



## Review article

## Pharmacological modulation of vascular ageing: A review from VascAgeNet

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## ABSTRACT

Vascular ageing, characterized by structural and functional changes in blood vessels of which arterial stiffness and endothelial dysfunction are key components, is associated with increased risk of cardiovascular and other age-related diseases. As the global population continues to age, understanding the underlying mechanisms and developing effective therapeutic interventions to mitigate vascular ageing becomes crucial for improving cardiovascular health outcomes. Therefore, this review provides an overview of the current knowledge on pharmacological modulation of vascular ageing, highlighting key strategies and promising therapeutic targets. Several molecular pathways have been identified as central players in vascular ageing, including oxidative stress and inflammation, the renin-angiotensin-aldosterone system, cellular senescence, macroautophagy, extracellular matrix remodelling, calcification, and gasotransmitter-related signalling. Pharmacological and dietary interventions targeting these pathways have shown potential in ameliorating age-related vascular changes. Nevertheless, the development and application of drugs targeting vascular ageing is complicated by various inherent challenges and limitations, such as certain preclinical methodological considerations, interactions with exercise training and sex/gender-related differences, which should be taken into account. Overall, pharmacological modulation of endothelial dysfunction and arterial stiffness as hallmarks of vascular ageing, holds great promise for improving cardiovascular health in the ageing population. Nonetheless, further research is needed to fully elucidate the underlying mechanisms and optimize the efficacy and safety of these interventions for clinical translation.

## 1. Introduction

Our fitness depends on a strong network of healthy blood vessels that efficiently delivers nutrients to organs and tissues and removes toxins. In that regard, the aorta serves an important purpose. Due to its elastic properties, it will dampen the pulsatile pressure resulting from the heart's contraction, thereby maintaining a continuous blood flow at the level of the arterioles (Laurent et al., 2001). Ageing induces both structural and functional changes in the vascular wall (Climie et al., 2023). Typical aspects include progressive stiffening of the blood vessel wall because of endothelial dysfunction, vascular smooth muscle cell (VSMC) proliferation, changes in extracellular matrix (ECM) composition (including elastin fragmentation and enhanced collagen production), increased oxidative stress, lipid accumulation and inflammation. As a consequence, the aorta loses its dampening function, leading to increased pulse pressure, further aggravating endothelial dysfunction and arterial stiffening. Importantly, both endothelial dysfunction and arterial stiffness are independent risk factors of cardiovascular morbidity and mortality (Bonetti et al., 2003; Laurent et al., 2001; Terentes-Printzios et al., 2017; Yoshii et al., 2020).

Thus, vascular ageing has major health consequences and leads to many age-related diseases that are responsible for both a social and economic burden in our society. It is estimated that cardiovascular disease (CVD), ischemic heart disease and cerebrovascular disease costs the EU health care system approximately 150 billion euro per year (Wilkins et al., 2017). This does not even include the costs related to hypertension and other forms of heart disease. Furthermore, according to the World Health Organization, the number of people over 60 years of age is projected to double from an estimated 1 billion in 2020 to nearly 2.1 billion in 2050, highlighting the need to promote healthy ageing. To increase health span, prevention and delay of arterial stiffening and endothelial dysfunction, as key indicators of vascular ageing, is highly relevant. But, it remains an overlooked strategy thus far. Currently, a number of cardiovascular risk factors, such as hypertension, hypercholesterolemia and diabetes, are being monitored and treated in clinical practice, thereby reducing CVD risk. However, therapeutic strategies specifically aimed at delaying vascular ageing by preventing arterial stiffening and endothelial dysfunction are lacking. To implement this, an adequate and routine monitoring of vascular function in a clinical setting is required. For instance, endothelial function can be assessed by measuring flow-mediated dilation (FMD). The primary technique used

to assess aortic stiffness is by calculating pulse wave velocity (PWV). This represents the speed of the pressure wave travelling along the arterial system, which is increased in stiffer arteries. Magnetic resonance imaging (MRI) is the current gold standard to measure PWV, because it can precisely reproduce morphology and function of the entire aorta, but it is a costly approach. Instead, the most widely employed method involves using tonometry on the carotid and femoral arteries to calculate the transit time of the pulse, specifically referred to as carotid-femoral PWV (Segers et al., 2020). However, these measurements are not routinely implemented in clinical practice. In addition, development of well-validated intervention strategies, targeting either arterial stiffness and/or endothelial dysfunction, are required. Therefore, the current review will highlight the mechanisms involved in the progression of vascular ageing, including related pharmacological and dietary interventions (Fig. 1). We will also focus on the challenges that exist to adequately monitor the efficiency of therapeutic strategies. Overall, we overview the knowledge on the progression of vascular ageing, with a focus on arterial stiffness and endothelial dysfunction, and the tools to modulate it. We believe this will emphasize the relevance of tackling vascular ageing to prevent age-related diseases and to improve the quality of life of the ageing population.

## 2. Pharmacological modulation of mechanisms and signalling pathways that contribute to vascular ageing

### 2.1. Oxidative stress

Reactive oxygen species (ROS) are natural by-products of aerobic cellular metabolism. Although ROS at moderate concentrations are key molecules in the regulation of biological functions, sustained or excessive production is harmful. To regulate ROS concentration, mammalian cells neutralise ROS to nontoxic forms through antioxidant systems (Valko et al., 2007) comprised of several enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx), as well as non-enzymatic molecules, including glutathione (GSH) and vitamins C and E (see Section 4.1).

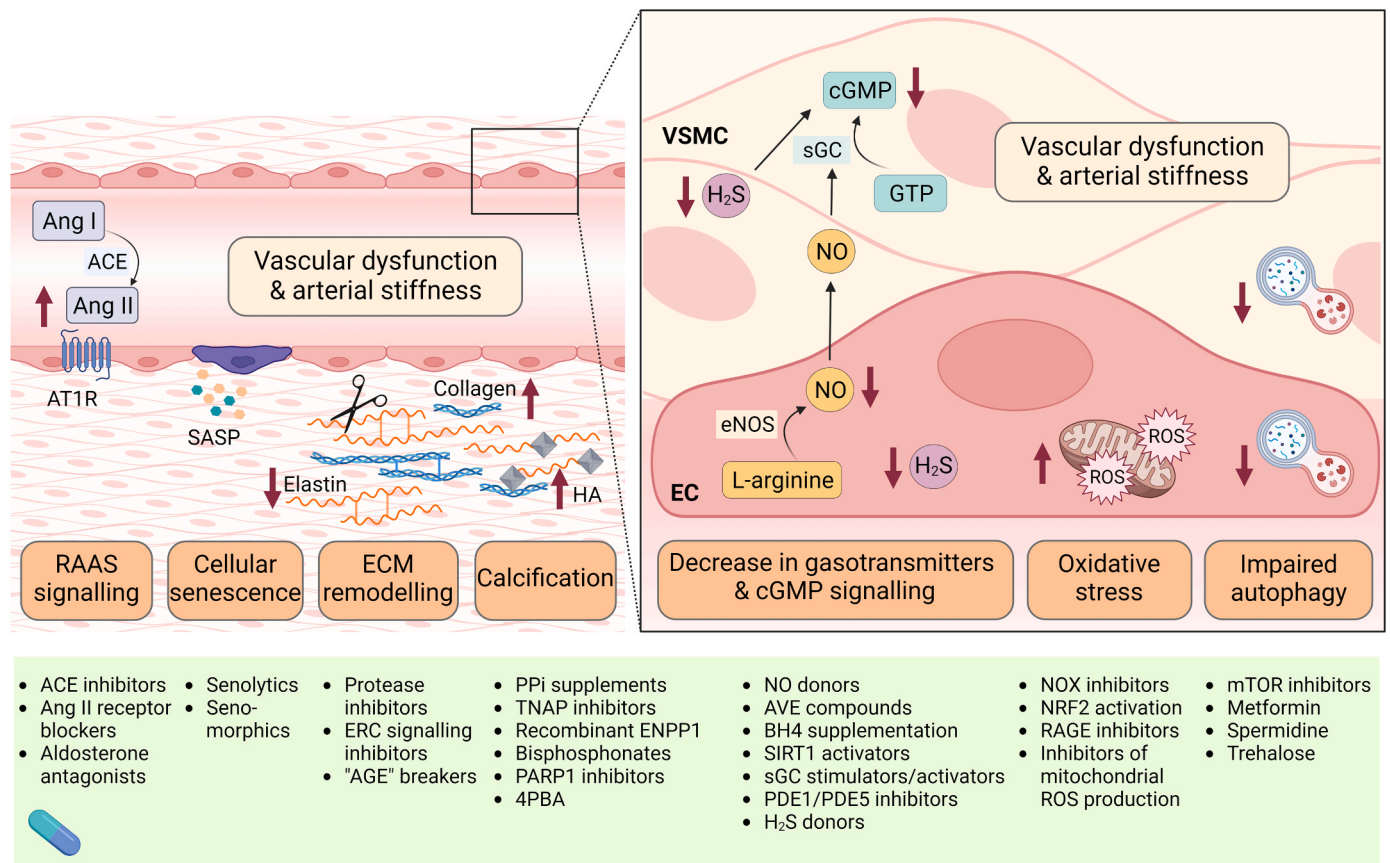
ROS are key players in vascular ageing. Upon ageing, mitochondria become dysfunctional, due to the oxidative damage of mitochondrial DNA (Mikhed et al., 2015). Consequently, the production of ROS increases to maintain sufficient ATP production (Mikhed et al., 2015) while in parallel, antioxidant mechanisms diminish (Inal et al., 2001).

This imbalance yields a hazardous oxidative stress state, resulting in modification of proteins, lipids, and DNA. (Wei and Lee, 2002). Accumulation of these damaged biomolecules can eventually lead to cellular senescence (Beckman and Ames, 1998) (see Section 2.3). Mounting evidence suggests high levels of oxidative stress predispose to vascular dysfunction and ageing through several still not fully elucidated molecular mechanisms, as reviewed elsewhere (Chandrasekaran et al., 2017). Mitochondrial ROS can activate transcription factors like nuclear factor kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1), thereby promoting inflammation (Ungvari et al., 2018). The activation of NF- $\kappa$ B and downregulation of nuclear factor erythroid 2-related factor 2 (NRF2) results in endothelial dysfunction with an ensuing reduction in the availability of nitric oxide (NO) and increase in vasoconstrictive molecules (Ungvari et al., 2018). In turn, this promotes more oxidative stress and inflammation, generating a vicious cycle, which ultimately leads to vascular dysfunction (Clapp et al., 2004; Kirkman et al., 2021).

### 2.1.1. Mitochondrial ROS

Targeting mitochondrial ROS production has been shown to have a beneficial effect on ageing. Resveratrol, a naturally occurring polyphenol, can attenuate mitochondrial-derived oxidative stress through targeted activation of sirtuin 1 (SIRT1) (Howitz et al., 2003), influencing

a broad range of mechanisms within the vasculature; many of which reduce the risk of cardiovascular complications (Barger et al., 2008). The multi-faceted nature of compounds like resveratrol promoted the development of therapeutics which specifically target mitochondrial-derived ROS. For instance, Euk-8; a mimetic of the free radical scavengers SOD and catalase, restores levels of oxidative stress to baseline conditions. It was shown that Euk-8 administration prevented the formation of oxygen-derived free radicals and improved NO production in the aortic root of *ApoE*<sup>-/-</sup> mice subjected to psychological stress (Andersson et al., 2010). In addition, it protected animals against the effects of surgically-induced high blood pressure, such as incidence of ventricular remodelling and cardiac decompensation (van Empel et al., 2006). Similarly, MitoQ; a mitochondria-targeting ubiquinone, infers protective effects in models of cardiovascular dysfunction (Graham et al., 2009) and vascular ageing (Gioscia-Ryan et al., 2018; Gioscia-Ryan et al., 2014). Mechanistically, Gioscia-Ryan et al. determined that the therapeutic effects of MitoQ supplementation on the vasculature stemmed partially from improvements to endothelial function (Gioscia-Ryan et al., 2014) and a reduction in elastin degradation (Gioscia-Ryan et al., 2018) in in vivo models of vascular ageing. Clinical examination of MitoQ by Rossman et al. translated many of these findings in their study, in which MitoQ treatment was found to improve



