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***In utero* vitamin D deficiency predisposes offspring to long-term adverse adipose tissue effects**

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Abstract

The fetal period represents an important window of susceptibility for later obesity and metabolic disease. Maternal vitamin D deficiency (VDD) during pregnancy is a global concern that may have long-lasting consequences on offspring metabolic health. We sought to determine whether a VDD *in utero* environment affects fetal adipose tissue development and offspring metabolic disease predisposition in adulthood. Furthermore, we sought to explore the extent to which the VDD intrauterine environment interacts with genetic background or postnatal environment to influence metabolic health. Eight-week-old P₀ female C57BL/6J mice were fed either a VDD diet or sufficient diet (VDS) from four weeks before pregnancy (periconception) then bred to male *A^{VY/a}* mice. Females were maintained on the diets throughout gestation. At weaning, *A^{VY/a}* and *a/a* male F₁ offspring were randomized to low-fat (LFD) or high-fat diet (HFD) until 19 weeks of age, at which point serum and adipose tissue were harvested for analyses. Mice born to VDD dams weighed less at weaning than offspring born to VDS dams but experienced rapid weight gain in the four weeks post weaning, and acquired a greater ratio of perigonadal (PGAT) to subcutaneous (SQAT) than control offspring. Additionally, these mice were more susceptible to HFD-induced adipocyte hypertrophy. Offspring of VDD dams also had greater expression of *Pparg* transcript. These novel findings demonstrate that *in utero* VDD, an easily correctable but highly prevalent health concern, predisposes offspring to long-term adipose tissue consequences and possible adverse metabolic health complications.

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Author contribution statement

A M B, C A P, C S R, and wrote the manuscript. A M B, the graduate student under CSR's and CAP's mentorship, and S A J were responsible for conducting the study and data acquisition. M E K and A M B performed the statistical analyses. All authors contributed to the final edits of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Keywords

pregnancy; fetal programming; metabolic health; agouti mouse; obesogenic models; *Pparg* transcript; nutrition; DOHaD

Introduction

Vitamin D deficiency (VDD) is a global health concern for pregnant women, with prevalence rates as high as 84% in some populations (Sachan *et al.* 2005). Given the strong association between maternal serum and placental/cord blood 25-hydroxyvitamin D (25[OH]D) concentrations, maternal vitamin D status is considered an excellent indicator of internal fetal vitamin D status (Novakovic *et al.* 2012). Reports on human pregnancies demonstrate an association between maternal VDD and fetal growth restriction (Miliku *et al.* 2016); however, this effect appears to be highly dependent on the severity of hypovitaminosis D and when during pregnancy the deficiency is experienced (Leffelaar *et al.* 2010, Burris *et al.* 2012, Schneuer *et al.* 2014, Eckhardt *et al.* 2015). Maternal VDD has also been independently linked to several negative metabolic health outcomes in humans, including excess adiposity and related insulin resistance (IR) in later life (Krishnaveni *et al.* 2011, Crozier *et al.* 2012). Further support for a causal relationship between maternal VDD and both impaired fetal development and adverse metabolic health outcomes has recently been documented in rodent models subjected to *in utero* VDD (Reichetzedder *et al.* 2014, Maia-Ceciliano *et al.* 2016). Yet, data are lacking on the specific effects of maternal VDD on offspring propensity for excess adiposity and/or associated metabolic complications.

Aims of this study presented herein were threefold: (1) to determine the consequences of maternal VDD on offspring whole body growth and adipose tissue-specific growth/development; (2) to explore the potential for the intrauterine VDD environment to interact with the postnatal environment and/or genetic background to influence metabolic health; and (3) to explore the molecular mechanisms by which maternal VDD might influence adipose tissue in offspring.

Materials and methods

Animals

All experiments were approved by the University of Missouri (MU) Animal Care and Use Committee. Eight-week-old female C57BL/6J (*a/a*) mice were obtained from Jackson Laboratory and maintained (2 littermates/ cage) at 24°C at the MU Animal Sciences Research Center on a 12-h light/darkness cycle (07:00–19:00 h). To minimize any other extrinsic confounders, animals were maintained under uniform animal husbandry conditions including same room, same water bottles and source, bedding, randomized shelf position and same personnel handling all animals; thus the only extrinsic variable was diet. To produce a maternal VDD model and VDS controls, female C57BL/6J mice were randomized to a refined VDD (Research Diets D1007301; New Brunswick, NJ, USA) or a refined VDS (AIN-93G; Research Diets D10012G) diet for a four-week deficiency induction period (Table 1). Vitamin D status of both groups was confirmed by measuring serum

concentrations of 25(OH) D weekly for the first four weeks after randomization. By the study period termination, VDD-fed animals achieved VDD (19.5 ± 7.7 nmol/L) and the animals on the VDS diet maintained sufficient vitamin D status (81.8 ± 14.4 nmol/L). While the levels of VDD in rodents are not clearly established, the observed concentrations are consistent with what is considered sufficient/ deficient in humans (Ross *et al.* 2011). Additionally, our vitamin D-deficient dams achieved serum 25(OH)D concentrations that were similar to other studies using rodent models to examine the effects of maternal VDD on offspring outcomes (Giulietti *et al.* 2004, O'Loan *et al.* 2007, Burne *et al.* 2011).

Animals were provided free access to diets and water. After a 4-week VDD inducement period, females from both groups were paired with male viable yellow *agouti* ($A^{vy/a}$) mice for breeding. This breeding scheme provides a/a and $A^{vy/a}$ F₁ progeny exhibiting a range of coat colors, from which only yellow coat color $A^{vy/a}$ male mice were used in the current studies. All dams were continued on their assigned diets until parturition, at which point, P₀ females were maintained on VDS diet through lactation. This timeline approach is similar to previously described reports that have shown maternal VDD can lead to offspring neurobehavioral disruptions (Almeras *et al.* 2007, Harms *et al.* 2008, Fernandes de Abreu *et al.* 2010, Burne *et al.* 2011). The lactating dams were fed the VDS diet because the enhanced calcium absorption that occurs with pregnancy goes back to the nonpregnant state after parturition, thus putting vitamin D-deficient dams at great risk for physiological stress due to disruptions in calcium homeostasis. Moreover, we have evidence that vitamin D-deficient female mice remain deficient for up to 3 weeks after returning to a VDS diet (Kent *et al.* 1991, Fudge & Kovacs 2010, Belenchia *et al.* 2017).

After weaning, $A^{vy/a}$ and a/a male offspring were randomly assigned to receive AIN-93G (LFD; Research Diets D10012G) or a high-fat diet (HFD, Research Diets D07101303B). We studied effects in males only, as males of this genotype and coat color are more likely to become obese and diabetic (Wolff *et al.* 1998, 1999). The treatment scheme creates a $2 \times 2 \times 2 = 8$ groups factorial treatment design (*in utero* environment (maternal VDS vs VDD) \times genetic background ($A^{vy/a}$ vs a/a offspring) \times postnatal environment (offspring low-vs high-fat diet)) (Fig. 1). This arrangement allowed us to examine the effects of maternal vitamin D status on offspring growth, adipose tissue health and other related metabolic parameters through two distinct models of excess adiposity (obesity): genetic ($A^{vy/a}$) and postnatal environmental (high-fat diet-induced). Additionally, it provided us with intra-litter genotype controls (a/a).

Offspring growth, body composition and energy balance

Offspring included in the study were weighed initially at weaning (21 days of age), and each week until study termination. The reason we did not weigh them prior to weaning was to minimize neonatal stress and potential maternal cannibalization. These data were used to plot growth curves. To pinpoint offspring growth patterns, the study period was divided into four distinct phases: neonatal (birth-3 weeks old), juvenile (3–7 weeks old), adolescence (7–11 weeks old) and adulthood (11–15 weeks old) (Fig. 1). Three-compartment body composition (lean tissue, fat tissue and water) was determined by EchoMRI at 19 weeks of age, just prior to study termination.

