Aortic Pathology and the Role of the Renin-Angiotensin System

Els Moltzer

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Moltzer, E.
Aortic Pathology and the Role of the Renin-Angiotensin Sytem
ISBN: 978-90-6464-456-6

Cover: Heart X-ray (www.dreamstime.com)
Lay-out and cover design: Maaike Sluijter and Pim Moltzer
Lay-out: Els Moltzer
Printed by: GVO drukkers en vormgevers b.v., Ponsen en Looijen

Aortic Pathology and the Role of the Renin-Angiotensin System

Aorta pathologie en de

rol van het renine-angiotensine systeem

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam

op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 23 maart 2011 om 11.30 uur
door

Els Moltzer

geboren te Rotterdam

Promotiecommissie:

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Financial support for the publication of this thesis was generously provided by:

Stichting Lijf en Leven

Erasmus University Rotterdam

J.E. Jurriaanse Stichting

Heart Medical Europe BV

Sanovi-Aventis Netherlands BV

AstraZenica BV

Novartis Pharma BV

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Voor mijn familie

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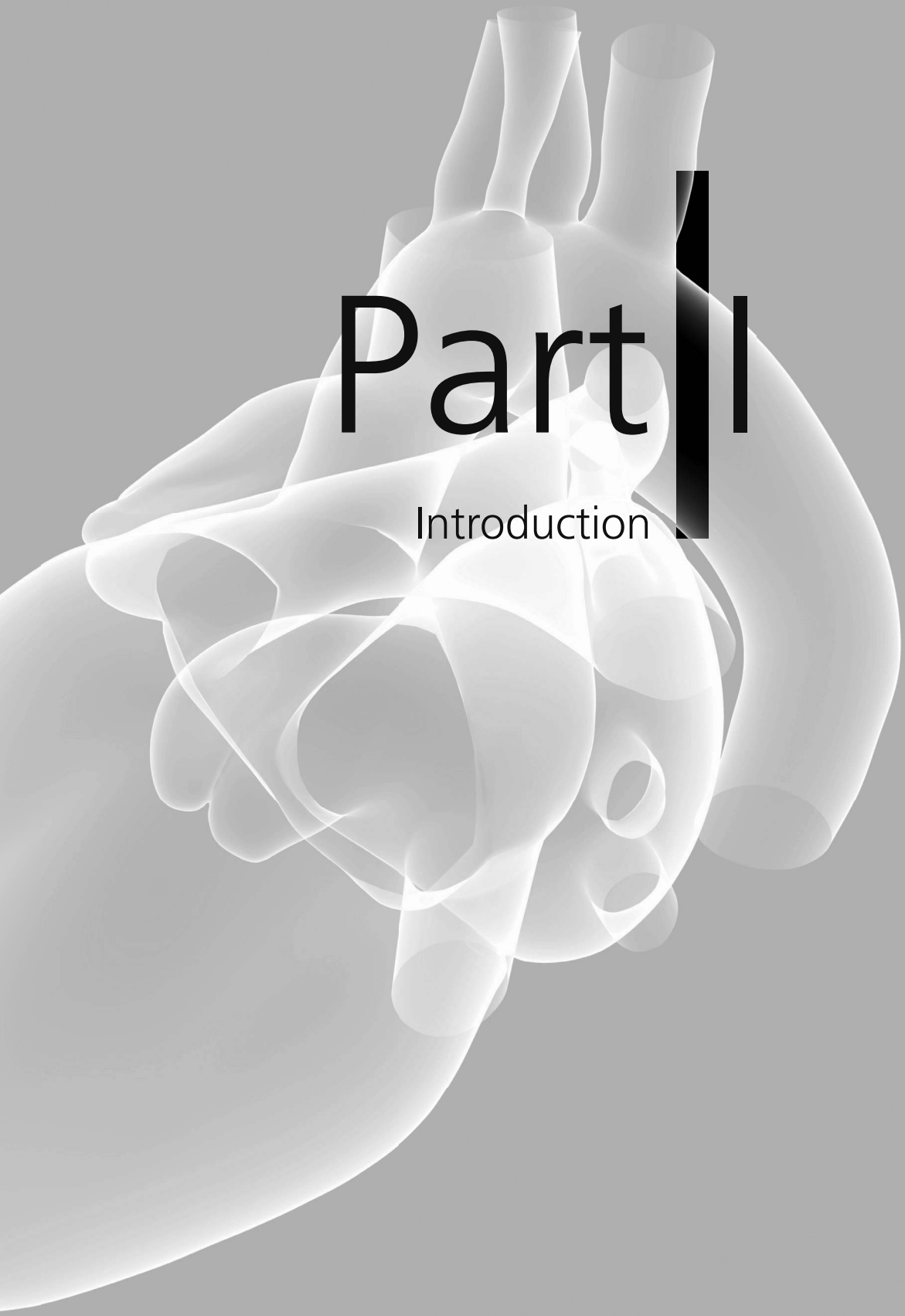

Part I

Introduction

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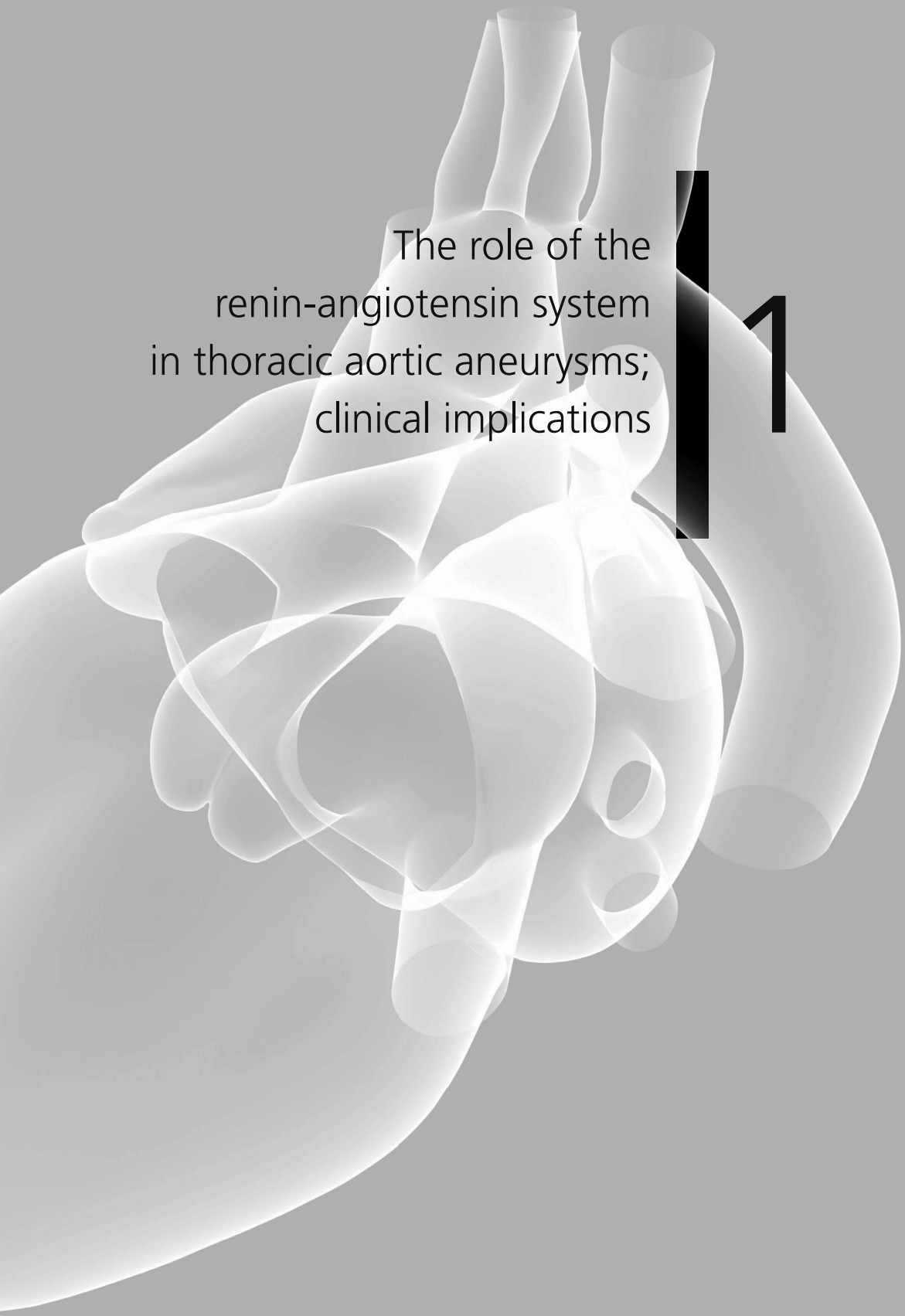

1 The role of the renin-angiotensin system in thoracic aortic aneurysms; clinical implications

1

ABSTRACT

Thoracic aortic aneurysms (TAAs) are a potential life-threatening disease with limited pharmacological treatment options. Current treatment options are aimed at lowering aortic hemodynamic stress, predominantly with beta-blockers. Increasing evidence supports a role for the renin-angiotensin system (RAS) in aneurysm development. RAS blockade would not only lower blood pressure, but might also target the molecular pathways involved in aneurysm formation, in particular the transforming growth factor-beta (TGF-β)- and extracellular signal-regulated kinase (ERK) 1/2 pathways. Indeed, the angiotensin II type 1 (AT_1) receptor blocker losartan was effective in lowering aortic root growth in mice and patients with Marfan syndrome. RAS inhibition, which is currently possible at three levels (renin, ACE and the AT_1 receptor), is always accompanied by a rise in renin due to interference with the negative feedback loop between renin and angiotensin II. Only during AT_1 receptor blockade will this result in stimulation of the non-blocked angiotensin II type 2 (AT_2) receptor. This review summarizes the clinical aspects of TAAs, provides an overview of the current mouse models for TAAs, and focuses on the RAS as a new target for TAA treatment, discussing in particular the possibility that AT_2 receptor stimulation might be crucial in this regard. If true, this would imply that AT_1 receptor blockers (and not ACE inhibitors or renin inhibitors) should be the preferred treatment option for TAAs.

INTRODUCTION

True aneurysms are defined as a local increase in diameter of over 50 percent of the normal diameter, including all three vessel layers.[1] Arterial aneurysms are most commonly located in the abdominal aorta. Thoracic aortic aneurysms (TAAs) are less common and occasionally appear as a pseudo-aneurysm or dissection, creating a false lumen between the aortic intima and media. Aneurysms are usually silent and unmasked by imaging; therefore, the true incidence of aortic aneurysms is hard to estimate. On the contrary, aortic dissections often present with symptoms, such as chest, back or flank pain. In 2007, the Centers for Disease Control and Prevention ranked aortic aneurysms as the 19th common cause of death in the USA.[2] Since many aortic aneurysms are silent, the true incidence for aortic disease is probably much higher. A large epidemiological project in a stable population shows that the true incidence of TAAs is increasing over time, even when correcting for the increase in imaging modalities.[3] Abdominal aortic aneurysms (AAAs) and TAAs share some important risk factors, such as increased age and hypertension, and pathological changes, including the loss of vascular smooth muscle cells. However, the pathophysiology underlying AAAs and TAAs differs to a large extent. AAAs are very uncommon under the age of 60, and their underlying cause is uncertain in most patients. On the contrary, in patients with an aneurysm in the ascending thoracic aorta, a genetic influence in an autosomal dominant pattern is often found.

There are substantial data providing evidence that the RAS is involved in the pathogenesis of AAAs. Infusion of angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system (RAS), in atherosclerotic apolipoprotein E and LDL receptor knockout mice provides an experimental model for AAAs and thereby implicates that the RAS could have a pivotal role in AAAs. This topic has been extensively reviewed by the group of Cassis and Daugherty.[4-7] In humans, few studies were able to genetically relate RAS to AAA disease. According to a meta-analysis, the D allele of the angiotensin-converting enzyme (ACE) represented a susceptibility factor for AAA.[8] In addition, the Ang II type 1 (AT_1) receptor 1166C polymorphism is associated with AAA in three case-control cohorts.[9] These two polymorphisms result, respectively, in increased ACE[10] and AT_1 receptor expression,[11] in agreement with a recent study in human AAA segments, where many RAS components were found to be more strongly expressed when compared to healthy controls.[12]

Limited data exist regarding the role of the RAS in TAAs. In this review, we will address this topic and focus on the basic science and subsequently discuss the clinical implications and perspectives.

THORACIC AORTIC ANEURYSMS

TAAs most often result from cystic medial degeneration, histologically seen as smooth muscle cell dropout and elastic fiber degeneration.[13] Cystic media degeneration occurs to some degree with aging and increases with hypertension. TAAs and thoracic aortic dissections are frequently familial diseases with an inherited pattern present in over 20% of the probands. The predominant inheritance pattern is autosomal dominant in more than three-quarters of the cases, with varying degrees of penetrance and expression, and a relatively early age of

onset.[14] TAAs can clinically be classified into syndromic, familial non-syndromic, or sporadic aneurysms.[13, 15] Table 1 provides an overview of the known mutations underlying syndromic and familial non-syndromic TAAs.

Marfan syndrome (MFS) is one of the most common hereditable connective tissue disorders, caused by a mutation in the fibrillin-1 gene.[16] Currently a large number of mutations have been identified, spread throughout the gene without signs of clustering.[17] Affected organs include the skeletal, ocular, pulmonary and cardiovascular system. The latter is featured by a severe dilation of the ascending aorta.

Loeys-Dietz syndrome shows some similarities with MFS, but also has other features and is caused by mutations in the transforming growth factor-beta (TGF-β) receptor type 1 and 2. Cardiovascular characteristics include arterial tortuosity and ascending aortic aneurysm and dissection.[18, 19]

Ehlers-Danlos syndrome type IV, the vascular type of Ehlers-Danlos syndromes, is caused by mutations in the COL3A1 gene. A mutation results in a deficiency in synthesis, secretion and structure of procollagen type III, making patients prone to aortic dissection and rupture.[20]

Cutis Laxa Type 1 is caused by mutations in a gene encoding fibulin-4, also results in arterial tortuosity and ascending aortic aneurysm and dissection.[21]

Turner syndrome is caused by the (partly) loss of an X-chromosome and results in a short stature of the affected women. Patients have an increased risk of cardiovascular disease, including a bicuspid aortic valve and aortic dilation and dissection.[22] In these patients, routine monitoring of the aorta is necessary and the aortic diameter should be corrected to body-surface area, to take into consideration the short stature.

Bicuspid aortic valves are the most frequent congenital anomaly of the heart and associated to TAAs, independent of hemodynamic differences, age or body size.[23, 24]

TREATMENT OF THORACIC AORTIC ANEURYSMS

Surgical and endovascular aortic repair

TAAs are a potential life-threatening disease and need close monitoring. Surgical therapy is often recommended profylactically to prevent aortic rupture and is usually performed with the interposition of a Dacron graft. The exact time to perform surgery is uncertain and also depends on the patient's condition. In general, a diameter ≥5.5 cm is considered for surgery. However, patients with a genetic disorder are more prone to aortic dissection or rupture and should be operated earlier.[25] Although mortality rates have improved over time, surgical repair of aortic aneurysms is still considered as a high risk-procedure with one-year mortality rates between 15-30%.[26-28] Perioperatively, vascular control is essential to maintain blood flow through the organs. Complications when flow is inappropriate include cerebral damage, renal failure and neurological damage like paraparesis/paraplegia.[28]

Table 1. Classification of TAAs, sporadic aneurysms are not listed in this table.

Disease	Gene	Chromosome	Reference
Syndromic			
Marfan Syndrome	*FBN1*	15q21.1	16
Marfan-like Syndrome	*TGFBR2*	3p24.2-p25	111
Loeys-Dietz Syndrome	*TGFBR1*	9q33-34	18, 19
	TGFBR2	3p24-25	18, 19
Ehlers-Danlos Syndrome type IV	*COL3A1*	2q24.3-31	112
Cutis Laxa Type 1	*Fibulin-4*	11q13	46
Arterial Tortuosity Syndrome	*SLC2A10*	20q13.1	113
Noonan Syndrome	*PTPN1*	12q24.1	114
Polycystic Kidney Disease 1	*PKD1*	16p13.3-p13.12	115
Turner Syndrome	Unknown	45, X0	116
Familial Non-Syndromic			
TAA + BAV	*NOTCH1*	9q34-35	117, 118
FAA1	Unknown	11q23.3-24	119
TAAD1	Unknown	5q13-q14	120
TAAD2	*TGFBR2*	3p24-25	121, 122
TAAD3	Unknown	15q24-26	123
TAAD4	*ACTA2*	10q22-q24	124
TAAD + PDA	*MYH11*	16p12.2-p13.13	125, 126

BAV, bicuspid aortic valve; PDA, patent ductus arteriosus; TAA, thoracic aortic aneurysm; TAAD, thoracic aortic aneurysm dissection.

For the treatment of descending TAAs, thoracic endovascular aneurysm repair became available in 2005. This technique, which is primarily used for relatively high risk-patients who can not tolerate open surgery, results in fewer complications,[29] but is still not suitable for the treatment of aneurysms located in the ascending thoracic aorta or aortic arch. Despite the fact that open surgery and endovascular aortic repair seem to be the only definite solution, asymptomatic patients may initially be treated medically to reduce aortic growth.

Historical medical management

Present evidence for drug therapy to regress or prevent TAAs is based upon studies performed in MFS patients. The first open labeled randomized trial was published in 1994, including 70 MFS patients between 12 and 50 years old.[30] Patients were randomly assigned to no treatment (the control group), or treatment with the beta-adrenergic blocker propranolol. After four years of follow-up, the propranolol-treated group had a 73% lower rate of aortic dilation and a lower mortality rate. The mechanical consequences of beta-blockade were hypothesized to underlie the beneficial effects. A lower blood pressure and left ventricular contractility together result in a reduction of hemodynamic stress on the aorta. In a retrospective study including two centers, a reduction in aortic root growth was observed in the beta-blocker-treated group as well.[31] In the years thereafter, a limited number of studies were conducted,

with contradictory results.[32-34] All studies were relatively small with virtually no long-term outcome data. A meta-analysis subsequently showed no evidence that beta-blockade has clinical benefit in patients with MFS.[35] Nevertheless, beta-blockade is currently standard clinical practice and recommended by the guidelines to treat aortic dilation in MFS patients.[25, 36]

Concurrently, other antihypertensive agents became subject of investigation. In one study, patients intolerant for beta-blockers were given the calcium antagonist verapamil.[32] The authors state that calcium antagonist therapy retards aortic root growth. However, this conclusion might be preliminary, since no sub-analysis was performed on the verapamil-treated patients and the patient number was very low (n=5). In a mouse model for MFS, preliminary experiments provided even arguments against the use of calcium antagonists, since treatment with amlodipine accelerated aortic root dilation and resulted in early mortality. Thus, calcium antagonists should be used with caution in MFS patients.[37]

Limited data are available for the medical treatment of TAAs in non-MFS patients. It is unknown whether results obtained from studies performed on MFS patients can be extrapolated to other TAAs. The ACC/AHA 2008 guidelines for valvular disease state that it is reasonable to give beta-blockers to patients with bicuspid aortic valves and dilated aortic roots (diameter >4.0 cm) whom are not candidates for surgical correction and whom do not have moderate to severe aortic regurgitation.[38] However, this is based on expert consensus only. In patients with vascular Ehlers-Danlos syndrome, the preventive effects of beta-blocker celiprolol, a selective β_1-adrenergic receptor blocker that simultaneously has β_2-adrenergic receptor agonistic effects, was studied. It was hypothesised that celiprolol could decrease vascular complications in Ehlers-Danlos syndrome by reducing heart rate and pulsatile pressure (through inhibition of the β_1-receptor) and by causing vasodilation (via stimulation of the β_2-receptor). Compared to patients that received no treatment, celiprolol induced a three-fold reduction in arterial events such as rupture or dissection and the trial was stopped early because of treatment benefit.[39]

Besides the fact that surgical and endovascular repair are life-saving in TAA patients and that pharmacological treatment is used to regress aortic growth, current treatment possibilities are far from optimal, with high morbidity and mortality rates in TAA patients. TAA mouse models are an attractive tool to study alternative pharmacological interventions in order to prevent aortic growth and rupture. The role of ACE inhibitors and AT_1 receptor blockers is based upon experimental studies in such models, and will be discussed later in this review.

MOUSE MODELS FOR THORACIC AORTIC ANEURYSMS

Mouse models that mimic human TAAs facilitate more in-depth analyses, ranging from pathology to molecular biology (i.e. RNA or protein expression analysis) and pharmacology (i.e. *ex vivo* vascular reactivity). Moreover, mouse models, if truly mimicking the human situation, allow the development of tools for disease monitoring and the investigation of effective alternative therapeutic approaches. To date, several mouse TAA models have been constructed that display many similarities with human TAAs.

Marfan syndrome

The vascular phenotype of MFS results from mutations in fibrillin-1, the major constituent of extracellular microfibrils. Microfibrils associated with elastin provide elasticity to the extracellular vascular network of the ascending aorta. Several mouse models have been developed based on mutations of the fibrillin-1 gene. Initially, mice with reduced expression of fibrillin-1 were generated. The first model was a deletion mutant in which exons 19-24 were replaced by a neomycin resistance gene. This gave rise to a mutant allele (mgΔ) and, through transcriptional interference caused by introduction of the neomycine resistence gene, resulted in a 10-fold reduced fibrillin-1 mRNA expression level from the targeted allele lacking exons 19-24.[40] Expression of both 10-fold reduced mutant transcript and wild type fibrillin-1 transcript in the heterozygous mgΔ/+ mice did not lead to vascular abnormalities. Homozygous deletion mutants (mgΔ/mgΔ), however, died within three weeks after birth due to cardiovascular complications such as haemothorax, haemopericardium or pulmonary haemorrhage, but interestingly these mice showed no fragmentation of elastic fibers. This led to the conclusion that fibrillin-1 was critical for vascular tissue homeostasis and proper composition of microfibrils rather than for proper elastogenesis. The premature death of these mice precluded further study of the pathophysiology of aneurysm development.

The broader pathogenic sequence for aneurysm formation including the hallmark of MFS, dissecting aortic aneurysm, was subsequently revealed in a mouse model underexpressing the wild type fibrillin-1 protein. Serendipitous aberrant gene targeting of the construct used to generate the mgΔ allele led to the hypomorphic fibrillin-1 allele (mgR), in which R stands for reduced expression of the wild type fibrillin-1 protein.[41] Mice homozygous for the mgR mutation (mgR/mgR) had a roughly a 5-fold reduction of fibrillin-1 mRNA and showed severe vascular defects, including medial calcification, inflammation and, importantly, fragmentation of elastic laminae. This model showed that fibrillin-1 not only affects tissue homeostasis, but that it can also compromise the structural integrity of the aortic wall, if insufficient levels of fibrillin-1 are produced, leading to aneurysms and dissections.

More recently, another approach was used to generate MFS mice. An important aspect of MFS is its dominant negative mode of inheritance, i.e. the presumably dominant-negative interference imposed by the protein derived from the mutant allele over the wild type protein.[42] This view was challenged through the generation of transgenic animals overexpressing the human fibrillin-1 protein with a cysteine substitution in an epidermal growth factor-like domain of fibrillin-1 at position 1663 (C1663R). This is the most common class of mutation in MFS. Although this human mutant protein was integrated in the microfibrils and the extracellular vascular network, such transgenic animals did not display any abnormalities.[42] Yet, mice heterozygous for a comparable missense mutation (C1039G) in the mouse fibrillin-1 gene did display vascular complications. This mutation also concerned a cysteine substitution in an epidermal growth factor-like domain of fibrillin-1. Cells heterozygous or homozygous for the C1039R fibrillin-1 mutation consistently showed histologic evidence for reduced deposition of microfibrils, and aortas of $Fbn1^{C1039G/+}$ mice appeared normal until two months of age, when elastic fiber fragmentation and loss of vascular smooth muscle cells, together with an increase of aortic wall thickness, were noted. The combined results of the healthy transgenic mice overexpressing the C1663R mutation and the aneurysmal $Fbn1^{C1039G/+}$ mice show that not the presence of the mutant protein, but rather the decreased levels of wild type fibrillin-1

results in MFS. These results challenge the dominant negative interference model, and show that also haploinsufficiency of fibrillin-1 might lead to MFS. In contrast to MFS patients, who suffer from a marked shortened life span,[43] Fbn1$^{C1039G/+}$ mice have a normal lifespan and do not die prematurely from aortic dissection.[42]

Fibulin-4 mutations

Besides the different mouse models mimicking MFS, other mouse models have been developed to study syndromic, non-MFS, TAAs, such as the fibulin-4 mouse models. Fibulin-4 is one of the seven-member family of extracellular matrix proteins that plays a role in elastic fiber assembly and function.[44] The earliest reports of mutations in fibulin-4 concern infants diagnosed with cutis laxa syndrome type 1 and describe vascular abnormalities including ascending TAAs and tortuosity of the descending thoracic aorta.[45-47] In a cohort of patients with arterial tortuosity, stenosis and aneurysms, three more mutations in fibulin-4 were identified.[21] The role of fibulin-4 in TAAs was discovered in a mouse model lacking fibulin-4 (Fibulin-4$^{-/-}$).[48] Fibulin-4$^{-/-}$ mice show arterial tortuosity, irregularity and aneurysms, and die perinatally due to aortic rupture precluding further analysis of the pathogenesis of aneurysm development in mature animals. A viable mouse model showing marked vascular abnormalities and allows studying the fibulin-4 deficiency was generated by reducing the expression of wild type fibulin-4 through transcriptional interference by placement of a neomycin selectable marker in opposite direction of the fibulin-4 coding sequence. A systemic 2-fold decreased expression (Fibulin-4$^{+/R}$) sporadically results in small arterial aneurysms, while a 4-fold reduced expression (Fibulin-4$^{R/R}$) results in severe aneurysm formation in the ascending thoracic aorta and arterial tortuosity and elongation of the descending thoracic aorta in all Fibulin-4$^{R/R}$ mice.[49] To investigate the tissue-specific role of fibulin-4 in arteries, two independent smooth muscle cell-specific conditional knockout models were constructed. Both conditional knockout models showed disrupted formation of elastic laminae, and developed TAAs.[50, 51]

Mouse models vs. human thoracic aortic aneurysms

Like in human TAAs, cystic media degeneration has been uncovered in most of the mouse models that were described earlier.[41, 42, 49-51] These models highly equate the human disease and are therefore most frequently used to study genetically-induced TAA formation. However, of these models developing cystic media degeneration, only Fibulin-4$^{R/R}$ mice die prematurely due to aortic rupture,[49] while the other mice display normal survival up to one year of age. Next to structural changes, the role of signaling pathways also seems to be comparable between human and mice with TAAs. Excessive TGF-β signaling has been found in TAA patients [18, 21, 52, 53] and is also described in TAA mice.[49, 54] Yet, mice do not spontaneously develop atherosclerosis as a result of high levels of anti-atherosclerotic HDL and low levels of pro-atherogenic LDL and VLDL, while in humans, aneurysm formation is associated with atherosclerosis and the risk factors for both conditions are similar.[55]. This contrast might be overcome by the use of atherosclerosis-prone apolipoprotein E-deficient or LDL receptor-deficient mice.

A role for the renin-angiotenin system?

The renin-angiotensin system

The RAS is an important regulator of blood pressure, electrolyte balance and body fluid homeostasis. The RAS is initiated by renin cleaving angiotensinogen into Ang I, which in turn is converted by ACE to Ang II, the main biological active component of the RAS (Figure 1). Ang II mediates its effects via AT_1 and Ang II type 2 (AT_2) receptors. Although the RAS traditionally has been viewed as a circulating system ("circulating RAS"), it is now widely accepted that angiotensin generation at tissue sites ("tissue" RAS) is of greater importance. Here, tissue Ang II generation occurs extracellular and depends on the uptake of liver-derived angiotensinogen and kidney-derived renin from the circulation.[56-60]

AT_1 receptors are widely expressed throughout the body and mediate the well-known effects of Ang II, including vasoconstriction, water and salt retention, growth and remodeling, and inflammation. From this point of view it is not surprising that RAS blockade, resulting in diminished Ang II-AT_1 receptor interaction, is highly effective in the treatment of hypertension and a wide range of cardiovascular diseases. The function of the AT_2 receptor is less well established. Generally, AT_2 receptors are believed to function as endogenous antagonists of the AT_1 receptor, thus potentially leading to vasodilation,[61, 62] natriuresis,[63] and suppression of growth,[64] fibrosis,[65] hypertrophy,[66] and inflammation.[67] Yet, under pathophysiological conditions, the AT_2 receptor function may become more AT_1 receptor-like and cause vasoconstriction.[68, 69]

RAS inhibition is currently possible at the level of renin, ACE and the AT_1 receptor (Figure 1). Due to the negative feedback loop between renin and Ang II (mediated via AT_1 receptors), RAS blockade will always be accompanied by high renin levels. During renin inhibition and ACE inhibition, this should not result in significant Ang II generation, but during AT_1 receptor high levels of Ang II will occur. Obviously, this Ang II cannot stimulate AT_1 receptors, but it may bind to the unoccupied AT_2 receptors, and thus activate alternative pathways that either further suppress the RAS[70] or that mimic AT_1 receptor-mediated effects. Clearly, not all RAS blockers are equal.

The renin-angiotensin system in thoracic aortic aneurysms

Evidence from mouse models

As discussed above, aneurysm formation is accompanied by a whole range of structural changes. In TAAs there is increasing evidence that structural elements can also act as important regulatory factors. Fibulin-4, usually considered as a structural protein, may be a regulatory factor in elastic fiber formation. Knocking down or knocking out fibulin-4 in mice results in a decreased tropoelastin expression in the aorta.[71] In fibrillin-1 deficient mice, increased TGF-β signaling is present in lungs with impaired distal airspace septation.[72] Fibrillin-1 was hypothesised to alter matrix sequestration of the large latent complex of TGF-β, thereby making it more accessible to activation. Increased phosphorylation and nuclear translocation of Smad2 (pSmad2, an intracellular mediator of the TGF-β signal) in both fibrillin-1 and fibulin-4 deficient aortas supports the hypothesis that TGF-β is important in TAAs (Figure 2).[49, 54] More signaling pathways were examined in smooth muscle cell-specific fibulin-4 knockdown mice

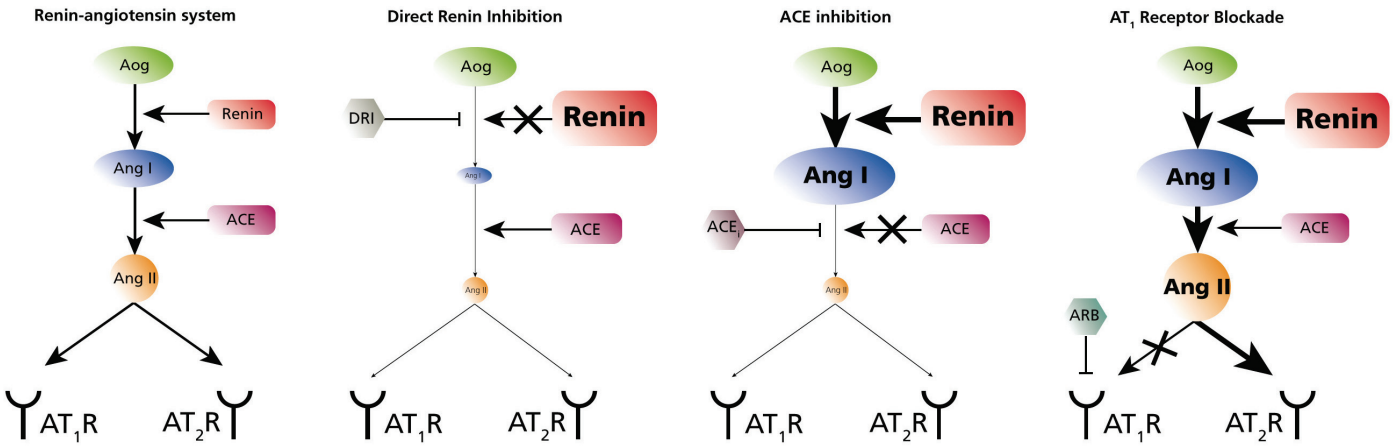

Figure 1. The renin-angiotensin system. During direct renin inhibition, levels of renin increase and Ang I and Ang II levels decrease. ACE inhibition results in high renin and Ang I levels, while Ang II levels are low. Finally, AT_1 receptor blockade levels of renin, Ang I and Ang II are high. ACE, angiotensin-converting enzyme; ACEi, angiotensin-converting enzyme inhibitor; Ang, angiotensin; Aog, angiotensinogen; ARB, angiotensin II type 1 receptor blockade, AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; DRI, direct renin inhibition.

(Fibulin-4^{SMKO}). Immunostaining and Western blot analysis demonstrate a significant increase of phosphorylated extracellular signal-regulated kinase (ERK) 1/2.[50] Taken together, therapeutic treatment for TAAs should not be limited to a reduction in hemodynamics; targeting the molecular pathways involved in aneurysm formation might be a valuable alternative. This concept is supported in fibrillin-1 deficient mice, where treatment with TGF-β neutralizing antibodies reduced elastic fiber fragmentation, aortic root enlargement, aortic wall thickness and TGF-β signaling in the aortic media.[54]

Ang II-induced hypertrophic, fibrotic and mitogenic responses are believed to involve TGF-β. In vascular smooth muscle cells, Ang II increases TGF-β mRNA and protein expression,[73] while in glomerular mesangial cells, Ang II enhances the conversion of latent TGF-β into its biologically active form.[74] Moreover, in infarcted hearts, an increase in AT_1 receptor density combined with enhanced cardiac Ang II generation is believed to underlie the increased expression of TGF-β_1 mRNA, which subsequently contributes to fibrous tissue formation.[75] Conversely, in uremic rats, AT_1 receptor blockade reduces serum creatinine and proteinuria in TGF-β-dependent manner.[76]

Based on these data, it was hypothesized that AT_1 receptor blocker losartan might provide similar effects in fibrillin-1 deficient mice as those induced by TGF-β neutralizing antibodies. Indeed, like TGF-β neutralizing antibodies, losartan treatment resulted in a reduced aortic wall thickness, less elastic fiber fragmentation, a reduction in aortic root growth and blunted TGF-β signaling in the aortic media. Beta-adrenergic blockade with propranolol, given in a similar antihypertensive dose, also reduced aortic root growth, but to a lesser extent.[54]

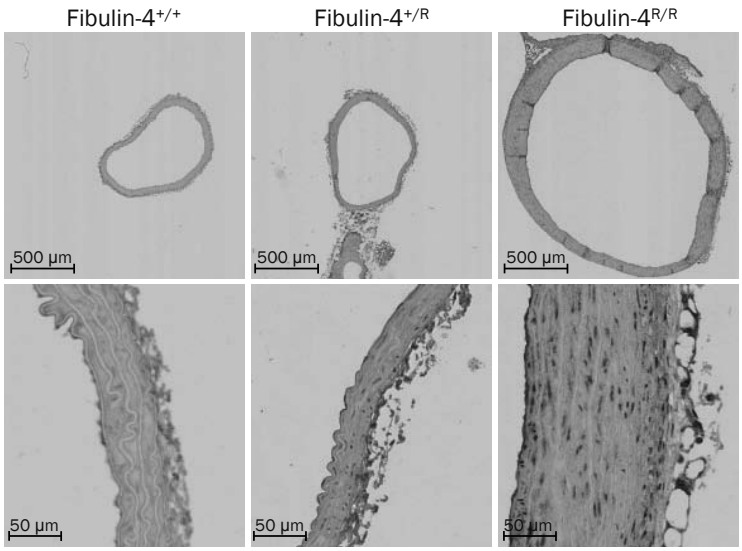

Figure 2. Ascending thoracic aortas from Fibulin-4$^{+/+}$, Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ mice. Staining with phosphorylated Smad2, a downstream of the TGF-β signal, demonstrated increased nuclear translocation of pSmad2 with decreasing expression of fibulin-4.

Thoracic aortic aneurysm patients

The involvement of the RAS is human TAAs has been studied in MFS patients. In aortic wall tissue of MFS patients, mRNA expression of the AT_1 receptor is lower, while AT_2 receptor expression is significantly higher when compared to controls.[77] Furthermore, the aortic tissue Ang II concentration is higher in MFS aortas than in control aortas.[77] Enhanced expression of the AT_2 receptor in the vasculature is also found in several other pathological conditions, such as hypertension and during aging.[78, 79] More recently, to identify a possible cause for smooth muscle cell hyperplasia in MYH11 mutant patients with TAAs, several genes of the RAS pathway were examined. In patient smooth muscle cells an increased expression of ACE was found, which could underlie the elevated vascular Ang II concentrations described above.[80]

Therapeutic implications

Cystic media degeneration, characteristic for the aortic wall in TAAs, is associated with apoptosis and loss of vascular smooth muscle cells,[81] elastic fiber degeneration and accumulation of proteoglycans. Ang II contributes to these phenomena via AT_1 and/or AT_2 receptors.[82] In cultured MFS aortas, ACE inhibition and AT_2 receptor blockade, but not AT_1 receptor blockade, inhibited serum deprivation-induced apoptosis of vascular smooth muscle cells.[77] Furthermore, arterial stiffening is an important complication in aneurysmal arteries, irrespective of aortic dilation and rupture. It is known that aortic distensibility and stiffness are abnormal in MFS patients,[83] and predict aortic dilation.[84] Increased arterial stiffness also is an independent risk factor for cardiovascular events in the general and hypertensive population.[85, 86] Several classes of anti-hypertensive drugs show beneficial effects on arterial stiffness, with RAS blockers being superior.[87] Treatment of MFS patients with ACE inhibitors was therefore of interest.

Two relatively small studies compared the effects of an ACE inhibitor with that of a beta-blocker. ACE inhibitors reduced arterial stiffness, improved aortic distensibility and slowed aortic root growth more when compared to a beta-blocker.[88, 89] Combined with the studies in fibrillin-1 deficient mice demonstrating improvement in the aortic wall structure and aortic size when treated with losartan,[54] it appeared that AT_1 receptor blockade might be even more successful. Eighteen paediatric patients with MFS that developed progressive aortic root enlargement on standard medical therapy were therefore additionally given an AT_1 receptor blocker for 12-47 months. Retrospective analysis revealed that, after adjusting for age and body-surface area, treatment with the AT_1 receptor blocker significantly slowed the rate of aortic root dilation.[90] To date, this small retrospective cohort study is the only study examining the role of AT_1 receptor blockade in TAA patients, and thus, clearly, confirmation in randomized trials is required.

Based on these results, recent guidelines state that it is reasonable to give losartan, while beta-blockade therapy is still recommended in all MFS patients with TAAs to reduce aortic root dilation. For non-MFS patients, the guidelines recommend blood pressure-lowering treatment to the lowest point patients can tolerate with beta-blockers and ACE inhibitors or AT_1 receptor blockers.[25] Thus, the recommendation for non-MFS patients still is to target hemodynamic stress, rather than disease progression by other mechanisms.

Table 2. Ongoing clinical trials to treat thoracic aortic aneurysms with AT_1 receptor blockers.

Pts	Age	Design	Treatment arms	F-up (mo)	N	Primary outcome	Secondary outcomes	Study name	Trial registration	Reference/ principal investigator
MFS	5-60 yrs	DB	losartan vs. atenolol		150	ao dilation progression		LO-AT-MARFAN01	NCT01145612	Forteza, A
MFS	≥25 yrs	DB	losartan vs. atenolol	6	50	ao biophysical properties	diastolic function, markers for ECM turnover and inflammation		NCT00723801	Creager, MA
MFS	≥10 yrs	DB	losartan vs. placebo	36	300	ao diameter	clinical events, drug tolerance, QoL	MARFAN SARTAN	NCT00763893	[127]
MFS	12-25 yrs	DB	losartan vs. atenolol	12	30	arterial stiffness (PWV)	ao biophysical properties, brachial artery reactivity, ao root dimensions		NCT00593710	Sandor, G
MFS	6 mo-25 yrs	SB	losartan vs. atenolol	36	604	adjusted ao root diameter change	ao stiffness, av and mv regurgitation, LV dimensions, weight, body mass, clinical events, adverse drug reactions		NCT00429364	[91]
MFS	≥18 yrs	SB	losartan vs. placebo (+ standard therapy)	36	300	ao diameter change	ao volume, ao stiffness, LV function, clinical events	COMPARE	NTR1423	[128]
MFS	≥1 yr	OL	losartan + atenolol or propranolol vs. atenolol or propranolol	4	44	echocardiographic			NCT00651235	Hsin-Hui, C
MFS	1-55 yrs	DB	losartan vs. nebivolol vs. losartan + nebivolol	24	291	adjusted ao root diameter change	arterial stiffness, av regurgitation, LV dimensions, serum TGF-β levels, pharmacokinetics and -genetics, quantification mutated gene, lung volumes, QoL	MaNeLo	NCT00683124	[93]
MFS	≥10 yrs	DB	losartan vs. placebo (+ standard therapy)	36	174	adjusted ao root diameter change	arterial stiffness, av and mv regurgitation progression, LV dimensions, skeletal and somatic traits, genetic polymorphisms clinical events, QoL		NCT00782327	Loeys, B
MFS	6-40 yrs	DB	irbesartan vs. placebo	60	490	ao root diameter change	adjusted ao root diameter change, av regurgitation and function, LV dimensions, cardiac rhythm, height, arm span, clinical events	AIMS	ISRCTN90011794	Mullen, M
BAV	≥18 yrs	DB	telmisartan vs. atenolol vs. placebo	60	352	change in ascending ao size (MRI)	change in ascending ao size (TEE)	BAV Study	NCT01202721	Therrien, J

ao, aortic; av, aortic valve; DB, double blinded; ECM, extracellular matrix; F-up, follow-up; MFS, Marfan syndrome; mo, months; MRI, magnetic resonance imaging; mv, mitral valve; N, number; OL, open labelled; Pts; patients; PWV, pulse wave velicity; QoL, quality of life; SB, single blinded; TEE, trans esophageal echocardiography; LV, left ventricular.

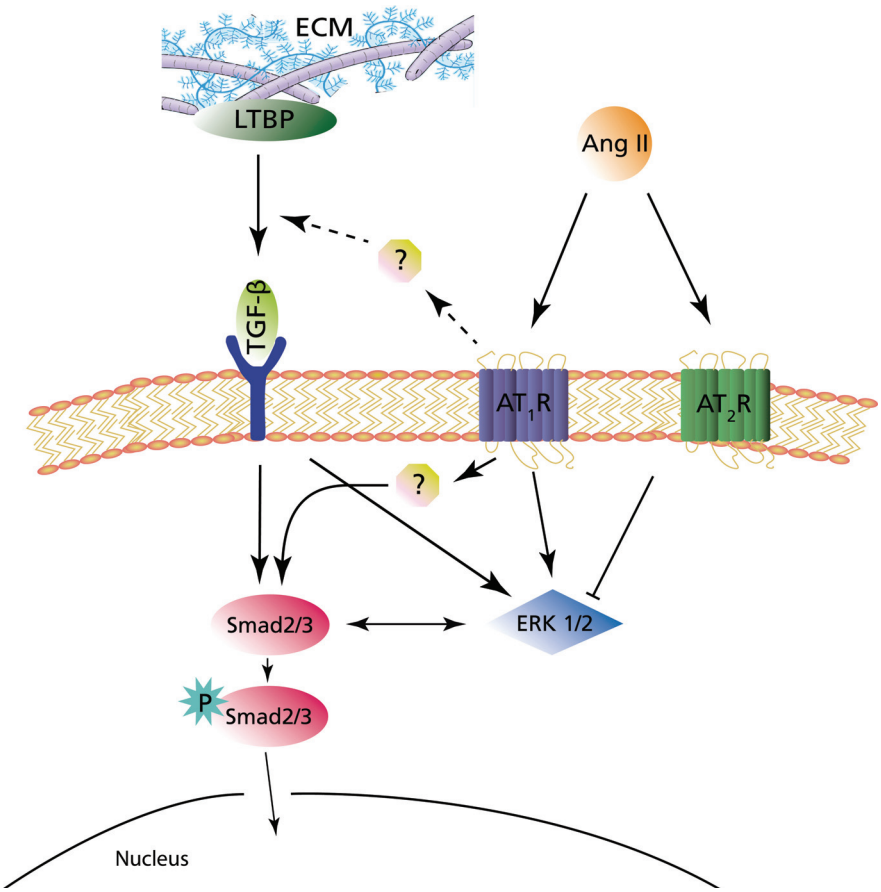

Figure 3. Schematic overview of signaling pathways involved in thoracic aortic aneurysms. Inactive TGF-β is stored as the large latent complex in the extracellular matrix, and after activation binds to the TGF-β receptor. Receptor binding results in activation of the Smad pathway. Ang II mediates its effects by binding to the AT_1 and/or AT_2 receptor and is able to activate the Smad pathway as well. AT_1 stimulation furthermore activates the ERK cascade, which can be inhibited by AT_1 receptor stimulation. Moreover, there is a cross-talk between Smad and ERK. Whether AT_1 receptor stimulation also affects TGF-β activity is unknown. Ang, angiotensin; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; LTBP, latent transforming growth factor-beta binding protein; P, phosphorylated; TGF-β, transforming growth factor-beta.

Future perspective

Current clinical trials

Multiple phase III studies are currently registered to compare the effects of AT_1 receptor blockade vs. beta-blockade on TAAs in a randomized manner (Table 2). The largest trial that has been announced is recruiting over 600 patients in the USA and Canada.[91] This study compares the rate in aortic root change adjusted for body surface area, after treatment with the beta-blocker atenolol or AT_1 receptor blocker losartan in MFS patients between 6 months and 25 years of age. This study is randomised, but is only blinded to the outcome investigator. No placebo-treated group is included, because it was considered unethical to withhold patients from the standard of care. Most upcoming trials compare the effect of AT_1 receptor blockade with the selective β_1-blocker atenolol, since blockade of the β_2 receptor will abolish the β_2 receptor-mediated vasodilator effects. Nebivolol, a selective β_1-blocker with vasodilating (NO-dependent) properties is administered in one study, because it potentially reduces arterial stiffness.[92, 93] Finally, to the best of our knowledge, only one trial is designed to study the effects of an AT_1 receptor blocker on TAAs in patients other than MFS patients. This study aims to determine whether the AT_1 receptor blocker telmisartan and/or the beta-blocker atenolol reduce the dilation of the aorta in patients with a bicuspid aortic valve.

Despite the wide range of new studies, none of these studies is designed to compare the effects of an AT_1 receptor blocker with an ACE-inhibitor. Two studies have already shown superiority of ACE-inhibitors over beta-blockers,[88, 89] but to fully understand these beneficial effects of ACE inhibition, a head-to-head comparison with an AT_1 receptor blocker is warranted. Does it simply reflect suppression of Ang II-AT_1 receptor interaction?

The promise of RAS blockade

The benefit of RAS blockade beyond its blood pressure-lowering effect involves at least three mechanisms that partly interact with each other (Figure 3). First, it is generally known that AT_1 receptor blockers and ACE inhibitors decrease TGF-β expression in animal models for renal fibrosis.[94] TGF-β predominantly transmits its signals through cytoplasmatic proteins, called Smads. Smads subsequently translocate into the cell nucleus, acting as transcription factors.[95] Nuclear translocation of pSmad2 can be used as a marker for TGF-β signaling. In MFS mice, losartan treatment reduced pSmad2 staining in the aortic media and prevented aortic wall changes.[54] Furthermore, in MFS patients, AT_1 receptor blockade induced a decrease in total circulating TGF-β levels.[52] Disturbed TGF-β signaling is also involved in other symptoms of MFS, such as lung emphysema, skeletal muscle myopathy and mitral valve prolaps.[72, 96, 97] Importantly, losartan successfully reduced these symptoms in MFS.[54, 97] The exact mechanism by which AT_1 receptor blockade antagonizes TGF-β signaling remains to be elucidated. In vascular smooth muscle cells, Ang II was able to activate the Smad pathway, independently of TGF-β.[98] It is unknown whether the effects of losartan on pSmad2 are mediated via TGF-β. In other pathogenic models, ACE inhibition and AT_1 receptor blockers reduced TGF-β to the same degree,[99] suggesting that Ang II-AT_1 receptor interaction is responsible for TGF-β activation.

Second, Ang II stimulates the ERK1/2 cascade via AT_1 receptors,[100] while the AT_2 receptor inactivates this cascade.[101] Increased ERK1/2 signaling has been demonstrated in different

subtypes of TAAs. In smooth muscle cell-specific fibulin-4 knockdown mice (Fibulin-4^{SMKO}) increased phosphorylated ERK1/2 was observed in the aneurysmal wall.[50] Preliminary results in MFS mice also point towards increased phosphorylated ERK1/2 levels, which increased even further in AT_2 knockout/MFS mice. Losartan, but not the ACE inhibitor captopril, reduced ERK activation, speculating that specific AT_1 receptor blockade/AT_2 receptor stimulation is favourable to reduce ERK in MFS mice. In patients with different mutations in the TGF-β receptor 2, both Smad and ERK signaling activity were increased.[53] A cross-talk between ERK and Smad makes a thorough understanding of these signaling pathways even more complex.

Third, as discussed, blockade of the AT_1 receptor is accompanied by a rise in Ang II levels, both in tissue and blood plasma.[102, 103] This Ang II might stimulate the non-blocked AT_2 receptor.[70] The actions of the AT_2 receptor are, as described above, thought to be opposite of the AT_1 receptor. Of relevance in TAAs, stimulation of the AT_2 receptor causes vasodilation,[61,104] inhibits cellular growth[64] and reduces extracellular matrix.[82] In apoE-/- mice with Ang II-induced AAAs, losartan inhibited aneurysm formation. AT_2 receptor blockade resulted in an increase in incidence and severity of the disease, and blunted many of the effects mediated by AT_1 signaling.[105] Taken together, it appears that AT_2 receptor stimulation during AT_1 receptor blockade is of major importance to obtain complete RAS suppression. However, not all AT_2 receptor-mediated effects described in the literature simply represent antagonism of AT_1 receptor-mediated effects, and according to some investigators, the AT_2 receptor phenotype becomes AT_1 receptor-like under pathophysiological conditions.[68, 106] Clearly therefore, the role of the AT_2 receptor in the pathophysiology of TAAs needs to be further elucidated.

