Thoracic aortic aneurysm, as occurs in Marfan syndrome, is generally asymptomatic until dissection or rupture, requiring surgical intervention as the only available treatment. Here, we show that nitric oxide (NO) signaling dysregulates actin cytoskeleton dynamics in Marfan Syndrome smooth muscle cells and that NO-donors induce Marfan-like aortopathy in wild-type mice, indicating that a marked increase in NO suffices to induce aortopathy. Levels of nitrated proteins are higher in plasma from Marfan patients and mice and in aortic tissue from Marfan mice than in control samples, indicating elevated circulating and tissue NO. Soluble guanylate cyclase and cGMP-dependent protein kinase are both activated in Marfan patients and mice and in wild-type mice treated with NO-donors, as shown by increased plasma cGMP and pVASP-S239 staining in aortic tissue. Marfan aortopathy in mice is reverted by pharmacological inhibition of soluble guanylate cyclase and cGMP-dependent protein kinase and lentiviral-mediated Prkg1 silencing. These findings identify potential biomarkers for monitoring Marfan Syndrome in patients and urge evaluation of cGMP-dependent protein kinase and soluble guanylate cyclase as therapeutic targets.
Thoracic aortic aneurysm and dissection (TAAD) is a major cause of morbidity and mortality in developed countries. TAAD is characterized by progressive vessel dilation associated with vascular smooth muscle cell (VSMC) dysfunction and destructive extracellular matrix remodeling. TAAD is often asymptomatic until the aorta dissects or ruptures. Approximately one in four cases of TAAD have a known genetic basis and can be categorized as either syndromic (showing prominent phenotypic features of a systemic connective tissue disorder such as Marfan syndrome [MFS]) or nonsyndromic, with an essentially vascular phenotype. TAAD and rupture account for >90% of MFS patient deaths. MFS is an inherited autosomal dominant disease caused by pathogenic variants of the fibrillin-1-encoding gene FBN1. Fibrillin-1 is a major component of extracellular microfibrils, providing a scaffold for elastic-fiber formation and maturation. Mutant fibrillin-1 disrupts microfibril formation, leading to medial degeneration, which destabilizes the aortic wall, rendering the aorta vulnerable to hemodynamic injury. Medial degeneration is characterized by poor alignment of elastin filaments, disorganization of lamellar units, VSMC death, and proteoglycan accumulation. Proteoglycans play essential roles in the preservation of aortic structure and function by regulating elastic fiber assembly and smooth muscle cell proliferation.

Current therapy for the cardiovascular complications of MFS consists of strict follow-up, lifestyle advice, medical treatment to slow the rate of aortic root dilation, and surgery to prevent dissection or rupture. Therapeutic strategy is usually based on β-adrenergic blockade. However, this treatment neither halts abnormal aortic growth nor prevents aortic dissection or death. Moreover, some studies suggest that β-adrenergic blockers do not even slow the rate of aortic growth in MFS. There is therefore a need for effective pharmacological strategies to manage TAAD in MFS patients.

MFS is accompanied by impaired aortic contractility, caused by as yet unknown molecular mechanisms. In small arteries, VSMC relaxation and vasodilatation are induced by nitric oxide (NO), produced in endothelial cells by constitutively expressed endothelial NO synthase (eNOS; also called NOS3). NO can also be produced by constitutively expressed NOS of neuronal origin (nNOS; also called NOS1) or by inducible NOS (iNOS; also called NOS2). NOS2 expression is induced in MFS patients and in a mouse model of MFS, and TAAD is reversed in the mouse model by pharmacological NOS2 inhibitors, raising the possibility that blocking NOS2 activity could be a promising treatment for TAAD. However, the mechanisms by which NOS2 contributes to TAAD in MFS remain unclear. Increased NOS2-derived NO levels stimulate soluble guanylate cyclase (sGC) to generate cGMP, which in turn activates cGMP-dependent protein kinase G (PRKG), which modulates the contractility of resistance vessels by regulating actin filament and myosin dynamics. NO also regulates numerous physiological processes in a cGMP-independent manner, including mechanisms based on S-nitrosylation and, in the presence or excess reactive oxygen species (ROS), protein tyrosine nitration.

In response to limited availability of the substrate L-arginine or the cofactor tetrahydrobiopterin (BH4), all high ROS levels, all NOS enzymes generate superoxide instead of NO, resulting in oxidative stress. As ROS levels are elevated in the MFS aorta, increased NOS2-derived NO levels stimulate soluble guanylate cyclase (sGC) to generate cGMP, which in turn activates cGMP-dependent protein kinase G (PRKG), which modulates the contractility of resistance vessels by regulating actin filament and myosin dynamics. NO also regulates numerous physiological processes in a cGMP-independent manner, including mechanisms based on S-nitrosylation and, in the presence or excess reactive oxygen species (ROS), protein tyrosine nitration.

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Results

NO signaling pathway activation dysregulates actin cytoskeleton dynamics in MFS VSMCs. To investigate whether the NO–sGC–PRKG signaling pathway is activated in MFS, we measured VASP–S239 phosphorylation (pVASP–S239) as a readout of PRKG activity in primary aortic VSMCs from wild-type (WT) mice and a mouse model of MFS (Fbn1C1039G/+ mice) cultured in the presence of inhibitors of NOS (L-NAME, sGC (ODQ), and PRKG (KT5823) (Fig. 1). pVASP–S239 levels were significantly increased in MFS VSMCs compared to WT VSMCs, indicating increased PRKG activity. Moreover, some studies suggest that β-adrenergic blockers do not even slow the rate of aortic growth in MFS. Therefore, there is a need for effective pharmacological strategies to manage TAAD in MFS patients.

MFS smooth muscle cells harboring the FBN1C1039Y pathogenic variant show defective deposition of fibrillin-1. In conclusion, we found no notable effect on extracellular fibrillin-1 deposition in WT VSMCs treated with 8-Br-cGMP (Supplementary Fig. 1). Together these results strongly suggest that overactivation of the NO–sGC–PRKG pathway in MFS VSMCs switches their phenotype by dysregulating the contractile machinery, including actin cytoskeleton dynamics, without affecting microfibril formation.