At 15–16 weeks of age, offspring energy expenditure was measured via indirect calorimetry to determine effects of each of its components (energy intake and expenditure) on growth and body composition. Mice from each group were individually adapted to metabolic chambers (Promethion; Sable Systems, Las Vegas, Nevada) for a minimum of 24 h. Afterward, all parameters were measured for 48 consecutive hours.

Vitamin D status and circulating cytokines in offspring

Whole blood was collected from offspring at 11 (saphenous vein) and 19 (cardiac puncture) weeks of age, representing mid- and endpoints of the study, and separated by centrifugation, and then stored at -80°C until analysis. Serum concentrations of 25(OH)D were measured using ELISA (Eagle Biosciences; Nashua, NH, USA; range 10–300 nmol/L, intra-assay CV = 4.2%), following the manufacturer's protocol. Serum leptin, adiponectin, interleukine-6 (IL6) and tumor necrosis factor-alpha (TNFA) were measured using a commercially available MILLIPLEX Kit that simultaneously measures all of these cytokines (Customized Mouse Adipokine Panel; EMD Millipore; intra-assay CV 5.5%).

Serum glucose assessments

At 15–16 weeks of age, following a six-hour fast (~04:00– 10:00 h), blood was drawn from the saphenous vein to measure fasting glucose (AlphaTRAK 2 glucometer; Zoetis; Florham Park, NJ, USA). Fasting intraperitoneal glucose tolerance tests (IPGTT) were then performed by giving mice an intraperitoneal injection of 20% glucose solution equivalent to 2.0/kg of body weight. Glucose levels were remeasured at 15, 30, 60 and 120 min. Glucose disposal was calculated by determining the area under curve (AUC) by trapezoidal method on a time \times concentration plot.

Adipose tissue collection

At study termination (19 weeks of age), mice were euthanized. Perigonadal (PGAT) and subcutaneous (SQAT; inguinal) adipose pads were excised and weighed. Both fat pads were dissected in half, with one-half fixed in 4% paraformaldehyde solution (for histological analysis) and the remaining half flash-frozen in liquid nitrogen and stored at -80°C for later RNA extraction (PGAT fat pad only). All excisions were performed by the same individual to minimize technical variation.

Perigonadal adipose tissue histology

Fixed samples of PGAT were sent to the MU Veterinary Diagnostic Lab, where they were paraffin-embedded, sectioned (4–5 μm) and stained with hematoxylin and eosin (H&E). Six to eight fields per sample were acquired with an Olympus DX51 light microscope with the 40 \times objective lens, from which a minimum of 100 cells were measured using ImageJ software as described previously. Briefly, captured images were processed using binary, gray mode and threshold commands. Adipocytes for which a complete border was not distinguishable (e.g. those on the edge of the frame) were deselected. From the processed images, mean adipocyte area was determined using the 'measure all' command (NIH). This and other similar methods have been previously demonstrated to provide a validated

estimate of mean adipocyte size and morphology (Yamauchi *et al.* 2001, Chen & Farese 2002, Wueest *et al.* 2012, Osman *et al.* 2013, Parlee *et al.* 2014).

Perigonadal adipose tissue RNA isolation, cDNA synthesis and gene expression analysis

RNA was isolated from 40 mg of PGAT using the AllPrep DNA/RNA/miRNA Microkit (Qiagen). Genomic DNA (gDNA) from isolated RNA was removed using DNase 1 (Qiagen). RNA purity and concentration were assessed by 260/280 and 260/230 readings using a NanoDrop Spectrophotometer (Thermo Fisher Scientific), and RNA integrity was determined by fragment analyzer (ABI 3730xl DNA Analyzer, Applied Biosystem). First-strand cDNA was prepared using 40 µg of perigonadal adipose tissue using the QuantiTect Reverse Transcription kit (Qiagen).

Real-time quantitative PCR (qPCR) was performed, using template cDNA, QuantiTect SYBR green master mix (Qiagen) and mouse-specific primers (Table 2). Forward and reverse primer pairs for *Vdr*, *Pparg*, *Lep*, *Adipoq*, *Cebpa*, and hypoxanthinephosphoribosyl-transferase-1 (*Hprt1*, reference control) were designed to amplify fragments spanning more than one exon using the NCBI primer design tool and confirmed using Primer3Plus. Primers were acquired from IDT (Coralville, IA, USA).

Reactions were run on the ABI 7500 (Applied Biosystems) with the following conditions: Polymerase activation: 15 min at 95°C, followed by 40 cycles of (a) denaturation 15 s at 94°C, (b) annealing: 35 s at 56°C and (c) extension 35 s at 72°C (data acquisition step). A dissociation melt curve analysis was performed from 60°C to 95°C to verify the specificity of the PCR products. Reaction data were analyzed using the comparative cycle threshold method. A serial dilution 'pool lane' that comprised equal amounts of template cDNA from each sample was set up to compare reaction efficiencies.

Normalized expression values ($-\Delta\Delta CT$) were determined by subtracting the detected CT value of the target gene from the detected CT value of the housekeeping gene (*Hprt1*). These values are such that an increase in $-\Delta\Delta CT$ corresponds to an increase in the relative expression.

$-\Delta\Delta CT$ linear model coefficients represent log-fold changes in gene expression and allow for pairwise comparisons.

Statistical analysis

There were three factors with two levels each, *in utero* environment (two levels reflecting maternal vitamin D status; VDD or VDS), genetic background (two levels of offspring genotype; *a/a* or *A^{Vy}/a*), and postnatal environment (two levels of offspring diet; LFD or HFD), yielding eight combinations. The general experimental design was a split-plot in space, with *in utero* environment serving as the main plot; and genetic background, postnatal environment and all possible interactions serving as subplots. As more than one offspring per litter was tested, we controlled for the possible confounding effect of litter by assigning a unique nominal value for each dam-sire pairing; offspring nested within litter was used as a random factor in all subsequent ANOVAs. For all dependent variables, the model included the main effects *in utero* environment, genetic background, offspring diet, and all possible two- and three-way interactions of these factors.

Since the primary research question involved the effects of *in utero* environment (maternal vitamin D status), for some outcomes (inflammatory markers and gene expression), data for the groups with the combined obesogenic models ($A^{VY/a}$ mice fed HFD) were not collected. In these experiments, genetic background and postnatal environment were combined into one factor, 'offspring group.' This combined factor had three levels of interest, LFD \times a/a , HFD \times a/a and LFD \times $A^{VY/a}$, yielding a 2×3 factorial design. In such analyses, the model included the fixed effects of *in utero* environment (two levels; VDS and VDD), the aforementioned 'offspring groups,' and the *in utero* environment*offspring group interaction.

All data were checked for normality using the Shapiro–Wilke test. Non-normally distributed data were log-transformed for analysis and subsequently back-transformed for data presentation. Two-way ANOVA was performed using PROC GLIMMIX. Litter within *in utero* environment was used as the denominator for the main plot and 'litter' within *in utero* environment*genetic background*postnatal environment was used as the denominator for all subplots. Fisher's protected least significant difference (LSD) was used to make all pairwise comparisons. All data are expressed as least square means \pm S.D. in text and least square means \pm S.E.M. in tables/figures, with $P < 0.05$ considered significant for main and simple main effects.

