Endothelial Senescence Contributes to Heart Failure With Preserved Ejection Fraction in an Aging Mouse Model

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Background—Because of global aging, the prevalence of heart failure with preserved ejection fraction (HFpEF) continues to rise. Although HFpEF pathophysiology remains incompletely understood, endothelial inflammation is stated to play a central role. Cellular senescence is a process of cellular growth arrest linked with aging and inflammation. We used mice with accelerated aging to investigate the role of cellular senescence in HFpEF development.

Methods and Results—Senescence-accelerated mice (SAM, n=18) and control mice with normal senescence (n=15) were fed normal chow or a high-fat, high-salt diet (WD). Vascular and cardiac function was assessed at 8, 16, and 24 weeks of age. At 24 weeks, both SAM on WD (SAM-WD) and SAM on regular diet displayed endothelial dysfunction, as evidenced by impaired acetylcholine-induced relaxation of aortic segments and reduced basal nitric oxide. At week 24, SAM-WD had developed HFpEF, characterized by diastolic dysfunction, left ventricular hypertrophy, left atrial dilatation, and interstitial fibrosis. Also, exercise capacity was reduced and lung weight increased. Cardiovascular inflammation and senescence were assessed by immunohistochemical and immunofluorescence staining of hearts and aortas. SAM-WD showed increased endothelial inflammation (intercellular adhesion molecule 1 expression) and increased endothelial senescence (acetyl-p53/CD31 costaining). The latter correlated with diastolic function and intercellular adhesion molecule 1 expression.

Conclusions—SAM develop endothelial dysfunction. Adding a high-salt, high-fat diet accelerates endothelial senescence and instigates endothelial inflammation. This coincides with hemodynamic and structural changes typical of HFpEF. Targeting endothelial senescence could be a new therapeutic avenue in HFpEF. (Circ Heart Fail. 2017;10:e003806. DOI: 10.1161/CIRCHEARTFAILURE.116.003806.)

Key Words: aging • endothelium • heart failure • heart failure with preserved ejection fraction • senescence

Heart failure (HF) with preserved ejection fraction (HFpEF) currently accounts for half of HF cases, and incidence is increasing because of global aging. Prognosis remains grim, with a 50% mortality rate at 5 years, because a specific therapy for HFpEF is lacking.1 Besides a preserved left ventricular (LV) systolic function, presence of diastolic dysfunction, structural heart disease, such as LV hypertrophy or left atrial dilatation, elevated natriuretic peptide levels, and signs and symptoms of HF are considered in the diagnosis of HFpEF.2

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Along with these cardiac changes, vascular alterations have been described in both animal and human HFpEF studies, including endothelial dysfunction, arterial stiffening, and vascular inflammation.3–5 Through ventricular–arterial coupling and cross talk between endothelium and cardiomyocytes, this vascular maladaptation can worsen diastolic function.6,7 Cellular senescence is a state of cell-cycle arrest associated with aging induced by mitogenic signals, DNA damage, and inflammation. Senescent cells actively secrete auto- and paracrine signals, such as inflammatory cytokines. This senescence-associated secretory phenotype initiates a vicious circle of inflammation and senescence, which is thought to lead to cardiovascular disease.8,9 By increasing systemic and vascular inflammation, cellular senescence could also contribute to HFpEF pathophysiology.

Although aging is a central risk factor for HFpEF, animal models have, until now, focused on the role of HFpEF comorbidities, such as arterial hypertension, diabetes mellitus, and obesity.4,10 The senescence-accelerated mouse (SAM) is an aging model showing early diastolic and endothelial dysfunction, and cross talk between endothelium and cardiomyocytes, this vascular maladaptation can worsen diastolic function.6,7

Drs Lemmens and Van Craenenbroeck contributed equally to this work.


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dysfunction.11,12 We aimed to induce HFpEF in SAM by exposure to a high-fat, high-salt diet, which is known to induce vascular inflammation.13 Also, we hypothesized that cellular senescence plays a role in the development of HFpEF in SAM.

Methods
An expanded Methods section can be found in the Data Supplement.

Animals and Experimental Procedures
Female senescence-accelerated prone mice (SAM, SAMp8/TaHsd; n=18) and control senescence-accelerated resistant mice (CON, SAMr1/TaHsd; n=15) were purchased (Envigo) and randomized at 8 weeks of age to either a Western-type diet (ABDiets) and 1% NaCl supplementation in drinking water (WD) or control chow and tap water. Echocardiography (Vevo 2100, Visualsonics), blood glucose (LifeScan), blood pressure measurement (Coda, Kent Scientific), and treadmill ergometry (Leica Systems) were performed at the age of 8, 16, and 24 weeks. At 24 weeks, invasive cardiac pressure–volume measurements were performed (Millar), animals were euthanized, and ex vivo vascular function was measured in an organ bath setup. Organ weight was recorded and heart and thoracic aorta samples were collected for histological analyses, quantitative polymerase chain reaction, and Western blotting. Details of these procedures can be found in the Data Supplement. All mice were exposed to a 12-hour light/dark cycle, with food and water provided ad libitum. The study was approved by the Ethical Committee of the University of Antwerp, and all experiments were performed in accordance with the Use of Laboratory Animals directive by the European Commission 86/609/EEC.

Statistical Analyses
Data are presented as mean±SD. Analyses were performed with GraphPad Prism v6 and IBM SPSS Statistics v23. For data collected at one time point, we computed 2-way analysis of variance. For data collected at 3 time points, generalized estimating equations with an unstructured correlation matrix were used. We used senescence and diet as relevant factors for 2-way analysis of variance and added time in generalized estimating equations. P values are reported accordingly (P-senescence, P-diet, P-time, and P-interaction). For fitting curves, nonlinear regression analysis with least squares fit was used. To compare pressure–volume curves, analysis of covariance was used on logarithmically transformed data as described by Burkhoff et al.14 Correlations were assessed by Spearman’s test. Holm–Šídák correction for multiple comparisons was used with all analyses. A 2-tailed P<0.05 was considered significant.

