Chapter X

Physiological and pathological vascular aging

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Abstract

Aging is a risk factor for cardiovascular diseases. Through aging, blood vessels become stiffer, less elastic and, thus, with less ability to contract. The objectives of this chapter are to review (i) recent progresses in the characterization of physiological and pathological vascular aging and (ii) *in vitro* platforms to study vascular aging. Initially, we will discuss the causes and biomarkers of vascular aging. Then we will discuss the main characteristics related to physiological and pathological aging including (i) altered ECM remodeling (e.g. composition, mechanical properties, degradation, calcification of the ECM during aging), (ii) enhanced fibrosis (e.g. causes and mechanisms), (iii) vascular cell dysfunction triggered by chronic oxidative stress, inflammation or senescence, and (iv) altered responses of vascular cells to flow shear stress. Finally, we will discuss *in vitro* systems to study vascular aging, particularly the effect of biomechanics in aged cells as well as the effect of drugs during vascular aging.

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1-Introduction

It is expected that by 2030 the number of people aged 60 years and over will grow by 56 percent [1]. Aging is recognized as the key factor in most chronic diseases, including neurodegenerative, cerebrovascular and cardiovascular disorders, through the accumulation of biological changes over time [2]. Thus, age-related biological changes have emerged as a serious issue, with increased socioeconomic and healthcare burdens. Several hallmarks of aging in mammals have been identified including telomere attrition, genomic instability, epigenetic modifications, loss of proteostasis, cellular senescence, mitochondrial dysfunction, altered intercellular communication, deregulated nutrient sensing and stem cell exhaustion [3][4][5].

A major trigger for cellular aging and senescence is telomere shortening [5, 6]. Indeed, a cell's finite replication capacity is promoted by telomere shortening [7]. Telomeres are repeats of highly conserved nucleotide sequences that compose the chromosome ends to prevent chromosome fusion [7]. Mammalian cells do not express telomerase, the enzyme responsible for telomere replication, and because the enzyme is consumed at each cell cycle, telomeres become shorter until they cannot prevent a DNA damage response [8, 9]. Another trigger for normal aging is related to genomic instability. The accumulation of DNA damage over time is the result of environmental factors (e.g. chemicals, UV/IR radiation) as well as endogenous agents (e.g. DNA replication errors, reactive oxygen species). DNA damage accumulates when intrinsic mechanisms cannot eliminate these dysfunctional cells and thus tissue and organism homeostasis become compromised [5]. However, more than being genetically predetermined, organismal lifespan is also epigenetically modified. Exposure to environmental stresses (e.g. smoking, pollution, sedentary lifestyles) during an individual's lifetime may induce epigenetic alterations that can compromise normal gene expression without altering the underlying DNA sequence [10]. These exogenous factors are major players in premature defects in mitochondrial functionality, insulin signaling, endothelial homeostasis and redox balance, promoting early senescent features [11]. To overcome intracellular damage that

accumulates with age, a quality control network, that maintains correctly folded proteins and degrades unfolded or misfolded proteins, is fundamental. This maintenance system, known as proteostasis, is supported by the heat shock family of proteins (namely chaperones) and by the proteolytic systems ubiquitin-proteasome and lysosome-autophagy, which determines cell fate [12]. However, protein homeostasis declines with age, promoting proteotoxicity that further leads to the development of age-related proteinophaties [13]. Another trigger of normal aging is cell senescence. Cellular senescence acts as an anticancer mechanism through the activation of tumor-suppressor mechanisms in response to oncogenic stimuli, including the p53/p21 and $p16^{INK4a}$/pRB pathways [14]. However, it was recently demonstrated that eliminating senescent cells from a mice model not only increased longevity but also improved overall health, thus suggesting that senescent cells are major drivers of aging [15, 16].

This chapter reviews the physiological and pathological aging process of vessels in Hutchinson-Gilford Progeria Syndrome, a disease characterized by premature aging in children (focusing on the biophysical and cellular changes that occur in the vessels during aging). Many aspects observed in physiological aging are shared by pathological aging. Therefore, the use of accelerated aging models may facilitate the study of vascular aging. Finally, we review the latest efforts to create suitable *in vitro* models to study the process of aging in the vascular system.

2-Vascular aging: general insights

Several molecular mechanisms are implicated in vascular aging including sirtuins, telomere shortening and telomerase, progerin, klotho gene, and JunD among others [17]. Vascular aging is characterized by collagen deposition, vascular remodeling, interstitial fibrosis, and inflammation which further leads to wall thickening, arterial stiffening and vessel dilatation [11].

Vascular aging is evaluated in multiple ways. Vascular stiffness increases with aging and is easily monitored by pulse wave velocity [18]. High levels of C-reactive protein, an inflammatory

marker, and low levels of adiponectin, an anti-atherogenic factor, are related with atherogenesis and consequently with vascular aging [19]. Lymphocyte telomere length, easily accessed through peripheral blood, may be used as a biomarker of vessel aging since it is related with stem cell and endothelial progenitor cells (EPCs) telomere length [20]. Inflammatory markers such as the nuclear factor-kappa B (NF-$_K$B) and insulin growth factor-1 (IGF-1) maybe also be used as biomarkers of vascular aging. Other biomarkers are strictly linked with senescence and can reflect cell cycle arrest (e.g. p53, p21, p16^{INK4a}), absence of cellular proliferation (e.g. lack of BrdU incorporation, Ki67), activation of double stranded brakes (e.g. H2AX, p53BP1 foci), expression of inflammatory factors (e.g. interleukin 6 and 8), cell senescence (SA-β-gal), loss of lamin B1, and activation of pathways that regulate the secretory phenotype (e.g. p-p65 or p-p38) [8, 21-23].

3- Physiological vascular aging

Vascular aging is characterized by biophysical changes. There is a fatigue of the vessels resulting from sustained mechanical stress-associated pressure caused by blood flow. The extracellular matrix (ECM) becomes stiffer, losing elasticity and, therefore, the ability to stretch [24]. In addition, endothelial cells (ECs) become dysfunctional as result of a pro-inflammatory environment and increased oxidative stress [25].

