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Role of microRNAs in inflammaging and age related diseases

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Summary

The elderly population is growing in the world due to increased life expectancy combined with declining birth rates. As people reach advanced age, they often face years of disability marked by multiple chronic diseases, poor mobility, and loss of independence. This burden of disability has caused many to worry about the impact of an aging population on social and economic stability. A huge challenge for modern biomedical research is to shorten as much as possible this period of frailty and disability to improve health span (Tchkonia et al., 2013) and to achieve "healthy aging" (Olivieri et al., 2017).

"Aging" is not a static but rather a dynamic phenotype that changes over time. Continuous interaction between individuals' genetic makeup and environmental factors results in a spectrum of states that range from healthy aging to age-related disease. A pervasive feature of aging is a chronic and systemic pro-inflammatory status, termed "Inflammaging". The source of this age-related systemic chronic inflammation was mainly attributed to the progressive activation of immune cells over time (Franceschi et al., 2000) even if today a variety of non-immune aging cells have been targeted as pro-inflammatory. Regardless of the cell types involved, there are growing epidemiological evidences that a state of bland inflammation is associated with and predicts several age-related diseases (ARDs), including

type 2 diabetes mellitus (T2DM) and its complications (Prattichizzo et al., 2016). Therefore, aging is the main risk factor for several pathological conditions.

Cellular senescence is historically associated with aging. However recently, the role of senescent cells in tissue remodelling during many physiological processes, including placental biology, embryonic patterning, wound healing, and tissue stress responses caused by cancer therapy has been suggested and it's getting a lot of interest (Malaquin et al., 2016). Besides growth arrest, a significant feature of senescent cells is their ability to modify their immediate microenvironment using a senescence-associated (SA) secretome, commonly termed the SA secretory phenotype (SASP) (Coppè et al., 2008). This pro-inflammatory status of senescent cells could be a fundamental additional contributor to systemic inflammation. The identification of pathways that control age related inflammation across multiple systems is therefore important in order to understand whether treatments that modulate inflammaging may be beneficial in old people. Potential strategies for mitigating the deleterious effects of senescent cells include interfering with pathways that lead to senescence-associated growth arrest, eliminating senescent cells, and interfering with the adverse effects of senescent cells by targeting the SASP (Tchkonia et al., 2013).

Mitochondria dysfunction is deeply involved in the aging process and with the pathogenesis of major ARDs. Hallmarks of mitochondrial dysfunction include decreased efficacy of the respiratory chain and ATP production, decreased mitochondrial membrane potential, damaged cristae, swollen organelles, increased oxidative stress, and decreased mitochondrial DNA (mt-DNA) copy number. In addition to energy production, mitochondria play a central role in apoptosis, buffering calcium release, retrograde signalling to the nuclear genome, producing reactive oxygen species (ROS), participating in steroid synthesis, signalling to the

immune system, as well as controlling the cell cycle and cell growth (Coppotelli and Ross, 2016). Furthermore, mitochondria can promote inflammation releasing pro-inflammatory molecules and inducing inflammasome activation.

The identification of the pathways that control age related inflammation across multiple systems is therefore important because their modulation by specific treatments may be beneficial in old people. Recently, microRNAs (miRNAs or miRs) were largely investigated.

MicroRNAs are small single-strand non-coding RNAs involved in gene expression that are increasingly recognized as epigenetic regulators of gene expression, modulating virtually all cellular processes, including senescence. MiRs can silence the mRNA target in most of cases by binding its 3'-untranslated region (UTR) in the cytoplasm, but they can also exert post-transcriptional control when bound to a region outside of the 3'UTR specifically within the 5'UTR and coding regions of the mRNA target. Moreover, in some cases, they activate transcription of a specific gene or stabilize the mRNA. A single miRNA has the ability to regulate multiple targets and, in turn, a single mRNA can be targeted by several miRs (Breving and Esquela-Kerscher, 2010). Over the years, a set of miRNAs with a well-recognized role in inflammaging, organismal aging, and development of ARDs has been defined (Olivieri et al., 2013; Olivieri et al., 2015); examples are miR-146a-5p, -21, -126a-3p. Interestingly, recent studies suggest that microRNAs can be found associated or inside mitochondria (mitomiRs) affecting their function, and that subset of these are SA-microRNAs (Bandiera et al., 2011). These miRs may play an important role in cellular senescence.

Interestingly, miRNAs can be released and also detected outside the cells. Recent evidence has confirmed that some features of aging are reflected in the profile of circulating miRNAs

and their shuttles (Olivieri et al., 2017). For these reasons, miRNAs are considered to be attractive candidates as diagnostic, prognostic and predictive biomarkers (Iorio and Croce, 2012), especially in ARDs such as T2DM (de Lucia C et al., 2017; Prattichizzo et al., 2015). The ARDs may well be characterised by molecule signatures whose identification would take us a little closer to discovering the biomarkers of health deterioration during aging. MiRNAs seem good candidates in this context.

1. Introduction

1.1 Cellular senescence contributes to onset and maintenance of inflammaging

Aging is an inevitable outcome of life, characterized by a gradual decline in tissue and organ function and increased risk of disease and death (López-Otín C et al., 2013). It is a complex phenomenon that results from environmental, stochastic, genetic, and epigenetic events in different cell types and tissues and their interactions all the lifelong (Franceschi and Campisi, 2014). Human aging is characterized by a chronic, low-grade inflammation and this phenomenon has been termed “inflammaging”. Inflammaging is a systemic condition in the absence of overt infection (“sterile” inflammation) and it represents a highly significant risk factor for both morbidity and mortality in the elderly people, as most if not all age-related diseases share an inflammatory pathogenesis (Franceschi, 2000).

Acute inflammation can be beneficial: transient immune response is necessary to counteract harmful conditions such as traumatic tissue injury or an invading pathogen. However, acute inflammatory responses may be defective during aging. Many of the features of acute inflammation continue as the inflammation becomes chronic, but this one is usually of low

grade and persistent, resulting in responses that lead to tissue degeneration (Franceschi and Campisi 2014; Freund et al., 2011).

The number of senescent cells increases in multiple tissues with chronological aging, and senescent cells are present at sites of age-related pathology (Herbig et al., 2006; Lawless et al., 2010; Krishnamurthy et al., 2006; Jeyapalan et al., 2007). Senescent cells are usually cleared in normal tissues by the immune system (Kang et al., 2011). However, in aged person immunosenescence, characterized by the loss of function of immune system, compromises the clearance of senescent cells and exacerbates inflammation (Franceschi et al., 2000; Shaw et al., 2010).

Because the number of senescent cells increases with aging, it has been widely assumed that senescence contributes to aging. This claim is mainly sustained by data on animal models showing that periodic clearance of senescent cells, using genetic systems or drugs, is accompanied by a mean lifespan and healthspan extension, coupled with a reduced inflammatory gene expression in multiple tissues, including kidney and the heart (Baker et al, 2011). Moreover, the accumulation of senescent cells has recently been suggested to be a crucial factor contributing to systemic inflammation. Mechanistically, senescent cells likely fuel chronic inflammation because of acquisition a specific phenotype named "senescence-associated secretory phenotype" (SASP), characterized by the enhanced secretion of pro-inflammatory molecules and mediators (Tchkonia et al., 2013). Thus, senescent cells burden can modify the tissue microenvironment in a SASP-mediated manner and these spots are prominent sites of many age-related pathologies. Clearly, preventing development of the SASP or ameliorating its effects can mitigate the deleterious effect of senescent cells.

1.2 The hallmarks of cellular senescence

Cellular senescence can be defined as a stable arrest of the cell cycle coupled to continued viability, metabolic activity and stereotyped phenotypic changes (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007; Kuilman et al., 2010). More than 50 years ago, Hayflick and Moorhead observed that fibroblasts from healthy human donors had a limited ability to proliferate in culture. The non-dividing cells remained viable for many weeks, but failed to duplicate despite the presence of space and nutrients in the medium. When these cells became irreversibly arrested, they reached the so-called "Hayflick limit" (Hayflick and Moorhead, 1961). Today, a similar irreversible cell cycle arrest, now designated as "cellular senescence" can be triggered by a myriad of stimuli, including activation of oncogenes, telomere erosion or damage, mitochondrial deterioration, oxidative stress, and excessive DNA damage (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007; Kuilman et al., 2010; Baker and Sedivy 2013).

Telomeres are regions of repeat sequences, at the end of each linear chromosome, bound by multiple telomeric proteins. In mammalian cells, telomere DNA contains double-stranded tandem repeats of TTAGGG followed by terminal 3′ G-rich single-stranded overhangs. Telomere DNA probably adopt the T-loop structure, where the telomere end folds back on itself and the 3′ G strand overhang invades into the double-stranded DNA (the so-called D-loop). Functional telomeric structure prevents the degradation or fusion of chromosome ends, and thus is essential for maintaining the integrity and stability of eukaryotic genomes (Lu W et al., 2013). Due to the intrinsic inability of the replication machinery to copy the ends of linear molecules, telomeric DNA is subject to attrition during mitosis and the replication process can go on until a critical threshold of telomere length is reached.

Telomeric DNA is also highly prone to oxidative damage, and the increase in oxidative stress induces its shortening (Rhee et al., 2011; von Zglinicki, 2000). For these reasons, telomeres shortening is a widely used indicator of replicative senescence and cumulative genomic damage in somatic cells (Allsopp RC and Harley CB, 1995).

Dysfunctional telomeres, and non telomeric-DNA damage, trigger a classical DNA damage response (DDR), which imply the recruitment and activation of an apical DDR kinase like ATM (ataxia-telangiectasia mutated), to the damage site (d'Adda di Fagagna et al., 2003). In addition, compounds (e.g. histone deacetylase inhibitors), which relax chromatin without physically damaging DNA, activate the DDR proteins (Bakkenist and Kastan, 2003). The DDR is an evolutionarily conserved signaling cascade, which directs cell fate toward cell cycle progression, senescence or apoptosis. Both damage- and telomere-initiated senescence depend strongly on p53 and are usually accompanied by expression of p21 (Campisi and d'Adda di Fagagna, 2007). In addition to p53, replicative senescence is linked to p16INK4A (a cyclin-dependent kinase inhibitor). P16INK4A is encoded, together with ARF, by the INK4a/ARF locus. Indeed, activation of both the p53 and p16INK4A–RB pathways is essential for induction of senescence in a variety of human cell types (Kuilman et al., 2010).

1.2.1 Senescence markers: What defines a senescent cell?

Senescent cells display several characteristics (Figure 1) which, together, can partially let us define the senescent state:

i. permanent growth arrest state, which cannot be reversed by known physiological stimuli;

ii. morphological changes, such as increased cell size and distinctive enlarged flat morphology;

iii. increased activity of SA–β-galactosidase (β-gal) (Dimri et al., 1995), which partly reflects the expansion of the lysosomal compartment, giving rise to an increase in β-gal activity that can be measured also at suboptimal pH 6 (Kurz et al., 2000);

iv. activation, by most senescent cells, of p16INK4a (Alcorta et al., 1996);

v. appearance of persistent nuclear foci DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) and accumulation of SA heterochromatic foci (SAHF) (Salminen et al., 2012). These foci contain activated DDR proteins, including phospho-ATM and phosphorylated ATM/ataxia telangiectasia and Rad3 related (ATR) substrates (d'Adda di Fagagna et al., 2003). This leads to the local phosphorylation of multiple ATM substrates in the chromatin surrounding the DNA lesion, usually including γH2AX (a phosphorylated form of the histone variant H2AX);

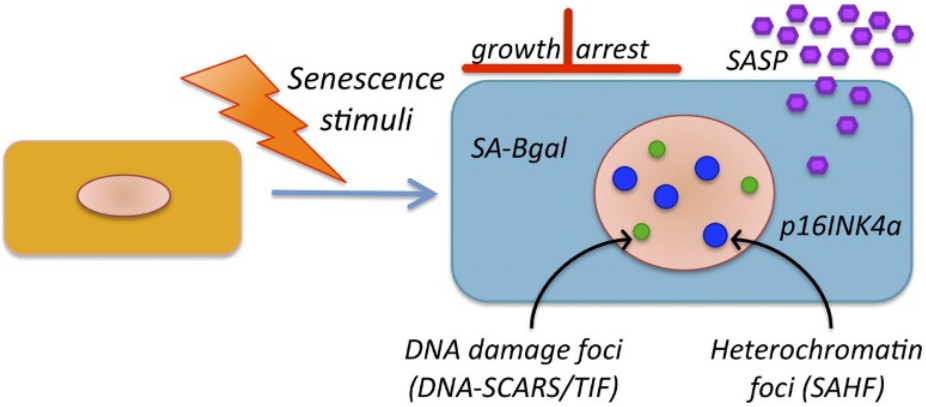


Figure 1. Hallmarks of senescent cells. Markers of senescent cells include an essentially irreversible growth arrest; expression of SA-β-gal and p16INK4a; robust secretion of numerous factors (SASP); and nuclear foci containing DDR proteins (DNA-SCARS/TIF) or heterochromatin (SAHF) (Rodier and Campisi, 2011).

vi. secretion of a plethora of proinflammatory factors, including growth factors, proteases, cytokines and chemokines, which have potent autocrine and paracrine activities (Coppé et al., 2008).

vii. altered susceptibility to apoptosis and un-efficient autophagy.

1.2.2 Senescence-associated secretory phenotype (SASP)

Cells undergoing senescence display dramatic alterations in their secretome, acquiring a typical secretory phenotype termed SASP (Coppé et al., 2008; Rodier et al., 2009). The SASP consists of a plethora of factors that includes cytokines (i.e. interleukin (IL)-1α/β, IL-6, IL-8), growth factors (i.e. EGF, FGF, VEGF), proteases (i.e. metalloproteinase MMP-1, -2, and -3) and other non-soluble extracellular matrix proteins (i.e. collagens, fibronectin, laminin). Although a core of pro-inflammatory factors is a feature of all senescent cells, the specific composition of the SASP depends on the cell type and senescence inducer (Coppé et al., 2010). The SASP is a temporally regulated program. In culture, cells develop a full SASP five days later the senescence induction. Not all SASP factors start to be secreted at the same time (Coppé et al., 2008). Indeed, most of them are released in small amounts until several days after the induction of genotoxic stress. Accordingly, recent studies have shown that a certain number of secreted factors can be expressed much earlier than others, and can influence the subsequent expression of other SASP factors (Malaquin et al., 2016). In particular, pro-inflammatory cytokines IL-1α and IL-1β play critical role in the early phase of the SASP. Indeed, IL-1α acts as upstream regulator of IL-6/IL-8 cytokine network (Orjalo et al., 2009). Of note, during senescence, IL-1α and IL-1β are both secreted at low levels compared to IL-6 and IL-8 (Bhaumik et al., 2009; Coppe et al., 2008), which can act in an autocrine manner to reinforce the senescence growth arrest (Acosta et al., 2008). IL-1β

originates from its precursor pro-IL1β and it is activated to its mature secreted form after a proteolytic cleavage mediated by a component of the inflammasome complex, the caspase-1 (Galliher-Beckley et al., 2013). IL-1α acts intracellularly or as a cell surface-bound protein. Beyond the early phase of the SASP, IL-1α has been implicated in the upregulation of microRNA-146a/b, which are responsible for the inhibition of interleukin-1 receptor-associated kinase 1 (IRAK-1) and the resulting reduction in IL-6 in senescent fibroblasts naturally expressing high levels of IL-6 in their mature SASP. This mechanism suggests the possibility that a negative feedback loop may restrict the activity of the late mature SASP to prevent pervasive chronic inflammation, and that this loop may be established or alternatively controlled via microRNAs after the appearance of the mature late SASP signature (Bhaumik et al., 2009).

The SASP program is triggered by a variety of genotoxic senescence-inducing stress (e.g. telomere shortening or damage, cytotoxic drugs, radiation, oncogene activation, oxidative stress) (Coppé et al., 2008). DNA damage activates DDR, which allows for the recruitment and activation of ATM and subsequent DDR signaling cascade. Importantly, when DNA lesions are irreparable and a DDR signal is persistent, cells activate the senescence program and consequently all SA phenotypes, including SASP. Persistent DNA damage signaling is required for maintaining senescence-associated inflammatory cytokine secretion (Rodier et al., 2009). However, when a senescent-like phenotype is triggered in cells that overexpress cell-cycle inhibitors, such as p16 or p21, cells undergo a growth arrest with many characteristics of senescent cells, but fail to activate DDR and do not produce SASP (Coppé et al., 2010). Notably, although the DDR is important for both the SASP and SA growth arrest, the molecular regulation of these two processes is quite different, as p53 plays no role in establishing and maintaining the SASP despite its crucial function in regulating the SA

growth arrest (Coppé et al., 2008). Passos *et al.* proposed that a persistent DDR triggers continued production of reactive oxygen species (ROS), forming a dynamic feedback loop that actively maintains a deep senescent state (Passos et al., 2010).

However, canonical DDR signaling seems not be sufficient for the SASP. Another important pathway activated in response to cell stress is the mitogen-activated protein kinase p38 (p38MAPK). P38MAPK phosphorylation is important for the establishment of the senescence growth arrest due to its ability to activate both the p53 and pRb/p16 pathways. Freund and colleagues showed that p38MAPK activity is necessary and sufficient for the development of a SASP in cells induced to senesce by direct DNA damage or oncogenic RAS. In these contexts, p38MAPK is not activated quickly, but rather with delayed kinetics characteristic of the SASP (Freund et al., 2011).

These different signaling pathways (DDR, p38MAPK and IL-1 pathway) converge toward the activation of the nuclear transcription factor-κB (NF-κB), necessary for the expression of genes encoding SASP factors (Salminen et al., 2012) and CCA AT/enhancer–binding protein beta (C/EPBβ) (Acosta et al., 2008). Interestingly, these transcription factors were previously known to be involved in the regulation of cellular stress and inflammatory signals by promoting the expression of many cytokines (Pahl, 1999). Recently, another transcription factor - GATA4 - has also been identified as a senescence and SASP regulator via indirect activation of NF-κB. Indeed, GATA4 is degraded by p62-mediated selective autophagy, except during senescence, where its activation depends on ATM action (Kang et al., 2015). Interestingly, mTOR-inhibitor rapamycin, which has long been known to extend lifespan and health span in mice (Harrison et al., 2009) controls SASP protein secretion by enhancing IL-1α translation (Laberge et al., 2015). Recently, sensing of cytoplasmic chromatin by the

cGAS/STING pathway has been proposed as a trigger for SASP induction (Dou et al., 2017; Glück et al., 2017).

A recent work by Wiley *et al.* has shown that mitochondrial dysfunctions cause cell senescence and a distinct secretory phenotype termed MiDAS (mitochondrial dysfunction-associated senescence). Senescent cells with mitochondrial dysfunction do not secrete inflammatory components typical of the SASP, such as IL-6, IL-8 or IL-1β. The authors show that MiDAS was not caused by accumulation of nuclear DNA damage or oxidative stress but rather by a decreased ratio of NAD^+/NADH and the resulting activation of AMP-activated protein kinase (AMPK), which in turn activates p53 (Wiley et al., 2015; Gallage and Gil, 2015).

SASP can have a beneficial role in a variety of processes modifying the microenvironment, triggering immune surveillance and enforcing senescent cell cycle arrest in an autocrine and paracrine manners (Rodier and Campisi, 2011). In the physiological setting, senescent cells play an important beneficial role in embryonic development and in the organization of tissue repair responses that promote wound healing (Demaria et al., 2014; Muñoz-Espín et al., 2013; Storer et al., 2013). Moreover, SASP can play critical role in removing the transient senescent cells inducing immune-mediated clearance and modulating the differentiation of neighboring cells (Parrinello et al., 2005; Krizhanovsky et al., 2008). In fact, it has been suggested that senescent cells can also promote cellular plasticity and tissue regeneration (Ritschka et al., 2017). In the context of cancer, SASP also contributes to the clearance of damaged senescent tumor cells by enhancing both innate and adaptive immunity (Xue et al., 2007; Kang et al., 2011).

In contrast, the effects of the SASP could be deleterious: it can transmit cellular senescence on nearby healthy cells, thus representing a non-cell-autonomous mechanism (Acosta et al.,

2013). This paracrine effect could potentially explain persistent chronic inflammation, also known as inflammaging that contributes to multiple age-related phenotypes (Franceschi and Campisi, 2014). In the case of cancer microenvironment, SASP of senescent stromal fibroblasts can sustain tumor growth and invasion and can even promote long-term cancer therapy resistance (Krtolica et al., 2001). Thus, we can conclude that the ultimate outcome of SASP and in general of senescence within a tissue seems to be highly dependent on the context (Figure 2).

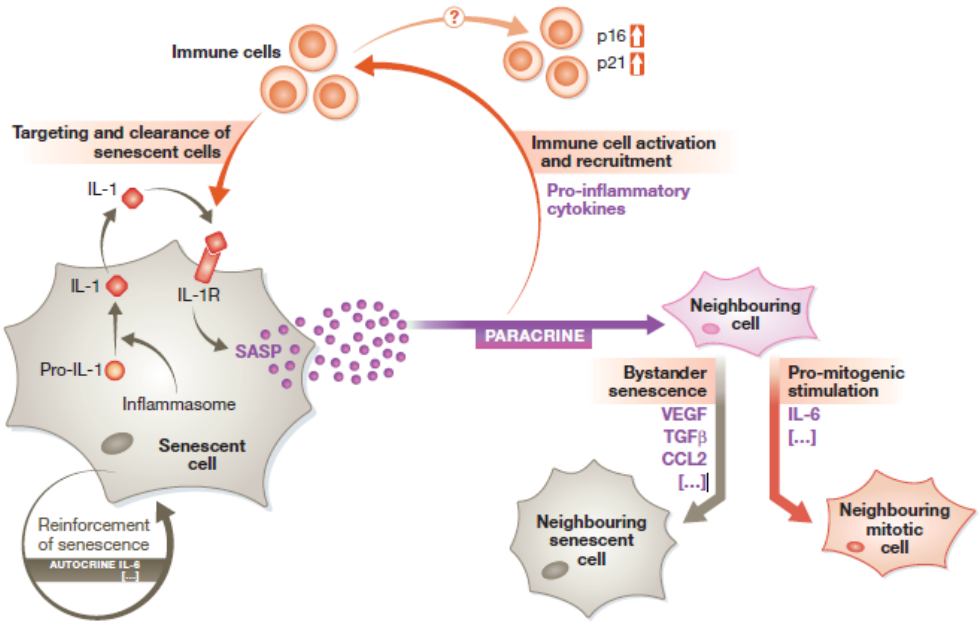


Figure 2. Cell autonomous and non-cell autonomous effects of cellular senescence. Stress stimuli can trigger normal mitotic cells to go into senescence. This involves inflammasome-mediated activation of IL-1 signaling, which initiates the SASP response. The SASP acts cell autonomously (autocrine) to reinforce the senescent phenotype via cytokines such as IL-6. The SASP also acts non-cell autonomously (paracrine) to influence the cells in the surrounding environment. Paradoxically, the SASP can also exert pro-mitogenic stimulation of neighboring cells via cytokines like IL-6, which appear to play dual roles depending on the context. Furthermore, the SASP can act on the immune system via pro-inflammatory cytokines, leading to immune cell recruitment and subsequent targeting and clearance of senescent cells. Alternatively, the SASP can trigger upregulation of p16 and p21 levels on neighboring immune cells, the functional consequences of which are not yet so clear (Tasdemir and Lowe, 2013).

