Effect of age and gender on the progression of adult vascular dysfunction in a mouse model of fetal programming lacking endothelial nitric oxide synthase

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Chiossi G, Costantine MM, Tamayo E, Orise P, Hankins GD, Saade GR, Longo M. Effect of age and gender on the progression of adult vascular dysfunction in a mouse model of fetal programming lacking endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 301: H297–H305, 2011. First published May 13, 2011; doi:10.1152/ajpheart.01284.2010.—The objective of this study was to investigate vascular function at different ages in a transgene murine model of fetal vascular programming using a model of uteroplacental insufficiency induced by lack of endothelial nitric oxide synthase. Homozygous NOS3 knockout (KO) and wild-type (WT) mice were cross bred to produce WT, KO, and heterozygous that developed in WT (KOP) or KO (KOM) mothers. Male/female offspring from the four groups were killed at 7, 14, and 21 wk of age (n = 5–10/group), and carotid arteries were used for in vitro vascular studies. Responses to phenylephrine (PE), with/without N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME), angiotensin (ANG), acetylcholine (ACh), sodium nitroprusside, and isoproterenol (ISO) were studied. At 7 wk, only KO offspring showed higher contractile response to PE, whereas, at 14 and 21 wk, both KO and KOM had a higher response. Incubation with L-NAME abolished these differences. ANG contraction was higher in male KO in all age groups and in 21-wk-old females. Relaxation to ACh and ISO was absent in KO, and significantly decreased in KOM offspring in all age groups compared with KOP and WT, independent of gender. Sodium nitroprusside was not different between groups. The effect of the altered intrauterine environment on the development of abnormal vascular function was limited at 7 wk of age and most evident at 14 wk; further deterioration was limited to ANG-mediated vascular contractility in KO offspring. Our findings provide some hope that at least the first seven postnatal weeks may be an appropriate therapeutic window to prevent cardiovascular disease later in life.

fetal programming; age process; cardiovascular development; kidney development; vascular structure

THE ASSOCIATION BETWEEN SUBOPTIMAL intrauterine environment and adult diseases was initially suggested in the 1960s by Robert McCance and Elsie Widdowson (48, 49) in their original work on the influence of early neonatal nutrition on growth, body size, and development. Their work was later highlighted by the epidemiological studies of David Barker in the 1980s, which showed that low birth weight is an important risk factor for the future development of cardiovascular diseases. Barker’s hypothesis, which was subsequently termed the “developmental origin of adult diseases,” suggests that insults to the fetus during critical periods of development lead to fetal programming and produce adaptive changes that have long-term consequences (1–4).

The milieu in which the fetus develops is determined by the interactions between the fetal genome and the intrauterine environment, which in turn may depend on the influences related to the maternal genetic information. Long-term health of the offspring is then determined by these intrauterine influences as well as by the genetic makeup of the individual, which is additionally dependent on inheritance of maternal genes. Therefore, a genetic trait that affects vascular function may influence uteroplacental perfusion if present in the mother, as well as influence long-term health of the offspring if inherited by her fetus. To study these complex interactions, we used a transgenic mouse model deficient in endothelial nitric oxide synthase (eNOS; or NOS3), the rate-limiting enzyme responsible for nitric oxide (NO) production from L-arginine in endothelial cells (15, 22). NO is a potent smooth muscle relaxant and an essential modulator of vascular tone (34, 38); it also contributes to the maintenance of adequate uteroplacental perfusion through its vasodilatory properties. Indeed, inhibition of NOS3 during pregnancy results in maternal hypertension and fetal growth restriction (37, 42, 47). Because it combines an adverse uterine environment with a genetic alteration affecting vascular function, this animal model is well-suited to study the contribution of each variable on the vascular function in adult life.

In prior work, transgenic male and female mice lacking a functional NOS3 gene [NOS3\textsuperscript{−/−} or knockout (KO)] were crossbred with wild-type mice [NOS3\textsuperscript{+/−} or wild type (WT)] to generate heterozygous offspring (NOS3\textsuperscript{+/−}) that developed either in a mother lacking a functional NOS3 (maternally derived heterozygous offspring; KOM) or in normal wild-type mothers (paternally derived heterozygous offspring; KOP). In that study, we demonstrated that the abnormal uterine environment in which KOM offspring developed altered fetal and postnatal growth as well as resulted in abnormal adult vascular function compared with KOP offspring, which were genonomically similar but developed in a normal uterine environment. Most importantly, the endothelium-dependent relaxation to acetylcholine (ACh) was absent in KOM offspring, similar to KO mice, whereas that of KOP mice was similar to the one observed in WT animals; this effect was evident in the carotid as well the mesenteric vascular bed (28). These striking differences were initially demonstrated in the offspring at 7–8 wk of age. Therefore, we hypothesized that the previously
demonstrated differences in vascular profile in adult offspring progress over time and are gender specific. Our objective is thus to evaluate the progression of the vascular dysfunction in this animal model over time, and to characterize the differences existing between genders.

