Novel Directions In Therapy Against Age-Related Vascular Disease

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ISBN: 978-94-6203-614-7
Cover : Photo from EisukeTachikawa
Design, cover desigen and layout: Haiyan Wu
Printing: CPI Koninklijke Wöhrmann

NOVEL DIRECTIONS IN THERAPY AGAINST

AGE-RELATED VASCULAR DISEASE

Nieuwe richting in therapie tegen

leeftijd-gerelateerde vasculaire ziekte

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

donderdag 25 september 2014 om 11.30 uur

door

Haiyan Wu

geboren te Guizhou (China)

Promotie commissie

Promotor:	Prof.dr. A.H.J. Danser
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Financial support for the publication of this thesis was generously provided by Erasmus University Rotterdam

Financial support by The Dutch Heart Foundation for the publication of this thesis is gratefully acknowledge

This studies described in this thesis were supported by a grant of the China Scholarship Council (2010624026)

This work is dedicated to my family

谨以此书献给我的家人

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General introduction

Partly published as: Genomic instability and vascular aging: a focus on nucleotide excision repair

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Trends Cardiovasc Med. 2014;24:61-68.

INTRODUCTION

Genomic instability is recognized as one of the primary mechanisms that lead to organismal aging, and leads to progeria when developing in an accelerated pace due to defective genomic maintenance systems, such as nucleotide excision repair, in humans and mouse models of progeroid syndromes. The role of genomic instability related to nuclear DNA is currently under investigation with respect to its role in cardiovascular disease, in particular in relation to vascular aging. In this review we highlight the first findings in this field of research that come from experiment in nucleotide excision repair-defective mouse models and from genetic studies. Possible mechanisms that mediate the consequences of genomic instability at the local, vascular and at the systemic level, such as cell senescence, mutations, mitochondrial damage, and sirtuin 1 and IGF-1 decrease, are discussed and important goals for future research are set.

Cardiovascular disease, DNA damage and the aging process

Cardiovascular disease (CVD) is the leading cause of death worldwide. The combat against age-related CVD represents one of the greatest challenges for limitation of socio-economic problems in modern civilization [1]. Comprehension, prevention and treatment of vascular aging is of utmost importance when addressing this challenge because of the causal relationship between vascular aging and CVD [2].
During aging, endothelial cells (EC) and vascular smooth muscle cells (VSMC), undergo functional changes that are highly associated with hypertension, disturbed organ perfusion, vascular stiffness, and atherosclerosis, four major hallmarks of age-related CVD [2]. The causes of these changes, especially when independent from classical cardiovascular risk factors, are unclear, but might relate to genomic instability.

Research into genomic maintenance brought us the understanding of the importance of unrepaired DNA damage in the aging process. Genomic integrity is constantly safeguarded by genomic maintenance systems that check, organise and repair DNA [3, 4]. Genomic instability caused by the disruption of genomic maintenance systems leads to human progeria syndromes as well as cancer, and this can be recapitulated in mouse models [5].

Several associations between genomic instability and vascular aging exist. Firstly, the progeria syndromes Werner's syndrome and laminopathies related to lamin A mutations (such as Hutchinson-Gilford Progeria Syndrome), are featured by CVD, and this can also be reproduced in animal models [6, 7]. Secondly, atherosclerotic plaques show increased DNA damage and DNA repair proteins, and increased cellular senescence, a cell-growth arrest that can relate to unrepaired DNA damage and that leads to deleterious functional changes [8-12]. Thirdly, statins, which protect against atherosclerosis, promote the detection and repair of DNA.[13] Fourthly, telomere attrition and mitochondrial DNA damage are associated with some aspects of cardiovascular aging, as described elsewhere [14-17]. These clues prompted us to investigate if genomic instability due to unrepaired nuclear DNA, a main driving mechanism of aging, could play a role in CVD. Here we review these

new insights, with a special focus on DNA lesions that relate to nucleotide excision repair (NER), and where important we refer to other DNA repair systems. In the remaining part of the review the term 'nuclear DNA' will refer to non-telomeric regions.

DNA repair systems

DNA is threatened by three main sources of damage: intrinsic molecular reactions within DNA molecules such as hydrolysis, attacks by reactive molecules that originate from our own metabolism, and damage by exogenous physical and chemical entities. The diverse lesion types that thus arise are repaired by diverse DNA repair pathways: 1) the direct reversal pathway, which allows the direct reversal of chemical modifications of nucleic acids, (2) mismatch repair (MMR), correcting DNA replication-induced base pair mismatches (3) nucleotide excision repair (NER, Figure 1), which repairs transcription-disturbing bulky adducts, (4) base excision repair (BER), which repairs mainly oxidized and alkylation lesions in the nucleus and mitochondria, and single strand breaks (5) homologous recombination (HR), and (6) non-homologous end joining (NHEJ), which repair single and double strand breaks [4, 18]. Telomere maintenance requires further specialized proteins [19]. DNA repair systems can to some degree overlap. For example, a combination of NHEJ and NER is needed to repair cross-linked complementary DNA. Furthermore, oxidative DNA lesions might be repaired by both BER and NER, depending on whether the lesion is helix-distorting (bulky) or not [20].

NER, cancer and aging

NER is involved in both cancer as well as aging, as witnessed by increased cancer susceptibility as well as progeroid phenotypes in NER-defective humans and mice [18, 21]. NER consists of two parts, transcription-coupled (TC-)NER and global genomic (GG-)NER (Figure 1). TC-NER removes bulky lesions that have stalled transcription by RNA polymerases due to helix distortion. GG-NER screens the genome for bulky lesions independently from transcription. The two subsystems are only separated at the level of DNA damage recognition, whilst converging at the point where the lesion is repaired. Typically, functional disturbance of components that are only involved in GG-NER leads to increased cancer susceptibility whilst defects in TC-NER-specific components induce accelerated aging. In agreement, defects in the NER components that participate in the unwinding of DNA, removal of damaged DNA and fill-in of the removed part, i.e. at the level where TC- and GG-NER pathways join, can lead to both increased cancer susceptibility and aging. Mouse models of defective NER display segmental progeria, i.e. many, but not all, organs show signs of accelerated aging [21].

NER and vascular ageing

To study the impact of NER on vascular aging, two mouse models of genomic instability were used, one being the XPD^{TTD} mouse, a model of moderately accelerated ageing, and the other being the $Ercc1^{d/-}$ mouse, which undergoes aging in a much faster pace [22-24]. Thorough

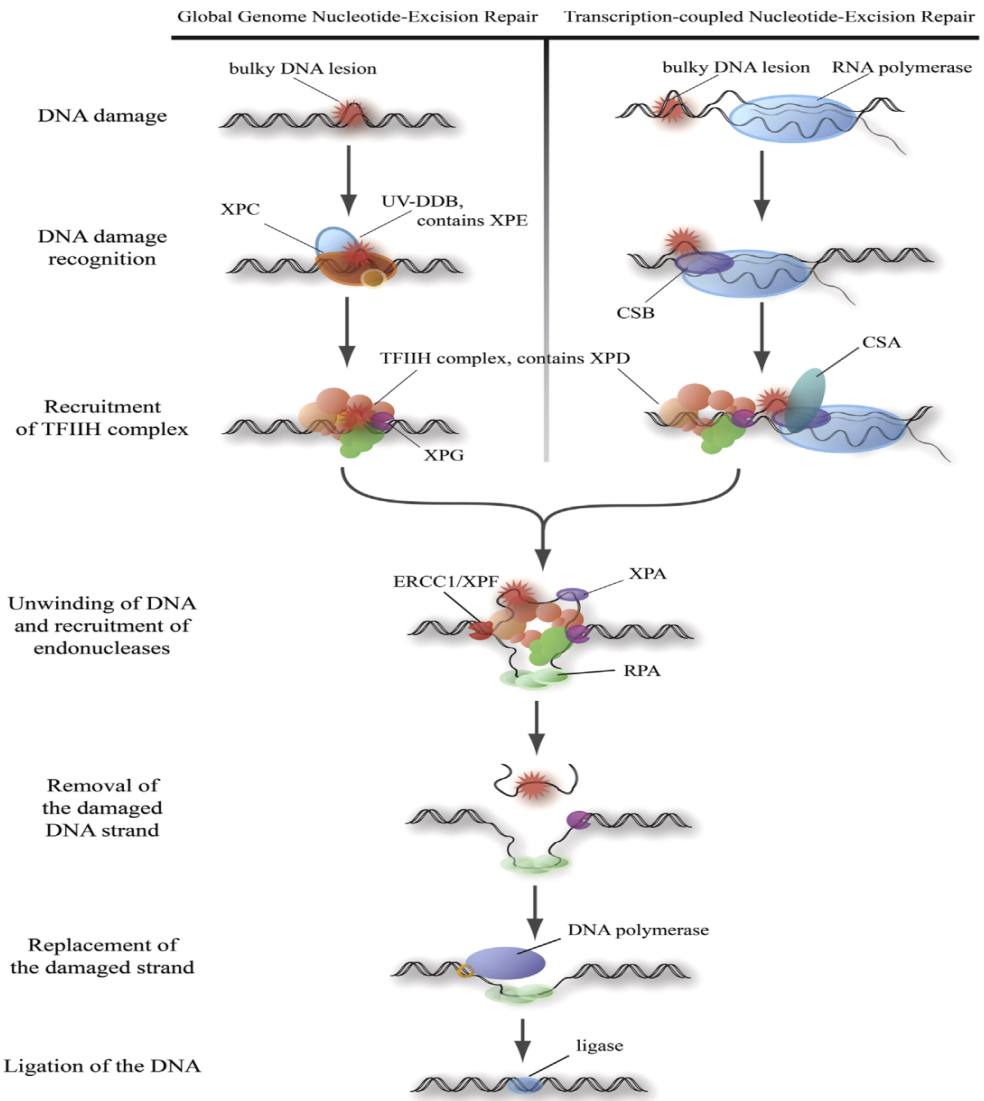

Figure1.Nucleotide excision repair (NER). Helix-distorting lesions are recognized in the GG-NER by Xeroderma Pigmentosum C (XPC) and the UV-DDB protein dimer. In case of stalled transcription, this damage is recognized by TC-NER proteins, Cockayne Syndrome A (CSA) and B (CSB). Recognition is followed by the recruitment of transcription factor II H (TFIIH) complex, opening of the DNA double helix by XPB and XPD helicases, and damage verification by XPA protein. Replication protein A (RPA) prevents reannealing of the single strand and damaged strand is excised by excision repair cross-complementing 1 (ERCC1)/XPF and XPG endonucleases. The excised strand is then reconstituted by the replication machinery and sealed by ligase (figure based on Hoeijmakers 2009).

evaluation of age-related vascular function changes and cellular aging was performed including measurements of blood pressure, in vivo and ex vivo vasomotor function, endothelial nitric oxide synthase activation, differentiation between endothelial versus vascular smooth muscle cell function, vascular stiffness, and markers of vascular aging [25]. Evaluation was done at the age of 26 weeks and 52 weeks in XPD^{TTD} (only ex vivo vasomotor function) and at the age of 8 and 16 weeks in $Ercc1^{d/-}$. Both animals showed decreased aortic vasodilator responses to the endothelium-dependent vasodilator acetylcholine at younger age than wild-type littermates (WT), and, importantly, the development of the endothelial dysfunction was much more accelerated in $Ercc1^{d/-}$ than in XPD^{TTD}, which is in accordance with the relative pace of (accelerated) aging in the two strains.

Analysis of microvascular function by hind leg reactive hyperemia measurements showed a decreased vasodilator response in $Ercc1^{d/-}$ as compared to WT [25]. The method that was used (laser Doppler technique) most probably measures superficial resistance vessels, and might represent both endothelium-dependent as well as endothelium-independent relaxations [26-28]. Similar microvascular changes are also observed in aged humans, and are independent from cardiovascular risk factors [26, 29].

Pressure-diameter relationships showed that carotids of $Ercc1^{d/-}$ displayed higher stiffness than WT [25]. Increased stiffness is also an important feature in human vascular aging, which again appears to be independent of classical cardiovascular risk factors [30].

Blood pressure, as measured by conscious tail cuff experiments, was increased in $Ercc1^{d/-}$. Systolic pressure show the clearest increase, which is in accordance with human aging during which systolic pressure keeps rising and diastolic pressure follows a biphasic trend [31]. Thus a defective NER in $Ercc1^{d/-}$ mouse faithfully reproduces some symptoms of vascular aging observed also in humans, already between 8 and 16 weeks after birth of the mice, which is strongly accelerated as compared to WT.

Organ bath studies and molecular analyses provided mechanistic insight in the diminished vasodilator responses [25]. These experiments showed that nitric oxide (NO)-mediated responses, endothelial NO synthase (eNOS) expression and eNOS activation were decreased. Since senescence markers p53, p21 and senescence-associated β-galactosidase were increased the diminished NO might relate to endothelial cell senescence [11, 32-35]. Increased generation of reactive oxygen species (ROS), a cause of age-related decreased NO-dependent vasodilation [15, 36], was partly responsible for the diminished vasodilations in $Ercc1^{d/-}$ mice since anti-oxidants N-acetylcysteïne and tertrahydrobiopterin (BH4) improved vasodilations. Uncoupling of eNOS, which leads to a switch from NO to ROS production by eNOS [37], did not appear to cause these changes [25]. Possible other ROS sources in cardiovascular tissue are mitochondria and nicotinamide adenine dinucleotide phosphate-(NADPH)-oxidase stimulation [15, 36, 38]. Interestingly, DNA damage induced by X, γ, or UV irradiation irradiation triggers activation of NADPH-oxidases, as shown in hematopoietic cells, and this is believed to perpetuate and increase DNA damage through ROS production [39, 40]. It would therefore be interesting to investigate the impact of DNA damage related to NER on NADPH-oxidase activation.

Vascular aging features that were not found in NER-defective mice

The aging vasculature shows several features that are not found in NER-defective mice. Aging-related arterial stiffening has been attributed to hypertrophy and extracellular matrix changes [2]. Hypertrophy was not found in NER-defective mice. This might be due to the suppression of the somatotropic axis in such animals [41-43], as also discussed later, which might prevent vascular hypertrophy. Furthermore, we found no indication of fibrosis. However, vascular aging is accompanied by a shift from an elastic to a rigid extracellular matrix [44, 45], and this aspect still needs to be studied in NER-defective mice.

Another feature that was not seen is atherosclerosis. This could simply relate to the fact that for development of atherosclerotic plaques in mice it is necessary to interfere with lipid metabolism, e.g. by genetic ablation of apolipoprotein E (ApoE) or lipoprotein receptor, and apply a high fat diet [46, 47]. Moreover, NER-defective mice, amongst which XpDTTD and Ercc1$^{d/-}$, and humans are featured by a strongly decreased amount of fat tissue, a feature that is partly related to changes in regulation of lipid handling by peroxisome proliferator-activating receptors (PPAR), as shown in XPD-mutated cells [48, 49]. Pharmacological modulation of PPAR signaling is a well-known strategy to influence atherogenesis [50, 51]. It will therefore be interesting to explore the effect of defective NER on atherogenesis, e.g. in mice with combined NER defects and atherosclerosis.

Possible mechanisms of NER-related vascular aging

It is not known how genomic instability related to nuclear DNA damage leads to the vascular aging phenotype that is observed in NER-deficient mice, because studies in this direction have not been done yet. However, some of the reported phenotypic changes in such mice provide clues for speculation on mechanisms. Important in this respect is the question if the observed changes are a consequence of local or of systemic genomic instability. Figure 2 provides a summary.

Local genomic instability: mutations, altered transcription and mitochondrial function

Local DNA damage, such as bulky adducts or even mutations, could explain certain changes, e.g. the reduced eNOS expression and activity, on the basis of either mutation of the gene product, stalled transcription, a change in expression of miRNA or endothelial senescence [42, 52-56].

Alternatively, local changes in mitochondrial function could arise from a defective NER. Some NER components are involved in regulation of mitochondrial BER, and the NER components Cockayne Syndrome A and B directly participate in maintenance of mitochondrial genomic integrity and transcription [49, 57, 58]. In addition, nuclear DNA repair problems lead to mitochondria-related vascular dysfunction, as demonstrated in mice with defective ataxia telangiectasia (ATM), an important DNA damage recognition protein [59]. Given the importance of mitochondrial (DNA) damage in CVD [15, 59], it cannot be excluded that

accelerated vascular aging in our NER-defective mice is due to mitochondrial instability. This remains a very speculative possibility however because the relation between the ERCC1 or XDP proteins and mitochondrial (genomic) maintenance is unknown.

Another important player might be sirtuin 1, a versatile acetylase that has been implicated in longevity, protection from DNA damage-induced endothelial cell senescence and dysfunction, and protection from atherosclerosis through its role in regulation of DNA repair partly through its role in damage recognition [60, 61]. We found sirtuin 1 mRNA to be downregulated in Ercc1$^{d/-}$ renal tissue (unpublished observations), and its down regulation in aged mice is associated with endothelial dysfunction [62], which prompts the question if this decrease can further jeopardize genomic integrity and thus contribute to (vascular) aging or even the reduced DNA repair capacity observed in aged organisms [63].

Effects of systemic genomic instability: metabolic adaptations

NER-defective mice as well as aged wild-type animals show a suppression of the somatotropic axis [41-43, 64]. Although it is hard to establish this suppression in aging humans due to many confounders [65], it is believed this metabolic change is induced to switch from a state of somatic growth to that of maintenance, and that this switch serves to promote survival, hence called 'survival response', amongst others by preventing tumorigenesis [18, 41, 42]. One of the key events is the suppression of GH/IGF-1 signaling.

GH and IGF-1 have been shown to play a role in cardiovascular disease, as thoroughly reviewed elsewhere [66]. Importantly, high IGF-1 levels are associated with improved endothelial function, increased eNOS activity and decreases vascular and mitochondrial oxidative stress, whilst lowered GH or IGF-1 leads to the opposite [15, 66]. In addition, BH4 and IGF-1 levels positively correlate with each other. Thus, low IGF-1 levels in our NER-defective mice could contribute to the observed vascular changes through various mechanisms.

The relationship between GH/IFG-1 signaling and blood pressure is more difficult to explain because of the contradicting observations that on the one hand low normal IGF-1 levels in humans and liver-specific IGF-deficiency in mice is associated with elevated blood pressure, whereas on the other hand GH knockout mice display low blood pressure and vice versa [67, 68]. Therefore, the role of suppression of the somatotropic axis, in particular of GH and IGF-1, in blood pressure regulation remains to be further elucidated.

It cannot be excluded that yet other survival response components contribute to the observed vascular changes. Of further importance, suppression of the somatotropic axis can also be found at the cellular level and might therefore also play a role in effects of local genomic instability [42].

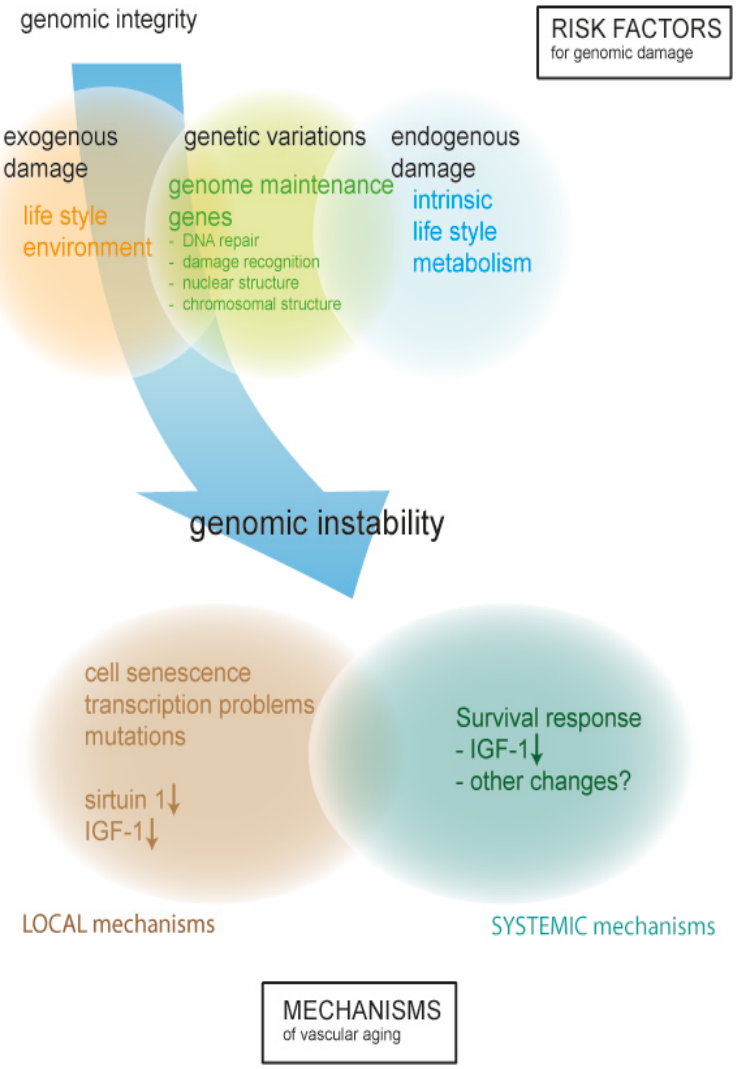

Figure 2. Hypothetical mechanisms that lead to nuclear genomic instability and to subsequent vascular aging. The interplay between DNA damage sources and genetic factors that influence genomic maintenance at different levels determine the rate of progression of genomic instability, and thus of vascular aging. The mechanisms that mediate the impact of genomic instability on vascular aging can be found both on a local and systemic level, and encompass genomic damage, cellular senescence and metabolic and signalling adaptations related to the survival response. See article text for further specification.

Susceptibility to DNA damage: a hidden risk factor?

It is important to realize that NER-defective mice are most of all tools to investigate the role of genomic instability and vascular aging, and the few NER-defective progeroid humans are as far as known not suffering from cardiovascular disease (no publicly available reports). What is more important is the question how susceptible the general population is to accumulating DNA damage, which in all likelihood is determined by genetic and environmental factors and the interaction between these variables (Figure 2). We showed that some single nucleotide polymorphisms (SNP) in NER component genes, in particular the rs2029298 SNP that relates to the XPE DNA damage recognition complex, appear to be associated with higher carotid-femoral pulse wave velocity [25], a marker for vascular stiffness that positively correlates with age. Interestingly, several reports show an interaction of SNP in the BER genes OGG1 and XRCC1, and the NER genes ERCC2 (XPD) and ERCC5 with genomic instability in coronary disease and with atherosclerosis and stroke risk [69-72]. Furthermore, genetic associations between NEHJ and the DNA damage-sensing proteins MRE11 and ATM with myocardial infarction risk have been found [73-75]. NEHJ is involved in double strand break repair, and, in extrapolation, the strong phenotype that we observed ERCC1-defective mice might result from a dual function of ERCC1 in NER and double strand break repair.

These observations indicate that cardiovascular risk may stand in relation to various DNA repair system implicating the involvement of diverse types of DNA damage and, hence, various sources of damaging agents. All three aforementioned main sources of damage could in fact contribute to vascular aging (Figure 2). Oxidative stress would be an important endogenous source which relates to classical risk factors [10, 15, 59], whereas exogenous sources remain to be largely identified, although some might already have been recognized, such as chronic exposure to cigarette smoke and air pollution [76, 77]. It is tempting to speculate that such exogenous sources are contributing to the aging process, and that the interplay with genetic variations in DNA repair molecules represent a hidden risk factor that partly explains the CVD risk factor age. Thus the risk factor age might be partly modifiable.

Prospective treatments of genomic instability-related vascular disease

Renin–angiotensin system-related pathways: crossroads between the aging process and cardiovascular disease.

In relation to age-related cardiovascular problems, over the past few decades, a large amount of studies has uncovered a multitude of deleterious cardiovascular and renal effects attributed to the renin-angiotensin system (RAS). The RAS, through its active agent angiotensin II (Ang II), has a blood pressure-, vascular hypertrophy- and inflammation-increasing function. Ang II, through its Ang II type 1 receptor (AT1) receptor, causes vasoconstriction, decreases sodium and water excretion directly (and also indirectly via stimulation of the synthesis and release of aldosterone), and facilitates sympathetic activity - all of these effects increase blood pressure [78]. This can be part of regular physiological

function, but when exacerbated it contributes to the pathogenesis of several age-related, human cardiovascular diseases, including hypertension, atherosclerosis, (myocardial) infarction, congestive heart failure, coronary artery disease (CAD) and diabetic nephropathy. Blockade of Ang II signaling, therefore, is a widely used strategy to control these diseases. In the clinic, angiotensin I-converting enzyme (ACE) inhibitors, which block Ang II production, and AT1 receptor blockers are commonly prescribed for the treatment of hypertension, attenuation of heart failure after myocardial infarction, and diabetic nephropathy [79, 80]. These therapies are, unfortunately, usually prescribed when the aging process has already exerted its devastating effects in the cardiovascular system.

However, there are clues that Ang II/AT1 receptor signaling is involved in vascular cell senescence [81], DNA damage [82], and longevity (Benigni et al. 2009), indicating that currently RAS inhibition is not optimally used to battle aging-related cardiovascular disease. Ang II-induced ROS production is predominantly regulated by three enzymes: NADPH oxidase, mitochondrial respiratory complex, and nitric oxide synthase (NOS) [83], and each of these ROS-generating systems activates appropriate signaling pathways via selective oxidation of specific proteins. These reactions are negatively regulated by ROS-scavenging enzymes or disulfide bridge reducing enzymes, and functional disorders of these enzymes are found to cause cardiovascular dysfunction. Therefore, Ang II could be involved in organ senescence given its ability to mediate the release of oxidant species. In support of this hypothesis, Ang II-induced ROS production via AT1 receptors promotes the onset of vascular senescence associated with functional and structural changes of blood vessels that contribute to age-related vascular disease [84]. Moreover, Ang II-mediated vasoconstriction is increased during aging, thus further increasing blood pressure, vascular wall hypertrophy, myocardial hypertrophy, and inflammation. Simultaneously, it is well-known that renin decreases with age.

There is evidence that the increased ROS production is involved in aging-related organ dysfunction, and this is even believed, although with reservations, to directly affect longevity [85, 86]. Homozygous mice deficient for AT1A receptors grow up (unaltered body weight) normally and outlive their wild-type littermates by 26% [84]. These $AT1A^{-/-}$ mice also develop fewer aortic atherosclerotic lesions and less cardiac injury during aging. Further study shows that oxidative stress is reduced in cardiomyocytes, aorta and kidneys of these mice as compared to aged wild-type mice. In addition, ultrastructural analysis of mitochondria in proximal renal tubular cells shows that $AT1A^{-/-}$ mice have an increased number of mitochondria. The authors therefore are of the opinion that effects on longevity observed in AT1A receptor-deficient mice are due to the reduction of mitochondrial damage and oxidative stress. These results suggest that AT1 receptor blockade, when started early in life, could be useful to prevent vascular aging and age-related cardiovascular. In a model of genomic instability this hypothesis can be readily tested.

Strongly related to the RAS, bradykinin (BK) is a peptide hormone that exerts vasodilatory effects. Bradykinin is metabolized by ACE, and as a consequence, ACE inhibition increases the levels of BK and its active metabolite des-Arg9-BK (DABK) [82, 87, 88]. In animal

models several protective effects of ACE inhibitors, such as the treatment of age-related cardiovascular disease (hypertension, congestive heart failure) and renal disease (diabetic nephropathy), have been shown to be at least partly mediated through BK elevation [89, 90]. In the heart, these beneficial BK effects may involve coronary vasodilation and the concomitant improvement of local myocardial blood flow. The effects of BK and DABK are mediated by activation of two distinct G protein-coupled receptors, the B1 and B2 receptor (B1R and B2R). Several studies have shown that the contribution of both receptors varies in different animals and vascular species and under different conditions [91]. BK exerts its vasodilatory effect through two pathways, namely production of NO by eNOS and the release of vasodilatory autacoids such as prostaglandin I2 produced from arachidonic acid. Thus, ACE inhibitors might be beneficial by stimulation of BK-induced NO and autocoids. Also for aging this might be an important protective pathway. It has shown that BK, through prostaglandin release, protects against ROS-induced DNA damage and thus prevents cellular senescence in endothelial cells [56]. BK signaling might therefore protect against vascular aging related to genomic instability. This might be important for the treatment effects of ACE inhibitors in CAD, an age-related disease that is featured by the presence of senescence and DNA damage in vascular cells [9, 72, 92].

