RESEARCH ARTICLE

Mild aerobic exercise blocks elastin fiber fragmentation and aortic dilatation in a mouse model of Marfan syndrome associated aortic aneurysm

Christine Gibson,1 Cory Nielsen,1 Ramona Alex,1 Kimbal Cooper,1 Michael Farney,1 Douglas Gaufin,1 Jason Z. Cui,3 Cornelis van Breemen,2 Tom L. Broderick,2 Johana Vallejo-Elias,2 and Mitra Esfandiarei1,3

1Department of Biomedical Sciences, College of Health Sciences; 2Department of Physiology, Arizona College of Osteopathic Medicine, Midwestern University, Glendale, Arizona; and 3Department of Anesthesiology, Pharmacology, and Therapeutics, University of British Columbia, Vancouver, British Columbia, Canada

Submitted 13 February 2017; accepted in final form 3 April 2017

Gibson C, Nielsen C, Alex R, Cooper K, Farney M, Gaufin D, Cui JZ, van Breemen C, Broderick TL, Vallejo-Elias J, Esfandiarei M. Mild aerobic exercise blocks elastin fiber fragmentation and aortic dilatation in a mouse model of Marfan syndrome associated aortic aneurysm. J Appl Physiol 123: 147–160, 2017. First published April 6, 2017; doi:10.1152/japplphysiol.00132.2017.—Regular low-impact physical activity is generally allowed in patients with Marfan syndrome, a connective tissue disorder caused by heterozygous mutations in the fibrillin-1 gene. However, being above average in height encourages young adults with this syndrome to engage in high-intensity contact sports, which unfortunately increases the risk for aortic aneurysm and rupture, the leading cause of death in Marfan syndrome. In this study, we investigated the effects of voluntary (cage-wheel) or forced (treadmill) aerobic exercise at different intensities on aortic function and structure in a mouse model of Marfan syndrome. Four-week-old Marfan and wild-type mice were subjected to voluntary and forced exercise regimens or sedentary lifestyle for 5 mo. Thoracic aortic tissue was isolated and subjected to structural and functional studies. Our data showed that exercise improved aortic wall structure and function in Marfan mice and that the beneficial effect was biphasic, with an optimum at low intensity exercise (55–65% \( V_{O_{2max}} \)) and tapering off at a higher intensity of exercise (85% \( V_{O_{2max}} \)). The mechanism underlying the reduced elastin fragmentation in Marfan mice involved reduction of the expression of matrix metalloproteinases 2 and 9 within the aortic wall. These findings present the first evidence of potential beneficial effects of mild exercise on the structural integrity of the aortic wall in Marfan syndrome associated aneurysm. Our finding that moderate, but not strenuous, exercise protects aortic structure and function in a mouse model of Marfan syndrome could have important implications for the medical care of young Marfan patients.

NEW & NOTEWORTHY The present study provides conclusive scientific evidence that daily exercise can improve aortic health in a mouse model of Marfan syndrome associated aortic aneurysm, and it establishes the threshold for the exercise intensity beyond which exercise may not be as protective. These findings establish a platform for a new focus on promoting regular exercise in Marfan patients at an optimum intensity and create a paradigm shift in clinical care of Marfan patients suffering from aortic aneurysm complications.

Marfan syndrome; aortic aneurysm; mild exercise; elastin fragmentation; aortic elasticity

MARFAN SYNDROME (MFS) is a connective tissue disorder caused by heterozygous mutations in the fibrillin-1 gene (\( FBN1 \)) with no sex or ethnic bias (14). MFS is considered a systemic disease of the connective tissue with a variety of clinical manifestations in the ocular (ectopia lentis), musculoskeletal (pectus deformity and scoliosis), and, most importantly, cardiovascular system (29). Cardiovascular complications include progressive growth and dilation of the aortic root, aortic wall dissection, aortic valve regurgitation, and mitral valve prolapse, with sudden death often due to dissection and rupture of the aorta in young adults (14, 23).

Fibrillin-1 is an extracellular matrix (ECM) glycoprotein that is widely distributed in the connective tissues of the body, and it has been shown to control cell function partially by sequestering latent transforming growth factor \( \beta \) (TGF-\( \beta \)) complexes through interaction with the latent TGF-\( \beta \) binding protein. Therefore, it is expected that mutations in \( FBN1 \) can lead to excessive TGF-\( \beta \) signaling, resulting in reduced mechanical compliance and progressive aortic aneurysm mainly due to unbalanced ECM remodeling (1). This abnormal signaling pattern is also thought to lead to vascular remodeling through the overexpression and increased activity of matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9 (29). It has been shown that MMPs degrade the collagen and elastin components of the matrix, leading to unfavorable conditions that seem to be the principal underlying causes of thoracic aortic aneurysm, leading to acute dissection or rupture (1). In a mouse model of MFS, inhibition of TGF-\( \beta \) and MMP mediated signaling pathways has been shown to improve aortic function and structure and to delay the progression of aortic aneurysm (2, 5, 28).