Unadjusted Pearson correlation coefficients (r) were determined to assess the relationship between offspring weight at weaning, juvenile weight gain and adipose distribution. Correlations between PGAT gene expression and the aforementioned variables were also determined. All correlation analyses were carried out using PROC REG (SAS 9.4). $P < 0.05$ was considered significant.

Results

Pregnancy and litter characteristics

There were no differences between the VDD and VDS *in utero* environment groups in gestation length (20.2 ± 0.6 vs 19.9 ± 0.7 ; $P = 0.73$) or litter size (7.8 ± 1.2 vs 7.2 ± 1.9 ; $P = 0.37$; Table 3). Offspring sex ratio did not differ between the two groups (male pups: 53 vs 55%, respectively, $P = 0.93$) nor were there any differences in percentage of $A^{VY/a}$ offspring (47.9%, respectively, $P = 0.81$).

Offspring vitamin D status

No differences existed in serum 25(OH)D, at either 11 or 19 weeks of age, in the offspring born to VDD dams vs VDS dams (63.7 ± 10.8 vs 64.8 ± 10.4 nmol/L, $P = 0.79$ and 65.6 ± 13.4 vs 65.8 ± 11.1 nmol/L, $P = 0.83$, respectively). However, mean serum 25(OH)D levels were lower in both obesogenic offspring models ($A^{VY/a} = 47.4 \pm 12.1$ nmol/L and HFD = 48.9 ± 14.2 nmol/L; $P = 0.72$) than non-obesogenic offspring.

Offspring growth and energy balance

Mice born to VDD dams weighed less at weaning than those born to VDS dams (9.94 ± 0.68 g vs 10.41 ± 0.62 g, $P = 0.014$; not shown). There was a significant three-way interaction

between *in utero* environment, genetic background and postnatal environment on overall offspring growth curves from weaning to 19 weeks of age ($P=0.002$, respectively; not shown). The simple effects of this interaction revealed that offspring of VDD dams were more susceptible to genetically induced than HFD-induced obesity. Furthermore, offspring born to VDD dams gained more weight during the juvenile period (three to seven weeks of age; $+21.2$ g vs $+19.1$ g, $P=0.003$; Fig. 2), while there was no effect of maternal vitamin D status on weight gain during any other period of life.

In utero environment (maternal vitamin D status) had no effect on overall feed intake (g/day; $P=0.138$) or total energy intake (kcal/day; $P=0.105$; not shown). No significant main or interaction effects were evident for any of the three factors (*in utero* environment, genetic background and postnatal environment) on energy expenditure or spontaneous physical activity. $A^{VY/a}$ mice consumed significantly greater amounts of feed than a/a mice (3.3 ± 0.4 vs 2.7 ± 0.4 g/day; $P=0.0461$). Due to the enhanced caloric density of the HFD and hyperphagic phenotype of $A^{VY/a}$ mice, both the $A^{VY/a}$ (14.7 ± 1.5 kcal, $P=0.033$) and HFD (14.9 ± 2.1 kcal, $P=0.044$) groups consumed significantly more total energy than *non-obesogenic* counterparts (not shown).

Total, percent and distribution of body fat

A significant three-way interaction existed between *in utero* environment, genetic background and postnatal environment on total fat mass ($P=0.026$, not shown). Determination of the simple effects revealed that *in utero* environment (maternal vitamin D status) was not responsible for this interaction at any level of genetic background \times postnatal environment. There were no significant main or interaction effects of *in utero* environment on body fat percentage. Both obesogenic groups ($A^{VY/a}$ and HFD) had body fat percentages that were significantly greater than non-obesogenic groups (47.2 ± 10.4 vs $29.8 \pm 10.1\%$; $P < 0.001$; and 45.0 ± 11.4 vs $32.0 \pm 9.45\%$, $P < 0.001$, respectively).

In utero environment did not significantly affect absolute or relative SQAT pad weights of offspring ($P=0.113$ and $P=0.366$; not shown). However, there was a significant interaction between *in utero* environment and genetic background on absolute PGAT (visceral) weight ($P=0.022$), wherein *in utero* VDD resulted in significantly larger PGAT pads only in $A^{VY/a}$ offspring (2.13 ± 0.32 vs 1.76 ± 0.36 g; $P=0.006$; Fig. 3). Although the effect was similar in direction for the relative (to body weight) weight of PGAT pads, it did not reach statistical significance, ($P=0.08$; not shown). Both genetic background and postnatal environment had a significant effect on SQAT pad weight, with both obesogenic groups ($A^{VY/a}$ and HFD) having significantly larger SQAT pads (both $P < 0.0001$; not shown). A main effect of *in utero* environment on the ratio of PGAT to SQAT existed, wherein offspring gestated in a VDD *in utero* environment had a significantly greater ratio of PGAT to SQAT (1.1 ± 0.19 vs 0.94 ± 0.21 ; $P=0.012$; Fig. 3).

Blood glucose disposal

Baseline fasting glucose concentrations indicated no main or interaction effects of *in utero* environment (not shown). Likewise, the circulating glucose response to glucose load was not statistically significant between the maternal VDS and VDD groups (not shown). There was

a significant interaction effect of genetic background and postnatal environment, wherein mice with combined obesogenic factors (HFD-fed and $A^{VY/a}$) had elevated fasting glucose concentrations and impaired glycemic response regardless of *in utero* environment (not shown).

Adipocyte histology and circulating markers of adipocyte health

There was a significant two-way interaction between *in utero* environment and postnatal environment ($P = 0.039$), wherein HFD-induced PGAT adipocyte hypertrophy was greater in mice gestated in a VDD-deficient intrauterine environment than in those born from a VDS dams ($P = 0.017$; Fig. 4). Adipocytes from $A^{VY/a}$ offspring were significantly larger than those from non-obesogenic groups ($P = 0.002$). However, *in utero* environment did not similarly affect adipocyte area in $A^{VY/a}$ offspring ($P = 0.26$; not shown).

There were no main or interaction effects of *in utero* environment on the circulating concentrations of IL6 or TNF α ($P = 0.425$ and $P = 0.589$, respectively; not shown). Similarly, there were no main or interaction effects involving *in utero* environment on circulating leptin concentrations ($P = 0.451$; not shown). However, there was a main effect of offspring group on leptin, wherein $A^{VY/a}$ ($15,854 \pm 3320$ pg/mL), but not HFD-fed ($12,934 \pm 4656$ pg/mL) offspring, had greater circulating concentrations than a/a ($10,742 \pm 3235$ pg/mL; $P = 0.007$ and $P = 0.173$, respectively; not shown) mice.

Perigonadal adipose tissue gene expression

There was a significant main effect of *in utero* environment on *Pparg* mRNA expression, wherein mice born to VDD dams had 3.7-fold increase over those born to VDS dams ($P = 0.008$; Table 4). The interaction between *in utero* environment and offspring group was not significant ($P = 0.058$); however, visual examination of the interactions called for further analysis. In response to *in utero* VDD (vs VDS), *Pparg* expression was upregulated in the PGAT of both a/a (6.7-fold increase, $P = 0.006$) and $A^{VY/a}$ mice fed a low-fat diet (4.2-fold increase, $P = 0.02$), but not HFD-fed a/a mice. However, *in utero* environment did not significantly affect the relative expression levels of *Vdr* ($P = 0.946$), *Lep* ($P = 0.121$), *Adipoq* ($P = 0.669$) or *Cebpa* ($P = 0.092$) mRNA in PGAT.

Correlations between body weight and adiposity

There was a weak but significant negative correlation between weight at weaning and the ratio of PGAT:SQAT ($r = -0.28$, $P = 0.038$; Table 5).

