Effect of Sildenafil citrate treatment in the eNOS knockout mouse model of fetal growth restriction on long-term cardiometabolic outcomes in male offspring

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Abstract

Fetal growth restriction (FGR) is associated with an increased risk of hypertension, insulin resistance, obesity and cardiovascular disease in adulthood. Currently there are no effective treatments to reverse the course of FGR. This study used the eNOS knockout mouse (eNOS$^{-/-}$), a model of FGR, to determine the ability of sildenafil, a potential new treatment for FGR, to improve cardiovascular and metabolic outcomes in adult offspring following a complicated pregnancy.

Pregnant eNOS$^{-/-}$ and C57BL/6J control dams were randomised to sildenafil treatment (0.2 mg/ml in drinking water) or placebo at day 12.5 of gestation until birth. After weaning, male offspring were randomised to either a high fat (HFD; 45% kcal from fat) or normal chow diet (ND), and raised to either postnatal day 90 or 150. Growth and body composition, glucose tolerance, insulin resistance, systolic blood pressure and vascular function were analysed at both time-points.

eNOS$^{-/-}$ offspring were significantly smaller than their C57BL/6J controls at weaning and P90 ($p<0.01$); at P150 they were a similar weight. Total adipose tissue deposition at P90 was significantly increased only in eNOS$^{-/-}$ mice fed a HFD ($p<0.001$). At P150 both C57BL/6J and eNOS$^{-/-}$ offspring fed a HFD demonstrated significant adipose tissue deposition ($p<0.01$), regardless of maternal treatment.

Both diet and maternal sildenafil treatment had a significant effect on glucose tolerance. Glucose tolerance was significantly impaired in eNOS$^{-/-}$ mice fed a HFD ($p<0.01$); this was significant in offspring from both sildenafil and vehicle treated mothers at P90 and P150. Glucose tolerance was also impaired in C57BL/6J mice fed a HFD at both P90 and P150 ($p<0.01$), but only in those also exposed to sildenafil. In these C57BL/6J mice, sildenafil was associated with impaired insulin sensitivity at P90 ($p=0.020$) but increased insulin resistance at P150 ($p=0.019$).

Exposure to sildenafil was associated with a significant increase in systolic blood pressure in eNOS$^{-/-}$ mice compared with their C57BL/6J diet controls at P150 ($p<0.05$). Exposure to sildenafil had differing effects on vascular function in mesenteric arteries; it increased vasodilation in response to ACh in C57BL/6J mice, but was associated with a more constrictive phenotype in eNOS$^{-/-}$ mice.

eNOS$^{-/-}$ mice demonstrate a number of impaired outcomes consistent with programmed cardiometabolic disease, particularly when faced with the 'second hit' of a HFD. Exposure to sildenafil treatment during pregnancy did not increase fetal growth or significantly improve adult metabolic or cardiac outcomes. Maternal sildenafil treatment was, however, associated with small impairments in glucose handling and an increase in blood pressure. This study highlights the importance of understanding the long-term effects of treatment during pregnancy in offspring from both complicated and healthy control pregnancies.

Key words: Fetal growth restriction, mouse, sildenafil citrate, DOHaD, developmental programming

Chemical compounds studied in this article Sildenafil citrate (PubChem CID: 62853); Phenylephrine hydrochloride (PubChem CID: 5284443); Acetylcholine chloride (PubChem CID 6060); Sodium nitroprusside (PubChem CID: 11953895).

1. Introduction

Fetal growth restriction (FGR), defined as a fetus which fails to achieve its genetic growth potential, is a serious complication of pregnancy associated with significant risk of perinatal morbidity and mortality [1,2]. Of equal concern is the growing body of evidence demonstrating the association between growth restriction and the development of obesity and cardio-metabolic diseases, such as diabetes and hypertension, later in life [3, 4]. Currently, there are no curative treatments for this condition; typically, the pregnancies are closely monitored, with early delivery indicated when there is significant risk of fetal demise. Recently, a number of agents have been identified as potential treatments, including sildenafil citrate [5]. Pre-clinical studies have demonstrated the ability of sildenafil to improve fetal growth in a number of models, and have supported the development of a series of multicentre, randomised controlled trials (e.g. STRIDER) [6]. Although the beneficial effects of sildenafil on fetal growth have been demonstrated in animal models [7-9], little is known regarding its ability to mitigate the long-term health risks associated with FGR. There is strong evidence demonstrating the effect of the intrauterine environment on fetal development and subsequent risk of adult disease [10-12], illustrating that the pre-natal period is one of remarkable plasticity. It follows, therefore, that pregnancy may also provide a unique window for intervention and a reduction in the risk of developing adult diseases.

The aim of this study was to utilise a mouse model of FGR to determine the effect of sildenafil treatment, during pregnancy, on cardio-metabolic outcomes in adult offspring. The model used in this study is the endothelial nitric oxide synthase knockout ($eNOS^{-/-}$) mouse. The $eNOS^{-/-}$ mouse shares several key features with human FGR, including the consistent production of growth restricted offspring [13-15]. As with many clinical cases of FGR,

impaired vascular adaptations to pregnancy are associated with this model, including impaired uterine artery remodelling, resulting in reduced uterine artery blood flow [16], and an impaired response to endothelium-dependent vasodilators. A placental deficit is also present, with reduced System A (i.e. amino acid) transport being observed [14], suggesting that the FGR seen in this model is likely due to a combination of impaired vascular and nutrient transport mechanisms.

As previously highlighted, sildenafil citrate is a promising potential therapy for FGR, as it is a potent enhancer of vasodilation and is proven to be safe for use during pregnancy [5]. Sildenafil functions by inhibiting the breakdown of the NO second messenger cGMP, increasing the bioavailability of NO. Recent animal studies have also suggested that sildenafil may mediate its effects on fetal growth at least partly via the placenta [7, 8]. It is possible, therefore, that sildenafil treatment of $eNOS^{-/-}$ mice during pregnancy may increase fetal growth via improved vascular relaxation and/or placental function. It should be highlighted that the $eNOS^{-/-}$ mouse, although lacking the endothelial NOS isoform, does possess both inducible and neuronal forms of NOS enzymes (iNOS and nNOS) which enable production of NO in this model.

We hypothesised that sildenafil citrate treatment during pregnancy would increase fetal growth in the $eNOS^{-/-}$ model. We further hypothesised that this increase in fetal growth would be associated with improved cardiac and metabolic outcomes in adult offspring.

2. Methods

2.1 Animal Care

Animal experiments were approved by the Animal Ethics Committee of the University of Auckland (Ethics approval R1097). Female eNOS deficient mice (strain B6.129P2-Nos3tm1Unc/J) and C57BL/6J control mice were obtained from Jackson Laboratories (Bar Harbour, ME). Animals had access to food and water *ad libitum*. Lighting conditions were 12:12hr light-dark cycle and temperature maintained at 20-22°C. Virgin females, of 8 to 12 weeks of age were used and only first pregnancies were studied.

An outline of the experimental protocol is provided in Figure 1. Mice were bred with age and genotype matched males, with the presence of a copulation plug denoting day 0.5 of pregnancy. At postnatal day 2, the litter size of both C57BL/6J and $eNOS^{-/-}$ mice was reduced to n=6 if necessary to ensure adequate and standardised supply of milk to all pups. Male offspring only were weaned at postnatal day 21 and randomised onto either a high fat diet (HFD, 45% kcal from fat; D12451, Research Diets Inc., NJ, USA) or normal chow diet (ND). Offspring from each litter were raised to 90 days and 150 days of age in cages of no more than 3 animals. Cages containing wheat kernel bedding and a small amount of paper nesting material were changed weekly.

2.2 Sildenafil treatment

Pregnant females were randomised onto sildenafil citrate treatment in drinking water (0.2mg/mL), or vehicle drinking water at gestational day 12.5 until being allowed to give birth naturally at day 19.5. The timing of intervention during pregnancy was determined by a number of factors. At day 12.5, placental development is largely complete, the utero-placental circulation is fully open and fetal growth is primarily driven by nutrients delivered via the utero-placental circulation. It was therefore hypothesised that any beneficial effects of sildenafil on fetal growth, either via increased vasodilation or direct placental effects, would

be observed from this time point, whilst minimising potential teratogenic effects. The dose administered (~1mg/day) is roughly equivalent to a dose of 100 mg/day in a 70 kg human after making the appropriate adjustments for altered pharmacokinetics in mice. Further, we have demonstrated the ability to increase fetal growth in other mouse models of FGR using this concentration of drug and dosing regime [10].

