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Molecular basis of Osteoarthritis and aspects of cellular senescence in disease

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Abstract

The aim of this study is to investigate on some molecular mechanisms contributing to the pathogenesis of osteoarthritis (OA) and in particular to the senescence of articular chondrocytes. It is focused on understanding molecular events downstream GSK3ϐ inactivation or dependent on the activity of IKKŭ, a kinase that does not belong to the phenotype of healthy articular chondrocytes. Moreover, the potential of some nutraceuticals on scavenging ROS thus reducing oxidative stress, DNA damage, and chondrocyte senescence has been evaluated *in vitro*.

The *in vitro* LiCl-mediated GSK3ϐ inactivation resulted in increased mitochondrial ROS production, that impacted on cellular proliferation, with S-phase transient arrest, increased SA-ϐ gal and PAS staining, cell size and granularity. ROS are also responsible for the of increased expression of two major oxidative lesions, i.e. 1) double strand breaks, tagged by ɔH2AX, that associates with activation of GADD45ϐ and p21, and 2) 8-oxo-dG adducts, that associate with increased IKKŭand MMP-10 expression. The pattern observed *in vitro* was confirmed on cartilage from OA patients.

IKKα dramatically affects the intensity of the DNA damage response induced by oxidative stress (H_2O_2 exposure) in chondrocytes, as evidenced by silencing strategies. At early time point an higher percentage of ɔH2AX positive cells and more foci in IKKα-KD cells are observed, but IKKα KD cells proved to almost completely recover after 24 hours respect to their controls. Telomere attrition is also reduced in IKKαKD. Finally MSH6 and MLH1 genes are up-regulated in IKKŭKD cells but not in control cells.

Hydroxytyrosol and Spermidine have a great ROS scavenging capacity *in vitro*. Both treatments revert the H_2O_2 dependent increase of cell death and ɔH2AX-foci formation and senescence, suggesting the ability of increasing cell homeostasis. These data indicate that nutraceuticals represent a great challenge in OA management, for both therapeutical and preventive purposes.

1- INTRODUCTION

1.1 Osteoarthritis (OA)

General characteristics.

Osteoarthritis (OA), the most common arthritis in western population, is a degenerative joint disease characterized by progressive loss of cartilage, synovial inflammation, osteophyte formation, joint space narrowing and subchondral bone sclerosis (Castaneda, Roman-Blas et al. 2012); (Sharma, Jagga et al. 2013), and is responsible for structural and functional alterations of articular cartilage. This disease mostly affects knee, hip and hand and is one of the most common source of pain and disability in the elderly (Arden and Nevitt 2006) (Fig.1). OA affects one in six adults, and by 2030 it is estimated that 20% of people in Europe and United States will be affected by this pathology (De Bari, Kurth et al. 2010).

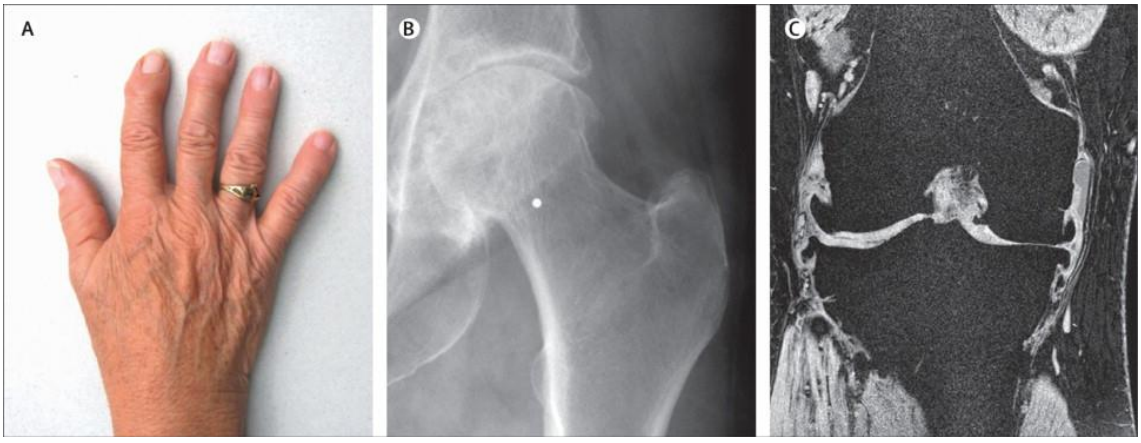


Fig.1 (A) Osteoarthritis at level of hand (deformations of the distal interphalangeal joints). **(B)** Plain radiograph of an osteoarthritic hip joint. **(C)** MRI of an osteoarthritic knee.
Image modified by Bijlsma et al., 2001

The aetiology of OA is multi-factorial and includes not only age, gender, weight but also joint injury and genetic factors. After the age of 50 years more women than men are affected (Bijlsma, Berenbaum et al. 2011). Osteoarthritis develops progressively over several years and chronic pain is frequently associated with significant functional impairment due to progressive degradation of articular cartilage, inflammation, stiffness and loss of mobility (Felson 2006). These symptoms diminish the patientsô quality of life.

On the biochemical level, OA is characterized by uncontrolled production of matrix-degrading enzymes, including aggrecanases (ADAMTSs) and matrix metalloproteinases (MMPs), which results in the destruction of cartilage extracellular matrix (Goldring and Goldring 2007). Other hallmarks of the disease are new bone formation at the joint margins (called *osteophytes*), limited inflammation of the synovial tissue (*synovitis*), and changes in the subchondral bone structure (*sclerosis and cysts*) (Fig. 2). This process is driven, in part, by signalling mechanisms induced by stressful or inflammatory mediators.

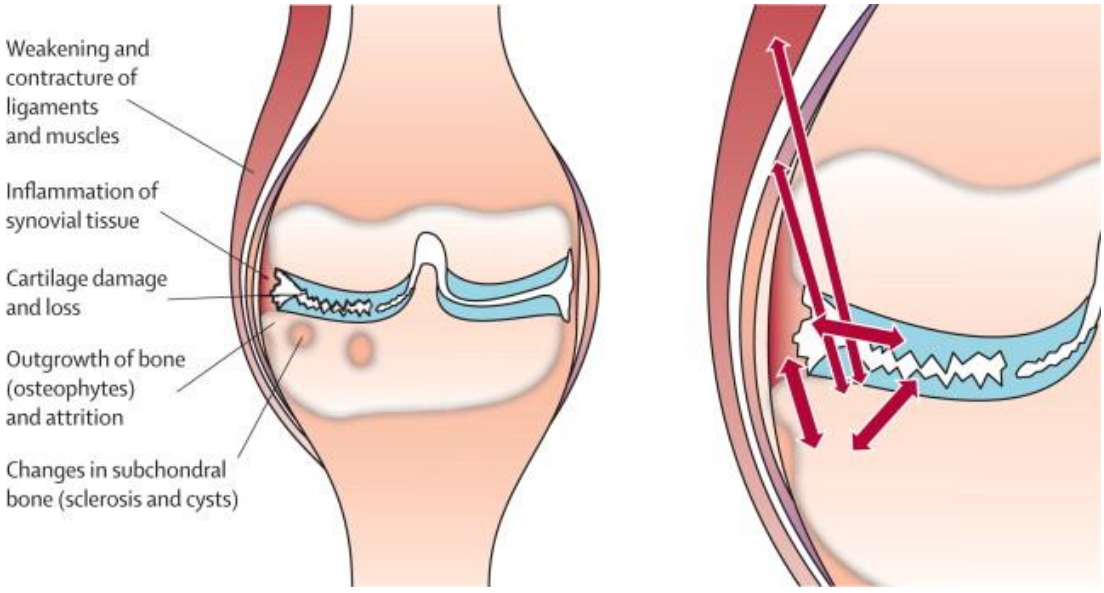


Fig.2 Schematic drawing of an osteoarthritic joint Clinical and structural hallmarks of the disease are shown on the left. On the right the interplay between cartilage, bone and synovial tissue is displayed.
Image modified by Lotz et al., 2012

Osteoarthritis affects not only articular cartilage but also subchondral bone, synovial membrane and joint capsule (Goldring and Goldring 2007). The very early histopathological features are characterized by hypertrophic repair (Dreier 2010). In this context articular chondrocytes increase proliferation rate so they form ñclustersò. Some anabolic activities are up-regulated such as an increased synthesis of proteoglycans and type II collagen. Besides this very early hypertrophic phase, the process is followed by a high catabolic activity, leading to an increased production of matrix metalloproteinases, aggrecanases, higher expression of regulatory proteins, increase of stress, senescence and apoptotic markers (Goldring and Marcu 2009). Expression of MMP-1, MMP-3, MMP-9, MMP-13 and MMP-14, as well as aggrecanases (ADAMTS-5, ADAMTS-4, and ADAMTS-9), drastically increases (van der Kraan, Blaney Davidson et al. 2010); (Madry, Luyten et al. 2012). The expression of MMP-13, known to be pivotal in OA pathology, is regulated by the interplay of two fundamental transcription factors (Runx-2 and Sox-9), contributing to the cartilage degeneration process (Orfanidou, Iliopoulos et al. 2009). These early alterations lead to a progressive loss of the cartilaginous structure, beginning with fibrillation of the superficial zone, followed by matrix loss, and appearance of simple and then more complex fissures (Goldring and Goldring 2007). Moreover, there is evidence of chondrocyte death and of chondrons, in which the cells are disoriented with lack of their normal spatial organization (Pritzker, Gay et al. 2006) . As a result, the thickness of the articular cartilage decreases.

In 2006, some OARSI (Osteoarthritis Research Society International) members proposed a grading and staging system for OA. This group has defined as ñ*grade***ò** a quantitative evaluation of the extent of OA progression into articular cartilage depth while the horizontal extent of the cartilage lesion is termed ñ*stage***ò**. The ñ*score***ò**, defined as the result of OA grade and stage, reflects OA severity and extent. The grade

value goes from 0.0 (articular cartilage surface is intact) to 6.0 (joint surface is deformed). On the other hand, with healthy cartilage being of stage 0, the stage value goes from 1 (less than 10% of joint involvement) to 4 (more than 50% of joint involvement) (Pritzker, Gay et al. 2006).

1.2 Articular Cartilage

Development of articular cartilage: from chondrogenesis to endochondral ossification.

Chondrocytes are the only cellular component of articular cartilage and their primary function is to maintain cartilage homeostasis. During fetal development, the greater part of the skeleton is cartilaginous. At the level of the long bones, this ñtemporaryò cartilage is gradually replaced by bone in a process called *endochondral ossification,* that ends at puberty, although the growth plates will complete their ossification only some years later. In contrast, the cartilage in the joints remains un-ossified during the whole life and is, therefore, ñpermanentò. The development of both temporary and permanent cartilage is called *chondrogenesis* and begins with the proliferation of chondroprogenitor from mesenchymal stem cells; at the completion of the process, in articular cartilage, the chondrocytes are kept in a state of maturational arrest and are prevented to complete the default route of their maturation process which ends with hypertrophy and terminal differentiation. Stage-specific transcription factors and signalling pathways play a key role in the process of chondrocyte maturation (Dreier 2010). As articular cartilage is formed to last for life, chondrocytes display only moderate metabolic activity under normal conditions to maintain their surrounding extracellular matrix (ECM) comprising collagens, proteoglycans and non-collagenous proteins. Under non-diseased conditions, the cells remain in a resting state and refrain from proliferation and terminal differentiation; this process is regulated by the

transcription factor Sox9. In a diseased OA state, however, some articular chondrocytes loose their differentiated phenotype and enter an endochondral ossification-like cascade of proliferation (Tallheden, Bengtsson et al. 2005) and hypertrophic differentiation followed by extracellular matrix calcification.

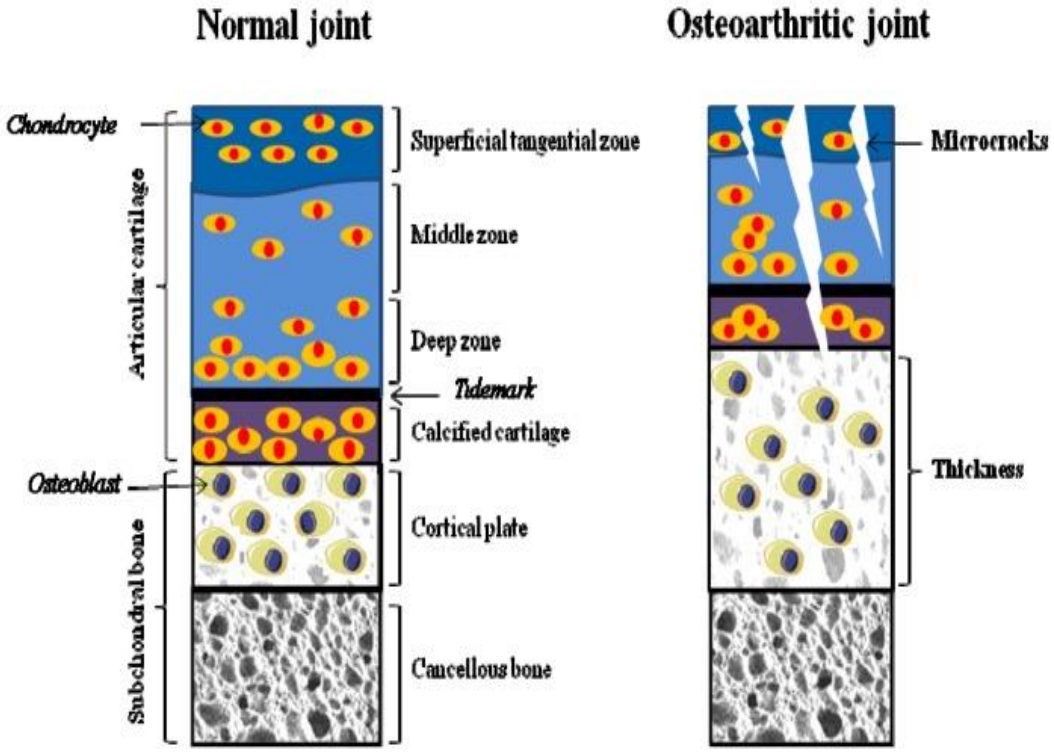


Fig.3 Anatomy of articular cartilage and subchondral bone in normal and OA joints. Normal articular cartilage is divided into four zones. These zones consist of a small number of chondrocytes trapped in collagen matrix. Alteration in OA joint is represented by collagen matrix disruption in articular cartilage and thickening of subchondral bone. Fissuring and flanking in articular cartilage induces vascularization of cartilage, leading to exposure of subchondral bone to external surface.
Image modified by Sharma et al., 2013

This process is associated with an increase of environmental stress, articular chondrocyte proliferation, expression of hypertrophy markers (for example, MMP-13 and collagen X), remodeling of the cartilage matrix by proteases, vascularization and focal calcification of joint cartilage with deposition of calcium hydroxyapatite crystals. This profound ECM perturbation and environmental stress can lead to chondrocyte programmed cell death. Hypertrophic differentiation and endochondral ossification are regulated by a transcription factor called Runx-2. Therefore some of the characteristics

of osteoarthritic cartilage resemble chondrocyte differentiation processes during skeletal development (Dreier 2010) and osteoarthritic chondrocytes undergo hypertrophy and terminal endochondral ossification, mimicking the pattern of differentiation of fetal skeletogenesis (Borzi, Olivotto et al. 2010); (Marcu, Otero et al. 2010)

Structure of articular cartilage.

In regard to its cellular composition, articular cartilage is less complex as compared to other tissues as it is neither vascularized nor innervated and does not contain tissue macrophages. Nevertheless it is a tightly regulated tissue, kept in a low homeostatic turnover by the chondrocytes that represent approximately 5% of the total tissue volume (Buckwalter, Mankin et al. 2005). Articular cartilage covers the end of bones of synovial or diarthrodial joints and acts bearing static and dynamic forces (shear, compression and tension). These mechanical loads are absorbed by the cartilage extracellular matrix (ECM) that is mainly constituted by collagens and proteoglycans. Type II collagen is composed of three polypeptide chains forming the fibrillar unit that polymerize outside the cells in a network with type XI and type IX collagen (Heinegard and Saxne 2011). Proteoglycans are mostly represented by large macromolecular aggregates of units called ñaggrecanò anchored to a hyaluronan molecule. Aggrecan are macromolecular complexes with a core protein which establishes several interactions with glycosaminoglycans (GAG). The network of type II collagen fibrils is essential for maintaining the volume and shape, while proteoglycans ensure elasticity of the tissue. Collagens and proteoglycans are differently organized across the ECM thus it is possible to distinguished a *territorial* and an *inter-territorial* region as a function of the proximity to chondrocytes. In the inter-territorial area, the collagen component is highly prevalent. Conversely, the proteoglycan component is much more represented in the

territorial region (Madry, Grun et al. 2011). In articular cartilage four zones can be distinguished; the superficial, middle, deep and calcified zone. From a structural point of view, the *superficial* layer of articular cartilage is composed by a network of extracellular matrix proteins, proteoglycans, collagen, and non-collagenous proteins, and maintains a high water content. This zone includes a large percentage of immature and progenitor cells (Grogan, Miyaki et al. 2009). *Middle* layer contains randomly oriented collagen fibers with larger chondrocytes. *Deep* stratum is formed by vertical columns separated by collagenous fibrils. The last layer of articular cartilage is represented by *calcified* cartilage with partial mineralization and hypertrophic chondrocytes. This zone is separated from the deep zone by the so called *tidemark* of collagen X fibrils (Poole, Kojima et al. 2001). Subchondral bone plate is placed under the deepest calcified area of the articular cartilage (Burr 2004) (Fig. 3). The unique composition and organization of the matrix in articular cartilage are determined during embryonic and postnatal development. Articular cartilage is avascular, alymphatic and aneural, and oxygen and nutrients arrive to chondrocytes by diffusion from the synovial space. Oxygen gradient is found in the tissue and surface chondrocytes have an aerobic metabolism while the deeper-layer chondrocytes have an almost complete anaerobic metabolism.

1.3 Role of senescence in OA

General features of senescence.

The process of cell senescence is classically defined as the loss of the ability of mitotic cells to further divide in culture after a reproducible number of population doublings (generally from 30 to 40), the so called ó6Hayflick limitò (Hayflick 1984). This process refers to the irreversible growth arrest that occurs when dividing cells encounter many

different stimuli. The limited growth of human cells in culture is due to telomere erosion at each S phase of cellular cycle; besides the gradual loss of DNA at the ends of chromosomes, the eroded telomeres generate a persistent DNA damage response (DDR), which initiates and maintains the senescence growth arrest (d'Adda di Fagagna, Reaper et al. 2003); (Takai, Smogorzewska et al. 2003); (Rodier, Coppe et al. 2009). Genomic damage affects both telomeric and non-telomeric sites; for example DNA double strand breaks are especially potent senescence inducers that generate the persistent DDR signaling needed for the senescence growth arrest (Nakamura, Chiang et al. 2008). Another cause of senescence is the so-called ñculture stressò, that includes not only inappropriate condition of culture (e.g., plastic, serum), but also an oxidative stress (Parrinello, Samper et al. 2003).

