Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Animal Breeding and Genetics

Impact of a maternal high-fat diet on mouse placental epigenetic programming

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Abstract

Objective:

To assess how nutrient intakes of mouse mothers during pregnancy influence placental and fetal growth, we fed pregnant mother with a high-fat diet (HFD) and collect fetus and placentas in the middle of fetal period and at term.

Material and methods:

We studied gene expression by RT-qPCR and DNA methylation by LUMA to understand the molecular mechanisms of placental epigenetic programming in the labyrinth layer of the placenta, where nutrient exchanges take place.

Results:

Food intake of the mother was adjusted for caloric intake during gestation. Fetal and placental weights were affected by the diet and sex of the fetus. At term, there was a diet effect on placental gene expression of *Dnmt3l*, coding for a Dnmt cofactor important for regulating DNA methylation. However, the expression of three other epigenetic enzymes *Suv39h1*, *Suv39h2* and *Jarid 1c*, did not change either between the diets nor between the sexes at the two stages 15.5 and 18.5 days. No differences in global DNA methylation was observed between diet or sex at the two stages.

Conclusion:

These findings suggest that a HFD intake during pregnancy has an impact on fetal and placental growth and gene expression. Such an effect could have long term consequences for the offspring's risk of metabolic syndrome by altering programming *in utero.*

Keywords: Epigenetic, high-fat diet, placenta, DOHaD, Sexual dimorphism

SUMMARY

Obesity is a global problem associated with high risks of cardiovascular diseases and type 2 diabetes. Despite the increase in prevalence of overweight and obesity all over the world, there are not many studies focusing on understanding the underlying mechanism of nutritional programming. The developmental origins of health and disease hypothesis (DOHaD) states that suboptimal maternal environment, including changes in diet, affect in developmental programming of predisposition to adult-onset diseases. The studies involved in this concept therefore have important medical, socioeconomical and biological implications.

A growing body of evidence suggests that the risk of developing complex diseases in adulthood is not only due to genetic factors but also environmental factors. Epigenetics is defined as inherited changes in gene expression that are not due to the changes in DNA sequence, which are responsible for regulating gene expression patterns. Environmental factors can impact epigenetic modifications, leading to long term changes in cell function, therefore predisposing to diseases. Implementation of epigenetic analysis in research could open a window into potential molecular mechanisms of gene-environment interactions.

The placenta is an extra-embryonic organ which regulates nutrient, oxygen, hormone traffic between mother and fetus. Changes in maternal diet can thus influence intrauterine growth and developmental programming of the fetus.

The aim of the study was to examine the impact of a maternal high-fat diet (HFD) on gene expression in time-, sex- and tissue-specific manner and to determine the link between developmental studies with molecular studies. The current study was performed on the labyrinth layer of placenta, where the nutrient exchanges take place, at different embryonic stages (middle of fetal period and term), in both HFD and control diet (CD) fed pregnant mice. Statistical analyses of the food and caloric intake showed that the pregnant dams under HFD, reduced their food intake leading to a balanced caloric intake with CD fed mothers. Our model is therefore a model of maternal malnutrition rather than overnutrition. Weight gain of the placenta in both stages showed sex differences, with male heavier than the female placentas. In addition, we observed sex-specifc different responses under the HFD in terms of fetal weights in the middle of the fetal period. Moreover, a molecular analysis showed time-dependent changes in gene expression within the labyrinth. Further investigations at different stages and on different layers of placenta will therefore be of interest. Examining epigenetic

alterations in the placenta should provide biomarkers of exposure, disease risk and important insights into the human development and disease. Thus, epigenetic alterations may be used in disease diagnosis and prognosis as well as novel drug treatment and disease prevention strategies.

Table 1: Classification of Overweight and Obesity by BMI, Waist circumference and associated disease risk in human

Classification	Degree of obesity	BMI (kg/m2) Principal cut-off points	Disease risk relative to weight waist circumference	
			M < 102	M ≥ 102
			W < 88	W ≥ 88
Underweight		< 18.50		
Normal range		18.50 - 24.99		
Overweight		≥ 25.00		
Pre-obese		25.00 - 29.99	Increased	High
Obese		≥ 30.00		
	I	30.0 - 34.9	High	Very high
	II	35.0 - 39.9	Very high	Very high
Extreme obesity	III	≥ 40	Extremely High	Extremely High

Adapted from WHO Global Database on Body Mass Index (2) and National Heart Lung and Blood Institute's report (3) M: Men, W: Woman

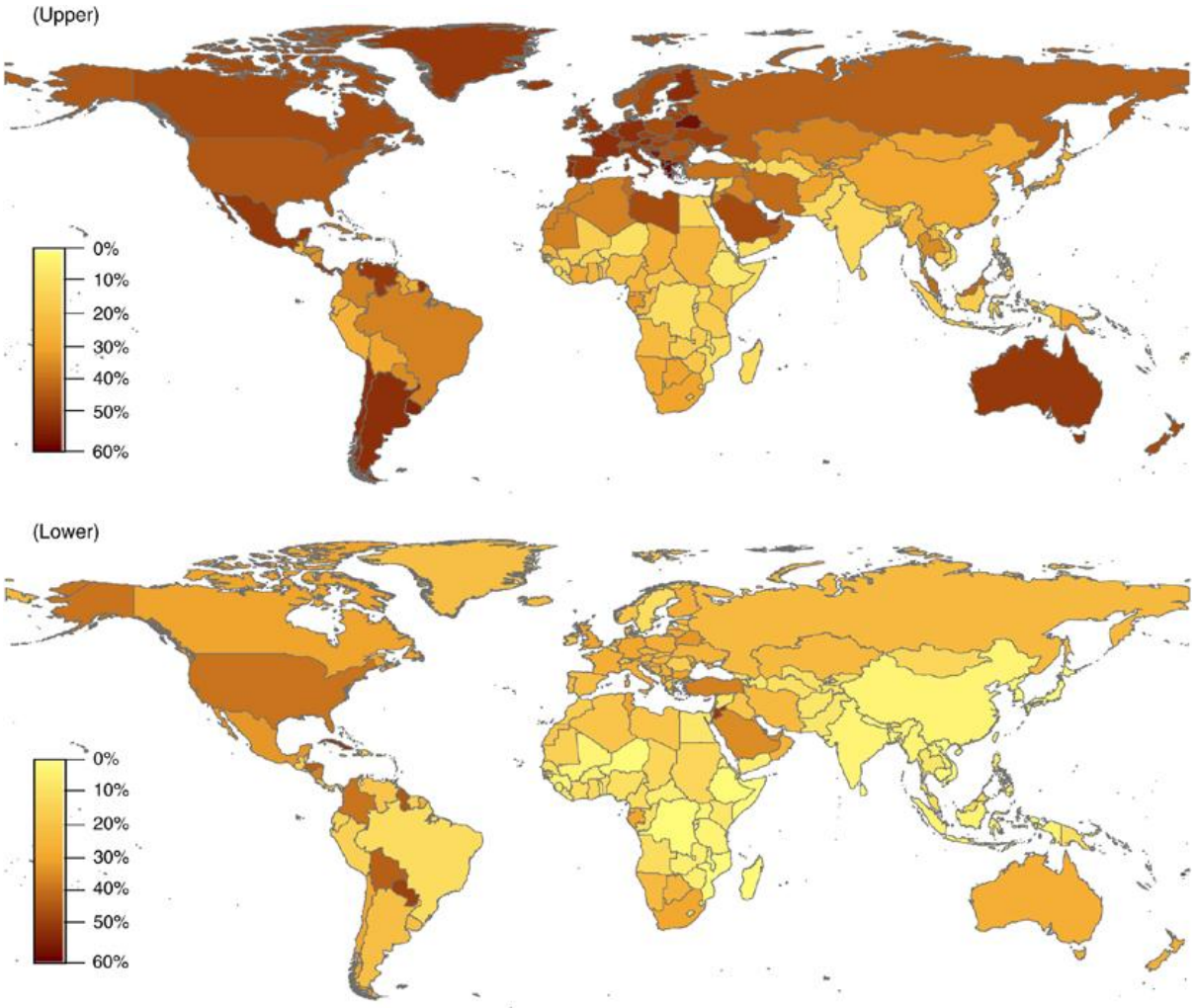
1. INTRODUCTION

The incidence of epidemic obesity and overweight has become a major public health concern since it has reached alarming levels worldwide. According to World Health Organization (WHO), the major criterium of overweight and obesity is body mass index (BMI), which can be simply calculated by comparing weight and height (BMI = weight (kg) / height $(m)^2$), that classifies an overweight or obese person when BMI is equal to or more than 25 or 30, respectively (Table 1). In addition to BMI, obesity is also evaluated through waist circumference or waist–hip circumference ratio measurements according to body fat distribution. Furthermore, obesity is characterized as an excess of fat cell numbers, sizes, and/or fat accumulation in adipocytes due to the energy imbalance between consumed and expended calories.

The prevalence of overweight and obesity has been increasing rapidly not only in industrialized countries but also in developing countries (Usfar et al., 2010). According to the recent statistics from the WHO, obesity has reached epidemic levels worldwide (Figure 1): in 2005, about 1.6 billion people were overweight and 400 million adults were obese (Kelly et al., 2008) (Figure 1). Moreover, according to WHO estimations, by the year 2015, there will be 2.3 billion overweight adults and more than 700 million of them will be obese in the world (1).

The current obesity epidemic is a result of complex interplay between multiple genetic, epigenetic, environmental and socio-economical factors (Flier, 2004; Hotamisligil, 2006; McAllister et al., 2009; Tamashiro and Moran, 2010). However, the 'fetal origins' hypothesis, which was first proposed by Barker and colleagues and evolved as 'Developmental Origins of Adult Health and Disease' (DOHaD), provides a complementary explanation for the obesity epidemic. The DOHaD hypothesis states that exposure to an unfavorable environment during critical window(s) of development (either prenatal or postnatal period), may have direct influence on fetal growth and vulnerability of the offspring to a broad range of non communicable diseases in adulthood including obesity, cardiovascular diseases, type 2 diabetes (T2D) and cancer (Gluckman et al., 2007). In this respect, many stress factors in the womb that lead to abnormal ‘programming’ have been identified including suboptimal diet, hypoxia, abnormal circadian rhythm, hormonal disrupters, increased maternal age, microorganisms, chemical/ pharmaceutical exposures (drugs, nicotine, alcohol etc.).

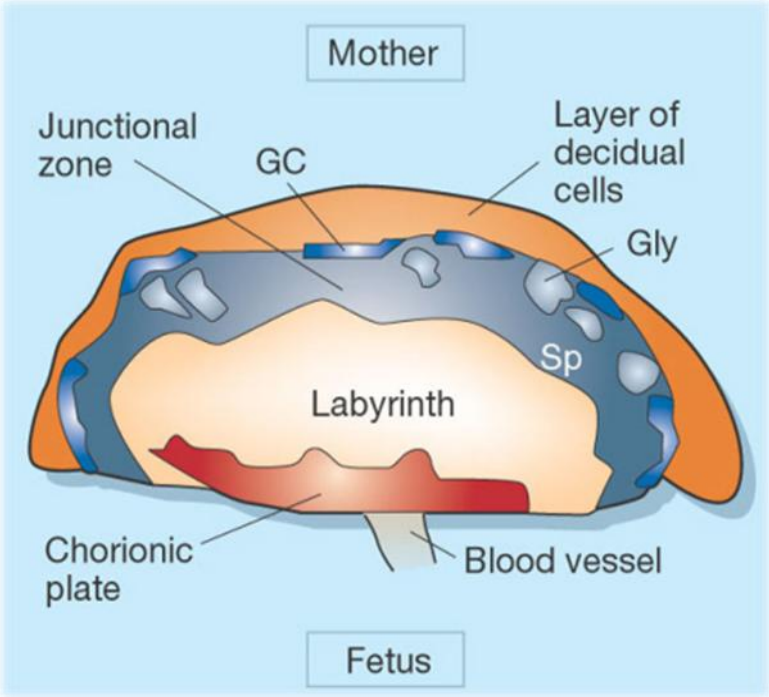
Fig.1. Country based prevalence of overweight (upper) and obesity (lower) in adults in 2005. Adapted from (Kelly et al., 2008)



