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ORIGINAL ARTICLE

Multigenerational obesity-induced perturbations in oocyte-secreted factor signalling can be ameliorated by exercise and nicotinamide mononucleotide

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STUDY QUESTION: Can maternal and offspring high-fat diet (HFD)-induced changes in mRNA expression levels in mice be ameliorated by interventions in female offspring?

SUMMARY ANSWER: Our results indicate that exercise and nicotinamide mononucleotide (NMN) can ameliorate the negative effects of maternal and post-weaning HFD in female offspring.

WHAT IS KNOWN ALREADY: Maternal and post-weaning HFD can perturb offspring developmental trajectories. As rates of maternal obesity are rising globally, there is a need for effective treatments in offspring to ameliorate the negative effects from a maternal obesogenic environment. Modulation of the nicotinamide adenine dinucleotide (NAD^+) pathway by exercise and the NAD^+ precursor NMN has previously been shown to reduce the effects of obesity.

STUDY DESIGN, SIZE, DURATION: This study consisted of a multigenerational study using C57Bl6 mice. Mice were fed a control (chow) or HFD *ad libitum* throughout mating, pregnancy and lactation ($n = 13–25$). Female offspring ($n = 72$) were then also supplied either a chow or HFD post-weaning. At 9 weeks of age offspring from HFD dams were subjected to exercise on a treadmill for 9 weeks or at 16 weeks of age administered NMN (i.p.) for 2.5 weeks. At 18.5 weeks mice were euthanized and ovaries and cumulus–oocyte complexes (COC) were collected to examine the possibility of ameliorating the negative effects of maternal and post-weaning HFD.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ovary and COC mRNA expression was analysed using RT-qPCR. An initial screen of candidate genes was developed to test which molecular pathways may be involved in generating adverse reproductive system effects. For histological analysis, ovarian tissue was fixed in paraformaldehyde and embedded in paraffin and stained with haematoxylin and eosin. The numbers of primordial, primary, secondary and antral follicles were counted.

MAIN RESULTS AND THE ROLE OF CHANCE: In the offspring's COC, maternal obesity increased both growth differentiation factor 9 (*Gdf9:* 2-fold; $P < 0.05$, HFD versus chow) and bone morphogenetic protein 15 (*Bmp15:* 4-fold; $P < 0.05$, HFD versus chow) mRNA expression levels while exercise and NMN interventions did not regulate *Gdf9* and *Bmp15* in the same manner. In whole ovary, maternal diet programmed a 25–50% reduction in FSH receptor and sirtuin-3 mRNA expression levels in daughter ovaries ($P < 0.05$, HFD versus chow). There was a significant interaction between HFD and intervention on the proportion of large preantral and preovulatory follicles ($P < 0.05$).

However, the increase in preovulatory follicles did not translate to increased oocyte yield. NMN administration resulted in reduced body weight in HFD-fed individuals.

LIMITATIONS, REASONS FOR CAUTION: It is unclear if the changes in oocyte mRNA expression levels reported here will impact oocyte quality and fertility in offspring. Offspring ovulation rate or fecundity could not be studied here and fertility trials are required to determine if the changes in gene expression do reduce fertility.

WIDER IMPLICATIONS OF THE FINDINGS: Our results demonstrate that maternal and offspring HFD perturbs key signalling pathways that are known to regulate fertility in mice, highlighting the importance of interventions in helping to prevent the declining rates of fertility in the context of the current obesity epidemic.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants and fellowships from the National Health and Medical Research Council to R.B.G. (APP1023210, APP1062762, APP1117538) and to M.J.M. and D.A.S. (APP1044295). DAS is a consultant to and inventor on patents licenced to Ovascience, Metrobiotech and GlaxoSmithKline. The other authors declare that there is no conflict of interest.

Key words: nicotinamide mononucleotide / NAD^+ biosynthesis / growth differentiation factor 9 / bone morphogenetic protein 15 / oocyte / obesity / oocyte / FSH receptor / sirtuin

WHAT DOES THIS MEAN FOR PATIENTS?

This article investigates whether the combined effects of a mother's and offspring's high-fat diet can be reversed. Obesity is increasing worldwide, and this has an impact on both men and women and on their children. When a mother is obese, it may affect her daughter's ovulation rate, embryo quality and menopause later in life.

Mice were used in this study, which looked at whether exercise and a drug called nicotinamide mononucleotide (NMN) can be used to reduce the impact of a mother's high-fat diet on her children. The researchers found that genes which are essential to egg quality and fertility were sensitive to a mother's diet, but that interventions such as exercise and NMN could make a difference to the impact this has. NMN reduced bodyweight in individuals consuming a high-fat diet.

Introduction

Obesity is considered by the World Health Organization as a global epidemic (WHO, 2003) and can lead to a wide range of co-morbidities such as infertility (Cordozo *et al.*, 2011). Maternal obesity prior to and during pregnancy can induce detrimental programming outcomes in the developing gamete and conceptus. As the prevalence of obesity in women of reproductive age is increasing (Heslehurst *et al.*, 2010; Fisher *et al.*, 2013), there is increased risk of immediate (Aune *et al.*, 2014) and delayed adverse outcomes for both mother and her offspring later in life (Reynolds *et al.*, 2013).

During foetal organogenesis, the developing reproductive system seems to be particularly sensitive to early life influences. Maternal diet during pregnancy affects numerous parameters of offspring reproductive physiology including the follicular reserve (Cheong *et al.*, 2014; Tsoulis *et al.*, 2016) and oocyte quality (Minge *et al.*, 2008). It is well established that gamete and foetal environments play a critical role in programming many aspects of physiology later in life (Bertoldo *et al.*, 2018). Once developmental programming is set, it remains throughout life and can be transmitted to successive generations (Aiken and Ozanne, 2014). Owing to the finite number of functional oocytes that form solely during foetal development, unfavourable conditions during this period may directly affect the fertility of offspring.

Folliculogenesis and oocyte development are regulated by numerous locally produced intra-ovarian factors that act in paracrine and autocrine manners, which in turn interact with endocrine cues particularly during the late stages of oocyte development (Scaramuzzi *et al.*, 2011). The oocyte plays an indispensable role in follicle development and the acquisition of oocyte developmental competence through the release of oocyte-secreted factors that act on the granulosa and cumulus cells (Gilchrist *et al.*, 2008). Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are two of these factors and belong to the transforming growth factor β (TGFβ) superfamily. Other members of the TGFβ superfamily, such as anti-Müllerian hormone (AMH), are synthesized by the granulosa cells and regulate the sensitivity of follicles to FSH (Durlinger *et al.*, 2001). Furthermore, a complex relationship exists within the follicle whereby AMH and the oocyte-secreted factors regulate each other's expression (Estienne *et al.*, 2015). GDF9 and BMP15 also act synergistically to modulate folliculogenesis (Otsuka *et al.*, 2011; Mottershead *et al.*, 2012) and their expression ratios and relative bioactivities are believed to regulate mammalian ovulation rate (Crawford and McNatty, 2012). The requirement of GDF9 and BMP15 for the acquisition of oocyte developmental competence is also widely accepted (Gilchrist *et al.*, 2008). Given that GDF9 and BMP15 are indispensable to female fertility, it is likely that maternally induced perturbations in their regulation could impact female offspring fertility.