As a side effect, however, BK is believed to be responsible for causing dry cough, which results in withdrawal from ACE inhibitors. Moreover, BK has pro-inflammatory actions, leading to increased vascular permeability [93, 94]. This generates questions to which of the functions of BK contribute to beneficial effects of ACE inhibitors, and whether all BK effects are protective. This is not only true for BK but can also be said for the adverse effects of Ang II, i.e., are these effects all deleterious? In the case of patients, most elderly, with diabetic nephropathy it has been observed that potentially too much suppression of Ang II signaling will lead to adverse effects; a topic of a vivid polemic [95]. How this relates to the earlier mentioned observation that Ang II signaling increases with age is unclear, but it is perceivable that elevated Ang II levels will perhaps increase blood pressure to warrant sufficient organ and skeletal muscle perfusion in the aging organism. In addition, Ang II is a prerequisite for normal renal function and glomerular filtration. The notion that high blood pressure in (biologically) very old subjects is associated with better vitality and not anymore with increased cardiovascular risk has led to such hypotheses, but hitherto conclusive experimental evidence for this concept is lacking [96].

Apart from possible side effects, RAS inhibition is also hampered by therapy resistance [97]. It is not entirely clear which mechanisms lead to disparities in responsiveness of patients to RAS inhibition. Some genetic studies have shown that gene variants of RAS components such as ACE, the AT1 receptor, angiotensinogen, ACE2 and the BK receptor are associated with the risk for age-related CVD or the treatment efficacy of RAS inhibition in such diseases [97-102]. Despite such findings, results are either inconsistent or do not seem to be reproducible in large genome wide association studies [101, 103, 104], and hitherto genotyping of RAS components, or on any other related gene for that matter, has not found its way to the clinic. Often, the contribution of genotype alone seems simply too small to be a useful basis for a treatment regime, and attempts are now made to refine methods

for searching clinically applicable genotyping methods [97, 105-107]. Also, mechanistic explanations for the role of a risk allele and, as a consequence, some sort of clinical laboratory test, are often unavailable.

As an example of the pharmacogenetic role of BK receptor gene variants, the EUROPA trial, which compared the effects of the angiotensin-converting enzyme (ACE) inhibitor perindopril versus placebo in patients with stable CAD, revealed an association between treatment benefit with respect to primary endpoints that relate to cardiac ischemia (cardiovascular mortality, non-fatal myocardial infarction and resuscitated cardiac arrest) and the single nucleotide polymorphism (SNP) rs12050217 [108]. This polymorphism is located in the bradykinin (BK) B1 receptor gene. However, a mechanistic explanation and, consequently, a functional test to confirm the potential risk within individuals harboring the rs12050217 allele are missing. Comparable conclusions have been drawn for various RAS components. For example, the ACE I/D, angiotensinogen M235T and AT1 receptor A1166C polymorphisms have compelled many researchers over the years, however, as time passed and more comprehensive analyses in larger populations were concluded the enthusiasm waned because the contribution of the I/D polymorphism to ACE activity and disease risk, and therefore its usefulness for clinical application, was not so evident anymore [97, 109-113]. A rather simple explanation for the disappearance of polymorphism effects in larger study populations is that many other genes and non-genetic factors might influence RAS activity [103]. In a large, heterogenous patient population differences in genetic composition, age range, co-morbidities, ethnicity, diet, social context, and environmental factors will lessen the contribution of a genotype that happened to be a perceptible risk factor in a small, less heterogenous population. One might then wish to explore the factors that decrease the contribution of genotype, but it seems more straightforward to immediately test the activity of the mechanism that communicates the pathogenesis associated with the risk allele in a simple, affordable clinical laboratory test.

Dietary restriction

Dietary restriction (DR) is a reduction of intake of food to the level that it results in low-normal levels of energy intake whilst maintaining sufficient levels of protein and micronutrients as to avoid malnutrition [114]. A DR regimen usually involves a gradually imposed 30% reduction in total food intake from estimated ad libitum (AL) levels [115]. Although the first records that witness of the believe that low food intake is associated with increased longevity dates back as far as 3 millennia ago, scientific research in this field is relatively young [116]. The first experiments showing that DR increases life expectancy were performed in rats [117, 118]. To date, DR is the most effective intervention known with respect to increasing life expectancy under experimental conditions, demonstrating effectiveness in slowing down the aging process and increasing longevity in a variety of organisms, including yeast, worms, flies, spiders, rotifers, fish and rodents [119-122]. DR can also contribute to protection against age-related cardiovascular disease in the following two ways: (1) it could prevent an excessive uptake of food constituents that contribute to cardiovascular risk, such as carbohydrates and polysatured fats [123], or (2) it could

act through its beneficial impact on the aging process itself [124]. Moreover, life-long DR preserves vascular function by improvement of vasodilator function, due to reduced ROS and increased NO production, leading to a reduced age-dependent increase of blood pressure and vascular wall remodeling, as shown in rodents [125, 126]. DR also protects against cardiovascular disease in nonhuman primates [127].

The question as to what would be the leading mechanism that mediates these effects of DR is still under investigation. Especially in relationship with genomic integrity it is not clear what DR could contribute. It was long believed that reduction of ROS, leading to less damage of DNA and other macromolecules, would be central in the aging process. However, many studies have shown discrepancies in the association between ROS and anti-oxidant enzyme levels on the one hand and longevity on the other hand [86]. Therefore, it seems more sensible to assume that although increased ROS levels are involved in the functional changes during aging, they are not a causative factor in aging. Other possible directions to search for mechanisms are the effects of DR on nutrient sensing pathways (in which Sirt-1 could play a role) and the associated reduction in IGF-1/GH. As mentioned earlier, genomic instability has a profound effect on such pathways that in turn might influence vascular function. It seems therefore worthwhile to study the interplay between vascular aging and DR in animal models of genomic instability since this might lead to novel insights leading to the core mechanisms that govern age-related vascular disease and treatment thereof.

Aim and scope of this thesis

Nowadays, the average life expectancy of humans is increasing, and the percentage of people that enters the 65 and even older age group is growing rapidly and will continue to do so in the next 20 years [128, 129]. Within this aging population, cardiovascular disease will remain the main cause of death, and the costs associated with treatment will continue to increase. Hypertension and increasing age are two leading risk factors for age-related vascular disease [130, 131]. As explained above, RAS activation and genomic instability respectively are the main causal mechanisms for these risk factors, and may even have a reciprocal relationship with each other. However, this relationship has not been studied yet. Moreover, it is not entirely clear how RAS activity and BK signaling can guide therapy responses, and, as a consequence, therapeutic regimes, in hypertension and age-related vascular disease. Related to a possible effective intervention that might influence genomic instability and a possible interaction with the RAS in relation to age-related vascular disease, either pharmacological blockade of AT1 receptor signaling or lifestyle intervention with DR might offer possibilities. It is an important and exciting question which of these interventions would be most effective.

To address the question regarding the usefulness of RAS activity as a predictive marker for RAS inhibition as an antihypertensive treatment we investigated, in Chapter 2, whether baseline ACE and/or renin levels can truly be used in the individual hypertensive patient. To this end, ACE and plasma renin were measured before onset of acute and chronic RAS blockade (either ACE inhibition alone or in combination with AT1 receptor antagonists

or hydrochlorothiazide) and their association with blood pressure and aldosterone suppression after treatment was tested.

To find out what mechanism possibly underlies the observation that the rs12050217 BK B1 receptor polymorphism partly determines the ACE inhibitor therapy response in CAD, the pathogenesis of which is strongly related to cellular and organismal aging, we examined the effect of this polymorphism on B1 receptor-mediated coronary artery dilation and peripheral blood mononuclear cell activation in Chapter 3. Such studies might be useful to develop better predictors of ACE inhibitor therapy response.

To evaluate if there is a connection between RAS and genomic instability, in Chapter 4 we have studied the effect of chronic RAS inhibition, achieved by oral delivery of the AT1 receptor antagonist losartan, on vasodilator function and blood pressure in Ercc1$^{d/-}$ mice. Furthermore, we evaluated vasoconstrictive responses to Ang II in these aging animals. Starting treatment at an age of 4 weeks, when the consequences of the accelerated aging process are still minimal, this study implicitly addresses the question if a lifelong treatment with an AT1 receptor antagonist could efficiently prevent the devastating consequences of genomic instability.

To answer the question if DR, an important anti-aging intervention, could prevent the consequences of genomic instability, we have explored in Chapter 5 if DR could improve vascular function in Ercc1$^{d/-}$ mice, using vasodilator function and Ang II-induced vasoconstrictions as most important read-outs. By starting DR at the age of 7 weeks, which is before the onset of weight loss due to the accelerated aging process, a life-long intervention approach is mimicked, referring to the accumulating effect of calorie intake on genomic integrity.

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Chapter 2

Renin-angiotensin system phenotyping as a guidance toward personalized medicine for ACE inhibitors: can the response to ACE inhibition be predicted on the basis of plasma renin or ACE?

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Cardiovasc Drugs Ther. 2014;28: 335-345

ABSTRACT

Background
Not all hypertensive patients respond well to ACE inhibition. Here we determined whether renin-angiotensin system (RAS) phenotyping, i.e., the measurement of renin or ACE, can predict the individual response to RAS blockade, either chronically (enalapril vs. enalapril+candesartan) or acutely (enalapril □ hydrochlorothiazide, HCT).

Methods and Results
Chronic enalapril+candesartan induced larger renin rises, but did not lower blood pressure (BP) more than enalapril. Similar observations were made for enalapril+HCT vs. enalapril when given acutely. Baseline renin predicted the peak changes in BP chronically, but not acutely. Baseline ACE levels had no predictive value. Yet, after acute drug intake, the degree of ACE inhibition, like Δrenin, did correlate with ΔBP. Only the relationship with Δrenin remained significant after chronic RAS blockade. Thus, a high degree of ACE inhibition and a steep renin rise associate with larger acute responses to enalapril. However, variation was large, ranging >50 mm Hg for a given degree of ACE inhibition or Δrenin. The same was true for the relationships between Δrenin and ΔBP, and between baseline renin and the maximum reduction in BP in the chronic study.

Conclusions
Our data do not support that RAS phenotyping will help to predict the individual BP response to RAS blockade. Notably, these conclusions were reached in a carefully characterized, homogenous population, and when taking into account the known fluctuations in renin that relate to gender, age, ethnicity, salt intake and diuretic treatment, it seems unlikely that a cut-off renin level can be defined that has predictive value.

INTRODUCTION

ACE inhibitors are widely used for the treatment of cardiovascular diseases. Not all patients respond to ACE inhibitors, and it has been suggested that genetic variation might be a useful marker to predict the therapeutic efficacy of these drugs [1-3]. Yet, although numerous studies have investigated the ACE insertion (I)/deletion (D) polymorphism in this regard, data remain inconclusive [4-7]. This relates to a wide variety of issues, including insufficient sample size and genotype misclassification, as well as confounding (e.g., renin-affecting) factors like gender, age, sodium intake and ethnicity [8-10]. Moreover, the percentage of ACE variation attributable to the I/D polymorphism appears to be much lower (<20%) than originally reported [11]. A much simpler approach might be to make use of ACE phenotyping as a first step toward personalized medicine for ACE inhibitors, in other words to measure the concentration of ACE in circulating blood as a starting point. Plasma (or serum) ACE concentrations are stable within one individual [12] and reasonably reflect tissue ACE [13], although obviously there are exceptions, for instance due to mutations that increase ACE shedding [14]. Furthermore, ACE is the most important, if not the only, angiotensin (Ang) II-generating enzyme in the intact human body [15, 16]. Measuring ACE allows one to take into consideration the full variation range of ACE, and not just the <20% that is accounted for by the ACE I/D polymorphism.

A similar predictive value has been attributed to plasma renin concentration or activity (PRC, PRA) [17, 18]. Here, it is generally believed that patients with high baseline renin levels display larger/better blood pressure (BP) responses [19, 20], and that steep renin rises during treatment accurately reflect the efficacy of RAS blockade [21]. However, as for the ACE I/D polymorphism, variation was high, and gender, age, salt intake and ethnicity were again important confounding factors.

In the present study we set out to determine whether ACE and/or renin levels can truly be used as determinants of the response to an ACE inhibitor in an individual hypertensive patient. We therefore extensively characterized the renin-angiotensin system (RAS) at baseline and during treatment in 2 small well-defined populations, undergoing, respectively, acute and chronic ACE inhibitor treatment, either alone or in combination with an Ang II type 1 receptor antagonist or a diuretic. We focused on the changes in BP and plasma aldosterone as efficacy parameters of RAS blockade.

PATIENTS & METHODS

Clinical studies

Chronic study. This study was conducted as a double blind cross-over trial lasting 11 weeks. A three week single-blind placebo run-in period was followed by eight weeks of double-blind active medication. Patients were recruited from the out-patient hypertension clinic of the Erasmus MC, and antihypertensive drugs, if taken, were discontinued for at least 2 weeks. Caucasian subjects with uncomplicated hypertension, aged between 18 and 75

years, could participate if they were willing to give written informed consent. The BP at the end of the placebo run-in period should be between 140-180 mm Hg systolic and 90-100 mm Hg diastolic (SBP, DBP). Patients with a history of cardiovascular disease other than hypertension, and patients with diabetes mellitus, malignancy, chronic obstructive lung disease, gastro-intestinal disease and liver disease or abuse of nicotine or alcohol were excluded. The study protocol was approved by the Ethical Committee of the Erasmus MC.

After the placebo run-in period the patients were randomised for treatment with enalapril 40 mg (E40), or enalapril 20 mg and 16 mg candesartan (E20+C16) once daily. Placebo, enalapril and candesartan capsules had a similar appearance. During each period two capsules were taken every morning. At the end of the placebo and active treatment periods Ang I infusions to assess degree of RAS blockade, blood sampling and ambulatory BP measurements were performed. For these investigations the patient came to the clinical research unit of the Erasmus MC at 7:30 am. On arrival the ambulatory BP monitor (ABPM, Spacelabs, model 90207, Redmond, USA) was fitted to the patient and a cannula for blood sampling and Ang I (Clinalpha, Darmstadt, Germany) infusion was placed in a forearm vein. Ang I instead of Ang II infusions were applied because they allow to measure the degree of RAS blockade during administration of both an ACE inhibitor and an Ang II type 1 receptor antagonist [22]. During the Ang I infusion BP was measured continuously by means of the Finapres BP monitor (Ohmeda 2300) while the patient was in recumbent position. During placebo, Ang I infusion rates were 2, 5, 10 and 20 ng/kg/min, with each infusion step lasting 10 minutes. During active treatment Ang I infusion rates were 10, 20, 40, 100, 200, 400 and 800 ng/kg/min. Ang I infusions were increased every 10 minutes until an increase in systolic BP of 20 mm Hg was achieved (Pd20). The Ang I infusions were performed 24-26 hours after intake of study medication (trough) and 4 hours after controlled intake of study medication (peak). ABPM was measured at 20-minute intervals during the day (7 am to 11 pm) and at 30-min intervals during the night (0-7 am). Blood samples for measurement of plasma enalaprilat, renin, ACE, Ang I and aldosterone were taken just before the start of Ang I infusions as described before.

Acute study. For this study, patients were recruited from the outpatient hypertension clinic of the Erasmus MC. Male and female subjects diagnosed with grade 1 or 2 essential hypertension (SBP 140-180 mm Hg and/or DBP 90-110 mm Hg, when off antihypertensive medication for at least two weeks), with an age between 18-75 years and of Caucasian or Asian ethnicity (n=1) were included in the study. Subjects were excluded from the study if any of the following conditions was present: grade 3 hypertension, secondary forms of hypertension, history of myocardial infarction or stroke, impaired renal function, defined as a serum creatinine concentration >120 µmol/L, a history of gout or allergy to an ACE inhibitor, diabetes mellitus, regular use of non-steroidal anti-inflammatory drugs, use of glucocorticoids or excessive use of alcohol. Pregnant or nursing women were also excluded. Written informed consent was obtained from all subjects. The study protocol was approved by the Ethical Committee of Erasmus MC.

All patients received individualized instructions for a diet containing 100 mmol of sodium

per day. Adherence to the diet was checked by the measurement of 24-hour urinary sodium excretion at baseline. Patients were studied 3 times at an interval of 3 weeks. According to a randomization list, each patient was given in a double-blinded way a single dose of enalapril 20 mg or placebo without prior treatment with a diuretic. In addition, each subject was given in a non-blinded way a single dose of enalapril 20 mg, after pre-treatment with a low dose of hydrochlorothiazide (HCT), 12.5 mg daily for one week. HCT pre-treatment makes BP more RAS-dependent, increasing the possibility to find associations between changes in RAS components and BP reduction in response to ACE inhibition. At each visit an intravenous cannula (BD VenflonTM Pro 20GA 1.26IN) for blood sampling was inserted in a forearm vein and a cuff for BP measurement was fitted at the non-dominant arm. BP was measured with the Spacelabs 90217 BP monitor. With the patient in a semi-supine position, BP was initially measured at intervals of 5 minutes for 30 minutes and values were averaged to obtain a baseline BP value. Subsequently BP was measured at intervals of 15 minutes during the day (6 am to 11 pm) and of 30 minutes during the night. After baseline BP measurements, blood was sampled, and immediately after blood sampling the patient took his/her study medication. Blood sampling was repeated 1, 2, 4, 6 and 24 hours after intake of study medication.

Biochemical measurements

Plasma renin concentration was measured by immunoradiometric assay (Nichols, Capistano, USA) [23]. Plasma ACE activity was measured by fluorimetric method using two different substrates: hippuryl-L-histidyl-L-leucine (Hip-His-Leu, HHL) (Sigma) and benzyloxycarbonyl-L-phenylalanyl-L-histidyl-L-leucine (Z-Phe-His-Leu, ZPHL) (Bachem, King of Prussia, USA). The ACE C-domain hydrolyzes HHL 9-fold faster than the N-domain, while ZPHL is hydrolyzed to the same degree by both domains [24]. ACE C-domain-selective ACE inhibitors like enalaprilat will therefore block HHL hydrolysis more strongly than ZPHL hydrolysis, so that the ZPHL/HHL ratio will vary depending on the enalaprilat concentration. On this basis, making use of commercially available enalaprilat (Apotex, Toronto, Canada) as a reference, we were able to determine the enalaprilat concentration in plasma [24]. Plasma ACE concentration was determined by an enzyme-linked immunoassay (Chemicon Int., Temecula, USA) making use of a monoclonal antibody against an epitope localized on the N-domain (9B9) as a detecting antibody and polyclonal antibodies to human ACE conjugated to peroxidase as revelation antibody [25, 26]. Plasma Ang I was measured by an in-house developed radioimmunoassay and plasma aldosterone by a solid phase radioimmunoassay using a commercially available kit (Coat-a-Count Diagnostic Products Corp) [27].

Statistical analysis

Data are presented as mean±SD or geometric means and interquartile ranges for non-normal distributed values. For each patient in the chronic study the dose of Ang I increasing SBP by 20 mm Hg was calculated by linear regression analysis of the dose-response curve. One way analysis of variance for repeated measurements and Newman-Keuls post hoc tests or Friedman test for non-parametric data was used for comparison between placebo and

the active treatment groups. Difference between active treatment groups were compared with Student's paired t-test or Wilcoxon signed rank test. Differences between men and women and between subjects >60 years and <60 years were compared with Student's unpaired t-test. Pearson correlation test was used for analysis of associations. Associations between RAS components and the effects on BP were limited to SBP, as this parameter was expected to display the largest changes. P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

RESULTS

Subjects

Chronic study. The mean age of the 14 patients (11 male, 3 female) was 57 years (range 50-65 years). Their body mass index (BMI) was 27.4±4.7 kg/m^2, and they had a known duration of hypertension of 9.5±6.5 years. Baseline serum creatinine, electrolytes and hemoglobin concentration were in the normal range (data not shown).

Acute study. The mean age of the 20 patients (13 male, 7 female) was 59 years (range 39-71 years). Their BMI was 28.7±1.0 kg/m^2. The 24-hour urinary sodium excretion was 113.9±5.5 mmol/24 hours, indicating excellent adherence to the diet. Baseline SBP and DBP (average of 6 measurements) during the 3 study days did not differ (P=0.45), and averaged to 147.0±3.9 and 89.0±2.2 mm Hg, respectively. Baseline serum creatinine, electrolytes and hemoglobin concentration were in the normal range (data not shown).

Renin, Ang I and aldosterone

Chronic study. Renin and Ang I increased during active treatment compared to placebo (Table 1), and the increments tended to be larger with dual RAS blockade. Aldosterone decreased comparably with both active treatments. During placebo, the infusion rate of Ang I to increase SBP by 20 mm Hg (Pd20) was 8.4±0.9 ng/kg/min at trough and 7.9±0.8 ng/kg/min at peak (P=NS). Trough and peak Pd20's were closely correlated (r=0.68, P<0.02). During active treatment Pd20 markedly increased. The Pd20 ratio (Pd20 during active treatment/Pd20 during placebo) was higher with E20+C16 than with E40: at trough, the respective ratios were 9.9±3.0 and 4.4±0.9 (P<0.05), and at peak 57.2±9.8 and 24.7±6.2 (P<0.01). This confirms that the degree of RAS blockade was substantially larger during dual RAS blockade. The reactive increments of renin and Pd20 ratio's at trough and peak were not correlated (r=0.35 and r=0.33 for E40 and r=0.37 and r=0.33 for E20+C16).
Acute study. HCT pretreatment increased renin (P<0.05) but did not alter aldosterone (Figures 1C and 1D, Table 2). The increase in renin induced by enalapril on top of HCT was larger than without HCT pretreatment (P<0.05), whereas the enalapril-induced decreases in aldosterone were identical with and without HCT pretreatment.

ACE

Chronic study. At peak, enalaprilat was twofold higher during E40 as compared to E20+C16, whereas at trough enalaprilat did not differ between the two treatment regimens (Table 1). The inter-individual variation of the plasma ACE concentration at baseline ranged from 262 to 713 ng/mL. During active treatment, plasma ACE increased by 39±4% with E40 and by 41±4% with E20+C16 (Table 1), and both increases were significantly correlated (r=0.84, P<0.001). The increase in ACE did not correlate with baseline ACE. For both the HHL and ZPHL substrates the degree of ACE inhibition at peak was higher with E40 than with E20+C16 (Table 1). At trough, the degree of ACE inhibition between the two treatments did not differ. As expected, the degree of ACE inhibition was substantially greater with HHL than with ZPHL as substrate.

Acute study. HCT did not alter the plasma ACE concentration, nor its activity when determined with either HHL or ZPHL (data not shown). No change in ACE concentration occurred during acute enalapril exposure (Figure 2A, Table 3). Enalapril reduced ACE activity independently of HCT pretreatment, reaching maximum effects within 2 hours, both when determined with HHL and ZPHL (Figures 2B and 2C, Table 3). The degree of inhibition was again larger with HHL than with ZPHL.

Blood pressure

Chronic study. Day and night-time values of ABPM at the end of the placebo period and the two active treatment periods are given in Table 4. Day- and night-time systolic and diastolic BP decreased during active treatment compared to placebo, but the fall in BP with the two active treatments did not differ. Compared to placebo, heart rate did not change with E40 or E20+C16.

Acute study. HCT pretreatment marginally reduced SBP (*P*=0.45; Figure 1A). The maximal SBP response to enalapril was observed 6 hours after dosing. At this time SBP had decreased by 13% (range -31 to 25%) with enalapril and by 17% (range -42 to 8%) with enalapril+HCT (*P*=0.25 for difference). Changes in DBP followed a similar pattern (Figure 1B). The BP responses without and with pre-treatment of HCT were closely related (*r*=0.74, *P*<0.001)

Table 1. Trough and peak values of plasma enalaprilat, ACE concentration, ACE inhibition, renin, angiotensin I and aldosterone during chronic treatment with placebo, enalapril 40 mg (E40) or enalapril 20 mg and candesartan 16 mg (E20+C16).

	Placebo	**E40**	**E20+C16**	**P-value**
[Enalaprilat], nmol/L				
Trough	-	0.9±0.4	1.0±0.4	0.40
Peak	-	4.9±1.3	2.4±0.7	0.02
[ACE], ng/ml				
Trough	426±126	583±143***	560±132***	0.36
Peak	not available	561±143	594±152	0.50
ACE inhibition(HHL), %				
Trough	-	18.1±11.6	18.0±8.3	0.44
Peak	-	66.9±9.1	51.4±7.8	<0.001
ACE inhibition (ZPHL), %				
Trough	-	4.6±2.4	4.4±2.1	0.20
Peak	-	19.7±7.8	14.0±6.4	0.03
Renin, mU/L				
Trough	14.5 (10.8-20.3)	28.2 (15.6-57.7)*	52.5 (19.3-172)***	0.28
Peak	16.7 (12.6-24.4)	40.4 (16.8-135)**	65.3 (17.4-356)***	0.46
Ang I, ng/L				
Trough	3.9 (1.6-3.1)	12.1 (6.4-26.9)***	20.7 (6.6-63.7)***	0.37
Peak	5.4 (1.9-9.7)	17.9 (5.5-90.4)***	27.3 (5.1-44.9)***	0.51
Aldosterone, ng/L				
Trough	68.9±13.5	40.8±6.1**	42.4±5.7**	0.63
Peak	73.1±13.7	35.3±5.4*	32.9±4.5**	0.76

Values are means ± SD or geometric means and interquartile ranges. *P<0.05, **P<0.01, ***P<0.001 versus placebo, the P-value in the last column of the Table refers to the difference between E40 and E20+C16.

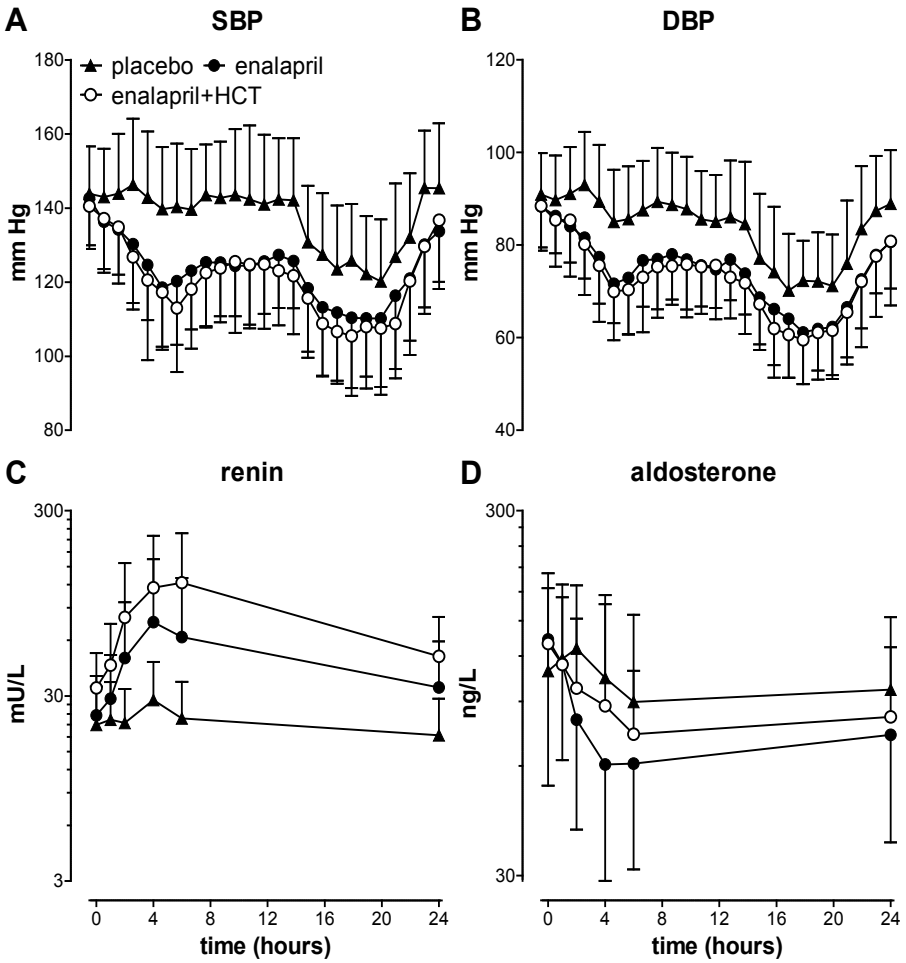

Figure 1. Changes in systolic blood pressure (A), diastolic blood pressure (B), renin (C), and aldosterone (D) following acute exposure to enalapril, with or without diuretic pretreatment. Data are mean±SD of n=20.

