

# Late gestational hypoxia and a postnatal high salt diet programs endothelial dysfunction and arterial stiffness in adult mouse offspring

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## Key points

- Maternal hypoxia is a common perturbation that leads to growth restriction and may program vascular dysfunction in adult offspring.
- An adverse prenatal environment may render offspring vulnerable to increased cardiovascular risk when challenged with a ‘second hit’, such as a high salt diet.
- We investigated whether maternal hypoxia impaired vascular function, structure and mechanics in mouse offspring, and also whether this was exacerbated by excess dietary salt intake in postnatal life.
- Maternal hypoxia predisposed adult male and female offspring to endothelial dysfunction.
- The combination of prenatal hypoxia and high dietary salt intake caused significant stiffening of mesenteric arteries, and also altered structural characteristics of the aorta consistent with vascular stiffening.
- The results of the present study suggest that prenatal hypoxia combined with a high salt diet in postnatal life can contribute to vascular dysfunction.

**Abstract** Gestational hypoxia and high dietary salt intake have both been associated with impaired vascular function in adulthood. Using a mouse model of prenatal hypoxia, we examined whether a chronic high salt diet had an additive effect in promoting vascular dysfunction in offspring. Pregnant CD1 dams were placed in a hypoxic chamber (12% O<sub>2</sub>) or housed under normal conditions (21% O<sub>2</sub>) from embryonic day 14.5 until birth. Gestational hypoxia resulted in a reduced body weight for both male and female offspring at birth. This restriction in body weight persisted until weaning, after which the animals underwent catch-up growth. At 10 weeks of age, a subset of offspring was placed on a high salt diet (5% NaCl). Pressurized myography of mesenteric resistance arteries at 12 months of age showed that both male and female offspring exposed to maternal hypoxia had significantly impaired endothelial function, as demonstrated by impaired vasodilatation to ACh but not sodium nitroprusside. Endothelial dysfunction caused by prenatal hypoxia was not exacerbated by postnatal consumption of a high salt diet. Prenatal hypoxia increased microvascular stiffness in male offspring. The combination of prenatal hypoxia and a postnatal high salt diet caused a leftward shift in the stress–strain relationship in both sexes. Histopathological analysis of aortic sections revealed a loss of elastin integrity and increased collagen, consistent with increased vascular stiffness. These results demonstrate that prenatal

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hypoxia programs endothelial dysfunction in both sexes. A chronic high salt diet in postnatal life had an additive deleterious effect on vascular mechanics and structural characteristics in both sexes.

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**Abbreviations** C, control; CHS, control offspring fed high salt; CNS, control offspring fed normal salt; CSA, cross-sectional area; CVD, cardiovascular disease; E, embryonic day; H, hypoxia; HS, high salt; HHS, hypoxia offspring fed high salt; HNS, hypoxia offspring fed normal salt; KPSS, high potassium physiological salt solution; KPSS<sub>max</sub>, maximum vessel contraction to KPSS; NO, nitric oxide; NS, normal salt; P, postnatal day; PE, phenylephrine; R<sub>max</sub>, maximum relaxation; SNP, sodium nitroprusside.

## Introduction

Cardiovascular disease (CVD) is recognized as the leading cause of global mortality. The development of CVD is associated with risk factors such as smoking, age, sex and hypertension (Mozaffarian *et al.* 2008). Epidemiological evidence suggests that CVD can also be linked to poor development in early life (Barker, 2002). Adverse conditions during fetal life may require the fetus and placenta to make adaptations to organogenesis so as to remain viable in the short-term, although these adaptations frequently lead to intrauterine growth restriction (Barker, 1998). If these adaptations occur during critical periods of development, permanent changes in organ structure and function can occur, leading to poor health outcomes and an increased susceptibility to disease in adulthood. This concept is known as developmental programming (Barker, 1995, 1998).

Impaired oxygen supply to the fetus is a common clinical complication during pregnancy and can arise from living at high altitude (Giussani *et al.* 1993), maternal smoking (Bulterys *et al.* 1990) and poor placentation leading to reduced fetoplacental perfusion (Krebs *et al.* 1996). Mild, acute forms of hypoxia initiate the centralization of blood flow away from the peripheral circulation to the heart and brain (Giussani *et al.* 1993; Baschat *et al.* 1997). Although this adaptation is necessary for the immediate preservation of critical organs, the decreased blood flow can impair development of peripheral organs resulting in significant adverse consequences in later life. If the hypoxic insult is prolonged, these immediate adaptations may be sustained and result in compromised growth *in utero*. Low birth weight and brain sparing following chronic *in utero* hypoxia have been observed across all species studied, including humans (Wladimiroff *et al.* 1986; Giussani *et al.* 2001), chicks (Mulder *et al.* 2002; Giussani *et al.* 2007) and the rat (Williams *et al.* 2005a; Williams *et al.* 2005b), and this is associated with an increased risk of CVD later in life.

Endothelial dysfunction and arterial stiffness are both associated with CVD in adulthood (Benetos *et al.* 2002). Interestingly, impaired peripheral vascular endothelial

function has been demonstrated in low birth weight infants (Norman & Martin, 2003), children (Martin *et al.* 2000) and young adults (Goodfellow *et al.* 1998; Leeson *et al.* 2001), and increased arterial stiffness has been shown to contribute to elevated blood pressure in the human population (te Velde *et al.* 2004). There is now substantial evidence from animal models to show that perturbations *in utero* such as hypoxia (Williams *et al.* 2005b), vitamin D deficiency (Tare *et al.* 2011) and undernutrition (Brawley *et al.* 2003) can also program vascular dysfunction in adulthood. Offspring born to rats that were exposed to chronic hypoxia during late pregnancy develop endothelial dysfunction (Williams *et al.* 2005b; Giussani *et al.* 2012) and enhanced myogenic tone (Hemmings *et al.* 2005). These factors may contribute to the increased arterial blood pressure observed in middle-aged rats exposed to chronic hypoxia *in utero* (Rook *et al.* 2014). Furthermore, ventricular (Camm *et al.* 2010) and aortic wall thickening (Rouwet *et al.* 2002; Giussani *et al.* 2012) and increased susceptibility to cardiac ischaemia-reperfusion injury (Xu *et al.* 2006; Xue & Zhang, 2009) have also been reported in adult rodents prenatally exposed to hypoxia. Together, these studies demonstrate that prenatal hypoxia can significantly elevate the propensity to develop CVD in adulthood.

