Resveratrol Inhibits Aortic Root Dilatation in the Fbn1C1039G/+ Marfan Mouse Model

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Objective—Marfan syndrome (MFS) is a connective tissue disorder caused by mutations in the fibrillin-1 gene. Patients with MFS are at risk of aortic aneurysm formation and dissection. Usually, blood pressure–lowering drugs are used to reduce aortic events; however, this is not sufficient for most patients. In the aorta of smooth muscle cell–specific sirtuin-1–deficient mice, spontaneous aneurysm formation and senescence are observed. Resveratrol is known to enhance sirtuin-1 activity and to reduce senescence, which prompted us to investigate the effectiveness of resveratrol in inhibition of aortic dilatation in the Fbn1C1039G/+ MFS mouse model.

Approach and Results—Aortic senescence strongly correlates with aortic root dilatation rate in MFS mice. However, although resveratrol inhibits aortic dilatation, it only shows a trend toward reduced aortic senescence. Resveratrol enhances nuclear localization of sirtuin-1 in the vessel wall and, in contrast to losartan, does not affect leukocyte infiltration nor activation of SMAD2 and extracellular signal–regulated kinases 1/2 (ERK1/2). Interestingly, specific sirtuin-1 activation (SIRT1720) or inhibition (sirtinol) in MFS mice does not affect aortic root dilatation rate, although senescence is changed. Resveratrol reduces aortic elastin breaks and decreases micro-RNA-29b expression coinciding with enhanced antiapoptotic Bcl-2 expression and decreased number of terminal apoptotic cells. In cultured smooth muscle cells, the resveratrol effect on micro-RNA-29b downregulation is endothelial cell and nuclear factor κB-dependent.

Conclusions—Resveratrol inhibits aortic root dilatation in MFS mice by promoting elastin integrity and smooth muscle cell survival, involving downregulation of the aneurysm-related micro-RNA-29b in the aorta. On the basis of these data, resveratrol holds promise as a novel intervention strategy for patients with MFS. (Arterioscler Thromb Vasc Biol. 2016;36:1618-1626. DOI: 10.1161/ATVBAHA.116.307841.)

Key Words: aortic aneurysm ■ extracellular matrix ■ Marfan syndrome ■ micro-RNAs ■ resveratrol ■ sirtuin-1

Marfan syndrome (MFS) is an autosomal connective tissue disorder caused by different mutations in the fibrillin-1 gene (FBN1) with an incidence of 1 of 5000 individuals.1 Patients with MFS have extended bones, develop scoliosis and ectopia lentis. Another major clinical problem for patients with MFS is their increased risk to develop aortic aneurysms, and often, lethal dissections. Until now, the most effective treatment to prevent aortic dissections is prophylactic aortic surgery.2 To evaluate the size and risk of aortic dissection, patients with MFS have their aorta monitored regularly by imaging. Current pharmacological treatment aims at blood pressure regulation using mostly β-blockers and losartan.3

An important role in the disease process has been attributed to angiotensin-II receptor type-1 (AT1R) signaling and subsequent overexpression of transforming growth factor-β (TGF-β), leading to canonical SMAD2 and noncanonical extracellular signal–regulated kinases 1/2 (ERK1/2) phosphorylation. Blockade of AT1R by losartan has a beneficial effect on aortic dilatation in the Fbn1C1039G/+ and Fbn1mgR/mgR MFS mouse models.4–6 However, in 3 out of 4 clinical trials in patients with MFS, losartan was not superior to β-blocker therapy.7–9 Only in the COMPARE trial (Cozaar in Marfan Patients Reduces Aortic Enlargement), we observed a significant effect of losartan when used on top of standard medication,7 which may be explained by the beneficial effect especially in MFS patients with a haploinsufficient FBN1 mutation (one third of the patients with MFS).10 This patient category was more sensitive to losartan treatment when compared with most patients with a

Received on: February 2, 2016; final version accepted on: May 25, 2016.
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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.307841/-/DC1.
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dominant-negative FBN1 mutation (two third). These observations strongly support the hypothesis that besides AT1R signaling, additional pathways are responsible for pathological aortic changes in patients with MFS.

Spontaneous aneurysm development and senescence were observed in a proatherogenic mouse model with sirtuin-1 (SIRT1) deficiency specifically in smooth muscle cells (SMCs), which may suggest that inhibiting senescence is a potential treatment approach to combat aneurysm development. Senescence is a cellular state of discontinued cell division, with a unique (inflammatory) cytokine profile. It can be induced by age and various stressors, such as DNA damage and oxidative stress. Interestingly, cultured porcine abdominal aortic aneurysm SMCs show increased senescence compared with healthy SMCs. In addition, enhanced oxidative stress is found in the aorta of MFS mice as a potential inducer of vascular senescence. Together, these data suggest that senescence could play a role in aortic aneurysm formation. Resveratrol (a polyphenol in skin of red grapes) reduces vascular senescence by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, thus decreasing oxidative stress, in a SIRT1-dependent fashion. Therefore, we hypothesize that modulation of aneurysm progression in MFS mice may be possible with resveratrol, the SIRT1 agonist SRT1720, and the SIRT1 antagonist sirtinol. In this study, we show that resveratrol decreases aortic root dilatation in the Fbn1C1039G/+ MFS mice, whereas specific SIRT modulation did not, which reveals a different mechanism of action than anticipated.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Senescence Is Correlated With Aortic Root Dilatation
To study aortic senescence, aortic arches of MFS mice were analyzed for senescence-associated (cytoplasmic) β-galactosidase–stained aorta, the blue senescent cells were localized throughout the vessel wall in endothelial cells, medial SMCs, and adventitial fibroblasts (Figure 1A).