Direct AT_2 receptor stimulation in absence of AT_1 receptor blockade has been a real challenge because of the lack of selectivity of the compounds used. The recent development of a selective AT_2 receptor agonist, compound 21 (C21) might provide new insights.[107] So far, several studies have shown that C21 induces beneficial anti-inflammatory and anti-fibrotic effects in various animal models.[67, 108, 109] Whether C21 mediates beneficial effects in TAA models such as mice with a reduced expression of fibulin-4 (Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$) remains to be investigated.

Habashi and co-workers investigated the role of the AT_2 receptor in $Fbn1^{C1039G/+}$ mice by disrupting the AT_2 receptor gene.[110] AT_2 receptor-deficient $Fbn1^{C1039G/+}$ mice showed worsened aortic wall architecture versus $Fbn1^{C1039G/+}$ controls and the beneficial effects of losartan on aneurysm progression were lost. These data, although preliminary, suggests a protective role of the AT_2 receptor in the development of TAAs and would support the concept that the beneficial effects of the AT_1 receptor blocker losartan rely on AT_2 receptor stimulation. A similar approach, by disrupting the AT_2 receptor in Fibulin-$4^{+/R}$ and/or Fibulin-$4^{R/R}$ mice might provide more insight in the role of the AT_2 receptor in TAAs.

CONCLUSION

TAA development involves both the RAS and TGF-β signaling pathways, and RAS blockers, being able to suppress both, are promising new tools for the treatment of TAAs. In addition, these drugs reduce hemodynamic load. Whether all RAS blockers are equal is currently uncertain, and if AT_2 receptor stimulation is a prerequisite for the beneficial effect of RAS blockade, AT_1 receptor blockers are the preferred type of RAS blocker in TAA patients. Mice models that closely mimic human TAAs allow a detailed investigation of the complex interplay between AT_1 receptors, AT_2 receptors, and TGF-β/ERK1/2 signaling. Such studies, combined with results of the ongoing clinical trials in TAA patients, will pave the way for better treatment modalities of this disease.

Acknowledgements: We thank Natasja WM Ramnath and Marcel Vermeij for performing the pSmad2 stainings.

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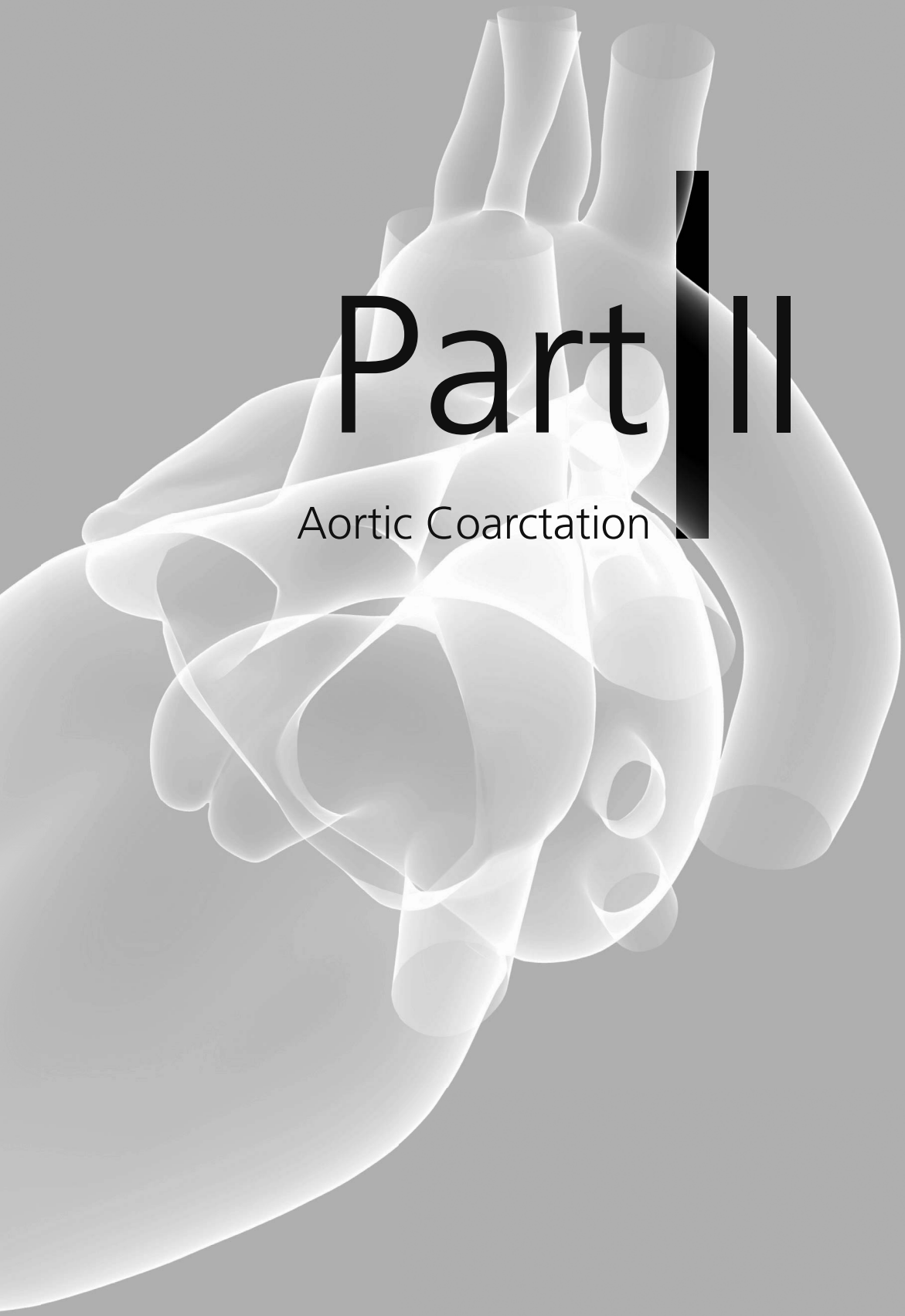
AIM OF THIS THESIS

The aorta is the largest and most important artery of the body. Diseases of the aorta include a local widening, so called aortic aneurysms or a local narrowing, the aortic coarctation. In addition, (high) blood pressure will affect the aorta. The renin-angiotensin system (RAS) affects aortic pathology locally, as well as via its effect on blood pressure. In this thesis, the role of the RAS was studied in the following pathological conditions of the aorta:

Aortic Coartation: An aortic coarctation (COA) is a congenital narrowing of the descending thoracic aorta. COAs are usually diagnosed in the neonatal period and require rapid medical care and surgical correction of the narrowed segment. Despite successful relief, the repaired aorta may develop aortic recoarctation (reCOA) and/or patients may develop hypertension. The underlying pathophysiology of hypertension in post-COA patients is still not clear. We aimed to provide more insight in the causative mechanism of post-COA hypertension by the therapeutic treatment of post-COA patients with either a beta-blocker or angiotensin (Ang) II type 1 (AT_1) receptor antagonist. Next, the blood pressure effect of percutaneous stent implantation was studied in adults with a significant native or recurrent COA (Chapter 2-4).

Hypertension: Inhibitors of the RAS are important therapeutics for the treatment of hypertension. Recently, a new type has been introduced, the renin inhibitors. The blood pressure-lowering effect of renin inhibitors is comparable to that of AT_1 receptor blockers and ACE inhibitors. Yet, their effect on end-organ damage is less well established; therefore we compared the effect of renin inhibition on the heart with that of other RAS blockers in spontaneously hypertensive rats (SHR). RAS blockade results in renin release. Only during AT_1 receptor blockade, this will result in high Ang II levels, allowing concomitant stimulation of Ang II type 2 (AT_2) receptors. Such stimulation is generally thought to counteract the effect of AT_1 receptor stimulation, although under pathophysiological conditions such as hypertension, contradictory results have been obtained. Therefore, we also evaluated the effects of AT_2 receptor stimulation in SHR (Chapter 5 and 6).

Thoracic Aortic Aneurysm: An aortic aneurysm is a degenerative disease of the aortic media. Nowadays, the progression rate of thoracic aortic aneurysms (TAAs) can be slowed down by the use of beta-blockers, but the only cure is surgery. Mice underexpressing fibulin-4 serve as a model for TAA development. After characterization of the model, mice were used to study the treatment effect of AT_1 receptor blockade. Besides, biomarkers for TAA development were identified by the combined use of aortic transcriptome and proteome analysis. Finally, fibulin-4 deficient mice display severe heart failure and aortic valve disease. We examined whether fibulin-4 mutations could predispose to human ascending aortic pathology and/or aortic valve stenosis (Chapter 7-9).



Part II

Aortic Coarctation

Based on:

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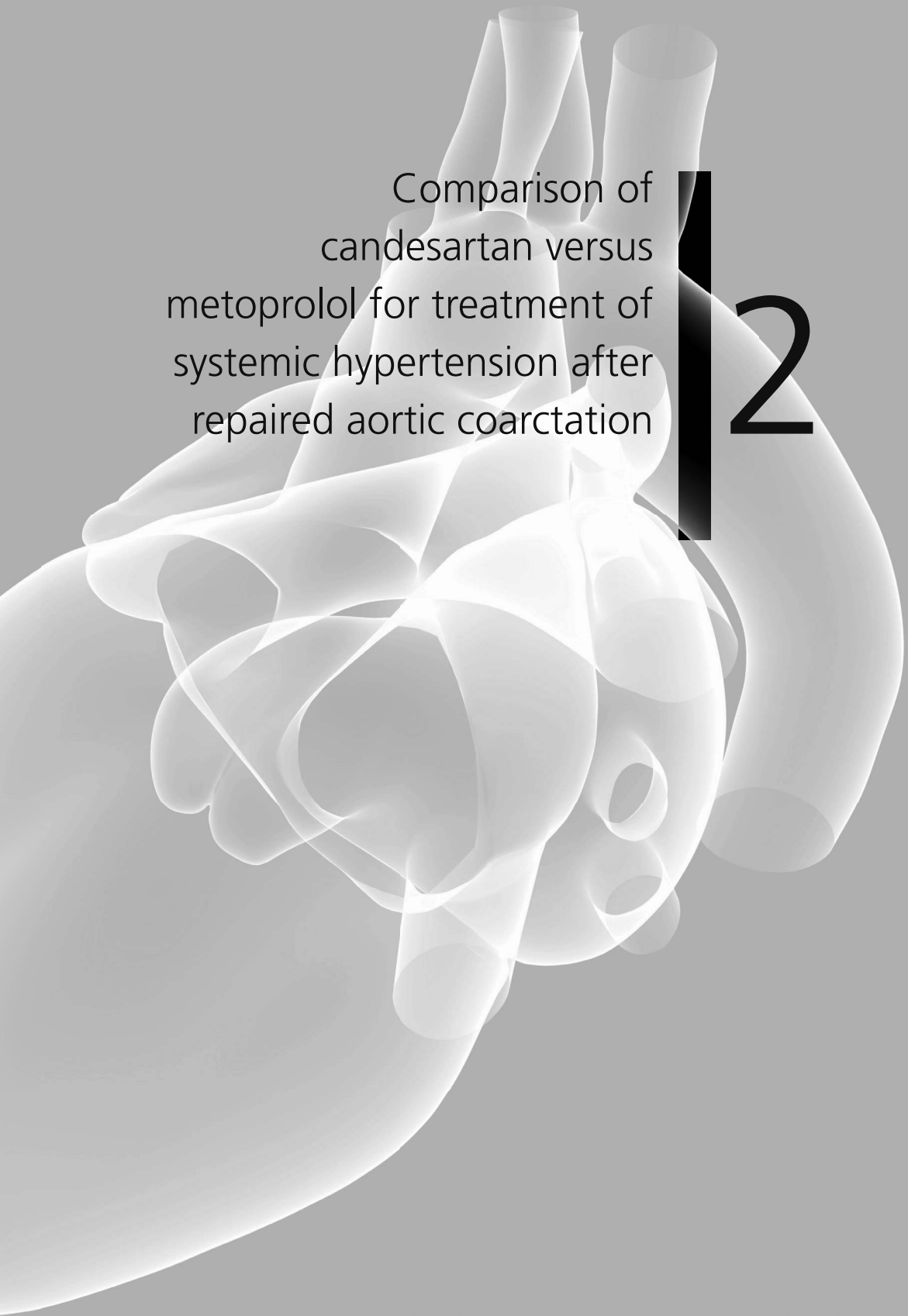
American Journal of Cardiology 2010 105:217-222

2

Comparison of candesartan versus metoprolol for treatment of systemic hypertension after repaired aortic coarctation



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ABSTRACT

Even after successful repair, hypertension is one of the main determinants of cardiovascular morbidity and mortality in patients with aortic coarctation (COA). We compared the effect of candesartan (angiotensin II type 1 receptor blockade) and metoprolol (beta-adrenergic receptor blockade) on blood pressure, large artery stiffness and neurohormonal status in hypertensive patients after repaired COA. In this open-label, crossover study, hypertensive post-COA patients were randomly assigned to treatment with candesartan 8 mg or metoprolol 100 mg once daily first. After 8 weeks of treatment with one of the drugs, the other treatment was given for 8 weeks. Treatment effects were assessed with 24-hour ambulatory blood pressure monitoring, measurements of large artery stiffness and neurohormonal plasma levels at baseline and after 8 weeks of either treatment. Sixteen patients (mean age 37±12 years, 26±15 years after repair, 63% male) completed the study. Twenty-four hour mean arterial pressure (MAP) at baseline was 97.7±6.2 mm Hg. Metoprolol (mean dose 163±50 mg daily) decreased MAP more than candesartan (mean dose 13±4 mg daily, resp. 7.0±4.2 and 4.1±3.6 mm Hg, p=0.018, 95% CI 0.6-5.5). Large artery stiffness did not change on either treatment. With metoprolol, plasma B-type natriuretic peptide increased, whereas plasma renin decreased. With candesartan, plasma renin and noradrenaline increased, whereas aldosterone levels decreased. In conclusion, in adult hypertensive post-coarctectomy patients, metoprolol had more antihypertensive effect than candesartan. Moreover, the neurohormonal outcome does not support a significant role for the renin-angiotensin-system in the causative mechanism of post-coarctectomy hypertension.

INTRODUCTION

Hypertension is one of the main determinants of cardiovascular morbidity in patients with successfully repaired coarctation of the aorta (COA).[1] The prevalence of late systolic hypertension in adults 10 to 20 years after repair is 30%, and 30 years postoperatively it increases to approximately 68%.[2, 3] The exact cause of this hypertension remains unknown. Potential causes are structural and functional abnormalities of the precoarctation arterial wall with decreased compliance.[4] For instance, increased collagen and decreased smooth muscle mass in the aortic wall are believed to result in diminished arterial wall compliance and increased rigidity.[4-7] Furthermore, hypertension could occur due to impaired baroreflex sensitivity.[5, 8, 9] Although extensive research on the causes of late hypertension has been performed, still no data are available on specific treatment for COA patients, despite the fact that such knowledge could contribute to the understanding of the causative mechanism. Beta-blockers are currently the most commonly used antihypertensive agents in post-COA patients. Some authors recommend angiotensin-converting enzyme (ACE) inhibitors as first-choice treatment based on evidence that ACE inhibitors improve pulsatile haemodynamics and regress left ventricular hypertrophy.[7] In other patient groups, angiotensin II type 1 (AT_1) receptor blockers (ARBs) are as effective and have fewer side effects. Moreover, ARBs, like ACE inhibitors, can have favourably affects on large artery stiffness.[10, 11] In the current crossover study we investigated the effect of the ARB candesartan and the beta-blocker metoprolol on blood pressure (BP) in patients after repaired COA. In addition the effect of the two drugs on large artery stiffness and changes in neurohormonal status was studied to define the optimal treatment and to provide further insight in the causative mechanism of late hypertension in post-COA patients.

METHODS

In this single-center, prospective, randomised, open-label crossover study, informed consent was obtained from each patient. The hospital's medical ethics committee approved the study. Eighteen adult post-coarctectomy patients diagnosed with hypertension defined as systolic BP (SBP) ≥140 mm Hg and/or diastolic BP (DBP) ≥90 mm Hg and/or current use of anti-hypertensive medication, were included. Patients were excluded with known intolerance to candesartan or metoprolol; significant liver insufficiency (ALAT or ASAT >3 ULN); current use of ≥3 anti-hypertensive drugs; pregnancy; wish to become pregnant during the study; evidence of recurrent (re)COA (arm-to-leg BP gradient ≥20 mm Hg) or general contraindications for candesartan or metoprolol. When a gradient ≥20 mm Hg was found, an MRI was performed (five patients) to exclude a reCOA.

Patients currently using anti-hypertensive medication were enrolled after a washout period of 3 weeks. Patients were randomly assigned to treatment with candesartan 8 mg (group A) or metoprolol 100 mg (selokeen zoc, group B) once daily first. After 8 weeks of treatment with one of the drugs another washout period of 3 weeks was arranged. Then, the other treatment was given for 8 weeks. Measurements of 24-hour ambulatory blood pressure monitoring (ABPM), aortic and carotid stiffness and vasoactive hormone plasma levels were performed after both periods of washout and after 8 weeks of medication use. After completing the study, patients were asked about adverse effects and preference of medication.

BP responses to treatment were measured after both periods of washout and after 8 weeks of either treatment on the right arm by 24-hour ABPM. ABPMs were recorded with a Spacelabs 90207-30 or Spacelabs 90-217-9Q device. The recordings were performed every 30 min during daytime (07.00 h - 22.00 h) and every 60 min during night (22.00 h – 07.00 h). The monitor display was switched off during ABPM to avoid possible impact on the BP readings. Four weeks after starting the medication a single BP measurement was performed at the patient's general practitioner or at the outpatient clinic. For ethical reasons, medication dose was doubled if SBP ≥140 mm Hg and/or DBP ≥90 mm Hg.

Aortic stiffness was measured with the patient in supine position using the carotid-femoral Pulse Wave Velocity (PWV). PWV was assessed with a non-invasive automatic device (Complior SP, Artech Medical, Pantin, France)[12] that measures the time delay between the beginning of the rapid upstroke of simultaneously recorded pulse waves in the carotid and the femoral artery. The distance between the jugulum and the femoral artery was measured. PWV was calculated as the ratio between that distance and the foot-to-foot time delay and was expressed in meters per second (m/s).

Carotid distensibility was assessed with the patients in supine position, with the head turned slightly in the controlateral position for measurement at the right common artery. The vessel

Table 1. Baseline patient characteristics

Patient no	Gender (F/M)	Age (yrs)	Age at repair (yrs)	BMI (kg/m²)	Repair type	Re-intervention	Additional congenital cardiac anomalies	Anti-hypertensive treatment before study	Arm-leg gradient (mm Hg)	Evicence of reCOA on MRI
1	M	20	0	31	E/E	+	ASD	+	10	0
2	M	20	1	24	E/E	0	VSD, MR	0	30	0
3	M	20	7	22	E/E	0	none	0	0	-
4	F	25	0	24	SCflap	0	none	+	9	-
5	M	28	19	22	E/E	0	BAV	+	NA	0
6	F	30	0	23	E/E	0	BAV, PDA	+	0	-
7	M	33	9	35	Patch	0	none	+	0	-
8	M	38	20	27	Patch	0	VSD	+	2	-
9	M	39	32	23	Graft	0	AR	+	0	-
10	F	41	40	29	Stent	0	BAV, SVAS	+	0	-
11	M	44	17	21	Patch	+	BAV	0	25	0
12	F	46	17	27	Patch	0	BAV	+	0	-
13	M	48	6	26	E/E	0	BAV, PDA, MR	+	0	-
14	F	49	5	34	Patch	+	none	+	20	0
15	F	50	5	24	E/E	0	none	+	0	-
16	M	61	7	27	-	-	none	+	0	-

+, yes; 0, no; -, unknown; AR, aortic regurgitation; ASD, atrial septal defect; BAV, bicuspid aortic valve; BMI, body mass index at time of study; E/E, end-to-end anastomosis; F, female; Graft, graft interposition; M, male; MR, mitral regurgitation; Patch, patch aortoplasty; PDA, patent ductus arteriosus; reCOA, recoarctation; SCflap, subclavian flap; Stent, stent placement; SVAS, subvalvular aortic stenosis; VSD, ventricular septal defect; yrs, years.

wall motion of the artery was measured by means of a duplex scanner (ATL Ultramark IV, 7.5 MHz probe, Soma Technology Inc, Bloomfield, CT, USA) connected to a vessel wall movement detector system. This is a validated technique for the measurement of carotid distensibility.[13] A region 1.5 cm proximal to the origin of the bulb of the carotid artery was identified with the use of B-mode ultrasound. The displacement of the arterial walls was obtained by processing the radiofrequency signals originating from two selected sample volumes positioned over the anterior and posterior walls. The end-diastolic diameter (D), the absolute change in diameter during systole (ΔD), and the relative change in diameter (ΔD/D) were computed as the mean of 4 cardiac cycles. The cross-sectional arterial wall distensibility coefficient was calculated according the following equation: distensibility coefficient = 2ΔD/(D*pulse pressure) (10^{-3}/kPa).[14]

Blood samples were taken for measurements of B-type natriuretic peptide (BNP), renin, aldosterone, catecholamines and endothelin-1 (ET-1). An intravenous cannula was inserted in one of the forearm veins and blood samples were obtained after 30 min in supine position. BNP, renin, aldosterone and ET-1 were determined with commercially available kits as previously described.[15, 16] Noradrenaline and adrenaline were measured with fluorimetric detection after high-performance liquid chromatography.[17]

Normally distributed continuous data are presented as mean±standard deviation (SD) and categorical variables as counts and percentages. Non-normal distributed data, including neurohormonal status, are presented as median with interquartile range (IQR, 25th and 75th percentiles). Baseline (pre-treatment) values were established after the washout period of 3 weeks. Delta's (Δ) were calculated as the value post-treatment minus the value pre-treatment. The delta of the two treatment deltas was used to analyse the difference between the treatment regimens by the paired Student's *t*-test for continuous variables, when normally distributed. The Wilcoxon signed-ranks test was applied for the skewed distributed neurohormonal status. All statistical tests were two-sided and a p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill, USA).

RESULTS

Baseline characteristics of the study population are shown in Table 1. Eighteen patients were included and 16 patients (89%, mean age 37±12 years, 63% male) completed the study successfully. Reasons for not completing the study were respiratory distress provoked by beta-blockade (n=1) and non-compliance (n=1). None of the participating patients were smokers or diabetics. Treatment dosage of both candesartan (mean dose 13±4 mg daily) and metoprolol (mean dose 163±50 mg daily) was doubled after 4 weeks in 10 patients, while in the residual 6 patients the initial dosage was continued for both drugs. Sixteen patients completed the study and in 15 patients (94%) all ABPM recordings were successful. Results are shown in Table 2 and Figure 1. No differences were found between the two baseline measurements. Both treatment strategies lowered day, night and 24-hour BP. Metoprolol reduced 24-hour MAP, 24-hour DBP, daytime SBP and daytime DBP more than candesartan. There was a trend towards improved reduction of 24-hour SBP in favour of metoprolol. Twenty-four hour heart rate (HR) was lower on metoprolol than on candesartan. Treatment rank had no influence on treatment efficacy (data not shown).

Table 2. Twenty-four hour ambulatory blood pressure measurements

	Treatment	N	Pre-treatment	Post-treatment	Δ	ΔC-ΔM	p-value	95% CI
Day								
MAP (mm Hg)	candesartan	16	103.2±6.2	98.8±7.5	-4.4±4.4*	3.9	0.006	1.3-6.5
	metoprolol	16	103.1±7.3	94.8±7.2	-8.3±4.3*			
SBP (mm Hg)	candesartan	16	148.9±1.9	144.4±2.3	-4.4±2.0*	5.7	0.008	1.8-9.6
	metoprolol	16	148.3±2.0	138.2±2.2	-10.1±1.7*			
DBP (mm Hg)	candesartan	16	82.9±2.2	78.3±2.1	-4.6±1.2*	4.2	0.032	0.4-8.0
	metoprolol	16	82.9±2.3	74.2±2.4	-8.8±1.2*			
PP (mm Hg)	candesartan	16	66.3±9.9	65.4±12.5	-0.9±6.6	0.5	0.843	-4.8-5.8
	metoprolol	16	65.6±9.5	64.2±12.2	-1.4±7.7			
HR (bpm)	candesartan	16	75.9±9.7	75.3±8.2	-0.6±7.0	12.8	<0.001	8.5-17.2
	metoprolol	16	73.3±9.2	59.9±8.7	-13.4±7.3*			
Night								
MAP (mm Hg)	candesartan	15	88.3±8.3	83.5±9.6	-4.7±7.7*	-0.3	0.902	-4.8-4.3
	metoprolol	16	88.6±8.0	84.4±8.4	-4.3±5.4*			
SBP (mm Hg)	candesartan	15	129.6±2.9	120.9±2.7	-8.5±1.9*	-2.1	0.576	-7.7-3.5
	metoprolol	16	128.6±3.2	122.6±3.5	-6.4±2.1*			
DBP (mm Hg)	candesartan	15	68.5±2.5	64.1±2.0	-4.1±1.2*	0.6	0.288	-2.6-4.4
	metoprolol	16	68.9±2.0	64.3±2.2	-5.1±1.4*			
PP (mm Hg)	candesartan	15	61.2±12.1	57.8±13.4	-3.4±4.9*	-2.0	0.352	-6.5-2.5
	metoprolol	16	59.8±12.4	58.5±14.5	-1.3±5.4			
HR (bpm)	candesartan	15	63.0±6.6	61.9±10.6	-1.1±7.9	6.9	0.029	0.8-12.9
	metoprolol	16	62.5±8.4	55.1±6.7	-7.4±7.5*			
24-hour								
MAP (mm Hg)	candesartan	15	97.5±6.1	93.5±6.1	-4.1±3.6*	3.1	0.018	0.6-5.5
	metoprolol	16	97.4±6.9	90.4±6.8	-7.0±4.2*			
SBP (mm Hg)	candesartan	15	141.7±2.0	135.7±2.3	-5.5±1.9*	3.8	0.071	-0.4-8.0
	metoprolol	16	140.9±2.2	131.9±2.5	-9.3±1.7*			
DBP (mm Hg)	candesartan	15	77.3±2.2	72.9±1.9	-4.1± 0.8*	3.3	0.046	0.1-6.5
	metoprolol	16	77.3±2.2	70.1±2.2	-7.3±1.1*			
PP (mm Hg)	candesartan	15	64.6±10.3	62.8±12.8	-1.8±5.6	-0.1	0.972	-4.0-4.1
	metoprolol	16	63.6±10.1	61.9±13.0	-1.7±5.4			
HR (bpm)	candesartan	15	71.1±8.0	70.3±8.2	-0.8±6.4	10.9	<0.001	6.6-15.1
	metoprolol	16	69.2±8.4	58.1±7.3	-11.1±5.9*			

Values are expressed as mean±SD. *p<0.05 vs. pre-treatment; Δ, post-treatment – pre-treatment; ΔC–ΔM, ΔC for candesartan – ΔM for metoprolol; bpm, beats per minure; CI, confidence interval; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; N, number; PP, pulse pressure; SBP, systolic blood pressure.

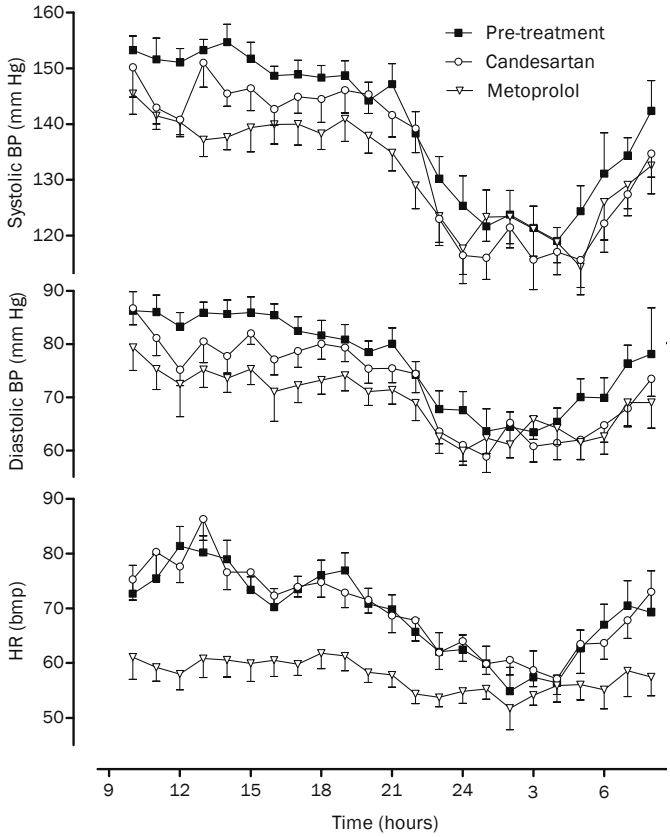

Figure 1. Twenty-four hour systolic blood pressure, diastolic blood pressure and heart rate before and after treatment with candesartan or metoprolol. The pre-treatment curve is calculated as the mean of the two pre-treatment measurements after washout. Error bars indicate SEM. Bpm, beats per minute.

Table 3. Measures of large artery stiffness and carotid diameter

		N	Pre-treatment	Post-treatment	Δ	ΔC–ΔM	p-value	95% CI
PWV (m/s)	candesartan	15	6.8±1.6	7.2±1.7	0.4±1.5	0.6	0.288	-0.6–1.8
	metoprolol	15	7.3±1.1	7.0±1.0	-0.2±1.3			
Distensibility coefficient	candesartan	15	29.0±11.7	29.5±11.1	-0.4±3.5	-2.7	0.176	-6.8–1.4
(Mpa-1)	metoprolol	15	27.1±10.1	30.3±11.3	-3.3±6.6			
Carotid diameter (mm)	candesartan	16	8.0±1.5	7.7±1.4	0.4±0.8	0.2	0.717	-0.6–0.4
	metoprolol	15	8.0±1.4	7.8±1.3*	0.2±0.4			

Values are expressed as mean±SD. *p<0.05 vs. pre-treatment; Δ, post-treatment–pre-treatment; ΔC–ΔM, Δ for candesartan – Δ for metoprolol; CI, confidence interval; N, number; PWV, pulse wave velocity.

Table 4. Neurohormones

		N	Reference value	Pre-treatment	Post-treatment	p-value
BNP (pmol/l)	candesartan	14	<6	2.0 (0.6–4.9)	2.1 (0.5–5.2)	0.124
	metoprolol	14		2.3 (0.6–6.1)	5.0 (0.8–14.3)*	
Renin (µU/ml)	candesartan	15	10-50	14.6 (10.1–30.6)	47.7 (41.5–125.7)*	0.001
	metoprolol	15		10.8 (7–24.3)	8.2 (3.4–10.6)*	
Aldosterone (pg/ml)	candesartan	15	50-200	60.5 (38.3–94.0)	50.0 (28.0–60.0)*	0.173
	metoprolol	15		47.0 (26.0–91.0)	41.0 (28.0–69.0)	
Noradrenaline (pg/ml)	candesartan	15	150-600	201.5 (187.3–293.0)	255.0 (215.0–294.0)*	0.281
	metoprolol	15		224.0 (178.0–307.0)	207.0 (171.0–294.0)	
Adrenaline (pg/ml)	candesartan	15	10-100	22.5 (17.5–37.5)	18.0 (11.0–39.0)	0.394
	metoprolol	15		21.0 (12.0–31.0)	24.0 (18.0–47.0)	
ET-1 (pg/ml)	candesartan	15	0.913 (ND–2.48)	0.9 (.8–1.1)	1.0 (0.7–1.2)	0.910
	metoprolol	15		1.1 (0.8–1.2)	1.1 (0.6–1.4)	

Pre-treatment and post-treatment values are expressed as median with interquartile range. Reference value of endothelin-1 is given as mean (range). *p<0.05 vs. pre-treatment; BNP, B-type Natriuretic Peptide; ET-1, endothelin-1; N, number; ND, non detectable.

Aortic PWV and carotid distensibility were measured successfully in 15 patients (94%). No differences were found at baseline for either PWV or carotid distensibility (Table 3). We found no effect of either treatment on both measurements of large artery stiffness. Carotid diameter decreased after treatment with metoprolol, but not after treatment with candesartan.

At both baseline investigations, there were no differences in the plasma concentrations of BNP, renin, aldosterone, noradrenaline, adrenaline and ET-1, and all values were within the normal range (Table 4). Plasma BNP concentrations increased with metoprolol. Renin concentrations increased after treatment with candesartan and decreased on metoprolol. Noradrenaline levels increased, and aldosterone levels decreased after treatment with candesartan.

Adverse effects during metoprolol use were dizziness (3), fatigue (3), complaints of cold (1) and sleep disturbances (1). On candesartan, dizziness (2), pressure on the chest (1), dyspnoea (1) and pain in the legs (1) were reported. After completing the study, 8 patients preferred treatment with candesartan, 5 metoprolol and 3 had no preference.

DISCUSSION

In this study performed in adult hypertensive post-coarctectomy patients, metoprolol in its used dosage was more effective in lowering 24-hour MAP, especially during daytime, than candesartan. Comparable clinically relevant doses were chosen for both drugs. We cannot exclude, however, that higher doses of candesartan are more effective and will be as effective as metoprolol without an increase in clinically relevant side effects. Recently, the role of ventricular stiffness in the complex mechanism of hypertension in post-COA patients has been highlighted.[18] As blockers of the renin-angiotensin system (RAS), rather than beta-blockers are the preferred treatment for ventricular stiffness, a preference for RAS-blockers rather than beta-blockers was expected.[18] However, In patients with left ventricular dysfunction, abnormal long-axis function is associated with subendocardial ischemia and here beta-blockers may be superior.[19] Furthermore, although we expected some effect especially of RAS-blockers, no effect on arterial stiffness of either treatment was found in this study. Possibly, our treatment period was too short. However such effects have been described by others after a 4-week period of treatment, albeit in other patients groups.[10, 11]

The increased levels of noradrenaline after treatment with candesartan were unexpected. One might argue that the rise in angiotensins during AT_1 receptor blockade[20] has affected sympathetic outflow via stimulation of non-AT_1 receptors, e.g. angiotensin II type 2 (AT_2) or angiotensin-(1-7) receptors.[21, 22] Indeed, angiotensin-(1-7), like angiotensin II, facilitated noradrenaline release from rat atria.[21] The effect of angiotensin-(1-7) could be blocked with the AT_2 receptor blocker PD123319, suggesting that it involved stimulation of AT_2 receptors on sympathetic nerve endings.

All neurohormonal levels at baseline were within normal ranges. Treatment with metoprolol modestly increased the concentrations of BNP, while no changes were observed after treatment with candesartan. This parallels earlier findings in hypertensive patients treated with the beta-blocker bisoprolol.[15] Thus, it does not appear to be specific for repaired COA patients, and is unlikely to provide insight in the causative mechanism of the hypertension. Furthermore, this study does not provide evidence that hyperactivity of the RAS caused by a reduced blood flow to the kidneys plays an important role in hypertension in post-COA patients.

The expected occurrence of adverse effects may influence the physician's choice of medication. Beta-blockers have a poor reputation, especially in relative young adult patients. In our small population, although not significant, more adverse effects were observed on metoprolol, and this may play a role in prescription behaviour.

Some limitations have to be mentioned about the present study. This was an open-labeled study; however, 24-hour ABPMs were blinded to the patients and investigators. Only patients with mild to moderate hypertension were included; users of ≥3 antihypertensive drugs were excluded for ethical reasons. Therefore, we cannot comment on similar patients with severe hypertension. Finally, a small population was studied and two out of 18 patients did not complete the study.

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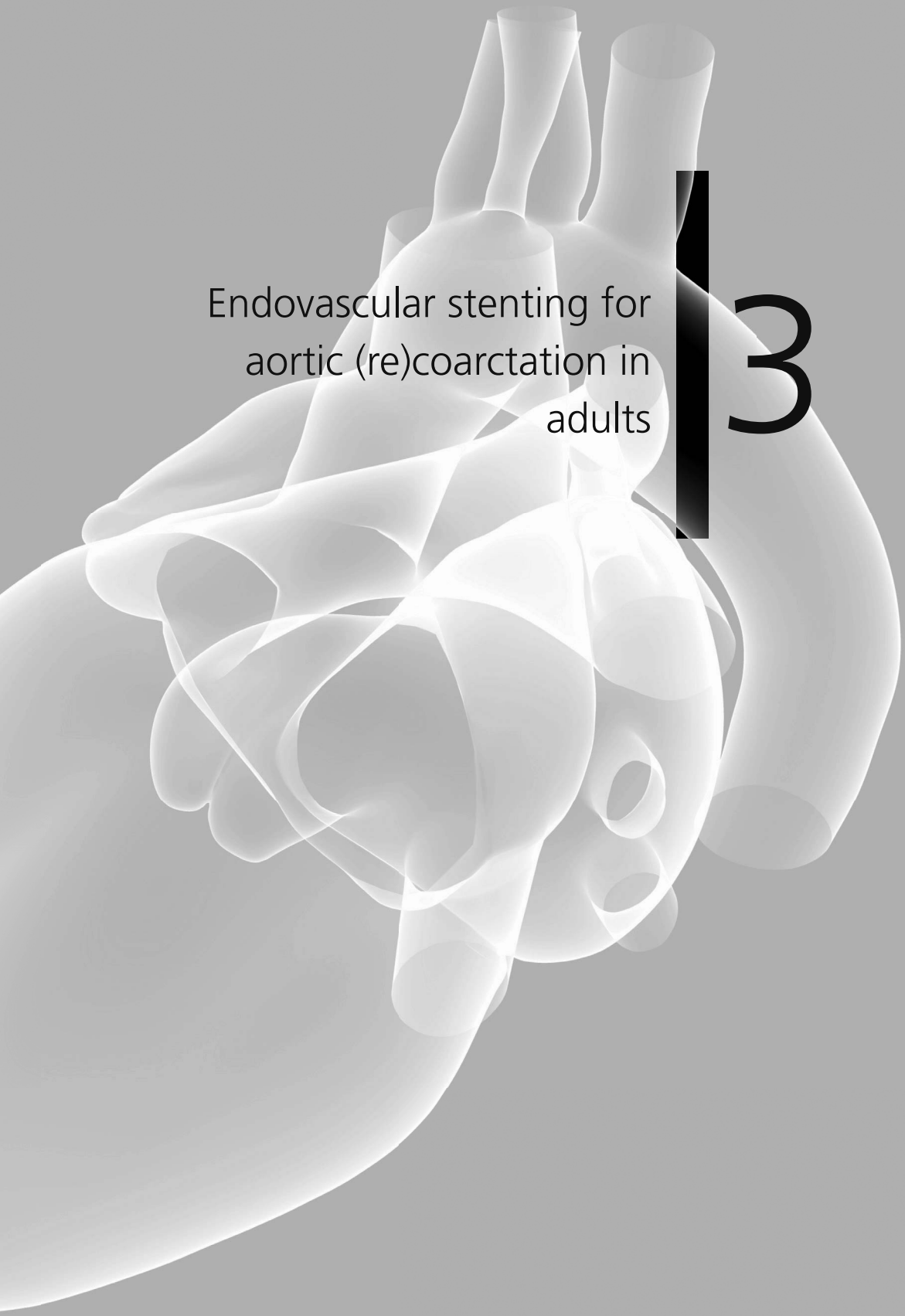
Netherlands Heart Journal 2010 18:430-436



3

Endovascular stenting for aortic (re)coarctation in adults

3

ABSTRACT

Stenting for native and recurrent aortic coarctation (COA) in adults has become an important therapeutic strategy. In this prospective observational study we evaluated the intermediate-term outcome of stent implantation for either native COA or recurrent COA (reCOA) in adults. All adults that underwent stent implantation between January 2003 and December 2008 in our institution were included. Diagnosis of (re)COA was based upon a combination of clinical signs, non-invasive imaging or invasive gradient measurements. NuMED stents were implanted under general anesthesia. Twenty-four patients (50% male) underwent stent implantation for native (n=6) or reCOA (n=18) at a median age of 36 (18-60) years. There was a significant improvement in pre-stent versus post-stent invasive systolic gradient (19 vs. 0 mm Hg, $p<0.001$) and COA diameter (10 vs. 16 mm, $p<0.001$). Acute complications (12.5%) included: death due to aortic rupture despite immediate successful coverage with a covered stent (n=1) and groin haematoma (n=2). During a median follow-up period of 33 (8-77) months (n=22), late complications occurred in 3 patients (13.6%): stent migration to the ascending aorta (n=1), pseudo-aneurysm at the site of the initial stent (n=1), and occluded external iliac artery (n=1). Stent implantation did not reduce the need for antihypertensive medication or blood pressure at last follow-up. We conclude that COA stenting results in a significant gradient decrease and increase in vessel diameter. However, serious complications do occur and hypertension remains in the majority of patients.

INTRODUCTION

Over the past 15 years endovascular treatment of native aortic coarctation (COA) and recurrent COA (reCOA) has gained widespread acceptance in older children and adolescents.[1, 2] In analogy of coronary angioplasty, there has been a shift in the interventional treatment of COA from balloon angioplasty to balloon expandable intravascular stent placement.[3-11] Although the acute hemodynamic improvements are well-known, concerns exist about aortic rupture, especially in patients with native COA. In the longer term, pseudo-aneurysm formation and reCOA may occur.[12, 13] In our institution stent implantation for COA was started in 2003. This prospective, single center, observational study reports the acute and intermediate results in adults who underwent endovascular stent placement for both native COA and reCOA.

PATIENTS AND METHODS

Study population

Between January 2003 and December 2008, a total of 24 adult patients received endovascular stent implantation for (re)COA. Diagnosis of (re)COA was based upon a combination of clinical signs (arm-leg blood pressure (BP) difference ≥20 mm Hg), non-invasive imaging (echocardiography, CT-scan or MRI) and/or invasive gradient measurements. The choice for stent implantation was made by a team of cardiac surgeons, interventional cardiologists and cardiologists specialized in adult congenital heart disease. In our institution there is a tendency to opt for surgical treatment in patients with native COA, unless specific conditions in the patient such as pregnancy exist. In reCOA, stenting is the preferred choice.

Intervention

All procedures were performed under general anesthesia and full heparinization. Retrograde aortic catheterization was performed. The peak systolic gradient was measured invasively before and after stent implantation. Biplane aortography was performed for optimal delineation of the stenosis, including measurements of the distal arch before the narrowing, the stenosis and the descending aorta. The procedure was considered successful if the systolic gradient was reduced to ≤10 mm Hg and/or the angiographic diameter had increased ≥50%. A covered or bare CP stent (NuMED CP stent, Heart Medical Europe BV, Best, The Netherlands) was hand crimped on a balloon (Balloon-in-Balloon, NuMED). The balloon-stent assembly was advanced through a long 11-14 F sheath (William Cook Europe, Bjaeverskov, Denmark). The inner balloon was inflated, and with angiographic control through the long sheath, the stent position could be adjusted before the outer balloon was inflated. Control angiography and pressure measurements were performed. When a waist persisted and the residual gradient was significant, further dilation with a high pressure balloon (Zymed or Mullins NuMED) was performed. At final angiography, special attention was given to possible signs of dissection. After the intervention, patients received acetylsalicylic acid for 6 months.

Follow-up

All patients were scheduled for echocardiography, 24-hour ambulatory blood pressure monitoring (ABPM) and CT-scan or MRI during clinical follow-up. Echocardiographic Doppler

study was performed to evaluate left ventricular function and flow velocity over the distal arch. A possible reCOA was diagnosed by a diastolic run-off on echocardiography. Ventricular function was evaluated as good, moderate or poor. Twenty-four hour ABPM was measured on the right arm (Spacelabs Healthcare, Buck, UK). Recordings were performed every 30 min during daytime (7.00 h–22.00 h) and every 60 min during the night (22.00 h–07.00 h). The monitor display was switched off during ABPM to avoid possible impact on the BP readings. Hypertension was defined as systolic BP ≥140 mm Hg and/or diastolic BP ≥90 mm Hg. Either a CT-scan or MRI was performed to document arch morphology and to demonstrate possible pseudo-aneurysm formation during follow-up.

Statistics

Normally distributed continuous data are presented as mean±standard deviation (SD) and nominal variables as counts and/or percentages. Non-normal distributed continuous data

Table 1. Baseline patient characteristics

	All patients (n=24)	Native COA (n=6)	ReCOA (n=18)
Age (years)	35±13	28±9	38±13
Gender (male)	12 (50)	4 (67)	8 (44)
BMI (kg/m^2)	24±4	24±6	24±4
Antihypertensive treatment (%)	18 (75)	3 (50)	15 (83)
Previous repair type (%)			
End-to-end anastomosis	13	-	13 (72)
Subclavian flap	3	-	3 (17)
Graft interposition	1	-	1 (6)
Patch angioplasty	1	-	1 (6)
Associated cardiac anomalies (%)			
Bicuspid aortic valve	7	3	4
Aortic valve stenosis	5	1	4
Ventricular septal defect	4	1	3
Patent arterial duct	2	0	2
Aortic regurgitation	2	2	0
Mitral valve insufficiency	2	1	1
Atrioventricular septal defect	1	1	0
Discrete subaortic stenosis	1	1	0
Tricuspid valve insufficiency	1	0	1
None	6 (25)	0 (0)	6 (33)
Associated comorbidities			
Turner's syndrome	2	1	1
Mohr syndrome	1	1	0

Values are expressed as mean±SD for continuous variables and numbers (percentages) for dichotomous variables. There are no significant differences between the native COA and reCOA group concerning age, gender, BMI and antihypertensive treatment. BMI, body mass index; COA, aortic coarctation; reCOA, recurrent aortic coarctation.

are presented as median and ranges. The (paired) Student's t test was used for continuous variables, when normally distributed. In case of skewed distributed data, the Wilcoxon signed-rank was considered for paired observations and the Mann-Withney U test for unpaired observations. Unpaired nominal variables were analysed using the Fisher's exact test. All statistical tests were two-sided and a p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill, USA).

RESULTS

Study population

Between January 2003 and December 2008, 24 patients were diagnosed with native COA (25%) or reCOA (75%) and accepted for endovascular stent implantation at our institution (Table 1). Three patients with repaired COA had previously been treated with endovascular balloon angioplasty for reCOA. One patient was diagnosed with a native COA at 9 weeks gestation and very high BP in the upper extremities. She underwent a two-step approach; a stent implantation at 12 weeks gestation and additional stent dilation 6 months after delivery. Other patients that underwent stent implantation for native COA had a history of multiple cardiac surgeries (n=3), mental retardation (n=1) and Turners syndrome (n=1).

Procedural characteristics

A total of 25 stents were implanted. Procedural characteristics are shown in Table 2. In one patient a second covered stent was placed immediately after the bare stent to treat aortic rupture. The median invasively measured systolic gradient decreased significantly (p<0.001, Figure 1 left panel) and reduced to ≤10 mm Hg in 21 patients (88%, 5 native COA, 16 reCOA). The diameter of the distal aortic arch was 17±3 mm, the distal aorta 20±4 mm. The

Table 2. Procedural characteristics

	All patients (n=24)	Native COA (n=6)	ReCOA (n=18)
Stent length (mm)	45 (34-45)	42 (34-45)	45 (34-45)
Balloon size (mm)	18 (12–24)	18 (12–24)	18 (14-22)
Covered (%)	6 (25)	1 (17)	5 (28)
Invasive gradient (mm Hg)			
Pre-stent	19 (6-50)	25 (18-30)	17 (10-50)
Post-stent	0 (0-35)*	1 (0–11)*	0 (0-35)*
Diameter lesion (mm)			
Pre-stent	10 (2-17)	9 (2-17)	10 (7-15)
Post-stent	16 (10-28)*	17 (10-28)*	16 (12-19)*
Diameter increase (%)	58 (20–400)	86 (58–400)	50 (20–99)†

Values are expressed as median (ranges) for continuous variables and number (percentages) for dichotomous variables. *p<0.05 vs. pre-stent; †p<0.05 vs. native COA; COA, aortic coarctation; reCOA, recurrent aortic coarctation.

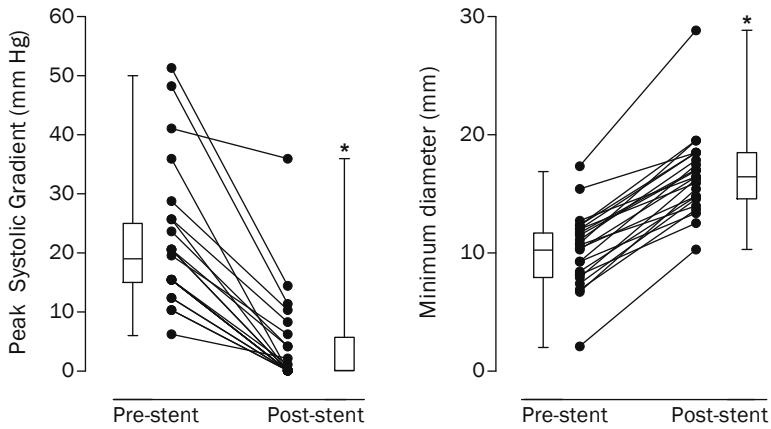


Figure 1. Invasively measured peak systolic gradient before and after stent implantation (left). Minimum diameter at the side of the lesion before and after stent implantation (right). Boxplots indicate the median, interquartile ranges, highest and lowest values; *p<0.001 vs. pre-stent.