**Fig. 1.** Overview of vascular ageing mechanisms and potential therapeutic strategies. The main pathophysiological mechanisms that are involved in vascular dysfunction and arterial stiffening include, enhanced renin-angiotensin-aldosterone (RAAS) signalling, cellular senescence, extracellular matrix (ECM) remodelling characterized by a decreased elastin/collagen ratio, deposition of hydroxyapatite (HA) crystals (calcification), a decrease in gasotransmitters (NO and H<sub>2</sub>S) and cGMP signalling, oxidative stress, and impaired autophagy. These pathways can be pharmacologically targeted by several classes of drugs mentioned in the green box. 4PBA: 4-phenylbutyrate; ACE: angiotensin-converting enzyme; AGE: advanced glycation end-product; Ang I: angiotensin I; Ang II: angiotensin II; AT1R: angiotensin II type 1 receptor; BH4: tetrahydrobiopterin; cGMP: cyclic guanosine monophosphate; EC: endothelial cell; eNOS: endothelial nitric oxide synthase; ENPP1: ecto-nucleotide pyrophosphatase/phosphodiesterase 1; ERC: elastin receptor complex; GTP: guanosine triphosphate; H<sub>2</sub>S: hydrogen sulphide; mTOR: mammalian target of rapamycin; NO: nitric oxide; NOX: NADPH oxidase; NRF2: nuclear factor erythroid 2-related factor 2; PARP1: poly [ADP-ribose] polymerase 1; PDE1: phosphodiesterase 1; PDE5: phosphodiesterase 5; PPI: inorganic pyrophosphate; RAGE: receptor for advanced glycation end-products; ROS: reactive oxygen species; SASP: senescence associated secretory phenotype; SIRT1: sirtuin 1; TNAP: tissue nonspecific alkaline phosphatase; VSMC: vascular smooth muscle cell. Created with BioRender.com.



brachial artery dilation and reduce aortic stiffness in an elderly population (Rossman et al., 2018). The correlative reduction in markers of oxidative stress in those who received MitoQ supplementation aligned with previous studies examining the compound (Gioscia-Ryan et al., 2018; Gioscia-Ryan et al., 2014), lending further credence to the idea that mitochondrial ROS is a pivotal player in age-related vascular dysfunction. Furthermore, a study by Park et al. revealed that treatment with MitoQ improved endothelial function in patients with peripheral artery disease (Park et al., 2020). Alternatively, the Szeto-Schiller (SS) peptides have shown efficacy in treating various ROS-induced pathologies; SS peptide-treated animals subjected to ischemic-reperfusion injury showed a significant reduction in cardiac damage, predominantly through the scavenging of hydrogen peroxide and peroxynitrite (Dai et al., 2011). At the moment it remains unknown whether these peptides can also improve age-related vascular dysfunction.

### 2.1.2. NADPH oxidase

The role of NADPH oxidase (NOX)-regulated ROS production in vascular dysfunction and vascular pathologies has been widely recognized (Frazziano et al., 2014). The NOX family of enzymes consists of seven isoforms, four of which (NOX-1, -2, -4, and -5) are expressed in vascular tissue (Drummond and Sobey, 2014). These isoforms are influenced by a wide range of stimuli such as proinflammatory mediators and cytokines, and increasing evidence indicates that their expression and activity increases with age (Sorescu et al., 2002; Vendrov et al., 2015). For instance, Vendrov et al. found that NOX expression was increased in an in vivo model of vascular ageing; a result which correlated with pathological changes in mitochondrial function and aortic stiffness (Vendrov et al., 2015).

Pan-NOX inhibitors, such as apocynin and S17834, and selective, isoform-specific NOX inhibitors have been evaluated in various studies using animal models of CVD, and exhibited beneficial effects (Cayatte et al., 2001; Wind et al., 2010). For instance, GKT136901 and GKT137831, both NOX1/4 inhibitors, were shown to significantly reduce atherosclerotic plaque development and cardiac remodelling (Vendrov et al., 2010; Zhao et al., 2015). However, direct effects on endothelial function and arterial stiffness still need to be determined. With the pre-clinical success of selective NOX inhibitors, clinical trials investigating the effects of the next generation of such i.e., setanaxib (GKT831) and GKT137831 (NOX1/4 inhibitors), are currently ongoing (Sylvester et al., 2022).

### 2.1.3. NRF2

NRF2, a transcription factor that regulates the expression of haem oxygenase, glutathione-S-transferase, etc., is a central factor in the regulation of redox homeostasis (Cui et al., 2016). It mitigates the pathophysiological effects of increased oxidative stress in several age-related diseases such as Alzheimer's disease, diabetes and CVDs (Kobayashi et al., 2016), and is thus of interest as a potential therapeutic target in the context of vascular ageing.

Studies have shown that in young subjects, the homeostatic response of NRF2 in preserving oxidative balance is a key mechanism in preventing oxidative stress, which may have implications within the vasculature specifically (Afonyushkin et al., 2010; Xue et al., 2008). In an in vivo model of ageing it was demonstrated that a progressive increase in ROS correlated with decreased NRF2 activity (Ungvari et al., 2011). In the same study, ex vivo cultures of aortas taken from aged animals showed an increased sensitivity to ROS-inducing stimuli in comparison to those taken from younger counterparts. This was directly attributable to the blunted NRF2 response in the aged group (Ungvari et al., 2011).

Many therapeutic strategies have focussed on disrupting the interaction of kelch-like ECH-associated protein 1 (KEAP1) with NRF2, since KEAP1 suppresses NRF2 activity by promoting its proteasomal degradation. For example, bardoxolone methyl, and sulforaphane have both been shown to covalently bind to KEAP1, releasing NRF2 and inducing

transcription of antioxidant genes. Both compounds have been shown to exert vasoprotective effects in the context of diabetes, atherosclerosis, and chronic kidney disease in preclinical and clinical trials (phase I and II) (Cakir et al., 2022; Pan et al., 2022; Pergola et al., 2011; Tan et al., 2014). However, a phase III clinical trial in patients with type 2 diabetes mellitus and stage 4 chronic kidney disease was prematurely terminated due to a higher rate of cardiac-related adverse events in the group treated with bardoxolone methyl (de Zeeuw et al., 2013). Alternative strategies targeting NRF2 independent from KEAP1 (Fao et al., 2019), showed therapeutic benefits in an inflammatory context that may be translated to the vascular system. Taken together, modulating NRF2 activity remains a promising approach to inhibit the influence of ROS within the vasculature, hence preventing/delaying vascular ageing.

### 2.1.4. RAGE

The receptor for advanced glycation end-products (RAGE) is expressed on a number of cell types implicated in arterial ageing. Studies have demonstrated that RAGE signalling has downstream consequences in inflammatory and oxidative stress events that promote arterial stiffness and consequently vascular ageing. For example, it was shown that RAGE activation on endothelial cells (ECs) promoted NADPH activity, leading to increased oxidative stress and an inflammatory phenotype (Wautier et al., 2001). Similar studies marked a relationship between RAGE activation and NADPH oxidase-derived oxidative stress (Gao et al., 2008), and as such, increases in RAGE expression at sites of inflammation subsequently create a vicious cycle (Lin et al., 2009), which leads to vascular remodelling, and promotes indices associated with vascular ageing. Increased serum levels of the soluble form of RAGE have been associated with an increased risk of mortality in older adults (Butcher et al., 2019), while accumulation of advanced glycation end-products (AGEs) that activate RAGE in aged tissues (Ahmed et al., 1997; Odetti et al., 1998; Schleicher et al., 1997) have also been noted. Together with existing evidence on RAGE activity in vascular disease, it is hypothesised that AGE/RAGE dynamics are a probable cause and/or a biomarker of vascular ageing. Simm et al. reported that in patients undergoing cardiac surgery, an age-dependent increase in RAGE was observed, with RAGE expression correlating with reduced vascular function (Simm et al., 2004).

Since RAGE signalling is driven through receptor activation by proinflammatory ligands, compounds targeting these ligands exert beneficial effects in RAGE-associated pathophysiological conditions (Farmer and Kennedy, 2009). For example, targeting of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) shows vascular benefits in a number of disease contexts through modulation of RAGE expression (also see Section 3). Conversely, thiazolidinediones (Peyroux and Sternberg, 2006), rosiglitazone (Wang et al., 2006), and angiotensin II type 1 receptor (AT1R) antagonists (Yamagishi et al., 2008), reduce RAGE-associated ROS production. Moreover, treatment with soluble RAGE, which is a soluble form of the receptor that can act as a ligand decoy, has also shown promise by improving endothelial function in diabetic mice (Gao et al., 2008).

## 2.2. Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone (RAAS) system constitutes a vital vasoactive system in the regulation of blood pressure, cardiovascular and renal function. Many cardiovascular and renal complications such as congestive heart failure, systemic hypertension, and chronic kidney disease are associated with chronic RAAS hyperactivation or hyperaldosteronism and arterial stiffness. Therefore, RAAS is considered as a potential therapeutic target (Jia et al., 2018). To date, RAAS inhibition is the cornerstone in treatment of diseases associated with arterial stiffness (Laurent et al., 2021). Clinical trials have demonstrated that pharmacological inhibitors of angiotensin II (Ang II) or aldosterone, i.e., angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) and aldosterone antagonists, all reduce arterial stiffness

(Li et al., 2020; Mahmud and Feely, 2002; Mahmud and Feely, 2004; Shahin et al., 2012) (see Section 3). Due to the multifactorial physiological characteristics of the RAAS, the effect of RAAS inhibition is complex, facilitating reduced inflammation, fibrosis, oxidative stress and vascular remodelling, and preserved vascular tone by release of mediators such as NO, prostacyclin, bradykinin, endothelium-derived hyperpolarizing factors (Pacurari et al., 2014), resulting in reduced vascular ageing.

Emerging pharmacological strategies and RAAS components for reduction of arterial stiffness and vascular ageing have been identified (Oparil and Schmieder, 2015). One such component is the prorenin receptor (PRR), which binds to the precursor molecule of renin, i.e., prorenin, and promotes Ang II generation in plasma and cardiovascular tissues (Nguyen et al., 2002; Simons et al., 2020). PRR activation leads to VSMC proliferation, endothelial dysfunction, neovascularization, and development of vascular diseases. In animal models, loss of soluble PRR reduced blood pressure at baseline and decreased Ang II-induced hypertension and renal injury (Ramkumar et al., 2021). Thus, PRR may be a novel therapeutic target for diseases associated with vascular ageing, such as hypertension, heart failure, and atherosclerosis (Xu et al., 2022). A second component of the RAAS is angiotensin-converting enzyme 2 (ACE2). Reduction of ACE2 promotes adverse vascular remodelling (Chamsi-Pasha et al., 2014; Wang et al., 2012) through production of ROS, activation of matrix metalloproteinases (MMPs), and induction of VSMC apoptosis, making it a critical mediator of vascular disease and leading to increased vascular stiffness in mice (Patel et al., 2014). ACE2 has also been found to be an independent predictor of arterial stiffness in diabetic patients (Srivastava et al., 2020). A third emerging component is Ang-(1–7), which is a Mas-receptor agonist, and has an opposite mechanism of action to Ang II, causing vasodilation. Animal models have shown a direct blood pressure lowering effect of Ang-(1–7), as well as reduced hypertrophy, fibrosis and atherosclerosis but clinical data are scarce (Medina and Arnold, 2019).

### 2.3. Cellular senescence

Cellular senescence consists of three main groups: oncogene-induced, stress-induced and replicative senescence (Regulski, 2017). Accumulation of senescent cells results in tissue dysfunction which may be the consequence of increased production, as well as decreased removal of senescent cells (Katzir et al., 2021). The most prominent feature of senescent cells, in addition to cell-cycle arrest, is the production of proinflammatory cytokines, growth factors and proteases commonly named as senescence associated secretory phenotype (SASP), that exert their effects in autocrine as well as in paracrine fashion (Kumari and Jat, 2021). In addition, despite their growth arrest, senescent cells do not undergo apoptosis and stay metabolically active (Csekes and Rackova, 2021). The most frequently used methods to detect cellular senescence in the cardiovascular apparatus include analysis of SA- $\beta$ -galactosidase activity, senescence-associated heterochromatin foci, cell cycle regulation markers p16<sup>Ink4A</sup>, p53-p21<sup>Cip1/WAF1</sup> and p27<sup>Kip1</sup>, chromatin arranging protein high mobility group box 1 (HMBG1), DNA double strand break markers lamin-B1 and histone  $\gamma$ H2AX, immature nuclear membrane component prelamin A (progerin), and the molecules of SASP (Munoz-Espin and Serrano, 2014). However, none of these markers is specific for senescence, and no one-fits-all marker is available. Preferentially, a combination of 3 or more markers from these diverse categories is tested to warrant senescence. Cell type specific mechanisms and biomarkers of cellular senescence are detailed in this review (Inci et al., 2022).

Focusing on the vasculature, a recent study reported that cellular senescence and SASP contribute to aortic stiffening and endothelial dysfunction in aged mice (Clayton et al., 2023). Specifically for ECs, an increased expression of senescence biomarkers (p53, p16, p21) has been shown in elderly, which was associated with defective endothelial function (Rossman et al., 2017). Furthermore, senescent ECs show

reduced eNOS expression and activity, resulting in impaired NO bioavailability (Hayashi et al., 2008). In VSMCs, senescence promotes vascular calcification and inflammation (Lin et al., 2023). Thus, accumulation of senescent cells leads to several cellular consequences and ultimately to age-related diseases including CVDs (Bozaykut, 2019). Therefore, therapies targeting senescent cells, which are called senotherapeutics, are emerging as a promising approach for age-related pathologies. Senotherapeutics are mainly classified into two groups; senolytics that selectively eliminate the senescent cells by inducing cell death, and senomorphics that mainly avoid detrimental effects of SASP without affecting the total number of senescent cells (Kim and Kim, 2019).

