1    **Paternal low protein diet affects adult offspring cardiovascular and metabolic**

2    **function in mice.**

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12    **Abbreviated Running Title**: Paternal low protein diet and adult offspring health in mice

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27      Whilst the association between maternal periconceptional diet and adult offspring

28      health is well characterised, our understanding of the impact of paternal nutrition at the time

29      of conception on offspring phenotype remains poorly defined. Therefore, we determined the

30      effect of a paternal preconception low protein diet (LPD) on adult offspring cardiovascular

31      and metabolic health in mice. Male C57BL/6 mice were fed either normal protein diet (18%

32      casein; NPD) or LPD (9% casein) for 7 weeks prior to mating. At birth, a reduced

33      male:female ratio (P=0.03) and increased male offspring weight (P=0.009) were observed in

34      litters from LPD compared to NPD stud males with no differences in mean litter size. LPD

35      offspring were heavier than NPD offspring at 2 and 3 weeks of age (P<0.02). However, no

36      subsequent differences in body weight were observed. Adult male offspring derived from LPD

37      studs developed relative hypotension (decreased by 9.2 mmHg) and elevated heart rate

38      (P<0.05), whilst both male and female offspring displayed vascular dysfunction and impaired

39      glucose tolerance relative to NPD offspring. At cull (24 weeks), LPD males had elevated

40      adiposity (P=0.04), reduced heart:body weight ratio (P=0.04) and elevated circulating TNF-$\alpha$

41      levels (P=0.015) when compared to NPD males. Transcript expression in offspring heart and

42      liver tissue was reduced for genes involved in calcium signalling (*Adcy, Plcb, Prkcb)* and

43      metabolism (*Fto*) in LPD offspring (P<0.03). These novel data reveal the impact of sub-

44      optimal paternal nutrition on adult offspring cardiovascular and metabolic homeostasis, and

45      provide some insight into the underlying regulatory mechanisms.

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**Introduction**

Studies in humans and animal models have identified strong associations between adult disease risk and environmental perturbations experienced during early development **(21)**. Gamete maturation and developmental events associated with fertilisation and pre-implantation embryo development appear particularly sensitive to changes in environmental conditions **(50)**. Studies from a diverse range of model species including rat **(24)**, sheep **(40)**, and human populations **(10)** have revealed similar phenotypic changes in offspring growth, cardiovascular and metabolic homeostasis following maternal periconceptional environment manipulation. In the sheep, half maintenance feeding prior to conception induced changes in blastocyst transcript levels for genes associated with metabolic activity **(33)**. In the sheep and cow, global maternal gestational undernutrition has been shown to elevate fetal blood pressure **(14)** impair adult offspring glucose tolerance **(45)** and affect offspring growth and adiposity in a sex specific manner **(26)**. Similarly in rodents, the feeding of a maternal low protein diet (LPD) during gestation induces significant changes in offspring birth weight and growth **(53)**, preferences for high-fat foods **(6)**, insulin resistance **(16)** and hypertension and vascular dysfunction **(37)**.

Whilst our understanding of the developmental consequences of manipulating the maternal environment is well defined, the impact of paternal physiology and nutritional status around conception remains largely under-investigated. Studies in humans and mice have demonstrated that increasing male BMI associates with reduced sperm motility **(19)**, increased incidences of sperm abnormality **(23)** and DNA fragmentation **(11)**, and reduced pregnancy rates **(17)**. In mice, offspring metabolic profiles including hepatic lipid and cholesterol biosynthesis at weaning, serum glucose, IGF-1 and corticosterone levels are altered in response to paternal LPD **(9)** or pre-mating fasting **(2)**. Consumption of a high fat **(27)** or high energy **(36)** diet impacts negatively on sperm motility, DNA integrity and blastocyst developmental rates and impairs offspring pancreatic ß-cell function **(28)**. In men, paternal obesity has been shown to associate with decreased blastocyst development

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84 and live birth rate **(5)** and the DNA methylation status of the *IGF2* differentially methylated region

85 in the cord blood of newborn children **(42)**.

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87     Whilst these studies identify intergenerational transmission of metabolic disorders in young

88 offspring through sperm mediated mechanisms, the impact on adult offspring cardiovascular and

89 metabolic phenotype remains unknown. Therefore, the aim of our current study was to determine

90 the impact of a paternal LPD on well-defined markers of adult offspring cardiovascular and

91 metabolic health, focusing on analysis of adult offspring blood pressure, arterial function, in vivo

92 glucose tolerance and the expression of cardiovascular and metabolic regulatory genes.

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94 **Materials and Methods**

95 *Animal Treatments*

96     All mice and experimental procedures were conducted using protocols approved by, and in

97 accordance with, the UK Home Office Animal (Scientific Procedures) Act 1986 and local ethics

98 committee at the University of Nottingham. Virgin male (9 week old) and female (5-9 week old)

99 C57BL/6 mice (Harlan Ltd, Belton, Leicestershire, UK) were maintained for 2 weeks at the

100 University of Nottingham's Bio Support Unit on a 07:00–19:00 light-dark cycle at a temperature of

101 20–22°C with *ad libitum* access to chow (2018 Teklad Global 18% Protein Rodent Diet; Harlan,

102 UK) and water. Weight matched male mice were housed singly and allocated to either a control

103 normal protein diet (NPD; 18 % casein, 42.5% maize starch, 21.3 % sucrose, 10% corn oil, 5%

104 cellulose; n = 8) or isocaloric (calories/gm) low protein diet (LPD; 9% casein, 48.5 maize starch,

105 24.3% sucrose, 10% corn oil, 5% cellulose; n = 8) offered *ad libitum* (Special Dietary Services Ltd,

106 UK; composition published previously **(24, 52)**) for 7 weeks prior to initiation of mating, and

107 maintained on respective diets until cull at 32 weeks of age (**Figure 1**).

108

109        Virgin, chow fed 7-9 week old C57BL/6 females were caged singly with either NPD or LPD

110    studs, with access *ad libitum* to the studs' respective diet. The presence of a vaginal plug the

111    following morning was taken as a sign of mating. Plug positive females were housed singly and

112    maintained on chow until offspring weaning at which time they were culled.  At birth, offspring

113    were weighed and the litter male:female ratio determined. At 3 weeks of age all offspring were

114    weaned, the sexes caged separately per litter with access to chow and water *ad libitum* and allocated

115    randomly tail marks with permanent marker for subsequent weekly tracking of individuals. All

116    offspring were weighed weekly from birth till 24 weeks of age. All studs generated 2 litters each,

117    however, during pre-weaning development 2 NPD and 2 LPD litters, each from separate studs, were

118    lost due to maternal infanticide. As such, a total of 14 litters per dietary treatment were analysed.

119

120    ***Blood Pressure Measurement***

121        Blood pressure (systolic and diastolic) and heart rate were measured in stud males at 11

122    (pre-diet feeding), 17 (pre-mating) and 27 weeks of age, and all generated offspring from all 14

123    litters per treatment group (n = 42 NPD males, 43 NPD females, 27 LPD males and 42 LPD

124    females in total) at 6, 10, 14 and 18 weeks of age using a computerised, non-invasive, tail-cuff

125    system (Kent Scientific, USA). All mice were acclimatised to the experimental room for at least one

126    hour followed by a minimum of 30 minutes warming at 27-30$^{o}$C prior to being placed within the

127    restraining and measurement apparatus for 5 minutes prior to measurement.

128

129    ***Glucose Tolerance Test (GTT)***

130        Offspring glucose tolerance was determined in at least one male and female per litter at 22

131    weeks of age. Offspring were fasted overnight, with access to water *ad libitum,* and weighed

132    immediately prior to GTT. After administration of local anaesthetic (EMLA cream, Eutectic

133    Mixture of Local Anaesthetics, Lidocaine/Prilocaine, AstraZeneca, UK), fasting blood glucose

134    levels were determined in a sample collected from the tail vein using a hand-held glucometer

135 (Freestyle Optium, UK) prior to an intraperitoneal glucose bolus (2g/kg body weight in PBS).

136 Blood samples were collected from the tail vein at 15, 30, 60 and 90 minutes post-bolus for

137 determination of glucose concentration. All animals were returned to their original cage with

138 accesses to food and water *ad libitum*.