To assess if senescence correlates with aortic root dilatation rate, the aortic arches were incubated with fluorescent fluorescein di-β-D-galactopyranoside substrate to quantify senescence. We calculated the aortic root dilatation rate from the 2- and 4-month aortic root diameters as determined by quantitative morphometry. A strong positive correlation was found between the aortic root dilatation rate and aortic senescence (r=0.772; P<0.001; Figure 1B), suggesting that senescence may be involved in aneurysm development.

Resveratrol Reduces Aortic Root Dilatation
To counteract senescence, MFS mice were treated with resveratrol for 2 months starting at 2 months of age. Interestingly, 2-month-old MFS mice already had a larger aortic root diameter (tissue sections) than wild-type (WT) mice of the same age (0.62±0.031 versus 0.55±0.035 mm; P=0.003). Thus, drug treatment aims at controlling disease progression, not prevention. At 4 months of age, MFS mice showed the expected increase in aortic root dilatation rate when compared with WT mice (Figure 2A; P=0.004), which was significantly reduced by losartan (positive control; P=0.018). This is in line with previous findings in MFS mice, as measured by ultrasound. Moreover, resveratrol treatment significantly reduced the aortic root dilatation rate (Figure 2A; P<0.001), even significantly better than losartan (P=0.007), suppressing it to WT level.

To relate our method of analysis of aortic root sections to in vivo diameters, we compared the histological diameters to the diameters obtained by ultrasound imaging in WT and MFS mice of different ages. Aortic root dimensions correlated well with ultrasound measurements in the same mice (Figure 1 in the online-only Data Supplement; r=0.709; P<0.001) although the diameter was 1.8-fold smaller in tissue sections, probably because of lack of arterial pressure and shrinkage on processing. Therefore, the absolute diameters obtained with ultrasound are considered as actual width. However, to monitor aortic dilatation and treatment efficacy, quantification via histology is reliable.

Resveratrol Has a Positive Effect on Aortic Wall Pathology
Given that resveratrol can activate SIRT1, we studied nuclear localization of SIRT1 as a measure of activation in the aortic root. Nuclear SIRT1 was similar in WT, MFS placebo, and MFS losartan-treated mice (Figure 2A); however, resveratrol treatment significantly increased the number of SIRT1-positive nuclei (Figure 2A and 2D; P<0.001). These data suggest that enhanced SIRT1 protects against aortic damage as resveratrol treatment diminished the aortic root dilatation rate.

Quantitative analysis of senescence in the aortic arch showed a significant increase in MFS placebo mice when compared with WT mice (Figure 2B; P=0.011). Interestingly, not only resveratrol- but also losartan-treated MFS mice showed a trend toward decreased senescence when compared with
the MFS placebo group (Figure 2B; P=0.087 and P=0.113, respectively).

Total medial area is known to increase in MFS mice (Figure 2B; P=0.002) because of excessive extracellular matrix production at sites of elastin loss, which is a sign of disease severity.\(^4\) Compared with MFS placebo mice, the medial area was significantly smaller in resveratrol-treated mice (P=0.013), suggesting that disproportionate extracellular matrix production was reduced by resveratrol, whereas losartan did not improve medial thickness significantly.

A characteristic feature of MFS is elastic lamina fragmentation in the aorta because these lamellae consist of elastin and fibrillin-1\(^{19,20}\); of which, the latter protein is defective in MFS.\(^21\) To determine if resveratrol has an effect...
on the integrity of these elastin fibers, the number of elastin breaks was quantified in aortic root sections. Although elastin breaks were high in MFS placebo (P=0.001), resveratrol treatment showed a decrease, even compared with losartan-treated MFS mice (Figure 2C and 2D; P=0.024 and P=0.021, respectively).

Resveratrol treatment showed the expected decrease in weight gain (Figure II in the online-only Data Supplement; P=0.024), indicating that resveratrol was metabolized.

**SIRT1 Modulation Does Not Alter Aortic Root Dilatation**

To delineate the mechanism by which resveratrol inhibits aortic root dilatation in MFS mice, we performed an experiment with the SIRT1 activator SRT1720 and the SIRT1 inhibitor sirtinol, as we observed increased nuclear SIRT1 staining in resveratrol-treated MFS mice. SRT1720 activates SIRT1 indirectly via intracellular increase of nicotinamide adenine dinucleotide (NAD). This mechanism of SIRT1 induction is similar to that of resveratrol. Sirtinol is known to enhance senescence through deactivation of SIRT1 and is used in cancer research to induce premature aging of cancer cells to limit tumor growth. Unexpectedly, the aortic root dilatation rate was not changed significantly by SRT1720 or sirtinol (Figure 3A). Because the SIRT1 agonist and antagonist do not show opposing or significant effects on aortic root dilatation, we conclude that the positive resveratrol effect is not an SIRT1-dependent process.