Correlations between PGAT gene expression and phenotypic assessments

Among the genes analyzed from PGAT, the expression levels of *Pparg* ($r = -0.43$, $P = 0.009$), *Cebpa* ($r = -0.38$, $P = 0.025$) and *Vdr* ($r = -0.42$, $P = 0.01$) were all positively associated with PGAT:SQAT ratio (Table 6). However, while significant, the strength of these relationships was moderate. Likewise, moderate but significant associations existed between PGAT *Pparg* expression and PGAT weight ($r = 0.34$, $P < 0.043$). PGAT *Lep* expression was positively associated with total fat mass ($r = -0.42$, $P = 0.04$), PGAT mass ($r = -0.48$, $P = 0.003$) and circulating leptin concentration ($r = -0.46$, $P = 0.006$; Table 6).

Discussion

Vitamin D is a key player in differentiation of mesenchymal stem cells into several cell types including adipocytes (Narvaez *et al.* 2013, Valle *et al.* 2016). However, there is a gap in our understanding of the consequences of maternal VDD during pregnancy on adipose tissue development and related parameters of metabolic health in offspring. Our results indicate that male murine offspring gestated in a VDD environment experience: fetal growth restriction, accelerated growth in early life, larger visceral body fat pads and greater susceptibility to HFD-induced adipocyte hypertrophy. Moreover, the fetal growth restriction and accelerated juvenile weight gain are associated with adverse metabolic-health-related phenotypic outcomes in adulthood (greater ratio of visceral to subcutaneous fat distribution and adult body fat mass, respectively); and these observations are associated with greater *Pparg* expression in visceral adipose tissue.

There is scientific support for a relationship between maternal VDD and impaired fetal growth (Bodnar *et al.* 2010, Gernand *et al.* 2014). Epidemiological studies show a strong correlation between maternal VDD and fetal growth restriction in several cohorts (Leffelaar *et al.* 2010, Walsh *et al.* 2012). Conversely, prospective studies have yielded conflicting results that may be attributed to the varying degrees of deficiency and when during embryonic development the deficiency is experienced (Hashemipour *et al.* 2014, Park *et al.* 2014, Reichetzedder *et al.* 2014). In the present study, maternal VDD resulted in impaired fetal growth with apparent 'catch-up' juvenile weight gain. Offspring born to these dams weighed ~5% less than those to controls at weaning regardless of genetic background ($A^{VY/a}$ or a/a) and gained ~11% more weight in the first four weeks after weaning. Differences in body weight at weaning suggest that they weighed less at birth and/or gained less in the first three weeks post parturition. However, we are unaware of any evidence demonstrating delayed neonatal growth and therefore, we believe the rapid weight gain observed in these mice during the first four weeks post weaning suggests that fetal growth restriction with VDD is more likely. Although maternal VDD did not affect total fat mass or body composition of adult offspring in this rodent study; the finding of a positive correlation between juvenile weight gain and total adult fat mass, even when corrected for genetic background and postnatal environment, is interesting as accelerated weight gain during the neonatal period in humans is associated with adiposity in adulthood (Stettler *et al.* 2003, 2005, Breij *et al.* 2014).

Human studies have indicated that accumulation of fat in visceral adipose depots poses a greater risk to metabolic health than that in subcutaneous sites (Despres *et al.* 1990, Bouchard *et al.* 1993, Despres 2001, Landsberg 2008, Lee *et al.* 2013), possibly more so than excess total fat mass. Many factors influence the location of adipose deposition and its metabolic actions (Lachance & Page 1953, Pascot *et al.* 1999), and there is some evidence for vitamin D to be an important mediator. For example, in a rodent experiment, F₁ progeny born to VDD dams were shown to have more total fat and visceral fat (Nascimento *et al.* 2013). Our study extends these findings by showing that although total fat mass was unaffected, a greater visceral-to-subcutaneous fat ratio of adult offspring developed in the VDD *in utero* environment, independent of genetic background or postnatal environment. This is noteworthy as it indicates that exposure to VDD *in utero* either directly or indirectly

influences the development of body fat toward a more unfavorable (in terms of metabolic health) distribution.

The genes for the vitamin D receptor (VDR) and vitamin D-metabolizing enzymes are located in animal and human adipocytes; and both the active form, 1,25 dihydroxyvitamin D (1,25[OH]₂D), and the parent molecule, vitamin D, are known to influence several key adipogenic genes and transcription factors as well as lipid accumulation (Ding *et al.* 2012, Zoico *et al.* 2014). Even so, the specific function of vitamin D in the adipogenic process remains equivocal, and often contradictory, with studies showing both stimulation and inhibition of adipogenesis (Mutt *et al.* 2014). With the aid of two obesogenic models, we were able to explore the interactive effects of intrauterine vitamin D availability with both postnatal environment and genetic background on the development of adiposity and related metabolic health measures in offspring. Our data reveal that maternal VDD exacerbates select metabolic parameters differently depending on the obesity model (Fig. 5). Offspring gestated in a VDD environment demonstrated greater susceptibility to HFD-induced adipocyte hypertrophy. Conversely, intrauterine VDD only affected the distribution of adipose tissue in the genetic model of obesity, with *A^{Y/a}*, but not HFD-fed mice possessing larger visceral fat pads.

Maternal VDD has been associated with autoimmune disease in offspring (Erkkola *et al.* 2011), and multiple studies have demonstrated that offspring gestated in a VDD intrauterine environment are more susceptible to HFD-induced inflammation (Villa *et al.* 2016). We did not detect any effects of *in utero* VDD on either circulating inflammatory marker, TNFA or IL6. However, neither of our obesogenic models demonstrated elevated levels of these markers, and thus it is possible that the study was not carried out for long time to induce significant immunological responses. Circulating leptin concentration, which is highly correlated with adipose mass/size and insulin response, was significantly greater in our genetic model of obesity but not in our HFD-induced model of obesity. Both obesogenic models had altered glucose homeostasis and glycemic responses, while the *in utero* environment (maternal VDD) had neither effect on circulating leptin concentration nor on glucose disposal. This is not surprising since *in utero* VDD did not affect overall fat mass of the offspring. Furthermore, subcutaneous adipose tissue has been shown to produce more leptin than visceral adipose tissue and in the current study, *in utero* VDD had greater effect on visceral (PGAT) mass.

The nuclear receptor PPARG plays integral roles in both adipogenesis and lipid storage (Spiegelman *et al.* 1997, Rosen & Spiegelman 2000, Lazar 2002). In this study, mice born to VDD dams had greater *Pparg* expression compared to offspring born to those developed in a VDS intrauterine environment. We have previously noted similar findings in non-obese mice born to VDD dams (Belenchia *et al.* 2016). Taken together, these PPARG expression observations in adult offspring provide evidence that maternal VDD during pregnancy has profound and long-lasting effects on adipose tissue and perhaps its metabolic activity. Although our study was limited by the lack of protein expression required to confirm an increase in the actual receptor, it is possible that modest increases in *Pparg* expression can cause significant downstream effects on adipose tissue mass.

Limitations of this investigation include restricting our analyses to male offspring. We chose this initial approach based on evidence that *A^{VY}/a* males have greater metabolic disruptions than females (Klebig *et al.* 1995, Wolff *et al.* 1999). Extending the phenotypic findings identified herein, our follow-up studies will include female *A^{VY}/a* and *a/a* offspring exposed to similar prenatal and postnatal diet alterations; and further exploration of the effects of *in utero* VDD on other metabolic tissues such as liver and muscle. Another limitation is the length of the study, which may have been too short to see changes in any of the secondary measures of metabolic health, such as inflammatory markers or glucose metabolism. Thus, in future work, we plan to expand assessments and extend study length in offspring exposed to *in utero* VDD to determine if these offspring are more susceptible to greater overall morbidity issues and decreased lifespan. Additionally, future works will aim to replicate the observed changes in adipose tissue distribution and adipocyte characterization using more concise methods, such as whole body densitometry and stereological assessment of adipocyte 3D structure. The use of other animal models (such as VDR knockout) might also provide additional insight into the pleiotropic roles of vitamin D. Another future study would be to extend the maternal VDD through lactation to determine if more pronounced offspring effects are observed.