Table 2. Changes in plasma renin and aldosterone levels following acute exposure to placebo or enalapril, with or without hydrochlorothiazide (HCT) pretreatment. Data are mean±SD of n=20. *P <0.01, #P<0.05 vs. t=0.

Time (hours)	Placebo		Enalapril		Enalapril + HCT	
	Renin (mU/L)	Aldosterone (ng/L)	Renin (mU/L)	Aldosterone (ng/L)	Renin (mU/L)	Aldosterone (ng/L)
0	21±13	109±75	24±15	133±80	33±18	130±72
1	22±13	118±71	29±21	114±52	44±30	114±60
2	21±11	126±62	48±48	80±40*	80±75	98±54
4	29±17	104±72	75±89*	61±31*	115±104*	88±79
6	23±13	90±66	62±68	61±30*	123±105*	73±36#
24	18±11	97±56	33±26	73±36*	49±31	82±45

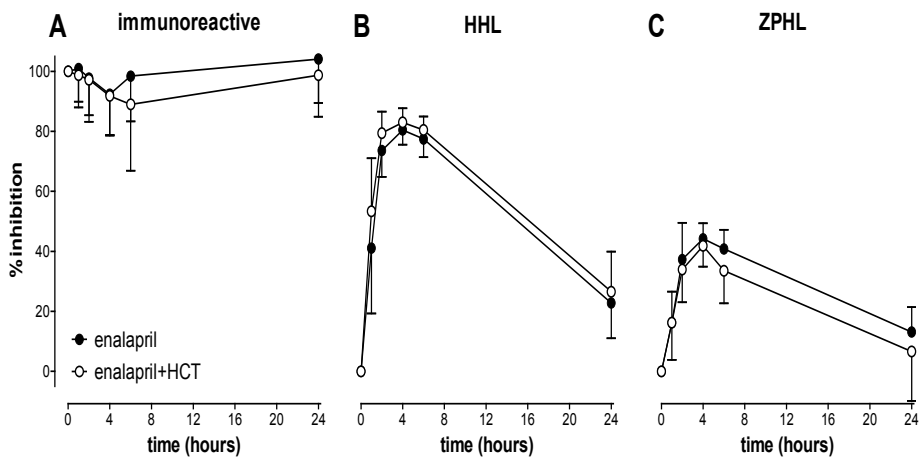

Figure 2. Changes in immunoreactive ACE (panel A) and ACE activity (determined with HHL or ZPHL; panels B and C) following acute exposure to enalapril, with or without diuretic pretreatment. Data are mean ± SD of n=20, and have been expressed as % of t=0.

Table 3. Changes in immunoreactive ACE and ACE activity (determined with HHL or ZPHL) following acute exposure to enalapril, with or without hydrochlorothiazide (HCT) pretreatment. Data are mean±SD of n=20, and have been expressed as % of t=0. *P<0.01, #P<0.05 vs. 100%.

Time (hours)	Enalapril			Enalapril + HCT		
	Immunoreactive ACE (% of t=0)	HHL (% of t=0)	ZPHL (% of t=0)	Immunoreactive ACE (% of t=0)	HHL (% of t=0)	ZPHL (% of t=0)
1	101±13	59±22#	84±10	99±9	46±18#	84±12
2	98±15	26±9*	63±12#	97±12	20±7*	66±11
4	92±14	19±5*	56±5#	92±13	16±5*	58±7#
6	98±15	22±6*	59±6#	89±22	19±5*	67±11
24	104±15	77±12	87±8	99±14	73±14	93±17

Table 4. Day- and nighttime ambulatory blood pressure and heart rate during placebo treatment and their changes following chronic treatment with enalapril 40 mg (E40) or enalapril 20 mg + 16 mg candesartan (E20+C16).

	Placebo	E40	E20+C16	P-value
Daytime				
SBP, mm Hg	153.9±3.2	-11.7±1.9***	-12.4±3.1***	0.44
DBP, mm Hg	98.2±2.1	-8.4±1.5**	-9.3±2.3***	0.15
HR, bpm	77.4±2.1	-0.6±1.4	0.3±1.5	0.93
Night-time				
SBP, mm Hg	138.5±3.6	-9.1±3.8*	-10.9±5.0*	0.70
DBP, mm Hg	85.1±2.5	-6.4±3.2*	-7.1±3.5*	0.58
HR, bpm	69.4±2.0	-0.1±1.6	0.6±1.7	0.81

Values are means ± SD. *P<0.05, **P<0.01, ***P<0.001 versus placebo, the P-value in the last column of the Table refers to the difference between E40 and E20+C16. *SBP* and *DBP*, systolic and diastolic blood pressure; *HR*, heart rate.

Gender and age

Chronic study. The inclusion of only 3 women and the narrow age range in this study did not allow a subanalysis of gender- and age-related differences.

Acute study. Subdividing the patients according to gender or age (< or > 60 years) revealed that, in general, women and older subjects had lower renin levels, and as a result, displayed more modest absolute renin changes (Table 5). However, due to the low n-number, none of these differences were significant, except for the difference between the baseline renin levels of men and women after HCT pretreatment (P<0.05). The degree of ACE inhibition was identical in all subgroups, both with and without HCT pretreatment. As expected, older subjects also displayed lower aldosterone levels (P<0.05), and their aldosterone response to ACE inhibition tended to be more modest (P=0.05). After HCT pretreatment, these differences were no longer significant. Of interest, despite their lower renin levels, the aldosterone levels of women were not different from those of men, nor did their decreases in aldosterone following ACE inhibition differ from those in men.

Pretreatment with HCT did not alter this outcome. The modest (and in most cases non-significant) differences in renin and aldosterone between men and women and between subjects below and above 60 years did not translate into differences in either baseline SBP, or peak SBP response to ACE inhibition. Therefore, in all subsequent analyses we did not separately consider gender or age.

Predictive value of plasma renin and ACE at baseline

Chronic study. Correlation coefficients for the placebo-corrected changes in SBP and

plasma aldosterone at trough (between 20-24 hours after drug intake) and peak (between 3-7 hours after drug intake) in response to E40 or E20+C16 and baseline values of plasma ACE and PRC are given in Table 6. There were no associations with plasma ACE at baseline. PRC correlated under almost all conditions (trough and peak) negatively with the decrease in aldosterone: in other words, the higher baseline renin, the larger the drop in aldosterone, irrespective of the type of RAS blockade. A similar negative association was found with the decrease in SBP at peak, although this was significant only during E20+C16.

Acute study. Correlation coefficients for the placebo-corrected changes in SBP and plasma aldosterone at trough and peak in response to enalapril with and without HCT pretreatment are given in Table 7. Although there were trends for negative associations between baseline renin or baseline ACE and the decrease in SBP or aldosterone, under no condition did any of these associations reach significance.

Table 5. Baseline levels of systolic blood pressure (SBP), renin and aldosterone and their changes at peak following acute exposure to placebo or enalapril, with or without hydrochlorothiazide (HCT) pretreatment, subdivided according to gender and age. Data are mean±SD. *P<0.05 vs. men, **P<0.05 vs. <60 years, #P=0.05 vs. <60 years.

	Men (n=13)	Women (n=7)	<60 years (n=9)	>60 years (n=11)
Enalapril				
Baseline SBP (mm Hg)	144±16	141±10	142±13	144±16
ΔSBP at peak (mm Hg)	-14±20	-24±5	-19±14	-15±20
Baseline Renin (mU/L)	25±16	21±13	26±18	21±12
ΔRenin at peak (mU/L)	54±75	50±84	75±95	25±34
% ACE inhibition	80±6	82±5	83±5	79±5
Baseline aldosterone (ng/L)	115±55	166±111	166±93	94±35**
ΔAldosterone at peak (ng/L)	-75±47	-119±97	-117±83	-58±27#
Enalapril + HCT				
Baseline SBP (mm Hg)	144±14	134±10	141±12	140±16
ΔSBP at peak (mm Hg)	-18±22	-22±14	-20±16	-18±24
Baseline Renin (mU/L)	38±19	24±13*	37±21	29±13
ΔRenin at peak (mU/L)	110±102	59±60	102±114	81±56
% ACE inhibition	85±4	83±5	85±4	84±5
Baseline aldosterone (ng/L)	122±59	144±96	153±82	102±50
ΔAldosterone at peak (ng/L)	-71±44	-81±61	-93±57	-52±26

Table 6 Correlation coefficients between the placebo-corrected changes in systolic blood pressure (SBP) and plasma aldosterone at peak and trough and the baseline plasma levels of ACE and renin in response to chronic treatment with enalapril 40 mg (E40) or enalapril 20 mg + 16 mg candesartan (E20+C16). Data are derived from 14 patients.

	E40		E20+C16	
Parameter	[ACE]	[Renin]	[ACE]	[Renin]
ΔSBP at peak	0.02	0.33	-0.15	-0.52*
Δ[aldosterone] at peak	-0.25	-0.24	-0.34	-0.52*
ΔSBP at trough	-0.16	0.08	-0.13	-0.20
Δ[aldosterone] at trough	-0.04	-0.55*	-0.11	-0.58*

*P<0.05

Table 7 Correlation coefficients between the placebo-corrected changes in systolic blood pressure (SBP) and plasma aldosterone at peak and trough and the baseline plasma levels of ACE and renin in response to acute exposure to enalapril 20 mg without or with HCT pretreatment. Data are derived from 20 patients.

	Enalapril		Enalapril+HCT	
Parameter	[ACE]	[Renin]	[ACE]	[Renin]
ΔSBP at peak	-0.02	-0.08	-0.20	-0.30
Δ[aldosterone] at peak	-0.28	-0.20	-0.27	-0.27
ΔSBP at trough	-0.10	-0.13	-0.18	-0.02
Δ[aldosterone] at trough	0.15	0.27	0.13	0.11

Predictive value of the changes in plasma renin and ACE activity during treatment

Chronic study. The change in PRC correlated inversely with the decrease in SBP (r=-0.31, P<0.05; Figure 3A), but not with the decrease in plasma aldosterone (r=0.02, P=NS; Figure 3C) or the degree of ACE inhibition (r=0.05, P=NS). The degree of ACE inhibition correlated neither with the decrease in SBP (r=-0.23, P=NS; Figure 3B) nor with the decrease in plasma aldosterone (r=-0.24, P=NS; Figure 3D).

Acute study. The change in PRC correlated inversely with the decrease in SBP (r=-0.21, P<0.01; Figure 4A) and plasma aldosterone (r=-0.25, P<0.001; Figure 4C), as well as directly with the degree of ACE inhibition (r=0.33, P<0.001). The degree of ACE inhibition also correlated inversely with the decrease in SBP (r=-0.15, P<0.05; Figure 4B), but not with the decrease in plasma aldosterone (r=-0.11, P=NS; Figure 4D).

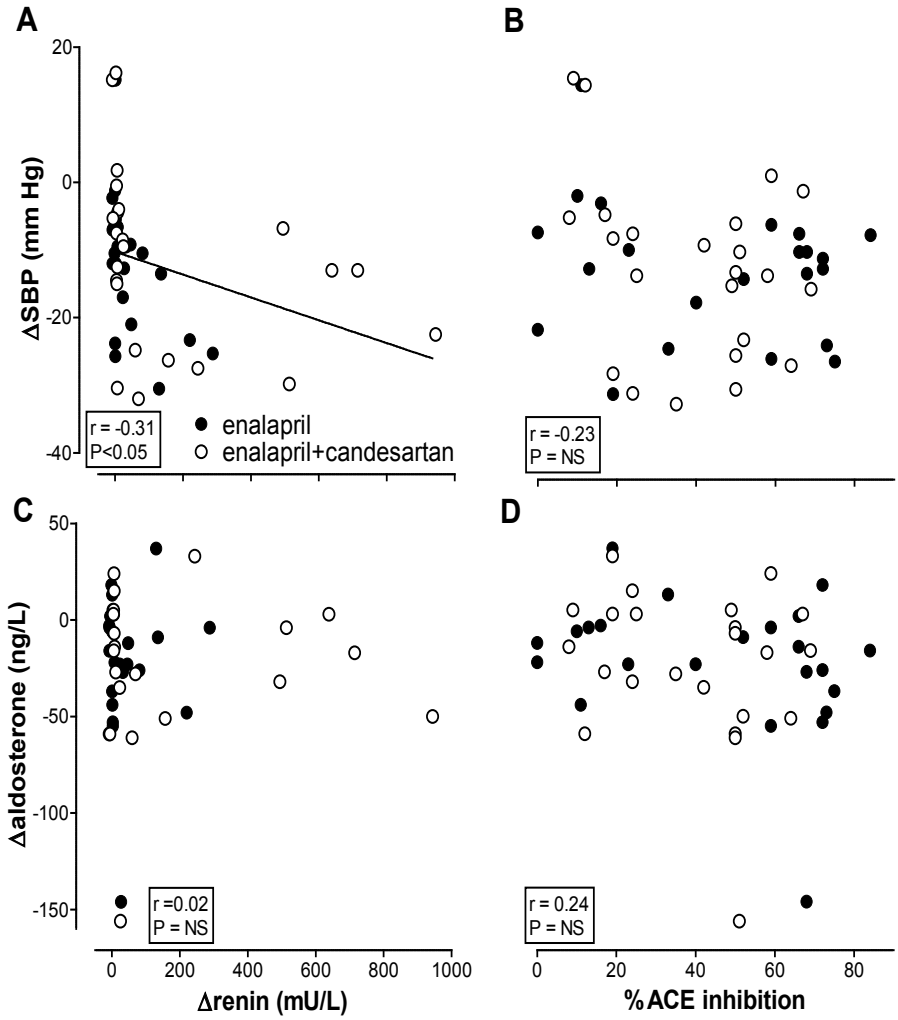

Figure 3. Relationship (or the absence thereof) between the change in systolic blood pressure (panels A and B) or aldosterone (panels C and D) and the change in plasma renin or ACE activity in 14 hypertensive patients treated for 4 weeks with 40 mg enalapril or 20 mg enalapril + 16 mg candesartan. Data represent the change versus t=0 and have been corrected for the effect of placebo.

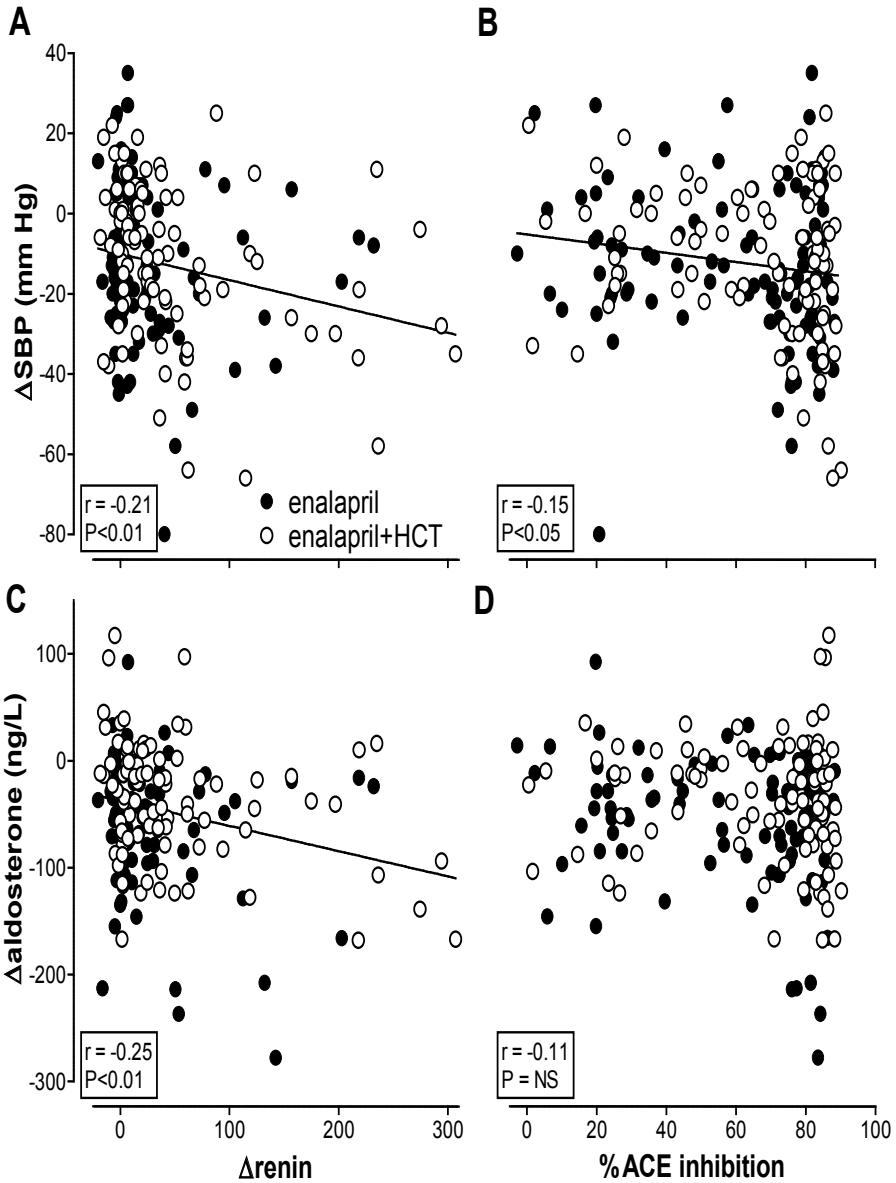

Figure 4. Relationship (or the absence thereof) between the change in systolic blood pressure (panels A and B) or aldosterone (panels C and D) and the change in plasma renin or ACE activity in 20 hypertensive patients exposed to 20 mg enalapril with or without diuretic pretreatment. Data represent the change versus t=0 and have been corrected for the effect of placebo.

DISCUSSION

This study shows that baseline plasma renin levels have predictive value with regard to the maximum reductions in plasma aldosterone and, to a lesser degree, BP following chronic, but not acute, RAS blockade. No such predictive power was observed for baseline plasma ACE levels. Nevertheless, the degree of ACE inhibition after acute drug intake did correlate with the change in BP. Likewise, the rise in PRC correlated with the decrease in BP following acute drug intake. This relationship between ΔPRC and ΔBP was also observed following chronic RAS blockade, while the relationship between the degree of ACE inhibition and ΔBP was then lost. Thus, as one would expect, a high degree of ACE inhibition and a steep rise in renin (the latter reflecting significant RAS blockade at the level of the juxtaglomerular cells) associate with a larger acute response to enalapril. However, like in previous studies [19, 20], variation was large (ranging >50 mm Hg for a given degree of ACE inhibition or PRC rise), and thus such knowledge cannot be used to accurately predict the acute BP response to ACE inhibition in an individual patient. The same was true for the relationships between ΔPRC and ΔBP, and between baseline PRC and the maximum reduction in BP and aldosterone, respectively, in the chronic study.

The lack of correlation between the degree of plasma ACE inhibition and the decrease in plasma aldosterone suggests that plasma ACE inhibition does not reflect adrenal ACE inhibition, neither acutely nor chronically. In contrast, the rise in renin correlated significantly with the degree of ACE inhibition following acute enalapril exposure, and thus renal ACE inhibition under those circumstances apparently parallels plasma ACE inhibition. This relationship was lost following chronic treatment, possibly due to the upregulation of renin and/or ACE in the kidney after long-term ACE inhibition [28-32]. Finally, the correlation between the changes in renin and aldosterone in the acute setting support a significant contribution of kidney-derived renin to adrenal aldosterone production prior to the start of RAS blockade. Like in the kidney, this relationship was lost after chronic enalapril exposure, suggesting that then non-renin driven stimulators of aldosterone (reflecting the so-called 'aldosterone escape' [33, 34]) come into play.

Chronic dual RAS blockade with E20+C16 significantly enhanced the degree of RAS inhibition, as reflected by higher PRC, Ang I levels and Pd20 values versus E40. Yet, it did not result in significantly larger effects on BP or aldosterone. The lack of an additional effect on aldosterone is not too surprising in view of the RAS-independent regulators of aldosterone synthesis that override following chronic RAS blockade. The lack of an additional effect on BP should be evaluated in view of the fact that the negative association between ΔBP and ΔPRC displays a wide variation. Clearly, the same degree of BP lowering can be observed at widely varying renin levels [19] and thus a specific renin rise cannot accurately predict the BP change in an individual patient. In general, the largest rises in PRC were observed in the dual RAS blockade group. Nevertheless, the BP-lowering effect of RAS blockade will have a limit [35]. RAS blockade on top of that may further suppress the RAS, thereby inducing additional rises in renin [23], but the consequence of this will rather be effects beyond BP, e.g., on kidney function, albumin excretion and K+- handling, as evidenced by ONTARGET

(ramipril + telmisartan) and ALTITUDE (aliskiren on top of an ACE inhibitor or an Ang II type 1 receptor blocker) [36, 37].

Diuretic pretreatment, by activating the RAS, normally enhances the response to a RAS blocker [38]. Such RAS activation as well as an enhanced renin response to ACE inhibition also occurred in the present study. However, this did not lead to larger decreases in BP or greater reductions in aldosterone, at least acutely. Thus, either this requires prolonged treatment, or the rises in renin were too modest to allow a significant increase in BP reduction and aldosterone suppression. The fact that we did see larger BP reductions upon chronic RAS blockade at higher baseline PRC supports the former.

Women and older subjects are known to display lower renin levels [38]. Aldosterone also decreases with age, while the aldosterone levels between men and women generally do not differ [38-40]. Our study fully confirms this view. It additionally suggests, like previous studies [19, 20], that the (absolute) changes in renin and aldosterone following RAS blockade depend on their initial levels, i.e., people with higher baseline aldosterone levels will display larger net aldosterone decreases, and people with high baseline renin levels show larger increases in renin. To what degree this implies that women and older patients respond less well to RAS blockade cannot be concluded from our results; addressing this question would have required much larger patient numbers and was beyond the goal of our study.

Chronic ACE inhibition upregulated plasma ACE by approximately 40%, in full agreement with earlier observations [41]. The ACE increase was identical in patients treated with E20 and E40, and thus is independent of the degree of ACE inhibition. This rise in ACE is known to be the consequence of increased ACE shedding (due to the altered conformation of ACE when bound to enalapril) [42], and might simultaneously involve a modest upregulation of ACE due to interference with the negative feedback by Ang II [43]. Such upregulation would explain why, following chronic ACE inhibition, the degree of ACE inhibition no longer accurately reflects the effects on BP or renin.

A weakness of this study are its relatively small patient numbers. However, even if additional correlations had been found in a larger population, the practical conclusions of such correlations, given their large variation, would not have been different from the conclusions we have reached now. Secondly, since enalapril is a prodrug, it cannot be excluded that there is a certain time discrepancy between its maximum effects on RAS parameters and BP. We have tried to counteract this issue by measuring RAS activity at various time points after an acute exposure to enalapril, and by additionally performing a chronic study.
In summary, our data do not support that RAS phenotyping will be of great value for predicting BP response to an ACE inhibitor at the level of the individual patient. At most, high renin levels are indicative of a larger BP response to RAS blockade. The novelty and importance of this study lie in the fact that we carefully characterized the responses to RAS blockade in a homogenous population, thus providing the optimal conditions to find a role for RAS phenotyping in hypertension. Unfortunately, no such role was found, since even in this population variations

in RAS component levels and BP responses were very large. Taking into account the additional, well-known fluctuations in renin that relate to gender, age, ethnicity, salt intake and diuretic treatment, it is clear that it is unlikely that a cut-off renin level can be easily defined that has predictive value. Generally, a high degree of ACE inhibition and a large rise in renin (reflecting excellent RAS blockade) are indicative of drug efficacy, but here the variations are again of such magnitude that they too cannot be of value at the individual level.

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Chapter 3

Genetic and gender differences determine bradykinin type 1 receptor responses in human tissue: implications for the ACE inhibitor-induced effects in patients with coronary artery disease

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Clin Sci. 2014;126: 441-449

ABSTRACT

Background
The efficacy of angiotensin-converting enzyme inhibitor perindopril in coronary artery disease associates with the rs12050217 A/G single nucleotide polymorphism in the bradykinin B1 receptor gene.

Methods and Results
To investigate the underlying mechanism, we examined the effect of this polymorphism on B1 receptor-mediated coronary artery dilation and peripheral blood mononuclear cell activation. Vasorelaxant responses of human coronary microarteries from subject without coronary disease to des-Arg9-bradykinin and to bradykinin were studied in organ bath experiments. Des-Arg9-bradykinin responses were endothelium-dependent and exclusively mediated by B1 receptors, whilst responses to bradykinin were induced through B2 receptors. The presence of the G allele reduced the response to des-Arg9-bradykinin, whereas the responses to bradykinin were unaffected by rs12050217 genotype. In freshly obtained human mononuclear cells pro-inflammatory 1 µmol/L des-Arg9-bradykinin increased CXC chemokine ligand (CXCL)-5 expression. The response was not affected by genotype but only occurred in blood cells from women, showing a positive correlation with their 17β-estradiol levels ($r^2=0.32$, $p=0.02$). IL-1β increased CXCL-5 expression in both genders, and this response was not associated with 17β-estradiol levels.

Conclusions
The observed decrease in coronary vasodilator response might explain the worsened treatment response to perindopril of G allele carriers found in PERGENE. The gender difference in responses to B1receptor stimulation in blood mononuclear cells might be of importance for gender differences in ACE inhibitor therapy, which need to be studied more comprehensively.

INTRODUCTION

Pharmacogenetic analysis of the EUROPA trial, which compared the effects of the angiotensin-converting enzyme (ACE) inhibitor perindopril versus placebo in patients with stable coronary artery disease (CAD), revealed an association between treatment benefit with respect to primary endpoints that relate to cardiac ischemia (cardiovascular mortality, non-fatal myocardial infarction and resuscitated cardiac arrest) and the single nucleotide polymorphism (SNP) rs12050217 [1]. This polymorphism is located in the bradykinin B1 receptor gene [1]. ACE inhibitors increase the levels of the hormone bradykinin (BK) and its active metabolite des-Arg9-BK (DABK) [2, 3]. Bradykinin preferentially binds the bradykinin type 2 (B2) receptor, thus acting as a potent endothelium-dependent vasodilator and pro-inflammatory hormone. DABK is the preferred bradykinin type 1 (B1) receptor agonist. B2 receptor-mediated effects have been studied widely, but the effects of the B1 receptor are less well-established. Like the B2 receptor, this receptor induces endothelium-dependent vasodilatation in coronary arteries [4-6]. This occurs fully independently of the B2 receptor. Next to its vasodilating effects, the B1 receptor also induces an immunogenic response in endothelial cells and inflammatory cells that comprises of increased nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity and the production of the inflammatory cytokines CXC chemokine ligand (CXCL)-5 and interleukin (IL) 6 [7-9]. Through these pathways, the B1 receptor might play a role in CAD, thereby potentially explaining the association found between ACE inhibitor efficacy and the rs12050217 SNP.

SNP rs12050217 is positioned in intron 1 of the B1 receptor (BDKRB1) gene on chromosome 14, in an area that is not a promoter region nor contains regulatory elements [10-12]. Recent 1000-genome data indicate that the rs12050217 SNP is in linkage disequilibrium with the rs870282 SNP in intron 2 of the BDKRB1 gene. Although this SNP has not been described in other studies, the SNP is located in an area that has been described as a secondary promoter region, located in front of exon 3, which contains the entire coding region of the BDKRB1 gene [12]. On the basis of this observation, we hypothesized that the presence of the minor allele for rs12050217 induces a difference in B1 receptor expression, thus affecting CAD via the above-described B1 receptor-mediated mechanisms. To investigate this possibility, we related the presence of the rs12050217 SNP to the DABK-induced 1) relaxation of human coronary microarteries (HCMAs) and 2) inflammatory response of peripheral blood mononuclear cells (PBMNCs).

