# DNA damage-dependent mechanisms of ageing and disease in the macro- and microvasculature

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**Abstract**
A decline in the function of the macro- and micro-vasculature occurs with ageing. DNA damage also accumulates with ageing, and thus DNA damage and repair have important roles in physiological ageing. Considerable evidence also supports a crucial role for DNA damage in the development and progression of macrovascular disease such as atherosclerosis. These findings support the concept that prolonged exposure to risk factors is a major stimulus for DNA damage within the vasculature, in part via the generation of reactive oxygen species. Genomic instability can directly affect vascular cellular function, leading to cell cycle arrest, apoptosis and premature vascular cell senescence. In contrast, the study of age-related impaired function and DNA damage mechanisms in the microvasculature is limited, although ageing is associated with microvessel endothelial dysfunction. This review examines current knowledge on the role of DNA damage and DNA repair systems in macrovascular disease such as atherosclerosis and microvascular disease. We also discuss the cellular responses to DNA damage to identify possible strategies for prevention and treatment.

**Keywords**: DNA damage, ageing, atherosclerosis, microvasculature, endothelial cells

## 1 Introduction

The vasculature of healthy individuals undergoes several important changes with increasing age, which increase the likelihood of developing cardiovascular disease. Both the onset and progression of vascular disease are related to age, in part because of common manifestations. For example, ageing is associated with endothelial dysfunction (Celermajer et al., 1994; Van Der Loo et al., 2000), arterial stiffening and remodelling (Lakatta and Levy, 2003), impaired angiogenesis (Rivard et al., 1999), defective vascular repair (Weinsaft and Edelberg, 2001), and an increasing prevalence of atherosclerosis (Eggen and Solberg, 1968; Erusalimsky and Kurz, 2005). The reasons for these associations are still unclear, but it is plausible that organismal ageing and vascular disease share common cellular mechanisms. One process that has been increasingly linked to both ageing and the development of vascular pathology is DNA damage (Fuster and Andrés, 2006; Minamino et al., 2002). Here, we review current knowledge on the role of DNA damage and DNA repair systems in atherosclerosis and microvascular disease, and discuss the cellular response to DNA damage to identify possible strategies for prevention and treatment.

## 2 DNA damage pathways and repair mechanisms

DNA damage is manifested in a variety of forms including single strand breaks, double strand breaks, base modification (e.g. 7,8-dihydro-8-oxoguanine (8-oxoG)) and mispairing, all of which need to be successfully repaired to avoid accumulation of mutations, cell cycle arrest and apoptosis. DNA damage activates a variety of DNA repair mechanisms, collectively termed the DNA-damage response (DDR). Many of these DNA repair mechanisms are activated in vascular disease, which repair the DNA, stall the cell cycle to allow repair to occur, or trigger apoptosis or cell senescence to prevent propagation of damaged DNA **(Fig. 1)**.

### 2.1 Oxidative damage and base excision repair

DNA bases are particularly susceptible to oxidation mediated by reactive oxygen species (ROS) (Neeley and Essigmann, 2006). The low redox potential of guanine makes this base particularly vulnerable and leads to a plethora of oxidised guanine products (Neeley and Essigmann, 2006). The most thoroughly studied guanine oxidation product is 8-oxoG (Neeley and Essigmann, 2006), and the presence of 8-oxoG is often used as a cellular biomarker to indicate the extent of oxidative stress. 8-oxoG is a highly mutagenic miscoding lesion that can lead to G:C to T:A transversion mutations (Grollman and Moriya, 1993).

The base excision repair pathway is the primary mechanism for repair of oxidative base lesions, such as 8-oxoG and formamidopyrimidines (4,6-diamino-5-formamidopyrimidine, FapyG)**(Fig. 1)**. Base excision repair is a critical process for genomic maintenance, as highlighted by the severe phenotypes seen in animals deficient in base excision repair function, including premature ageing and metabolic defects (Vartanian et al., 2006). Base excision repair involves the concerted effort of several repair proteins that recognize and excise specific DNA damage, eventually replacing the damaged moiety with a normal nucleotide and restoring the DNA back to its original state (Hoeijmakers, 2001). Base excision repair facilitates the repair of damaged DNA via two general pathways – which involve either the replacement of one (short-patch base excision repair) or more nucleotides (long-patch base excision repair) at the lesion site (David et al., 2007). The initial step in base excision repair uses DNA glycosylases, which cleave the N-glycosyl bond between the sugar and the base, thus releasing the damaged base to form an abasic site, also termed apurinic/apyrimidinic (AP) site. The efficient repair of abasic sites is critical because they are highly mutagenic (Loeb, 1985). There are several different glycosylases specific for certain lesions. Individual glycosylases may recognize more than one type of damage, and each specific modification may be recognized by more than one type of glycosylase, giving a degree of redundancy in the process. For example, 8-oxoguanine DNA glycosylase (OGG1) has strong specificity for 8-oxoG lesions, whereas Nei-like DNA glycosylase 1 (NEIL1) efficiently removes FapyG lesions (Parsons et al., 2005). OGG1 and NEIL1 are

classified as bifunctional glycosylases as they possess both glycosylase and AP lyase activity, namely base excision and an incision 3' to the AP site can occur, resulting in a single strand break.

**2.2 Single strand breaks and repair**

DNA single-strand breaks are among the most frequent DNA lesions, arising directly from damage to the deoxyribose moieties or indirectly as intermediates of base excision repair (Beckman and Ames, 1997; Lindahl, 1993). Left unrepaired, single-strand breaks are a major threat to genetic stability and cell survival, accelerating mutation rates and increasing levels of chromosomal aberrations (Caldecott, 2008). The pathways for single-strand break repair in mammalian cells involve a number of co-ordinated, sequential reactions responsible for damage detection, end processing, gap filling and ligation. Three excision repair pathways exist to repair single stranded DNA damage: base excision repair, nucleotide excision repair, and DNA mismatch repair. While the base excision repair pathway, as discussed, recognizes specific non-bulky lesions in DNA, nucleotide excision repair removes bulky DNA adducts induced by UV, and the DNA mismatch repair pathway targets mismatched base pairs (Caldecott, 2008)**(Fig. 1)**.