To investigate treatment effectiveness, we measured senescence, medial area, and weight gain. On analysis of aortic senescence, we observed increased senescence by sirtinol (Figure 3B; P=0.050). Interestingly, the increase in senescence did not result in detrimental aortic growth. Significant reduction in medial area and weight gain was observed by sirtinol (Figure 3C; P=0.001).
measured upon SRT1720 treatment (Figure 3C; Figure III in the online-only Data Supplement; \( P=0.003 \) and \( P<0.001 \), respectively), which is similar to that shown for resveratrol. In conclusion, SRT1720 and sirtinol were provided in an effective dose; yet, they did not influence aortic root dilatation rate significantly.

**Resveratrol Does Not Change Inflammation, TGF-β Signaling, and Cardiac Phenotype**

Given that SIRT1 is not the mechanism whereby resveratrol inhibits aortic dilatation, we considered whether resveratrol reduces inflammation or TGF-β signaling. Because resveratrol is known to reduce inflammation\(^2\) and inflammation is observed in MFS aortic tissues,\(^2\) we therefore quantified aortic leukocytes (CD45). In addition, we quantified the amount of nuclear phosphorylated SMAD family member-2 (pSMAD2) and mitogen-activated protein kinase ERK1/2 (pERK1/2), representing the canonical and noncanonical TGF-β pathway\(^1\) because TGF-β–mediated signaling is considered a typical feature of MFS. Leukocyte and pERK1/2 positive area were significantly increased in MFS placebo mice (Figure 4A and 4C; \( P<0.001 \) and \( P=0.015 \)). Losartan effectively decreased the inflammatory state and nuclear pSMAD2 and pERK1/2 in the aortic root, as expected\(^4\) (Figure 4A through 4C; \( P=0.005 \), \( P=0.039 \), and \( P=0.013 \)), whereas the resveratrol-treated mice revealed a similar level of inflammation and pSMAD2 and ERK1/2 activation as the placebo MFS mice.

Because MFS may also affect the heart, the effect of resveratrol on cardiac stress markers atrial natriuretic peptide and brain natriuretic peptide was analyzed. No cardiac stress could be detected by in situ hybridization for atrial natriuretic peptide mRNA or serum brain natriuretic peptide in these relatively young MFS mice (Figure IV in the online-only Data Supplement).

These data thus demonstrate that resveratrol does not affect accumulation of inflammatory cells in the aortic root, leaves TGF-β signaling intact, and does not affect the cardiac phenotype, yet does reduce aortic root dilatation.

**Resveratrol Affects Aneurysm-Related Micro-RNAs**

Many relevant micro-RNAs (miRs) have been described for aortic aneurysm formation\(^2\); therefore, we investigated the effect of resveratrol on these miRs (Figure 5A). MiR-21a and miR-195 were upregulated after resveratrol treatment (\( P=0.014 \) and \( P=0.016 \), whereas miR-23b, miR-24, and miR-712 were unaffected. Interestingly, the miR-29 family members \( a \) to \( c \) were all downregulated by resveratrol (Figure 5A; \( P=0.030 \), \( P=0.038 \), and \( P=0.030 \), respectively). MiR-29b downregulation has actually been successful in preventing aneurysm formation in different murine abdominal and thoracic (MFS) aortic aneurysm models.\(^5\) MiR-29b has been reported to increase SMC apoptosis; therefore, we measured expression of antiapoptotic factors Bcl-2 and Mcl-1.\(^5\) Clearly, these prosurvival genes were more abundant after resveratrol treatment when compared with WT and MFS placebo mice (Figure 5C; \( P=0.010 \), \( P=0.041 \), \( P=0.002 \), and \( P=0.002 \), respectively), which may contribute to increased aortic integrity. Subsequently, apoptosis was investigated by performing a terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. Resveratrol-treated MFS mice showed less apoptotic cells in the aortic root (Figure 5C; right; \( P=0.016 \)), indicating reduced SMC loss.

**Endothelial Cell–Dependent Effect of Resveratrol on SMC miR-29b Expression**

To further delineate the mechanism whereby resveratrol modulates miR-29b expression, we cultured aortic mouse
SMCs with resveratrol, as it is the major cell type in the vessel wall. However, no difference in miR-29b expression was observed (Figure VA in the online-only Data Supplement). Hereafter, human umbilical cord endothelial cells (HUVECs) were cultured with resveratrol as these cells communicate with SMCs and are dysfunctional in MFS.32–35 Again, we did not observe a difference in expression of miR-29b upon direct resveratrol stimulation (Figure VB in the online-only Data Supplement). However, resveratrol-treated HUVECs expressed increased shear stress–responsive transcription factor KLF2, as also observed by others,36,37 which represents an improved endothelial phenotype (Figure VC in the online-only Data Supplement; P=0.012). Subsequently, conditioned medium derived from resveratrol-treated HUVECs, given to SMCs (Figure 6A), did downregulate miR-29b and upregulate Bcl-2 expression in SMCs, similar to those in the resveratrol-treated mouse aortae (Figure 6A; P=0.011 and P=0.025, respectively). To delineate the role of KLF2 in SMC miR-29b expression, lentiviral overexpression of KLF2 in HUVECs was performed (Figure VIA in the online-only Data Supplement; P=0.044), and hereafter, SMCs were stimulated with the conditioned medium. No decrease of miR-29b or increase in Bcl-2 mRNA could be observed (Figure VIB and VIC), indicating no KLF2-dependent regulation of miR-29b.

