Effects of Late Gestational High Fat Diet on Body Weight, Metabolic Regulation and Adipokine Expression in Offspring

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Abstract

Aims/Hypothesis—Gestational exposures such as dietary changes can alter offspring phenotype through epigenetic modifications and promote increased risk for specific diseases, such as metabolic syndrome. We hypothesized that high fat diet (HFD) during late gestation would lead increased risk for insulin resistance and hyperlipidemia via associated epigenetic alterations in tissue adipocytokine genes.

Methods—Offspring mice of mothers fed a HFD during late gestation (HFDO) were weighed and their food intake measured weekly till age 20 weeks at which time glucose and insulin tolerance tests, plasma lipid and adipocytokine levels were assessed, as well as mRNA expression in visceral fat. Adipocytokine gene methylation levels in visceral fat, liver, and muscle were also assayed.

Results—HFDO mice had increased weight accrual and food intake, and exhibited insulin resistance, hyperlipidemia, and hyperleptinemia, as well as hypoadiponectinemia. Furthermore, increased methylation of adiponectin and leptin receptor, and decreased methylation of leptin genes with unchanged GLP-1 methylation patterns emerged in HFDO mice.

Conclusions—Taken together, late gestational HFD induces increased risk of metabolic syndrome in the progeny, which is coupled with hypoadiponectinemia as well as with leptin resistance, and concomitant presence of selective tissue-based epigenetic changes among adipocytokine genes.

Keywords

high fat diet; obesity; insulin resistance; leptin; adiponectin; epigenetics; DNA methylation; gestation

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Introduction

During pregnancy, interactions between mother and fetus are critical for optimal growth and development. Deviations from the ideal gestational trajectory may impose long-term adverse consequences for the physiological and behavioral health of the offspring (1). These assumptions have been corroborated significantly by studies involving prenatal stress (2-4) and induced maternal malnutrition (5).

There is compelling evidence of a sizeable contribution of early-life influences on the risk of becoming an obese adolescent or adult, and of developing obesity-associated diseases in adulthood (6). Indeed, the rapidly rising incidence of childhood obesity is testimony to the contributions of early-life processes. Developmental factors can affect adult disease risk via several pathways, all of which depend on developmental plasticity (7), and many of these effects are now believed to be mediated by epigenetic modifications of the genome.

For example, early-life exposures such as maternal under- or overnutrition and neonatal overfeeding have been shown experimentally to affect satiety, food preference, muscle mass, and insulin resistance in the offspring. These changes, accompanied by modulations in body composition and cardiovascular and metabolic function, are associated with alterations in the offspring's epigenetic state (8-10).

Exposure to a high-fat diet (HFD) during gestation can induce a type 2 diabetes phenotype in the progeny (11). Furthermore, mice born to HFD-induced obese dams were heavier, had increased blood pressure, and were hyperglycemic (12). Adipocytokines such as leptin, adiponectin, and glucagon-like peptide-1 (GLP-1) play important roles in appetite regulation and glucose homeostasis. A recent study showed the presence of significant alterations in glucose homeostasis and epigenetic-mediated changes in the genes encoding for adiponectin and leptin expression in the offspring of mice fed HFD for a 4-week period preceding the onset of pregnancy and further extending throughout gestation (13). In the present study, we examined whether HFD during late stages of pregnancy could induce transgenerational changes in somatic growth, feeding patterns, insulin sensitivity, adipocytokine and lipid homeostasis. We further correlate these phenotypic alterations with epigenetic modifications and significant changes in gene expression, with special emphasis on adipocytokines in visceral adipose tissues of the adult offspring mice.

Methods

All experiments were approved by the University of Chicago's animal care committee (IACUC). Male and female of wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) for breeding. After arrival, all animals were allowed to recover within the animal care facility for 7 days. The animals were housed in cages with 12:12 h light–dark cycles (lights on at 07:00) in constant temperature ($24\pm0.2°C$) with *ad libitum* access to food and water. Adult breeding pairs aged 3 months were used to generate only one litter. The males were removed once pregnancy was detected usually around day 5 to 6 of gestation. Day 0 of gestation was defined as the day of plug observation. Mice were checked every morning and the body weight of the female mouse was monitored daily for a

consistent increase, such as to confirm pregnancy. Following 12 days of pregnancy, dams were fed with either low fat diet containing 10 kcal% fat (LFD; Research Diet, New Brunswick) or a high fat diet containing 60 kcal% fat (HFD; Research Diet, New Brunswick). After birth, litter size was limited to 6 pups per litter to assure adequate and standardized nutrition until weaning. During lactation, all mothers were fed with their respective diets, and pups were kept with their mother until weaning at 21 days of age. Once weaned, offspring mice (males and females) were placed in individual cages. All pups had access to water and were fed with LFD for 18 weeks, and were sacrificed at age 21 weeks. Offspring mice were housed in standard conditions in a temperature-controlled room (23 ± 2 °C) with 12:12 h light–dark cycles (lights on at 07:00 AM).

Body Weight and Food Intake

Body weight was assessed weekly for a period of 21 weeks always at the same time of the day (middle of the light cycle period). Food intake was carefully recorded daily for each cage starting at week 4 after birth.

GTT and ITT

Both tests were performed at week 20 or 21 after birth in a random order. In both tests, animals were fasted for 3 hours with water available *ad libitum*. An intraperitoneal (IP) injection (26G 3/8" needle) of sterile glucose (2mg/g of body weight for GTT) or an IP injection of sterile humulin (0.25units/kg of body weight for ITT) was administered. At the beginning of both tests, the tip of the tail was nicked using a sterile surgical blade. Blood recovered from the tip of the tail at different time points (for GTT: 0, 15, 30, 60, 90 and 120 min following injection; ITT: 0, 15, 30, 60, 75, 90, 105, 120 min after injection) was tested for glucose levels using an OneTouch Ultra2 glucometer (Life Scan, Inc; Milpitas, CA). At the indicated time points, venous blood samples were collected in heparin-coated capillary tubes from the tail vein.