1.3 Mitochondria in aging cells and their role in inflammaging

Mitochondria play an essential role in energy generation, cell signaling, differentiation, death and obviously in the aging process. Any given mitochondrion is not a discrete, autonomous organelle. In fact, the identity of an individual mitochondrion is short-lived, because it will fuse with a neighboring mitochondrion in the near future. Therefore, the entire mitochondrial population is in constant flux, driven by continual fusion and division of mitochondria (Chan, 2006).

Recently, Correia–Melo *et al*. have shown that mitochondria are required for the pro-oxidant and pro-inflammatory phenotypes of senescent cells. A DDR pathway converging on mTORC1 phosphorylation promoted peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC-1β) dependent mitochondrial biogenesis, contributing to a ROS-mediated activation of the DDR and cell cycle arrest. Of note, the reduction in mitochondrial content *in vivo*, by either mTORC1 inhibition or PGC-1β deletion, prevented senescence in the aging mouse liver of the senescent phenotype (Correia-Melo et al., 2016).

1.3.1 Electron transport chain and mitochondrial ROS production

Mitochondria are the principal functional players of energy metabolism, as they provide most of the cellular energy through oxidative phosphorylation (OXPHOS). OXPHOS operates through five complexes, embedded in the inner mitochondrial membrane, that altogether represent the electron transport chain (ETC). The ETC is assembled from the genes of both the nuclear DNA and the mitochondrial DNA (mtDNA). The mtDNA is a circular, double-strand molecule, which encodes 13 polypeptides essential for OXPHOS, 2 rRNAs and 22 tRNAs for mitochondrial protein synthesis. Mammalian cells can have

hundreds to thousands of mitochondria, and each mitochondrion contains several mtDNA genomes. In physiological condition, during aerobic respiration, a variable percentage of electrons leaks from the ETC, particularly from complexes I and III, prematurely reduce oxygen and generate ROS (Bratic and Trifunovic, 2010). This process is exacerbated in senescent cells and leads to an over-production of ROS with concomitant ATP production decrease (Zwerschke et al., 2003). Low levels of ROS may improve systemic defense mechanisms by inducing an adaptive response, acting as signaling molecules. This concept is termed "mitohormesis". On the contrary, high levels of ROS have been implicated into cellular damage and telomeres shortening hence promoting aging process (Ristow and Schmeisser, 2014; von Zglinicki et al., 2002; Parrinello et al., 2003). In the 50s, Harman with his Free Radical Theory of Aging (FRTA) theorized increased formation of ROS to be a major cause of aging (Harman, 1956). Because of mitochondria are the dominant source of cellular ROS, he extended his initial FRTA theory to the Mitochondrial Free Radical Theory of Aging (MFRTA) (Harman, 1972). One of the current opinions is that the relationship between ROS levels and aging is not always linear and oxidative stress is not *per se* the major determinant of aging. Indeed, ROS-lowering interventions did not give expected results (Sesso et al., 2012; Rautiainen et al., 2012) and as a matter of fact, as opposed to severe mitochondrial dysfunction, mild mitochondrial stress has an evolutionarily conserved anti-aging effect. However, excessive or uncontrolled free radical production can induce an inflammatory response, and free radicals are themselves inflammation effectors (Naik and Dixit, 2011). Thus, in aging cells, mitochondria-derived ROS and oxidative stress should be considered for their role as pro-inflammatory triggers rather than damaging molecules that progressively disrupt cell components and cellular homeostasis alone. Accordingly, ROS can partly mediate the senescence "bystander effect",

i.e. the transition of the senescent phenotype from a senescent cell (SC) to neighbor cells (Nelson et al., 2017).

Even if ROS production is the best-known mitochondria biochemical change, other important phenomena occur which alter mitochondrial function. One of this concerns mitochondrial genome mutation: a general age-unrelated rule is the higher mitochondrial genome mutation rate than the nuclear genome (Trifunovic, 2006); Greaves and colleagues have shown that during aging selected pathogenic mutations increase and clonally expand but not the frequency of mutation (Greaves et al., 2014). Of note, mtDNA mutation accumulation in mouse tissues is influenced by the nuclear genetic background and correlates to neither cellular ROS content nor tissue senescence. MtDNA presents a number of genetic variants, called haplogroups. These haplogroups contain a functional, single-base polymorphism (SNPs) and can thus confer to their mitochondria a differential efficiency in the oxidative metabolism. Interestingly, they were associated with lifespan and longevity (Salvioli et al., 2006).

1.3.2 Mitochondria fuel Inflammaging through activation of receptors of the innate immune system

Mitochondria play a major role in inflammaging via not only ROS but also releasing several molecules inducing the inflammasome activation. This is partly due to the mitochondrial molecules conserved similarities to bacteria, as phylogenetically bacterial symbionts of early eukaryotic cells: mtDNA, N-formyl peptides and cardiolipin, a lipid present in the inner mitochondrial membrane, can act as damage-associated molecular pattern (DAMP)s activating pattern recognition receptors (PRRs), a large family of proteins that include Toll-

like receptors (TLRs) and NOD-like receptors (NLRs) (Franceschi et al., 2017). In particular, oxidized mtDNA and cardiolipin, released into the cytosol, could be recognized by Nlrp3 inflammasome, leading to IL-1β release (Shimada et al., 2012; Iyer et al., 2013). MtDNA is also recognized by TLR9, which senses DNA of bacterial and viral origin (Lamphier et al., 2006), inducing activation of NF-kB (Zhang et al., 2014). Further, mtDNA can even activate the cytosolic DNA sensor cyclic GMP-AMP Synthase (cGAS)/Stimulator of Interferon Genes (STING) pathway thus triggering expression of SASP factors (de Galarreta and Lujambio, 2017; Yuan et al., 2017).

1.3.3 Mitochondria proteins: the potential role of Bcl-2 family members in regulating apoptosis and autophagy in aging cells

Aging cell show an altered susceptibility to apoptosis and a reduction of autophagy efficiency. Consequently, cells accumulate dysfunctional organelles, which in turn contribute to the aging cell phenotype. Among the master regulators of both processes, the Bcl-2 family members should be considered. These proteins can be associated to the outer mitochondrial membranes. They are functionally described as either pro-apoptotic (e.g. BAX and BAK), which promote the mitochondrial outer membrane permeabilization (MOMP) or anti-apoptotic (e.g. Bcl-2, Bcl-xL and Mcl-1), which preserve outer mitochondrial membrane (OMM) integrity by directly inhibiting the pro-apoptotic proteins of the family. The balance of BCL-2 members and the regulation of their interactions dictate survival or commitment to apoptosis (Chipuk et al., 2010).

During programmed cell death MOMP allows soluble proteins (e.g. cytochrome c and the apoptosis initiating factor AIF) sequestered in the mitochondrial intermembrane space (IMS)

to diffuse into the cytosol. Cytochrome *c* release is a key event leading to activation of initiator caspase-9, cysteine proteases necessary for cell death, which then activates executioner caspases-3 and -7 (Chan, 2006).

In addition Bcl-2, Bcl-xL and Mcl-1 negatively regulates autophagy through the interaction with Beclin-1, which has a central role in this process (Liang et al., 1998).

Macroautophagy (or simpler autophagy) is a self-degradative mechanism that involves the engulfment of cytoplasm material and intracellular organelles within a double-layered membrane structure that then fuses with lysosomes (referred to as an autolysosome). Autophagy is a basic mechanism for cellular homeostasis that is important for balancing sources of energy in response to nutrient stress, in removing misfolded or aggregated proteins, clearing damaged organelles - such as mitochondria, endoplasmic reticulum and peroxisomes - as well as eliminating intracellular pathogens (Glick et al., 2010). Apoptosis and autophagy are often regulated by similar pathway and engage common sub-cellular sites and organelles, leading to the suggestion that these two processes are mechanistically linked.

As both processes are altered in aging cells the intriguing idea of the role of Bcl-2 family member in cellular dysfunction during aging has been suggested in my laboratory (Rippo et al, 2014) and by others (Uraoka M et al., 2011).

1.4 Epigenetic biomarkers of cellular senescence and aging

The term epigenetics includes several phenomena such as DNA methylation, histone tail modifications, and microRNAs mediated mechanisms, which are able to mould the chromatin structure and/or gene expression levels, without altering the primary DNA

sequence (Stoccoro et al., 2016). Epigenetic markers can be changed by environmental influences and can then be passed on to daughter cells or via the germ line to offspring. Epigenetic modifications of chromatin and DNA have been recognized as important permissive and suppressive factors in controlling the expressed genome via gene transcription. A variety of changes in epigenetic biomarkers have been found in ARDs (Ospelt, 2016). Global DNA methylation is often lost in repetitive sequences, while in several specific promoter regions hyper-methylation is found. DNA methylation is achieved by the action of DNA methyltransferases. In addition, noncoding RNA can be considered as epigenetic markers and changes in microRNAs levels were observed during senescence and in ARDs (Olivieri et al., 2013). Understanding their emerging role as regulators of inflammaging and cellular senescence will enhance our knowledge of the molecular mechanisms involved in physiological and pathological aging and will provide new diagnostic tools and treatment options for patients with the major ARDs.

1.5 MicroRNAs

MicroRNAs are versatile regulators of gene expression. Since their discovery, thousands of miRNAs have been identified in humans, as well as in other species. In the human genome alone more than 2580 have been estimated miRNA genes (miRBase Registry, release 21.0). Online sources for sequences, such as the *miRbase* database (www.mirbase.org), were also made available since then. Recent evidence in the last year suggests that miRNA-mediated activities are surprisingly complex and that an intricate network of factors stringently control miRNA processing and biological activities in both negative and positive ways (Breving and Esquela-Kerscher, 2010).

1.5.1 miRNA Biogenesis and Function

The microRNA biogenesis begins with genomic DNA transcription by RNA polymerase II (Pol II). RNA Pol II produces long pri-miRNAs from independent genomic transcription units or from the introns of protein-coding genes. Splicing is not required for production of miRNAs arising from introns. Furthermore, co-transcriptional processing of pri-miRNA into precursor miRNAs (pre-miRNAs) does not affect the splicing of the host pre-mRNA. In animals, the RNase III enzyme Drosha converts pri-miRNAs into pre-miRNAs, which are ~60 nucleotide stem–loop structures. Drosha acts as a component of a larger complex, the microprocessor, which includes a dsRNA-binding protein, named DGCR8 in mammals and Pasha in other animals. The nuclear transport receptor exportin 5 recognizes the ends and the stem of the pre-miRNA and exports the pre-miRNA from the nucleus to the cytoplasm via the nuclear pore. In the cytoplasm, the pre-microRNA is further processed by a second RNase III enzyme, Dicer, to double-stranded anti-parallel RNA (miRNA–miRNA*). This duplex is loaded into an Argonaute (AGO) protein. Subsequent maturation steps expel the miRNA*, producing a mature RNA-induced silencing complex (RISC). The RISC contains miRNA, an Argonaute protein and other protein factors. Finally, miRNA guides the RISC complex to complemental mRNA sequences to repress their expression (Bartel, 2004).

The specificity of microRNAs is based on their mature sequence and is achieved through complementarity of target mRNAs with the “seed” region of the microRNA that is only six nucleotides in length. Typically, miRNA-binding sites of animal mRNAs reside in their 3’UTRs, resulting in down regulation of gene expression via translational repression and/or mRNA degradation (Ameres and Zamore, 2013). There is also increasing evidence that animal miRNAs can also exert post-transcriptional control when bound to sites located

outside of the 3'UTR and specifically within the 5'UTR and coding regions of the mRNA target (Duursma et al., 2008; Lytle et al., 2007; Tay et al., 2008). Moreover, accumulating evidences indicate that small RNAs bound to promoter regions in the nucleus can activate gene transcription, a phenomenon referred to as RNA activation (RNAa) (Janowski et al., 2007; Li et al., 2006) (Figure 3).

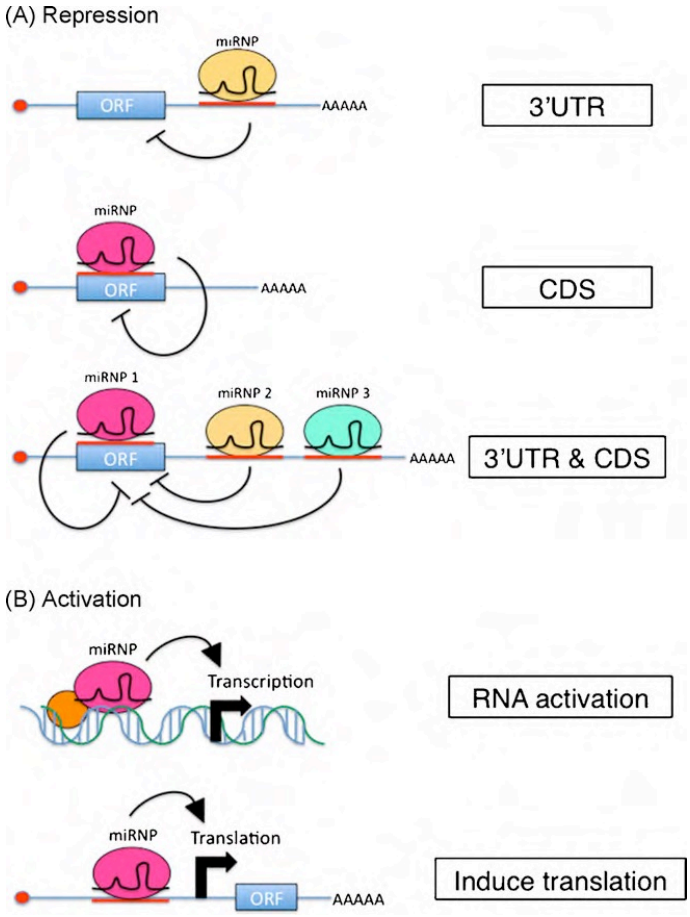


Figure 3: miRNAs can function to repress or activate target mRNA expression. (A) The canonical mode of miRNA-mediated repression occurs when a miRNA binds to mRNA regions of the 3'UTR, leading to down-regulation of target expression via translational repression and/or mRNA degradation. MiRNAs can also associate with binding sites located in the amino acid coding sequence (CDS) of mRNA transcripts to repress target expression (middle panel). Complex networks of distinct miRNAs can function together and bind to multiple miRNA complementary regions located both in the coding and non-coding regions of the mRNA target in order to fine-tune expression (bottom panel). (B) Recently, it has been shown that miRs can associate with incomplete complementarity to promoter elements of protein-coding genes and activate/enhance target transcription and protein expression via the phenomena RNA activation (RNAa) (top panel). Optimal promoter conditions (i.e., cofactors, chromatin structure) are apparently required for this activating effect to occur. MiRNAs can also induce protein translation by associating specifically with 5'UTR elements of mRNA targets (Breving and Esquela-Kerscher, 2010).

Of note, a single microRNA might control hundreds of distinct targets. These highly complex target networks pose a significant challenge to the mechanistic dissection of miRNA-mediated phenotypes. With such widespread control of gene regulation, miRNAs appear to have an influence on virtually every genetic pathway. Indeed, the growing number of miRNAs characterized to date reveals that they control a large range of essential biological processes related to cellular differentiation and growth, apoptosis, as well as aging, metabolic and immune responses.

1.5.2 Inflamma-miRs

The inflammatory response comprises complex biological reactions that require a fine-tuned integration between a range of immune system cell classes and an extensive network of biomolecules, which until recently had been thought to be largely cytokines. However, identification of the vast repertoire of miRNAs in the mammalian genome has completely revolutionized our understanding of most biological processes, including inflammation (Nilsen, 2007). Sensing of dangerous signals by the innate immune system involves a number of germline-encoded pattern recognition receptors (PRRs) that can detect both conserved pathogen- associated molecular profiles (PAMPs) expressed on microorganisms and altered endogenous ligands, mostly released by necrotic, senescent, and/or damaged cells (DAMPs). Among PRRs, toll-like receptors (TLRs) play a central role, since their engagement activates a potent pro-inflammatory pathway **(**Kawai and Akira, 2011). TLR signalling initiates from different adaptor proteins, such as myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter protein-inducing interferon-β, which in turn activate several downstream pathways, leading to activation of NF-κB, mitogen-activated protein kinases (MAPKs), and members of the interferon regulatory factor family**.** Fine-

tuning of TLR signalling prevents generation of harmful and inappropriate inflammatory responses without lowering the surveillance for potentially dangerous signals. Deregulation of the whole network can have destructive effects and lead to tissue damage: this is a hallmark of chronic inflammation, which is often associated with ARDs development and progression (Olivieri et al., 2013a and b). A mounting body of evidence has been documenting a relatively small number of miRNAs that are involved in regulating inflammation: their prototypes are miR-155, miR-21, and miR-146a (Quinn and O'Neill, 2011), after referred to as *inflamma-miRs*. In physiological conditions, transcription of miR-155, miR-21, and miR-146a is at baseline levels; however, initiation of pro-inflammatory TLR signalling immediately results in strong co-induction of their expression through a mechanism that is largely NF-κB-dependent (Boldin and Baltimore, 2012). Although the importance of *inflamma-miRs* in innate immune response regulation is widely accepted, the molecular mechanism of their action has proved to be highly complex. Early investigations disclosed that miR-146a acts as a negative regulator of TLR signalling by targeting both tumour necrosis factor receptor-associated factor 6 (TRAF6) and IL-1receptor-associated kinase 1 (IRAK-1) (Taganov et al., 2006). MiR-21 was found to down-regulate the expression of IRAK and MyD88**,** as well as of programmed cell death protein 4 (PDCD4), switching the cell program from pro-inflammatory to anti-inflammatory, mainly as reflected by IL-10 production (Sheddy et al., 2010). Overall, these finding have generated a model where *inflamma-miRs* operate as a negative feedback loop to protect the organism against overwhelming inflammation. Subsequent research has disclosed that *inflamma-miRs* could play a dual function, inhibiting as well as inducing TLR signalling. This is the case of miR-155 and miR-21, which can function as an agonist of single-stranded RNA-binding TLRs and can therefore induce NF-κB activation and secretion of inflammatory molecules (Fabbri

et al., 2012). Although *inflamma-miRs* are co-induced during TLR signalling, several observations suggest that they do not act redundantly and simultaneously, but rather cooperate to control TLR signalling through functionally different performances. Such coordination of *inflamma-miRs* could go beyond suppression of TLR signalling and contribute to regulating the immunity/inflammation balance. Knockout mouse models have been instrumental in shedding light on the role of *inflamma-miRs* in inflammation and inflammation-related diseases. Transcriptome analysis of bic/miR-155-deficient CD4+ T cells in mice identified a wide spectrum of miR-155-regulated genes, including cytokines, chemokines, and transcription factors, suggesting that bic/miR-155 plays a key role in immune system homeostasis and function (Rodriguez et al., 2007). Knockout of miR-146a gene in C57BL/6 mice involves increased transcription of NF-κB-regulated genes (Zhao et al., 2011). These animals also develop myeloid sarcomas and lymphomas as well as chronic myeloproliferation in bone marrow. Genetic ablation of NF-κB p50 suppresses the myeloproliferation, demonstrating that NF-κB dysregulation is responsible for the myeloproliferative disease (Zhao et al., 2011). Details on the role of *inflamma-miRs* in controlling TLR signalling are just beginning to be explored, and further investigations are warranted to gain insights not only into their individual contribution to the homeostasis of the innate immune response, but also into the consequences of their deregulation in conditions characterized by chronic inflammation.

1.5.3 Cellular Senescence Associated (SA)-miRs

As mentioned above, several miRNAs have been shown to be involved in the regulation of pathways implicated in cellular senescence and exert important effects on cell cycle progression.

A particular aspect concerns p53, a critical regulator of cellular senescence. It enhances the post-transcriptional maturation of several miRNAs with growth-suppressive function (Suzuki et al., 2009; Liu et al., 2012; Munk et al., 2017) and it can be itself a target of microRNAs (Park et al., 2009; Inomata et al., 2009). P53 activation can be regulated by the NAD-dependent deacetylase Sirtuin-1 (SIRT-1), a gene that regulates cellular senescence and limits longevity, whose expression is under miR-34a. The miR-34 family regulates cell cycle progression, cellular senescence and apoptosis. One of the best-characterized SA-miR is just miR-34a. Expression of miR-34a leads to reduced SIRT1 levels, lead to an increase in p53 acetylation and activation. P53 in turn promotes the expression of the miRNA family miR-34; this creates a positive p53-activating feedback loop leading to increased p21 levels, thereby triggering senescence (Yamakuchi and Lowenstein, 2009).

Moreover, it has been demonstrated that miRs can also modulate p16 expression and telomere length (Lal et al., 2008; Benetti et al., 2008) and that miR-20a and the other miRs of the same cluster (miR-17-92 cluster) contribute to regulating cell cycle progression (Pickering et al., 2009; Cloonan et al., 2008; Ivanovska et al., 2008). Interestingly, ablation of miRNAs expression by knockdown of Dicer has been shown to result in the induction of premature senescence phenotype in embryonic fibroblast through activation of the p53 pathway (Mudhasani et al., 2008). Finally, several miRs that appear to be modulated during cellular senescence and aging are found in a range of ARDs, including cardiovascular diseases (CVD) and neurodegenerative diseases (Dimmeler and Nicotera, 2013).