To study apoptosis, SMCs were stimulated with HUVEC-conditioned medium +/- apoptosis-inducer staurosporin. SMCs showed a decrease in apoptotic cells when HUVECs were incubated with resveratrol (Figure 6A; ***P<0.001 and ***P=0.004). However, the effect of resveratrol-stimulated HUVEC-conditioned medium on SMCs was no longer significantly reduced (Figure 6B).

Pooled serum from resveratrol-treated mice also downregulated miR-29b expression in SMCs (Figure 6C; P=0.010), illustrating the indirect effect of resveratrol on SMCs via (among others) endothelial cells. NF-xB inhibitor Ikka is upregulated in SMCs stimulated with MFS placebo serum, whereas this effect was no longer seen in SMCs stimulated with resveratrol-treated mouse serum (Figure 6C; P=0.049). Typical downstream genes of NF-xB, cytokine Il6 and chemokine Il8, were downregulated in SMCs with MFS placebo mouse serum (Figure 6C, bottom; P=0.031, P=0.005, P<0.001, and P=0.005) and normalized with MFS resveratrol mouse serum, indicating reduced NF-xB activity in MFS placebo mice, which is rescued after resveratrol treatment. This suggests that enhanced NF-xB activity in SMCs may be responsible for the reduction in miR-29b, as shown before.5

Our data demonstrate that resveratrol has a protective effect on elastin integrity and SMC survival, presumably by increasing SMC NF-xB signaling and thereby reducing miR-29b expression, which protects against aortic dilatation (Figure 6D).

Figure 6. Resveratrol-mediated regulation of micro-RNA-29b (miR-29b) in the aorta. A. Scheme of experimental set-up; smooth muscle cells (SMCs) are stimulated with conditioned medium of human umbilical cord endothelial cells (HUVECs), which were treated with/without resveratrol. Medium of resveratrol-treated HUVECs inhibits miR-29b and upregulates Bcl-2 expression in cultured SMCs compared with medium of control HUVECs. The number of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cells is decreased by resveratrol-treated HUVEC medium, also after staurosporin (stauro) incubation. B. Nuclear factor xB (NF-xB) activity is decreased in HUVECs and SMCs, which are treated with resveratrol directly. In SMCs, treated with HUVEC-conditioned medium, no difference can be observed with/without resveratrol. C. SMCs stimulated with serum from wild-type (WT), Marfan syndrome (MFS) placebo, and resveratrol-treated MFS mice show an increase in miR-29b expression with MFS placebo serum, which is inhibited with MFS resveratrol serum. NF-xB inhibitor Ikka mRNA expression is increased with MFS serum, which is no longer significant with MFS resveratrol serum. Hallmark NF-xB downstream genes Il6 and Il8 are decreased in expression with MFS serum and normalized with MFS resveratrol serum. D. Resveratrol downregulates miR-29b in SMCs in an endothelial cell–dependent manner, probably via enhancing NF-xB signaling, resulting in decreased SMC apoptosis. This indirect effect could be mimicked when using serum from the different mouse groups. In addition, resveratrol improves the extracellular matrix integrity by decreasing the number of elastin breaks. Collectively, this leads to enhanced aortic repair. OD indicates optical density.
Discussion

In this study, we demonstrate a positive correlation between aortic senescence and aortic root dilatation. Treatment of Fbn1C1039G/+ MFS mice with losartan and resveratrol inhibited aortic dilatation, with resveratrol having a more pronounced effect than losartan. Both compounds reduced aortic senescence such that it was not significantly different from the WT mice. Losartan treatment diminished vascular inflammation and pSMAD2 and pERK1/2 signaling, whereas resveratrol increased SIRT1 activation and reduced medial thickening, and elastin breaks. Yet, direct SIRT1 activation or inhibition did not affect aortic root dilatation, indicating that the beneficial effect of resveratrol is SIRT1 and senescence independent. Resveratrol did attenuate miR-29b expression in vivo and in vitro in SMCs in an indirect, endothelial cell–dependent manner. Interestingly, losartan has also been reported to reduce miR-29b, which was TGF-β dependent.5 Given that TGF-β signaling (pSMAD2 and pERK1/2) remained unaltered in response to resveratrol, we conclude that resveratrol reduces miR-29b not via affecting TGF-β, yet via increasing NF-κB activity.

