AT1 receptor blockade, but not renin inhibition, reduces aneurysm growth and cardiac failure in Fibulin-4 mice

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ABSTRACT

Aims: Increasing evidence supports a role for the angiotensin (Ang) II-AT_1 receptor axis in aneurysm development. Here we studied whether counteracting this axis via stimulation of AT_2 receptors is beneficial. Such stimulation occurs naturally during AT_1 receptor blockade with losartan, but not during renin inhibition with aliskiren.

Methods and Results: Aneurysmal homozygous Fibulin-$4^{R/R}$ mice, displaying a 4-fold reduced fibulin-4 expression, were treated with placebo, losartan, aliskiren, or the β-blocker propranolol from day 35-100. Their phenotype includes cystic media degeneration, aortic regurgitation, left ventricular (LV) dilation, reduced ejection fraction, and fractional shortening. While losartan and aliskiren reduced hemodynamic stress and increased renin similarly, only losartan increased survival. Propranolol had no effect. No drug rescued elastic fiber fragmentation in established aneurysms, although losartan did reduce aneurysm size. Losartan also increased ejection fraction, decreased LV diameter, and reduced cardiac pSmad2 signaling. None of these effects were seen with aliskiren or propranolol. Longitudinal microCT measurements, a novel method in which each mouse serves as its own control, revealed that losartan reduced LV growth more than aneurysm growth, presumably because the heart profits both from the local (cardiac) effects of losartan and its effects on aortic root remodeling.

Conclusions: Losartan, but not aliskiren or propranolol, improved survival in Fibulin-$4^{R/R}$ mice. This most likely relates to its capacity to improve structure and function of both aorta and heart. The absence of this effect during aliskiren treatment, despite a similar degree of blood pressure reduction and renin-angiotensin system blockade, suggests that it might be due to AT_2 receptor stimulation.

INTRODUCTION

Thoracic aorta aneurysms (TAA) show degeneration of the medial layer of the aortic wall, characterized by elastic fiber fragmentation, loss of smooth muscle cells, and the accumulation of amorphous extracellular matrix.[1] Such aortic wall degeneration is often a consequence of inherited connective tissue disorders. The most common inherited TAA disease, Marfan syndrome (MFS), is due to a mutation in the *FBN1* gene, which encodes the extracellular matrix (ECM) glycoprotein fibrillin-1. *FBN1* mutations result in a disorganized ECM assembly in the aortic wall[2], leading to all above described key features of TAA in MFS patients. Mice heterozygous for a cysteine substitution in an epidermal growth factor-like domain of fibrillin-1 (*Fbn1*$^{C1039G/+}$ mice), i.e., a mutation which is prototypical for the *FBN1* mutations in humans, similarly develop TAA.[3]

Another factor in the elastic fiber assembly of the vessel wall, heart valves and myocardial interstitium, is the ECM protein fibulin-4, encoded by the FBLN4 gene.[4, 5] In humans, a mutation in this gene causes cutis laxa syndrome, that besides cutis laxa (loose skin), bone fragility and lung emphysema is characterized by vascular tortuosity and aneurysms similar to those observed in MFS.[6-11] Moreover, mice with a systemic 4-fold reduced fibulin-4 expression (Fibulin-4$^{R/R}$) share similar key features as seen in MFS and cutis laxa syndrome, i.e., cystic media degeneration, aortic regurgitation, and impaired cardiac morphology and function[12, 13], while complete fibulin-4 gene knock-out mice (Fibulin-4$^{-/-}$) die perinatally from aortic rupture.[14]

Recent studies have shown that transforming growth factor (TGF)β signaling is upregulated in TAAs of MFS.[13, 15, 16] While direct regulators of TGFβ signaling include TGFβ and bone morphogenetic protein ligands, indirect stimulation of TGFβ signaling is accomplished by angiotensin (Ang) II, via its type 1 receptor (AT_1R). In support of this concept, both TGFβ-neutralizing antibodies and the AT_1R blocker losartan exerted beneficial effects in rodent TAA models, including Fibulin-4$^{R/R}$ mice when treated prenatally.[13, 15] Yet, clinical studies with losartan in MFS did not yield uniformly positive results.[17, 18] Blocking AT_1R results in a counterregulatory rise in renin, thereby increasing Ang II levels. This Ang II cannot stimulate the blocked AT_1R, but it may still bind to the unoccupied Ang II type 2 receptors (AT_2R), which antagonizes AT_1R-mediated effects.[19, 20] Such AT_2R stimulation is potentially beneficial in TAA[20], and will not occur during other forms of renin-angiotensin system (RAS) blockade, i.e., inhibition of the enzymes that generate Ang I (renin) or Ang II (ACE).

In the present study, we hypothesized that losartan outperforms the renin inhibitor aliskiren in the treatment of Fibulin-4$^{R/R}$ mice, given its additional AT_2R-stimulating effects. Both drugs were compared with placebo and the β-blocker propranolol, a MFS drug that is often used in the clinic because it is expected to reduce heart rate, blood pressure and dP/dt. ACE inhibitors were not included, since such drugs, in addition to suppressing

Ang II, also increase bradykinin, thus introducing interference with yet another hormonal system. Treatment started postnatally at a clinically relevant age: day 35, when the aneurysm is already present, and lasted up to 100 days. Moreover, we used a novel *in-vivo* µCT-technique allowing longitudinal measurement that monitors the therapeutic treatment effects on both aneurysm progression as well as cardiac growth in time simultaneously.

Our data show that losartan, but not aliskiren or propranolol, independently of its blood pressure-lowering effect, improved survival in Fibulin-$4^{R/R}$ mice. The absence of this effect during aliskiren treatment suggests that it might involve AT_2R stimulation.

MATERIAL AND METHODS

Experimental animals

Generation of Fibulin-$4^{R/R}$ mice has been described previously.[12] Heterozygous (Fibulin-$4^{+/R}$) mice in a mixed C57Bl/6x129 background were mated to obtain Fibulin-$4^{+/+}$ (wild-type) and Fibulin-$4^{R/R}$ littermates. Animals were housed in the institutional animal facility. Both males and females were included in the study, and since no apparent sex-related differences were observed, data from both sexes were pooled. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC, Rotterdam, The Netherlands (protocol number 139-11-09 and 139-13-11). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, revised 2011).