ELISA Assays

Plasma insulin, leptin, adiponectin and GLP-1 assays were carried out using enzyme-linked immunosorbent assay kits (Ultra Sensitive Mouse Insulin ELISA Kit; Millipore, Billerica, MA) according to the manufacturer's protocol. For the insulin assay, the appropriate range of the assay was 0.2 - 10 ng/mL, with the limit of sensitivity at 0.2 ng/mL, and intra- and inter-assay variations at 3.73% and 10.52%, respectively, within the assay range. Insulin resistance was assessed using the homeostasis model assessment (HOMA) equation (fasting insulin $\times$ fasting glucose/22.5). For the leptin assay, the appropriate range was 0.2 - 30 ng/mL, with the sensitivity threshold at 0.05 ng/mL, and intra- and inter-assay variations at 1.49% and 3.85%, respectively, within the assay range. For the adiponectin assay, the appropriate range was 1 - 50 ng/mL, with the sensitivity threshold at 0.2 ng/mL, and intra- and inter-assay variations at 5.75% and 5.98%, respectively, within the assay range. For the GLP-1 assay, the appropriate range was 4.1 - 1000 pM, with the sensitivity threshold at 1.5 pM, and intra- and inter-assay variations at 1.5% and 11%, respectively, within the assay range.

For unbiased analyses of GTT and ITT curves, we initially performed calculation of the homeostatic model of insulin resistance (HOMA-IR) for baseline fasting conditions, and slope analyses as previously described (14). Slope A obtained from the GTT was calculated using the glucose levels measured at times 0-15min after glucose injection. Slope B from the same test was computed between the peak serum glucose levels (15min) and 120 min after glucose injection. In contrast, only Slope A was calculated for the ITT, and included glucose levels measured at time 4 min till nadir glucose levels (60min) after insulin injection.

Biochemical analyses

The collected fresh blood was centrifuged at $2000 \times g$ for 20 min at 4°C, subsequently plasma was centrifuged for 5 minutes at 13,000 rpm to remove remaining cells and platelets, and immediately frozen at -80°C until further analysis. Lipid profiles, including total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and triglycerides (TG) were measured in plasma using Infinity kits (Thermo Scientific). Free fatty acid levels were determined by enzymatic assay (Wako, Richmond, VA).

Total RNA extraction and quantitative RT-PCR

White adipose tissue (visceral fat) was dissected from non-fasted mice at 21 weeks of age. Total RNA were isolated using automated RNA extraction (Promega, Madison, WI) according to manufacturer's protocol. The RNA quality and integrity were determined using the Eukaryote Total RNA Nano 6000 LabChip assay (Agilent Technologies) on the Agilent 2100 Bioanalyzer. Gene expression assays for leptin, adiponectin, and glucagon-like peptide-1 (GLP-1 (TaqMan; Applied Biosystems) were developed for RT-PCR analysis. Eukaryotic 18S rRNA (Hs99999901_s1) served as an internal control.

DNA quantification and bisulfite conversion

Genomic DNA from visceral fat was quantified (Applied Biosystems, Forest City, CA). In a total reaction volume of 25 μl, 2 μl of genomic DNA was used for absolute quantification for the RNase P assay on the ABI 7900HT Real Time PCR System, according to the manufacturer's protocol. After genomic DNA quantification, 500 ng of genomic DNA underwent bisulfite modification utilizing the EZ DNA Methylation-Direct Kit (Zymo Research, Orange, CA). The bisulfite-converted DNA was resuspended in 12 μl TE buffer and stored at -80°C until the samples were ready for analysis.

Gene-specific DNA Methylation Analysis

Genomic DNA from visceral fat, skeletal muscle, and the liver were analyzed for CpG methylation patterns in the genes coding for leptin, leptin receptor, GLP-1, and adiponectin, using Pyrosequencing methylation assays performed by EpigenDx (Hopkinton, MA). Briefly, 500 ng of sample DNA was bisulfate-treated using the Zymo DNA Methylation Kit (Zymo research, Orange, CA). Bisulfate treated DNA was then eluted in 20 μl volume and 1 μl of it was used for PCR amplification of each region of interest. PCR was performed with one of the PCR primers biotinylated to allow purification of single-stranded DNA templates and the reaction contained the following components: 3.0 mM $MgCl_2$, 200 μM dNTPs, 0.2

μM primers, 1.25 U HotStarTaq DNA polymerase (Qiagen Inc., Valencia, CA), and ~10 ng of bisulfite-converted DNA per 50 μl reaction. PCR cycling conditions were: 94 °C ×15 min; 45 cycles of 94 °C ×30 s, 56 °C ×30 s, and 72 °C ×30 s; and final extension of 72 °C ×5 min. Following purification, PCR products (10 μl) were sequenced by the Pyrosequencing PSQ96 HS System (PSQ H96A, Qiagen Pyrosequencing). The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (PSQ H96A, Qiagen Pyrosequencing). Assays performed in this study included leptin (Mouse Lep/ADS1820m), leptin receptor (Mouse LepR/ADS928), GLP-1 (Mouse Gcg/ADS2706 and ADS2707), and adiponectin (Mouse Adipoq/ADS2704, ADS2705, and ADS2718). CpG target regions that were covered by these assays are listed in supplemental Figure 1.

Statistical analyses

Data are reported as mean ± S.E. All analyses were conducted using SPSS software (version 18; Chicago, IL), and consisted of either 2-way ANOVA for repeated measures followed by post-hoc Bonferroni corrections or unpaired t-tests as appropriate. A two-tailed P-value < 0.05 was considered statistically significant.

Results

Effect of HFD during late gestation on weight trajectory and caloric intake in offspring

At birth, there were no differences in weight among HFD-exposed offspring mice (HFDO; n=36/experimental group) and those born to dams fed low fat diet (LFDO; Figure 1; n=36/ experimental group). However, starting at post-natal week 6 of age, HFDO had increased body weights (p<0.001 ANOVA), and this was particularly prominent in males (Figure 1 upper panels; p<0.001). Furthermore, HFDO mice consumed more calories when compared to LFDO (Figure 1 mid panels; p<0.001 ANOVA). Thus, there was an effect of age on diet-induced body weight accrual and on food intake patterns. However, since the gender differences were manifest only related to timing rather than the actual directions of changes, most subsequent results are presented with male. s and females merged, except when significant differences were apparent. In a subset of 12 mice/experimental group, careful dissection and weighing of subcutaneous and visceral fat was performed by a blinded investigator (AP). The weights of these 2 adipose tissue compartments are shown in Table 1, and clearly illustrate the significantly larger fat mass in both subcutaneous and visceral adipose tissues.

