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Allopurinol blocks aortic aneurysm in a mouse model of Marfan syndrome via reducing aortic oxidative stress

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ABSTRACT

Background: Increasing evidence indicates that redox stress participates in MFS aortopathy, though its mechanistic contribution is little known. We reported elevated reactive oxygen species (ROS) formation and NADPH oxidase NOX4 upregulation in MFS patients and mouse aortae. Here we address the contribution of xanthine oxidoreductase (XOR), which catabolizes purines into uric acid and ROS in MFS aortopathy.

Methods and results: In aortic samples from MFS patients, XOR protein expression, revealed by immunohistochemistry, increased in both the tunicae intima and media of the dilated zone. In MFS mice (*Fbn1*$^{C1041G/+}$), aortic *XOR* mRNA transcripts and enzymatic activity of the oxidase form (XO) were augmented in the aorta of 3-month-old mice but not in older animals. The administration of the XOR inhibitor allopurinol (ALO) halted the progression of aortic root aneurysm in MFS mice. ALO administrated before the onset of the aneurysm prevented its subsequent development. ALO also inhibited MFS-associated endothelial dysfunction as well as elastic fiber fragmentation, nuclear translocation of pNRF2 and increased 3′-nitrotyrosine levels, and collagen maturation remodeling, all occurring in the tunica media. ALO reduced the MFS-associated large aortic production of H_2O_2, and NOX4 and MMP2 transcriptional overexpression.

Conclusions: Allopurinol interferes in aortic aneurysm progression acting as a potent antioxidant. This study strengthens the concept that redox stress is an important determinant of aortic aneurysm formation and progression in MFS and warrants the evaluation of ALO therapy in MFS patients.

1. Introduction

Marfan syndrome (MFS) is a relatively common inherited rare disease of the connective tissue caused by mutations in the gene encoding the extracellular matrix glycoprotein fibrillin-1 [1]. This multisystem disease mainly affects the skeleton (abnormally long bones and spine deformations), eyes (lens dislocation) and aorta (aortic root aneurysm). The latter commonly leads to aortic dissection and rupture, the main cause of reduced life expectancy in patients [2].

Reactive oxygen species (ROS) consist of radical (anion superoxide/O_2^- and hydroxyl radical/HO) and non-radical (hydrogen peroxide/H_2O_2) oxygen species, which are highly reactive chemicals formed by the partial reduction of oxygen. O_2^- and H_2O_2 are the most frequently formed ROS and, under physiological concentrations, have important signaling functions (oxidative eustress) [3]. However, when ROS

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Nonstandard abbreviations

ALO	allopurinol
CRCs	cumulative concentration-response curves
3-NT	3′-nitrotyrosine
PA	palliative experimental treatment with allopurinol
PE	preventive experimental treatment with allopurinol
PE < ALO and PA < ALO	respective PE and PA treatments with subsequent allopurinol withdrawal

overwhelm the intrinsic cellular antioxidant system, either via an abnormal overproduction of ROS or reduction of their antioxidant capacity, they contribute to pathogenesis (oxidative stress), causing transient or permanent damage to nucleic acids, proteins, and lipids [4,5].

In the cardiovascular system, ROS are involved in, among other dysfunctions, the development of aortic abdominal aneurysms (AAA) through the regulation of inflammation induction, matrix metalloproteinases (MMPs) expression, vascular smooth muscle cell (VSMC) apoptosis and phenotypic changes and modifying extracellular matrix properties [6–10]. However, the impact of ROS on thoracic aortic aneurysm (TAA) of genetic origin, such as in MFS and Loeys-Dietz syndrome (LDS), is less known [11]. We and others have previously shown that ROS production is increased in MFS [12–18], although the specific generators of ROS and their respective impact on the formation and/or progression in either human or murine MFS aortic aneurysms is still poorly understood [19,20]. We reported the involvement of upregulated NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 4 (NOX4) both in human and mouse MFS aortic samples and cultured VSMCs [17]. Nonetheless, besides NOX4, another important source of ROS in the cardiovascular system is xanthine oxidoreductase (XOR) [21].

XOR is involved in the final steps of nucleic acid-associated purine degradation and, particularly, in the conversion of hypoxanthine into xanthine and xanthine into urate. XOR exists in two forms that are derived from a single gene (*XDH*) [22]. The reduced form of XOR is referred to as xanthine dehydrogenase (XDH), and the oxidized form as xanthine oxidase (XO). XDH can be post-translationally modified to XO via proteolysis or oxidation of critical cysteines [23]. The XDH form has greater abundance and affinity for NAD^+ as the electron acceptor to generate NADH, which is a critical substrate for NADPH oxidases. Likewise, the XO form is mainly associated with the production of large amounts of O_2^- and H_2O_2 by preferentially using oxygen as the electron acceptor [24,25]. Under healthy conditions, XDH is constitutively expressed, and XO levels are low both in plasma and heart [26]. However, XDH conversion into XO is favored by hypoxia, low pH, ischemia, inflammation, and the presence of peroxynitrite and H_2O_2 itself [27–29]. XOR is widely distributed throughout the organism mainly in the liver and gut, but also present in intestine, lung, kidney, myocardium, brain and plasma [30]. In the vascular endothelium, XOR is bound to cell surface glycosaminoglycans [26]; the enhancement of endothelium attached XOR favors local ROS production with subsequent endothelial dysfunction [31]. XOR-derived anion superoxide (O_2^-) easily interacts with endothelial cell-generated nitric oxide (NO) forming peroxynitrite ($ONOO^-$), which in the endothelium of the tunica intima and VSMCs of the media irreversibly generates reactive nitrogen species (RNS) residues such as 3′-nitrotyrosine [32,33], which is usually used as a redox stress marker. Increased XOR levels have been reported in aortic samples from adult MFS mice [14] and LDS patients [34], even though the extent to which XOR contributes to TAA pathogenesis is unknown.

On the other hand, concomitantly to ROS production, XOR generates uric acid (UA), which in humans can pathologically accumulate in the plasma and some tissues. In rodents, UA is rapidly catabolized by uricase (absent in humans) to allantoin [35]. UA has a dual role in redox biology, acting as an antioxidant (both *in vitro* and *in vivo*) accounting for as much as 50% of the total antioxidant capacity of biological fluids in humans [36]. However, when UA accumulates in the cytoplasm or in acidic/hydrophobic milieu, it acts as a pro-oxidant promoting redox stress [36,37]. UA was found in the wall of human aortic aneurysms and atherosclerotic arteries [38] and there was a positive correlation between serum UA levels and aortic dilation and dissection [39,40]. Epidemiologic and biochemical studies on UA formation have demonstrated that it is not only UA itself that leads to a worse prognosis and increased cardiovascular events, but also ROS formed during XOR activity. Therefore, the resulting combined action of excessively formed UA and ROS surely contribute to oxidative stress-linked cardiovascular pathological events [41].

The aim of this study was to investigate the participation of XOR in MFS aortopathy in more detail and evaluate the effectiveness of the XOR inhibitor allopurinol (ALO) on the formation and/or progression of MFS-associated aortopathy. ALO is an analogue of hypoxanthine and, therefore, a competitive inhibitor of XOR [42]; it is a drug routinely prescribed to treat hypouricemic and hypertensive patients [43]. XOR also functions beyond its basic housekeeping role in purine catabolism, acting in oxidant signaling, which could contribute to exacerbating oxidative stress-associated MFS aortopathy. Therefore, since XOR is an important source of ROS in the cardiovascular system, we hypothesized that, together with upregulated NOX4 [17,44], the formation and/or progression of aortic aneurysm in MFS is favored by abnormal aortic XOR activity with the consequent dysfunctional increase in ROS levels (and likely UA and/or allantoin as well) exacerbating the oxidative stress-associated MFS aortopathy.

2. Material and methods

Please see Methods in Supplemental Materials [92–96].

3. Results

3.1. XOR is augmented in the dilated aorta of MFS patients

We evaluated protein levels by immunohistochemistry with specific anti-XOR antibodies in the aorta of MFS patients subjected to aortic reparatory surgery. Unlike the tunica adventitia, both the tunicae intima and media of the dilated aortic zone presented a significant increase in immunostaining compared with adjacent non-dilated and healthy aortae (Fig. 1), which is demonstrative of an increased protein expression of XOR associated with aortic aneurysm in MFS patients. No correlation between the intensity of the immunostaining and the age of the patients analyzed was observed.