Poly (ADP-ribose) polymerase 1 (PARP1) is one of the key proteins in single-strand break repair, as it is a nick sensor and also binds to single-strand breaks, short gaps in duplex DNA, double-strand breaks and other abnormal DNA structures, and initiates the efficient repair of single-strand breaks (Lan et al., 2004; Okano et al., 2003). After activation of PARP at single-strand breaks, the X-ray repair cross-complementing protein 1 (XRCC1) accumulates at poly(ADP-ribose) (pAR) sites (Lan et al., 2004; Okano et al., 2003). XRCC1 is thought to act as a scaffold protein for other repair factors and has been shown to physically interact with several enzymes known to be involved in the repair of single-strand breaks, including DNA ligase IIIα, DNA polymerase β, APE1, polynucleotide kinase/phosphatase, PARP-1 and OGG1 (Marsin et al., 2003; Masson et al., 1998; Schreiber et al., 2002). Key mediator proteins involved in nucleotide excision repair include excision repair cross complementing-group (ERCC)-1 and ERCC4, and the DNA mismatch repair pathway is mediated by MutL homolog 1 (MLH1) and MutS homolog 2 (MSH2) (Caldecott, 2008)**(Fig. 1)**.

**2.3 Double strand breaks and recombinational repair**

Double-strand breaks are produced by ROS, ionising radiation, and chemicals that generate ROS. DNA double-strand breaks are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed (Hartlerode and Scully, 2009). As a consequence, the two DNA ends generated by a double-strand break are liable to become physically dissociated from one another, making ensuing repair difficult to perform and providing the opportunity for inappropriate recombination with other sites in the genome. Unrepaired or improperly repaired double-strand breaks have serious potential consequences, including cell death, senescence, dysregulation of cellular function, genomic instability, and, in higher organisms, oncogenic transformation (Chapman et al., 2012).

Double-strand breaks are repaired either by homologous recombination or non-homologous end joining mechanisms. These pathways are largely distinct from one another and function in complementary ways to effect double-strand break repair (Cromie et al., 2001; Haber, 2000; Takata et al., 1998). During homologous recombination the damaged chromosome enters into synapsis with, and retrieves genetic information from, an undamaged DNA molecule with which it shares extensive sequence homology. In contrast, non-homologous end joining, which brings about the ligation of two DNA double-strand breaks without the requirement for extensive sequence homology between the DNA ends, does not need synapsis of the broken DNA with an undamaged partner DNA molecule. Both pathways are highly conserved throughout eukaryotic evolution but their relative importance differs from one organism to another.

A crucial component of the DNA double-strand break signalling cascade in mammalian cells and one of the first proteins to respond to double-strand breaks is the protein kinase ATM (ataxia telangiectasia mutated), a member of the phosphoinositide-3 kinase-like kinase (PIKK) family (Abraham, 2001; Shiloh, 2001). ATM deficiency leads to the neurodegenerative syndrome ataxia-telangiectasia. ATM substrates include H2AX, a nucleosomal histone variant, and p53 binding protein-1 (53BP1), which facilitate checkpoint activation and repair. Phosphorylated H2AX (γ-H2AX) and 53BP1 rapidly localise to double-strand breaks, forming characteristic foci. ATM also phosphorylates the DDR kinase checkpoint kinase-2 (CHK2), which promotes growth arrest; p53, a tumour suppressor and transcriptional regulator that orchestrates repair and cell-cycle arrest, and NBS1 (Nijmegen breakage syndrome), a member of the MRN (MRE11–Rad50–NBS1) complex (Zhou and Elledge, 2000). The MRN complex and the Ku70-Ku80 proteins are among the first recruited to double-strand breaks, initiating the DDR and facilitating interplay between repair mechanisms, activation of cell cycle checkpoints, and effector pathways (Lamarche et al., 2010). The Ku70-Ku80 heterodimer promotes further recruitment of the catalytic subunit of DNA-dependent protein kinase (DNA-PK) and other factors essential for non-homologous end joining (Rivera-Calzada et al., 2007). Double-strand breaks that cannot be repaired cause constitutive DDR signalling, prolonged p53-dependent growth arrest, and eventually an essentially irreversible senescence arrest (Rodier et al., 2005)**(Fig. 1).**

**2.4 Mitochondrial DNA damage and repair**

Although much attention has been paid to the effects of DNA damage on nuclear DNA, mitochondria harbour a critical component of the total cellular DNA, which can also become damaged. For example, the oxidative production of ATP required for cellular function generates ROS that damage mitochondrial DNA (mtDNA)(Cadet et al., 2010). Additionally, environmental chemicals can induce mtDNA damage, both by amplifying the production of endogenous DNA lesions and by generating unique DNA adducts (Swenberg et al., 2010).

8-oxoG accumulates with age in mtDNA both *in vivo* and *in vitro* (Barreau et al., 1996; Mecocci et al., 1993; Sohal et al., 1994), and lesions or mutations in mtDNA can drastically alter mitochondrial function. Furthermore, mtDNA contains a higher steady-state level of oxidative damage compared with nuclear DNA (Croteau and Bohr, 1997). This has been ascribed to the localisation of mtDNA near the inner mitochondrial membrane where oxidants are formed, as well as lack of histones and diminished DNA repair activities. For example, unlike nuclei that have multiple mechanisms for DNA repair, mitochondria appear more limited in their ability to repair all possible forms of mtDNA damage (Liu and Demple, 2010), although 8-oxoG is repaired more efficiently in mitochondria than in the nucleus (Thorslund et al., 2002). Several base excision repair proteins have been identified in mitochondria and these are generally either identical to nuclear DNA repair proteins or isoforms of nuclear proteins arising from differential splicing or alternative transcription initiation sites. Non-homologous end joining and homologous recombination activities that repair double-strand breaks have also been detected in mammalian mitochondria, and recent studies suggest a role for the RecQ helicase, RECQL4, in mitochondrial double-strand break repair (Bacman et al., 2009; Singh et al., 2012).

**2.5 Telomere shortening and maintenance**

Telomeres consist of tandem repeats of the sequence TTAGGG at the end of chromosomes (de Lange, 2009). To shield the telomere from unwanted interactions with DNA repair and damage sensing molecules, the cell uses a six-protein complex termed shelterin to protect and cap the telomere (De Lange, 2005). This complex includes telomeric repeat binding factor (TRF)-1 and TRF2, and loss of either protein has detrimental effects on telomere function and stability (Hohensinner et al., 2014). For example, a recent study described age-related loss of telomere protection via TRF1

reduction and a subsequent increase in telomere damage in ageing endothelial cells (ECs) *in vitro* (Hohensinner et al., 2016).

Human cells generally do not express sufficient telomerase to counteract telomere shortening caused by the inability of the DNA replication machinery to fully replicate chromosomal ends. Thus, human telomeres generally shorten with each cell division until some retain hardly any terminal telomeric repeats. These then fail to act as telomeres and are instead recognised as double-strand breaks, triggering chromosomal fusions and ensuing breakage–fusion–breakage cycles. Under such situations of chronic DDR activation, cells undergo apoptosis or senescence (di Fagagna et al., 2004; Longhese, 2008). Telomeres were reported to be persistent targets of DNA damage *in vitro* and *in vivo* (Hewitt et al., 2012). Both natural ageing and circumstances of high cell turnover, such as chronic inflammation, show telomere shortening leading to apoptosis and senescence.

