SUPPLEMENTAL MATERIAL

ALLOPURINOL BLOCKS AORTIC ANEURYSM IN A MOUSE MODEL OF MARFAN SYNDROME BY REDUCING AORTIC OXIDATIVE STRESS

Short title: *Allopurinol inhibits Marfan syndrome aortopathy*

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CONTENT

1. MATERIAL AND METHODS

Human tissue collection, mice, and experimental designs

Healthy ascending aortic tissue was collected from heart donors via the organ donation organization at the Hospital Clínic i Provincial (Barcelona, Spain) and Hospital de Bellvitge (L'Hospitalet de Llobregat, Barcelona, Spain). The age and gender of heart donors were unknown because Spanish law protects personal information about organ donors. Ascending aortic aneurysm samples were collected from patients with Marfan syndrome (MFS) (with ages ranging from 17 to 60 years) undergoing aortic aneurysm repair surgery. All the patients in whom aortae were resected fulfilled MFS diagnostic criteria according to Ghent nosology, but no genetic information regarding putative *FBN1* mutations was available. For each patient, we obtained a 3 x 3 cm sample from two areas: the dilated zone, corresponding to the sinuses of Valsalva, and the adjacent virtually non-dilated aorta (according to the surgeon's opinion) corresponding to the distal ascending aorta. The aortae were maintained in cold saline solution or cardioprotective solution before delivery to the laboratory.

MFS mice with a fibrillin-1 mutation *(Fbn1*$^{C1041G/+}$*;* hereafter C1041G*)* (hereafter, MFS mice) were purchased from The Jackson Laboratory (B6.129-Fbn1tm1Hcd/J; Strain #012885/Common name: C1039G; Bar Harbor, ME 04609, USA). MFS and sex- and age-matched wild-type littermates (WT mice)

were maintained in a C57BL/6J genetic background. All mice were housed according to the University of Barcelona institutional guidelines (constant room temperature at 22ºC, controlled environment 12/12-hour light/dark cycle, 60% humidity and *ad libitum* access to food and water).

WT and MFS mice were administered allopurinol (hereafter ALO) (A8003, Sigma-Aldrich) diluted in drinking water (20 mg/kg/day; 125 mg/mL) [92,93]. ALO was fully replaced each third day of treatment. We performed two experimental ALO treatment approaches: palliative (PA) and preventive (PE). For PA treatments, ALO was administered to mice of 2 months of age until 6-month-old (PA1) or 9-month-old (PA2) being a respective effective treatment of 4 and 7 months, respectively (Fig. S1). For PE treatment, ALO was administered to the pregnant WT mother, maintained after giving birth (lactation period of 25 days) and thereafter maintained in drinking water to weaned babies until three months of age. To evaluate whether ALO's effect on aortopathy was transient or permanent, the inhibitor was withdrawn from drinking water (<ALO) for a period of three months following the PE and PA1 experimental treatments with 6- and 9-month age endpoints (PE<ALO and (PA1<ALO, respectively; Fig. S1). At each outcome time points, mice were subjected to echocardiographic analysis. Both the ascending aorta and liver were dissected and fixed for paraffin embedding for (immuno)histological studies or immersed in RNA Later (R-0901, Sigma Aldrich), frozen in liquid nitrogen and stored at -80ºC for molecular tests.

Study approvals

Human tissues were collected with the required approval from the Institutional Clinical Review Board of Spanish clinical centers, and the patients' written

consent conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Patients were informed about the use for research studies of their extracted aortic samples. All aortic tissues described in the manuscript were those obtained from Spanish Marfan patients and heart donors. Due to the Spanish Data Protection Act, we do not have access to their clinical history or personal data.

Animal care and colony maintenance conformed to the European Union (Directive 2010/63/EU) and Spanish guidelines (RD 53/2013) for the use of experimental animals. Ethical approval was obtained from the local animal ethics committee (CEEA protocol approval number 10340).

Echocardiography

Two-dimensional transthoracic echocardiography was performed in all animals under 1.5% inhaled isoflurane. Each animal was scanned 12–24 hours before sacrifice. Images were obtained with a 10–13 MHz phased array linear transducer (IL12i GE Healthcare, Madrid, Spain) in a Vivid Q system (GE Healthcare, Madrid, Spain). Images were recorded and later analyzed offline using commercially available software (EchoPac v.08.1.6, GE Healthcare, Madrid, Spain). Proximal aortic segments were assessed in a parasternal long-axis view. The aortic root diameter was measured from inner edge to inner edge in end-diastole at the level of the sinus of Valsalva. All echocardiographic measurements were carried out in a blinded manner by two independent investigators at two different periods, and with no knowledge of genotype or treatment.

Histology and histomorphometry

Paraffin-embedded tissue arrays of mice aortae from different experimental sets were cut into 5 µm sections. Elastic fiber ruptures were quantified by counting the number of large fiber breaks in tissue sections stained with Verhoeff-Van Gieson. Breaks larger than 20 µm were defined as evident large discontinuities in the normal circumferential continuity (360º) of each elastic lamina in the aortic media[45]. They were counted along the length of each elastic lamina in four different, representative images of three non-consecutive sections of the same ascending aorta. The number of sections studied for condition were usually of three, spacing 10 µm between them (2 sections). All measurements were carried out in a blinded manner by two different observers with no knowledge of genotype and treatment. Images were captured using a Leica Leitz DMRB microscope (40x oil immersion objective) equipped with a Leica DC500 camera and analyzed with Fiji Image J Analysis software.

Immunohistochemistry and immunofluorescence staining

For immunohistochemistry and/or immunofluorescence, paraffin-embedded aortic tissue sections (5 µm thick) were deparaffinized and rehydrated prior to unmasking the epitope. Horseradish peroxidase (HRP)-based immunohistochemistry was used to stain aortic tissue sections for XOR and 3'-nitrotyrosine (3-NT). To unmask XOR epitopes, aortic tissue sections were treated with a retrieval solution (10 mM sodium citrate, 0.05% Tween, pH 6) for 30 min in the steamer at 95ºC. No antigen retrieval was used for 3-NT. Next, sections were incubated for 10 min with peroxidase blocking solution (Dako Real Peroxidase-blocking solution), rinsed three times with PBS and then incubated with 1% BSA in PBS prior to overnight incubation at 4ºC with the respective

primary polyclonal antibodies anti-XOR (1:50; Rockland 200-4183S) or anti-3-NT (1:200; Merck Millipore 06-284). On the next day, sections were incubated with the manufacturer's goat anti-rabbit secondary antibody solution (1:500; Abcam ab97051) for 1 h followed by the Liquid DAB+Substrate Chromogen System (Dako System HRP) for 1 min at room temperature. HRP-stained non-consecutive sections were visualized under a Leica Leitz DMRB microscope (40x immersion oil objective).

Immunofluorescence was used to stain pNRF2 in aortic sections. Sections were treated first with heat-mediated retrieval solution (1 M Tris-EDTA, 0.05% Tween, pH 9) for 30 min in the steamer at 95ºC. Next, sections were incubated for 20 minutes with ammonium chloride (NH_4Cl, 50 mM, pH 7.4) to block free aldehyde groups, followed by a permeabilization step using 0.3% Triton X-100 for 10 min and then treated with 1% BSA blocking buffer solution for 2 h prior to overnight incubation with monoclonal anti-pNRF2 (1:200; Abcam ab76026) in a humidified chamber at 4°C. On the next day, sections were rinsed with PBS, followed by 60 min incubation with the secondary antibody goat anti-rabbit Alexa 647 (1:1.000, A-21246, Invitrogen). Sections were counterstained with DAPI (1:10.000) and images were acquired using an AF6000 widefield fluorescent microscope.

For quantitative analysis of immunostainings, four areas of each ascending aorta section were quantified with Image J software. All measurements were carried out in a blinded manner by two independent investigators.

Uric acid, allantoin and hydrogen peroxide in blood plasma and in ascending aortic rings

Blood from mice was collected directly from the left ventricle just before the aortic tissue was dissected. Thereafter, the blood plasma was obtained by centrifugation of the blood at 3,000 rpm for 10 min at 4ºC and immediately stored frozen at -80 ºC.