1.5.4 MitomiRs

Recently, through different experimental approaches in different mammalian species, a number of studies allow identification of "signatures" of miRNAs located in the mitochondria (Table 1). These miRs were collectively called "mitomiRs". Indeed, this subset includes both nuclear-encoded miRNAs that translocate into the mitochondria and target either mitochondrial or nuclear mRNA and a small number of mitochondrial-encoded microRNA (mit-mitomiRs). Early studies on the subject were mostly descriptive to detect the presence of miRNAs within the mitochondrion (Giuliani et al., 2017b). Kren and co-workers first detected 15 nuclear-encoded mitomiRs from adult rat livers that seemed to be involved in the modulation of genes associated with apoptosis, cell proliferation, and differentiation. They also postulated that given the central role that mitochondria play in apoptosis, they might serve as reservoirs of select miRNAs that may modulate these processes in a coordinate fashion (Kren et al., 2009). A subsequent study identified a pool of 20 miRs highly expressed in mitochondria of mice liver. Interestingly, mitochondria have a unique population of miRNAs, independent of the total cellular abundance of miRs: the miRs that were highly abundant in mitochondria were not those that were highly expressed in liver (Bian et al., 2010). For the first time, in 2011 Barrey and colleagues showed the presence of pre-miRs inside mitochondria, postulating that here some pre-miRNA sequences could be processed to mature miRNAs, which could be immediately active on the mitochondrial transcripts or exported in the cytosol in order to interfere with genomic mRNA (Barrey et al, 2010). Subsequently, other groups identified new mitomiRs from different tissue and cell types (Bandiera et al., 2011b; Mercer et al., 2011; Sripada et al., 2012; Dasgupta et al., 2015; Jagannathan et al., 2015). Overall these data indicate that

mitochondria have a discrete pool of mitomiRs and the association of miRs with mitochondria is species and cell type-specific (Geiger and Dalgaard 2017).

Human				Rat	Mouse					
Barrey 2011		Bandiera 2011	Sripada 2012	Kren 2009	Bian 2010	Jagannathan 2015				
human skeletal/muscular cells		HeLa cells	HeLa/HEK293	Rat liver	mouse liver	mouse heart				
let-7b	miR-193b	miR-328-5p	let-7b-5p	miR-130a-3p	let-7f-5p	let-7b	miR-151-3p	miR-1934-3p	miR-130a	miR-574-5p
let-7g	miR-197	miR-494-3p	let-7g-5p	miR-130b-3p	miR-101-5p	let-7a	miR-203-3p	miR-211-3p	miR-497	miR-148a-3p
miR-19b	miR-199a-5p	miR-513a-5p	miR-107	miR-140-5p	miR-122-5p	let-7c	miR-212-3p	miR-3072-3p	miR-188-5p	miR-200c-3p
miR-20a	miR-210	miR-638	miR-181a-5p	miR-320-3p	miR-181b-5p	let-7f	miR-5112	miR-320-3p	miR-3098-5p	miR-300-3p
miR-23a	miR-221	miR-1201	miR-221-5p	miR-494-3p	miR-181d-5p	miR-149-3p	miR-135a-1-3p	miR-1199-5p	miR-30c-1-3p	miR-181b-5p
miR-23b	miR-324-3p	miR-1246	miR-320a	miR-671	miR-188-5p	miR-149-5p	miR-721	miR-5108	miR-712	miR-5131
miR-24	miR-324-5p	miR-1275	miR-494-3p		miR-29a-3p	miR-23b	miR-125a-3p	miR-375-3p	miR-3102-5p	
miR-34a	miR-365	miR-1908	miR-1275		miR-29c-3p	miR-1	miR-1904	miR-203-3p	miR-877-3p	
miR-92a	miR-423-3p	miR-1972	miR-1973		miR-361-5p	miR-29a	miR-1894-3p	miR-126-3p	miR-3963	
miR-93	miR-484	miR-1973	miR-		miR-432	miR-125b-5p	miR-3102-5p	miR-26a	miR-341-3p	
miR-103	miR-486-5p	miR-1974			miR-494-3p	miR-29b	miR-494	miR-23a	miR-342-3p	
miR-106a	miR-490-3p	miR-1977			miR-680	miR-709	miR-1939	miR-27b	miR-423-3p	
miR-107	miR-503	miR-1798			miR-689	miR-22	miR-3470a	miR-99a	miR-3081-5p	
miR-125b	miR-501-3p		Zhang 2014	Das 2012	miR-690	miR-24	miR-144	miR-139-3p	miR-1895	
miR-125a-5p	miR-532-3p		human	rat	miR-705	miR-680	miR-3107	miR-378	miR-720	
miR-127-3p	miR-542-5p		skeletal muscle	cardiomyocites	miR-711	miR-21	miR-451	miR-27a	miR-1897-5p	
miR-133b	miR-574-3p		miR-1	miR-181c	miR-721	miR-133a-3p	miR-1224	miR-29c	miR-3085-3p	
miR-133a	miR-598	Mercer 2011			miR-720	miR-133a-5p	miR-2861	miR-30a	miR-3092	
miR-134	miR-720	143B cells	Dasgupta 2015		miR-762	miR-133b	miR-2137	miR-30d	miR-2145	
miR-149	miR-1974	miR-16	206 ρ° cells		miR-805	miR-128-3p	miR-1937c	miR-30e	miR-652-5p	
miR-151-5p	miR-1979	miR-146a	miR-181c		miR-671-5p	miR-3095-3p	miR-466i-5p	miR-3082-5p	miR-1187	
miR-181a	miR-675*	miR-103	mR-146a		miR-1982-5p	miR-1937b	miR-705	miR-483-5p	miR-466h-3p	

Table 1. List of miRs found within mitochondria (extracted from Giuliani et al, accepted for publication in Mediators of Inflammation, 2017)

1.6 A miRs subset belonging to inflamma-miRs, SA-miRs, and mitomiRs

Since mitochondria play a key role in aging, it is reasonable to surmise that some mitomiRs are involved in the process, and that the most likely candidates should be found among the miRs involved both in aging and in inflammation (Rippo et al., 2014). Figure 4 shows a Venn diagram illustrating the intersections of the three different sets of miRNAs (inflamma-miRs, SA-miRs and mitomiRs). These three subtypes of miRs share a discrete pool of miRs (SA-inflamma-mitomiRs) that includes 10 microRNAs (miR-19b, miR-20a, miR-24, miR-34a, miR-125b, miR-146a, miR-181a and miR-181c, miR-210, and miR-221).

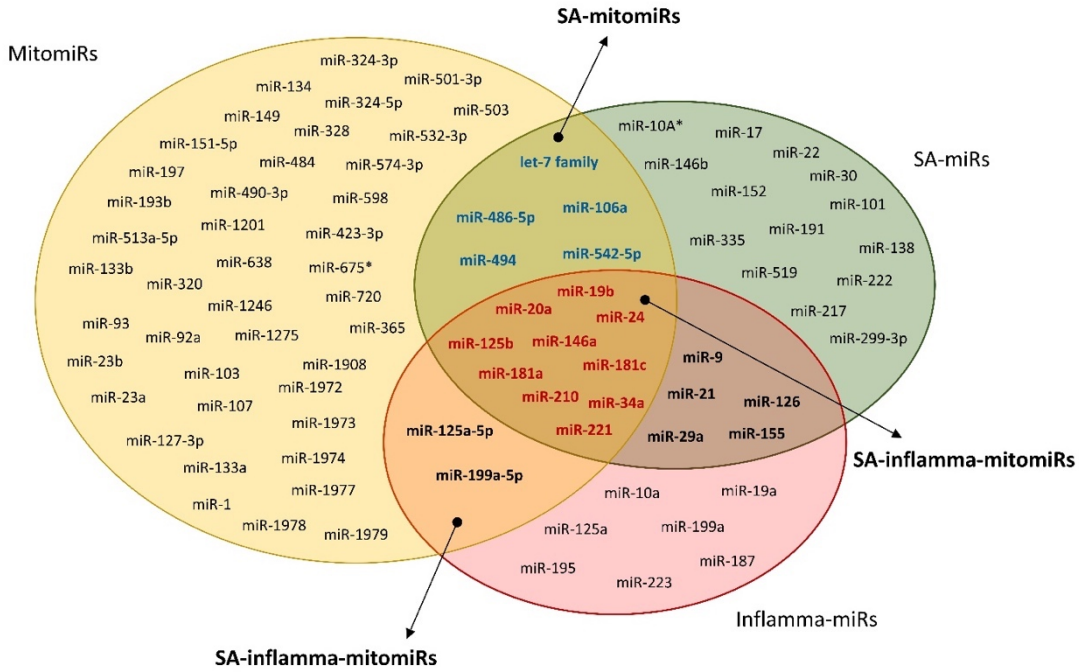


Figure 4. Venn diagram showing the mitomiRs involved in both aging and inflammation. Mitochondria-related (mitomiRs), senescence-associated (SA-miRs) and inflammation-related (inflamma-miRs) miR sets. SA-mitomiRs: miRs found both in the mitomiR and the SA-miR set; SA-inflamma-mitomiRs: miRs found in all three sets (modified by Giuliani et al., 2017b).

In our laboratory, by using the bioinformatics tools Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) the hypothesis that one role of SA-inflamma-mitomiRs may be the control mitochondria-associated protein expression was tested. They limited the research approach to human tissues and cell lines and to cytosolic targets observed experimentally, though not necessarily validated. This approach demonstrated that most SA-mitomiRs are involved in controlling the expression of at least one Bcl-2 family member (Rippo et al., 2014). Literature has also shown that several SA-mitomiRs e inflamma-mitomiRs can modulate Bcl-2 family members.

The above considerations suggest i) that in aging cells modulation of several mitomiRs should result in Bcl-2 deregulation and consequently alteration of Bcl-2-mediated processes

and ii) the hypothesis that SA-mitomiRs could influence mitochondrial function and, as a consequence, may promote ROS production and inflammation (summarized in Figure 5).

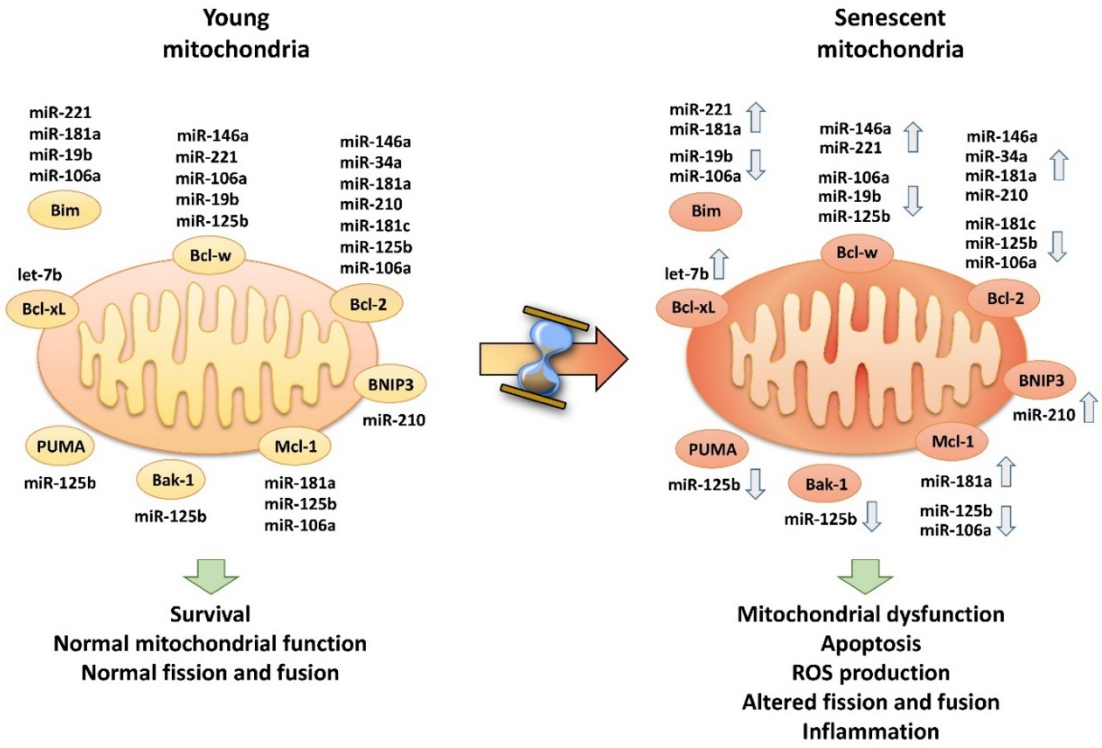


Figure 5. SA-mitomiRs and SA-inflamma-mitomiRs appear to be involved in controlling the expression of one or more Bcl-2 family member. In young HUVECs mitomiRs may control the expression of Bcl-2 family members, resulting in their fine tuning and optimum function at the mitochondrial level. In senescent HUVECs, mitomiR deregulation may affect the balance of Bcl-2 family members, resulting in mitochondrial dysfunction (Giuliani et al., 2017b).

This hypothesis is supported by experimental evidence that miR-34a, miR-181a and miR-146a control Bcl-2 expression (Yang et al. 2014; Ouyang et al., 2012; Zhang F et al., 2015). In addition, IPA of "molecular and cellular functions" identified the main functions in which these SA-mitomiRs are involved. Most are among those affected by aging: cell cycle, cell growth and proliferation, survival, function, and maintenance. These data suggest that SA-inflamma-mitomiR deregulation, by altering these key functions, should be associated with

cell dysfunction and disease. Indeed, IPA of miR-146a, miR-34a, and miR-181a in relation to the pathway "diseases and disorders", showed their association with several important age-associated diseases, including cancer, CVD, and neurological and metabolic disorders (Rippo et al. 2014). The authors proposed a model of how a mitomiRs subset (miR-181a, miR-34a and miR-146a) may control mitochondrial function/dysfunction and inflammation during cell aging via modulation of Bcl-2 family members.

1.7 Circulating microRNAs

MiRNAs are not confined within the cell. Accumulating evidence indicates that miRNAs are secreted in extracellular spaces in microvesicle-encapsulated form or are released in vesicle-free form and can enter into target cells. To move into extracellular circulatory biofluids, they take advantage of different systems:

1) miRNAs are bound to high-density lipoprotein (HDL) particles in a non-vesicle form,
2) they form complex with Ago2 proteins,
3) they are placed in exosomes,
4) they are encapsulated in micro-vesicles (MVs),
5) miRNAs accumulate in apoptotic bodies (Kumar and Reddy, 2016).

All miRNA transport strategies described to date allow communication between cells found in different organs. Exosomes may be the simplest and most robust way to realize a systemic miRNA-based signal network. However, little is known of how miRNA species are sorted into exosomes and which miRNA binding proteins are involved. HDL is another well-established circulating miR carrier, shuttling the potent gene regulators to distant tissues.

The report that miRNAs carried by HDL may be altered in disease states had further broadened our understanding of the complex effects that the lipoprotein can exert on target cells and tissues. The delivery of lipid-associated miRNAs to recipient cells is achieved by various routes, including endocytotic uptake, membrane fusion, and scavenger receptors. Circulating miRNA levels can be used as biomarkers for the major age-related diseases with important diagnostic and prognostic implications but their clinical relevance is still controversial (Cortez and Calin, 2009; D'Alessandra et al., 2010; Zampetaki et al., 2012; Olivieri et al., 2013c).

2. Aim of the work

Aging is one of the major risk factor for pathologies characterized by chronic inflammation status such as cardiovascular diseases, T2DM and neurodegenerative diseases – commonly termed ARDs. The understanding of mechanisms that regulates these inflammatory processes is one of the main target of the current scientific research to both prevent aging related pathologies and improve their outcome.

Non-coding nucleic acids, among which miRNAs represent one of the most studied category, contribute to these mechanisms by controlling and fine tuning protein expression. Since miRNAs expression can be regulated, they represent an intriguing opportunity as targets of new treatments; indeed, combinatorial therapies with conventional drugs and miRNA- or anti-miRNA-treatments are already in progress (van Beijnum, et al., 2017).

Here three different aspects for which miRNAs are involved in cellular senescence, Inflammaging and ARDs were investigate by addressing three specific aims:

1. Assess whether age-related mitomiRs might play a direct role in controlling mitochondrial function by regulating mitochondrial protein expression.

Current research suggests that a large number of microRNAs are involved in the regulation of mitochondrial activities (mitomiRs) and some of them are modulated during senescence and inflammatory processes. Mitochondria have long been recognized for their canonical role in cellular respiration and oxidative phosphorylation. However, it is becoming increasingly evident that they represent key participants in sensing and integrating signal from the environment to trigger adaptive and compensatory responses in cells (Aon and Camara, 2015). Thus, acting as pleiotropic organelles, mitochondria might regulate aging and age-related diseases on several aspects and appear as indispensable for the pro-inflammatory and the pro-aging features of senescent cells (Correia-melo et al., 2015). Understanding the multitude of mechanisms that lead these organelles to be the "bad" players of aging could shed light on new potential druggable pathways. We focused on three mitomiRs (miR-34a, -146a and 181a) that can target Bcl-2 in different cellular systems and pathological context and that may be linked each other and to mitochondrial activity by an intriguing molecular network (Rippo et al., 2014). The aim of my PhD study was then to assess whether aging-related mitomiRs might play a direct role in controlling mitochondrial function by regulating mitochondrial protein expression. Their modulation could thus mediate the loss of mitochondrial canonical function in aging cells, inducing or contributing to the inflammatory response and to age-related diseases.

2. Evaluate whether and how prevention of TNF-α activity can restrain SASP.

Several miRNAs have been associated to the SASP phenotype. SASP attenuation, SASP factors modulation and selective senescent cells removal or killing are all potential strategies (senotherapies) for mitigating the deleterious effects of senescent cells. Highly promising results are coming from work on SASP suppressor and senolytic agents (Childs et al., 2015;

Chang et al., 2016). The second aim of this study was thus to evaluate whether and how adalimumab, a monoclonal antibody directed against tumor necrosis factor-α (TNF-α), a major SASP component, can prevent the SASP of endothelial cells in replicative senescence. The ability of adalimumab to restrain or delay SASP acquisition was tested by evaluating two of the senescence/SASP-associated miRNAs (miR-126-3p and miR-146a-5p) and the respective targets (Spred1 and Irak1).

3. Explore the potential of these a subset of circulating miRs as biomarkers of T2DM complications.

Increasing interests toward miRNA is justified by their role as reliable circulating markers of aging related disease. They have also the advantage to be easily accessible, rapidly analysable, highly stable and resistant so that their use for diagnostic and prognostic purpose could reduce the necessity of invasive procedures in patients introducing the concept of "liquid biopsy". One of the ARDs, which arouses major interest for innovative diagnostic/prognostic biomarkers, is T2DM. T2DM is a chronic multi factorial metabolic disease, caused by environmental and genetic factors. A meta-analysis of randomised controlled trials has demonstrated that the benefit-risk ratio of intensive glucose lowering treatment, administered to prevent diabetic vascular complications, remains uncertain (Boussageon et al., 2011). This suggests that the parameters currently used to monitor T2DM progression do not adequately predict the probability of developing complications; therefore, innovative biomarkers are required to manage patients with T2DM and its severe complications. Circulating miR-126-3p levels have been associated with endothelial function (Wang et al., 2008) and circulating miR-21-5p with systemic inflammatory status (Olivieri et al., 2013a), two conditions that are closely related to glycaemia control. The last

aim of this work was therefore to explore the potential of these two miRs as biomarkers of T2DM complications.

3. Results and discussion

3.1 MitomiR-34a, -146a and -181a/Bcl-2 axis in senescence endothelial cells affects mitochondrial function and autophagy

3.1.1 Human senescent endothelial cells show altered mitochondrial activity and morphology

Senescent cells show altered phenotype and function. Among the subcellular compartments, mitochondria undergo profound morphological and biochemical changes reflecting their dysfunction. To characterize mitochondrial activity during senescence, we used replicative senescence of human primary endothelial cells (HUVECs) as a model. HUVECs were sub-cultured until complete growth arrested (Figure 6A, upper panel).

To confirm the acquisition of the senescent phenotype at later passages (Cell population doubling, CPD>25) SA-β Gal staining was performed: senescent cells displayed a high SA-β Gal positivity (Figure 6A, bottom panels). In addition, p16(Ink4a), the prototypical protein characterizing senescent cells, showed a significant increase in both intermediate and senescent cells compared to younger cells (Figure 6B). We also evaluated the increase of Caspase-1 (Casp-1) activity and the production of its substrate IL-1β, a cytokine typically released by senescent cells and that characterizes the acquisition of SASP (Figure 6C).