3.1- Altered ECM remodeling

The vessel wall is mainly composed of an ECM which provides structural support, defines the vessel's mechanical properties. By interacting with vascular cells, the ECM is able to act as a signal transductor to modulate cell proliferation, survival, differentiation and gene expression. The major components of the vascular ECM are collagen and elastin, complemented by other molecules including fibronectin, microfibrils, proteoglycans and glycoproteins [26]. Different sections of the

blood vessel wall have different compositions of ECM proteins [27]. In the tunica intima, ECs are lined the vessel luminal surface, attaching to a basement membrane containing mainly laminin, type IV collagen, nidrogen, perlecan, type XV and VIII collagens and fibronectin [26, 28]. Between the intima and the tunica media, arteries and veins are supported by the internal elastic lamina [27, 29]. In the tunica media, vascular smooth muscle cells (SMCs) and elastins are the major components. Elastin forms concentric fenestrated sheets, intercalated with collagen fibers and proteoglycans, which connects with SMCs [30]. Elastin is an elastic fiber produced by SMCs, presenting low tensile strength that contributes to the elasticity of the vessels and to store the recoiling energy, contributing to vessel compliancy [26]. The percentage of lamellar units present in the vessel varies with the tensile strength that the vessel is subjected to, being higher in the larger and more proximal vessels that withstand higher wall tension [31]. Finally, tunica adventitia, the outside layer of the vessels, is rich in collagen type I and III. Collagen provides high tensile strength, which prevents wall rupture due to blood pressure. The production of the adventitia proteins is mainly done by fibroblasts [26, 32].

In aged blood vessels, the endothelium, the SMCs and the ECM suffer structural and functional changes that lead to arterial stiffness, fibrosis and endothelial dysfunction [33]. The ECM in the vascular wall becomes thicker and stiffer with aging, due to several factors including (i) increase of the collagen to elastin ratio, (ii) impairment of the balance between ECM degradation and production and (iii) dysfunction of newly synthesized ECM [34]. Collagens and elastin are the major components of the blood vessel's ECM and the absolute and relative quantities of these proteins define the biomechanical properties of the vessels [35]. Collagen provides the tensile strength while elastin the elastic properties for the vessels [36]. Elastin represents approximately 50% of the arterial wall dry weight and it is mainly produced by SMCs and fibroblasts, which have a low turnover rate during their life [37].

As mentioned above, through aging, changes in the composition and structure of collagen, as well as the ratio between elastin to collagen, contribute to a decrease in the total arterial compliance [38]. An increase in the content of collagen type I and III across the vessel wall [39] occurs during aging, namely in the adventia, causing the stiffening of the vessels [40]. In addition, increased cross-linking between collagen fibers leads to more insoluble fibers and thus, less availability for enzymatic degradation, with an increased tensile strength [41]. The cross-linking process may be driven by enzymes, such as lysyl oxidases, which promote the formation of inter- and intramolecular cross-links [42] or by the accumulation of advanced glycation end products (AGEs) [43]. AGEs are formed by non-enzymatic glycation of proteins and lipids and their production is accelerated with aging. Collagen and elastin, present in the vessels, have a low turnover rate and become more susceptible to glycation [44, 45]. With aging, the elastin content of blood vessels decreases, thereby increasing the collagen to elastin ratio [36]. Moreover, elastin suffers structural changes, due to the repeated mechanical forces during stretches and relaxation in the cardiac cycle, as well as increased oxidative stress that concomitantly contributes to fragmentation and rupture of elastin fibers [46]. Elastin cross-linking with AGEs also contributes to an increase in fragility and fragmentation of this protein [47].

Another feature, that contributes to ECM remodeling with aging, is the imbalance between the synthesis and degradation of ECM components. Matrix metalloproteinases (MMPs) are endopeptidases capable of degrading ECM components [48]. With age, an increase in the activity of MMP-2/-7/-9/-14 in the aortic walls of rodents, non-human primates and humans has been reported [49]. Increases in MMP-2 activity in the aorta are associated with elastin fragmentation [50]. Furthermore, MMP-2 expression leads to the stimulation of transforming growth factor (TGF-β1) signaling, increased production of collagen I, II and III by vascular SMCs, and increased secretion of fibronectin [49]. Activation of MMP-9, by pro-hypertensive factors, shear stress, pressure and TGF-β1/SMAD signaling, is associated with increased oxidative stress, inflammation, fibrosis and

DNA damage [51]. Altogether, changes in the expression of MMP's with aging contributes to the increased fibrosis and stiffening of the vessels.

Vascular calcification is a marker of vascular aging. It typically occurs after deposition of calcium-phosphate in distinct layers of the arteries. This is an active process, similar to bone formation, that involves the differentiation of SMCs into "osteoblast-like" cells that present a secretory phenotype [52]. SMCs synthesize proteins such as alkaline phosphatase, osteopontin, osteocalcin and collagen. Inflammation and activation of the NF-κB pathway play the main role in triggering SMCs into the osteogenic phenotype, by increasing levels of IL-6, tumor necrosis factor alfa (TNF-α), MMP-2, MMP-9 and cathepsin S [53, 54]. The fragmentation of elastin, described above, also contributes to SMCs differentiation and the deposition of calcium [46]. The age associated cell senescence of SMCs and their secretory phenotype, associated with activation of the NF-κB process, contribute to the differentiation of SMCs into the osteogenic phenotype [55]. Calcification in the intima, present in atherosclerosis, reduces the lumen vessel diameter and causes arterial dysfunction. In the media, calcification is concentric, with diffuse mineral deposits and promotes an increase in the arterial stiffness [52].

3.2- Enhanced fibrosis

Fibrosis is defined as the formation of excessive fibrous tissue, due to increased deposition of ECM components [56]. It is an adaptive response that gradually extends to the surrounding spaces and leads to increased arterial stiffening. Aging-associated factors, such as reduced nitric oxide (NO) availability, oxidative stress, calcification, ECM remodeling and a pro-inflammatory environment, all contribute to increased fibrosis. Pro-hypertensive factors, such as angiotensin II (Ang II), endothelin-1 (ET-1) and aldosterone, induce the activation of the signaling pathways p38 MAPK and TGF-β/SMAD, further promoting synthesis of fibrotic tissue [57, 58]. Another factor that induces fibrosis is transglutaminase (TG2), a protein that interacts with the ECM, regulates

fibroblast activity and ECM organization. The deregulation of the activity of TG2 leads to increased stiffness of the vessels [59]. Higher levels of MMP-2 and MMP-9 also contribute to the release of TGF-β1, resulting in higher ECM deposition [60].