In conclusion, *in utero* VDD affects adipose tissue physiology and distribution into adulthood. Regardless of genetic background or postnatal environment, our results show that adult offspring gestated in a VDD environment gain more weight early in life, and have a more metabolically unfavorable fat distribution. Furthermore, the phenotypic response to *in utero* VDD is dissimilar between the two obesogenic models. These differences were associated with differences in *Pparg* mRNA expression in visceral fat, providing evidence that some metabolic disturbances of *in utero* VDD are mediated through direct effects on adipose tissue. These novel findings demonstrate that *in utero* VDD, an easily correctable but highly prevalent health concern, predisposes offspring to long-term adipose tissue consequences and possible adverse metabolic health complications.

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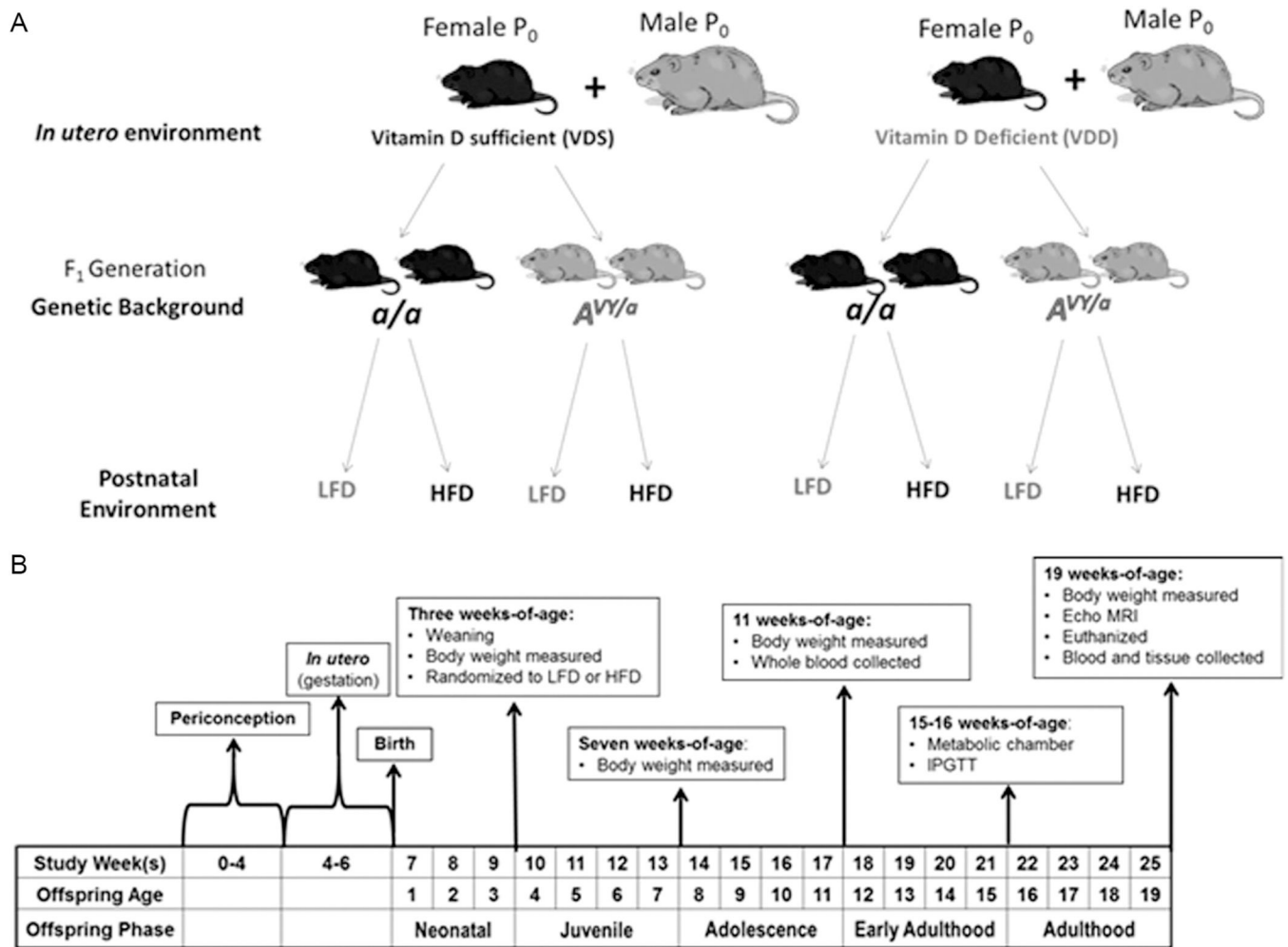


Figure 1.
 (A) General experimental design. Black mice represent *a/a* and yellow colored mice represent *A^{VY}/a*. Female *a/a* (C57BL/6) mice were fed either a vitamin D-sufficient (VDS) or deficient (VDD) diet to generate vitamin D sufficient or deficient *in utero* environments for pups. Female *a/a* were then bred with male *A^{VY}/a* to generate litters containing mice of both genetic backgrounds. After weaning, offspring from each of these genetic backgrounds were randomized to receive either a high-fat diet (HFD) or low-fat diet (LFD) to create two distinct postnatal environments. (B) Experimental timeline for analyses and offspring age/ life phase classifications of male F₁ offspring.