The increased prevalence of obesity can be explained by combining an increasing energy intake with decreased energy expenditure. Therefore, lifestyle interventions such as diet and exercise are commonly employed to reduce obesity. Nicotinamide adenine dinucleotide

(NAD^+) is recognized as a central regulator of fundamental biological processes including energy metabolism, lifespan regulation, DNA repair and apoptosis (Belenky *et al.*, 2007). NAD^+ biosynthesis is severely compromised in obese individuals (Yoshino *et al.*, 2011) and studies, including our own, have demonstrated that exercise increases NAD^+ levels (Canto *et al.*, 2010; Koltai *et al.*, 2010; Uddin *et al.*, 2016), and reverses the adverse effects of obesity in numerous organs (Yoshino *et al.*, 2011; Uddin *et al.*, 2016). Therefore, the positive effects of exercise are thought to be mediated, at least in part, through increasing NAD^+, making it a promising target for pharmacological intervention. Recently we reversed the negative impacts of maternal obesity in offspring through exercise and promotion of NAD^+ biosynthesis by using a NAD^+ precursor, nicotinamide mononucleotide (NMN) (Uddin *et al.*, 2017). Currently there is substantial interest in NMN as an anti-aging drug and it is undergoing Phase I clinical trials in males (UMIN, 2016). Importantly, NMN can be purchased as a 'supplement' and alarmingly IVF patients are already self-medicating even though its effects on fertility, oocyte function and offspring health are largely unknown. Consequently, research using animal models is a high priority to define the effects of NMN on oocytes and fertility.

The interplay between maternal diet during gamete and conceptus development and post-weaning offspring diet in generating adverse programming effects has been investigated by numerous groups (Chen *et al.*, 2008; Watkins *et al.*, 2008; Jungheim *et al.*, 2010; Bahari *et al.*, 2013). A post-weaning high-fat diet (HFD) can exacerbate the effects of a developmental programme set by an adverse maternal diet (Chen *et al.*, 2008). Conversely postnatal interventions such as exercise (Bahari *et al.*, 2013; Uddin *et al.*, 2016, 2017) or NMN (Uddin *et al.*, 2016, 2017) could assist in the prevention of adverse phenotypes later in life. Protection from adverse programming is not well explored in the reproductive system. The aim of this study was to assess the impact of maternal and post-weaning diet in mice on the mRNA expression levels of key genes regulating oocyte quality and ovarian function in offspring. In addition, we investigated the ameliorating effects of exercise and NMN intervention on HFD-induced oocyte gene expression.

Materials and Methods

Animal experimentation

All animal procedures were approved by the UNSW Animal Ethics Committee; ethics number 13/25B. Three-week-old C57BL6/J female mice were purchased from the Animal Resources Centre, Western Australia, and housed at 21 ± 2°C (12:12 h light/dark) at the Biological Resources Centre facility, UNSW, Australia. Mice were acclimatized for 1 week on standard chow diet. A week later groups of mice with similar average body weight were assigned to either a control chow or HFD (Supplementary Table SI).

The chow diet consisted of 11 kJ/g, 4% maximum crude fat of total food weight (Supplementary Table SI; Gordon's Stock Feeds, Yanderra, NSW, Australia). The HFD pellets were a semi-pure dietary formulation for laboratory rats and mice based on Research Diets D12451 (Specialty Feeds, Glen Forrest, Western Australia) containing 23.5% of total weight as fat and 19 kJ/g digestible energy (Specialty Feeds SF 04-001). The diets were available *ad libitum*.

The experimental design is presented in Fig. 1A, which is the identical design and the same mice as recently described (Uddin *et al.*, 2017). Following 6 weeks on the different diets, one adult male mouse maintained on a chow diet was introduced into each cage of four females for 4 days. There was no significant difference in body weights of male mice between the two female dietary groups. All dams were virgins at the time of mating and had only their first litter included in the study. Two weeks after mating pregnant mice were housed individually and the pre-pregnancy diet was continued throughout pregnancy and lactation. All pups were born over a 3-day period and were left undisturbed for the first week of life to prevent stressing the dam. At 3 weeks of age, female offspring were weaned and allocated either a chow or HFD, thereby generating four dietary groups in the offspring (Fig. 1B). After weaning, female offspring were allocated to different dietary and intervention groups in a manner ensuring no differences in group average body weights. Offspring body weight and white adipose tissue (WAT) were collected when mice were euthanized at 18 weeks of age.

Only offspring of HFD dams were subjected to exercise or NMN interventions as previously described (Uddin *et al.*, 2017). Six weeks after introducing the two diet regimes to offspring from HFD dams, these animals were further distributed into either an exercise, NMN or no intervention group, creating a total of eight groups (Fig. 1B). At 9 weeks of age, a cohort of the offspring from HFD dams began exercise as described below. It is possible to achieve the same effect of 8.5 weeks of exercise with 2.5 weeks of NMN injections, as we have previously demonstrated (Uddin *et al.*, 2016). Therefore at 16 weeks of age, a second cohort was administered NMN as described below. Vehicle (phosphate-buffered saline) was administered to the remaining groups. To avoid the confounding effects of many siblings in any treatment, care was taken to ensure that siblings were distributed to different interventions (Supplementary Table SII).

After 5 days of training, the exercise groups underwent treadmill use (Columbus Instruments Exer 3/6 (0257–901 M), OH, USA) running 6 days per week for 9 weeks. Prior to each session the exercised mice had a warm-up period of increasing running speed from 6 to 12 m/min. At 9 min the speed was increased to 15 m/min. After 400 m, the speed was reduced to 6 m/min for 5 min then returned to 15 m/min for another 20 min. Exercise was carried out 1 h before the end of the light period. All of the mice in the non-exercise groups were transported to the exercise room every day and experienced the treadmill with the belt turned off for 12 min, 5 days per week. This exercise regime was selected as it has previously been shown to ameliorate the pathophysiological effects of HFD in metabolic tissues (Uddin *et al.*, 2016, 2017).

NMN (Sigma N3501) was dissolved in PBS and injected i.p. daily from 16 to 18.5 weeks of age (18 days). The dose was 500 mg/kg body weight as previously reported and has previously been demonstrated to partly ameliorate the pathophysiology of HFD-induced obesity in female mice (Yoshino *et al.*, 2011; Uddin *et al.*, 2016, 2017). All non-NMN-treated mice received a vehicle i.p. injection of PBS daily at the end of the light period (19:00 h).

RNA extraction

Ovarian tissue and cumulus–oocyte complexes (COCs) were thawed on ice and solubilized in Qiazol (Qiagen, Germantown, MD, USA) before RNA extraction. Total RNA was extracted using the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. DNA that may have been co-extracted was removed by the addition of a DNase treatment (Qiagen). RNA was eluted in 10 µl of RNase-free water and stored at –80°C. Final RNA quality and concentrations were determined using an Agilent 2100 electrophoresis bioanalyser (Agilent Technologies, Santa Clara, CA, USA). All sample RNA integrity numbers were >7.4.