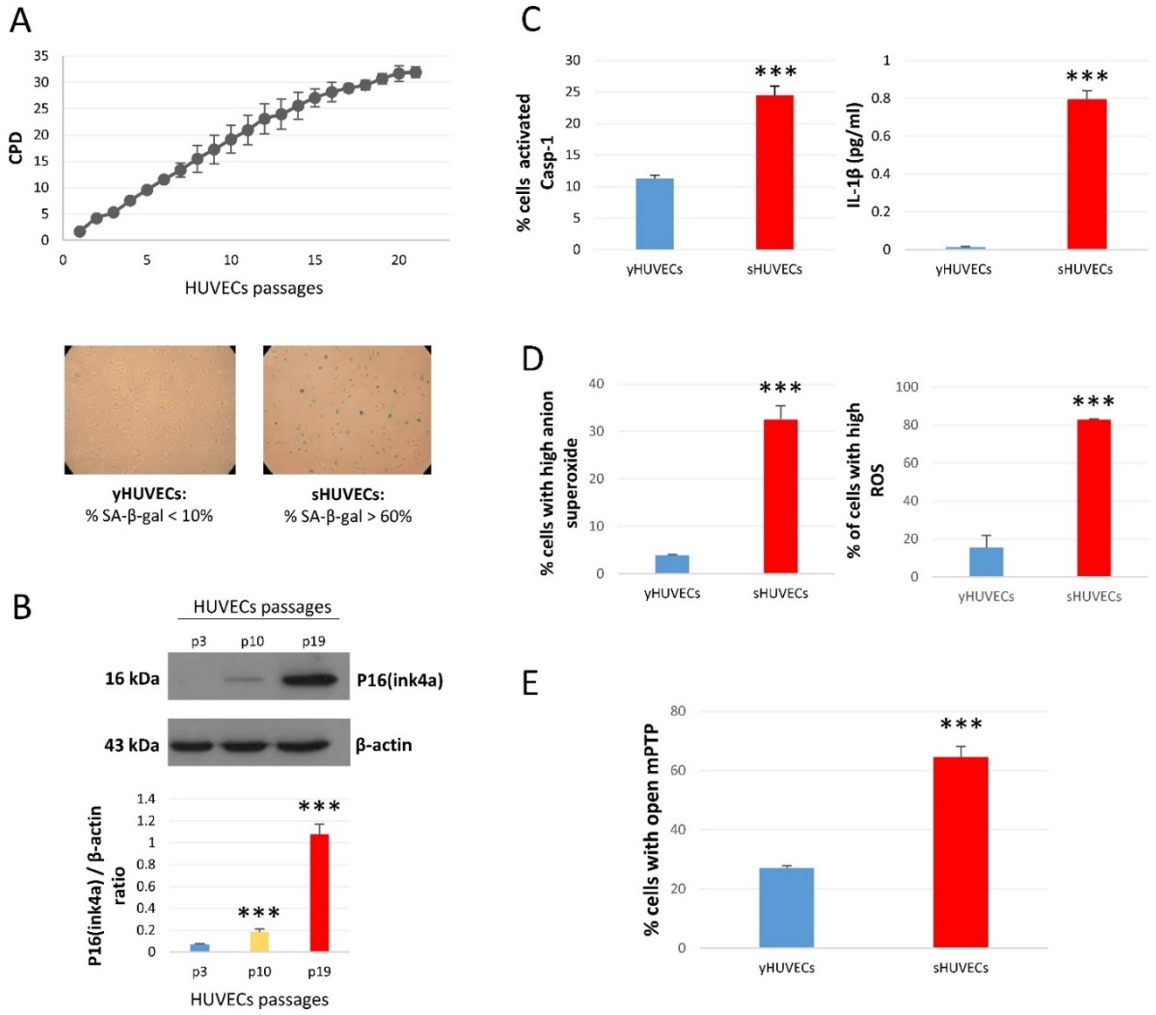
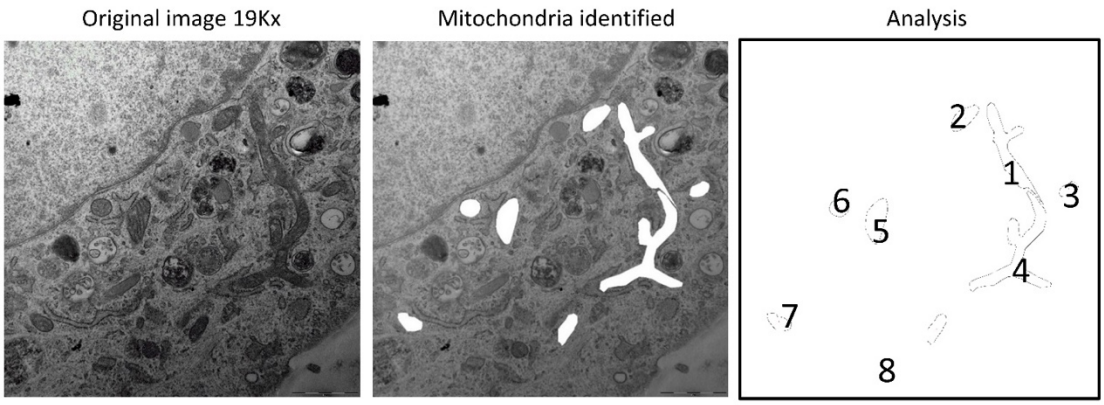


Figure 6. Characterization of endothelial cells in replicative senescence. (A) Growth curve of three different pool oh HUVECs - Y-axis: Cumulative Population Doubling (CPD); X-axis: cell passages from P1 to P21; Cells passages (upper panel) two representative photos of SA-β-Gal staining of young and senescent HUVECs. Cells with percentage of SA-β-Gal <10% were considered young cells (yHUVECs), while those with % of SA-β-Gal > 60% were identified as senescent cells (sHUVECs). (B) P16(ink4a) protein level in young (P3), intermediate (P10) and senescent (P19) HUVECs. (C) Percentage of HUVECs with active Casp-1 and IL-1β concentration (pg/ml) in culture media. (D) Percentage of HUVECs with high levels of anion superoxide (MitoSOX) and ROS (DFCDA). (E) Percentage of HUVECs with open mPTP. *** p<0.001 intermediate or senescent cells *vs* young cells. Bar graphs indicate means±SD of three independent experiments.

Furthermore, ROS and superoxide anion, markers of oxidative stress, increased in senescent (sHUVECs) compared to younger HUVECs (yHUVECs) (Figure 6D). As ROS in young cells are directly related to the opening of the permeability transition pore (mPTP) and both, in turn have a central role in in alterations of mitochondrial structure (Batandier et al., 2004),

we assayed for the first time this biochemical event. As shown in Figure 6E more than 60% of sHUVECs have got mPTP opened compared to 20% of yHUVECs. Altogether, these data support the assumption that in senescent cells mitochondria show altered biochemical functions.

Finally, we evaluated mitochondrial morphology in young and senescent HUVECs in order to provide evidence on their dysfunction. Indeed, mitochondrial perimeter positively correlates with mitochondria about to undergo a fission event. Similarly, mitochondrial solidity (compact shape) positively correlates with mitochondria about to undergo a fusion event (Westrate et al., 2014). TEM analysis showed that sHUVECs have higher perimeter and lower solidity than younger cells (Figure 7). We also measured the area, a third morphological feature that positively correlates with mitochondrion propensity to fragment.



	yHUVECs (n=24)	sHUVECs (n=22)
% Mitochondrial solidity < 0.8	1.3	2.7
Err. St.	0.21	0.32
Ttest	P < 0.05	

Label	Area	Perim.	Solidity
1	0.289	4.466	0.509
2	0.095	1.319	0.969
3	0.046	0.863	0.962
4	0.449	6.405	0.394
5	0.157	1.735	0.984
6	0.061	0.94	0.981
7	0.065	1.079	0.953
8	0.074	1.232	0.948

Figure 7. TEM analysis of mitochondrial morphology in endothelial cell. A representative image of mitochondria in a senescent cell (upper panel). Mitochondrial Area, Perimeter and Solidity are measured (lower panel).

3.1.2 MiR-34a, -146a and -181a are up-regulated in senescent endothelial cells and modulate Bcl-2 expression

Bcl-2 family members are regulators of mitochondrial physiological functions; therefore, the expression of Bcl-2 protein, the master of the family, has been analyzed in both young and senescent cells. Bcl-2 showed a consistent down-regulation during senescence process (Figure 8 A and B). MiR-34a, miR-146a and miR-181a can modulate Bcl-2 and other Bcl-2 family members (Ouyang et al., 2012; Yang et al., 2014, Zhang et al., 2015), thus we hypothesized a role of these three miRs in Bcl-2 modulation during senescence (Rippo et al., 2014). Here we show that beside the already demonstrated up-regulation of miR-146a (Olivieri et al., 2013c), senescent HUVECs express higher miR-34a and miR-181a levels (Figure 8C) compared to younger cells. Then, to corroborate our hypothesis that these miRs could be defined mitomiRs in our experimental model, we have analyzed their expression in isolated mitochondria from young and senescent cells. All three miRs were found expressed within mitochondria and notably, their levels were increased in sHUVECs mitochondrial fraction compared with the cytoplasmic one (Figure 8D).

In order to demonstrate a relationship between miR-34a, miR-146a and miR-181a and Bcl-2, yHUVECs were singularly transfected with the corresponding miRNA mimic. After 24 hours, over-expression of all the three mature miRs resulted in Bcl-2 down-regulation: miR-34a induced the more relevant decrease (from 100% of control to 60%), followed by miR-146a (from 100% to 65%) and by miR-181a (from 100% to 80%) (Figure 8E).

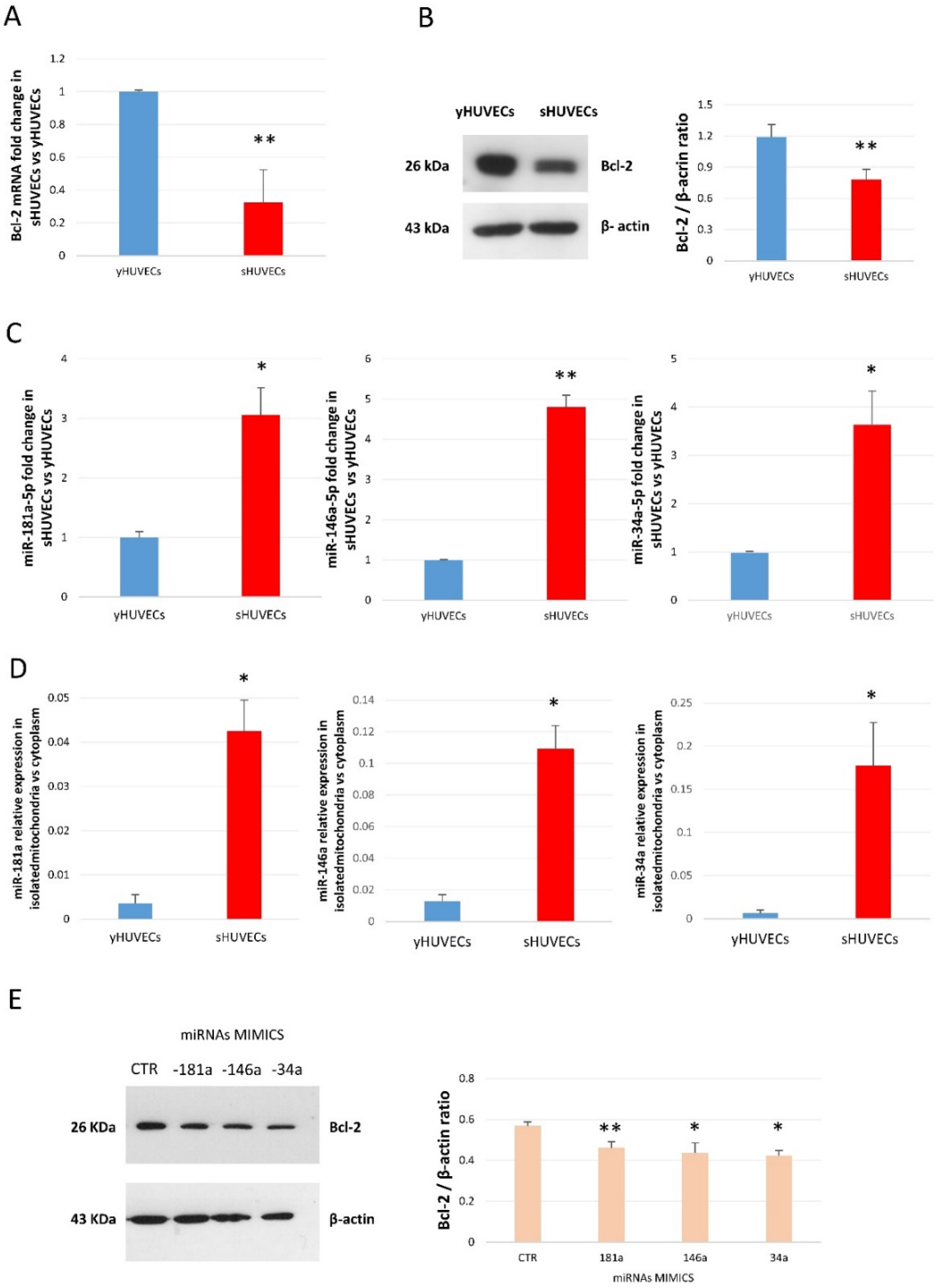


Figure 8. MiR-34a, -146a and -181a are up-regulated in endothelial cells and modulate Bcl-2. Bcl-2 mRNA (A) and protein (B) levels are down-regulated in sHUVECs compared to younger cells. (C) MiR-34a, -146a and -181a are up-regulated in replicative senescent HUVECs. (D) MiRs relative expression in isolated mitochondria vs cytoplasm. (E) Representative western blot of Bcl-2 protein expression after 24 h of miRNAs mimics transfection. CTR: miRNA negative control. *$p < 0.05$; **$p < 0.01$; *** $p<0.001$ intermediate or senescent cells *vs* young cells. Bar graphs represent means±SD of three independent experiments.

3.1.3 Overexpression of Mito-miR-146a, -34a and -181a induces mPTP opening, apoptosis and caspase-1 activation in young endothelial cells

The Bcl-2 family of proteins controls a critical step in commitment to apoptosis by regulating permeabilization of the mitochondrial membrane (mPTP) (Shamas-Din et al., 2014**)**. Given the role of miR-34a, miR-146a, and miR-181a in the regulation of Bcl-2, we analyzed mitochondrial transition pore and apoptosis rate in miRNA mimic transfected yHUVECs. MiRNAs forced expression of each of the three miRs promotes mPTP opening (Figure 9A) and apoptosis, analyzed by casp-3 activation and annexin V positivity (Figure 9B). Moreover, we also tested Survivin expression, a negative regulator of programmed cell death (Johnson, 2000). Western blot showed a significant mitomiR-mediated down regulation of the protein, consistent with the increased susceptibility to programmed cell death. Finally, Caspase-1 displayed an increased activation following miR-34a and -146a but not -181a overexpression (Figure 9C), possibly because of mitochondrial dysfunction or an accelerated cellular aging (Rimessi et al., 2016**)**. These data collectively indicate that at least three senescence-modulated mitomiRs are each alone sufficient to functionally affect mitochondrial function and apoptosis in young endothelial cells.

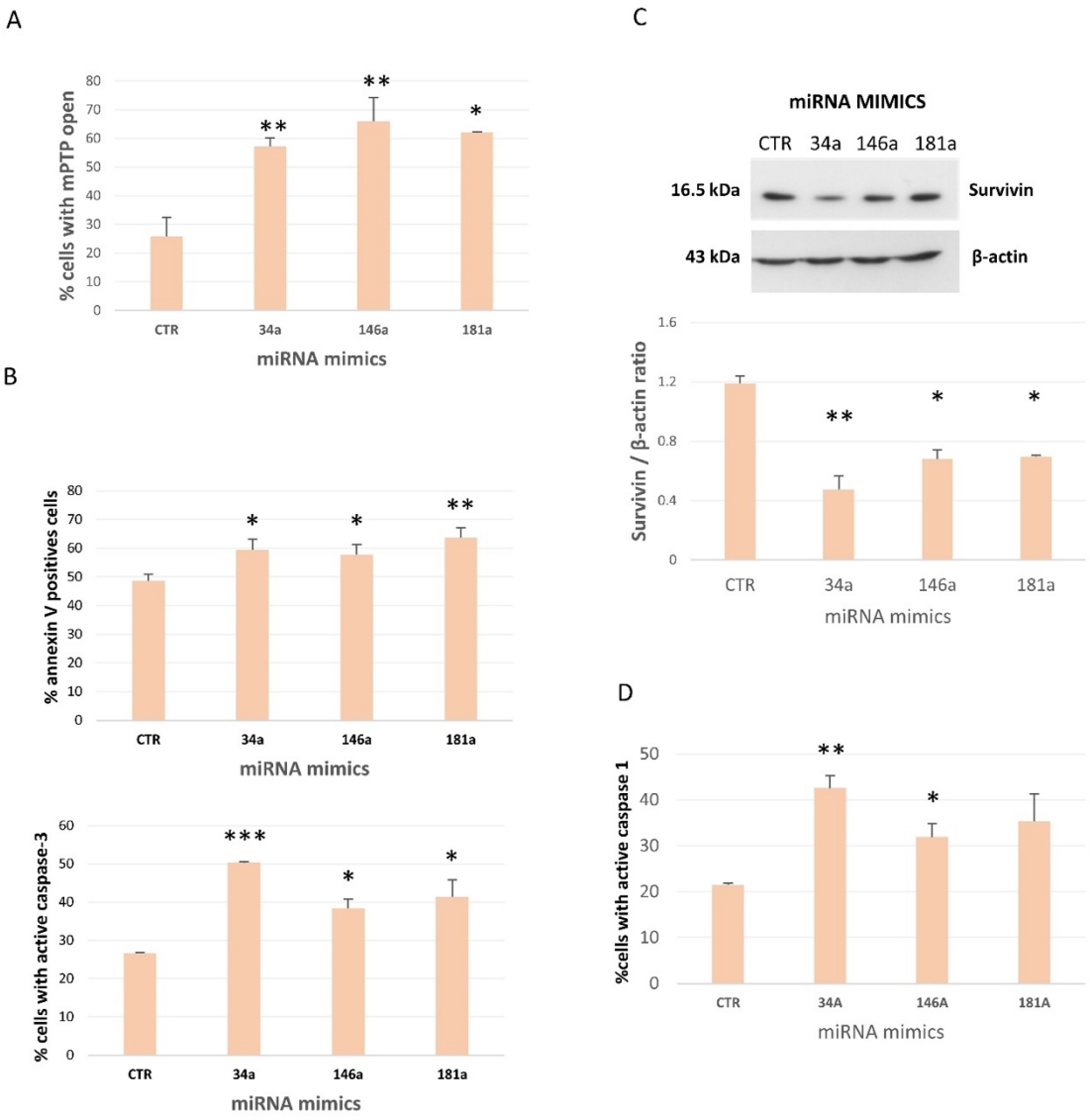


Figure 9. Overexpression of mitomiR-146a, -34a and -181a induces mPTP opening, apoptosis and casp-1 activation in yHUVECs. (A) Percentage of transfected young cells with open mPTP. (B) Percentage of transfected yHUVECs with Annexin V positive and with active Casp-3. (C) Survivin protein expression in transfected yHUVECs. (D) Percentage of transfected yHUVECs with active casp-1. CTR: miRNA negative control. *$p < 0.05$; **$p < 0.01$; *** $p<0.001$ intermediate or senescent cells vs young cells. Bar graphs represent means±SD of three independent experiments.

3.1.4 Human senescent endothelial cells are more resistant to apoptosis than younger cells

Since forced expression of SA-mitomiR-34a, -146a, -181a in young Endothelial Cells (ECs) had a pro-apoptotic effect, whereas endogenous expression in the senescent cells apparently does not affect their survival, we evaluated whether young and senescent HUVECs show a different susceptibility to both spontaneous and induced programmed death. Both were cultured in the presence or absence of serum and apoptosis was evaluated by annexin V and casp-3 activation after 48 and 72 hours. At early time point, young and senescent cells displayed similar spontaneous apoptosis rate. However, when apoptosis was induced by serum withdrawal senescent cells showed a significantly lower proportion of Annexin V positivity (Figure 10A). The analysis at 72 hours followed the same trend (Figure 10A). Similar results of fold increase were obtained for Caspase-3 activation (Figure 10 A and B right panels) but sHUVECs displayed a higher basal caspase-3 activity compared to young cells in absence of serum (Figure 10B left panel), suggesting a potential active role of this enzyme in processes different from programmed cell death. All these data indicate that senescent ECs are more resistant to serum withdrawal-induced apoptosis, despite Bcl-2 down-regulation during replicative senescence.

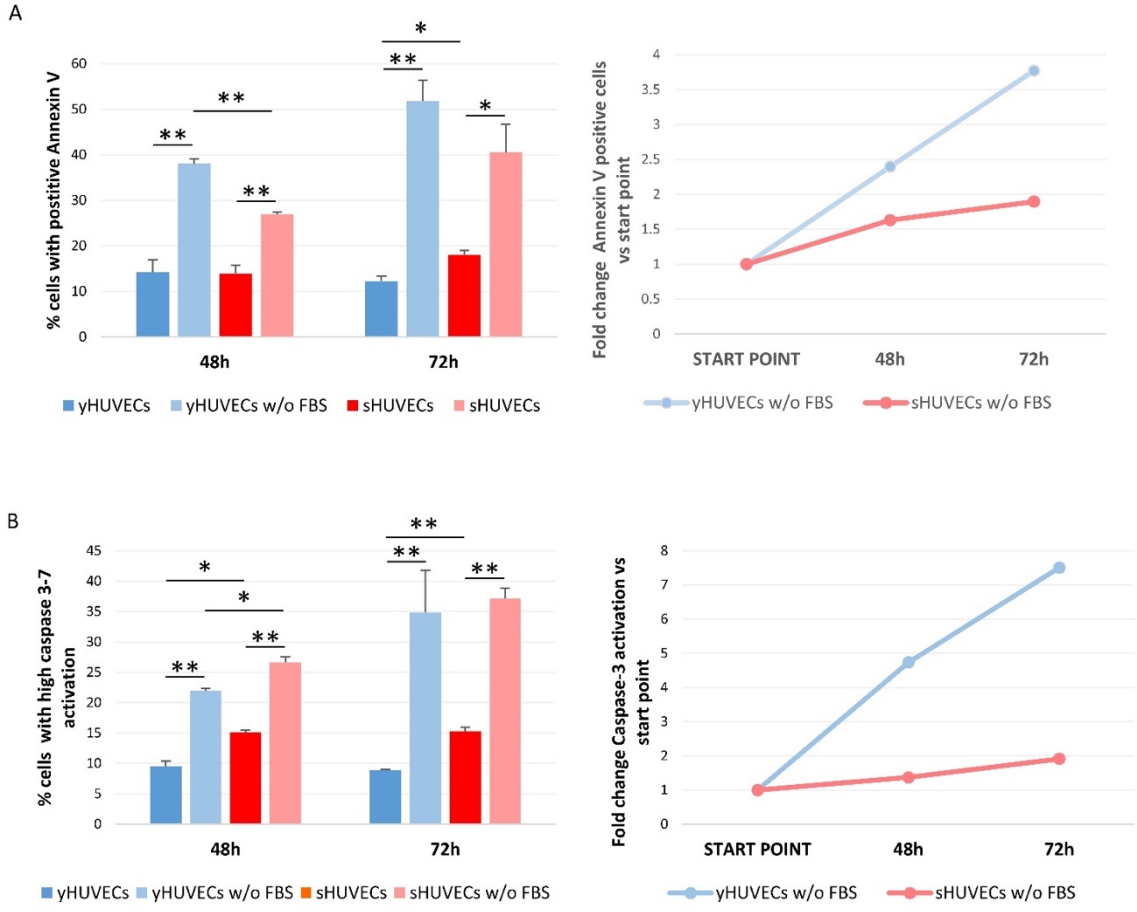


Figure 10. Human senescent endothelial cells are more resistant to apoptosis than younger cells. (A) Percentage of Annexin V positive cells with or without serum (left panel). Fold change of Annexin V positive cells vs "start point" of the experiment. *p < 0.05; **p < 0.01; *** p<0.001 intermediate or senescent cells vs young cells. Data are the means±SD of three independent experiments.