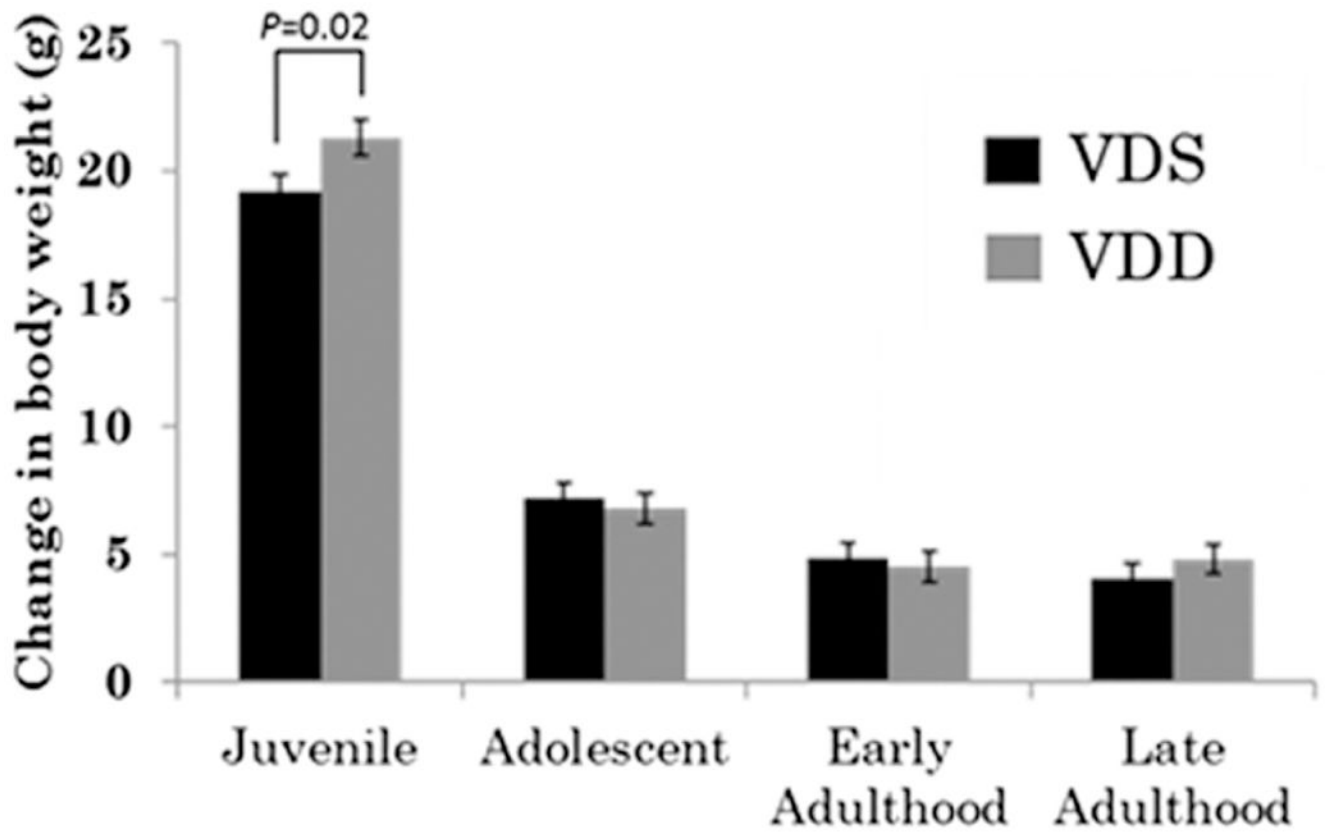


Figure 2. Body weight (BW) changes in offspring during various life phases: juvenile (weaning through 7 weeks of age), adolescent (7 through 11 weeks of age), early adulthood (11 through 15 weeks of age) and adulthood (15 through 19 weeks of age). Different shaded bars represent different *in utero* environment groups (i.e., maternal vitamin D status, vitamin D deficient (VDD) or sufficient (VDS)). Data are presented as marginal mean \pm S.E.M.

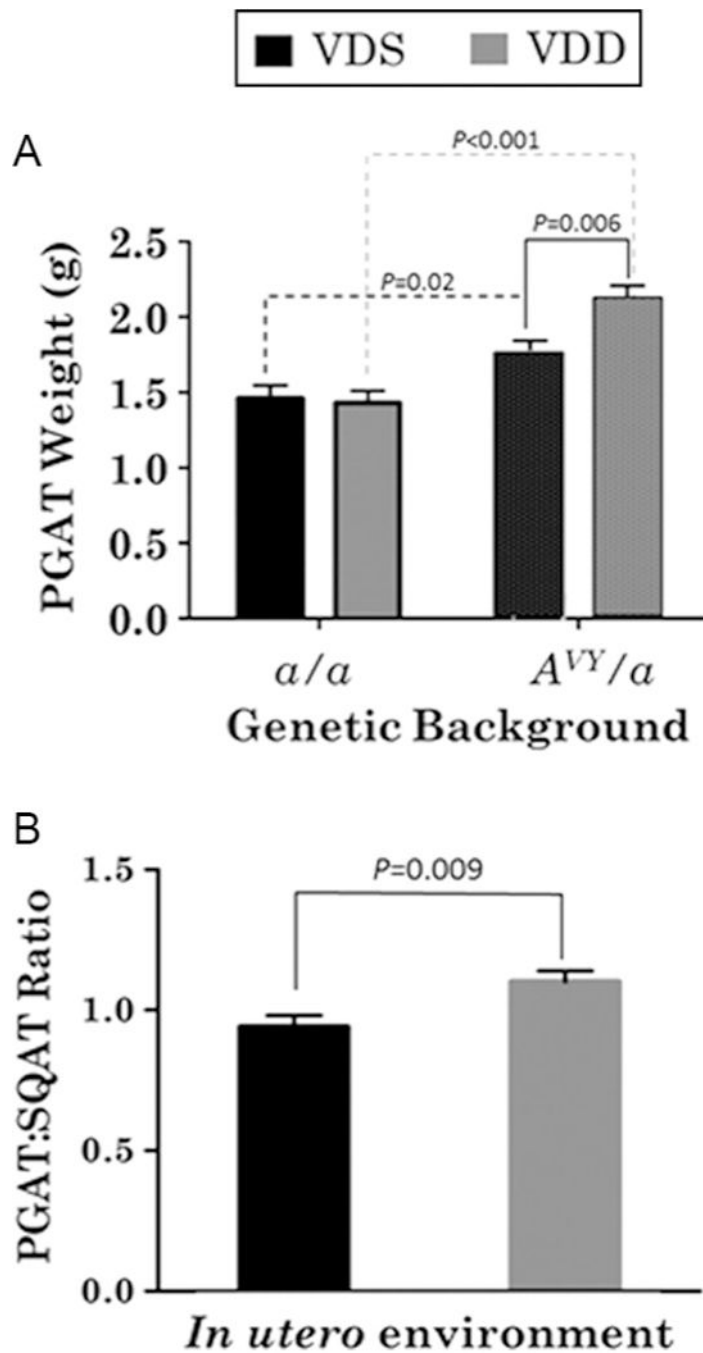


Figure 3. (A) Perigonadal adipose pad (PGAT) weights. Different shaded bars represent different *in utero* environment groups. (B) Ratio of perigonadal (PGAT) to subcutaneous (SQAT) adipose tissue weights. Different shaded bars represent different *in utero* environment groups (i.e., maternal vitamin D status, vitamin D deficient (VDD) or sufficient (VDS)). Data are presented as marginal mean \pm S.E.M.

