Paternal Diet Impairs F1 and F2 Offspring Vascular Function Through Sperm and Seminal Plasma Specific Mechanisms in Mice

Hannah L. Morgan[a], Panaigota Paganopoulou[a], Sofia Akhtar[b], Natalie Urquhart[b], Ranmini Philomin[b], Yasmin Dickinson[b], Adam J. Watkins[a,b]

[a] Division of Child Health, Obstetrics and Gynaecology, Faculty of Medicine, University of Nottingham, Nottingham. NG7 2UH, UK.

[b] School of Life and Health Sciences, Aston University, Birmingham, B4 7ET, UK.

Corresponding Author:

Adam J. Watkins: Division of Child Health, Obstetrics and Gynaecology, Faculty of Medicine, University of Nottingham, Nottingham. NG7 2UH, UK.

E mail: adam.watkins@nottingham.ac.uk

Tel: +44 (0)115 8230699

Short title: Paternal diet and offspring vascular dysfunction

Key Points

- A low protein diet had minimal effects on paternal cardiovascular function or renin-angiotensin system activity.
- Paternal low protein diet modified F1 neonatal and adult offspring renin-angiotensin system activity and cardiovascular function in a sperm and/or seminal plasma specific manner.
- Paternal low protein diet modified F1 male offspring testicular expression of central epigenetic regulators.
- Significant changes in F2 neonatal offspring growth and tissue angiotensin converting enzyme activity were programmed by paternal low protein diet in a sperm and/or seminal plasma specific manner.

Abstract

Whilst the impact of maternal diet on adult offspring health is well characterised, the role a father's diet has on his offspring's health remains poorly defined. We establish the significance of a sup-optimal paternal low protein diet for offspring vascular homeostasis and define the sperm and seminal plasma specific programming effects on cardiovascular health. Male C57BL6 mice were fed either a control normal protein diet (NPD; 18% protein) or an isocaloric low protein diet (LPD; 9% protein) for a minimum of 7 weeks. Using artificial insemination, in combination with vasectomised male mating, we generated offspring derived from either NPD or LPD sperm (devoid of seminal plasma) but in the presence of NPD or LPD seminal plasma (devoid of sperm). We observed that either LPD sperm or seminal fluid at conception impaired adult offspring vascular function in response to both vasoconstrictors and dilators. Underlying these changes in vascular function were significant changes in serum, lung and kidney angiotensin converting enzyme (ACE) activity, established in F1 offspring from 3 weeks of age, maintained into adulthood and present also within juvenile F2 offspring. Furthermore, we observed differential expression of multiple central Renin-Angiotensin System regulators in adult offspring kidneys. Finally, paternal diet modified the expression profiles of central epigenetic regulators of DNA methylation, histone modifications and RNA methylation in adult F1 male testes. These novel data reveal the impact of sub-optimal paternal nutrition on offspring cardiovascular well-being, programming offspring cardiovascular function through both sperm and seminal plasma specific mechanisms over successive generations.

Keywords: Developmental programming; Cardiovascular health; Renin-Angiotensin System; Paternal diet.

Introduction

The association between poor maternal diet and the predisposition of her offspring to developing cardiovascular dysfunction and metabolic disease is well established (Fleming *et al.*, 2018). Animal model studies show that sub-optimal maternal diet during specific stages of pregnancy result in offspring displaying hypertension (Torrens *et al.*, 2009; Watkins *et al.*, 2010) and vascular dysfunction (Gray *et al.*, 2015; Loche *et al.*, 2018). Underlying many of these impairments in blood pressure regulation are changes in the renin-angiotensin system (RAS) pathway function. A central mediator of the RAS's control on blood pressure is angiotensin-converting enzyme (ACE). ACE cleaves angiotensin I to form angiotensin II, a potent regulator of vascular function and blood volume (Dasinger *et al.*, 2016). In rodents, offspring of dams fed diets high in fat (Zhang *et al.*, 2018), salt (Mao *et al.*, 2013) or low in protein (Watkins *et al.*, 2010) have all been shown to program offspring cardiovascular dysfunction via impaired RAS regulation.

There is growing evidence indicating paternal health and well-being at the time of conception also impacts on offspring long-term cardio-metabolic health (Hur *et al.*, 2017; Lucas & Watkins, 2017). In mice, paternal undernutrition perturbs embryonic development and metabolism, fetal growth and adult offspring cardio-metabolic ill-health (Carone *et al.*, 2010; McPherson *et al.*, 2016; Watkins *et al.*, 2017). Similarly, paternal over nutrition and type 2 diabetes in mice affect offspring metabolic health over multiple generations (Ng *et al.*, 2010; Wei *et al.*, 2014; Cropley *et al.*, 2016). Furthermore, paternal obesity results in over-activation of the RAS in offspring adipose tissue, associated with poor glucose metabolism and increased inflammatory status (Ornellas *et al.*, 2018). Changes in sperm quality and genomic integrity are one potential mechanism through which paternal ill-health impacts on offspring development. In men, obesity, smoking, excessive alcohol consumption and type 2 diabetes all impact negatively on sperm quality and DNA integrity (Borges *et al.*, 2018) and embryo development (Stuppia *et al.*, 2015). Sperm from obese men display different patterns of DNA methylation to men of normal weight (Soubry *et al.*, 2016), while sperm from infertile men display

different profiles of protamines (Aoki *et al.*, 2006), DNA methylation (Jenkins *et al.*, 2016) and micro RNAs (Liu *et al.*, 2012) to sperm from healthy men. In animals, significant changes in sperm epigenetic status are observed in response to paternal obesity (Fullston *et al.*, 2013), under-nutrition (McPherson *et al.*, 2016) and diabetes (Pavlinkova *et al.*, 2017).

Separate to the genetic/epigenetic programming mechanisms directed by the sperm, the seminal plasma also influences embryonic, fetal and offspring development. Seminal plasma is critical for the initiation of uterine immunological and inflammatory responses which are essential in early pregnancy establishment (Sharkey *et al.*, 2012). Furthermore, removal of the seminal vesicles in mice results in significant impairments male fertility, preimplantation embryo development and adult offspring growth, metabolism and blood pressure regulation (Bromfield *et al.*, 2014).

Together, these studies demonstrate offspring metabolic ill-health is programmed in response to poor paternal diet at the time of conception via sperm- and seminal plasma-specific mechanisms. However, few studies have explored the link between paternal diet and offspring cardiovascular health. Furthermore, to our understanding, no studies have defined the relative sperm- and seminal plasma-specific contributions to offspring cardiovascular health. Recently, we characterised the relative sperm and seminal plasma-specific impact on offspring growth and metabolic homeostasis (Watkins *et al.*, 2018). In the present study, we extend our analysis of offspring metabolic health to define the impact of paternal diet on offspring vascular function, angiotensin converting enzyme (ACE) activity and expression of central RAS pathway regulators. Furthermore, we assess whether changes in blood pressure regulatory mechanisms programmed within F1 offspring are retained within an F2 generation.

Materials and Methods

Ethical approval

All experimental procedures were conducted under the UK Home Office Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012, which transposed Directive 2010/63/EU into UK law, with approval of the local ethics committee at Aston University and conform to principles and regulations for reporting animal experiments as described previously (Grundy, 2015). Intact and vasectomised 8 week old C57BL/6 male mice (Harlan Ltd, Belton, Leicestershire, UK) were maintained as described previously (Watkins *et al.*, 2018). Briefly, males were fed either control normal protein diet (NPD; 18% casein; n = 16 intact and 8 vasectomised males) or isocaloric low protein diet (LPD; 9% casein; n = 16 intact and 8 vasectomised) for a period of 8-12 weeks to ensure all stages of spermatogenesis and spermiogenesis were exposed to the diets (Oakberg, 1956). Diets were manufactured commercially (Special Dietary Services Ltd; UK) and their composition published previously (Watkins *et al.*, 2018).

Generation of F1 and F2 offspring

Offspring were generated as described previously (Watkins *et al.*, 2018). Briefly, virgin 8 week old female C57BL/6 mice (Charles River, UK) were superovulated by intraperitoneal injections of pregnant mare serum gonadotrophin (1 IU; Interve, UK) and human chorionic gonadotrophin (1 IU; Intervet, UK). Intact NPD and LPD fed males were culled by cervical dislocation after a minimum of 8 weeks on respective diets. Sperm were isolated from caudal epididymi NPD and LPD stud males as described previously (Watkins *et al.*, 2018) and allowed to capacitate in pre-warmed medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30mM HEPES; supplemented freshly with 10 mM lactic acid, 1 mM sodium pyruvate, 20 mg/ml BSA, 25 mM $NaHCO_3$). Females were artificially inseminated 12 hours post human chorionic gonadotrophin injection with approximately 10^7 sperm and subsequently housed overnight with a vasectomised C57BL/6 male fed either NPD or LPD. Females were weighed regularly (every 4-5 days) for the detection of weight gain. Four groups of offspring were generated termed NN (NPD sperm and NPD seminal plasma), LL (LPD sperm and

LPD seminal plasma), NL (NPD sperm and LPD seminal plasma) and LN (LPD sperm and NPD seminal plasma). See Figure 1 for diagrammatic representation of the study design and n numbers. Details on number of females inseminated, pregnancy rates, gestation lengths and litter parameters have been published previously (Watkins *et al.*, 2018).

All dams and offspring received standard chow and water *ad libitum* throughout the study. For the production of an F2 generation, 16 week old F1 males (n = 6 males per treatment group; each from separate litters) were mated naturally to virgin, 8 week old female C57BL/6 mice (Charles River, UK) acquired separately for mating with F1 males. Females were allowed to develop to term and all dams and F2 offspring received standard chow and water *ad libitum*. F1 offspring were culled by cervical dislocation at either 3 (juvenile) or 16 (adult) weeks of age while all F2 offspring were culled at 3 weeks of age. Blood samples were taken via heart puncture, centrifuged at 10,000 rpm (4^{o}C, 10 minutes), and the serum aliquoted and stored at -80^{o}C. Kidneys and lungs were dissected, weighed, snap frozen and stored at -80^{o}C.

Analysis of vascular function by fine wire myography

Stud male and offspring vascular function was assessed using wire myograph (Danish Myo Technology A/S, Denmark) in F1 male and female offspring at 16 weeks of age as described previously (Watkins & Sinclair, 2014). Mesenteric artery segments from stud males and adult male and female F1 offspring were maintained at 37^{o}C in physiological salt solution (NaCl, 119; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.17; NaHCO3, 25; KH2PO4, 1.18; EDTA, 0.026; and D-glucose, 5.5 mM) and gassed with 95% O_2 and 5% CO_2 (Watkins & Sinclair, 2014). Following normalisation (setting internal vessel circumference to 90% of the IC_{100} (Docherty *et al.*, 2001), initial assessment of vessel integrity and maximal response to excess K^+ (125mM), cumulative concentration response curves were conducted for the α_1-adrenergic agonist phenylephrine (PE; 10^{-9} to 10^{-4} mol/L). Subsequently, vessels were pre-constricted to 80% of maximal PE response with the thromboxane mimetic U46619

(10 µmol/L) prior to analysis of vascular responsiveness to the vasodilators acetylcholine (ACh) (10^{-9} to 10^{-4} mol/L) and isoprenaline (stud males only; 10^{-9} to 10^{-4} mol/L). Cumulative concentration responses to ACh were repeated in the presence of the nitric oxide (NO) synthase inhibitor $N^{\acute{o}}$-nitro-Larginine methyl ester (L-NAME; 100 µmol/L). Finally, following pre-constriction with U46619, vasodilatation responses to the nitric oxide donor sodium nitroprusside (SNP; 10^{-10} to 10^{-4} mol/L) were assessed. All drugs and chemicals were purchased from Sigma (UK).