3.1.5 Senescent endothelial cells show increased accumulation of autophagosomes and miR-34a, -146a and -181a modulate LC3

Beyond their major role in the apoptotic process, Bcl-2 proteins family contributes to the regulation of numerous non-apoptotic functions, including autophagy. In particular, Bcl-2 is able to inhibit autophagy (Gross and Katz, 2017). Thus, in order to elucidate the potential consequence of Bcl-2 down-regulation, we looked for alterations in the autophagic process in our model of endothelial cells senescence. Electron microscopy analysis and microtubule-

associated protein 1A/1B-light chain 3 (LC3) assessment through western blotting are the gold standard to monitor autophagic activity (Tanida et al., 2008). TEM qualitative and semi-quantitative analysis of senescent ECs showed a largest number of autophagosomic vesicle compared to young cells (Figure 11A). Moreover, LC3-II expression progressively and significantly increased during endothelial cells senescence (Figure 11B). Transfection of yHUVECs with miR-34a, -146a and -181a mimics promoted the conversion of LC3-I to LC3-II (Figure 11C) thus suggesting a simulation of the process occurring in senescent cells with increased autophagosomal accumulation.

3.1.6 Discussion

Mitochondria seem to be one of the major determinants of aging (Correia-melo et al., 2015). Various microRNAs, which are associated to senescence and inflammatory process, are known to influence mitochondrial functions (mitomiRs). Recently, Rippo and colleagues unearthed three different SA-inflamma-mitomiRs (miR-34a, -146a, -181a) that can target Bcl-2 protein (Rippo et al., 2014). Bcl-2 modulation during senescence is largely studied, but results achieved are contrasting (Zhu et al., 2016; Uraoka et al., 2011), maybe due to senescence inducers and cellular types. The role of Bcl-2 in cellular senescence makes sense due to Bcl-2 family central players of cell death by diverse mechanisms, including apoptosis and autophagy. This hypothesis took form and strength recently as several Bcl-2 proteins have been individuated as targets of senolytics (Zhu et al., 2016).

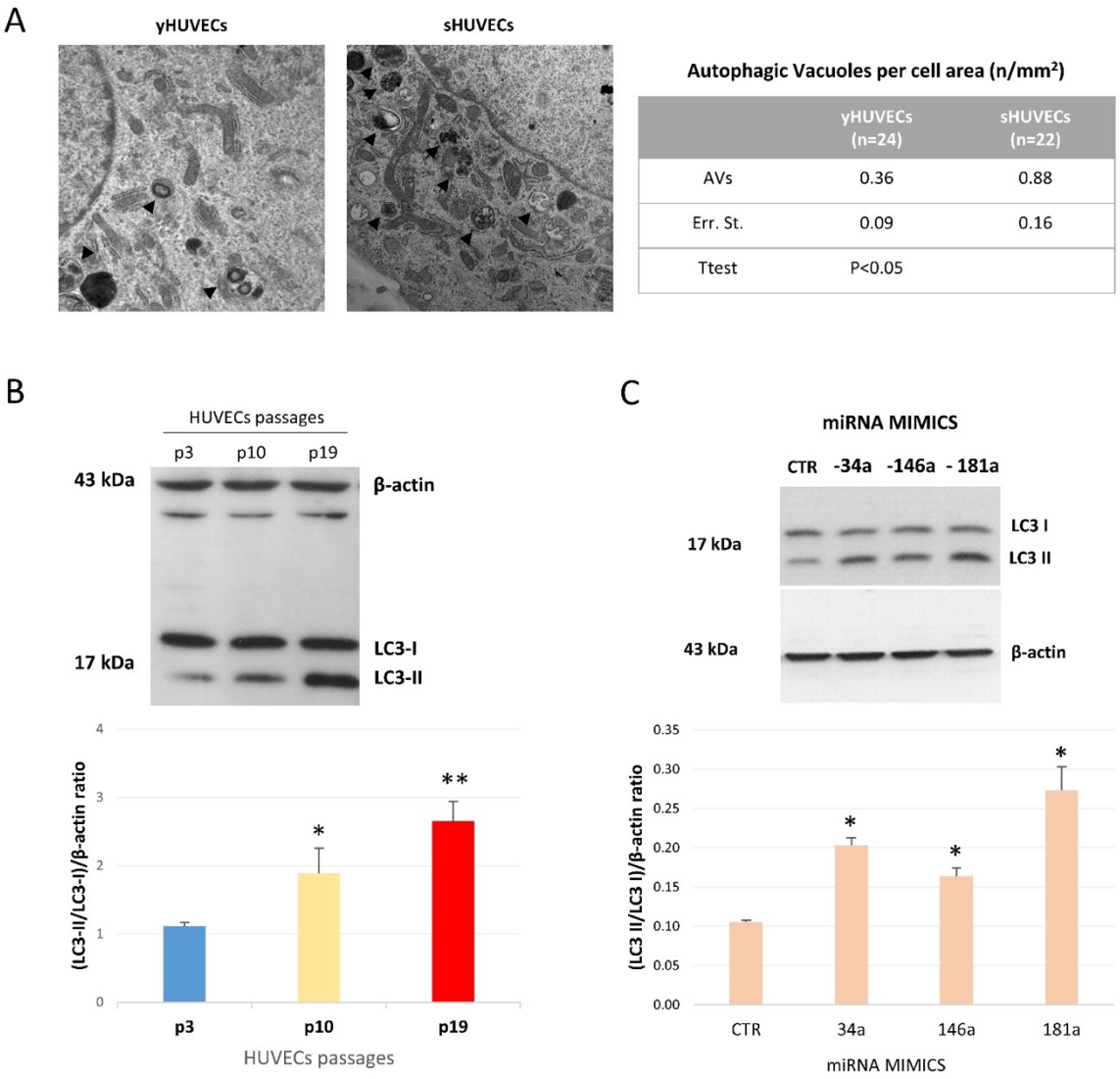


Figure 11. Senescent endothelial cells show increased autophagosomes accumulation and miR-34a, -146a and -181a modulate LC3 I-II expression. (A) TEM images of autophagic vacuoles (AV) in young and senescent HUVECs (left panel); quantification of AVs per cell area (right panel). (B) LC3-II/LC-I ratio. (C) LC3-II/LC-I ratio in transfected young cells. *p < 0.05; **p < 0.01; *** p<0.001 intermediate or senescent cells vs young cells. Data are the means±SD of three independent experiments.

With this work we demonstrated that during endothelial replicative senescence mitomiR-34a, -146a and -181a regulate Bcl-2 expression. Their increased expression correlates with the down-regulation of the anti-apoptotic target Bcl-2. Surprisingly, we observed that even though senescent endothelial cells express lower levels of Bcl-2 and show most of the

biochemical features of a dying cell related to Bcl-2 downregulation, they are more resistant to pro-apoptotic stimuli, such as serum deprivation compared to young cells. Here we show for the first time that these biochemical changes in aging cells can depend on mitomiR up-regulation. In fact, forced and acute expression of miR-34a, -146a and -181a in young cells affects mitochondrial function provoking mPTP opening, casp-3 and casp-1 activation, survivin down-regulation and, as expected, increased susceptibility to apoptotic death, annexin V positivity, all phenomena consistent with Bcl-2 downregulation. The property of senescent cell population resistance to cell death appears therefore contradictory. One conceivable explanation could be given by the inhibitory role of Bcl-2 on the early phases of autophagy: its progressive and chronic downregulation in senescent cells could promote the auophagic process, thus representing a senescent cell adaptation to extreme conditions and protecting cells from damage accumulation and apoptosis. However, the efficiency of the autophagic flux has been shown to decline during aging in an evolutionarily conserved manner and we observed autophagic vacuoles accumulation in senescent endothelial cells and acute mitomiR up-regulation by transfection in young cells leads to LC3II over-expression. Whether autophagic flux efficacy is actually improved or impaired in our senescent ECs remain to be clearly established. Thus, unless ECs display peculiar autophagic features during aging, it is plausible that the observed cargo of autophagic vacuoles progressively accumulates without fusing with lysosomes in aging cells. The altered autophagic flux would not be consistent with the inhibitory role of Bcl-2 on the early phases of autophagy and would instead indicate either no role or interestingly novel potential role of Bcl-2 during aging. One hypothesis currently under investigation in our lab is that aging cells could isolate those organelles (or part of them) highly damaged in waste dumps isolated from the cytoplasm.

Overall, in this first part, data demonstrated how at the molecular level some SA-inflamma-mitomiRs affect mitochondrial functions during replicative senescence, and suggest an axis connecting mitochondrial dysfunction, cell death and autophagy machineries and inflammation.

3.2 Anti-TNF-α treatment modulates SASP and SASP-related microRNAs in endothelial and circulating angiogenic cells

3.2.1 Anti-TNF-α treatment of THP-1 cells

One of the factors characterizing the SASP phenotype is TNF-α, which exerts autocrine and paracrine pro-inflammatory effects. Therefore we were interested to test the hypothesis that adalimumab, a specific antibody anti-TNF-α, could be effective in modulating SASP and the expression of associated microRNAs in senescent endothelial cells and their circulating precursors. As a positive control of treatment efficiency, we used LPS-stimulated THP-1cells because activated monocytes are among the major producers of TNF-α and LPS is a known TLR4 agonist. LPS-stimulated THP-1 show a secretory phenotype similar to sHUVECs. Therefore, we first assessed their "SASP like" phenotype analyzing i) the expression of miR-126-3p and miR-146a-5p with their respective markers, Spred1 and Irak1; ii) IL6 release in culture medium and iii) SA-β-Gal activity. Cells were treated with LPS (1 μg/ml), for 30 minutes or 5 hours. (Figure 12A). The levels of the two miRs and the amount of target proteins determined in cells not exposed to LPS were considered as baseline.

LPS stimulation induced a significantly increased release of TNF-α which was highest at 5 hours; it also increased miR-146a-5p expression, and reduced miR-126a-3p levels (Figure 12A, B and C). Irak1 is expressed at low level in basal condition and it rose significantly at 30 minutes; Spread1 expression increased after 5 hours of LPS treatment, but the latter change was modest (Figure 12D).

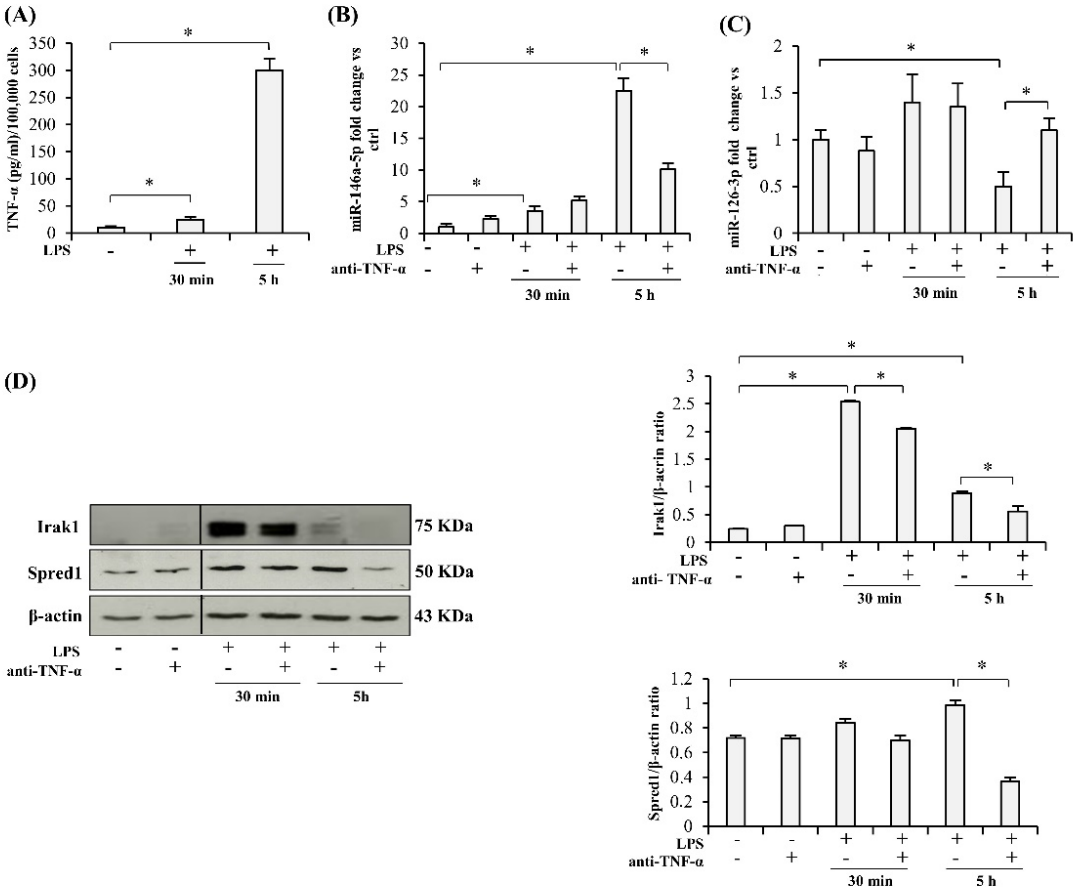


Figure 12. Effect of TNF-α blockade on the expression of miRs and their target proteins in LPS-exposed THP-1 cells. **(A)** TNF-α release into the culture medium by THP-1 cells after LPS treatment (1 μg/ml), expressed as pg/ml per 100,000 cells. (B) MiR-146a-5p expression in THP-1 cells after 30 min or 5 h (hours) LPS exposure, with/without 24 h anti-TNF-α pretreatment, measured as fold change *vs* ctrl. (C) MiR-126-3p expression in THP-1 cells after 30 min or 5 h LPS exposure, with/without 24 h anti-TNF-α pretreatment, measured as fold change *vs* ctrl. (D) Irak1 and Spred1 expression levels and densitometry data in THP-1 cells after 30 min or 5 h LPS stimulation, with/without 24 h anti-TNF-α pretreatment. * Student's *t* test, $p < 0.05$. Data are mean ± S.D. of 3 independent experiments.

Interestingly, pretreatment with anti-TNF-α for 24 hours significantly inhibited LPS-induced miR-146a-5p up-regulation and miR-126-3p down-regulation (Figure 12B and C), it attenuated the increase in Irak1 levels (Figure 12D), and completely abolished the slight increase in Spred1 protein (Figure 12D).

3.2.2 Anti-TNF-α treatment of HUVECs

3.2.2.1 Effects of TNF-α inhibition on young and senescent HUVECs

Young HUVECs release small amount of TNF-α in culture medium but their stimulation with LPS induced TNF-a release as well as during their replicative senescence (Figure 13A). MiR-126-3p and miR-146a-5p follow the same trend during replicative senescence (Figure 13D and 13B) as previously reported (Olivieri et al., 2013c; Olivieri et al., 2014). Treatment with anti-TNF-α alone for 24 hours significantly decreased miR-146a-5p only in senescent HUVECs (Figure 13B) while no significant change in miR-126-3p was observed in either young or senescent HUVECs. MiR-146a-5p increased after LPS stimulation both in young and senescent HUVECs (Figure 13C), while anti-TNF-α treatment prevented miR-146a-5p increase after 5 hours of LPS treatment only in senescent HUVECs (Figure 13C). Irak1 expression parallels miR-146a-5p trend with a significant reduction obtained with anti-TNF-α treatment both alone and after 5 hours of LPS stimulation only in senescent cells (Figure 13F). MiR-126-3p expression decreases after 5 hours of LPS treatment only in senescent HUVECs and this effect is prevented by anti-TNF-α treatment (Figure 13E). Previous papers showed no modulation of miR-126-3p in neither LPS nor TNF-α treated endothelial cells (Harris et al., 2008). These data are in agreement with our data on young HUVECs (Figure 13E). Concordantly, Spred1 expression is significantly lower in senescent cells treated with

anti-TNF-α, after 5 hours of LPS stimulation (Figure 13F). No significant modulation of Irak1 and Spred1 was observed in young cells (data not shown).

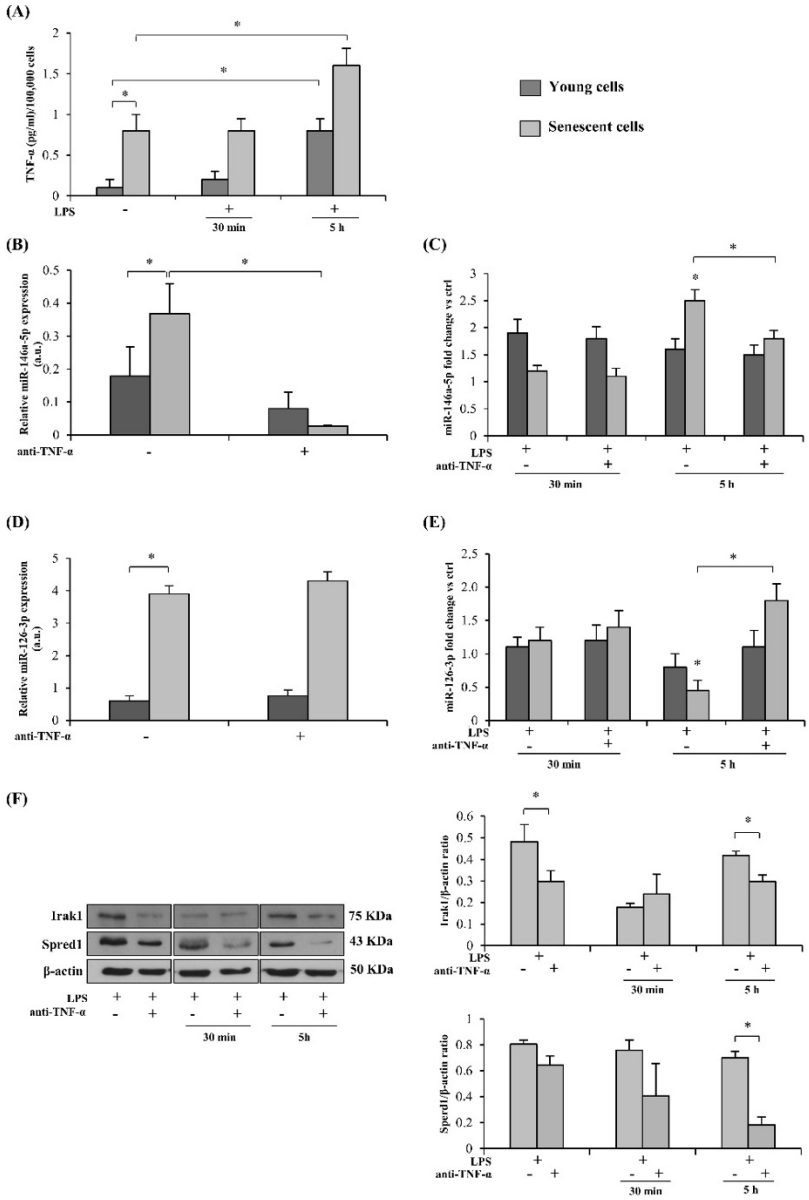
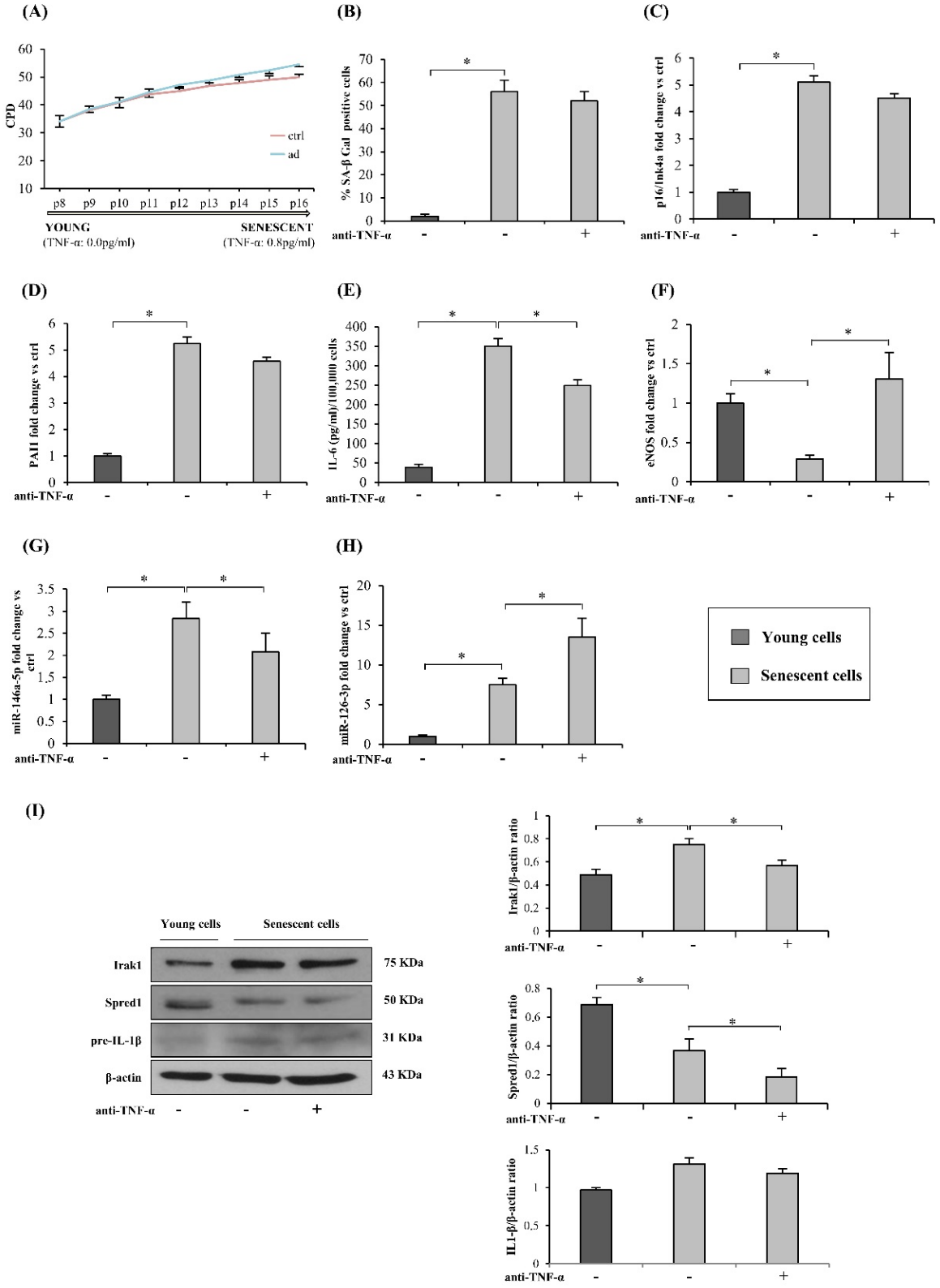


Figure 13. Effects of TNF-α inhibition on young and senescent HUVECs. **(A)** TNF-α release into the culture medium by LPS-exposed (1 µg/ml) young and senescent HUVECs, expressed as pg/ml per 100,000 cells. **(B)** MiR-146a-5p expression in young and senescent HUVECs with/without 24 h anti-TNF-α pretreatment, measured as relative expression (a.u). (C) MiR-146a-5p expression in young and senescent HUVECs after 30 min or 5 h LPS stimulation, with/without 24 h anti-TNF-α pretreatment, measured as fold change *vs* ctrl. (D) MiR-126-3p expression in young and senescent HUVECs with/without 24 h anti-TNF-α pretreatment, measured as relative expression (a.u). (E) MiR-126-3p expression in young and senescent HUVECs after 30 min or 5 h LPS stimulation, with/without 24 h anti-TNF-α pretreatment, measured as fold change *vs* ctrl. (F) Irak1 and Spred1 expression and densitometry data in young and senescent HUVECs after 30 min or 5 h LPS stimulation, with/without 24 h anti-TNF-α pretreatment. * Student's *t* test, $p < 0.05$. Data are mean ± S.D. of 3 independent experiments.