139

140 *Mesenteric Artery Vasoreactivity*

141 Offspring (n = 8 pairs of male and female offspring per treatment group, each pair from

142 separate litters) vascular function was assessed at 24 weeks of age in isolated small mesenteric

143 artery segments as described previously **(51)** on a wire myograph (Danish Myo Technology A/S,

144 Denmark). Cumulative concentration response curves (CRCs) were measured for the $\alpha_1$-adrenergic

145 agonist phenylephrine ($10^{-9}$ to $10^{-4}$ mol/L), and after submaximal ($EC_{80}$) pre-constriction with the

146 thromboxane mimetic U46619 (10 mmol/L), the vasodilators acetylcholine (ACh; $10^{-9}$ to $10^{-5}$

147 mol/L) and isoprenaline (ISO; $10^{-10}$ to $10^{-6}$ mol/L) and the nitric oxide donor sodium nitroprusside

148 (SNP; $10^{-11}$ to $10^{-5}$ mol/L) in that order in the same arteries. All drugs were purchased from Sigma

149 (UK).

150

151 *Tissue Sampling*

152 All mice were culled by cervical dislocation. At 32 weeks of age, stud males were culled

153 and blood samples, taken via heart puncture, were allowed to clot on ice prior to centrifugation at

154 10,000 rpm, $4^{o}$C for 10 minutes, after which serum was aliquoted and stored at $-80^{o}$C. Liver,

155 kidneys, heart, lungs, testes and retroperitoneal, gonadal, inguinal and interscapular fat (anatomical

156 locations defined previously **(52))** were removed, weighed and stored at $-80^{o}$C. Left and right

157 caudal epididymi were removed and placed within a pre-warmed 200 µl drop of sperm motility

158 medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30mM HEPES; freshly

159 supplemented with 10 mM lactic acid, 1 mM sodium pyruvate, 20 mg/ml BSA, 25 mM $NaHCO_3$).

160 Epididymi were slashed several times using a 23 gauge needle and left for 15 minutes at $37^{o}$C for

161     sperm to swim out. A sample of sperm was taken for counting using a Neubeur counting chamber

162     prior to assessment of motility. Collected sperm were pipetted under 2 ml of pre-warmed motility

163     medium and left to swim up for one hour at $37^{o}C$. Sperm within the top 1.5 ml of medium were

164     collected and counted as above. At 24 weeks of age, offspring were culled for collection of blood

165     and somatic tissues (as described above).

166

167     *Metabolite and Hormone Measurements*

168     Following cull at 32 weeks of age, stud serum glucose was analysed using a commercial

169     glucose oxidase assay (Sigma, UK) and serum insulin and testosterone levels determined by ELISA

170     (Millipore and R&D Systems respectively, UK). Stud testes were homogenised (50 mM HEPES,

171     150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% SDS) prior to protein level determination (DC

172     assay, Bio-Rad Laboratories, CA, USA). Testicular testosterone levels were determined using a

173     commercial ELISA (R&D Systems). Levels of adiponectin and TNF-α were determined in

174     offspring serum at cull by ELISA (R&D Systems). All assays were conducted in accordance with

175     the manufacturer's instructions and measured on a Benchmark microplate reader (Bio-Rad

176     Laboratories).

177

178     *RNA extraction and transcript expression*

179     RNA was extracted from offspring heart and liver tissues using the RNeasy Mini Kit

180     (QIAGEN, UK) according to manufacturer's instructions. Contaminating genomic DNA was

181     removed by on-column DNase I digestion prior to cDNA synthesis using the ImProm™II kit

182     (Promega, UK) using the included random primers. For Real-Time PCR (RTqPCR), 1 µl (5 ng

183     RNA equivalent) of cDNA was added to a mastermix comprising 10 µl mastermix (2X Precision

184     SYBRgreen Mastermix; PrimerDesign, UK), 0.7 µl primer mix (5 µM forward and reverse primers)

185     and 8.3 µl water per reaction. Water was used in place of cDNA as a no template control.

186     Amplification and detection was performed using a Lightcycler 480 (Roche) and data acquired

187 using the LightCycler SW 1.5.lnk software. A post-amplification melting curve confirmed the

188 presence of specific products for each primer set. Ct values were converted to relative expression

189 values using the delta-delta Ct method with offspring heart data normalised to the expression of

190 *Ppib* and *Sdha* and liver data normalised to *Pgk1* and *Tbp*. geNorm software **(48)** was used to

191 determine these to be the most stable reference genes. Primer sequences and amplification

192 efficiencies are provided in **Table 1**.

193

194 *Statistical analyses*

195 Where appropriate, stud male data were analysed using independent samples or repeated

196 measures *t*-tests, following assessment for normality, and Pearson correlation for analysis of

197 correlation between phenotypic measurements (SPSS version 17). Offspring litter sex ratios were

198 analysed using a binomial test (SPSS version 17). Analysis of offspring vascular responsiveness

199 was conducted using GraphPad Prism 6, with the log effective concentration equal to 50% of the

200 maximal response (pEC50) and maximum response for each of the CRCs analysed with an

201 independent samples *t*-test. All other offspring data were analysed using a multilevel random effects

202 regression model (SPSS version 18) **(53)**, accounting for paternal origin of litter, gestational litter

203 size, offspring sex and body weight. Significance was taken at P<0.05.

204

205 **Results**

206 *Paternal LPD reduces stud growth*

207 Prior to experimental feeding, there was no difference in mean NPD and LPD stud body

208 weight (**Figure 1**). During the first 3 weeks of feeding, LPD stud males displayed a trend (P<0.1)

209 towards a lower body weight (i.e. at 12 -14 weeks of age), becoming lighter (P<0.05) during weeks

210 4 - 9 (i.e. at 15 - 20 weeks of age, mating initiated at 18 weeks of age). Throughout the study, LPD

211 studs grew more slowly (P=0.004) (by 12%) than NPD studs. NPD and LPD stud systolic and

212 diastolic blood pressure and heart rate did not differ throughout the entire study (data not shown).

213   Analysis of stud male organ weights at 32 weeks of age revealed a significantly lighter kidney

214   (NPD 0.171 ± 0.004g, LPD 0.149 ± 0.003g; P=0.0005) and carcass (body weight minus the weight

215   of collected organs and fat pads) (NPD 29.09 ± 0.27g, LPD 27.59 ± 0.47g; P=0.015) weight in LPD

216   males. These differences remained when calculated as a proportion of body weight (P <0.05; data

217   not shown). No difference between NPD and LPD stud testosterone (combined serum and

218   testicular; NPD 9.30 ± 5.24 ng/ml, LPD 11.40 ± 3.92 ng/ml) or serum insulin (NPD 2.26 ± 0.61

219   ng/ml, LPD 1.44 ± 0.12 ng/ml) were observed. However, serum glucose was higher in LPD studs

220   (NPD 1.32 ± 0.08 mg/ml, LPD 1.55 ± 0.07 mg/ml) at a trend level (P=0.06). No differences in the

221   total number of sperm (NPD $12.90x10^6 ± 2.30x10^6$/ml, LPD $11.62x10^6 ± 1.99x10^6$/ml), or number

222   of sperm collected following swim-up (NPD $4.99x10^6 ± 1.29x10^6$/ml, LPD $4.68x10^6 ±$

223   $0.72x10^6$/ml) were observed between stud groups.

224

225   ***Paternal LPD affects offspring sex ratios, birth weight and adult phenotype***

226   Mean maternal weight prior to conception (16.28 ± 0.11 g), after 2 weeks of pregnancy

227   (23.80 ± 0.29 g) and mean litter size (5.7 ± 0.4) did not differ between treatment groups. However,

228   the proportion of male pups at birth was reduced in the LPD compared to the NPD treatment group

229   (NPD 0.54 ± 0.04, LPD 0.40 ± 0.06; P=0.03), and male offspring birth weight was increased (NPD

230   1.26 ± 0.02 g, LPD 1.33 ± 0.02 g; P=0.05). LPD offspring were also heavier than NPD offspring at

231   2 (NPD 6.54 ± 0.07 g, LPD 7.13 ± 0.08 g; P=0.006) and 3 (NPD 7.82 ± 0.10 g, LPD 8.61 ± 0.14 g;

232   P=0.019) weeks of age. At weaning, the sexes were caged separately (mean of 3 NPD males, 2 LPD

233   males, 3 NPD females and 3 LPD females per cage) with no further differences in body weights

234   being observed between NPD and LPD offspring for up to 24 weeks of age (data not shown).

235

236   Analysis of correlations between paternal phenotype at the time of mating and offspring

237   early postnatal development revealed significant negative correlations between LPD stud body

238   weight and mean litter male:female ratio (r=-0.444, P<0.0001) and offspring weight at 1 (r =-0.27,

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239    P =0.023) and 2 weeks of age (r =-0.257, P =0.030). In NPD offspring, a positive correlation was

240    observed between stud body weight and litter male:female ratio (r =0.191, P =0.085) and offspring

241    weight at 2 weeks (r =0.210, P =0.06), which was significant at 1 week of age (r =0.224, P =0.046).

