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Therapy-induced senescence in normal fibroblasts promotes *in vitro* tumour cell growth and invasiveness: study of the role of Quercetin in modulating these processes.

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ABSTRACT

Aging is a complex biological phenomenon representing the major risk factor for developing age-related diseases, such as cardiovascular pathologies, neurodegenerative diseases, and cancer. Geroscience, the new vision of gerontology, identifies cellular senescence as an interconnected biological process that characterises aging and age-related diseases. Therefore, many strategies have been employed in the last years to reduce the harmful effects of senescence, such as *senolytic* drugs that selectively kill senescent cells, or *senomorphic* ones that interfere with the production of the senescence-associated secretory phenotype (SASP) factors. Here we show that a pre-treatment with Quercetin, a bioactive flavonoid present in many fruits and vegetables, increasing cellular antioxidant defence, can reduce Doxorubicin (Doxo)-induced cellular senescence in human normal WI-38 fibroblasts. Moreover, exerting an excellent senomorphic action, Quercetin reduces the SASP and consequently reduces the pro-tumour effects of conditioned medium (CM) from Doxo-induced senescent fibroblasts on Osteosarcoma cells.

Furthermore, we also analyse the effect of Quercetin after the induction of senescence to investigate its senolytic activity, and we show that it can reduce the number of Doxo-induced senescent fibroblasts without affecting the proliferating ones, even if more investigations about the mechanism of action are necessary. Finally, our data show that, also in this condition, the treatment with Quercetin can cancel the pro-tumour effects of Doxo-induced senescent fibroblasts CM on U2OS cells. Overall, our findings demonstrate that Quercetin is an excellent senomorphic agent and a potential senolytic substance that can protect normal fibroblasts against the off-target induction of senescence by the chemotherapy drug Doxorubicin, and it can potentially eliminate the formed senescent cells, reducing their pro-tumour and deleterious effects on Osteosarcoma cells.

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LIST OF ABBREVIATIONS

7-AAD	7-Amino-Actinomycin
AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
APO-C	Apoprotein C
ARD	Age-related disease
ATM	Ataxia Telangiectasia Mutated
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CCFs	Cytoplasmic chromatin fragments
CCN1	Cellular communication network factor 1
CDKi	Cyclin-dependent kinase inhibitors
cGAMP	Cyclin GMP-AMP
cGAS	GMP-AMP synthase
CRMs	Calorie restriction mimetics
CRP	C-reactive protein
CSFs	Colony stimulating factors
CTGF	Connective tissue growth factor
CXCR4	C-X-C motif chemokine receptor 4
DAMPs	Damage-associated molecular patterns
DNA-SCARS	DNA segments with chromatin alterations reinforcing senescence
DDR	DNA damage response
ECM	Extracellular matrix
EFS	Event free survival
ER	Endoplasmic reticulum
EVs	Extracellular vesicles
FasL	Fas ligand
H3K9me3	Histone H3 trimethylated at lysine 9
HAPLN1	Hyaluronan and proteoglycan link protein 1
HGF	Hepatocyte growth factor
HMGA2	Non-histone chromatin protein
HP1	Heterochromatin protein 1
HSC	Hematopoietic stem cells
HUVECs	Human umbilical vein epithelial cells
IDD	Intervertebral disc degeneration
IGF-1	Insulin-like gene 1
IGF	Insulin-like growth factor
IGBP-7	Pleiotropic protein IGF binding protein-7
IL-6	Interleukin 6
IR	Ionizing radiation
IS	Immune system
LDH	Lactate dehydrogenase
MiRNA	MicroRNA
MMPs	Matrix metalloproteinases

MSCs	Mesenchymal stromal cells
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
NAMPT	Nicotinamide phosphoribosyl transferase
NF-κB	Nuclear factor- κB
OIS	Oncogene-induced senescence
OS	Osteosarcoma
OXPHOS	Oxidative phosphorylation
PAI-1	Protease inhibitor plasminogen activator inhibitor-1
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
pH2AX	Phosphorylated H2A histone family member X
PI3K	Phosphoinositide-3-kinase
PM	Plasma membrane
p-p53	Phosphorylated p53
PRR	Pattern recognition receptors
pRB	Retinoblastoma protein
QM	Conditioned medium from proliferating fibroblasts treated with Quercetin
QSM	Conditioned medium from Quercetin pre-treated Doxo-induced senescent fibroblasts
ROS	Reactive oxygen species
SA-β-gal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatin foci
SFK	Src-family tyrosine kinase
SIPS	Stress-induced premature senescence
SM	Doxo-induced senescent fibroblasts conditioned medium
SOD	Superoxide dismutase
SQM	Conditioned medium from Doxo-induced senescent fibroblasts post-treated with Quercetin
TASCC	TOR autophagy spatial coupling compartment
TIS	Therapy-induced senescence
VEGF	Vascular endothelial growth factor

ABSTRACT

Aging is a complex biological phenomenon representing the major risk factor for developing age-related diseases, such as cardiovascular pathologies, neurodegenerative diseases, and cancer. Geroscience, the new vision of gerontology, identifies cellular senescence as an interconnected biological process that characterises aging and age-related diseases. Therefore, many strategies have been employed in the last years to reduce the harmful effects of senescence, such as *senolytic* drugs that selectively kill senescent cells, or *senomorphic* ones that interfere with the production of the senescence-associated secretory phenotype (SASP) factors. Here we show that a pre-treatment with Quercetin, a bioactive flavonoid present in many fruits and vegetables, increasing cellular antioxidant defence, can reduce Doxorubicin (Doxo)-induced cellular senescence in human normal WI-38 fibroblasts. Moreover, exerting an excellent senomorphic action, Quercetin reduces the SASP and consequently reduces the pro-tumour effects of conditioned medium (CM) from Doxo-induced senescent fibroblasts on Osteosarcoma cells.

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1 INTRODUCTION

1.1 Aging

Aging is a natural and universal feature of most living organisms, and in humans has become a medical and social priority due to increased life expectancy and consequent high prevalence of elderly subjects in the population. However, it is one of the most complex biological phenomena, and it can be defined as the accumulation of unrepaired or damaging modifications produced by endogenous and/or exogenous stimuli, occurring in molecules, cells, tissues, and organs. In addition, to compensate for the adverse effects of such damages, the body promotes a series of adaptive mechanisms, changing its compositions and the microenvironment, that become an essential part of the aging process (Ostan et al., 2008). Unfortunately, all these modifications concur to increase the risk of developing the age-related diseases.

In the past years, over 300 theories were formulated to explain why aging takes place (Medvedev, 1990), and among these, the most prominent are:

1. *The molecular cross-linkage theory* (Bjorksten, 1968) attributed changes in aging to the presence of crosslinking materials in the body.
2. *The gene regulation theory* proposed that gene expression changes during life trigger and drive the aging process (Kanungo, 1975). Gene expression studies applied to aging, carried out in the following years, identified a signalling pathway characterized by the activation of the insulin-like gene 1 (IGF-1), which can regulate lifespan in nematodes, insects, and mice. Indeed, Tatar et al. demonstrated that the reducing IGF-1 mediated signal increases life expectancy in *C. Elegans* (Tatar et al., 2003). Moreover, studies conducted on human centenarians and their family members showed that "healthy aging" is supported and also influenced by a strong genetic component, which regards lifespan-associated loci, as insulin/IGF-1 pathway genes, Apoprotein C (APO-C) gene, and pro-and anti-inflammatory cytokine genes (Barbieri et al., 2003; Franceschi & Bonafè, 2003; McElwee et al., 2007; Testa et al., 2011).
3. *The evolutionary theories* tried to explain the remarkable differences observed in aging and longevity records among different biological species through the interaction between mutation and selection processes (Gavrilov & Gavrilova, 2002). Among these, the three most important were:

   a. *The theory of programmed death*, proposed by August Weisman, according to which a specific death mechanism designed by natural selection would eliminate the old members of a population (Weismann, 1882);
   b. *The mutation accumulation theory* proposed that from the evolutionary perspective aging is the inevitable result of the reducing force of natural selection and accumulation of late-acting deleterious mutations (Medawar, 1946, 1952);
   c. *The antagonistic pleiotropy theory* suggested that late-acting deleterious genes could be accumulated and favoured by natural selection for their beneficial effects early in life (Williams, 1957).

4. *The neuroendocrine theory* proposed that changes in neuroendocrine functions affect coordination and the body's response to the external environment. Therefore, aging would result in a progressive decrease in the ability to adapt and survive to exogenous stresses (physical, chemical, and emotional stimuli) that can lead to the onset of diseases and death (McEwen & Lasley, 2002).
5. *The free radical theory* postulated that aging is caused by free radical reactions, characterized by the transient presence of highly reactive intermediates, known as free radicals (Harman, 1956, 1993). These unstable compounds, which can be produced by internal metabolism and environment (such as ionizing radiation, γ-, X-, ultraviolet rays, and ultrasound), can trigger and promote the damage of DNA molecules, proteins, and lipids, altering the structure of the cell. Defence mechanisms that have evolved to cope with free radicals' production include enzymes such as heme-containing and glutathione peroxidases, superoxide dismutase (SOD), and antioxidants such as carotenes, carnosine, and tocopherols. To support this theory, different studies proved that removing antioxidant enzymes, as SOD, reduces the health/lifespan of multiple model organisms (Sohal & Orr, 2012; Van Remmen et al., 2004; Y. Zhang et al., 2009). However, other studies demonstrated that over-expression of several antioxidant enzymes was insufficient to impact lifespan (Huang et al., 2000; Mele et al., 2006; Pérez et al., 2009; Pomatto & Davies, 2018). Over the years, contradictory findings have led to the conclusion that the binary idea proposed by Harman that free radicals are only harmful, while antioxidants must always be beneficial, is not a universal phenomenon.
6. *The network theory,* formulated by Franceschi in 1989, suggested that a network of cellular and molecular defence mechanisms, including heat shock proteins, DNA repair mechanisms, apoptosis, immune and neuroendocrine systems, indirectly controls the aging process (Franceschi, 1989). Underlying these mechanisms, there was the theory of

Immunosenescence, a dynamic process that affects both innate and acquired immunity and results in a low-grade, chronic inflammation, called *inflammaging* (Franceschi et al., 2000, 2006), involved in aetiology and progression of age-related diseases (Furman et al., 2019). In the past years, studies on healthy centenarians have demonstrated that the activities of lymphocytes, NK cells, and complement system were preserved during aging, suggesting that the Immunosenescence process mainly involved acquired immunity (Franceschi et al., 1995). However, several studies have subsequently shown that fundamental components of the innate immune system (IS) change with age and are correlated to increased risk of infection and infection mortality (Santoro et al., 2021). All these findings suggest that both branches of the IS, innate and adaptive, change during aging but not to the same extent or the same consequences. Therefore, it could be conceptualized that age-related immune changes may be a mix of adaptation/resilience and maladaptation, closely related to the immunobiography (the combination of type, dose, intensity, and temporal sequence of antigenic stimuli to which each individual is exposed) (Franceschi et al., 2017; Fulop et al., 2004). Furthermore, successful aging is determined by a lower predisposition to mount an inflammatory response combined with an efficient anti-inflammatory network (Santoro et al., 2021).

7. *The cellular senescence theory* was formulated in 1961 by Hayflick, which noted that human diploid fibroblasts in culture could reach a maximum number of cell divisions ("Hayflick's limit") before arrest (Hayflick & Moorhead, 1961). It postulated that aging phenotypes were caused by an increase in the frequency of senescent cells (Weinert & Timiras, 2003). The theory will be discussed in Section 1.3.

More recently, however, these theories have been replaced by the idea that aging is a complex multifactorial process encompassing genes, performance repair systems, milieu, and chance (Da Costa et al., 2016).

1.2 Age-related diseases

The increase in human life expectancy and the consequent population aging will increase age-related diseases (ARDs), such as cardiovascular disease, diabetes, arthritis, osteoporosis, blindness, sarcopenia, neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), and several other chronic conditions (Franceschi et al., 2018). Moreover, many types of cancer are considered ARDs, as their incidence increase with near

exponential kinetics at approximately the mid-point of the lifespan (e.g., 50-60 years of age) (Siegel et al., 2021).

Aging is the most important known risk factor for many chronic diseases, and "Geroscience", the new vision of gerontology, aims to understand the relationship between aging and ARDs and improve the mechanisms that can prevent, delay, and/or counteract them. Therefore, it is likely that the future trend of medicine will try to decrease the prevalence of diseases by slowing the aging rate, thus allowing people to stay healthy and active as long as possible (Franceschi et al., 2018; Sierra & Kohanski, 2015). One of the major achievements of Geroscience has been to identify nine selected and interconnected biological processes that represent the critical pillars of aging and ARDs, and which therefore determine the aging phenotype (Kennedy et al., 2014; López-Otín et al., 2013). Among these, shown in detail in **Figure 1**, we remind:

- ✓ Genomic instability: nuclear DNA, mitochondrial DNA, nuclear architecture
- ✓ Telomere attrition
- ✓ Epigenetic alterations: histone modifications, DNA methylation, chromatin remodelling, transcriptional alterations, reversion of epigenetic changes
- ✓ Loss of proteostasis: chaperone-mediated protein folding and stability, proteolytic systems
- ✓ Deregulated nutrient sensing: the insulin and IGF-1 signalling pathway, other nutrient-sensing systems (mTOR, AMPK, sirtuins)
- ✓ Mitochondrial dysfunction: reactive oxygen species, mitochondrial integrity, and biogenesis, mitohormesis
- ✓ Cellular senescence
- ✓ Stem cell exhaustion
- ✓ Altered intercellular communication: inflammation, other types of intercellular communication.

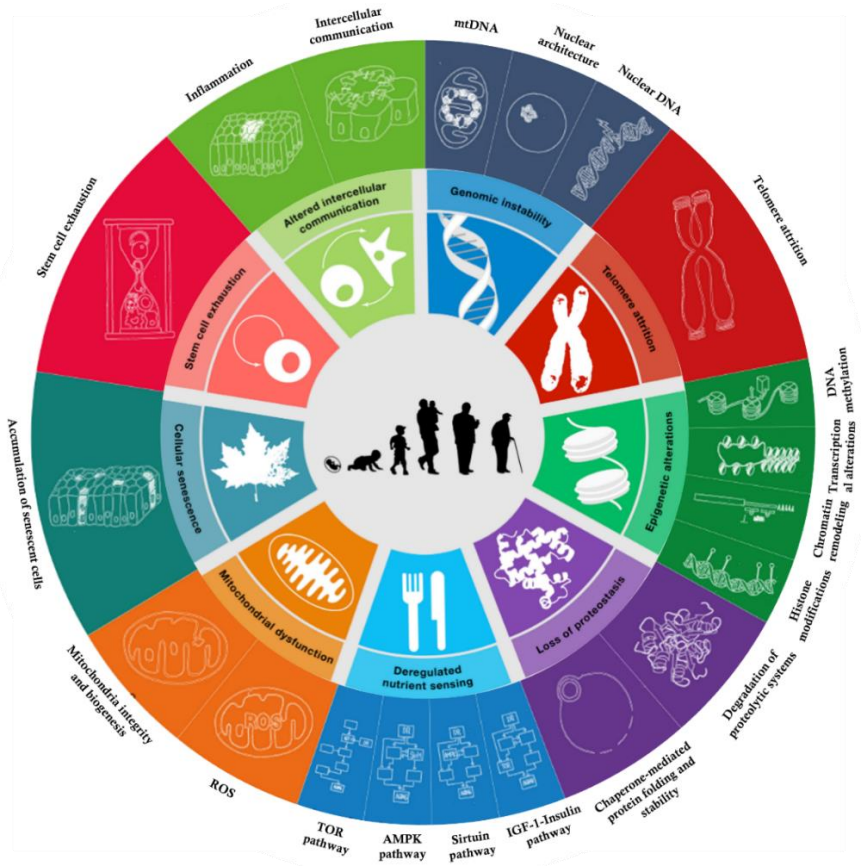

Figure 1. **The Hallmarks of aging.** The internal wheel represents the 9 hallmarks of aging, proposed by López-Otín et al. (López-Otín et al., 2013). The extension of the wheel describes the detailed subcategories of these hallmarks. Abbreviations: *mtDNA*: mitochondrial DNA; *ROS*: reactive oxygen species (Lemoine, 2021).

Since many chronic disease and pathological conditions are at least in part determined by some of these mechanisms, it seems acceptable to think that the difference between aging and diseases may lie in the combination of the rate/speed/intensity of aging processes, and the specific genetic and lifestyle/habit predisposition. Accordingly, aging and ARDs have to be considered different trajectories of the same process, but with a different rate depending on diverse genetic backgrounds and lifestyles (Franceschi et al., 2018).

Below, the involvement of some of the processes, identified as the nine pillars, in several age-related pathologies are briefly listed (**Figure 2**):

1. *Stem cells exhaustion*. The functional decline of adult stem cells does not occur only as a quantitative reduction in their number but rather as a qualitative change and reduced functional capacity. For example, loss of regenerative capacity of satellite cells (the muscle's stem cells) is a feature shared with aging and may be involved in developing sarcopenia (Franceschi et al., 2018). Also, in chronic obstructive pulmonary disease, the reduced regenerative capacity of basal progenitor cells required for airway epithelial differentiation participate in the pathology (Ryan et al., 2014). Moreover, due to their two

principal properties (self-renewal and differentiation), stem cells represent an ideal target for accumulating pre-cancerous damages that can be propagated to self-renewing progeny and downstream progenitors. The activity of tumour suppressor proteins that arrest the malignant clones copes with mutagenic accumulation. Nevertheless, tumour suppressor pathways in tissues with a constant demand for stem cell and progenitor activity may lead to stem cell erosion, thus contributing to aging (Rossi et al., 2008).

2. *Accumulation of senescent cells*. Different studies showed that senescent cells accumulate with age, and this accumulation was related to several ARDs, including atherosclerosis, AD, PD, and osteoarthritis (Campisi & Robert, 2014). Moreover, despite cell senescence arising as an anti-neoplastic mechanism, the acquisition of the pro-inflammatory secretory phenotype (Coppé et al., 2008) can in part contribute to the promotion of the malignant and metastatic features of cancer cells (Lecot et al., 2016). The link between senescence and cancer will be deeply discussed in Section 1.3.3.
3. *Inflammaging*. Aging is associated with a low-grade, sterile chronic inflammation, called "inflammaging", that is crucially involved in the aetiology and progression of ARDs (Furman et al., 2019). Indeed, the analysis of epidemiologic data revealed a correlation between elevated levels of inflammatory factors, such as interleukin 6 (IL-6), and C-reactive protein (CRP), and multiple morbidities of the elderly (Franceschi & Campisi, 2014; Rea et al., 2018). Accordingly, also in neurodegenerative diseases inflammation plays an important role. For example, neuroinflammation in AD involves resident cells, such as microglia, astrocytes, and neurons, but also cells or soluble factors of the peripheral IS that can enter into the brain developing inflammation and stimulating neurodegeneration (Perry, 2004). In the same manner, it has been shown that in PD, a strong detrimental inflammatory response can be fueled by neuronal degeneration due to α-synuclein aggregates. Interestingly, IL-6 is one of the most important components of inflammaging, and can promote the progression toward the malignancy of cancer cells (Hirano, 2021). Nowadays, it is widely accepted that inflammation is involved in cancer onset and progression. Moreover, since it appears to be a universal feature of human aging, it can be hypothesized that the effect of inflammation on a long-lived individual may increase his/her probability of developing cancer (Franceschi et al., 2018).
4. *Immunosenescence*. During aging the IS undergoes profound transformations, included in the concept of Immunosenescence, that affect multiple aspects of immunity, such as susceptibility to infections, autoimmunity, response to vaccination, and cancer development (Panda et al., 2009; Santoro et al., 2021). Indeed, aging is characterized by thymic

involution and reduced T cell diversity, with a decreased number and frequency of naïve CD4 + and CD8+ T cells and a reciprocal increase of CD8+ memory T cells. These changes in T cells composition during aging compromise their ability to be activated, recognized, and to destroy or control cancer growth, helping to create a more permissive environment towards cancer evasion of immunosurveillance (Pawelec, 2017). Moreover, emerging epidemiological and molecular studies indicate that age-related changes in innate IS cells strongly contribute to the pathophysiology of sarcopenia, increasing systemic inflammation (Wilson et al., 2017), and in chronic obstructive pulmonary disease development and progression (John-Schuster et al., 2016).

5. *Autophagy*. Autophagy, derived from the Greek meaning "eating of self", is an evolutionary self-preservation mechanism for recycling intracellular proteins and organelles via lysosome-mediated degradation, promoting homeostasis, differentiation, adaptation to stress, and survival. Due to its role in removing defective organelles, cellular debris, and misfolded protein aggregates, autophagy operates as an anti-aging mechanism. Indeed a growing body of evidence suggests that autophagic activity declines with the aging process (Aman et al., 2021). Moreover, autophagy has also a central role in removing intracellular carcinogenic agents, maintaining genome integrity, and tumorigenesis prevention. Thus, the decline in autophagy capacity during aging seems to contribute to the process of tumour initiation, although the role of autophagy in tumour progression reveals a great complexity (Zinger et al., 2017). The reduction or dysregulation of autophagy plays an important role also in maculopathy, osteoarthritis, chronic obstructive pulmonary disease and can participate in AD pathogenesis (Franceschi et al., 2018).
6. *Microbiome*. The human microbiome is the microbial ecosystem associated with the human body. One of the most extensive interfaces between the human host and microbe is the human intestinal mucosa. During aging intestinal barrier functions change, accompanied by the alteration of microbiome composition. Several studies on different experimental models demonstrated the correlation between aging, barrier function, and microbiome (Heintz & Mair, 2014; Rera et al., 2012; Zapata & Quagliarello, 2015). Aging-related intrinsic functional changes of gastrointestinal tracts include decreased intestinal motility, diverticular disease, alterations in salivary function, and poor dentition. In addition, the impairment of mucosal integrity can increase commensal bacteria invasiveness, triggering the systemic secretion of cytokines, contributing to inflammaging and associated pathologies. In some cases, these age-associated changes in inflammatory responses can create a pro-tumorigenic environment that overcomes the only local effect in the intestine

(Zinger et al., 2017). Interestingly, recent studies showed that also PD is associated with gut dysbiosis, showing a significantly reduced faecal concentration of short-chain fatty acids that could impact central nervous system alterations (Mulak & Bonaz, 2015; Tremlett et al., 2017).

7. *MicroRNAs*. MicroRNA (miRNAs) are a class of endogenous small non-coding RNA molecules that play an essential role in regulating numerous biological processes and networks. They act as negative regulators of gene expression, targeting specific mRNAs for degradation or preventing their translation into protein. In addition, they can be used in intercellular communications among adjacent cells via gap junctions or endocrine communication via microvesicles. These features and their relatively low-binding specificity, which permits one miRNA to target multiple genes, make miRNAs perfect candidates for regulators of critical systemic processes, such as aging, inflammatory response, and tumorigenesis (Schroen & Heymans, 2012; Schwarzenbach et al., 2014). In this context, three classes of miRNAs were described: the ones that can be associated with senescence (*SA-miRs*), inflammation (*inflamma-miRs*), and cancer (*onco-miRs*). Several studies have identified miRNAs through these three categories that are involved in the regulation of different pathways, as Nuclear factor (NF)-κB, mechanistic target of rapamycin (mTOR), TGF-β, Wnt, and Sirtuins signalling, that link aging, senescence, and cancer (Olivieri et al., 2013).

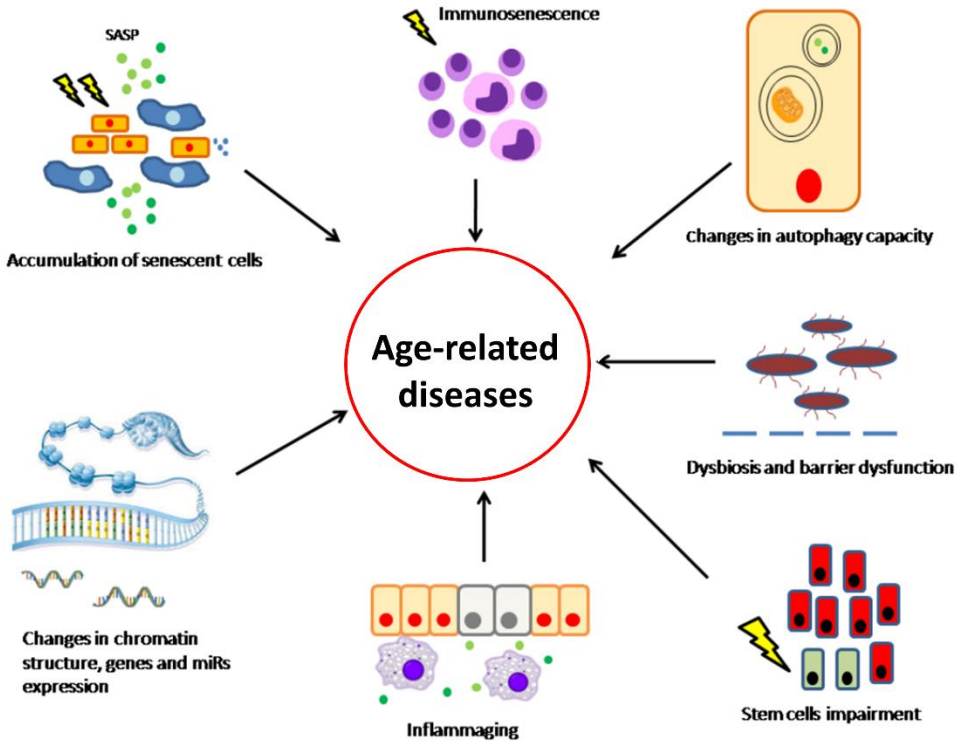

***Figure 2*. Mechanisms that link aging to ARDs.** The figure shows some cell-autonomous and non-cell-autonomous mechanisms identified as critical factors that link age-related diseases and aging. Exposure to endogenous and exogenous stressors causes multiple cellular and tissue function changes during life. For example, the accumulation of senescent cells leads to tissue degeneration and changes the microenvironment through the SASP factors. In addition, changes in the immune system with age (immunosenescence) contribute to the development of *inflammaging*. This inflammatory process joints intracellular processes, such as reduction of autophagy, changes in chromatin function, microbiome modification, and intestinal barrier dysfunction, to ensemble a detrimental milieu favouring ARDs (adapted from Zinger et al., 2017).

All these described processes are involved in several and distinct types of ARDs. Since these pathologies are so diverse, it is likely that one or a few of those mechanisms mainly drive most, if not all, of them. Cellular senescence is one perfect candidate for such a role.

1.3 Cellular Senescence

The term senescence derives from the Latin word *senex*, which means “old” or “elderly”. However, senescence was used in biological organisms to describe deteriorative processes that follow cell development and maturation (Campisi & D’Adda Di Fagagna, 2007). Senescence is now defined as a highly dynamic multi-step process, during which senescent cells that undergo an irreversible cell cycle arrest evolve and diversify their properties continuously in a context-dependent manner (Kumari & Jat, 2021; van Deursen, 2014). Senescent cells experienced structural changes at molecular and cellular levels, which allow them to remain

viable, resistant to apoptosis, and metabolically actives (Childs et al., 2014; E. Wang, 1995; Zhu et al., 2015).