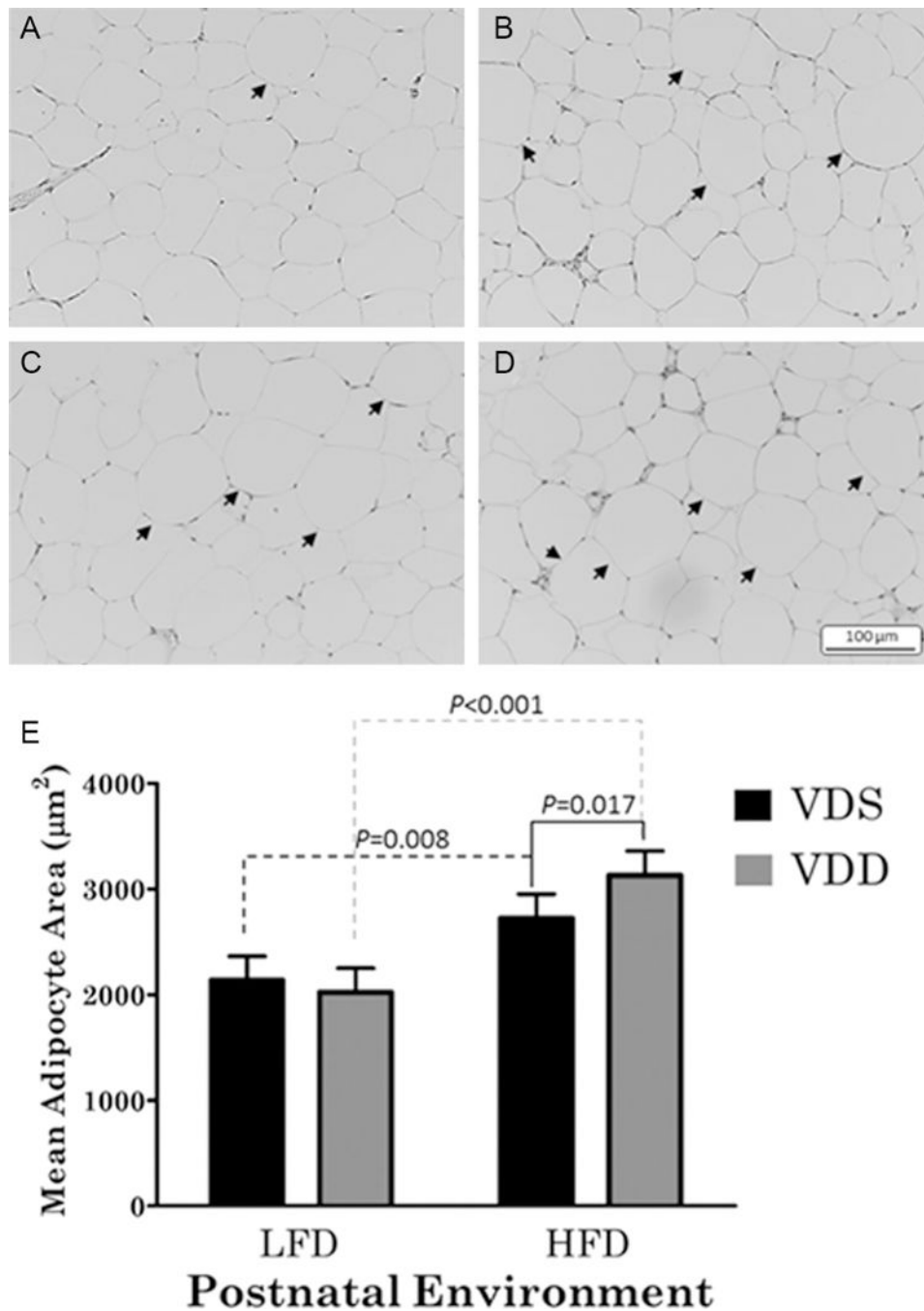


Figure 4. (A, B, C and D) Micrographs of representative H&E-stained perigonadal adipose tissue samples from vitamin D sufficient-low fat (VDS-LF), vitamin D sufficient-high fat (VDS-HF), vitamin D deficient-low fat (VDD-LF) and vitamin D deficient-high fat (VDD-HF) offspring groups, respectively. Arrows indicate larger adipocytes (hypertrophied) compared to the mean of the VDS-LF group. All images were obtained from *a/a* mice within the respective *in utero* (maternal vitamin D status) and postnatal environment combination (low- or high-fat diet). (E) Mean perigonadal adipocyte area. Images were captured with a 40×

objective lens. Mean values for each sample were obtained from at least 100 cells taken from a minimum of six fields of view. Different shaded bars represent different *in utero* environment groups (i.e., maternal vitamin D status, vitamin D deficient (VDD) or sufficient (VDS)).

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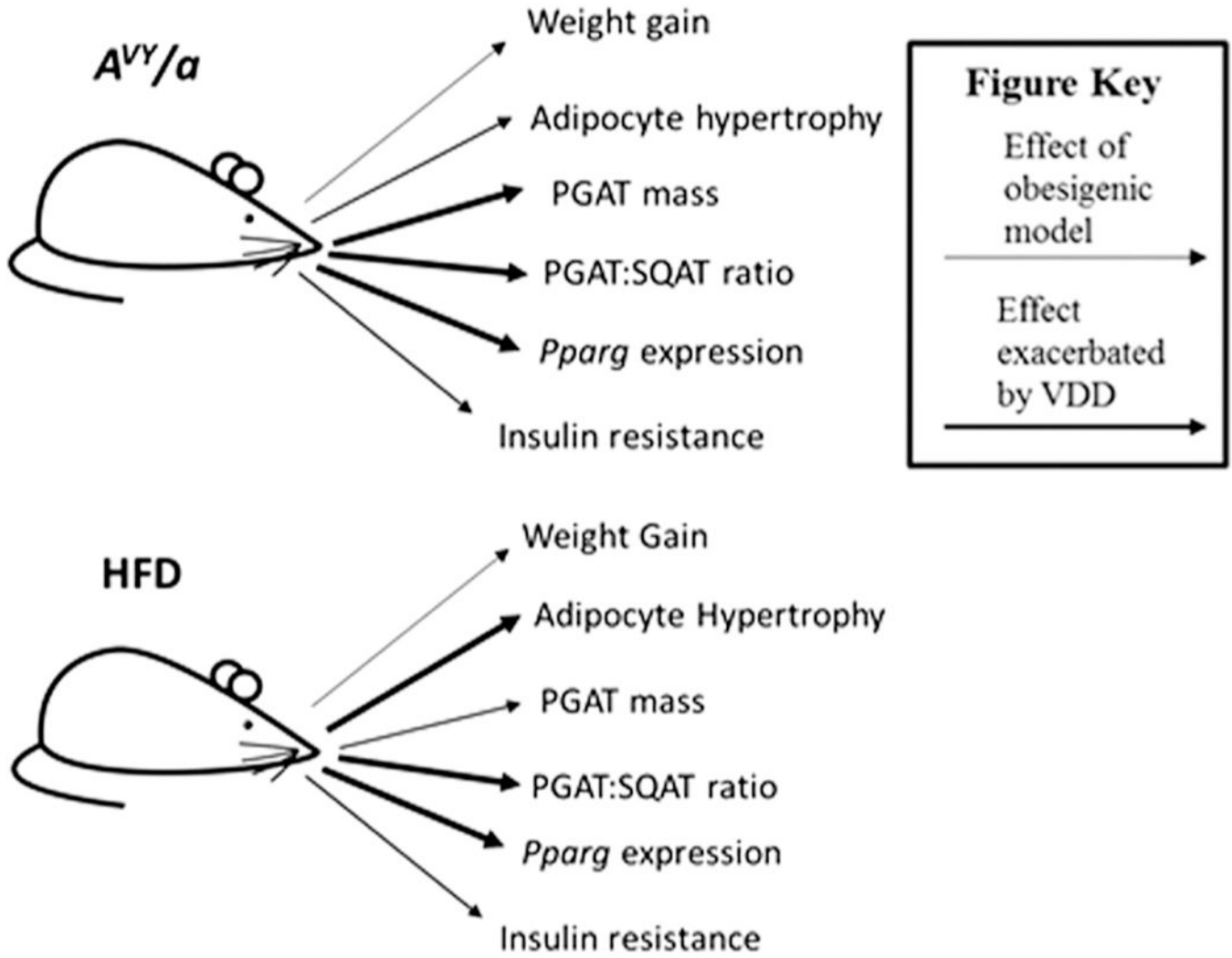


Figure 5. Summary of observed metabolic effects of both offspring obesogenic models, i.e., genetic (A^{VY}/a) and diet-induced (HFD). Arrows indicate a significant difference between obesogenic vs non-obesogenic groups for specific measurements ($P < 0.05$). Bold arrows indicate an effect that was exacerbated in offspring subjected to a vitamin D deficiency (VDD) *in utero* environment.

Table 1

Maternal and offspring diet compositions.

Ingredient	VDS/LFD		VDD		HFD	
	g%	kcal%	g%	kcal%	g%	kcal%
Protein	20.0	20	20	20	20	20
Carbohydrate	64.0	64	64	64	41	35
Fat	7.0	16	7	16	24	45
kcal/g	4.0		4.0		4.8	
	IU	Amount	IU	Amount	IU	Amount
Vitamin D ₃	10	1 IU/g	–	0 IU/g	10	1 IU/g
Calcium		5 mg/g		5 mg/g		5 mg/g
Phosphorous		3 mg/g		3 mg/g		3 mg/g

HFD, high-fat diet; LFD, low-fat diet; VDD, vitamin D deficient; VDS, vitamin D sufficient.