3.2. XOR mRNA expression and enzymatic activity are only increased in the aorta of young MFS mice

We next evaluated whether XOR was also increased in the MFS mouse aorta. Firstly, we evaluated XOR transcriptional levels by RT-PCR in 3- and 6- and 9-month-old WT and MFS mice. Aortae from 3-month-old MFS mice showed significantly increased levels of *XOR* transcripts compared with age-matched WT animals. This increase was not observed in 6- or 9-month-old mice (Fig. 2A). Immunohistochemistry with anti-XOR antibodies confirmed the increased expression of XOR in the endothelial cell layer (intima) and in the media only in 3-month-old MFS aortic wall (Fig. 2B). The adventitia was also stained for XOR, but just like in the human aorta, nonsignificant changes were observed over the age.

To differentiate the functional contribution of the XDH and XO protein forms of XOR in the MFS aorta, we next measured their respective enzymatic activities in aortic lysates (see a representative assay in Fig. S2). The XO/XDH enzymatic activity ratio was significantly higher in 3-month-old but not in 6- or 9-month-old MFS mice (Fig. 2C),

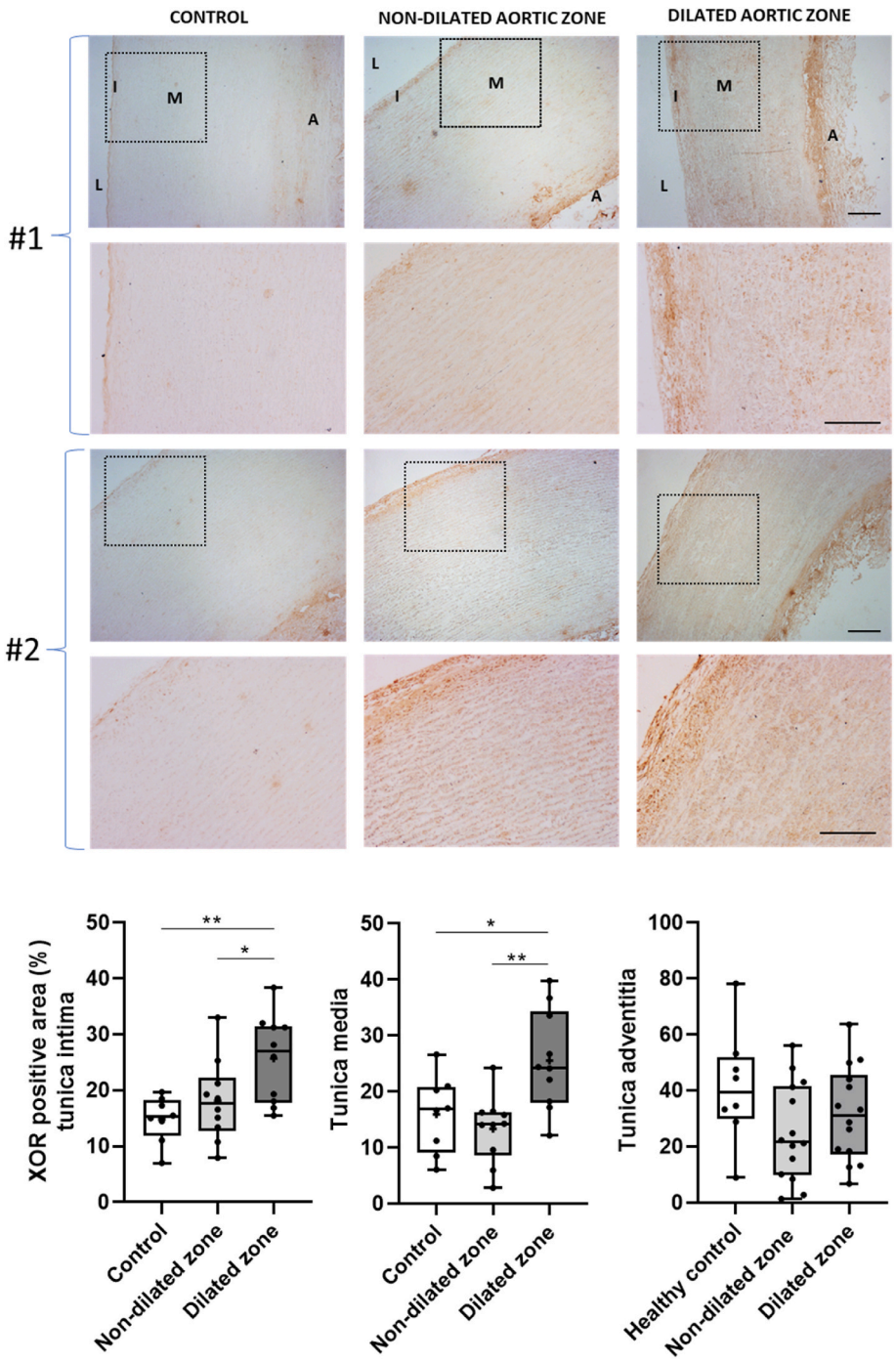

Fig. 1. Xanthine oxidoreductase is upregulated in the aorta of MFS patients. Representative images of XOR immunostaining in the ascending aorta from two healthy heart donors (control) and in the dilated and adjacent non-dilated aneurysmal zones of the aorta from two representative MFS patients (#1 and #2). The lower panels of each aortic sample are magnified images from the corresponding upper sample (black squares). L = lumen; I: tunica intima; M: tunica media; A: tunica adventitia. Below of images, quantitative analysis of immunolabeling results evaluated in tunicae intima, media, and adventitia. Bars, 100 μm. Statistical test analysis: One-way ANOVA; $*p \leq 0.05$ and $**p \leq 0.01$.

which is in accordance with transcriptional results (Fig. 2A). In this manner, results complement the previously reported upregulation of XOR in MFS mouse aortae [14].

3.3. Allopurinol inhibits the progression of aortic aneurysm and prevents its formation in MFS mice

The upregulation and activity of XOR in MFS aortae suggest its contribution to oxidative stress in MFS aortopathy. We hypothesized that the inhibition of its activity could ameliorate aortic aneurysm progression. To test this hypothesis, we treated WT and MFS mice with ALO. We first palliatively treated 2-month-old mice with ALO until 6 months of age (PA1; Fig. S1) and then evaluated aneurysm progression by ultrasonography. ALO significantly reduced the characteristic enlarged aortic root diameter occurring in MFS mice, the diameter obtained being highly similar to WT animals (Fig. 3A and Table S2). ALO did not cause any alteration in the aortic root diameter of WT mice. Moreover, no sex differences were observed regarding the effectiveness of ALO (Table S2).

We also evaluated aortic wall organization by histomorphology, quantifying the number of large elastic fiber ruptures as previously defined [45]. ALO treatment in MFS mice caused an apparent normalization of elastic fiber organization and integrity, their overall morphology being indistinguishable from non-treated WT animals (Fig. 3B).