The evidence of links between environmental conditions and disease risk in offspring is now compelling (Gluckman et al., 2008). While Barker' hypothesis has led to extensive research on the role of undernutrition during gestation, it has been reported that, maternal overnutrition can also promote occurrence of metabolic problems in adulthood. However, only a handful of animal experiments have been reported about the impact of maternal overnutrition during pregnancy. Offspring from mice fed with an obesogenic diet (16% fat, 33% sugar) before mating and throughout pregnancy and lactation exhibit higher blood pressure, increased adiposity and insulin resistance in comparison with control offspring (Samuelsson et al., 2008). In another study, exposure to a high-fat diet (HFD, 60% fat) during pregnancy was shown to result in increased risk of metabolic diseases in the offspring of mice despite their being fed standard chow diet (10% fat) throughout postnatal life (Liang et al., 2009). Similarly, feeding rat mothers with a cafeteria diet (19.15% fat, 47.04% carbohydrates) during pregnancy and lactation leads to increased adiposity and hyperphagia in the offspring (Bayol et al., 2005). The impact of maternal overnutrition during pregnancy has been also studied in sheep models in order to understand early programming of later metabolic problems (George et al., 2010; Igwebuike, 2010; Zhang et al., In press). In a sheep model of maternal overnutrition, 20 day-old lambs showed correlation between hypothalamic leptin pathway gene expression and increased adiposity (Bispham et al., 2003). The link between maternal obesity and/or overnutrition and accelerated fetal overgrowth with altered angiogenic gene expressions has been demonstrated (Ma et al., 2010). Similarly, a recent article suggests that the early programming of obesity may result from maternal overnutrition during the peri-conceptional period or increased fetal nutrition in late pregnancy in sheep (Zhang et al., In press). Furthermore, the effect of maternal HFD was demonstrated in primate models, where it disrupts *in utero* hemodynamics and results in altered gene expression (Aagaard-Tillery et al., 2008; Frias et al., 2011). Collectively, these studies demonstrate that overnutrition during early prenatal and postnatal life can promote long-term metabolic changes and increased risk of metabolic diseases in later life.

The placenta is an interface between fetus and mother, responsible for nutrient and gas exchange of the fetus, removal of harmful waste products from the fetus, secretion of pregnancy-associated hormones and growth factors, and also serving as an immune barrier to protect the fetus from the immune system of the mother (Frost and Moore, 2010; Georgiades et al., 2002; Nelissen et al., 2010). During pregnancy, balanced maternal nutrition and normal placental functions are essential for the intrauterine development and growth of the healthy fetus. Previous studies have demonstrated that the placenta has a key role as a programming

Fig.2. The mature placenta consists of three major cell layers according to the cell types: the labyrinth, the junctional zone and maternal decidua. The labyrinth layer contains epithelial cells and vascular cells, the junctional zone including spongiotrphoblasts, glycogen cells and giant cells respectively. The maternal decidua contains trophoblast giant cells and glycogen trophoblast cells and the junctional zone contains spongiotrophoblast cells, glycogen cells and secondary giant cells (Tycko and Efstratiadis, 2002).



agent for non-communicable diseases including the metabolic syndrome, cardiovascular diseases and cancer. Therefore, perturbations of the dialogues between mother and fetus have a negative impact on fetal growth and developmental programming (Barker et al., 2010; Godfrey, 2002; McMillen and Robinson, 2005; Thornburg et al., 2010a). The links between altered maternal nutrition and placental characteristics have been investigated by a number of studies. Maternal HFD in mouse causes up-regulation of placental nutrient transport, resulting in fetal overgrowth, which increases the risk for developing metabolic syndrome later in life (Jones et al., 2009). In another study, it has been shown that prenatal HFD alters glucose metabolism and elevates placental oxidative stress in mice (Han et al., 2005; Liang et al., 2010). Therefore, the placenta is an appropriate organ to study how environmental factors *in utero*, such as maternal diet, thereby influence the fetal development (Gallou-Kabani et al., 2010; Jansson and Powell, 2007; Mao et al., 2010).

Due to the similarities between underlying structures and genetic mechanisms with human, mouse is a useful model for studying placental development. From an evolutionary standpoint, human and rodent placentation is analog, both are 'hemochorial', which means that there is direct interaction of maternal blood and trophoblasts (Knox and Baker, 2008). In mice, the mature placenta consists of three major cell layers according to the cell types: the labyrinth, the junctional zone and maternal decidua (Gallou-Kabani et al., 2010; Watson and Cross, 2005) (Figure 2). The maternal decidua is responsible for maintaining maternal immune tolerance for fetus derieved antigens (Vacca et al., 2010). The labyrinth is responsible for nutrient transfer, gas exchange between mother and fetus and waste elimination from fetus to mother (Watson and Cross, 2005). The junctional zone includes giant cells that secrete cytokines and hormones and glycogen cells which are providing a substantial glycogen energy source for the fetal growth (Coan et al., 2006; Gardner and Davies, 1993). Despite the remarkable roles of the placenta, very few studies have investigated how maternal overnutrition affects transcriptomic and epigenetic signatures in the placenta (Gallou-Kabani et al., 2010; Jones et al., 2009; Mao et al., 2010).

It is well-known that predisposition to childhood and adult diseases exhibits sex bias: females are more prone to obesity whereas males are more prone to cardiovascular diseases. Animal studies indicate that the maternal programming, which affects the later phenotype, depends not only on the timing and intensity of the insult, but also on the sex of the offspring. For example, high calorie diets are known to favor male births over females by a sex ratio of almost 2:1 (Fountain et al., 2008; Mao et al., 2010; Rosenfeld et al., 2003). Obesity induced programming may give rise to sex differences in insulin secretion and glucose tolerance, with

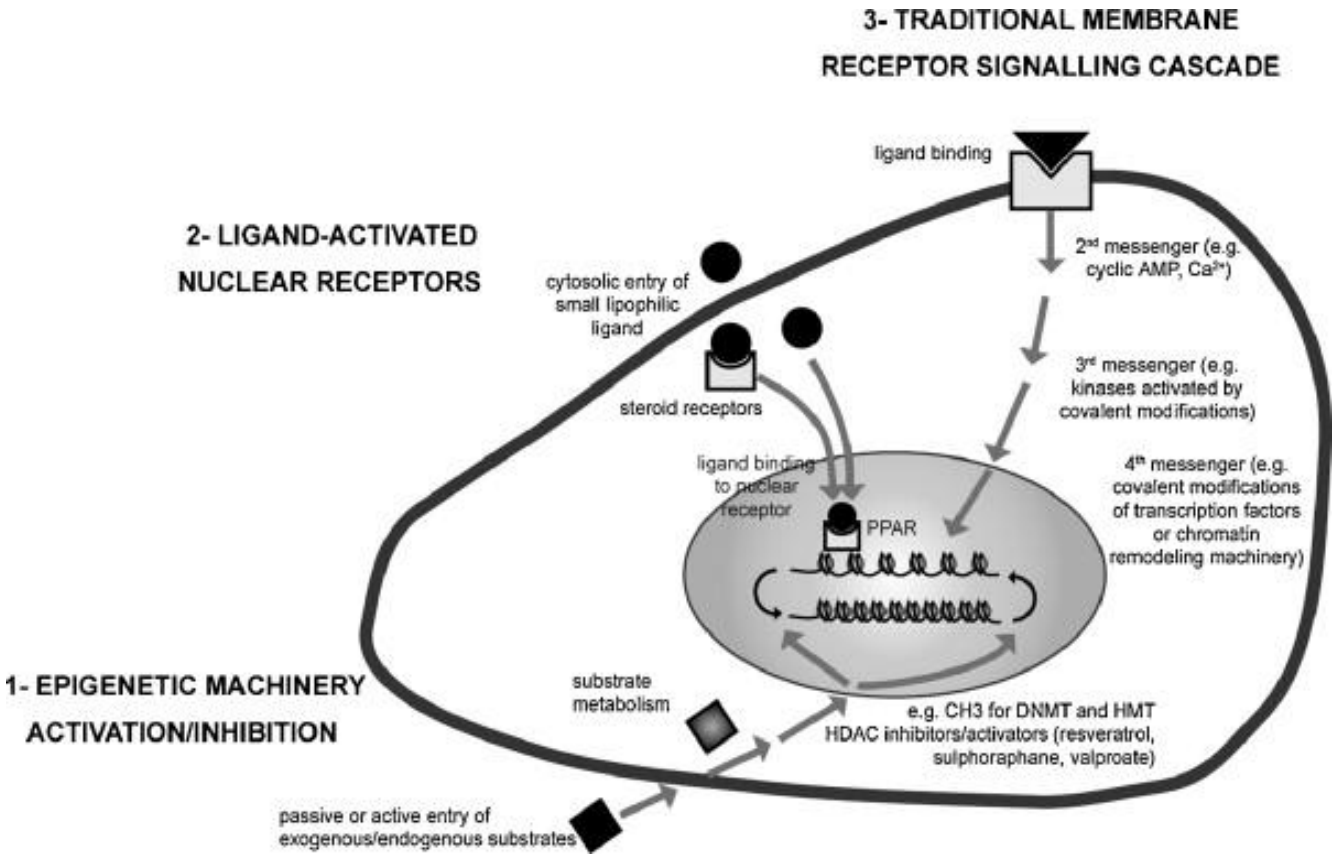
females being more sensitive to environmental insults than males (Han et al., 2005). Thus, current evidence suggests that sex-specific adaptation of the placenta may play a critical role in differences in fetal response to the maternal environment, in contrast to the previous thoughts that the placenta was an asexual organ. Since the placenta is an extra-embryonic organ, the sex of the embryo is actually the sex of the placenta. Global gene expression patterns are sex-specific in human placenta (Sood et al., 2006). Mao et. al. showed that gene expression in the mouse placenta is affected by maternal diet and differs between the sex, where placenta of the females are more sensitive than placenta of the males to the suboptimal nutrition *in utero* (Mao et al., 2010). Furthermore, there is a sex-specific contribution of prenatal stress on gene expression levels during murine fetal programming of development in early gestation. Males showed a non-significant trend to increased glucocorticoid-inactivating enzyme 11 β-hydroxysteroid dehydrogenase type II (11β-HSD2) gene expression, whereas female placentas had a significant reduction in this enzyme (Pankevich et al., 2009). According to Clifton et al., male and female fetuses and neonates develop different strategies to cope with environmental stress factors where male fetuses attempt to grow normally which is then associated with a greater risk of compromising for second adverse event. In contrast, female fetuses adapt to this insult by reducing growth, thus allowing them to further compromise for environmental insults (Clifton, 2010). However, there are still a limited number of studies investigating the link between sexual dimorphism, diet and gene expression in placenta (Gallou-Kabani et al., 2010; Mao et al., 2010; Pankevich et al., 2009; Sood et al., 2006). Further molecular investigation of to what extent sexual dimorphism is shaped by maternal malnutrition is therefore of interest.

While genome-wide association studies have identified several genomic loci that are associated with obesity, the rapid shift of populations towards a more overweight phenotype, within few generations, suggests that this change can not be explained only by genetic factors (Maher, 2008; Manolio et al., 2009). Moreover it has been hypothesized that in early life, the influence of environmental exposures, such as diet, can have a profound effect on epigenetic marks (Thornburg et al., 2010b). The term ‘epigenetics’ means heritable changes in gene expression that are not due to the changes in DNA sequence itself. The major epigenetic marks are DNA methylation and posttranslational histone modifications. It leads to long term covalent modifications, resulting in changes in gene expression through the complex cross-talk between and within epigenetic machinery enzymes and epigenetic marks. Epigenetic processes act in a tissue-specific, stage/time dependent manner to direct development (Gheorghe et al., 2010; Ollikainen et al., 2010; Schneider et al., 210). DNA methylation is

which is involved in variable key mechanisms including silencing of genes and repetitive elements, DNA repair and replication, genomic imprinting, and X chromosome inactivation. DNA methylation is catalyzed by DNA methyltransferases (Dnmts) that transfer a methyl group to the 5'- C of cytosine in CpGs using S-adenosylmethionine (SAM) as a methyl donor (Fuso et al., 2011; Klose and Bird, 2006; Ross, 2003). Dnmt1 is responsible for maintenance of DNA methylation as it converts hemi-methylated into the fully methylated sites during DNA replication. Dnmt3a and Dnmt3b are *de novo* methyltransferases. They recognize unmethylated CpG sites and are responsible for the process by initiation of cytosine methylation. Dnmt3-like (Dnmt3l) is another protein involved in DNA methylation. It has no DNA methyltransferase activity but interacts with Dnmt3a and Dnmt3b and plays regulatory role in DNA methylation. Furthermore, the epigenetic marks are complemented by posttranslational histone modifications. Such modifications include acetylation, methylation, phosphorylation, and ubiquitination. These modifications are driven by several enzymes including histone methyltransferases (HMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), histone demethylases (HDMs) or phosphatases. Histone acetylation usually loosens chromatin packaging and correlates with transcriptional activation. Epigenetic marks are recognized by binding proteins that trigger either formation of heterochromatin and gene silencing or recruitment of transcription factors for gene activation. Overall, these complex interactions are involved in gene regulation, according to the cell type and time with consequences for programming cell fate decisions during the development.