242    Additional negative correlations were observed between the number of days studs were on LPD and

243    litter male:female ratio (r =-0.277, P =0.018), offspring birth weight (r =-0.421, P <0.0001), weight

244    at 1 (r =-0.363, P =0.002) and 2 weeks of age (r =-0.348, P =0.003) which were not observed in

245    NPD offspring.

246

247        No difference in mean systolic or diastolic blood pressure or heart rates were observed

248    between NPD and LPD offspring at 6, 10 or 14 weeks of age (data not shown). However, at 18

249    weeks of age, LPD males displayed lower diastolic (NPD 84.80 ± 1.82 mmHg, LPD 75.88 ± 2.16

250    mmHg), systolic (NPD 113.13 ± 2.19 mmHg, LPD 103.93 ± 2.27 mmHg) and mean (NPD 93.93 ±

251    1.91 mmHg, LPD 84.86 ± 2.16 mmHg) blood pressure, and elevated mean heart rate (NPD 682 ±

252    11 beats per minute, LPD 711 ± 16 beats per minute; P<0.05) (**Figure 2**).

253

254        At 22 weeks of age, both male and female LPD offspring displayed elevated blood glucose

255    concentrations following an intraperitoneal glucose bolus. At 15 and 60 minutes post injection, LPD

256    males had significantly elevated blood glucose concentrations, with a reduced overall clearance at

257    60 and 90 minutes (area under the curve, AUC; P=0.034 and 0.029 respectively) (**Figure 3A**). LPD

258    females similarly had elevated blood glucose concentrations at 15 and 30 minutes post injection,

259    and impaired overall clearance at 60 and 90 minutes (AUC, P=0.022 and 0.080 respectively)

260    (**Figure 3B**).

261

262        At 24 weeks of age, significantly attenuated vasoconstrictive responses (pEC50) to the α-1

263    adrenergic agonist phenylephrine (PE) and maximal vasodilatory responses to isoprenaline (ISO)

264    and the nitric oxide donor SNP were observed in arteries from LPD males (**Figure 4A,** P<0.05).

10

265   Significantly attenuated pEC50 and maximal responses to SNP were also observed in LPD females

266   (**Figure 4B,** P<0.05).

267

268        Analysis of offspring organ and fat pad weights at 24 weeks of age revealed significantly

269   increased inguinal (NPD 0.59 ± 0.04g, LPD 0.77 ± 0.07g; P=0.017) and total fat (combined

270   individual fat pads, NPD 2.02 ± 0.01g, LPD 2.46 ± 0.18g; P=0.035) weights in LPD males but not

271   females. When expressed as a percentage of body weight, reduced heart (NPD 0.53 ± 0.01, LPD

272   0.51 ± 0.02; P=0.04) and elevated inguinal fat (NPD 2.00 ± 0.12, LPD 2.51 ± 0.20; P =0.02) and

273   total fat (NPD 6.81 ± 0.34, LPD 8.06 ± 0.53; P=0.04) proportions were observed in LPD males. As

274   global adiposity levels influence metabolic state and glucose homeostasis, we performed additional

275   retrospective analyses incorporating body weight and adiposity measurements as 'random effects'

276   within our regression analyses of offspring glucose tolerance. We observed a positive interaction

277   between offspring body weight and overall glucose clearance (AUC) such that AUC increased by

278   40.57 per g increase in body weight (P =0.004), however, no interaction between total fat weight

279   and AUC (P =0.98) was observed. In males, a significant positive interaction between body weight

280   and AUC was observed (AUC increased by 39.11 for each g increase in body weight; P =0.04), but

281   no interaction with total fat and AUC (P =0.41) was present. Female offspring displayed no

282   interaction between body weight and mean AUC (P =0.48), however, a positive interaction with

283   total fat and AUC at a trend level (AUC increased by 219.76 for each g increase in total fat; P

284   =0.07) was observed. No interaction between blood glucose and total adiposity were observed in

285   male or female offspring at each individual time point post glucose bolus. However, body weight

286   was observed to interact positively with mean blood glucose levels in male offspring at 60 (0.621

287   mmol increase for every g increase in body weight; P =0.017) and 90 minutes (0.682 mmol increase

288   for every g increase in body weight; P =0.008). No such interactions in female offspring were

289   observed. Additional analyses of offspring adiposity and adult health revealed a negative correlation

290   in LPD offspring between BMI (weight g/length from nose to base of tail (cm$^2$)) and diastolic blood

291  pressure (r =-0.276, P=0.027) and a positive correlation between total fat (g) and heart rate (r

292  =0.279, P =0.026) at 18 weeks of age which were not present within NPD offspring. Serum

293  adiponectin concentrations were greater (P<0.001) in female than male offspring but were

294  unaffected by paternal diet (**Table 2**). In contrast, there was a paternal-diet by offspring-sex

295  interaction (P = 0.015) for TNF-α which indicated that this cytokine was elevated in LPD male

296  compared to NPD male offspring, with no differences between paternal dietary treatments in female

297  offspring. Analysis of correlation between adiponectin, TNF-α and offspring phenotype revealed

298  significant negative correlations between adiponectin levels and body weight at cull in NPD and

299  LPD offspring (P <0.0001). Additionally, adiponectin levels correlated negatively with body weight

300  at 3 weeks of age (r =-0.384; P =0.033) and TNF-α levels (r =-0.349; P =0.05) in LPD offspring.

301  Finally, a positive correlation between TNF-α levels and body weight at cull (r =0.471; P =0.008)

302  was also observed in LPD offspring.

303

304  Gene expression analyses in offspring heart for receptors involved in regulation of cardiac

305  function revealed no differences for adrenergic receptor beta 1 (*Adrb1*), angiotensin II receptor type

306  1a (*Agtr1a*), bone morphogenetic protein receptor type II (*Bmpr2*), cholinergic receptor muscarinic

307  2 (*Chrm2*) or the solute carrier family 2 (facilitated glucose transporter), member 4 (*Glut4*) between

308  NPD and LPD offspring (data not shown). However, analysis of genes involved in calcium

309  signalling revealed significantly decreased expression of adenylate cyclase 5 (*Adcy5*; P=0.026),

310  phospholipase C beta1 (*Plcb1*; P=0.027) and protein kinase C beta (*Prkcb*; P=0.008) in offspring

311  heart tissue (**Table 3**). As well as having a role in regulation of cardiovascular function, ADCY5,

312  along with FTO (fat mass and obesity associated) have been identified as genes that are altered in

313  type 2 diabetes **(3, 25)**. Therefore, in response to the observations of impaired glucose tolerance and

314  elevated adiposity in LPD offspring, we analysed the expression of *Adcy5* and *Fto* in offspring liver

315  tissue. We observed no change in the expression of *Adcy5,* however, *Fto* was decreased (P=0.006)

316   (**Table 3).** *Fto* expression in offspring cardiac tissue was also reduced ($P<0.001$) in NPD offspring

317   (**Table 3**).

318

319   **Discussion**

320       To date, the majority of studies detailing the developmental programming of offspring

321   health have focused on manipulation of the maternal environment. As such, our understanding of

322   the impact of paternal nutrition on offspring development and long-term adult health remains poorly

323   defined. In the present study, we have shown that LPD has minimal effects on paternal physiology

324   and fertility (between 18 and 32 weeks of age), but that adult offspring derived from them display

325   significantly impaired cardiovascular and metabolic homeostasis. Our results provide evidence of

326   an intergenerational modification of adult offspring phenotype in response to paternal diet.

327

328       Our results identify a series of offspring growth, cardiovascular and metabolic phenotypes

329   whose regulation is compromised by paternal LPD. At birth, we observed a significantly reduced

330   litter male:female ratio and increased weight of male offspring from LPD studs. In mice, a low

331   calorie diet fed to females results in a selective loss of male embryos during preimplantation

332   development and subsequent skewing of litter sex ratios in favour of males **(38)**. However, no such

333   effects are observed when low calorie diets are fed to males. In contrast, no effects on litter sex ratio

334   have been reported following maternal LPD in the mouse  **(53, 54)**. Based on previous reports **(38)**,

335   we do not believe LPD induces a differential production in the number of X and Y bearing sperm in

336   studs. However, differences in capacitation rates or motility between X and Y bearing sperm might

337   explain these effects. Alternatively, LPD semen may induce a uterine environment more favourable

338   to female preimplantation embryos, resulting in a selective loss of male embryos **(38)**. However,

339   additional studies are necessary to determine whether functional differences exist between X and Y

340   bearing sperm, and at what developmental stage offspring sex ratio is established.

341

342    Dysfunctional regulation between constriction and dilatation responses within resistance

343    arteries has been identified in rodent models of cardiovascular programming **(37, 51, 53)**.