ALO has also been safely administered during the late stages of

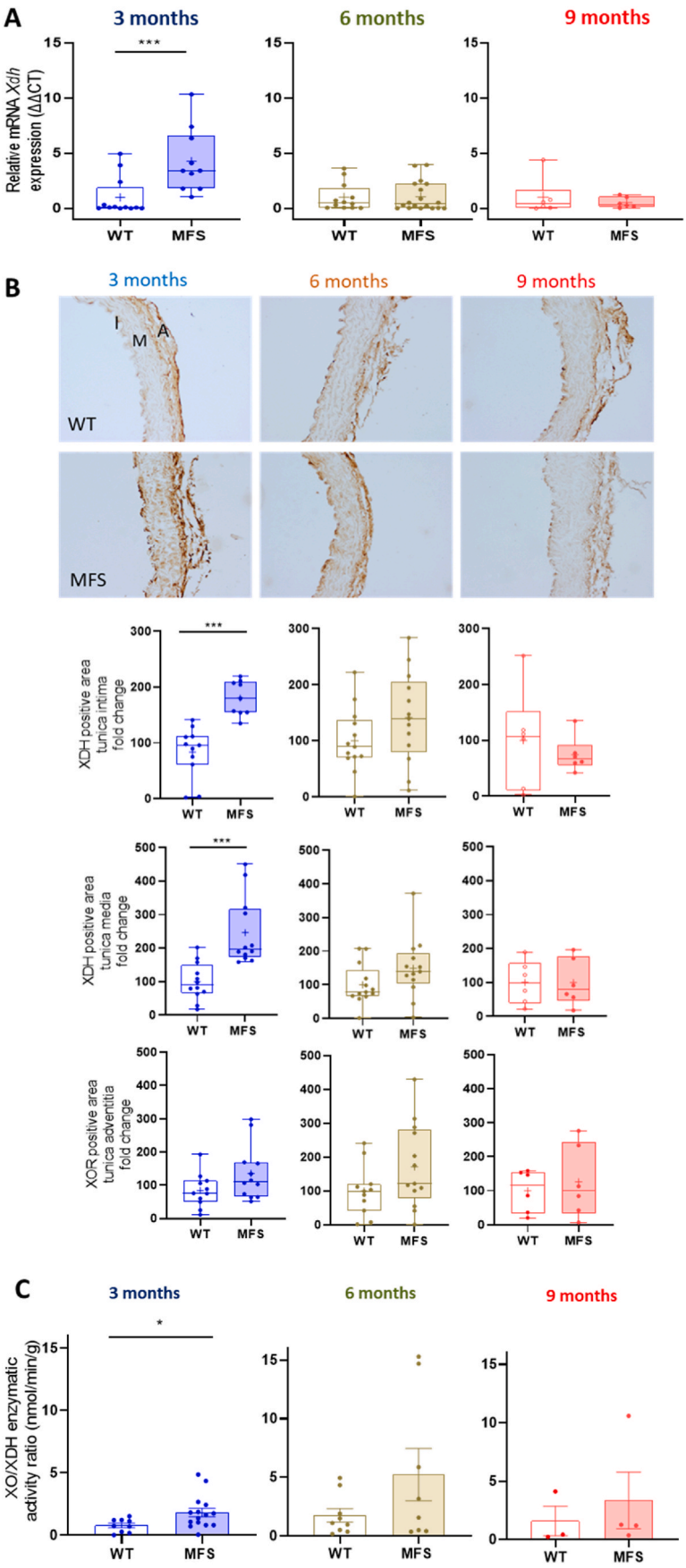

Fig. 2. XOR expression levels and enzymatic activity in the MFS mouse ascending aorta. **(A)** mRNA expression levels of XOR (*Xdh*) in WT and MFS mice of different ages (3-, 6- and 9-month-old). **(B)** XOR protein levels revealed by immunohistochemistry with anti-XOR antibodies in paraffin-embedded aortae from 3-, 6- and 9-month-old WT and MFS mice. Below of images, the quantitative analysis of the respective HRP immunostaining in tunicae intima (endothelial cell layer) (I), media (M) and adventitia (A). Bar, 100 μm. **(C)** XO/XDH enzymatic activity ratio in WT and 3-, 6-, and 9-month-old MFS mice. Data represented as boxplots. Statistical test analysis: Mann Whitney *U* test (A–C). ***$p \leq 0.001$ and *$p \leq 0.05$.

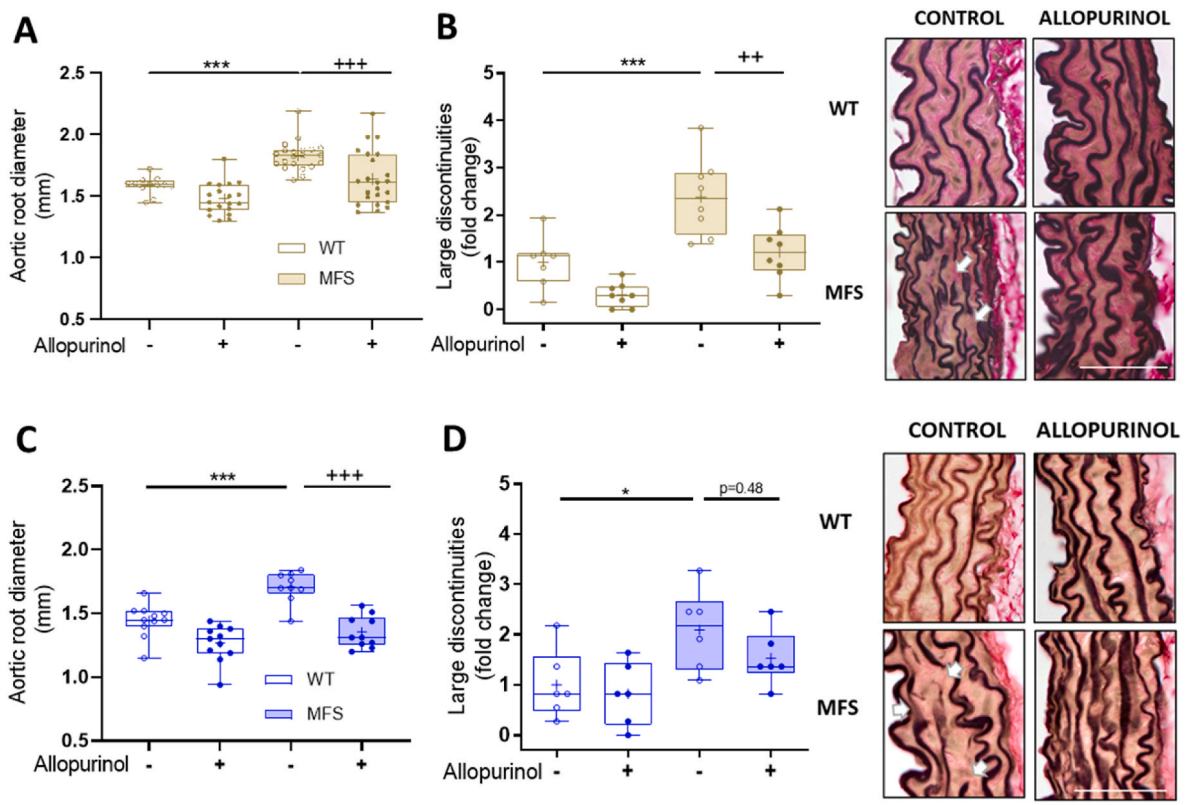

Fig. 3. Allopurinol prevents both the formation and progression of aortic root dilation in MFS mice. (A) Allopurinol halts the progression of aortic root dilatation in MFS mice measured by ultrasonography in WT and MFS mice of 6 months of age treated with allopurinol for 16 weeks (PA1). See also Table S2. **(B)** The number of large discontinuities in the elastic lamina of the aortic media from WT and MFS mice palliatively treated with allopurinol. **(C)** Allopurinol prevents the formation of aortic root dilatation in MFS mice measured by ultrasonography in 3-month-old WT and MFS mice preventively treated with allopurinol for 12 weeks (PE). See also Table S3. **(D)** Number of large discontinuities in the elastic lamina of the aortic media from WT and MFS mice preventively treated with allopurinol. On the right of panels B and D, representative Elastin Verhoeff-Van Gieson staining in paraffin sections of the ascending aorta. White arrows in B and D indicate examples of the large discontinuities analyzed. Bar, 100 μm. Data represented as boxplots. Statistical test analysis: Two-way ANOVA and Tukey post-test. $^{***/+++}p \leq 0.001$, $^{++}p \leq 0.01$ and $^{*}p \leq 0.05$; *effect of genotype; $^{+}$effect of treatment.

pregnancy, demonstrating efficiency in reducing uric acid, hypertension, and oxidative stress levels [46–49]. Therefore, we next evaluated whether ALO also prevented the formation of the aneurysm. ALO was administered to pregnant and lactating female mice and then to weaning offspring until the age of 3 months (PE; Fig. S1). ALO fully prevented aneurysm formation (Fig. 3C and Table S3), also showing a trend to reduce elastic fiber breaks (Fig. 3D).

We also analyzed the diameter of the tubular portion of the ascending aorta. In the PA1 treatment, ALO also normalized the diameter of MFS aortae (Fig. S3A; Table S4). However, this was not the case for the PE treatment (Fig. S3B; Table S5), likely because of the shorter ALO treatment and the younger age of the MFS mice treated, in which the ascending aorta was still not sufficiently affected as in the PA1 treatment.