Senescence has been associated to several phenotypes that could be resumed in some points:

1- The senescence associated growth arrest is permanent and not reversible.
2- Senescent cells increase in size, enlarging more than two-fold relative to the size of non senescent cells (Hayflick 1965).
3- Senescent cells express a senescence-associated ß-galactosidase (SA-ßgal) (Dimri, Lee et al. 1995).
4- Most senescent cells express $p16^{INK4a}$, that causes formation of senescence-associated heterochromatin foci (SAHF) (Narita, Nunez et al. 2003).
5- DDR-induced senescence is characterized by persistent nuclear foci that contain activated DDR proteins (d'Adda di Fagagna, Reaper et al. 2003).
6- Senescent cells with persistent DDR signaling secrete growth factors, proteases, cytokines, and other factors that have potent autocrine and paracrine activities

(Coppe, Patil et al. 2008) called senescence-associated secretory phenotype (SASP).

The phenomenon of óintrinsicõ or ñreplicativeò senescence.

According to its classical definition, senescence is a phenomenon affecting proliferating cells which loose their proliferative activity after a period of expansion in culture (óintrinsicõ or ñreplicativeò senescence). It is accompanied by a reduction in proliferative potential and it is not only associated with an alteration of the morphology and cellular functions, but also with shortening of the total length of telomeres and the possibility of accumulating DNA damage (Lawless, Wang et al. 2010). Replicative senescence is characterized by a period of normal growth followed by cessation of cell divisions. The cells placed in long-term culture show a series of physiological, molecular and functional changes that lead to senescence (Bonab, Alimoghaddam et al. 2006). So this mechanism may have evolved in tissues to prevent cells with damaged DNA from being replicated and thus to prevent tumor formation (Loeser 2009). *In vivo* studies have demonstrated the relevance of replicative senescence for tissues with rapid turnover (such as skin), showing that fibroblasts isolated from older humans or animals reach replicative senescence sooner than cells isolated from younger individuals (Muller 2009). Replicative senescence is associated with changes in DNA structure and function including telomere shortening accompanied by telomere dysfunction (Itahana, Campisi et al. 2004); (Goyns 2002).

Telomeres are big nucleoprotein complex found at the ends of chromosomes (Blackburn 1994). In humans, the repetitive sequence of telomeric DNA is **d(TTAGGG)** (Meyne, Ratliff et al. 1989) and most of that DNA is organized in nucleosomes. Telomeric DNA is always oriented 5'-3' towards the terminal portion of the chromosome and has a

protrudent extreme of ~200 nucleotides as consequence of the problem of terminal replication. In humans, telomeres are bound by a six-protein complex called *Shelterin*,

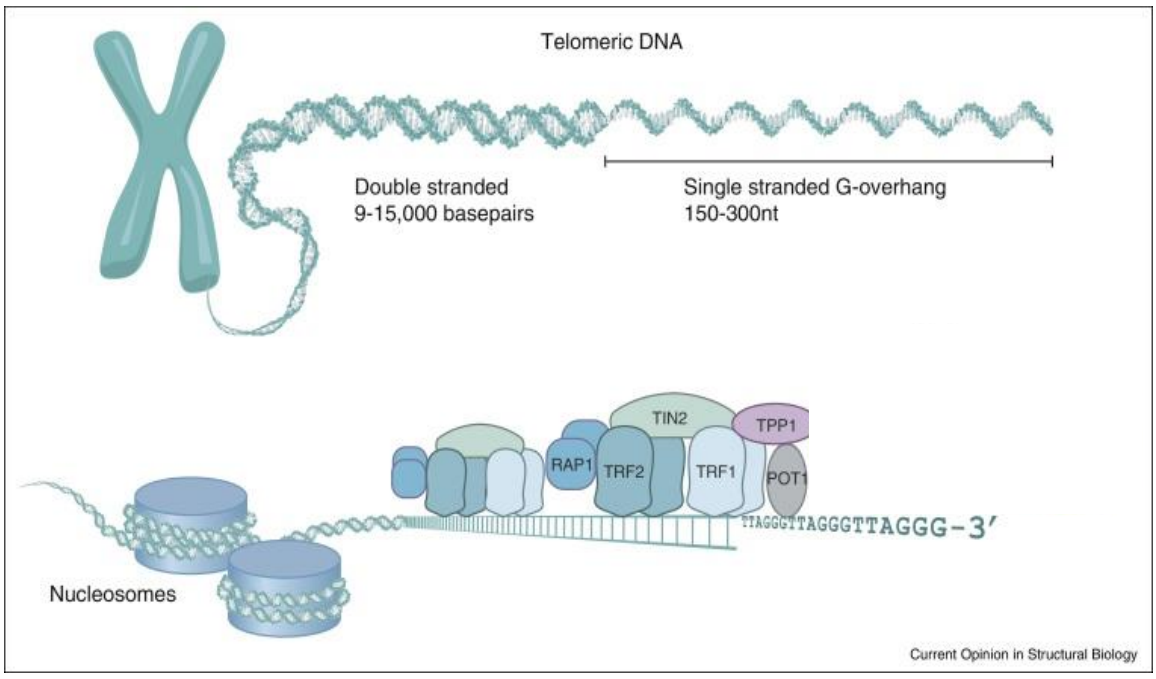


Fig.4 Human telomeric structure. A schematic representation of telomeric DNA bound by the *Shelterin* complex consisting of six telomere binding proteins: TRF1, TRF2, Rap1, TIN2, TPP1 and POT1.
Image modified by Sandin et al., 2014

comprised of TRF1 and TRF2 which in turn recruit RAP1, TIN2, TPP1 and POT1 which interacts with single-stranded and double-stranded telomeric DNA (Fig.4). This structure forms a large protective loop called T-loop which serves to sequester the chromosome terminus. At the very end of the T-loop, the single-stranded telomere DNA is held onto a region of double-stranded DNA creating a triple-stranded structure called D-loop (Gomez, Armando et al. 2012). These structures prevent chromosome ends from being recognised as DNA break (de Lange 2002). Telomeric DNA is gradually lost with each S phase because DNA polymerases are unidirectional and cannot initiate a new DNA strand, resulting in progressive shortening of DNA near the end of a chromosome; additionally, most normal differentiated cells do not express

telomerase, the specialized enzyme that can restore telomeric DNA sequences *de novo* (Harley, Futcher et al. 1990); (Bodnar, Ouellette et al. 1998). Progressive telomere erosion with ongoing proliferation can therefore results in cell cycle arrest and replicative senescence. However, cell senescence appears to be much more complex than simple cell-cycle arrest occurring after a finite number of cell divisions. Replicative senescence has been associated with features of senescent phenotype such as enlarged flattened cells in culture, expression of the senescence-associated ß-galactosidase (SA-ßgal), and presence of markers of DNA damage (Rodier, Munoz et al. 2011).

The phenomenon of óextrinsicôo or ñstress-inducedò senescence.

Progressive telomere shorting due to repeated cycles of cell division does not explain senescence in post-mitotic tissue like articular cartilage. It is known that cellular senescence might be provoked by various cellular events such as oxidative damage to DNA or other cell components. Oxidative damage, activated oncogenes, and inflammation can also damage telomeres and this is a much more likely mechanism for senescence in cartilage. This model of senescence may be called óextrinsicôo or stress-induced and is relevant for post-mitotic tissues, in which damaged proteins, lipids and DNA accumulate over time. This type of senescence can occur following diverse stimuli including ultraviolet radiation, oxidative damage, activated oncogenes, and chronic inflammation (Itahana, Campisi et al. 2004); (Patil, Mian et al. 2005). It has been shown that post-mitotic cells are more vulnerable to accumulation of aberrant protein and metabolic waste (Vicencio, Galluzzi et al. 2008). Oxidative damage to DNA can directly contribute to stress-induced senescence and, because the ends of chromosomes are particularly sensitive to oxidative stress, this can result in telomere

shortening similar to that seen with replicative senescence (Itahana, Campisi et al. 2004); (Goyns 2002).

Senescence in Aging and Osteoarthritis.

Since the incidence of osteoarthritis increases with age, the aging of chondrocytes is accompanied by progressive cellular senescence and reduced ability to maintain and to restore articular cartilage. In fact chondrocytes from older adults share many of the changes exhibited by senescent cells, like telomere shortening, SA-ß galactosidase staining, increase in p53 and p21 activity and in DNA damage (Mowla, Lam et al. 2014); (Martin 2001). Adult articular chondrocytes rarely, if ever, divide in normal tissue in vivo (Aigner, Zien et al. 2001) so it would seem unlikely that they could experience telomere shortening due to classical replicative senescence *in vivo*. However, chondrocyte proliferation is a feature seen in OA tissues and both telomere shortening and the presence of SA-ß galactosidase have been observed in chondrocytes in OA lesions. Thus, it is much more likely that chondrocyte senescence is induced by chronic stress. This process, besides limiting replicative potential of the cells, is also associated to the so-called ñSenescence associated Secretory phenotypeò (SASP) sharing many features with the OA chondrocyte phenotype, such as increased production of IL-6 and IL-1, MMP-3 and MMP-13 and growth factors (Freund, Orjalo et al. 2010). During aging and osteoarthritis, articular cartilage extracellular matrix changes with respect to the total amount and composition, and undergoes proteolysis and other posttranslational modifications (Fig. 5).

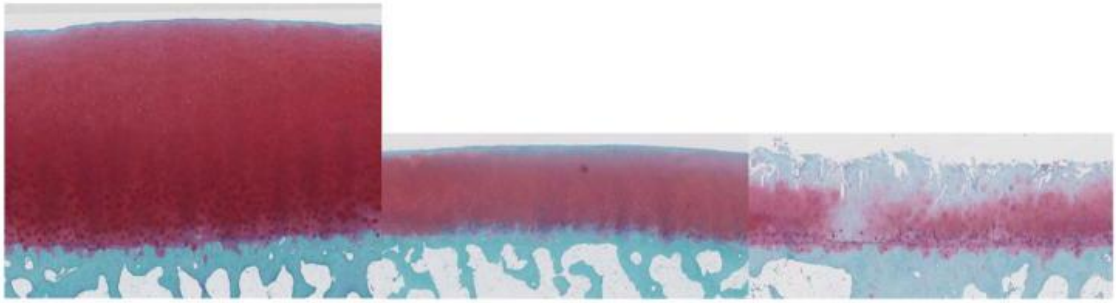


Fig. 5 Safranin O staining of human cartilage derived from young normal donor, old normal donor and OA patient.
Image modified by Lotz et al., 2012

In OA, increased proteolytic activity in cartilage and synovial fluid causes cartilage matrix changes, with an increase in degraded collagen molecules. This degradation is accompanied by the loss of cartilage in the superficial zone and loss of aggrecan (Lotz and Loeser 2012). Moreover, during aging and OA, chondrocytes are less responsive to anabolic growth factors like IGF-1 and TGF-ϐ (Fortier, Barker et al. 2011) able to regulate their synthetic activity. With OA progression, an increased catabolic activity causes imbalance of cartilage homeostasis and cartilage matrix breakdown. These events are largely mediated not only by proinflammatory cytokines and matrix metalloproteinases (Sandell and Aigner 2001) but also by reactive oxygen species (ROS).

SASP: ñSenescence associated Secretory phenotype".

Judith Campisi and collaborators demonstrated that genotoxic stress in cells induces senescence and is accompanied by secretion of a myriad of factors associated with inflammation (Coppe, Patil et al. 2008). They called this cellular state the senescence-associated secretory phenotype (SASP). Genotoxic stress, provoked by exhaustive replication, ionizing radiation or oxidative stress induced a similar type of SASP in

normal human fibroblasts and epithelial cells. Secreted factors included interleukins and chemokines, e.g. IL-1Ŭƃ, IL-6, IL-8, MCP-2 and MIP-1Ŭ, growth factors, such as bFGF, EGF and VEGF, and several matrix metalloproteinases (Coppe, Desprez et al. 2010).

The signaling regulation of cellular senescence seems characterized by several positive, cell type specific feedback loops driven by secreted inflammatory mediators which can boost senescence via transcriptional regulation acting in an autocrine and/or paracrine manner. In fact many of the secreted compounds have effects not only in the tissue microenvironment but also at the systemic level. For example some of the released proinflammatory cytokines and chemokines can arrest cell growth (Salminen, Kauppinen et al. 2012). Moreover, inflammatory cytokines stimulate NF-əB signaling in senescent cells and can prevent apoptosis and thus maintain their senescent phenotype. Several studies have confirmed that apoptosis is reduced in senescent cells (Salminen, Ojala et al. 2011) . However, chronic inflammation is associated with many age-related diseases, e.g. metabolic disorders, cardiovascular and neurodegenerative diseases and osteoarthritis and the aging process itself also involves a low grade inflammation (Salminen, Huuskonen et al. 2008).

1.4 Role of oxidative stress in OA

Reactive Oxygen Species (ROS) are oxygen-containing molecules which are produced during normal metabolism. Oxidative stress results when the amount of ROS exceeds the anti-oxidant capacity of the cell. This process induces cell senescence not only *in vitro* but also *in vivo*, in fact there is evidence of age related oxidative stress in many tissues (Muller 2009). ROS are by-products of aerobic metabolism and include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OHĀ), all of

which have specific chemical properties for different biological targets (Schieber and Chandel 2014). ROS are also generated by enzymes such as NADPH oxidase and 5-lipoxygenase in response to specific cell signaling pathways. These ROS serve as secondary messengers that regulate signal transduction by activating redox-sensitive kinases and inhibiting redox-sensitive phosphatases (Finkel and Holbrook 2000).

Oxidative stress may play a major role in the link between the process of aging and the development of osteoarthritis: it has been reported that oxidative stress plays a role in chondrocyte senescence *in vitro*; exogenous addition of hydrogen peroxide or IL-1 (to mimic chronic inflammation) to cultured chondrocytes was found to induce markers of the senescent phenotype (Dai, Shan et al. 2006) and accelerates telomere shortening (Brandl, Hartmann et al. 2011). In this context ROS are often associated with the principles of oxidative stress contributing directly to cell senescence by causing damage to proteins, lipids, and DNA.

Increased levels of intracellular ROS were detected in cartilage derived from old rats when compared to young adults (Jallali, Ridha et al. 2005) and make human chondrocytes and rat chondrocytes more susceptible to cell death mediated by oxidants (Carlo and Loeser 2003) in an age-related manner. Not only aging and inflammation, but also other stressful factors contribute to production of ROS and induction of oxidative stress in chondrocytes of articular cartilage. In fact it has been reported that injurious mechanical loading may be a stimulus for excessive ROS production in cartilage (Green, Noble et al. 2006) and could thus contribute to stress-induced chondrocyte senescence and osteoarthritis.

There are many clues about the fact that oxidative stress may involve not only articular cartilage, but also subchondral bone and synovial membrane. Different clinical presentations of OA are associated with cellular senescence that might occur in the

different joint tissues: fibrosis and stiffness of joint capsule is one of these. It is documented that the cellular mechanism of fibrosis control is accompanied by cellular senescence (Krizhanovsky, Xue et al. 2008) and since fibrosis is a feature of OA, it is conceivable that intra-articular paracrine ROS messaging might promote senescence of fibroid cells to limit fibrosis (Fig.6). Moreover, joint inflammation is a characteristic of OA so it is likely that ROS induced senescence of synovial and cartilage cells can promote the inflammatory transition of the osteoarthritic joint (Ziskoven, Jager et al. 2010). Indeed senescent chondrocytes have paracrine effects on articular cartilage (Freund, Orjalo et al. 2010).

It has also been reported that mitochondrial respiratory chain is one of the major sites of ROS production and that inhibition of Complex III not only induces ROS production but also pro-inflammatory molecules (Turrens 2003). Moreover in aged and OA cartilage, a decrease of enzymes responsible of the cellular anti-oxidant defense (such as mitochondrial superoxide dismutase (SOD2)and glutathione peroxidase) has been documented.

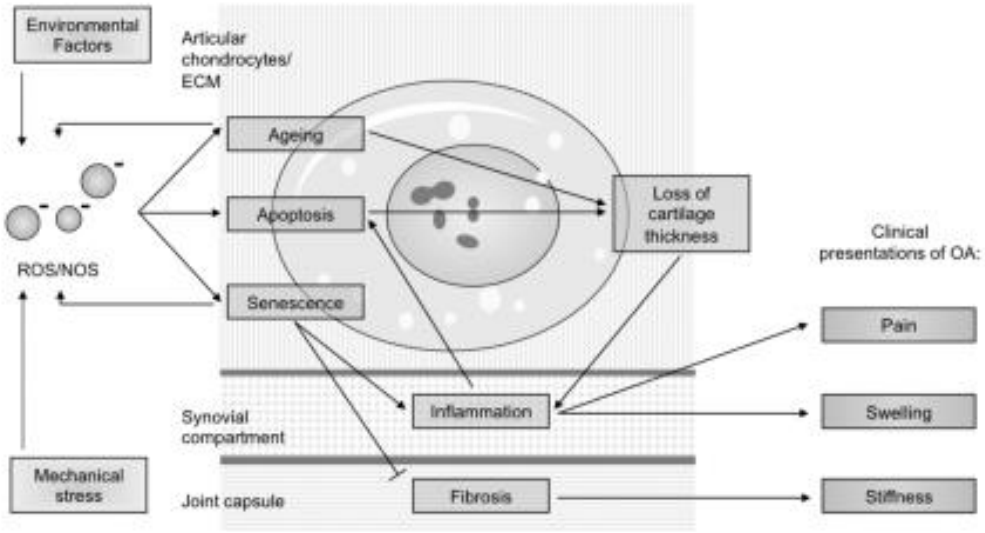


Fig.6 Hypothetical relation between oxidative stress and osteoarthritis**.**
Image modified by Ziskoven et al., 2010

1.5 Involvement of oxidative stress in DNA damage

When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress induces damage to DNA, proteins and lipids which consequently becomes implicated in the pathology of various diseases. Various forms of DNA damage were discovered such as those caused by oxidative damage that stimulate damage signals, activating checkpoint responses and in certain cases premature senescence (Colavitti and Finkel 2005). Regarding DNA damage, it is estimated that 80% of ROS attack bases while the remaining 20% act on sugar moieties generating multiple lesions that may lead to double strand breaks (Sedelnikova, Pilch et al. 2003). Another type of oxidative DNA lesion is the adduct 7,8-dihydro-8-oxo-2ô-deoxyguanosine (8-oxo-G) that mostly affects purines (Brierley and Martin 2013) in genomic and mitochondrial DNA, as well as in RNA.

To deal with oxidative damage to DNA from various endogenous and exogenous sources, mammalian cells have evolved many mechanisms to first detect, and subsequently repair such damage.