3.2.2.2 Effects of TNF-α inhibition on HUVECs undergoing replicative senescence

We next tested if continuous TNF-α blockade during replicative senescence could reduce senescence/SASP markers in HUVECs. Since no detectable TNF-α is released in culture medium of young cells, exogenous addiction of adalimumab to endothelial cells culture started when HUVECs reached 34 CPD, (Figure 14A). Unfortunately, the treatment failed to reduce the percentage of SA-β-Gal-positive cells (Figure 14A and B); in addition, it did not significantly affect the increase of p16/Ink4a and PAI1 expression, two classic markers of endothelial cells senescence (Figure 14C and D). However, IL6 release in medium was significantly lower in anti-TNF-α treated cells at the end of the growth curve (Figure 14E), reflecting the reduction of SASP-related cytokines. Furthermore, the dramatic decrease of eNOS expression observed during cell senescence is completely abolished in presence of anti-TNF-α treatment (Figure 14F). Moreover, we observed a decreased miR-146a-5p and Irak1 expression (Figure 14G and 14I), together with increased miR-126-3p and decreased Spred1 expression (Figure 14H and 14I). IL-1β in Western Blot analysis was unchanged between the two groups (Figure 14I), while IL-1β in culture medium was undetectable (data not shown).

Figure 14 (next page). Effects of TNF-α inhibition on HUVECs undergoing replicative senescence. **(A)** Cumulative population doublings (CPDs) of HUVECs exposed to continuous anti-TNF-α treatment and of control cultures from 34 CPDs to complete growth arrest. Y axis, CPD; x axis, number of passages. **(B)** Percentage of SA-β-Gal-positive cells at the beginning of the curve (young cells) and at its end (senescent cells), with/without (w/o) anti-TNF-α treatment. **(C)** p16/Ink4a mRNA expression in young and senescent cells w/o anti-TNF-α treatment. Data expressed as fold changes *vs* young cells. **(D)** PAI1 mRNA expression in young and senescent cells w/o anti-TNF-α treatment. Data expressed as fold changes *vs* young cells. **(E)** IL-6 release (pg/ml per 100,000 cells) into the culture medium by young and senescent cells w/o anti-TNF-α treatment. **(F)** eNOS mRNA expression in young and senescent cells w/o anti-TNF-α treatment. Data expressed as fold changes *vs* young cells. **(G)** MiR-146a-5p expression in young and senescent cells with/without anti-TNF-α treatment. Data expressed as fold changes *vs* young cells. **(H)** MiR-126-3p expression in young and senescent cells with/without anti-TNF-α treatment. Data expressed as fold changes *vs* young cells. **(I)** Irak1, Spred1 and IL1β expression and densitometry data (normalized to β-actin) in young and senescent cells with/without anti-TNF-α treatment. * Student's *t* test, $p < 0.05$. Data are mean ± S.D. of 3 independent experiments.


(A)
CPD
ctrl
ad
p8 p9 p10 p11 p12 p13 p14 p15 p16
YOUNG
(TNF-α: 0.0pg/ml)
SENESCENT
(TNF-α: 0.8pg/ml)
(B)
% SA-β Gal positive cells
anti-TNF-α
(C)
p16/Ink4a fold change vs ctrl
anti-TNF-α
(D)
PAI1 fold change vs ctrl
anti-TNF-α
(E)
IL-6 (pg/ml)/100,000 cells
anti-TNF-α
(F)
eNOS fold change vs ctrl
anti-TNF-α
(G)
miR-146a-5p fold change vs ctrl
anti-TNF-α
(H)
miR-126-3p fold change vs ctrl
anti-TNF-α
Young cells
Senescent cells
(I)
Young cells
Senescent cells
Irak1
Spred1
pre-IL-1β
β-actin
75 KDa
50 KDa
31 KDa
43 KDa
anti-TNF-α
Irak1/β-actin ratio
anti-TNF-α
Spred1/β-actin ratio
anti-TNF-α
IL1-β/β-actin ratio
anti-TNF-α

3.2.2.3 *Effects of TNF-α inhibition on the SASP bystander effect*

To test the ability of TNF-α inhibition to induce a bystander effect, conditioned medium from senescent HUVECs (SA-β Gal > 50 %) was mixed with 1/3 of fresh medium (with 30% of serum) and used to treat young endothelial cells, with or without TNF-α blocking antibody, for 2 weeks (Fig 15A). Exposure of young HUVECs to conditioned medium from senescent cells induced a doubling of SA-β-Gal-positive cells, including adalimumab-treated ones (Figure 15B). Moreover, senescence conditioned medium induced eNOS down-regulation and p16/INK4a up-regulation, neither effect was significantly reversed by anti-TNF-α (Figure 15C and 15D).

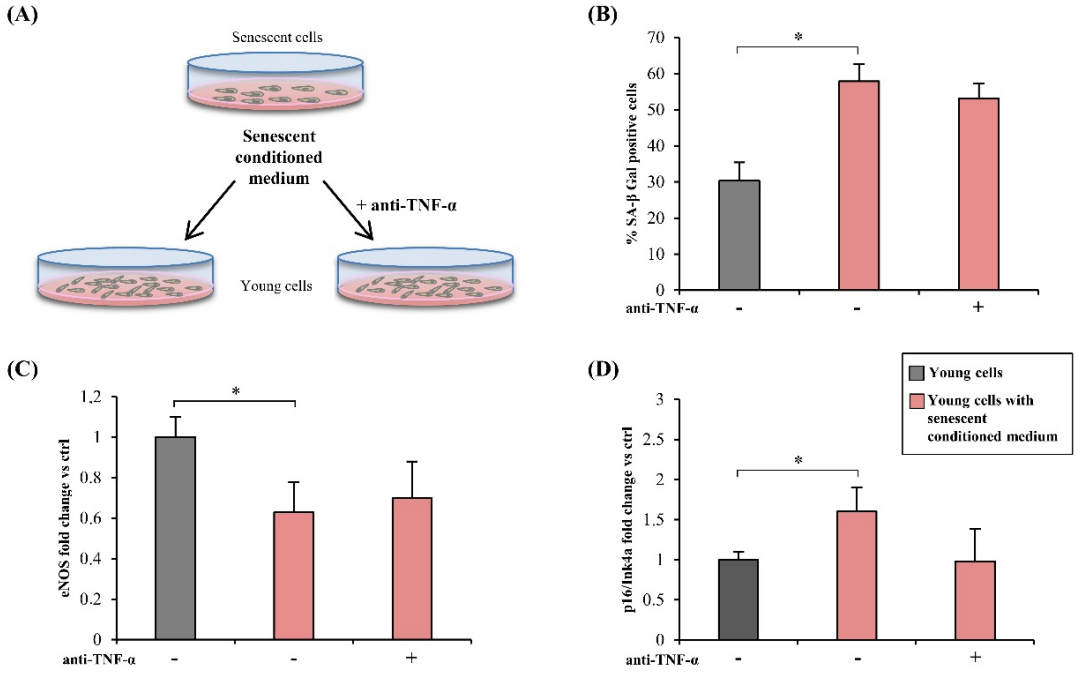


Figure 15. Effects of TNF-α inhibition on the SASP bystander effect. **(A)** Drawing showing the experimental design. Conditioned medium from senescent cells (SA-β-Gal > 50 %) was mixed with 1/3 of fresh medium (with 30 % serum) and used to treat young cells (SA-β-Gal < 5 %) for 2 weeks, with/without anti-TNF-α treatment. Conditioned medium from young cells was used as control. **(B)** Percentage of SA-β-Gal positive cells after 2 week exposure to conditioned media, with/without anti-TNF-α treatment. **(C)** and **(D)** eNOS and p16/Ink4a expression in cells treated with conditioned medium, with/without anti-TNF-α treatment. Data expressed as fold change *vs* control cells. * Student's *t* test, $p < 0.05$. Data are mean ± S.D. of 3 independent experiments.

3.2.3 Adalimumab treatment of sHUVECs affects their secretome ability to promote tumor cells migration

To test whether long-term anti-TNF-α treatment reduces the cell non-autonomous functions of the endothelial SASP, a wound healing assay was conducted using MCF-7 breast cancer cells and A549 lung cancer cells cultured in presence of conditioned medium from senescent HUVECs. Notably, the MCF-7 cell line is responsive to IL-6 (Zacarias-Fluck et al., 2015). Senescent cells subjected to long-term adalimumab treatment and untreated were placed in fresh medium for 24 h and their conditioned medium was mixed with 1/3 fresh DMEM. These mixtures, senescence conditioned medium (SEN-CM), and anti-TNF-α treated senescence conditioned medium (ATT SEN-CM) were used to treat MCF-7 cells in the wound healing assay (Figure 16). Conditioned medium from young cells mixed with DMEM was used as control. SEN-CM strongly promoted MCF-7 migration, whereas ATT SEN-CM did not (Figure 16B and C). This experiment clearly demonstrates that continuous blockade of TNF-α in endothelial cells lead to a less consistent SASP. The same experiment performed with A549, a lung tumor cell line, did not achieve the same results (data not shown), suggesting a different sensitivity of various tumors to SASP components.

3.2.4 Effects of TNF-α inhibition in the secretome of senescent HUVECs on mammospheres assay

The mammosphere assay allows to propagate a population of putative cancer-stem-cells as floating spheroids. This assay represents a useful surrogate test to screen the tumorigenic and metastatic potential of breast cancer cells (Sansone et al., 2007). The same conditioned media obtained from young cells (CTRL-CM), senescent cells (SEN-CM), and anti-TNF-α

treated senescent cells (ATT SEN-CM) used to treat MCF-7 cells in the wound healing assay were used for MCF-7 mammospheres (MS) assay (Figure 16D and E). Primary MS formation was assessed after 3 and 7 days. SEN-CM strongly promoted the formation of MCF-7-derived mammospheres, whereas in ATT SEN-CM this promotion is significantly reduced both at 3 and at 7 days ($p > 0.05$) (Figure 16D and E).

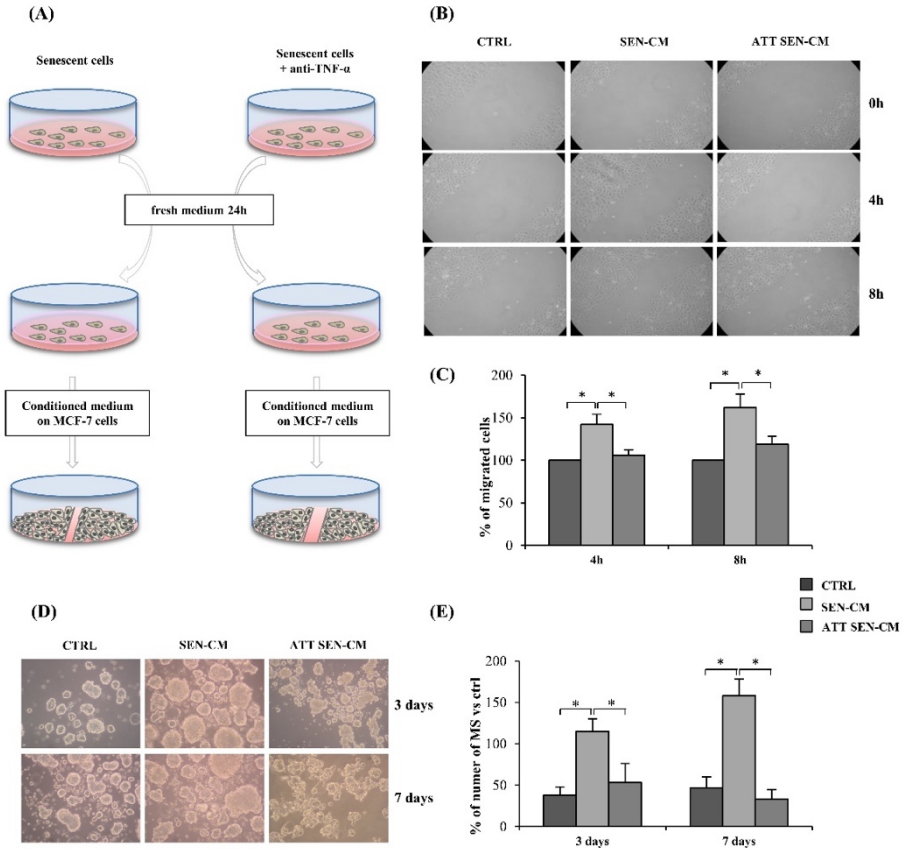


Figure 16. Anti-TNF-α treatment effect on pro-motility activity and mammospheres (MS) promotion of HUVECs secretome on MCF-7 tumor cell. **(A)** Drawing showing the experimental design. Senescent cells exposed to anti-TNF-α long-term treatment and senescent control cells were switched to fresh medium for 24 h and then their conditioned medium was mixed with 1/3 fresh DMEM. These mixtures, senescence conditioned medium (SEN-CM), and anti-TNF-α treated senescence conditioned medium (ATT SEN-CM) were used to treat MCF-7 in a wound healing assay. Conditioned medium from young cells was mixed with DMEM and used as control. **(B)** Light microscopic photographs showing MCF-7 migration in the wound healing assay 0, 4 and 8 h after treatment with SEN-CM or ATT SEN-CM. **(C)** Percentage of migrating cells after 4 and 8 h exposure to SEN-CM and ATT SEN-CM. Data expressed as percent of control. **(D)** Representative pictures of MCF7-derived mammosphers (MS) promotion induced by conditioned media obtained from young cells (CTRL-CM), senescent cells (SEN-CM), and anti-TNF-α treated senescent cells (ATT SEN-CM). (E) Quantification of MCF7-derived mammospheres in presence of different conditioned media. Data are presented as number of MS per well, at 3 and 7 days. Data are mean ± S.D. of 3 independent experiments. Scale bar 50 µm. * $p < 0.05$. * Student's *t* test, $p < 0.05$. Data are mean ± S.D. of 3 independent experiments.

3.2.5 Effects of TNF-α inhibition on CACs from psoriatic patients

Psoriatic patients show elevated circulating levels of TNF-α and psoriasis have been associated to an increased incidence of ARDs (Henseler and Christophers, 1995; Hugh et al., 2014). Given that Circulating angiogenic cells (CACs) are monocytes capable of releasing pro-angiogenic cytokines, CACs from 10 psoriatic patients, receiving adalimumab after a washout period, were analyzed to establish whether anti-TNF-α monotherapy for 3 months is able to reduce miR-126-3p and miR-146a-5p expression. TNF-α inhibition affected miR-146a-5p levels (Figure 17A), as also noted in the experiments with THP-1 cells and HUVECs, suggesting that the epigenetic modulation of inflammatory pathways induced by adalimumab *in vitro* resembles the modulation induced *in vivo*. Notably, miR-126-5p expression was not significantly affected in psoriasis patients, suggesting that the anti-inflammatory effects of adalimumab prevail on its proangiogenic effects (Figure 17B).

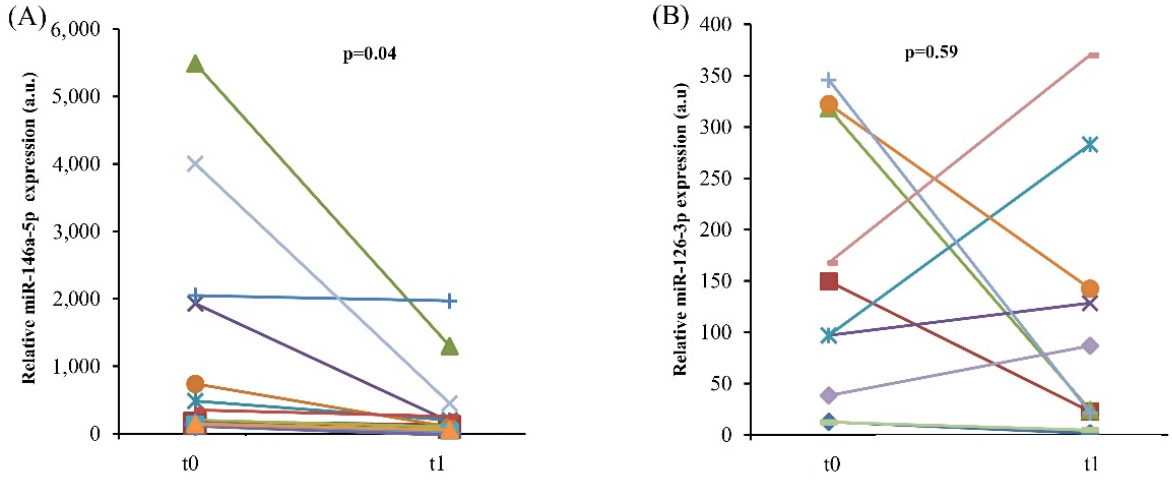


Figure 17. In vivo anti-TNF-α treatment and endothelial senescence-associated miR expression levels in psoriatic patients. MiR-146a-5p **(A)** and miR-126-3p **B.** expression in CACs from 13 psoriasis patients before and after 3 month anti-TNF-α monotherapy with adalimumab. P from Student's t test for paired samples.

3.2.6 Discussion

Selective senescent cells removal or SASP attenuation is one of the major goals of present and future aging research. Avoiding deleterious effect associated to SASP could provide an incredible prompt to successful aging (Tchkonia et al., 2013). Here I have shown the data published in Oncotarget (Prattichizzo et al., 2016b) that suggest how continuous blockade of TNF-α in endothelial cells undergoing senescence attenuates SASP. In fact, we observed decreased IL6 secretion, suppression of miR-146a-5p and increase of miR-126-3p, respectively an inflamma-miR and an endo-miR, and their targets. Moreover, our data suggest that this effect is accompanied by increased eNOS expression, suggesting an enhanced endothelial function. Interestingly, this effect is not associated with significant decrease of classic senescence biomarkers, such as SA-β-Gal, p16/Ink4a, and PAI1. Previous studies have shown the possibility of experimentally dissociate SASP from senescence (Acosta et al., 2013; Laberge et al., 2012). Some reports show that SASP modulation influences the rate of senescence (Acosta et al., 2013; McCarthy et al., 2013; Hubackova et al., 2012), while others do not (Laberge et al., 2012). In our experimental model, i.e. HUVECs replicative senescence, the number of senescent cells was not significantly influenced by continuous anti-TNF-α treatment, suggesting that TNF-α is not closely associated with the arrest of replicative growth. Although it has been demonstrated that IL-1 or TGF-β blockade can attenuate SASP spread in different senescence models (Acosta et al., 2013; Hubackova et al., 2012), data on anti-TNF-α treatment were scarce and inconclusive (Zhang Y et al., 2009). The present findings indicate that anti-TNF-α treatment can restrain the SASP without significantly affecting senescence signal transmission, either autocrine or paracrine. We block only TNF-α spontaneously produced by endothelial cells

undergoing senescence, which never increases over 1 pg/ml per 100.000 cells, whereas in other experimental models cells have been treated with greater (up to 10 fold) amounts of TNF-α (Zhang Y et al., 2009). This suggests a relevant role for TNF-α both in physiological endothelial aging (with low level of circulating TNF-α) and accelerated endothelial/general aging in presence of chronic diseases (characterized by high circulating levels of various cytokines including TNF-α). In particular, TNF-α seems to have a relevant role in endothelial inflammation during aging (Zhang H et al., 2009), since its detrimental role for endothelial function is exacerbated in chronic conditions linked to accelerated aging such as diabetes, cardiovascular diseases or autoimmune diseases (Steyers and Miller, 2014). Psoriasis is one of such conditions (Hugh et al., 2014). Our data add other molecular mechanisms of TNF-α induced endothelial dysfunction and inflammation. Interestingly, increasing molecular and statistical evidences suggest chronic inflammation that accompanies most age related diseases as a main driver of accelerated aging (Jurk et al., 2014; Stepanova et al., 2015).