METHODS

Myograph studies

HCMAs were obtained from 21 organ donors, who died of non-cardiac diseases (3 suicide, 3 cerebrovascular accident, 4 head trauma, 4 subarachnoid bleeding, 6 post-anoxic encephalopathy; see Table 1 for demographic data) maximally 24 hr before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The study was approved by the

Ethics Committee of the Erasmus MC, and studies occurred according to the principles outlined in the Declaration of Helsinki. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (inner diameter 260–600 mm, mean 420 mm) was removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3; pH 7.4.

Following overnight storage, HCMAs were cut into segments of 2 mm length and mounted in a Mulvany myograph (J.P. Trading) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2, and maintained at 37°C. Tissue responses were measured as changes in isometric force, using a Harvard isometric transducer. Following a 30-min-stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure. Endothelial integrity was verified by observing relaxation to 10 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane A2 analogue U46619. Segments displaying <60% relaxation to substance P were not used. Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, segments were pre-incubated for 30 min with the B1 receptor antagonist DALBK (10 µmol/L), the B2 receptor antagonist Hoe140 (1 µmol/L), or no antagonist, and preconstricted to >50% of the maximum response to KCl with 10 nmol/L U46619. Thereafter, concentration-response curves (CRCs) to DABK were constructed, followed by exposure to 100 nmol/L bradykinin. In a selected set of vessels, the endothelium was removed in one segment by gently rubbing the lumen with a roughened paperclip. The cyclooxygenase inhibitor indomethacin (5 mmol/L) and/or the calcium antagonist nifedipine (100 nmol/L) were present during the entire experiment to suppress spontaneously occurring contractions and relaxations [13, 14]. Nifedipine was added only in case of remaining spontaneous vasomotor activity (despite the addition of indomethacin). DABK CRCs with (n=5) and without (n=5) nifedipine were identical (pEC50 -8.8 ± 0.4 vs. -8.3 ± 0.2, Emax -43.5 ± 5.2 % vs. -42.4 ± 4.8), and data with and without nifedipine were therefore combined. All drugs were from Sigma-Aldrich.

Human PBMNCs

Human PBMNCs were isolated from 20 mL blood donated by 44 young healthy volunteers (see Table 2 for demographic data), collected in EDTA tubes and processed immediately. PBMNCs were isolated by density centrifugation by Histopaque 1077 (Sigma), according to the manufacturer's protocol. Erythrocytes were removed by incubating the PBMNCs in red blood cell lysis solution (VWR) for 10 minutes at 4°C. After counting, cells were seeded at a density of 1x106 cells per mL of RPMI 1640 (Gibco) + 2% Pen Strep and 10% FCS (Thermo) medium in a 37°C 5% CO_2 incubator. On the following day, the cells were exposed for 8 hours to 1 µmol/L DABK, 1 ng/mL IL-1β, or 10 ng/mL 17β--estradiol, with or without a 20-min pre-exposure to 10 µmol/L DALBK. Cells used in the 17β--estradiol experiments were obtained from female donors only, and had been seeded in RPMI without phenol red, making use of

charcoal-stripped FCS. The study was approved by the independent Ethics Committee of the Erasmus MC, and studies occurred conform the principles outlined in the Declaration of Helsinki.

Genotyping

DNA was isolated from left ventricular tissue with the Qiagen DNA isolation kit according to the manufacturer's protocol. Genotyping for the rs12050217 SNP was done in 10 ng DNA with probes from Life Technologies according to the manufacturer's protocol.

Protein analysis

Coronary arteries were lysed with 50 mmol/L Tris HCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L Igepal CA-630, 5 mmol/L deoxycholic acid, and 1 mmol/L sodium dodecyl sulphate, in the presence of protease inhibitor cocktail (Roche) and serine-threonine phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Lysates were analyzed by standard Western blotting techniques under denaturating conditions, making use of an anti-B1 receptor antibody (Origene). Normalization occurred by reprobing the blot against anti-actin (C4, Millipore). Detection was by enhanced chemiluminescence detection method and quantification by densitometry.

Real-time quantitative reverse transcription PCR

Total RNA isolation was performed with the NucleoSpin RNA II kit (Machery-Nagel). RNA was reverse-transcribed by use of the Quantitect Reverse Transcription Kit (Qiagen). 10 ng of cDNA was amplified by real-time polymerase chain reaction, and normalized to RPLP0 ribosomal protein, large, P0 forward (36B4) as endogenous control. Each reaction was performed in duplo with SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: CXCL-5 forward 5'- GAGAGCTGCGTTGCGTTTG-3, reverse 5'- TTTCCTTGTTTCCACCGTCCA -3; 36b4: 5'- AACGGGTACAAACGAGTC-3' reverse 5'-AGATGGATCAGCCAAGAAG-3'. mRNA expression changes, expressed as fold changes versus untreated controls, were calculated by Δ-Δ-Ct method, and the distributions of the PCR data were normalized by performing a log2 transformation.

17β-Estradiol measurement

17β-Estradiol levels were determined in plasma by Cayman assay (estradiol-acetylcholinesterase conjugate-based tracer, 96-well estradiol ELISA kit, item # 582251), according to the manufacturer's protocol.

Statistics

Data are presented as mean ± SEM. Relaxant effects were expressed as the percentage of

the U46619-induced preconstriction. CRCs were analyzed with GraphPad Prism 5 software, non-linear regression analysis function, to obtain pEC50 (-10logEC50), and Emax values were calculated by averaging the maximal responses taken from each individual CRC. To determine genotype-dependent alterations in the DABK CRC, a two-way ANOVA was applied. In addition, response differences at individual, equimolar agonist concentrations of the CRCs were compared by Student's t-test. The difference between the DABK Emax and the response to 100 nmol/L bradykinin in each vessel was tested by paired t-test. PCR data were tested by Student's t-test or one-sample t-test (versus baseline variable 0).

RESULTS

Studies in human coronary microarteries

DABK concentration-dependently relaxed preconstricted HCMAs by maximally 64.3 ± 4.0 % (pEC50 -8.4 ± 0.2, n=6; Figure 1). Endothelium removal (n=6) and pretreatment with the B1 receptor antagonist DALBK (n=6), but not pretreatment with the B2 receptor antagonist Hoe140 (n=14), fully inhibited the effects of DABK. Bradykinin (100 nmol/L), when given on top of DABK, exerted no further effect in vessel segments pretreated with Hoe 140, but caused a further relaxation of 20.8 ± 5.1 % (P< 0.0006, paired t-test vs. DABK Emax) in the segments in the non-pretreated control segments. Moreover, bradykinin caused an almost complete relaxation (-80.6 ± 6.8 %) in the DALBK-pretreated segments that did not respond to DABK. Subdividing the DABK CRCs according to B1 receptor genotype (Table 1, Figure 2), revealed that subjects with the AA genotype tended to respond more strongly than subjects with the AG+GG genotype (two-way ANOVA, P=0.06). At a DABK concentration of 30 nmol/L, the larger relaxation in AA's reached statistical significance (P=0.03, t-test). No differences with regard to potency (pEC50 -8.2 ± 0.2 vs. -7.8 ± 0.2) were observed. The bradykinin B2 receptor-mediated response was unrelated to the B1 receptor genotype. Western blot analysis of 19 vessel segments revealed no difference in HCMA B1 receptor protein content between the 2 genotypes.

Table 1. rs12050217 genotype of human coronary microarteries.

	AA	**AG+GG**
n	13	8
Age	41±4	48±7
Male/Female	8/5	4/4

Figure 1

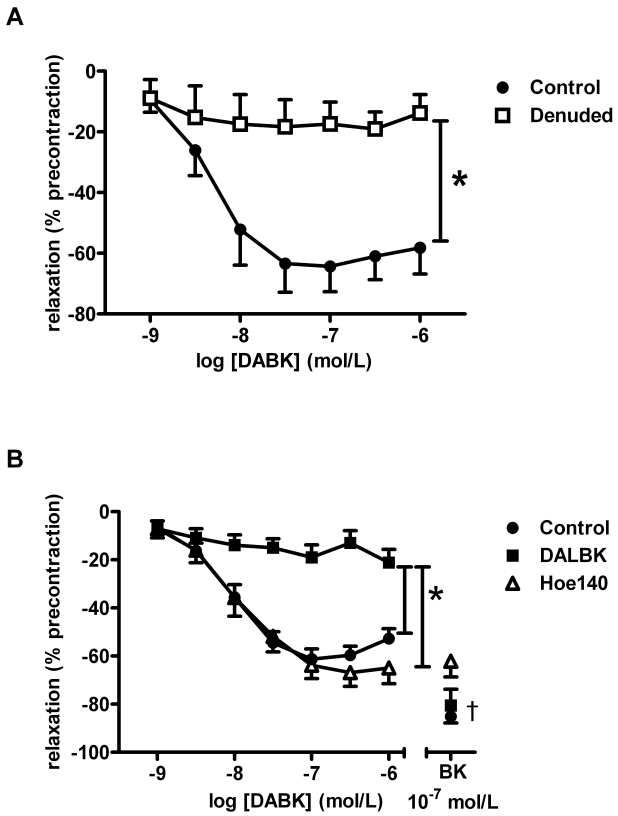

Figure 1. Effect of DABK and/or bradykinin in intact and endothelium-denuded HCMAs (panel A; n=6 each) and in intact arteries after pretreatment with either no antagonist (Control), the B1 receptor antagonist DALBK, or the B2 receptor antagonist Hoe140 (panel B; n=14-21). Data (mean±SEM) have been expressed as a percentage of the contraction induced by U46619. *p <0.05 (GLM-RM). † p < 0.05 BK 100 nmol/L vs E_{max} DABK in Control and DALBK (paired t-test).

Figure 2

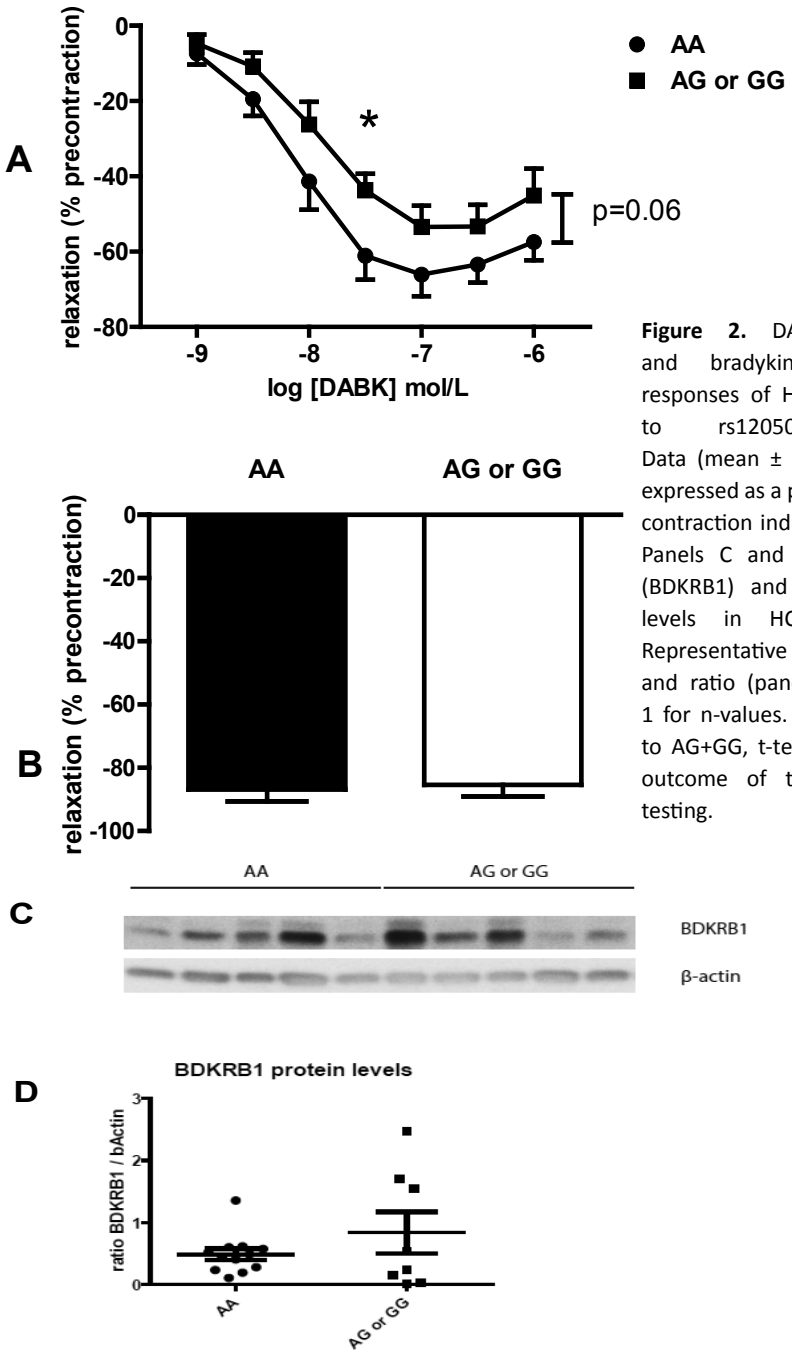

Figure 2. DABK (panel A) and bradykinin (panel B) responses of HCMAs according to rs12050217 genotype. Data (mean ± SEM) have been expressed as a percentage of the contraction induced by U46619. Panels C and D. B1 receptor (BDKRB1) and β-actin protein levels in HCMA segments. Representative blot (panel C) and ratio (panel D). See Table 1 for n-values. * $p<0.05$ AA vs. to AG+GG, t-test. P=0.06 is the outcome of two-way ANOVA testing.

Studies in human peripheral blood mononuclear cells

Both IL-1 β and DABK increased CXCL-5 gene expression in PBMNCs (P<0.05; Figure 3). Unexpectedly, the DABK-induced increase occurred exclusively in women. DALBK prevented this effect. Subdividing the women according to B1 receptor genotype (Table 2) revealed no difference (data not shown). The 17 β-estradiol plasma levels of these women correlated positively (P=0.02) with the effect of DABK on CXCL-5 expression in PBMNCs (Figure 4A) but showed no significant correlation with the CXCL-5 response to IL-1β (Figure 4B). 17 β-Estradiol did not affect CXCL-5 gene expression (data not shown, n=6).

Figure 3

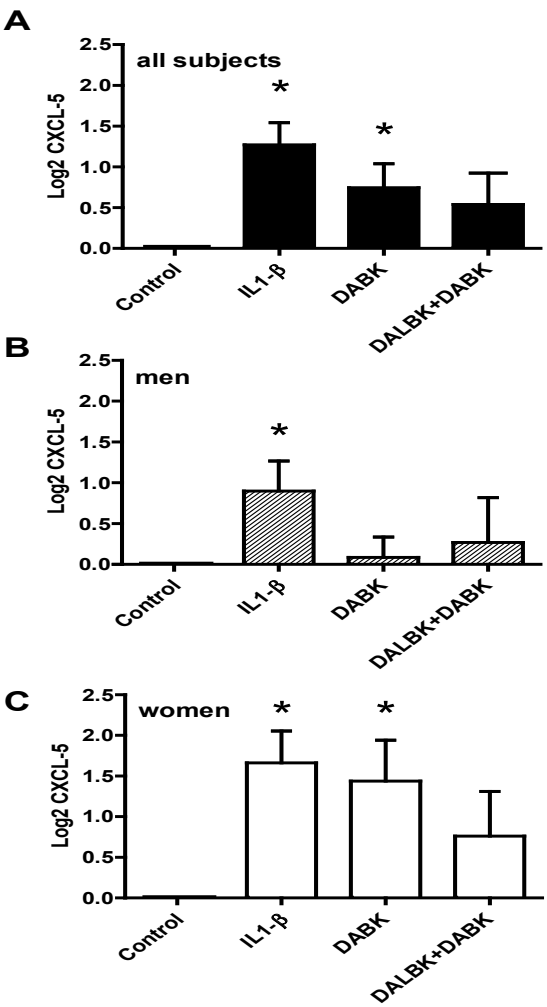

Figure3. CXCL-5 gene expression in human PBMNCs exposed to IL-1β or DABK with or without pretreatment with DALBK in all subjects (panel A), men (panel B) or women (panel C). Data are expressed as mean log2 fold changes ±SEM, * p<0.05 vs. control

Table 2. rs12050217 genotype of human PBMNCs

	AA	AG+GG
n	27	17
Age	34±2	32±2
Male/Female	13/14	9/8

Figure 4

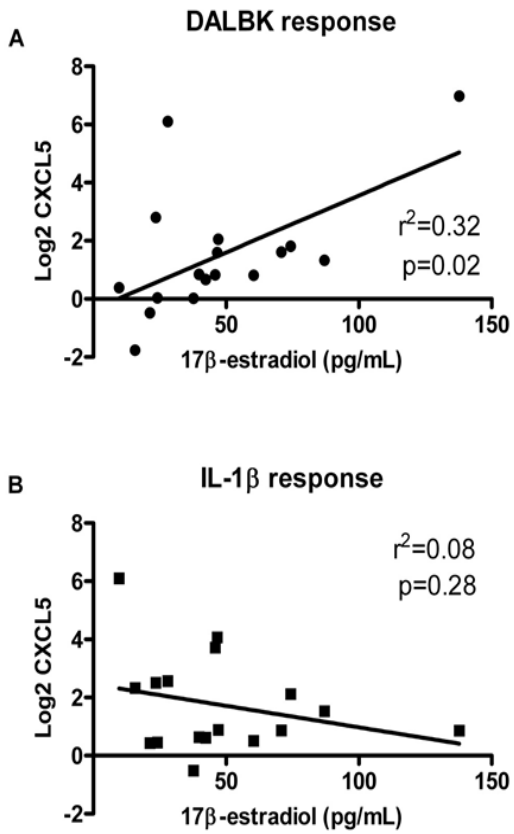

Figure4. CXCL-5 gene expression in human PBMNCs exposed to DABK (panel A) or IL-1 β (panel B) in relation to plasma 17β-estradiol levels of the female donors.

DISCUSSION

Based on the observation that differential treatment efficacy to perindopril of CAD patients is partly based on genetic determinants [1], we investigated the hypothesis that the A/G alleles of the rs12050217 SNP influence the endothelium-dependent vasodilation to bradykinin B1 receptor stimulation. To the best of our knowledge this is the first study describing B1 receptor-mediated vasodilation in HCMAs. Similar to B1 receptor-mediated vasodilation in large human coronary arteries, this effect was endothelium-dependent and did not involve B2 receptor activation [4]. B1 receptor desensitization started to occur at DABK levels >100 nmol/L (Figure 1), in agreement with previous studies [15]. The AA genotype of the rs12050217 SNP was associated with a larger relaxant effect in response to DABK than the AG and GG genotype, whereas no difference in DABK potency was observed. This is in agreement with the result of the PERGENE study that showed that the G allele was unfavourable as compared to the A allele with respect to the treatment effect of perindopril, since a decreased B1 receptor-mediated coronary vasodilation would attenuate the responsiveness to the (des-Arg9-)BK-augmenting effect of ACE inhibition. Importantly, the genotype distribution in the current study was comparable to that in the EUROPA trial [1]. Since the alleles from SNP rs12050217 are in linkage disequilibrium with those from rs870282 in intron 2 of the BDKRB1 gene, which acts as a secondary promoter region, we speculated that the differential response between the genotypes might be explained by a difference in BDKRB1 expression. However, we were unable to demonstrate such upregulation at the protein level in HCMA segments. Thus, alternatively, the enhanced response may be due to post-receptor mechanisms, e.g. alterations in the signaling pathway.

Apart from coronary artery vasodilation, inflammation plays a role in CAD. The B1 receptor has a reciprocal relationship with cytokines, being a modulator of their production as well as being regulated itself by cytokines, and both processes are NF-κB-dependent [7, 16-18]. We tested the hypothesis that the A/G alleles of rs12050217 would affect the pro-inflammatory response of PBMNC from healthy donors to B1 receptor stimulation. Although our data do not support this concept, unexpectedly, B1 receptor stimulation of PBMNCs with DABK increased the pro-inflammatory cytokine CXCL-5 exclusively in women. This was not due to the inability of male PBMNCs to display an inflammatory response, since their response to IL-1β was identical to that in women. The inflammatory response to DABK in women was unrelated to the B1 receptor A/G genotype, although it did relate to the plasma levels of 17β -estradiol.

Thus, instead of a genotype-related effect we encountered a marked gender-related difference in the DABK inflammatory response that was associated with the in-vivo 17β-estradiol levels. The role of estrogens in relation to gender differences in cardiovascular disease and side effects of steroid hormones used as anticonceptives has been a longstanding topic of research. In general, female sex steroids are considered to protect women from cardiovascular disease until they become post-menopausal – thereafter, the reverse may be true [19, 20]. Importantly, pre-menopausal protection may involve, at least

in part, anti-inflammatory effects of estrogens [20]. Yet, the effect of estrogens on cytokine production by leucocytes has been poorly investigated, let alone the modulatory effect of 17β-estradiol on monocyte responses to cytokines [20]. 17β-estradiol has both pro- and anti-inflammatory effects, depending on the organ that is investigated, the onset and duration of treatment with this steroid, age, the interaction with other (anti-)inflammatory substances, and, in the case of cell culture experiments, the cell type, the culture medium and the culturing conditions. When added to cultured monocytes the acute effect of 17β-estradiol is mostly anti-inflammatory, amongst others decreasing IgG-induced IL-1β production through CD16 downregulation [20]. However, pro-inflammatory 17β-estradiol effects in such cells have also been reported [20, 21], and the effects of 17β-estradiol on vascular and neuronal BK and DABK responses are similarly conflicting [22-26]. Our study in premenopausal women is in two ways different from previous reports because we studied (1) priming effects of 17β-estradiol in PBMNC, as 17β-estradiol is largely withdrawn when transferring the cells from blood to bio-assay conditions (and in fact had no effects of its own when added directly to PBMNCs), and (2) the modulation of cytokine responses instead of direct effect on cytokine release. Since no association of the plasma 17β-estradiol levels with the inflammatory response to IL-1β was found, our results imply a modulatory role of 17β-estradiol in pro-inflammatory B1 receptor signalling. This role may range from affecting B1 receptor expression, B1 receptor signalling pathways, or the interaction of B1 receptors with other receptors. For instance, given the well-known effects of glucocorticoids on B1 receptor signalling [27-29], the observation that estrogen regulates both glucocorticoid receptor expression [30] and glucocorticoid release in vivo [31] might underlie our findings.

Estrogens have been identified as important regulators of the RAS, causing downregulation of renin and ACE activity and a decrease of AT1 receptors in favour of AT2 receptor signalling [32-35]. More related to our findings, the effect of gender in therapeutic efficacy of ACE inhibitor has not been investigated extensively in human settings, women being mostly underrepresented in clinical studies. The limited data that are available from meta-analyses do not confirm the results from animal models in which estrogens favour ACE inhibitor effects [33, 36]. Nevertheless, in spontaneously hypertensive rats, 17β-estradiol was found to improve the hypotensive effect of ACE inhibition, and this was partly related to bradykinin B2 receptor activity [37]. Studies with respect to the involvement of bradykinin B1 receptor signalling in gender or estrogen-induced changes in ACE inhibition efficacy are to our knowledge non-existent.

In summary, our present study was prompted by our previous finding in the EUROPA study population that the rs12050217 SNP determines the treatment benefit of the ACE inhibitor perindopril. Our results show that the G allele attenuates the B1 receptor-mediated coronary vasodilator response, which might play a role in the reduced treatment benefit of perindopril. The proinflammatory response of PBMNC to B1 receptor stimulation, measured as increased CXCL-5, was not influenced by genotype. However, this response was only observed in PBMNC of female donors, and associated with plasma 17β-estradiol levels. The role of female sex hormones in bradykinin responses and ACE inhibition in relation to inflammation is a relevant topic with implications for cardiovascular disease.

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Chapter 4

The effect of genomic instability on responsiveness to angiotensin II type 1 receptor stimulation and blockade

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Manuscript in preparation

ABSTRACT

Background
Ageing is the dominant risk factor for cardiovascular disease. Recently, we have shown that genomic instability, a causative mechanism of ageing in general, induced by functional mutation of the ERCC1 DNA repair protein in mice (Ercc1$^{d/-}$ mice) causes accelerated age-dependent vascular dysfunction and hypertension [1]. The renin–angiotensin system (RAS) has been implicated in vascular ageing and hypertension, in which an excessive stimulation of angiotensin II type 1 receptors (AT1R) plays a central role by both causing detrimental vascular remodeling as well as genomic instability due to DNA damage. We here test the hypothesis that genomic instability can cause the enhanced responsiveness to angiotensin II, and that this plays a role in the development of vascular dysfunction. To this end angiotensin II and vasodilator responses were tested in iliac arteries of untreated and losartan-treated Ercc1$^{d/-}$ mice.

Methods and Results
Male and female Ercc1$^{d/-}$ and wild type (WT) mice from 5 week of age were divided into two groups per strain, one group for a 7-week treatment with the AT1R antagonist losartan and one untreated group. Blood pressure (BP) was measured in a subgroup of conscious mice by tail-cuff technique. Systolic blood pressure tended to be increased in Ercc1$^{d/-}$ mice vs. WT, and was normalized by losartan. Angiotensin II responses of iliac arteries were increased in Ercc1$^{d/-}$ vs. WT, and this was also normalized by losartan. The AT2R antagonist PD123319 only increased Ang II responses in WT mice. The guanylyl cyclase inhibitor ODQ did not significantly increase Ang II responses. Ercc1$^{d/-}$ displayed lower acetylcholine and sodium nitroprusside responses than WT, and this was not improved by losartan treatment.

Conclusions
The results show that genomic instability increases AT1R-mediated vasoconstriction, but that this does not contribute to the development of vasodilator dysfunction. A loss of counterregulatory AT2R function contributes to the enhanced AT1R response in Ercc1$^{d/-}$ mice. The increased angiotensin II activity is largely independent of nitric oxide–cGMP signaling.

INTRODUCTION

Ageing is a major risk factor for cardiovascular diseases (CVDs). It prolongs the exposure to risk factors for CVDs, such as hyperglycemia, dyslipidemia, and smoking, but on the other hand, ageing also acts independently from these risk factors [2]. Comprehension, prevention, and treatment of vascular ageing are of utmost importance when engaging in this challenge because of the causal relationship between vascular ageing and CVD [3, 4]. Organismal ageing is a process that can arise from adaptive mechanisms that are switched on in the organism with the purpose of protecting itself against genomic instability, one of the putative main causes of the ageing process [5, 6]. How this process is involved in vascular ageing is now beginning to be explored [7]. Ageing is associated with accumulation of DNA damage in the body. Among others this has been observed in arterial plaques [6, 8]. DNA damage in vascular cells can lead to cellular ageing, which is accompanied by a pathological phenotype of the affected cells that refers to age-related vascular pathology [9].

Mutations in proteins that safeguard genomic integrity lead to an increased susceptibility to DNA damage and can cause premature ageing, or progeria. Progeroid syndromes arising from such mutations can be observed in the clinic, as well as in mouse models, and can be accompanied by features of accelerated cardiovascular ageing. Examples are Hutchinson-Gilford progeria syndrome and Atypical Werner Syndrome, and several mouse models with DNA repair defects [7, 10-12]. In a recent study we found that Ercc1$^{d/-}$ mice, a strain which develops a progeroid phenotype as a consequence of a functional mutation in the excision repair cross-complementing 1 (ERCC1) endonuclease (which is involved in nucleotide excision repair, crosslink repair and non-homologues end-joining), display accelerated age-dependent vasodilator dysfunction, increased vascular stiffness, increased blood pressure, and vascular cell senescence [1, 13]. It is therefore believed that genomic instability, a causative hallmark of ageing, might also be pivotal in age-related cardiovascular disease [7].