Tissue angiotensin converting enzyme (ACE) activity assay

Samples of offspring kidney and lung tissue were homogenised in 200µl sodium borate buffer (0.5 M H_3BO_4, 1.125 M NaCl, pH 8.3) prior to centrifugation at 13,000 rpm for 10 minutes at 4 ^{O}C. Kidney lysate supernatant (30-50 µL) was mixed with 0.5 M sodium borate buffer and 20 µL of N-Hippuryl-His-Leu (20 mM in 0.5 M sodium borate buffer) to a final volume of 100 µL and incubated for 30 minutes at 37°C alongside blanks (containing only borate buffer and N-Hippuryl-His-Leu) and a standard curve of His-Leu (0 to 2 mM). After incubation, 400 µl of 1M NaOH was added to all samples prior to 400 µl of O-phthalaldehyde (20mg/ml in methanol). All samples were mixed and left at room temperature for 10 minutes prior to the addition of 400µL 1 M HCl. Samples, blanks and the standard curve were spun and measured colormetrically at 380 nm. Samples of offspring serum (10 - 20 µl) were mixed with 60 - 70 µl of sodium borate buffer and 20 µL of N-Hippuryl-His-Leu and incubated for 15-30 minutes at 37°C. Following incubation, serum samples were analysed as for the kidney and lung tissue samples. Serum ACE activity was expressed as nmol His-Leu formed, while kidney and lung samples were expressed as nmol His-Leu formed per mg of protein. All drugs and chemicals were purchased from Sigma (UK).

Offspring testicular and kidney gene expression

RNA was extracted from F1 and F2 (juvenile and adult) offspring kidneys and adult F1 offspring testes using the RNeasy Mini Kit (QIAGEN, UK), with on-column DNase I digestion, according to

manufacturer's instructions. Following determination of RNA concentration and contamination by Nanodrop, RNA was converted to cDNA using the NanoScript (PrimerDesign, UK) according to the manufacturer's instructions. For Real-Time PCR (RT-qPCR), 5 ng cDNA was added to 2X mastermix (Precision SYBRgreen Mastermix; Primerdesign, UK), 5 µM forward and reverse primers (Eurofins) and water. Analysis of renal gene expression was performed using a Stratagene Mx 3000P System (Agilent Technologies, USA), while offspring testicular expression was conducted using an Applied Biosystems 7500 system. Offspring relative kidney RAS expression data were calculated using the $\Delta\Delta C_T$ method following normalisation to the expression of *Sdha* and *Tuba* while relative testicular expression was normalised to *Pgk1* and *Tbp*. Using geNorm software (Watkins *et al.*, 2018), these reference genes were identified as being the most stable across all groups (from a total panel of 6 reference genes) with their primers displaying high efficiency (<1.9) of amplification (Lucas *et al.*, 2011). Primer sequences are given in Table 1.

Offspring testicular DNA methylation analysis

DNA was isolated from adult offspring testes using the DNeasy Blood & Tissue Kit (Qiagen, UK) according to the manufacturer's instructions and diluted to a final concentration of 100 ng/µl in water. Quantification of global DNA methylation was conducted using the Methylated DNA Quantification Kit (Catalogue # ab117128, Abcam, UK) according to the manufacturer's instructions. All samples were measured in triplicate and analysed alongside appropriate negative controls.

Statistical analyses

Stud male data were analysed using independent samples or repeated measures *t*-tests, where appropriate, following assessment for normality (Shaprio-Wilk and Kolmogorov-Smirnov tests) with GraphPad Prism (version 7). All offspring data were analysed using a multilevel random effects regression model (SPSS version 23) as described previously (Watkins *et al.*, 2018) followed by Bonferroni post-hoc test. Correlations between parameters was conducted using Pearson correlation.

Data were analysed for interactions between treatment group and offspring sex by two way ANOVA and reported where appropriate. Significance was taken at $P<0.05$.

Results

3.1 LPD has minimally effect on paternal growth and vascular function

When compared to the NPD, LPD has no significant effect on paternal body weight (Figure 2A). Similarly, there was no effect of LPD on the weight of paternal fat, liver, heart or lungs, relative to body weight (Figure 2B). However, kidney weight (average of left and right kidney; NPD = 0.48 ± 0.03 gm, LPD = 0.42 ± 0.01 gm; P = 0.004) and kidney:body weight ratio (Figure 2B, P = 0.007) were significantly lower in LPD males than NPD males. Paternal LPD had no effect on mesenteric artery responsiveness to the vasoconstrictor phenylephrine (PE) or the vasodilators acetylcholine (ACh), isoprenaline (IOS) or sodium nitroprusside (SNP) (Figure 2C-F). Finally, while paternal LPD had no effect on either serum or lung tissue angiotensin converting enzyme (ACE, Figure 2E, I) activity, a lower kidney ACE activity was observed in males fed LPD (Figure 2H; P = 0.003).

Impact of paternal diet on F1 offspring growth and ACE activity

Previously, we showed that at 16 weeks of age, LL, NL and LN offspring mice all became overweight, glucose intolerant and displayed metabolic dysfunction when compared to NN offspring (Watkins *et al.*, 2018). In our current study, at 3 weeks of age, LL males were heavier than NN and NL males at 3 weeks of age (Figure 3A; $P < 0.003$). However, no difference in kidney or lung weights were observed between groups (Data not shown). While no difference in mean serum (Figure 3B) or kidney (Figure 3C) ACE activity were observed between males, NL and LN males displayed reduced lung ACE activity when compared to NN males (Figure 3D; $P < 0.05$). Similar to the males, LL females displayed an elevated body weight when compared to NN and LN females (Figure 3E; $P < 0.02$). NL females were also heavier when compared to NN females (P = 0.003). Analysis of juvenile female

organ weights revealed increased kidney:body weight ratio in LN females when compared to LL and NL females (Data not shown). At 3 weeks of age, LL females displayed elevated serum ACE activity (Figure 3F; $P = 0.004$) while NL and LN females displayed reduced lung ACE activity (Figure 3H; $P < 0.05$) when compared to NN offspring.

In adult (16 weeks of age) offspring, no difference in male body weight (Figure 2I), kidney or lung:body weight ratios (Data not shown) were observed. However, NL males had a higher kidney ACE activity when compared to LL males (Figure 3K; $P < 0.04$), while LN males had a lower lung ACE activity when compared to NN, LL and LN males (Figure 3L; $P < 0.02$). In females, both NL and LN females were heavier than NN females (Figure 3M; $P < 0.05$). NL females also displayed an elevated lung:body weight ratio when compared LL females (Data not shown). LL females displayed an elevated serum ACE activity when compared to NN females (Figure 3N; $P = 0.013$), while LL and LN females had a lower kidney ACE activity when compared to NN and NL females (Figure 3O; $P < 0.04$). No difference in female lung ACE was observed between groups (Figure 3P).

As we observed significant differences in offspring ACE activity, we analysed relative expression of several RAS pathway genes in adult offspring kidneys. As no significant effect of sex was observed, expression data for males and females were combined ($P < 0.1$). We observed LL offspring displayed increased expression of angiotensin converting enzyme (*Ace*) when compared to NN offspring (Figure 4A; $P <0.03$). Both NL and LN offspring also displayed elevated expression of *Ace2* (Figure 4B), the type-1A angiotensin II receptor (*Agtr1a;* Figure 4C) and the renin receptor (*Atp6ap2;* Figure 4F) when compared to NN offspring ($P < 0.05$). LN offspring also displayed elevated expression of the type-1B angiotensin II receptor (*Agtr1b*; Figure 4D), while LL offspring displayed elevated expression of the type-2 angiotensin II receptor (*Agtr2;* Figure 4E) when compared to NN offspring ($P < 0.05$).

Impact of paternal diet on F1 adult offspring vascular function

We observed no effect of paternal diet on mesenteric artery responsiveness to the vasoconstrictor phenylephrine (PE; Figure 5A-C) or the vasodilator acetylcholine (Ach; Figure 5D-F) in male offspring. However, LL and LN offspring vessels displayed an increased insensitivity to pre-incubation with the nitric oxide synthase inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME), displaying an elevated maximal responses to ACh (Figure 5G, H; $P < 0.02$) while LN vessels also displayed a reduced pEC50 responses (Figure 5I; $P = 0.037$). While minimal differences in male offspring vascular responses were observed to the nitric oxide donor sodium nitroprusside (SNP; Figure 5J), an elevated maximal response was observed in LL males vessels when compared with NN males (Figure 5K; $P = 0.004$).

In females, both NL and LN offspring vessels displayed reduced maximal constriction responses to phenylephrine when compared to NN and LL offspring vessels (Figure 6A and B; $P < 0.001$). A reduced pEC50 to phenylephrine was also observed for NL female offspring vessels when compared to NN and LL vessels (Figure 6C; $P = < 0.05$). While no difference in maximal vasodilatory responses to ACh were observed between female groups (Figure 6D and E), a significantly reduced pEC50 was observed in LL and NL female offspring (Figure 6F; $P < 0.035$). Following pre-incubation with L-NAME, increased NO-independent vasodilation in arteries from LL, NL and LN female offspring was observed when compared to NN females (Figure 6G and H; $P \leq 0.01$). Furthermore, responses of NL and LN vessels were higher than that of the LL vessels (Figure 6H; $P \leq 0.006$), though there was no difference in the pEC50 between groups (Figure 6I). No difference in response to sodium nitroprusside were observed between females (Figure 6J-L).