Supraphysiological NO levels induce an MFS-like aortic pathology. VSMCs from MFS patients and the Fbn1C1039G/+ mouse model (MFS mice) express high levels of NOS2, a NOS enzyme that produces large amounts of NO. We therefore investigated whether supraphysiological NO levels affect aortic homeostasis in healthy mice. As an NO source, we used isosorbide mononitrate (ISMN), a long-lasting nitrate that is meta-

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Fig. 1 Pharmacological inhibition of signaling components of the NO-sGC-PRKG pathway decreases pVASP-S239 induction in VSMCs from MFS mice. 

a NO signaling components and the targets of pharmacological stimuli (green) and inhibitors (red). 

b Representative images of pVASP-S239 immunofluorescence (red) and DAPI-stained nuclei (blue).

c Quantification of pVASP-S239 immunofluorescence in WT and MFS VSMCs treated with 300 μM L-NAME, 10 μM ODQ, or 1 μM KT5823 for 1 h before stimulation for 5 minutes with 100 μM 8-Br-cGMP or 100 μM DetaNO, as indicated. 

b, c Five independent cell batches were used for all conditions except for MFS VSMCs (n = 4). Scale bar, 50 μm. Data are shown relative to untreated WT cells as mean ± s.e.m. Each data point denotes the mean value from an independent experiment. Differences were analyzed by one-way ANOVA with Dunnett’s post-hoc test (p-values are shown). Source data are provided in the Source Data file.
We performed in vivo longitudinal dose-response studies to characterize the effects of infusing ISMN for 7 days on the aortic phenotype of WT C57BL/6 mice (Fig. 3a). Since there were no previous reports on the effect of ISMN on aortic phenotype, and the normal clinical dose of ISMN used to treat chronic stable angina pectoris ranges from 0.5 to 4 mg/kg/day, we tested several doses from 1 to 50 mg/kg/day. ISMN induced a dose-dependent decrease in systolic blood pressure (BP) (Fig. 3b) and dilation of both the ascending aorta (AsAo) and the abdominal aorta (AbAo) (Fig. 3c, d). Aortic diameters at the highest dose were similar to those in MFS mice (Fig. 3e). ISMN doses above 50 mg/kg/day did not induce larger aortic dilations or BP drops (Supplementary Fig. 2), and this dose was therefore used for further experiments.

Fig. 2 The NO–sGC–PRKG pathway modulates the contractility phenotype of VSMCs. a, b Representative images of F-actin staining (red) and DAPI-stained nuclei (blue) and F-actin quantification in a WT VSMCs treated as indicated for 5 min (n = 4 independent cell batches per condition) and b WT and MFS VSMCs untreated or treated with KT5823 for 24 h (n = 5 independent cell batches per condition). Scale bar, 50 μm. Data are shown relative to untreated WT cells as mean ± s.e.m. Each data point denotes the mean value from an independent experiment. Differences were analyzed by one-way ANOVA with Tukey’s post-hoc test (p-values are shown). c, d RT-qPCR analysis of Acta2, Cnn1, and Tagln2 mRNA expression in c WT VSMCs treated as indicated for 4 h and d WT or MFS VSMCs treated as indicated for 24 h (n = 4 independent cell batches per group in c and n = 5 independent cell batches per group in d). mRNA amounts were normalized to Gapdh expression (mean ± s.e.m.). Each data point denotes the mean value from an independent experiment. Differences were analyzed by one-way ANOVA followed by Dunnett’s post-hoc test (p-values are shown). Source data are provided in the Source Data file.
To determine if other NO donors had a similar effect on BP and aortic dilation, we treated WT mice with DetaNO. Unlike ISMN, DetaNO is unstable at 37 °C, and we therefore used osmotic minipumps to infuse it for just 2 days. Like ISMN, DetaNO decreased systolic BP (Supplementary Fig. 3a) and induced marked AsAo and AbAo dilations (Supplementary Fig. 3b-c).

FBN1 pathogenic variants cause not only aortic dilation, but also medial degeneration characterized by elastic-fiber fragmentation and disarray and proteoglycan accumulation in the aorta, a feature that might predispose to dissection. Histological analysis of the AsAo in ISMN-infused WT mice revealed both proteoglycan accumulation and elastic-fiber fragmentation and disorganization as early as 7 days after treatment initiation, as shown by Alcian Blue and modified Verhoeff elastic-Van Gieson (EVG) staining, respectively (Fig. 3f, g). After 28 days, these features were more pronounced and were comparable to those in age-matched MFS mice (Fig. 3f, g). Similar elastic-fiber alterations were found upon treatment of WT mice with DetaNO (Supplementary Fig. 3d-3e). These results thus indicate that supraphysiological levels of NO are sufficient to induce MFS-like aortopathy in WT mice.

In humans, sustained high plasma nitrate concentrations induce a clinically relevant decay of the vasodilatory effect,
known as nitrate tolerance41. To investigate if continuous ISMN delivery in mice loses efficacy over the long term, we performed a longitudinal study in which ISMN (50 mg/kg/day) was infused for 28 days (Supplementary Fig. 4a). In line with the results shown in Fig. 3, ISMN sharply decreased systolic BP and increased AsAo and AbAo diameters after 1 day of infusion; moreover, these effects were sustained over the remaining days of treatment (Supplementary Fig. 4b-4c), ruling out a tolerance phenomenon.

The sGC–PRKG signaling pathway is activated in MFS mice and patients. We next investigated whether the elevated NO2 expression found in MFS patients and mice results in activation of the sGC–PRKG signaling pathway. sGC generates cGMP, and intracellular cGMP pools are in a dynamic steady-state relationship with plasma cGMP levels42,43. We found substantially elevated plasma cGMP in both MFS mice and ISMN-treated WT mice (Fig. 4a), strongly suggesting that sGC is indeed activated in MFS mice. Similar plasma cGMP increases were found upon DetaNO treatment (Fig. 4b). As a readout of PRKG activity, we determined pVASP-S239 levels in aortic tissue. pVASP-S239 levels were very high in the aortas of MFS mice or WT mice treated with ISMN for 7 or 28 days (Fig. 4c, d and Supplementary Fig. 5). The increase in plasma cGMP and aortic pVASP-S239 was not due to increased sGC or Prkg mRNA or protein expression in MFS (Supplementary Fig. 6), suggesting that sGC and Prkg are activated in MFS by post-translational mechanisms.