It is not known how genomic instability is translated into the typical changes of the circulatory system that one can observe during ageing. We here propose that one of the possibilities is an increased activation of the renin-angiotensin system (RAS). Angiotensin II (Ang II), a primary effector peptide of the RAS, plays a role in several age-related cardiovascular diseases, and has been implicated in vascular cell senescence and organismal longevity. Ang II binds to the G protein-coupled Ang II type 1 receptor (AT1R), thereby promoting the pathogenesis of several human diseases, including hypertension, congestive heart failure, coronary artery disease, and diabetic nephropathy. This involves its vasoconstrictive effects and effects on sodium and water transport in the kidneys and its contribution to cellular growth, migration and senescence of cardiovascular cells, fibrosis and inflammation [14-17]. Signaling via an alternative receptor, the Ang II type 2 receptor (AT2R), is believed to be protective against detrimental AT1R signaling [18]. The vasoconstrictive responsiveness to Ang II is enhanced in aged individuals, and vascular angiotensin-converting enzyme expression is increased, which should lead to increased local Ang II production [16]. Moreover, Ang II causes cellular senescence in cultured endothelial cells (EC) and vascular smooth muscle cells (VSMC), which is associated with DNA damage in the latter [16, 19, 20]. Conversely, a chronic decrease of AT1R signaling by treatment with antagonists or AT1R gene knockout

decreases cardiac markers of genomic damage in diabetic mice and increases longevity [21, 22]. It is therefore reasonable to propose that Ang II is involved in vascular ageing. The damage in DNA or other macromolecules caused by reactive oxygen species (ROS), which are increased by AT1R, would be a main causative mechanism [16, 19]. Moreover, ROS might also be causal in the decrease of nitric oxide synthase (NOS) activity in the aorta and NO production that takes place with ageing, and that is believed to be pivotal in the pathogenesis of age-related disease [23]. Indeed, chronic long-term administration of Ang II inhibitors maintains endothelial NOS activity in old animals and simultaneously reduces ROS [24]. Thus, the potent ability of Ang II to activate NADPH oxidase in the cardiovascular system, thus augmenting ROS production, likely is a major contributor to the pathogenesis of vascular and also other age-related diseases [25-28].

There are, however, several important questions remaining. Firstly, it is unknown what causes the ageing-related increased RAS activation. Secondly, there is no *in vivo* evidence that under physiological conditions Ang II contributes to genomic instability-related vascular ageing. Thirdly, the believe that ROS are involved in the ageing process is controversial since there is growing evidence that there is no relationship between activity of anti-oxidant pathways, ROS production and longevity [29]. In addition, we only observed a modest contribution of ROS in the endothelial dysfunction in animals with accelerated vascular ageing due to genomic instability [1]. Fourthly, until now wildtype (WT) rodent models do not unequivocally mimic the increased response to Ang II observed in humans, which prompts the question if human vascular ageing is well-represented by WT rodent models and whether mouse models for genomic instability might perhaps be more completely representing human ageing, as is also the case for other features of ageing [30-32].

In the present study we explored if genomic instability leads to increased AT1R responsiveness in the vascular wall, and if AT1R blockade with losartan would prevent the accelerated development of endothelial dysfunction in Ercc1$^{d/-}$ mice. Furthermore, we have explored AT2R function, which has been described as being influenced by ageing as well [33].

METHODS

Animals

Male and female Ercc1$^{d/-}$ (n=28) and wild type mice (n=17) were bred at the Erasmus MC animal facility. From 5 weeks of age, Ercc1$^{d/-}$ and WT mice were divided into two groups per strain, which were either treated with losartan (100 mg/kg/day) in drinking water, or drinking water only. Thus, the following groups were created: Ercc1$^{d/-}$ losartan (n=15); Ercc1$^{d/-}$ water (n=13); WT losartan (n=7); WT water (n=10). Ang II responses in rodents are highly dependent on normal development and ageing [32]. To study the progressive changes in Ang II responses, a small 18-week old group without losartan treatment was included as well (n=3). The animals were housed in individually ventilated cages with access to normal chow and water *ad libitum.* All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Assessment of blood pressure in vivo

At the age of 11 weeks blood pressure was measured in a subgroup of conscious Ercc1$^{d/-}$ mice and WT littermates using the tail cuff technique. The animals were first trained for 4 consecutive days to acclimatize to measurement conditions. On the subsequent 5th day representative blood pressures were measured, which are presented in the Results section. Thereafter, the animals were sacrificed and vascular tissue was taken out to measure vasomotor function in organ bath set-ups.

Mulvany myography

Tissue harvesting and preparation
Thoracic aorta and iliac arteries were collected from mice within 5 minutes after sacrifice by asphyxiation, and stored overnight in cold, oxygenated (5% CO_2; 95% O_2) Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,$CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3 in distilled water; pH7.4). The following day, vessel segments were mounted in 6-mL small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer at 37℃ and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized by adjusting the transversal length of the suspended vessel segments to 90% of the estimated length at which the tension is equivalent to an effective transmural pressure of 100 mmHg. Viability of the tissue was tested though induction of contractions by exposure to respectively 30, 60, and 100 mmol/L KCl.

Testing of vasodilator function
After washout of KCl, pre-constriction was elicited with 30 nmol/L U46619, a thromboxane mimetic, resulting in 50-100% of the previously obtained contraction to 100 mmol/L KCl. Following preconstriction, relaxation concentration-response curves (CRCs) for the endothelium-dependent vasodilator acetylcholine (ACh) were constructed by giving cumulative doses (10^{-9}-10^{-5} mol/L), followed by exposure to the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). To further explore the endothelium-independent dilator we cumulatively added SNP (10^{-9}-10^{-5} mol/L) in parallel rings preconstricted with 30 nmol/L U46619. In the case that sufficient aortic tissue was available, the involvement of NO and cGMP in ACh responses was investigated by performing the experiments in the presence of endothelial nitric oxide synthase inhibitor N^G-Methyl-L-Arginine acetate salt (L-NMMA, 10^{-4} mol/L). Inhibitors were added to the organ bath 10 minutes prior to U46619.

Testing of Ang II vasoconstrictor function
To detect the vasoconstrictor function in iliac arteries, the segments were exposed to increasing concentrations of KCl (30, 60, 100 mmol/L) for viability testing. Subsequently, after three times washout of KCl, Ang II (10^{-10}-10^{-6} mol/L) CRCs were constructed. To investigate the involvement of cGMP signaling, a subgroup of segments was pre-incubated with the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4] Oxadiazolo [4,3]-a quinoxalin-1-one (ODQ, 10^{-5} mol/L). AT2R effects were measured by pre-incubation of iliac artery segments with PD123319 (10^{-6} mol/L), an AT2R antagonist. Inhibitors were added to the

organ bath at least 20 minutes prior to Ang II.

Table 1. Numbers of 12 week-old animals included in the diverse evaluations**.**

Parameter	WT water (n=10)	Ercc1$^{d/-}$ water (n=13)	WT losartan (n=7)	Ercc1$^{d/-}$ losartan (n=15)
Vasodilator function	6	10	4	7
Ang II constrictions	9	13	5	11
BLP	9	9	7	15

Statistical methods

Data are presented as mean ± SEM. SNP-corrected ACh responses were calculated as follows: (response to ACh as % of U46619 preconstriction / response to 10^{-4} mol/L SNP as % of U46619) x 100 (to convert into percentage) x -1 (to indicate that it is a relaxation). Statistical testing for differences between single values expressed in bar graphs was performed by t-test or 1-way ANOVA followed by indicated post-hoc tests, where appropriate ($^{\dagger}p<0.05$ with respect to WT water; $^{\#}p<0.05$ with respect to Ercc1$^{d/-}$ water). To test the hypothesis that blood pressure would be increased in Ercc1$^{d/-}$ mice and attenuated by losartan we employed a one-sided t-test. Differences in dose-response curves were tested by general linear model for repeated measures (GLM-RM, sphericity assumed). Differences were considered significant at $p<0.05$.

RESULTS

Blood pressure measurements

Blood pressure was measured in 11 week-old Ercc1$^{d/-}$ and WT mice. In agreement with our previous study [1] blood pressure tended to be slightly higher in Ercc1$^{d/-}$ mice, mostly reflected by systolic blood pressure (SBP), and to a lesser extent by diastolic and mean arterial blood pressure (DBP, MAP) (Figure 1). The difference reached borderline significance for SBP ($p = 0.08$, 1-tailed t-test). Losartan significantly lowered SBP, DBP and MAP in both mouse strains.

Vasomotor responses to Ang II

In iliac arteries of 12 week-old Ercc1$^{d/-}$ mice vasoconstriction to Ang II was elevated as compared to WT mice (Figure 2A), although borderline significance was reached over the whole curve (GLM-RM $p = 0.069$), and significant increases were only reached for the last 4 concentrations when tested individually. At the age of 18 weeks, however, the difference between Ercc1$^{d/-}$ and WT became significant over the entire curve (Figure 2A). Strikingly, Ang II responses in WT animals appeared to become somewhat lower, whereas in Ercc1$^{d/-}$ the responses appeared to increase when passing from 12 to 18 weeks of age.

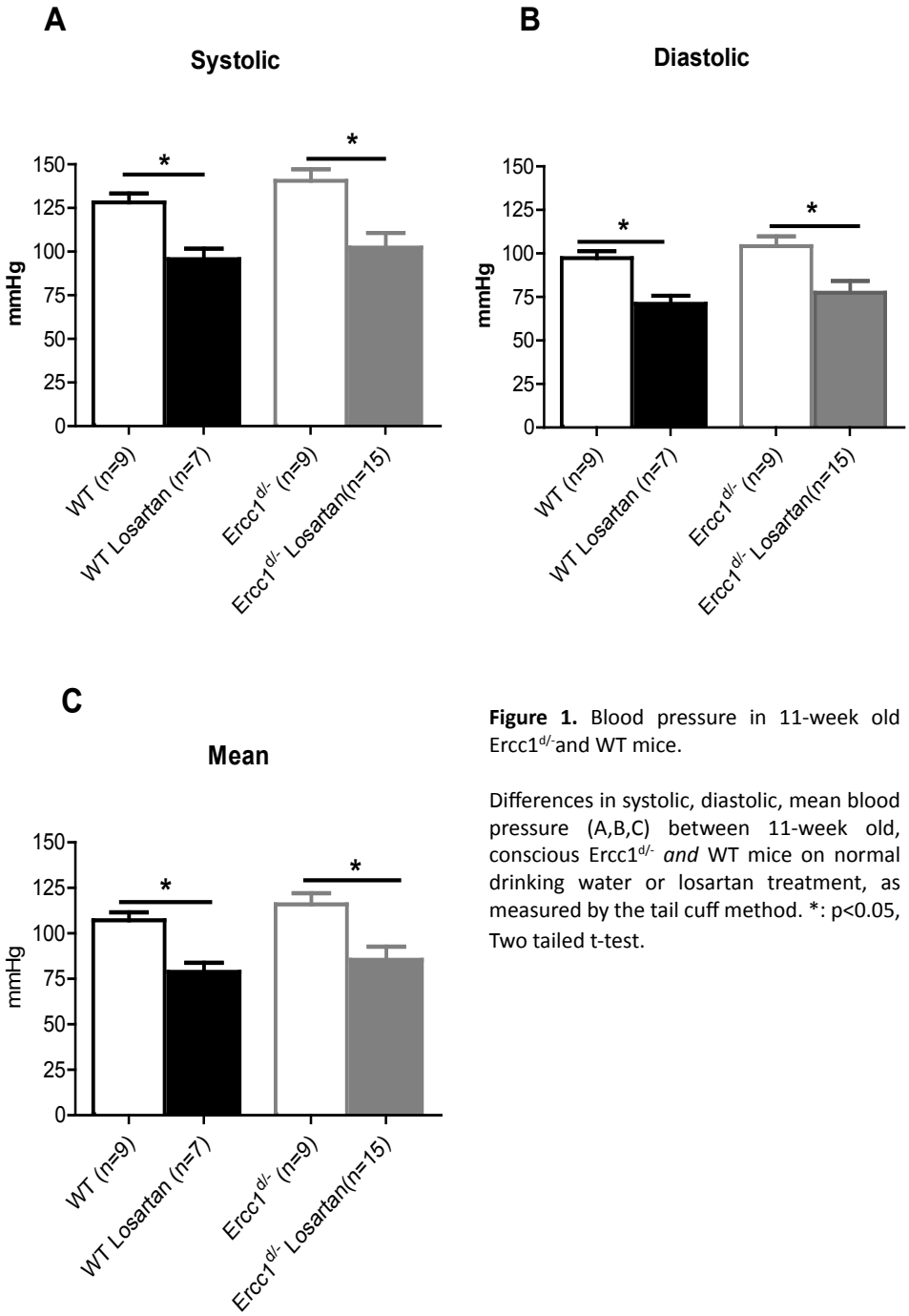

Figure 1. Blood pressure in 11-week old Ercc1$^{d/-}$and WT mice.

Differences in systolic, diastolic, mean blood pressure (A,B,C) between 11-week old, conscious Ercc1$^{d/-}$ *and* WT mice on normal drinking water or losartan treatment, as measured by the tail cuff method. *: $p<0.05$, Two tailed t-test.

Chronic losartan treatment in the 12 week group led to decreased Ang II responses in Ercc1$^{d/-}$ animals, but not in WT animals (Figure 2B and C).

To explore the possible involvement of counterregulation of Ang II-induced constriction, or absence thereof, by NO-cGMP signalling, Ang II responses were studied in the presence of the guanylyl cyclase inhibitor ODQ. This approach, rather than adding an eNOS inhibitor, was chosen because Ercc1$^{d/-}$ mice show both changes in endothelial NO production as well as in cGMP responses of VSMC [1]. Although the presence of ODQ tended to increase Ang II responses, the increase was very modest and did not reach significance in either WT or Ercc1$^{d/-}$ animals (Figure 2B and 2C).

AT2R has been reported to inhibit AT1R-mediated vasoconstriction [34, 35]. To explore the effect of genomic instability on AT2R activity, Ang II responses in Ercc1$^{d/-}$ and WT in the presence of AT2R antagonist PD123319 were compared to those in the absence of this antagonist (Figure 2D). In 12 weeks-old mice, PD123319 increased the Ang II response in WT but not in Ercc1$^{d/-}$. In 18 weeks-old animal PD123319 decreased Ang II responses in both mouse strains (Figure 2E).

Effect of chronic losartan treatment on accelerated age-related vasodilator dysfunction

In 12 weeks-old Ercc1$^{d/-}$ vasodilator responses to ACh were decreased as compared to WT animals (Figure 3A). The response to a single dose of SNP 10^{-4} mol/L, when applied just after completion of the ACh CRC, was also decreased in Ercc1$^{d/-}$ (Figure 3C), and so were responses to SNP (full CRC) without the prior application of ACh (Figure 3D). Even when correcting for the difference in endothelium-independent NO/cGMP-mediated response, the ACh responses were still decreased in Ercc1$^{d/-}$ mice (Figure 3B). Similar to 12 weeks-old animals, 18 weeks-old Ercc1$^{d/-}$ mice showed significantly decreased responses to ACh, SNP 10^{-4} mol/L single dose, SNP CRCs and SNP-corrected ACh responses ($p<0.05$, n=3 observations per group, data not shown).

Chronic AT1R blockade with losartan in vivo did not significantly change any of the responses except for responses to 10^{-4} mol/L SNP after ACh in Ercc1$^{d/-}$. Compared to Ercc1$^{d/-}$ treated with losartan and untreated WT, the response to this SNP concentration in Ercc1$^{d/-}$ mice fed with normal drinking water was smaller (Figure 3C, $p<0.05$, 1-way ANOVA Dunnett's post-hoc test for multiple comparisons). This beneficial effect could, however, not be seen when studying full SNP CRCs in the absence of ACh (Figure 3D).

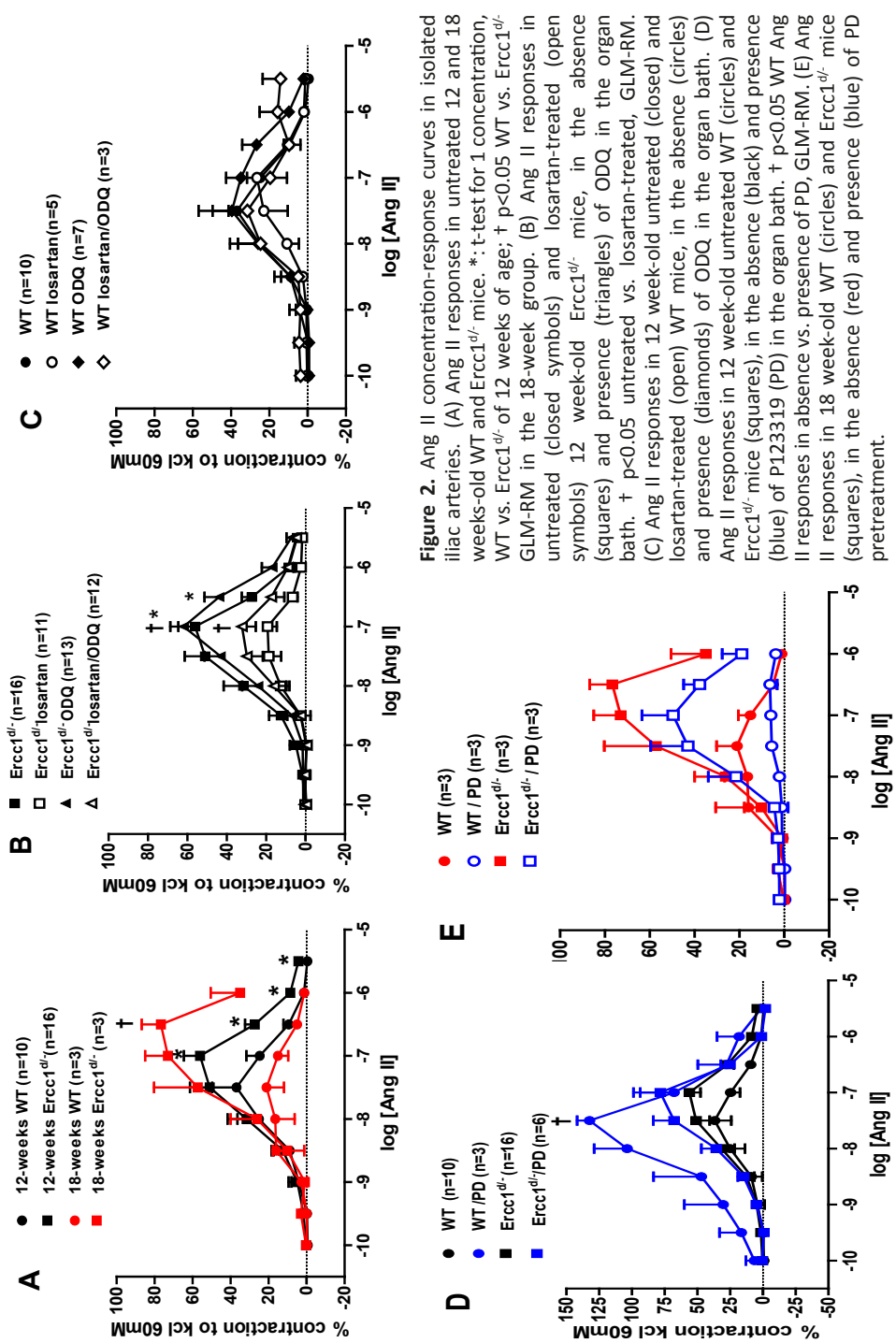

Figure 2. Ang II concentration-response curves in isolated iliac arteries. (A) Ang II responses in untreated 12 and 18 weeks-old WT and Ercc1$^{d/-}$ mice. *: t-test for 1 concentration, WT vs. Ercc1$^{d/-}$ of 12 weeks of age; † p<0.05 WT vs. Ercc1$^{d/-}$ GLM-RM in the 18-week group. (B) Ang II responses in untreated (closed symbols) and losartan-treated (open symbols) 12 week-old Ercc1$^{d/-}$ mice, in the absence (squares) and presence (triangles) of ODQ in the organ bath. † p<0.05 untreated vs. losartan-treated, GLM-RM. (C) Ang II responses in 12 week-old untreated (closed) and losartan-treated (open) WT mice, in the absence (circles) and presence (diamonds) of ODQ in the organ bath. (D) Ang II responses in 12 week-old untreated WT (circles) and Ercc1$^{d/-}$ mice (squares), in the absence (black) and presence (blue) of P123319 (PD) in the organ bath. † p<0.05 WT Ang II responses in absence vs. presence of PD, GLM-RM. (E) Ang II responses in 18 week-old WT (circles) and Ercc1$^{d/-}$ mice (squares), in the absence (red) and presence (blue) of PD pretreatment.

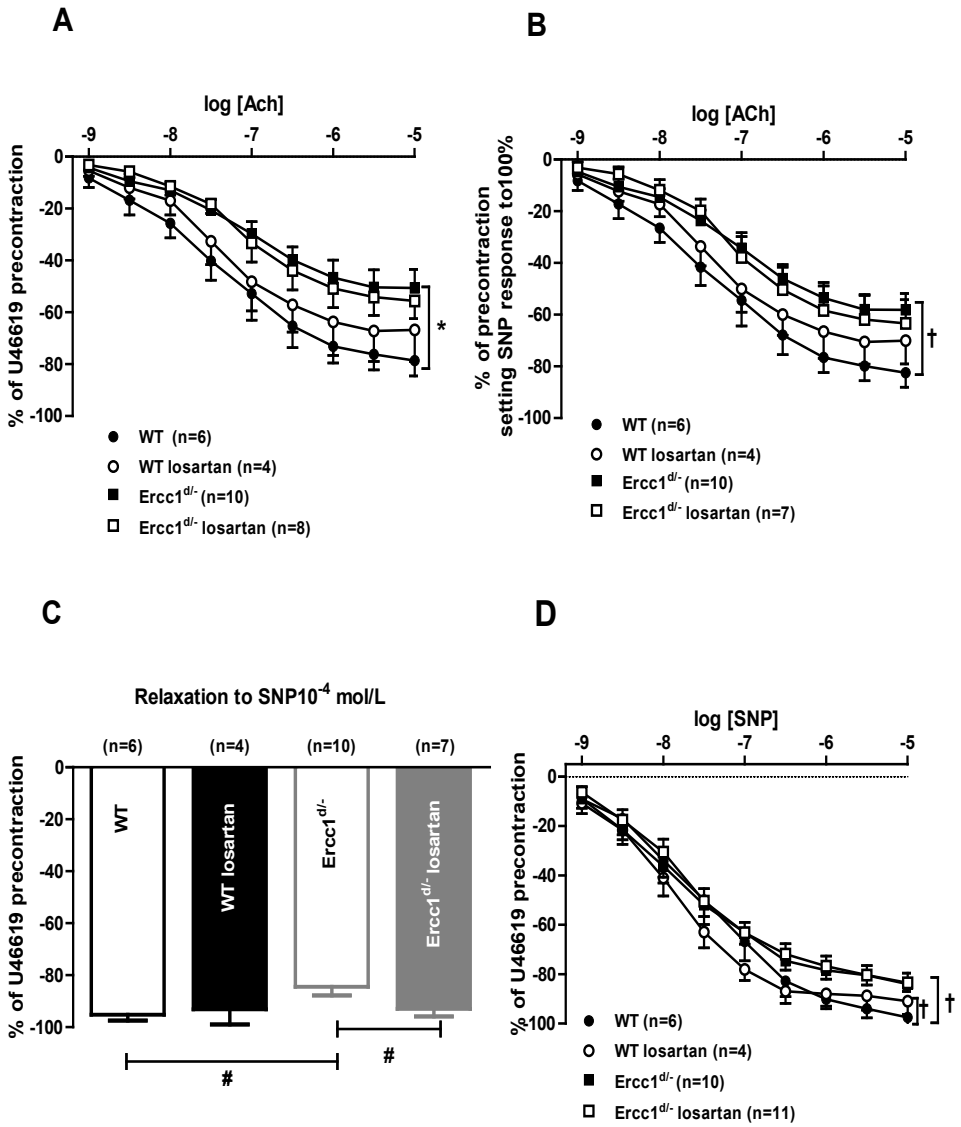

Figure 3. Ex-vivo vascular function in 12-week old Ercc1$^{d/-}$ and WT Mice.
Vasodilation to (A) acetylcholine and (B) sodium nitroprusside (SNP) in thoracic aorta of 12-week old Ercc1$^{d/-}$and WT mice. Acetylcholine responses expressed as % of the sodium nitrioprusside response (C). Relaxation of thoracic aortas to endothelium-independent vasodilator sodium nitroprusside (D), both genotype mice were under water and losartan treatments respectively. † p<0.05 : GLM-RM; # p<0.05: 1-way ANOVA, Dunnett's post-hoc test.

DISCUSSION

In this study we explored the role of genomic instability in effects of AT1R activation and blockade in the vasculature. The results show that genomic instability in mice causes an increased arterial vasoconstrictor responsiveness to Ang II, which increases with age. Since the present cohort of Ercc1$^{d/-}$ mice showed at most a modest increase in blood pressure, reaching borderline significance for SBP only, it is unlikely that blood pressure is an important factor in determining the increased AT1R sensitivity. Chronic losartan treatment lowered blood pressure, but this did not improve the vasodilator dysfunction in Ercc1$^{d/-}$ mice. This leads to the conclusion that Ang II signaling, although increased in Ercc1$^{d/-}$, does not appear to play an important role in the accelerated development of age-related vascular vasodilator dysfunction due to genomic instability observed in this mouse model.

Looking further into the mechanisms that might explain the increased response to Ang II, a possible pathway is the loss of physiological antagonism through NO-cGMP signaling. Indeed, Ercc1$^{d/-}$ mice showed a decreased NO-cGMP-dependent vasorelaxation in the present study, like in our previous study [1]. However, since ODQ only marginally, and not significantly, increased Ang II responses and only partially eliminated the difference in Ang II response between Ercc1$^{d/-}$ and WT mice, the decreased NO-cGMP signaling cannot fully explain the Ang II hyper-responsiveness in Ercc1$^{d/-}$. Instead, AT2R antagonist PD123319 dramatically increased Ang II contractions in 12 weeks-old WT but not in Ercc1$^{d/-}$, and therefore loss of counterregulatory AT2R function might explain the increased Ang II responses. For Ercc1$^{d/-}$ mice the results in 18 weeks-old animals further support this involvement of AT2R, as the PD123319 data now even support a constrictor role of AT2R at this age. Such a switch in AT2R function is in full agreement with observations in old rats as well as in hypertensive rats [36]. However, since blood pressure was only marginally increased in 12-week Ercc1$^{d/-}$ mice it is likely that it is predominantly the accelerated ageing process that is causative for the AT2R function switch.

It should be noted, however, that in WT animals at the age of 18 weeks, Ang II responses appeared to be reduced, and that also in these animals there appeared to be a switch in AT2R function. Such a change of Ang II responsiveness is a puzzling phenomenon that has been regularly encountered [32]. Partly this might relate to pathophysiological conditions, but otherwise Ang II responses are under the control of an intricate interplay of diverse signaling pathways, including production of ROS, NO-cGMP, and eicosanoids, and direct receptor interactions arising from receptor heterodimerization [36]. As a further complication, the variation in AT1R and AT2R signaling during life can also be vessel type- and species-dependent. As a consequence, the full characterization and understanding of age-related changes in Ang II signaling will require many years of study in different animal models and vessel types. In general, however, it seems plausible that due to the switch of AT2R from an antagonistic to an enhancing role towards AT1R, the possibilities to counterregulate AT1R are partly lost and genomic instability thus leads to a progressive vasoconstrictive response to Ang II. This situation thus reflects pathological conditions, and these conditions might contribute to the increased blood pressure in Ercc1$^{d/-}$, as supported by the observation that chronic losartan treatment lowers blood pressure to the level in

WT mice. Diminished NO-cGMP signaling per se does not appear to underlie the increased Ang II responsiveness, although such signaling did decrease in the ageing animals. In the 18 week-old WT littermates, it is expected that the full abilities to adapt Ang II responsiveness are entirely intact, and the switch of AT2R function in these animals is therefore likely to represent a physiological condition rather than a pathological one.

The role of AT2R in the ageing cardiovascular patient has been studied too poorly to find support for the hypothesis presented in the previous paragraph. In one study it has been shown that counterregulation of AT1R by AT2R is lost in ageing WT rats [33]. The question is, however, if aged WT rodents are a genuine model for human ageing since they do not have many features in common. Better representatives, or at least more complete ones, seem to be models in which important mechanisms for biological ageing have been modified. This can be models in which genetic manipulation has augmented characteristics of human-like ageing by modulation of genes that increases the primary causative ageing hallmark, genomic instability, or adaptive or integrative ageing hallmarks [5, 12]. Interestingly, growth hormone knockout mice, which have a prolonged life span due to suppression of the somatotropic axis (a recognized adaptive pathway to protect against biological ageing), display decreased AT1R expression and increased AT2R expression in the heart [37]. Combined with our results it seems therefore worthwhile to apply models of accelerated and decelerated ageing when trying to study the contribution of ageing to AT2R function. Combining this with WT or non-aged cardiovascular disease animal models, ageing-induced changes can be discriminated from developmental or disease-related AT2R changes.