Results
SAM-WD Develop HFpEF at 24 Weeks
In SAM on normal chow, we observe no changes in diastolic function besides a small but significant shift of the end-diastolic pressure–volume relationship (EDPVR) to the left (P=0.006 versus CON; Figure 1A). SAM-WD display diastolic dysfunction both on invasive hemodynamics (Table 1) and echocardiography (Table 2). The EDPVR shows a shift upwards and to the left, accompanied by an increase in LV end-diastolic pressure (LVEDP; both P<0.001 versus SAM; Figure 1A and 1B). This shift is mainly caused by a slowing of the first phase of diastole, active relaxation, assessed by k and Tau measurements (P=0.023 and P=0.002 versus SAM, respectively). Although changes in passive myocardial stiffness are less prominent, SAM-WD show several signs of decreased LV compliance. When comparing stiffness constant β, while accounting for changes in k because of their interdependence, results are significant (P=0.005 versus SAM). Also, LVEDP is higher in SAM-WD group for a comparable LV end-diastolic volume, implying reduced end-diastolic distensibility (Table 1).

Echocardiographically, E/e’ starts to increase in SAM-WD after 16 weeks of age, reaching a significant difference with all other groups at 24 weeks (P=0.045 versus SAM; Figure 1C and 1D). Mitral E/A ratio and isovolumic relaxation time were not different between groups (P=0.995 and P=0.573 versus SAM). Additional echocardiographic data are displayed in Table I in the Data Supplement.

Also, only SAM-WD display structural cardiac changes associated with HFpEF. Left atrial diameter is increased at 24 weeks (P<0.001 versus SAM; Figure 2A). LV hypertrophy is evident from increased heart weight at euthanizing, increased LV mass on echocardiography, and increased cardiomyocyte cross-sectional area on histology (P<0.001, P=0.033, and P=0.010 versus SAM; Figure 2B and Tables 2 and 3). Myocardial fibrosis is markedly increased in SAM-WD (P=0.010 versus SAM; Figure 2C and 2D). This is confirmed on quantitative polymerase chain reaction of heart tissue, showing increased expression of mRNA associated with hypertrophy and fibrosis (Figure 2E). Also, quantitative polymerase chain reaction reveals increased B-type natriuretic peptide synthesis (P<0.001 versus SAM).

Finally, SAM-WD exhibit signs and symptoms of HF. Lung wet/dry ratio is increased, indicating lung edema at euthanizing (P=0.023 versus SAM; Table 3). At 24 weeks, treadmill running time is reduced to 71.5% of baseline, indicating reduced exercise capacity (P<0.001 versus SAM; Table II in the Data Supplement). Blood pressure, blood glucose, and body weight were not different between groups (Table II in the Data Supplement). WD had very few effects in CON.

To summarize, SAM-WD display diastolic dysfunction, structural cardiac changes, and signs and symptoms that are compatible with HFpEF. These changes occurred independently of comorbidities.

Vascular Dysfunction Is Present in SAM Regardless of Diet, but Endothelial Inflammation Is Only Seen in SAM-WD
Aortic segments of both SAM and SAM-WD display vascular dysfunction in an ex vivo organ bath setup (Table III in the Data Supplement). Acetylcholine-induced vasorelaxation, an endothelium-dependent mechanism, is reduced compared with that in CON and CON-WD (P-senescence <0.005; Figure 3A). Also, maximal contractions to phenylephrine (Emax) are increased in SAM and SAM-WD groups (P-senescence <0.001). When measuring Emax with and without presence of L-N(3)-nitroarginine methyl ester, the difference in maximal contraction (ΔEmax) is reduced in SAM and SAM-WD (P-senescence <0.001; Figure 3B). Our group has previously shown that ΔEmax is an indicator of basal nitric oxide (NO) content.15 A lower ΔEmax in SAM and SAM-WD indicates a reduced amount of basal NO counteracting phenylephrine. Endothelial-independent vasorelaxation, assessed using the NO donor diethylamine nitric oxide (DEANO), was also slightly reduced in both SAM and SAM-WD (P-senescence =0.010; Table III in the Data Supplement).

Arterial stiffness was assessed by ultrasound (pulse wave velocity), invasive hemodynamics (arterial elastance) and
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Remarkably, stiffness was not increased in this model (P=0.837, P=0.625, and P=0.286 for senescence, respectively; Table 1; Tables I and III in the Data Supplement).

Endothelial NO synthase (eNOS) protein expression was decreased in SAM-WD compared with SAM (P=0.038) and controls (P=0.008; Figure 3C and 3D).

Increased intercellular adhesion molecule 1 (ICAM) expression, indicating endothelial inflammation, is seen in SAM-WD aortas (P=0.002 versus SAM; Figure 3E and 3F) and hearts (P=0.018 versus SAM; Figure 3G and 3H). This is confirmed by quantitative polymerase chain reaction of ICAM and vascular cell adhesion molecule 1 in heart tissue (mean fold change: SAM-WD, 1.23±0.02; SAM, 1.16±0.04; P=0.007 for ICAM and SAM-WD, 1.42±0.06; SAM, 0.99±0.08; P=0.003 for vascular cell adhesion molecule 1).

Endothelial Cellular Senescence Is Present in Both SAM and SAM-WD and Is Most Prominent in SAM-WD

Senescent cells exhibit acetylation of p53 and upregulation of SA-βgal activity.8 SAM show increased cellular senescence regardless of their diet: SA-βgal staining is increased in SAM and SAM-WD aortas (P-senescence <0.001; Figure 4A and 4B) and hearts (P-senescence <0.001; Figure 4C and 4D). Expression of acetyl-p53 is also increased in SAM and SAM-WD hearts and aortas (P-senescence both <0.001).

When only endothelial cells are considered, senescence is additionally increased in SAM-WD (heart P<0.001, aorta P=0.042 versus SAM; Figure 4F and 4I). In contrast, in nonendothelial cells (ie, vascular smooth muscle cells in the aorta and cardiomyocytes and fibroblasts in the heart), there is no evidence of an additional increase in acetyl-p53 expression in SAM-WD (heart P=0.281, aorta P=0.609 versus SAM; Figure 4G and 4J).