In an abdominal aortic aneurysm model in rats, resveratrol effectively inhibited aortic dilatation by counteracting the inflammatory response.25 We observed that losartan reduces vascular inflammation in MFS mice; however, resveratrol did not. This finding indicates that inflammation per se does not need to be reduced to inhibit aortic root dilatation. In line with these observations, we demonstrated that anti-inflammatory medication diminishes vascular inflammation, yet it did not reduce aortic root dilatation.29 One may speculate that not the number of inflammatory cells is relevant but that the type of inflammatory cells in the vasculature is decisive on aortic growth outcome.

Interestingly, it has been described that resveratrol can suppress the expression of AT1R and thereby the detrimental pathways downstream of AT1R,40 such as increased senescence.16 However, if this would be the primary mechanism of action in our study, we would expect a similar outcome between the AT1R blocker losartan and resveratrol on vascular inflammation and phosphorylation of SMAD2 and ERK1/2, which we did not observe.

Excessive oxidative stress is detrimental for the vessel wall16 and is observed in the Fbn1C1039G/+ MFS mouse model.15 Resveratrol was shown to inhibit aortic dilatation in the oxidative stress–induced CaCl₂ aneurysm mouse model by attenuation of inflammation, oxidative stress, and matrix degradation.42 This is further illustrated in rat aortic tissue, where senescence is reduced by an SIRT1-dependent decrease in oxidative stress.16 In the current study, we show a significant increase in nuclear SIRT1 and a modest decrease in aortic senescence after resveratrol. However, modulation of SIRT1 activity with SRT1720 or sirtinol did change senescence, yet did not change aortic root dilatation and thus seems insufficient as a drug target in MFS.

We demonstrated that resveratrol influenced aortic repair, indicated by decreased elastin degradation, increased cell survival (Bcl-2/Mcl-1/less apoptotic cells), and enhanced NF-κB signaling. These characteristics fit with the described features of inhibition of miR-29b in MFS mice3 and thus may be considered as the working mechanism of resveratrol in MFS mice. Of interest, the increase in miR-21a and miR-195 after resveratrol may also contribute to the protective aortic phenotype,29,43 but this requires further investigation in MFS.

In conclusion, resveratrol has a beneficial effect on the vasculature, resulting in improved elastin integrity and cell survival by downregulating miR-29b expression. With the knowledge that inhibition of miR-29b is effective in MFS mice,5,29–31 it now becomes feasible to apply resveratrol as a novel treatment strategy.

In patients with MFS, blood pressure regulation is still the only pharmacological treatment available.1 Here, we show that resveratrol is effective at inhibiting the aortic root dilatation rate in Fbn1C1039G/+ MFS mice, affecting a mechanism different from AT1R or TGF-β signaling. Several resveratrol trials have been performed in humans, mostly in diabetic and obese men.44,45 Positive effects of resveratrol were reported on reduced systolic blood pressure and body fat, affecting lipid profiles, inflammation markers, and glucose metabolism.44 Taken together, no harmful effects were reported in these human studies, supporting the use of resveratrol as a potential drug candidate to treat patients with MFS.

Acknowledgments

We thank Peter ten Dijke for providing the pSMAD2 antibody (Department of Molecular Cell Biology, Cancer Genomics Center Netherlands and Center for BioMedical Genetics, Leiden University Medical Center, NL) and Dr Fontijn for providing the KLF2 lentiviral particles used in this study (Department of Molecular Cell Biology and Immunology, VU University Medical Center, NL).

Sources of Funding

This study was supported by the AMC Graduate School (AMC PhD Scholarship), ZonMW (project, 114024034), and the Netherlands Heart Foundation (grant, 2008B115).

Disclosures

None.

References


**Highlights**

- Resveratrol inhibits aortic root dilatation in the Marfan mouse (Fbn1<sup>C1039G/+</sup> ).
- The number of elastin breaks in the aortic wall is reduced by resveratrol.
- Micro-RNA-29b expression is downregulated by resveratrol.
- Resveratrol upregulates antiapoptotic micro-RNA-29b target Bcl-2 and decreases the number of apoptotic cells.
- Nuclear factor κB signaling is induced in smooth muscle cells by resveratrol-treated endothelial cell medium and mouse serum.
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Arterioscler Thromb Vasc Biol. 2016;36:1618-1626; originally published online June 9, 2016; doi: 10.1161/ATVBAHA.116.307841
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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In the article by Hibender et al, which appeared in the August 2016 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2016;36:1618–1626. DOI: 10.1161/ATVBAHA.116.307841), a correction was needed.

In the Figure 6 legend, panel D description, a word was erroneously deleted. The corrected sentence reads as follows: “D. Resveratrol downregulates miR-29b in SMCs in an endothelial cell–dependent manner, probably via enhancing NF-κB signaling, resulting in decreased SMC apoptosis.”

The publisher apologizes for the error.

The online version of the article has been corrected and is available at [http://atvb.ahajournals.org/content/36/8/1618](http://atvb.ahajournals.org/content/36/8/1618).
Figure SI. Aortic dilatation by echocardiography and histology correlates. A positive correlation is found between the aortic root diameter measured by echocardiography and the aortic root diameter obtained by histology (in mm; \( r=0.7087 \) and \( P<0.0001 \)). The diameters are 1.8-fold smaller in tissue sections, probably due to tissue handling.