The first formal description of cellular senescence was done in 1961 by Hayflick, who showed that human fibroblasts in culture undergone irreversible proliferative arrest after a finite number of divisions (Hayflick & Moorhead, 1961). This replicative limit was defined as "Hayflick's limit" and identified the so-called *replicative senescence* that was later attributed to telomere shortening (Hayflick, 1965). Subsequent studies have shown that other types of stimuli, such as severe or irreparable DNA damage, oxidative stress, mitochondria deterioration, exposition to ionizing radiations (IR), activated oncogene, and chemotherapy can trigger *in vitro* a "stress-induced premature senescence (SIPS)" (Campisi, 2001; Campisi & D'Adda Di Fagagna, 2007). Moreover, it is also accepted that senescence plays a crucial role *in vivo.* Indeed a large body of evidence from animal models had demonstrated the importance of senescence in different physiological and pathological processes (Burton & Krizhanovsky, 2014). In physiological conditions, senescent cells are involved in embryonic development (Muñoz-Espín & Serrano, 2014; Storer et al., 2013), tissue remodelling and repair (Yun et al., 2015), wound healing (Demaria et al., 2014; Krizhanovsky et al., 2008), and tumour suppression, by preventing transformed and damaged cells to propagate their dysfunctional genome to the next generation (Faget et al., 2019; Hanahan & Weinberg, 2011; Serrano et al., 1997). Usually, senescent cells are removed by the IS, allowing them to perform their physiological functions correctly. However, during chronological aging, senescent cells have been found to accumulate exponentially in multiple tissues (López-Otín et al., 2013), either because an aged IS fails to eliminate them, or the rate of senescent cells formation is increased, having the potential to promote pathological conditions and age-related disorders (Baker et al., 2011; Burton & Krizhanovsky, 2014). This apparent paradox can be clarified by understanding how aging phenotypes evolved. Evolutionary theories explain that most organisms, evolving in a high-risk environment, rarely reach old age, and this causes a decline of natural selection for processes that promote disabilities in advanced age, including ARDs (Campisi, 2011). The concept of "antagonistic pleiotropy" extends this theory and indicates that a biological process selected to be beneficial early in life can be deleterious in old organisms (Williams, 1957). This theory can explain the two faces of senescence that evolved to promote tissue repair and suppress cancer development (selected activities) early in life but showed a "dark side" (unselected activities) that is revealed only late in life, when the accumulation of senescent cells could endorse chronic inflammation, promoting the creation of a pro-tumorigenic milieu (Campisi, 2011) (**Figure 3**).

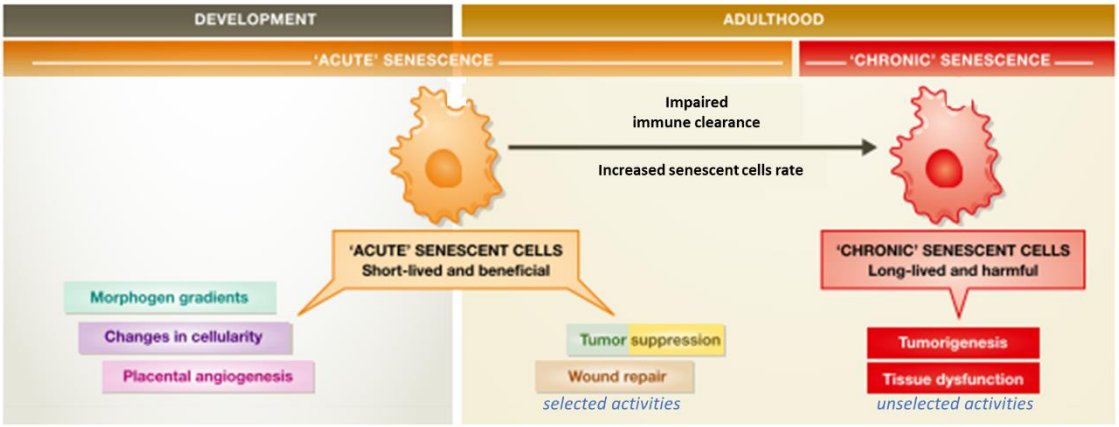

Figure 3. **Cellular senescence as a double-edged sword during lifespan.** During development cells can undergo acute senescence, to cope with physiological processes, as tumour suppression and tissues repair (selected traits). However, upon IS dysfunction, acute senescent cells may accumulate and contribute to tissues disfunction and ARDs development, including cancer (adapted from Childs et al., 2014)

1.3.1 Senescence hallmarks

Several aspects characterize senescent phenotype, none of which unequivocally identify senescent cells independently. Moreover, not all senescent cells and not all types of senescence-induced stimuli lead to the expression of the same hallmarks (Rodier & Campisi, 2011). Therefore, to establish if a cell is entered in a senescent state, it is necessary to use a combination of these markers.

The stable cell cycle and proliferation arrest, due to the engagement of various cyclin-dependent kinase inhibitors (CDKi) in senescent cells, is accompanied by other typical features, including the activation of a chronic DNA damage response (DDR), induction of anti-apoptotic genes, altered metabolic rates, endoplasmic reticulum (ER) stress, and enhanced secretion of pro-inflammatory and tissue-remodelling factors. As a consequence of these modifications, senescent cells also show a structural aberration, including enlarged and flattened morphology, the altered composition of the plasma membrane (PM), accumulation of lysosomes and mitochondria, and nuclear alterations (Campisi & D'Adda Di Fagagna, 2007; Coppé et al., 2008; Hernandez-Segura et al., 2018; Muñoz-Espín & Serrano, 2014). The main senescence features are described in **Figure 4**.

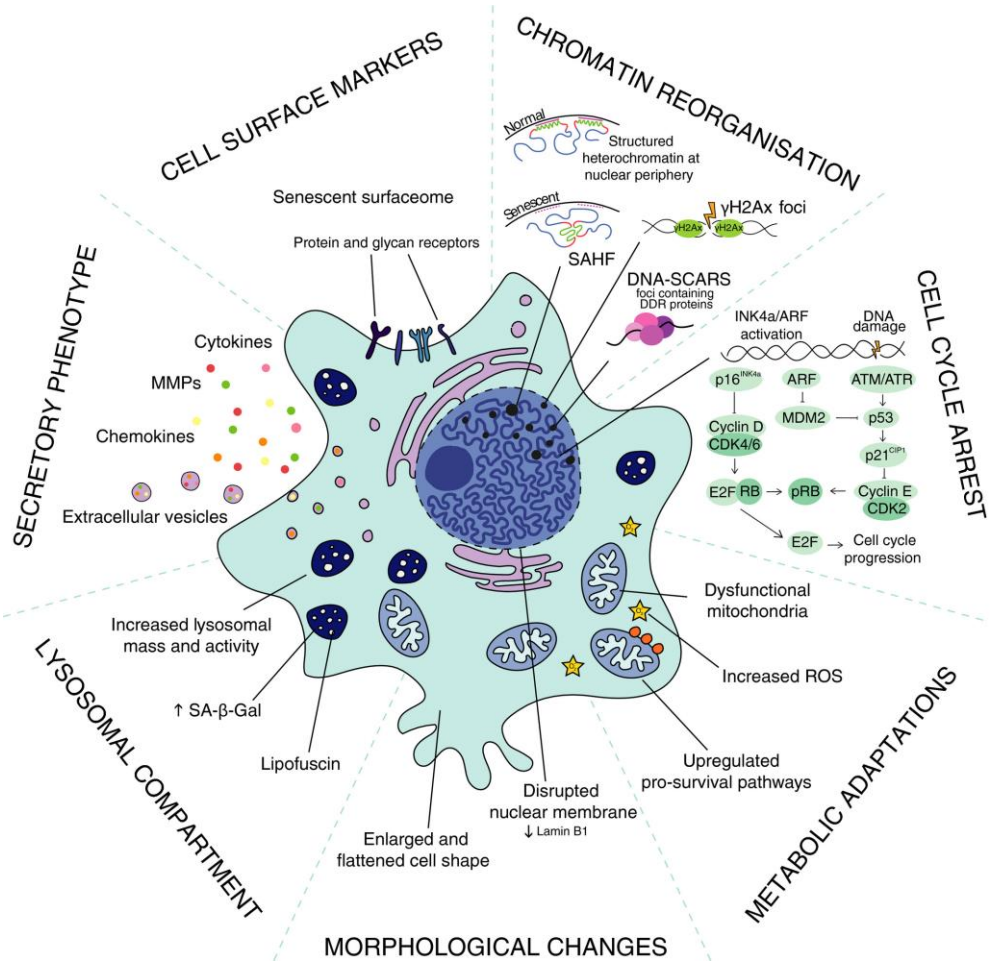

Figure 4. **Hallmarks of cellular senescence.** The molecular pathways of senescence result in morphological alterations that constitute senescence hallmarks. These include a flattened and enlarged morphology, stable cell cycle arrest, driven by $p16^{INK4a}$/Rb and/or $p21^{CIP1}$/p53 axis, chromatin alterations and reorganization, including SAHF, and γH2AX foci, and remodelling of the nuclear envelope, that comprises the loss of Lamin B1. Moreover, other typical features are macromolecular damage (increased ROS levels), metabolic changes (large and dysfunctional mitochondria), increased lysosomal compartment, which implies the overexpression of the SA-β-gal, and the secretion of a vast spectrum of cytokines, chemokines, proteases, and growth factors, referred to as "senescence-associated secretory phenotype" (SASP). Abbreviations: *DNA-SCARS*: DNA segments with chromatin alterations reinforcing senescence; *MMPs*: Matrix metalloproteinases; *ROS*: Reactive oxygen species; *SA-β-gal*: Senescence-associated β galactosidase; *SAHF*: Senescence-associated heterochromatin foci (González-Gualda et al., 2021).

1.3.1.1 Cell cycle arrest

The cell cycle is a sequence of coordinates events in a cell as it grows and divides. A stable cell cycle and proliferation arrest are two essential markers to define senescent cells. Contrary to what happens in the phenomenon of quiescence, in which cell cycle is reversibly arrested in G0 phase, senescent cell cycle arrest occurs in G1 or G2 phase (Di Leonardo et al., 1994). The two most crucial tumour suppressor pathways that drive and stabilize this cell cycle arrest are $p53/p21^{WAF1/CIP1}$ and $p16^{INK4a}$/pRB. These pathways are complex and involve many upstream regulators and downstream effectors that are interlinked with extensive crosstalk

(Kumari & Jat, 2021). The senescent state is mainly maintained by p53 and pRB, which are key transcriptional regulators that induce extensive gene expression changes. $p21^{WAF1/CIP1}$ and $p16^{INK4a}$ are also equally fundamental components of such pathways as they are cyclin-dependent kinase inhibitors (CDKIs) and act as negative regulators of cell cycle progression. In both networks, the final downstream target is pRB, which in its hypophosphorylated state binds and blocks E2F (a transcriptional factor that promotes cell cycle progression), preventing the transcription of replicative genes (González-Gualda et al., 2021) (**Figure 5**).

1. *$p16^{INK4A}$/pRB pathway*. $p16^{INK4A}$/pRB pathway is usually activated in replicative senescence, ROS-induced senescence, and oncogene-induced senescence (OIS), and it is thought to play an essential role in maintaining the senescent state (Beauséjour et al., 2003; Campisi & D'Adda Di Fagagna, 2007; González-Gualda et al., 2021). $p16^{INK4A}$ is a CDKI that directly binds to CDK4/6 and blocks the formation of cyclin D-CDK4/6 complexes, preventing the phosphorylation of pRB. Typically, pRB is maintained in the inactive phosphorylated form by such complexes. The inhibition of CDKI-cyclin complexes leads to the activation of pRB through dephosphorylation and consequently to the sequestering of E2F, inhibiting transcription of cell cycle progression genes (Kumari & Jat, 2021). Interestingly, it seems that $p16^{INK4A}$/pRB pathway is crucial to generating senescence-associated heterochromatin foci (SAHFs), maybe due to the ability of pRB to complex with histone-modifying enzymes that form repressive chromatin and silence the genes required for proliferation, establish a self-maintaining senescence state (Campisi & D'Adda Di Fagagna, 2007; Narita et al., 2003; R. Zhang et al., 2005).
2. *P53/$p21^{WAF1/CIP1}$ pathway.* P53/$p21^{WAF1/CIP1}$ pathway is activated in response to DNA damage caused by telomere attrition, oncogenic activation, or oxidative stress, and it is thought to be activated early on in the senescence program (Beauséjour et al., 2003; Dulić et al., 2000; González-Gualda et al., 2021). At the basal level, p53 is in an inactivated state in the cytoplasm linked to MDM2, an E3 ubiquitin ligase, which allows its degradation. The stress triggers constitutive DDR signalling ATM (Ataxia Telangiectasia Mutated)/ATR that chronically activates p53 by phosphorylation (p-p53), inhibiting the binding with MDM2. Furthermore, the phosphorylation of p53 upregulated the transient expression of the CDKI $p21^{WAF1/CIP1}$, which inhibits CDK2-cyclin E, which permits the dephosphorylation of RB, leading to the sequester of E2F and the arrest of the cell cycle (Kumari & Jat, 2021). Moreover, one exciting study seems to suggest that p53 is upregulated in the initial state of senescence, but when cells reach the late stage of senescence, its expression decreases

gradually, and the $p16^{INK4A}$/pRB axis takes over to establish the irreversible senescence phenotype (Beauséjour et al., 2003; Lagoumtzi & Chondrogianni, 2021).

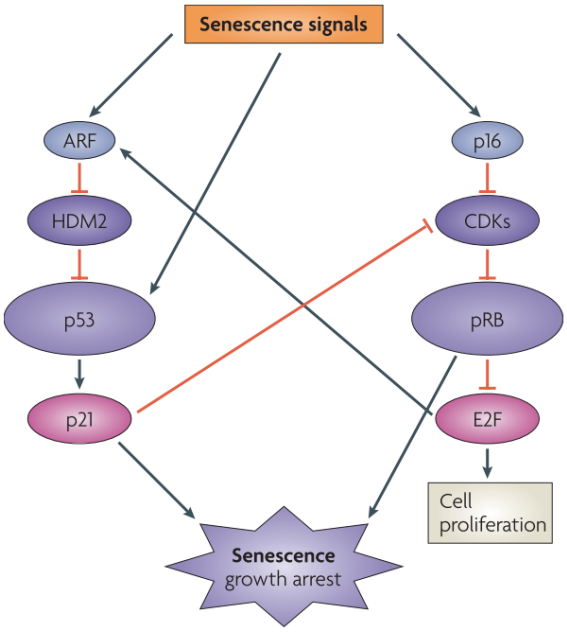

Figure 5. **Senescence cell cycle arrest.** Senescence-inducing stimuli, including DNA damage, telomere attrition, oxidative stress, etc, engage either p53/p21 and/or p16/pRB tumour suppressor pathways. P53 is negatively regulated by E3 ubiquitin-protein ligase HDM2, which facilitates its degradation, and it is in turn negatively regulated by alternate-reading-frame protein (ARF). Active p53 instituites the senescence growth arrest inducing the expression of p21, a CDKI, that among other activities, suppresses the phosphorylation of pRB, stimulating its activation. Senescence triggers can also activate p16, another CDKI, that prevent pRB phosphorylation and inactivation too. pRB binds and sequesters E2F, a transcription factor that stimulates the expression of genes required for cell cycle progression, suppressing cell proliferation (Campisi & D'Adda Di Fagagna, 2007).

1.3.1.2 Altered morphology and granularity

A key feature of *in vitro* senescence is the large and flat morphology (Hernandez-Segura et al., 2018). This event is strictly linked to changes in cytoskeletal structure and composition. Indeed, senescent cells present an accumulation of vimentin, which determines the formation of large bundles of thick and crossed-bridged filaments, reducing the motility (Hwang et al., 2009), and upregulation of caveolin-1 (Hernandez-Segura et al., 2018). Interestingly, recent findings suggest that this increased size consequently leads to an increased cytoplasm to DNA ratio and cytoplasmic dilution, which delays the protein diffusion and binding required for translation, thus contributing to cell cycle arrest (Neurohr et al., 2019). Moreover, changes in nuclear morphology can be observed in senescent cells, probably driven by the loss of the Lamin B1, a structural protein of the nuclear lamina that contributes to the size, shape, and stability of the nucleus (Sadaie et al., 2013). The consequent destabilization of the nucleus, following Lamin B1 loss, results in other nuclear changes, such as the formation of the SAHF

and the appearance of cytoplasmic chromatin fragments (CCFs), enriched by markers associated with DNA damage (Ivanov et al., 2013). Granularity also increases in senescent cells, probably due to the increased size and number of lysosomes, easily detected by light microscopy and flow cytometry-based analysis (González-Gualda et al., 2021).

1.3.1.3 Increased lysosomal content and senescence-associated SA-β-Gal activity

Another typical feature of senescent cells is the increase in lysosomes' number, size, and content (Cho & Hwang, 2012). Sometimes these altered lysosomes accumulate auto-fluorescent materials, as lipofuscins, referred to as "residual bodies" (Georgakopoulou et al., 2013), and they seem to determine a reduced turnover of damaged organelles, including mitochondria, that can lead to an enhanced ROS production, which in turn damages the cell (Hwang et al., 2009). However, the most widely used senescence marker is the increased levels of SA-β-Gal activity (Dimri et al., 1995). SA-β-Gal is a hydrolase enzyme that catalyses the hydrolysis of β-galactosidase into monosaccharides. Usually, its activity has been detected at a pH of 4, but since it is upregulated during senescence, its residual activity can be measured at a suboptimal pH of 6 as a marker of senescence state (Hernandez-Segura et al., 2018; Lee et al., 2006). The primary staining to assess the presence of senescent cells consisting in incubating the cells with a substrate for SA-β-Gal, 5- bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal), which is catalysed into galactose and 5-bromo-4-chloro-3-hydroxyindole-1, which then dimerizes and forms the blue coloured precipitate indigo inside cells cytoplasm (González-Gualda et al., 2021). Therefore, SA-β-Gal activity is used as a surrogate marker for the enhanced lysosomal content of senescent cells.

1.3.1.4 Nuclear changes and senescence-associated heterochromatin foci (SAHF)

The macromolecular alteration also occurs in the nucleus, where heterochromatin organization and associated structural proteins change. In non-senescent dividing cells, heterochromatin is structurally less dense and located at the nuclear periphery. However, in senescence, to silence proliferation-promoting genes, the chromatin rearranges and forms dense structures known as SAHF (González-Gualda et al., 2021), whose formation is promoted by pRB (Narita et al., 2003). These structures, enriched in heterochromatin-forming proteins, such as heterochromatin protein 1 (HP1), histone H3 trimethylated at lysine 9 (H3K9me3), non-histone chromatin protein (HMGA2), and the histone H2A variant macroH2A, incorporate the promoter of E2F, resulting in stable repression of its target genes (Scurr et al., 2017; R. Zhang

et al., 2005). Moreover, senescent cells can also exhibit DNA-SCARS, nuclear structures containing several proteins associated with DDR, including ATM, ATR, and phosphorylated H2A histone family member X (pH2AX) foci, even without the presence of a DNA-damaging agent (Pospelova et al., 2009). All these structures can be identified by immunoblotting or imaging techniques.

1.3.1.5 Metabolic alterations and pro-survival pathways

Senescent cells are metabolically active and increase the import of glucose and the activity of many glycolytic enzymes (Ogrodnik, 2021). Moreover, they showed a reduction in oxidative phosphorylation (OXPHOS) and in NAD^+/NADH ratio, lower content of ATP, and a higher AMP/ATP ratio (James et al., 2015; Nacarelli et al., 2019). AMP prevents AMP-activated protein kinase (AMPK) dephosphorylation, causing its activation. AMPK act as a sensor of the reduced energetic state and activates catabolic pathways, inhibiting the biosynthetic ones (Hernandez-Segura et al., 2018). The increase in glycolysis usually leads to augmented lactate production, but this correlation is unclear in senescent cells. Different studies, indeed, showed that senescent cells do not show a linear relationship between glucose consumption and lactate production (Unterluggauer et al., 2008; Zwerschke et al., 2003). The use of glucose, which is neither used for lactate production nor OXPHOS, in senescent cells is probably employed for an increase in biomass production (Ogrodnik, 2021). An example of this phenomenon can reside in the glycolytic dispensation of carbon for the production of lipid precursors (Lunt & Vander Heiden, 2011), that are accumulated in senescent cells (Ogrodnik, 2021; Ogrodnik et al., 2017).

It should be noted that senescent cells also present changes in mitochondria number and structure. Indeed, mitochondria accumulate in senescence, primarily due to the reduction in their degradation (mitophagy) (Korolchuk et al., 2017), as a mechanism to protect senescent cells from apoptosis (Correia-Melo & Passos, 2015). Furthermore, these mitochondria also present alteration in their structure, grow larger with distinct changes in their crista, and show a decreased membrane potential. Thus, the presence of these abundant and dysfunctional organelles leads to an intensified ROS production (Victorelli & Passos, 2019), which in some cases can also trigger senescence in an autocrine and paracrine manner when released (Nelson et al., 2012). An additional hallmark of senescence, related to mitochondria is the resistance to apoptosis, resulting from the upregulation of pro-survival pathways (González-Gualda et al., 2021). Indeed, senescent cells were found to upregulate anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-xL, Bcl-W, located on mitochondrial membrane and endoplasmic reticulum

(Yosef et al., 2016). The increased expression of these proteins prevents the release of cytochrome C and thus the assembling of the *apoptosome*, the complex structure that activates caspase signalling pathways leading to intrinsic apoptosis (González-Gualda et al., 2021).

1.3.2 The Senescence-Associated Secretory Phenotype (SASP)

Although SASP is too unspecific and heterogeneous to be used as an unequivocal senescence marker, it is the most intriguing one because it is thought to be the reason why senescent cells contribute to tissue homeostasis or dysfunction (González-Gualda et al., 2021). Indeed, the SASP comprises several inflammatory cytokines, chemokines, growth factors, metalloproteases, and extracellular vesicles (EVs) that are secreted from senescent cells, and whose expression vary widely, changes over time, and depends on the duration and/or inducer stimuli of senescence as well as the cell type (Hernandez-Segura et al., 2017; Jakhar & Crasta, 2019). Furthermore, SASP factors include several families of soluble and insoluble factors and can be globally divided into 3 major categories (Coppé et al., 2010):

1. *Soluble signalling factors*, including
    a. *Interleukin*, such as IL-6, IL-1, IL-7, IL-13, and IL-15;
    b. *Chemokines (CXCL and CCL)*, as IL-8 (CXCL-8), GROα and GROβ (CXCL-1 and -2), MCP-2, -3, -4, -1 (CCL-8, -7, -13, -2), CXCL-12;
    c. *Growth factors*, as insulin-like growth factor (IGF), different colony-stimulating factors (CSFs), EGF, HGF, FGF, VEGF;
2. S*ecreted proteases*, as MMP family members, including MMP-1, -2, -3, -9 -10, and serine proteases and regulators of the plasminogen activation pathway (PAI-1 and -2);
3. *Secreted insoluble proteins/extracellular matrix (ECM) components*, as fibronectin.

Although the events that activate the SASP are still poorly understood, we know that they are typically connected to the DDR and induce a transcriptional program required for its generation (Birch & Gil, 2020). Among these several events, we can mention the macromolecular damage sensor, as RIG-1, a sensor for cytoplasmic RNA, which mediates senescence-associated inflammation (Liu et al., 2011), or the inflammasomes (Acosta et al., 2013), that are groups of pattern recognition receptors (PRR) capable of recognizing different damage-associated molecular patterns (DAMPs) and activating IL-1 inflammatory cascade (Schroder & Tschopp, 2010). Furthermore, cytosolic DNA is also an important inducer of SASP, and it is mainly present in senescent cells, as retrotransposable elements like LINE-1, that was found transcriptionally downregulated in late senescent (De Cecco et al., 2019), mitochondrial DNA, and CCFs (Ivanov et al., 2013), that originate from the blebbing of the nuclear membrane

following the loss of Lamin B1 expression. Moreover, cytosolic DNA activates cGAS/STING pathway, considered a key regulator of SASP induction, in which DNA is sensing by the cyclin GMP-AMP synthase (cGAS), that produces cyclin GMP-AMP (cGAMP), activating STING, the stimulator of interferon genes, leading to inflammatory responses (Glück et al., 2017; Yang et al., 2017). Moreover, other pathways such as p38MAPK (Freund et al., 2011), JAK2/STAT3 (Xu et al., 2015), mTOR (Laberge et al., 2015), phosphoinositide-3-kinase (PI3K) (Zhang et al., 2018), HSP90 (Di Martino et al., 2018), GATA4/p62-mediated autophagy (Kang et al., 2015), macroH2A1 and ATM (Chen et al., 2015), are involved in the development and regulation of the SASP, and most of them ultimately converge on the activation of NF-κB and CEBPβ (Kumari & Jat, 2021). **Figure 6** shows all these different mechanisms involved in SASP regulation.

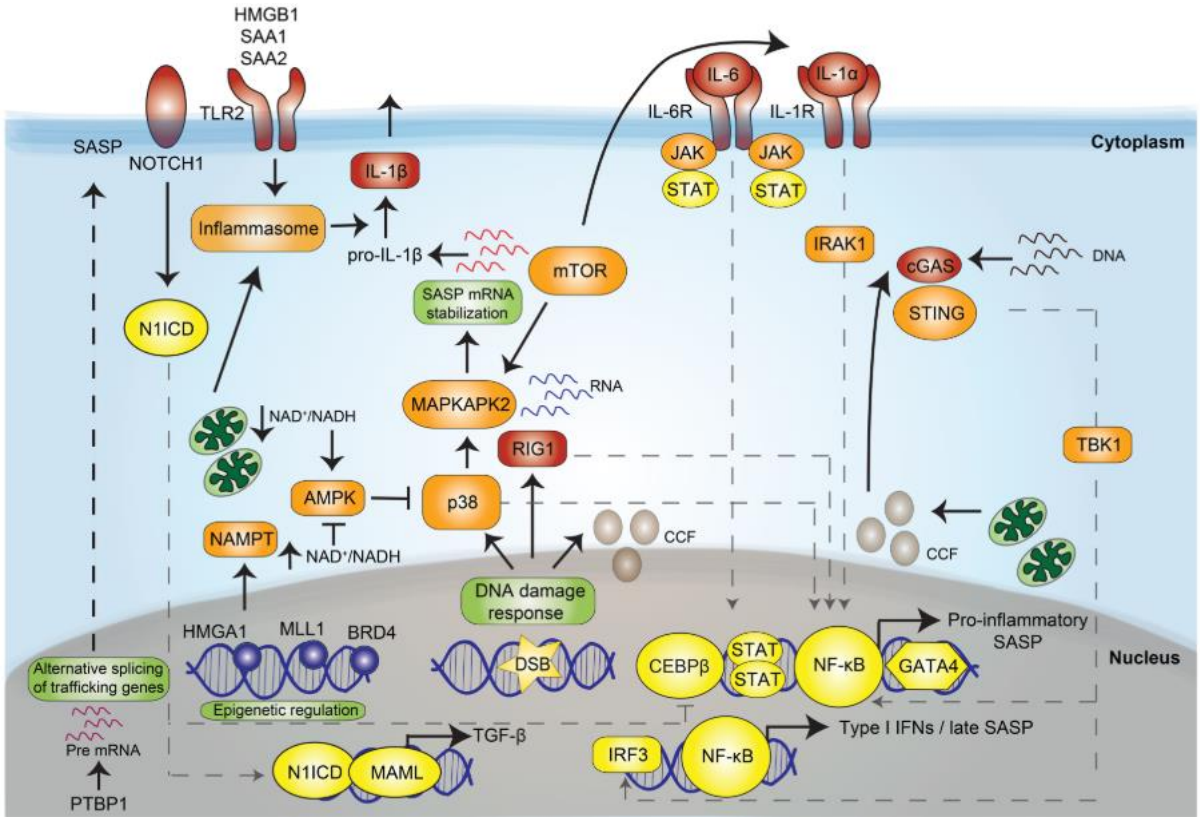

***Figure 6*. The crucial pathways that regulate SASP**. The scheme shows different factors contributing to SASP induction and production. Transcription factors are yellow, intracellular signalling components are orange, and sensors, receptors, and ligands are red. Abbreviations: *CCF*: chromatin cytoplasmic fragments; *DSB*: Double-strand breaks; *SASP*: Senescence-associated secretory phenotype (Birch & Gil, 2020).