Table 3. Echocardiographic follow-up

	All patients (n=24)	Native COA (n=6)	ReCOA (n=18)
Peak systolic velocity (proximal of lesion, m/s)			
Pre-stent	1.3±0.5	1.2±0.2	1.3±0.4
Post-stent	1.2±0.3	1.5±0.1	1.3±0.3†
Peak systolic velocity (maximum, m/s)			
Pre-stent	3.6±0.6	3.6±0.7	3.6±0.6
Post-stent	2.4±0.7*	2.0±0.4*	2.3±0.7*
Diastolic run-off (%)			
Pre-stent	67	75	65
Post-stent	15*	17	14*
Left ventricular function (%)			
Pre-stent			
Good	79	50	89
Moderate	14	17	-
Poor	17	33	11
Post-stent			
Good	90	67	100†
Moderate	10	33	-
Poor	-	-	-

Values are expressed as mean±SD for continuous variables and percentages for dichotomous variables. *p<0.05 vs. pre-stent; †p<0.05 vs. native COA; COA, aortic coarctation; reCOA, recurrent aortic coarctation.

median minimum diameter at the side of the COA increased significantly from 10 (2–17) to 16 (10–28) mm (p<0.001, Figure 1 right panel). An increase in diameter of ≥50% after the procedure was seen in 15 patients (65%, all reCOA). The procedure was considered successful in 22 patients (92%, 6 native COA, 16 reCOA).

Acute complications

One major complication occurred in a patient with reCOA in whom the aorta ruptured. Despite immediate successful placement with a covered stent, the clinical condition remained critical due to an unexplained massive pulmonary hemorrhage into the left main bronchus, which, despite selective ventilation of the right lung, could not be controlled and the patient died the same day. Post mortem examination excluded an aortic bronchial fistula as possible cause for the bleeding. Two other patients developed a groin hematoma after the procedure.

Follow-up

During a median follow-up period of 33 (range 8-77) months, data were collected for 22 patients. Echocardiographic follow-up was collected in 20 (91%) patients, 28 (2-77) months after implantation (Table 3). Maximum peak systolic velocity, measured at the side of the

Table 4. Blood pressure

	All patients (n=24)	Native COA (n=6)	ReCOA (n=18)
BP systolic (mm Hg)			
Pre-stent	162±22	160±18	162±23
Post-stent	154±12	157±12	153±13
BP diastolic (mm Hg)			
Pre-stent	84±16	87±26	82±12
Post-stent	80±9	88±6	77±8†
Arm-to-leg gradient (mm Hg)			
Pre-stent	23 (0-54)	12 (3-21)	27 (0-54)
Post-stent	0 (0-44)	0 (0-21)	0 (0-44)
Hypertension (%)			
Pre-stent	79	63	78
Post-stent	82	83	81
Use of antihypertensive medication (%)			
Pre-stent	75	50	83
Post-stent	67	67	67
Post-stent 24-hour ABPM (mm Hg)			
Daytime	135 (±23)/79(±10)	133 (±19)/81 (±8)	135 (±25)/78 (±11)
Nighttime	121 (±21)/68 (±10)	119 (±23)/68 (±9)	121 (±21)/68 (±11)
24-Hour	130 (±22)/75 (±10)	129 (±19)/77 (±7)	129 (±23)/74 (±11)

Values are expressed as mean±SD or median (ranges) for continuous variables and percentages for dichotomous variables. †p<0.05 vs. native COA; ABPM, ambulatory blood pressure monitoring; BP, blood pressure; COA, aortic coarctation; reCOA, recurrent aortic coarctation.

lesion decreased from 3.6±0.6 to 2.4±0.7 m/s and no differences were found between the native vs. reCOA group. The percentage of patients with a diastolic run-off decreased from 67 to 15%. Left ventricular function improved slightly after the procedure (not significant).

Additional imaging by CT-scan or MRI was obtained in 20 patients (91%) at a median follow-up of 8 (0.3-33) months. No pseudo-aneurysm or dissection was found. Late complications occurred in 3 patients (13.6%, all reCOA). One patient had a stent migration to the ascending aorta one and a half year after the procedure which was recognized by echocardiography and confirmed by a CT-scan. This was treated by surgical correction of the aortic arch by means of graft interposition. The second patient had drug-resistant hypertension and a pseudo-aneurysm was discovered during a diagnostic angiography for assessment of the invasive gradient over the stent. This was treated by a covered stent. One patient with groin hematoma after the index procedure was diagnosed with an occluded external iliac artery and neurological complaints on the right leg. The occlusion was opened by urokinase treatment followed by iliac stent placement.

BP data are shown in Table 4. Before stenting, 19 patients (79%) were hypertensive and 18 (75%) were treated with antihypertensive agents. After a median follow-up of 27 (1-65) months, BP data was available of 22 patients. A small decrease in BP was observed (not significant) and 14 patients (67%) were treated with antihypertensive drugs. There was a trend (p=0.066) towards a decreased median arm-to-leg BP gradient after stent implantation. Twenty patients also underwent 24-hour ABPM, 15 (6-44) months after the procedure, of whom 7 (35%) were hypertensive during daytime. No BP differences were found between the native COA and reCOA group, except a lower diastolic BP in the reCOA group after stenting (p=0.038).

DISCUSSION

In this study we report our 6 years experience of stent implantation for native COA and reCOA in adults. Several studies about stent implantations for COA have been published in the past two decades.[3-10] Some conclude that, in a selected patient group, stent implantation is feasible, safe and an effective alternative to balloon angioplasty or surgery.[6, 7, 10-12] Others describe very promising results regarding BP reduction, but only after short-term follow-up.[8] Since hypertension is a main determinant of cardiovascular morbidity and mortality in patients with repaired COA,[14, 15] BP reduction is a major goal in this population. Stenting was successful in all, but two patients. In addition, the arm-to-leg systolic gradient fell sufficiently. These findings both point to a good relief of the obstruction, however, we found only a small decrease in upper body systolic BP after stenting (not significant). Our data parallel other reports that hypertension may persist after COA stenting in adult life, probably due to structural and functional abnormalities of the arterial wall, which can result in diminished arterial wall compliance and increased rigidity.[16-20] Our study confirms that COA is not only a located narrowing of the aorta, but indeed a systemic cardiovascular disease.[13]

COA stenting may result in serious complications, as illustrated in our series. We observed 3 major complications: fatal vessel rupture, pseudo-aneurysm formation during follow-up

and stent migration. Vascular complications have been reported in the literature.[21] The most common complication described is pseudo-aneurysm formation occurring in 0–11%.[5, 11, 22] The use of a covered stent as primary choice remains somewhat controversial.[23] It aims to prevent pseudo-aneurysm formation, however, it may result in unwanted occlusion of side branches, also when it has to be deployed in the descending aorta after accidental embolization. Whether the fatal outcome in the patient with vessel rupture could have been prevented by using a covered stent is unclear. Deployment of a covered stent within the bare stent proved effective on angiographic control, but due to secondary pulmonary haemorrhage with severe respiratory failure the clinical outcome was dramatic. Direct availability of covered stents in the catheterization laboratory is a prerequisite for interventional treatment of COA. Distal stent migration or embolization also has been described before.[21] It is usually managed by positioning the stent lower in the descending aorta. Proximal migration of the stent has not been described to our knowledge. Surgical removal of the stent with arch reconstruction was even more imminent, because during surgery an ulceration of the ascending aortic wall due to the stent was found. This case illustrates the wide variety in aortic arch morphology in these patients. The balloon expandable stents that are available at present are all closed cell types that adapt poorly to curved vessels. Newer stents should be developed to overcome this problem. The patient with delayed pseudo-aneurysm formation underlines the need for CT-scan or MRI during follow-up. In the one patient in whom the pseudo-aneurysm occurred, this could be successfully treated with a covered self expanding stent.

Some limitations have to be noted about this study. Only a small number of patients underwent stent implantation since we started this procedure in 2003. Furthermore, our population included both native and reCOA. Third, this was a single center report and patients were not compared to surgery or balloon angioplasty alone. Finally, 24-hour BP monitoring before stenting was not performed in the majority of the patients. Post-stent 24-hour ABPM is therefore difficult to translate in terms of BP reduction.

In conclusion, (re)COA stenting in adults results in significant gradient decrease and increase in vessel diameter. However, serious complications do occur and hypertension remains in the majority of patients. Large studies comparing surgical techniques, ballooning or ballooning with stent placement are warranted.

Acknowledgements: We would like to thank all referring cardiologists for their help in completing the follow-up data.

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Based on:

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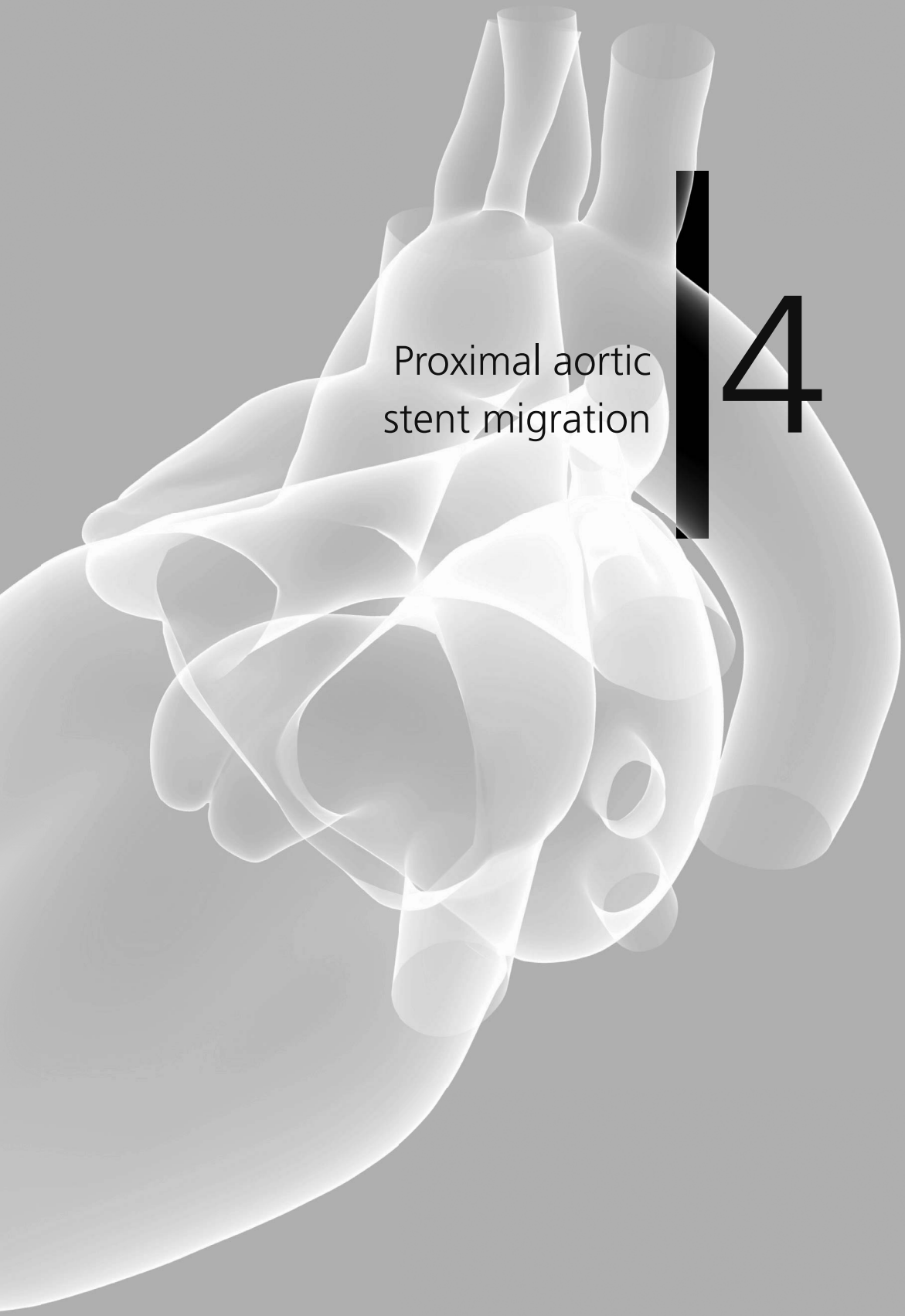
Submitted



4 Proximal aortic stent migration

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ABSTRACT

A 21-year old man underwent stent implantation for recurrent aortic obstruction, after repair of an aortic interruption type A. Stent implantation was uncomplicated with complete relieve of the systolic gradient. One year later, follow-up by transthoracic echocardiography identified a more proximal position of the stent, which was confirmed by a CT-scan. Additional stent placement was considered, yet surgical reconstruction was our final decision. During surgery, an unexpected ulceration of the ascending aortic wall due to an eroding effect of the proximal stent end was found. Repair of this potentially lethal lesion was included in the surgical reconstruction as well.

CASE

A 21-year old man underwent stent implantation (8Z45CP stent on 18 mm BiB-balloon NuMED, Heart Medical Europe BV, Best, The Netherlands) for a recurrent aortic obstruction, after previous repair of an aortic interruption type A. At the age of two weeks an end-to-side anastomosis of the descending aorta on the distal arch (left subclavian artery) was performed. Diagnosis of restenosis was based upon upper limb hypertension, a diastolic run-off with echo-doppler, a 30% narrowing of the aorta on MRI and an invasive systolic gradient of 36 mm Hg (Figure 1 left). Stenting was followed by post-dilation with a 20 mm high pressure balloon. The peak systolic gradient decreased from 15 to 0 mm Hg and the diameter increased from 11 to 17 mm (Figure 1 middle). One year later, transthoracic echocardiography (TTE) suggested a more proximal position of the stent, which was confirmed with a CT-scan. Systolic gradients were measured during diagnostic catheterization (Figure 1 right) and the position of the stent was further visualized with TTE (Figure 2 upper). The case was discussed with internal and external experts. Two treatment options were considered. One option was fixation of the stent with one or two distal stents including redilation of the narrowed segment. The second option was surgical removal of the stent with reconstruction of the aortic arch. Although votes were divided, surgical intervention was our final decision. An aortic arch reconstruction was performed with removal of the stent and the stenotic part of the aorta, with interposition of a 24 mm vascular graft. During surgery, an unexpected ulceration of the ascending aortic wall due to an eroding effect of the proximal stent end was found. This potentially lethal lesion was included in the reconstruction. Surgery was successful and postoperative recovery was uncomplicated (Figure 2 lower).

DISCUSSION

Migration of aortic stents for recurrent or native aortic coarctation (COA) is not uncommon. In a large multi-institutional study including 565 procedures for recurrent or native COA, stent migration was reported as the most frequent technical complication, occurring in 5% of the procedures.[1] These were all immediate stent migrations, and stents generally moved

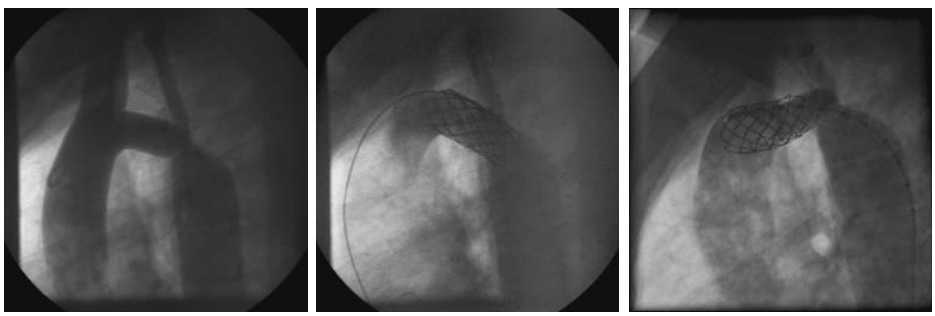

Figure 1. Lateral projections of the aortic arch. Left: Aortic arch before stent implantation, demonstrating the local narrowing in the distal aortic arch, followed by a post-stenotic dilation of the descending thoracic aorta. Middle: The aortic arch immediately after stent implantation. Right: The aortic arch at follow-up, the stent has migrated to the transverse arch and is proximal free from the aortic wall.

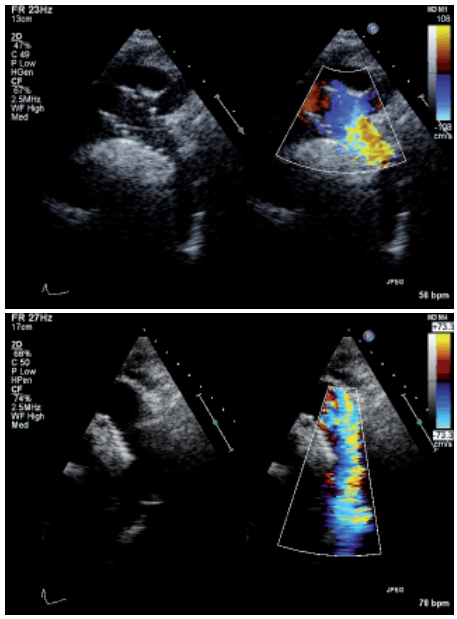

Figure 2. Transthoracic echocardiography. Before surgical reconstruction; the stent has migrated proximally (upper). Follow-up after surgical aortic arch reconstruction with a vascular graft shows no residual narrowing over the aorta (lower).

antegrade in the direction of the flow to a more distal position in the descending aorta. Such a complication can be corrected either by repositioning of the stent in the optimal position or deployment of the stent in the descending aorta, followed by a new stent implantation at the narrowed site.[1-3] The occurrence of late migration has been reported sporadically. For example, one asymptomatic delayed stent migration was detected 3 weeks after the procedure and was displaced distally.[4] Pilla et al described two cases of late stent migration; one showed slight distal migration, associated with small aneurysm formation, but the stent was still covering the COA site. The second was found in the abdominal aorta, requiring new stent implantation.[5] These authors suggest that late stent migration probably occurs early after implantation, but is detected later during follow-up. Therefore, accurate imaging is necessary before discharge and during follow-up. To our knowledge, retrograde migration of an aortic stent for COA into the ascending aorta has only been reported once. In a 15-year-old girl, a stent was placed for recurrent COA; during final pressure measurement the stent was dislodged and moved into the ascending aorta, just above the aortic valve. The stent was removed surgically and the aortic arch was reconstructed with a patch.[6] Our decision for a surgical approach was based upon the concern of the presence of stents in the transverse arch, with a possible risk of cerebral embolization of vascular tissue fragments during a new catheter intervention. Also, the sharp angle in the distal arch made it unattractive for placement of rigid and overlapping stents, which probably would not result in a nice curved distal aortic arch. This case illustrates the need for the development of large stents that combine good radial strength with more flexibility for use in curved vessels. It also illustrates that an inappropriately positioned stent may result in a potentially life threatening eroding aortic lesion and that surgical reconstruction of the aortic arch was safe and sound. This should be taken into account in the decision for surgery versus a new catheter intervention in similar cases.

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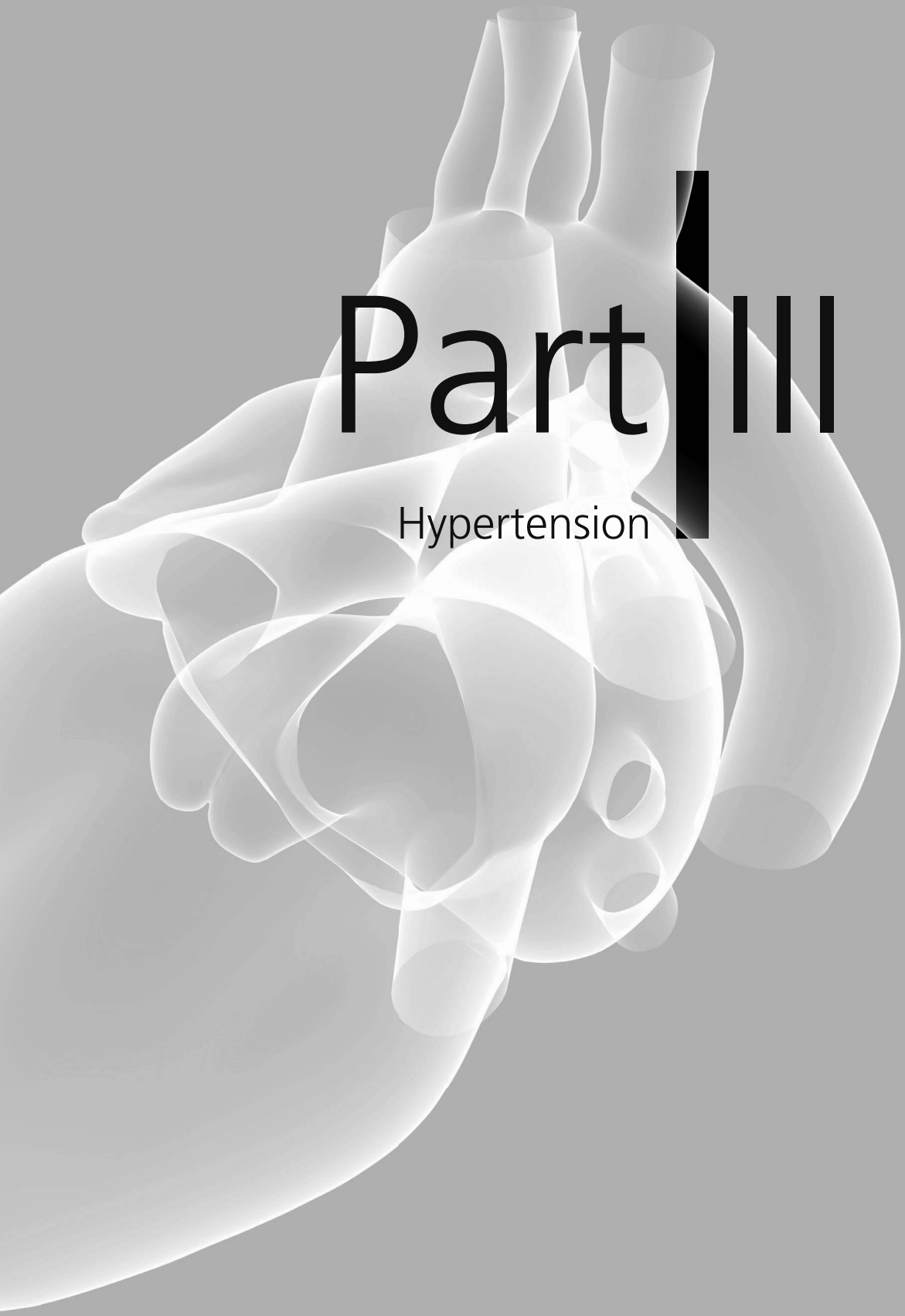

Part III

Hypertension

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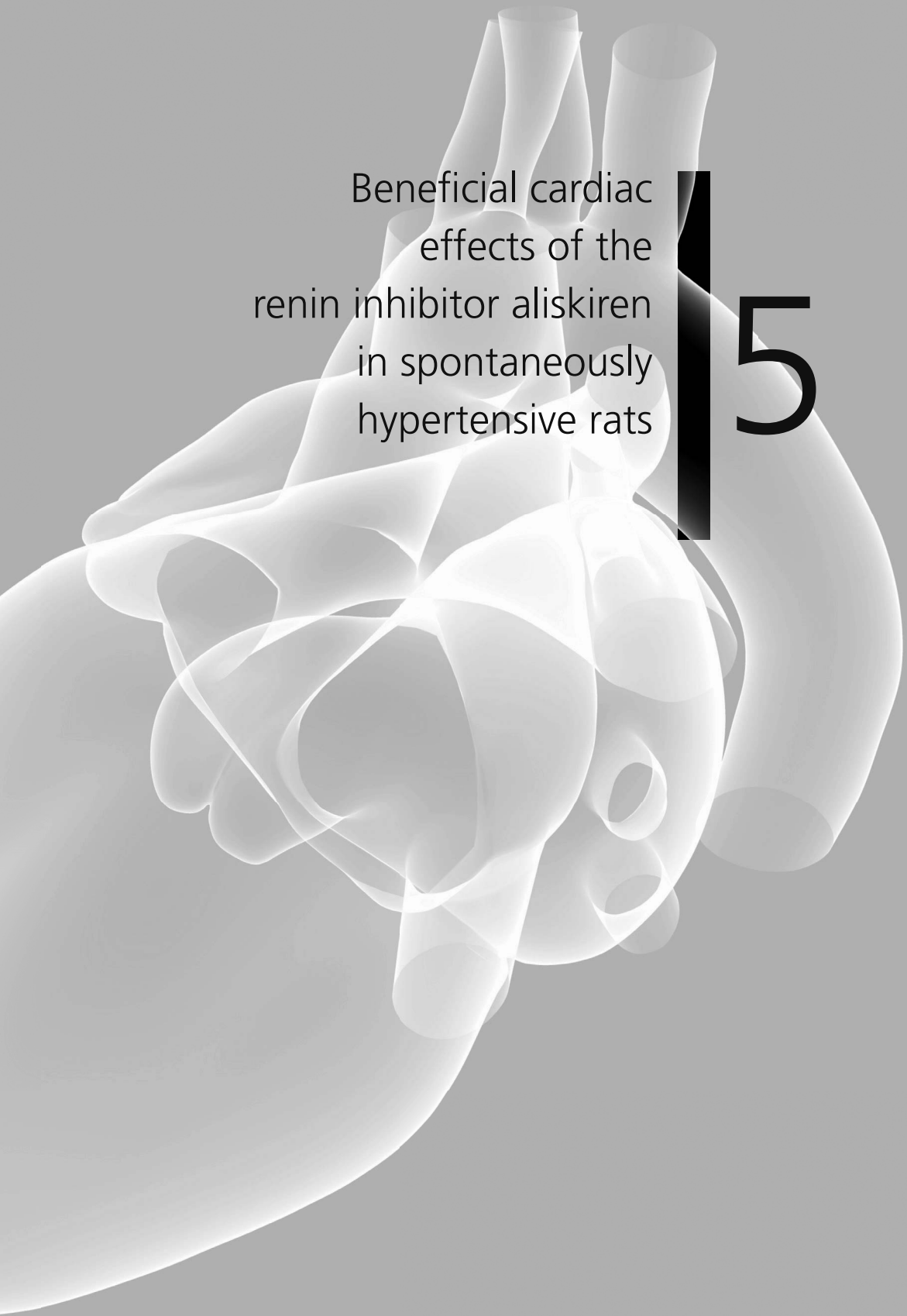
Journal of Hypertension 2010 28:2145-2155

Beneficial cardiac effects of the renin inhibitor aliskiren in spontaneously hypertensive rats

5



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ABSTRACT

The blood pressure-lowering effect of the renin inhibitor aliskiren equals that of ACE inhibitors and angiotensin (Ang) II type 1 (AT_1) receptor blockers. Whether aliskiren offers end-organ protection remains to be investigated. Here, we compared the cardiac effects of aliskiren, the AT_1 receptor blocker irbesartan and the ACE inhibitor captopril in spontaneously hypertensive rats (SHR) at equi-hypotensive doses. SHR were treated for 1-3 weeks with vehicle, aliskiren, captopril or irbesartan (100, 3 and 15 mg/kg per day, respectively) using an osmotic minipump, and compared to vehicle-treated Wistar-Kyoto controls. All drugs lowered (but not normalized) mean arterial pressure in SHR equi-effectively, as monitored by radiotelemetry, without altering heart rate. All drugs also reduced the increased cardiomyocyte area in SHR, and tended to normalize the elevated B-type natriuretic peptide plasma levels. In the Langendorff setup, all drugs normalized the diminished endothelium-dependent vasodilator response to bradykinin in SHR. Moreover, aliskiren and irbesartan, but not captopril, decreased the enhanced coronary Ang II response in SHR. Aliskiren reduced plasma renin activity and the plasma and tissue angiotensin levels after one week of treatment; yet, after 3 weeks of aliskiren treatment, only the cardiac angiotensin levels remained suppressed, while no tissue angiotensin reductions were seen with captopril or irbesartan. In conclusion, for a given decrease in blood pressure, aliskiren improves coronary endothelial function and decreases cardiac hypertrophy in SHR to at least the same degree as ACE inhibition and AT_1 receptor blockade. In addition, aliskiren diminishes the enhanced Ang II response in the coronary circulation of SHR and offers superior longterm cardiac angiotensin suppression.

INTRODUCTION

Aliskiren is a direct renin inhibitor that lowers blood pressure and promotes left ventricular mass reduction at least as effectively as other blockers of the renin-angiotensin system (RAS).[1-3] When given on top of angiotensin (Ang) II type 1 (AT_1) receptor blockade, it has incremental blood pressure-lowering[1] and left ventricular mass-reducing effects,[2] although only the former were statistically significant from that of the AT_1 receptor blocker alone. In patients with symptomatic heart failure, aliskiren, when added on top of an angiotensin-converting enzyme (ACE) inhibitor (or an AT_1 receptor antagonist) and a beta-blocker for 3 months, improved B-type natriuretic peptide (BNP), urinary aldosterone, and mitral regurgitation.[3] These additive effects of aliskiren on top of what is currently considered 'optimal' RAS blockade may relate to the capacity of this drug to suppress the consequences of the reactive rise in renin that occurs normally when blocking the RAS.[4] In fact, at a dose of 300 mg, within 5 hours the plasma aliskiren levels are high enough to block plasma renin by more than 99%, even when renin levels have increased more than 10-fold.[5]

Due to the high species-specificity of the renin-angiotensinogen reaction, animal data allowing a more detailed insight into the cardioprotective effects of aliskiren are scarce. In double transgenic (dTGR) rats, expressing both the human renin and angiotensinogen gene, aliskiren decreases blood pressure and cardiac hypertrophy at clinically relevant doses.[6] However, given the exceptionally high Ang II levels in these animals (up to 20 times normal), causing them to die within 7 weeks without treatment, as well as the absence of RAS feedback mechanisms in dTGR, it is uncertain to what degree such data reflect 'normal' physiology. In spontaneously hypertensive rats (SHR), a well-established rat model that responds favorably to RAS blockade, aliskiren lowers blood pressure only when used at a high dose of 100 mg/kg per day.[7]

In the present study, we used of this high dose in SHR to compare the effects of aliskiren with those of the AT_1 receptor blocker irbesartan and the ACE inhibitor captopril on cardiac hypertrophy, coronary hemodynamics, and tissue and plasma RAS components. Great care was taken to select doses of irbesartan and captopril that lowered blood pressure to the same degree as aliskiren, in order to rule out differences in cardiac efficacy that are the consequence of differences in antihypertensive efficacy.

METHODS

Animals and drugs

Male SHR (280-300 g; n=74) and their normotensive Wistar-Kyoto controls (WKY: 280-300 g; n=15) were obtained from Charles River (Germany). SHR and WKY were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands. Captopril, Ang II, bradykinin and sodium nitroprusside (SNP) were purchased from Sigma. Irbesartan and aliskiren were kind gifts of Sanofi-Synthelabo (Gouda, The Netherlands) and Novartis Pharmaceuticals (Basel, Switzerland), respectively. To allow administration via osmotic minipumps, irbesartan (87.5-175 mg/ml) was dissolved in saline in the presence of 4% vol/

vol NaOH (after which the pH was adjusted to 7-9 by equilibration with carbogen).[8] Captopril (17.5-35 mg/ml) and aliskiren (583.3 mg/ml) were dissolved in saline. Ang II and bradykinin for in-vitro use were dissolved in water, and stock solutions were stored in aliquots at -80ºC.

In vivo studies

Rats were anesthetized by inhalation of isoflurane (Rhodia Organique Fine Limited) in air. A radio-telemetry transmitter (TA11PA-C40, Datascience Inc) was implanted into the abdominal cavity with the fluid-filled catheter placed into the lower abdominal aorta. After surgery, rats were treated with Temgesic® (buprenorphine, Reckitt and Colman) for two days (0.1 and 0.05 mg/kg on the first and second day, respectively) and were allowed to recover for an additional 9 days. Next, baseline telemetry measurements (mean arterial pressure (MAP) and heart rate (HR)) were obtained during the next two days. Then (day 0) osmotic minipumps (2ML4 Alzet) were implanted subcutaneously under isoflurane anesthesia to infuse vehicle (saline) or drugs. Infusions lasted 1-3 weeks, i.e., until day 7 or day 21.

At the end of the infusion period, animals were anesthetized by inhalation of isoflurane. The hepatic portal vein was cannulated to collect 2 ml of blood; 1 ml was centrifuged for 5 min at 13000 rpm to obtain blood plasma and 1 ml was collected in 10 ml of 4 mol/L guanine thiocyanate. Both were stored at -80ºC until further processing. Hearts were rapidly excised and either placed in ice-cold Tyrode's buffer (for the Langendorff studies) or stored at -80ºC (for angiotensin measurements). Kidneys were also rapidly excised and stored at -80ºC (to study AT_1 receptor expression).

In vitro studies and histology

Hearts were buffer-perfused according to Langendorff as described before.[9] Coronary flow was measured with a flow probe (Transonic systems). After a stabilization period of 30 min, baseline values of coronary flow were obtained. Next, bolus injections (100 µl) of Tyrode's buffer were applied 3 times to determine injection-induced changes in coronary flow. Concentration-response curves to bradykinin and Ang II (0.1 nmol/L to 10 mmol/L) were constructed by bolus injections, after which the maximum coronary flow was determined by injecting 10 mmol/L SNP. Next, the hearts were collected, and the ventricular heart weight was determined after removal of the atria and large vessels to allow the calculation of the heart weight/body weight (HW/BW) ratio. Ventricles were then cut into 3 transversal slices and fixed in a 3.5-4% formaldehyde solution (Boom). After fixation, the slices were dehydrated and paraffin-embedded. Gomori's silver staining was applied to deparaffinized 5-µm thick sections of the left ventricle to visualize individual cardiomyocytes.[10] Only transversally cut cells showing a nucleus were used to determine the cardiomyocyte area.

Biochemical measurements

Plasma renin activity (PRA) and plasma renin concentration (PRC) were measured as described before.[11] PRC was determined after diluting the samples 1:10 in assay buffer, to dissociate aliskiren from renin.[12] Plasma and tissue angiotensin levels were determined by radioimmunoassay following SepPak extraction and high-performance liquid chromatography separation.[13] Plasma BNP-45 (BNP-45) was measured by enzyme immunoassay method

(Phoenix Pharmaceuticals Inc). Western blot analysis was applied to quantify the renal AT_1 receptor content. In brief, kidneys were homogenized with a Polytron PT2100 (Kinematica AG) in ice-cold Lysisbuffer. SDS-PAGE was performed on 10% polyacrylamide gels. Each lane was loaded with 20 µg protein. After transfer, nonspecific sites were blocked with 5% milk in PBS-Tween. Then, the blots were incubated overnight with 1:400 anti AT_1 receptor (N-10; Santa Cruz Biotechnology) and 1:20.000 anti actin (C4; Millipore). After washing, the sites of antibody-antigen reaction were visualized with 1:5000 horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) using the enhanced chemiluminescence Western blotting detection system (Pierce Biotech). Signal intensities were quantified by scanning densitometry (Bio-rad Laboratories).

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated from kidneys using the Trizol reagent (Gibco-BRL) and reverse transcribed. The resulting cDNA was amplified in 40 cycles (denaturation at 95° C for 10 min; thermal cycling at 95°C for 15 sec, annealing/extension at 60°C for 1 min) with a Step-One cycler (NYSE, Applied Biosystems) using the SYBR Green Q-PCR core KIT (Eurogentec). Primers (forward 5'-ACTGCCTGAACCCTCTGTTC-3', reverse 5'-TCGTAGACAGGCTTGAGTGG-3') were from Invitrogen. The comparative cycle time method (ΔΔCT) was used for relative quantification of gene expression. Messenger RNA expression was normalized versus GAPDH and actin and expressed as the ratio of target to control value.[14]

Table 1. Heart rate (HR) and mean arterial blood pressure (MAP) at baseline, the maximum decrease in MAP (ΔMAP) and ΔMAP at day 7, 14 and 21 after the start of treatment, the area over the curve (AOC), and body weight (BW), heart weight (HW) and the HW/BW ratio at the end of the treatment period, in SHR and WKY rats treated for 3 weeks with vehicle, aliskiren, captopril, or irbesartan.

	WKY Vehicle	**SHR Vehicle**	**SHR Aliskiren**	**SHR Captopril**		**SHR Irbesartan**	
	Control	Control	100 mg/kg per day	3 mg/kg per day	6 mg/kg per day	15 mg/kg per day	30 mg/kg per day
N	5	10	12	7	2	10	5
Baseline HR (bpm)	366±2	326±4†	328±4	329±4	341±4	334±5	332±8
Baseline MAP (mm Hg)	102±2	146±3†	151±3	143±4	158±22	147±3	149±5
ΔMAP							
Maximum	-3±4	-1±1	-29±2*	-22±6*	-29±7*	-20±2*	-53±2*
Day 7	-1±2	-3±1	-22±3*	-17±6*	-28±8*	-18±3*	-46±7*
Day 14	-1±2	3±1	-14±2*	-16±6*	-28±6*	-10±2*	-43±9*
Day 21	-1±1	4±1	-10±3	-13±6*	-25±5*	-7±2*	-45±2*
AOC MAP (mm Hg x days)	70±30	68±10	388±36*	356±128	546±143*	325±39	923±100*
BW (g)	431±5	385±4†	360±4*	367±6*	369±3	372±3*	371±7
HW (g)	1.17±0.04	1.39±0.04†	1.29±0.04	1.26±0.04	1.40±0.02	1.31±0.04	1.24±0.06*
HW/BW (g/kg)	2.71±0.08	3.61±0.08†	3.57±0.09	3.42±0.09	3.79±0.08	3.52±0.10	3.49±0.26

Data are mean±SEM. †p<0.05 vs. vehicle-treated WKY rats; *p<0.05 vs. vehicle-treated SHR.

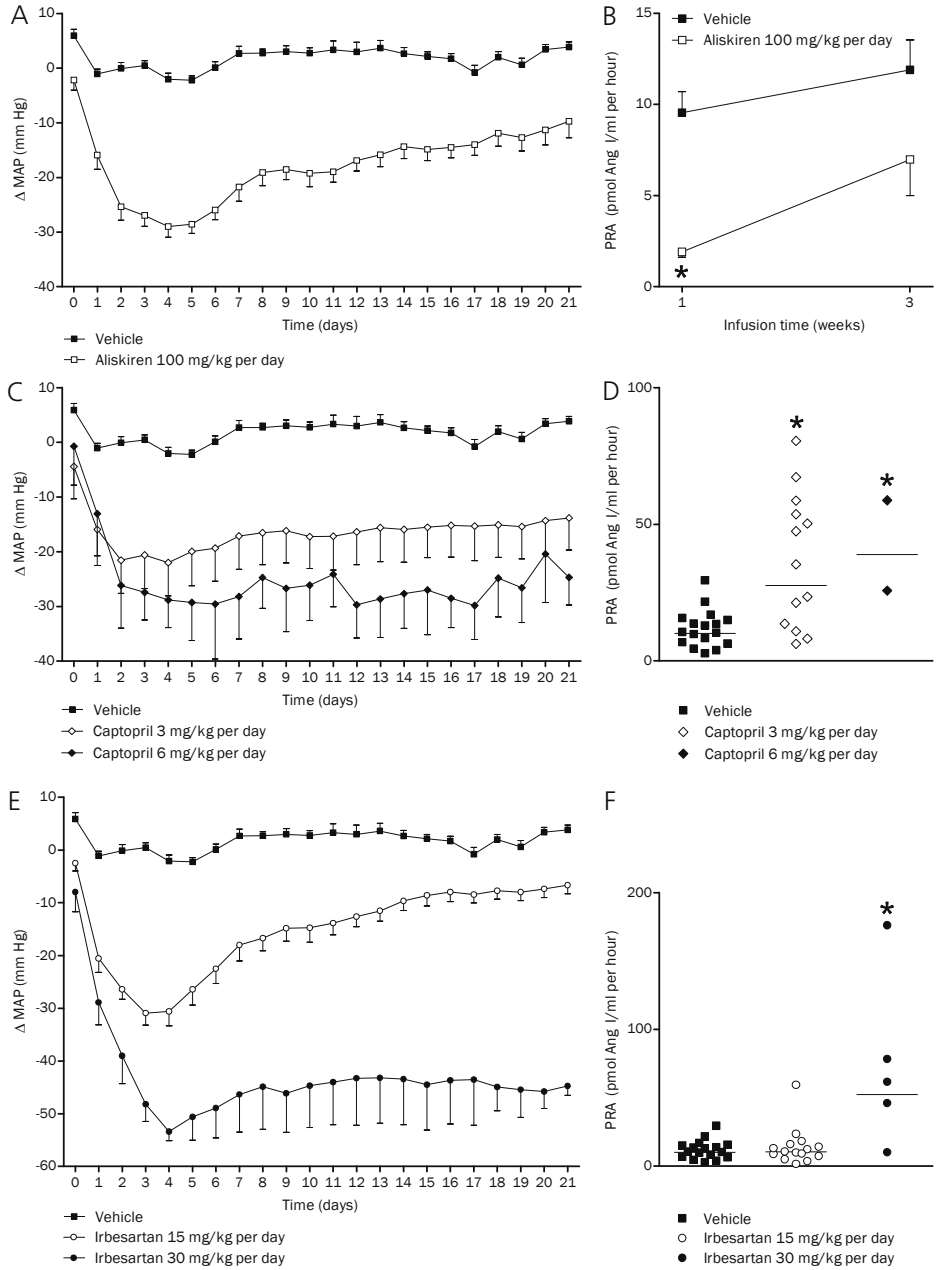

Figure 1. ΔMAP during a 3-week infusion of aliskiren (**A**), captopril (**C**) and irbesartan (**E**) versus vehicle in SHR. Plasma renin activity (PRA) on day 7 and 21 after treatment of aliskiren (**B**), and after a 3-week infusion of captopril (**D**) or irbesartan (**F**). Data are mean±SEM or individual values and geometric mean (horizontal bar). *p<0.05 vs. vehicle.

Data analysis

Telemetric data were recorded and digitalized using the Dataquest Acquisition & Analysis system (DQ ART 3.1 Silver, Datascience Inc). Each animal was sampled for 10 sec at 10-min intervals for a period of 23 days. All recordings were averaged per day and baseline values were calculated using the data from the first two days of measurement before treatment was started. Changes in blood pressure from baseline were analyzed by comparison of the areas over the curve (AOC), as calculated by the trapezoidal method (mm Hg x days).[7, 15] Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments). After manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc). Six consecutive beats were selected for determination of coronary flow. Statistical analysis between groups was performed by Student's t test or one-way analysis of variance (ANOVA), followed by post-hoc evaluation according to Dunnet, $p<0.05$ was considered significant.

RESULTS

Baseline hemodynamics and effect of vehicle treatment

At the start of the study, body weights of SHR (280±2 g, n=74) and WKY (280±5 g, n=15) were identical. Baseline MAP was higher in SHR than WKY, whereas the opposite was true for baseline HR (Table 1). Vehicle did not affect MAP or HR in either strain. At the end of the 21 day-treatment period WKY had gained more weight than SHR, in full agreement with previous findings.[16, 17]

Hemodynamics after RAS blockade in SHR

Aliskiren 100 mg/kg per day lowered MAP (Table 1 and Figure 1A), without affecting HR (data not shown). MAP was maximally reduced at the fourth day after the start of infusion.

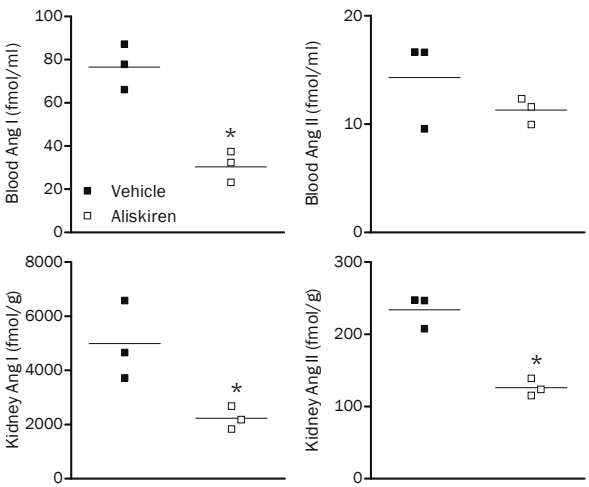

Figure 2. Ang I and Ang II levels in blood plasma and kidney after a 1-week treatment of SHR with vehicle or aliskiren. Data (n=3) are represented as scatter dot plot. The horizontal bar represents the geometric mean. *p<0.05 vs. vehicle.

Thereafter, the effect of aliskiren leveled off, but MAP remained reduced at day 7, 14 and 21. Irbesartan (15-30 mg/kg per day) and captopril (3-6 mg/kg per day) reduced MAP at all tested doses (Table 1 and Figures 1C and E, respectively). The blood pressure-lowering effects of the two highest doses of irbesartan and captopril, estimated from the AOC values and the MAP decreases at day 7, 14 and 21 (Table 1), were larger than that of aliskiren, whereas the effects of the two lowest doses of both drugs were comparable to that of aliskiren. HR was unaltered during treatment with these lower doses (data not shown). Based on these findings, all further studies were performed with aliskiren 100 mg/kg per day, irbesartan 15 mg/kg per day and captopril 3 mg/kg per day.

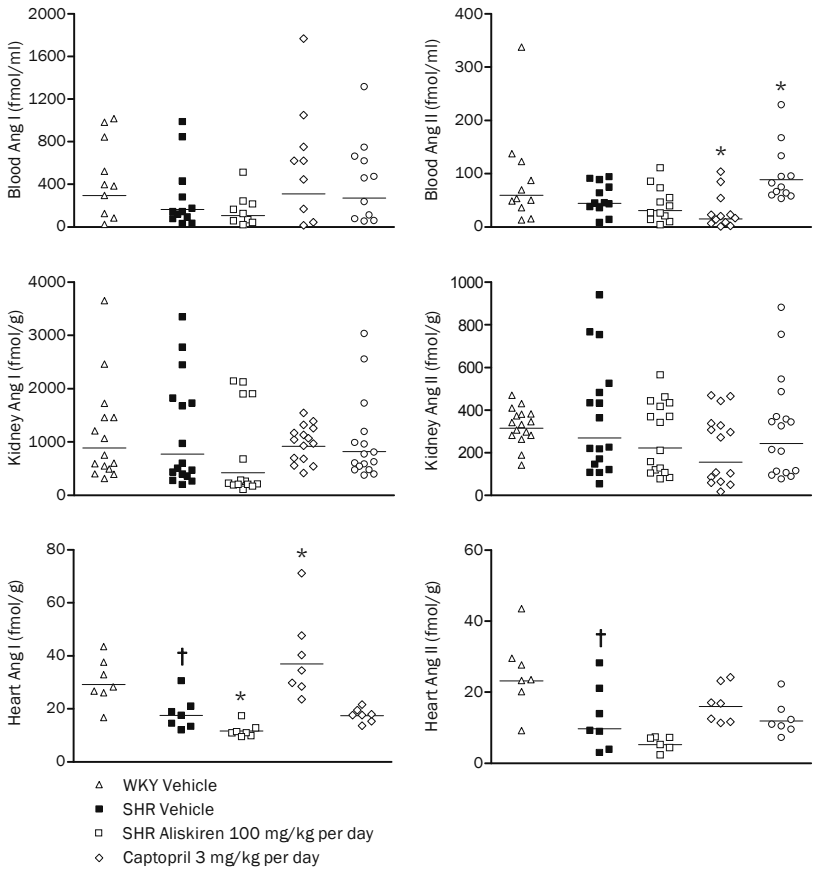

Figure 3. Ang I and Ang II levels in blood plasma, kidney and heart after a 3-week treatment of SHR with vehicle, aliskiren, captopril, or irbesartan versus WKY treated with vehicle. Data (n=6-16) are represented as scatter dot plot. The horizontal bar represents the geometric mean. †p<0.05 vs. vehicle-treated WKY rats; *p<0.05 vs. vehicle-treated SHR.

Plasma renin activity, plasma renin concentration, angiotensins and BNP

After 3 weeks of vehicle treatment, PRA levels in WKY and SHR (12.7±2.0 vs. 11.9±1.7 pmol Ang I/ml per h) were identical. Aliskiren reduced PRA levels in SHR after one week by 80% ($p<0.0001$; Figure 1B) in comparison to the vehicle treated group. This effect tended to disappear after 3 weeks of treatment. In comparison to vehicle-treated SHR, both doses of captopril and the highest dose of irbesartan increased PRA after 3 weeks of treatment (Figures 1D and 1F, respectively). PRC levels after 3 weeks in untreated SHR, and in SHR treated with aliskiren, captopril (3 and 6 mg/kg per day) or irbesartan (15 and 30 mg/kg per day) were 9.3±0.9, 11.3±1.9, 27.5±23.1, 87.1±61.0 ($p<0.05$ vs. untreated), 12.0±4.3, and 143±33.5 ($p<0.05$) pmol Ang I/ml per h, respectively.

In agreement with the aliskiren-induced reduction in PRA after one week, plasma Ang I, renal Ang I and renal Ang II were also reduced after one week of aliskiren exposure ($p<0.05$; Figure 2). The modest reduction in plasma Ang II at this time point was not significant. Like the effect on PRA, these effects had disappeared after 3 weeks of treatment (Figure 3). In contrast, cardiac Ang I in SHR remained reduced after 3 weeks of aliskiren exposure ($p<0.05$; Figure 3), and a similar trend was observed for cardiac Ang II ($p=0.09$; Figure 3). The total cardiac angiotensin content (i.e., the sum of Ang I and II) was also reduced after aliskiren ($p<0.02$).

