CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Inhibition of Marfan Syndrome Aortic Root Dilation by Losartan

Role of Angiotensin II Receptor Type 1—Independent Activation of Endothelial Function

Stephanie L. Sellers,^{*¶} Nadia Milad,^{*¶} Rayleigh Chan,^{*¶} Michael Mielnik,^{*¶} Una Jermilova,^{*¶} Paul L. Huang,[‡] Rini de Crom,[§] Jeremy A. Hirota,^{¶†} James C. Hogg,^{¶††} George G. Sandor,^{||} Casey Van Breemen,^{||} Mitra Esfandiari,^{**} Michael A. Seidman,^{¶††} and Pascal Bernatchez^{*¶}

From the Department of Anesthesiology, Pharmacology and Therapeutics,^{*} the UBC Centre for Heart Lung Innovation and St. Paul's Hospital,[¶] the Division of Respiratory Medicine, Department of Medicine, Chan-Yeung Centre for Occupational and Environmental Respiratory Disease,[‡] the Department of Pathology and Laboratory Medicine,^{††} Providence Health Care, and the Child and Family Research Institute,^{||} University of British Columbia (UBC), Vancouver, British Columbia, Canada; the Cardiovascular Research Centre,[‡] Massachusetts General Hospital, Harvard University, Charlestown, Massachusetts; the Department of Cell Biology and Genetics,[§] Erasmus University Medical Center, Rotterdam, the Netherlands; and the Department of Biomedical Sciences,^{**} Midwestern University, Glendale, Arizona

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Address correspondence to
Pascal Bernatchez, Ph.D.,
Department of Anesthesiology,
Pharmacology and Therapeutics,
University of British
Columbia, Centre for Heart and
Lung Innovation, St. Paul's
Hospital, 1081 Burrard St.,
Room 166, Vancouver, BC,
Canada V6Z 1Y6. E-mail:
pascal.bernatchez@ubc.ca.

Marfan syndrome (MFS) is a genetic disorder that frequently leads to aortic root dissection and aneurysm. Despite promising preclinical and pilot clinical data, a recent large-scale study using anti-hypertensive angiotensin II (AngII) receptor type 1 (ATR1) blocker losartan has failed to meet expectations at preventing MFS-associated aortic root dilation, casting doubts about optimal therapy. To study the deleterious role of normal ATR1 signaling in aortic root widening, we generated MFS mice lacking ATR1a expression in an attempt to preserve protective ATR2 signaling. Despite being hypotensive and resistant to AngII vasopressor effects, MFS/ATR1a-null mice showed unabated aortic root enlargement and remained fully responsive to losartan, confirming that blood pressure lowering is of minor therapeutic value in MFS and that losartan's antiremodeling properties may be ATR1 independent. Having shown that MFS causes endothelial dysfunction and that losartan can activate endothelial function in mice and patients, we found that nitric oxide synthase (NOS) inhibition renders losartan therapeutically inactive, whereas multiple transgenic and pharmacologic models of endothelial NOS activation block aortic root dilation by correcting extracellular signal-regulated kinase signaling. *In vitro*, losartan can increase endothelial NO release in the absence of AngII and correct MFS NO levels *in vivo*. Our data suggest that increased protective endothelial function, rather than ATR1 inhibition or blood pressure lowering, might be of therapeutic significance in preventing aortic root disease in MFS. (*Am J Pathol* 2018, 188: 574–585; <https://doi.org/10.1016/j.ajpath.2017.11.006>)

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Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder caused by mutations in the fibrillin-1 (*FBN1*) gene, with an estimated prevalence of 1:5000.¹ *FBN1* plays a key role in formation and stabilization of elastic fibers that provide aortic recoil during systole¹ and in regulating quiescence of transforming growth factor- β (TGF- β), a pluripotent cytokine believed to be a causative factor in the pathogenesis of MFS.² Of the diverse manifestations of MFS, progressive aortic root dilation is the most serious because it is associated with increasing risk of acute aortic dissection^{3,4} and death.^{5–8} Hence, pharmacotherapy aimed at slowing the rate of dilation is key to patient management. The antihypertensive medication losartan, an angiotensin II (AngII) receptor type 1 (ATR1) blocker,⁹ is recommended by current treatment guidelines because of unique antiremodeling properties⁷ that are not observed with either angiotensin-converting enzyme inhibitors or β blockers.^{9,10} Losartan was shown to decrease the rate of aortic root dilation in rodent models of MFS and in pediatric patients refractory to β -blocker treatment.^{7,11} Yet, a large multicenter trial failed to show the expected superiority of losartan treatment over atenolol,¹² which further stirred debate about optimal MFS patient management.