Telomere shortening can also be affected by oxidative stress, as the high incidence of guanine residues in telomeric DNA sequences makes the telomere a preferred target for oxidative damage. Moreover, repair of single-strand breaks (Petersen et al., 1998), and to some extent also of UV-induced damage (Kruk et al., 1995), is significantly less efficient in telomeres compared to the bulk of the genome. The presence of 8-oxoG inhibits telomerase activity and decreases the binding of telomeric proteins to the telomere sequence, leading to the disruption of telomere length, maintenance and function (Opresko et al., 2005).

## 3 Cellular consequences of DNA damage

Genomic instability at the cellular level can directly affect vascular function as the consequences of persistent genomic and mitochondrial DNA damage include growth arrest/senescence, inflammation and apoptosis, all of which are present in vascular disease **(Fig. 2)**.

### 3.1 Cellular senescence

Cell senescence is defined as irreversible cell cycle arrest, or loss of the ability of cells to divide (Ben-Porath and Weinberg, 2004). Cell senescence may be triggered by two general mechanisms; replicative senescence and stress-induced premature senescence (SIPS). Replicative senescence occurs with exhaustion of proliferative lifespan over time, characteristic of an ageing phenotype and is associated with reduction in telomere length at chromosomal ends, which induces a DNA damage response. In contrast, SIPS is triggered by external stimuli, including oxidising agents and radiation, which activate the intracellular senescence cascade prematurely. Unlike replicative senescence, SIPS is not usually characterised by telomere shortening.

Senescence can be recognised in tissues by expression of a number of markers, including senescence-associated beta galactosidase (SaβG), a lysosomal enzyme that increases with cellular ageing (Dimri et al., 1995). Recent studies have shown that senescent cells in culture and *in vivo* also develop a complex senescence-associated secretory phenotype (SASP), where cellular senescence is accompanied by a striking increase in the secretion of 40–80 factors that participate in intercellular signalling (Coppé et al., 2010)**(Fig. 2)**. SASP factors are generally induced at the level of mRNA (Coppé et al., 2008) and can be divided into the following major categories: soluble signalling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins/extracellular matrix components. These factors can affect surrounding cells by activating various cell-surface receptors and corresponding signal transduction pathways that may lead to multiple pathologies. A persistent DDR appears to be critical for regulation of the SASP (Coppé et al., 2010). Loss of ATM or other factors responding to DNA damage (such as NBS1 and CHK2) leads to reductions in some SASP factors, such as interleukin (IL)-6 and IL-8 (Rodier et al., 2009). SASP factors IL-6 and IL-8 are of particular interest: these cytokines are among the most robustly induced and

secreted factors and initiate inflammatory responses, such as those associated with age-related pathologies (Caruso et al., 2004).

### 3.2 Apoptosis

Cells undergo apoptosis when DNA damage is too much to repair **(Fig. 2)**, which serves to prevent further replication of mutated DNA. DNA damage-mediated cell death is thought to be primarily regulated by the ATM-mediated phosphorylation of p53. This activates downstream pro-apoptotic genes including Puma and Bax that cause permeabilisation of the outer membrane of the mitochondria, resulting in the release of cytochrome c from the mitochondrial intermembrane space (reviewed in Norbury and Zhivotovsky, 2004). The released cytochrome c assembles a multiprotein caspase-activating complex, referred to as the "apoptosome" (Green and Kroemer, 2004). The central component of the apoptosome is Apaf1, a caspase-activating protein that oligomerizes and then binds procaspase-9. The caspase proteins cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

## 4 DNA damage in vascular disease

DNA damage affecting both genomic and mitochondrial DNA is present in a variety of both acquired and inherited vascular diseases affecting both large vessels and the microvasculature. Multiple cell types show persistent DNA damage and exhibit a range of lesions. Recent evidence has indicated that DNA damage occurs early, is progressive, and is sufficient to impair function of cells comprising the vascular wall.

### 4.1 Macrovascular disease- Atherosclerosis

Acquired macrovascular disease, in particular atherosclerosis, is associated with DNA damage and defective repair. Atherosclerosis is characterised by enhanced nuclear and mitochondrial DNA damage and enhanced DDR signalling, leading to cell senescence and cell death in atherosclerotic plaques (Bennett et al., 1995; Matthews et al., 2006).

#### 4.1.1 Evidence of DNA damage in atherosclerosis

The advanced atherosclerotic plaque comprises a fibrous cap containing VSMCs and infiltrating macrophages overlaid by a dysfunctional EC layer. The cap separates a 'necrotic core' comprising foam cells of macrophage and VSMC origin, dead cells, extracellular lipid, and cellular debris. Both cells comprising the advanced lesion and circulating cells from elderly subjects and patients with atherosclerosis contain increased DNA damage compared to control subjects (Botto et al., 2001; Mahmoudi et al., 2008; Martinet et al., 2001, 2002). Plaque-derived VSMCs retain increased DNA damage *in vitro* compared with normal VSMCs, as shown by increased expression of phosphorylated forms of ATM and Histone 2A protein X proteins ($\gamma$-H2AX) (Mahmoudi et al., 2008). DNA strand breaks are also significantly higher in patients with coronary artery disease than controls (Botto et al., 2001), and human plaques show markers of oxidative DNA damage, including expression of 8-oxoG, an oxidative modification of guanine residues in DNA (Martinet et al., 2001, 2002). Strong nuclear and cytoplasmic immunoreactivity for 8-oxoG is detected in plaque VSMCs, macrophages and ECs, but not in VSMCs of adjacent normal media or normal arteries (Martinet et al., 2001, 2002).

Telomere attrition is linked with both vascular disease and vascular ageing (Matthews et al., 2006; Yamada, 2003). In atherosclerosis, shortened telomeres are observed in plaque VSMCs (Matthews et al., 2006). Telomere attrition and low levels of telomerase expression and activity are functionally important in VSMC senescence, since ectopic telomerase expression can dramatically increase lifespan of both plaque and normal VSMCs (Matthews et al., 2006). Reduced telomere length has also been shown in plaque ECs (Minamino et al., 2002) and cultured endothelial progenitor cells

from coronary artery disease patients (Satoh et al., 2008). Additionally, telomeres in circulating blood leukocytes and bone marrow-derived cells are shorter in patients with cardiovascular disease compared with controls (Brouilette et al., 2003; Brouilette et al., 2007; Spyridopoulos et al., 2008; van der Harst et al., 2007), and are inversely correlated to cardiovascular disease risks in patients with subclinical diseases (Panayiotou et al., 2010; Willeit et al., 2010). In leukocytes from coronary heart disease patients, telomere length was shorter in all subfractions studied compared to age-matched controls, including CD34+ peripheral blood stem cells and progenitor cells, monocytes, granulocytes, B lymphocytes, and CD4+ T cells, including their memory and naive subpopulations (Spyridopoulos et al., 2009).