An increasing body of evidence from animal studies and epidemiological studies support that epigenetic marks acts as a memory of exposure to unfavorable environmental insults (chemical, nutritional or metabolic) during early life (Attig et al., 2010). Nutrition can influence gene expression and development, by triggering epigenetic changes of the genome (Dolinoy et al., 2007). Furthermore, as a result of aberrant demethylation, incomplete erasure of the epigenetic marks may lead to such 'memory' of epigenetic state at specific alleles (Angiolini et al., 2006). A well-known example is the agouti mouse, which carries the agouti viable yellow (A^{vy}) epiallele, which is responsible for coat color. The brown phenotype is due to the increased methylation at the (A^{vy}) locus. It has been shown that the offspring born from methyl-supplemented mothers had brown coat color rather than yellow (Waterland and Jirtle, 2003). Recently, Dolinoy et al. showed that maternal supplementation with genistein resulted in phenotypic changes in agouti viable yellow mice due to the increased methylation at (A^{vy}) locus of agouti mouse (Dolinoy et al., 2006). Three types of mechanistic pathways have been proposed to explain how environmental factors, including diet, induce epigenetic changes

Fig.3. Mechanistic pathways for environmental factors involved in epigenetic reprogramming (Gabory et al., 2009)



(Figure 3). Nutrient metabolites can be direct activators or inhibitors of the epigenetic machinery, ligands of nuclear receptors that triggers local epigenetic changes of their target genes, or ligands of classical membrane receptor signaling cascades where the last messengers are the epigenetic machinery enzymes (Gabory et al., 2009). The consequences can be altered tissue-, stage-, sex-, and age- specific epigenetic landscapes.

Previously, the impact of maternal HFD during pregnancy on the gene expression of total mouse placenta at E15.5 was investigated by our laboratory using an Affymetrix microarray approach. This analysis revealed that diet altered the expression of genes involved in many pathways including metabolic, cellular, and immune response. These findings demonstrate how a single environmental challenge, maternal HFD, has an impact on programming in a sexually dimorphic manner. Furthermore, this study provided several candidate genes, including genes encoding epigenetic machinery enzymes, which are dysregulated by high-fat HFD and/or altered depending on the sex of the fetus, namely: *Suv39h1, Suv39h2, Dnmt3L, Jarid 1c.*

In this project, in order to answer the question of how epigenetic marks and machinery enzymes and their interactions with each other evolve during early development, we focussed on tissue-, sex- and stage-dependent epigenetic marks and on kinetic aspects during different stages in mouse embryogenesis. In addition, since epigenetic modifications are cell-specific, we studied gene expression and epigenetic signature changes in the labyrinth, in contrast to the previous study that was performed on total placenta. Therefore, our aim was to analyze the labyrinth layer of the mouse placenta, at three different embryonic stages: E12.5, which is the beginning of the fetal period, E15.5 as a middle of fetal period and E18.5, since the term placenta may carry valuable information about the pregnancy to transfer this information from animal experiments to on the human placenta.

Fig.4. The experimental design of the study

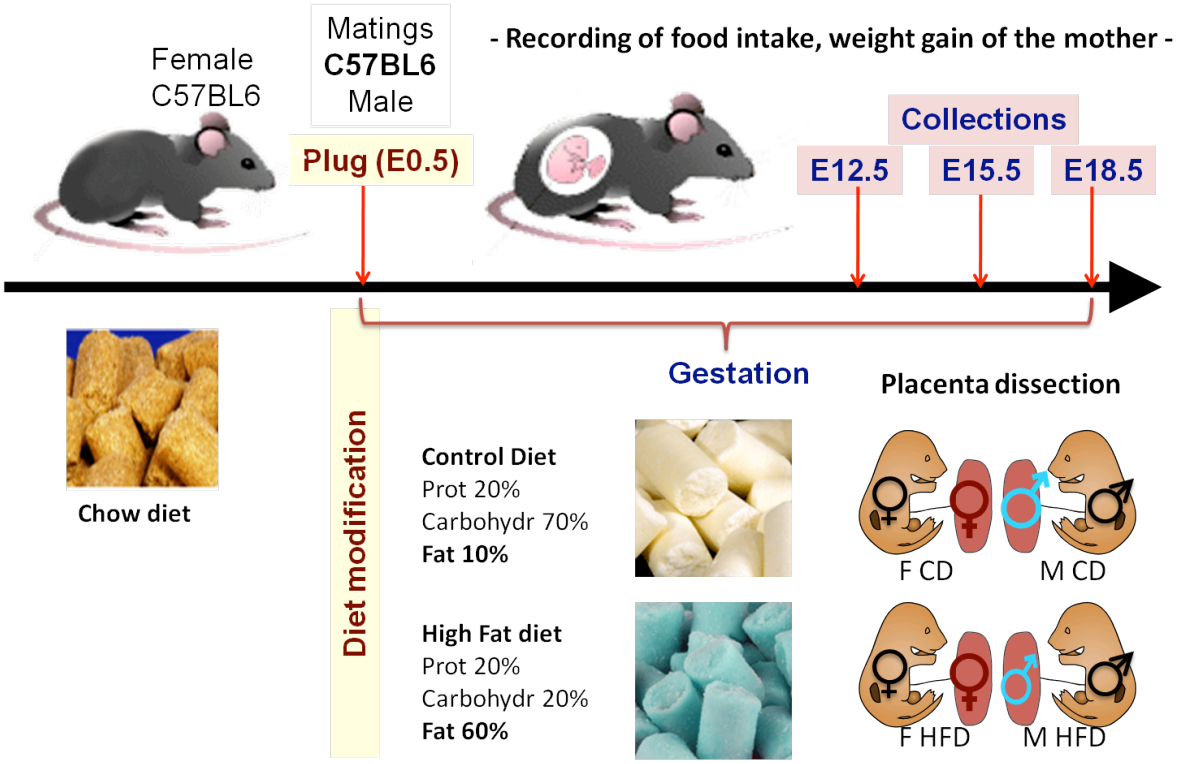


Table 2. Diet compositions (Research Diets, New Brunswick, USA; HFD: D12492, CD: D12450B)

	Control diet	High-Fat Diet
Lipids	**10%**	**60%**
	Saturated fatty acids: 25,1%	Saturated fatty acids: 35%
	Monounsaturated fatty acids: 34,7%	Monounsaturated fatty acids: 43,4%
	Polyunsaturated fatty acids: 40,2%	Polyunsaturated fatty acids: 15,9%
Carbohydrates	**70%**	**20%**
	Corn starch: 45%	Maltodextrin: 65%
	Maltodextrin: 5%	Sucrose: 35%
	Sucrose: 50%	
Proteins	**20%**	**20%**

2. MATERIAL AND METHODS

2.1. Animal model and experimental design

All experiments on animals were done according to the European Communities Council Directive of 1986 (86/609/EEC) and the laboratory has accreditation from the French Ministry of Agriculture for experimentation with mice (No. A 75-15-02).

C57BL/6JOlaHsd mice (10 males, 80 females) were obtained from Harlan® Laboratory (The Netherlands) at 7 weeks of age and housed in groups until mating. All animals were housed under controlled light (12-hour light/ 12-hour dark cycle) and temperature (22°C ± 2ºC). The animals had free access to water and chow diet during the 2-weeks of adaptation period. Males and females were mated during the night. Evidence of a vaginal plug denoted day 0.5 of gestation and the female mice housed individually either received ad libitum CD (10% of calories from fat, 20% proteins and 70% carbohydrates) or HFD (60% of calories from fat, 20% proteins and 20% carbohydrates) throughout the gestation (Figure 4) (Gallou-Kabani et al., 2010). Diets were supplied in pellet form from Research Diets (New Brunswick, USA; HFD: D12492, CD: D12450B) (Table 2). Daily weight gain and food intake were recorded throughout gestation. Daily weight gain was estimated according to the following formula:

% Weight gain = ([Weight of day (n) - weight of day 0.5] / weight of day 0.5)

Daily food intake was estimated by subtracting the amount of food left on the grid from initial food weight. Calorie intakes were calculated on the basis of 3.8 kcal/g for CD and 5.2 kcal/g for HFD.

The pregnant females were sacrified at E12.5, E15.5 or E18.5. Before dissection, maternal blood was collected from the jugular vein, centrifuged at 3500 rpm and plasma samples were stored at –20°C for later analysis. Blood samples were taken from the tail-tip for blood glucose measurement performed with glucometer (Optium Xceed™, Abbott).

Following dissection, the fetuses and placentas were removed rapidly and placed in a petri dish containing PBS 1X solution. Entire placentas were isolated via dissection and maternal deciduas and endometrial tissues were removed under the binocular loop before recording weights of the placentas. The labyrinth and junctional zone were mechanically separated with forceps under the loop, snap frozen in liquid nitrogen, and stored at -80°C. Placental labyrinth tissue was powdered by grinding in liquid nitrogen. The tissue powder was separated in three tubes, two of them for later DNA and RNA extraction, respectively. The absence of any

external malformations and developmental stages of the embryos were confirmed by visual inspection according to a modified Theiler staging system (4). Wet weight of embryos was recorded and sexes were determined by visual observation of gonads under the loop for E15.5 and E18.5 stages.

2.2. DNA precipitation and genotyping for embryonic sex determination

Tissue lysis was performed from the body of the E12.5 mouse fetuses, by incubation in lysis buffer (2M Tris-HCl pH 8.0, 0.5M EDTA, 20% SDS, 5 M NaCl) and 0.4 µg/µl of proteinase at 56 °C overnight. DNA was precipitated with isopropanol and pellet was washed in 70% ethanol. Pellet was dried in the SpeedVac centrifuge and resuspended in 100µl dH_2O (Versol).

In order to determine the sex of the fetus at E12.5, primers *ZFY3* (5'-cctattgcatggactgcagcttatg-3'), *ZFY5* (5'-gactagacatgtcttaacatctgtcc-3') and *Rtl1F* (5'-gcccaggaacactatgtggaactc-3') and *Rtl1R*(3'-aagtctcatcatctgcctccctcg-5') were used for PCR amplification in 12.5µl reaction, containing 0.15µM of each primer, ~50 ng of genomic DNA, 0.2mM dNTP,1.5 mM $MgCl_2$, 1X PCR buffer and 0.0625U *Taq* polymerase (Go Taq®, Promega, USA). The *Zfy* primers (Zfy-3 and Zfy-5) assayed the sex of mouse embryos (i.e., presence of Y chromosome) whereas primers detecting the autosomal *Rtl1* gene served as a control for DNA extraction. The PCR amplification was carried out following cycling parameters: 94 °C, 30 s; 30 cycles 94 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; followed by a final cycle at 72 °C for 10 min.

2.3. Extraction of genomic DNA from mouse placentas

20 mg of labyrinth tissue powder was resuspended in 200 µl Lysis Buffer (1M Tris-HCl pH 8.0, 0.5M EDTA, 10% SDS, 5 M NaCl) and incubated with 0.02 µg/µL proteinase K at 55 °C overnight. The DNA was isolated using standard phenol:chloroform:isoamyl alcohol (24:24:1) extraction method: aqueous and phenol phases were separated by centrifugation and the aqueous phase, containing DNA, was isolated. The DNA was precipitated in 0.2 M NaCl with 100% ethanol, washed in 70% ethanol, dryed at room temperature and resuspended in 60 µl dH_2O (Versol) and stored at 4°C.