Mechanistic and pharmacologic studies have suggested that losartan decreases aortic root dilation by blocking deleterious AngII-ATR1 signaling while preserving protective AngII-ATR2 interactions, something not achieved by angiotensin-converting enzyme inhibitors because of their inhibitory effect on AngII synthesis.⁷ Both ATR1 blockers and angiotensin-converting enzyme inhibitors can attenuate canonical ATR1-driven TGF- β signaling, which is Smad dependent. However, AngII-ATR2 signaling preserved by losartan results in lower noncanonical TGF- β activation of the extracellular signal-regulated kinase pathway and protection against aberrant growth and rupture of the aorta.¹³ Whether this unique property of losartan can rationalize its unexpected therapeutic properties in non-AngII-driven lung disease¹⁴ remains to be determined.

To better understand losartan's complex antiremodeling properties, MFS mice were generated with blunted cardiac and aortic root AngII/ATR1 signaling on the basis of the premise that the resultant animals would not only be protected against MFS-associated cardiomyopathy.¹⁵ They would also be protected against aortic root dilation through lower pathological ATR1 signaling and preserved ATR2-dependent protection of the aortic wall. To our surprise, despite conferring protection against MFS cardiomyopathy,¹⁵ loss of ATR1a did not confer any protection against the aortic manifestations of MFS, despite causing hypotension, blunted AngII pressor responses, and decreased heart rate (HR). Moreover, these animals were neither resistant nor hypersensitive to losartan, suggesting that its mechanism of action is more complex than a simple ATR2-protective¹³ switch in AngII signaling and may include ATR1-independent effects. Instead, inhibition of nitric oxide synthase (NOS) activity renders losartan

inactive, whereas multiple transgenic and pharmacologic approaches that activate the endothelial NOS-derived NO pathway can attenuate MFS aortic root remodeling *in vivo*. Hence, we suggest that losartan can decrease the rate of aortic root remodeling via endothelial function activation, possibly in an off-target manner, and that reversing MFS-associated endothelial dysfunction may be of therapeutic value in MFS.

Materials and Methods

Animals

Marfan mice (fibrillin C1039G^{+/-}) were originally supplied from the laboratory of Dr. Harry Dietz (Johns Hopkins School of Medicine, Baltimore, MS) and maintained in the Genetically Engineered Models facility at the Center for Heart Lung Innovation. ATR1a-knockout mice (ATR1a^{-/-}; stock number 002682) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Genetically Engineered Models facility at the Center for Heart Lung Innovation. Marfan mice lacking ATR1 expression were generated by crossing of MFS and ATR1a^{-/-} mice to give MFS ATR1^{+/-} mice, which were subsequently crossed together to generate MFS ATR1a^{-/-} mice and littermate controls. MFS phosphorylated mimetic S1176D knock-in mice and phosphorylated inactive S1176A knock-in mice were generated in a similar manner, with knock-in strains originally supplied by Dr. Paul Huang (Harvard Medical School, Boston, MA).¹⁶ MFS containing endothelial NOS (eNOS) transgenes and controls were generated using littermates from crosses of MFS with eNOS hemizygous transgenic animals supplied by R.d.C.¹⁷ Animals were randomly assigned to groups on weaning by a technician blind to genotype; group sizes were based on estimates derived from previously noted phenotypes of the C1039G model.⁷ Animals were excluded on weaning in the rare event of abnormality (eg, malocclusion) as predetermined under the University of British Columbia Animal Care Guidelines. All animals were housed in the Genetically Engineered Models facility at the Center for Heart Lung Innovation on a standard 12-hour light/dark cycle, fed standard laboratory chow, and maintained using breeding and procedures approved by the University of British Columbia Animal Care Committee.

Losartan, L-NAME, and CavNOxin Treatment

Losartan treatment was provided via drinking water (0.6 g/L) starting at 6 weeks of age (MFS, $n = 11$; MFS/ATR1a^{-/-}, $n = 5$). L-NG-Nitroarginine methyl ester (L-NAME; Sigma, St. Louis, MO; 0.5 mg/mL) was given alone (control) or in combination with losartan in drinking water starting at 6 weeks of age and was also administered via i.p. injection (10 mg/kg) biweekly for the first 2 weeks (MFS, $n = 5$ per group).

CavNOxin [MFS, $n = 7$; wild type (WT), $n = 6$] was given via i.p. injections, as previously described.^{18,19}

ATR1a Expression and AngII Blood Pressure Response

Disruption of the *ATR1a* gene was confirmed in *ATR1a*^{-/-} mice via PCR (primer sequences: 5'-TGAGAACACCAATATCACTG-3', 5'-TTCGTAGACAGGCTTGAG-3', and 5'-CCTTCTATCGCCTTCTTGACG-3'; obtained from the Jackson Laboratory). Loss of functional *ATR1a* response to AngII was confirmed by blood pressure response to acute infusion of AngII. Unsedated baseline systolic and diastolic blood pressure was averaged over a 5-day period using the Kent Scientific Corp. CODA2 (Torrington, CT) in *ATR1a*^{+/+} ($n = 8$), MFS *ATR1a*^{-/-} ($n = 7$), MFS ($n = 5$), and *ATR1a*^{-/-} ($n = 5$) mice. Mice were injected i.p. with AngII (0.2 mg/kg; Sigma), and changes in blood pressure were measured over a 10-minute period to determine peak response to AngII. Changes in blood pressure are expressed as change in systolic and diastolic pressure from baseline at peak response to AngII. Peak blood pressure values were averaged for each mouse over three consecutive measurement cycles.