Double-strand breaks and ɔH2AX.

DSBs are formed when both DNA strands are broken in sufficiently close proximity (<20 bp). They are one of the most critical lesions with respect to preservation of genomic integrity because affect the continuity of the genome. Cells undergo rapid and efficient error correction to preserve genomic stability by activating complex DNA repair pathways collectively known as the DNA damage response (DDR) (Khanna, Lavin et al. 2001 (Khanna, Lavin et al. 2001).

Phosphorylation of H2AX is one of the earlier cellular response to DSBs that accumulates in DNA foci and activates a complex molecular response (Gire, Roux et al.

2004). H2AX is a minor histone H2A variant which makes up between 2 to 25% of the H2A pool, depending on the cell type (Rogakou, Pilch et al. 1998). The H2AFX gene encodes H2AX; it is mapped onto position 23 on the q arm of chromosome 11 (Mah, El-Osta et al. 2010) and the sequence is highly conserved. In mammalians, Ser-139 at the C-terminus of the H2AX molecule is phosphorylated to produce ɔH2AX, which in recent years has emerged as one of the most well known markers of DNA damage and repair (Sedelnikova, Rogakou et al. 2002). This phosphorylation is mediated by the phosphatidylinositol 3-kinase-related kinase (PIKK) family of proteins. The three proteins potentially involved in the process are ataxia telangiectasia mutated (ATM), DNA-protein kinase catalytic subunit (DNA-PKcs) and ATM and RAD3-related (ATR) that act at the level of the highly conserved Serine-Glutamine (SQ) motif of H2AX following damage occurring after either UV exposure or metabolic stress or reactive oxygen species (Falck, Coates et al. 2005), but ATM seems the main kinase involved in H2AX phosphorylation (Fernandez-Capetillo, Chen et al. 2002). One aspect of H2AX phosphorylation is that it is not limited to the immediate vicinity, but spreads to a large chromatin region surrounding the 2Mbp region of chromatin around DSB and comprises about 2000 ɔH2AX molecules (Rogakou, Pilch et al. 1998).

Each ɔH2AX focus acts as a platform for the accumulation of DDR and repair factors and functions by altering chromatin structure to increase its accessibility (Paull and Lee 2005). Accumulating multiprotein complexes consist of other DNA repair and signaling molecules such as 53BP1, BRCA1, MDC1, NBS1, MRE11, RAD50 and RAD51 and form nuclear foci that have been also found to co-localize with ɔH2AX foci (Mah, El-Osta et al. 2010) (Fig.7). These proteins are recruited not only for their high affinity for ɔH2AX but also thanks to changes in chromatin conformation induced by the phosphorylation process (Andegeko, Moyal et al. 2001). Along with the accumulation

of DSB response and repair factors, a small fraction of ATM responsible for H2AX phosphorylation is retained in the nucleus and is found associated with ɔH2AX foci (Bonner, Redon et al. 2008).

H2AX phosphorylation is a crucial step in the DDR, because it induces DSB repair initiation by increasing DNA accessibility and facilitating the recruitment and accumulation of specific DDR proteins. The DDR is a cascade that senses DNA damage, induces cell cycle arrest and initiates DNA damage repair. Moreover, ɔH2AX modulates G2/M checkpoint responses and prevents cell cycle progression also inducing cellular senescence. This process avoids the duplication of damaged DNA into daughter cells to impede the propagation of corrupted genetic information (d'Adda di Fagagna 2008). Two types of ɔH2AX foci have been identified: one type is ñtransientò and DSBs are repaired and removed from the nucleus, the other type involves the formation of ñpersistentò foci where DSBs accumulate and remain unrepaired (Sedelnikova, Horikawa et al. 2004). Low levels of ROS are important for signal transduction and modulation of gene expression but high concentrations or prolonged exposures to ROS may have deleterious effects (Colavitti and Finkel 2005) including unrepairable DSBs likely due to persistent oxidative stress caused by defects in redox homeostasis (Marnett 2000).

The DNA damage response (DDR) is also responsible of the expression of a subset of SASP factors, including IL-6 and IL-8 (Rodier, Coppe et al. 2009). If the extent of DNA damage is broad, cells undergo either senescence or apoptosis, depending on the level of the DNA damage itself. In the case of senescence, cells arrest growth and maintain chronic low-level DDR signaling (d'Adda di Fagagna 2008).

This low-level DDR is persistent and necessary for a robust SASP. It is probable that the DDR stimulates the SASP by activating NF-əB that is a known target of ATM (Elkon, Rashi-Elkeles et al. 2005)

DNA mismatch repair (MMR) pathway.

DNA mismatch repair (MMR) is the main post-replicative correction pathway playing a key role in maintaining genomic stability and is therefore crucial for proliferating cells. The system corrects mispairs generated during replication, recombination and DNA damage and its efficiency can be monitored by assessing genomic instability at microsatellite loci (microsatellite instability or MSI) (Neri, Mariani et al. 2011). It has also been reported that it plays an important role in preventing mutations associated with the oxidative DNA lesion, 8-oxo-dG. The eukaryotic MMR pathway is formed by two components, both functioning as heterodimeric complexes, called *MutS* and *MutL*. The most abundant mismatch-binding heterodimer is MutSŭ, composed of MSH2 and MSH6 (Kolodner, Tytell et al. 1999). MutSɓ is the other heterodimer composed of MSH2 and MSH3. Similarly, the MutL complexes are formed by different heterodimeric complexes, thus yielding MutLŭ (made up of MLH1 and PMS2 proteins), MutLɓ (composed of MLH1 and PMS1 proteins) and MutLɔ (containing MLH1 and MLH3) (Brierley and Martin 2013).

The initial recognition of mismatches is carried out by MSH2 bound to either MSH6 or MSH3. A second heterodimer of MLH1 bound to either PMS2 or PMS1 mediates the recruitment of additional proteins completing the repair process (Fig.8). Oxidative DNA damage can increase the frequency of MSI (Jackson, Chen et al. 1998) due to a functional inactivation of MMR by non-cytotoxic levels of H_2O_2 (Chang, Marra et al. 2002); (Lee, Chang et al. 2003).

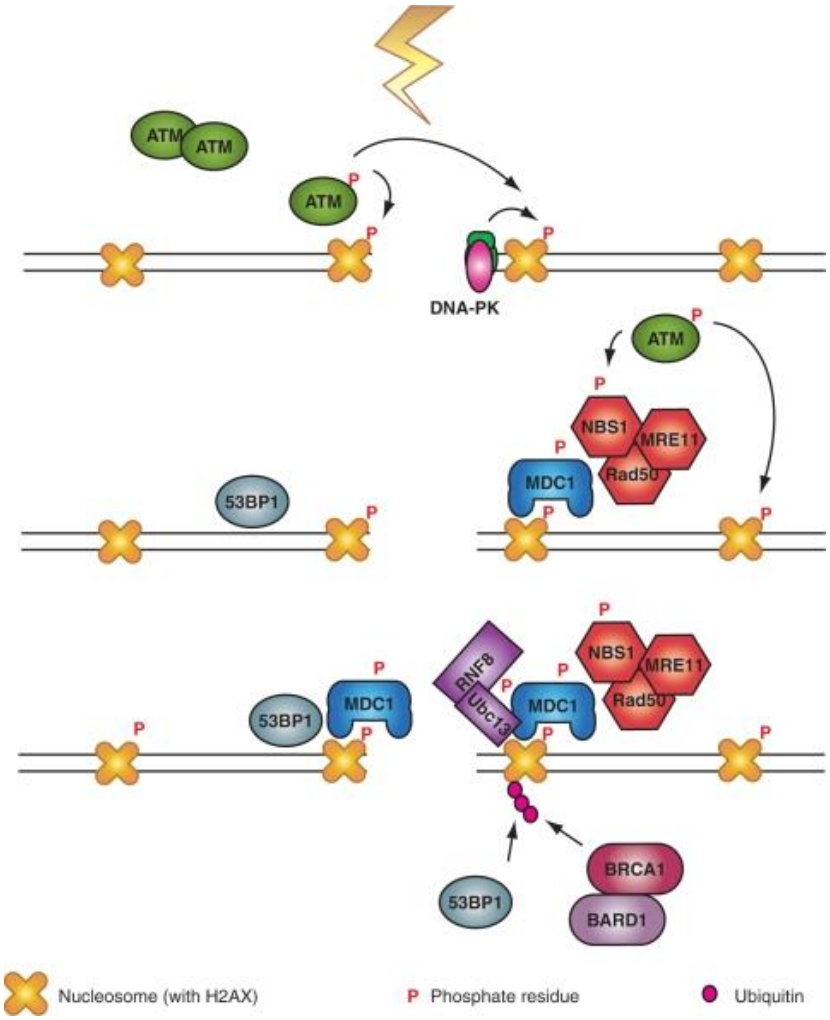


Fig.7 Schematic representation of ɔH2AX-mediated DSB repair. DSBs induce the initial phosphorylation of H2AX mediated by ATM, or DNA-PK, that activates a nucleation reaction with the recruitment of the proteins of the complex.
This generates a feedback loop that leads to further phosphorylation of H2AX and the chromatin modifications required for the recruitment of 53BP1. The activation cascade culminates with the recruitment of RNF8 to phosphorylated MDC1 and the polyubiquitinylation of H2AX to recruit BRCA1/BARD1. ɔH2AX efficiently coordinates DDR signaling.
Image modified by Kinner et al., 2008

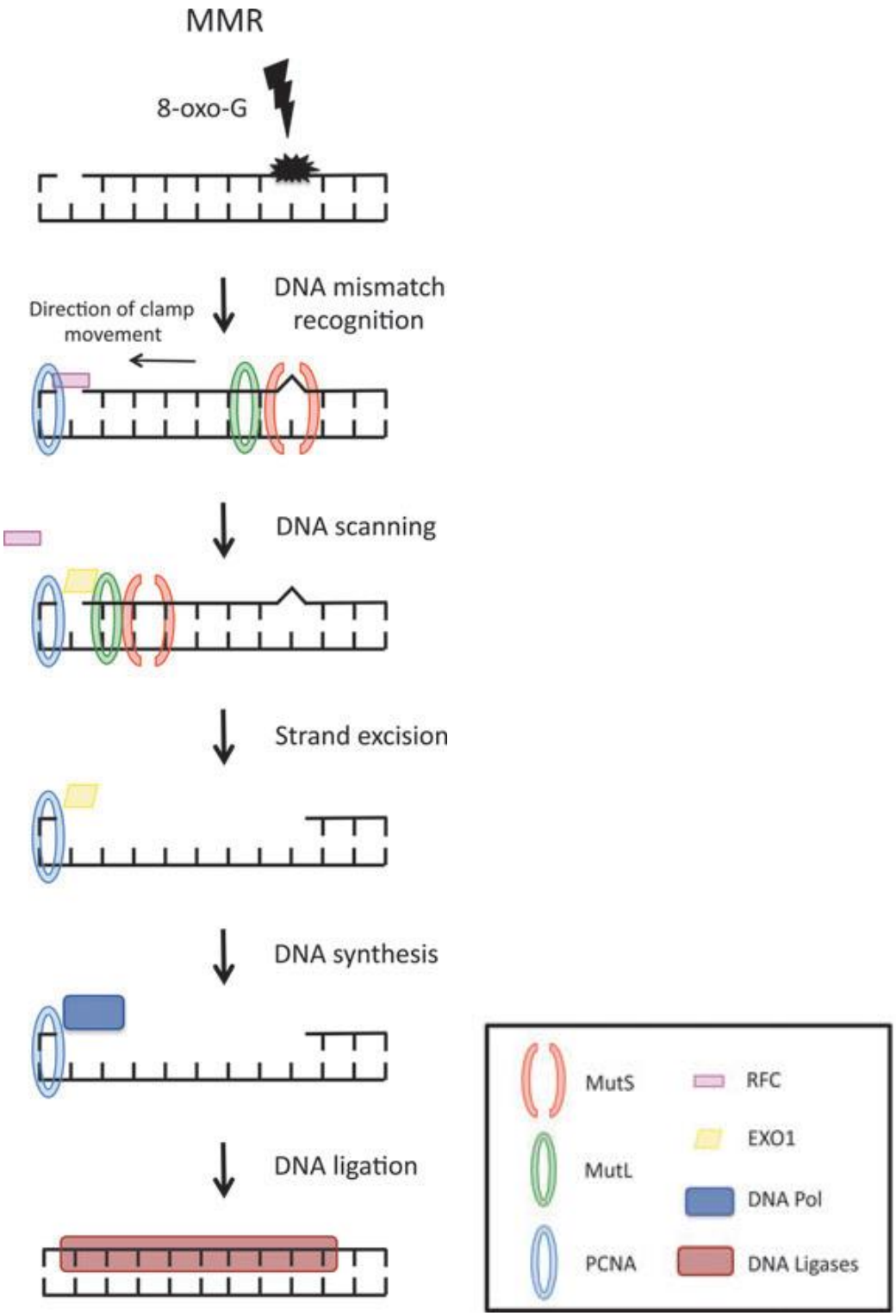


Fig.8 Schematic representation of MMR pathway in response to oxidative stress..
MutS detects the oxidative damaged DNA bases and recruits MutL. The MutS/MutL complex leaves the damaged site and slides along the DNA double helix and eventually encounters a single-strand gap on the daughter strand bound by accessory proteins (PCNA and RFC). This encounter displaces RFC and allows EXO1 to access the daughter-strand DNA to degrade DNA across the site of oxidative damage, before becoming inactivated by MutL. The oxidative damaged DNA is then excised, followed by the synthesis of new DNA by a DNA polymerase. Finally, the new DNA strand is ligated onto the existing daughter strand by a DNA ligase. EXO1, DNA exonuclease.
Image modified by Brierly et al., 2013

*Role of Gadd45**β** and p21 in DNA damage response and senescence.*

The gadd45 is a family of genes including gadd45Ŭ, gadd45ɓ, and gadd45ɔ that encode for the Growth Arrest and DNA damage-inducible 45 proteins that are key players in cellular stress responses. These are small (18 kDa), evolutionarily conserved proteins that are highly homologous to each other (55ï 57% overall identity at the aminoacid level), highly acidic, and localized within both the cell cytoplasm and nucleus (Liebermann, Tront et al. 2011). Expression of gadd45Ŭ, gadd45ɓ, and gadd45ɔ is induced in response to environmental and physiological stresses (Gupta, Gupta et al. 2006) and it has been shown that GADD45 proteins participate in cell cycle arrest, DNA repair, cell survival and apoptosis in response to these stresses, as well as having a role in development.

In fact, GADD45ɓ has been identified as an essential mediator of *Col10a1* and *Mmp13* gene expression in late-stage hypertrophic chondrocytes in the mouse embryo, acting as a cell survival factor during terminal differentiation, driving chondrocyte hypertrophy via p38 MAPK activation and preventing apoptosis of hypertrophic chondrocytes (Ijiri, Zerbini et al. 2005).

During cellular senescence cells show a variety of associated phenotypic changes and one of the most prominent initiator of senescence is the DNA damage response, with induction of cell cycle arrest through the activation of checkpoint proteins, notably p21 (Passos, Nelson et al. 2010). In the process of cellular senescence, a clear relationship has been shown between p21 and GADD45ɓ that interact in a feedback loop mechanism. This process starts with mitochondrial dysfunction, production of reactive oxygen species (ROS) and p21 activation and results in the establishment of the senescent phenotype (Passos, Nelson et al. 2010). It has also been reported that p21 is a down-stream target of GADD45ɓ suggesting the involvement of both proteins in the

molecular mechanism of chondrocyte senescence in mice (Shimada, Sakakima et al. 2011).

1.6 Role of GSK3ϐ in OA

GSK3ϐ (Glycogen Synthase kinase 3ϐ) was initially identified as a key regulator of insulin-dependent glycogenesis, because of its role as a protein kinase able to phosphorylate and inhibit glycogen synthase (Embi, Rylatt et al. 1980). However, it is currently well known that it is a multi-functional kinase that performs a regulatory role in several cellular functions, including embryonic development, cell metabolism, proliferation and intracellular signaling (Cohen and Frame 2001). GSK3ϐ in cells is present in a multiprotein complex with axin, APC (Adenomatosus Polposis Coli) and ϐ-catenin. It is involved in the so called *Wnt canonical signaling*: in the absence of secreted Wnt glycoproteins, GSK3ϐ is active and phosphorylates the other proteins of the complex at the level of cytoplasm. The principal function of this complex is to maintain ϐ-catenin in an inactive state via its phosphorylation, thus preventing its nuclear translocation and transcriptional activation of TCF/LEF complex (Seidensticker and Behrens 2000).

Wnt binding to its receptors instead leads to stabilization of β-catenin via inhibition of GSK-3β (that occurs by phosphorylation of serine-9 that significantly decreases active site availability) (Fig. 9). Uncontrolled triggering of the Wnt pathway is linked to the development of a number of age-related pathologies such as osteoarthritis and an aberrant Wnt signaling can promote cell senescence (Maiese, Li et al. 2008). Disruption of the control of Wnt signaling has also been associated with altered joint formation, chondrogenesis, and OA (Dell'accio, De Bari et al. 2008); (Staines, Macrae et al. 2012).

For these reasons, in the context of skeletogenesis, GSK3ϐ is considered among the major molecular constraints which keep chondrocytes in an ñarrested stateò, avoiding the process of endochondral ossification at the level of articular cartilage (Lories, Corr et al. 2013). It has also been reported that inhibition of GSK3ϐ is a key event for chondrocyte maturation at the level of temporary cartilage during skeletogenesis. This process is under the control of regulatory kinases such as Akt (Rokutanda, Fujita et al. 2009) and cGMP dependent protein kinase II, that drive the process towards hypertrophy and terminal differentiation (Kawasaki, Kugimiya et al. 2008).

Moreover, OA-like changes occur in mice following both under- and over-activation of the Wnt pathway (Zhu, Tang et al. 2009). A fine regulation of GSK3ϐ activity is therefore fundamental for the chondrogenesis and skeletal development because it guarantees the correct balance between cytoplasmatic and nuclear ϐ-catenin (Lories, Corr et al. 2013).

In fact, evidence indicates that not only GSK3ϐ inhibition induces OA changes in articular chondrocytes (Miclea, Siebelt et al. 2011); (Litherland, Hui et al. 2014), but also the ablation of ϐ-catenin signaling pathway is associated with cartilage degeneration in transgenic mice (Zhu, Chen et al. 2008).

Collectively, functional genomics data about the effect of over- or under- activity of ϐ-catenin suggest that the a fine tuning of ϐ-catenin signaling via GSK3ϐ regulation must be maintained to avoid osteoarthritis features at the level of human articular cartilage. Moreover it has been reported that GSK3 is also present at the level of mitochondria, and inactivation of ñmitochondrial GSK3ϐò contributes to decreasing mitochondrial complex IV activity thus leading to subsequent ROS generation responsible for cell senescence in human diploid fibroblasts (Seo, Jung et al. 2008); (Byun, Jung et al. 2012).

