Nucleotide Excision DNA Repair Is Associated With Age-Related Vascular Dysfunction

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Background—Vascular dysfunction in atherosclerosis and diabetes mellitus, as observed in the aging population of developed societies, is associated with vascular DNA damage and cell senescence. We hypothesized that cumulative DNA damage during aging contributes to vascular dysfunction.

Methods and Results—In mice with genomic instability resulting from the defective nucleotide excision repair genes Ercc1 and Xpd (Ercc1<sup>−/−</sup> and Xpd<sup>−/−</sup> mice), we explored age-dependent vascular function compared with that in wild-type mice. Ercc1<sup>−/−</sup> mice showed increased vascular cell senescence, accelerated development of vasodilator dysfunction, increased vascular stiffness, and elevated blood pressure at a very young age. The vasodilator dysfunction was due to decreased endothelial nitric oxide synthase levels and impaired smooth muscle cell function, which involved phosphodiesterase activity. Similar to Ercc1<sup>−/−</sup> mice, age-related endothelium-dependent vasodilator dysfunction in Xpd<sup>−/−</sup> animals was increased. To investigate the implications for human vascular disease, we explored associations between single-nucleotide polymorphisms of selected nucleotide excision repair genes and arterial stiffness within the AortaGen Consortium and found a significant association of a single-nucleotide polymorphism (rs209298) in the putative promoter region of DDB2 gene with carotid-femoral pulse wave velocity.

Conclusions—Mice with genomic instability recapitulate age-dependent vascular dysfunction as observed in animal models and in humans but with an accelerated progression compared with wild-type mice. In addition, we found associations between variations in human DNA repair genes and markers for vascular stiffness, which is associated with aging. Our study supports the concept that genomic instability contributes importantly to the development of cardiovascular disease. (Circulation. 2012;126:468-478.)

Key Words: aging cardiovascular diseases endothelium nitric oxide synthase vasodilation

Vascular and endothelial function deteriorates with age and is considered a key factor in the development and progression of age-related cardiovascular disease (CVD).<sup>1−3</sup>

The high prevalence of CVD-related mortality resulting from increasing life expectancy highlights the necessity of understanding how aging influences vascular function. Currently, aging is viewed as a consequence of the prolonged exposure to risk factors, eg, an unfavorable lipid profile, smoking, and...
diabetes mellitus, during which accumulation of damage increases the risk of developing vascular dysfunction and associated disease. At the cellular level, this could be related to the increased production of reactive oxygen species (ROS) and a resulting increase in lipid oxidation or interference with cellular metabolism. This can directly affect vascular function and/or lead to apoptosis or cellular senescence, a state in which the cell remains in cell cycle arrest and has lost its optimal function. In the case of endothelial cells, this functional change results in a provasoconstrictor and proinflammatory phenotype.

The concept that unrepaired DNA damage has dramatic effects on the aging phenotype stems from multiple lines of evidence, including the fact that the majority of human progeroid syndromes are due to mutations in DNA repair and response genes. One of the DNA repair systems that is very important in this regard is nucleotide excision repair (NER), which removes a wide class of helix-distorting DNA lesions induced by ultraviolet (UV) but also by numerous manmade or natural chemical compounds and ROS. The process of DNA damage removal consists of (1) DNA damage recognition by XPC for some lesions assisted by the UV-DDB1/2 (XPE) complex, (2) local unwinding of DNA provided by the multisubunit TFIHH complex, (3) damage verification by XPA, (4) excision of the damaged DNA section by endonucleases XPG and ERCC1/XPF, and (5) replacement of the excised DNA using the intact strand as template. Mutation of factors involved in NER can have severe consequences for human health as demonstrated by several human progeroid syndromes. Examples are the rare autosomal-recessive genetic disorders Cockayne syndrome, trichothiodystrophy, and the recently described XP-E/ERCC1 syndrome. For instance, trichothiodystrophy is caused by point mutations in the XPD, XPF, or TTDA gene, affecting DNA repair function and stability of the dual-functional NER/basal transcription initiation factor TFIH. This causes UV sensitivity and accelerated segmental aging symptoms, including early cessation of growth, cachexia, osteoporosis, progressive neurological abnormalities, and premature death. Likewise, several mouse models with NER defects show a segmental premature aging phenotype in which the severity depends on the extent to which the DNA repair system is affected.

To investigate whether DNA damage plays a role in age-related vascular dysfunction, we studied vasomotor function and cellular senescence in 2 NER-defect mouse models, differing in type and severity of the DNA repair defect. In Ercc1<sup>−/−</sup> animals, 1 allele of the NER-DNA crosslink repair (XLR) endonuclease ERCC1 is mutated, resulting in a truncated protein (lacking the C-terminal 7 amino acids), and the other allele is completely inactivated. In Xpd<sup>TTD</sup> mice, the XPD helicase of the TFIH core complex carries a homozygous R<sup>223</sup>W functional point mutation as found in a trichothiodystrophy patient. The NER-XLR defect in the Ercc1<sup>−/−</sup> animals is more severe, resulting in very early cessation of growth; very premature liver, kidney, bone marrow, and neurological aging phenotype; and a reduced lifespan of ≈5 to 6 months. The milder phenotype of Xpd<sup>TTD</sup> mice results in retarded growth, cachexia, an age-related osteoporosis, and a slightly reduced lifespan.

To investigate whether NER gene variations could have an impact on human vascular disease and in line with our murine phenotype, we performed genetic studies to examine the association of genetic variation in genes coding for proteins involved in NER with carotid-femoral pulse wave velocity (CFPWV). The associations between genetic variation in selected NER genes and the vascular phenotype were assessed within the framework of the AortaGen Consortium.