3.3- Vascular cell dysfunction

Impairment in EC vasodilatation capacity is one of the first signs of vessel aging [61]. The main agents responsible for vasodilatation are endothelium-derived hyperpolarizing factor (EDHF), prostacyclin and NO [62]. EDHF contributes to endothelial vasodilatation and declines with age [63]. Prostacyclin is a cyclooxygenase (COX)-derived vasodilator and its contribution to endothelial vasodilatation is lost with age, in humans [64]. On the other hand, the contribution of COX-derived contractile factors, such as thromboxane A_2, increases with age [65, 66]. NO is synthesized by endothelial nitric oxide synthase (eNOS), whose activity is decreased with aging, leading to reduced availability of NO and decreased endothelial vasodilatation. This lowered availability of NO is also associated with increased reactive oxygen species (ROS) production and consequent excessive oxidative stress, by modulating the production of superoxide in human vessels [67]. Moreover, NO reacts with superoxide to produce peroxynitrite, a highly reactive specie that is cytotoxic and contributes to vascular aging [68].

One hallmark of vascular aging is EC dysfunction, triggered by chronic oxidative stress [69]. An increase in oxidative stress on aged vessels has been observed both in animal models and in humans [70, 71]. Part of this effect is mediated by ROS. An imbalance in ROS production can lead to the accumulation of damaged or misfolded proteins, DNA mutations, inflammation [72] and EC senescence [73]. The main sources of ROS that lead to oxidative stress in aged vessels are NADPH oxidases, xanthine oxidase, uncoupled NO synthase and the mitochondrial respiratory chain [62]. NADPH oxidases are involved in the generation of superoxide and are upregulated in the presence of cardiovascular risk factors, including aging [74, 75]. Xanthine oxidase is an enzyme

capable of producing ROS and its accumulation in the aortic wall has been associated with aging [76]. The synthesis of NO by nitric oxide synthase is done by catalyzing the conversion of L-arginine to L-citruline. For the reaction to occur, dimerization of the enzyme L-arginine and the cofactor tetrahydrobiopterin (BH_4) have to be present. Uncoupling of NO synthase happens when L-arginine or BH_4 are not present. When this occurs, it has been described an increase in ROS production [77]. The mitochondria are primarily responsible for ROS production, through the respiratory chain [78]. With age, there is an accumulation of impaired mitochondria that leads to oxidative stress and contributes to vascular aging and impaired vasodilatation.

EC dysfunction triggered by inflammation is another hallmark of vascular aging [69]. In aged individuals, there is an increase of pro-inflammatory factors such as TNF-α, IL-1β, IL-6, CRP, Ang II, MMPs, calpain-1, monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN-γ) and intercellular adhesion molecules (ICAM) [79, 80]. Upregulation of TNF-α has been described and associated with oxidative stress, endothelial dysfunction, apoptosis and impairment of endothelium dilatation. The proinflammatory cytokine IL-6 is also associated with vascular diseases in aging. The inflammatory response in vascular cells is mainly mediated by the transcription factor NF-κB. When activated, it promotes the transcription of proinflammatory cytokines that are shown to be highly active and related with increased oxidative stress and endothelial dysfunction [79, 81].

Another hallmark of vascular aging is EC dysfunction triggered by senescence. The loss of the replicative capacity of ECs impairs the response to injury and the repair of dysfunctional endothelium [82]. The senescent phenotype in ECs can be due to replicative senescence or stress-induced premature senescence [82]. Replicative senescence occurs due to the limited potential of cell division and is characterized by the shortening of telomere size and loss of the proliferative capacity of ECs [83]. Stress-induced premature senescence occurs after cell exposure to stressful conditions such as altered glucose levels, oxidized low density lipoprotein, homocysteine, ceramide, Ang II, elevated blood pressure, increased ROS levels and inflammation [84]. Senescent ECs

display alterations in gene transcription and protein profile expression. Levels of transcription of IL-1α, IL-8, fibronectin, ICAM-1, p1, p53 and iNOS are shown to be upregulated, while eNOS is downregulated. Protein degradation of endothelial differentiation-related factor-1 (EDRF-1) and cyclin-dependent kinase 2 (CDK2) are increased in senescent ECs [83, 85, 86].

3.4- Altered response of vascular cells to flow shear stress

Blood flow in vessels creates a parallel friction force in the endothelium, called fluid shear stress, which influences the phenotype of ECs. ECs sense the shear stress and dynamically respond, by converting mechanical forces into intracellular signals [87]. This process is carried out by mechanotransducers such as glycocalyx, ion channels, G proteins, adhesion molecules and the cytoskeleton [87]. The prolonged exposition to blood flow causes structural changes, including elongation of ECs in the direction of the blood flow, and the orientation of the actin cytoskeleton, microtubules and intermediate filaments in the flow direction [88]. During aging, there is an altered response of ECs to shear stress, which can contribute to the development of atherosclerotic plaques [89]. As mentioned above, one of the main contributors for endothelial dysfunction is the reduction of NO availability due to the reduced activity of eNOS. Activation of eNOS can be carried out by fluid shear stress created by the blood flow in the endothelial layer [90]. In aged vessels, the reduced availability of NO can be associated with the lack of response of ECs to the shear stress and consequent decrease in NO production. The inflammatory process is also influenced by shear stress. During aging low levels of shear stress can promote arterial inflammation, through the induction of NK-κB expression in ECs and can contribute to the formation of atherosclerotic plaques [91]. An altered response of SMCs to shear stress also occurs in aged cells. In this case, increased aortic intraluminal pressure causes activation of ERK1/2, p38 MAPK, and JNK proteins [92].