#### 2.3.1. Senolytics

Senescent cells are commonly resistant to cell death by their significant expression of prosurvival proteins. However, preclinical studies have shown that inhibitors of these prosurvival proteins such as BCL-2 family inhibitors, p53 binding inhibitors, kinase inhibitors, and some natural compounds, are able to kill senescent cells (Niedernhofer and Robbins, 2018). For instance, preclinical studies using ABT737, which is a BCL-2 family inhibitor, showed senolytic potential by promoting apoptosis (Ovadya and Krizhanovsky, 2018). Navitoclax (ABT263), which is the orally bioavailable analogue of ABT737, was shown to affect human ECs and lung fibroblasts, and to remove senescent cells in the context of atherosclerosis (van Deursen, 2019). A very recent study demonstrated the potential of navitoclax to reduce cardiovascular ageing, since it was able to diminish aortic stiffness and to improve endothelial function in old mice (Clayton et al., 2023). Therefore, navitoclax might have the potential to address age-related CVDs (Childs et al., 2016). Although clinical trials for the senolytic use of navitoclax are lacking, its safety and efficacy has been confirmed in cancer patients (Cleary et al., 2014; Harrison et al., 2022). On the other hand, a well-studied senolytic therapy is the combination of dasatinib (D), a tyrosine kinase inhibitor, and quercetin (Q), a natural flavonoid that inhibits the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. D+Q combination was shown to reduce senescent cells, vasomotor dysfunction and aortic calcification in old mice (Roos et al., 2016). Another study demonstrated the effects of D+Q on cardiovascular ageing through improved cardiac function in old mice, as well as extended health span and reduced senescence markers in *Ercc1*<sup>-/-</sup> progeroid mice (Zhu et al., 2015). In addition, D+Q combination therapy decreased senescent cell load and ameliorated cardiovascular and physical function in progeroid mice (Inci et al., 2022). A first in-human trial evaluating D+Q treatment (NCT02874989) demonstrated positive effects on frailty and physical functions of idiopathic pulmonary fibrosis patients (Justice et al., 2019). Other clinical trials (phase I and/or phase II) evaluating the effects of D+Q, fisetin or UBX0101 (nutlin-3a) are currently ongoing in patients suffering from Alzheimer's disease, diabetic kidney disease, osteoarthritis or COVID-19 (Chaib et al., 2022).

#### 2.3.2. Senomorphics

Another strategy to therapeutically target senescent cells is to modulate the secretory phenotype of these cells (Fuhrmann-Stroissnigg et al., 2017). Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitor, that is used in patients to reduce cholesterol levels, also affects SASP status in senescent fibroblasts and cell cycle arrest in endothelial progenitor cells (Liu et al., 2015). Being extensively studied as a longevity drug, and, as a potential senomorphic, rapamycin, which is a mammalian target of rapamycin (mTOR) inhibitor, has demonstrated its ability to increase lifespan in mice (Harrison et al., 2009). Additionally, rapamycin has also been shown to reduce the harmful impact of oxidative stress on vascular contractility of mouse aortic rings, partly by preserving stem cell functionality and exerting anti-inflammatory effects (Gao et al., 2011). Moreover, a SIRT1 activator, resveratrol, inhibited both inflammation within the arterial wall and stiffening of arteries in nonhuman primates (Mattison et al., 2014).

Other studies have demonstrated several senomorphic candidates including mTOR inhibitors (Liu et al., 2015), antioxidants (Si and Liu, 2014), sirtuin activators (Hubbard and Sinclair, 2014), anti-inflammatory agents (Soto-Gamez and Demaria, 2017), autophagy activators (Nakamura and Yoshimori, 2018) and proteasome activators (Kim and Kim, 2019).

Currently, there are no clinical trials for the use of senotherapeutics in CVDs and vascular ageing, despite its significant impact on age-related health. The challenge lies in the limited tolerance for side effects in cardiovascular medicine, making some senolytics unsuitable. While senolytics affect overall senescence, questions remain about their mechanism of action and long-term effects. Nonetheless, both senolytic and senomorphic-based therapies hold promise to prevent or treat age-related vascular dysfunction, but further research is required.

## 2.4. Macroautophagy

Macroautophagy (hereafter referred to as autophagy) is a complex intracellular process that delivers cytoplasmic constituents for degradation into lysosomes. It occurs at basal levels in most tissues to allow constitutive turnover of cytosolic components, but is stimulated by environmental stress-related signals (e.g., nutrient deprivation, oxidative injury) to recycle nutrients and to generate energy for maintenance of cell viability in unfavourable conditions. Systemic or tissue-specific deletion of autophagy-related (*Atg*) genes in various organisms may lead to serious malformations and even death, supporting the general hypothesis that autophagy is an essential process that contributes to health and overall well-being. Along these lines, there is increasing interest in the role of autophagy in maintaining normal vessel wall biology and a growing suspicion that autophagic dysregulation may be a common pathway through which vascular ageing and associated pathologies develop (Nussenzweig et al., 2015). Preclinical data demonstrate that changes in lifestyle as well as nutritional factors may exert their known health benefits through the autophagy pathway. Unfortunately, autophagic activity declines with age due to impaired lysosomal function and reduced expression of several *Atg* genes that are pivotal for both autophagy initiation and activity, thereby contributing to the accumulation of damaged macromolecules and organelles during vascular ageing. To elucidate the tangible impact of defective autophagy on the vasculature and the development of age-related vascular pathologies, several groups created mouse models carrying a cell-type specific deletion of an essential autophagy gene (e.g., *Atg5*, *Atg7*). Defective autophagy in VSMCs, that were isolated from *Atg7<sup>F/F</sup> SM22 $\alpha$ -Cre<sup>+</sup>* mice, triggers stress-induced premature senescence (Grootaert et al., 2015). Moreover, autophagy defective VSMCs are characterized by an augmented migration potential, upregulation of inflammasome components and increased collagen synthesis. The inverse relationship between autophagy and senescence seems highly conceivable when they are considered as two cytoprotective pathways. When autophagy is impaired, senescence can be engaged as a back-up mechanism to protect the cell while, vice versa, autophagy may prevent cellular senescence by facilitating the removal of damaged organelles and promoting self-renewal. Besides the development of senescence, *Atg7<sup>F/F</sup> SM22 $\alpha$ -Cre<sup>+</sup>* VSMCs are highly resistant to oxidative stress-mediated cell death (Grootaert et al., 2015). This phenomenon is attributed to activation of the transcription factor NRF2 resulting in upregulation of several anti-oxidative enzymes. Possibly, the NRF2 pathway is activated in autophagy defective VSMCs as a protective back-up mechanism to maintain cell survival against oxidative insults. Recent evidence shows that autophagy is also involved in controlling contractile function and Ca<sup>2+</sup> homeostasis (De Munck et al., 2020; De Munck et al., 2022; Michiels et al., 2015). In addition, aorta segments of mice with a VSMC autophagy defect reveal higher arterial stiffness (De Munck et al., 2022). Passive aortic wall remodelling, rather than differences in VSMC tone, is responsible for these phenomena. Autophagy is also an essential process supporting EC function, and autophagy loss in ECs is linked with

vascular disease (Mameli et al., 2022). Indeed, impaired endothelial autophagy compromises shear stress-induced NO generation (Bharath et al., 2014), which is essential to establish vasodilation, and increases the permeability of the endothelium via a ROS-dependent mechanism (Patella et al., 2016).

Many detrimental effects related to arterial ageing are normalized by enhancing autophagy with specific drugs. Treatment of old mice with the autophagy-enhancing agent trehalose rescues NO-mediated endothelium-dependent dilation (EDD) by reducing oxidative stress, and normalizes inflammatory cytokine expression (LaRocca et al., 2012). In a similar way, administration of trehalose ameliorates vascular function and arterial stiffening in spontaneously hypertensive rats (McCarthy et al., 2019). Spermidine, a natural polyamine that promotes autophagy through inhibition of acetyltransferases (Pietrocola et al., 2015), may also be a promising nutraceutical treatment for arterial ageing. Supplementation of spermidine normalizes arterial stiffness, restores NO-mediated EDD and reduces markers of oxidative stress in old mice (LaRocca et al., 2013). Several other naturally occurring compounds or traditional medicines, including large-leaf yellow tea, curcumin, caffeine and resveratrol have beneficial effects by inducing autophagy in vascular cells and can act on arterial ageing. Although we should take into consideration that many of these compounds have multiple cellular targets beyond autophagy induction, that can contribute to their vascular anti-ageing effects.

ClinicalTrials.gov shows > 200 clinical trials related to autophagy. Many use lysosomotropic agents such as hydroxychloroquine to inhibit autophagy in the context of cancer, but other trials aim to induce autophagy in many different conditions using a variety of drugs including trehalose, spermidine or metformin. Inducing autophagy may become a game changer in the treatment of many pathologies, yet unequivocal validation of autophagy induction as a translatable therapeutic strategy remains very difficult. An important reason is the absence of reliable and selective compounds that pharmacologically induce autophagy in vivo. Rapamycin and its derivatives are the best-known autophagy inducers working via inhibition of mTOR and strongly induce autophagy in various model systems but were not developed for this purpose and consequently lack target specificity. Although dietary rapamycin supplementation reverses age-related vascular dysfunction and oxidative stress in mice (Lesniewski et al., 2017), chronic use of mTOR inhibitors results in off-target-effects such as inhibition of translation, cell growth and proliferation. Apart from target specificity, several other issues need to be solved before autophagy inducers can be clinically used to prevent vascular ageing. Given the chronic nature of arterial ageing, we would like to administer autophagy-inducing drugs over long time spans. However, little is known about the efficacy of drug-mediated autophagy induction following long-term treatment. Growing evidence suggests that intermittent and episodic induction of autophagy, rather than chronic upregulation, is more effective and applicable in the practice of promoting healthy ageing and longevity (Ulgherait et al., 2021). Indeed, continuous administration of the autophagy inducer everolimus induces tolerance and decreases autophagy in mice (Kurdi et al., 2016). Thus, episodic stimulation of autophagy can overcome tolerance and may also prevent the potential risk of exhausting the autophagy-lysosome machinery or inducing autophagy-associated cell death.

## 2.5. Extracellular matrix remodelling

The extracellular matrix (ECM) is a three-dimensional architectural network of macromolecules that determines the morphological and physical properties of tissues and organs and that governs cellular processes. Any modification in ECM composition and structure, called ECM remodelling, has a major impact on the morphology, mechanical and functional properties of the arterial wall (Wagenseil and Mecham, 2009). Elastin and collagens are the two major matrix components of the arterial wall. While elastic fibres, made of elastin and microfibrils,



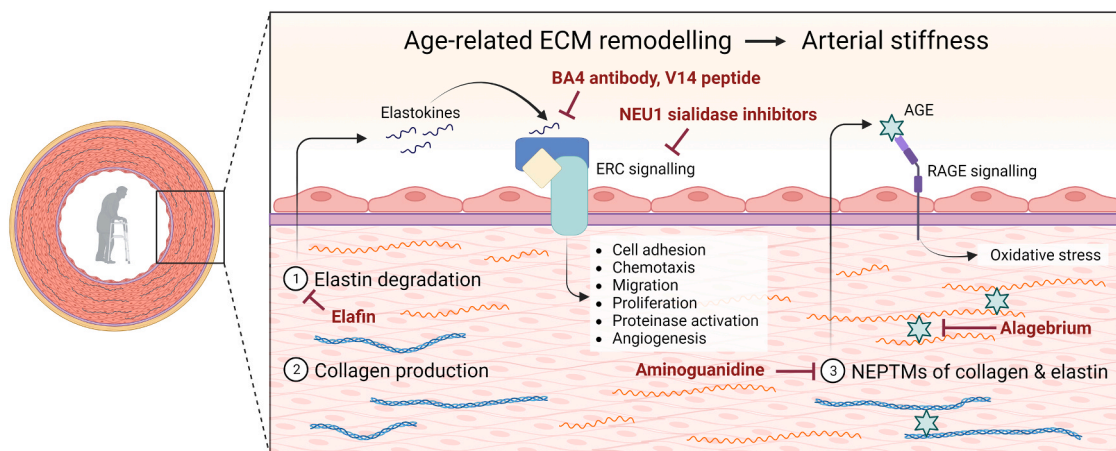
provide elasticity and resilience to the vessel, collagens, especially type I collagen, convey tensile strength and rigidity. Along with ageing, elastin and collagen are prone to several alterations and chemical reactions, especially due to their long-half-life.

