However, the prevalence of OA rises directly with age, which represents the most prominent risk factor for the initiation and progression of primary OA, but it is important to underline that OA is not a simple “wearing out in time” of the joints and the degenerative changes related to age can be distinguished from those due to the disease (Musumeci et al., 2013c). The relationship between the development of OA and aging is not completely understood and it is thought that the age-related changes are correlated to other risk factors, which may occur concurrently or in conjunction with it. In reality, not all older adults develop OA and OA-like changes can also develop without a significant contribution of aging. Thus, aging and OA are inter-related but not inter-dependent (Loeser, 2004). However, there is also a possibility that the chondrocytes undergo premature aging due to several factors, such as excessive mechanical load or oxidative stress. In the latter case, aging and the development of OA are both inter-related and inter-dependent (Martin et al., 2004a; Akagi et al., 2010).



**Figure 3.** Moderate OA articular knee cartilage from rat. Hematoxylin and Eosin staining. Articular knee hyaline cartilage layers at early OA stage: superficial zone, intermediate (or middle zone), radial zone (or deep zone) and calcified cartilage (or calcified zone). Tidemark, the border between non-calcified and calcified cartilage. Clear deep fissures in the articular surface, the cells from the superficial, intermediate and deep zone, where chondrocytes are not arranged in columns. The tidemark is not intact in all its extension and the subchondral bone shows little fibrillation. Magnification x20. Scale bars: 100 µm.



**Figure 4.** Severe OA articular knee cartilage from rat. Hematoxylin and Eosin staining. Articular knee hyaline cartilage layers at advanced OA stage, due to aging: superficial zone, intermediate (or middle zone), radial zone (or deep zone) and calcified cartilage (or calcified zone). Tidemark, the border between non-calcified and calcified cartilage. Cells are arranged in clusters especially around fissures or disappear completely as the disease progresses. The organization of cartilage is completely disordered and replaced by fibrocartilaginous, scar-like tissue with fibroblast like cells. Magnification x20. Scale bars: 100 µm.

#### 4. Cellular aging

Cellular aging, or cell senescence, refers to the limited capacity of mitotic cells to further multiply in time (over 30–40 divisions). This limit is known as the “Hayflick limit” (Hayflick, 1984). This form of senescence is called “replicative senescence”, also known as intrinsic senescence, which results from an arrest in cell-cycle progression. Some of the changes exhibited by cells, which have undergone replicative senescence can be found in cells in older adults, such as shortened telomeres, formation of senescence-associated (SA) heterochromatin (Muller, 2009) and changes of phenotype with an alteration in gene expression (Bodnar et al., 1998). It has been hypothesized that the telomere length could be considered as a marker for replicative senescence. Telomeres cannot be completely replicated in primary cells and become shorter with each round of cell division. Telomeres are nucleoprotein structures (TTAGGG repeats) that cap the ends of the linear eukaryotic chromosomes and thereby protect their stability and integrity during replication by protecting chromosome ends against exonucleases (Fig. 1). Telomeres are replicated by a special reverse transcriptase called telomerase, in a complex mechanism that is coordinated with the genome's replication. Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA sequences and comprises two essential components. One is the functional RNA component (in humans called hTERC), which serves as a template for telomeric DNA synthesis. The other is a catalytic protein (hTERT) with reverse transcriptase activity and the primary determinant for the enzyme activity (Bryan and Cech, 1999; Kupiec, 2014). The level of telomerase in normal human somatic tissues is insufficient to prevent telomere shortening. Telomeres can be lengthened through increasing telomerase activity by exogenous expression of hTERT or hTR (the RNA template) (Greider, 1998). As proof of this concept the chondrocytes transduced with hTERT proved to be able to increase telomere length and therefore to prolong cell lifespan, increasing in this way the efficacy of cartilage repair (Martin and Buckwalter, 2003). Another type of senescence is “stress-induced senescence”, also known as extrinsic senescence, which is independent of telomere length. In quiescent cells such as chondrocytes, this type of senescence may be more important than the replicative version, because progressive telomere shortening cannot completely explain senescence in these post-mitotic cells (Ben-Porath and Weinberg, 2005; Chen and Goligorsky, 2006). The various types of stress responsible for this kind of senescence include DNA damage, oxidative stress, oncogene activity, ultraviolet radiation and chronic inflammation (Itahana et al., 2004; Campisi, 2005). Oxidative stress is thought to play a major role as a stressor. It results when the amount of reactive oxygen species (ROS) exceeds the anti-oxidant capacity of the cell. ROS are generated by intracellular enzymes such as nicotinic amide adenine dinucleotide phosphate (NADPH) oxidase and 5-lipoxygenase in response to activation of specific cell signaling pathways

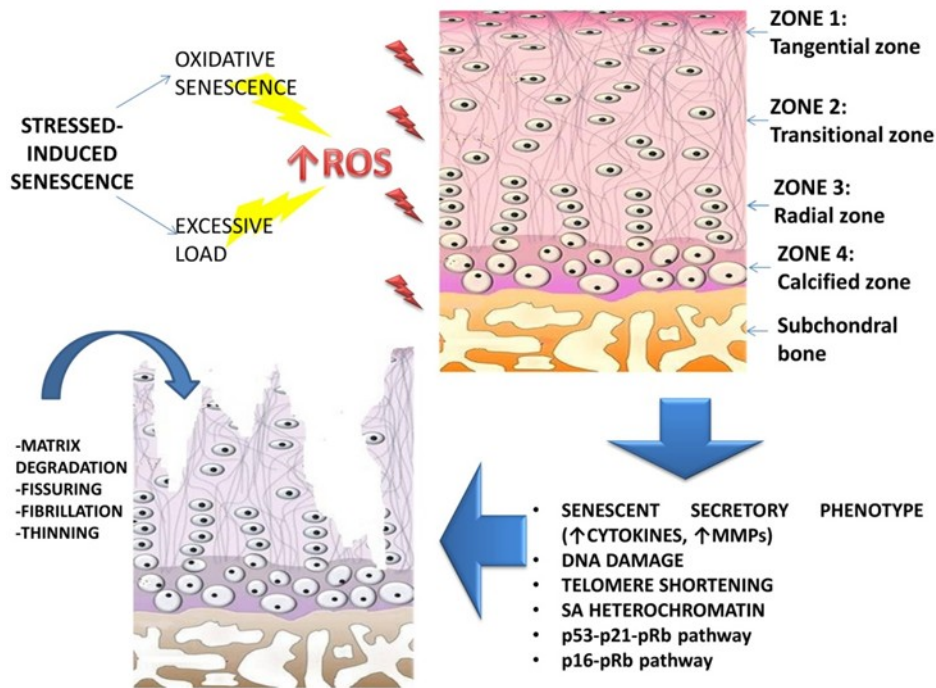
(Kamata and Hirata, 1999; Finkel and Holbrook, 2000). A direct role for increased ROS levels in promoting cell senescence is a positive feedback activation of the ROS-protein kinase C delta (PKC $\delta$ ) signaling pathway, which cooperates with the p16<sup>INK4A</sup>-retinoblastoma protein (Rb) pathway, which plays an important role in the control of cell-cycle progression (Takahashi et al., 2006). Telomere shortening is also observed in stress-induced senescence and it is due to oxidative damage to DNA caused by ROS. The ends of chromosomes are particularly sensitive to oxidative damage, which causes telomere erosion similar to that seen with replicative senescence (Yudoh et al., 2005). Also, the ROS generated from excessive mechanical loading and stimulation of cytokines contribute to DNA damage, which subsequently results in telomere shortening (Tomiya et al., 2007; Davies et al., 2008). Cellular senescence, as well as apoptosis, can be viewed as a powerful tumor-suppressor mechanism that withdraws cells with irreparable DNA damage from the cell cycle (de Lange and Jacks, 1999; Artandi and DePinho, 2000; Puzzo et al., 2014) through the intrinsic or mitochondrial (Caltabiano et al., 2013; Loreto et al., 2011a) and extrinsic apoptosis pathway (Cardile et al., 2013; Loreto et al., 2011b). Several recent studies report that cartilage degeneration also coincides with increased apoptotic chondrocytes (Musumeci et al., 2011a; Musumeci et al., 2011b; Galanti et al., 2013). Therefore, the senescence signals, that is, a telomere-based one or a stress-based one, trigger a DNA damage response and this response shares a common signaling pathway that converges on either or both of the well-established two tumor-suppressor proteins, p53 (the p53-p21-pRb pathway) (Martin and Buckwalter, 2003; Herbig and Sedivy, 2006) and RB and pRb proteins (the p16-pRb pathway) (Musumeci et al., 2010; Musumeci et al., 2011c). In the p53-p21-pRb pathway, senescence stimuli activate the p53, which then can induce senescence by activating pRb through p21, which is a transcriptional target of p53. This senescence can be reversed upon subsequent inactivation of p53. In the p16-pRb pathway, senescence stimuli induce p16, which activates pRb. Once the pRb pathway is engaged by p16, the senescence cannot be reversed by subsequent inactivation of p53, silencing of p16 or inactivation of pRb (Beauséjour et al., 2003). The difference between these two pathways is that the p53-p21-pRb pathway mediates the senescence due to telomere shortening and the p16-pRb pathway is thought to mediate premature senescence (Beauséjour et al., 2003). Once cells have entered senescence, they are arrested in the G1 phase of the cell cycle and display a characteristic morphology (vacuolated, flattened cells) and altered gene expression (Cristofalo et al., 2004). The senescent cells exhibit the so-called “senescent secretory phenotype” (SSP), which could be also correlated with the development of OA. It is interesting to note that the senescent cells, which are mitotically inactive, are biologically active (Campisi, 2005). These cells are able to increase the expression of genes that inhibit proliferation and to increase the secretion of several proteins, including

inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 $\beta$  (IL-1 $\beta$ ), degradative enzymes such as metalloproteinases (MMPs) and growth factors such as epidermal growth factor (EGF) that regulate cell proliferation and all of which may stimulate tissue aging and tumorigenesis (Zhang et al., 2003). Recent studies reported that the increased expression of the IL-8 receptor CXCR2 and insulin-like growth factor binding protein 7 (IGFBP7) could also contribute to cell aging (Acosta et al., 2008; Wajapeyee et al., 2008). The accumulation of cells expressing the SSP can also contribute to tissue senescence by impairing the extracellular matrix due to the increased secretion of degradative enzymes (Campisi and d'Adda di Fagagna, 2007). Another important feature of senescent cells is represented by the epigenetic changes related to the formation of foci of heterochromatin, referred to as senescence-associated heterochromatin foci (SAHF), which include histone variants such as the macro-H2A (Zhang and Adams, 2007).

## **5. Age-related articular cartilage degeneration.**

Articular cartilage matrix undergoes several changes with age, including structural, molecular and mechanical ones, surface fibrillation, alterations in structure and composition of proteoglycans, increased collagen cross-linking and decreased tensile strength and stiffness (Hollander et al., 1995; Squires et al., 2003) (Fig. 5). Deterioration in chondrocyte function accompanies these changes also in the extracellular matrix (Aurich et al., 2002). Several reports revealed that chondrocyte senescence contributes to the risk for cartilage degeneration by the decreased ability of chondrocytes to maintain and repair the articular cartilage tissue (Martin and Buckwalter, 2001a; Aigner et al., 2002). There is clinical evidence from Magnetic Resonance Imaging (MRI) studies that the articular cartilage in the knee thins with aging, especially at the patella and at the femoral side of the joint (Hudelmaier et al., 2001; Ding et al., 2005). The progressive articular cartilage thinning with age is related to gradual loss of cartilage matrix and decrease in cartilage hydration and cellularity. This kind of damage stimulates a chondrocyte specific synthetic and proliferative response that may maintain or even restore the articular cartilage. This response may continue for years. However, in instances of progressive joint degeneration the anabolic response eventually declines and the imbalance between chondrocyte synthetic activity and degradative activity leads to progressive thinning of articular cartilage. These alterations may further accelerate the loss of articular cartilage (Buckwalter et al., 2000). Different changes observed in articular cartilage with aging are probably due to chondrocyte senescence, which results in the progressive decrease in cell function. In fact, the mitotic and synthetic activity of human chondrocytes decline with age. They become less responsive to anabolic mechanical stimuli, to anabolic cytokine and to insulin-like growth factor I (IGF-I). The cells synthesize smaller

aggrecans and less functional link proteins leading to the formation of smaller and more irregular proteoglycan aggregates. The latter is the most striking change in articular cartilage matrix related with age. Aggrecans are the molecules that give articular cartilage its stiffness to compression, resilience and durability, thus their alteration makes the tissue more vulnerable to injury and development of progressive degeneration (Buckwalter et al., 1986; Buckwalter and Rosenberg, 1988; Bolton et al., 1999; Martin and Buckwalter, 2000).



**Figure 5.** Stress-induced senescence and Osteoarthritis. The telomere shortening process in senescent chondrocytes is more probably due to the stress-induced type of senescence. Oxidative stress and excessive mechanical loading are thought to be the major stressors that induce the increased production of ROS, which are responsible for DNA damage and for the subsequent senescence of the cells. Once cells have entered senescence, they are arrested in the G1 phase of the cell cycle and they display a characteristic gene expression called “senescent secretory phenotype”, which is strongly correlated with the development of OA.

Moreover, the senescent cartilage matrix appears more susceptible to the accumulation of advanced glycation end-products (AGEs) in cartilage collagen, which results in increased cross-linking and in subsequent increased stiffness, making the cartilage more susceptible to fatigue failure (Verzijl et al., 2002). In addition, the increased levels of AGEs in articular cartilage may also affect chondrocyte function by decreasing its anabolic activity. The mechanism proposed to be responsible for this alteration is the expression of the receptor for the advanced glycation end-products (RAGE) by chondrocytes, which proves to be increased both with aging and in development of OA (DeGroot et al., 1999; Loeser et al., 2005). Stimulation of chondrocyte RAGE results in increased production of MMPs and in modulation of the chondrocyte phenotype to

hypertrophy, which represent two hallmarks of OA (Cecil et al., 2005; Yammani et al., 2006). Furthermore, RAGE signaling is also associated with increased levels of ROS, providing another link between oxidative stress, aging and OA (Loeser, 2004). Another important feature of the aged articular cartilage is its increased calcification, as demonstrated radiographically (Felson et al., 1989). This could be associated with the increased activity of transglutaminase, involved in the biomineralization process (Rosenthal et al., 1997) and with increased production of the inorganic pyrophosphate in response to transforming growth factor  $\beta$  (TGF- $\beta$ ) stimulation (Felson et al., 1989). Chondrocalcinosis is strongly associated with OA, but there is evidence of older people with asymptomatic chondrocalcinosis, thus it proves not to be inter-dependent with the development of OA and its role is not completely clear (Doherty and Dieppe, 1988; Rosen et al., 1997).

## **6. Chondrocyte senescence**

Chondrocytes from older adults exhibit many changes, typical of cell senescence, when compared with cells isolated from young adults. The most evident change is represented by telomere shortening, characteristic of replicative senescence. This evidence is controversial as adult articular chondrocytes rarely, if ever, divide in normal tissue in vivo. The lack of cell division in normal adult articular cartilage suggests that the chondrocytes present in the cartilage of an older adult are likely to be the same cells that were present decades earlier. This fact makes these cells more susceptible to the accumulation of changes from both aging and extrinsic stress. In fact, it is most likely that chondrocyte senescence is the extrinsic type, induced by different stressors. The telomere shortening in adult chondrocytes could be due to DNA damage from ROS as discussed further above (Mankin, 1963; Martin and Buckwalter, 2001b; Martin et al., 2004b). The increased ROS levels could be both age-related (Del Carlo and Loeser, 2003) and generated from excessive mechanical loading and/or stimulation by cytokines (Kurz et al., 2005; Davies et al., 2008). There is also evidence for reduced levels of antioxidant enzymes in cartilage with aging and in OA that would contribute to chondrocyte senescence and oxidative damage. In human articular chondrocytes, decreased levels of mitochondrial superoxide dismutase were found both with aging and in OA cells (Finkel and Holbrook, 2000). Moreover, it has been hypothesized that joint injury accelerates chondrocyte senescence and that this acceleration plays a role in the joint degeneration responsible for post-traumatic OA. Indeed, excessive loading of articular surfaces due to acute joint trauma or post-traumatic joint instability, incongruity or mal-alignment increases release of ROS, and the increased oxidative stress on chondrocytes accelerates chondrocyte senescence (James et al., 2004). Other important features of chondrocyte senescence are the exhibition of SSP, which has important implications in development and progression of OA and the decline in the proliferative



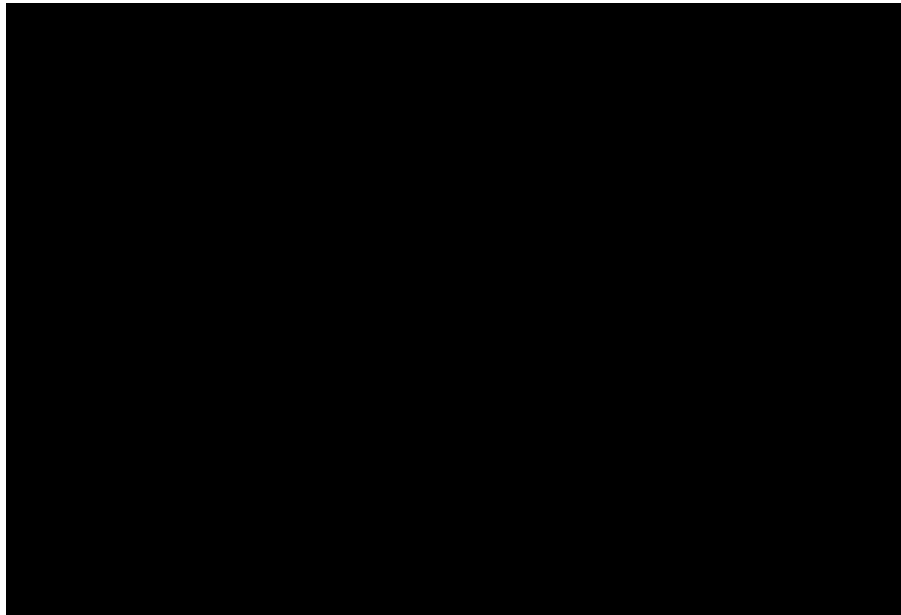
and anabolic response to growth factors, as well as their reduction in cartilage. It has been noted that senescent chondrocytes lose the ability in response to: IGF-I, which is known to be an important autocrine survival factor that stimulates cartilage matrix synthesis (Martin et al., 1997); TGF- $\beta$ , an important cartilage anabolic factor (Scharstuhl et al., 2002) and to bone morphogenetic protein 6 (BMP-6), known to stimulate proteoglycan synthesis (Bobacz et al., 2003). Chondrocyte senescence also contributes to the decline in the cell number within the cartilaginous tissue, due to increased cell death. Several studies demonstrated the loss of cellular density in cartilage with aging or/and in OA (Vignon et al., 1976; Adams and Horton, 1998; Horton et al., 1998; Aigner et al., 2004a; Kuhn et al., 2004). These findings provide evidence to support the concept that chondrocyte senescence may be involved in the progression of cartilage degeneration, because of their decreased ability to maintain or restore the articular cartilage (Fig. 5).

## 7. Chondrocyte senescence markers

An altered gene expression pattern on the cellular level appears to be one potentially important facet of chondrocyte behavior in OA cartilage (Aigner et al., 2004b; Aigner et al., 2007). The diversification of gene expression in senescent chondrocytes is due to stochastic DNA damage, which represents a core mechanism in cellular aging in general and in OA cartilage degeneration in particular. The evidence of senescence in chondrocytes can be investigated using several senescence markers, such as senescent-associated- $\beta$ -galactosidase (SA- $\beta$ gal), highly condensed domains of facultative heterochromatin SAHF, increased p53, p21, pRb and p16<sup>INK4a</sup> (Fig. 5). Staining for SA- $\beta$ gal has been shown to be present in articular chondrocytes from older adults and in OA chondrocytes (Martin and Buckwalter, 2001b; Price et al., 2002). SA- $\beta$ gal is related to the detection of increased levels of the lysosomal enzyme  $\beta$ -galactosidase at pH 6.0 rather than at the normal pH 4.5. Detection of its activity at pH 6.0 is thought to be due to an increase in lysosomal mass (Itahana et al., 2007). Chondrocyte SA- $\beta$ gal staining, as well as telomere shortening, has also been noted after treatment in vitro with IL-1 $\beta$  or H<sub>2</sub>O<sub>2</sub> consistent with stress-induced senescence (Dai et al., 2006). Although SA- $\beta$ gal is a useful senescence marker, its activity is critically dependent on the detection conditions, and SA- $\beta$ gal is also expressed in the non-senescent cells that have a high lysosomal content (Kurz et al., 2000; Matthews et al., 2006). Multiple markers of senescence are therefore recommended to demonstrate senescence in vivo. SAHF are thought to repress expression of proliferation-promoting genes, thereby contributing to senescence-associated proliferation arrest. Inclusion of proliferation-promoting genes, such as cyclin A, into these compact chromatin foci is thought to silence expression of those genes, which are associated with cell cycle arrest (Adams, 2007). *Ink4a* encodes an archetypical cyclin-dependent inhibitor (CKI) associated

with senescence. Indeed, the over-expression of p16<sup>INK4a</sup> in chondrocytes is associated with SSP, which includes increased production of pro-inflammatory cytokines (such as IL-6, IL-8, IL-1 $\beta$ ) and matrix remodeling regulatory metalloproteinases (such as MMP1, MMP13, etc) (Leonardi et al., 2008; Loreto et al., 2013). As mentioned above, all these factors are deleterious for cartilage integrity. According to this finding, the repressed levels of miR-24, a negative regulator of p16<sup>INK4a</sup>, was also found in OA cartilage (Philipot et al., 2014). Recently, the expression of Caveolin1, a protein that participates in premature cellular senescence, was also investigated in human OA cartilage. It was observed that the treatments with catabolic factors of oxidative stress (H<sub>2</sub>O<sub>2</sub>) and IL-1 $\beta$ , which simulate the OA environment, was able to up-regulate the expression of caveolin1. The over-expression of caveolin1 is associated with cartilage degeneration and the mediation of the premature senescence in OA chondrocytes by activating p38 MAPK, which impair the ability of chondrocytes to produce type II collagen and aggrecan (Dai et al., 2006). Other important senescence markers are represented by telomere length and telomerase activity. As discussed in detail above, telomere shortening is the most representative feature of cellular aging, and it is due to the decreased expression of telomerase with time which leads to telomere instability. Telomere length can be measured by using the Single Telomere Length Assay (STELA), Southern blot analysis, Q-PCR and the more recent Quantitative-Peptide Nucleic Acid-Fluorescent *in situ* Hybridization assay (Q-PNA-FISH) (Cukusic et al., 2014). Telomerase activity can be measured for example by using a Telomere Repeat Amplification Protocol (TRAP) (Zhou and Xing, 2012) (Table 1). Recently, several studies have been focused on the expression of lubricin, also known as proteoglycan 4 (PRG4) or superficial zone protein (SZP), in different experimental conditions, in particular in conjunction with physical activity (Musumeci et al., 2013d). Lubricin is a chondroprotective glycoprotein that serves as a critical boundary lubricant between opposing cartilage surfaces (Musumeci et al., 2013e; Leonardi et al., 2011). It has a major protective role in preventing cartilage wear and synovial cell adhesion, proliferation, and in reducing the coefficient of friction of the articular cartilage surface (Musumeci et al., 2013f; Leonardi et al., 2012a,b). Since the lubricin has a fundamental role in maintaining the homeostasis of the articular cartilage and in preventing its degeneration, we hypothesized that its expression would decrease in senescent chondrocytes and that it could be evaluated as a new specific chondrocyte senescence marker as confirmed in our recent and interesting study (Musumeci et al., 2014d).

**Table 1.** An overview of the key markers for the senescent chondrocytes and their functions.



## 8. Conclusions

Although the direct relationship between the aging process and the development of OA is not completely understood, we may surmise that chondrocyte senescence contributes to cartilage degeneration by impairing the ability of these cells to maintain and repair the cartilage tissue. Moreover, we have also seen that these two processes (aging and OA) could be inter-dependent. There are several lines of evidence that suggest chondrocytes exposed to the “osteoarthritic environment” are characterized by oxidative stress and production of cytokines, and this induces the so-called stress-induced senescent state, which may contribute to cartilage degeneration as we have discussed above. All these observations suggest that a better understanding of the changes arising with age in articular cartilage and how they influence the response of the tissue to different stressors, as well as the identification of new increasingly sensitive senescence markers, would be very useful in the preventive detection of the disease and in its consequent treatment. Further research is required to unravel the detailed mechanisms of senescence related to the pathogenesis of OA. Strategies aimed at preventing chondrocyte senescence could have a positive impact on the development of new therapies for OA and on halting the progression of this severe disease.

**Conflict of Interest.** The authors have no conflict of interest.

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## CHAPTER III

## **Physical activity ameliorates cartilage degeneration in a rat model of aging: A study on lubricin expression**

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## 1. Abstract

Osteoarthritis (OA) is a common musculoskeletal disorder characterized by slow progression and joint tissue degeneration. Aging is one of the most prominent risk factors for the development and progression of OA. OA is not, however, an inevitable consequence of aging and age-related changes in the joint can be distinguished from those that are the result of joint injury or inflammatory disease. The question that remains is whether OA can be prevented by undertaking regular physical activity. Would moderate physical activity in the elderly cartilage (and lubricin expression) comparable to a sedentary healthy adult? In this study we used physical exercise in healthy young, adult, and aged rats to evaluate the expression of lubricin as a novel biomarker of chondrocyte senescence. Immunohistochemistry and western blotting were used to evaluate the expression of lubricin in articular cartilage, while enzyme-linked immunosorbent assay was used to quantify lubricin in synovial fluid. Morphological evaluation was done by histology to monitor possible tissue alterations. Our data suggest that moderate physical activity and normal mechanical joint loading in elderly rats improve tribology and lubricative properties of articular cartilage, promoting lubricin synthesis and its elevation in synovial fluid, thus preventing cartilage

**Keywords:** Articular cartilage, chondrocyte, senescence, physical exercise, joint degeneration, lubricin, osteoarthritis.degradation compared with unexercised adult rats.

## 2. Introduction

Osteoarthritis (OA) is an extremely common musculoskeletal disorder characterized by slow, progressive, and degenerative changes in load-bearing synovial joints, most commonly in hand, hip, and knee joints (Loeser, 2010, 2011).

Age is classified as a prominent risk factor for OA disease, which is the most common cause of chronic disability in older adults. It is believed that chondrocyte senescence plays a major role in the development and progression of OA in aging cartilage, since accumulation of senescent cells impairs the intrinsic ability of the tissue to maintain and repair the extracellular matrix (ECM) of cartilage, resulting in a mechanically compromised tissue (Loeser, 2009). However, OA is not an inevitable consequence of aging and aging-related changes in the joint are different from those due to injury or inflammatory disease (Loeser, 2009).

Evidence of cell senescence in tissues from older adults can be obtained by examining for the presence of cellular senescence markers (Loeser, 2009). These markers currently include

histological staining for senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal), senescence-associated heterochromatin (SA heterochromatin), increased p53 (p53), cyclin-dependent kinase inhibitor 1 (p21), and cyclin-dependent kinase inhibitor 2A (p16) and reduced Wnt2 or Irp (int-related protein) (Campisi & d'Adda di Fagagna, 2007). In this study we examined whether the expression of lubricin, also known as proteoglycan 4 (PRG4) or superficial zone protein (SZP), could be used as a marker of chondrocyte senescence and/or OA. Lubricin is a chondroprotective glycoprotein that serves as a critical boundary lubricant between opposing cartilage surfaces (Leonardi et al., 2012a). It has a major protective role in preventing cartilage wear and synovial cell adhesion, proliferation, and in reducing the coefficient of friction of the articular cartilage surface (Leonardi et al., 2012b, c). Several recent studies found that treatment with recombinant lubricin could protect articular cartilage and prevent the process of OA in animal models (Teeple et al., 2011). Lubricin is also upregulated by mechanical compression and shear under homeostatic conditions in cartilage explant cultures and in whole joints (Nugent-Derfus et al., 2007). Specific stimuli that mimic the kinematics of natural joints, in particular surface motion obtained by bioreactor, may promote the upregulation of lubricin gene expression (Grad et al., 2005). Moreover, dynamic shear stimulation increased lubricin secretion and also the total number of cells expressing lubricin (Nugent et al., 2006). Physical activity increases lubricin expression and reduces inflammation in articular cartilage damaged by glucocorticoids (Musumeci et al., 2013a). Moreover, a healthy diet, olive oil-based, in conjunction with physical activity, improves the expression of lubricin in the articular cartilage surface, preventing OA (Musumeci et al., 2013b). Furthermore, physical activity increases energy metabolism, contributes to increase bone and skeletal muscle mass, strengthens the joints and can be useful in controlling pain, and improves cardiorespiratory efficiency by promoting psychological well-being (Kragstrup et al., 2011; Pichler et al., 2013). However, immobility, age, malnutrition, and other disabilities can interfere with optimal physical activity for patients with problems (Cordeiro et al., 2014). We studied the expression of lubricin in groups of animals at different ages, with and without physical activity. The overall objective of this work was to subject healthy young, adult, and aged rats to a protocol of physical exercise, adapted to various age groups, for a period of at least 8 weeks to evaluate the expression of lubricin as a new chondrocyte senescence marker. The fundamental questions that we posed at the inception of this study were as follows: could an elderly person undertake regular physical activity that can protect cartilage (by increasing lubricin expression)? Can we use rats as a model to examine the potential benefits of moderate physical activity on articular cartilage, using lubricin as a marker? Thus, the hypothesis that we tested

in this study was whether physical activity can modify the natural history of joint degeneration, promoting enhanced lubrication in adult joints, preventing the occurrence of OA.

### 3. Materials and methods

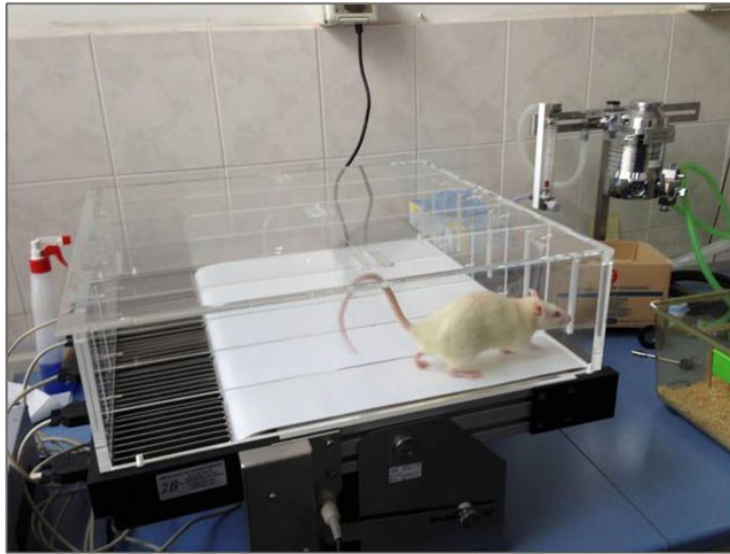
#### 3.1. Animals

Thirty male albino Wistar rats (Charles River Laboratories, Milan, Italy), five rats for each group, with different body weights and ages, were divided in six groups: (a) young rats, 3 months old, average body weight of  $160 \pm 20$  g, common diet, sedentary; (b) young rats, 3 months old, average body weight of  $160 \pm 20$  g, common diet, subject to physical activity; (c) adult rats, 12 months old, average body weight of  $200 \pm 20$  g, common diet, sedentary; (d) adult rats, 12 months old, average body weight of  $200 \pm 20$  g, common diet, subject to physical activity; (e) older rats, 24 months of age, average body weight of  $240 \pm 20$  g, common diet, sedentary; (f) older rats, 24 months of age, average body weight of  $240 \pm 20$  g, common diet, subject to physical activity. Rats were individually housed in polycarbonate cages (cage dimensions: 10.25"W  $\times$  18.75"D  $\times$  8"H) during the entire stabling period of the study and were housed at controlled temperature (20–23 °C) with a relative humidity of 40–60%, with free access to water and food and photoperiod of 12 h light and dark. Antibiotic, analgesic, muscle relaxant, and non-steroidal anti-inflammatory drugs (NSAID) therapy were not administered so as not to alter the results as previously described (Musumeci et al., 2013b). All procedures conformed to the guidelines of the Animal Care and Use Committee of the University of Catania, in accordance with the European Community Council Directive (86/609/EEC) and Italian Animal Protection Law (116/1992).

#### 3.2. Experimental design

Common diet: rats were fed *ad libitum*, with standard rat chow containing carbohydrates (40%), proteins containing all essential amino acids (30%), and lipids (30%). Lipids were a mixture of neutral fatty acids, saturated fatty acids, and unsaturated fatty acids. Diets were prepared by Laboratorio Dottori Piccioni, Gessate (Milan), Italy, and were administered for the entire period of the experiment. Groups 1, 3, and 5 rats were left free without immobilization in their cages. Groups 2, 4, and 6 rats performed physical activity for 8 weeks. For the first 2 weeks, rats were subjected to exercise on treadmill (Fig. 1) (2Biological instrument, Varese, Italy), at a speed of 10–20 m/min (type of exercise: moderate interval training, depending on the characteristics and on the age of the rats), without any inclination, 5 days a week for 10 min daily. During the next 6 weeks,

the same groups (2, 4, and 6) were subjected to treadmill exercise (2Biological instrument), 5 days a week for 20 min daily. The treadmill inclined at 3° (between 2 and 6 degrees) set at a speed of 10–30 m/min (type of exercise: interval training, between moderate and strong depending on the characteristics and on the age of the rats). A minimal electric shock (0.2 mA) forced the rat to walk on the treadmill at a speed and inclination adapted to it. The shock serves to stimulate the rat to walk and to instruct it. Usually the rat learns this activity in the first 2 min of the exercise. This type of exercise is used to stimulate the muscles, joints, and bones in the work of flexion-extension of the limbs. All electric shock data were acquired by data acquisition software (2Biological instrument). Before and after exercise, rats were left free without immobilization in their cages. During the exercise, the possible suffering of the animal was evaluated. The evaluated parameters were based on the clinical aspect of the animals (lucidity of the fur, presence of periocular secretion, weight, food and water consumption). These parameters were monitored at least once a day. Potential rats discarded from the trial: rats that showed a progressive loss of 10% of their total body weight within 3 days from the start of weight loss; rats that exhibited difficulties in walking; rats that exceeded the number of five electric shocks (0.2 mA) without learning the work to be done on the treadmill. None of the rats presented any of these characteristics and thus no animals were excluded from the study. The day following the last training (after 8 weeks of experiment), the animals were humanely sacrificed by an intravenous lethal injection of anesthetic overdose using a mixture of Zoletil 100 (Virbac, Milan, Italy) at a dose of 80 mg/kg and DEXDOMITOR (Virbac) at a dose of 50 mg/kg. After euthanasia, both femurs were explanted, cleaned of soft tissues. Articular cartilage samples from distal epiphysis of the femur (condyles) were evaluated by histochemical, immunohistochemical, and western blot techniques; synovial fluid was used to quantify lubricin levels by enzyme-linked immunosorbent assay (ELISA).



**Figure 1.** Rat subjected to physical exercise on a treadmill. Performance of rats subjected to mild exercise on treadmill (2Biological instrument, Varese, Italy) at a speed of 10 m/min without any inclination.

### 3.3. Histochemistry

Samples from distal epiphysis of the femur (condyles) were rinsed in phosphate buffered saline (PBS) and fixed in 10% buffered formalin. After an overnight wash, specimens were dehydrated in a graded series of ethanol baths, cleared in xylene and paraffin-embedded, preserving their orientation and anatomical ultrastructure. Sections (4–5- $\mu\text{m}$  in thickness) were cut from paraffin blocks using a microtome, mounted on saline-coated slides, and stored at room temperature. The sections were stained with Alcian Blue pH 2.5 periodic acid Schiff (PAS) to assess the synthesis of sulfated glycosaminoglycans as constituents of proteoglycans (the assessment was made on the intensity of staining). The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photomicrographs were captured using a digital camera (AxioCam MRc5; Carl Zeiss).

### 3.4. Immunohistochemistry

For immunohistochemical analysis, cartilage tissue from distal epiphysis of the femur (condyles) was processed as previously described (Musumeci et al., 2013c). Briefly, the slides were dewaxed in xylene, hydrated using a graded series of ethanol baths, and incubated for 30 min in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to quench endogenous peroxidase activity before being rinsed for 20

min with PBS (Bio-Optica, Milan, Italy). The sections were heated (5 min × 3) in capped polypropylene slide holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica), using a microwave oven (750 W) to unmask antigenic sites as previously described (Musumeci et al., 2013e). The protein-blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA; Sigma, Milan, Italy) in PBS for 1 h in a humidified chamber. BSA was used as a blocking agent to prevent non-specific binding of the primary and secondary antibodies to the tissue sections. Following blocking, the sections were incubated overnight at 4 °C with rabbit polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma) overnight at 4 °C. Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-rabbit was used as secondary antibody) and then detected with peroxidase-labeled streptavidin, both incubated for 10 min at room temperature (LSAB+System-HRP, K0690: Dako, Glostrup, Denmark). Immunoreactivity was visualized by incubating the sections for 2 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in glycerol vinyl alcohol (Zymed Laboratories, San Francisco, California, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5; Carl Zeiss).

### 3.5. Evaluation of immunohistochemistry

The lubricin-staining status was identified as either negative or positive. Positive immunohistochemical staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described (Musumeci et al., 2013d). Staining intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0–4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of lubricin immunopositive cells [ES (extent score)] was independently evaluated by three investigators (two anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: < 5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and > 75% (++++). Counting was performed at ×200 magnification. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at the protein



level. Positive controls consisted of tissue specimens with known antigenic positivity. Sections treated with PBS without the primary antibody served as negative controls.

### **3.6. Computerized morphometric measurements and image analysis**

Fifteen fields from articular cartilage (femoral condyles) randomly selected from each section were analyzed; and the percentage area stained with lubricin antibody was calculated using image analysis software (AxioVision Release 4.8.2 – SP2 Software; Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabeling in each field. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, using objective lens of magnification  $\times 20$ , i.e., total magnification  $\times 400$ ) fitted with a digital camera (AxioCam MRc5; Carl Zeiss); evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement.

### **3.7. Biochemical studies**

Joints were washed by injecting 100  $\mu\text{L}$  of normal saline in the joint capsule, then aspirating 20  $\mu\text{L}$  of synovial fluid. Concentrations of lubricin in the synovial fluid were measured using a commercially available ELISA kit (Pierce Biotechnology, Rockford, Illinois, USA) as previously described (Ludwig et al., 2012) according to the manufacturer's instructions for the quantitative determination of lubricin. The experiments were repeated in triplicate.

### **3.8. Western blot analysis**

Crude extracts were prepared by homogenizing cartilage tissue in a buffer containing 20 mM Tris (pH 7.4), 2 mM ethylenediaminetetraacetic acid, 0.5 mM ethylene glycol tetraacetic acid, 50 mM mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana, USA) using a Teflon glass homogenizer and then sonicated. Protein concentrations were determined using the Bradford protein assay (Bradford, 1976), using BSA as a standard. Sample proteins (50  $\mu\text{g}$ ) were diluted in 4 $\times$  sodium dodecyl sulphate (SDS) protein gel loading solution (Invitrogen, Monza, Italy), boiled for 5 min, separated on 4–12% Bis-tris gel (Invitrogen), and electroblotted to nitrocellulose membrane (Invitrogen). Nonspecific binding was

blocked for 2 h at 37 °C with 10% nonfat dry milk in Tween-Tris buffered saline (TTBS; Invitrogen). Membranes (Invitrogen), were incubated overnight at 4 °C with the following antibodies: rabbit polyclonal anti-lubricin antibody (ab28484; Abcam) diluted 1:1000, molecular weight 280 kDa, and rabbit anti- $\beta$ -tubulin (Santa Cruz Biotechnology, California, USA) diluted 1:200, molecular weight 48 kDa, which was used as loading control. HRP-conjugated anti-rabbit secondary antibody (ECL Western Blotting System; GE Healthcare, Milan, Italy) were diluted at 1:10 000 for  $\beta$ -tubulin and 1:7500 for lubricin. All antibodies were diluted in 10% nonfat dry milk solution in TTBS. Blots were developed using the enhanced chemiluminescence technique (GE Healthcare) and relative band densities were quantified using ImageQuantTL 7.0 software (GE Healthcare). No signal was detected when the primary antibody was omitted (data not shown). The experiments were repeated in triplicate.