3.4 Paternal diet modifies testicular expression of epigenetic regulators in F1 offspring

Studies have shown that parental programming of offspring health can span multiple generations, in response to both maternal and paternal health (Vickers, 2014). Changes in epigenetic status of F1

299 testicular tissue could provide one potential mechanism for the transgenerational transfer of
300 environmental information across generations. We observed significant changes in the transcript
301 expression of central regulators of RNA methylation, histone modification and DNA methylation in
302 the testes of adult F1 male offspring. Specifically, LN males displayed an elevated expression of the
303 RNA methylation regulators Alpha-ketoglutarate-dependent dioxygenase (*Fto;* Figure 7A, P =
304 0.045), N6-adenosine-methyltransferase subunit (*Mettl3*; Figure 7B, P = 0.051) and N6-adenosine-
305 methyltransferase non-catalytic subunit (*Mettl14*; Figure 7C, P = 0.05) when compared to LL males.
306 Similarly, elevated expression of Histone deacetylase 1 (*Hdac1*; Figure 7D, P = 0.011), Histone
307 deacetylase 2 (*Hdac2*; Figure 7E, P = 0.055), and Lysine-specific demethylase 3A (*Kdm3a*; Figure
308 7E, P = 0.055) were observed in LN male testes when compared to LL males. Differences between
309 LL and LN offspring were also observed for the testicular expression of DNA methytransferase 1
310 (*Dnmt1*; Figure 7G, P = 0.003) and DNA methytransferase 3B (*Dnmt3b;* Figure 7I, P = 0.004), though
311 no difference in the expression of *Dnmt3a* (Figure 7H) or *Dnmt3b* (Figure 7J) were observed.
312 Analysis of testicular tissue DNA revealed NL testes had a lower percentage global DNA methylation
313 when compared to LL and LN testes (Figure 7K; P < 0.025) which was not different to NN testes.
314 Finally, while NN, LL and NL testes displayed non-significant positive correlations between *Dnmt1*
315 expression and DNA methylation (NN r = 0.38, P = 0.35; LL r = 0.63, P = 0.09; NL r = 0.28, P =
316 0.51) LN offspring displayed a non-significant negative correlation (r = -0.44, P = 0.28) (Figure 7L).
317
318 ***3.5 Impact of paternal diet on F2 offspring growth and ACE activity***
319 We observed differential expression of epigenetic regulators in F1 male offspring testes. Furthermore,
320 we identified significant changes in F1 offspring physiology at 3 weeks of age that perpetuated into
321 adulthood mirroring our previous studies (Watkins *et al.*, 2008; Watkins & Sinclair, 2014; Watkins
322 *et al.*, 2018) in which early (first three weeks of life) perturbations in development and physiology
323 correlate significantly with adult growth, adiposity, metabolic ill-health and cardiovascular
324 dysfunction. Therefore, we determined whether changes in offspring growth and RAS phenotype

were propagated into an F2 generation via F1 males, and were evident at three weeks of age, suggestive of long-term changes in F2 offspring phenotype. We observed that between birth and three weeks of age, LL, NL and LN offspring (males and females combined) all displayed elevated body weight when compared to NN offspring (Figure 8A; $P < 0.05$). Furthermore, we observed elevated activities of serum ACE in LL, NL and LN offspring (Figure 8B; $P < 0.05$) and kidney ACE in LL offspring (Figure 8C; $P = 0.0004$) when compared to NN offspring. No differences in offspring lung ACE activity were observed across groups (Figure 8D).

4. Discussion

The impact of poor maternal diet on offspring cardiovascular health has been studied in detail (Fleming *et al.*, 2018). However, the consequences of poor paternal diet for the long-term health of his offspring remain poorly defined. In the current study, we combine artificial insemination with vasectomised male mating (Watkins *et al.*, 2018) to define specific sperm and/or seminal plasma programming mechanisms on offspring cardiovascular health. We show that offspring derived from either paternal LPD sperm and/or seminal plasma displayed altered tissue ACE activity, RAS pathway gene expression and vascular dysfunction. Furthermore, we observed juvenile F2 offspring displayed similar changes in growth and tissue ACE activity as for F1 offspring. However, the effects observed differed between the sexes, the offspring at different ages and different treatment groups, such that the effect size tended to be greatest in LN and NL offspring. These results provide new insight into the impact of poor paternal diet at the time of conception on the cardiovascular health of his offspring over multiple generations (Figure 8).

4.1 Effect of paternal diet on offspring RAS homeostasis

The RAS is a hormonal mechanism central to the regulation of bodily sodium levels, blood volume and cardiovascular function and health (Otto, 2017). Data from a range of animal models

manipulating maternal diet during specific periods of gestation reveal significant changes in offspring RAS function and homeostasis correlating with elevated blood pressure (Moritz *et al.*, 2010). Previously, we have shown that paternal LPD results in offspring overgrowth, hypotension, vascular dysfunction and impaired glucose tolerance in mice (Watkins & Sinclair, 2014). Our current study extends these observations identifying significant changes in tissue ACE activity and kidney RAS gene expression. Of significance was the observation that changes in ACE activity became manifest in offspring tissues and serum from just 3 weeks of age. These data suggest that poor paternal diet programs RAS homeostatic set-points either during fetal development or during early juvenile life. Indeed, the RAS has been shown to be important during gestation for both placental (Pringle *et al.*, 2011) and kidney (Pentz *et al.*, 2004) development. Furthermore, fetal and juvenile kidney development and angiotensin receptor expression in pregnant rats and sheep are sensitive to both maternal LPD and glucocorticoid exposure (Moritz *et al.*, 2010). Interestingly, we observed that NL and LN juvenile (both males and females) tended to have lower lung ACE activity profiles when compared to NN and LN offspring. However, in the adult kidney, NL and/or LN offspring had elevated expression profiles for the RAS pathway genes *Ace2, Agtr1a, Agtr1b* and *Atp6ap2*. It is widely appreciated that gene transcript levels are not always reflective of expression at the protein level or in protein activity. Furthermore, while we explored the expression of multiple RAS pathway genes, we only analysed the activity of a single enzyme, ACE. Therefore, further studies would be necessary to confirm the impact of paternal diet fully on offspring RAS regulation and their connection of offspring blood pressure. Furthermore, in contrast to our previous study (Watkins & Sinclair, 2014), we employed the use of superovulation and artificial insemination in our current study. As all treatment groups were generated using the same artificial and superovulation techniques, it is likely that any differences in postnatal phenotype between treatment groups can be attributed to the dietary origin of the sperm and/or seminal plasma. However, further studies would be needed to separate the superovulation and artificial insemination effects from those programmed by paternal dietary-modified semen.

4.2 Paternal diet impairs offspring vascular function

A second key observation was that poor paternal diet altered adult offspring vascular function. Significant impairments in offspring vaso-constriction and -dilatory responses have been observed in multiple models of developmental programming (Watkins *et al.*, 2010; Loche *et al.*, 2018; Yeung *et al.*, 2018). Recently, studies have begun to define the association between poor paternal diet and offspring cardiovascular health (Bromfield *et al.*, 2014; McPherson *et al.*, 2016). Previously, we demonstrated that paternal LPD resulted in adult offspring hypotension in association with reduced vascular responsiveness (Watkins & Sinclair, 2014). In our current study, we observed that pre-incubation of mesenteric artery segments with the nitric oxide synthesis inhibitor, L-NAME, had minimal impact on ACh-mediated vasodilation responses in LL and LN male offspring. However, in NN and NL males, following L-NAME preincubation, ACh-mediated vasodilatation was significantly reduced. In contrast, all experimental female groups displayed significantly greater ACh-mediated vasodilatory responses following L-NAME pre-incubation when compared to NN females. Interestingly, NL and LN females also displayed a significantly greater vasodilation responses when compared to LL females. These observations suggest an additive effect in vascular impairment when the sperm and the seminal fluid are from males fed different diets. We also observed increased responsiveness in male arteries to the nitric oxide donor SNP.

The differential cholinergic and nitric oxide vasodilatory responses observed in both male and female offspring suggest an augmented role of endothelium derived hyperpolarization (EDH). ACh-mediated vasodilatory response is a result of signalling through multiple pathways including eNOS, gap junctions, CYP450 and voltage-gated potassium channels (Freed & Gutterman, 2017). In eNOS knockout mice, upregulation of signalling through the EDH pathway is observed in order to maintain moderate vasodilator responses (Manicam *et al.*, 2017). Furthermore, studies show different isoforms of nitric oxide synthase e.g. nNOS can compensate for the lack of endothelial nitric oxide synthase

to maintain vascular function (Lamping *et al.*, 2000). Interestingly, in other disease states associated with microvascular complications, such as type 2 diabetes, enhanced EDH-mediated vasodilatation is also observed (Pannirselvam *et al.*, 2006). Indeed, we demonstrated previously that LL, NL and LN offspring all displayed glucose intolerance and non-alcoholic fatty liver disease phenotypes (Watkins *et al.*, 2018). Interestingly, mirroring impairments in offspring metabolic health, offspring derived from sperm and seminal plasma from males fed different diets (NL and LN) displayed the largest impairments in vascular function. We propose that a dichotomy between the sperm mediated programming of the embryo and the seminal plasma mediated priming of the uterine environment has the greatest impact on offspring growth, metabolism and vascular health. However, as the same phenotype or direction of effect was not evident in all our analyses, it is conceivable that other mechanisms of programming may underlie our paternal diet model. From our initial analysis, we can cannot discount roles of phosphodiesterase, adenylate cyclase, calcium-activated potassium channels, prostacyclins and/or eicosanoids. Therefor a series of additional experiments with specific activators/inhibitors such as cilostamide , cilostazol, forskolin, apamin, charybdotoxin or the removal of the endothelium from the vessels would be needed to define not only the precise roles of EDH, NO and non-NO signalling mechanisms in offspring cardiovascular homeostasis but also define the precise sperm and seminal plasma programming mechanisms involved.

4.3 Paternal diet alters F1 testicular epigenetic status

Paternal transgenerational programming of offspring phenotype has been observed in both human (Pembrey *et al.*, 2006) and animal model studies (Pentinat *et al.*, 2010; Fullston *et al.*, 2013). Epigenetic mechanisms afford a plausible pathway by which paternal well-being can impact on multiple generations. Previously, we demonstrated that sperm from LPD males displayed global hypomethylation in association with reduced testicular expression of DNA methyltransferases and 1-carbon metabolism regulators (Watkins *et al.*, 2018). Further to changes in sperm DNA methylation, changes in histone and RNA modifications are believed to afford additional layers of epigenetic

regulation. In our study, we observed modified expression of central epigenetic regulators in F1 adult offspring testes, programmed in a sperm and/or seminal plasma specific manner. We observed a persistent profile whereby LN males displayed higher relative expression compared to other groups. Specifically, LN males had a significantly higher expression when compared to LL males for the RNA methylation regulators *Fto*, *Mettl3* and *Mettl14*, the histone modifiers *Hdac1*, *Hdac2* and *Kdm3a* and the DNA methylation regulators *Dnmt1* and *Dnmt3b*. N^6-adenosine methylation (m^6A) is the most prevalent modification to mammalian RNA and is regulated by a series of methyltransferases and demethylases (Yue *et al.*, 2015). In humans, increased expression of *FTO, METTL3* and *METTL14*, as well as levels of m^6A, have been identified in the sperm of infertile men (Yang *et al.*, 2016), in addition to significant changes in both histone modification and DNA methylation status (Rahiminia *et al.*, 2018). Therefore, disruption of the testicular epigenetic status in F1 male offspring could provide one mechanism through which paternal transgenerational programming occurs. Indeed, we observed significantly altered growth, serum ACE activity and kidney ACE activity in juvenile F2 offspring suggesting poor paternal diet could affect offspring phenotype across more than one generation. Interestingly, growth and ACE activity profiles in F2 neonates did not match that of F1 offspring. Similar effects have been observed in response to paternal hyperglycemia (Pentinat *et al.*, 2010), suggesting offspring phenotype may be more influenced by dynamic epigenetic mechanisms as opposed to static, heritable modifications to DNA sequences. However, as we only assessed F2 programming via the male lineage and only up to 3 weeks of age, further studies are needed to confirm whether similar modes of programming are observed via the female lineage and the precise epigenetic changes programmed within F1 offspring. In addition, further genome-wide characterisation of F1 generation testicular tissue would be needed to define fully the epigenetic changes linking F1 and F2 generation physiology in response to paternal diet.