NF-κB and CEBPβ, two transcription factors overexpressed in the chromatin fraction of senescent cells, cooperatively control the transcription of crucial SASP proteins, such as IL-1α, IL-6, and IL-8, which in turn can positively regulate NF-κB and CEBPβ activity, forming an

autocrine feed-forward circle that enhances SASP signalling (Kumari & Jat, 2021; Orjalo et al., 2009; Rodier et al., 2009; Zhang et al., 2018). Moreover, as is mentioned above, SASP can also regulate at epigenetic level, through the decrease of H3K9 demethylation (Takahashi et al., 2012) or the increased expression of macroH2A1 (Chen et al., 2015), but also at the post-transcriptional level. mTOR is a key regulator of protein translation in senescent cells (Birch & Gil, 2020), and indeed its inhibition by rapamycin can suppress the secretion of senescent inflammatory cytokines (Herranz et al., 2015; Laberge et al., 2015). mTOR can regulate SASP expression via two mechanisms: (1) promoting the translation of IL-1α, which in turn activates NF-κB and CEBPβ (Laberge et al., 2015), and (2) regulating the translation of MAPKAPK2, a kinase downstream from p38, which in turn phosphorylates and inhibits the RNA-binding protein ZFP36L1 preventing the degradation of mRNA-encoding SASP factors (Herranz et al., 2015). Moreover, its accumulation with lysosomes at the trans side of the Golgi in TOR autophagy spatial coupling compartment (TASCC) involves its activation in the autophagy process, which increases the SASP during OIS (Narita et al., 2011).

Interestingly, a recent study shows that the HMGA-NAMPT-NAD+ signalling axis can influence SASP production (Nacarelli et al., 2019). HMGA1 modifies chromatin structures during senescence, upregulating the nicotinamide phosphoribosyl-transferase (NAMPT) through an enhancer element that promotes SASP in response to an increased NAD+/NADH ratio (Nacarelli et al., 2019). Taken together, the data suggested that an increase in the ratio of NAD+/NADH is capable of enhancing and reinforcing SASP factors production (Kumari & Jat, 2021).

Collectively, the SASP is the characteristic of senescent cells that confers most of its beneficial and detrimental biological effects and therefore is the main instrument through which senescent cells regulate normal physiology and pathology (Kumari & Jat, 2021). The main beneficial and detrimental effects of the SASP are described in the subsequent sections and are shown in **Figure 7**.

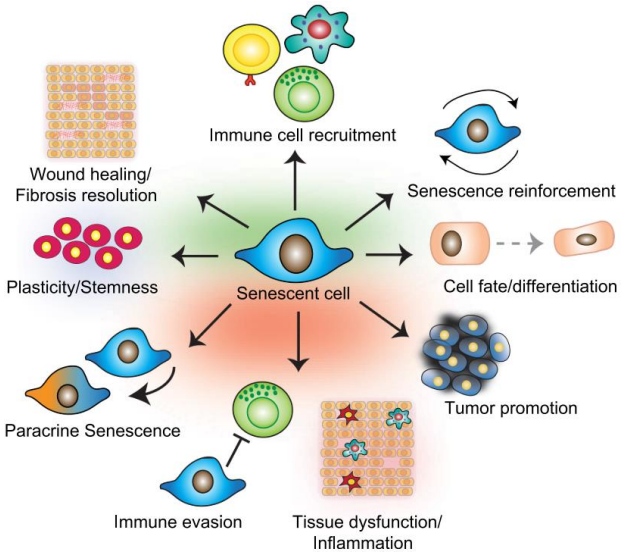

Figure 7. **The pleiotropic effects of the SASP.** The picture summarises the effects exerted by senescent cells (in the middle) through the SASP. The effects in green are beneficial, whereas those in red are detrimental consequences of the SASP (Birch & Gil, 2020).

1.3.2.1 The beneficial effects of the SASP

The composition of the SASP is heterogeneous, and its functions are quite diverse and depend on the cell type, the senescence-induced stimuli, the genetic context of cells exposed to SASP, and the neighbouring environment (Kumari & Jat, 2021). Because the SASP is primarily a genomic damage response (Rodier et al., 2009, 2011), one of its beneficial functions may reinforce senescence in an autocrine and paracrine manner and contribute to tumour suppression, limiting the proliferation of damaged cells, and enhancing the subsequent immune-clearance of the pre-malignant cells (Acosta et al., 2008; Kuilman et al., 2008; Kumari & Jat, 2021; Nelson et al., 2012). Factors involved in these actions include the pro-inflammatory cytokines IL-6 and IL-8, the protease inhibitor plasminogen activator inhibitor-1 (PAI-1), and the pleiotropic protein IGF binding protein-7 (IGBP-7) (Acosta et al., 2008; Kortlever et al., 2006; Kuilman et al., 2008; Wajapeyee et al., 2008). These secreted proteins act by engaging intracellular signalling that activates the tumour suppressor pathways that establish and maintain senescence growth arrest (Campisi et al., 2011). The SASP also includes chemokines and cytokines that can attract and activate IS cells, like natural killer cells, to target and kill senescent cells, thus promoting and facilitating their clearance from tissues (Krizhanovsky et al., 2008; Xue et al., 2007). Moreover, they can communicate cellular damage or dysfunction to surrounding cells and stimulate repair (Rodier & Campisi, 2011). Indeed, in addition to its supporting role in tumour suppression, SASP components play several beneficial roles, such as accelerating wound healing by secreting factors such as platelet-derived growth factor (PDGF)

and cellular communication network factor 1 (CCN1) (Demaria et al., 2014). Moreover, SASP promotes stemness and tissue plasticity to maintain tissue homeostasis (Ritschka et al., 2017), contributing to embryonic development (Storer et al., 2013) and fibrotic scar degradation (Krizhanovsky et al., 2008).

1.3.2.2 The detrimental effects of the SASP

The evolutionary theory of antagonistic pleiotropy explains why a tumour suppressive mechanism with many physiological and beneficial functions can also have deleterious effects, especially late in life (Rodier & Campisi, 2011). Senescent cells accumulate with aging, probably due to failed clearance by IS or increased production due to age-dependent acceleration of tissue damage (Campisi, 2005). These chronic senescent cells secreting several proteins with potent biological activity can alter tissue structure and microenvironment (Rodier & Campisi, 2011). Indeed the progressive accumulation of senescent cells can destroy tissue homeostasis in different manners, including depleting the organism of stem and progenitors cells required for tissue repair and regeneration (Birch & Gil, 2020). For example, the senescence of neural progenitors contributes to age-related decline in neurogenesis (Molofsky et al., 2006), or that of muscle stem cells can be responsible for their age-related decline of regenerative potential (García-Prat et al., 2016). However, these deleterious effects are intrinsic to cells, but in many contests, senescent cells exert their effects in a paracrine manner through the SASP. Around 40% of SASP factors in human plasma are associated with age (Basisty et al., 2020); thus, SASP likely contributes to inflammaging, that along with the increase of immune infiltration, drives the loss of resilience and the risk of developing ARDs (Franceschi & Campisi, 2014). Consistent with this, some studies showed that the clearance of senescent cells could reduce the levels of secreted inflammatory factors known to drive pathologies (Baker et al., 2011, 2016). However, in addition to the systemic level, SASP factors can locally induce tissue dysfunction (Birch & Gil, 2020). For example, in this framework, haemostatic factors, as SERPINs and PAI-1, may link the SASP with Doxorubicin-associated thrombosis in mice (Wiley et al., 2019) or TGFβ1 drives the spreading of senescence during hepatocyte injury decreasing liver regeneration (Bird et al., 2018). SASP produced by senescent bone cells are involved in the induction of osteoporosis, increasing osteoclast progenitor survival and impairing bone synthesis, resulting in an imbalance in bone turnover rate in favour of resorption (Calcinotto et al., 2019).

Moreover, in atherosclerosis, senescence of monocytes and macrophages limits plaques growth, but their secreted SASP factors can also induce the progression of the disease (He & Sharpless,

2017). SASP can also affect neurodegeneration; indeed, pieces of evidence are emerging on the involvement of astrocytes senescence in neuroinflammation and neurodegenerative diseases, like Parkinson's and Alzheimer's diseases (Bhat et al., 2012; Chinta et al., 2018). At last, the increased expression of CCL2 and TNF-α, along with the reduced production of anti-inflammatory adiponectin in senescent adipocytes, leads mice to develop impairments in insulin sensitivity and glucose tolerance, linking senescence and the SASP also to type 2 diabetes (Minamino et al., 2009) (**Figure 8**).

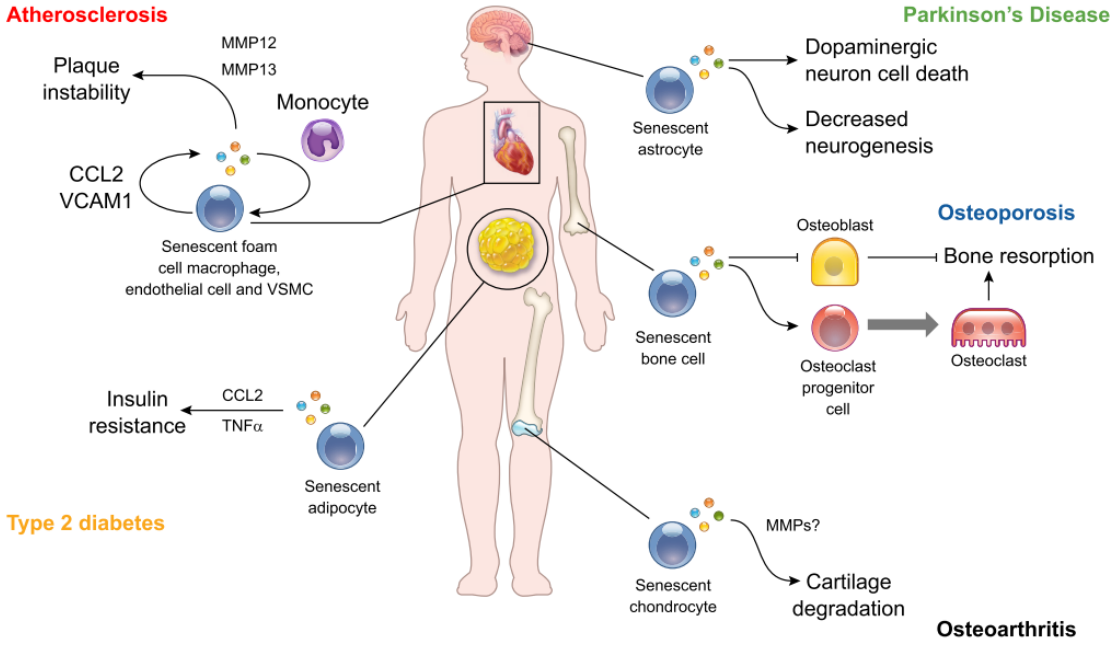

Figure 8. **Senescent cells and SASP effects in age-related diseases.** In atherosclerosis, senescent macrophages secrete CCL2 and VCAM1 to recruit monocytes and convert them into senescent cells. Senescent endothelial and vascular smooth muscle cells secrete MMP12 and MMP13 to promote plaque instability. In osteoarthritis, senescent chondrocytes are involved in cartilage degradation likely via MMPs activity, while in osteoporosis, SASP from senescent bone cells promotes osteoclast progenitor survival and inhibits osteoblast activity, inducing bone resorption. The SASP secreted from senescent astrocytes triggers dopaminergic neuronal cell death and decreases neurogenesis in Parkinson's disease. Senescent adipocytes secrete factors, such as CCL2 and TNFα, promoting insulin resistance in type 2 diabetes. Abbreviations: *CCL2*: C-C motif chemokine ligand 2; *MMP*: metalloprotease; *TNFα*: Tumour necrosis factor α; *VCAM1*: Vascular cell adhesion protein 1; *VSMC*: Vascular smooth muscle cells (Calcinotto et al., 2019).

1.3.3 Senescence and cancer

Nowadays, it is accepted that senescent cells play an essential role in different phases of tumorigenesis, such as in tumour initiation, establishment, progression, and escape.

Oncogene activation in cells can result in proliferative stress and senescence induction, named OIS, limiting tumour growth and inhibiting the progression from benign lesions to malignant tumours (Calcinotto et al., 2019). Several types of oncogene are shown to induce OIS in primary

and tumour cells, including RAS (Courtois-Cox et al., 2008), HER2 (Angelini et al., 2013), EGFR (Garbers et al., 2013), BRAF (Wajapeyee et al., 2008), and MYC (Courtois-Cox et al., 2006). Moreover, also several chemotherapy drugs, such as Docetaxel, Bleomycin, Cyclophosphamide, Doxorubicin, Vincristine, Etoposide, and Cisplatin, can induce the so-called therapy-induced senescence (TIS) in different types of cancer and normal cells (Demaria et al., 2017; Ewald et al., 2010; Saleh et al., 2020). Although the induction of cellular senescence in neoplastic cells is classically considered a tumour defence barrier, several findings show that in certain conditions, these senescent tumour cells can favour tumour progression through the SASP, educating and modifying the microenvironment (Calcinotto et al., 2019; Rodier & Campisi, 2011). In this framework, the SASP acts as a double-edged sword because it can recruit and activate immune cells or induce paracrine senescence in neighbouring cells, acting as a barrier against tumour growth, but it can also promote tumour progression, boosting cell proliferation and driving tumour vascularization (Calcinotto et al., 2019; Coppé et al., 2006). Indeed, although TIS can be initially beneficial in blocking tumour cells proliferation, it also impairs the elimination of senescent cells by IS, leading to their accumulation (Marcoux et al., 2013; Ness et al., 2015). This accumulation of senescent cells adds to what already occurs with age and creates a pro-inflammatory microenvironment favourable for cancer development or progression (Campisi, 2013; Campisi & D'Adda Di Fagagna, 2007). To confirm the critical role of senescence in cancer, an interesting study conducted on a transgenic mouse that enables the inducible killing of senescent cells expressing $p16^{INK4a}$, has shown that eliminating senescent cells reduced spontaneous tumour formation (Baker et al., 2016). This finding could be due to removing incipient senescent tumour cells that might later seed a tumour or eliminating tumour-promoting senescent stromal cells, decreasing the possible stromal support for tumour initiation (Faget et al., 2019). However, the most crucial role in this scenario is performed by the SASP, which provides the neoplastic cells to acquire invasive and metastatic features.

IL-6, one of the principal SASP factors, produced from different models of senescent cells that can support the growth of breast tumours (Di et al., 2014; Zacarias-Fluck et al., 2015), or increase the proliferation of several types of cancer cells, including skin carcinoma (Lederle et al., 2011), Osteosarcoma (C. Zhang et al., 2019), lung (Song et al., 2011), prostate (Rojas et al., 2011), and triple-negative breast cancer cells (Hartman et al., 2013). Other SASP factors can promote tumour growth, as well as GRO-α, which stimulates proliferation in an oesophageal tumour model (B. Wang et al., 2006). MMPs produced by senescent fibroblasts induced early tumour growth in a breast xenograft model and hepatocyte growth factor (HGF), which

increased the proliferation of MDA-MB-231 breast cancer cells (Liu & Hornsby, 2007). Moreover, SASP factors can drive and promote several processes involved in tumour progression and metastasis. Accordingly, vascular endothelial growth factor (VEGF) produced by senescent fibroblasts can drive angiogenesis (Oubaha et al., 2016), promote endothelial cell invasion and increase vascularization (Coppé et al., 2006). Similarly, connective tissue growth factor (CTGF) promoted angiogenesis and tumorigenesis in a mouse prostate model (Yang et al., 2005). In addition, also SASP molecules produced by senescent colorectal cancer cells can trigger EMT in colon and rectal cancer cell lines (Tato-Costa et al., 2016), complementing their ability to induce cancer cell migration and invasion (Aifuwa et al., 2015; Farsam et al., 2016). Some of the most critical SASP factors that can drive metastatic progression are MMPs (Egeblad & Werb, 2002; Malaquin et al., 2013; Qian et al., 2002; Tsai et al., 2005). MMP-1 and MMP-2 secreted by replicative senescent skin fibroblasts activated PAR-1 on tumorigenic keratinocytes, stimulating their invasive activity (Malaquin et al., 2013). Moreover, it is interesting to note that MMPs were found to be overexpressed in aged human skin, which accumulates senescent fibroblasts (Dimri et al., 1995; Ruhland et al., 2016), compared to young skin. Furthermore, these upregulated MMPs were adjacent to PAR-1 positive dysplastic tissue (Malaquin et al., 2013), suggesting that these mechanisms are also active *in vivo* (Faget et al., 2019). Furthermore, also senescent cell-derived ECM remodelling can influence metastasis. For example, the reduced expression of hyaluronan and proteoglycan link protein 1 (HAPLN1) in aged fibroblasts leads to a more aligned ECM, promoting metastasis of melanoma cells by increasing cell motility (Kaur et al., 2020). However, the metastatic potential can also be triggered by senescent tumour cells in neighbouring neoplastic cells, as shown in breast cancer, where senescent cancer cells could promote proliferating breast cancer cells to metastasize (Angelini et al., 2013). The effects of SASP factors can also extend to the pre-metastatic niche. For example, IL-6 secreted by senescent osteoblasts directly stimulated osteoclastogenesis within the pre-metastatic niche and thus facilitates breast tumour cell seeding to the bone (Luo et al., 2016), suggesting that senescent cells can create a "fertile" niche that may facilitate tumour growth in the bone (Faget et al., 2019). Finally, cells induced to senescence by chemotherapy drugs can play a fundamental role in cancer promotion. Interestingly, their elimination prevented or delayed cancer relapse and spread to distal tissues, further eliminating some of the damaging side-off effects of chemotherapy (Demaria et al., 2017).

Overall, senescent cells can exert many different functions in the cancer framework that depend on cell type, senescence-inducing stress, tissues involved, and recipient cells (**Figure 9**). However, the increasing of senescent cells with age, due to either their uncomplete clearance

or their faster generation in aged individuals, can create and sustain inflammaging through SASP factors release, creating a fertile ground for seeding cancer cells.

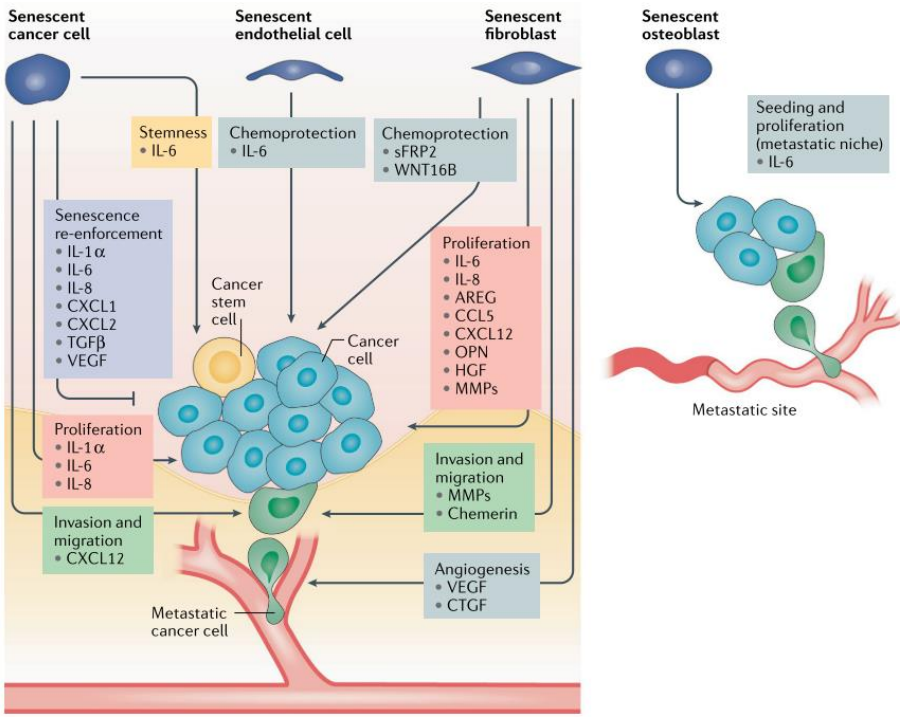

Figure 9. **Functions of SASP factors during tumour initiation and progression.** Senescent cells secrete pro-tumorigenic SASP factors in the microenvironment, regulating tumour growth and progression. Senescence cancer cells produce SASP factors that reinforce senescence arrest. Several molecules secreted from senescent fibroblasts, and cancer cells can stimulate proliferation, migration, and invasion of tumour cells. Additionally, some SASP factors can stimulate angiogenesis in tumour milieu. Molecules secreted from senescent stromal cells, such as fibroblasts or endothelial cells, can induce stemness in cancer cells and offer chemoprotection to tumour cells. Moreover, in metastatic sites, osteoblasts create a pro-metastatic niche supporting the seeding and growth of metastatic cancer cells. Abbreviation: *AREG*: amphiregulin; *CCL*: C-C motif chemokine ligand; *CTGF*: connective tissue growth factor; *CXCL*: C-X-C motif chemokine ligand; *HGF*: hepatocyte growth factor; *MMPs*: matrix metalloproteinases; *OPN*: osteopontin; *sFRP2*: secreted frizzled-related protein 2; *TGFβ*: transforming growth factor β; *VEGF*: vascular endothelial growth factor (Faget et al., 2019).

1.4 Osteosarcoma

Osteosarcoma (OS) is a primary malignant bone tumour with mesenchymal histogenesis, characterized by the deposition of immature bone or osteoid matrix (Durfee et al., 2016). Although OS is the most common primary malignant bone cancer, it is a rare disease and has an annual incidence of 3-4 patients per million (Smeland et al., 2019). It is characterized by a bimodal age distribution (**Figure 10**), with the first peak at 15-19 years of age (8 cases/million/year) and the second at 75-79 years (6 cases/million/year), with middle lower plateau (1-2 cases/million/year) in persons aged 25-59 years (Czarnecka et al., 2020; Rickel et al., 2017; Savage & Mirabello, 2011). Males are more likely to be affected than females, with

a male to female ratio of 1.28:1 in 25-59 age group and 1.43:1 in 0-24 group age. (Klein & Siegal, 2006; Smeland et al., 2019). However, in the elderly group (60+), the worldwide male to female ratio was 1.01:1 (Mirabello et al., 2009b).

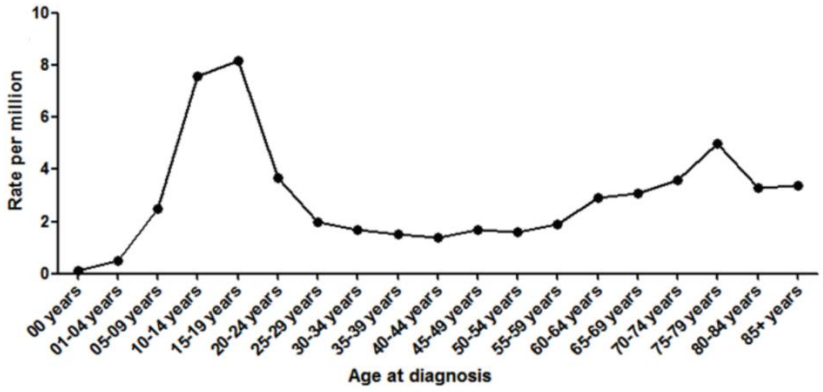

Figure 10. **Osteosarcoma incidence by age.** Rate of osteosarcoma according to the age of diagnosis (Nie & Peng, 2018).

OS can arise in any bone, but it preferentially affects the metaphysis of long bones, such as the femur (42%), tibia (19%), humerus (10%) (Sadykova et al., 2020), especially in young patients. The occurrence of OS in these sites of remarkably rapid growth during adolescence underlines the link between bone growth and OS formation. Indeed, probably due to the high cell turnover during puberty, the metaphyseal area becomes more vulnerable to neoplastic lesions (Savage & Mirabello, 2011). Furthermore, the tendency of OS to occur in the extremity bones decreases with age, and in old patients, other bones were also identified as tumour sites, such as cranial, facial, and axial skeleton, which represent about 40% of all OS in over-60 aged individuals (Sadykova et al., 2020).

Because OS may produce various kinds of extracellular matrix and has a different degree of differentiation, its histologic pattern varies significantly, from case to case, but also from area to area in the same case (Klein & Siegal, 2006). Thus, depending on the tumour's features and predominant stromal differentiation, we can identify six subtypes, i.e., osteoblastic, fibroblastic, chondroblastic, small-cell, telangiectatic high-grade surface, and extraskeletal. Moreover, following the histological appearance, we can distinguish three more categories: (1) high-grade, which includes most of the subtypes; (2) intermediate-grade; (3) low-grade, including periosteal and parosteal ones (Czarnecka et al., 2020; Lindsey et al., 2017). The so-called conventional OS is a high-grade tumour growing intramedullary, and it is the most frequent type (Rickel et al., 2017).

Otherwise, clinically OS can be divided into two stages, localized OS, which only affects the bone and the tissues in which it developed, and metastatic one, which has spread from the original site to other organs (Sadykova et al., 2020). Metastatic disease has a high frequency and occurs in over 80% of patients despite chemotherapy and surgical resection of the primary tumour, principally localizing to the lung (Osasan et al., 2016), and it is considered the first cause of death (Osasan et al., 2016). Furthermore, in older patients, the localization of OS primarily to the axial skeleton, the larger tumour size, and the lower socio-economic status seem to be associated with a higher likelihood of metastatic disease (**Figure 11**) (Miller et al., 2013; Smrke et al., 2021).

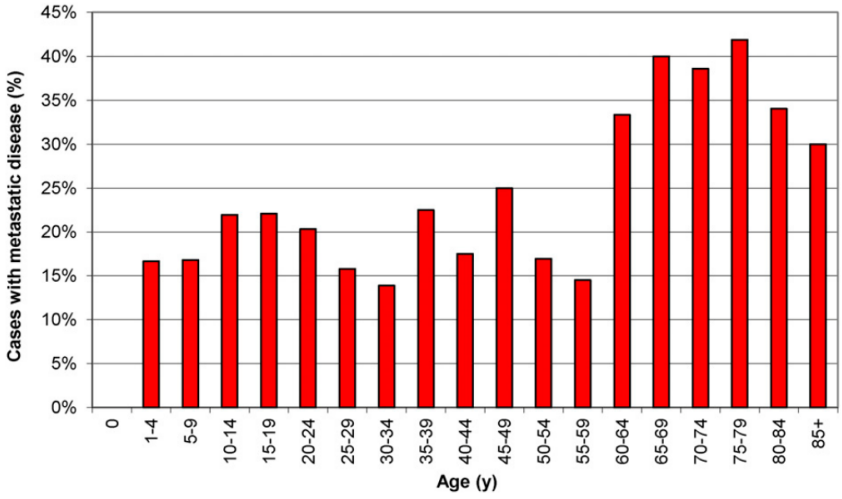

Figure 11. **Percentage of high-grade osteosarcoma cases with distant metastatic disease at presentation according to age at diagnosis.** Data are provided by Surveillance, Epidemiology, and End Results (SEER) database (Miller et al., 2013).