### 3.9. Statistical analysis

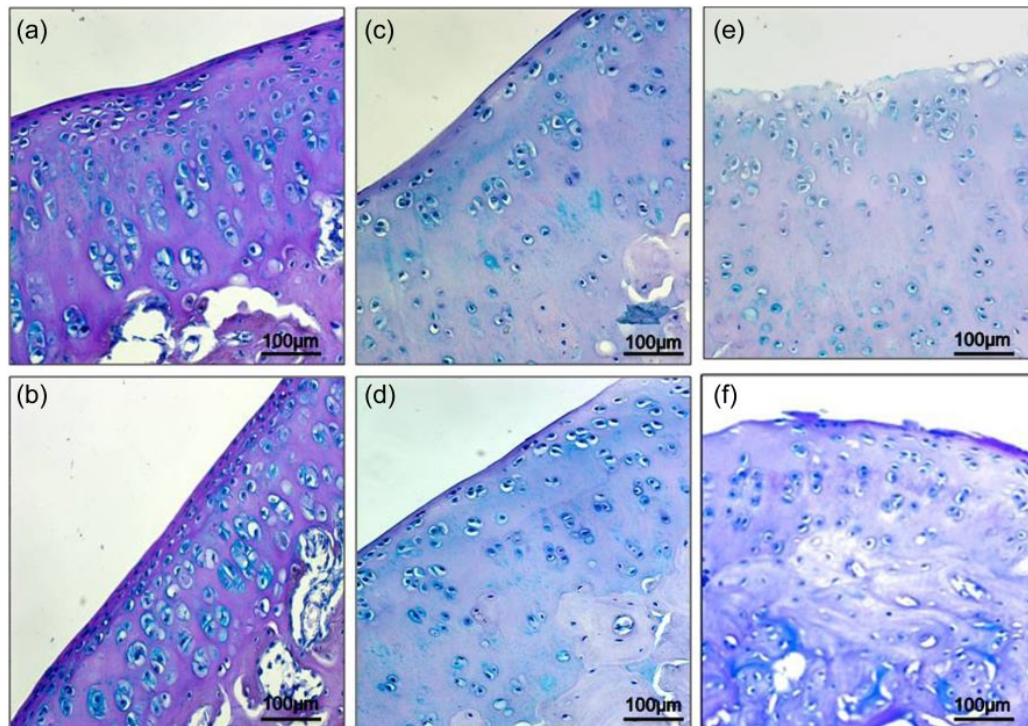
Statistical analysis was performed using SPSS software (SPSS® release 16.0, Chicago, Illinois, USA). Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's *t*-test, while comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. *P*-values of less than 0.05 were considered statistically significant; *P*-values of less than 0.01 were considered highly statistically significant. Data are presented as the mean  $\pm$  standard error of the mean (SEM). Cohen's kappa was applied to measure the agreement between the three observers and averaged to evaluate overall agreement as previously described (Musumeci et al., 2013c,d).

## 4. Results

### 4.1. Histochemistry

Histochemical staining (Alcian Blue pH 2.5 PAS) demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from the first four groups: (1) young sedentary rats; (2) young rats subject to physical activity; (3) adult sedentary rats; and (4) adult rats subject to physical activity. Clones of chondrocytes were evident. The group 5 (older sedentary rats) demonstrated slight structural alteration with fragmentation and clefts in cartilage tissue, reminiscent of an early stage of OA, and only rare clones of chondrocytes were evident. Conversely, group 6

(older rats subject to physical activity) demonstrated absence of structural alterations and clones of chondrocytes were again apparent. The chondrocytes from groups 1 and 2 (Fig. 2(a,b)) did not show any sign of cellular degeneration demonstrated by an intense staining, while the chondrocytes from groups 3 and 4 (Fig. 2(c,d)) showed early signs of cellular degeneration demonstrated by a moderate staining. The chondrocytes from group 5 (Fig. 2(e)) showed clear signs of cellular degeneration demonstrated by reduced/weak staining, although the chondrocytes from group 6 (Fig. 2(f)) showed a moderate staining.



**Figure 2.** Histochemical staining. (a–f) Histochemical staining (Alcian Blue pH 2.5 periodic acid Schiff) demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from the first four groups. Cartilage tissue samples from group 5 demonstrated slight structural alterations, including fragmentation and clefts in cartilage tissue, comparable to changes seen in the early stages of osteoarthritis, while group 6 demonstrated absence of structural alterations, due to recovery and improvement after physical activity. (a,b) The chondrocytes from groups 1 and 2 did not show any sign of cellular degeneration demonstrated by intense Alcian Blue staining. (c,d,f) The chondrocytes from groups 3, 4, and 6 showed early signs of cellular degeneration as demonstrated by moderate Alcian Blue staining. (e) The chondrocytes from group 5 showed clear signs of cellular degeneration demonstrated by reduced/weak Alcian Blue staining.

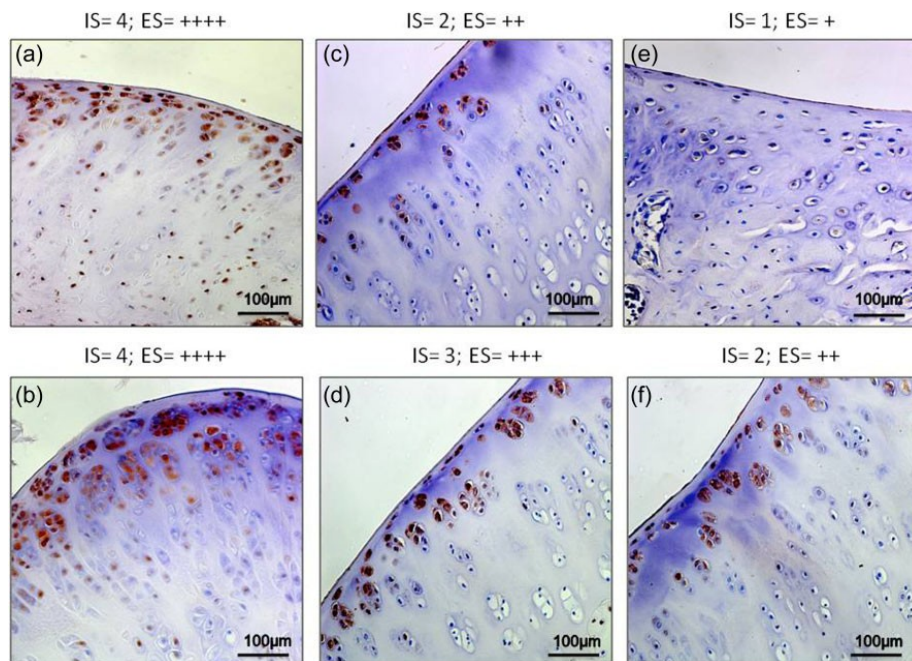
## 4.2. Observations

Lubricin immunohistochemical staining in explanted tissue was observed in the ECM of cells of all groups. Articular cartilage displayed a layer of lubricin on the surface of tissues (superficial zone). However, we observed different patterns of immunopositive cells in the specimens examined. Expression of lubricin was investigated via immunohistochemistry, in vivo explanted tissue (Table 1). A very strong lubricin staining (IS = 4; ES = ++++) was observed in groups 1 and 2, where almost all chondrocytes were immunolabeled in the superficial zone (Fig. 3(a,b)). On the other hand, a moderate lubricin immunoreactivity (IS = 2; ES = ++) was noted in group 3, where less than half of the chondrocytes were immunostained in the superficial zone (Fig. 3(c)). Furthermore, a strong lubricin immunoreactivity (IS = 3; ES = +++) was seen in group 4, where more than half of the chondrocytes were immunostained in the superficial zone (Fig. 3(d)). In the other groups 5 and 6, the expression of lubricin significantly decreased compared with groups 1 and 2. In fact, lubricin expression in chondrocytes was weak or absent in group 5 (IS = 1; ES = +), where a few chondrocytes were immunostained in the superficial zone (Fig. 3(e)), while in group 6 the lubricin expression increased comparable to group 3, indeed the expression was moderate (IS = 2; ES = ++) where less than half of the chondrocytes were immunostained in the superficial zone (Fig. 3(f)). No immunoreactivity was observed in the negative control (ES = 0; IS = 0) treated with PBS without the primary antibodies (Fig. 4(a)). The percentage of lubricin-positive cells was observed among groups. Both ES and IS were significantly greater ( $P < 0.05$ ). Statistical analysis showed that the percentage of lubricin-positive cells was statistically significant in groups 3, 4, and 6 ( $P < 0.05$ ), while in group 5 was highly statistically significant ( $P < 0.01$ ), equated to groups 1 and 2, (Fig. 4(b)). Interobserver agreement, measured as Kappa coefficient, was 0.96 (almost perfect).

**Table 1.** Evaluation of lubricin immunostaining.

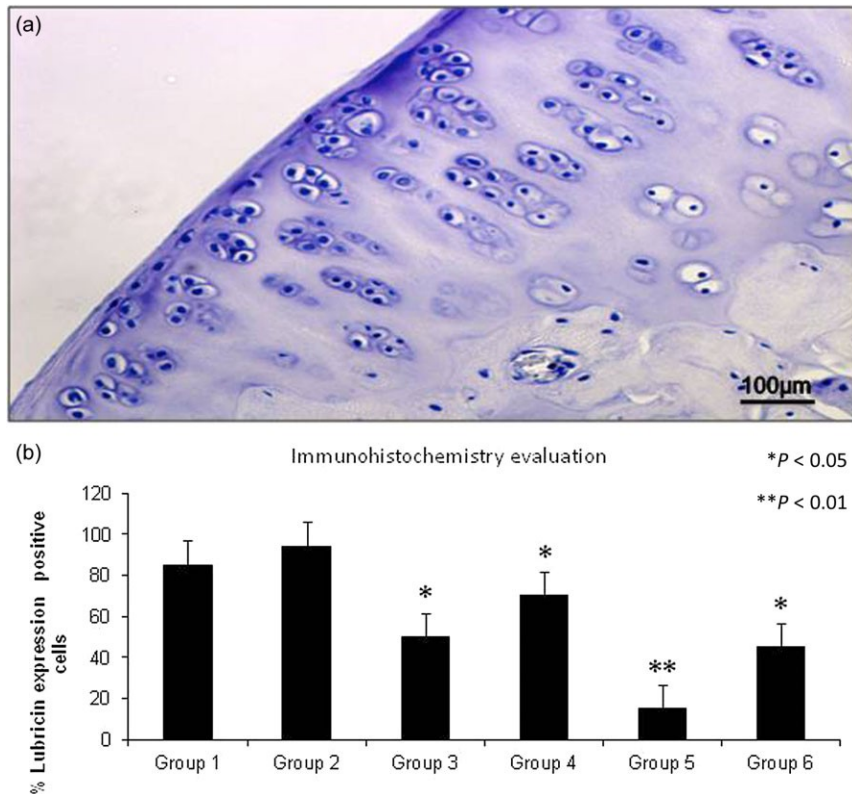
Groups (five rats for each group)	Intensity of lubricin immunostaining (IS)	Percentage of lubricin immunopositive cells (ES)
1) Young sedentary rats	Very strong lubricin immunostaining (IS = 4)	Very strong lubricin immunostaining (ES = ++++)
2) Young rats subject to physical activity	Very strong lubricin immunostaining (IS = 4)	Very strong lubricin immunostaining (ES = ++++)
3) Adult sedentary rats	Moderate lubricin immunostaining (IS = 2)	Moderate lubricin immunostaining (ES = ++)
4) Adult rats subject to physical activity	Strong lubricin immunostaining (IS = 3)	Strong lubricin immunostaining (ES = +++)
5) Older sedentary rats	Weak lubricin immunostaining (IS = 1)	Weak lubricin immunostaining (ES = +)
6) Older rats subject to physical activity	Moderate lubricin immunostaining (IS = 2)	Moderate lubricin immunostaining (ES = ++)

Intensity of staining (IS) was graded on a scale of 0–4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of lubricin immunopositive cells [ES (extent score)] was independently evaluated by three investigators (two anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and >75% (++++).



**Figure 3.** Immunohistochemical staining. Lubricin immunostaining in cartilage explants was visualized throughout the extracellular matrix in samples from all groups: (a) young sedentary rats; (b) young rats subject to physical activity; (c) adult sedentary rats; (d) adult rats subject to physical activity; (e) older sedentary rats; (f) older rats subject to physical activity. Panels a–f: magnification  $\times 20$ ; scale bars 100  $\mu\text{m}$ . (a,b) A very strong lubricin staining [intensity of staining (IS) = 4; extent score (ES) = ++++] was observed in groups 1 and 2, where almost all chondrocytes displayed immunolabeling

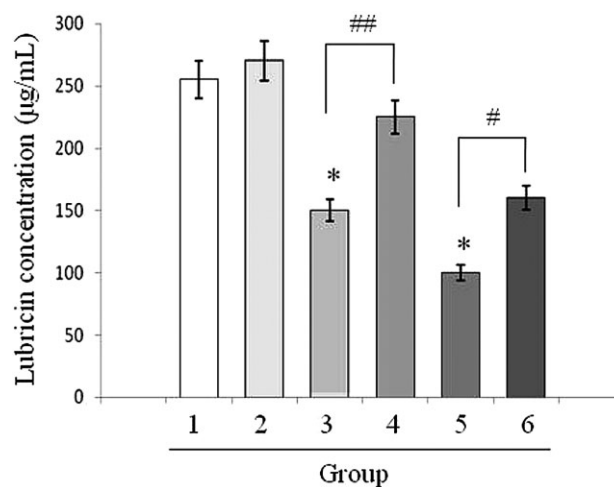
in the superficial zone. (c) Moderate lubricin immunoreactivity (IS = 2; ES = ++) was noted in group 3, where less than half of the chondrocytes were immunostained in the superficial zone. (d) Strong lubricin immunoreactivity (IS = 3; ES = +++) was detected in group 4, where more than half of the chondrocytes exhibited immunostaining in the superficial zone. (e) Weak lubricin immunoreactivity (IS = 1; ES = +) was observed in group 5, where a few chondrocytes were immunostained in the superficial zone. (f) Moderate lubricin immunoreactivity (IS = 2; ES = ++) was detected in group 6, where less than half of the chondrocytes were immunostained in the superficial zone.



**Figure 4.** Immunohistochemical evaluation. (a) No immunoreactivity was observed in negative controls [extent score (ES) = 0; intensity of staining (IS) = 0] treated with phosphate buffered saline in the absence of the primary antibody. Magnification  $\times 20$ ; scale bars 100  $\mu\text{m}$ . (b) Immunohistochemical evaluation. The percentage of lubricin-positive cells was observed among groups. Both ES and IS were significantly greater ( $P < 0.05$ ). Statistical analysis showed that the percentage of lubricin-positive cells was statistically significant in groups 3, 4, and 6 ( $P < 0.05$ ), while in group 5 was highly statistically significant ( $P < 0.01$ ), equated to groups 1 and 2. Interobserver agreement, measured as Kappa coefficient, was 0.96 (almost perfect). Results were presented as the mean  $\pm$  standard error of the mean. Analysis of variance was used to evaluate the significance of the results. \*  $P < 0.05$  and \*\*  $P < 0.01$ , when compared with group 1.

### 4.3. Biochemical analysis

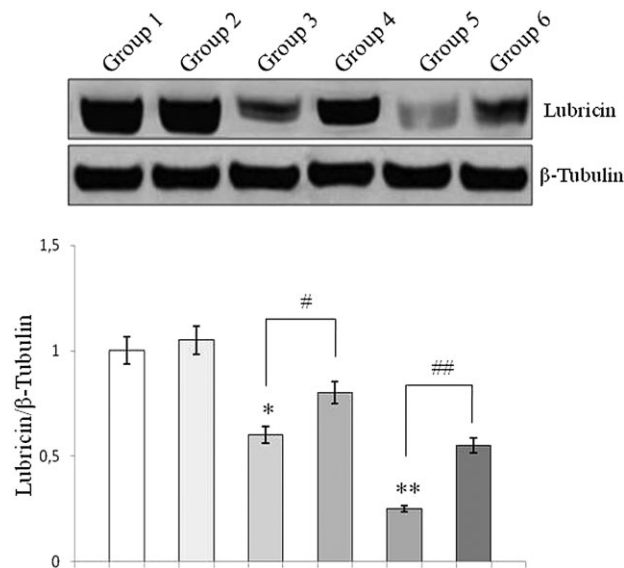
In order to investigate the effects of physical activity on lubricin levels in the synovial fluid of aging rats, we employed the ELISA method. Data obtained from these analyses confirmed that lubricin levels in rat synovial fluid were strikingly decreased with aging (Fig. 5). As indicated, group 1 showed higher lubricin levels ( $255 \pm 13$ ) when compared with groups 3 ( $150 \pm 7.5$ ) and 5 ( $100 \pm 5$ ). Interestingly, physical activity provoked a relevant enhancement of lubricin concentration as compared with synovial fluid of untrained rats. Such an increase, although less evident in group 2, reaches significant values both in group 4 ( $##P < 0.05$  vs group 3) and group 6 ( $#P < 0.5$  vs group 5).



**Figure 5.** Enzyme-linked immunosorbent assay (ELISA). Lubricin levels in the synovial fluid from all groups of rats were determined by ELISA. Lubricin levels significantly decreased in groups 3 and 5 ( $*P < 0.05$ ) in comparison to group 1. Lubricin levels significantly increased in groups 4 ( $##P < 0.01$ ) and 6 ( $#P < 0.05$ ) rats in comparison to group 3 and 5, respectively. Results are presented as the mean  $\pm$  standard error of the mean. Analysis of variance test was used to evaluate the significance of the results.  $*P < 0.05$  vs group 1;  $#P < 0.05$  vs group 5;  $##P < 0.01$  vs group 3.

### 4.4. Western blot analysis

To evaluate whether physical activity affects lubricin protein expression in cartilage of aging rats, we also performed western blot analysis. The representative western blot bands displayed in Fig. 6 show a high degree of correlation with the ELISA data from synovial fluid. In fact, lubricin protein expression was the strongest in group 1, while in groups 3 and 5 it was the weakest ( $*P < 0.01$ ). In groups 2, 4, and 6 (trained rats), the expression of lubricin increased in comparison to group 1, 3, and 5 (untrained rats), respectively, although significant values were reached only in groups 4 ( $#P < 0.5$  vs group 3) and 6 ( $##P < 0.05$  vs group 5).



**Figure 6.** Western blot analysis. Lubricin protein expression in cartilage from all groups was determined by western blot analysis. In groups 3 and 5, the expression of lubricin decreased compared with group 1. In groups 4 and 6, lubricin expression significantly increased when compared with groups 3 and 5, respectively. Relative band optical densities were quantified using ImageQuantTL software and normalized values were plotted in the histogram. \*P < 0.05 or \*\*P < 0.01 vs group 1; #P < 0.05 vs control group 3 or ###P < 0.01 vs group 5. B-Tubulin was used as loading control in each experiment.

## 5. Discussion

Cell senescence may be a mechanism to prevent the replication of cells with damaged DNA and thus tumor formation. OA is probably not a direct consequence of aging, but rather, aging affects the ability of the articular cartilage to maintain homeostasis when stressed. Age-related oxidative stress and damage may play a central role in cartilage aging through modulation of cell signaling pathways that regulate anabolic and catabolic activity.

Lubricin is a boundary lubricant, present in synovial fluid and on the superficial layer of articular cartilage, producing low friction and protecting surfaces from wear (Musumeci et al., 2013a; Chang et al., 2014). This protein is expressed less with aging and during OA (Musumeci et al., 2013b; Waller et al., 2013). Compromising boundary lubrication will cause increased friction, load amplification, and ultimately, greater cartilage damage. Lubricin is involved in several joint diseases and may play a beneficial role against the degradation of articular cartilage (Elsaid et al., 2012; Musumeci et al., 2013c). Lubricin has been noted to be sensitive to mechanical loading, as indeed running induces maximal expression of lubricin in the superficial zone of knee joint articular (Ogawa et al., 2014).

The impact of exercise on cartilage health depends on the extent of injury, the method, and the intensity of exercise (Musumeci et al., 2014a). While a running wheel exercise protocol



protects articular cartilage and stimulates cartilage matrix synthesis in an uninjured animal model (Otterness et al., 1998), a forced joint exercise regimen accelerates cartilage degeneration in a rat model (Appelton et al., 2008). In another study, strenuous joint exercise has resulted in cartilage degeneration similar to early OA changes in normal rats (Pap et al., 1998), while an exercise regimen following anterior cruciate ligament transection provided a protective effect on articular cartilage (Galois et al., 2004), as also enhanced by an olive oil-based diet (Musumeci et al., 2013b). In recent studies authors confirmed the beneficial effect of the physical activity in bone (Pichler et al., 2013) and cartilage (Musumeci et al., 2013a) glucocorticoid-induced diseases.

Published data in the literature have showed a link between physical activity and inflammatory biomarkers, although increasing physical activity could be effective for reducing chronic inflammation, especially in individuals with chronic diseases associated with a state of elevated inflammation (Beavers et al., 2010). In other recent and interesting studies our group demonstrated the effect of the combination of extra virgin olive oil diet and physical activity in skeletal muscle, preventing the oxidative stress from exhaustive exercise in muscles (Musumeci et al., 2014b). Our present study confirms the beneficial effects of physical activity (treadmill training) on articular cartilage. Alcian Blue staining demonstrated absence of structural alterations, fragmentation, and clefts in cartilage tissue from young and adult rats. The group 5 (old sedentary rats) showed a slight structural alteration with fragmentation and clefts in cartilage tissue, and a reduction of clones of chondrocytes comparable to an early stage of OA. Conversely, the group 6 (old rats subject to physical activity) demonstrated absence of structural alterations and increased number of clones of chondrocytes, due to a slight recovery and improvement after physical activity. The chondrocytes from groups 1 and 2 did not show any sign of cellular degeneration demonstrated by an intense staining, while the chondrocytes from groups 3 and 4 presented early signs of cellular degeneration demonstrated by a moderate staining. The chondrocytes from group 5 showed clear signs of cellular degeneration demonstrated by reduced/weak staining, although the chondrocytes from group 6 had moderate staining. This data mirrored the results obtained with immunohistochemistry in the study of lubricin expression. Lubricin immunohistochemical staining in explanted tissue was observed in an overall ECM of cells in all groups. A very strong lubricin staining was observed in groups 1 and 2, moderate lubricin immunoreactivity was observed in group 3, strong lubricin immunoreactivity was noted in group 4, while in the other groups 5 and 6 the expression of lubricin significantly decreased compared with groups 1 and 2. In fact, lubricin expression in chondrocytes was weak or absent in group 5, where a few chondrocytes were immunostained in the superficial zone, while in group 6 the lubricin

expression increased comparable to group 3, indeed the expression was moderate where less than half of the chondrocytes were immunostained in the superficial zone. The western blot and ELISA methods have confirmed these results.

## 6. Perspectives

Our findings suggest that moderate physical exercise, normal joint loading, and mechanical stimulation in elderly rats improve lubrication and prevent cartilage degeneration, promoting lubricin synthesis in synovial fluid, compared with unexercised adult rats. Physical activity increases joint mobility, consequently, both synovial fluid and chondrocytes express more lubricin, resulting in enhanced lubrication of articular surfaces. This protective lubrication mechanism help to prevent the onset of OA in aging. Our results also support the hypothesis that physical activity could also be used in aging as a natural therapeutic treatment for cartilage diseases to prevent OA and limit the deleterious effects of chondrocyte senescence. Further longer term *in vitro*, *in vivo*, and clinical studies using athletic cohorts are needed to elucidate the mechanisms that contribute to chondrocyte and cartilage aging. Although this study highlights the benefits of moderate exercise for articular cartilage, more research is needed to understand the precise role of lubricin in the context of cartilage aging. With our results we can also hypothesize that lack of lubricin could be a possible biomarker for the detection of chondrocytes senescence and in chondrocytes affected by OA. This knowledge may help us develop novel, innovative, and preventive strategies to slow or halt the aging process and the development of OA in joint tissues.

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## CHAPTER IV

## **REVIEW**

# **Altered joint tribology: reduced lubricin synthesis as a consequence of inflammatory process. New horizon for the therapeutic approaches in osteoarthritis.**

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## 1. Abstract

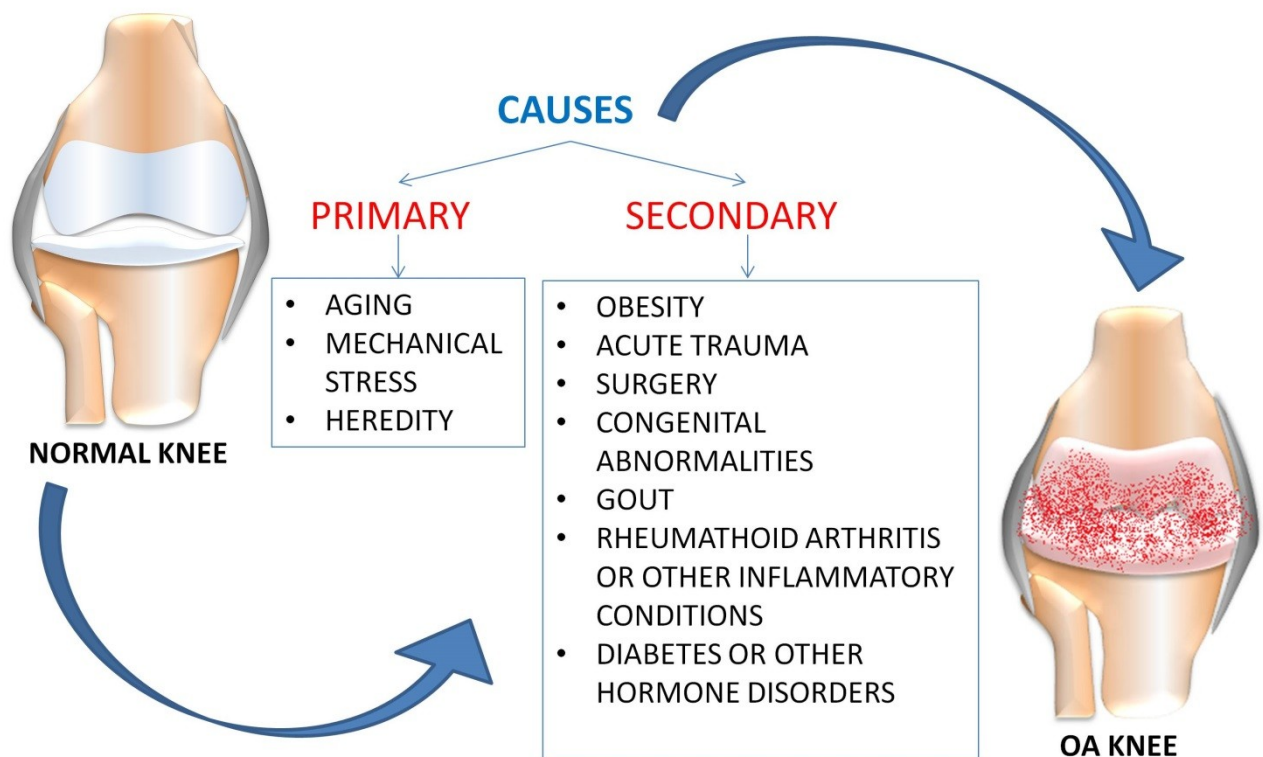
Osteoarthritis (OA) is the most common form of joint disease. The aim of the present review was to consolidate the current evidence that implicates inflammatory process in the attenuation of synovial lubrication and joint tissue homeostasis in OA. Moreover, based on these findings, some evidences for novel therapeutic strategies for the prevention and/or treatment of this complex disorder are examined and proposed. A literature search was conducted on different index database using appropriate keywords. The studies reviewed and considered in this paper support the idea that inflammatory mediators participate in the onset and progression of OA after joint injury. The evidences from literature indicate that the flow of pro-inflammatory cytokines following the acute injury seems to be directly associated with the altered lubricating ability occurring in the joint tissue. The latter is associated with the reduction of lubricin, which is one of the major joint lubricants. Future research in this field should focus on the development of new therapies that attenuate the inflammatory process and restore lubricin synthesis and function. This approach could be able to support joint tribology and synovial lubrication leading to improve joint function and pain relief.

**Keywords:** inflammation, pro-inflammatory cytokines, lubricin, osteoarthritis, joint injury

## 2. Introduction

Osteoarthritis (OA) has long been considered a “wear and tear” disease. Traditionally, the etiology of OA has been linked with increased mechanical overload on weight bearing joints, anatomical joint incongruence and fragility of articular cartilage [1]. However, this concept is gradually being challenged as evidence accumulates to support the ‘inflammatory’ basis of OA. The capacity for joint repair gradually diminishes with aging. The articular cartilage component of the joint is often damaged in focal or more extensive areas following joint injury. Cartilage is a connective tissue, which is neither vascularized nor innervated and for this reason it cannot respond to acute injuries with the usual cycle of reparative responses [2]. Chondrocytes, the unique cells that are present in cartilage, are sparsely distributed within the tissue and have a low reparative capacity since they have a very low metabolic activity. OA is a very complex disease with a multifactorial etiology, which includes aging, synovitis, “low grade” systemic inflammation, obesity, prior joint injuries, gender, genetic factors and metabolic syndrome among the most prominent risk factors for its development and progression [3, 4, 5] (Fig. 1). Another fundamental aspect of the OA pathophysiological process is the reduced boundary-lubricating ability of synovial fluid. The latter is associated with the reduction of lubricin, one of the major joint lubricants [6]. In this review we

discuss the most important findings regarding the inflammatory process and the altered lubricating ability occurring in the joint tissue following acute injury, highlighting a possible cross-link between these two pathological aspects of this complex disease. The key observations of this review should provide further motivation for studying the link between these two important features of OA, laying the foundation for its novel therapeutic approaches and innovative treatments.



**Figure 1.** Illustration representing several factors responsible for the development of primary or secondary OA.

### 3. Inflammatory Theory of Osteoarthritis

The ‘inflammatory theory’ of OA onset is certainly not a recent concept. Indeed, in a paper published in 1975, George Ehrlich described a cohort of predominantly menopausal females presenting a deforming and inflammatory OA, some of whom developed changes characteristic of rheumatoid arthritis (RA), [7]. This was probably the first paper that emphasized the importance of inflammation as a key component of OA. Although his original observations were published more than forty years ago, the importance of Ehrlich’s findings has not been fully appreciated until very recently. The major players in the field of OA research have realized the importance of this aspect and proposed a connection between inflammation, synovitis and structural changes in OA. The

advent of molecular biology and its introduction to bone and joint research dates back to 1990s. Numerous soluble mediators of inflammation, such as cytokines and prostaglandins, were discovered and found to be associated with increased production of matrix metalloproteinases (MMPs), primary enzymes responsible for cartilage degradation [8]. More recent data from literature indicate subchondral bone, cartilage and synovium as a source of inflammatory mediators implicated in OA progression and cartilage degeneration [2, 9]. This emphasizes the complexity of the disease, which implicates the entire joint as an organ and not only cartilage as a joint tissue. Such findings are supported by a recent discovery concerning the influence of pro-inflammatory cytokines on the reduced production of lubricin and the consequent decreased boundary lubricating ability of synovial fluid in OA joints, suggesting its important role in the development of this complex disease [10].

### **3.1. Inflammatory cytokines**

A primarily destructive impact on cartilage is due to the effect of inflammatory cytokines associated with biomechanical factors. The latter have a multilevel impact on joint tissues, involving premature aging, chondrocyte apoptosis and decrease in synthesis of key components of extracellular matrix (ECM). Inflammatory cytokines contribute also to increase the synthesis of many proteolytic enzymes, responsible for cartilage degradation and determine a reduced lubricating ability of synovial fluid. Among the inflammatory cytokines, which determine the loss of metabolic homeostasis of joint tissues by promoting catabolic and destructive processes, the greatest importance is attributed to interleukin 1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-6, IL-15, IL-17, and IL-18 (Fig.2 and Table 1) [11].

### **3.2. Synovitis**

Synovitis has been recognized as a critical feature of OA and many studies are focused on this condition as a key driver of the disease process. Synovitis is defined as the local inflammation of synovial membrane, usually painful, characterized by joint swelling due to synovial thickening or effusion. Synovial inflammation occurs frequently after traumatic joint injury and is associated with increased pain and dysfunction [12]. It probably occurs as degraded cartilage fragments and degraded ECM macromolecules are released into the joint and come into contact with synovium. Synovial cells react to the release of these fragments/molecules and become activated, producing inflammatory mediators [8]. The latter stimulate chondrocytes in the superficial layer of cartilage and the synovium itself to synthesize MMPs and other matrix degrading enzymes that increase cartilage degradation. These mediators are also responsible for synovial angiogenesis and



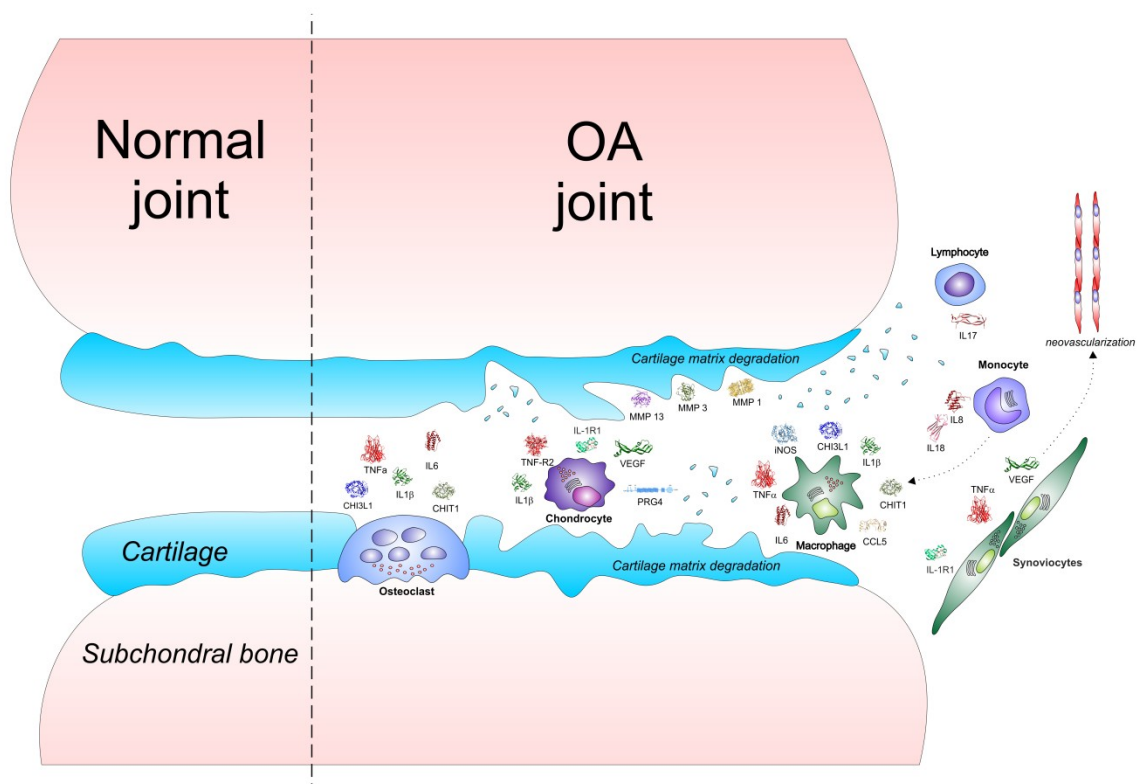
subsequent increase of synthesis of inflammatory cytokines and MMPs by synovial cells themselves, establishing a vicious cycle [13]. Clearly, the described events influence the lubricating ability of the joint as confirmed by Jay et al. in the study focused on lubricating ability of aspirated SF from patients with knee joint synovitis. The latter study demonstrated a non-lubricating bearings and increased catabolism of collagen type II in SF aspirates of these patients, suggesting that it may play a fundamental role in acute cartilage destruction ultimately resulting in post-traumatic (PT)-OA [14].

### 3.3. Inflammaging

Age is the most important risk factor for OA onset. The aging process does not necessarily need to be reported to the time that passes. Indeed, there are two different types of cellular senescence: the replicative and the stress-induced ones. The former is associated with an arrest in cell-cycle progression, resulting from a natural telomere shortening process and found in cells in older adults. The latter is independent of telomere length and is associated with several kinds of stresses, especially oxidative stress and inflammatory processes that establish during the OA onset [15]. "Inflammaging" refers to low-grade inflammation that occurs during physiological aging. Accordingly to this concept, the inflammatory process, as well as all the events closely linked to it, contributes to the chondrocyte senescence, which results in the age-related degradation of cartilage, subchondral bone, and synovium, determining the early development of OA [16]. Interestingly, the link between inflammation and aging appears to be interdependent as it not only determines the early senescence of chondrocytes but, at the same time it appears to be its direct consequence. Indeed, senescent chondrocytes show impaired activity compared with normal chondrocytes, with evidence of the so-called 'senescent secretory phenotype', which is characterized by increased expression of genes encoding for inflammatory cytokines such as IL-6, IL-1 $\beta$  and several members of MMP family [17]. The increased expression of both advanced glycation end-products (AGEs) and AGE receptors (RAGEs) in OA chondrocytes, has also been associated with dysregulated signalling pathways, altered synthetic activity and enhanced sensitivity to cytokines and chemokines, which in turn trigger the expression of MMPs and other inflammatory mediators [18]. Interestingly, also the lubricin production has been evaluated in the aged rats and it appeared to be decreased, suggesting that also the chondrocyte senescence has an important role in the lubricating properties of cartilage tissue [16]. This is probably a direct consequence of the above suggested strict link existing between aging and inflammation.

### 3.4. Chitinases

















Lately, in OA pathophysiology much interest has been given to some members from the family of chitinases such as chitotriosidase (CHIT1) and chitinase 3-like-1 (CHI3L1). Elevated levels of these proteins have been reported in several chronic inflammatory and degenerative disorders [19]. In our recent study we observed an increased expression of these proteins in a rat model of OA. Their production has been closely related to inflammatory processes and pro-inflammatory cytokines, and it was suggested that their over-expression could be involved in cartilage remodelling and degradation processes in OA joints [20]. Moreover, in another our recent study, we demonstrate that there is an inversely proportional correlation in the expression of CHI3L1 and lubricin in the normal and osteoarthritic rat articular cartilage. What we observed is that the levels of lubricin increase in normal cartilage and decrease in OA cartilage and the levels of CHI3L1 increase in OA cartilage and decrease in normal cartilage. This finding suggests that these two glycoproteins are functionally associated with the development of OA and underlines one more time the important link between the inflammation and lubricating properties of the articular cartilage tissue in the OA onset [21].



**Figure 2.** The pro-inflammatory cytokines, including IL-1 $\beta$ , TNF- $\alpha$ , IL-8, IL-18, IL-6 and CHI3L1 and CHIT1, are elevated in OA. These cytokines contribute to the pathogenesis of OA by mediating the destruction of articular cartilage

within the joint. The degenerative processes involve different types of cells including monocytes, macrophages, lymphocytes, osteoclasts, chondrocytes and synoviocytes.

**Table I.** Target genes involved in OA. Molecular analyses was performed using the UCSF Chimera package. Chimera is graphics software developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (Pettersen et al., 2004).