Importantly, prenatally-programmed vulnerability to CVD may be exacerbated by a postnatal 'second-hit' such as a chronic high salt (HS) diet (Ruta *et al.* 2010), a high fat diet (Rueda-Clausen *et al.* 2012) and ageing. In a rat model of prenatal hypoxia, vascular alterations such as increased myogenic tone only arose in animals aged >6 months (Hemmings *et al.* 2005). In the same model, a combination of prenatal hypoxia and the postnatal consumption of a high fat diet increased susceptibility to cardiac and vascular pathologies (Rueda-Clausen *et al.* 2012). In Western societies, diets are often not only high in fat, but also high in salt (Cordain *et al.* 2005). A chronic increase in dietary sodium intake has been shown to result in increased vascular stiffness in mice (Yu *et al.* 2004) and enhanced vasoconstrictor responsiveness in the rat (Sofola *et al.* 2002). Although a HS diet has been shown to

exacerbate disease outcomes following maternal protein restriction (Woods *et al.* 2004) and a congenital nephron deficit (Ruta *et al.* 2010), no study has yet investigated whether the adverse vascular outcomes following prenatal hypoxia are exacerbated by a HS diet.

In the present study, we used our recently reported model of prenatal hypoxia in the mouse (Cuffe *et al.* 2014a; Cuffe *et al.* 2014b) to assess the impact of chronic late gestational hypoxia on microvascular structure and function in aged male and female offspring. In particular, we aimed to examine whether chronic high dietary sodium intake could exacerbate any deficits in microvascular structure and function. We hypothesized that arteries from hypoxia-exposed offspring would have endothelial dysfunction and increased vascular stiffness. In addition, we hypothesized that these alterations would be exacerbated by high dietary sodium intake.

## Methods

### Ethics approval

All experiments were approved in advance by the University of Queensland animal ethics committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

### Animal treatment: maternal hypoxia

At embryonic day (E) 14.5 of pregnancy, time-mated CD-1 mice were randomly allocated to normoxic room conditions (control, C;  $n = 21$ ) or housed inside a hypoxic chamber continuously flushed with nitrogen gas to maintain an oxygen concentration of 12% (hypoxia, H;  $n = 22$ ) for the remainder of pregnancy. Food and water was provided *ad libitum*. Body weight, food and water consumption were monitored daily throughout the experimental protocol. This experimental protocol does not result in changes in maternal food intake and weight gain, and is thus not compromised by maternal undernutrition (Cuffe *et al.* 2014a). The dams were removed from the hypoxic chamber upon littering down (postnatal day, P0). A subset of dams and offspring (C:  $n = 10$ ; H:  $n = 11$ ) were culled at P0. Pup body weight was recorded, and maternal and pup blood were collected for measurement of haematocrit. The remaining offspring remained with their mothers until weaning at P21, and were then weighed weekly for the duration of the study.

### Dietary intervention: chronic HS diet

A subset of animals aged 10 weeks ( $n = 11$  per sex per treatment, one or two animals from each litter) was randomly allocated to receive a HS (HS) diet (5% NaCl,

wt/wt; modified AIN 93M, SF05-023; Specialty Feeds, Memphis, TN, USA). The remaining animals ( $n = 11$  per sex per treatment) were maintained on a matched control normal salt (NS) diet (0.26% NaCl, wt/wt; AIN 93M; Specialty Feeds). Food and water consumption were monitored for the first 5 days of diet administration to ensure the animals adjusted to the dietary change. Animals were maintained on the appropriate diet until post-mortem at 12 months of age.

### Urinalysis

Aged offspring (mean  $\pm$  SEM:  $12 \pm 0.5$  months) were housed in individual metabolic cages for 24 h (with food and water provided *ad libitum*) to measure urinary flow rates and electrolyte excretion. All animals were acclimatized to the metabolic cages on two consecutive days prior to the experiment. Urine was collected and stored at  $-20^{\circ}\text{C}$  for subsequent analysis. Urinary sodium, chloride and potassium concentrations were measured using a COBAS Integra 400 Plus analyser (Roche Diagnostics Ltd, Rotkreuz, Switzerland).

### Pressurized myography

Following completion of renal function measurements, animals were killed by  $\text{CO}_2$  inhalation (six to 10 per group). Second-order mesenteric arteries were dissected from connective tissue, placed in physiological salt solution (composition in  $\text{mmol l}^{-1}$ : 118 NaCl, 4.65 KCl, 1.18  $\text{MgSO}_4$ , 1.18  $\text{KH}_2\text{PO}_4$  and 2.5  $\text{CaCl}_2$ ; 25 mM  $\text{NaHCO}_3$ , 5.5 mM glucose and 0.026 mM EDTA), mounted in a pressurized myograph and the intraluminal pressure gradually increased to 45 mmHg. Maximal vessel contractility was determined using high potassium physiological salt solution (KPSS) containing an equimolar substitution of KCl for NaCl. Vessel contractility was assessed by cumulative concentration–response curves to phenylephrine (PE;  $10^{-9}$  to  $10^{-4}$   $\text{mol l}^{-1}$ ). The contractile responses were expressed as a percentage of the maximum response to KPSS ( $\text{KPSS}_{\text{max}}$ ). Endothelium-dependent and endothelium independent relaxations were assessed in vessels submaximally constricted with PE (70% of  $\text{KPSS}_{\text{max}}$ ), using ACh ( $10^{-9}$  to  $10^{-4}$   $\text{mol l}^{-1}$ ) and sodium nitroprusside (SNP;  $10^{-10}$  to  $10^{-4}$   $\text{mol l}^{-1}$ ), respectively. Relaxation responses were expressed as the percentage relaxation from the PE-induced contraction. Non-linear regression (Prism 6; GraphPad Software Inc., San Diego, CA, USA) was used to determine the  $\text{pEC}_{50}$  and maximum response ( $R_{\text{max}}$ ) for each vessel. To measure vascular structure and mechanics, vessels were superfused with  $\text{Ca}^{2+}$ -free physiological salt solution containing 1  $\text{mmol l}^{-1}$  of EGTA to remove intrinsic tone. Intraluminal pressure was increased stepwise from 3 to

140 mmHg and lumen diameter and wall thickness were measured at each pressure. Medial cross-sectional area (CSA), distensibility, circumferential stress and strain were calculated as described previously (Virdis *et al.* 2002; Iglarz *et al.* 2003).

### Histomorphometric analyses of the aorta

Thoracic aortas were fixed in 4% paraformaldehyde before processing to paraffin. Transverse aortic sections were taken at 5  $\mu\text{m}$ . Sections were stained with Masson's trichrome to assess collagen content and Verhoeff's van Gieson elastin stain to detect elastin expression. Slides were imaged using a 20 $\times$  objective on a BX61 microscope (Olympus, Tokyo, Japan). Masson's trichrome-stained aortic sections were graded by a blinded observer as showing 0–10% media collagen content (grade 0), 10–25% media collagen content (grade 1), 25–50% media collagen content (grade 2) or 50–75% media collagen content (grade 3). Elastin content was evaluated by subtracting background, converting image to binary and calculating the percentage of black (elastin) and white pixels.