An important observation is that chronic AT1R blockade did not prevent the accelerated development of vasodilator dysfunction that we have now repeatedly demonstrated in Ercc1$^{d/-}$ mice [1]. This is in contrast with the observation that Ang II can induce DNA damage and senescence in cultured EC and VSMC [19, 20, 38, 39]. Interestingly, genetic knockout of AT2R further promotes Ang II-induced senescence in cultured VSMC, implying that the observed loss of beneficial AT2R should facilitate the vascular ageing process [40]. A possible explanation for the lack of effect of losartan might be that vascular dysfunction in Ercc1$^{d/-}$ mice is largely ROS-independent, while normally the detrimental effects of Ang II involve ROS formation [1]. This not only supports the hypothesis that we need to explore the involvement of other sources of DNA damaging agents than ROS in vascular ageing [41], but it is also an invitation to the performance of studies that are comparable to the present study in models of ROS-dependent genomic instability. Although such models do not show decreased longevity, it has been shown that genetic knockout of anti-oxidant enzymes increases oxidative DNA damage in various organs [29, 42]. Moreover, genetic knockout of manganese superoxide dismutase also enhances the endothelial dysfunction induced by a non-pressor dose of Ang II [43, 44]. The relationship with DNA damage, however, has not been studied in the latter study. Taking all the arguments in consideration, it seems that although ROS is not a causative factor in organismal ageing, we cannot exclude that DNA (or macromolecular) damage by ROS contributes to Ang II-induced changes during vascular ageing.

In summary, our present study shows that genomic instability increases AT1R vasoconstrictor activity, and that this might be explained by decreased counterregulation by AT2R independent from NO-cGMP signaling. Since this enhancement could be blocked by chronic losartan treatment, the RAS itself is involved in its progression. The increased AT1R activity did not contribute to the accelerated development of vasodilator dysfunction in Ercc1$^{d/-}$ mice, because losartan treatment was ineffective at this point. Future studies should address the questions which sources of DNA damage cause an increased RAS activity and how this takes place. That in such studies an emphasis should be put on oxidative damage follows from the fact that it might be both cause as well as consequence of increased Ang II / AT1R signaling.

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Chapter 5

Dietary restriction improves vasomotor changes caused by accelerated vascular aging due to genomic instability

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Manuscript in preparation

ABSTRACT

Background:
Recently it was found that genomic instability, a causative hallmark of aging, contributes to vascular aging. This was amongst others demonstrated by the observation that mice with a dysfunction of the endonuclease Ercc1 (so called Ercc1$^{d/-}$ mice), which display a progeroid phenotype, showed accelerated development of age-related vascular problems and elevated blood pressure. In this study we explored if diet restriction (DR), a well-known anti-aging intervention, could prevent genomic instability-related vascular changes.

Methods and Results
Ercc1$^{d/-}$ and wildtype littermate mice were fed with DR (increasing nutrient restriction from 10 to 30%) or ad libitum (AL) diets for 4 to 9 weeks, whereafter thoracic aortas were isolated and used for ex vivo organ bath experiments. To investigate endothelial and vascular smooth muscle dilator function, aorta preconstricted with U44619 (thromboxane analogue) was exposed to acetylcholine (ACh: 10^{-9}-10^{-5}M) and sodium nitroprusside (SNP: 10^{-9}-10^{-4}M), respectively. Contribution of nitric oxide and prostaglandins was assessed by performing the experiments in the presence and absence of eNOS inhibitor L-NMMA and indomethacine, respectively. In addition, concentration-related vasoconstrictions to Ang II were determined in iliac arteries.

Ercc1$^{d/-}$ mice of 16 weeks of age, but not Ercc1$^{d/-}$ of 11 weeks of age nor wildtype mice until the age of 20 weeks, showed a reduction of ACh and SNP-mediated responses. DR partly prevented the loss of ACh responsiveness, an effect that lasted for at least 30 weeks, an age at which all AL-fed Ercc1$^{d/-}$ have already died. SNP responses were entirely preserved by DR. ACh responses that were corrected for SNP effects were only slightly and not significantly better after DR. L-NMMA considerably and significantly reduced ACh response in all mice (between 40 to 100% reduction), but indomethacine only had a significant effect in aorta of DR-treated in the Ercc1$^{d/-}$ mice. This was accompanied by reduced PDE4B and increased COX-1 mRNA levels in lung of Ercc1$^{d/-}$, which was strongest after DR. Ang II vasoconstrictions were increased in Ercc1$^{d/-}$ as compared with wildtype mice in AL fed animals. DR reduced Ang II constriction in Ercc1$^{d/-}$ to the level of wildtype mice and was without effect in wildtype mice.

Conclusions: DR decreases the accelerated vasodilator dysfunction caused by genomic instability most probably due to an enhanced prostaglandin/cAMP signaling cascade. Enhancement of Ang II vasoconstriction is also prevented by DR. DR is therefore an effective and versatile vascular protective intervention that can protect against genomic instability-related vascular aging.

INTRODUCTION

Ageing is the major risk factor for the development of cardiovascular diseases (CVD). It is becoming evident that ageing renders the cardiovascular system prone to disease even in the absence of traditional risk factors (e.g. hypertension, diabetes, and smoking) [1, 2]. One of the many factors that contribute to organismal ageing is genomic instability. We recently found that genomic instability also induces vascular ageing, and proposed that, in humans, this might act independently of classical cardiovascular risk factors [3, 4]. A better understanding of how genomic instability affects vascular ageing might lead to new treatment targets for cardiovascular diseases.

Dysfunction of genes needed for genomic maintenance results in accelerated ageing (progeria) [5], suggesting that the molecular basis of ageing involves genomic instability. In general, DNA damage detection and repair enzymes of 7 different repair systems are recruited to safeguard genomic integrity by repairing specific lesions types, helped by nuclear envelope proteins called lamins. The repair pathways are: 1) the direct reversal pathway, which allows the direct reversal of chemical modifications of nucleic acids, (2) mismatch repair (MMR), repairing base pair mismatches (3) nucleotide excision repair (NER, Figure 1), which repairs transcription-disturbing bulky adducts, (4) base excision repair (BER), which repairs mainly oxidized and alkylation lesions in the nucleus and mitochondria, and single strand breaks (5) homologous recombination (HR), and (6) non-homologous end joining (NHEJ), which repair single and double strand breaks [5, 6]. DNA repair systems show functional overlap and some individual repair factors are involved in multiple repair pathways. For example, ERCC1 is implicated as ERCC1/XPF dimeric endonuclease in NER, cross-link repair and some subpathways of homologous recombinant repair [5, 7]. Similar progeroid features have been observed in mice with mutations of genome maintenance genes, for example ERCC1-defective Ercc1$^{d/-}$ mice [5, 8, 9]. DNA damage occurs continuously due to exogenous and endogenous insults, and this should be increased by defects in genomic maintenance. Indeed, during ageing there is an increase of damaged DNA observed in many organisms [10]. Although more evidence for the role of DNA damage in ageing is certainly required [11], it is becoming increasingly clear that the ageing process seems to be driven by a defined set of metabolic changes that is aimed at sustaining organismal maintenance instead of growth; an adaptation that is presumably evoked to reduce further accumulation of DNA damage and that is called the survival response [12, 13].

The notion that genomic instability also plays a role in vascular ageing originates from our observations in mice with a defective XPD gene (XPD^{TTD} mice) and in Ercc1d/- mice, which both display an accelerated development of age-related vasodilator dysfunction due to decreased endothelial and vascular smooth muscle responses [4, 13]. Ercc1$^{d/-}$ further showed a decreased release of the endothelium-derived relaxing factor (EDRF) nitric oxide (NO), a decreased endothelial NO synthase (eNOS) expression and activation, increased vascular cell senescence, increased vascular stiffness, and elevated blood pressure, all at a very young age [4, 13]. These results recapitulate several important features of human vascular ageing [2, 14]. Furthermore, we and other observed an association between DNA repair gene polymorphisms and markers for cardiovascular ageing in humans [4, 15]. These

data suggest that genomic instability is involved in vascular ageing, and the question is how this mechanism of vascular ageing can be inhibited.

Diet restriction (DR) has been recognized as long as from premedieval times as a possible way to prolong life span [16, 17]. Rodents have been identified as animal models that respond well to DR [18-21]. DR is a moderate reduction of intake of food with an otherwise normal nutrient composition that increases (healthy) life span, and is thus distinct from severe reductions that shorten life span through malnutrition [22]. To date, DR has been the most widely studied and most effective experimental strategy for increasing longevity [23-26].

Since several decades it is known that this life extension can be partly explained on the basis of reduced tumor growth [16, 17]. DR might also rescue from overconsumption of an inappropriate diet, e.g. high-lipid or high salt diet, but given its effectiveness in healthy rodents it is believed that DR not merely rescues from illness [20, 27, 28]. The mechanisms of the DR effect might strongly relate to the survival response because there is a paradoxical similarity between DR-fed wild-type mice and progeroid mice in that both exhibit a reduced somatic growth. This is most probably the consequence of repression of the somatotrophic axis, since reduced levels of IGF-1 are observed in both DR-fed and progeroid mice, and genetic repression of IGF-1 signaling reduces growth and increases longevity [17, 22, 27, 29-31].

DR has also been reported to exert beneficial effects on the ageing cardiovascular system, some of which are likely related to reduction in inflammation and oxidative stress [31-33]. In the vasculature, DR appears to protect against endothelial dysfunction and arterial stiffness and attenuates atherogenesis by reducing several cardiometabolic risk factors such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 [34, 35]. During the process of ageing, and in several types of vascular disease and hypertension, the endothelium becomes dysfunctional, which is evidenced by an impairment in endothelium-dependent relaxation due to a reduced release of EDRF and an increase in endothelium-derived constrictor factors (EDCF) [36]. DR was reported to attenuate the endothelium dysfunction by improving the bioavailability of NO and by restoring eNOS expression [37, 38]. On the basis of these observations, DR might also protect against vasodilator dysfunction caused by genomic instability.

In contrast, the DR-induced suppression of IGF-1 signaling is expected to be harmful for the circulatory system because decreased IGF1 reduces eNOS and increases reactive oxygen species (ROS) inducing a pro-inflammatory phenotype of the endothelial cell. Therefore, we suggested that IGF-1 reduction could contribute to the poor endothelial function in progeroid mice [3]. Moreover, DR was suggested to improve CV health through the increase of anti-oxidant pathways in wild-type animals [39], but these pathways are already increased in ad lib fed progeroid mice with a defective NER [40]. Based on these paradoxes we wondered if DR could improve vasodilator dysfunction caused by genomic instability or might be detrimental. Furthermore, we have investigated the responses to the vasoconstrictor angiotensin II (Ang II) that are importantly related to vasodilator dysfunction

[41].

METHODS

Animals

The animals used in this study were 7-, 11-, and 16-week-old Ercc1$^{d/-}$ mice, and 11-, 20- week-old wildtype (WT) mice that were either treated ad libitum (AL) or with DR respectively. DR was started at 7 weeks of age with a 10% restriction, and then increased with 10% weekly until 9 weeks of age after which 30% DR was maintained until the day of sacrifice. As the DR group allowed longer survival of the Ercc1$^{d/-}$ mice, an additional group of 30-week old mice was explored to assess if any protective effect on the vasculature would last at least as long as the maximal life expectancy of AL-fed Ercc1$^{d/-}$.

Organ bath experiments

Tissue harvesting and preparation

Thoracic aorta and iliac arteries were collected from mice within 5 minutes after sacrifice by asphyxiation, and stored overnight in cold, oxygenated (5% CO_2; 95% O_2) Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7,$CaCl_2$ 2.5, $MgSO_4$ 1.2 , KH2PO4 1.2, $NaHCO_3$ 25 and glucose 8.3 in distilled water; pH 7.4) solution. The following day, vessels segments were mounted in 6-mL small wire myograph organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer at 37°C and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at which the effective transmural pressure is 100 mmHg. Viability of the tissue was tested though induction of contractions by exposure to respectively 30, 60, and 100 mmol/L KCl.

Testing of vasodilator function

After washout of KCl, pre-constriction was elicited with 30 nmol/L U46619, a thromboxane mimetic, resulting in 50-100% of the previously obtained contraction to 100 mmol/L KCl. Following pre-constriction, relaxation concentration-response curves (CRCs) for the endothelium-dependent vasodilator acetylcholine (ACh) were constructed by giving cumulative doses (10^{-9}-10^{-5} mol/L), followed by exposure to the endothelium-independent vasodilator sodium nitroprusside (SNP, 10^{-4} mol/L). To further explore the endothelium-independent dilator we cumulatively added SNP (10^{-9}-10^{-5} mol/L) in parallel rings preconstricted with 30 nmol/L U46619. In the case that sufficient aortic tissue was available, the involvement of nitric oxide (NO) and prostaglandins in ACh responses was investigated by performing the experiments in the presence of the endothelial nitric oxide synthase inhibitor NG-Methyl-L-Arginine acetate salt (L-NMMA, 10^{-5} mol/L), the cyclo-oxygenase (COX) inhibitor indomethacin (INDO, 10^{-5} mol/L) or both inhibitors. Inhibitors were added to the organ bath 10 minutes prior to U46619.
Testing of vasoconstrictor function

To detect the vasoconstrictor function in different diet groups, increasing concentrations of KCl (15, 30, 60, 100 mmol/L) were given without prior viability testing. Subsequently, after three times washout of KCl, thoracic aortas were stimulated with cumulative concentrations of U46619 (10^{-8}-10^{-6} mol/L). In iliac arteries, following viability testing by constriction with KCl 100 mmol/L and washout, Ang II (10^{-10}-10^{-7} mol/L) CRCs were constructed.

Quantitative real-time PCR

Total RNA isolation was performed with the Nucleospin RNA II kit (Machery-Nagel). RNA was reverse transcribed by use of Quantitect Rev.Transcriptiom Kit (Qiagen). Four nanograms of cDNA was amplified by real-time polymerase chain reaction (qPCR) and normalized to β-actin as an endogenous control (values were excluded of Ct ≥ 31). Each reaction was performed in duplo with SYBR Green PCR Master Mix (Applied Biosystems). The relative amount of genomic DNA in DNA samples was determined as follows: RQ = $2^{(-\Delta\Delta Ct)}$. COX-1 and -2, adenylate cyclase (AC) -5 and 6, phosphodiesterase (PDE) 4B and 4D, and the prostaglandin-I receptor (PTGI) were studied. Details of primers are provided in Table 1.
Statistical methods

Data are presented as mean ± SEM. SNP-corrected ACh responses were calculated as follows: (response to ACh as % of U46619 preconstriction / response to 10-4mol/L SNP as % of U46619) x 100 (to indicate as a percentage) x -1 (to indicate that it is a relaxation).

Table1. Primer sequences for qPCR

Marker	Forward primer	Reverse primer
COX-1	TACTCACAGTGCGGTCCAAC	GTACAGAGGGCAGAATGCGA
COX-2	GGGCCATGGAGTGGACTTAAA	TCCATCCTTGAAAAGGCGCA
AC5	CCAGTGTACTGCCCAAGAACT	GTAAACAGTGATTCTCCGCAGC
AC6	CTGCGGTGAGGGAGAATCACT	AGCCCTGACACGCAGTAGT
PDE4B	ACTGATGCACAGCTCAAGCC	CCAGCTCCTTGGCTAGATGA
PDE4D	GCAGACTTGCGAAGCGAATC	CCATTGTCCACATCGAAACCAC
PTGI	ATGTACCGCCAACAGAGACG	CCTCGGATCATGAGAGGCAG

RESULTS

The effect of DR on acetylcholine responses in WT and Ercc1$^{d/-}$ mice

To find out what is the effect of DR on accelerated vascular ageing caused by genomic instability, the vasodilator effect of ACh was measured in both male and female *Ercc1$^{d/-}$* mice in the age from 7 to 30 weeks. First, the age-dependent responses to ACh were assessed in AL- vs. DR-fed Ercc1$^{d/-}$ mice to see if vasodilator function worsened (Figure 1A). The results demonstrate that under AL diet there was an age-related deterioration of dilator response to ACh, the 16-week-old Ercc1 mutant mice showing the most severe vasodilation. WT did not show any change in ACh response from 11 to 20 weeks (Figure 1B), as expected in view of their young age. Comparing WT with Ercc1$^{d/-}$ a trend towards declining vasodilator function is seen at week 11 ($p=0.325$, GLM-RM, WT n=6, Ercc1$^{d/-}$ n=11) and this becomes more pronounced and significant from the age of 16 weeks (*$p<0.05$, GLM-RM, WT n=8, Ercc1$^{d/-}$ n=11). Since in the 11-week-old Ercc1$^{d/-}$ no significant changes in ACh responses had yet occurred, these results are not further discussed.

Comparing the diets, in animals from the age of 16 weeks, DR significantly prevented the aggravation of ACh responses in AL-fed 16 week-old Ercc1$^{d/-}$ (Figure 1C) whilst being without effect in 20-week-old WT (Figure 1D). Vasodilations were not fully preserved to the level of WT mice (*$p<0.05$ Ercc1$^{d/-}$ 16 week AL vs. WT 20 week AL or DR, GLM-RM), but did not differ from vasodilations in Ercc1$^{d/-}$ of 7-weeks of age, the age at which DR was started in Ercc1$^{d/-}$. At the age of 30 weeks vasodilations to ACh in DR-fed Ercc1$^{d/-}$ were still significantly better compared with 16-week-old AL-fed Ercc1$^{d/-}$ (Figure 1C). At that age all AL-fed Ercc1$^{d/-}$ mice had already died while all DR-fed Ercc1$^{d/-}$ mice were still alive (data not shown).

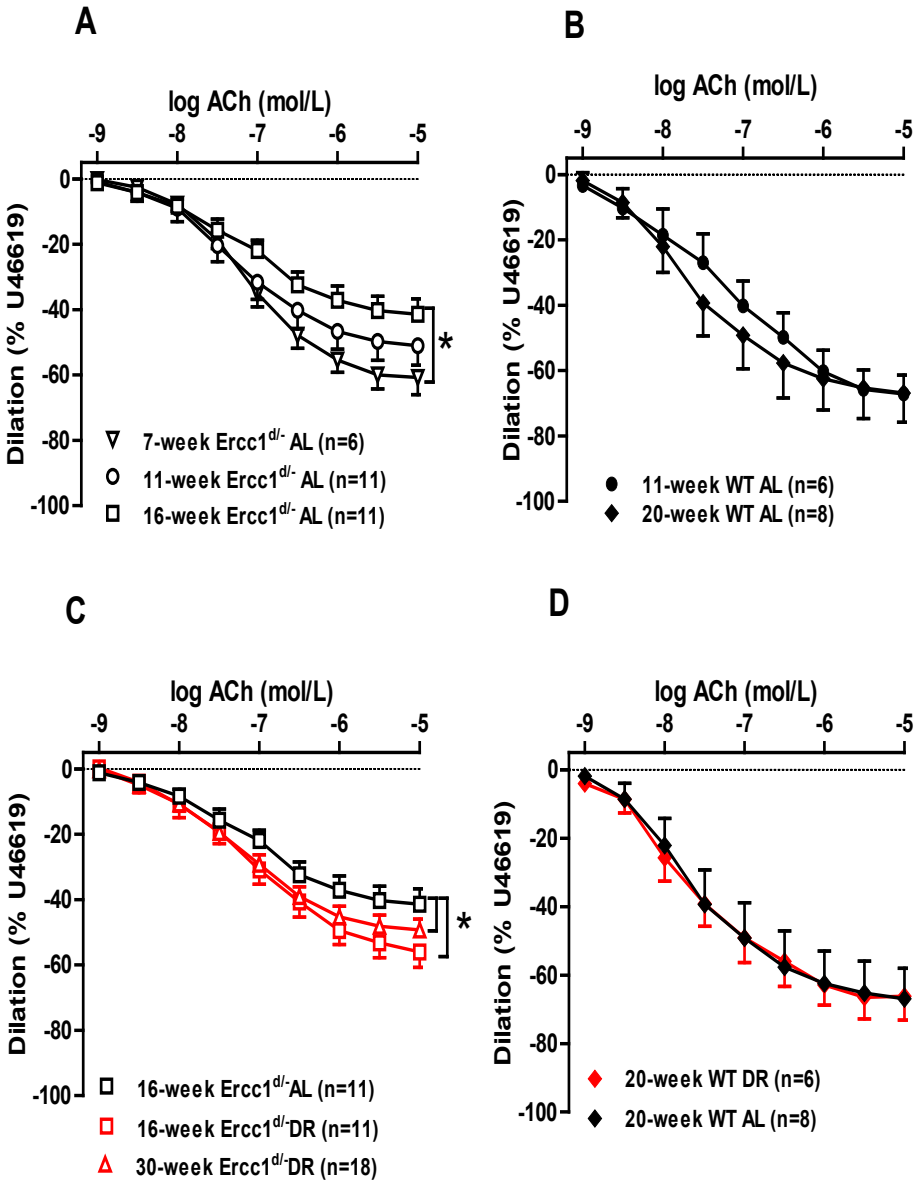

Figure 1. Age-dependent acetylcholine (ACh)-induced vasodilations in aortic segments from ad libitum (AL) fed wildtype (WT) and Ercc1$^{d/-}$ mice as measured ex vivo in small wire organ baths. ACh was given in cumulative concentrations after preconstriction with the thromboxane analogue U46619. Responses are expressed as % relaxation of the U46619 preconstriction. Error bars: S.E.M. *:p<0.05, general linear model for repeated measures (GLM-RM).

The role of endothelial signalling compounds in genotype and diet-related effects

In our previous study [4] we found that ACh-mediated dilations were predominantly mediated by NO release, and that the loss of vasodilation in 16-week old Ercc1$^{d/-}$ was predominantly due to loss of NO signalling and eNOS expression and activation. In the current study we therefore performed experiments in aortic rings with L-NMMA and indomethacin. In aortic tissue from 16-week-old AL-fed Ercc1$^{d/-}$ vs. 20-week-old AL-fed WT ACh responses were almost completely inhibited by L-NMMA, but the inhibition was relatively more pronounced in WT than in Ercc1$^{d/-}$ (Figure 2A). INDO did not inhibit dilations significantly in WT, and had no further effect in the presence of L-NMMA (Figure 2A vs. 2B).

In 20-week-old DR-fed WT mice L-NMMA completely blocked ACh responses and, like in AL-fed mice, INDO alone or on top of L-NMMA had no effect (Figure 2C and D). In 16-week-old DR-fed Ercc1$^{d/-}$ mice L-NMMA and INDO both blocked ACh responses by about 50% (Figure 2C and D). When given combined these inhibitors totally abolished ACh responses (Figure 2D, $p<0.05$ GLM-RM vs. L-NMMA or INDO alone). This indicates that the enhanced ACh response following DR involved dilatory prostaglandins.

To find out which component could be responsible for the increased response to prostaglandins the expression of cyclo-oxygenase (COX) -1 and -2, phosphodiesterase (PDE) 4B and D, adenylyl cyclase 5/6 (AC 5/6) and the IP prostaglandin receptor were tested. This was done in the blood vessel-rich lung tissue. Ct values for IP receptors, PDE4D and AC6 mRNA levels were on average > 34, and therefore we considered these levels too low to be reliably measured. COX-1 mRNA showed a trend to increase ($p<0.05$ one-way ANOVA for all 4 groups) after DR in WT and in Ercc1$^{d/-}$ mice, and was therefore maximally and significantly increased in DR-fed Ercc1$^{d/-}$ mice as compared with AL-fed WT mice (Figure 3); PDE4B mRNA was decreased in both Ercc1$^{d/-}$ mouse groups compared with AL-fed WT mice (Figure 3). COX-2 and AC 5/6 mRNA but did not show significant changes among the groups.

Endothelial vs. non-endothelial responses

To investigate to which extent genotype and diet influenced non-endothelial functions, we measured responses to SNP, which releases NO immediately in vascular smooth muscle cells (VSMC) leading to a maximal production of cGMP and a maximal vasodilation. Since SNP responses could be totally blocked with the guanylyl cyclase inhibitor ODQ the effects entirely relied on cGMP (data not shown). The results show that with increasing age, responses to 10^{-4} mol/L SNP, which was given on top of ACh, progressively decreased in AL-fed Ercc1$^{d/-}$ mice, reaching statistical significance in 16-week-old mice as compared to 7- week-old mice (Figure 4A, One-way ANOVA on 7-, 11- and 16-week AL-fed mice with Dunnett post-hoc test). DR significantly prevented the age-dependent decline in dilator responses in 11- and 16-week-old mice (Figure 3B, t-test). Even 30-week-old DR-fed Ercc1$^{d/-}$ still tended to display a better SNP response as compared to 16-week AL-fed Ercc1$^{d/-}$ mice ($p=0.038$, t-test), although being almost twice as old. In WT animals no age- or diet-related changes were observed (Figure 4B).

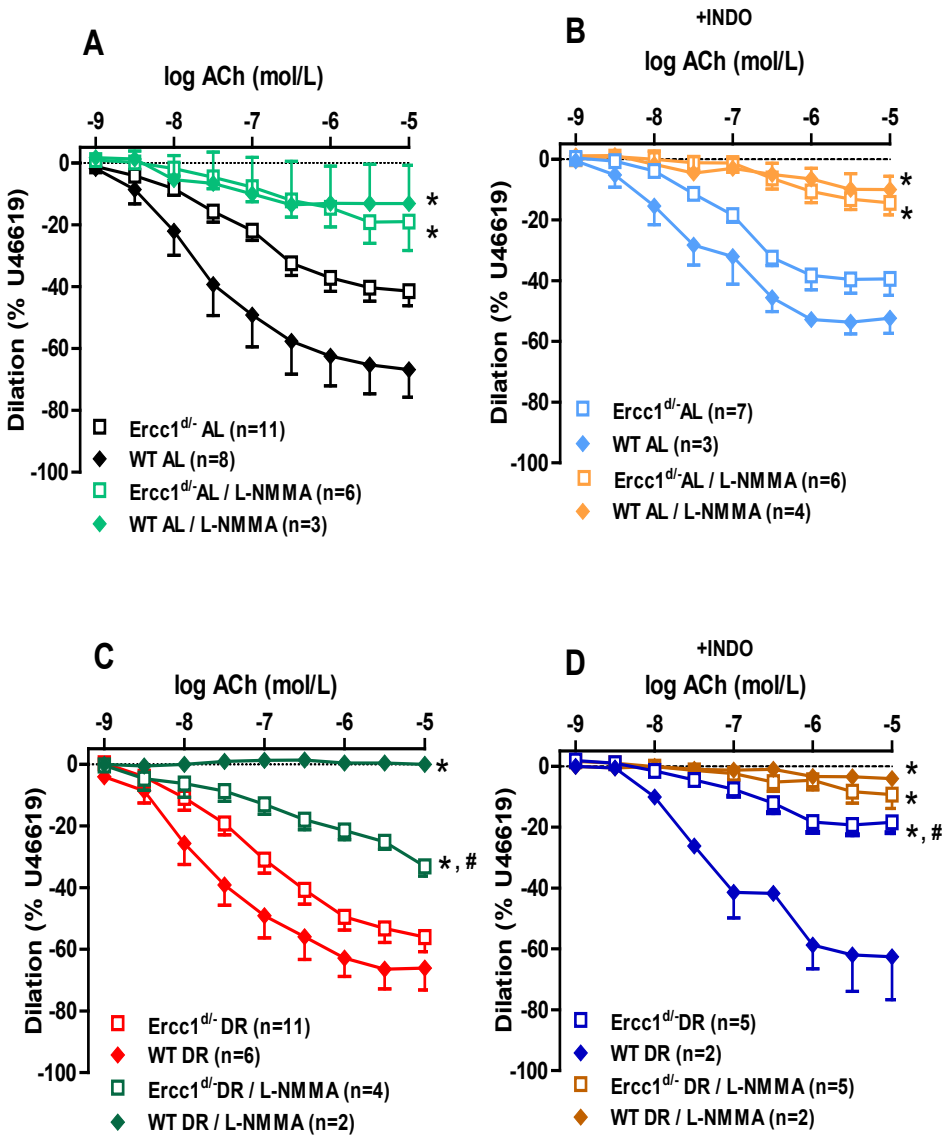

Figure 2. Role of NO and prostaglandins in ACh-induced vasodilations of aortic segments from 20 weeks-old WT and 16 weeks-old Ercc1$^{d/-}$ mice measured in organ baths. L-NMMA (10^{-5} mol/L) and INDO (10^{-5} mol/L) resp. inhibit NO and prostaglandin synthesis, and were added to the organ baths 10 minutes before U46619. Responses are expressed as % relaxation of the U46619 preconstriction. Error bars: S.E.M. *:p<0.05 vs. non-pretreated segments of the same strain depicted in panel A (for AL-fed mice) or C (for DR-fed mice). #:p<0.05 vs. L-NMMA+INDO-treated Ercc1$^{d/-}$ segments.