Furthermore, endothelial senescence correlates well with measurements of cardiac and vascular function. The percentage of endothelial cells positive for acetyl-p53 correlates with LVEDP (r=0.53; P=0.007), Tau (r=0.72; P<0.001), and k (r=0.49; P=0.035). For vascular function, the percentage of endothelial cells positive for acetyl-p53 correlates inversely with the maximal vasorelaxation to acetylcholine (r=−0.58; P=0.001) and ∆Emax (r=−0.54; P=0.004). Also, endothelial senescence correlates with endothelial inflammation as measured by ICAM positivity on immunohistochemical staining (r=0.63; P<0.001).

Discussion

When fed WD from 8 to 24 weeks of age, SAM develop diastolic dysfunction, structural cardiac changes, including LV hypertrophy and left atrial dilatation, signs and symptoms of HF, and elevated levels of B-type natriuretic peptide, while ejection fraction is preserved. This novel model fulfills all criteria needed for the diagnosis of HFpEF. Furthermore, our study confirms certain important findings reported in other HFpEF models.
First, endothelial inflammation and reduced NO-dependent signaling were recently reported in hearts of ZSF1 obese rats with HFpEF. This observation is confirmed in our model and extended toward the vascular system. We show endothelial inflammation, reduced basal NO, and reduced stimulated NO production in thoracic aorta segments of SAM-WD. These findings reflect the systemic nature of comorbidity-induced inflammation.

Second, endothelial dysfunction because of eNOS downregulation was reported in a hypertensive rat HFpEF model. We confirm the reduced eNOS protein expression in aortas of SAM compared with controls, which is contrary to the reduced basal NO amount we found in SAM. However, our group has previously shown that eNOS protein expression does not relate to the amount of basal NO. A possible explanation for eNOS downregulation in SAM-WD mice is increased endothelial inflammation. Indeed, the eNOS expression pattern closely resembles the pattern seen for aortic ICAM expression seen in Figure 3F.

Third, myocardial fibrosis has been described in most HFpEF models, especially when pressure overload is induced. In SAM-WD, fibrosis developed independently of hypertension. This is in accordance with the paradigm by Paulus and Tschöpe that endothelial inflammation alone is able to induce changes in adjacent cells, including fibroblasts.

Furthermore, we add insights on the contribution of cellular senescence to HFpEF pathophysiology. Cellular senescence is strongly linked to other age-related diseases, such as osteoarthritis and atherosclerosis. Also, increased cardiomyocyte senescence is linked to HF with reduced ejection fraction in older patients. The role of cellular senescence in HFpEF has not been investigated to date.

We show that WD induces endothelial senescence in the heart and vasculature of SAM, manifested by increased p53 acetylation, while senescence in other cell types is comparable between SAM and SAM-WD. It has previously been shown that the p53 pathway is crucially involved in the transition from LV hypertrophy to HF. Recently, an endothelium-specific knockout of p53 showed reduced cardiac hypertrophy and fibrosis when subjected to pressure overload. These studies and our data support a role of endothelial senescence in HFpEF pathophysiology.

In our study, endothelial senescence and endothelial inflammation correlated well, providing an interesting link between both phenomena. When cells become senescent, they secrete inflammatory mediators to signal their removal to the immune system. This senescence-associated secretory phenotype has the ability to induce senescence in neighboring cells, initiating a chain reaction of cellular senescence. As such, cellular senescence can be proposed as a link between comorbidity-driven systemic inflammation and endothelial inflammation (Figure 5). In line with a recent call for endothelial-specific therapies in HFpEF, targeting endothelial senescence could be a new therapeutic avenue in HFpEF.

Our study has, however, some limitations. We did not find a change in mitral E/A ratio or isovolumic relaxation time.
Table 2. Echocardiography at Different Time Points

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CON-WD</th>
<th>SAM</th>
<th>SAM-WD</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 wk</td>
<td>16 wk</td>
<td>24 wk</td>
<td>8 wk</td>
<td>16 wk</td>
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<tr>
<td>LV diastolic function</td>
<td></td>
<td></td>
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<tr>
<td>E wave/A wave (ratio)</td>
<td>1.28±0.10</td>
<td>1.38±0.17</td>
<td>1.44±0.12</td>
<td>1.80±0.28</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td>E wave/e’ wave (ratio)</td>
<td>36.5±5.4</td>
<td>27.4±5.3</td>
<td>30.5±2.3</td>
<td>22.5±0.4</td>
<td>21.1±3.4</td>
</tr>
<tr>
<td>Isovolumetric relaxation time, ms</td>
<td>13.7±0.7</td>
<td>14.5±0.9</td>
<td>13.6±0.8</td>
<td>13.3±0.8</td>
<td>18.3±2.2</td>
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<tr>
<td>LV systolic function</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>35.7±2.1</td>
<td>35.0±2.8</td>
<td>35.5±1.6</td>
<td>31.2±3.3</td>
<td>36.9±4.3</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>66.0±2.8</td>
<td>64.6±3.6</td>
<td>65.5±2.3</td>
<td>59.5±5.0</td>
<td>67.3±5.9</td>
</tr>
<tr>
<td>Cardiac structure</td>
<td></td>
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<tr>
<td>LV mass, ASE formula, mg</td>
<td>92.8±7.8</td>
<td>105.0±9.2</td>
<td>102.2±4.3</td>
<td>100.9±9.5</td>
<td>125.6±9.6</td>
</tr>
<tr>
<td>Left atrial diameter, mm</td>
<td>1.43±0.15</td>
<td>1.61±0.12</td>
<td>1.64±0.11</td>
<td>1.61±0.12</td>
<td>1.53±0.21</td>
</tr>
</tbody>
</table>

Values are expressed in mean±SD. Generalized estimating equations, n=5–9 per group. ASE indicates American Society of Echocardiography; CON, control mice with normal senescence; LV, left ventricular; SAM, senescence-accelerated mouse; and WD, Western-type diet.