344    Augmentation of peripheral vascular function is characterised by the presence of altered

345    endothelium-dependent vasodilation and/or changes in activity of signalling mechanisms regulating

346    vascular smooth muscle function. We identified significant impairments in mesenteric artery

347    responses to the $\alpha_1$-adrenergic agonist phenylephrine (PE), the ß-adrenoreceptor agonist

348    isoprenaline (ISO) and the nitric oxide donor sodium nitroprusside (SNP) in LPD offspring,

349    however, no impairment in response to endothelial-dependent vasodilator acetylcholine (ACh) was

350    observed. Within the resistance vasculature, endothelium-dependent vasodilatation is mediated

351    predominantly through the action of endothelium-derived hyperpolarizing factor (EDHF) via small

352    and intermediate calcium-activated potassium channels rather than NO **(31)**. Indeed, in conditions

353    of reduced NO bio-availability, up-regulation of EDHF activity has been observed **(39)**. As ACh

354    induces vascular smooth muscle cell hyperpolarization through both eNOS and EDHF pathways, a

355    functional EDHF component would mask any impairment in NO signalling present. This could

356    provide one mechanism through which altered responsiveness to SNP, but not Ach, could be

357    manifest. Calcium homeostasis is central to the regulation of vascular smooth muscle function. PE

358    induces vasoconstriction though the activation of phospholipase C and the mobilisation of calcium

359    from intracellular stores, activating myosin light chain kinase. Conversely, isoprenaline elevates

360    intracellular cAMP levels, activating protein kinase A, which inhibits myosin light chain kinase,

361    causing vasodilatation. Isoprenaline also acts in concert with endothelial nitric oxide synthase,

362    stimulating soluble guanylate cyclase to increase cGMP levels within the vascular smooth muscle,

363    also inhibiting myosin light chain kinase activity. Therefore, impairment in vascular smooth muscle

364    cell calcium signalling would impact negatively on both vaso-constriction and –dilatation function,

365    as observed in LPD offspring. However, from the present study, the exact roles of NO, soluble

366    guanylate cyclase, adenylate cyclase, prostaglandins and calcium-activated potassium channels are

367    uncertain and would require further investigation.

14

368

369     Similarly, modulation of myocardial intracellular calcium signalling through altered

370     sympathetic or parasympathetic innervation, inhibition of calcium entry or AT1 receptor antagonists

371     (angiotensin II) would result in a lowering of blood pressure **(56)**. As no difference in cardiac

372     expression of the ß1-adrenergic, angiotensin II type 1a or the cardiac muscarinic cholinergic

373     receptors were observed in offspring, we assessed the expression profiles of central regulators of

374     intracellular calcium signalling in offspring cardiac tissue. Here, we observed significantly

375     decreased expression of *Adcy5*, *Plcb*, and *Prkcb* in LPD offspring. Adenylate cyclase, in particular

376     Adcy5*,* plays a central role in modulating cardiac contractility, transducing the signal from the ß-

377     adrenergic receptor, elevating cAMP-protein kinase A signalling and ultimately the influx of

378     calcium ions through voltage-dependent L-type calcium channels. Disruption of cardiac *Adcy5*

379     expression results in decreased in vivo responsiveness to isoprenaline, elevated heart rate through

380     reduced parasympathetic regulation, and reduced hypertrophy and apoptosis **(29, 30)**. Plcb catalyses

381     the hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP2) to generate inositol (1,4,5)

382     triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 initiates an increase in intracellular calcium,

383     whereas DAG activates protein kinase C beta (Prkcb). In turn Prkcb, a serine and threonine kinase,

384     phosphorylates a wide range of protein targets. Elevated expression of Prkcb isoforms, specifically

385     in the myocardium, is associated with hypertrophy, fibrosis, impairment of left ventricular

386     performance, progressive cardiomyopathy and heart failure **(49, 55)**. In contrast, Prkcb inhibition

387     preserves cardiac contractility by attenuating diastolic dysfunction, myocyte hypertrophy, and

388     collagen deposition **(12)**. Therefore, our observed relative hypotension, tachycardia and reduced

389     heart:body weight ratios observed in LPD offspring may result from impaired parasympathetic

390     stimulation of cardiac tissues providing decreased baroreflex restraint coupled  with a reduced rate

391     of age related cardiomyopathy and fibrosis in response to reduced calcium signalling gene

392     expression **(7)**, however, additional studies would be required to verify these conclusions.

393

394       In our current study, LPD offspring also exhibited reduced glucose tolerance and elevated

395    adiposity in adulthood. Broad effects on the metabolism of young offspring following paternal

396    nutritional manipulation have been reported. Offspring pancreatic ß-cell function and gene

397    expression are affected by paternal preconception high fat diet in rats **(28)**, with paternal

398    preconception fasting elevating offspring glucose levels in mice **(2)**. Similarly, elevated expression

399    of hepatic lipid and cholesterol biosynthesis genes, with decreased levels of cholesterol esters, at

400    weaning have been observed in offspring mice from LPD fed studs **(9)**, however, adult  sex-specific

401    cardiovascular, metabolic and glucogenic phenotype was not assessed. A causal link does exist

402    between cardiovascular and metabolic phenotype, with increased adiposity and altered gene

403    expression profiles, vascular dysfunction and hypertension being observed in female offspring from

404    mouse dams fed a LPD exclusively during preimplantation development **(52, 53)**. Impaired

405    glycaemic homeostasis and increased adiposity are chronic inflammatory conditions associated with

406    elevated levels of adipokines, inflammatory cytokines and oxidised low-density lipopropoteins, all

407    known to impair vascular smooth muscle and cardiac function **(35, 46)**. Therefore, we measured the

408    circulating levels of adiponectin and TNF-α in NPD and LPD offspring. Adiponectin levels

409    correlate negatively with adiposity, with low levels being associating with cardio-metabolic

410    disorders including endothelial dysfunction, type 2 diabetes and blood pressure **(18)**. Conversely,

411    elevated levels of the pro-inflammatory cytokine TNF- α are associated with insulin resistance and

412    impact negatively on vascular function **(4)**. We observed no significant difference in either

413    adiponectin concentration between NPD and LPD males, or between NPD and LPD females.

414    However, female offspring did display significantly higher adiponectin levels than males, reflective

415    of previous reports **(15)**. We also observed a significant paternal-diet by offspring-sex interaction

416    indicating significant differences in the impact of paternal diet on offspring inflammatory responses

417    dependent on sex. As such, the comparatively low levels of adiponectin, coupled with elevated

418    levels of TNF- α, may contribute to the increased vascular dysfunction and impaired glucose

419    homeostasis observed in LPD males. Conversely, in LPD females, the opposite relationship may

16

420 provide some protection against developing cardiovascular impairments to the same magnitude as

421 those observed in the LPD males. Interestingly, we observed specific correlations between early

422 postnatal body weight, adiponectin and TNF-α levels and adult body weight in LPD offspring.

423 These data highlight the importance of early development and physiological characteristics (e.g.

424 body weight) and adult markers of metabolic health, a central concept of the Developmental Origins

425 of Health and Disease (DOHaD) hypothesis **(21)**. Indeed, we have demonstrated previously similar

426 associations between early postnatal weight and adult cardiovascular dysfunction in a mouse

427 maternal model of gestational LPD fed exclusively during preimplantation development **(53)**. We

428 also observed a negative correlation between BMI and diastolic blood pressure in LPD offspring,

429 whilst total fat weight correlated positively with heart rate at 18 weeks of age, highlighting

430 additionally the interaction between adult adiposity and cardiovascular regulation within our model.

431

432       Human genome-wide associated studies of have identified significant associations between

433 genetic polymorphisms and the risk prediction for type II diabetes including *ADCY5* and Fat Mass

434 and Obesity associated gene (*FTO*) **(1, 3)**. We observed significantly decreased expression of *Fto* in

435 the livers of LPD offspring, with no change in *Adcy5* expression. The *FTO* gene is an AlkB-like,

436 Fe(II)- and 2-oxoglutarate–dependent nucleic acid demethylase, acting on single-stranded DNA and

437 RNA, and has been showed to predispose individuals to diabetes through an effect on body mass

438 index **(43)**. In mice, fasting increases *Fto* expression in the liver within an inverse correlation

439 between *Fto* mRNA and glucose levels **(34)**, whilst knock out of the Fto gene results in reduced

440 lean mass and elevated fat mass **(25).** In mouse models of obesity, reduced hepatic *Fto* expression

441 has been reported **(44).** As such, the reduced hepatic expression of *Fto* observed in NPD mice may

442 disrupt regulation of energy metabolism, predisposing to impaired glucose tolerance and elevated

443 adiposity.