2.3 Body weight and Food intake

Following weaning at P21, weights were taken weekly to assess the growth trajectory of the offspring. Food consumption was measured weekly and the average consumption per mouse was analysed in kcals consumed.

2.4 Oral Glucose Tolerance Test

Glucose tolerance was assessed via an oral glucose tolerance test (OGTT). Mice were fasted for 6 hours prior to commencing the test. Blood obtained from a tail snip was taken at baseline, and blood glucose concentration measured using a glucometer (Freestyle Optium, Abbott). Glucose solution (0.2g/ml) was dosed via oral gavage (2mg glucose per g body weight) immediately after (t=0). Blood samples were then obtained from the tail snip at 30 minute intervals thereafter until 2 hours post baseline (30, 60, 90 and 120 mins) and blood glucose concentration measured using a glucometer.

2.5 Systolic Blood Pressure

Blood pressure was measured one week before culling using tail cuff plethysmography with no prior training. Mice were placed in a warming cupboard at 28°C for an hour before being persuaded into a restraining tube with an inflatable tail cuff, which connected to a pressure and recording unit (Model 179 with an automatic cuff inflation pump (NW20), IITC Life Science, Woodland Hills, CA). The restraining tube was placed into an enclosed area which was warmed to 30-32°C. The mice were allowed 5 minutes to adjust to the surroundings

before readings were taken at 5 minute intervals until 3 successful readings were taken. The tail cuff was inflated to 180mmHg, and subsequently released at ~5mmHg/sec. Readings were undertaken in a room separate from housing and at the same time of day for all animals.

2.6 Tissue Collection

Mice were fasted for 6 hours prior to culling via cervical dislocation. A terminal blood sample was obtained via cardiac puncture and plasma samples stored. Blood glucose concentration was measured immediately using a glucometer. The following tissues were systematically collected: pancreas, spleen, mesentery, kidneys, adipose tissue (gonadal, perirenal, and retroperitoneal fat depots), liver (large lobe), skeletal muscle, and tail snip. The entire mesentery was collected and immediately placed in ice cold physiological saline solution (PSS mmol/L; 10 HEPES, 142 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.6 $CaCl_2$, 1.18 KH_2PO_4, 5.5 Glucose, 0.034 EDTA; pH7.4 at 37°C) in preparation for wire myography. All other tissues were placed on ice until weighed.

% body fat was calculated as the weight of the three adipose depots (gonadal, perirenal and retroperitoneal), divided by body weight then multiplied by 100. Lean weight was calculated as body weight minus the weight of the three adipose depots.

2.7 Insulin concentration via ELISA

Plasma insulin concentration was assessed in samples collected at either P90 or P150, using a commercial mouse insulin kit (Ultra sensitive mouse Insulin ELISA kit, Chrystal Chem, IL, USA).

188 **2.8 Myography**

189 Vascular function was assessed using a DMT wire myography (610m, Danish Myotech,
190 Aarhus, Denmark) and interpreted using LabChart software (ADInstruments, v8.1.2,
191 Dunedin, New Zealand). Second order mesenteric arteries were dissected from the
192 surrounding adipose and connective tissues and mounted on 25µm tungsten wire
193 (ADInstruments, Dunedin, New Zealand). The vessels were kept at 37°C in 5 mL of PSS and
194 at pH 7.4, bubbled constantly with air.

195 Once mounted, the vessels were set at zero (where there is no resistance detected on the
196 myograph) followed by normalisation to a luminal pressure of 13.3kPa as determined by
197 LabChart software, to establish *in vivo* conditions which corresponds an internal
198 circumference equal to a transmural pressure of 90 mmHg. After normalisation the integrity
199 of the vessels was assessed; criteria for vessels to be included in further analysis were
200 constriction of >2mN to phenylephrine (Pe) 10^{-5} M, followed by a notable decrease in force
201 in response to addition of acetylcholine (ACh) 10^{-5} M.

202 Vessel function was then assessed by the construction of dose response curves to
203 vasoconstrictor and vasodilator agents. For all dose response curves, the vasoactive agents
204 were added in increasing doses at logarithmic steps to produce a final bath concentration
205 ranging from 10^{-10} to 10^{-5} M. For the U46619 and ACh dose response curves, however,
206 smaller incremental additions were made between 10^{-8} and 10^{-5} M to correspond with 0.5 log
207 steps, to increase the sensitivity of response information.

208 The Pe constriction curve was initiated with 10^{-10} followed by 10^{-9}, 10^{-8}, 3x 10^{-8}, 10^{-7}, 3x 10^{-7},
209 10^{-6}, 3x 10^{-6}, and finally 10^{-5} M, before washing the vessels in twice with PSS and letting
210 them rest for 20 mins. Each subsequent dose was added once the constriction response had
211 plateaued or after 2mins if there was no response. The concentration at which the vessels

reached 80% of their maximum constriction was noted (EC_{80}) and was the concentration used to achieve pre-constriction prior to the construction of relaxation curves.

After completion of the vasoconstriction curve, vessels were washed twice with PSS and allowed to rest for 20 mins, before being constricted using their EC_{80} concentration and allowed to plateau before beginning the ACh relaxation curve. The same final bath concentrations of ACh were used as in the constriction curve. However, each dose was added at 2 minute intervals regardless of activity; at completion the baths were washed and the vessels rested for 20 mins.

Sodium Nitroprusside (SNP), a vasodilator that acts by anabolising to NO without any receptor interaction and used to assess endothelium-independent vasodilation, was also used. The SNP curve was constructed by the addition of 10^{-10} to 10^{-5} M in 6 steps at 2 min intervals as per the ACh curve. At the completion of the SNP curve, the PSS in the bath was replaced by KPSS, a PSS containing a High K^+ concentration solution.

2.9 Homeostatic model assessment index (HOMA)

As described [17], HOMA is a method used to quantify insulin resistance and estimate pancreatic beta cell function. Fasting glucose concentration (mmol/L) taken from a post mortem cardiac puncture sample, together with plasma insulin concentration (mU/L), as determined via ELISA measurement, were used to calculate this measure.

$$HOMA\ IR = \frac{(Glucose\ \times Insulin)}{22.5}$$

2.10 Statistical analyses

All data are presented as mean ± SEM, with degrees of freedom (F), unless stated otherwise. All data are normally distributed as assessed by Shapiro Wilk's test of normality ($p<0.05$), unless otherwise stated. Data were analysed using a three-way analysis of variance (ANOVA), with genotype, postnatal diet and exposure to sildenafil *in utero* as independent

variables. The relationship between fat mass, fasting glucose and systolic blood pressure was explored using linear regression analysis. A value of $p \leq 0.05$ was considered significant for three-way ANOVA and linear regression, while a value of $p \leq 0.025$ was considered significant for two- and one-way ANOVA.

Graphpad Prism 6 (Graphpad Software Inc., La Jolla, CA) software was used for all graphing purposes. All statistical analyses were undertaken using SPSS Statistics (IBM Corp. 2013, IBM SPSS Statistics for Windows, Version 22.0., Armonk, NY).

3. Results

All data are presented as averages over a litter if applicable. Litter size for analyses ranges from n=1-3 individual mice, with a total of 4-7 litters studied per group. Postnatal diet is denoted as ND (Normal chow diet) or HFD (High fat diet, 45kcal/g derived from fat), followed by a + or – symbol representing sildenafil treatment presence or absence. E.g. $eNOS^{-/-}$ ND+ refers to eNOS knockout mice on a normal chow diet, exposed to sildenafil citrate treatment *in utero*. P21, P90 and P150 denote postnatal age in days.

3.1. Litter size, body weight and food intake

There was a significant effect of genotype ($p<0.0001$), but not treatment ($p=0.606$), on litter size at P2, with $eNOS^{-/-}$ mice having significantly smaller litters than C57BL/6J controls in both the vehicle (5.18 ± 0.42 vs. 8 ± 0.58 pups) and sildenafil treated groups (4.57 ± 0.56 vs. 8 ± 0.52 pups).