<b>CHIMERA IMAGE</b>	<b>GENE</b>	<b>PDB code</b>	<b>NAME</b>
	CCL15	1U4L	<b>Chemokine (C-C motif) ligand 15</b>
	CHI3L1	4P8U	<b>Chitinase-3-like protein 1</b>
	CHIT1	1GUV	<b>chitotriosidasi</b>
	IL1β	1TWM	<b>Interleukin-1 beta</b>
	IL1βR	1F20H	<b>Interleukin-1 beta receptor</b>
	IL6	1ALU	<b>Interleukin-6</b>
	IL18	1IL8	<b>Interleukin-18</b>
	iNOS	1NSI	<b>Nitric oxide synthases</b>
	MMP1	3SH1	<b>Matrix metalloproteinase-1</b>
	MMP3	1B3D	<b>Matrix metalloproteinase-3</b>
	MMP13	1CXV	<b>Matrix metalloproteinase-13</b>
	TGFB1	3KFD	<b>Transforming growth factor beta 1</b>
	TNF $\alpha$	1TNF	<b>tumor necrosis factor alpha</b>
	TNFR1	3ALP	<b>Cluster of Differentiation 120a</b>
	TNFR12	3ALQ	<b>Cluster of Differentiation 120b</b>
	VEGF	4KZM	<b>Vascular endothelial growth factor</b>

## **4. Inflammation and lubricin synthesis**

### **4.1. Lubricin**

Great importance in the biology of OA has been attributed to changes in lubrication at the surface of articular cartilage. This aspect of pathophysiological process of OA has not been entirely understood yet and it needs to be deepened. Lubricin is a surface-active mucin-like glycoprotein, encoded by the proteoglycan 4 (PRG4) gene, specifically synthesized by chondrocytes located at the surface of articular cartilage [22-24]. As a lubricating glycoprotein, the production of lubricin has been found in SF [25], menisci [25, 26], superficial layer of articular cartilage [27, 28], tendons [29], temporomandibular joint disc [30, 31] and periodontal ligament [32]. Lubricin, also referred to as superficial zone protein (SZP), has been reported to be a proteoglycan too, specifically PRG4. Lord and colleagues demonstrated that lubricin present in human synovial fluid is a heterogeneous population with both glycoprotein and proteoglycan forms [33]. PRG4 has been also identified as megakaryocyte stimulating factor (MSF) and the expression of human and mouse PRG4 genes was similar and found not only in cartilage, but also in liver, heart, lung, and bone [34]. Ludwig and others [35] demonstrated that the synovial fluid of human patients with symptomatic OA had lower levels of PRG4 and reduced boundary lubrication properties. In contrast, and perhaps unexpectedly, Neu and colleagues [36] found elevated levels of SZP in patients suffering from advanced OA, suggesting that SZP may be ineffective in reducing joint friction in the boundary lubrication mode at an advanced stage of OA, where other mechanisms may dominate the observed tribological behaviour. Lubricin contains multiple protein domains. The largest central mucin-like domain (high content of proline, serine and threonine) consists of imperfectly repeated sequences of EPATTPK, which provide the scaffold for O-glycosylation [37]. This domain contains C-terminal haemopexin domain and two somatomedin domains at the N-terminus. The boundary lubricating ability of lubricin has been attributed to its O-glycans, which affect its physical properties such as high viscosity and low friction [37]. Glycomic studies have shown that lubricin presents abundant sialylated and unsialylated core 1 and several sialylated, fucosylated and sulfated core 2 oligosaccharides [37].

### **4.2. Lubricin and OA**

It has been shown that lubricin is critical to normal joint function, providing boundary lubrication of congruent articular surfaces under conditions of high contact pressure and near-zero sliding speed. Furthermore, it has an important role in preventing chondrocytes apoptosis and in synovial cell adhesion and proliferation [38]. It has been shown that lubricin knockout mice develop

clinical and radiologic signs of joint disease and histologic abnormalities in their articulating joints with increasing age. The most important features are represented by synovial hyperplasia and subintimal fibrosis, proteinaceous deposits on the cartilage surface, and irregular cartilage surface and endochondral growth plates, abnormal calcification in tendon sheaths and osteophytes [39]. Furthermore, its decreased synthesis has been observed in several studies in both OA joints and PT conditions [26]. In an interesting study [40] the increased joint friction and cellular apoptosis have been noted in lubricin knockout mice when compared with wild-type mice. The same study reported that addition of lubricin in the in vitro bovine explant cartilage-on-cartilage bearing system significantly lowered the coefficient of friction and chondrocyte apoptosis in superficial layers of cartilage, confirming its crucial role in prevention of cartilage degeneration [40]. Supplementation of lubricin by intra-articular injection has also been shown to improve weight bearing in studies that employ measurements of hind limb force. Lubricin reduces the severity of PT-OA and the level of urinary C-terminal cross-linked telopeptide type II collagen (CTX-II), without affecting the Osteoarthritis Research Society International (OARSI) scores [41]. Over-production of lubricin in transgenic mice reduces the severity of both age-related and PT-OA and that it is due to the fact that lubricin inhibits the expression of genes involved in cartilage catabolism and chondrocyte hypertrophy, as it up-regulates HIF-3 $\alpha$ , which is a negative regulator of HIF-1 $\alpha$  and HIF-2 $\alpha$ , genes responsible for catabolic and anabolic activity that promotes OA [42]. The early stages following ACL injury is characterized by changes in levels of sulfated glycosaminoglycans (sGAG). The significantly increased sGAG and aquaporins levels in synovial fluid following joint injury may be indicative of articular cartilage damage [43]. It has been suggested that proteoglycan turnover increases in presence of low lubricin values as its decreased levels have been consistently associated with high sGAG concentrations [6]. Lubricin levels appear then to recover within 1 year after injury [6], however once initiated cartilage tissue damage, it appears to persist in time. These findings support the idea that the initial reduction of lubricin synthesis may initiate a cascade of events leading in time, to the onset of OA. Anyhow, this observation needs the further studies to support this hypothesis.

#### **4.3. OA-related lubricin reduction mechanisms**

The principal mechanism proposed for the reduction of lubricin synthesis is represented by the degradation activity of neutrophil-derived enzymes and inflammatory mediators present in the PT-SF [6]. It was shown in fact, that some cytokines (IL-1 $\beta$ , TNF- $\alpha$  and IL-6), are associated with the up-regulation of proteolytic enzymes such as procathepsin-B, neutrophil elastase and MMPs, that degrade lubricin and lead to the loss of SF chondroprotection, especially in early stages after

injury [6] (Fig.3). The proteolytic activity seems to be an important link between the inflammatory process and the decreased synovial fluid lubricating ability, suggesting an intimate correlation between these two pathological aspects of OA. In the later stages of injury, other factors, such as joint utilization and loss of intra-articular surface congruence, may contribute to potentiate this damage. This notion may complicate understanding of the real sequence of catabolic events that occur during the onset of OA [42]. Suggestions regarding the involvement of lubricin in OA disease and inflammation can be identified from its glycosylation. An interesting study [44] reported that lubricin presents an altered glycosylation profile in OA samples, where the decrease in sialylation was observed. This phenomenon was associated with the decreased lubricating ability, due to the reduced negative charge density on the surface of lubricin, given by the disialylated structures. It was observed, in fact, that more acid residues may enhance, through the increased repellent charge forces, the lubricating ability of lubricin [45]. Moreover, analysis showed that approximately 50% of lubricin O-glycans contain terminal galactose, a potential ligand for galectins. Galectin-3 was reported to be increased in OA chondrocytes and it has been suggested that it may be involved in the cartilage remodelling process [46]. Another attractive glyco-epitope on lubricin is represented by Sialyl Lewisx (sLex), which indicates the L-selectin binding ability. L-selectin has been shown to be involved in leukocyte trafficking. It was discovered that lubricin is able to bind to polymorphonuclear granulocytes (PMN) or neutrophils, which use L-selectin to roll along endothelium in the initial phase of adhesion cascade [47]. Furthermore, data from literature report that PMN recruited to the inflamed synovial area, where they probably play an important role in the cartilage degeneration, maintain a coat of lubricin. These findings suggest the implication of lubricin in PMN-mediated inflammation in an L-selectin-dependent and independent manner [48].



**Figure 3.** Schematic representation of the mechanism proposed for the reduction of lubricin synthesis in knee OA. The increased production of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$  and IL-6) is associated with the up-regulation of proteolytic enzymes (procathepsin-B, neutrophil elastase and MMPs) that degrade lubricin and lead to the loss of synovial fluid chondroprotection and the consequent onset of OA.

## 5. Therapeutic approaches for OA

### 5.1. Anticytokine therapy

The most evident approach in this field is represented by the anticytokine therapy based on the fact that initiation and progression of articular cartilage destruction, primarily involve pro-inflammatory and catabolic cytokines, especially IL-1 $\beta$  and TNF- $\alpha$  [48]. Animal models of OA confirmed that IL-1 $\beta$  inhibition, by IL-1 receptor antagonist (IL-1RA), showed a reduction of cartilage destruction [49]. The experimental OA was also inhibited by IL-1RA gene transfer, which results in the increased expression of IL-1RA in synovial membrane, resulting in diminished oedema, pain and radiological alterations in horses [50]. In humans, a case report described the successful treatment of inflammatory knee OA by using adalimumab, TNF- $\alpha$  inhibitor, with significant reduction of synovitis, synovial effusion, and complete resolution of bone marrow oedema [51]. In the study conducted on rats, blocking effects of TNF- $\alpha$  leads to an increase in total lubricin in the joint, suggesting improved chondroprotective ability. Early inhibition of effects of TNF- $\alpha$ , restores lubricin in SF and on the surface of articular cartilage, lowers whole joint coefficient of friction and limits cartilage damage [10]. Another approach is represented by the use

of antibodies against nerve growth factor (NGF). Studies have shown that NGF is a major mediator of inflammatory and neuropathic pain, providing a new therapeutic target [52]. In a study published in 2010, in a phase II trial, tanezumab showed to reduce pain and stiffness in patients with knee OA [53]. Subsequently, a phase III trial program initiated to study in detail the efficacy and safety of this monoclonal antibody. Other monoclonal antibodies against NGF have also been developed and two, fulranumab and fasinumab, have been studied in relation to OA. Inhibition of NGF by these antibodies has been demonstrated to reduce the pain and increase the function and well-being of individuals with symptomatic OA. All three antibody preparations have reported efficacy, although additional studies are required for fulranumab and fasinumab to determine the optimal dose for clinical use and to limit their adverse side-effects [54]. Indeed, increasing doses of anti-NGF antibodies has been associated with the syndrome of rapid progression of OA, characterized by chondrolysis and bone destruction [55]. Recently, also the anti-inflammatory effect of Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide with trophic effects, has been assessed in OA rat models. It was shown that PACAP levels decreased in OA cartilage and SF when compared with the control ones. Moreover, it was found that *in vitro*, PACAP was able to prevent IL-1 $\beta$ -induced chondrocyte apoptosis, suggesting its possible therapeutic use in the treatment of OA [56].

## 5.2. Recombinant lubricin

Intra-articular recombinant lubricin supplementation has also been advocated as a potential new therapeutic modality for this pathology [57, 58]. In a rat model of OA, an intra-articular injection of a novel recombinant lubricin protein construct, proved to bind effectively to cartilage surfaces, and facilitated both cartilage boundary lubrication and inhibition of synovial cell adhesion, resulting in significant, chondroprotective effects during the progression of OA [59]. The positive results for the intra-articular recombinant lubricin injection, has been also confirmed by the study on a rat model of ACL injury, where the reduction in cartilage damage and collagen type II degradation have been shown after treatment [60, 61]. In a recent study on ovariectomized rats, it was found that both early and late recombinant lubricin treatments attenuated the onset of OA by balancing the interplay between articular cartilage and subchondral bone. The best results were shown for the early treatment. Specifically, recombinant lubricin treatment protected articular cartilage from degeneration, demonstrated by lower proteoglycan loss, lower OARSI scores, less calcification cartilage zone and reduced immunostaining for collagen X and MMP-13, but increased the expression of lubricin. Chondroprotective effects of lubricin normalized bone remodelling in subchondral bone underneath, which in turn attenuated the articular cartilage erosion [62].



However, this kind of therapeutic approach is often limited by its short half-life. To remedy this problem and to ensure a long-term expression of Prg4 and chondroprotection in OA, the use of intra-articular, helper-dependent adenoviral virus (HDV) gene transfer delivering Prg4, has been proposed in a recent study [41]. The latter showed protection against the development of both PT and age-related OA, without significant adverse effects on cartilage development. Recently authors evaluated the binding of recombinant human proteoglycan 4 (rhPRG4) to CD44 receptor and its consequences on cytokine-induced synoviocyte proliferation. They concluded that PRG4 (lubricin) is a novel putative ligand for CD44 and may control synoviocyte overgrowth in inflammatory arthropathies via a CD44-mediated mechanism [63].

### 5.3 Physical activity

Physical activity covers not just sports but also simple everyday movements such as housework, walking and playing. Regular exercise has a great importance in maintaining good health, balance, posture indeed inactivity is a risk factor for different chronic diseases [64]. Regular physical exercise is normally suggested in cases of non-communicable chronic disease (NCD) for its specific effects in reducing cardiovascular risk factors and also has an anti-inflammatory effect that is well known, as this is the principal component of many chronic diseases [65]. In our recent study, we investigated a possible preventive treatment for OA through the combination of the Mediterranean diet, based on consumption of olive oil, and of the mild physical activity. The beneficial effects of extra-virgin olive oil have been widely studied thanks to its anti-inflammatory properties. For this reason we studied the role of extra-virgin olive oil-based diet coupled with moderate physical activity on inflammation and expression of lubricin in articular cartilage of rats after induced OA. It was observed that the effects of injury decrease drastically the expression of lubricin and increase the expression of IL-1 in rats, while after physical activity and extra-virgin olive oil supplemented diet, the levels returned to normality when compared to the control group [11]. These data suggest that mild physical activity improves lubrication by promoting lubricin synthesis and prevents cartilage degeneration in rats. These findings are supported also by our two previous studies. Indeed, we demonstrated that physical activity increases joint mobility and lubricin expression, resulting in enhanced lubrication of articular surfaces in aged rats [66] and we assessed the beneficial effects of physical activity on the articular cartilage of rats with glucocorticoid-induced osteoporosis [38]. In both cases the expression of lubricin was increased after the moderate physical activity treatment. These findings are also supported by several other studies, for example Ogawa and colleagues demonstrated that running induces maximal expression of lubricin in the superficial zone of articular cartilage in a COX-2-dependent manner, underlying a positive effect of

mechanical motion on lubricin expression [67]. Furthermore, the evidence of the influence of mechanical factors on lubricin metabolism in vivo has been also reported by Ni and coauthors in an interesting study aimed to understand alterations in cartilage lubricin expression and immunolocalisation after treadmill treatment with different intensities in a rat model. What was observed is that there is a marked intensity-specific effect of running on the lubricin immunolocalisation and gene expression in cartilage, which is inversely proportional to the Mankin score [68]. Another remarkable study of Elsaid and coauthors evaluated the impact of forced joint exercise following acute joint injury on lubricin metabolism, which resulted in decreased lubricin expression and increased cartilage degeneration. The same study was also aimed to assess a single-dose purified human lubricin injection in exercised injured joints, resulting in chondroprotection and preserved superficial zone chondrocyte viability [69]. All these results suggest that it might be possible that mechanical stimulation in the articular cartilage could induce the expression of lubricin, which is capable of preventing cartilage degeneration and might be used to slow down the development of OA in joint tissues [70-72]. To conclude and to further underline the importance of the physical activity in the treatment of OA, we report also some of the numerous on-going clinical studies regarding this practice. In an interesting trial by Murphy and coauthors [73], the effectiveness of a tailored activity pacing intervention on fatigue, pain, and physical function is examined in people with knee and hip OA. The activity pacing intervention tested in the trial has been designed to help people modulate their activity levels and reduce OA symptom associated with too much or too less physical activity. As a result of this trial, it will be possible to determine if activity pacing is more effective than usual care, and if an individually tailored approach improves fatigue and pain more than a general activity pacing approach. Another interesting recent on-going pilot randomized controlled trial centered on the physical activity approach is the one carried out by Linda Li at the University of British Columbia. The primary goal of this trial is to assess the feasibility and preliminary efficacy of a multi-component intervention/model of care involving a group education session, use of the Fitbit Flex (a wireless physical activity tracking device), and weekly telephone counselling by a physiotherapist to improve physical activity and reduce sedentary time in patients with knee OA (ClinicalTrials.gov Identifier: NCT02313506 ).

## **6. Conclusions**

Recent research has uncovered the multiplicity, complexity, and multilevel nature of the inflammatory and degradative processes that occur in OA. It is becoming increasingly apparent that inflammatory mediators participate in the onset and progression of OA. As outlined in this paper, the studies reviewed support the idea that the development of this disease is closely related to

inflammatory processes, reduced levels of lubricin and impaired lubricating ability of synovial fluid. Several therapeutic approaches aimed to solve, slow down or improve the joint condition after acute injury have been taken into account, including anticytokine therapy, intra-articular supplementation of recombinant lubricin and physical activity, suggesting the new horizons for the treatment of this complex disease. Future research in this field should focus on the development of new therapies that attenuate inflammation and stimulate lubricin production. This approach may support joint tribology and synovial lubrication leading to improved joint function and pain relief.

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## CHAPTER V

**Expression of CHI3L1 and Chitotriosidase in osteoarthritic rat cartilage model. A morphological study.**

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## 1. Abstract

Osteoarthritis is a degenerative joint disease, which affects millions of people around the world. It occurs when the protective cartilage at the end of bones wears over time, leading to loss of flexibility of the joint, pain and stiffness. The cause of osteoarthritis is unknown, but its development is associated with different factors, such as metabolic, genetic, mechanical and inflammatory ones. In recent years the biological role of chitinases has been studied in relation to different inflammatory diseases and more in particular the elevated levels of CHI3L1 and Chitotriosidase have been reported in a variety of diseases including chronic inflammation and degenerative disorders. The aim of this study was to investigate, by immunohistochemistry, the distribution of CHI3L1 and Chitotriosidase in osteoarthritic and normal rat articular cartilage, to discover their potential role in the development of this disease. Immunohistochemical analysis showed that CHI3L1 and Chitotriosidase staining was very strong in osteoarthritic cartilage, especially in the superficial areas of the cartilage most exposed to mechanical load, while it was weak or absent in normal cartilage. These findings suggest that these two chitinases could be functionally associated with the development of osteoarthritis and could be used as useful serum markers for this disease. However, the longer-term *in vivo* and *in vitro* studies are needed to understand the exact mechanism of these molecules, their receptors and activities on cartilage tissue.

**Keywords:** CHI3L1, Chitotriosidase, Osteoarthritis, ACLT, Immunohistochemistry.

## 2. Introduction

Osteoarthritis (OA) also known as degenerative arthritis or degenerative joint disease or osteoarthrosis is the most common form of arthritis, affecting millions of people around the world. It affects about 8 million people in the United Kingdom and nearly 27 million people in the United States.<sup>1</sup> Often called wear-and-tear arthritis, OA occurs when the protective cartilage at the ends of bones wears down over time.<sup>2</sup> Although OA can damage any joint in the body, the disorder most commonly affects joints in the hands, neck, lower back, knees and hips. OA is a group of mechanical abnormalities involving degradation of joints including not only the articular cartilage but also the subchondral bone.<sup>3</sup> OA gradually worsens with time, and no cure exists. Treatments can slow the progression of the disease, relieve pain and improve joint function. Symptoms of OA include loss of flexibility, limited movement, pain and swelling within the joint.<sup>4</sup> The condition results from injury to the cartilage, which normally absorbs stress and covers the bones, so they can move smoothly. A variety of causes, hereditary, developmental, metabolic, and mechanical deficits,

may initiate processes leading to loss of cartilage. When bone surfaces become less protected by cartilage, bone may be exposed and damaged. As a result of decreased movement secondary to pain, regional muscles may atrophy, and ligaments may become more lax. The main symptom is pain, causing loss of ability and often stiffness. Pain is generally described as a sharp ache, or a burning sensation in the associate muscles and tendons. Treatment generally involves a combination of exercise, lifestyle modification, and analgesics.<sup>4</sup> If pain becomes debilitating, joint replacement surgery may be used to improve the quality of life.<sup>5</sup> For most people, the cause of OA is unknown, but metabolic, genetic, chemical, inflammation and mechanical factors play a role in its development.<sup>6</sup> For this reason, in the present study, we investigated, in an “in vivo” osteoarthritic rat model, the human cartilage glycoprotein 39 (GP-39, YKL-40), also known as CHI3L1 and the Chitotriosidase (CHIT1). Mammalian chitinases belong to the glycohydrolase family 18, which have evolved to hydrolyze chitin, a polymer of N-acetylglucosamine.<sup>7,8</sup> The family of chitinases includes members both with and without glycohydrolase enzymatic activity against chitin. Chitotriosidase (CHIT1) is a true chitinase possessing chitinolytic (glycohydrolase) activities.<sup>9</sup> In contrast, chitinase-like-lectins (Chi-lectins) or chitinase-like proteins (C/CLPs), including chitinase 3-like-1 (CHI3L1, YKL40, HC-gp39), show enzymatic activity despite the retention and conservation of the substrate-binding cleft of the chitinases.<sup>10</sup> For the majority of the mammalian chitinases important biological roles in chronic inflammatory diseases have been identified.<sup>11-13</sup> So far, CHIT-1 is the best-characterized true chitinase from a clinical and biological perspective. Elevated levels have been reported in a variety of diseases including infections, chronic inflammation and degenerative disorders.<sup>14,15</sup> The sources of secreted CHIT1 are abnormal lipid-laden macrophages formed in tissues of patients with Gaucher's disease.<sup>16</sup> This molecule correlated strongly with disease symptoms and is used to monitor the efficacy of therapy.<sup>17</sup> Recently, it was hypothesized that cellular alteration in Gaucher's disease produced a proinflammatory milieu leading to bone destruction through enhancement of monocyte differentiation to osteoclasts and improvement of osteoclasts resorption activity.<sup>18</sup> To confirm this data it was demonstrated that the chitinases, CHIT1 and CH3L1, are closely related to the process of osteoclastogenesis and the digestion of bone matrix via MMP9.<sup>19</sup> Despite various theories having been proposed to explain the disruption of bone homeostatic balance in Gaucher's disease, implying dysfunction of osteoclasts, osteoblasts and mesenchymal cells,<sup>20-22</sup> to date the effect of CHIT1 remains nearly unexplored. Only one study has shown that, in periprosthetic soft tissue from patients with osteolysis the expression of alternative macrophage activation markers (CHIT1, CCL18) was increased in comparison to OA controls.<sup>23</sup> Interestingly this finding indicated that the activation of alternative macrophage is involved in osteolysis and suggested a correlation between CHIT1 and osteolytic lesions [21]. In

contrast to CHIT1, some evidence reports increased levels of CHI3L1 protein and/or mRNA in patients with a wide spectrum of pathologies.<sup>24</sup> The CHI3L1 is a glycoprotein secreted by articular chondrocytes, synoviocytes and macrophages. Serum and synovial fluid CHI3L1 levels are elevated in inflammatory diseases and correlate with the degree of joint destruction in rheumatoid arthritis. CHI3L1 is a candidate auto antigen in rheumatoid arthritis and is important in the capacity of cells to respond to and cope with changes in their environment.<sup>25</sup> Recently Einarsson and coauthors stated that chondrocytes of human osteoarthritic cartilage secrete the inflammation associated chitolectin CHI3L1.<sup>26</sup> CHI3L1, is a major secretory protein of human chondrocytes in cell culture. CHI3L1 mRNA is expressed by cartilage from patients with rheumatoid arthritis, but is not detectable in normal human cartilage.<sup>27</sup> Moreover, it was observed that in patients with myeloma elevated serum concentrations of CHI3L1 aggravated bone destruction and were associated with an increase of bone resorption activity hastening the progression of bone disease.<sup>28</sup> The aim of this study was to investigate, by immunohistochemistry the distribution of CHI3L1 and Chitotriosidase in osteoarthritic (n=20) and normal (n=10) rat articular cartilage, collected from femoral condyles after anterior cruciate ligament transection (ACLT), to discover a potential role for CHI3L1 and Chitotriosidase in osteoarthritic cartilage and to improve a new possible therapeutic concept for treating inflammatory joint diseases. The induced OA in articular cartilage was demonstrated by histomorphometric and histological analysis.

### **3. Materials and methods**

#### **3.1. Breeding and housing of animals**

In our study, we used thirty 6-months-old healthy male Sprague Dawley rats (Charles River Laboratories, Milan, Italy), with an average body weight of  $200 \pm 40$  g. Rats were individually housed in polycarbonate cages (cage dimensions: 10.25"W x 18.75"D x 8"H) during the entire period of the study and were housed at controlled temperature (20-23°C) and humidity, with free access to water and food and photoperiod of 12 hours light/dark. All surgical procedures for anterior cruciate ligament transection were performed in accordance with the method previously described.<sup>29,30</sup> The 30 animals were distributed in two different groups: 10 rats for control group (without ACLT) and 20 rats for OA group (knee articular cartilage of both distal femoral epiphyses was submitted to ACLT to induce OA model and left untreated). Antibiotic, analgesic, muscle relaxant and NSAID therapy was not performed not to alter the results. Postoperatively, the animals were permitted free cage activity without joint immobilization. The animals at 3 months which underwent ACLT were sacrificed by intracardial Pentothal® injection 30-40 mg/kg (Biochemie, Kundl, Austria); under Furane 2% ®-narcosis (Abbott Laboratories, Maidenhead, Berks., UK).

Both femurs were explanted, cleaned of soft tissues and the samples were used to perform histomorphometric evaluations. Cartilage tissue were used to perform histological and immunohistochemical analyses. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania. The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

### **3.2. Histomorphometric analysis**

Femurs were explanted, cleaned of soft tissues as previously described.<sup>31</sup> Histomorphometric analysis was performed on the total number of rats used and specifically on both medial and lateral femoral condyles from all groups (untreated and treated animals). Histomorphometry was performed with image analysis, Kontron KS 300 software (Kontron Electronics, Eching bei Munchen, Germany). Two blinded investigators (2 anatomical morphologists) made the analyses. We assumed that the evaluations were correct if there were no statistically different values between the investigators. Fifteen fields randomly selected from each section were analyzed. The semi-quantitative histological grading criteria of Kraus' modified Mankin score<sup>32,33</sup> and histopathology OARSI system<sup>34</sup> were used.

### **3.3. Histology**

Samples were fixed in 10% neutral buffered-formalin (Bio-Optica, Milan, Italy), following overnight washing and routinely embedded in paraffin as previously described.<sup>35</sup> After wax infiltration the tissue samples were orientated in the cassettes in the same direction. Sections 4–5 µm thick were cut from paraffin blocks using a rotary manual microtome (Leica RM2235, Milan, Italy) and mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained as previously described<sup>36</sup> for routine histological evaluation by hematoxylin and eosin (H&E) staining for the morphological structure. The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### **3.4. Immunohistochemistry (IHC)**

For immunohistochemical analysis slides were processed as previously described.<sup>37</sup> Briefly, the slides were dewaxed in xylene, hydrated using graded ethanol and were incubated for 30 min in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to quench endogenous peroxidase activity and then rinsed for 20 min with

phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA, Sigma, Milan, Italy) in PBS for 1 hour in a moist chamber. BSA was used as a blocking agent to prevent non-specific binding of the antibody. Following blocking, the sections were incubated overnight at 4 °C with goat polyclonal GP-39 antibody (CHI3L1), work dilution in PBS 1:100 (sc-30465, Santa Cruz Biotechnology, Inc. USA) and rabbit polyclonal Chitotriosidase antibody, work dilution in PBS 1:100 (sc-99033, Santa Cruz Biotechnology, Inc. USA). Immune complexes were then treated with a biotinylated link antibody and then detected with peroxidase labeled streptavidin, both incubated for 10 min at room temperature (LSAB+ System-HRP, K0690, Dako, Denmark). The immunoreaction was visualized by incubating the sections for 2 minutes in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The sections were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### 3.5. Evaluation of immunohistochemistry

The CHI3L1 and Chitotriosidase-staining status was identified as either negative or positive. Immunohistochemical positive staining was defined as the detection of brown chromogen on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane and evaluated as previously described.<sup>37</sup> Stain intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-4, according to the following assessment: no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, very strong staining = 4. The percentage of antibodies immunopositive cells (Extent Score=ES) was independently evaluated by 2 investigators (2 anatomical morphologists) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5-30% (+); 31-50% (++); 51-75% (+++), and >75% (++++). Counting was performed under Zeiss Axioplan light microscope at x200 magnification. In case of disputes concerning the interpretation, the case has been revised to reach a unanimous agreement, as previously described.<sup>38</sup> Digital pictures were photographed with a digital camera (Canon, Japan) at 20x, 40x and 60x magnification. Positive and negative controls were performed to test the specific

reaction of primary antibodies used in this study at a protein level. Positive control for both antibodies consisted of rat liver sections. Sections treated with PBS without the primary antibodies served as negative controls. Positive immunolabeling for antibodies were nuclear/cytoplasmic.

### **3.6. Computerized morphometric measurements and image analysis**

Fifteen fields, randomly selected from each section, were analyzed and the percentage area stained with CHI3L1 and Chitotriosidase antibodies was calculated using image analysis software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany, using objective lens of magnification x20 i.e. total magnification 400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany); evaluations were made by two blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement.

### **3.7. Statistical analysis**

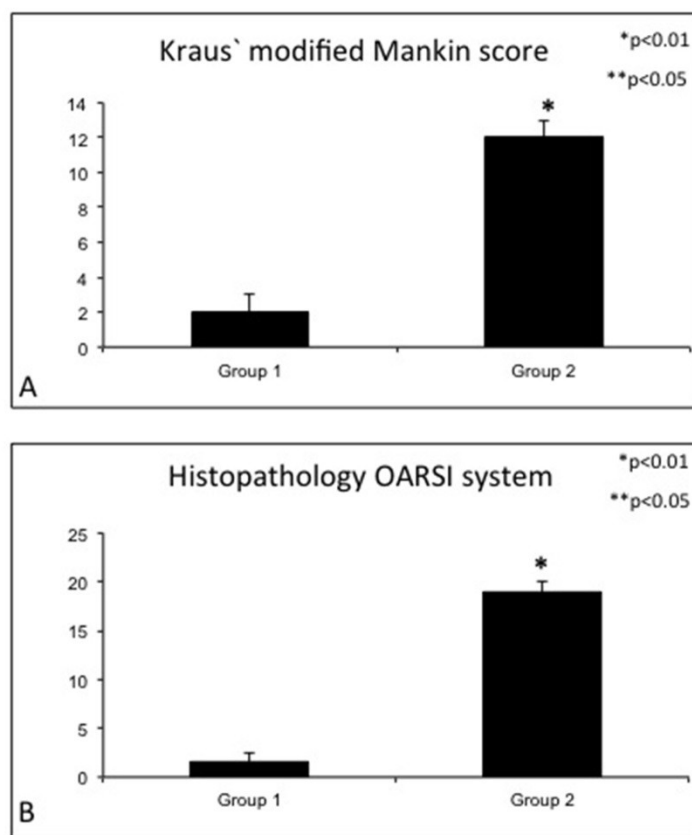
Statistical analysis was performed using SPSS software (SPSS® release 16.0, IBM, Chicago, IL, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's t test, whilst comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. P-values of less than 0.05 were considered statistically significant; p-values of less than 0.01 were considered highly statistically significant. Data are presented as the mean  $\pm$  SEM as previously described.<sup>37</sup> Cohen's kappa was applied to measure the agreement between the two observers and averaged to evaluate overall agreement as previously described.<sup>37</sup>

## **4. Results**

### **4.1. Histomorphometric analyses**

The histomorphometric parameters performed in group 1 (without ACLT), confirmed that the animals demonstrated no sign of cartilage degeneration with an intact and normal cartilage structure, whilst in group 2 (with ACLT) the animals demonstrated more serious pathological changes to the cartilage, OA moderate and severe, in fact horizontal cleavage tears or flaps and deep

lesions were present. Group 2 confirmed the development of articular degenerative processes, which were significantly different from the control group, as confirmed by Kraus' modified Mankin score (Fig. 1A), and histopathology OARSI system (Fig. 1B). The inter-observer variability among three observers for the MANKIN system showed a similar good intra-class correlation coefficient ( $ICC > 0.82$ ) as for the OARSI system ( $ICC > 0.72$ ). Repeat scoring by investigators showed very good agreement ( $ICC > 0.96$ ). The surface represented by lesion depth was the parameter where all investigators showed an excellent agreement. Other parameters such as cellularity and tidemark had greater inter-reader disagreement.

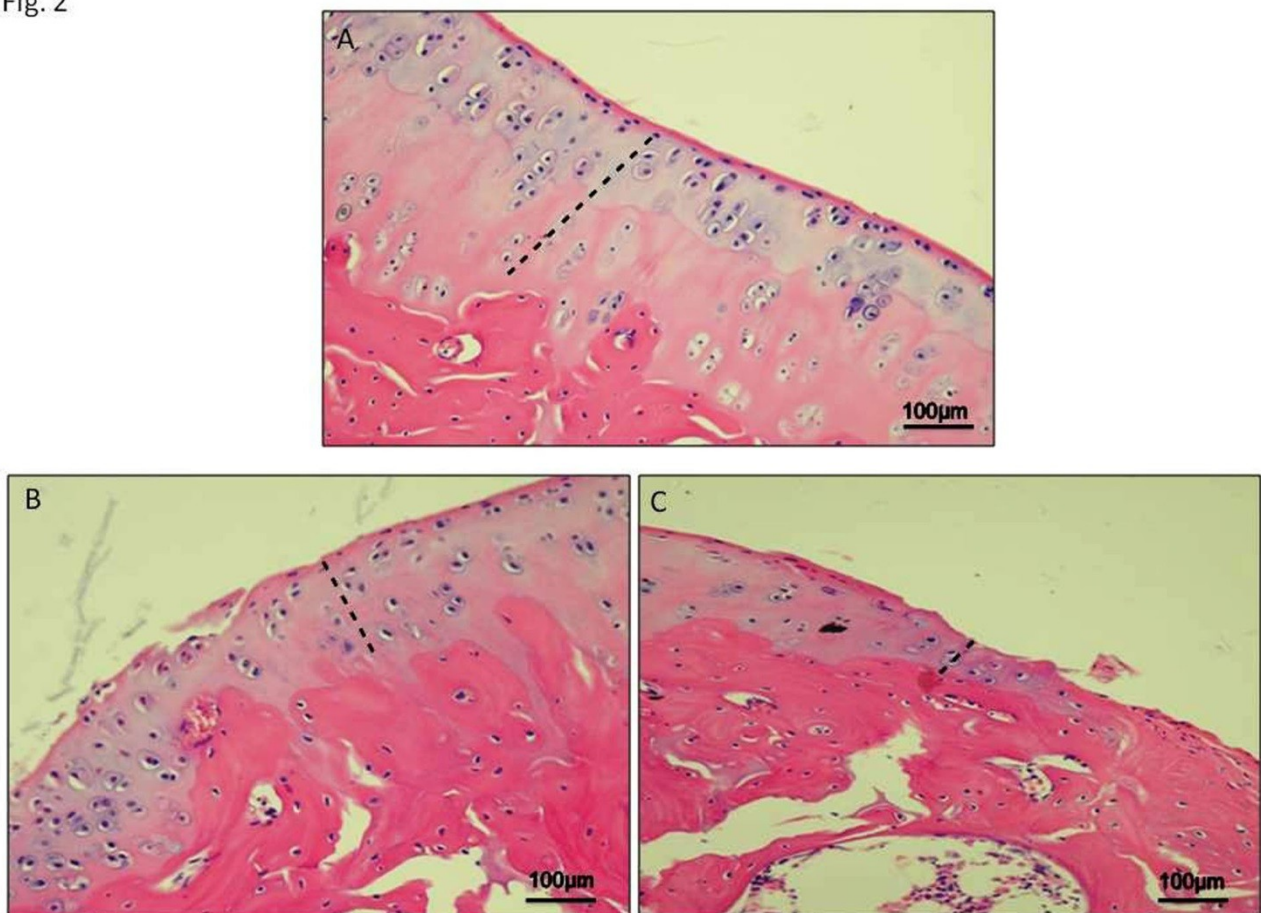


**Figure 1.** Graph A. Kraus' modified Mankin score among groups. Results are presented as the mean  $\pm$  SEM. Student's t test, was used to evaluate the significance of the results. \*\*p < 0.05 and \*p < 0.01, when compared to the control group. Graph B. Histopathology OARSI system among groups. Results are presented as the mean  $\pm$  SEM. Student's t test, was used to evaluate the significance of the results. \*\*p < 0.05 and \*p < 0.01, when compared to the control group.

## 4.2. Histology

The histological (H&E staining) analysis of cartilage from group 1 (without ACLT), showed a preserved morphological structure (Fig. 2 A). This contrasts with group 2 (with ACLT), moderate OA cartilage where structural alterations included a reduction of cartilage thickness of the superficial and the middle zones (Fig. 2 B). The structure of the collagen network is damaged, which leads to reduced thickness of the cartilage. The chondrocytes are unable to maintain their repair activity with subsequent loss of the cartilage tissue. In severe OA, group 2 (with ACLT), the cartilage demonstrated deep surface clefts, disappearance of cells from the tangential zone, cloning, and a lack of cells in the intermediate and radial zone, which are not arranged in columns. The tidemark is no longer intact and the subchondral bone shows fibrillation (Fig. 2 C). Moreover, while the surface of healthy hyaline cartilage appears white, shiny, elastic and firm, in OA the surface becomes dull and irregular.

Fig. 2



**Figure 2. A.** H&E staining demonstrated absence of structural alterations in control cartilage (group 1 without ACLT). The dashed line represents the layers (thickness) of hyaline healthy cartilage. In the superficial zone, cells are flat and small; in the middle and deep zone, cells are organized in columns; the tidemark is evident. Magnification x20; Scale bars: 100µm. **B.** H&E staining demonstrated signs of structural alterations in moderate OA cartilage (group 2 with



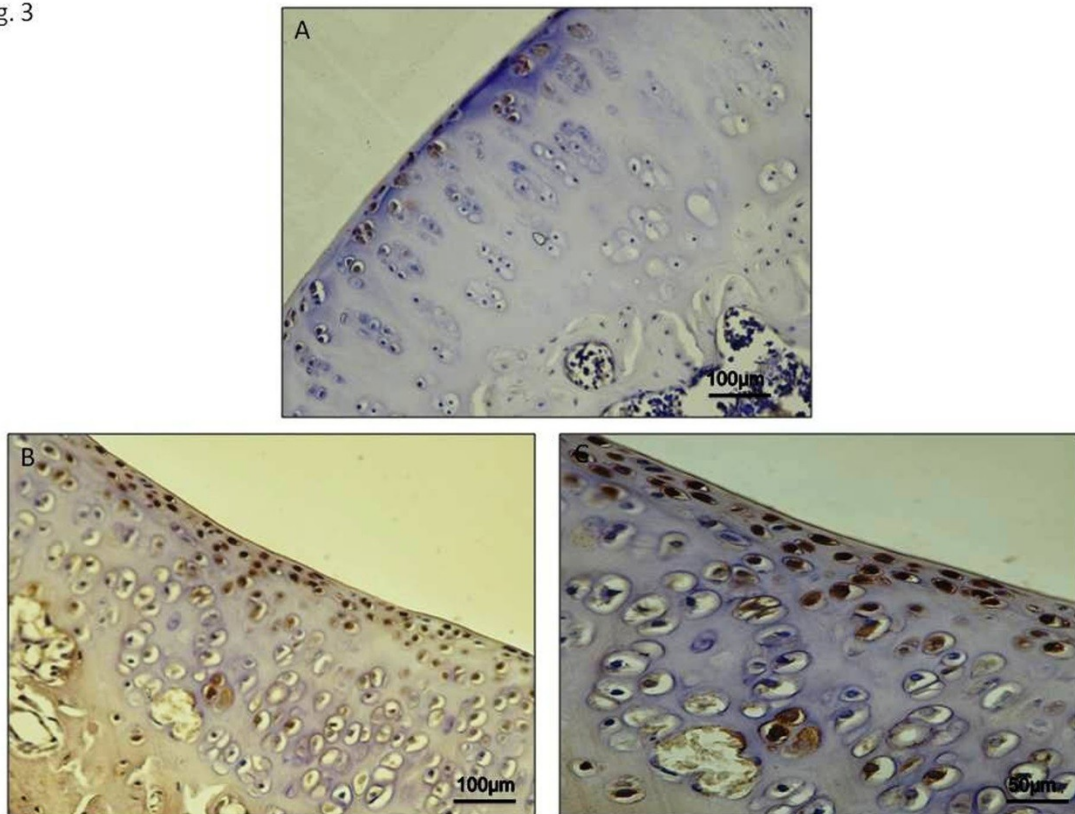
ACLT). The dashed line represents the layers (thickness) of hyaline cartilage. The structural alterations included a reduction of cartilage thickness of the superficial and the middle zones. The tidemark is almost intact. Magnification x20; Scale bars: 100µm. C. H&E staining demonstrated signs of structural alterations in severe OA (group 2 with ACLT). The dashed line represents the layers (thickness) of hyaline cartilage. Severe OA cartilage, demonstrated deep surface clefts, disappearance of cells from the superficial zone, cloning, and a lack of cells in the intermediate and deep zone, which are not arranged in columns. The tidemark is no longer intact and the subchondral bone shows fibrillation. Cartilage is completely replaced by fibrocartilaginous, scar-like tissue with fibroblast like cells. Magnification x20; Scale bars: 100µm.