Treatment

Fibulin-$4^{R/R}$ mice and wild-type mice were treated postnatally from the age of 35 days up to 100 days with placebo, losartan (60 mg/kg p.o. per day; a kind gift of MSD, Haarlem, The Netherlands), aliskiren (62.5 mg/kg p.o. per day; a kind gift of Novartis Pharmaceuticals, Basel, Switzerland), or propranolol (50 mg/kg p.o. per day; Sigma, St. Louis, USA) in drinking water, as described before.[13, 15, 21, 22]

Histology

Mice (age 100 days) were weighed, euthanized by an overdose of CO_2, and necropsied according to standard protocols. Perfusion-fixed aortas and hearts were isolated and paraffin-embedded. Next, 4 µm-aorta sections were haematoxylin and eosin (HE)-stained, stained for elastin (Verhoeff van Gieson), glycosaminoglycans (Alcian Blue) or vascular smooth muscle cells (VSMCs, α-smooth muscle actin). Immunohistochemistry for phosphorylated Smad2 (pSmad2) was performed as described previously[23], using rabbit antiphospho-smad2 antibodies (Cell Signaling Technology, Danvers, USA). Positively stained pSmad2 nuclei were divided by the total number of nuclei to obtain relative

amounts. HE-stained aorta slides were scanned with a nanozoomer (Hamamatsu, Almere, The Netherlands), and subsequently aortic wall diameter and aortic wall area were analyzed with NanoZoomer Digital Pathology view (Hamamatsu). Finally, 5-µm heart sections were stained with Gomori's silver staining to visualize individual cardiomyocytes of the left ventricle (LV).[24] Only transversally cut cells showing a nucleus were used to determine the cardiomyocyte area.

Biochemical measurements

RAS components were measured in kidneys (Ang II) and blood plasma (renin). Blood was collected from the left ventricle immediately prior to euthanization in heparin-coated tubes, centrifuged at 5500 RPM, and plasma was stored at -80°C. Kidneys were removed after the animals had been euthanized, frozen in liquid nitrogen, and stored at -80°C. Tissue Ang II was measured by radioimmunoassay, after SepPak extraction and reversed-phase HPLC separation as previously described.[25, 26] Plasma renin concentration (PRC) was determined by enzyme-kinetic assay in the presence of excess angiotensinogen as described before.[26] Additionally, B-type natriuretic peptide-45 (BNP-45) was measured in plasma, making use of a commercially available enzyme immuno-assay (Phoenix Pharmaceuticals Inc., Karlsruhe, Germany).

Ultrasound and hemodynamic measurements

To evaluate the treatment of the different compounds on aneurysm formation and cardiac function, cardiac geometry, echocardiographic and hemodynamic measurements were performed in 100-days old Fibulin-$4^{+/+}$ (wild type) and Fibulin-$4^{R/R}$ mice. Mice were anesthetized with 2.5% isoflurane and ventilated with 35% O_2. Anesthesia did not affect heart rate (data not shown). Echocardiography of the ascending aorta and LV was performed using a Vevo2100 (VisualSonics Inc., Toronto, Canada). Ascending aorta and LV lumen diameter, aortic distensibility, ejection fraction and fractional shortening were obtained from M-Mode images. Ejection fraction and fractional shortening were defined as the relative differences between end-diastolic and end-systolic volumes and diameter, respectively.[13] Subsequently, a 1.4-Fr microtipped manometer (Millar Instruments, Houston, USA) was inserted into the right carotid artery to measure aortic pressure.[27] Hemodynamic data were recorded and digitized using an online 4-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, USA), analysis was performed with a program written in Matlab.[28] Ten consecutive beats were selected for determination of systolic and diastolic blood pressure, subsequent mean arterial pressures (MAP) were calculated.

Western blot

LV tissue samples were used for immunoblotting of extracellular signal-regulated kinases (ERK1/2), phosphorylated ERK1/2 (pERK1/2), Smad2 and pSmad2 (Cell Signaling Technol-

ogy). Ratios of phosphorylated protein levels to loading control β-actin were calculated and corrected for the ratios in wild-type mice.

Quantitative real-time reverse transcription polymerase chain reaction

Expression of angiotensin II type 1a, type 1b and type 2 receptors ($AT_{1a}R$, $AT_{1b}R$ and AT_2R) was analyzed in LV tissue. Total RNA was isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands). cDNA samples were subjected to 40 cycles real-time PCR analysis using SYBR Green qPCR Master Mix 2x (Bio-Rad) and primers; β-actin 5'-AGCCATGTACGTAGCCATCCA-3', 5'-TCTCCGGAGTCCATCACAATG-3'; β_2-microglobin 5'-CTCACACTGAATTCACCCCCA-3', 5'-GTCTCGATCCCAGTAGACGGT-3'; $AT_{1a}R$ 5'-CCCACGTGTCCCTGTTACTAC-3', 5'-TTTGGGGACAGTACAGGTTTC-3'; $AT_{1b}R$ 5'-CTGTGAAATTGCGGACGTAGT-3', 5'-AAGCCATAAAACAGAGGGTTCAG-3'; AT_2R 5'-TACCCGTGACCAAGTCCTGA-3', 5'-TACCCATCCAGGTCAGAGCA-3'. Gene expression was calculated using β-actin and β_2-microglobin as housekeeping genes and the comparative Ct method (ΔΔCt) was used for relative quantification of gene expression.

FMT-CT Imaging

We used vascular Computed Tomography (CT) and fluorescent mediated tomography (FMT)-CT imaging with near-infrared fluorescent protease activatable probes as previously described.[29] In short, mice subjected to FMT-CT were shaved and depilated to remove all hair that otherwise would absorb light and interfere with optical imaging. Mice subjected to vascular CT and FMT-CT mice received 5 mL/kg body weight Exia 160 contrast agent (Binitio Biomedical Inc., Ottawa, Canada) through injection in the tail vein for subsequent CT analysis. Mice only subjected to vascular CT imaging were anesthetized (2.5% isoflurane) and scanned directly with the microCT scanner (Quantum FX system, Perkin Elmer Inc., Akron, USA). The thoracic aorta diameter, thoracic aortic volume and left ventricular volume were analyzed with a rendering program 'microCT Tools by Analyze 11.0 software' (AnalyzeDirect Inc., Overland Park, USA). Fibulin-4 mice which were also subjected to FMT imaging, were scanned with an FMT 2500 system (Perkin Elmer Inc.) at 680 nm excitation and emission wavelengths, at 24 hours after tail vein injection of 5 nmol of MMPSense680™ (Perkin Elmer Inc.). Mice were anesthetized (2.5% isoflurane) and fixed into the portable animal imaging cassette that lightly compressed the anesthetized mouse between optically translucent windows, thereby preventing motion during FMT and CT imaging. After FMT imaging, anesthetized mice were scanned with the microCT scanner to identify heart and aortic root region of the animals. After FMT-CT imaging, complete aortas were harvested and fluorescence was quantified using the FMT 2500 and Odyssey imaging systems (LI-COR Inc.). Near infrared images were obtained in the 680 nm channel.

