Published in final edited form as:

Mol Cell Endocrinol. 2013 December 5; 381(0): 160–167. doi:10.1016/j.mce.2013.07.024.

Tissue-specific Leptin promoter DNA methylation is associated with maternal and infant perinatal factors

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Abstract

Leptin a regulator of body weight is involved in reproductive and developmental functions. Leptin promoter DNA methylation (*LEP*) regulates gene expression in a tissue-specific manner and has been linked to adverse pregnancy outcomes. In non-pathologic human pregnancies, we assessed *LEP* methylation, genotyped the single nucleotide polymorphism (SNP) rs2167270 in placental (n=81), maternal and cord blood samples (n=60), and examined the association between methylation, genotype, and perinatal factors. Maternal blood *LEP* methylation was lower in pre-pregnancy obese women ($P=0.01$). Cord blood *LEP* methylation was higher in small for gestational age (SGA) ($P=4.6 \times 10^{-3}$) and A/A genotype ($P=1.6 \times 10^{-4}$), lower (-1.47 , $P=0.03$) in infants born to pre-pregnancy obese mothers and correlated ($P=0.01$) with maternal blood *LEP*. Gender was associated with placental *LEP* methylation ($P=0.05$). These results suggest that *LEP* epigenetic control may be influenced by perinatal factors including: maternal obesity, infant growth, genotype and gender in a tissue-specific manner and may have multigenerational implications.

Keywords

leptin; epigenetics; DNA methylation; rs2167270; pregnancy; maternal obesity; small for gestational age

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Conflict of interest: The authors declare there are no conflicts of interest.

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1. Introduction

Obesity is a global public health concern, responsible for substantial morbidity, mortality and increasing health-care costs (Swinburn et al., 2011). In the United States, it is estimated that one in three adults are obese (Flegal et al., 2012); women of reproductive age do not escape this epidemic (Lawlor et al., 2012). Obesity during pregnancy and increased gestational weight gain (GWG) have been linked to obstetric pathology and early and adult life offspring outcomes (Oken, 2009; Poston et al., 2011). Simultaneously, small for gestational age (SGA) or large for gestational age (LGA) infants are at increased risk of obesity and other chronic diseases (Mehta et al., 2011; Rinaudo and Wang, 2012; Saenger et al., 2007). The relation between birthweight and adult disease gave rise to the “thrifty phenotype” hypothesis that posits that a suboptimal *in utero* environment “programs” the organism to adapt to low-resource conditions that if mismatched in adult life can result in metabolic disease (Hales and Barker, 2001). Later, this evolved into the developmental origins of adult health and disease (DOHaD) paradigm (Gluckman et al., 2005). Hence, it is possible that metabolic fetal programming of adult disease could contribute to the rising obesity epidemic.

Recent evidence has identified genetic and epigenetic factors could be involved in fetal programming; since it requires plasticity, epigenetic mechanisms are likely candidates, as increasing evidence has demonstrated their susceptibility to modification by environmental cues but their relative persistence following development (Christensen and Marsit, 2011; Michels and Waterland, 2012; Novakovic and Saffery, 2012). DNA methylation is an epigenetic modification that involves the addition of methyl group to a cytosine base in the context of a CpG dinucleotide, these to cluster in high density regions, often in gene promoters where increased methylation has been linked to decreased expression (Jones, 2012). DNA methylation marks are reset during embryonic development, and are then critical in establishing cellular differentiation through their restitution in a tissue-specific manner (Novakovic and Saffery, 2012).

Leptin, a 16 kD peptide hormone encoded by the gene *LEP*, predominantly produced by adipose tissue, is involved in food intake, energy expenditure, and reproduction amongst many other functions. Serum leptin correlates with adipose mass and body mass index (BMI) and induces satiety. Obesity is usually associated with hyperleptinemia, but obese women commonly have satiety deregulation because of a concomitant leptin resistance (Caprio et al., 2001; Friedman, 2011). Serum leptin increases during pregnancy (Lepercq et al., 2001) when it is produced by the placenta (Masuzaki et al., 1997) as well as both maternal and fetal adipose tissues. Leptin placental production starts early in development where it has paracrine and endocrine functions (Ashworth et al., 2000). DNA methylation at the *LEP* promoter follows an inverse relationship with *LEP* tissue expression in human cells *in vitro* (Melzner et al., 2002; Noer et al., 2006) and in primary tissue (Bouchard et al., 2010; Marchi et al., 2011). Placental *LEP* promoter methylation has been associated with pregnancy complications such as impaired glucose metabolism (Bouchard et al., 2010) and early onset pre-eclampsia (Hogg et al., 2013). During development, both excess and reduced nutrient availability could increase obesity risk, possibly by disruption of leptin-regulated food intake behaviors programmed *in utero*, but the mechanisms by which this happens are not understood (Vickers and Sloboda, 2012). In summary, *LEP* is epigenetically regulated and exerts metabolic and reproductive functions. Thus, *LEP* DNA methylation is a plausible factor to be involved in fetal metabolic programming. Using a quantitative methylation technique, the present study aimed to investigate the associations between *LEP* promoter DNA methylation in placental tissue, maternal blood, and infant cord blood, and perinatal maternal and infant factors in a population of healthy term infants.