### Methods

**Clinical Perspective on p 478**

Despite extensive research into oxidative stress–induced cellular damage and senescence, the main causative mechanism of aging and age-related CVD remains unknown. For instance, it is unclear why vasomotor function declines with aging, even in the absence of apparent risk factors. Moreover, in the vast majority of epidemiological studies, aging remains the most significant risk factor for CVD, even after correction for classic cardiovascular risk factors.

For details of the experimental setup, see the online-only Data Supplement.

**Animals**

The animals used in experiments were 8- and 16-week-old Ercc1<sup>−/−</sup> mice, their wild-type (WT) littermates of the same age, and 16-, 26-, and 52-week-old mice of the same background—F1 hybrid between FVB and C57Bl/6 and 26- and 52-week-old Xpd<sup>TTD</sup> mice and their WT controls in a C57Bl/6 background. All animal studies were approved by an independent animal ethics committee.

**Isolation and Culture of Endothelial Cells**

Endothelial cells were isolated from 16-week-old Ercc1<sup>−/−</sup> mice and cultured under mouse lung endothelial cell medium under an atmosphere of normal air enriched with 5% CO<sub>2</sub>.

**Senescence-Associated β-Galactosidase Staining**

Senescence was determined by senescence-associated β-galactosidase staining (SA-β-gal staining) at pH 6.0.

**Quantitative Real-Time Polymerase Chain Reaction**

Relative expression of cyclin-dependent kinase inhibitor 1A (p21) and tumor protein 53 (p53) genes was measured in thoracic aortas of 16-week-old Ercc1<sup>−/−</sup> and WT mice.

**Assessment of Blood Pressure and Vasodilator Function In Vivo**

In vivo hind-leg vasodilator function was measured by laser Doppler perfusion imaging of reactive hyperemia after transient blood flow interruption in 8-week-old Ercc1<sup>−/−</sup> and WT mice. Simultaneously, blood pressure was measured in conscious Ercc1<sup>−/−</sup> mice and WT littermates with the tail cuff technique.

**Organ Bath Experiments**

The responses of aortic tissue from 8- and 16-week-old Ercc1<sup>−/−</sup> mice and their 8-, 16-, 26-, and 52-week-old WT littermates, as well as 26- and 52-week-old Xpd<sup>TTD</sup> mice and their WT littermates, were measured in small wire myograph organ baths containing oxygenated Krebs-Henseleit buffer at 37°C. After preconstriction with 30 nmol/L U46619, relaxation concentration-response curves to acetylcholine were constructed, followed by an exposure to 100 µmol/L sodium nitroprusside. N<sup>ω</sup>-nitro-L-arginine-methyl ester (L-NAME) 100 µmol/L pretreatment was used to investigate the involvement of nitric oxide (NO). 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol; 100 µmol/L) and N-acetyl-cysteine (NAC; 50 µmol/L) were used as scavengers of ROS. Tetrahydrobiopterin (BH₄;...
100 µmol/L) was used to prevent the uncoupling of endothelial NO synthase (eNOS). Vinpocetine (100 µmol/L) was used to investigate phosphodiesterase activity.

**Measurement of Mechanical Properties of the Vascular Wall**

Carotid arteries were explanted from 16-week-old Ercc1<sup>−/−</sup> and WT mice and mounted in the perfusion myograph. The vessel diameter–pressure relationship was determined and stress-strain relationships were constructed.13

**Immunoprecipitation and Immunoblotting of eNOS**

Aortas and hearts were used to investigate the levels of eNOS and the fraction of eNOS phosphorylated at position Tyr-657. Ser-1177 eNOS phosphorylation was investigated in lungs either at baseline or after 10 minutes of stimulation with 10 µmol/L acetylcholine.

**Statistical Methods of Animal Studies**

Data are presented as mean±SEM. Statistical analysis between the groups of single values was performed by 2-sided t test, 2-sided t test after log transformation of the data, Mann–Whitney U test, or 1-way ANOVA followed by the Bonferroni post hoc test when appropriate. To test the hypothesis that blood pressure would be increased in Ercc1<sup>−/−</sup> animals, we used a 1-sided t test. Differences in dose–response curves were tested by ANOVA for repeated measures (sphericity assumed). Differences were considered significant at P<0.05.

**Human Studies**

In accordance with the phenotype observed in mice, we investigated the association of single-nucleotide polymorphisms (SNPs) in NER components with CFPWV, a measure of vascular stiffness. CFPWV, a well-known marker of age-related vascular disease in humans, is strongly associated with increased risk for major CVD events. To investigate this association, we used the data from the AortaGen Consortium, which consists of 20 634 participants from 9 cohort studies. A detailed description of the AortaGen Consortium is provided in the online-only Data Supplement.

**Statistical Methods of Human Studies**

Genes coding for NER components that belong to the machinery that binds the DNA to either recognize or repair damage were selected. These were the following NER components: ERCC8 (CSA); ERCC6 (CSB); DDB1; DDB2 (XPE); ERCC1; GTF2H1 (p62); GTF2H3 (p54); GTF2H4 (p52); GTF2H5 (TTDA, TF5); RAD23A (hHR23A); RAD23B (hHR23B); ERCC3 (XPB); XPC; ERCC2 (XPD); ERCC4 (XPF); and ERCC5 (XPG). To test for association with CFPWV, we selected the tag SNPs that cover the variation in the genes of interest 50 kb region around that were nonredundant at a linkage disequilibrium threshold of r<sup>2</sup>≥0.7 using the Tagger program of Haplovew. Our selection resulted in 310 SNPs. We decided a priori on a significance threshold of P<1.61×10<sup>−4</sup>, which corresponds to the Bonferroni-adjusted P value for the number of tested SNPs.