Before the 1970s, patients with OS were treated with surgery alone, with event-free survival (EFS) estimated at 20% (Smrke et al., 2021). Nowadays, the treatment requires a multidisciplinary approach that combines the surgery with preoperative and postoperative multimodal chemotherapy, effectuated with three or four cytotoxic agents, i.e., Cisplatin, Doxorubicin, and high-dose Methotrexate/Ifosfamide (Czarnecka et al., 2020). The introduction of this new approach increased the disease-free survival for high-grade OS patients from 10-20% to 60%. However, although the 5-year survival rate in patients with localized OS reaches 70-75%, the long-term survival for metastatic disease is only 30% (Rickel et al., 2017). Tumour metastasis is primarily responsible for the stagnation in developing new therapeutic targets for OS. Thus, the scientific community is engaged in the search for new targeted

therapeutic strategies that can improve the efficacy of the treatment, especially for metastatic disease, and reduce its toxicity.

In OS, as in other types of cancer, the multiple cell-cell interactions between OS cells and tumour stromal cells are important for tumour development and progression. OS tumour niche is constituted by OS cells, bone cells, including osteocytes, osteoclasts, and osteoblasts, stromal cells, such as fibroblasts and mesenchymal stromal cells (MSCs), endothelial progenitors and cells, pericytes, IS cells, including monocytes, macrophages, and lymphocytes, and ECM components (Czarnecka et al., 2020). All these components interact to promote tumour growth and dormancy, invasion and metastasis, and resistance to therapy (**Figure 12**). These effects are mediated in part by stromal cells secreted multiple cytokines, growth factors, and chemokines, as well as their receptors, which promote, in an autocrine and paracrine manner, cell division and differentiation of OS cells, but also of osteoblasts, MSCs, and endothelial cells (Corre et al., 2020). One example is represented by OS intratumoral IL-6, IL-8, CXCL12, CCL5, and VEGF, which promote tumour growth and angiogenesis, as well as the metastatic spread (Kawano et al., 2018; Zheng et al., 2018). In particular, CXCL12/CXCR4 pathway was identified as one of the most important actors in promoting OS metastasis, along with overexpression of ezrin and MET and induction of Src-family tyrosine kinase (SFK) (Czarnecka et al., 2020). Since metastatic disease is the first cause of death for OS patients, metastasis development mechanisms are all considered potential therapeutic targets.

Thus, the objective of the scientific community is nowadays to investigate the components of OS niche and the processes that lead to metastasis formation, recurrence, and therapy resistance, to improve new therapeutical strategies, or to find new ones that can ameliorate OS prognosis.

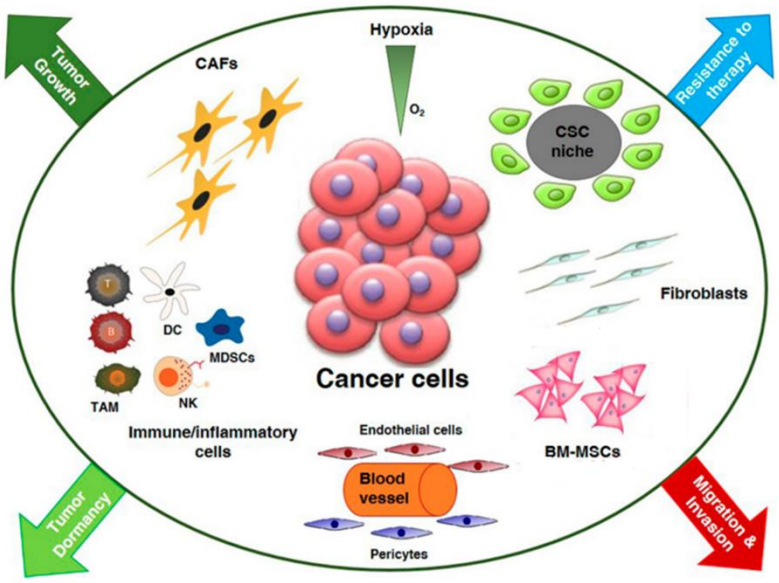

Figure 12. **OS microenvironment.** Cancer cells in the primary tumour are surrounded by a complex microenvironment that comprises several stromal cell types that cooperate to promote tumour dormancy, growth, resistance to therapy, and metastasis. Abbreviations: *BM-MSCs*: Bone marrow-derived mesenchymal stem cells; *CAFs*: Cancer-associated fibroblasts; *CSC*: Cancer stem cell; *DC*: Dendritic cells; *MDSCs*: Myeloid-derived-suppressor cells; *NK*: Natural killer cells; *TAM*: Tumour-associated macrophages (adapted from Pelagalli et al., 2016).

1.5 Senotherapeutics

Nowadays, the scientific community well knows the involvement of cellular senescence in aging and several types of ARDs. Genetic models enabling the specific elimination of senescent cells demonstrated that their selective ablation could delay age-related phenotypes in adipose tissue and muscle (Baker et al., 2011), and even more exciting could increase the median lifespan and healthspan, attenuating age-related functional and structural deterioration of multiple organs, without any detrimental side effects (Baker et al., 2016). Subsequently, several studies with a transgenic mouse model have proven that the genetic ablation of senescent cells improved age-related lipodystrophy (M. Xu, Palmer, et al., 2015), hepatic steatosis (Ogrodnik et al., 2017), age-related cardiac function, and bone loss (Farr et al., 2017), and tau-mediated cerebral pathologies (Bussian et al., 2018). Thus, in recent years, many pharmacological interventions targeting cellular senescence, named senotherapeutics, have been proposed and are classified as *senolytics* that selectively kill senescent cells, *senomorphics*, which modulate senescence blocking the SASP, and *senoinflammation*, referred to the IS-mediated clearance of senescent cells (**Figure 13)** (E. C. Kim & Kim, 2019).

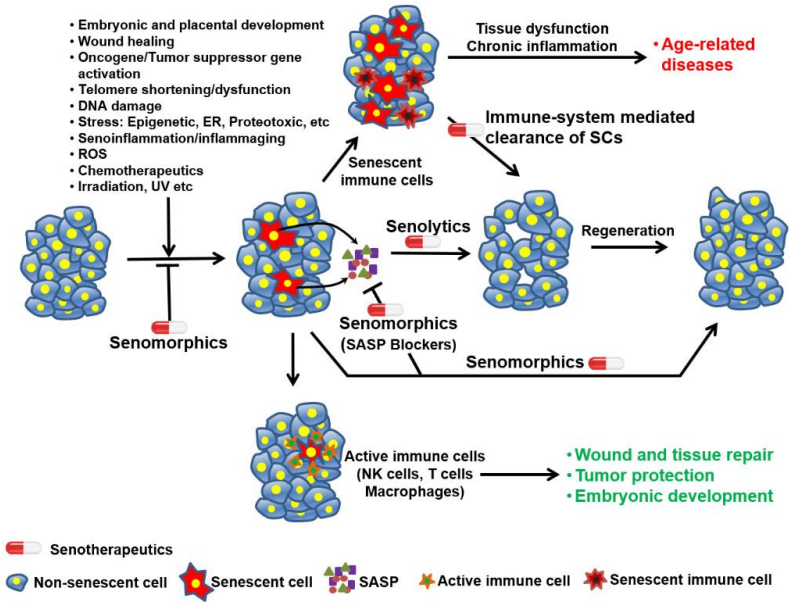

***Figure 13*. Senotheraputics targeting senescent cells.** Different factors can induce a senescence state that is involved in many biological processes, such as embryonic development, tissue homeostasis, and dysfunction, and that can contribute to aging and ARDs. Senotherapeutics comprise senolytics, which selectively kill senescent cells, senomorphics which modulate or reverse the senescent phenotypes by interfering with senescence triggers or blocking SASP factors production/secretion, and mediators of IS clearance of senescent cells. Abbreviations: *ER*: endoplasmic reticulum; *NK*: natural killer; *ROS*: reactive oxygen species; *SASP*: Senescence-associated secretory phenotype; *SCs*: senescent cells; *UV*: ultraviolet ray (E. C. Kim & Kim, 2019).

1. *Senolytics*. Resistance of senescent cells to apoptosis and cell death due to the upregulation of Bcl-2 and Bcl-xL suggested the inhibitors of these anti-apoptotic proteins as valuable candidates for senolytics. In this framework, several molecules, as ABT-263 (Navitoclax), ABT-737, A1221852, and A1155463, were tested *in vitro* and *in vivo* for their senolytic activity (E. C. Kim & Kim, 2019). ABT-263 binds the inhibitory domain of Bcl-2, Bcl-xL, and Bcl-W, eliminating different types of senescent cells (J. Chang et al., 2016) and decreasing viability of senescent human umbilical vein epithelial cells (HUVECs) and human lung fibroblasts, but not of primary human preadipocytes (Zhu et al., 2016). ABT-737 was able to selectively kill *H-Ras*-, etoposide- or replicative-induced senescent fibroblasts *in vitro* and cleared lung epithelial cells in a mouse model, increasing hair follicle stem cell proliferation (Yosef et al., 2016). Moreover, A1331852 and A1155463, selective Bcl-xL inhibitors, induced apoptosis of irradiation-induced senescent HUVECs, fibroblasts, but not preadipocytes (Zhu et al., 2017). Other novel candidates to senolytics are compounds involved in autophagy regulation, as inhibitors of HSP90, which were shown to induce apoptosis in different types of senescent cells *in vitro* (Fuhrmann-Stroissnigg et al., 2017). Finally, also the co-administration of Dasatinib and Quercetin seems to exert prominent senolytic activities, but these will be discussed in Section 1.5.1;

2. *Senomorphics.* Senomorphics are a wide range of agents that can modulate the phenotypes of senescent cells, interfering with senoinflammation/inflammaging, senescence-related signal pathways, and SASP, without inducing apoptosis in senescent cells (E. C. Kim & Kim, 2019). This category includes well known anti-aging compounds, such as telomerase activator (P. Liu et al., 2017), calorie restriction mimetics (CRMs) (Roth & Ingram, 2016), caloric restriction diets (Wei et al., 2017), sirtuins activator (Hubbard & Sinclair, 2014), mTOR inhibitors (Lamming et al., 2013), antioxidants (Si & Liu, 2014), anti-inflammatory agents (Soto-Gamez & Demaria, 2017), and autophagy and proteasome activators (Chondrogianni et al., 2015; Hansen et al., 2018). Furthermore, novel substances which exhibit their senomorphic activity targeting selective markers of cellular senescence, as the SASP, are emerging. Indeed, a main strategic plan of potential therapies was targeting the pathways related to SASP expression, including p38MAPK, PI3k/Akt, JAK/STAT, and transcription factors, as NF-κB and CEBPβ (Lagoumtzi & Chondrogianni, 2021). An example is a peptide inhibitor of IKK, the NF-κB activating kinase, that was able to reduce cellular senescence *in vitro* and delay age-related symptoms and pathologies in a progeroid mouse (Tilstra et al., 2012), and inhibitors of JACK that could suppress senoinflammation alleviated age-related tissue dysfunction (M. Xu, Tchkonia, et al., 2015). Instead, another approach is based on neutralising the activity and function of specific SASP factors, such as IL-1α, IL-6, and IL-8, with specific antibodies (von Kobbe, 2019).

However, among all these molecules, the research is focalized especially on those natural substances beneficial for health, such as nutraceutical compounds, which can favour the so-called “healthy aging”.

Flavonoids are polyphenol compounds, generally ubiquitous in Plant Kingdom (Simioni et al., 2018) that have been demonstrated to provide many health benefits and influence exercise performance for athletes and older people (Fusco et al., 2007). This group includes flavonols (Quercetin), flavones (Luteolin), flavanones (Naringenin), anthocyanidins (Cyanidin), and isoflavones (Genistein) (Simioni et al., 2018). In addition, some of these compounds exert an anti-inflammatory action (Middleton et al., 2000) and inhibit the chronic low-grade inflammation (Csiszar et al., 2012; Ding et al., 2010), downregulating the expression of pro-inflammatory molecules, as IL-6 and TNF-α, *via* blocking the inflammatory pathways (Kim et al., 2004). Due to these abilities, they are excellent candidates to exert senomorphic action, particularly inhibiting the SASP. Indeed, recent findings showed that many naturally occurring flavonoids, such as kaempferol, apigenin, and wogonin, can suppress cellular senescence and its associated secretory phenotype (Bian et al., 2020; Velarde & Demaria, 2016).

1.5.1 Quercetin

Quercetin (3,3',4',5,7-pentahydroxylflavone) is a natural bioactive flavonoid found in various cultivated plants and derived foods, such as nuts, grapes, onions, broccoli, apples, and black tea (Simioni et al., 2018). This flavonoid is known to exert a valuable antioxidant activity (Wu et al., 2017) due to its chemical structure, particularly to the presence and position of the hydroxyl (-OH) groups (**Figure 14)**, responsible for the radical scavenging mechanism that protects against ROS injury (Santos & Mira, 2004).

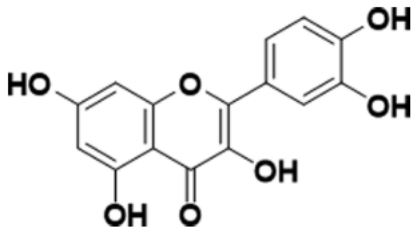

Figure 14. **Chemical structure of Quercetin** (Salehi et al., 2020)**.**

Moreover, it is also known for its anticancer (Hashemzaei et al., 2017) and cardioprotective activity (Bartekova et al., 2016). Its actions are also known in the framework of senescence. Indeed, Quercetin has been shown to extend the cellular lifespan of human primary fibroblasts (HFL-1) and to promote a rejuvenated phenotype when they become senescent (Chondrogianni et al., 2010), as well as to extend organismal lifespan in *C. elegans* (Kampkötter et al., 2008). Nevertheless, the most investigated action of Quercetin in senescence is its senolytic activity. In recent years its combination with Dasatinib (D+Q), a multityrosine kinase inhibitor, has been tested in multiple *in vitro* and *in vivo* models (Lagoumtzi & Chondrogianni, 2021). D+Q drove to senolysis senescent HUVECs, and mouse bone marrow-derived mesenchymal stem cells (BM-MSCs), inducing apoptosis mainly through PI3k/Akt and p53/p21/serpine pathways. Conversely, their activity was not effective against senescent human preadipocytes (Zhu et al., 2015). Moreover, D+Q administration was shown to reduce senescent cells burden in aged, radiation-exposed and progeroid mice, improving healthspan parameters, as cardiovascular and physical function (M. Xu et al., 2018), and improving vasomotor function in aged and hypercholesterolemic mice (Roos et al., 2016), lung function in a mouse model of idiopathic pulmonary fibrosis (Schafer et al., 2017), and in a mouse model of hepatic steatosis (Ogrodnik et al., 2017). Furthermore, in a mouse model of osteoporosis, the co-administration of Dasatinib and Quercetin reduced p16 mRNA levels in bones and reduced the number of senescent

osteocytes (Farr et al., 2017). Due to the *in vitro* and *in vivo* research's success, the combination D+Q has already been inserted in clinical trials as a senolityc agent in idiopathic pulmonary fibrosis (Justice et al., 2019).

Interestingly, Quercetin has also been suggested to act as a senomorphic agent since it was shown to attenuate intervertebral disc degeneration (IDD) progression through Nrf2-mediated suppression of NF-κB (Shao et al., 2021). However, this potential activity of Quercetin has yet to be deeply investigated.

2 AIMS OF THE STUDY

Aging is a complex biological phenomenon that represents the major risk factor for the development of age-related diseases (ARDs). Among the processes involved in aging and ARDs, cellular senescence is one of the most intriguing. Cellular senescence is defined as a multi-step process that different stimuli can induce. The senescent cells undergo an irreversible cell cycle arrest and develop several modified features, along with a multi-faceted senescence-associated secretory phenotype (SASP). The demonstrated chronic accumulation of senescent cells during aging and their finding in tissues of patients affected by ARDs, suggest that they can have a detrimental role. The SASP factors production mainly mediates the "dark side" of cellular senescence. Indeed, the secretion of a vast plethora of proteins with potent biological activities can fuel both local and systemic inflammation, contributing to the inflammaging and the development and progression of some ARDs, such as atherosclerosis, Alzheimer's disease, osteoarthritis and cancer.

Furthermore, the SASP includes factors promoting cancer cell proliferation, invasion, and metastasis. In addition, it was demonstrated that some common chemotherapy drugs, such as Doxorubicin, that successfully kill cancer cells could also induce a senescence response in both tumour and normal cells and fuel the development of metastatic phenotype in residual living tumour cells. Thus, in recent years, one of the main goals of the research to eliminate the detrimental effects of senescent cells is to find molecules, especially natural substances beneficial for health, that can selectively eliminate senescent cells (*senolytic* effect) or can block the SASP (*senomorphics* effect).

The first aim of this project was to verify the ability of Doxorubicin, a chemotherapy drug used in the treatment of several types of tumours, including Osteosarcoma, in inducing senescence in normal human fibroblasts *in vitro.* Fibroblasts commonly belong to cancer stroma, and the treatment with chemotherapy could transform them into cancer-associated fibroblasts (CAFs) that favouring malignant transformation, contributing to therapy failure and metastatic spread.

The second aim of the study was to test the ability of Quercetin, a natural bioactive flavonoid, in preventing Doxo-induced senescence and in reducing SASP factors production, decreasing the possible damaging effects of senescent cells.

Accordingly, the third aim of the study was to verify if Doxo-induced senescent fibroblasts could promote cancer cells through the expression of the SASP and if Quercetin pre-treatment could mitigate these effects. As cancer model, we chose to study Osteosarcoma, a primary malignant bone tumour in which stromal cells perform a fundamental role. Osteosarcoma

presents a high mortality rate due to metastatic spread and therapy toxicity, also driven by Doxorubicin, one of the common chemotherapy drugs used in the treatment.

Interestingly, Osteosarcoma incidence is characterized by bimodal age distribution, with the first peak in puberty, and the second peak in elderly patients, which showed a high tendency to present metastatic disease and worse prognosis. Therefore, we hypothesized that senescence induced by Doxorubicin as an off-target effect in stromal fibroblasts could act as a pro-tumorigenic mechanism, especially in elderly patients that already present a high number of senescent cells and an inflammaging status. In this context, we aimed to investigate the effects of conditioned medium from Doxo-induced senescent fibroblasts on Osteosarcoma cells growth and invasiveness. Moreover, we verified if Quercetin pre-treatment, reducing the onset of Doxo-induced senescence and the SASP, could limit their deleterious effects.

Finally, we evaluated if the conditioned medium from Doxo-induced senescent fibroblasts pre-treated or not pre-treated with Quercetin exerted similar effects (augment of cell growth and migration) in normal cells like the fibroblasts.

Furthermore, in the last section of the project, we have evaluated a post-treatment with Quercetin after the induction of senescence by Doxorubicin to analyse the potential senolytic action of the flavonoid. Finally, we have evaluated whether the post-treatment with Quercetin of Doxo-induced senescent fibroblasts could modify their pro-tumorigenic influence on Osteosarcoma cells.

3 MATERIALS AND METHODS

3.1 Reagents

Doxorubicin (#5927S Cell Signaling Technology) was dissolved in Dimethyl Sulfoxide (DMSO, D8418, Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 10 mM and stocked at -20°C until use. Quercetin (purity ≥ 95%, Q4951) powder was purchased from Sigma-Aldrich and was resuspended in DMSO to a concentration of 30 mg/mL (99 mM). 5-Bromo-4-Chloro-3-Indolyl B-D-*Galactop B4252 was purchased from Sigma-Aldrich.

3.2 Cell lines and culture conditions

Human fetal lung fibroblasts WI-38 (AG06814-N, Coriell Institute for Medical Research, Camden, New Jersey), and human OS cell line U2OS (pRB +/+, p53+/+) (HTB-93, ATCC, Manassas, VA, USA) were grown in complete medium (DMEM HIGH supplemented with 10% of heat-inactivated FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 0.25 µg/mL Amphotericin B, and 2mM L-Glutammin) at 37°C in 5% CO_2 humidified incubator. WI-38 cells with Population Doubling Level (PDL) <30 were used in this study to avoid replicative senescence.

3.3 Induction of senescence and Quercetin treatments

Before starting experiments, an adequate number of WI-38 cells ($3x10^4$ for well) were plated for 24h in complete medium at 37°C, in a 5% CO_2 humidified incubator. Subsequently, WI-38 cells were treated with Doxorubicin 50 nM for 48h to induce senescence, and then cells were cultured in fresh complete medium for 3 days. The control was represented by cells incubated for 48h in complete medium with 0.0005% of DMSO (used as diluent for Doxo) and then maintained in complete fresh medium for the following 3 days. At this time, senescence markers were analyzed.

WI-38 cells were pre-treated in complete medium with Quercetin 40 µM for 24h, then washed and exposed to Doxo-induced senescence for 48h and then cultured for 3 days. Control samples were represented by WI-38 cells treated with DMSO (C) or Quercetin (Q) for 24h and then cultured in fresh complete medium.

To test the senolytic activity of Quercetin, WI-38 cells were exposed to Doxorubicin for 48h and then incubated in fresh complete medium for 72h to induce senescence; finally, cells were treated with Quercetin 40 µM for 3 days. Control samples were represented by WI-38 cells treated only with DMSO (C) or Quercetin (Q) for 3 days.

3.4 Conditioned medium preparation and treatment of U2OS and WI-38 cells

WI-38 cells after the pre-treatment with Quercetin (24h) were incubated for 48h with Doxo, then washed and cultured for 3 days with fresh complete medium. The supernatant was collected, centrifuged and frozen at -80°C.

To collect conditioned medium (CM) from Quercetin post-treated cells, WI-38 fibroblasts were treated with Doxo for 48h, then washed and cultured 3 days in fresh complete medium to induce senescence. Then, cells were treated with Quercetin for 3 days, washed, and incubated for the following 3 days in fresh complete medium. At the end, the supernatant was collected, centrifuged and frozen at -80°C.

CM was used to treat U2OS and WI-38 cells.

3.5 Cell proliferation

Trypan Blue exclusion assay was used to determine cell growth and vitality. Briefly, WI-38 cells or U2OS cells were trypsinized and stained with Trypan Blue. Then, live cells and dead cells were counted using a Bürker chamber under a light microscope.

3.6 Senescence-Associated β-Galactosidase Staining

SA-β-Gal staining was performed as described previously (Dimri et al., 1995). WI-38 were plated $8x10^3$ cells/cm^2 and assessed for SA-β-Gal activity at various times after the different treatments. A minimum of 300 cells for each group was counted. Positive (blue) cells were expressed as a percentage of the total cell number.

3.7 Flow-cytometric analysis of cell-cycle

Cell cycle analysis was performed at different times with Propidium Iodide staining. Briefly, WI-38 cells were trypsinized, counted, and aliquoted in FACS tubes. The cells were washed in PBS 1X and then incubated in a hypotonic solution of Propidium Iodide (0.1% w/v Sodium citrate tribasic, 0.1% Triton X-100, and 50 µg/mL Propidium Iodide) at 4°C for 30 min. The cell cycle was analysed using FACS CANTO II and BD FACSDIVA Software (Becton, Dickinson and Company). The data were analysed using FlowJo Software.

3.8 Western blotting

The whole-cell lysate was obtained using Laemmli Buffer. Protein concentration was determined using BCA Protein assay kit (ThermoFisher Scientific). 50 µg of protein per sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

blotted to nitrocellulose membrane (Amersham Protran, GE Healthcare Life Science) by electroblotting. After being blocked with Bovine Serum Albumin (BSA) 5%, the membrane was incubated with primary antibody against phospho(Ser807/811)-RB (#8516, Cell Signalling Technology), Lamin B1 (ab16048, Abcam), p21 (sc-817, Santa Cruz Biotechnology), NF-κB p65 (#4764, Cell Signalling Technology), SOD-1 (GTX100659, Genetex), SOD-2 (ADI-SOD-110, Enzo Life Sciences) and α-tubulin (sc-32293, Santa Cruz Biotechnology), respectively, at 4°C overnight. Washed membranes were incubated for 1h at room temperature with IRDye800CW–conjugated or IRDye680–conjugated secondary antibody (LI-COR Biosciences) and antibody-coated bands were visualised by Odyssey Infrared Imaging System Densitometry (LI-COR Biosciences).

3.9 Immunostaining

WI-38 cells were plated on sterile coverslips placed insight 12-well plates, and the treatments were performed as described above. At the end of experiments, cells were fixed in PFA 4% for 10 min or in iced Methanol 100% at -20°C for 15 min, permeabilised with 0.2% Triton X-100 for 5 min, quenched with Sodium Borohydride, washed, and blocked for 1 h with 1% BSA 10% horse serum at room temperature. Slides were incubated overnight at 4°C with primary antibodies in BSA 1% (p21: sc-817, Santa Cruz Biotechnology; Lamin B1: #68591, Cell Signalling Technology; Histone Macro H2A.1: ABE215, Merck Millipore; pH2AX: #9718, Cell Signalling Technology). Slides were washed three times and incubated at room temperature for 40 min with Alexa Fluor 488, or cy3 labeled fluorescent conjugated antibodies (goat anti-rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Thermofisher Scientific). Nuclear DNA was stained with ProLong™ Gold Antifade Mountant with DAPI (Thermofisher Scientific). Slides were visualised using Leica SP8 Confocal Microscope.

3.10 ROS assessment by Flow Cytometry Analysis

ROS production was assessed in WI-38 fibroblasts after 24 and 48h of Doxo-treatment. Briefly, cells were trypsinised, washed with PBS1X, and incubated with H2DCF-DA 2.5 μM (Invitrogen, Carlsbad, CA, USA) in DMEM high glucose without serum and phenol red for 30 minutes at 37°C 5% CO_2. After labelling, the samples were examined immediately using FACS CANTO II flow cytometer, and the data were analysed using FACSDiva software (Becton-Dickinson, San Jose, CA, USA).

3.11 Total Antioxidant Capacity (TAC) Assay

The oxygen radical absorbance capacity (ORAC) method was performed as previously described (Emmi et al., 2019; Sofi et al., 2018) on WI-38 fibroblasts protein lysates extracted 24h after Quercetin treatment. Briefly, fluorescein solution (6 nM) was prepared in 75 mM sodium phosphate buffer (pH 7.4), and Trolox (250 µM) was used as standard. 100 µL of fluorescein was pre-incubated with 70 µL of each sample for 30 minutes at 37°C, in each well, before rapidly adding AAPH solution (19 mM final concentration). Fluorescence was then measured using Synergy H1 microplate reader (BioTek, Winooski, VT), and the results were expressed as Trolox Equivalents (µM) and then normalized for protein concentration.

3.12 Evaluation of cells death/apoptosis

Apoptosis was evaluated by flow cytometric analysis with PE Annexin V Apoptosis Detection Kit I (BD Pharmigen^{TM}) following manufacturer's instruction. Prior to beginning, 10X Annexin V Binding Buffer (containing 0.1 M Hepes/NaOH pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) was diluted to a working 1X solution with distilled water. Then, cells were trypsinized, counted, aliquoted in FACS tubes, and washed once with PBS 1X. The cells pellet was resuspended in 100 µL of 1X Binding Buffer, 5 µL of PE Annexin V and 5 µL of 7-Amino-Actinomycin (7-AAD) were added. Tubes were incubated for 15 minutes at room temperature in the dark. At the end, 400 µL of 1X Binding Buffer was added to each tube. Samples were analysed using FACS CANTO II flow cytometer. Cells that resulted positive only for Annexin V staining were considered early apoptotic, cells positive for both Annexin V and 7-AAD were considered in late apoptosis state, and cells positive only for 7-AAD staining were considered necrotic. The cells negative for each staining were considered alive.