MATERIALS AND METHODS

Animals. Mature cycling female and male mice that are homozygous for disruption of the NOS3 gene (NOS3 knockout, strain B6.129P2-Nos3tm1Unc, stock no. 002684, KO) and their age-matched wild-type controls (NOS3 wild type, strain C57BL/6J, stock no. 000664, WT) were purchased from Jackson Laboratory (Bar Harbor, ME). Approval for the study was obtained from the Institutional Animal Care and Use Committee (IACUC). The mice were housed separately in temperature- and humidity-controlled quarters at the University of Texas Medical Branch. Female and male KO mice with constant 12:12-h light-dark cycles in the animal care facility at the University of Texas Medical Branch. Female and male KO mice were treated as sisters (SNP), and isoproterenol (ISO) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Stock solutions of all of the drugs (10\(^{-5}\) to 10\(^{-10}\) mmol/l) were prepared in deionized water and stored at –20°C. The composition of Krebs solution was as follows (in mmol/l): 119 NaCl, 4.7 KCl, 1.2 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), 0.026 ethylenediaminetetraacetic acid, and 11.5 glucose.

Preparations were bathed in a physiological salt solution that was bubbled continuously with a mixture of 95% O\(_2\) and 5% CO\(_2\), with the temperature maintained at 37°C and the pH kept at 7.4. Vascular tension was recorded continuously by an isometric force transducer. Vessels were allowed to stabilize under a passive tension up to 3.5 mN and then contracted two times with 60 mmol/l KCl to enhance reproducibility of the responses. After 1 h equilibration, the \(\alpha\)-adrenergic agonist PE was added at a final concentration of 10\(^{-7}\) to 10\(^{-6}\) mmol/l to produce matching contractions in the study groups. Vascular responses to the endothelium-dependent vasorelaxant ACh (10\(^{-10}\) to 10\(^{-5}\) mmol/l), the endothelium-independent vasorelaxant SNP (10\(^{-5}\) to 10\(^{-5}\) mmol/l), and the \(\beta\)-adrenoreceptor agonist ISO (10\(^{-10}\) to 10\(^{-5}\) mmol/l) were studied. Contractile responses were evaluated for PE (10\(^{-10}\) to 10\(^{-5}\) mmol/l) in the presence or absence of the nonselective NOS inhibitor L-NAME (10\(^{-5}\) mmol/l) and ANG (10\(^{-10}\) to 10\(^{-5}\) mmol/l). The samples were washed with Krebs solution and left to recover for ≥30 min after each agent was tested.

Data analysis. Vascular tension was recorded continuously by an isometric transducer, analyzed with Power laboratory data acquisition software (DataQ Instruments, Akron, OH), and presented as means ± SE. Dose-response curves were generated for each vasoactive agent. The maximal effect was calculated and expressed as the mean ± SE. The second response to KCl was used as a reference to calculate the percent vascular tension achieved by the contractile agents, whereas PE-induced contraction was used to measure the percent relaxation produced by the vasorelaxant agents. Continuous data were tested for normality using the Kolmogorov-Smirnov test and then compared using one-way ANOVA followed by the Newman-Keuls multiple-comparisons test. A P < 0.05 was considered statistically significant.

RESULTS

Seven-week-old KO offspring had significantly higher contractile responses to PE compared with WT, KOP, and KOM (Table 1 and Fig. 1, A and B; P < 0.05), whereas at 14 and 21 wk of age, both KO and KOM offspring had increased contractile responses to PE compared with WT, KOP, and KOM.

### Table 1. Maximal contractile effect to PE, with and without L-NAME, and angiotensin in female and male KO, KOM, KOP, and WT at 7, 14, and 21 wk of age