*P<0.05 for senescence.
†P<0.05 for time.
‡P<0.05 for senescence–diet interaction.
§P<0.05 for senescence–time interaction.
||P<0.05 for diet–time interaction.
¶P<0.05 for diet.
#P<0.05 for senescence–diet–time interaction.
in SAM-WD, even though invasive hemodynamics clearly show diastolic dysfunction and delayed relaxation. This could reflect the lower sensitivity of mitral flow Doppler in the diagnosis of diastolic dysfunction, compared with tissue Doppler or invasive hemodynamics.23

Although a shift of the EDPVR is clearly seen in SAM-WD, there is no significant difference in stiffness constant $\beta$ when comparing groups with analysis of variance. However, it is more appropriate to compare $\beta$ using analysis of covariance, taking into account the other EDPVR constant $k$, because of their interdependence.14 Using this approach, significant differences exist between SAM-WD and all other groups. Most, but not all, other HFpEF rodent models show increased stiffness constant $\beta$.24 Nonetheless, increased $\beta$ is

Table 3. Structural Cardiopulmonary Changes at Euthanizing

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CON-WD</th>
<th>SAM</th>
<th>SAM-WD</th>
<th>Significance</th>
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</thead>
<tbody>
<tr>
<td>Tissue morphometry</td>
<td></td>
<td></td>
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<tr>
<td>Heart weight/TL, mg/mm</td>
<td>6.84±0.91</td>
<td>6.19±0.76</td>
<td>6.88±0.43</td>
<td>9.16±0.92</td>
<td>*†‡</td>
</tr>
<tr>
<td>Lung weight/TL, mg/mm</td>
<td>8.13±2.23</td>
<td>6.91±1.10</td>
<td>7.72±0.97</td>
<td>9.88±2.00</td>
<td>*‡</td>
</tr>
<tr>
<td>Lung wet/dry ratio</td>
<td>3.67±0.68</td>
<td>3.78±0.42</td>
<td>3.80±0.88</td>
<td>4.82±0.51</td>
<td>*†</td>
</tr>
<tr>
<td>Liver weight/TL, mg/mm</td>
<td>68.3±13.5</td>
<td>74.8±8.5</td>
<td>75.6±7.9</td>
<td>103.2±23.7</td>
<td>*†</td>
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<tr>
<td>Histology</td>
<td></td>
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<tr>
<td>Cardiomyocyte cross-sectional area, mm²</td>
<td>259.4±46.4</td>
<td>250.1±61.3</td>
<td>302.2±109.7</td>
<td>508.5±120.8</td>
<td>*†‡</td>
</tr>
<tr>
<td>Collagen on Sirius Red staining, %</td>
<td>0.85±0.67</td>
<td>0.48±0.30</td>
<td>0.66±0.36</td>
<td>5.10±3.39</td>
<td>*‡‡</td>
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</tbody>
</table>

Values are expressed in mean±SD. Two-way analysis of variance, n=4–9. CON indicates control mice with normal senescence; SAM, senescence-accelerated mouse; TL, tibia length; and WD, Western-type diet.

*P<0.05 for senescence.
†P<0.05 for diet.
‡P<0.05 for interaction.
not a prerequisite to obtain elevated LVEDP because active relaxation can contribute substantially to LVEDP increases.25

Visually, a slight change in inclination of the EDPVR curve seems to appear in CON-WD (Figure 1A). However, when comparing curves, there is no statistical difference with CON animals (P>0.05), stiffness coefficient β is similar between CON and CON-WD, and end-diastolic volumes and pressures are comparable. We conclude that no hemodynamic differences exist between CON and CON-WD.

In contrast to an earlier study by Reed et al, we did not find compelling evidence of diastolic dysfunction or myocardial fibrosis in 24-week-old SAM on normal diet. Changes in hemodynamics and fibrosis were rather moderate in their study compared with the changes we observed in SAM-WD animals in this study. Furthermore, Reed et al used a linear curve fit for the EDPVR, which does not accurately reflect diastolic properties of the heart.14

Despite assessment by 3 different techniques, we did not find increased arterial stiffness in this model of HFP EF. Even though a clear relation between arterial stiffness and diastolic function exists in humans;26 at rest both increased, and normal arterial stiffness has been observed in HFP EF patients.3,27
Figure 4. Increased endothelial senescence in SAM-WD compared with SAM. A–D. Cellular senescence in SAM but not CON genotypes. A, Representative histological images of thoracic aorta stained for senescence-associated β-galactosidase (blue, arrows). Scale bar = 5 μm. B, Digital quantification of β-galactosidase-positive area relative to total aorta area. N=4 to 9. C, Representative histological images of hearts stained for senescence-associated β-galactosidase (blue). Scale bar = 5 μm. D, Digital quantification of β-galactosidase-positive pixels relative to total pixels. N=4 to 9. E–J, Increased acetyl-p53 expression in endothelial nuclei but not in other cell types. E, Representative fluorescence microscopy images of thoracic aorta stained for cellular senescence using acetyl-p53 (red), CD31 (green), and DAPI (blue). Scale bar = 50 μm. F, Digital quantification of the number of nuclei positive for acetyl-p53 staining in aortic endothelial cells. N=4 to 9. G, Digital quantification of the number of nuclei positive for acetyl-p53 staining in vascular smooth muscle cells. N=4 to 9. H, Representative fluorescence microscopy images of hearts stained for cellular senescence using acetyl-p53 (red), CD31 (green), and DAPI (blue). Scale bar = 50 μm. I, Digital quantification of the number of nuclei positive for acetyl-p53 staining in cardiac endothelial cells. N=4 to 9. J, Digital quantification of the number of nuclei positive for acetyl-p53 staining in cardiac nonendothelial cells (i.e., cardiomyocytes and fibroblasts). N=4 to 9. CON indicates control mice with normal senescence; DAPI, 4',6-diamidino-2-phenylindole; ns, not significant; SAM, senescence-accelerated mouse; WD, Western-type diet; and VSMC, vascular smooth muscle cells. *P<0.05, **P<0.01, ***P<0.001 vs CON; †P<0.05, ††P<0.001 vs SAM.
However, during exercise, an inappropriate increase in stiffness indexes is uniformly seen. As we did not incorporate cardiac stress testing in our protocol, either there is no arterial stiffness or increased stiffness is only evident on exertion in this model.