Histology

Histology was performed on hearts and lungs fixed in 10% buffered formalin. Hearts were bisected parallel to the atrio-ventricular plane and embedded in paraffin. Sections were analyzed every 50 μ m throughout the aortic root. Elastic fibers were assessed at the sinus of Valsalva on slides stained with Movat's pentachrome by a blinded cardiac pathologist. Elastic fiber fragmentation was scored on a scale of 0 to 3, wherein 0 indicates no fragmentation; 1, mild fragmentation (0% to 20%); 2, moderate fragmentation (20% to 40%); and 3, extensive fragmentation (>40%). Observable webbing, defined as interlinking of elastic fibers between concentric layers, was also scored on a scale of 0 to 3 using the same parameters as fragmentation. Histology of the left lung was performed, as previously described, via inflation and paraffin embedding, and air space enlargement was analyzed by mean linear intercept.²⁰ Processing, staining, and quantification of histology were performed by technicians blinded to genotype and treatment group. Staining of biotin-conjugated AngII used AngII with avidin-biotin horseradish peroxidase detection and was performed on frozen sections (30 μ m thick) of aortic root and

descending aorta in the presence and absence of losartan. Extracellular signal-regulated kinase phosphorylation assays were performed using rabbit polyclonal phosphorylated extracellular signal-regulated kinase 1/2 (202/204) antibodies (Cell Signaling, Danvers, MA) on paraffin-embedded sections exposed to citrate antigen retrieval solutions.

Echocardiograms

Echocardiograms were performed on mice anesthetized with isoflurane using a VisualSonics (Toronto, ON, Canada) Vevo 2100 system with an MS-550D 40-MHz probe by a technician blinded to genotype and treatment group. Aortic root measurements were averaged from multiple measurements taken in both M and B mode at the level of the sinus of Valsalva.²¹ Data for the MFS *ATR1a*^{-/-} strain and controls were analyzed at 3 (WT, $n = 11$; MFS, $n = 9$; MFS/*ATR1a*^{-/-}, $n = 6$) 6 (WT, $n = 6$; MFS, $n = 7$; MFS/*ATR1a*^{-/-}, $n = 6$), and 9 (WT, $n = 6$; MFS, $n = 8$; MFS/*ATR1a*^{-/-}, $n = 6$) months of age. Echocardiograms for the MFS S1176D and MFS eNOS transgene-positive strains and controls were analyzed at 3 months of age (WT, $n = 11$; MFS, $n = 15$; MFS S1176D, $n = 13$; S1176D, $n = 9$; and WT, $n = 7$; MFS, $n = 10$; MFS eNOS transgene positive, $n = 7$; eNOS transgene positive, $n = 7$, respectively) and at 6 months in the S1176A strain (WT, $n = 9$; MFS, $n = 10$; MFS S1176A, $n = 5$).

Cell Culture, TGF- β , Nitric Oxide Levels, and Western Blot Analysis

Nitric oxide levels from cell supernatants were quantified by measuring soluble nitrite levels, a stable breakdown product of NO, in the supernatant of cultured bovine aortic endothelial cells (ECs) using a GE/Sievers (Boulder, CO) Nitric Oxide analyzer. Bovine aortic ECs were isolated from freshly harvested aortas, cultured in Dulbecco's modified Eagle's medium (Life Technologies, Burlington, ON, Canada) containing 5% fetal bovine serum (Hyclone Laboratories, Logan, UT) and antibiotics. Bovine aortic ECs were characterized by their cobblestone monolayer morphology, factor VIII immunohistochemistry, and by diiodo-indocarbocyanide acetylated low-density lipoprotein uptake, as described previously. ECs were grown to confluence, starved, and treated as previously described²²; absence of *Mycoplasma* was