**Results**

**Vascular Cell Aging in Ercc1<sup>−/−</sup> Mice**

SA-β-gal staining of aortas from 16-week-old Ercc1<sup>−/−</sup> mice compared with their WT littermates showed that, macroscopically, senescence staining clearly dominated in aortas from Ercc1<sup>−/−</sup> (Figure 1A). Microscopically, stained cells were detected in both the endothelium and media of Ercc1<sup>−/−</sup> aortas. The quantity and visibility of senescent cells allowed reliable counting in the media, showing a marked increase in Ercc1<sup>−/−</sup> animals (Figure 1B). Similarly, RNA levels of the genes composing DNA damage–related CDK inhibition p21 (Cdkn1a) and p53 (Trp53) were increased in the aorta of
Ercc1<sup>dr−</sup>, however only p21 reached statistical significance (Figure 1C and 1D).

To further investigate the effect of defective NER on proliferative senescence in endothelial cells, we measured the percentage of SA-β-gal-positive cells after 20 days in culture. The levels of SA-β-gal–positive endothelial cells in the lung were on average 10.3 times higher in cultures from Ercc1<sup>dr−</sup> compared with WT mice (Figure 1E and 1F).

On the basis of the robust proliferation of freshly cultured lung endothelial cells, we decided to test endothelial-dependent angiogenic outgrowth potential in aortic explants of 16-week-old Ercc1<sup>dr−</sup> and WT mice. No significant difference in outgrowth was observed (Figure I in the online-only Data Supplement).

**In Vivo Vascular Function of NER-Defective and WT Mice**

To determine possible functional changes in the vasculature, we assessed vasodilator function in response to reactive hyperemia in the hind limbs of 8-week-old WT (Figure 2A) and Ercc1<sup>dr−</sup> mice (Figure 2B). In 8-week-old Ercc1<sup>dr−</sup> animals, we observed a decreased reactive hyperemia (Figure 2C and 2D). In addition, we observed a significant increase in systolic pressure, mean arterial pressure, and pulse pressure in Ercc1<sup>dr−</sup> mice (Figure 2E–2H). Diastolic blood pressure tended to increase, albeit without statistical significance (Figure 2G).

**Age-Dependent Change of Ex Vivo Vascular Function in WT and NER-Defective Mice**

To reveal the mechanisms of vasodilator dysfunction in Ercc1<sup>dr−</sup> mice and to address the question of age dependency, we compared vasodilator function in 8- and 16-week-old mice. At both ages, mice show signs of progeria without obvious deterioration in general health. Ercc1<sup>dr−</sup> animals showed progressive reduction of acetylcholine-induced aortic relaxation at these ages (Figure 3A). Sodium nitroprusside responses were reduced in 16-week-old Ercc1<sup>dr−</sup> mice and
tended to be decreased in 8-week-old Ercc1d∥ mice (Figure 3B). To estimate the contribution of the endothelium to vasodilator dysfunction, acetylcholine-induced relaxations were corrected for sodium nitroprusside responses, revealing that the endothelial contribution to the response to acetylcholine was reduced in Ercc1d∥ mice compared with WT mice at both ages (Figure 3C).

DNA repair–competent WT animals 16, 26, and 52 weeks of age showed a much slower age-dependent reduction in acetylcholine responses than Ercc1d∥ mice, becoming statistically significant after 52 weeks (Figure 3D). Endothelial-independent responses to sodium nitroprusside did not change in WT mice (Figure 3E).

To determine whether a slower onset of progeria could delay the onset of vascular dysfunction, we assessed vascular function in the NER-impaired XpdTTD mouse that displays a milder phenotype. Vasodilator responses to acetylcholine in U46619-precontracted aortic rings were significantly reduced in 52-week-old XpdTTD mice compared with those at 26 weeks and more markedly than in WT littermates (Figure 3F). The noticeable, modest difference between 52-week-old XpdTTD and WT animals did not reach significance. Endothelial-independent responses to sodium nitroprusside were equal (Figure 3G).

Pretreatment with the eNOS inhibitor L-NAME abolished all acetylcholine-induced relaxations in all animals (data not shown), indicating that acetylcholine responses depended entirely on eNOS/NO.

**Mechanisms of Endothelial Vasodilator Dysfunction in NER-Defective Mice**

In aortas from 16-week-old Ercc1d∥ mice, eNOS levels were reduced by ∼67% compared with WT (Figure 4A). Phosphorylation of the tyrosine residue at position 657 (pTyr657-eNOS) inhibits eNOS, and this tyrosine phosphorylation tended to be increased in Ercc1d∥ hearts (Figure 4B).
eNOS-activating phosphorylation of the serine residue at position 1177 (pSer1177-eNOS) was comparable in explanted WT and Ercc1<sup>−/−</sup>/H11002 lungs at baseline, but 10 μmol/L acetylcholine increased pSer1177-eNOS only in WT mice (Figure 4E).

Uncoupling of eNOS results in a switch from NO to ROS production. It can be a consequence of the decreased availability of the essential cofactor BH4, and its reconstitution can restore NO production. BH4 had no effect on the acetylcholine-induced relaxation of aortic rings from WT mice but restored that of rings from Ercc1<sup>−/−</sup>/H11002 mice (Figure 5A). BH4 also increased endothelium-independent sodium nitroprusside responses (Figure 5B) in Ercc1<sup>−/−</sup> mice.

ROS coming from various sources can scavenge NO, thereby leading to vasodilator dysfunction that can be rescued by ROS scavengers. Whereas tempol was without effect (Figure II A and II B in the online-only Data Supplement), NAC caused a modest and significant improvement of the acetylcholine and sodium nitroprusside responses in Ercc1<sup>−/−</sup> aortas (Figure 5C and 5D). After correction for sodium nitroprusside responses, acetylcholine responses were not changed by BH4 or NAC (Figure 5E and 5F). Therefore, the BH4- and NAC-induced improvement in relaxations most likely represents improved vascular smooth muscle cell (VSMC) sensitivity.