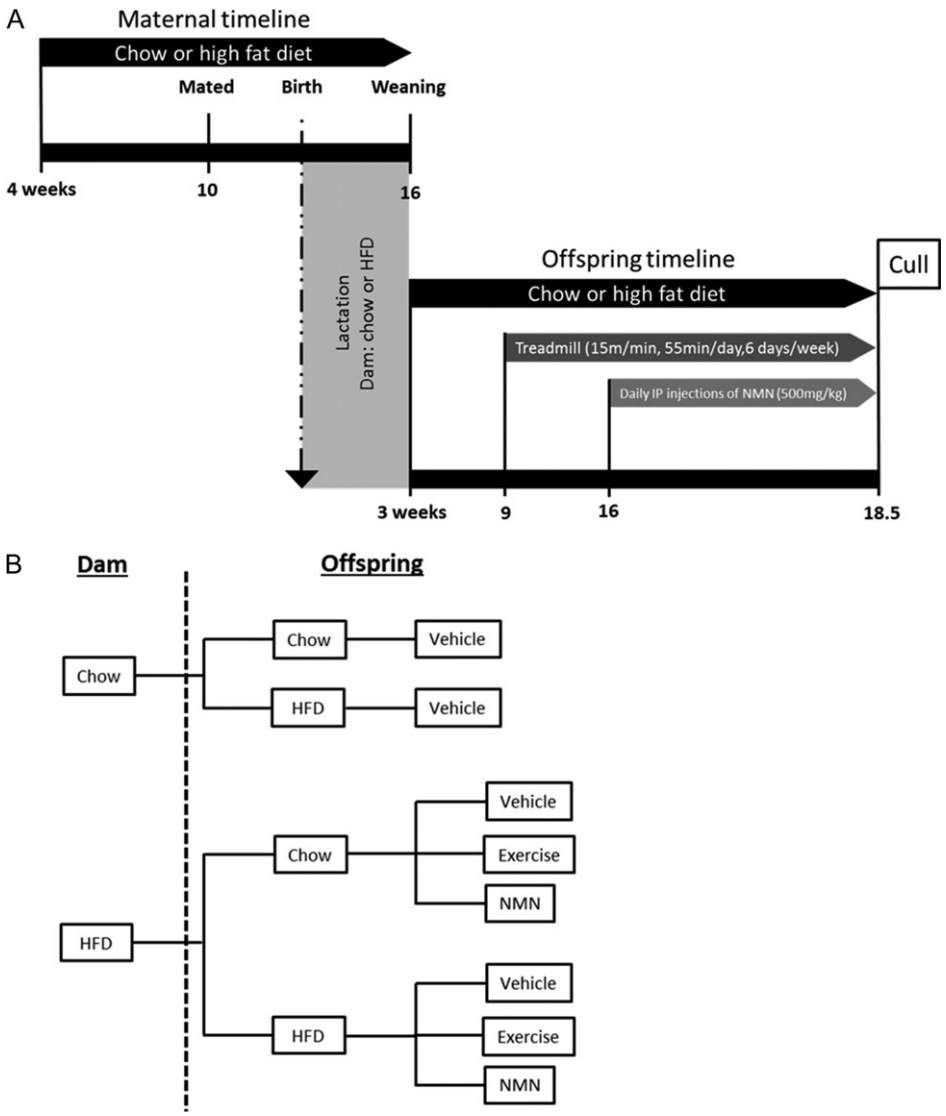

Figure 1 The experimental mouse treatment protocol and cohort design. In the treatment protocol (**A**) and cohort design (**B**), from 4 weeks of age, dams were fed a chow or high-fat diet (HFD) for 6 weeks before mating and for the duration of parturition and weaning. The dams were maintained on their respective diets until sacrifice at 30 weeks of age. Following weaning, female offspring were fed a chow or HFD. At 9 and 16 weeks of age, cohorts of animals were either exercised on a treadmill or administered nicotinamide mononucleotide (NMN), respectively. Offspring were culled at 18.5 weeks of age.

Quantitative RT-PCR

An initial screen of candidate genes was developed to test which molecular pathways may be involved in generating adverse reproductive system effects (primer sequences are listed in Supplementary Table SIII). These genes were chosen based on previous work on reproductive physiology and knowledge of programming mechanisms in other organ systems. Relative gene expression methods were calculated using the $2^{-(\Delta\Delta CT)}$ method.

COCs

An equal amount of total RNA (1 μg) from each sample was reversed transcribed using SuperScript®III Reverse Transcriptase (Life Technologies #18080085). Briefly, following the manufacture's protocol, the RNA was mixed with Random Primers (1 μL) and dNTPs (1 μL) followed by 5 min incubation at 65°C. To this mix, the following was added; 5× first strand buffer (4 μL), 0.1 M dithiothreitol (1 μL) and enzyme (1 μL) on ice. The samples were then incubated for 10 min at 25°C, 60 min at 50°C and 15 min at 70°C. Primers (2.5 μL Forward and Reverse) and cDNA (3 μL) were then added to a 20 μL total reaction volume with SYBR Green (10 μL). PCRs were performed using the LightCycler 480 II (Roche, Sydney). Thermal cycling conditions were set at 95°C for denaturing, then 40 cycles at 95 and 60°C for 15 and 60 s, respectively, for annealing and extension, followed by 95–60°C ramp for melt curve analysis.

Ovarian tissue

An equal amount of total RNA (1 μg) from each sample was reversed transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Gladesville,

Australia). Briefly, following the manufacture's protocol, RNA was mixed with Master-Mix (4 µL), enzyme (1 µL) and made to 20 µL with dH_2O. This reaction was incubated in a thermocycler for 10 min at 25°C, 30 min at 42°C and 5 min at 85°C. Quantitative PCR conditions for ovarian tissue were the same as those described for COCs. Primers are listed in Supplementary Table SIV.

Statistical analysis

Statistical analyses were performed using Prism 6 (GraphPad Software Inc. La Jolla, CA, USA). Data that were not normally distributed were log transformed before analysis. Data that were not normally distributed after log transformation were analysed using a non-parametric Kruskal–Wallis test and a Dunn's multiple comparison test. Statistical differences were detected using a Student's *t* test for maternal phenotype traits. To investigate the effect of diet and intervention on mRNA expression levels, follicle distribution and oocyte number in offspring, a two-way ANOVA with a Bonferonni correction for multiple comparisons followed by a Tukey's post-hoc test were used. When no interaction was detected ($P > 0.05$) the simple ANOVA main effects (diet or intervention) were assessed and reported. A value of $P < 0.05$ was considered significant; however, if the *P* value approached significance, it is provided in the figures.

Results

Dams consuming a HFD have heavier daughters at weaning

Dams that were fed a HFD for 6 weeks prior to mating were 15% heavier at the time of mating compared to those dams that consumed a chow diet ($P < 0.0001$; Fig. 2A). At weaning there was no effect of diet on litter size (chow: 6.0 ± 0.4 versus HFD: 6.0 ± 0.4) or offspring sex ratios. However, the daughters whose dams consumed a HFD were heavier compared with those whose dams consumed a chow diet ($P < 0.0001$; Fig. 2B). Male offspring showed similar effects of maternal obesity on body weight as their female siblings (data not shown).

Offspring body weight and tissue composition

Offspring body weight and WAT mass are shown in Fig. 3. Significant post-weaning diet effects were observed on final body weight. Post-weaning HFD increased offspring body weight significantly ($P < 0.001$; Fig. 3A). Additive effects of maternal and post-weaning HFD were also observed (Fig. 3A). Offspring final body weight was dependent on the exercise and NMN interventions when offspring were fed a HFD ($P < 0.05$; Fig. 3C). Post-weaning HFD increased offspring WAT mass significantly ($P < 0.001$; Fig. 3B and D). There was no effect of either intervention on WAT mass (Fig. 3D).

Diet and intervention effects on ovarian follicle distribution and oocyte yield

The proportion of primordial, small preantral, small antral, large antral and the number of corpora lutea were not affected by either maternal or offspring diet, or intervention. There was a significant interactive effect between offspring HFD and intervention on the proportion of large preantral and preovulatory follicles ($P < 0.05$; Table I), whereby exercise and NMN increased the number of large preantral follicles when both dam and daughter consumed a HFD. However in these animals, only exercise increased the number of preovulatory follicles ($P < 0.05$; Table I). The increase in preovulatory follicles did not translate to increased oocyte yield (Supplementary Table SV). There were no simple main effects of diet on the ovaries and all ovaries appeared normal. Representative cross-sections of ovaries from all treatment groups are shown in Supplementary Fig. S1.