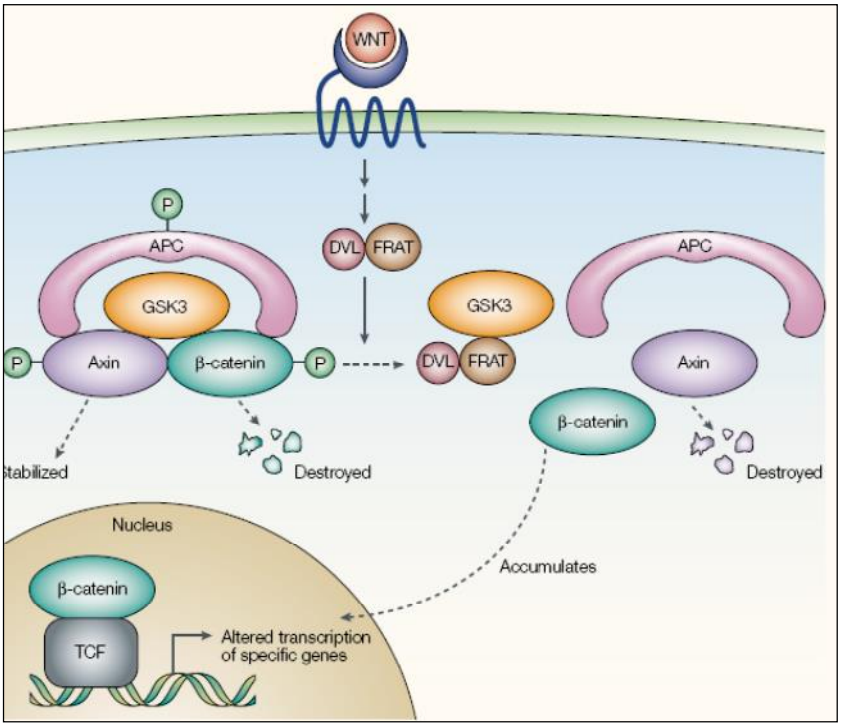


Fig.9 Schematic representation of Wnt canonic pathway. The presence of Wnt protein induces GSK3ɓ inactivation and dissociation of the cytoplasmatic complex. This process is followed by nuclear translocation of ɓ-catenin and transcription activation of specific target genes.
Image modified by Cohen et al., 2001

1.7 NF-əB pathway: the role of IKKŬ

With regards to cell senescence, the secretory component of SASP (Senescence Associated Secretory Phenotype) involves cytokines, chemokines, growth factors and matrix metalloproteinases and NF-əB signaling is recognized as the master regulator of this kind of inflammatory responses. The NF-əB system is a conserved signaling pathway which is activated in response to a wide variety of insults and cellular stress to facilitate innate immunity responses and to establish cellular defense in order to maintain cell and tissue homeostasis (Vallabhapurapu and Karin 2009). NF-əB transcription factors guide a wide range of inflammatory responses, regulating cell differentiation and development programs and ultimately control cell growth (Bonizzi and Karin 2004); (Karin and Greten 2005); (Basak, Kim et al. 2007).

NF-əB mediated transcriptional control is driven by the assembly of homodimers and heterodimers of 5 different NF-əB proteins (RelA/p65, RelB, c-Rel, NFəB1/p105 and

NFəB2/p100). In the absence of any stimulus, NF-əB dimers are sequestered in the cytoplasm and their transcriptional activities blocked by one out of three small inhibitory NF-əB proteins (IəBŭ, IəBɓ, IəBů). NF-əB transcription factors are then activated by N-terminal phosphorylation of inhibitor IəBs, that releases the NF-əB dimers so they are free to migrate to the nucleus and activate target genes. The kinases responsible for this process are IKKŭ and IKKɓ. IKKɓ is essential for the nuclear translocation of NF-əB while IKKŭ acts only occasionally as kinase of IəBŭ (Marcu, Otero et al. 2010). Interestingly, most of the signaling pathways acting in the cells in response to stressful conditions (genotoxic, environmental or inflammatory stress) target the IKK complex, which means that IKKŭ and IKKɓ can even activate NF-əB independent targets, e.g. ɓ-catenin, histone H3, TSC1, FOXO3a and several nuclear co-activators (Salminen, Kauppinen et al. 2012). In addition, the NF-əB system participates in crosstalk with several transcription factors which join together different signaling pathways, e.g. p53 and Wnt (Mengel, Hunziker et al. 2010).

Cellular senescence involves several morphological and energetic-metabolic changes during the generation of the senescent phenotype. It is known that NF-əB signaling can regulate energy homeostasis (Kawauchi, Araki et al. 2008) and the major housekeeping system, i.e. the autophagic cleansing system (Baldwin 2012). Cellular stress induces autophagocytosis, a self-eating process, which has many crucial functions in cellular survival. Moreover, the autophagic degradation capacity declines with aging in many tissues (Salminen and Kaarniranta 2009). In conclusion, it seems that the IKK complex, in particular IKKŭ, is an important player in the regulation of cellular senescence induction (Tilstra, Robinson et al. 2012).

It has also been reported that ablation of IKKŭ has peculiar effects on extracellular matrix remodeling (reducing the level of bioactive ECM degradation products), on

chondrocyte proliferative potential and cell cycle distribution, on hypertrophic differentiation and viability of the cells in maturing micromasses in conjunction with subcellular features of cell health (mitochondrial morphology and cell-cell or cell-ECM specialized junctions) thus suggesting that this kinase might contribute to the abnormal phenotype of osteoarthritic chondrocytes (Olivotto, Borzi et al. 2008).

1.8 Nutraceuticals: a new way to counteract cell oxidative stress and senescence in osteoarthritic chondrocytes

Osteoarthritis prevention is a major challenge for the research in this clinical field. Available pharmacological treatments are very expensive and almost not very effective; overall, there is currently no cure for OA, and there are no therapies which prevent, slow or arrest OA progression (Le Graverand-Gastineau 2010). Most treatments are focused on the control of the secondary symptoms of the disease, but fail to address the complex nature of OA, and have no beneficial effects on chondroprotection and therefore on OA prevention and modification. Furthermore, long-term use of available therapies is often associated with side effects at gastrointestinal, renal, and cardiovascular level (Cheng and Visco 2012).

In this scenario an alternative and safe opportunity is represented by nutraceuticals, defined as *"Food, or parts of food, that provide medical or health benefits, including the prevention and/or treatment of disease"*. Indeed, it has been recognized that nutraceuticals may exert effects on molecular targeting of OA (Leong, Choudhury et al. 2013;(Henrotin, Lambert et al. 2011).

Recently, it has been reported that natural compounds found in fruits, teas, spices, wine, and vegetables such as phytoflavonoids, polyphenols, and bioflavonoids, have a great potential in modifying OA disease and symptoms thanks to their anti-

inflammatory and anti-catabolic actions, and their protective effects against oxidative stress (Shen, Smith et al. 2012). Another interesting compound with a protective effect on chondrocytes is sulforaphane, an isothiocyanate derived from cruciferous vegetables (Facchini, Stanic et al. 2011). An emerging role in reducing oxidative stress and DNA damage has been reported for hydroxytyrosol (HT), a phenolic compound found in the fruits of olive tree and in olive oil and highly present in Mediterranean diet, with an high anti-oxidant and cytoprotective activity (Facchini, Cetrullo et al. 2014). Spermidine (SPD), a natural dietary compound also found in high concentrations in Mediterranean diet (Soda 2010), belongs to the class of polyamines, naturally occurring polycations. It has been reported that supplementation with spermidine reduces oxidative stress and extends lifespan in yeast and flies by an autophagy-dependent mechanism (Eisenberg, Knauer et al. 2009; Guo, Harada et al. 2011; Minois, Carmona-Gutierrez et al. 2012).

These data open new perspectives for the study of possible ways to prevent, control or even revert osteoarthritis and for the development of a nutraceutical-based molecular targeting strategy for chondroprotection as an alternative to classical treatments.

2- AIMS OF THE STUDY

The main aim of this study is to investigate about the molecular bases of osteoarthritis with particular regards to aspects of cellular senescence in disease. This study will focus on the molecular mechanisms whereby two major enzymatic systems in chondrocytes may affect oxidative stress and DNA damage leading to chondrocyte senescence. More in detail we plan to investigate on the effects of pharmacological inhibition (LiCl) of GSK3ϐ and of the activity of IKKŭ, by comparison with chondrocytes bearing a retroviral mediated IKKŭ Knock Down (KD). Moreover we will evaluate the ability of selected nutraceuticals of scavenging oxidative stress thus limiting senescence induction of osteoarthritic chondrocytes.

The first aim of the study will focus on the effect of GSK3ϐ pharmacological inactivation on cellular senescence by evaluation of :

- ROS production by mitochondria;
- induction of "intrinsic" oxidative damage (assessed by increased 8-oxo-guanine adducts) and DNA damage response (evaluated by ɔH2AX foci formation) in osteoarthritic chondrocytes;
- induction of transient S phase arrest, reduced proliferation, and increased percentage of hypertrophic chondrocytes;
- induction of increased expression of the senescence marker GADD45ϐ, p21, SA-ϐ galactosidase and PAS staining.

The second aim of the project will focus on the DNA Damage Response in primary cultures of IKKŭ Wild Type or Knock Down osteoarthritic chondrocytes after induction of "extrinsic" oxidative stress using H_2O_2 and measuring:

- telomere length by Flow-FISH;
- ɔH2AX foci formation;
- expression of the DNA Mismatch repair system genes (Msh2-Msh6-Msh3-Mlh1-Pms1- Pms2).

The third aim of the project will focus on the ability of some nutraceuticals (i.e. hydroxytyrosol and spermidine) to attenuate oxidative stress inducted using H_2O_2 and measuring:

- effects of pre-treatment with hydroxytyrosol on ɔH2AX foci formation and cellular senescence;
- effects of pre-treatment with spermidine on cellular viability and ɔH2AX foci formation.

3- MATERIALS AND METHODS

3.1 Effects of GSK3β pharmacological inactivation

Chondrocytes isolation.

After Istituto Ortopedico Rizzoli Ethics Committee approval and patientsô informed consent, primary chondrocytes were obtained from 13 OA patients undergoing knee arthroplasty. Cartilage was cut from the subchondral bone using a sterile sharp scalpel blade and placed in serum free Dulbeccoôs Modified Eagleôs Medium (DMEM) supplemented with antibiotics. Once all the cartilage was obtained, it was diced as finely as possible. In fact dicing the tissue finely improved the efficiency of the subsequent enzymatic digestion. All the cartilage obtained was put into a sterile Petri. Chondrocytes were isolated by mean of a sequential enzymatic digestion. The first step was a proteolytic digestion of ECM, my mean of cartilage incubation with Protease from Streptomyces griseus 26,5U/ml (Sigma-Aldrich) in serum free DMEM for 1hour at 37°C 5% CO_2. At the end of this step supernatant was eliminated and cartilage was put into a sterile bottle containing Collagenase from Clostridium histolyticum 545U/ml (Sigma-Aldrich) in serum free DMEM and incubated for 1hour at 37°C in continuous stirring. Once digested, the cell suspension was strained through commercially available cell strainers (100µm pore size) and centrifuged (1800 rpm for 10 min) to obtain a cell pellet. This pellet was washed in DMEM containing 10% FBS and the cells counted. To obtain standard monolayer cultures, the cells were plated on culture plastic flasks at a density of 20,000 cells/cm^2. Chondrocytes were expanded in culture up to passage 1 (p1) and then used as described below for monolayer culture.

ROS production evaluation downstream GSK3β inactivation.

Reactive oxygen species (ROS) production at the level of mitochondria was assessed in live OA chondrocytes undergoing GSK3β inactivation. More in detail, the accumulation of ROS within mitochondria was assessed by mean of the overlap of the signals of selective fluorescent dyes for both mitochondria and ROS. Primary chondrocytes were plated in petri dishes with 0.17 mm thin glass well (Cell Culture Dish, World Precision Instruments Germany GmbH), suitable for signal detection at the confocal microscope. The cells were left to adhere for 72 hours, and then were either left unstimulated or treated with 5 or 10 mM LiCl or the highly selective GSK3β inhibitor SB216763 at 10 µM (Coghlan, Culbert et al. 2000) for 4 hours.

Analysis of the mitochondrial involvement was approached by ñreal timeò and ñtime lapseò 30 µM DCHF-DA staining of increased ROS generation, overlapping with Mitotracker Orange CMTMRos mitochondrial staining (Molecular Probes) along with Hoechst 33342 nuclear counterstaining.

Fluorescent signals were acquired by NIKON confocal microscope system A1 equipped with a Nikon Eclipse T*i* microscope and an Argon Ion laser for 488 nm line, a DPSS laser for 561nm line and a diode laser for 640mn line. Emission signals were detected by a photomultiplier tube (DU4) preceded by emission filters BP 525/50 nm, BP 595/50 nm and BP 700/75 nm for Sybr green, Alexa Fluor555 or Dy Light 647, respectively. Laser scanning, image acquisition and processing were performed with Nikon Imaging Software NIS Elements AR-4 (Nikon Inc., USA). Fields of 210 µm x 210 µm (acquired with a Nikon plan apo 60x 1.40 oil objective) were acquired and analyzed.

Effects of GSK3β inhibition on proliferation and cell cycle.

Some experiments were carried out to investigate the effect of GSK3β inhibition on growth and cell cycle distribution of chondrocytes as well as downstream effects on cell senescence. At this purpose, experiments were established with p1 chondrocyte cultures obtained from 13 different patients. Cells were plated at low density (10,000-15,000 cells per cm^2) in order to avoid culture conditions which could bias evaluation of senescence (Severino, Allen et al. 2000) and left for 72 hours. Then parallel cultures were kept either unstimulated or stimulated with 5 mM LiCl for 8, 16, 24, 48 and 72 hours. In some cases cultures were also stimulated with SB216763 (Coghlan, Culbert et al. 2000) which, used at 10µM, behaves as a rather selective inhibitor for GSK3. At the end, for each experimental conditions cells were trypsinized, counted and either fixed (10 min at RT with 2% PFA and stored at 4°C) for Flow Cytometry or senescence detection or pelleted for western blot analysis.

Cell cycle distribution was evaluated by flow cytometry by mean of DNA staining (Sytox green, Molecular Probes, at 5µM) of cells that had been previously fixed with 2% PFA and then post-fixed with 100µl methanol 90% on ice for 10minutes. A RNAse treatment was then applied by resuspending the cells with 100µl RNAse ONE buffer (Promega), preheating at 65°C 10 min and then addition of 1 µl (2.5 U) of RNAse One (Promega) and 1µL RNAse A (Sigma-Aldrich). Digestion was left for 30ô at 37 °C. Analyses were performed using a FACS Canto II flow cytometer (BD).

Light scattering properties of the cells were also analyzed by assessing both the forward scatter (FSC) of the cells which evaluate the cell size and therefore the hypertrophy promoting activity of LiCl as well as the side scatter (SSC), which correlates with granularity and has been reported to increase in cell senescence. The median values of several thousands of cells values were obtained and separated for each cell cycle phase

for both normal and lithium chloride treated cells, and normalized to the median size of control cells in the G1 phase.

Real time PCR analysis.

Cells from 4 patients were also dedicated for Real Time PCR analysis of gene expression. Total RNA was isolated from pellets obtained from cells either unstimulated or stimulated with 5 mM LiCl for 8, 16 and 24 hours. Total RNA was extracted from cells using TRIZOL (Invitrogen). RNA (0.5 εg) was reverse-transcribed using the Superscript VILO cDNA Synthesis kit (Invitrogen) and DNA treatment and removal was performed with DNA-free (Ambion). Each PCR reaction was carried out on 25 ng of cDNA sample using SYBR Premix EX Taq (Takara). The following conditions were used: 95 °C for 30 sec; 40 cycles at 95 °C for 5 s, 60 °C for 20 s and 65 °C for 15 s in a LightCycler 480 system (Roche diagnostics) and analyzed with the dedicated software. All values were normalized to GAPDH housekeeping gene and expressed as relative expression or fold change using the respective formulae $2^{\bar{\imath}\ \varphi CT}$ or $2^{\bar{\imath}\ \varphi\varphi Ct}$. Primers were as follows: GAPDH (NM_002046, forward 579ï 598 and reverse 701ï 683); IKKŬCHUK (NM_001278, forward 1803-1826 and reverse 1865-1888); MMP10 (NM_002425.2, forward 1278ï 1298 and reverse 1472ï 1449).

Evaluation of senescence by mean of SA-ß Gal activity and Glycogen content.

Senescence was evaluated by both assessment of SA-ß Gal activity with the Senescent Cells Staining kit (SIGMA) and assessment of the increased glycogen content with the PAS staining (Sigma-Aldrich). Nearly 10,000 cells were cytospinned on a glass slide and further processed as recommended by the manufacturer. Development time was overnight for Senescent Cells Staining kit (Sigma-Aldrich). At the end, to facilitate their

automatic counting, the cells were treated with SyBr Green nuclear counterstaining. Then, an automatic analysis procedure was employed exploiting the NIS software which combined colorimetric and fluorescent staining. A threshold was set in order to take into account only cells above a given intensity of senescent staining. At least four fields were counted for each condition. The same cell suspension underwent evaluation of glycogen accumulation by mean of the PAS staining.

Immunoblotting.

Evaluation of the expression levels of proteins that are induced upon DNA damage was carried out by western blotting. Lysis buffer volumes were adjusted in order to load an equivalent of 150,000 cells. To achieve effective extraction of proteins, including those bound to DNA, radioimmunoprecipitation (RIPA) buffer with the addition of benzonase and protease inhibitor cocktail (PIC; Sigma-Aldrich) was used to extract proteins. The composition of the buffer was as follows: Tris-HCl 50mM pH 7.4, NaCl 150mM, Nonidet P-40 1%, SDS 0.1%, Na deoxicolate 0.5%, NaF 1mM, Na3VO4 1mM, PMSF 1mM, 1:200 PIC, and 100U/mL benzonase. Briefly, total cellular lysates in RIPA buffer were obtained from monolayers solubilized by keeping the pellet on ice. Samples were loaded in the wells of Nu-Page precast 4%ï 10% polyacrylamide gels (Invitrogen), which were subsequently transferred onto polyvinylidene fluoride membranes by a dry electroblotting method using I-Blot (Invitrogen) and then subjected to immunodetection exploiting the SNAP-ID device (Merck Millipore). Signals were detected with appropriate secondary antibodies and revealed with ECL Select kit (GE Healthcare), using the CCD camera acquisition system of Image Station 4000 MM and Carestream Molecular Imaging Software 5.0. (Carestream Health, Inc.). Immunoblot experiments were designed to kinetically assess the correlated protein expression of

phospho GSK3ϐ (phospho-GSK-3-beta (Ser9), D85E12, XP Rabbit monoclonal, Cell Signaling Technology), total GSK3ϐ (Rabbit monoclonal, Cell Signaling Technology), ɔH2AX (phospho-Histone H2A.X, Ser139, rabbit polyclonal antibody, Upstateï Millipore), GADD45ϐ (sc-8776) and cyclin-dependent kinases inhibitor p21 (Santa Cruz Biotechnology, rabbit polyclonal sc-756). IKKŬwas detected by mean of an IKKŬ mouse monoclonal IgG2B (BD Pharmingen, code 556532). Monoclonal anti-GAPDH (clone 6C5, Chemiconï Millipore) or beta-actin (Sigma) served as loading controls. At least 4 different experiments were carried out for each analysis.