3.13 Wound healing assay

The wound-healing assay was performed to evaluate U2OS and WI-38 cell migration under the effect of CM. U2OS and WI-38 cells were plated on 24 well plates 72h before the treatment. A vertical line was scratched on adherent cell monolayer in the center with a sterile 200 µl micropipette tip. Then, cells were washed with PBS 1X and incubated with CM. Wound closure was monitored at 6, 24, 48 hrs. The scratch closure area was measured using ImageJ software.

3.14 Invasion assay

U2OS cells invasiveness was evaluated using Boyden chamber assay. The method is based on the passage of the cells across 8 µm pore size polyvinyl pyrrolidone (PVP)-free polycarbonate filters (Neuro Probe, Inc., USA) precoated with Matrigel (50 µg/filter), placed between the two

wells of the Boyden chamber. CM from Doxo-treated fibroblasts (SM), Quercetin pre-treated Doxo-treated fibroblasts (QSM), Quercetin treated fibroblasts (QM), and control cells (CM) were placed in the bottom wells of the chamber. U2OS cells resuspended in complete DMEM HIGH supplemented with 2% FBS were seeded in the upper chamber ($2x10^4$ cells/well) and incubated overnight. The filters were removed and fixed in Methanol for 1 h. Non-migrated cells attached to the upper surface of the filter were scraped, while migrated cells, adherent on the lower filter surface, were stained with Crystal violet 0.01% and counted using a light microscope (40x magnification). Each point was performed in triplicate, and mean values of migrated cells for each point were calculated and expressed in relation to control.

3.15 Colony formation assay

U2OS colony formation under treatment with CM was evaluated as follows: $2x10^3$ cells were seeded into a 60 mm cell culture dish in complete medium, and after 72h, the medium was removed, and the cells were incubated with the different conditioned media for 7 days. Then, cells were washed with PBS 1X and stained with 0.1% Crystal Violet for 10 minutes. After washing twice with water, the dishes were left to dry overnight. The next day, a picture of the whole plates was taken, and the number and area of colonies were evaluated by ImageJ software.

3.16 RNA extraction and quantitative real-time PCR

Total RNA extraction was performed 3 days after Doxo treatment with Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada) following the manufacturer's instruction. cDNA synthesis was carried out using ImProm-IITM Reverse Transcription System (Promega Corporation, Madison, WI, USA), and quantitative real-time PCR (qPCR) was performed using GoTaq® qPCR Master Mix (Promega). For primer sequances see **Table 1**. The qPCR analysis was carried out in triplicate using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Gercules, CA, USA). mRNA was quantified with the ΔΔCt method, and mRNA levels were normalised to GAPDH as endogenous control.

Gene	Forward Primer	Reverse Primer
IL-6	5'-AGACAGCCACTCACCTCTTCAG-3'	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
IL-8	5'-GAGAGTGATTGAGAGTGGACCAC-3'	5'- CACAACCCTCTGCACCCAGTTT-3'
GROα	5'- AGCTTGCCTCAATCCTGCATCC-3'	5'- TCCTTCAGGAACAGCCACCAGT-3'
IL-1α	5'- TGTATGTGACTGCCCAAGATGAAG-3'	5'-AGAGGAGGTTGGTCTCACTACC-3'
MMP2	5'- AGCGAGTGGATGCCGCCTTTAA-3'	5'- CATTCCAGGCATCTGCGATGAG-3'
CXCL12	5'- CTCAACACTCCAAACTGTGCCC-3'	5'- CTCCAGGTACTCCTGAATCCAC-3'
TNF-α	5'- CTCTTCTGCCTGCTGCACTTTG-5'	5'- ATGGGCTACAGGCTTGTCACTC-3'
GAPDH	5'- GTCTCCTCTGACTTCAACAGCG-3'	5'- ACCACCCTGTTGCTGTAGCCAA-3'

Table 1. Primer Sequences used for qPCR evaluation of the indicated gene expression.

3.17 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean ± SD calculated on at least three independent experiments. P values were calculated using the Student's t-test or one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

4 RESULTS

4.1 Section I

In the first part of this work, we focused on senescence induction in WI-38 human fetal lung fibroblasts by the chemotherapy drug Doxorubicin. After several experiments with different doses and times of treatment (data not shown), we induced the senescence treating the cells with the dose of 50 nM for 48h, followed by incubation in fresh complete medium for 72h, to permit the development of senescence. Our results showed that after Doxo treatment, fibroblasts undergo proliferative arrest and accumulate in G2/M phase of cell cycle. Moreover, they showed the typical senescence features, such as flattened and enlargened morphology, increased SA-β-Gal activity and SAHF formation, upregulated expression of p21, and decreased phospho-RB and Lamin B1 expression. Thus, the results obtained in this part confirm that Doxorubicin can induce cellular senescence in normal human fibroblasts.

4.1.1 Doxorubicin treatment-induced senescence in WI-38 fibroblasts

Doxorubicin-induced senescence was used as a model of therapy-induced senescence (TIS). To check the induction of senescence, we analysed some senescence markers at 72h after the treatment for 48h with Doxo 50 nM (**Figure 15A**). Our results showed that WI-38 cells after 48h of Doxo treatment significantly decreased their growth compared to control (C), and the proliferation arrest was maintained even for the following 72h (**Figure 15B**). Cytofluorimetric analysis of cell-cycle showed that Doxo-treated cells exhibited a significantly predominant block in G2/M phase at both times (67,4% and 71,5%, respectively), compared to the control (21,7% and 16,6%, respectively) (**Figure 15C**). Concurrently, 72h after Doxo-treatment, we observed that cells were enlarged in size, and at least 60% were positive for SA-β-Gal activity, compared to control cells (**Figure 15D**).

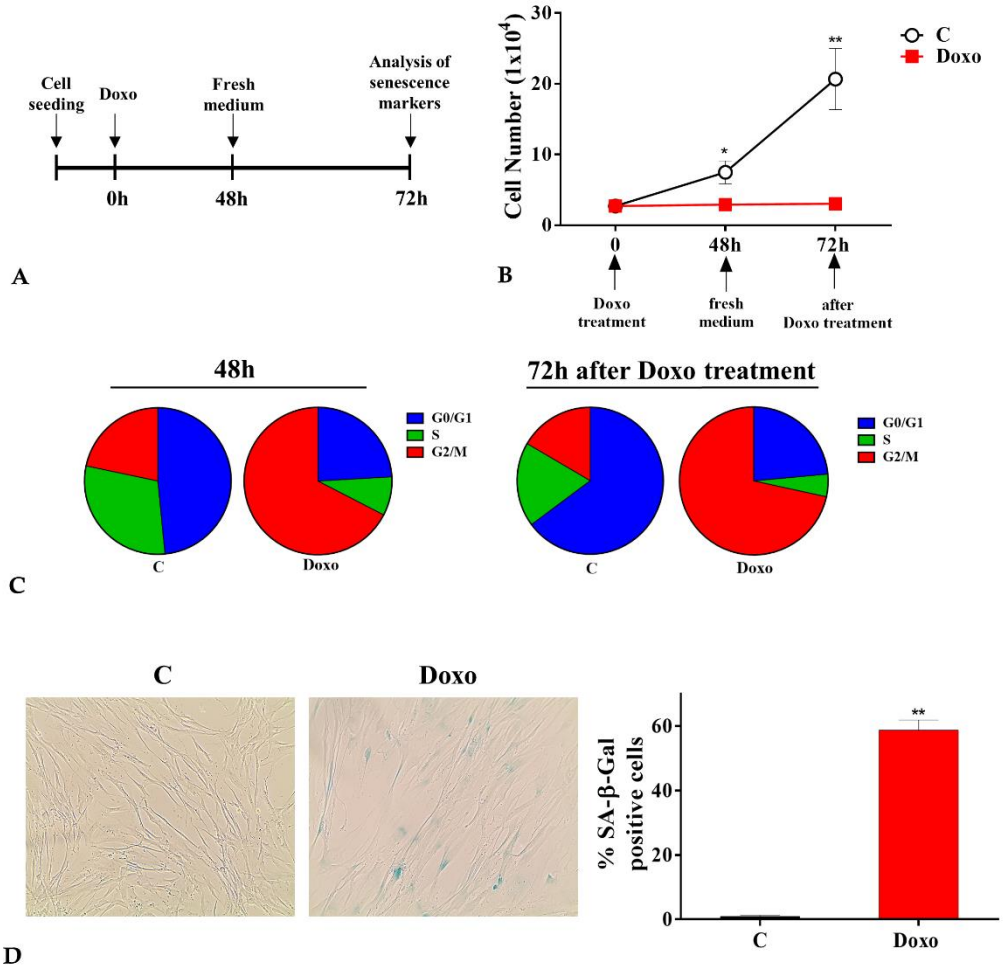

Figure 15. **Doxorubicin treatment induces premature senescence in Wi-38 fibroblasts**. **(A)** Experimental scheme. Cells were exposed to Doxorubicin 50 nM for 48h and then cultured in fresh complete medium for the following 72h. **(B)** Trypan Blue exclusion assay was performed at 48h of Doxo treatment, and at the following 72h, in Doxo-treated cells (Doxo) and vehicle control cells (C). Data are represented as the mean of at least four independent experiments ± standard deviation (SD). P values refers to differences with respect to control C (*p<0.05; ** p<0.001). **(C)** Doxo treatment induces a cell cycle arrest in G2/M phase in most of treated cells. Each pie chart section represents the percentage of cells in a specific cell cycle phase. Data are expressed as the mean of at least four independent experiments. The differences between G0/G1, S, and G2/M phase of Doxo sample and

control resulted significant ($p<0.001$ for all three phases) at 48h of Doxo treatment, and 72h after Doxo treatment ($p<0.001$, $p<0.05$, $p<0.001$, respectively). **(D)** Representative images of SA-β-Gal activity (left) were performed 72h after Doxo treatment. The percentage of SA-β-Gal positive cells (blue ones) with respect to the total number of cells/well was evaluated in three independent experiments, and the results are represented as the mean ± SD (right). P values refers to difference compared to control C (** $p<0.001$). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Doxo*: Doxo-induced senescent fibroblasts.

We have also analysed in Western Blot the expression of the two important pathways closely related to cellular senescence, p21-p53, p16-pRB and the expression of Lamin B1, a structural component of the nucleus reduced in senescent cells. As shown in **Figure 16A**, treated cells significantly reduced phospho-Rb and Lamin B1 and increased p21 expression compared to control cells. Concomitantly, we have analysed by confocal microscopy the presence in Doxo-treated cells of pH2AX nuclear foci, a marker of DNA double-strand breaks (DSB) frequently present in senescent cells, and macroH2A1, an histonic variant component of SAHF. Notably, most of the treated cells (71,3%) were positive for both p21 and pH2AX foci, and 75% of cells were simultaneously negative for Lamin B1 and positive for macroH2A1 (**Figure 16B**). The control showed a low presence of p21-pH2AX foci positive cells (4,4%) and Lamin B1 negative-macroH2A1 positive cells (13,2%). These results indicated that after Doxo-treatment, the most WI-38 cells were senescent.

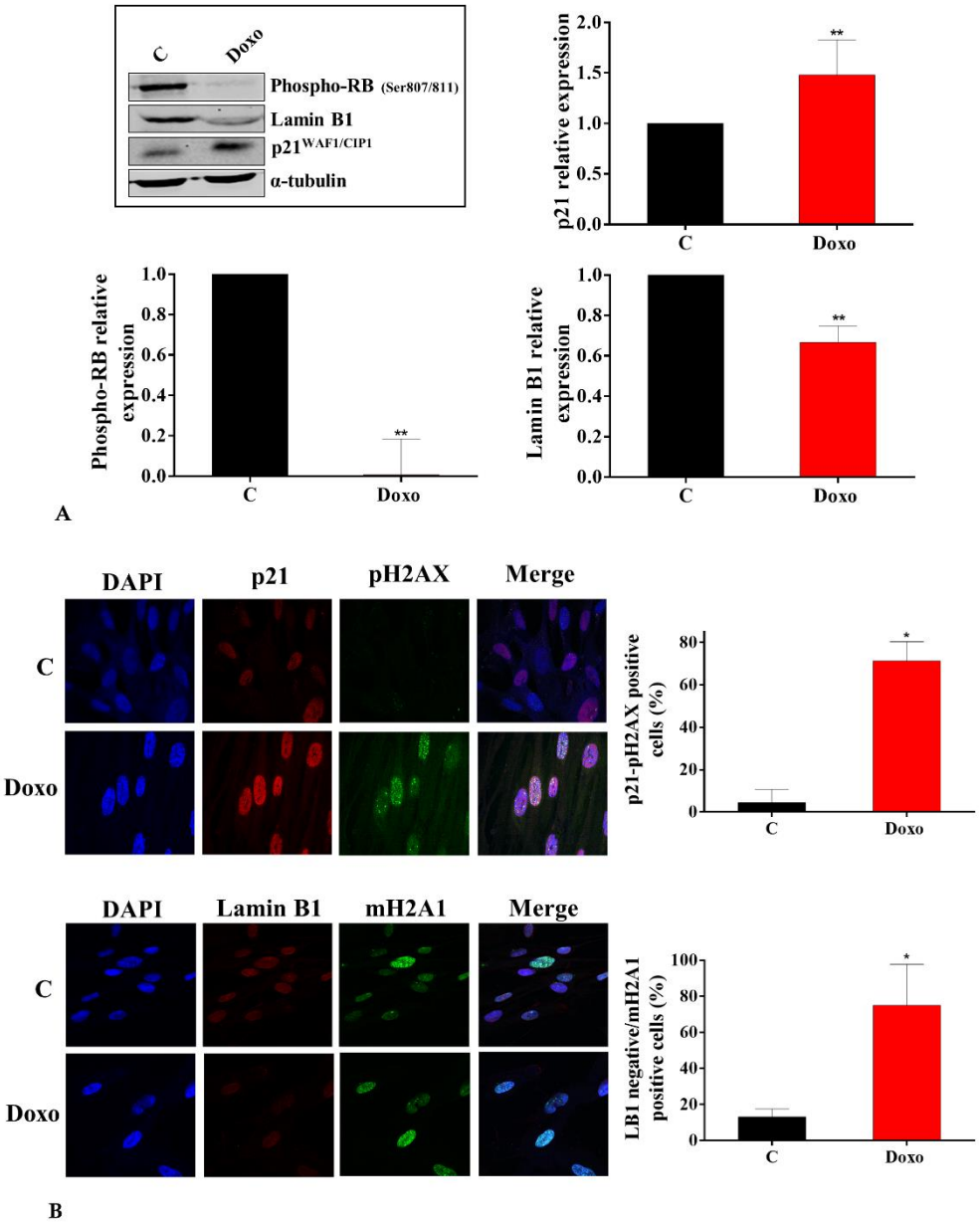

Figure 16. **Doxorubicin treatment induces the expression of senescence markers and senescence-associated heterochromatin foci (SAHF). (A)** Immunoblot representative image (left) and quantification of the expression of phosho-RB, Lamin B1 and p21 in Doxo-treated (Doxo) and vehicle control (C) Wi-38 fibroblasts. Lysates were collected 72h after Doxo treatment. Protein band intensity was normalized to α-tubulin and expressed in relation to control. Data are expressed as the mean ± SD of triplicate experiments. P values refer to differences compared to control (** $p<0.001$). **(B)** Confocal microscopy images of indirect immunofluorescence of pH2AX foci (green) and p21 (red), and macroH2A1 foci (green) and Lamin B1 (red), in-vehicle control cells (C) and Doxo-induced senescent fibroblasts (Doxo). DNA was counterstained with DAPI (blue). Confocal images from three independent experiments were analysed to quantify p21-pH2AX positive cells and Lamin B1 negative-macroH2A1 positive cells with respect to the total number of cells. P values refers to differences regarding control (*$p<0.05$). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Doxo*: Doxo-induced senescent fibroblasts.

4.2 Section II

In the second part of the study, we performed a pre-treatment with Quercetin before the induction of senescence by Doxorubicin to evaluate if the substance could prevent Doxo-induced senescence and damage in WI-38 fibroblasts. The range of the non-toxic doses of Quercetin most used in literature in vitro studies was from 10 to 40 µM. After some experiments conducted with these different doses (data not shown), we proceeded with the 40 µM one, which showed better results. Herein we showed that a pre-treatment of 24h with Quercetin 40 µM can reduce the onset of Doxo-induced senescence, as proved by the partially recovered proliferative capacity and the reduced G2/M cell cycle block. Moreover, we demonstrated that Quercetin pre-treated cells reduced SA-β-Gal activity and SAHF formation, with an increased phospho-RB and Lamin B1 level and a decreased p21 expression.

Furthermore, we demonstrated that Quercetin could protect the cells from Doxo-induced damage, increasing cellular antioxidant defence and reducing Doxo-associated ROS production. Considering these results, we analysed Quercetin action in reducing SASP factors production. Our results showed that Quercetin pre-treatment reduced NF-κB level and decreased the gene expression of some known SASP factors, which are conversely upregulated in Doxo-induced senescent fibroblasts. Overall, these results showed that Quercetin exerts an excellent senomorphic action, protecting the cells from Doxo-induced senescence and reducing the SASP.

This section has also investigated the effects of Doxo-induced senescent and Quercetin pre-treated fibroblasts conditioned medium on OS cell line (U2OS) and WI-38 fibroblasts. Our data have proven that conditioned medium of Doxo-induced senescent fibroblasts (SM) increase U2OS cells growth, migration, and invasiveness. On the contrary, CM of Quercetin pre-treated fibroblasts (QSM) did not induce the same aggressiveness in OS cells.

Moreover, we showed that SM seems to reduce the proliferation of WI-38 cells and that conversely, QSM did not alter their growth. Furthermore, both SM and QSM did not induce changes in WI-38 migration, suggesting that the promoting effects were specific to neoplastic cells.

4.2.1 Quercetin pre-treatment reduces Doxorubicin-induced senescence in WI-38 fibroblasts

To verify the ability of Quercetin to reduce Doxo-induced senescence, WI-38 fibroblasts were pre-treated with the flavonoid for 24h, before the induction of senescence with Doxo (**Figure 17A**). Doxo-treated cells underwent a proliferative arrest, and, on the contrary, Quercetin pre-treated cells recovered their proliferative capacity partially from day 3, and their growth continued for the following 10 days after Doxo treatment (**Figure 17B**). Indeed, at day 3, Doxo-treated cells presented an accumulation in G2/M phase of cells cycle (61,5%) along with a minimal S phase fraction (8.5%), and reduced G0/G1 phase (30%), while Quercetin pre-treated cells showed similar values for G0/G1 phase (30.4%), but a reduced G2/M phase (50.4%) and a significantly increased S fraction (19.2%) compared to Doxo-treated cells ($p<0.05$). At day 10, Doxo-induced senescent fibroblasts did not resume cell cycle and presented similar percentages to day 3 values (35,9% G0/G1, 5.6% S, 58,5% G2/M). Conversely, as shown in **Figure 17C,** the cell cycle of Quercetin pre-treated fibroblasts varied with respect to day 3, and at day 10 the cells showed an increased G0/G1 and S phase (45% and 13% respectively), and a significant reduction in G2/M fraction (41.7%) compared to Doxo-induced senescent cells ($p<0.05$).

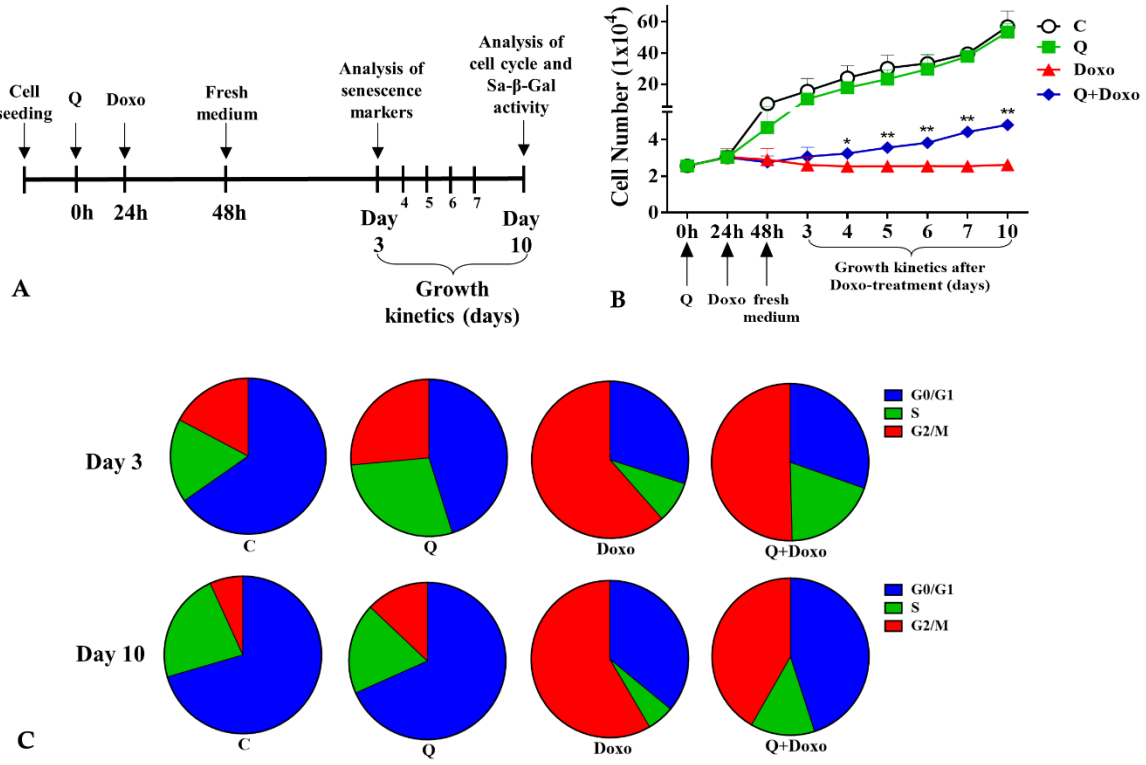

Figure 17. **Quercetin pre-treatment reduces Doxorubicin-induced senescence in Wi-38 fibroblasts. (A)** Experimental scheme of the preventive effect of Quercetin. Cells were pre-treated with Quercetin 40µM for 24h and then exposed to the induction of senescence by Doxorubicin. **(B)** Trypan Blue exclusion assay was performed at different times during the treatments, and a growth curve was done from day 3 onwards. Data are represented

as the mean ± SD of at least three independent experiments. The differences in cell proliferation between Doxo-treated cells (Doxo) and vehicle control (C) or Quercetin treated fibroblasts (Q) resulted significant at all times ($p<0.001$). P values, shown in the figure, refer to differences between Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo) and Doxo-treated cells (Doxo) (*<0.05; ** $p<0.001$). **(C)** Cell cycle analysis was performed at day 3 and 10 from the end of Doxo treatment. Each pie chart section represents the percentage of cells in a specific cell cycle phase. Data are represented as the mean of at least three independent experiments. The differences between G0/G1, S, and G2/M phase of Doxo sample and control resulted significant at both times ($p<0.001$, $p<0.05$, and $p<0.001$, respectively). Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo) presented a significant increased S phase ($p<0.05$) at day 3, and a significant decreased G2/M phase ($p<0.05$) at day 10 with respect to Doxo-treated cells (Doxo). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: Quercetin treated fibroblast; *Doxo*: Doxo-induced senescent fibroblasts; *Q+Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

Notably, the analysis of Sa-β-Gal activity showed that at day 3, cells pre-treated with Quercetin presented a significantly lower number of positive cells (16.8%) than Doxo-treated cells (60.8%). On day 10, although the percentage of positive cells increased in the pre-treated sample (36.8%), this was still significantly lower than Doxo-treated cells (91.5%) (**Figure 18**).

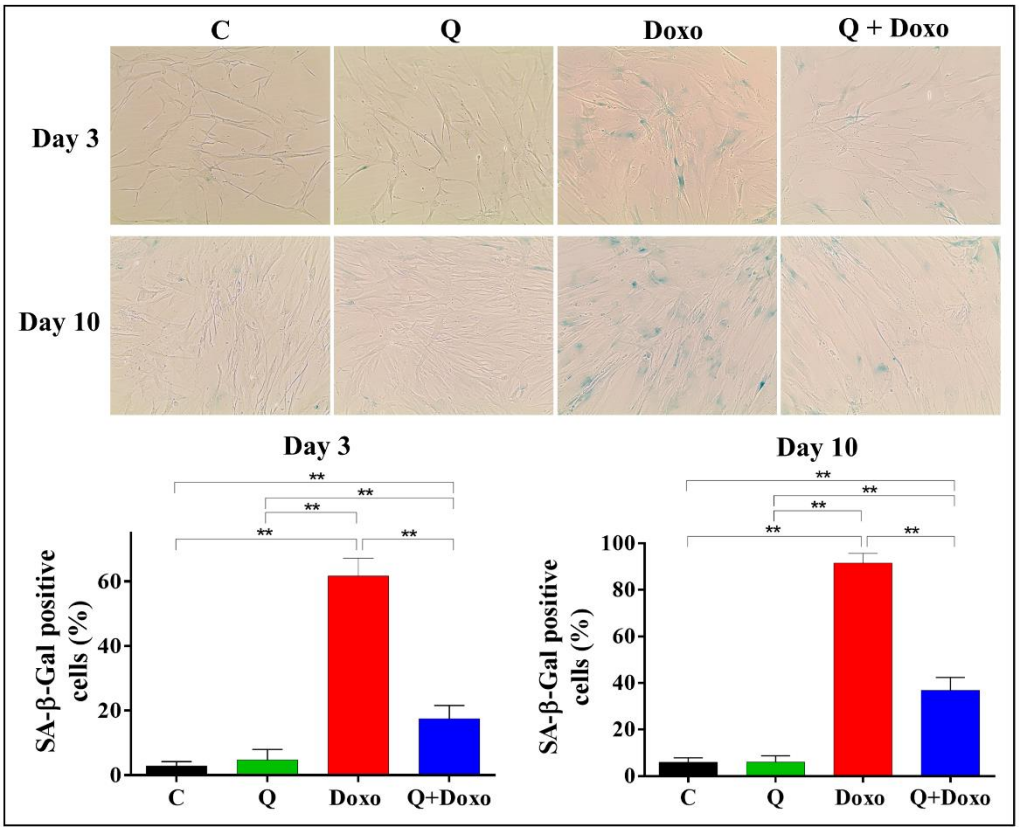

Figure 18. **Quercetin pre-treatment reduces the number of SA-β-Gal positive cells.** Representative images of SA-β-Gal activity (upper) performed 3 and 10 days after Doxo treatment. The percentage of SA-β-Gal positive cells (blue ones) with respect to the total number of cells/well (down) was evaluated in at least three independent experiments, and the results are represented as the mean ± SD (** $p<0.001$). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: Quercetin treated fibroblast; *Doxo*: Doxo-induced senescent fibroblasts; *Q+Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

Next, to verify the effect of the Quercetin on senescent phenotype, we evaluated the changes in the expression of senescent molecular markers. Phospho-RB and Lamin B1 expression was significantly up-regulated, and p21 significantly decreased in Quercetin pre-treated cells compared to Doxo-treated cells (**Figure 19**).