Besides endothelial inflammation, other factors can influence the reduced vascular reactivity to acetylcholine and phenylephrine we found in SAM mice. Aging also reduces smooth muscle cell numbers and density and causes smooth muscle cell hypertrophy.

Finally, there is a rather large variation in several measurements in the SAM-WD group. This is most likely because of variability in food and water intake associated with a diet intervention in mice.

Conclusions

SAM develop cellular senescence and endothelial dysfunction regardless of their diet. Adding a high-fat, high-salt diet accelerates endothelial senescence and instigates endothelial inflammation. It also leads to diastolic dysfunction, structural cardiac changes, and signs and symptoms typical of HFpEF, independent of comorbidities. We propose a link between endothelial senescence and endothelial inflammation through the senescence-associated secretory phenotype. As such, endothelial senescence could be of pathophysiological significance in HFpEF and form a new therapeutic target.

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Disclosures

None.

References

Endothelial Senescence in HFpEF


CLINICAL PERSPECTIVE

Heart failure with preserved ejection fraction (HFpEF) is a cardiovascular disorder with increasing prevalence and high mortality. Because targeted therapy is lacking and pathophysiology remains unclear, HFpEF is currently considered one of the biggest unmet clinical needs. A much-needed, aging-focused, preclinical model of HFpEF is obtained by exposing senescence-accelerated mice to a high-fat high-salt diet. Also, we add insights to the pathophysiology of HFpEF by demonstrating endothelial senescence in both the vascular and cardiac tissue of these mice, correlating with inflammation and indices of cardiovascular function. We propose endothelial senescence as a novel therapeutic target in HFpEF.
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Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2017/06/13/CIRCHEARTFAILURE.116.003806.DC1

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Echocardiography

Transthoracic echocardiography (Vevo 2100, VisualSonics) was performed on spontaneously breathing mice under light anesthesia (1.5 – 2% isoflurane). Body temperature was maintained at 36-38°C and heart rate at 500 ±50 beats/min. Cardiac measurements were performed with a 55-MHz transducer. First, in a left parasternal position, left atrial (LA) area and diameter were measured in long axis view at the start of the P wave on the electrocardiogram. The M-mode cursor was positioned at the level of the aortic valve to measure LA diameter. Left ventricular (LV) wall thickness and cavity diameters were measured in short axis view, both at end-diastole and end-systole, with the M-mode cursor perpendicular to the LV anterior and posterior walls at the level of the papillary muscles. LV ejection fraction, fractional shortening and LV mass were calculated from these parameters. A pulse wave Doppler sample volume was placed in the LA parallel to the pulmonary vein inflow signal, to obtain pulmonary vein systolic and diastolic peak velocities and calculate the systolic/diastolic ratio. Pulse wave Doppler signals were also recorded from the pulmonary artery at the site of the most intense flow signal, mean pulmonary artery pressure was calculated using the formula 58.7 – (1.21 • pulmonary artery acceleration time) reported elsewhere.1

The transducer was shifted to an apical four-chamber position. Pulsed wave Doppler tracings were obtained from a sample volume in the LV inflow signal at the site of the most intense flow. Mitral valve early wave peak (E), atrial wave peak (A), isovolumic relaxation time and E wave deceleration time were measured. Early (e’) and atrial contraction (a’) myocardial relaxation velocities were recorded at the level of basal septal LV segment using tissue Doppler signals. Mitral valve E/A ratio and E/e’ ratio were calculated.

Pulse wave velocity

Abdominal aorta pulse wave velocity (PWV) was determined using the method developed by Di Lascio and colleagues.2 In the same ultrasound session, a 24-MHz transducer was positioned abdominally. 700 frames-per-second B-mode images of the abdominal aorta were obtained using the EKV imaging mode (VisualSonics) to measure aortic diameter (D). A pulse wave Doppler tracing was obtained to measure aortic flow velocity (V). Velocity was plotted against the natural logarithm of the diameter, and the slope of the linear part of the resulting ln(D)-V loop was used to calculate PWV.
All measurements were obtained at end-respiration and in triplicate. All calculated parameters except PWV were automatically computed by VevoLab software v1.7.1 (VisualSonics). PWV was computed with Mathlab v2014 (Mathworks).

**Blood Glucose Measurement**

By cutting the distal end of the tail, a minimal amount of blood was obtained to determine blood glucose with a OneTouch Ultra test strip and glucose meter (LifeScan) in a non-fasting state.

**Blood Pressure Measurement**

Mice were placed consciously in restrainers on a heating pad (37°C) and kept in the dark. Flow sensors and occluding cuffs, controlled by a programmed electrosphygmomanometer (Coda, Kent Scientific), were placed at the distal end of the tail. Systolic and diastolic blood pressures were obtained automatically from flow data using Coda software v4.0 (Kent Scientific). To familiarize mice with the procedure, measurements were repeated on three subsequent days of which only the final measurement was recorded. 10-25 consecutive pressure measurements from each animal were averaged. Sessions with <10 successful measurements were excluded from analysis.

**Treadmill Ergometry**

Exercise performance was measured on a treadmill at 10° inclination with adjustable speed and an electrical stimulation grid at the bottom (Leica Systems). After an accommodation period of 5 minutes, mice started the test at a speed of 15 m/s. Speed was increased every 2 minutes with 4 m/s increments, until exhaustion. Exhaustion was defined as the mouse staying on the grid despite electrical stimulation, or the mouse receiving >10 electrical stimulations in 1 minute. To familiarize mice with the procedure, on two days preceding the test mice could run in the treadmill at 15 m/s for 10 minutes.