Measurement of uric acid (UA) in blood plasma was evaluated by high-performance liquid chromatography (HPLC) with ultraviolet detection. The method used for UA extraction from biological samples was an adaptation of a method previously described[94]. The plasma (100 µl) was deproteinized with 10% trichloroacetic acid. Ten µl of supernatant was injected into the HPLC system consisting of a Perkin Elmer series 200 Pump, a 717 plus Autosampler, a 2487 Dual λ absorbance detector, and a reverse-phase ODS2 column (Waters, Barcelona, Spain; 4.6 mm·200 mm, 5 µm particle size). The mobile phase was methanol/ammonium acetate 5 mM/acetonitrile (1:96:3 v/v), which was run with an isocratic regular low flow rate of 1.2 mL/min and the wavelength UV detector was set at 292 nm. UA eluted at a retention time of 2.9 minutes. Quantification was performed by external calibration. The UA detection limit in plasma was 10 ng/mL

For the determination of allantoin in blood plasma, an adapted protocol was used as previously described [95]. Briefly, plasma (60 µl) was deproteinized with acetonitrile (25 µl). Samples were centrifuged (5 min, 12,000 *g*). Ten µl of supernatant was injected into the HPLC system. Separation of allantoin was performed on a Synergy Hydro-RP C-18 reversed-phase column (250 × 4.6 mm I.D., 5 m particle size) from Phenomenex (Torrance, CA, USA). Allantoin elution (at 4 min) was performed with potassium dihydrogen phosphate (10 mM, pH 2.7): acetonitrile (85:15) and ultraviolet detection (at 235 nm).

Hydrogen peroxide (H_2O_2) was measured both in blood plasma and in freshly dissected aortic tissue (aortic rings) utilizing a commercial assay kit (ab102500 Abcam, Cambridge, UK). Non-deproteinized blood plasma from WT and MFS mice (50 µL) and standard dilutions were mixed with 50 µL of reaction Mix (composed of 48 µL of the assay buffer plus 1 µL OxiRed Probe + 1 µL HRP for fluorometric measures) and incubated (protected from daylight) for 10 min at room temperature. In the case of the aortic tissue, two types of measurements were carried out. On the one hand, H_2O_2 was measured in the aorta from mice that followed the preventive treatment with ALO (*in vivo* treatment); on the other hand, H_2O_2 was measured in the aorta in which ALO was directly added to the assay (*in vitro* treatment) Freshly dissected ascending aorta (the adventitia was quickly removed) were cut in two portions (3-4 mm thick each) corresponding to the proximal (the half close to the heart) and distal (the half close to the aortic arch) and maintained immersed in DMEM in the culture incubator at 37ºC and 5% CO_2 until time of the assay. Subsequently, each aortic portion was incubated with the reaction mix of the kit as indicated above for the blood plasma. For the *in vitro* approach, the dissected ascending aorta was treated as above with the difference that ALO (100 µM) was added to one of the two aortic portions while the other portion received the vehicle (physiological serum/PS). Fluorometric readings were obtained at different times, reaching a plateau at 120 min. Results were normalized to the weight of the respective aortic portion. Fluorescence was measured with a Synergy fluorimeter (Ex/Em = 535/587 nm).

Myography tissue preparation and vascular reactivity

Segments of the ascending aorta from 9-month-old mice treated or not with ALO were dissected free of fat and placed in a cold physiological salt solution (PSS; composition in mM: NaCl 112; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.1; $MgSO_4$ 1.2; $NaHCO_3$ 25 and glucose 11.1) gassed with 95% O_2 and 5% CO_2. Ascending aortic segments (2-3 mm) were set up on an isometric wire myograph (model 410A; Danish Myo Technology, Aarhus, Denmark) filled with PSS (37ºC; 95% O_2 and 5% CO_2) as previously described [96]. The vessels were stretched to 6 mN as reported [93], rinsed and allowed to equilibrate for 45 min. Tissues were then contracted twice with KCl (100 mM) every 5 min. After rinsing, vessels were left to equilibrate for an additional 30 min before starting the experiments. Vasodilatation caused by nitric oxide (NO) produced either by the endothelium itself (triggered by acetylcholine/ACh) or by the NO donor sodium nitroprusside (NTP) were determined by cumulative concentration-response curves (CRCs) of respective relaxation to ACh (10^{-9}-10^{-5} M) or NTP (10^{-10}-10^{-5} M) after phenylephrine (Phe; 3×10^{-6} M)-induced precontracted vessel. To study the impact of ALO on contractile responses triggered by α_1-adrenergic stimulation, the CRCs of Phe (10^{-9} to 3×10^{-5} M)-induced contraction were evaluated. Relaxations to ACh are expressed as a percentage of Phe-precontracted level. Contractions to Phe are expressed as a percentage of the tone generated by KCl. Data from CRCs were plotted using Graph Pad Software version 8.0 (San Diego, CA, USA) with sigmoid curve fitting (variable slope) performed by non-linear regression. These curves were used to derive the values for E_{max} (the maximal relaxant response) and pEC_{50} (-log of the agonist concentration needed to produce 50% of E_{max}).

Quantitative Real-Time PCR

Total RNA from ascending aortae was extracted using Trizol© following manufacturer's recommendations (Invitrogen, USA). RNA concentration was quantified using Nanodrop (Agilent, USA). mRNA expression levels were determined by quantitative real-time PCR (qRT-PCR) using the SYBR green detection kit. mRNA levels encoding for XOR were expressed relative to *Gadph*, which was used as the housekeeping gene. qPCR reactions were performed following the protocol guidelines of the SYBR green master mix (ThermoFisher Scientific, Waltham, MA, USA). Briefly, reactions were performed in a total volume of 10 µL, including 5 µL of SYBR green PCR master mix, 2 µM of each primer, 2 µL of nuclease-free water, and 1 µL of the previously reverse-transcribed cDNA (25 ng) template on a 368-well iCycler iQ PCR plater (Bio-Rad). All reactions were carried out in duplicate for each sample. The thermocycling profile included 45 cycles of denaturation at 95 ºC for 15 seconds and annealing and elongation at 60ºC for 60 seconds. Cycle threshold (Ct) values for each gene were referenced to the internal control (comparative Ct (ΔΔCt)) and converted to the linear form relative to corresponding levels in WT aortae. The primer sequences for the murine genes used in this study are shown in Table S1.

Fluorometric assay for measuring the enzymatic activity of xanthine dehydrogenase (XDH) and xanthine oxidase (XO) forms

XO activity was determined in WT and MFS mice from liver and aorta (in which the adventitia was previously removed) lysates using a fluorimetry-based method [97]. Part of the liver and total aorta were homogenized with five volumes per gram of tissue of 0.25 M sucrose, 10 mM DTT, 0.2 mM PMSF, 0.1 mM EDTA and

50 mM K-phosphate, pH 7.4. Homogenates were centrifuged for 30 min at 15,000 *g* and the supernatants were obtained for XO activity. XO activity was measured by calculating the slope of the increase in fluorescence after adding pterin (0.010 mmol/L), which measures the conversion of pterin to isoxanthopterin. Total activity (XO+XDH) was likewise determined after adding methylene blue (0.010 mmol/L), which replaces NAD+ as an electron acceptor. The reaction was stopped by adding allopurinol (50 µmol/L). To calibrate the fluorescence signal, the activity of a standard concentration of isoxanthopterin was measured. The extent of dehydrogenase (XDH)-to-oxidase (XO) conversion was calculated from the proportion of XO activity divided by the total activities of XDH+XO. Values were expressed as nmol/min per g of protein. The protein concentration of homogenates was determined with the Bradford assay.

Blood pressure measurements

Systolic blood pressure measurements were acquired in 9-month-old animals by the tail-cuff method and using the Niprem 645 non-invasive blood pressure system (Cibertec, Madrid, Spain). Mice were positioned on a heating pad and all measurements were carried out in the dark to minimize stress. All animals were habituated to the tail-cuff by daily training one week prior to the final measurements. Then, the systolic blood pressure was recorded over the course of three days. For quantitative analysis, the mean value of three measurements per day was used for each animal. All measurements were carried out in a blinded manner with no knowledge of genotype or experimental group.