A major hallmark of aged conduit arteries is fragmentation of elastic fibres (Duca et al., 2016). One important cause is the mechanical fracture of elastin lamellae (Fig. 2) due to fatigue failure caused by the repetitive stretches and relaxations experienced by the aorta over the human lifespan. These impairments or even ruptures lead to a decrease in elastic fibre function and to the transfer of mechanical stress to collagens, increasing stiffness and decreasing arterial distensibility (Greenwald, 2007; Hodis and Zamir, 2009). Elastin fragmentation is enhanced by the action of proteinases (elastases) and leads to the release of bioactive peptides (elastokines) that modulate a plethora of biological activities mainly through a membrane receptor called elastin receptor complex (ERC) (Tembely et al., 2022). This includes modulation of cell adhesion, chemotaxis, migration, proliferation, proteinase activation, angiogenesis, and apoptosis (Fig. 2). The ERC is a heterotrimeric receptor containing the elastin-binding protein (EBP), a protective protein/cathepsin A, and neuraminidase 1 (NEU1) as the catalytic subunit (Bennasroune et al., 2019).

ECM proteins also undergo chemical reactions such as non-enzymatic post-translational modifications (NEPTMs, Fig. 2), which correspond to the binding of metabolites to their functional groups (Jaisson and Gillery, 2010). These irreversible reactions are cumulative and especially affect long-lived proteins (Goris et al., 2016). The most important NEPTMs are glycation (binding of oses), and carbamylation (binding of isocyanic acid, a by-product of urea). Glycation generates oxidative reactions leading to the formation of complex products called advanced glycation end-products (AGEs) (Singh et al., 2001). NEPTMs cause alterations of structural and functional properties of ECM proteins and the irreversible accumulation of post-translational modification-derived products (PTMDPs) leads to vascular tissue disorganization (Goldin et al., 2006) (Fig. 2). Furthermore, PTMDPs can interact with cell receptors, such as AGEs with their major receptor RAGE, leading to increased oxidative stress (Fig. 2 and Section 2.1) (Stirban et al., 2014). Both carbamylation and glycation have been demonstrated to participate in vascular ageing and in long-term complications of chronic diseases including diabetes and chronic kidney disease (Machowska et al., 2016; Wang et al., 2007). For example, carbamylation of elastic fibres has been shown to be a molecular substratum for

aortic stiffness (Doue et al., 2021). Therefore, targeting elastin proteolysis and NEPTMs of ECM proteins may represent valuable therapeutic strategies to delay vascular complications associated with ageing.

Targeting elastin degradation using elastase inhibitors remains challenging, since several proteinases can degrade elastin. Matrix metalloproteinases (MMPs) have long been regarded as attractive targets and numerous synthetic MMP inhibitors have been designed and tested in animal models. To date, few MMP inhibitors demonstrated potential for clinical utility mainly due to their broad substrate spectra and off-target effects (Vandenbroucke and Libert, 2014). Cathepsins, mainly cathepsin S, have also been the target of pharmacological drugs (Figueiredo et al., 2015; Lai et al., 2020). Promising results in animal models have been reported with preserved elastic fibre integrity and reduced vascular complications. However, more research is required to determine whether these cathepsin S inhibitors can be pharmacologically effective in humans. Another strategy is based on the use of endogenous inhibitors of the neutrophil elastase. Preclinical observations have suggested that elafin could be a promising candidate (Alam et al., 2015; Zaidi et al., 2000). Another suitable but challenging way is restoring elastic fibre loss during ageing. Indeed, neosynthesis of functional elastic fibres is exceedingly tricky (Kielty et al., 2002). Striking results have been reported with the ATP-dependent  $K^+$  channel opener minoxidil. Chronic treatment for 3 months in aged mice was shown to preserve elastic lamellae integrity along with formation of newly synthesized elastic fibres and significant improvement of arterial biomechanical properties (Coquand-Gandit et al., 2017; Fhayli et al., 2019). Another promising option is counteracting the deleterious effects of the elastokines by blocking elastokines/ERC interaction by either a 14-mer synthetic peptide (V14 peptide) that encompasses the ligand binding domain on EBP (Blanchevoys et al., 2013) or a blocking antibody (BA4 antibody) (Wrenn et al., 1986). Chronic administration of BA4 antibody for 2 months in a murine model of Marfan syndrome was shown to rescue elastin fragmentation and to prevent aortic macrophage infiltration in the aorta (Guo et al., 2013). Finally, inhibition of NEU1 catalytic activity is also a promising alternative. This can be achieved by broad-spectrum sialidase inhibitors or more selective NEU1 inhibitors. Two of them, C9-BA-DANA and CG14601, have been recently evaluated in atherosclerosis-prone mice and were shown to significantly delay formation of fatty streaks in aortic roots (Demina et al., 2021). Finally, NEU1 has been shown to form dimers at the plasma membrane (Maurice et al., 2016) and blocking NEU1 dimerization by interfering peptides



**Fig. 2.** Impact of ageing on extracellular matrix remodelling and potential therapeutic strategies. Ageing is associated with strong modifications of vascular ECM, with elastin degradation (1) and increased production of collagen (2) being the two major hallmarks. Elastin degradation leads to the production of elastokines that bind to ERC leading to modulation of a plethora of cellular responses. In addition, NEPTMs (mainly glycation and carbamylation) of long half-life ECM proteins, such as collagen and elastin, are increased during ageing and induce the formation of AGEs and carbamylation-derived products (3). AGEs can bind to RAGE, leading to increased oxidative stress. All these events also contribute to decreased compliance and increased stiffness in aged conduit arteries. The main pharmacological blocking strategies are depicted in red. AGE: advanced glycation end-product; ECM: extracellular matrix; ERC: elastin receptor complex; NEPTMs: non-enzymatic post-translational modifications; NEU1: neuraminidase 1; RAGE: receptor for advanced glycation end-products. Created with BioRender.com.

was shown to decrease membrane NEU1 catalytic activity (Albrecht et al., 2020). Although the therapeutic potential of these interfering peptides remains to be demonstrated in animal models, they should open new avenues for selective NEU1 inhibition.

Most of the experiments devoted to counteracting the deleterious effects of NEPTMs have focused on limiting their formation. The major strategy has been based on competing agents, like aminoguanidine, a glycation inhibitor, which proved to be efficient in reducing arterial stiffness in rats assessed by measurement of aortic input impedance (Chang et al., 2006) and vasodilatory response to acetylcholine (Li et al., 1996). However, these promises were not confirmed by clinical trials, mainly because of safety concerns (Bolton et al., 2004). Carbamylation has been targeted by using amino acids as scavengers, which reduce protein carbamylation in uremic patients (Kalim et al., 2015). Additionally, compounds able to break AGE crosslinks ("AGE breakers") have been developed, like alagebrium. Despite some positive results obtained in patients with CVDs, this molecule is not commercially available as a drug, mainly because of financial and licensing difficulties (Toprak and Yigitaslan, 2019), but also because disparate effects have been reported (Oudegeest-Sander et al., 2013).

## 2.6. Calcification

Vascular calcification in the media layer of large and middle-sized arteries, mainly in the form of hydroxyapatite crystals, is a hallmark in common multifactorial disorders (e.g., chronic kidney disease, CVDs) and ageing. Therefore, anti-calcifying mechanisms may serve as novel therapeutic targets. One of the potent endogenous circulatory inhibitors of vascular calcification is plasma inorganic pyrophosphate (PPI), generated by the concerted action of three enzymes from nucleotide triphosphates, mainly ATP. Approximately, 60–70% of circulatory ATP is released from the liver, by cellular transport via the ATP Binding Cassette (ABC) transporter ABCC6 (Jansen et al., 2014). The majority of the remaining 30–40% is provided by transport via the ubiquitously expressed ankylosis homologue (ANKH) (Szeri et al., 2022). ATP is hydrolysed to PPI and AMP by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) (Rutsch et al., 2003), the sole enzyme able to generate extracellular PPI, thereby inhibiting the growth of calcification nidi. Plasma PPI is eventually converted to the pro-calcifying inorganic phosphate (Pi) by membrane bound and circulatory alkaline phosphatases, mainly tissue nonspecific alkaline phosphatase (TNAP) and placental alkaline phosphatase during pregnancy (Veiga-Lopez et al., 2020). AMP, generated from ATP by ENPP1 and/or via hydrolysis by CD39, is further degraded to adenosine and Pi by CD73/NT5E. Adenosine inhibits the expression of TNAP and is a potential novel pharmacological target for vascular calcification (Goettsch et al., 2022). On another note, the DNA damage response, induced by oxidative stress (see Section 2.1), is also linked to vascular medial calcification via the accumulation of its by-product poly [ADP-ribose] (PAR), which initiates biomineralization by concentrating calcium to form nucleation nidi (Müller et al., 2019). In an accelerated ageing mouse model, the Hutchinson-Gilford progeria syndrome model, excessive vascular calcification presents with increased CD39 and TNAP activity, accompanied by ~90% reduction in plasma ATP and PPI levels (Villa-Bellosta, 2019). This underlines the relationship between ageing and disturbances in the Pi/PPI equilibrium. To further understand the mechanisms of vascular calcification and how it can be targeted, lessons can be learned from rare genetic diseases such as pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI).

Several promising approaches to improve vascular calcification and associated age-related diseases, aim to restore PPI/Pi balance. Oral PPI supplementation (NCT04868578) and the TNAP inhibitor lansoprazole (NCT04660461) are investigated in PXE, while the soluble recombinant ENPP1 enzyme INZ-701 is in clinical trial for GACI (NCT04686175). Furthermore, myo-inositol-hexaphosphate (SNF-472), a small molecule mimicking the effect of PPI, is in phase 2/3 trials for common diseases (e.

g., Calciphylaxis) (Sinha et al., 2022). Non-hydrolysable stable PPI analogues, bisphosphonates (e.g., etidronate), used in osteoporosis, have been successfully applied in GACI (Rutsch et al., 2008) and are currently the first line of treatment. Etidronate is currently also investigated in PXE (Bartstra et al., 2020; Kranenburg et al., 2018).

Besides restoring the PPI/Pi balance, also the release of ATP can be modulated by targeting ABCC6. The pharmacochaperone 4-phenylbutyrate (4-PBA), approved for urea cycle disorders, has a potential to correct intracellular localization of mislocalized ABCC6 mutants (Le Saux et al., 2011). The oral administration of 4-PBA has already been used effectively for the treatment of other ABC transporter-related rare diseases (Gonzales et al., 2015) and could lead to allele specific therapy in PXE (Le Saux et al., 2011; Pomozi et al., 2017).

In addition, oxidative stress mediated calcification can be reduced by inhibiting poly [ADP-ribose] polymerase 1 (PARP1), the predominant PAR-producing enzyme. Minocycline, a second-generation, semi-synthetic tetracycline, currently in use against acne, is an inhibitor of PARP1 and was shown to effectively reduce ectopic mineralization in mouse and zebrafish PXE models (Bouderlique et al., 2022; Huang et al., 2022; Nollet et al., 2022).

Overall, the current studies have focused on targeting vascular calcification in rare genetic diseases. Thus, further research in vascular ageing models is required to validate the use of these treatment strategies in the context of CVD and other age-related diseases.

## 2.7. Gasotransmitters

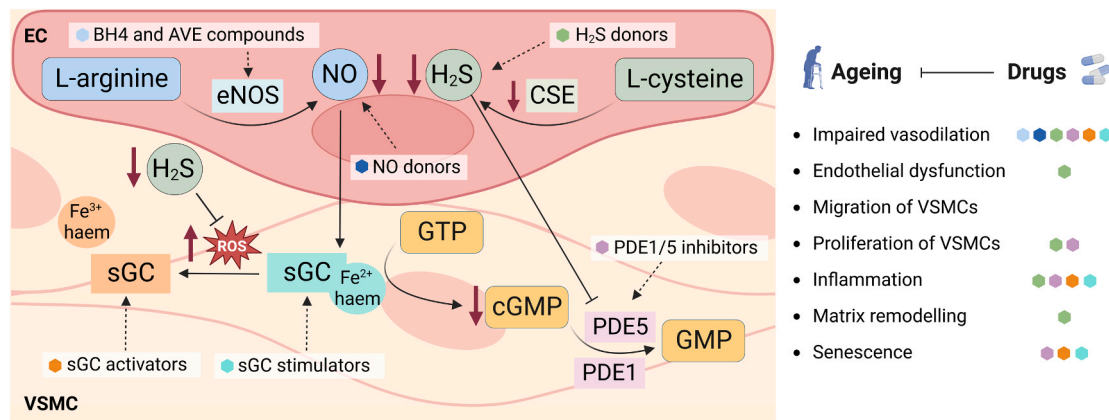
Gasotransmitters are a class of small, gaseous molecules that act as signalling molecules in various physiological processes in the body. In the context of vascular ageing, particularly NO and hydrogen sulphide (H<sub>2</sub>S), play significant roles.