3.4. *The inhibition of aortopathy by allopurinol is reversible after drug withdrawal*

We next examined whether the progression of the aortopathy inhibited by ALO was permanent or transient. To this end, we evaluated the aortic root diameter after the withdrawal of ALO following PA1 and PE treatments (PA1<ALO and PE < ALO, respectively; Fig. S1). When 6-month-old MFS animals were subjected to PA1 treatment with ALO and subsequently withdrawn from the drug for three months, until reaching 9 months of age, no statistical difference was obtained between their respective aortic root diameters (dashed red boxes of PA1<ALO 9-month-old MFS groups in Fig. S4A; Table S6). On the other hand, the 3-month-old animals subjected to PE treatment with ALO were withdrawn from the drug until reaching 6 months of age (WT and MFS groups labeled with red circles in Fig. S4B). There was no statistical difference between ALO-treated MFS groups regardless of whether ALO was subsequently withdrawn or not ($p = 0.08$) (dashed brown boxes of PE < ALO 6-month-old MFS groups in Fig. S4B; Table S7). Therefore, results show that the inhibitory effect of ALO on the aneurysm is only effective while the drug is administered.

3.5. *Allopurinol prevents endothelial dysfunction in MFS ascending aorta*

ROS generated by endothelial XOR could mediate alterations in the endothelial-associated vascular function by reducing NO bioavailability via direct interaction with O_2^- [32,33,50]. Previous evidence indicated that the MFS aorta shows endothelial dysfunction [14,51,52]. Thus, we analyzed the potential therapeutic effects of ALO treatment on endothelial function (Fig. 4). To this aim, we used wire-myography to measure the reactivity of endothelium-intact ascending aortas from 9-month-old MFS mice (PA2; Fig. S1), age at which, in our hands, endothelial dysfunctions were more clearly observed [52]. Firstly, we examined aortic root diameter in this mouse subset and the aneurysm was also inhibited after seven months of ALO treatment (PA2) (Fig. S5 and Table S8). Thereafter, ascending aortae were isolated, vessels were settled in the myograph and contracted with KCl to check their functionality (Fig. 4A). All aortae responded similarly to KCl (Fig. 4B and Table S10). The relaxant response to acetylcholine (ACh; Fig. 4A), mostly mediated by activation of endothelial NOS (NOS3), is an indicator of endothelial function. Untreated MFS mice showed a reduced sensitivity (pEC_{50}) to endothelium-dependent ACh-induced relaxation

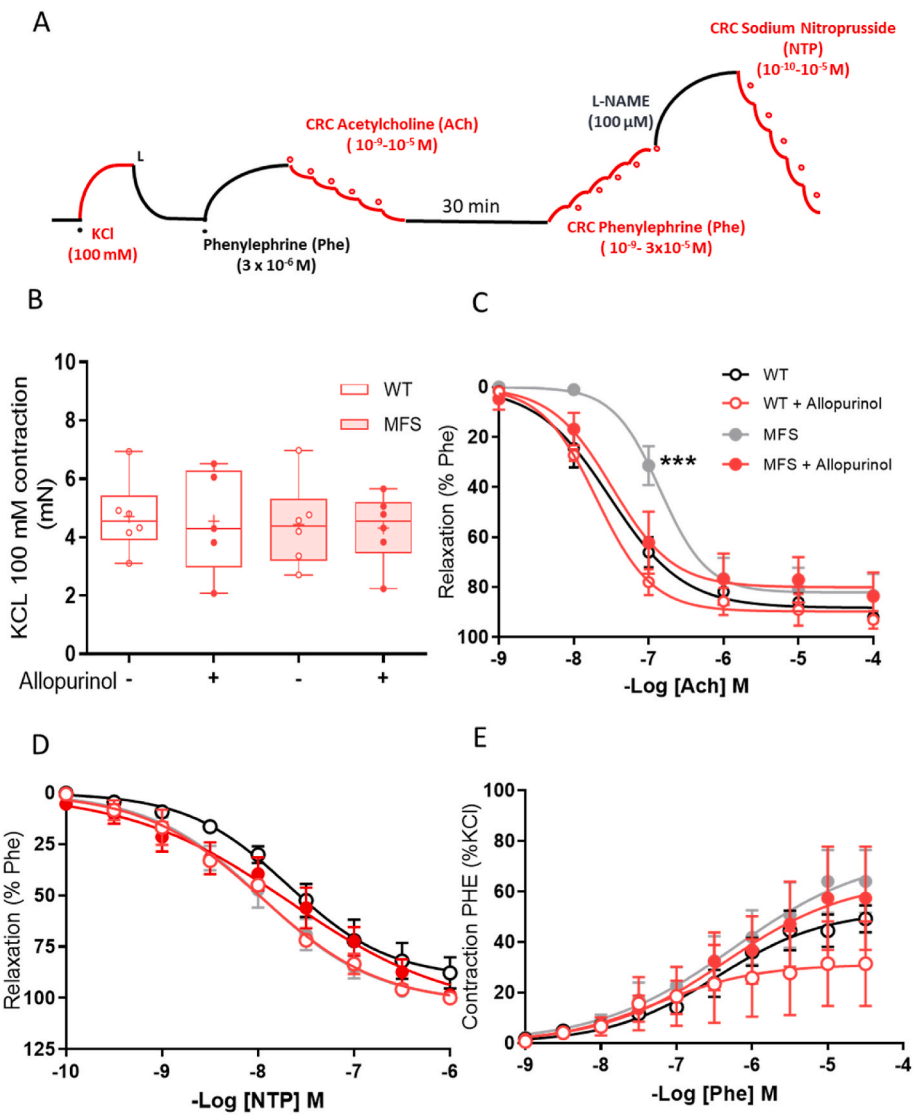

Fig. 4. Allopurinol prevents the endothelial vasodilation dysfunction of MFS aorta. (A) Experimental protocol for wire-myography experiments carried out in isolated 9-month-old ascending aorta from the diverse experimental groups indicated in the following panels. Red lines indicate where myographic measurements were obtained. **(B)** Physiological state test of aortic reactivity following the addition of KCl (100 mM). Data represented as boxplots. See also Table S10. **(C)** Relaxation of aortic reactivity following ACh addition. **(D)** Relaxation of reactivity after the addition of the NO donor sodium nitroprusside. **(E)** Contraction of aortic reactivity after the addition of phenylephrine. Data in C-E are mean ± SD. See also Tables S9 and S10. Statistical test tests: Two-way ANOVA and Tukey post-test (B); non-linear regression of data by fitting Phe/ACh/NTP concentration-response curves to a sigmoidal dose-response (C–E). ***$p \leq 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared with WT (the curve shifted to the right in Fig. 4C/asterisks; Table S9), which indicated that ACh-mediated aortic relaxation was impaired in MFS mice and, therefore, demonstrative of dysfunctional endothelium. The treatment with ALO showed an ACh-induced relaxation in MFS aortae that was indistinguishable from WT animals (pEC_{50} 7.49 ± 0.19 and 7.52 ± 0.10, respectively) (Fig. 4C; Table S9), proving that ALO treatment normalizes endothelial function in MFS mice.

To check the participation of the NO/GC/cGMP pathway in the VSMCs of untreated MFS mice, we next added increased concentrations of the NO donor sodium nitroprusside (NTP) to aortae pre-contracted with phenylephrine (Phe) and the non-selective NOS inhibitor N (gamma)-nitro-L-arginine methyl ester (L-NAME; Fig. 4A), resulting in similar concentration-dependent aortic vasodilatations in untreated MFS and WT mice (Fig. 4D). No changes in the vasorelaxant response to NTP were observed following ALO administration either in WT or MFS mice (Fig. 4D; Table S9). We also evaluated the α_1-associated contractile response of ascending aortae when stimulated with different concentrations of Phe. Results were highly similar between non-treated MFS and WT ascending aortae or following ALO treatment (Fig. 4E; Table S10).

At this point of the study, there is an apparent inconsistency knowing that allopurinol is greatly inhibiting aortic aneurysm over the age in MFS mice (examined at 3-to-9 months old animals), but nevertheless XOR is only significantly increased in young animals (3 months-age). This means that other mechanisms directly or indirectly mediated by allopurinol must be affected. This conundrum is resolved below.