Collagen content measurements

Collagen content was evaluated with the PicroSirius Red Staining method. Briefly, paraffin-embedded tissue arrays of mice aortae from different experimental sets were cut into 5 µm sections. Deparaffination was performed with xylene, and rehydration in 100% ethanol three times for 5 minutes, three times in 96% ethanol for 5 min and dH_2O for 5 min. After rehydration, samples were immersed in phophomolybdic acid 0,2% for 2 min and rinsed with distilled H_2O. Then, samples were immersed in picrosirius red (previously prepared with picric acid (Fluka 74069) and direct red 80 (Aldrich 365548) for 2 h. Afterward, samples were rinsed with distilled H_2O for 5 min and immersed in HCl 0,01 N for 2 min. To avoid background, unstaining was performed with ethanol 75º for 45 sec. Finally, slides were dehydrated with absolute ethanol for 5 min and fixated with xylene twice for 10 min each. Mount with DPX with coverslips. Images were captured using a Leica Leitz DMRB microscope (10x and 40x oil immersion objective) equipped with a Leica DC500 camera and analyzed with Fiji Image J Analysis software with a predesigned macro program.

Statistics

Data were presented either as bars showing mean ± standard error of the mean (SEM) or median±interquartile range (IQR) boxplots, in which the error bars represent minimum and maximum values, the horizontal bars and the crosses indicate median and mean values, respectively, and the extremities of the boxes indicate interquartile ranges. Firstly, normal distribution and equality of error variance data were verified with Kolmogorov-Smirnov/Shapiro Wilk tests and Levene's test, respectively, using the IBM SPSS Statistics Base 22.0 before parametric tests were used. Differences between three or four groups were evaluated using one-way or two-way ANOVA with Tukey's *post-hoc* test if data

were normally distributed, and variances were equal or Kruskal-Wallis test with Dunn's *post-hoc* test if data were not normally distributed. For comparison of two groups, the unpaired t-test was utilized when the data were normally distributed, and variances were equal or the Mann-Whitney U test if data did not follow a normal distribution. A value of $P \leq 0.05$ was considered statistically significant. Data analysis was carried out using GraphPad Prism software (version 9.1.2; GraphPad Software, La Jolla, CA). Outliers (ROUT 2%, GraphPad Prism software) were removed before analysis.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

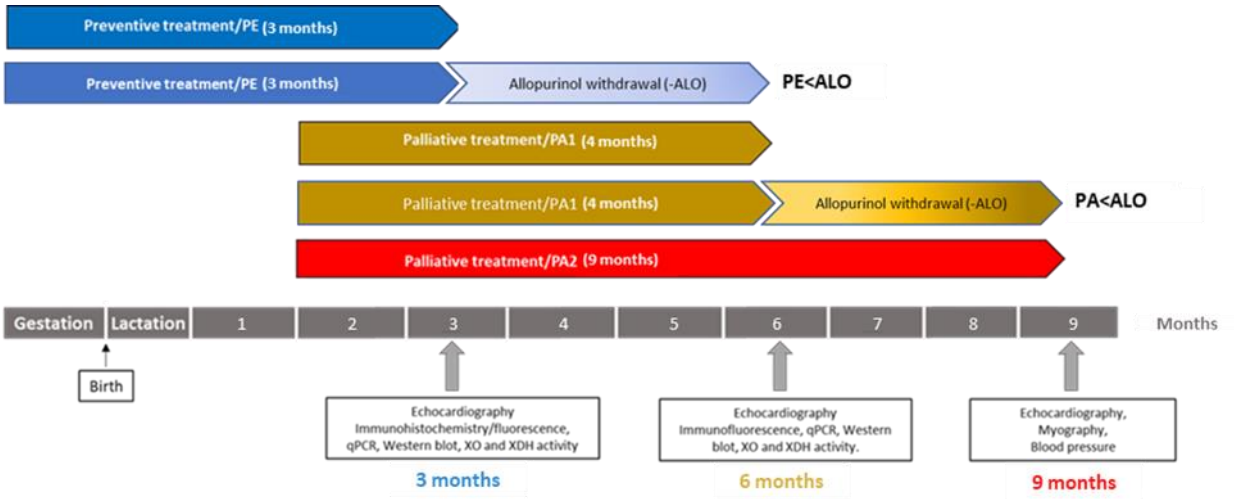

Figure S1. **Representative scheme of the experimental protocols for allopurinol treatments.** PE: preventive allopurinol (ALO) treatment (endpoint at 3-month-old); PA: palliative allopurinol treatments PA1 and PA2 whose difference between them being the duration of treatment, which was 4 months (endpoint at 6-month-old mice) and 7 months (endpoint at 9-month-old mice), respectively. PE<ALO: preventive allopurinol treatment followed by the subsequent drug withdrawal for 3 months (endpoint at 6-months-old); PA1<ALO: palliative allopurinol treatment followed by the subsequent drug withdrawal for 3 months (endpoint at 9-month-old). Note that each endpoint duration protocol has a different code color, which is maintained for the figures and supplemental figures shown throughout the manuscript: blue (PE), brown (PA1) and red (PA2).

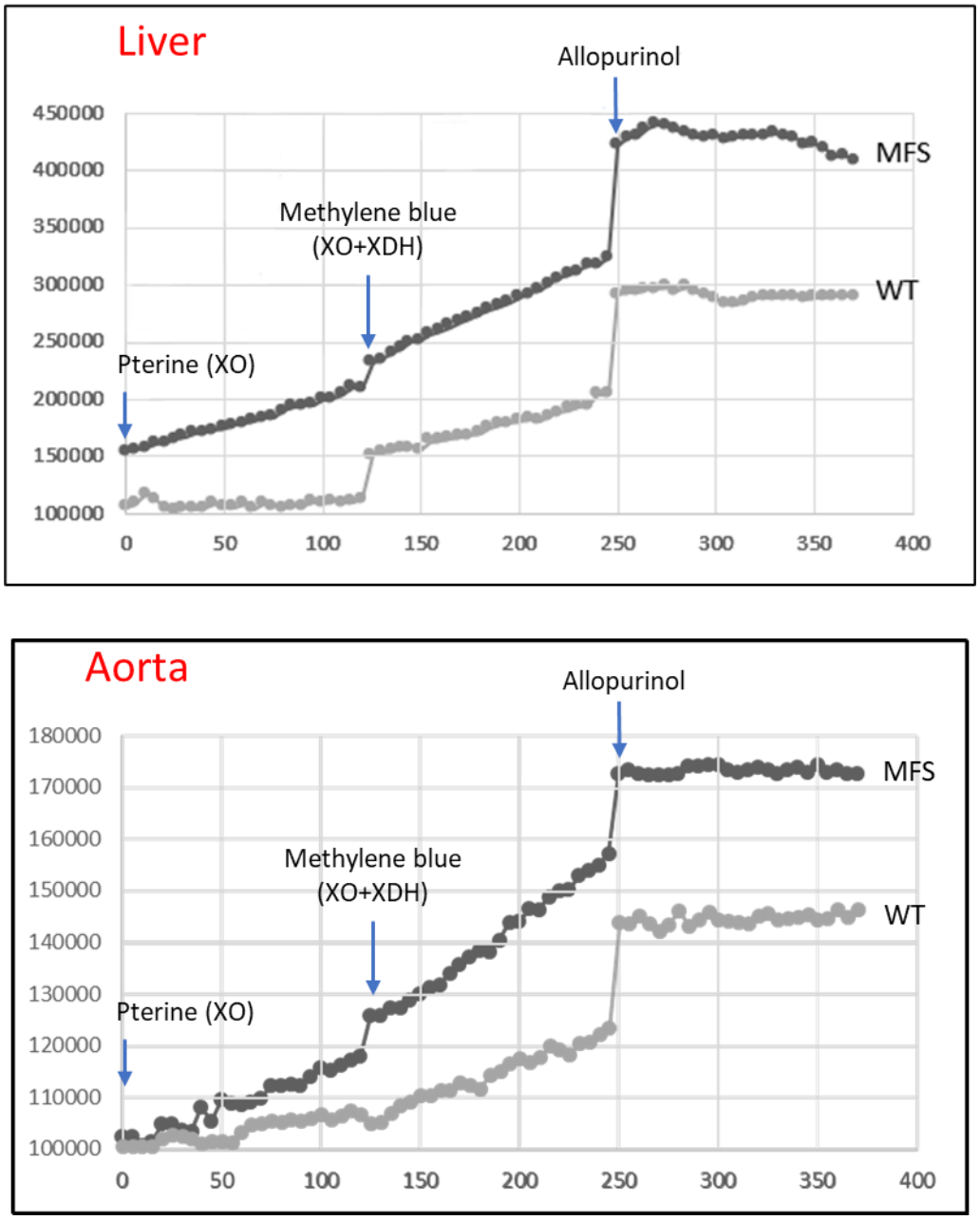

Figure S2. Fluorometric assay for measuring xanthine dehydrogenase (XDH) and oxidase (XO) enzymatic activity in liver and aortic tissues. Liver (as positive control of the assay) and aortic WT and MFS lysates had pterine added as a specific substrate for XO and methylene blue for total XOR (XO+XDH) activity. Allopurinol was added at the end of the assay to check its effectiveness in inhibiting total XDH activity. Units in the Y axis are fluorescence arbitrary units.