**Mechanisms of VSMC Vasodilator Dysfunction in NER-Defective Mice**

The phosphodiesterase inhibitor vinpocetine improved sodium nitroprusside responses in Ercc1<sup>−/−</sup> mice (Figure 6). The phosphodiesterase 5–specific inhibitor sildenafil had similar effects (data not shown; n=5). The responses to an activator of protein kinase G were identical in Ercc1<sup>−/−</sup> mice and WT littermates (n=9 per group; logarithm of concentra-
tion needed to reach 50% dilation, \(-5.268 \pm 0.2353\) versus \(-5.569 \pm 0.1957\), respectively).

Mechanical Properties of Conductive Vessels in Ercc1
d Under similar perfusion pressure increments, differential increases in the vessel lumen were observed in Ercc1
compared with WT mice (Figure 7A). Recalculation of measured values demonstrated a significantly lower strain, indicating lower elasticity, in Ercc1
to acetylcholine (A) and sodium nitroprusside (B) in aortic tissue of 16-week-old Ercc1
and wild-type (WT) mice. E. The acetylcholine responses after correction for individual responses to sodium nitroprusside. The effect of short-term N-acetyl-cysteine (NAC) supplementation on responses of Ercc1
to acetylcholine (C) and sodium nitroprusside (D). Acetylcholine responses expressed as percent of the sodium nitroprusside response (F). \(P<0.05\) (Mann–Whitney U test).

Figure 5. Effects of reactive oxygen species scavenging and prevention of endothelial nitric oxide synthase uncoupling on ex vivo vascular function. Effects of acute tetrahydrobiopterin (BH4) supplementation on responses to acetylcholine (A) and sodium nitroprusside (B) in aortic tissue of 16-week-old Ercc1
and wild-type (WT) mice.

Figure 6. Effect of phosphodiesterase inhibition vinpocetine (Vinpo) on endothelium-independent vasodilatation. Ercc1
mice show reduced vasodilatory response to sodium nitroprusside 10
mol/L compared with wild-type (WT) littermates. Phosphodiesterase blockade abolishes this difference. \(P<0.05\) (t test).

Human Studies
Previous human studies indicate that NER-component SNPs influence the risk of developing cancer, suggesting that these SNPs modulate NER function. Ercc1
mice showed an increased vascular stiffness. We hypothesized that genetic variation in the NER components might also influence vascular stiffness in humans. Increased vascular stiffness is an important feature of human vascular aging, represented by the variable CFPWV. Therefore, we studied the effect of SNPs in NER components that, like ERCC1 and XPD, belong to the machinery that binds the DNA to either recognize or repair damage on variation of CFPWV in the cohorts from the AortaGen Consortium.

The AortaGen Consortium used a sex-specific standardized regression residual for 1000/CFPWV that was adjusted for age, age squared, height, and weight for meta-analysis.
Genome-wide association analyses were conducted with the use of an additive gene-dose model. The results for the association of the genetic variants in the NER components with CFPWV in humans are presented in the Table. The allele dose effect is expressed as standard deviations of inverse CFPWV per coded allele. After Bonferroni correction for multiple testing, 1 locus reached an association with CFPWV at the threshold of \( P = 1.61 \times 10^{-4} \), namely SNP rs2029298 (\( \beta = -0.05 \); SE, 0.01; \( P = 1.04 \times 10^{-4} \)). The closest gene to this SNP is \( \text{DDB2 (XPE)} \). Another SNP in this region, rs3781619, located within the \( \text{DDB2 (XPE)} \) gene, showed a suggestive association with the CFPWV measure (\( \beta = -0.03 \); SE, 0.02; \( P = 3.80 \times 10^{-2} \)). In addition, we found suggestive associations for 8 other SNPs located within or near \( \text{ERCC5 (XPG)}, \text{ERCC6 (CSB)}, \text{GTF2H3}, \text{GTF2H1}, \) and \( \text{ERCC2 (XPD)} \). To summarize, \( \text{DDB2 (XPE)} \) was the most important gene associated with CFPWV.

**Discussion**

The present study shows that mice with an increased susceptibility to DNA damage resulting from a defect in NER as in \( \text{XpdTTD} \) and NER and XLR as in \( \text{Ercc1d} \) display an

<table>
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<tr>
<th>SNP</th>
<th>Chromosome</th>
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<th>Closest Gene</th>
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SNP indicates single nucleotide polymorphism.

* Coded allele is the minor allele. The analyses were adjusted for age, age squared, sex, height, and weight. The allele dose effect is the standard deviation of inverse carotid-femoral pulse wave velocity (CFPWV) per coded allele. Because of the inverse transformation of CFPWV, a negative \( \beta \) represents a higher CFPWV for each dose of the minor allele. For all of the SNPs in the table, \( n = 20,634 \), except for rs2340693, rs9586010, and rs6488885, for which \( n = 16,418 \).
increased number of senescent vascular cells, an increased susceptibility of endothelial cells to become senescent, and accelerated worsening of vasodilator function during aging. The worsening of vasodilator function involves both endothelial cell and VSMC dysfunction. Furthermore, Ercc1<sup>ΔΔ</sup>-mice display increased vascular stiffness, systolic blood pressure, and pulse pressure and reduced reactive hyperemia, which are typical features of vascular aging in humans. In addition, genetic association studies from the AortaGen Consortium suggest that SNPs in NER genes coding for components that participate in DNA recognition and repair contribute to vascular stiffness, as measured by CFPWV, in humans. These results suggest that genomic instability is involved in the development of vascular aging and warrant further investigations into the involvement of the DNA repair systems in age-related CVD.