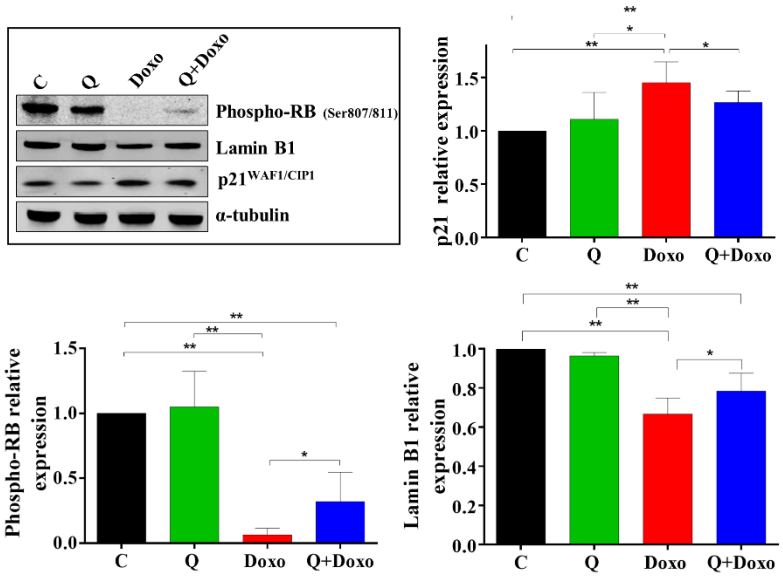

***Figure 19*. Quercetin pre-treatment changes the expression of senescence markers.** Immunoblot representative image (left) and quantification of the expression of phosphor-RB, Lamin B1 and p21 in vehicle control (C), Quercetin treated (Q), Doxo-treated (Doxo) and Quercetin pre-treated Doxo-treated (Q+Doxo) Wi-38 fibroblasts. Lysates were collected 3 days after Doxo treatment. Protein band intensity was normalized to α-tubulin and expressed in comparison to control. Data are represented as the mean ± SD of six independent experiments (*p<0.05; ** p<0.001). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: Quercetin treated fibroblast; *Doxo*: Doxo-induced senescent fibroblasts; *Q+Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

Moreover, by confocal microscopy, we have analysed the presence of pH2AX and macroH2A1 foci combined with p21 and Lamin B1 expression, respectively. As shown in **Figure 20,** p21-pH2AX foci positive cells and Lamin B1 negative-macroH2A1 foci positive cells were significantly reduced in Quercetin-pretreated cells (30.2% and 16%, respectively), compared to Doxo-treated cells (79%, and 73%, respectively).

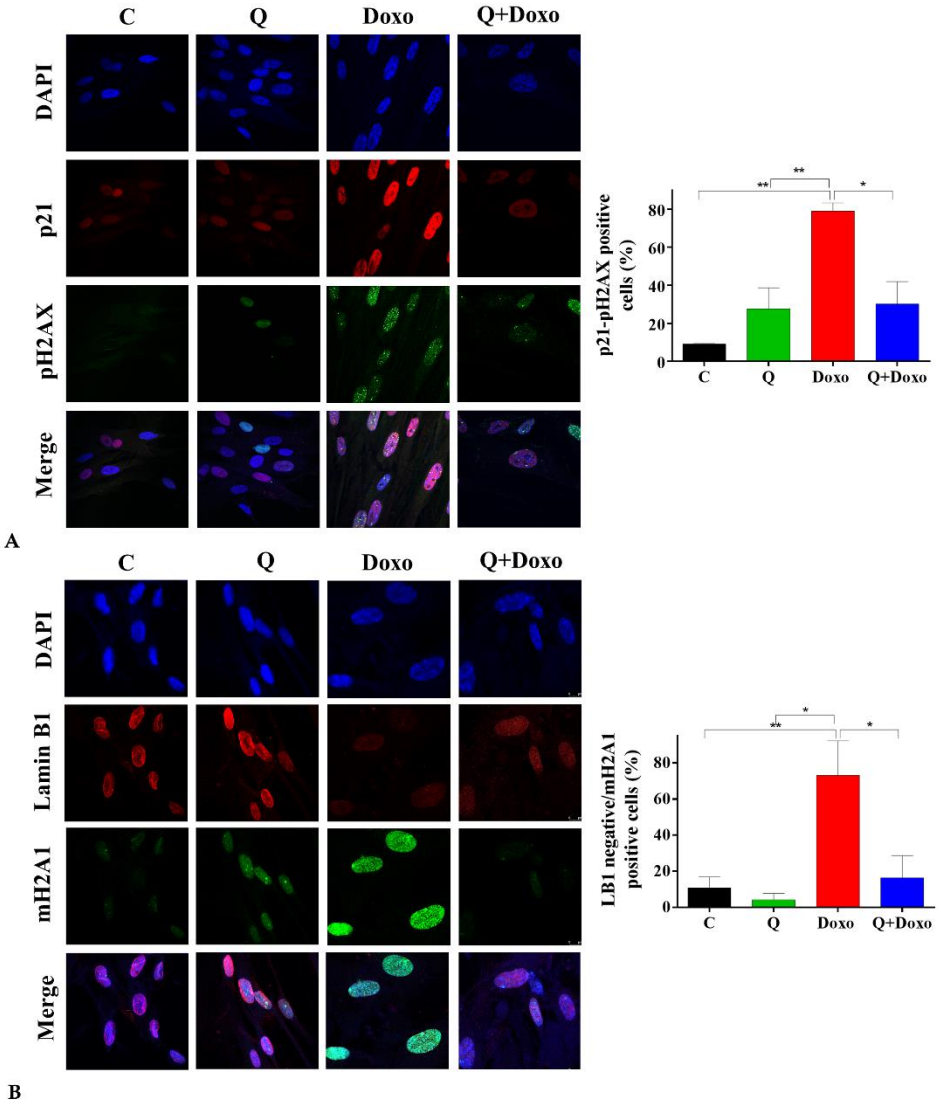

Figure 20. **Quercetin pre-treatment reduces SAHF. (A)** Confocal microscopy images (left) of indirect immunofluorescence of pH2AX foci (green) and p21 (red) and the percentage of p21/pH2AX positive cells (right), in vehicle control (C), Quercetin treated cells (Q), Doxo-induced senescent fibroblasts (Doxo), and Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo). DNA was counterstained with DAPI (blue). Confocal images from three independent experiments were analysed to quantify p21-pH2AX positive cells with respect to the total number of cells (*p<0.05; ** p<0.001). **(B)** Confocal microscopy images (left) of indirect immunofluorescence of macroH2A1 foci (green) and Lamin B1 (red), and the percentage of Lamin B1 negative/macroH2A1 positive cells (right). DNA was counterstained with DAPI (blue). Confocal images from three independent experiments were analysed to quantify Lamin B1 negative-macroH2A1 positive cells with respect to the total number of cells (*p<0.05; ** p<0.001). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: Quercetin treated fibroblast; *Doxo*: Doxo-induced senescent fibroblasts; *Q+Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

In conclusion, our results showed that Quercetin pre-treated cells partially recovered their proliferative capacity, reduced cell cycle arrest in G2/M, decreased Sa-β-Gal activity, and showed a reduced formation of pH2AX and macroH2A1 foci, suggesting that Quercetin pre-treatment could protect the cells from Doxo-induced senescence.

4.2.2 Quercetin pre-treatment increases cellular antioxidant defence

To further investigate the mechanisms underlying the protective effect of Quercetin against Doxo-induced senescence, we evaluated the production of ROS after 24 and 48h of Doxo-treatment. Cellular ROS levels were significantly increased by 1.3 and 1.7 times, respectively, after 24 and 48h of Doxo treatment, compared to control (**Figure 21A**). Furthermore, Quercetin pre-treatment significantly reduced ROS production compared to Doxo-treated cells (0.7 at 24h and 0.8 at 48h). Moreover, we measured the Total Antioxidant Capacity (TAC) of protein lysates after 24h of Quercetin treatment, and the results showed that it was increased in WI-38 cells exposed to Quercetin compared to control (**Figure 21B**). In particular, Quercetin pre-treatment significantly up-regulated SOD-1 and SOD-2 enzymes expression (**Figure 21C**). These results suggested that Quercetin pre-treatment increased cellular antioxidant defences, partially protecting cells from Doxo-induced damage and senescence.

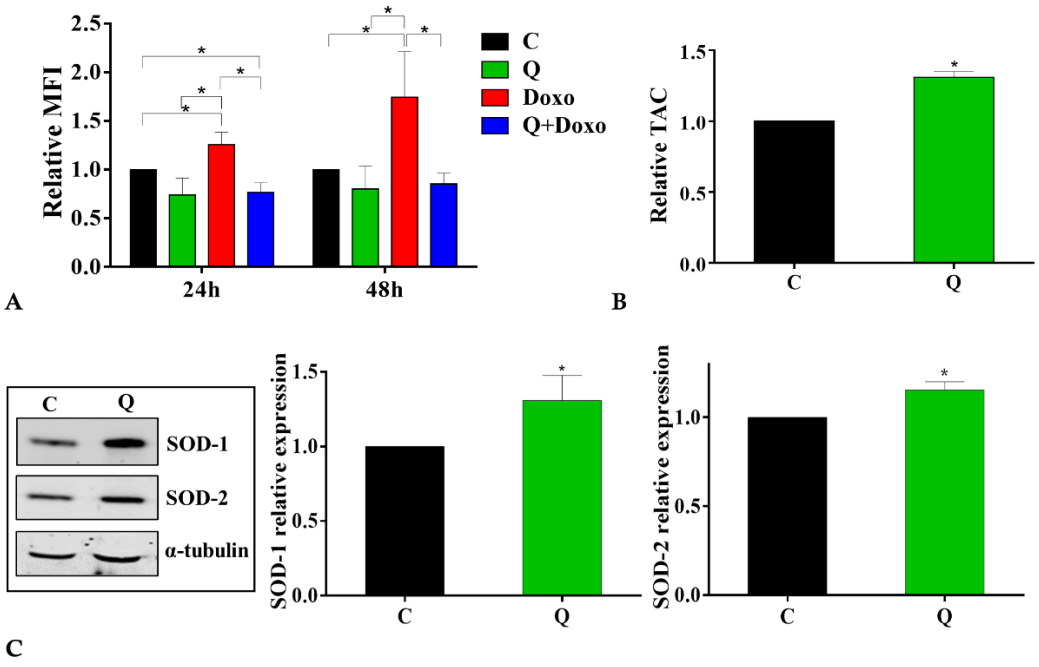

Figure 21. **Quercetin pre-treatment increases cellular antioxidant defence. (A)** Analysis of ROS production by H2DCF-DA assay, after 24 and 48h of Doxo-treatment. Data are represented as the mean of Median Fluorescent Intensity relative to control of three independent experiments ± SD (*p<0.05). **(B)** Total antioxidant capacity of protein lysates collected after 24h of Quercetin treatment. Data are expressed relative to control (assumed as value 1) and as the mean of three independent experiments ± SD. P values refer to differences with respect to control C (*p<0.05). **(C)** Immunoblot representative image (left) and quantification of the expression of SOD-1 and SOD-2 enzymes in vehicle control (C) and Quercetin treated cells (Q). Data are represented as the mean of three independent experiments ± SD. P values refer to differences with respect to control C (*p<0.05). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; Q+*Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

4.2.3 Quercetin pre-treatment reduces NF-κB level and gene expression of some SASP factors

We have analyzed the expression of NF-κB, one of the major transcription factors that control the production of SASP molecules, after Doxo treatment. Doxo-treated cells presented a slight but significant increase of NF-κB expression compared to control cells, and interestingly Quercetin pre-treated cells showed an expression significantly reduced compared to Doxo-treated cells (**Figure 22A**).

Moreover, gene expression levels of some known SASP factors, named IL-1α, IL-6, IL-8, GROα, MMP2, TNFα, and CXCL12, were evaluated 3 days after Doxo treatment. Real-Time PCR experiments indicated that IL-6 and IL-8, two of the most prominent SASP products, IL-1α, MMP2, TNFα, and CXCL12, were significantly up-regulated in Doxo-induced senescent fibroblasts (**Figure 22B**). Although the pre-treatment with Quercetin reduced the increase in expression of all 6 genes, only for IL-6, IL-8, CXCL12, IL-1α, the differences were significant ($p<0.05$). Moreover, the expression of GROα was not modified in Doxo-induced senescent fibroblasts compared to control but was significantly reduced in both samples treated with Quercetin, respect to control, and to Doxo-induced senescent fibroblasts. These results suggest that Quercetin could decrease SASP production, maybe through the reduction of NF-κB level, revealing itself as an excellent senomorphic agent.

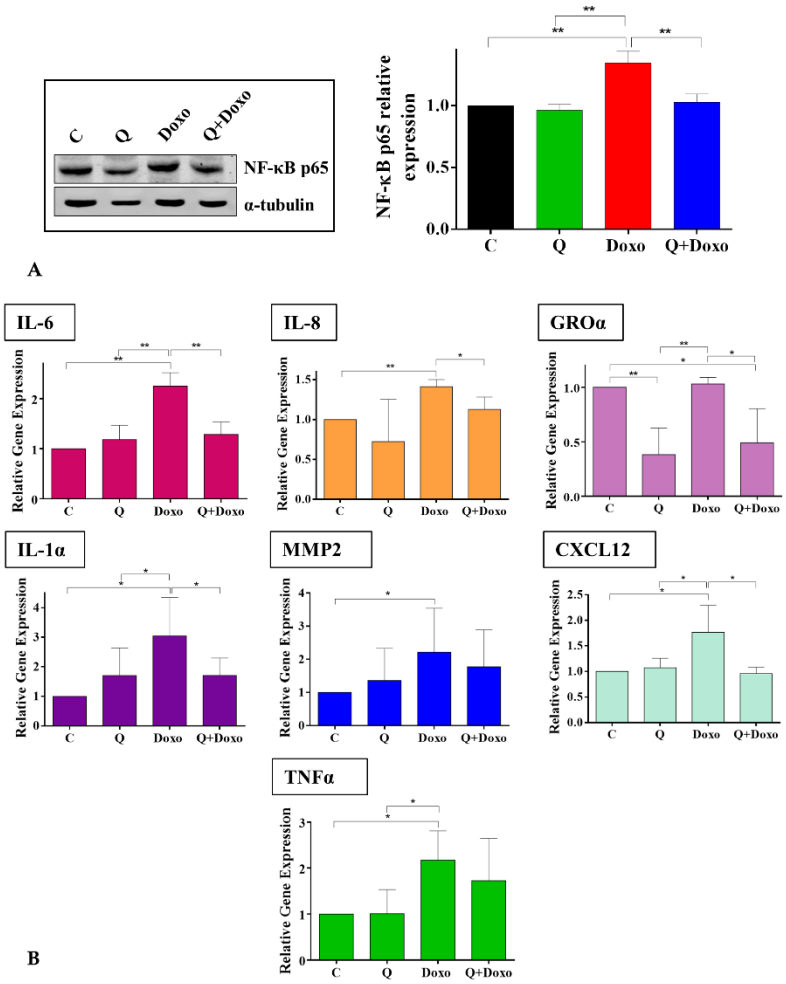

Figure 22. **NF-κB level and SASP factors gene expression. (A)** Immunoblot representative image (left) and quantification of the expression of NF-κB in vehicle control (C), Quercetin treated (Q), Doxo-treated (Doxo) and Quercetin pre-treated Doxo-treated (Q+Doxo) WI-38 fibroblasts. Lysates were collected after Doxo treatment (48h). Protein band intensity was normalized to α-tubulin and expressed in relation to control. Data are represented as the mean ± SD of three independent experiments (*p<0.05). **(B)** Gene expression analysis was performed on vehicle control (C), Quercetin-treated cells (Q), Doxo-induced senescent fibroblasts (Doxo), and Quercetin pre-treated Doxo-treated fibroblasts (Q+Doxo) 3 days after Doxo treatment. GAPDH was used as the housekeeping gene, and data were normalized to vehicle control C (assumed as value 1). Data are expressed as the mean ± SD of triplicate experiments (*p<0.05; **p<0.001). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *Q*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Q+Doxo*: Quercetin pre-treated Doxo-induced senescent fibroblasts.

4.2.4 Effects of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on Osteosarcoma cells (U2OS)

After verifying the increase in gene expression of some SASP factors by Doxo-induced senescent fibroblasts and the senomorphic action of Quercetin, we verified whether the conditioned medium from Doxo-induced senescent fibroblasts (SM) and Quercetin-pretreated Doxo-treated fibroblasts (QSM) could have effects on U2OS proliferation and invasion

capacity. SM significantly enhanced U2OS proliferation over time (**Figure 23A**) and colonies number and area (**Figure 23B and C**) compared to QSM.

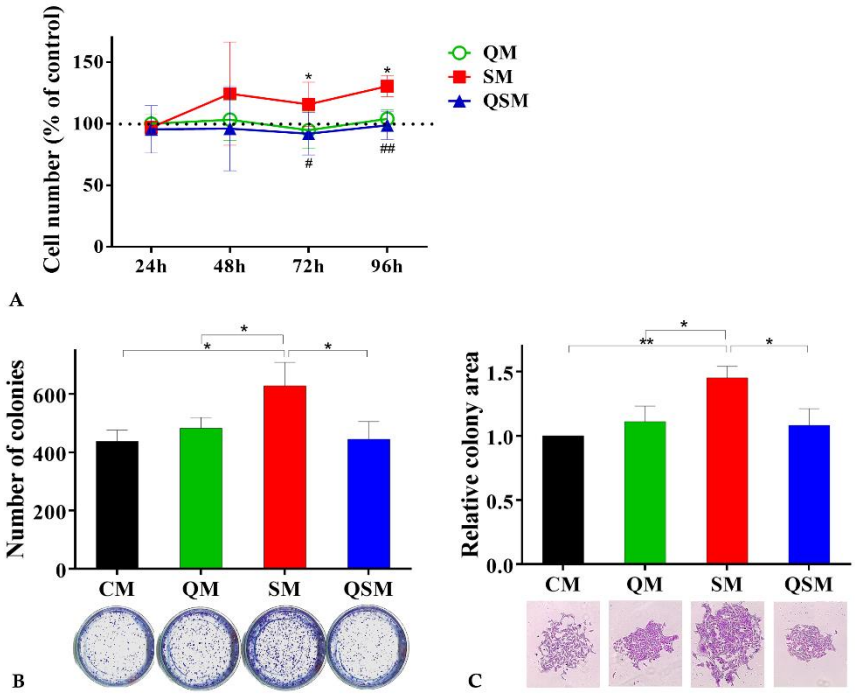

Figure 23. **Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on U2OS growth. (A)** Trypan Blue exclusion assay on U2OS cells treated with conditioned medium from Quercetin treated cells (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM). Data are shown as the mean percentage of three independent experiments ± SD relative to U2OS treated with vehicle control conditioned medium (CM). P values refers to differences respect to control (*p<0.05) and to SM ($^{\#}$p<0.05; $^{\#\#}$p<0.001). **(B)** Representative images (down) and calculated number of U2OS cells (upper) colonies formed after 7 days of treatment with conditioned media. Data represented the mean of three independent experiments ± SD (*p<0.05). **(C)** Colonies area (representative images below) was calculated with Image J Software, and data are expressed relative to control (CM) and represented as the mean of three independent experiment ± SD (*p<0.05; **p<0.001). Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from Quercetin treated fibroblasts; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *QSM*: conditioned medium from Quercetin pre-treated Doxorubicin-treated fibroblasts.

Moreover, the wound healing assay showed that SM induced a significant increase in U2OS cells migration (p<0.05), while as expected, QSM reduced this effect (**Figure 24A**). Similar results were obtained evaluating the invasion capacity of U2OS cells (**Figure 24B**). SM induced a 1.6-fold increase of invasive index, while U2OS treated with QSM showed an invasive grade like those of controls (p <0.05). Overall, these results demonstrate that SM, but not QSM, could increase the proliferation, migration, and invasiveness of U2OS cells. On the other hand, Quercetin pre-treatment, reducing the senescent cells, and modulating the expression and secretion of SASP factors, reduce SM's promoting effects.

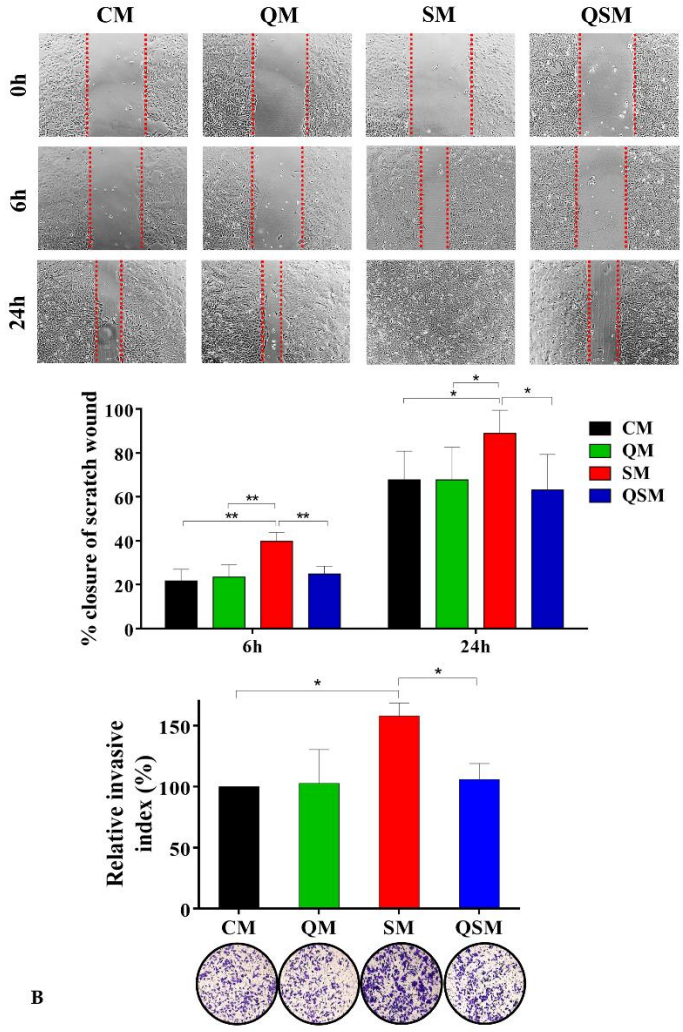

***Figure 24*. Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on U2OS cells migration and invasiveness. (A)** Representative images (upper) and the percentage of scratch wound area (down) of Wound Healing Assay of U2OS cells treated with conditioned media. Data are shown as the mean percentage of three independent experiments ± SD of wound closure area with respect to initial scratch (*p<0.05; **p<0.001). **(B)** Boyden chamber invasion test on U2OS cells. Conditioned media from control fibroblasts (CM), Quercetin-treated fibroblasts (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM) were placed in the bottom well of the chamber. Data are represented as a relative invasive index with respect to control, and the mean of three independent experiments ±SD (*p<0.05). Representative images of invasive cells are shown below the graph. Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from Quercetin treated fibroblasts; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *QSM*: conditioned medium from Quercetin-pre-treated Doxo-treated fibroblasts.

4.2.5 Effects of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-induced senescent fibroblasts on WI-38 fibroblasts

To verify if the increased aggressiveness induced by SM was specific to cancer cells or it was a general effect, we analysed the proliferation and migration of non-tumoral cells as WI-38 fibroblasts. Interestingly, SM significantly reduced WI-38 cells proliferation, especially at 72h ($p<0.05$) and 96h ($p<0.001$), compared to CM and QSM (**Figure 25A)**. This reduction of

the proliferation induced by SM could be due to the induction of paracrine senescence, even if our findings did not demonstrate it. Furthermore, the migration analysis did not show significant differences between samples (**Figure 25B**). These results showed that the promoting effects of CM from Doxo-induced senescent fibroblasts are specific to OS cells and did not affect normal fibroblasts.

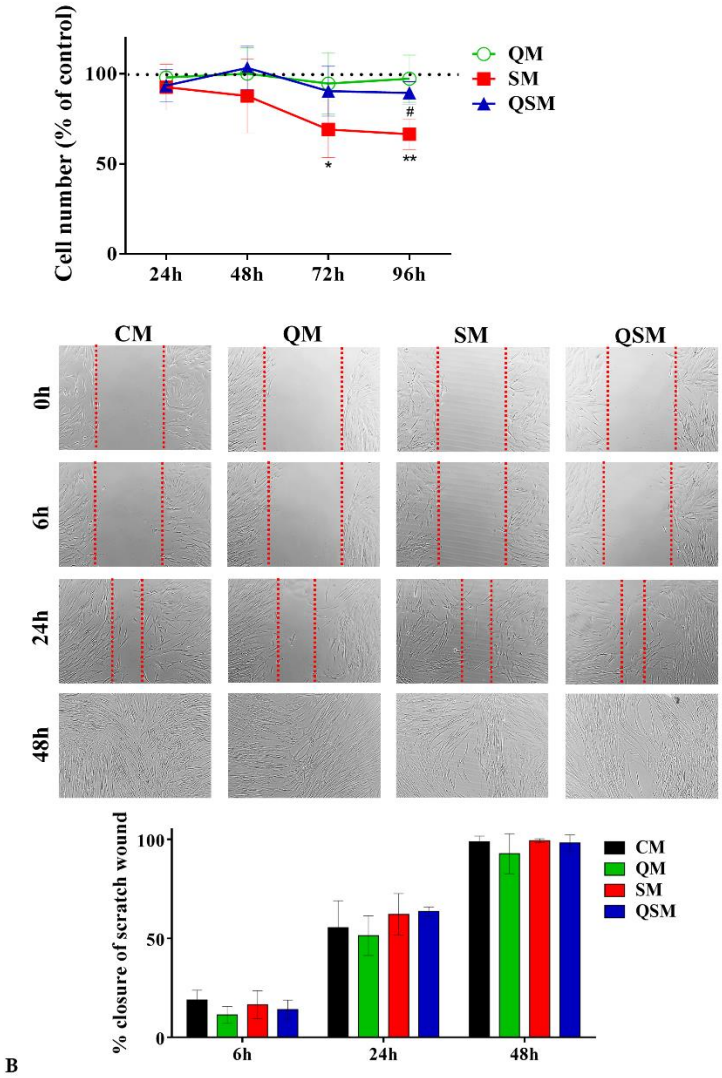

Figure 25. Effect of Conditioned Medium from Doxo-induced senescent fibroblasts and Quercetin pre-treated Doxo-treated fibroblasts on WI-38 fibroblasts. (A) Trypan Blue exclusion assay on WI-38 cells treated with conditioned medium from Quercetin treated cells (QM), Doxo-induced senescent fibroblasts (SM), and Quercetin pre-treated Doxo-treated fibroblasts (QSM). Data are shown as the mean percentage of three independent experiments ±SD relative to WI-38 treated with vehicle control conditioned medium (CM). P values refers to differences with respect to control (*$p<0.05$; **$p<0.001$) and to SM ($^{\#}p<0.05$). **(B)** Representative images (upper) and the percentage of scratch wound area (down) of Wound Healing Assay of WI-38 cells treated with conditioned media. Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from Quercetin treated fibroblasts; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *QSM*: conditioned medium from Quercetin pre-treated Doxo-treated fibroblasts.

4.3 Section III

In the last section of this work, we analysed the senolytic activity of Quercetin against Doxo-induced senescent fibroblasts. After the induction of senescence by Doxorubicin, we treated the cells with Quercetin 40 µM, for 3 days. Our results showed that the treatment with Quercetin reduced the number of Doxo-induced senescent fibroblasts and decreased the percentage of cells in G2/M phase. Conversely, normal proliferating fibroblasts' growth and cell ceycle were not affected. Moreover, after Quercetin treatment, there was a reduction in the number of SA-β-Gal positive cells, an increase in Lamin B1 and phospho-RB, and a decrease in p21 expression.