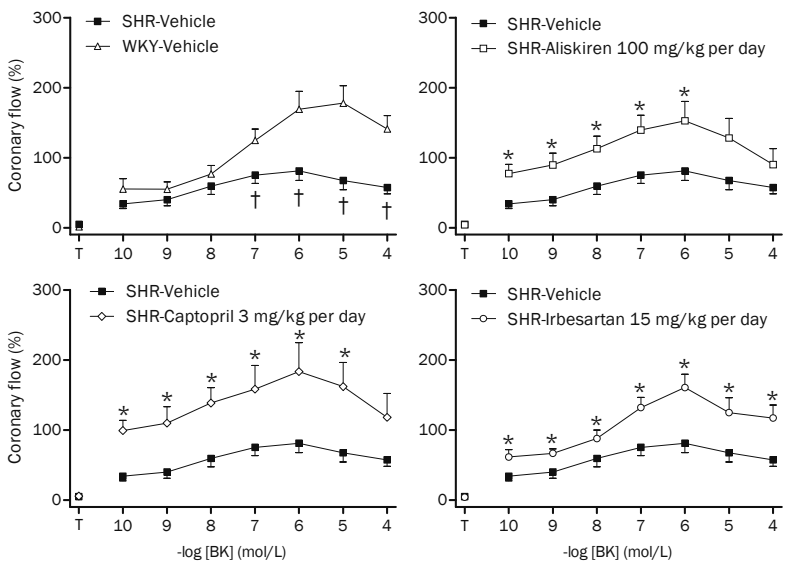

Figure 4. Effect of bradykinin (BK) bolus injections (100 µL) on coronary flow after a 3-week treatment of WKY rats with vehicle vs. SHR treated for 3 weeks with vehicle (upper left), aliskiren (upper right), captopril (lower left) or irbesartan (lower right). Data (mean±SEM, n=6-10) were obtained using the Langendorff heart preparation, and represent % change from baseline. T represents the effect of a bolus injection with Tyrode's buffer. †$p<0.05$ vs. vehicle-treated WKY rats; *$p<0.05$ vs. vehicle-treated SHR.

As expected, irbesartan increased plasma Ang II, and captopril decreased plasma Ang II after 3 weeks of treatment ($p<0.05$ for both). With the exception of a captopril-induced increase in cardiac Ang I ($p<0.05$), no significant changes in tissue Ang I and II levels were observed after either captopril or irbesartan, nor did these drugs affect the total cardiac angiotensin content. After 3 weeks of vehicle treatment, the plasma and renal Ang I and II levels in SHR were identical to those in WKY, whereas the cardiac Ang I and II levels in SHR were lower than those in WKY ($p<0.05$; Figure 3).

BNP levels in vehicle-treated SHR (87±7 pg/ml, n=7) were higher than in WKY (55±2 pg/ml n=10; $p<0.01$). A three-week treatment with aliskiren, captopril or irbesartan tended to normalize BNP (65±6, 71±4 and 64±3; not significant vs. vehicle treatment).

Langendorff studies

Baseline coronary flow of vehicle-treated WKY (8.4±0.6 ml/min, n=6), vehicle-treated SHR (11.6±0.8 ml/min, n=9), aliskiren-treated SHR (9.6±0.6 ml/min, n=10), irbesartan-treated SHR (10.2±1.3 ml/min, n=6) and captopril-treated SHR (10.4±0.9 ml/min, n=7) were identical. Bolus injections with Tyrode's buffer injections did not significantly affect coronary flow (Figures 4 and 5). Bradykinin increased coronary flow in both vehicle-treated WKY and SHR

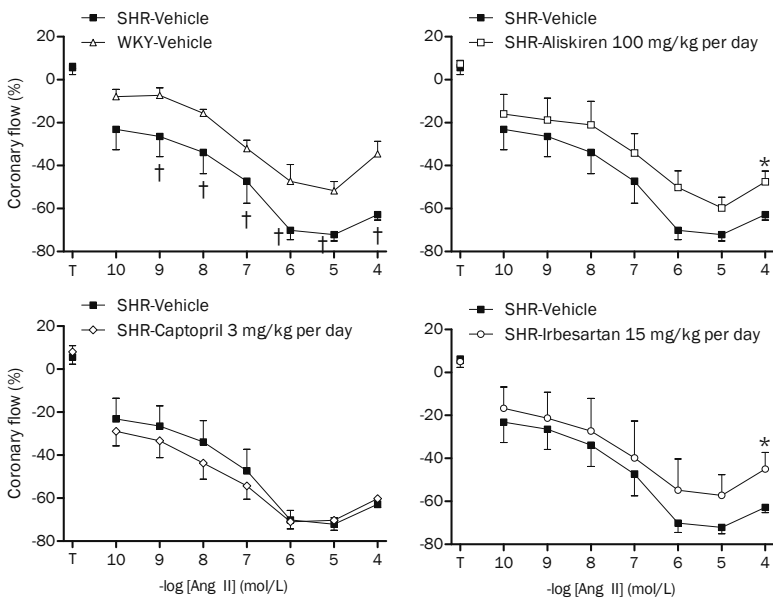

Figure 5. Effects of angiotensin (Ang) II bolus injections (100 μL) on coronary flow after a 3-week treatment of WKY rats with vehicle vs. SHR treated for 3 weeks with vehicle (upper left), aliskiren (upper right), captopril (lower left) or irbesartan (lower right). Data (mean±SEM, n=5-8) were obtained using the Langendorff heart preparation, and represent % change from baseline. T represents the effect of a bolus injection with Tyrode's buffer. †$p<0.05$ vs. vehicle-treated WKY rats; *$p<0.05$ vs. vehicle-treated SHR.

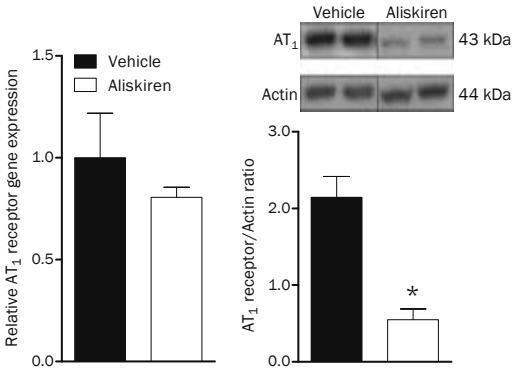

Figure 6. Expression of AT_1 receptor mRNA (left) and protein (right, ratio vs. actin; see insert for representative example) in the kidney of SHR treated for 3 weeks with vehicle or aliskiren. Data are mean±SEM (n=7-13). *p<0.05 vs. vehicle-treated SHR.

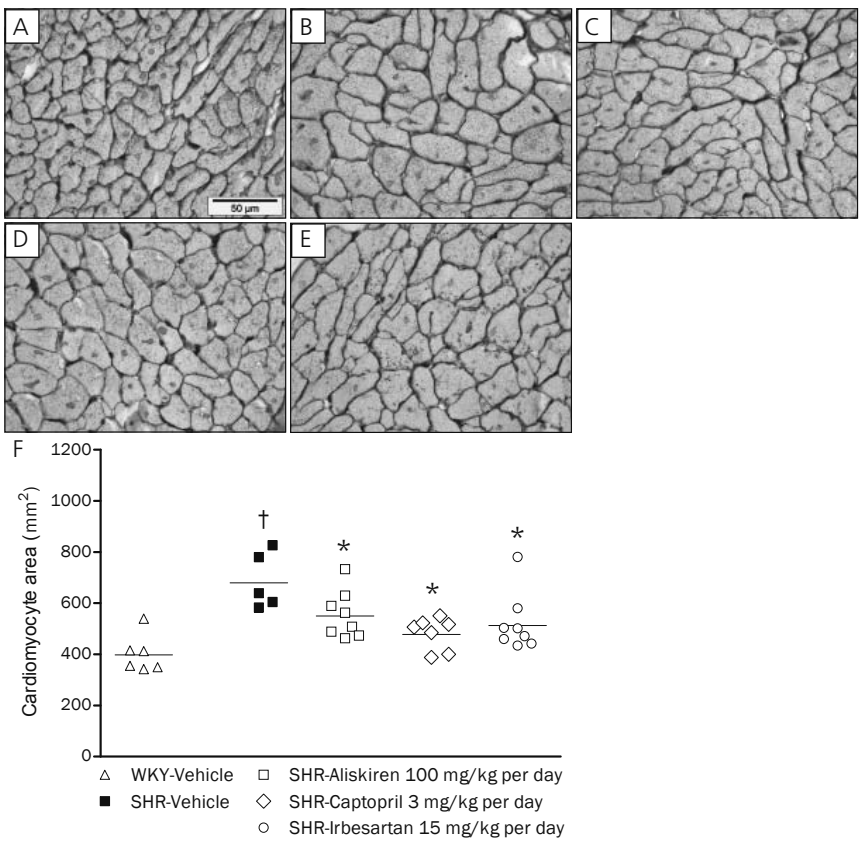

Figure 7. Gomori-stained sections showing cardiomyocytes in the left ventricular wall of hearts from WKY rats treated with vehicle (**A**), or SHR rats treated with vehicle (**B**), aliskiren (**C**), captopril (**D**), or irbesartan (**E**). The bar in picture **A** represents 50 µm. Panel F summarizes the findings on the cardiomyocyte area (mean±SEM n=5-8) in the 5 groups. †p<0.05 vs. vehicle-treated WKY rats; *p<0.05 vs. vehicle-treated SHR.

(Figure 4), but its effects were much larger in the former (E_{max}: +179±25% vs. +82±13%, p<0.05). Treatment of SHR with aliskiren, irbesartan or captopril increased the effect of bradykinin to WKY values (p<0.05 for all).

Ang II dose-dependently decreased coronary flow in both vehicle-treated WKY and SHR (Figure 5), with larger effects occurring in the latter (E_{max}: -52±4% vs. -75±3%, p<0.05). Treatment of SHR with aliskiren or irbesartan, but not captopril, normalized the effect of Ang II to WKY values (p<0.05 for both). The maximum coronary flow values (determined with SNP) were identical in vehicle-treated WKY and SHR (30.7±3.0 ml/min vs. 26.0±2.3 ml/min), and treatment of SHR with aliskiren, irbesartan or captopril did not alter these values (data not shown).

Renal AT_1 receptor expression

Western blot analysis revealed that aliskiren reduced the renal AT_1 receptor content (p<0.05; Figure 6B). A similar tendency was observed for renal AT_1 receptor gene expression (p=NS; Figure 6A).

Cardiac hypertrophy

HW/BW ratio (Table 1) and cardiomyocyte area (Figure 7) were larger (p<0.0001) in vehicle-treated SHR than in vehicle-treated WKY. Treatment of SHR with aliskiren, irbesartan or captopril did not significantly alter the HW/BW ratio, but reduced the cardiomyocyte area (p<0.05).

DISCUSSION

This study is the first to compare, head-to-head, the 3 types of RAS blockade in a well-established cardiovascular model, the SHR. This is of importance now that the renin inhibitor aliskiren has entered the clinical arena. We show that, for a given decrease in blood pressure, aliskiren improves coronary endothelial function and decreases cardiac hypertrophy to at least the same degree as ACE inhibition and AT_1 receptor blockade. Like irbesartan, it decreases the coronary vasoconstrictor effects of Ang II, although it is not an AT_1 receptor blocker. Finally, it allows cardiac angiotensin generation to remain suppressed even after 3 weeks of treatment, despite the counterregulatory mechanisms that will then have upregulated the RAS.[18-20]

The aliskiren-induced decrease in blood pressure in SHR in the present study was identical to that observed by Wood et al. using the same dose in SHR.[7] The decrease in blood pressure reached its maximum at day 4 and leveled off at the end of the 3-week infusion period, although at that time it was still reduced in comparison to vehicle-treated SHR. It is clear that this dose was insufficient to normalize blood pressure. The reason for this modest effect is the high species-specificity of the renin-angiotensinogen reaction. As a consequence, human renin inhibitors are much less potent towards rat and mouse renin,[7, 21] so that testing these drugs in rodents requires doses that are up to 100 times higher than in humans. It is for this reason that dTGR are frequently used to evaluate the consequences of human renin inhibitor treatment at 'human' doses.[6] However, given the very high angiotensin levels in these rats,

as well as the absence of normal RAS feedback mechanisms in this model, the physiological relevance of such studies is limited.

In view of the modest effect of aliskiren on blood pressure in SHR we took great care to apply doses of captopril and irbesartan that caused comparable decreases in blood pressure. Not surprisingly, these doses caused barely detectable effects on plasma and tissue angiotensin levels, whereas higher doses exerted much stronger effects, both with regard to RAS blockade (as evidenced by a larger rise in PRA) and the decrease in blood pressure. Nevertheless, aliskiren at 100 mg/kg per day reduced PRA by 80% after one week of treatment and lowered Ang I and II in blood plasma and kidney. Similar decreases in plasma and tissue Ang II levels have been observed with ACE inhibitors during short-term exposure.[20, 22] Long-term exposure usually resulted in a return of the Ang II levels to baseline or even to higher levels ('Ang II escape').[19, 20] This is due to the many feedback mechanisms, allowing rises in renin, ACE and non-ACE converting enzymes, among others, to restore Ang II generation.[23, 24] Our PRC measurements confirm such a rise in renin during high doses of captopril and irbesartan, but not during aliskiren treatment. To measure PRC in aliskiren-treated rats, we diluted plasma 10-fold. Previous studies in mice have shown that a 100-fold dilution of mouse plasma is sufficient to dissociate aliskiren from mouse renin,[12] thereby allowing quantification of mouse renin by an enzyme-kinetic assay. Unfortunately, rat renin levels are substantially lower than mouse renin levels, and thus a 10-fold dilution was the maximum we could apply to still yield detectable Ang I generation. A 10-fold dilution may not have been sufficient to fully dissociate all aliskiren from renin, and thus our PRC measurements are likely to underestimate renin. To observe a renin rise during aliskiren treatment in rats, non-enzyme-kinetic (i.e., immunoreactive) assays are required, which currently exist for human renin only.[25]

Angiotensin generation at cardiac and vascular tissue sites depends on uptake of renal renin.[13, 26] Renin inhibitors may thus have a pharmacokinetic advantage over ACE inhibitors and AT_1 receptor blockers in that they may bind to plasma renin on its way to tissue sites, allowing subsequent accumulation of the renin-renin inhibitor complex in tissues, whereas ACE inhibitors and AT_1 receptors blockers first have to get to the tissues to exert an effect.[4] Chronic treatment with aliskiren will eventually replace tissue renin by aliskiren-bound renin. This may explain why the effect of this drug lasts longer than expected on the basis of its plasma half life.[25, 27, 28] Our data are compatible with this concept, since the cardiac angiotensin content remained suppressed after 3 weeks, when counterregulatory effects had already increased PRA and normalized renal angiotensin levels. Clearly, the feedback mechanisms had not yet been able to replace all aliskiren-blocked renin at cardiac tissue sites by 'free' renin. Given this outcome, it would be of interest to see whether treatment with higher doses would also have kept the plasma and renal angiotensin levels suppressed. Unfortunately however, such studies in rats are technically impossible.

Importantly, despite the modest blood pressure-lowering effects of the 3 RAS blockers, all blockers restored the impaired coronary endothelial function in SHR (determined as the response to bradykinin) to normal, WKY levels. Although in the case of captopril part of this effect may relate to the fact that ACE degrades bradykinin, the most likely explanation of this effect is that all 3 blockers prevent Ang II from inducing oxidative stress in the vascular wall.[29, 30] Interestingly in this regard, aliskiren has been reported to suppress cardiac oxidative stress

in a more pronounced manner than other RAS blockers.[31]

Ang II exerted much stronger contractile effects in SHR than WKY, which could relate to the absence of Ang II type 2 (AT_2) receptor-mediated vasorelaxation in the coronary circulation of the SHR.[9, 32-34] Unexpectedly, aliskiren, like irbesartan, diminished the enhanced response to Ang II in SHR. If anything, one would expect an increase in Ang II potency when suppressing Ang II formation (as tended to occur during captopril treatment), since normally lowering the agonist concentration will upregulate receptor expression. However, tissue AT_1 receptor expression was found to be reduced in the present study. Given the rise in renin that normally occurs during aliskiren treatment, it is tempting to speculate that its stimulation of the recently discovered (pro)renin receptor somehow underlies this phenomenon.[35-37] Importantly, a similar decrease in AT_1 receptor density was recently observed in skeletal muscle and kidneys of transgenic TG(mRen-2)27 rats following aliskiren treatment,[38, 39] and thus AT_1 receptor suppression may be a general consequence of renin inhibition.

Given the modest effect on blood pressure, it is not surprising that none of the 3 drugs reduced HW/BW, a parameter that, at least in part, depends on blood pressure. Yet, all 3 drugs significantly decreased the enhanced left ventricular cardiomyocyte area in SHR, and, in parallel with this observation, tended to decrease BNP levels. These observations are in full agreement with those on renin inhibition in humans.[2, 3] Our study is the first to relate this to a decrease in cardiac angiotensin content, and to reveal the accompanying changes in the coronary vascular bed that may underlie these beneficial effects.

In summary, RAS blockade in SHR at the level of renin, ACE or AT_1 receptors, when using equi-hypotensive doses, offers similar cardioprotection over a 3-week period. Yet, only renin inhibition allowed cardiac angiotensins to remain suppressed after 3 weeks. Therefore, the longterm effects of renin blockade might be superior to those of ACE or AT_1 receptor blockade. Importantly, the aliskiren doses used in this study were far above those applied in humans. Nevertheless, the similarity of the aliskiren-, captopril-, and irbesartan-induced effects, as well as aliskiren's capacity to suppress angiotensins, strongly suggests that the efficacy of the renin inhibitor is related to its RAS-blocking properties rather than to non-specific effects. Ongoing long-term clinical trials will soon reveal whether our findings hold in humans as well.

Acknowledgement: This work was financially supported by a grant of the Netherlands Heart Foundation (NHF-2007B019).

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Based on:

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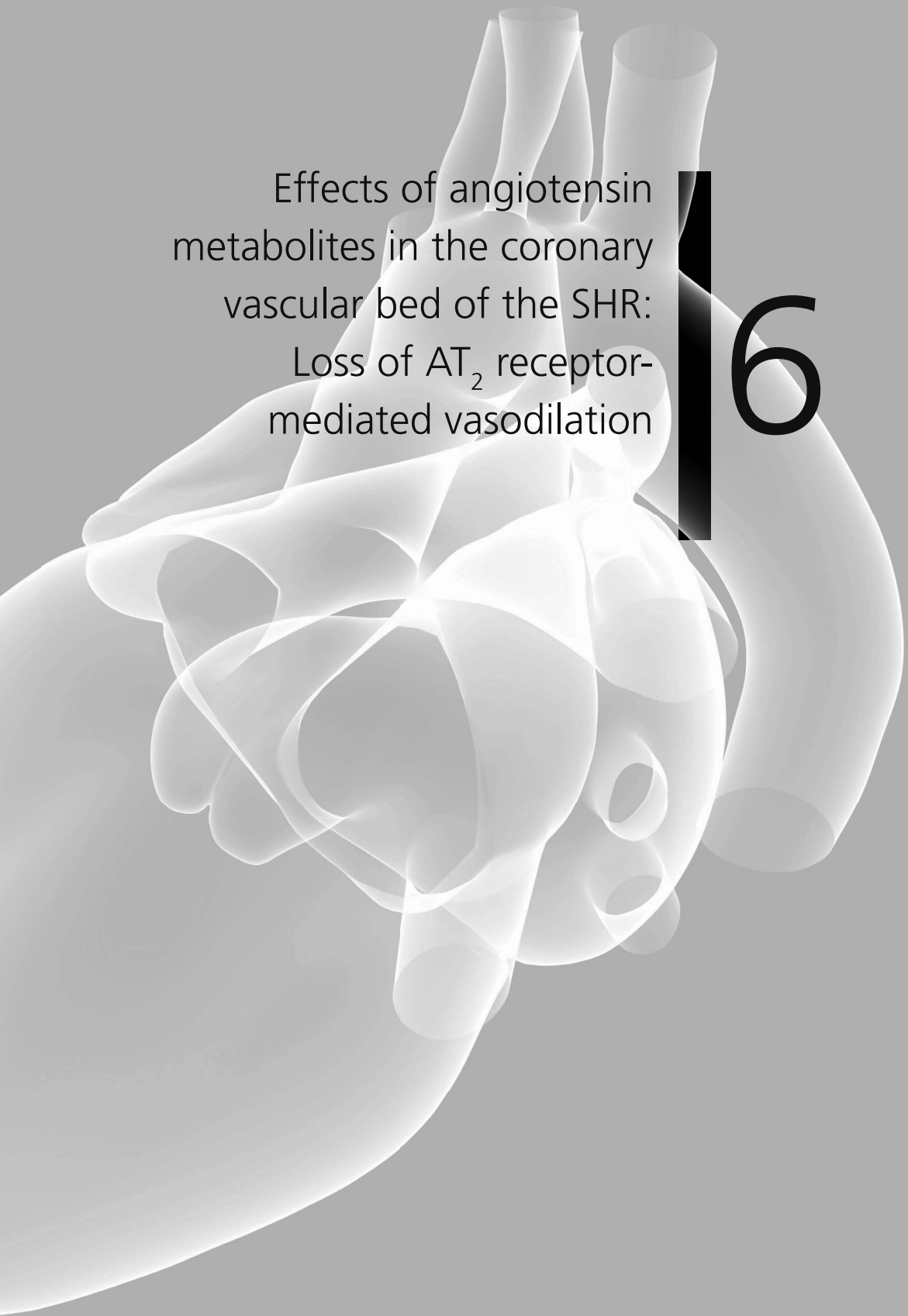
Hypertension 2010 55:516-522



6

Effects of angiotensin metabolites in the coronary vascular bed of the SHR: Loss of AT_2 receptor-mediated vasodilation

6

ABSTRACT

Since angiotensin (Ang) metabolites mediate functions independently of Ang II, we investigated their effects on coronary flow in spontaneously hypertensive rats (SHR). Results were compared to those in iliac artery and abdominal aorta and the coronary circulation of the Wistar rat. Ang II, III and IV decreased coronary flow in SHR and Wistar rats, Ang III and IV being ≈10 and ≈1000 times less potent than Ang II. Ang-(1-7) decreased coronary flow at concentrations >1 µmol/L in SHR. The Ang II type 1 (AT_1) receptor antagonist irbesartan blocked the effects of Ang II, III and IV, whereas the Ang II type 2 (AT_2) receptor antagonist PD123319 blocked the effects of Ang-(1-7). The maximal Ang II- and III-induced decreases in coronary flow in SHR were twice as large as those in Wistar rats. PD123319 enhanced the constrictor effects of Ang II and III in Wistar rats so that, in the presence of this drug, their effects were comparable to those in SHR. In contrast, PD123319 did not alter the Ang II- and III-induced responses in SHR, and blocked the constrictor effect of Ang II in iliac arteries. AT_2 receptor-mediated relaxation did not occur in iliac arteries and abdominal aortas, and the constrictor effects of angiotensin metabolites in these vessels were identical in Wistar rats and SHR. In conclusion, coronary constriction induced by Ang II, Ang III and Ang-(1-7) is enhanced in SHR as compared with Wistar rats. This is attributable to the absence of counterregulatory AT_2 receptor-mediated relaxation and/or a change of AT_2 receptor phenotype from relaxant to constrictor.

INTRODUCTION

Angiotensin (Ang) I and II are metabolized by a whole range of peptidases,[1] resulting in the generation of Ang III, Ang IV and Ang-(1-7). Ang II exerts its effects via Ang II type 1 (AT_1) and type 2 (AT_2) receptors, whereas Ang III, Ang IV and Ang-(1-7) mediate functions of their own by stimulating AT_1, AT_2 and/or newly discovered receptors.[2-7] For instance, Ang III appears to be the preferred agonist of the AT_2 receptor both in the heart and kidney, inducing, respectively, coronary vasodilation[8] and natriuresis.[5] In addition, Ang III regulates blood pressure via central AT_1 receptor activation.[9] Ang IV mediates relaxant effects via AT_4 receptors,[10] whereas Ang-(1-7) activates vasodilatory Mas receptors.[11]

AT_2 receptor upregulation and/or AT_1 receptor downregulation (resulting in a relative AT_2 receptor upregulation) is generally believed to induce protective effects under pathophysiological conditions.[12-14] However, such beneficial effects have not been found consistently by all investigators. For instance, AT_2 receptors mediate constriction in the renal medulla of 2-kidney, 1-clip rats[15] and in mesenteric arteries of spontaneously hypertensive rats (SHR),[16] and the AT_2 receptor-induced natriuresis by Ang III no longer occurs in SHR.[17] Interestingly, blood pressure-lowering in SHR restored the vasodilatory function of the AT_2 receptor.[18] Chronic treatment of ApoE knockout mice with Ang IV reversed vascular dysfunction, possibly by enhancing NO bioavailability in an AT_2 and/or AT_4 receptor-dependent manner.[7] Finally, Ang-(1-7) exerts vasodepressor[19, 20] and anti-remodeling[21] effects under pathological conditions. Although this has been attributed to its capacity to activate Mas receptors,[22] it may also involve AT_2 receptor activation,[20] ACE inhibition,[23] and/or AT_1 receptor blockade.[8, 24]

Given the conflicting data regarding the endogenous agonist and effect(s) of the AT_2 receptor under pathophysiological conditions, we compared the effects of Ang II, Ang III, Ang IV and Ang-(1-7) in the coronary arteries, iliac artery and aorta of the SHR under carefully standardized conditions, both with and without blockade of AT_1 or AT_2 receptors. These vascular beds were chosen since they had been studied previously in Wistar rats,[8] thus allowing a detailed comparison of AT_2 receptor function between normotensive and hypertensive rats.

METHODS

Experimental animals

Sixty-three 3-month-old, male SHR (mean arterial blood pressure 146±3 mm Hg, n=10; body weight 296±3 gram, n=63) were obtained from Charles River (Germany). All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Tissue collection

Male SHR were anesthetized with pentobarbital (60 mg/kg i.p.). Hearts were rapidly excised and placed in ice-cold Tyrode's buffer,[8] gassed with 95% O_2 and 5% CO_2. Subsequently, the iliac arteries and abdominal aorta were removed and either used directly or after overnight storage in cold, oxygenated Krebs-Henseleit solution.[8] Overnight storage did not affect responsiveness.[25, 26]

Langendorff preparation

Hearts were perfused according to Langendorff as described before.[8] Coronary flow was measured with a flow probe (Transonic systems, Ithaca, NY). After a stabilization period of 30 min, baseline values of coronary flow were obtained. Next, bolus injections (100 µl) of Tyrode's buffer were applied 3 times to determine injection-induced changes in coronary flow. Subsequently, concentration-response curves (CRCs) to angiotensins were constructed by applying bolus injections, in the absence or presence of 1 µmol/L irbesartan or PD123319 in the perfusion buffer.[6, 27]

Mulvany myographs

Iliac arteries (diameter 954±8 µm, n=149) and abdominal aortas (diameter 1279±15 µm, n=145) were cut into ring segments of approximately 2 mm length and mounted in a Mulvany myograph with separated 6-ml organ baths containing gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C. No anti-oxidants were added. The tension was normalized to 90% of the estimated diameter at 100 mm Hg effective transmural pressure.[28] Following a 30-min stabilization period, the maximal contractile response was determined by exposing the vessels to 100 mmol/L KCl. Thereafter, the vessels were pre-incubated for 30 min in fresh buffer in the absence or presence of 100 µmol/L L-NAME, 1 µmol/L irbesartan, or 1 µmol/L PD123319, and CRCs to angiotensins were constructed. In order to study vasorelaxation, vessels were preconstricted with U46619 (10-100 nmol/L) prior to their exposure to angiotensins.

Data analysis

Data obtained with the Langendorff preparation were recorded and digitalized using WinDaq waveform recording software (Dataq Instruments). After a manual selection of the desired signals pre- and post-injection, data were analyzed using Matlab (Mathworks Inc). Six consecutive beats were selected for coronary flow determination. CRCs were analyzed as described before,[29] using Graph Pad Prism 3.01 (Graph Pad Software Inc), to determine the maximum effect (E_{max}) and pEC_{50} (=-$^{10}\log EC_{50}$) values. The pEC_{50} values refer to the agonist concentration in injection fluid of the Langendorff model and do not reflect the actual concentrations seen by the receptor. In the Mulvany myograph studies, Ang III and Ang IV did not reach E_{max} at the highest concentrations used. We therefore determined the concentration required to obtain 5% of the K^+-induced contraction ($EC_{5\%K^+}$) in order to calculate $pEC_{5\%K^+}$ values.[25] Statistical analysis was by two-way ANOVA, followed by post hoc evaluation according to Dunnet and $p<0.05$ was considered significant.

RESULTS

Langendorff preparation

Baseline coronary flow (9.6±0.4 ml/min; n=60) was similar in all groups. Bolus injections with Tyrode's buffer injections did not significantly affect coronary flow (Figure 1). Ang II, Ang III, Ang IV and Ang-(1-7) concentration-dependently decreased coronary flow, by maximally 66±5%, 74±3%, 42±9% and 24±3%, respectively (Figure 1A-D). Ang III (pEC_{50} 6.9±0.1, n=6), Ang IV (pEC_{50} 5.4±0.2, n=5) and Ang-(1-7) (pEC_{50} 5.0±0.3, n=4) were, respectively,

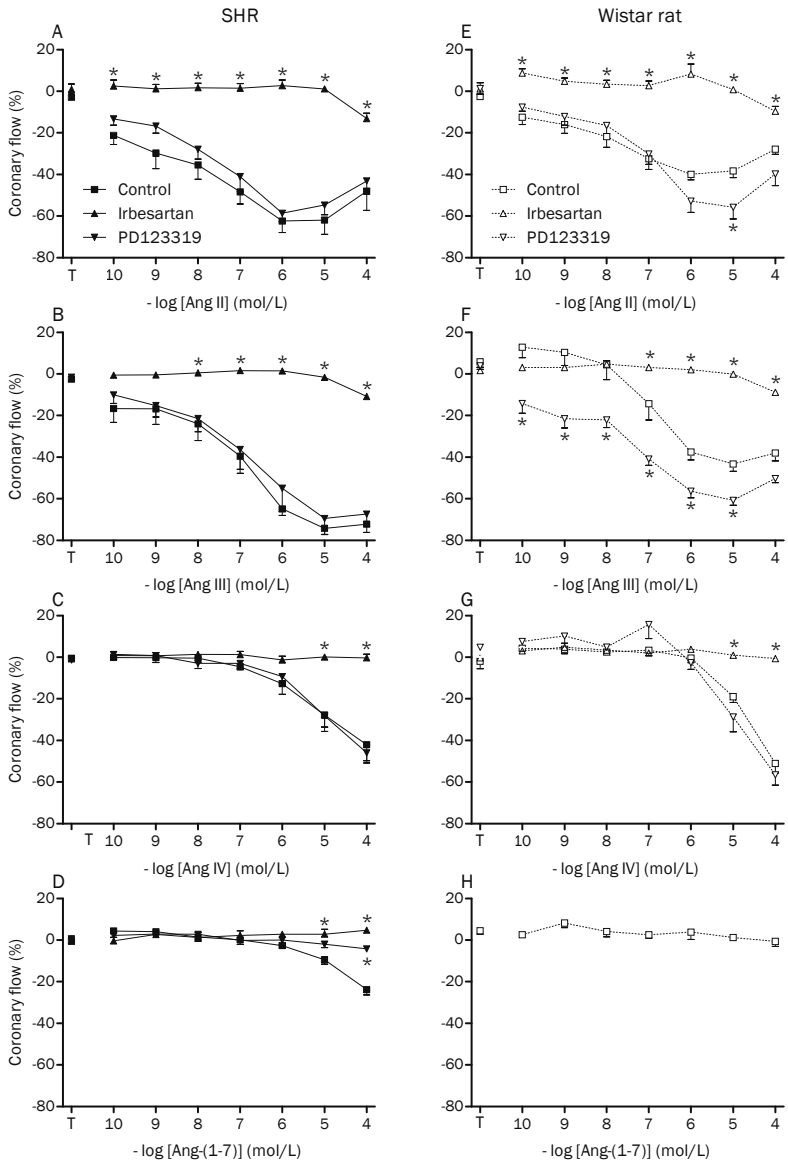


Figure 1. Effects of angiotensins on coronary flow in SHR (left panels) and Wistar rats (right panels; redrawn from van Esch et al. 2008[8]) with or without irbesartan or PD123319. The x-axis displays the concentration of the agonist in the injection fluid. Data (mean±SEM of n=4-7) represent percentage change from baseline. T, bolus injection of Tyrode's buffer. *p<0.05 vs. control within the same strain.

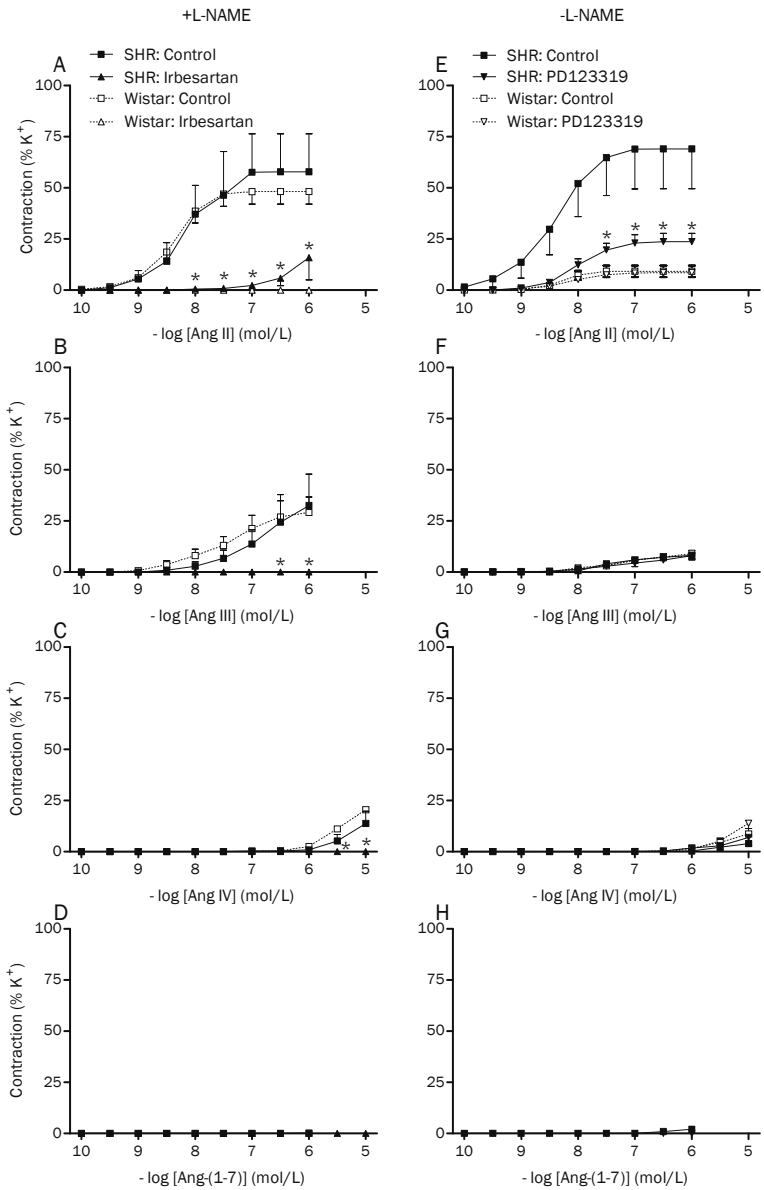


Figure 2. Effects of angiotensins in iliac arteries of SHR and Wistar rats (redrawn from van Esch et al. 2008[8]) with or without L-NAME, irbesartan or PD123319. The x-axis displays the concentration of the agonist in the organ bath fluid. Data (mean±SEM of n=4-9) are expressed as a percentage of the response to 100 mmol/L KCl. *p<0.05 vs. SHR control. The effects of irbesartan in Wistar rats were identical to those in SHR.

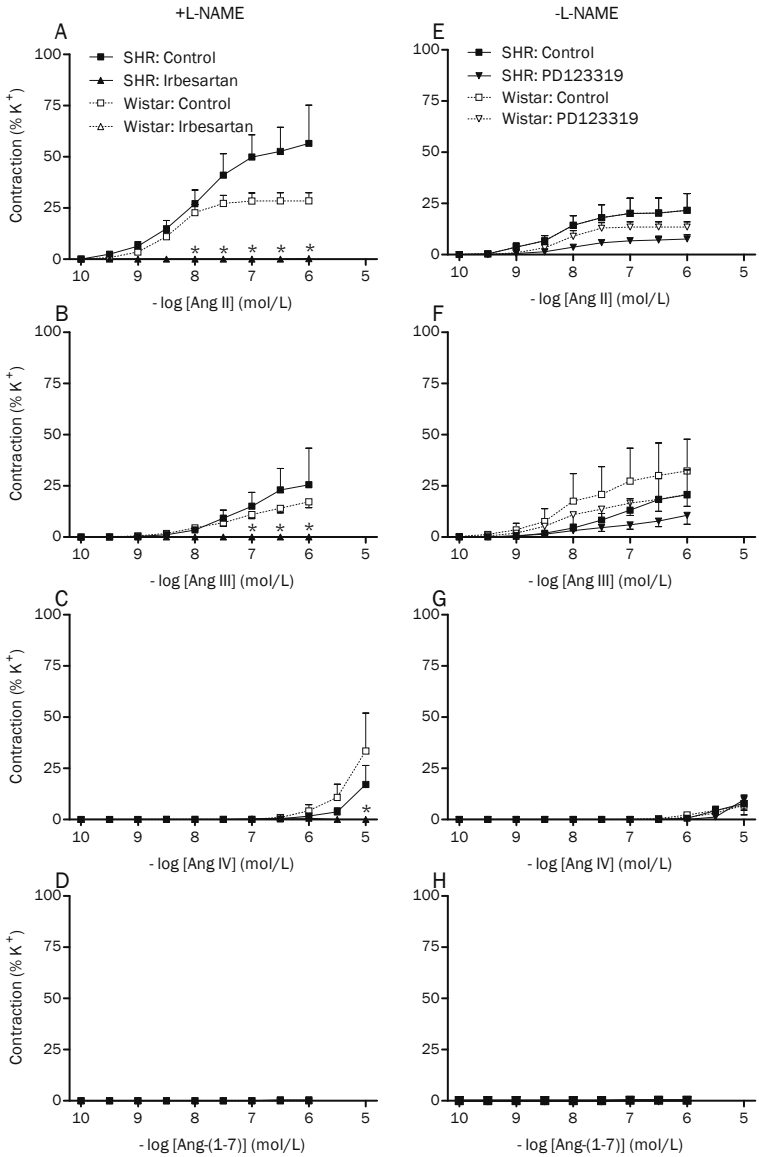


Figure 3. Effects of angiotensins in abdominal aortas of SHR and Wistar rats (redrawn from van Esch et al. 2008.8) with or without L-NAME, irbesartan or PD123319. The x-axis displays the concentration of the agonist in the organ bath fluid. Data (mean±SEM of n=4-10) are expressed as a percentage of the response to 100 mmol/L KCl. *p<0.05 vs. SHR control. The effects of irbesartan in Wistar rats were identical to those in SHR.

~16, ~501 and ~1259-fold less potent ($p<0.05$ for all) than Ang II (pEC_{50} 8.1±0.4, n=7). Irbesartan abolished all Ang II, Ang III, Ang IV and Ang-(1-7)-induced flow changes (n=4), while PD123319 abolished the constrictor effects of Ang-(1-7) only (n=4, $p<0.05$).

Mulvany myographs

Ang II, Ang III and Ang IV constricted iliac arteries (Figure 2A-H) and abdominal aortas (Figure 3A-H) in a concentration-dependent manner, whereas Ang-(1-7) had no effect. In the abdominal aorta, but not in the iliac artery, L-NAME greatly enhanced ($p<0.05$) the response to Ang II. In iliac arteries, in the presence of L-NAME, Ang III and Ang IV ($pEC_{5\%K}{}^{+}$'s: 7.3±0.2, n=5 and 5.5±0.2, n=4) were, respectively, ~25 and ~1584-fold ($p<0.001$ for both) less potent than Ang II ($pEC_{5\%K}{}^{+}$: 8.7±0.3, n=5). Potencies in the absence of L-NAME (n=5-6) were identical to those in the presence of L-NAME. In abdominal aortas (n=5-6), both with and without L-NAME, the potencies of Ang II, III and IV were identical to those in iliac arteries.

Irbesartan abolished all angiotensin-induced contractions in the presence of L-NAME in both iliac arteries (n=5-7; Figure 2A-C) and abdominal aortas (n=4-10; Figure 3A-C), but did not unmask Ang-(1-7) effects (Figure 2D and 3D, respectively). Without L-NAME, PD123319 lowered the contractile response to Ang II ($p<0.05$, n=8-9; Figure 2E) in iliac arteries. It did not affect the responses to Ang III, Ang IV and Ang-(1-7) in iliac arteries (n=4-8; Figure 2F-H), nor did it alter any of the responses in abdominal aortas (n=5-10; Figure 3F-H). No relaxant responses to Ang II, Ang III, Ang IV or Ang-(1-7) were observed in preconstricted iliac arteries and abdominal aortas in the absence or presence of irbesartan (n=4-6; data not shown).

DISCUSSION

All angiotensin metabolites evaluated in this study caused coronary vasoconstriction in SHR via AT_1 receptor stimulation. Although their potencies were identical to those in Wistar rats, the coronary constrictor efficacy of both Ang II and III was much larger in SHR (Figure 1). PD123319 did not enhance the coronary effect of Ang II and III in SHR, as opposed to its potentiating effects in the hearts of Wistar rats. In fact, the coronary constrictor effects of Ang II and III in SHR in the absence of PD123319 were as large as their coronary constrictor effects in Wistar rats in the presence of PD123319 (Figure 1). This suggests that the main reason for the enhanced coronary constrictor effects in SHR is the lack of counterregulatory, AT_2 receptor-mediated coronary vasodilation. Such vasodilation is endothelium-dependent and involves bradykinin B_2 receptor activation, endothelial NO synthase, NO and cGMP.[30-32] Both in the coronary circulation[8] and kidney,[5] Ang III appeared to be the preferred agonist of the AT_2 receptor. The absence of this vasodilator effect in SHR may relate to the endothelial dysfunction in this model, as observed in the coronary[33] and other vascular beds.[34, 35] This dysfunction is believed to be the result of enhanced reactive oxygen species (ROS) production under pathological conditions.[36] ROS may in fact directly downregulate AT_2 receptors.[37] If significant, it will no longer allow the previously described AT_1-AT_2 receptor heterodimerization, which is responsible, at least in part, for AT_2 receptor-mediated effects.[27, 38]

Alternatively, the phenotype and/or location of the AT_2 receptor may change under pathophysiological conditions. For instance, AT_2 receptor stimulation induced constriction in

mesenteric arteries of SHR,[16, 18] as opposed to relaxation in WKY rats.[18] Since this contractile response was not affected by removal of the endothelium,[18] the site of AT_2 receptor expression apparently had changed from the endothelium to the smooth muscle cell.

In the present study Ang-(1-7) caused coronary constriction in SHR in an AT_2 receptor-dependent manner (Figure 1D), confirming that the function of this receptor in the coronary vascular bed had also changed from vasodilator to vasoconstrictor. Moreover, the Ang II-induced constriction of iliac arteries obtained from SHR partly involved AT_2 receptors (Figure 2E). Preliminary experiments in endothelium-denuded iliac arteries (n=2; van Esch et al., unpublished observations 2009) furthermore revealed that this AT_2 receptor-mediated contractile response, like that in the SHR mesenteric artery,[18] occurred in an endothelium-independent manner. Finally, in iliac arteries, L-NAME greatly enhanced the constrictor effect of Ang II in Wistar rats (Figures 2A and 2E), whereas in SHR the effect of Ang II in the absence of L-NAME was already as large as that in Wistar rats in the presence of L-NAME, with the addition of L-NAME causing no further effect. This illustrates the presence of endothelial dysfunction in SHR, no longer allowing endothelial NO to counteract Ang II-induced constriction. Such dysfunction had not yet occurred in the abdominal aortas of the SHR, since in these vessels L-NAME still enhanced the effect of Ang II, while PD123319 exerted no (significant) blocking effect towards Ang II-induced vasoconstriction.

The constrictor effects of Ang III and IV in iliac arteries and abdominal aortas were much more modest than those of Ang II, at least at concentrations up to 10 µmol/L. No differences occurred between SHR and Wistar rats, nor were these responses affected by L-NAME or PD123319 (Figures 2 and 3). The complete blockade of these effects by irbesartan suggest that they are exclusively AT_1 receptor-mediated. Clearly therefore, these modest responses reflect the reduced potency of Ang III and IV towards AT_1 receptors as compared to Ang II.[39, 40] Moreover, given the absence of Ang III-induced (constrictor) responses mediated via AT_2 receptors, it appears that Ang II is the preferred agonist of this AT_2 receptor-mediated constriction, as opposed to the relaxant effect of AT_2 receptor stimulation in normotensive animals, where Ang III is the preferred agonist.[8]

Under no condition did Ang-(1-7) exert constrictor or (following preconstriction) dilator effects in the iliac arteries or abdominal aortas of either SHR or Wistar rats. The vasodilator effects of Ang-(1-7) that have been described in vitro[11, 41] may, therefore, be limited to certain vascular beds.

In conclusion, angiotensin-induced coronary constriction is enhanced in SHR as compared to Wistar rats, because the counterregulatory AT_2 receptor-mediated relaxant effects are either absent or have been reversed into constrictor effects. Similarly, in iliac arteries of SHR, AT_2 receptors mediate contractile responses. In addition, the lack of endogenous endothelial NO production in these vessels greatly increases the response to Ang II.

Perspectives

AT_2 receptor function changes under hypertensive conditions from relaxant to constrictor, and Ang II rather than Ang III then becomes its endogenous agonist. It remains to be determined

whether this is due to ROS-induced endothelial dysfunction, resulting in the disappearance of endothelial (dilator) AT_2 receptors and/or alternative expression of (constrictor) AT_2 receptors in smooth muscle cells. It might also be the consequence of hypertension per se, since blood pressure-lowering reversed the AT_2 receptor-mediated vasoconstriction into vasodilation.[18] The fact that AT_2 receptors are capable of inducing vasoconstriction raises concern regarding the clinical application of AT_2 receptor agonists like compound 21,[42] at least in the absence of AT_1 receptor blockade. AT_2 receptor agonists would allow a direct evaluation of AT_2 receptors function, thereby potentially overcoming the disadvantage of the indirect approach in the present study, which relies on the use of antagonists with limited specificity.

Acknowledgement: This work was financially supported by a grant of the Netherlands Heart Foundation (NHF-2007B019).