Offspring COC mRNA expression level is regulated by both maternal and offspring diet

When both dam and daughter were fed a HFD, both *Gdf9* (Fig. 4A) and *Bmp15* (Fig. 4B) mRNA expression level was upregulated compared to those COCs when both consumed a chow diet ($P < 0.05$), while if either consumed a HFD, gene expression values were intermediate. There were no differences among groups in the expression ratio between *Gdf9* and *Bmp15* mRNA (data not shown). There was a significant effect of offspring diet on COC AMH receptor (*Amhr*) mRNA expression levels ($P < 0.01$; Fig. 4H), but no effect on *Amh* expression (Fig. 4G). While there were no overall main effects of either maternal and offspring HFD on COC sirtuin (*Sirt*)*1* and *Sirt3* (Fig. 4C and I) mRNA expression, the mitochondrial genes peroxisome proliferator-activated receptor gamma alpha (*Pparg1ca*) and mitochondrial pyruvate carrier 1 (*Mpc1*) were non-significantly upregulated, or were upregulated, if the dam consumed a HFD and the daughter consumed a chow diet ($P = 0.075$, Fig. 5A; $P < 0.05$, Fig. 5B). There were no significant effects of maternal or post-weaning diet on the mRNA expression levels for any of the remaining genes of interest (Supplementary Table SVI).

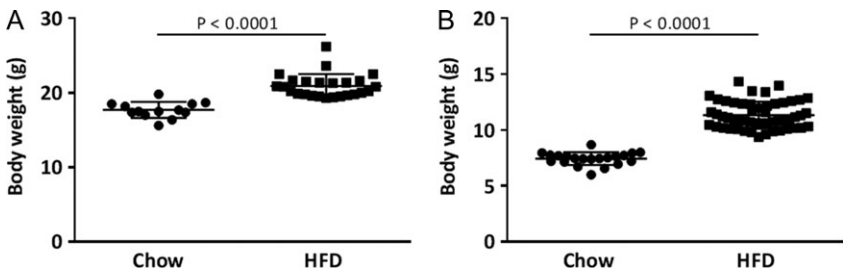

Figure 2 Maternal HFD leads to increased body weight in murine offspring at weaning. The body weights at mating of dams maintained on a control ($n = 13$) or HFD ($n = 25$) for 6 weeks (**A**). At weaning, offspring exposed to a maternal HFD ($n = 52$) during gestation and lactation are heavier than those exposed to a chow diet ($n = 21$) (**B**). Analyses calculated using a Student's *t* test.

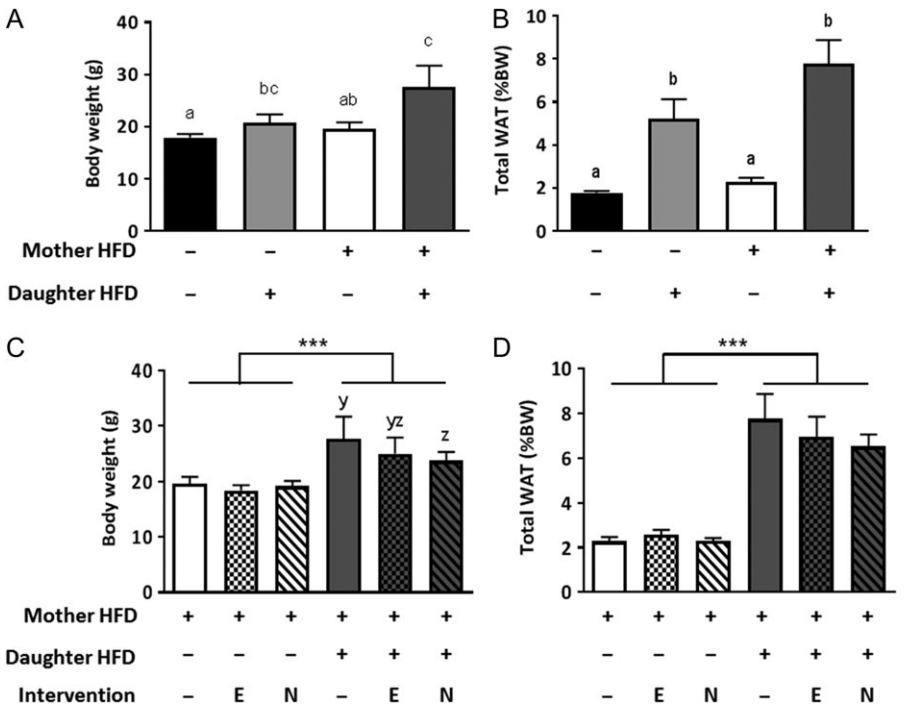

Figure 3 Effect of diet and intervention on female murine offspring phenotype. Effect of maternal and offspring HFD (**A**) or intervention (**C**) on offspring body weight and white adipose tissue mass (WAT) (**B**, **D**). All data are the mean ± SEM, $n = 7–10$ offspring from $n = 7–9$ dams per treatment. All animals were 18 weeks of age. Data analysis for body weight was performed using a Mann Whitney test. WAT mass was analysed using a two-way ANOVA followed by a Tukey's multiple comparison post-hoc test. a, b, c; different letters represent statistical significance at $P < 0.01$ for simple main effects; ***$P < 0.001$ additive maternal and offspring diet effect, x, y; different letters represent statistical significance at $P < 0.05$ intervention effect.

Table I ***In vivo*** **follicle development in mice.**

Maternal diet	Offspring diet	Intervention	Primordial	Small preantral	Large preantral	Small antral	Large antral	Preovulatory	Corpora lutea
Chow	Chow	Vehicle	10.4 ± 4.7	40.7 ± 8.0	18.5 ± 4.7	17.9 ± 2.9	10.6 ± 0.5	1.7 ± 2.0	3.7 ± 1.3
Chow	HFD	Vehicle	17.6 ± 1.2	31.9 ± 3.0	23.0 ± 2.0	13.4 ± 2.9	8.4 ± 2.7	5.7 ± 2.9	5.3 ± 0.3
HFD	Chow	Vehicle	11.9 ± 7.0	35.0 ± 6.4	23.6 ± 1.8	12.3 ± 2.3	12.3 ± 2.3	5.0 ± 1.4	2.3 ± 1.9
HFD	Chow	Exercise	16.6 ± 6.0	31.6 ± 1.8	24.9 ± 10.2	15.8 ± 4.1	7.3 ± 2.2	3.8 ± 2.7	3.0 ± 1.2
HFD	Chow	NMN	26.8 ± 2.4	33.3 ± 6.7	16.2 ± 3.9	11.8 ± 2.0	5.5 ± 0.6	6.4 ± 2.1	2.5 ± 0.5
HFD	HFD	Vehicle	25.0 ± 5.7	34.5 ± 8.5	11.7 ± 4.0‡	18.5 ± 4.7	6.8 ± 2.4	3.8 ± 0.3	3.3 ± 0.3
HFD	HFD	Exercise	22.6 ± 5.4	26.4 ± 4.9	16.4 ± 5.0	18.9 ± 2.8	7.1 ± 4.2	8.5 ± 1.3‡	3.5 ± 1.3
HFD	HFD	NMN	28.1 ± 3.7	25.7 ± 2.8	18.4 ± 3.7	13.9 ± 1.2	11.0 ± 5.4	2.9 ± 1.8	3.5 ± 0.5

Data are shown as mean number per ovary ± SEM, $n = 3–4$ animals per group. Two comparisons were made, first to assess the effect of maternal and post-weaning diet, and a second to assess the effect of intervention. Data were assessed by a two-ANOVA followed by a Tukey's multiple comparison post-hoc test. Significant interactions are presented as: ‡$P < 0.05$. No simple main effects were observed. No siblings were used in this analysis.
HFD, high-fat diet.
NMN, nicotinamide mononucleotide.