Effects of HFD late gestational exposures on glucose intolerance and insulin resistance

At post-natal age 20-21 weeks, offspring mice underwent GTT and ITT (n=12/experimental group for each of the tests). Significant differences in HOMA-IR were present at baseline under fasting conditions, with higher values in HFDO mice, suggestive of insulin resistance. Furthermore, markedly altered GTT curves with significantly higher peak glycemic levels after glucose injection and slower glucose level decline kinetics were apparent in HFDO mice (p<0.001; n=12/group; Figure 2). ITT further confirmed the presence of peripheral tissue insulin resistance as evidenced by significantly less reductions in glycemic concentrations over time after insulin administration in HFDO mice (p<0.001; n=12/group; Figure 2)

The effect of late HFD exposure in utero on serum lipids, leptin, adiponectin, and GLP-1 levels

At 20 weeks of age, HFDO mice had significantly higher fasting total cholesterol, LDL cholesterol and triglyceride serum levels, and lower HDL cholesterol concentrations when compared to LFDO (Table 2). Furthermore, serum free fatty acid levels were also increased in HFDO mice (Table 2; p<0.001; n=12/group). Some male to female differences also emerged (Table 2).

Plasma fasting leptin concentrations were higher in HFDO mice, and conversely adiponectin levels were lower, with no significant differences in GLP-1 concentrations between the 2 groups when both genders of offspring were included as a single group (Table 2). However, male HFDO mice exhibited higher GLP-1 levels when compared to male LFDO (p<0.01; n=12/group), and such differences were absent in female offspring.

Effects of HFD exposure in utero of leptin, adiponectin, and GLP-1 gene expression in the adipose tissues of offspring at 20 weeks of age

Expression of leptin mRNA was significantly up-regulated in adipose tissue derived from HFDO when compared to LFDO mice. In contrast, decreased expression of the leptin receptor gene was apparent in visceral fat, whereas the adiponectin gene was down-regulated in the visceral adipose tissue of HFD compared with LFD mice at 20 wk of age (p< 0.01; Table 3). GLP-1 mRNA expression in visceral fat was significantly elevated only in HFDO male mice (Table 3).

Effects of HFD exposure in utero on methylation of the promoter regions of the adiponectin, leptin, leptin receptor and GLP-1 genes in the adipose, liver, and muscle tissues of offspring

Individual CpG island methylation levels for each of the genes of interest in HFDO and LFDO are shown in Table 4 for the 3 tissues that were assessed in this study. Differences in methylation between LFDO and HFDO mice emerged that were both tissue- and CpG site-specific. Globally, HFDO mice exhibited enhanced methylation of the adiponectin and leptin receptor loci, with reduced methylation of the leptin gene, and no significant changes in GLP-1 gene methylation (Table 4).

Leptin—Two CgP sites in the leptin gene were hypomethylated in HFDO in visceral fat: at -266 from TSS (53.7±1.3% vs. 47.4±0.9% in LFDO, p<0.004), and -214 from TSS (49.1±2.3% vs. 42.0±1.4% in LFDO, p<0.03). No significant differences between the 2 groups emerged for all other CpG sites in this tissue.

In liver, only one CpG site was significantly different among the 2 experimental conditions, located at -252 from TSS (48.6±1.1% vs. 37.9±1.1% in LFDO, p<0.0001). In muscle, again one CpG site exhibited significantly different methylation levels, located at -266 from TSS (55.4±1.3% vs. 47.8±1.1% in LFDO, p<0.004).

Leptin Receptor—In visceral fat, 4 CpG sites showed evidence of significant increases in methylation in HFDO when compared to LFDO (Table 4). Similarly, there were 5 CpG sites

(of which 1 was the same as in visceral fat) that showed increased methylation levels in HFDO (Table 3). Interestingly, no significant differences emerged in skeletal muscle for leptin receptor methylation among the 2 groups.

Adiponectin—In visceral adipose tissues, adiponectin promoter showed 2 CpG sites that were hypermethylated at positions +128 and +76, with no other significant changes found among the other 5 additional CpG sites (Table 4). No changes in methylation emerged in the liver, and only one CpG site showed evidence of hypermethylation in skeletal muscle (Table 3).

GLP-1—When compared to LFDO, no significant changes in methylation emerged in visceral fat, liver, or skeletal muscle (Table 4).

Discussion

This study shows that a short fetal exposure to high fat diet during late gestation and during the lactation period in mice is accompanied by significant increases in post-natal caloric intake and weight accrual in the offspring. This diet is associated with profound metabolic alterations, such as insulin resistance, reduced adiponectin plasma levels, leptin resistance, and abnormal serum lipids, which although present in all HFDO-exposed mice, was more pronounced in male mice. The mechanisms underlying this apparent gender dymorphism are unclear. Furthermore, methylation patterns among several of the well-established adipocytokines in visceral fat, liver, and skeletal muscle revealed selective changes in methylation levels that may not only underlie the concurrent abnormalities in tissue expression of these genes, but may also account for the metabolic dysfunction elicited by a dietary modification during late pregnancy.

Before we discuss the overall implications of our findings, a technical issue deserves comment. We used an exploratory pyrosequencing strategy focused on determining the methylation patterns of only a restricted number of genes known to be associated with metabolic regulation, namely adipocytokines. Therefore, it is likely that a large number of other genes may be affected as well, and such assumption certainly merits further exploration using whole genome approaches. Notwithstanding, our findings are compatible with the concept that HFD during the late phases of gestation and during lactation induces extensive epigenetic alterations in the offspring that may be involved in the increased risk for development of obesity and metabolic dysfunction in these animals.

Previously described murine models of maternal overnutrition using a high fat diet have emphasized offspring development (15-19). Such models consistently described the presence of glycemic and lipidemic abnormalities in the offspring, and current findings about the effect of HFD exposure *in utero* during a short period in late gestation are consistent with these previous studies. Furthermore, our data indicate that pregnant female mice fed HFD, even for a relatively short period, show permanent detrimental effects in body composition and metabolism in their offspring, predisposing them to the metabolic syndrome later in life, even when they are fed standard chow, a finding that corroborates some antecedent reports (16, 20, 21). Different from previous studies however, was the fact

that we provided continued HFD to the lactating mothers that received HFD during late gestation. Based on such approach, we cannot readily differentiate between the specific effects of late gestational HFD and those, if any, induced by HFD during the lactating period. Indeed, there were no differences in body weight among HFDO and LFDO mice at weaning, and such differences emerged only at age 6 weeks. Separation of diet during gestation and lactation contributions will have however to be explored in future studies.