444

445     Our observations raise the question as to the underlying developmental mechanisms through

446     which paternal diet modifies adult offspring cardiovascular and metabolic phenotype. Recent

447     studies have identified sperm hypomethylation and histone-enrichment at the promoters of

448     developmental regulatory genes in both mice and men **(8, 20)**. Carone et al., **(9)** also observed

449     significant changes in the epigenome of sperm isolated from stud mice fed a LPD, correlating with

450     weaning offspring metabolic phenotype. These observations highlight the potential that sperm

451     epigenetic status could influence both sperm function and post fertilisation development and gene

452     expression patterns. However, as the sperm epigenome is dramatically remodelled at fertilisation by

453     the cytoplasm of the oocyte, the persistence of sperm epigenetic marks and their effects on offspring

454     phenotype remains unknown. Recently, it has been demonstrated that paternal sensory environment

455     prior to conception, influenced the sensory nervous system structure and function in F1 and F2

456     generations in mice **(13)**. Interestingly, bisulfite sequencing analysis of paternal sperm and offspring

457     tissue DNA revealed similar patterns of hypomethylation of the olfactory receptor *Olfr151*, proving

458     potential evidence of an epigenetic basis of transgenerational inheritance of phenotype. Secondly,

459     the relative contribution of sperm genomic-mediated programming and that determined by the

460     composition of seminal plasma on long-term development and wellbeing of offspring remains to be

461     established. It is known that seminal plasma cytokines stimulate maternal reproductive tract

462     immunological responses, influencing embryonic, placental and offspring development **(41).** In

463     human assisted reproductive cycles, there is an increasing awareness of the benefits of seminal

464     plasma exposure on appropriate uterine responses and pregnancy outcomes following embryo

465     transfer **(47)**. Whether paternal LPD modifies the composition of the seminal plasma, and the

466     impact this may have on uterine physiology following mating remains to be determined.

467

468     Our data extend the concept of developmental programming revealing the role of paternal

469     nutrition in the early origins of adult offspring cardiovascular and metabolic health. These data are

470     timely and relevant to human disease as paternal diet and lifestyle not only contribute to male-factor

471  infertility **(5)**, but can also influence the cardiovascular and metabolic disease risk in subsequent

472  generations **(22, 32)**. Our observations highlight the need for a greater understanding of the

473  underlying mechanisms through which parental diet and physiology affect gamete maturation,

474  semen quality, and ultimately, long-term offspring health.

475

476

485 **Disclosures**

486 The authors declare no conflicts of interest, financial or otherwise.

487

488　**References**

489　1.　**Abbas S, Raza ST, Ahmed F, Ahmad A, Rizvi S, and Mahdi F**. Association of Genetic

490　polymorphism of PPARgamma-2, ACE, MTHFR, FABP-2 and FTO genes in risk prediction of

491　type 2 diabetes mellitus. *J Biomed Sci* 20: 80, 2013.

492　2.　**Anderson LM, Riffle L, Wilson R, Travlos GS, Lubomirski MS, and Alvord WG**.

493　Preconceptional fasting of fathers alters serum glucose in offspring of mice. *Nutrition* 22: 327-331,

494　2006.

495　3.　**Andersson EA, Pilgaard K, Pisinger C, Harder MN, Grarup N, Faerch K, Poulsen P,**

496　**Witte DR, Jorgensen T, Vaag A, Hansen T, and Pedersen O**. Type 2 diabetes risk alleles near

497　ADCY5, CDKAL1 and HHEX-IDE are associated with reduced birthweight. *Diabetologia* 53:

498　1908-1916, 2010.

499　4.　**Aroor AR, McKarns S, Demarco VG, Jia G, and Sowers JR**. Maladaptive immune and

500　inflammatory pathways lead to cardiovascular insulin resistance. *Metabolism* 62: 1543-1552, 2013.

501　5.　**Bakos HW, Henshaw RC, Mitchell M, and Lane M**. Paternal body mass index is

502　associated with decreased blastocyst development and reduced live birth rates following assisted

503　reproductive technology. *Fertil Steril* 95: 1700-1704, 2011.

504　6.　**Bellinger L, Lilley C, and Langley-Evans SC**. Prenatal exposure to a maternal low-protein

505　diet programmes a preference for high-fat foods in the young adult rat. *Br J Nutr* 92: 513-520, 2004.

506　7.　**Boyle AJ, Shih H, Hwang J, Ye J, Lee B, Zhang Y, Kwon D, Jun K, Zheng D, Sievers**

507　**R, Angeli F, Yeghiazarians Y, and Lee R**. Cardiomyopathy of aging in the mammalian heart is

508　characterized by myocardial hypertrophy, fibrosis and a predisposition towards cardiomyocyte

509　apoptosis and autophagy. *Exp Gerontol* 46: 549-559, 2011.

510　8.　**Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C,**

511　**Schubeler D, Stadler MB, and Peters AH**. Repressive and active histone methylation mark

512　distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol* 17: 679-687, 2010.

513  9.    **Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, Li R, Bock C, Li C, Gu H,**

514  **Zamore PD, Meissner A, Weng Z, Hofmann HA, Friedman N, and Rando OJ**. Paternally

515  induced transgenerational environmental reprogramming of metabolic gene expression in mammals.

516  *Cell* 143: 1084-1096, 2010.

517  10.   **Ceelen M, van Weissenbruch MM, Prein J, Smit JJ, Vermeiden JP, Spreeuwenberg M,**

518  **van Leeuwen FE, and Delemarre-van de Waal HA**. Growth during infancy and early childhood

519  in relation to blood pressure and body fat measures at age 8-18 years of IVF children and

520  spontaneously conceived controls born to subfertile parents. *Hum Reprod* 24: 2788-2795, 2009.

521  11.   **Chavarro JE, Furtado J, Toth TL, Ford J, Keller M, Campos H, and Hauser R**. Trans-

522  fatty acid levels in sperm are associated with sperm concentration among men from an infertility

523  clinic. *Fertil Steril* 95: 1794-1797, 2011.

524  12.   **Connelly KA, Kelly DJ, Zhang Y, Prior DL, Advani A, Cox AJ, Thai K, Krum H, and**

525  **Gilbert RE**. Inhibition of protein kinase C-beta by ruboxistaurin preserves cardiac function and

526  reduces extracellular matrix production in diabetic cardiomyopathy. *Circ Heart Fail* 2: 129-137,

527  2009.

528  13.   **Dias BG, and Ressler KJ**. Parental olfactory experience influences behavior and neural

529  structure in subsequent generations. *Nat Neurosci* 2013.

530  14.   **Edwards LJ, and McMillen IC**. Periconceptional nutrition programs development of the

531  cardiovascular system in the fetal sheep. *Am J Physiol Regul Integr Comp Physiol* 283: R669-679,

532  2002.

533  15.   **Eglit T, Lember M, Ringmets I, and Rajasalu T**. Gender differences in serum high-

534  molecular-weight adiponectin levels in metabolic syndrome. *Eur J Endocrinol* 168: 385-391, 2013.

535  16.   **Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, and Ozanne**

536  **SE**. Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein

537  expression in 21-mo-old female rat offspring. *Am J Physiol Regul Integr Comp Physiol* 288: R368-

538  373, 2005.

539     17.     **Ghanayem BI, Bai R, Kissling GE, Travlos G, and Hoffler U**. Diet-induced obesity in

540     male mice is associated with reduced fertility and potentiation of acrylamide-induced reproductive

541     toxicity. *Biol Reprod* 82: 96-104, 2010.

542     18.     **Gu P, and Xu A**. Interplay between adipose tissue and blood vessels in obesity and vascular

543     dysfunction. *Rev Endocr Metab Disord* 14: 49-58, 2013.

544     19.     **Hammoud AO, Gibson M, Stanford J, White G, Carrell DT, and Peterson M**. In vitro

545     fertilization availability and utilization in the United States: a study of demographic, social, and

546     economic factors. *Fertil Steril* 91: 1630-1635, 2009.

547     20.     **Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, and Cairns BR**. Distinctive

548     chromatin in human sperm packages genes for embryo development. *Nature* 460: 473-478, 2009.

549     21.     **Hanson M, Godfrey KM, Lillycrop KA, Burdge GC, and Gluckman PD**.

550     Developmental plasticity and developmental origins of non-communicable disease: theoretical

551     considerations and epigenetic mechanisms. *Prog Biophys Mol Biol* 106: 272-280, 2011.

552     22.     **Kaati G, Bygren LO, and Edvinsson S**. Cardiovascular and diabetes mortality determined

553     by nutrition during parents' and grandparents' slow growth period. *Eur J Hum Genet* 10: 682-688,

554     2002.