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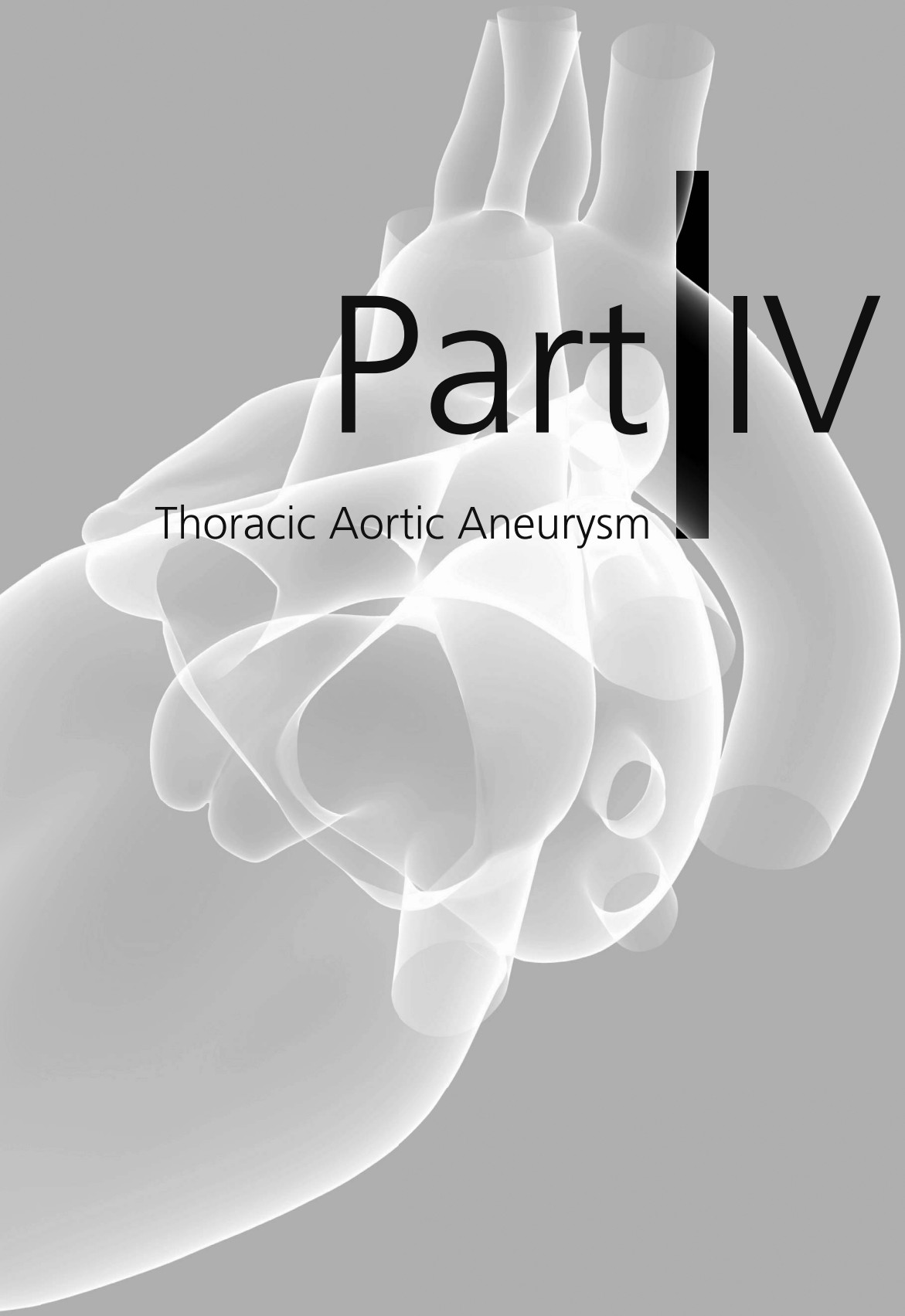

Part IV

Thoracic Aortic Aneurysm

Based on:

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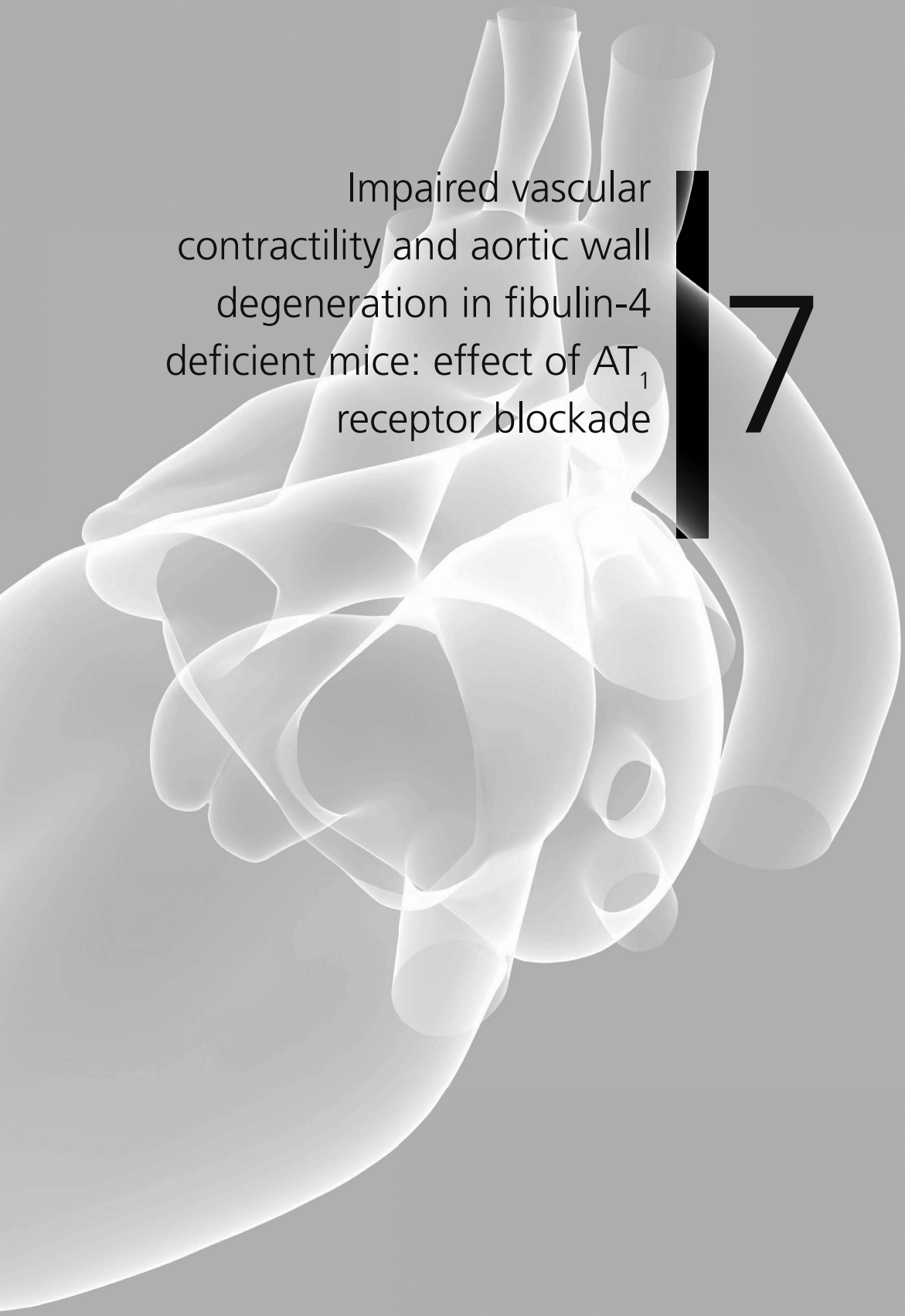
Submitted



7

Impaired vascular contractility and aortic wall degeneration in fibulin-4 deficient mice: effect of AT_1 receptor blockade

7

ABSTRACT

Medial degeneration is a key feature of aneurysm disease and aortic dissection. In a murine aneurysm model we investigated the structural and functional characteristics of aortic wall degeneration in adult fibulin-4 deficient mice and the potential therapeutic role of the angiotensin (Ang) II type 1 (AT_1) receptor antagonist losartan in preventing aortic media degeneration. Adult mice with 2-fold (heterozygous Fibulin-$4^{+/R}$) and 4-fold (homozygous Fibulin-$4^{R/R}$) reduced expression of fibulin-4 displayed the histological features of cystic media degeneration as found in patients with aneurysm or dissection, including elastin fiber fragmentation, loss of smooth muscle cells, and deposition of ground substance in the extracellular matrix of the aortic media. The aortic contractile capacity, determined by isometric force measurements, was diminished and was associated with dysregulation of contractile genes as shown by aortic transcriptome analysis. These structural and functional alterations were accompanied by upregulation of TGF-β signaling in aortas from fibulin-4 deficient mice, as identified by genome-scaled network analysis as well as by immunohistochemical staining for phosphorylated Smad2, an intracellular mediator of TGF-β. Tissue levels of Ang II, a regulator of TGF-β signaling, were increased. Prenatal treatment with the AT_1 receptor antagonist losartan, which blunts TGF-β signaling, prevented elastic fiber fragmentation in the aortic media of Fibulin-$4^{R/R}$ mice. In conclusion, the AT_1 receptor blocker losartan can prevent aortic media degeneration in a non-Marfan syndrome aneurysm mouse model. These findings may extend the potential therapeutic application of inhibitors of the renin-angiotensin system to the treatment of aneurysm disease.

INTRODUCTION

Degeneration of the medial layer of the aorta is a key feature of aneurysm disease and aortic dissection.[1] Cystic medial degeneration is characterized by elastic fiber fragmentation, loss of smooth muscle cells (SMC), and accumulation of amorphous extracellular matrix (ECM) in the aortic wall. Although media degeneration occurs to some degree with aging, excessive aortic wall degeneration may lead to dilation of the aorta and aneurysm formation, or, alternatively, aortic dissection.[2, 3] In addition, advanced aortic degeneration may be part of inherited disorders of the connective tissue. One of the most common of these syndromes is Marfan syndrome (MFS), resulting from a mutation in the FBN1 gene which encodes the ECM glycoprotein fibrillin-1.[4] MFS is characterized by elastic fiber fragmentation, loss of elastin content, and accumulation of amorphous matrix components in the aortic wall, resulting in

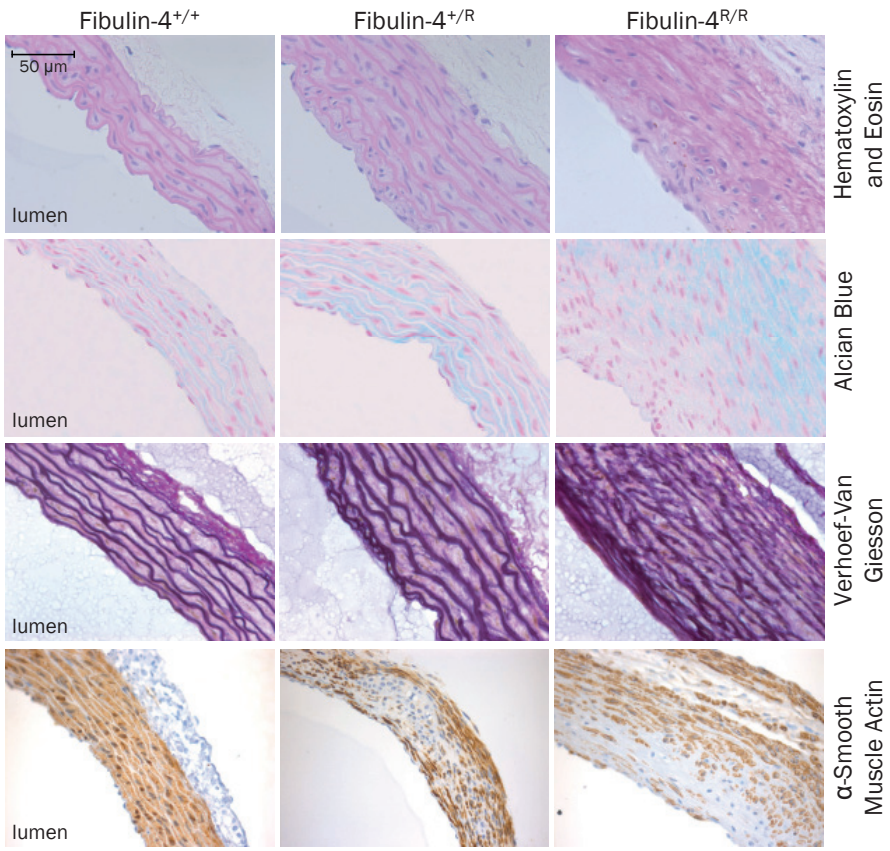


Figure 1. Architecture of ascending thoracic aortas. In adult Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ aortas there is an increase in aortic wall thickness (Hematoxylin and Eosin), glycosaminoglycan depositions (blue areas in Alcian Blue staining), elastic fiber fragmentation (Verhoef-Van Giesson) and loss of smooth muscle cells in the media (α-Smooth Muscle Actin).

the formation of thoracic aortic aneurysms (TAAs).[5] Mice with a mutation in the fibrillin-1 gene are widely used to study the pathophysiologic mechanisms underlying MFS and its treatment options.[6]

Several mutations in other genes encoding ECM proteins have also been identified in patients with TAAs, including mutations in the fibulin-4 gene.[7 8] Fibulin-4 is one of the seven-member family of ECM proteins that play a role in elastic fiber assembly and function.[9] Fibulin-4 is highly expressed in the medial layers of blood vessel walls, including the aortic media.[10] It has been shown that mutant mice lacking fibulin-4 (Fibulin-$4^{-/-}$) die perinatally from aortic rupture.[11] Furthermore, newborn mice with a systemic 4-fold reduced expression of fibulin-4 (Fibulin-$4^{R/R}$) display elastic fiber fragmentation and develop aneurysms in the ascending thoracic aorta. Interestingly, even a 2-fold reduced expression of fibulin-4 in the heterozygous Fibulin-$4^{+/R}$ mice already induces similar, though milder, changes in the aorta.[12]

Since aneurysm disease is a condition of the aging population, the present study first focused on the structural and functional characterization of aortic wall degeneration in adult fibulin-4 deficient mice. Recent studies have shown that antagonizing transforming growth factor-β (TGF-β) by either TGF-β neutralizing antibodies or the angiotensin (Ang) II type 1 (AT_1) receptor antagonist losartan can slow the progression rate of aortic root dilation in an MFS mouse model[6] and in patients with MFS.[13] Therefore, we next investigated the role of the renin-angiotensin system (RAS) in aneurysm formation in fibulin-4 deficient mice. We show that the AT_1 receptor blocker losartan can prevent aortic media degeneration in this non-MFS aneurysm mouse model. These findings extend the potential therapeutic application of inhibitors of the RAS to the treatment of aneurysm disease.

METHODS

Experimental animals

We previously generated a fibulin-4 allele with reduced expression by transcriptional interference through placement of a TKneo targeting construct in the downstream Mus81 gene.[12] Heterozygous (Fibulin-$4^{+/R}$) mice in a mixed C57Bl/6J;129Sv background were mated to obtain Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibullin-$4^{R/R}$ littermates and were housed in the institutional animal facility. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Histology and immunohistochemistry

Mice (age 100 days) were euthanized by an overdose CO_2, fixed by perfusion fixation with 4% formaldehyde, and necropsied according to standard protocols. Perfusion-fixed aortas were isolated and paraffin embedded. Next, 4-μm sections were haematoxylin and eosin stained and stained for elastin (Verhoeff-van Gieson), glycosaminoglycans (Alcian Blue) and SMCs (α-SMA). Immunohistochemistry for phosphorylated Smad2 (pSmad2) was performed as described previously [14] using rabbit antiphospho-Smad2 antibodies.

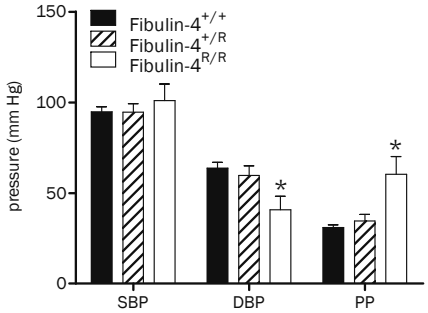


Figure 2. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) measured using an intra-aortic microtip pressure transducer catheter. With decreasing expression of Fibulin-4, DBP decreased and pulse pressure (PP) increased (p for trend 0.009 and <0.001 resp.). Data are mean±SEM of 4-17 mice. *p<0.05 vs. Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ (two-way ANOVA).

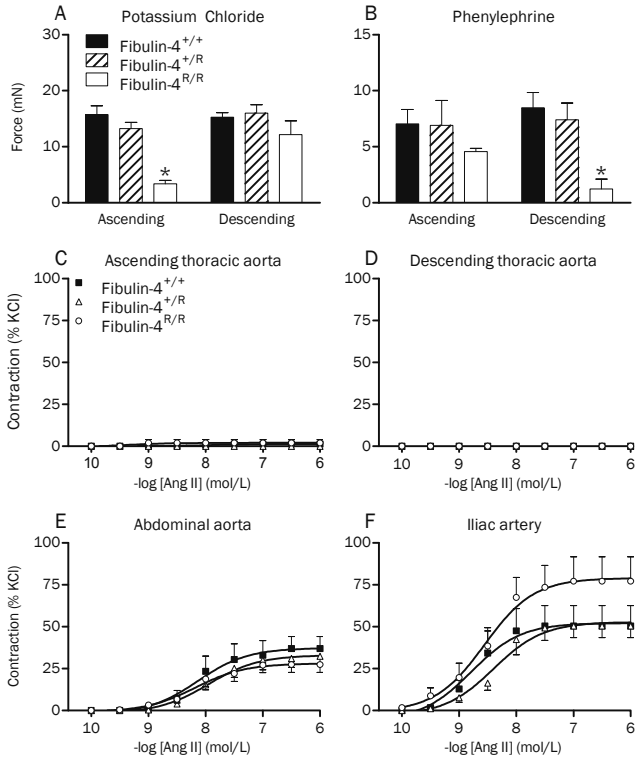


Figure 3. Contractility mediated by KCl, phenylephrine and angiotensin (Ang) II. (**A**) In ascending aortas, KCl-induced contractility decreased in a gene dose-dependently in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice (p for trend <0.001). (**B**) In descending aortas, phenylephrine-induced contractility decreased gene dose-dependently in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice (p for trend 0.004). Data are mean±SEM of 6-18 experiments, *p<0.05 vs. Fibulin-$4^{+/+}$ mice. (**C-F**) Effect of angiotensin II on (**C**) ascending thoracic aortas, (**D**) descending thoracic aortas, (**E**) abdominal aortas and (**F**) iliac arteries. Data (mean±SEM of n=3-6) are shown as a percentage of the response to 100 mmol/L KCl.

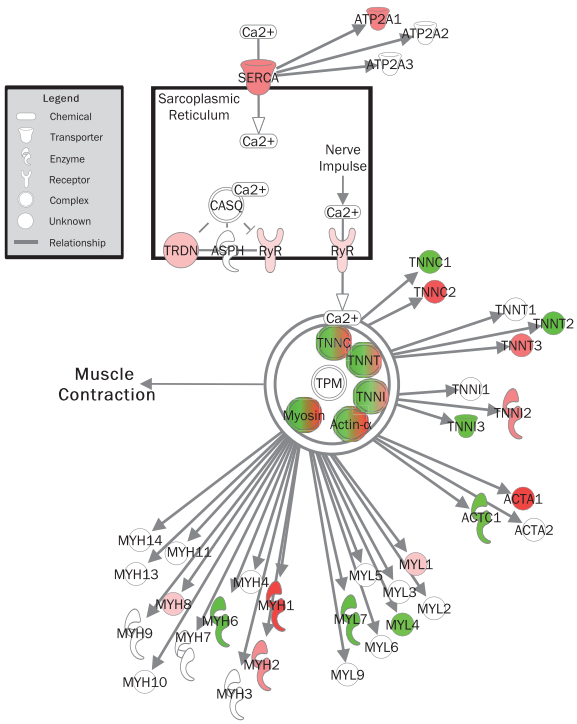


Figure 4. Calcium signaling pathway in a resting muscle cell (Fibulin-4$^{+/R}$ vs. Fibulin-4$^{+/+}$ aortas). Colors show up- (red) and downregulation (green) of molecules involved in muscle cell contraction.

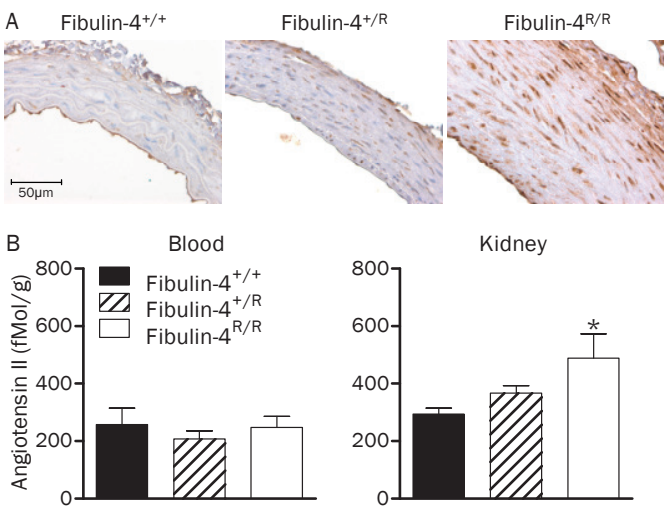


Figure 5. Increased levels of pSmad2 and angiotensin II in fibulin-4 mutant aortas. (A) Immunhistochemistry reveals a graded increase in expression and nuclear translocation of pSmad2 in the aortic media of adult fibulin-4 deficient mice. (B) With reduced fibulin-4 expression, tissue (but not blood) angiotensin II levels increase (p for trend 0.004). Data are shown as mean±SEM of 4–18 experiments. *p<0.05 vs. Fibulin-4$^{+/+}$.

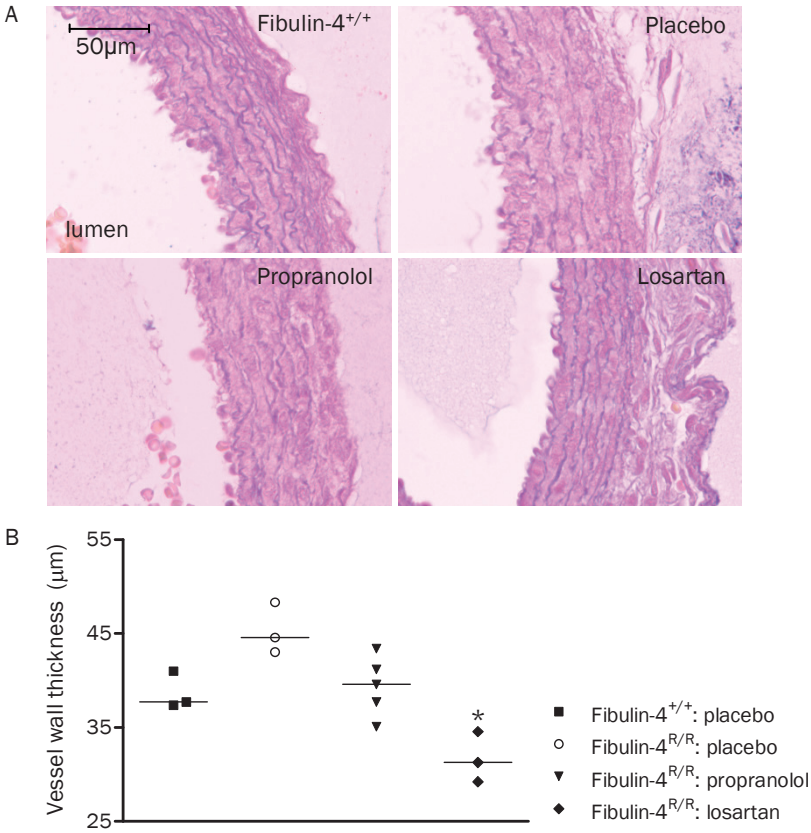


Figure 6. Prenatal treatment with propranolol, losartan or placebo. (**A**) Elastic fiber fragmentation in Fibulin-$4^{R/R}$ mice could be prevented with losartan, but not with propranolol or placebo. (**B**) Vessel wall thickness of thoracic aortas of Fibulin-$4^{+/+}$ and treated Fibulin-$4^{R/R}$ mice. Losartan treatment of Fibulin-$4^{R/R}$ mice recovered vessel wall thickness. Horizontal lines indicate the median of 3-5 mice, *p<0.05.

Microarray hybridizations

Standard procedures were used to obtain total RNA (Qiagen) of two Fibulin-$4^{+/+}$, two Fibulin-$4^{+/R}$ and four Fibulin-$4^{R/R}$ aortas (10 days old). Synthesis and hybridization was performed as described before.[12] To examine the quality of the various arrays, several R packages (including affyQCreport) were run starting from the CEL files. All created plots, including the percentage of present calls, noise, background, and ratio of GAPDH 3′ to 5′ (<1.4) indicated a high quality of all samples and an overall comparability, except for two samples, which were excluded from further analysis. Of the 45101 probe sets, ~55% was called present in all samples. Raw intensities values of all samples were normalized by robust multichip analysis normalization (background correction and quantile normalization) using Partek version 6.4 (Partek Inc., St. Louis, MO). The normalized data file was transposed and imported into OmniViz version 6.0.1

(Biowisdom, Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and ^{2}log transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using ANOVA (Partek) and SAM (OmniViz). Cut-offs values for significantly expressed genes were the FDR and a fold change of 1.5. Functional analysis was done using Ingenuity Pathway Analysis ((IPA) Ingenuity, Mountain View, CA). Microarray experiments have been previously described and complied with the regulations for Minimum Information of Microarray Experiments (MIAME) and can be retrieved from ArrayExpress (www.ebi.ac.uk/arrayexpress/ , accession code: E-MEXP-840).[12]

Hemodynamic measurements

Mice (15-20 weeks old) were sedated with 4% isoflurane and intubated as previously described.[15] For measuring systolic and diastolic BP, mice were instrumented with a calibrated high fidelity 1.4 F microtip pressure transducer catheter (SPR-671, Millar Instruments), which was inserted into the left carotid artery and advanced into the aortic arch.[12] Hemodynamic data were recorded and digitized using an online 4-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, Ohio, USA), for later analysis with a program written in Matlab. Ten consecutive beats were selected for determination of BP.

Mulvany myographs

Male mice (age 120 days) were euthanized with an overdose of pentobarbital i.p. (60 mg/kg). Thoracic aorta, abdominal aorta and iliac artery were isolated and stored overnight in cold, oxygenated Krebs-Henseleit buffer solution. The following day, vessel segments were mounted in 6-ml organ baths (Danish Myograph Technology, Aarhus, Denmark) containing Krebs-Henseleit buffer (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) at 37^{o}C and oxygenated with 95% O_2 and 5% CO_2. The tension was normalized to 90% of the estimated diameter at 100-mm Hg effective transmural pressure.[16] Maximum contractile responses were determined using 100 mmol/L KCl. Concentration response curves (CRCs) were constructed to phenylephrine and Ang II (Sigma); the latter with a 30-min incubation with the NO synthase inhibitor L-NAME (100 µmol/L; Sigma).

Biochemical measurements

Kidneys were excised and blood was collected from the left ventricle and stored in 4 mol/L guanine thiocyanate as described before.[17] Both were immediately frozen in liquid nitrogen and stored at -80^{o}C. Ang II was determined by using radioimmunoassay, following SepPak extraction and high-performance liquid chromatography separation.[18]

Prenatal treatment

Fibulin-4$^{+/R}$ mice were bred to produce Fibulin-4$^{+/+}$ and Fibulin-4$^{R/R}$ mice. Pregnant mice received either propranolol (0.5 g/L, Sigma), losartan (0.6 g/L, Sigma) or placebo in their drinking water as described before.[6] Treatment was started at embryonic day (E)14.5 and

continued for 5 days. At E19.5 the pregnant mice were euthanized by an overdose CO_2 and a caesarian section was performed to collect the fetuses. Aortas from the fetuses were isolated and paraffin embedded. Next, 4-µm sections were stained for elastin (Verhoeff-van Gieson). Aortic wall thickness of the ascending aorta was measured in the presented section at 6 sites and averaged using Leica QWin software (Leica, Glattburg, Switzerland).

Data-analysis

Normally distributed data are presented as mean±SEM. CRCs were analyzed using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, California, USA) to determine the maximum effect (E_{max}) as described before.[19] Analysis of the differences between CRCs was performed by two-way ANOVA. The one-way ANOVA was considered for the analysis of E_{max}, blood pressures, Ang II levels and vessel wall thickness. Both analyses were followed by post hoc evaluation according to Bonferroni. To evaluate the dose-dependent effect of fibulin-4 expression, a linear regression analysis was performed to obtain a p for trend. The latter statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill, USA). All statistical tests were two-sided and a p-value <0.05 was considered statistically significant.

RESULTS

Adult fibulin-4 deficient mice display aortic wall degeneration

Although 10-days-old Fibulin-$4^{R/R}$ mice showed severe TAAs,[12] number of Fibulin-$4^{R/R}$ mice survived towards adult age. The structural alterations resulting from reduced fibulin-4 in adult mice were characterized in 100-days-old mice. All aneurysms of Fibulin-$4^{R/R}$ mice were located in the ascending thoracic aorta. Aortic wall thickness was increased in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ as compared with Fibulin-$4^{+/+}$ mice (Figure 1). The increase in aortic wall thickness was, at least in part, due to increased deposition of glycosaminoglycans in the ECM, as demonstrated by Alcian blue staining. Aortas of wild type mice displayed a normal pattern of elastic lamellae forming dense parallel sheets. In contrast, the thickened aortic walls in fibulin-4 deficient mice displayed changes in elastic fiber organization, varying from moderate elastic fiber fragmentation in Fibulin-$4^{+/R}$ mice to complete destruction of elastin lamellar organization in Fibulin-$4^{R/R}$ mice. In addition to changes in elastin structure, aortic walls of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice displayed loss of SMCs, as evidenced by α-smooth muscle actin (SMA) staining and increased numbers of apoptotic cells (data not shown).

Functional consequences of fibulin-4 deficiency

Increased aortic pulse pressure

Since elastic fiber fragmentation may be associated with loss of elasticity and increased stiffness of the aortic wall, we next determined the *in vivo* aortic BP using a microtip pressure catheter. In Fibulin-$4^{R/R}$ mice a slightly increased systolic BP and decreased diastolic BP was observed compared to wild type animals resulting in a higher aortic pulse pressure in Fibulin-$4^{R/R}$ mice compared to controls (Figure 2), which is consistent with increased arterial stiffness.[20]

Reduced aortic contractility

To evaluate the functional effects of SMC loss, *in vitro* vascular contractility was studied in different segments of the aorta and the iliac arteries. In line with the reduced numbers of SMCs in the thoracic aorta, the maximum contractility of thoracic aortas in response to KCl (100 mmol/L) was more than 3-fold lower in Fibulin-$4^{R/R}$ mice than in Fibulin-$4^{+/+}$ mice (Figure 3A). Similarly, receptor-mediated vasoconstriction in response to phenylephrine (100 µmol/L) was significantly lower in thoracic aortic rings of Fibulin-$4^{R/R}$ mice than in Fibulin-$4^{+/+}$ mice (Figure 3B). The contractile responses of the abdominal aorta and the iliac arteries did not differ between groups (data not shown). Increasing doses of Ang II, following a 30-min incubation with N^{ω}-nitro-L-arginine methyl ester (L-NAME), did not induce vasoconstriction in the thoracic aorta (Figure 3C-D). The contractile responses of the abdominal aorta and iliac arteries in response to Ang II were not different between fibulin-4 deficient and wild type mice (Figure 3E-F). This difference probably relates to the lower AT_1 receptor levels in the thoracic aorta than in other large arteries in the mouse.[21, 22]

Disturbed calcium signaling in fibulin-4 deficient mice

Next, genome-scaled network analysis from Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ aortas was performed using dedicated microarray statistics with a focus on canonical pathway analysis. Differentially expressed genes were initially identified using statistical analysis of microarrays ANOVA (false discovery rate (FDR) 0.5 and 1.5-fold change up- or downregulation). Transcriptomes of Fibulin-$4^{+/+}$ and Fibulin-$4^{+/R}$ full length aortas were compared and 26 probe sets were identified. With IPA, a list of involved canonical pathways was constructed (Table 1). The calcium signaling showed up as the top canonical pathway. Next, an independent SAM analysis was performed (FDR of 0.0032 (falsely called <1) and 1.5-fold change up- or downregulation). This approach identified 279 probe sets, from which a second top list of canonical pathways was constructed (Table 3). Again, the calcium signaling pathway was highly significant. Interestingly, very specific genes involved in muscle cell contraction were up- or downregulated (Figure 4).

Next, differences between transcriptomes of Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ aortas were analyzed. Statistical analysis of microarrays ANOVA was performed with the same selection criteria as for Fibulin-$4^{+/+}$ vs. Fibulin-$4^{+/R}$ aortas. Canonical pathway analysis identified mainly pathways involved in immunological and inflammatory diseases (Table 2) and after analysis with SAM (FDR 0.2 and 1.5-fold change up- or downregulation) a table with principally similar pathways was constructed (Table 4). These analyses identified a few genes involved in the aforementioned calcium signaling pathway.

Fibulin-4 deficient mice show dysregulation of TGF-β signaling and increased tissue angiotensin II

In a mouse model of MFS, it has been demonstrated that dysregulation of TGF-β activation and the RAS play an important role in aneurysm formation.[6, 23, 24] Hence, we next investigated the involvement of TGF-β signaling and Ang II in fibulin-4 deficient mice. First, genome-scaled network analysis from Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ aortas identified the upregulation of TGF-β in Fibulin-$4^{R/R}$ mice compared to Fibulin-$4^{+/+}$ mice (Table 2 and 4). Next,

immunohistochemical staining for phosphorylated Smad2 (pSmad2), an intracellular mediator of the TGF-β signal, in ascending thoracic aortas was performed. A graded increase in the nuclear translocation of pSmad2 in the aortic media of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice was observed (Figure 5A), indicating increased TGF-β signaling in adult fibulin-4 deficient mice. Ang II is important in TGF-β signaling, by stimulating TGF-β1 mRNA and protein expression, which leads to TGF-β activation. This indicates that TGF-β acts downstream of Ang II signaling.[5] Therefore, we subsequently measured Ang II levels in blood and in kidney tissue of fibulin-4 deficient mice. Plasma Ang II levels were identical in the 3 genotypes (Figure 5B). In contrast, renal tissue Ang II levels displayed a clear gene dose-dependent increase in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice (Figure 5B; $p<0.004$ for gene deletion effect), which may be due to increased AT_1 receptor binding at this site, resulting in increased receptor-bounded Ang II levels.[26]

Prenatal treatment with losartan reduces aortic wall degeneration

In genetically engineered MFS mice with abnormal fibrillin-1, blocking TGF-β, either by TGF-β neutralizing antibody or by the AT_1 receptor blocker losartan, has been shown to prevent aortic root dilation, elastic fiber degeneration, and pSmad2 activation.[6] Since dysregulation of TGF-β signaling and activation of the RAS were also observed in fibulin-4 deficient mice, we next investigated the potential therapeutic effect of losartan. To prevent Fibulin-$4^{R/R}$ mice for premature drop-out due to aortic rupture, mice were treated as early as possible. Thus, mice were prenatally treated with placebo, beta-adrenergic receptor blocker propranolol, or AT_1 receptor blocker losartan. Propranolol, used as standard therapy to slow progression rate of aortic root growth in patients with MFS, served as control agent in an equihypotensive dosage.[6] Cross-sections of ascending aortas collected from Fibulin-$4^{+/+}$ newborn mice, revealed the presence of intact elastic layers (Figure 6A). As expected, placebo-treated Fibulin-$4^{R/R}$ mice showed severe fragmentation and an increased aortic wall thickness in this area (Figure 6A-B). Treatment of Fibulin-$4^{R/R}$ mice with propranolol did not change elastic fiber fragmentation, but slightly lowered vessel wall thickness. Yet, treatment with losartan improved elastic fiber fragmentation and greatly reduced vessel wall thickness.

DISCUSSION

Adult fibulin-4 deficient (Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$) mice display gene-dose-dependent elastic fiber fragmentation, dropout of SMCs, and deposition of mucopolysaccharide ground substance in the ECM of the aortic media. The structural changes observed in adult fibulin-4 deficient mice reflect the key histological features of cystic medial degeneration in patients with aortic aneurysm or dissection.[1, 27, 28] In patients, medial degeneration is histologically characterized by fragmentation and loss of elastin, loss of SMCs, and formation of areas devoid of elastin that are filled with amorphous ECM. Cystic medial degeneration characterizes the final common pathway for various processes that affect the integrity of the aortic media. These findings support the use of the fibulin-4 deficient murine model for the study of aortic degeneration and aneurysm formation and its pharmacotherapeutical intervention.

The ECM provides the structural and functional platform of the aorta. In normal healthy aorta, elastin and collagen account for 50% of the dry weight and provide the aortic wall with non-linear elasticity properties.[29] One of the critical elements of the ECM are the elastic

lamellae. Elastin is incorporated in elastic fibers on a scaffold of microfibrils. The elastic fibers in normal healthy aorta are arranged in concentric elastic lamellae and, together with vascular SMCs, form lamellar units.[30] Deposition of elastin is not uniform in the aorta, with a decrease in the number of elastin lamellar units from the ascending aorta to the abdominal aorta.[29] The circumferentially aligned collagen and elastin fibers in the aortic media provide tensile strength, permitting the aorta to withstand pulsatile flow and BP delivered by the heart and to limit distal shear stress.

The loss of elastic fiber integrity in the aortic wall observed in fibulin-4 deficient mice was associated with an increase in aortic pulse pressure, mainly due to a decline in diastolic BP, reflecting diminished aortic resilience and tensile strength. Similar stiffening of the aortic wall with increased pulse pressure has been found in the well-characterized genetically engineered mouse model of MFS with a mutation in the FBN1 gene ($Fbn1^{C1039G/+}$) and in patients with MFS.[31 32] The rise in aortic pulse pressure in conjunction with aortic dilation will further increase arterial wall stress over the cardiac cycle and thereby extend elastic fiber fragmentation. In MFS patients it has been shown that elevated aortic pulse-wave velocity, as a measure for reduced aortic elasticity, is a predictor for aortic dilation and dissection.[33]

The changes in aortic media structure were accompanied by impaired contractile function. Both adrenergic-receptor and receptor-independent vascular contractility were reduced in fibulin-4 deficient aortic rings. The decreased contractile capacity could, at least in part, be explained by the loss of SMCs in fibulin-4 deficient aortas. In addition, loss of fibulin-4 is assumed to disrupt the interaction between elastic fibers and SMCs, leading to alterations in actin cytoskeleton organization.[34] Third, altered calcium signaling may contribute to disturbed vascular contractile capacity.

Using aortic transcriptome analysis, we identified altered expression pattern of genes encoding for proteins involved in calcium signaling in Fibulin-$4^{+/R}$ as compared with Fibulin-$4^{+/+}$ aortas. These data indicate that fibulin-4 deletion not only affects aortic media structure, but also affects contractile function, as was also predicted based on fibulin-4 conditional knockout mice.[34] It has been suggested that aortic contractility contributes to the overall tensile strength and structural integrity of the aortic wall.[35] The observed disturbances in the biomechanical properties of the aorta are in line with findings in the genetic mouse model of MFS.[36] The altered load-bearing capacity of the aorta due to disturbances in the synthesis and breakdown of the aortic medial ECM as well as impaired aortic contractility culminates in increased aortic wall stress, which may contribute to dissection and aneurysm formation.

As in the MFS mouse model, the alterations in aortic structure and function were associated with increased TGF-β signaling in adult aneurysmal fibulin-4 deficient mice, as evidenced by a graded increase in the expression of pSmad2, an intracellular mediator of the TGF-β signal, in the aortic media of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice. Augmented TGF-β activation is associated with upregulation of matrix metalloproteinases and degradation of the aortic media, as shown in both MFS mice and in newborn fibulin-4 deficient mice.[36-38] Furthermore, altered TGF-β signaling has also been reported in humans with cardiovascular malformations due to fibulin-4 deficiency.[39] The importance of TGF-β signaling in aneurysm formation is further supported by the recent demonstration of increased circulating TGF-β concentrations

in patients and mice with MFS, and the correlation between increased serum TGF-β and aortic root dilation.[40]

It is still unclear how fibulin-4 deficiency correlates with increased TGF-β signaling. Increased TGF-β production may be due to Ang II.[41-44] For example, in human vascular SMCs, stimulation with Ang II induced a 6-fold increase in TGF-β production.[42] The contribution of the RAS in the fibulin-4 mouse model was investigated by measuring circulating and renal tissue Ang II. Changes in renal Ang II content mirror changes in the Ang II content of other tissues, including the aorta.[17, 26] However, renal Ang II levels are generally much higher than Ang II levels in blood vessel walls, and can thus be measured with much greater accuracy. Therefore, we determined renal tissue Ang II levels as a reflection of changes in aortic Ang II content in adult fibulin-4 deficient mice. While Ang II levels were preserved in plasma, renal Ang II levels increased with decreasing expression of fibulin-4. Tissue Ang II levels depend, at least in part, on AT_1 receptor binding and internalization of extracellularly generated Ang II.[17, 26] However, aortic transcriptome analysis did not show increased expression of AT_1 receptor density in fibulin-4 deficient mice. Alternatively, upregulated tissue Ang II levels may be due to increased renin uptake at tissue sites,[45] and thus future studies should investigate vascular (pro)renin receptor density. Evidence is accumulating that the RAS plays an important role in the pathogenesis of aneurysm formation.[6, 24, 46, 47] Ang II and AT_2 receptor expression are increased in MFS aortic tissue and have been associated with cystic medial degeneration.[24] The increased tissue Ang II levels observed in fibulin-4 deficient mice are in line with these findings and support the role for the RAS in this model.

Drugs that interfere with the RAS may reduce aortic media degeneration. In cultured aortic cells from MFS, angiotensin-converting enzyme inhibition and AT_1 receptor antagonism significantly inhibited SMC apoptosis.[24] Interestingly, blockade of the AT_1 receptor by losartan has been shown to diminish TGF-β signaling, with a reduction in free TGF-β levels, tissue expression of TGF-β responsive genes, and levels of mediators within the TGF-β signaling cascade, and to prevent aortic aneurysm development in the MFS mouse model.[6] Furthermore, treatment with losartan reduced circulating TGF-β levels and slowed the rate of aortic root dilation both in MFS mice and in MFS patients.[13, 40] Based on these findings, we investigated whether aortic media degeneration in the fibulin-4 aneurysm model is associated with increased TGF-β signaling and could be prevented by the TGF-β antagonist losartan. Losartan, but not β-blocker propranolol, treatment of pregnant mice prevented elastic fiber fragmentation and disarray in the aortic media of newborn Fibulin-$4^{R/R}$ mice. In addition to interfering with TGF-β signaling, AT_1 receptor blockade will indirectly lead to stimulation of the AT_2 receptor. Through a negative feedback-mechanism, Ang II levels rise and can bind the AT_2 receptor, which can have positive effects on the vascular remodeling. Results obtained in MFS mice further demonstrate that losartan is able to improve phenylephrine-induced contractility.[48] Whether or not aortic function is also affected by losartan treatment in fibulin-4 deficient mice remains to be elucidated.

The present study is the first to show that losartan is effective for treatment of non-MFS based aneurysms. Together with previous reports, these data suggest that the antihypertensive drug losartan, an AT_1 receptor blocker that blunts TGF-β activation, may be an effective drug in the secondary prevention of aortic media degeneration and aneurysm formation.

Table 1. Top ten Ingenuity Canonical Pathways following ANOVA (Fibulin-$4^{+/+}$ vs. Fibulin-$4^{+/R}$)

Ingenuity Canonical Pathways	p-value	Ratio	Genes
Calcium Signaling	$3.5*10^{-10}$	0.034	ACTA1↑*, ATP2A1↑*, MYH1↑*, MYH2↑*, TNNC2↑*, TNNT3↑*, TNNI2↑*
Actin Cytoskeleton Signaling	$2.5*10^{-6}$	0.021	ACTA1↑*, MYH1↑*, MYH2↑*, MYLPF↑, PIK3CD↑
C21-Steroid Hormone Metabolism	$2.0*10^{-4}$	0.029	HSD11B1↑, HSD3B2↑
Tight Junction Signaling	$5.5*10^{-4}$	0.018	ACTA1↑*, MYH1↑*, MYH2↑*
Leukocyte Extravasation Signaling	$7.9*10^{-4}$	0.015	ACTA1↑, NCF2↑, PIK3CD↑
Calcium-induced T Lymphocyte Apoptosis	$1.3*10^{-3}$	0.032	HLA-DMA↑, ATP2A1↑*
Androgen and Estrogen Metabolism	$1.6*10^{-3}$	0.015	HSD11B1↑, HSD3B2↑
IL-4 Signaling	$2.0*10^{-3}$	0.028	HLA-DMA↑, PIK3CD↑
Nitric Oxide Signaling in the Cardiovascular System	$2.3*10^{-3}$	0.022	ATP2A1↑*, PIK3CD↑
CTLA4 Signaling in Cytotoxic T Lymphocytes	$3.0*10^{-3}$	0.023	HLA-DMA↑, PIK3CD↑

Top canonical pathways of aortic transcriptome changes in Fibulin-$4^{+/R}$ mice compared to Fibulin-$4^{+/+}$ littermates. When comparing Fibulin-$4^{+/R}$ and Fibulin-$4^{+/+}$ aortas, mostly genes involved in calcium signaling were identified (*).

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Table 2. Top ten Ingenuity Canonical Pathways following ANOVA (Fibulin-$4^{+/+}$ vs. Fibulin-$4^{R/R}$)

Ingenuity Canonical Pathways	p-value	Ratio	Genes
Complement System	$3.5*10^{-7}$	0.167	C1QA↑, C1QB↑, C1QC↑, C3AR1↑, C5AR1↑, CFB↑
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	$9.3*10^{-7}$	0.091	C1QA↑, C1QB↑, C1QC↑, C3AR1↑, C5AR1↑, CASP1↑, IRF1↑, TLR2↑
IL-12 Signaling and Production in Macrophages	$6.2*10^{-6}$	0.061	ALOX12↑, IRF1↑, MAF↑, PRKCD↑, SPI1↑, STAT1↑, TGFB1↑*, TLR2↑
TREM1 Signaling	$1.5*10^{-5}$	0.087	CASP1↑, CCL2↑, CD86↑, FCGR2B↑, TLR2↑, TYROBP↑
IL-10 Signaling	$3.2*10^{-5}$	0.085	CCR1↑, CCR5↑, CD14↑, FCGR2A↑, FCGR2B↑, SOCS3↑
Dendritic Cell Maturation	$4.6*10^{-5}$	0.049	CD86↑, FCGR2A↑, FCGR2B↑, HLA-B↑, HLA-DMA↑, STAT1↑, TLR2↑, TYROBP↑
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	$3.5*10^{-4}$	0.058	ACTG2↓, FCGR2A↑, FGR↑, PLD4↑, PRKCD↑, VAV1↑
Hepatic Fibrosis/ Hepatic Stellate Cell Activation	$1.6*10^{-3}$	0.044	CCL2↑, CCR5↑, CD14↑, TGFB1↑*, MYH9↑, STAT1↑
Leukocyte Extravasation Signaling	$1.7*10^{-3}$	0.036	ACTG2↓, CLDN3↑, CYBB↑, MMP14↑, NCF2↑, PRKCD↑, VAV1↑
Natural Killer Cell Signaling	$1.9*10^{-3}$	0.044	CD300A↑, FCER1G↑, PRKCD↑, TYROBP↑, VAV1↑

Top canonical pathways of aortic transcriptome changes in Fibulin-$4^{R/R}$ mice compared to Fibulin-$4^{+/+}$ littermates. Analysis of Fibulin-$4^{R/R}$ mice revealed mainly genes associated with immunological or infectious diseases. TGF-β showed upregulation (*).

Table 3. Top ten Ingenuity Canonical Pathways following Statistical Analysis of Microarrays (Fibulin-$4^{+/+}$ vs. Fibulin-$4^{+/R}$)

Ingenuity Canonical Pathways	p-value	Ratio	Genes
Antigen Presentation Pathway	$6.3*10^{-22}$	0.359	B2M↑, CD74↑, HLA-B↑, HLA-C↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, HLA-DQA1↑, HLA-DQB2↑, HLA-DRB1↑, HLA-E↑, PSMB8↑, PSMB9↑, TAP1↑
Dendritic Cell Maturation	$7.9*10^{-17}$	0.115	B2M↑, CD86↑, FCGR2A↑, FCGR2B↑, FCGR3A↑,HLA-B↑, HLA-C↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, HLA-DQA1↑, HLA-DQB2↑, HLA-DRB1↑, NFKBIE↑, PIK3CA↓, PIK3CD↑, STAT1↑, STAT4↑, TYROBP↑
Calcium Signaling	$1.6*10^{-13}$	0.087	ACTA1↑*, ACTC1↓*, ATP2A1↑*, MYH1↑*, MYH2↑*, MYH6↓*, MYH8↑*, MYL1↑*, MYL4↓*, MYL7↓*, RYR1↑*, TNNC1↓*, TNNC2↑*, TNNI2↑*, TNNI3↓*, TNNT2↓*, TNNT3↑*, TRDN↑*
CD28 Signaling in T Helper Cells	$6.3*10^{-11}$	0.105	CARD11↑, CD247↑, CD4↑, CD86↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, NFKBIE↑, PIK3CA↓, PIK3CD↑, PTPRC↑, SYK↑, VAV1↑
Role of NFAT in Regulation of the Immune Response	$1.0*10^{-10}$	0.080	BLNK↑, CD247↑, CD4↑, CD86↑, FCER1G↑, FCGR2A↑, FCGR2B↑, FCGR3A↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, NFKBIE↑, PIK3CA↓, PIK3CD↑, SYK↑
IL-4 Signaling	$7.8*10^{-10}$	0.139	B2M↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, HLA-DQA1↑, HLA-DQB2↑, HLA-DRB1↑, INPP5D↑, PIK3CA↓,PIK3CD↑
Natural Killer Cell Signaling	$2.0*10^{-9}$	0.096	CD247↑, FCER1G↑, FCGR3A↑, INPP5D↑, KLRD1↑, PIK3CA↓, PIK3CD↑, PRKCB↑, SYK↑, TYROBP↑, VAV1↑
B Cell Receptor Signaling	$3.2*10^{-9}$	0.084	BLNK↑, CD22↑, FCGR2A↑, FCGR2B↑, INPP5D↑, NFKBIE↑, PIK3AP1↑, PIK3CA↓, PIK3CD↑, PRKCB↑, PTPRC↑, SYK↑, VAV1↑
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	$3.7*10^{-9}$	0.106	ACTA1↑*, ACTC1↓*, FCGR2A↑, FCGR3A↑, FGR↑, HCK↑, INPP5D↑, PLD4↑, PRKCB↑, SYK↑, VAV1↑
CTLA4 Signaling in Cytotoxic T Lymphocytes	$6.5*10^{-9}$	0.112	CD247↑, CD4↑, CD86↑, HLA-DMA↑, HLA-DMB↑, HLA-DOA↑, PIK3CA↓, PIK3CD↑, PTPN22↑, SYK↑

Top canonical pathways of aortic transcriptome changes in Fibulin-$4^{+/R}$ mice compared to Fibulin-$4^{+/+}$ littermates. Molecules involved in muscle contractility showed high significance (*). Furthermore, many genes involved in immune responses and infectious diseases were identified.

Table 4. Top ten Ingenuity Canonical Pathways following Statistical Analysis of Microarrays (Fibulin-$4^{+/+}$ vs. Fibulin-$4^{R/R}$)

Ingenuity Canonical Pathways	p-value	Ratio	Genes
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	$7.9*10^{-12}$	0.140	C1QA↑, C1QB↑, C1QC↑, C5AR1↑, C3AR1↑, CASP1↑, CLEC7A↑, IRF1↑, OAS1↑, SYK↑, TLR1↑, TLR2↑
Complement System	$1.0*10^{-8}$	0.194	C1QA↑, C1QB↑, C1QC↑, C3AR1↑, C4B↑, C5AR1↑, CFB↑
Atherosclerosis Signaling	$4.3*10^{-8}$	0.091	ALOX12↑, ALOX15↑, CCL2↑, CCR2↑, ITGB2↑, MSR1↑, SELP↑, TGFB1↑*, VCAM1↑, WISP2↑
Dendritic Cell Maturation	$7.6*10^{-8}$	0.063	FCER1G↑, FCGR1A↑, FCGR2A↑, FCGR2B↑, FCGR3A↑, TLR2↑, HLA-B↑, HLA-C↑, HLA-DMA↑,TNFRSF1B↑, TYROBP↑
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	$2.9*10^{-7}$	0.089	HCK↑, FCGR1A↑, FCGR2A↑, FCGR3A↑, FGR↑, PLD4↑, PRKCD↑, SYK↑, VAV1↑
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	$4.2*10^{-7}$	0.044	C5AR1↑, CCL2↑, CFB↑, FCGR1A↑, FCGR3A↑, FOS↑, FRZB↑, LRP1↑, PRKCD↑, SOCS3↑, TGFB1↑*, TLR1↑, TLR2↑, TNFRSF1B↑, VCAM1↑
IL-12 Signaling and Production in Macrophages	$5.6*10^{-7}$	0.067	ALOX12↑, ALOX15↑, FOS↑, IRF1↑, MAF↑, PRKCD↑, SPI1↑, TGFB1↑*, TLR2↑
Systemic Lupus Erythematosus Signaling	$8.7*10^{-7}$	0.060	CD72↑, FCER1G↑, FCGR1A↑, FCGR2A↑, FCGR2B↑,FCGR3A↑, FOS↑, HLA-B↑,HLA-C↑
TREM1 Signaling	$9.5*10^{-7}$	0.101	CASP1↑, CCL2↑, FCGR2B↑, LAT2↑, TLR1↑, TLR2↑,TYROBP↑
IL-10 Signaling	$2.0*10^{-6}$	0.100	CCR1↑, CCR5↑,CD14↑, FCGR2A↑,FCGR2B↑, FOS↑, SOCS3↑

Top canonical pathways of aortic transcriptome changes in Fibulin-$4^{R/R}$ mice compared to Fibulin-$4^{+/+}$ littermates. Mainly genes involved in immune responses and infectious diseases were identified. TGF-β showed upregulation (*).