Our observations on the dysregulation of serum lipids and adipocytokine levels coupled with abnormal glucose metabolism and peripheral tissue insulin resistance has been recently reported as well (13). The remarkable analogy between those findings and the current results would indicate that the hyperleptinemia and decreased expression of leptin receptor in visceral fat may underlie the increased calorie intake and concomitant weight accrual and adiposity observed among the HFDO mice, possibly via hypothalamic pathways (22-24). Furthermore, we postulate that the hypoadiponectinemia associated with HFDO may be an important determinant of insulin resistance (25). Interestingly, we did not find evidence of any alteration in GLP-1 gene expression in HFDO mice, although males displayed increased serum GLP-1 levels and also exhibited a trend towards higher GLP-1 receptor expression in visceral adipose tissues (Tables 2 and 3). This finding was surprising considering the putative multiplicity of roles played by this peptide and its cognate receptor in the regulation of glucose, lipid, and body weight homeostasis (26-29). Indeed, peripheral and central GLP-1 combine to regulate both short-term and long-term energy balance, and the significance of increased versus reduced GLP-1 levels in obesity or diabetes remains elusive, such that the gender dimorphic features identified in HFDO will undoubtedly merit further investigation (30, 31).

Of note, exposures to HFD *in utero* may lead to reprogramming of the gluconeogenic capacity of offspring through epigenetic modifications, and potentially lead to excessive glucose production and reduced insulin sensitivity in adulthood (9). Moreover, epigenetic regulation of gene expression emerged as an important contributor to the changes in size, cytology, and morphology undergone by adipocytes during obesity (32). Therefore, future studies should focus on the impact of HFDO on additional organs such as pancreas, particularly the endocrine pancreas, and the hypothalamus, as well as delineate in greater detail the ultra-structural and functional changes that develop in different adipose tissue compartments in HFDO mice.

Subsequent to reports suggesting that histone-based epigenetic modifications in the promoter region of adiponectin might play important roles in adipogenesis (33), and also that DNA methylation in the promoter region of leptin might contribute to its expression (33-38), the current findings showing increased methylation and reduced expression of adiponectin and inverse methylation and expression of leptin were anticipated, based on the phenotypic characteristics exhibited by HFDO mice. Indeed, Masuyama and Hiramatsu showed that offspring of HFD-fed pregnant mice exhibited modifications of H3K9 leading to alteration in the methylation to acetylation status of the adiponectin promoter region in visceral fat, and changes in the methylation of H4K20 in the leptin promoter region, suggesting that histone modifications may modulate a reduction in adiponectin expression and enhanced leptin transcription in HFDO mouse adipose tissue (13). In addition to the

confirmatory nature of our findings, additional hitherto novel observations deserve mention. Indeed, differential CpG methylation of leptin receptor regulatory sequences in both visceral fat and liver of HFDO mice coincided with reduced mRNA expression of this receptor in visceral adipose tissue. Thus, epigenetically-mediated leptin resistance via down-regulation of leptin receptor transcription could account for the increased plasma leptin levels found in HFDO mice, and the differential weight trajectories seen in these mice. Similar evidence for leptin resistance has been described in perturbations occurring during early life, such as nicotine exposures or overnutrition (39, 40). It will be of great interest to explore whether interventions such as restricted caloric diets or exercise training can reverse the maternally-derived long-term effects of HFD on the offspring (41). In addition, as mentioned above, separation of the effects of HFD during gestation and during lactation will need to be elucidated.

Of importance, not all genes with relevance to glycemic homeostatic regulation and other metabolic functions were epigenetically modified. Indeed, there were no discernible changes in the methylation status of the GLP-1 gene in any of the 3 tissues examined. GLP-1 plays a significant role in appetite regulation, adipocyte cell fate, and glycemic control (42-44), and the increased food intake and metabolic alterations observed in HFDO mice would therefore support the presence of *a priori* increases in methylation of this gene. However, such a possibility was not completely excluded since GLP-1 biogenesis originates in gut enterocytes (45), and these cells were not assessed in our pyrosequencing strategy.