To determine if the NO-sGC–PRKG pathway is activated in MFS patients, we measured serum or plasma cGMP and PRKG activity in aortic tissue from MFS patients. Plasma or serum cGMP from three independent MFS patient cohorts was measured in aortic tissue from MFS patients. Plasma or serum cGMP from three independent MFS patient cohorts was markedly higher than in healthy donors (Fig. 5a). PRKG activity was also higher in aortic sections from two MFS patient cohorts than in sections from multiorgan transplant donors. Immunohistochemistry and immunofluorescence revealed higher pVASP-S239 levels in MFS samples (Fig. 5b, c and Supplementary Fig. 7). Together, these data strongly suggest that the sGC–PRKG pathway is activated in MFS patients.

MFS mice and patients have elevated levels of protein nitration in aortic tissue and plasma. The lipophilic properties of NO allow it to diffuse from the vessel wall to the vessel lumen22,33,44, where it can potentially trigger NO-derived modifications of plasma proteins. These include Cys S-nitrosylation, a labile modification that cannot be directly detected unless a stable derivative is generated45, and nitration, a stable modification that takes place under pro-oxidant conditions46. We carried out a high-throughput proteomics analysis to explore the relative nitration levels of plasma proteins in seven MFS mice and seven WT littermates. As a positive control, we also included 6 DetaNO-treated WT mice. Mass spectrometry analysis of plasma protein digests quantified 43 nitrated peptide species identified in more than 1 MS/MS spectrum from 19 nitrated proteins that included 47 nitrated sites (NS) (Supplementary Data 1 and 2). Of the NS, 88% correspond to nitro-Tyr and 12% to nitro-Trp. The cumulative distribution of nitro-protein quantifications in plasma from NO-donor-treated mice was significantly shifted toward positive values in relation to other plasma proteins (Fig. 6a), indicating that NO treatment produced a generalized increase in nitrated protein abundance. In contrast, the cumulative distribution of all remaining plasma proteins closely followed the expected null-hypothesis distribution (Fig. 6a), demonstrating the accuracy of the model and that the nitration profile was not biased due to the quantitative analysis applied. Interestingly, the relative abundance of nitrated proteins was also elevated in plasma from MFS mice (Fig. 6a), demonstrating an increased nitration in these mice that can be clearly detected in plasma.

To determine if increased aortic NO levels in MFS patients is also indicated by elevated plasma-protein nitration, we performed a quantitative proteomics analysis in plasma samples from 30 healthy donors and 23 MFS patients, identifying 40 nitrated peptide species from 18 nitrated proteins, many of which were also found in the analysis of mouse plasma (Supplementary Data 3 and 4). Among 43 NS identified in these peptides, 66% correspond to nitro-Tyr and 34% to nitro-Trp. Reproducing the results obtained in mice, plasma from MFS patients showed a generalized elevation in the abundance of nitrated proteins relative to controls (Fig. 6b), suggesting that a highly nitrated plasma profile may be a signature of MFS and strongly supporting the notion that NO levels are increased in MFS. To provide a set of candidate nitrated peptides as biomarkers for clinical diagnosis or prognosis, we selected the nitrated peptides that were significantly upregulated in MFS patients (Fig. 6c). The quantitative values of these peptides were combined to obtain a nitrated plasma index (NPI), which provides a measure of the increase in nitration. Mean NPI was clearly increased in the MFS population, with most MFS patients remaining above the mean of the healthy controls (Fig. 6d).

We additionally searched for aortic tissue proteins whose nitration levels could be affected by NO-sGC–PRKG pathway activation. Quantitative proteomics analysis in pooled aorta samples from 12 WT (n = 6 pools) and 12 MFS (n = 6 pools) mice identified 124 nitrated peptide species from 104 nitro-proteins, which included 147 NS (Supplementary Data 5). Of these NS, 75% correspond to nitro-Tyr and 25% to nitro-Trp. MFS mice showed a consistent and significant upregulation of 24 aortic nitro-proteins (Fig. 6e and Supplementary Fig. 8). This analysis revealed a sharp increase of Acta2 nitration as well as substantial increases in the nitration of seven additional
cytoskeletal and extracellular matrix proteins that might play a role in contractility regulation (Fig. 6f).

The NO–sGC–PRKG pathway mediates aortopathy in MFS. Activation of the NO–sGC–PRKG signaling pathway in MFS patients and mice suggested its involvement in MFS aortopathy. However, increased plasma-protein nitration in MFS patients and mice is also consistent with a cGMP-independent contribution of NO to aortic disease. To ascertain whether sGC–PRKG activation plays a causal role in MFS aortopathy, we treated MFS mice with pharmacological inhibitors of these enzymes. Daily intraperitoneal (i.p.) administration of the sGC inhibitor ODQ (20 mg/kg/day) to 14-week-old MFS mice (Fig. 7a) markedly decreased plasma cGMP (Fig. 7b), inhibited Prkg activity determined by pVASP-S239 aortic staining (Fig. 7c, d), and completely reversed AsAo and AbAo dilation after 7 days without inducing arterial hypertension (Fig. 7e, f). Histological analysis of AsAo cross-sections after 21 days of treatment showed that pharmacological sGC inhibition partially reverted elastic-fiber fragmentation and decreased aortic wall thickness (Supplementary Fig. 11).

Daily i.p. treatment of MFS mice with the PRKG inhibitor KT5823 (2 μmol/kg/day) (Fig. 7g) had similar effects on Prkg activity (Fig. 7h, i), AsAo and AbAo dilation, and BP (Fig. 7j, k), suggesting that sGC and PRKG mediate aortic disease in MFS. However, no reversion of medial degeneration was observed after 7 days of KT5823 treatment (Supplementary Fig. 11a), suggesting that tissue regeneration might require a longer period of Prkg suppression.