Figure SII. Resveratrol affects weight gain. Weight gain over 2 months (in grams). Resveratrol shows effectiveness by decreasing the weight gain of MFS mice to a lower rate than in MFS placebo mice.
Figure SIII. SIRT1 modulation effects weight gain. SRT1720 decreased the weight gain over 2 months (in grams) of MFS mice when compared to MFS placebo mice.

A

ANP mRNA

WT MFS placebo MFS resveratrol

B

NT Pro-BNP [ng/L]

WT MFS placebo MFS resveratrol

Figure SIV. MFS mice and resveratrol-treated MFS mice show no differences in ANP mRNA expression in the heart and NT Pro-BNP serum levels. A. In situ hybridisation for ANP mRNA in the hearts (25x, scale bar=6.25 µm) and B. NT Pro-BNP serum levels in of WT, MFS placebo and MFS mice treated with resveratrol.
Figure SV. MiR-29b mRNA expression is not changed in resveratrol-treated SMCs and HUVECs. A. SMCs directly stimulated with resveratrol do not show a difference in miR-29b expression. B. When HUVECs are stimulated with resveratrol, miR-29b mRNA expression is not influenced. C. KLF2 mRNA expression is upregulated when HUVECs are stimulated with resveratrol.
Figure SVI. KLF2 overexpression in HUVECs does not lead to downregulation of \textit{miR-29b} mRNA expression in SMCs. \textbf{A.} Lentiviral KLF2 overexpression is established in HUVECs. \textbf{B.} When SMCs are stimulated with HUVECs conditioned medium from control and KLF2 overexpressing cells, \textit{miR-29b} and \textbf{C.} \textit{Bcl-2} mRNA expression are not influenced.
Materials and methods

Animal studies
Two-month old \( Fbn1^{C1039G/+} \) male and female mice and age-matched wild type (WT) littersmates were used for the resveratrol study (n\( \geq 11 \) per group). The mice are on a C57Bl6 background and maintained as a heterozygous breeding colony in our breeding facility. Losartan (0.6 mg/ml; losartan potassium, Teva Pharmachemie, The Netherlands) or resveratrol (0.1 mg/ml; R5010, Sigma-Aldrich) was administered for two months via the drinking water, which was refreshed three times a week. The number of mice used for the resveratrol study were: WT, n=12; MFS placebo, n=17; MFS losartan, n=12; MFS resveratrol, n=11. In addition, SIRT1 activator SRT1720 (1,5 mg/kg, 567860, Merck Millipore, in n=7 male mice), or the SIRT1 inhibitor sirtinol (15 mg/kg, CAS 410536-97-9, Santa Cruz Biotechnology, in n=8 male mice), dissolved in 17% DMSO (Sigma-Aldrich) in 0.9% NaCl was administered subcutaneously (sc) (3x/week). To correct for the effect of sc injections, four MFS placebo mice were taken along, which responded in all measurements as the non-injected MFS placebo and were thus added to this group in the analyses. At four months of age, the mice were euthanized, perfusion fixed, and hearts and aortas were collected to prepare for analyses. In order to calculate weight gain and aortic dilatation rate, a separate group of untreated MFS and WT mice (n=7 and n=6 per group, respectively) were harvested at two months of age, corresponding with the age of onset of treatment in the other groups. The average weight and aortic root diameter (sex matched) were subtracted from the treated four month old mice to calculate weight gain and aortic root dilatation rate. For mRNA analysis of the ascending aorta, a similar experimental setup as described above was used; WT, MFS placebo and resveratrol-treated MFS mice (two months treatment) were harvested at four months of age (n=6 per group). Animal care and experimental procedures were approved by the independent animal experimental committee for Animal Welfare of the Amsterdam Medical Center according to the guidelines of the Amsterdam Medical Center and the Directive 2010/63/EU of the European Parliament.

Echocardiography
To validate aortic root cross-sections in relation to in vivo aortic root diameters, we measured cross-section diameters and compared them to echocardiography data from the same mice. Transthoracic echocardiograms (WT, n=7 vs MFS mice n=18 of 2-8 months old) were performed in isoflurane-anaesthetized mice (4% isoflurane for induction and 2-3% isoflurane for maintenance of anesthesia) by using the Vevo770 high-resolution imaging system with a 40-MHz probe (RMV 704 scanhead) (VisualSonics Inc, Toronto, Canada). A two-dimensional short-axis view of the aorta was obtained at the level of the aortic root. Thereafter, the mice were euthanized, perfused with phosphate buffered saline (PBS) and fixative, hearts and aorta were harvested for cross-section analysis.
Senescence associated-β-galactosidase (SA-β-Gal) staining
Aortic arches (containing the ascending aorta, arch and proximal thoracic descending aorta) were fixed with 4% neutral buffered formaldehyde (Shandon Formal-Fixx, Thermo Scientific). After rinsing with PBS, arches were stained for 48 hours at 37°C in buffer containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (Invitrogen), 150 mmol/L NaCl, 2 mmol/L MgCl₂, 5 mmol/L potassium hexacyanoferrate(III) (244023, Sigma-Aldrich), 5mM potassium hexacyanoferrate(II) trihydrate (P3289, Sigma-Aldrich) and 40 mmol/L citric acid/sodium hydrogen phosphate buffer pH6, to visualize aortic senescence. Chemicals without further specifications were purchased from Merck.