2. Materials and methods

2.1. Study population and design

The subjects involved in this study are part of the ongoing Rhode Island Child Health Study (Marsit et al., 2012) that recruits mother-infant pairs following delivery at Women and Infants Hospital in Providence Rhode Island, USA starting in March 2009 and continuing to enroll. Briefly, term infants born small (10th percentile) and large (90th percentile) for gestational age, according to the Fenton growth charts (Fenton, 2003) were gender and gestational age matched with an infant appropriate for gestational age (AGA). Post-recruitment all infants were re-classified into birthweight categories using recently developed growth charts (Fenton and Kim, 2013). All subjects provided written informed consent following protocols approved by the Institutional Review Board at Women and Infants Hospital and Dartmouth College. Exclusion criteria included non-singleton pregnancy, maternal age <18 years, maternal life-threatening complications and infant congenital or chromosomal abnormalities. Clinical information was collected using a structured chart review form, followed by an interviewer-administered questionnaire to gather information on socio-demographic variables, personal and family medical, lifestyle and exposure history. Maternal pre-pregnancy body mass index (BMI), tobacco use during pregnancy and pregnancy hypertension were obtained from medical charts records and maternal GWG was self-reported in the study questionnaire.

2.2. Sample collection and nucleic acid extraction

Fetal placental samples were collected from all subjects within two hours following delivery; twelve fragments, three from each quadrant were obtained two centimeters (cm) from the umbilical cord and free of maternal decidua. Collected tissue was immediately placed on RNAlater solution (Life Technologies, Grand Island, NY, USA) and stored at 4°C. Subsequently, tissue segments from each placental region were blotted dry, snap frozen in liquid nitrogen, homogenized by pulverization using a stainless steel cup and piston unit (Cellcrusher, Cork, Ireland) and stored at -80°C until needed. When available, paired maternal and infant cord blood was obtained from residual samples collected for clinical purposes, making them suitable for total genomic DNA extraction but not for mRNA or protein measurements. Maternal blood was collected via venipuncture at the time of admission for delivery, and infant blood was obtained with a syringe from the umbilical cord after delivery. DNA was extracted from homogenized placental samples and from blood using the DNAeasy Blood & Tissue Kit (Qiagen, Inc, Valencia, CA, USA). The resulting DNA was quantified using the ND 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). All procedures were performed following manufacturer's protocols.

2.3. DNA Bisulfite Modification and Pyrosequencing Analysis

DNA samples (500 ng) were sodium bisulfite-modified using the EZ DNA methylation Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's protocols. For methylation detection, bisulfite pyrosequencing was employed. Primers (Integrated DNA Technologies, Inc, Coralville, IA) were designed using the PyroMark Assay Design software version 2.0.1.15 (Qiagen) in a region previously associated with leptin expression (Bouchard et al., 2010; Melzner et al., 2002; Yokomori et al., 2002). The PyroMark PCR kit (Qiagen) and PCR primers (Table S1) were used to amplify a 383 base pair region in the *LEP* promoter; cycling conditions were 94°C for 15 min followed by 50 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min with a final extension of 10 min at 72°C. Pyrosequencing was performed in triplicate using the Pyromark MD (Qiagen) instrument with two forward assays covering a total of 23 CpG loci (Tables S1 and S2). Non-CpG cytosines within each read served as internal controls to verify bisulfite DNA modification efficiency (95% in all samples) and each pyrosequencing run included a no template

control; all samples were sequenced by the same operator. DNA methylation results were analyzed with the PyroMark CpG software, version 1.0.11 (Qiagen).

2.4. SNP Genotyping

Samples were genotyped for rs2167270 (+19 G>A), a common SNP in the *LEP* promoter region studied. Genotypes calls were made by analyzing the pyrograms and comparing peak heights for each allele; triplicate wells were called independently and compared for quality control. Additionally, we confirmed the pyrosequencing genotypes by performing an allelic discrimination Taqman (Life Technologies) assay (ID:C__15966471_20) for maternal and cord blood DNA samples, using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Genotype calls from the two methods were equivalent in all samples compared.