### 4.3. Immunohistochemical observations

CHI3L1 and Chitotriosidase were evaluated by immunohistochemical staining in cartilage of all groups. Different patterns of immunopositive cells in the sets of specimens were seen. This immunohistochemical staining was found in chondrocytes of osteoarthritic cartilage mainly in the superficial and middle zone of the cartilage rather than the deep zone, while it was weak/absent in normal cartilage.

CHI3L1 immunolabeling was weak/absent in cartilage tissue samples from group 1, without ACLT, [(Fig. 3A); (ES=+; IS=1)], and very strong in group 2, with ACLT, moderate/severe OA [(Fig. 3B, C); (ES=++++; IS=4)].

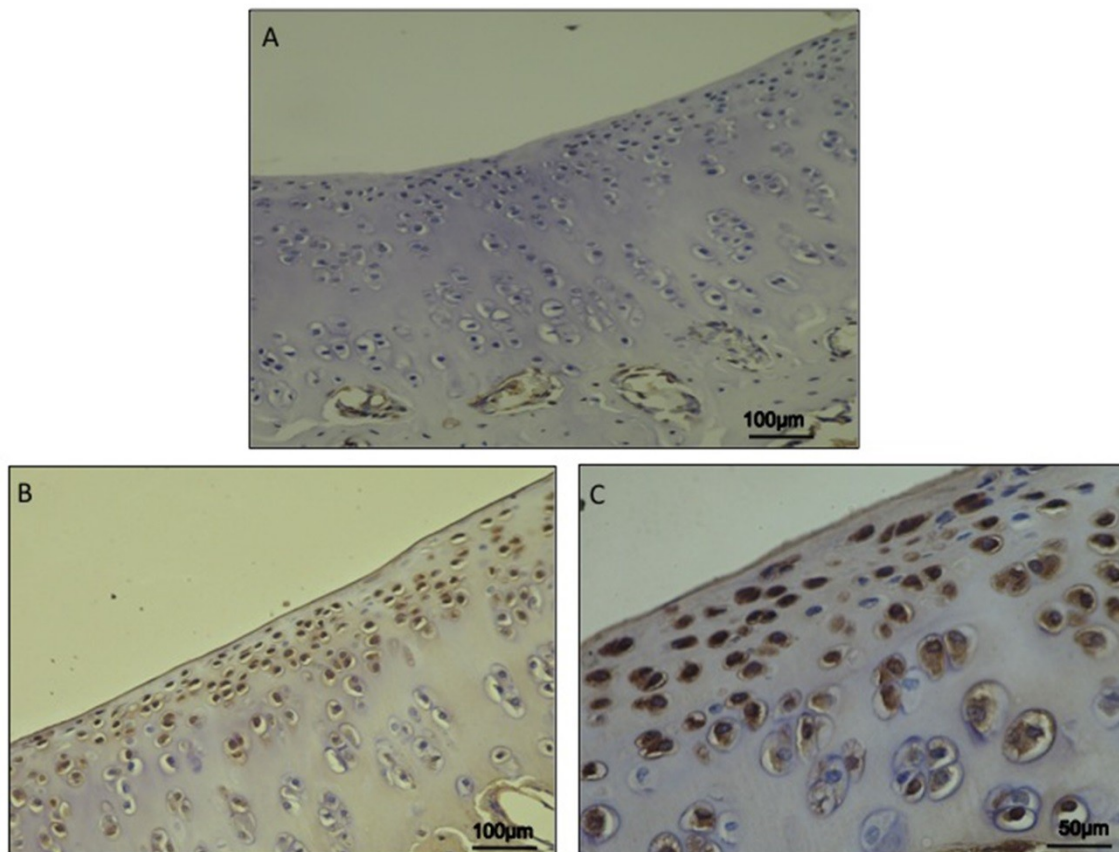
Fig. 3



**Figure 3. A.** CHI3L1 immunohistochemistry specimen from control cartilage (group 1, without ACLT) exhibited a weak/absent (ES=+; IS=1) immunostaining in chondrocytes from rat femoral articular cartilage. Magnifications x20; Scale bars: 100µm. **B.** CHI3L1 immunohistochemistry specimen from moderate/severe OA cartilage (group 2, with ACLT) exhibited a very strong (ES=++++; IS=4) immunostaining in chondrocytes from rat femoral articular cartilage (superficial and middle zone). Magnification x20; Scale bars: 100µm. **C.** Magnification of the figure B. Magnification x40; Scale bars: 50µm.

No immunostaining was observed in the negative control (ES=0; IS=0) treated with PBS without the primary antibody (data not shown). The percentage of CHI3L1-positive cells was observed among groups ( $p < 0.01$  vs. others) (Fig. 5A). Interobserver agreement, measured as Kappa coefficient, was 0.95 (almost perfect).

Chitotriosidase immunolabeling was weak/absent in cartilage tissue samples from group 1, without ACLT, [(Fig. 4A); (ES=+; IS=1)], and very strong in group 2, with ACLT, moderate/severe OA [(Fig. 4B, C); (ES=++++; IS=4)].

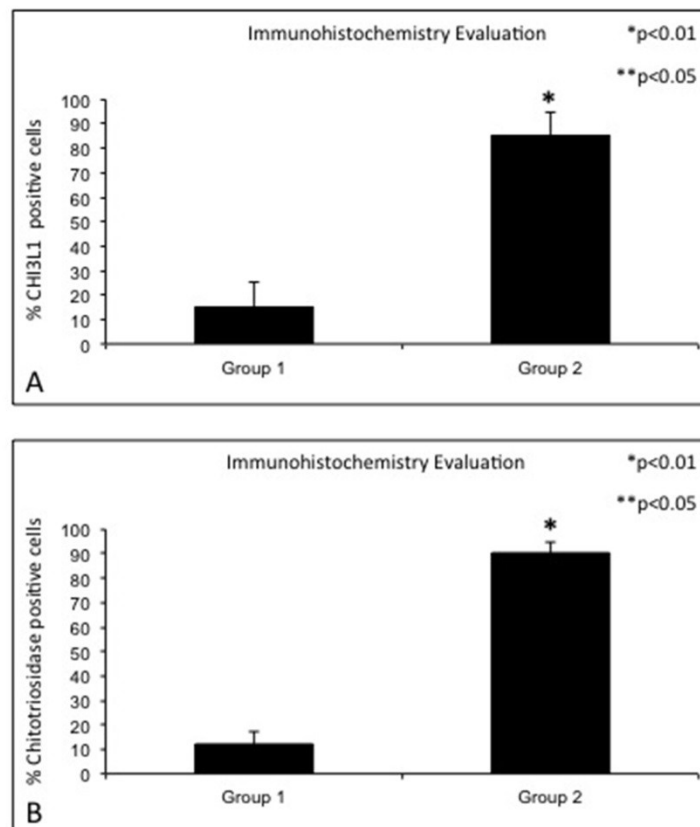


**Figure 4. A.** Chitotriosidase immunohistochemistry specimen from control cartilage (group 1, without ACLT) exhibited a weak/absent (ES=+; IS=1) immunostaining in chondrocytes from rat femoral articular cartilage. Magnifications x20; Scale bars: 100µm. **B.** Chitotriosidase immunohistochemistry specimen from moderate/severe OA cartilage (group 2, with ACLT) exhibited a very strong (ES=++++; IS=4) immunostaining in chondrocytes from rat

femoral articular cartilage (superficial and middle zone). Magnification x20; Scale bars: 100µm. C. Magnification of the figure B. Magnification x40; Scale bars: 50µm.

No immunostaining was observed in the negative control (ES=0; IS=0) treated with PBS without the primary antibody (data not shown). The percentage of Chitotriosidase-positive cells observed among groups ( $p < 0.01$  vs. others) (Fig. 5B). Interobserver agreement, measured as Kappa coefficient, was 0.92 (almost perfect).

Fig. 5

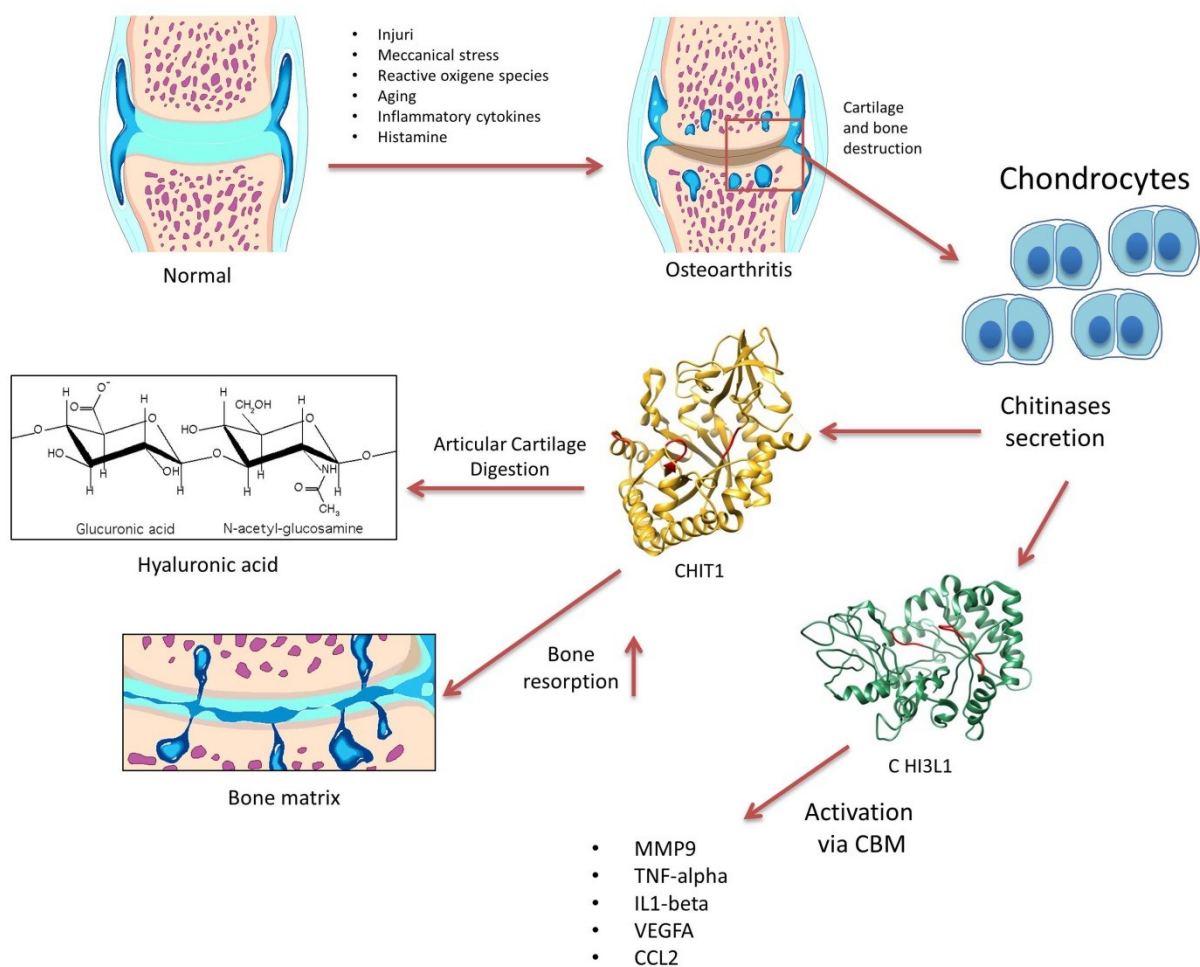


**Figure 5.** Graph A. Immunohistochemistry: percentage of CHI3L1 positive cells out of the total number of cells counted in control group and in treated group. Results are presented as the mean  $\pm$  SEM. Student's t test, was used to evaluate the significance of the results. \*\*p < 0.05 and \*p < 0.01, when compared to the control group. Graph B. Immunohistochemistry: percentage of Chitotriosidase positive cells out of the total number of cells counted in control group and in treated group. Results are presented as the mean  $\pm$  SEM. Student's t test, was used to evaluate the significance of the results. \*\*p < 0.05 and \*p < 0.01, when compared to the control group.

## 5. Discussion

Articular cartilage injuries are one of the most challenging problems in musculoskeletal medicine due to the poor intrinsic regenerative capacity of this tissue.<sup>39</sup> OA represents a major clinical challenge to clinicians and a scientific challenge to cartilage biologists due to the limited repair capacity of articular cartilage and the avascular nature of the tissue. Articular cartilage defects are increasingly common among the elderly population causing pain, reduced joint function and significant disability.<sup>40,41</sup>

In this study, we have demonstrated that CHI3L1 and Chitotriosidase are expressed in osteoarthritic rat cartilage model (Fig. 6).



**Figure 6.** Graphic representation of CHI3L1 and Chitotriosidase expression in osteoarthritic chondrocytes.

CHI3L1 had been linked to tissue remodeling<sup>42,43</sup>, joint injury<sup>44</sup>, and significantly elevated levels of CHI3L1 protein have been detected in serum and synovial fluid from OA patients.<sup>45,46</sup> The plethora of evidence showing that CHI3L1 stimulates proliferation of connective tissue cells and modulates expression levels of chemokines and metalloproteases in inflammatory fibroblasts, and

that enhances chemotaxis of endothelial cells<sup>47,48</sup> strongly indicate that CHI3L1 plays a crucial role in stromal cells not only in inflammatory conditions. Additionally, *in vitro* studies demonstrated that CHI3L1 is secreted by osteosarcoma<sup>49</sup> and during osteoclast differentiation and bone digestion.<sup>50</sup> The findings showing a correlation between CHI3L1 expression and the development of primary and metastatic tumors further support the idea that CHI3L1 plays a role in the development and progression of a variety of malignancies.<sup>51,52</sup> As regards the other chitinase, the Chitotriosidase is synthesized as a 50-kDa protein with a PI of about 6.5 and 7.2. It is predominantly secreted, but in part processed into a 39-kDa form with a PI of 8.0 that accumulates in lysosomes. The C-terminus present in the 50-kDa chitotriosidase, but absent in the 39-kDa isoform, was found to mediate tight binding to chitin. In both isoforms, the enzymatic activity is still present. Much evidence reports the central role of CHIT-1 in the expanding spectrum of disorders suggesting that over- production of CHIT-1 could exert deleterious effect in many degenerative disorders.<sup>53</sup> This concept is also sustained by our previous findings in which we observed that genetic variation within the CHIT-1 gene was strongly associated with human longevity and with several phenotypes of healthy aging<sup>54</sup>, and that a functional polymorphism in the CHIT-1 gene protects from NAFLD progression.<sup>55</sup> In our results the histomorphometric parameters performed in group 1 (without ACLT), confirmed that the animals demonstrated no sign of cartilage degeneration with an intact and normal cartilage structure, whilst in group 2 (with ACLT) the animals demonstrated more serious pathological changes to the cartilage, OA moderate and severe, in fact horizontal cleavage tears or flaps and deep lesions were present, as confirmed by Kraus' modified Mankin score and histopathology OARSI system. These results were corroborated by histological examination.

Immunohistochemical analysis showed that CHI3L1 and Chitotriosidase staining was found in chondrocytes of osteoarthritic cartilage mainly in the superficial and middle zone of the cartilage rather than the deep zone. There was a tendency for a high number of positive chondrocytes in areas of the femoral condyles with a considerable biomechanical load. The number of chondrocytes with a positive staining for both CHI3L1 and Chitotriosidase was weak/absent in normal cartilage, while the expression for both CHI3L1 and Chitotriosidase was very strong in osteoarthritic cartilage with ACLT. The two chitinase are typically produced in the lysosomes and subsequently secreted. Their production is closely related to an inflammatory process, and pro-inflammatory cytokines.<sup>56</sup> CHIT1 and CHI3L1 present Carbohydrate-binding motif (CBM), then the ability to bind carbohydrates, in particular glycosaminoglycans. This ability could explain the altered levels of the two molecules during a chronic inflammatory process such as OA. In fact, this observation is consistent with the evidence showing that CHI3L1 inhibition restrains tumor growth and metastasis by its own CBM.<sup>57</sup> Furthermore, treatment with both CHIT1 and CHI3L1 siRNAs in osteoclast *in vitro* model, induced

a significant reduction of MMP9.<sup>50</sup> Recent articles confirm that the gelatinases influence OA onset and progression regulating the subchondral bone remodelling. In particular, a predominant role of MMP-9 emerged during last year. Among various MMPs, the total MMP-9 level is positively correlated with the total MMP-13 level in OA<sup>58,59</sup>, and it has been hypothesized that this gelatinase might be involved in the activation of pro-MMP-13 through yet unknown mechanisms. Notably, MMP-13 has long been considered as the major enzyme involved in cartilage erosion during OA, thus MMP-9 might play a role, at least cooperatively, in joint degradation. That being so, it would seem plausible that the chitinase may be involved in the resorption of articular cartilage in OA through different pathways. The chitotriosidase could be involved in the resorption of cartilage and bone matrix via its catalytic site and the CBM, while the CHI3L1 could activate pro-inflammatory cytokines through its CBM.

The present findings suggests that CHI3L1 and Chitotriosidase may be of importance in cartilage remodeling/degradation of osteoarthritic joints. To our knowledge this is the first study reporting that CHIT1 and CHI3L1 may emerge as useful serum markers for OA, but also that it is functionally involved in the tissue degeneration. The expression of CHIT1 and of CHI3L1 in chondrocytes of osteoarthritic cartilage has a detrimental role in the cellular remodelling during the OA. Although these results are in accordance with Connor and co-authors<sup>60</sup>, they are preliminary and further, longer-term *in vivo* and *in vitro* studies are needed to understand the exact mechanism of CHI3L1 and Chitotriosidase production and regulation in cartilage tissue and to define in detail these molecules, their receptors and activities on cells involved in OA.

**Contribution.** All authors have made substantial intellectual contributions to the conception and design of the study, data acquisition, analysis and interpretation. MD carried out the experimental work, study execution and manuscript writing. MAS contributed to data collection, technical assistance, interpretation and analysis. LM and DT manuscript discussing and proofreading. GM conceived the study design, manuscript writing and planning, results discussing and supervised. All authors contributed to data interpretation and manuscript preparation. All authors have approved the final submitted version.

**Competing interests.** No competing interests were disclosed.

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## CHAPTER VI

## **Co-Expression and Co-Localization of Cartilage Glycoproteins CHI3L1 and Lubricin in Osteoarthritic Cartilage: Morphological, Immunohistochemical and Gene Expression Profiles**

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## 1. Abstract

Osteoarthritis is the most common human arthritis characterized by degeneration of articular cartilage. Several studies reported that levels of human cartilage glycoprotein chitinase 3-like-1 (CHI3L1) are known as a potential marker for the activation of chondrocytes and the progression of Osteoarthritis (OA), whereas lubricin appears to be chondroprotective. The aim of this study was to investigate the co-expression and co-localization of CHI3L1 and lubricin in normal and osteoarthritic rat articular cartilage to correlate their modified expression to a specific grade of OA. Samples of normal and osteoarthritic rat articular cartilage were analyzed by the Kellgren–Lawrence OA severity scores, the Kraus’ modified Mankin score and the Histopathology Osteoarthritis Research Society International (OARSI) system for histomorphometric evaluations, and through *CHI3L1* and *lubricin* gene expression, immunohistochemistry and double immunostaining analysis. The immunoexpression and the mRNA levels of lubricin increased in normal cartilage and decreased in OA cartilage (normal vs. OA,  $p < 0.01$ ). By contrast, the immunoexpression and the mRNA levels of CHI3L1 increased in OA cartilage and decreased in normal cartilage (normal vs. OA,  $p < 0.01$ ). Our findings are consistent with reports suggesting that these two glycoproteins are functionally associated with the development of OA and in particular with grade 2/3 of OA, suggesting that in the future they could be helpful to stage the severity and progression of the disease.

**Keywords:** Lubricin; CHI3L1; Osteoarthritis; anterior cruciate ligament transection (ACLT); Immunohistochemistry; mRNA

## 2. Introduction

Osteoarthritis (OA) is the most common human arthritis characterized by deterioration and loss of articular cartilage [1]. OA is the most prevalent joint condition resulting in physical disability resulting in a high economic burden largely attributable to the effects of disability, comorbid disease, and the expense of treatment [2]. The main risk factors involved in the pathogenesis of OA are genetics, aging, obesity, injury and biomechanical stress [3]. This condition is associated with progressive hyaline articular cartilage loss, low-grade synovitis and alterations in subchondral bone and periarticular tissues [4].

The causes behind OA development and progression continue to remain largely undefined and understanding the molecular pathogenesis of the disease remains a priority. Recent studies have shown that two glycoproteins may be particularly relevant to OA pathogenesis. The human cartilage glycoprotein chitinase 3-like-1 (CHI3L1) is associated with mediators of inflammation [5–7] and

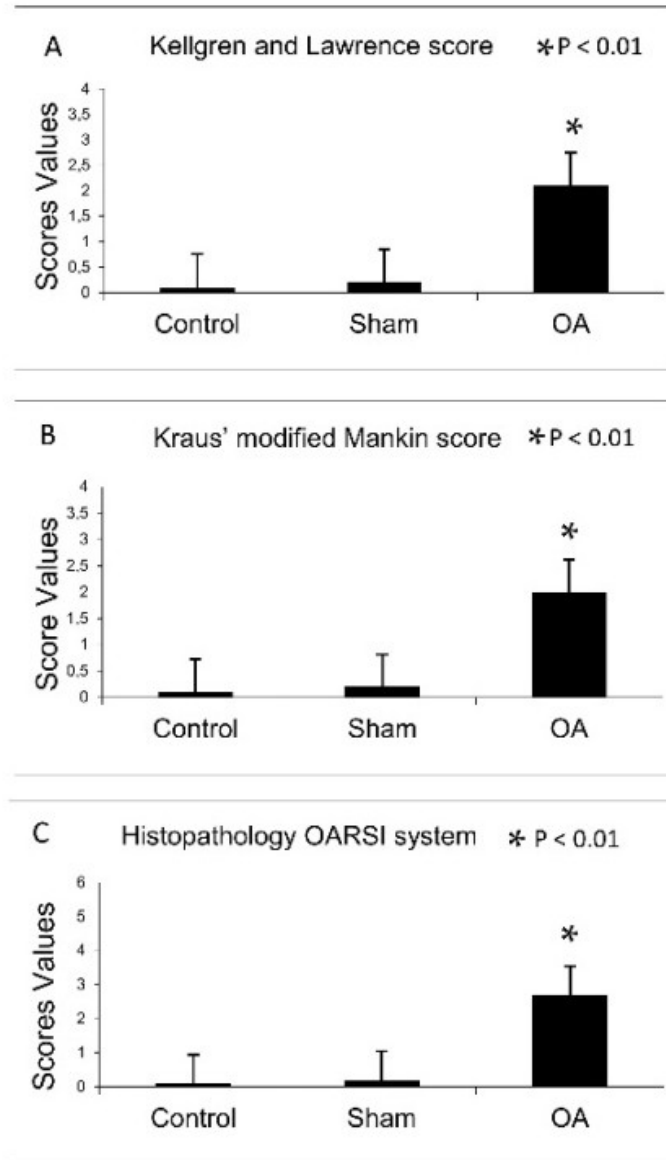
cartilage damage involved in the pathogenesis of OA [8]. We have earlier reported an increased expression of this protein in the OA rat model when compared to the control group [9]. Its production has been correlated to joint inflammation and it was suggested that its over-expression could be involved in remodelling and degradation of cartilage in OA joints [9].

Another fundamental aspect of the OA pathophysiological process is represented by the reduced boundary-lubricating ability of synovial fluid [10]. The latter is associated with the reduction of lubricin, one of the major joint lubricants, in both acute and chronic conditions [11]. In this regard lubricin is a glycoprotein that has received considerable attention such as chondroprotective molecule [10–13]. The association between cartilage, boundary lubrication and evident changes in cartilage tissue after injury has not yet been clearly understood, but considerable evidence from the literature indicates that it may predispose the articular cartilage to degenerate and develop OA [12,14]. Hyper expression of lubricin in transgenic mice has been shown to reduce the severity of both age-related OA as well as in a cruciate ligament transection model of OA [15]. The aim of the present study was to investigate, for the first time, the co-expression and co-localization of CHI3L1 and lubricin in normal and osteoarthritic rat articular cartilage from femoral condyles after anterior cruciate ligament transection (ACLT), morphologically by both immunohistochemistry and double immuno-staining to correlate their modified expression to a specific grade of OA. The purpose of this study was to discover potential roles for both glycoproteins in OA pathophysiology and possibly to improve knowledge in this field in order to find new treatments for inflammatory joint diseases. To strengthen our morphological results we also performed gene expression analyses for both glycoproteins. The experimental model adopted in this study was the induction of a moderate OA in rat by ACLT. This model exhibits a number of characteristics similar to human post-traumatic OA and is widely used in this field of research [9]. In order to evaluate the experimental induction of OA, we performed the radiographic Kellgren–Lawrence OA severity scores, by photographic examination and X-ray microtomography imaging, and the histomorphometric evaluation, by the macroscopic Kraus’ modified Mankin score and the microscopic histopathology Osteoarthritis Research Society International (OARSI) system.

### **3. Results**

#### **3.1. Radiographic Analysis**

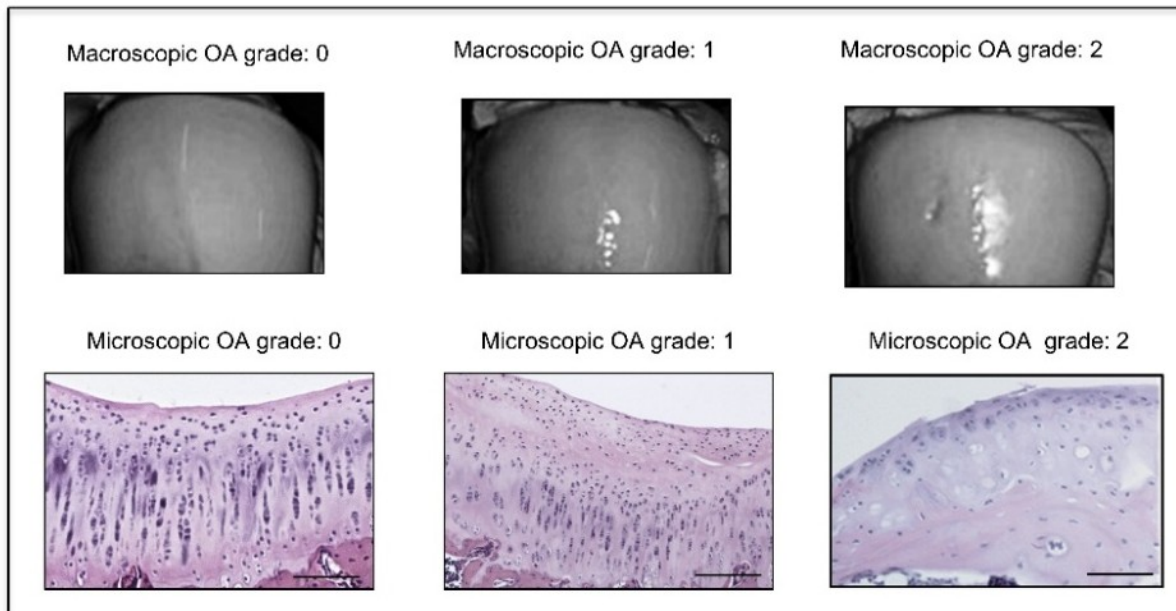
In agreement with the Kellgren and Lawrence classification, rats from control and sham groups (without ACLT) showed intact and normal cartilage structure without signs of cartilage degeneration (Kellgren–Lawrence score, Grade 0), while in the OA group (with ACLT) animals showed moderate OA of the knee (Kellgren–Lawrence score, Grade 2) as shown in Figure 1A.



**Figure 1.** (A) Kellgren and Lawrence score among groups; (B) Kraus' modified Mankin score among groups; and (C) Histopathology OARSI system among groups. Results are presented as the mean  $\pm$  SEM. Analysis Of Variance (ANOVA), was used to evaluate the significance of the results. \*  $p < 0.01$ , when compared to the control groups. OA: Osteoarthritis.

### 3.2. Histomorphometric Analyses

The histomorphometric parameters made in both control and sham groups, confirmed the presence of intact and normal cartilage structure without signs of cartilage degeneration indeed both Kraus' modified Mankin and Histopathology OARSI system scores were 0. In the OA group, instead, cartilage showed more serious pathological changes, consistent with moderate OA with a Kraus' modified Mankin score of 2 and Histopathology OARSI system score between 2 and 3. Thus, the OA group articular cartilage showed signs of degeneration significantly different from the control groups, as confirmed by Kraus' modified Mankin score (Figures 1B and 2), and histopathology OARSI system (Figures 1C and 2).



**Figure 2.** Macroscopic and microscopic articular cartilage degeneration between OA grade 0 to OA grade 2 according to macroscopic Kraus' modified Mankin score and microscopic histopathology OARSI system, Magnifications  $\times 20$ , scale bars 100  $\mu\text{m}$ .

### 3.3. Immunohistochemistry (IHC) Observations

CHI3L1 and lubricin were assessed by immunohistochemical staining in cartilage of all groups. Different patterns of immunopositive cells in the sets of specimens were observed (Table 1).

**Table 1.** Evaluation of lubricin and chitinase 3-like-1 (CHI3L1) immunostaining. Intensity immunostaining (IS); Percentage of immunopositive cells expressed by Extent Score (ES): <5% (0); 5%–30% (+); 31%–50% (++); 51%–75% (+++), and >75% (++++). ACLT: anterior cruciate ligament transection; OA: Osteoarthritis.

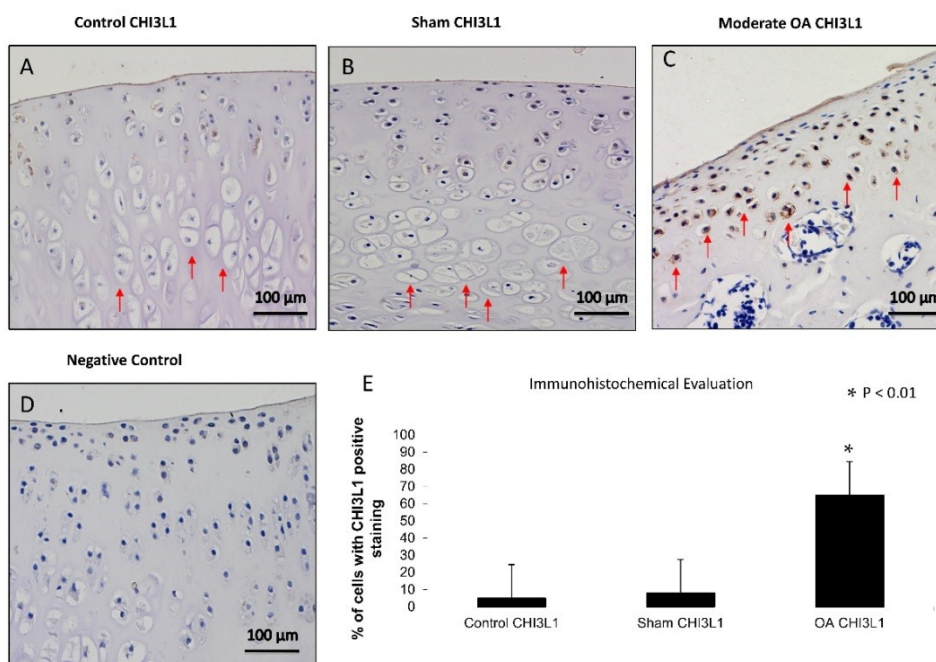
Groups	Lubricin	CHI3L1
Control rats without ACLT	Very strong immunostaining (ES = +++; IS = 4)	Weak/absent immunostaining (ES = +; IS = 1)
Sham operated control rats	Very strong immunostaining (ES = +++; IS = 4)	Weak/absent immunostaining (ES = +; IS = 1)
Experimental rats with ACLT (OA)	Weak/absent immunostaining (ES = +; IS = 1)	Strong immunostaining (ES = +++; IS = 3)

CHI3L1 overexpression was found in chondrocytes from the OA group mainly in the middle and deep zone of the cartilage rather than the superficial zone, while it was weakly expressed in

cartilage from superficial, middle and deep zone of control and sham groups (Figure 3). CHI3L1 immunolabeling was weak/absent (ES = +; IS = 1) in control and sham groups (Figure 3A,B) and was strong (ES = +++; IS = 3) in cartilage from OA group (Figure 3C). The negative control treated with PBS without the primary antibody (CHI3L1) did not show immunostaining (ES = 0; IS = 0) as shown in Figure 3D. The percentage of CHI3L1-positive cells was identified among groups ( $p < 0.01$  vs. others) as shown in Figure 3E. Interobserver agreement, measured as Cohen's kappa coefficient, was 0.88.

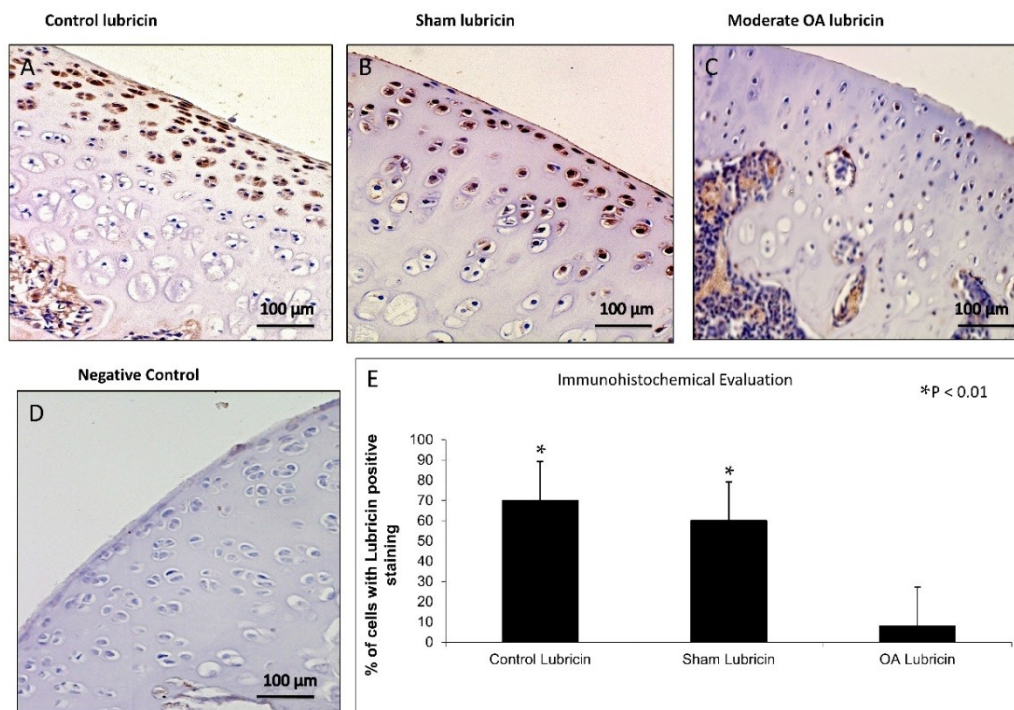
Moderate OA cartilage structural variations included a reduction of cartilage thickness of the superficial and the middle zones, clear deep fissures in the articular surface, and reduction of cells from the superficial, intermediate and deep zone, where chondrocytes are not arranged in columns. The tidemark is not intact in all its extension and the subchondral bone shows fibrillation.

In control and in sham groups lubricin overexpression was found mainly in chondrocytes from the superficial and middle zone of the cartilage rather than the deep zone, while it was weakly expressed in cartilage from superficial, middle and deep zone of osteoarthritic cartilage (Figure 4). Lubricin immunolabeling was very strong (ES = +++; IS = 4) in the control and in the sham groups (Figure 4A,B) and was weak/absent (ES = +; IS = 1) in cartilage from the OA group as shown in Figure 4C. The negative control treated with PBS without the primary antibody (lubricin) did not show immunostaining (ES = 0; IS = 0) as shown in Figure 4D. The percentage of lubricin-positive cells was observed among groups ( $p < 0.01$  vs. others) as shown in Figure 4E. Interobserver agreement, measured as Cohen's kappa coefficient, was 0.92.





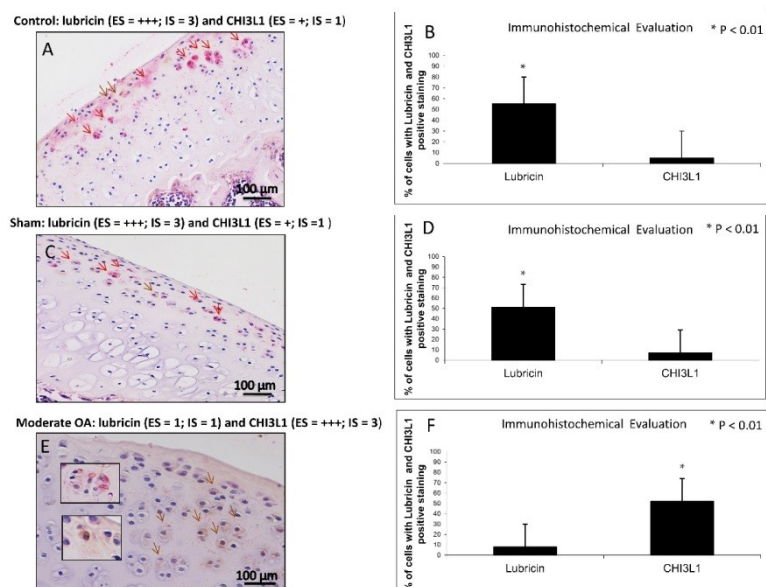
**Figure 3.** Evaluation of chitinase 3-like-1 (CHI3L1) immunostaining. (A,B) CHI3L1 immunohistochemistry specimen from control (A) and sham (B) rat femoral articular cartilage (without anterior cruciate ligament transection (ACLT)) showed a weak/absent (Extent Score (ES) = +; Intensity immunostaining (IS) = 1) immunostaining in cartilage superficial, middle and deep zone in which hypertrophic chondrocytes are evident (red arrows); (C) CHI3L1 immunohistochemistry specimen from moderate OA rat femoral articular cartilage (with ACLT) exhibited a strong (ES = +++; IS = 3) immunostaining in middle and deep cartilage zone (red arrows) and a reduction of cartilage thickness of the superficial and the middle zones is evident and in the deep zone the chondrocytes are not hypertrophic and are not arranged in columns; (D) The negative control treated with PBS without the primary antibody (CHI3L1) did not show immunostaining (ES = 0; IS = 0). (A–D) Magnifications  $\times 20$ ; Scale bars: 100  $\mu\text{m}$ ; (E) Immunohistochemical evaluation graph: percentage of CHI3L1 positive cells out of the total number of cells counted in control groups and in OA group. Results are presented as the mean  $\pm$  SEM. ANOVA was used to evaluate the significance of the results. \*  $p < 0.01$ , when compared to the control groups.



**Figure 4.** Evaluation of lubricin immunostaining. (A,B) Lubricin immunohistochemistry specimen from control (A) and sham (B) cartilage (without ACLT) showed a very strong (ES = +++; IS = 4) immunostaining in chondrocytes from superficial and middle zone of rat femoral articular cartilage; (C) Lubricin immunohistochemistry specimen from moderate OA cartilage (with ACLT) showed a weak/absent (ES = +; IS = 1) immunostaining in chondrocytes from rat femoral articular cartilage (superficial, middle and deep zone); (D) The negative control treated with PBS without the primary antibody (lubricin) did not show immunostaining (ES = 0; IS = 0). (A–D) Magnifications  $\times 20$ ; Scale bars: 100  $\mu\text{m}$ ; (E) Immunohistochemical evaluation graph: percentage of lubricin positive cells out of the total number of cells counted in control groups and in the OA group. Results are presented as the mean  $\pm$  SEM. ANOVA was used to evaluate the significance of the results. \*  $p < 0.01$ , when compared to the control groups.