Body weight and energy intake curves after weaning are shown in Figure 2. A three-way ANOVA analysing strain, diet, and treatment was conducted for P90 and P150 time-points. $eNOS^{-/-}$ mice were significantly lighter in body weight than C57BL/6J offspring at P21 ($p<0.05$; Figure 2A). A significant effect of genotype remained at P90 ($p=0.008$), with $eNOS^{-/-}$ offspring lighter in weight. The introduction of a HFD to offspring resulted in increased body weight regardless of strain or treatment ($p=0.001$). By P150, HFD offspring remained heavier than ND offspring ($p<0.001$), but there was no longer a difference between $eNOS^{-/-}$ or C57BL/6J offspring overall, suggesting by this time $eNOS^{-/-}$ offspring had “caught up” in growth. There was no effect of sildenafil treatment on offspring weight at any time.

3.2. Adipose tissue deposition

There was a significant two-way interaction between strain and diet in adipose tissue deposition at P90 (Figure 3A). This was observed in both vehicle ($F(1,26)=9.33$, $p=0.005$) and sildenafil treated offspring ($F(1,23)=8.65$, $p=0.007$). HFD increased adipose deposition in eNOS$^{-/-}$ offspring, but had no effect in C57BL/6J offspring. There was no effect of strain, diet or treatment on lean weight (Figure 3B). At P150, the interaction between strain and diet was no longer present, with only a main effect of diet, in which HFD was associated with increased adipose tissue deposition ($p<0.001$; Figure 3C). Sildenafil had no effect on adiposity at either time point. There was no effect of strain, diet or treatment on lean weight (Figure 3D).

3.3. Glucose tolerance

3.3.1. OGTT at P84

Both genotype (eNOS$^{-/-}$) and a HFD were associated with increased fasting blood glucose concentration, but only in vehicle-exposed offspring i.e. it was only observed in eNOS$^{-/-}$ HFD- mice. HFD was also associated with increased blood glucose concentration at 90 and 120 minutes in eNOS$^{-/-}$ but not C57BL/6J offspring. Sildenafil had an effect in C57BL/6J mice on HFD only, significantly lowering blood glucose concentrations at 60, 90, and 120 minutes (Figure 4A, B).

When glucose tolerance was expressed as area under the OGTT curve (AUC), there was a significant three-way interaction between treatment, strain and diet. HFD was associated with increased AUC in both sets of eNOS$^{-/-}$ offspring, as well as C57BL/6J offspring exposed to sildenafil (Figure 4C).

3.3.2. OGTT at P144

As with P84 animals, eNOS$^{-/-}$ was associated with increased fasting blood glucose in HFD-fed vehicle-exposed offspring only. HFD increased glucose concentrations at 0, 60, 90 and 120 minutes in eNOS$^{-/-}$ vehicle-treated offspring. This effect of HFD was also present in eNOS$^{-/-}$ offspring exposed to sildenafil, but only at the 90 minute time point. HFD also significantly increased blood glucose at all time points in C57BL/6J offspring, although this was only significant in those exposed to sildenafil in utero (Figure 5A, B).

There was a significant interaction between treatment, strain and diet on AUC at P144. HFD was again associated with an increased AUC in C57BL/6J sildenafil-treated mice as well as both sets of eNOS$^{-/-}$ offspring. There was no effect of strain or sildenafil on AUC of the OGTT (Figure 5C).

3.4. Plasma insulin and HOMA-IR

Plasma insulin at P90 was not affected by strain, diet, or treatment. There was a three-way interaction in HOMA-IR. Sildenafil was associated with reduced insulin resistance in C57BL/6J offspring fed HFD ($F(1,4)=14.11$, $^{*}p=0.020$) . The same was not observed in eNOS$^{-/-}$ offspring.

Plasma insulin at P150 was not affected by strain, diet, or treatment. There was a three-way interaction in HOMA-IR ($F(1,11)=9.10$, $p=0.012$). In contrast to the results observed at P90, sildenafil was associated with increased insulin resistance in C57BL/6J offspring fed HFD (C57BL/6J: HFD- vs HFD+: 7.66 ± 1.35 vs 21.46 ± 0.06, $p=0.019$). eNOS$^{-/-}$ was also associated with reduced insulin resistance compared to C57BL/6J in those offspring fed ND (ND-: C57BL/6J vs eNOS-/-; 5.66 ± 0.27 vs 2.53 ± 0.07; $p=0.008$).

3.5. Systolic blood pressure

There was no effect of strain, diet, or treatment on systolic blood pressure at P83 (Figure 6A). There was a two-way interaction between strain and treatment at P143 (Figure 6B). Of those mice exposed to sildenafil, blood pressure was elevated in $eNOS^{-/-}$ offspring compared to C57BL/6J offspring. There was no independent effect of exposure to sildenafil on offspring blood pressure.

3.6. Linear regression analysis of fat mass, body weight and blood pressure

Linear regression analysis was carried out to determine any relationship between fat mass, fasting plasma glucose concentration and systolic blood pressure at P90 and P150.

3.6.1 P90

At P90, a significant correlation was observed between fat mass and fasting plasma glucose concentration.

Regression coefficients:

Fat mass vs. fasting glucose – $R=0.647$, $R^2=0.419$, $p<0.001$

Fat mass vs. systolic blood pressure – not significant

Fasting glucose vs. systolic blood pressure – not significant

When fat mass vs. fasting glucose was further explored, split by exposure to treatment and genotype, then an effect of genotype, but not treatment, was observed. A significant relationship remained in $eNOS^{-/-}$ mice (no treatment $p=0.0358$, exposed to sildenafil $p=0.0326$), regardless of treatment. No such relationship was observed in C57BL/6J mice (no treatment $p=0.2716$, exposed to sildenafil $p=0.7215$).

3.6.2 P150

Again, a significant relationship was observed between fat mass and fasting plasma glucose concentration.

Regression coefficients:

Fat mass vs. fasting glucose - $R=0.41$, $R^2=0.17$, $p<0.05$

Fat mass vs. systolic blood pressure – not significant

Fasting glucose vs. systolic blood pressure – not significant

When the fat mass vs. fasting glucose relationship was further examined, split by exposure to treatment and genotype, no effect of treatment or genotype was observed, although a trend was seen in $eNOS^{-/-}$ mice (no treatment $p=0.0862$, exposed to sildenafil $p=0.0825$).

In summary, a strong association between fat mass and fasting plasma glucose concentration at P90 was observed in $eNOS^{-/-}$, but not C57BL/6J mice. This was independent of treatment. This effect was lost when split by genotype and treatment at P150.

3.7. Mesenteric artery function

3.7.1. Constriction

At P90, $eNOS^{-/-}$ was associated with increased constriction response to U46619 in offspring fed HFD (C57BL/6J vs $eNOS^{-/-}$: 6.91 ± 0.19 vs 7.93 ± 0.19, $p=0.014$; Figure 7A, C). There was no effect of diet or treatment at P90. At P150, HFD was associated with a reduced constriction response to U46619 in $eNOS^{-/-}$ offspring exposed to sildenafil ($eNOS^{-/-}$: ND+ vs HFD+: 101.55 ± 26.26 vs 3.06 ± 1.67%, $p=0.010$; Figure 8A, C). Additionally, sildenafil was associated with increased constriction in $eNOS^{-/-}$ ND offspring at P150 ($eNOS^{-/-}$: ND+ vs ND-: 101.55 ± 26.26 vs 26.31 ± 7.13%, $p=0.018$).

370

371 **3.7.2. Relaxation**

372 At P90, eNOS$^{-/-}$ was associated with a reduced maximal relaxation response to ACh in HFD-
373 fed offspring not exposed to sildenafil (HFD-: C57BL/6J vs eNOS$^{-/-}$: 48.90 ± 0.62 vs 24.10 ±
374 1.43%, p=0.001; Figure 7B, D). HFD was associated with an increased maximal relaxation
375 response to ACh in C57BL/6J offspring not exposed to sildenafil (C57BL/6J: ND- vs HFD-:
376 28.97 ± 1.28 vs 48.90 ± 0.62%, p=0.007; Figure 7B). HFD also increased relaxation response
377 to SNP in eNOS$^{-/-}$ mice exposed to sildenafil (p=0.017). Sildenafil exposure had no effect on
378 relaxation response to ACh or SNP at P90. At P150, eNOS$^{-/-}$ was associated with reduced
379 maximal relaxation to ACh in HFD-fed offspring exposed to sildenafil (HFD+: C57BL/6J vs
380 eNOS$^{-/-}$: 34.11 ± 5.00 vs 15.16 ± 4.01, p=0.025; Figure 8B, D). Sildenafil increased
381 relaxation response to ACh in C57BL/6J mice on ND (C57BL/6J: ND- vs ND+: 16.69 ± 2.36
382 vs 35.81 ± 3.41, p=0.008; Figure 8B). There were no interactions or differences between
383 groups in response to SNP at P150.