3.6. *Allopurinol prevents increased levels of H_2O_2 in MFS aorta*

Considering that ALO is clinically prescribed to treat hyperuricemia, we first measured UA and allantoin levels in blood plasma in WT and MFS mice to see potential changes associated with genotype or age or both. We noticed that neither UA nor allantoin, nor their ratio, showed any change between WT and MFS mice over age (Figs. S6A–C). Next, we analyzed whether ALO (PA1) reduced UA in blood plasma, but we observed that UA levels remained unaltered (Fig. S7). The possibility that the inhibition of aneurysm progression was mediated by reducing blood pressure as previously reported in other studies [53] was also discarded since systolic blood pressure values also remained unaltered (Fig. S8).

The aortopathy inhibition by ALO inhibiting XOR activity only happens in the early ages of MFS mice (3 months), but it cannot be explained in older animals. Therefore, other mechanism(s) must significantly participate in which redox stress should be directly or indirectly affected by ALO. In this regard, it was reported that ALO could directly scavenge ROS [54], thus, we measured ROS levels in blood

plasma and aorta from WT and MFS mice. We chose to measure H_2O_2 because it is the major ROS produced both by XOR under aerobic conditions [55], which occur in the heart and aorta, and by NOX4, which is upregulated in MFS aorta [17]. In blood plasma, we observed no differences with age between WT and MFS mice (Fig. S9). In contrast, ascending aorta rings from MFS mice showed significantly higher levels of H_2O_2 compared with WT mice of different ages (Fig. 5A). The *in vitro* administration of ALO to these aortic rings normalized H_2O_2 levels. This was also the case when ALO was administered *in vivo* in MFS mice (PE), whose aortic H_2O_2 levels were highly similar to those of untreated WT aortae (Fig. 5B). Therefore, ALO either prevented or inhibited the MFS-associated high levels of H_2O_2 in the ascending aorta irrespectively of *in vivo* or *in vitro* administration.

3.7. Allopurinol downregulates NOX4 and MMP2 expression in MFS aorta

Knowing that ALO blocks aortic aneurysm progression with age (3-, 6- and 9-month- old MFS mice), that aortic H_2O_2 levels also remain significantly elevated at these ages, and that XOR is only upregulated at early ages (3 months), we postulated that, regardless of its scavenging action, ALO could be directly or indirectly affecting other enzymatic H_2O_2 sources at the MFS aorta. In this regard, we next examined whether ALO treatments affected NOX4 expression, which greatly and directly produces H_2O_2 and whose expression and activity are abnormally increased in MFS aortae [17,18]. Thus, we evaluated NOX4 mRNA levels in the aorta of WT and MFS mice palliatively (PA1) and preventively (PE) treated with ALO. In both cases, ALO-treated MFS mice inhibited the characteristic upregulation of NOX4 in MFS aorta (Fig. 6A and B).

Another potential and complementary mechanism by which ALO, acting as an indirect antioxidant, could contribute to explaining the large reduction of MFS-associated aortic wall disarrays is by inhibiting the ROS-mediated activation of MPPs, as ROS are potent activators of MMPs [56]. MMP2 and MMP9, two well-known MMPs involved in MFS aortopathy [57] can be indirectly activated by ROS from their inactive-zymogen isoforms and via direct activation of their gene expression [58]. As expected, MFS aortae showed increased MMP2 protein levels in the tunica media compared with WT aorta. Both PE and PA1 ALO treatments inhibited the increased expression of MMP2 (Fig. 7).

3.8. Allopurinol prevents redox stress-associated injuries in the aortic tunica media of MFS mice

RNS can be produced due to the interaction of endothelial-generated NO and XDH-induced ROS [32,33]. Peroxynitrite and 3′-nitrotyrosine (3-NT) products are reliable redox stress biomarkers; immunohistochemical evaluation showed greater levels of 3-NT in the tunica media of MFS aortae. In both PA1 and PE approaches, ALO significantly prevented this increase (Figs. S10A and B).

The nuclear factor erythroid 2-related factor 2 (NRF2) is a key transcription factor that regulates the expression of several antioxidant defense mechanisms. Oxidative stress triggers its phosphorylation (pNRF2), being subsequently translocated to the nucleus to activate the expression response of physiological antioxidant enzymes [59]. Thus, we evaluated the nuclear presence of pNRF2 in aortic paraffin sections from WT and MFS mice treated with ALO after PA1 and PE treatments (Fig. 8A and B, respectively). Aortic media showed a higher presence of nuclear pNRF2 in MFS than WT VSMCs. This result demonstrated that

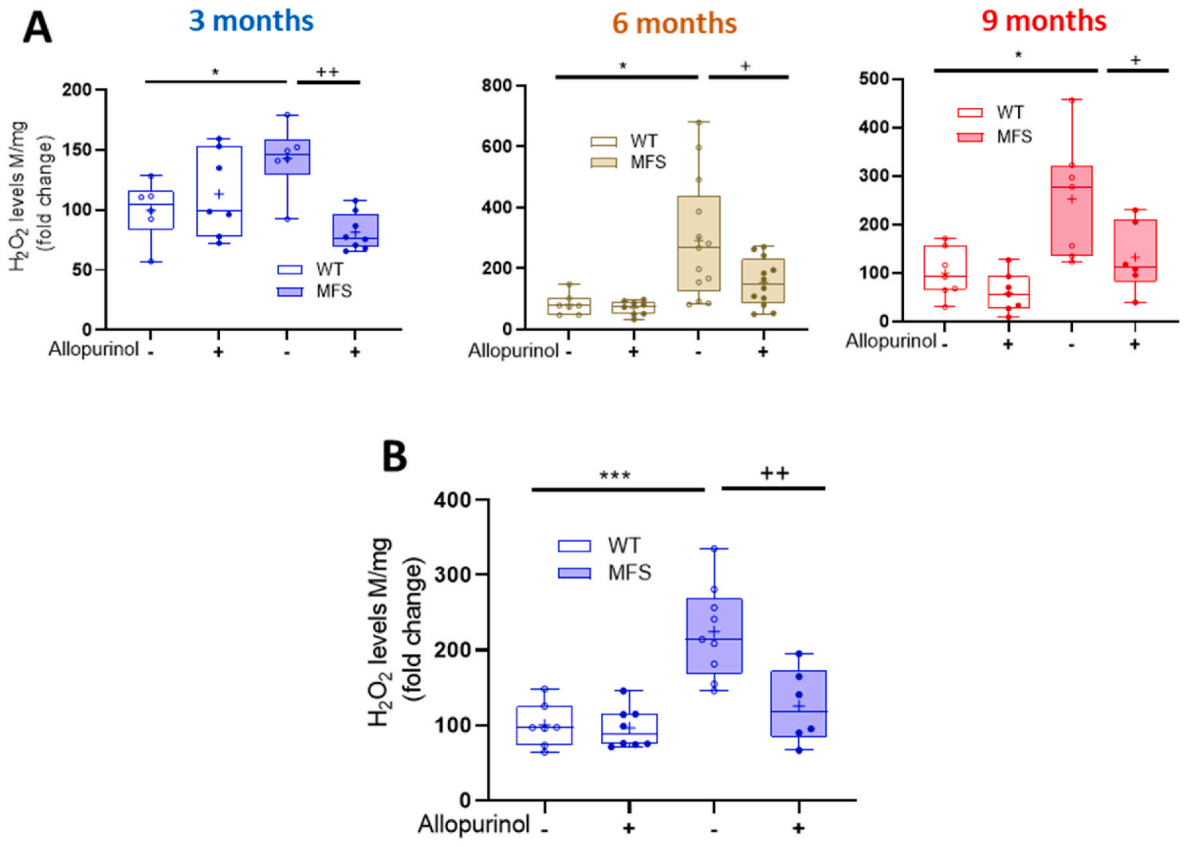

Fig. 5. High H_2O_2 levels generated in the ascending aorta of MFS mice are inhibited by allopurinol when administered both *in vitro* and *in vivo*. (**A**) H_2O_2 levels produced in ascending aortic rings over age in WT and MFS mice. Allopurinol was added *in vitro* to the assay containing aortic rings and the fluorescence signal measured after 2 h. (**B**) H_2O_2 levels measured in ascending aortic rings of WT and MFS mice preventively (PE) treated with allopurinol (*in vivo*). Data represented as boxplots. Statistical test analysis: Two-way ANOVA and Tukey's post-test $^{***}p \leq 0.001$, $^{++}p \leq 0.01$, $^{*/+}p \leq 0.05$; *effect of genotype; $^{+}$effect of treatment.