### 3.4. Double Immunostaining Observations

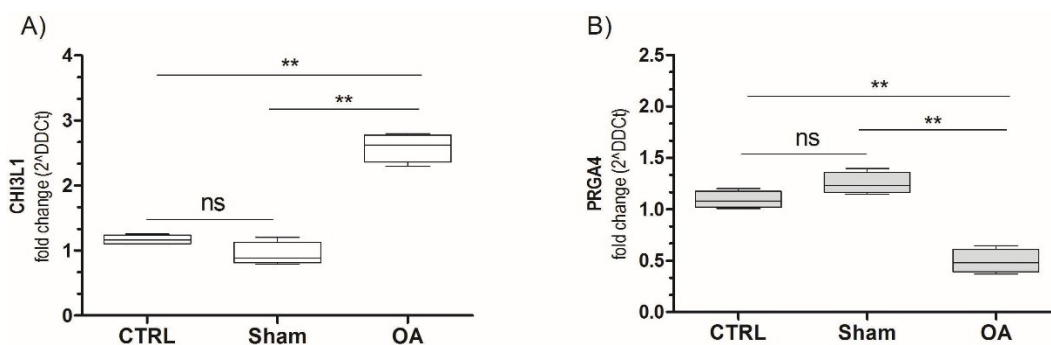
Double staining was performed with the specific antibodies against lubricin (red) and CHI3L1 (brown) to investigate their expression and to assess their distribution in normal and osteoarthritic articular cartilage tissue. This double stain technique allows us to identify the localization of these two studied proteins. With this technique, we have strengthened and confirmed our previous results, as can be seen from the data presented subsequently. In the control (Figure 5A) and in the sham (Figure 5C) groups, lubricin immunolabeling was strong (ES = +++; IS = 3, red staining), instead the expression of CHI3L1 was weak/absent (ES = +; IS = 1, brown staining). The percentage of lubricin and CHI3L1-positive cells was observed in the control group (lubricin vs. CHI3L1,  $p < 0.01$ ) as shown in Figure 5B. Interobserver agreement, measured as Cohen's kappa coefficient, was 0.90. The percentage of lubricin and CHI3L1-positive cells was observed in the sham group (lubricin vs. CHI3L1,  $p < 0.01$ ) as shown in Figure 5D. Interobserver agreement, measured as Cohen's kappa coefficient, was 0.86. In the OA group (Figure 5E) lubricin immunolabeling was weak/absent (ES = +; IS = 1, red staining), instead the expression of CHI3L1 was strong (ES = +++; IS = 3, brown staining). The expression of CHI3L1 increases with the intensification of the cartilage damage. The percentage of lubricin and CHI3L1-positive cells was observed in moderate OA (lubricin vs. CHI3L1,  $p < 0.01$ ) as shown in Figure 5F. Interobserver agreement, measured as Cohen's kappa coefficient, was 0.96. Lubricin/CHI3L1 ratio was quantified in control and OA groups. The ratio clearly demonstrated the increased expression of lubricin in control cartilage and the increased expression of CHI3L1 in OA cartilage, conversely the decreased expression of lubricin was found in OA cartilage and the decreased expression of CHI3L1 was found in the control cartilage.



**Figure 5.** Evaluation of lubricin and CHI3L1 double staining in control and in moderate OA cartilage. (A,C) In control and in sham groups lubricin immunolabeling was strong (ES = +++; IS = 3, red staining, red arrows), instead the expression of CHI3L1 was weak/absent (ES = +; IS = 1, brown staining, brown arrow); (E) In moderate OA, lubricin immunolabeling was weak/absent (ES = +; IS = 1, red staining, red arrows), instead the expression of CHI3L1 was strong (ES = +++; IS = 3, brown staining, brown arrows); (A,C,E) Magnifications  $\times 20$ ; Scale bars: 100  $\mu\text{m}$ ; inserts: magnifications  $\times 40$ ; Scale bars: 50  $\mu\text{m}$ ; (B) Immunohistochemical evaluation graph: percentage of lubricin and CHI3L1-positive cells out of the total number of cells counted in the control group; (D) Immunohistochemical evaluation graph: percentage of lubricin and CHI3L1-positive cells out of the total number of cells counted in the sham group; (F) Immunohistochemical evaluation graph: percentage of lubricin and CHI3L1-positive cells out of the total number of cells counted in the moderate OA group. Results are presented as the mean  $\pm$  SEM. Student's *t* test, was used to evaluate the significance of the results. \*  $p < 0.01$ , when compared lubricin vs. CHI3L1.

### 3.5. Chitinase 3-Like-1 (CHI3L1) and Lubricin mRNA Expression in Osteoarthritic Rat Cartilage Model

The PCR analysis demonstrated that the CHI3L1 mRNA expression significantly increased in OA cartilage (fold 3.2,  $p < 0.001$ ) compared to the sham and control groups ( $p < 0.001$ ) as shown in Figure 6A. Opposite results were obtained for lubricin, in fact lubricin mRNA expression was significantly reduced in OA cartilage (fold 0.47,  $p < 0.001$ ) compared to the sham and control groups ( $p < 0.001$ ) as shown in Figure 6B. Non-significant results were obtained comparing the sham and the control. These data unequivocally confirm the results obtained by immunohistochemistry, supporting the possible relationship between the opposite CHI3L1- and lubricin-expression and the progression of OA, in particular grade 2/3 of OA.

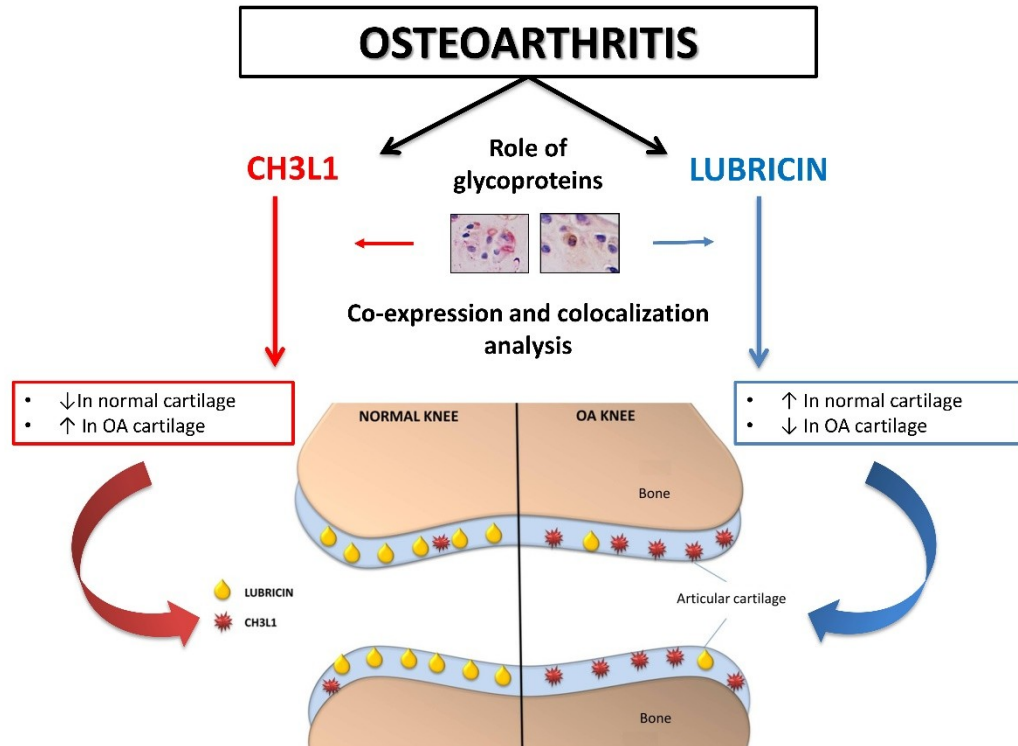


**Figure 6.** CHI3L1 (A) and PRG4 (B) mRNA expression in osteoarthritic rat cartilage model. Expression levels of CHI3L1 and PRG4 (lubricin) in cartilage of osteoarthritis rats. Total RNA was extracted as indicated in Materials and Method and CHI3L1/PRG4 expression was measured by real-time PCR. Data are expressed as mean  $\pm$  SD of at least three independent experiments. \*  $p < 0.01$ , \*\*  $p < 0.001$ , compared to sham and control. CTRL: control; ns: not significant.

#### 4. Discussion

In this study the histomorphometric results in control and sham groups (without ACLT), showed an intact and normal cartilage structure without signs of cartilage degeneration (Kellgren–Lawrence OA severity scores, Grade 0; Kraus’ modified Mankin score, Grade 0; Histopathology OARSI system, Grade 0), whilst in the OA group (with ACLT) cartilage showed more serious pathological changes, as horizontal cleavage tears or flaps and deep lesions, confirming moderate OA (Kellgren–Lawrence OA severity scores, Grade 2; Kraus’ modified Mankin score, Grade 2; Histopathology OARSI system, Grades 2/3). The development of articular degenerative processes in OA group was clear and significantly different from the control groups, as confirmed by the Kellgren–Lawrence OA severity scores, Kraus’ modified Mankin score and histopathology OARSI system. These results were supported by immunohistochemistry, double-staining and mRNA examination. In this study the immunoexpression and the mRNA levels of lubricin increased in normal cartilage and decreased in OA cartilage, while the immunoexpression and the mRNA levels of CHI3L1 increased in OA cartilage and decreased in normal cartilage (Figure 7). We found a negative correlation between the expression of the chondroprotective lubricin and the pro-inflammatory CHI3L1 in normal cartilage and in an experimental model of OA cartilage.

OA is a disease with a high incidence and prevalence, with an expected increase in the number of affected individuals, particularly due to the aging of the population, but also due to the increasing prevalence of obesity and a sedentary lifestyle [16,17]. It is becoming clear that articular tissues other than cartilage play an important role in the process of OA; it is regarded as a whole joint disease. In the last decades the interest of biochemists and biologists focused on glycoproteins and it is well known that there are many biomarkers and glycoproteins involved in the OA pathophysiological process [18–23]. This improved attention is partly due to the fact that glycoproteins were revealed to be abundant in living organisms [24]. Many glycoproteins have structural functions, form connective tissues such as collagen, and are used as protective agents and lubricants [24–26]. These molecules are composed of a peptide chain with one or more carbohydrate moieties linked *N*-glycosidically or *O*-glycosidically to their constituent protein. According to these structures, glycoproteins are divided in two categories. Fine structural differences within these broader categories, account for the variety of functions among glycoproteins. Specific enzymes regulate glycoprotein synthesis and degradation [24–26].



**Figure 7.** Graphical representation of the involvement of two glycoproteins (CHI3L1 and lubricin) in normal and osteoarthritic articular cartilage. Red arrows (CHI3L1 pathway); Blue arrows (lubricin pathway).

In this study we investigated the co-expression and the co-localization of two fundamental glycoproteins [27,28] involved in cartilage, CHI3L1 and lubricin. CHI3L1, or YKL-40, is a glycoprotein produced by articular chondrocytes, synoviocytes and macrophages. Serum and SF (Synovial fluid) levels of CHI3L1 are increased in inflammatory diseases and correlate with the grade of cartilage degeneration in rheumatoid arthritis. CHI3L1 is a candidate auto antigen in rheumatoid arthritis related to the ability of cells to respond to and cope with variations in their environment [29]. Recently, authors stated that chondrocytes of human osteoarthritic cartilage secrete the inflammation associated chitolectin CHI3L1 [30]. CHI3L1 is a major secretory protein of human chondrocytes in cell culture. CHI3L1 mRNA is not detectable in normal human cartilage, though it is expressed in cartilage from patients with rheumatoid arthritis [31]. Moreover, CHI3L1 levels in SF but not serum were independently and positively related to clinical findings, such as pain and physical disability, in knee OA patients; thus it could represent a potential biomarker of severity of OA [32].

Another important glycoprotein is represented by lubricin. Lubricin is a chondroprotective, mucinous glycoprotein, the product of the *proteoglycan 4 (PRG4)* gene [33]. It has been found in several tissues including the synovial membranes and SF [34], the superficial zone of articular

cartilage [35], tendon and ligament [36], disc and meniscus [37,38]. The lubricin role is maintaining joint integrity. Lubricin is a lubricating glycoprotein present in SF, specifically produced and expressed by articular chondrocytes of the superficial zone. It is recognized as playing a major protective role, preventing cartilage wear and synovial cell adhesion and proliferation and reducing the amount of friction of the articular cartilage surface [39]. It has been also demonstrated that physical activity promotes the expression of lubricin and attenuates the cartilage degeneration process, suggesting again its important role in chondroprotection [40,42]. Indeed, the lack of lubricin secretion may be involved in the pathology of OA. Authors demonstrated that considerable negative regulation of lubricin, as well as of other proteoglycans and SF biomarkers, develops in the human knee meniscus and anterior cruciate ligament (ACL) in the acute phase of joint injury, highlighting its involvement in articular injury [38,43]. When recombinant lubricin was injected in injured joints, in a study on animal model of OA, its improved chondroprotection, suggesting its potential use in new approaches for the treatment of OA and other cartilage disease [44–48].

The results of the present research are in accordance with our previous studies [9,35,39]. Here, we demonstrated for the first time the co-expression and the co-localization of these two glycoproteins in *in vivo* normal cartilage, and in an experimental model of OA cartilage. CHI3L1 overexpression was found in chondrocytes from the OA group mainly in the middle and deep zone of the cartilage rather than the superficial zone, while it was weakly expressed in cartilage from the superficial, middle and deep zones of control and sham group cartilage. In contrast, in the control and in the sham groups, lubricin overexpression was found mainly in chondrocytes from the superficial and middle zone of the cartilage rather than the deep zone, while it was weakly expressed in cartilage from superficial, middle and deep zone of osteoarthritic cartilage.

Our results indicate that CHI3L1 and lubricin might be considered as potential natural agents for providing therapeutic protective effects in joint inflammation, and/or may promote cartilage preservation in degenerative disorders of articular cartilage.

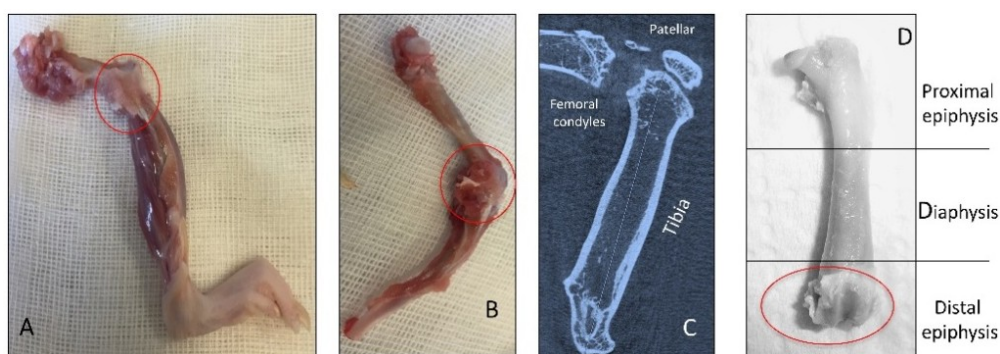
The findings of our study suggest that the two glycoproteins, CHI3L1 and lubricin, could be functionally associated with the development of OA, in particular with grade 2/3 of OA evidenced in histomorphometric analysis of our samples, and could be used as biomarker matches, suggesting that in the future they could be helpful to stage the severity and progression of the disease.

## **5. Materials and Methods**

### **5.1. Breeding and Housing of Animals**

Thirty 3-month-old healthy male Wistar Outbred Rats (Charles River Laboratories, Milan, Italy), with an average body weight of  $160 \pm 80$  g, were used for this study. Rats were housed in

polycarbonate cages (cage dimensions: 10.25"W × 18.75"D × 8"H) at controlled temperature (20–23 °C) and humidity during the whole period of the research, with free access to water and food and photoperiod of 12 h light/dark. Surgical procedures for ACLT were performed in accordance with the method previously described [1,9,49]. The ACLT surgery procedure was made under total anesthesia, 30 mg/kg Zoletil 100 + altadol 5 mg/kg + maintenance mixture of O<sub>2</sub> and isoflurane 2%–2.5%, (Vibrac, Milan, Italy). The anterior portion of the left hind limb was shaved with an electric clipper, and cleaned with povidone iodine (Sceptre Medical, New Delhi, India). The skin around the knee cap was vertically incised along the medial border of the knee cap. The patella was displaced laterally to expose the anterior cruciate ligament. Then, the anterior cruciate ligament was cut with surgical scissors without injury to the cartilage of the tibia. The patella was then replaced back, and the fascia and skin were closed with a 3–0 polydioxanone suture. A single dose of antibiotic Convenia<sup>®</sup> 0.1 mL/kg, (Vibrac, Milan, Italy) cream was applied to avoid postoperative infection. After surgery, free cage movement without joint immobilization was permitted to all animals. The 30 animals were divided in two groups: the control group without ACLT (10 rats) and the OA group with ACLT (20 rats). The control group was composed of two subgroups: control normal group (5 rats) without surgical treatment and sham-operated control group (5 rats), receiving the same surgical procedure as the experimental group, without ACLT. The OA group instead consisted of 20 rats submitted to ACLT surgical treatment inducing the OA model. During the experiment the possible suffering of the animals was monitored through the clinical conditions of the animal (fur appearance, weight, consumption of food and water, lameness) evaluated once a day. The animals from all groups at 2 months after the surgical procedures were sacrificed by intracardial Pentothal<sup>®</sup> injection 30–40 mg/kg (Biochemie, Kundl, Austria); under Furane 2%<sup>®</sup>-narcosis (Abbott Laboratories, Maidenhead, Berks, UK). The pre-operative examinations included physical examination, photographical examination and X-ray microtomography imaging (Figure 8C). The radiographic analyses demonstrating the joint pathology were preliminary to explantation of both femurs that were cleaned from soft tissues and used to perform histomorphometric evaluations (Figure 8A,B,D). Each sample of articular cartilage, from the three groups, was divided in fragments in order to perform histological, immunohistochemical and gene expression analyses. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania (Protocol n. 125 of the 01.07.2011, Italian Ministry of Health). The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).



**Figure 8.** Representation of *in vivo* explanted knee joint bones (red circles). (A,B) Knee OA bones joint after explantation, not cleaned of soft tissues; (C) X-ray microtomography imaging of the OA knee bones joint; (D) OA femur cleaned of soft tissues.

## 5.2. Radiographic Analysis

The pre-operative examinations included physical examination, photographical examination and X-ray microtomography imaging (Bruker, Milan, Italy). We chose our samples in accordance with the radiographic OA severity evaluated by the Kellgren and Lawrence score [50] to classify the severity of knee OA that showed radiographic worsening over time. Two blinded investigators (two anatomical morphologists) made the analyses, and the evaluations were assumed correct if no statistically significant difference was showed between the investigators. The Kellgren and Lawrence system provides a score of severity from 0 to 4: Grade 0, no radiographic features of OA are present; Grade 1, doubtful joint space narrowing (JSN) and possible osteophytic lipping; Grade 2, definite osteophytes and possible JSN on anteroposterior weight-bearing radiograph; Grade 3, multiple osteophytes, definite JSN, sclerosis, possible bony deformity; Grade 4, large osteophytes, marked JSN, severe sclerosis and definite bony deformity. The inter-observer variability between two observers for the Kellgren–Lawrence score showed a similar good intra-class correlation coefficient ( $ICC > 0.84$ ). Repeat scoring by investigators showed very good agreement ( $ICC > 0.90$ ).

## 5.3. Histomorphometric Analysis

The femurs explantation procedure and the subsequent cleaning of soft tissues was performed as previously described [51]. Samples from all rats (both medial and lateral femoral condyles of untreated and surgically treated animals) were used for the histomorphometric analysis. Histomorphometry was performed with image analysis, Kontron KS 300 software (Kontron Electronics, Eching bei Munchen, Germany) by three blinded investigators (two anatomical



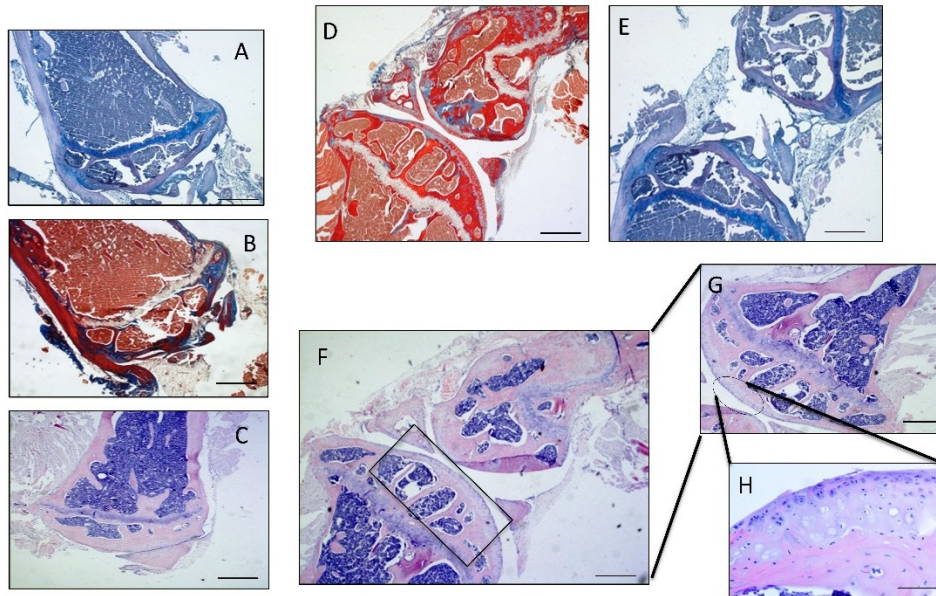
morphologists and one histologist). Evaluations were assumed correct if there were no statistically significant differences between the investigators. Fifteen fields randomly selected from each section were analyzed. The semi-quantitative grading criteria of macroscopic Kraus' modified Mankin score [52,53] and microscopic histopathology OARSI system [54,55] were used. The inter-observer variability among 3 observers for the Mankin system showed a good intra-class correlation coefficient ( $ICC > 0.92$ ) as for the OARSI system ( $ICC > 0.89$ ). Repeat scoring by investigators showed very good agreement ( $ICC > 0.94$ ).

The Kraus' modified Mankin score provides grades from 0 to 4: Grade 0, normal cartilage; Grade 1, minimal articular damage; Grade 2, articular cartilage damage affecting up to 30% of the articular surface; Grade 3, loss of up to 50% of the articular cartilage; Grade 4, severe loss of cartilage affecting more than 50% of the articular surface.

The Histopathology OARSI system provides grades from 0 to 6: Grade 0, normal articular cartilage; Grade 1, intact surface; Grade 2, surface discontinuity; Grade 3, vertical fissures extending into mid zone; Grade 4, erosion; Grade 5, denudation; Grade 6, deformation.

#### **5.4. Histology and Histochemistry Analysis**

Some fragments of articular cartilage samples were fixed in 10% neutral buffered-formalin (Bio-Optica, Milan, Italy), following overnight washing and routinely embedded in paraffin as previously described [1]. Samples were positioned in the cassettes in the same direction after wax infiltration. A rotary manual microtome (Leica RM2235, Milan, Italy) was used to cut 4–5  $\mu\text{m}$  thick sections from paraffin blocks that were mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. After dewaxing in xylene, the slides were hydrated using graded ethanol, and stained for histological evaluation by Hematoxylin and Eosin (Figure 9C,F–H) and Masson's Trichrome (Figure 9B,D) (Bio-Optica) staining for cell identification and the detection of structural alterations. Then the slides were analysed through toluidine blue staining (Figure 9A,E) (Fluka, St. Louis, MO, USA) to assess synthesis of sulfated glycosaminoglycan (GAG) containing proteoglycans (basing on the intensity of staining), in order to evaluate the experimental induction of OA according to the histopathology OARSI system. The samples were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and a digital camera (AxioCam MRc5, Carl Zeiss) was used to take the pictures.



**Figure 9.** (A) Micrograph of the OA distal epiphysis of the femur stained with Toluidine Blue, Magnification  $\times 10$ ; (B) Micrograph of the OA distal epiphysis of the femur stained with Masson's Trichrome, Magnification  $\times 10$ ; (C) Micrograph of the OA distal epiphysis of the femur stained with Hematoxylin and Eosin, Magnification  $\times 10$ ; (D) Micrograph of the OA knee joint stained with Masson's Trichrome, Magnification  $\times 5$ ; (E) Micrograph of the OA knee joint stained with Toluidine Blue, Magnification  $\times 5$ ; (F) Micrograph of the OA knee joint stained with Hematoxylin and Eosin, Magnification  $\times 5$ ; (G) Magnification of J  $\times 10$ ; (H) Magnification  $\times 20$ , Scale bars: 100  $\mu\text{m}$ .

### 5.5. Immunohistochemistry (IHC) Analysis

Some fragments of articular cartilage were processed for immunohistochemical analysis as previously described [56]. Briefly, after dewaxing in xylene, the slides were hydrated through graded ethanol and incubated for 30 min in 0.3%  $\text{H}_2\text{O}_2$ /methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica). The sections were then heated (5 min  $\times$  3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica), using a microwave oven (750 W) to unmask antigenic sites. The blocking step to prevent non-specific binding of the antibody was performed before application of the primary antibody with 5% bovine serum albumin (BSA, Sigma, Milan, Italy) in PBS for 1 h in a moist chamber. After blocking, the sections were incubated overnight at 4  $^\circ\text{C}$  with goat polyclonal GP-39 antibody (CHI3L1), work dilution in PBS 1:100 (sc-30465, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and with rabbit polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Bio-Optica) for 10 min. Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-goat and anti-rabbit were used as secondary antibodies) and then detected with peroxidase labeled streptavidin, both incubated for 10 min at room temperature (LSAB+ System-HRP, K0690, Dako, Denmark). The

immunoreaction was visualized by incubating the sections for 2 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The samples were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed, Laboratories Inc., San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss).

## 5.6. Double Immuno-Staining Analysis

Double staining was performed with the specific antibodies against lubricin (red) and CHI3L1 (brown) to investigate their expression and to assess their distribution in normal and osteoarthritic articular cartilage tissue. The procedure was performed according to the manufacturer's instructions using kit EnVision™ G/2 Doublestain System (Dako, Glostrup, Denmark), Rabbit/Mouse DAB+/Permanent Red (K5261; Dako, Glostrup, Denmark). Briefly, the slides were incubated with Dual Endogenous Enzyme Block solution (kit EnVision™ G/2 Doublestain System), containing 0.5% hydrogen peroxide, detergents, enzyme inhibitors and preservative, pH 2, for 5 min before being rinsed with PBS (Bio-Optica) for 5 min. After, the sections were incubated with goat polyclonal GP-39 antibody (CHI3L1; sc-30465, Santa Cruz Biotechnology, Inc.) working dilution 1:100 in PBS. Immune complexes were then treated with dextran polymer conjugated with horseradish peroxidase and affinity-isolated immunoglobulins, Polymer/HRP (kit EnVision™ G/2 Doublestain System), for 10 min. Immunoreactivity was visualized by incubating the sections for 2 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB+ Working Solution, prepared by thoroughly mixing 1 mL of DAB+ Substrate Buffer with 1 drop (25–30 µL) of DAB+ Chromogen; kit EnVision™ G/2 Doublestain System). Then the slides were treated with blocking solution, Doublestain Block (kit EnVision™ G/2 Doublestain System), for 3 min, before incubating them with the second rabbit polyclonal anti-lubricin antibody (ab28484; Abcam), diluted 1:100 in PBS (Bio-Optica) for 10 min. The slides were then incubated with dextran polymer coupled with secondary antibodies against goat and rabbit immunoglobulins, Rabbit/Mouse link (kit EnVision™ G/2 Doublestain System), for 10 min and after, with dextran polymer conjugated with alkaline phosphatase and affinity-isolated immunoglobulins, Polymer/AP (kit EnVision™ G/2 Doublestain System), for 10 min. At this point, the immunoreactivity for the second antibody was visualized by using the Permanent Red Working Solution, prepared by thoroughly mixing 100 parts of Permanent Red Substrate Buffer with 1 part of Permanent Red Chromogen (kit EnVision™ G/2 Doublestain System), for 10 min. The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden)

and mounted in Dako Glycergel™ Mounting Medium (C0563; Dako, Glostrup, Denmark). The stained slides were observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss).

### 5.7. Evaluation of Immunohistochemistry

The CHI3L1 and lubricin-staining status were identified as either negative or positive. As previously described, immunohistochemical staining was defined positive if brown or red chromogens were detected on the edge of the hematoxylin-stained cell nucleus, within the cytoplasm or in the membrane [56]. Light microscopy was used to evaluate stain intensity and the percentage of immunopositive cells. Intensity of staining (IS) was evaluated on a 4 grades scale (0–4), as following: no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, very strong staining = 4. Three investigators (2 anatomical morphologists and one histologist) independently evaluated the percentage of antibodies immunopositive cells through the five categories of Extent Score (ES): <5% (0); 5%–30% (+); 31%–50% (++); 51%–75% (+++), and >75% (++++). Counting was performed under Zeiss Axioplan light microscope at x200 magnification. If disputes concerning the interpretation occurred, the case was revised to reach a unanimous agreement, as previously described [9]. A digital camera (Canon, Tokyo, Japan) at x20, x40 and x60 magnifications was used to take digital pictures. In this study positive controls, consisted of rat cartilage tissue, and negative control sections, treated with PBS without the primary antibodies, were performed to test the specific reaction of primary antibodies used at a protein level. Positive immunolabeling for antibodies were nuclear/cytoplasmic.

### 5.8. Computerized Morphometric Measurements and Image Analysis

Image analysis software (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling, was used to calculate the percentage area stained with CHI3L1 and lubricin antibodies in 15 fields, randomly selected from each section. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, using objective lens of magnification  $\times 20$  *i.e.*, total magnification 400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss). Three blinded investigators (2 anatomical morphologists and one histologist) made the evaluations that were assumed to be correct if values have not statistically significant difference [57]. If disputes concerning interpretation occurred, unanimous agreement was reached after sample re-evaluation.

## 5.9. RNA Isolation and Preparation

Some fragments of articular cartilage were immersed in QIAzol (Qiagen, Mississauga, ON, Canada). After homogenization, total RNA was isolated using the Lipid Tissues Mini Kit (Qiagen, Mississauga, ON, Canada), according to the instructions of the manufacturer (Qiagen). RNA quantity was evaluated using the RiboGreen Assay (Molecular Probes, Burlington, ON, Canada), and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

## 5.10. Gene Expression Analysis by Real-Time PCR (qRT-PCR)

Total RNA extracted (500–800 ng per sample) from articular cartilage was reverse-transcribed with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Milan, Italy) in a 20  $\mu$ L reaction solution. Quantitative RT-PCR was performed using one-twentieth of the RT products and platinum SYBR Green qPCR SuperMix UDG with Rox (Invitrogen Life Technologies, Milan, Italy). The primers used are shown in Table 2. The reaction was followed by a melting curve protocol according to the specifications of the ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA). Rat  $\beta$ -Actin (ACTB) was used as a housekeeping gene for normalization. Data are presented as mean  $\pm$  SD of at least three independent experiments. Differences were analyzed by Student's *t* test, with  $p < 0.01$  being considered statistically significant.

**Table 2.** Primers used in gene expression analysis by real-time PCR (qRT-PCR). PRG4: proteoglycan 4; ACTB:  $\beta$ -Actin; Ta: thymine and adenine.

Primers	Forward	Reverse	Ta	Size
PRG4	CTACAACAGCTTCTGCGAAGAA	GATTTGGGTGAACGTTTGGTGG	60	117
CH13L1	GAGCTGCTTCCCAGATGCCC	CATGCCATACAGGGTTACGTC	60	121
ACTB	CATGTACGTAGCCATCCAGG	CTCTCAGCTGTGGTGGTGAA	57	225

## 5.11. Statistical Analysis

Statistical analysis was performed using GraphPad InStat<sup>®</sup> Biostatistics version 3.0 software (GraphPad Software, Inc. La Jolla, CA, USA). Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Student's *t* test was used for comparisons between two means, while analysis of variance (ANOVA) and Bonferroni's test were used for comparison between more than two groups. *p*-values of less than 0.05 ( $p < 0.05$ ) was considered statistically significant; *p*-values of less than 0.01 ( $p < 0.01$ ) was considered highly statistically significant; *p*-values of less than 0.001 ( $p < 0.001$ ) was considered extremely statistically significant. Data are presented as the mean  $\pm$  SEM. Cohen's  $\kappa$  was applied to measure the agreement between the two-blinded observers and averaged to evaluate overall agreement.

## 6. Conclusions

The therapeutic management of OA remains a challenge for physicians. In this setting the basic sciences are involved in finding information regarding the pathophysiological process of this common and severe disease. In this study we highlighted a possible cross-link between two pathological aspects of the inflammatory process and the altered lubricating ability occurring in the joint tissue following chronic disease, such as OA. Our findings might motivate further studies about the link between the two important aspects of OA, inflammatory process through the study of CHI3L1 and lubricating ability through the study of lubricin. However, further studies are needed to understand the exact mechanisms in which these molecules are involved in order to design therapies or treatments to prevent or attenuate the osteoarthritic process.

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**Author Contributions:** All authors have made substantial intellectual contributions to the conception and design of the study as well as data acquisition, analysis and interpretation. Michelino Di Rosa and Francesca Maria Trovato carried out the experimental *in vitro* work, study execution and literature research. Marta Anna Szychlinska and Lidia Puzzo carried out the experimental *in vivo* work. Paola Castrogiovanni and Rosy Leonardi carried out the study execution, contributed to data collection and provided technical assistance. Giuseppe Musumeci conceived the study design, supervised the manuscript writing, planning and editing, coordinated the execution of experimental procedures and the analysis and discussion of results. All authors contributed to data interpretation and manuscript preparation. All authors approved the final submitted version.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Abbreviations:** ACLT: Anterior cruciate ligament transection; CHI3L1 YKL-40: Chitinase 3-like-1; GP-39: Glycoprotein 39; OA: Osteoarthritis; PRG4: Proteoglycan 4; SF: Synovial fluid; SZP: Superficial zone protein.

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## CHAPTER VII

## **Biosynthesis of collagen I, II, RUNX2 and lubricin at different time points of chondrogenic differentiation in a 3D *in vitro* model of human adipose tissue derived mesenchymal stem cells**

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## 1. Abstract

The first aim of the study was to identify the most appropriate time for differentiation of adipose tissue-derived mesenchymal stem cells (MSCs) in chondrocytes, through the self-assembly process. For this purpose, the expression of some chondrocyte markers, such as collagen type I, collagen type II, RUNX2 and lubricin was investigated at different times (7, 14, 21 and 28 days) of chondrogenic differentiation of MSCs, by using immunohistochemistry and Western blot analysis. The second aim of the study was to demonstrate that the expression of lubricin, such as the expression of collagen type II, could be a possible biomarker for the detection of chondrocytes well-being and viability in the natural self-assembling constructs, called ‘cell pellets’. Moreover, histology (hematoxylin and eosin) and histochemistry (alcian blue staining) methods were used to assess the chondrogenic differentiation of MSCs. The results showed that after 21 days the differentiated chondrocytes, when compared with MSCs cultured without chondrogenic medium (CD44, CD90 and CD105 positive; CD45, CD14 and CD34 negative), were able to produce significant quantities of collagen type I, collagen type II, and lubricin, suggesting the hyaline cartilage formation. Moreover, during the differentiation phase, the cells showed a reduced expression of RUNX2, protein expressed by osteoblasts. Therefore, it has been proved that 21 days is the optimum time for the implantation of chondrocytes differentiated from adipose tissue-derived MSCs. This information could be useful for the future development of cell-based repair therapies for degenerative diseases of articular cartilage.

**Keywords:** Adipose tissue, Cartilage, Chondrocytes, Collagen, Lubricin, Mesenchymal Stem Cells, Regeneration, RUNX2

**Abbreviations:** ANOVA, analysis of variance; AML-3, acute myeloid leukemia 3 protein; BCA, bicinchoninic acid assay; BMP-2, bone morphogenetic protein-2; BSA, bovine serum albumin; CBF-alpha-1, core-binding factor subunit alpha-1; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; ES, Extent Score; FBS, Fetal bovine serum; FGF-23, fibroblast growth factor-23; GAG, glycosaminoglycans; H&E, Hematoxylin and Eosin; IHC, Immunohistochemistry; IS, intensity of staining; MFI, Mean fluorescence intensity; MSCGS, Mesenchymal Stem Cells growth supplement; MSCs, mesenchymal stem cells; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RUNX2, Runt-related transcription factor 2; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TBST, TBS-Tween; T-PER, Tissue protein extraction reagent.

## 2. Introduction

Pluripotent stem cells offer the possibility as a renewable source of cells and tissues, to be used in a myriad of diseases, conditions and disabilities, including osteoarthritis (OA). Defects of load-bearing connective tissues, such as articular cartilage, often result from trauma, degenerative disease or just as a result of normal wear and tear associated with aging. The poor self-repair capacity of cartilage tissue, has prompted the development of a variety of therapeutic approaches, such as tissue engineering (Musumeci et al., 2014a; Mobasheri et al., 2009; Johnstone et al., 2013).

Mesenchymal stem cells (MSCs) can be easily withdrawn from a variety of tissues including bone marrow, synovial membranes, umbilical cord blood, adipose tissue, etc. (Winter et al., 2003; Guilak et al., 2010) and the involved procedures are less invasive and destructive than those used in the articular cartilage sampling (Musumeci et al., 2013a). The differentiation of MSCs into different cell types, in this case in chondrocytes, depends on the local microenvironment, growth factors, extracellular matrix and mechanical forces (Wang et al., 2003; Miyanishi et al., 2006; Richardson et al., 2010; Johnstone et al., 2013). Adipose tissue is made up of several depots located in two compartments of the body: some of them are placed below the skin (subcutaneous depots) and other in the trunk (visceral depots) (Cinti, 2011). Furthermore, adipose tissue is an alternative stem cell source that can be obtained by less invasive methods and in larger quantities than bone marrow (Musumeci et al., 2011a). Moreover, the adipose tissue-derived stem cells can be easily grown under standard culture conditions. In our recent study, we focused on the isolation, cultivation and characterization of human MSCs from adipose tissue, but their differentiation in chondrocytes has been evaluated only at 24<sup>th</sup> day (Musumeci et al., 2011a).

Lubricin is a chondroprotective mucinous glycoprotein that plays an important role in joint lubrication and synovial homeostasis, preventing cartilage wear and synovial cell adhesion (Drewniak et al., 2012; Musumeci et al., 2013b; Waller et al., 2013). This important protein is found in several joint tissues including synovial membranes and fluid (Elsaid et al., 2005; Rhee et al., 2005), superficial zone of articular cartilage (Schumacher et al., 1994; Jones et al., 2007; Musumeci et al., 2013c), tendon (Rees et al., 2002; Funakoshi et al., 2008), ligament (Teeples et al., 2011; Leonardi et al., 2012a), disc (Leonardi et al., 2012b; Leonardi et al., 2012c) and meniscus (Zhang et al., 2011; Musumeci et al., 2013d; Musumeci et al., 2014b). Lubricin was also studied in an “*in vitro study*” directly on cells and in combination with artificial three-dimensional scaffolds (Musumeci et al., 2011b). Recently, several studies have been focused on the expression of lubricin in different experimental conditions, in particular in conjunction with physical activity (Musumeci et al., 2013b; Musumeci et al., 2013c; Musumeci et al., 2014c). Since lubricin has a fundamental role in maintaining the homeostasis of the articular cartilage and in preventing its degeneration,

authors hypothesized that its expression would decrease in senescent chondrocytes and that it could be evaluated as a new specific chondrocyte senescence marker (Musumeci et al., 2014c; Musumeci et al., 2014d; Musumeci et al., 2014e).