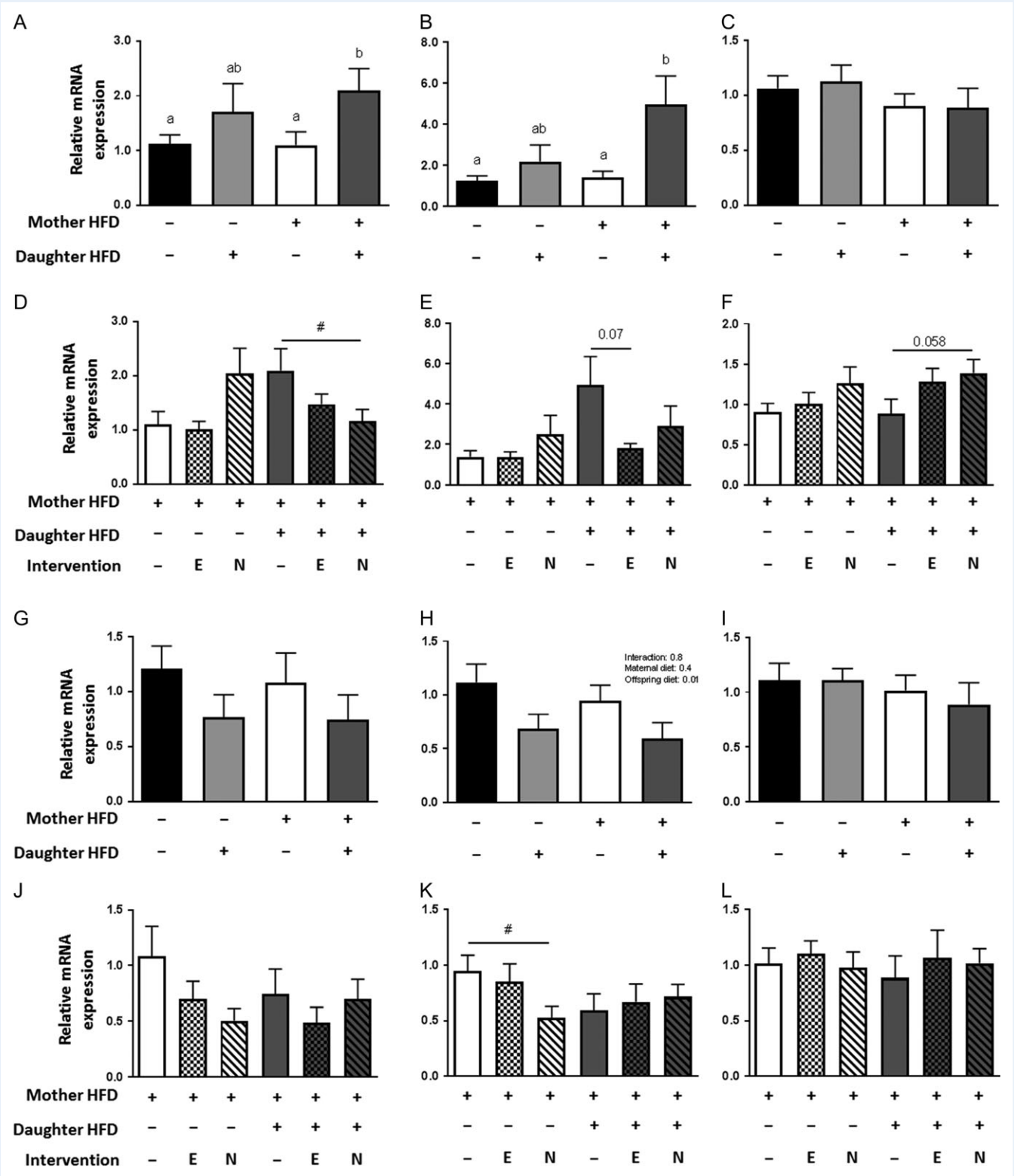

Figure 4 Effect of HFD and intervention on cumulus–oocyte complex gene expression in murine offspring. Effect of maternal and offspring high HFD on *Gdf9* (**A**), *Bmp15* (**B**), *Sirt1* (**C**), *Amh* (**G**), *Amhr* (**H**) and *Sirt3* (**I**) mRNA expression. Effect of intervention on *Gdf9* (**D**), *Bmp15* (**E**), *Sirt1* (**F**), *Amh* (**J**), *Amhr* (**K**) and *Sirt3* (**L**) mRNA expression. All data are the mean ± SEM, $n = 6$–10 offspring, from 6 to 9 dams per treatment. Values are expressed as relative fold change normalized to the control group. To investigate the effect of diet and intervention on mRNA expression, data were compared using a two-way ANOVA followed by a Tukey's multiple comparison post-hoc test. Different letters represent statistical significance at $P < 0.05$ for simple main effects; $^{\#}P < 0.05$ intervention effect. *Gdf9*, growth differentiation factor 9; *Bmp15*, bone morphogenetic protein 15; *Sirt1*, sirtuin 1; *Sirt3*, sirtuin 3; *Amh*, anti-müllerian hormone; *Amhr*, anti-müllerian hormone receptor.

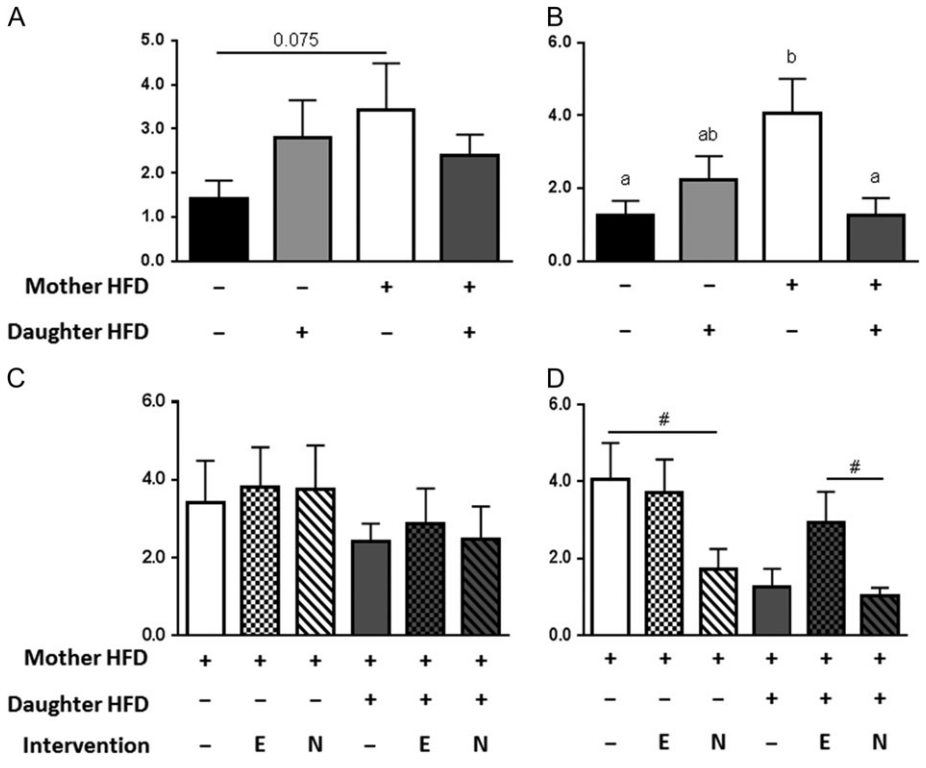

Figure 5 Cumulus–oocyte complex mitochondrial gene expression in response to diet and intervention in mice. Effect of maternal and offspring HFS and intervention on offspring cumulus–oocyte complex *Pparg1ca* (**A**, **C**) and *Mpc1* (**B**, **D**) mRNA expression. Values are expressed as relative fold change normalized to the control group. To investigate the effect of diet and intervention on mRNA expression, data were compared using a two-way ANOVA followed by a Tukey's multiple comparison post-hoc test. Different letters represent statistical significance at $P < 0.05$; $^{\#}P < 0.05$ intervention effect. All data are the mean ± SEM, $n = 6$–9 offspring per treatment from 6 to 9 dams. *Pparg1ca*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Mpc1*, mitochondrial pyruvate carrier 1.