2.5. Statistical Analysis

The *LEP* promoter mean methylation distributions were assessed with Shapiro-Wilk test, confirming normality in the analyzed tissues. Pairwise Pearson correlations were used to compare methylation between each of the 23 CpG loci within each sample type. Bivariate associations between methylation extent and covariates were initially compared with a Student's *t*-test or ANOVA. Fisher exact or χ^2 tests were used accordingly to evaluate frequency differences between groups. Models were constructed with methylation as the dependent variable and either birthweight group, maternal pre-pregnancy BMI category or maternal blood *LEP* methylation as the independent variable. Covariates evaluated as possible confounders included variables that could potentially affect leptin methylation or birthweight (cord blood and placenta models) and/or maternal BMI (maternal blood models). Infant birthweight was evaluated as a categorical variable because of the known association of serum leptin with birthweight group, the clinical relevance of these groupings, and as the relationship between cord blood *LEP* methylation and infant weight was not linear. Self-reported maternal GWG data was combined with pre-pregnancy BMI to construct a categorical variable (adequate, inadequate or excessive GWG) following the Institute of Medicine (IMO) cutoffs (Rasmussen et al., 2010). The final adjusted regression models were corrected for maternal factors (pre-pregnancy BMI, GWG, race, tobacco use during pregnancy, pregnancy hypertension, rs2167270 genotype) and or infant factors (birthweight group, gender, infant genotype, delivery method). The maternal blood *LEP* regression model was adjusted only for the mentioned maternal factors and the cord blood and placental *LEP* methylation models we adjusted for both maternal and infant factors). Sensitivity analyses were performed to test that associations obtained from the linear models were not driven by outlying subjects. All analyses were conducted in RStudio version 0.97.314 (RStudio, Boston, MA, USA). All tests were two-sided and statistical significance was determined at *P*-value <0.05.

3. Results

3.1. Socio-demographic characteristics

The maternal and infant characteristics of the study population (n=81) are described in Table 1. The mean gestational age was 39 weeks with a similar proportion of female and male infants. In accordance with the planned composition of the cohort, our sample is overrepresented for LGA (28%, n=23) and SGA (27%, n=22) infants. The average maternal age was 29 years with the majority (73%) of the participants reporting Caucasian race. Only 5% reported tobacco use during pregnancy. Mothers were grouped according to World Health Organization classification of adult BMI categories: normal (18.5–24.9 kg/m²), overweight (25–29.9 kg/m²) and obese (≥30 kg/m²); the average BMI was 25.5 kg/m² (standard deviation (SD) 5.9) and ranged from 17–41 kg/m². Only five women had BMI

<18.5 kg/m² and were included in the normal category because of the low prevalence and their maternal blood *LEP* methylation values did not differ from those considered normal BMI ($P=0.64$, data not shown). In this study, we did not have any participants with gestational, type I or type II diabetes. Of note, in this population we observed a positive correlation between pre-pregnancy maternal BMI and infant birthweight ($r=0.37$, $P=8.4\times10^{-4}$).

3.2. *LEP* methylation distributions across tissues

We examined *LEP* promoter methylation using pyrosequencing of 23 CpG sites across different tissues: maternal blood (n=60), cord blood (n=60) and placentas (n=81). A group of 39 mother-infant pairs had matched maternal, cord blood and placental data, the remaining (n=42) had placental with either cord or maternal blood *LEP* methylation data. We observed a moderate correlation between all the CpG sites surveyed across the region in each of the examined tissues (Fig. S1, S2, S3). Due to this level of correlation and a prior report which utilized the mean methylation across the whole region (Bouchard et al., 2010), we utilized the mean of these 23 sites in subsequent analysis. The mean *LEP* methylation distribution from each tissue is displayed in Fig. 1. Placental methylation showed higher variability across samples than cord and maternal blood methylation. The average *LEP* mean promoter methylation across tissues differed significantly ($P<5\times10^{-16}$); cord blood methylation was the lowest (14.1%, SD 1.9) followed by maternal blood (20.1%, SD 3.1) and placenta (23.7%, SD 5.5) (Table S3).

3.3. rs2167270 genotype, birthweight and maternal BMI

To examine if a common genetic variant (rs2167270) in the *LEP* promoter region analyzed was associated with methylation, we genotyped infant and maternal samples. Genotype frequencies in both mothers and infants were in Hardy-Weinberg equilibrium. Overall, 35% of the infants were homozygous for the wild type allele (G/G), 52% heterozygous (G/A) and 13% homozygous for the variant (A/A). Infant genotype frequencies did not differ between birthweight groups (Fisher's exact test, $P=0.23$). For the subset of participants with maternal blood samples (n=60) we observed the following maternal genotype frequencies: 43% (G/G), 45% (G/A) and 12% (A/A), and these did not differ between BMI categories ($P=1$).

3.4. Maternal blood *LEP* DNA methylation

Considering the association of serum leptin and body weight during pregnancy (Highman et al., 1998; Misra and Trudeau, 2011), we performed multivariable linear regression analysis to model the association of maternal blood *LEP* promoter methylation and pre-pregnancy BMI category in the subset of participants with available maternal blood samples (Table 2, Fig. S4). The population characteristics of this group are shown in Table S4. We observed differences in maternal blood *LEP* methylation between BMI categories, which remained significant after adjusting for potential confounders including maternal age, genotype, ethnicity, tobacco use during pregnancy and pregnancy weight gain. Obese women had 2.95% ($P=0.01$) lower maternal blood *LEP* promoter methylation compared to normal weight women, controlling for confounders. In addition, although not statistically significant ($P=0.17$), we observed that overweight women had 1.65% lower methylation in blood, and a test for trend suggested significantly less methylation with increasing BMI class ($P<0.01$). The mean maternal blood *LEP* methylation was not different between rs2167270 genotypes as demonstrated in the model or in specific analyses comparing each genotype: A/A (21%), G/A (19.8%) and G/G (20.1%) ($P=0.68$, Table S5).