Currently, the only available and recommended therapeutic approach is treatment of MFS patients with blood pressure lowering medications such as beta blockers (atenolol) or angiotensin-II (ANG II) receptor blockers (ARBs) such as losartan, to reduce the hemodynamic overload in the aortic arch. However, such treatments can only delay the onset of the disease, without preventing the ultimate need for aortic replacement surgery (15, 22). The therapeutic approach is more challenging in younger MFS patients (3–18 yr old), as there are no conclusive data on potential side effects of long-term use of such medications in children and young adults. Considering the well-known beneficial effects of exercise on cardiovascular performance, it seems worthwhile to explore its potential value in the management of MFS. Several high-profile cases of sudden aortic rupture in Marfan patients during highly com-
petitive sports events have dramatically emphasized the risks associated with vigorous exercise. Nevertheless, we have considered the possibility that, in the case of MFS, there exists an optimal level of exercise which can benefit the vasculature and that increasing the level of exercise beyond this optimum could diminish benefit and increase risk. Therefore, in this report, we studied the effects of several aerobic exercise regimens of different intensity on the progression of aortic aneurysm, aortic function, and aortic wall structural integrity in a well-established mouse model of MFS.

**METHODS**

**Experimental animals and exercise protocol.** All surgical procedures and animal care were conducted according to the guidelines of the National Institutes of Health for the care and use of laboratory animals and compiled with the guidelines of the Midwestern University Institutional Animal Care and Use Committee (Approved IACUC Applications Nos. 2424 and 2444). The mouse model of MFS used in this study was in the C57BL/6 background and carried a heterozygous mutation in an Fbn1 allele encoding a cysteine substitution (Fbn1C1039G/+), which recapitulates classic aortic manifestations common in human MFS patients (12, 21, 30). The Fbn1C1039G/+ mouse (Jackson laboratory stock 012885) was back-crossed to wild-type C57BL/6 mice (wild-type littermates) for at least three generations to breed enough control and MFS mice for the study. We used 4-wk-old male mice for this study. At 4 wk of age, control (wild-type littermates) and MFS mice (n = 6–16) were subjected to voluntary cage-wheel exercise, forced treadmill exercise, or a sedentary lifestyle for 5 mo. Throughout this paper, the forced exercise refers to mild aerobic exercise at 55% intensity (55% VO$_2$max), unless otherwise indicated. The exercise protocol was continued until the mice reached 6 mo of age. For the sedentary (no exercise) groups, mice were placed in cages without exercise wheels or on a stationary treadmill for 30 min. Voluntary wheel exercise groups had continuous access to the running wheel during both diurnal and nocturnal cycles. The running wheel system was equipped with a 0.24-m diameter wheel and a 0.05-m wide running surface attached to a digital magnetic counter that was activated by wheel revolutions. Daily values for the rotations and distance covered (km/day) were recorded for the duration of the study. The magnetic counter was set to zero after each day’s recording to ensure accurate reading for the following day.

For the forced treadmill running groups, exercising at 55% VO$_2$max, acclimation training occurred over three consecutive days, each beginning at 5 m/min and gradually increasing to 8 m/min for a period of 20 min. For the actual training, mice were exercised 5 days/wk for 30 min/day as follows: for the first 5 min gradually increasing from 5 m/min to the maximum 8 m/min, and for the remaining 25 min at 8 m/min. The forced groups exercising at 65%, 75%, and 85% VO$_2$max followed the same basic acclimation protocol, except that the speeds were gradually increased to 10 m/min, 1.5 m/min, and 20 m/min, respectively, over the course of the acclimation period. During the actual training periods, the mice were exercised 5 days/wk for 30 min/day or until exhaustion as follows: for the first 5 min gradually increasing from 5 m/min to the maximum speed for the designated group for the remaining 25 min of the protocol.

**Aortic tissue preparation.** At the age of 6 mo, mice were euthanized with 5% isoflurane inhalant followed by cervical dislocation. Thoracic aorta segments (2 mm in length) were dissected from the thoracic cage, placed in ice-cold oxygenated (95% O$_2$–5% CO$_2$) HEPES Physiological Salt Solution (HEPES-PSS), and cleaned of connective tissue and blood, with special care taken to protect the endothelium for functional studies. The aortic root was collected and fixed in formalin for histological staining.

**Histological studies of the aortic wall.** Segments from the ascending aorta and arch were formalin fixed and embedded in paraffin. Specimens were cut into 10-µm-thick cross-sections. Tissue sections were deparaffinized in xylene, rehydrated in graded ethanol, and then stained with the Elastic Stain Kit (Sigma-Aldrich, St. Louis, MO) as previously described (10). Image acquisition was performed using an Olympus light microscope and an Axiozess digital camera. The length and counts of elastin fiber fragments within the aortic wall were determined using the line profiling tool, while the elastin thickness was determined utilizing the count and measure objects tool in Image-Pro Premier 9.1 (Media Cybernetics, Bethesda, MD). Elastin fibers were traced or outlined, and then their length or thickness measured in pixels followed by a unit conversion to either micrometers (for fiber length) or micrometers squared (for fiber area). For immunohistochemical studies, the rehydrated aerobic sections were stained using the EXPOSE mouse and rabbit specific horseradish peroxidase/3,3’-diaminobenzidine tetrahydrochloride (HRP/DAB) detection immunohistochemistry (IHC) kit (Abcam, Cambridge, MA). Primary antibodies detecting TGF-β, phospho-Smad2, MMP-2, and MMP-9 were purchased from Abcam. Slides were washed with Tris-buffered saline and counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO). Digital images were obtained at 400× magnification using an Olympus Vanox AH-3 microscope and AxioVision v4.8.2 imaging software. Image analysis was performed using National Institutes of Health Image J 1.43j software to measure the area of regions of interest (4 per section), and the mean intensity of positive signal after background noise was subtracted.