Exercise and NMN intervention can differentially regulate mRNA expression in COCs from offspring of HFD fed dams

As the effects of excessive maternal weight gain prior to and during pregnancy exacerbate the problems of over-nutrition in the offspring (Chen *et al.*, 2008) we investigated the effect of exercise or NMN treatment on offspring COC gene expression. When both dam and daughter were fed a HFD, NMN reduced COC *Gdf9* mRNA levels when compared to the no intervention control (Fig. 4D; $P < 0.05$). When daughters consumed a chow diet, there was no effect of intervention on *Gdf9* or *Bmp15* mRNA expression in her COCs (Fig. 4D and E). However, when both dam and daughter consumed a HFD, there was a tendency for exercise to ameliorate the HFD effect on daughter COC *Bmp15* mRNA expression ($P = 0.07$; Fig. 4E). There was no significant effect of NMN on *Bmp15* mRNA expression (Fig. 4E). When the dam was fed a HFD and the daughter chow, NMN reduced COC *Amhr* mRNA expression ($P < 0.05$; Fig. 4K). However, *Amhr* mRNA expression was not affected by intervention when both dam and daughter were fed a HFD (Fig. 4K).

While there was no effect of maternal and offspring HFD on *Sirt1* mRNA expression (Fig. 5A), NMN induced a non-significant increase of *Sirt1* mRNA in offspring oocytes when dam and daughter consumed a HFD ($P = 0.058$; Fig. 5F). Maternal HFD increased mitochondrial pyruvate carrier 1 (*Mpc1*) mRNA expression in offspring COCs ($P < 0.05$; Fig. 5B). However, this appeared to be ameliorated by their offspring also consuming a HFD, or administration of NMN, or both ($P < 0.05$; Fig. 5D). If both dam and daughter consumed a HFD, exercise was significantly less effective than NMN at normalizing COC *Mpc1* mRNA expression ($P < 0.05$; Fig. 5D). There were no significant effects of intervention on expression levels of any of the remaining genes of interest (*Sirt3*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pparg1ca*), inhibitor of DNA binding *(Id)1*, *Id2*, gap junction alpha-1 protein (*Gja1*), protein kinase AMP activated alpha 2 catalytic subunit (*Prkaa2*), Fatty acid synthase (*Fasn*), Lactate dehydrogenase A *(Ldha)*; Supplementary Table SVI).

Offspring ovary mRNA expression level is regulated by both maternal and offspring diet

There was a significant interaction of dam and offspring diet on *Gdf9* mRNA expression such that when dams were fed a chow diet and offspring a HFD, ovarian *Gdf9* mRNA level increased ($P < 0.05$; Fig. 6A). However, when dams and offspring consumed a HFD, there was a significant decrease in *Gdf9* mRNA ($P < 0.001$; Fig. 6A). There was no effect of diet on ovarian *Bmp15* mRNA (Fig. 6B). When both dam and offspring consumed a HFD, FSH receptor *(Fshr)* mRNA expression was significantly reduced compared to controls ($P < 0.05$; Fig. 6C).

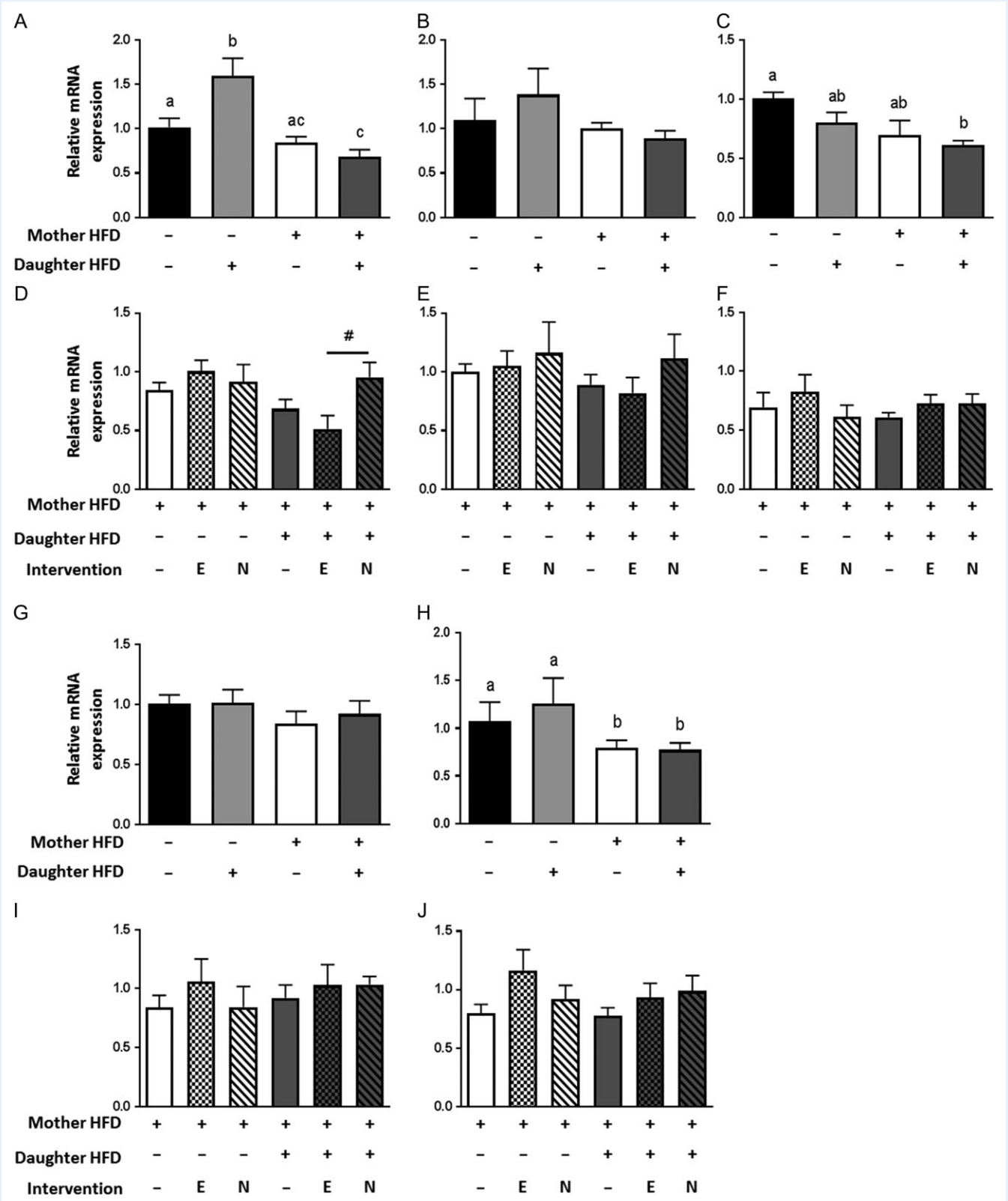

Figure 6 Effect of HFD and intervention on ovarian tissue gene expression in murine offspring. Effect of maternal and offspring high HFD on *Gdf9* (**A**), *Bmp15* (**B**), *Fshr* (**C**), *Sirt1* (**G**) and *Sirt3* (**H**) mRNA expression. Effect of intervention on *Gdf9* (**D**), *Bmp15* (**E**), *Fshr* (**F**), *Sirt1* (**I**) and *Sirt3* (**J**) mRNA expression. All data are the mean ± SEM, $n = 6$–10 offspring from 6 to 9 dams per treatment. Values are expressed as relative fold change normalized to the control group. To investigate the effect of diet and intervention on mRNA expression, data were compared using a two-way ANOVA followed by a Tukey's multiple comparison post-hoc test. Different letters represent statistical significance at $P < 0.05$ for simple main effects; $^{\#}P < 0.05$ intervention effect. *Fshr*: FSH receptor.