3.2 Cartilage explants studies

Osteochondral specimen (full thickness section of cartilage and subchondral bone obtained with a biopsy needle from knee articular cartilage derived from arthroplasty in respect of the Institutional Ethical Commitee) were established from seven patients with a detailed characterization of metabolic features. After removal of the subchondral bone tissues, identified by visual inspection, the cartilage cylinders were embedded in OCT, snap frozen and kept at-80 °C for future sectioning at 5µm and processing, essentially as described in (Borzi, Olivotto et al. 2010).

Immunohistochemistry and immunofluorescence.

Immunohistochemistry or immunofluorescence experiments were performed essentially as described in (Borzi, Olivotto et al. 2010) to detect expression and subcellular localization of: phosphorylated GSK3ϐ (Anti-phospho-GSK3 ϐ, Ser9, clone EPR2286Y, rabbit monoclonal antibody, Millipore), 8-hydroxy-2ô-deoxyguanosine (8-oxo-dG, a marker of oxidative damage, Trevigen, clone 2E2, mouse monoclonal), GADD45ϐ (GADD45ϐ goat polyclonal sc-8776, Santa Cruz Biotechnology), p21 (Santa

Cruz Biotechnology, rabbit polyclonal sc-756), senescence associated ß-galactosidase (Santa Cruz Biotechnology, goat polyclonal sc-19119) in the articular cartilage tissue from superficial to deep zones. These samples were also processed for immunofluorescent staining and confocal microscopy analysis of GADD45ß (GADD45ß goat polyclonal sc-8776, Santa Cruz Biotechnology). GADD45beta signals were reported as Mean Intensity (i.e. mean of intensity values of pixels, NIS Elements AR2 Image Analysis) per cell positive area. The subcellular distribution of each signal was evaluated by reference to nuclear counterstaining (Sybr green or DAPI 1:10000, Molecular Probes).

3.3 IKKŬ Knock Down cultures

Chondrocyte retroviral transduction.

Chondrocytes from 10 OA patients undergoing knee replacement surgery were isolated by sequential enzymatic digestion as described before and then expanded in vitro at a density of 20,000 cells per cm^2 up to confluence. IKKŬ Knockdown was then obtained by transduction with retroviral vectors containing IKK-Ŭ-specific short hairpin RNAs as described (Olivotto et al., 2008). Knock-downs (KDs) of IKKŬ were achieved by transduction of early passage primary chondrocytes with retroviral vectors containing IKKŬ specific shRNA. IKKŬ specific oligonucleotides for each shRNA (shOligos) had been previously subcloned into the pSuper.retro(Puro) moloney retroviral vector according to the manufacturer (OligoEngine, Seattle, WA). To avoid potential off-target effects, multiple shOligos had been designed containing 19-22 nt complementary to sequences in different exons of IKKŬ. These were IKKŬ3 (19mer starting at Nt 1288) and IKKŬ4 (22 mer starting at Nt 1474). The phenotypes of chondrocytes stably transduced with IKKŬ specific shRNAs were compared with that of a negative control

(GL2), compromising cells obtained from the same patient infected by a retroviral vector harboring a firefly luciferase-specific shRNA (GL2). Early-passage primary OA chondrocytes were transduced by spinoculation with amphotyped retroviruses prepared from Phoenix A packaging cells (provided by Dr. Gary Nolan at Stanford University). Briefly viral supernatants were applied to cells by centrifugation at 1100×g at 32°C for 45 minutes with continued incubation for 5 hours at 32°C in 5% CO_2 followed by replacement with regular growth media. Seventy-two hours later, shRNA expressing cells were selected for puromycin resistance (1.5 ε g/ml) with 3 changes of media over 6 days. At the end of the selection, the cells were collected and 300.000 pelleted for western blot evaluation of the efficiency of the knockdown. IKK KD efficiency was determined with rabbit anti-human IKKŬ (Cell Signaling Technology, Beverly, MA) immunoblotting of total cellular protein, with bands visualized by chemiluminescence. Protein extraction, electrophoresis, blotting and immunodetection was performed essentially as described in (Guidotti, Facchini et al. 2013). Signals were revealed with ECL Select kit (GE Healthcare), using the CCD camera acquisition system of Image Station 4000 MM and Carestream Molecular Imaging Software 5.0. (Carestream Health, Inc.). Semi-quantitative analysis of bands was performed by using the ñoptical densityò of each band and using QuantityOne software (BioRad). Ratio values between target and housekeeping protein in GL2 control cells were obtained and put as 100%. IKKŬ KD of chondrocytes was calculated by mean of the formula: (IKKŬ vs housekeeping expression ratio of GL2 control cells ï IKKŬ vs housekeeping expression ratio of IKKŬ KD cells) / (IKKŬ vs housekeeping expression ratio of GL2 control cells) * 100. Values for all specimens were pulled and presented as the mean with standard error.

Effect of oxidative stress induction on IKKŬKD and control cells.

IKKŬ KD and control cells cultured in monolayer at low density were exposed to 100 µM H_2O_2 for 1 h at 37°C, 5% CO_2. Cells were partly harvested and partly incubated in fresh medium for another 6h, 24h or 72h. Specificity of H_2O_2 effect was confirmed by exposure of the cells to 5mM N-acetyl-cysteine (NAC) 30min prior to H_2O_2 incubation. Double strand breaks (DSB) quantification was evaluated by ɔH2AX foci formation in chromatin immediately after H_2O_2 exposure and after 24h recovery in fresh medium. Cells fixed in 2%PFA and permeabilized in 90% methanol were stained with anti-ɔH2AX mouse MoAb (clone JBW301, Millipore 05-636) followed by Alexa Fluor 647 Donkey anti-mouse IgG secondary antibody (#715496150, Jackson Labs). Then, DNA was stained with the picogreen dye, and the cells underwent a flow cytometry analysis of the amount of ɔH2AX per cell cycle phase.

Mismatch repair gene expression analysis.

Total RNA was extracted and reverse-transcribed from untreated and H_2O_2 treated IKKŬKD and GL2 cells pellets, after 6h recovery in fresh medium as described above. MMR enzyme (MLH1, MSH2, MSH6, MSH3, PMS1 and PMS2) expression was evaluated by semi-quantitative real-time RT-PCR using Sybr green dye and GAPDH as a reference control (Neri, Pawelec et al. 2007) .

Flow-FISH determination of Telomere Length.

150,000 IKKŬ KD and GL2 chondrocytes in low density monolayer cultures were exposed to 100 µM H_2O_2 for 1 h at 37°C, 5% CO_2. After detachment with trypsin, cells were treated with RNasi ONE (Promega) and then washed and resuspended in hybridization buffer (70% deionized formamide, 20 mM Tris buffer pH 7.0, 1%BSA)

containing 2.0 µM FITC labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Biosynthesis INC, USA), specific for the telomeric sequence. Chondrocyte samples incubated in hybridization mixture without probe were used as negative controls. Samples and controls were heat-denatured for 7 min at 80°C and left to hybridize 2 hours at room temperature in the dark. The cells were washed, DNA was stained with SYTOX 7-AAD (Invitrogen) and then analyzed by a FACSCanto II flow cytometer equipped with a 488 nm laser; FITC emission collected through 525/40BP; 7-AAD emission collected through 660/20BP; 5000 events were acquired for each sample. Telomere length was estimated on the basis of the fluorescence signal, and quantitatively assessed as the difference between the geometric mean fluorescence intensity (MFI) of the cells hybridized with telomere PNA-probe and that of the corresponding unstained control sample.

3.4 Nutraceutical treatments

Hydroxytyrosol treatment of chondrocytes and protection from oxidative stress. Chondrocytes from 3 OA patients undergoing knee replacement surgery were isolated by sequential enzymatic digestion as described before, and then expanded in vitro in 10% FBS D-MEM. After seeding in monolayer culture, cells were treated with 100µM H_2O_2 for 1 to 4 hours; 100µM hydroxytyrosol (Sigma-Aldrich) was added 30 minutes before H_2O_2 treatment. At the end of the incubation the cells were trypsinized, collected and fixed with 2% PFA. Detection of ɔH2AX at both 1 and 4 hours was performed by flow cytometry as described above. Senescence was evaluated only on the samples stimulated for 4 hours measuring SA-ɓGal activity by mean of the Senescent Cells Staining kit (SIGMA) as described before.

Spermidine treatment of chondrocytes and protection from oxidative stress.

Chondrocytes from 3 OA patients undergoing knee replacement surgery were isolated by sequential enzymatic digestion as described before, and then expanded in vitro in 10% FBS D-MEM until confluence. Then chondrocytes were seeded into high density (62500 cells/cm^2) monolayer culture, cultured for 72 hours, starved for 24 hours and then treated or not with 100nM spermidine (SPD) for additional 24 hours. The different samples type of samples under analysis were: 1) unstimulated, 2) 500µM H_2O_2 3) 100 nM SPD. Then, all the cells received Sytox Green to allow for a real time detection of dying cells (Molecular Probes, at 5µM). At the same time some prior to exposure to 500µM H_2O_2. At the end of this incubation some cells were treated at the same time with 500µM H_2O_2 and for 24 hours while control cells were only treated with Sytox Green. This dye is a dead-cell staining with a high-affinity for nucleic acid and that easily penetrates cells with compromised plasma membranes while is uncapable of penetrating the membranes of live cells. At the end of the incubation, the cells were fixed with 2% PFA, and cellular viability was evaluated by flow cytometry. On the same cells DNA damage due to the exogenous oxidative stress, was evaluated by measuring ɔH2AX foci by flow cytometry as described before.

3.5 Statistics

All data shown in graphs are expressed as mean ± standard error of the mean (S.E.M.) of *n* separated determinations performed in different experiments and then analyzed by GraphPad Prism 5.0 (GraphPad software, San Diego, CA). Means of groups were compared with paired Student T Test (one-tailed P value) and considered significant when $P < 0.05$, with *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

4- RESULTS

4.1 Effects of GSK3ϐ inactivation *in vitro*

GSK3ϐ inactivation in vitro determines ROS production and oxidative damage in chondrocytes.

The effects of GSK3ϐ inactivation in articular chondrocytes from OA patients have been investigated. GSK3ϐ phosphorylation was induced in low density monolayer cultures by treatment with either LiCl (at 5 mM or 10 mM)- or specific inhibitor SB216763 (at 10 µM) for 4 hours. Confocal microscopy analysis of cells showed an endogenous production of reactive oxygen species at the level of mitochondria. This phenomenon was detected by combining the signal of the ROS-specific probe dichlorofluorescein diacetate with the red Mitotracker Orange CMTMRos mitochondrial staining (Figure 1A). In keeping with this observation, at 16 hours post stimulation, the LiCl treated cells but not the SB216763 treated cells accumulated a significantly higher level of 8-oxo-dG ($p=0.032$, $n=6$) compared to unstimulated cells (Figure 1A graph).

GSK3ϐ inactivation affects cell proliferation with S-phase arrest of chondrocytes cultured in monolayer.

GSK3ϐ inactivation has effects on chondrocyte proliferation as demonstrated on cells at passage 1 (p1) of culture across 8, 16, 24, 48 and 72 hours of incubation with or without GSK3ϐ inhibitors. Cells at the various time points were counted and showed that LiCl-dependent GSK3ϐ inactivation impacts on cellular proliferation, with an evident and significant reduction at 8 hours (0.0122, $n=12$), 16 hours ($p=0.0004$, $n=12$), 24 hours

(p=0.0004, n=12), 48 hours (0.0193, n=4) post stimulation, as shown in figure 1B, reporting the cumulative evaluation of experiments performed with cells of different patients, normalized to the 8 hours count of control cells. The percentage reduction compared to control cells was maximal at 24 hours, both in 5 mM LiCl and 10 µM SB216763 treated cells (Figure 1C). In subsequent time points the difference between control and GSK3ϐ inhibited cells was partially recovered. Moreover, the DNA staining (Sytox green) of the LiCl treated cultures showed a significant accumulation in the S phase at 24 hours (Fig. 1D, p=0.0320, n=5; with a representative example) coincident with the time point with maximal difference in cell counts between control cells and cells who had underwent LiCl mediated GSK3ϐ inhibition.

*GSK3**ϐ** inactivation in monolayer cultures induces senescence and hypertrophy in chondrocytes.*

Experiments performed with a specific hystochemical kit for SA-ϐ galactosidase activity demonstrate that only LiCl-mediated GSK3ϐ inhibition induces cell senescence. In fact, the treatment with 5mM LiCl significantly increases the level of ϐ-galactosidase; and noteworthy, larger cells, exhibiting a ñhypertrophicò phenotype, were more positive to the staining than the others. The quantitative analysis of the increased percentage of senescent/hypertrophic cells indicated a significant increase of senescent, SA-ϐgal positive cells already at the time point of 8 hours (Figure 2A). On the other hand, the analysis of the percentage of the PAS positive cells indicated, as expected, that GSK3ϐ inactivation in chondrocytes achieved by either LiCl or SB216763 leads to a progressive increase of glycogenesis so that the percentage increase of PAS positive cells became significant at 24 hours for both 5mM LiCl (n=5, p=0.0201) and 10µM SB216763 (n=4, p=0.0025). Similar to the analysis of SA-ϐ gal activity (Figure 2B) also

the PAS staining confirmed a more positive signal in larger cells. Flow cytometric analysis confirmed that at each cell cycle phase, LiCl determines the accumulation of larger cells respect to the controls (size of the cells was quantitatively assessed by forward scatter), and richer of intracellular granularity (a feature quantitatively assessed by side scatter, sometimes used as a marker of senescence) already at 8 hours stimulation as showed in Figure 2C; confirming the observation that LiCl treatment at the same time induces hypertrophy and senescence.

GSK3β inactivation in monolayer determines the activation of DNA damage response and increases expression of IKKΫand MMP10.

The time course assessment of the total protein lysates following GSK3β inactivation indicated that LiCl treatment leads to a DNA damage response in monolayer chondrocytes. Figure 3A shows a representative western blot analysis demonstrating a LiCl-dependent increased level of γH2AX at each time point. Interestingly, Figure 3A shows that at the very early time point of 8 hours, the oxidative stress leads to the induction of GADD45β, and to an increased expression of p21.

Moreover, we investigated gene and protein regulation of IKKΫ. The treatment with 5mM LiCl but not with 10 μM SB216763 significantly increased IKKΫ mRNA at 16 hours as demonstrated in Figure 3B. Western blot analysis confirmed an increased IKKΫprotein expression at 16 hours. At the same time point, we also observed an up-regulation of MMP-10 mRNA (Figure 3C) an IKKΫtarget gene, as previously reported (Olivotto, Otero et al. 2013).

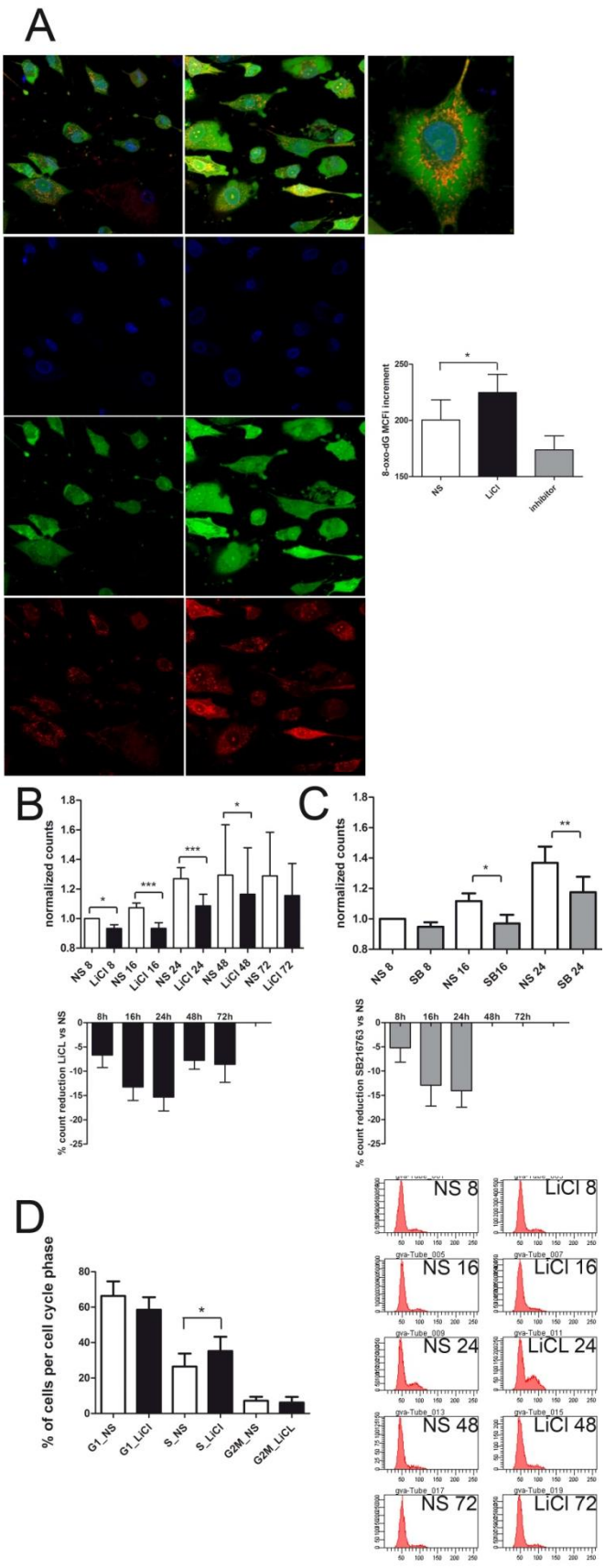


Figure 1. ***In vitro*** **phosphorilation of GSK3ϐ determines ROS production, oxidative damage, growth inhibition and activation of an intra-S checkpoint. 1A:** 5 mM LiCl increases ROS production and mitochondria activation at 4 hours post stimulation (right column) compared to control samples (left column). On the right: high magnification detail of a LiCl treated cell. **Right Graph**: fluorescence intensity (MCFI) increment of the oxidative stress marker 8-oxo-dG, difference between the median channel of fluorescence intensity of the cells stained for 8-oxo-dG and that of the same cells probed with the negative control (isotype IgG2 control). At 16 hours, the LiCl treated cells (black histogram) but not the SB216763 treated cells (grey histogram) accumulated a significantly higher level of 8-oxo-dG (p=0.032, n=6) compared to unstimulated cells (white histogram). **1B and C**: longitudinal assessment of the effects of GSK3ϐ inhibition on cell growth versus the control (white column). **B**: LiCl (black histograms) and **C**: SB216763 (grey column): upper graphs indicate counts normalized versus the 8 hours count; lower graph indicate at each time point the percentage count reduction due to either LiCl or SB216763. *P< 0.05; **P<0.01; ***P<0.001(see results for details). **1D** Left: DNA staining (Sytox green) of the cells indicates that LiCl determines a significant increased of cells in the S phase (p=0.0320, n=5) at 24 hours. Right: a representative example with cell cycle analysis of control (left) versus 5mM LiCl (right) treated cells at each time point.