Senescence-associated Fluorescein Di-β-D-Galactopyranoside (FDG) analysis
After SA-β-Gal staining, the aortic arches were incubated in the same buffer as described above, however, the X-Gal was substituted for FDG (Life Technologies); which was diluted 200 times from stock solution (200 nmol/L in 1:1 H₂O:DMSO) into H₂O and incubated at 37°C for 10 minutes. The aortic arches were incubated for 24 hours at 37°C, after which the reaction buffer was measured with a Synergy HT at Excitation/Emission: 485/528 nm, to quantify substrate conversion as a measure for aortic senescence.

Immunohistochemistry
Mouse hearts were embedded in paraffin and cross-sections were prepared (7 µm). Aortic root sections were taken for analysis at the location just proximal of the aortic valve area, which results in a limited number of sections before entering the ascending aorta. After deparaffinization and rehydration hematoxylin and eosin staining was used to visualize the aortic wall and Lawson staining (Klinipath) was performed to visualize the integrity of the elastic laminae. Immunohistochemical stainings were initiated by quenching endogenous peroxidase activity for 20 min in 1% H₂O₂ and incubated for 8 min with Superblock (ScyTek). Antigen retrieval was performed by heating the sections for 10 min in citrate buffer pH6. Overnight incubation at 4°C with antibodies recognizing pSMAD2 (kindly provided by Prof. P. ten Dijke), pERK1/2 (p44/42, Cell Signaling), CD45 (clone 30 F-11, eBioscience) or SIRT1 (07-131, Millipore) was performed. Subsequently, the sections were washed with TRIS-buffered saline (TBS) and incubated with a rabbit anti-rat IgG antibody (E0468, DAKO) for 1 hour (for CD45), and/or 30 min with a horseradish peroxidase (HRP) conjugated anti-rabbit IgG polymer (BrightVision, ImmunoLogic) (for pSMAD2, pERK1/2, CD45 and SIRT-1). Diaminobenzidine tetrachloride (DAB) was used as substrate for HRP. Hereafter, sections were rinsed in water, dehydrated and embedded in pertex (HistoLab). Measurements of aortic root diameters and medial area (mm²) were performed using Adobe Photoshop CS5. Elastic lamina breaks were counted by a researcher blinded to the genotype or treatment. Quantification of CD45, pSMAD2, pERK1/2 and SIRT-1 was performed with QWin software (Leica Microsystem) (all mice per group; ratio of positive stained area/total area).
To reveal positive blue senescent cells, cryosections were generated from the ascending aortas, which had been stained for SA-β-Gal.

For the Atrial Natriuretic Peptide (ANF) mRNA in situ hybridization heart sections at the left ventricle from WT, MFS placebo and MFS mice treated with resveratrol were used and deparaffinized, rehydrated in a graded series of alcohol and incubated with 10 mg/ml proteinase K dissolved in PBS for 15 min at 37°C. The proteinase K activity was blocked by rinsing the sections in 0.2% glycine in PBST (PBS+0.05%Tween-20) for 5 min. After rinsing in PBS, the sections were postfixed for 10 min in 4% PFA and 0.2% glutaraldehyde in PBS, followed by rinsing in PBS. After prehybridization for at least 1 hr at 70°C in hybridization mix (50%formamide, 5xSSC (20xSSC; 3 M NaCl, 0.3 M tri-sodium citrate, pH 4.5), 1% blocking solution (Roche), 5 mM EDTA, 0.1% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma), 0.5 mg/ml heparin (BD Biosciences), and 1 mg/ml yeast totalRNA (Roche), a digoxigenin (DIG)-labeled probe was added to the hybridization mix in a final concentration of 1 ng/ml. A probe specific for ANP was used. After overnight hybridization, the sections were rinsed with 2xSSC, followed by two washes with 50% formamide, 2xSSC, pH 4.5, at 65°C, and rinsing in TNT (0.1 M Tris-HCl, pH=7.5, 0.15 M NaCl, 0.05% Tween-20) at room temperature. Subsequently, the sections were incubated for 1 hour in MABT-block (100 mM Maleic Acid, 150 mM NaCl, pH 7.4, 0.05% Tween-20, 2% blocking solution), followed by 2 hours incubation in MABT-block containing 100 mU/ml alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche, 1093274). After rinsing in TNT and subsequently in NTM (100 mM Tris pH 9.0, 100 mM NaCl, 50 mM MgCl2), probe binding was visualized using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche, 1681451). Color development was stopped by rinsing in double-distilled water. The sections were dehydrated in a graded ethanol series, rinsed in xylene, and embedded in Entellan (Merck). Images were recorded using QWin software (Leica Microsystem) on a Zeiss Axiophot microscope.