**Invasive Cardiac Pressure-Volume Measurement**

Mice were anesthetized with a 10 µL/g urethane (Sigma-Aldrich) and 10 µL/g etomidate (GlaxoSmithKline) intraperitoneal injection and 1 µL/g morphine subcutaneous injection (Sterop Laboratories) as previously reported. Fully anesthetized mice were placed on a heat pad, intubated and ventilated. After cannulation of
the left internal jugular vein, a left-sided partial thoracotomy was performed and a 1.4F pressure conductance catheter (Millar) was inserted into the LV through the apex. Pressure and volume signals were continuously acquired in LabChart software via a PowerLab 8/30 interface (AD Instruments). Serial measurements were obtained in steady state, during occlusion of the inferior caval vein (to obtain load-independent hemodynamic parameters), and during administration of serial 3 µL boluses of hypertonic (15%) NaCl (to calibrate conductance measurements). Ventilation was paused during acquisition to minimize artifacts. Mice were euthanized by exsanguination during blood collection from the inferior caval vein, under full anesthesia. The blood was used for volume calibration in a dedicated Millar cuvette. Data was analyzed using the PV Loop 2.0 module in LabChart v7.3.7 (AD Instruments). Cuvette and bolus data were used for volume calibration. 7-15 stable pressure-volume loops were averaged for each analysis, and 3-5 analyses were obtained for each animal. End-systolic pressure-volume relationship (ESPVR) and end-diastolic pressure-volume relationship (EDPVR) were automatically calculated by a curve fit through end-systolic and end-diastolic points on the pressure-volume plot, respectively.

**Ex-Vivo Vascular Function Measurement**

Aortic segments were cut at equal lengths (1.5 mm), mounted between two parallel hooks in 8 ml organ baths filled with Krebs Ringer solution and connected to a data acquisition system (PowerLab 8/30 and LabChart v7.2.5, AD Instruments). Four organ baths were used. In the first, isometric force was acquired and reported in mN and endothelial nitric oxide synthase (eNOS) was inhibited by 300 µM N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich). In the second, isometric force was measured but eNOS was not inhibited. In these setups, segments were gradually stretched until a stable preload of 12 mN was reached, as described earlier.\textsuperscript{4,5} Vascular smooth muscle cells were stimulated in both setups using increasing concentrations of phenylephrine (PE, Sigma-Aldrich), whereby the amount of basal nitric oxide (NO) was estimated by using the difference in isometric force between the two organ baths.\textsuperscript{6} Segments were washed and re-immersed in Krebs Ringer solution free of Ca\textsuperscript{2+} and supplemented with 1 mM triethylene glycol diamine tetraacetic acid (EGTA) as chelating agent, subsequently 3.5 mM Ca\textsuperscript{2+} was added to induce maximal contraction. In the setup without L-NAME, the aortic segment was relaxed using increasing concentrations of acetylcholine (ACh, Sterop Laboratories) to determine endothelial-dependent vasorelaxation.
In the setup with L-NAME, the aortic segment was relaxed using increasing concentrations of the NO donor diethylamine nitric oxide (DEANO, Sigma-Aldrich) to determine endothelial-independent vasorelaxation.

In the third and fourth bath, force and displacement of the upper hook were controlled and measured with a force-length transducer to determine mechanical properties of the aortic segment while being stretched at physiological pressure and frequency (Rodent Oscillatory Tension Set-up to study Arterial Compliance). Hereby the Peterson modulus of elasticity ($E_p$) is obtained, a frequently used, vessel size-independent measure of arterial stiffness calculated as $E_p = D_0 \cdot (\Delta P / \Delta D)$ with $D_0$ the diastolic diameter of the vessel, $\Delta P$ the pressure difference ($\pm 40$ mmHg in this study) and $\Delta D$ the difference between systolic and diastolic diameter. Diameters were derived from the displacement of the upper hook, being directly proportional to the inner circumference of the vessel. In the third setup eNOS was inhibited by addition of L-NAME, in the fourth eNOS was not inhibited.

**Histology, immunohistochemistry and fluorescence microscopy**

Transverse sections of the heart were formalin-fixed for 24 hours, dehydrated overnight in 60% isopropanol and embedded in paraffin. Serial 5 µm cross-sections of the heart were obtained and stained with hematoxylin and eosin for quantification of cardiomyocyte cross-sectional area. Transverse sections of both heart and thoracic aorta were immersed in Neg-50 frozen section medium (ThermoFisher), frozen with liquid nitrogen and stored at -80°C until analysis. Serial 5 µm cross-sections were cut and stained with Sirius red to assess collagen content; for immunohistochemistry with CD54 (BD Pharmingen 550287) to assess endothelial inflammation; for fluorescence microscopy with acetyl-p53 (Sigma-Aldrich SAB4503014) and CD31 (BD Pharmingen 550274) to assess endothelial cellular senescence; and for senescence-associated β-galactosidase staining (SA-βgal, Sigma-Aldrich CS0030). Conventional images were digitally acquired with Universal Grab 6.1 software (IDL) using an Olympus BX40 microscope. Fluorescent images were digitally acquired using an EVOS FL Auto Cell Imaging System (ThermoFisher). Quantification was performed on 10 random images per slide with Image J software v2.0.0.

**Western blotting**

Western blotting was performed as described previously. Briefly, aortic tissue was homogenized (Precellys, Bertin Technologies) in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche).
Protein concentration was determined using the bicinchoninic acid method (ThermoFisher). Then, equal amounts of protein were separated on 4-12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were immersed in Odyssey blocking buffer (LI-COR) for one hour, incubated overnight at 4°C with primary antibodies diluted in blocking buffer, and subsequently incubated with infrared dye labeled secondary antibodies for one hour. Antibodies used: mouse anti-eNOS (BD Pharmingen 610297, 140 kDa), mouse anti-ß-actin (Sigma-Aldrich A5441 clone AC-15, 42 kDa), anti-mouse (LI-COR, IgG926-68070). Antibodies were detected using infrared imaging (Odyssey SA, LI-COR) and protein band intensity was quantified with Image Studio v4.0 (LI-COR).