4.4 Conclusions

Our current study demonstrates sub-optimal paternal diet at the time of conception programs offspring vascular function and RAS pathway function via sperm and/or seminal plasma specific mechanisms. These observations are in agreement with our recent study where offspring growth and metabolic health were also programmed via both sperm epigenetic and seminal plasma-specific processes (Watkins *et al.*, 2018). We feel that our study has relevance for human health in relation to perturbed early developmental processes. Many human assisted reproductive techniques occur either in seminal plasma-free environments and/or where embryos are transferred into a uterine environment that has not been primed by seminal plasma. Indeed, studies are now identifying significant changes in cardio-metabolic health of children derived from assisted reproductive techniques (Meister *et al.*, 2018).

References

Aoki VW, Emery BR, Liu L & Carrell DT. (2006). Protamine levels vary between individual sperm cells of infertile human males and correlate with viability and DNA integrity. *J Androl* **27,** 890-898.

Borges E, Jr., Braga D, Provenza RR, Figueira RCS, Iaconelli A, Jr. & Setti AS. (2018). Paternal lifestyle factors in relation to semen quality and in vitro reproductive outcomes. *Andrologia***,** e13090.

Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ & Robertson SA. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci U S A* **111,** 2200-2205.

Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, Bock C, Li C, Gu H, Zamore PD, Meissner A, Weng Z, Hofmann HA, Friedman N & Rando OJ. (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* **143,** 1084-1096.

Cropley JE, Eaton SA, Aiken A, Young PE, Giannoulatou E, Ho JWK, Buckland ME, Keam SP, Hutvagner G, Humphreys DT, Langley KG, Henstridge DC, Martin DIK, Febbraio MA & Suter CM. (2016). Male-lineage transmission of an acquired metabolic phenotype induced by grand-paternal obesity. *Mol Metab* **5,** 699-708.

Dasinger JH, Davis GK, Newsome AD & Alexander BT. (2016). Developmental Programming of Hypertension: Physiological Mechanisms. *Hypertension* **68,** 826-831.

Docherty CC, Kalmar-Nagy J, Engelen M & Nathanielsz PW. (2001). Development of fetal vascular responses to endothelin-1 and acetylcholine in the sheep. *American journal of physiology Regulatory, integrative and comparative physiology* **280,** R554-562.

Fleming TP, Watkins AJ, Velazquez MA, Mathers JC, Prentice AM, Stephenson J, Barker M, Saffery R, Yajnik CS, Eckert JJ, Hanson MA, Forrester T, Gluckman PD & Godfrey KM. (2018). Origins of lifetime health around the time of conception: causes and consequences. *Lancet* **391,** 1842-1852.

Freed JK & Gutterman DD. (2017). Communication Is Key: Mechanisms of Intercellular Signaling in Vasodilation. *J Cardiovasc Pharmacol* **69,** 264-272.

Fullston T, Ohlsson Teague EM, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, Print CG, Owens JA & Lane M. (2013). Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27,** 4226-4243.

Gray C, Harrison CJ, Segovia SA, Reynolds CM & Vickers MH. (2015). Maternal salt and fat intake causes hypertension and sustained endothelial dysfunction in fetal, weanling and adult male resistance vessels. *Scientific reports* **5,** 9753.

Grundy D. (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. *The Journal of physiology* **593,** 2547-2549.

Hur SSJ, Cropley JE & Suter CM. (2017). Paternal epigenetic programming: evolving metabolic disease risk. *J Mol Endocrinol* **58,** R159-R168.

Jenkins TG, Aston KI, Meyer TD, Hotaling JM, Shamsi MB, Johnstone EB, Cox KJ, Stanford JB, Porucznik CA & Carrell DT. (2016). Decreased fecundity and sperm DNA methylation patterns. *Fertility and sterility* **105,** 51-57 e51-53.

Lamping KG, Nuno DW, Shesely EG, Maeda N & Faraci FM. (2000). Vasodilator mechanisms in the coronary circulation of endothelial nitric oxide synthase-deficient mice. *American journal of physiology Heart and circulatory physiology* **279,** H1906-1912.

Liu WM, Pang RT, Chiu PC, Wong BP, Lao K, Lee KF & Yeung WS. (2012). Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proceedings of the National Academy of Sciences of the United States of America* **109,** 490-494.

Loche E, Blackmore HL, Carpenter AA, Beeson JH, Pinnock A, Ashmore TJ, Aiken CE, de Almeida-Faria J, Schoonejans JM, Giussani DA, Fernandez-Twinn DS & Ozanne SE. (2018). Maternal diet-induced obesity programmes cardiac dysfunction in male mice independently of post-weaning diet. *Cardiovasc Res* **114,** 1372-1384.

Lucas ES & Watkins AJ. (2017). The Long-Term Effects of the Periconceptional Period on Embryo Epigenetic Profile and Phenotype; The Paternal Role and His Contribution, and How Males Can Affect Offspring's Phenotype/Epigenetic Profile. *Advances in experimental medicine and biology* **1014,** 137-154.

Lucas ES, Watkins AJ, Cox AL, Marfy-Smith SJ, Smyth N & Fleming TP. (2011). Tissue-specific selection of reference genes is required for expression studies in the mouse model of maternal protein undernutrition. *Theriogenology* **76,** 558-569.

Manicam C, Ginter N, Li H, Xia N, Goloborodko E, Zadeh JK, Musayeva A, Pfeiffer N & Gericke A. (2017). Compensatory Vasodilator Mechanisms in the Ophthalmic Artery of Endothelial Nitric Oxide Synthase Gene Knockout Mice. *Scientific reports* **7,** 7111.

Mao C, Liu R, Bo L, Chen N, Li S, Xia S, Chen J, Li D, Zhang L & Xu Z. (2013). High-salt diets during pregnancy affected fetal and offspring renal renin-angiotensin system. *The Journal of endocrinology* **218,** 61-73.

McPherson NO, Fullston T, Kang WX, Sandeman LY, Corbett MA, Owens JA & Lane M. (2016). Paternal under-nutrition programs metabolic syndrome in offspring which can be reversed by antioxidant/vitamin food fortification in fathers. *Sci Rep* **6,** 27010.

Meister TA, Rimoldi SF, Soria R, von Arx R, Messerli FH, Sartori C, Scherrer U & Rexhaj E. (2018). Association of Assisted Reproductive Technologies With Arterial Hypertension During Adolescence. *J Am Coll Cardiol* **72,** 1267-1274.

Moritz KM, Cuffe JS, Wilson LB, Dickinson H, Wlodek ME, Simmons DG & Denton KM. (2010). Review: Sex specific programming: a critical role for the renal renin-angiotensin system. *Placenta* **31 Suppl,** S40-46.

Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA & Morris MJ. (2010). Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature* **467,** 963-966.

583 Oakberg EF. (1956). Duration of spermatogenesis in the mouse and timing of stages of the cycle of
584 the seminiferous epithelium. *Am J Anat* **99,** 507-516.
585
586 Ornellas F, Bringhenti I, Mattos B, Mandarim-de-Lacerda CA & Aguila MB. (2018). Father's obesity
587 programs the adipose tissue in the offspring via the local renin-angiotensin system and
588 MAPKs pathways, especially in adult male mice. *European journal of nutrition* **57,** 1901-
589 1912.
590
591 Otto CM. (2017). Heartbeat: Renin-angiotensin system blockade for prevention of cardiovascular
592 disease. *Heart* **103,** 1305-1307.
593
594 Pannirselvam M, Ding H, Anderson TJ & Triggle CR. (2006). Pharmacological characteristics of
595 endothelium-derived hyperpolarizing factor-mediated relaxation of small mesenteric arteries
596 from db/db mice. *Eur J Pharmacol* **551,** 98-107.
597
598 Pavlinkova G, Margaryan H, Zatecka E, Valaskova E, Elzeinova F, Kubatova A, Bohuslavova R &
599 Peknicova J. (2017). Transgenerational inheritance of susceptibility to diabetes-induced male
600 subfertility. *Sci Rep* **7,** 4940.
601
602 Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, Golding J & Team AS.
603 (2006). Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* **14,**
604 159-166.
605
606 Pentinat T, Ramon-Krauel M, Cebria J, Diaz R & Jimenez-Chillaron JC. (2010). Transgenerational
607 inheritance of glucose intolerance in a mouse model of neonatal overnutrition. *Endocrinology*
608 **151,** 5617-5623.
609
610 Pentz ES, Moyano MA, Thornhill BA, Sequeira Lopez ML & Gomez RA. (2004). Ablation of renin-
611 expressing juxtaglomerular cells results in a distinct kidney phenotype. *American journal of*
612 *physiology Regulatory, integrative and comparative physiology* **286,** R474-483.
613
614 Pringle KG, Tadros MA, Callister RJ & Lumbers ER. (2011). The expression and localization of the
615 human placental prorenin/renin-angiotensin system throughout pregnancy: roles in
616 trophoblast invasion and angiogenesis? *Placenta* **32,** 956-962.
617
618 Rahiminia T, Yazd EF, Fesahat F, Moein MR, Mirjalili AM & Talebi AR. (2018). Sperm chromatin
619 and DNA integrity, methyltransferase mRNA levels, and global DNA methylation in
620 oligoasthenoteratozoospermia. *Clin Exp Reprod Med* **45,** 17-24.
621
622 Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K & Robertson SA. (2012). Seminal
623 fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the
624 human cervix after coitus. *J Immunol* **188,** 2445-2454.
625
626 Soubry A, Guo L, Huang Z, Hoyo C, Romanus S, Price T & Murphy SK. (2016). Obesity-related
627 DNA methylation at imprinted genes in human sperm: Results from the TIEGER study. *Clin*
628 *Epigenetics* **8,** 51.
629
630 Stuppia L, Franzago M, Ballerini P, Gatta V & Antonucci I. (2015). Epigenetics and male
631 reproduction: the consequences of paternal lifestyle on fertility, embryo development, and
632 children lifetime health. *Clin Epigenetics* **7,** 120.
633

Torrens C, Snelling TH, Chau R, Shanmuganathan M, Cleal JK, Poore KR, Noakes DE, Poston L, Hanson MA & Green LR. (2009). Effects of pre- and periconceptional undernutrition on arterial function in adult female sheep are vascular bed dependent. *Experimental physiology* **94,** 1024-1033.

Vickers MH. (2014). Developmental programming and transgenerational transmission of obesity. *Ann Nutr Metab* **64 Suppl 1,** 26-34.

Watkins AJ, Dias I, Tsuro H, Allen D, Emes RD, Moreton J, Wilson R, Ingram RJM & Sinclair KD. (2018). Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. *Proc Natl Acad Sci U S A* **115,** 10064-10069.