<table>
<thead>
<tr>
<th>Agents</th>
<th>Age, wk</th>
<th>KO</th>
<th>KOM</th>
<th>KO</th>
<th>KO</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>7</td>
<td>70.1 ± 6.2</td>
<td>84.5 ± 7.0</td>
<td>150.3 ± 6.9*</td>
<td>97.2 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>71.7 ± 8.1</td>
<td>153 ± 9.9#</td>
<td>174.8 ± 1.9*</td>
<td>102.2 ± 11.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>77.5 ± 6.7</td>
<td>139 ± 6.1#</td>
<td>172.7 ± 7.5*</td>
<td>102.7 ± 10.8</td>
<td></td>
</tr>
<tr>
<td>PE + L-NAME</td>
<td>7</td>
<td>142.4 ± 7.7</td>
<td>201.03 ± 12</td>
<td>202.57 ± 17.1</td>
<td>155.8 ± 19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>173.1 ± 8.8*</td>
<td>201.89 ± 3.8</td>
<td>237.08 ± 7.9</td>
<td>211.9 ± 10.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>172.4 ± 5.1*</td>
<td>226.2 ± 8.4</td>
<td>206 ± 8.8</td>
<td>214.6 ± 8.8</td>
<td></td>
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<tr>
<td>ANG</td>
<td>7</td>
<td>0.9 ± 0.5</td>
<td>2.0 ± 0.6</td>
<td>2.9 ± 1.4</td>
<td>0.6 ± 0.07</td>
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<tr>
<td></td>
<td>14</td>
<td>2.1 ± 0.7</td>
<td>5.5 ± 2.3</td>
<td>6.1 ± 2.5</td>
<td>4.5 ± 1.9</td>
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<tr>
<td></td>
<td>21</td>
<td>3.8 ± 0.8</td>
<td>5.13 ± 3.01</td>
<td>2.8 ± 12.7*</td>
<td>3.3 ± 0.7</td>
<td></td>
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<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PE</td>
<td>7</td>
<td>69.4 ± 4.6</td>
<td>80.2 ± 3.1</td>
<td>136.3 ± 3.5*</td>
<td>89.2 ± 9.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>71.4 ± 8.9</td>
<td>133.6 ± 9.9#</td>
<td>159.4 ± 9.4*</td>
<td>89.6 ± 6.3</td>
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<td></td>
<td>21</td>
<td>84.7 ± 12.6</td>
<td>138.7 ± 6.7#</td>
<td>164.3 ± 8.9*</td>
<td>98.7 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>PE+L-NAME</td>
<td>7</td>
<td>164.4 ± 7.2</td>
<td>184 ± 9.1</td>
<td>212.3 ± 5.4</td>
<td>192.1 ± 9.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>174.7 ± 3.1</td>
<td>205.1 ± 8.1</td>
<td>225.7 ± 5.9</td>
<td>186.9 ± 17.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>170.3 ± 7.3</td>
<td>191.4 ± 10.4</td>
<td>219.3 ± 17.1</td>
<td>208.4 ± 10</td>
<td></td>
</tr>
<tr>
<td>ANG</td>
<td>7</td>
<td>0.11 ± 0.06</td>
<td>-0.57 ± 0.36</td>
<td>9.0 ± 2.42*</td>
<td>0.53 ± 0.31</td>
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<tr>
<td></td>
<td>14</td>
<td>1.54 ± 0.57</td>
<td>3.59 ± 1.30</td>
<td>13.2 ± 2.98*</td>
<td>0.21 ± 0.85</td>
<td></td>
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<tr>
<td></td>
<td>21</td>
<td>3.01 ± 0.94</td>
<td>4.76 ± 1.16</td>
<td>30.7 ± 6.2*</td>
<td>2.76 ± 0.90</td>
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</tr>
</tbody>
</table>

Results are expressed as percent of the reference KCl contraction (mean ± SE). PE, phenylephrine; L-NAME, N\(^2\)-nitro-L-arginine methyl ester; KO, knockout; WT, wild type; KOM, maternally derived heterozygous; KOP, paternally derived heterozygous; ANG, angiotensin. P < 0.05 for KO vs. WT, KOM, and KOP (*) and for KOM vs. WT and KOP (#).
tractile responses to PE compared with WT and KOP (Table 1 and Fig. 1, C–F; *P < 0.05). These responses were seen in both female and male offspring groups. Additionally, when compared with offspring of different ages or who had previously developed in a different intrauterine environment, both male and female KOM had increased vascular responses to PE at 14 and 21 wk that were even more prominent than the ones presented at 7 wk (*P < 0.05). Incubation with L-NAME abolished the differences mediated by the α1-adrenergic agonist in the male offspring (Table 1). In contrast, vessels from female KOP preincubated with L-NAME showed decreased vascular contraction to PE compared with WT, KOM, and KO.
DISCUSSION