Data analysis

Normally distributed data are presented as mean±SEM. One-way ANOVA was applied for the analysis between groups, followed by a post-hoc Dunnett's test when appropriate. All statistical tests were two-sided and P<0.05 was considered statistically significant.

RESULTS

Losartan increases survival of adult Fibulin-4$^{R/R}$ animals independently of its effect on blood pressure and the degree of RAS blockade

Reduced fibulin-4 expression resulted in severe TAA, cardiac hypertrophy, and diminished survival (Fig. 1A-1B), in full agreement with previous observations.[12, 13] Losartan, but not aliskiren treatment, significantly improved survival (Fig. 1C). Propranolol even tended to diminish survival (P=0.25), and no animal survived up to 100 days with this treatment. As a consequence, blood pressure data could not be obtained in propranolol-treated mice, and in only 3 surviving aliskiren-treated mice versus 5 losartan-treated mice. MAP tended to be diminished in Fibulin-4$^{R/R}$ mice (P=0.17). Both RAS blockers similarly reduced MAP at 100 days (Fig. 1D). PRC and renal Ang II were higher in fibulin-4$^{R/R}$ mice than in wild-type animals (Fig. 1E-1F). Losartan and aliskiren comparably increased PRC versus placebo, suggesting a similar degree of RAS blockade. Losartan, but not aliskiren, additionally suppressed renal Ang II.

Losartan improves aneurysm size and aortic distensibility without affecting structural changes and matrix metalloproteinases (MMPs)

At the age of 100 days, the ascending aortic diameter in Fibulin-4$^{R/R}$ mice was almost 3 times enlarged compared to wild-type mice (Fig. 2A). This widening was accompanied by an approximately 50% decrease in distensibility (Fig. 2B) and an increased wall thickness (Fig. 2C). Losartan improved diameter and distensibility without affecting thoracic aortic wall thickness, whereas aliskiren had no significant effect on any of these parameters (Fig. 2A-2C). For reasons discussed above, similar data could not be obtained for propranolol. Neither losartan nor aliskiren affected the disturbed aortic wall morphology, the severe alterations in elastic fiber organization, or the increased glycosaminoglycan deposition in Fibulin-4$^{R/R}$ mice (Fig. 2D-2F). These drugs also did not significantly improve the reduced VSMC content, or diminish the increased pSmad2-signaling in these animals (Fig. 2G-2H). Non-canonical (pERK) TGFβ signaling was similarly unaffected (data not shown).

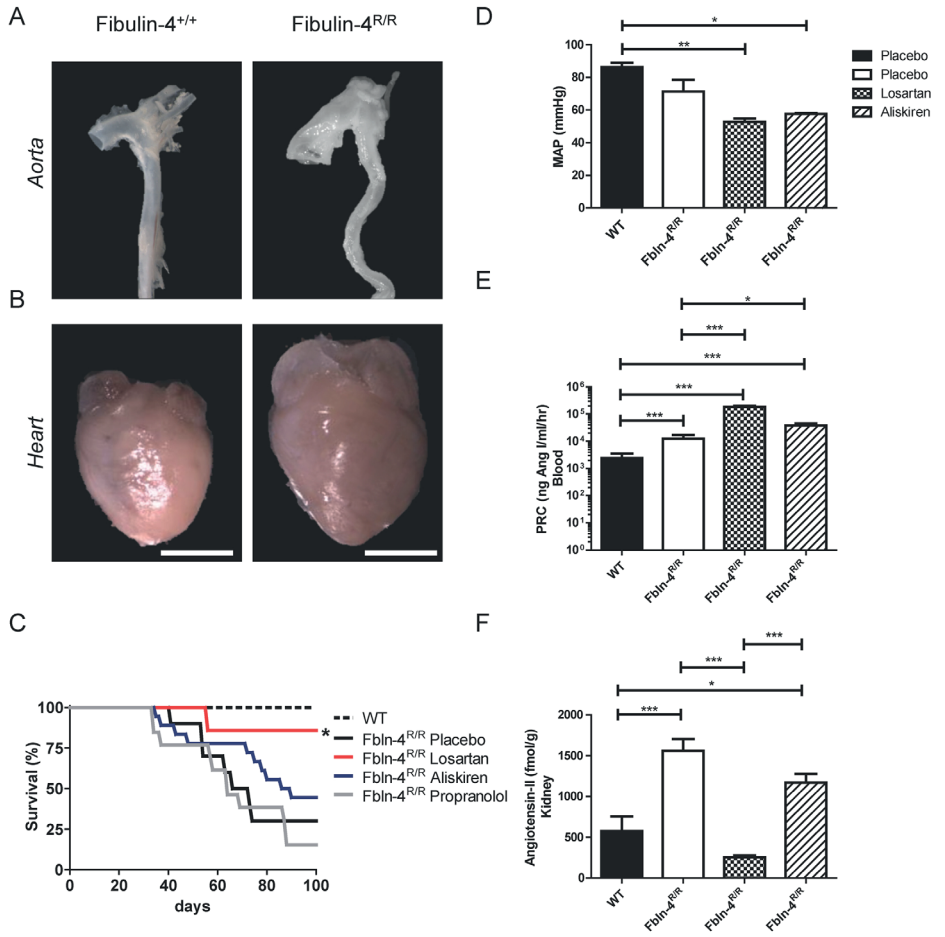

Figure 1. (A-B) Reduced fibulin-4 expression results in thoracic aorta aneurysms and cardiac hypertrophy in 100-day old Fibulin-$4^{R/R}$ mice (white bars represent 4 mm). (C) Kaplan-Meier survival curves of WT and treated Fibulin-$4^{R/R}$ mice (n=7-19). *P<0.05 vs. placebo. (D-F) Mean arterial pressure (MAP; n=3-5), plasma renin concentration (PRC; n=10-18), and renal angiotensin II levels (n=5) in Fibulin-$4^{R/R}$ mice treated for 65 days with placebo, losartan, aliskiren or propranolol vs. untreated age-matched WT mice. Data are mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

In vivo MMP activity, measured by 3D FMT-CT, was undetectable in aortas of wild-type mice, but greatly increased in the aortic arch of placebo- or losartan-treated Fibulin-$4^{R/R}$ mice (Fig. 3A). Abdominal aorta MMP measurements were inaccurate due to the high fluorescent signal from the liver. Removal of the aortas after sacrifice allowed ex-vivo imaging at much great sensitivity (Fig. 3B), and confirmed the in-vivo observations. Losartan did not affect MMP activity as compared to placebo (Fig. 3C). Consequently, MMP activity was not determined in aliskiren-treated mice.