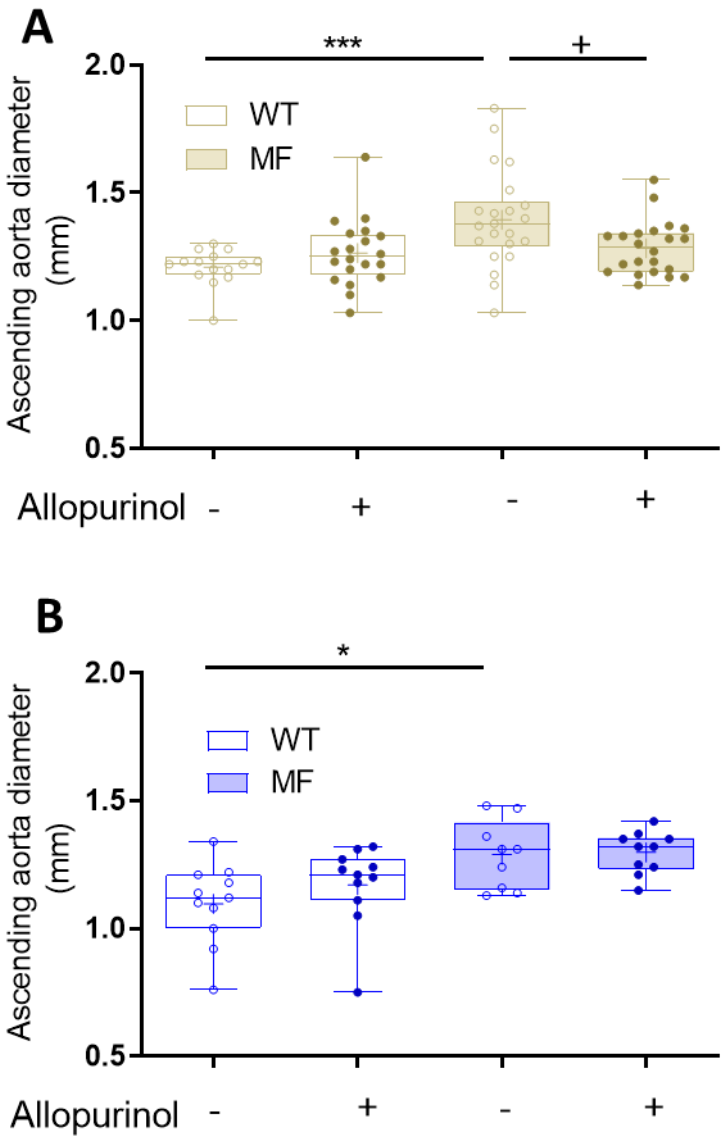

Figure S3. **Ascending aorta diameter in WT and MFS mice treated with allopurinol. (A)** Ultrasonography in WT and MFS mice of 6 months of age palliatively treated with allopurinol (PA1). See also Table S4. (**B)** Ultrasonography in 3 months of age WT and MFS mice preventively treated with allopurinol (PE). See also Table S5. Data represented as boxplots. Statistical analysis: Two-way ANOVA and Tukey post-test (A and B). ***$P \leq 0.001$ and $^{*}P \leq 0.05$; *effect of genotype; $^{+}$effect of treatment.

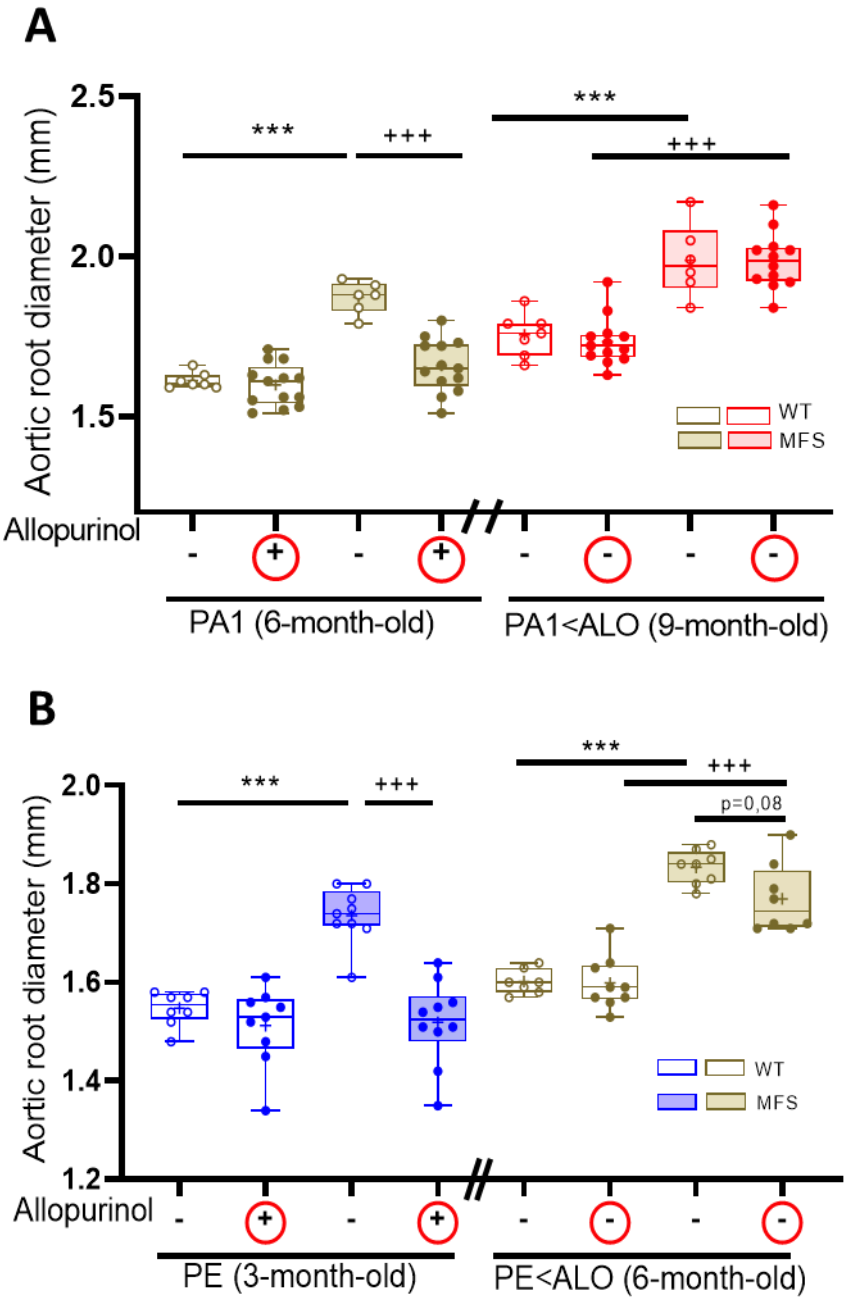

Figure S4. Reversion of aortic root dilation following the withdrawal of allopurinol. (**A**) WT and MFS mice were treated with allopurinol (ALO) following the palliative treatment from 2 to 6 months of age (PA1; 6-month-old). Thereafter, ALO was withdrawn from WT and MFS mice (+ inside red circles) until 9 months of age (PA1<ALO) (- inside red circles). The aortic root diameter was measured by ultrasonography at 6- and 9-month-old (brown and red boxes, respectively). N= 6-13. See also Table S6. (**B**) WT and MFS mice were treated with allopurinol

following the preventive treatment (PE, 3-months-old). Thereafter, allopurinol was withdrawn from WT and MFS mice (+ inside red circles) until 6 months of age (PE<ALO) (- inside red circles). The aortic root diameter was measured by ultrasonography at 3- and 6-month-old (blue and brown boxes, respectively). N= 6-10. See also Table S7. Statistical analysis in A and B: Two-way ANOVA and Tukey post-test. ***, $^{+++}P \leq 0.001$; *effect of genotype; $^{+}$effect of treatment.