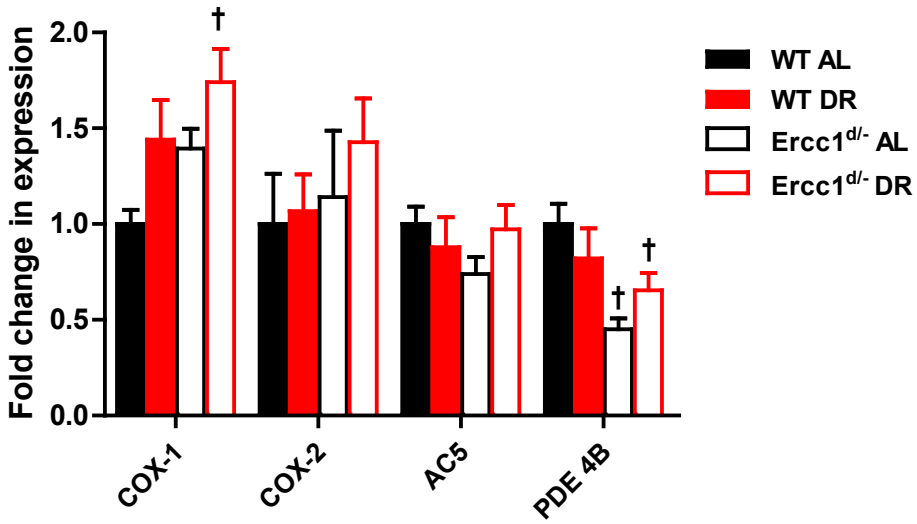

Figure 3. RNA expression levels in lung tissue of COX-1, COX-2, AC 5/6, and PDE4B in WT and Ercc1$^{d/-}$ mice after AL and DR feeding. †=P<0.05 vs. WT AL (one way- ANOVA followed by Dunnett's post-hoc test vs. WT-AL).

To exclude any influence of ACh on SNP responses and to explore dose-related effects of SNP, we generated SNP titration curves in 16-week-old Ercc1$^{d/-}$ and in 20-week-old WT (Figure 4C). The data confirmed that in AL-fed Ercc1$^{d/-}$ mice SNP responses were strongly reduced, and these were fully restored to the level of WT animals by DR. In WT animals no significant changes occurred.

The response to ACh depends on the amount of EDRF that is released from the endothelium as well as the responsiveness of the VSMC to the released EDRF. To estimate the proportion of the ACh effect that was mediated by EDRF on the one hand vs. maximum VSMC responsiveness on the other, we corrected ACh responses for SNP responses. This revealed that the endothelial contribution to the worsening response to acetylcholine in Ercc1$^{d/-}$ was undetectable between the age of 7 to 11 weeks (Figure 5A), but emerged between the age of 11 and 16 weeks (Figure 5A). Indeed, at the age of 16 weeks AL-fed Ercc1$^{d/-}$ had a significantly deteriorated SNP-corrected ACh response as compared to AL-fed WT of 20 weeks (Figure 5B).

Since the effect of DR on ACh responses was observed in 16-week-old Ercc1$^{d/-}$ mice, SNP-corrected values are best-studied at that age. DR only showed a very marginal, non-significant increase of the response at the highest concentrations of ACh in Ercc1$^{d/-}$, both in 16 and 30-week DR-fed mice as compared to 16-week AL-fed (Figure 5B). The responses to ACh remained significantly reduced as compared to WT (Figure 5B), in which DR had no effect.

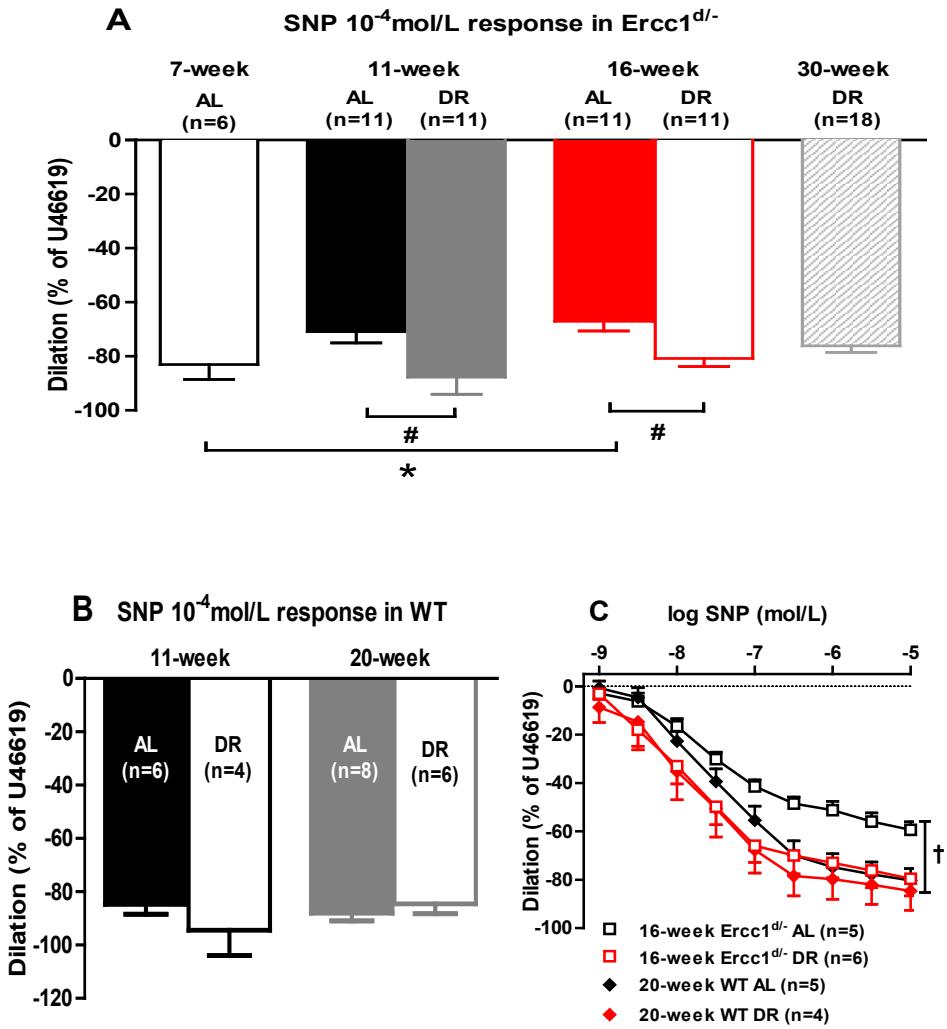

Figure 4. Effect of diet restriction (DR) on age-dependent sodium nitroprusside (SNP)-induced vasodilations in U46619-preconstricted aortic segments from WT and Ercc1d/- mice measured in organ baths. SNP was either given as a bolus concentration of 10-4mol/L SNP after performing ACh concentration response curves (A,B) or administered in cumulative concentrations immediately after preconstriction (C). Responses are expressed as % relaxation of the U46619 preconstriction. Error bars: S.E.M. *: $p<0.05$ 16-week vs. 7-week Ercc1$^{d/-}$ AL, one-way ANOVA, Dunnett post-hoc test, #: $p<0.05$ t-test, †: $p<0.05$, 16-week Ercc1$^{d/-}$ AL compared to all other groups, GLM-RM.

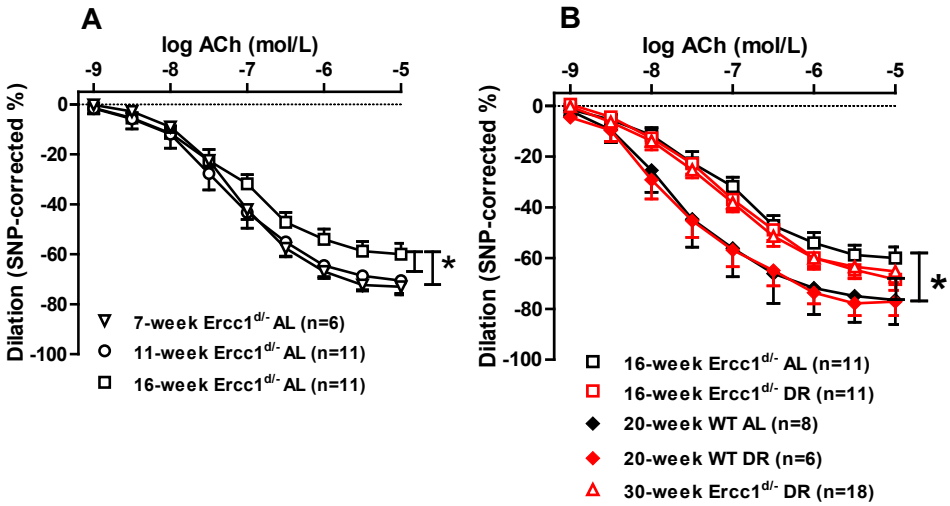

Figure 5. Effect of diet restriction (DR) on age-dependent acetylcholine (ACh)-induced vasodilation in aortic segments from U46619-preconstricted WT and Ercc1$^{d/-}$ mice measured in organ baths. Responses are expressed as % of the U46619 preconstriction and corrected for relaxation to 10^{-4} mol/L SNP given after the final dose of ACh (see methods). Error bars: S.E.M. *:p<0.05 as indicated with brackets; GLM-RM.

Effects of DR on Ang II responses

Vasoconstriction to Ang II (Fig. 6) tended to be higher in Al-fed Ercc1$^{d/-}$ vs. WT mice, but this did not reach the significance level of p<0.05 for the whole concentration-response curve (GLM-RM). However, DR-fed Ercc1$^{d/-}$ showed a trend for a decrease as compared to AL-fed Ercc1$^{d/-}$, reaching a significant difference for the maximal response occurring at $3x10^{-8}$ mol/L Ang II, and other concentrations, and reaching a borderline significance of p=0.059 over the entire concentration-response curve (GLM-RM).

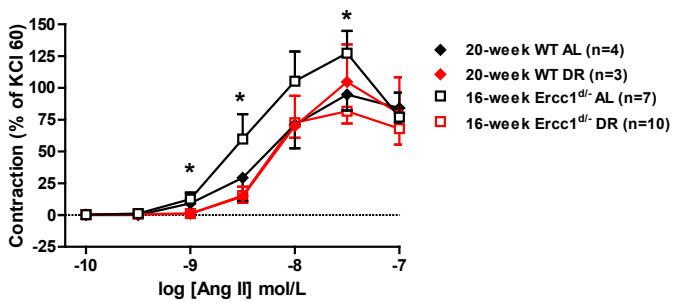

Figure 6. Vasconstrictions to Ang II of iliac arteries, (measured in organ baths. *p<0.05 Ercc1$^{d/-}$ AL vs. Ercc1$^{d/-}$ DR, t-test per concentration).

DISCUSSION

In this study we investigated if DR would protect against the accelerated development of vasodilator dysfunction caused by genomic instability. The results show that DR both protected against the development of endothelium-dependent as well as endothelium-independent vasodilator dysfunction. The preservation of ACh responses was due to the appearance of a prostaglandin-mediated relaxant response. A possible explanation for this emerging prostaglandin response is the increase of COX-1 in combination with a decreased PDE4B level, which should lead to improved vasodilator cAMP signaling. Preservation of endothelium-dependent responses by DR has been previously reported in aging WT rodents [42, 43], and this was due to reduced vascular oxidative stress, possibly involving nuclear factor erythroid-2-related factor-2 (Nrf2)-mediated upregulation of antioxidants [38]. Importantly, in Ercc1-mutated and other NER-defective models anti-oxidants that are regulated by Nrf2 are increased in a way that is similar to DR-fed WT mice[44]. Moreover, ROS inhibition only marginally improves ACh responses and smooth muscle NO-cGMP signaling in Ercc1$^{d/-}$ mice, and the decreased response to endothelium-mediated NO signaling in Ercc1$^{d/-}$ was virtually entirely due to reduced eNOS expression and activation by phosphorylation at serine 1177 [4]. Therefore, it seems reasonable to assume that anti-oxidant pathways are already upregulated in Ercc1$^{d/-}$ mice, so that no further increase can be expected after DR feeding. Apparently these mice have to recruit another system to improve vasodilator function mediated through the endothelium. This is also suggested by our present observation that in AL-fed Ercc1$^{d/-}$ there is already a downregulation of PDE4B, and a tendency to increased COX-1 expression in order to improve cAMP signaling. However, only DR allows full access to optimization of vasodilatory prostaglandin signaling and therefore optimal responses to ACh. Although SNP-correction of ACh concentration-related responses leads to a loss of statistical significance of the DR effect, the difference between AL and DR-fed Ercc1$^{d/-}$ of 16 weeks of age does not seem to fully disappear (Figure 5B). Therefore it appears that combined improvement of VSMC responses to NO-cGMP and increased prostaglandin-cAMP by the endothelium are both needed for an optimal effect of DR.

Another important observation in our study is that Ang II responses tended to be increased in untreated Ercc1$^{d/-}$ mice compared to untreated WT, although the n-number in the WT group was too low for rigorous statistical comparison. This observation corresponds to our earlier findings in these mice (Chapter 4), and the well-known fact that in the elderly blood pressure and blood flow responses to Ang II are elevated, especially in the presence of diabetes or the absence of counterregulation by AT2 receptors [46-49]. Also key features of arterial aging, like growth, inflammation and matrix responses, are expected to be linked to increased Ang II signaling in the aging organism, and increased expression of RAS components is often observed in the arterial wall [50]. While conclusive evidence for such presumptions is still needed, it should be emphasized that there are also studies that could not detect an elevated age-related Ang II vasoconstrictor response [51]. However, this absence of a detectable age-related effect could be due to the use of brachial artery as a model, which is an artery that is relatively protected against age-related remodeling, or simply the lack of sufficient amounts of elderly people [48, 52-54]. Further confusion about

age-related Ang II vasoconstrictor activity comes from studies in various WT rodents in which both an increase as well as a decrease of Ang II responses can be observed in aging animals [51]. Disparities in animals and vessel species that were used and the fact that aged WT animals poorly mimic human aging, or at least worse than DNA repair mutant mice, raise the question if all studies in WT animals are well-representative for vascular aging in humans. Whatever reasons there are for the contrasting findings, our present study shows that genomic instability, a main causative hallmark of aging, leads to an increased Ang II activity. This strongly supports the view that Ang II is importantly involved in aging-related vascular disease.

To our knowledge this is the first study to show that DR leads to a decrease of age-related enhancement of Ang II signaling. Reminiscent of our observation are previous studies showing that DR decreases plasma renin activity in obese persons, decreases cardiac ACE and AT1 receptor expression in a rat model for metabolic syndrome, and decreases detrimental cardiac and vascular remodeling in transgenic rats that overexpress the human renin and angiotensinogen genes [55-57]. The cardiac effects of DR in animals with an activated RAS are associated with improvement of mitochondrial function. This is interesting in light of the fact that both DR as well as RAS blockade show similar beneficial effects on mitochondrial function [58]. It raises the important possibility that some DR effects might be due to decreased mitochondrial damage through an activated RAS. Other possible mechanisms that might contribute to suppression of Ang II vasocontrictions by DR in Ercc1$^{d/-}$ mice are increased counterregulation of AT1 receptor by an increase of AT2 receptors [59], and the observed improvement of NO-cGMP-mediated responses, which again might partly relate to beneficial effects on mitochondria [40].

Taking all the observations together the results emphasize the versatile and powerful treatment effect of DR. In addition it stresses the need to further explore the molecular mechanisms that allow such a flexibility to adapt to aging-induced loss of vascular function, and that this goes beyond the effects on ROS and NO signaling that are observed in aged WT animals.

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Chapter 6

Summary, general discussion and perspectives

SUMMARY

Genomic instability and cardiovascular diseases

Age-related cardiovascular diseases (CVD) are responsible for the majority of deaths and a major cause of disability and frailty in developed countries. Research endeavours to define why advancing age affects cardiovascular structure and function and why, as a consequence, CVD is formidable, are required. It is still a daunting task to unravel the contribution of all factors that either co-vary with age, or that are not related to age per se, such as genetic predisposition. In this thesis, of which the main findings are summarized in **Table 1**, CVD has been approached on the basis of a duality in the factors that govern age-related vascular dysfunction, namely the contribution of the aging process as caused by genomic instability, a main causative hallmark of biological aging, versus the contribution of dysregulation of homeostasis that, as time progresses, increasingly damages the vasculature; a process that we here call chronological aging. With respect to the latter, we have focused on RAS activation and genetic variability in signalling of bradykinin.

The proposed duality is a simplification of the labyrinth of processes that play a role in vascular aging. However, this simplification is a tool that has helped to discover novel mechanisms in the pathogenesis of CVD. **Chapter 1** provides a summary of recent discoveries that resulted from pioneering work that was based on the assumption that genomic instability is a causative factor in the pathogenesis of age-related CVD. In parallel, it proposes that the constituents of each part in this bimodal model interact; it is not a strict dichotomy. The ways these interactions take place and the consequences thereof are starting to be unravelled. This thesis addresses some questions that result in the expansion of knowledge on this topic, as will be summarized below.

While a vast amount of knowledge related to the role of genomic instability in CVD still lies beyond our field of view, the implementation of knowledge arising from investigation of classical paradigms appears to become increasingly complicated. Two chapters of this thesis cater to this problem, focusing on the prediction of the responsiveness in individual patients with age-associated CVD to pharmacotherapy based on RAS modulation.

In **Chapter 2** the question was addressed whether measurement of ACE activity and/or renin concentration before onset of pharmacological intervention aimed at reducing Ang II production and AT1 receptor-mediated signalling could predict the acute and chronic antihypertensive responsiveness in individual patients. In general, renin activity associated better with this responsiveness than ACE activity did. Neither of the measurements, however, allowed per-patient predictions because the interindividual variability of the values is simply too high. Thus, despite earlier epidemiological studies that support the predictor value of either renin or ACE, when bringing this down to the individual patient, even in a very homogenous population, it is of limited or no use. The main reason for this is that multiple factors additionally affect these parameters, including gender, ethnicity, salt intake, antihypertensive medication and age. As a consequence, it is impossible to define a uniform limit that can be generally applied to all hypertensive patients.

The studies in **Chapter 3** focused on the question what could be the mechanism that explains the contribution of the bradykinin (BK) B1 receptor rs12050217 polymorphism to ACE inhibitor therapy responsiveness in patients with coronary artery disease (CAD). The results suggest that only the variability in coronary vasomotor response to the B1 receptor agonist DABK is associated with the genotype, and not the inflammatory response, which was apparently more dependent on gender differences. Hypothetically, an increase of BK peptide upon intake of ACE inhibitors would lead to a better relaxant effect in patients with the AA genotype because of their higher sensitivity of B1 receptors. B1 receptors levels did not differ between the genotypes, and therefore we were unable to find a straightforward explanation for the improved responsiveness to DABK.

The second part of the thesis focused on the effect of genomic instability on vasomotor responses to Ang II and the treatment response to AT1 receptor blockade. In addition, the effect of an established anti-aging therapy, diet restriction, on vasomotor dysfunction caused by genomic instability was evaluated.

Excessive Ang II signalling has been implicated in age-related cardiovascular disease. Blockade of this excessive signalling is a currently applied clinical intervention strategy in such diseases. The relationship with genomic instability is unknown. In **Chapter 4** the question was addressed whether genomic instability would change the responsiveness to Ang II and AT1 receptor blockade. Ercc1$^{d/-}$ mice display an increased vasocontrictive response to Ang II in the iliac artery. This increased response appears to depend at least partly on a loss of AT2 receptor counterregulation. Loss of NO-cGMP signaling, which also occurs both due to endothelial and smooth muscle cell changes, does not appear to play an important role in the enhanced Ang II response. Chronic treatment with the AT1 receptor antagonist losartan prevented the enhancement of Ang II-induced vasoconstriction, again without improvement of NO-cGMP signaling. Apparantly, both genomic instability as well as AT1 receptor signalling are involved in the sustained enhancement of Ang II-induced vasoconstriction. Since losartan could not prevent the accelerated loss of NO-cGMP-mediated vasodilation in Ercc1$^{d/-}$ mice, Ang II-generated reactive oxygen species (ROS) apparently do not contribute to endothelial dysfunction nor to vascular genomic instability in Ercc1$^{d/-}$ mice. This might be expected in view of the fact that repair of oxidative lesions is largely intact in these animals.

Prompted by the results in Chapter 4 one could wonder if vascular aging due to genomic instability can be prevented at all. In **Chapter 5** we explored if diet restriction (from 10% increasing to 30% reduction of the estimated ad lib intake) would prevent vasomotor changes in Ercc1$^{d/-}$ mice. These mice showed both a decreased endothelium-dependent and -independent response to NO-cGMP signalling. These changes were prevented by a 9-week diet restriction intervention. The diet-restricted Ercc1$^{d/-}$ mice lived at least up until an age of 30 weeks, an age at which ad lib-fed Ercc1$^{d/-}$ mice had all perished, and moreover the protective effect on vasodilator function was still present. Upregulated prostaglandin/cAMP signaling was most probably the mechanism that allowed improvement of vasodilator responses, as evidenced by the blocking effects of the cyclo-oxygenase (COX) inhibitor indomethacin, and the alterations in vascular COX-1 and PDE4B expression. In addition,

the enhanced vasoconstrictive responsiveness to Ang II, observed earlier in Chapter 4, was also prevented by diet restriction. Therefore, diet restriction is a very effective and versatile intervention against vascular problems related to aging.

General discussion and perspectives

Predictive markers for treatment efficacy of RAS modulation in age-related cardiovascular disease: can they be improved?

In the beginning of the thesis the question was addressed if the response to RAS blockade in age-related cardiovascular disease could be predicted. As pointed out in Chapter 2 biochemical assays to determine plasma renin concentration or ACE activity might predict the antihypertensive response to RAS-modulating drugs in human populations, but on a per patient level this does not appear to be feasible. This might be due to the large variability in the values, even when limiting the analysis to a homogenous patient population within a narrow age range. On the other hand, it might also relate to the fact that plasma values are not directly representative for Ang II function in the target tissue. Therefore, taking all factors into account that govern blood pressure control and RAS activity (gender, age, ethnicity, salt intake and diuretic treatment) it seems as a mission impossible to define uniform values that predict responsiveness in individual hypertensive patients. Similarly, pharmacogenomic approaches to predict RAS inhibitor efficacy seem also inconclusive at best. It is obvious that RAS inhibitor pharmacogenomics in CVD is still in its infancy and that there is room for improvement.

Perhaps the combination of biochemical with pharmacogenomic assays might be a platform to refine predictions. Also the use of assays that represent vascular function improvement or a decrease of disease markers might lead to further refinement. Previous studies have tried to use baseline values of pro-inflammatory or prothrombotic markers as predictors in CAD. In the case of antihypertensive treatment this might also have priority since in the majority of the patients chronic reduction of cardio- and cerebrovascular risk is the most relevant goal, not blood pressure lowering *per se*, which is more relevant in acutely threatening forms of hypertension. Although the response of biomarkers for cardiovascular risk to RAS-modulating drugs corresponds with improvement of vascular function, and despite the fact that RAS-modulating drugs improve endothelial progenitor cell levels, the predictive value of such biomarkers for the therapeutic effect of such drugs has not yet been established by strong clinical end points [1-5]. Until today there have been no reports that have demonstrated the usefulness of such markers, let alone when used in combination with genetic analyses. This might therefore represent an option for improvement.

Table 1. Summary of previous and newly obtained knowledge.

What was already known
Age is the major independent risk factor for the development of CVD, but the underlying mechanisms still need to be unraveled.
Genomic instability is a major causative factor in the process of ageing.
DNA repair defect in mice leads to accelerated vascular ageing.
Ang II can induce DNA damage by AT1 receptor-induced ROS production, causing vascular cell senescence.
Renin and ACE activity predict the antihypertensive response on RAS modulation at the population level.
Bradykinin B1 receptor genotype predicts ACE inhibitor therapy responsiveness in coronary artery disease, but the mechanism is unknown.
Dietary restriction prolongs life span in wild type animal models.
Dietary restriction can improve cardiovascular function. Reduction of salt and oxidative stress are potential mechanisms, which act on a relatively short notice.
What this thesis adds
Novel hypothesis: factors that influence genomic instability are amongst the mechanisms that constitute the independent risk factor age.
Renin and ACE activity do not predict the antihypertensive response on RAS modulation at the individual patient level.
Bradykinin B1 receptor genotype determines the vasodilator responsiveness to receptor agonists in coronary arteries, but not inflammatory responses in leucocytes.
Genomic instability increases vascular Ang II responses, and this is prevented by chronic treatment with the AT1 receptor antagonist losartan.
Chronic losartan treatment does not improve vascular NO-cGMP signaling caused by genomic instability.
Dietary restriction protects against the accelerated vasodilator dysfunction and enhancement of vascular Ang II responses caused by genomic instability.

Chapter 3 suggests that the variability in response of endothelial cells to B1 receptor stimulation in combination with receptor genotype might be of use when trying to improve predictions. However, the development of a clinically applicable bioassay is not at all easy if not impossible if it would require measurement in cells or blood vessels of individual patients. Circulating endothelial and endothelial progenitor cells have been proposed as markers for cardiovascular risk [6-8], and theoretically they might be used for the development of bioassays. The rarity of such cells, the absence of a clear definition with respect to their phenotypical markers and the possible lack of precision of derived bioassays, which impedes per patient risk stratification, are hurdles that will make the development of bioassays a daunting task. Perhaps technological developments are a first requirement to overcome such hurdles. In the case of genotyping, improvement of sequencing techniques has indeed shown that with technological developments a dramatic increase in throughput, reliability and cost-effectiveness can be achieved [9-11]. The recent foundation of the Medical Delta in the Netherlands illustrates the awareness of the possibilities that can be offered by specific integration of technical and medical research disciplines.

The next steps to further establish the role of genomic instability in vascular aging

Of more importance in the scientific hierarchy than issues about specific clinical markers is the question what are the fundaments of age-related cardiovascular disease. Especially the identity of the biological mechanisms that explain the risk posed by the factor 'age' alone, independently from classical cardiovascular risk factors such as diabetes, smoking, hyperlipidemia etc., is entirely obscure. Insight in this matter is very likely to open possibilities for finding novel markers and treatments. In the ideal case the vascular aging process could be followed in individuals from early age on and thus allow for preventive measures to be taken.

As discussed in Chapter 1, genomic instability and the associated response might be a leading biological mechanism to focus on to resolve these issues. The logical first step was to study the role of genomic instability in age-related vascular changes that are known to occur in humans. This was recently done by Durik et al. (2012), as thoroughly discussed in Chapter 1. Although this previous work strongly suggests a role of genomic instability, it is entirely unknown what mechanisms convey its harmful effects on the vasculature. Chapter 4 and 5 of the present thesis studied, as a hypothesis-based next step, the impact of a classical pharmacological intervention in age-related cardiovascular disease and a classical anti-aging intervention, the outcome of which has been summarized the thesis Summary section. The paragraphs below highlight the possible next questions and steps in the sequel of research endeavors along these lines. This will gradually provide more insight into the mechanisms that link genomic instability to vascular aging.