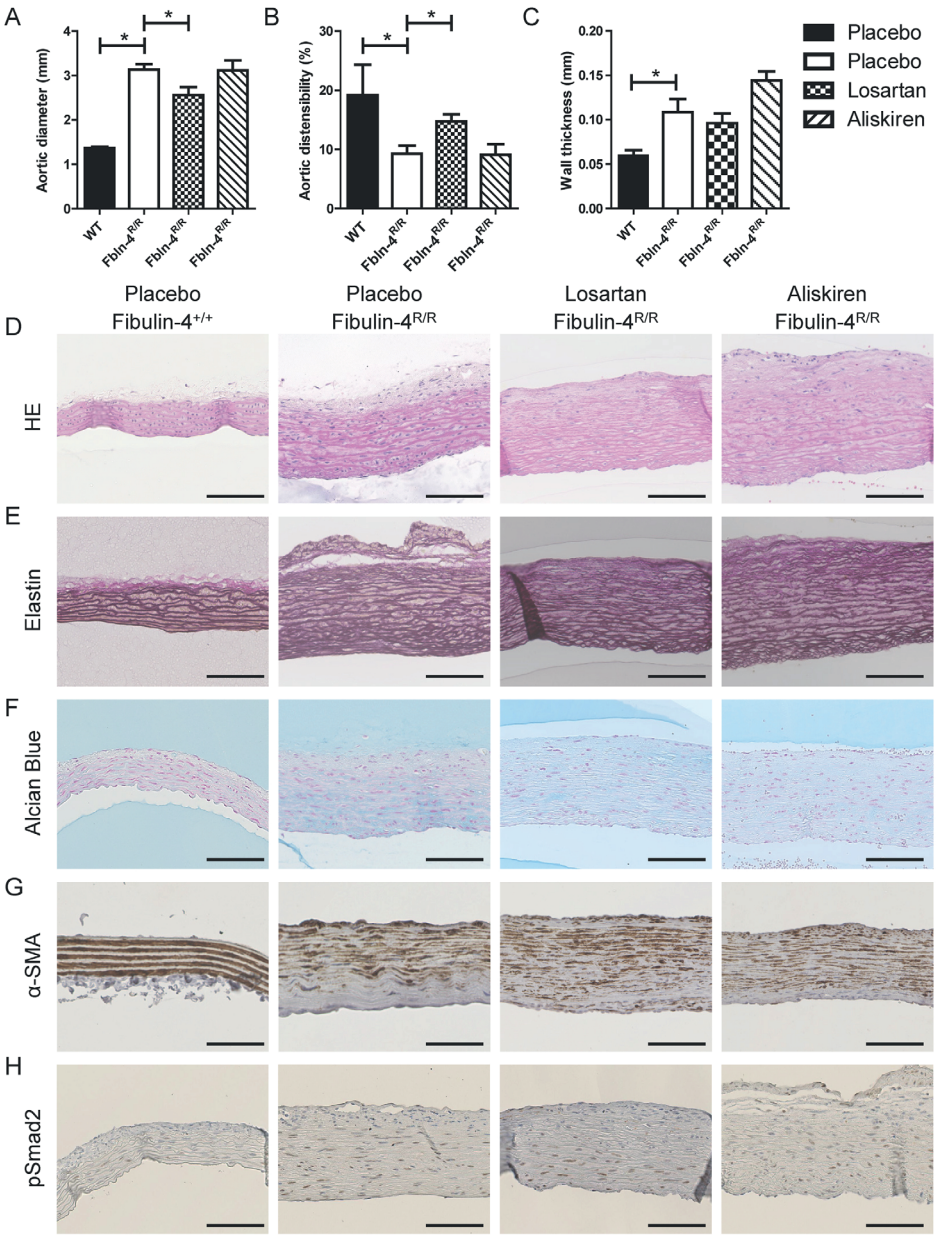

Figure 2. (A-C) Aortic diameter, distensibility and wall diameter in Fibulin-4$^{R/R}$ mice treated for 65 days with placebo, losartan or aliskiren vs. age-matched untreated WT mice (mean±SEM of n=6-10) (black bars represent 100 μm); *P<0.05 vs. placebo. Treatment did not affect aortic wall morphology (D), elastic fiber fragmentation (E), extracellular matrix deposition (Alcian Blue) (F), α-smooth muscle actin (SMA) deposition (G), or pSmad2-signaling (H).