4. Pathological vascular aging: the example of Hutchinson-Gilford Progeria Syndrome (HGPS)

HGPS is a rare disease in which patients exhibit accelerated aging-related symptoms such as alopecia, osteoporosis, subcutaneous fat loss, lipodystrophy and skin wrinkling [5]. Cardiovascular complications are the most devastating symptoms of this syndrome. Children develop progressive arteriosclerosis of the coronary and cerebrovascular arteries, eventually leading to fatal myocardial infarction or stroke at a mean age of 13 years old [5]. Classical HGPS is caused by a "*de novo*" point mutation in the LMNA gene, leading to the production of an aberrant protein named progerin [93]. Progerin accumulates in the nuclear membrane, prompting nuclear morphology abnormalities, misregulated gene expression, loss of peripheral heterochromatin, mitochondrial dysfunction, defects in DNA repair, alternate splicing, epigenetic changes, accelerated telomere shortening and premature senescence [94]. The same molecular mechanisms occur during normal aging, supporting the notion that HGPS mimics at least some aspects of physiological aging (**Fig. 1**). This can be partially explained by the fact that levels of progerin increase during physiological aging, although not at a same degree as in HGPS-affected cells [95].

Although not all features of physiological aging are manifest in this syndrome, from a cardiovascular standpoint, the case reports seem to be very consistent with a premature aging phenotype [96]. In fact, it has been shown that progerin accumulates mainly in the nucleus of vascular cells such as ECs, SMCs and fibroblasts. It has also been shown that progerin is widely present in the arterial walls and intimal arteriosclerotic plaques of HGPS patients, similar to healthy aged individuals [97, 98]. This evidence partially explains the severity of the cardiovascular phenotype in HGPS children. Development of advanced fibrotic arteriosclerosis with calcification and overall thickening and stiffening of the arterial walls, as well as mild systemic inflammation levels, are some of the HGPS vascular symptoms that also typically occur in normal aging [98].

4.1- Altered ECM remodeling

HGPS patients show vessel walls with marked fibrosis, having high stiffness and a decreased compliance of the vessels. Autopsies of HGPS patients reveal an accumulation of type I and type IV collagen and of proteoglycans, such as decorin and versican, as well as deposition of hyaluron in the arteriosclerotic lesions [98]. Genome-scale expression profiling of HGPS and aged-donor fibroblasts have shown altered expression of genes involved in ECM synthesis or modification [99, 100]. There is an up-regulation of proteoglycan cell adhesion proteins, which are important for ECM stability and for binding other proteoglycans, hyaluron and fibrous matrix proteins such as collagen [99, 100]. Laminin, a protein that forms essential interactions with collagen type IV and associates with cell-binding proteins, is also up-regulated, thereby influencing cell attachment, morphology and survival. A mouse model of HGPS showed increased arterial hyaluron content with age [101]. Hyaluron is an important ECM component, highly related to SMC proliferation and migration, and has been demonstrated to accumulate in early arteriosclerosis. The increased expression of fibrous proteins of the ECM, including collagen fibers, contributes to a decline in vessel elasticity [99, 100].

The ECM remodeling profile in HGPS is affected by enzyme expression. Studies have shown a specific downregulation of MMP-3 expression in HGPS cell lines and an associated donor age-dependent decline in secreted MMP-3 and MMP-2 proteins, which might contribute to the altered ECM in HGPS [102]. Differences in MMP-3 activity have also been reported in other vascular pathologies in the normal aging population [102]. Overall, there is an excess of ECM deposition, with increased expression of ECM components and decreased expression of ECM remodeling enzymes, which leads to structural effects on tissue function and also lead to signaling imbalances.

Several pathways regulating ECM are affected in HGPS. Mutant lamin A is accountable for the inhibition of the canonical Wnt signaling pathway, which is implicated in the regulation of

genes encoding ECM proteins [103]. TGF-β1/SMAD pathway has also been implicated in HGPS. This pathway can be activated through integrins, via mechanical force, contributing to increased collagen synthesis and, therefore, fibrosis [104]. Expression of TGFβ1 increases in an age-dependent manner in both HGPS and healthy cell lines. In postnatal tissues, especially those subjected to extensive mechanical stress, such as the skeletal and cardiovascular systems, the ECM assumes a structural role in maintaining tissue integrity and homeostasis [105]. In HGPS and physiological aging there is a concomitant alteration of ECM components and pathways that regulate ECM synthesis and organization, leading to a vicious cycle of matrix remodeling that contributes to the vasculopathy of aging.

4.2- Arterial stiffening and fibrosis

Progressive development of fibrotic arteriosclerosis, associated with an abnormal ECM content, reveals a major role of the ECM in the vasculopathy of HGPS patients [109, 116]. Similar to normal aged individuals, HGPS children show typically intimal fibrotic arteriosclerotic plaques in the main cardiac arteries. These lesions have a complex morphology, including thinning of the medial layer of the vessels, subjacent to the thick arteriosclerotic plaque in the intimal region, a necrotic core and foci of chronic inflammation. HGPS patients show evidence of marked fibrosis of the adventitial layer of large vessels, with a dense perimeter of collagen. In contrast, the same adventitial changes are not observed in normal aged individuals [109, 110]. HGPS fibroblasts have increased levels of ROS and protein oxidation, as well as decreased proteasome activity, thus contributing to cell dysfunction and altered production of ECM proteins [105]. Extensive calcification of the arteries is also frequently observed in HGPS.

4.3- Vascular cell dysfunction

Arteriosclerosis progression and severity in HGPS vascular systems are correlated with the accumulation of vascular senescent cells [105]. Senescent ECs in arteriosclerotic patients are characterized by low EC growth potential, as well as increased DNA damage and oxidative stress [105]. Moreover, ECs with a HGPS phenotype release inflammatory molecules and express higher levels of ICAM-1, after TNF-α stimulation, than non-HGPS ECs [106]. The increase in ICAM-1 increases the adhesion of monocytes, described as the first event in the initiation of arteriosclerosis [106]. This may explain the increased inflammation and macrophage localization in arteriosclerotic plaques of HGPS large vessels, contributing to the development of early arteriosclerosis [98]. The accumulation of both prelamin A and progerin at the nuclear envelope result in increased NF-$_{K}$B activation and systemic inflammation, revealing a possibility that this pathway plays a critical role in premature vascular aging [107]. It is also possible, though not yet studied, that HGPS-ECs also have an impaired NO synthesis function. Overall, EC dysfunction reduces EC contribution to arterioprotection and vascular repair.