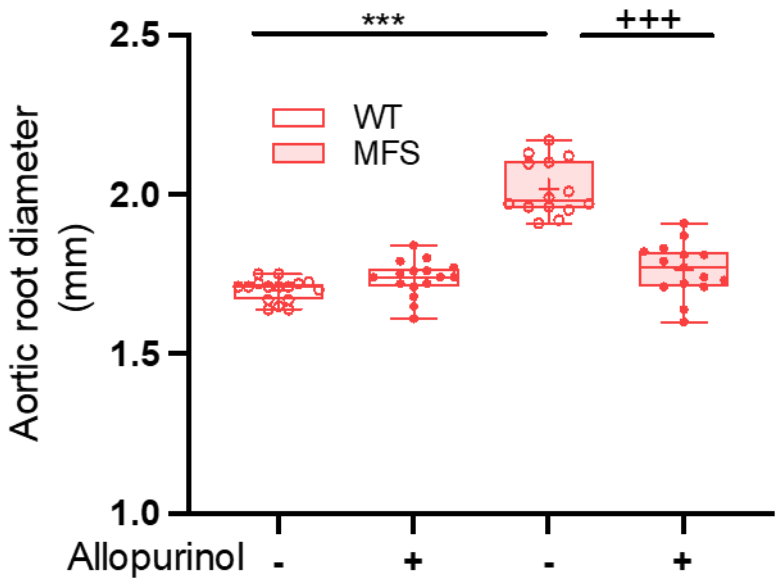

Figure S5. Aortic root diameter in WT and MFS mice palliatively treated with allopurinol (PA2). Data represented as boxplots. Statistical analyses: See also Table S6. Two-way ANOVA and Tukey's post-test. ***/+++$P \leq 0.001$; *effect of genotype; $^{+}$effect of treatment. n=14-16.

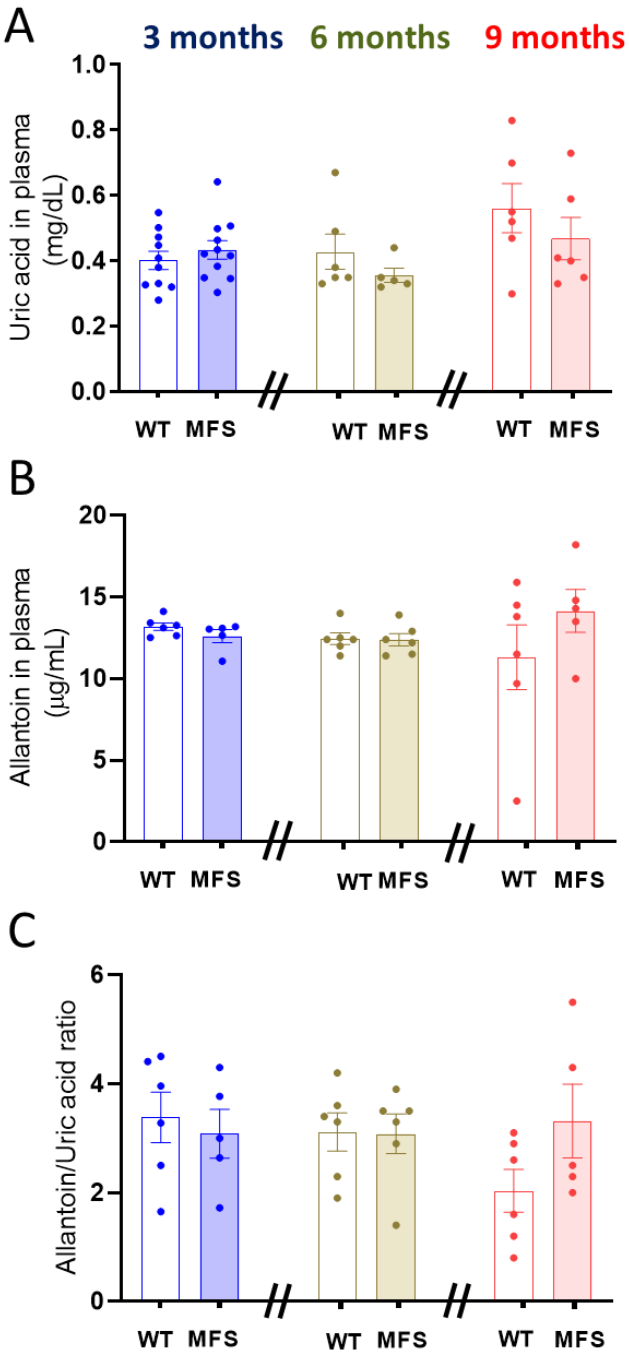

Figure S6. Uric acid and allantoin blood plasma levels are not altered in MFS mice. Blood plasma levels of uric acid (A), its catabolite allantoin (B) and their ratio (Allantoin/uric acid) (C) measured in WT and MFS mice of different ages (3-, 6-, and 9-month-old). Data as the mean ± SEM. Statistical analysis: Kruskal-Wallis with Dunn's multiple comparison test. n= 5-12.

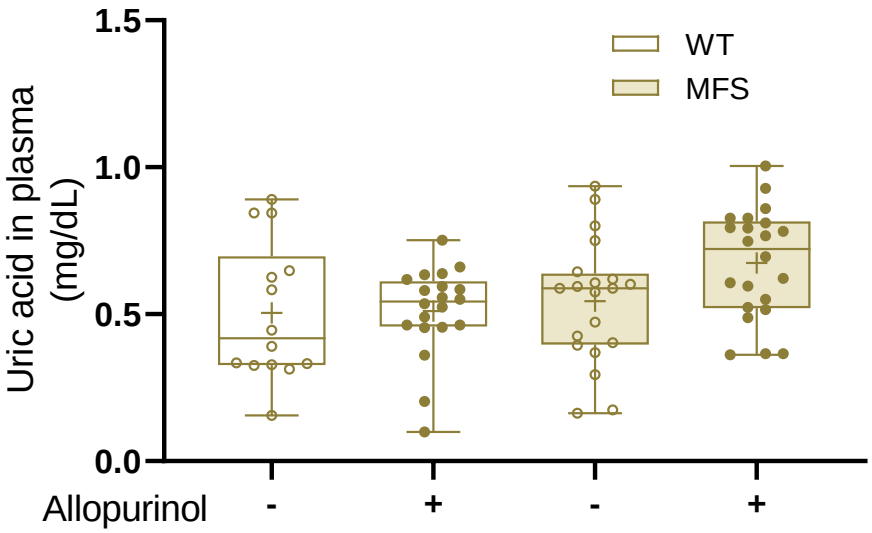

Figure S7. Plasma levels of uric acid do not change following allopurinol administration in WT and MFS mice. Uric acid blood plasma levels in WT and MFS mice palliatively treated with allopurinol (PA1) (n=14-22). Data represented as boxplots. Statistical test analysis: Two-way ANOVA with Tukey's post-test.

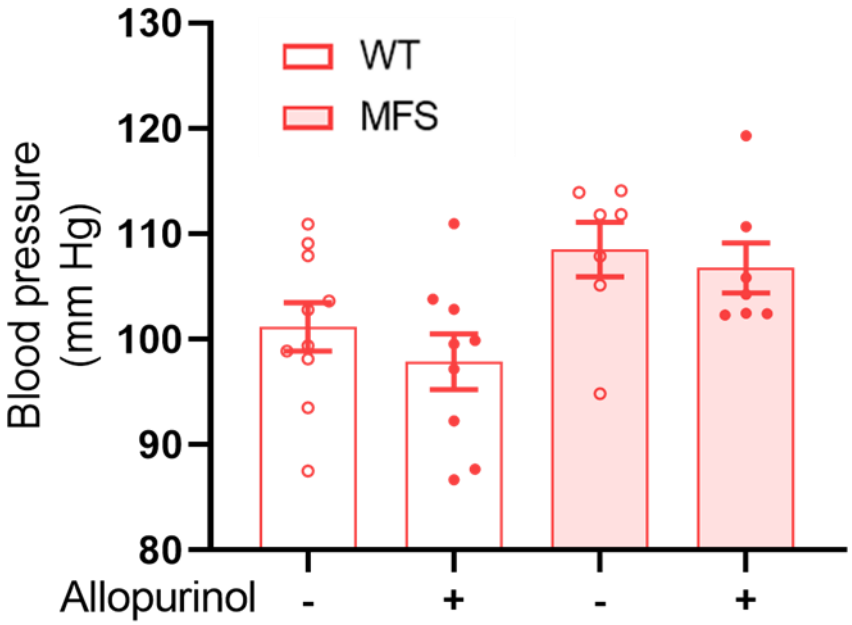

Figure S8. Allopurinol does not alter systolic blood pressure. Systolic blood pressure measurements in 9-month-old WT and MFS mice palliatively treated with allopurinol for 28 weeks (PA2) (n=6-10). Data as the mean ± SEM. Statistical test analysis: Kruskal-Wallis with Dunn's multiple comparison test.