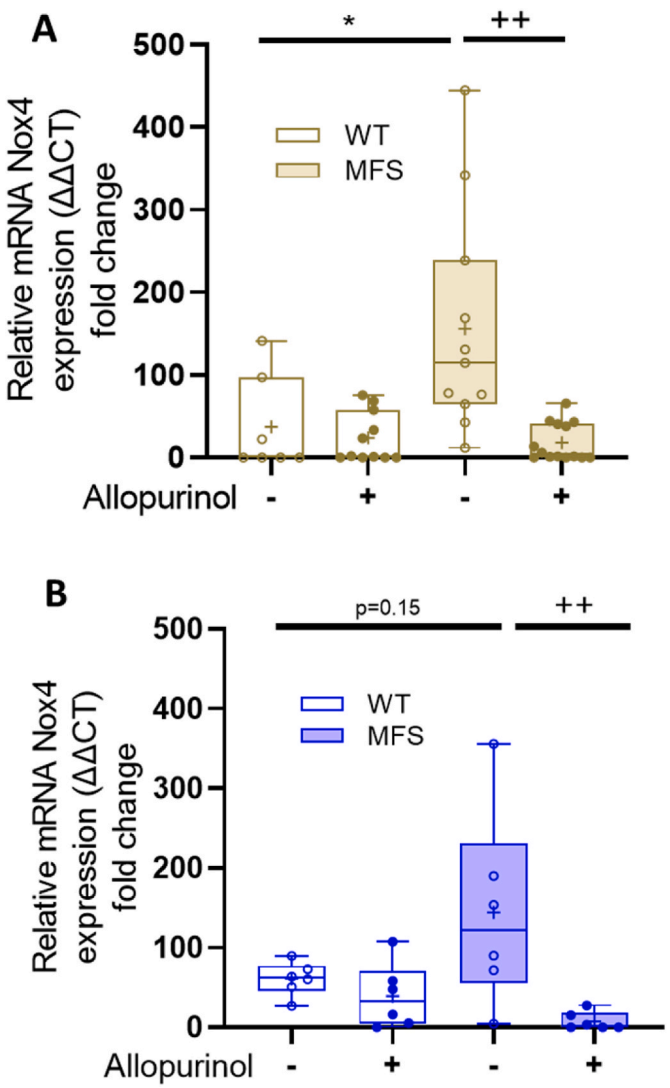

Fig. 6. Allopurinol prevents the upregulation of NADPH oxidase NOX4 in MFS aorta. mRNA expression levels of NOX4 in WT and MFS mice following the PA1 **(A)** or PE **(B)** allopurinol treatments. Statistical test analysis: Kruskal-Wallis and Dunn's multiple comparison test. $^{*/+}p \leq 0.05$ and $^{++}p \leq 0.01$; *effect of genotype; $^{+}$effect of treatment.

the MFS aorta suffered redox stress, which in turn triggered the endogenous antioxidant response. However, when ALO was administered to MFS mice, the number of pNRF2 stained nuclei was indistinguishable from WT aortae, regardless of the experimental treatment followed. This result is a logical consequence of the ALO-induced inhibition of the oxidative stress occurring in the MFS aorta.

Elastic fiber disruption in the MFS aorta is often accompanied by compensatory fibrotic-like remodeling supported by collagen overexpression and/or organization rearrangements occurring mainly in the tunica media [60]. This remodeling can be visualized under a polarized light microscope in paraffin-embedded aortae cross-sections stained with Picrosirius red [61]. We first evaluated total collagen content in the media quantifying Picrosirius staining and we did not observe any significant change (Fig. S11). We next analyzed green and red collagen fibers, which is illustrative of their different thickness and assembly compaction and, therefore, of their degree of maturity [62]. We observed an increase in immature (green) collagen fibers in the aortic tunica media of 6-month-old MFS mice (PA1 treatment). Notably, this collagen maturation remodeling was not produced after ALO treatment (PA1; Fig. 9). No differences were achieved in preventive ALO administration in MFS mice even though similar changes in green fibers tend to occur as well (Fig. S12). In PA1 and PE treatments, ALO did not reduce the increased trend of mature (red) collagen fiber formation (Fig. 9 and Fig. S12, respectively). Altogether, results indicated that ALO also reduced the collagen remodeling taking place in the aortic media of MFS mice.

4. Discussion

Over the last decade, significant progress has been made regarding the molecular mechanisms that participate in the formation and progression of aortic aneurysms in MFS but, until recently, current pharmacological approaches with angiotensin receptor blockers (ARBs; losartan), β blockers (atenolol) or both administered together have afforded uncertain results regarding halting or mitigating aortic aneurysm progression. Nonetheless, in a recent collaborative individual patient data meta-analysis of randomized trials for both treatments in MFS patients not submitted to aortic surgery, collected data showed that ARBs reduced the rate of increase of the aortic root Z score, even among those taking a β blocker [63]. Authors suggested that their additive combination would provide greater reductions in the rate of aortic enlargement than either treatment alone. Being undoubtedly good news, this does not rule out the possibility of using other new or repositioned complementary therapeutic options that specifically target other molecular processes having a relevant impact on aortopathy progression. With this idea in mind, the aim of our study was to obtain: (a) further insights into the contribution of redox stress to the molecular pathogenesis of aortic aneurysms. We have focused on XOR, which, jointly with NADPH oxidases, is a potent ROS-generating system in the cardiovascular system in health and disease; and (b) solid experimental evidence in a representative murine model of MFS to support a new pharmacological approach targeting redox stress (allopurinol), which could be easily translated to MFS patients.

This article produced several main findings: (i) XOR is upregulated in the aorta of both MFS patients and young mice; (ii) this upregulation is accompanied by increased enzymatic activity of the oxidase (XO) form in detriment of the dehydrogenase (XDH) from; (iii) the XOR inhibitor allopurinol (ALO) prevents both the formation and progression of aortic root dilation in MFS mice; (iv) this inhibitory effect is non-permanent since the withdrawal of ALO causes the reappearance of the aneurysm; (v) ALO prevents the characteristic MFS endothelial-dependent vasodilator dysfunction; (vi) ALO inhibits the MFS-associated increase of aortic H_2O_2 levels both *in vivo* and *in vitro* as well as the subsequent accompanying redox stress-associated reactions, such as accumulation of 3-NT, augmented nuclear translocation of pNRF2 and collagen remodeling of the aortic media; and (vii) ALO inhibits MFS-associated upregulation of NOX4 and reduces MMP2 expression in the aortic tunica media. Mechanistically, we postulated that ALO mitigates MFS aortopathy progression by acting as a potent antioxidant, both directly inhibiting XOR activity and scavenging ROS and indirectly downregulating NOX4 and MMP2 expression.

In aortic samples from MFS patients, XOR expression increased compared with healthy controls. This result was confirmed in MFS mice both at the protein and mRNA level. These changes were only observed in young (3-month-old) but not in older mice (6- and 9-month-old). It is possible that XOR upregulation only occurs while the aortic dilation undergoes rapid growth, which we found to occur until 3 months of age, becoming slower in older animals [45]. Nonetheless, no differences between WT and MFS aortae were observed regarding XOR activity in the study of the reactivity of MFS aorta [14], and after nitro-oleic acid treatment as a mediator in ERK1/2 and NOS2 expression in MFS aortopathy [64]. Explanations for this apparent discrepancy may be related to the different murine MFS models used (mgR/mgR and AngII infusion-induced acceleration of the aortopathy in MFS mouse, respectively) or the different XOR activity assays utilized, and/or even to local animal facility conditions.