In summary, late gestational HFD in pregnant mice followed by HFD during the lactating period is associated with epigenetic modification of genes regulating in metabolic pathways in their offspring, particularly in the adipocytokines adiponectin, leptin, and leptin receptor. These alterations are tissue-selective and coincide with the presence of a metabolically abnormal phenotype at 20 weeks of age, manifesting as increased body weight starting at 6 weeks of age, insulin resistance, elevated serum lipids, and unbalanced plasma adipocytokines. Early intervention strategies aiming to identify subjects at risk for these changes, and potentially reverse the complex interactions leading to metabolic syndrome during adulthood should be definitely pursued.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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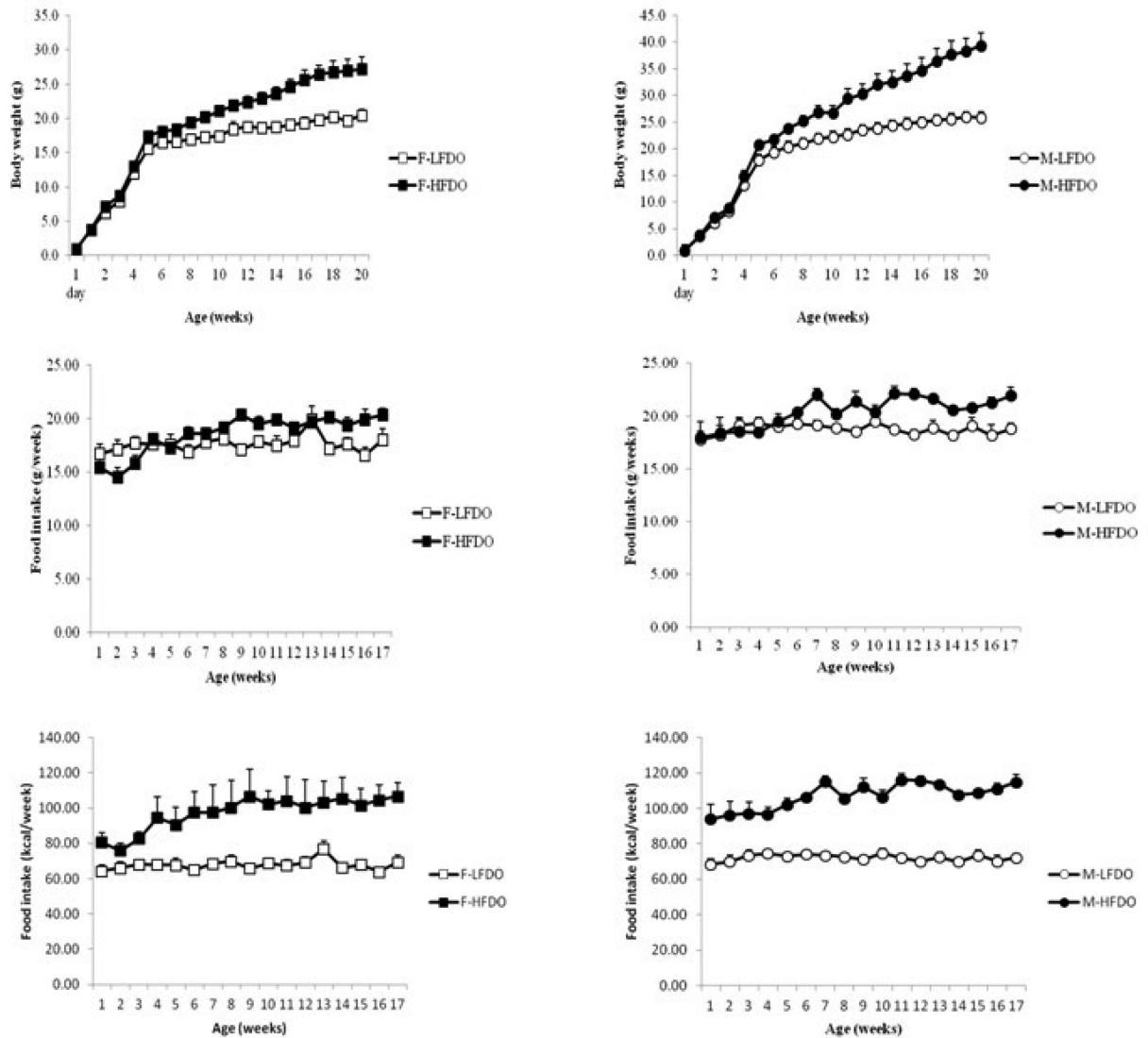
Figure 1.

Top Panels: Body weight evolution in HFDO and LFDO female and male mice from birth till 20 weeks of age (n=36 per experimental group). Differences between HFDO and LFDO reached statistical significance after week 6-7 of age in males, and week 8 in females. (* p<0.001).

Mid Panels: Food intake in male and female HFDO and LFDO mice. (HFDO vs. LFDO, p<0.0001)

Lower Panels: Food intake expressed as Kcal/week/g body weight in male and female HFDO and LFDO mice. (HFDO vs. LFDO, p<0.0001)