3.5. Cord blood *LEP* DNA methylation

Cord blood leptin has previously been shown to be associated with infant birthweight, and SGA infants have lower serum leptin (Karakosta et al., 2011; Ren and Shen, 2010; Schubring et al., 1997). Therefore, we sought to investigate possible relationships between *LEP* promoter methylation in cord blood and infant birthweight in the group of participants with available data (Table S4). First, we examined the effect of genotype on cord blood *LEP* DNA methylation. The mean extent of methylation was higher in A/A infants (16.5%) compared to G/A (13.8) or G/G (13.7%) (ANOVA, $P=6.13 \times 10^{-4}$) (Table S5). Since the cord blood methylation values between the G/G and G/A genotypes did not differ (Tukey's test, $P=0.98$), we grouped the homozygous (G/G) and heterozygous (G/A) and compared those to the homozygous variant group (A/A) in all further analyses (A/A= 16.6% versus G/G and G/A 13.8%, Student's t-test, $P=5 \times 10^{-3}$, Figure 2A). We did not observe a significant difference in *LEP* methylation extent by birthweight group (ANOVA, $P=0.29$, Figure 2B), but this analysis suggested a trend of higher *LEP* methylation in SGA (14.8%) infants compared to AGA (13.8%) infants and LGA (14.1%). Of note, in our sample we did not have any infants in the SGA group with the A/A genotype. Because we observed higher cord blood *LEP* methylation in the A/A genotype and the SGA group, we repeated the bivariate analyses between birthweight group and cord blood *LEP* excluding the A/A infants. In this analysis we detected significantly higher *LEP* methylation in SGA (14.8%) infants (ANOVA, $P=0.02$) compared to AGA (13.5%, Tukey's test, $P=0.04$) or LGA (13.2%, Tukey's test, $P=0.02$). In multivariable linear regression analyses (Table 3) adjusted for possible confounders (maternal and infant factors), we observed a positive association between cord blood *LEP* methylation and birthweight group; SGA infants had 1.78% greater methylation than AGA infants ($P=4.6 \times 10^{-3}$). Similarly, infants with A/A genotype at rs2167270 variant had significantly higher methylation (2.68%) levels compared to those with at least one G allele ($P=1.2 \times 10^{-4}$) independent of birthweight status and other factors included in the model. Interestingly, cord blood *LEP* methylation was significantly lower (−1.47%, $P=0.03$) in infants born to obese mothers and higher (1.06%, $P=0.04$) in women with excessive GWG. In our study, obese and overweight women were more likely to gain excessive weight during pregnancy than normal BMI women (73%, 68% and 35%, respectively; Fisher's exact test, $P=0.007$). Additionally, we observed that infants born to women who reported smoking during pregnancy had higher (2.22%, $P=0.03$) cord blood *LEP* methylation than infants born to non-smokers, but this result should be interpreted with caution as we only have three mothers that reported smoking during pregnancy in this data set. Because of the known association between SGA and cigarette smoking, we repeated the regression analysis excluding the mothers that reported tobacco use during pregnancy; the observed effects of SGA birth weight group, infant genotype, pre-pregnancy obese BMI and excessive GWG remain similar in size and statistically significant (data not shown).

3.6. Placental *LEP* DNA methylation

The placenta produces leptin and this adipokine has shown to have a variety of roles in this tissue (Gambino et al., 2012). In contrast to the results obtained in cord blood, we did not observe a significant association between birthweight and placental *LEP* DNA methylation in either bivariate or multivariable linear regression models controlled for confounders (Table 3). Likewise, we did not observe an association between infant genotype and mean placental *LEP* methylation (Table S5); A/A (23.7%), G/A (24.2%) and G/G (22.9%) (ANOVA, $P=0.67$). However, we detected a significant association between gender and *LEP* methylation ($P=0.05$) in this tissue after adjusting for possible confounders (infant and maternal factors). Male infants had 2.91% higher placental *LEP* methylation compared to females. Additionally, SGA status and infant A/A genotype showed higher placental *LEP* methylation similar to the results obtained in cord blood, but without statistical significance. Interestingly and although not significant, we observed opposite effect of pre-pregnancy

obesity; obese women have infants with higher placental *LEP* methylation. Nonetheless, in this study infant gender was the only covariate that we could identify that significantly predicted *LEP* DNA methylation in placental tissue.