Runt-related transcription factor 2 (RUNX2), also known as core-binding factor subunit alpha-1 (CBF-alpha-1) or acute myeloid leukemia 3 protein (AML-3), is an essential transcription factor for osteoblast differentiation, bone formation and skeletal morphogenesis (Enomoto et al., 2000). RUNX2-deficient mice showed a complete lack of bone formation due to the maturational arrest of osteoblasts. Furthermore, in this model the chondrocyte maturation was disturbed. Indeed, RUNX2 regulates also hypertrophic chondrocyte differentiation (Enomoto et al., 2000). Authors demonstrated that chondrocyte maturation is dependent on RUNX2 and RUNX3, and that the absence of both of them causes a complete lack of chondrocyte maturation (Yoshida et al., 2004). The first aim of this study was to determine the best time required to obtain functionally mature chondrocytes from adipose tissue-derived MSCs treated with chondrogenic medium. For this purpose we assessed the expression of some markers, such as collagen type I, II, RUNX2 and lubricin, during the MSCs differentiation phase at 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> day by immunohistochemistry and Western blot analysis. The second aim of this study was to demonstrate that the presence of lubricin could be used as a biomarker for the detection of chondrocytes well-being and viability in a natural self-assembly constructs, called ‘cell pellets’. Hopefully in the future the well-differentiated chondrocytes could be used as a therapeutic treatment to reduce the progression of cartilage degenerative diseases.

### **3. Materials and methods**

#### **3.1. Patients**

Adipose tissue was collected from 40 donors, 10 males and 30 females (from 22 to 30 years of age and mean body mass index of 27 + 3.8) undergoing liposuction procedures for cosmetic surgery. Lipoaspirates were obtained under an approved Institutional Review Board protocol and after the informed consent from the patients of the Cannizzaro Hospital in Catania.

#### **3.2. Culture of human mesenchymal stem cells (MSCs) from adipose tissue**

The lipoaspirate from each patients (50-100 ml) was washed with sterile phosphate-buffered saline (PBS) (Invitrogen, Milan, Italy) to remove red blood cells and debris, incubated for 3 hours at 37 °C with an equal volume of serum-free DMEM low glucose (DMEM-1g; Lonza, Milan, Italy) containing 0.075% of type I collagenase (Invitrogen, Milan, Italy). Collagenase was then inactivated by an equal volume of DMEM-1g containing 10% of fetal bovine serum (FBS; Lonza,

Milan, Italy). Successively, the digested lipoaspirate was centrifuged at 1,200 x g for 10 minutes. Pellets were resuspended in PBS and filtered through a 100 µm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). Filtered cells were again centrifuged at 1,200 x g for 10 minutes, plated in T-75 culture flasks (Falcon BD Biosciences, Milan, Italy) with DMEM-1g (10% FBS, penicillin/streptomycin 1%) containing 1% of Mesenchymal Stem Cells Growth Supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy) and incubated at 37 °C with 5% CO<sub>2</sub>. Twenty-four hours after the initial plating, non-adherent cells were removed by exhaustive washing of the plates.

### **3.3. Determination of markers for MSCs**

In order to identify MSCs derived from lipoaspirates, flow cytometry was carried out to identify specific cell surface markers of this cell. After reaching confluence (80% of total flask surface), cells were trypsinized (Sigma-Aldrich, Milan, Italy) and sub-cultured in 6-well culture plates for 2 days. Cells were first washed with PBS, again trypsinized, fixed with 2% paraformaldehyde (PFA) for 20 minutes at 4 °C and permeabilized with 1× Triton (Sigma-Aldrich, Milan, Italy) for 5 minutes at 4 °C. Subsequently, cells were washed with PBS/BSA 1% and incubated with primary antibodies (Millipore, Milan, Italy) for 60 minutes at room temperature. Antibodies used in the different samples were anti-CD44 (1:200), anti-CD90 (1:100), anti-CD105 (1:100), anti-CD14 (1:200), anti-CD34 (1:200) or anti-CD45 (1:200). Cells were then washed with PBS/1% BSA and incubated for 60 minutes at room temperature in the dark with secondary antibodies (goat anti-mouse or goat anti-rabbit) conjugated with fluorescein (FITC; 1:200; Millipore, Milan, Italy). Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA). A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at 488 nm and fluorescence was monitored at 525 nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically.

### **3.4. Differentiation of human MSCs in chondrocytes**

MSCs were trypsinized and diluted to a final concentration of 2.5 x 10<sup>5</sup> cells/ml medium. Then, 0.5 ml of cell suspension was transferred in a 15 ml polypropylene conical tube. They were then centrifuged at 150 g for 5 minutes at room temperature and washed with 1 ml incomplete chondrogenic medium (Lonza, Milan, Italy) containing 1% penicillin/streptomycin. Cells were resuspended in forty cell pellets with 0.5 ml of complete chondrogenic medium and TGF-β3 at a final concentration of 10 ng/ml and centrifuged at 150 g for an additional 5 minutes. With no further

resuspension, the forty cell pellets, measuring 1 mm<sup>2</sup> each, were cultured for 7, 14, 21 and 28 days at 37 °C and 5% CO<sub>2</sub> in 0.5 ml of complete chondrogenic medium (Lonza, Milan, Italy), replaced every 3 days.

### 3.5. Histology and histochemistry

After 7, 14, 21 and 28 days, samples (10 cell pellets) were rinsed in PBS, fixed in 10% buffered-formalin (Bio-Optica, Milan, Italy). After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded. After wax infiltration, samples (10 cell pellets) were orientated in the cassettes in the same direction. Sections (4-5 µm in thickness) were cut from paraffin blocks using a rotary manual microtome (Leica RM2235, Milan, Italy), mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. The sections were stained with Hematoxylin and Eosin (H&E), as previously described (Musumeci et al., 2013e), for general cell identification and for the presence or absence of morphological alterations. Alcian blue pH 2.5 periodic acid shift (PAS) was used to assess synthesis of sulphated GAG containing proteoglycans (assessment was made on the intensity of staining). The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photomicrographs were captured using a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### 3.6. Immunohistochemistry (IHC)

For immunohistochemical analysis, samples (15 cell pellets) were processed as previously described (Pichler et al., 2013). Briefly, the slides were dewaxed in xylene, hydrated using graded ethanols and incubated for 30 minutes in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to quench endogenous peroxidase activity before being rinsed for 20 minutes with PBS (Bio-Optica, Milan, Italy). The sections were heated (5 minutes x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites as previously described (Pichler et al., 2013). The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA, Sigma-Aldrich, Milan, Italy) in PBS for 1 hour in a humid chamber. BSA was used as a blocking agent to prevent non-specific binding of the antibody to the tissue sections. Following blocking, the sections were incubated overnight at 4 °C with diluted rabbit polyclonal antibodies against types I (ab34710-Abcam, Cambridge, UK) and II (ab855266-Abcam, Cambridge, UK) collagen, both diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy), with mouse monoclonal anti-RUNX2 antibody (ab76956; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy) and with rabbit



polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy). Immune complexes were then treated with a biotinylated link antibodies (HRP-conjugated anti-rabbit and anti-mouse were used as secondary antibodies) and then detected with peroxidase labeled streptavidin, both incubated for 10 minutes at room temperature (LSAB+ System-HRP, K0690, Dako, Glostrup, Denmark).

Immunoreactivity was visualized by incubating the sections for 2 minutes in a 0.1% 3,3'-diaminobenzidine (DAB) and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### **3.7. Evaluation of immunohistochemistry**

The collagen type I, II, RUNX2 and lubricin-staining status was identified as either negative or positive. Immunohistochemical positive staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described (Pichler et al., 2013). Staining intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of collagen type I, II, RUNX2 and lubricin immunopositive cells (Extent Score= ES) was independently evaluated by 3 investigators (2 anatomists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5–30% (+); 31–50% (++); 51-75% (+++), and >75% (++++). Counting was performed at x200 magnification. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at the protein level. Positive controls consisted of tissue specimens with known antigenic positivity (cartilage tissue). Sections treated with PBS without the primary antibodies served as negative controls.

### **3.8. Computerized morphometric measurements and image analysis**

Fifteen fields, randomly selected from each section, were analyzed and the percentage area stained with collagen type I, II, RUNX2 and lubricin antibodies was calculated using image analysis software (AxioVision Release 4.8.2; SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany),

which quantifies the level of staining intensity of positive immunolabelling in each field. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany, using objective lens of magnification x20 i.e. total magnification 400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany); evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement.

### **3.9. Western blot analysis**

At 7, 14, 21 and 28 days the production of collagen type I, and II, RUNX2 and lubricin, were evaluated by Western blot. Briefly, samples (15 cell pellets) were rinsed twice with PBS and protein were extracted on ice with a lysis buffer solution (T-PER Tissue Protein Extraction Reagent, Thermo Scientific, Milan, Italy) in presence of protease inhibitor cocktail (Roche, Milan, Italy). After 10 minutes, sonication was applied and supernatant was collected by centrifugation (15,000 x g, 15 minutes, and 4 °C). Protein quantification for each sample was obtained with bicinchoninic acid assay (BCA assay; Pierce, Fisher Scientific, Milan, Italy). Equal amount of proteins was resolved on 10% SDS-PAGE. Then, proteins were transferred to nitrocellulose membrane (Invitrogen, Milan, Italy), blocked for 1 hour at RT with 5% skimmed milk powder in TBS-Tween 0.1% (TBST) buffer, probed with appropriate primary antibody anti-lubricin (1:500; ab28484-Abcam, Cambridge, UK), anti-collagen types I and II (1:1000; ab34710 and ab85266-Abcam, respectively, Cambridge, UK), anti-RUNX2 antibody (1:1,000; ab76956; Abcam, Cambridge, UK) and anti- $\alpha$ -tubulin (1:5,000; Sigma-Aldrich, Milan, Italy). Blots were rinsed three times in TBST and the appropriate HRP-conjugated secondary antibody was incubated for 1 hour at RT (goat anti-rabbit, 1:20,000; goat anti-mouse, 1:5,000, both from Santa Cruz Biotechnology; DBA, Milan, Italy). The blots were developed using enhanced chemiluminescent solution (Pierce, Fisher Scientific, Milan, Italy) and visualized with autoradiography film. Densitometrical measurements were made by using Image J software (NIH, Bethesda, MD, USA). Normalization was carried out with respect to the  $\alpha$ -tubulin amount in the same sample.

### **3.10. Statistical analysis**

Statistical analysis was performed using SPSS software (SPSS® release 16.0, IBM, Chicago, IL, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the

Student's t-test, whilst comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. p-values of less than 0.05 were considered statistically significant; p-values of less than 0.01 were considered highly statistically significant. Data are presented as the mean  $\pm$  SEM. Cohen's kappa was applied to measure the agreement between the three observers and averaged to evaluate overall agreement as previously described (Pichler et al., 2013).

## 4. Results

### 4.1. Identification of MSCs markers

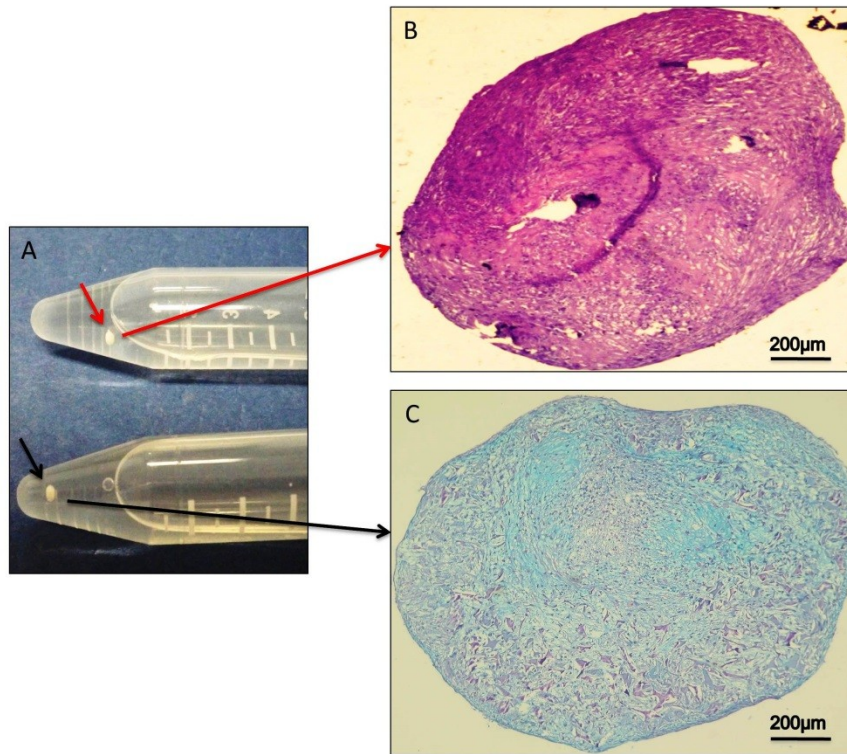
After the first passage, flow cytometry analysis was carried out to identify MSCs markers. The results demonstrated that MSCs did not present labeling for CD45, CD14 and CD34 and were positive for CD44 (H-CAM), CD90 (Thy 1), and CD105 (Endoglin) although to a different extent (Table 1).

**Table I.** MSCs markers identified by flow cytometric analysis

<b>Cell Surface (Cluster of Differentiation) Marker</b>	<b>% positive cells</b>
<b>CD44</b>	<b>95.5 <math>\pm</math> 0.3</b>
<b>CD90</b>	<b>88.6 <math>\pm</math> 0.5</b>
<b>CD105</b>	<b>80.2 <math>\pm</math> 1.6</b>
<b>CD14</b>	<b>8.3 <math>\pm</math> 6.2</b>
<b>CD34</b>	<b>15.5 <math>\pm</math> 6.3</b>
<b>CD45</b>	<b>9.7 <math>\pm</math> 6.2</b>

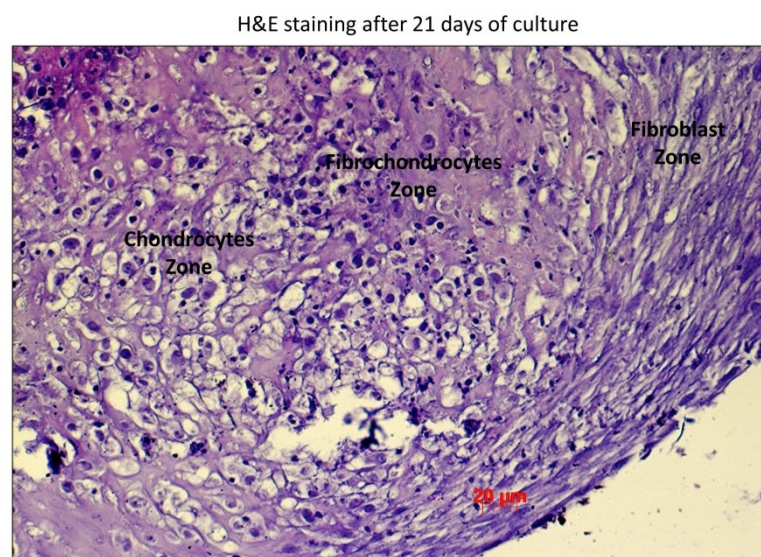
### 4.2. Histology and histochemistry

Macroscopic morphology (Fig. 1 A), histology (H&E staining, Fig. 1 B) and histochemistry (Alcian blue, pH 2.5 PAS staining, Fig. 1 C) results demonstrated absence of morphological aberrations into the 1 mm<sup>2</sup> implantable size cell pellets.



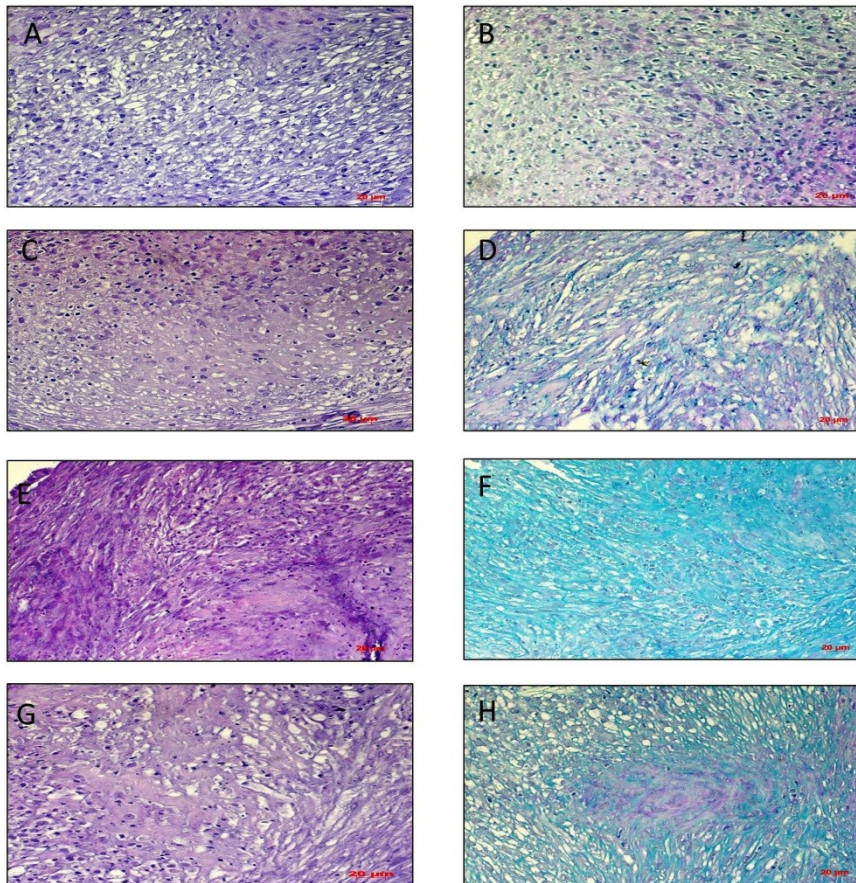
**Figure 1.** A). Macroscopic morphology of cell pellets by measuring 1 mm<sup>2</sup> each, black and red arrows. B). Histology (H&E staining) results demonstrated absence of morphological aberrations in the cell pellets. Magnification x5; Scale bars: 200 µm. C). Histochemistry (Alcian Blue pH 2.5 PAS staining) results demonstrated absence of morphological alterations within cell pellets. Magnification x5; Scale bars: 200 µm.

Microscopically at 21 days after culture, we found different populations of cells in the cell pellets, like dense fibrocartilage, composed of sparsely populated cells called fibrochondrocytes because they exhibit characteristics of both fibroblasts and chondrocytes (Fig. 2). Indeed, in the new tissue formed we morphologically identified three zones, chondrocytes zone, fibrochondrocytes zone and fibroblasts zone.



**Figure 2.** Histology (H&E staining) at 21 days after culture. Within the cell pellets we morphologically identified three distinct zones, chondrocyte zone, fibrochondrocyte zone and fibroblast zone. Magnification x20; Scale bars: 20  $\mu$ m.

The H&E staining was used for determining the time-dependent cell morphology in differentiated chondrocytes in cell pellets, at 7, 14, 21 and 28 days after culture (Fig. 3 A, C, E, G). The new chondrocytes showed an increase in proliferation, cellular aggregations (a hyaline cartilage-like structure) and extracellular matrix (ECM) production as observed in figure 3. They did not show any signs of cellular damage at any of the times investigated as demonstrated by H&E staining (Fig. 3 A, C, G). The best time of culture, demonstrated by a strong H&E staining (IS: 4), was 21 days (Fig. 3 E).



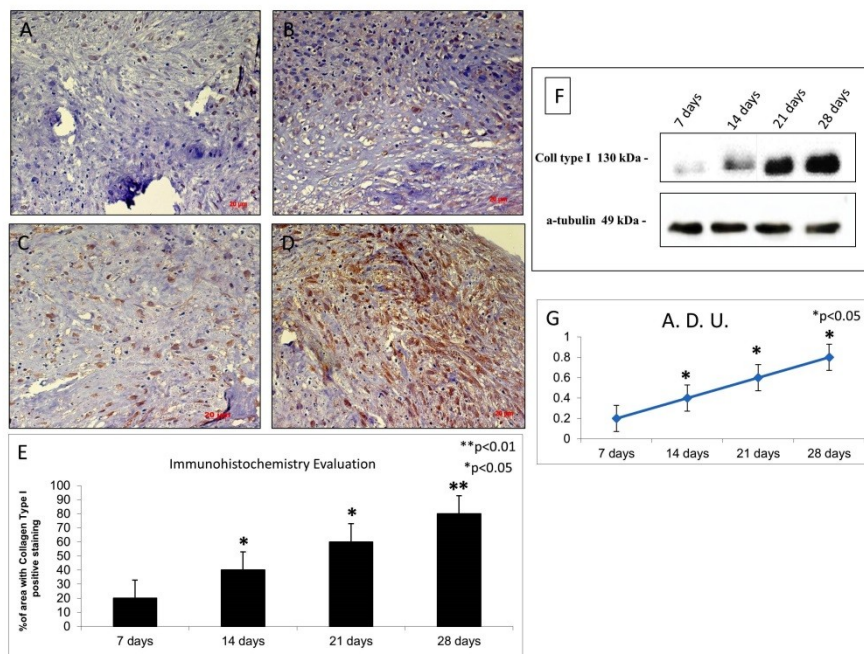
**Figure 3.** A, C, E, G). H&E staining was used to determine the time dependent cell viability in differentiated chondrocytes in cell pellets, at 7 (A), 14 (B), 21 (C) and 28 (D) days after culture. The new chondrocytes showed an increase in proliferation, cellular aggregations (a hyaline cartilage-like structure), ECM production. They did not show any signs of cellular damage demonstrated by an intense H&E staining. The best time of culture was 21 days as demonstrated by a more intense H&E staining. Panel A, C, E, G: Magnification x20; Scale bars: 20  $\mu$ m. B, D, F, H). Alcian Blue pH 2.5 PAS, was used to assess synthesis of GAG, including proteoglycans (the assessment was made on the intensity of staining) in differentiated chondrocytes in the cell pellets, at 7 (B), 14 (D), 21 (F) and 28 (H) days after culture. We found an intense Alcian Blue pH 2.5 PAS staining at all times (B, D, H) showing the presence of

proteoglycans, although the best time of culture was 21 days as demonstrated by a strong Alcian Blue pH 2.5 PAS staining (F). Panel B, D, F, H: Magnification x20; Scale bars: 20  $\mu$ m.

Specific chemical stains, such as Alcian blue pH 2.5 PAS, were used to assess synthesis of glycosaminoglycans (GAG), including proteoglycans (the assessment was made on the intensity of staining), in differentiated chondrocytes, at 7, 14, 21 and 28 days after culture. We found an intense Alcian blue pH 2.5 PAS staining (IS: 3) at all times (Fig. 3 B, D, H) showing the presence of proteoglycans, although the best time of culture was 21 days as determined by a stronger Alcian blue pH 2.5 PAS staining (IS: 4) (Fig. 3 F).

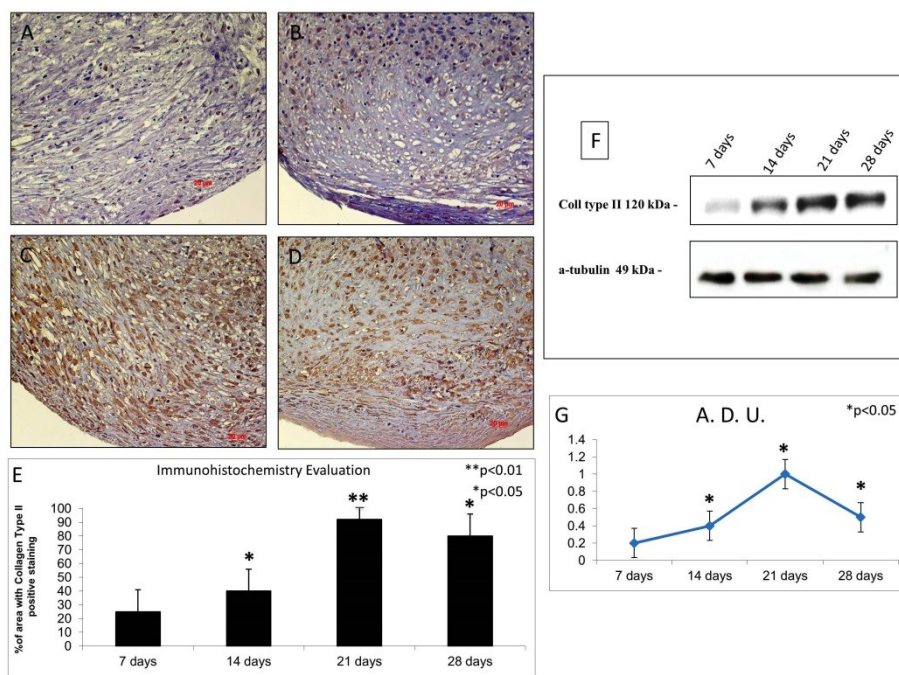
### 4.3. Immunohistochemistry (IHC)

Collagen type I immunohistochemical staining in pellets was evaluated in morphologically different cells, fibrochondrocytes, fibroblasts, chondrocytes and relative ECM. Expression of collagen type I is indicative of fibrocartilage formation. Weak collagen type I staining was observed at 7 days (IS= 1; ES= +, Fig. 4 A). Moderate collagen type I staining was seen at 14 days (IS= 2; ES= ++, Fig. 4 B). Strong collagen type I staining was observed at 21 days (IS= 3; ES= +++, Fig. 4 C). Very strong collagen type I staining was found at 28 days (IS= 4; ES= +++++, Fig. 4 D). No immunoreactivity was demonstrated in the negative control treated with PBS without the primary antibodies (data not shown). At 21 days both ES and IS were significantly greater compared to other time points ( $p < 0.01$ ). Inter-observer agreement, measured using the Kappa coefficient, was 0.88 (Fig. 4 E).



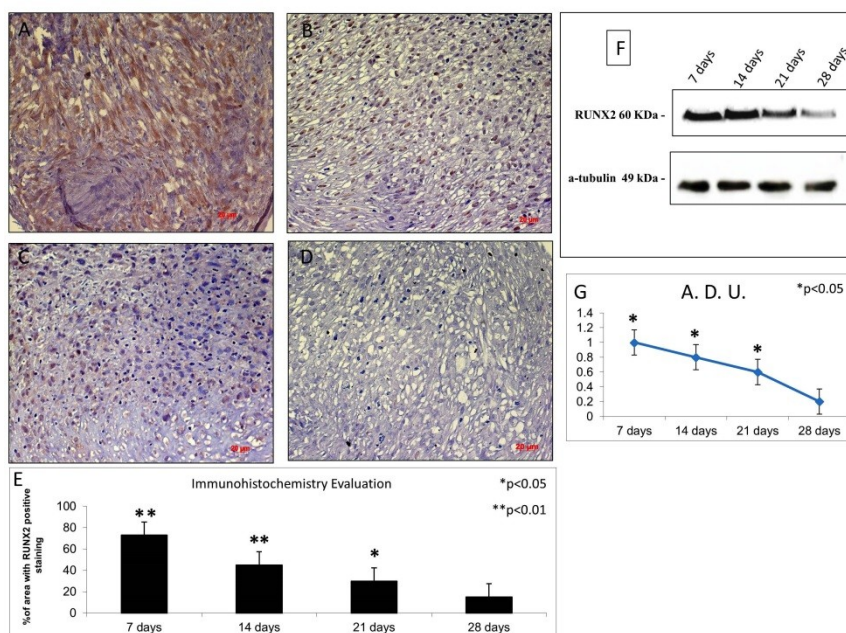
**Figure 4.** A-D. Immunohistochemical evaluation of collagen type I in the cell pellets. A). Weak collagen type I staining was showed at 7 days (IS= 1; ES= +). B). Moderate collagen type I staining was observed at 14 days (IS= 2; ES= ++). C). Strong collagen type I staining was found at 21 days (IS= 3; ES= +++). D). Very strong collagen type staining was demonstrated at 28 days (IS= 4; ES= ++++). Panel A-D: Magnification x20; Scale bars: 20µm. E). Graphical representation of the immunohistochemical data. Percentage of collagen type I positive cells out of the total number of cells counted at different times (7, 14, 21, and 28 days). Results are presented as the mean ±SEM. ANOVA-test was used to evaluate the significance of the results. \* p < 0.05 and \*\* p < 0.01, when compared between different times of culture. F). Collagen type I production at 7, 14, 21 and 28 days determined by Western blotting. G). Data presented show the relative expression (mean + SEM) calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. \*p<0.05 compared to cells treated with adipogenic medium at 7 days.

Collagen type II immunohistochemical staining in pellet was evaluated in morphologically different cells, fibrochondrocytes, fibroblasts, chondrocytes and relative ECM. Expression of collagen type II, was indicative of hyaline cartilage formation. Weak collagen type II staining was found at 7 days (IS= 1; ES= +, Fig. 5 A). Moderate collagen type II staining was shown at 14 days (IS= 2; ES= ++, Fig. 5 B). Very strong collagen type II staining was demonstrated at 21 days (IS= 4; ES= ++++, Fig. 5 C). Strong collagen type II staining was observed at 28 days (IS= 3; ES= +++, Fig. 5 D). No immunoreactivity was observed in the negative control treated with PBS without the primary antibodies (data not shown). At 21 days both ES and IS were significantly greater compared to other time points ( $p<0.01$ ). Inter-observer agreement, measured using the Kappa coefficient, was 0.92 (Fig. 5 E).



**Figure 5.** A-D. Immunohistochemical evaluation of collagen type II in the cell pellets. A). Weak collagen type II staining was showed at 7 days (IS= 1; ES= +). B). Moderate collagen type II staining was observed at 14 days (IS= 2; ES= ++). C). Very strong collagen type II staining was demonstrated at 21 days (IS= 4; ES= ++++). D). Strong collagen type II staining was found at 28 days (IS= 3; ES= +++). Panel A-D: Magnification x20; Scale bars: 20µm. E). Graphical representation of the immunohistochemical data. Percentage of collagen type II positive cells out of the total number of cells counted at different times (7, 14, 21, and 28 days). Results are presented as the mean ±SEM. ANOVA-test was used to evaluate the significance of the results. \*  $p < 0.05$  and \*\*  $p < 0.01$ , when compared between different times of culture. F). Collagen type II production at 7, 14, 21 and 28 days determined by Western blotting. G). Data presented show the relative expression (mean + SEM) calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. \* $p < 0.05$  compared to cells treated with adipogenic medium at 7 days.

RUNX2 immunohistochemical staining in cell pellets was evaluated in different kind of cells, fibrochondrocytes, fibroblasts, chondrocytes and relative ECM. Strong RUNX2 staining was observed at 7 days (IS= 3; ES= +++, Fig. 6 A). Moderate RUNX2 staining was observed at 14 days (IS= 2; ES= ++, Fig. 6 B). Weak RUNX2 staining was observed at 21 days (IS= 1; ES= +, Fig. 6 C). Absence of RUNX2 staining was observed at 28 days (IS= 0; ES= 0, Fig. 6 D). No immunoreactivity was observed in the negative control treated with PBS without the primary antibodies (data not shown). Both ES and IS were significantly greater ( $p < 0.01$ ). Inter-observer agreement, measured using the Kappa coefficient, was 0.94 (Fig. 6 E).

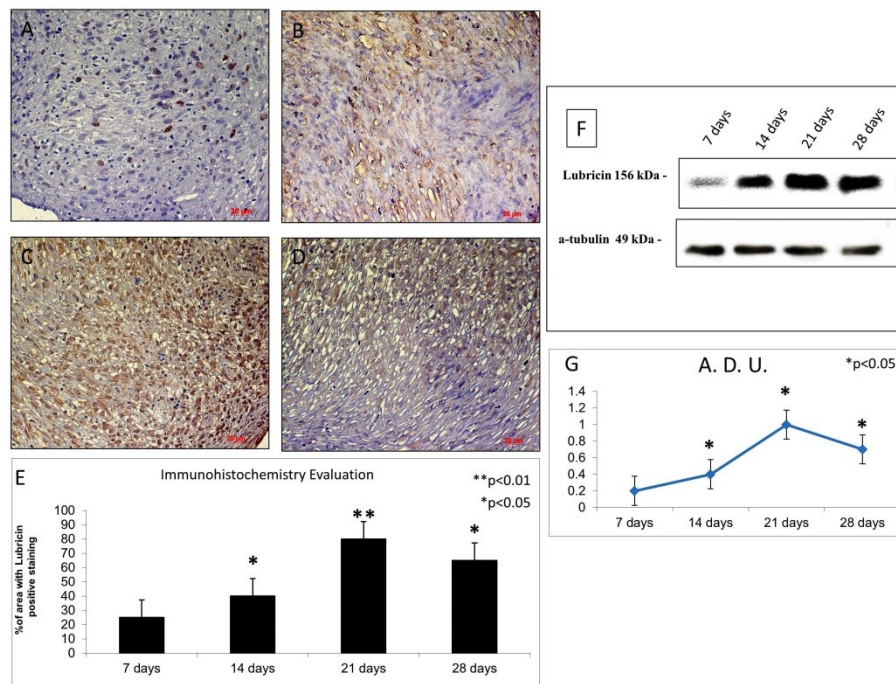


**Figure 6.** A-D. Immunohistochemical evaluation of RUNX2 in the cell pellets. A). Strong RUNX2 staining was observed at 7 days (IS= 3; ES= +++). B). Moderate RUNX2 staining was observed at 14 days (IS= 2; ES= ++). C). Weak RUNX2 staining was observed at 21 days (IS= 1; ES= +). D). Absent RUNX2 staining was observed at 28 days



(IS= 0; ES= 0). Panel A-D: Magnification x20; Scale bars: 20µm. E). Graphical representation of the immunohistochemical data. Percentage of RUNX2 positive cells out of the total number of cells counted at different time points (7, 14, 21, and 28 days). Results are presented as the mean ±SEM. ANOVA-test was used to evaluate the significance of the results. \*  $p < 0.05$  and \*\*  $p < 0.01$ , when compared between different times of culture. F). RUNX2 production at 7, 14, 21 and 28 days determined by Western blotting. G). Data presented show the relative expression (mean + SEM) calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. \* $p < 0.05$  compared to cells treated with adipogenic medium at 7 days.

Lubricin immunohistochemical staining in pellets was evaluated in morphologically different cells, fibrochondrocytes, fibroblasts, chondrocytes and relative ECM. Expression of lubricin is indicative of cartilage lubrication. Weak lubricin staining was observed at 7 days (IS= 1; ES= +, Fig. 7 A). Moderate lubricin staining was showed at 14 days (IS= 2; ES= ++, Fig. 7 B). Very strong lubricin staining was observed at 21 days (IS= 4; ES= +++++, Fig. 7 C). Strong lubricin staining was observed at 28 days (IS= 3; ES= ++++, Fig. 7 D). No immunoreactivity was demonstrated in the negative control treated with PBS without the primary antibodies (data not shown). At 21 days both ES and IS were significantly greater compared to other time points ( $p < 0.01$ ). Inter-observer agreement, measured using the Kappa coefficient, was 0.96 (Fig. 7 E).



**Figure 7.** A-D. Immunohistochemical evaluation of lubricin in the cell pellets. A). Weak lubricin staining was observed at 7 days (IS= 1; ES= +). B). Moderate lubricin staining was showed at 14 days (IS= 2; ES= ++). C). Very strong lubricin staining was demonstrated at 21 days (IS= 4; ES= +++++). D). Strong lubricin staining was found at 28 days (IS= 3; ES= ++++). Panel A-D: Magnification x20; Scale bars: 20µm. E). Graphical representation of the immunohistochemical data. Percentage of lubricin positive cells out of the total number of cells counted at different time points (7, 14, 21, and 28 days). Results are presented as the mean ±SEM. ANOVA-test was used to evaluate the

significance of the results. \*  $p < 0.05$  and \*\*  $p < 0.01$ , when compared between different times of culture. F). Lubricin production at 7, 14, 21 and 28 days determined by Western blotting. G). Data presented show the relative expression (mean + SEM) calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments.

\* $p < 0.05$  compared to cells treated with adipogenic medium at 7 days.

#### 4.4. Western blot analysis

In this study, we examined, by Western blotting, the production of collagen type I (Fig. 4 F, G) and type II (Fig. 5 F, G), RUNX2 (Fig. 6 F, G) and lubricin (Fig. 7 F, G) during the chondrogenic differentiating of adipose derived MSCs. The results confirmed those obtained by immunohistochemistry. At 7 days, differentiating MSCs expressed negligible amounts of lubricin and collagen type I and II in a small quantity, instead the RUNX2 was highly expressed. At 14 days, collagen type I and II and lubricin increased, while RUNX2 decreased; at 21 days we found a very strong expression of lubricin and collagen type II, while the expression of collagen type I was strong and the RUNX2 was weak; at 28 days, the expression of lubricin and collagen type II was lower although strong, while the expression of collagen type I was very strong and the expression of RUNX2 was absent. At the same time, MSCs untreated with chondrogenic differentiating medium synthesized undetectable quantities of collagen type I and II, RUNX2 and lubricin (data not shown). Data presented show the relative expression (mean + SEM) calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments.

#### 5. Discussion

The future of regenerative medicine depends on fully understanding the regenerative properties of stem cells, carefully identifying and studying the molecules required for their differentiation into various cell types (Mobasher et al., 2014). In this study, we focused on the possible use of MSCs to improve current technologies and to bring cell therapies to clinical care. In particular, we focused on isolation, cultivation, characterization of MSCs derived from adipose tissue and on their differentiation in chondrocytes in a three-dimensional environment, through the stimulation of chondrogenic signalling pathways. In this study we corroborated and strengthened our previous results (Musumeci et al., 2011a), and the innovation of this research is given by the evaluation of the different time points (7, 14, 21 and 28 days) in order to find the best time of chondrogenic differentiation of MSCs. Moreover, we evaluated the role of the RUNX2 during the chondrogenic process. During the differentiation phase, matrix production in the cell pellets was evaluated through histology using hematoxylin and eosin staining, and the synthesis of ECM molecules, such as GAG, was evaluated through histochemistry using Alcian blue staining. Immunohistochemistry and Western blot analysis were used to detect and quantify collagen type I,

type II, RUNX2 and lubricin. According to histology, histochemistry, immunohistochemistry and Western blot results we clearly showed that 21 days of culture is the best time to obtain functionally mature chondrocytes differentiated from adipose tissue-derived MSCs. At this time, chondrogenic cell pellets could be a useful tool for an eventual cartilage implantation because they produce a strong quantity of collagen type I, II, and lubricin, instead the production of RUNX2 decreases.

In particular, we observed that the expression of lubricin and collagen type II, which is specific of hyaline cartilage, was very high at 21<sup>st</sup> day of chondrogenic culture. At 28<sup>th</sup> day the expression of lubricin and collagen type II decreased slightly, while that of collagen type I increased significantly, indicating the transition of cells from chondrocytic phenotype to fibrochondrocytic one. These data indicate that the best time to obtain functionally mature fibrochondrocytes differentiated from adipose tissue-derived MSCs is 28 days of chondrogenic culture. This information could be useful for the treatment of fibrocartilage degenerative diseases such as degenerative disc disease, but further studies are needed. Moreover, our results indicate that almost the totality of the cells expresses the RUNX2 transcription factor during the first two weeks of culture, probably because osteoblasts and chondrocytes have the same mesenchymal progenitor and the commitment to the osteoblast fate is dependent on this transcription factor (Skalska et al., 2014). An interesting and recent study evaluated RUNX2 expression in osteoarthritic and normal chondrocytes treated with human recombinant Fibroblast Growth Factor 23 (FGF-23). In osteoarthritic chondrocytes compared to normal ones, the expression of RUNX2 was higher. Moreover, the inhibition of Sox9, molecule that is vital for chondrogenesis in the embryonic development and allows to maintain the chondrocytic phenotypes, resulted in increased RUNX2 expression in normal chondrocytes (Kim et al., 2013). Instead the silencing of RUNX2 did not affect Sox9 levels in osteoarthritic chondrocytes. Moreover simultaneous down-regulation of Sox9 and up-regulation of FGF-23 expressions in normal chondrocytes resulted in additive up-regulation of RUNX2 expression (Orfanidou et al., 2009). According to our results, the RUNX2 expression decreases in a time-dependent manner during the chondrogenic differentiation phase, underlining the progressive loss of osteoblastic features in the newly differentiated chondrocytes.