### Statistical analysis

All data are presented as the mean  $\pm$  SEM. Maternal haematocrit was analysed via a Student's *t* test. Offspring haematocrit and body weight at P0 were analysed by two-way ANOVA examining the effects of prenatal hypoxia ( $P_{\text{trt}}$ ) and sex ( $P_{\text{sex}}$ ). Data for body and organ weights, urinalysis and vascular parameters of male and female offspring at 12 months of age were analysed separately. These data were analysed by two-way ANOVA examining the effects of prenatal hypoxia ( $P_{\text{trt}}$ ) and postnatal HS diet ( $P_{\text{diet}}$ ). Bonferroni *post hoc* tests were used when necessary.  $P < 0.05$  was considered statistically significant.

## Results

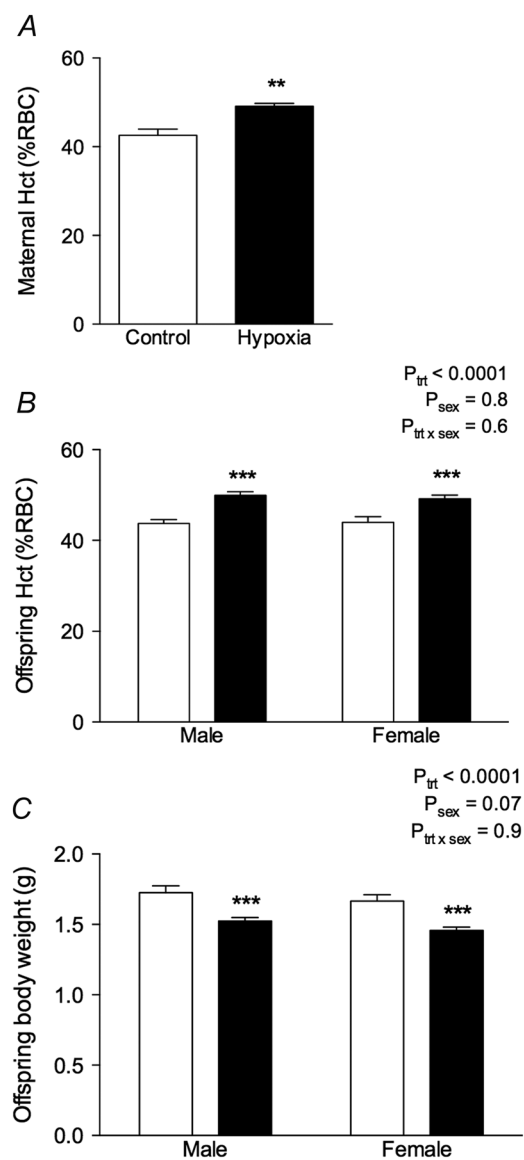
### Offspring weights and growth

Maternal ( $P = 0.007$ ) (Fig. 1A) and pup haematocrit ( $P_{\text{trt}} < 0.0001$ ) (Fig. 1B) was elevated in hypoxia-exposed animals. Litter size did not differ between treatment groups (C:  $14 \pm 1$ , H:  $14 \pm 1$ ). At P0, body weights for both males and females (litter averages) were reduced by  $\sim 12\%$  in animals from hypoxia-exposed dams (male C:  $1.73 \pm 0.5$  g, male H:  $1.52 \pm 0.03$  g, female C:  $1.67 \pm 0.04$  g, female H:  $1.46 \pm 0.02$  g;  $P_{\text{trt}} < 0.0001$ ) (Fig. 1C). Hypoxia-exposed animals remained lighter than control counterparts by  $\sim 7\%$  at the weaning age of P21 (male C:  $11.9 \pm 0.3$  g, male H:  $11.0 \pm 0.3$  g, female C:  $11.2 \pm 0.3$  g, female H:  $10.4 \pm 0.4$  g) ( $P_{\text{trt}} = 0.01$ ). There

was no difference in body weights between treatment groups in male (Fig. 2A) or female (Fig. 2B) offspring from 3 to 12 months of age.

### Urinalysis

Urinary excretion of electrolytes did not differ between control and hypoxic-exposed animals. However, sodium and chloride excretion was significantly elevated in male



**Figure 1. Maternal and offspring parameters at day of birth** Maternal haematocrit (Hct, % packed red blood cell volume) (A), offspring haematocrit (B) and offspring body weight (C) were determined at P0. Control: white bars; hypoxia: black bars. Values are the mean  $\pm$  SEM ( $n = 8$ – $10$  per group). \*\* $P < 0.001$  from a *t* test, \*\*\* $P < 0.0001$  (from Bonferroni *post hoc* compared to same sex controls).



and female offspring (~3- to 5-fold) fed the HS diet compared to those on the control NS diet (data not shown). Potassium excretion did not differ between dietary groups.

### Microvascular function

The maximal contractile response to high potassium physiological salt solution (KPSS) was not affected by maternal hypoxia but was enhanced in male offspring maintained on the HS diet compared to the NS diet (C, control; H, hypoxia) (CNS:  $47.4 \pm 1.0$ ; HNS:  $47.4 \pm 1.0$ ; CHS:  $52.7 \pm 0.9$ ; HHS:  $55.6 \pm 2.2\%$ ;  $P_{\text{diet}} < 0.0001$ ). The maximum contractile response to phenylephrine was unaffected by prenatal hypoxia treatment or dietary HS intake (Fig. 3A). However, sensitivity to phenylephrine was increased in animals maintained on the HS diet, irrespective of the maternal treatment ( $P = 0.008$ ) (Fig. 3A and Table 1). The maximum dilator response to ACh was significantly attenuated in male hypoxia-exposed offspring (Fig. 3C and Table 1), whereas the sensitivity to ACh was similar in all groups. The reduced response to ACh indicates impairment in endothelium-dependent relaxation because responsiveness to the nitric oxide donor SNP was statistically similar in all treatment groups (Fig. 3E and Table 1). There was a trend towards reduced maximum relaxation to SNP in male offspring fed the HS diet, although this was not statistically significant ( $P = 0.07$ ) (Table 1). In female offspring, the maximal response to KPSS did not differ between treatment and dietary groups (CNS:  $52.2 \pm 1.9$ ; HNS:  $50.2 \pm 2.3$ ; CHS:  $48.4 \pm 1.4$ ; HHS:  $51.0 \pm 1.8\%$ ). Arteries from female offspring showed similar responsiveness to phenylephrine, regardless of treatment or dietary groups (Fig. 3B and Table 1). Evidence of endothelial dysfunction was also seen in female hypoxia-exposed offspring, which had reduced maximum responses to ACh (Fig. 3D and Table 1). Sensitivity to ACh (Fig. 3D and Table 1) and responsiveness to SNP (Fig. 3F and Table 1) did not differ between groups.