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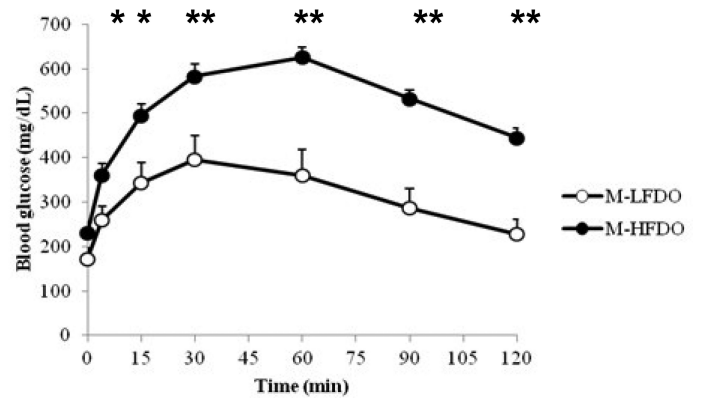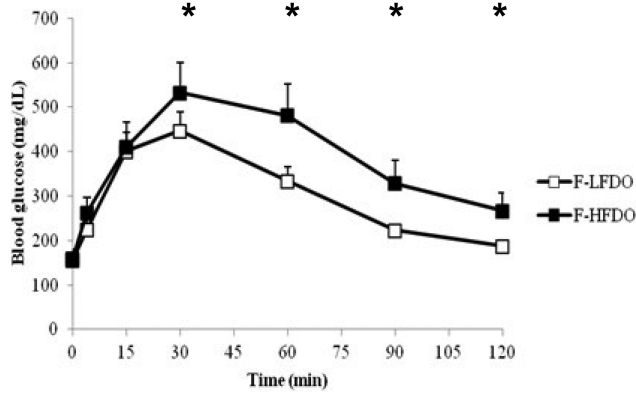
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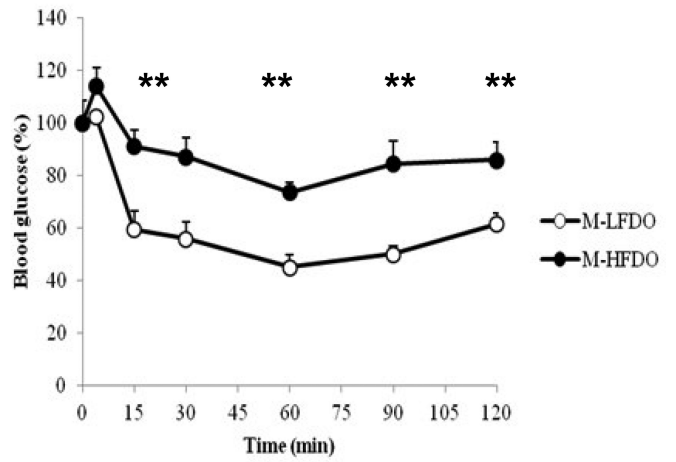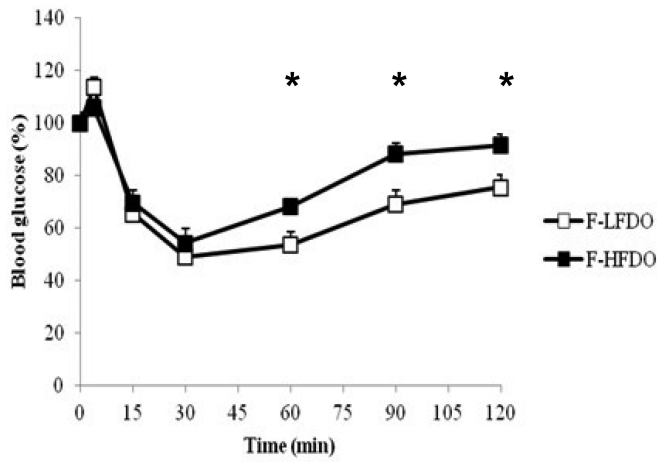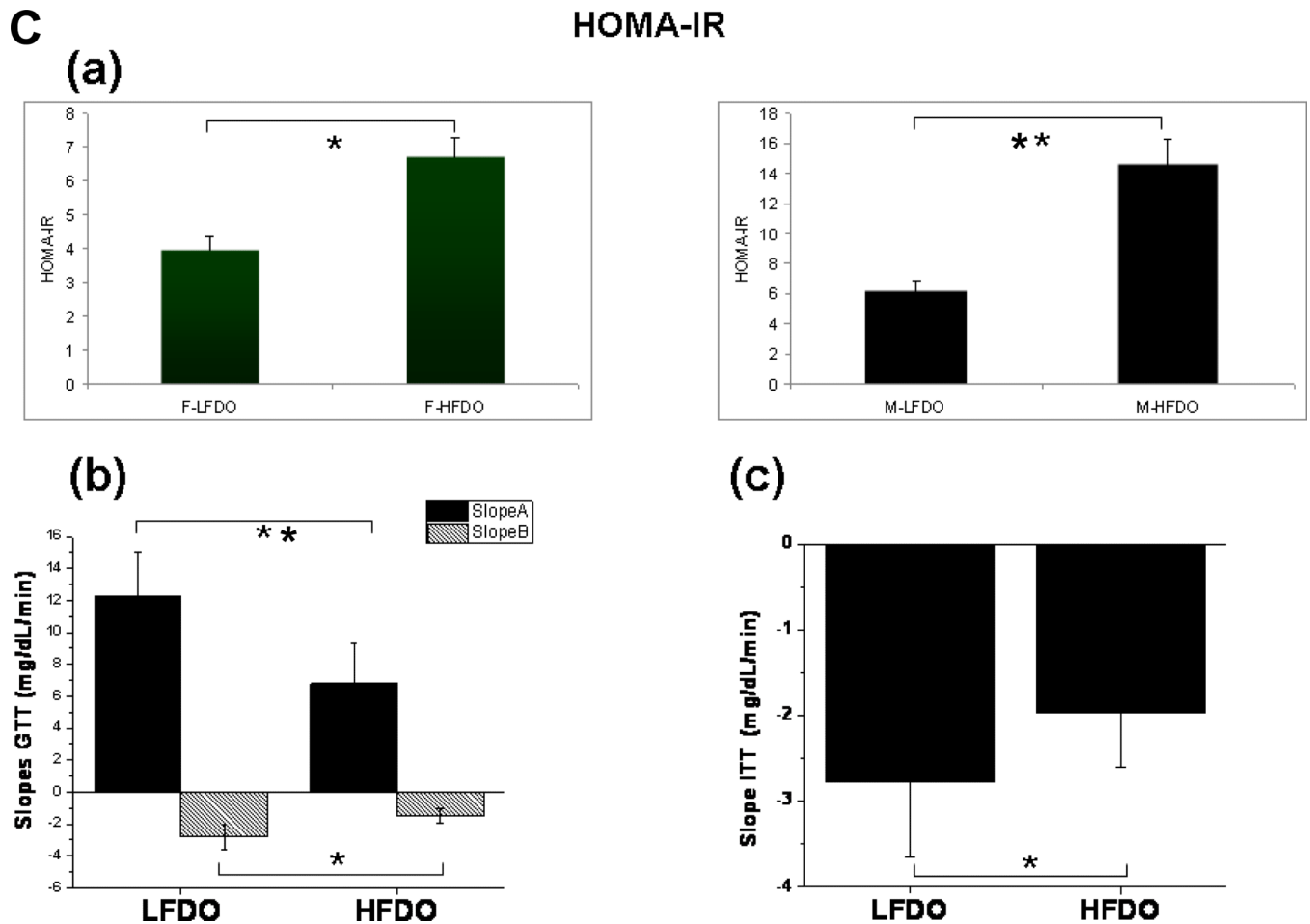
Figure 2.
Plasma glucose concentrations over 2h during the intraperitoneal glucose tolerance test (2mg glucose/g body weight) (Panel A) and intraperitoneal insulin tolerance test (0.25units/kg body weight) (Panel B) following fasting for 3h in HFDO and LFDO mice. HOMA-IR for HFDO and LFDO female and male mice are also shown (Panel Ca). Dynamic slopes for GTT (Panel Cb) and for ITT (Panel Cc) are shown for HFDO and LFDO mice. Slope A was calculated using the glucose levels measured at times 0-15min after glucose injection in GTT, while Slope B was computed between times 15-120min after glucose injection during GTT. In contrast, Slope A was measured between times 4-60min after insulin injection. Slopes results are mean ± SE (n=12 per group); * p<0.001 ANOVA; ** p<0.01 ANOVA.

Table 1

Mass of subcutaneous and visceral adipose tissues at 20 weeks of age in the offspring from pregnant mice fed with low fat diet or high fat diet during late gestation.

Group	Average Body weight (g)	Subcutaneous (g)	P-value	Visceral (g)	P-value
F-LFDO	20.33 ± 0.73	0.21 ± 0.02	<0.002	0.24 ± 0.031	<0.001
F-HFDO	29.13 ± 1.46	0.63 ± 0.09		0.81 ± 0.18	
M-LFDO	25.82 ± 0.43	0.26 ± 0.01	<0.002	0.44 ± 0.03	<0.0001
M-HFDO	36.83 ± 1.63	1.1 ± 0.25		2.28 ± 0.35	

n=12/group

Table 2

Serum lipid profiles and plasma adipocytokine concentrations in 20week-old offspring from pregnant mice fed with low fat diet (LFDO) or high fat diet (HFDO) during late gestation and lactation period.