3.7. *LEP* DNA methylation between tissues

Placental *LEP* methylation was not correlated with maternal or cord blood methylation ($r = -0.04$, $P = 0.79$; $r = 0.04$, $P = 0.75$), respectively. In contrast, maternal blood was positively correlated with cord blood methylation ($r = 0.4$, $P = 0.01$) (Table S3, Fig. 3). In a linear regression model of cord blood methylation as the dependent variable and maternal *LEP* methylation and genotype as independent variables, we identified a significant association after adjusting for possible infant and maternal confounders (Table S6). In the 37 mother-infant pairs examined, a 1% increase in maternal blood methylation is associated with a 0.25% increase in infant blood methylation ($\beta = 0.25$; Std. Error = 0.08; $P = 0.01$). Similar to our previous result, infants with A/A genotype had a significantly higher cord blood *LEP* methylation compared to infants with at least one G allele and we observed a marginally significant effect of obesity on cord blood *LEP* methylation, despite the sample size reduction (Table S6).

4. Discussion

In this study, we found evidence of associations between perinatal maternal and infant factors and *LEP* promoter methylation. Specifically, maternal blood methylation was associated with pre-pregnancy obesity. Cord blood methylation was associated with infant SGA status, A/A (rs2167270) genotype, excessive maternal GWG and pregnancy smoking and negatively associated with pre-pregnancy obesity. In placental tissue, *LEP* DNA methylation was associated with infant gender. Finally, we found that maternal blood *LEP* DNA methylation correlates with infant blood *LEP* methylation.

We detected a moderate correlation between methylation at individual CpGs and the overall mean *LEP* promoter methylation for the analyzed region. Similar results were obtained in a recent study (Bouchard et al., 2010) that assessed *LEP* promoter methylation in human placental biopsies. Correspondingly, the average methylation (24%) values we observed in placenta resemble those obtained in the prior study ($\approx 28\%$); the slight difference in the results could be due to partial overlap in the examined region, a different analysis technique, and different population characteristics. Our findings indicate that there are differences between mean and distribution of methylation values between the analyzed tissues; this complements previous reports that described tissue-specific *LEP* DNA methylation (Marchi et al., 2011; Stöger, 2006). DNA methylation extent in the examined region of the *LEP* promoter has been previously associated with leptin expression in mouse (Yokomori et al., 2002), human adipocytes (Melzner et al., 2002), and human placenta (Bouchard et al., 2010). Moreover, DNA binding proteins such as specificity protein (SP1) and CAAT-enhancer binding protein (C/EBP β) (Mason et al., 1998) are known to bind to this area, and C/EBP β binding affinity is reduced in methylated probes compared to non-methylated ones as assessed by electrophoretic mobility shift assay in LiSa-2 cells nuclear extracts (Melzner et al., 2002).

Studies from the Dutch Famine Birth Cohort have found higher blood *LEP* methylation levels in famine-exposed adult men compared to unexposed siblings, although they did not comment on the association with current BMI (Tobi et al., 2009); whereas, other studies from the same cohort have observed that famine exposure increases the risk of obesity later in life (Ravelli et al., 1976; Roseboom et al., 2006). Additionally, previous studies (Highman et al., 1998; Misra and Trudeau, 2011) have reported associations between serum leptin and maternal weight and adiposity, but the association between maternal blood *LEP* methylation

extent and maternal BMI category has not been described before. To our knowledge this is the first report that has established an association between pre-pregnancy obesity and maternal blood *LEP* methylation at term. Our results showed lower blood *LEP* methylation amongst obese women, thus we can hypothesize that peripheral blood cells may also contribute to increased serum leptin in obesity, and that peripheral blood cells may mimic the metabolic changes occurring in adipocytes. This would need to be more thoroughly examined in samples appropriate to examine leptin expression in peripheral blood cells. Prior studies have not specifically correlated blood *LEP* DNA methylation with serum leptin or measurements of leptin in adipocytes in humans, and the samples collected for our study could not appropriately address this issue, thus highlighting a need for future studies to assess this connections. Our results should be replicated in different population studies to further evaluate the observed association, and if possible, the relationship between adipocyte and peripheral blood *LEP* methylation and serum levels specifically interrogated.