To test this hypothesis, we silenced Prkg expression in the aortas of adult MFS mice. There are 2 Prkg isoforms: Prkg1, which is ubiquitously expressed but particularly abundant in vascular smooth muscle cells; and Prkg2, which is mainly expressed in non-vascular tissues. We therefore screened candidate shRNAs specific for Prkg1 in cultured VSMCs, identifying shPrkg1-A and shPrkg1-B as having high silencing capacity (Fig. 8a). The shRNA-encoding lentivirus also encoded green fluorescent protein (GFP) to facilitate assessment of transduction efficiency. In vitro transduction of MFS VSMCs with control shRNA (shScr), shPrkg1-A, or shPrkg1-B lentivirus yielded similar GFP mRNA expression levels, and Prkg1 expression was specifically silenced by shPrkg1-A and shPrkg1-B lentiviruses (Supplementary Fig. 12a).
Consistent with the results obtained upon pharmacological Prkg inhibition, Prkg1 knockdown decreased the expression of the contractility markers Acta2, Tagln2, and Cnn1 in MFS cells to normal levels (Supplementary Fig. 12a) while increasing actin fiber formation to normal levels (Supplementary Fig. 12b). Prkg1 knockdown did not, however, restore the capacity of MFS VSMCs to generate extracellular fibrillin-1 fibers (Supplementary Fig. 12c). Intrajugular delivery of lentivirus encoding shPrkg1-A or shPrkg1-B into MFS mice (Fig. 8b) yielded efficient transduction of all aortic layers, determined by GFP immunostaining of aortic sections 4 weeks after lentivirus delivery (Fig. 8c). Prkg1 protein expression was almost undetectable in aortic samples from these mice (Fig. 8c).
d). Consistent with the results obtained with the PRKG inhibitor, Prkg1 silencing in the aortas of MFS mice markedly decreased pVASP-S239 aortic staining (Fig. 8e, f) and reversed AsAo and AbAo dilation (Fig. 8g). Prkg1 knockdown did not raise BP above normotensive values in untreated littermate controls (Fig. 8h). Histological analysis of aortic cross-sections showed that Prkg1 silencing for 28 days led to an almost complete reversal of medial degeneration, determined by the thickness of the aortic wall (Fig. 8i, j) and the regression of elastic-fiber fragmentation and disarray (Fig. 8i, k). Together, these results urge the evaluation of sGC and PRKG as potential targets for therapeutic intervention.

**Discussion**

Our results demonstrate that the NO–sGC–PRKG signaling pathway mediates aortopathy in a mouse model of MFS and is activated in MFS mice and MFS patients. These findings identify potential targets for intervention in human MFS, as well as circulating activation markers of this pathway that might be useful for MFS disease monitoring and clinical follow-up.

Our previous work in MFS mice showed that NOS2, whose expression is induced in VSMCs from MFS mice and patients, is an important mediator of medial degeneration and aortic dilation, key features of MFS aortopathy that are efficiently reverted by NOS2 inhibition. NOS2 activity generates much larger...
Fig. 6 Quantitative proteomics shows that increased protein nitration is a signature of MFS in mice and humans. Quantitative proteomics analysis of plasma samples from a untreated WT mice (n = 7), MFS mice (n = 7), and DetaNO-treated WT mice (n = 6) and b MFS patients (n = 23) and healthy donors (n = 30). Data were analyzed using the SanXoT package. Theoretical, normal distribution; Nitro-proteins, cumulative distributions of indicated nitrated protein values corrected to the values for nonmodified peptides from the same protein; All-proteins, cumulative distributions of nonmodified peptides. Data are expressed in standardized log2-ratios (Zq) relative to a untreated WT or b healthy donors (Control). Differences were analyzed by two-tailed Kolmogorov-Smirnov test (p-values are shown; n.s., not significant). Representative annotated fragmentation spectra for 2 mouse nitro-peptides are provided in Supplementary Fig. 7. All M5/MS spectra of identified tryptic nitro-peptides are provided in the Data availability section. c List of nitrated peptides showing significantly higher abundance in MFS patients. The heatmap shows standardized log2-ratios (Zpq) and statistical significance calculated using limma analysis. The annotated fragmentation spectra of these 7 nitro-peptides are provided in Supplementary Fig. 10. d Nitrated plasma index, defined as the weighted mean of the nitro-peptides listed in c, for 23 MFS patients and 30 healthy donors (Control). Each data point denotes an individual, boxes enclose the interquartile range (IQR), the line in the box shows the centre (median), and whiskers extend 1.5 times above and below the IQR. Differences were analyzed by unpaired two-tailed Student’s t-test. e Quantitative proteomics analysis of pooled aortic samples from 12 untreated WT mice (n = 6 pools) and 12 MFS mice (n = 6 pools). Nitro-protein distributions are shown as in a and b. Differences were analyzed by two-tailed Kolmogorov-Smirnov test. f Barplots showing standardized log2-ratios (Zq) integrated by condition (untreated WT and MFS) for significantly upregulated nitro-proteins related to the cytoskeleton: thrombospondin type-1 domain-containing protein 4 (THSD4), coronin 1-C (COROC1), latent-transforming growth factor beta 2 and 4 (LTBP2, LTBP4), acitin smooth muscle (ACTA2), tensin 3 (TNS3), dynein heavy chain 10 (Dnah10), and tubulin beta 4 chain (TUBB4B). Differences were analyzed by limma statistical analysis (exact p-values are shown). Source data are provided in the Source Data file.

amounts of NO than NOS3 or NOS1, and therefore its induction in VSMCs might lead to the sustained production of high NO levels. This may in turn activate a number of signaling pathways, with the major candidate being the sGC–PRKG pathway. However, NO can also signal through sGC–PRKG-independent pathways, including those involving nitrosative stress. Our results indicate that supraphysiological NO levels, generated by administration of ISMN or DetaNO, suffice to trigger an MFS-like aortopathy in WT mice. These NO donors induce dose-dependent aortic dilation, elastic-fiber fragmentation, and a drop in BP, as well as recapitulating the sGC–PRKG signaling activation seen in MFS. The association of increased NO–sGC–PRKG signaling with aortopathy warrants consideration of the potential detrimental effects of drugs in clinical use that elicit chronic stimulation of this pathway. This applies not only to NO donors, but also to drugs that impair cGMP degradation, including phosphodiesterase type 5 (PDE5) inhibitors. NO donors have been extensively used to treat angina pectoris and heart failure, and PDE5 inhibitors to treat pulmonary hypertension and erectile dysfunction, with, to our knowledge, no reported pathological consequences related to aortic homeostasis or dilation. Based on accumulated clinical experience, we do not anticipate that patients treated with these agents will develop harmful aortic dilation. Nevertheless, our results with high NO-donor doses in mice suggest that these patients may be at risk of mild aortic dilation. Our results therefore indicate the need of caution with drugs that chronically activate the NO–sGC–PRKG signaling pathway, since they may be detrimental to aortic homeostasis. These drugs include advanced sGC stimulators such as Vericiguat (BAY 1021189) and Riociguat (BAY 63–2521), the latter having received FDA approval for pulmonary hypertension and heart failure, as well as novel short-acting sGC activators that lack hypertensive side effects, such as TY-55002, a candidate for the treatment of patients with acute decompensated heart failure.