Acknowledgments: We thank Ed Lansbergen, Diana Ensink and Dennis de Meulder for their assistance with the losartan treatment. This work was financially supported by the stichting Lijf en Leven and the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NWO).

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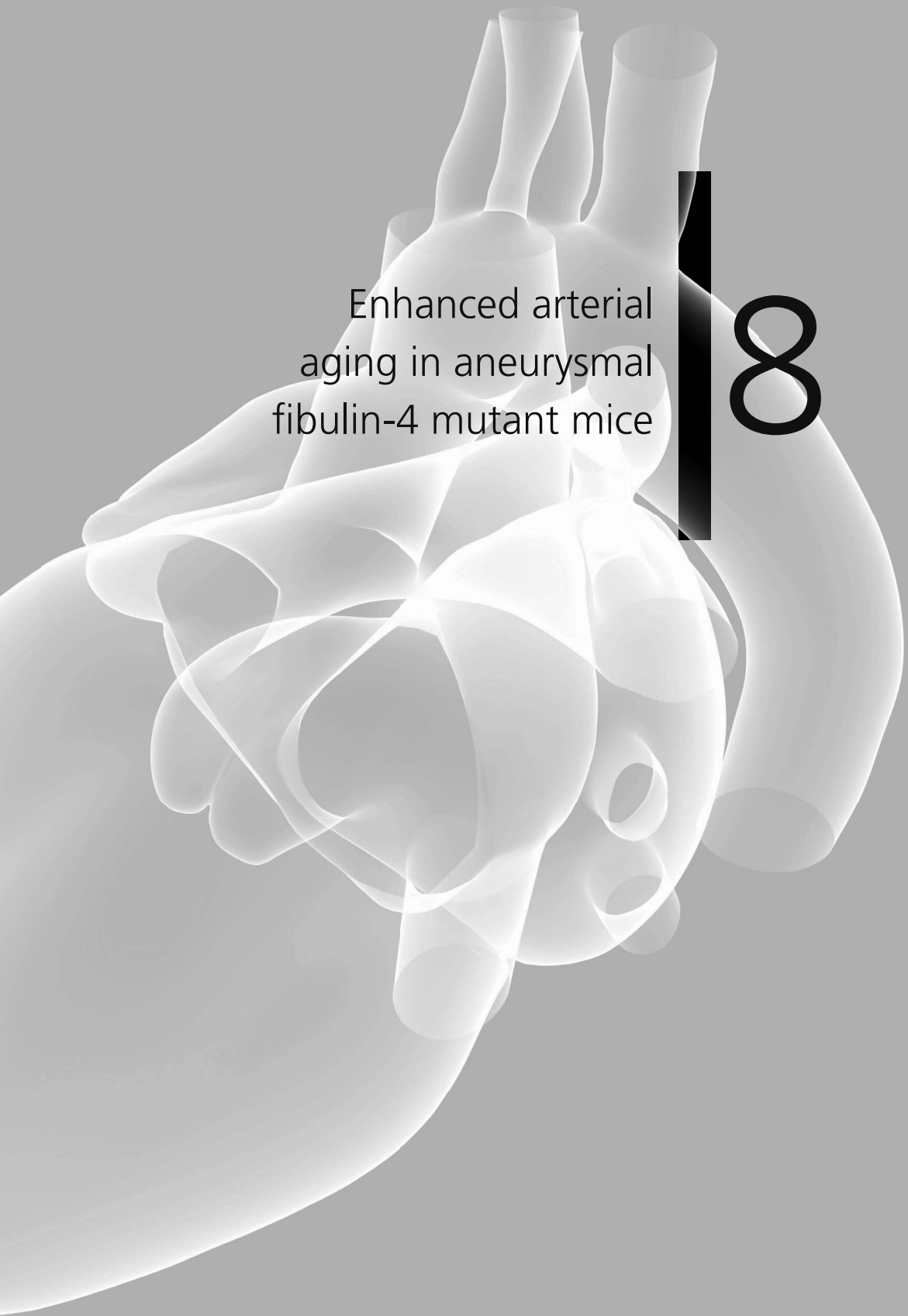
Enhanced arterial aging in aneurysmal fibulin-4 mutant mice



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ABSTRACT

Fibulin-4 is a secreted glycoprotein, which is expressed in medial layers of blood vessels. All six reported fibulin-4 patients suffer from cardiovascular complications including aortic aneurysms, arterial tortuosity and elastin abnormalities. We used fibulin-4 mouse models that express reduced levels of the fibulin-4 protein to identify primary changes in global aortic protein expression patterns that lead to the development of these aortic abnormalities. Interestingly, heterozygous Fibulin-$4^{+/R}$ mice show only mild arterial abnormalities, while the homozygous Fibulin-$4^{R/R}$ mice display elongated and 2-3 fold dilated ascending aortas. To get insight into the underlying molecular pathways involved in aneurysm formation and response to aortic failure, we determined the aorta proteome of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ animals. A full un-biased qualitative MS/MS proteomic screen of the aorta protein extracts identified an increase in mitochondrial oxidative phosphorylation as the major dysregulated pathway in both the Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ animals. Next, an overlay of the aortic proteome and transcriptome was made, which yielded a limited set of biomarkers. In the Fibulin-$4^{R/R}$ mice this narrow set of biomarkers pointed towards altered regulation of 17β-estradiol and the inflammation associated TNF-α pathway. Interestingly, deregulation of the 17β-estradiol pathway was also found in the Fibulin-$4^{+/R}$ mice. The signaling molecule 17β-estradiol is a metabolite, known to deregulate production of reactive oxygen species (ROS) by poorly understood mechanisms. Notably, both mitochondrial function and the 17β-estradiol pathway are deregulated by oxidative stress, which is a hallmark of aging and age-related cardiovascular diseases. Increased arterial aging was subsequently demonstrated by a gradual increase in ROS production, endothelial dysfunction and reduced aortic distensibility in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice. These results uncover new regulatory pathways likely to be associated with enhanced arterial aging in aneurysmal fibulin-4 mice.

INTRODUCTION

Thoracic aortic aneurysm is a life threatening condition with high patient mortality. The molecular mechanism of aortic aneurysm formation is complex and not well understood. Till date, surgery is the most effective way of patient care. Genetic predisposition, aging and hypertension are important contributors of disease onset and progression. Importantly, aging is a ubiquitous contributor of aortic aneurysm in elderly patients.

Fibulin-4 is a secreted glycoprotein, which is expressed in medial layers of blood vessels and a critical component for the structural integrity and elasticity of the aortic wall.[1, 2] Six patients have been reported with mutations in fibulin-4 and all suffer from cardiovascular complications including aortic aneurysms, arterial tortuosity and elastin abnormalities.[3-6] Insufficient levels of fibulin-4 compromise the structural integrity of the aortic wall, which can lead to aneurysm formation. Mice with reduced levels of the fibulin-4 protein develop aortic abnormalities similar to fibulin-4 patients, such as a severe dilation of the ascending aorta, characteristic for aortic aneurysms.[7-9] Fibulin-4 is an essential gene, but previously we have generated an allele referred to as fibulin-4^{R}, which reduces the fibulin-4 protein expression two-fold.[7] Our Fibulin-$4^{R/R}$ mouse model mimics the aneurysm of genetically affected patients due to 4-fold reduced levels of fibulin-4 expression. Fibulin-$4^{+/R}$ mice with a 2-fold decrease in fibulin-4 expression, show the onset of aneurysm formation, a situation that is comparable with the development of an aneurysm in aging humans.

Fibulin-4 protein levels in the aorta are essential for vascular maturation, wherein the protein tethers elastic fibers to smooth muscle cells via integrin-mediated binding at regions of the cell membrane, occupied by intracellular membrane-associated dense plaques anchoring sites for actin filaments. Consistent with this finding, 4-fold reduction of the fibulin-4 protein in the aorta of the Fibulin-$4^{R/R}$ homozygous mice causes a 2-3 fold dilated ascending thoracic aorta, while heterozygous Fibulin-$4^{+/R}$ mice show mild symptoms of aneurysm formation, like incidental ballooning of the right subclavian artery and mild elastin abnormalities. Interestingly, the heterozygous mutant mouse provides a model for late aneurysm onset, caused in patients with diverse genetic predisposition and by environmental factors. Notably, our genome-wide aorta transcriptome and histological findings revealed TGF-β signaling as the critical event in the pathogenesis of aneurysm formation in the heterozygous Fibulin-$4^{+/R}$ and homozygous Fibulin-$4^{R/R}$ animals.[7]

In this study, we performed a combined proteome and transcriptome analysis to identify underlying molecular changes that precede (using Fibulin-$4^{+/R}$ mice) and accompany (using Fibulin-$4^{R/R}$ mice) aneurysm formation. Since both Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice show clear signs of mitochondrial dysfunction and altered 17β-estradiol associated metabolism, we determined whether Fibulin-4 mice display premature onset of key features of enhanced arterial aging such as increased production of reactive oxygen species (ROS) in the aorta, endothelial dysfunction and reduced aorta distensibility. Interestingly, both Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice show enhanced ROS-induced arterial aging, which is accompanied by increased inflammation in the Fibulin-$4^{R/R}$ animals.

MATERIALS AND METHODS

Experimental animals

We previously generated a fibulin-4 allele with reduced expression by transcriptional interference through placement of a TKneo targeting construct in the downstream Mus81 gene.[7] Heterozygous (Fibulin-$4^{+/R}$) mice in a mixed C57Bl/6J;129Sv background were mated to obtain Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibullin-$4^{R/R}$ littermates and were housed in the institutional animal facility. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands.

Preparation of the aorta protein extracts and MS/MS protein identification

Fibulin-4 mutant mice age 80-90 days were sacrificed and ascending thoracic aortas were collected and isolated. Next, aorta protein extracts were made and aortas and protein concentration were determined as described previously.[10] Equal amounts of total proteins were loaded and size separated on the gradient (5-20%) SDS gel. Subsequently, the gels were stained with Coomassie Brilliant Blue, and all stained bands were cut horizontally from the SDS gel and treated with trypsin. LC-MS/MS mass spectra were recorded in positive ion mode on an applied biosystem 4700 LC-MS spectrometer. Inbuilt software on the mass spectrometer was used to process MS/MS data, which generated m/z peak lists. Finally, using these peak lists on the Mascot database searches protein identification was obtained.

Microarray analyses

Transcriptome datasets were collected from the GEO database.[7] Collected datasets were analyzed by various R packages and Partek version 6.4 (Partek Inc., St. Louis, MO) as described previously.[7] Previously described datasets for two Fibulin-$4^{R/R}$ mice were discarded in the present study, as they failed to fulfill the quality control criteria. Rejection of these two datasets also improved the overall PCA map (data not shown). The final six datasets consisted of two for each genotype. Differentially expressed genes were identified using ANOVA (Partek). Cut-off values for significantly expressed genes were the false discovery rate of 0.05 and a fold change of 1.5.

Pathway design and analysis

For integrating gene and protein datasets, web-based open source tools were used to ensure correct annotation of genes and proteins. Uniport (www.uniport.org) archive batch retrieval tool to translate proteins and Entrez gene ID for transcriptome (affymetrix) annotations were consulted. Finally, the corrected annotated lists of proteins and genes were uploaded on the Ingenuity web server (www.ingenuity.com) and functional pathways were designed and analyzed with Ingenuity Pathway Analysis (IPA version: Pre 8.0, Ingenuity, Mountain View, CA). First, an automatic analysis was done and next, the runs were manually checked for relevant molecular pathways.

Histology and immunohistochemistry

Mice (age 90-100 days) were euthanized by an overdose CO_2, and necropsied according to

standard protocols. Aortas were isolated and paraffin embedded. Next, 5-µm sections were stained. BrdU staining was performed on perfusion-fixed aortas, according to the protocol of the manufacturer (Roche, Basel).

Mulvany myographs

Male mice (age 120 days) were euthanized with pentobarbital i.p. (150 µl, 60 mg/ml). Thoracic aorta, abdominal aorta and iliac artery were isolated and stored overnight in cold, oxygenated Krebs-Henseleit buffer solution. The following day, vessel segments were mounted in 6-ml 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 100-mm Hg effective transmural pressure.[11] Relaxation concentration response curves (CRCs) were constructed upon addition of acetylcholine and sodium nitroprusside (SNP) following preconstriction with 30 nmol/L U46619.

Aortic distensibility

Mice (age 70-120 days) were sedated with 4% isoflurane and intubated as previously described.[12] The mice were ventilated with a mixture of O_2 and N_2O (1/2, vol/vol) with a pressure controlled ventilator (Vevo Compact Anaesthesia System, VisualSonics Inc) to which 2% isoflurane was added for anaesthesia. Ventilation rate was set at 90 strokes/min with a peak inspiration pressure of 18 cm H_2O and a positive end expiration pressure (PEEP) of 4 cm H_2O. The mice were placed on a heating pad to maintain body temperature at 37°C. *In vivo* transthoracic echocardiography of the aorta was performed with a Vevo2100 (VisualSonics Inc., Toronto, Canada) using a 40-MHz linear interfaced array transducer (MS550S). Measurement of aortic distensibility was performed using the displacement of the arterial walls. The end-diastolic diameter (DD), the absolute change in diameter during systole (DD-SD), and the relative change in diameter ((DD-SD)/DD) were computed.

In vivo registration of reactive oxygen species

Mice were anaesthetized (isoflurane 1.5%, O_2 2 L/min) and visualized using an imaging system (Caliper). L-012 (8-amino-4-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H) dione, a chemitransluminescent probe and derivative of luminol,[13] was purchased from Wako Chemical (Neuss, Germany) and dissolved in H_2O. A concentration of 25 mg/kg in a volume of 100 µl was administered intravenously and *in vivo* imaging was performed within 5 min after administration L-012. For *ex vivo* imaging, mice were sacrificed by an overdose of anaesthesia and the chest was opened according to standard necropsy protocols. Data acquisition and analysis was performed using IVIS imaging software Living Image (Caliper).

Data-analysis

Data are presented as mean±SEM. CRCs were analyzed using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, California, USA). The CRCs allowed us to compare the maximum effect (E_{max}) and to calculate the concentration that leads to 50% maximal response ($-^{10}\log EC_{50}$) as described before.[14] Analysis of the differences between CRCs was performed by two-way ANOVA and followed by post hoc evaluation according to Bonferroni. To evaluate the dose-

dependent effect of fibulin-4 expression, a linear regression analysis was performed to obtain a p for trend. The latter statistical analysis was performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill, USA). All tests were two-sided, and a p-value <0.05 was considered significant.

RESULTS

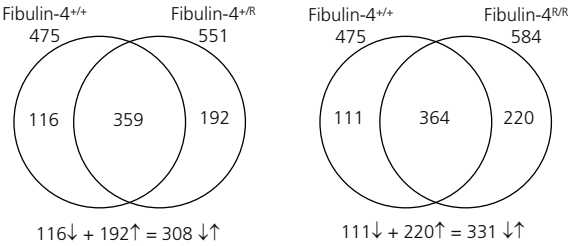
Aorta protein expression profiles of fibulin-4 mutant mice

Previously, when we compared the elastic fiber network of the aortas of wild type and homozygous mutant fibulin-4 mice, we observed significant changes in the architecture of the elastic laminae, consisting of a granular appearance of elastin in the outer layers of the aorta. In addition, the inner layers showed fragmentation and disarray of elastic fibers and the accumulation of amorphous matrix between fibers leading to aortic wall thickening. While the integrity of elastic fibers in heterozygous Fibulin-$4^{+/R}$ mice was preserved, we observed increased accumulation of amorphous matrix material between layers, indicating a gene-dosage effect. Aorta transcriptome analysis of heterozygous and homozygous Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ animals identified excessive TGF-β signaling as the critical event in the pathogenesis of aneurysm formation. Immunolabeling of aortic tissue slices from the different mice revealed a graded increase in TGF-β signaling in comparison to wild type animals, as evidenced by increased phosphorylation and nuclear translocation of Smad2.[7]

Next, we sought to identify changes leading to aortic degeneration and aortic failure at the protein level. Therefore, we analyzed proteome expression profiles using a 1D gradient gel of total aorta protein extract. Proteins separated on the gel were trypsin digested and identified by a MS/MS method, coupled with the mascot database searches. We determined the aorta proteome profiles of Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice (Tables 1-3). Figure 1 shows a full unbiased qualitative MS/MS proteomic screen of the aorta protein extracts. In the wild type Fibulin-$4^{+/+}$ aorta, 475 proteins were identified, in the Fibulin-$4^{+/R}$ aorta 551 and finally 584 in the Fibulin-$4^{R/R}$ aorta. A duplicate analysis of the samples identified more than 75% of the proteins in the first analysis. Analysis of an independent set of aorta extracts identified more than 65% of the previously identified proteins.

Subsequently, we performed a pairwise comparison of the identified proteins between Fibulin-$4^{+/R}$ vs. wild type and Fibulin-$4^{R/R}$ vs. wild type mice. In both cases, the majority of proteins were present in the aortas of both genotypes. However, in the first comparison 116 proteins were identified in the wild type aorta that were not identified in the aorta of heterozygous Fibulin-$4^{+/R}$ animal. Therefore, we assume that the level of these proteins is reduced in the heterozygous mutant mice. We considered these 116 proteins as downregulated in the heterozygous mice. Similarly, 192 proteins were considered to be upregulated in the heterozygous mutant animal. We followed the same strategy for comparison of Fibulin-$4^{R/R}$ vs. Fibulin-$4^{+/+}$ aortas, resulting in two lists of identified proteins. The first list contained 308 identified proteins deregulated in the Fibulin-$4^{+/R}$ mutant aortas, while the second contained 331 proteins deregulated in the Fibulin-$4^{R/R}$ aortas. Using the two lists we performed analysis to identify biological pathways mediated by these proteins. At first, the clustering of proteins under molecular functions was tested. Remarkably, for both set of comparisons a common cluster that shows up is oxidative phosphorylation, a mitochondrial electron transport process involved in energy production.[15] Since the majority of identified proteins in this cluster are upregulated, this suggests that the

A



B

Fibulin-4$^{+/R}$/Fibulin-4$^{+/+}$	
Mitochondria	**Proteins (↑↓)**
Oxidative Phosphorylation	ATP5J2↑, ATP5L↑, COX5B↑, COX6B1↑, COX7A2↑, UQCRHL↑, ND1↑, ND3↑, NDUFA1↑, NDUFA3↑, DUFA5↑, NDUFA11↑, UFB7↑, NDUFB8↑, UFB11↑, NDUFC2↑, NDUFA6↑, NDUFA9↓, NDUFS6↑, NDUFS7↑, NDUFV1↑, PPA1↓, UQCR11↑, UQCRB↑

Fibulin-4$^{R/R}$/Fibulin-4$^{+/+}$	
Mitochondria	**Proteins (↑↓)**
Oxidative Phosphorylation	ATP5J2↑, ATP5L↑, COX6B1↑, COXA7A1↑, COX7A2↑, NDUFA5↑, NDUFA6↑, NDUFA9↑, NDUFB11↑, NDUFS5↑, NDUFS7↑, NDUFS6↑, PPA1↑, UQCRB↑

Figure 1. Proteome screen of aortas identified mitochondrial dysfunction and deregulated oxidative phosphorylation in Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ aortas compared to wild type animals (**A**) Venn diagram showing the overlap and differences between proteins identified from the indicated genotypes. (**B**) Ingenuity pathway analysis of 308 deregulated proteins in Fibulin-4$^{+/R}$ and 331 deregulated proteins in Fibulin-4$^{R/R}$ mice identified oxidative phosphorylation regulation as the major biological pathway affected.

decrease of fibulin-4, a structural protein, may enhance mitochondrial activity, and thereby induce cellular stress, i.e oxidative stress.[15]

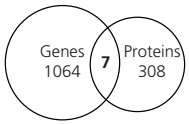
Connection between fibulin-4 deficiency and 17β-estradiol

In the above section, proteome analysis of the fibulin-4 mutant mice unveiled deregulated proteins and associated molecular functions. In particular, pathway analysis pointed towards an altered mitochondrial metabolism. However, our previous aortic transcriptome analysis identified excessive TGF-β signaling as the critical event in the pathogenesis of aneurysm formation.[7] Thus, aortic transcriptome and proteome analyses independently identified different deregulated biological processes as their top hits.

To discover common processes identified by both transcriptome and proteome approaches we combined both types of analyses (Figure 2A). Combining transcriptome and proteome data provided a great advantage as it narrowed the potential identified biomarkers by each separate approach to just a few. Figure 2A shows 7 and 11 potential biomarkers, respectively, that are differentially affected in the heterozygous and homozygous animals compared to wild type animals. Next, these potential biomarkers were used to find altered pathways associated with aortic aneurysms using pathway analysis. Interestingly, for the homozygous mutant we discovered connections to beta-estradiol and TNF pathways (Figure

A

Fibulin-4$^{+/R}$ / Fibulin-4$^{+/+}$



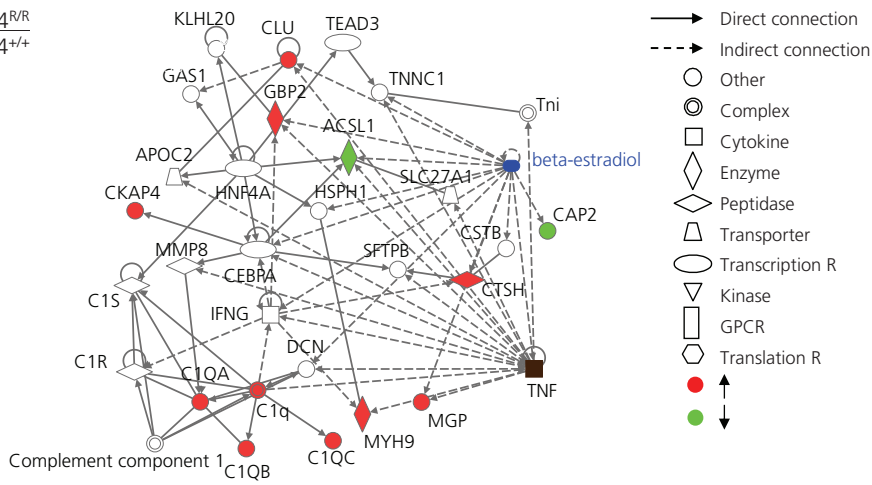
ARRB2	Arrestin, beta 2
CAP2	Adenylate cyclase-associated protein 2
CLU	Clusterin
DSP	Desmoplakin
FABP3	Fatty acid binding protein 3
HSD11B1	Hydroxysteroid dehydrogenase 1
NEB	Nebulin

Fibulin-4$^{R/R}$ / Fibulin-4$^{+/+}$

ACSL1	Acyl-CoA synthetase long-chain family member 1
C1QA C1QB C1QC	Complement component 1, q subcomponent, A, B, C chain
CAP2	Adenylate cyclase-associated protein, 2
CKAP4	Cytoskeleton associated protein 4
CLU	Clusterin
CTSH	Cathepsin H
GBP2	Guanylate binding protein 2, interferon-inducible
MGP	Matrix-Gla protein
MYH9	Myosin Heavy Chain 9

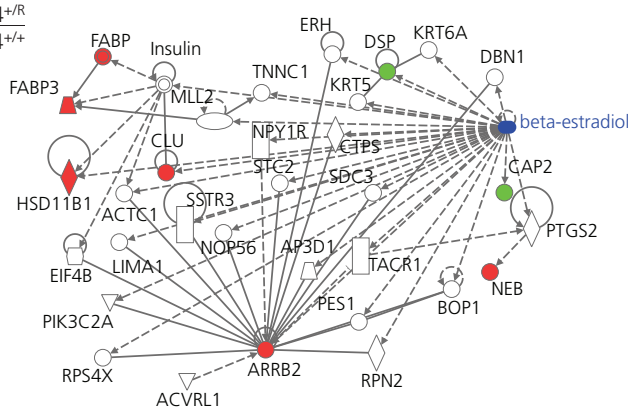
B

Fibulin-4$^{R/R}$ / Fibulin-4$^{+/+}$



C

Fibulin-4$^{+/R}$ / Fibulin-4$^{+/+}$



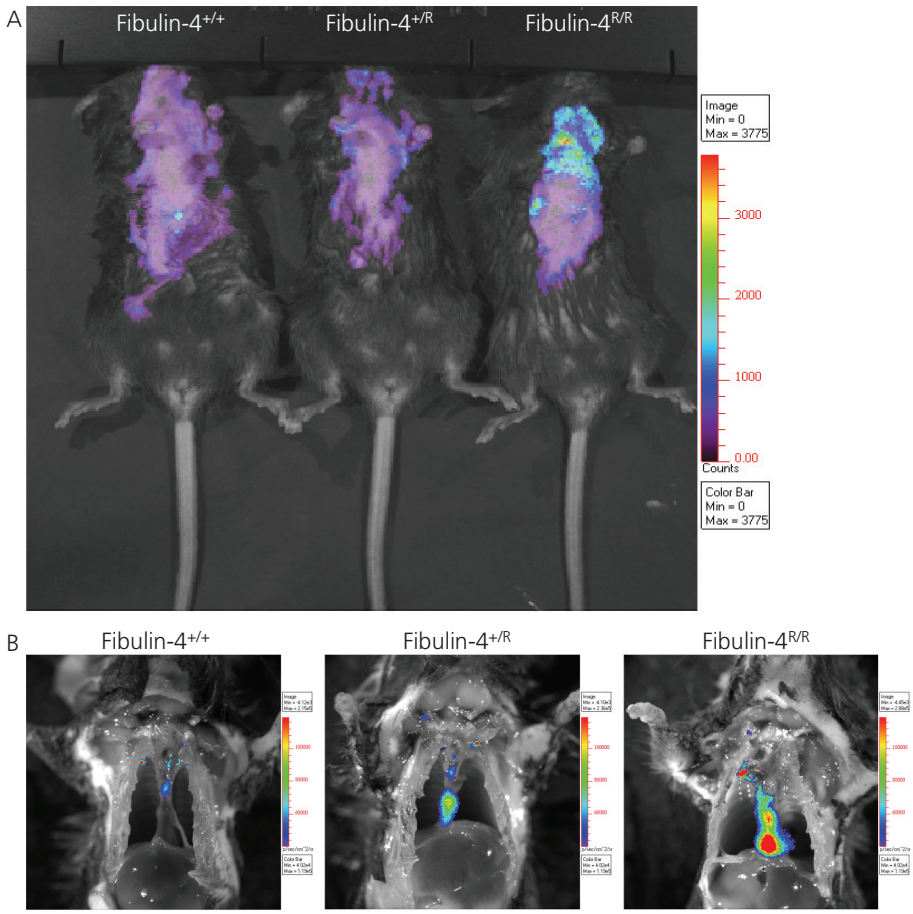


Figure 3. Increased ROS levels detected by molecular imaging using chemiluminescent probe L-012. (**A**) ***In vivo*** imaging shows excessive signaling in the Fibulin-4$^{R/R}$ mouse. (**B**) *Ex vivo* analysis of the chest region demonstrated increased signaling in the aortas of Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ mice compared to wild type mice.

Figure 2 (left). Combined gene expression analysis and proteome expression analysis identified deregulation of the17β-estradiol pathway in both Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ mice. (**A**) List of biomarkers identified by gene expression analysis and proteome expression analysis in Fibulin-4$^{+/R}$ (7) and Fibulin-4$^{R/R}$ (11) mice. (**B-C**) Gene ingenuity network analysis of biomarkers identified in Fibulin-4$^{R/R}$ (**B**) and Fibulin-4$^{+/R}$ mice (**C**). Both networks point towards a deregulated 17β-estradiol pathway. The identification of, among others, complement components results in the additional recognition of TNF-α associated pathways in Fibulin-4$^{R/R}$ mice (**B**).

2B). Importantly, 17β-estradiol is a metabolite, signaling molecule and an aging marker that deregulates the production of ROS by a poorly defined mechanism, while TNF is a cytokine and an inflammation marker.[16] Additional inflammation markers that were upregulated were the different components of complement 1Q. This led us to hypothesize that aging and inflammation might be a coupled feature of fibulin-4 homozygous mutant mice. In the much more mildly affected heterozygous mutant mice we also found links to 17β-estradiol. In addition, we noticed connections to the Insulin hub, which is important for aging-related IGF1-signaling (Figure 2C).[17] This demonstrates that 17β-estradiol is a common element between heterozygous and homozygous mutant mice. In both these mice levels of 17β-estradiol and estrogen may deregulate the oxidative stress induced production of ROS in the aorta.[18] Importantly, oxidative stress-induced ROS causes intracellular and extracellular damage as is commonly found in aging animal models,[19] and age-induced cardiovascular animal models.[15]

Non-invasive visualization of reactive oxygen species in fibulin-4 mutant mice

To test our hypothesis of aging and inflammation-induced altered ROS production in the aortas of fibulin-4 mutant mice, we indirectly imaged the *in vivo* levels of ROS by a chemiluminescence probe (L-012), which is a modified luminol derivative and is a sensitive marker of ROS.[13, 20] L-012 was simultaneously injected intravenously in anaesthetized Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice and chemiluminescence was subsequently recorded *in vivo*. In the presence of L-012, in aortic cells that produce ROS, generates a marked chemiluminescence with negligible background.[13, 20] Levels of ROS were examined by monitoring the emitted light. *In vivo* images showed increased luminescence in the chest region of the Fibulin-$4^{R/R}$ mouse, indicating increased ROS production in this area (Figure 3A). To elucidate the origin of the signal, mice were sacrificed and imaged *ex vivo*. *Ex vivo* images showed a steady increase of ROS levels in the aortas of Fibulin-$4^{+/R}$ and an even further increase in Fibulin-$4^{R/R}$ mice (Figure 3B). Notably, this increase in chemiluminesence was also observed in the dissected lungs, but not in other organs (data not shown), and thus emphasises that this relative overproduction of ROS was mainly present in tissue containing fibulin-4 and elastic fibers.[21]

Endothelial dysfunction

Increased oxidative stress induces endothelial dysfunction by impairing the bioactivity of endothelial nitric oxide (NO). In addition, endothelial dysfunction is used as a marker of aging.[22] Proliferation of endothelial cells, indicating endothelial cell damage, was visualized by BrdU staining. Indeed, increased proliferation was observed specifically for endothelial cells in Fibulin-$4^{R/R}$ mice (Figure 4A).

Next, the functional consequences of the endothelial cell damage in fibulin-4 mutant mice were tested in several segments of the large arterial tree. CRCs were constructed to examine endothelium-dependent and –independent relaxation with acetylcholine and SNP, respectively. In the ascending thoracic aorta of Fibulin-$4^{R/R}$ mice, acetylcholine-induced vasorelaxation was greatly reduced (Figure 4B). This was evidenced by both a reduction in E_{max} and pEC_{50}, the latter also visible by a rightward shift of the CRC. The E_{max} decreased from 22±2% in Fibulin-$4^{+/+}$ to 16±2% in Fibulin-$4^{+/R}$ and 8±2% in Fibulin-$4^{R/R}$ mice. Interestingly,

a reduced endothelium-dependent relaxation was not limited to the aneurysmal area, but also present in the abdominal aortas and iliac arteries. There was a trend towards endothelial dysfunction in Fibulin-$4^{+/R}$ mice, since their acetylcholine CRCs were always in between those of Fibulin-$4^{+/+}$ and Fibulin-$4^{R/R}$ mice. Finally, endothelium-independent relaxation was tested in the same setup, using the exogenous NO donor SNP. SNP-induced relaxation was increased in the descending thoracic aorta of Fibulin-$4^{R/R}$ mice compared to controls, and preserved in the other segments. Thus, endothelial dysfunction in fibulin-4 deficient mice was evidenced by impaired endothelial NO and exogenous donation of NO induces normal vasorelaxation.

Impaired aortic distensibility

In the section above we determined vasorelaxation features of mutant mice aortas under *in vitro* pharmacological conditions. To determine *in vivo* dynamic properties of aortas, we measured aortic wall displacements by means of transthoracic echocardiography. Aortic wall displacement or distensibilty is an elasticity index of the aorta, which normally decreases with aging.[23] Aortic distensibility inversely correlates with aortic stiffness and can be measured using *in vivo* transthoracic echocardiography. Figure 5 shows ascending thoracic aortas of fibulin-4 mutant and wild type mice in M-mode. The graph shows the comparison of the calculated aortic distensibility on the y-axis between the mutant mice on the x-axis. Aorta distensibility gradually decreases with decreasing expression of fibulin-4 in a gene-dose dependent manner (p for trend <0.05). This data suggests that a 2- and 4-fold reduced expression of fibulin-4 protein in the aortas of mutant mice results in increased arterial stiffness, which is an important marker for vascular aging.

DISCUSSION

In mammals, aging is accompanied by progressive cardiovascular diseases. Aortic aneurysms can be classified as an age-associated disease just as hypertension and atherosclerosis. The mechanism that governs the formation of aortic aneurysm in humans and mice is still poorly understood. The disease pathology in the context of aortic aneurysm is recognized by a bulge in the aorta structure, which is resistant to mechanical stress. A mechanically challenged aorta has several detrimental effects on the physiology of the heart and other circulatory organs to a point that essential function of these organs fail. Therefore, modeling age-related aneurysms and associated organ failure is a major, but necessary, challenge on the path to cure or increase the quality of life of elderly patients.

Altered mitochondrial activity in aortas of fibulin-4 mutant mice

The comparison of aorta proteome profiles of wild type and fibulin-4 mutant mice identified numerous proteins involved in mitochondrial function as differentially affected (Figure 1B). In this regard, a biological process of interest is oxidative phosphorylation in mitochrondria. Mitochondrial electron transport process, which fuels cells by producing ATP, is controlled by a cluster of five multi protein complexes (I-V).[24] We have identified several complex I-V protein components with increased expression in fibulin-4 mutant mice (Figure 1B). These findings are indicative of altered mitochondrial activity in aortas with reduced fibulin-4 expression. Altered mitochondrial activity is associated with oxidative stress-induced production of ROS, which

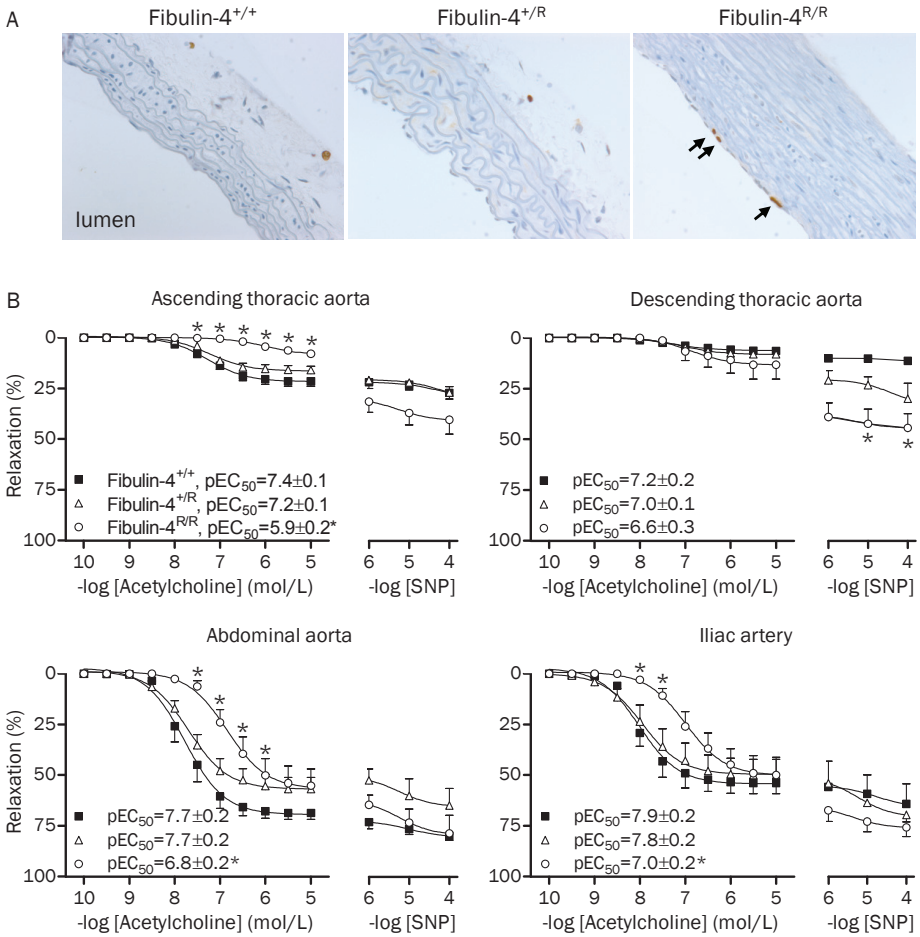


Figure 4. Endothelial dysfunction in fibulin-4 mutant aortas. (**A**) Increased proliferation of endothelial cells are present in aortas from Fibulin-$4^{R/R}$ mice only and indicated by the arrows. (**B**) Relaxant responses to the endothelium-dependent vasodilator acetylcholine and the endothelium-independent vasodilator sodium nitroprusside (SNP) in ascending thoracic, descending thoracic and abdominal aortas, as well as iliac arteries. Data (mean±SEM of 2-7 experiments) are shown as a percentage of the preconstriction induced by U46619. *p<0.05 vs. Fibulin-$4^{+/+}$ mice.

is a natural byproduct of mitochondrial activity. To obtain experimental evidence that altered complex I-V protein expression does affect ROS levels, we visualized the ROS levels in aortas of fibulin-4 mutant mice by indirectly imaging it (Figure 3B). Indeed, using a luminescence probe to detect ROS *in vivo* and *ex vivo* an increase in ROS is seen in a reduced fibulin-4 gene dose-dependent manner, mainly in tissues containing fibulin-4 and elastic fibers. The results of the pathway analysis and animal imaging indicate that the increased ROS levels in response to reduced fibulin-4 expression are the result of altered mitochondrial activity.

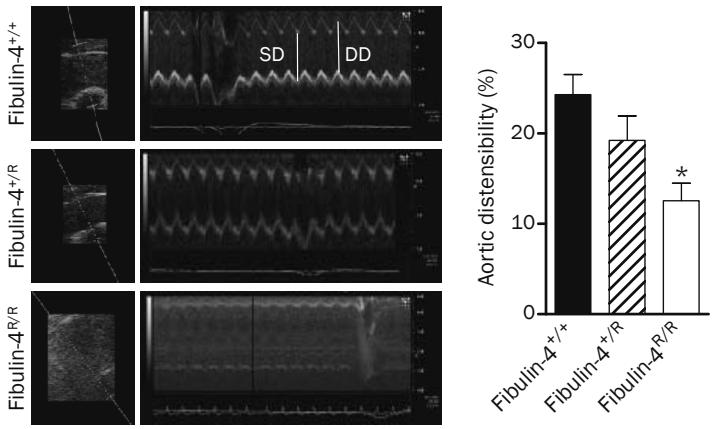


Figure 5. Aortic wall distensibility as assessed by small animal ultrasound imaging using a visualsonics Vevo 2100 at 25 MHz. Systolic and diastolic ascending aortic diameters were recorded in M-mode. Transthoracic echocardiography of the thoracic aorta identified a gene-dose dependent decrease in aortic distensibility in fibulin-4 mutant mice (p for trend <0.05). *p<0.001 vs. wild type.

Gene dose-dependent inflammation in fibulin-4 mutant mice

A fully developed aneurysm is often accompanied by inflammation.[25] This is also seen in mouse models for aortic aneurysms.[26-28] The homozygous Fibulin-4$^{R/R}$ animals are no exception, as the bioinformatics analysis of our combined microarray expression and proteome experiments reveals upregulated expression of complement factors (Figure 2A and B) and connections to the TNF pathway (Figure 2). TNF is a cytokine and a well-known inflammation marker. Cytokines are classic example of inflammatory genes.[29] Although inflammation is a healing process for repairing damaged tissue, overproduction of cytokines by immune cells in response to local tissue damage can induce changes in the vasculature, thereby triggering responses that are no longer the direct result of the aneurysm. In this regard, the Fibulin-4$^{+/R}$ mice provide an important model for aneurysm formation because inflammation does not play a prominent role (Figure 2A and C).

Production of ROS in aneurysm development

In contrast to inflammation, which is limited to the Fibulin-4$^{R/R}$ animals, our experiments revealed an altered oxidative metabolism in the aortas of both Fibulin-4$^{R/R}$ and Fibulin-4$^{+/R}$ mice. First, comparison of the proteome profiles of wild type and mutant mice shows upregulated mitochondrial activity in response to lower fibulin-4 levels (Figure 1). Second, *in vivo* imaging of aortas reveals an increase in ROS in a fibulin-4 dose-dependent manner (Figure 3). Third, bioinformatics analysis reveals an altered 17β-estradiol pathway in both the Fibulin-4$^{R/R}$ and Fibulin-4$^{+/R}$ mice (Figure 2B and C). 17β-estradiol is implicated in deregulation of ROS production.[18] How 17β-estradiol levels control ROS production is poorly understood. 17β-estradiol, a sex steroid, binds and affects estrogen receptors (ER), which are widely expressed

in several tissues, including vascular smooth muscle and endothelial cells. They are involved in increasing cardiac mitochondrial efficiency. Recently, the association between ERα and the risk of cardiovascular diseases has been demonstrated.[30] Common ERα polymorphisms are significantly associated with age-related changes in left ventricular structure.[30, 31] However, it is mechanistically unclear how hormonal changes affect vascular permeability and aortic aneurysm formation. One hypothesis is that the estradiol-ER complex[32] directly deregulates the mitochondrial electron transport processes by increasing the synthesis and consumption of ATP by cells, thereby progressively damaging mitochondria biogenesis.[33] Alternatively, this complex might deregulate the expression of antioxidant enzymes in cells, and an imbalance in the level of antioxidant enzymes and estrogens may enhance the production of ROS.[18] To unravel this puzzle in an animal model, a potential solution would be to treat aneurysmal fibulin-4 mutant mice with an estrogen agonist and demonstrate that the drug can reverse the aorta damage. Thus, by using a combined transcriptomics and proteomics bioinformatics analysis we gained new insights in the molecular affects of fibulin-4 deficiency on the aorta. Using transcriptomics we previously identified upregulated TGF-β signaling in fibulin-4 mutant animals. By using proteomics we here show that fibulin-4 deficiency leads to altered mitochondrial metabolism. Thus, the separate analyses identified different biological processes. However, the power of combining both the transcriptomics and proteomic data in one analysis is demonstrated by the fact that the number of affected biological pathways is narrowed to just a few and an additional affected pathway, 17ß-estradiol signaling, is identified, that escaped detection by each separate approach.

Functional analysis of arterial aging in fibulin-4 mutant mice

Next, we investigated whether fibulin-4 deficiency affected the protective role of the aortic endothelium. Intact endothelium protects vessels from atherosclerosis, while damage to endothelial cells causes platelet aggregation and inflammatory responses. Indeed, combined transcriptome and proteome analysis shows that inflammatory factors are upregulated in Fibulin-4$^{R/R}$ aortas (Figure 2B). Consistent with this observation the endothelial cell layer of Fibulin-4$^{R/R}$ aortas appears to be damaged and/or stressed since it displays enhanced proliferation (Figure 4A). An important question is whether the endothelium of fibulin-4 mutant mice is also functionally affected. Endothelium-dependent relaxation is an important feature of the endothelium. This relaxation is characterized by an imbalance between vasodilator and vasoconstrictor agents, mainly due to a reduced availability of NO and/or endothelium-derived hyperpolarizing factors.[34] Indeed, impaired endothelium-dependent vasorelaxation is seen in a gene dose-dependent manner along the length of the aorta (Figure 4B). Furthermore, fibulin-4 levels affected aortic wall function *in vivo* as demonstrated by transthoracic echocardiography, which shows that aorta distensibility is reduced in mutant mice. Above, we showed that aortas of fibulin-4 mutant mice have higher ROS levels, which could accelerate aging of their aortas. Indeed, our analyses of endothelial function and aorta distensibility measurements provide functional evidence for premature aging of fibulin-4 defective aortas (Figures 4 and 5). Our results are consistent with the observations in human patients, including the fact that fibulin-4 patients suffer from aortic stenosis[6] and the detrimental effect that endothelial dysfunction can have on the aorta structure of Marfan patients[35] and in Marfan-model mice.[36]

Fibulin-4 mutant mice and the effect of gene dose on aortic aneurysm

The results presented here underscore the importance of the fibulin-4 mutant mice as a tool in aneurysm research. The fibulin-4 (FBLN4) protein is a critical component for the structural integrity and elasticity of the aortic wall. Insufficient levels of fibulin-4 compromise the structural integrity of the aortic wall, which can lead to aneurysm formation. Fibulin-4 is an essential gene, but the mutant allele that we generated (fibulin-4^{R}) reduces its expression. Homozygous mutant mice (Fibulin-$4^{R/R}$) have 4-fold lower Fibulin-4 levels, while the heterozygous mice (Fibulin-$4^{+/R}$) display a 2-fold reduction. Fibulin-$4^{R/R}$ mice develop a fully thoracic aortic aneurysm, while Fibulin-$4^{+/R}$ mice are only mild affected. Thus, the magnitude of the aortic decay appears to be fibulin-4 dose dependent, indicating that our Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mouse models are sensitive and might be helpful to identify underlying molecular changes preceding and accompanying aneurysm formation. For example, an important distinction between the two models concerns the inflammatory response. While the Fibulin-$4^{R/R}$ mice display signs of inflammation, the Fibulin-$4^{+/R}$ mice do not and therefore they might be useful to study aneurysm formation without interference brought about by inflammation.

Acknowledgements: The authors are grateful to Diederik Kuster, Erikjan Rijkers and Sigrid Swagemakers for the assistance and discussions during the initial stage of the project. This work was financially supported by the stichting Lijf en Leven and the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NWO).

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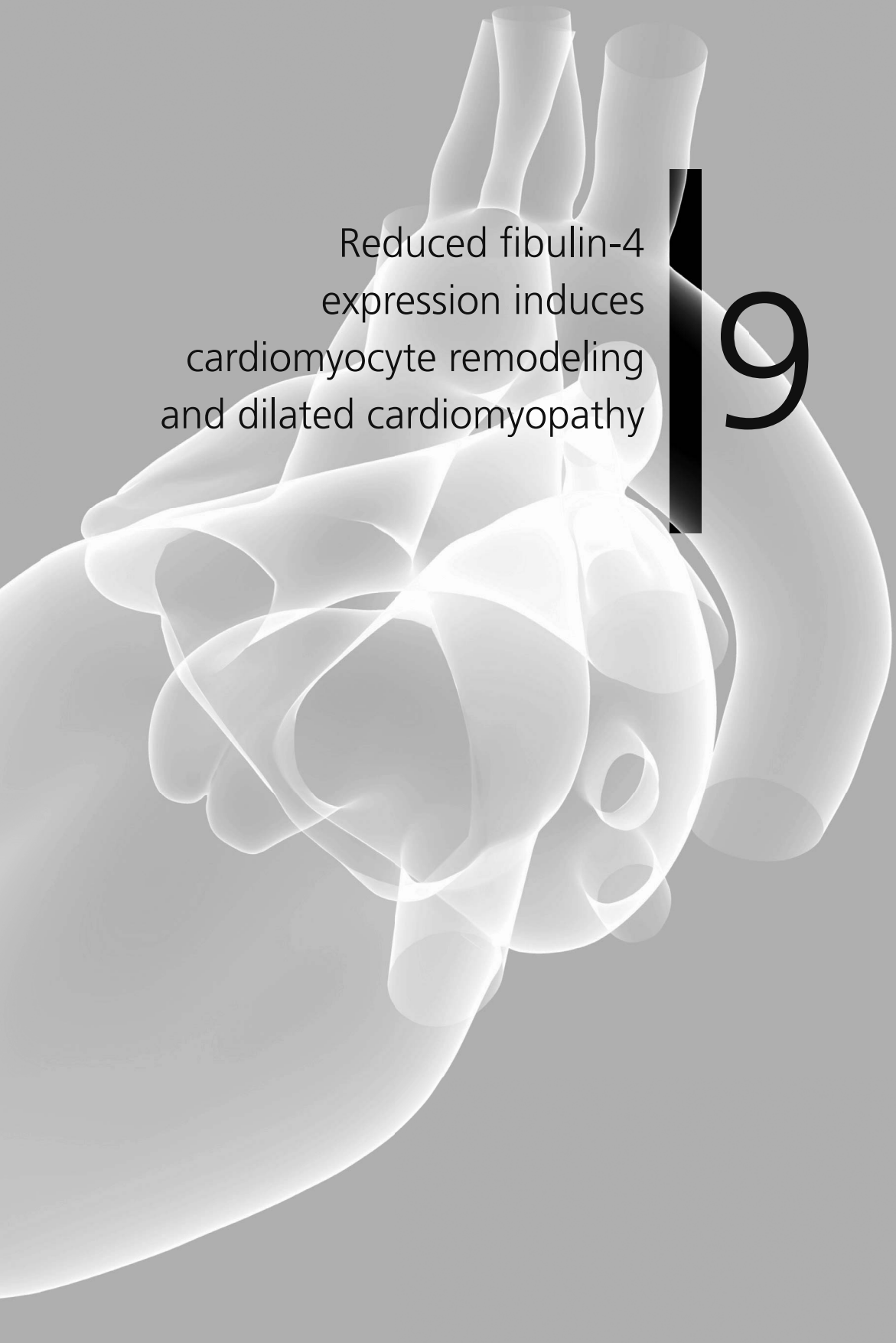

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Reduced fibulin-4 expression induces cardiomyocyte remodeling and dilated cardiomyopathy

9

ABSTRACT

Thoracic aortic aneurysms are commonly caused by connective tissue disorders, such as Marfan syndrome. Marfan syndrome patients often develop severe aortic dilation, which is associated with cardiac valve disease. However, the existence of primary cardiomyopathy is still not clear. Patients with a mutation in the extracellular matrix protein fibulin-4 display a Marfan-like phenotype, including thoracic aortic aneurysm and aortic regurgitation. We used mice with a reduced expression of fibulin-4 to evaluate *ex vivo* and *in vivo* cardiac parameters. Fibulin-$4^{R/R}$ mice, with a 4-fold reduced expression level of fibulin-4, suffered from severe aortic regurgitation and stenosis, most likely contributing to the left ventricular dilation and hypertrophy, and both impaired ejection fraction and fractional shortening. In addition, these mice showed a severe increase in cardiomyocyte size. In contrast, Fibulin-$4^{+/R}$ mice, with a 2-fold reduced expression level of fibulin-4 do not show signs of cardiac valve disease. However, careful pathological and hemodynamic analysis showed that also Fibulin-$4^{+/R}$ mice showed a moderate increase in cardiomyocyte size, and reduced ejection fraction and fractional shortening compared to wild type Fibulin-$4^{+/+}$ mice. Based on these cardiac abnormalities, we investigated whether fibulin-4 mutations could genetically predispose to aortic stenosis in humans. This was tested in a cohort of 400 patients with aortic dilation and/or aortic valve stenosis cohort. Although exon sequencing of fibulin-4 revealed no pathogenic mutation, we cannot rule out that subtle mutations affecting the expression level of fibulin-4 are present in these patients.