384

4. Discussion

FGR is a leading contributor to perinatal mortality and morbidity globally. The lack of a curative treatment option is a significant concern, and is the urgent focus of a number of research groups. A number of new treatments, including sildenafil citrate, are currently being investigated, and if successful would have an immediate impact on perinatal health.

Of particular interest in this study, however, is the potential that by improving fetal growth and development, a treatment administered during pregnancy might also mitigate the increased later risk of metabolic and cardiovascular disease facing individuals that were growth restricted *in utero*. This study demonstrates that sildenafil, a potential treatment for FGR, may have small beneficial effects on metabolic and cardiovascular indices in a model of FGR. It also, however, highlights some potential risks of treatment in control mice, highlighting the importance of understanding any long-term effects that may occur in offspring following maternal treatment during pregnancy.

4.1 Body weight

Previous studies of the eNOS$^{-/-}$ mouse by ourselves and others have demonstrated a consistent reduction in fetal growth of around 10% at day 18.5 of a 19.5-day gestation [13, 14]. Due to concerns of neonatal loss (maternal neonatal cannibalism caused be handling of pups), the weight of offspring was not assessed in this study between the time of birth and weaning as first intended. Therefore, we were unable to assess the extent of growth restriction present in the eNOS$^{-/-}$ pups, nor the effect of sildenafil on rescuing fetal growth at birth. However, given that eNOS$^{-/-}$ offspring were significantly smaller than their C57BL/6J counterparts at weaning (P21), it seems reasonable to assume that a significant degree of FGR was present.

Litter size was significantly reduced in eNOS$^{-/-}$ mice in this study. This is in line with other studies, which have observed significant reductions in litter size both in late gestation [15, 16], as well as in the early postnatal period [18]. A smaller litter size might be expected to augment fetal growth, but this does not appear to be the case in the eNOS$^{-/-}$ mouse; detailed studies during gestation suggest a combination of impaired vascular development and adaptations to pregnancy [13, 15, 16], allied to impaired placental transport mechanisms [14] contribute to the growth restriction observed in this model, as opposed to physical restrictions in the uterus. Treatment with sildenafil citrate did not affect litter size in either C57BL/6J or eNOS$^{-/-}$ mice, suggesting it does not adversely affect later stage prenatal development or survival in these strains.

We observed that eNOS$^{-/-}$ offspring exposed to sildenafil treatment remained smaller than C57BL/6J mice at P21, suggesting that sildenafil was not able to rescue fetal growth in this model. Despite the positive effect of sildenafil in some models, such as the COMT$^{-/-}$ mouse, other investigators have failed to demonstrate a positive effect of sildenafil on fetal growth in models similar to the eNOS$^{-/-}$ mouse, such as the L-NAME treated rat model [19]. This is perhaps not surprising, as treatment with L-NAME inhibits all 3 isoforms of NOS, and presumably significantly reduces the production of NO / cGMP, thereby rendering sildenafil ineffective. The eNOS$^{-/-}$ mouse in comparison does still produce active inducible (iNOS or NOS1) and neuronal (nNOS or NOS2) NOS, and therefore still produces NO. We hypothesised that treatment with sildenafil would be able to potentiate the effects of NO produced by NOS1 and NOS2, and still have a positive effect on fetal growth. Expression of NOS1 has been observed in the placenta, particularly during early gestation, suggesting an important role in placental development [20]. NOS1 has also been observed in the radial arteries of the placenta, as well as at the maternal/fetal interface, suggesting roles in vascular development and transport mechanisms [20, 21]. Interestingly, other investigators have seen

436 positive effects on pregnancy and birth outcomes in the L-NAME model, despite total
437 inhibition of all NOS isoforms. Effects observed included a trend towards increased fetal and
438 placental weight [22] as well as increased offspring weight gain and survival [23]. These
439 results suggest it should, therefore, have been possible to demonstrate increased fetal growth
440 following sildenafil treatment in eNOS$^{-/-}$ mice. We have previously demonstrated the ability
441 of sildenafil to increase fetal weight in an alternative mouse model, the COMT$^{-/-}$ mouse [10],
442 which was associated with a reduction in placental resistance as well as an increased
443 sensitivity of the uterine artery to the vasodilator ACh [10]. Given that the eNOS$^{-/-}$ mouse
444 exhibits impaired uterine artery blood flow as well as reduced placental nutrient transport, we
445 hypothesised that sildenafil may increase fetal growth in this model via increased utero-
446 placental perfusion and increased nutrient delivery to (and therefore transport across) the
447 placenta. The lack of effect on fetal growth observed in this study may be due to a number of
448 reasons. Sildenafil exerts its effects by preventing the degradation of cGMP, a second
449 messenger of NO. Although the eNOS$^{-/-}$ mouse can still produce NO (via iNOS and nNOS),
450 and a component of NO-mediated vasodilation remains in arteries from these mice [24], this
451 may be significantly reduced in the eNOS$^{-/-}$ mouse, thus potentially reducing any beneficial
452 effects of sildenafil on utero-placental perfusion. Further, it is unclear to what extent the
453 reduced placental amino acid transport observed in eNOS$^{-/-}$ mice is due to reduced nutrient
454 availability (due to reduced uterine blood flow) or to an impairment in the transport system
455 itself. If amino acid transporter expression is reduced, or function impaired, increased
456 delivery of substrate to the placenta may not be enough to increase fetal growth.
457 At P90 weight was still reduced in eNOS$^{-/-}$ offspring, only achieving similar body weight to
458 their equivalent C57BL/6J counterparts at P150. FGR is often associated with significantly
459 accelerated “catch-up” growth in the immediate post-weaning period. This was not observed
460 in our eNOS$^{-/-}$ offspring. This may be due to the pathological mechanisms underlying the

development of FGR, as not all growth-restricted offspring maintain accelerated growth trajectories and 'overtake' control offspring. It is possible that, had we studied them for longer, the eNOS$^{-/-}$ offspring would have overtaken the C57BL/6J controls and become significantly heavier. However, it should be noted that following the addition of a HFD, the differences in body weight at P90 between the two strains was no longer significantly different, although the weights of eNOS$^{-/-}$ offspring did remain lower than their controls. This suggests that the growth restricted offspring are more susceptible to a HFD, which is in line with previous findings [25]. Exposure to sildenafil during pregnancy, however, had no effect on this finding.

4.2 Adipose tissue deposition

Diet had a greater effect on total adipose tissue deposition in eNOS$^{-/-}$ offspring. At P90, eNOS$^{-/-}$ mice on HFD had a significantly higher proportion of body weight due to adipose deposition; likely explaining why they were no longer smaller than their C57BL/6J controls. At P150, however, there was no difference between strains. This suggests an increased susceptibility to adipose deposition in eNOS$^{-/-}$ offspring, predisposing these mice to deposit fat at an earlier age in response to a HFD. An increase in adipose tissue deposition, as well as altered adipocyte function, has previously been observed across a range of animal models of FGR [26, 27]. Earlier adiposity rebound has been found to be associated with a higher incidence of type 2 diabetes in adulthood [28], suggesting that earlier adipose tissue deposition in eNOS$^{-/-}$ offspring may indicate a predisposition to insulin resistance in these animals.

It should be noted that although all offspring on a HFD in this study had increased adiposity, eNOS$^{-/-}$ offspring exhibited an earlier response, most likely due to programming effects or

altered metabolic function / adiposity due to a reduced litter size. There was no effect of sildenafil treatment during pregnancy on this measure.