Watkins AJ, Lucas ES, Torrens C, Cleal JK, Green L, Osmond C, Eckert JJ, Gray WP, Hanson MA & Fleming TP. (2010). Maternal low-protein diet during mouse pre-implantation development induces vascular dysfunction and altered renin-angiotensin-system homeostasis in the offspring. *The British journal of nutrition* **103,** 1762-1770.

Watkins AJ & Sinclair KD. (2014). Paternal low protein diet affects adult offspring cardiovascular and metabolic function in mice. *American journal of physiology Heart and circulatory physiology* **306,** H1444-1452.

Watkins AJ, Sirovica S, Stokes B, Isaacs M, Addison O & Martin RA. (2017). Paternal low protein diet programs preimplantation embryo gene expression, fetal growth and skeletal development in mice. *Biochimica et biophysica acta* **1863,** 1371-1381.

Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, Wilkins A, Perry VH, Sheth B, Kwong WY, Eckert JJ, Wild AE, Hanson MA, Osmond C & Fleming TP. (2008). Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. *Biology of reproduction* **78,** 299-306.

Wei Y, Yang CR, Wei YP, Zhao ZA, Hou Y, Schatten H & Sun QY. (2014). Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals. *Proceedings of the National Academy of Sciences of the United States of America* **111,** 1873-1878.

Yang Y, Huang W, Huang JT, Shen F, Xiong J, Yuan EF, Qin SS, Zhang M, Feng YQ, Yuan BF & Liu SM. (2016). Increased N6-methyladenosine in Human Sperm RNA as a Risk Factor for Asthenozoospermia. *Scientific reports* **6,** 24345.

Yeung KR, Sunderland N, Lind JM, Heffernan S, Pears S, Xu B, Hennessy A & Makris A. (2018). Increased salt sensitivity in offspring of pregnancies complicated by experimental preeclampsia. *Clin Exp Pharmacol Physiol*.

Yue Y, Liu J & He C. (2015). RNA N6-methyladenosine methylation in post-transcriptional gene expression regulation. *Genes & development* **29,** 1343-1355.

Zhang YP, Huo YL, Fang ZQ, Wang XF, Li JD, Wang HP, Peng W, Johnson AK & Xue B. (2018). Maternal high-fat diet acts on the brain to induce baroreflex dysfunction and sensitization of angiotensin II-induced hypertension in adult offspring. *American journal of physiology Heart and circulatory physiology* **314,** H1061-H1069.

Additional information

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Author contributions

Conception and design of the experiments: H.L.M. and A.J.W.

Collection and analysis of data: H.L.M, P.P, A.A, N.U, R.P, Y.D and A.J.W.

Interpretation of data: H.L.M, P.P, A.A, N.U, R.P, Y.D and A.J.W.

Drafting the article: H.L.M. and A.J.W.

Revising article critically for intellectual content: H.L.M. and A.J.W.

All authors read and approved the manuscript before submission. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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711 T**able 1: List of primers used for RT-qPCR studies**
712
713

Gene Name	Gene Symbol	Accession Number	Primer Sequences		Amplicon Length
			Forward Primer	Reverse Primer	
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	*Ace*	NM_207624.5	tctgcttccccaacaagact	aggatgttggtgagctctgg	61
Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	*Ace2*	NM_001130513.1	tgtagaacgtaccttcgcagag	gggctgatgtaggaagggta	99
Angiotensin II receptor, type 1a	*Agtr1a*	NM_177322.3	actcacagcaaccctccaag	ctcagacactgttcaaaatgca	62
Angiotensin II receptor, type 1a	*Agtr1b*	NM_175086.3	cgccagcagcactgtaga	gggggtgaattcaaaatg	93
angiotensin II receptor, type 2	*Agtr2*	NM_007429.4	ggagctcggaactgaaagc	ctgcagcaactccaaattctt	131
ATPase, H+ transporting, lysosomal accessory protein 2	*Atp6ap2*	NM_027439.4	gggtggataaactggcacttc	tggaatttgcaacgctgtc	93
Renin 1 structural	*Ren1*	NM_031192.3	cccgacatttcctttgacc	tgtgcacagcttgtctctcc	96
DNA methyltransferase 1	*Dnmt1*	NM_010066.3	gctaccagtgcacctttggt	atgatggccctccttcgt	73
DNA methyltransferase 3a	*Dnmt3a*	NM_007872.4	acacagggcccgttacttct	tcacagtggatgccaaagg	65
DNA methyltransferase 3b	*Dnmt3b*	AF151969.	gcctgcaagacttcttcactact	ggtacaacttgggtggctca	63
DNA methyltransferase 3L	*Dnmt3L*	NM_001081695.1	aaccgacggagcattgaa	ccgagtgtacacctggagagt	60
Alpha-ketoglutarate-dependent dioxygenase FTO	*Fto*	NM_011936.2	tctgtctgccatcctggtc	tggtaaagtccggacgactc	94
N6-adenosine-methyltransferase subunit METTL3	*Mettl3*	NM_019721.2	taaaccacgggaaggaacac	ttatgactggtggaacgaacc	112
N6-adenosine-methyltransferase non-catalytic subunit	*Mettl14*	NM_201638.2	gcagcacctcggtcatttat	tcttctgtaaccccactttcg	93
Histone deacetylase 1	*Hdac1*	NM_008228.2	tggtctctaccgaaaaatggag	tcatcactgtggtacttggtca	78
Histone deacetylase 2	*Hdac2*	NM_008229.2	tgctgttcatgaagacagtgg	tttgtctgatgctcgaatgg	76
Lysine (K)-specific demethylase 3A	*Kdm3a*	NM_173001.3	tctgggatggatttgaagatg	aaacctggaaggcatcatgt	125
Phosphoglycerate kinase 1	*Pgk1*	NM_008828	tacctgctggctggatgg	cacagcctcggcatatttct	65

Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	*Sdha*	NM_023281	tgttcagttccaccccaca	tctccacgacacccttctgt	66
TATA box binding protein	*Tbp*	NM_013684.3	gggagaatcatggaccagaa	gatgggaattccaggagtca	90
Tubulin alpha-1A chain	*Tuba1a*	NM_011653	ctggaacccacggtcatc	gtggccacgagcatagttatt	114

Figure legends

Figure 1: Schematic diagram of the findings of this study showing the programming of offspring vascular function and RAS activity in response to paternal diet via sperm and seminal plasma specific mechanisms. F1 offspring were derived from the artificial insemination of females with sperm from either NPD or LPD fed males prior to them being mated overnight with NPD or LPD fed vasectomised males. In total, 8 F1 litters per treatment group were created. All stud (both sperm donor and vasectomised) males and each dam was only used to create one litter. For the generation of F2 offspring, 6 F1 males per treatment group (each from separate litters) were mated to females (not from within this study). One litter per F1 male was generated.

Figure 2: Impact of NPD and LPD diet on male growth (A), organ weights (B) vascular responsiveness to phenylephrine (C), acetylcholine (D), isoprenaline (E) and sodium nitroprusside (F) and serum ACE (G), kidney ACE (H) and lung ACE (I) activities of stud males fed either normal protein diet (NPD) or low protein diet (LPD). Data are mean ± SEM. N = 8 males per dietary group. Data were analysed by independent samples or repeated measures *t*-tests, where appropriate. *** $P < 0.001$.

Figure 3: Impact of paternal diet on NN, LL, NL and LN offspring body weight and activity of serum ACE, kidney ACE and lung ACE respectively in male (A-D) and female (E-H) F1 juveniles (3 weeks of age) and male (I-L) and female (M-P) adult offspring. Data are mean ± SEM. N = 9 – 30 males and females for each dietary group from 7 separate litters. Data were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. Different letters denote statistical significance at $P < 0.05$.

Figure 4: Impact of paternal diet on F1 adult (16 weeks of age) NN, LL, NL and LN offspring relative kidney gene expression for *Ace* (A), *Ace2* (B), *Agtr1a* (C), *Agtr1b* (D), *Agtr2* (E), *Atp6ap2* (F) and *Ren1* (G). Data are mean ± SEM. N = 10 (5 males and 5 females for each dietary group from 5 separate litters). Data were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. Different letters denote statistical significance at $P < 0.05$.

Figure 5: Impact of paternal diet on F1 adult (16 weeks of age) NN, LL, NL and LN offspring vascular function. Adult male offspring vascular responses to cumulative concentration responses to phenylephrine (PE) (A), maximal constriction response (B) and pEC50 (C). Adult male offspring vascular responses to cumulative concentration responses to acetylcholine (Ach) (D), maximal relaxation response (E) and pEC50 (F). Adult male offspring vascular responses to cumulative concentration responses to acetylcholine following pre-incubation with L-NAME (Ach+L-NAME) (G), maximal relaxation response (H) and pEC50 (I). Adult male offspring vascular responses to cumulative concentration responses to sodium nitroprusside (SNP) (J), maximal relaxation response (K) and pEC50 (L). Data are mean ± SEM. N = 7 males for each dietary group, each from a separate litter. Data were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. Different letters denote statistical significance at $P < 0.05$.

Figure 6: Impact of paternal diet on adult (16 weeks of age) NN, LL, NL and LN offspring vascular function. Adult female offspring F1 vascular responses to cumulative concentration responses to phenylephrine (PE) (A), maximal constriction response (B) and pEC50 (C). Adult female offspring vascular responses to cumulative concentration responses to acetylcholine (Ach) (D), maximal relaxation response (E) and pEC50 (F). Adult female offspring vascular responses to cumulative concentration responses to acetylcholine following pre-incubation with L-NAME (Ach+L-NAME) (G), maximal relaxation response (H) and pEC50 (I). Adult female offspring vascular responses to cumulative concentration responses to sodium nitroprusside (SNP) (J), maximal relaxation response

(K) and pEC50 (L). Data are mean ± SEM. N = 7 females for each dietary group, each from a separate litter. Data were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. Different letters denote statistical significance at $P < 0.05$.

Figure 7: Impact of paternal diet on adult (16 weeks of age) male NN, LL, NL and LN offspring testicular gene expression. Relative expression of the RNA methylation regulators Fto (A), Mettl3 (B) and Mettl14 (C), the histone modifiers Hdac1 (D), Hdac2 (E) and Kdm3a (F) and the DNA methyltransferases Dnmt1 (G), Dnmt3a (H), Dnmt3b (I) and Dnmt3L (J). Offspring testicular DNA methylation (K) and correlation between Dnmt1 expression and DNA methylation (L). Data are mean ± SEM. N = 8 males for each dietary group, taken from across all litters. Data in A-K were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. Data in L were analysed by Pearson's correlation. Different letters denote statistical significance at $P < 0.05$.

Figure 8: Impact of paternal diet on juvenile (3 weeks of age) NN, LL, NL and LN F2 offspring growth (A), serum ACE (B), kidney ACE (C) and lung ACE (D) activities. Data in A are Z-scores of weight from 40-26 offspring taken from 4-6 litters per treatment group. Data in B, C and D are mean ± SEM, n = 10 offspring (5 males and 5 females) per dietary group. Data were analysed by random effect regression analysis followed by Bonferroni post hoc test for multiple comparisons. No significant differences in litter sex ratio or effect of sex on body weight, was observed between groups. Different letters denote statistical significance at $P < 0.05$.