Interestingly, our findings showed also a decrease in the number of macroH2A1 positive/Lamin B1 negative cells, quite comparable to the reduction of SA-β-Gal positive cells. Considering this evidence, we analysed the apoptosis and cell death rate, but contrary to what we expected, the results did not show a significant difference in either apoptosis or necrosis between senescent cells treated or not with Quercetin. However, further investigations are needed.

Moreover, we have evaluated the effects of conditioned medium from Doxo-induced senescent fibroblasts treated (SQM) and non-treated (SM) with Quercetin on U2OS cells to verify whether the Quercetin could modify the promoting effects of senescent cells on U2OS. Our data showed that treating Doxo-induced senescent fibroblasts with Quercetin reduced their influence on U2OS cells proliferation and migration.

4.3.1 Quercetin treatment reduces the number of Doxo-induced senescent fibroblasts

To verify the senolytic activity of Quercetin, after the induction of senescence by Doxorubicin (as described in Section I), WI-38 senescent cells were treated with Quercetin 40 µM for 3 days (**Figure 26A**). As shown in **Figure 26B**, the number of Doxo-induced senescent fibroblasts treated with Quercetin (Doxo+Q) significantly decreased compared to non-treated senescent cells (Doxo) from the first day of treatment ($p<0.05$) until the third day, when the decrease has reached the highest level ($p<0.05$). Notably, Quercetin treatment did not affect the proliferation and the number of non-senescent normal fibroblasts (Q) at any time. In the same manner, while proliferating fibroblasts treated with Quercetin did not show an altered cell cycle phase distribution compared to control cells, the treatment with Quercetin in senescent fibroblasts increased G0/G1 and S phases and decreased the G2/M fraction (41,8%, 13,3 %, and 38,4%, respectively), with respect to senescent non-treated cells (30.6%, 5.3%, and 64%, respectively; $p<0.05$), that continued to be consistently arrested in G2/M phase (**Figure 26C**).

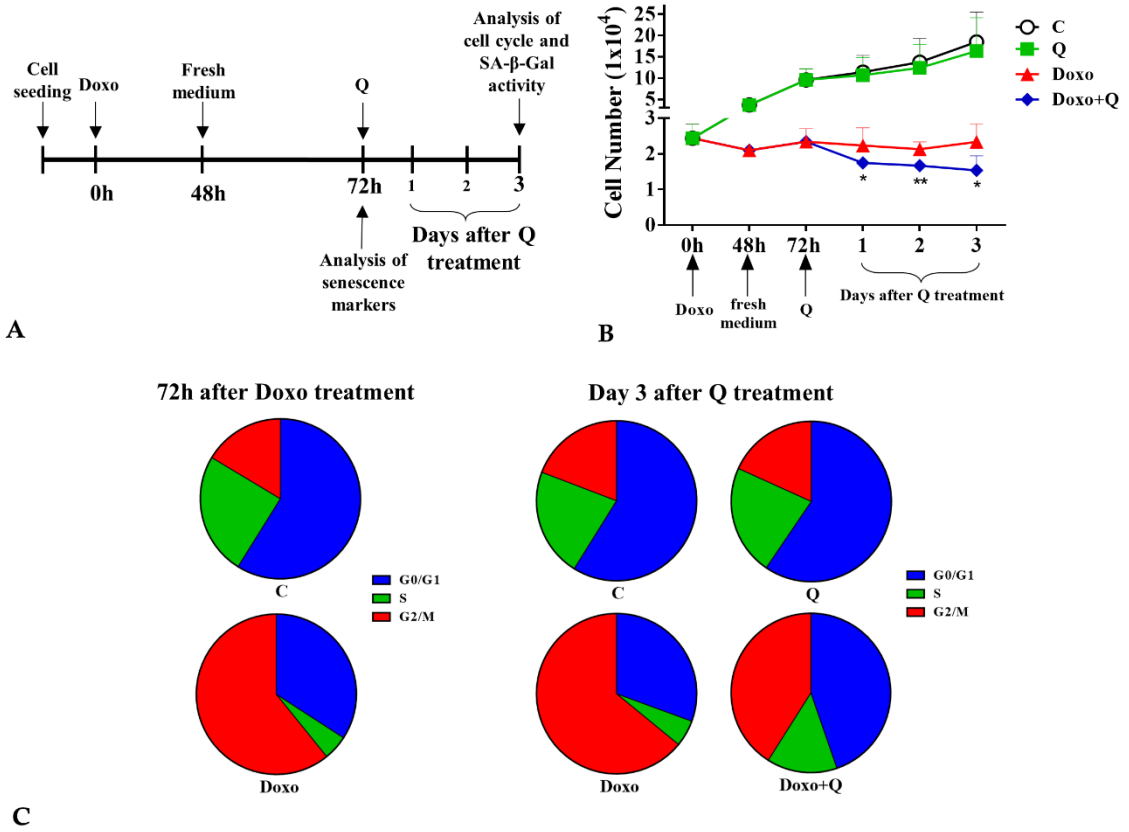

Figure 26. **Quercetin reduces the number of Doxo-induced senescent Wi-38 fibroblasts. (A)** Experimental scheme. After the induction of senescence by Doxorubicin, cells were treated with Quercetin 40µM for 3 days. **(B)** Trypan Blue exclusion assay was performed at different times during the treatments. Data are represented as the mean ± SD of at least four independent experiments. The differences in cell proliferation between Doxo-treated cells (Doxo) and vehicle control (C) or Quercetin treated fibroblasts (Q) resulted significant at all times ($p<0.001$).

P values, shown in the figure, refer to differences between Doxo-induced senescent fibroblasts treated (Doxo+Q) and non-treated with Quercetin (Doxo) (*<0.05; ** p<0.001). **(C)** Cell cycle analysis performed 72h after Doxo treatment and 3 days after Quercetin treatment. Each section of pie chart represents the percentage of cells in a specific cell cycle phase. Data are represented as the mean of four independent experiments. The differences between G0/G1, S, and G2/M phase of Doxo sample and control resulted significant at both times (p<0.001). Quercetin treated senescent cells (Doxo+Q) presented a significant increased S phase (p<0.05), and a significant decreased G2/M phase (p<0.05) with respect to non-treated senescent cells (Doxo). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *QM*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Doxo+Q*: Doxo-induced senescent fibroblasts treated with Quercetin.

The analysis of Sa-β-Gal activity, confirming what we have shown in Section I, indicated that 72h after Doxo treatment 57% of cells were positive for the staining. Notably, 3 days after Quercetin treatment, the number of positively stained cells, the senescent ones, was significantly reduced in Quercetin treated senescent fibroblasts (Doxo+Q) compared to senescent non-treated cells (Doxo) (40.9% and 72%, respectively; p<0.001) (**Figure 27**).

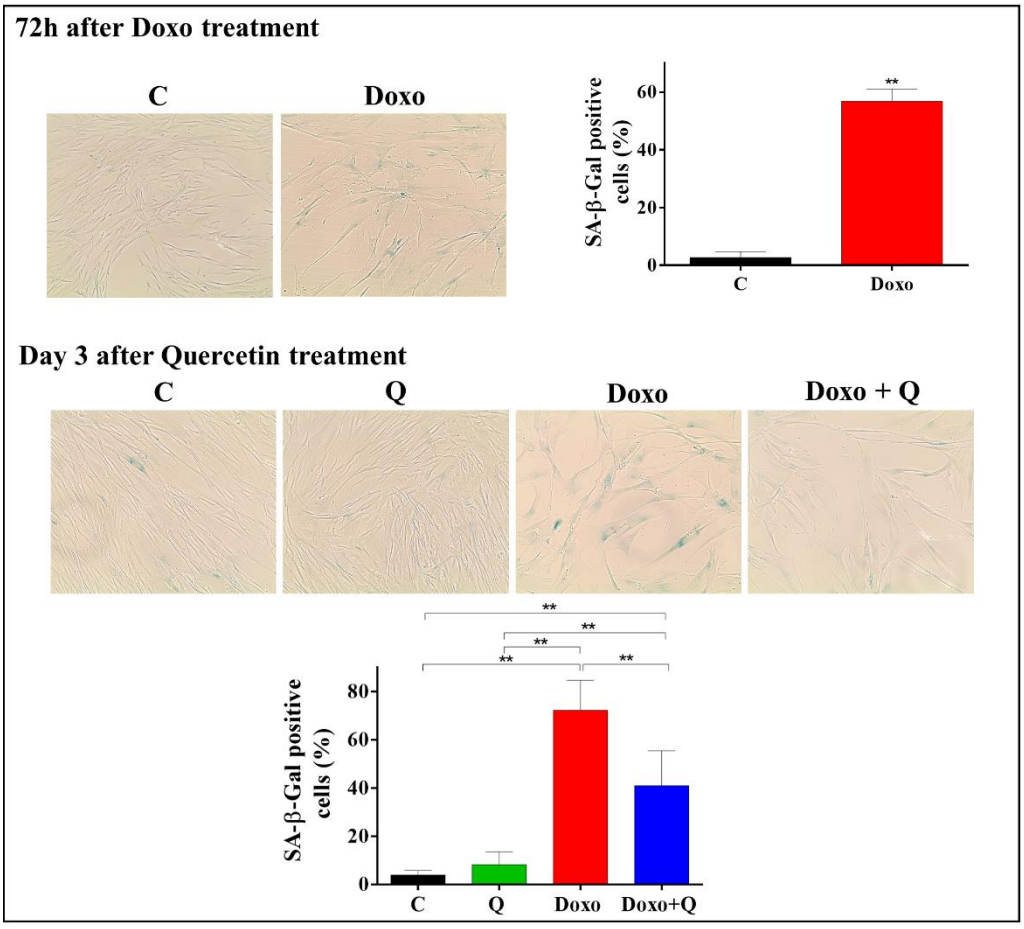

Figure 27. **Quercetin treatment reduces the number of SA-β-Gal positive cells.** Representative images of SA-β-Gal activity (upper) performed 72h after Doxo treatment and 3 days after the subsequent Quercetin treatment. The percentage of SA-β-Gal positive cells (blue ones) with respect to the total number of cells/well (down) was evaluated in at least four independent experiments, and the results are represented as the mean ± SD (** p<0.001). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *QM*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Doxo+Q*: Doxo-induced senescent fibroblasts treated with Quercetin.

To confirm these results we have analysed the presence of macroH2A1 foci in combination with the absence of Lamin B1 protein. Our preliminary data showed that the number of macroH2A1 positive/Lamin B1 negative cells was lower in senescent fibroblasts treated with Quercetin than in non-treated senescent cells (40% and 79%, respectively), and resembled the number of Sa-β-Gal positive cells (**Figure 28**).

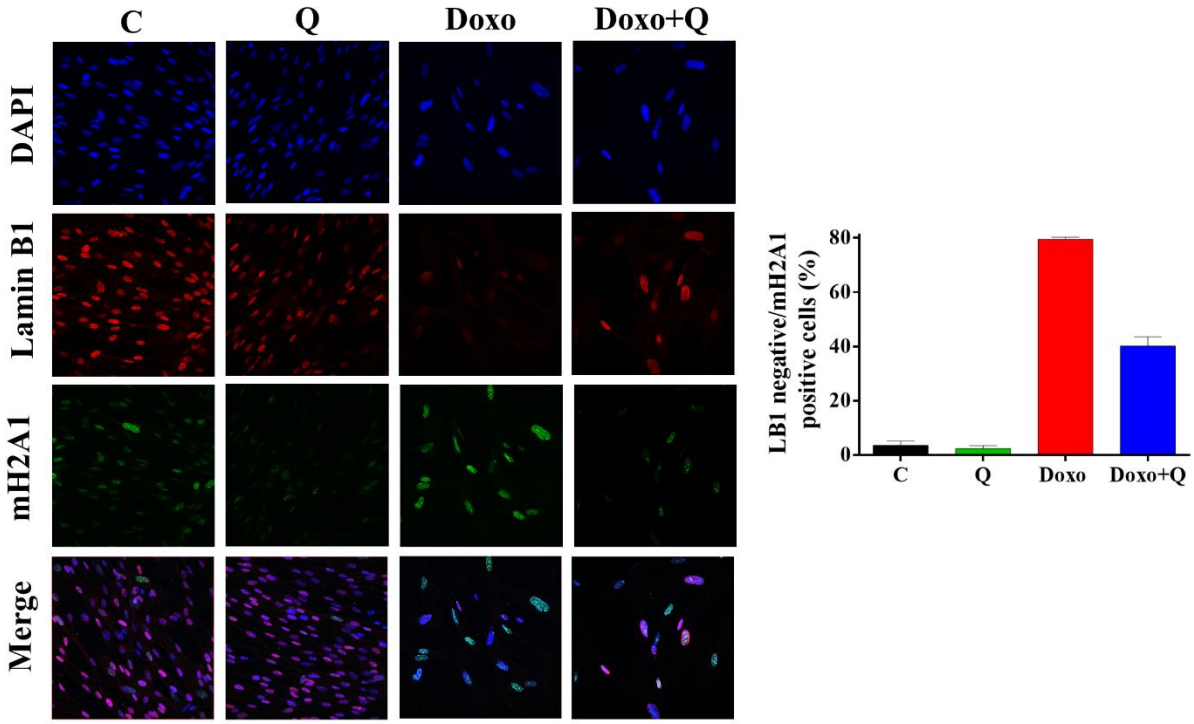

Figure 28. **Quercetin treatment reduces SAHF.** Confocal microscopy images (left) of indirect immunofluorescence of macroH2A1 foci (green) and Lamin B1 (red), and the percentage of Lamin B1 negative/macroH2A1 positive cells (right). DNA was counterstained with DAPI (blue). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *QM*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Doxo+Q*: Doxo-induced senescent fibroblasts treated with Quercetin.

Next, we have evaluated the changes in the expression of senescent molecular markers. Accordingly to the showed results, phospho-RB and Lamin B1 expression was significantly up-regulated, and p21 significantly decreased in Doxo-induced senescent cells treated with Quercetin, compared to senescent non-treated cells (**Figure 29**).

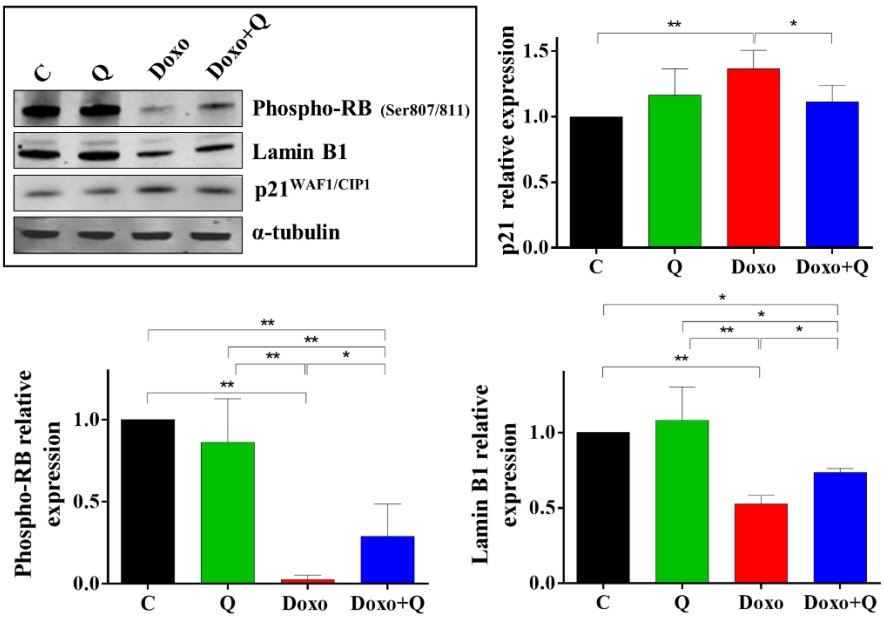

***Figure 29*. Quercetin treatment changes the expression of senescence markers.** Immunoblot representative image (left) and quantification of the expression of phospho-RB, Lamin B1 and p21 in vehicle control (C), Quercetin treated (Q), Doxo-induced senescent fibroblasts non-treated (Doxo) and treated with Quercetin (Doxo+Q). Lysates were collected 3 days after Quercetin treatment. Protein band intensity was normalized to α-tubulin and expressed in relation to control. Data are represented as the mean ± SD of at least four independent experiments (*p<0.05; ** p<0.001). Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *QM*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Doxo+Q*: Doxo-induced senescent fibroblasts treated with Quercetin.

Taken together, these results demonstrated that Quercetin was able to reduce the number of Doxo-induced senescent cells, as supported by the comparable reduced number of SA-β-Gal and macroH2A1 positive/Lamin B1 negative cells, along with the increase in phospho-RB and Lamin B1 level, and the reduced p21 expression, which suggests a reduction of senescent cells that express or not these markers. Notably, no effect was noted on normal proliferating fibroblasts, suggesting that Quercetin exert a detrimental action only on senescent cells.

4.3.2 Evaluation of cell death after Quercetin treatment

The data reported in paragraph 4.3.1 demonstrated that Quercetin can reduce the number of Doxo-induced senescent cells. In order to assess whether this reduction was due to an increase in cell death of senescent cells, we evaluated by flow cytometry the presence of apoptotic and necrotic cells after 3 days of Quercetin treatment, when we have seen the highest reduction in senescent cell number. However, our preliminary data showed that even if Doxo-induced senescent cells treated with Quercetin presented a slight increase in early and late apoptosis with respect to non-treated senescent cells, the differences were not significant (**Figure 30**). Nevertheless, this aspect will be deeply investigated with subsequent studies.

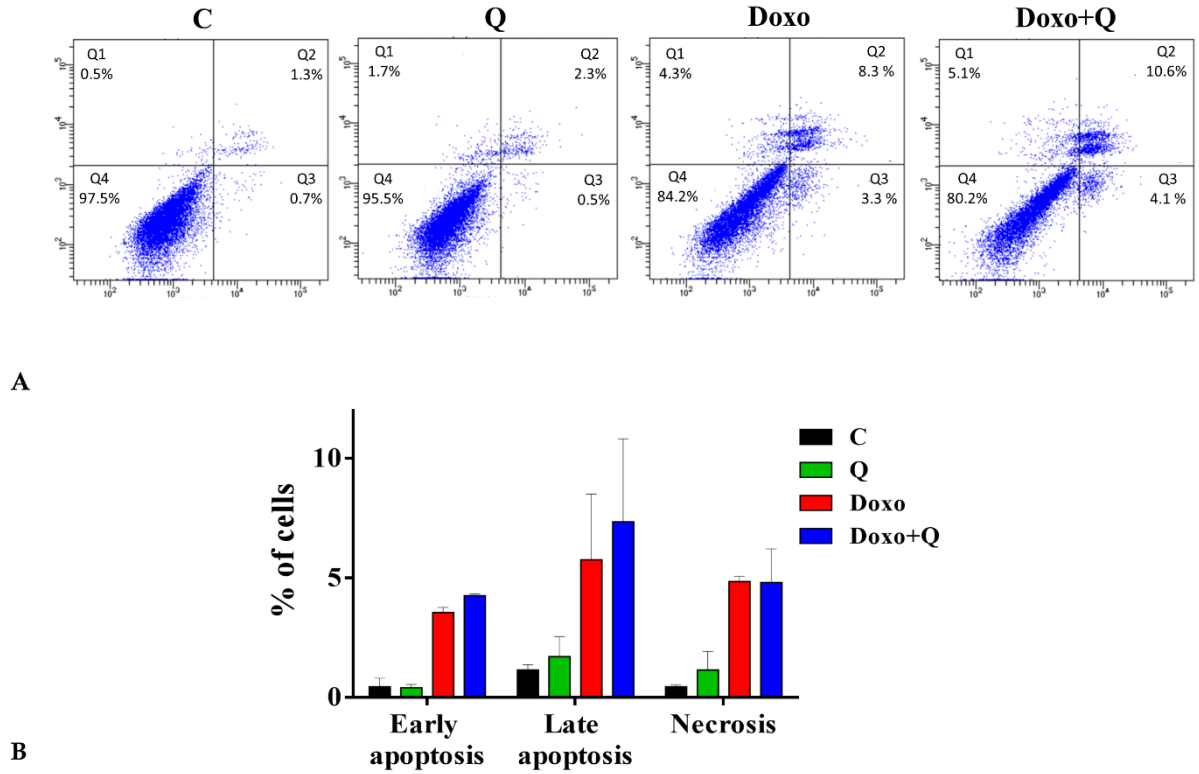

Figure 30. **Determination of early apoptosis, late apoptosis, and necrosis after 3 days of Quercetin treatment on Doxo-induced cell senescence. (A)** Representative dot plots obtained from flow cytometry analysis using PE Annexin V Apoptosis Detection Kit I shown the following populations: necrotic (Q1-upper left), late apoptosis (Q2-upper right), early apoptosis (Q3-lower right), and viable cells (Q4-lower left). **(B)** The percentage of cells in any state is expressed as a mean ± SD of two independent experiments. Abbreviations: *C*: control proliferating fibroblasts treated with vehicle; *QM*: proliferating fibroblasts treated with Quercetin; *Doxo*: Doxo-induced senescent fibroblasts; *Doxo+Q*: Doxo-induced senescent fibroblasts treated with Quercetin.

4.3.3 Effects of Conditioned Medium from Quercetin treated and non-treated Doxo-induced senescent fibroblasts on Osteosarcoma cells

In Section II, we have demonstrated that SM can increase U2OS cell proliferation, migration and invasiveness and that Quercetin pre-treatment, reducing cell senescence and the SASP, can reduce these effects. In this section, we have also shown that Quercetin treatment after the induction of senescence could reduce senescent cells. Thus, in the last part of this work we wanted to evaluate whether conditioned medium from Doxo-induced senescent cells post-treated with Quercetin (SQM) could exert different effects on U2OS cells compared to that of Doxo-induced non-treated senescent fibroblast (SM). As we expected, SM significantly enhanced U2OS proliferation over time compared to control (CM) ($p<0.05$ at 72h and 96h) as well as colony formation and area (**Figure 31**). Interestingly, SQM significantly reduced U2OS cell proliferation compared to SM ($p<0.05$ at 72h and 96h) (**Figure 31A**) and induced a colony

formation rate similar in number and area to that induced by CM, and thus significantly reduced compared to SM ($p<0.05$) (**Figure 31B and C**).

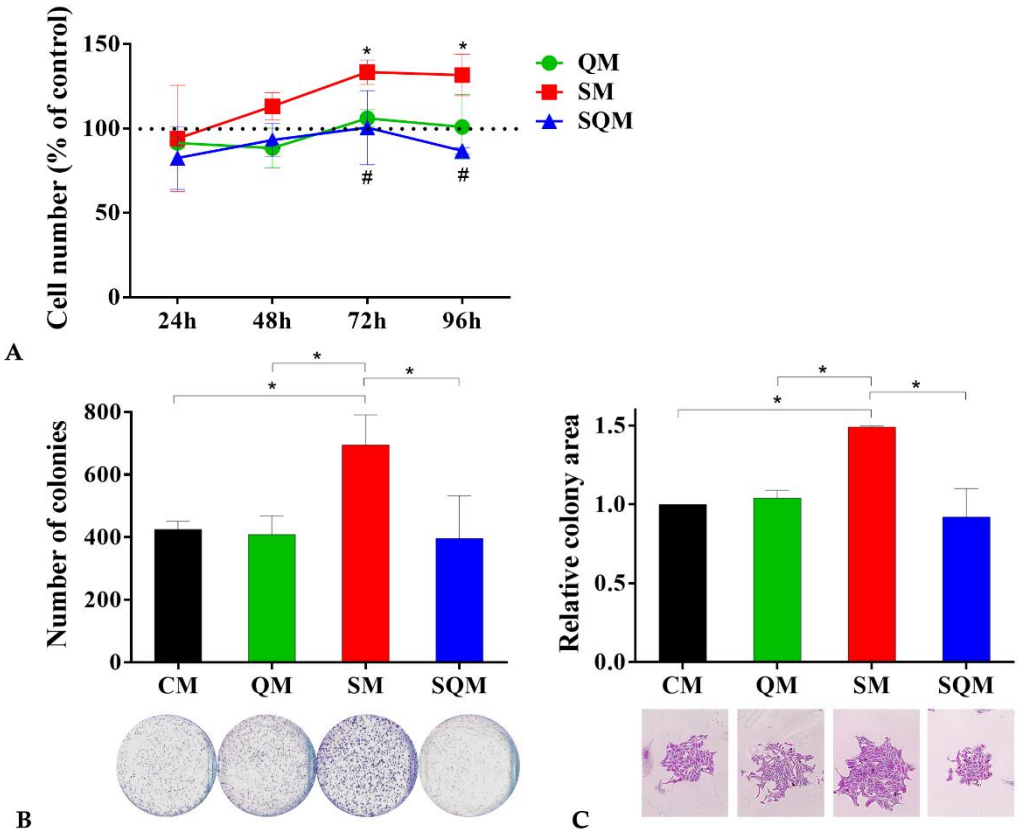

Figure 31. **Effect of Conditioned Medium from Doxo-induced senescent fibroblasts treated and non-treated with Quercetin on U2OS cells growth. (A)** Trypan Blue exclusion assay on U2OS cells treated with conditioned medium from Quercetin treated cells (QM), Doxo-induced senescent fibroblasts (SM), and Doxo-induced senescent fibroblasts treated with Quercetin (SQM). Data are shown as the mean percentage of two independent experiments ± SD relative to U2OS treated with vehicle control conditioned medium (CM). P values refers to differences respect to control (*$p<0.05$) and to SM ($^{\#}p<0.05$). **(B)** Representative images (down) and calculated number of U2OS colonies (upper) formed after 7 days of treatment with conditioned media. Data are represented as the mean of two independent experiments ± SD (*$p<0.05$). **(C)** Colonies area (representative images below) was calculated with Image J Software, and data are expressed relative to control (CM) and represented as the mean of two independent experiments ± SD (*$p<0.05$). Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from proliferating fibroblasts treated with Quercetin; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *SQM*: conditioned medium from Doxo-induced senescent fibroblasts treated with Quercetin.

Moreover, we have analysed the effects of conditioned media on U2OS migration. Our data showed that, as expected, SM produced a significant increase in U2OS cells migration compared to CM ($p<0.05$). Conversely, SQM did not enhance cell migration, which remained significantly lower than that induced by SM ($p<0.05$) (**Figure 32**).