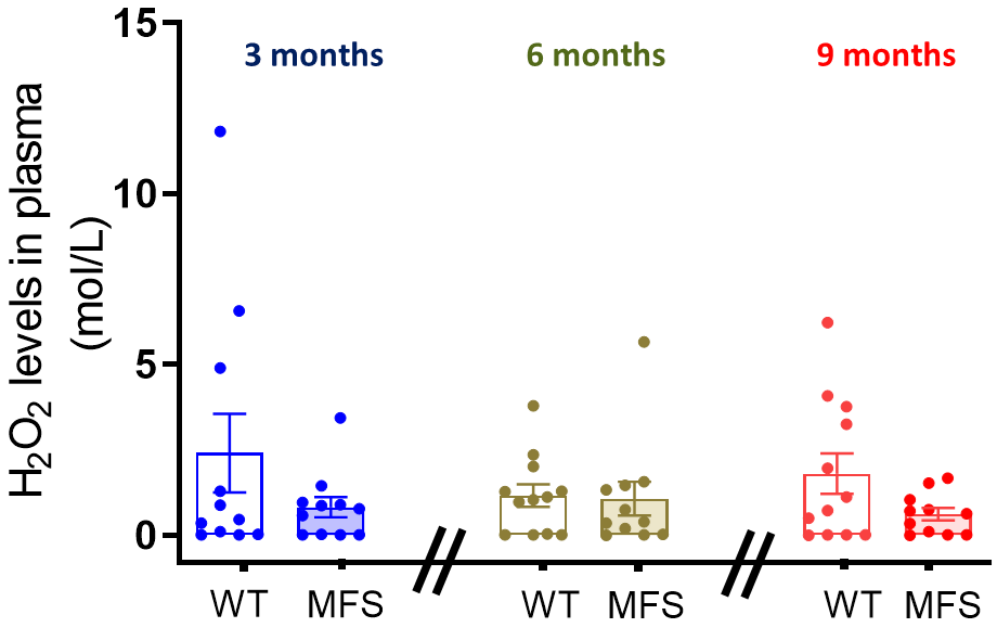

Figure S9. H_2O_2 plasma levels do not change with age in MFS mice. H_2O_2 levels in the blood plasma of WT and MFS of different ages (3-, 6-, and 9-monthold) (n=12-14). Data as the mean ± SEM. Statistical test analysis: Kruskal-Wallis with Dunn's multiple comparison test.

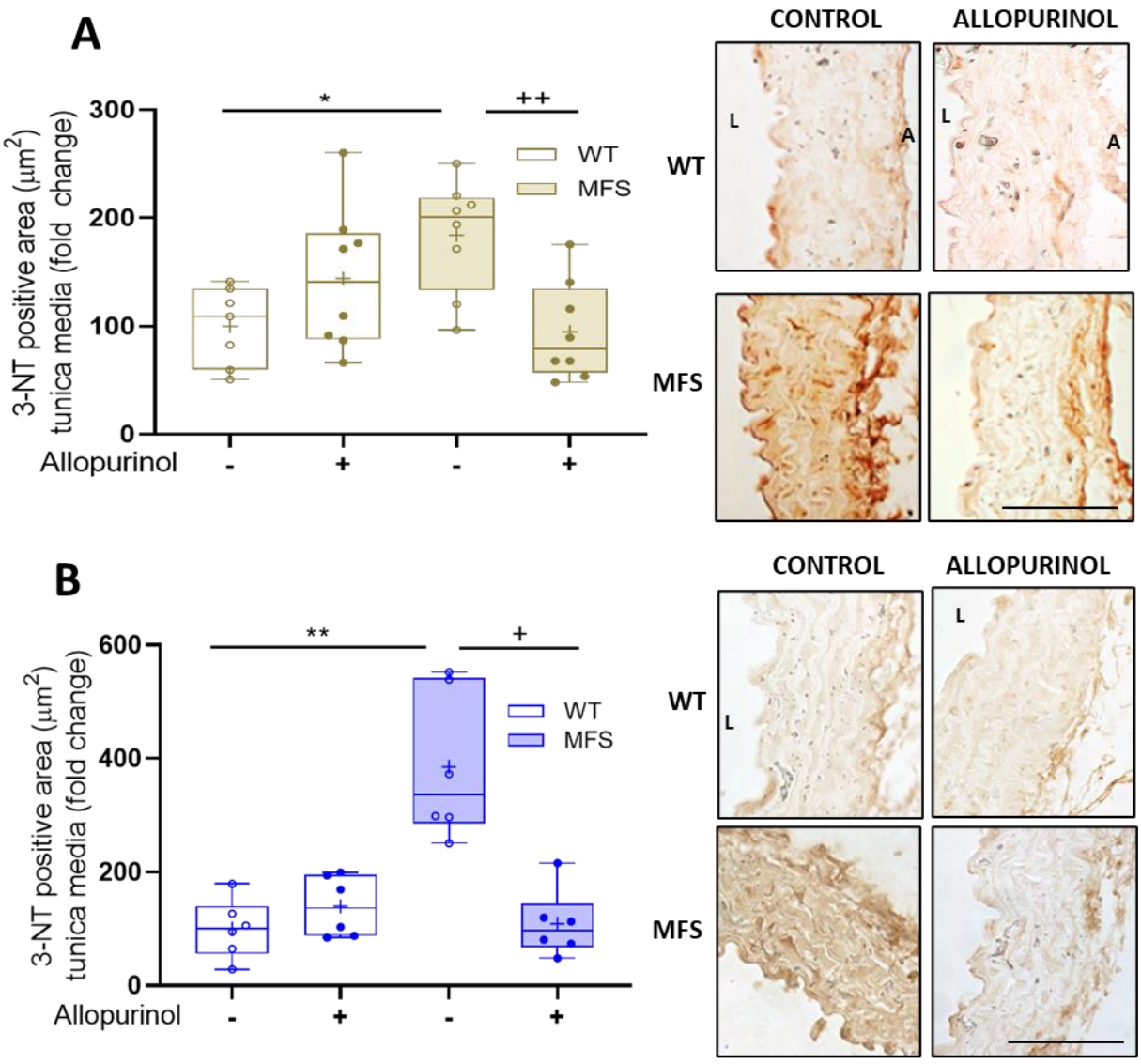

Figure S10. Allopurinol reduces redox stress-associated 3´-nitrotyrosine levels in the tunica media of MFS aorta. Quantitative analysis and representative images of the 3-NT levels in the aortic tunica media evidenced by immunohistochemistry with anti-3-NT antibodies after palliative (PA1) **(A)** and preventive (PE) **(B)** treatments with allopurinol in WT and MFS mice. Bar, 100 µm. Data represented as boxplots. Statistical test analysis: Two-way ANOVA and Tukey's post-test (A); Kruskal-Wallis and Dunn's multiple comparison tests (B). $^{**/++}P \leq 0.01$ and $^{*/+}P \leq 0.05$; *effect of genotype; $^{+}$effect of treatment.