Figure 1

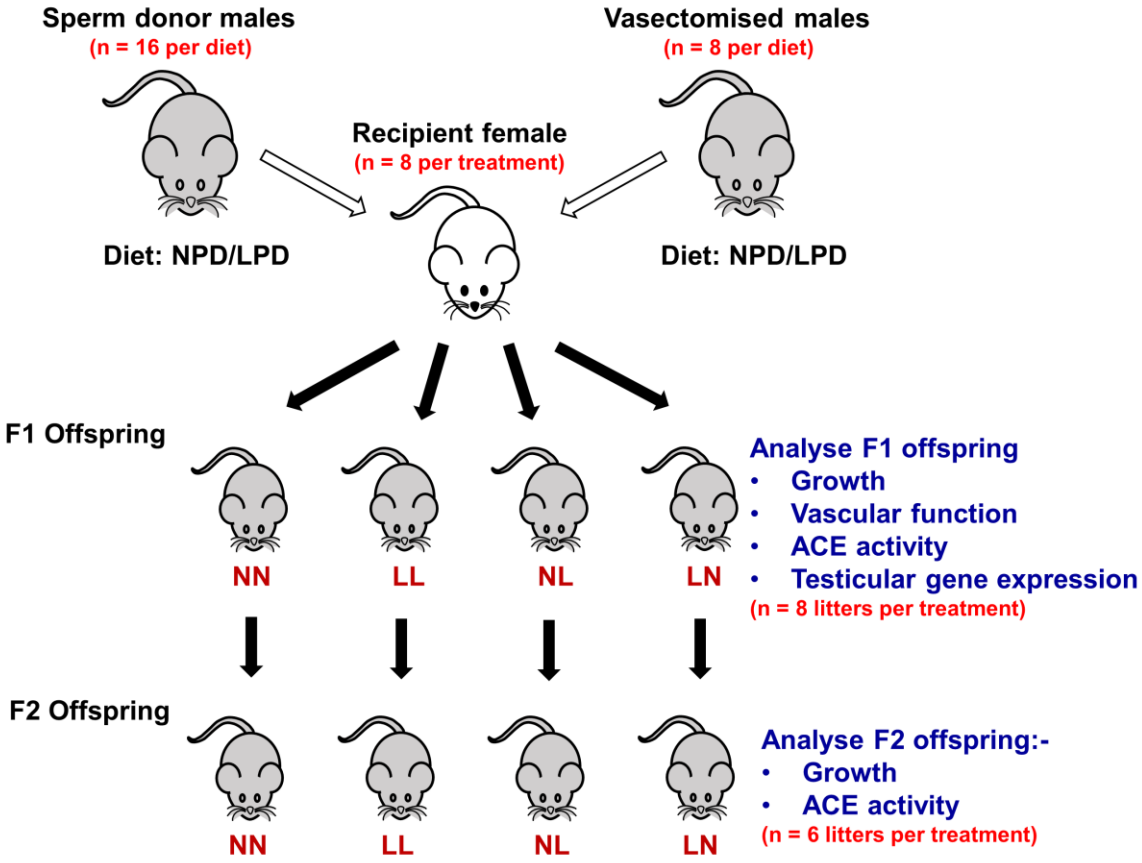

Sperm donor males
(n = 16 per diet)
Vasectomised males
(n = 8 per diet)
Recipient female
(n = 8 per treatment)
Diet: NPD/LPD
Diet: NPD/LPD
F1 Offspring
NN
LL
NL
LN
Analyse F1 offspring
• Growth
• Vascular function
• ACE activity
• Testicular gene expression
(n = 8 litters per treatment)
F2 Offspring
NN
LL
NL
LN
Analyse F2 offspring:-
• Growth
• ACE activity
(n = 6 litters per treatment)

Figure 2

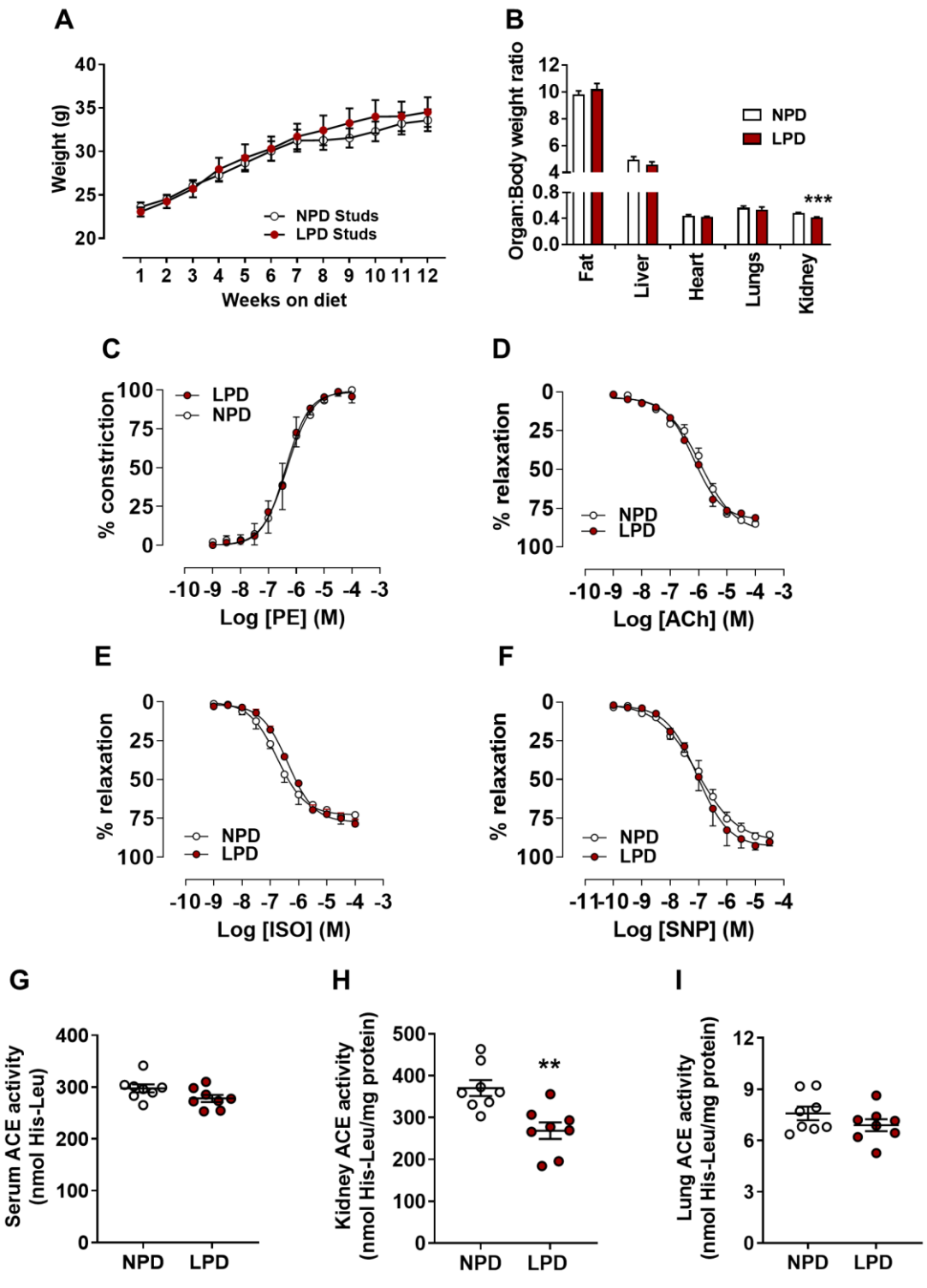

A
Weight (g)
Weeks on diet
NPD Studs
LPD Studs
B
Organ:Body weight ratio
NPD
LPD
***
Fat
Liver
Heart
Lungs
Kidney
C
% constriction
LPD
NPD
Log [PE] (M)
D
% relaxation
NPD
LPD
Log [ACh] (M)
E
% relaxation
NPD
LPD
Log [ISO] (M)
F
% relaxation
NPD
LPD
Log [SNP] (M)
G
Serum ACE activity (nmol His-Leu)
NPD
LPD
H
Kidney ACE activity (nmol His-Leu/mg protein)
**
NPD
LPD
I
Lung ACE activity (nmol His-Leu/mg protein)
NPD
LPD