Maternal HFD significantly reduced *Sirt3* mRNA expression in offspring ovaries (Fig. 6H; $P < 0.05$).

Genes involved in cell cycle and metabolic regulation were affected by maternal and offspring diet (Table II). Maternal HFD significantly down-regulated daughter ovary *Nfkb1* mRNA expression, whereas insulin receptor (*Insr*) and cAMP responsive element binding protein 1 (*Creb1*) were increased when daughters consumed a HFD (Table II; $P < 0.05$). There were no significant effects of maternal or post-weaning diet on expression levels of any of the remaining genes of interest (Supplementary Table SVII).

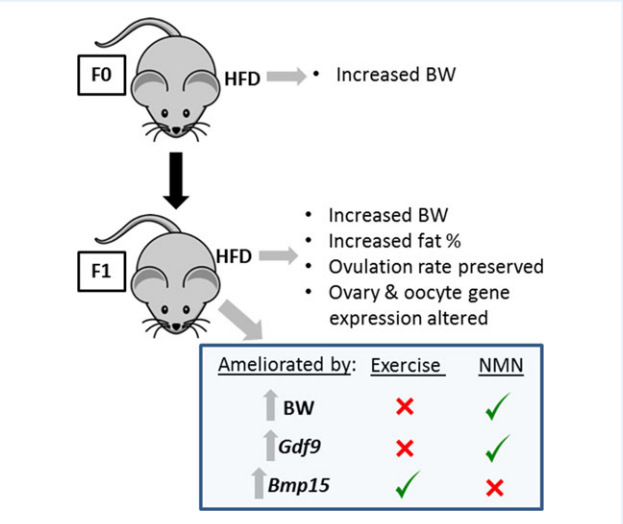

Figure 7 Diagramatic illustration summarizing the main findings from the study. Maternal and post-weaning HFD in mice led to obesity and abnormal ovarian and oocyte gene expression in female offspring. The increased offspring body weight and oocyte *Gdf9* expression induced by HFDs were ameliorated by administering offspring with NMN, whilst exercise normalized oocyte *Bmp15* expression. BW, body weight. FO, first generation. F1, second generation.

Exercise and NMN intervention differentially regulate mRNA levels in offspring ovaries

When daughters from HFD dams were fed a chow diet and administered NMN, ovarian *Pparg1ca* mRNA expression was significantly upregulated compared to the exercise intervention or no intervention ($P < 0.001$; Table II). If both dam and daughter consumed a HFD, NMN significantly upregulated *Prkaa2*, cryptochrome 1 (*Cry1*) (Table II, $P < 0.05$) and *Gdf9* (Fig. 6D, $P < 0.001$) mRNA expression level, compared to the exercise intervention group. There was no effect of exercise or NMN treatment on ovarian *Sirt1* or *Sirt3* mRNA (Fig. 6I and J). None of the remaining molecular markers measured at the mRNA expression level analysed in this study showed significant expression differences in ovarian tissue in response to diet or intervention.

Discussion

Modern society has been transformed, whereby individuals have a more sedentary lifestyle, with reduced physical activity and an increase in energy dense diets. This has significantly increased the prevalence of obesity worldwide, resulting in considerable consequences for fertility in both males and females and their offspring. We have previously shown that various interventions imposed on offspring have the greatest effect on those animals exposed to both a maternal and post-weaning HFD (Bahari *et al.*, 2013; Uddin *et al.*, 2017). We have also demonstrated that strategies increasing energy utilization either through pharmacological interventions or physical activity can be effective at reducing the effects of maternal and post-weaning HFD (Uddin *et al.*, 2017). Specifically in mice, we observed that 2.5 weeks of NMN supplementation had similar beneficial effects to 9 weeks of exercise for ameliorating the pathophysiological effects of HFD (Uddin *et al.*, 2017). For some of our genes of interest, we have shown here

Table II mRNA expression levels for metabolic markers in mouse offspring ovaries.

Maternal diet	Offspring diet	Intervention	Cell cycle regulation			Metabolism		
			Creb1	Cry1	Nfkb1	Pparg1ca	Prkaa2	Insr
Chow	Chow	Vehicle	1.2 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2
Chow	HFD	Vehicle	2.7 ± 0.5[b]	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	2.1 ± 0.5[c]
HFD	Chow	Vehicle	1.3 ± 0.2	0.9 ± 0.1	0.8 ± 0.1[a]	0.7 ± 0.1	0.8 ± 0.1	1.5 ± 0.1
HFD	Chow	Exercise	1.5 ± 0.3	1.4 ± 0.2[d]	1.2 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	2.2 ± 0.4
HFD	Chow	NMN	1.7 ± 0.2	0.8 ± 0.3	0.9 ± 0.1	1.4 ± 0.5[e]	1.1 ± 0.1	1.5 ± 0.4
HFD	HFD	Vehicle	1.6 ± 0.1	0.9 ± 0.2	0.8 ± 0.1[a]	0.8 ± 0.1	0.7 ± 0.1	1.7 ± 0.2
HFD	HFD	Exercise	1.5 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	1.5 ± 0.3
HFD	HFD	NMN	1.6 ± 0.4	1.2 ± 0.2[d]	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1[d]	1.8 ± 0.3

To assess the effect of exercise or NMN on ovarian gene expression, cohorts of females were exercised or treated with NMN as described in the methods. Data are shown as mean ± SEM, $n = 4$–6 animals from 4 to 6 dams per group. Data were compared by a two-way ANOVA followed by a Tukey's multiple comparison post-hoc test. The significant effects (simple main effects) are presented as: [a]$P < 0.05$ maternal diet effect; [b]$P < 0.001$ maternal diet effect; [c]$P < 0.05$ offspring diet effect; [d]$P < 0.05$ intervention effect; [e]$P < 0.001$ intervention effect.

Creb1, cAMP responsive element binding protein 1; *Cry1*, cryptochrome 1; *Nfkb1*, nuclear factor kappa b1; *Pparg1ca*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Prkaa2*, AMP-activated protein kinase alpha-2; *Insr*, insulin receptor.

for the first time that maternal and post-weaning HFD induced perturbations in key fertility gene signalling pathways can be ameliorated by interventions in the offspring (Fig. 7). We also demonstrated clear maternal HFD programming of *Fshr* in offspring for the first time. The two interventions utilized in the present study (exercise and NMN administration) reduced body weights to the same degree. This suggests that the differential effects observed for mRNA expression levels cannot be attributed to the differences in the degree of body weight loss between groups but to the distinct physiological effects induced by each intervention. Furthermore, the insight provided by this study has important clinical implications at a population level.