555     23.     **Kort HI, Massey JB, Elsner CW, Mitchell-Leef D, Shapiro DB, Witt MA, and**

556     **Roudebush WE**. Impact of body mass index values on sperm quantity and quality. *J Androl* 27:

557     450-452, 2006.

558     24.     **Kwong WY, Wild AE, Roberts P, Willis AC, and Fleming TP**. Maternal undernutrition

559     during the preimplantation period of rat development causes blastocyst abnormalities and

560     programming of postnatal hypertension. *Development* 127: 4195-4202, 2000.

561     25.     **McMurray F, Church CD, Larder R, Nicholson G, Wells S, Teboul L, Tung YC,**

562     **Rimmington D, Bosch F, Jimenez V, Yeo GS, O'Rahilly S, Ashcroft FM, Coll AP, and Cox**

563     **RD**. Adult onset global loss of the fto gene alters body composition and metabolism in the mouse.

564     *PLoS Genet* 9: e1003166, 2013.

565 26. **Micke GC, Sullivan TM, Gatford KL, Owens JA, and Perry VE**. Nutrient intake in the

566 bovine during early and mid-gestation causes sex-specific changes in progeny plasma IGF-I,

567 liveweight, height and carcass traits. *Anim Reprod Sci* 121: 208-217, 2010.

568 27. **Mitchell M, Bakos HW, and Lane M**. Paternal diet-induced obesity impairs embryo

569 development and implantation in the mouse. *Fertil Steril* 95: 1349-1353, 2011.

570 28. **Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, and Morris MJ**. Chronic high-fat diet

571 in fathers programs beta-cell dysfunction in female rat offspring. *Nature* 467: 963-966, 2010.

572 29. **Okumura S, Kawabe J, Yatani A, Takagi G, Lee MC, Hong C, Liu J, Takagi I,**

573 **Sadoshima J, Vatner DE, Vatner SF, and Ishikawa Y**. Type 5 adenylyl cyclase disruption alters

574 not only sympathetic but also parasympathetic and calcium-mediated cardiac regulation. *Circ Res*

575 93: 364-371, 2003.

576 30. **Okumura S, Takagi G, Kawabe J, Yang G, Lee MC, Hong C, Liu J, Vatner DE,**

577 **Sadoshima J, Vatner SF, and Ishikawa Y**. Disruption of type 5 adenylyl cyclase gene preserves

578 cardiac function against pressure overload. *Proc Natl Acad Sci U S A* 100: 9986-9990, 2003.

579 31. **Ozkor MA, and Quyyumi AA**. Endothelium-derived hyperpolarizing factor and vascular

580 function. *Cardiol Res Pract* 2011: 156146, 2011.

581 32. **Pembrey ME, Bygren LO, Kaati G, Edvinsson S, Northstone K, Sjostrom M, and**

582 **Golding J**. Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* 14:

583 159-166, 2006.

584 33. **Pisani LF, Antonini S, Pocar P, Ferrari S, Brevini TA, Rhind SM, and Gandolfi F**.

585 Effects of pre-mating nutrition on mRNA levels of developmentally relevant genes in sheep oocytes

586 and granulosa cells. *Reproduction* 136: 303-312, 2008.

587 34. **Poritsanos NJ, Lew PS, and Mizuno TM**. Relationship between blood glucose levels and

588 hepatic Fto mRNA expression in mice. *Biochem Biophys Res Commun* 400: 713-717, 2010.

589 35. **Porter KE, and Riches K**. The vascular smooth muscle cell: a therapeutic target in Type 2

590 diabetes? *Clin Sci (Lond)* 125: 167-182, 2013.

591    36.    **Rato L, Alves MG, Dias TR, Lopes G, Cavaco JE, Socorro S, and Oliveira PF**. High-

592    energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male

593    reproductive parameters. *Andrology* 1: 495-504, 2013.

594    37.    **Rodford JL, Torrens C, Siow RC, Mann GE, Hanson MA, and Clough GF**. Endothelial

595    dysfunction and reduced antioxidant protection in an animal model of the developmental origins of

596    cardiovascular disease. *J Physiol* 586: 4709-4720, 2008.

597    38.    **Rosenfeld CS**. Periconceptional influences on offspring sex ratio and placental responses.

598    *Reprod Fertil Dev* 24: 45-58, 2011.

599    39.    **Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ,**

600    **and    Ahluwalia    A**. Investigation of vascular responses in endothelial nitric oxide

601    synthase/cyclooxygenase-1    double-knockout    mice:    key    role    for    endothelium-derived

602    hyperpolarizing factor in the regulation of blood pressure in vivo. *Circulation* 111: 796-803, 2005.

603    40.    **Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A,**

604    **Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG, and Young LE**. DNA

605    methylation, insulin resistance, and blood pressure in offspring determined by maternal

606    periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A* 104: 19351-19356,

607    2007.

608    41.    **Sjoblom C, Roberts CT, Wikland M, and Robertson SA**. Granulocyte-macrophage

609    colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth

610    trajectory and placental morphogenesis. *Endocrinology* 146: 2142-2153, 2005.

611    42.    **Soubry A, Schildkraut JM, Murtha A, Wang F, Huang Z, Bernal A, Kurtzberg J,**

612    **Jirtle RL, Murphy SK, and Hoyo C**. Paternal obesity is associated with IGF2 hypomethylation in

613    newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC Med* 11: 29, 2013.

614    43.    **Sovio U, Mook-Kanamori DO, Warrington NM, Lawrence R, Briollais L, Palmer CN,**

615    **Cecil J, Sandling JK, Syvanen AC, Kaakinen M, Beilin LJ, Millwood IY, Bennett AJ, Laitinen**

616    **J, Pouta A, Molitor J, Davey Smith G, Ben-Shlomo Y, Jaddoe VW, Palmer LJ, Pennell CE,**

Cole TJ, McCarthy MI, Jarvelin MR, and Timpson NJ. Association between common variation at the FTO locus and changes in body mass index from infancy to late childhood: the complex nature of genetic association through growth and development. *PLoS Genet* 7: e1001307, 2011.

44. Stratigopoulos G, Padilla SL, LeDuc CA, Watson E, Hattersley AT, McCarthy MI, Zeltser LM, Chung WK, and Leibel RL. Regulation of Fto/Ftm gene expression in mice and humans. *Am J Physiol Regul Integr Comp Physiol* 294: R1185-1196, 2008.

45. Todd SE, Oliver MH, Jaquiery AL, Bloomfield FH, and Harding JE. Periconceptional undernutrition of ewes impairs glucose tolerance in their adult offspring. *Pediatr Res* 65: 409-413, 2009.

46. Trayhurn P, and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 92: 347-355, 2004.

47. Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, Norman RJ, Robertson SA, and Simon C. The effect of intercourse on pregnancy rates during assisted human reproduction. *Hum Reprod* 15: 2653-2658, 2000.

48. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, and Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.

49. Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Ways DK, Hoit BD, Walsh RA, and King GL. Targeted overexpression of protein kinase C beta2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci U S A* 94: 9320-9325, 1997.

50. Watkins AJ, and Fleming TP. Blastocyst environment and its influence on offspring cardiovascular health: the heart of the matter. *J Anat* 215: 52-59, 2009.

51. Watkins AJ, Lucas ES, Torrens C, Cleal JK, Green L, Osmond C, Eckert JJ, Gray WP, Hanson MA, and Fleming TP. Maternal low-protein diet during mouse pre-implantation development induces vascular dysfunction and altered renin-angiotensin-system homeostasis in the offspring. *Br J Nutr* 103: 1762-1770, 2010.

643    52.    **Watkins AJ, Lucas ES, Wilkins A, Cagampang FR, and Fleming TP**. Maternal

644    periconceptional and gestational low protein diet affects mouse offspring growth, cardiovascular

645    and adipose phenotype at 1 year of age. *PLoS One* 6: e28745, 2011.

646    53.    **Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, Wilkins A,**

647    **Perry VH, Sheth B, Kwong WY, Eckert JJ, Wild AE, Hanson MA, Osmond C, and Fleming**

648    **TP**. Adaptive responses by mouse early embryos to maternal diet protect fetal growth but

649    predispose to adult onset disease. *Biol Reprod* 78: 299-306, 2008.

650    54.    **Watkins AJ, Wilkins A, Cunningham C, Perry VH, Seet MJ, Osmond C, Eckert JJ,**

651    **Torrens C, Cagampang FR, Cleal J, Gray WP, Hanson MA, and Fleming TP**. Low protein diet

652    fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular

653    abnormalities in offspring. *J Physiol* 586: 2231-2244, 2008.