### 2.7.1. Nitric oxide - cGMP

The NO – cGMP signalling cascade is one of the most important vasodilator mechanisms and is consistently found to be disturbed in ageing (Golshiri et al., 2019). The signalling cascade starts with Ca<sup>2+</sup> increase in ECs, which activates endothelial NO synthase (eNOS) to release NO from L-arginine in a reaction involving nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), enzyme-linked haem and tetrahydrobiopterin (BH4) as cofactors (Golshiri et al., 2019). NO transfers to VSMCs where it binds soluble guanylyl cyclase (sGC) at its ferrous haem-group, thus increasing the rate of cGMP production by sGC. The resulting increase of cGMP activates protein kinase G (PKG) and subsequently vasodilator-stimulated phosphoprotein (VASP), resulting in relaxation (Schafer et al., 2003). Furthermore, cGMP decreases RhoA/Rho kinase activation, thus inhibiting constriction (Marinko et al., 2015; Schafer et al., 2003). Besides acting as a vasodilator via cGMP production, NO inhibits migration and proliferation of VSMCs, neointima formation and ECM alterations, which are involved in vascular remodelling (Fernández-Varo et al., 2003). Also, cGMP signalling has been implicated in vascular remodelling as an anti-fibrotic and anti-hypertrophic messenger system (Ataei Ataabadi et al., 2020). Upon ageing, NO release, activity and expression of eNOS, substrate/cofactor availability is decreased and NO inactivation is increased (Pourbagher-Shahri et al., 2021), all leading to decreased NO availability and vascular dysfunction. A recent study reported that a pronounced and early CVD phenotype is present in eNOS knockout mice (De Moudt et al., 2022). In elderly, early dysfunction of the NO system and subsequent oxidative stress cause age-related reduction in EDD. Therefore, NO and cGMP modulation could be versatile strategies to attenuate vascular ageing (Fig. 3).

Because dysfunctional NO is a major contributing factor to age-related vascular dysfunction, treatment strategies aiming at increasing the bioavailability of NO might be promising. The NO pathway can be pharmacologically modulated by increasing expression and/or activity





**Fig. 3.** Pharmacological targeting of gasotransmitters in vascular ageing. Upon ageing, nitric oxide (NO) levels are decreased, leading to reduced soluble guanylyl cyclase (sGC) activation and subsequent cyclic guanosine monophosphate (cGMP) synthesis. Furthermore, due to an age-related increase in reactive oxygen species (ROS), the Fe<sup>2+</sup> haem group of sGC will be oxidized, resulting in a haem-free form of sGC which is insensitive to activation by NO. Lower levels of hydrogen sulphide (H<sub>2</sub>S) further contribute to vascular dysfunction by sustaining oxidative stress and promoting phosphodiesterase 5 (PDE5) activity, leading to the conversion of cGMP to GMP and thus inhibiting cGMP-related signalling. Overall, this will lead to impaired vasodilation, endothelial dysfunction, migration and proliferation of vascular smooth muscle cells (VSMCs), inflammation, matrix remodelling and cellular senescence. To overcome these age-related changes in vascular function, several classes of compounds were developed. AVE compounds can induce endothelial nitric oxide synthase (eNOS) production and chronic tetrahydrobiopterin (BH4) supplementation might be able to re-couple eNOS, thereby increasing NO levels. Also, NO donors can potentially be implemented. Furthermore, slow release H<sub>2</sub>S donors, and sGC stimulators and activators have shown potential. In addition, inhibitors of PDE1 and PDE5 appear to be promising, mainly due to PDE1 inhibition. Red arrows indicate age-related changes. CSE: cystathionine  $\gamma$ -lyase; EC: endothelial cell; GTP: guanosine triphosphate. Created with BioRender.com.

of eNOS, preventing eNOS uncoupling, diminishing scavenging of NO and restoring substrate/cofactor availability (Fig. 3). AVE compounds, such as AVE 9488, are small molecules, found by high throughput screening, that enhance eNOS transcription, thus augmenting eNOS protein (Wohlfart et al., 2008). These compounds have been shown to correct endothelium-dependent relaxation in naturally aged mice but have not yet been tested in relation to other features of vascular ageing.

Oestrogen is also known to activate eNOS via the PI3K/AKT pathway (Simoncini et al., 2002) and to increase NO availability. Furthermore, it can attenuate the production of the eNOS endogenous inhibitor, asymmetric dimethylarginine (ADMA) (Monsalve et al., 2007), and decreases O<sub>2</sub> concentrations (Wassmann et al., 2001). Oestrogen replacement was shown to improve NO-mediated dilation in aged and ovariectomized rats (LeBlanc et al., 2009). On the other hand, due to ageing-associated methylation of the oestrogen receptor gene, it should be emphasized that oestrogen might have little ability to overcome the effect of ageing on vascular function in older women (Herrington et al., 2001). It has also been reported that oestrogens can adversely affect triglyceride levels and have pro-thrombotic properties which could increase CVD risk and mortality. However, the 'Women's Health Initiative Estrogen Plus Progestin' and 'Estrogen-Alone' Trials showed that oestrogen treatment was not associated with an increased risk of all-cause or cardiovascular mortality during a cumulative follow-up of 18 years (Manson et al., 2017).

Deficiency in the cofactor BH4 has been implicated in age-related vascular dysfunction (Kuzkaya et al., 2003) and thus might be a potential target. However, contrasting results are described regarding the acute or chronic effect of BH4 supplementation on age-related NO impairment. Whereas acute BH4 supplementation was reported to have no effect on FMD and uncoupling of eNOS (Bisconti et al., 2022), chronic supplementation has been shown to re-couple NOS and improve vascular function in animal and clinical CVD studies (Bendall et al., 2014). However, other studies investigating BH4 supplementation show no improvement in FMD, both after acute (Gates et al., 2007) and long-term treatment (Huang et al., 2020).

Sirtuins play a critical role in modulating arterial remodelling during vascular ageing. SIRT1 and eNOS potentiate each other through positive feedback mechanisms (Man et al., 2019), in normal but not in pathological conditions. Under normal conditions SIRT1 activates eNOS due

to its deacetylase activity acting on the lysines 496 and 506 of eNOS, which play a modulatory role in eNOS activity (Mattagajasingh et al., 2007). Conversely, adenoviral vector-mediated suppression of SIRT1 decreases eNOS activity. Caloric restriction and resveratrol, two important anti-ageing, SIRT1 activating interventions, mimic the eNOS deacetylation effect (Mattagajasingh et al., 2007). Caloric restriction also increases eNOS expression, but it is unknown if this is SIRT1-deacetylation-dependent. In contrast, in senescent ECs, uncoupling of eNOS decreases the expression of SIRT1 (Lemarie et al., 2011). Also, in young vs. aged mice and in humans, SIRT1 expression is positively correlated with endothelium-dependent vasorelaxation (Donato et al., 2011). Thus, the coupling of SIRT1 to eNOS increase and activation is an important target in interventions in vascular ageing. The pathway can be approached by small molecule drugs. SRT1720, an activator of SIRT1, elevates eNOS expression and protein levels (Gano et al., 2014; Li et al., 2016). Also naturally occurring molecules have been identified. Resveratrol, a compound found in grapes, acts on vascular ageing by activating SIRT1, increasing NO bioavailability by decreasing ROS, and decreasing iNOS expression (Labinskyy et al., 2006). We should note that resveratrol is a multi-target molecule and other cellular mechanisms and signalling pathways are likely involved in its protective effects in vascular ageing. Another natural substance, anthocyanin-rich mulberry extract, has been shown to improve eNOS function through SIRT1 in ageing rats (Lee et al., 2020).

Another strategy might be to supply NO through nitrite (NO<sub>2</sub>) and nitrate (NO<sub>3</sub>). Inorganic nitrite supplementation by using oral sodium nitrite has been tested in old mice and humans and was found to improve endothelial function, yet without affecting blood pressure (Rossman et al., 2021). Dietary interventions with beetroot juice are another proposed option. It contains several active phytochemicals as well as inorganic nitrate, which is converted to nitrite by bacteria in the gastro-intestinal tract (Mirmiran et al., 2020). In short lasting studies, beetroot juice has shown to lower blood pressure and to enhance vasodilation (Lundberg et al., 2008; Mirmiran et al., 2020). In addition, a phase 2 study in hypertensive subjects (aged 15 – 85 years) showed promising results with beetroot juice, lowering blood pressure and vascular stiffness (Kapil et al., 2015). However, this form of inorganic nitrate supply has not become part of the standard clinical armament yet. In contrast, organic nitrates are used clinically as a vasodilating

treatment. However, their chronic use is hindered by nitrate tolerance and induction of endothelial dysfunction (Sage et al., 2000; Schulz et al., 2002). Consequently, their use is momentarily limited to angina pectoris and acute hypertensive emergencies.

Next to modulating NO bioavailability, also cGMP is a promising target. The production of cGMP can be augmented by stimulating sGC. In that regard, nitrates are given clinically to supply NO and thus activate sGC. As an alternative, sGC stimulators and activators have been designed to respectively increase activity of NO-bound or oxidation-inactivated sGC (Fig. 3). With respect to hypertension, Adempas® (riociguat) has been clinically registered as an sGC stimulator for the treatment of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension. There is ample evidence that these compounds increase cGMP and vasodilation, attenuate high blood pressure, and display a protective effect in patients with acute heart failure (Armstrong et al., 2020; Ataei Ataabadi et al., 2020). Recently, it was shown in the *Ercc1*<sup>Δ/Δ</sup> mouse, a model of accelerated ageing that mimics a wide array of human-like non-atherosclerotic vascular ageing features (Durik et al., 2012), that chronic treatment with sGC activator BAY 58–6544 improved cGMP-driven arterial vasodilation and microvascular reactive hyperaemia, even improving longevity in these short-lived mice (Ataei Ataabadi et al., 2022). Being safe, clinically approved medicines for chronic treatment, sGC stimulators and activators are promising for treatment of vascular ageing.

The intracellular levels of cGMP are tightly controlled by phosphodiesterases (PDEs) (Ataei Ataabadi et al., 2020). For vascular ageing, PDE1 and PDE5 have been identified as important cGMP-metabolizing subtypes in the vascular system (Ataei Ataabadi et al., 2020) (Fig. 3). PDE5 inhibitors (sildenafil, vardenafil, and tadalafil) are prescribed clinically for erectile dysfunction, a specific form of vascular ageing (Terentes-Printzios et al., 2022). Recently, it was shown that the dual PDE1/5 inhibitor sildenafil, which acts preferentially on PDE5, has modest effects on cGMP-mediated vasodilation in aged vasculature of *Ercc1*<sup>Δ/Δ</sup> mice. Interestingly, PDE1 seems to become dominant over PDE5 in regulating vasodilation to cGMP when arteries age (Golshiri et al., 2020). Indeed, chronic treatment with the specific PDE1 inhibitor lenrispodun had a stronger effect on ageing-related aortic and microvascular vasodilator dysfunction. Moreover, lenrispodun treatment attenuated vascular inflammation, and reduced senescence markers p16 and p21 in *Ercc1*<sup>Δ/Δ</sup> mouse aortic tissue and in human cultured VSMCs (Golshiri et al., 2021a; Golshiri et al., 2021b). Since PDE1 metabolizes both cGMP and cAMP, it is not certain if the effects on vascular ageing are exclusively through cGMP. Lenrispodun was shown to be safe during Phase 2 clinical studies in patients with heart failure ([www.intracellulartherapies.com](http://www.intracellulartherapies.com)). Nevertheless, the efficacy of PDE1 inhibitors in reduction of hypertrophy, vascular permeability, and arterial stiffness still needs to be tested.

### 2.7.2. Hydrogen sulphide

H<sub>2</sub>S, previously known as a poisonous gas, is the most recently recognized gasotransmitter, next to NO and carbon monoxide (Wang, 2002). H<sub>2</sub>S is endogenously generated by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and the pyridoxal 5-phosphate (PLP)-dependent cysteine aminotransferase (CAT) - 3-mercaptopyruvate sulphur transferase (MST) axis. CSE and CBS use L-cysteine as a substrate and PLP as a cofactor.

H<sub>2</sub>S levels are reported to decrease upon ageing and in age-related diseases (Testai et al., 2020). H<sub>2</sub>S levels were lower in heart, liver and kidney of D-galactose induced aged mice (Wu et al., 2017) as well as in plasma and aorta of atherosclerotic *ApoE*<sup>-/-</sup> mice (Wang et al., 2009b). Furthermore, in patients with atherosclerosis and coronary artery disease H<sub>2</sub>S levels are significantly decreased (Jiang et al., 2005). This might be caused by deficiency of CSE, the major H<sub>2</sub>S producing enzyme in the vascular system. CSE expression is reduced in human aortic ECs stimulated by oxidized low-density lipoprotein (oxLDL) as well as in the aorta of *ApoE*<sup>-/-</sup> mice (Leucker et al., 2017). A deficiency in CSE is known

to impair EDD, increase blood pressure (Mani et al., 2013), and expression of vascular adhesion molecules, which leads to aortic intimal proliferation and consequently development of early atherosclerosis in mice (Mani et al., 2013).