Cord blood methylation is higher in SGA infants and those with the A/A (homozygous variant) genotype (rs2167270) in the *LEP* promoter, and our multivariable model suggests these are independent associations. Serum cord blood leptin has been consistently correlated with birthweight in different populations (Ren and Shen, 2010), moreover SGA infants have lower leptin levels (Ren and Shen, 2010) and adipose tissue mass (Martinez-Cordero et al., 2006). Hence, we can hypothesize that the higher *LEP* promoter methylation observed in SGA infants reflect lower infant body fat, similar to maternal blood *LEP* DNA methylation levels reflecting maternal adiposity. Further research is needed to assess the connections between fetal adipose mass at birth and *LEP* DNA methylation in cord blood. In addition, it is recognized that being born SGA carries a higher risk of metabolic disease such as obesity later in life (Saenger et al., 2007), hence it would be interesting to investigate *LEP* DNA methylation at different time points over the life course to assess if there are differences between SGA individuals that eventually develop obesity and those who do not. Additionally, our results show an association of maternal pre-pregnancy obesity and lower cord blood methylation levels, results that are consistent with those observed in maternal blood. We can hypothesize that this association is derived from the known relationship between maternal weight and birthweight (Frederick et al., 2008; Hull et al., 2008). Obese mothers have larger infants and this resulted in an opposite association on cord blood *LEP* from the observed effects of SGA. Concomitantly, the adjusted regression model for cord blood *LEP* methylation shows a direct association of GWG and *LEP* methylation; excessive GWG increases *LEP* methylation. This result is unexpected as it has been reported that women with excessive GWG have infants with increased adiposity and LGA (Johnson et al., 2013; Poston et al., 2011). However, we can hypothesized that this results from an infant adaptive response to the augmented maternal adiposity levels. Nonetheless, from our results the effect size of SGA on cord blood *LEP* methylation was higher than that of excessive GWG. Hence, specific mechanistic research is needed to address these adaptive mechanisms *in utero*, while further population-based research is needed to confirm and extend these associations.

Interestingly, we observed an association between genotype and *LEP* DNA methylation in cord blood that was not detected between genotype and methylation in maternal blood or placental tissue. DNA sequence variation acting in *cis* can exert effects on DNA methylation across the genome, and allele specific methylation has been reported to differ between tissues within loci (Schalkwyk et al., 2010). This may explain both the association between genotype and methylation in cord blood as well as the lack of association between genotype and methylation in the other tissues examined. These associations should be explored further, and in a larger population which is more statistically powered to examine interactions between birthweight group and genotype on *LEP* promoter methylation.

Placental DNA methylation in other loci has been previously associated with infant birthweight (Filiberto et al., 2011; Hogg et al., 2012; Marsit et al., 2012), hence, we hypothesized that placental *LEP* DNA methylation would be associated with birthweight. Surprisingly, we did not find evidence of a significant association. Our findings indicate an association between gender and placental *LEP* methylation, which was not detected in cord blood, further supporting the concept of placenta as a unique pregnancy tissue that can be influenced by fetal factors, but one that is epigenetically distinct from cord blood. Gender differences in *LEP* DNA methylation have been observed previously (Tobi et al., 2009) in peripheral blood of adults exposed to wartime famine; men had higher methylation than women. Additionally, a different study (Chan et al., 2006) found elevated leptin levels in amniotic fluid of female fetuses compared to males in normal and pre-eclamptic pregnancies. This observation supports our *LEP* methylation finding; since lower methylation levels in placentas from female infants could account for these increases in this hormone in amniotic fluid. Moreover, 17 β -estradiol increases leptin expression in placental cells through actions on the estrogen receptor- (Maymo et al., 2011), although, we are aware that this can occur through other mechanisms of gene regulation. Our findings, while plausible, need to be validated in other studies, especially as a recent study (Hogg et al., 2013) did not observe differences in placental *LEP* methylation by infant gender.

In the 39 mother-infant pairs for which we have paired cord and maternal blood information, we detected a significant, moderate, positive correlation between cord *LEP* methylation and maternal blood *LEP*. This result is likely related to the positive correlation between infant birthweight and pre-pregnancy maternal BMI observed in this study and other reports (Frederick et al., 2008; Hull et al., 2008). However, this association persisted after adjusting for pre-gestational BMI and birthweight group. Overall, our findings from maternal and cord blood suggest that pre-pregnancy obesity influences not only maternal blood *LEP* methylation but perhaps also infant cord blood *LEP* methylation. Interestingly, evidence from a murine study (Jousse et al., 2011) demonstrated a similar effect, but with opposite exposure; maternal undernutrition results in *LEP* hypomethylation in offspring adipose tissue. The results from our study suggest a potential for multigenerational epigenetic inheritance in humans which has been proposed (Daxinger and Whitelaw, 2012), and add to the increasingly strong evidence (Hogg et al., 2012) of associations between *in utero* environmental influences and altered DNA methylation.

Our study has a number of strengths: we have mother-infant paired *LEP* methylation data in different tissues and we are the first study to evaluate *LEP* methylation in infant and maternal blood and placental tissue in non-pathologic pregnancies. Notably, this study is also the first to find a correlation between maternal and infant DNA methylation in humans. However, this work has several limitations: (1) our sample size is small, (2) we lack serum leptin measurements, (3) we did not directly measure anthropometric data and this could potentially introduce self-report bias and (4) we studied only one gene in a myriad of possible candidates that contribute to complex phenotypes such as maternal obesity and SGA birthweight. In addition, like all population-based studies we are unable to elucidate the exact cellular mechanisms that link perinatal maternal and infant factors studied with epigenetic alterations.