Metabolic parameters	Females		P value	Males		P value	Diet × Gender effect P value
	LFDO (n=12)	HFDO (n=12)		LFDO (n=13)	HFDO (n=12)		
Trigycerides (mg/dL)	78.05 ± 1.48	112.95 ± 1.23	<0.01	81.31 ± 1.83	119.11 ±0.04	<0.01	NS
Total cholesterol (mg/dL)	99.17± 3.32	127.34± 1.25	<0.01	86.91 ± 3.59	133.09± 2.56	<0.01	NS
HDL cholesterol (mg/dL)	78.94 ± 1.87	69.51 ± 1.44	<0.05	88.14 ± 2.07	82.23± 2.69	<0.05	NS
LDL cholesterol (mg/dL)	58.94 ± 2.22	83.46 ± 1.62	<0.01	66.57 ± 2.2	77.54 ± 2.83	<0.01	NS
NEFA (mM/L)	0.59 ± 0.03	0.81 ± 0.07	<0.01	0.48 ± 0.02	0.75 ± 0.04	<0.01	NS
Glucose (ng/mL)	155.11 ± 5.26	159.43 ± 20.59	NS	187.12 ± 9.25	231.58 ± 23.69	<0.01	NS
Insulin (pmole/L)	77.82 ± 2.85	118.06 ± 9.29	<0.01	95.56 ± 4.95	192.41 ± 30.12	<0.01	<0.05
HOMA	3.96 ± 0.38	6.7 ± 0.62	<0.01	6.2 ± 0.64	14.63 ± 1.61	<0.01	<0.05
Leptin (ng/mL)	3.21 ± 0.42	12.76 ± 0.19	<0.01	3.59 ± 0.18	18.33 ± 1.0	<0.01	<0.05
Adiponectin (µg/mL)	41.54 ± 1.77	32.51 ± 1.56	<0.01	44.14± 1.12	28.38± 1.99	<0.01	=0.055
GLP-1 (pmole/L)	36.93 ± 1.67	34.69 ± 1.53	NS	29.86± 0.73	41.86 ± 1.83	<0.01	<0.01

LFDO vs. HFDO: p<0.01; NEFA – non-esterified fatty acids; n=12/group; NS – not significant

Table 3

qRT-PCR for gene expression profiles in visceral fat of LFDO and HFDO female and male mice.

Gene name	Gene symbol	RefSeq #	Relative expression							
			Female			Male				
			LFDO	HFDO	p-value	LFDO	HFDO	p-value		
Leptin	Lep	NM_008493.3	1	2.95	0.001	1	4.03	0.001		
Leptin receptor	Leptr	NM_46146.2	1	0.69	0.01	1	0.63	0.01		
Adiponectin	AdipoQ	NM_009605.4	1	0.58	0.001	1	0.26	0.001		
GLP-1	GCG	NM_008100.3	1	1.19	>0.05	1	1.18	>0.05		
Glucagon-like peptide-1 receptor	GLP-1r	NM_021332.2	1	1.1	>0.05	1	1.54	0.006		

N=12/group; expression levels for each of the genes is normalized against LFDO average expression; unpaired t test, 2-tailed p value reported.

Table 4

Adipocytokine gene methylation levels in 20week-old offspring from pregnant mice fed with low fat diet (LFDO) or high fat diet (HFDO) during the 3rd trimester of gestation.

(A) Adiponectin:

		Adiponectin							
		ADS2704RS - 5'UTR			ADS2718FS2-intron 1	ADS2705FS1 - intron 1	ADS2705FS2 - exon 2		
		CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7	Mean Region
From ATG		-8562	-8595	-8614	-6960	-131	+51	+60	
From TSS		+128	+95	+76	+1730	+8559	+8740	+8749	
Visceral Fat	LFDO	61.5±1.3	52.2±1.5	63.2±0.4	65.9±1.0	79.6±2.3	71.8±1.5	66.5±2.4	71.0±1.2
	HFDO	70.3±1.9	54.5±3.1	69.7±2.6	66.9±1.4	83.3±3.8	75.2±2.6	72.9±4.1	75.8±2.2
Liver	LFDO	89.4±0.9	72.2±2.8	82.5±1.5	77.0±1.2	95.8±1.4	77.7±2.2	83.2±1.7	83.4±1.0
	HFDO	90.7±1.2	71.6±2.7	82.5±2.5	76.2±1.1	93.7±1.0	83.7±1.6	85.9±1.9	84.9±1.0
Muscle	LFDO	70.5±2.8	65.4±2.3	75.9±1.7	65.9±1.1	83.8±1.7	74.4±1.8	69.8±2.1	73.5±0.8
	HFDO	65.9±2.0	55.8±2.4	72.2±2.0	62.8±2.3	78.1±1.8	73.2±1.0	71.2±0.7	71.3±1.1

(B) Leptin:

		Leptin											
		ADS1820mFS2											
		CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7	CpG #8	CpG #9	CpG #10	CpG #11	Mean Region
From ATG		-8997	-8983	-8962	-8955	-8945	-8931	-8913	-8907	-8905	-8888	-8883	
From TSS		-266	-252	-231	-224	-214	-200	-182	-176	-174	-157	-152	
Visceral Fat	LFDO	53.7±1.3	36.4±1.1	43.2±0.9	37.9±0.9	49.1±1.3	33.4±1.4	48.7±1.7	48.4±2.8	24.1±1.7	53.0±1.8	46.0±2.5	43.6±2.7
	HFDO	47.4±0.9	39.6±1.8	44.9±2.9	42.7±2.9	42.0±1.4	40.4±2.9	38.3±1.5	52.7±3.6	24.0±1.5	58.8±2.8	47.2±2.8	42.4±2.2
Liver	LFDO	64.9±2.1	48.6±1.1	61.9±1.2	58.2±2.2	63.2±1.8	38.0±2.3	53.2±2.4	62.5±2.9	27.2±1.5	69.1±1.4	48.2±2.1	54.1±2.6
	HFDO	62.0±6.6	37.9±1.1	62.1±3.0	64.6±2.8	66.0±2.2	44.4±3.2	59.0±3.3	64.8±3.2	29.3±1.3	67.2±1.6	53.0±4.8	55.5±2.5
Muscle	LFDO	55.4±1.3	37.8±1.4	41.6±1.2	41.8±1.4	45.8±2.3	35.6±2.8	39.6±2.1	51.9±2.3	24.3±1.2	56.5±1.7	44.8±3.5	43.2±2.0
	HFDO	47.8±1.0	32.6±2.4	38.3±1.3	37.1±1.0	42.6±2.1	36.9±1.9	39.3±1.9	53.0±2.1	24.4±1.0	55.9±1.4	43.7±4.3	41.1±2.1