Figure 1 Blood pressure reduction and loss of *ATR1a* expression does not prevent aortic root dilation or progression in Marfan syndrome (MFS). **A:** Mean arterial pressure was evaluated in wild-type (WT) and MFS mice with *ATR1a* and without *ATR1a* (*ATR1a*^{-/-}) expression, as determined by repeated measurements. **B:** Lack of systolic or diastolic blood pressure (BP) responses to angiotensin II (AngII) injection is seen in *ATR1a*^{-/-} deficient groups, with data presented as differential between pre- and post-AngII values. **C and D:** Echocardiograms and average aortic root diameters (double-headed arrows) of WT, MFS, and MFS *ATR1a*^{-/-} mice at 3 and 6 months, as performed by a blinded technician. Data represent average aortic root diameter, wherein diameter was determined by averaging multiple measurements taken in both M and B modes. **E–H:** Histology of aortic root sections at the level of the sinus of Valsalva. Sections were stained with Movat's pentachrome at 6 and 22 months of age (**E**) to determine elastic fiber fragmentation (**F**), elastic fiber webbing (**G**), and aortic wall thickness (**H**). Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 7$ WT (**A** and **B**) and WT at 6 months (**E–H**); $n = 6$ MFS *ATR1a*^{-/-} and MFS (**A** and **B**), MFS/*ATR1a*^{-/-} (**C** and **D**), and MFS *ATR1a*^{-/-} at 22 months (**E–H**); $n = 11$ WT (**C** and **D**); $n = 9$ MFS (**C** and **D**); $n = 10$ MFS at 6 months (**E–H**); $n = 5$ MFS/*ATR1a*^{-/-} at 6 months and WT and MFS at 22 months (**E–H**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (analysis of variance and *t*-test, with Bonferroni's correction). Scale bar = 100 μ m (**E**).

A

B

C

D

E

F

confirmed. Confluent bovine aortic ECs were starved in fetal bovine serum-free Dulbecco's modified Eagle's medium for 24 hours and treated with losartan (10^{-8} , 10^{-7} , or 10^{-6} mol/L, as indicated). For studies with vascular endothelial growth factor receptor-2 (VEGFR2) knockdown, ECs were treated with VEGFR2 siRNA (Qiagen, Hilden, Germany; target sequence, CAGAAGTGATTGGAAATGATA). Knockdown was confirmed via Western blot analysis (VEGFR2/Flk-1; 1:1000; Santa Cruz Biotechnology, Dallas, TX) and quantified relative to heat shock protein 90 loading control (1:1000; BD Biosciences, San Jose, CA). Total nitric oxide levels from plasma were assayed using the Enzo Griess reaction kit (Ann Harbor, MI; ADI917-020), as previously described,²² using plasma collected from mice fasted for 12 hours at 6 months of age (WT, $n = 5$; MFS, $n = 9$; MFS + losartan, $n = 5$). Total plasma NO levels were correlated to aortic root diameters at 6 months of age in WT and MFS mice.

Statistical Analysis

Animal experiments were designed to have, with an α level of 0.05, 90% power to detect a 20% reduction in aortic root dilation as the wanted end point, although the University of British Columbia's reduction of animal use policy encourages early termination of experiments if statistical significance is reached. Groups were compared via a one-way analysis of variance, followed by *t*-test, whereas multiple comparisons were tested with Bonferroni's correction based on normal distributions, with equal variance confirmed by Bartlett's test (significance level of 0.05).

Results

The contribution of ATR1a to MFS aortic root pathology was evaluated by generating MFS mice (C1039G^{+/-} MFS) lacking the AngII/ATR1a axis (ATR1a-null or ATR1a^{-/-} mice), which has been shown to be the lead regulator of endogenous AngII resting mean arterial pressures and heart rate²³ and the pathogenic AngII receptor in a cardiac-centric model of MFS.¹⁵ Both WT and MFS mice lacking ATR1a showed lower basal mean arterial pressure (Figure 1A)²⁴ and a similar decrease in resting heart rate (28 and 30 minutes⁻¹, respectively; data not shown) compared with their ATR1a-expressing controls, as previously demonstrated.²³ These mice also lacked pressure responses after acute AngII infusion (0.2 mg/kg) (Figure 1B). Although we hypothesized that loss of ATR1a would protect mice against

MFS aortic root dilation as a result of blunted deleterious ATR1a signaling and intact protective ATR2 signaling,^{13,15} we observed comparable aortic root widening in MFS and MFS/ATR1a^{-/-} mice (1.84 ± 0.04 mm versus 1.88 ± 0.03 mm, respectively) compared with 1.45 ± 0.04 mm in WT (Figure 1, C and D) or ATR1a^{-/-} (Supplemental Figures S1 and S2). Similar aneurysm progression was seen in MFS and MFS/ATR1a^{-/-} mice at 6 and 9 months (Figure 1D and Supplemental Figure S1). Histological analyses revealed similar increases in elastic fiber fragmentation at 6 months of age in MFS and MFS/ATR1a^{-/-} mice (Figure 1, E–G). These two groups also demonstrated a trend of increased webbing of elastic fibers (Figure 1, E–G) and a significant increase in aortic wall thickness (Figure 1E), a pattern of pathology also seen at later time points (Figure 1H). Collectively, this demonstrates that on both a histological and whole organ level, MFS mice do not benefit from deletion of ATR1a, which challenges our initial hypothesis of protection against the aortic manifestations of MFS in ATR1a-null mice. In contrast to MFS-associated cardiomyopathy,¹⁵ hypotension, lower HR, and blunted ATR1a signaling, with the expected shift toward protective ATR2 signaling,¹³ do not prevent MFS-associated aortic root pathology.