DNA damage can also occur in the mitochondrial genome. Indeed, mtDNA damage is associated with the extent of atherosclerosis observed in human aortas and aortas from apolipoprotein E (ApoE)$^{-/-}$ mice (Ballinger et al., 2002; Yu et al., 2013), providing evidence that mtDNA damage may have a causal role in atherosclerosis. In addition, circulating cells in patients with severe coronary atherosclerosis display a significantly higher incidence of the mitochondrial deletion mtDNA 4977 (Botto et al., 2005). This deletion is associated with mitochondrial dysfunction (James and Murphy, 2002), although it is not clear whether the levels of the deletion observed in atherosclerosis are sufficient alone to cause mitochondrial dysfunction. Whereas gradual loss of mitochondrial function occurs with age, it can be accelerated by oxidative stress, for example due to increased oxidised LDL (oxLDL) during atherogenesis (Fujimoto et al., 2010; Tatarkova et al., 2011).

Patients with some progeria syndromes develop premature atherosclerosis in addition to manifestations of premature ageing. Recent studies have revealed that most progeria syndromes are caused by mutations in genes involved in the DNA damage response; thus a common feature of progeria syndromes is an enhanced accumulation of DNA damage, arising from a compromised repair system. The manifestation of premature vascular disease in these syndromes is a strong indication that DNA damage and inefficient repair is directly causal in atherosclerosis, in the absence of any other risk factors for atherosclerosis. The progeria syndromes associated with macrovascular disease include Werner syndrome, Hutchinson-Gilford syndrome and Ataxia telangiectasia (Capell et al., 2007)

In addiiton to findings that atherosclerosis is associated with different forms of DNA damage, *in vivo* studies utilising genetic manipulation of DNA repair components suggest a causative role for DNA damage in development and progression of atherosclerosis. For example, ATM haploinsufficiency caused defects in cell proliferation, apoptosis and mitochondrial dysfunction in VSMCs and macrophages; this failure of DNA repair promoted atherosclerosis and a metabolic syndrome phenotype (Mercer et al., 2010). Similarly, human plaque VSMCs show reduced expression and telomere binding of the telomere protein TRF2, which is associated with increased DNA damage and DDR activation at telomeres (Wang et al., 2015). Gain- and loss-of-function studies of TRF2 in ApoE$^{-/-}$ mice showed that VSMC senescence promotes both plaque progression and features of unstable plaques (Wang et al., 2015). Inhibition of signalling from the proximal DDR complex NBS1 in VSMCs also results in marked effects *in vitro* on DDR signalling, cell proliferation, and apoptosis (Gray et al., 2015); however, neither accelerating nor retarding DSB repair in VSMCs affected plaque burden, but had pronounced effects on fibrous cap structure and thus plaque stability (Gray et al., 2015). Finally, the inhibition of PARP1 (see section 2.2) can improve endothelial function in ApoE$^{-/-}$ mice fed a high-fat diet (Benko et al., 2004), while an elevated level of PARP1 was found in human atherosclerotic plaques (Martinet et al., 2002).

The effects of mtDNA lesions have been studied using mitochondrial mutator mice, a model of premature ageing with extensive mtDNA damage. These mice have a homozygous mutation of the proofreading enzyme polymerase gamma (Polg$^{m/m}$)(Trifunovic et al., 2004). Polg$^{m/m}$ mice that were

also deficient for ApoE showed that mtDNA defects promote atherosclerosis and plaque vulnerability without any increase in ROS (Yu et al., 2013). The mtDNA damage in this model caused VSMC and monocyte apoptosis and a pro-inflammatory monocyte phenotype (Yu et al., 2013). Furthermore, mtDNA lesions were associated with high-risk atherosclerotic plaques in humans (Yu et al., 2013). Simialarly, low-density lipoprotein receptor $(LDLR)^{-/-}$ mice lacking the base excision repair protein OGG1 in all tissues showed increased oxidized mtDNA, inflammasome activation, and apoptosis (Tumurkhuu et al., 2016), with increased plaque and necrotic core areas, pointing to a protective role for OGG1 in atherogenesis (Tumurkhuu et al., 2016). Combined, these studies provide the best evidence to date that alteration in the DDR can play a causative role in atherosclerosis.

**4.1.2 Evidence of cellular senescence in atherosclerosis**

SAβG-positive vascular smooth muscle cells (VSMCs) and ECs are observed in aged large vessels and atherosclerotic lesions when compared with their respective young and normal controls (Childs et al., 2016; Gardner et al., 2015; Matthews et al., 2006; Minamino et al., 2002). Although macrophages also show SAβG activity such that this marker is not specific for senescent cells, *in vitro* studies have shown that plaque VSMCs show reduced cell proliferation rates and earlier induction of senescence compared to medial VSMCs (Matthews et al., 2006). Characterisation of lesion development also confirms that cell proliferation is low in advanced plaques compared to non-atherosclerotic aorta or coronary artery VSMCs (Bennett et al., 1995). Together, this supports the idea that atherosclerosis is associated with premature cellular senescence.

However, whether the premature senescence in atherosclerosis represents replicative or stress-induced premature senescence is not clear, as both forms accompany cardiovascular disease. For example, subcultured plaque VSMCs have an enlarged, flattened and stellar shape with high amounts of cytoplasmic vacuoles and lysosomes; features that are also present in normal VSMCs undergoing replicative senescence in culture (Gorenne et al., 2006). As mentioned previously, plaque cells exhibit shortened telomeres (Matthews et al., 2006; Minamino et al., 2002), also characteristic of cells undergoing replicative arrest. However, chronic oxidative stress is a feature of atherosclerosis and increased levels of ROS can induce premature senescence both via and independently of telomere attrition, suggesting that ROS within plaques may promote cellular senescence through both replicative and stress-induced premature senescence pathways (Matthews et al., 2006). Therefore, the distinction between replicative and oxidative stress-induced senescence is not clearly delineated.

**4.1.3 Evidence of apoptosis in atherosclerosis**

Apoptosis is increased in aged vascular cells and in vascular cells in atherosclerotic plaques. Plaque stability is determined by its mechanical properties, in particular the number of VSMCs and their ability to synthesize extracellular matrix (Davies et al., 1993). Indeed, VSMC cell death results in multiple features of plaque vulnerability, including thinning of the fibrous cap, a larger necrotic core, and an overall acceleration of plaque growth (Clarke and Bennett, 2006; Clarke et al., 2008). Increased levels of VSMC apoptosis are also seen in mature plaques when compared to control vessels (Geng and Libby, 1995), and in unstable versus stable plaques or patients (Bauriedel et al., 1999). VSMC apoptosis has also been associated with numerous other features within plaques including inflammation, calcification, thrombosis (reviewed in Littlewood and Bennett, 2003) and aneurysm formation (Lopez-Candales et al., 1997). EC death is also implicated in both atherogenesis and plaque erosion (Durand et al., 2004). Similarly, macrophage apoptosis can be identified in advanced lesions. Cell death can be induced by ROS and oxidised lipids in vascular cells, whereas p53 expression in an experimental neointima can induce apoptosis and promote plaque rupture (von der Thüsen et al., 2002).