The involvement of DNA damage and repair in age-related vascular disease is also apparent from previous clinical studies showing that senescent cells and oxidative DNA damage are present in atherosclerotic plaques, the beneficial effects of statins on DNA repair, the increased levels of 8-hydroxy-2,9-deoxyguanosine in urine of hypertensive patients with diabetes mellitus. These SNPs, which are located in the coronary artery disease, intracranial aneurysm, and type 2 diabetes mellitus. These SNPs, which are located in the ANRIL noncoding RNA, lie in the vicinity of the INK4A genes that code for the cyclin-dependent kinase inhibitors p15/Cdkn2B, p16/Cdkn2A, and p14/ARF, which may affect the induction of cellular senescence in response to DNA damage. Deletion of ANRIL in mice reduces Cdkn2a and Cdkn2b expression and increases cultured smooth muscle cell proliferation. Vascular function has not been assessed in these mice. No previous studies have examined vascular senescence parallel to vascular function, but the studies cited here support our hypothesis that DNA damage and cellular senescence contribute to the pathogenesis of age-related CVD.

Ercc1<sup>ΔΔ</sup>-mice exhibited reduced reactive hyperemia, suggesting vasodilator dysfunction in resistance vasculature. Increased peripheral resistance may lead to increased mean arterial pressure, which was observed in Ercc1<sup>ΔΔ</sup>-mice. Because systolic blood pressure also increased, we suspected reduced compliance of conduit vessels. The observation that aortic dilator function was decreased in Ercc1<sup>ΔΔ</sup>- and Xpd<sup>TTD</sup> supports this concept. However, Ercc1<sup>ΔΔ</sup>- dilator function seems to depend on both VSMC and endothelial function, whereas Xpd<sup>TTD</sup> showed no impaired VSMC relaxation. Naturally aging mice also showed endothelial dysfunction without VSMC impairment. Thus, the observed VSMC dysfunction in Ercc1<sup>ΔΔ</sup>- might represent an extreme aging phenotype, as might be expected from a combined defect of NER and XLR. Although the senescence marker prelamin A is found in aortic VSMCs of aged humans outside atherosclerotic plaques, the role of VSMC senescence in human vasodilator function remains unclear, whereas a role in atherosclerosis has been proposed. In contrast, the role of endothelial (progenitor) cell senescence has been amply addressed.

We explored the role of eNOS in vasodilator dysfunction and found that aortic NO function and eNOS levels are decreased in Ercc1<sup>ΔΔ</sup>- mice. This is in agreement with previous observations in 26- to 28-month-old mice and with a diminished NO release and eNOS expression in cultured senescent endothelial cells and atherosclerotic plaque samples. Our pharmacological studies with BH4, tempol, and NAC show that ROS production resulting from eNOS uncoupling does not play a major role in the observed aortic endothelial dysfunction. At most, ROS modestly affected VSMC function. Interestingly, BH4 and NAC improved vasodilator function to a similar extent. This might suggest a similar action of both compounds, which would be in line with ROS scavenging properties of BH4.

Our observation that BH4 improved mainly VSMC instead of endothelial cell vasodilator function in aorta is in sharp contrast to the observation that the BH4 precursor sepiapterin improved dilations to acetylcholine but not to NaNO2 in mesenteric arteries of 24-month-old mice and warrants exploration of vasodilator mechanisms in resistance vessels from our mice.

Exploration of the endothelium-independent vasodilator dysfunction revealed that phosphodiesterase inhibition almost completely rescued VSMC vasodilator function. This strongly suggests the existence of a relative phosphodiesterase overactivity in Ercc1<sup>ΔΔ</sup>- mice. Interestingly, the substrate of several phosphodiesterase enzymes, cGMP, also regulates VSMC proliferation and extracellular matrix composition. Therefore, long-term changes in cGMP levels could lead to vascular changes, including increased stiffness and a higher number of senescent cells. A prolonged increase in phosphodiesterase activity and reduction in NO production could thus lead to an increased vascular stiffness, as supported by the increased vascular stiffness, despite an increased sensitivity to NO, in eNOS knockout mice. In Ercc1<sup>ΔΔ</sup>- mice, the reduced sensitivity to NO will contribute even further to reduced cGMP levels.

Our human genetic study intended to explore whether there is a possible relationship between genetic variants in NER genes and risk for increased vascular stiffness, a typical marker for human vascular aging that was recapitulated in the Ercc1<sup>ΔΔ</sup>- mice. Besides finding 1 SNP, rs2029298, upstream of the start codon of DDB2 (XPE) that passed the Bonferroni-corrected significance level, we found suggestive associations of SNPs in or close to ERC5, ERCC6, GTF2H3, GTF2H1, and XPD for CFPWV. The finding that CFPWV is significantly associated with DDB2 is compelling because CFPWV is the human equivalent of the mouse variables for vascular stiffness that were measured and is negatively associated with vasodilator function in aging mice and humans. DDB2 encodes the smaller subunit of a heterodimeric DNA binding protein with high affinity to UV–damaged DNA (UV-DDB) and has been studied in both rodents and humans. XPE is a component of NER, and its activity is not tied to transcription, but it acts in the whole genome. The role of XPE is to identify UV-induced lesions, ie, cyclobutane pyrimidine dimers and polycyclic aromatic hydrocarbon adducts. The function of the upstream region in which the SNP for DDB2 was found has not been characterized, although the gene in
humans has been shown to be under control of p53, the guardian of the genome, and to be inducible after genotoxic stress. Hence, DDB2 (XPE) may at least in part control global genome NER.

As is usual for common genetic variants, the effect sizes were small. The effect size of our top SNP, rs2029298, is $-0.05 \pm 0.01$ SD per allele after correction for age, age squared, sex, height, and weight. Extrapolation of this value of $-0.05$ SD per allele to an absolute age value for an individual with the use of CFPWV-age association graphs is difficult because these relations are not linear. Moreover, the impact of the SNP will depend on other risk factors that are present. It is possible, however, to compare the relative contribution of the risk allele of SNP rs2029298 with others found in genome-wide association studies. In the AortaGen Consortium study, the locus, found in a BCL11B gene desert, was the most strongly associated with CFPWV and found with others in genome-wide association studies. In the present study, the effect size of the DDB2 SNP found in the present study is in a comparable range.