### Vascular structure

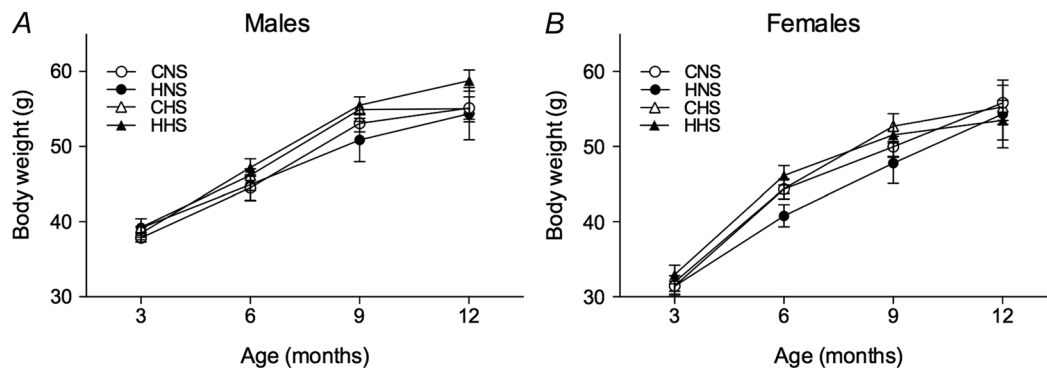
Mesenteric arteries from male offspring all had similar lumen diameters (Fig. 4A) and media:lumen ratios (Fig. 4E) across increasing intraluminal pressures. No differences in lumen diameters and media:lumen ratios were observed at 45 mmHg, which is the pressure at which the functional experiments were conducted (Table 2). Male animals fed the HS diet, had decreased medial CSA ( $P_{\text{diet}} = 0.03$ ) (Fig. 4C) compared to those on a NS diet. CSA did not differ in offspring from the hypoxia and control groups. In female offspring, neither the maternal hypoxia, nor the HS diet had an effect on lumen diameter (Fig. 4B), medial CSA (Fig. 4D) or media:lumen ratio (Fig. 4F) at any pressure (Table 2).

### Vascular mechanics

There was a leftward shift in the stress–strain curve in arteries from males fed the HS diet compared to those on the NS diet, indicating increased vessel stiffness (Fig. 5A). This leftward shift was greatest in male HHS mesenteric arteries compared to NS arteries (Fig. 5A). The rate constant of the stress–strain curve was greater in both male offspring fed a HS diet and hypoxia-exposed male offspring ( $P_{\text{trt}} = 0.03$ ,  $P_{\text{diet}} = 0.02$ ) (Table 3). By contrast, in female offspring, the combination of hypoxia and a HS diet shifted the stress–strain curve to the left, indicating increased vessel stiffness. This was accompanied by a significant increase in the rate constant of the stress–strain curve in HHS female arteries compared to other treatment groups ( $P_{\text{diet}} = 0.008$ ,  $P_{\text{trt} \times \text{diet}} = 0.03$ ) (Table 3).

### Aorta histology

Representative sections of male aortas from all treatment groups stained with Masson's trichrome to detect collagen and Verhoeff's Van Gieson to detect elastin are shown in Fig. 6. Significant collagen deposition can be observed in

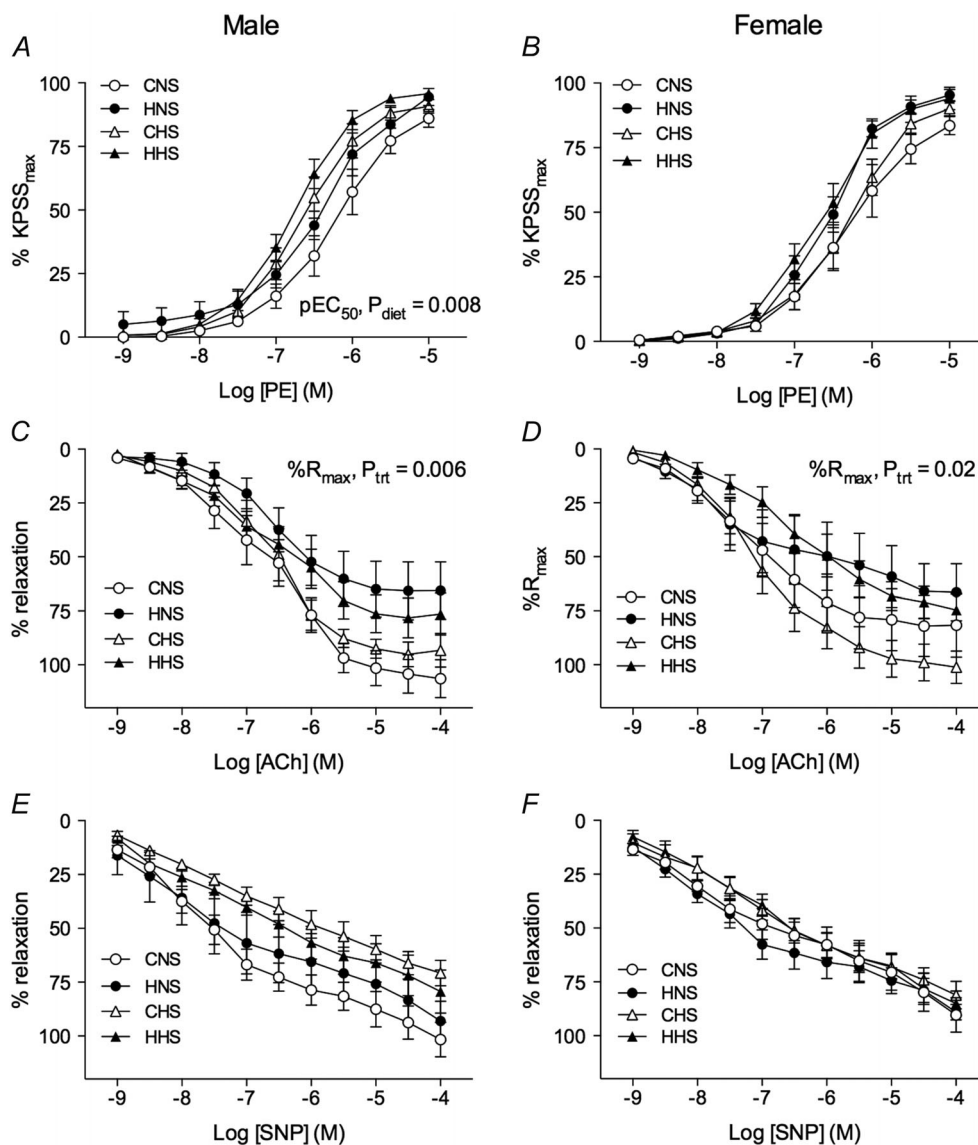


**Figure 2. Growth curves of offspring**

Growth curves of male (A) and female (B) offspring either on a NS (0.26% NaCl) or HS diet (5% NaCl) from the age of 3–12 months. Values are the mean  $\pm$  SEM (of  $n = 11$  litters per group). Control: white points; hypoxia: black points.

the media of aortas taken from control male offspring fed the HS diet. Equivalent levels of collagen deposition can be seen in aortas taken from hypoxia males fed NS and HS diets. When estimated semi-quantitatively, the aortic collagen content score was increased  $\sim 2$ -fold in male ( $P_{\text{diet}} = 0.02$ ) (Fig. 7A) and female ( $P_{\text{diet}} = 0.007$ ) (Fig. 7B) offspring fed the HS diet (Fig. 6). Male but not female hypoxia-exposed offspring fed a NS diet also showed increased aortic collagen content.