**Measurement of isometric force.** Aortic segments (2 mm) were prepared from each animal and gently mounted isometrically in a small vessel myograph (DMT-USA, Ann Arbor, MI) for measurement of contractility. The segments were equilibrated for 30 min at 37°C in HEPES-PSS solution, which was aerated continuously with 95% O$_2$–5% CO$_2$. The vessels were stretched to the optimal tension (6.0 mN) for 30 min. Optimal tension was determined in preliminary experiments by subjecting arterial segments to different resting tensions and stimulating with 60 mmol/l KCl. The optimal tension measured as the submaximal force generated in response to 60 mmol/l KCl was identical for both control and Marfan mice. To determine the viability and reactivity of the aortic smooth muscle, the vessels were then challenged twice with 60 mmol/l KCl buffer as previously described (6, 9, 25). Concentration-response curves of phenylephrine-induced contraction were then constructed by adding phenylephrine in a consecutive, cumulative manner (1 nmol/ml to 50 µmol/ml) (4, 8, 9), and percent contraction was computed as the percent increase or decrease in force with respect to the initial phenylephrine-induced contraction, where recorded response in control mouse aorta was set arbitrarily as 100% contraction. To study the effect of NO–nitro-L-arginine methyl ester (L-NAME; 200 µmol/ml), vessels were incubated with this compound for 30 min before phenylephrine (5 µmol/ml) application.

**Measurement of aortic wall elasticity or stiffness.** The stress-strain relationship was used to evaluate vessel wall strength and elasticity or stiffness. Aortic segments (2 mm) were mounted isometrically in a small vessel myograph in the HEPES-PSS solution at 37°C, which was gassed continuously with 95% O$_2$–5% CO$_2$ for the duration of the experiment.

Aortic segments were then gradually stretched, by increasing the distance between the two stainless steel pins (200 µm diameter) in the myograph chamber, and held at each length for 30 s. The distance between the two pins was then gradually increased by 100 µm, which correlates with an increase in the estimated length of vascular smooth muscle cells (SMCs) within the vessel wall. The stretching protocol was repeated until the blood vessel could not maintain its tone, and the force (stress point) at which the anchored aortic segment ruptured was recorded as the ultimate point (rupture point). Reversibility of elasticity was measured by performing three consecutive stress-strain measurements with 30-min intervals (resting periods). The aortic rings were stretched by 100 µm until the recorded generated force reached 50% below the point at which the aortic ring ruptures. This value was
derived from preliminary experiments performed in our laboratory. The engineering strain was plotted in relation to calculated wall stress as described below.

The circumference of the vessel ($L_C$) was calculated as the number of adjustments or turns multiplied by the distance between the pins (0.100 mm) all multiplied by two, plus four times the radius of the pins (0.100 mm), plus two times one-half the circumference of the pins ($2 \pi r$), where the radius ($r$) is 0.100 mm.

$$L_C = \left[ 2 \cdot \left( \text{number of turns} \cdot 0.100 \text{ mm} \right) \right] + \left( 4 \cdot 0.100 \text{ mm} \right) + \left[ 2 \cdot \left( \frac{1}{2} \cdot 2 \pi \cdot 0.100 \text{ mm} \right) \right]$$

To calculate the amount of stress endured by the aortic wall (WS), the force ($F$) produced by the stretched vessel was multiplied by the natural logarithm of one plus the engineering strain ($\epsilon$), all divided by the area ($A$) of the vessel.

$$WS = \frac{F \cdot \ln(1 + \epsilon)}{A} = \frac{\text{mN}}{\text{mm}^2}$$

The engineering strain ($\epsilon$) was calculated by the change in circumference of the vessel ($\Delta L_C$) as the increase in distance between the pins divided by the initial vessel circumference ($L_{C0}$).

$$\epsilon = \frac{\Delta L_C}{L_{C0}}$$

The area ($A$) of the vessel was calculated as two times the thickness of the vessel wall ($t$), which is constant at 0.075 mm, and then multiplied by the length of the vessel ($L_V$), which is constant at 2 mm.

$$A = 2 \cdot t \cdot L_V$$

**Buffers and reagents.** For all experimental protocols, aortic sections were perfused with HEPES-PSS buffer (pH 7.4) containing 10 mmol/l HEPES, 6 mmol/l glucose, 1.8 mmol/l CaCl$_2$, 130 mmol/l NaCl, 4 mmol/l KCl, 4 mmol/l NaHCO$_3$, 2.2 mmol/l MgSO$_4$, 1.2 mmol/l KH$_2$PO$_4$, and 0.03 mmol/l EDTA. High-K$^+$ buffer (60 mmol/l KCl) was identical to HEPES-PSS buffer, with the exception that it contained 74 mmol/l NaCl and 60 mmol/l KCl. All pharmacological agonists and inhibitors used in this study were purchased from Sigma-Aldrich.