As said above, collagen type II and lubricin are mainly expressed at the 21<sup>st</sup> day of culture and their levels decreased at 28<sup>th</sup> day, instead the collagen type I expression increases at 28<sup>th</sup> day, probably because after 21 days, as in monolayer culture, the new chondrocytes dedifferentiate in fibroblasts-like-cells or fibrochondrocytes, losing their morphological and phenotypic features (Musumeci et al., 2011c; Musumeci et al., 2013a; Musumeci et al., 2013f; Musumeci et al., 2014a). The transplantation of autologous adipose tissue-derived chondrocytes producing lubricin, could be of great interest for repairing cartilage lesions in patients affected by OA since it has been

demonstrated that lubricin is able to reduce the progression of several articular diseases (Flannery, 2010). With our results we can also strengthen the hypothesis that the presence of lubricin could be a possible biomarker, such as collagen type II, for the detection of chondrocytes well-being and viability (Musumeci et al., 2014a; Musumeci et al., 2014b; Musumeci et al., 2014c). Articular cartilage injuries are one of the most challenging problems in musculoskeletal medicine due to the poor intrinsic regenerative capacity of this tissue thus, new approaches to repair joint structures need to be established (Luyten&Vanlauwe, 2012). Although the use of chondrocytes in cartilage tissue engineering is still prevalent, concerns associated with donor-site morbidity, cell de-differentiation and the limited span of these cells, have led to the use of MSCs (Musumeci et al., 2011a) as alternative cell source for cartilage defect repair (Musumeci et al., 2013f; Mobasher et al., 2014). The possibility of generating chondrocytes from adipose tissue in patients with OA and other degenerative joint disorders is a less invasive way to collect MSCs compared to bone marrow aspiration (Musumeci et al., 2011a).

Our study, in accordance with the literature (Rajmohan et al., 2014; Shen et al., 2014), tries to reproduce a cartilage tissue without the use of the artificial scaffolds, but directly through a natural self-assembly process (old technique but still very timely and effective) where MSCs form by themselves a three-dimensional structure (cell pellets). Tissue regeneration through self-assembly process of MSCs was a breakthrough in the field of tissue engineering and even today could be a possible valid method. This is definitely an advantage from the point of view of the human experimentation, where there is the need to evaluate biocompatibility, biodegradability and biotoxicity of artificial scaffolds. Moreover, this study suggests that it is possible to regenerate a cartilage tissue, through the use of MSCs withdrawn from adipose tissue with a minimally invasive technique, such as liposuction, without going to impair the already injured cartilage tissue such for eg. the autologous chondrocytes transplantation (ACT) through arthroscopy (more invasive technique).

The use of adipose-derived MSCs for chondrocytes differentiation and subsequently for autologous cell transplantation is an interesting field of study in order to reduce disease progression. We believe that these findings could be clinically relevant for future therapeutic approaches. In conclusion, we showed that 21 days is the best time of culture to differentiate mature chondrocytes from adipose tissue-derived MSCs that could be used in cell-based repair strategies for OA and degenerative pathologies of articular cartilage. The present study is preliminary and more functional assays, *in vitro* and *in vivo* studies in this field are necessary so that this could be considered as a therapeutic approach.

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## CHAPTER VIII

**Assessment of osteoblast and chondrocyte biomarkers in adipose tissue derived-mesenchymal stem cells and relative study of Lubricin and Caspase-3 expression. A bioengineering model for cartilage regeneration.**

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## 1. Abstract

The poor self-repair capacity of cartilage tissue in degenerative conditions, such as osteoarthritis (OA), has prompted the development of a variety of therapeutic approaches, such as cellular therapies and tissue engineering based on the use of mesenchymal stem cells (MSCs). The aim of this study is to demonstrate, for the first time, that the chondrocytes differentiated from rat adipose tissue derived-MSCs (AMSCs), are able to constitute a morphologically and biochemically healthy hyaline cartilage after 6 weeks of culture on a Collagen Cell Carrier (CCC) scaffold. In this study we evaluated the expression of some osteoblasts (Runt-related transcription factor 2 (RUNX2) and osteocalcin), chondrocytes (collagen I, II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated AMSCs, AMSCs-derived chondrocytes cultured in monolayer and AMSCs-derived chondrocytes seeded on CCC scaffolds, by different techniques such as immunohistochemistry, ELISA, Western blot and gene expression analyses. Our results showed the increased expression of collagen II and lubricin in AMSCs-derived chondrocytes cultured on CCC scaffolds, whereas the expression of collagen I, RUNX2, osteocalcin and caspase-3 resulted decreased, when compared to the controls. In conclusion, this innovative basic study could be a possible key for future therapeutic strategies for articular cartilage restoration through the use of CCC scaffolds, to reduce the morbidity from acute cartilage injuries and degenerative joint diseases.

**Keywords:** Adipose tissue; Apoptosis; Cartilage; Chondrocytes; Collagen Cell Carrier; Lubricin; Mesenchymal Stem Cells; Regeneration; RUNX2

## 2. Introduction

Articular cartilage (AC) is a specialized connective tissue that covers joint surfaces and facilitates the transmission of loads with a low frictional coefficient, allowing friction-free movement. Nevertheless, AC has very poor healing potential and is prone to both acute injury and degenerative conditions, such as OA, leading to severe histopathological changes and to an increased apoptosis rate and a decreased lubricin expression within the AC [1, 2].

Mesenchymal stem cells (MSCs) offer an option as a renewable source of cells and tissues, to be used in many diseases related to AC such as OA. The poor self-repair capacity of cartilage tissue has prompted the development of a variety of therapeutic approaches, such as tissue engineering and cellular therapies [3,4]. Tissue engineering is a part of biomedicine in which the knowledge of function of cells enables us to control and promote the repair of damaged and diseased cartilage tissues. Cellular therapies could be one option to improve the patient quality of life. Currently, autologous cell therapies such as autologous chondrocyte implantation (ACI) and

matrix assisted ACI (MACI) require tissue to be explanted from a healthy area of cartilage, either from an intact portion of the damaged joint or from another of the patients' joints. ACI is considered a promising therapeutic option for cartilage repair, however, allogeneic (unrelated cell donor) cell therapy has significant advantages over ACI including no donor site morbidity, healthy donors and reduced costs [5]. We have recently examined a potential allogeneic cell type for the treatment of OA, namely the chondrocyte-differentiated from adipose tissue derived-MSCs (AMSCs) [6,7]. AMSCs are an adult stromal cell population possessing potent differentiation capacity into chondrocytes and osteocytes representing a resource for use across major histocompatibility complex (MHC) barriers [8,9]. The involved procedures are less invasive and destructive than those used in AC specimens [10]. The differentiation of AMSCs into different cell types, in this case into chondrocytes, depends on the local microenvironment, growth factors, extracellular matrix and mechanical forces [11–13]. Cell death with morphological and molecular features of apoptosis has been detected in OA cartilage, suggesting a key role for chondrocyte death/survival in the pathogenesis of the disease. The identification of biomarkers of chondrocyte apoptosis may facilitate the development of novel therapies that may eliminate the cause of, or, at least, slow down the degenerative processes in OA [14]. Apoptosis is defined as physiologic cell death in order to remove harmful, damaged or unwanted cells without inducing an inflammatory response and without the release of cell contents as observed during necrotic cell death [15]. The two main mechanisms regulating apoptosis include the intrinsic pathway mediated by mitochondria and the extrinsic pathway [16] induced by death signaling ligands, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) or FasL. These ligands bind their receptor and induce the initiator protease caspase-8, which in turn activates executioner proteases such as caspase-3. The caspase cascade plays a vital role in the induction, transduction, amplification and execution of apoptotic signals within the cell [17].

Lubricin is a chondroprotective mucinous glycoprotein that plays an important role in joint lubrication and synovial homeostasis, preventing cartilage wear and synovial cell adhesion [18, 19]. As the goal of today's medicine shifts more and more towards disease prevention rather than treatment, lubricin might represent an attractive candidate therapy target molecule in the context of OA [20,21]. This important protein is found in several joint tissues including synovial membranes and fluid [22], the superficial zone of AC [23, 24], tendons [25], ligaments [26], discs [27,28] and menisci [29,30]. Thanks to its boundary lubricating properties, lubricin prevents synoviocyte overgrowth, protects cartilage surfaces and prevents cartilage wear. Lubricin was also studied in an *in vitro* study directly on cells and in combination with artificial three-dimensional scaffolds [6,7,31].

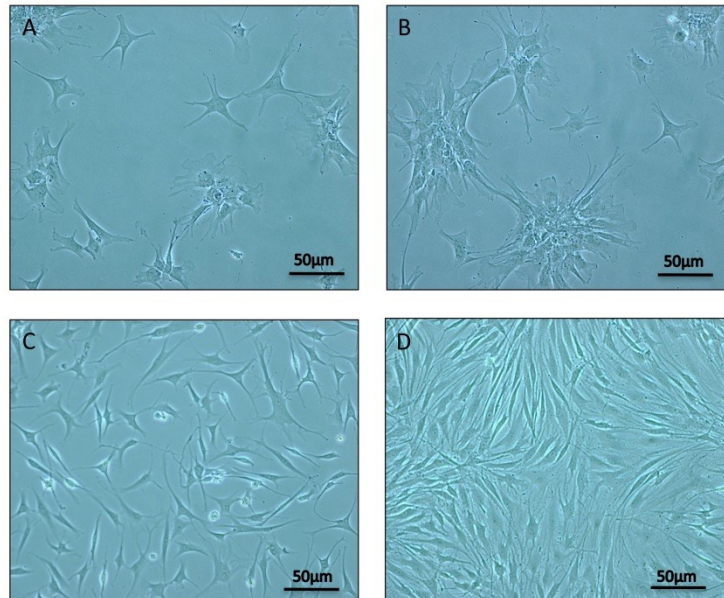
In this study we obtain AMSCs from rat adipose tissue. In order to identify AMSCs derived from lipoaspirates, flow cytometry was carried out to identify specific cell surface markers of these cells (CD44, CD90 and CD105 positive; CD45, CD14 and CD34 negative). We differentiated AMSCs in chondrocytes through chondrogenic medium, after that the chondrocytes were seeded in a novel collagen scaffold for cell culture applications named Collagen Cell Carrier (CCC) [32,33]. These scaffold samples were preconditioned in chondrogenic medium for 6 weeks. In this study we assessed the expression of some osteoblasts (runt-related transcription factor 2 (RUNX2) and osteocalcin), chondrocytes (collagen type I, collagen type II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated AMSCs and in differentiated chondrocytes by ELISA, Western Blot and gene expression analyses. With the same molecules we evaluated also through immunohistochemical, biochemical and gene expression analyses the chondrocytes into the CCC scaffolds. These scaffolds were also morphologically examined. The aim of this study is to demonstrate, morphologically and biochemically, for the first time, a healthy hyaline cartilage structure after 6 weeks of culture of differentiated chondrocytes from AMSCs, growing into CCC Scaffold, and the increased or decreased expression of lubricating ability through the study of lubricin and the apoptosis through the study of caspase-3. The presence or absence of lubricin and caspase-3 could be used as a biomarker match for the detection of chondrocyte's well-being and viability in CCC Scaffold indicating a healthy hyaline cartilage structure. Our study hypothesis is that these two molecules are inversely proportional and this data could help better understand this complex phenomenon for possible CCC scaffold implantation. Hopefully in the future the well-differentiated chondrocytes within CCC scaffolds could be used in therapeutic treatment to reduce the progression of cartilage degenerative diseases such as OA.

### **3. Results**

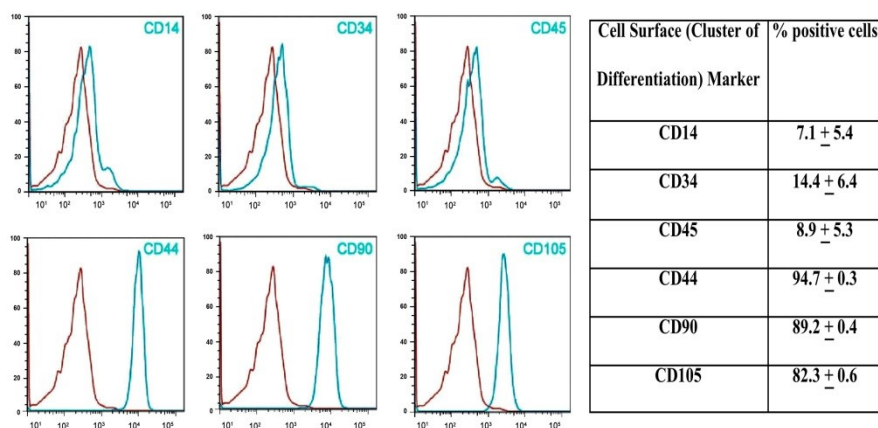
#### **3.1. Identification of adipose tissue-derived MSC markers**

After the first passage, flow cytometry analysis was carried out to identify AMSC markers. To isolate rat AMSCs, adipocytes cells were lipoaspirated from adipose tissue of subcutaneous fat. The cell suspension was purified on Ficoll plaque gradient. The purified cells were then cultured overnight in DMEM for identification of AMSCs. The cultured cells in vitro showed a fibroblast-like morphology in monolayer culture (Figure 1). Antibodies against CD14, CD34, CD45, CD44, CD90 and CD105 were used for immunostaining and species matched isotype antibodies were used as negative controls. Flow cytometric analysis showed that AMSCs expressed high levels of CD44, CD90 and CD105. The positive cells were over 95%, demonstrating a high purity of AMSCs.

However, expression of hematopoietic markers CD14, CD34 and CD45 was low indicating a low contamination of hematopoietic cells in the isolated MSCs (Figure 2).



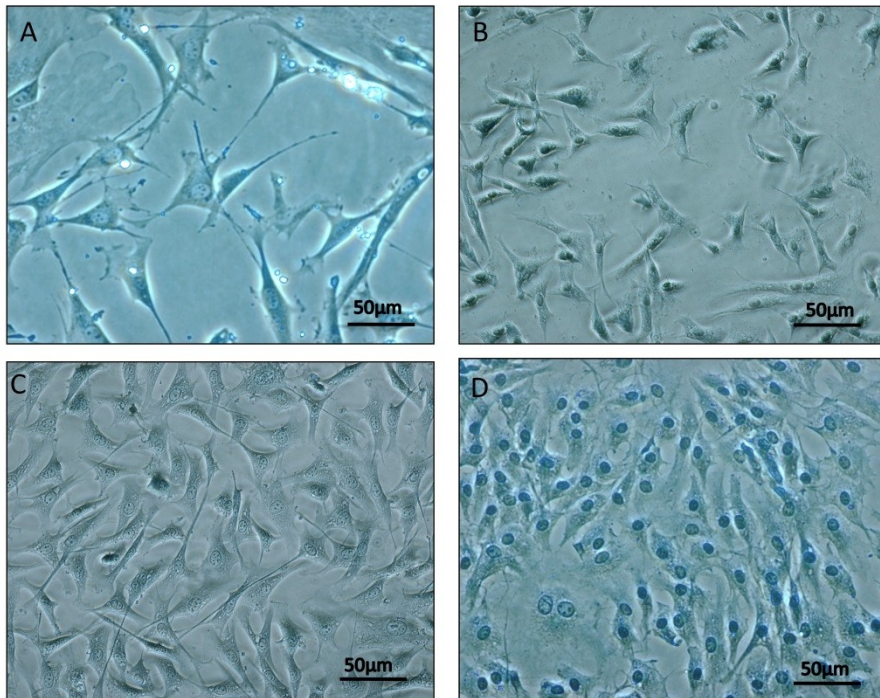
**Figure 1.** AMSCs at day 3 (A), 6 (B), 9 (C) and 12 (D) of culture (Mesenchymal Stem Cells Growth Medium, MSCGM) with fibroblast-like morphology. A-D: Scale bars 50  $\mu$ m.



**Figure 2.** AMSC markers identified by flow cytometric analysis. The MSCs from adipose tissue were isolated from rats. After purification by Ficoll plaque, the cells were cultured for 24 hours. The cells were then stained with antibodies against CD14, CD34, CD45, CD44, CD90 and CD105 (Blue line). The species matched isotype antibodies were used as negative controls (red line). The cultured cells in vitro showed a fibroblast-like morphology in monolayer culture with positive MSC specific markers CD44, CD90 and CD105, but the hematopoietic markers CD14, CD34 and CD45 were negative in the isolated MSC.

### 3.2. Differentiation of AMSCs into chondrogenic lineages

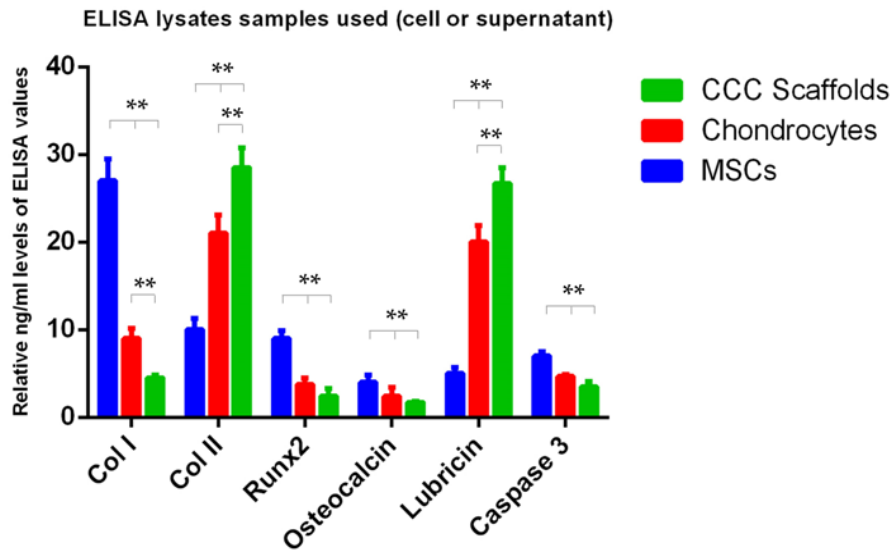
One day after the MSCs isolation from adipose tissue, we added the chondrocyte permissive medium containing growth factors into the cell medium and we maintained this condition until 14 days after initial cell isolation. The AMSCs after 14 days of culture showed chondrocyte morphology (Figure 3).



**Figure 3.** Chondrocytes from AMSCs at day 3 (A), 6 (B), 9 (C) and 12 (D) of culture (Complete Chondrogenic Medium, CCM) with typical chondrocyte round morphology. A-D: Scale bars 50  $\mu\text{m}$ .

### 3.3. Biochemical studies (ELISA)

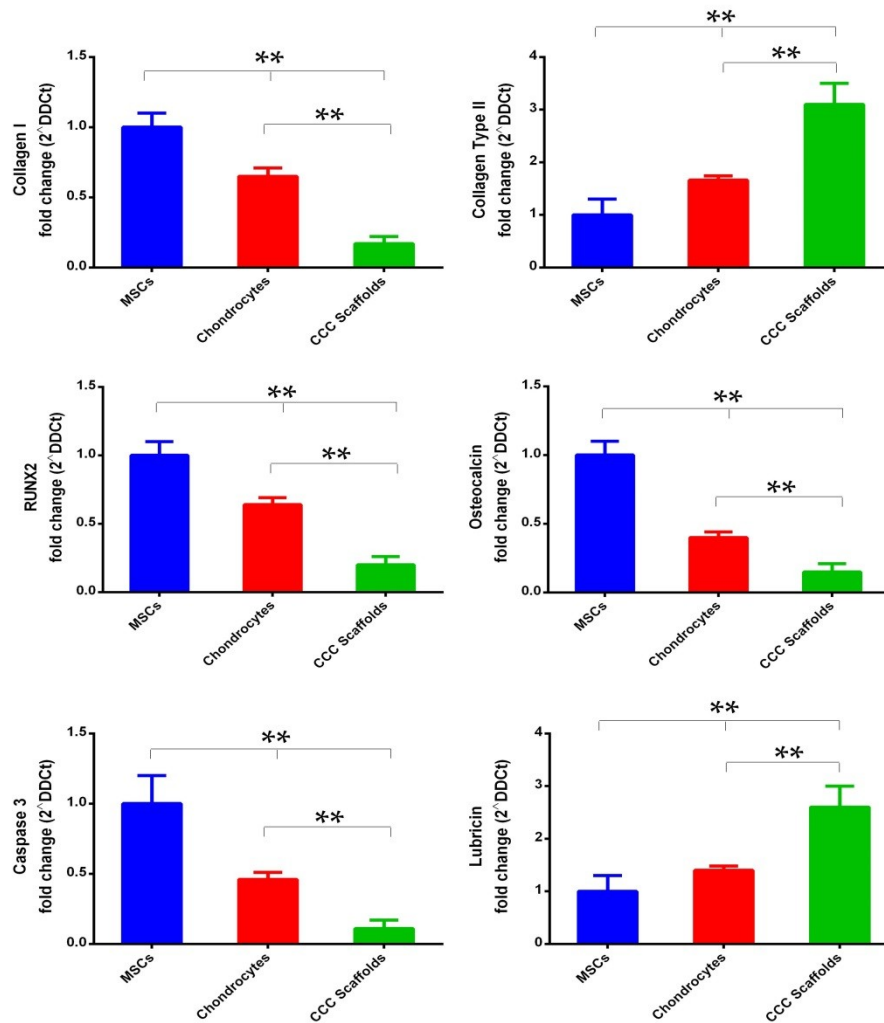
Concentrations of RUNX2 in cell lysates and the concentration of collagen type I, II, osteocalcin, lubricin and caspase-3 in lysed cell supernatant of AMSCs, chondrocytes and CCC scaffolds were measured (Figure 4). The levels of collagen type II and lubricin significantly increased between the comparison of the lysate samples used (supernatant) of AMSCs, chondrocytes and CCC scaffolds ( $p < 0.01$ ). Instead the levels of collagen type I, RUNX2, osteocalcin and caspase-3, significantly decreased between the comparison of the lysates samples used (cell or supernatant) of AMSCs, chondrocytes and CCC scaffolds ( $p < 0.01$ ).



**Figure 4.** ELISA: Collagen type I, II, RUNX2, osteocalcin, lubricin and caspase-3 levels were quantified in lysates samples used (cell or supernatant) of AMSCs, chondrocytes and CCC scaffolds. Data are expressed as mean  $\pm$  SD of at least three independent experiments. ANOVA and Bonferroni's test, were used to evaluate the significance of the results. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $P < 0.001$  when compared to the samples.

### 3.4. Gene expression analysis by real-time PCR (qRT-PCR)

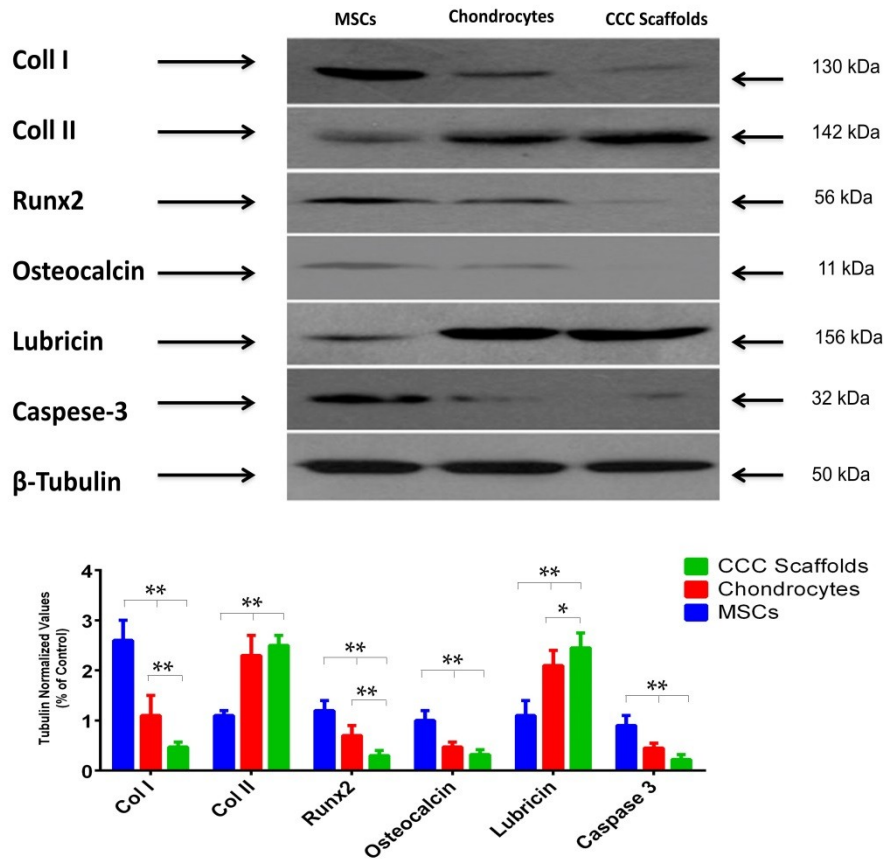
Quantitative RT-PCR analyses were measured in AMSCs, chondrocytes and CCC scaffolds (Figure 5). The results showed that the collagen type II and lubricin significantly increased between the comparison of AMSCs, chondrocytes and CCC scaffolds ( $p < 0.01$ ), whereas collagen type I, RUNX2, osteocalcin and caspase-3 decreased between the comparison of AMSCs, chondrocytes and CCC scaffolds ( $p < 0.01$ ).



**Figure 5.** Real-time PCR. Collagen type I, II, RUNX2, osteocalcin, lubricin and caspase-3 mRNA expression levels in AMSCs, chondrocytes and CCC scaffolds. Total RNA was extracted as indicated in Materials and Method and Collagen type I, II, RUNX2, osteocalcin, lubricin and caspase-3 expression were measured by real-time PCR. Data are expressed as mean  $\pm$  SD of at least three independent experiments. ANOVA and Bonferroni's test, were used to evaluate the significance of the results. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $P < 0.001$  when compared to the samples.

### 3.5. Western blot analysis

The results of ELISA and gene expression analysis were confirmed by Western blot analysis (Figure 6). In fact, the representative bands displayed in the Western blot figure show high correspondence with the results obtained with ELISA and Gene expression analysis. Collagen type II and lubricin significantly increased ( $p < 0.01$ ), between the comparison of AMSCs, chondrocytes and CCC scaffolds, whereas collagen type I, RUNX2, osteocalcin and caspase-3 decreased ( $p < 0.01$ ), suggesting a possible inverse trend between lubricin protein levels and the apoptosis.

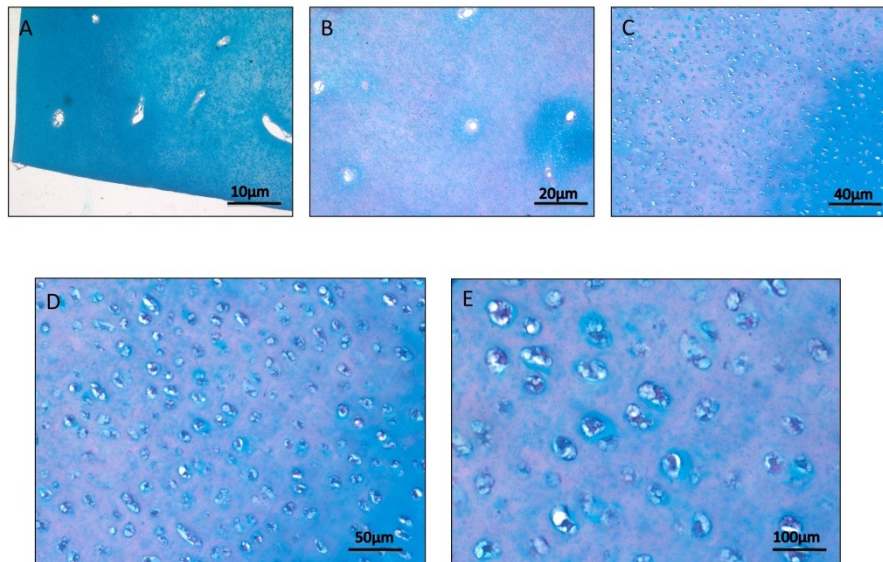


**Figure 6.** Western blot analysis. The expression of collagen type I, II, RUNX2, osteocalcin, lubricin, caspase-3 and  $\beta$ -tubulin protein was evaluated in undifferentiated AMSCs, chondrocytes and CCC scaffolds. Representative immunoblots shown indicate the protein expression levels and were obtained using 50  $\mu$ g of cell homogenates for each experimental group.  $\beta$ -Tubulin was used as loading control in each experiment. The bar graph represents the percentage of tubulin normalized integrated density of collagen type I, II, RUNX2, osteocalcin, lubricin and caspase-3 bands from AMSCs, chondrocytes and CCC scaffolds. Data are expressed as mean  $\pm$  SD of at least three independent experiments. ANOVA and Bonferroni's test were used to evaluate the significance of the results. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $P < 0.001$  when compared to the samples.

### 3.6. Histochemistry

Microscopic morphology was seen through histochemistry (Alcian blue, pH 2.5 PAS staining), demonstrating absence of morphological aberrations in CCC scaffolds after 6 weeks of culture. Microscopically after 6 weeks of culture, we found chondrocytes in the CCC scaffolds. The new chondrocytes grown inside the CCC scaffolds clearly showed an increase in proliferation, cellular aggregations (a hyaline cartilage-like structure) and extracellular matrix (ECM) production as observed in Figure 7. They did not show any signs of cellular damage as demonstrated by the staining (Figure 7). This staining was used also to assess synthesis of glycosaminoglycans (GAG), including proteoglycans (the assessment was made on the intensity of staining (IS)). We found an intense Alcian blue pH 2.5 PAS staining (IS: 3/4) showing the presence of proteoglycans, confirming that the cells in the scaffolds are chondrocytes.





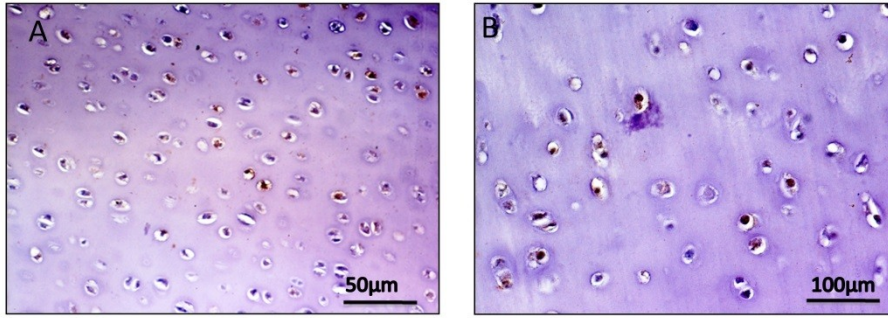
**Figure 7.** Histochemistry (Alcian Blue pH 2.5 PAS staining) results demonstrated absence of morphological alterations in CCC scaffolds. The chondrocytes in the CCC scaffolds showed an increase in proliferation, cellular aggregations (a hyaline cartilage-like structure), ECM production including proteoglycans. (A) Scale bars = 10 µm; (B) Scale bars = 20 µm; (C) Scale bars = 40 µm; (D) Scale bars = 50 µm; (E) Scale bars = 100 µm.

### 3.7. Immunohistochemistry (IHC)

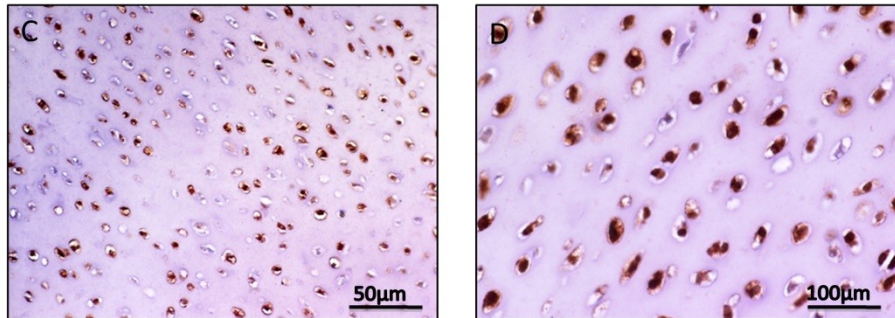
Immunohistochemical results confirmed the previous ones shown in the other previous methods. Indeed, the expression of collagen type II and lubricin significantly increased in CCC scaffolds after 6 weeks of culture, whereas the expression of collagen type I, RUNX2, osteocalcin and caspase-3 decreased. These data demonstrated that in the scaffolds after 6 weeks of culture we can observe a healthy hyaline cartilage, suggesting an inverse trend between lubricin and apoptosis immunoexpression. In particular: Collagen type I immunohistochemical staining was evaluated in CCC scaffolds. Expression of collagen type I is indicative of fibrocartilage. Moderate collagen type I staining was observed (Extent Score (ES) = ++; IS = 2, Figure 8A and B). Both ES and IS were significantly greater ( $p < 0.05$ ) compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.88 (Figure 9C and D).

Collagen type II immunohistochemical staining was evaluated in CCC scaffolds. Expression of collagen type II is indicative of hyaline cartilage. Very strong collagen type II staining was observed (ES = ++++; IS= 4, Figure 8C and D). Both ES and IS were significantly greater ( $p < 0.01$ ) compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.92 (Figure 9C and D).

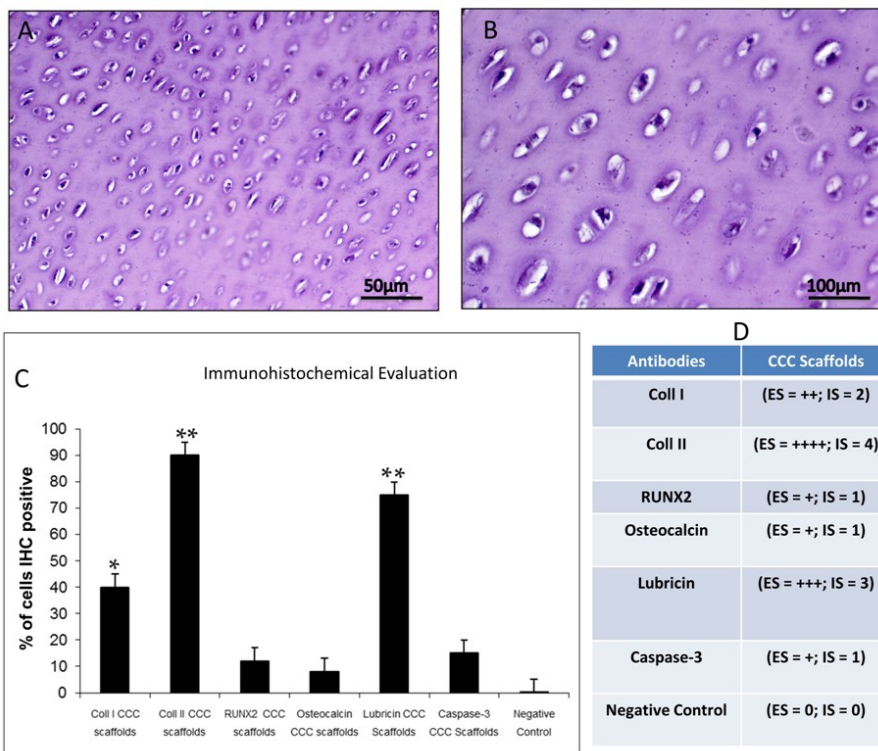
Coll I, Moderate IHC Expression



Coll II, Very strong IHC Expression



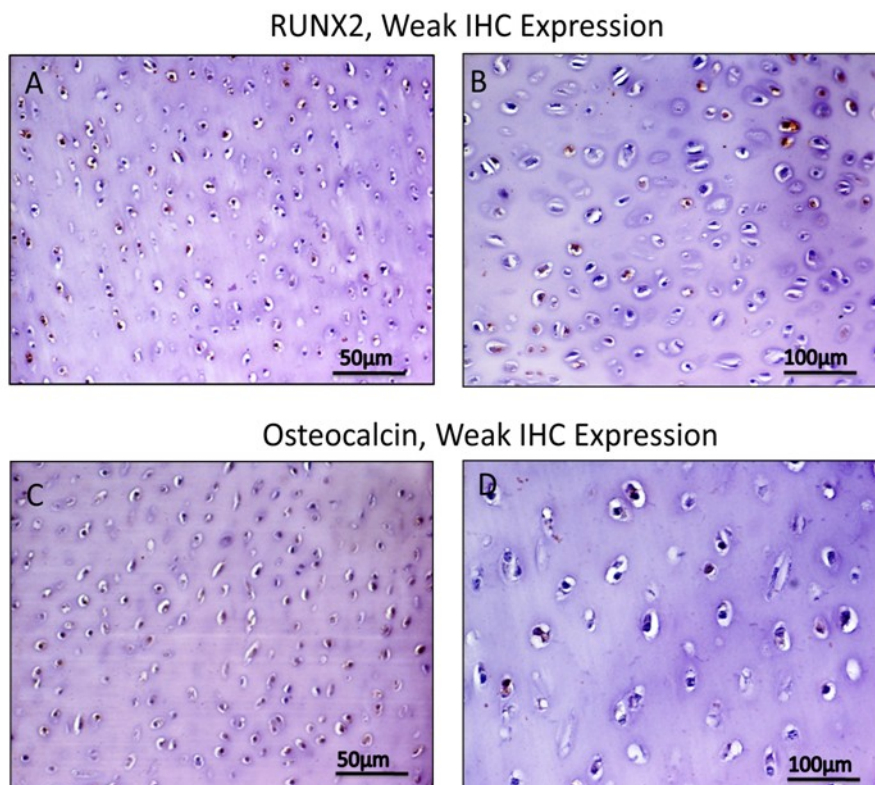
**Figure 8.** Immunohistochemical evaluation of collagen type I and II in CCC scaffolds. Moderate collagen type I staining was observed (ES = ++; IS = 2). Both ES and IS were significantly greater ( $p < 0.05$ ) compared to negative control. (A) Scale bars = 50  $\mu\text{m}$ ; (B) Scale bars = 100  $\mu\text{m}$ . Very strong collagen type II staining was observed (ES = ++++; IS = 4). Both ES and IS were significantly greater ( $p < 0.01$ ) compared to negative control. (C) Scale bars = 50  $\mu\text{m}$ ; (D) Scale bars = 100  $\mu\text{m}$ .



**Figure 9.** Immunohistochemical evaluation of all antibodies. (A,B) Negative control. No immunoreactivity was demonstrated in the negative controls treated with PBS without the primary antibodies (ES = 0; IS= 0). A), Scale bars = 50  $\mu$ m; B) Scale bars = 100  $\mu$ m; (C) Evaluation of immunostaining. Data are expressed as mean  $\pm$  SD of at least three independent experiments. ANOVA and Bonferroni's test, were used to evaluate the significance of the results. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $P < 0.001$  when compared to the negative control; (D) Evaluation of immunostaining. Intensity of staining (IS) was graded on a scale of 0-4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), and very strong staining (4). ES scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5–30% (+); 31–50% (++); 51-75% (+++), and >75% (++++).

RUNX2 immunohistochemical staining was evaluated in CCC scaffolds. Expression of RUNX2 is indicative of osteoblastic differentiation. Weak RUNX2 staining was observed (ES = +; IS= 1, Figure 10A and B). Both ES and IS were not significant compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.87 (Figure 9C and D).

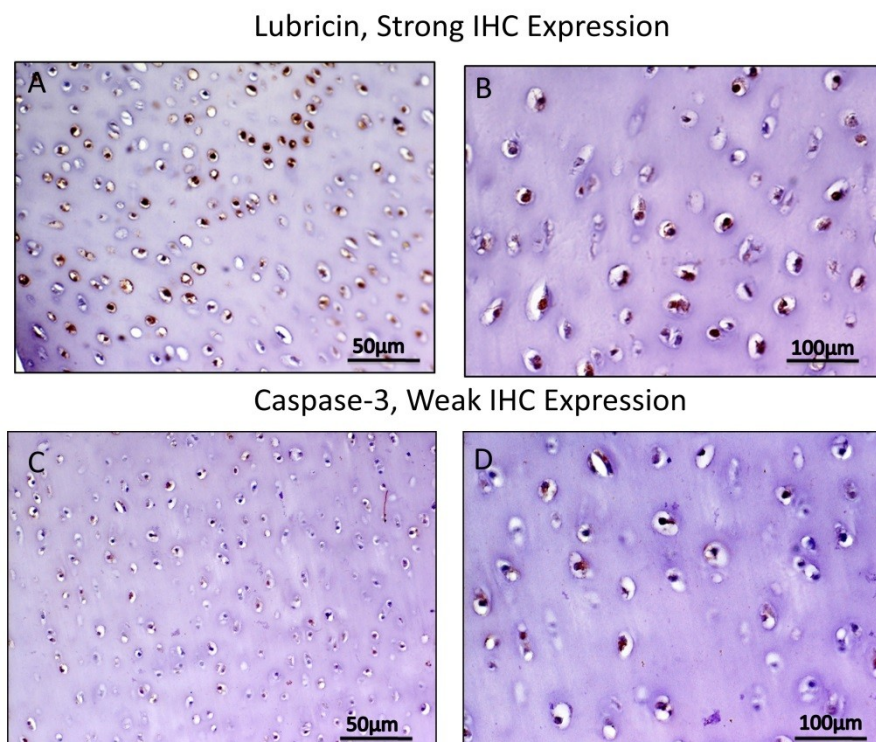
Osteocalcin immunohistochemical staining was evaluated in CCC scaffolds. Expression of osteocalcin is indicative of bone formation. Weak osteocalcin staining was observed (ES = +; IS= 1, Figure 10C and D). Both ES and IS were not significant compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.89 (Figure 9C and D).