Progressive loss of SMCs is a characteristic of HGPS patients, as well as in progeria mouse models [108]. SMCs depletion occurs mainly in arteries exposed to high flow shear stress such as the aorta and carotid [101, 109]. The loss of SMCs occurs in the media of the artery, with accumulation of matrix proteoglycan [101, 110]. The SMCs remaining in the vessels of HGPS show marked premature senescence and, it is thought, that SMC dysfunction is a major trigger of vascular calcification, another hallmark of both physiological and accelerated aging. Progerin and prelamin A accumulation in the nucleus of SMCs leads to nuclear lamina dysfunction, including impaired DNA damage response and repair. This in turn leads to the activation of senescence pathways in prelamin A-expressing SMCs, with induction of senescence-associated secretory phenotype (SASP). SASP includes inflammatory factors, extracellular matrix remodeling proteases, and proteins implicated in the regulation of SMCs calcification, such as BMP2, Runx2, osteocalcin, osteopontin, and osteoprotegerin [111]. Moreover, exposure of SMCs to calcifying medium leads to

upregulation of lamin A and prelamin A expression, accompanied by an increase of pro-calcification factors and increased calcium deposition [108]. Besides the transition of SMCs to an "osteoblastic-like" phenotype, there are other factors that contribute to vascular calcification. There is enhanced formation of calcium-phosphate deposits in blood vessels likely due to mitochondrial dysfunction and ATP production. Progerin-expressing SMCs showed impaired mitochondrial dysfunction and ATP production, leading to reduced synthesis of pyrophosphate (PPi), a potent inhibitor of the formation of calcium-deposits. Progerin-expressing SMCs also showed upregulation of several enzymes responsible for PPi hydrolysis and of phosphate synthesis [112].

Although not yet explored, it is possible that the remodeled ECM, that evolves the HGPS SMCs, has a large role in the development of vascular calcification. It has already been shown that metalloproteinases are up-regulated in human arteriosclerotic plaques and that by inhibiting their activity it is possible to reduce arterial calcification [113]. These evidences demonstrate the importance of the altered ECM in the vasculopathy of physiological and accelerated aging.

4.4- Altered response of vascular cells to flow shear stress

Cells of the cardiovascular system are exposed to various types of hemodynamic stress. Nucleus shape has been associated with adaptation to shear stress, an alteration that minimizes the total force exerted on the nuclei. Normally, when a cell is exposed to shear stress, there is a reorganization and up-regulation of nuclear laminas which protect the DNA and the nucleus interior from the effects of shear stress [114, 115]. Since lamins are involved in several nuclear functions including regulation of gene expression, it is likely that alterations in the nuclear lamina response to shear stress impacts gene expression. It is also hypothesized that a cellular phenotype, activated in response to well-defined laminar shear stress, resists the development of arteriosclerotic lesions, by activating protective signaling pathways. In HGPS there is an abnormal nuclear lamina that leads to a more stiffened nucleus and failure to respond normally to shear stress [114]. Therefore, progerin-

expressing cells, that do not respond normally to shear stress, contribute to the early development of arteriosclerosis in HGPS patients, evident by the fact that arteriosclerotic plaques develop preferentially in regions of elevated shear flow [115]. Moreover, it has been shown that HGPS neighboring, but unaffected cells, also have an altered response to shear stress, further contributing to the severity of the vasculopathy of HGPS [115].

Loss of SMCs in regions exposed to high fluidic shear stress, such as the aortic and carotid arteries, is strong evidence that their normal adaptation to shear stress is altered in HGPS. These altered responses are linked to changes in mechanotransduction pathways [116]. Progerin-expressing SMCs, exposed to high *in vivo* hemodynamic forces, have a significant reduction in the expression of mechanotransduction-related proteins such as vinculin and vimentin.

In contrast to SMCs, progerin-expressing ECs seem to be more resistant to shear stress than ECs without progerin. Indeed, an intact monolayer of vimentin-positive ECs is typically observed in progeria mouse models [116]. In aortic regions presenting SMCs loss, ECs show more than an eight-fold level of vimentin expression, in comparison with ECs overlying adjacent aortic regions not depleted of SMCs. This up-regulation in vimentin helps ECs withstand the same mechanical forces that are degenerative to SMCs. Furthermore, vimentin filaments in ECs, associate with integrins to form cell-matrix adhesions through flow-induced focal contacts [116].

5– *In vitro* systems to study vascular aging

Animal models are important tools to study aging, since they share common molecular mechanisms of pathophysiology with aged people, providing insight into the molecular mechanisms of aging. Therefore, a wide range of *in vivo* vascular aging models have been developed, such as mouse models of accelerated aging [117-121]. Nevertheless, vascular aging is a complex biological phenomenon in which several components are involved. Thus, it is very unlikely that an individual gene mutation in mice would recapitulate all the features of human aging. Furthermore, it is

difficult to identify cellular and molecular key players to disease in whole-animal models. These limitations of vascular aging animal models, lead to the development of new *in vitro* vascular aging models.

Two (2D) and three (3D)-dimensional *in vitro* models may be used to study vascular aging. 2D models are relatively cheap and simple; however, they may not recapitulate important aspects of vascular biology [122] and *in vivo* predictivity [123] because cells are not exposed to normal mechanical signals, including fluid shear stress, tension and compression. These limitations have led to increased interest in 3D models which provide more predictive data for *in vivo* tests [124]. 3D models may replicate both anatomical macro and microstructures, including appropriate cell types, ECM, and suitable physiological cues [125].

Microfluidic devices offer the possibility of culturing living cells, in continuously perfused micrometer chambers, in order to model physiological functions of tissues and organs [123]. Advantages of microfluidic devices comprise: i) the possibility of incorporating physical forces, including fluid shear stress, cyclic strain and mechanical compression, ii) the possibility of making a 3-D microenvironment by using hydrogels as scaffolds, iii) the possibility of mimicking relevant tissues by incorporating human cells (cell lines, primary cells, stem cells differentiated to specific lineages as well as the differentiation process itself), and iv) controlling the distribution of chemical variables.