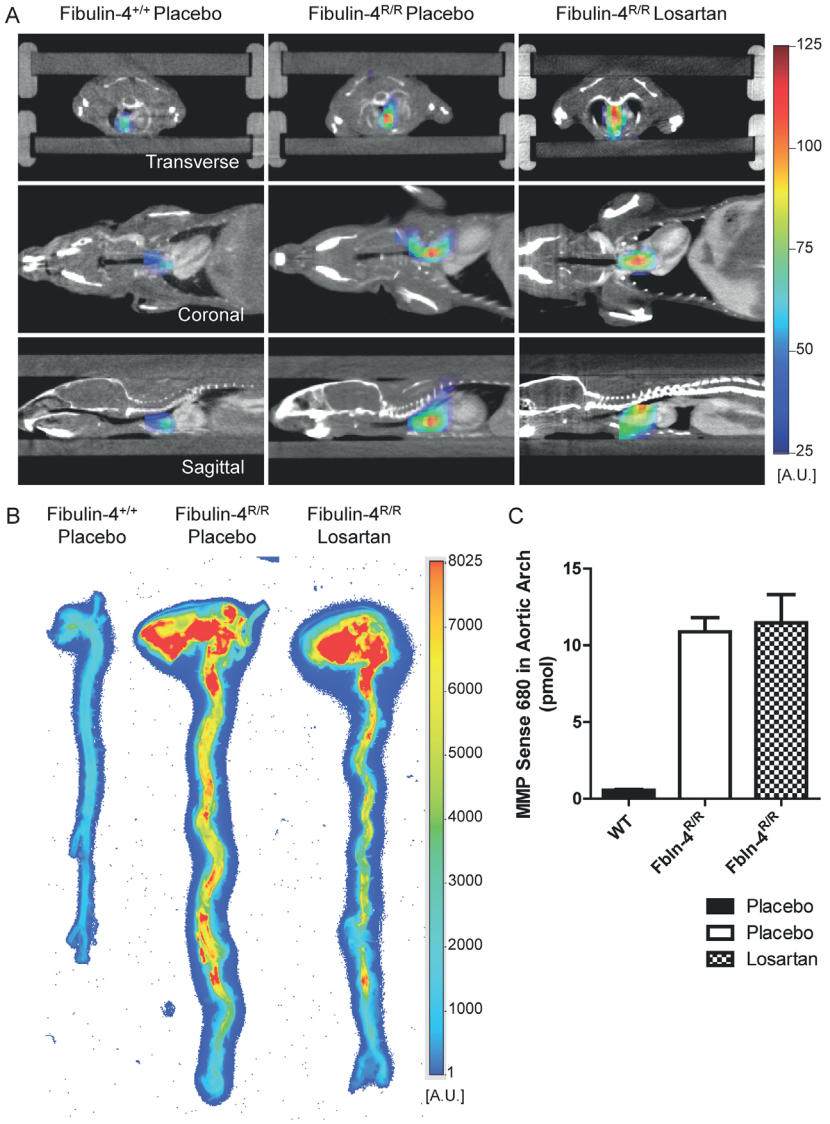

Figure 3. (A) *In-vivo* three-dimensional FMT-CT co-registration of heart and aorta in Fibulin-$4^{R/R}$ mice treated for 65 days with placebo or losartan vs. age-matched untreated WT mice, after injection of MMPSense 680 to determine matrix metalloproteinase (MMP) activity. (B) MMP activity determined *ex vivo* in whole aortas, and (C) its quantification (mean±SEM of n=2).

Losartan improves cardiac morphology and function

Transthoracic echocardiography in placebo-treated Fibulin-$4^{R/R}$ mice revealed a tripling of LV mass and a doubling of LV diameter versus wild-type mice (Fig. 4A-4B) at the age of 100 days. Ejection fraction and fractional shortening were both greatly reduced (Fig.

4C-4D). Losartan improved all parameters, although significance was not reached for LV mass. Aliskiren affected none of these parameters. Data for propranolol in 100-day old mice could not be obtained.

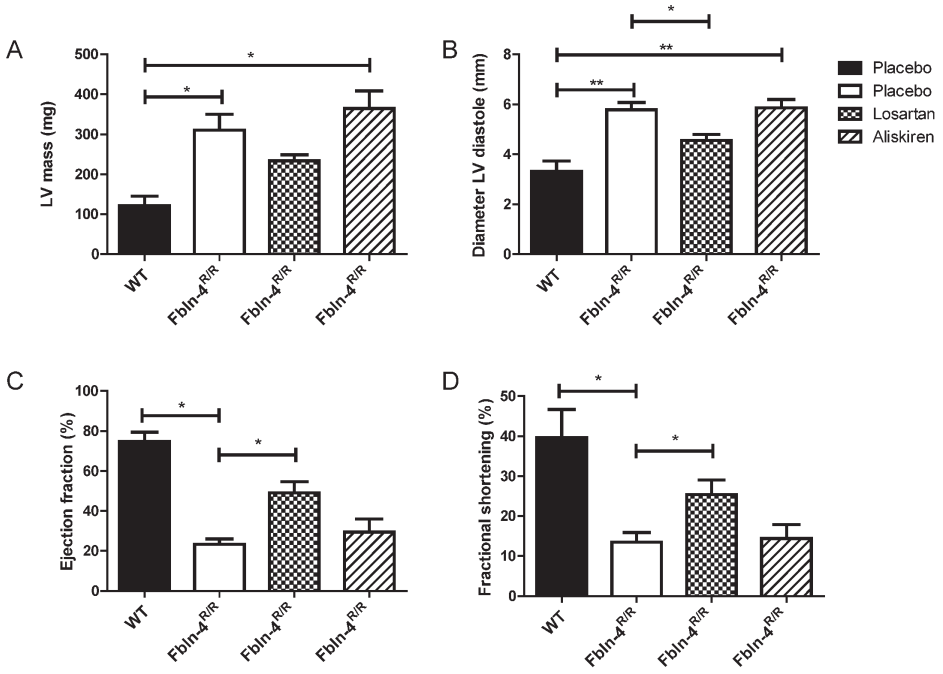

Figure 4. (A-D) Left ventricular (LV) mass, LV diameter, ejection fraction and fractional shortening determined by *in-vivo* transthoracic echocardiography in Fibulin-$4^{R/R}$ mice treated for 65 days with placebo, losartan or aliskiren vs. age-matched untreated WT mice (mean±SEM of n=6-10). *P<0.05, **P<0.01.

Losartan prevents cardiomyocyte hypertrophy and reduces canonical TGFβ signaling

Cardiomyocyte area doubled in Fibulin-$4^{R/R}$ versus wild-type mice, and losartan (but not aliskiren) fully prevented this hypertrophic response (Fig. 5A-5B). As expected, changes in plasma BNP paralleled this pattern, although no significant differences were observed for this parameter (Fig. 5C). Both canonical (pSmad2) and non-canonical (pERK) TGFβ signaling were upregulated in hearts of Fibulin-$4^{R/R}$ mice, but losartan reduced only the former to wild-type levels (Fig. 5D-5E). Smad2 and ERK levels were identical under all conditions (data not shown). LV $AT_{1a}R$ -, $AT_{1b}R$ -, and AT_2R expression were downregulated in Fibulin-$4^{R/R}$ mice versus wild-type mice, and losartan treatment exclusively normalized $AT_{1a}R$ expression (Fig. 5F). Unfortunately, due to scarcity of available tissue, similar data could not be obtained in aliskiren- or propranolol-treated mice.