Table 3. Primer sequences and corresponding annealing temperatures used in qPCR

Gene	Sequence (5'-3')	Annealing temperature (°C)
Eif4A2 (mouse)_R	ACA-CCA-TCG-GGG-TCC-ATT-CC	**62**
Eif4A2 (mouse)_F	CCT-GTC-TTT-TCA-GTC-GGG-CG	
SDHA (mouse)_R	AGG-TCT-GTG-TTC-CAA-ACC-ATT-CC	**64**
SDHA (mouse))_F	TTC-CGT-GTG-GGG-AGT-GTA-TTG-C	
TBP (mouse)_R	CAA-GTT-TAC-AGC-CAA-GAT-TCA-CGG	**66**
TBP (mouse)_F	TAT-GAC-CCC-TAT-CAC-TCC-TGC-CAC	
Jarid1c_F	GAA-GGA-ACC-ACA-GCA-ATG	**60**
Jarid1c_R	CAG-CCA-TCA-CAC-AGT-AAG-AG	
Dnmt3l_F	TGA-CTG-AGG-ATG-ACC-AAG-AGA-CAA-C	**62**
Dnmt3l_R	TCC-ACA-CCC-GCA-TAG-CAT-TC	
Suv39h1_F	GCA-CAA-GTT-TGC-CTA-CAA-TG	**60**
Suv39h1_R	TTC-TGG-ACT-ACA-CGG-TTT-GG	
Suv39h2_F	ACC-TGA-ATG-TCC-CAA-TAG-G	**62**
Suv39h2_R	TTT-TAC-ACC-CCA-ACC-ACA-GC	

2.4. RNA extraction

Labyrinth powder was disrupted and homogenized by Mixer Mill MM 300 (Qiagen) in RLT buffer (Qiagen, France). Total RNA was extracted from mouse placenta with the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, France). RNA was resuspended in 50 µl RNase-free water.

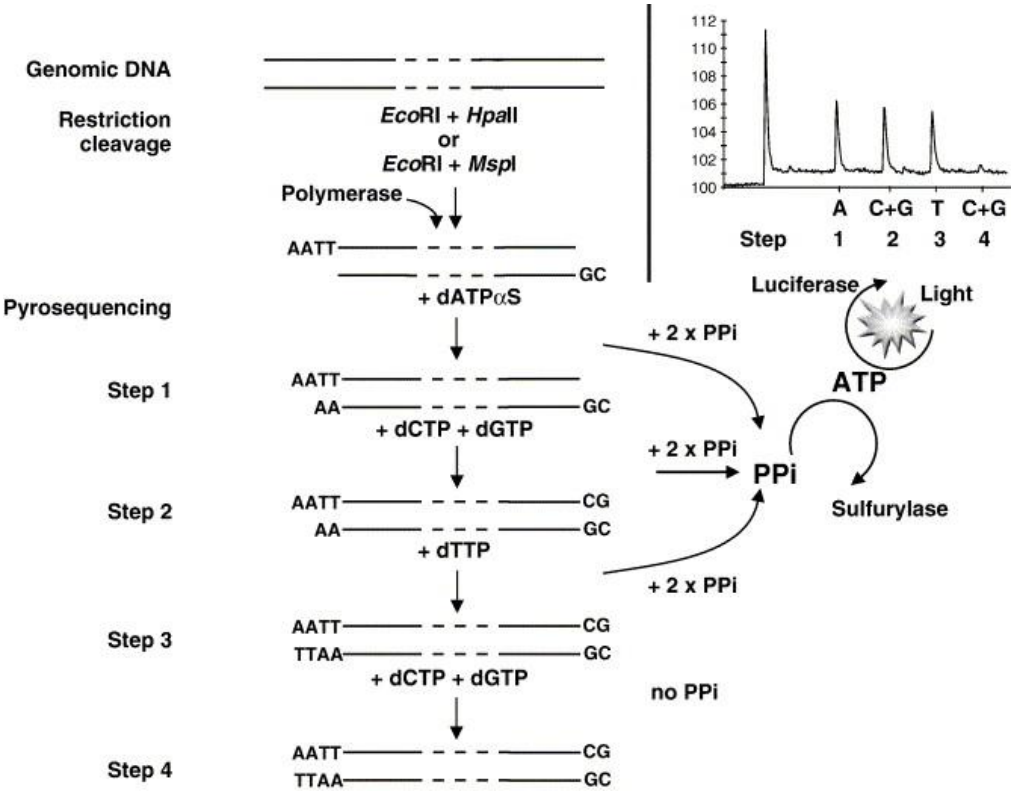
2.5. Nucleic Acid quality and quantity

A NanoDrop® ND2000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to measure the nucleic acid concentration and the absorbance ratios (A260/A280 and A260/A230). Nucleic acid quality was further assessed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light by E-BOX- VX2 photodocumentation system (Vilber Lourmat, France).

2.6. Reverse transcription-quantitive PCR (RT-qPCR)

RNA samples of the same litter were pooled separately according to their sexes (n=5, litter per diet). After incubation with 0.0625 U DNAse I (Ambion) 37°C 15 minutes, 75°C 10 minutes, the RNAs were reverse transcribed in duplicates from 6 µg of total RNA in the presence of 50ng random primers (hexamers, Invitrogen), 500nM dNTPs, 10nM DTT (Invitrogen) and 10U of Supercript II, reverse transcriptase (Invitrogen). The cDNA duplicates were pooled. One –RT control was also performed for each sample. SYBR Green based RT-qPCR was set up in 96-well plate in a total volume of 15µl containing 0.3 µM of each primer, 1x Absolute Blue qPCR SYBR Green Rox Mix (Thermo Scientific) and 2ng of the cDNA sample. Each amplification was carried out in duplicates, and –RT controls in simplicate, in the presence of 10-fold dilutions (8 points) of standart cDNA in an ABI PRISM 7300 apparatus (Applied Biosystems), under the following conditions: 15 minutes incubation at 95°C, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at the annealing temperature corresponding to the gene of interests (Table 3). The house-keeping genes of *Eif4a2, TBP, Sdha* were used as reference genes and normalization factor was obtained using the GeNorm software to normalize the qPCR. Data were analyzed in Microsoft Excel. The primer sequences are shown in the Table 3.

Fig.5. LUminometric Methylation Assay- A high throughput method to the analysis of genomic DNA methylation (Karimi et al., 2006b)



2.7. Luminometric Methylation Assay (LUMA)

This method is used to quantify global DNA methylation (Karimi et al., 2006a; Karimi et al., 2006b). The principle of the technique is based on digestion of genomic DNA by CpG methylation sensitive and insensitive restriction enzymes, namely Hpa II and Msp I, respectively. EcoRI was included in all reactions as a normalization reference. MspI and HpaII both leave 5'-CG overhangs after DNA cleavage, whereas EcoRI produces 5'-AATT overhangs. Using the pyrosequencing technology, the digestion sites were filled separately by polymerase extensions through the sequential addition of dNTPs (Figure 5): A, C+G, T. For each extension step, pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase. Luciferin is converted by luciferase using ATP into oxy-luciferin, which produces light detected by a charge coupled device (CCD) camera. Light emission is proportional to the quantity of extended nucleotide. Peak heights were calculated by PyroMark Q24™ Software (Qiagen). A and T peaks represents EcoRI digestion profile and C+G peak corresponds to MspI or HpaII digestion profile, respectively (Figure 5). The assay was carried out in duplicates. Briefly, a total of 500ng genomic DNA was cleaved with HpaII+EcoRI or MspI+EcoRI in separate reactions. The enzymatic reaction incubated at 37°C for 4 h. Following the digestion, 20µl the annealing buffer was added to 20µl of the digestion products. Enzyme mix, substrate mix and dNTPs were added by the apparatus (Pyrosequencer Q24, Qiagen). (C+G)/A ratio were calculated for all digestion, representing MspI/EcoRI or HpaII/EcoRI ratio, depending on the digestion mix. MspI/EcoRI ratio represents the total amount of CCGG restriction sites on the genome, and HpaII/EcoRI represents the amount of unmethylated CCGG restriction sites. As a quality control of the experiment, MspI/EcoRI ratios must be constant in all samples. Global DNA methylation level was calculated from the (HpaII/EcoRI) / (MspI/EcoRI) ratio. Fully methylated DNA gives a ratio close to 0 whereas unmethylated DNA ratio is close to 1. Each experiment was carried out in duplicates. The HpaII/MspI ratio for each sample corresponds to the average of the two values.

2.8. Statistical Analysis

All data were expressed as means ± standard error of the mean (SEM). The effects of sex and diet on development (weight gain of fetus, placenta and mother), on expression of the candidate genes and differences between methylation profiles were assessed by using a non-parametric Mann-Whitney test with StatEl (ADscience, France). All statistical tests were considered to be statistically significant at $P<0.05$ (Table 4 and Table 5).

Fig.6. Fetus, placental weight and Fetus to Placenta weight ratio Index (FPI) of E15.5 (17 FCD, 17 FHFD, 18 MCD, 19 MHFD) and E18.5 (19 FCD, 13 FHFD, 26 MCD, 19 MHFD (*P* values are shown below in the Table 1).

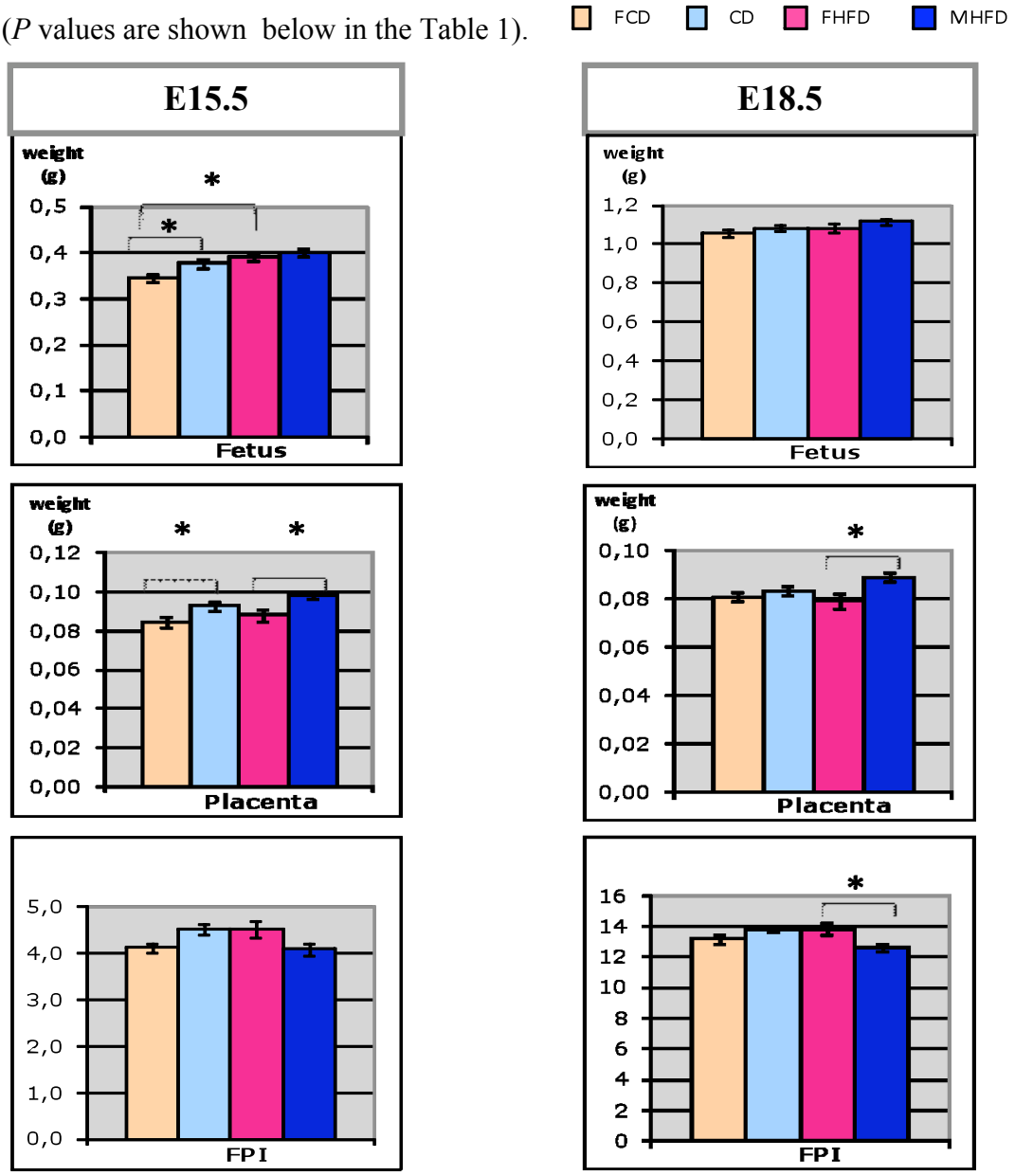


Table 4. *P* values according to Mann & Whitney test for fetus weight, placental weight and FPI at E15.5 and E18.5