The most noticeable effect of HFD was the modulation of oocyte-specific mRNA expression levels and that diet-dependent changes occur in multiple oocyte mRNAs, suggesting that obesity induces abnormal gene transcription during oocyte development. *Gdf9* and *Bmp15* are oocyte-specific genes central to fertility; controlling species-specific fecundity, folliculogenesis and oocyte quality (Gilchrist *et al.*, 2008; McNatty *et al.*, 2014). In the present study in mice obesity altered oocyte mRNA expression levels of *Gdf9* and *Bmp15*. Programming of oocyte *Gdf9* and *Bmp15* mRNA expression was compounded by combined maternal obesity and post-weaning over-nutrition. While the magnitude of response between *Gdf9* and *Bmp15* mRNA is different, the overall pattern of response is similar, and expected, as expression of *Gdf9* and *Bmp15* appear to be tightly co-regulated (Crawford and McNatty, 2012). Cheong *et al.* (2014) observed increases in *Gdf9* mRNA expression in offspring ovaries when both the dam and daughter consumed a HFD. While Tsoulis *et al.* (2016) did not observe an effect of maternal HFD on offspring ovary *Bmp15* expression, this may be related to the lack of observed differences in bodyweight between treatments in that study. However, additives to maternal diets have previously been shown to alter offspring oocyte *Gdf9* and *Bmp15* mRNA expression in rabbits (Abadjieva and Kistanova, 2016). To our knowledge the only other report of HFD altering *Bmp15* mRNA expression is from Sohrabi *et al.* (2015), who observed a 15-fold increase in *Bmp15* mRNA expression in mature oocytes compared to those oocytes from females consuming chow. Together, these results suggest that not only are GDF9 and BMP15 sensitive to the metabolic state of the individual but also are affected by maternal HFD. Interestingly, our ability to normalize *Gdf9* and *Bmp15* mRNA expression in a clinically relevant cohort is significant in that the effects of maternal diet-induced perturbations could be ameliorated with either exercise or NMN.

Alterations in oocyte expression of *GDF9* and *BMP15* are typically associated with changes in ovulation rate and fecundity (McNatty *et al.*, 2004). Reduced expression or function of GDF9 or BMP15 slightly reduces fertility in mice, but increases fertility in mono-ovular species (Galloway *et al.*, 2000; Yan *et al.*, 2001). Transgenic mice with oocyte-specific over-expression of *Bmp15* exhibit no change in ovulation quota or litter sizes but have accelerated follicle growth leading to early onset reproductive senescence (McMahon *et al.*, 2008). In the current study we were not able to study offspring ovulation rate or fecundity. However, we did observe an increase in the number of pre-antral and preovulatory follicles, but no concomitant increase in oocyte yield collected from antral follicles from unstimulated animals in response to maternal and offspring HFDs. Other studies have observed an increase in ovulation rate in response to HFD (Minge *et al.*, 2008). Nonetheless, ovulation rate is governed by the expression ratios of *GDF9* and *BMP15* (Crawford and McNatty, 2012). In our study, the oocytes from HFD females exhibited an increase in both *GDF9* and *BMP15* mRNA expression, but no change in the expression ratios of these two genes. We speculate that oocyte yield and corpus luteum number did not change in these animals, as the ratio of these genes remained constant despite diet. The prevailing hypothesis by which *GDF9* and *BMP15* gene dosage in oocytes affects fecundity is via the effects of the differences in their ratio on the expression and function of the gonadotrophin receptors on granulosa cells (McNatty *et al.*, 2014). In mice, BMP15 suppresses *Fshr* and *Lhr* (LH receptor) mRNA expression in granulosa cells (Otsuka *et al.*, 2001). This is consistent with our finding of reduced *Fshr* expression in ovarian tissue from mice over-expressing oocyte *Bmp15* and *Gdf9* as a result of maternal and offspring exposure to HFD. The finding that FSHR regulation is in part under dietary control, and intriguingly can be programmed as a result of maternal HFD, is notable as FSH signalling is fundamental to female fertility. To our knowledge, this is the first evidence of maternal diet programming *Fshr* mRNA expression in offspring ovaries.

Clearly the multigenerational effects of such dietary insults on the fertility of the next generation in humans is currently unknown, but based on the fundamental role of *GDF9* and *BMP15* in regulating mammalian fecundity, we speculate that it may have an effect. Based on the discussion above, maternal obesity, either with or without offspring obesity, may affect the ovulation rate or ovarian reserve of the next generation of mothers, and hence timing of the menopause or oocyte, and hence embryo quality. Oocyte-secreted factor mRNA expression levels cannot be directly related to oocyte developmental competence as measurement of mRNA requires destruction of the oocyte and there are currently no reliable measures for the oocyte-secreted proteins. Although there is no consensus on the association of *Gdf9* and *Bmp15* mRNA and oocyte developmental competence (Li *et al.*, 2014; Ashry *et al.*, 2015; Pawlak *et al.*, 2015), addition of these growth factors to oocyte culture systems dramatically improves oocyte developmental competence (Hussein *et al.*, 2006; Mottershead *et al.*, 2015).

Detrimental reproductive phenotypes of young female offspring after exposure to an adverse maternal diet have been observed in a number of animal models (Connor *et al.*, 2012; Aiken *et al.*, 2013) and humans (de Bruin *et al.*, 1998; Boynton-Jarrett *et al.*, 2011; Chan *et al.*, 2015). Defence against adverse programming is not well explored in the reproductive system. Exercise and pharmacological interventions have previously been used to ameliorate the direct effects of a HFD in the individual (Yoshino *et al.*, 2011; Uddin *et al.*, 2016) and in the offspring of obese dams (Uddin *et al.*, 2017). Indeed we observed differential effects between exercise and NMN treatment in oocyte and ovary gene expression that may be related to the higher levels of NAD^+ resulting from the NMN intervention. This is the first study in the area of NAD^+ therapy directly comparing and evaluating the effects of an NAD^+ promoting drug and exercise on oocyte and ovary gene expression. We were not prepared to superovulate the animals because of the potential confounding effects of superovulation on oocyte gene expression (Chu *et al.*, 2012), and potential unknown interactions with NMN. However, oocyte yield from antral follicles, and the corpus luteum, can provide useful indications of follicle development, even though we did not count directly the number of ovulated oocytes. Furthermore, as the primary focus was on the molecular changes in tissues, it is anticipated that the non-exercise

control would not confound the results as the control mice experienced transport to the exercise room every day and the stationary treadmill 5 days a week. However, whilst this study demonstrates the benefit of exercise, the exercise regime used in the present study cannot be equated to exercise in women, and a similar exercise intervention for overweight women would need to be designed and evaluated.

The results of the present study advance our understanding of how subfertility can occur in the offspring of obese dams by demonstrating that genes critical to fecundity and oocyte quality are sensitive to maternal diet. This study suggests that an obesogenic environment during gamete and conceptus development has lasting consequences on female mouse oocyte biology, which may have implications for the estimated 15–30% of cases of idiopathic infertility in women (Quaas and Dokras, 2008). Further research is required to determine the full impact of HFD-induced perturbed oocyte mRNA levels of *Gdf9* and *Bmp15* on fecundity, reproductive senescence, oocyte quality and developmental programming of embryos. However, we were able to show a normalization of expression of these key oocyte genes following exercise and pharmacologic intervention in the offspring exposed to maternal and post-weaning HFD. As we know that multigenerational increases in disease risk exist (Frias and Grover, 2012; Chan *et al.*, 2015), understanding how the female germline is impaired by the maternal environment is critical as this may reveal a mechanism by which we could prevent intergenerational disease transmission (Lane *et al.*, 2015). NMN is a highly topical drug in the context of infertility owing to its broad-ranging anti-ageing effects and its current use by IVF patients as a supplement. This study provides important new knowledge on the effects of NMN intake on ovarian and oocyte function. If proven safe, such interventions have the potential to provide cost-effective strategies at a population level for preventing diseases associated with early-life insults.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

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Authors' roles

R.B.G., M.J.M. and D.A.S. secured funding for the project. M.J.M., R.B.G., D.A.S., M.J.B. and N.A.Y. conceived and designed the study. M.J.B., G.M.U., N.A.Y., D.A. performed experiments. MJB and NAY analysed data. M.J.B., R.B.G., M.J.M., K.A.W. and N.A.Y. interpreted data. M.J.B., R.B.G. and M.J.M. wrote the article which was reviewed by all authors.

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Conflict of interest

D.A.S. is a consultant to and inventor on patents licenced to Ovascience, Metrobiotech and GlaxoSmithKline. The other authors declare that there is no conflict of interest.

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