**Combined benefit score.** The Combined Benefit Score is the combined effect of exercise on elastin fiber length, elastin fragmentation, and elasticity (measured as wall stress). These components were expressed as a percentage of the measures for the control and MFS mice relative to the corresponding values for the control mice exercising at 55% $\text{VO}_{2\text{max}}$, which we considered as the gold standard for desired outcomes. For example, both elastin fiber length and elasticity were calculated as sedentary MFS mice divided by control mice exercising at 55% $\text{VO}_{2\text{max}}$ (the gold standard). These numbers were then multiplied by 100 to be expressed as a percentage of efficacy compared to the gold standard. These calculations were applied to both control and MFS mice exercising at 55%, 65%, 75%, and 85%, or living under sedentary conditions. The elastin fragmentation ratio was inverted, because it is an inverse relationship compared with the other two variables. The three components of the score were each computed as a percentage of the healthy exercising control. The three components were averaged to obtain the final score. The Combined Benefit Score was plotted in relation to exercise intensity.

**Statistical analysis.** Statistical significance, dose-response curves, and stress-strain curves were prepared using GraphPad Prism software (San Diego, CA). A two-tailed Student’s $t$-test was used for comparison between two groups. For comparisons involving three or more groups, a two-way ANOVA followed by a Tukey multiple comparison test was utilized. Data are presented as means ± SE with a $P < 0.05$ value considered significant.

**RESULTS**

**Voluntary cage-wheel performance in control and Marfan mice.** To investigate whether MFS mice were willing to run on a voluntary basis and able to tolerate bouts of exercise, we compared the recorded daily distance covered by MFS and control mice for the duration of our study (5 mo). When the average daily distance covered in each experimental group was compared month to month (Fig. 1A), we observed different trends in each group of mice. In the control group, mice covered a much longer daily distance (4.667 ± 0.442 km during the first month and 5.017 ± 0.441 km during the second month) compared with MFS mice (2.518 ± 0.555 km during the first month and 3.703 ± 0.473 km during the second month). However, during the last 3 mo of the exercise protocol there was no significant difference between the average daily distance covered by MFS and control mice (Fig. 1A). In general, the performance of MFS mice on the wheel was fairly consistent over time, while control mice covered significantly less distance on the wheel as the study progressed. The same pattern was observed when we plotted the average daily distance covered by mice in each group for each week of the study (Fig. 1B). We also investigated the effects of both voluntary wheel and forced exercise routines on mouse body weight. In the voluntary cage-wheel control and MFS groups, total body weight increased consistently as mice aged without any significant differences between groups, while in the treadmill control and MFS groups, forced routine exercise decreased the average body weight in a similar manner in both groups (Fig. 1C).

**Effects of exercise on aortic elastin fragmentation.** To investigate the effects of exercise on elastin fragmentation and organization within the aortic wall, several sections from sedentary and exercised control and MFS aorta were subjected to conventional van Gieson staining. As expected, in sedentary control mice, the elastin fibers appear sigmoidal in shape without any fragmentation or breakage, whereas in sedentary MFS mice they appeared severely fragmented and disorganized (Fig. 2A). The line and object profiling tools in Image Pro Premier software were used to trace the length, number, and total area occupied by elastic fiber fragments within multiple regions of interest on aortic cross-sections. In both voluntary and forced exercise MFS mice, elastic fibers appeared to have normal sigmoidal shape with a significant increase in fiber length (Fig 2B) and a marked decrease in elastin fragment count (Fig. 2C). Both exercise routines also improved the thickness of elastic fibers within the aortic wall of MFS mice (Fig. 2D), confirming the beneficial effects of both exercise routines in improving the architecture of elastic fibers within the aortic wall of MFS mice.

**Effects of exercise on aortic wall stress and diameter (dilation).** In a myograph chamber, the total force generated in response to stretch is expected to increase exponentially as a function of strain. To further investigate the structural integrity and stability of the aorta, we measured the breaking stress or rupture point at which the association between the elastic fibers and SMCs is disrupted and, therefore, the vessel can no longer maintain its tension. As expected, the maximum stretch-induced force (breaking stress) was significantly lower in MFS mouse aorta than in aorta isolated from control mice, and both voluntary and forced exercise routines prevented aortic wall weakening and increased the breaking stress in MFS aorta back.
to the levels observed in control mice aorta (Fig. 3A). One extra piece of information that can be deduced from the recorded trace in a myograph chamber is the diameter of the aortic ring. This is possible because the distance between the two pins (on which the aortic ring is anchored) at the initial point, at which generated force can be recorded for the first time (when the aortic wall initially touches the pin), is an indicator of the aortic diameter in the resting condition. As reported before, a significant increase in aortic diameter (aortic dilation) was observed in MFS aorta compared with control, but both voluntary wheel and forced treadmill exercise routines reduced aortic diameter in MFS mice to the level observed in control mice aorta (Fig. 3B).