Seemingly in conflict with our results, increased NO production by constitutively active Nos3 has been reported to exert a beneficial effect on the aorta in MFS mice. It should be noted, however, that NO levels generated by Nos3 are almost 1000-fold lower than those generated by Nos2. Given that Nos2 inhibitors regress aortic disease in MFS mice, our data suggest that the overproduction of NO by Nos2 underlies MFS aortopathy through sGC–PRKG pathway activation. It therefore seems that Nos2 activity might overcome the potential positive effects of Nos3 in MFS aorta.

When uncoupled, Nos2 can lead to overproduction of superoxide anion and peroxynitrite, which activate many signaling pathways. Nos2-mediated ROS overproduction can trigger a positive feedback loop via oxidation of the NOS cofactor BH4, further uncoupling Nos2 and increasing superoxide production. In this scenario, high NO and superoxide anion levels would generate peroxynitrite, leading to nitrosative stress accompanied by Tyr/Trp-nitration and S-nitrosylation of proteins and oxidative damage to other biomolecules. Oxidative stress has been linked to syndromic and nonsyndromic familial aortic diseases; moreover, in MFS mice redox stress is associated with NOX4 (NAPDH oxidase 4), which is upregulated in the aortas of MFS patients and whose deficiency protects MFS mice from elastic-fiber fragmentation and aortic dilation.

Because NO diffuses from the vascular wall to the lumen, the rise in nitrated proteins in MFS mice and patients indicates increased NO production in MFS and suggests that, despite the presence of high ROS levels, Nos2 generates high amounts of NO in MFS mice and patients. These data are also consistent with a recent report showing significant elevation of NO-derived metabolites such as nitrates in the plasma of MFS patients. Nitration on Tyr and Trp is considered a stable NO-derived post-translational modification, and only a few studies have reported these modifications in plasma. Given the low abundance of nitration, its detection in complex samples such as plasma is challenging with currently available technology, and we were able to detect Tyr/Trp-nitration only in some abundant proteins. Nevertheless, our data show that nitration is markedly upregulated in MFS mice and that MFS mice and NO-treated WT mice have similar nitration profiles, further supporting the notion that excessive NO production underlies MFS. Moreover, given the upregulation of plasma-protein nitration in MFS patients, our data also suggest that a highly nitrated plasma profile may be a signature of MFS, warranting future assessment of this post-translational modification as a biomarker of the disease.

Other markers of MFS identified in our study include cGMP, whose plasma levels are substantially upregulated in MFS mice and three MFS patient cohorts. Circulating cGMP appears to be a good indicator of sGC activity that can be easily determined in patient plasma or serum. Indeed, elevated cGMP has been reported in other diseases, including congestive heart failure and several cancers.

Given that pVASP-S239 appears to be a good marker of PRKG activity, its increase in the aortas of MFS patients strongly suggests enhanced PRKG activity in this tissue. This idea is supported by the marked drop in pVASP-S239 in MFS VSMCs treated with the PRKG inhibitor KT5823 and in the aortas of MFS mice treated with ODQ, KT5823, or lentivirus encoding...
Prkg1-shRNA. These findings suggest that analysis of pVASP-S239 in TAAD tissue samples obtained during elective or emergency aortic root surgery could be used to assess the extent of PRKG activation in these tissues.

Aortic dissection is the major cause of morbidity and mortality in MFS. Unfortunately, dissection often occurs when the aortic diameter is below the recommended threshold for elective prophylactic surgery\(^65\),\(^66\). This is also the case for type B dissections in MFS patients with prior prophylactic aortic surgery\(^67\). The identification of biomarkers that predict the risk of dissection would help in surgical decision-making. Plasma cGMP and Tyr/Trp-nitrated proteins could help to monitor the course of the disease or the efficacy of future treatments in clinical trials; however, future studies involving a larger clinical sample will be needed to support the prognostic potential of plasma cGMP and protein nitration. It is important to interpret changes in the identified markers with caution because our comparison of circulating markers was limited to MFS patients and healthy donors. Upregulated levels of the markers we describe here are likely a feature of other diseases involving NO pathway activation, such as infection, inflammation, allergy, or sepsis\(^58\),\(^69\). Although the identity of nitrated plasma proteins may differ depending on the
Fig. 7 Pharmacological inhibition of sGC or PRKG reverts aortopathy in Marfan syndrome. a Experimental design. 14-week-old MFS mice were treated daily for 21 days with 20mg/kg/day ODQ. Longitudinal ultrasound and BP analysis (Eco-BP) was performed at the indicated times (empty triangles). b Plasma cGMP at 21 d (n = 8 WT mice, n = 10 MFS mice, and n = 9 ODQ-treated MFS mice). c Representative pVASP-S239 immunofluorescence (red) in mouse aortic sections. Yellow dashed lines delineate the lumen boundary. IgG staining served as a negative control. Scale bar, 50 µm. d Quantification of pVASP-S239 immunofluorescence in aortic sections from 10 untreated WT mice (--), 10 MFS mice, and 9 MFS mice treated with 20 mg/kg/day ODQ for 21 d. IgG staining served as a negative control. Scale bar, 50 µm. Data are mean ± s.e.m. Each data point denotes an individual mouse. Differences were analyzed by one-way ANOVA with Tukey’s post-hoc test (p-values are shown). e, f Systolic BP (e) and maximal AsAo and AbAo (f) diameter at the indicated times (n = 5 mice each for untreated and ODQ-treated MFS groups; n = 7 mice for WT group). Data are mean ± s.e.m. **P < 0.01, ***P < 0.001, and ****P < 0.0001 (versus WT mice); #P < 0.05, ###P < 0.001, and ####P < 0.0001 (versus untreated MFS) by repeated-measurements two-way ANOVA with Tukey’s post-hoc test. g Experimental design. 14-week-old MFS mice were treated daily for 7 days with 2µmol/kg/day KT5823 and monitored for aortic dilation and BP before treatment and 3 and 7 d post-treatment. h Representative pVASP-S239 immunofluorescence (red) in mouse aortic sections. Yellow dashed lines delineate the lumen boundary. IgG staining served as a negative control. Scale bar, 50 µm. I Quantification of pVASP-S239 immunofluorescence in aortic sections from untreated WT mice (--) (n = 5), MFS mice (n = 5), and MFS mice treated with 2µmol/kg/day KT5823 for 7 d (n = 7). Data are shown relative to untreated WT mice as mean ± s.e.m. Each data point denotes an individual mouse. Differences were analyzed by one-way ANOVA with Tukey’s post-hoc test (p-values are shown). j, k Systolic BP (j) and maximal AsAo and AbAo (k) diameter at the indicated times (n = 10 for untreated WT group; n = 0 per each MFS group). Data are mean ± s.e.m. **P < 0.01 and ****P < 0.0001 (versus WT), #P < 0.05 and ##P < 0.01 (versus untreated MFS) by repeated-measurements two-way ANOVA with Tukey’s post-hoc test. Source are provided in the Source Data file.