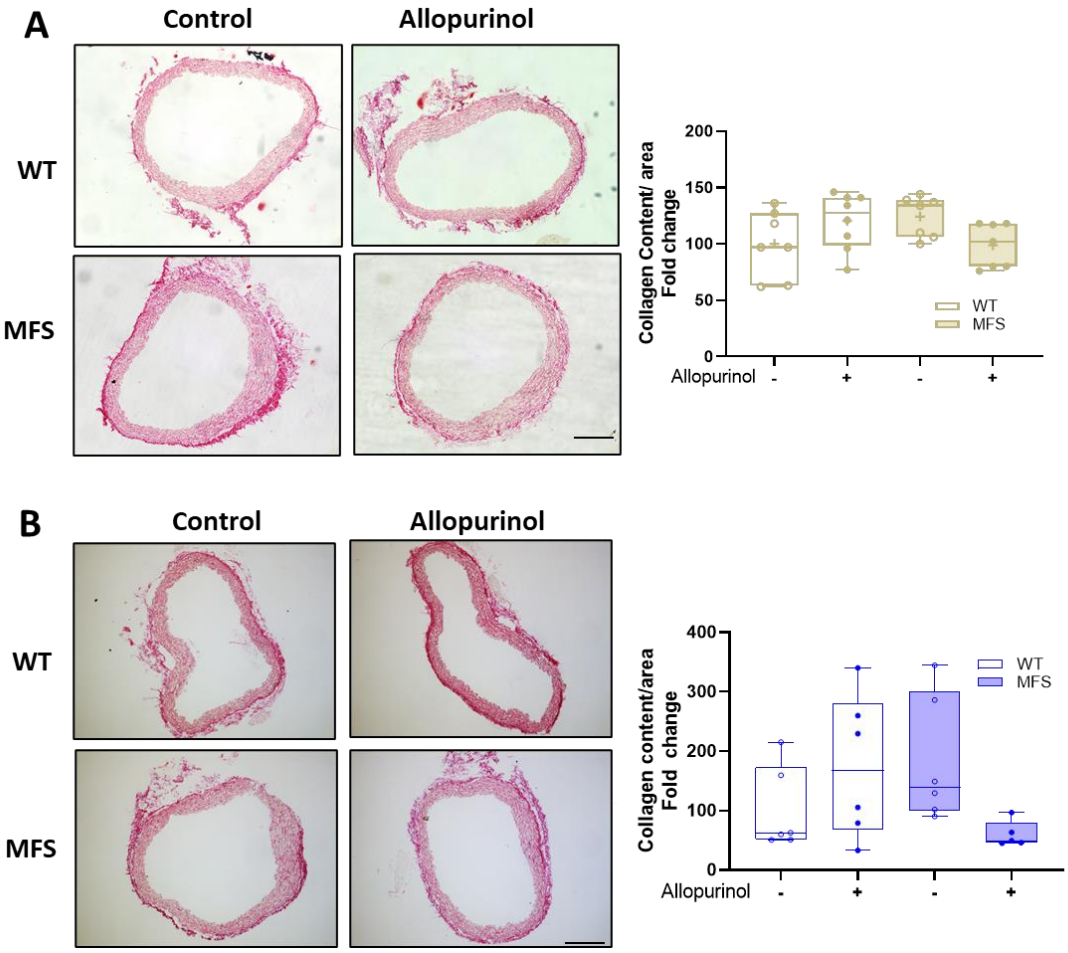

Figure S11. Collagen total content in the aortic tunica media of MFS mice following the administration of allopurinol. Picrosirius red staining of WT and MFS aortae from non-treated (control) or treated with allopurinol following its palliative (PA1) or preventive (PE) administration (A and B, respectively). At their respective side, the quantitative analysis of the staining at the media. Bar, 100 µm.

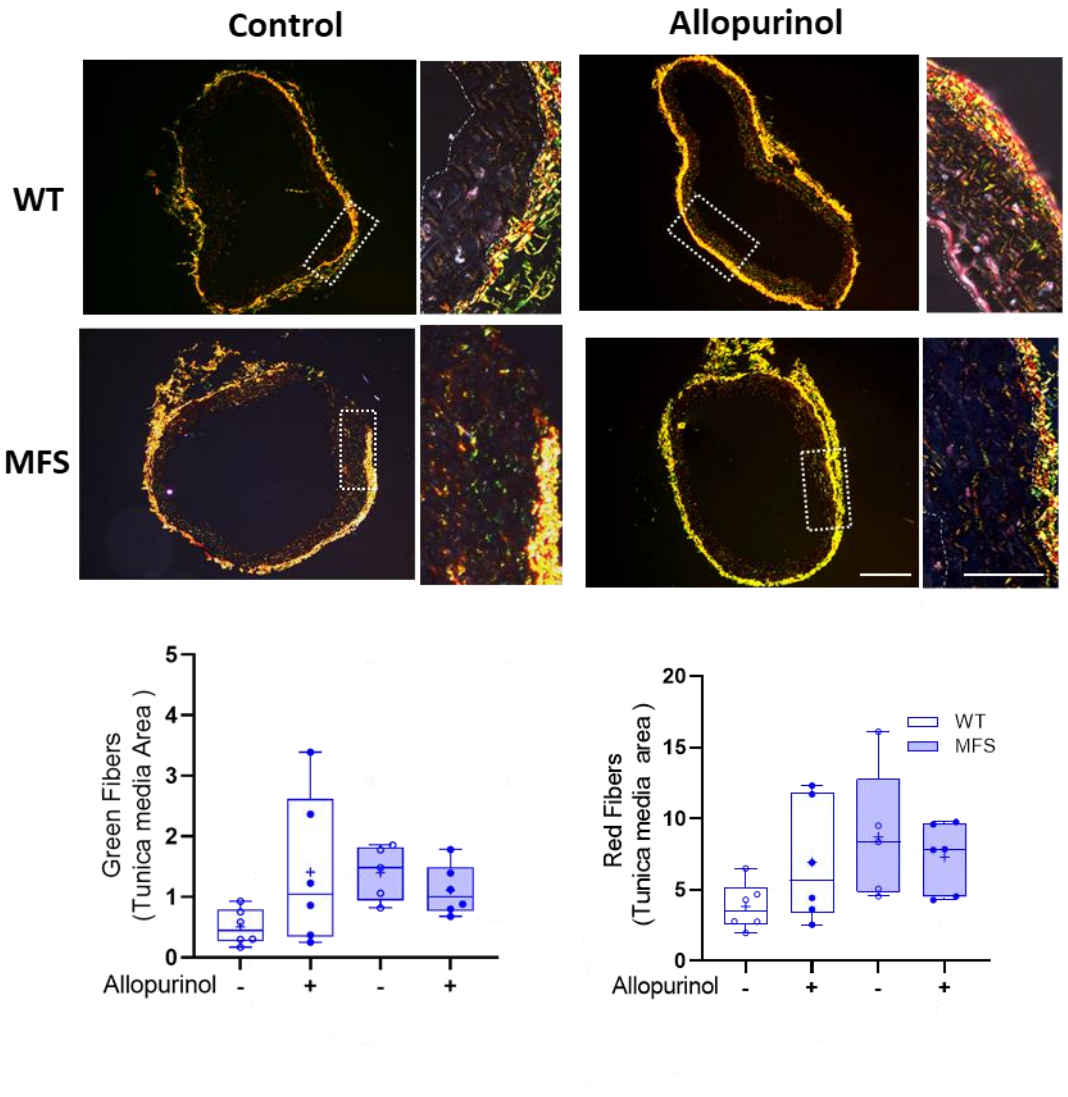

Figure S12. Collagen maturation state in the aortic tunica media of MFS mice following the preventive treatment with allopurinol. Immature (green) and mature (red) collagen fibers of the tunicae media and adventitia of WT and MFS aortae stained with Picrosirius red visualized under the polarized microscope see Fig. S11). WT and MFS mice were treated allopurinol in a preventive manner (PE) (n=5-6).). Representative fluorescence images of the whole aorta and enhanced indicated regions (white dashed lines). In enhanced images on the right of each panel, the media is at the center and the adventitia is on its right. The respective quantitative analysis of both types of collagen fibers is shown below images. Bar, 100 µm. Data represented as boxplots. Statistical test analysis: Kruskal-Wallis and Dunn's multiple comparison tests.

3. SUPPLEMENTAL TABLES

Table S1. Primers used in RT-PCR analysis in MFS mice.

Gene	Primer sequences
Xdh	Fw: 5´- GGAGATATTGGTGTCCATTGTG -3´ Rv: 5´- CCTGCTTGAAGGCTGAGAAA -3´
Nox4	Fw: 5'- ACGTCCTCGGTGGAAACTT-3' Rv: 5'-AGTGAATTGGGTCCACAACAG-3'
Gapdh	Fw: 5´- TTGATGGCAACAATCTCCAC -3´ Rv: 5´- CGTCCCGTAGACAAAATGGT-3´

Table S2. Echocardiographic values of the aortic root diameter (in mm) of WT and MFS mice subjected to palliative treatment (PA1) in the presence (+) or absence (-) of allopurinol after treatment for 4 months (from 2-to-6 month-old mice). Graphic shown in Fig. 3A. Data pooled by sex is included.