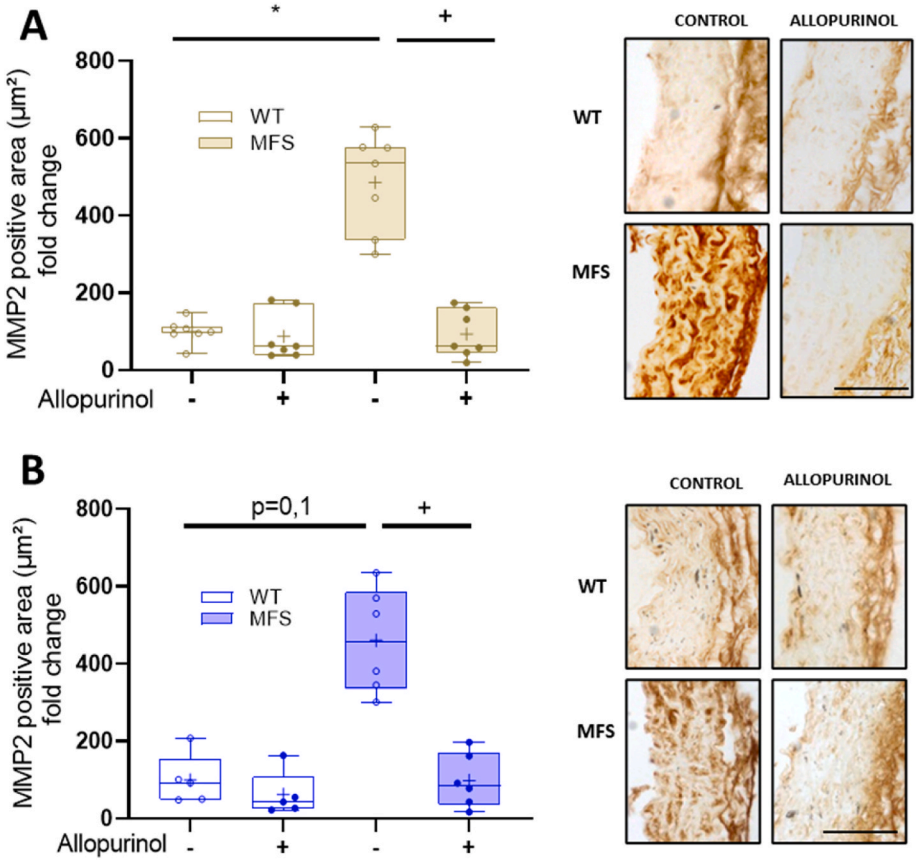

Fig. 7. Allopurinol reduces increased MMP2 levels in the tunica media of MFS aorta. MMP2 levels in the tunica media revealed by immunohistochemistry with anti-MMP2 antibodies in paraffin-embedded aortae from WT and MFS mice following palliative (PA1 **(A)** or preventive (PE) **(B)** administration of allopurinol. On the right, quantitative analysis of the respective HRP immunostaining. Bar, 100 µm. Statistical test analysis: Two-way ANOVA and Tukey's post-test $^{*/++}p \leq 0.05$. *Effect of genotype; $^{++}$effect of treatment.

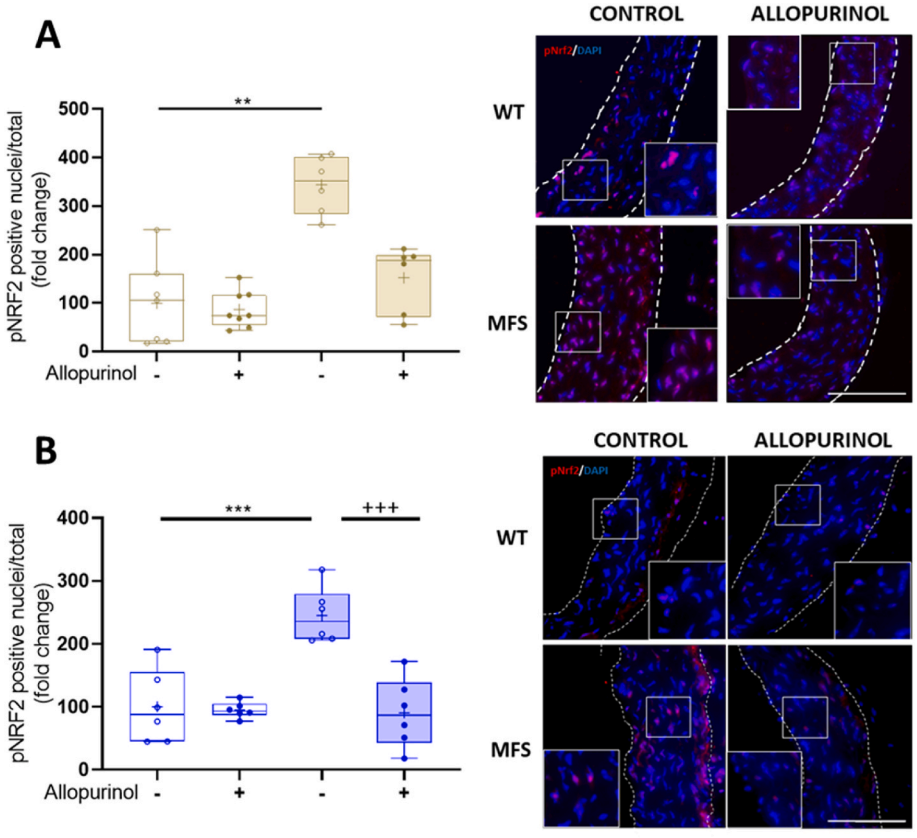

Fig. 8. Allopurinol inhibits the oxidative stress-associated increased nuclear translocation of pNRF2 in MFS aorta. Quantitative analysis and representative images of the nuclear translocation of the phosphorylated form of NRF2 in VSMCs of the tunica media of WT and MFS mice treated with allopurinol palliatively (PA1) **(A)** and preventively (PE) **(B)**. Insets in the immunofluorescent images detail the nuclear localization of pNRF2. Bar, 100 µm. Statistical test: Kruskal-Wallis and Dunn's multiple comparison tests. $^{***/+++}p \leq 0.001$ $^{**}p \leq 0.01$; *effect of genotype; $^{+}$effect of treatment.

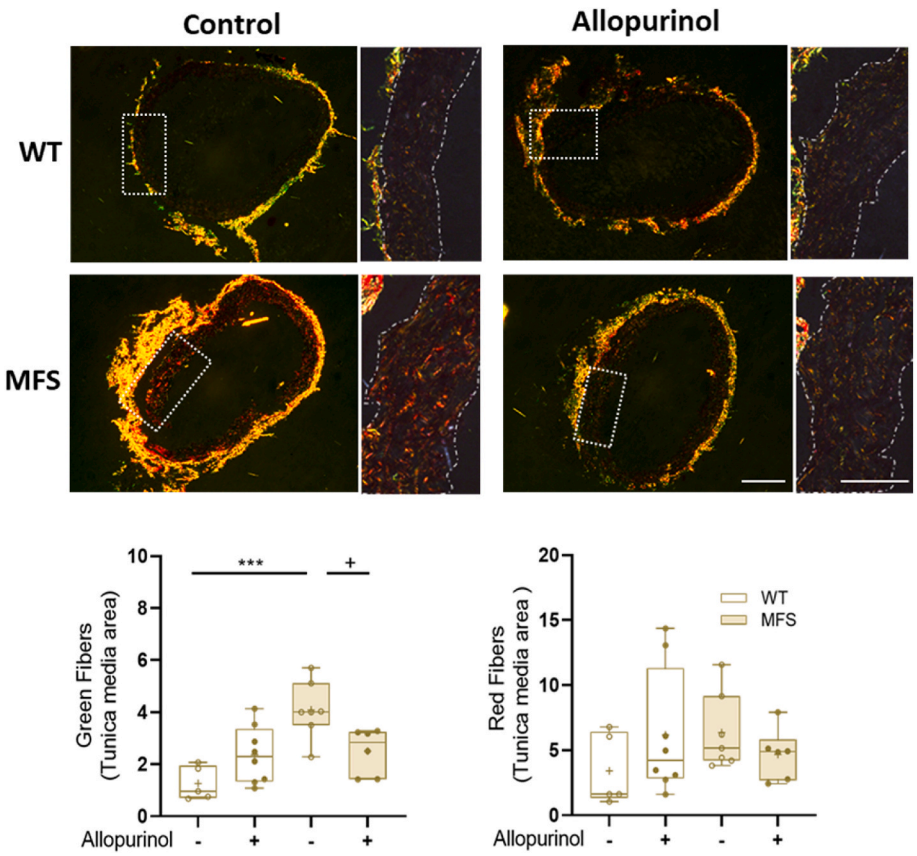

Fig. 9. Allopurinol reduces the MFS-associated collagen maturation remodeling of the tunica media. Immature (green) and mature (red) collagen fibers of the tunicae media and adventitia of WT and MFS aortae stained with Picrosirius red (see Fig. S11) visualized under the polarized microscope. WT and MFS mice were palliatively treated (PA1) or not with allopurinol. Representative fluorescent images of the whole aorta. On the right, images show the enhanced media outlined with white dots. Bar, 100 μm. The respective quantitative analysis of both types of collagen fibers is shown under the images. Statistical test: Kruskal-Wallis and Dunn's multiple comparison tests. ***$p \leq 0.001$ and $^{+}p \leq 0.05$; *effect of genotype; $^{+}$effect of treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ALO is a well-characterized specific inhibitor of XOR activity, mainly of the XO form [42], which was confirmed in the XOR enzymatic assay (Fig. S2). The main clinical actions of ALO are widely linked to UA-associated pathologies [65,66]. UA plasma levels were highly similar between MFS and WT animals, which at first glance discarded a UA-mediated effect in the aortopathy in MFS mice. Curiously, ALO did not cause the expected reduction of UA and/or allantoin plasma levels. However, it is important to highlight that the equivalent dose of ALO used here in mice (20 mg/kg/day) if administered to humans (~120 mg/day calculated for a weight of 70 Kg) [67] does not reduce normal plasma UA levels (≤6–7 mg/dL) [68]. Therefore, the blockade of MFS aortopathy by ALO is, in principle, not directly related to changes in UA, but to another accompanying mechanism(s).