INTRODUCTION

Heritable connective tissue disorders such as Marfan syndrome (MFS) are, among others, characterized by cardiovascular manifestations. Because of their lethality, aortic complications in MFS have been studied extensively. Aortic root dilation leads to aortic regurgitation, and together with aortic dissection contributes to morbidity and mortality in MFS.[1] Echocardiographic studies have demonstrated that pediatric and adult patients with MFS also display mitral valve prolapse and tricuspid valve dysfunction, causing secondary functional complications when associated with valvular incompetence.[2-5] Furthermore, it has been postulated that there is primary myocardial impairment in patients with MFS.[6] In particular, diastolic dysfunction has been reported in children with MFS, while both diastolic and systolic dysfunction have been observed in adults.[7, 8] Analogous to the human situation, the genetically engineered fibrillin-1 deficient MFS mouse has been shown to display cardiac valve abnormalities, consisting of myxomatous changes of the mitral valves and mitral valve prolapse with left atrial and ventricular enlargement and mitral valve regurgitation.[9] These alterations in atrioventricular valve architecture were associated with excess transforming growth factor-β (TGF-β) activation and signaling.[9] These findings suggest that extracellular matrix remodeling and an abnormal TGF-β biological pathway may underlie impaired cardiac function.

In addition to the fibrillin-1 gene, mutations in other genes encoding extracellular matrix proteins have also been identified in patients with thoracic aortic aneurysms, including mutations in the fibulin-4 gene.[10-12] Fibulin-4 is an extracellular glycoprotein that is expressed in blood vessel walls, heart valves, along basement membranes, and around cardiomyocytes.[13] Fibulin-4 plays a role in elastic fiber assembly and function and is also a regulatory factor in elastogenesis.[14, 15] We have previously demonstrated that mice with a systemic 4-fold (Fibulin-$4^{R/R}$) or 2-fold (Fibulin-$4^{+/R}$) reduced expression of fibulin-4 share a number of key features with the MFS mouse model, including aortic wall degeneration, aneurysm formation, and increased TGF-β expression.[16, 17] In addition, fibulin-4 deficient mice have impaired vascular contractility (chapter 7), increased arterial stiffness (chapter 8), left ventricular hypertrophy and aortic stenosis and regurgitation.[16]

The combination of aortic stenosis and ascending aorta dilation is well known in patients with a bicuspid aortic valve and occurs irrespective of altered hemodynamics.[18, 19] This clinical syndrome can also be familial and some gene mutations, for instance in NOTCH-1 have been found in specific cases.[20] However, in the majority of patients no clear genetic underlying cause could be identified. As aortic stenosis was found in fibulin-4 deficient mice, it might be expected to be present also in patients. However, this has not been described so far.

The aim of the present study was to characterize the effect of fibulin-4 deficiency on cardiac structure and function. Fibulin-4 deficient mice showed cardiomyopathy as evidenced by left ventricular (LV) dilation, LV cardiomyocyte hypertrophy, decreased LV ejection fraction, and increased plasma BNP levels. In addition, we investigated whether fibulin-4 mutations could genetically predispose to aortic disease. To this end, we developed a PCR protocol for human fibulin-4 sequence analysis and subsequently sequenced DNA of 400 patients with ascending aortic pathology and/or aortic stenosis. To validate the newly found Single Nucleotide Polymorphism's (SNP's), the gene was also sequenced in 190 healthy controls.

Materials and methods

Experimental animals

We previously generated a fibulin-4 allele with reduced expression by transcriptional interference through placement of a TKneo targeting construct in the downstream Mus81 gene.[16] Heterozygous (Fibulin-$4^{+/R}$) mice in a mixed C57Bl/gJ;129Sv background were mated to obtain Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibullin-$4^{R/R}$ littermates and were housed in the institutional animal facility. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cardiac histology

Mice (age 14 weeks) were weighed, euthanized by an overdose of CO_2, and necropsied according to standard protocols. Perfusion fixation was applied to those hearts that were used for transverse cross-sections and stained for elastin (Verhoeff-van Gieson). For all other analyses, atria were removed and ventricles were weighted and subsequently fixed in 4% formaldehyde solution. After fixation, ventricles were cut into 4 slices, dehydrated and paraffin-embedded. Next, 5-µm sections were stained with Gomori's silver staining to visualize individual cardiomyocytes of the LV.[21] Only transversally cut cells showing a nucleus were used to determine the cardiomyocyte area. Collagen content was visualized with Picrosirius Red stainings and determined as the red positive area.

Echocardiography

Mice (8 female, 3 male, age 16 weeks) were sedated with 4% isoflurane and intubated as previously described.[22] The mice were ventilated with a mixture of O_2 and N_2O (1/2, vol/vol) with a pressure controlled ventilator (CWE, SAR-830/P) to which 2.5% isoflurane was added for anesthesia. Ventilation rate was set at 90 strokes/min with a peak inspiration pressure of 18 cmH_2O and a positive end expiration pressure (PEEP) of 4 cmH_2O. The mice were placed on a heating pad to maintain body temperature at 37°C. *In vivo* transthoracic echocardiography of the aortic arch and left ventricle was performed with a Vevo2100 (VisualSonics Inc., Toronto, Canada) using a 40-MHz linear interfaced array transducer (MS550S). B-mode and M-mode echocardiograms were captured of the ascending thoracic aorta at its biggest diameter and of the LV short axis at midpapillary level and long-axis plane with simultaneous ECG. Colour Doppler was used to visualize aortic regurgitation. LV mass was calculated using the following formula: $1{,}05*((5/6\text{PI}*(r+wt)^2*(L+wt))-(5/6\ \text{PI}*r^2*L))$ where r = end-diastolic inner radius, wt = end-diastolic LV thickness and L = LV end-diastolic length, as described previously.[23] Ejection fraction and fractional shortening were defined as the relative differences between end-diastolic and end-systolic volumes and diameter, respectively.

Plasma BNP levels

After echocardiography, mice were sacrificed by an overdose of isoflurane. The chest was opened according to standard necropsy protocols and blood was collected directly from the

heart. Plasma levels of B-type natriuretic peptide-45 (BNP-45) were measured by enzyme immunoassay methods using a commercially availably kit (Phoenix Pharmaceuticals, Inc.).

Human fibulin-4 sequence analysis

Sequencing protocol for the human Fibulin-4 gene

Primers (Table 1) were designed with the Fibulin-4 gene sequence from the Ensemble database (RefSeq IDs: NM_016938 and Accession Number: AF109121).[24] The exons and approximately 80-100 bp flanking each exon on both sides were selectively amplified. An M13-DNA sequence was added to each primer for simple and efficient direct sequencing. A M13-DNA sequence was added to the 5'-end of all the forward primers (5'-TGTAAAACGACGGCCAGT-3') and the 5'-end of the reverse primers (5'-CAGGAAACAGCTATGACC-3') for subsequent sequence analysis. Exon 11 was split into 2700 bp fragments (exon 11.1 and exon 11.2). Amplification reactions were performed in a total volume of 25 µl, containing 10x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 1.325 µl DMSO (Dimethyl Sulfoxide 99,9%, Sigma-Aldrich), 200 µM of each dNTP (Invitrogen), $4x10^{-4}$ µM forward primer, $4x10^{-4}$ µM reverse primer, 0.1 units Platinum Taq DNA polymerase (Invitrogen) and 100 ng genomic DNA. The PCR conditions were: 2' 94°C, 35 cycles of 1' 94°C, 1' 60°C, and 90" 72°C with a final extension for 10' 72°C and 5' 20°C. Sequence analysis was done with Seqscape (Applied Biosystems). Non-coding exon 1 was left out of the analysis.

Table 1. Sequences of the primers used for the amplification of the different exons of the human fibulin-4 gene.

Exons	M13-tail – Primer	
2&3	TGTAAAACGACGGCCAGT-CCCAAGCACGGTCCTGAG	F
	CAGGAAACAGCTATGACC- ACGGCAGTTCTTGGAGGTG	R
4	TGTAAAACGACGGCCAGT-TTGTTGAGCAGAGTGGGAAG	F
	CAGGAAACAGCTATGACC-CGGCAGGTCCTAAACAACAT	R
5	TGTAAAACGACGGCCAGT-GTGCTGTTGAGAGAACCGAAT	F
	CAGGAAACAGCTATGACC-GAATGGGGGTCAGGTGCTA	R
6&7	TGTAAAACGACGGCCAGT-GCTCCTCGGACACAGGATTA	F
	CAGGAAACAGCTATGACC-GGGTGCACAGTGACAGGAG	R
8&9	TGTAAAACGACGGCCAGT-TTGTCTCATCCCCCTCTGTC	F
	CAGGAAACAGCTATGACC-TGATTCCCATCATCCCTCA	R
10	TGTAAAACGACGGCCAGT-CCCTGAGGGATGATGGGAATC	F
	CAGGAAACAGCTATGACC-ATAGCAGCCTGGCCGAGT	R
11.1	TGTAAAACGACGGCCAGT-TCAAAGAGCTGCATGAGAGG	F
	CAGGAAACAGCTATGACC-CCCCATTTAGGTGAACTTGG	R
11.2	TGTAAAACGACGGCCAGT-CCTCCCTGCAGCTACCCTA	F
	CAGGAAACAGCTATGACC-GCATTGCAGCTTGGAATCTA	R

M13-tails are displayed before the forward and reverse primers. F, Forward, R, Reverse.

Patient samples

From the CONgenital CORvitia (CONCOR), the Dutch registry and DNA-bank for adults with congenital heart disease,[25] 400 samples could be obtained of patients with aorta and/or aortic valve abnormalities. Of those, 94 had a similar phenotype as MFS patients, but had no fibrillin-1 mutation, 70 patients had ascending aorta dilation, 173 aortic coarctation (COA), 133 congenital aortic stenosis, 9 aortic stenosis and ascending aorta dilation and 15 patients had combined COA and ascending aorta dilation. 399 DNA samples of the CONCOR patients could be sequenced successfully. One sample had a bad quality and was therefore excluded. DNA samples of 190 healthy subjects were obtained from the department of Clinical Genetics of the Erasmus MC. All patients and healthy controls have a Caucasian background.

SNP analysis

First, the ensemble database was used to identify the SNP's found in the CONCOR patients. The SNP's found in the ensemble database were selected with their reference sequence (rs) numbers, which are linked to the dbSNP database. From this database the frequencies of the SNP's in certain populations could be obtained, which were compared to the frequencies of the SNP's in the CONCOR patient group, determined by dividing the number of found SNP's by the total number of DNA samples. All the found SNP's were tested on the probability of creating additional splice sites and functional conservation using Netgene2 and Fruitfly, SIFT and Polyphen.

Data analysis

Normally distributed data are presented as mean±SEM. The one-way ANOVA was applied for the analysis between groups, followed by a post hoc Dunett test if ANOVA demonstrated significant. Non-normally distributed data are expressed as median and analysis was performed using a Mann-Whitney test. To evaluate the dose-dependent effect of fibulin-4 deletion, a linear regression analysis was performed to obtain a p for trend. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, Ill, USA). All statistical tests were two-sided and a p-value <0.05 was considered statistically significant.

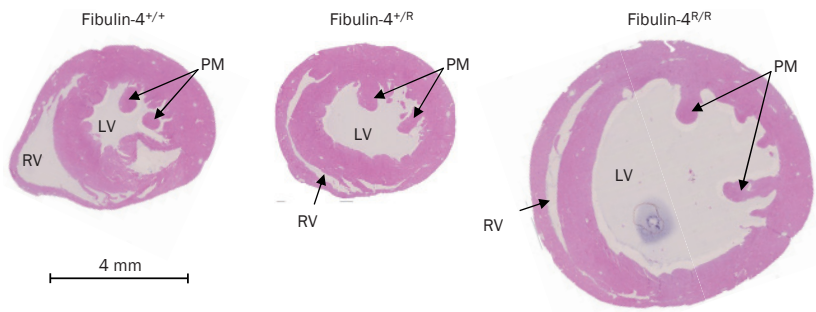

Figure 1. Transversal cross-sections of Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ hematoxylin and eosin stained hearts. Note the large increase in left ventricular diameter in the Fibulin-$4^{R/R}$ heart. PM, papillary muscle; RV, right ventricle; LV, left ventricle.

RESULTS

Cardiac abnormalities and cardiomyocyte size in Fibulin-4 mice

To characterize cardiac abnormalities due to fibulin-4 insufficiency, we performed histological analysis on hearts of Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ and wild type mice. Macroscopic transversal cross-sections of hearts obtained from fibulin-4 mutant mice displayed severe enlargement of the homozygous Fibulin-$4^{R/R}$ mice when compared to wild type Fibulin-$4^{+/+}$ and heterozygous Fibulin-$4^{+/R}$ mice (Figure 1). Increased cardiac size resulted in approximately a 2-fold increase in combined ventricular weight/body weight ratio in Fibulin-$4^{R/R}$ mice only (Figure 2A). This was furthermore associated with a similar increase in cardiomyocyte area (Figure 2B-C). Interestingly, cardiomyocyte area increased in a gene-dose dependent manner with decreasing expression of fibulin-4, already evident in Fibulin-$4^{+/R}$ mice (p for trend <0.001, Figure 2B-C).

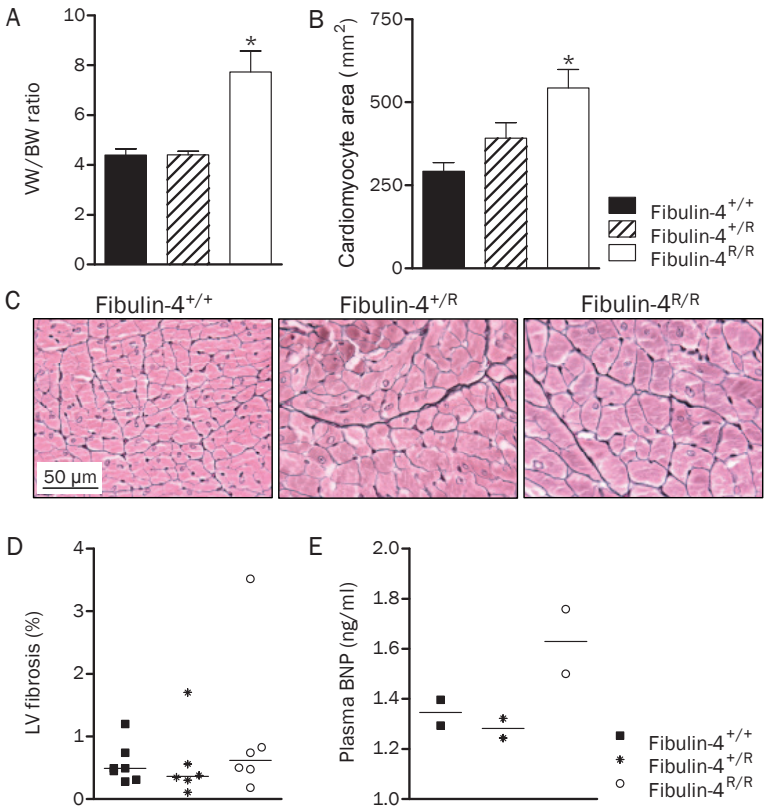

Figure 2. (**A**) The ratio of combined ventricular weight/body weight (VW/BW) is much larger in Fibulin-$4^{R/R}$ mice. (**B**) Cardiomyocyte area was enlarged, already in Fibulin-$4^{+/R}$ mice when compared to Fibulin-$4^{+/+}$ mice and further increased in Fibulin-$4^{R/R}$ mice (p for trend <0.05), calculated from Gomori's silver staining (**C**). (**D**) No difference in the amount of fibrosis was observed between the three groups. (**E**) Plasma BNP, a marker for heart failure, tends to increase in Fibulin-$4^{R/R}$ mice. *p<0.05 vs. Fibulin-$4^{+/+}$.

This intermediate increase in cardiomyocyte size in Fibulin-4$^{+/R}$ mice did, however, not result in a significant increase in total heart weight. We therefore suggest that not only the increase in cardiomyocyte size, but also an increase in cell number, and thus cardiac remodeling leads to the strong increase in heart weight in the Fibulin-4$^{R/R}$ mice. Indeed, we observed no signs of extensive cardiac remodeling in the Fibulin-4$^{+/R}$ mice hearts.

Next, to determine whether cardiac remodelling was associated with increased fibrosis in fibulin-4 deficient mice, we analyzed the amount of collagen in Picrosirius Red stainings. Although we observed no clear differences between the different genotypes, we identified a few outliers in both the Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ group (Figure 2D). To determine whether the cardiac abnormalities observed, resulted in a measurable systemic response we determined plasma BNP levels. Plasma BNP, used as a marker for heart failure, was higher in Fibulin-4$^{R/R}$

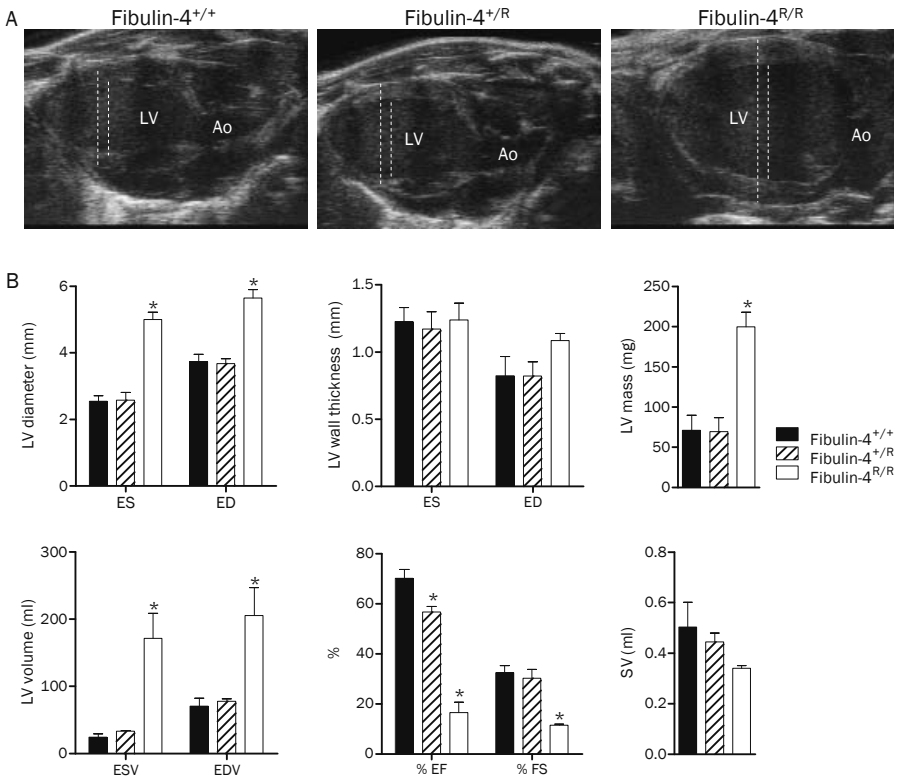

Figure 3. *In vivo* transthoracic echocardiography. (**A**) Representative examples of long-axis images obtained from Fibulin-4$^{+/+}$, Fibulin-4$^{+/R}$ and Fibulin-4$^{R/R}$ mice, with enlargement of the Fibulin-4$^{R/R}$ left ventricle. White dotted lines indicate the cross-sectional area to obtain diameters. (**B**) When compared to wild type littermates, Fibulin-4$^{R/R}$ mice demonstrate increased left ventricular volumes and mass with reduced ejection fraction and fractional shortening. Ejection fraction is also significantly lower in Fibulin-4$^{+/R}$ mice when compared to Fibulin-4$^{+/+}$ mice. Abbreviations: *p<0.05 vs. Fibulin-4$^{+/+}$, Ao, aorta, ED, end-diastolic; EF, ejection fraction; ES, end-systolic; FS, fractional shortening; SV, stroke volume; LV, left ventricle.

mice (Figure 2E), but not in Fibulin-$4^{+/R}$ mice. In conclusion, we observed a graded increase in cardiomyocyte size in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ compared to wild type controls. Only in Fibulin-$4^{R/R}$ mice this increased cardiomyocyte size was associated with LV remodelling and changes in BNP levels.

Functional consequence of cardiac abnormalities and increased cardiomyocyte size in fibulin-4 mice

Cardiac parameters were evaluated in vivo using transthoracic echocardiography. Figure 3A shows representative examples of long-axis images obtained from Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice. Figure 3B presents measurements obtained from the long-axis plane; all parameters were comparable to those obtained in short-axis plane (data not shown). LV end-systolic diameter changed from 2.5±0.2 to 2.6±0.2 and 5.0±0.2 mm and LV end-diastolic diameter from 3.7±0.2 and 3.7±0.1 to 5.7±0.3 mm in Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice respectively (Figure 3B upper panel).

LV thickness did not differ significantly between groups, although a slight increase in LV end-diastolic thickness might be present in Fibulin-$4^{R/R}$ mice. No differences in echocardiographic LV mass were observed between wild type (90±15 mg) and Fibulin-$4^{+/R}$ mice (87±11 mg) while in Fibulin-$4^{R/R}$ mice LV mass was much larger (218±59 mg).

Table 2. Variations detected in the different patients from the CONCOR database.

Exon nr:	Coarctation (188)	Aortic Valvar Stenosis (142)	Ascending Aorta Dilatation (No Marfan Syndrome) (94)
2&3	**c.160 +19 C>T heterozygous** c.139 C>T heterozygous rs 2234457	**c. 160 +19 C>T heterozygous**	**c.111 +29 G>C heterozygous**
4	c.277 G>A heterozygous rs 2234462 c.276 C>T homo/heterozygous rs 633800	c.276 C>T homo/heterozygous rs 633800	c. 276 C>T homo/heterozygous rs 633800
5	**c.368 -11 G>A heterozygous** **c.368 -4 G>A heterozygous** c.490 +23 G>C homo/heterozygous rs 630394	**c.368 -11 G>A heterozygous** **c.368 -4 G>A heterozygous** c.490 +23 G>C homo/heterozygous rs 630394	**c.368 -4 G>A heterozygous** **c.490 +30 C>T heterozygous** c.490 +23 G>C homo/heterozygous rs 630394
8&9	**c.728 -3 C>T heterozygous** c.775 A>G homo/heterozygous rs 601314	c. 775 A>G homo/heterozygous rs 601314	**c.728 -3 C>T heterozygous** c.775 A>G homo/heterozygous rs 601314
11.1	c.1188 C>T heterozygous rs 2234473	c.1188 C>T heterozygous rs 2234473	c.1188 C>T heterozygous rs 2234473
11.2	**159-165 del G (3'UTR)** **187 G>A heterozygous (3'UTR)**	**159-165 del G (3'UTR)**	**159-165 del G (3'UTR)**

Found SNP's for the different patient groups are displayed. New detected SNP's are indicated in bold. SNP's found in the Ensemble database are indicated with a reference sequence (rs) number.

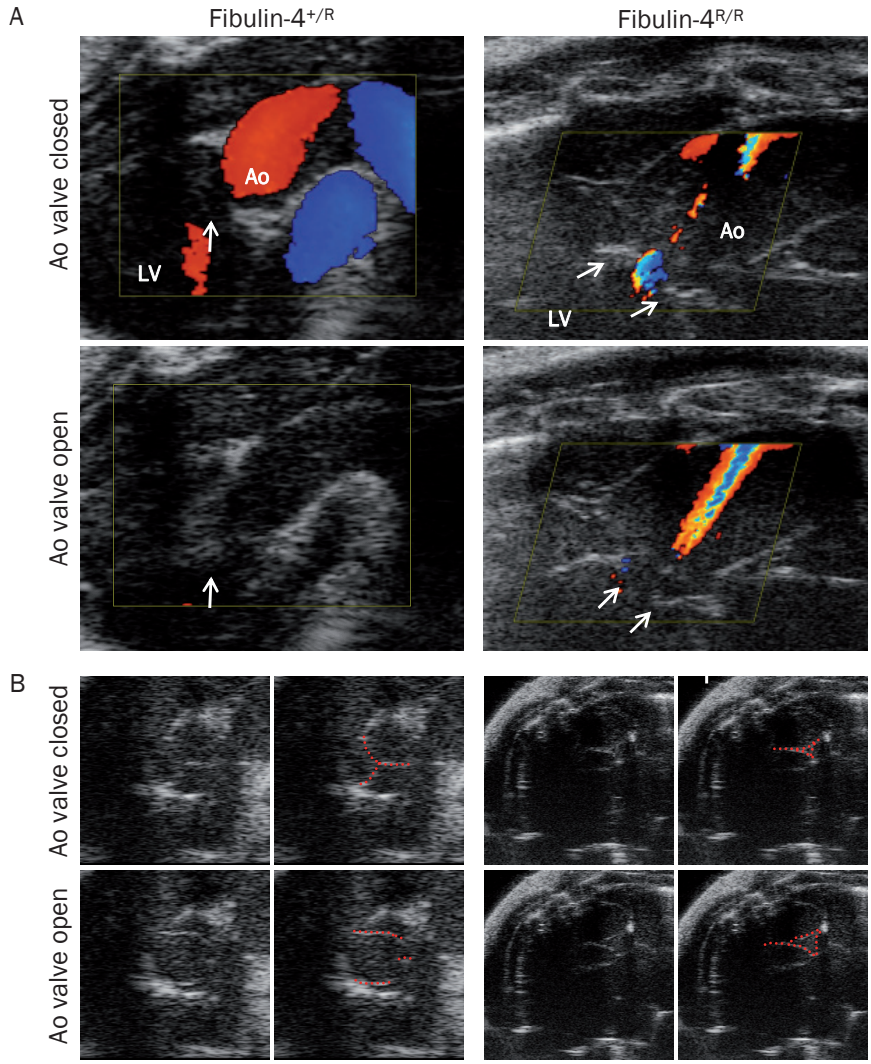

Figure 4. Aortic valve disease is present in Fibulin-4$^{R/R}$ mice only. (**A**) Aortic valve insufficiency, evidenced by reverse flow through the valve is demonstrated in Fibulin-4$^{R/R}$ mice. When the aortic valve is open, a narrow jet indicates severe aortic stenosis. Aortic valves are indicated by the white arrows. (**B**) In Fibulin-4$^{+/R}$ mice, the aortic valve has three leaflets of equal size that open and close accurately. Inadequate closure and only marginal opening of the aortic valve underlie aortic valve insufficiency and stenosis in Fibulin-4$^{R/R}$ mice. Note that three leaflets are present, but one is smaller than the other two. Ao, aorta; LV, left ventricle.

Table 3. Frequencies of the known variations in different populations from dbSNP and the CONCOR patient group.

Reference sequence		HapMap CEU (%) n=180	HapMap HCB (%) n=90	HapMap JPT (%) n=91	HapMap YRI (%) n=18	AGI ASP (%) n=41	KHP1 (%) n=90	AFD Afr PANEL (%) n=23	SC12 AA (%) n=12	CONCOR patients (%) n=400
2234457	C/C					94.1				99.5
139 C>T	C/T					5.9				0.25
	T/T					-				0
633800	G/G	25.9	75.6	61.4	84.7					24.25
276 G>A	A/G	62.1	24.4	31.8	15.3					50.3
	A/A	12.1	-	6.8	-					25
2234462	G/G						1			99.25
277 G>A	A/G						-			00.75
	A/A						-			0
630394 ex.5+23 G>C	G/G	33.3	-	13.3	7.4		1.2			11.8
	G/C	52.6	36.4	33.3	44.4		37.2			44
	C/C	14	63.6	53.3	48.1		6.16			43.5
601314	T/T	-	-	-	15		-	8.7	11.1	0
775 A>G	T/C	-	-	-	51.7		-	34.8	22.2	1
	C/C	100	100	100	33.3		100	56.5	66.7	98.5
2234473	G/G									99.25
1188 C>T	A/G									0.75
	A/A									0

Frequencies of the known SNP's in different populations could be obtained from the dbSNP website and were compared to the frequencies in the CONCOR patients. In case no frequency was determined, the box is empty, frequencies that could not be detected are indicated with a horizontal stripe (-). n, number; HapMap CEU, European; HapMap HCB, Asian; HapMap JPT, Asian; HapMap YRI, Sub-saharan Africa; AGI ASP population, African American; KHP1, Korean; AFD Afr PANEL, African American; SC12 AA. African American.

LV volumes increased in a gene-dose dependent manner both end-systolic (from 25±5 to 33±1 and 172±37 ml (p for trend 0.003)) and end-diastolic (from 71±23 to 78±4 and 206±42 ml (p for trend <0.05)) in Fibulin-$4^{+/+}$, Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice respectively (Figure 3B lower panel). Relatively, the difference between end-systolic and end-diastolic diameter and volume declined, resulting in a reduced ejection fraction from 70±4% in Fibulin-$4^{+/+}$ to 57±2% in Fibulin-$4^{+/R}$ and 17±4% in Fibulin-$4^{R/R}$ mice (p for trend <0.001). Such a trend was also observed for fractional shortening which was 33±3% in Fibulin-$4^{+/+}$, 30±4% in Fibulin-$4^{+/R}$ and 11±0.4% in Fibulin-$4^{R/R}$ mice (p for trend <0.004). Finally, stroke volume tended to lower with fibulin-4 deficiency, although this failed to reach statistical significance. There were no differences in heart rate (data not shown).

Table 4. Frequencies of the SNP's from the CONCOR patients compared to the frequencies in the control samples.

Region	Variation		Frequency CONCOR (%)	Frequency Controls (%)
Exon 4	277G>A	**G/G**	99.3	98.9
	rs 2234462	**A/G**	0.8	1.1
		A/A	0	0
	276 G>A	**G/G**	24.6	22.9
	rs 633800	**A/G**	50.4	54.3
		A/A	25	22.9
Exon 5		**G/G**	99.2	98.4
	-11 G>A	**A/G**	0.8	1.6
		A/A	0	0
		G/G	98.0	98.9
	-4 G>A	**A/G**	2.0	1.1
		A/A	0	0
Exon 8&9	+23 C>T	**G/G**	11.8	8.5
	rs 630394	**G/C**	44	44.7
		C/C	43.5	45.7
	755 A>G	**T/T**	0	0
	rs 601314	**T/C**	1.5	2.7
		C/C	98.5	96.3
	1188G>A	**G/G**	99.3	99.5
Exon 11.1	rs 2234473	**A/G**	0.8	0.5
		A/A	0	0
Exon 11.2	160 del G	**G/G**	94	92
		Del/G	6	8

Found SNP's for the different patient groups are displayed. New detected SNP's are indicated in bold. SNP's found in the Ensemble database are indicated with an rs number. rs, reference sequence.

Aortic valve disease in Fibulin-4$^{R/R}$ mice only

In Fibulin-4$^{R/R}$ mice, severe aortic valve disease has been described previously, and predisposes to cardiac abnormalities.[16] Whether aortic valve disease is also responsible for reduced cardiac function in Fibulin-4$^{+/R}$ mice was evaluated in the present study. With echocardiographic colour Doppler, we did not observe aortic regurgitation in Fibulin-4$^{+/R}$ mice (Figure 4A). In Fibulin-4$^{R/R}$ mice colour Doppler clearly demonstrated that inadequate closure of the aortic valve results in aortic regurgitation. The narrow flow when the aortic valve is open points towards aortic valve stenosis (Figure 4A). With a short-axis view of the aortic valves, accurate closure and opening of the three valve leaflets in Fibulin-4$^{+/R}$ mice could be observed (Figure 4B). In Fibulin-4$^{R/R}$ mice, inadequate closure and marginal opening of the aortic valve were observed.

Human fibulin-4 mutation analysis

Comparison of the sequenced fragments of the fibulin-4 gene to the reference sequence (Ensemble database) resulted in the detection of in total 14 SNP's in introns, exons and non-coding regions (Table 2). To determine whether these SNP's had introduced alternative splice sites these were analyzed using Netgene2 and Fruitfly. We found no consistent prediction for alternative splice sites. In addition, predictions based on SIFT and Polyphen indicated that the amino-acid substitutions are common and have no major effects on protein function.

To determine whether the SNP's in the CONCOR patients could be specifically associated with aortic and heart pathology, the frequency of the found SNP's were compared to frequencies of the SNP's in control populations. Six SNP's were described earlier and could therefore be compared with previously determined frequencies (Table 3).

The frequencies of the SNP's that were not described previously were compared to the SNP frequency in a control group that was sequenced for this purpose specifically (Table 4). No significant differences were detected between the frequencies of the SNP's in the reported populations, control group and the CONCOR cohort. We found no indications for direct pathogenic mutations in the fibulin-4 gene of the 399 CONCOR patients. Analysis for functional consequences of the found SNP's for both creating additional splice sites and the effect of amino-acid substitutions indicated no functional abnormalities. Finally, no SNP's were found with a higher or exclusive frequency in the CONCOR patients, indicating that the found SNP's are benign.

DISCUSSION

In the current study, we demonstrated that fibulin-4 deficiency in mice results in deterioration of cardiac function. In Fibulin-$4^{+/R}$ mice, this was evident from a reduced ejection fraction and fractional shortening, which were accompanied with an increase in cardiomyocyte size. In Fibulin-$4^{R/R}$ mice, also large geometric changes such as an increase in LV diameter and mass, as well as an increase in plasma BNP, a marker for heart failure, were present. The absence of aortic valve disease in Fibulin-$4^{+/R}$ mice suggests a primary myocardial defect in fibulin-4 mutant mice, while in Fibulin-$4^{R/R}$ mice this phenotype is aggravated by severe aortic stenosis and regurgitation. However, subsequent sequence analysis in a cohort of 400 patients with similar ascending aortic and/or aortic valve pathology, revealed no evidence of a pathogenic mutation in the fibulin-4 gene.

In healthy mice, fibulin-4 expression is present in heart valves.[13] We demonstrated that Fibulin-$4^{R/R}$ mice display both aortic stenosis and aortic regurgitation, probably commonly caused by fibulin-4 deficiency and ascending aortic dilation,[16, 17] the latter also being a common cause for aortic regurgitation in humans.[26] In general, aortic valve stenosis results in LV hypertrophy, to overcome the resistance caused by the obstructed valve.[27] Conversely, aortic regurgitation results in a volume overload and subsequently produces increased LV volumes.[27] In the present study clear dilated cardiomyopathy was observed in Fibulin-$4^{R/R}$ mice, resulting from aortic valve disease. However, in Fibulin-$4^{+/R}$ mice mild deterioration of cardiac function was not associated with aortic valve disease. This suggests that primary myocardial impairment

might underlie this mild phenotype. In MFS patients, heart failure is frequently found as a consequence of cardiac valve disease and ascending thoracic aneurysms. The presence of fibrillin-1, the defective gene in MFS patients, in myocardium[28] suggested the possibility of primary cardiomyopathy as well. This has been evaluated in MFS patients without significant aortic regurgitation or cardiovascular surgery. One quarter of the population had reduced LV ejection fraction and this supported the concept of primary cardiomyopathy in a subset of MFS patients.[6] Like fibrillin-1, fibulin-4 is also present in the myocardium. Immunohistochemical analysis of healthy adult mouse tissue shows fibulin-4 depositions around cardiac veins, arteries and cardiomyocytes.[13] Furthermore, besides its role as a structural protein, fibulin-4 functions also as a regulatory factor.[29] Thus, disturbance of the fibulin-4 regulatory function might affect cardiomyocyte function.

In the various Fibulin-$4^{R/R}$ mice analyzed, we occasionally observed concentric hypertrophy (data not shown), while dilated cardiomyopathy was present in the large majority of 100-day old animal. This is similar to patients with aortic stenosis, whom also develop hypertrophic cardiomyopathy. Whether dilated cardiomyopathy is preceded by concentric hypertrophy in Fibulin-$4^{R/R}$ mice has to be evaluated by analyzing cardiac development over time and could have been missed in the current cross-sectional study, in which only adult fibulin-4 mice (100-120 days) were examined. A longitudinal study, with a follow-up of young mice until adulthood, should provide more insight in the underlying mechanism.

Congenital aortic valve stenosis is characterized by aortic leaflet thickening, calcification and inflammation,[30] and Fibulin-$4^{R/R}$ mice display a very similar phenotype.[16] In humans, congenital aortic stenosis is most often due to a bicuspid aortic valve. To date, few mouse models have been developed to study congenital aortic valve disease, but none showed the characteristic bi-leaflet, instead of the normal tri-leaflet aortic valve. Families with heterozygous mutations in NOTCH1 develop early and severe calcification of the aortic valve, and most valves are bicuspid.[20] However, Notch1+/- mice show increased aortic valve calcification compared to wild type littermates, but tri-leaflet valves were present in all mice.[31] Mice overexpressing Pkd1, model human autosomal dominant polycystic disease. These mice develop a range of abnormalities, and some presented with calcified aortic valves and hemodynamic signs of aortic stenosis.[32] The variable presence of cardiovascular manifestations, together with the severe phenotype, makes this model less suitable to study aortic stenosis. To study non-congenital calcific aortic valve disease, hypercholesterolemic low density lipoprotein receptor-deficient apolipoprotein B100-only mice can be used.[33] These mice are prone to develop calcification and oxidative stress in the aortic valve, resulting in valve dysfunction, mimicking aortic valve disease in the elderly. In light of these data, Fibulin-$4^{R/R}$ mice are an interesting model. Pathological examination did not show aberrant attachment of the three valve leaflets in any of these animals, thus excluding bicuspid aortic valves. Echocardiographic analyses, however, showed aberrations in the leaflet composition as one of the leaflets was much smaller compared to the others (Figure 4B), contributing very little to aortic valve function. It therefore appears that in Fibulin-$4^{R/R}$ mice the aortic valve function is reduced, like in human bicuspid valve disease.

Although clear aortic valve disease was present in Fibulin-$4^{R/R}$ mice, no aortic valve abnormalities have been reported for patients with a mutation in the fibulin-4 gene.[10, 12, 34, 35]

In two cases, ventricular hypertrophy was diagnosed.[34, 35] According to our data, it is possible that these patients mimic the mildly affected Fibulin-4$^{+/R}$, rather than Fibulin-4$^{R/R}$ mice. Also in a specific selected cohort of 400 ascending aortic pathology and/or aortic valve disease patients, no pathogenic mutation was found after sequencing of the coding exons of the fibulin-4 gene. It is possible that in humans, fibulin-4 mutations do not underlie the combined existence of ascending aortic aneurysms and aortic valve stenosis, however, we cannot rule out the occurrence of milder mutations affecting the expression level of the protein. This remains to be determined by genomic sequencing of the fibulin-4 locus or by fibulin-4 RNA expression analysis. It is also possible that our set of patients was too heterogeneous to find such mutation. Aortic dilation was not present in all patients and only a small subset of the population had the combined diagnosis of aortic dilation and aortic stenosis. A pathogenic fibulin-4 mutation might be found in a larger and more homogeneous population.

In conclusion, fibulin-4 deficiency results in dilated cardiomyopathy, which is severely aggravated by aortic valve disease in Fibulin-4$^{R/R}$ mice. With comparison to human analysis, we were not yet able to find an association between combined existence of aortic pathology and aortic valve stenosis, and pathogenic fibulin-4 gene mutations.

Acknowledgement: This work was financially supported by the stichting Lijf en Leven and the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research (NWO).

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Part V

Summary and Discussion

SUMMARY AND DISCUSSION

The aorta is the largest and most important artery that supplies oxygen throughout the human body. Pathophysiological conditions of the aorta include structural malformations and conditions affecting hemodynamic stress on the aortic wall, like hypertension. The two most common structural malformations are an aortic coarctation (COA) and aortic aneurysm

SUMMARY

Aortic Coarctation

A COA is a congenital narrowing of the descending thoracic aorta, just distal of the aortic arch, resulting in a significant blood pressure gradient between the upper and lower body. Usually, a COA is diagnosed during the neonatal period, and surgical repair is often performed within weeks. Despite successful surgery, many patients eventually develop hypertension, leading to a reduced life expectancy. Little is known about the underlying mechanism for post-COA hypertension. Hyper-activation of the renin-angiotensin system (RAS) by underperfusion of the kidneys is one hypothesis that has been postulated,[1] and is supported by increased plasma renin activity in postoperative patients.[2] Concomitantly, hyperactivity of the RAS could underlie endothelial dysfunction in these patients.[3, 4] Angiotensin (Ang) II, the major component of the RAS, suppresses nitric oxide (NO) production, which is characteristic for endothelial impairment. Currently, no data are available concerning the most effective class of anti-hypertensive medication in hypertensive post-COA patients. A better understanding of the disease could contribute to the optimization of such treatment. Furthermore, several drugs have been proposed that might have beneficial effects on vascular stiffness,[5] which is commonly seen in post-COA patients.[6-8] In a cross-over design, the effects of beta-blocker metoprolol and Ang II type 1 (AT_1) receptor blocker candesartan were evaluated in post-COA patients (**chapter 2**). Effects on blood pressure, arterial stiffness and neurohormonal status were evaluated. Metoprolol was more effective in lowering 24-hour mean arterial pressure (MAP) than candesartan, especially during daytime. We expected superior effects of candesartan over metoprolol on arterial stiffness, based on studies in essential hypertensive subjects.[5] However, no improvement was observed after either treatment. This could be explained by the structural changes in the aortic wall, including the loss of smooth muscle cells and increase in collagen fibers.[9] The decrease in arterial stiffness by anti-hypertensive drugs is partly mediated by smooth muscle cell relaxation, and it is comprehensible that the loss of smooth muscle cells hinders the aimed favorable effects. Although we could not demonstrate a substantial role for the RAS in post-COA hypertension, it is still possible that the hyperactivation of the RAS results in Ang II-induced NO suppression causing endothelial dysfunction. Others reported that a four-week treatment with angiotensin-converting enzyme (ACE) inhibitor ramipril improves endothelial dysfunction in successfully repaired COA patients.[10] It is hypothesized that ramipril improves NO bioavailability by decreasing the breakdown of bradykinin, thus indirectly inducing bradykinin type 2 receptor-mediated release of endothelial NO.[11]

Upper body hypertension is also commonly found in adult (post-)COA patients with a significant aortic gradient. However, interventional gradient relief only occasionally results

in blood pressure normalization. Stent implantation is widely accepted to treat native or recurrent COA in adult patients, but concerns exist about aortic ruptures and data on long-term effects are scarce. In **chapter 3** the effect of endovascular stenting on blood pressure was evaluated in patients with hemodynamically significant native or recurrent COA. COA stenting resulted in a significant gradient decline and increase in vessel wall diameter. A calamitous procedural complication resulted in the death of one patient on the day of stent implantation. Follow-up with echocardiography demonstrated a reduction in maximum peak systolic velocity and a modest increase in left ventricular function. Blood pressure decreased slightly, but the need for anti-hypertensive drugs was unchanged. One pseudoaneurysm occurred and could successfully be treated with a covered stent. Proximal migration of a stent into the aortic arch is a rare complication and such a case is described in **chapter 4**. After discussing the case with (inter)national experts, the stent and narrowed segment of the aorta were removed and a graft was positioned. This case illustrates the need for larger stents that are flexible and can adapt to the aortic shape.

Hypertension

Classes of anti-hypertensive drugs include diuretics, calcium-channel antagonists, beta-blockers and inhibitors of the RAS. Recently, a new type of RAS inhibitor, the direct renin inhibitor aliskiren, showed safety and tolerability in mild-to-moderate hypertensive patients.[12] In **chapter 5**, we investigated whether aliskiren also mediates end-organ protection. In spontaneously hypertensive rats (SHR), we compared the cardiac effects of aliskiren (100 mg/kg per day), with those of the AT_1 receptor blocker irbesartan (15 mg/kg per day) and the ACE inhibitor captopril (3 mg/kg per day) for 3 weeks. Treatment with all drugs significantly lowered MAP without effecting heart rate; the drugs reduced the cardiomyocyte area, as well as B-type natriuretic peptide levels, a marker for congestive heart failure, and improved endothelial function in the coronary arteries. Direct renin inhibition by aliskiren reduced plasma renin activity as well as plasma and tissue angiotensin levels after one week of treatment. After 3 weeks, only cardiac angiotensin levels remained repressed. Renal AT_1 receptor expression was reduced, which may contribute to lower renal angiotensin levels.[13] No changes in tissue angiotensin levels were observed during treatment with irbesartan or captopril. Thus, aliskiren is as least as effective as AT_1 receptor blockade and ACE inhibition in terms of cardiac protection, and the suppressed cardiac angiotensin levels after prolonged treatment with aliskiren might support superior long-term cardiac effects for direct renin inhibition.

Ang II exerts its main effects via AT_1 receptors. AT_2 receptors are generally believed to function as an endogenous antagonist of the AT_1 receptor, contributing to vasodilation and tissue remodeling. Under pathophysiological conditions, such as hypertension, contradictory data exists regarding the AT_2 receptor function. We compared the effects of Ang II, III, IV and 1-7 in the coronary and iliac arteries as well as the aorta of SHR to those in normotensive Wistar rats in **chapter 6**. Like in normotensive rats, all angiotensin metabolites induced coronary vasoconstriction in the SHR via stimulation of the AT_1 receptor. The efficacy of Ang II and Ang III was much larger in SHR compared to Wistar rats. Blockade of the AT_2 receptor with PD123319 did not enhance Ang II and Ang III-induced vasoconstriction in the coronary arteries in SHR, while it did in Wistar rats. Thus, during hypertension there is either an absence

of the counterregulatory vasodilator effect of the AT_2 receptor or its effect is changed into vasoconstriction. Furthermore, vasoconstriction to angiotensin metabolites in iliac arteries and abdominal aortas were identical in SHR and Wistar rats and could be prevented with AT_1 receptor blocker irbesartan. PD123319 blocked vasoconstrictor effects in iliac arteries of SHR, again highlighting the vasoconstrictor effects of the AT_2 receptor under pathophysiological conditions.

Thoracic Aortic Aneurysm

There is increasing evidence that the RAS plays an important role in thoracic aortic aneurysm (TAA) development. This has been demonstrated in a mouse model and patients with Marfan syndrome, a genetic disorder predisposing for TAA formation.[14, 15] The functional role of the RAS in TAAs and its therapeutical implications is reviewed in **chapter 1**. Degeneration of the aortic media is characteristic for TAAs.[16, 17] Mice with a systemic two- (Fibulin-$4^{+/R}$) and four-fold (Fibulin-$4^{R/R}$) reduced expression of fibulin-4 display similar histological features as TAA patients.[18] This model was used to study structural alterations in TAAs (**chapter 7**). Cellular changes included an increase in aortic wall thickness at least partly due to increased extracellular matrix depositions, fragmentation of elastic laminae and a loss of smooth muscle cells. Functionally, this resulted in decreased diastolic pressure and increased pulse pressure, and a reduced contractility of the thoracic aorta. Impaired contractility could be due to an altered calcium-signaling pathway as identified with aortic transcriptome analysis. This analysis furthermore pointed towards a role for TGF-β in the pathogenesis of TAA formation. Indeed Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ presented a gene-dose dependent increase of TGF-β signaling in the aortic wall, accompanied with increased tissue Ang II levels, which is a regulator of TGF-β signaling. Next, mice were treated with AT_1 receptor blocker losartan, since AT_1 receptor blockade can indirectly reduce TGF-β signaling.[19] Treatment with losartan largely improved aortic wall degeneration in newborn aneurysmal Fibulin-$4^{R/R}$ mice. Whether an altered aortic gene expression level of fibulin-4 results in a change of the aortic proteome was evaluated in **chapter 8**. We performed an aortic proteomic screen for wild type Fibulin-$4^{+/+}$, heterozygous Fibulin-$4^{+/R}$ and homozygous Fibulin-$4^{R/R}$ aortas. Biological pathway analysis identified increased oxidative phosphorylation, resulting in mitonchondrial dysfunction and increased oxidative stress in fibulin-4 deficient mice. To identify those genes that were found dysregulated in both the aortic transcriptome and proteomic screen, an overlay of both analyses was made. Overlapping biomarkers all pointed towards the 17β-estradiol pathway in both Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ mice. In aneurysmal Fibulin-$4^{R/R}$ aortas, TNF-α, a marker for inflammation, was also identified. Mitochondrial dysfunction and estrogens result in increased reactive oxygen species (ROS) and are both markers for age-related diseases. In vivo imaging demonstrated a gradual increase of ROS in Fibulin-$4^{+/R}$ and Fibulin-$4^{R/R}$ aortas. Enhanced arterial aging was furthermore evidenced by endothelial dysfunction and impaired aortic distensibility as a marker of arterial stiffness.