**Effects of exercise on aortic wall elasticity.** Elastin contributes to elasticity and structural stability of the aortic wall. Therefore, it is expected that the loss of elastic fiber organization and structural integrity observed in MFS aorta would have a negative impact on elasticity. The true elasticity of the aorta is defined by the ability of the blood vessel to return to its original diameter and conformation. Therefore, the reversibility of aortic elasticity is a significant indicator of its normal functionality. To investigate the true elasticity of the aortic segments, three consecutive stress-strain measurements were performed in both control and MFS aortic rings. Our data showed that the slope for the stress-strain curve in MFS aorta was decreased as the vessel was stretched three consecutive times, indicating a significant tearing and weakening and reduced stretch-induced force generation due to elastic fiber fragmentation and disorganization that impacts aortic wall integrity and elasticity (Fig. 4A). Both voluntary and forced exercise routines significantly improved aortic wall elasticity (Fig. 4, B and C). As highlighted in Fig. 4D, it is important to...
emphasize that, after each stress-strain measurement in aortic rings isolated from MFS mice, the maximum force recorded for any given strain markedly decreased, highlighting the inability of the aorta to generate the same amount of force while responding to stretch and in returning to its original conformation, a contractile defect that was corrected in MFS mice subjected to exercise routines.

**Effects of exercise on aortic contractility.** Vascular SMCs contribute to the integrity and tensile strength of the aortic wall; therefore, loss or dysfunction of SMCs can contribute to the formation of aneurysms (27). SMC contraction is controlled by calcium ($\text{Ca}^{2+}$) entry from the extracellular space and its release from the endoplasmic reticulum. We first measured vasoconstriction in response to 60 mmol/l KCl-
induced depolarization in the aorta from control and MFS mice subjected to sedentary lifestyle, voluntary exercise, or forced exercise, and we found no significant differences in the maximum force generated (Fig. 5A). We then studied the vasoconstriction response to receptor-mediated stimulation in our experimental groups by generating concentration-response curves (0.5 nmol/ml to 50 µmol/ml) for phenylephrine-induced tonic contraction (Fig. 5B). In 6-mo-old sedentary MFS mice, the maximal force (\(E_{\text{max}}\)) induced by phenylephrine was significantly reduced compared with sedentary control mice (Fig. 5B). Interestingly, both voluntary and forced exercise regimens reduced the maximal response in the control aorta, with no apparent effect on maximum force generated in MFS aorta (Fig. 5C). In regard to the sensitivity of the tissue to phenylephrine, the sedentary MFS mice demonstrated a significant increase in the dose of phenylephrine needed to reach 50\% maximal contraction (\(EC_{50}\)), and, while forced exercise regimens significantly reduced the \(EC_{50}\), voluntary wheel exercise had no effect (Fig. 5D).

To determine the underlying mechanism responsible for the significant reduction in phenylephrine-induced vasoconstric- tion response in MFS aortic segments, we pretreated the aortic rings with 200 μmol/ml L-NAME, a general blocker of NO production, 30 min before phenylephrine application. L-NAME potenti ated the \(E_{\text{max}}\) in the aortas from both control and MFS voluntary exercise groups by 121\% and 155\%, respectively (Fig. 6A). \(E_{\text{max}}\) was potentiated by 113\% and 115\% in the control and MFS forced exercise groups, respectively (Fig. 6B). Interestingly, in the sedentary MFS aortas, treatment with L-NAME restored phenylephrine-induced vasoconstriction by 111\% and to levels no different from the sedentary control aortas treated with L-NAME, indicating a significantly higher level of basal NO production in aortic segments isolated from sedentary MFS mice. We further calculated the difference in phenylephrine-induced force generation in the absence and presence of L-NAME in both control and MFS mice subjected to sedentary life style or voluntary cage-wheel and forced treadmill exercise regimens. As shown in Fig. 6C, inhibition of NO production in both control and MFS mice aorta significantly increased the recorded force generation; however, the difference between MFS and control was much more pronounced in the sedentary groups, indicating that MFS aortic tissue has higher basal levels of NO compared with control.

**Threshold for the protective effects of exercise on aortic wall structure.** To determine the effects of increasing exercise intensity on aortic wall structure and elasticity, we subjected 4-wk-old control and MFS mice to increasing levels of exercise intensity (8, 10, 15, and 20 m/min, corresponding to 55\%, 65\%, 75\%, and 85\% \(V_{\text{O2max}}\), respectively) on a treadmill (30 min/day, 5 days/wk) for the duration of the study. At 6 mo of age, aortic tissue was isolated and elastin fragmentation and aortic wall elasticity were measured. Our data showed that exercise training at intensity levels between 55\% and 65\% is beneficial and significantly reduces elastin fragmentation and disorganization within the aortic wall; however, much less recovery is observed in 75–85\% MFS groups (Fig. 7, A and B). The reversibility of elasticity was also significantly restored in MFS mice subjected to 55–65\% intensity, but the recovery was less pronounced in MFS mice subjected to 75–85\% intensity exercise regimens (Fig. 7C), indicating that high-intensity exercise is not as beneficial and effective in restoring aortic wall structural integrity and elasticity as the mild exercise regimen.