Aortas of male CNS offspring presented elastin fibres laid down as concentric layers and occupying the greatest proportion of media compared to other treatment groups (Figs 6 and 7C). Modest degeneration of elastin fibre integrity was observed in male CHS aortas (Fig. 6). Elastin content was  $\sim 30\%$  lower in aortas from male CHS aortas compared to male CNS aortas ( $P_{\text{diet}} = 0.04$ ) (Fig. 7C). Prenatal hypoxia caused marked fragmentation and disorganization of the elastin fibres (Fig. 6) and



**Figure 3. Mesenteric arteries: functional responses**

Mesenteric arteries were obtained from aged male (left) and female (right) control and hypoxia-exposed offspring fed a NS or HS diet. Functional responses to cumulative additions of (A and B) phenylephrine, (C and D) ACh and (E and F) SNP were measured in arteries pressurized to 45 mmHg. Data in A and B are expressed as a percentage of the maximum contraction to high potassium physiological salt solution (KPSS<sub>max</sub>). C–F, vessels were contracted to  $\sim 70\%$  of KPSS<sub>max</sub> with phenylephrine, and relaxation responses to the vasodilator agonists expressed as a percentage of the PE-induced contraction. Values are the mean  $\pm$  SEM ( $n = 6$ –10 per group). Control: white points; hypoxia: black points.

**Table 1. Vascular sensitivities (expressed as pEC<sub>50</sub>) and maximum responses to phenylephrine (% KPSS<sub>max</sub>), ACh (% R<sub>max</sub>) and SNP (% R<sub>max</sub>)**

		NS		HS		Two-way ANOVA		
		Control	Hypoxia	Control	Hypoxia	P <sub>trt</sub>	P <sub>diet</sub>	P <sub>trt × diet</sub>
<b>Males</b>								
PE	pEC <sub>50</sub>	-6.2 ± 0.2	-6.4 ± 0.1	-6.6 ± 0.1	-6.8 ± 0.1	0.2	0.008	0.9
	% KPSS <sub>max</sub>	101.1 ± 6.2	102.7 ± 2.7	96.1 ± 1.9	100.0 ± 2.1	0.5	0.4	0.8
ACh	pEC <sub>50</sub>	-6.6 ± 0.2	-6.7 ± 0.2	-6.6 ± 0.1	-6.6 ± 0.2	0.6	0.8	0.9
	% R <sub>max</sub>	107.3 ± 9.2	66.3 ± 13.2	95.4 ± 5.0	77.2 ± 9.5	0.006	0.96	0.3
SNP	pEC <sub>50</sub>	-7.4 ± 0.1	-6.9 ± 0.4	-6.5 ± 0.4	-6.6 ± 0.3	0.6	0.09	0.4
	% R <sub>max</sub>	89.1 ± 7.2	93.7 ± 8.5	67.2 ± 5.5	74.2 ± 8.9	0.9	0.07	0.5
<b>Females</b>								
PE	pEC <sub>50</sub>	-6.4 ± 0.2	-6.5 ± 0.1	-6.3 ± 0.1	-6.7 ± 0.1	0.07	0.7	0.5
	% KPSS <sub>max</sub>	92.9 ± 4.3	105.2 ± 5.1	94.1 ± 2.2	97.6 ± 4.5	0.06	0.4	0.3
ACh	pEC <sub>50</sub>	-7.0 ± 0.2	-6.7 ± 0.5	-7.0 ± 0.2	-6.4 ± 0.2	0.2	0.6	0.8
	% R <sub>max</sub>	89.4 ± 13.4	65.7 ± 12.4	98.5 ± 8.2	71.8 ± 6.9	0.02	0.5	0.9
SNP	pEC <sub>50</sub>	-7.5 ± 0.3	-7.8 ± 0.3	-7.7 ± 0.1	-7.7 ± 0.2	0.6	0.9	0.6
	% R <sub>max</sub>	70.0 ± 6.9	76.3 ± 4.4	66.6 ± 5.3	68.0 ± 6.1	0.5	0.3	0.7

Values are the mean ± SEM (n = 6–10 per group). The effect of treatment (trt), diet or their interaction (trt × diet) was evaluated by two-way ANOVA.

**Table 2. Structural parameters of mesenteric arteries from aged offspring when pressurized to 45 mmHg**

		NS		HS		Two-way ANOVA		
		Control	Hypoxia	Control	Hypoxia	P <sub>trt</sub>	P <sub>diet</sub>	P <sub>trt × diet</sub>
<b>Males</b>								
	Lumen diameter (μm)	212 ± 6	237 ± 18	207 ± 16	232 ± 12	0.1	0.8	0.97
	Medial CSA (μm <sup>2</sup> )	18666 ± 1042	19577 ± 1814	15962 ± 1855	16143 ± 752	0.7	0.03	0.8
	Media:lumen	21.5 ± 1.0	22.5 ± 1.0	21.8 ± 2.4	18.1 ± 1.2	0.3	0.1	0.09
<b>Females</b>								
	Lumen diameter (μm)	227 ± 14	225 ± 10	226 ± 12	210 ± 11	0.5	0.5	0.5
	Medial CSA (μm <sup>2</sup> )	16472 ± 970	15992 ± 947	18206 ± 1123	15921 ± 1648	0.3	0.5	0.5
	Media:lumen	19.0 ± 1.1	18.7 ± 1.2	21.0 ± 1.6	21.2 ± 3.6	0.98	0.4	0.9

Values are the mean ± SEM (n = 6–10 per group). The effect of treatment (trt), diet or their interaction (trt × diet) was evaluated by two-way ANOVA.

**Table 3. Rate constant, β, calculated from the exponential function fitted to stress-strain curves of mesenteric arteries from aged offspring**

		NS		HS		Two-way ANOVA		
		Control	Hypoxia	Control	Hypoxia	P <sub>trt</sub>	P <sub>diet</sub>	P <sub>trt × diet</sub>
<b>β, rate constant</b>								
Males		4.1 ± 0.3	4.8 ± 0.5	4.9 ± 0.6	6.0 ± 0.3	0.03	0.02	0.6
Females		5.4 ± 0.4	5.0 ± 0.4	5.8 ± 0.5	7.9 ± 0.7*	0.1	0.008	0.03

Values are the mean ± SEM (n = 6–10 per group). The effect of treatment (trt), diet or their interaction (trt × diet) was evaluated by two-way ANOVA. \*P < 0.01 (from Bonferroni post hoc compared to HS controls).

reduced elastin content in male (P<sub>trt</sub> = 0.03) (Fig. 7C) and female offspring (P<sub>trt</sub> = 0.03) (Fig. 7D). Degeneration of the elastin fibres was most pronounced in aortas of the HHS male, where disorganization and discontinuity of the elastin fibre segments can clearly be observed in