### 4.2 Microvascular disease

The microcirculation provides the interface for the delivery of oxygen and nutrients and the removal of waste products and carbon dioxide (Tooke and Lowe, 1996). Organs, tissues and most cells are dependent on adequate perfusion by the microcirculation, and adequate microvessel function is therefore essential (Tooke and Lowe, 1996). As such, changes in microvessel function with age may have important consequences for organ function. In ageing, upregulation of pro-oxidants and downregulation of antioxidants results in an increase in ROS (Li and Fukagawa, 2010; Ungvari et al., 2010) and development of vascular dysfunction in both animal models and in humans (Moon et al., 2003). Abnormalities of the microcirculation have been reported in a number of disease states associated with ageing, including hypertension (Feihl et al., 2006; Tooke et al., 1991), insulin resistance (Serné et al., 2007) and type I and II diabetes (Tooke et al., 1996). However, in contrast to macrovascular disease, there are few studies defining the role, if any, of DNA damage in microvascular disease. Age-related impairment of endothelial function has been described, but currently our understanding of whether ageing affects other vascular cell types such as VSMCs and pericytes in the microvasculature is incomplete.

#### 4.2.1 Microvascular dysfunction and DNA damage in ageing

##### 4.2.1.1 Endothelial cells

Microvascular endothelial dysfunction manifests as reduced angiogenic capacity and impaired vasodilatory function, in large part because of reduced nitric oxide (NO) bioavailability. Indeed, decreased NO bioavailability caused by impaired endothelial NOS (eNOS) activity is one of the causes of age-related microvascular endothelial dysfunction in animal models (Chatziantoniou et al., 1998; Scioli et al., 2014). Consequently, vasodilatory responses decline with age in coronary, skeletal, and cerebral vascular beds (Bauersachs et al., 1998; Farkas et al., 2006; Jayaweera et al., 1999; Kuo et al., 1990; Scioli et al., 2014; Viner et al., 1996). Although there are few studies that identify whether DNA damage *per se* induces these features, late-generation mice lacking the Telomerase RNA component (TERC) have short telomeres and display reduced angiogenesis in both Matrigel implants and murine melanoma grafts (Franco et al., 2002), suggesting that telomere attrition leads to microvascular EC senescence, which likely impairs angiogenesis in the aged organism. However, it is unclear whether the normal telomere attrition with ageing is sufficient to impair microvascular function.

Oxidative stress in ageing is associated with an increased rate of endothelial apoptosis (Csiszar et al., 2004; Pearson et al., 2008), which may also contribute to microvascular rarefaction impairing the blood supply of the heart (Anversa et al., 1994) and brain (Sonntag et al., 1997). Cerebrovascular ECs are rich in mitochondria, and normal mitochondrial function is essential for maintaining the integrity of the blood–brain barrier (Dai et al., 2012). However, mitochondrial-derived ROS may also reduce endothelium-dependent vasodilation (Gao et al., 2009; Wenzel et al., 2008), suggesting that age-related mitochondrial dysfunction may contribute to breakdown of the blood–brain barrier, promoting neuroinflammation in ageing (Farrall and Wardlaw, 2009). Further studies are required, however, to determine whether agents that reduce mitochondrial oxidative stress are able to prevent the development of endothelial apoptosis, improve vasodilator function in the aged vasculature, and/or maintain the integrity of the blood brain barrier.

##### 4.2.1.2 Smooth muscle cells and pericytes

There are marked changes in multiple vascular cell types in older versus younger individuals, including in VSMCs (Hariri et al., 1988; Stemerman et al., 1982). A higher proportion of senescent VSMCs, characterised by a flattened shape, abnormal nuclear morphology and positive staining for the SAβG, are observed in primary medial VSMCs isolated from peripheral arteries of aged human donors compared to younger controls (Ragnauth et al., 2010). However, the exact mechanisms and

direct effects of VSMC senescence in microvasculature ageing and disease remain unclear and require further investigation. The effect of age on VSMC apoptosis has also not been extensively studied, although increased VSMC apoptosis with age has been observed in mouse VSMCs *in vitro* (Wang et al., 2011). In contrast, reduced vulnerability to apoptosis has been reported in isolated rat VSMCs challenged with elevated glucose (Fukagawa et al., 2001).

Pericytes, the mural cells of capillaries, play an important role in vessel stabilisation, by regulating endothelial cell proliferation and stabilising nascent vessels (Gerhardt and Betsholtz, 2003). Previous work reported a reduction in pericyte number in aged capillaries (Kovacic et al., 2011). However, although suggestive that alterations in pericytes with ageing contribute to development of age-related morphological and physiological abnormalities of the microvasculature, their precise role is unproven.

**4.2.2 Microvascular endothelial dysfunction and DNA damage in disease**

Diabetes is strongly associated with both macrovascular complications, including atherosclerosis, and microvascular complications, including retinopathy, nephropathy, and neuropathy. Diabetic microvascular complications are caused by prolonged exposure to high glucose levels, termed hyperglycaemia (Control and Group, 1993; Group, 1998) and oxidative stress is considered a crucial mediator of its damaging effect (Giugliano et al., 1996; Nishikawa et al., 2000). For example, monocytes from type 2 diabetes patients display significantly shorter telomeres compared with control subjects and telomere length correlated inversely with oxidative DNA damage (Sampson et al., 2006). Several studies have shown accumulation of 8-oxoG and increased oxidative DNA damage in leukocytes of patients with diabetes (Choi et al., 2005; Dinçer et al., 2002; Hinokio et al., 1999; Pitozzi et al., 2003; Sampson et al., 2006), and higher levels of 8-hydroxyguanine (8-OHG), a precursor to 8-oxoG, are found in the serum of diabetic patients with advanced microvascular complications, including proliferative diabetic retinopathy and advanced nephropathy (Shin et al., 2001). Similarly, urinary 8-oxoG or 8-OHG has been reported as a biomarker of oxidative DNA damage in diabetic nephropathy patients, although which cell type within the kidney sustaining oxidative damage is unknown (Hinokio et al., 1999; Tatsch et al., 2015; Wu et al., 2004; Xu et al., 2004). Interestingly, treatment using a variety of antioxidant compounds can reduce retinopathy and neuropathy in human and experimental diabetes (DeRubertis et al., 2007; Kowluru et al., 2006; Zhang et al., 2006), suggesting that ROS are involved in their pathogenesis.