**Effects of aerobic exercise on MMP-2 and MMP-9 expression in the aortic wall.** To investigate the potential underlying mechanism for the observed protective effects of mild exercise on aortic wall structure and function, we measured the expression levels for MMP-2, MMP-9, TGF-β, and phospho-Smad2 (an indicator of TGF-β activity) in the aortic tissue sections isolated from control and MFS mice subjected to sedentary life style, mild aerobic exercise (55\% \(V_{\text{O2max}}\)), or high-intensity aerobic exercise (85\% \(V_{\text{O2max}}\)). Our data reconfirmed our previous report that expression of MMP-2 and MMP-9 was significantly higher in MFS aortic rings compared with control mice (4). Furthermore, compared with high-intensity exercise (85\% intensity), mild exercise (55\% intensity) was more effective in reducing
MMP-2 and MMP-9 expression levels within the aortic rings (Fig. 8, A and B). Interestingly, neither mild (55%) nor high-intensity (85%) exercise had any effect on TGF-β and downstream phospho-Smad2 expression in the MFS mouse aorta (Fig. 8, C and D).

**Optimal exercise intensity.** To illustrate the overall beneficial effects of exercise in protecting MFS mice from aortic aneurysm progression, we combined three of the measures into a single Combined Benefit Score. The score combines the effects of exercise on aortic function and structure measured as elastin fiber length, elastin fragmentation, and aortic wall elasticity (wall stress). The components were expressed as a percentage of the measures obtained from the sedentary control, sedentary MFS, and exercising MFS mice relative to the corresponding values for the control mice exercising at 55% V\textsubscript{O\textsubscript{2 max}} (arbitrarily set as the optimum condition). The elastin fragmentation ratio was inverted because it is an inverse relationship compared with the other three measurements. Thus we have three components of the score, each computed as a percentage of the healthy exercising control. The three components were averaged to obtain the final score. As shown in Fig. 9A, exercise presented clear overall protective effects on function and structure of the aorta in MFS mice, which featured an optimum when MFS mice were subjected to an exercise intensity of 55% (of \textsubscript{V}O\textsubscript{2 max}).
DISCUSSION

The aim of this study was to determine whether voluntary wheel or forced treadmill exercise could prevent or delay the degradation of aortic structure and function, which has been documented in a mouse model that recapitulates the aortic phenotypic changes in human MFS patients. To this effect, we used a daily aerobic exercise routine to determine the morphological and functional changes in elastic fibers, maximum aortic wall stress and elasticity, and the aortic vasoconstriction response in a mouse model of MFS. The application of both treadmill and cage-wheel exercise protocols allowed us to compare the effects of tightly controlled mild aerobic (treadmill) with voluntary exercise that was not strictly controlled with respect to duration or intensity (cage wheel) on aortic structure and function in the MFS mouse. Often in cardiovascular diseases such as aortic aneurysms, intense aerobic exercise can cause patients to become fatigued, unwilling to continue the exercise routine, or face the risk of aortic dissection and rupture (16). For this reason, it was of interest to assess whether Marfan mice were willing to voluntarily run on a wheel despite the progression of their disease. We observed that, compared with age-matched controls, voluntary exercising MFS mice covered significantly less distance during the early months of the study period. However, because the control mice also tended to slow down, the difference between MFS and control mice

Fig. 5. Effects of exercise on aortic smooth muscle cell (SMC) contractility. A: aortic SMCs response to KCl-induced contraction was not different among groups, indicating that mechanisms controlling SMC membrane depolarization are not affected. B: phenylephrine dose-response curves between sedentary, voluntary, and forced exercise groups. C: in the sedentary MFS aortas, a significant decrease in maximal force was observed compared with sedentary control aortas. Neither voluntary nor forced exercise MFS aortas exhibited any improvement in phenylephrine (PE)-induced contraction; however, both types of exercise significantly reduced the maximal response in the control aorta. D: sedentary MFS mice aortic rings demonstrated a significant decrease in sensitivity in response to phenylephrine-induced constriction. In the forced exercising MFS mice, there was a significantly reduced 50% of maximal contraction ($EC_{50}$), whereas voluntary wheel exercise had no effect on MFS aortas. Values are means ± SE, n as indicated on each bar, P as indicated.
was not evident as the study progressed through the third month of the protocol.

Elastin fragmentation and truncation have been extensively recognized in MFS patients with aortic aneurysms, as well as in the mouse model of the disease, and has been linked to the weakening of the aortic wall in response to the force of arterial pressure (4, 5, 24, 28). In the ascending thoracic aorta of the sedentary MFS mouse, we have confirmed the distinct disruption of the elastic fibers presenting as shortened and disorganized morphology, which is clearly different from the wavy, sigmoidal, and organized pattern that is observed in the sedentary control mouse. Both voluntary cage-wheel and forced treadmill exercise improved elastin fiber organization in MFS mice with less fragmentation and increased fiber length. Exercise caused the elastic fibers of the MFS mice to become indistinguishable from the sedentary or forced exercise control groups. Two major components of the aortic wall are elastic fibers and collagen fibrils that provide elasticity and tensile strength to the wall. With the fragmented and disorganized elastin in the MFS aorta, it is expected that the aortic wall will be weaker compared with controls. In our laboratory, we have observed that the stress-strain relationship of the 6-mo sedentary MFS aorta has a decreased slope compared with the sedentary control aorta, indicating a weakening of the aortic wall and a decreased maximum generated force and rupture point (ultimate stress). In addition, the aorta of the sedentary MFS mice demonstrated more strain (deformation) compared with sedentary controls (indicative of aneurysm formation). Both voluntary cage-wheel and forced treadmill exercise can improve the strength of the aortic wall while reducing the strain of the aorta, indicating that both exercise regimens blocked the progression of aortic dilation in the MFS mouse aorta.