E15.5				E18.5			
	Fetus	Placenta	FPI		Fetus	Placenta	FPI
Diet	0,0005	0,19	0,74	Diet	0,24	0,12	0,88
Sex	0,024	0,0004	0,82	Sex	0,087	0,0099	0,03
Diet in F	0,0008	0,45	0,12	Diet in F	0,79	0,62	0,88
Diet in M	0,79	0,23	0,27	Diet in M	0,26	0,051	0,11
Sex in CD	0,0034	0,0022	0,28	Sex in CD	0,42	0,41	0,88
Sex in HFD	0,24	0,0081	0,15	Sex in HFD	0,13	0,0014	0,00054

3. RESULTS

3.1. Developmental Studies

C57BL/6 mice were crossed and diet was changed when a vaginal plug was observed (Embryonic day (E) 0.5 = vaginal plug). The mice received either a control diet (CD, 10% fat) or a high-fat diet (HFD, 60% fat) (Table 2). Dams were sacrified at three different stages (E12.5, E15.5 and E18.5), fetus and placenta were collected and empty dams were weighted. The data from E12.5 samples is excluded since we could not collect sufficient number mothers. At E15.5, we collected 19 female and 22 male fetuses from 6 CD mothers, and 18 female and 20 male fetuses from 6 HFD mothers. At E18.5, we obtained 18 female and 26 male fetuses from 6 CD mothers and 16 female and 20 male fetuses from 6 HFD mothers. Neither the CD nor the HFD litters resulted in external physical malformations. Pregnancy rate was not affected by diet modification. Litter size (8 in CD, 8.3 in HFD at E12.5; 7 in CD, 6.3 in HFD at the stage of E15.5 and 7.3 in CD, 6 in HFD at E18.5) and female/male ratio (86.36 % in CD and 90% in HFD at E15.5, 69.2% in CD, 80% in HFD at E18.5) were not affected by the maternal diet.

3.1.1. Fetus, placental weight and Fetus to Placenta Weight ratio Index (FPI)

At E15.5, the fetus weight was significantly affected by diet *($p<0.01$)* and sexually dimorphic *($p<0.05$)*, with an increased weight under the HFD and male fetuses were heavier than female fetuses (Figure 6). In addition, the impact of maternal HFD was significant particularly on female fetuses, with increased weight under the HFD *($p<0.01$)*. When sexual dimorphism was estimated on CD and HFD separately, male fetuses were significantly heavier than the female on CD only. No effect of diet nor sex was observed on fetus weight at E18.5.

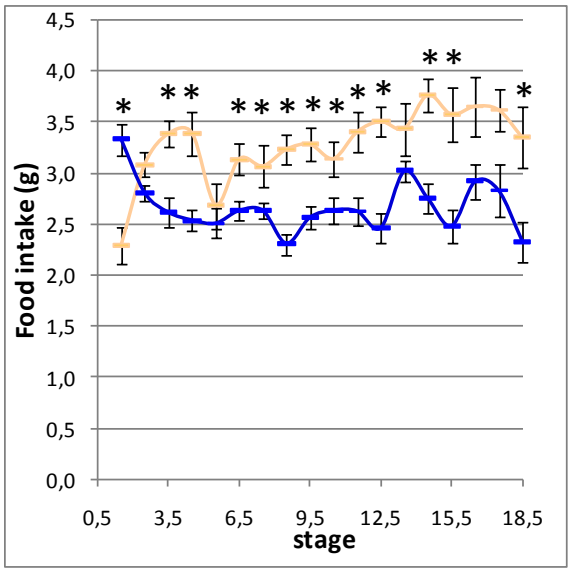
At stages E15.5 and E18.5, there was a significant sex effect on placental weight: male placentas were heavier than the female placentas, regardless of diet *($p<0.01$)*. Moreover, at E15.5, male placentas were heavier than the female ones *($p<0.01$)* under the CD and HFD, when analyzed separately but at E18.5 (Figure 6), the sexual dimorphism on placental weight was significant only under the HFD *($p<0.01$)*. Diet did not affect placenta weight at term.

No sex nor diet effect was observed at E15.5 on the FPI, which reflects nutrient transfer from mother to the fetus through the placenta. Remarkably, at E18.5, the FPI is sexually dimorphic, regardless of the diet or under the HFD, with higher FPI values in female than in males *($p<0.05$)*.

Fig.7. Food (a) and caloric (b) intake of the pregnant dam (n = 13 CD, 13 HFD from E1.5 to E12.5; n = 12 CD, 10 HFD from E12.5 to E15.5, n = 6 CD, 5 HFD from E15.5 to E18.5)

CD HFD

a)



b)

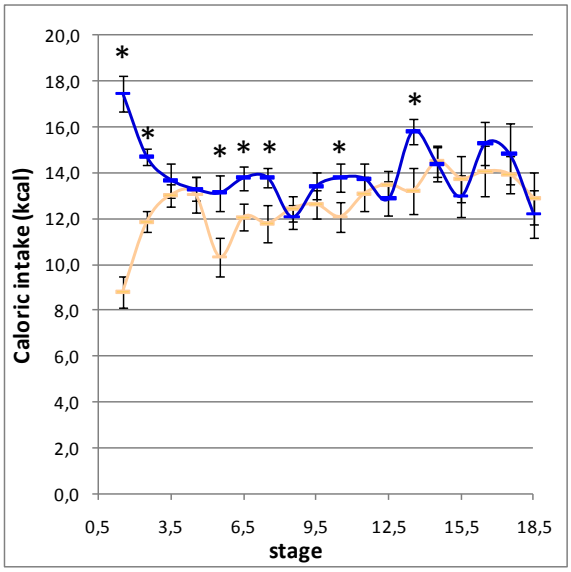
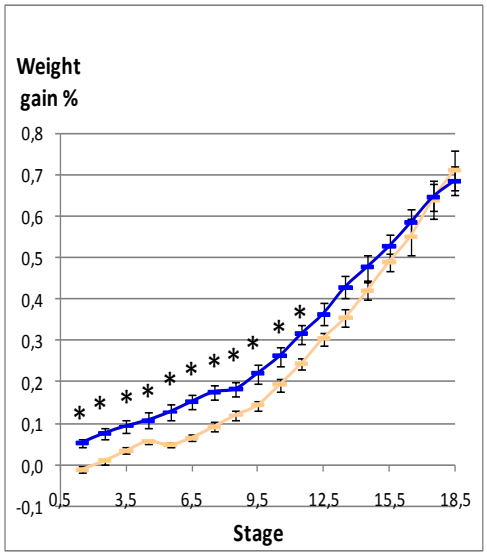


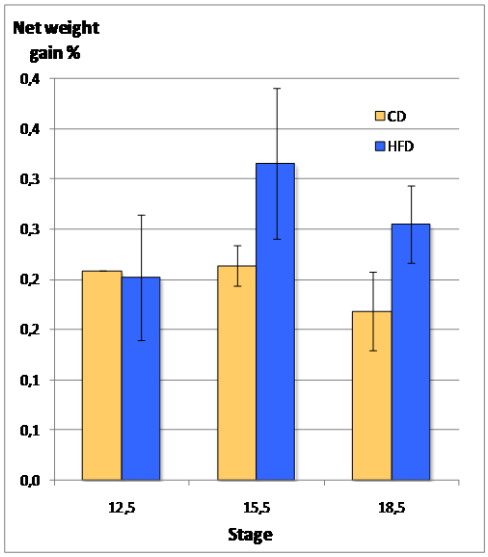
Fig.8. Body weight gain of the pregnant mice (n=28) (a) and net weight gain after gestation (b) (n = 13CD, 13 HFD at E12.5, n = 12 CD, 10 HFD at E15.5, n = 6 CD, 5 HFD at E18.5)

CD HFD

a)



b)



3.1.2. Food intake of the pregnant dam

The daily food intake of HFD mothers was higher than that of the CD mothers on the first day and then the food intake of HFD dams decreased while food intake of the CD dams increased. We then observed a significant difference in food intake throughout the gestational period (Figure 7), with HFD mothers eating 22 % less than the CD group. Daily caloric intake was calculated from the food intake records, on the basis of 3.85kcal/g for the CD diet and 5.24kcal/g for the HFD. Overall, no differences in caloric intake were found between HFD and CD groups, except for days E1.5, E2.5, E5.5, E6.5, E7.5, E10.5 and E13.5.

3.1.3. Body weight gain of the dam

We observed that weight gain of the HFD pregnant dam was increased compared to the CD dams until E11.5 ($P< 0.05$). After this stage, the difference between the two groups was no longer significant (Figure 8 [a]). Moreover, there was no statistically significant difference in maternal net weight gain after removal of the fetuses by dissection section at E12.5, E15.5 and E18.5 stages (Figure 8 [b]).

3.2. Analysis of gene expression by Reverse Transcription-quantitative PCR

In a precedent Affymetrix microarray analysis, four genes encoding epigenetic machinery enzymes were dysregulated on total mouse placenta by the HFD at E15.5 (Gabory et al., submitted): the DNA methyltransferase3 cofactor *Dnmt3l*, the H3K9 trimethyltransferases *Suv39h1* and *Suv39h2* and the H3K4 demethylase *Jarid1c*. To understand the underlying mechanism of programming under the effect of maternal HFD throughout pregnancy, we analyzed these four candidate genes by RT-qPCR in the mouse labyrinth tissue at two different embryonic stages (E15.5 and E18.5). There was a significant decrease of *Dnmt3l* expression under the HFD regardless of the sex *($p< 0.05$)* at E18.5 but no differences was found at E15.5 (Table 5). There was a non-significant tendency to downregulation of *Suv39h1* in the HFD placentas vs. CD placentas when the analysis was done regardless of fetus sex at both stages. No differences were found for *Suv39h2* and *Jarid 1c*, neither between the diet nor the sexes (Figure 9).

Table 5. *P* values for the gene expression analysis by qPCR

a)

	P values for E15.5 gene exression			
	Jarid1c	***Suv39h1***	***Suv39h2***	***Dnmt3l***
sex effect	0,82	0,29	0,65	0,23
diet effect	0,17	0,60	0,82	0,76
diet effect on females	0,69	1,00	1,00	0,69
diet effect on males	0,22	0,69	0,84	1,00
sex effect in CD	0,42	0,69	0,84	0,42
sex effect in HFD	0,31	0,22	0,84	0,55

b)

	P values for E18.5 gene exression			
	Jarid1c	***Suv39h1***	***Suv39h2***	***Dnmt3l***
sex effect	0,13	0,71	0.94	0,45
diet effect	0,41	0,10	0.36	**0,05**
diet effect on females	0,69	0,42	1.00	0,22
diet effect on males	0,15	0,22	0.31	0,22
sex effect in CD	0,84	0,55	0.42	0,69
sex effect in HFD	0,15	0,42	0.69	0,55

Fig.9. Gene expression profiles analyzed by Reverse Transcription-quantitative PCR (6 FCD, 5 MCD, 4 HFD, 5MHFD at E15.5 vs 5FCD, 5MCD, 5HFD, 5MHFD at E18.5). There is a significant diet effect on *Dnmt3l* expression at E18.5 *(p<0.05)*.