654    55.    **Way KJ, Isshiki K, Suzuma K, Yokota T, Zvagelsky D, Schoen FJ, Sandusky GE,**

655    **Pechous PA, Vlahos CJ, Wakasaki H, and King GL**. Expression of connective tissue growth

656    factor is increased in injured myocardium associated with protein kinase C beta2 activation and

657    diabetes. *Diabetes* 51: 2709-2718, 2002.

658    56.    **Zubcevic J, Waki H, Raizada MK, and Paton JF**. Autonomic-immune-vascular

659    interaction: an emerging concept for neurogenic hypertension. *Hypertension* 57: 1026-1033, 2011.

660

661

662

663 **Figure Legends**

664 **Figure 1.** Mean weekly body weight of NPD (open circles, n = 8) and LPD (closed circles, n = 8)

665 stud males. Experimental feeding began at 11 weeks of age and continued till cull at 32 weeks.

666 Mating to chow fed females began at 18 weeks of age. Error bars are S.E.M, *P<0.05.

667

668 **Figure 2.** Systolic (A), diastolic (B) and mean (C) blood pressure and heart rate (beats per minute;

669 BPM) (D) in NPD (white bars) and LPD (black bars) offspring at 18 weeks of age. n = 34 NPD

670 males, 36 NPD females, 24 LPD males and 42 LPD females. Error bars are S.E.M, *P<0.05.

671

672 **Figure 3.** Mean changes in blood glucose levels following an intraperitoneal glucose bolus (2g/kg

673 body weight) in male (A), and female (B), NPD (open circles) and LPD (closed circles) offspring at

674 22 weeks of age. n = 15 male and 15 female offspring of each diet group representing all litters.

675 Error bars are S.E.M, *P<0.05.

676

677 **Figure 4.** Mean vasoreactivity of isolated mesenteric arteries from male (A), and female (B), NPD

678 (open circles) and LPD (closed circles) offspring at 24 weeks of age. Cumulative additions of

679 phenylephrine (PE) and, after pre-constriction, of the vasodilators acetylcholine (ACh), isoprenaline

680 (ISO) and sodium nitroprusside (SNP). n =8 males and 8 females of each diet group, each pair from

681 separate litters. Error bars are S.E.M. *P<0.05.
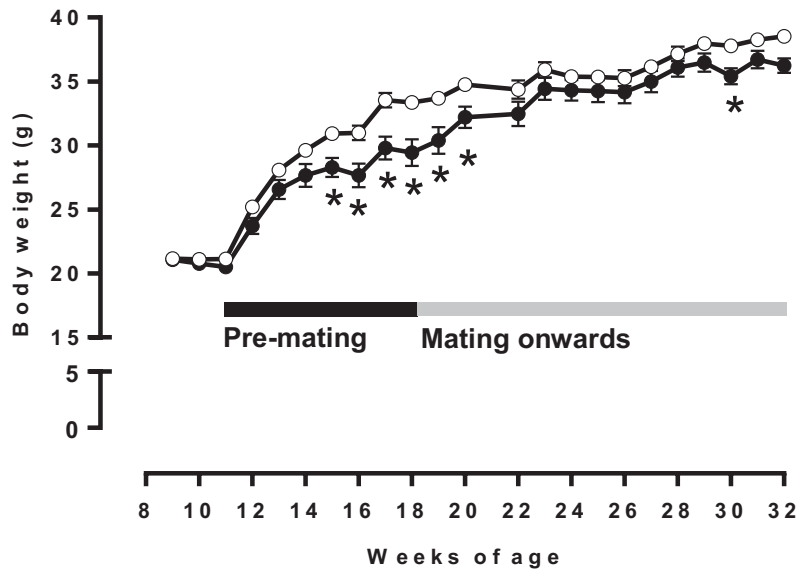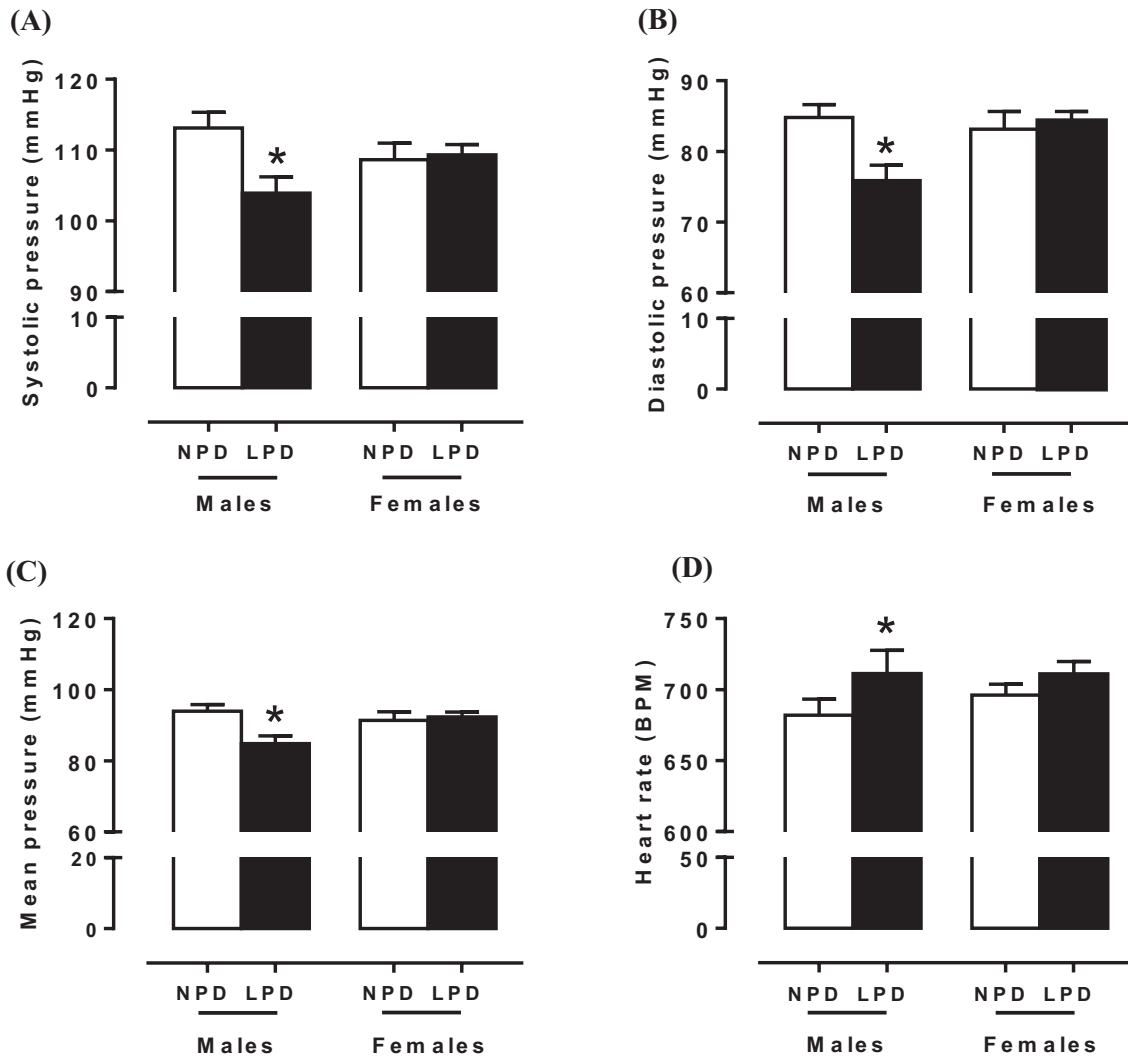
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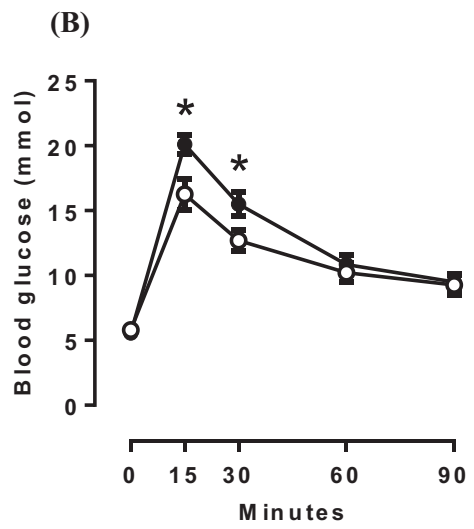684
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**Figure 1**