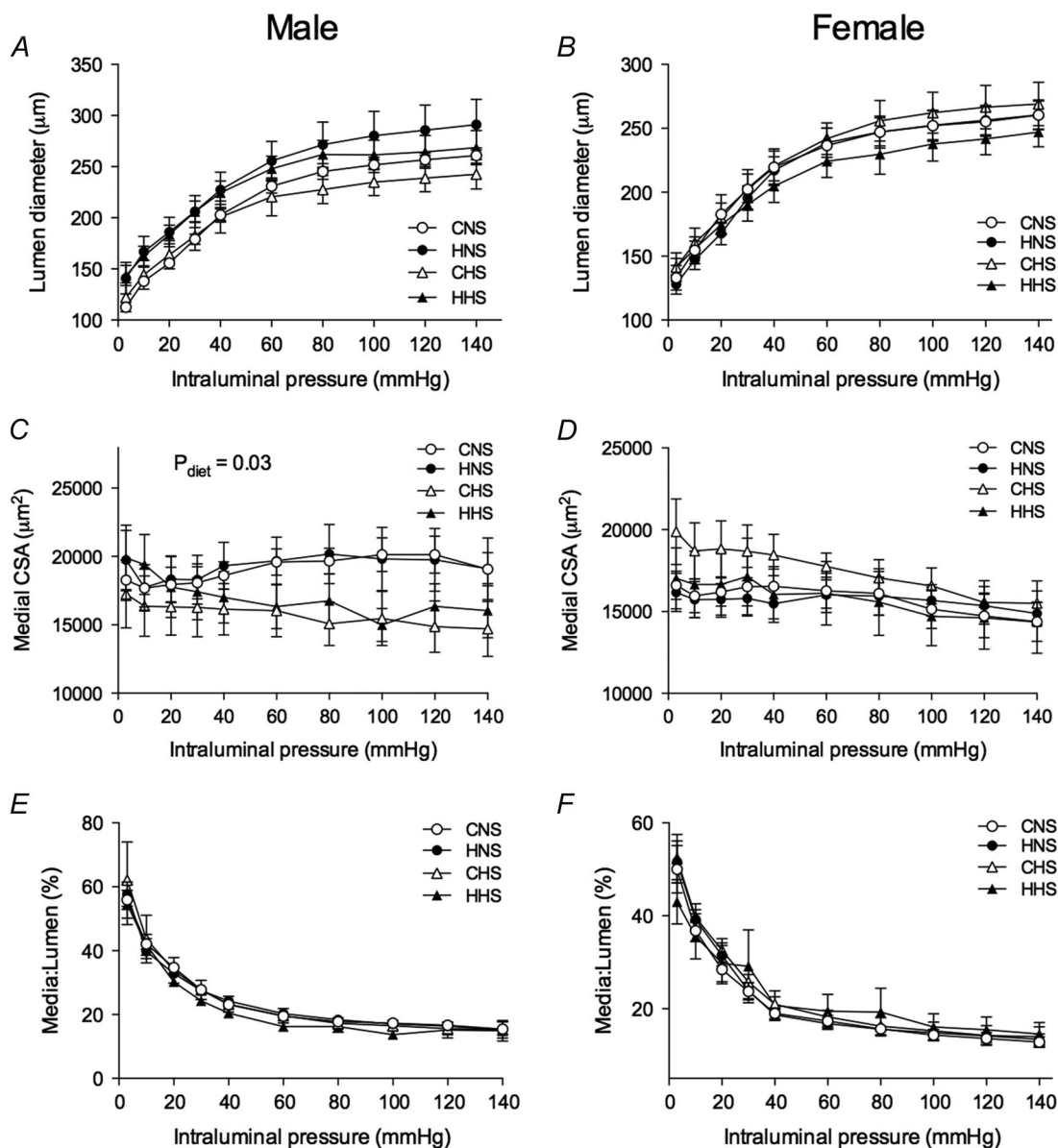
Fig. 6. Elastin content in aortas of male HHS offspring was ~42% lower compared to elastin content in aortas of CHS offspring (Fig. 7C). The HS diet reduced aortic elastin significantly in both female control and hypoxia-exposed offspring (P<sub>diet</sub> < 0.0001) (Fig. 7D).

## Discussion

The present study examined the effects of maternal hypoxia during late gestation, and determined how this gestational insult interacted with the postnatal consumption of a chronic HS diet to affect vascular function, structure and mechanics in adult offspring. We show for the first time that, although prenatal hypoxia exposure alone caused mild endothelial dysfunction in mesenteric vessels, the combined insult of prenatal hypoxia and a chronic postnatal HS diet caused significant stiffening of

the mesenteric vasculature and altered extracellular matrix composition in the aorta; most notably a loss of elastin fibre integrity. Our results suggest that, although prenatal hypoxia may be unavoidable, consuming a healthy diet, particularly with respect to sodium intake, may prevent or at least limit adverse vascular outcomes in adulthood.

During early life, suboptimal influences such as maternal low protein diet (Brawley *et al.* 2003), maternal high fat diet (Khan *et al.* 2004) and vitamin D insufficiency (Tare *et al.* 2011) can program vascular dysfunction.



**Figure 4. Structural properties of mesenteric arteries**

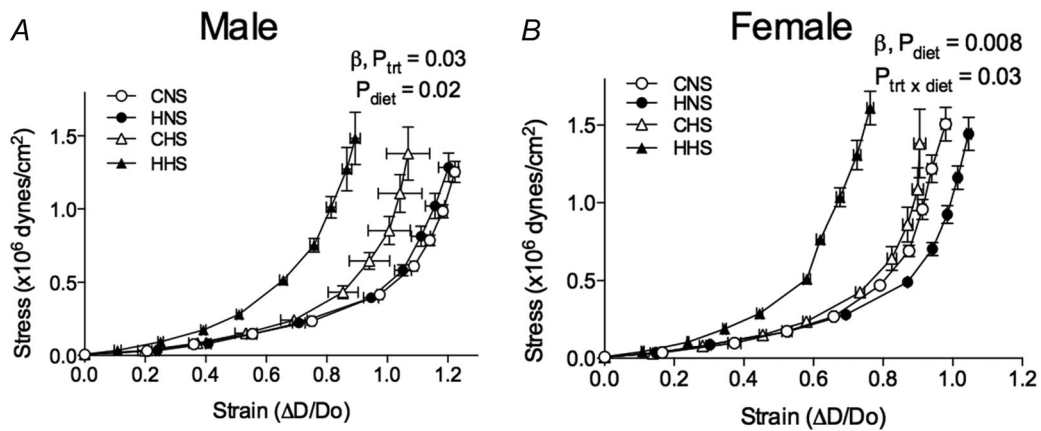
Arteries were obtained from aged male (left) and female (right) control and hypoxia-exposed offspring fed a NS or HS diet. Lumen diameter in male (A) and female (B) offspring, medial CSA in male (C) and female (D) offspring, as well as media:lumen ratio in male (E) and female (F) offspring in response to increasing intraluminal pressure in deactivated mesenteric arteries. Values are the mean  $\pm$  SEM ( $n = 6-10$  per group). Control: white points; hypoxia: black points.