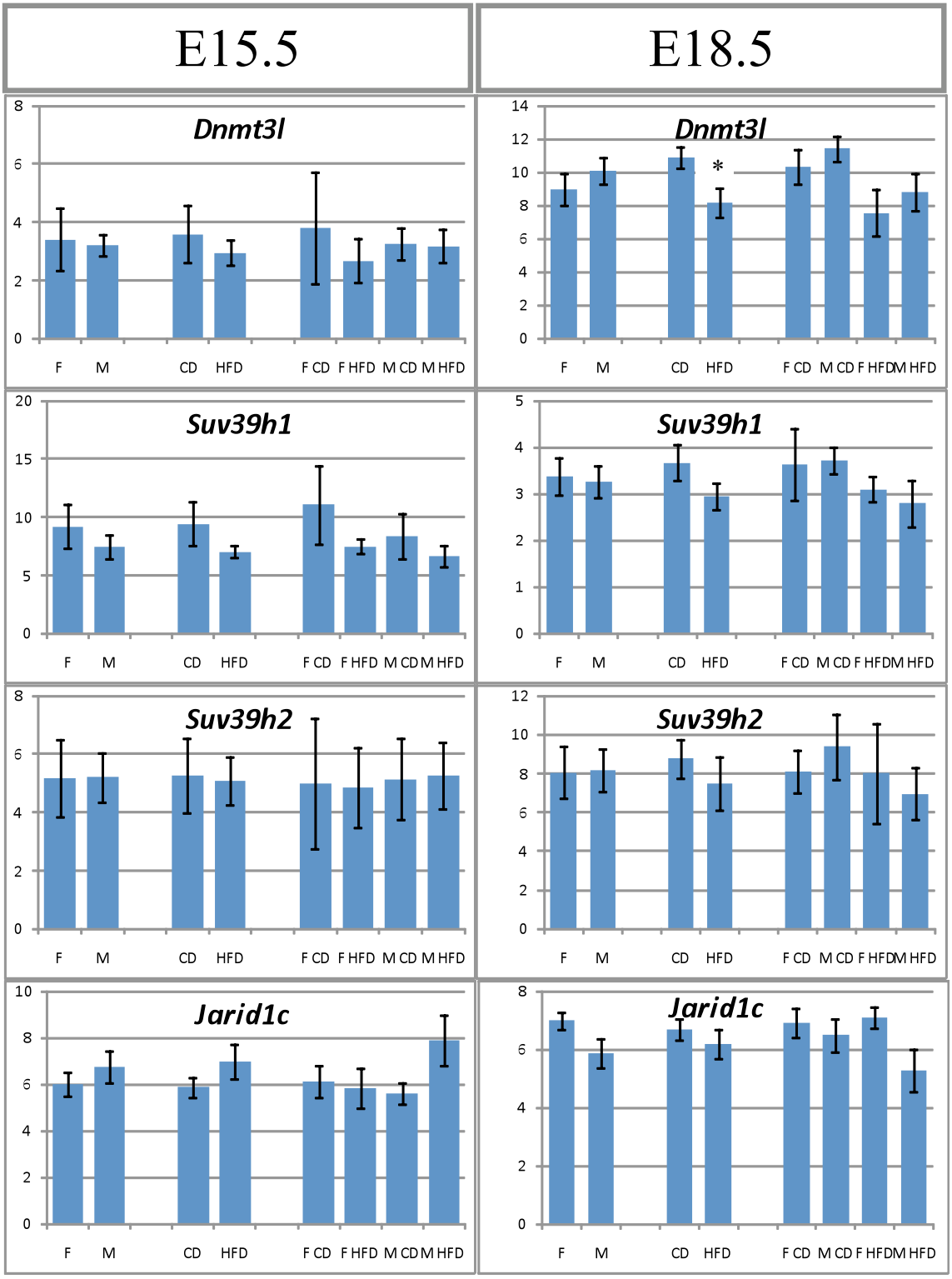
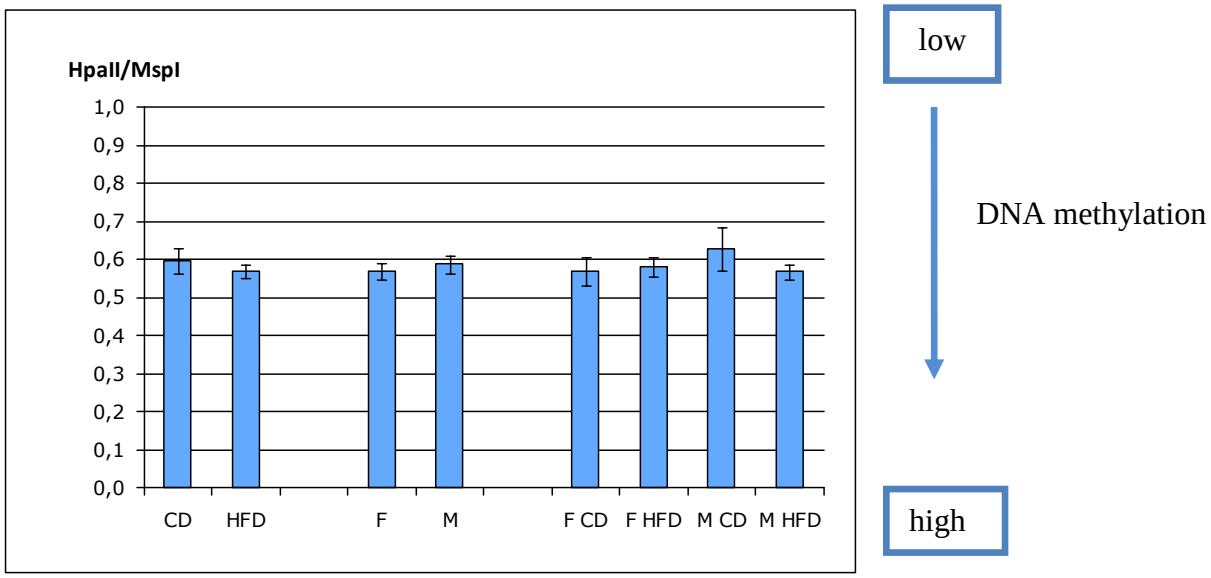
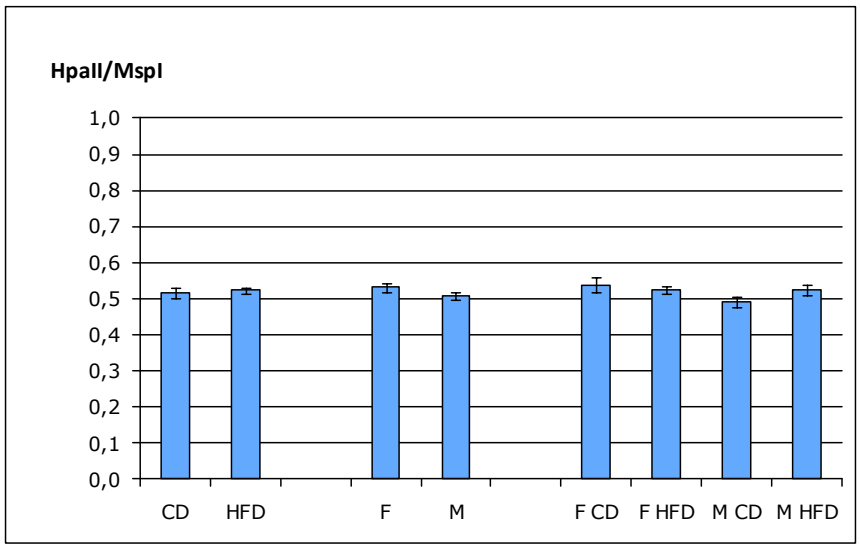


Fig.10. LUMA analysis of global DNA methylation levels at E15.5 (5 FCD, 13 FHFD and 5 MCD, 11 MHFD) **(a) and E18.5** (9 FCD and 9 FHFD and 8 MCD, 9 MHFD) **(b)**. The ratio of HpaII/MspI would be 1.0 if DNA is completely unmethylated and approach to zero if DNA is fully methylated.

a)



b)



3.3 Analysis of global DNA methylation by Luminometric Methylation Assay (LUMA)

Global DNA methylation levels were analyzed by the LUMA assay. Figure 10 shows the distribution of methylation levels in the placentas of E15.5 and E18.5. We did not observe any statistically significant differences on global DNA methylation. However, there was a trend towards hypomethylation in males versus females under the CD at E15.5 (Figure 10).

4. DISCUSSION

In order to understand the impact of maternal malnutrition on the underlying molecular mechanisms of programming *in utero*, we developed a HFD fed mouse model during gestation. We studied C57BL/6J mice, a strain susceptible to diet-induced obesity (Gallou-Kabani et al., 2007; Tallman et al., 2009). Moreover, unlike previous reports using diets composed of mainly saturated fatty acids, we chose a HFD composed of 20% protein, 20% carbohydrate, and 60% fat including different levels of monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) in order to better represent Western diet (Table 1).

In this study, we could analyse E15.5 and E18.5 but not E12.5 since the collections are ongoing. However, it will be important to make analysis at this stage for having a complete picture of kinetics aspect of placental development. The main observations that we obtained regarding developmental features and epigenomic analysis were the following:

- The pregnant mice fed with HFD, reduced their food intake and subsequently had the same caloric intake as CD fed mice.
- E15.5 fetal weight was both affected by the diet and sexually dimorphic. However, E18.5 fetal weight was neither influenced by diet nor sexually dimorphic.
- Under the CD, fetal weight and placental weight were both affected by the sex of the fetus in the middle of fetal period (males were heavier than the females) but no more at term. Moreover, while female fetal weight was affected by the HFD, male fetal weight was not affected in the middle of fetal period.
- We observed sexual dimorphism in placental weight in both stages with males having larger placenta the females.
- There was a sexual dimorphism in FPI (fetus to placental weight ratio index) at term under the HFD -regardless of diet- with average female FPI higher than average male FPI when analyzed seperately.
- The HFD had an influence on the gene expression in *Dnmt3l* at E18.5: there was a significant decrease of *Dnmt3l* expression, regardless of the sex, at the end of the gestation. The other candidate epigenetic machinery genes were not differentially expressed between the 4 groups (F CD, F HFD, M CD and M HFD).
- The global methylation pattern, analysed by LUMA, was not statistically different between the groups at the two different stages in the labyrinth.

4.1. Developmental Studies

4.1.1. The link between placental and fetal growth and structure

Our model is not a model of maternal obesity, the diet being changed after mating. According to the reports on food intake of HFD fed rodents during gestation, the mothers either adjust their food intake in terms of calories regardless of the diet (Theys et al., 2010) or do not adjust and increase the caloric intake (Jones et al., 2009; Samuelsson et al., 2008). In our model, we observed that dams that were fed with HFD, decreased their food intake leading to a balanced caloric intake. Our model is therefore not a model of overnutrition but a model of maternal malnutrition, with disrupted lipid and carbohydrate metabolism. This switch in HFD dams may provide clues in adaptation to environmental insults by altering response of metabolism during pregnancy. This result is in contrast to findings in overweight and obese pregnant women, who have increased insulin and intake of fat, carbohydrates, and total energy (Jones et al., 2009).

One of the main observation in this study was the sexual dimorphism in placental weight at both stages with male placentas being heavier than females placentas. Moreover, while female fetal weight was affected by the HFD in the middle of fetal period, male fetal weight was not affected. We can speculate that females respond to HFD more robustly than the males. This is consistent with previous findings showing that females are more sensitive to the environmental exposures *in utero* (Clifton, 2010; Han et al., 2005; Mao et al., 2010). Despite the observation of E15.5 fetal weight being affected by the diet and sexually dimorphic, E18.5 fetal weight was neither influenced by diet nor was sexually dimorphic. In addition, female fetal weight was no more affected at the term of gestation. We observed neither intrauterine growth restriction nor macrosomia at term. This raises the question as to whether there is any fetal adaptation. If there is, this supports the statement that developmental plasticity and fetal adaptations arise from the impact of maternal nutrients on the placenta which plays a key role in the delivery of nutrients to the fetus (Godfrey, 2002).

Experimental and epidemiological studies in humans and animal models have demonstrated that predisposition to metabolic syndrome is associated with either low or high FPI (Gallou-Kabani et al., 2010; Godfrey, 2002; Thornburg et al., 2010a). In our malnutrition model, we could observe a sexual dimorphism in FPI at term -regardless of diet- with female FPI higher than the male FPI. It has been suggested that lower FPI is driven primarily by higher placental weight (Nelson et al., 2008) and the lower FPI in males might be associated with adult risk of cardiovascular disease (Risnes et al. 2009, Eriksson et al., 2000, Eriksson et al.,

2010, Barker et al.,1993). This finding is consistent with the previous findings in the laboratory which demonstrates that FPI is significantly higher in female mice than male mice, however in contrast to the findings of HFD reducing the FPI (Gallou-Kabani et al., 2010).