The renin-angiotensin system, genomic instability and aging: where to go now?

Our result in Chapter 4 that AT1 receptor antagonist treatment does not improve endothelial function is in contrast with several studies in animal models and patients that RAS blockade can protect the endothelium in several age-associated human cardiovascular diseases [12,

13]. On the one hand this could be explained by immediate effects that are not directly related to biological aging as based on genomic instability (e.g. lowering of oxidative stress). In other words: this is the effect of RAS blockade on the first (chronological) mode of the duality that was introduced in the thesis Summary. Implicitly, this would mean that RAS blockade has no effect on the accelerated aging as caused by a functional defect in ERCC1 endonuclease. In Chapter 4, and partly also addressed in Chapter 5, this lack of effectiveness might be explained by the relative independency of Ercc1$^{d/-}$ mice on oxidative stress. In fact, AT1A receptor knockout mice are believed to live longer due to reduced oxidative stress, but also due to preservation of mitochondrial function and an increase of mitochondria-related pro-survival genes [14]. Also losartan treatment starting two weeks after weaning in Wistar rats prolongs life span [15], so that a role of embryonal development can be excluded. Vascular protection in these two studies was observed as a reduced age-dependent increase of the size and of fibrosis of the aorta and aortic arch. Endothelial function studies were not performed. In summary, the discrepancies with our study, which appears to point to a lack of effect on aging, can have the following reasons:

- AT1 receptors and ROS are not involved in genomic instability that arises from ERCC1 dysfunction.
- The consequences of genomic instability cannot be further improved by AT1 receptor blockade because anti-oxidant and other pro-survival pathways, especially those involved in mitochondrial protection, are already increased.
- A disparity in the vascular variables.

The first two issues can be addressed by studying the effect of lifelong AT1 receptor blockade on longevity of animals that are deficient of ERCC1 or related proteins and in animals that lack a proper repair of oxidative and mitochondrial DNA damage. Also, models in which the pro-survival pathways are suppressed can be very useful. Further study on the third issue should focus on the effect of RAS blockade on composition of the extracellular matrix and vascular dimensions in such models. Moreover, in Ercc1$^{d/-}$ mice the increased vascular stiffness has not yet been explained, and a change in extracellular matrix composition is an intelligible explanation.

Importantly, evaluation of mitochondrial function and expression of markers that are related to mitochondrial activity and protection, such as energy and nutrient-sensing proteins can further illuminate the mechanisms that connect the RAS with the process of (vascular) aging. Amongst these markers nicotinamide phosphoribosyltransferase and Sirt3 might be useful because these have been implicated in the anti-aging effect of AT1A receptor blockade [14]. In fact, such markers might be explored as possible markers to test the effectiveness of RAS blockade in age-related vascular disease. The application of mitochondrial function-related markers would represent an entirely novel approach to predict treatment effectiveness of RAS blockade. At the moment, however, the usefulness of such markers remains highly speculative, both because the importance of mitochondrial function in vascular aging needs to be further explored, especially in humans, and because the relationship between Ang II signaling and mitochrondrial function and aging is a rather uncharted area [16-18].

Another important question is why genomic instability leads to an increased Ang II responsiveness. Since decreased endothelial dilator function cannot be an explanation,

there is no clue as yet that can lead directly to an answer on this question. Naturally, one could study if the individual DNA damage response components are involved, but there is a broad array of candidate mechanisms (Chapter 1). Perhaps it is needed to narrow down the options first by investigating if Ang II activation takes place on a local or on a systemic level by comparison of the RAS in vascular-specific DNA repair-defective mice with our currently used whole body –transgenic models such as Ercc1$^{d/-}$.

Pharmacological intervention in the RAS: can it ever be used as an anti-aging therapy?

What if AT1 receptor blockade is effective in increasing lifespan by slowing down the aging process and in improving vascular dysfunction in certain models of genomic instability? Can this be translated to application in humans? And would one choose for AT1 receptor blockade, ACE inhibition, renin inhibition or perhaps even AT2 receptor stimulation? Should treatment be started at early age to be optimally effective? The task of addressing these questions is particularly challenging and depends on the following requirements:

- Sufficient evidence that demonstrates and explains the role of AT1 and AT2 receptors in modification of health span and lifespan.
- Identification of (vascular) aging markers that justify treatment.
- Prospective cohort studies spanning several generations and applying diverse RAS modulating drugs.
- Public acceptance of lifelong pharmacotherapy against diseases of affluence.

Despite the fact that RAS modulation is to some degree successfully applied in patients that already have an age-related disease, and leads to longer event-free survival in coronary artery disease [20-21], there is hardly evidence from animal studies and none at all from human studies regarding the question whether AT1 or AT2 receptor interference can prevent or delay the occurrence of age-related disease, let alone increase health or life span. Concerning markers, Chapter 2 is an example of how difficult it is to find suitable markers on a per patient base. Perhaps large scale genetic profiling in combination with large cohort association studies might eventually identify the required markers so that it will be possible to start prospective pharmacological studies to prove this concept. However, financing bodies for such long-lasting studies will be hard, if not impossible, to find. Even more threatening might be the public opinion that is fueled by the awareness that health care costs due to aging of the population are ever increasing, and that there might be a better alternative: the alternative that is presented in Chapter 5.

Nutritional intervention: a powerful tool for further research in vascular aging

In Chapter 5 it becomes clear that dietary restriction is superior to RAS inhibition, at least, when it comes to prevention of vasomotor dysfunction due to genomic instability. DR not only increased life span in Ercc1$^{d/-}$ animals dramatically, but it also improved the diminished acetylcholine and SNP responses. Moreover, it prevented the increase in Ang II responses, and it might thus also prevent vascular dysfunction related to the aforementioned first mode of vascular aging. In relation with Chapter 4 it seems worthwhile to investigate the mechanisms by which DR suppresses increased Ang II responsiveness: it might automatically

lead to identification of the mechanisms by which genomic instability increases Ang II responsiveness. At least, the preservation of antagonism of AT1 receptor signaling by AT2 receptors, however that may result from DR, is involved, suggesting a shift in AT1 to AT2 receptor ratio. Indeed, in a previous study it was shown that AT1 receptor expression but not AT2 receptor increases in the rat adrenal medulla with age [22]. DR increased AT2 receptor expression, whilst AT1 receptors were only decreased by DR in combination with exercise. AT2 receptor increase therefore seems to be the prominent protective effect of DR, but this needs to be further investigated in relation to vascular function. Strikingly, the AT1 receptor-blocking activity of AT2 receptors in human coronary arteries increased with age, albeit in relatively healthy arteries only [23]. Perhaps this result suggests that AT2 activity indeed contributes to healthy vascular aging in humans, and a comparison with diseased arteries would be of importance to establish this possibility. In addition, effects of DR on RAS components needs to be explored as there is a general lack of knowledge on this topic, with the exception perhaps of the effect of salt intake, which is a classical topic in the field of hypertension research.

Apart from modulation of angiotensin receptors DR can again be related to RAS at the level of mitochondria. As mentioned in Chapter 5 DR has beneficial effects on cardiac dysfunction in RAS-activated animals, and this is associated with mitochondrial improvement [24]. Interestingly, DR and RAS blockade have similar beneficial effects on mitochondria [25], and in Chapter 5 it was argued that some of the DR effects might be due to decreased RAS activity. As alternative explanations, the effects of DR might either be more completely protecting mitochondria, or taking place at a hierarchically more important level, e.g. DR might preserve genomic integrity whereas RAS blockade would fail to do this. This hypothesis warrants the exploration of the effect of DR and of RAS modulation on genomic and mitochondrial integrity in cardiovascular tissue. The possible transgenic models to be used as tools were already mentioned in the previous section.

With respect to clinical development, adaptation of life style might seem more acceptable for the general public than lifelong pharmacotherapy. Adherence to a healthy life style is unfortunately not easy to accomplish in 'developed and developing countries'. However, again with increasing health care costs as motivation, governmental financing for campaigns and improvement of research to battle the problems related to the western diet is increasing. This is for instance reflected in the Dutch Life Science and Health program. As the idea of (re) introduction of more healthy food is becoming increasingly popular, also the food industry is more inclined to investing in such research. An important scientific question related to this topic is what food constituents need to be adapted to improve healthy (vascular) aging. One of the consistent findings is that calorie reduction alone cannot explain the effects of DR on lifespan. As has become clear from studies in invertebrate organisms such as C. elegans and D. melanogaster, and from studies in mice, the ratio of proteins and carbohydrates in food, as established at the macronutrient level importantly affects longevity as well as cardiovascular parameters. Moreover, a high carbohydrate: protein ratio is beneficial independently from calorie intake and even when applying *ad lib* feeding [28]. Although in primates this still has to be established, an intriguing observation in monkeys indicates that a role of macronutrient ratio might apply here as well. In two independent studies the effect

of DR on longevity and age-related pathologies, amongst which cardiovascular, was tested in rhesus monkeys [28, 29]. One study found an effect on longevity, but the other study did not. One of the most likely explanations is that the balance in food constituents was different between the two studies [30]. It would therefore be interesting to include cardiovascular variables, including neurohormonal measurements such as levels of RAS components, in nutritional intervention studies that explore the contribution of specific food constituents. Again, DNA repair-defective mice could be useful in preclinical studies. A threat for such research, however, might be that there are many ratios (and individual constituents if micronutritional levels would be implicated) that should be included, extending beyond the current financial possibilities. But at least public acceptance and funding appears to be growing. Of course, when conducting research on the effect of anti-aging nutritional or related nutriceutical interventions on cardiovascular disease one has always to bear in mind the duality that was presented in the thesis Summary. In other words, these anti-aging nutritional interventions will be acting complementary to pharmacological interventions that reduce the damage inflicted by high salt and high lipid diets, such as treatment with RAS-modulating drugs, antidiuretics, statins etc., which are already applied successfully in the clinic to reduce events caused by vascular aging independently of the effects that such drugs might have on the genomic integrity or the aging process itself.

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Nederlandse samenvatting

SAMENVATTING

Genetische instabiliteit en cardiovasculaire ziekten

Leeftijds-gerelateerde cardiovasculaire ziekten (CVZ) zijn verantwoordelijk voor de meerderheid van de sterfte, en een voorname oorzaak van invaliditeit en fragiliteit in mensen uit ontwikkelde landen. Er is veel onderzoek gedaan om te zien hoe ouderdom invloed heeft op de cardiovasculaire structuur en functie, en waarom als gevolg daarvan CVZ ontstaan. Het is nog steeds een flinke taak om de bijdrage te ontrafelen van alle factoren die ofwel variëren met leeftijd, ofwel niet *per se* gerelateerd zijn aan leeftijd, zoals genetische aanleg. In dit proefschrift zijn CVZ benaderd op basis van een dualiteit in factoren die leeftijds-gerelateerde disfunctie beïnvloeden, namelijk de bijdrage van het verouderingsproces dat veroorzaakt wordt door genetische instabiliteit, een voorname oorzaak van biologische veroudering, versus de bijdrage van disregulatie van homeostase die naarmate de tijd vordert de bloedvaten steeds verder aantast, hetgeen hier chronologische veroudering wordt genoemd. In het laatste geval ligt in dit proefschrift de focus op RAS activatie en genetische variabiliteit in de signaalfunctie van bradykinine.

De voorgestelde dualiteit is een vereenvoudiging van het labyrint van processen die een rol spelen in vasculaire veroudering. Deze vereenvoudiging is echter een hulpmiddel dat al geholpen heeft bij de ontdekking van nieuwe mechanismen in de pathogenese van CVZ. **Hoofdstuk 1** biedt een samenvatting van recente ontdekkingen die tot stand zijn gekomen uit pionierswerk dat gebaseerd was op de aanname dat genetische instabiliteit een causatieve factor is in de pathogenese van leeftijds-gerelateerde CVZ. In parallel hiermee vermijdt dit hoofdstuk ook een té beperkte visie door te stellen dat de componenten van elk onderdeel in dit bimodale model op elkaar inspelen; er is geen strikte tweedeling. Momenteel staan we aan het begin van ontdekkingen met betrekking tot hoe deze interacties plaatsvinden en wat de gevolgen daarvan zijn. Dit proefschrift gaat in op enkele vragen die resulteerden in meer kennis en inzicht in dit onderwerp, wat hieronder is samengevat.

Hoewel veel kennis over de rol van genetische instabiliteit in CVZ nog buiten ons gezichtsveld ligt, lijkt de toepassing van kennis die opgedaan wordt in het onderzoek naar klassieke paradigma's steeds ingewikkelder te worden. Twee hoofdstukken in dit proefschrift gaan over dit probleem, waarbij de nadruk ligt op de voorspelling van de response van individuele patiënten met leeftijds-gerelateerde CVZ op farmacotherapie gebaseerd op RAS modulatie.

In **hoofdstuk 2** is ingegaan op de vraag of meting van de ACE activiteit en/of renine concentratie voor aanvang van farmacologische interventie gericht op een reductie van Ang II productie en AT1 receptor-gemedieerde signaalfunctie een voorspelling kon geven van de acute en chronische anti-hypertensieve respons bij individuele patiënten. Over het algemeen associeert renine activiteit beter met deze respons dan de ACE activiteit. Geen van de metingen gaven echter patiënt-specifieke voorspellingen, omdat de individuele variatie van de waarden gewoonweg te hoog is. Ondanks eerdere epidemiologische studies die de voorspellende waarde van renine of ACE ondersteunen, is heeft het nauwelijks of zelfs geen waarde wanneer dit teruggebracht wordt tot de individuele patiënt, zelfs in een

erg homogene populatie. De voornaamste reden hiervoor is dat er meerdere bijkomende factoren zijn die invloed hebben op deze parameters, waaronder geslacht, etniciteit, zoutinname, anti-hypertensieve medicatie en leeftijd. Als gevolg daarvan is het onmogelijk om tot een uniforme grenswaarde te komen die algemeen toegepast kan worden op alle, individuele hypertensiepatiënten.

De studies in **hoofdstuk 3** gaan in op de vraag wat het mechanisme kan zijn dat de bijdrage van bradykinine (BK) B1 receptor rs12050217 polymorfisme aan de response op therapie met ACE remmers in patiënten met coronaire arterie ziekte (CAZ). De resultaten wijzen erop dat de variabiliteit in coronaire vasomotor respons op de B1 receptor agonist DABK geassocieerd is met het genotype, en niet de inflammatoire respons, die waarschijnlijk meer samenhangt met geslachtsverschillen. In theorie leidt een toename van BK na inname van ACE remmers tot een beter relaxerend effect in patiënten met het AA genotype omdat zij gevoeliger zijn voor stimulatie van B1 receptoren. De B1 receptor eiwit niveau's verschilden niet tussen de genotypen en daardoor waren we niet in staat een logische verklaring te vinden voor de verbeterde respons op DABK.

Het tweede deel van het proefschrift concentreert zich op het effect van genetische instabiliteit op de vasomotorische respons op Ang II en het effect van de behandeling op de blokkade van de AT1 receptor. Daarnaast is het effect bekeken van een bestaande anti-verouderingstherapie, dieet restrictie, op vasomotorische disfunctie veroorzaakt door genetische instabiliteit.

Excessieve Ang II signaalfunctie staat in verband met leeftijds-gerelateerde cardiovasculaire ziekten. Blokkade van deze excessieve signaalfunctie wordt momenteel toegepast als klinische interventie strategie bij zulke ziekten. De relatie met genetische instabiliteit is onbekend. In **hoofdstuk 4** is ingegaan op de vraag of genetische instabiliteit de respons op Ang II en AT1 receptor blokkade zou veranderen. Ercc1$^{d/-}$ muizen laten een toegenomen vaatvernauwende reactie op Ang II zien in de arteria iliaca. Deze verhoogde respons lijkt in ieder geval deels afhankelijk te zijn van een verlies van AT2 receptor counterregulatie. Verlies van NO-cGMP signaalfunctie, wat ook voorkomt door veranderingen in zowel endotheel als gladde spiercellen, lijkt geen belangrijke rol te spelen in de verbeterde reactie op Ang II. Chronische behandeling met AT1 receptor antagonist losartan voorkwam de toename van Ang II-geïnduceerde vaatvernauwing, maar verbeterde de NO-cGMP signaalfunctie niet. Blijkbaar zijn zowel genetische instabiliteit als de AT1 receptor signaalfunctie betrokken bij de aanhoudende verbetering van Ang II-geïnduceerde vaatvernauwing. Aangezien losartan het versnelde verlies van NO-cGMP-gemedieerde vasodilatatie in Ercc1$^{d/-}$ muizen niet kon voorkomen, dragen door Ang II gestimuleerde vorming van vrije zuurstof radicalen blijkbaar niet bij aan endotheel disfunctie noch aan vasculaire genetische instabiliteit bij Ercc1$^{d/-}$ muizen. Dit zou te verwachten zijn gezien het feit dat reparatie van oxidatieve DNA beschadigingen door vrije radicalen grotendeels intact is bij deze dieren.

Door de resultaten in hoofdstuk 4 zou men zich kunnen afvragen of vasculaire veroudering als gevolg van genetische instabiliteit al met al voorkomen kan worden. In **hoofdstuk 5** verkenden we of dieetrestrictie (van 10% toenemend tot 30% reductie van de geschatte *ad*

libitum inname) vasomotoriche veranderingen in Ercc1$^{d/-}$ muizen kunnen voorkomen. Deze muizen toonden een afgenomen endotheel-afhankelijke en -onafhankelijke reactie op de NO-cGMP signaalfunctie. Deze veranderingen werden voorkomen door een dieetrestrictie gedurende 9 weken. De Ercc1$^{d/-}$ muizen met dieetrestrictie leefden tenminste tot een leeftijd van 30 weken, een leeftijd waarop alle *ad lib*-gevoerde Ercc1$^{d/-}$ muizen reeds overleden waren. Bovendien was het beschermend effect op de vaatverwijdende functie nog steeds aanwezig in de muizen met dieetrestrictie. Een toenemende prostaglandine/cAMP signaalfunctie was hoogstwaarschijnlijk het mechanisme dat zorgde voor verbetering van de vaatverwijdende respons, zoals bleek uit de blokkerende effecten van de cyclo-oxygenase (COX) blokker indomethacine en de veranderingen in COX-1 en PDE4B expressie, gemeten in de long. Bovendien werd de verhoogde vaatvernauwende reactie op Ang II, die eerder gezien werd in hoofdstuk 4, ook voorkomen met dieetrestrictie. Daarom is dieetrestrictie een erg effectieve en veelzijdige interventie bij leeftijds-gerelateerde vasculaire problemen.

Curriculum Vitae

Haiyan Wu （吴海燕） was born on June 15th 1985 in Guizhou, China. In the summer of 2007 she received her Bachelor of Medicine degree at Sichuan University, Chengdu. After receiving the second prize for her high score on the entrance exams, she entered in 2007 as an Excellent Graduate Student to study pharmacology, and obtained her Master of Science degree in 2010. Her master research project was focused on antibiotics. Thereafter, she was awarded with a national grant, the “China Scholarship Council Grant”, and she came to the Netherlands in the same year to start her work as a PhD candidate at the department of Pharmacology at the Erasmus MC Rotterdam. Under the supervision of dr. A.J.M. Roks and prof. dr. A.H.J. Danser she performed her research on novel approaches to develop diagnostical tools for and develop against age-related vascular disease.

Publications

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PhD Portfolio

Novel Direction In Therapy Against Age-related Vascular Disease

H.Wu

Erasmus MC Department:	Internal Medicine, Division of Pharmacology and Vascular Medicine
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotor:	Prof. dr. A. H. J. Danser
Co-Promotor:	Dr. A. J. M. Roks
PhD period:	2010-2014

PhD training	**Year**
Academic skill (22 ECTS)	
Principles of laboratory animal course	2012
English Biomedical Writing and Communication	2012
NHS course vascular biology	2011
NHS course Thrombosis and Hemostatic	2012
COEUR Courses	2010-2014
Conferences (12.5 ECTS)	
Second Benelux Congress on Physiology and Pharmacology, Maastricht**	2014
Angiotensin Gordon research Conference, Lucca (Barga),Italy**	2014
23th European Society of Hypertension Meeting, Milan, Italy **	2013
Wetenschapsdagen Interne Geneeskunde, Antwerpen, België*	2011-2014
FIGON Dutch Medicines Days, Lunteren, The Netherlands**	2012-2013
Seminars and Symposia (4.8 ECTS)	
Current Cardiac and Vascular Aging Research at EMC	2014
Angiotensin Gordon research Conference, Lucca (Barga),Italy	2014
COEUR seminars	2010-2014
Dutch Pharmacology Spring Meetings	2012-2013

***** Poster presentation, ****** Oral presentation

Research seminar march Focus on Aging	2012
Research seminar April Healthy Aging	2011
Research seminar Heart valve implantation	2012
Research seminar Identification of novel regulators of vessels formation	2011
COEUR PhD days	2011-2014

Lectures (0.7 ECTS)

Erasmus MC/COEUR Lectures	2011-2014

Teaching (2.4 ECTS)

Supervise practicum Pharmacological control of the autonomous nervous system	2012-2013

Prizes and grants

Dutch NVF Travel Grant	2014
Trustfonds Travel Grant	2014
Accommodation Grant, 23th European Society of Hypertension Meeting	2013
China Scholarship Council Grant, Graduate Student overseas study program	2010-2014
Excellent Graduate Student, Sichuan University	2008-2009
Second Prize of Scholarship, Sichuan University	2007-2010
Second Prize of Scholarship, Sichuan University	2004

ACKNOWLEDGEMNTS

Four years have flown by!

First and foremost, I would like to thank my research supervisor Anton Roks. I was so lucky to have had you as my boss: so kind and gentle, and always so patient to guide and teach me. Especially in the first year, when I got lost in the complications that scientific research entails, your generosity and enthusiasm led me out of my feelings of desperation. I particularly appreciate your help that was necessary to develop the understanding and confidence needed to perform science. Thank you for your encouragements that stimulated me to think critically about my own research. I particularly enjoyed our discussions that we regularly had in your office regarding the data. Not only because you inspired me during these occasions, but also because I am very much enjoyed your homemade coffee prepared from freshly ground Doppio's beans, for which you used your dear grandmother's antique coffee mill. I admire your exceptional memory for almost aspect of the research background and your ability to notice even the tiniest error present in my data just from a glance at the graphs. You always helped me so much, thank you!

Jan Danser, as my promoter, thank you for all the guidance you gave me in these years. Without your assistance and dedicated involvement in every step throughout the process, this thesis would have never been accomplished so efficiently. I would like to thank you very much for your support and understanding over these past four years. I would like to thank you for your trust and for giving me the opportunity to do my PhD training at your department. Thanks to you I learned how to work more systematically, and each discussion with you brought me back on the right track when needed.

I would also like to show gratitude to the reading committee, prof. dr. Jan Hoeijmakers, prof. dr. Eric Sijbrands and prof. dr. Rob Henning. Thank you for all your efforts and the priceless feedback that helped me to improve my thesis. I feel very honoured to have you in the defence committee.

In the remaining part of this acknowledgements section I would like to thank my colleagues from the department.

Hisko Oeseburg, thanks to your strict teaching I learned to operate various molecular and cell culturing tools, I learned from you to always write down what I have done in my logbook at any time that it is necessary!

Matej Durik, with your help, I could start to do the mulvany experiments as soon as possible, and your detailed considerations in the lab made a deep impression on me. Your optimistic attitude towards life taught me how to face the problems which I would meet in everyday' s life.

Kayi, thank you for taking care of when I started my work in our department. Your encouragements and comforting gave me confidence. You helped me a lot, especially, but not exclusively, about how to adapt my way of living to the new circumstances that at the time were so foreign to me. I deeply appreciate what you did for me.

Bruno Seva Pessoa, thank you for your helpfulness and discussion about the mice, and our discussions about our homelands.

Xifeng, thanks for you guidance for western blot and cell culturing experiments, which helped me a lot.

Joep van Esch, t-shirt-man of our lab, you deeply impressed me with your work and the

questions you asked, and during my lab presentations also gave me lot of inspiration. Beside that you taught me many things about how to design the protocol that helped me saving quite a lot of time.

Wendy Batenburg, I learned a lot from your experience with previous experiments and data, which give me enormously helpful clues for the design of my experiment protocol. Thank you for your care when I was be invited to give an oral presentation at the Gordon Conference in Italy; your 'smiling face painting' took away much of my nervousness.

René de Vries, thanks for helping me so many times with the mouse and vascular experiments. One can always trust the data you generate!

Richard van Veghel, with your patient help when performing the tail cuff and laser Doppler experiments I learned how to measure these valuable variables in the mice. You are a such kind and joyful colleague. I wish you all fun with your family life, which I know you cherish!

Frank Leijten, I learned from you how to do qPCR. Thanks to your help nice and important data were generated!

Usha Bhaggoe, thank you so much for generating important western blot data.

Ingrid Garrelds, thank you for your guidance regarding the collection of samples for angiotensine and renin measurements.

Lodi Roksnoer, I particularly enjoyed having you as my travel companion at the Gordon Conference, and I will always remember our surprise when experiencing the generosity of Munich airport….

Khatera Ibrahimi, thank you for our conversations, which made me feel so good because you were always so positive to life and you have such a nice sense of humour.

Stephanie Lankhorst, I will always remember the nice time we had in the Papendal course, and our discussions about how to avoid boredom during long writing periods, especially when composing the thesis.

Paula Bautista Nino, you were my roommate at the Physphar conference in Maastricht, we had an amazing dinner in an Italian restaurant and shared many interesting stories and you told me many things about your homeland Colombia. I wish that someday in the future I can visit you there.

Sieneke Labruijere, Bibi van Thiel, Luuk te Riet, Arthur Moes, Mahdi Salih, Koen Verdonk, Yanto Ridwan…Thank you for also being my great colleague who made my working time full of joy!

谨将我最深切最真挚的感谢，献给我亲爱的父亲母亲，如果没有你们一如既往的无私的爱的支持，我不可能将这四年的旅程痛并快乐的享受下来。自幼时起，是你们的谆谆教导，让我懂得如果要实现自己的理想必得要珍惜自己的时间，点点滴滴中懂得了，不经一番寒彻骨，哪得梅花扑鼻香？你们的耐心教导，让我学会了去享受自己的生活，无论是苦与甜，我都甘之如饴。正是由于你们的苦心培养，在成长的道路上，我才不致因软弱而放弃，不致因懦弱而逃避，若说今日我能有一星半点成就，皆是父母心血。

感谢我的爱人，一波，谢谢你对我的感情上的疼惜和性格上的包容。这一路上，相扶相携，十四年的岁月中，你是我的同学，朋友，兄长，爱人，谢谢你的倾听，支持，鼓励，教导，你不仅是我情感上的港湾，也是我成长道路上至为难得的良师益友。谢谢有你的陪伴，这一路的风景才格外的美丽，希望我们能一起翻开生活的新的一页，共同书写将来的生活。

感谢我的硕士导师，刘小康老师，正是在您的教导和鼓励下，我才能从一个站在科研大门之前的懵懵懂懂的小孩子渐渐明白科研的真正意义是什么。是您，帮我揭开了那一层朦胧的科研面纱，让我意识到作为一个科研人员所需的素质和素养，也让我明白了，想要真正了解科学的魅力必须要有严谨的科研思维，不能只作为一个匠人而是要去成为一个project的设计师和艺术家。

谢谢在荷兰的四年里，给予我学习上的帮助和生活中快乐的小伙伴们：倩华姐，谢谢你对我的生活上的帮助和工作上的指导，你的为人处世对我的影响极深；宝月，记得在海边挖生蚝，在雨中烧烤，那段经历何等有趣？！魁魁，你是我最佳午餐搭档，我们互相吐槽的时刻是我最放松的时刻，很难忘那段一起在巴黎的旅程，希望将来还能和你一起游荡在某一个城市，无拘无束。稚超兄，谢谢你的热心和实验上的指导，我从你身上学到很多思辨的方法；莹莹，林肯，舟桥，世豪，　栾盈…和你们的趣事太多太多没办法一一说完，但我想说的是，有缘相识于荷兰，有幸共享一段人生的时光，我很感谢你们在我四年的荷兰生活里，画下了一道道绚丽多彩的痕迹，在我将来的岁月里，我必定会不断想念你们，想念这段温柔而短暂的经历。