Cells cultured under flow conditions have different biological properties. Shear stress, which is the tangential force to the cells surface, is known to induce substantial morphological and biochemical changes in vascular cells through mechanotransduction [126]. The necessary flow rates, to reach typical shear stress forces, vary from vessel to vessel. In healthy conditions, the rate is estimated to be between 1 to 6 dyn/cm^2 in the venous system and between 10 to 70 dyn/cm^2 in the arterial system [90]. There are a variety of techniques used to achieve this, ranging from pneumatic and syringe pumps to electro-kinetics to control shear stress in microfluidic devices.

Constant flow, typical of many capillaries can be applied through gravity flow, where a height difference between inlet and outlet reservoirs is used to provide steady differential pressure [127]. However, if flow rates are extremely high, other fluid systems such as using liquid level sensing and computer-controlled valves to transmit the proper amount of media, may be valuable in maintaining a certain pressure and flow rate [128]. Pulsatile flow can be achieved using an elastomeric microfluidic cell shearing chamber, interfaced with computer-controlled movement of piezoelectric pins [129]. Additional approaches to generate high shear stress in the fluidics include the incorporation of solenoid pinch valves into gravity flow to turn vessel flow on and off electronically [130], external peristaltic pumps [131] and pneumatic micropumps [132].

Microfluidic systems with progeria cells have been instrumental in the study of pathological aging [116, 133]. In one case, a microfluidic system was developed with a top fluidic channel, a middle thin polydimethylsiloxane (PDMS) membrane and a bottom vacuum channel (**Fig. 2a**). SMCs derived from human induced pluripotent stem cells obtained from HGPS patients (HGPS-iPSC-SMCs) were cultured on top of the membrane which was deformed by applying different amount of pressures on the bottom channel. This aged model combined biomechanical strain and flow, and thus allowed for a better understanding of the inflammatory response of aged SMCs to strain, characterized by an increase in levels of inflammation markers as well as DNA damage (**Fig. 2b, c**) [133]. In a separate study, a microfluidic system was developed to study the response of SMCs (HGPS vs wild type) to flow shear stress [116]. Aortas were exposed to high fluidic shear stress (75 dynes/cm^2) for 30 min. Using this model, it was possible to verify that high fluidic shear stress produced a substantial decrease in vimentin of progeria aortas but not in the wildtype controls. This model showed that a decrease of this protein may contribute to development of vasculopathy in the ascending aorta in progeria syndrome. Previous models are focused mainly on aged SMCs and do not take into account the interaction with aged ECs. Co-cultures of SMCs and ECs may be used as platforms to predict functional and pathological disease characteristics [134].

The importance of flow shear stress in aging was also addressed by a functional 3D model of HGPS that replicated an arteriole-scale tissue engineered blood vessel (TEBV) (**Fig. 3a**) [134]. HGPS-iPSC-SMCs and human cord blood-derived endothelial progenitor cells (hCB-EPCs) from a healthy donor were used. TEBVs were incorporated into a flow loop and perfused with steady laminar flow at a shear stress of 6.8 dynes/cm^2 for 1 to 4 weeks, for maturation and functional characterization studies. TEBVs fabricated from HGPS-iPSC-SMCs and hCB-EPCs showed reduced vasoactivity, increased medial wall thickness, increased calcification and apoptosis relative to control TEBVs (**Fig. 3b, c, d**) [134]. When HGPS-iPSC-SMCs were subjected to repeated pulses of electrical stimulation, they rapidly senesced [135].

6- Conclusions and future directions

This review highlights the similarities and differences in vascular aging between physiological and pathological aging, as well as the suitability of HGPS as a model of vascular aging. Some of the similarities between physiological and HGPS aging are due to the fact that progerin accumulates in vascular cells and other cells during physiological aging [136]. The derivation of iPSCs from HGPS fibroblasts has created an excellent platform to obtain cells otherwise difficult to isolate, due to the rarity of the disease [137, 138]. The use of vascular cells derived from HGPS iPSCs may provide further insights into vascular aging [133]. Studies have demonstrated that the accumulation of progerin in HGPS-iPSC derived SMCs led to a downregulation of DNA-dependent protein kinase catalytic subunit expression [137] and poly(ADP-ribose) polymerase 1 [139], which resulted in low cell proliferation. In addition, the use of ECs from HGPS-iPSCs showed that these cells had higher mechanosensitivity, likely due to an elevation of channel V2 expression upon mechanical stimulation [140]. It is expected that the combination of these HGPS cells in bioengineered systems will facilitate the discovery of new

molecular targets. Future studies should evaluate the effect of flow shear stress in vascular cells and to determine the mechanisms behind their mechanosensitivity.

This review also highlights a set of markers and features that characterize vascular aging, both in physiological and pathological conditions. It is known that molecular aging is controlled by multiple pathways and their impact, as well as how they intertwine, is not completely understood [17]. Although it is known that the ECM is significantly altered during aging, it remains to be determined its effect in the aging process, as well as its effects in inducing "aging" in young vascular cells. The creation of substrates with variable stiffness using recent technologies [141], as well as the identification of new ECM molecules that mediate the aging process, might facilitate the *in vitro* study of this process.