More detailed sequencing might pinpoint genetic variants with stronger associations. This is an important consideration in view of the evolutionary pressure against genetic variations that severely impair the function of DNA repair systems. Severe DNA repair dysfunctions are often lethal or lead to infertility, which makes them more likely to be present only as minor alleles. Furthermore, the involvement of a DNA repair defect in vascular damage might depend strongly on the presence of factors that induce DNA damage. Nonetheless, increased PWV is prominently present in Hutchinson-Gilford progeria, a very rare syndrome caused by genomic instability resulting from mutation of the LMNA gene, which is featured by premature cardiovascular death. 12, 39

**Conclusions**

We explored the possibility of whether the reduced efficiency of 2 DNA repair pathways, NER and interstrand crosslink repair, contributes to vascular aging in mice. We observed functional changes in the vasculature (worsened vasodilator function and increased vascular stiffness) and hypertension, which are reminiscent of changes in aging humans and animals. We also found an association between SNPs of genes that encode for relevant NER components and increased vascular stiffness. From these observations, we conclude that DNA repair capacity is associated with accelerated vascular aging in mice and that there may be implications for risk stratification in humans with respect to age-dependent CVD. Whether this relates to oxidative stress, classic risk factors, and/or local damage or even extends beyond these limits will be a central question in studies to come.

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**Disclosures**

None.

**References**

Aging strongly contributes to cardiovascular disease. It prolongs exposure to classic cardiovascular risk factors such as hypertension and diabetes mellitus but also acts as an independent risk factor. Recent evidence suggests that gradually accumulating DNA damage, leading to genomic instability, is a main cause of aging. This study is the first to show that mice with a defective DNA repair system not only age fast but also display accelerated development of vascular problems mimicking those in aging humans: increased blood pressure, increased vascular stiffness, decreased vascular relaxation, and cellular aging. Of interest, phosphodiesterase inhibition acutely improved the diminished relaxation in vitro, suggesting that enhanced breakdown of cGMP may underlie this phenomenon. Furthermore, in humans, variations in DNA repair genes were associated with markers for vascular aging. Taken together, these results indicate that genomic instability plays a central role in vascular aging. Genomic instability may also explain the high prevalence of cardiovascular death in Hutchinson-Gilford progeria and Werner progeroid syndrome, both of which feature genomic instability. Because oxidative stress is an important inducer of DNA damage, future aging-suppressor agents may involve drugs that improve genomic integrity (eg, statins and rapamycin) and drugs that prevent oxidative stress (eg, renin-angiotensin system blockers and antioxidants). In addition, drugs facilitating the nitric oxide–soluble guanylate cyclase–cGMP–phosphodiesterase pathway might be of value. The successful application of such treatments requires proper risk stratification, preferably at younger ages. This might include analyses of genetic variations in DNA repair genes and the identification of all possible sources of cardiovascular DNA damage.


Nucleotide Excision DNA Repair Is Associated With Age-Related Vascular Dysfunction


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SUPPLEMENTAL MATERIAL

Expanded materials and methods

Animals. Ercc1<sup>−/<sup>−</sup></sup> mice of 8 and 16 weeks in an F1 hybrid Fvb/C57Bl/6, background, their
wild-type littermates (WT) of the same age, 16, 26 and 52 week old phenotypically equivalent
WT and 26 and 52 week old XPD<sup>TTD</sup> mice and their WT in a C57Bl/6 background, were bred
at the Erasmus MC animal facility. The animals were housed in individually ventilated cages
with access to normal chow and water <i>ad libitum</i>. As required by Dutch law, all animal
studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the
Institutional Animal Care and Use Committee).

Isolation and culture of endothelial cells. Endothelial cells of 16 week old Ercc1<sup>−/<sup>−</sup></sup> mice
were isolated according to previously reported methods.<sup>1</sup> After isolation the cells were seeded
on 12 well culture plates (Costar) coated with 1% gelatine (225 bloom, Sigma Chemicals, the
Netherlands) and cultured under mouse lung endothelial cell medium (MLEC; DMEM/F12
(Gibco), containing 20 % FCS (Invitrogen) and endothelial cell growth supplement (ECGS-H
8 µL/mL, Promocell, cat # C30120). The atmosphere in the culture cabinet consisted of
normal air enriched with 5% CO<sub>2</sub>. Cells were generally used after passage 1 or 2, and never
beyond passage 3. Cells were passaged before they reach confluence.

Senescence-associated β-galactosidase staining. Senescence was determined by senescence-
associated β-galactosidase staining (SA-β-gal staining) at pH 6.0 with previously described
methods and reagents.<sup>2-3</sup> After staining cells were covered by Vectashield containing 2-(4-
amidinophenyl)-1H-indole-6-carboxamidine (DAPI) to visualize the nuclei and pictures were
taken under a microscope using normal and ultra-violet light. An overlay of these pictures was made and nuclei, combined with blue staining were counted as a positive cell.

SA-β-Gal-stained aortic tissue of 16 week old Ercc1d/− mice was embedded in paraffin, cut transversally and the number of SA-β-gal positive cells in the lamina media was counted under the microscope at 200x magnification.

**Quantitative real-time PCR.** Relative expression of cyclin-dependent kinase inhibitor 1A (p21) and tumor protein 53 (p53) genes was measured in thoracic aortas of 16 week old Ercc1d/− and WT mice. Quantitative real-time PCRs were conducted using a Step-One cycler Applied Biosystems (UK, Applied Biosystems), and the SYBR ® Green PCR Master Mix (UK, Applied Biosystems) as per manufacturer's recommendations. β-actin and HPRT-1 DNA quantitation was performed in parallel on all samples in order to determine the actual input amount of cDNA and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency. The sense and antisense primers for mouse p21 were 5’-GCC-CAA-GGT-CTA-CCT-GAG-CCC-3’; 5’-TCT-TGC-AGA-AGA-CCA-ATC-TGC-GCT-3’, and the sense and antisense primers for mouse p53 were 5’-CTC-CAG-CTG-GGA-GCC-GTG-TC-3’; 5’-GCT-CCC-TGG-GGG-CAG-TTC-AG-3’respectively.