Figure 3

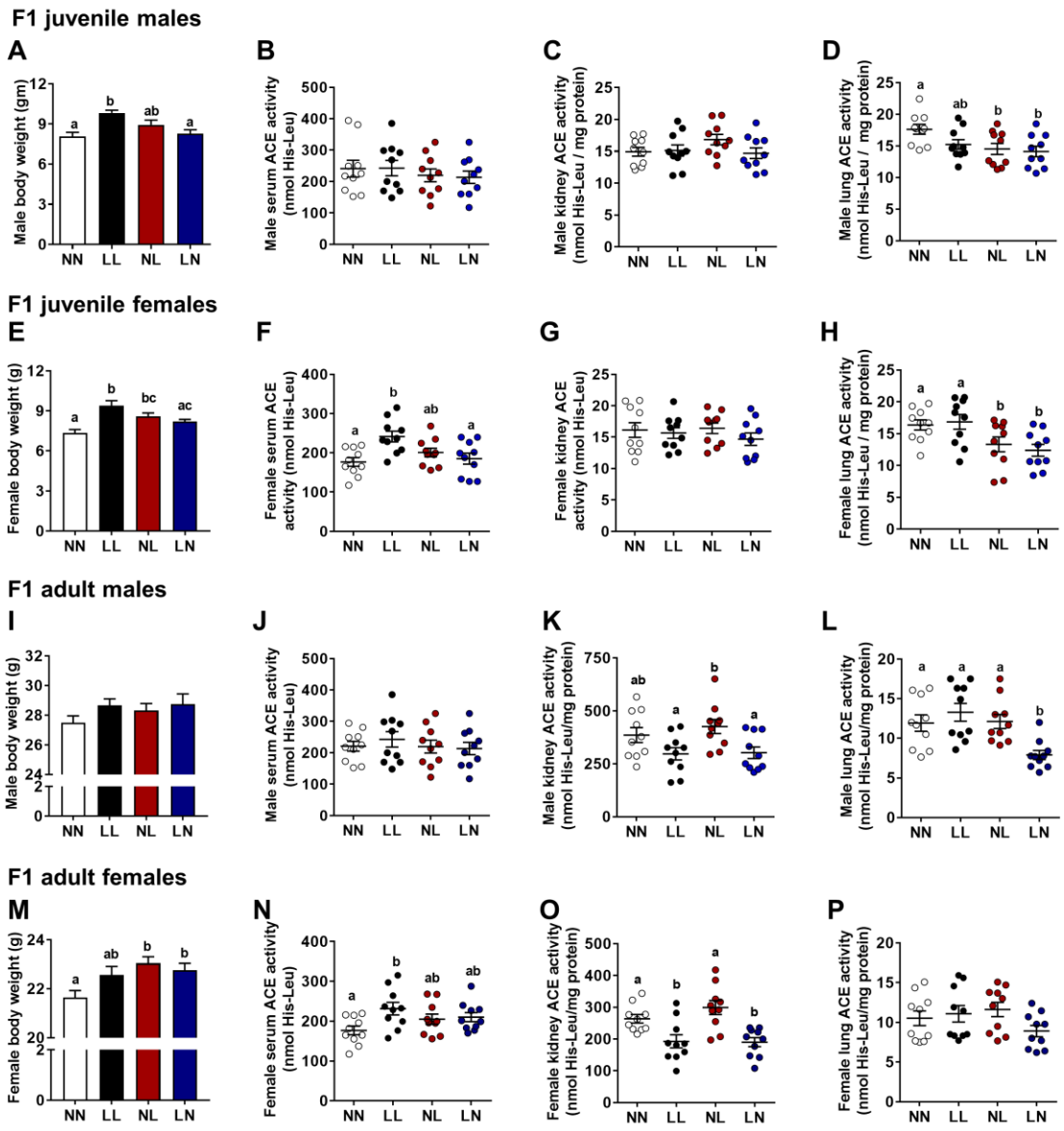

F1 juvenile males
A
Male body weight (gm)
B
Male serum ACE activity (nmol His-Leu)
C
Male kidney ACE activity (nmol His-Leu / mg protein)
D
Male lung ACE activity (nmol His-Leu / mg protein)
F1 juvenile females
E
Female body weight (g)
F
Female serum ACE activity (nmol His-Leu)
G
Female kidney ACE activity (nmol His-Leu)
H
Female lung ACE activity (nmol His-Leu / mg protein)
F1 adult males
I
Male body weight (g)
J
Male serum ACE activity (nmol His-Leu)
K
Male kidney ACE activity (nmol His-Leu/mg protein)
L
Male lung ACE activity (nmol His-Leu/mg protein)
F1 adult females
M
Female body weight (g)
N
Female serum ACE activity (nmol His-Leu)
O
Female kidney ACE activity (nmol His-Leu/mg protein)
P
Female lung ACE activity (nmol His-Leu/mg protein)
NN
LL
NL
LN

Figure 4

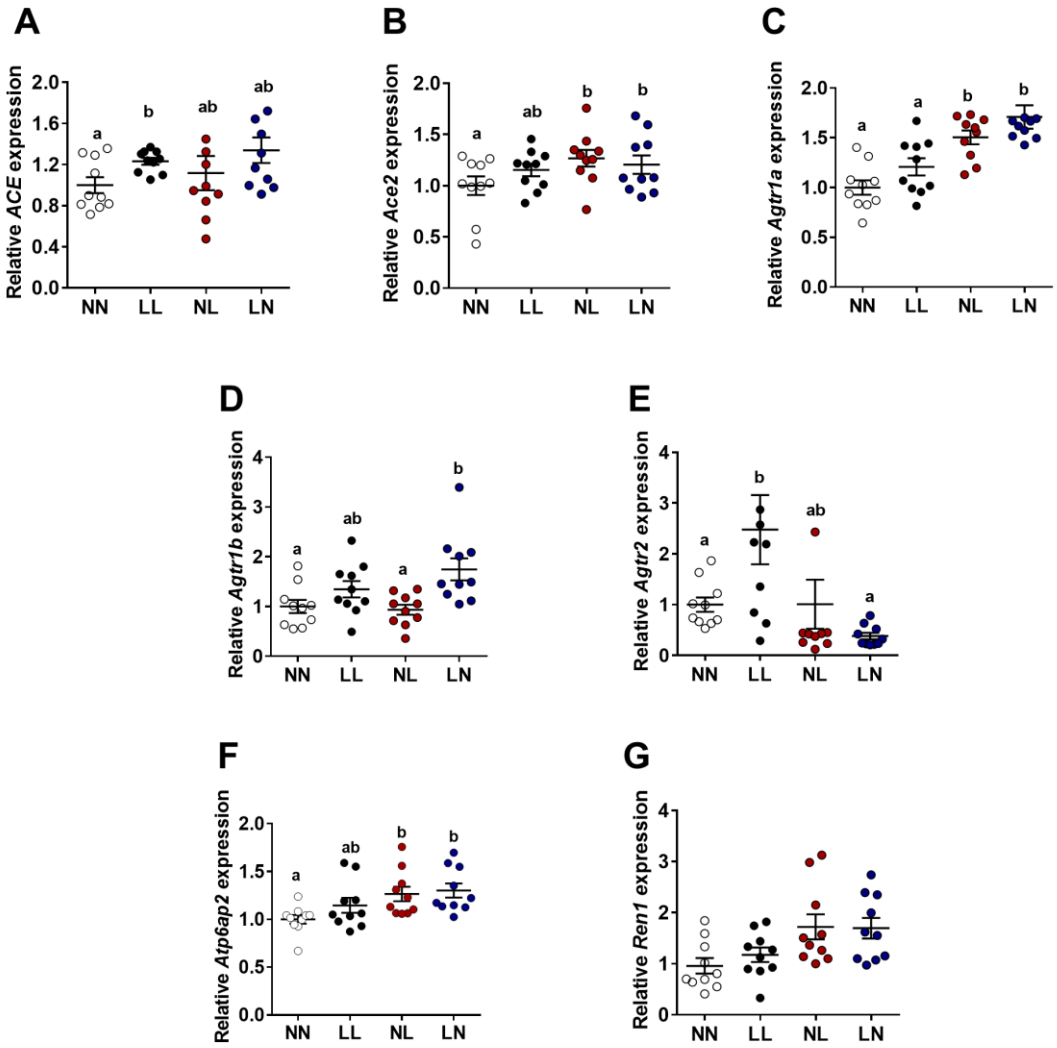

A
Relative ACE expression
2.0
1.6
1.2
0.8
0.4
0.0
a
b
ab
ab
NN
LL
NL
LN
B
Relative Ace2 expression
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
NN
LL
NL
LN
C
Relative Agtr1a expression
2.0
1.5
1.0
0.5
0.0
a
a
b
b
NN
LL
NL
LN
D
Relative Agtr1b expression
4
3
2
1
0
a
ab
a
b
NN
LL
NL
LN
E
Relative Agtr2 expression
4
3
2
1
0
a
b
ab
a
NN
LL
NL
LN
F
Relative Atp6ap2 expression
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
NN
LL
NL
LN
G
Relative Ren1 expression
4
3
2
1
0
NN
LL
NL
LN

Figure 5

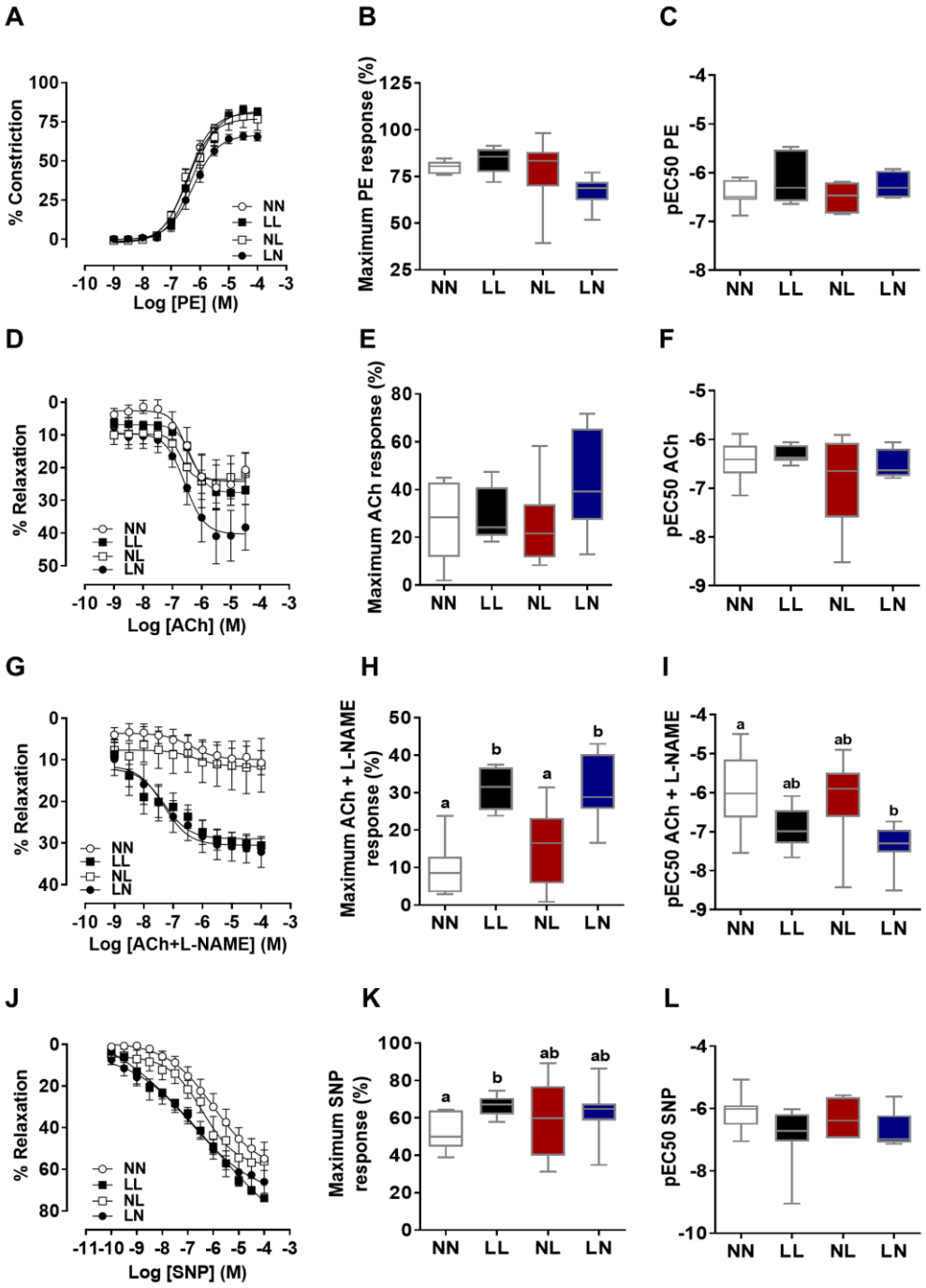

A
% Constriction
Log [PE] (M)
NN
LL
NL
LN
B
Maximum PE response (%)
C
pEC50 PE
D
% Relaxation
Log [ACh] (M)
E
Maximum ACh response (%)
F
pEC50 ACh
G
% Relaxation
Log [ACh+L-NAME] (M)
H
Maximum ACh + L-NAME response (%)
I
pEC50 ACh + L-NAME
J
% Relaxation
Log [SNP] (M)
K
Maximum SNP response (%)
L
pEC50 SNP