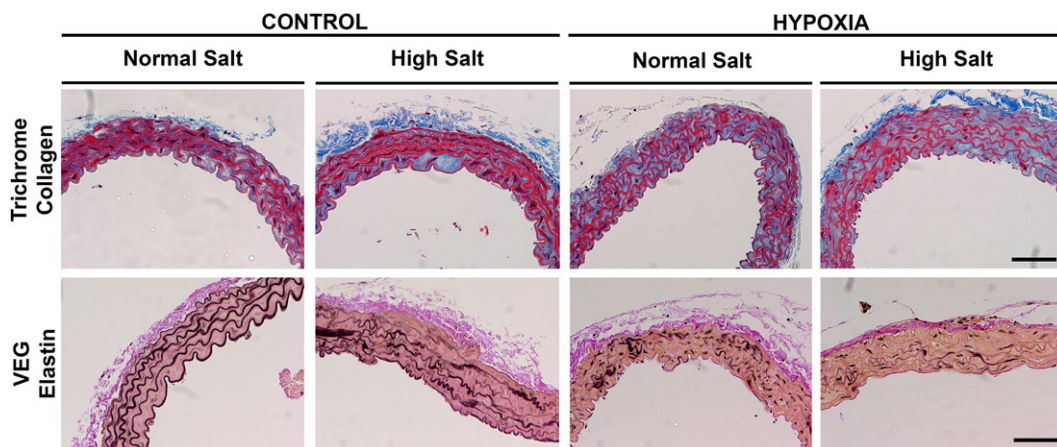
In particular, endothelial dysfunction as a result of impaired responsiveness to endothelium-dependent vasodilators such as ACh is commonly reported in models of developmental programming (McMillen & Robinson, 2005). In our model of prenatal hypoxia, there was reduced ACh-induced vasodilatation in the offspring of both sexes, although there were no differences in vasodilator responses to the NO donor SNP. This suggests that the impaired vascular response to ACh in hypoxia-exposed offspring is a result of impaired endothelial function rather than decreased vascular smooth muscle sensitivity to NO-induced relaxation. This is consistent with previous reports using a model of prenatal hypoxia in the rat showing impaired endothelial function in the resistance vasculature of hypoxia-exposed adult offspring (Hemmings *et al.* 2005; Williams *et al.* 2005b; Morton *et al.* 2011). Notably, Morton *et al.* (2011) used Sprague–Dawley

rats at 12 months (the same age used in the present study) to show that prenatal hypoxia impaired flow-mediated vasodilatation in both sexes. Although, in the present study, we did not directly determine the mechanisms underlying this endothelial dysfunction, it may be a result of reduced NO bioavailability associated with increased superoxide anion production, as previously reported by Williams *et al.* (2005b). Increased oxidative stress in the fetal rat vasculature has been associated with endothelial dysfunction (Giussani *et al.* 2012), suggesting that hypoxia-induced oxidative stress during fetal development can have adverse cardiovascular outcomes in later life.

In both the human population and animal models, endothelial dysfunction is associated with low birth weight (Leeson *et al.* 2001). Our model of chronic hypoxia in the mouse led to low birth weight, and resulted in endothelial dysfunction in aged offspring. Importantly, our model of



**Figure 5. Mechanical properties of mesenteric arteries**  
Arteries were obtained from aged male (left) and female (right) control and hypoxia-exposed offspring fed a NS or HS diet. Stress–strain relationship in vessels of male (A) and female (B) offspring. Values are the mean ± SEM (n = 6–10 per group). Control: white points; hypoxia: black points.

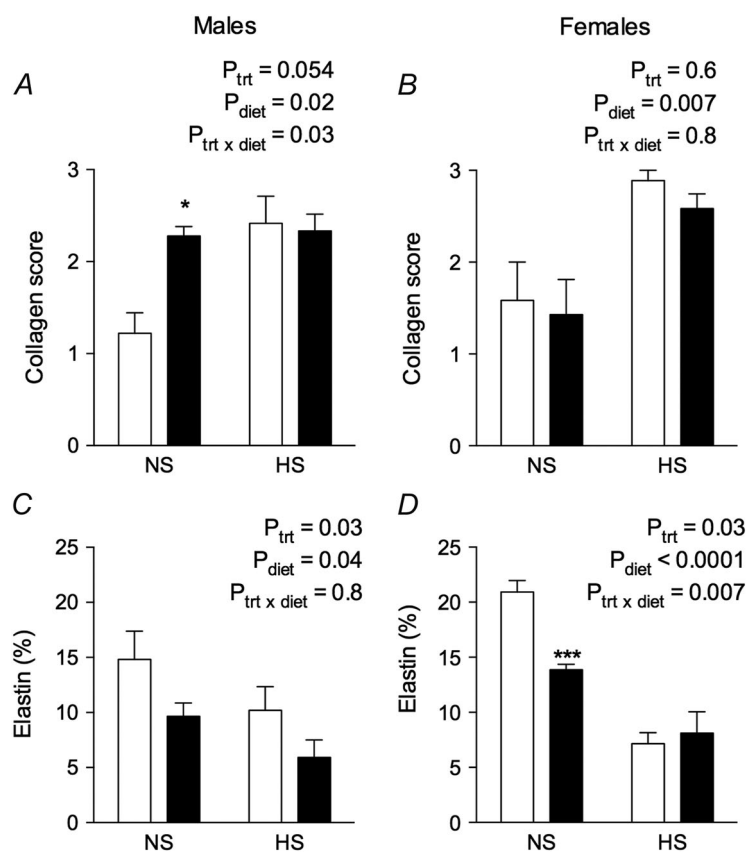


**Figure 6. Microphotographs of aortic sections**  
Sections were stained with Masson’s trichrome to detect collagen (blue) and Verhoeff’s Van Gieson to detect elastin (black). Aortas were obtained from aged male hypoxia-exposed offspring fed a NS or HS diet. Scale bar = 50 μm.

gestational hypoxia using the mouse is not confounded by maternal undernutrition (Cuffe *et al.* 2014a). Similar models of maternal hypoxia during late gestation in the rat have reported a significant reduction in maternal food intake during the time spent inside the hypoxia chamber (Williams *et al.* 2005b; Camm *et al.* 2010). Under-nourishment during pregnancy alone has been shown to alter vascular function (Brawley *et al.* 2003; Torrens *et al.* 2003). Therefore, the combination of reduction in maternal oxygen supply and reduced food consumption provides a dual insult to the developing fetus. Similarly, the vascular dysfunction seen in rat models of uteroplacental insufficiency may be attributable to a reduction in both oxygen and nutrient supply to the fetus (Tare *et al.* 2012). Our findings suggest that fetal hypoxia alone is able to result in vascular dysfunction in offspring. Growth restriction was sustained in our model during lactation but substantial catch-up growth was observed post-weaning, both of which are predictors of adult-onset cardiovascular dysfunction. Reduced body weight pre-weaning may signify an impaired lactational environment, which has previously been reported in a model of uteroplacental insufficiency in the rat (O'Dowd *et al.* 2008). However, in our model, absolute growth during lactation was similar in the control and prenatal hypoxia groups. Sensitivity to vasodilators and resistance artery stiffness was improved when these growth-restricted offspring

were cross-fostered onto healthy mothers to improve the lactational environment (Tare *et al.* 2012). These studies highlight the importance of a healthy diet throughout lactation for growth-restricted offspring.