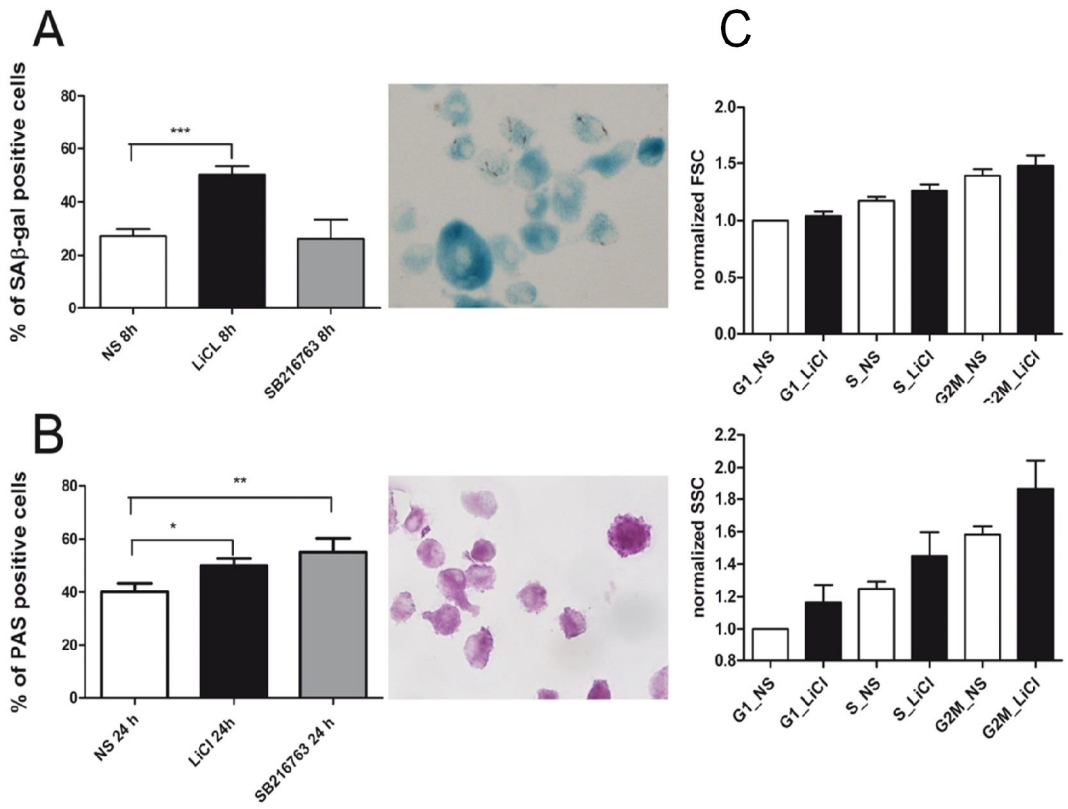


Figure 2. GSK3b inactivation in monolayer cultures induces senescence and hypertrophy in chondrocytes. 3A and B SA-bGalactosidase activity and PAS staining were quantitatively evaluated by image analysis (NIS software) in multiple experiments with different patients, examining at least four fields (with 40-160 cells each), with cells automatically detected by mean of DAPI nuclear counterstaining and whose staining intensity was assessed in order to objectively determine the percentage of cells above a given threshold of signal intensity. **3A. SA-bGalactosidase activity.** Graph indicates the mean percentage of cells with staining intensity above a given threshold. 5mM LiCl increases the percentage of SA-bGal positive cells already at 8 hours post stimulation. (n=6; p=0.0008). **Right image**: a representative picture showing that hypertrophic cells also show the strongest level of SA-b Gal activity. **3B. PAS staining**. Graph indicates the mean percentage of cells with staining intensity above a given threshold. At 24 hours post treatment, both 5mM LiCl (p=0.0201, n=5) and SB216763 (p=0.0025, n=4) resulted in significantly increased PAS staining compared to control cells. **Right image**: a representative picture showing that hypertrophic cells also show the strongest level of PAS staining. **3C** Cell cycle phase distribution of Forward Scatter (upper graph, a parameter related to cell size) and Side scatter (lower graph, a parameter related to cell granularity) of 4 different experiments, with the values normalized to the value of each control G1 phase cells. 5mM LiCl treatment determines an increase of scatter values at G1, S and G2/M phase.

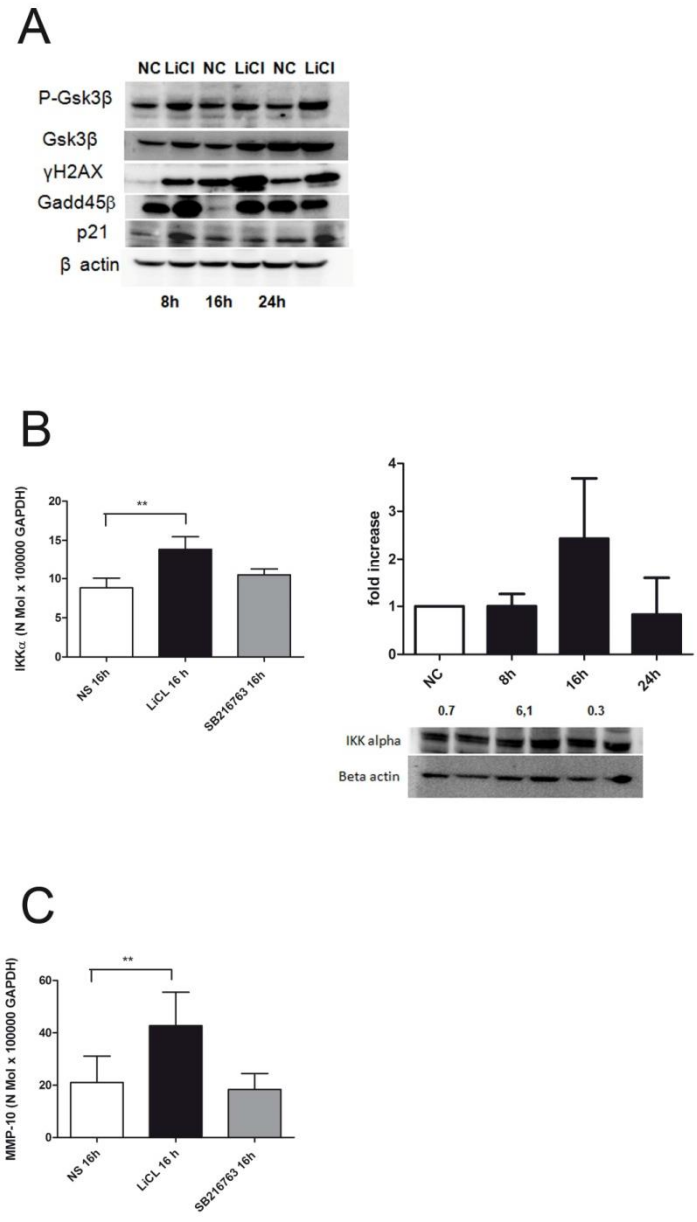


Figure 3. GSK3ϐ inactivation in monolayer determines the activation of a DNA damage response and increases expression of IKKŬ and MMP10. 3A Representative example of a Western Blot analysis : LiCl induces an increased GSK3ϐ phosphorylation leading to a DNA damage response (DDR). The DDR includes markers of DNA damage (double strand breaks evidenced as ɔH2AX), increased expression of GADD45ϐ and p21. **3B** 16 hours LiCl treatment significantly increased IKKŬ gene expression (p=0.0058, N=3) and at this time point an increased IKKŬ protein expression was also appreciated (n=4). **3C** 16 hours LiCl treatment significantly increased gene expression of MMP-10 (p=0.0092, N=3), a target gene of IKKŬ.

4.2 GSK3ϐ phosphorilation in cartilage explants

*Evaluation of GSK3**ϐ** inactivation in cartilage from OA patients.*

The extent of *in vivo* GSK3ϐ inactivation was investigated on knee cartilage samples derived from 7 osteoarthritic patients. Most phospho(Ser9) GSK3ϐ positive cells were localized in mid-deep cartilage layers. PhosphoGSK3ϐ in cartilage had only an extranuclear pattern (Figure 4) and an high phosphoGSK3ϐ staining was also found in calcified cartilage areas.

*DNA damage response is associated with GSK3**ϐ** inactivation in cartilage from OA patients.*

In samples derived from OA patients we found evidence that, even *in vivo*, GSK3ϐ inactivation is responsible for the triggering of the pathway oxidative DNA damage>GADD45ϐ>p21, that we previously observed downstream pharmacological *in vitro* GSK3ϐ inactivation. Figure 5 shows a representative immunohistochemistry experiment derived from an OA sample with a high prevalence of phosphorylated (Ser9) GSK3ϐ positive cells.

The presence of oxidative stress was evidenced at the tissue level by using 8oxo-d-G antibody, that gave a positive staining in mid-deep layers, therefore correlated with the distribution of phosphoGSK3ϐ. The expression of GADD45ϐ, was also found in mid-deep layers with an exclusive cytoplasmic distribution. The same pattern was found for p21, particularly evident in mid-deep layers. With regards to the intensity, in the same samples showing association of stronger staining of phosphoGSK3ϐ, 8-oxo-dG, GADD45ϐ and p21, we also detected higher staining of the senescence associated ϐ-galactosidase, suggesting that not only in monolayer cultures, but also at the level of the

cartilage tissue, there is a mechanistical link between GSK3β inactivation, DNA damage response and chondrocyte senescence and that in both settings GSK3β inactivation is responsible of senescence and hypertrophy.

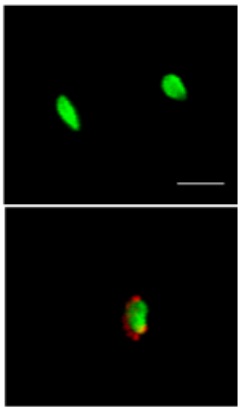


Figure 4. In vivo detection of phosphoGSK3ϐ in articular chondrocytes derived from an osteoarthritic patient. High magnification images of chondrocytes in the superficial (upper picture) or mid-deep layers (lower picture) of cartilage derived from an OA patient indicates a extranuclear pattern of staining.

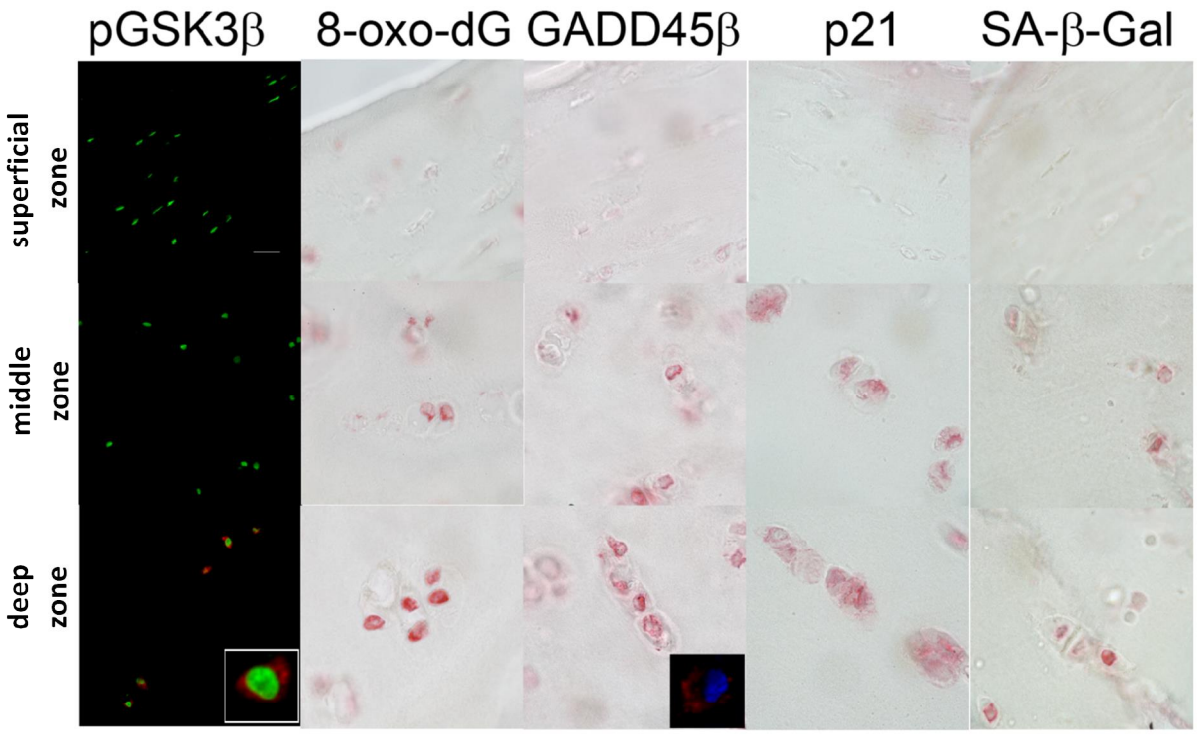


Figure 5. DNA damage response is associated with GSK3ϐ inactivation in cartilage from OA patients. A representative case of knee cartilage from an OA patient. pGSK3ϐ was analyzed with immunofluorescence and images were taken at the confocal microscope (bar=25µm); 8-oxo-dG, GADD45ϐ, p21 and SA-ϐ-Gal were assessed with immunohistochemistry and colorimetric detection, and the bright field images were all taken at 400x magnification. In the upper panel high magnification insets show the prevalent cytoplasmic localization of pGSK3ϐ or GADD45ϐ signals.

4.3 Functional genomics analysis of IKKŬ effects on oxidative DNA damage

*IKK**Ŭ** impacts on double strand breaks (DSB) induction after an oxidative stress.*

In the second part of the work the role of IKKŬ on the chondrocyte response to an exogenous oxidative stress has been investigated by using IKKŬ Knock Down (KD) chondrocyes.

Double strand breaks induction was evaluated by measuring ɔH2AX foci formation. In fact ɔH2AX serves as a marker of double strand breaks since it specifically labels sites of H2AX phosphorylation, which can subsequently lead to the recruitment of DNA repair factors. Moreover, ɔH2AX can be considered a marker of the senescent-associated phenotype (d'Adda di Fagagna 2008).

We saw that in the absence of any stimulus, the amount of ɔH2AX DNA foci as evaluated by the Flow Citometry (Figure 6C) is comparable in IKKα Knock Down and controls cells (Figure 6A). Data are showed as MCFI increment representing the difference between the median channel (MC) fluorescence intensity of the sample labelled with the anti-ɔH2AX antibody and the MC fluorescence intensity of the sample labelled with the isotype control antibody. Immediately after H_2O_2 exposure, an higher percentage of ɔH2AX positive cells and more foci per cell are found in IKKα Knock Down cells as compared to their GL2 controls (Figure 6A). We then coupled ɔH2AX staining with cell cycle analysis and the results indicated that G2-M cells are the more susceptible to DNA damage in both IKKα Knock Down and control chondrocytes (Figure 6B). However, IKKα KD cells proved to almost completely repair Double Strand Breaks after 24 hours of recovery after H_2O_2 treatment, as demonstrated by a sensitive reduction of the ɔH2AX signal in IKKŬKD cells at this time point. Moreover, NAC incubation prior to H_2O_2 completely reverts ɔH2AX induction (Figure 6B) thus confirming the specificity of the signal with regards to ROS induction.

IKKŮ impacts on telomere length after an oxidative stress.

We aimed to assess the effect of exogenous oxidative stress on the induction of "extrinsic senescence" by performing a Flow-FISH analysis in order to measure telomere length by using a FITC-PNA probe (Figure 7C), in IKKα Knock Down and their control chondrocytes after exposure to H_2O_2. The results we obtained demonstrated that under basal conditions, in the absence of any exogenous oxidative stress, there is no difference in the telomere length between control and IKKα KD chondrocytes (Figure 7A). We then measured the effects of oxidative stress on telomere shortening 72 hours after H_2O_2 exposure. In order to evaluate the effect of a specific stimulus on telomere attrition, 72 hours of recovery are necessary to allow the cells to replicate at least one time. Our data show that H_2O_2 exposure shortens telomere length particularly in the G2-M fraction of control cells but not in their IKKŮ KD counterparts that proved to be more resistant to the effect of "extrinsic senescence" following an extrinsic oxidative stress (Figure 7B).

IKKŮ impacts on DNA Mismatch Repair (MMR) system after an oxidative stress.

DNA Mismatch Repair (MMR) is the main post-replicative correction pathway and playing a key role in maintaining genomic stability is therefore crucial for actively proliferating cells. If this system does not work properly, for example because of an oxidative stress, a Microsatellite instability (MSI) might be observed (Neri, Mariani et al. 2011), leading to genomic aberrations that can lead to mutations. We evaluated the effect of hydrogen peroxide on the expression of MMR genes in IKKŮ Knock Down and control cells by semi-quantitative Real-Time PCR.

The analysis involved all the major genes of the pathway (MSH2, MSH3, MSH6, MLH1, PMS1 and PMS2) and showed that only two of them are up-regulated after

oxidative stress induction. MSH6 and MLH1 mRNA are higher in IKKŬ Knock Down cells than control cells after H_2O_2 exposure (Figure 8) indicating a higher capacity of IKKŬ KD cells to avoid the Microsatellite instability that occurs after an exogenous oxidative stress thus preventing the process of "extrinsic senescence".