Figure 6

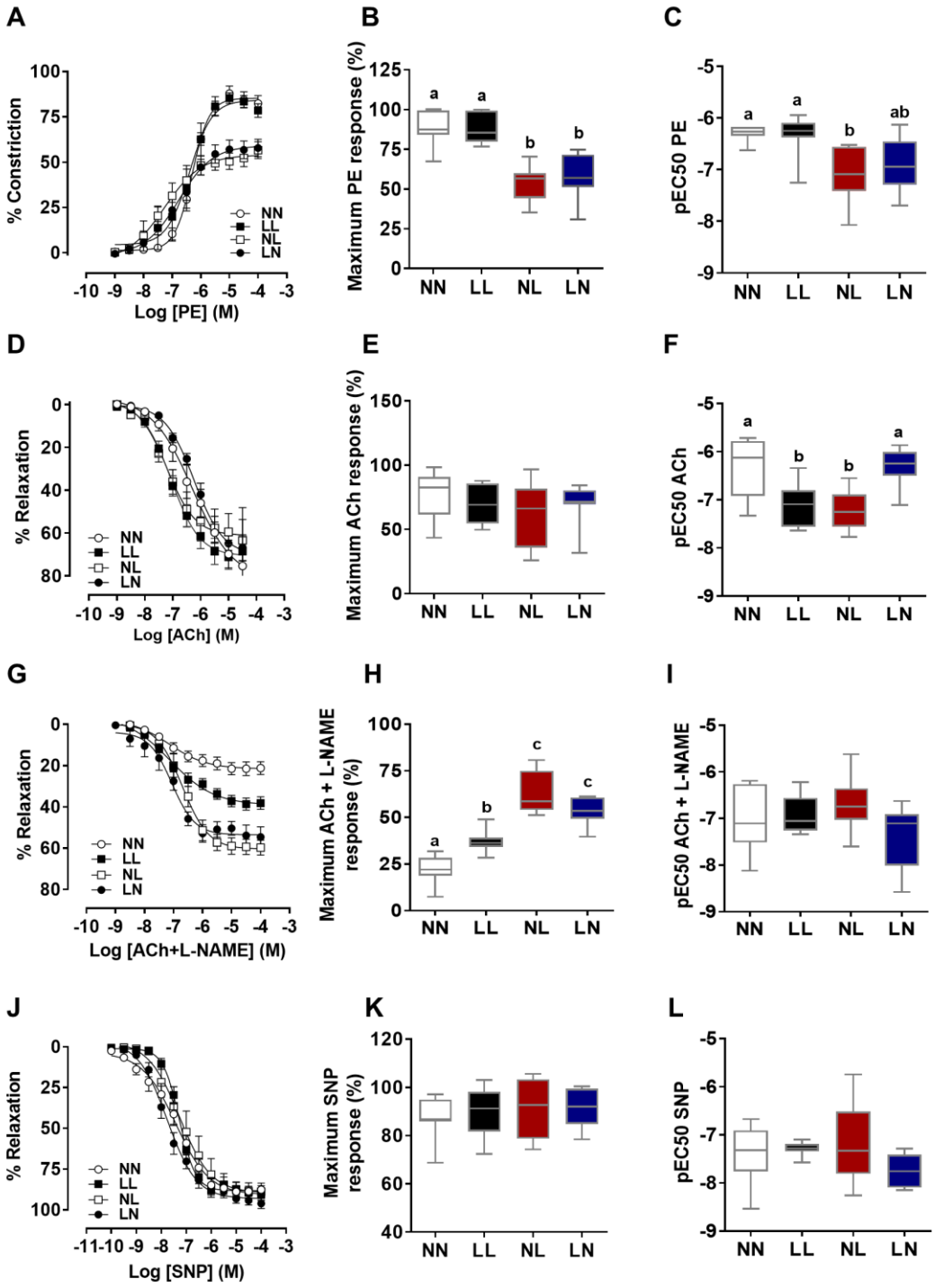

A
% Constriction
Log [PE] (M)
NN
LL
NL
LN
B
Maximum PE response (%)
C
pEC50 PE
D
% Relaxation
Log [ACh] (M)
E
Maximum ACh response (%)
F
pEC50 ACh
G
% Relaxation
Log [ACh+L-NAME] (M)
H
Maximum ACh + L-NAME response (%)
I
pEC50 ACh + L-NAME
J
% Relaxation
Log [SNP] (M)
K
Maximum SNP response (%)
L
pEC50 SNP

Figure 7

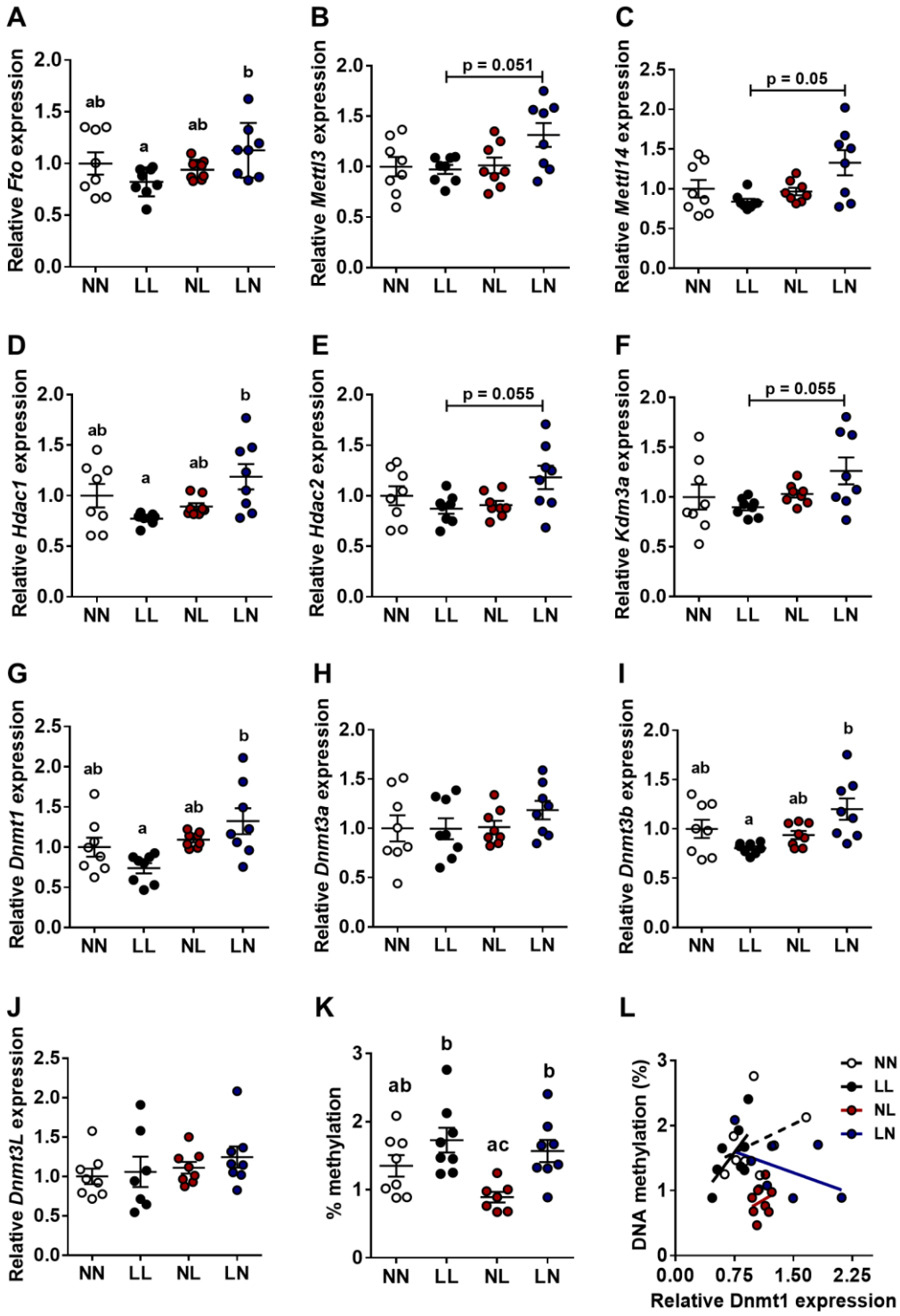
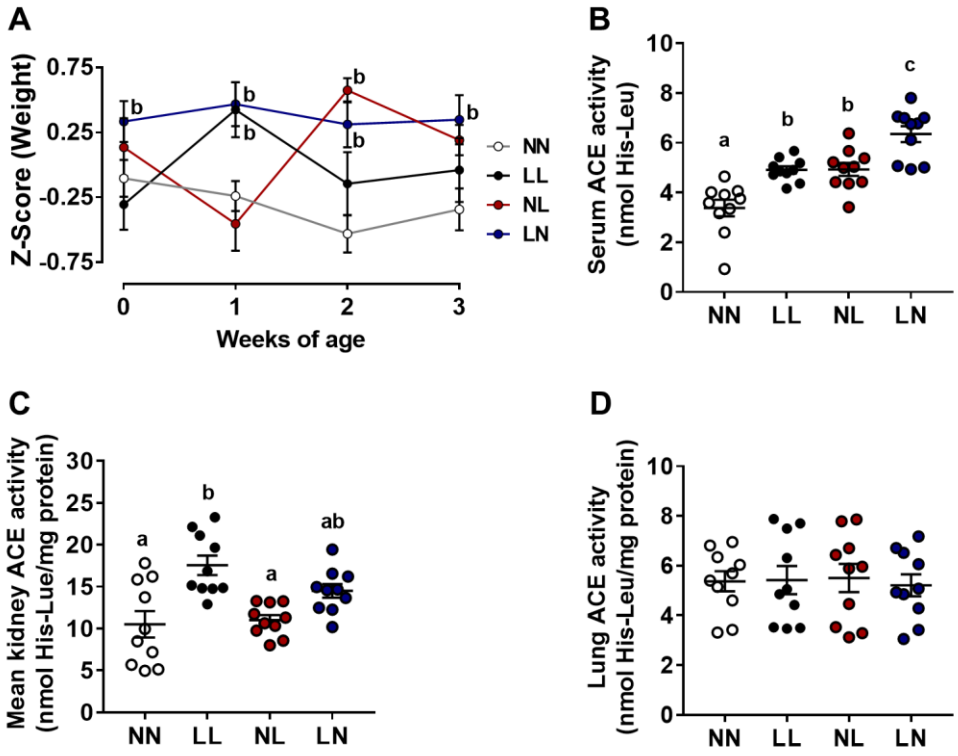

A
Relative Fto expression
ab
a
ab
b
B
Relative Mettl3 expression
p = 0.051
C
Relative Mettl14 expression
p = 0.05
D
Relative Hdac1 expression
E
Relative Hdac2 expression
p = 0.055
F
Relative Kdm3a expression
p = 0.055
G
Relative Dnmt1 expression
H
Relative Dnmt3a expression
I
Relative Dnmt3b expression
J
Relative Dnmt3L expression
K
% methylation
ac
L
DNA methylation (%)
Relative Dnmt1 expression
NN
LL
NL
LN

Figure 8


A
Z-Score (Weight)
0.75
0.25
-0.25
-0.75
0
1
2
3
Weeks of age
NN
LL
NL
LN
B
Serum ACE activity (nmol His-Leu)
C
Mean kidney ACE activity (nmol His-Lue/mg protein)
D
Lung ACE activity (nmol His-Leu/mg protein)