**Figure 2**

**Figure 3**

**(A)**



**(B)**

**Figure 4**



**(A)**

**(B)**
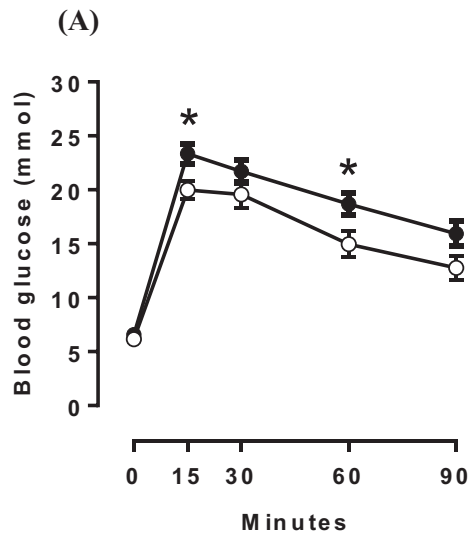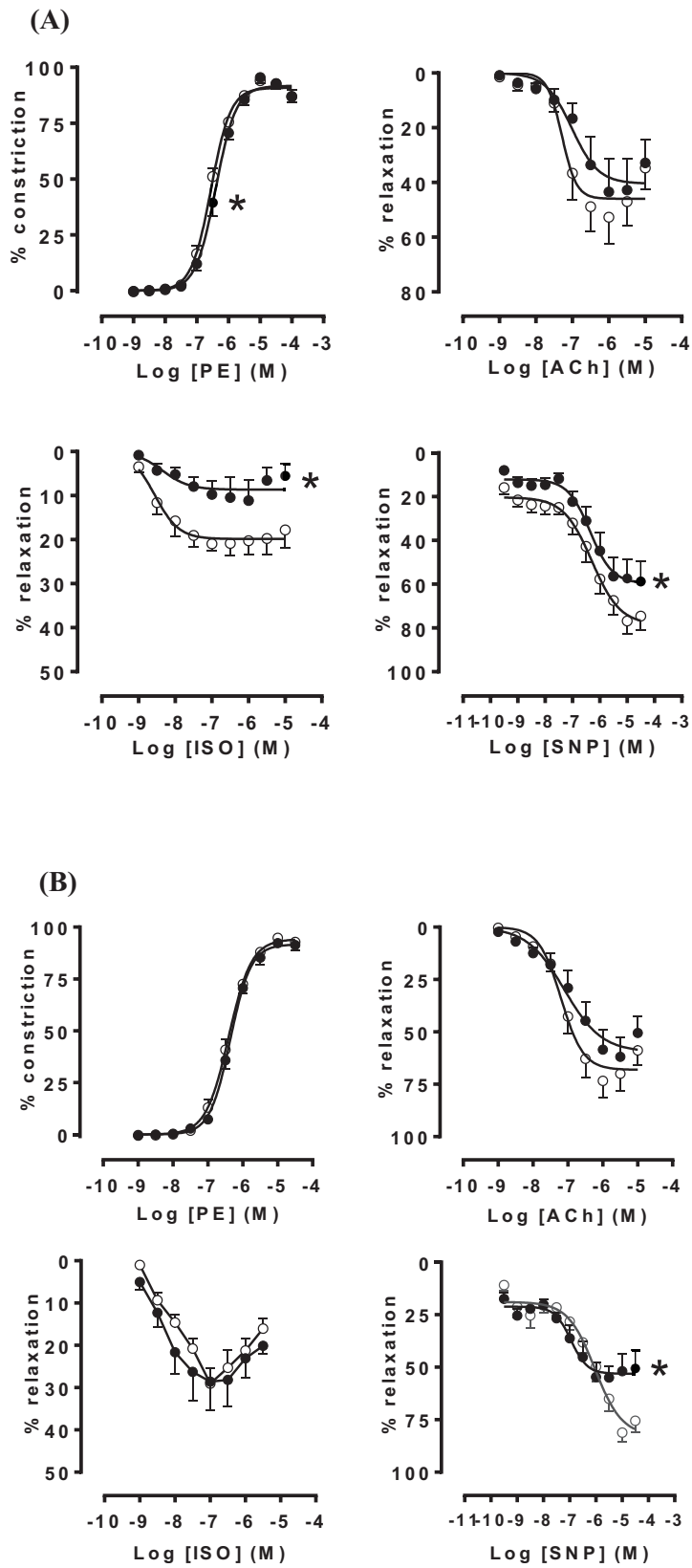
**Table 1.** Real-time qPCR primer details.

| Gene Name | Gene Symbol | Accession Number | Primer Sequences Forward Primer | Reverse Primer | Amplicon Length | Primer Efficiency |
|---|---|---|---|---|---|---|
| Adrenergic receptor, beta 1 | *Adrb1* | NM_007419.2 | ggatcgcctcttcgtcttct | cagtagatgatggggttgaagg | 69 | 1.70 |
| Angiotensin II receptor, type 1 alpha | *Agtr1a* | NM_177322.3 | actcacagcaaccctccaag | ctcagacactgttcaaaatgcac | 62 | 1.84 |
| Bone morphogenetic protein receptor, type II | *Bmpr2* | NM_007561.3 | gagccctcccttgacctg | gtatcgaccccgtccaatc | 60 | 1.80 |
| Cholinergic receptor, muscarinic 2, cardiac | *Chrm2* | NM_203491.3 | tcggtgttaactgtcatcttcc | tcaggttggaccggtttg | 109 | 1.85 |
| Solute carrier family 2, member 4 | *Glut4* | NM_009204.2 | gacggacactccatctgttg | gccacgatggagacatagc | 115 | 1.83 |
| Adenylate cyclase 5 | *Adcy5* | NM_001012765.4 | atggaagctggtggcaag | cacctcatagtccccattcag | 78 | 1.70 |
| Protein kinase C beta | *Prkcb* | NM_008855.2 | aagcgagggcaatgaaga | cttctggagccttggtacctt | 74 | 1.70 |
| Phospholipase C beta 1 | *Plcb1* | NM_001145830.1 | tcgatgagaagcccaagc | ggcagcctttgaacttgtc | 67 | 1.75 |
| Fat mass and obesity associated | *Fto* | NM_011936.2 | tctgtctgccatcctggtc | tggtaaagtccggacgactc | 94 | 1.72 |
| Phosphoglycerate kinase 1 | *Pgk1* | NM_008828 | tacctgctggctggatgg | cacagcctcggcatatttct | 65 | 1.70 |
| Peptidylprolyl isomerase B | *Ppib* | NM_011149 | ttcttcataaccacagtcaagacc | accttccgtaccacatccat | 92 | 1.80 |
| Succinate dehydrogenase complex, subunit A, flavoprotein | *Sdha* | NM_023281 | tgttcagttccaccccaca | tctccacgacacccttctgt | 66 | 1.88 |
| TATA box binding protein | *Tbp* | NM_013684.3 | gggagaatcatggaccagaa | gatgggaattccaggagtca | 90 | 1.70 |

**Table 2.** Offspring serum adiponectin and TNF-α concentrations.

| Sex | Males | | Females | | Significance (P) | | |
|---|---|---|---|---|---|---|---|
| Diet | NPD | LPD | NPD | LPD | Diet | Sex | Diet x Sex |
| Adiponectin (ug/ml) | 8.82 ± 0.29 | 8.95 ± 0.30 | 13.62 ± 0.29 | 13.20 ± 0.29 | - | < 0.001 | - |
| TNF-α (pg/ml) | 4.02 ± 1.82 | 9.38 ± 1.89 | 5.63 ± 1.86 | 3.64 ± 1.86 | - | - | 0.015 |

Mean serum adiponectin and TNF-α concentrations. n = 16 males and 16 females from each dietary group, with all litters sampled. Values are mean ± S.E.M.

**Table 3.** Offspring tissue transcript expression.

| Tissue | Gene | NPD | LPD | P value |
|---|---|---|---|---|
| Heart | *Adcy5* | 1.00 ± 0.02 | 0.93 ± 0.02 | 0.026 |
| | Fto | 1.00 ± 0.01 | 0.88 ± 0.02 | <0.001 |
| | *Plcb* | 1.00 ± 0.03 | 0.89 ± 0.03 | 0.027 |
| | *Prkcb* | 1.00 ± 0.08 | 0.78 ± 0.03 | 0.008 |
| Liver | *Adcy5* | 1.00 ± 0.06 | 0.90 ± 0.06 | 0.285 |
| | *Fto* | 1.00 ± 0.02 | 0.90 ± 0.02 | 0.006 |

Mean relative transcript expression (± S.E.M.) for selected genes involved in calcium signalling and metabolic regulation from NPD and LPD offspring heart and liver tissue.  n = 10 males and 10 females from each dietary group, with each pair from separate litters. Transcript expression normalised to that of *Ppib* and *Sdha* (heart) and *Pgk1* and *Tbp (liver),* and adjusted to NPD values of 1.00.