4.3 Glucose tolerance

Metabolic disease, such as impaired glucose tolerance, has typically been observed in both humans [29, 30] and animals born growth restricted [31, 32]. The mechanisms underlying this phenomenon include impaired insulin secretion and increased insulin resistance, which in turn may be mediated by increased adipose tissue deposition and altered adipocyte function [33]. At P90, eNOS$^{-/-}$ offspring on a high fat diet, in the vehicle group, exhibited impaired glucose tolerance compared to normal diet offspring. As discussed above, these mice had a greater proportion of adipose tissue deposition, which may explain the greater impairment of glucose tolerance. The same degree of impairment was not observed in eNOS$^{-/-}$ mice exposed to sildenafil, then fed a high fat diet. This relative improvement in glucose tolerance may be related to the subtle decrease in adipose tissue deposition observed in this group; however, this is something that would need to be investigated further as the changes observed were not statistically significant in this study. At the P150 time point, both groups of eNOS$^{-/-}$ mice fed a high fat diet have significant glucose intolerance, regardless of treatment. At this time point, total adipose tissue deposition is very similar between groups, again suggesting that adipose tissue is at least in part playing a role in mediating impaired glucose tolerance.

When the relationship between adiposity and glucose tolerance was explored, a strong association was observed in eNOS$^{-/-}$ mice at P90. Although this was reduced at P150, a trend was still apparent in eNOS$^{-/-}$ mice. Although small group sizes mean these data should be treated cautiously, they do support the hypothesis that eNOS$^{-/-}$ offspring display programmed metabolic dysfunction.

It should be noted that C57BL/6J offspring on high fat diet that had been exposed to sildenafil *in utero* demonstrated a significant impairment in glucose tolerance at both P90 and P150. This group had the highest degree of adipose tissue deposition relative to body weight of any of the groups, providing further evidence that increased adipose tissue is associated with impaired glucose tolerance in this model. It should be noted that in this group we were not able to attain 6 litters (n=4), so data from this group will have greater variance. Despite this limitation, this finding does require further attention to determine the possible mechanisms by which sildenafil exposure predisposes offspring from normal pregnancies to glucose intolerance.

4.4 Insulin resistance

Size at birth has a very clear association with an increased risk of developing insulin resistance and type 2 diabetes in adulthood [34, 35]. Although there has been a tremendous research effort to understand the mechanisms of insulin resistance, an understanding in the context of FGR has not yet been reached. What is known, however, is that altered pancreatic beta cell mass, as well as adiposity, each plays a role in mediating insulin resistance. Using the homeostatic model assessment of insulin resistance (HOMA-IR) to assess pancreatic beta cell function and essentially determine a measure of insulin resistance, we observed a significant level of insulin resistance in C57BL/6J HFD+ offspring at P150. Mice in this group had fasting insulin concentrations far above what was observed in other groups, in addition to a HOMA-IR score which naturally shared a similar trend. As highlighted previously, this group of mice had the greatest degree of adiposity, and taken together, these results indicate that C57BL/6J offspring fed a high fat diet after being exposed to sildenafil treatment *in utero* display the most pronounced aberrations in metabolic dysfunction.

Increased adiposity, impaired glucose tolerance, and significant insulin resistance in this group justifies further investigation into the role sildenafil treatment may play in a normal pregnancy.

4.5 Blood pressure

An inverse relationship between size at birth and systolic blood pressure is well documented [36, 37]. The mechanism(s) behind this phenomenon, however, remain somewhat elusive, although there are links to a reduction in kidney size and associated nephron deficit [38, 39], as well as impaired vascular function [40-42]. Other mechanisms include tissue remodelling [43], reduced angiogenesis [44] and increased vascular oxidative stress [45, 46]. The eNOS$^{-/-}$ mouse has been extensively studied, and typically adult males demonstrate hypertension, although the severity can vary significantly from an increase of 14 to 50mmHg compared with control mice [47-49]. It was surprising, therefore, that we found no differences in blood pressure between eNOS$^{-/-}$ and C57BL/6J mice in our study at P90. A difference was observed at P150 in mice exposed to sildenafil, with eNOS$^{-/-}$ mice having a greater blood pressure than their C57BL/6J counterparts.

Blood pressure in this study was measured by tail cuff plethysmography. This procedure can be associated with increased stress to the animals, and thus the readings obtained may be more variable than those obtained from telemetered animals, whose readings are made in unrestrained animals in their home cage. It is possible that this variability, coupled with a relatively small sample size, was enough to mask any hypertension in eNOS$^{-/-}$ mice at the earlier time point, especially if the increase is as small as 14mmHg.

There was evidence that exposure to sildenafil was associated with a small but significant increase in blood pressure in eNOS$^{-/-}$ mice at the later time point. In our study, although there were no associated changes in kidney weight, there were some subtle changes in mesenteric artery function, which might explain the changes in blood pressure observed. An increase in sensitivity to the vasoconstrictor U46619 was observed in arteries from eNOS$^{-/-}$ ND+ mice, whilst reduced maximal endothelium-dependent relaxation was noted in arteries from eNOS$^{-/-}$ HFD+ mice. In the rodent, the mesenteric vascular bed plays an important role in the regulation of blood pressure, and the small changes in vascular function observed here, leading to a more constrictive phenotype, likely contributed to the increase in blood pressure.

Further studies could investigate other mechanisms which may explain the changes seen, such as remodelling of the conduit arteries or increased vascular oxidative stress. It should be noted, however, that the increases in blood pressure were small, and may be of limited physiological consequences.

4.6 Vascular function

Peripheral resistance arteries play an important role in the maintenance of blood pressure, and altered responses to vasoactive agents in these arteries may play a role in the pathogenesis and pathophysiology of hypertension [50, 51], whilst changes in response can serve as an indicator of overall cardiovascular health [52, 53]. Significant endothelial dysfunction has previously been observed in adults born growth restricted, including a blunted response to ACh [54]; this impairment is associated with hypertension [40, 41] and may represent one mechanism by which affected individuals are at greater risk of cardiovascular disease.

At postnatal day 90, eNOS$^{-/-}$ offspring on a high fat diet, which were not exposed to sildenafil treatment, had a significantly enhanced constriction response to U46619 compared to

C57BL/6J offspring on the same diet and treatment. This in line with previous studies which have shown a strong association with low birth weight, teamed with hypercaloric postnatal nutrition, in the incidence of hypertension and cardiovascular disease in adulthood in human populations [55, 56]. Interestingly, this difference was not seen in mice from sildenafil-treated dams, suggesting that sildenafil exposure may be attenuating, to a mild degree, a sensitivity to constriction that is inherent in eNOS$^{-/-}$ offspring which are then challenged with high fat diet. This did not, however, translate into a reduction in blood pressure. In contrast, an increase in sensitivity to U46619 was observed in eNOS$^{-/-}$ mice exposed to sildenafil and fed a normal diet; this change was associated with an increase in blood pressure.

Small differences in response to ACh were also noted in this study, including a reduction in maximal relaxation in arteries from eNOS$^{-/-}$ HF- offspring at P90, and in eNOS$^{-/-}$ HF+ offspring at P150. Little is known about the long-term effects of exposure to sildenafil on vascular function in offspring. One previous study has examined the effect of a higher dose of sildenafil on fetal vascular function. Using a different mouse model of FGR, but with a similar route of administration and timing of treatment, fetal abdominal vascular function was assessed at gestational day 18.5 [57]. Arteries from mice exposed to sildenafil exhibited impaired endothelium-dependent and independent vasodilation. The potential mechanism(s) underlying the changes in vascular function observed in these studies is not clear, although other investigators have highlighted the potential role of increased oxidative stress, increased inflammation or changes in vascular telomere length in mediating programmed vascular dysfunction in animal models of FGR [58, 59]. It is not clear if exposure to sildenafil increases inflammation or oxidative stress in the vasculature. Further interrogation of the components of the vasodilatory response to ACh is needed, as well as an assessment of oxidative stress and inflammatory markers in these peripheral arteries, to determine the

potential mechanisms by which exposure to sildenafil *in utero* affects offspring vascular function.

There was no effect of genotype or exposure to sildenafil on the response to sodium nitroprusside, indicating that there were no effects of being born FGR, or exposure to sildenafil, on the ability of vascular smooth muscle to relax. This is in contrast to a previous study, in which exposure to sildenafil was associated with reduced endothelium independent vasodilatory responses in the fetal abdominal aorta [57]. That study differed, not only in the vascular bed but also the length of time since exposure to the drug (0 days vs. 90 or 150 days), and may be indicative of the differences between immediate and long-term changes in vascular function.

It should be noted that exposure to sildenafil was associated with an increased response to ACh in arteries from control mice. Again, further investigation of the response to ACh, including its components and indicators of oxidative stress / inflammation, would help to better understand the mechanisms by which sildenafil effects vascular function in offspring. Overall, the changes in vascular function were small, but together suggest a more constrictive phenotype in eNOS$^{-/-}$ mice exposed to sildenafil. This likely contributes to the small increases in systolic blood pressure observed in this group of mice.