To rationalize the unexpected observation of persistent aortic disease in MFS mice lacking ATR1a, their response to losartan was studied. A similar reduction in aortic root diameter was observed in both MFS and MFS/ATR1a^{-/-} mice (Figure 2A), and prevention of elastic fiber fragmentation (Supplemental Figure S3) was observed with losartan in drinking water (0.6 g/L). Considering the dose of losartan used is submaximal,²⁵ loss of ATR1a does not increase sensitivity to losartan, which would be expected in a shift toward protective ATR2 signaling in animals lacking ATR1a. Conversely, this may also indicate that the effect of losartan on MFS aortic root dilation may be ATR1a independent or that ATR1a^{-/-} tissues show ATR1b compensation, an unlikely possibility based on past reports.²⁶ With the knowledge that ATR antibodies are notorious for their established non-specificity,^{27,28} the lack of ATR1b compensation in ATR1a tissues was confirmed by comparing the losartan-sensitive binding of biotin-AngII on frozen aortic root sections from animals with normal or lacking ATR1a gene. In MFS myocardium (positive control for high ATR1ab and ATR2 expression), robust biotin-AngII binding was detected (Figure 2B), whereas pretreatment of sections with losartan (which does not block ATR2)

Figure 2 Loss of ATR1a expression does not prevent systemic manifestations of Marfan syndrome (MFS) or losartan activity. **A:** MFS and MFS/ATR1a^{-/-} groups were treated with losartan. Echocardiogram and averaged quantification of aortic root diameter (**double-headed arrows**) show similar reduction in both MFS and MFS/ATR1a^{-/-} mice treated with losartan at 3 months of age. **B:** After convincing control experiments using myocardium tissues, biotin-conjugated angiotensin II (AngII) is bound to wild-type (WT) or ATR1a-null aortic root with (+losartan) and without losartan. The differential signal is interpreted as the total level of ATR1 (in WT tissues) or ATR1b (in ATR1a^{-/-} tissues) present. **C:** Lateral radiographs to assess kyphosis (**white arrows**) in C1039^{+/-} and MFS/ATR1a^{-/-} groups relative to control (WT) in representative images. **D–F:** Histology of inflated lungs relative to control (Mason's trichrome stain) at 6 and 22 months of age, as quantified by mean linear intercept. $n = 9$ MFS (**A**); $n = 11$ MFS + losartan (**A**) and MFS at 6 months (**D–F**); $n = 5$ MFS ATR1a^{-/-} and MFS ATR1a^{-/-} + losartan (**A**) and WT and MFS ATR1a^{-/-} at 6 months and WT and MFS at 22 months (**D–F**); $n = 6$ MFS ATR1a^{-/-} at 22 months (**D–F**). * $P < 0.05$, ** $P < 0.01$ (analysis of variance and *t*-test). Scale bars = 100 μ m (**D**). L, lumen.

decreased biotin-AngII binding (Figure 2B). This indicated high losartan-sensitive ATR1 expression (a and/or b isoforms) and significant ATR2 expression (resistant to losartan). However, use of MFS/ATR1a^{-/-} myocardium decreased biotin-AngII binding compared with MFS myocardium (Figure 2B) and completely blunted losartan's effect, indicating lack of ATR1b compensation in ATR1a^{-/-} myocardium, with normal ATR2 expression (myocardium controls without biotin-AngII show no staining; data not shown). In MFS aortic root tissues, biotin-AngII binding was detected (Figure 2B), albeit to lower levels than in myocardium; however, losartan preincubation almost completely blocked biotin-AngII detection, indicating significant ATR1 expression in MFS aortic root with low levels of ATR2. Most important, use of MFS/ATR1a^{-/-} aortic root sections showed marginal biotin-AngII binding and rendered losartan inactive, indicating lack of both ATR1a expression and ATR1b compensation in MFS aortic root with an inactivated *ATR1* gene. This supports the concept of off-target effects by losartan because *ATR1a* gene knockout blunts all ATR1 expression in the aortic root. Moreover, the low aortic root expression of ATR2, indicated by the marginal biotin-AngII detection in MFS aortic root preincubated with losartan, suggests that the protective shift toward ATR2 signaling theory in MFS animals treated with losartan might be of less importance than expected.¹³