**Quantitative polymerase chain reaction**

Total RNA was prepared from frozen heart samples using the Nucleospin RNA kit (Filter Service). Samples were analyzed using TaqMan gene expression assays (ThermoFisher): COL1A1 (Mm00801666_g1), COL3A1 (Mm01254476_m1), ICAM (Mm00492566_m1), MYH7 (Mm00600555_m1), NPPB (Mm01255770_g1), VCAM (Mm01320970_m1). GAPDH (Mm9999915_g1) was used as reference gene. Real time quantitative RT-PCR was performed on an ABI Prism 7300 sequence detector system (ThermoFisher). The parameters for PCR amplification were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. mRNA expression was normalized to CON group and GAPDH expression according to the $2^{\Delta\Delta C_t}$ method.
Supplementary Tables

Table S1. Additional echocardiographic parameters at different time points

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>CON-WD</th>
<th>SAM</th>
<th>SAM-WD</th>
<th>Significance</th>
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<td>8w</td>
<td>16w</td>
<td>24w</td>
<td>8w</td>
<td>16w</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>473 ± 14</td>
<td>466 ± 11</td>
<td>495 ± 13</td>
<td>497 ± 24</td>
<td>458 ± 9</td>
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<tr>
<td>LV Diastolic function</td>
<td></td>
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<tr>
<td>E wave deceleration time (ms)</td>
<td>23.5</td>
<td>25.6</td>
<td>20.8</td>
<td>14.9</td>
<td>25.8</td>
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<td>Pulmonary vein systolic / diastolic velocity (ratio)</td>
<td>0.40</td>
<td>0.33</td>
<td>0.44</td>
<td>0.43</td>
<td>0.48</td>
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<tr>
<td>LV Systolic function</td>
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<td>Global longitudinal strain (%)</td>
<td>-21.9</td>
<td>-22.6</td>
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<td>Cardiac structure</td>
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<td>Interventricular septum width in diastole (mm)</td>
<td>0.91</td>
<td>0.96</td>
<td>0.94</td>
<td>0.93</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>± 0.06</td>
<td>± 0.09</td>
<td>± 0.05</td>
<td>± 0.09</td>
<td>± 0.05</td>
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<td>LV internal diameter (mm)</td>
<td>3.50 ± 0.11</td>
<td>3.62 ± 0.11</td>
<td>3.73 ± 0.12</td>
<td>3.76 ± 0.13</td>
<td>3.44 ± 0.20</td>
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<td>LV posterior wall width in diastole (mm)</td>
<td>0.94</td>
<td>0.99</td>
<td>0.92</td>
<td>0.89</td>
<td>1.23</td>
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<td>Left atrial area (mm²)</td>
<td>2.61 ± 0.08</td>
<td>1.98 ± 0.08</td>
<td>1.78 ± 0.04</td>
<td>2.33 ± 0.02</td>
<td>2.09 ± 0.11</td>
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<tr>
<td>Right ventricle</td>
<td></td>
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<tr>
<td>Mean pulmonary artery pressure (mmHg)</td>
<td>34.5 ± 1.3</td>
<td>32.9 ± 0.9</td>
<td>32.1 ± 1.2</td>
<td>36.0 ± 0.5</td>
<td>41.6 ± 1.6</td>
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<td>Arterial stiffness</td>
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<td>Pulse wave velocity at abdominal aorta (m/s)</td>
<td>1.82 ± 0.18</td>
<td>2.36 ± 0.27</td>
<td>1.91 ± 0.33</td>
<td>1.47 ± 0.32</td>
<td>1.92 ± 0.25</td>
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</table>

LV = left ventricular. Generalized estimating equations, n= 5-9 per time point. * p<0.05 for senescence, † p<0.05 for diet, ‡ p<0.05 for time, § p<0.05 for senescence-diet interaction, || p<0.05 for senescence-time interaction, # p<0.05 for diet-time interaction, ** p<0.05 for senescence-diet-time interaction.
Table S2. Other noninvasive tests at different time points