4.7 Limitations

Our model of growth restriction, as is a limitation with many animal models, did not satisfy all the phenotypic aspects observed in human adult growth restricted offspring. The eNOS$^{-/-}$ model utilised the absence of an enzyme to induce the maternal phenotype, at the cost of maintaining this same deletion in offspring. It is therefore difficult to separate any strain

637 effects identified which may be due to fetal programming or lack of eNOS expression. Thus,
638 additional studies are required to provide more conclusive evidence around the long-term
639 effects of maternal sildenafil treatment on offspring. Utilising another animal model would be
640 beneficial, as no one model can satisfy all aspects of FGR.
641 It should also be highlighted that other investigators have noted sex-specific effects of
642 programming in adult offspring. This current study was limited to looking at the effects in
643 male offspring only, but we acknowledge that different programming effects, or indeed
644 effects of sildenafil exposure, may be present in female offspring, and we would seek to
645 include animals of both sexes in any further study.
646
647 **4.8 Summary and conclusions**
648 We have demonstrated that the eNOS$^{-/-}$ model of growth restriction exhibits a number of
649 features associated with developmental programming of cardiovascular and metabolic
650 disease, particularly when faced with a 'second hit' of a high fat diet. This includes
651 alterations in body composition which favours energy storage when faced with a high fat diet,
652 impaired glucose tolerance and a small increase in systolic blood pressure. Exposure to
653 sildenafil treatment *in utero* had little effect on these outcomes, although small beneficial
654 effects on vascular function and glucose tolerance were observed at the earlier P90 time-
655 point. It should be noted, however, that exposure to sildenafil was also associated with a
656 more constrictive vascular phenotype and a small increase in blood pressure in eNOS$^{-/-}$ mice
657 and increased insulin resistance in C57BL/6L mice.
658
659 The potential benefits of improving fetal growth, and therefore reducing perinatal morbidity
660 and mortality are enormous, and any successful treatment would likely have tremendous
661 health, financial and societal benefits. This study highlights, however, the importance of

understanding the long-term effects of treatment during pregnancy on affected individuals. Further, a better understanding of the underlying mechanisms of any effects seen in offspring will allow for the mitigation of any potential adverse effects, and also to optimise any possible benefits they may provide in reducing the programming of cardio-metabolic diseases.

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6. References

1. Simchen MJ, Beiner ME, Strauss-Liviathan N, Dulitzky M, Kuint J, Mashiach S, Schiff E. (2000) Neonatal outcome in growth-restricted versus appropriately grown preterm infants. *Am J Perinatol*. 17(4):187-192.

2. Bernstein IM, Horbar JD, Badger GJ, Ohlsson A, Golan A. (2000) Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. the vermont oxford network. *Am J Obstet Gynecol*. 182(1 Pt 1):198-206.

3. Barker DJ. (2004) The developmental origins of chronic adult disease. *Acta Paediatr Suppl*. 93(446):26.

4. Gluckman PD, Hanson MA, Spencer HG (2005). Predictive adaptive responses and human evolution. *Trends in Ecology and Evolution*, 20(10):527–533. http://doi.org/10.1016/j.tree.2005.08.001

5. von Dadelszen P, Dwinnell S, Magee LA, Carleton BC, Gruslin A, Lee B, Lim KI, Liston RM, Miller SP, Rurak D, Sherlock RL, Skoll MA, Wareing MM, Baker PN; Research into Advanced Fetal Diagnosis and Therapy (RAFT) Group. (2011). Sildenafil citrate therapy for severe early-onset intrauterine growth restriction. *BJOG,* 118(5):624-8. doi: 10.1111/j.1471-0528.2010.02879.x.

6. Ganzevoort W, Alfirevic Z, von Dadelszen P, Kenny L, Papageorghiou A, van Wassenaer-Leemhuis A, Gluud C, Mol BW, Baker PN. (2014). STRIDER: Sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction--a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. *Syst Rev*, 3:23 doi: 10.1186/2046-4053-3-23.

695 7. Stanley JL, Andersson IJ, Poudel R, Rueda-Clausen CF, Sibley CP, Davidge ST, Baker PN
696 (2012). Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase
697 knockout mouse model. *Hypertension*, 59(5):1021-1028.

698 8. Oyston C, Stanley J, Oliver M, Bloomfield F, Baker PN (2012). Maternal administration of
699 sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in
700 the growth restricted ovine fetus. *Hypertension*, 68(3):760-767.

701 9. Satterfield MC, Bazer FW, Spencer TE, Wu G (2010). Sildenafil citrate treatment enhances
702 amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine
703 growth restriction. *J Nutr*, 140(2):251-258.

704 10. Katantie E, Osmond C, Barker DJ, Forsen T, Phillips DI, Eriksson JG (2005). Size at
705 birth as a predictor of mortality in adulthood: a follow-up of 350 000 person-years. *Int J*
706 *Epidemiol,* 34(3):655-663.
707
708 11. Yzydorczyk C, Armengaud JB, Peyter AC, Chehade H, Cachat F, Juvet C, Siddeek B,
709 Simoncini S, Sabatier F, Dignat-George F, Mitanchez D, Simeoni U (2017). Endothelial
710 dysfunction in individuals born after fetal growth restriction: cardiovascular and renal
711 consequences and preventative approaches. *J Dev Orig Health Dis*, 8(4):448-464.
712
713 12. Gluckman PD, Lillycrop KA, Vickers MH, Pleasants AB, Phillips ES, Beedle AS,
714 Burdge GC, Hanson MA (2007). Metabolic plasticity during mammalian development is
715 directionally dependent on early nutritional status. *Proc Natl Acad Sci USA*,
716 104(31):12796-127800.
717

718 13. Stanley JL, Andersson IJ, Hirt CJ, Moore L, Dilworth MR, Chade AR, Sibley CP, Davidge
719 ST, Baker PN (2012). Effect of the Anti-Oxidant Tempol on Fetal Growth in a Mouse
720 Model of Fetal Growth Restriction. *Biology of Reproduction*, 87(1):25–25.
721 http://doi.org/10.1095/biolreprod.111.09619

722 14. Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ, Glazier JD
723 (2012). eNOS knockout mouse as a model of fetal growth restriction with an impaired
724 uterine artery function and placental transport phenotype. *AJP: Regulatory, Integrative*
725 *and Comparative Physiology*, 303(1):R86–R93.
726 http://doi.org/10.1152/ajpregu.00600.2011

727 15. Kulandavelu S, Whiteley KJ, Bainbridge SA, Qu D, Adamson, SL (2013). Endothelial
728 NO synthase augments fetoplacental blood flow, placental vascularization, and fetal
729 growth in mice. *Hypertension*, 61(1):259–266.
730 http://doi.org/10.1161/HYPERTENSIONAHA.112.201996

731 16. Kulandavelu S, Whiteley KJ, Qu D, Mu J, Bainbridge SA, Adamson SL (2012).
732 Endothelial nitric oxide synthase deficiency reduces uterine blood flow, spiral artery
733 elongation, and placental oxygenation in pregnant mice. *Hypertension*, 60(1):231–238.
734 http://doi.org/10.1161/HYPERTENSIONAHA.111.187559
735

736 17. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R (1985). Homeostasis
737 model assessment: insulin resistance and beta-cell function from fasting plasma glucose
738 and insulin concentrations in man. *Diabetologia*, 28(7):412–419.
739

740 18. Hefler LA, Reyes CA, O'Brien WE, Gregg AR (2001). Perinatal development of
741 endothelial nitric oxide synthase-deficient mice. *Biol Reprod*, 64(2):666-673.

19. Nassar AH, Masrouha KZ, Itani H, Nader KA, Usta IM (2012). Effect of sildenafil in Nω-nitro-L-arginine methyl ester-induced intrauterine growth restriction in a rat model. *Am J Perinatol*, 29(6):429-434.

20. Khan H, Kusakabe KT, Wakitani S, Hiyama M, Takeshita A, Kiso Y (2012). Expression and localization of NO synthase isoenzymes (iNOS and eNOS) in the development of the rabbit placenta. *J Reprod Dev*, 58(2):231-236.