A 2-fold dilution series was created from a random pool of cDNA from our sample groups. The PCR efficiency and correlation coefficients (R2) of each primer pair were generated using the slopes of the standard curves. The efficiencies were calculated by the formula:

\[ \text{efficiency} = \left(10^{-\frac{1}{\text{slope}}} - 1 \right) \times 100 \]

For a correct interpretation of the real-time PCR results all data has been normalized which is achieved by calculating the geometric mean of the two stable reference genes.
**Ring outgrowth assay.** For the ex vivo analysis of endothelium dependent sprouting of aortas, tissue of 16 week old Ercc1<sup>d<sub>c</sub></sup> and WT mice were harvested, cut into 2 mm thick segments, and placed in serum-reduced Matrigel in a 96 well plate (BD Biosciences, The Netherlands). Embedded aortic segments were then incubated with 200μL fibroblast-conditioned EGM2 medium per well for 4 days before evaluation by phase-contrast microscopy. Tube length was analysed with a commercial image analysis system (Impak C, Clemex Technologies, Canada).

**Assessment of blood pressure and vasodilator function in vivo.** To measure in vivo vasodilator function, a Laser Doppler perfusion imaging was used to determine the increase of the hindleg perfusion after transient occlusion of the blood flow (reactive hyperemia). This measurement was done at least 3 days and maximally 7 days after blood pressure measurement with tail cuff.

Twenty-four hours before measuring blood flow, the hair of the left hind leg was removed by a hair removal cream. At the day of measurement, mice were anesthetized by 2.8% isoflurane/O<sub>2</sub> ventilation, and kept on a heating pad regulated by a rectal thermometer to keep the body temperature stable between 36.4 and 37.0 °C. The hindleg in which the measurements were performed was kept in a steady position with the use of a fixation device that was designed for this particular purpose. After 20 minutes of equilibration, 5 minutes baseline perfusion was recorded. Then, 2 minutes of occlusion was applied with the use of an exogenous tourniquet. After release of the tourniquet, blood flow was monitored for 10 minutes during which hyperaemia and return of the blood flow to the post-occlusion baseline occurred. For analysis, the region of interest containing the whole of the leg was chosen. The results were expressed as relative values compared to the post-occlusion baseline. To compare the Ercc1<sup>d<sub>c</sub></sup> mice with WT mice the results were expressed as the maximum response to
occlusion or the area under the response curve (only the area above the baseline was considered, values below the baseline were considered to be 0).

To address if impaired peripheral vasodilator function results in increased blood pressure we measured the blood pressure in conscious Ercc1<sup>1/1</sup> mice and WT littermates using the tail cuff technique (using CODA High-Throughput device from Kent Scientific). Stress-induced responses were minimized by a 4-days period of acclimatization sessions. At day 5, 30 measurement cycles were recorded, and the mean of all correct measurements, as determined on the basis of pressure-flow relationships, were taken for each mouse.

**Organ bath experiments.** Ercc1<sup>1/1</sup> and WT mice were asphyxiated in a CO<sub>2</sub> chamber. The thoracic aortas were isolated and stored overnight in cold, oxygenated Krebs-Henseleit buffer solution. Sizes of the aortas were consistently lower in Ercc1<sup>1/1</sup> than in WT. The following day, vessel segments were mounted in 6-mL organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25 and glucose 8.3; pH 7.4) at 37°C and oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure. Maximum contractile responses were determined using 100 mmol/L KCl. Preconstriction with 30 nmol/L U46619 resulted in 50-100% of 100 mmol/L KCl precontraction. Following precontraction, relaxation concentration-response curves (CRCs) were constructed to acetylcholine, and responses to 100 µmol/L sodium nitroprusside were determined. L-NAME 100 µmol/L, given 10 minutes before U46619, was used to investigate the involvement of nitric oxide (NO) in the relaxant responses. Similarly, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol), 100 µmol/L and N-acetyl-cysteine (NAC, 30 µmol/L) were used as scavengers of reactive oxygen species. Tetrahydrobiopterine (BH4, 100 µmol/L) was used to prevent the uncoupling of eNOS. To investigate the
involvement of increased PDE enzyme activity in VSMC dysfunction of \textit{Ercc1}^{d/c} mice, 10 \(\mu\)mol/L vinpocetine was used. This PDE inhibitor displays selectivity towards PDE1.\textsuperscript{6} vinpocetine was used together with 100 \(\mu\)mol/L L-NAME, to allow the measurement of the response to exogenous sodium nitroprusside (100 \(\mu\)mol/L) in the absence of endogenous NO. To test for the differences in protein kinase G (PKG) sensitivity, dose-response curves to 8-Bromo-\(\beta\)-phenyl-1,N\(^2\)-ethenoguanosine-3',5'-cyclicmonophosphate (8-Br-PET-cGMP) were constructed under L-NAME inhibition (100 \(\mu\)mol/L).

**Measurement of mechanical properties of the vascular wall.** The explanted thoracic aortas were fixed for 48 hours in formalin and consequently embedded in paraffin. Embedded tissue was transversally cut into 5 \(\mu\)m thick sections, and wall thickness and lumen diameter were measured.

The carotid arteries were explanted from 16 week old \textit{Ercc1}^{d/c} and WT animals. The region selected was between the aortic arch and first carotid branch, 2-3 mm of length. The carotid artery was mounted in pressure myograph (Danish Myograph Technology, Aarhus, Denmark) in calcium free buffer (in mmol/L: NaCl 120, KCl 5.9, EGTA 2, MgCl\(_2\) 3.6, NaH\(_2\)PO\(_4\) 1.2, glucose 11.4, NaHCO\(_3\) 26.3; pH 7.4), to measure the passive properties of the vessels. The intraluminal pressure of the vessel was increased stepwise by 10 mm Hg starting at 10 mm Hg and reaching 120 mm Hg. Each step was kept for 3 minutes for the vessel to equilibrate and at the end of the step, lumen and vessel diameter was measured. Using these data, mediastress and mediasitry were calculated and a stress-strain relationship was constructed.