Vulnerability to CVD programmed *in utero* may be exacerbated by a postnatal 'second-hit', such as a HS (Ruta *et al.* 2010) or high fat diet (Rueda-Clausen *et al.* 2012). The chronic HS diet produced modest alterations to microvascular function in aged control and hypoxia-exposed male (but not female) offspring. To our knowledge, the present study is the first to examine the impact of a chronic HS diet in the CD-1 mouse strain and, importantly, to examine these effects in both sexes. Our findings therefore warrant further investigation. Dietary salt loading to assess microvascular function has overwhelmingly been performed in the rat (Liu *et al.* 1999; Sofola *et al.* 2002). Sprague–Dawley rats fed a HS diet have increased small artery reactivity to vasoconstrictors but no overall impairment in endothelial function. However, the mechanism of ACh-induced dilatation was altered (Sofola *et al.* 2002). Reduced endothelium-dependent responses associated with HS intake have been reported in skeletal muscle arterioles (Boegehold, 1995), cerebral arterioles and small feed arteries of rat gracilis muscle (Liu *et al.* 1997). However, many of these vascular function studies were performed following exposure to extremely high levels of sodium (7–10% NaCl) in the diet over an



**Figure 7. Semi-quantification of aortic histology**

Collagen scores in aorta sections stained with Masson's trichrome to detect collagen in male (A) and female (B) control and hypoxia exposed offspring fed a NS or HS diet. Elastin (%) in aorta sections stained with Verhoeff's Van Gieson in male (C) and female (D) offspring. Values are the mean  $\pm$  SEM ( $n = 6-8$  per group). \* $P < 0.01$ , \*\*\* $P < 0.0001$  (from Bonferroni *post hoc* compared to control offspring fed the NS diet).

acute period, which has limited relevance to the human population. In the present study, we have employed a more modest increase in dietary sodium (from 0.26% to 5%) across the adult lifespan of the mouse, which makes our studies of greater relevance. However, we cannot discount the possibility that more overt vascular deficits may be observed with a further increase in dietary sodium.

Vascular stiffening is a major risk factor for cardiovascular dysfunction. Increased vascular stiffness is not only associated with a low birth weight in humans, but also increases with age (Martin *et al.* 2000; te Velde *et al.* 2004) and other cardiovascular risk factors such as hypertension, diabetes and tobacco smoking (Benetos *et al.* 2002). A striking finding to emerge from the present study was that vascular stiffness was markedly exacerbated by the combination of prenatal hypoxia and a postnatal HS diet. Importantly, these changes in the stress–strain relationship of the microvasculature were geometry independent because no changes were observed in media:lumen ratios. Prenatal hypoxia alone had a limited effect on vascular stiffness, which is consistent with observations in aged rats exposed to hypoxia during late gestation (Morton *et al.* 2011). Given that vascular stiffening occurred in the absence of geometric changes, we examined the deposition and organization of aortic extracellular matrix proteins, particularly collagen and elastin, to determine the mechanical properties of vessel walls. We found reduced elastin content and significant disorganization and fragmentation of the elastin layers in hypoxia-exposed offspring. The synthesis of elastin fibres responsible for vessel elasticity is restricted to fetal and early postnatal life and is therefore vulnerable to influences early in life such as chronic hypoxia (Davis, 1995). However, during the postnatal period, elastin fibres are sensitive to degradation by matrix metalloproteinases and elastases produced by inflammatory cells during the pathogenesis of vascular lesions such as aneurysms and arteriosclerosis (Galis & Khatri, 2002). Although the HS diet reduced elastin content in both treatment groups, the effect was significantly greater in hypoxia-exposed offspring, particularly males. The significant disorganization and loss of elastin fibres may be the result of a prenatal impairment in elastin synthesis and enhanced postnatal degradation of fibres, consistent with vascular injury. Substantial collagen accumulation was observed in hypoxia-exposed male offspring, although it was most prominent in offspring fed the HS diet irrespective of prenatal treatment. Dietary salt intake is a known contributor to fibrosis in the kidney, heart and vasculature (Yu *et al.* 2004), which in turn leads to vascular stiffening and increased cardiovascular risk. We observed slight differences between males and females in the histological changes in response to prenatal hypoxia and a postnatal HS

diet. Most notably, hypoxia alone did not increase aortic collagen deposition in females, although it did in male offspring. Because the female hormonal milieu contains oestrogen, a hormone with vasoprotective effects, female offspring may be protected against a mild prenatal insult such as hypoxia (Mendelsohn & Karas, 1999). The postnatal HS diet increased aortic collagen deposition in both sexes, suggesting that the protective effects of oestrogen on the vasculature may be diminished in the ageing female mouse. This is observed in the human population where rates of CVD are higher in postmenopausal women than premenopausal women, associated with the declining plasma oestrogen levels with age (Khalil, 2010). Because the loss of elastin fibre integrity and increased media collagen content were observed in a compliance vessel, we cannot discount the possibility that the pathophysiology could present differently in the resistance vasculature. However, these changes are indicative of vascular stiffening and are consistent with the reduced vascular compliance observed in the microvasculature of hypoxia-exposed offspring fed the HS diet.

In conclusion, we report for the first time that vascular stiffening in aged offspring exposed to gestational hypoxia is markedly exacerbated by a postnatal HS diet. The changes in aortic extracellular matrix composition probably underlie the increase in vascular stiffness in hypoxia-exposed offspring fed the chronic HS diet. Prenatal hypoxia alone impaired endothelial function in adult male and female mice, demonstrating that the endothelium, a vital regulator of vascular tone, is susceptible to adverse environmental conditions *in utero*. Because high dietary salt intake is endemic in Western societies, such adverse changes in vascular function and wall stiffness may be inadvertently enhanced by suboptimal dietary choices. This may have serious consequences for individuals predisposed to CVD because of their *in utero* environments. It is advised that individuals born with a low birth weight take precautions to ensure that dietary sodium intake in adult life is not excessive so as to limit adverse cardiovascular outcomes.

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## Additional information

### Competing interests

The authors declare that they have no competing interests.

### Author contributions

SLW, RRS, TMP and KMM were responsible for conception and design of the experiments. SLW and TT were responsible for the collection, analysis and interpretation of data. All authors were involved in drafting the article and revising it critically for intellectual content. All authors approved the final version of the manuscript submitted for publication.

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