Due to the lower H<sub>2</sub>S availability as we age, its vasoprotective effects diminish. Persulphidation (or S-sulphydration) of target proteins is considered one of the main protective mechanisms of H<sub>2</sub>S in vascular ageing. H<sub>2</sub>S can directly interact with and activate NRF2 via S-sulphydration (Corsello et al., 2018), and protect against cellular senescence (Hourihan et al., 2013). Another mechanism by which H<sub>2</sub>S can inhibit senescence is via increasing deacetylase activity of SIRT1 and SIRT3 (Du et al., 2019a; Liu et al., 2021). Furthermore, H<sub>2</sub>S has vasorelaxant effects in several vascular beds (Hosoki et al., 1997) proposedly by activation of K<sub>ATP</sub> potassium channels (Zhao et al., 2001), transient receptor potential channel V4 and A1 (TRPV4, TRPA1) (Naik et al., 2016; Pozsgai et al., 2012), endothelial calcium activated potassium channels, and inhibition of PDE5 (Bucci et al., 2010; Coletta et al., 2012) and phospholipase A<sub>2</sub> (d'Emmanuele di Villa Bianca et al., 2011; di Villa Bianca et al., 2010). H<sub>2</sub>S may also have additional vascular protective effects through its anti-fibrotic, anti-apoptotic, anti-inflammatory and antioxidant properties (Wang, 2002).

Considering the many beneficial effects of H<sub>2</sub>S in the vascular system, it has become a promising therapeutic target to tackle vascular ageing and related diseases. H<sub>2</sub>S releasing donors have been considered as the first approach to restore declined H<sub>2</sub>S levels (Fig. 3). Aqueous solutions of sulphur salts, such as sodium hydrogen sulphide (NaHS), and sodium sulphide, provide direct and rapid release of free H<sub>2</sub>S and are therefore most frequently used in research. NaHS treatment reduces VSMC proliferation, collagen fibres, Ang II levels and oxidative stress (Qiao et al., 2014; Yetik-Anacak et al., 2016). Unfortunately, in vivo administration of sulphur salts causes a rapid and high release of H<sub>2</sub>S, followed by a steep decline. This pharmacokinetic profile, with very high peak plasma concentrations, may cause toxic effects and limits the clinical usage of H<sub>2</sub>S donors (Li et al., 2018). Thus, other H<sub>2</sub>S-targeted small molecules, where H<sub>2</sub>S release is controlled by endogenous H<sub>2</sub>S formation or by a slow-release formula, may have more potential. One of these slow-release donors, GYY4137, causes relaxation, by opening ATP-dependent potassium channels (Li et al., 2008), protects ECs against hydrogen peroxide-induced oxidative stress and cell death (Qabazard et al., 2013), inhibits vascular inflammation and oxidative stress (Liu et al., 2013), thereby improving endothelial function in *ApoE*<sup>-/-</sup> mice (Liu et al., 2013). Another slow release H<sub>2</sub>S donor, triphenylphosphonium-derived dithiolethion (AP39), has been reported to significantly reduce systolic blood pressure, heart rate and arterial stiffness in hypertensive rats (Tomasova et al., 2015). Oral administration of SG-1002, a synthetic H<sub>2</sub>S prodrug containing > 90% sulphur, results in a more sustained and consistent increase in H<sub>2</sub>S levels. SG-1002 was tested in a phase I clinical study (ID: NCT01989208) and found safe and effective to normalize H<sub>2</sub>S and NO levels in patients with heart failure. In addition, the ACE inhibitor zofenopril is a prodrug and quickly hydrolyses to a sulfhydryl-containing active metabolite, zofenoprilat. Zofenoprilat normalizes vascular response to acetylcholine in spontaneously hypertensive mice based on H<sub>2</sub>S release, which was independent from ACE inhibition (Bucci et al., 2014).

Also, stimulating H<sub>2</sub>S release might be a therapeutic option. Resveratrol is known to induce endogenous H<sub>2</sub>S formation, although the mechanism is still unknown. Interestingly, resveratrol causes aortic relaxation in a H<sub>2</sub>S-dependent manner (Yetik-Anacak et al., 2016). Furthermore, synthetic analogues of resveratrol have recently been shown to promote H<sub>2</sub>S production and aortic vasorelaxation in mice (Ozbek et al., 2023).

## 3. Approved drugs with off-label effects on vascular ageing

Several approved drugs represent potential strategies for maintaining or restoring healthy vascular ageing, including antihypertensive

agents, statins, mTOR inhibitors, AMP-activated protein kinase (AMPK) activators, anti-inflammatory cytokine therapies, PPAR $\gamma$  activators, and anti-fibrotic drugs (Nowak et al., 2018). These drugs modulate functional or structural components of arterial stiffness.

Hypertension and arterial stiffening are associated with unhealthy vascular ageing (Nowak et al., 2018). Antihypertensive drugs improve mean arterial blood pressure, endothelial function and vessel tone, and reduce structural remodelling (Jani and Rajkumar, 2006). Accordingly, they improve carotid-femoral PWV, augmentation index, and systemic arterial compliance over and above blood pressure reduction in long-term studies.  $\beta$ -blockers are less useful, because slowing the heart rate may increase pulse pressure (Nowak et al., 2018). The best evidence is presented for ACE inhibitors and ARBs (Nowak et al., 2018). In rodents, inhibition of the RAAS system has marked anti-ageing effects, extending life span and reversing age-related changes in the vasculature (Benigni et al., 2009) (also see Section 2.2). The effect of ACE inhibitors on arterial ageing is at least partially independent of blood pressure reduction (Shahin et al., 2012). ACE inhibitors reduce oxidative stress and increase NO bioavailability by increasing bradykinin in the vessel wall. Moreover, both ACE inhibitors and ARBs reduce collagen deposition, leading to improved arterial compliance and delayed arterial ageing (Neves et al., 2018). Aldosterone receptor antagonists can reduce arterial stiffness by increasing the elastin-collagen ratio (Lunder et al., 2021). Thus, pharmacological inhibition of RAAS reduces arterial stiffness in old animals and elderly humans independent of changes in blood pressure (Hayashi et al., 2006; Ungvari et al., 2018).

In addition to cholesterol lowering, statins upregulate the eNOS/NO pathway, reduce inflammation and stimulate NRF2, thereby lowering oxidative stress. Accordingly, they favourably act on endothelial function, reduce arterial stiffness and major cardiovascular events, especially in the ageing population (Liao and Laufs, 2005). Moreover, statins can slightly activate longevity genes such as sirtuins, AMPK and klotho (Lunder et al., 2021; Zhang et al., 2013). They decrease carotid-femoral PWV without altering systolic blood pressure (Jani and Rajkumar, 2006). Together with antihypertensive drugs, statins should be considered to maintain or restore healthy vascular ageing (Nowak et al., 2018).

With advancing age, nutrient sensing pathways, including mTOR, AMPK, and sirtuins, become dysregulated (Lopez-Otin et al., 2013). Interventions targeting these pathways may help maintain or restore healthy vascular ageing. mTOR inhibition has protective, anti-ageing effects on vessels, delaying EC senescence (Wang et al., 2009a) and promoting endothelium-mediated, NO-dependent vasodilation (Parlar et al., 2010; Ungvari et al., 2018). The mTOR inhibitor rapamycin improves endothelial function by increasing NO bioavailability and reducing oxidative stress in mice (Lesniewski et al., 2017; Martínez-Cisuelo et al., 2016). Moreover, it reduces inflammatory processes in the arterial wall, particularly through downregulation of NF- $\kappa$ B-mediated processes (Lunder et al., 2021). Rapamycin significantly reduces PWV in aged mice without altering blood pressure, activates arterial AMPK and thus may modulate the functional regulation of arterial stiffness. mTOR inhibition regulates the phenotypic switch of VSMCs (Ha et al., 2015) and decreases collagen and AGEs in the aorta of aged mice, indicating reduced cross-linking of collagen by AGEs (Lesniewski et al., 2017). However, rapamycin and its analogues (rapalogs) have important adverse effects, which limit their clinical use in the context of healthy vascular ageing (Nowak et al., 2018).

Several antidiabetic drugs have potential to maintain or restore healthy vascular ageing. The AMPK activator metformin can reduce carotid-femoral PWV in women with polycystic ovary syndrome (Agarwal et al., 2010). By activating AMPK in the endothelium, metformin promotes the activation of eNOS, inhibits NF- $\kappa$ B signalling, and reduces inflammation. Moreover, it reduces ROS formation via mTOR inhibition, leading to a reduction in superoxide that could cause DNA damage (Lunder et al., 2021; Valencia et al., 2017). Metformin-induced weight loss may also improve arterial stiffness. The clinical trial TAME (Targeting Ageing With Metformin) aims to determine whether

metformin can delay the onset of age-related diseases in humans (Kulkarni et al., 2020). The PPAR $\gamma$  agonist pioglitazone reduces PWV in diabetic patients without changing blood pressure and reduces circulating markers of inflammation (Ryan et al., 2007). However, the effects of these agents on carotid-femoral PWV in age-related arterial stiffening are not known (Nowak et al., 2018). The dipeptidyl peptidase-4 (DPP-4) inhibitor vildagliptin reduces vascular stiffness by increasing NO synthesis, thereby improving vascular relaxation and decreasing blood pressure (Wicinski et al., 2020). Therefore, vildagliptin may have a protective role in vascular ageing. In addition, sodium-glucose cotransporter-2 (SGLT-2) inhibitors increase NO bioavailability (Chilton et al., 2015; de Oliveira et al., 2022; Mancina et al., 2016; Shigiyama et al., 2017) and restore EDD by decreasing ROS production (Uthman et al., 2019). SGLT-2 inhibitors can decrease central systolic blood pressure, central pulse pressure, PWV and augmentation index in patients with diabetes, all parameters of arterial stiffness (Lunder et al., 2021). However, contradictory findings have been reported, showing no effect of dapagliflozin treatment on PWV (Karalliedde et al., 2022). The effects of SGLT-2 inhibitors on the expression of longevity genes have not yet been studied in detail. Possibly, SGLT-2 inhibitors could activate the SIRT1 and/or AMPK signalling pathways and suppress the AKT/mTOR signalling pathway (Packer, 2020). Glucagon like peptide-1 (GLP-1) receptor agonists improve endothelial dysfunction, inflammatory markers, oxidative stress and vascular activation in patients with obesity and pre-diabetes (Saraiva and Sposito, 2014). They decrease systolic and diastolic blood pressure and reduce PWV in patients with diabetes (Lambadiari et al., 2018). GLP-1 receptor stimulation leads to cAMP generation and activation of downstream pathways, including AMPK (Lunder et al., 2021). The combination of metformin and a GLP-1 receptor agonist or an SGLT-2 inhibitor (Neutel et al., 2023) could be of interest for the treatment of arterial ageing (Lunder et al., 2021). In particular GLP-1 receptor agonists such as semaglutide are interesting in this context, whose beneficial effects on the cardiovascular system are becoming increasingly evident, including in non-diabetics (Lenharo, 2023). In addition, GLP-1/GIP agonists, such as terzepatide, and GLP-1/GIP/glucagon agonists, such as retatrutide, are also promising, but further research is needed.

Presumably, anti-inflammatory cytokine therapies can reduce arterial stiffness via anti-inflammatory effects (Angel et al., 2010). However, the potential side effects of anti-inflammatory cytokine therapies limit their use in healthy ageing populations (Nowak et al., 2018).

Some drugs specifically target structural components of arterial stiffness. The anti-fibrotic agent pirfenidone is used clinically to treat idiopathic pulmonary fibrosis. It inhibits TGF- $\beta$ , TNF- $\alpha$  and other growth factors, and acts on matrix formation (Nowak et al., 2018). In rodents, pirfenidone reduces cardiac stiffness without altering blood pressure (Miric et al., 2001) and could therefore be promising for reducing age-related aortic stiffness.

#### 4. The effects of dietary supplements on vascular ageing

To minimize vascular damage caused by ageing, one of the best suggested methods is controlling the diet, especially the level of calorie intake and macronutrient composition. Calorie restriction is the process of limiting energy intake without causing malnutrition. It is reported to decrease inflammation and oxidative stress (Flanagan et al., 2020), thereby delaying vascular ageing and increasing lifespan (Weiss and Fontana, 2011). The ratio between different macronutrients is important as well. When protein to carbohydrate ratio is reduced, an increase in lifespan up to 30% is observed in mice (Solon-Biet et al., 2014). Dietary supplements have also drawn quite some attention, since it is significantly less effortless to implement in a daily routine, compared to restricting calorie intake or regulating macronutrient distribution. Therefore, we focus below on the vascular anti-ageing effects of the most common dietary supplements that have been investigated in clinical trials in the context of CVD.