Unfortunately, anti-TNF-α therapy cannot be proposed for clinical use as anti-aging agent in old patients, due to its consistent side effects. However, the present findings suggest that it is possible to dissociate the SASP from senescence, in line with earlier reports (Acosta et al., 2013; Laberge et al., 2012) and encourage the research for natural or synthetic substances that not only suppress but also restrain the SASP.

3.3 MiR-21-5p and miR-126a-3p levels in plasma and circulating angiogenic cells: relationship with type 2 diabetes complications

The chemical-clinical, and anthropometric characteristics of the studied groups, 107 healthy CTR subjects and 193 T2DM patients, 76 patients without (T2DM NC) and 117 patients with (T2DM C) diabetic complications, are reported in Table 2. Since T2DM group included a greater proportion of males than the CTR group, T2DM C patients were older than CTR subjects and males were a greater proportion than among T2DM NC patients as well as CTR subjects, all subsequent analyses were adjusted for age and sex.

Variables	CTR (N=107)	T2DM NC (n=76)	T2DM C (n=117)
Age, (yrs)	64.25±7.56	65.56±6.96	66.51±7.48#
Males, n (%)	49 (45.79)	36 (47.37)	69 (58.97)*#
BMI, kg/m2	26.67±5.4	28.47±4.34	28.57±3.46*
Glucose, mg/dL	92.23±8.41	154.63±40.78*	178.89±56.78*#
HbA1c, %	5.96±0.41	7.34±1.28*	7.77±1.19*#
Insulin, mcU/mL	5.66±3.75	6.63±4.65	6.78±4.73
HDL cholesterol, mg/dL	59.29±15.34	55.94±17.85	51.43±15.14#
Total cholesterol, mg/dL	212.72±42.21	215.17±37.35	202.19±39.20
Creatinine, mg/dL	0.82±0.22	0.84±0.17	1.01±0.43*#
Hs-CRP, mg/L	2.52±3.77	3.79±4.25	3.54±4.24
MiR-126-3p, RE	0.33±0.31	0.24±0.24*	0.23±0.19*
MiR-21, RE	0.43±0.42	0.33±0.42*	0.31±0.34*

Table 2. Chemical, clinical, and anthropometric characteristics of CTR subjects and patients without and with T2DM complications. T2DM NC: patients without diabetic complications; T2DM C: patients with diabetic complications; RE=relative expression. The chi square test was applied for dichotomous variables. ANCOVA adjusted for age and sex or for variables significantly correlating with miRNAs was applied for continuous variables: *p<0.05, reference group: CTR; #p<0.05, reference group: T2DM NC (diabetic patients without diabetic complications).

MiR-126a-3p and miR-21-5p declined significantly from CTR to T2DM NC and T2DM C (linear trend for miR-126a-3p, F = 9.51, $p = 0.002$; linear trend for miR-21-5p: F = 5.33, $p = 0.02$; miR-126a-3p, ANCOVA adjusted for age and sex; F-test = 3.82, $p = 0.023$; miR-21-5p, ANCOVA adjusted for age, sex, glucose, and HbA1c; F-test = 4.52, $p = 0.012$) (Figure 18). However, the differences in miR-21-5p and miR-126a-3p levels were not significant between patients without and with diabetes complications (ANCOVA adjusted for age, sex, glucose and HbA1c: F-test = 0.005, $p = 0.945$ and F-test = 0.612, $p = 0.435$, respectively).

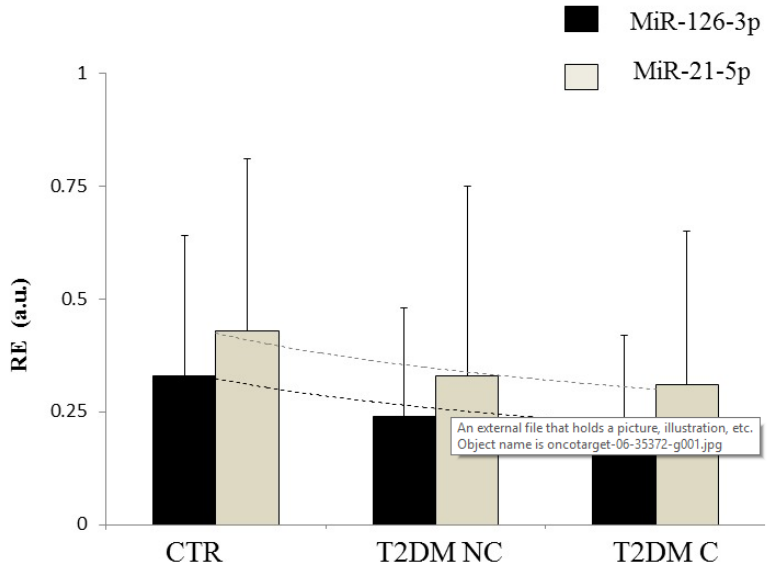


Figure 18. Circulating levels of miR-21-5p and miR-126a-3p in CTR subjects and T2DM patients. Linear trend for miR-126a-3p, F = 9.51, $p = 0.002$; linear trend for miR-21-5p: F = 5.33, $p = 0.02$. CTR: healthy control subjects; T2DM NC: patients without diabetic complications; T2DM C: patients with diabetic complications. RE = relative expression. Dotted line shows the exponential trend.

Analysis in relation to the different T2DM complications showed no significant differences for neuropathy, nephropathy, chronic renal failure, retinopathy, or lower limb arteriopathy (Table 3). However, the circulating levels of both miRNAs were significantly different between patients with and without major adverse cardiovascular events or MACE (miR-21-5p: 56 patients with *vs.* 137 patients without MACE, 0.46±0.44 *vs.* 0.26±0.33, $p < 0.001$;

miR-126a-3p: 0.21±0.21 *vs*. 0.28±0.22, p = 0.032) (Table 2). Multivariate covariance analysis with miR-21-5p and miR-126a-3p as the dependent variables confirmed the significance of MACE complications (Wilk's lambda = 0.943, p = 0.007).

	MiR-21 levels		
Diabetes complications	**Patients with a specific complication *vs*. all the other patients**	**Mean ± SD**	***p***
neuropathy	37 *vs*. 156	0.21±0.27 vs. 0.34±0.39	0.089
nephropathy	27 *vs*. 166	0.26±0.28 vs. 0.33±0.39	0.489
chronic renal failure	10 *vs*. 183	0.39±0.41 vs. 0.31±0.38	0.375
retinopathy	84 *vs*. 109	0.27±0.30 vs. 0.35±0.43	0.165
lower limb arteriopathy	11 *vs*. 182	0.37±0.33 vs. 0.31±0.38	0.534
MACE	56 *vs*. 137	0.46±0.44 vs. 0.26±0.33	**<0.001**
	MiR-126 levels		
neuropathy	37 *vs*. 156	0.18±0.20 vs. 0.24±0.22	0.122
nephropathy	27 *vs*. 166	0.20±0.16 vs. 0.23±0.22	0.512
chronic renal failure	10 *vs*. 183	0.23±0.19 vs. 0.23±0.22	0.993
retinopathy	84 *vs*. 109	0.23±0.20 vs. 0.23±0.23	0.863
lower limb arteriopathy	11 *vs*. 182	0.23±0.21 vs. 0.29±0.24	0.351
MACE	56 *vs*. 137	0.21±0.21 vs. 0.28±0.22	**0.032**

Table 3. Circulating miR-21-5p and miR-126a-3p levels in T2DM patients with different diabetic complications. ANCOVA adjusted for age, sex, and number of complications. Levels lower than 0.05 in bold.

Since among the 117 T2DM C patients enrolled for our study, 60 patients had more than one complications (Figure 19), to confirm the significant differences in circulating miR-21-5p and miR-126a-3p found in patients with MACE compared with those suffering from other complications, the total 193 T2DM patients were divided into 4 groups: no complications (NC, n = 76), MACE only (MACE, n = 18); MACE and other complications (MACE+OC, n = 38); complications other than MACE (OC, n = 62). Circulating miR-21-5p and miR-126a-3p levels were significantly different in the 4 groups of T2DM patients (Figure 19)

(ANCOVA adjusted for age, sex, glucose, and HbA1c; miR-21-5p: F-test = 8.375, p < 0.001; miR-126a-3p: F-test = 6.655, p = 0.002).

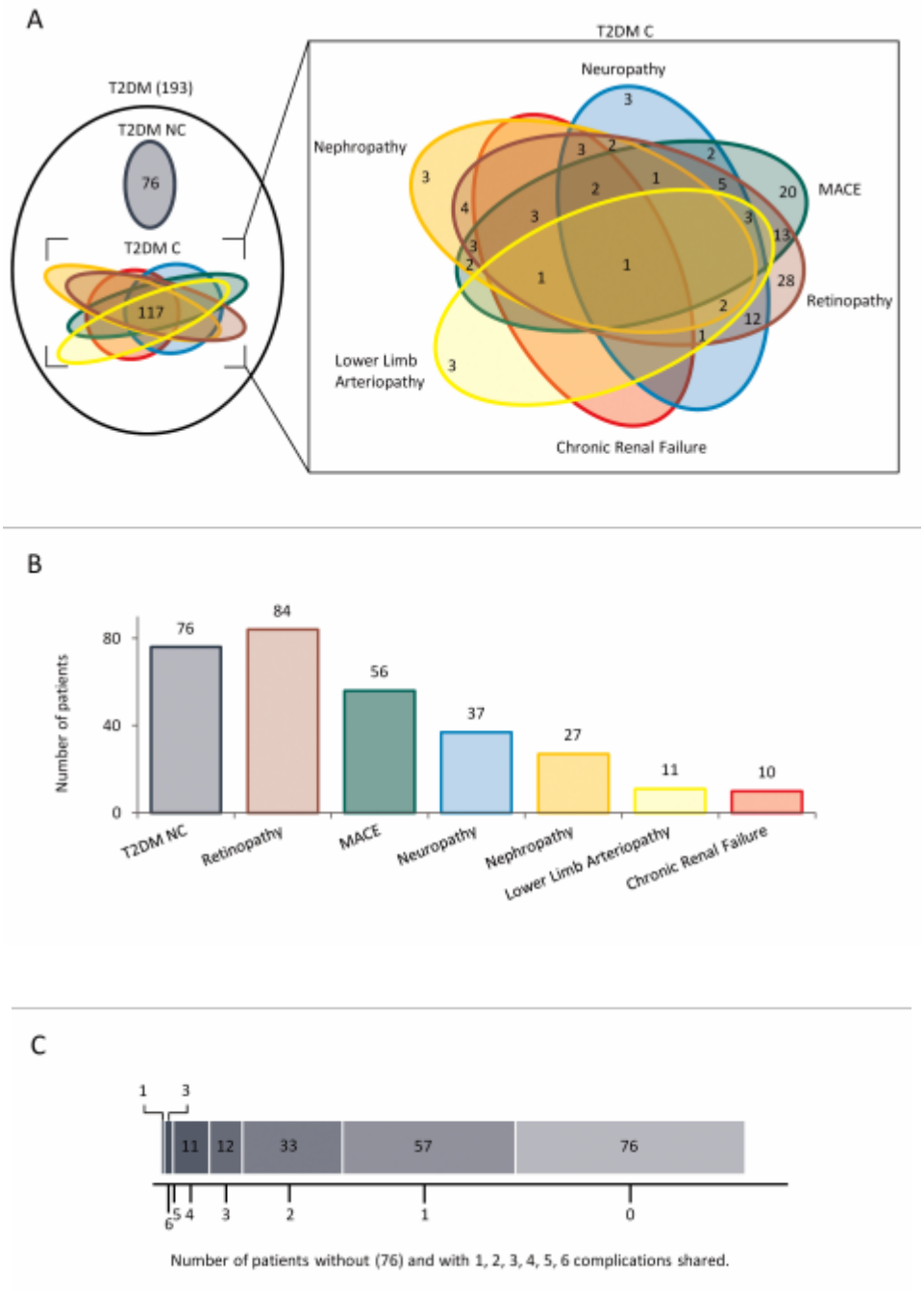


Figure 19. Overview of diabetic patients without or with complications. (A) Venn diagramm showing the overlaps between different complications (T2DMC). (B) Histogram displaying the size of each group of T2DM. **C.** Charts showing number of T2DM patients without complications and with one or more complications.

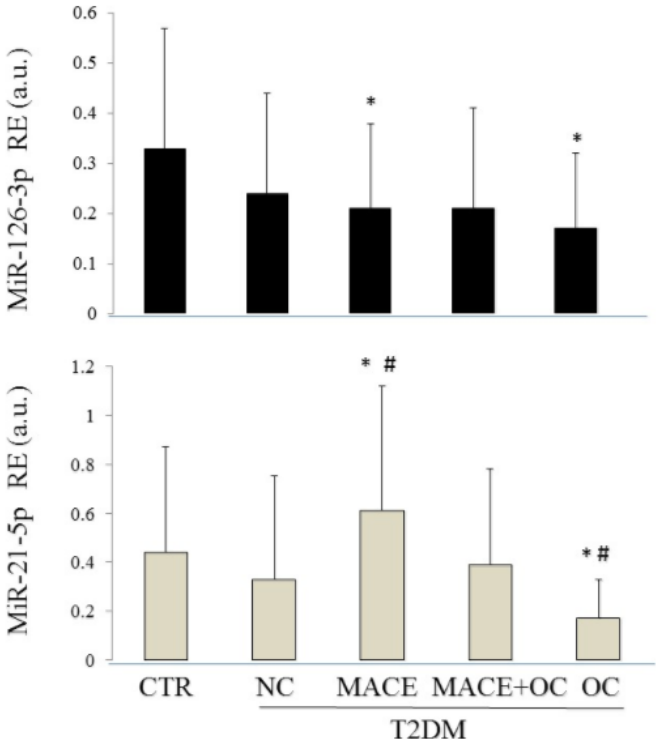


Figure 20. Circulating levels of miR-21-5p and miR-126a-3p in CTR and T2DM patients. CTR: healthy control subjects; T2DM NC: patients without diabetic complications; T2DM C: patients with diabetic complications; RE = relative expression; MACE: T2DM patients with a previous MACE; MACE+OC (other complications): T2DM patients with a previous MACE and at least another diabetic complication; OC (other complications): T2DM patients with complications other than MACE. RE: relative expression; CTR: healthy control subjects; NC: diabetic patients without diabetic complications; MACE: T2DM patients with a previous MACE; MACE+OC (other complications): T2DM patients with a previous MACE and at least another diabetic complication; OC (other complications): patients with diabetic complications other than MACE. MiR-21-5p: ANCOVA adjusted for age, sex, BMI, glucose, and HbA1c; F-test = 6.44, $p < 0.001$; ANCOVA adjusted for age and sex; F-test = 8.375, $p < 0.001$. MiR-126a-3p: ANCOVA adjusted for age, sex, glucose, and HbA1c; F-test = 4.55, $p = 0.01$; miR-126a-3p: ANCOVA adjusted for age and sex; F-test = 6.655, $p = 0.002$. #Bonferroni's correction, alpha = 0.05/10, $p < 0.005$, reference group: T2DM NC; *Bonferroni's correction, alpha = 0.05/10, $p < 0.005$, reference group: CTR.

Circulating miR-21-5p and miR-126a-3p were compared also between CTR and T2DM patients groups and were found to be significantly different (ANCOVA adjusted for age, sex, BMI, glucose, and HbA1c; circulating miR-21-5p: F-test = 6.44, $p < 0.001$; circulating miR-126a-3p: F-test = 4.55, $p = 0.001$). Application of Bonferroni's correction using CTR as reference group confirmed that miR-21-5p levels were significantly higher and miR-126a-3p levels were significantly lower in patients with MACE. MiR-126a-3p and miR-21-5p

levels were both significantly lower in patients with complications other than MACE (T2DM + OC). Correlation analysis, controlled for sex and age, between circulating miR-21-5p and miR-126a-3p and the variables that were found to be significantly different between T2DM patients and healthy CTR showed weak but significant positive correlation between miR-21-5p and BMI (partial correlation coefficient, PCC; 0.12, $p = 0.049$) and a weak but significant negative correlation between miR-126a-3p and glycaemia parameters (miR-126a-3p/glucose, PCC, −0.13, $p = 0.038$; miR-126a-3p/HbA1c, PCC, −0.13, $p = 0.033$). Notably, a positive correlation was observed between circulating miR-21-5p and glycaemia parameters (glucose and HbA1c levels) when correlation analysis was confined to T2DM patients (miR-21/glucose, PCC, 0.14, $p = 0.049$; miR-21-5p/HbA1c, PCC, 0.15, $p = 0.045$). Since it has been reported that aspirin treatment may affect circulating levels of miRNAs (de Boer et al., 2013), we asked our MACE patients whether they were taking aspirin treatment and found that they accounted for only 3 %. To confirm the results obtained on plasma we analyzed miR-126a-3p and miR-21-5p expression levels in CACs from a subgroups of CTR, T2DM NC and T2DM + MACE. MiR-126a-3p levels were significantly lower in CACs from T2DM NC and T2DM+MACE compared to CTR and miR-21-5p levels were higher in CACs from T2DM, both T2DM+MACE and T2DM NC, compared with CTR (Figure 21). Notably, the highest expression levels of miR-21 were observed in CACs from T2DM+MACE patients.

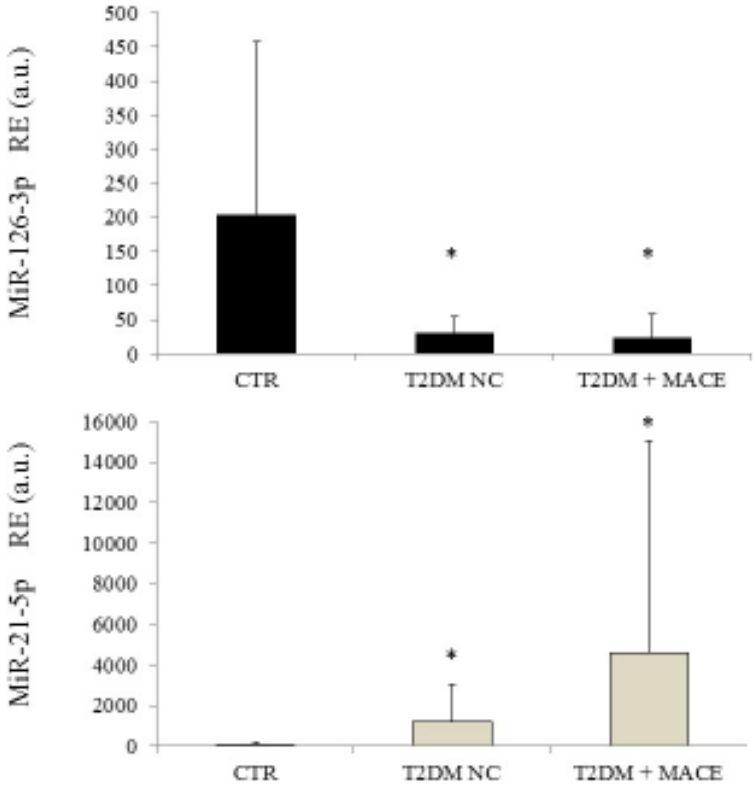


*Figure 21. MiR-21-5p and miR-126a-3p expression levels in CACs from CTR, T2DM NC and T2DM +MACE patients.*T2DM NC: patients without diabetic complications; T2DM + MACE: T2DM patients with a previous MACE; RE = relative expression.

3.3.1 Discussion

T2DM represents the most common metabolic disorder worldwide. Because of population aging and increasing trends toward obesity and sedentary lifestyles, the number of affected individuals is increasing at worrisome rates. While both environmental and genetic factors are known to contribute to the development of T2DM, continuous research is needed to identify specific biomarkers that could aid both in prevention of the disease and development of newer therapeutic options (Villard et al., 2015). Early exposure to hyperglycemia can drive the development of late complications, so that the progression of the disease persists despite improved glycemic control, indicating a memory of the metabolic insult,

phenomenon named “metabolic memory” (Ceriello, 2012). Epigenetic signatures, including circulating miRNAs, are emerging as important biomarkers of T2DM progression (Paneni et al., 2013; Reddy et al., 2015). Hundreds of miRNAs are passively or actively released into the circulation to facilitate metabolic crosstalk between organs and tissues, and could be applied to evaluate health status and ARDs progression. This study measured the circulating levels of miR-126a-3p and miR-21-5p to test their potential to be used as biomarkers of T2DM and related complications. MiR-126a-3p is the most extensively studied circulating miRNA in T2DM. It promotes vascular regeneration by functioning as an angiomiR and by modulating the mobilization of hematopoietic stem/progenitor cells. These miRNAs are associated with endothelial dysfunction and inflammation, two milestones of T2DM progression. We found that both miRNAs decreased from CTR subjects to patients without and with diabetes complications. We observed that miR-126a-3p and miR-21-5p levels declined significantly from CTR to T2DM non complicated and T2DM complicated patients. When the analysis focused on patients with specific complications, significantly higher miR-21-5p and lower miR-126a-3p levels were seen in T2DM patients with previous episode of myocardial infarction (MACE). These observations are in accordance with the results of a previous prospective study showing that higher circulating and microvesicles-contained miR-126a-3p levels are protective against MACE (Jansen et al., 2014). Notably, we analyzed in our retrospective study only a group of T2DM patients survived to the previous MACE. Moreover, our and others previous data suggest that MACE is a potent inducer of miR-21-5p release in the circulation (Olivieri et al., 2013a; Olivieri et al., 2013d; D’Alessandra et al., 2013). MiR-21 transfer/hyperexpression has been hypothesized as mechanism promoting cardiomyocyte survival during acute ischemic events, and cardiac fibroblasts proliferation after the acute event (Tu et al., 2013). However, it was recently

highlighted a link between miR-21 expression levels and angiogenic properties of endothelial cells; miR-21 hyper-expression suppresses endothelial progenitor cells (EPC) proliferation (Zuo et al., 2015) and contribute to endothelial cell senescence (Dellago et al., 2013).

When we assessed miR-126a-3p and miR-21-5p levels in CACs, a reduced miR-126a-3p-5p levels and an increased miR-21-3p expression levels were observed in T2DM patients (both T2DM NC and T2DM + MACE) compared to CTR. Previous data on endothelial progenitor cells from diabetic patients shoved a reduced miR-126a-3p expression (Olivieri et al., 2014b) and an overexpression of miR-21 was reported in response to high glucose in endothelial cells (Zeng et al., 2013).