(C) Leptin Receptor:

Leptin Receptor

		ADS928FSre								ADS928FS2								
From ATG		-10671	-10665	-10663	-10661	-10656	-10650	-10647	-10645	-10619	-10614	-10611	-10609	-10605	-10603	-10599	-10591	
From TSS		-45	-39	-37	-35	-30	-24	-21	-19	8	13	16	18	22	24	28	36	
		CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7	CpG #8	CpG #9	CpG #10	CpG #11	CpG #12	CpG #13	CpG #14	CpG #15	CpG #16	Mean
Visceral	LFDO	8.6±1.2	7.5±0.3	**12.6±1.8**	10.2±1.4	13.0±1.6	16.2±1.4	9.1±0.7	8.0±1.1	6.2±0.5	5.0±0.6	**3.6±0.8**	**3.6±0.5**	4.4±0.8	**3.3±0.4**	4.3±0.4	4.5±0.3	7.5±0.4
Fat	HFDO	10.3±1.0	11.5±0.7	**19.4±1.3**	8.1±1.2	11.3±1.0	18.3±2.3	10.9±1.6	10.2±1.2	5.6±1.5	7.7±1.5	**6.1±1.5**	**6.2±0.2**	5.7±1.0	**6.4±0.8**	5.1±1.4	3.9±0.7	8.5±0.6
	LFDO	7.2±0.8	**6.4±1.0**	9.8±1.4	**8.6±1.0**	10.2±1.5	**17.4±1.7**	13.8±2.9	11.4±1.2	**5.1±0.9**	4.4±0.5	2.4±1.2	4.6±1.2	2.9±1.2	**3.1±0.5**	3.2±0.9	1.8±0.8	7.1±0.6
Liver	HFDO	12.8±3.2	**11.6±1.7**	13.9±2.4	**17.0±2.2**	12.8±2.7	**29.5±3.7**	15.8±3.1	17.6±1.5	**9.3±1.8**	3.6±1.3	2.2±1.1	5.0±1.1	2.8±1.3	**6.5±0.5**	2.6±1.0	2.4±1.5	10.3±1.2
	LFDO	9.1±1.1	6.9±1.5	9.0±1.1	11.0±1.0	10.1±0.5	19.8±1.2	12.4±1.1	6.9±1.0	7.3±1.6	6.1±0.6	3.6±1.3	3.3±0.7	4.9±0.4	6.7±1.8	3.9±0.5	4.0±1.2	7.8±0.4
Muscle	HFDO	12.2±1.6	7.9±0.7	10.2±0.8	11.8±1.0	9.1±1.0	19.1±1.5	11.0±0.9	9.7±0.8	6.9±0.6	4.2±1.0	2.8±0.7	3.3±0.1	3.2±1.3	4.8±0.9	3.1±0.8	2.8±1.0	7.5±0.6

(D) GLP-1:

		ADS2706FS1 - Promoter						ADS2706FS2 - Promoter				Promoter	ADS2707FS - Exon 1 5'UTR			
From ATG		-3397	-3361	-3359	-3345	-3275	-3265	-3264	-3254	-3245			-3028	-2992	-2989	
GLP-1		-303	-267	-265	-251	-181	-171	-170	-160	-151			+67	+103	+106	
		Pos #1	Pos #2	SNP T>C, rs28006 820 Gain CpG in C allele	Pos #3	Pos #4	Pos #5	SNP G>C, rs28006 821 Lost CpG in C allele	Pos #6	Pos #7	Pos #8-#9		Pos #10	Pos #11	Pos #12	Mean
Visceral	LFDO	72.6±0.7	71.1±1.4	X	86.0±0.8	84.5±1.1	72.4±1.3	X	72.6±1.5	76.8±1.3	X		97.2±0.8	69.1±1.5	79.9±1.7	78.2±1.6
Fat	HFDO	69.6±1.7	66.6±2.5	X	86.6±0.7	85.0±1.2	69.2±1.5	X	76.0±1.7	77.7±1.8	X		96.5±0.9	72.4±1.9	82.8±2.3	78.2±1.8
Liver	LFDO	45.8±1.1	42.9±1.3	X	58.3±1.3	48.6±1.4	44.7±1.0	X	42.9±0.9	48.0±1.1	X		95.0±0.9	71.7±1.0	77.9±1.4	57.6±1.2
	HFDO	48.6±0.7	45.5±0.8	X	57.3±1.0	47.9±1.1	42.2±0.8	X	41.4±0.8	48.8±1.2	X		93.2±0.9	67.9±1.2	75.6±1.3	56.9±1.1
Muscle	LFDO	64.3±1.2	68.5±1.3	X	83.5±1.5	81.4±1.6	69.4±1.1	X	70.0±1.2	73.5±1.3	X		94.4±1.4	73.0±1.2	77.1±1.0	75.5±1.2
	HFDO	61.6±1.3	69.7±0.9	X	80.2±1.7	83.4±1.7	67.7±0.9	X	69.8±1.0	74.5±1.2	X		92.8±1.4	71.4±1.1	72.3±1.1	74.3±1.3

Cells in bold indicate p value <0.01 for HFDO vs. LFDO using unpaired t-tests; n=8/group; p values remained significant after adjusting for multiple comparisons based on the number of CpG sites assessed and using the mean for the region as the denominator, as shown in shaded areas. Please note that each tissue was treated separately for statistical comparisons.

Cells in bold indicate p value <0.01 for HFDO vs. LFDO; n=8/group; ; p values remained significant after adjusting for multiple comparisons based on the number of CpG sites assessed and using the mean for the region as the denominator, as shown in shaded areas. Please note that each tissue was treated separately for statistical comparisons.

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Cells in bold indicate p value <0.01 for HFDO vs. LFDO; n=8/group; ; p values remained significant after adjusting for multiple comparisons based on the number of CpG sites assessed and using the mean for the region as the denominator, as shown in shaded areas. Please note that each tissue was treated separately for statistical comparisons.

None of the comparisons for HFDO vs. LFDO achieved statistical significance (i.e., p<0.01); n=8/group