21. Purcell T, Buhimschi IA, Given R, Chwalisz K, Garfield RE (1997). Inducible nitric oxide synthase is present in the rat placenta at the fetal-maternal interface and decreases prior to labour. *Mol Hum Reprod*, 3(6):485-491.

22. Ramesar SV, Mackraj I, Gathiram P, Moodley J (2010). Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. *Eur J Obstet Gynecol Reprod Biol*, 149(1):22-26.

23. Herraiz S, Pellicer B, Serra V, Cauli O, Cortijo J, Felipo V, Pellicer A (2012). Sildenafil citrate improves perinatal outcome in fetuses from pre-eclamptic rats. *BJOG*, 119(11):1394-1402.

24. Chlopicki S, Kozlovski VI, Lorkowska B, Drelicharz L, Gebska A (2005). Compensation of endothelium-dependent responses in coronary circulation of eNOS-deficient mice. *Journal of Cardiovascular Pharmacology*, 46(1):115–123.

25. Bellinger L, Sculley DV, Langley-Evans SC (2006). Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. *International Journal of Obesity*, 30(5):729–738. http://doi.org/10.1038/sj.ijo.0803205

26. Ozanne SE, Lewis R, Jennings BJ, Hales CN (2004). Early programming of weight gain in mice prevents the induction of obesity by a highly palatable diet. *Clinical Science (London, England: 1979)*, 106(2):141–145. http://doi.org/10.1042/CS20030278

27. Zambrano E, Bautista CJ, Deás M, Martínez-Samayoa PM, González-Zamorano M, Ledesma H, Morales J, Larrea F, Nathanielsz, P. W. (2006). A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. *The Journal of Physiology*, 571(Pt 1):221–230. http://doi.org/10.1113/jphysiol.2005.100313

28. Eriksson JG, Forsén T, Tuomilehto J, Osmond C, Barker DJP (2003). Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. *Diabetologia*, 46(2):190–4. http://doi.org/10.1007/s00125-002-1012-5

29. Forsén T, Eriksson J, Tuomilehto J, Reunanen A, Osmond C, Barker D (2000). The fetal and childhood growth of persons who develop type 2 diabetes. *Annals of Internal Medicine*, 133(3):176–182.

30. Robinson JS, Moore VM, Owens JA, McMillen IC (2000). Origins of fetal growth restriction. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*,

92(1):13–19.

31. Garg M, Thamotharan M, Dai Y, Lagishetty V, Matveyenko AV, Lee WNP, Devaskar S U (2013). Glucose intolerance and lipid metabolic adaptations in response to intrauterine and postnatal calorie restriction in male adult rats. *Endocrinology*, 154(1):102–113. http://doi.org/10.1210/en.2012-1640

32. Tran M, Gallo LA, Jefferies AJ, Moritz KM, Wlodek ME (2013). Transgenerational metabolic outcomes associated with uteroplacental insufficiency. *The Journal of Endocrinology*, 217(1):105–118. http://doi.org/10.1530/JOE-12-0560

33. Shoelson SE, Herrero L, Naaz A (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology*, 132(6):2169–2180. http://doi.org/10.1053/j.gastro.2007.03.059

34. Barker DJP, Hales CN, Fall CHD, Osmond C, Phipps K, Clark PMS (1993). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia*, 36(1):62–67. http://doi.org/10.1007/BF00399095

35. Rich-Edwards JW, Colditz G, Stampfer MJ, Willett WC, Gillman MW, Hennekens CH, Speizer FE, Manson JE (1999). Birthweight and the risk for type 2 diabetes mellitus in adult women. *Annals of Internal Medicine*, 130:278–284. http://doi.org/199902160-00005 [pii]

36. Langley SC, Jackson AA (1994). Increased systolic blood pressure in adult rats induced

by fetal exposure to maternal low protein diets. *Clinical Science (London, England : 1979)*, 86(2):217–222.

37. Huxley RR, Shiell W, Law CM (2000). The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature. *Journal of Hypertension*, 18(7):815–831. http://doi.org/10.1097/00004872-200018070-00002

38. Luyckx V, Shukha K, Brenner B (2011). Low Nephron Number and Its Clinical Consequences. *Rambam Maimonides Medical Journal*, 2(4):1–16. http://doi.org/10.5041/RMMJ.10061

39. Hughson M, Farris AB, Douglas-Denton R, Hoy WE, Bertram JF (2003). Glomerular number and size in autopsy kidneys: The relationship to birth weight. *Kidney International*, 63(6):2113–2122. http://doi.org/10.1046/j.1523-1755.2003.00018.x

40. Gray C, Li M, Reynolds CM, Vickers MH (2013). Pre-weaning growth hormone treatment reverses hypertension and endothelial dysfunction in adult male offspring of mothers undernourished during pregnancy. *PloS One*, 8(1):e53505. http://doi.org/10.1371/journal.pone.0053505

41. Ozaki T, Nishina H, Hanson MA, Poston L (2001). Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *The Journal of Physiology*, 530(Pt 1):141–152.

841 42. Brawley L, Poston L, Hanson MA (2003). Mechanisms underlying the programming of
842 small artery dysfunction: review of the model using low protein diet in pregnancy in the
843 rat. *Archives of Physiology and Biochemistry*, 111(1):23–35.
844 http://doi.org/10.1076/apab.111.2.5.23.17506
845
846 43. Pladys P, Lahaie I, Cambonie G, Thibault G, Lê NLO, Abran D, Nuyt AM (2004). Role
847 of brain and peripheral angiotensin II in hypertension and altered arterial baroreflex
848 programmed during fetal life in rat. *Pediatric Research*, 55(6):1042–1049.
849 http://doi.org/10.1203/01.PDR.0000127012.37315.36
850
851 44. Alves JG, Vilarim JN, Figueiroa JN (1999). Fetal influences on neonatal blood pressure.
852 *Journal of Perinatology: Official Journal of the California Perinatal Association*, 19(8
853 Pt 1):593–595.
854
855 45. Hadoke PWF, Lindsay RS, Seckl JR, Walker BR, Kenyon CJ (2006). Altered vascular
856 contractility in adult female rats with hypertension programmed by prenatal
857 glucocorticoid exposure. *The Journal of Endocrinology*, 188(3):435–442.
858 http://doi.org/10.1677/joe.1.06506
859
860 46. Li J-M, Shah AM (2004). Endothelial cell superoxide generation: regulation and
861 relevance for cardiovascular pathophysiology. *Am J Physiol Regul, Integr Comp Physiol*,
862 287(5):R1014–1030. http://doi.org/10.1152/ajpregu.00124.2004
863
864 47. Stauss HM, Gödecke A, Mrowka R, Schrader J, Persson PB (1999). Enhanced blood
865 pressure variability in eNOS knockout mice. *Hypertension*, 33(6):1359–1363.

48. Stauss HM, Nafz B, Mrowka R, Persson PB (2000). Blood pressure control in eNOS knock-out mice: comparison with other species under NO blockade. *Acta Physiologica Scandinavica*, 168(1):155–160. http://doi.org/10.1046/j.1365-201x.2000.00639.x

49. Wagner C, Gödecke A, Ford M, Schnermann J, Schrader J, Kurtz A (2000). Regulation of renin gene expression in kidneys of eNOS- and nNOS-deficient mice. *Pflügers Archiv: European Journal of Physiology*, 439(5):567–572.

50. Aalkjaer C, Heagerty AM, Petersen KK, Swales JD, Mulvany MJ (1987). Evidence for increased media thickness, increased neuronal amine uptake, and depressed excitation--contraction coupling in isolated resistance vessels from essential hypertensives. *Circulation Research*, 61(2):181–186.

51. Schiffrin EL (1992). Reactivity of small blood vessels in hypertension: relation with structural changes. State of the art lecture. *Hypertension*, 19(2 Suppl):II1–9.

52. Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA (1993). Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*, 87(5):1468–1474.

53. Yoshida M, Imaizumi T, Ando S, Hirooka Y, Harada S, Takeshita A (1991). Impaired forearm vasodilatation by acetylcholine in patients with hypertension. *Heart and Vessels*, 6(4):218–223.