5. Conclusion

In summary, in this study we demonstrated that *LEP* DNA methylation is associated in a tissue-specific manner with maternal (pre-pregnancy obesity, pregnancy smoking and GWG) and infant factors (SGA, genotype and gender) in non-pathological pregnancies. We also provide evidence for an association between maternal and infant *LEP* DNA methylation. Future research in larger studies is needed to further elucidate and confirm the

detected associations. However, this investigation contributes to the increasing number of studies that have identified the importance of leptin in pregnancy and to the small number of studies in *LEP* DNA promoter methylation. Identifying maternal factors that can produce potentially life-long epigenetic alterations in offspring is of crucial importance, as this supports the need for perinatal intervention studies to decrease adverse infant and adult-life outcomes such as obesity and cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Thanks to Joyce Lee for her hard work in recruitment of subjects into this study and the support of the staff of the Brown Center for the Study of Children at Risk for their efforts. This work was funded by the National Institute of Health (NIH) through the following grants: R01MH094609 (NIH-NIMH), P20GM103537 (NIH-NIGMS) and P30CA23108 (NIH-NCI). Its contents are the responsibility of the authors and do not necessarily represent the official views of the funding institutions.

Abbreviations

LEP	leptin promoter DNA methylation
SGA	small for gestational age
SNP	single nucleotide polymorphism
LGA	large for gestational age
AGA	adequate for gestational age
GWG	gestational weight gain
DOHaD	developmental origins of adult health and disease
LGA	large for gestational age, BMI, body mass index
SD	standard deviation
Std.Error	standard error
SP1	specificity protein
C/EBP	CAAT-enhancer binding protein alpha

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Highlights

Maternal and infant blood *LEP* methylation are associated with pre-pregnancy obesity.

SGA and A/A (rs2167270) infants have higher cord blood *LEP* DNA methylation.

LEP DNA methylation is higher in placentas from male infants.

Maternal blood *LEP* DNA methylation correlates to infant cord blood *LEP* methylation.

Fig. 1. *LEP* promoter methylation by tissue

Distribution of *LEP* methylation (y-axis) in samples by tissue (x-axis): cord blood (n=60), maternal blood (n=60) and placenta (n=81). The black bars represents the mean within each tissue; cord blood (14.1%), maternal blood (20.1%) and placenta (23.7%) The *P*-value is the result of ANOVA test.

Fig. 2. *LEP* promoter methylation in cord blood

(A) Cord blood *LEP* promoter methylation by rs2167270 genotype. Distribution of *LEP* methylation (y-axis) in cord blood samples by genotype (x-axis): G/G and G/A (n=52) and A/A (n=8). Black bars represent the mean value for each group; G/G/and G/A (13.8%) and A/A (16.6%). The *P*-value is the result of Student's t-test.

(B). Cord blood *LEP* promoter methylation by birthweight group. Distribution of *LEP* methylation (y-axis) in study samples by birthweight (x-axis): SGA (n=15), AGA (n=26) and LGA (n=19). Black bars represent the mean value for each group; SGA (14.8%), AGA (13.8%) and LGA (14.1%). The *P*-value is the result of ANOVA.

Fig. 3. Maternal and cord blood *LEP* promoter methylation
Scatterplot of maternal blood *LEP* methylation (y-axis) and cord blood *LEP* methylation (x-axis) in 39 mother-infant pairs. Depicted is r and the Pearson correlation P -value.

Table 1
Study population characteristics

Clinical and socio-demographic characteristic of the infants and mothers enrolled in the study (n=81) with placental *LEP* methylation information. Infant and maternal characteristic are described for specific parameters. Infants were classified according to gestational age and birthweight into three groups: adequate for gestational age (AGA), large for gestational age (LGA) and small for gestational age (SGA). SD: standard deviation.

	n	%	Mean	SD
Infant variables				
Birthweight group	81			
AGA	36	44		
LGA	23	28		
SGA	22	27		
Birthweight (g)	81		3406	714
Gestational age (weeks)	81		39	1.1
Gender	81			
Female	40	49		
Male	41	51		
IUGR	77			
No	67	87		
Yes	10	13		
Maternal variables				
Age (yrs)	81		29	6.2
Pre-pregnancy BMI (kg/m ²)	81		25.5	5.9
Normal	47	58		
Overweight	19	23		
Obese	15	19		
Pregnancy Weight Gain	80		14.1	5.9
Inadequate	18	22.5		
Adequate	22	27.5		
Excessive	40	50		
Maternal race	81			
Other	22	27		
White	59	73		
Tobacco during pregnancy	81			
No	77	95		
Yes	4	5		
Delivery Method	81			
Vaginal	46	57		
C-Section	35	43		
Pregnancy Hypertension	80			
No	74	92.5		
Yes	6	7.5		

SD: standard deviation; AGA: adequate for gestational age; LGA: large for gestational age; SGA: small for gestational age

Table 2

Multivariable linear regression models of maternal blood *LEP* methylation
 Multivariable linear regression models of maternal blood *LEP* methylation as the dependent variable and pre-pregnancy BMI category as the independent variable. Models are provided unadjusted and adjusted for possible confounder variables included in the table. * P for trend<0.01.^a Data missing on gestational weight gain and pregnancy hypertension.