Aortic root diameter at 6-month-old mice (PA1)				
Allopurinol	**WT**		**MFS**	
-	**1.59±0.02**	**12**	**1.83±0.03*** **	**21**
	♂ 1.58±0.03	8	♂1.85±0.05*** **	11
	♀1.59±0.05	4	♀1.74±0.06*** **	10
+	**1.51±0.04**	**20**	**1.64±0.05^{++}**	**23**
	♂1.56±0.07	10	♂1,75±0.09	9
	♀1.45±0.03	10	♀1.57±0.04^{+}	14

Statistical analysis: Three-Way ANOVA. *Effect of genotype; $^{+}$effect of treatment. ***p≤0.001; ^{++}p≤0.01; ^{+}p≤0.05. The number of animals is indicated on the right of each column. Sex: ♂ males, ♀ females.

Table S3. Echocardiographic values of the aortic root diameter (in mm) of WT and MFS mice subjected to preventive treatment (PE) in the presence (+) and absence (-) of allopurinol. Graphics shown in Fig. 3C.

Aortic root diameter at 3-month-old mice (PE)				
Allopurinol	**WT**		**MFS**	
-	1.45±0.04	11	1.71±0.04***	9
+	1.27±0.04	11	1.35±0.04^{+++}	10

Statistical analyses: Two-Way ANOVA followed by Tukey post-test. *effect of genotype; $^{+}$effect of treatment. ***, ^{+++}p≤0.001; ^{++}p≤0.01; *p≤0.05. The number of animals is indicated on the right of each column.

Table S4 Echocardiographic values of the ascending aorta diameter (in mm) of WT and MFS mice subjected to palliative treatment (PA1) in the presence (+) or absence (-) of allopurinol after treatment for 4 months (2-to-6-month-old mice). Graphic shown in Fig. S3A.

Ascending Aorta diameter at 6-month-old mice (PA1)				
Allopurinol	**WT**		**MFS**	
-	**1.21±0.02**	**15**	**1.39±0.04*** **	**22**
	♂1.21±0.04	7	♂1.50±0.06	11
	♀1.21±0.01	8	♀1.28±0.04	11
+	**1.26±0. 03**	**20**	**1.32±0.05^{+}**	**22**
	♂1.34±0.04	10	♂1.47±0.11	9
	♀1.19±0.02	10	♀1.24±0.02	13

Statistical analyses: Two-way ANOVA followed by Tukey post-test. *Effect of genotype; $^{+}$effect of treatment. ***$p\leq0.001$; $^{+}p\leq0.05$. The number of animals is indicated on the right of each column.

Table S5. Echocardiographic values of the ascending aorta diameter (in mm) of WT and MFS mice subjected to preventive treatment (PE) in the presence (+) and absence (-) of allopurinol. Graphics shown in Fig. S3B.

Ascending aorta diameter at 3-month-old mice (PE)				
Allopurinol	**WT**		**MFS**	
-	1.10±0.16	11	1.29±0.13*	9
+	1.17±0.16	11	1.30±0.08	10

Statistical analyses: Two-way ANOVA followed by Tukey post-test. *Effect of genotype; $^{+}$effect of treatment. *$p\leq0.05$. The number of animals is indicated on the right of each column.

Table S6. Echocardiographic values of the aortic root diameter (in mm) of WT and MFS mice subjected to preventive treatment with allopurinol from gestation until endpoint at 3-month-old /PE). Thereafter, allopurinol was withdrawn for 3 months, and mice subjected to ultrasonography (endpoint at 6-month-old/PE<ALO). Graphics shown in Fig. S4A.

	Aortic root diameter at 3-month-old mice (PE)				**Aortic root diameter at 6-month-old mice (PE<ALO)**			
Allopurinol	**WT**		**MFS**		**WT**		**MFS**	
-	1.54±0.01	8	1.73±0.02***	9	1.61±0.01	7	1.84±0.01***	8
+ (in PE only)	1.51±0.03	9	1.52±0.03^{+++}	10	1.60±0.01	9	1.77±0.02	8

Statistical analyses: Two-way ANOVA followed by Tukey post-test. *Effect of genotype; $^{+}$effect of treatment. ***,$^{+++}$$p$≤0.001. The number of animals is indicated on the right of each column.

Table S7. Echocardiographic values of the aortic root diameter (in mm) of WT and MFS mice subjected to palliative treatment with allopurinol (from 2-to-6-month-old/PA1). Thereafter, allopurinol was withdrawn for a period of 3 months (until 9-month-old), and mice were subjected to endpoint ultrasonography (9 month-old/PA1<ALO). Graphics shown in Fig. S4B.

	Aortic root diameter at 6-month-old mice (PA1)				**Aortic root diameter at 9-month-old mice (PA1<ALO)**			
Allopurinol	**WT**		**MFS**		**WT**		**MFS**	
-	1.61±0.01	7	1.87±0.02***	6	1.76±0.03	7	1.98±0.05***	6
+ (in PA1 only)	1.60±0.02	13	1.66±0.02^{+++}	13	1.73±0.02	13	1.97±0.02	12

Statistical analyses: Two-way ANOVA followed by Tukey post-test. *Effect of genotype; $^{+}$effect of treatment. ***,$^{+++}$$p$≤0.001. The number of animals is indicated on the right of each column.

Table S8. Echocardiographic values of the aortic root diameter (in mm) of WT and MFS mice subjected to palliative treatment (PA2) with allopurinol for 6 months (from 2- to 9-month-old mice). Data pooled for sex is also shown. Graphics shown in Fig. S5.

Aortic root diameter at 9-month-old mice (PA2)				
Allopurinol	**WT**		**MFS**	
-	**1.70±0.01**	**16**	**2.02±0.02*****	**14**
	♂ 1.69±0.02	7	♂2.07±0.03***	8
	♀1.72±0.02	9	♀1.94±0.01***	6
+	**1.74±0.01**	**16**	**1.76±0.02^{+++}**	**15**
	♂1.75±0.02	7	♂1.74±0.03^{++}	8
	♀1.73±0.02	9	♀1.79±0.03^{+++}	7

Statistical analysis: Three-Way ANOVA. *Effect of genotype; $^{+}$effect of treatment. ***, $^{+++}p\leq0.001$; $^{++}p\leq0.01$; $^{+}p\leq0.05$. The number of animals is indicated on the right of each column. Sex: ♂ males, ♀ females.

Table S9. Potency (pEC_{50}) and maximum response (E_{max}) of the concentration–response curves (CRCs) values for ACh- and NTP-induced relaxation response (%) in the ascending aorta from WT and MFS mice (9-months-old) in the presence (+) or absence (-) of allopurinol. Graphic shown in Fig. 4D.

Ascending aorta		**WT**				**MFS**			
Allopurinol		**-**		**+**		**-**		**+**	
CRC	E_{max} ***(%FE)***	88.25±2.99	6	89.70±2.52	4	82.09±4.65	6	80.06±5.45	6
ACh	pEC_{50}	7.52±0.10		7.69±0.08		6.85±0.13***		7.49±0.19	
CRC	E_{max} ***(%FE)***	90.21±4.75	5	102.8±2.94	4	102.1±3.45	4	106.7±7.27	5
NTP	pEC_{50}	7.69±0.11		7.96±0.07		7.96±0.08		7.66±0.18	

*Effect of genotype; ***$p\leq0.001$. The number of animals is indicated on the right of each column. Two-way ANOVA followed by Tukey post-test.

Table S10. KCl vascular tone was expressed as force units (mN). Potency (pEC_{50}) and maximum response (E_{max}) of the concentration-response curve (CRC) values for Phe-induced contraction response (%) in the ascending aorta of 9-month-old WT and MFS mice in the presence (+) or absence (-) of allopurinol. The number of animals is indicated on the right of each column. Graphic shown in Figs. 4B and 4E.

Ascending aorta		**WT**				**MFS**			
Allopurinol		**-**		**+**		**-**		**+**	
KCl	***E_{max} (mN)***	4.70±0.52	6	4.56±0.80	5	4.43±0.60	6	4.32±0.49	6
CRC	***E_{max} (%KCl)***	52.67±6.60	6	31.18±9.44	5	75.32±19.06	6	64.70±20.21	5
Phe	***pEC_{50}***	6.46±0.28		7.28±0.80		6.25±0.61		6.35±0.72	