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Table 2

Primer sequences used in the real-time quantitative PCR analysis.

Name	Sequence (5'→3')	GenBank accession	NCBI gene ID
<i>Pparg2 F</i>	TCGCTGATGCACTGCCTATG	NM_011146.3	19,016
<i>Pparg2 R</i>	GAGAGGTCCACAGAGCTGATT		
<i>Vdr F</i>	GTGCAGCGTAAGCGAGAGAT	NM_009504.4	22,337
<i>Vdr R</i>	GGATGGCGATAATGTGCTGTTG		
<i>Cepba F</i>	TTCGGGTCGCTGGATCTCTA	NM_007678.3	12,606
<i>Cepba R</i>	TCAAGGAGAAACCACCACGG		
<i>Lep F</i>	TTCACACACGCAGTCGGTATC	NM_008493.3	16,846
<i>Lep R</i>	GGCTGGTGAGGACCTGTTG		
<i>adipoq F</i>	GTTCCAATGTACCCATTTCGC	NM_009605	11,450
<i>adipoq R</i>	TGTTGCAGTAGAACTTGCCAG		
<i>Hprt1 F</i>	GTTGGGCTTACCTCATGCT	NM_013556	15,452
<i>Hprt1 R</i>	TCATCGCTAACACGACGCT		
<i>Gapdh F</i>	TGGCCTCCGTGTTCTCTAC	NM_008084	14,433
<i>Gapdh R</i>	GAGTTGCTGTTGAAGTCGCA		

Table 3Litter characteristics of *in utero* environment (maternal vitamin D deficiency) groups.

Parameter	VDS	VDD	P Value
Pups per litter ^a (mean ± S.E.M.)	7.8 ± 0.4	7.3 ± 0.7	0.37
Male (%)	53	55	0.93
<i>a/a</i> (%)	48.2	47.9	0.81
<i>A^{vy}/a</i> (%) ^b	51.7	52.1	0.85
Gestation length (d) ^a	20.2	19.9	0.73

^aTogether, the lack of difference in litter size and gestation length demonstrate that VDD dams did not demonstrate dystocia or birthing difficulty (i.e. calcium-dependent processes were not disrupted)

^brepresents full range of coat colors that span from pseudo-agouti to yellow. In the current study, only yellow male offspring were examined. *a/a*, non-agouti (c57BL/6); *A^{vy}/a*, viable yellow agouti; VDD, vitamin D deficient; VDS, vitamin D sufficient.

mRNA expression in perigonadal adipose tissue of mice offspring at 19 weeks of age^a.

Table 4

	<i>In utero environment and obesogenic group</i>							
	VDS	VDD	VDS	VDD	VDS	VDD	VDS	VDD
	<i>a/a</i> -LFD	<i>a/a</i> -LFD	<i>A^{Vγ/a}</i> -LFD	<i>A^{Vγ/a}</i> -LFD	<i>A^{Vγ/a}</i> -LFD	<i>A^{Vγ/a}</i> -LFD	<i>a/a</i> -HFD	<i>a/a</i> -HFD
<i>Pparg</i>	9.0 ± 0.5	6.0 ± 0.6 ^b	7.9 ± 0.6	5.7 ± 0.7 ^b	6.3 ± 0.5	6.3 ± 0.5	6.6 ± 0.4	6.6 ± 0.4
<i>Cebpa</i>	5.7 ± 1.0	4.0 ± 0.9	4.2 ± 1.0	3.0 ± 0.9	3.5 ± 0.9	3.5 ± 0.9	3.4 ± 0.8	3.4 ± 0.8
<i>Vdr</i>	12.2 ± 0.6	10.8 ± 0.5	12.3 ± 0.6	11.3 ± 0.6	11.4 ± 0.8	11.4 ± 0.8	11.7 ± 0.6	11.7 ± 0.6
<i>Lep</i>	6.3 ± 0.8	4.8 ± 0.8	4.8 ± 0.9	3.0 ± 0.6	3.9 ± 0.8	3.9 ± 0.8	3.4 ± 0.4	3.4 ± 0.4
<i>Adip</i>	0.77 ± 0.4	0.84 ± 0.4	0.18 ± 0.3	0.67 ± 0.4	0.61 ± 0.3	0.61 ± 0.3	0.39 ± 0.5	0.39 ± 0.5

^aData are presented as means ± S.E.M.; value shown is Ct (gene of interest – housekeeping gene), therefore lower values indicate higher expression

^bsignificantly different than value to the left (between *in utero* environment; $P < 0.05$).

a/a, non-agouti (c57BL/6); *Adip*, adiponectin; *A^{Vγ/a}*, viable yellow agouti; *Cebpa*, CCAAT/enhancer-binding protein-alpha; HFD, high-fat diet; *IU*, *in utero*; *Lep*, *leptin*; LFD, low-fat diet; *Pparg*, peroxisome proliferator-activated receptor gamma; OG, offspring group (genetic background × postnatal environment); VDD, vitamin D deficient; *Vdr*, vitamin D receptor; VDS, vitamin D sufficient.

Correlations of body weight and adipose tissue mass measurements of mice offspring at 19 weeks of age^a.

Table 5

	Weight at weaning	Juvenile weight gain	Total fat mass	PGAT:SQAT ratio
Weight at weaning	<i>R</i>	–	ns	–0.28
	<i>P</i> value	–	ns	0.038
Juvenile weight gain	<i>R</i>	ns	0.84	ns
	<i>P</i> value	ns	<0.001	ns
Total fat mass	<i>R</i>	ns	–	0.84
	<i>P</i> value	ns	–	<0.001
PGAT:SQAT ratio	<i>R</i>	–0.28	ns	–
	<i>P</i> value	0.038	ns	–

^aData are *r*-unadjusted Pearson correlation coefficients; *P* value represents the significance of the correlation

^bns, non-significant correlation (*P* > 0.05); bold values indicate statistical significance, *P* < 0.05. PGAT, perigonadal adipose tissue; SQAT, subcutaneous adipose tissue.

Correlation of perigonadal adipose tissue gene expression with various phenotypic assessments of mice offspring body fat at 19 weeks of age.^a

Table 6

	Total fat mass	Perigonadal pad weight	Subcutaneous pad weight	PGAT:SQAT ratio	Circulating leptin
<i>Pparg</i>	<i>R</i> ns ^b	0.34	Ns	0.43	0.42
	<i>P</i> -value	0.043	Ns	0.009	0.005
<i>Cebpa</i>	<i>R</i>	0.43	Ns	0.38	ns
	<i>P</i> -value	0.009	Ns	0.025	ns
<i>Vdr</i>	<i>R</i>	Ns	Ns	0.42	ns
	<i>P</i> -value	Ns	Ns	0.010	ns
<i>Lep</i>	<i>R</i>	0.42	Ns	ns	0.46
	<i>P</i> -value	0.044	Ns	ns	0.007
<i>Adip</i>	<i>R</i>	ns	Ns	ns	ns
	<i>P</i> -value	ns	Ns	ns	ns

^aData are *r*-unadjusted Pearson correlation coefficients; *P*-value represents the significance of the correlation

^bns, non-significant correlation ($P > 0.05$); bold values indicate statistical significance, $P < 0.05$. *Adip*, adiponectin; *Cebpa*, CCAAT/enhancer-binding protein-alpha; *Lep*, leptin; PGAT, perigonadal adipose tissue; *Pparg*, peroxisome proliferator-activated receptor gamma; SQAT, subcutaneous adipose tissue; *Vdr*, vitamin D receptor.