Nonaortic, non-ATR1-driven MFS pathology was also investigated, and it was found that kyphosis was easily appreciated in both MFS mice and MFS/ATR1a^{-/-} mice (Figure 2C), as expected. Moreover, airspace enlargement was notable at 6 months (Figure 2D), with progression seen at 22 months of age (Figure 2, E and F, and Supplemental Figure S3) in both MFS mice and MFS/ATR1a^{-/-} mice compared with wild-type controls, with similar airspace widening by histology (Figure 2, D–F). Finally, evaluation of ATR1a^{-/-} mice showed no confounding phenotype (Supplemental Figure S2). Therefore, these findings suggest that skeletal and pulmonary pathologies of MFS in the C1039G model are independent of ATR1a, as suggested by others.⁷

Our group and others have reported abnormal endothelial function in murine MFS models and patients,^{29,30} which may lead to abnormal cardiac and vascular remodeling.^{19,31} Most important, we have described how losartan can normalize vascular function, including acetylcholine-induced vasorelaxation EC₅₀,³² in early MFS aortic disease. Losartan can increase MFS patient flow-mediated dilation,³³ a marker of endothelial function and heightened NO bioavailability, two biological properties not shared by atenolol.³⁴ Hence, we investigated whether losartan's effect on MFS aortic root disease may be NO dependent. Treatment of MFS mice with NOS inhibitor L-NAME rendered losartan therapeutically inactive (Figure 3, A and B), confirming that losartan has endothelial function-dependent effects, as suggested by others.³⁵ *In vivo*, MFS mice showed decreased plasma NO levels relative to control, which were corrected with losartan treatment (Figure 3C). Furthermore,

increasing aortic root diameter was found to correlate with reduced plasma NO ($R^2 = 0.8$) (Figure 3D). *In vitro*, cultured aortic ECs were challenged with losartan under acute and chronic conditions in absence of exogenous AngII, which should render an AngII receptor antagonist inactive and abrogate the possibility of an ATR2-dependent protective shift in signaling activities. However, losartan triggered a dose- and time-dependent increase in NO release (Figure 4, A and B). A report has shown how losartan can directly activate the VEGFR2-eNOS pathway in an AngII/ATR1-independent manner,³⁵ which may activate eNOS-dependent NO release and inhibit pathological TGF- β signaling.³⁶ VEGFR2 knockdown blocked such effect (Figure 4, C and D), confirming the dependence on a VEGFR2-dependent complex in losartan's NO-releasing activities, as suggested.³⁵ ATR2 blocker PD 123319 had no effect, as expected (data not shown). This suggests that losartan can activate eNOS and NO release in an ATR1-independent manner, as shown by others.³⁵

The protective role of eNOS was confirmed by breeding MFS mice to a strain expressing a form of endogenous eNOS with the phosphorylated mimetic S1176D mutation.³⁷ This caused a 46% decrease in aortic root enlargement compared with MFS mice (Figure 5A) and elastic fiber fragmentation similar to losartan treatment and corrected noncanonical TGF- β signaling (extracellular signal-regulated kinase phosphorylation),⁹ with near-complete inhibition of ascending aorta enlargement, another phenotype of the MFS mouse used (Figure 5B and Supplemental Figure S4). In contrast, substitution with phosphorylated inactive S1176A eNOS caused a nonsignificant increase in MFS aortic root diameter (Figure 5C). Finally, the hemizygous eNOS overexpression model¹⁷ (Figure 5D) significantly reduced (by 58%) MFS aortic root widening, further supporting the capacity of eNOS to block aortic root remodeling. None of the eNOS transgenic lines caused a significant change in WT aortic root diameter. eNOS activity was confirmed by using a pharmacologic approach that targets endogenous eNOS. Indeed, endogenous eNOS-specific activation with CavNOxin, a caveolin-1 peptide antagonist (Figure 5E) capable of activating eNOS specifically,¹⁹ significantly decreased MFS-associated aortic root aneurysm by 60%. Taken together, our results show that increased eNOS-dependent endothelial function can prevent aortic root disease in this MFS model (Figure 5F). In addition, this supports the hypothesis that losartan may have eNOS-driven off-target effects, as suggested by the absence of protective ATR2 shift in MFS/ATR1a^{-/-} mice (Figures 1 and 2) and L-NAME-dependent inhibition of NO release, which renders losartan inactive.

Discussion

Overall, the findings presented herein illustrate the broad aspect of losartan-based pharmacotherapy in MFS and help

Figure 3 Losartan acts through a nitric oxide synthase (NOS)—dependent mechanism. **A** and **B**: Combination treatment with losartan and NOS inhibitor L-NAME blocks the effect of losartan on aortic root widening (double-headed arrows) at 3 months of age, wherein aortic root diameters are determined from multiple measurements by a blinded technician. **C**: Total plasma NO levels in wild-type (WT), Marfan syndrome (MFS), and losartan-treated MFS mice were determined at 6 months of age in fasted mice and show significant reduction in NO in MFS mice that is corrected with losartan. **D**: A relationship is seen between aortic root diameter and total plasma NO at 6 months of age. Data are expressed as means $\pm$ SEM (**B**). $n = 9$ MFS (**A–C**); $n = 5$ MFS + L-NAME, MFS + losartan, and MFS + losartan + L-NAME (**A–C**) and WT (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (analysis of variance and *t*-test).