**Figure 10.** Immunohistochemical evaluation of RUNX2 and osteocalcin in CCC scaffolds. Weak RUNX2 staining was observed (ES = +; IS= 1, Fig. 10 A and B). Both ES and IS were not significant compared to negative control. (A) Scale bars = 50  $\mu\text{m}$ ; (B) Scale bars = 100  $\mu\text{m}$ . Weak osteocalcin staining was observed (ES = +; IS= 1, Fig. 10 C and D). Both ES and IS were not significant compared to negative control. (C) Scale bars = 50  $\mu\text{m}$ ; (D) Scale bars = 100  $\mu\text{m}$ .

Lubricin immunohistochemical staining was evaluated in CCC scaffolds. Expression of lubricin is indicative of cartilage lubrication. Strong lubricin staining was observed (ES = +++; IS= 3, Figure 11A and B). Both ES and IS were significantly greater ( $p < 0.01$ ) compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.91 (Figure 9 C and D).

Caspase-3 immunohistochemical staining was evaluated in CCC scaffolds. Expression of caspase-3 is indicative of apoptosis. Weak caspase-3 staining was observed (ES = +; IS= 1, Figure 11C and D). Both ES and IS were not significant compared to negative control (Figure 9A and B). Inter-observer agreement, measured using the Kappa coefficient, was 0.88 (Figure 9C and D). No immunoreactivity was demonstrated in the negative controls treated with phosphate-buffered saline (PBS) without the primary antibodies (ES = 0; IS= 0, Figure 9A and B).



**Figure 11.** Immunohistochemical evaluation of lubricin and caspase-3 in CCC scaffolds. Strong lubricin staining was observed (ES = +++; IS= 3). Both ES and IS were significantly greater ( $p < 0.01$ ) compared to negative control. (A) Scale bars = 50  $\mu\text{m}$ ; (B) Scale bars = 100  $\mu\text{m}$ . Weak caspase-3 staining was observed (ES = +; IS= 1). Both ES and IS were not significant compared to negative control. (C) Scale bars = 50  $\mu\text{m}$ ; (D) Scale bars = 100  $\mu\text{m}$ .

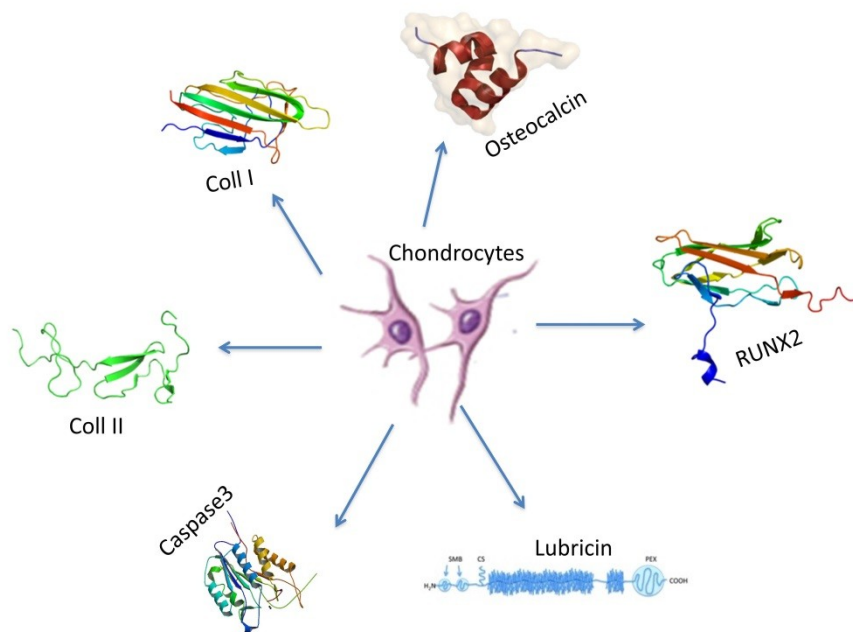
#### 4. Discussion

OA is a disabling disease characterized by progressive degeneration of the cartilage tissue that leads to stiffness and pain in joints [34]. The most important risk factors are age, joint injury, obesity and metabolic diseases. AC is an avascular tissue with limited ability for self-regeneration and the current clinical treatments have restricted capacity to restore damages induced by trauma or diseases [35]. Therefore, new techniques are being tested for cartilage repair, using scaffolds and/or stem cells. The future of regenerative medicine depends on fully understanding the regenerative properties of stem cells, carefully identifying and studying the molecules required for their differentiation into various cell types [36]. In this study, we focus on the possible use of AMSCs to improve current technologies and to bring cell therapies to clinical care. In particular, we focused on isolation, culture and characterization of MSCs derived from adipose tissue and on their differentiation into chondrocytes in a CCC three-dimensional scaffold, through the external stimulation of chondrogenic signalling pathways. These cells showed that, after the differentiation process, they did not lose their morphologic and phenotypic characteristics and, even more important, that, after 6 weeks of culture on CCC scaffolds, they did not become hypertrophic, ensuring a stable healthy cartilage, suitable for a possible implantation. These data have been unequivocally confirmed by our results. Significant attention was paid to improve chondrogenic differentiation capacity but, unfortunately, their hypertrophic potential was largely overlooked. Hypertrophic chondrocytes play a critical role in endochondral bone formation as well as in the progression of OA. The hypertrophic differentiation, occurring in the late stages of MSCs chondrogenesis, is the first challenge that needs to be overcome in cartilage tissue engineering, to obtain adequate, high quality chondrocytes in vitro. Understanding a series of signaling pathways and biophysical factors, which are involved in the process of chondrocyte hypertrophy, could be beneficial in controlling the cartilage regeneration [37].

The aim of this study is to evaluate the expression of some osteoblasts (RUNX2 and osteocalcin), chondrocytes (collagen I, II and lubricin) and apoptosis (caspase-3) biomarkers in undifferentiated AMSCs, differentiated AMSCs-derived chondrocytes cultured in monolayer and AMSCs-derived chondrocytes seeded on CCC scaffolds. The morphological, immunohistochemical, biochemical and gene expression profiles were assessed to test if after 6 weeks of culture.

Our results, corroborated by different scientific methods (ELISA, Gene Expression, Western blot and Immunohistochemistry analyses), and the study of different molecules (Figure 12), showed the increased expression of collagen type II (marker of hyaline cartilage) and lubricin (marker of cartilage lubrication) in differentiated AMSCs-derived chondrocytes cultured on CCC scaffolds

after 6 weeks of culture, whereas the expression of collagen type I (marker of fibrocartilage), RUNX2 (a protein expressed by the hypertrophic chondrocytes in the late stages of differentiation and normally expressed by osteoblasts), osteocalcin (marker of bone formation) and caspase-3 (marker of apoptosis) were shown to be decreased in AMSCs-derived chondrocytes cultured on CCC scaffolds and increased in undifferentiated AMSCs.



**Figure 12.** Target genes involved in this study. Molecular analyses were performed using the UCSF Chimera package. Chimera is graphics software developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco [38].

Our data also demonstrated that AMSCs-derived chondrocytes culture on CCC scaffolds after 6 weeks of culture showed: increased proliferation, cellular aggregations, ECM production including proteoglycans, and an inverse trend between lubricin and apoptosis expression, demonstrated a healthy hyaline cartilage structure. The transplantation of autologous adipose tissue-derived chondrocytes producing lubricin, could be important for repairing cartilage lesions in patients affected by OA since it has been demonstrated that lubricin is able to reduce the progression of several articular diseases [39]. Our results also strengthen the hypothesis that the presence of lubricin, such as collagen type II, could be a possible biomarker for the detection of chondrocyte well-being and viability. RUNX2 is an essential transcription factor for osteoblast differentiation, bone formation, skeletal morphogenesis and also regulates the differentiation of hypertrophic chondrocytes [40,41]. Molecular markers and signaling pathways associated with chondrocyte apoptosis may turn out to be therapeutic targets in OA and approaches aimed at

neutralizing apoptosis-inducing molecules may at least delay the progression of cartilage degeneration in OA [2]. AC injuries are one of the most challenging problems in musculoskeletal medicine owing to the poor intrinsic regenerative capacity of this tissue, consequently novel approaches to repair joint structures need to be established [42]. Although the use of chondrocytes in cartilage tissue engineering is still prevalent, concerns associated with donor-site morbidity, cell de-differentiation and the limited span of these cells have led to the use of MSCs as an alternative cell source for cartilage defect repair [43]. In a recent interesting and innovative study, authors demonstrated the mechanically induced chondrogenesis, through bioreactor system, of bone marrow derived-MSCs seeded into fibrin-poly(ester-urethane) scaffolds, without the use of external growth factors [44]. The latter strengthen the importance of the biomechanical effects for the MSCs chondrogenesis. The possibility of generating chondrocytes from adipose tissue in patients with OA and other degenerative joint disorders is a less invasive way to collect MSCs compared to bone marrow aspiration [6,7]. This study suggests that it is possible to regenerate cartilage tissue, through the use of MSCs drawn from adipose tissue with a minimally invasive technique, such as liposuction, without the need to impair the already injured cartilage tissue, such as in the autologous chondrocytic transplantation (ACT) technique [6,7]. With our results, through the use of a novel CCC scaffold, we show preliminary results of a good scientific impact, that the differentiated AMSCs do not undergo the hypertrophic dedifferentiation, maintaining a stable healthy hyaline cartilage structure, which could be used for a possible transplantation. The use of AMSCs for chondrocyte differentiation and subsequently for ACT is an interesting field of study in order to reduce disease progression. We believe that these findings could be clinically relevant for future therapeutic approaches, even if the present study presents some limitations, such as the absence of mechanical tests to check the mechanical stability of the new cartilage grown in the CCC scaffold and the *in vivo* scaffold implantation procedure to check the *in vivo* biocompatibility, biodegradation and possible rejection of this scaffold inside the joint. However, these limitations have already been overcome positively by other authors, as previously described [32,33].

## **5. Materials and Methods**

### **5.1. Animals**

Adipose tissue of subcutaneous fat was collected from twenty 10-month-old healthy female Wistar Outbred Rats (Charles River Laboratories, Milan, Italy), with an average body weight of  $340\pm 60$  g undergoing liposuction procedures as previously described [34]. Rats were individually housed in polycarbonate cages at controlled temperature (20-23°C) and humidity during the entire period of the study, with free access to water and food and a photoperiod of 12 hours light/dark. The

liposuction procedure was made under total anesthesia, 30 mg/Kg Zoletil 100 + altadol 5 mg/kg + maintenance mixture of O<sub>2</sub> and isoflurane 2-2,5%, (Vibrac, Milan, Italia). Following anesthesia, the abdominal skin was shaved. We used a tumescent technique and a mixture (30–35 ml) of Ringer's lactate (Vibrac, Milan, Italia), lidocaine, adrenaline, and bicarbonate was injected into the rats undergoing liposuction. As in clinical practice, liposuction was performed using the syringe method. Instead of an aspirator, a 10-cc syringe connected to the cannula was used. Aspirated material (a mixture of fat tissue, blood, and serous fluid) was collected in the syringes and transferred into 15ml sterile conical centrifuge tubes (Falcon, Milan, Italy) after the procedures. For liposuction, a small incision was made in the groin and a minimal dissection was made to facilitate aspiration of fat deposits in the subcutaneous region and to avoid trauma to the tissues. Liposuction was performed for 30 min on the right side and 30 min on the left side. The animals after liposuction procedures were sacrificed by intracardial Pentothal® injection 30-40 mg/kg (Biochemie, Kundl, Austria); under Furane 2%®-narcosis (Abbott Laboratories, Maidenhead, Berks, UK). All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee (I.A.C.U.C.) of the University of Catania (Protocol n. 125 of the 1 July 2011, Italian Ministry of Health). The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

## **5.2. Culture of adipose tissue-derived MSCs**

The lipoaspirate (a mixture of fat tissue, blood, and serous fluid) from each rat (1-5 ml) was washed with sterile PBS (Invitrogen, Milan, Italy) to remove red blood cells and debris, incubated for 3 hours at 37 °C with an equal volume of serum-free DMEM low glucose (DMEM-1g; Lonza, Milan, Italy) containing 0.075% of type I collagenase (Invitrogen, Milan, Italy). Collagenase was then inactivated by an equal volume of DMEM-1g containing 10% of fetal bovine serum (FBS; Lonza, Milan, Italy). Subsequently, the digested lipoaspirate was centrifuged at 1,200 x g for 10 minutes. The 20 pellets from each donor (rats) were divided in 40 flasks (2 flasks for each donor) and resuspended in PBS and filtered through a 100 µm nylon cell strainer (Falcon BD Biosciences, Milan, Italy). Filtered cells were again centrifuged at 1,200 x g for 10 minutes, plated in 40 T-75 culture flasks (Falcon BD Biosciences, Milan, Italy) with DMEM-1g (10% FBS, penicillin/streptomycin 1%) containing 1% of Mesenchymal Stem Cells Growth Supplement (MSCGS; ScienCell Research Laboratories, Milan, Italy) and incubated at 37 °C with 5% CO<sub>2</sub>. Twenty-four hours after the initial plating, non-adherent cells were removed by exhaustive washing of the plates.

## **5.3. Determination of markers for AMSCs**



In order to identify AMSCs derived from lipoaspirates adipose tissue, flow cytometry was carried out to identify specific cell surface markers of this cell. After reaching confluence (80% of total flask surface), cells from 20 flasks were trypsinized (Sigma-Aldrich, Milan, Italy) and sub-cultured for 2 days. Cells were first washed with PBS, again trypsinized, fixed with 2% paraformaldehyde (PFA) for 10 minutes at 4 °C and permeabilized with 1× Triton (Sigma-Aldrich, Milan, Italy) for 5 minutes at 4 °C. Subsequently, cells were washed with PBS/BSA 1% and incubated with primary antibodies (Millipore, Milan, Italy) for 60 minutes at room temperature. Antibodies used in the different samples were anti-CD44 (1:200), anti-CD90 (1:100), anti-CD105 (1:100), anti-CD14 (1:200), anti-CD34 (1:200) or anti-CD45 (1:200). Cells were then washed with PBS/1% BSA and incubated for 60 minutes at room temperature in the dark with secondary antibodies (goat anti-mouse or goat anti-rabbit) conjugated with fluorescein (FITC; 1:200; Millipore, Milan, Italy). Samples were analyzed using a Coulter Epics Elite ESP flow cytometer (Coulter, Miami, FL, USA). A minimum of 10,000 forward and side scatter gated events were collected per specimen. Samples were excited at  $\gamma=488$  nm and fluorescence was monitored at  $\gamma=525$  nm. Fluorescence was detected using logarithmic amplification. Mean fluorescence intensity (MFI) values were calculated and recorded automatically. After the flow cytometry procedure the cells from the other 20 flasks were grown for 2 weeks and the medium was replaced every 2/3 days. At the end of two weeks of culture, cells from 20 donors (rats) were collected for ELISA, Western blot, gene expression analyses and for chondrocyte differentiation.

#### **5.4. Differentiation of AMSCs in chondrogenic lineage**

AMSCs were trypsinized and diluted to a final concentration of  $2.5 \times 10^5$  cells/ml medium. Then, 0.5 ml of cell suspension was transferred to a 15 ml polypropylene conical tube. They were then centrifuged at  $150 \times g$  for 5 minutes at room temperature and washed with 1 ml growth medium (Lonza, Milan, Italy) containing 1% penicillin/streptomycin. Briefly, at passage 2, growth medium of MSCs was replaced with complete chondrogenic medium that included serum-free DMEM-HG (4.5 g/L) supplemented with 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, 1 mM sodium pyruvate (Gibco, Carlsbad, CA, USA), 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 1% ITS (insulin 25  $\mu\text{g/ml}$ , transferrin 25  $\mu\text{g/ml}$ , and sodium selenite 25 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) and 10 ng/ml recombinant human transforming growth factor- $\beta$ 1 (Invitrogen, Carlsbad, CA, USA). With no further resuspension, the cells were cultured for 14 days at 37 °C and 5% CO<sub>2</sub> in 0.5 ml of complete chondrogenic medium, replaced every 2/3 days. At the end of

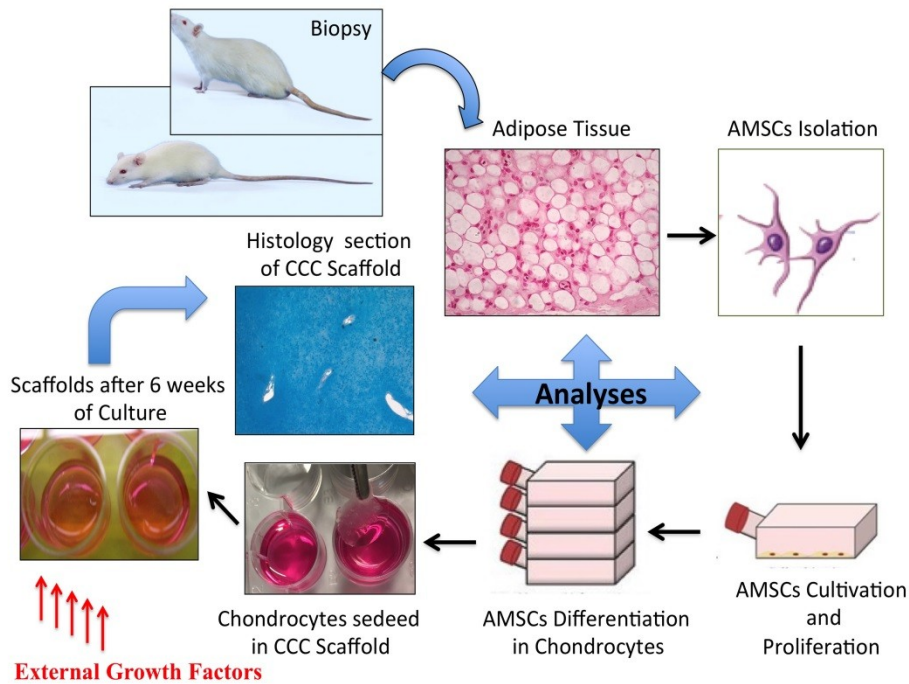
two weeks of culture, cells from 20 donors (rats) were collected for ELISA, Western blot, gene expression analyses and for scaffold seeding.

#### **5.5. CCC pre-treatment for cell culture experiments**

For cell culture experiments, CCC sheets were equilibrated overnight in distilled water (200mL per sheet) at 37°C. Afterward, the wet biomaterial was stamped out into circular discs with a diameter of 12 mm. Discs were transferred into 12-well plates (Corning) preloaded with distilled water. Care was taken that discs were positioned free from creases to the center of the bottom of the well. After removal of residual water, CCC containing culture plates were dried overnight at room temperature under sterile conditions in a laminar air flow. Before cell seeding, dried CCCs were equilibrated with culture medium for 10 min at 37°C. Due to the drying process, the collagen discs were firmly attached to the plastic well bottom without gaps even after subsequent wetting with culture medium. Thus, seeded cells could only adhere to the upper surface of the CCC. Nevertheless, after culturing, the attached CCC could be mechanically detached residue-free from the well bottom with forceps. To culture cells on both sides of the scaffold, attached CCC was removed from the bottom of the well after the preincubation step with culture medium.

#### **5.6. CCC Scaffold Seeding**

After density gradient centrifugation, AMSCs-derived chondrocytes after two weeks of culture, from 20 donors (rats), at passages 2/3 were seeded into 60 CCC scaffolds (3 for each donor) (Figure 13) (50,000 cells/cm<sup>2</sup>) and cultured for up to 6 weeks in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The complete chondrogenic medium (as described above) was renewed every 2/3 days. At the end of 6 weeks of culture, the 60 CCC scaffolds were collected for ELISA (15 scaffolds) Western blot (15 scaffolds), gene expression (15 scaffolds), histochemistry and immunohistochemistry (15 scaffolds) analyses.



**Figure 13.** Schematic design of this study. In this graphical abstract we shown the different steps of this research: (1) Adipose tissue biopsy from rats; (2) Explanted adipose tissue; (3) Cell Isolation from adipose tissue (AMSCs); (4) AMSCs cultivation and proliferation; (5) AMSCc differentiation in Chondrocytes; (6) Chondrocytes seeded in CCC scaffolds; (7) CCC scaffold after 6 weeks of culture; Notice the difference in size between the scaffold at day one and after 6 weeks of culture. The size doubled over the culture period. This suggests that there is cellular growth in the scaffolds; (8) Histology section of CCC scaffold after 6 weeks of culture.

### 5.7. Biochemical analysis (ELISA)

Concentrations of RUNX2 in cell lysates and the concentration of collagen type I, II, osteocalcin, lubricin and caspase-3 in lysed cell supernatant of AMSCs (after 2 weeks of culture), chondrocytes (after 2 weeks of culture) and CCC scaffolds (after 6 weeks of culture) were measured using a commercially available ELISA kit (antibodies-online Inc, 11 Dunwoody Park, Suite 145, Atlanta, GA 30338, USA) according to the manufacturer's instructions for the quantitative determination of all samples used (ThermoFisher Scientific, 81 Wyman Street Waltham, MA, USA). Absorbance was measured at 450nm against 630nm as reference with an ELISA reader (Dynatec, MR 5000; Dynatech Laboratories, LabX, Canada).

### 5.8. RNA isolation and preparation

All samples (AMSCs, chondrocytes and CCC scaffolds) were immersed in QIAzol (Qiagen, Mississauga, Ontario, Canada). After homogenization, total RNA was isolated using the Lipid Tissues Mini Kit, according to the instructions of the manufacturer (Qiagen, Mississauga, Ontario,

Canada). RNA quantity was evaluated using the RiboGreen Assay (Molecular Probes, Burlington, Ontario, Canada), and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

### 5.9. Gene expression analysis by real-time PCR (qRT-PCR)

Total RNA extracted (500–800 ng per sample) was reverse-transcribed with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Milan, Italy) in a 20- $\mu$ l-reaction solution. Quantitative RT-PCR was performed using one-twentieth of the RT products and platinum SYBR Green qPCR SuperMix UDG with Rox (Invitrogen Life Technologies, Italy). The primers used are shown in Table 1. The reaction was followed by a melting curve protocol according to the specifications of the ABI 7900 instrument (Applied Biosystems). Rat  $\beta$ -Actin was used as a housekeeping gene for normalization. Data are presented as mean  $\pm$ SD of at least three independent experiments. Differences were analyzed by ANOVA, with  $p < 0.01$  being considered statistically significant.

**Table 1.** Primers used in gene expression analysis by real-time PCR (qRT-PCR)

<b>Primers</b>	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>
<b><i>RUNX2</i></b>	GAC GAG GCA AGA GTT TCA CC	GGA CCG TCC ACT GTC ACT TT
<b><i>Osteocalcin</i></b>	CAC TCC TCG CCC TAT TGG C	CCC TCC TGC TTG GAC ACA AAG
<b><i>PRG4</i></b>	CTA CAA CAG CTT CTG CGA AGA A	GAT TTG GGT GAA CGT TTG GTG G
<b><i>Caspase-3</i></b>	AGA ACT GGA CTG TGG CAT TGA G	GCT TGT CGG CAT ACT GTT TCA G
<b><i>ACTB</i></b>	GAC AGG ATG CAG AAG GAG ATT ACT G	CCA CCG ATC CAC ACA GAG TAC TT

### 5.10. Western blot analysis

Western blot analysis was performed to analyze the production of collagen type I, II, RUNX2, osteocalcin, lubricin and caspase-3. Briefly, all samples (AMSCS, chondrocytes and CCC scaffolds) were homogenized in a buffer containing T-Per buffer (Thermoscientific, Rockford, IL, USA) and a protease inhibitor cocktail (Roche Diagnostics) in a Teflon-glass homogenizer followed

by sonication. Protein concentrations were determined using Bradford's method using bovine serum albumin as a standard. Sample proteins (50 µg) were diluted in sodium dodecyl sulphate protein gel loading solution (Invitrogen, Monza, Italy), boiled for 5 minutes, separated on 4%–12% Bis-tris gel (Invitrogen) and electroblotted onto nitrocellulose membranes (Invitrogen). We used the following antibodies: anti-collagen types I and II (1:1000; ab34710 and ab85266; Abcam, respectively, Cambridge, UK), anti-RUNX2 antibody (1:1,000; ab76956; Abcam, Cambridge, UK), anti-osteocalcin (1:500; ab133612; Abcam, Cambridge, UK), anti-lubricin (1:500; ab28484; Abcam, Cambridge, UK), anti-caspase-3 antibody (1:500; ab4051; Abcam, Cambridge, UK) and  $\beta$ -tubulin (Santa Cruz Biotechnology Inc, Bergheimer, Germany; 1:20,000), which was used as loading control. The HRP-conjugated secondary antibody (Santa Cruz Biotechnology Inc, Bergheimer, Germany) were diluted 1:10.000. Nonspecific binding was blocked for 2 hours at 37 °C with 5% nonfat dry milk in Tween-Tris-buffered saline. All antibodies were prepared in 5% nonfat dry milk solution in Tween-Tris-buffered saline. The blots were developed using enhanced chemiluminescent solution (Pierce, Fisher Scientific, Milan, Italy) and visualized with autoradiography film. Densitometrical measurements were made by using Image J software (NIH, Bethesda, MD, USA). No signal was detected when the primary antibody was omitted (data not shown).

### **5.11. Histochemistry**

After 6 weeks of culture, CCC scaffold samples were rinsed in PBS, fixed in 10% buffered-formalin (Bio-Optica, Milan, Italy). After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded. After wax infiltration, samples were orientated in the cassettes in the same direction. Sections (4-5 µm thick) were cut from paraffin blocks using a rotary manual microtome (Leica RM2235, Milan, Italy), mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. The sections were stained with Alcian blue pH 2.5 for general cell identification, for the presence or absence of morphological alterations and to assess synthesis of sulphated GAG containing proteoglycans (assessment was made on the IS). The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photomicrographs were captured using a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### **5.12. Immunohistochemistry (IHC)**

For immunohistochemical analysis, CCC scaffold samples were processed as previously described [46]. Briefly, the slides were dewaxed in xylene, hydrated using graded ethanols and

incubated for 30 minutes in 0.3% H<sub>2</sub>O<sub>2</sub>/methanol to quench endogenous peroxidase activity before being rinsed for 20 minutes with PBS (Bio-Optica, Milan, Italy). The sections were heated (5 minutes x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites as previously described [47,48]. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA, Sigma-Aldrich, Milan, Italy) in PBS for 1 hour in a humid chamber. BSA was used as a blocking agent to prevent non-specific binding of the antibody to the tissue sections. Following blocking, the sections were incubated overnight at 4°C with diluted rabbit polyclonal antibodies against types I collagen (ab34710; Abcam, Cambridge, UK) and type II collagen (ab855266; Abcam, Cambridge, UK) collagen, both diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy) with mouse monoclonal anti-RUNX2 antibody (ab76956; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy), with rabbit polyclonal anti-osteocalcin antibody (ab93876; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy), with rabbit polyclonal anti-lubricin antibody (ab28484; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy) and with rabbit polyclonal anti-caspase-3 antibody (ab4051; Abcam, Cambridge, UK), diluted 1:100 in PBS (Sigma-Aldrich, Milan, Italy). Immune complexes were then treated with biotinylated link antibodies (HRP-conjugated anti-rabbit and anti-mouse were used as secondary antibodies) and then detected with peroxidase labeled streptavidin, both incubated for 10 minutes at room temperature (LSAB+ System-HRP, K0690, Dako, Glostrup, Denmark). Immunoreactivity was visualized by incubating the sections for 2 minutes in a 0.1% 3,3'-diaminobenzidine (DAB) and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Glostrup, Denmark). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

### **5.13. Evaluation of immunohistochemistry**

The collagen type I, II, RUNX2, osteocalcin, lubricin and casapse-3-staining status was identified as either negative or positive. Immunohistochemical positive staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distribution within the cytoplasm or in the membrane using light microscopy as previously described [49]. Staining intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-4, according to the following

assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), and very strong staining (4). The percentage of collagen type I, type II, RUNX2, osteocalcin, lubricin and caspase-3 immunopositive cells (Extent Score, ES) was independently evaluated by 3 investigators (2 anatomists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5–30% (+); 31–50% (++); 51–75% (+++), and >75% (++++). Counting was performed at x200 magnification. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at the protein level. Positive controls consisted of tissue specimens with known antigenic positivity (cartilage and bone tissues). Sections treated with PBS without the primary antibodies served as negative controls.

#### **5.14. Computerized morphometric measurements and image analysis**

Fifteen fields, randomly selected from each section, were analyzed and the percentage area stained with collagen type I, type II, RUNX2, osteocalcin, lubricin and caspase-3 antibodies was calculated using image analysis software (AxioVision Release 4.8.2; SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany, using objective lens of magnification x20 i.e. total magnification x400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany). Evaluations were made by three blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement as previously described

#### **5.15. Statistical analysis**

Statistical analysis was performed using GraphPad InStat® Biostatistics version 3.0, (GraphPad Software, Inc. La Jolla, CA, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. One way ANOVA with Sidak's multiple comparison test were used for comparisons between two means (immunohistochemistry), while two-way ANOVA with Tukey's multiple comparisons test were used for comparison between more than two means (groups/samples). P-values of less than 0.05 (\*  $P < 0.05$ ) was considered statistically significant; p-values of less than 0.01 (\*\*  $P < 0.01$ ) was considered highly statistically significant; p-values of less than 0.001 (\*\*\*)  $P < 0.001$ ) was considered extremely statistically significant. Data are presented as the mean  $\pm$  SD. Cohen's kappa was applied to measure the

agreement between the two/three-blinded observers and averaged to evaluate overall agreement for the morphological observations as previously described [50-54]. All experiments made were replicated in triplicate.

## 6. Conclusions

The pathway for developing a clinical strategy for OA has been complicated by the fact that OA is a heterogeneous disease process and by the inability to predict which patients will progressively lose function and develop more joint damage [55-56]. In conclusion this innovative basic study could be a possible key for future therapeutic strategies for AC restoration through the use of CCC scaffolds, to reduce the morbidity from acute cartilage injuries and degenerative joint disease. This could be a biological roadmap for therapies to repair cartilage defects and damage from OA, and hopefully to reduce the need for joint replacement surgery in the near future. The results of this study can also be helpful in overcoming the problems faced during transplantation of MSCs such as hypertrophy. We believe that with this study we have added just a little but important piece to the puzzle for a possible treatment of OA, however, further research in this field is necessary to develop a therapeutic approach.

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**Conflicts of Interest:** The authors declare no conflict of interest.



**Abbreviations:**

AC	Articular cartilage
ACI	Autologous chondrocyte implantation
ACT	Autologous chondrocytic transplantation
AMSCs	Adipose tissue derived-MSCs
CCC	Collagen cell carrier
CCM	Complete chondrogenic medium
DAB	3,3'-diaminobenzidine
ECM	Extracellular matrix
ES	Extent Score
FBS	Fetal bovine serum
GAG	Glycosaminoglycans
IS	Intensity of staining
MACI	Matrix assisted ACI
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MSCGS	Mesenchymal stem cells growth Supplement
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RUNX2	Runt-related transcription factor 2
TNF $\alpha$	Tumor necrosis factor-alpha

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## CHAPTER IX

## 1. General Discussion

OA is a disabling degenerative disorder which affects the articular cartilage of diarthrodial joints, leading to severe stiffness and pain. It is almost the most common global musculoskeletal disease affecting men and women as they age (7). The most important risk factors triggering the initiation and progression of OA are age, acute joint injury (such as cruciate ligament rupture), excessive mechanical load, gender, genetic predisposition, obesity and metabolic diseases (diabetes, etc) (21). Due to the poor self-repairing ability of articular cartilage, due mainly to its avascular and aneural nature, OA is a challenging disorder with limited treatment options. Traditional pharmacologic therapies such as, non-steroidal anti-inflammatory drugs (NSAID) and opioids are effective in relieving pain, but are incapable of reversing cartilage damage and are normally associated with adverse events (22). Therefore, further insights regarding the morpho-molecular mechanisms and studies focused on pathophysiological changes occurring in cartilage tissue, seem of fundamental importance for the future preventive and curative therapies for this complicated and almost global disease. Regenerative methodologies, such as cellular therapies and tissue engineering-based approaches for OA, are emerging as promising alternatives and are widely investigated. The purpose of the present research was to improve the understanding of the morpho-molecular mechanisms occurring in the osteoarthritic and normal cartilage and to attempt to find the possible therapeutic solutions for OA. Primarily, we focused on the detailed and observational study based on histochemical staining of the articular cartilage of rats in different conditions, both pathological and normal ones, as well as in the cartilage obtained from the adipose tissue-derived MSCs. In this way we were able to identify and confirm the evidences found in literature, regarding the structural differences observable between the normal and diseased osteoarthritic cartilage at different stages. Furthermore, we dealt with the identification of the possible markers of the healthy hyaline cartilage such as lubricin, which has been shown to be chondroprotective (20,23,24), and we evaluated its expression in different conditions. What we observed in the first study, was the increased expression of lubricin in articular cartilage of aged rats undergoing the mild physical activity, when compared to the cartilage of old sedentary rats. This finding confirms that lubricin is sensitive to mechanical loading (25) and suggests that mild physical activity may play a beneficial role against the degradation of articular cartilage, potentially opening novel therapeutic or preventive avenues for OA treatment. Moreover, in the other studies, we evaluated the expression of CHIT1 and CHI3L1, which have been widely studied and associated with many degenerative disorders (26), in osteoarthritic and normal rat articular cartilage and we found that their expression increases significantly in the diseased cartilage, suggesting their role in the OA disease. Afterwards, we hypothesised that the expression of CHI3L1 might be directly related to the

expression of lubricin. Indeed, we observed that the lubricin expression decreases with the increase of CHI3L1 expression in the osteoarthritic cartilage, suggesting an interesting link between the inflammatory process and the altered lubricating ability occurring in the joint tissue in OA. We also investigated the expression of lubricin among others, as a marker of healthy cartilage, first in the chondrogenic pellets derived from adipose tissue-MSCs and then in the same cells cultured for 6 weeks on CCC scaffolds. We found that 21 days of pellet culture is the best time to obtain the functionally mature MSC-derived chondrocytes and we demonstrated that chondrocytes cultured on CCC result in a healthy stable hyaline cartilage and they might represent an optimum alternative for the cartilage regeneration approach.

## **2. General Conclusions**

The therapeutic and/or preventive solutions for OA are still a big challenge for the clinical world. The basic biomedical sciences are strictly involved in improving the knowledge about the pathophysiological process and morpho-molecular mechanisms occurring in this severe degenerative disorder. The present study focused on the most important facets of OA. Primarily, we took into account its principal risk factor and triggering element represented by aging process, to pass subsequently to investigate about its main cause given by the inflammatory process and altered lubricating ability of the tissue, to end with the investigation of the solution to the problem given by the tissue engineering approach based on adipose tissue-derived MSCs. In conclusion, the present research highlights some important aspects concerning molecular alterations of articular cartilage tissue occurring in the pathological conditions and provides new insights for the treatments aimed to prevent, attenuate or solve the osteoarthritic process, as well as to restore the lost cartilage tissue.

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## 4. List of publications and scientific contributions

### 4.1. Other Publications

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## 4.2. Posters

1. Musumeci G, Trovato FM, **Szychlinska MA**, Catalano D, Imbesi R, Castrogiovanni P, Trovato GM. Beneficial Effects of Mediterranean Diet and Physical Activity on Osteoarthritis. Morphomolecular Study on Lubricin Expression. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.

2. Musumeci G, Trovato FM, **Szychlinska MA**, Catalano D, Trovato GM. The Effects of Exercise and Kinesio Tape on Physical Limitations in Patients with Knee Osteoarthritis. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.
3. **Szychlinska MA**, Trovato FM, Guglielmino C, Castrogiovanni P, Musumeci G. Immunohistochemical and Gene Expression Profiles of Glycoproteins Chitinase-3-like protein 1 and Lubricin in Osteoarthritic Cartilage. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.
4. Trovato FM, **Szychlinska MA**, Guglielmino C, Castrogiovanni P, Musumeci G. The Effects of Physical Activity (treadmill and vibration stimulation training) in Rats with Glucocorticoid-Induced Osteoporosis. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.
5. Trovato FM, **Szychlinska MA**, Guglielmino C, Castrogiovanni P, Musumeci G. The Effects of Physical Activity (treadmill and vibration stimulation training) in Rats with Glucocorticoid-Induced Osteoarthritis. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.
6. **Szychlinska MA**, Trovato FM, Guglielmino C, Castrogiovanni P. Chondrogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells in a 3D in Vitro Model. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Brescia (Italy), 18-19 Novembre 2016.
7. Musumeci G, Loreto C, Castrogiovanni P, Imbesi R, **Szychlinska MA**, Castorina S. CHI3L1 and Lubricin Expression in Osteoarthritic Cartilage. IJAE. vol. 121, Roma (Italy), 15-17 September 2016.
8. **Szychlinska MA**, Castrogiovanni P, Imbesi R, Giunta S, Musumeci G. Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells at different time points. IJAE. vol. 121, Roma (Italy), 15-17 September 2016.
9. Musumeci G, Trovato FM, **Szychlinska MA**, Catalano D, Trovato GM. Beneficial Effects of Mediterranean Diet and Physical Activity on Osteoarthritis. Morphomolecular Study on Lubricin Expression. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Cesena (Italy), 16-17 Ottobre 2015.
10. Musumeci G, Trovato FM, **Szychlinska MA**, Catalano D, Trovato GM. The Effects of Exercise and Kinesio Tape on Physical Limitations in Patients with Knee Osteoarthritis. Congresso Nazionale Società Italiana di Medicina dello Sport e dell'Esercizio (SIMSE), Cesena (Italy), 16-17 Ottobre 2015.
11. Musumeci G, C Loreto, Giunta A, Castorina A, **Szychlinska MA**, R Imbesi, Castrogiovanni P, Castorina S. Beneficial effects of PACAP in osteoarthritis cartilage. An "in vivo" and an "in vitro" morphological and biochemical study. IJAE. vol. 120, 69° Congresso SIAI 2015, Ferrara (Italy), 17-19 September 2015. (comunicazione orale G. Musumeci)
12. **Szychlinska MA**, Castrogiovanni P, Castorina A, Giunta A, Imbesi R, Pichler K, Mobasher A, Alini M and Musumeci G. Lubricin expression in an osteoarthritis rat model with Mediterranean Diet and mild physical activity to prevent cartilage degeneration. IJAE. vol. 119, Ancona (Italy), 18-20 September 2014.

### 4.3. Awards

1. **2016.** At the National Congress of Italian Society of Sports Medicine and Exercise (SIMSE), in Brescia on 18 to 19 November 2016, the Selection Jury awarded the Best Poster Award 2016 to Marta Szychlinska for the poster entitled “Immunohistochemical and Gene Expression Profiles of Glycoproteins Chitinase-3-like protein 1 and Lubricin in Osteoarthritic Cartilage”.
2. **2016.** The Editorial Board of the International Journal of Molecular Sciences (IJMS, ISSN 1422-0067), Impact Factor 3.257, assigned the Best Original Research Article prize of the "International Journal of Molecular Sciences 2016 Best Paper Award": Ameliorative Effects of PACAP against Cartilage Degeneration. Morphological, Immunohistochemical and Biochemical Evidence from in Vivo and in Vitro Models of Rat Osteoarthritis *Int. J. Mol. Sci.* 2015, 16(3), 5922–5944; doi:10.3390/ijms16035922 of which Marta A. Szychlinska is one of the coauthors.