Pathology, these markers would in principle be more suitable for monitoring or predicting disease course than for diagnosis. Again, future studies in larger clinical cohorts will be important in determining whether pharmacological treatment can modify the profile of nitrated proteins and the levels of cGMP. The presence of increased plasma cGMP and protein nitration in patients and MFS mice indicates that medical treatment does not induce this increase, but it will be important to confirm that medication does not affect the protein nitration profile. Regardless of their potential as biomarkers in familial aortopathies, cGMP and protein nitration appear to be useful tools for determining if this pathway is also activated in other TAADs.

Tissue levels of pVASP-S239 could also be used to determine if PRKG activation is a signature of other TAADs. Recent findings show that a gain-of-function mutation in the human PRKG1 gene generates a constitutively activated kinase and predisposes to TAA in affected families; moreover, the equivalent activating mutation in heterozygous mice (Prkg1R177Q+/− mice) triggers an age-dependent aortic dilation56,70,71. In affected families, the increased PRKG1 activity stimulates myosin regulatory light chain phosphatase, which can alter VSMC contractility34. It is thus clear that PRKG activation can mediate aortopathy in syndromic and nonsyndromic familial TAA. It remains to be seen whether sGC–PRKG pathway activation also mediates disease in syndromic diseases other than MFS or in nonsyndromic TAADs not caused by PRKG1 mutation.

An essential role of NO–sGC–PRKG signaling in aortopathy does not exclude roles in the disease for NOS2-derived signals independent of this pathway. Indeed, oxidants such as NOS2-derived superoxide anion can also activate PRKG29, and signals downstream of PRKG involving oxidative stress and JNK activation have been shown to mediate aortopathy in Prkg1R177Q+/− mice, in which antioxidants prevent age-dependent aortic dilation56. However, the action of PRKG appears to be mediated mainly through its regulation of contractility via myosin light chain phosphatase activation and decreased calcium influx in VSMCs54,72. The drop in cellular calcium levels might contribute to aortic disease, as suggested by the deleterious effect of calcium channel blockers (CCBs) in MFS mice and by the increased risk of aortic dissection and need for aortic surgery in MFS patients treated with CCBs compared with patients receiving other anti-hypertensive drugs73. Indeed, the aortic damage caused by CCBs in MFS might be attributable to its cooperation with PRKG activation in depleting cellular calcium stocks. Guideline recommendations for CCBs as an alternative blood pressure regulator in patients at risk of aortic aneurysm should perhaps be revisited. Our data showing VASP-mediated modulation of actin fiber formation suggest that PRKG regulation of contractility might be regulated not only through the myosin components of the actomyosin cytoskeleton, but also through actin. Since actin Tyr nitration impairs actin polymerization dynamics74, our finding that numerous Acta2 Tyr residues are nitrated in the aortas of MFS mice reveals an additional mechanism for NO-mediated regulation of actomyosin cytoskeleton dynamics tuning and for contractility dysregulation. In this scenario, the induction of contractility markers might be seen as an adaptive response to a deficient capacity of the aorta for contraction.

Our results suggest that the control of NO levels and signaling in the aorta is critical for VSMC homeostasis and implicate abnormally high NO–sGC–PRKG signaling as the underlying cause of aortopathy in MFS mice and patients. PRKG can be activated by superoxide anion; however, our data showing that sGC pharmacological inhibition reduces pVASP-S239 in vitro and in vivo strongly suggest that PRKG activation in MFS is dependent on sGC activity. Furthermore, the regression of aortic dilation in MFS mice treated with ODQ supports a causal role for sGC activation in the disease. Similarly, the reversion of aortic dilation and medial degeneration in MFS mice treated with PRKG inhibitors or aortic Prkg1 knockdown also strongly suggests that MFS is mediated by PRKG activation.

Although prophylactic surgery has increased the lifespan of MFS patients, there are currently no pharmacological treatments available to arrest aortic growth or prevent dissections14,75. There is therefore an urgent need to develop effective therapies. Our data lay the basis for exploring the implication of NO–sGC–PRKG signaling in other forms of TAAD and determining the potential of sGC or PRKG inhibition in the treatment of patients with MFS or other forms of TAAD.

Methods

Animal procedures. Animal procedures and experiments complied with all relevant ethical regulations, were approved by the CNIC Ethics Committee and the Madrid regional authorities (ref. PROEX 80/16) and conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005. Overall mouse health was assessed by daily inspection for signs of discomfort, weight loss, or changes in behavior, mobility, and feeding or drinking habits. Mice were housed in a pathogen-free animal facility under a 12 h light/dark cycle at constant temperature and humidity, and fed standard rodent chow and water ad libitum. Fbn1+/-;Prkg1−/− mice58, which harbor a mutation in the Fbn1 gene, were obtained from Jackson Laboratories (JAX mice stock #012885). This strain had been previously backcrossed to the C57BL/6

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background for more than nine generations. All mice were genotyped by tail-sample PCR using the following primers: 5′-CTCATCATTTTTGGCCAGTTG-3′ and 5′-GCACTTGATGCACATTCACA-3′. Wild-type (WT) mice were on the C57BL/6 background, and 12–15-week-old males and females were used for all experiments. Mice were treated with isosorbide mononitrate (ISMN; MedChem Express; Monmouth Junction, NJ, USA) or DetaNONOate (DetaNO; Enzo Life Sciences; Farmingdale, NY, USA) using subcutaneous osmotic minipumps (Alzet Corp; Cupertino, CA, USA). The specific inhibitors of sGC (ODQ) and PRKG (KT5823), both from Focus Biomolecules (Plymouth Meeting, PA, USA), were dissolved in dimethylsulfoxide (DMSO), diluted in 0.9% NaCl (DMSO content was <0.5%), and administered i.p. at 20 mg/kg/day for 21 days and 5 mg/kg/day for 7 days, respectively. Control mice received the corresponding intraperitoneal vehicle.