rationalize the pathogenesis and controversy surrounding management of MFS given recent large clinical trial results.¹² Our findings that chronically hypotensive MFS mice with a decreased HR, lacking AngII vasopressor responses and aortic root ATR1a expression, undergo normal MFS progression support the ill-defined concept that lowering of blood pressure and afterload to attenuate aortic wall stress is not of therapeutic significance in MFS.¹³ A protective shift toward ATR2 signaling or increased losartan sensitivity was not observed in these MFS mice with blunted ATR1a expression. These findings suggested that protective ATR2 signaling might be more complex to trigger than initially believed, particularly if aortic root expression of ATR2 is as low as these data suggest. Considering others have also observed the antiaortic root remodeling effect of losartan in MFS without changes in blood pressure,¹³ these data depict a smaller contribution of the AngII system and its receptors in the vascular manifestations of MFS. This further supports the widespread belief that angiotensin-converting enzyme inhibitors are not relevant for MFS aortic root disease

management.^{7,13} In contrast, data on MFS cardiomyopathy obtained with the cardiac-centric MgR MFS model¹⁵ show a clear dependence on ATR1a signaling activities. This suggested that the heart and aortic root behave differently in MFS, which may be explained by their different function, type of muscle tissues, and even embryonic origins.³⁸ The unique clinical challenge posed by aortic root pathology is further exemplified by a recent report showing protection against MFS-associated ascending and thoracic aorta dilation with L-NAME³⁹ because of the presence of proinflammatory inducible NOS. These data support the complexity of aortic root disease management compared with other more predictable sections of the aorta and suggest that the enzymatic source of NO is as important as the tissue from which it originates.

Because MFS mice lacking ATR1a do not show protection against MFS aortic root disease or increased losartan sensitivity—key components of a protective ATR2-dependent switch in AngII signaling—and because L-NAME can block losartan's effect on aortic root widening, off-target

Figure 4 Losartan increases endothelial cell nitric oxide release in a vascular endothelial growth factor receptor-2 (VEGFR2)—dependent manner. **A** and **B**: Losartan increases dose dependently ionophore-induced nitrate release (NO₃⁻) in culture endothelial cells under acute (1 hour) and chronic (48 to 72 hours) conditions, with control values subtracted. **C** and **D**: Western blots showing decreased VEGFR2 expression (250-kDa marker) in endothelial cells treated with VEGFR2 silencing RNA. Heat shock protein 90 is used as loading control (Ctrl). VEGFR2 siRNA-treated endothelial cells showed no increase in ionophore-induced nitrate with acute losartan (Los) treatment. For ionophore-induced nitrate studies, values represent 1-hour accumulation of nitrate for pretreated cells and 15-minute accumulation for acutely treated cells. Data show release, with each condition performed in triplicate. Data are expressed as means ± SEM (**A**, **B**, and **D**). **P* < 0.05 versus ionophore (analysis of variance and *t*-test); †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 versus unstimulated cells (analysis of variance and *t*-test); ‡*P* < 0.05 versus ctrl siRNA (analysis of variance and *t*-test).

NO-dependent actions of losartan were examined. This is a concept supported by reports from our group describing how losartan can correct vascular function in MFS mice and increase flow-mediated dilation in MFS patients.^{32,33} Losartan can trigger activation of the eNOS NO release independently of AngII, which is consistent with reports of ATR1 blockers triggering ATR1-independent signaling activities,⁴⁰ and losartan can activate the VEGFR2 and Akt pathways in absence of AngII in cells lacking ATR1.³⁵ The *in vitro* and *in vivo* mechanisms are poorly understood, because losartan's off-target signaling activities are complex.⁴¹ Besides known secondary effectors,⁴² VEGFR2 signals downstream through a range of adapter molecules that include c-Src and VRAP, among many others.⁴³ Which one binds to losartan and whether losartan alone can activate eNOS or only potentiate the signaling activity of a primary NO activator, such as laminar flow, warrant further investigation, particularly *in vivo*. Use of anti-VEGF therapeutic bevacizumab comes with a Food and Drug Administration warning of aortic aneurysm and is linked to lung airspace enlargement, two phenotypes of MFS.^{44–46} Because of lack of eNOS-specific NO activating medications, our knowledge of the vascular effects of reversing endothelial dysfunction remains fragmental. Using the experimental eNOS-specific CavNOxin, we and others have shown that increased eNOS NO release can promote aortic smooth muscle cell quiescence, decreased inflammation, remodeling, and oxidative stress known to decrease TGF-β signaling.^{19,31} TGF-β is a controversial effector in MFS,⁴⁷ despite antiaortic remodeling effects observed with anti-TGF-β blocking antibodies⁸