In order to determine structural differences in the placenta under the high-fat exposure, histological analysis was performed and revealed no differences in the proportion of the different layers nor shape of the placenta between the 4 groups (Gabory et al., submitted). A more detailed analysis of histological sections in different tissues and embryonic stages, immunocytochemistry based analysis (for detecting leptin, adiponectin, insulin, vimentin and cytokeratin) is therefore of interest. Insulin and leptin have been proposed as a regulator of placental growth (Nelson et al., 2009). Leptin and adiponectin are reported to be influenced by body fat status and associated with increased risk of metabolic diseases. In addition, leptin is found to be negatively correlated with insulin and adiponectin negatively correlates with plasma triglycerides (Smith et al., 2006). The transcript and protein levels of adiponectin are reported to be down-regulated in obesity and diabetes, both in animal models and in humans (Wong et al., 2008). Vimentin and cytokeratin are markers of blood vessels at maternal-fetal interface in the placenta (Litwin et al., 2010).

4.2. Epigenomic Analysis

4.2.1. Changes in expression of genes involved in the epigenetic machinery

In a previous study, *Dnmt3l*, *Suv39h1 and Suv39h2* were downregulated in the HFD placentas (Gabory et al submitted). Moreover, sexual dimorphism was observed for *Suv39h2* and *Jarid1c*, with higher expression of *Suv39h2* in male than female placentas, and higher expression of *Jarid1c* in female than male placentas. On the other hand, the expression of *Dnmt3a*, *Dnmt3b* and *Dnmt1* did not differ between the sexes or between diets (Gabory et.al, submitted). *Suv39h1* and *Suv39h2* encode methyltransferases involved in trimethylation of the lysine 9 of histone H3, thereby resulting in a repressive chromatin structure (Ozdag et al., 2006). *Jarid1c*, which has a crucial role in H3K4me3 demethylation (H3K4me3 is an active chromatin mark), is X chromosome linked, escapes X inactivation and is more strongly expressed in females than in males (Li and Carrel, 2008). *Dnmt3l* has been shown to recruit histone deacetylases and to interact with H3K4me3 (Deplus et al., 2002; Ooi et al., 2007). Moreover, *Suv39h1* was recently shown to be involved in the 'metabolic memory' in the endothelial cells of diabetic patients, suggesting that dysregulation of the epigenetic machinery maybe a major underlying factor in disease phenotype (Villeneuve et al., 2008).

Therefore, consistent with previous studies, such a complex network includes cross-talks between epigenetic marks.

It is well-known that the epigenetic modifications regulate gene expression in a cell- and sex-specific and time-dependent manner. At the middle of the fetal period, neither diet nor sex had impact on gene expression in our current analyses in the labyrinth whereas in total placenta, it was observed that the genes dysregulated under HFD, due to effects of diet, sex or both, included four genes encoding enzymes from an important network of the epigenetic machinery (Gabory et al., submitted). *Dnmt3l, Suv39h1 and Suv39h2* were downregulated in the HFD total placentas in the previous study gene expression analysis which was conducted at E15.5 (Gabory et al., submitted). We had the same trends for *Dnmt3l, Suv39h1 and Suv39h2*, in the labyrinth, at the same stage, however it was not statistically significant. *Jarid1c* was more expressed in females than males (Gabory et al., submitted) whereas we had observed contrast trend for this gene in the labyrinth, although it was not significant (Figure 9). The differences in the gene expression profiles with the previous study, can be explained mainly by stage and tissue specifity in our current model. Therefore, in addition to studies in the labyrinth tissue, it would be essential to study gene expression patterns within the junctional zone. One other potential limitation of our study was the small sample size in our current study, which may not be sufficient to reach statistical power. Another factor leading to different gene expression profiles could be due to the use of C57BL/6J males instead of DBA2/J males for mating in this animal model. The different genetic bakground may respond differently to the HFD. This is consistent with the previous findings showing that C57BL/6J are comparatively more resistant than the DBA2/J to the HFD (Li and Churchill, 2010; Tortoriello et al., 2004).

In addition, it will be important to determine how the crosstalk between key repressive or activating epigenetic marks and their modifying enzymes can be disturbed by environmental insults, leading to developmental and metabolic malprogramming at different embryonic stages. Moreover, besides the gene expression analysis, the identification of modifications on histone marks will be another ongoing aspect of research.

4.2.2. Coupling gene expression profile with global methylation

The present study has demonstrated that exposure to HFD during pregnancy contribute to epigenetic regulation of Dnmt3l in the labyrinth tissue, at E18.5. Among the genes studied, only *Dnmt3l* was significantly decreased under the HFD, regardless of the sex. In parallel, there was no significant difference in global DNA methylation at E18.5. Therefore, the

decreased Dnmt3l is not linked with a change in global DNA methylation in term placenta. This can be explained by at least four reasons.

First, Dnmt3l itself lacks DNA methylase activity and function as a cofactor recruiting Dnmt3a and Dnmt3b to stimulate *de novo* methylation. Since we were analysing global DNA metylation, it can be important to test further Dnmt3a and Dnmt3b with Dnmt1. Moreover, Dnmt3l appears to require properly packaged DNA within chromatin for stimulating de novo methylation which is consistent with the findings that Dnmt3l binds to histones directly (Ooi et al., 2007; Wienholz et al., 2010). Therefore, in order to better understand the implications of diet, histone post-translational modifications need to be further analysed.

Second, decreased *Dnmt3l* expression was analysed by RT-qPCR which detect mRNA level. An expression analyse at the Dnmt3l protein level would allow us to verify if the active form of this protein is reduced.

Third, there might be differences in global methylation but this might not be detectable by LUMA in which we analysed CpG-specific metylation. So, different approaches including high-performance liquid chromatography (HPLC) and MALDI-TOF MS) (Ragoussis et al., 2006; Sulewska et al., 2007).

Last, there could be differences in methylation but in specific sequences in the genome, targeted by Dnmt3l, but not detectable by a global approach. Here, the right choice of the method also depends on the amount, quality, and type of the biological material to be analyzed, the equipment in the laboratory and specialists.

5. CONCLUSION

In parallel to the obesity epidemic, the increasing prevalence of obese or overweight women of childbearing age is becoming an alarming public health concern. Even more alarming is the adverse consequences of obesity, not only on pregnancy outcome and maternal health, but also on the quality of life and further health of the next generation. The prevention of obesity must therefore begin in early stages of life. However, few studies focus on both sex- and diet-dependent differences in epigenetic programming of predisposition to metabolic syndrome *in utero*. In human studies, it is difficult to assess the impact of overnutrition on the placenta and developing embryo at certain critical stages important for programming of metabolic syndrome due to the difficulties related to dietary questionnaires and ethical issues. The animal models therefore provide valuable information about epigenetic modifications in response to the early environmental events resulting in modified gene expression patterns and phenotypes later in life in a sex-specific manner. Increasing evidence reveals a placental signature of maternal HFD impact that clearly differs according to the fetus sex. Our current data also support an epigenetic basis of nutritional placental programming. Research on nutritional programming of obesity has many avenues of research (physiology, metabolism, epigenetics), paving the way to new hypotheses about the origin of metabolic diseases. With the transferring the data obtained in our study, to human health research, we want to decipher how epigenetic marks could be used as bio- markers, since they provide an "archive" of the effects of deleterious maternal metabolism and diet during development. In this respect, evidence- based dietary recommendations during the prenatal period via clinical counseling could help women of childbearing age and also the next generations to improve their health.

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REFERENCES

Aagaard-Tillery, K. M., Grove, K., Bishop, J., Ke, X., Fu, Q., McKnight, R. and Lane, R. H. (2008). Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. *J Mol Endocrinol* **41**, 91-102.

Angiolini, E., Fowden, A., Coan, P., Sandovici, I., Smith, P., Dean, W., Burton, G., Tycko, B., Reik, W., Sibley, C. et al. (2006). Regulation of placental efficiency for nutrient transport by imprinted genes. *Placenta* **27 Suppl A**, S98-102.

Attig, L., Gabory, A. and Junien, C. (2010). Early nutrition and epigenetic programming: chasing shadows. *Curr Opin Clin Nutr Metab Care* **13**, 284-93.

Barker, D. J., Thornburg, K. L., Osmond, C., Kajantie, E. and Eriksson, J. G. (2010). The prenatal origins of lung cancer. II. The placenta. *Am J Hum Biol* **22**, 512-6.

Bayol, S. A., Simbi, B. H. and Stickland, N. C. (2005). A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J Physiol* **567**, 951-61.

Bispham, J., Gopalakrishnan, G. S., Dandrea, J., Wilson, V., Budge, H., Keisler, D. H., Broughton Pipkin, F., Stephenson, T. and Symonds, M. E. (2003). Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* **144**, 3575-85.

Clifton, V. L. (2010). Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta* **31 Suppl**, S33-9.

Coan, P. M., Conroy, N., Burton, G. J. and Ferguson-Smith, A. C. (2006). Origin and characteristics of glycogen cells in the developing murine placenta. *Dev Dyn* **235**, 3280-94.

Deplus, R., Brenner, C., Burgers, W. A., Putmans, P., Kouzarides, T., de Launoit, Y. and Fuks, F. (2002). Dnmt3L is a transcriptional repressor that recruits histone deacetylase. *Nucleic Acids Res* **30**, 3831-8.

Dolinoy, D. C., Das, R., Weidman, J. R. and Jirtle, R. L. (2007). Metastable epialleles, imprinting, and the fetal origins of adult diseases. *Pediatr Res* **61**, 30R-37R.

Dolinoy, D. C., Weidman, J. R., Waterland, R. A. and Jirtle, R. L. (2006). Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect* **114**, 567-72.

Flier, J. S. (2004). Obesity wars: molecular progress confronts an expanding epidemic. *Cell* **116**, 337-50.

Fountain, E. D., Mao, J., Whyte, J. J., Mueller, K. E., Ellersieck, M. R., Will, M. J., Roberts, R. M., Macdonald, R. and Rosenfeld, C. S. (2008). Effects of diets enriched in omega-3 and omega-6 polyunsaturated fatty acids on offspring sex-ratio and maternal behavior in mice. *Biol Reprod* **78**, 211-7.

Frias, A. E., Morgan, T. K., Evans, A. E., Rasanen, J., Oh, K. Y., Thornburg, K. L. and Grove, K. L. (2011). Maternal High-Fat Diet Disturbs Uteroplacental Hemodynamics and Increases the Frequency of Stillbirth in a Nonhuman Primate Model of Excess Nutrition. *Endocrinology*.

Frost, J. M. and Moore, G. E. (2010). The importance of imprinting in the human placenta. *PLoS Genet* **6**, e1001015.

Fuso, A., Nicolia, V., Cavallaro, R. A. and Scarpa, S. (2011). DNA methylase and demethylase activities are modulated by one-carbon metabolism in Alzheimer's disease models. *J Nutr Biochem* **22**, 242-51.