#### 4.1. Vitamin B3, C, E and D

Vitamin B3 is a precursor for nicotinamide adenine dinucleotide (NAD)<sup>+</sup> and accumulating evidence suggests that decreased NAD<sup>+</sup> availability has been causally linked to multiple disease conditions, including ageing (Covarrubias et al., 2021). The mechanisms leading to age-related NAD<sup>+</sup> decline are likely attributed to altered NAD<sup>+</sup> synthesis, increased NAD<sup>+</sup> consumption, or both (Schultz and Sinclair, 2016). In this regard, the expression of nicotinamide phosphoribosyltransferase (NAMPT), which catalyses the rate-limiting step in the biosynthesis of NAD<sup>+</sup>, is downregulated in ageing (Yoshino et al., 2011). Increased NAD<sup>+</sup> consumption is caused by age-mediated induction of PARP1 (Pacher et al., 2002), and the NADase CD38 (Camacho-Pereira et al., 2016; Tarrago et al., 2018). Endothelial dysfunction in the aged vasculature has been, at least in part, linked to NAD<sup>+</sup> depletion (Csizsar et al., 2019). As ECs express the enzymes involved in NAD<sup>+</sup> biosynthesis, it could be suggested that endothelial NAD<sup>+</sup> may be positively influenced by the administration of NAD<sup>+</sup> precursors or be sensitive to other NAD<sup>+</sup>-increasing approaches to rescue EC dysfunction. Nicotinamide (NAM) and their nicotinamide derivatives, such as nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), are common forms of vitamin B3 and physiological sources of NAD<sup>+</sup> (Bogan and Brenner, 2008). NMN supplementation to elderly mice has been shown to improve EC function and blood flow, which was SIRT1-dependent (Das et al., 2019). Likewise, increasing endothelial NAD<sup>+</sup> content may be beneficial in scenarios requiring EC-based vascular repair and regeneration during ageing, such as impaired carotid artery EDD. In this context, NMN supplementation reversed arterial dysfunction by decreasing oxidative stress in the vasculature of aged mice (de Picciotto et al., 2016). Consistently, aged ECs exposed to NMN are protected against oxidative stress in a SIRT1-dependent fashion (Kiss et al., 2019). Interestingly, NMN-mediated protection against endothelial dysfunction could have been at least in part explained by an extracellular conversion of NMN to NR at the luminal surface of ECs (Mateuszuk et al., 2020). In agreement, the administration of NR concomitantly prevented oxidative stress in ECs (Hong et al., 2018). Supporting this, in clinical studies supplementation of 125 mg NMN in healthy middle-aged participants significantly decreased brachial-ankle PWV in subjects with above average BMI and blood glucose levels (Katayoshi et al., 2023).

Vitamin C (ascorbate) and E (tocopherol) are particularly known for their antioxidant properties. They are shown to reverse endothelial dysfunction in rats by regulating eNOS and NADPH oxidase (see Section 2.1.2) (Ulker et al., 2003). In addition, they are suggested to regulate the antioxidant response by activating transcription factors such as NRF2 (see Section 2.1.3) (Jomova et al., 2023). Although, clinical studies using only one antioxidant vitamin reported no effect on arterial stiffness, short-time combined application of vitamin C and E has been shown to significantly reduce central PWV and to improve endothelial function (Plantinga et al., 2007). This is also in line with the fact that vitamin C and E act synergistically, since vitamin C is needed for vitamin E regeneration (Packer et al., 1979). In another clinical trial, acute supplementation of vitamin C reduced PWV in both young and old healthy participants (Ashor et al., 2020). However, it should be noted that in randomized trials, long-term vitamin E or vitamin C supplementation was not able to reduce the occurrence of major cardiovascular events (Cook et al., 2007; Sesso et al., 2008).

Vitamin D is known to regulate antioxidant mechanisms (see Section 2.1), cell proliferation, differentiation, and apoptosis, thereby maintaining homeostasis (Bhutia, 2022). However, there are other potential pathways such as the inhibition of RAAS (see Section 2.2) and activation of autophagy (see Section 2.4) that might affect vascular ageing (Bhutia, 2022; Li, 2003). Various clinical studies have reported the effects of vitamin D on arterial stiffness and endothelial function. A recent meta-analysis demonstrated that daily vitamin D supplementation ( $\geq 2000$  IU) improved carotid-femoral PWV in adults with vitamin D deficiency (Chen et al., 2020). Raed et al., reported that vitamin D

supplementation significantly improved arterial stiffness in overweight African American patients (Raed et al., 2017). Another recent meta-analysis showed that vitamin D3 reduced arterial stiffness when supplemented longer than 12 weeks (Saz-Lara et al., 2022). Furthermore, a randomized, double-blinded, placebo-controlled clinical trial demonstrated that oral vitamin D3 significantly improved endothelial function (Harris et al., 2011). In contrast to the previous trials, a meta-analysis showed that vitamin D supplementation was not able to reduce PWV (Mirhosseini et al., 2018). Moreover, an editorial paper from the American Heart Association indicated the lack of clear evidence for the beneficial effects of high-dose vitamin D supplementation on arterial stiffness and central blood pressure (Arora and Wang, 2017).

Thus, it is clear that further research is needed to evaluate in which circumstances significant benefit from vitamin supplementation can be obtained. The implementation of a pharmacogenomic approach to the use of vitamins may help identify individuals for whom vitamins provide a significant clinical benefit. As vitamins have a systemic effect, they will not specifically target the vasculature, which should also be taken into consideration when evaluating their potential use in CVD prevention and treatment.

#### 4.2. Fatty acids

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are considered essential, since humans cannot synthesize them. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the two major n-3 PUFAs found abundantly in fish oils. Instead, alpha-linolenic acid (ALA) is found in plant oils, such as flaxseed oils. The initial observation that the consumption of n-3 PUFAs protects from CVD was conducted almost fifty years ago in Greenland Eskimos (Bang et al., 1976). Since then, several epidemiological studies were conducted. Results from prospective cohort studies indicated that consuming fish or fish oil containing EPA and DHA is associated with decreased cardiovascular death, whereas consumption of ALA is not as effective (Breslow, 2006; Engler and Engler, 2006; Manson et al., 2020). Interestingly, randomized control trials in the context of secondary prevention also showed that the consumption of EPA plus DHA at a dose of  $> 3$  g/day improved vascular reactivity, hypertension, and inflammation. The effects of n-3 PUFAs on modulation of vascular tone have been widely reviewed (Colussi et al., 2017; Daci et al., 2020; Du et al., 2019b; Tucci et al., 2022). In pre-clinical trials, the effect of n-3 PUFAs was mainly studied in isolated vessels obtained from experimental animals such as aortic rings, mesenteric arteries, and coronary arteries, among others. Specifically, ion channel activity (calcium channels or potassium channels), which plays a crucial role in controlling vascular tone, was assessed, as well as the frequency and amplitude of action potentials in vascular cells. Also, patch clamp techniques helped quantifying the electrical changes associated with NO release, influencing vascular tone and promoting vasodilation. Interestingly, these studies observed multiple mechanisms of action by which DHA and EPA can control vascular tone such as modulating the NO pathway (see Section 2.7.1), activating potassium channels, or reducing intracellular calcium levels (Daci et al., 2020). Moreover, antihypertensive effects of DHA were reported in rats (Engler et al., 2003). One possible mechanism that has been suggested is the reduction of vascular wall thickness (Engler et al., 2003). Although other mechanisms such as a reduction in aldosterone (see Section 2.2), changes in renal amino acid metabolism (Engler et al., 1999), and modulation of calcium release have also been proposed (Engler, 1992). n-3 PUFAs also have anti-inflammatory properties, thereby attenuating the expression of EC adhesion molecules (Collie-Duguid and Wahle, 1996; De Caterina et al., 2000; De Caterina and Massaro, 2005) or inhibiting VSMC proliferation (Terano et al., 1997). This could be caused by a diminished activation of the NF- $\kappa$ B transcription factor system, along with a decreased production of intracellular hydrogen peroxide and a lowering of cyclooxygenase-2 expression. Although this suggested hypothesis remains to be experimentally confirmed. In

clinical studies, fish oil rich EPA and DHA has been shown to lower blood pressure in a dose and time dependent manner (Appel et al., 1993; Morris et al., 1993). Furthermore, n-3 PUFA supplementation for 12 weeks decreased carotid-femoral PWV in an old population, but no effect was observed in a young population (Monahan et al., 2015). Shen et al., reported, even in groups with chronic heart disease, independent from the stage, that n-3 PUFA supplementation significantly prevented myocardial infarction occurrence and reduced the rate of major adverse cardiac events (Shen et al., 2022). However, some clinical trials failed to demonstrate beneficial effects of n-3 PUFAs. A double blinded, placebo-controlled investigation, including 12,513 patients with known cardiovascular risk factors, showed no benefit of daily n-3 PUFA supplementation (Risk Prevention Study Collaborative Group et al., 2013). The administration route of n-3 PUFAs, their source, the insufficient length of the follow-up, the different patient characteristics, the number of participants and their age seems to be involved in conflicting results between clinical studies. Moreover, the importance of the pharmacokinetic profile of n-3 PUFAs has to be taken into consideration, since several factors might affect the bioavailability. Thus, additional research is needed to determine which conditions and individuals may benefit from n-3 PUFA supplementation in order to improve vascular ageing.

#### 4.3. Prospects of dietary supplements for vascular ageing therapy

Besides the dietary supplements and vitamins discussed in the current review, several other emerging compounds have recently shown potential to improve vascular function in ageing. For instance, pycnogenol, an extract of French maritime pine bark, has shown to improve endothelial function and to reduce oxidative stress (Enseleit et al., 2012). Furthermore, many dietary polyphenols (resveratrol, curcumin, cocoa, green tea, etc.) have shown potential to prevent arterial stiffening and vascular dysfunction by inhibiting oxidative stress, inflammation and glycation, and by enhancing autophagy (De Bruyne et al., 2019). In addition, taurine supplementation can affect vascular health by inhibiting RAAS and by promoting H<sub>2</sub>S production (Ozsarlak-Sozer et al., 2016; Qaradakhhi et al., 2020). Also beetroot juice, which is rich in nitrates and improves NO production, has been shown to improve vascular function (see Section 2.7.1) (Mirmiran et al., 2020). However, the efficacy and safety of these dietary compounds and supplements may vary, and as such, it is evident that further fundamental and clinical research is essential to elucidate their impact on the aged vasculature. Drawing conclusions based on current research findings is challenging due to a vast variability in study design, dosages and duration. This is further complicated by the heterogeneity of the chosen study populations, which vary in age and disease status, hampering the comparison of data across studies. Thus, future clinical investigations must be meticulously conducted, focussing on optimizing the dosage of dietary supplements and determining long-term effects in appropriate patient cohorts. Moreover, it might be of interest to study a possible synergistic effect by combining multiple dietary supplements. Overall, this comprehensive approach is vital to optimize the efficacy of dietary supplements in clinical studies and determine their potential to tackle age-related vascular dysfunction.

#### 5. Biomechanical, methodological considerations when measuring arterial stiffness and endothelial dysfunction in preclinical studies

Animal models of CVD are commonly used for hypothesis forming and testing in the first stages of the identification of new pharmacological targets and for the development of novel therapeutic strategies. Different animal models of CVD have been used in preclinical studies to identify therapies that are able to slow down or revert abnormal vascular ageing (Ataei Ataabadi et al., 2021; Dou et al., 2021; Karuppagounder et al., 2017; Kelynack and Holt, 2016; Mojiri et al., 2021; Spronck and Humphrey, 2022; Vasquez et al., 2012). Given that arterial

behaviour highly depends on the applied loading condition (Giudici et al., 2021; Spronck and Humphrey, 2019; van der Bruggen et al., 2021), ex vivo measurements of vascular ageing in preclinical studies offer important advantages over in vivo measurements such as PWV, which provide only a narrow view of arterial function (Spronck and Humphrey, 2019). Taking arterial stiffness as an example, by controlling the loading conditions, ex vivo measurements facilitate disentangling the complex bi-directional relationship between blood pressure and arterial stiffness, where short-term changes in blood pressure acutely modulate the artery wall stiffness due to its non-linear mechanical properties (Spronck et al., 2015), and vascular remodelling may induce long-term changes in blood pressure (and vice versa) (Humphrey et al., 2016; Najjar et al., 2008). Conversely, in vivo measurements are hampered by the inherent confounding effect of blood pressure at the time of measurement and statistical corrections do not suffice to disentangle this complex pressure-stiffness relationship (Spronck, 2021), which can be solved using blood pressure-corrected metrics such as CAVI/CAVIO (Shirai et al., 2006; Spronck et al., 2017), or methods that track stiffness with blood pressure over time (Gosse et al., 2023). Nonetheless, ex vivo techniques must mimic the in vivo loading conditions as closely as possible to maximise physiological relevance. Two experimental aspects should be considered. First, in vivo, arteries are subjected to biaxial loading, where 1) a dynamic circumferential pressure load is superimposed to 2) a constant elongation along the vessel's main axis (van Loon et al., 1977). Widely used mechanical testing techniques (e.g., wire and pressure myography) totally or partially neglect the vessel's axial elongation (Ataei Ataabadi et al., 2021; Boutouyrie et al., 1998; del Campo and Ferrer, 2015). As such, the measured ex vivo response cannot be used to directly infer information about arterial (dys)function in vivo (Caulk et al., 2019). Conversely, more complex biaxial testing systems, which thoroughly replicate the in vivo loading configuration and simultaneously measure arterial behaviour in two directions, yield information more directly relatable to in vivo physiology/pathology (Bersi et al., 2017; Gleason et al., 2004; Spronck et al., 2021). Second, because of viscoelasticity, arterial wall behaviour is strongly affected by the loading frequency/speed (Franchini et al., 2021; van der Bruggen et al., 2021). While in vivo, the pressure load acting on arteries is pulsatile (i.e., dynamic), most experimental set-ups allow only to impose quasi-static (i.e., at a negligible speed) loading conditions (Ataei Ataabadi et al., 2021; Boutouyrie et al., 1998; del Campo and Ferrer, 2015; Gleason et al., 2004). This means that in such set-ups, the experimentally measured mechanical properties are only partially representative of the artery behaviour in vivo (van der Bruggen et al., 2021). Set-ups that do test the arteries under in vivo-realistic dynamic conditions are, e.g., ROTSAC (Leloup et al., 2016) and DynamX (van der Bruggen et al., 2021).