Previous studies have shown that hyperglycaemic culture conditions induce EC apoptosis and generation of ROS (Ceriello et al., 2002; Inoguchi et al., 2000). Similarly, compared to controls, cells cultured in high glucose conditions show increased oxidative DNA damage (Kasai et al., 1986). In addition, oxidative damage occurs in the cerebral microvessels of a diabetic mouse model (Liao et al., 2005), and mitochondrial 8-oxoG and mtDNA with a 4,834-bp deletion are significantly increased in the kidneys of diabetic rat models (Kakimoto et al., 2002). Furthermore, diabetes induces mitochondrial damage in the retina and mitochondrial dysfunction is considered to play a significant role in the development of retinopathy. For example, high glucose exposure of retinal ECs induces mtDNA damage and affects base excision repair enzyme machinery, which functions to repair oxidative DNA damage (Madsen-Bouterse et al., 2010). Although together these data suggest that DNA damage and microvascular complications in diabetes co-exist, it is unclear whether nuclear or mitochondrial DNA damage, mitochondrial dysfunction, or increased ROS actually cause the microvascular dysfunction that occurs in diabetes. Similarly, the mechanism of action of DNA damage and microvascular dysfunction is not known, although a recent study showed increased SA$\beta$G reactivity and accelerated cell senescence in the retinas of aged and diabetic rat models compared to controls (Lamoke et al., 2015).

Increased oxidative DNA damage has also been described in other chronic diseases associated with atherosclerosis and vascular dysfunction. For example, 8-oxoG is elevated in leukocytes and sera of patients with rheumatoid arthritis (RA)(Bashir et al., 1993; Hajizadeh et al., 2003), whilst premature telomere shortening is observed in RA bone marrow-derived progenitor cells (Colmegna et al., 2008; Koetz et al., 2000; Schönland et al., 2003). RA tissue also displays evidence of microsatellite instability reflecting on-going mutagenesis (Lee et al., 2003). Such mutagenesis is normally corrected by DNA repair systems including the mismatch repair (MMR) system; however, the MMR system appears defective in RA (Lee et al., 2003). Furthermore, mtDNA damage has been implicated in inflammatory arthritis; studies demonstrate that extracellular mtDNA is increased in RA synovial fluid and plasma (Hajizadeh et al., 2003) and that oxidatively damaged mtDNA can induce murine arthritis (Collins et al., 2004). Studies have also shown that end-stage renal disease, which may be due in part to small vessel disease (Kang et al., 2002; Nakagawa et al., 2003), is associated with oxidative stress (Palleschi et al., 2007; Vaziri, 2004). Increased DNA damage in peripheral lymphocytes of patients with chronic kidney disease, studied by comet assay, correlated with severity of renal impairment (Stopper et al., 2001).

Finally, idiopathic pulmonary arterial hypertension (IPAH) is a life-threatening disorder characterised by progressive loss of pulmonary microvessels (Rabinovitch, 2008). Whole-exome sequencing has identified topoisomerase DNA binding II binding protein 1(TopBP1) as a key gene involved in protecting the pulmonary endothelium against injury (de Jesus Perez et al., 2014). TopBP1 has been implicated in initiation of DNA replication and the response to DNA damage (Kumagai et al., 2006; Nam and Cortez, 2011). Compared with healthy controls, IPAH pulmonary microvascular ECs display increased phosphorylated $\gamma$-H2AX foci per nuclei, indicative of DNA strand breaks, which inversely correlate with levels of TopBP1 in each cell type (de Jesus Perez et al., 2014).

Apoptosis of microvascular ECs has also been shown in response to chemotherapy and radiation therapy (Garcia-Barros et al., 2003; Geng and Libby, 1995; Paris et al., 2001), where it is likely to be at least in part due to induction of DNA damage. For example*, in vitro* treatment of human umbilical vein ECs with the chemotherapeutic agent Pemetrexed or irradiation induced cell apoptosis and decreased microvessel density in tumour models *in vivo*. Combined therapy induced cumulative effects on EC death. Microvascular endothelial apoptosis has also been demonstrated to play a critical role in radiation therapy-induced complications, such as pneumonitis (Fuks et al., 1994) and, more recently, gastrointestinal tract damage (Paris et al., 2001). Endothelial apoptosis has been observed within the central nervous system following radiation therapy, and a recent study suggests that early apoptosis of the microvascular EC population initiates the development of acute blood brain barrier disruption after radiation therapy (Li et al., 2003).

## 5 Endogenous and exogenous causal agents in vascular disease

### 5.1 Oxidative stress

A crucial mechanism involved in driving atherosclerosis and ageing-associated microvascular dysfunction is oxidative stress, a state in which the generation of ROS exceeds cellular antioxidant defence systems, resulting in DNA damage, cellular dysfunction and apoptosis. ROS include the superoxide anion ($*O2^-$), hydrogen peroxide, hydroxyl radical, peroxynitrite and lipid peroxides, with their reactivity and half-life varying according to species. Major sources of cellular ROS production are the mitochondria, peroxisomes and cytochrome p450 enzymes.

ROS can cause lipid peroxidation, protein damage, and can induce several types of DNA lesions to both nuclear and mitochondrial DNA, including oxidative base modifications, SSBs and DSBs. Increased intracellular ROS induces oxidative damage to mitochondrial DNA which leads to mitochondrial dysfunction, while hydrogen peroxide induces mitochondrial DNA damage and

dysfunction in ECs and VSMCs (Ballinger et al., 2000). As mentioned previously, mtDNA damage correlates with the extent of atherosclerosis observed in human aortas and aortas from $ApoE^{-/-}$ mice, whilst disruption of manganese superoxide dismutase, a mitochondrial antioxidant enzyme, show increased mtDNA damage and accelerated atherogenesis at arterial branch points (Ballinger et al., 2002). Oxidative stress is also associated with accelerated telomere shortening and subsequently reduced telomere length, leading to premature cellular senescence (Matthews et al., 2006).

Although the presence of ROS within the atherosclerotic plaque is not disputed, the major target cells of ROS *in vivo* are unclear. Reactive oxygen species are known to induce apoptotic cell death in various cell types, including endothelial and smooth muscle cells, suggesting a role for ROS in vascular cell apoptosis. Indeed, multiple studies show that addition of exogenous ROS or stimulation of ROS formation in ECs by pro-atherosclerotic or pro-inflammatory factors promotes apoptotic cell death of ECs (reviewed in Dimmeler and Zeiher, 2000). In vitro, $H_2O_2$ addition or generation of $H_2O_2$ by glucose/glucose oxidase can indeed stimulate apoptosis of VSMC (Li et al., 1997a, b). Furthermore, the pro-apoptotic protein p53 upregulates the levels of intracellular ROS, suggesting that ROS may also be involved in intracellular pro-apoptotic signalling (Johnson et al., 1996). Increased oxidative stress is also a major feature of Type II diabetes, a major risk factor for atherosclerosis. For example, circulating mononuclear cells from patients with type II diabetes exhibit increased production of reactive oxygen species (Orie et al., 2000) and enhanced lipid peroxidation (Ahmed et al., 2006) resulting in DNA damage (Song et al., 2007) and mitochondrial dysfunction.