54. Torrens C, Hanson MA, Gluckman PD, Vickers MH (2009). Maternal undernutrition leads to endothelial dysfunction in adult male rat offspring independent of postnatal diet. *The British Journal of Nutrition*, 101(1):27–33. http://doi.org/10.1017/S0007114508988760

55. Barker DJ (1986). Infant Mortality, Childhood Nutrition, and Ischaemic Heart Disease in England and Wales. *The Lancet*, 327(8489):1077–1081. http://doi.org/10.1016/S0140-6736(86)91340-1

56. Barker DJ, Osmond C, Simmonds SJ, Wield GA (1993). The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *Bmj*, 306(6875):422–6. http://doi.org/10.1136/bmj.306.6875.422

57. Renshall LJ, Dilworth MR, Greenwood SL, Sibley CP, Wareing M (2014). In vitro assessment of mouse fetal abdominal aortic vascular function. *Am J Physiol Regul Integr Comp Physiol,* 307(6):R746-754.

58. Cooke CM, Shah A, Kirschenman RD, Quon AL, Morton JS, Care AS, Davidge ST (2018). Increased susceptibility to cardiovascular disease in offspring born from dams of advanced maternal age. *J Physiol*, doi: 10.1113/JP275472.

59. Allison BJ, Kaandorp JJ, Kane AD, Camm EJ, Lusby C, Cross CM, Nevin-Dolan R, Thakor AS, Derks JB, Tarry-Adkins JL, Ozanne SE, Giussani DA (2016). Divergence of mechanistic pathways mediating cardiovascular aging and developmental programming of cardiovascular disease. *FASEB J*, 30(5):1968-1975.

916 **Figure Legends**

917

918 **Figure 1. Outline of experimental design**

919 Pregnant C57BL/6J and eNOS$^{-/-}$ mice were randomized to sildenafil or vehicle control groups
920 at gestational day (gd) 12.5. Treatment was provided until the dam had given birth. Male
921 offspring were weaned at postnatal day (P) 21, and randomized to a normal or high fat diet
922 and were studied at either P90 or P150.

923

924 **Figure 2. Growth trajectory and food intake over the course of 21 weeks.**

925 A) There was a significant effect of genotype on body weight at P21. eNOS$^{-/-}$ mice were
926 significantly smaller than their treatment counterparts. B) C57BL/6J offspring growth was
927 altered by diet but not by sildenafil. C) C57BL/6J offspring cumulative energy intake was not
928 affected by diet or sildenafil. D) eNOS$^{-/-}$ offspring growth was altered by diet but not sildenafil.
929 E) eNOS$^{-/-}$ offspring cumulative energy intake was not affected by diet or sildenafil. F)
930 C57BL/6J and eNOS$^{-/-}$ offspring growth comparison for normal diet groups. G) C57BL/6J and
931 eNOS$^{-/-}$ offspring growth comparison for high diet groups
932 + sildenafil treatment, - no sildenafil treatment, ND Normal chow diet, HFD High fat diet,
933 Data presented as mean ± SEM (n=4-7), post-hoc analysis: *$p<0.05$, **$p=0.003$ vs.
934 C57BL/6J -, #$p<0.001$ vs. C57BL/6J +

935

936 **Figure 3. Total adipose tissue deposition relative to body weight at P90 and P150.**

937 A) Adipose deposition at P90. There was a significant interaction between strain and diet in
938 both vehicle ($F(1,26)=9.33$, $p=0.005$) and sildenafil treated offspring ($F(1,23)=8.65$,
939 $p=0.007$). eNOS$^{-/-}$ offspring on HFD had significantly higher total adiposity than C57BL/6J

HFD-fed counterparts; $p=<0.001 vs. C57BL/6J HF-, *p=0.014 vs. C57BL/6J HF+, #p<0.001 vs. treatment and diet control group e.g. eNOS$^{-/-}$ HFD- vs. ND- and HFD+ vs. ND-

B) There was no effect of strain, treatment or diet on lean weight at P90.

C) Adipose deposition at P150. Addition of HFD significantly increased total fat deposition in both C57BL/6J and eNOS$^{-/-}$ mice when compared to normal diet / treatment controls.

D) There was no effect of strain, treatment or diet on lean weight at P150.

+ sildenafil treatment, - no sildenafil treatment, ND Normal chow diet, HFD High fat diet, Data presented as mean ± SEM (n=4-7), post-hoc analysis: # p=≤0.001vs. treatment and diet control groups within strain, e.g. C57BL/6J HFD- vs. ND-

Figure 4. **Glucose tolerance is impaired in HFD fed offspring at P84**

A) C57BL/6J OGTT curve - HFD+ offspring had significantly elevated plasma glucose levels at 60, 90 and 120 mins compared with HFD- offspring.

B) eNOS$^{-/-}$ OGTT curve - Sildenafil null offspring on high fat diet had significantly elevated fasting blood glucose compared to ND eNOS$^{-/-}$ controls.

C) AUC P90 – Impaired glucose tolerance was observed in HFD fed eNOS$^{-/-}$ offspring compared with C57BL/6J mice from vehicle treated dams. In contrast, impaired glucose tolerance was observed in C57BL/6J HFD offspring exposed to sildenafil compared with their vehicle counterparts (HFD+ vs. HFD-).

+ sildenafil treatment, - no sildenafil treatment, ND Normal chow diet, HFD High fat diet, Data presented as mean ± SEM (n=4-7), post-hoc analysis: * p=≤0.05, **p=≤0.01¸ # p=≤0.001.

Figure 5. Glucose tolerance is impaired in HFD fed offspring at P144

A) C57BL/6J OGTT curve - HFD+ offspring had significantly elevated plasma glucose levels at 0, 30, 60, 90 and 120 mins compared with ND+ mice.
B) eNOS$^{-/-}$ OGTT curve - Sildenafil null offspring on high fat diet had significantly elevated fasting blood glucose compared to ND eNOS$^{-/-}$ controls.
C) AUC – Impaired glucose tolerance was observed in HFD fed eNOS$^{-/-}$ offspring compared with their ND controls; this was seen in mice from both vehicle and sildenafil treated dams. Impaired glucose tolerance was also observed in C57BL/6J HFD offspring, but only in those exposed to sildenafil compared with their normal diet C57BL/6J controls.
+ sildenafil treatment, - no sildenafil treatment, ND Normal chow diet, HFD High fat diet, Data presented as mean ± SEM (n=4-7), post-hoc analysis: * p=≤0.05, **p=≤0.01¸ # p=≤0.001.

Figure 6. Systolic blood pressure at P83 and P143.
A) Blood pressure at P83. There were no significant differences in systolic blood pressure between groups, regardless of strain, diet or exposure to sildenafil.
B) Blood pressure at P143. Systolic blood pressure was significantly increased in eNOS$^{-/-}$ mice exposed to sildenafil compared to their C57BL/6J diet controls ND+(*) and HFD+(**).
+ sildenafil treatment, - no sildenafil treatment, ND Normal chow diet, HFD High fat diet, Data presented as mean ± SEM (n=4-7), post-hoc analysis: * p=≤0.05, **p=≤0.01.

Figure 7. Vascular reactivity of mesenteric arteries at P90
A) There was no effect of diet or treatment on the ability of mesenteric arteries from C57BL/6J mice to constrict in response to U46619, or their sensitivity to the drug. B) Vehicle treated C57BL/6J mice fed a high fat diet showed increased maximal relaxation to ACh (p=0.007), but there was no effect on sensitivity to ACh. C) Arteries from eNOS$^{-/-}$ mice fed a

high fat diet demonstrated increased maximal constriction to U46619 compared to their C57BL/6J counterparts (p=0.014), regardless of treatment. D) Maximal relaxation was reduced in vehicle treated eNOS$^{-/-}$ mice on a high fat diet compared with their C57BL/6J counterparts (p=0.001).

Data presented as mean ± SEM (n=4-7).

Figure 8. Vascular reactivity of mesenteric arteries at P150

A) There was no effect of diet, strain or treatment on the constrictor response of arteries from C57BL/6J mice to U46619. B) Exposure to sildenafil was associated with increased endothelium-dependent relaxation in arteries from C57BL/6J mice fed a normal diet vs. their vehicle treated controls (p=0.008). C) Constriction in response to U46619 was reduced in arteries from sildenafil-exposed eNOS$^{-/-}$ mice fed a high fat diet vs. their normal diet counterparts (p=0.010). D) Relaxation was reduced in HFD-fed eNOS$^{-/-}$ mice exposed to sildenafil compared with their C57BL/6J counterparts (p=0.025).

Data presented as mean ± SEM (n=4-7).