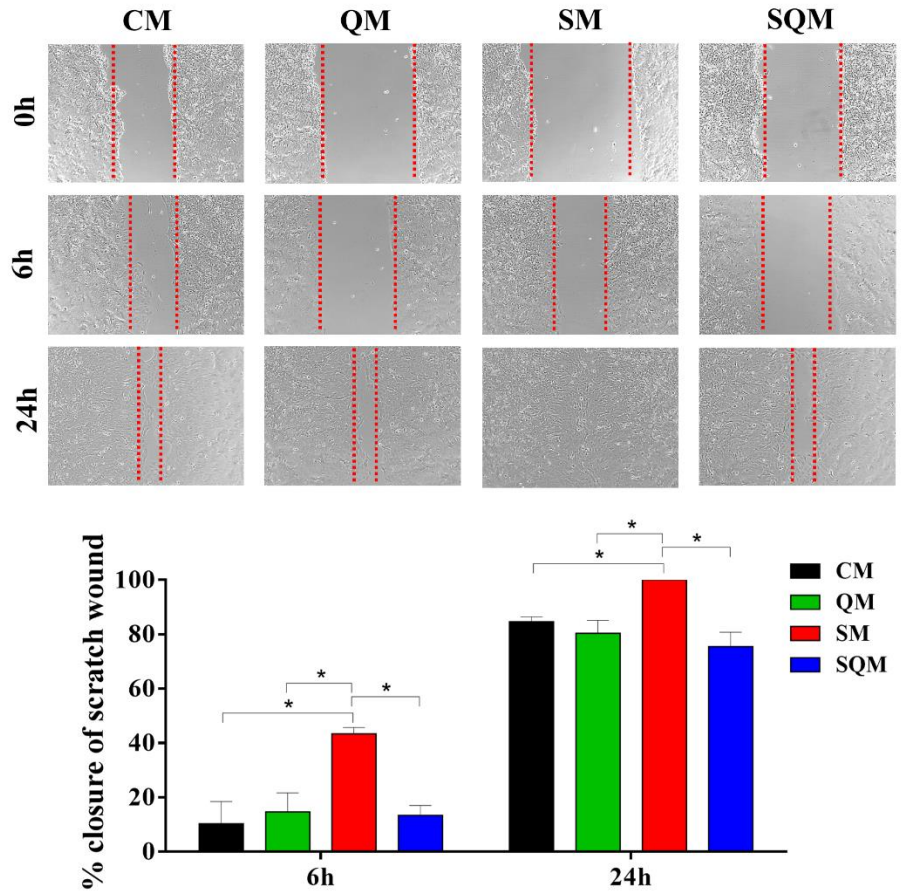

Figure 32. **Effect of Conditioned Medium from Doxo-induced senescent fibroblasts treated and non-treated with Quercetin on U2OS cells migration.** Representative images (upper) and the percentage of scratch wound area (down) of Wound Healing Assay of U2OS cells treated with conditioned media. Data are shown as the mean percentage of two independent experiments ± SD of wound closure area compared to initial scratch (*$p<0.05$). Abbreviations: *CM*: conditioned medium from control proliferating fibroblasts; *QM*: conditioned medium from proliferating fibroblasts treated with Quercetin; *SM*: conditioned medium from Doxo-induced senescent fibroblasts; *SQM*: conditioned medium from Doxo-induced senescent fibroblasts treated with Quercetin.

Overall, these data suggest that Quercetin, reducing the number of senescent cells after senescence induction, can also mitigate the effects on U2OS proliferation and migration. However, further investigations are necessary.

5 DISCUSSION

Aging is a complex biological phenomenon characterized by the progressive accumulation of unrepaired or damaging modifications occurring in molecules, cells, tissues, and organs, which the body tries to counteract by implementing a series of adaptive mechanisms that became an essential part of the aging process (Ostan et al., 2008). Moreover, it represents the major risk factor for several pathologies that occur more frequently in people getting older, named age-related diseases (ARDs). Geroscience identified cellular senescence as one of the nine selected and interconnected biological processes representing the critical pillars of aging and ARDs and determining the aging phenotype (Kennedy et al., 2014; López-Otín et al., 2013). Senescent cells accumulate in different aged tissues over time and contribute to the development and progression of several ARDs, such as cardiovascular diseases, neurodegenerative diseases, and cancer (Collado et al., 2007; He & Sharpless, 2017). Different studies showed that senescent cell clearance *in vivo* extends lifespan and healthspan and attenuates age-related pathologies (Baker et al., 2011, 2016; Childs et al., 2016; Chinta et al., 2018). The deleterious biological effects of senescence are driven mainly by the SASP (Coppé et al., 2010), which can contribute to inflammaging, creating a microenvironment favourable for developing different ARDs, including cancer. In this framework, interestingly, also several chemotherapy drugs have been found to induce the so-called therapy-induced senescence (TIS) in different types of cancer and normal cells (Demaria et al., 2017; Ewald et al., 2010; Saleh et al., 2020). The induction of senescence in cancer cells may be considered a positive outcome for the chemotherapy because it arrests tumour growth, resulting in inhibitory or growth-neutral tumour effects (Ewald et al., 2008; Xue et al., 2007). Conversely, in some situations, senescent cancer cells can promote tumour growth and recurrence, sustaining the disease progression (Hou et al., 2019; Saleh et al., 2019), suggesting that TIS can generate a heterogeneous response that depends on cell types, tissues, organs, and on the type of stimulus that induce the senescence response (Ewald et al., 2010). Recently, different studies have suggested that one of the central mechanisms leading to chemotoxicity is the premature and excessive induction of TIS in non-malignant cells. Findings demonstrated that genetic or pharmacological TIS removal was able to alleviate different therapy-induced toxicity, as fatigue, myelosuppression, cardiomyopathy, bone loss and frailty, and interestingly also cancer progression and relapse (Baar et al., 2017; Demaria et al., 2017; Murali et al., 2018; Sun et al., 2012; Yao et al., 2020). Accordingly, our results showed that the treatment with 50 nM of chemotherapy drug Doxorubicin successfully induced cellular senescence in WI-38 normal human foetal lung

fibroblasts, as evidenced by the acquisition of large and flattened morphology, the arrest of the cell cycle, the downregulation of phosphor-RB and LaminB1, and the increase of p21 expression, SA-β-Gal activity, and SAHF formation. Our results were consistent with several studies that reported the ability of Doxo to induce senescence in different types of cells (Bielak-Zmijewska et al., 2014; Hou et al., 2019; Kozhukharova et al., 2018; Maejima et al., 2008; Piegari et al., 2013), including WI-38 fibroblasts (Liu et al., 2019). Doxorubicin, belonging to the anthracycline family, acts by interacting with DNA by intercalation, causing the inhibition of topoisomerase II and generating free radical species that induce DNA double-strand breaks (DSBs) and cellular damages (Rivankar, 2014). Conversely to what happens in replicative senescence, in which cells preferentially accumulate in the G1 phase of cells cycle, our data, according to other studies (Bielak-Zmijewska et al., 2014; Chang et al., 2002; Roger et al., 2021; M.-Y. Yang et al., 2012), showed that Doxo-induced senescent fibroblasts preferentially accumulate in G2/M phase. This phenomenon could be explained by considering that cells in the S phase, the most targeted by the drug, are damaged and blocked in the subsequent phase, the G2/M. Interestingly, the arrest of the cell cycle in this phase was likely reinforced by the observed high level of p21, which seems to inhibit the mitotic cyclin B1-CDK1 complex, and lead to the reduced phosphorylation of pRB protein with consequently repression of E2F-dependent G2/M regulators (Cherrier-Savournin et al., 2004; Krenning et al., 2014).

Due to the proven important role of cellular senescence in aging and ARDs, recent advances encouraged a search for “senolytic” drugs, that can selectively eliminate senescent cells, or “senomorphic” ones, that can reduce the SASP. The research is focalized especially on those natural substances beneficial for health, such as nutraceutical compounds, which can favour the so-called “healthy aging”. In this framework, in the second part of our work, we demonstrated that a pre-treatment with Quercetin showed a senomorphic activity, partially protecting the cells from Doxo-induced senescence and reducing the SASP. Quercetin is a bioflavonoid, widely distributed in fruits and vegetables, known for its antioxidant, anti-inflammatory, anti-cancer activity (Andres et al., 2018; D’Andrea, 2015), and recently used in combination with the tyrosine kinase inhibitor Dasatinib as senolytic compound (Hickson et al., 2019; Justice et al., 2019; Saccon et al., 2021; M. Xu et al., 2018). Our findings demonstrated that Quercetin pre-treatment reduced the number of SA-β-Gal positive cells, decreasing cell cycle arrests and proliferation block, increasing the expression of phospho-RB, and reducing p21 level. Moreover, WI-38 pre-treated fibroblasts showed an increased Lamin B1 expression, together with a reduction of pH2AX and macroH2A1 foci. Since the formation of SAHF is associated with the stable repression of genes involved in cell cycle progression, and it seems to be strictly

related to the hypo phosphorylation of RB (Narita et al., 2003), the increased expression of phospho-RB in Quercetin pre-treated cells is likely related to the decreased presence of SAHF and consequently to the increased proliferation. Some other works have investigated the role of Quercetin as a geroprotective agent that can delay or reduce the onset of replicative senescence in human cells. Chondrogianni et al. have shown that Quercetin treatment on human embryonic fibroblasts for several days delayed the appearance of replicative senescence, and restored in part their proliferation, maybe due to its antioxidant and proteasome activating properties (Chondrogianni et al., 2010). Moreover, in an *in vitro* model of accelerated aging, Quercetin reduces replicative senescence onset, restoring heterochromatin architecture and decreasing ROS levels (Geng et al., 2019). For the first time, our study demonstrated that a pre-treatment with Quercetin was able to reduce Doxo-induced senescence and DNA damage, exerting its protective effects enhancing the total cellular antioxidant activity and particularly the expression of SOD-1 and SOD-2.

Moreover, we showed that the pre-treatment with Quercetin decreased Doxo-induced ROS production. Consistent with our data, some studies have demonstrated that Quercetin has a high total antioxidant capacity and efficiently protects against oxidative stress (Crascì et al., 2018; Kim et al., 2011; LaManna et al., 2011). Consistent with our data, many evidences from human and animal studies demonstrated that the antioxidant activity of Quercetin is mainly exerted through its effects on antioxidant enzymes, including the upregulation of SOD activity (Dong et al., 2014), signal transduction pathways, and ROS (Xu et al., 2019).

Furthermore, we evaluated whether Quercetin could also inhibit the SASP, which is the principal responsible for the damaging activities of senescent cells (Coppé et al., 2006; Coppé et al., 2008; Dilley et al., 2003; Gonzalez-Meljem et al., 2018; Krtolica et al., 2001; Liu & Hornsby, 2007).

Our data showed that NF-κB protein expression was significantly enhanced in Doxo-treated fibroblasts and that Quercetin pre-treatment prevented this increase. These findings are in accord with other studies showing that Quercetin exerts an anti-inflammatory action suppressing NF-κB expression and its nuclear translocation in different kinds of cells (Chen et al., 2020; Cheng et al., 2019; Lee et al., 2018). We have also investigated the expression of several SASP-related molecules, including IL-6, IL-8, IL-1α, TNF-α, MMP2, and CXCL12, significantly increased in Doxo-induced senescent cells and reduced in Quercetin pre-treated fibroblasts. In addition, another factor as GROα was interestingly reduced by the pre-treatment with Quercetin, although its expression was not found up-regulated in Doxo-treated cells compared to control. This missed increase in GROα expression may be due to the plastic

phenotype of the SASP, which can vary among cell types and stimuli, but also presents fluctuation over time into the same cells (Campisi, 2013; Hernandez-Segura et al., 2018; Jakhar and Crasta, 2019). Interestingly, the production of IL-1α seems to happen in the early stage of senescence, when it plays a fundamental role in the development and maintenance of the SASP (Acosta et al., 2008; Roger et al., 2021). Thus, the reduced expression of this interleukin in Quercetin pre-treated fibroblasts can reinforce SASP suppression by the flavonoid.

Subsequently, we measured the ability of conditioned medium of Doxo-induced senescent fibroblasts (SM) to promote human Osteosarcoma (OS) cells growth and invasiveness and modify the proliferation and migration of normal cells. Osteosarcoma (OS) is a primary malignant bone tumour with a high mortality rate and a bimodal incidence observed worldwide, with the first peak in puberty, and the second peak in old age, mainly in the seventh and eight decades of life (Mirabello et al., 2009b). The appearance of OS in the elderly requires aggressive treatment with both neoadjuvant and adjuvant chemotherapy, and it is frequently associated with a greater incidence of metastasis and worse prognosis (Ek et al., 2006; Mirabello et al., 2009a; Savage & Mirabello, 2011). Doxorubicin is one of the mainstay drugs in the treatment of OS, and our data confirmed its known role as a senescence inducer genotoxic agent. As well as other cancer types, the interaction between OS cells and cells that belong to tumour stroma, such as fibroblasts or mesenchymal cells, is vital for tumour development and progression (Czarnecka et al., 2020). Consistent with this, our results showed, for the first time, that SM was able to significantly increase proliferation and colony formation of U2OS cells and that QSM did not modify OS cells growth compared to the control. In accord with our data, different studies have shown that co-culture of senescent fibroblasts, induced by various stimuli, can promote the growth of pre-neoplastic and malignant cells from several types of tumours (Bhatia et al., 2008; Gonzalez-Meljem et al., 2018; Lawrenson et al., 2010; Liu & Hornsby, 2007; Luo et al., 2011; Pazolli et al., 2009). Overall, the pro-tumorigenic action of SM that we have seen on U2OS cells could probably be due to the SASP, particularly to those factors we have investigated. Indeed, both IL-6 and IL-8 were shown to be essential factors in the malignant transformation of OS cells due to their ability in increasing proliferation, colony formation, migration and invasiveness of U2OS and MG-63 cells (Cortini et al., 2016; Gross et al., 2018; Lin et al., 2013; Zhang et al., 2019).

Furthermore, some studies showed that serum levels of IL-6, IL-8, and TNFα were higher in OS patients than controls (Kushlinskii et al., 2014; Xiao et al., 2014; Zhang et al., 2019) and that IL-6 increased level was associated with worse TNM stages, and presence of metastasis,

while TNFα and IL-8 levels correlated with bigger tumour size and maybe the progression of the disease (Xiao et al., 2014).

The increased mortality and worse prognosis observed in older people affected by cancer could be fueled by a chronic, low-level inflammatory state typical in the elderly, named "inflammaging", and could represent one of the links between ageing and cancer. Considering these data, the further increase of pro-inflammatory cytokines due to Doxo-induced senescent fibroblasts could increase the inflammaging of elderly patients, potentially favouring the creation of a pro-tumour microenvironment that could contribute to the worse prognosis observed in older. In this perspective, Quercetin, which is already recognized as an anti-inflammatory agent, reducing IL-6, IL-8 and TNF-α expression and consequently the pro-tumorigenic activity of Doxo-induced senescent fibroblasts, could potentially be used to counteract the detrimental effect of therapy-induced senescence in cancer treatment.

Furthermore, we have also demonstrated that SM stimulated U2OS migration and invasiveness and that QSM, did not induce the same aggressive behaviour. Accordingly, different studies showed that senescent cells had been found to contribute also to the acquisition of invasive and metastatic properties of several types of cancer cells (Alspach et al., 2013; Coppé et al., 2008; Farsam et al., 2016; Hassona et al., 2014; Y. H. Kim et al., 2017). Our results showed that Doxo-induced senescent fibroblasts overexpressed CXCL12, a chemokine that functions as a chemoattractant, confirming what was seen in other senescent cells types (Choi et al., 2021; Jiang et al., 2019; Y. H. Kim et al., 2017). Several studies have shown the importance of CXCL12/CXCR4 (C-X-C motif chemokine receptor 4) pathway in OS progression and prognosis (Laverdiere et al., 2005; Lu et al., 2015; Neklyudova et al., 2016; Oda et al., 2006; Perissinotto et al., 2005; Pollino et al., 2019), and the overexpression of CXCR4 was detected in OS patients, with high-grade disease, and also in two different lines of OS cells, 143-B and U2OS (Pollino et al., 2019). Thus, the proven upregulation of CXCR4 in U2OS cells (Pollino et al., 2019) and the overexpression of its ligand CXCL12 by Doxo-induced senescent fibroblasts identifies this axis as one of the best candidates for driving SM effects on U2OS cells migration and invasion. Conversely, the reduction by Quercetin pre-treatment of CXCL12 expression maybe explains the reduced aggressiveness of U2OS cells incubated with QSM. Otherwise, another important SASP factor that can promote the invasive activity of several cancer cells, including U2OS (Hassona et al., 2014; Liu et al., 2016; Malaquin et al., 2013), is MMP2. MMP2 belongs to the family of metalloprotease, and it can be synthesized by different cell types, including that belonging to tumour stroma (Egeblad & Werb, 2002) and several senescent cell models (Hassona et al., 2014; Malaquin et al., 2013). Accordingly, Doxo-induced

senescent fibroblasts upregulated MMP2 expression, which can contribute to the increased invasiveness of U2OS, although Quercetin did not significantly reduce its expression compared to senescent cells.

Furthermore, in accord with other studies (Hou et al., 2019; Krtolica et al., 2001), the promoting effects of senescent fibroblasts did not affect normal human cells, in which the different CM did not alter the migration, while the proliferation was significantly reduced by SM compared to control. This reduction in proliferation could be due to the induction of senescence via "bystander effect", as shown in other works (Acosta et al., 2013; da Silva et al., 2019; Nelson et al., 2012, 2018), but our preliminary data (not shown) did not demonstrate it.

These data suggest that the senescent microenvironment can synergize only with neoplastic cells favouring their malignant features. Therefore, Quercetin pre-treatment, acting as a senomorphic agent, can reduce the effect of Doxo-induced senescent fibroblasts both on OS cells, reducing their aggressiveness and maybe blocking the spread of senescence on normal fibroblasts.

In the third section of this work, we evaluated whether Quercetin could also exert a senolytic action on Doxo-induced senescent fibroblasts, and it could reduce, through the elimination of senescent cells, their pro-tumorigenic effects on U2OS. Our results showed that Quercetin treatment of 3 days after Doxo-induction of senescence reduced the number of senescent fibroblasts, decreasing the percentage of SA-β-Gal positive cells, along with the expression of other senescence markers, without affecting proliferating cells. The senolytic activity of Quercetin has been previously studied in different types of cells (Hohmann et al., 2019; Hwang et al., 2018; Lewinska et al., 2020; Zhu et al., 2015). Zhu and colleagues tested Quercetin first as senolytic substance because it was selected among the drugs that potentially target overexpressed gene products that protect senescent cells from apoptosis (Zhu et al., 2015). The treatment with Quercetin for 3 days at different doses (from 5 to 50 μM), showed that the flavonoid reduced the vitality and caused cell death of irradiated-senescent HUVECs and mouse BM-MSCs to a greater extent than proliferating cells (Zhu et al., 2015).

Conversely, Quercetin treatment was less effective on preadipocytes and did not significantly affect primary mouse embryonic fibroblasts (Zhu et al., 2015). In the same work, the authors also tested the senolytic action of Dasatinib, a tyrosine kinase inhibitor. They found that Dasatinib was preferentially effective on preadipocytes than on senescent HUVECs and did not affect either mouse fibroblasts or mouse BM-MSCs, suggesting that the senolytic action may also be considered cell-type specific (Zhu et al., 2015). Consistent with the data obtained on HUVEC and mouse BM-MSCs, our results showed that Quercetin could specifically reduce the

number of Doxo-induced senescent fibroblasts and did not affect the viability of proliferating cells, demonstrating to have a senescent-specific effect. However, our preliminary data did not evidence a significant enhancement of cell death in senescent fibroblasts treated with Quercetin, but further investigations are necessary.

Conversely, a study on arterial vascular endothelial cells revealed that Quercetin at a concentration that reduced senescent cells number also caused significant cell death in proliferating cells, evidencing that there was no evidence of senescent cell-specific cell death (Hwang et al., 2018). Consistent with these data, also hydrogen peroxide-induced senescent human foreskin fibroblasts BJ treated with Quercetin for 24h and then incubated in fresh medium for the subsequent seven days, presented increased apoptosis and cell death comparable to that of proliferating cells and did not show changes in the number of SA-β-Gal positive cells (Lewinska et al., 2020). Instead, a study conducted on normal senescent lung and idiopathic pulmonary fibrosis fibroblasts showed that the treatment with Quercetin 50 µM for 24h did not induce significant changes in apoptosis and viability of senescent cells but demonstrated an increase of Fas expression in senescent cells. However, interestingly, Quercetin could render senescent fibroblasts susceptible to apoptosis induced by FasL and TRAIL (Hohmann et al., 2019). Overall, these findings demonstrate that the role of Quercetin alone as senolytic may be quite controversial and had to be considered cell-type specific (Demirci et al., 2021). Furthermore, our results showed for the first time a potential action of the flavonoid against Doxo-induced senescent fibroblasts, even if the mechanisms through Quercetin exert its senolytic effect required more investigations.

In the last part of this work, we have analysed whether Quercetin post-treatment of Doxo senescent fibroblast could have the same outcomes seen in Section II on U2OS cells. Our results confirmed that SM increased U2OS cells growth and migration, and interestingly that CM from senescent fibroblasts treated with Quercetin (SQM) reduced these effects. Several studies have demonstrated that senescent cells clearance can exert an anti-cancer effect. For example, a study on a transgenic mouse that enables the inducible killing of senescent cells expressing $p16^{INK4a}$, has shown that eliminating senescent cells reduced spontaneous tumour formation (Baker et al., 2016). Moreover, senolytic compounds have also been proven to delay tumour recurrence and metastasis in mouse cancer models after chemotherapy (Demaria et al., 2017) and ameliorate side effects associated with the treatment (Baar et al., 2017).

Interestingly, Guerrero et al. showed that broad-spectrum senolytics could eliminate senescent cancer cells and senescent pre-neoplastic and normal cells, like fibroblasts, induced by anti-cancer therapy (Guerrero et al., 2019). However, it could be possible that in our model, the post-

treatment of senescent fibroblasts with Quercetin could have a senolytic effects, reducing senescent cells rate, but at the same time it could potentially exert also at this level a senomorphic action reducing the SASP. Thus, the reduced growth and migration of U2OS due to SQM could be the outcome of combining these two activities of the flavonoid.

6 CONCLUSION

This study was the first to show that Quercetin, a natural bioactive flavonoid, can prevent Doxo-induced senescence as a senomorphic agent that reduces the effects of the SASP on OS and normal cells. Increasing cellular antioxidant defence, Quercetin was able to protect in part normal fibroblasts to the damaging effect of ROS produced by Doxo treatment, decreasing SAHF formation, Lamin B1 loss, NF-κB expression, and consequently the levels of some SASP molecules (**Figure 33A**), suppressing the pro-tumour effects of senescent cells. Moreover, our work demonstrated that Quercetin can also exert a potential senolytic activity on Doxo-induced senescent fibroblasts, reducing their number, along with the percentage of SA-β-Gal positive cells, without affecting the viability of proliferating cells. Although the mechanisms by which Quercetin carried out its damaging action had to be clarified, our preliminary data demonstrated that it could neutralize, also in this condition, the pro-tumour effects of Doxo-induced senescent fibroblasts, decreasing U2OS cells growth and migration (**Figure 33B**).

Although other studies are necessary to deepen the role of Quercetin as a senomorphic and senolytic agent against Doxo-induced senescence, this work may pave the way to investigate the use of Quercetin as an adjuvant in the treatment of OS, trying to reduce the damaging effects of therapy-induced senescence, especially in elderly patients, in which senescent cells are already abundant in several tissues and organs.

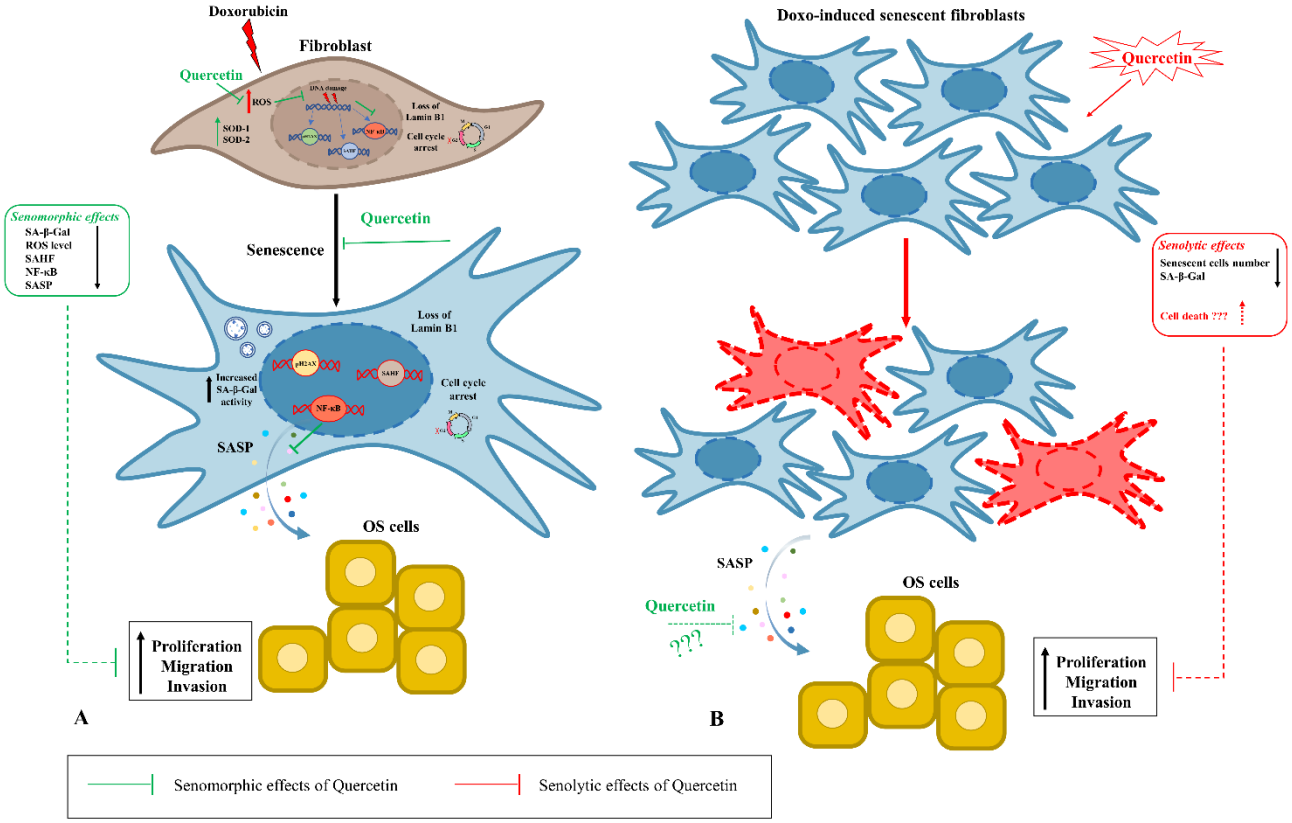

Figure 33. **The senomorphic action and the potential senolityc effect of Quercetin against Doxo-induced senescence hypothesized in our work. (A)** Quercetin, enhancing cellular antioxidant defence, was able to protect the cells from Doxo-induced senescence and damage, reducing SA-β-Gal positive cells number, SAHF formation, and cell cycle arrest. Moreover, it exerted its senomorphic action decreasing NF-κB level and consequently the expression of some SASP factors, inhibiting the promoting effects of senescent cells on U2OS cells growth and invasiveness. **(B)** The treatment with Quercetin after the induction of senescence by Doxorubicin reduced the number of senescent fibroblasts, and the percentage of cells positive for SA-β-Gal staining, decreasing the presence of senescence markers in senescent treated cells, without affecting proliferating fibroblasts. Moreover, the post-treatment with Quercetin was able to abrogate the pro-tumour effects of Doxo-induced senescent fibroblasts conditioned medium on U2OS cells. This suggest that Quercetin could have a senolytic effects, reducing senescent cells rate, but at the same time it could potentially exert also at this level a senomorphic action reducing the SASP. Abbreviations: *OS*: Osteosarcoma; *ROS*: Reactive oxygen species; *SA-β-Gal*: Senescence-associated β-galactosidase activity; *SAHF*: Senescence-associated heterochromatin foci; *SASP*: Senescence-associated secretory phenotype; *SOD*: Superoxide dismutase.

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