Cells have evolved protection strategies against oxidative damage relying on anti-oxidant enzymes such as superoxide dismutases (SODs), which convert the superoxide radical to hydrogen peroxide and water, and glutathione peroxidase, which scavenges and removes ROS from the cellular environment. As normal by-products of metabolism, ROS are a potential source of chronic, persistent DNA damage in all cells, which may also contribute to ageing (Sohal and Weindruch, 1996). For example, levels of 8-oxoG accumulate with age (Hamilton et al., 2001) and in models of atherosclerosis (Martinet et al., 2001). Indeed, normalization of ROS has been shown to prevent age-related vascular dysfunction (Francia et al., 2004), in part through JunD, a member of the activated protein-1 family of transcription factors (Paneni et al., 2013). For example, *in vivo* genetic deletion of JunD was associated with premature endothelial dysfunction and vascular aging via ROS generation (Paneni et al., 2013). The mitochondrial adaptor protein $p66^{Shc}$ also plays a role in redox metabolism and is a key determinant of ageing (Camici et al., 2015; Migliaccio et al., 1999); genetic deletion of $p66^{Shc}$ in mice lowers ROS levels and oxidative DNA damage and prolongs lifespan by 30% (Migliaccio et al., 1999; Napoli et al., 2003).

**5.2 Cytotoxic cancer therapeutics and radiotherapy**

Chemotherapeutics and radiotherapy target the mechanisms involved in regulating DNA and cell replication. For example, alkylating agents such as cyclophosphamide prevent cell division by introducing new bonds into the DNA structure (Colvin, 1999). These agents can also induce ROS generation that in turn increases the levels of DNA damage and mitochondrial injury (Velez et al., 2011). Similarly, radiation therapy directly induces single-strand breaks and double-strand breaks, resulting in cellular growth arrest and apoptosis. In cancer, the increased proliferative capacity of tumour cells results in incomplete DNA repair after radiotherapy, resulting in consequent damage accumulation through multiple divisions.

Although chemo- and radiotherapy are widely used in the treatment of cancer, both modalities have side effects owing to extensive DNA damage to 'off-target' healthy cells, including vascular cells (Curigliano et al., 2010). Indeed, there is now strong causative evidence between DNA damage-inducing treatments for cancer and cardiovascular disease from large clinical cohort studies (Aleman et al., 2007; van den Belt-Dusebout et al., 2006). Cytotoxic chemotherapy and ionising radiation

induce DNA damage and cell death in ECs and VSMCs, with subsequent endothelial dysfunction and inflammation, resulting in cell senescence, apoptosis, thrombosis formation, mitochondrial dysfunction and fibrosis, all of which promote vascular disease.

## 6 Therapeutic targeting of vascular ageing

The consequences of macrovascular and microvascular ageing contribute directly to disease, and therefore become targets for both drug therapy and amelioration of risk factors. However, none of these approaches are either specific or particularly effective alone, and at present, a combination of approaches should be recommended.

### 6.1 Anti-oxidant therapy

Expression of naturally occurring anti-oxidants is reduced with age and in vascular disease (Collins et al., 2009), and ROS are increased in vascular disease and can induce premature ageing. Antioxidants could therefore be beneficial as potent therapeutics to prevent cardiovascular diseases and ageing **(Fig. 2)**. Indeed, numerous studies have shown that antioxidant treatment with various agents reduces the development of atherosclerosis in hypercholesterolemic animal models (Steinberg and Witztum, 2002), and epidemiological data in humans also supports a protective role for antioxidant supplementation (Jha et al., 1995). Despite this, prospective clinical trials with multiple antioxidant vitamins, such as vitamins C, E and beta carotene, in patients with pre-existing atherosclerosis have thus far been disappointing (Yusuf et al., 2000). Although there may be many reasons for this, including pharmacokinetic profiles and penetration of agents, antioxidants currently have no place in established guidelines for treatment or prevention of large artery disease.

In contrast, anti-oxidant agents have showed more promise in reducing the progression of end-organ microvascular dysfunction. Antioxidants and free radical scavengers such as ascorbic acid and N-acetyl-cysteine showed clinical efficacy in patients with endothelial dysfunction by inducing beneficial effects on oxidative stress and vascular dysfunction (May and Harrison, 2013; Ozkanlar and Akcay, 2012; Radomska-Leśniewska et al., 2006)**(Fig. 2)**. Similarly, Propionyl-L-carnitine has also been reported to modulate nuclear factor (NF)-κB activity in vascular cells and to reduce age-related microvascular dysfunction and post-ischaemic endothelial dysfunction (Orlandi et al., 2007), and ascorbate is essential for normal endothelial function and prevents microvascular dysfunction and hydrogen peroxide-mediated injury in cultured microvascular ECs (Stasi et al., 2010). Other natural substances such as resveratrol have been reported to minimise oxidative stress and stimulate endothelial NO generation (Csiszar et al., 2009b; Csiszar et al., 2008). *In vitro* studies suggest that the molecular mechanisms of resveratrol-mediated vasoprotection involve NF-κB inhibition, upregulation of eNOS and antioxidant enzyme levels, and the prevention of oxidative stress–induced apoptosis (Csiszar et al., 2009b; Csiszar et al., 2008). Together these studies suggest that antioxidant regimens could be effective in counteracting adverse clinical effects of age-related microvascular endothelial dysfunction, although proof will require large randomised controlled studies.

### 6.2 Changes of lifestyle, anti-atherosclerotic and anti-ischemic treatments

Diet and nutritional status are associated with atherosclerosis and age-related vascular changes (Fontana et al., 2012). Manipulating diet via caloric restriction (CR) has also been shown to have beneficial effects on vessel ageing *in vivo* (Fornieri et al., 1999; Guo et al., 2001; Rippe et al., 2010; Zanetti et al., 2010), with improved EC function through augmenting NO generation, reducing inflammation and reducing oxidative stress by upregulating antioxidant expression and protecting mitochondrial function (Csiszar et al., 2009a; Ungvari et al., 2008)(**Fig. 2)**. In particular, the sirtuins (SIRT1–7) are a family of nicotinamide adenine dinucleotide $(NAD)^+$-dependent deacetylases and adenosine diphosphate–ribosyltransferases that may be partially responsible for the age-delaying effects of CR. In some experimental models CR increases SIRT1, leading to improved endothelial

function (Rippe et al., 2010; Zanetti et al., 2010), while knocking down SIRT1 disrupts CR-mediated antioxidant and anti-inflammatory vascular effects (Csiszar et al., 2009a).