Although the aforementioned considerations mainly focused on passive arterial mechanics (i.e., arterial stiffness), the points raised also have direct implications for active arterial mechanics (i.e., vaso-reactivity) and, consequently, endothelial (dys)function. This is the case because 1) smooth muscle contraction (typically assumed to be in the circumferential direction) will affect axial mechanics due to the aforementioned biaxial coupling, but also because 2) the vasoreactive response of an artery has been shown to depend on its biaxial loading state (e.g., the EC50-value for potassium chloride-induced vasoconstriction differs by a factor of 4 between uniaxial and biaxial ex vivo loading regimens) (Caulk et al., 2019). Likely, the same holds for endothelium-mediated responses to e.g., acetylcholine.

Thus, compared to in vivo biomechanical markers, an adequate ex vivo approach provides a much more detailed assessment of the mechanical behaviour of the arterial wall and yields vast amounts of data. However, interpreting and drawing conclusions from such data requires integration and interpretation through in silico constitutive modelling. The fundamental assumption of constitutive modelling is that a tissue's mechanical behaviour results from the summed contributions of its constituents (e.g., elastin, collagen, and smooth muscle cells), each

governed by a mathematical function (Holzapfel and Ogden, 2010; Humphrey, 2002). By fitting the constitutive model to the experimental data, a set of model parameters can be identified (Giudici et al., 2021; Spronck et al., 2021). Constitutive models with varying degrees of complexity have been proposed to capture the mechanical behaviour of arteries (Giudici et al., 2021; Giudici et al., 2023; Holzapfel and Ogden, 2010; Humphrey, 2002; Spronck et al., 2021) and how it is affected by ageing (Giudici et al., 2022; Haskett et al., 2010; Hopper et al., 2021; Jadidi et al., 2020; Weisbecker et al., 2012; Zulliger and Stergiopoulos, 2007). Constitutive model fitting is typically performed on datasets obtained from extensive ex vivo biaxial testing (Ferruzzi et al., 2013; van der Bruggen et al., 2021), but it is also feasible using in vivo human data (Reesink and Spronck, 2019), which allows to assess the performance of vascular drug therapies (Heusinkveld et al., 2018). Notably, constitutive modelling does not add information but rather provides a means to extract meaningful information from the measured data. Overall, pre-clinical studies with detailed ex vivo measurements combined with constitutive modelling offer key advantages in studying disease models of vascular ageing and potential therapies.

## 6. Open challenges in the development and application of drugs targeting vascular ageing

### 6.1. Inconsistent interaction of drugs with co-prescribed exercise training

There is overwhelming evidence showing that regular aerobic exercise training (AET), and the concomitant increase in cardiorespiratory fitness (e.g.,  $VO_{2max}$ ), significantly reduces ageing-associated CVD risk (Pettee Gabriel et al., 2023), CVD-related mortality (Imboden et al., 2018; Pettee Gabriel et al., 2023) and all-cause mortality (Dutta et al., 2012). AET has a profound therapeutic effect on systemic vascular function and structure through improvements in endothelial function, arterial stiffness, and circulating inflammatory markers (Lavie et al., 2015). As exercise likely elicits these vascular benefits via alternative mechanisms compared to traditional pharmaceuticals, both treatment strategies are often co-prescribed to offer 'additive' cardiovascular protection. However, the interaction between exercise and traditional pharmaceuticals on cardiovascular health markers appears to be inconsistent, with some reporting a blunting of exercise-induced benefits by pharmacological administration in certain disease states.

Exercise and statin combination therapy offers greater protection against all-cause mortality than either treatment in isolation (Kokkinos et al., 2013). Similarly, combination therapy can yield greater improvements in cholesterol management and overall cardiometabolic health than statin therapy alone (Baptista et al., 2018; Gui et al., 2017). Although the benefit of statin treatment outweighs potential drawbacks, statins can have a number of side effects, including diabetes risk, mitochondrial dysfunction, and myalgia/myopathies, which have implications on exercise adherence and physiological adaptations to training. Indeed, statins have been reported to blunt exercise-induced increases in  $VO_{2max}$  and skeletal muscle mitochondrial content in obese individuals (Mikus et al., 2013). Only two studies have examined the combined effect of exercise and statins on vascular function. Walsh et al. reported that exercise training improved endothelium-dependent vascular function in dyslipidaemia patients taking statins, but only improved basal NO bioactivity in untreated patients (Walsh et al., 2003). Whereas Ivey et al. demonstrated that AET improved cerebrovascular reactivity in stroke survivors, but only in those not taking a statin (Ivey et al., 2011). Accordingly, there is evidence that statins can both augment and blunt the (cardio)vascular benefits of AET, however, more research into the interactive effects at various regions of the vascular tree is required.

Metformin administration has been reported to attenuate the increase in  $VO_{2max}$  and whole-body insulin sensitivity following 12 weeks of AET by ~50% (Konopka et al., 2019). Similarly, exercise-induced improvements in skeletal muscle insulin sensitivity and AMPK

activation were also reduced when co-prescribed with metformin, indicating an interference with exercise-induced cellular signalling and glucose uptake (Malin et al., 2012; Sharoff et al., 2010). In contrast, some studies have found exercise combined with metformin augments  $VO_{2max}$ , and improves overall exercise capacity (Boulé et al., 2013; Jadhav et al., 2006). Nevertheless, metformin inhibits complex I-dependent respiration, resulting in increased lactic acid formation and signalling abnormalities (Feng et al., 2022), which may increase perceived exertion and oppose exercise-induced adaptations (Kraus and Slentz, 2009).

Distinct anti-hypertensive drugs may also interact with exercise and reduce exercising levels of cardiac output, peripheral vasodilation, tissue perfusion and shear stress. For example, beta-blockade affects the physiological response to sub-maximal exercise by lowering heart rate, blood pressure, cardiac output and peripheral blood flow, whilst increasing muscle fatigue and perception of effort (Derman and Schwellnus, 1998). Not only can these present considerable difficulties when prescribing exercise intensity via heart rate parameters, these alterations in local (shear stress) and systemic (haemodynamic) vascular stimuli may also impact future training adaptations. Indeed, whilst the combination of exercise and varying anti-hypertensive drugs appears to augment post-exercise hypotension beyond exercise alone (Ramirez-Jimenez et al., 2018a; Ramirez-Jimenez et al., 2018b; Ramirez-Jimenez et al., 2019), the chronic interactive effects on blood pressure are inconsistent (Ramirez-Jimenez et al., 2021; Sjutharson et al., 2022). However, Motoyama et al. reported that any additional blood pressure lowering effects of AET in existing anti-hypertensive drug users rapidly disappear upon cessation of training despite continuation of the drugs, indicating that combination therapy can offer additive benefit (Motoyama et al., 1998).

The inconsistent interaction between co-prescribed exercise and pharmaceuticals warrants further investigation. Whether a synergistic or inhibitory effect is observed may be dependent on disease type and severity, medication type/dose/combination, exercise adherence and the (cardio)vascular outcome examined. Future investigations should utilize systematic vascular phenotyping to examine disease-specific interactions between pharmaceutical therapies and exercise to help tailor future primary and secondary CVD prevention options.

### 6.2. Sex and gender-related effects

Although differences in the epidemiology and pathophysiology of vascular ageing are increasingly being reported for women and men, limited sex and gender-disaggregated data exist for prospective studies (Seeland et al., 2021). Up until menopause, vascular ageing appears to be slower in women than men, at which point it accelerates to the same level observed in men (DuPont et al., 2019; Lu et al., 2020). Menopause transition has been shown to augment age-dependent increase in arterial stiffness in middle aged women (Samargandy et al., 2020), and oestrogen replacement therapy in postmenopausal women is associated with reduced blood pressure and arterial stiffness (Scuteri et al., 2001). On the other hand, men with low levels of testosterone have impaired endothelial function and increased arterial stiffness (Moreau et al., 2020). Because of these discrepancies in the pathophysiology and chronological development of vascular ageing, both male and female subjects should be included equally in preclinical and clinical trials to adequately assess differential sex and gender-related effects of potential anti-ageing treatments.

## 7. Conclusion and future perspectives

Vascular ageing will gain importance in the upcoming years due to the growing number of elderly people. This will not only increase the costs associated with treatment of age-related diseases, such as CVD, but will also impact the quality of life. It is therefore highly relevant to develop methods or treatment protocols to promote healthy ageing. At



the moment pathophysiological processes such as oxidative stress, enhanced RAAS signalling, cellular senescence, impaired autophagy, ECM remodelling, vascular calcification, a decline in H<sub>2</sub>S, and decreased NO-cGMP signalling, all play a role in vascular ageing. Although life-style- and pharmacologically-based strategies targeting these mechanisms are available, the multi-faceted nature of vascular ageing complicates their evaluation and implementation. Accordingly, several of the therapeutic approaches and drugs that are discussed in the current review, have been validated in preclinical and/or clinical studies, but not specifically to be used in the context of vascular ageing. This is mainly because important vascular ageing-related parameters, such as arterial stiffness and endothelial dysfunction, are not routinely measured in clinical practice. Furthermore, there are no criteria to define high-risk patients that would be eligible for treatment. So, efforts should be made to implement monitoring of vascular function and stiffness in clinical practice, and to establish cut-off values that can be used as a diagnostic criterion.

Overall, the field of vascular ageing research is gaining more and more interest, resulting in a growing number of potential drug targets and related lifestyle and pharmacological interventions. Although there are no approved drugs available to specifically tackle arterial stiffness and endothelial dysfunction, many therapies for CVDs and type 2 diabetes have shown to modulate age-related vascular changes. Also newly developed compounds (e.g., NOX inhibitors, senotherapeutics, autophagy inducers, NEU1 inhibitors...) have shown promise and require further clinical validation in terms of their safety and efficacy to modulate vascular ageing.

#### CRediT authorship contribution statement

**LR:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **SD:** Conceptualization, Writing - original draft, Writing - review & editing. **BGT:** Conceptualization, Writing - original draft, Writing - review & editing. **TA:** Writing - original draft, Writing - review & editing. **SB:** Writing - original draft, Writing - review & editing. **MBP:** Writing - original draft, Writing - review & editing. **PB:** Writing - original draft, Writing - review & editing. **GRYDM:** Writing - original draft, Writing - review & editing. **LD:** Writing - original draft, Writing - review & editing. **ND:** Writing - original draft, Writing - review & editing. **DF:** Writing - original draft, Writing - review & editing. **EF:** Writing - original draft, Writing - review & editing. **PG:** Writing - original draft, Writing - review & editing. **AG:** Writing - original draft, Writing - review & editing. **SJ:** Writing - original draft, Writing - review & editing. **MJ:** Writing - original draft, Writing - review & editing. **JJ:** Writing - original draft, Writing - review & editing. **AKLH:** Writing - original draft, Writing - review & editing. **WM:** Writing - original draft, Writing - review & editing. **PM:** Writing - original draft, Writing - review & editing. **BJM:** Writing - original draft, Writing - review & editing. **ENO:** Writing - original draft, Writing - review & editing. **GP:** Writing - review & editing. **CJAP:** Writing - original draft, Writing - review & editing. **KDR:** Writing - original draft, Writing - review & editing. **AJMR:** Writing - original draft, Writing - review & editing. **NR:** Writing - original draft, Writing - review & editing. **JS:** Writing - original draft, Writing - review & editing. **YS:** Writing - review & editing. **BS:** Writing - original draft, Writing - review & editing. **FS:** Writing - original draft, Writing - review & editing. **DTP:** Writing - review & editing. **ETA:** Writing - original draft, Writing - review & editing. **OTC:** Writing - original draft, Writing - review & editing. **EU:** Writing - original draft, Writing - review & editing. **GYA:** Conceptualization, Visualization, Writing - original draft, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data Availability

No data was used for the research described in the article.

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