Of note, ALO has a ROS lowering effect not necessarily related to its XOR inhibition activity, acting accordingly as a direct ROS scavenging moiety [68–71]. We demonstrated this possibility when ALO was added *in vitro* to MFS aortic rings, quickly attenuating their intrinsic increased H_2O_2 production levels, regardless of mouse age. Importantly, this reduction was also observed in aortic rings from MFS mice with the drug administered *in vivo*. In addition to the direct scavenging role of ALO, its effectiveness as a direct antioxidant can be reinforced, reducing the characteristic aortic upregulation of NOX4, knowing that NOX4 is the NADPH oxidase that directly generates H_2O_2 [72]. Pathological processes that usually accompany redox stress, such as the formation of 3-NT, pNRF2 nuclear translocation and fibrotic remodeling responses were all reduced or inhibited by ALO. The pathophysiological significance of these results in the MFS aortic media suggested several conclusions: (i) the accumulation of 3-NT is representative of abnormal RNS formation because of the pathological uncoupling of NO, which in turn leads to protein nitration via the formation of the highly reactive intermediate peroxynitrite and its subsequent product 3-NT [73]. This 3-NT upsurge in MFS aortic media is consistent with the recent demonstration of NO uncoupling in aneurysm formation in both MFS mice and patients [64,74,75]. Along this line of evidence, we identified actin as a nitrated protein target in MFS aorta from MFS patients [17], contributing, in this manner, to the reported damaged contractile properties of VSMCs in MFS [76]; (ii) redox stress is so elevated in MFS aorta that the intrinsic physiological antioxidant response mediated by the nuclear translocation of pNRF2 [60] is not high enough to compensate it. In contrast, ALO treatment allows this compensation, normalizing, to a large extent, the endogenous redox levels in MFS aorta and associated modifications; and (iii) ALO normalized the content of immature collagen (green) fibers, hence mitigating the collagen remodeling response that usually accompanies the aneurysm to compensate elastic fiber disarray. It would be interesting to know whether ALO is also able to prevent the characteristic phenotypic switch of VSMCs to a mixed contractile-secretory phenotype [77,78].

Of notice, ALO normalized ACh-stimulated vasodilator function, improving endothelial function. It is not a surprise given the reported effects of ALO on increasing NO bioavailability, totally or partially attributable to a reduction in ROS production [32]. Of notice, restoring the endothelial function is important since it has been reported in patients with MFS that flow-mediated dilatation (a noninvasive measurement of endothelial function) correlated with aortic dilatation [33].

In reference to the normalization by ALO treatments (PA1 and PE) of the structural aortic wall organization evidenced by the normalization of elastic laminae breaks, this effect could be explained by the inhibition of the ROS-mediated activation of MMP2 upregulation, which, together with MMP9, are two well-known MMPs upregulated and overactivated in MFS aorta [58]. Nonetheless, XOR itself can directly activate MMPs in a ROS-independent manner [79].

Finally, it cannot be discarded that ALO could also mediate its inhibitory action on aortopathy by acting as an anti-inflammatory agent [80] and/or also indirectly blocking the ROS-induced heat shock protein expression generating endoplasmic reticulum (ER) stress, which has an impact on abdominal and thoracic aortic aneurysms [81–84]. These two

possibilities deserve future attention.

We are aware that our study has some limitations: (i) ALO has a short half-life in plasma and is rapidly metabolized to oxypurinol, which has a longer lifespan [42]. It is possible that oxypurinol rather than allopurinol might be the primary metabolite that directly mediates the results we report herein. Nonetheless, in this respect, it is important to take into account that it is not recommended to administer higher doses of ALO in humans (>300 mg/day) because the high concentration of oxypurinol generated then acts as a pro-oxidant instead of antioxidant, favoring oxidative stress [68]; (ii) the MFS model used in our study (C1041G) is a very useful model to evaluate the temporal course of aortopathy (besides other clinical manifestations), but it is not the most suitable to evaluate the survival rate after ALO treatments. Other, more severe MFS murine models for aortic dissection and rupture, such as the *Fbn1* hypomorphic mouse (mgR/mgR) [85] or the AngiotensinII-induced accelerated model of MFS aortopathy [64], both leading to aortic dissection and rupture, would be more appropriate experimental murine models for this aim; and (iii) the aortic arch and descending thoracic aorta have not been studied; (iv) we have only studied ALO's effects on the cardiovascular system (TAA more specifically) because it is responsible for the life-threatening complication in MFS; and (v) even though ALO has been widely proven to be a safe and well-tolerated drug, it would also be of interest to evaluate if ALO has further beneficial or detrimental effects on other affected organ systems as MFS is a multisystemic disorder.

5. Conclusion

Our results definitively place redox stress among the molecular mechanisms that significantly participate in the pathogenesis of aortic aneurysm in MFS by the persistent high production levels of ROS mediated by upregulation of XOR (current study), the NADPH oxidase NOX4 [17], eNOS dysfunction [74] and mitochondrial electron transport-associated redox stress [86], all from studies performed both in MFS murine models and patients. How, and to what extent, do each of these interlinked mechanisms participate in aortic injury is currently unknown, but most likely they all act additively or synergistically to damage the aorta severely and permanently. We here demonstrated ALO's effectiveness, acting as a strong antioxidant drug in MFS aortae directly both as an inhibitor of XOR activity in early ages when XOR is overexpressed, and as ROS scavenger in the absence of XOR activity, and indirectly by downregulating NOX4 and MMP2 overexpression. ALO is an economic drug, which has been widely prescribed in clinical practice since the latter half of the last century and, most importantly, has been proven to be effective, safe, and usually well tolerated. ALO has been reported to present lower rates of cardiovascular events (related to hypertension) compared with non-treated patients [87]. Of note, a preprint study reported that febuxostat has similar effects to ALO, blocking MFS aortopathy [88]. Febuxostat is another specific XOR inhibitor not resembling purines or pyrimidines [89]. Some concerns regarding cardiovascular safety have been reported for febuxostat compared with ALO [90], but a recent study seems to refute this concern [91]. In sum, our results in MFS mice support the design of a clinical trial with ALO as having a potential beneficial effect in the pharmacological clinical practice of MFS patients. Last, but not least, such a potential benefit could only be obtained when the drug is administered chronically.

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CRediT authorship contribution statement

Isaac Rodríguez-Rovira: Methodology, Investigation, Writing – review & editing. **Cristina Arce:** Methodology, Data curation, Formal analysis, Investigation, Writing – review & editing. **Karo De Rycke:** Methodology, Investigation, Writing – review & editing. **Belén Pérez:** Methodology, Investigation, Writing – review & editing. **Aitor Carretero:** Methodology, Writing – review & editing. **Marc Arbonés:** Methodology, Writing – review & editing. **Gisela Teixidò-Turà:** Supervision, Writing – review & editing. **Mari Carmen Gómez-Cabrera:** Funding acquisition, Supervision, Writing – review & editing. **Victoria Campuzano:** Investigation, Supervision, Writing – review & editing. **Francesc Jiménez-Altayó:** Methodology, Investigation, Funding acquisition, Supervision, Writing – review & editing. **Gustavo Egea:** Conceptualization, Funding acquisition, Supervision, Data curation, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.11.001.

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