and similarity of MFS to Loeys-Dietz syndrome. However, the potential off-target activity of losartan helps rationalize its anti-lung remodeling properties,^{14,48} although whether this therapeutic effect stems from activation of the NOS system remains to be confirmed. Lung epithelial cells express neuronal NOS and may express eNOS, whereas inducible NOS may be connective tissue disease dependent,^{48,49} further stressing the complexity of the NOS system in tissue remodeling. Losartan has been linked to ATR1-independent activation of other cellular pathways⁴¹; whether these pathways play a role in losartan signaling in ECs or other cell types at the center of aortic wall remodeling, such as smooth muscle cells and adventitial fibroblasts, is unknown and was not investigated in the current study. The off-target effects of ATR1 blockers may also include anti-lung tissue remodeling properties because losartan was shown to attenuate cigarette smoke-induced emphysema, although loss of ATR1a in our current study did not prevent MFS-associated airspace widening. The nonvascular antiremodeling effects of losartan, which may also affect the skeletal manifestation of MFS, warrant further investigations.

From a patient outcome perspective, our data support the concept that blood pressure lowering in MFS is of little therapeutic value and propose to focus on gains in endothelial function. We and others have shown endothelial dysfunction in MFS aortas, which can be corrected with losartan in patients,³³ and eNOS-specific up-regulation of endothelial function can trigger profound antiremodeling effects.^{29,50,51} Hence, prophylactic treatment of MFS aortic root disease may benefit from endothelial

Figure 5 Increased endothelial nitric oxide synthase (eNOS) activity is therapeutically relevant in Marfan syndrome (MFS) models. **A** and **B**: MFS mice expressing a constitutively active eNOS mutant [eNOS 1176 Ser-to-Asp knock-in (S1176D)] show attenuated aortic root and ascending aorta widening. **C**: An inactive eNOS mutant [eNOS 1176 Ser-to-Ala knock-in (S1176A)] causes nonsignificant increase in aortic root diameter (MFS S1176A) relative to MFS controls. **D** and **E**: Overexpression of eNOS transgene (eNOS Tg) also reduces aortic root aneurysm (**D**), as does treatment with eNOS-activator caveolin-1 peptide mimetic (CavNOxin; **E**). Quantification of aortic root diameter via echocardiograms represents mean group aortic root diameter and quantification of total NO. **A** represents averages of $n > 5$ biological replicates averaged from assays completed in technical duplicate. **F**: Losartan may act in a vascular endothelial growth factor receptor-2 (VEGFR2)-dependent manner to stimulate NO production through eNOS—a key antiaortic root remodeling enzyme, as confirmed using multiple models—and this effect is likely independent of its ATR1 blocking activities. Data are expressed as means $\pm$ SEM (**A–E**). $n = 11$ WT (**A** and **B**); $n = 15$ MFS (**A** and **B**); $n = 13$ MFS S1176D (**A** and **B**); $n = 9$ S1176D (**A** and **B**), WT (**C**), and MFS (**E**); $n = 10$ MFS (**C** and **D**), $n = 5$ MFS S1176A and S1176A (**C**); $n = 7$ WT, MFS eNOS Tg⁺, and eNOS Tg⁺ (**D**); $n = 6$ WT and WT + CavNOxin (**E**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (analysis of variance and t -test). WT, wild-type.

function—optimized approaches, a claim also supported by the protective effect of mild aerobic exercise on MFS aortic root disease.⁵² Although exercise also reduces resting HR, the effect of ATR1a deletion on C1039G mice HR did not reduce aortic root remodeling, further supporting the role of endothelial NO in MFS. Hence, exercise and other well-optimized endothelial function-enhancing approaches may prove superior to commonly used therapies, a controversial topic in a recent large-scale trial.¹² Our groups have shown that losartan and atenolol differ in their effect on endothelial function,³³ which raises the possibility of titrating losartan against endothelial function, similarly to atenolol titration against HR, as suggested by current guidelines. Such difference in mechanism of action may rationalize the synergies observed with a high dose of losartan in combination with atenolol compared with atenolol alone in MFS patients.⁵³ To err on the side of caution, the mouse experiments did not include blood NO levels in ATR1a-null mice after losartan treatment. In addition, the data were obtained in a standardized MFS model mimicking the most common type of human FBN1 with homogeneous response to losartan, whereas MFS patients may show greater heterogeneity on the basis of their underlying FBN1 mutation. To our knowledge, this study may be the first to identify an animal model of human disease in which a

clinically available medication triggers an entirely endothelial function—based therapeutic effect. Our findings may have implications beyond our understanding of MFS, because they also support potential off-target effects of losartan on protective endothelial function in non-AngII-driven diseases.

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Supplemental Data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ajpath.2017.11.006>.

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