Finally, fibulin-4 deficiency results in a heart failure and aortic valve disease, as described in **chapter 9**. Fibulin-$4^{R/R}$ mice demonstrate a more than two-fold increase in left ventricular diameter compared to wild type and Fibulin-$4^{+/R}$ littermates, accompanied with higher end-systolic and end-diastolic volumes, an impaired ejection fraction and fractional shortening. Cardiac remodeling resulted in an increased left ventricular mass and cardiomyocyte area.

Interestingly, also in heterozygous Fibulin-4$^{+/R}$ mice we found a slightly lower ejection fraction, fractional shortening and larger cardiomyocyte area. Primary cardiomyopathy might underlie this phenomenon and in Fibulin-4$^{R/R}$ mice severe aortic valve stenosis and regurgitation further aggravate cardiomyopathy. The combination of TAA and aortic valve stenosis is well known in patients with bicuspid aortic valves. Whether human fibulin-4 mutations could underlie this clinical syndrome is currently unknown. Therefore, a PCR protocol was developed for human fibulin-4 sequence analysis. Four hundred patients with ascending aortic arch pathology and/or aortic valve stenosis were screened and newly found single nucleotide polymorphisms (SNPs) were compared to the general population and a control group. No indication was found for direct pathogenic mutations in the fibulin-4 gene in this cohort.

DISCUSSION

It is clear that aortic pathology is a major risk factor for cardiovascular morbidity and mortality. Timely treatment is warranted, but even then, accurate intervention may not prevent all patients from complications and premature death. Over the last decades, surgical techniques have been improving, resulting in increased survival. In aortic coarctation (COA) patients, the median age of death without surgery is estimated to be 35 years,[20] while surgical repair increased survival rates significantly to almost 90% at age 20.[21] Despite, or maybe due to, successful surgery, long term complications like hypertension do occur and special attention is warranted to further improve life expectancy. In aortic aneurysms, a lot of effort has been made to develop more effective, preventive treatment, to protect patients from aortic dissection or rupture. This has been managed by the development of new surgical techniques, such as a retroperitoneal approach for abdominal aortic surgery, which might be associated with fewer post-operative complications than the traditional transabdominal approach.[22] Moreover, in abdominal, thoraco-abdominal and descending thoracic aortic aneurysms, endovascular stent implantation has become an attractive alternative, especially in high risk patients.[23] In aneurysms of the ascending thoracic aorta, beta-blockade therapy has long been standard of care to regress hemodynamic stress, especially in Marfan syndrome (MFS) patients.[24]

Recently, increased TGF-β signaling has been correlated to thoracic aortic aneurysm (TAA) formation in MFS,[14, 25] fibulin-4 mutations,[18, 26] Loeys-Dietz syndrome,[27, 28] bicuspid aortic valves with aortic aneurysm,[29] and arterial tortuosity syndrome.[30] In MFS mice this could be inhibited by losartan treatment.[14] However, controversy exists regarding the precise role of TGF-β in aortic aneurysms.[31] For example, Loeys-Dietz syndrome is caused by a mutation in either TGF-β receptor 1 or 2, and many disease-causing mutated forms of TGF-β receptor 2 theoretically should result in attenuation or loss of cellular TGF-β responsiveness,[27, 31] while increased TGF-β signaling (assessed by the amount of phosphorylated Smad2) was observed in the aortic wall.[27] More controversy for the role of TGF-β in the development of aortic aneurysms has been derived from abdominal aortic aneurysms (AAAs) models. Angiotensin (Ang) II infusion in hyperlipidemic apolipoprotein E knockout (ApoE$^{-/-}$) mice is a well established model for AAA development.[32] More recently, is was discovered that Ang II infusion in normocholesterolemic C57Bl/6 mice also resulted in AAA formation, but at a lower incidence.[33] Adding TGF-β neutralizing antibodies to Ang II-infused C57Bl/6 mice resulted to an increase from 20% to 80% in the development of AAAs.[34] Finally, in ApoE$^{-/-}$ mice

with overexpression of active TGF-β1 in the heart, elevated cardiac and plasma TGF-β1 were accompanied with less aortic root dilation and fewer pseudoaneurysms.[35] Thus, dysregulation of TGF-β in aneurysmal disease is well established, but yet controversial, and depends largely of the model that has been used.

Losartan, an Ang II type 1 (AT_1) receptor blocker and antagonist of the TGF-β signal, is effective in the treatment of aortic root growth in MFS patients[14] and our data support the beneficial effect of this drug in aneurysmal Fibulin-$4^{R/R}$ mice (**chapter 7**). Possibly, the effects of losartan can be mimicked by other inhibitors of the renin-angiotensin system (RAS). Angiotensin-converting enzyme inhibitors have only been tested to a limited extent in MFS patients.[36, 37] These drugs, as well as the direct renin inhibitors might reduce the elevated Ang II levels observed in MFS patients.[38] Such elevated levels are in agreement with the increased tissue Ang II in fibulin-4 mutant mice that we found (**chapter 7**). A comparison of TGF-β signaling after treatment with the various RAS blockers is warranted. In a preliminary experiment prenatal treatment of Fibulin-$4^{R/R}$ mice with the renin inhibitor aliskiren reduced aortic wall thickness as effectively as losartan. Yet elastic fiber architecture did not improve with aliskiren (Moltzer et al, unpublished data 2010). These findings suggest an important role for concomitant Ang II type 2 (AT_2) receptor stimulation during AT_1 receptor blockade, as has been hypothesized earlier.[39] Therefore, administration of compound 21 (C21), a specific AT_2 receptor agonist, in a TAA animal model would be of great interest. In addition to exclude the possibility of a losartan-specific effect, other AT_1 receptor antagonists should be tested as well.

Although the first non-randomized study on losartan treatment in MFS patients largely reduced aortic root growth,[15] the need for even better drugs to treat TAAs in MFS patients and non-MFS patients remains. Especially for the treatment of non-MFS patients, more research is warranted, and the development of other mouse models will facilitate this. In humans, atherosclerosis plays an important role in aneurysm formation, mainly in the descending thoracic and abdominal aorta. Mice do not develop atherosclerosis, but crossing $ApoE^{-/-}$ with Fibulin-$4^{+/R}$ mice is an attractive approach to overcome this problem. Fibulin-$4^{+/R}$ mice display enhanced arterial aging, but do not develop severe aneurysms spontaneously (**chapter 8**). Inducing atherosclerosis might trigger aneurysm formation, and subsequently can be used as a model for atherosclerosis-associated aneurysms of the elderly population. Atherosclerosis-associated aneurysms might benefit from other pharmacological treatment than the pure genetic-induced TAAs and thus treatment effects have to be studies independently.

Finally, in patients with a genetic predisposition for TAAs, disease development is often early in life. Pregnancy is a major concern for many young women with TAAs, since it is a high-risk period for aortic dissection and rupture. In MFS, women are at increased risk of aortic dissection, even without the preconceptional presence of aortic root dilation, and hormonal changes may even aggravate the loss of elastic fibers.[40] During pregnancy, medical management is necessary to minimize aortic root dilation and the risk of aortic dissection. Although losartan appears to be the preferred treatment in MFS patients, it is teratogenic and thus contraindicated during pregnancy. Pregnant women are still dependent on beta-blockers.[24] With the development of newer drugs, research should also focus on their applicability during pregnancy.

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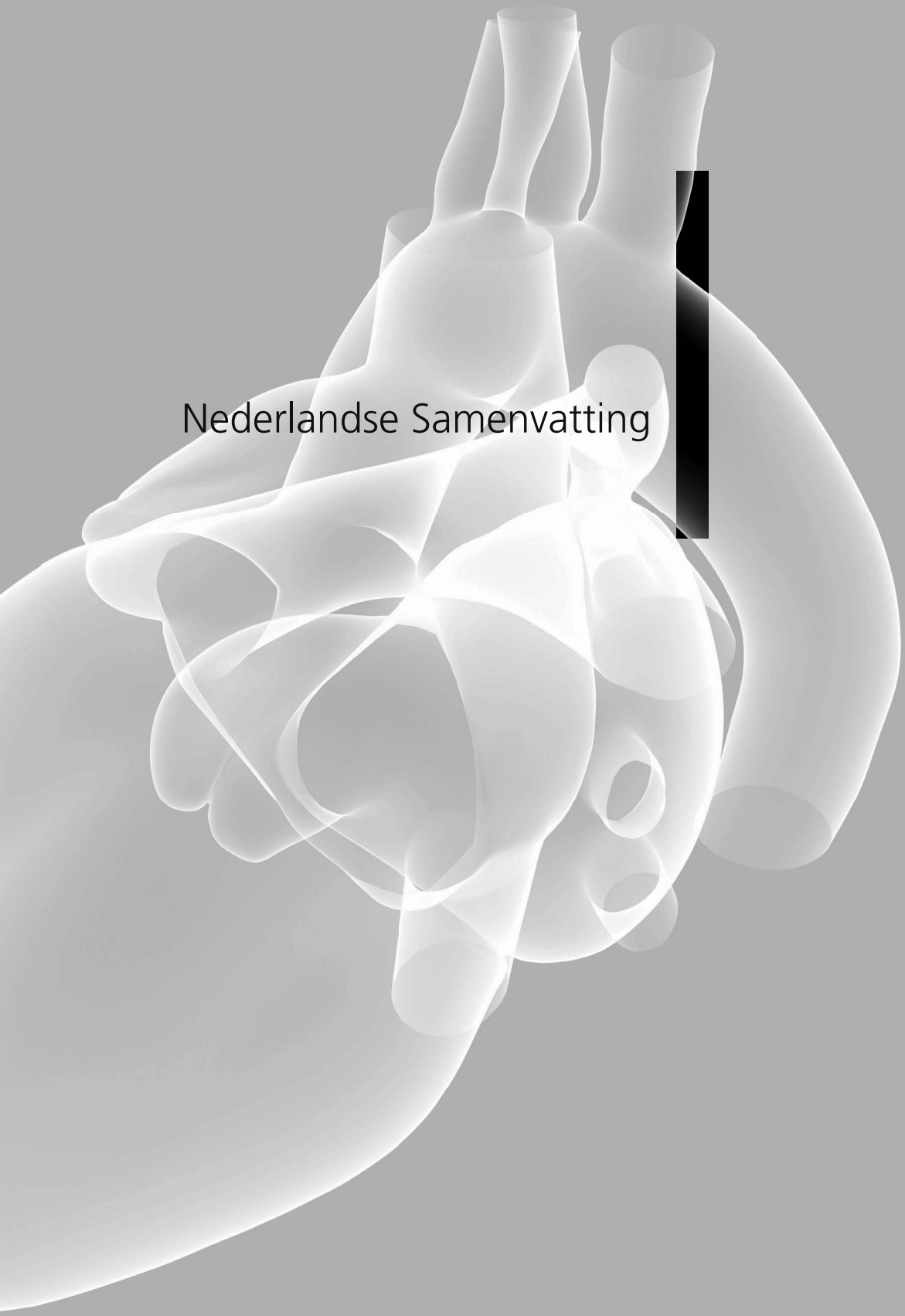


Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

De aorta is het grootste en belangrijkste bloedvat dat zuurstof door het menselijk lichaam vervoert. Pathofysiologische omstandigheden van de aorta kunnen structurele afwijkingen zijn, en omstandigheden waarbij hemodynamische stress de aortawand kan beïnvloeden, zoals tijdens hypertensie. De twee meest voorkomende structurele afwijkingen zijn een aangeboren vernauwing (aorta coarctatie) en een verwijding (aorta aneurysma) van de aorta.

Aorta Coarctatie

Een aorta coarctatie is een aangeboren vernauwing van de aorta, net na de aortaboog, waardoor er een bloeddruk gradiënt tussen de bovenste en onderste lichaamshelft ontstaat. Over het algemeen wordt de diagnose van een aorta coarctatie gesteld in de neonatale periode, en wordt een chirurgische correctie uitgevoerd binnen enkele weken. Ondanks succesvolle chirurgie ontwikkelen veel patiënten op latere leeftijd hypertensie, waardoor zij een verminderde levensverwachting hebben ten opzichte van de gezonde populatie. Er is weinig bekend over het onderliggende mechanisme voor deze hypertensie. Eén van de hypotheses is dat een onvoldoende doorbloeding door de nieren een overactiviteit van het renine-angiotensine systeem (RAS) veroorzaakt. Momenteel is er niets bekend over de meest effectieve klasse van bloeddrukverlagende medicijnen voor de behandeling van late hypertensie bij patiënten met een geopereerde aorta coarctatie. Zulke gegevens zouden bij kunnen dragen aan een beter inzicht in het ziekteproces. Bovendien zouden sommige medicijnen een gunstig effect kunnen hebben op vaatwandstijfheid, die verhoogd is bij deze patiënten. In **hoofdstuk 2** hebben wij de effecten van beta-blokker metoprolol en angiotensine II type 1 (AT_1) receptor blokker candesartan vergeleken in patiënten met een geopereerde aorta coarctatie en hypertensie. Alle patiënten kregen beide medicijnen, allebei voor een periode van 8 weken en de effecten op bloeddruk, arteriële vaatwandstijfheid en enkele hormonen werden vergeleken. Beide medicijnen verlaagden de bloeddruk effectief in vergelijking met de periode waarin de bloeddruk niet werd behandeld. Echter, metoprolol was effectiever dan candesartan in het verlagen van 24-uurs bloeddruk en dit verschil was voornamelijk overdag zichtbaar. Er werd geen effect waargenomen op metingen van arteriële vaatwandstijfheid. En op basis van de hormoonwaarden kon geen aanwijzing worden gevonden voor een overactiviteit van het RAS. Hypertensie van de bovenste lichaamshelft wordt ook vaak waargenomen bij volwassenen met een aorta coarctatie waarbij de vernauwing nog of weer aanwezig is. Het opheffen van de vernauwing leidt slechts in enkele gevallen tot een normalisatie van de bloeddruk. Het inbrengen van een stent is een wereldwijd geaccepteerde manier om volwassenen met een aorta coarctatie te behandelen. Er is echter nog weinig bekend over de langetermijnuitkomsten.

In **hoofdstuk 3** zijn de effecten van endovasculaire stent implantatie op de bloeddruk onderzocht in patiënten met een hemodynamisch significante (rest)vernauwing. Stent

plaatsing resulteerde in een significante afname van de bloeddrukgradiënt en een toename van de aortadiameter. Eén patiënt ontwikkelde een ernstige complicatie tijdens de ingreep en overleed diezelfde dag. Alle patiënten werden vervolgd met echocardiografie van het hart en de aorta, waarbij een daling in de maximale pieksnelheid over de aorta en een kleine verbetering in de hartfunctie te zien waren. De bloeddruk nam slechts matig af en het aantal patiënten dat behandeld moest worden voor hypertensie was na de ingreep onveranderd. Tijdens het vervolgen van de patiënten werd één pseudoaneurysma gediagnosticeerd, die behandeld kon worden met een nieuwe, gecoate (covered) stent. Tot slot was er één bijzondere complicatie, waarbij de stent proximaal was verplaatst naar de aortaboog en dit is beschreven in **hoofdstuk 4**. Nadat de casus was besproken in binnen- en buitenland werden de stent en het vernauwde gedeelte van de aorta chirurgisch verwijderd en werd er een vaatprothese geplaatst.

Hypertensie

Verschillende vormen van bloeddrukverlagende medicijnen zijn diuretica, calciumkanaal antagonisten, beta-blokkers en remmers van het RAS. Onlangs is er een nieuwe remmer van het RAS ontwikkeld, de directe renine remmer aliskiren. In **hoofdstuk 5** hebben wij onderzocht of aliskiren ook bescherming kan bieden tegen orgaanschade. In spontaan hypertensieve ratten (SHR) hebben wij de effecten van aliskiren (100 mg/kg per dag), AT_1 receptor blokker irbesartan (15 mg/kg per dag) en de ACE remmer captopril (3 mg/kg per dag) op het hart vergeleken. Alle medicijnen verlaagden de bloeddruk zonder de hartslag te beïnvloeden, reduceerden de grootte van hartspiercellen, verlaagden het B-type natriuretisch peptide (een marker voor hartfalen) en verbeterde de endotheelcel functie in de kransslagaders. Zoals verwacht resulteerde behandeling met aliskiren in een verlaging van de plasma renineactiviteit en zowel de plasma als weefsel angiotensinewaardes na één week. Na drie weken waren alleen de cardiale angiotensine waardes nog verlaagd. AT_1 receptorexpressie in de nieren was afgenomen, wat mogelijk bijdraagt aan lagere angiotensinespiegels in de nieren. Geen veranderingen in weefsel angiotensinespiegels werden waargenomen na behandeling met irbesartan of captopril. Hieruit hebben wij geconcludeerd dat aliskiren minstens even effectief is als AT_1 receptor blokkade en ACE remming ter bescherming van het hart. De lagere angiotensinewaardes in het hart na langere behandeling met aliskiren zou kunnen duiden op een betere langetermijnbescherming van het hart bij directe remming van renine.

De effecten van angiotensine II worden voornamelijk bepaald door de AT_1 receptor. Over het algemeen wordt aangenomen dat AT_2 receptoren endogene antagonisten zijn van de AT_1 receptor, bijvoorbeeld via vaatverwijding en herstructurering van weefsel. Echter de rol van de AT_2 receptor is controversieel tijdens ziekteprocessen, zoals hypertensie. Wij hebben de effecten van angiotensine II, III, IV en 1-7 vergeleken in de kransslagaders, arteria iliaca en de abdominale aorta van SHR en normotensieve Wistar ratten in **hoofdstuk 6**. Alle angiotensine metabolieten induceerden AT_1 receptor-afhankelijke vaatvernauwing in de kransslagaders in

SHR, vergelijkbaar met de effecten in normotensieve Wistar ratten. Blokkade van de AT_2 receptor met PD123319 versterkte de angiotensine II en angiotensine III-geïnduceerde vaatvernauwing wel in Wistar ratten, maar niet in SHR. Dus, tijdens hypertensie is er een tegenovergesteld effect van de AT_2 receptor, of deze is veranderd van vaatverwijdend naar vaatvernauwend. Vaatvernauwing die werd geïnduceerd door angiotensine metabolieten in arteria iliaca en aorta's was vergelijkbaar tussen SHR en Wistar ratten en kon worden geblokkeerd met AT_1 receptor blokker irbesartan. PD123319 blokkeerde de vaatvernauwende effecten in arteria iliaca in SHR, waarmee wederom de vaatvernauwende functie van de AT_2 receptor werd aangetoond tijdens hypertensie.

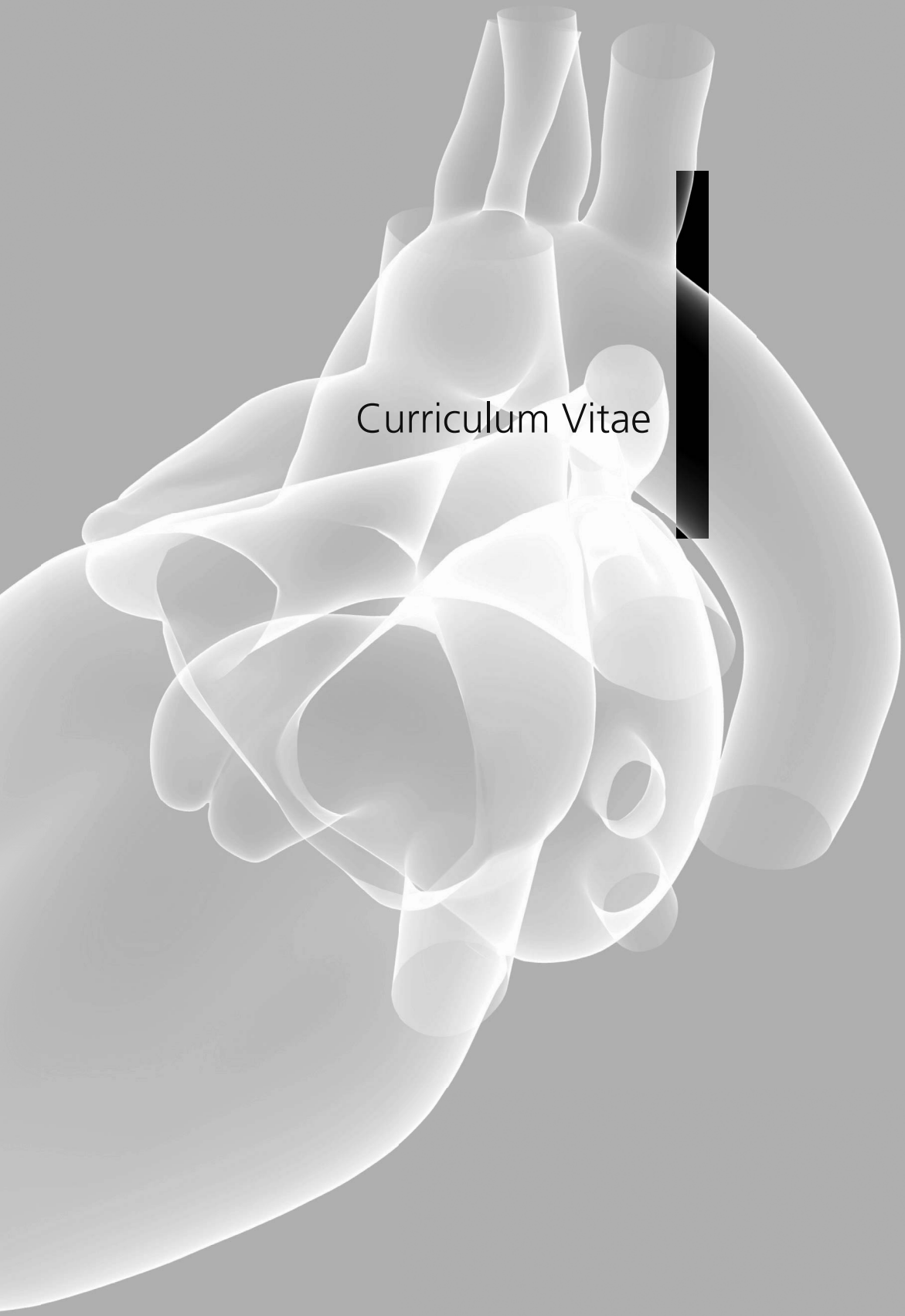
Thoracale Aorta Aneurysma's

Er is toenemend bewijs dat het RAS een belangrijke rol kan spelen in de ontwikkeling van thoracale aorta aneurysma's (TAA). Een literatuuroverzicht over dit onderwerp is beschreven in **hoofdstuk 1**. Schade van de middelste laag van de aorta vaatwand is karakteristiek voor TAAs. Muizen met een 2-maal (Fibulin-$4^{+/R}$) en 4-maal (Fibulin-$4^{R/R}$) verlaagde expressie van fibuline-4 hebben dezelfde histologische kenmerken als TAA patiënten. Dit muismodel werd gebruikt om de structurele afwijkingen van TAAs te onderzoeken (**hoofdstuk 7**). Cellulaire veranderingen waren een dikkere aortawand, onder andere als gevolg van een toename in extracellulaire matrix tussen de cellen, onderbreking van elastine lagen en een verlies van gladde spiercellen. Functioneel resulteerde dit in een verlaagde diastolische druk en een verhoogde polsdruk, en een verminderde vaatvernauwende werking van de thoracale aorta. Een verminderde contractiliteit kan het gevolg zijn van een verstoorde calciumsignalering, die werd gevonden met een aorta transcriptoom analyse. Uit deze analyse, waarbij veranderingen in genen worden bekeken, bleek ook een rol voor TGF-β weggelegd in de ontwikkeling van TAAs. Een histologische kleuring van TGF-β in de aortawand liet zien dat er meer TGF-β signalering was in aorta's van Fibulin-$4^{+/R}$ en Fibulin-$4^{R/R}$ muizen. Bovendien was ook angiotensine II, wat tevens een regulator van het TGF-β signaal is, in het weefsel van deze muizen verhoogd. De muizen werden vervolgens behandeld met AT_1 receptor blokker losartan, omdat AT_1 receptor blokkade indirect ook TGF-β signalering kan remmen. In pasgeboren Fibulin-$4^{R/R}$ muizen met een ernstige TAA verbeterde behandeling met losartan de afwijkingen aan de vaatwand.

Of een verandering in gen expressie van fibuline-4 deficiënte aorta's ook resulteert in veranderingen op eiwitniveau werd onderzocht in **hoofdstuk 8**. Eiwitten aanwezig in de aorta's van wild type Fibuline-$4^{+/+}$, Fibulin-$4^{+/R}$ en Fibulin-$4^{R/R}$ muizen werden vergeleken. Met de analyse van de biologische signaleringroutes werd een verhoogde oxidatieve fosforylatie gevonden, resulterend in mitochondriale dysfunctie en verhoogde oxidatieve stress in fibuline-4 deficiënte muizen. Om genen te identificeren die zowel een verandering laten zien op genniveau en eiwitniveau in fibuline-4 deficiënte muizen werd een overlap van beide analyses gemaakt. De geïdentificeerde biomarkers verwezen allemaal naar de 17β-estradiol signaleringsroute in zowel de Fibulin-$4^{+/R}$ en Fibulin-$4^{R/R}$ muizen. In aorta's van Fibulin-$4^{R/R}$

muizen werd ook TNF-α, een marker voor ontsteking gevonden. Een slechte functie van de mitochondrieën en oestrogenen resulteren in meer reactieve zuurstof radicalen en zijn beide markers voor verouderingsgerelateerde ziekteprocessen. Met een fluorescerende marker kon een graduele toename van reactieve zuurstofradicalen worden gezien in Fibulin-$4^{+/R}$ en Fibulin-$4^{R/R}$ aorta's. Meer tekenen van veroudering werden bovendien gezien door een verminderde endotheelcel functie, en toegenomen stijfheid van de aorta's.

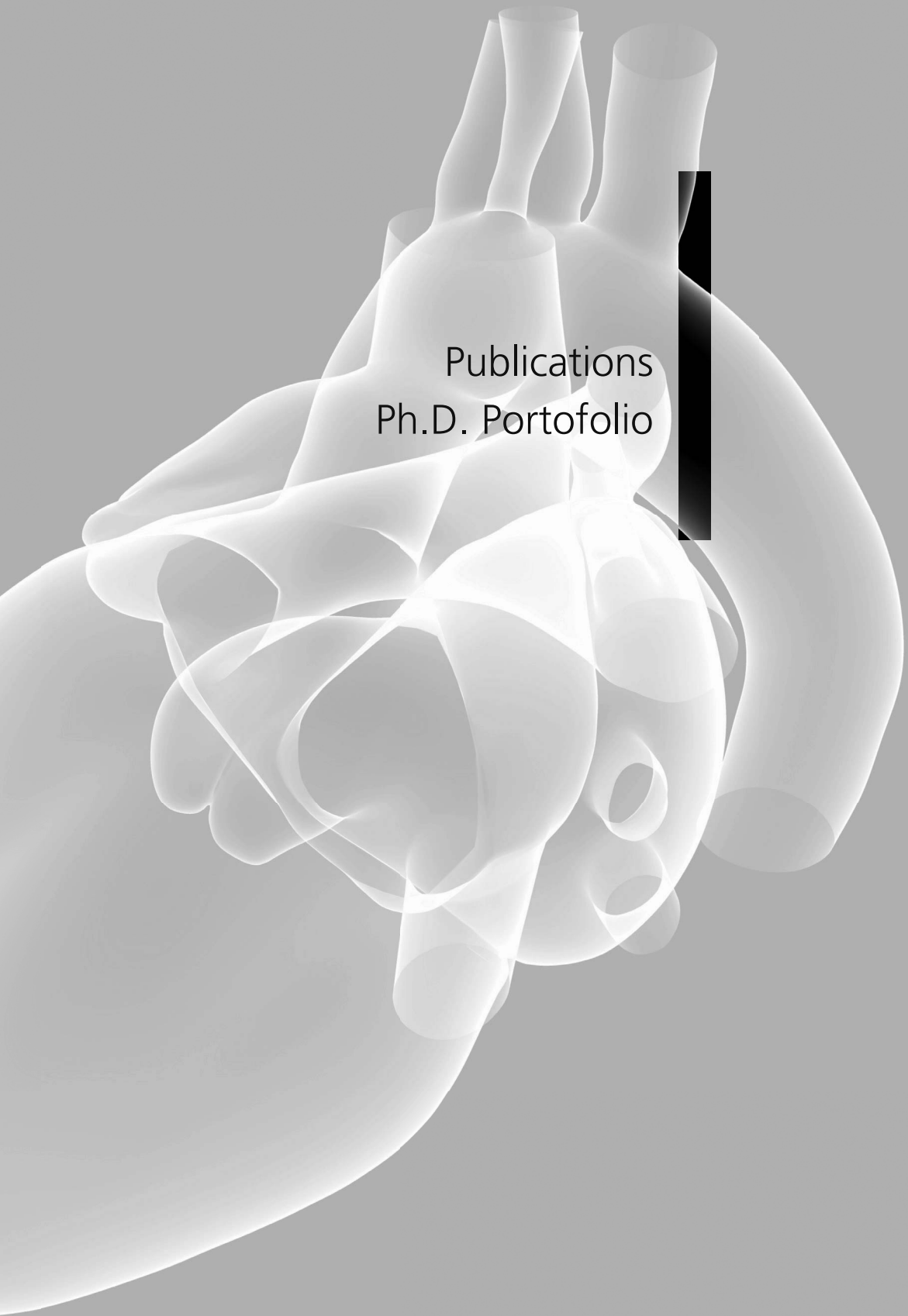
In **hoofdstuk 9** wordt beschreven dat fibuline-4 deficiëntie resulteert in hartfalen en aortaklep afwijkingen. In Fibulin-$4^{R/R}$ muizen is er een grote toename in de diameter van het linker ventrikel, in vergelijking met Fibulin-$4^{+/+}$ en Fibulin-$4^{+/R}$ muizen. Dit wordt vergezeld met verhoogde eind-systolische en end-diastolische volumes, een verminderde ejectie fractie en een afname in de verhouding tussen end-systolische en eind-diastolische dikte van het linker ventrikel. Bovendien resulteert een aanpassing van het hart tot een grotere massa van het linker ventrikel en grotere hartspiercellen. Opmerkelijk is dat Fibulin-$4^{+/R}$ muizen wel een afname laten zien van de ejectie fractie en de verhouding tussen eind-systolische en eind-diastolische dikte van het linker ventrikel en een toename in cardiomyocytgrootte, maar geen aortaklep afwijkingen hebben. Wellicht is hier sprake van een primaire cardiomyopathie, welke in Fibulin-$4^{R/R}$ muizen wordt verergerd door de aanwezigheid van ernstige aortklep stenose en lekkage. De combinatie van TAA en aortaklep stenose is welbekend in patiënten met een bicuspide aortaklep. Het is onbekend of patiënten met een mutatie in het fibuline-4 gen deze combinatie ook laten zien. Daarom hebben wij een PCR protocol ontwikkeld om het humane fibuline-4 gen te bekijken. In totaal werden 400 patiënten met een afwijking aan de thoracale aorta en/of aortaklep stenose gescreend op nieuwe puntmutaties en dit werd vergeleken met gegevens uit gezonde populaties en een controle groep. In dit cohort hebben wij geen aanwijzingen gevonden voor een puntmutatie die de directe aanleiding zou zijn voor aorta en/of aortaklep afwijkingen.



Curriculum Vitae

CURRICULUM VITAE

Els Moltzer werd geboren op 30 november 1985 te Rotterdam en behaalde daar in 2003 haar VWO-diploma aan het Melanchthon College. In hetzelfde jaar begon zij aan de studie Geneeskunde aan de Erasmus Universiteit in Rotterdam. In 2005 werd daarnaast gestart met de Master of Science in Clinical Research met een deeltijdprogramma voor geneeskundestudenten. De doctoraalfase van Geneeskunde en de M.Sc. in Clinical Research werden gezamenlijk afgesloten met een wetenschappelijke stage op de afdeling Cardiologie in het Erasmus Medisch Centrum (Prof.dr. M.L. Simoons en Prof.dr. J.W. Roos-Hesselink) naar hypertensie bij patiënten met een geopereerde aorta coarctatie, resulterend in het tweede hoofdstuk van dit proefschrift. Gelijktijdig werd er, in samenwerking met de afdeling Celbiologie en Genetica (dr. J. Essers), onderzoek gedaan op de afdeling Inwendige Geneeskunde – Divisie van Vasculaire Geneeskunde en Farmacologie (Prof.dr. A.H.J. Danser) naar een muismodel voor aorta aneurysma's. Door een samenwerking van de genoemde afdelingen kon zij in 2008 officieel beginnen als onderzoeker in opleiding aan het promotietraject naar aorta pathologie en de effecten van het renine-angiotensine systeem.



Publications
Ph.D. Portofolio

PUBICATIONS

Full papers

Moltzer E, Verkuil AV, van Veghel R, Danser AHJ, van Esch JHM. Effects of angiotensin metabolites in the coronary vascular bed of the spontaneously hypertensive rat: loss of angiotensin II type 2 receptor-mediated vasodilation. Hypertension. 2010;55:516-522.

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Ph.D. PORTOFOLIO

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Erasmus MC Departments:	Internal Medicine, Division of Vascular Medicine and Pharmacology Cardiology
Research School:	Cardiovascular Research School (COEUR), Erasmus MC, Rotterdam, The Netherlands
Promotors:	Prof.dr. A.H.J. Danser Prof.dr. J.W. Roos-Hesselink
Copromotor:	Dr. J. Essers

Education

2008 – 2011	Ph.D., Erasmus MC Rotterdam, The Netherlands Thesis: Aortic pathology and the role of the renin-angiotensin system
2005 – 2008	M.Sc. in Clinical Research, Nihes, Erasmus MC Rotterdam, The Netherlands
2003 – 2007	Doctorate in Medicine, Erasmus MC, Rotterdam, The Netherlands

PhD training

In-depth courses (17.9 ECTS)

2005 – 2011	COEUR lectures, seminars and courses, Erasmus MC, Rotterdam, The Netherlands
2009	Venture Challenge Workshops, Netherlands Genomics Institute, Rockanje, The Netherlands
2008	Course on Laboratory Animal Science, Erasmus MC, Rotterdam, The Netherlands
2007	Interactieve basis echocursus: aangeboren hartafwijkingen, Rotterdam, The Netherlands

Teaching (3.4 ECTS)

2008-2010	Supervising practical 'Farmacologische beinvloeding van het autonome zenuwstelsel', 1st year medical students, Erasmus MC, Rotterdam, The Netherlands
2009-2010	Supervising practical, students Junior Med School, Highschool, MSc in Molecular Medicine, Erasmus MC, Rotterdam, The Netherlands
2009-2010	Supervising 2nd year medical students in writing a review, Erasmus MC, Rotterdam, The Netherlands
2008	Supervising, Erasmus Summer Programme, Principles of Research in Medicine, Nihes, Erasmus MC, Rotterdam, The Netherlands

Symposia and conferences (16.1 ECTS)

Oral presentation

2011	Wetenschapsdagen Inwendige Geneeskunde, Antwerp, Belgium
2010	Stafdag Heelkunde, Delft, The Netherlands
2010	COEUR Research Seminar on Coarctation of the Aorta, Erasmus MC, Rotterdam, The Netherlands
2010	COEUR Research Seminar on Extracellular Matrix in Vascular Disease, Erasmus MC, Rotterdam, The Netherlands
2010	Figon Dutch Medicine Days, Lunteren, The Netherlands
2010	20th European Meeting on Hypertension, European Society of Hypertension, Oslo, Norway
2009	Figon Dutch Medicine Days, Lunteren, The Netherlands
2009	Nederlands Hypertensie Genootschap Meeting, Maastricht, The Netherlands
2009	European Society of Cardiology Congress, Barcelona, Spain
2009	Microcirculation and Vascular Biology/ Nederlands Hypertensie Genootschap Meeting, Biezenmortel, The Netherlands

Poster presentation

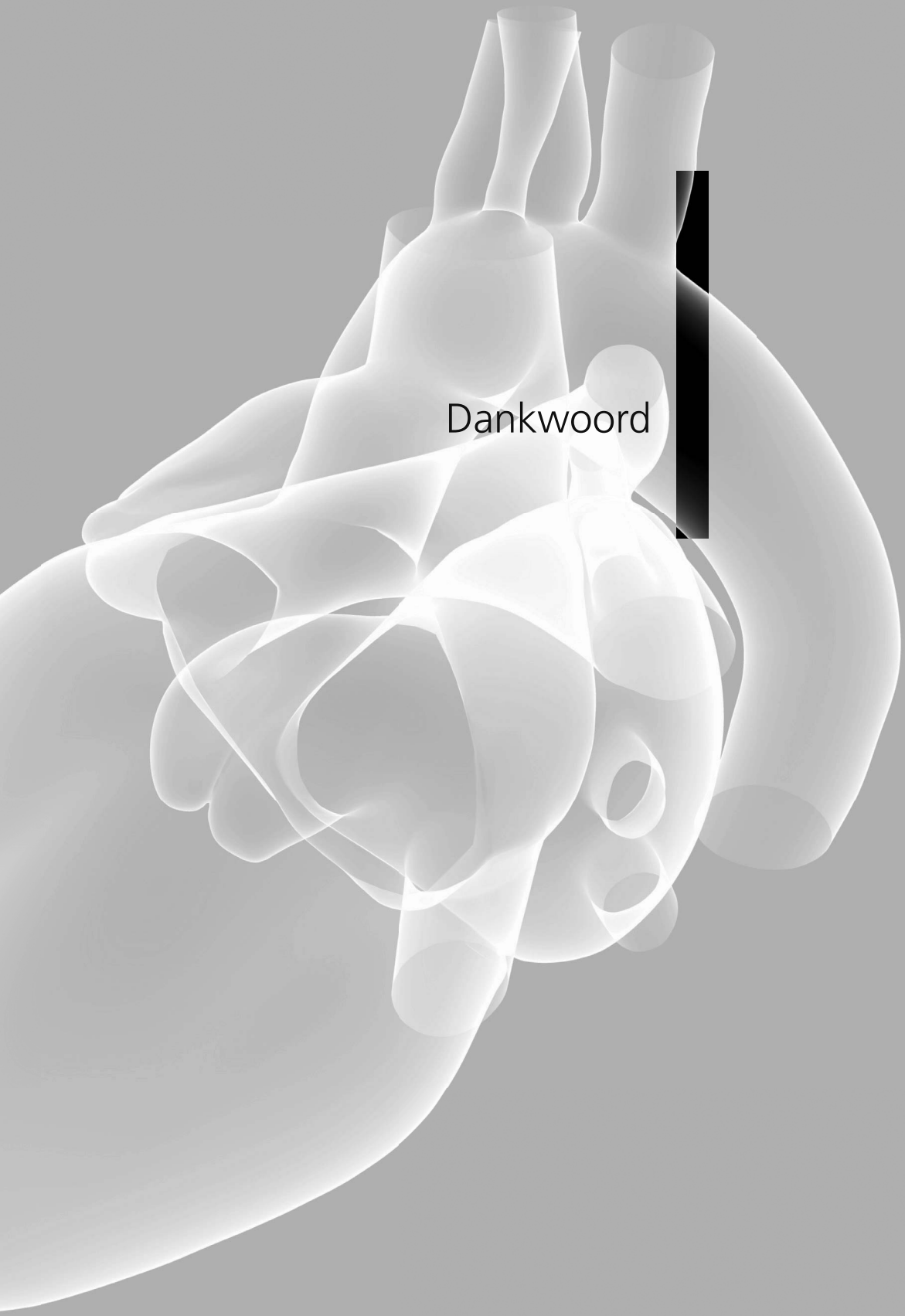
2010	Wetenschapsdagen Inwendige Geneeskunde, Antwerp, Belgium
2009	American Heart Association Scientific Sessions 2009, Orlando, USA
2009	Figon Dutch Medicine Days, Lunteren, The Netherlands
2009	63th High Blood Pressure Research Conference, Chicago, USA
2009	European Society of Cardiology Congress, Barcelona, Spain
2009	19th European Meeting on Hypertension, European Society of Hypertension, Milan, Italy
2009	Nederlandse Vereniging voor Cardiologie, jubileumcongres, Amsterdam, The Netherlands
2009	Wetenschapsdagen Inwendige Geneeskunde, Antwerp, Belgium

Attended

2010	American Heart Association Scientific Sessions 2010, Chicago, USA
2010	Cardiology and Vascular Medicine update and perspective, Rotterdam, The Netherlands
2008	Behandeling van aangeboren hartafwijkingen: indicaties, technische aspecten en late gevolgen, Utrecht, The Netherlands
2007	Figon Dutch Medicine Days, Lunteren, The Netherlands
2007	Cardiology and Vascular Medicine update and perspective, Rotterdam, The Netherlands
2007	Long may we live: causes of diseases in the elderly, Utrecht, The Netherlands
2006	Cardiology and Vascular Medicine update and perspective, Rotterdam, The Netherlands

Awards

2010	Best Basic Science Abstract, Stafdag Heelkunde
2010	Accomodation grant, 20th European Meeting on Hypertension
2009	3rd Poster Prize, Figon Dutch Medicine Days
2009	Traval grant, 19th European Meeting on Hypertension
2009	2nd Poster Prize, Wetenschapsdagen Inwendige Geneeskunde
2009	Young Investigator Award, Nederlands Hypertensie Genootschap Meeting



Dankwoord

DANKWOORD

Ruim vier jaar geleden startte ik als student-onderzoeker aan mijn eigen onderzoek. Studeren werd bestuderen en vervolgens voortgezet als promotieonderzoek, wat uiteindelijk heeft geresulteerd in dit proefschrift. Ik ben trots op het resultaat, maar dit proefschrift zou nooit tot stand zijn gekomen dankzij de hulp van velen. In het bijzonder ben ik diegenen dankbaar die mij de laatste maanden hebben geholpen de deadline te halen, ondanks het werken met één hand.

Prof.dr. Roos-Hesselink, Jolien, via Prof.dr. Simoons kwam ik bij de congenitale cardiologie terecht. Van jou kreeg ik, als student nog, de verantwoordelijkheid om een nieuw patiëntgebonden onderzoek op te starten. Het begin was onwennig maar al snel voelde ik mij in het onderzoek thuis. Jouw kijk is altijd positief en dat heeft mij gestimuleerd om met het onderzoek door te gaan. Dankzij jou, Prof.dr. Simoons en Prof.dr. Danser kreeg ik de kans om mijn onderzoeksstage te vervolgen in een promotietraject, waar ik absoluut geen spijt van heb, bedankt daarvoor.

Prof.dr. Danser, Jan, met jou samenwerken is zoals ik mij de colleges farmacie herinner, georganiseerd en duidelijk. Ik kan mij steeds opnieuw verbazen over jouw georganiseerde kamer, vol dossiermappen, en slechts zelden trek je de verkeerde la open om een referentie op te zoeken. Na een gesprek met jou lijken onderzoeksproblemen opgelost, maar help je ons altijd wel herinneren aan al die andere dingen die ook, liefst gisteren, nog gedaan moeten worden. Bedankt voor je hulp en optimisme de afgelopen jaren!

Dr. Essers, Jeroen, met jouw enthousiasme heb je mij ook aangestoken. Jouw deur staat altijd open om even binnen te lopen, en daar heb ik veel gebruik van gemaakt. Tijdens mijn promotietraject heb ik veel congressen mogen bezoeken, maar de congressen waar ik met jou ben geweest waren het meest intensief. Ondanks een vol programma moet er natuurlijk ook worden hard gelopen en souvenirs worden gekocht. Van jou heb ik geleerd het werk van anderen kritisch te beoordelen en dit toe te passen op ons eigen onderzoek. Bedankt voor je hulp bij het maken van de eindsprint voor het inleveren van dit proefschrift.

Prof.dr. Duncker, Dirk, bedankt voor je zitting in de leescommissie. Ik ben blij dat ik met jou heb mogen samenwerken, je bent mijn voorbeeld als clinicus op het lab, en zal je heldere kijk op de statistiek niet vergeten. Prof.dr. Doevendans, Prof.dr. Verhagen, dank voor uw zitting in de leescommissie en de beoordeling van mijn proefschrift. Prof.dr. Zijlstra en Prof.dr. Loeys, dank voor uw deelname aan de grote commissie.

Mijn collega's van de Farmacologie; Joep, samen hebben we de afgelopen jaren veel sport- en onderzoeksuitslagen (en natuurlijk de lay-out) doorgenomen. Bedankt voor alle adviezen tijdens de koffie, en hulp tijdens experimenten. Al vind je zelf van niet, jouw bijdrage was en is onmisbaar. Kayi, bedankt dat je mijn paranimph wil zijn. Het is handig dat we samen wat zaken kunnen regelen, nu we zo kort na elkaar promoveren, maar het levert ons beiden ook extra druk op. Gelukkig blijf je altijd vrolijk en sta je altijd klaar met iets lekkers voor ons. Bedankt voor je adviezen en hulp bij het maken van mijn proefschrift. En die hotelkamer delen we nog wel een keer!

Richard, bedankt voor je gezelligheid, muziek en hulp bij computerproblemen en experimenten; op de één of andere manier vind jij altijd wel tijd om even te helpen. Matej, thanks for the English/Dutch intermezzo's during boring workdays. Luuk, ik wens je veel succes met het voortzetten van dit project. En alle andere (oud) collega's van de 14e verdieping: Hisko, Goran, Haiyan, Birgitte, Koen, Ilse, Wendy, Manne, Sieneke, René de V., Marcel, Akis, Anton en Antoinette, zonder jullie allen zou het werken op deze afdeling niet zo leuk zijn. Het is niet gek dat veel promovendi aanblijven als post-doc en ik zal de gezelligheid van de afdeling erg missen. Alle andere collega's: Frank, Ingrid, Usha, Angelique, Jeanette, René de B., Nils, Christina, Eric, Edith P., Mieke, Edith V., Marjolein, Evelien, Serieta, Bianca, Luit, Mariëtte, Pieter, Joost en Ton, bedankt voor de gezelligheid op het werk en tijdens congressen en labdagen.

Prof.dr. Villalón, Carlos, thank you for giving me the opportunity writing a review for publication.

De collega's van de Cardiologie: Heleen, met jouw analytische kijk heb je vaak dingen voor mij verhelderd. Ik wens je een mooie dag toe wanneer jij je proefschrift mag verdedigen. Celeste, bedankt voor het opsporen van verdwenen statussen, terwijl jij zelf onverstoorbaar doorwerkt. Maarten Witsenburg, dank dat ik gebruik mocht maken van uw expertise op het gebied van aorta stents. Sing en Yusuf, jullie hebben mij als student onder jullie hoede genomen en geholpen met het opstarten van mijn eigen onderzoek, bedankt! Dr. Meijboom, Tineke, Judith en René, bedankt voor jullie hulp waar nodig. Nog velen anderen hebben mij geholpen met het verzamelen van patiëntengegevens, bedankt daarvoor.

Willeke, bedankt dat jij mijn promotiezaken op je wilde nemen, ondanks het feit dat we geen directe collega's meer zijn. Met veel vragen kon ik vanaf het begin van mijn onderzoek bij jou terecht, en hoewel je mij soms zenuwachtig kon maken met alles wat er nog moest gebeuren, ben ik je daar dankbaar voor.

De 'fibuline-4 groep': Paula, dankzij jou heb ik steeds de juiste muizen kunnen gebruiken. Natasja, bedankt voor het delen van de data in mijn laatste hoofdstuk, hopelijk kunnen we er een mooi paper van maken. Devashish, also thanks for combining our data. Nicole, bedankt voor je assistentie bij het verzamelen van de laatste gegevens. Ellen, bedankt voor je enthousiasme en kritische blik op mijn artikelen.

Marcel Vermeij; veel data is verzameld, dankzij jouw enthousiasme voor dit project. Beter te veel dan te weinig moet jouw motto zijn, want ik kreeg nooit wat ik je had gevraagd. Jij levert alles in veelvoud; extra weefsel en extra kleuringen in de hoogste kwaliteit. Dank voor alles. Lambert en Miranda, zonder jullie kennis en ervaring zouden de muizen echo's niet zo veel informatie hebben opgeleverd.

De studies met muizen zouden niet gedaan kunnen worden zonder de ondersteuning vanuit het EDC. Voornamelijk wil ik Ed Lansbergen, Dennis de Meulder en Diana Ensink bedanken. Zonder jullie hulp en flexibiliteit zou dit werk niet mogelijk zijn geweest.

Marie-Chantal, bedankt voor de gezellige koffie breaks.

Mijn studievriendinnen; Jacqueline, Germaine, Annemieke (en Pieter natuurlijk) en Marijn. Na een lange werkweek is er altijd genoeg gebeurd om even bij elkaar uit te blazen. Jac, bedankt dat je mijn paranimph wil zijn. Bij jou kan ik altijd terecht. We hebben de afgelopen jaren veel mooie momenten mogen delen en ik ga er vanuit dat er nog veel zullen volgen. Bedankt voor je steun en adviezen. Ik kijk al uit naar jouw promotie! Germaine, samen zijn we aan dit avontuur begonnen om eerst te promoveren en straks zullen we samen coassistent zijn. We hebben veel onderzoeksfrustraties en -successen gedeeld, en ik weet zeker dat jouw proefschrift ook snel in mijn kast zal staan.

Tot slot wil ik mijn familie bedanken, die in het bijzonder de laatste maanden, veel voor mij hebben gedaan, waardoor ik snel weer aan het werk kon en dit proefschrift er nu ligt. Papa en mama, jullie hebben mij vrij gelaten te studeren wat ik zelf wilde, maar het werd toch Geneeskunde. Jullie interesse in alles wat wij doen motiveert mij om er het beste uit te halen. Bedankt voor jullie steun en liefde. Pim en Benjamin, ik vind het een eer dat jullie komen spelen met de band. Pim, dankzij jouw grafische hulp heb ik een paar mooie plaatjes en een prachtig proefschrift! (En weet jij wat een receptor is). Pieter en Ouyan, Annelies en David, bedankt voor jullie steun en gezelligheid. Annelies, jij staat altijd voor mij klaar en bij jou kan ik al mijn verhalen kwijt. Bedankt voor je begrip. Ik ben er trots op jullie familie te zijn!

Els Moltzer

Dalam rangka memperingati nenek saya.