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<td>24w</td>
<td>8w</td>
<td>16w</td>
<td>24w</td>
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<td>Body weight (g)</td>
<td>24.3</td>
<td>26.7</td>
<td>28.0</td>
<td>24.4</td>
<td>30.6</td>
<td>30.8</td>
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<td>25.7</td>
<td>28.2</td>
<td>23.7</td>
<td>28.4</td>
<td>29.4</td>
<td>† ‡ **</td>
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<tr>
<td>± 0.5</td>
<td>± 0.8</td>
<td>± 1.0</td>
<td>± 0.7</td>
<td>± 1.5</td>
<td>± 1.5</td>
<td>± 0.5</td>
<td>± 1.0</td>
<td>± 0.7</td>
<td>± 1.0</td>
<td>± 1.4</td>
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<tr>
<td>Blood glucose (mmol/L)</td>
<td>7.98</td>
<td>7.89</td>
<td>9.01</td>
<td>7.69</td>
<td>7.59</td>
<td>7.71</td>
<td>7.66</td>
<td>8.09</td>
<td>8.59</td>
<td>7.52</td>
<td>8.31</td>
<td>8.80</td>
<td>† **</td>
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<td>± 0.27</td>
<td>± 0.30</td>
<td>± 0.35</td>
<td>± 0.57</td>
<td>± 0.27</td>
<td>± 0.09</td>
<td>± 0.50</td>
<td>± 0.56</td>
<td>± 0.75</td>
<td>± 0.98</td>
<td>± 0.82</td>
<td>± 0.64</td>
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<td>Tail cuff blood pressure</td>
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<tr>
<td>Systolic blood</td>
<td>96.5</td>
<td>89.1</td>
<td>101.1</td>
<td>99.8</td>
<td>97.6</td>
<td>100.1</td>
<td>94.1</td>
<td>96.8</td>
<td>87.5</td>
<td>90.7</td>
<td>83.2</td>
<td>85.9</td>
<td>* § **</td>
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<tr>
<td>pressure (mmHg)</td>
<td>± 3.2</td>
<td>± 1.8</td>
<td>± 3.1</td>
<td>± 6.0</td>
<td>± 5.1</td>
<td>± 4.8</td>
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<td>Diastolic blood</td>
<td>66.9</td>
<td>60.6</td>
<td>72.8</td>
<td>67.3</td>
<td>69.6</td>
<td>68.2</td>
<td>68.9</td>
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<td>59.9</td>
<td>60.2</td>
<td>54.8</td>
<td>59.2</td>
<td>* **</td>
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<tr>
<td>pressure (mmHg)</td>
<td>± 2.8</td>
<td>± 2.0</td>
<td>± 2.3</td>
<td>± 5.3</td>
<td>± 5.2</td>
<td>± 3.1</td>
<td>± 9.9</td>
<td>± 6.4</td>
<td>± 3.9</td>
<td>± 3.6</td>
<td>± 4.1</td>
<td>± 3.7</td>
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<tr>
<td>Treadmill ergometry</td>
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<tr>
<td>Running distance (% of baseline)</td>
<td>100.0</td>
<td>75.7</td>
<td>61.6</td>
<td>100.0</td>
<td>105.1</td>
<td>99.9</td>
<td>100.0</td>
<td>101.4</td>
<td>86.2</td>
<td>100.0</td>
<td>65.0</td>
<td>57.7</td>
<td>† § **</td>
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<td>± 0.0</td>
<td>± 6.5</td>
<td>± 4.2</td>
<td>± 0.0</td>
<td>± 17.7</td>
<td>± 13.8</td>
<td>± 0.0</td>
<td>± 4.8</td>
<td>± 4.8</td>
<td>± 0.0</td>
<td>± 5.4</td>
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<tr>
<td>Running time (% of baseline)</td>
<td>100.0</td>
<td>84.6</td>
<td>74.8</td>
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<td>102.8</td>
<td>98.9</td>
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<td>100.0</td>
<td>77.1</td>
<td>71.5</td>
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<tr>
<td>± 0.0</td>
<td>± 4.4</td>
<td>± 3.3</td>
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<td>± 10.5</td>
<td>± 8.1</td>
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<td>± 0.0</td>
<td>± 3.8</td>
<td>± 5.0</td>
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</table>
Generalized estimating equations, n=5-9 per time point. * p<0.05 for senescence, † p<0.05 for diet, ‡ p<0.05 for time, § p<0.05 for senescence-diet interaction, || p<0.05 for senescence-time interaction, # p<0.05 for diet-time interaction, ** p<0.05 for senescence-diet-time interaction.
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<th>SAM</th>
<th>SAM-WD</th>
<th>Significance</th>
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<td><strong>Isometric organ bath</strong></td>
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<tr>
<td>ACh: maximal relaxation (%)</td>
<td>95.3 ± 10.7</td>
<td>93.3 ± 7.6</td>
<td>83.5 ± 3.8</td>
<td>85.2 ± 6.3</td>
<td>*</td>
</tr>
<tr>
<td>ACh: EC&lt;sub&gt;50&lt;/sub&gt; (log M)</td>
<td>7.34 ± 0.19</td>
<td>7.37 ± 0.07</td>
<td>6.98 ± 0.12</td>
<td>7.12 ± 0.14</td>
<td>*</td>
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<tr>
<td>ACh: area under the curve</td>
<td>210.3 ± 36.7</td>
<td>213.2 ± 19.7</td>
<td>157.3 ± 11.8</td>
<td>173.4 ± 20.4</td>
<td>*</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td></td>
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<tr>
<td>DEANO: maximal relaxation (%)</td>
<td>103.7 ± 2.2</td>
<td>102.9 ± 2.4</td>
<td>101.4 ± 2.4</td>
<td>100.6 ± 1.4</td>
<td>*</td>
</tr>
<tr>
<td>DEANO: EC&lt;sub&gt;50&lt;/sub&gt; (log M)</td>
<td>8.06 ± 0.26</td>
<td>7.85 ± 0.47</td>
<td>7.90 ± 0.08</td>
<td>7.66 ± 0.17</td>
<td>†</td>
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<tr>
<td>DEANO: area under the curve (arbitrary units)</td>
<td>275.9 ± 32.2</td>
<td>281.3 ± 45.2</td>
<td>281.0 ± 11.2</td>
<td>250.8 ± 15.4</td>
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<tr>
<td>PE: maximal contraction (mN)</td>
<td>1.35 ± 0.70</td>
<td>1.21 ± 1.02</td>
<td>5.63 ± 1.60</td>
<td>3.87 ± 1.50</td>
<td>*</td>
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<tr>
<td>PE: difference in maximal contraction with and without L-NAME (mN)</td>
<td>7.92 ± 1.07</td>
<td>9.36 ± 1.27</td>
<td>5.27 ± 1.55</td>
<td>6.10 ± 1.50</td>
<td>*</td>
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<tr>
<td><strong>ROTSAC</strong></td>
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<td></td>
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<tr>
<td>Peterson modulus (mmHg)</td>
<td>277.7 ± 17.3</td>
<td>286.6 ± 7.9</td>
<td>269.6 ± 52.7</td>
<td>269.0 ± 7.5</td>
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</tr>
</tbody>
</table>

Two-way analysis of variance, n=4-6 per group. ACh = acetylcholine, DEANO: diethylamine nitric oxide, EC<sub>50</sub> = half maximal effective concentration, L-NAME = L-N<sup>5</sup>-nitroarginine methyl ester, PE = phenylephrine, ROTSAC = Rodent Oscillatory Tension Set-up to study Arterial Compliance. * p<0.05 for senescence, † p<0.05 for diet, ‡ p<0.05 for interaction.
References


8. Kurdi A, De Doncker M, Leloup A, Neels H, Timmermans J-P, Lemmens K, Apers S, De Meyer GRY, Martinet W. Continuous administration of the mTORC1 inhibitor everolimus induces tolerance and...