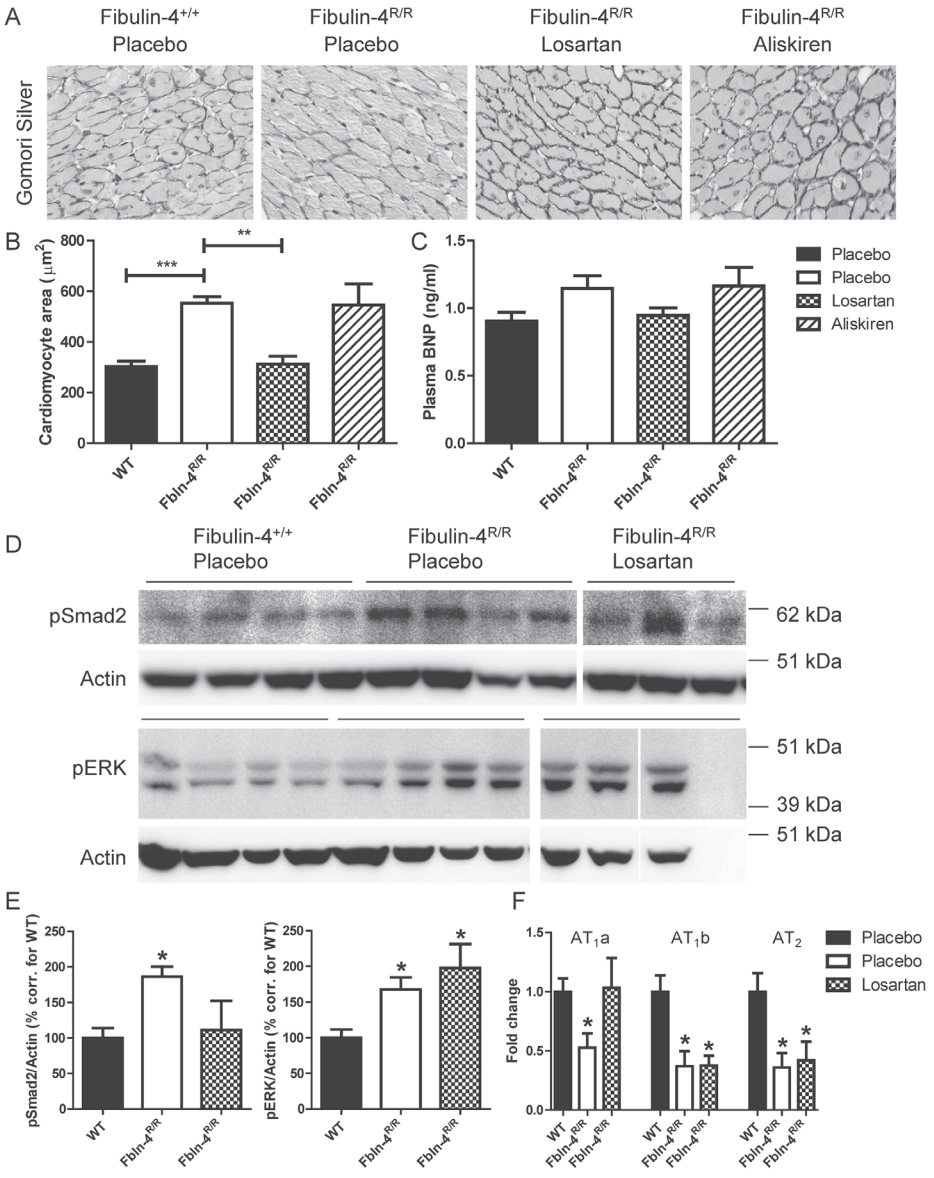

Figure 5. (A-B) Cardiomyocyte area (n=5-12; panel A shows a representative example) and (C) plasma brain natriuretic peptide (BNP; n=10-18) levels in Fibulin-$4^{R/R}$ mice treated for 65 days with placebo, losartan or aliskiren vs. age-matched untreated WT mice. Data are mean±SEM, **P<0.01, ***P<0.001 vs. WT or placebo. (D-E) pSmad2, pERK, and β-actin protein levels in hearts of Fibulin-$4^{R/R}$ mice treated for 65 days with placebo or losartan vs. age-matched untreated WT mice (n=3-4). *P<0.05 vs. WT. (F) Relative gene expression of LV Ang II receptors (n=3-10). *P<0.05 vs. WT.

Losartan prevents LV and aneurysm growth rate

We used a novel microCT method in combination with the vascular contrast agent Exia160, yielding longitudinal 3D data sets in which each animal serves as its own baseline control (Fig. 6A). At the start of treatment, both aortic volume and LV volume were not different in placebo- and losartan-treated Fibulin-$4^{R/R}$ mice (Fig. 6B-6C). Both volumes increased