Maternal Factors	Unadjusted (n=60)			Adjusted (n=58) ^{a,b}		
	Estimate	Std.Error	P	Estimate	Std.Error	P
Pre-pregnancy BMI (kg/m ²) category *						
Normal	Reference			Reference		
Overweight	-1.74	1.06	0.11	-1.65	1.18	0.17
Obese	-2.75	0.92	4.1×10 ⁻³	-2.95	1.15	0.01
Gestational weight gain						
Adequate	Reference			Reference		
Inadequate	-	-	-	-0.23	1.17	0.84
Excessive	-	-	-	0.46	1.03	0.66
Tobacco use during pregnancy						
No	Reference			Reference		
Yes	-	-	-	1.57	2.36	0.51
Pregnancy hypertension						
No	Reference			Reference		
Yes	-	-	-	-0.96	1.37	0.49
Race						
White	Reference			Reference		
Other	-	-	-	0.81	0.96	0.38
Maternal age (yrs)	-	-	-	0.02	0.08	0.80
Genotype rs2167270						
G/G and G/A	Reference			Reference		
A/A	-	-	-	0.84	1.28	0.51

^aModel adjusted all variables included in table.

^bData missing on gestational weight gain and pregnancy hypertension.

$P < 0.05$ are shown in bold,
 $*$ P for trend < 0.01

Table 3

Multivariable linear regression models of cord blood and placental *LEP* methylation

Multivariable linear regression models of cord blood *LEP* methylation and placental *LEP* methylation as the dependent variable and birthweight group as the independent variable. Models are provided unadjusted and adjusted for possible confounder variables included in the table. AGA: adequate for gestational age; LGA: large for gestational age; SGA: small for gestational age; Std. Error: standard error; ^a Data missing on gestational weight gain and pregnancy hypertension.

	Cord Blood Methylation				Placental Methylation			
	Unadjusted (n=60)	Adjusted (n=58) ^{a,b}	Unadjusted (n=81)	Adjusted (n=79) ^{a,b}	Unadjusted (n=81)	Adjusted (n=79) ^{a,b}	Unadjusted (n=81)	Adjusted (n=79) ^{a,b}
Infant Factors	Estimate	Std.Error	P	Estimate	Std.Error	P	Estimate	Std.Error
Birthweight Group								
AGA	Reference			Reference			Reference	
LGA	0.11	0.49	0.82	0.48	0.53	0.31	0.17	1.48
SGA	1.47	0.54	8.3×10⁻³	1.78	0.60	4.6×10⁻³	2.56	1.50
rs2167270								
G/G and G/A	Reference			Reference			Reference	
A/A	3.11	0.64	9.8×10⁻⁶	2.68	0.63	1.2×10⁻⁴	0.14	1.68
Infant Gender								
Female	Reference			Reference			Reference	
Male	-	-	-	-0.42	0.43	0.33	-	2.91
Maternal Factors								
Pre-pregnancy BMI (kg/m ²) category								
Normal	Reference			Reference			Reference	
Overweight	-	-	-	-0.32	0.49	0.51	-	0.48
Obese	-	-	-	-1.47	0.65	0.03	-	2.30
Pregnancy weight gain								
Adequate	Reference			Reference			Reference	
Inadequate	-	-	-	-0.03	0.65	0.97	-	1.11
Excessive	-	-	-	1.06	0.50	0.04	-	0.02
Race								
White	Reference			Reference			Referent	
Other	-	-	-	0.41	0.48	0.40	-	0.67
								1.54
								0.67

	Cord Blood Methylation				Placental Methylation			
	Unadjusted (n=60)	Adjusted(n=58) ^{a,b}			Unadjusted (n=81)	Adjusted (n=79) ^{a,b}		
	Estimate	Std.Error	P	Estimate	Std.Error	Estimate	Std.Error	P
Tobacco use during pregnancy								
No	-	-	-	Reference	-	Reference	-	0.58
Yes	-	-	-	2.22	0.99	-1.77	3.15	
Pregnancy hypertension								
No	-	-	-	Reference	-	Reference	-	
Yes	-	-	-	1.18	0.84	-	-	
Maternal age (yrs)								
				0.05	0.04	-0.84	2.54	0.74
Delivery method								
Vaginal				Reference	-	Reference	-	
C-section	-	-	-	-0.39	0.45	-0.16	1.41	0.91

^aModel adjusted for all variables included in table.

^bData missing on gestational weight gain and pregnancy hypertension, $P < 0.05$ are shown in bold.