**Blood pressure measurements.** Arterial blood pressure (BP) was measured in mouse tails using the automated BP-2000 Blood Pressure Analysis System and
isolated from 4 echography device (Fuji ± s.e.m. Each data point denotes an individual mouse. (
indicated times. IgG staining served as a negative control. Scale bar, 50 µm. (e) Quantification of Prkg1 immunofluorescence in AsAo sections of control WT and shScr-, and shPrkg1-B-transduced MFS mice (n = 9 in WT mice and MFS mice infected with shPrkg1-B, n = 10 in MFS mice infected with shScr or shPrkg1-A). Data are shown relative to WT mice as mean ± s.e.m. Each data point denotes an individual mouse. (f) Differences were analyzed by one-way ANOVA with Tukey’s post-hoc test (p-values are shown). (g, h) Maximal AsAo and AbAo (g) diameter and systolic BP (h) at the indicated times (n = 10 per group). Data are mean ± s.e.m. **P < 0.05, ***P < 0.001, ****P < 0.0001 versus shScr by repeated-measurements two-way ANOVA with Tukey’s post-hoc test. I Representative images showing staining with hematoxylin and eosin (HE) and elastic van Gieson (EVG) in AsAo of the indicated mice (n = 9–10 per group). Yellow arrowheads indicate elastin breaks. Scale bar, 50 µm. J Wall thickness and K elastin breaks in AsAo sections from the mouse cohorts shown in I. Data are mean ± s.e.m. Each data point denotes an individual mouse. j, Differences were analyzed by one-way ANOVA with Tukey’s post-hoc test (p-values are shown). Source data are provided in the Source Data file.

software (Visittech Systems, Apex, NC, USA).27,28 BP measurements were recorded in mice located in a tail-cuff restrainer over a warmed surface (37 °C). Mice underwent a training period, with BP monitored every day for 7 days. After that, baseline BP was measured 1 day before the beginning of the treatment in each mouse cohort. Fifteen consecutive systolic BP measurements were made, and the last 10 readings per mouse were recorded, tested for outliers using the Chauvenet criterion and averaged. BP monitoring was repeated during experiments as indicated in the Figure legends.

In vivo ultrasound imaging. Images of the aorta were obtained in isoflurane-sedated mice (2% isoflurane) by high-frequency ultrasound with a VIVO 2100 echography device (Fujifilm-VisualSonics, Toronto, Canada) with a transducer that provides 30-micron axial resolution. Maximal internal aortic diameters were measured at systole using VIVO 2100 software, version 1.5.0 (VisualSonics). Measurements were taken before treatment initiation to determine the baseline diameters and were repeated several times during the experiment.

Cell procedures. Primary mouse vascular smooth muscle cells (VSMCs) were isolated from 4–6-week-old mice and grown as described.29,30 Briefly, tissue was digested with a solution of collagenase and elastase until a single-cell suspension was obtained. Cells were then cultured at 37 °C, 5% CO2 in growth medium (Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 20% fetal bovine serum [FBS]). All experiments were performed during passages 3–7. VSMCs were FBS-starved 36 h before stimulation experiments. The HEK-293T (CRL-1373) and Jurkat (Clone E6-1, TIB-152) cell lines, required for high-titer lentivirus production and lentivirus titration, respectively, were purchased from ATCC. All cells were mycoplasma-negative.

VSMCs were incubated with 100 µM DetANO or 100 µM 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; Biolog; Bremen, Germany). In some experiments, the NOS inhibitor N′-nitro-L-arginine methyl ester (LNAME; 300 µM Sigma-Aldrich; St. Louis, MO, USA), ODQ (10 µM), or KTS823 (1 µM), were added to VSMCs 1 h before the addition of DetANO or 8-Br-cGMP. In another set of experiments, MFS VSMCs were incubated for 24 h with KTS823.

For cell immunostaining, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 in PBS for 30 min. Samples were incubated overnight in a 1:200 dilution of anti-phiVASP antibody (D-20; sc-101439; Santa Cruz Biotechnology; Santa Cruz, CA, USA) or rabbit anti-fibrin(ogen)-1 polyclonal antibody31 (1:2000; PA9543; kindly donated by Dr. L. Sakai). The secondary antibody was AlexaFluor568-conjugated goat anti-mouse (1:500; A-11031; Molecular Probes; Carlsbad, CA, USA). To determine F-actin formation, cells were stained with Texas Red-X-conjugated phalloidin (1:2000; T7471; Thermo Fisher Scientific, Bremen, Germany) in at least three independent batches, with three technical triplicates per condition and batch and at least three images captured per triplicate. Images were acquired at 1024 × 1024 pixels, 8 bits, using a Confocal TCS Leica SP5 microscope (Leica Microsystems GmbH; Wetzlar, Germany) fitted with a ×60 oil-immersion objective and Leica LAS AF V2.7.3. acquisition software. All images were processed for presentation with Photoshop (Adobe) according to the guidelines of this journal and analyzed with ImageJ software (version 1.52a, NIH, http://rsb.info.nih.gov/ij/). Staining was quantified after setting an intensity threshold to include only specific signals. For intracellular staining, total intensity was relativized to cell area. For extracellular staining of Fn1, the signal was calculated as the stained area. Each condition’s value was relativized to the average staining of the experiment.