Mouse serum measurement
ANP levels were measured in serum from WT, MFS placebo and resveratrol-treated mice by the department of Clinical Chemistry at the Academic Medical Center in Amsterdam.

HUVECs and SMCs culture experiments
Human umbilical cord endothelial cells (HUVECs) were isolated and cultured on fibronectin (Sanquin) coated culture surfaces with M199 medium (GIBCO, Invitrogen), supplemented with 100 U/ml penicillin/streptomycin (P/S, GIBCO, Invitrogen), 20% heat-inactivated FCS (GIBCO, Invitrogen), 0.05 μg/ml heparin (Sigma-Aldrich), 2 mM L-Glutamine (GIBCO, Invitrogen) and 25 μg/ml endothelial cell growth supplement (ECGS, Sigma-Aldrich). The cells were grown as a non-confluent monolayer and used at passage 2-3. The cells were then serum-starved for 16 hours (M199 medium with P/S and 1% albumin (Albuman, Sanquin)) and thereafter stimulated for 24 hours with 1 μM resveratrol (Sigma-Aldrich). Mouse aortic SMCs
were isolated and cultured on 0.1% gelatin (Merck) coated culture surfaces with DMEM/F12 medium (GIBCO, Invitrogen) with 100 U/ml P/S and 20% heat-inactivated FCS. SMCs underwent 24 hours of serum starvation at 100% confluency before stimulation with 50% HUVEC conditioned culture medium or mouse serum (2%) for 24 hours or 0.25 μM staurosporin. A serum pool was made for each treatment group by pooling 100 μl serum of each mouse within the group. For both resveratrol experiments, different pooled serum samples were made. HUVECs and SMCs were harvested by adding 1 ml TRI reagent (Sigma-Aldrich) to the cells.

RNA isolation and reverse transcription (RT) PCR in vivo and in vitro
Total RNA was extracted from frozen ascending aorta by freeze-thawing the tissue in TRI reagent and from cell culture samples. iScript cDNA synthesis kit (Bio-Rad) and miScript II RT Kit (Qiagen) were used to synthesize complementary DNA (cDNA). RT-PCR was performed using the LightCycler 480 Real-Time PCR System (Roche) with SensiFast Sybr No-Rox Mix (Bioline) and specific forward and reverse primers (Table on last page). Expression of mRNA transcripts for microRNA(miR)-21a, miR-23b, miR-24, miR-29a, -b, -c, miR-195a, miR-712, shear stress marker Kruppel-like factor 2 (KLF2) and anti-apoptosis markers B-cell CLL/lymphoma 2 (Bcl-2) and myeloid cell leukemia 1 (Mcl-1) (primers in Table 1) were studied. mRNA expression calculations were performed with the LinRegPCR software. The average values of the housekeeping genes (small nucleolar RNA, C/D box 48 (SNORD48), small nucleolar RNA MBII-202 (snoRNA-202), small nucleolar RNA MBII-234 (snoRNA-234) and large ribosomal phosphoprotein (RPLP0) were used to normalize for cDNA content.

TUNEL staining
Aortic root sections and SMCs cultured on glass slides were stained immunofluorescently to quantify apoptosis positive cells. Immunofluorescence with the was used. Slides were incubated for 15 min at 37°C, quenched with 1% H₂O₂ for 20 min and blocked with Superblock for 8 min. Following one hour incubation at 37°C with Terminal Deoxynucleotidyl Transferase from the In Situ Cell Death detection Kit (Roche) and HOECHST (1 μg/ml) for 15 min. Slides were embedded in MOWIOL and positive and total amount of cells were counted.

KLF2 overexpression
HUVECs were transduced twice with empty vector and lentivirus (kindly provided by Dr. R. Fontijn) containing the complete human open reading frame for LKLF, produced as described previously.

Nuclear factor κB (NF-kB) activity assay
Whole cell extracts were used to measure the NF-kB activity in HUVEC and SMC protein samples using the TransAM NF-kB p65transcription factor assay kit (Active Motif) following manufacturer’s protocol.
Statistical analysis
Statistical tests were performed with GraphPad Prism 5 software. Graphs represent the mean +SEM. The Pearson correlation calculation was used to quantify strength of association between variables. To compare variables between groups of animals in the various conditions the one-way analysis of variance (Gaussian distribution) or Kruskal-Wallis test (non-Gaussian distribution) was used. When these indicated a significant difference, the unpaired students t-test (Gaussian distribution) and Mann-Whitney U-test (non-Gaussian distribution) were used. A P-value of 0.05 was considered significant, represented in the graphs by * = P≤0.05, ** = P≤0.01 and *** = P≤0.001. In the SRT1720 and sirtinol study a P-value of 0.025 was considered significant as two new compounds were tested.

References


Table 1. Primer sequences used for RT-PCR. *Mus musculus* and *homo sapiens* specific primers for Bcl-2, Ikka (Chuk), Il6, Il8 (Cxcl15), KLF2, Mcl-1, miR-21a, miR-23b, miR-24, miR-29a, miR-29b, miR-29c, miR-195a, miR-712, RPLP0, Rplp0, SNORD48, snoRNA-202 and snoRNA-234.

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