**Lung eNOS activation experiments.** Freshly excised lungs from \textit{Ercc1}^{d/c} or WT mice were cut into approximately 1 mm\(^3\) pieces, divided into equally sized portions and kept in the Krebs-Henseleit buffer at 37\(^\circ\)C and oxygenated with 95\% O\(_2\) and 5\% CO\(_2\). After 1 hour of
equilibration, some portions of the lungs were exposed to 10 μmol/L acetylcholine for 10 minutes to determine the phosphorylation after stimulation, whilst others were not treated, to assess the baseline phosphorylation. After 10 minutes of treatment tissue was taken out of the buffer and snap-frozen in liquid nitrogen.

**Immunoprecipitation and immunoblotting of eNOS.** Aortas and pulverized hearts were lysed in Triton X-100 buffer. Tyr657-phosphorylated eNOS was immunoprecipitated with a specific antibody (Eurogentec, Seraing, Belgium, 1:1000). Detergent-soluble proteins or immunoprecipitates were heated with SDS-PAGE sample buffer, separated by SDS-PAGE, blotted on nitrocellulose filters and incubated with specific antibodies against pSer1177-eNOS (sc-21871-R, Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000), eNOS (sc-654, Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000), β-actin (a1978, Sigma-Aldrich, 1:20,000) or pan-actin (DLN-07273, Dianova, Hamburg, Germany, 1:1000).

**Statistical methods.** Data are presented as mean±SEM. Statistical analysis between the groups of single values was performed by two-sided t-test or 1-way ANOVA followed by Bonferroni’s post-hoc test, where appropriate. To test our assumption that blood pressure would be increased in Ercc1d/− animals, we employed a one-sided t-test. Differences in dose-response curves were tested by ANOVA for repeated measures (sphericity assumed). Differences were considered significant at p<0.05.

**Human studies.** To address the possible involvement of DNA repair capacity in development of CVD in humans, we investigated the association of SNPs in NER components to arterial stiffness. In accordance with the phenotype observed in mice, we examined the association of 310 selected NER SNPs with carotid-femoral pulse wave velocity (CFPWV) within the
framework of the AortaGen Consortium. CFPWV is a measure of aortic stiffness that is strongly associated with increased risk for major cardiovascular disease events.

**AortaGen Consortium.**

**a. Consortium Organization:** The AortaGen Consortium consists of 20,634 participants from 9 cohort studies that completed genome-wide genotyping and had measured carotid-femoral CFPWV⁸; including: the Age, Gene/Environment Susceptibility-Reykjavik Study (AGES-RS), the Baltimore Longitudinal Study of Aging (BLSA), the Erasmus Rucphen Family (ERF) study, the Framingham Heart Study (FHS), the Health, Aging and Body Composition (Health ABC) Study, the Heredity and Phenotype Intervention (HAPI) Heart Study, the Rotterdam Study I (RS-I), the Rotterdam Study II (RS-II)⁹, and the SardiNIA Study.

**b. Carotid-femoral pulse wave velocity (CFPWV) as the phenotype:** CFPWV is the gold standard method for assessment of arterial stiffness and is determined from the time taken for the arterial pulse to propagate from the carotid to the femoral artery. Different cohorts included in the AortaGen Consortium used different methods to ascertain carotid-femoral transit distance. To control for such differences, the AortaGen Consortium performed the genetic association analyses using a sex-specific standardized residual that was based on the inverse of CFPWV, which normalizes the distribution, and that was further adjusted for age, age², height and weight. As a result of these transformations, the cohorts had a highly comparable distribution of CFPWV (mean of 0 and standard deviation of 1 with a normal distribution)

**c. Genotyping and Imputation:** Genotyping and imputation methods for all of the 9 cohorts included in the AortaGen Consortium have been described previously⁸. For genome-wide SNP sets, genotyping was carried out using commercially available arrays. Prior to
imputation, quality control measures were applied. MACH was used by all cohorts for imputation of genotypes to the HapMap set of approximately 2.5 million SNPs.

**d. Statistical Analyses:** The AortaGen Consortium used a sex-specific standardized regression residual for 1000/CFPWV, adjusted for age, age², height and weight for meta-analysis. Genome-wide association analyses were conducted within each cohort using an additive gene-dose model. Linear mixed effects models were fitted to account for relatedness in pedigrees. Within-study associations were combined by prospective meta-analysis using inverse-variance weighting. Meta-analyses were performed using the software program MetABEL (http://www.genabel.org/packages/MetABEL). During meta-analysis, SNPs were excluded if weighted mean minor allele frequency was <1%, resulting in 2.41 million SNPs for analysis. The genomic control parameter was calculated to adjust each study and after meta-analysis, was recalculated to adjust for among-study heterogeneity.
Supplemental figure 1. Tube outgrowth from aortic tissue under culture conditions. The endothelium-dependent angiogenic potential is preserved in Ercc1<sup>−/−</sup> when compared with their WT littermates.
Supplemental figure 2. Effects of acute tempol supplementation in Ercc1<sup>d−c</sup>. Responses to acetylcholine (A) and sodium nitroprusside (B) after pretreatment with tempol show no improvement.
Supplemental figure 3. Wall thickness and wall to lumen ratio. Vessel dimensions are reduced in Ercc1<sup>d/-</sup> compared to WT mice. This can be also seen on the vessel wall thickness (A), however when related to its diameter (B), the ratio remains the same indicating, that the proportions are conserved and there is no relative hypertrophy in Ercc1<sup>d/-</sup> animals. * = p<0.05