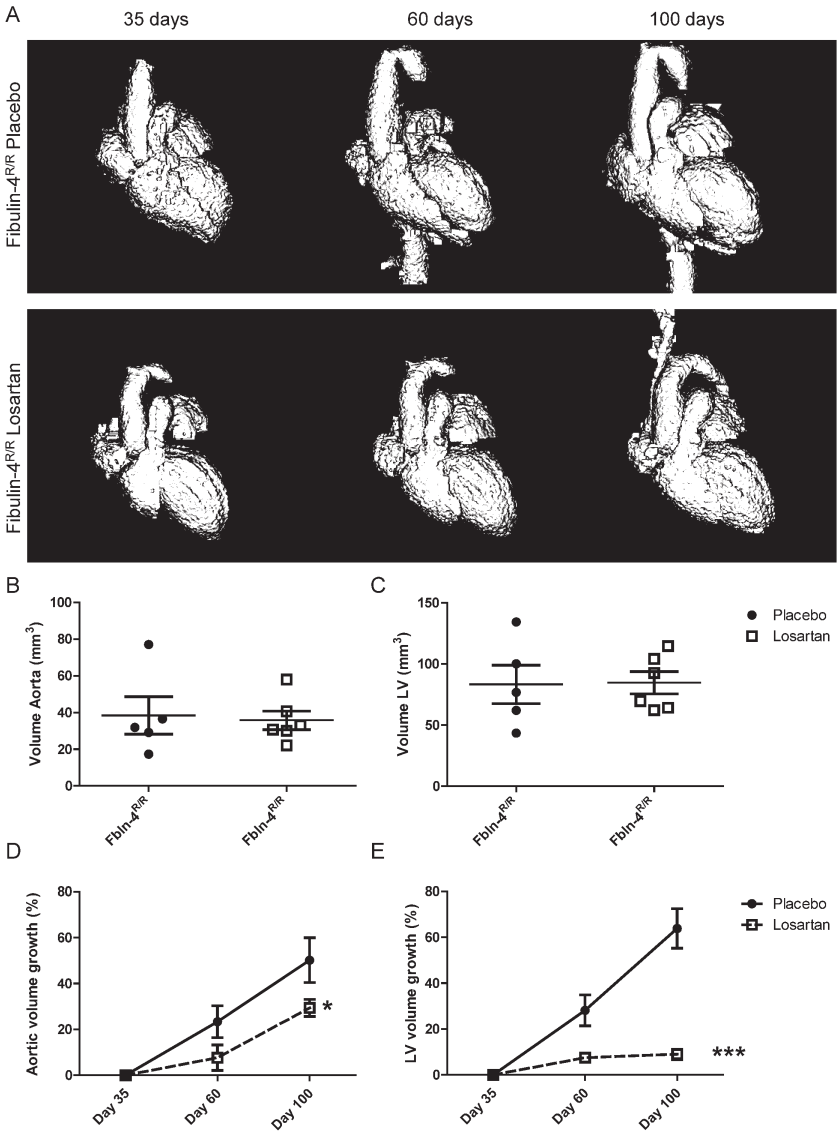

Figure 6. (A) 3D overview of CT-angiography with contrast agent Exia160. (B-C) Aortic and LV volume of placebo and losartan treated Fibulin-$4^{R/R}$ mice at baseline. (D-E) Percentage growth of ascending aortas and left ventricle (LV). Data are mean±SEM of n=4-6. *P<0.05, ***P<0.001 vs. placebo.

by approximately 60% during placebo treatment, and losartan largely (aortic volume), if not completely (LV volume) prevented this (Fig. 6D-6E).

DISCUSSION

The present study shows that losartan, but not aliskiren or propranolol, increased survival in Fibulin-$4^{R/R}$ mice, and that this predominantly related to its capacity to improve cardiac function and structure. Although losartan also stabilized aortic growth, these effects were more modest than its effects on LV growth, and they did not result in any change in aortic wall morphology, TGFβ-signaling, or MMP-activity. Nevertheless, there was an improvement in aortic distensibility. The larger effects on the heart most likely reflect the fact that the heart profits both from the local (cardiac) effects of losartan and its effects on aortic root remodeling. Since none of these effects were seen with aliskiren, despite the fact that this RAS blocker lowered blood pressure and inhibited the RAS to the same degree as losartan, we conclude that they are blood pressure-independent, and that losartan exerts effects beyond blockade of the classical Ang II-AT_1R axis. This most likely concerns its unique capacity to induce AT_2R stimulation. A second possibility would be activation of the angiotensin-(1-7)-Mas receptor axis. However, a study making use of *Fbn1*$^{C1039G/+}$ mice (an alternative, albeit less severe, TAA model) supports the former only, since it observed no effect of an ACE inhibitor, although such a drug, like an AT_1R blocker, activates the angiotensin-(1-7)-Mas receptor axis.[20, 30] Our study is the first to directly compare renin inhibition and AT_1R blockade in a mouse TAA model.

RAS activation, both in the circulation and at the tissue level, is an established characteristic of Fibulin-$4^{R/R}$ mice.[13, 31] Given the low Ang II levels in the aorta and its relatively small size[32], we measured Ang II in renal tissue to confirm the upregulated tissue RAS activity in this model. Increased Ang II levels will facilitate TGFβ-signaling, which is known to be enhanced in patients and mice with MFS.[29, 33-36] In fact, increased serum TGFβ levels correlated directly with aortic root dilation.[33] In agreement with the causative role of Ang II, we showed in an earlier study that prenatal treatment with losartan successfully improved elastic fiber fragmentation and reduced vessel wall thickness in Fibulin-$4^{R/R}$ mice.[13] Moreover, in mice that lack fibulin-4 in VSMCs (Fbln4^{SMKO} mice), aneurysm formation could be prevented completely when RAS blockade was started within a narrow therapeutic window during the first month of life.[31] In this latter study, ACE inhibition with captopril and losartan treatment were equally effective. Yet, in contrast with our study, no cardiac phenotype was reported in Fbln4^{SMKO} mice.

The present study in Fibulin-$4^{R/R}$ mice now evaluated postnatal losartan versus aliskiren treatment, started on day 35, i.e., when aneurysm formation is already present. This is not only more clinically relevant, as treatment in TAA patients usually starts in

the presence of an aneurysm, but also more realistic given the fact that such blockade is contraindicated during pregnancy. Propranolol, a classical MFS drug, was used as a comparator, but exerted no effect, in agreement with its lack of effect at the same dose (50 mg/kg p.o. per day) in *Fbn1*$^{C1039G/+}$ mice.[15] All drugs were given orally, since the fragility of our model, resulting in a very low survival, was not compatible with the operation required to implant osmotic minipumps. Although aliskiren displays a low bioavailability[37], and is highly species-specific[38], it blocks mouse renin at the same concentration range as human renin.[39] Consequently, by applying oral doses that were over 10 times higher than those used in humans (62.5 mg/kg p.o. per day versus 150-300 mg/day in humans), we were able, as in previous studies[21, 22], to achieve a degree of RAS blockade that yielded the same blood pressure-lowering effects as losartan at 60 mg/kg p.o. per day. Importantly, as an indication of RAS blockade, losartan and aliskiren increased circulating renin similarly. Probably as a consequence of this rise in renin release, aliskiren did not significantly decrease renal Ang II. Similar observations were made previously in the rat kidney.[40] Yet, losartan decreased renal Ang II, in agreement with the fact that tissue Ang II largely reflects Ang II that is bound to, or has been internalized via, AT_1R.[41, 42] Therefore, during losartan treatment, the reduction in tissue Ang II is an indication of the degree of AT_1R blockade. Unfortunately, we were unable to obtain comparable data for propranolol-treated mice, since none of these mice survived until the age of 100 days, i.e., the day of sacrifice for our RAS component measurements, at which timepoint blood pressure was measured. Nevertheless, it might be speculated that propranolol, given its modest renin-suppressing effects[43], did reduce Ang II. Long-term treatment with propranolol was feasible in *Fbn1*$^{C1039G/+}$ mice, in which aneurysm formation starts only at the age of 2 months.[3, 44] Propranolol affected blood pressure in *Fbn1*$^{C1039G/+}$ mice to the same extent as losartan.[15] Even if this had also been the case in our model, e.g., based on Ang II reduction, this effect would have resembled that of aliskiren, i.e., it could not have resulted in enhanced AT_2R stimulation. Thus, once TAA are established, both renin suppression with propranolol and renin inhibition with aliskiren lack the beneficial effects of losartan. In contrast, when treatment is started before the onset of TAA, like in the Fbln4^{SMKO} mice model described above[31], captopril yielded the same effects as losartan. Since captopril does not allow AT_2R stimulation, these data suggest that, at a very early stage of TAA, AT_1R are predominant, while at a later stage AT_2R may additionally come into play. This correlates well with the widely accepted phenomenon that AT_2Rs normally display low-to-undetectable levels, which increase only under pathological conditions, e.g., post-myocardial infarction, during hypertension-induced remodeling, and in heart failure.[45-47] Clearly, timing of treatment is of utmost importance, and different ages at the start of treatment (e.g. children/adolescents versus adults) may explain the success (or lack thereof) of different RAS blockers in clinical trials.[17, 18, 48] Moreover, when classifying *FBN1* mutations into 'haploinsufficiency' (decreased amount of normal fibrillin-1), and 'dominant negative' (normal fibrillin-1 abundance with mutant