Lentivirus production and infection. A lentiviral plasmid encoding GFP and shRNA targeting mouse Prkg1 was engineered by cloning the following shRNA sequences into the pH1-DUAL lentiviral vector: shPrkg1-A (sense) 5′-GATCCCCGTCCGGATATGTACGCAGACACCATCCTTCTGGATATAGTCGAGAACAGAAGGTTTTTTGC-3′; shPrkg1-B (sense) 5′-GATCCCCGAGGGCTGTTCTCCACCTTCTGAGACTATGAGAGACATCCTTGG-3′; shPrkg1-C (antisense) 5′-GGCGGCCAAAATACGAGTGAGAGCACATTCGGTGGGTTCGTCGAAGGTTTAACTC-3′; shPrkg1-D (antisense) 5′-GGCGGAAAACCGGAGAAATCTCCGCGAG-3′.

VSMCs were infected by transduction with HEK-293T cells with a plasmid encoding GFP and shPrkg1-specific shRNA. Viruses were concentrated from culture supernatant by ultracentrifugation (2 h at 121,896 × g; Ultraclear Tube; SW28 rotor and Optima L-100 XP Ultracentrifuge; Beckman, Brea, CA, USA), suspended in cold sterile PBS, and titrated by infection of Jurkat cells. Jurkat cells were seeded in a 96-well plate and infected with the desired lentiviral dilution (1:10-1:100000). After 48 h, cells were centrifuged 5 min at 1800 × g and resuspended in 200 µmL of ice-cold PBS with 1:100000 propidium iodide. Transduction efficiency (% of GFP-expressing cells) and cell death (propidium iodide incorporation) were quantified by flow cytometry, as illustrated in Supplementary Fig. 14. Flow cytometry data were collected using a Canto III F Hayes cytometer and the BD FACSDiva Software Version 6.1.3, and analyzed using FlowJo 10.7.1 software.

VSMCs were infected (multiplicity of infection = 10) overnight at 37 °C in growth medium. Medium was then replaced with fresh growth medium, and cells were cultured for 7 additional days and then processed for mRNA expression analysis and cell immunostaining or stimulated for 5 min with DetANO for immunoblot assays. For in vivo transduction experiments, animals were anesthetized (with ketamine and xylazine), and a small incision was made to expose the right jugular vein. Virus solution (100 µL, 106 particles/mL in PBS) was injected directly into the right jugular vein. Transduction efficiency was analyzed in aortic samples by GFP immunohistochemistry and Prkg1 immunofluorescence.

cGMP immunostaining. cGMP was measured in mouse and human plasma by competitive enzyme immunoassay (RKG005, R&D Systems; Minneapolis, MN, USA). To obtain mouse plasma, blood was extracted after sacrifice by CO2 inhalation using the cardiac puncture method, collected in EDTA tubes, and centrifuged for 15 min at 13,000 × g. Plasma and serum samples from MFS patients and healthy blood donors were obtained and processed following standard operating procedures.

Histology. After sacrifice of mice by CO2 inhalation, aortas were perfused with saline, isolated, fixed in 10% formalin overnight at 4 °C, and paraffin embedded. Paraffin cross-sections (5 µm) from fixed ascending aortas (AsAo) were prepared for immunohistochemistry or immunofluorescence, or stained with hematoxylin and eosin, Alcian blue, or modified Verhoeff elastic Van Gieson (EVG) kit (Sigma-Aldrich). Images were acquired under a Leica DM2500 microscope fitted with a ×40 HCX PL Fluor objective or with a scanner NanoZoomer-2. ORSC110730. Leica Application Suite V3.5.0 acquisition software and NDP.view 2 V2.7.43 software were used, respectively. Elastic lamina breaks, defined as interruptions in elastic fibers, were counted in the entire medial layer of six non-consecutive cross-sections per mouse, using 4–12 mice per experiment. The mean number of breaks was calculated. The exact number of mice per group is indicated in the figure legends.
For immunofluorescence, deparaffinized sections were rehydrated, boiled 3 min to retrieve antigens in 10 mM citrate buffer containing 0.05% Tween-20, pH 9.5, and blocked for 1 h with 10% goat serum plus 2% BSA in PBS. Samples were incubated with the following antibodies for immunohistochemistry or immunofluorescence: mouse monoclonal anti-pV-PAS (1:50; sc-101439, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-p-V-PAS (1:25; SAB4300129, Sigma–Aldrich, St. Louis, MO, USA), rabbit polyclonal anti-PFK (1:50, ADI-KAP-PK005-F, Enzo Life Sciences), and rabbit polyclonal anti-GFP (1:100, A11122, Invitrogen; Carlsbad, CA, USA). Specificity was determined by substituting the primary antibody with unrelated IgG (Santa Cruz) at the same dilutions as the antigen-specific antibodies. For immunohistochemistry, color was developed with DAB (Vector Laboratories, Burlingame, CA, USA), and sections were counterstained with hematoxylin and mounted in DPX (Casa Álvarez, Madrid, Spain). For immunofluorescence, secondary antibodies were polyclonal Alexa-Fluor-647-conjugated goat anti-rabbit or polyclonal Alexa-Fluor-647-conjugated chicken anti-mouse (1:300, Molecular Probes). Sections were mounted with DAPI in Citifluor AF4 mounting medium (Anatech, Madrid, Spain). For immuno-
were used: UniProtKB, Swiss-Prot and TrEMBL (https://www.uniprot.org/uniprot/), that support the repository (http://www.peptideatlas.org/PASS/PASS01528). The following databases human reference proteome database July 2018, are publicly available in the Peptide Atlas Research Reporting Summary linked to this article.

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**Data availability**

Data were provided with this paper. For further details, please contact the corresponding author.

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Author contributions
M.R.C. and J.M.R. conceived the study; M.T., A.d.I.F.-A., M.R.C., and J.M.R. designed the study and analyzed the data; A.d.I.F.-A. and M.T. performed most of the experiments, with contributions from S.M.-M., A.A., M.J.R.-R., M.J.M.-O., and D.L.-M.; A.A., E.B.-K., I.G.-V., performed the proteomics and bioinformatics analysis under the supervision of J.V., who also provided ideas for the project; C.E.M., E.G.-I., A.F., L.M.-M., J.D.B., J.F.N., G.T.-T., and A.E. provided human tissue samples and ideas for the project; M.R.C. and J.M.R. wrote the manuscript with contributions from A.d.I.F.-A., M.T., E.B.-K., A.A., and J.V. All authors read and approved the manuscript.

Competing interests
The authors declare no competing interests.

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