Gabory, A., Attig, L. and Junien, C. (2009). Sexual dimorphism in environmental epigenetic programming. *Mol Cell Endocrinol* **304**, 8-18.
Gallou-Kabani, C., Gabory, A., Tost, J., Karimi, M., Mayeur, S., Lesage, J., Boudadi, E., Gross, M. S., Taurelle, J., Vige, A. et al. (2010). Sex- and diet-specific changes of imprinted gene expression and DNA methylation in mouse placenta under a high-fat diet. *PLoS One* **5**, e14398.
Gallou-Kabani, C., Vige, A., Gross, M. S., Rabes, J. P., Boileau, C., Larue-Achagiotis, C., Tome, D., Jais, J. P. and Junien, C. (2007). C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity (Silver Spring)* **15**, 1996-2005.
Gardner, R. L. and Davies, T. J. (1993). Lack of coupling between onset of giant transformation and genome endoreduplication in the mural trophectoderm of the mouse blastocyst. *J Exp Zool* **265**, 54-60.
George, L. A., Uthlaut, A. B., Long, N. M., Zhang, L., Ma, Y., Smith, D. T., Nathanielsz, P. W. and Ford, S. P. (2010). Different levels of overnutrition and weight gain during pregnancy have differential effects on fetal growth and organ development. *Reprod Biol Endocrinol* **8**, 75.
Georgiades, P., Ferguson-Smith, A. C. and Burton, G. J. (2002). Comparative developmental anatomy of the murine and human definitive placentae. *Placenta* **23**, 3-19.
Gheorghe, C. P., Goyal, R., Mittal, A. and Longo, L. D. (2010). Gene expression in the placenta: maternal stress and epigenetic responses. *Int J Dev Biol* **54**, 507-23.
Gluckman, P. D., Hanson, M. A., Cooper, C. and Thornburg, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* **359**, 61-73.
Godfrey, K. M. (2002). The role of the placenta in fetal programming-a review. *Placenta* **23 Suppl A**, S20-7.
Han, J., Xu, J., Epstein, P. N. and Liu, Y. Q. (2005). Long-term effect of maternal obesity on pancreatic beta cells of offspring: reduced beta cell adaptation to high glucose and high-fat diet challenges in adult female mouse offspring. *Diabetologia* **48**, 1810-8.
Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* **444**, 860-7.
Igwebuike, U. M. (2010). Impact of maternal nutrition on ovine foetoplacental development: A review of the role of insulin-like growth factors. *Anim Reprod Sci* **121**, 189-96.
Jansson, T. and Powell, T. L. (2007). Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. *Clin Sci (Lond)* **113**, 1-13.
Jones, H. N., Woollett, L. A., Barbour, N., Prasad, P. D., Powell, T. L. and Jansson, T. (2009). High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *Faseb J* **23**, 271-8.
Karimi, M., Johansson, S. and Ekstrom, T. J. (2006a). Using LUMA: a Luminometric-based assay for global DNA-methylation. *Epigenetics* **1**, 45-8.
Karimi, M., Johansson, S., Stach, D., Corcoran, M., Grander, D., Schalling, M., Bakalkin, G., Lyko, F., Larsson, C. and Ekstrom, T. J. (2006b). LUMA (LUminometric Methylation Assay)--a high throughput method to the analysis of genomic DNA methylation. *Exp Cell Res* **312**, 1989-95.
Kelly, T., Yang, W., Chen, C. S., Reynolds, K. and He, J. (2008). Global burden of obesity in 2005 and projections to 2030. *Int J Obes (Lond)* **32**, 1431-7.
Klose, R. J. and Bird, A. P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31**, 89-97.
Knox, K. and Baker, J. C. (2008). Genomic evolution of the placenta using co-option and duplication and divergence. *Genome Res* **18**, 695-705.
Li, N. and Carrel, L. (2008). Escape from X chromosome inactivation is an intrinsic property of the Jarid1c locus. *Proc Natl Acad Sci U S A* **105**, 17055-60.

Li, R. H. and Churchill, G. A. (2010). Epistasis contributes to the genetic buffering of plasma HDL cholesterol in mice. *Physiol Genomics* **42A**, 228-34.
Liang, C., DeCourcy, K. and Prater, M. R. (2010). High-saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice. *Metabolism* **59**, 943-50.
Liang, C., Oest, M. E. and Prater, M. R. (2009). Intrauterine exposure to high saturated fat diet elevates risk of adult-onset chronic diseases in C57BL/6 mice. *Birth Defects Res B Dev Reprod Toxicol* **86**, 377-84.
Litwin S, Cortina ME, Barrientos GL, Prados MB, Roux ME, Miranda SE. (2010). Multiparity increases trophoblast invasion and vascular endothelial growth factor expression at the maternal-fetal interface in mice. *J Reprod Immunol*. **85**(2):161-7.
Ma, Y., Zhu, M. J., Zhang, L., Hein, S. M., Nathanielsz, P. W. and Ford, S. P. (2010). Maternal obesity and overnutrition alter fetal growth rate and cotyledonary vascularity and angiogenic factor expression in the ewe. *Am J Physiol Regul Integr Comp Physiol* **299**, R249-58.
Maher, B. (2008). Personal genomes: The case of the missing heritability. *Nature* **456**, 18-21.
Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M. I., Ramos, E. M., Cardon, L. R., Chakravarti, A. et al. (2009). Finding the missing heritability of complex diseases. *Nature* **461**, 747-53.
Mao, J., Zhang, X., Sieli, P. T., Falduto, M. T., Torres, K. E. and Rosenfeld, C. S. (2010). Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. *Proc Natl Acad Sci U S A* **107**, 5557-62.
McAllister, E. J., Dhurandhar, N. V., Keith, S. W., Aronne, L. J., Barger, J., Baskin, M., Benca, R. M., Biggio, J., Boggiano, M. M., Eisenmann, J. C. et al. (2009). Ten putative contributors to the obesity epidemic. *Crit Rev Food Sci Nutr* **49**, 868-913.
McMillen, I. C. and Robinson, J. S. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev* **85**, 571-633.
Nelissen, E. C., van Montfoort, A. P., Dumoulin, J. C. and Evers, J. L. (2010). Epigenetics and the placenta. *Hum Reprod Update* **17**, 397-417.
Nelson, S. M., Coan, P. M., Burton, G. J. and Lindsay, R. S. (2009). Placental structure in type 1 diabetes: relation to fetal insulin, leptin, and IGF-I. *Diabetes* **58**, 2634-41.
Ollikainen, M., Smith, K. R., Joo, E. J., Ng, H. K., Andronikos, R., Novakovic, B., Abdul Aziz, N. K., Carlin, J. B., Morley, R., Saffery, R. et al. (2010). DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome. *Hum Mol Genet* **19**, 4176-88.
Ooi, S. K., Qiu, C., Bernstein, E., Li, K., Jia, D., Yang, Z., Erdjument-Bromage, H., Tempst, P., Lin, S. P., Allis, C. D. et al. (2007). DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature* **448**, 714-7.
Ozdag, H., Teschendorff, A. E., Ahmed, A. A., Hyland, S. J., Blenkiron, C., Bobrow, L., Veerakumarasivam, A., Burtt, G., Subkhankulova, T., Arends, M. J. et al. (2006). Differential expression of selected histone modifier genes in human solid cancers. *BMC Genomics* **7**, 90.
Pankevich, D. E., Mueller, B. R., Brockel, B. and Bale, T. L. (2009). Prenatal stress programming of offspring feeding behavior and energy balance begins early in pregnancy. *Physiol Behav* **98**, 94-102.
Ragoussis, J., Elvidge, G. P., Kaur, K. and Colella, S. (2006). Matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry in genomics research. *PLoS Genet* **2**, e100.
Rosenfeld, C. S., Grimm, K. M., Livingston, K. A., Brokman, A. M., Lamberson, W. E. and Roberts, R. M. (2003). Striking variation in the sex ratio of pups born to mice according

to whether maternal diet is high in fat or carbohydrate. *Proc Natl Acad Sci U S A* **100**, 4628-32.
Ross, S. A. (2003). Diet and DNA methylation interactions in cancer prevention. *Ann N Y Acad Sci* **983**, 197-207.
Samuelsson, A. M., Matthews, P. A., Argenton, M., Christie, M. R., McConnell, J. M., Jansen, E. H., Piersma, A. H., Ozanne, S. E., Twinn, D. F., Remacle, C. et al. (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. *Hypertension* **51**, 383-92.
Schneider, E., Pliushch, G., El Hajj, N., Galetzka, D., Puhl, A., Schorsch, M., Frauenknecht, K., Riepert, T., Tresch, A., Muller, A. M. et al. (210). Spatial, temporal and interindividual epigenetic variation of functionally important DNA methylation patterns. *Nucleic Acids Res* **38**, 3880-90.
Smith, J., Al-Amri, M., Sniderman, A. and Cianflone, K. (2006). Leptin and adiponectin in relation to body fat percentage, waist to hip ratio and the apoB/apoA1 ratio in Asian Indian and Caucasian men and women. *Nutr Metab (Lond)* **3**, 18.
Sood, R., Zehnder, J. L., Druzin, M. L. and Brown, P. O. (2006). Gene expression patterns in human placenta. *Proc Natl Acad Sci U S A* **103**, 5478-83.
Sulewska, A., Niklinska, W., Kozlowski, M., Minarowski, L., Naumnik, W., Niklinski, J., Dabrowska, K. and Chyczewski, L. (2007). Detection of DNA methylation in eucaryotic cells. *Folia Histochem Cytobiol* **45**, 315-24.
Tallman, D. L., Noto, A. D. and Taylor, C. G. (2009). Low and high-fat diets inconsistently induce obesity in C57BL/6J mice and obesity compromises n-3 fatty acid status. *Lipids* **44**, 577-80.
Tamashiro, K. L. and Moran, T. H. (2010). Perinatal environment and its influences on metabolic programming of offspring. *Physiol Behav* **100**, 560-6.
Theys, N., Ahn, M. T., Bouckenooghe, T., Reusens, B. and Remacle, C. (2010). Maternal malnutrition programs pancreatic islet mitochondrial dysfunction in the adult offspring. *J Nutr Biochem*.
Thornburg, K. L., O'Tierney, P. F. and Louey, S. (2010a). Review: The placenta is a programming agent for cardiovascular disease. *Placenta* **31 Suppl**, S54-9.
Thornburg, K. L., Shannon, J., Thuillier, P. and Turker, M. S. (2010b). In utero life and epigenetic predisposition for disease. *Adv Genet* **71**, 57-78.
Tortoriello, D. V., McMinn, J. and Chua, S. C. (2004). Dietary-induced obesity and hypothalamic infertility in female DBA/2J mice. *Endocrinology* **145**, 1238-47.
Tycko, B. and Efstratiadis, A. (2002). Genomic imprinting: piece of cake. *Nature* **417**, 913-4.
Usfar, A. A., Lebenthal, E., Atmarita, Achadi, E., Soekirman and Hadi, H. (2010). Obesity as a poverty-related emerging nutrition problems: the case of Indonesia. *Obes Rev* **11**, 924-8.
Vacca, P., Cantoni, C., Vitale, M., Prato, C., Canegallo, F., Fenoglio, D., Ragni, N., Moretta, L. and Mingari, M. C. (2010). Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci U S A* **107**, 11918-23.
Villeneuve, L. M., Reddy, M. A., Lanting, L. L., Wang, M., Meng, L. and Natarajan, R. (2008). Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. *Proc Natl Acad Sci U S A* **105**, 9047-52.
Waterland, R. A. and Jirtle, R. L. (2003). Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* **23**, 5293-300.

Watson, E. D. and Cross, J. C. (2005). Development of structures and transport functions in the mouse placenta. *Physiology (Bethesda)* **20**, 180-93.
Wienholz, B. L., Kareta, M. S., Moarefi, A. H., Gordon, C. A., Ginno, P. A. and Chedin, F. (2010). DNMT3L modulates significant and distinct flanking sequence preference for DNA methylation by DNMT3A and DNMT3B in vivo. *PLoS Genet* **6**.
Wong, G. W., Krawczyk, S. A., Kitidis-Mitrokostas, C., Revett, T., Gimeno, R. and Lodish, H. F. (2008). Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. *Biochem J* **416**, 161-77.
Zhang, S., Rattanatray, L., McMillen, I. C., Suter, C. M. and Morrison, J. L. (In press). Periconceptional nutrition and the early programming of a life of obesity or adversity. *Prog Biophys Mol Biol*.

WEB SOURCES

(1) WHO, NMH Fact Sheet (2009) Unhealthy diets **&** Physical Inactivity http://www.who.int/nmh/publications/fact_sheet_diet_en.pdf [consulted on July 14th, 2011].

(2) WHO (2004) Global database on Body Mass Index, BMI classification http://apps.who.int/bmi/index.jsp?introPage=intro_3.html [consulted on July 14th, 2011].

(3) National Institutes of Health, The Evidence Report (1998) Clinical Guidelines on the identification, evaluation and treatment of overweight and obesity in adults http://www.nhlbi.nih.gov/guidelines/obesity/ob_gdlns.pdf [consulted on July 14th, 2011].

(4) Richard Baldock, Jonathan Bard, Duncan Davidson and Kirstie Lawson (1998) Theiler Stage Definition http://www.emouseatlas.org/emap/ema/theiler_stages/StageDefinition/stagecriteria.html [consulted on July 14th, 2011].