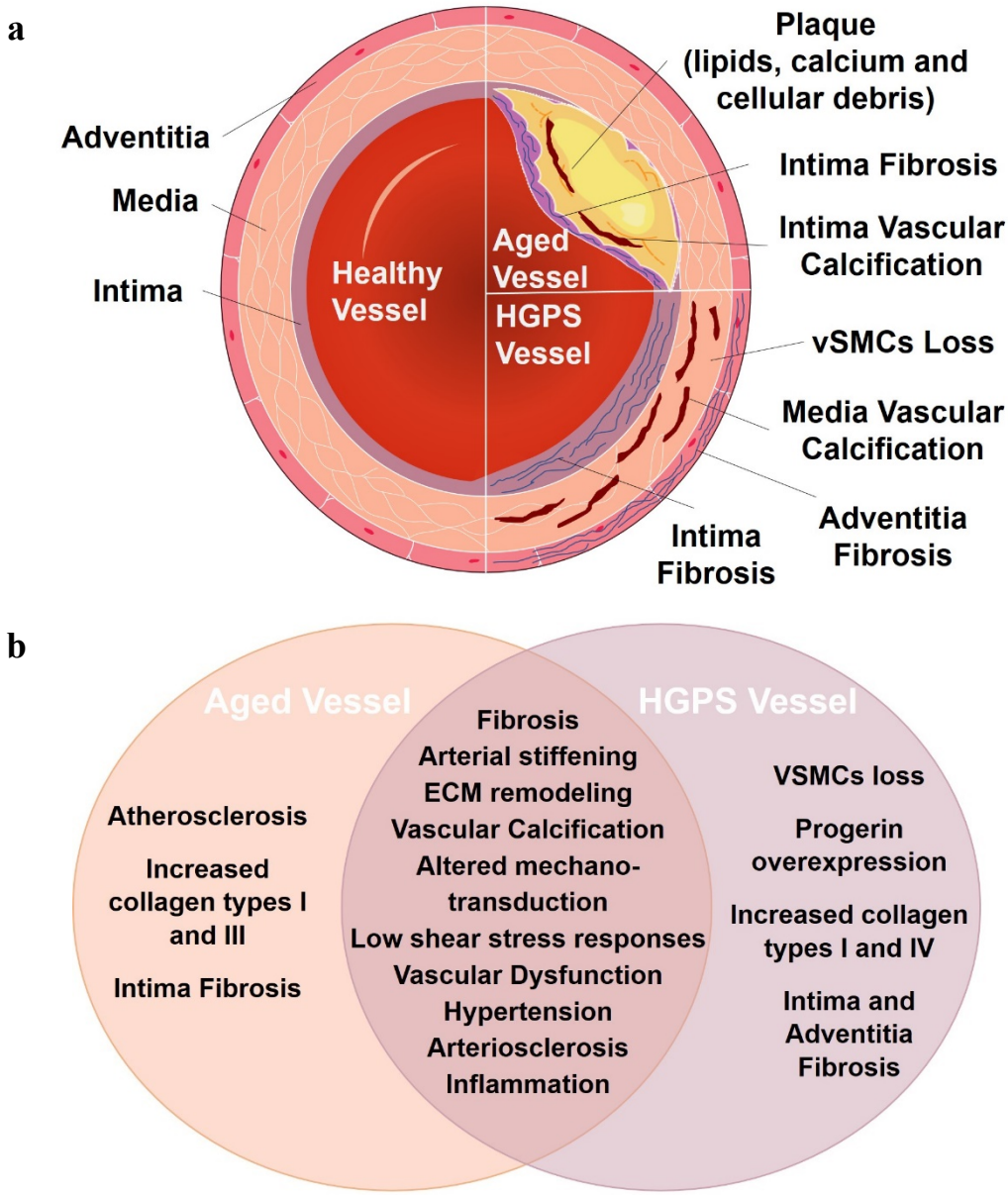

Figure 1- (a) Schematic representation of the differences and similarities between physiological, HGPS aged, and young healthy vessel. Both aged and HGPS vessels develop marked arteriosclerotic fibrosis and calcification, with an overall thickening and stiffening of the walls. Aged vessels develop a particular type of arteriosclerotic plaques, with the accumulation of lipids, which is absent in HGPS individuals. **(b)** Similarities and differences between cellular, molecular and biophysical components of physiological and HGPS-accelerated aging. HGPS represents a highly reliable model of vascular aging, showing similar symptoms that include vascular cell dysfunction, disruption of the ECM and altered mechanotransduction responses.