Physical activity and regular exercise training has been shown to play a significant role in the prevention of cardiovascular disease (Barry et al., 2014; Joyner and Green, 2009; Morris et al., 1953). Physical activity and regular exercise training is anti-atherogenic (Szostak and Laurant, 2011) and reduces oxidative stress through upregulation of antioxidants, such as SOD (Fukai et al., 2000; Miyazaki et al., 2001). Regular exercise also promotes mitochondrial health, which may also contribute to its vasoprotective properties during ageing **(Fig. 2)**. For example, a recent study on mitochondrial mutator mice ($Polg^{m/m}$), shows that endurance exercise induces mitochondrial biogenesis, prevents mtDNA mutations, increases mitochondrial oxidative capacity and blunts pathological levels of apoptosis in multiple tissues, including the heart (Safdar et al., 2011). However, further clinical studies are needed to determine whether mitochondrial revival through exercise is an effective therapeutic approach to mitigate vascular mitochondrial dysfunction associated with "healthy" macrovascular ageing in humans. Similarly, it has been reported that skin microvessel function is worse in older sedentary compared with older active or younger men, and this was attributable to impaired nitric oxide signalling (Black et al., 2008). The mechanisms by which exercise exerts its beneficial endothelial effects also include attenuation of hyperlipidaemia and protection against inflammatory tumour necrosis factor (TNF)-α-induced vascular impairment (Ungvari et al., 2010). However, exercise has also been reported to attenuate the age-related decline in vessel endothelial function through a nitric-oxide-dependent mechanism (DeSouza et al., 2000).

The Hydroxy-methylglutaryl-coenzyme A reductase inhibitors (statins) reduce progression of established plaques, and cardiovascular risk. Besides their well-documented primary role in reducing cholesterol synthesis, statins improve endothelial function by lipid-independent, anti-inflammatory and antioxidant properties and the capacity to restore microvascular NO availability (Bonetti et al., 2003). For example, Statins target mechanisms inducing premature ageing, such as increasing telomere protection through induction of TRF2 (Spyridopoulos et al., 2004), decreasing DNA damage by accelerating DNA damage repair (Pernice et al., 2006)**(Fig. 2**), and suppressing oxidative stress by reducing NAD(P)H oxidase activation and superoxide production (Schupp et al., 2008). Indeed, statins are associated with higher telomerase activity and longer telomere length in peripheral blood mononuclear cells when compared with control subjects not receiving statin treatment (Boccardi et al., 2013). Statins accelerate DNA repair via NBS1 stabilization and more rapid ATM and H2AX phosphorylation (Mahmoudi et al 2008), suggesting that the anti-atherogenic action of statins may be partly ascribed to their ability to provide protection against genomic damage.

The role of the pro-protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, another type of LDL-lowering drug, has not thus far been studied in the context of vascular function and ageing. However, PCSK9 is associated with inflammation and endothelial cell apoptosis, suggesting that PCSK9 inhibitors may improve endothelial function. Indeed, knockdown of PCSK9 reduces ox-LDL-induced apoptosis of human ECs, implying that PCSK9 can impair EC survival (Wu et al., 2012), and PCSK9 is markedly induced in EC sand VSMCs by inflammatory stimuli such as LPS, TNFα and oxLDL via the NF-κB signalling pathway (Ding et al., 2015). In VSMCs, knockdown of PCSK9 was associated with reduced mtROS, whilst mtROS itself induces PCSK9 levels (Ding et al., 2015), which may also affect downstream mtDNA damage and inflammatory pathways, and thus play a role in both microvascular ageing and macrovessel disease. Although atherosclerotic plaques do show PCSK9-positive VSMCS (Ding et al., 2015), further clarification of the functional role of PCSK9 in vascular cell types is required.

**7. Conclusions and future perspectives**

Research focused on understanding the prevalence and repair of the many forms of DNA damage in human tissues will advance our understanding of DNA repair, but also of human ageing and

progression of cardiovascular disease. Further insight into the basic biology of DNA damage and repair is therefore fundamental to identifying targets for novel therapeutic approaches to antagonise premature vascular ageing. The new therapeutic strategies can be addressed in different directions. For example, therapies aimed at both reducing DNA damage and potentiating the DDR, as observed with statins, may be effective in reducing vascular ageing.

The role of DNA damage in macrovascular ageing and disease is now well established. Atherosclerosis is associated with widespread DNA damage and both nuclear and mitochondrial DNA damage promote atherosclerosis. However, our understanding of the mechanisms by which DNA damage promotes atherosclerosis is still limited. Challenges for the future would be to determine how the activity of DDR proteins is regulated and why DDR impacts cellular function in vascular systems. In contrast, the evidence for DNA damage and its consequences in the microvasculature is far more limited, but no less important. Ageing elicits several structural and functional changes within the microvasculature. ROS and the concomitant oxidative stress play an important role in the process of microvascular disease. Although progress has been achieved in describing the age-related alterations of microvascular function, major gaps in our knowledge exist as to whether DNA damage mechanisms mediate this loss of function and how 'aged' cells promote disease. Understanding the mechanisms contributing to such changes in function is therefore crucial for both the prevention and the development of treatment for microvascular disease.

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**Legends to Figures**

**Fig. 1 Types of DNA damage that accumulate during ageing and vascular disease and their associated DNA repair pathways**
Extrinsic or intrinsic insults can stimulate a variety of DNA lesions, from single-strand breaks to telomere attrition. Key mediator proteins involved in each DNA damage repair mechanism are shown. Both nuclear DNA and mitochondrial DNA undergo age-associated genomic alterations. Excessive DNA damage or insufficient DNA repair favours the ageing process and can be a cause or consequence of vascular disease. HR, homologous recombination; NHEJ, non-homologous end joining. Adapted from (Lord and Ashworth, 2012).

**Fig. 2 Major components that contribute to age-related accumulation of DNA damage and subsequent consequences that lead to macro- and microvascular disease**
Evidence for DNA damage in macro- and microvascular disease (left), common manifestations of ageing in vascular cells (middle), and strategies to target and reverse these mechanisms (right). Age- and disease-related increases in oxidative stress and decline in DNA repair activity cause accumulation of DNA damage. DNA damage results in apoptosis or cell senescence, which in turn can lead to a senescence-associated secretory phenotype (SASP) and activate inflammatory processes. Ultimately vascular function declines, which leads to increased cardiovascular risk. Adapted from (Oh et al., 2014).