The improvement of the maximum force generated in exercised MFS mice can likely be attributed to several factors including decreased elastin fragmentation, increased elastin...
deposition, and decreased proteolytic activity of proteases involved in elastic fiber degradation, especially MMPs (17, 18). We tested the hypothesis that the protective effects of exercise in blocking elastin fragmentation and aneurysm progression are largely due to a significant decrease in MMP-2 and MMP-9 expression in the aorta. Our current data clearly show that mild exercise (55% \( \dot{V}O_{2\text{max}} \)) decreases the expression of MMPs (MMP-2 and MMP-9) within the aortic wall, underscoring the critical role of these proteinases in MFS aneurysm pathogenesis. It is of importance that the observed protective effect diminishes as the intensity of exercise increases to 85% \( \dot{V}O_{2\text{max}} \), suggesting that high-intensity

Fig. 7. Threshold for protective effects of exercise on aortic wall structure. A: representative images showing structure of elastin and collagen (van Gieson staining) in cross-sections of ascending aorta from sedentary control (a) and MFS (b) mice, as well as control and MFS mice forced treadmill exercised at 55% (c and d), 65% (e and f), 75% (g and h), and 85% (i and j) of \( \dot{V}O_{2\text{max}} \) at 6 mo of age. Elastin fibers were stained dark purple. Elastin fiber disorganization and fragmentation were evident in sedentary MFS aorta compared with controls. B: length of elastin fiber and number of elastin fiber segments in aortic sections from each group of exercised control and MFS mice. Exercise training at intensity levels between 55 and 65% was beneficial and significantly reduced elastin fragmentation and disorganization within the aortic wall; however, much less recovery was observed in 75–85% exercised MFS groups. C: reversibility of elasticity was also significantly restored in aortic rings isolated from MFS mice subjected to 55–65% intensity, but recovery was less pronounced in MFS mice subjected to 75–85% intensity exercise regimens. Values are means ± SE, \( n = 6 \), \( P \) as indicated.
Fig. 8. Effects of aerobic exercise on signaling protein expression in mice aorta. Representative immunohistochemistry staining and quantification of matrix metalloproteinase (MMP)-2, MMP-9, transforming growth factor β (TGF-β), and p-Smad expression levels within cross-sections of aortic wall in control and MFS mice subjected to sedentary life style, mild exercise (55% VO₂max), or high-intensity exercise (85% VO₂max) for 5 mo. A: although both types of exercise regimens increased MMP-2 levels in control mice aorta, only mild exercise (55% VO₂max) was able to decrease MMP-2 expression levels in MFS aorta. B: both types of exercise decrease MMP-9 expression levels in MFS aorta. C: although both types of exercise regimens increased TGF-β levels in control mice aorta, they had no effect on TGF-β expression levels in MFS aorta. D: both types of exercise did not affect Smad2 phosphorylation in control and MFS aortas. Values are means ± SE, n = 4 mice, data are average of 5 regions of interest across aortic sections, P as indicated.

Exercise does not provide the desired protective effect. The observation that expression levels of TGF-β and p-Smad2 proteins in the aortic walls were not affected by exercise was not unexpected, because our previous published report showed that MMP inhibition by doxycycline could significantly improve aortic structure and function in the mouse, without having any effects on TGF-β expression (5). These observations open a new discussion about the importance of the TGF-β pathway during the late phase of aneurysm progression in Marfan mice.

This enhanced integrity of the elastic fibers provides protection against the hemodynamic pressure changes that contribute to aneurysm formation in the mouse model and MFS patients. Although the magnitude of contractile force generated in response to SMC membrane depolarization (in response to 60 mmol/l KCl) was not different between MFS and controls in both sedentary and exercise groups, the phenylephrine-induced aortic contractile response was significantly decreased in sedentary MFS mice at 6 mo of age. We have shown in this study that this is partially due to reduced tissue sensitivity to phenylephrine, as well as a significant increase in basal NO levels in MFS aortic tissue. The latter observation warrants further investigation to explore potential involvement of NO regulatory mechanisms during the pathogenesis of MFS associated aneurysm.