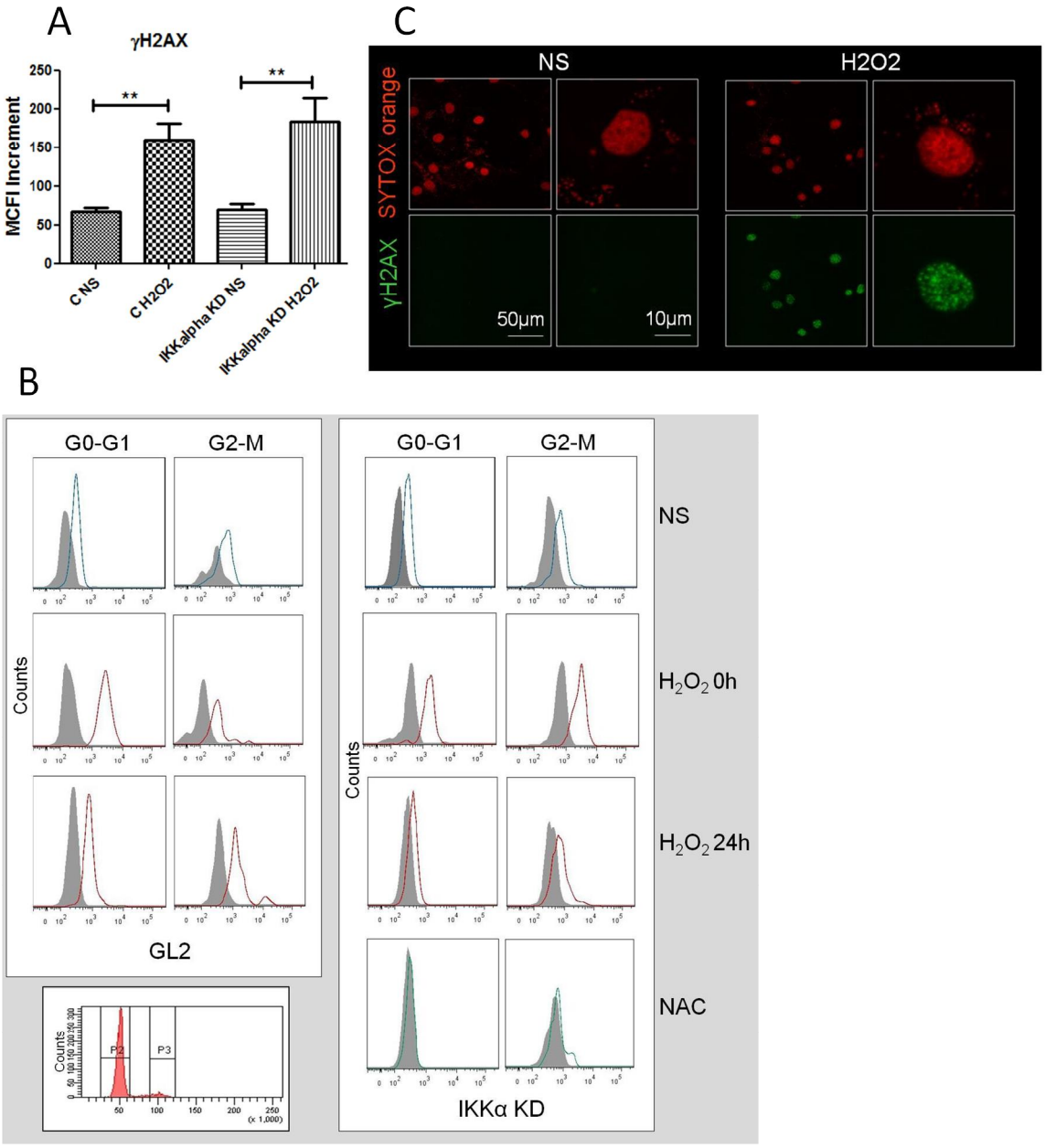
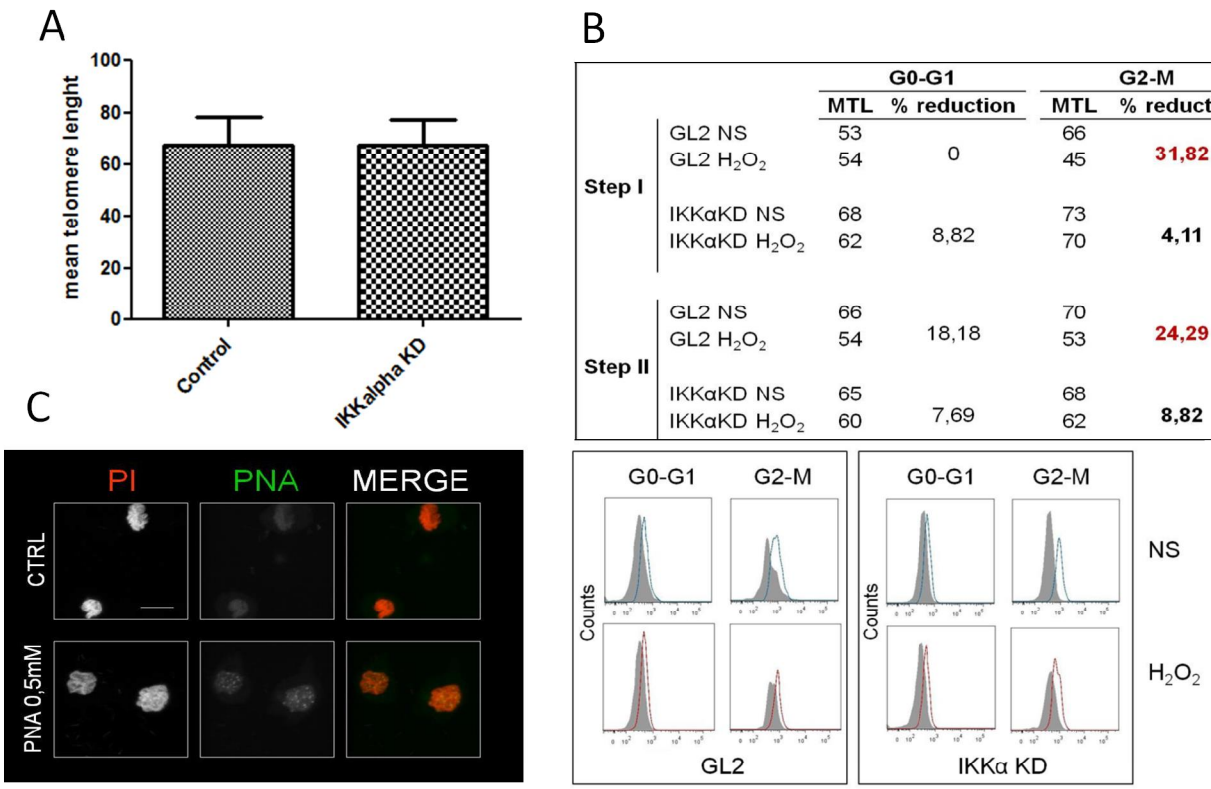


Figure 6. Effect of oxidative stress on double strand breaks (DSB) induction in IKKŬ Knock Down chondrocytes. 6A Cumulative ɔH2AX MCFI derived from the samples (controls and IKKalpha Knock Down cells) analiyzed by flow cytometry in basal conditions and immediately after H_2O_2 treatment are represented in the graph (n=7; p<0.001). **6B** Representative flow cytometric analysis of ɔH2AX coupled with cell cycle cell by picogreen staining in control (GL2) and IKKŬ KD chondrocytes under basal conditions (NS) and after H_2O_2 exposure (immediately post stimulation and after 24h recovery). Isotype controls are in grey. Results are shown for G0-G1 and G2-M. **6C** Representative confocal analysis of ɔH2AX foci in control and H_2O_2 treated chondrocytes. Nuclei were stained with Sytox green and ɔH2AX antibody was revealed with a DyLight 649 labelled secondary antibody. Images magnification: 60X.



		G0-G1		G2-M	
		MTL	% reduction	MTL	% reduction
Step I	GL2 NS	53		66	
	GL2 H_2O_2	54	0	45	31,82
	IKKαKD NS	68		73	
	IKKαKD H_2O_2	62	8,82	70	4,11
Step II	GL2 NS	66		70	
	GL2 H_2O_2	54	18,18	53	24,29
	IKKαKD NS	65		68	
	IKKαKD H_2O_2	60	7,69	62	8,82

Figure 7. Effect of oxidative stress on telomere length in IKKŭ Knock Down chondrocytes. 7A Cumulative flow cytometry evaluation of telomere length in control and IKKŭ KD chondrocytes under basal condition (n=7). **7B** Upper panel: telomere length reduction after 72 hours recovery following H_2O_2 exposure of GL2 and IKKŭKD cells onto two subsequent culture passages. MTL=Mean Telomere Length (upper panel). **7B** Lower panel: representative Flow-FISH analysis of telomere length coupled with cell cycle profile by picogreen staining in control (GL2) and IKKŭ KD chondrocytes under basal conditions (NS) and after H_2O_2 exposure (72h recovery). Isotype controls are in grey. Results are shown for G0-G1 and G2-M. **7C** Representative confocal analysis of FITC-PNA telomere probe. Nuclei were stained with Sytox AAD. Image magnification: 60X.

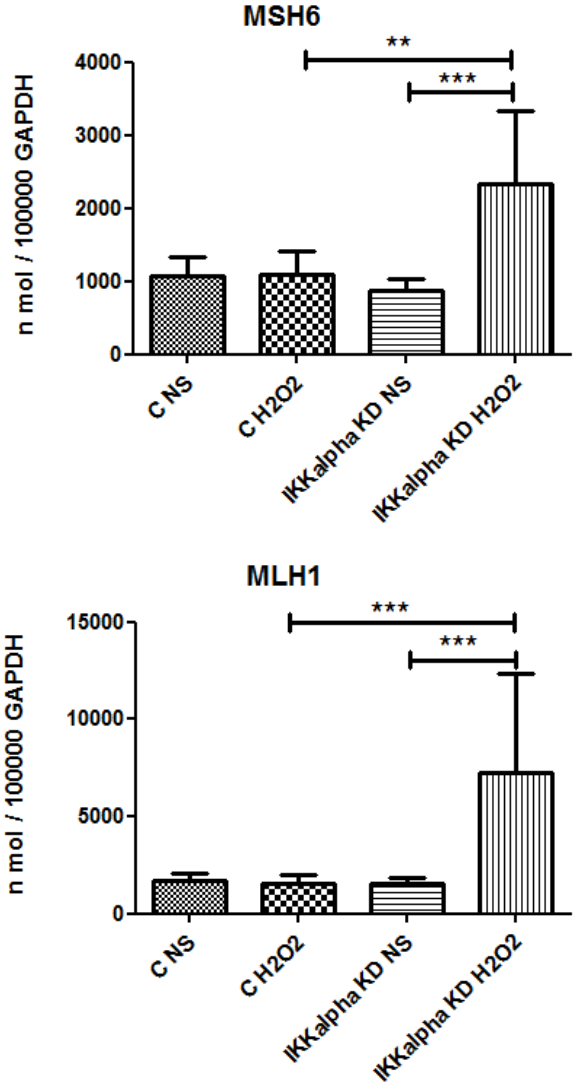


Figure 8. Effect of oxidative stress on the DNA Mismatch Repair (MMR) system in control and IKKŭ KD chondrocytes. Semi-quantitative real time PCR analysis of gene expression of two MMR enzymes in IKKŭ KD and control cells. MSH6 expression is significantly higher in IKKŭ KD cells compared to control cells after H_2O_2 treatment (n=10; **p=0.0028, ***p<0.0001). MLH1 is significantly higher in IKKŭ KD cells compared to control cells after H_2O_2 treatment treatment (n=10; ***p<0.0001).

4.4 Nutraceutical treatments and anti-oxidant activity

Hydroxytyrosol attenuates ɔH2AX-foci formation and senescence after H_2O_2 exposure in chondrocytes.

In the third part of the work the potential protective role of some nutraceuticals compounds and their ability in scavenging Reactive Oxygen Species in monolayer culture has been investigated. We observed the effect of hydroxytyrosol (HT) after the treatment of chondrocytes in low density monolayer with 100µM H_2O_2 for 1 or 4 hours. Figure 9A shows the induction of DNA damage due to oxidative stress after 1 hour stimulation as indicated by the increased signal of the phosphorylated form of H2AX. Figures 9A and 9B also show that the treatment with HT completely reverts the increase of ɔH2AX-foci at 1 hour incubation, the time point corresponding to the greater extent of damage, which then undergoes repair so that at 4 hours the signal is greatly reduced. Data are showed as MCFI increment representing the difference between the MC fluorescence intensity of the sample labelled with the anti-ɔH2AX antibody and the MC fluorescence intensity of the sample labelled with the isotype control antibody. The same cells were processed for the SA-ɓ Gal staining to evaluate the occurrence of senescence after oxidative stress and the protective effect of HT treatment. Figures 10A and 10B show how H_2O_2 treatment for 4 hours induces a significant augmentation of the percentage of senescent cells respect to the control, unstimulated cells. Nevertheless, the pre-treatment with HT induces an almost complete recovery of the normal phenotype. Interestingly, the treatment with HT alone induces a significant reduction of the percentage of senescent cells in comparison to unstimulated cells, indicating an anti-oxidant effect of the compound that is exerted in the absence of an oxidative stimulus.

Spermidine pre-incubation reduces cell death and ɔH2AX-foci formation after H_2O_2 exposure in chondrocytes.

In this part of the work the effect of 100 nM spermidine (SPD) pre-treatment prior to the exposure of high density chondrocyte monolayer to 500µM H_2O_2 for 24 hours has been investigated. Preliminary experiments to date have been only carried out with primary chondrocytes derived from two patients. Figure 11A shows the percentage of dead cells after treatment with H_2O_2 with or without SPD pre-treatment. The measure was performed by Sytox green staining and flow cytometry analysis. The data obtained indicate a marked augmentation of cell death after H_2O_2 incubation, but the pre-treatment with SPD strongly reduces cell death respect to H_2O_2 alone. On the same cells, the extent of ɔH2AX-foci formation was evaluated. Figure 11B shows that H_2O_2 induces ɔH2AX-foci augmentation due to oxidative stress, but pre-treatment with spermidine completely reverts this phenotype. Interestingly, the treatment with SPD alone induces a reduction of the extent of ɔH2AX-foci respect to control cells, indicating an anti-oxidant effect of the compound independently of the exposure to the oxidative stimulus, as previously seen for hydroxytyrosol. Flow cytometric data are showed as MCFI increment, indicating the difference between the MC fluorescence intensity of the sample labeled with the anti-ɔH2AX antibody and the MC fluorescence intensity of the sample labeled with the isotype control antibody.

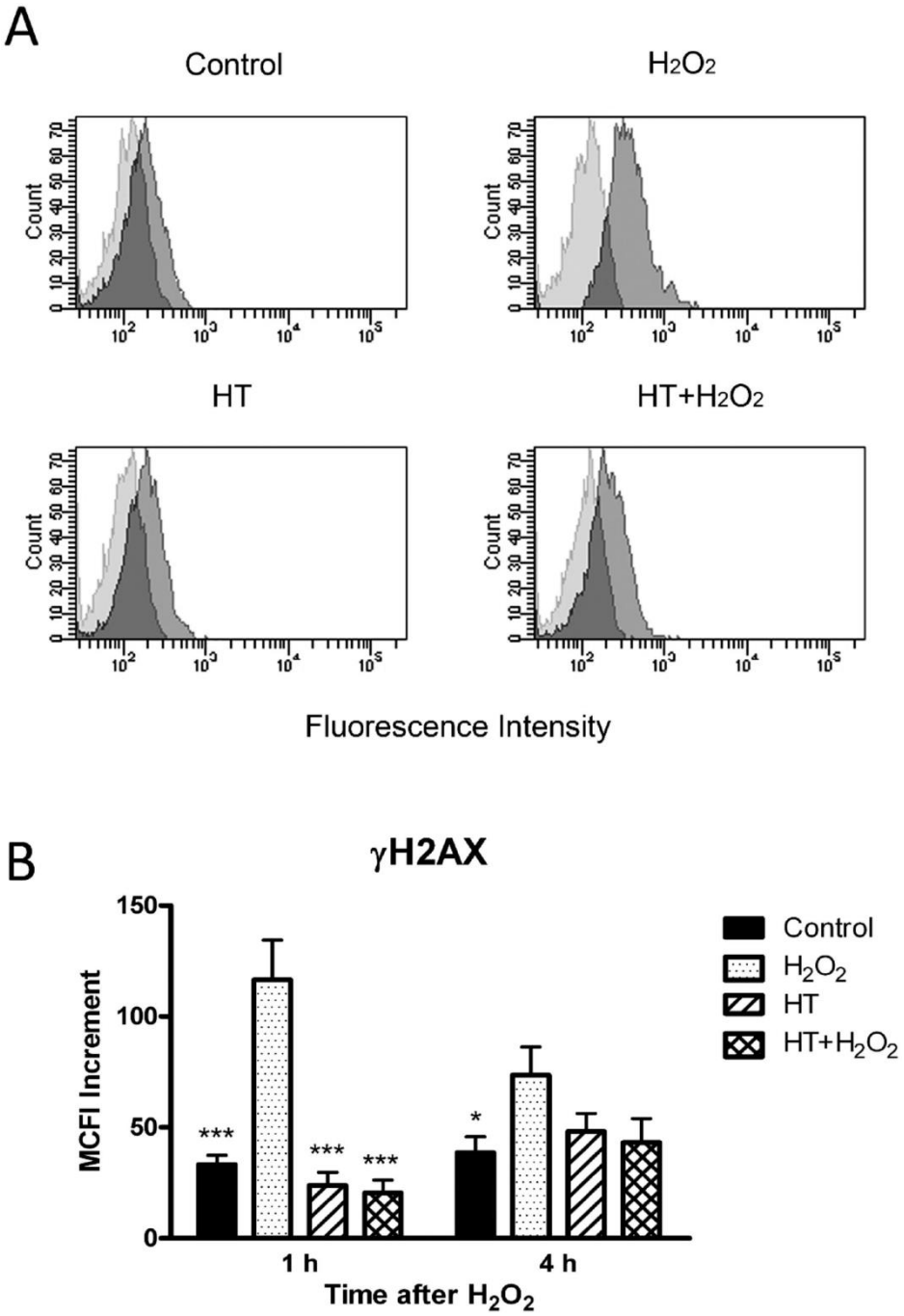


Figure 9. Hydroxytyrosol attenuates ɔH2AX-foci formation after H_2O_2 exposure in chondrocytes. 9A Representative flow cytometric analysis of ɔH2AX of chondrocytes incubated for 1 hour with H_2O_2. Light grey histograms represent isotype while dark grey histograms represent ɔH2AXï specific antibody. **9B** Cumulative MCFI derived from several samples analyzed by flow cytometry after 1 hour (n=5; p***<0.001) and 4 hours (n=6; p*<0.05) incubation with H_2O_2 are represented in the graph.