At 7 wk of age, only KO offspring showed higher contractile response to PE, whereas at 14 and 21 wk of age, both KO and KOM (male and female) had significantly higher PE-induced vascular contractility compared with WT and KOP offspring. Incubation with 
\( \text{L-N}
\text{NAME} \) abolished the differences seen in response to PE in all groups, especially in male offspring, indirectly suggesting that the exaggerated PE responses are mediated by the nitric oxide pathway. On the other hand, ANG-induced vascular contractility was higher in male KO offspring in all age groups and in 21-wk-old female offspring compared with WT, KOM, and KOP. The relaxation induced by ACh and ISO was absent in KO and significantly decreased in KOM offspring in all age groups compared with KOP and WT, independent of gender. ACh is an endothelium-dependent vasorelaxant. The lack of relaxation observed in the eNOS-knockout group (KO) provides further evidence that ACh-mediated vascular relaxation is NO dependent, and irrespective of age and gender. The altered vascular response detected in KOM at 14 wk and the normal response observed in KOP provide evidence of the timing of the altered endothelial function (being age dependent) and that only one copy of the eNOS gene is sufficient for normal responses to ACh in carotid arteries (12, 21).

From these data, we conclude that the effects of the altered intrauterine environment on the development of abnormal vascular function were limited at 7 wk of age in both male and female offspring and became well established at 14 wk, and further deterioration was limited to ANG-mediated vascular contractility in KO offspring at 21 wk. Our findings provide insight about a potential window in the first seven postnatal weeks when interventions may be directed to prevent cardiovascular disease later in life.

In an attempt to explain our findings of different vascular profile between KOP and KOM offspring, we previously looked at eNOS gene expression in the vasculature of these offspring and found no differences between KOP and KOM (8). Additionally, we have shown that the abnormal vascular phenotype of the adult KOM offspring (born to a KO mother) was abolished when KOM embryos were transferred to develop in wild-type (NOS3\(^{+/+}\)) surrogate mothers (28). These findings suggest that the differences in vascular profile between heterozygous offspring are related to the uterine environment in which the pups develop, rather than genetics. Although transmission of epigenetic traits across both maternal and paternal lines has been described, our data do not support such a mechanism in our animal model. Indeed, in support of the presence of abnormal uterine environment, it has been demonstrated that structural and cellular changes characteristic of uterine artery remodeling during pregnancy are markedly reduced in NOS3-deficient mice, contributing to poorer pregnancy outcome (43).

Our findings support the importance of in utero effects on vascular programming as one of the mechanisms by which growth-restricted infants are at increased risk of hypertension, cardiovascular morbidity, and mortality (3, 4). Our study also suggests a time frame for the development of these adverse outcomes. Identifying such a window in infants at risk of adverse outcomes when adults may help define when interven-
Table 2. Maximal relaxant effect to ACh, ISO, and SNP in female and male KO, KOM, KOP, and WT at 7, 14, and 21 wk of age

<table>
<thead>
<tr>
<th>Agents</th>
<th>Age, wk</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KOP</td>
<td>KOM</td>
</tr>
<tr>
<td>ACh</td>
<td>7</td>
<td>101.1 ± 3.2</td>
<td>70.6 ± 4.4#</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>114.9 ± 9.1</td>
<td>23.6 ± 5.0#</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>98.1 ± 2.6</td>
<td>39.9 ± 6.8#</td>
</tr>
<tr>
<td>ISO</td>
<td>7</td>
<td>98.9 ± 2.8</td>
<td>91.3 ± 2.7#</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>101.2 ± 6.0</td>
<td>45.2 ± 8.900#</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>97 ± 2.3</td>
<td>85.9 ± 2.3</td>
</tr>
<tr>
<td>SNP</td>
<td>7</td>
<td>110.2 ± 4.7</td>
<td>101.0 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>101.5 ± 1.7</td>
<td>98.7 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>100.6 ± 1.7</td>
<td>103.06 ± 3.5</td>
</tr>
</tbody>
</table>

Results express as percent of the reference PE contraction (mean ± SE). ACh, acetylcholine; ISO, isoproterenol; SNP, sodium nitroprusside. P < 0.05 for KO vs. WT, KOM, and KOP (*) and for KOM vs. WT and KOP (#).
tions may succeed the most in preventing the development of these morbidities.