Although improvement in structural integrity of the aortic wall could be expected to increase agonist-induced contraction, we found that implementation of aerobic exercise had no significant effect on maximum phenylephrine-induced vasoconstriction in MFS mice aorta, whereas both cage-wheel and treadmill exercise protocols seemed to significantly decrease the Eₘₐₓ for phenylephrine in control mice aorta. We thus considered the likelyhood that both MFS pathology and exercise modulate NO release and/or bioavailability within the aortic wall (3, 11, 13, 26). Pretreatment of aortic rings with L-NAME, a general inhibitor of NO synthesis, significantly increased the maximal response to phenylephrine in all experimental groups. Surprisingly, in both sedentary and exercised MFS aortic rings, L-NAME increased phenylephrine-induced force to levels comparable with those observed in control aortic rings, indicating that, in the absence of exercise, the basal level of NO was much higher in aortic rings of MFS aorta than in the control group. This finding presents a striking paradox by showing that the disruptive effects of Marfan in the sedentary mice and the protective effects due to exercise are both associated with increased levels of basal NO. An important point is
that, although exercise markedly increased the difference in phynylephrine-induced force generation in control aorta, it did not cause the same effects in MFS aortic rings, suggesting that the level of basal NO in MFS aortic tissue had already reached the maximum threshold, and therefore was not affected by exercise.

The present study not only demonstrates the benefit of mild exercise in MFS, but, in addition, establishes an optimum level for putative exercise therapy and shows that the observed benefit is lost when exercise becomes excessive. Our data provide the first evidence that high-intensity exercise (75–85% $V_{O2\text{max}}$) has some detrimental effects on elastic fiber structure and aortic wall elasticity in MFS-associated aortic aneurysm. Such detrimental effects were not observed in 55% and 65% groups. This observation should be taken into consideration when planning and designing future clinical trials to investigate the potential therapeutic value of exercise in delaying or blocking the progression of aortic aneurysm in human MFS patients.

Presently, the optimal approach for prevention and treatment of cardiovascular complications in MFS is not clear. Current therapy aims to reduce hemodynamic stress on the aorta without directly targeting the pathogenic basis for the disease. Although beta-blockers (e.g., atenolol) and ANG II receptor blockers (e.g., losartan) slow the rate of aortic dilation, they do not produce the desired hemodynamic effects in patients with advanced aortic dilation, and, in most cases, these medications only seem to delay rather than eliminate the ultimate need for aortic replacement surgery. The unsatisfactory outcomes of current therapies warrant further studies to explore more effective therapeutic targets. Most importantly, the therapeutic approach is much more challenging in young MFS patients (e.g., 3–18 yr old), because medications such as atenolol and losartan at the normal to high dosages recommended in adult patients have serious side effects in children and young adults. If, as presented in this study and in the mouse model, a mild exercise routine proves to be effective in reducing the expression levels of MMPs (and possibly other disease markers within the aortic tissue), it may provide a potential therapeutic approach to improve life expectancy in MFS patients.

In this study, we have presented evidence that a mild aerobic exercise regimen can preserve the elastin fiber organization and improve the tensile strength of the aortic wall in a mouse model of MFS. Although there have been previous studies in which the safety and efficacy as well as tolerance for exercise training were studied in elderly abdominal aortic aneurysm patients (19, 20), to our knowledge this is the first time that an aerobic exercise routine has been utilized as a nonpharmaceutical therapeutic option to prevent or delay the progression of aortic aneurysm associated with MFS. However, this study has generated more questions that need to be addressed by further studies. One such missing piece of information is a comprehensive study of endothelial function, and the effects that exercise may have on endothelium-dependent and -independent vasodilation in MFS mice aorta. Here, we have studied a mild aerobic exercise routine, which appears to be beneficial in mice, but the important question that remains to be answered is: Would a moderate-to-intense exercise protocol be beneficial or detrimental to MFS-associated aneurysm in human patients? These remaining important questions warrant carefully designed longitudinal studies (up to 12 mo), in which MFS mice are subjected to different exercise intensity levels, and in vivo measurements of cardiac and aortic function, heart rate, and blood pressure, to determine an exercise intensity that will be beneficial in delaying the progression of aortic aneurysm. It is also important to investigate the effects of exercise in combination with common blood-pressure-lowering medications such as atenolol or losartan that are commonly prescribed in MFS patients.

In conclusion, we believe that the present study provides helpful insights into the potential protective effects of a mild exercise routine in MFS patients in the absence of pharmacological interventions, especially in young adults at risk of cardiovascular complications and aortic aneurysm. In recent
years, clinical care has focused on what type of exercise MFS patients should avoid due to risk for aortic aneurysm; however, little clinical emphasis has been placed on encouraging these patients to engage in routine and safe exercise such as walking. In this study, we have provided proof-of-concept for the impact of exercise on aortic health in MFS mice, suggesting a potential pathway for the observed positive effects (Fig. 9B), and have proposed an optimal intensity at which exercise could have potential beneficial and therapeutic effects. Further investigation of experimental groups that receive lower doses of recommended medications (e.g., atenolol and losartan), while being subjected to a mild exercise regimen, will provide valuable information about the possible beneficial value of exercise as an adjunct therapy in adult MFS patients diagnosed in a more advanced stage of aortic aneurysms. Finally, further studies need to be conducted to establish a clear set of exercise guidelines for human MFS patients.

GRANTS

This work was supported by The Marfan Foundation and Midwestern University’s Biomedical Sciences Program.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

24. Syyong HT, Chung AWY, Yang HHC, van Bree men C. Dysfunction of endothelial and smooth muscle cells in small arteries of a mouse model of