fibrillin-1 incorporated in the matrix), Franken et al. observed that Marfan patients with haploinsufficient *FBN1* mutations were more responsive to losartan.[49] Since the *Fbn1*$^{C1039G/+}$ and Fibulin-4$^{R/R}$ TAA models closely correspond with the haploinsufficiency situation, it appears that the underlying mutation is an additional determinant of the success of AT_1R blockade in Marfan patients. Taken together, simultaneous AT_2R stimulation may not always offer an additional advantage, and thus selective AT_2R agonists should not by definition be preferred over AT_1R antagonists.

Given the predominant effects of losartan on the heart, we focused on canonical (pSmad2) and non-canonical (pERK) TGFβ signaling in cardiac tissue. Both were upregulated in Fibulin-4$^{R/R}$ mice, comparable to their upregulation in aortic tissue in Fbln4^{SMKO} and *Fbn1*$^{C1039G/+}$ mice.[20, 31] Yet, although losartan suppressed both types of signaling in aortic tissue in these latter models, in the hearts of our mice only the canonical signaling was found to be suppressed after losartan, while no pSmad2 suppression was seen in the aortic wall (Fig. 5E). These findings concur with the heart-specific effect of this AT_1R antagonist in our model, and suggest that the AT_2R stimulatory effects, if occurring, result in reduced canonical TGFβ signaling in the heart. Studies in transgenic animals support the concept that AT_2Rs are antihypertrophic and prevent remodeling.[50, 51] The lack of effect on pERK signaling in our Fibulin-4$^{R/R}$ mice is in agreement with a recent study by Cook et al.[52], who demonstrated that ERK1/2 activation peaks at a very early stage of the disease only, while pSmad2 remains elevated throughout the disease. From this perspective, effects of losartan on pERK1/2 are no longer expected after 100 days, simply because pERK1/2 is not activated anymore at that stage.

Gene expression studies in LV tissue revealed a reduction of all Ang II receptor types in Fibulin-4$^{R/R}$ mice compared to wild type mice. It should be noted that mice, unlike humans, display two AT_1R subtypes, $AT_{1a}R$ and $AT_{1b}R$, and that losartan blocks both AT_1Rs equally well. AT_1R downregulation is also known to occur in heart failure patients.[53] It was not observed in the aortic arch or kidney of our Fibulin-4$^{R/R}$ mice[13], implying that its downregulation was cardiac-specific. Importantly, although the raw Ct values for the $AT_{1b}R$, the AT_2R and the housekeeping genes β-actin and β_2-microglobin were identical in LV tissue and aorta (B.S. van Thiel, data not shown), the raw Ct values for the $AT_{1a}R$ in the LV were approximately 6 cycles lower than in the aorta. This suggests that $AT_{1a}R$ expression in the heart greatly exceeds that in the aorta. Losartan treatment exclusively normalized cardiac $AT_{1a}R$ expression in Fibulin-4$^{R/R}$ mice. Such upregulation is a well-known physiological response to receptor antagonism, once again supporting effective $AT_{1a}R$ blockade by losartan in the heart. Yet, it does not imply that $AT_{1a}R$ activation had now normalized (due to the simultaneous presence of losartan), and thus predominant AT_2R stimulation by the elevated levels of Ang II during losartan treatment is still highly likely.

Our data are the first to show the losartan-induced stabilization of LV growth over time with longitudinal microCT measurements. Using each animal as its own baseline

control, this novel approach enabled us to conclude that the effects of losartan on LV growth exceeded those on aortic growth. Combined with the FMT to co-registrate MMP-activity, this approach allows monitoring of cardiac and aortic remodeling in a unique, non-invasive manner. It would also reduce the required number of animals. Given the major limitation of our animal model, i.e. a complicated breeding scheme and a high death rate resulting in low n-numbers, this is an important advantage.

In conclusion, losartan, but not aliskiren or propranolol, improved survival in Fibulin-$4^{R/R}$ mice, by simultaneously stabilizing aortic growth, reducing aortic distensibility, and improving cardiac function and structure. The absence of these effects during aliskiren treatment, despite a similar reduction in blood pressure and degree of RAS blockade, suggests that it might be due to AT_2R stimulation and/or activation of the angiotensin-(1-7)/Mas receptor axis. Future studies, making use of AT_2R/Mas receptor knockout animals, AT_2R/Mas receptor antagonists (e.g., PD123319 and A779, respectively) or AT_2R/Mas receptor agonists (e.g., C21 and AVE0991, respectively) may help to substantiate this view. However, given the non-specific effects of the latter types of drugs[54, 55], the possibility that AT_2R heterodimerize with Mas receptors[56], and the consequences of AT_2R deletion on cardiac development and remodeling[57], the results of such studies may not be straightforward. In addition, none of these approaches is currently feasible in humans.

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