Several mechanisms have been previously proposed and are thought to lead to abnormal fetal vascular programming, although are speculative when applied to our model and require further testing. One mechanism involves structural changes in resistant vessels. Arterial wall stiffness is associated with adult hypertension and has been recognized as an early marker of cardiovascular disease. Vessel wall development is affected by blood flow and elastin synthesis, which in fetuses peaks in the last weeks of development (24, 27, 33). Vascular dysfunction and hypertension in the adult may therefore be the result of decreased arterial compliance originating in fetal life secondary to reduced elastin deposition (6, 16, 17). In fact, elastin

Fig. 4. A–F: concentration-response curves to acetylcholine (ACh, $10^{-10}$ to $10^{-5}$ mmol/l) in the carotid artery of female and male KO, WT, KOM, and KOP offspring at 7, 14, and 21 wk of age (female/male n = 5–10/age group).
knockout mice manifest high blood pressure and stiff stenotic arteries leading to impaired ventricular function (11, 46, 47). Adverse structural vascular changes are known to occur with aging, and this may explain the limited vascular dysfunction at 7 wk but established dysfunction at 14 wk in our animal model.

An adverse fetal environment may also lead to altered renal structure and function, leading to hypertension in adulthood (2, 10, 17, 51). Human studies also show that low-birth-weight infants have lower kidney weight, fewer nephrons (39), and higher rates of progressive glomerular sclerosis predisposing them to renal insufficiency and hypertension (7, 36). Animal models support this hypothesis as well.

Different animal models of growth restriction, such as protein restriction, decreased placenta perfusion, or those using

Fig. 5. A–F: concentration-response curves to isoproterenol (10⁻¹⁰ to 10⁻⁵ mmol/l) in the carotid artery of female and male KO, WT, KOM, and KOP offspring at 7, 14, and 21 wk of age (female/male, n = 5–10/age group).
corticosteroids, show that fetal growth restriction was associated with permanent nephron deficit (5, 25). Specifically, in the NOS3 knockout mouse model, we have shown that the glomeruli of 14-wk-old KOM and KO offspring are decreased and have altered structure compared with KOP and WT offspring (29). Others have shown that KO mice have altered renal function associated with accelerated glomerular and tubulointerstitial injury with a loss of glomerular and peritubular capillaries (35). These findings associated with the endothelial dysfunction seen in these KO and KOM mice lead to hypertension. Because nephrogenesis in mice is not completed until 2 wk after birth, further hemodynamic changes related to the postnatal environment may contribute to the abnormal renal and vascular development occurring later in life.

Studies have shown that treatment with NO inhibitors results in increased tissue angiotensin-converting enzyme expression in the perivascular areas, as well as cardiac superoxide production, thereby contributing to the long-term vascular effects of NO inhibition (18, 19, 20). In our animal model, KO offspring had significant contractile responses to ANG, but no differences were detected between KOM and KOP. Therefore, the renin-angiotensin system may play a role in the regulation of vascular tone homeostasis during fetal kidney development; its role later in life is unclear and needs further investigation.

Important gender differences in vascular reactivity profiles have been observed, since KO male offspring had higher responses to contractile and relaxant agents at any age, whereas the differences in female offspring became apparent only later in life. Such variation may be related to differences in estrogen concentrations between the different groups and as these female offspring age. This is, however, speculative and requires further investigation. Estrogen improves vascular tone by decreasing myointimal smooth muscle cell proliferation (40), inhibiting tumor necrosis factor-α-induced apoptosis in endothelial cells, (32), and inducing NOS3 in different tissues (45, 50).

Another mechanism is the response to stress later in life. We (9) have previously demonstrated that KOM offspring had significantly higher blood pressure compared with KOP after the introduction of stressful stimuli. Stress is believed to be a major risk factor in the development of cardiovascular disease in mice, since it alters the cardiovascular function (13, 14). The cardiovascular effects of stress are mediated by increased sympathetic activity and baroreflex sensitivity, leading to a reduction in blood pressure buffering (23, 41). As previously mentioned, KOM offspring had better vascular reactivity profiles as KO at 7 wk of age but not later in life. This could be related to an increased baseline sympathetic tone that accounts for worsening vascular function in response to shear stress as it is upregulated from 7 to 14 wk of age. Such findings are consistent with previous reports investigating the same animal model at a different age (16 wk) (44).

This study has limitations. Our findings in the carotid artery may not apply to other vascular beds. However, we have previously shown similar vascular responses to contractile and relaxant between carotid and mesenteric vessels (30). Others have demonstrated that, although the downstream vasculature contributes to the resistance in their vascular beds because of their small caliber, the differences when compared with larger vessels such as carotids are mostly quantitative (magnitude of effect) and not qualitative (nature of response) (26).

We demonstrated that altered vascular development starts early in life, and a window for potential interventions may exist. Our findings worsened with aging and were also gender dependent. This emphasizes the complexity of mechanisms involved in fetal cardiovascular development. Several pathways have been proposed and may explain our findings. These include vessel structure, endothelial function, kidney development, and central vascular tone regulation. These are speculative and require further research, however; identifying putative mechanisms is fundamental for any prevention strategy.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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