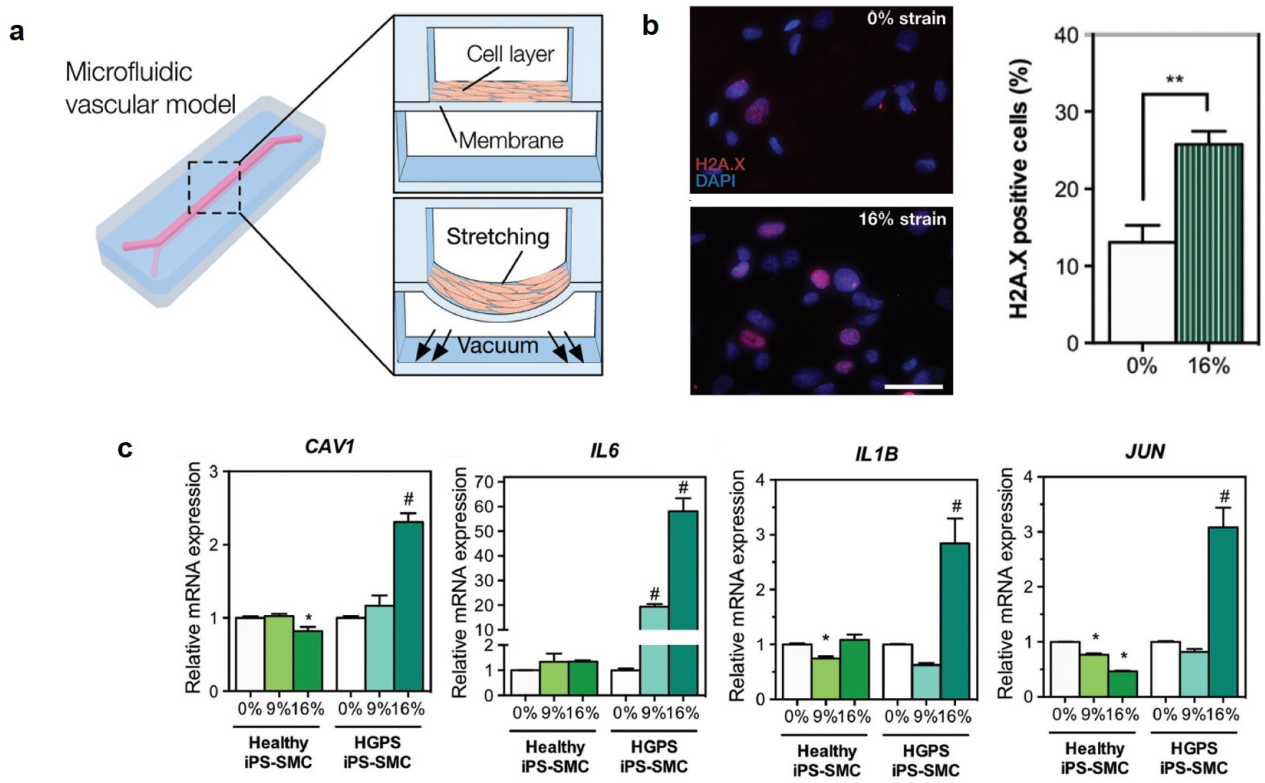

Figure 2- Recapitulation of blood vessel dynamics on a chip and exacerbated response to biomechanical strain in HGPS iPS-SMCs. (Copyright 2017, Small, USA, [133]). **(a)** Biomimetic microfluidic vascular model containing two overlapping channels. A cross-sectional view of the microfluidic device shows the cell layer cultured on top of the PDMS membrane and a view during vacuum stimulation regarding the downward membrane deformation. **(b)** DNA damage was evaluated in HGPS iPS-SMCs with H2A.X immunostaining and quantified (mean +/- SD of $n = 3$). **(c)** Injury marker *CAV1* and inflammation markers *IL6*, *IL1B*, and *JUN* were evaluated in HGPS iPS-SMCs and healthy iPS-SMCs (*$P < 0.01$ against 0% healthy iPS-SMCs, and # indicates $P < 0.01$ against 0% HGPS iPS-SMCs; bars represent mean +/- SD of n=5).

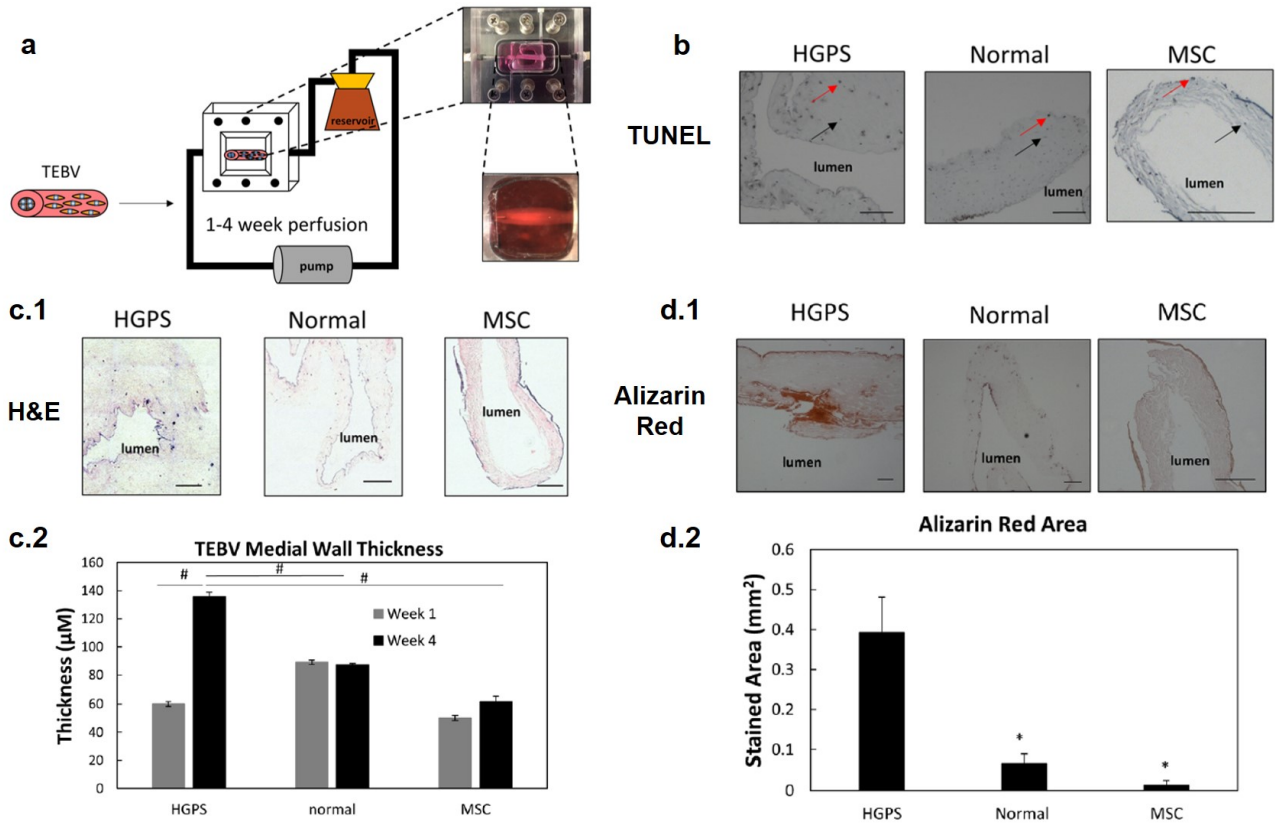

Figure 3- (a) Schematic diagram of the procedure to produce iPSC-derived SMC TEBVs from healthy and HGPS patients (Copyright 2017, Scientific Reports, USA, [134]). SMCs were incorporated into a dense collagen gel construct and were then incorporated into a flow loop and perfused with steady laminar flow at a shear stress of 6.8 dynes/cm2 for 1 to 4 weeks for maturation and functional characterization studies. **(b)** Apoptosis. Histochemical analysis of MSC, normal SMC and HGPS SMC TEBVs at week 4 with TUNEL staining. Red arrows indicate TUNEL positive cells and black arrows indicate TUNEL negative cells (Scale bar, 200 μm). **(c)** Thickness. **(c.1)** Histochemical analysis of HGPS SMC, normal SMC, and MSC TEBVs at week 4 with H&E (Scale bar, 200 μm). **(c.2)** The average thickness of MSC, normal SMC and HGPS SMC TEBVs at week 1 and week 4 based on H&E images. **(d)** Calcification. **(d.1)** Histochemical analysis of HGPS SMC, normal SMC, and MSC TEBVs at week 4 with Alizarin Red staining (Scale bar, 200 μm). **(d.2)** Quantification of the total area positive for Alizarin Red.

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