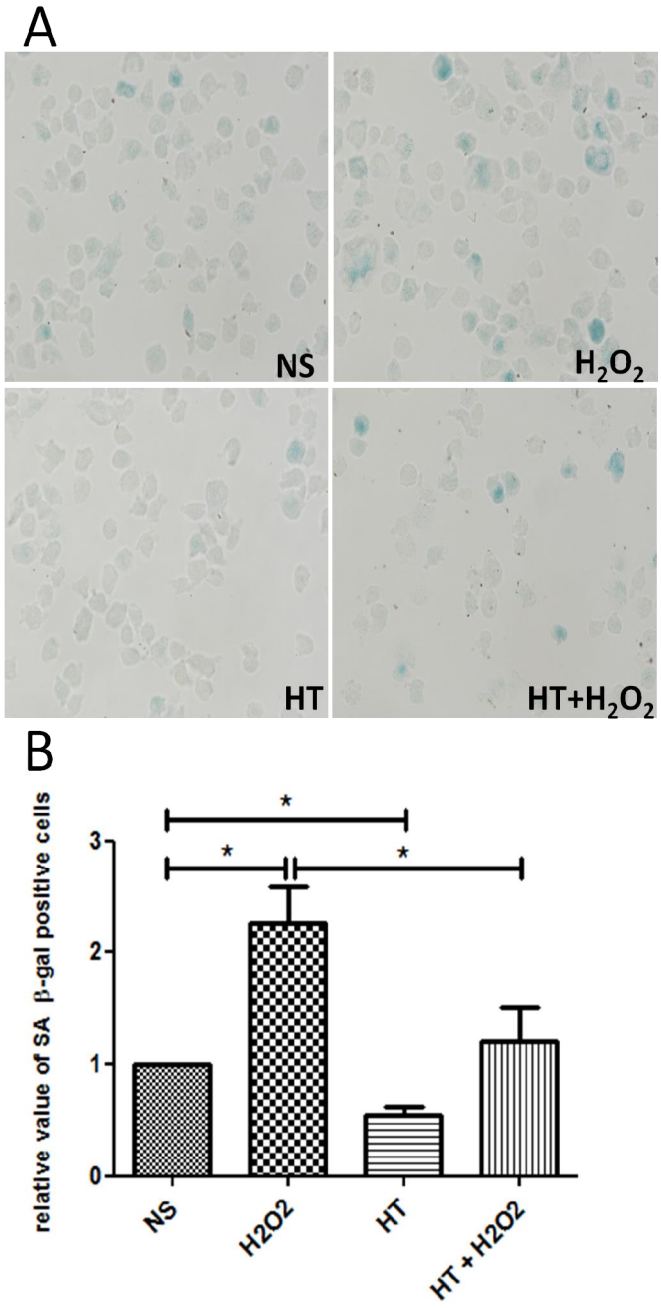


Figure 10. Hydroxytyrosol reduces senescence after H_2O_2 exposure in chondrocytes. 10A Representative pictures showing the increased SA-ß Gal activity in. H_2O_2 treated chondrocytes. HT pre-treatment reduces this staining. **10B** Graph indicating the ñrelative value (i.e. the value normalized to that of control cells)ò of SA ß-Gal staining across different treatments. H_2O_2 significantly increases the percentage of SA-ßGal positive cells already at 4 hours post stimulation and HT almost completely reverts this phenotype. (n=3; p*<0.05).

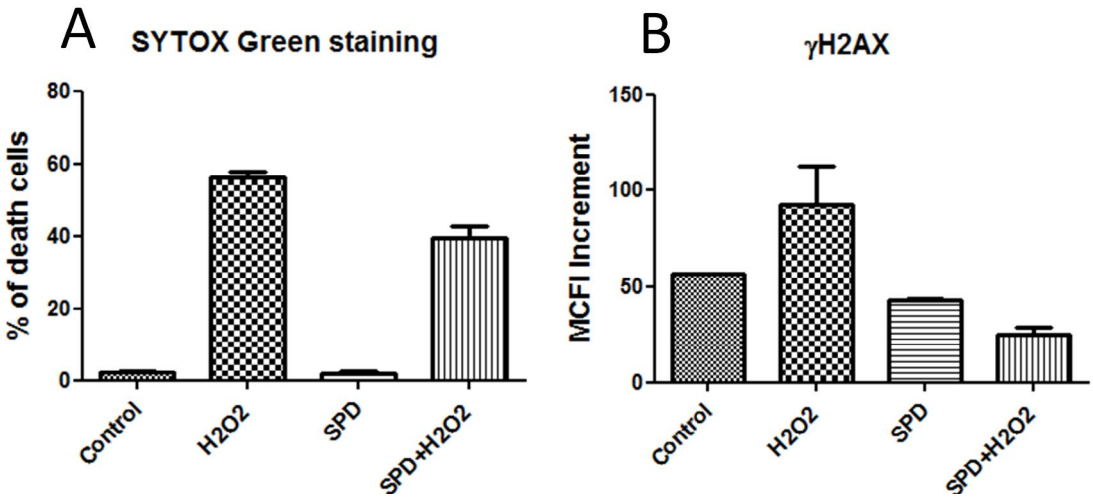


Figure 11. Spermidine pre-incubation reduces cell death and ɔH2AX-foci formation after H_2O_2 exposure in chondrocytes. 11A Graph represents the percentage of dead cells in basal conditions and after treatment with H_2O_2 with or without SPD pretreatment, as measured by Sytox green staining and flow cytometry analysis (n=2). **11B** Graph represents cumulative MCFI of ɔH2AX flow cytometry analysis in basal conditions and after treatment with H_2O_2, with or without SPD pretreatment (n=2)

5- CONCLUSIONS AND DISCUSSION

In this study we investigated some molecular mechanisms that contribute to aspects of cellular senescence in osteoarthritis. Since chondrocyte senescence is mainly of the extrinsic or ñstress inducedò type, the study was mainly focused on the evaluation of the role of oxidative stress and DNA damage in cellular senescence. We evaluated the impact on this phenomenon of two major enzymatic systems, GSK3ϐ and IKKŬ, that we manipulated either pharmacologically or by means of retroviral mediated Knock Down (KD) and we also assessed the ability of selected nutraceuticals on scavenging oxidative stress, avoiding the senescent phenotype of osteoarthritic chondrocytes.

The process of stress-induced senescence at the level of articular chondrocytes has a great impact on their ability to maintain and to restore tissue homeostasis and often leads to tissutal degeneration. In fact, articular cartilage is a post-mitotic tissue and it is very susceptible to extrinsically-delivered or intrinsically-generated reactive oxygen species.

In this work we performed *in vitro* experiments to understand the effects of GSK3ϐ inactivation in articular chondrocytes derived from osteoarthritic patients. GSK3ϐ pharmacological phosphorylation was induced by using two canonical inhibitors, LiCl and SB216763, both administered at the same concentrations used by Colghlan and collaborators (Coghlan, Culbert et al. 2000). It is likely that GSK3ϐ inactivation can be responsible of the chronic impairment found in articular chondrocytes of osteoarthritic chondrocytes, via an alterated mitochondrial mechanism (Byun, Jung et al. 2012; Byun, Jung et al. 2012) linked to ROS production and cellular senescence.

We first investigated the mitochondrial effects downstream LiCl- and SB216763-mediated GSK3β inactivation demonstrating, by time lapse confocal observation, that this process is responsible for the production of reactive oxygen species at the level of chondrocyte mitochondria, affecting their functions. This phenomenon is likely due to mitochondrial complex IV defects as previously reported by Byun and collaborators (Byun, Jung et al. 2012). Moreover Rose and collaborators have recently studied the level of DNA damage in chondrocytes, founding high levels in osteoarthritic samples, in conjunction with a progressive stress-induced senescence (Rose, Soder et al. 2012). Our data support the observation that chondrocytes are strongly susceptible to oxidative stress, in fact while reactive oxygen species (ROS), are responsible of damage at the level of several different intracellular compartments, nucleus and DNA seem to be highly susceptible to this kind of molecules in chondrocytes. One major ROS effect on DNA is represented by Double Strand Breaks (DSB), that occur when both DNA strands are broken closely. Cells respond to DSB activating ɔH2AX that regulates the recruitment and accumulation of multiple DNA damage response factors (DDR) (Haiko van Attikum et al., 2009).

As we hypothesized, we demonstrated that ROS are able to induce Double Strand Breaks and are responsible of activation of a DNA damage response that starts with histone H2AX phosphorylation and culminates with GADD45ϐ and p21 induction, driving chondrocytes to cellular senescence.

DNA damage was assessed by measuring not only ɔ-H2AX, marker of double strand breaks, but also 8-oxo-G, that is a known stable marker of oxidative stress because it stains 8-oxoguanine adducts caused by ROS at the level of both genomic and mitochondrial DNA (Markkanen, Hubscher et al. 2012). Mitochondrial DNA is notoriously more susceptible to oxidative damage than genomic DNA because ROS are

produced exactly in this compartment and because in mitochondria there is not a proper system of DNA repair (Yakes and Van Houten 1997). Also unstressed cells present a ñsteady stateò, basal level of 8-oxo-dG that can increase when intracellular ROS level exceeds the cellular antioxidant systems and the cells become oxidatively stressed. We easily demonstrated both ɔH2AX and 8-oxo-dG increase following GSK3ɓ inhibition, but the second marker seems enhanced only after LiCl and not SB216763 treatment.

DNA damage induced by reactive oxygen species downstream GSK3ɓ phosphorylation also activates the stress sensor GADD45ɓ, a protein involved in cell cycle arrest that may be responsible for the intra S checkpoint (Bhattacharjee, Park et al. 2005), a phenomenon that we have observed after LiCl treatment of chondrocytes. GADD45ɓ is also a mediator of p21 expression (Shimada et al., 2011), that is itself a cell-cycle regulator in response to DNA-damage (d'Adda di Fagagna 2008). In fact, the expression of p21 increases as a function of senescence in a GADD45ɓ-dependent manner in senescence-accelerated mice (Shimada, Sakakima et al. 2011). Our experiments evidenced an increased expression of p21 downstream GSK3ɓ inactivation, in keeping with a report showing that even in healthy tissue, there is an association between increased p21 expression and increased chondrocyte hypertrophy at the level of articular cartilage (Stewart, Farnum et al. 1997).

The pattern just described has been shown not only in *in vitro* chondrocyte cultures, but also in chondrocytes *in vivo* within articular cartilage, where we have highlighted a similar mechanism occurring in the middle-deep layers of articular cartilage as also confirmed by correlated staining of pGSK3ɓ, 8oxo-dG, GADD45ɓ and p21. Moreover, the data obtained indicate that GSK3ɓ activity in chondrocytes from osteoarthritic patients is essential for the maintenance of their proliferative potential and cell cycle distribution, as evidenced by the reduction of their proliferation potential and by the

accumulation in the S phase of the cell cycle observed in our experiments. Overall, this process leads to the survival of chondrocytes but to their functional impairment via induction of hypertrophy and senescence.

Extrinsic senescence is mostly due to oxidative stress and is responsible of the processes of aging in chondrocytes (Shane Anderson and Loeser 2010; Loeser 2011). The most common senescence markers are senescence associated ϐ-gal staining (Price, Waters et al. 2002) and glycogen accumulation (Seo, Jung et al. 2008). We found that GSK3ϐ inactivation is responsible for chondrocyte senescence as demonstrated by the impairment of proliferation and by the expression of senescence markers. We demonstrated that the accumulation of SA-ϐGal and glycogen in chondrocytes do not overlap, but instead follow different kinetics. More precisely, LiCl treatment induces an increased cell expression of SA-ϐGal already after 8 hours and, at the same time point, a flow cytometric analysis indicates that lithium chloride is responsible for both the increased cell size and granularity as measured by the forward and side scatter respectively. The other aspect that we evaluated concerns the glycogen content as measured by the PAS staining in conjunction with the image analysis evaluation of the signal; as we expected, both LiCl and SB216763 treatment of chondrocytes resulted in increased percentage of glycogen containing cells after 24 hours, due to the increased activity of glycogen synthase, in keeping with findings previously reported by (Coghlan, Culbert et al. 2000) in other cell types.

It has been reported that GSK3ϐ inhibition in cartilage induces osteoarthritic features due to the activation of the canonical Wnt pathway following the use of a selective GSK3ϐ inhibitor (Miclea et al., 2011). Indeed, chondrocyte stimulation with LiCl has been associated with the nuclear translocation of ϐ-catenin which is responsible of the chondrocyte terminal differentiation (Ryu, Kim et al. 2002). These observations are

collocated in the context of the deleterious effects of LiCl-induced GSK3ϐ inhibition on cartilage homeostasis.

Our data indeed indicate that LiCl treatment has greater effects than SB216763 on gene and protein expression not only of IKKŬ but also of its target gene MMP-10, a pivotal molecule in the process of chondrocyte differentiation and extracellular matrix remodeling (Olivotto, Otero et al. 2013) in both human and murine chondrocytes. IKKŬ itself has effects on extracellular matrix remodeling and on chondrocyte proliferative potential suggesting that this kinase might contribute to the abnormal phenotype of chondrocytes from OA patients (Olivotto et al., 2008).

Starting from the idea that IKKŬ is involved in the regulation of cellular senescence and in particular in the induction of SASP (Senescence Associated Secretory Phenotype) we investigated how IKKŬ precisely impacts on various types of oxidative damage by comparing wild type and IKKŬ KD chondrocytes and measuring the response of chondrocytes to an extrinsic oxidative stress, responsible of a stress-induced senescence. Our data indicate that IKKα controls the intensity of primary human OA chondrocyte DNA damage response induced by oxidative stress. In fact, IKKα KD induces an immediate increase of cell susceptibility to reactive oxygen species as demonstrated by higher ɔ-H2AX positivity, that is an indication of the presence of double strand breaks. Noteworthy, this phenomenon is also related to the capacity of the cells to respond to the oxidative stress and to repair the DNA breaks. In fact, IKKα KD cells show the capacity to completely recovery the DNA damage 24 hours after the oxidative stimulus. Thus, IKKα KD confers to the chondrocytes an higher capacity to repair respect to control osteoarthritic chondrocytes.

Telomeres protect the ends of chromosomes, and short telomere length is associated with poor health and mortality. This structures are very susceptible to oxidative stress,

that may function as a common trigger for activation of the senescence programs (Kawanishi et al., 2006). In this perspective we have assessed the effect of exogenous oxidative stress on the induction of "extrinsic senescence" by measuring telomere length in IKKα KD chondrocytes. As expected, IKKŬ KD cells undergo less shortening in telomere length respect to control cells, demonstrating a greater resistance to oxidative stress. This scenario is completed by the knowledge that, after an oxidative stress, cells need to restore DNA damage. DNA mismatch repair (MMR) system is one of the main post-replicative correction pathway playing a key role in maintaining genomic stability and is therefore crucial for proliferating cells. We demonstrated that after an exogenous oxidative stress only IKKŬ KD chondrocytes increase expression of two of the major genes of the system: MSH6, one components of the MutSŬ, that is the most abundant mismatch-binding heterodimer (Kolodner, Tytell et al. 1999) and MLH1, that with PMS1 constitutes the MutLɓ heterodimer (Brierley and Martin 2013). The activation of the expression of two genes codifying for components of both the major heterodimers of the DNA mismatch repair system seems to confirm the impact of IKKŬ in the complete and coordinate response to exogenous oxidative stress.

When ROS production exceeds the detoxification and scavenging capacity of the cell, oxidative stress induces damage to DNA, proteins and lipids with production of several by-products that consequently becomes implicated in the pathology of various diseases. To deal with oxidative DNA damage from various endogenous and exogenous sources, mammalian cells have evolved many mechanisms firstly to detect, and subsequently to repair such damage. However, often cells alone are not able to eliminate ROS and necessitate the help from some exogenous scavenging systems. Natural compounds found in fruits and vegetables, collectively known as ñNutraceuticalsò, could have a great potential as anti-oxidant thanks to their direct protective effects as well as their

ability to potentiate some cellular homeostatic mechanisms, thus modifying OA disease (Shen, Smith et al. 2012).

The ability of hydroxytyrosol (HT) to attenuate ɔH2AX-foci formation and senescence in chondrocytes after H_2O_2 exposure is due to its anti-oxidant and cytoprotective actions, according to some recent findings (Facchini, Cetrullo et al. 2014). Also spermidine (SPD) is able to reduce ɔH2AX-foci formation after H_2O_2 exposure in chondrocytes and is responsible of a marked reduction of cell death, a finding that is in agreement with the observation that supplementation with spermidine reduces oxidative stress and extends lifespan in yeast and flies (Eisenberg, Knauer et al. 2009; Guo, Harada et al. 2011; Minois, Carmona-Gutierrez et al. 2012).

Mediterranean diet is rich of food that provide health benefits, such as olive oil, and it is possible that the nutraceuticals contained in this kind of alimentation may exert effects on molecular targeting of osteoarthritis (Leong, Choudhury et al. 2013; (Henrotin, Lambert et al. 2011).

OA is a complex disease with an unclear etiology and multiple risk factors; recent studies suggest some critical events for OA initiation and disease progression: over activated catabolic activity primarily mediated by pro-inflammatory cytokines; deleterious stresses such as oxidative stress as well as the impaired defense mechanisms against these stress factors (*i.e.*, oxidative stress); proteolytic enzymes which directly degrade the cartilage matrix such as matrix metalloproteinases. There is currently no cure for OA, and there are no therapies which prevent, slow or arrest its progression (Le Graverand-Gastineau 2010). Prevention of this pathology is a very intriguing field and only the rescue or activation of intrinsic homeostatic mechanisms could provide an effective solution for a robust chondroprotection.

Reactive oxygen species, which regulate many signaling pathways, are important mediators in the pathogenesis of OA (Lo et al., 1996), therefore ROS can be categorized as one among the key molecular therapeutic targets in OA management. This target provides a significant rational foundation for pursuing nutraceuticals with anti-oxidative stress properties for an anti-OA nutraceutical selection and formulation. Nutraceuticals have been demonstrated to effectively suppress oxidative stress-induced deleterious responses, such as DNA damage, cell death and senescence (Facchini et al, 2014; Leong et al., 2014).

In conclusion, in this study the role of cellular senescence in the pathology of osteoarthritis has been extensively examined considering many points of view. The first one concerns the possible basal mechanism responsible of intrinsic reactive oxygen species production, that we observed in vitro downstream GSK3β inactivation, and that has the potential to drive articular chondrocytes to hypertrophy and senescence. The second one considers another basal mechanism; the involvement of IKKΰ in chondrocyte response to oxidative stress and induction of extrinsic senescence. Noteworthy, both the inactivated GSK3β and the increased IKKΰ expression are markers of the hypertrophic chondrocyte phenotype, and therefore contribute to the improper differentiation progression of osteoarthritic chondrocytes. The third one is focused on the use of some nutraceuticals to prevent DNA damage and cellular senescence.

Overall these data provide new perspectives for the study of viable ways to prevent osteoarthritis and its classical treatments by exploiting multiple approaches to restore the correct chondrocyte phenotype, by either preventing GSK3β phosphorylation, silencing IKKΰ or using an alternative nutraceutical-based molecular targeting strategy for chondroprotection.

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7- PUBLICATIONS

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