Since miR-21-5p and miR-126a-3p modulate inflammation and angiogenic pathways, our results suggest that CACs from T2DM are "proinflammatory cells" rather than "proangiogenic cells". These conditions could depend from an increased "senescence status" of these circulating cells reached under hyperglycemic conditions. The exposure to high glucose levels could induce the accumulation of dysfunctional endothelial senescent cells in T2DM patients shifting the transcriptional program of CACs from a "pro-angiogenic" to a "pro-inflammatory" profile, and increasing in turn the risk of developing micro and macrovascular complications.

These data have been published in Oncotarget (Olivieri et al., 2015).

4. Conclusion

A main feature of the aging process is a chronic progressive increase in the pro-inflammatory status –Inflammaging - deeply associated with many aging-related pathologies, such as neurodegenerative diseases, atherosclerosis, heart disease, T2DM, and cancer. Chronic inflammation is commonly due to continuous immune cell activation; however, senescent cells can be important contributors because of the acquisition of a secretory phenotype (SASP). SASP is characterized by the release of pro-inflammatory mediators, which, in turn, may act, in a paracrine or systemic manner, affecting the functionality of organs and systems, especially in the elderly. However, senescent cells can have a beneficial role in some instances, such as during wound healing, muscle regeneration, suppression of pancreatic and liver fibrosis, and prevention of tumorigenesis in young organisms. Thus, the rising interest in understanding the molecular regulators of senescence is mainly focused to the possible develop and futuristic therapeutic approaches that alleviate its harmful effects. MicroRNAs represent exciting new clinical biomarkers and targetable molecules. Several miRNAs are modulated during aging and inflammatory processes in different tissues and organisms. They are both target and are targeted by a number of factors important for senescence through positive and negative feedback loops (Olivieri et al., 2013a). In this script, I have depicted a brief excursus of my PhD work and collaborations concerning several aspect of miRNA role

in aging, from their functions in changing molecular and organelles function within the aging cells, passing through the secretome and its effect on bystander cells to conclude with an attempt to modulate their pro-inflammatory effects with drugs already used for human therapies. Here I showed that several SA-inflamma-miRs, specifically mitomiR-146a, -181a and -34a can regulate mitochondrial activities and perhaps autophagy during senescence through Bcl-2 and LC3 protein modulation (*manuscript in preparation*). MiRNAs actions result in a myriad of changes in alteration of cellular homeostasis. The identification of miRNAs in inflammaging is the first step towards understanding the specifics of their mechanisms in that process and potentially using this pathway for therapeutic advantage. Indeed, strategies aimed to regulate their expression could eventually be devised to treat a number of age-related diseases (Beg at al., 2017; Nana-Sinkam and Croce 2013). Some studies testing miR modulation in vivo have already reached the clinical trial stage; the most advanced among them involves miR-122 targeting in hepatitis C (Machlin et al., 2012) and a mimic of miR-34a (MRX34) incorporated into a lipid-based nanoparticle formulation (Beg et al., 2017). This suggests that despite the complexity of *in vivo* miR targeting, several new studies are expected to be designed to exploit this promising treatment approach.

With this work we showed that adalimumab, an anti-inflammatory monoclonal antibody, dampens SASP and modulates miR-146a levels (Prattichizzo et al., 2016b). MiR-146a is directly suppressed by IRAK1, a key regulator of the IL-1α receptor signaling, leading to inhibition of IL6 and IL8 production. Interestingly, high levels of secreted cytokines upregulated MiR-146a/b expression, which in turn prevented excessive SASP activity, targeting NF-kB (Bhaumik et al., 2009). NF-kB signalling is not only the master regulator of inflammatory responses, but can also regulate several homeostatic responses such as apoptosis, autophagy, and tissue atrophy, all of which are dysregulated during aging and

cellular senescence. Because of NF-kB is a target of multiple miRNAs, critical use of miRNAs holds promise as a strategy to improve health-span.

Changes in miRNAs expression have been proposed as potential biomarkers in chronic inflammatory diseases, such as T2DM. Indeed, miRNAs are stable and can be easily isolated and measured from tissues and body fluids. Nevertheless, circulating miRNAs detection may be hampered by several intrinsic characteristics including size, low abundance, and not fully standardized quantification. Finally, a chronic nonspecific inflammatory response typical of ARDs could interfere with disease-specific biomarker discovery (Chechlinska et al., 2010).

5. *Methods*

HUVEC and THP-1 culture

HUVECs derived from 3 donor pools were purchased from Clonetics (Lonza, Basel, Switzerland) and cultured in EGM-2 endothelial growth medium (Lonza). Briefly, fresh cells were seeded at a density of 5000/cm^2 in T 75 flasks (Corning Costar, Sigma Aldrich, St. Louis MO, USA); the medium was changed at 48 h intervals. Cultures reached confluence after 6-7 days, as assessed by light microscopic examination, and were passaged weekly. After trypsinization and before replating, harvested cells were counted using a hemocytometer. Replicative senescence was studied by culturing cells up to the 15/16th passage. Cumulative population doubling (CPD) was calculated as the sum of all PD changes. Cells were divided into young (SA-β-Gal < 10 %) and senescent (SA-β-Gal > 50 %).

Senescence-associated expression of β-galactosidase (SA-β-Gal) activity was detected by using Senescence Detection Kit (Catalog #K320 - BioVision Inc., Milpitas, CA, USA). Briefly, non-confluent HUVECs cultured in 12-wells plates were fixed for 15 minutes at room temperature, and then washed twice in phosphate-buffered saline (PBS). Cells were incubated overnight at 37°C with Staining Solution Mix (containing X-gal). SA-β-Gal was

assessed by light microscopic examination. The percentage of β-gal-positive cells was determined by counting at least 500 cells per well.

Human monocytic THP-1 cells were purchased from ATCC (Rockville, MD, USA) and maintained in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 1 % penicillin/streptomycin, and 1 % L-glutamine (all from Euroclone, Milano, Italy).

For serum deprivation experiments, cells were washed twice with PBS and cultured in EGM-2 without FBS (serum-free) for 24, 48 and 72 hours.

Anti-TNF-α and LPS treatment

After testing different pharmacologically appropriate adalimumab doses in BrDU and MTT assays (Supplementary Figure 1), 8 μg/ml of the human TNF-α inhibitor (Humira, Abbott, Lake Forest, IL, USA) was used in both short- and long-term experiments. This is the concentration commonly found in patient sera following injection of 0.8 ml adalimumab at a concentration of 50 mg/ml [40]. A random IgG at the same dose was always added to control cultures to avoid phenomena involving Fc receptor.

Lipopolysaccharide (LPS; Sigma-Aldrich, Taufkirchen, Germany) was added (1 μg/ml) to the medium as appropriate for the short-term experiments, i.e. 30 min and 5 h.

Cell viability assay

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test cell viability. Cells were grown in 96-well plates at a density of 2×103 cells/well. After 18 h they were washed with fresh medium then treated with differential doses of

adalimumab. After 24 h pretreatment, 100 µl MTT (1 mg/ml) was added and incubated for 4 h; the formazan salt that formed was solubilized by adding 200 µl dimethyl sulfoxide and its amount was determined by measuring optical density at 540 nm using a microplate reader (MPT Reader, Invitrogen, Milano, Italy).

Mitochondria isolation

A specific kit based on Anti-TOM22 MicroBeads (130-094-532 - Miltenyi Biotec Inc., Auburn, USA) was used to mitochondria isolation, according to the manufacturer's protocol. Briefly, Lysis Buffer containing the protease inhibitors was added to 3 x 10^6 collected HUVECs and the lysis was performed using 30 strokes by 27G needle. Then the mitochondria were magnetically labeled adding Anti-TOM22 MicroBeads to the lysate and incubating in a roller mixer for 1 hour at 4°C. After labelling incubation was proceed to magnetic separation. Each sample of eluted labeled mitochondria was divided in two aliquots: one corresponding on 1 x 10^6 cells for the miRNA analysis and the other one with the remaining volume for the proteins analysis in order to evaluate the purity of the isolated mitochondria.

In the latter case, the mitochondria were washed twice with storage buffer and the pellets were stored at -80°C until the proteins extraction and analysis.

While the fractions for miRNA analysis were decontaminated of extra-mitochondrial RNA by RNAse A treatment as described by Barrey et al. After the reaction stopped by proteinase K, the mitochondria were washed with the storage buffer and the pellets were stored at -80°C until the RNA extraction.

Cell transfection

8×10^4 cells were plated in six-well plates and let attached overnight before transfection with miR-34a, -146a and -181a miRVANA miRNA mimics (MC13089, MC10722, MC10421 - TermoFisher Scientific CA, USA) and mirVana miRNA mimic negative control #1 (4464058 - TermoFisher Scientific) at a concentration of 30nM. Transient transfection was performed with TransIT-2020 Transfection Reagent (MIR 5404 – Mirus Bio LLC) transfection reagent, according to the manufacturer's instructions. The mirus transfection method was optimised testing different quantities of reagent and miRNA mimics. In particular, Transfection Reagent (microlitre)/miR (microgram) ratios of 3:1 were found as optimal. Transfection Reagent–miR complex was prepared in serum-free medium. Analyses were performed 24 hours after transfection.

Cytokine production

Culture supernatants were collected at the end of each incubation, centrifuged, and stored at −20°C until use in the assays. IL-6 and TNF-α concentrations were measured using a commercially available, high-sensitivity ELISA kit (Invitrogen) or a 4 custom cytokine multiplex (Tema Ricerca, Castenaso, Bologna).

Psoriasis patients

A total of 10 psoriatic patients were enrolled in the study. The mean age was 53 ±14 years, 8 males and 2 females. The study protocol was approved by the Ethics Committee of UNIVPM (Ancona, Italy) and all enrolled patients provided a written informed consent. All

patients received 3 months of adalimumab monotherapy after a 12 weeks-wash-out period from previous therapy.

CAC isolation and RNA extraction

CACs were isolated from approximately 14 ml heparinized peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation with Ficoll (Ficoll-Paque™ PLUS, GE Healthcare Bio-Sciences Uppsala, Sweden) within 2 h of collection. Then 5×106 PBMCs were plated on 24-well fibronectin-coated plates (BD Biosciences, Mountain View, CA, USA) and maintained in endothelial basal medium (EBM; Clonetics-Lonza, Walkersville, MD USA) supplemented with EGM SingleQuots and 20 % fetal calf serum for 4 days. After 4 days in culture, non-adherent cells were removed by washing in PBS, whereas adherent cells were lysed directly in the culture wells.

The CAC phenotype was confirmed by cellular uptake of acetylated LDL (DiI-acLDL) and binding of FITC-conjugated lectin from *Ulex europaeus* (UEA-1) by fluorescence microscopy. Briefly, to detect DiLDL uptake, cells were incubated with DiLDL (2.4 μg/ml) (Molecular Probes, Eugene, OR, USA) at 37°C for 2 h. To detect UEA-1 binding, cells were fixed with 2 % paraformaldehyde for 15 min and incubated with FITC-labeled UEA-1 (10 μg/ml) (UEA-1, Sigma, St. Louis, MO, USA) for 1 h. Double-stained cells positive for both UEA-1 and DiLDL were regarded as CACs. RNA was purified according to the instruction manual of the Total RNA Extraction kit (Norgen Biotek).

Cultures and generation of MCF7 mammospheres (MS)

MCF7 were grown in RPMI 1640 +10% FBS medium. MCF7-derived mammospheres (MS) were obtained by plating 2500 cells into 3-cm^2 low-attachment wells (Corning, NY, USA) filled with mammary epithelial growth medium (MEGM), supplemented with MEGM bullet kit (Lonza Ltd, Basel, Switzerland). Primary MS formation was assessed after 3 and 7 days and photographed using a inverted microscope (Olympus CKX41, digital cameras Olympus C-5060, Japan). Only MS with an apparent diameter of ≥50 μm were scored for statistical analysis, as previously described (Sansone et al., 2007).

RNA isolation

Total RNA from HUVECs was isolated using the Norgen Biotek Kit (#37500 , Thorold, ON, Canada), according to the manufacturer's instructions. RNA was stored at −80 °C until use.

Quantitative RT-PCR of mature miRNAs

MiRNAs expression was quantified by quantitative real-time PCR (RT-qPCR) using TaqMan miRNA assay (Catalog #4427012 - TermoFisher Scientific), according to the manufacturer's protocol. Briefly, miRNA was reverse transcribed with the TaqMan MicroRNA reverse transcription kit (4366596 – ThermoFisher Scientific), using miR-specific stem-loop primer. 10 μl of RT mix contained 2 μl of each miR-specific stem-loop primer, 3.34 μl of input RNA, 1 μl of 10 mM dNTPs, 0.67 μl of reverse transcriptase, 1 μl of 10× buffer, 1.26 μl of RNase inhibitor diluted 1:10, and 0.73 μl of H2O. The mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min. The 10 μl qRT-PCR reaction mix included 0.5 μl 20x TaqMan MicroRNA Assay, which contained the PCR

primers and probes (5'-FAM), 5 µl 2x TaqMan Universal Master mix no UNG (4440040 – ThermoFisher Scientific), and 2.66 µl RT product. The reaction presented an initial step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data were analyzed with Rotor Gene Q (Qiagen, Hilden, Germany) with the automatic comparative threshold (Ct) setting for adapting baseline. qRT-PCR data were standardized to RNU44. The $2^{-\Delta CT}$ method was used to determine miRNA expression.

Quantitative RT-PCR of mRNA

RNA amount was determined by spectrophotometric quantification with Nanodrop ONE (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. qRT-PCR was performed in a Rotor-Gene Q (Qiagen) using SYBR® Green JumpStart™ *Taq* ReadyMix (S4438 Sigma) in a 10 μl reaction volume. Cycling conditions were: 95 °C for 2 m, and 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s (40 cycles). Samples were run in duplicate. mRNA quantification was assessed using the 2^{-DDCt} method.Gapdh and Beta-actin were used as an endogenous control.

Protein extraction and immunoblotting

Cells were washed twice in cold phosphate buffered saline (PBS). Total protein was extracted using RIPA buffer (150 mMNaCl, 10 mM Tris, pH 7.2, 0.1 % SDS, 1.0 % Triton X-100, 5 mM EDTA, pH 8.0) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Protein concentration was determined using Bradford Reagent (Sigma-Aldrich, Milano, Italy). Total protein extracts (40 µg) were separated by 10

% SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight with primary anti-Spred1 antibody diluted 1:1000 (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA), anti-Irak1 antibody diluted 1:250 (MBL International Corporation, Nagoya, Japan), and anti-IL-1β antibody diluted 1:1000 (Cell Signaling Technology, Beverly, MA, USA); subsequently they were incubated with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Immunoreactive proteins were visualized using ECL Plus chemiluminescence substrate (GE Healthcare, Pittsburgh, PA, USA). Membranes were incubated with anti β-actin diluted 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as an endogenous control.

Flow cytometer analysis

Analyses of the intracellular ROS level and mitochondrial functionality, apoptosis and inflammation were conducted by flow cytometric technique using special probes and Guava EasyCyte flow cytometer with GuavaSoft 2.7. All assays tested needed the excitation wavelength of 488nm and the fluorescence intensity was recorded in different channels on an average of 5,000 cells from each sample. Each condition was performed in triplicate and each sample was repeated twice.

Intracellular ROS assay. ROS levels were evaluated by carboxy-2,7-dichlorofluorescein diacetate (carboxy-H_2DCFDA) (Invitrogen). This probe diffuse across cell membranes where it is hydrolyzed to the non-fluorescent compound (H2DCF) which is oxidized by ROS to DCF, the fluorescent form of the dye. The cells were incubated with the dye (10μM in PBS) in the dark for 30 min at 37°C, washed, detached and then an aliquot of each sample was added to a solution of Guava Via-count (Merck Millipore). This is a fluorescent stain formulation which detect live and dead cells. Therefore the analysis of ROS and vitality were

conduct simultaneously on flow cytometer. Counterstaining with Via-count was necessary in order to evaluate intracellular levels of ROS only in viable cells, because cells with compromised cell membrane integrity can loss carboxy-H_2DCFDA and then result in false negative. To analyze data, two regions on the green channel were identified, corresponding to Low and High ROS define on base to the fluorescent distribution of young of yHUVECs.

Mitochondrial superoxide anion assay. To detect the mitochondrial superoxide anion generation, we used MitoSOX™ Red (Invitrogen; M36008), a fluorogenic dye highly selective for mitochondria superoxide in live cells. Cell staining protocol was performed following manufacturer's protocol. Briefly, MitoSOX was added to confluent cells and incubated at 37°C for 10 min in the dark, cells were detached and analyzed recording fluorescence emission in the yellow channel. For the analysis, fluorescence was divided in two regions, corresponding to Low and High level of superoxide anion. Data are shown as percentage of cells with High levels of superoxide anion production.

Permeability Transition Pore (mPTP) opening assay. Acetoxymethyl ester of calcein (Calcein AM) is a non-fluorescent dye that diffuses to all cellular compartments where intracellular esterases release the polar fluorescent calcein dye. It can't cross the mitochondrial and plasma membranes and the added cobalt quenches cytosolic calcein, while the mitochondrial calcein is not accessible, unless the mitochondrial membrane is permeabilized. mPTP opening was determined using a MitoProbe^{TM} Transition Pore Assay Kit (Invitrogen) based on manufacture's protocol. Adherent cells were loaded with 250nM Calcein AM in presence of 400μM cobalt chloride ($CoCl_2$) for 15min at 37°C. Then the cells were detached, washed twice and the green fluorescence was quantified by a flow cytometer. On the distribution of the fluorescence was identified a section corresponding on cells with mPTP open.

Annexin V assay. The Annexin V is calcium-dependent phospholipid binding protein which binds to phosphatidylserine (PS) on the surface of apoptotic cells. When it is used in association with 7-Aminoactinomycin (7-AAD), a cell impermeant DNA intercalator, allow to distinguish early and late apoptotic cells in addition to live and dead cells. Floating and attached cells were harvested, washed with PBS and 10^5 cells were resuspended in 200µl of Annexin binding buffer containing 5µl of Annexin V (Merck Millipore). After incubation in the dark for 15 min at 37°C the cells were wash twice, 200µl of buffer and 5µl of 7-AAD were added and the samples analyzed within 1 hour by flow cytometry.

Caspase 3/7 and 1 detection. Caspase3/7 and caspase-1 activation was assayed by FLICA probes FAM conjugated (ImmunoChemistry Technologies). FLICA is cell permeant, covalently binds to active caspases and revealed by green fluorescent signal. Cell staining was performed following manufacturer's instructions. Briefly, the cells were detached, washed and stained with 1x FLICA solution in the dark for 50min at 37°C. Cells were then counterstained with Propidium Iodide and immediately analyzed. The fluorescence results from the two channel was analyzed together and four regions were identified: live negative or positive caspase cells and dead negative or positive caspase cells.

TEM analysis

Cells were plated on aclar films (Ted Pella CA, USA) for flat embedding. Cells were fixed for 1 h at rt with a solution of 2.5 Glutaraldeyde in 0.1 M cacodylate buffer (pH 7.4) then post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min at rt followed by dehydration in acetone series and embedded in epoxy resin (Sigma #43359). Ultrathin (40 nm) section were stained with led citrate and uranyl acetate and imaged in a Philips CM12 TEM at 100 KV.

Patients

A total of 193 T2DM patients from central Italy and 107 healthy control subjects (CTRs) gave their informed consent to be enrolled in the study. The study protocol was approved by the Ethics Committee of INRCA-IRCCS (Ancona, Italy). T2DM was diagnosed according to American Diabetes Association Criteria [20]. Inclusion criteria were: a body mass index (BMI) < 40 kg/m2, age 35 to 85 years, and ability and willingness to provide a written informed consent and to comply with study requirements. The information collected included information on vital signs, anthropometric data, medical history and behaviours, and exercise.

The presence/absence of diabetic complications was established as follows:

- retinopathy was defined as dilated pupils detected on fundoscopy and/or fluorescence angiography;
- incipient nephropathy was a urinary albumin excretion rate > 30 mg/24 h and normal creatinine clearance;
- chronic renal failure was defined as an estimated glomerular filtration rate < 60 mL/min per 1.73 m2;
- neuropathy was established by electromyography;
- ischemic heart disease was diagnosed by clinical history and/or ischaemic electrocardiographic alterations; these patients had had ST- or non-ST elevation myocardial infarction, which was defined as a major acute cardiac event (MACE). Mean time from the MACE was 9±8 years;

- peripheral vascular disease, including arteriosclerosis obliterans and cerebrovascular disease, was diagnosed based on history, physical examination, and Doppler imaging.

Of the 193 T2DM patients with at least one complication, 37 had neuropathy, 11 had lower limb arteriopathy, 56 had MACE, 27 had nephropathy, 10 had chronic renal failure, and 84 had retinopathy.

Control samples (CTR) were selected among the husbands and wives of T2DM patients enrolled for the study. As microRNAs are epigenetic biomarkers, their expression is expected to be related with lifestyle and environment. Therefore, the choice of CTR among people living with diabetic patients is expected to minimize the effect of environment as confounding factor.

All study subjects reported dietary habits consistent with a Mediterranean-style diet.

Laboratory assays

Blood concentrations of total and HDL cholesterol, triglycerides, fasting glucose, HbA1c, fasting insulin, creatinine, and hs-CRP were measured by standard procedures.

Statistical analysis

Summarized data are shown as mean ± SD or as frequency (%). Analysis of covariance (ANCOVA) was used to compare the mean differences in chemical, clinical, and anthropometric variables after adjustment for age and sex (CTR subjects, and patients without and with diabetes complications). Multivariate analysis of covariance with miR-21-

5p and miR-126-3p as the two dependent variables was performed to confirm the statistical significance of MACE complications.

Partial correlation, adjusted for age and sex, was used to test for correlations between miRNAs and the others variables. The association of T2DM complications with circulating miR-21-5p and miR-126-3p was also assessed for linear trend.

Data analysis was performed using IBM SPSS Statistics for Windows, version 20 (IBM Corp, Armonk, NY, USA). The significance level was a P value < 0.05. Bonferroni's correction for multiple testing was applied, alpha = 0.05/10, $p < 0.005$.

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